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Phenotype and expression profile analysis of *Staphylococcus aureus* biofilms and planktonic cells in response to licochalcone A

Fengge Shen · Xudong Tang · Yang Wang · Zhiqiang Yang · Xiaochen Shi · Chao Wang · Qiaoli Zhang · Yanan An · Wei Cheng · Kunqi Jin · Mingyuan Liu · Na Guo · Lu Yu

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Abstract *Staphylococcus aureus* is one of the most important pathogens in humans and animals. The formation of biofilm by *S. aureus* is considered an important mechanism of antimicrobial resistance. Therefore, finding effective drugs against the biofilm produced by *S. aureus* has been a high priority. Licochalcone A (LAA), a natural plant product, was reported to have antibacterial activities and showed good activity against all 21 tested strains of *S. aureus* biofilm and planktonic cells. To detect the possible molecular mechanism of LAA against *S. aureus* biofilm or planktonic cells, Affymetrix GeneChips were used to determine the global comparative transcription of *S. aureus* biofilm and planktonic cells triggered by treatment with sub-bactericidal and sub-inhibitory concentrations of LAA, respectively. LAA significantly altered (greater than a 2- or less than −2-fold change) the expression of 693 genes in planktonic cells and 817 genes in biofilm. The levels of genes encoding autolysis-associated proteins, cell wall proteins, pathogenic factors, protein synthesis genes, and enzymes involved in capsule synthesis were significantly altered in LAA-treated *S. aureus*. Furthermore, some differences observed in the microarray analysis were verified by real-time RT–PCR. To our knowledge, this is the first observation of phenotype and expression profiles of *S. aureus* biofilm and planktonic cells in response to LAA treatment.

Keywords *Staphylococcus aureus* · Licochalcone A · Biofilm · GeneChip · Transcription

Fengge Shen and Xudong Tang contributed equally to this work.

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F. Shen · Z. Yang · X. Shi · C. Wang · Q. Zhang · Y. An · W. Cheng · K. Jin · M. Liu · N. Guo (✉) · L. Yu (✉)
Key Laboratory of Zoonosis Research, Ministry of Education, Institute of Zoonosis, College of Veterinary Medicine, College of Animal Science and Department of Food Quality and Safety, Jilin University, Changchun, China
e-mail: abas1980@163.com
e-mail: yulu225@sohu.com

X. Tang
Key Lab for New Drug Research of TCM, Research Institute of Tsinghua University in Shenzhen, Shenzhen, China

Y. Wang
Department of Infectious Diseases, First Hospital of Jilin University, Changchun, China

M. Liu
Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou, China

Introduction

Staphylococcus aureus is an important worldwide human pathogen that leads to a number of diseases including endocarditis, cellulitis, impetigo, etc. (Cameron et al. 2012). Greater than 60 % of *S. aureus* isolates are now resistant to methicillin, and some strains have developed resistance to more than 20 different antimicrobial agents. Resistance in clinical isolates is mostly related to the capacity for biofilm formation. The main component of the biofilm extracellular matrix in *S. aureus* is polysaccharide intercellular adhesin (PIA), which adheres the bacterial cells together and renders an increase in resistance to antimicrobial agents and host defenses (Rohde et al. 2001). Therefore, finding effective drugs against *S. aureus* biofilm has been a high priority.

Licochalcone A (LAA) (Fig. 1), a main phenolic component of the licorice species, *Glycyrrhiza inflata*, has been reported to have various biological activities, e.g., anti-inflammatory (Shibata 2000), anti-protozoal (Chen et al. 2001), anti-tumor (Shibata 2000), anti-oxidative (Wu et al. 2011), and antimicrobial (Liu et al. 2008) effects. LAA has

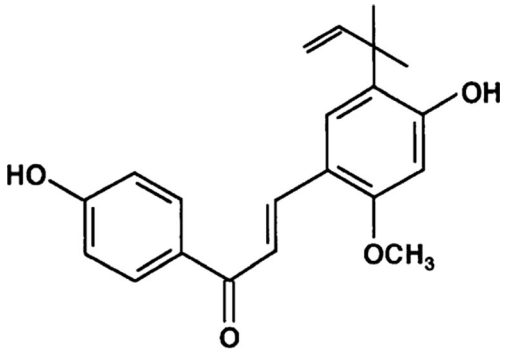

Fig. 1 Structure of LAA

antibacterial activities against both methicillin-sensitive and methicillin-resistant *S. aureus* (Liu et al. 2008). Our preliminary study has found that LAA has anti-biofilm activity against *S. aureus* strains (unpublished). However, the inhibitory characteristics and mechanism of LAA against *S. aureus* biofilms needs thorough investigation. Transcriptional profiles generated by GeneChip analysis of bacteria are a tool with which to investigate differential gene expression, exploring possible mechanisms of antimicrobial activity (Smith et al. 2010). Transcriptional profiles of *S. aureus* response to tigecycline, berberine, triclosan, and rhein have been previously performed (Wang et al. 2008; Jang et al. 2008). Moreover, virulence factors of *S. aureus* and *Staphylococcus epidermidis* were detected using an electrical protein array chip technology (Quiel et al. 2010). Although new techniques (such as tiling microarrays, protein biochips, and microfluidics) are gaining popularity, the reliability of results from cDNA GeneChip analyses has ensured that this widely used technique still has a place in gene expression studies.

In this study, we found than LAA had antimicrobial activity against *S. aureus* in planktonic or biofilm forms. Transcriptomic analysis showed the levels of genes encoding autolysis-associated proteins, cell wall proteins, pathogenic factors, protein synthesis, and enzymes involved in capsule synthesis were significantly altered in LAA-treated *S. aureus* biofilm and planktonic cells, and some of the microarray results were validated using real-time RT–PCR.

Materials and methods

Bacterial strains and materials

S. aureus ATCC 29213 was obtained from the China Medical Culture Collection Center. Twenty clinical samples of *S. aureus* (1078, 1018, 1524, 1628, 1932, 2001, 2005, 2027, 2233, 2441, 2484, 2750, 2796, 2871, 3076, 3218, 3198, 3212, 3701, and 3808) were isolated from the First Hospital of Jilin University, Changchun, China. LAA was purchased from Sigma-Aldrich (St Louis, MO, USA), and a stock solution was made in dimethyl sulfoxide (DMSO; Sigma-Aldrich). The final concentration of DMSO applied to culture systems was adjusted to 0.1 % (v/v), also in control groups.

Planktonic antimicrobial susceptibility testing

The minimum inhibitory concentrations (MICs) of LAA against the 21 *S. aureus* strains described above were determined according to the standard NCCLS procedures (National Committee for Clinical Laboratory Standards, also called CLSI 2005). The MIC was defined as the lowest concentration at which no visible growth was observed. The minimum bactericidal concentrations (MBCs) were identified as the lowest concentration to show no microbial growth on agar plates for 24 h at 37 °C. The assays were repeated in triplicate.

Establishment of microbial biofilms

Biofilms were established as previously described (Yu et al. 2008). To confirm slime production, microorganisms were cultured on Congo Red agar (CRA) (Yu et al. 2008), and the morphology of biofilms was observed under a microscope by staining with crystal violet, silver, and a LIVE/DEAD BacLight Bacterial Viability kit (Molecular Probes, Inc., Eugene, OR, USA).

Biofilm antimicrobial susceptibility testing

Biofilm antimicrobial susceptibility tests were performed as previously described (Yu et al. 2008). The minimum biofilm inhibition concentration (MBIC) was determined as the lowest concentration to show growth below or equal to that of the control. The minimum biofilm bactericidal concentration (MBBC) was identified as the lowest concentration demonstrating no bacterial growth.

Confocal laser scanning microscopy (CLSM)

Biofilm staining with a LIVE/DEAD BacLight Bacterial Viability kit (Invitrogen Molecular Probes, Inc., Eugene, OR, USA) was performed as previously described (Yu et al. 2008). CLSM images were collected using an Olympus FV1000 confocal laser scanning microscope (Olympus, Tokyo, Japan) with a ×40 objective lens. For detection of SYTO 9 (green channel), we used 488 nm excitation and 520 nm emission filter settings. For PI detection (red channel), we used 543 nm excitation and 572 nm emission filter settings. Image analyses and export were performed in Olympus Fluoview software version 1.7.3.0.

Growth curves

The growth curves of planktonic *S. aureus* ATCC 29213 were described in a previous study (Xing et al. 2012). The planktonic cell growth was spectrophotometrically monitored as the optical density (OD) at 600 nm, and the biofilm cell growth was spectrophotometrically monitored using an XTT assay at 540 nm, which was recorded at specific time intervals.

GeneChip analysis of planktonic and biofilm *S. aureus* with LAA treatment

The *S. aureus* strain ATCC 29213 was treated with LAA as previously described (Xing et al. 2012). In brief, *S. aureus* planktonic and biofilm cells were treated with LAA for 60 min at final concentrations of 1/2× MIC (2 μg mL^{-1}) and 4× MIBC (64 μg mL^{-1}), respectively. Construction of a genome-wide DNA microarray for *S. aureus*, RNA preparation, cDNA labeling, the GeneChip hybridization procedure, and microarray data processing were performed as described previously (Hutter et al. 2004). To select the differentially expressed genes, we used threshold values of ≥2 and ≤−2-fold change between three LAA-treated planktonic or biofilm samples and their controls. The false discovery rate (FDR) significance level was <5 %.

Quantitative real-time RT–PCR

RT–PCR was used to verify the microarray results. The RNA preparations from LAA-treated and control samples used in the microarray experiments were also used for RT–PCR follow-up studies. The cDNA was subjected to real-time PCR using the primer pairs listed in Table 1. RT–PCR was performed in triplicate using the 7000 Sequence Detection System (Applied Biosystems, USA) (Hutter et al. 2004).

Table 1 Primers used in real-time RT–PCR

Primer	Gene	Sequence
16S rRNA for		CGTGCTACAATGGACAATACAAA
16S rRNA rev		ATCTACGATTACTAGCGATTCCA
atl for	*atl*	TACCGTAACGGCGTAGGTCGT
atl rev	*atl*	CATAGTCGTGTGTGTGTACGA
sle1 for	*Sle1*	GTAGCCGTCCATCAACGAACT
sle1 rev	*Sle1*	CTATTGCTCGCAGCGTTACT
cidA for	*cidA*	CTTAGCCGGCAGTATTGTTG
cidA rev	*cidA*	TGAAGATAATGCAACGATAC
fnbB for	*fnbB*	TTCTGCATGACCTTCTGCA
fnbB rev	*fnbB*	AACTTGGAAAAATGGCGTTG
clfB for	*clfB*	TTGCCGCCATAAATGTGTTA
clfB rev	*clfB*	TCACCACAAACGATTTCCAA
lytM for	*lytM*	ATGCCAATGGAAGCGGCCA
lytM rev	*lytM*	TTCGCATGACCACTAGCTGT
isaA for	*isaA*	ACAGCTGCGTTGATTTGTTG
isaA rev	*isaA*	CTGCAGGTGCTACTGGTTCA
lrgA for	*lrgA*	CTGGTGCTGTTAAGTTAGGCG
lrgA rev	*lrgA*	GTGACATAGCCAGTACAAAT
lrgB for	*lrgB*	CGGTACAGTTGTAGCGTTATTA
lrgB rev	*lrgB*	AGTGCTAATCCTCGGGCAATA
cidB for	*cidB*	GACGTCATTGTAACGTTATTGC
cidB rev	*cidB*	TGAACTAAATGCACCGGATTC
RNAIII for	*RNAIII*	ATGAGTTGTTTAATTTTAAGAAT
RNAIII rev	*RNAIII*	CACTGTGTCGATAATCCA
agrA for	*agrA*	TGAAATTCGTAAGCATGACCC
agrA rev	*agrA*	CATCGCTGCAACTTTGTAGAC
capC for	*capC*	CATCCAGAGCGGAATAAAGC
capC rev	*capC*	GTGTTATGCGCATCTGAACC
capG for	*capG*	CAAGGCCTGAAATCATTCGT
capG rev	*capG*	CGCAATAATATTCCCCATCG

Results

Phenotype analysis of *S. aureus* biofilms and planktonic cells with LAA treatment

The most important step in biofilm research should be the construction of biofilm models. The ability of *S. aureus* to form biofilms was tested using the CRA method. As shown in Fig. 2a, biofilm producers generated black colonies. The morphology of the biofilms was further ascertained by microscopy using crystal violet, silver, and LIVE/DEAD bacterial viability kit staining (Fig. 2b–d).

In the assessment of the antimicrobial activities of LAA, the MIC and MBC values for the drug treatment against planktonic cells for all 21 strains ranged from 1 to 8 μg mL^{-1} and from 2 to 16 μg mL^{-1}, respectively; in biofilms, the MBIC and MBBC values for LAA treatment ranged from 8 to 64 μg mL^{-1} and ≥1,024 μg mL^{-1}, respectively (Table 2). This result showed that LAA effectively inhibited *S. aureus* planktonic cells and biofilm.

The effect of LAA against *S. aureus* ATCC 29213 biofilm grown on cover slides was observed by CLSM (Fig. 3a–b). With LAA treatment, Fig. 3 shows that there was a greatest decrease in Syto9 (green) staining (live cells) of biofilms, and there was a largest increase in PI (red) staining (dead cells) of biofilms, with changed drug concentrations (0–128 μg mL^{-1}) applied for the same time period. The thickness of biofilm after treatment of LAA was quantified by CLSM by one-way ANOVA analysis (Fig. 4). Figure 4 shows that the biofilm had become thinner with treatment of 0 to 128 μg mL^{-1} LAA for same times, which suggested that LAA prevented the biofilm from reaching the same thickness as the intreated control

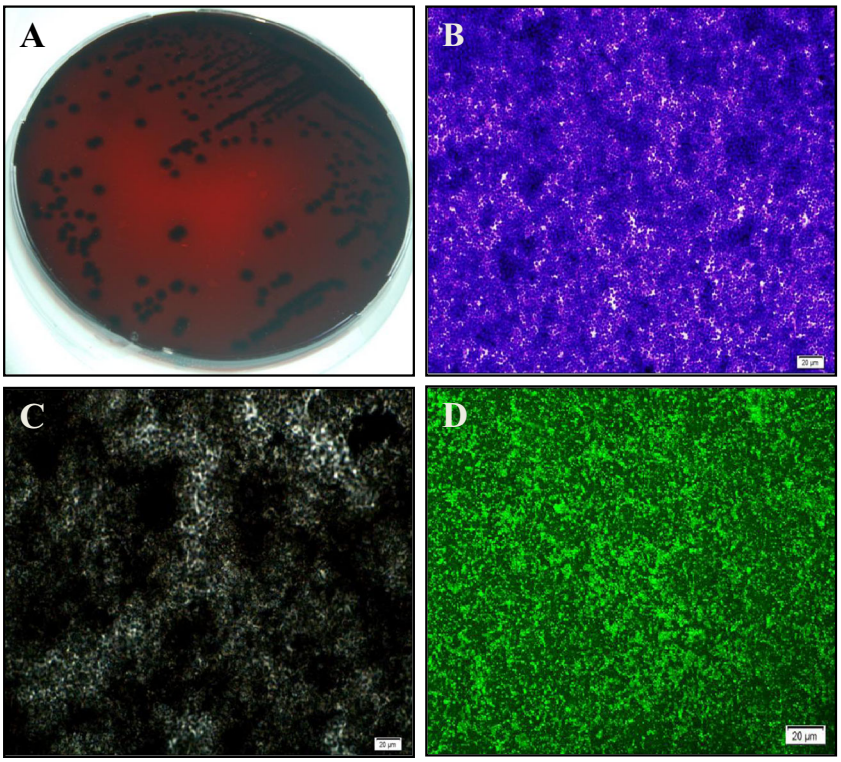

Fig. 2 The images of stained *S. aureus* 29213 biofilm grown on cover slide discs. **a** Ability to form biofilm was tested by the Congo red agar (CRA) method, which produced black colonies. **b** Two-day-old biofilm was washed gently three times with PBS and stained with 1 % crystal violet. **c** Two-day-old biofilm was washed gently three times with PBS with silver-staining. **d** Two-day-old biofilms were stained with the LIVE/DEAD bacterial viability kit. Live cells are stained with SYTO 9 and shown in *green*

Table 2 Antimicrobial activities of LAA against 21 *S. aureus* strains growing in planktonic and biofilm

Strain	Planktonic		Biofilm	
	MIC (μg/mL)	MBC (μg/mL)	MIBC (μg/mL)	MBBC (μg/mL)
S. aureus ATCC29213	2	4	16	>1,024
S. aureus 1078	4	8	32	>1,024
S. aureus 1018	4	16	64	>1,024
S. aureus 1524	4	8	32	>1,024
S. aureus 1628	4	8	16	>1,024
S. aureus 1932	8	16	16	>1,024
S. aureus 2001	2	4	16	>1,024
S. aureus 2005	2	4	8	>1,024
S. aureus 2027	4	8	16	>1,024
S. aureus 2233	8	16	64	>1,024
S. aureus 2441	4	8	64	>1,024
S. aureus 2484	4	8	32	>1,024
S. aureus 2750	2	8	16	>1,024
S. aureus 2796	2	4	16	>1,024
S. aureus 2871	4	8	64	>1,024
S. aureus 3076	8	16	64	>1,024
S. aureus 3218	8	16	64	>1,024
S. aureus 3198	2	4	32	>1,024
S. aureus 3212	4	8	16	>1,024
S. aureus 3701	4	8	32	>1,024
S. aureus 3808	1	2	8	1,024

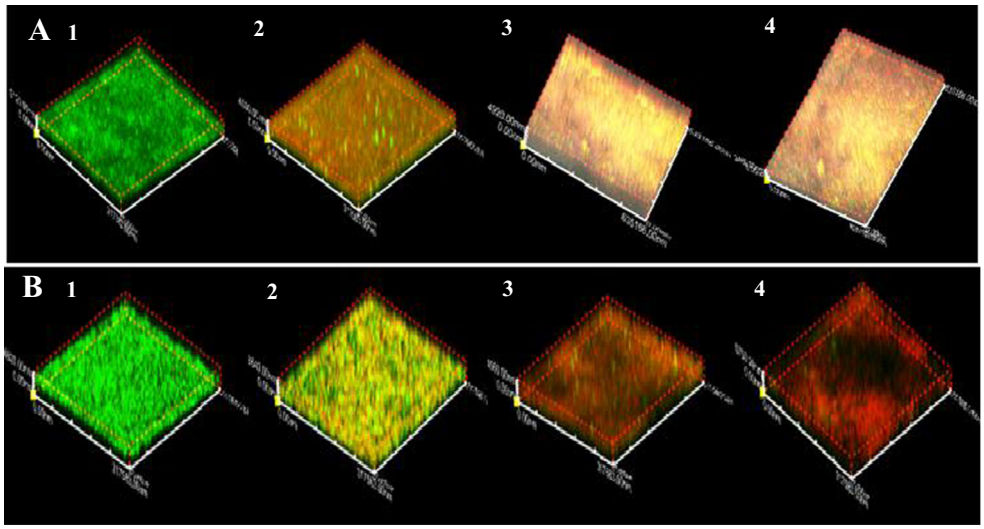

Fig. 3 Confocal laser scanning microscopy image of LIVE/DEAD®-stained *S. aureus* 29213 biofilm treated with LAA and grown on cover slide discs. **a** *S. aureus* 29213 were incubated at 37 °C for 24 h and treated with LAA. *1* 24 h growth control; *2* 1-day-old biofilm of *S. aureus* 29213 treated with 16 μg mL^{-1} LAA; *3* 1-day-old biofilm of *S. aureus* 29213 treated with 64 μg mL^{-1} LAA; *4* 1-day-old biofilm of *S. aureus* 29213 treated with 128 μg mL^{-1} LAA. **b** *S. aureus* 29213 were incubated at 37 °C for 48 h and treated with LAA. *1* 48 h growth control; *2* 2-day-old biofilm of *S. aureus* 29213 treated with 16 μg mL^{-1} LAA; *3* 2-day-old biofilm of *S. aureus* 29213 treated with 64 μg mL^{-1} LAA; *4* 2-day-old biofilm of *S. aureus* 29213 treated with 128 μg mL^{-1} LAA

groups (only containing 0.1 % v/v DMSO). As previously shown, this concentration of DMSO did not interfere with the testing system (You et al. 2013). The results highlighting the damage caused to the biofilm by LAA treatment was time dependent and concentration dependent.

In order to choose the suitable low LAA concentration against the *S. aureus* strain to be used in the transcription analysis, we determined growth curves of *S. aureus* ATCC 29213 with LAA. The growth curves of *S. aureus* ATCC 29213 with LAA treatment showed the OD of bacterial cultures increased steadily with 1, 2, and 2.5 μg mL^{-1} LAA treatment, but bacteria almost did not grow at 3 and 4 μg mL^{-1} of LAA (Fig. 5a). Compared with the control, the growth curve of biofilm showed that the optical density of cells decreased steadily with the addition of sub-MBBC concentrations of LAA (Fig. 5b). The results showed that there was a dose-dependent inhibitory activity of LAA against *S. aureus* and a low LAA concentration displayed a bacteriostatic action against *S. aureus* planktonic cells and biofilm.

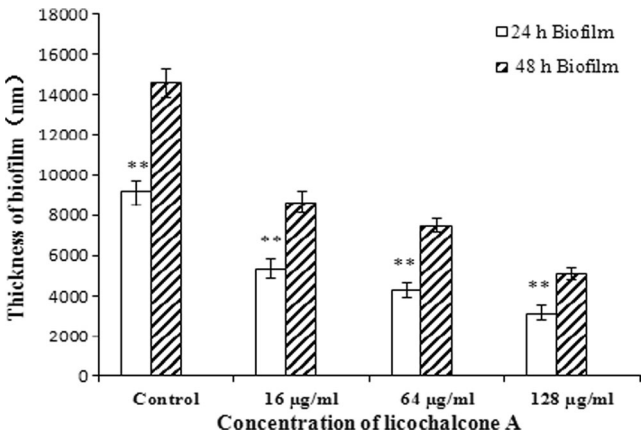

Fig. 4 The quantification of biofilm thickness (from *S. aureus* 29213 treated with LAA) by confocal laser scanning microscopy. Comparisons of mean values from three experiments were statistically evaluated by analysis of variance, followed by one-way ANOVA analysis. $^{**}p<0.01$ significant difference between 1-day-old biofilm and 2-day-old biofilm treated with LAA

Total alteration of gene transcription responses to LAA exposure

To study the effects of low LAA concentrations on the *S. aureus* ATCC 29213 strain, we used a microarray to examine the transcription of planktonic cells and biofilm at a sub-MIC concentration (1/2× MIC, 2 μg mL^{-1}) and a sub-MBBC concentration (4× MIBC, 64 μg mL^{-1}) of LAA at the 60 min time point. The GeneChip analysis of LAA against *S. aureus* planktonic cells and biofilm revealed that 693 and 817 genes were differentially regulated, respectively. Of these, the expression levels of 375 and 355 genes were markedly elevated in planktonic cells and biofilm, respectively, and 318 and 462 genes were significantly decreased. The distribution of LAA-responsive genes and their biological roles in planktonic cells and biofilm are shown in Figs. 5 and 6, respectively (not including hypothetical proteins and unknown genes). The microarray-related data were submitted to Gene Expression Omnibus under accession number GSE58938. A relatively complete list of all differentially expressed genes from bacteria treated by LAA can be found in the supplementary Tables S1 and S2 in the Supplementary Material.

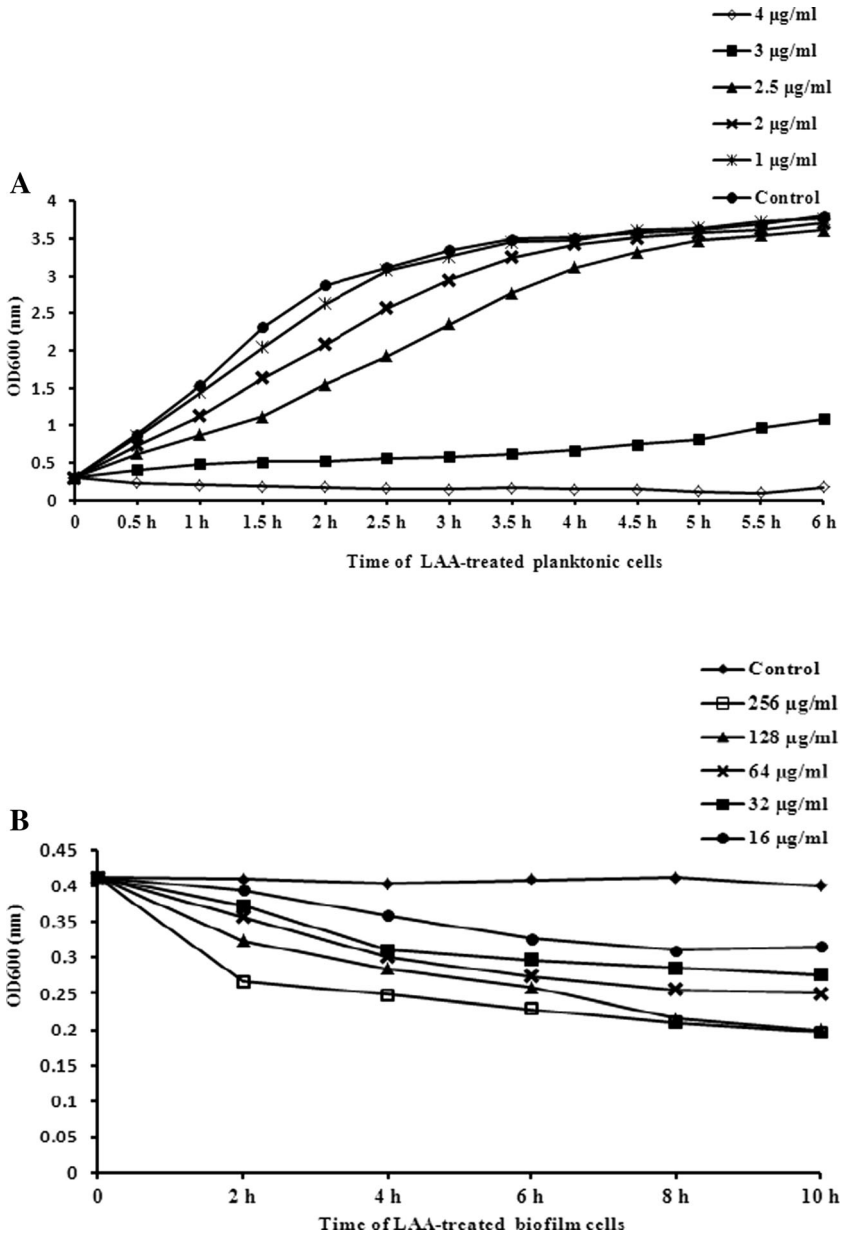

Fig. 5 Growth curve for *S. aureus* ATCC 29213 treated with LAA

Expression levels of autolysis-associated genes following treatment with LAA

To determine the effect of LAA against *S. aureus* planktonic cells and biofilm, autolysis genes were analyzed using the GeneChip. In planktonic cells, the main autolysin gene *lytM* was significantly down-regulated by 4.0-fold. The transcript levels of the negative regulator of autolysis *lrgB* was markedly increased by 5.1-fold, while the levels of the positive regulators, *agrA*, *lytR*, and *RNAIII*, were significantly decreased. The down-regulation of *lytM* and *agrA* and up-regulation of *lrgB* expressions were confirmed by RT–PCR (Table 3). Recent reports have demonstrated that extracellular DNA (eDNA), an essential matrix molecule in *S. aureus* biofilm, is released in cell autolysis (Otto 2012). The levels of the major autolysin genes *atl*, *slel*, and *lytM* were markedly down-regulated within LAA-treated biofilm. In *S. aureus*, cell death and lysis are controlled by the *cid* and *lrg* operons. The *cidA* gene encodes a murein hydrolase regulator that promotes cell lysis during biofilm development, whereas the *lrg* operon inhibits cell lysis (Sadykov and Bayles 2012). The transcript levels of the positive regulator *cidA* markedly decreased 2.6-fold, but the transcript levels of negative regulators of autolysis *lrgA* and *lrgB* notably increased 3.3- and 4.4-fold, respectively. In addition, the expression of *agrA*, a positive regulatory gene, was also down-regulated 1.5-fold. Surprisingly, transcription of the autolysin genes *cidBC* was significantly up-regulated. RT–PCR confirmed the decreased levels of *atl*, *sle1*, *cidA*,

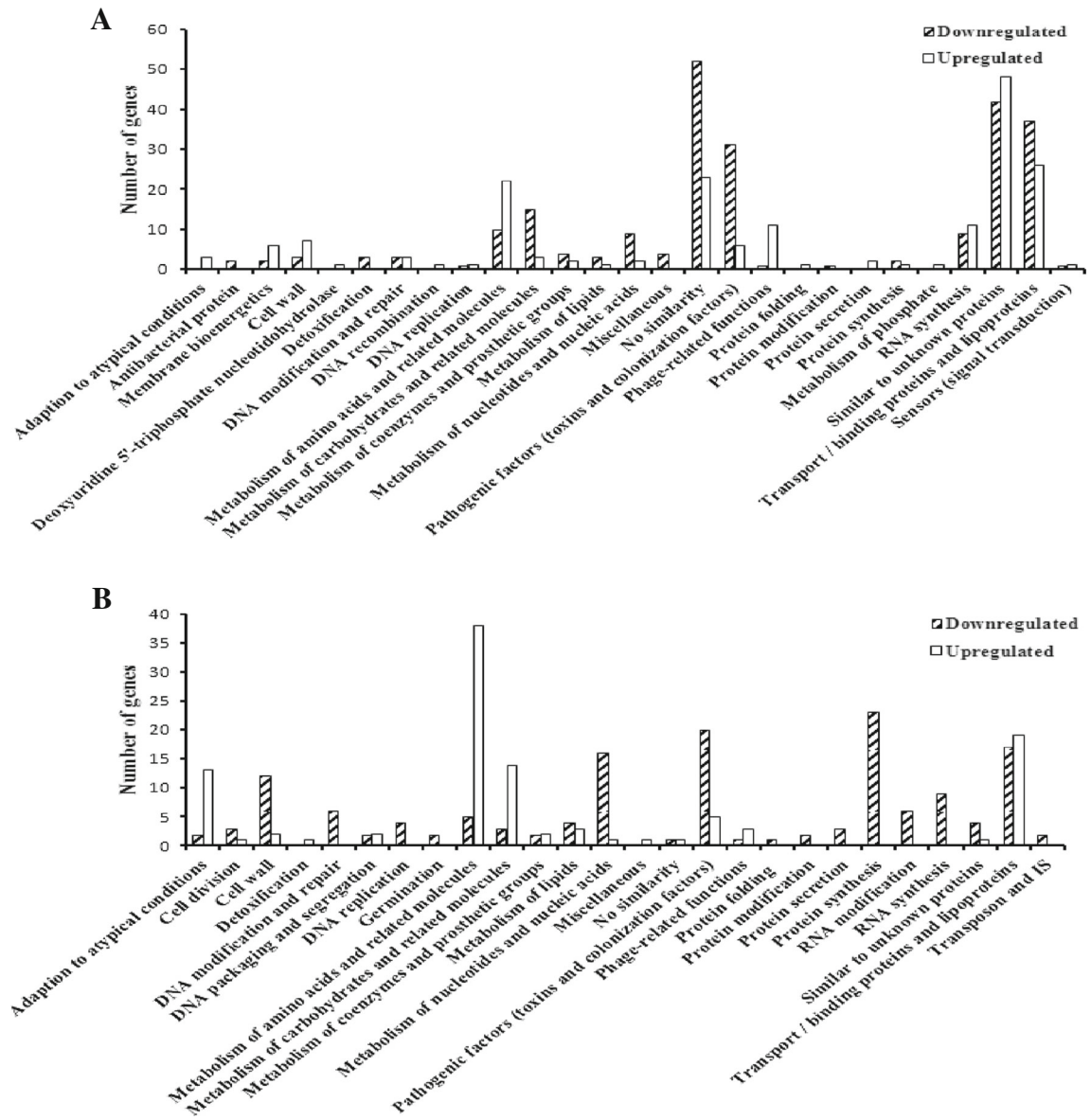

Fig. 6 Functional classification. **a** Differentially expressed genes from *S. aureus* 29213 planktonic cells treated with LAA (2 μg mL^{-1}) are grouped by functional classification. The differentially regulated genes were divided into 27 functional categories. The number of genes up-regulated and down-regulated for each functional group is represented. **b** Differentially expressed genes from *S. aureus* 29213 biofilm treated with LAA (64 μg mL^{-1}) are grouped by functional classification. The differentially regulated genes were divided into 26 functional categories. The number of genes up-regulated and down-regulated for each functional group is represented

lytM, and *agrA* and observed increases in *lrgA*, *lrgB*, and *cidB* transcription levels (Table 4). This result suggests that LAA reduces *S. aureus* biofilm production by inhibiting autolysis in vitro, similar to our previous report in which magnolol reduced *S. aureus* biofilm production in vitro (Wang et al. 2011).

Influence of LAA on capsule synthesis and cell wall synthesis genes

To investigate whether LAA could undermine the protective barrier of *S. aureus* planktonic cells and biofilm, capsule synthesis and cell wall synthesis genes were analyzed. GeneChip analysis data showed that the expression levels of capsule synthesis genes *betA*, *gbsA*, and SA2175 were increased in planktonic cells (Table 3). Capsule synthesis has an important role not only in planktonic cells but also in the adherence and formation stages of biofilm formation. Compared with planktonic cells, levels of genes (*capABCDEFGHIJKLMNOP*) encoding the capsule polysaccharide synthesis enzymes were significantly up-regulated, but the levels of *cap1A* and *cap1C* were down-regulated in biofilm. The levels of *asp23*, which codes for alkaline shock protein 23, was increased. This result

Table 3 Selected *S. aureus* genes that displayed altered expression after LAA treatment of planktonic cells, as determined by microarray analysis and real-time RT–PCR

Type of proteins	N315 open reading frame	Name	Product	Fold change in expression measured by:	
				Microarray analysis	Real-time RT–PCR
Cell wall-associated proteins	SA0265	*lytM*	Peptidoglycan hydrolase	−4.0	−7.4
	SA0205	Hypothetical protein	Protein similar to lysostaphin precursor	14	
	SA0522	Hypothetical protein	Protein similar to poly (glycerol-phosphate) α-glucosyltransferase	−2.2	
	SA0523	Hypothetical protein	Protein similar to poly (glycerol-phosphate) α-glucosyltransferase	−2.3	
	SA0598	*pbp4*	Penicillin binding protein 4	2.7	
	SA1691	*sgtB*	Protein similar to penicillin-binding protein 1A/1B	6.7	
	SA1926	*murZ*	UDP-*N*-acetylglucosamine 1-carboxylvinyl transferase 2	3.4	
	SA1935	*hmrA*	Similar to amidase (HmrA)	2.1	
	SA2288	*gtaB*	UTP-glucose-1-phosphate uridyltransferase	2.1	
	SA2480	*drp35*	Drp35	2.4	
Autolysis related proteins	SA0253	*lrgB*	Holin-like protein LrgB	5.1	12.8
	SA1844	*agrA*	Accessory gene regulator A	−2.5	−2.9
	SA0251	*lytR*	Two-component response regulator	−2.3	
Pathogenic factors (toxins and colonization factors)	SA0091	*plc*	1-Phosphatidylinositol phosphodiesterase precursor	−8.5	
	SA0102		Myosin-crossreactive streptococcal antigen homologue	−2.0	
	SA0107	*spa*	Immunoglobulin G binding protein A precursor	−8.5	
	SA0270	Hypothetical protein	Protein similar to secretory antigen Precursor SsaA	−4.7	
	SA0276	Hypothetical protein	Protein similar to diarrheal toxin	−4.6	
	SA0309	*geh*	Glycerol ester hydrolase	−7.0	
	SA0393	*set15*	Exotoxin 15	−4.6	
	SA0519	*sdrC*	Ser-Asp rich fibrinogen-binding, bone sialoprotein-binding protein	−3.3	
	SA0521	*sdrE*	Ser-Asp rich fibrinogen-binding, bone sialoprotein-binding protein	−2.4	
	SA0587		Lipoprotein, streptococcal adhesin PsaA homologue	−2.7	
	SA0610	Hypothetical protein	Protein similar to lipase LipA	−3.1	
	SA0746	*nuc1*	Staphylococcal nuclease	−8.2	
	SA0900	*sspB*	Cysteine protease precursor	−2.8	
	SA0901	*sspA*	Serine protease; V8 protease; glutamyl endopeptidase	−3.3	
	SA0909	*fmtA*	FmtA, autolysis, and methicillin-resistant-related protein	2.1	
	SA0977	*isdA*	Cell surface protein	−2.0	
	SA1000	Hypothetical protein	Protein similar to fibrinogen-binding protein	−2.5	
	SA1003	Hypothetical protein	Protein similar to fibrinogen-binding protein	−3.7	
	SA1004	Hypothetical protein	Protein similar to fibrinogen-binding protein	−2.8	
	SA1007		α-Hemolysin precursor	−3.0	
	SA1577	Hypothetical protein	Protein similar to FmtB protein	2.2	
	SA1627	*splF*	Serine protease SplF	−2.1	
	SA1629	*splC*	Serine protease SplC	−2.1	
	SA1631	*splA*	Serine protease SplA	−2.5	
	SA1758	*sak*	Staphylokinase precursor	−4.3	
	SA1812	Hypothetical protein	Protein similar to synergohymenotropic toxin precursor	−4.8	

Table 3 (continued)

Type of proteins	N315 open reading frame	Name	Product	Fold change in expression measured by:	
				Microarray analysis	Real-time RT–PCR
	SA1813	Hypothetical protein	Protein similar to leukocidin chain lukM precursor	−8.7	
	SA1898	Hypothetical protein	Protein similar to SceD precursor	8.2	
	SA2003	*hysA*	Hyaluronate lyase precursor	−5.7	
	SA2097	Hypothetical protein	Protein similar to secretory antigen precursor SsaA	2.8	
	SA2206	*sbi*	IgG-binding protein SBI	−8.9	
	SA2208	*hlgC*	γ-hemolysin component C	−2.3	
	SA2290	*fnbB*	Fibronectin-binding protein homolog	2.1	3.5
	SA2423	*clfB*	Clumping factor B	−2.0	−4.6
	SA2463	*lip*	Triacylglycerol lipase (EC 3.1.1.3) precursor	−2.3	
	SAS065	*RNAIII*	δ-Hemolysin	−2.5	−5.7
Adaptation to atypical conditions	SA2175	Hypothetical protein	Protein similar to small heat shock protein	2.0	
	SA2405	*betA*	Choline dehydrogenase	5.8	
	SA2406	*gbsA*	Glycine betaine aldehyde dehydrogenase gbsA	5.7	
Protein synthesis	SA0009	*sers*	Seryl-tRNA synthetase	−2.8	
	SA1036	*ileS*	Ile-tRNA synthetase	−2	
	SA1579	*leuS*	Leucyl-tRNA synthetase	3.4	

This GeneChip includes N315, Mu50, NCTC 8325, and COL

shows that biofilm production was affected by LAA treatment. RT–PCR confirmed the up-regulation of *capC* and *capG* levels (Table 4). The cell wall is important to resist damage from external factors. In planktonic cells treated with LAA, the transcript levels of cell wall synthesis genes SA0522 and SA0523 were significantly decreased, but the transcript levels of *pbp4*, *sgtB*, *murZ*, *hmrA*, *gtaB*, *drp35*, and SA0205 were markedly increased (Table 3). Resch et al. (2005) suggested that processes related to cell wall synthesis also play a key role in biofilm persistence. In biofilm cells challenged with LAA, the expression levels of cell wall synthesis genes *lytM*, *lytH*, *tagA*, *tag*, *pbp3*, *pbp4*, *llm*, *dltA*, *dltC*, *murE*, *uppS*, and *fmhA* were significantly down-regulated, but the transcript levels of *drp35* and *fmtB* were significantly increased by 3.6- and 2.1-fold, respectively (Table 4). This suggests that bacterial activation of the stress response in an attempt to withstand the antimicrobial challenge was also inhibited by LAA.

Effect of LAA on protein synthesis genes

We further observed the effects of LAA on protein synthesis genes in *S. aureus* planktonic cells and biofilm. The expression levels of protein synthesis genes *serS* and *ileS* were significantly decreased, but transcript levels of *leuS*, encoding leucyl-tRNA synthetase, were up-regulated 3.4-fold (Table 3) in planktonic cells. As shown in Table 4, nine genes encoding ribosomal proteins in biofilm cells after LAA treatment were down-regulated by 2.0- to 4.3-fold. At the same time, expression of the initiation factor gene *infC* and the genes of the transcription factors EF and T were notably decreased; moreover, the expression levels of protein synthesis genes *fmt*, *gatA*, *gatC*, *glyS*, *hisS*, *lepA*, *pheS*, *prfA*, *prfC*, *pth*, and *tyrS* were significantly down-regulated. This result showed that LAA inhibited *S. aureus* planktonic cells or biofilm by inhibiting genes associated with protein synthesis.

Expression alteration of virulence-associated genes after LAA treatment

The presence of LAA also reduced the level of virulence-associated genes in *S. aureus*. GeneChip analysis data showed significant down-regulation of *plc*, *spa*, *geh*, *set15*, *sdrCE*, *sbi*, *hlgC*, *clfB*, *lip*, *RNAIII*, *hysA*, *nuc1*, *splAFC*, *sak*, SA0102, SA0587, SA1007, and eight additional hypothetical virulence genes in planktonic cells after LAA treatment. Furthermore, the levels of *sspA* and *sspB* were markedly decreased (2.8- and 3.3-fold, respectively). The *S. aureus* V8 protease, encoded by *sspA*, is a major serine protease (Rice et al. 2001). This serine protease affects autolytic activity via the expression of the

Table 4 Selected *S. aureus* genes that displayed altered expression after LAA treatment of biofilm, as determined by microarray analysis and real-time RT–PCR

Type of proteins	N315 open reading frame	Name	Product	Fold change in expression measured by:	
				Microarray analysis	Real-time RT–PCR
Cell wall	SA0265	*lytM*	Peptidoglycan hydrolase	−4.7	
	SA0592	*tagA*	Teichoic acid biosynthesis protein	−3.1	
	SA0594	*tagG*	Teichoic acid translocation permease protein	−2.1	
	SA0598	*pbp4*	Penicillin-binding protein 4	−2.8	
	SA0702	*llm*	Lipophilic protein affecting bacterial lysis rate and methicillin resistance level	−3.8	
	SA0793	*dltA*	D-alanine–D-alanyl carrier protein ligase	−6.2	
	SA0795	*dltC*	D-alanyl carrier protein	−2.3	
	SA0876	*murE*	UDP-*N*-acetylmuramoylalanyl-D-glutamate–2, 6-diaminopimelate ligase	−2.6	
	SA1090	*lytN*	LytN protein	1.4	
	SA1103	*uppS*	Undecaprenyl pyrophosphate synthetase	−2.4	
	SA1381	*pbp3*	Penicillin-binding protein 3	−2.1	
	SA1458	*lytH*	*N*-acetylmuramoyl-L-alanine amidase	−2.3	
	SA2199	*fmhA*	FmhA protein	−3.1	
	SA2480	*drp35*	Drp35	3.6	
Autolysis and biofilm-related proteins	SA0905	*atl*	Autolysin (*N*-acetylmuramyl-L-alanine amidase and endo-b-*N*-acetylglucosaminidase)	−3.7	−9.1
	SA0423	*sle1*	*N*-Acetylmuramyl-L-alanine amidase	−3.5	−8.9
	SA2329	*cidA*	Protein similar to transcriptional regulator	−2.6	−4.1
	SA0265	*lytM*	Peptidoglycan hydrolase	−4.7	−14.8
	SA1090	*lytN*	LytN protein	1.4	
	SA0252	*lrgA*	Holin-like protein LrgA	3.3	8.7
	SA0253	*lrgB*	Antiholin-like protein LrgB	4.4	12.7
	SA1248	*arlR*	Truncated (putative response regulator ArlR)	−1.1	
	SA0573	*sarA*	Staphylococcal accessory regulator A	1.2	
	SA2328	*cidB*	Conserved hypothetical protein	2.4	3.2
	SA2327	*cidC*	Protein similar to pyruvate oxidase	2.7	
	SA1844	*agrA*	Accessory gene regulator A	−1.5	−1.8
	SA0251	*lytR*	Two-component response regulator	−1.1	
	SA0250	*lytS*	Two-component sensor histidine kinase	−1.5	
	SA0641	*mgrA*	Transcriptional regulator MgrA	−1.3	
	SA2459	*icaA*	Intercellular adhesion protein A	−1.8	
	SA2460	*icaD*	Intercellular adhesion protein D	−1.8	
	SA2461	*icaB*	Intercellular adhesion protein B	−1.5	
	SA2462	*icaC*	Intercellular adhesion protein C	−1.2	
Pathogenic factors (toxins and colonization factors)	SA0222	*coa*	Staphylocoagulase precursor	−5.0	
	SA0393	*set15*	Exotoxin 15	−3.3	
	SA0519	*sdrC*	Ser-Asp rich fibrinogen-binding, bone sialoprotein-binding protein	−2.1	
	SA0521	*sdrE*	Ser-Asp rich fibrinogen-binding, bone sialoprotein-binding protein	−6.5	
	SA1267	*ebhA*	Protein similar to streptococcal adhesin emb	2.9	
	SA1268	*ebhB*	Protein similar to streptococcal adhesin emb	2.3	
	SA1643	*sen*	Enterotoxin SeN	−2.1	
	SA1644	*yent2*	Enterotoxin YENT2	−2.5	
	SA1645	*yent1*	Enterotoxin Yent1	−2.3	

Table 4 (continued)

Type of proteins	N315 open reading frame	Name	Product	Fold change in expression measured by:	
				Microarray analysis	Real-time RT–PCR
	SA1646	*sei*	Extracellular enterotoxin type I precursor	−2.1	
	SA1647	*sem*	Enterotoxin SEM	−2.4	
	SA1648	*seo*	Enterotoxin SeO	−4.4	
	SA1750	*mapW* (truncated)	Truncated map-w protein	−2.1	
	SA1751	*mapW* (truncated)	Truncated map-w protein	−4.9	
	SA1752	*hlb* (truncated)	Truncated β-hemolysin	−2.4	
	SA1964	*fmtB* (*mrp*)	FmtB protein	2.1	
	SA2093	*ssaA*	Secretory antigen precursor SsaA homolog	−5.9	
	SA2206	*sbi*	IgG-binding protein SBI	−9.0	
	SA2209	*hlgB*	γ-hemolysin component B	2.1	
	SA2290	*fnbB*	Fibronectin-binding protein homolog	−6.9	−4.9
	SA2356	*isaA*	Immunodominant antigen A	−4.5	−3.3
	SA2423	*clfB*	Clumping factor B	−3.6	−2.4
	SA2459	*icaA*	Intercellular adhesion protein A	−1.8	
	SA2460	*icaD*	Intercellular adhesion protein D	−1.8	
	SA2463	*lip*	Triacylglycerol lipase (EC 3.1.1.3) precursor	4.6	
Adaptation to atypical conditions	SA0144	*capA*	Capsular polysaccharide synthesis enzyme Cap5A	9.2	
	SA0145	*capB*	Capsular polysaccharide synthesis enzyme Cap5B	8.1	
	SA0146	*capC*	Capsular polysaccharide synthesis enzyme Cap8C	8.0	13.2
	SA0147	*capD*	Capsular polysaccharide synthesis enzyme Cap5D	6.2	
	SA0148	*capE*	Capsular polysaccharide synthesis enzyme Cap8E	4.6	
	SA0149	*capF*	Capsular polysaccharide synthesis enzyme Cap5F	4.9	
	SA0150	*capG*	Capsular polysaccharide synthesis enzyme Cap5G	5.1	7.4
	SA0151	*capH*	Capsular polysaccharide synthesis enzyme *O*-acetyl transferase Cap5H	5.3	
	SA0152	*capI*	Capsular polysaccharide synthesis enzyme Cap5I	4.8	
	SA0153	*capJ*	Capsular polysaccharide synthesis enzyme Cap5J	3.8	
	SA0154	*capK*	Capsular polysaccharide synthesis enzyme Cap5K	3.8	
	SA0155	*capL*	Capsular polysaccharide synthesis enzyme Cap5L	3.1	
	SA0156	*capM*	Capsular polysaccharide synthesis enzyme Cap5M	3.0	
	SA0157	*capN*	Capsular polysaccharide synthesis enzyme Cap5N	2.8	
	SA0158	*capO*	Capsular polysaccharide synthesis enzyme Cap8O	2.4	
	SA0159	*capP*	Capsular polysaccharide synthesis enzyme Cap5P	2.1	
	SA1984	*asp23*	Alkaline shock protein 23, ASP23	2.1	
	SA2455	*cap1C*	Capsular polysaccharide biosynthesis, capC	−2.0	
	SA2457	*cap1A*	Capsular polysaccharide biosynthesis, capA	−2.3	
Protein synthesis	SA1359	*efp*	Translation elongation factor EF-P	−2.6	
	SA1059	*fmt*	Methionyl-tRNA formyltransferase	−2.8	
	SA1716	*gatA*	Glutamyl-tRNA Gln amidotransferase subunit A	−4.4	
	SA1717	*gatC*	Glutamyl-tRNA Gln amidotransferase subunit C	−4.2	
	SA1394	*glyS*	Glycyl-tRNA synthetase	−3.1	
	SA1457	*hisS*	Histidyl-tRNA synthetase	−2.8	
	SA1504	*infC*	Translation initiation factor IF-3 infC	−2.5	
	SA1413	*lepA*	GTP-binding protein	−2.6	
	SA0985	*pheS*	Phe-tRNA synthetase α chain	−2.8	
	SA1920	*prfA*	Peptide chain release factor 1	−5.0	

Table 4 (continued)

Type of proteins	N315 open reading frame	Name	Product	Fold change in expression measured by:	
				Microarray analysis	Real-time RT–PCR
	SA0877	*prfC*	Peptide chain release factor 3	−2.0	
	SA0460	*pth*	Peptidyl-tRNA hydrolase	−3.0	
	SA0496	*rplA*	50S ribosomal protein L1 (BL1)	−2.5	
	SA0498	*rplL*	50S ribosomal protein L7/L12	−2.4	
	SA1502	*rplT*	50S ribosomal protein L20	−4.3	
	SA1922	*rpmF*	Ribosomal protein L31	−2.0	
	SAS033	*rpmF*	Ribosomal protein L32	−2.0	
	SA1503	*rpmI*	50S ribosomal protein L35	−2.2	
	SA1099	*rpsB*	30S ribosomal protein S2	−3.5	
	SA1081	*rpsP*	30S ribosomal protein S16	−3.4	
	SA1414	*rpsT*	30S ribosomal protein S20 (BS20)	−2.2	
	SA1100	*tsf*	Elongation factor TS (EF-TS)	−2.2	
	SA1550	*tyrS*	Tyrosyl-tRNA synthetase	−3.2	

This GeneChip includes N315, Mu50, NCTC 8325, and COL

autolysin gene *atl* and the proteolytic maturation of the cysteine protease SspB coded by *sspB* (Komatsuzawa et al. 2001). However, the levels of expression of *fnbB*, *fmtA* SA1577, SA1898, and SA2097 were significantly up-regulated in LAA-treated biofilm. RT–PCR confirmed the observed decreases in *clfB* and *RNAIII* levels and the observed increase in *fnbB* levels (Table 3). These data suggest that LAA could suppress planktonic cells by affecting virulence-associated genes.

In staphylococci, toxins are responsible for its lethal pathogenicity. Until now, 20 serologically different staphylococcal superantigens have been described, including TSST-1, the staphylococcal enterotoxins (enterotoxins A to E and G to J), and the staphylococcal enterotoxin-like toxins (Smith et al. 2010). As shown in Table 4, the levels of toxin genes *set15*, *sen*, *yent2*, *yent1*, *sei*, *sem*, *seo*, and truncated (*hlb*) were notably decreased in biofilm cells. In *S. aureus*, virulence gene expression is mainly regulated by at least seven two-component systems (TCSs) (ArlRS, SaeRS, AgrAC, SrrAB, LytRS, YycFG, and VraRS), the DNA-binding protein SarA, the SarA family (SarS, SarR, SarU, SarT, SarV, MgrA, and TcaR) (Novick 2003), and an alternative sigma factor (SigB). The accessory gene regulator *agr* suppresses the post-exponential phase expression of cell surface binding proteins and enhances the expression of secreted proteins. Unlike *agr*, the *sar* locus activates the synthesis of both extracellular and surface-bound proteins in *S. aureus* (Cheung and Zhang 2002) The two-component system *lytSR* is involved in the regulation of peptidoglycan hydrolases, and *lrgA* and *lrgB* are positively regulated by *lytSR* (Sadykov and Bayles 2012). Recent reports have revealed that *lytSR* and *lrgAB* are down-regulated by ArlRS (Liang et al. 2005). The levels of the response regulators *agrA*, *lytR*, *lytS*, *mgrA*, and *arlR* were reduced after LAA treatment in biofilm. In addition, the expression levels of the virulence genes *coa*, *sdrCE*, *mapW* (truncated), *mapW* (truncated), *hlb* (truncated), *sbi*, and *isaA* were markedly down-regulated, while *icaA* and *icaD* were both slightly down-regulated (1.8-fold) in biofilm after LAA exposure. This result showed that LAA could inhibit the expression of biofilm virulence-associated genes.

Virulence genes have been implicated in biofilm formation. The attachment of *S. aureus* cells to abiotic or biological surfaces is the key step in biofilm development, and this is mediated by the protein adhesins on the microbial surface (Smith et al. 2010). The well-characterized adhesins in *S. aureus* are the structurally related fibrinogen binding proteins ClfA and ClfB, fibronectin-binding proteins FnbA and FnbB, and the collagen adhesin Can (Wann et al. 2000). We observed that the virulence gene *fnbB* (encoding FnbB) is down-regulated 6.9-fold in biofilm cells treated with LAA. Previous reports have shown that expression of the *fnb* genes is enhanced by SarA (encoded by *sarA*) through promoter binding in vitro (Chien et al. 1999). The level of *sarA* decreased 5.9-fold in the presence of LAA, and this may have directly influenced the transcription of *fnbB* but not *fnbA*. The level of the virulence gene *clfB*, which encodes ClfB, a protein that mediates the adherence of *S. aureus* to immobilized and soluble fibrinogen (Entenza et al. 2000), was decreased 3.6-fold in biofilm. However, the levels of expression of *ebhAB*, *fmtB* (*mrp*), *hlgB*, and *lip* were significantly up-regulated.

Real-time RT–PCR confirmed the decreased *fnbB*, *isaA*, and *clfB* levels (Table 4). This result showed that LAA might affect *S. aureus* biofilm by inhibiting the initial adhesion step.

Discussion

In a biofilm, a microbial community is attached to a surface and embedded in a self-produced matrix composed of extracellular polymeric substances. The bacterial cells grown in a biofilm have an increased resistance to grazing, desiccation, and antimicrobial agents compared to planktonic cells. In addition, the resistance of *S. aureus* biofilms were impacted of environmental conditions or sessile cells membrane fluidity with drug treatment (e.g., disinfectants) (Abdallah et al. 2014). In this study, we observed lawn-like bacteria that crumbled and overspread on cover slide discs visualized with crystal violet, silver, or SYTO 9-staining (Fig. 2b–d). These results indicated the successful formation of *S. aureus* biofilms in vitro. Previous reports have shown that LAA has antibacterial activities against *S. aureus*. We found that LAA not only strongly inhibited bacterial activity against 21 microorganisms tested as planktonic cells but also against biofilm (Table 2). We further corroborated the inhibitory activity of LAA against *S. aureus* biofilm by the CLSM (Fig. 3a–b).

Our previous reports have revealed that the compound concentration is of crucial importance for data quality in transcriptome analysis, and the best results were obtained with sub-inhibitory concentrations (concentrations that are just low enough not to affect the growth of the organism) (Yu et al. 2008). We chose the 60 min time point for drug treatment to avoid confounding secondary drug effects, and it has been claimed that compounds should be at a low concentration to lessen the effect on the growth of the organism and obtain optimal microarray results (Hutter et al. 2004).

When the *S. aureus* planktonic cells and biofilm were treated by LAA, the transcriptome analysis showed the expression of genes coding autolysis-associated protein, cell wall-associated protein, pathogenic factors, capsule synthesis, and protein synthesis were significantly regulated. Some of the same pathways were affected by LAA exposure in both *S. aureus* biofilm and planktonic cells, but most concrete genes were differentially regulated in biofilm compared with planktonic cells.

In staphylococcal biofilm, PIA is an adhesive molecule, and it is synthesized by the *icaADBC*-encoded proteins; the *ica* operon appears to be present in all *S. aureus* strains (Otto 2012). The *ica* genes, required for adhesion and biofilm formation, are up-regulated only at the beginning of biofilm formation, rather than during maturation and persistence (Resch et al. 2005). In this study, the microarray of LAA-treated *S. aureus* biofilm results showed that the levels of *icaADBC* decreased less than 2-fold. However, we used LAA to treat a mature 2-day biofilm; thus, the level of *ica* was only slightly changed by LAA treatment in this study.

In planktonic cells, the expression of *fnbB* was enhanced 2.1-fold, and *clfB* was decreased 2.0-fold after LAA treatment. Blickwede et al. (2005) reported similar findings that *fnbB* was up-regulated 2.0-fold in the *S. aureus* strain Newman exposed to a sub-lethal concentration clindamycin. At the same time, LAA treatment of biofilm appeared to increase the expression of the adhesin genes *ebhA* and *ebhB*. Their up-regulation was also reported in a study on tigecycline influence on the expression of virulence factors in biofilm cells in methicillin-resistant *S. aureus* (Smith et al. 2010). This result suggests that the binding capacity of *S. aureus* may be altered during drug-induced stress.

In addition, LAA also markedly inhibited the expression of autolysis-associated genes. The genes *fmtA* and *fmtB* were recently described as positive regulators of autolysis (Manna et al. 2004). Surprisingly, LAA treatment increased the level of *fmtB* by 2.1-fold in *S. aureus* planktonic cells, and the levels of *fmtA* were increased by 2.1-fold in *S. aureus* biofilm cells.

The majority of *S. aureus* isolates produce either a serotype 5 or 8 capsular polysaccharide that has been shown to enhance bacterial virulence. The expression of the *cap5*, *cap8*, and *asp23* genes was up-regulated in LAA-challenged biofilms. In addition, LAA-treated *S. aureus* planktonic cells also showed up-regulated capsular gene (SA2175, SA2405, SA2406) expressions. Other studies have also shown that exposure of sensitive *S. aureus* to sub-inhibitory levels of vancomycin leads to the elevation of capsular gene expression. This up-regulation of genes may be part of the bacterial stress response, an attempt to withstand the antimicrobial challenge. However, LAA treatment significantly down-regulated the levels of the *cap1A* and *cap1C* gene expression. Thus, the reduction in the levels of capsule gene expression may be a consequence of the decrease in virulence factors in the presence of LAA, which renders the organism easily cleared by the host phagocytic immune response.

Unlike antibiotics, anti-virulence agents diminish bacterial virulence and may not lead to drug resistance. Many anti-virulence agents had a good effect against persistent *S. aureus* infection, e.g., indole derivatives (Lee et al. 2013). In this study, *S. aureus RNAIII*, encoding δ-hemolysin, plays a key role in the quorum-sensing-dependent central regulatory circuit and coordinately regulates several virulence-associated genes (Novick 2003). Previous studies have shown that *RNAIII* inhibits the expression of surface protein A (*spa*), which is one of the major virulence factors during exponential phase (Gao and Stewart 2004). In this study, the expression of *RNAIII* and *spa* in LAA-treated *S. aureus* planktonic cells decreased 2.5- and 8.5-fold, respectively. Our study has also shown that the expression of toxin genes was inhibited by LAA in *S. aureus* in planktonic cells and biofilm. At the same time, the differential expression of virulence factors in

response to LAA may be coordinately regulated by these two-component signal transduction systems.

Unlike biofilm, planktonic cells challenged with LAA responded with significantly increased expression levels of the cell wall synthesis genes *vraS*, *vraR*, *pbp4*, *sgtB*, *murZ*, *hmrA*, *gtaB*, *drp35*, *fmtA*, SA1577, and SA0205. Previous studies of the *S. aureus* responses to the cell wall-active antibiotic vancomycin (Kuroda et al. 2003), oxacillin, bacitracin, and D-cycloserine (Utaida et al. 2003) identified a series of gene expression changes involved in cell wall synthesis, which seems to be predominantly regulated by the VraSR two-component regulatory system. McAleese et al. (2006) described that vancomycin treatment caused a core cell wall stress stimulation of 17 genes (including *pbp2, pbp4*, *sgtB*, *murZ*, *hmrA*, *gtaB*, and *drp35*). This result implies that although LAA harmed *S. aureus* biofilm, it also stressed *S. aureus* planktonic cells.

In addition to biofilm, the transcriptome analysis showed that LAA significantly inhibited the expression of initiation factors, elongation factors, ribosomal proteins, peptide chain release factor, tRNA synthetases (including glycyl-tRNA synthetase, histidyl-tRNA synthetase, and tyrosyl-tRNA synthetase), glutamyl-tRNA (Gln) amidotransferase, and GTP-binding protein of *S. aureus*. Previous reports indicated that the drug may elicit its antimicrobial effect on bacteria by binding to the 30S ribosomal subunit, preventing the incorporation of amino acid residues into the elongating peptide chain and inhibiting protein synthesis (Smith et al. 2010). In planktonic cells, LAA at a sub-lethal concentration inhibited the expression of tRNA synthetases (including glycyl-tRNA synthetase, histidyl-tRNA synthetase, and tyrosyl-tRNA synthetase). These results show that LAA also had effects on protein synthesis in *S. aureus*.

Bacterial biofilm infections can be very difficult to address, and the implanted (infected) device often has to be removed or replaced (Xing et al. 2012). Developing novel therapeutic agents or antibiotic alternatives may help solve the problem of biofilm infection. Plants and other natural materials may prove to be possible sources of new antibacterial and synergistic antibacterial compounds. In this study, we observed that LAA showed strong activity against *S. aureus* biofilm and planktonic cells, and it does influence the expression of some important genes in *S. aureus* biofilm and planktonic cells. These findings may have important implications for understanding the response mechanisms of *S. aureus* to LAA, and the results facilitate the further development of LAA as an antibacterial compound.

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Conflict of interest The authors declare no conflicts of interest.

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