



## *Staphylococcus aureus* glucose-induced biofilm accessory proteins, GbaAB, influence biofilm formation in a PIA-dependent manner



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### ABSTRACT

The Gram-positive bacteria *Staphylococcus aureus* and *Staphylococcus epidermidis* are capable of attaching to a biomaterial surface and forming resistant biofilms. The identification of biomolecular and regulatory factors involved in staphylococcal adhesion and biofilm formation is needed to understand biofilm-associated infection in humans. Here, we have identified a new operon, *gbaAB* (glucose induced biofilm accessory gene), that affects biofilm formation in *S. aureus* NCTC8325. Real-time reverse transcription PCR (RT-PCR) and electrophoretic mobility shift assay showed that GbaA and GbaB are transcribed from the same transcript, and GbaA directly inhibits the transcription of the *gbaAB* operon through self-repression. Our results indicated that the *gbaA* mutant displayed enhanced biofilm formation compared with the wild type. However, the *gbaB* and the *gbaAB* double mutant displayed reduced biofilm formation, suggesting that the *gbaAB* operon is involved in biofilm formation and that *gbaB* might be the key gene in biofilm regulation. Phenotypic analysis suggested that the *gbaAB* operon mediated biofilm formation of *S. aureus* at the multicellular aggregation stage rather than during initial attachment. In addition, real-time RT-PCR analysis showed that *icaA* was upregulated in the *gbaA* mutant and downregulated in the *gbaB* and *gbaAB* mutants compared with the wild type. In addition, the *gbaA* and the *gbaB* mutants affected the induction of biofilm formation by glucose. Our results suggest that the *gbaAB* operon is involved in the regulation of the multicellular aggregation step of *S. aureus* biofilm formation in response to glucose and that this regulation may be mediated through the *ica* operon.

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### Introduction

*Staphylococcus aureus* is a versatile human pathogen that causes a wide range of infections. Many *S. aureus* infections, such as endocarditis and osteomyelitis, are thought to be associated with the formation of biofilms (George and Muir, 2007). A biofilm is an adherent community of bacteria with an organized structure and chemistry, surrounded by an extracellular biochemical polymer. In addition to aiding the bacterial colonization of surfaces, biofilms are believed to increase the tolerance to antibiotics and host immune defences (Fux et al., 2005; Leid et al., 2002; Otto, 2008).

Biofilms are composed of layers of bacteria within a glycocalyx composed of polysaccharides, DNA, and proteins (Costerton et al., 1987; Laverty et al., 2013; O'Neill et al., 2008; Otto, 2008; Rice et al., 2007). Polysaccharides, predominantly polysaccharide intercellular adhesin (PIA/PNAG), are the most common components of *S. epidermidis* and *S. aureus* biofilms. PIA/PNAG synthesis is mediated

by the intercellular adhesion (*ica*) operon, which encodes three membrane proteins with enzymatic activity (IcaA, IcaD, and IcaC) and one extracellular protein (IcaB) (Cramton et al., 1999; Heilmann et al., 1996; Vuong et al., 2004). Moreover, a decreased PIA level is considered to be the main factor leading to the destructive ability of biofilm formation in *S. aureus* NCTC8325. In addition to the *ica* locus and PIA/PNAG, proteinaceous intercellular adhesins including Bap, SasG, Aap, AtlE, and MSCRAMMs play a role in staphylococcal biofilm formation (Cucarella et al., 2001; Fey, 2010; O'Gara, 2007; O'Neill et al., 2008). However, the regulation of these factors is complex and variable in different stains.

Several proteins have been reported to contribute to the regulation of expression of the staphylococcal *ica* locus, including IcaR, SigB, TcaR, SarA, SrrAB, CcpA, PurR, SarX, and Rbf (Cue et al., 2013; Fujimoto et al., 2009; Jefferson et al., 2004; Knobloch et al., 2001; O'Gara, 2007; Seidl et al., 2008; Ulrich et al., 2007). The most important factor involved in *icaADBC* regulation is IcaR, a member of the TetR family of regulatory proteins, which represses *ica* operon expression by binding to the *icaA* promoter region (Ramos et al., 2005). The inactivation of *icaR* has been shown to significantly increase *icaADBC* expression and PIA/PNAG production

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(Conlon et al., 2002a). In recent years, many factors including increased temperature, osmotic shock, low oxygen tension, ethanol, glucose, glucosamine, N-acetylglucosamine (GlcNAc), autoinducer 2 (AI-2), and sub-inhibitory concentrations of some antibiotics have been identified to influence the expression of PIA in vitro (Conlon et al., 2002a,b; Dobinsky et al., 2003; Gerke, 1998; Gotz, 2002; Knobloch et al., 2001; Mack et al., 1992; Rachid et al., 2000a,b; Yu et al., 2012; Zhang et al., 2011). Ethanol was shown to induce biofilm formation through *icaR*, whereas the effects of glucose and NaCl are not mediated through *icaR* (Conlon et al., 2002a,b). Recently, a series of studies conducted by the Lee group found that Rbf and SarX represent a regulatory cascade that promotes PIA-dependent biofilm formation in response to glucose and NaCl in *S. aureus* (Cue et al., 2013, 2009; Lim et al., 2004). Additionally, glucose affects *icaADBC* expression and PIA/PNAG production, and CcpA is required for aggregation and biofilm formation (Seidl et al., 2008). However, the mechanism has not yet been thoroughly characterized. Our previous work demonstrated that AI-2 can decrease biofilm formation in *S. aureus* via an *icaR*-activation pathway (Yu et al., 2012).

Thus, the genetic basis of biofilm formation in staphylococci is multifactorial, and much remains to be explored. In this study, we identified a novel operon, *gbaAB*, from *S. aureus* NCTC8325

and demonstrated that the *gbaAB* operon is involved in the biofilm formation of this bacterium on both polystyrene and glass. *gbaA* and *gbaB* are corresponding to open reading frame (ORF) SAOUHSC\_02897 (GeneID: 3921349) and ORF SAOUHSC\_02898 (GeneID: 3921350) of strain NCTC8325 at the National Center for Biotechnology Information (NCBI), respectively. Our data suggest that *gbaA* and *gbaB* are located in the same operon, and GbaA represses the transcript level of *gbaB* through direct self-repression of the *gbaAB* operon. Additionally, we found that GbaAB can modulate biofilm formation in response to glucose and that this regulation may be mediated by the *ica* operon in *S. aureus* NCTC8325.

## Materials and methods

### Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are described in Table 1. *Staphylococcus aureus* and *Escherichia coli* were grown in Luria–Bertani (LB; BD, Franklin Lakes, NJ, USA) medium, tryptic soy broth (TSB; BD) medium, tryptic soy broth without glucose (TSBg–; BD) or tryptic soy agar (TSA; BD) medium with the appropriate antibiotics for plasmid selection and maintenance. The

**Table 1**  
Strains and plasmids used in this study.

Strain or plasmid	Relevant genotype	Reference or source
<i>Strains</i>		
NCTC8325	Wild-type	NARSA <sup>a</sup>
RN4220	8325-4 <i>r</i> -initial recipient for modification of plasmids which are introduced into <i>S. aureus</i> from <i>E. coli</i>	NARSA
SH1000	8325-4 <i>rsbU</i> repaired	Horsburgh et al. (2008)
SY1	8325 <i>gbaA::ermB</i>	This study
SY2	8325 <i>gbaA::ermB</i> pLI50	This study
SY3	8325 <i>gbaA::ermB</i> pLIgbaA	This study
SY4	8325 <i>gbaB::ermB</i>	This study
SY5	8325 <i>gbaB::ermB</i> pLI50	This study
SY6	8325 <i>gbaB::ermB</i> pLIgbaB	This study
SY7	8325 <i>gbaAB::ermB</i>	This study
SY8	8325 <i>gbaAB::ermB</i> pLI50	This study
SY9	8325 <i>gbaAB::ermB</i> pLIgbaAB	This study
SY10	8325 pLI50	This study
SY11	8325 pLIgbaA	This study
SY12	8325 pLIgbaB	This study
SY13	8325 pLIgbaAB	This study
SY14	8325 pRMC2	This study
SY15	8325 pRMCgbaA	This study
SY16	8325 pRMCgbaB	This study
SY17	SH1000 pRMC2	This study
SY18	SH1000 pRMCgbaA	This study
SY19	SH1000 pRMCgbaB	This study
SY20	8325 pGFP	This study
SY21	8325 <i>gbaA::ermB</i> pGFP	This study
SY22	8325 <i>gbaB::ermB</i> pGFP	This study
DH5 $\alpha$	Clone host strain, <i>supE44</i> $\Delta$ <i>lacU169</i> ( $\phi$ 80 <i>dlacZ</i> $\Delta$ M15) <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i>	TransGen
BL21 (DE3)	Express strain, F <sup>-</sup> <i>ompT</i> <i>hsdS<sub>B</sub></i> ( <i>r<sub>B</sub></i> <sup>-</sup> <i>m<sub>B</sub></i> <sup>-</sup> ) <i>gal</i> <i>dcm</i> (DE3)	TransGen
<i>Plasmids</i>		
pEasy-blunt simple	Clone vector, Kan <sup>r</sup> Ap <sup>r</sup> b	TransGen
pET28a(+)	Expression vector with a hexahistidine tag, Kan <sup>r</sup>	Novagen
pGbaA	pET28a(+) with the <i>gbaA</i> coding sequence, Kan <sup>r</sup>	This study
pEC1	pUC18 derivative, source of the <i>ermB</i> gene, Ap <sup>r</sup>	Bruckner
pBT2	Shuttle vector, temperature sensitive, Ap <sup>r</sup> Cm <sup>r</sup> b	Bruckner
pBTgbaA	pBT2 containing upstream and downstream fragments of <i>gbaA</i> and <i>ermB</i> gene, for <i>gbaA</i> mutagenesis, Ap <sup>r</sup> Cm <sup>r</sup> Em <sup>r</sup> b	This study
pBTgbaB	pBT2 containing upstream and downstream fragments of <i>gbaB</i> and <i>ermB</i> gene, for <i>gbaB</i> mutagenesis, Ap <sup>r</sup> Cm <sup>r</sup> Em <sup>r</sup>	This study
pBTgbaAB	pBT2 containing upstream and downstream fragments of <i>gbaAB</i> and <i>ermB</i> gene, for <i>gbaAB</i> mutagenesis, Ap <sup>r</sup> Cm <sup>r</sup> Em <sup>r</sup>	This study
pRMC2	anhydrotetracycline-inducible expression vector for <i>S. aureus</i> , Ap <sup>r</sup> Cm <sup>r</sup>	Corrigan and Foster (2009)
pRMCgbaA	pRMC2 with <i>gbaA</i> ORF, Ap <sup>r</sup> Cm <sup>r</sup>	This study
pRMCgbaB	pRMC2 with <i>gbaB</i> ORF, Ap <sup>r</sup> Cm <sup>r</sup>	This study
pLI50	Shuttle cloning vector, Ap <sup>r</sup> Cm <sup>r</sup>	Addgene
pLIgbaA	pLI50 with <i>gbaA</i> ORF and its promoter, Ap <sup>r</sup> Cm <sup>r</sup>	This study
pLIgbaB	pLI50 with <i>gbaB</i> ORF and the promoter of <i>gbaA</i> , Ap <sup>r</sup> Cm <sup>r</sup>	This study
pgfp	gfp expression with the promoter of S10 ribosomal gene, Ap <sup>r</sup> , Cm <sup>r</sup>	

<sup>a</sup> NARSA, Network on Antimicrobial Resistance in *Staphylococcus aureus*.

<sup>b</sup> Kan<sup>r</sup>, kanamycin-resistant; Ap<sup>r</sup>, ampicillin-resistant; Cm<sup>r</sup>, chloramphenicol-resistant; Em<sup>r</sup>, erythromycin-resistant.

**Table 2**  
Primers used in this study.

Primer name	Oligonucleotide (5'–3') <sup>a</sup>	Application
up-gbaA-f	GCGgtcgacCTAGTTAATGCACCTACATA	<i>gbaA</i> deletion
up-gbaA-r	GCGgaattcTTGTGCGCTATTTCTTCAAT	<i>gbaA</i> deletion
down-gbaA-f	GCGggatccACGAAGTTCAAAACAGATAT	<i>gbaA</i> deletion
down-gbaA-r	GCGgaagcttGCTAATGAATTCACCTTAGAT	<i>gbaA</i> deletion
up-gbaB-f	GCGgtctacTAAGGCACTACTGCAATGTA	<i>gbaB</i> deletion
up-gbaB-r	GCGgaattcTACTTCTTCAACGTCACGAG	<i>gbaB</i> deletion
down-gbaB-f	GCGggatccTTAGAAAAATCTGAACAACC	<i>gbaB</i> deletion
down-gbaB-r	GCGgaagcttCATTTGTAAAACCTGTCTTT	<i>gbaB</i> deletion
up-gbaAB-f	GCGgtcgacCTAGTTAATGCACCTACATA	<i>gbaAB</i> deletion
up-gbaAB-r	GCGgaattcTTGTGCGCTATTTCTTCAAT	<i>gbaAB</i> deletion
down-gbaAB-f	GCGggatccTTAGAAAAATCTGAACAACC	<i>gbaAB</i> deletion
down-gbaAB-r	GCGgaagcttCATTTGTAAAACCTGTCTTT	<i>gbaAB</i> deletion
Em-f	GCGgaattcGATACAAATTCGCCGTAGGC	<i>airSR</i> deletion
Em-r	GCGggatccCGAAATAGATTTAAAAATTTCCG	<i>airSR</i> deletion
g-gbaA-f	GCGcatatgCGAAAAGATGCAAAAGAGAAT	Expression of GbaA
g-gbaA-r	GCGctcgagTTAGTCATTACGTCCACCT	Expression of GbaA
c-gbaA-f	GCGgaattcAATCGTTGTAAATGATGACT	$\Delta$ <i>gbaA</i> complementation
c-gbaA-r	GCGggatccATAAACTTTGTAACCAATG	$\Delta$ <i>gbaA</i> complementation
p-gbaAB-f	GCGgaagcttAATTTGATGACTAAAAATGCC	$\Delta$ <i>gbaB</i> complementation
p-gbaAB-r	GCGgtaccGATTACACCTACTTAAAT	$\Delta$ <i>gbaB</i> complementation
c-gbaAB-f	GCGgaagcttAATCGTTGTAAATGATGACT	$\Delta$ <i>gbaAB</i> complementation
c-gbaAB-r	GCGgagctcCTACCAAGGCATCTCTCCAT	$\Delta$ <i>gbaAB</i> complementation
i-gbaA-f	GCGgtaccAATTTAAGTAGGTGTAATCATGCC	<i>gbaA</i> induced expression
i-gbaA-r	GCGgagctcTTAGTCATTACGTCCACCT	<i>gbaA</i> induced expression
i-gbaB-f	GCGgtaccTTTAGATGAGGTGGACGTAAT	<i>gbaB</i> induced expression
i-gbaB-r	GCGgagctcCTACCAAGGCATCTCTCCAT	<i>gbaB</i> induced expression
rt-icaA-f	TTTCGGGTGCTTCACTCTAT	RT-real-time PCR
rt-icaA-r	CGTAGTAATACTTCGTGTCC	RT-real-time PCR
rt-gbaA-f	GCAAGTGTGCTTAGATTATC	RT-real-time PCR
rt-gbaA-r	CGTGTAAAGTGTGTCATC	RT-real-time PCR
rt-gbaB-f	TTAATCACAGGAGGCAATA	RT-real-time PCR
rt-gbaB-r	AGTAGTCACTTGTAACTACTA	RT-real-time PCR
rt-hu-f	AAAAAGAAGCTGGTTCAGCAGTAG	RT-real-time PCR
rt-hu-r	AAAAAGAAGCTGGTTCAGCAGTAG	RT-real-time PCR
p-gbaA-f	AATTGATGACTAAAAATGCC	EMSA
p-gbaA-r	GATTACACCTACTTAAAAAT	EMSA

<sup>a</sup> The sequences in lowercase letters refer to the restriction endonuclease recognition sites.

mutants were constructed using a method previously described (Bruckner, 1997). All primers used in this study are listed in Table 2.

#### Construction of complementation or over-expression strains

To create plasmids pLI*gbaA* and pLI*gbaAB*, the target genes and their promoters from *S. aureus* NCTC8325 were amplified by PCR with primers c-gbaA-f and c-gbaA-r for *gbaA* and primers c-gbaAB-f and c-gbaAB-r for *gbaAB*. The PCR products were cloned into pLI50 (Addgene). For pLI*gbaB* construction, before it was cloned into pLI50, the *gbaB* gene fragment was amplified with primers c-gbaA-f and c-gbaA-r and ligated to the *gbaAB* promoter fragment (amplified with primers p-gbaAB-f and p-gbaAB-r). To create plasmids pRMC*gbaA* and pRMC*gbaB*, the target genes were amplified by PCR with primers i-gbaA-f and i-gbaA-r for *gbaA* and primers i-gbaB-f and i-gbaB-r for *gbaB* and then cloned into pRMC2 (Corrigan and Foster, 2009). The plasmids were transformed into *E. coli* DH5 $\alpha$  and transformed by electroporation into *S. aureus* RN4220 and subsequently transferred to their mutant or wild-type strains. All primers used in this study are listed in Table 2.

#### Total RNA isolation, cDNA generation, real-time RT-PCR, and microarray processing

For the total RNA isolation, the overnight cultures of *S. aureus* were diluted 1:100 in TSB and then grown at different growth phase until collected. The cells were processed with 1 ml of TRIzol (TaKaRa, Kyoto, Japan) in combination with 0.1-mm-diameter-silica beads in a FastPrep-24 Automated system (MP Biomedicals Solon, OH, USA), and residual DNA was removed with RNase free

DNaseI (TaKaRa, Kyoto, Japan). For the reverse-transcription, the cDNAs were synthesized using a PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa). The real-time PCR was performed with SYBR Premix Ex Taq (TaKaRa) using the StepOne Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). The quantity of cDNA measured using real-time PCR was normalized to the abundance of hu cDNA (Valihrach and Demnerova, 2012). The real-time PCR assays were repeated a minimum of three times. To investigate the expression of genes that affect biofilm formation, bacterial cells were grown statically in the 24-well plate at different time courses (4 h, 8 h, 12 h) until collected. Bacteria in the wells of biofilm formation at 12 h were collected for microarray analysis. The microarray processing and data analysis were conducted by the Biochip Company of Shanghai, China. The microarray data was uploaded to Gene Expression Omnibus (GEO) with accession number: GSE53432.

#### Purification of GbaA

The 6-His-tagged GbaA protein was expressed and purified using standard procedures. The full-length *gbaA* ORF was amplified using PCR with the g-gbaA-f and g-gbaA-r-primers from *S. aureus* NCTC8325 genomic DNA, cloned into the expression vector pET28a (+) (Novagen, Merck, Darmstadt, Germany), and transformed into *E. coli* BL21 (DE3). The transformant was grown in LB at 37 °C to an OD600 of 0.4 and induced with 0.5 mM isopropyl- $\beta$ -D-1-thiogalactopyranoside (IPTG) at 37 °C for additional three hours. The cells were harvested and lysed by sonication in a lysis buffer (20 mM Tris-HCl, pH 7.5, 200 mM NaCl). GbaA was purified with a nickel-nitrilotriacetic acid agarose solution (Qiagen, Valencia, CA, USA) following the manufacturer's recommendation. The bound

protein was eluted with an elution buffer (200 mM imidazole, 20 mM Tris-HCl, pH 7.5, 200 mM NaCl). The imidazole in the eluent was removed using a Centrifuge Biomax-5 column (Millipore, Billerica, MA, USA), and the GbaA protein solution was supplemented with 30% glycerol and stored at  $-80^{\circ}\text{C}$  until use. The purity of the proteins was analyzed using SDS-PAGE, and the protein concentration was determined using the BCA assay with bovine serum albumin as the standard.

#### Electrophoretic mobility shift assay (EMSA)

The DNA fragments containing the promoter region were amplified from the *S. aureus* NCTC8325 genomic DNA. The PCR products were labeled using a digoxigenin (DIG) gel shift kit (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions. The labeled fragment was incubated at  $25^{\circ}\text{C}$  for 15 min with various amounts of GbaA in  $10\ \mu\text{l}$  of incubation buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA). After incubation, the mixtures were electrophoresed in a 5% native polyacrylamide gel in  $0.5\times$  Tris-borate-EDTA (TBE) buffer. The band shifts were detected and analyzed according to the manufacturer's instructions. The images were obtained using ImageQuant LAS 4000mini (GE, Piscataway, NJ, USA). The unlabeled fragments of the *pta* ORF region were added as non-specific DNA.

#### Biofilm formation and analysis

Biofilm formation under static conditions was determined by the microtitre plate assay based on the method described previously (Beenken et al., 2003). Briefly, the overnight cultures were made at a 1:100 dilution using fresh TSB medium. The diluted cell suspension was inoculated into flat-bottom 96-well polystyrene plates (Costar 3599, Corning Inc., Corning, NY),  $200\ \mu\text{l}$  for each well. The plates were incubated at  $37^{\circ}\text{C}$  for different time courses and the wells were rinsed gently with water five times to remove non-adherent cells. Subsequently, the plates were stained with 0.5% crystal violet for 15 min, and then rinsed again with water to remove unbound stain. After that, the plates were dried, and the optical density at 560 nm ( $\text{OD}_{560}$ ) was determined with an enzyme-linked immunosorbent assay reader in a  $3\times 3$  scan model. For assay of the effect of glucose, TSBg- medium was supplemented with glucose with a concentration range of 0–5.6%. To investigate the biofilm formation using pRMC2 anhydrotetracycline-inducible expression plasmid, the TSB medium was supplemented with anhydrotetracycline with a concentration range of 0–400 ng/ml.

The biofilm assay on glass was carried out essentially as described (Lim et al., 2004). In brief, a  $30\ \mu\text{l}$  sample of the cell suspension diluted as described above for the polystyrene assay was placed on sterile glass slides and incubated in a petri dish on a wet paper towel at  $37^{\circ}\text{C}$  overnight. The slides were rinsed gently with distilled water, air dried, and stained with 0.5% crystal violet for 15 min. Biofilm formation was also examined in a flow cell (Stovall, Greensboro, USA), which was assembled and prepared according to the manufacturer's instructions. Flow cell experiments and laser scanning confocal microscope (CLSM) were performed as described previously (Yu et al., 2012). Overnight cultures of different strains were adjusted to  $\text{OD}_{600}$  of 5.0 and made at a 1:100 dilution in fresh 0.5% TSB. Flow cells were inoculated with 4 ml of these culture dilutions and incubated at  $37^{\circ}\text{C}$  for 1 h, and then laminar flow ( $250\ \mu\text{l}/\text{m}$ ) was initiated. Biofilms of different strains were cultivated at  $37^{\circ}\text{C}$  in 0.5% TSB in three individual channels. The strains were transformed with the GFP plasmid for fluorescence detection, thus chloramphenicol was added to the flow cell medium to maintain plasmid selection. CLSM was performed on a Zeiss LSM710 system (Carl Zeiss, Jena, Germany) with a  $20\times 0.8$  n.a. apochromatic objective. Z-stacks were collected at

$1\ \mu\text{m}$  intervals. Confocal parameters set for wild type biofilm detection were taken as standard settings. Selected confocal images stood for similar areas of interest and each confocal experiment was repeated four times. The confocal images were acquired from Zeiss ZEN 2010 software package (Carl Zeiss, Jena, Germany) and the three-dimensional biofilm images were rendered with Imaris 7.0 (Bitplane, Zurich, Switzerland).

#### Primary attachment assay on polystyrene

Primary attachment assay on polystyrene was carried out essentially as described (Lim et al., 2004). In brief, overnight cultures were diluted in TSB, and about 300 CFU was added to polystyrene petri dishes. After incubation at  $37^{\circ}\text{C}$  for 1 h, the petri dishes were rinsed gently with 5 ml of sterile PBS (pH 7.5) three times and covered with 15 ml of molten 0.8% TSA maintained at  $48^{\circ}\text{C}$ . Primary attachment was expressed as a percentage of CFU remaining on the petri dishes after washing. Each experiment was repeated three times.

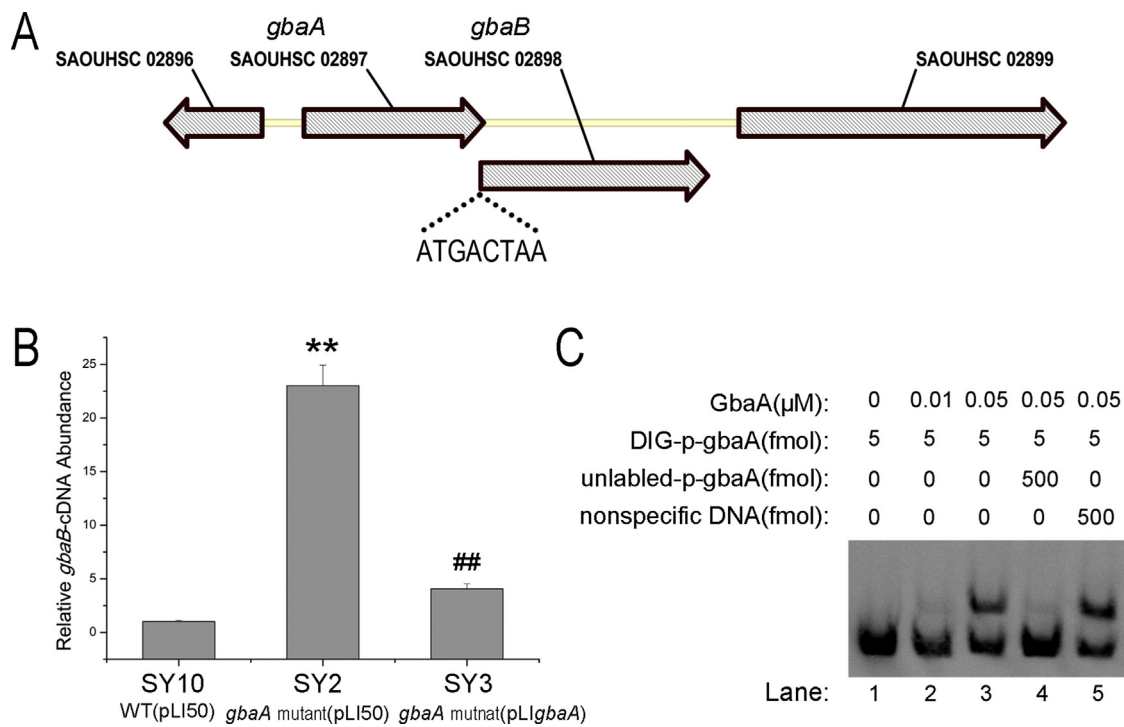
#### Detection of PIA

The PIA extracted from *S. aureus* was blotted onto PVDF membrane (GE Healthcare) using a 96-well dot-blot apparatus according to a method described elsewhere (Lin et al., 2012). After blotting, the membrane was dried and soaked in a solution containing 3% bovine serum albumin and 0.05% Tween-20 in phosphate-buffered saline (PBS). The membrane was then incubated at room temperature for 1 h in solution containing 0.8 mg/ml wheat germ agglutinin conjugated with biotin (WGA-biotin) (Sigma-Aldrich). After washing four times with PBS, PIA was detected using horseradish peroxidase-conjugated streptavidin followed by chemiluminescence detection (Thermo).

## Results

#### *GbaA* represses the transcription of *gbaAB* through a self-repression manner

Bioinformatics analysis revealed that GbaA contained a consensus region signature of the TetR family of regulators (Fig. S1), suggesting that GbaA is perhaps a transcriptional regulator (Ramos et al., 2005). In addition, GbaB is described as a putative short chain oxidoreductase in GenBank, suggesting that GbaB may participate in metabolic pathways. The available genomic information shows the organization of the *gbaAB* operon. As shown in Fig. 1A, the *gbaA* gene is located upstream of *gbaB*. There are eight short common base pairs at the adjacent site, suggesting that GbaA and GbaB might be in the same operon. To determine whether *gbaA* and *gbaB* are co-transcribed in the WT, the *gbaA* cDNA and *gbaB* cDNA was amplified by reverse transcription PCR from the same mRNA of WT using rt-*gbaA*-r and rt-*gbaB*-r specific primers (Fig. S5), respectively, and then the relative *gbaA*-cDNA abundance between *gbaA* cDNA and *gbaB* cDNA was compared using real-time PCR assays (Fig. S2C). The results showed the same cDNA level and  $T_m$  ( $77.58^{\circ}\text{C}$ ), indicating that *gbaA* and *gbaB* were transcribed at the same time. We also observed that the transcript levels of both *gbaA* and *gbaB* significantly decreased when the cells were grown to the early exponential phase ( $\text{OD}_{600} = 1.0$ , as shown in Fig. S4) in TSB medium (Fig. S2B), suggesting that the transcription of *gbaAB* might be repressed by another regulator to maintain the low transcript level. Because GbaA is a TetR family regulator containing a helix-turn-helix DNA-binding domain, we proposed that GbaA might have a negative regulatory effect on the transcription of the *gbaAB* operon through direct binding to the *gbaAB* promoter region.



**Fig. 1.** Both *gbaA* and *gbaB* are in the *gbaAB* operon, and GbaA represses the transcription of the *gbaAB* operon through direct self-repression. (A) Organization of the *gbaAB* operon in *S. aureus*. The arrows indicate the directions of translation as determined from the nucleotide sequence. (B) The regulatory effect of *gbaA* on the transcription of *gbaB*. The transcript levels of *gbaB* were compared between SY10 (the wild type with a blank pLI50), SY2 (the *gbaA* mutant with a blank pLI50), and SY3 (the *gbaA* mutant with a pLI50 encoding GbaA). (C) The ability of GbaA to bind to the *gbaAB* promoter as determined by EMSA. All of the real-time RT-PCR assays were repeated five times with similar results. Error bars indicate standard deviations. \*\* $P < 0.01$  vs SY10, ## $P < 0.01$  vs SY2.

Analysis of microarray results revealed that approximately 172 up-regulated genes and 172 down-regulated gene had a fold change less than 2.0, and the expression levels of *gbaB* was significantly up-regulated more than 177-fold in *gbaA* mutant (Table S1). In addition, we confirmed that the transcript level of *gbaB* in the *gbaA* mutant was significantly higher than that in the wild type using real-time RT-PCR analysis (Fig. 1B), suggesting that GbaA represses the transcription of *gbaAB* throughout the growth phase in *S. aureus*. The same results were obtained in other growth phases (data not shown). Furthermore, as shown in Fig. 1C, GbaA appears to have a strong ability to bind to the promoter regions of the *gbaAB* operon (Fig. S3) in vitro, confirming that GbaA represses *gbaAB* expression by directly binding to its promoter region.

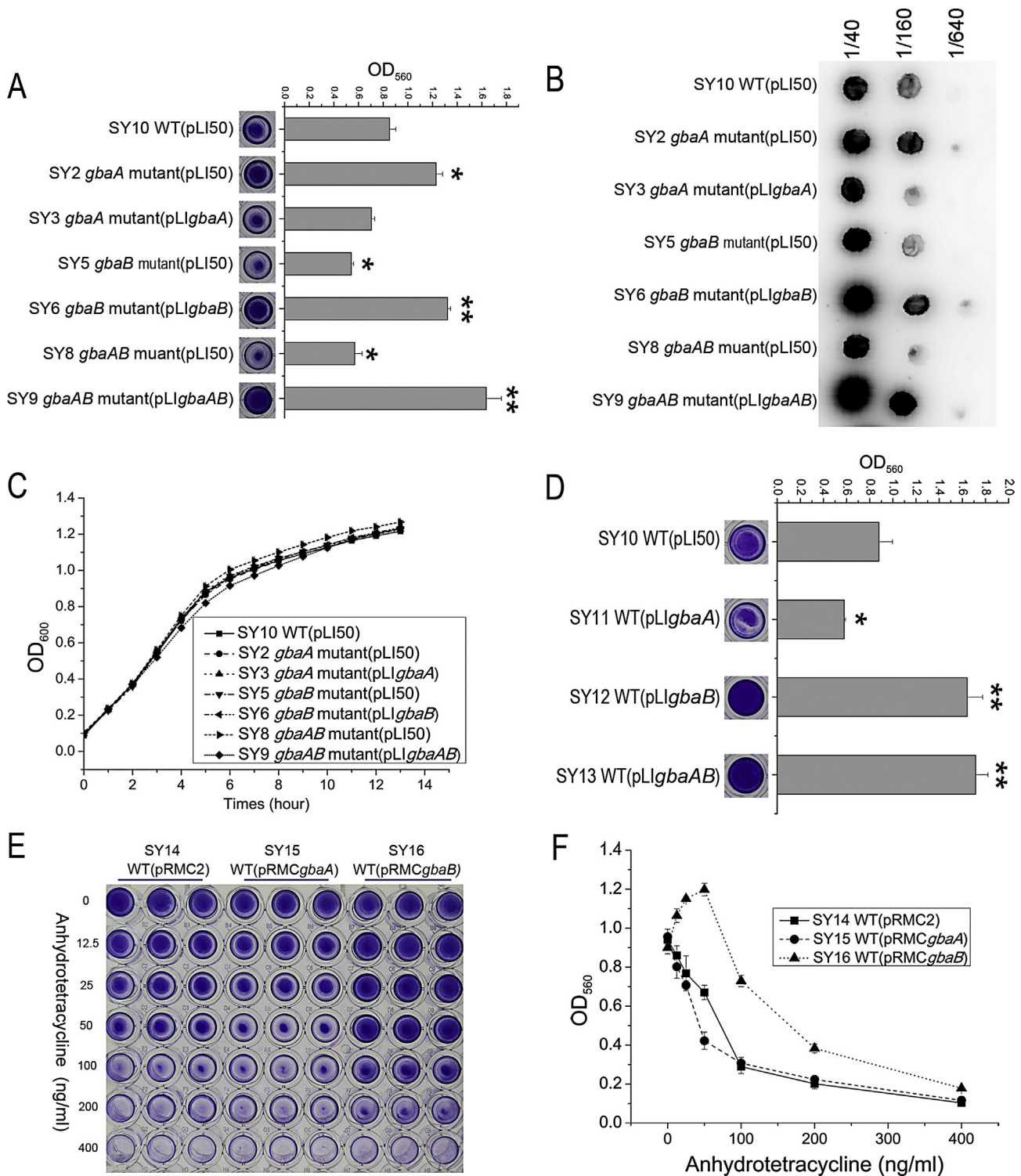
#### *GbaA* inhibits biofilm formation via *gbaB*, which is the biofilm-induced gene required for the multicellular aggregation of biofilm formation

QacR and IcaR are TetR family regulators and are known to be involved in multidrug resistance and biofilm formation in staphylococci. Therefore, it was reasonable to investigate whether *S. aureus* GbaA also has this common feature. We first compared the biofilm formation of the wild type, the *gbaA* mutant, and the *gbaA*-complemented strain on both polystyrene and glass. As shown in Fig. S6A and B, the *gbaA* mutation resulted in an increased biofilm formation ability on both polystyrene and glass, which could be complemented by pLI50 containing the wild type *gbaA* gene. These results indicated that GbaA is involved in biofilm formation. Because *gbaA* and *gbaB* are in the same operon, it is suggested that GbaB may also be involved in biofilm formation. We first tested the biofilm formation of the *gbaA* mutant, the *gbaA*-complemented strain, the *gbaB* mutant, the *gbaB*-complemented strain, the *gbaAB* mutant, the *gbaAB*-complemented strain, and the wild type using microtitre plate assays (Fig. 2A). The results showed

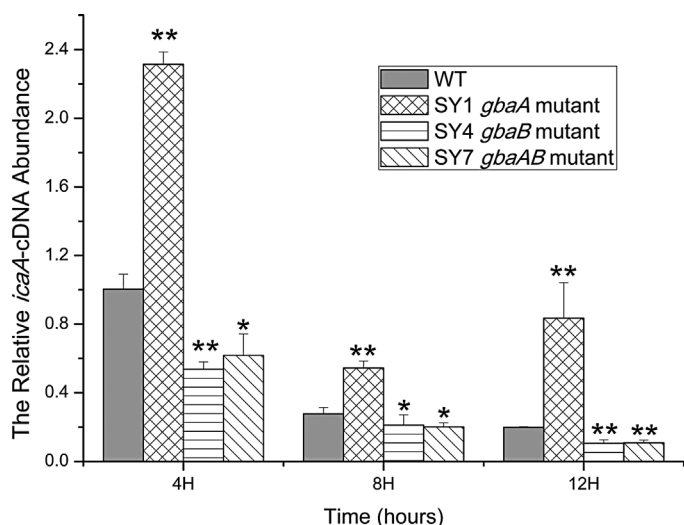
that both the *gbaB* and *gbaAB* mutants resulted in decreased biofilm formation abilities compared with that of the wild type. Additionally, as shown in Fig. 2A, the amounts of biofilm formed by the *gbaB*- and *gbaAB*-complemented strains were more than that formed by the wild-type strain. This finding was most likely due to the overexpression of the target gene because complementation was performed with a multicopy plasmid vector. In addition, we compared the growth rates of the *gbaA* mutant, the *gbaA*-complemented strain, the *gbaB* mutant, the *gbaB*-complemented strain, the *gbaAB* mutant, the *gbaAB*-complemented strain, and the wild type. The results showed no remarkable difference between the seven strains (Fig. 2C).

To confirm the regulatory role of GbaA and GbaB in biofilm formation, we individually overexpressed *gbaA* and *gbaB*, respectively, in the wild type by using pLI50 and pRMC2 plasmids. pLI50 is a stable expression plasmid that overexpresses the target gene by using its own promoter, and pRMC2 is an anhydrotetracycline (aTet)-inducible expression plasmid that can express the target gene by using the *pxyl*/teto promoter (Bateman et al., 2001; Corrigan and Foster, 2009). As shown in Fig. 2D, the NCTC8325 strain containing pLI50 and pLI50 showed significantly increased biofilm formation compared with the strain NCTC8325 containing pLI50 (vector). In contrast, the strain NCTC8325 containing pLI50 and pLI50 exhibited decreased biofilm formation. Also shown in Fig. 2E and F, the biofilm formation ability of the strain NCTC8325 containing pRMC2-*gbaB* was stronger than that of the strain NCTC8325 with pRMC2 (vector) induced by aTet ranging from 0 to 400 ng/ml. In contrast, the biofilm formation of the strain NCTC8325 containing pRMC2-*gbaA* was weaker from 0 to 100 ng/ml aTet and inappreciably different from 100 to 400 ng/ml aTet compared with the NCTC8325 cells containing pRMC2 (vector).

In addition, we checked the biofilm formation of the SH1000 strain, which contains the pRMC2-*gbaA*, pRMC2-*gbaB*, and pRMC2 plasmid, and the results showed that there were no notable differences



**Fig. 2.** *gbaA* regulates biofilm formation through *gbaB* in *S. aureus* NCTC8325. (A) Comparison of biofilm formation of SY10 (wild type with a blank pLI50), SY2 (the *gbaA* mutant with a blank pLI50), and SY3 (the *gbaA* mutant with a pLI50 encoding GbaA), SY5 (the *gbaB* mutant with a blank pLI50), SY6 (the *gbaB* mutant with a pLI50 encoding GbaB), SY8 (the *gbaAB* mutant with a blank pLI50), and SY9 (the *gbaAB* mutant with a pLI50 encoding GbaAB) on a polystyrene microtitre plate. \* $P < 0.05$ , \*\* $P < 0.01$  vs SY10. (B) Quantification of PIA of SY10, SY2, SY3, SY5, SY6, SY8 and SY9. PIA was extracted from overnight cultures of each strain, serially diluted, and applied to a PVDF membrane. PIA was detected using WGA-biotin. After incubation with HRP-streptavidin, the spots were visualized by chemiluminescence detection. Numbers at the top of the figure indicate PIA dilutions. (C) Comparison of the growth rates of SY10, SY2, SY3, SY5, SY6, SY8 and SY9. (D) Comparison of the biofilm formation of SY10, SY11 (the wild type with a pLI50 encoding GbaA), SY12 (the wild type with a pLI50 encoding GbaB) and SY13 (the wild type with a pLI50 encoding GbaAB). \* $P < 0.05$ , \*\* $P < 0.01$  vs SY10. (E) Comparison of the biofilm formation of SY14 (the wild type with a blank pRMC2), SY15 (the wild type with a pRMC2 encoding GbaA) and SY16 (the wild type with a pRMC2 encoding GbaB). (F) Quantitation of biofilms. The cells that adhered to the plate after staining with crystal violet were measured by OD<sub>560</sub>. All of the biofilm formation assays were repeated five times with similar results. Error bars indicate standard deviations. OD<sub>560</sub>, optical density at 560 nm.



**Fig. 3.** The *gbaAB* operon modulates the transcription of *icaA*. Relative *icaA* transcript levels of wild type, SY1 (the *gbaA* mutant), SY4 (the *gbaB* mutant), and SY7 (the *gbaAB* mutant) were measured. All of the real-time RT-PCR assays were repeated five times with similar results. Error bars indicate standard deviations. \* $P < 0.05$ , \*\* $P < 0.01$  vs WT.

between these three strains (Fig. S7). Strain SH1000 belongs to the NCTC8325 lineage (Herbert et al., 2010); however, SH1000 is a PIA-independent biofilm formation strain. Taken together, our data suggest that the *gbaAB* operon could affect biofilm formation in strain NCTC8325, and GbaA might regulate the biofilm formation via GbaB, which is the biofilm-induced protein involved in biofilm formation. To determine at which step the GbaAB affects biofilm formation on polystyrene, a primary attachment experiment was performed with Petri dishes as described in section “Materials and methods”. We found no significant differences between the *gbaA* mutant, the *gbaB* mutant, the *gbaAB* mutant, and wild type (data not shown), suggesting that the *gbaAB* operon affects multicellular aggregation or biofilm dispersal rather than primary attachment.

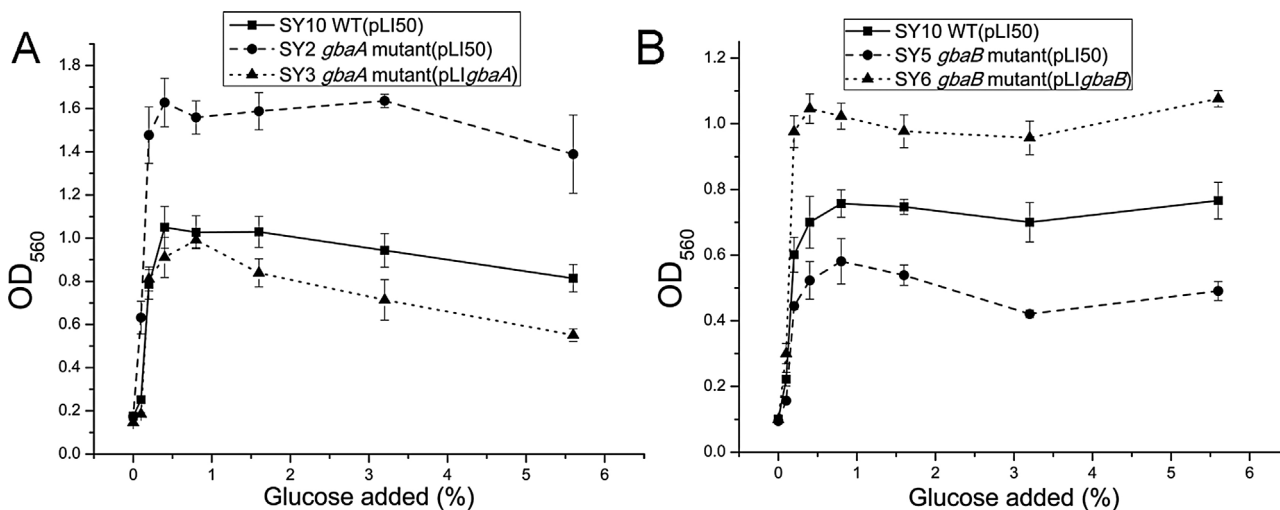
#### GbaAB regulates the expression of *ica* operon and the production of PIA

The *ica* operon is responsible for generating PIA, which is essential for biofilm formation in *S. aureus* NCTC8325. To examine

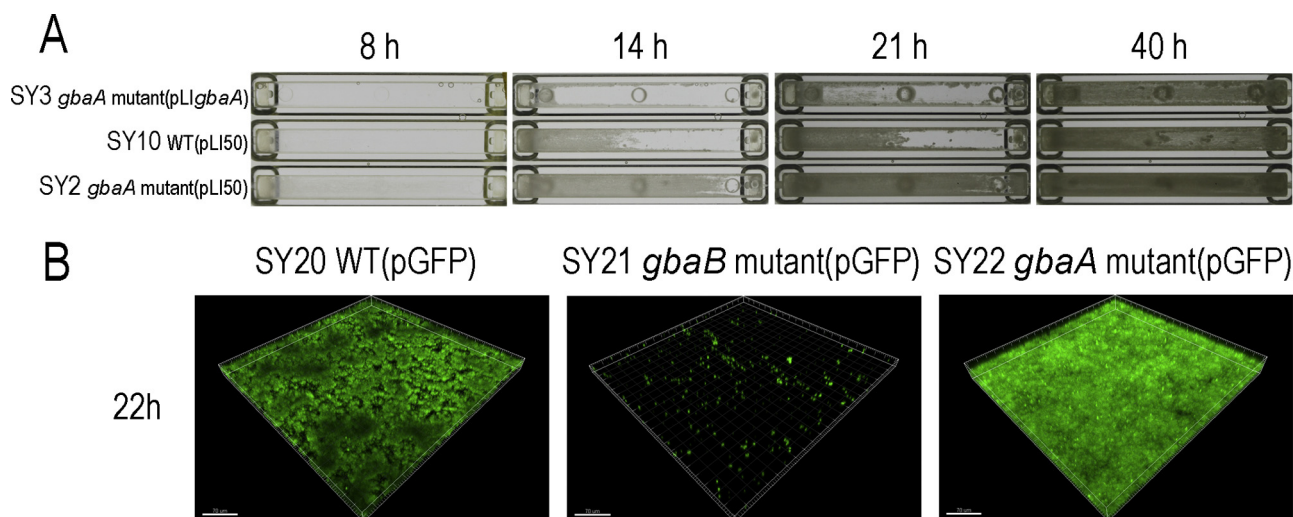
whether GbaAB regulates biofilm formation through activating the expression of the *ica* operon and subsequent PIA production, we measured the transcript level of *icaA* in the biofilm bacteria at different growth phases using real-time RT-PCR (Fig. 3) and PIA production (Fig. 2B). Interestingly, the transcript level of *icaA* was remarkably increased in the *gbaA* mutant compared to that in the wild type, whereas the transcript levels of *icaA* in the *gbaB* and *gbaAB* mutants were more decreased compared with the wild type (Fig. 3), suggesting that *gbaB*, which was repressed by GbaA, might indirectly induce the *ica* transcription. In addition, we found no remarkably different transcript levels of *icaR* between the *gbaA* mutant, the *gbaB* mutant, the *gbaAB* mutant, and wild type (data not shown). As shown in Fig. 2B, the PIA production was consistent with the biofilm formation ability (Fig. 2A), indicating that the effect of *gbaAB* on biofilm formation is due largely to PIA production. In conclusion, our results suggest that the *gbaAB* operon regulates biofilm formation via the *ica* operon and PIA in *S. aureus* NCTC8325.

#### Involvement of the *gbaAB* operon in biofilm induction by glucose

Glucose has been shown to induce the multicellular aggregation step of biofilm formation. To investigate whether biofilm induction by glucose is mediated through the *gbaAB* operon, biofilm assays were performed in the wild type, the isogenic *gbaA* and *gbaB* mutants, and the *gbaA*- and *gbaB*-complemented strains in the presence of various amounts of glucose. As shown in Fig. 4A, the mutation of *gbaA* resulted in increased glucose-induced biofilm formation at all glucose concentrations, and the *gbaB* mutant (Fig. 4B) showed a decrease of glucose-induced biofilm formation. The effect of the *gbaA* and *gbaB* mutants could be complemented by the pLI50 plasmid containing the *gbaA* and *gbaB* genes, respectively. Additionally, the *gbaA*-complemented strain produced less biofilm than the wild-type strain, while the *gbaB*-complemented strain produced more biofilm. These results indicated that GbaA might modulate biofilm formation on polystyrene in response to glucose through GbaB. We also checked whether the *gbaAB* operon regulates biofilm induction by NaCl and ethanol; however, the results showed no significant differences between the wild type and the mutants, suggesting that the *gbaAB* operon does not affect biofilm in response to NaCl and ethanol induction (data not shown).



**Fig. 4.** Biofilm formation in response to glucose. (A) Biofilm formation of SY10 (the wild type with a blank pLI50), SY2 (the *gbaA* mutant with a blank pLI50), and SY3 (the *gbaA* mutant with a pLI50 encoding GbaA) in TSBg<sub>-</sub> medium added with glucose from 0% to 5.6%. (B) Biofilm formation of SY10, SY5 (the *gbaB* mutant with a blank pLI50), SY6 (the *gbaB* mutant with a pLI50 encoding GbaB) in TSBg<sub>-</sub> medium added with glucose from 0% to 5.6%. The results represent the averages of at least three experiments with similar results. Error bars indicate standard deviations.



**Fig. 5.** Biofilm formation in a flow cell system. (A) Biofilm formation of SY10 (the wild type with a blank pLI50), SY2 (the *gbaA* mutant with a blank pLI50), and SY3 (the *gbaA* mutant with a pLI50 encoding GbaA) in flow cells after incubation for 8.0, 14.0, 21.0 and 40 h. (B) Biofilm integrity and GFP fluorescence of SY19 (the wild type with GFP), SY20 (the *gbaA* mutant with GFP) and SY21 (the *gbaB* mutant with GFP) were monitored after incubation for 22 h by CLSM.

#### Inactivation of *gbaA* resulted in increased biofilm formation ability in a flow cell, whereas the *gbaB* mutant reduced biofilm formation

To compare biofilm formation abilities in the different strains at the dynamic state, biofilm formation assays were performed by using a flow-cell system. Consistent with the static biofilm assays, the *gbaA* mutation increased biofilm formation in the flow cells, and the increase was complemented by plasmid pLI50 carrying the wild type *gbaA* gene (Fig. 5A). In addition, we checked the biofilm formation after incubation for 22 h in the flow cells by CLSM, and the results indicated that the mutation of *gbaA* indeed increased biofilm formation and that the mutation of *gbaB* dramatically reduced biofilm formation (Fig. 5B). Consistently, our results suggest that GbaAB affects biofilm formation in the dynamic state.

#### Discussion

Many staphylococcal infections, such as endocarditis and osteomyelitis, are associated with biofilm formation (George and Muir, 2007). For that reason, intensive research into the new regulatory mechanism of biofilm formation in *S. aureus* could facilitate the development of novel therapeutic devices. In this study we attempted to define the role of a novel operon, *gbaAB*, in *S. aureus* biofilm formation.

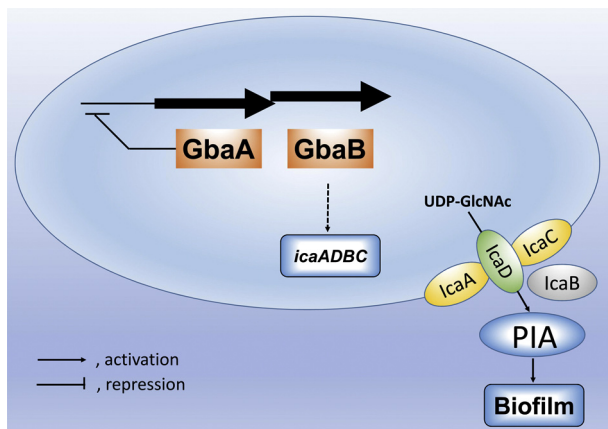
Both *gbaA* and *gbaB* can be found in all other *S. aureus* strains at the NCBI database. The predicted GbaA amino acid sequence alignments suggest that GbaA belongs to the TetR family transcriptional regulators, which are involved in a wide variety of gene regulatory networks, either as transcription activators or repressors (Ramos et al., 2005). Real-time RT-PCR and EMSA results showed that *gbaA* and *gbaB* are in the same operon and that *gbaA* inhibits the transcript level of *gbaB* by direct self-repression. In addition, the TetR family transcriptional regulators, which include the QacR repressor of the QacA multidrug efflux pump system and the IcaR repressor of the *ica* operon in *S. aureus*, are characterized by conserved helix-turn-helix DNA-binding domains at the amino-terminal ends and divergent carboxy-terminal domains, which may be involved in interactions with compounds that modulate their regulatory activity (Aramaki et al., 1995; Conlon et al., 2002a; Grkovic et al., 1998; Jeng et al., 2008; Ramos et al., 2005). Recently, other researchers using microarray analysis found that the transcript level of *gbaA* and *gbaB* can be modulated under some environmental stimuli in *S. aureus*. For instance, the transcription of *gbaA* and *gbaB* was

up-regulated under a tea tree oil (TTO) challenge, acid conditions (pH 4.0) and heat stress (48 °C), and down-regulated in response to diclofenac (80 µg/ml) and alkaline conditions (pH 10.0) (Anderson et al., 2010; Cuaron et al., 2013; Riordan et al., 2011). It is tempting to speculate that GbaA may also be modulated by interacting with inducing compounds and may then regulate the expression and facilitate *S. aureus* to adapt to stress, such as high or low temperature, antibiotics, alkaline or acid conditions.

Static and dynamic biofilm formation analysis indicated that *gbaA* regulates biofilm formation through *gbaB* in *S. aureus* NCTC8325. Interestingly, our results also showed that the over-expression of *gbaB* in *S. aureus* SH1000 did not induce biofilm formation (Fig. S7). SH1000 is a PIA-independent biofilm development strain (Boles and Horswill, 2008), whereas NCTC8325 is a PIA-dependent biofilm formation strain. Thus, we speculated that the *gbaAB* operon might regulate biofilm formation through activating the expression of the *ica* operon in NCTC8325. Real-time RT-PCR results confirmed that *gbaA* modulates the expression of the *ica* operon via *gbaB*. The expression of the *ica* operon can be regulated by many regulators, which can be classified by whether they directly bind to the promoter regions of the *ica* operon. Among these regulators, IcaR, TcaR, SarA, SrrA, and SarX could directly bind to the promoter regions of the *ica* operon, whereas SigB, CcpA, Rbf, and PurR could not. IcaR is the most important regulator. However, there was no significant difference in *icaR* expression in the *gbaA* mutant, the *gbaB* mutant, the *gbaAB* mutant, and the wild type (data not shown). Since both *tcaR* and *sigB* are inactive in *S. aureus* NCTC8325 (Herbert et al., 2010), it appears that the inactivation of *tcaR* or *sigB* is not required for biofilm formation in this strain. Although there were no significant differences in other regulator genes in the microarray analysis of the *gbaA* mutant and the wild type, these results still need to be confirmed by real-time RT-PCR assays in future studies.

In addition to the *ica* operon, some enzymes that provide biosynthetic intermediates or energy are also required for PIA synthesis (Dobinsky et al., 2003). The sugar nucleotide UDP-GlcNAc, which can be synthesized from glucose, fructose, glucosamine, and GlcNAc, is an immediate precursor required for the production of PIA (Gerke, 1998). Additionally, glucose, fructose, glucosamine, and GlcNAc are reported to possibly influence PIA expression (Gotz, 2002). As shown in Fig. S8, we found that biofilm formation of the *gbaA* mutant (*gbaB* high-expression) was stronger than that of the wild type (*gbaB* low-expression) when these biosynthetic





**Fig. 6.** Model of regulation of PIA synthesis by GbaAB in *S. aureus*. PIA is the most common components of biofilm in *S. aureus*. PIA synthesis is mediated by the intercellular adhesion (*ica*) operon which encodes three membrane proteins (IcaA, IcaD, and IcaC) with enzymatic activity and one extracellular protein (IcaB). *ica* operon can be induced by GbaB which is repressed by GbaA through self-repression. PIA, polysaccharide intercellular adhesion; UDP-GlcNAc, UDP-N-acetylglucosamine.

intermediates were added. In addition to biosynthetic intermediates, the amount of NADH also determines the amount of PIA that will be produced (Vuong et al., 2005). Bioinformatics predictions revealed that GbaB might be a putative NAD(P)(+)-binding oxidoreductase, suggesting that GbaB may synthesize NAD(P)H in metabolic pathways. As shown in Fig. S9, the intracellular concentrations of free NADH of *gbaA* mutant significantly increased compared with those of wild type. In addition, we found an NmrA-like NADH sensor protein SAOUHSC\_02895, which is near to *gbaB* (SAOUHSC\_02898), and the transcript levels of SAOUHSC\_02895 was significantly up-regulated more than 101-fold in *gbaA* mutant using microarray analysis (Table S1). It appears reasonable to speculate that GbaB may participate in the synthesis of NADH or may be a substrate for PIA synthesis.

Although many questions remain, these findings demonstrate that the *gbaAB* operon is involved in multicellular aggregation of biofilm formation via the *ica* operon (Fig. 6).

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ijmm.2014.04.003>.

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