

## Molecular Characterization of Regulatory Genes Associated with Biofilm Variation in a *Staphylococcus aureus* Strain

Kim, Jong-Hyun<sup>1</sup>, Cheorl-Ho Kim<sup>2</sup>, Jörg Hacker<sup>3</sup>, Wilma Ziebuhr<sup>3</sup>, Bok Kwon Lee<sup>1</sup>, and Seung-hak Cho<sup>1\*</sup>

<sup>1</sup>Division of Enteric Bacterial Infections, Center for Infectious Diseases, National Institute of Health, Seoul 122-701, Korea

<sup>2</sup>Department of Biological Science, Sungkyunkwan University, Suwon 440-746, Korea

<sup>3</sup>Institut für Molekulare Infektionsbiologie, D-97070 Würzburg, Germany

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**Biofilm formation in association with the intercellular adhesion (*icaADBC*) gene cluster is a serious problem in nosocomial infections of *Staphylococcus aureus*. In all 112 *S. aureus* strains tested, the *ica* genes were present, and none of these strains formed biofilms. The biofilm formation is known to be changeable by environmental factors. We have found about 30% of phase variation in these strains with treatment of tetracycline, pristinamycin, and sodium chloride. However, this phenotype disappeared without these substances. Therefore, we have constructed stable biofilm-producing variants through a passage culture method. To explain the mechanism of this variation, nucleotide changes of *ica* genes were tested in strain *S. aureus* 483 and the biofilm-producing variants. No differences of DNA sequence in *ica* genes were found between the strains. Additionally, molecular analysis of three regulatory genes, the accessory gene regulator (*agr*) and the staphylococcal accessory regulator (*sarA*), and in addition, alternative transcription factor  $\sigma^B$  (*sigB*), was performed. The data of Northern blot and complementation showed that SigB plays an important role for this biofilm variation in *S. aureus* 483 and the biofilm-producing variants. Sequence analysis of the *sigB* operon indicated three point mutations in the *rsbU* gene, especially in the stop codon, and two point mutations in the *rsbW* gene. This study shows that this variation of biofilm formation in *S. aureus* is deduced by the role of *sigB*, not *agr* and *sarA*.**

**Keywords:** Biofilm, variation, *Staphylococcus aureus*, regulatory genes, *sigB*

Bacteria colonize prosthetic implants as a biofilm, multiple layers of sessile cells that adhere to the implant surface

\*Corresponding author

Phone: 82-2-380-1464; Fax: 82-2-352-4767;

E-mail: skcho38@nih.go.kr

as well as to each other. Biofilms produced by bacteria provide a protected environment for host defenses [3, 12]. *S. aureus* infections occur at a high frequency in association with certain types of prosthetic devices [11, 29, 33]. The ability of *S. aureus* to form biofilms helps the bacterium to survive hostile environments within the host, and is considered to be responsible for chronic or persistent infections [6]. It has been observed that biofilm-associated *Staphylococcus aureus* cause many diseases including septicemia, endocarditis, and osteomyelitis [16, 20].

Biofilm formation in staphylococci is thought to be a two-step process. The first step involves attachment of bacteria to a substrate surface. The second step of biofilm development includes bacteria accumulation by cell-cell adhesion using polysaccharide intercellular adhesin (PIA), which has linear  $\beta$ -1,6-linked glucosaminylglycans [23]. PIA is found to be mediated by the products of a gene locus comprising four intercellular adhesion (*icaADBC*) genes that are organized in an operon structure [17]. The sequence of *ica* products between *S. epidermidis* and *S. aureus* revealed the identity of 59% to 78% [7].

Another important property of staphylococci is their capacity to change specific phenotypic features rapidly. The presence of phase variation in biofilm formation of *S. aureus* has been reported before [1]. However, the mechanism of the phenomenon for *S. aureus* has not been investigated yet. The *ica* expression was found to be strongly influenced by environmental factors [8, 28]. Several stimuli, such as subinhibitory concentrations of antibiotics, osmolarity, and growth in anaerobic conditions, are known as inducers for the biofilm formation via enhancement of *ica* expression. Moreover, *ica* operon expression can be turned on and off by the insertion and excision of the insertion sequence IS256 [4, 36].

It has been proposed that biofilm formation by *S. aureus* may be regulated in a density-dependent manner by loci such as the accessory gene regulator (*agr*) and the staphylococcal

accessory regulator (*sarA*) and, in addition, alternative transcription factor  $\sigma^B$  [26, 27, 31]. However, the regulatory mechanisms involved in the *S. aureus* biofilm development remain poorly understood.

Therefore, in this study, we have carried out a phase variation of biofilm formation in a *S. aureus* strain. To further investigate the mechanism involved in the process of biofilm formation by *S. aureus*, this study was carried out to examine the phase variants of biofilm formation of *S. aureus* in relation to the regulatory genes.

## MATERIALS AND METHODS

### Bacterial Strains and Species Identification

Ninety-five clinical *S. aureus* strains obtained from patients in association with plastic-caused infections and 17 skin strains isolated from healthy volunteers were investigated. Saprophytic strains were isolated from the healthy people with Wab-Stab (Mast Diagnostica, Reinfeld, Germany), brought into the laboratory, and cultured on blood agar plates. For the identification of *Staphylococcus* species, the catalase test, coagulase test with staphylect plus-kit (Oxoid, Wesel, Germany), and API-Staph Ident System (bioMérieux, Nürtingen, Germany) were used.

### PCR Primers and Amplification

Primers for the amplification and sequence analyses are summarized in Table 1. The primers for the amplification of the *ica* genes and regulator genes were *icaAD*, B, C, *agr*, *sarA*, *sigB*, and *asp23*. For the sequence analyses of *rsb* and *sigB* genes, *rsb-seq-for.*, *rsb-seq-rev.* and *sigB-seq-rev.* were used.

### Biofilm Assay

Bacteria were grown overnight in Tryptic soy broth (TSB; Oxoid Ltd., Basingstoke, Hampshire, England) at 37°C. Cultures were then

diluted in 1:200 ratio and incubated in a tissue culture microtiter plate overnight (Greiner, Nürtingen, Germany). Microtiter wells were washed three times with phosphate-buffered saline (PBS; 7 mM Na<sub>2</sub>HPO<sub>4</sub>, 3 mM NaH<sub>2</sub>PO<sub>4</sub>, 130 mM NaCl [pH 7.4]), dried in an inverted position, and stained for 10 min with 0.4% crystal violet solution [5]. The absorbance at 490 nm was determined. An OD<sub>490</sub> of >0.120 was regarded as biofilm positive.

### Isolation of Chromosomal DNA and Southern Hybridization

The bacterial cells were grown in TSB medium overnight at 37°C. The overnight cultures were diluted in 1:100 ratio and grown to the mid-log phase in Luria-Bertani broth (Difco) supplemented with 1% glycine at 37°C. Bacteria were harvested by centrifugation of 1.5 ml of the culture. The bacterial pellet was resuspended in 100  $\mu$ l of 10 mM Tris-HCl (pH 7.5) containing 25% sucrose, 15  $\mu$ l of 500 mM EDTA (pH 8.0), and 10  $\mu$ l of lysostaphin (2 mg/ml) (Sigma, Deisenhofen, Germany), and incubated for 10 min at 37°C. After lysis of the bacterial cells, the chromosomal DNA was isolated by standard procedures [30]. After restriction endonuclease digestion, the DNA was analyzed by agarose gel electrophoresis (1% agarose in Tris-phosphate-EDTA). Gels were blotted on nylon membranes (Hybond-N<sup>+</sup>, Amersham Life Science, Little Chalfont, England) by standard methods [30]. Labeling of DNA probe and hybridization were performed by using the nonradioactive ECL direct nucleic acid-labeling and detection system (Amersham Life Science) according to manufacturer instructions. For hybridization, the hybridization buffer contained 0.5 M NaCl, and high-stringency washing steps were performed at 55°C in a buffer containing 0.1 $\times$  standard saline citrate (SSC) and 0.4% sodium dodecyl sulfate.

### Northern Blot Analyses

Total RNA of the *S. aureus* strains was isolated from exponentially growing cells with a "FastRNA" kit (Dianova, Germany). Eight  $\mu$ g of total RNA of each sample was electrophoresed through a 1.2% agarose-0.66 M formaldehyde gel in morpholinepropanesulfonic acid (MOPS) running buffer (20 mM MOPS, 10 mM sodium acetate, 2 mM EDTA). Gel electrophoresis was performed at 5 V/cm for 3 h. The gel was blotted onto a nylon membrane (Hybond-N<sup>+</sup>; Amersham Life Science) and hybridized with an ECL-labeled (Amersham Life Science) DNA probe.

### Induction Test for the Biofilm Formation

Bacteria (*ica* gene +, biofilm -) were grown overnight in TSB medium. The fresh TSB media were mixed with each of various subinhibitory concentrations of two antibiotics (tetracycline or pristinamycin) or sodium chloride. The tetracycline and pristinamycin ranged from 16  $\mu$ g/ml to 0.03  $\mu$ g/ml for the sensitive isolates, but for the resistant isolates from 120  $\mu$ g/ml to 0.9  $\mu$ g/ml, and sodium chloride ranged from 2% to 5%. The overnight bacteria culture were diluted at 1:200 ratio with each mixed media, as described previously, and incubated overnight in a tissue culture microtiter plate. After that, we performed biofilm assay.

### Isolation of Biofilm-positive Variants by Passage Culture Method

To obtain adhering *S. aureus* variants, single colonies were picked and incubated in fresh sterile TSB in a tissue culture flask (Greiner) at 37°C. After 24 h, the medium was replaced. This procedure was repeated until a biofilm of adhering bacteria became visible on the bottom of the flask. Colony morphology of the variants was studied

**Table 1.** Primers used in this study.

Target gene	Primer sequence (5' to 3')
<i>icaAD</i>	TACCGTCATACCCCTTCTCTG GACAAGAACTACTGCTGCGT
<i>icaB</i>	CCCAACGCTAAAATCATCGC ATTGGAGTTCGGAGTGACTGC
<i>icaC</i>	CATGAAAATATGGAGGGTGG TCAAACCTGATTCGCCACCG
<i>agr</i>	CATAGCACTGAGTCCAAG ATCCCTAATCGTACTTGC
<i>sarA</i>	CCCAGAAATACAATCACTGTG AGTGCCATTAGTGCAAACC
<i>sigB</i>	GGACAATCACATCACGAAG CTAGCAGTGTAAATACTGC
<i>asp23</i>	GCATACGACAATCAAACCTGG CACTAAAATGGTGTGCCGAG
<i>rsb-seq-for.</i>	TCAGTTAGAGGTAGAGTGTT
<i>rsb-seq-rev.</i>	CACAGCTTCACTAACTGCAA
<i>sigB-seq-rev.</i>	CTGATAGAAGGTGAACGCT

on CRA, which was prepared by adding 0.8 g of Congo red (Serva, Heidelberg, Germany) and 36 g of saccharose (Roth, Karlsruhe, Germany), both of which had been previously autoclaved separately, to 1 l of brain heart infusion agar (Oxoid, Basingstoke, England). Plates were incubated for 24 h at 37°C and subsequently overnight at room temperature. Colony morphology was examined with a plate microscope.

#### Complementation of *sigB*

For the complementation, the *sigB* gene was subcloned in the pBluescript vector and transformed into *E. coli* TOP10F'. The pRSETc expression vector (Invitrogen) of the 6× Histidine Fusion System was used for cloning the SigB. The plasmid encoding SigB was constructed by inserting the BglIII-EcoRI DNA fragment into the multiple cloning site of the vector. A biofilm-producing variant was transformed with this ligation product.

#### Pulsed-Field Gel Electrophoresis

DNA isolation from *S. aureus* and pulsed-field gel electrophoresis were performed as described previously [15, 22].

#### Detection of PIA Expression by Use of Immunoblot

PIA detection was performed as described previously [6]. Cells were grown overnight in TSB supplemented with 0.25% glucose, the optical density was determined, and an equal number of cells (1.5 to 2 ml) from each culture was resuspended in 50 µl of 1.5 M EDTA (pH 8.0). Cells were then incubated for 5 min at 100°C and centrifuged to pellet the cells, and 40 µl of the supernatant was incubated with 10 µl of 10 mg/ml proteinase K (Boehringer GmbH, Mannheim, Germany) for 1 h at 37°C to minimize nonspecific background. The extracts were then spotted onto a nitrocellulose membrane, and the membrane was dried, blocked with 3% bovine serum albumin, and incubated overnight with an anti-*S. epidermidis* PIA antibody. Anti-PIA antibodies were detected using a biotin-conjugated anti-rabbit immunoglobulin G (IgG) antibody (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) diluted 1:5,000, horseradish peroxidase-conjugated streptavidin (Amersham Buchler GmbH & Co. HG, Braunschweig, Germany) diluted 1:3,000, and the Amersham ECL (enhanced chemiluminescence) Western blotting system.

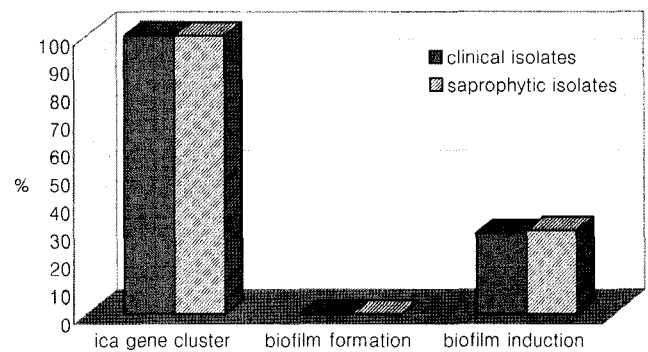
#### Sequence Analysis

Nucleotide sequence analysis was performed on an ABI Prism 310 sequence by capillary electrophoresis using the ABI Prism dGTP BigDye Terminator Ready Reaction kit (Perkin-Elmer Corporation, Weiterstadt, Germany). Nucleotide sequences were subsequently analyzed with HUSAR software (DKFZ, Heidelberg, Germany).

## RESULTS AND DISCUSSION

#### Biofilm Formed by *ica* Gene Cluster and Induction for the Biofilm Expression in *S. aureus*

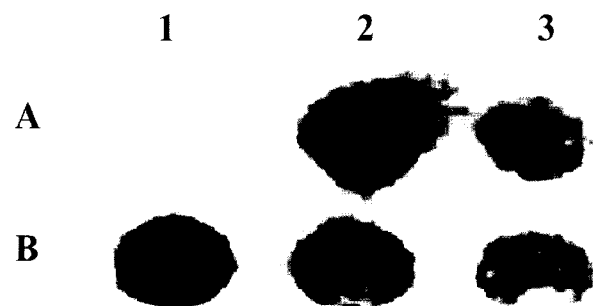
To compare the presence of the *ica* gene cluster on chromosomal DNA among *S. aureus* isolates from several sources, chromosomal DNA was isolated from the strains, digested with EcoRI, and tested by Southern hybridization. The chromosomal DNA was hybridized with the PCR



**Fig. 1.** Distribution of the *ica* gene cluster, biofilm formation, and induction for the biofilm expression in the tested *S. aureus* strains.

product of each *icaAD, B*, and *C*. For this experiment, 112 *S. aureus* strains (*i.e.*, 95 clinical isolates from patients and 17 saprophytic isolates) were tested. In *S. aureus*, no difference of the *ica* gene locus presence between the clinical and saprophytic isolates could be observed. The *ica* genes were present in all strains tested. To obtain information on the biofilm formation of the clinical and saprophytic isolates in *S. aureus*, the quantitative biofilm assay in polystyrol microtiter plates was used. In *S. aureus*, none of these strains formed biofilms (Fig. 1). These data indicate that the *ica* gene locus in *S. aureus* is not expressed in regular laboratory media, although this gene was commonly present in all *S. aureus* strains.

*Staphylococcus* is an extremely adaptable microorganism, capable of changing rapidly its phenotypic and genotypic characteristics [1, 10, 35]. Antibiotics have been shown to alter both the structure and physiology of *Staphylococcus* when the organism is exposed to concentrations below the minimum inhibitory concentration (MIC) [13]. We analyzed the coordinate expression of the *ica* gene cluster from these strains by induction for the biofilm formation with tetracycline, pristnamycin, or natrium chloride. The bacteria were grown in TSB medium supplemented with the subinhibitory concentrations of the antibiotics or the



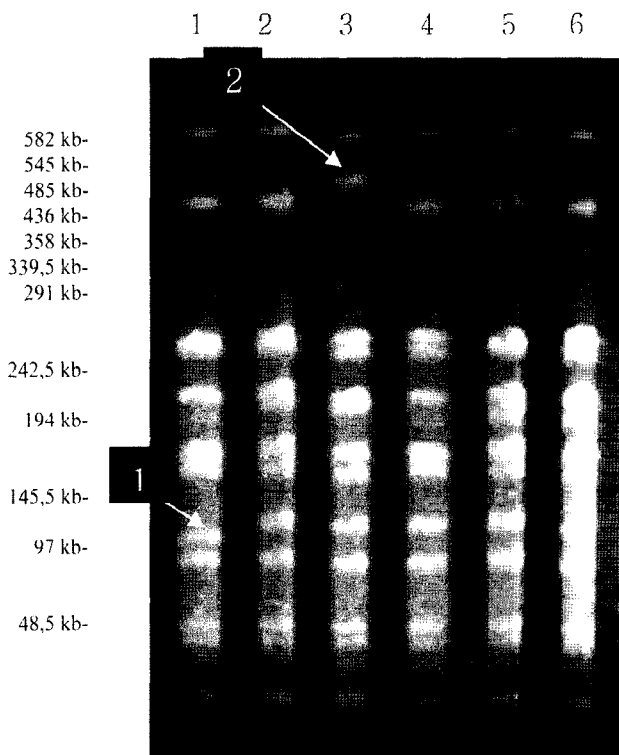
**Fig. 2.** PIA production of the non-adhering wild-type strains and biofilm-producing variants. Lane A1, non-adhering wild-type strains; lanes A2–3, B1–3, biofilm-producing variants.

concentrations of 2%–5% sodium chloride. As seen in Fig. 1, in the treatment of these inducing substances, the induction of biofilm formation showed 29% in the clinical isolates and 30% in the saprophytic isolates. However, this phenotype was temporary, because the biofilm induction disappeared without treatment of the induction substance during continued cultivation.

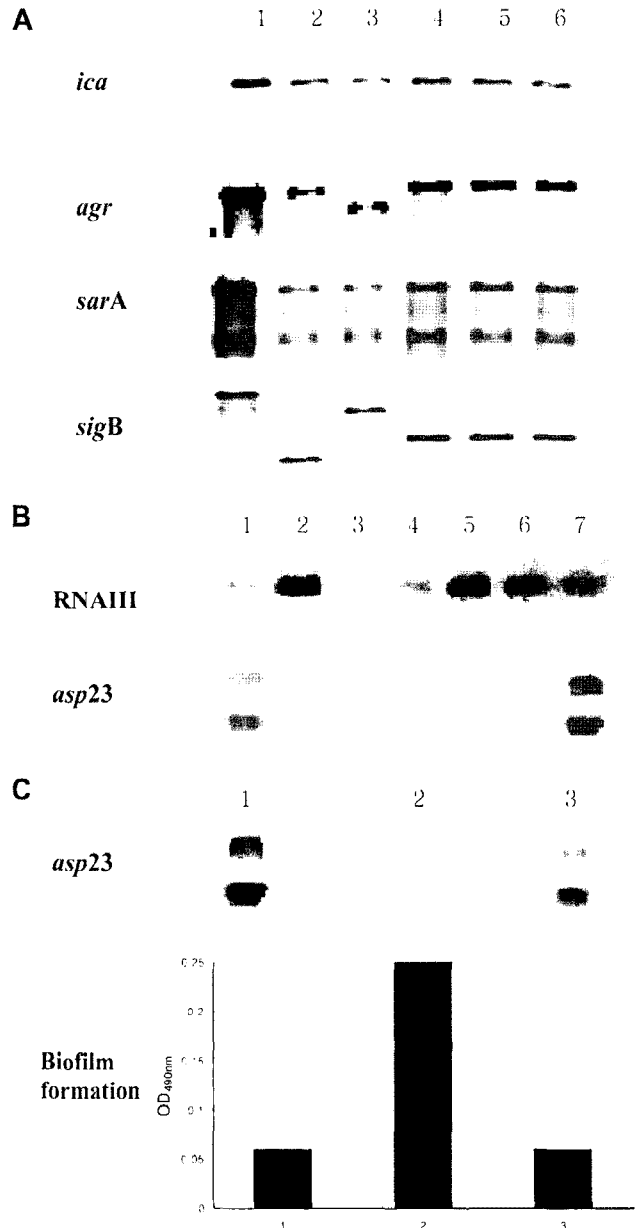
**Isolation and Characterization of Adhering *S. aureus* Variants**

In order to obtain consistently induced isolates, passage culture method was used. From one strain, *S. aureus* 483, we have isolated five biofilm-producing variants through repeated passage cultivation and subcultivation on Congo red agar. Additionally, *ica* expression of these strains was tested with the immunoblot method. The expression of *ica* genes was detected only in five variants (Fig. 2). To decide whether the five variants represent contamination or real variants from the original parental strains, the restriction length polymorphism was tested by pulsed-field gel electrophoresis. These experiments revealed some detectable differences between these strains (Fig. 3). In the arrow 1 of Fig. 3, the DNA size of wild-type strain (lane 1) was different from the other strains between 97 kb and 145.5 kb. Between the sizes of 436 kb and 485 kb, the

upper second band of lane 3 was some higher than those of other lanes (arrow 2). However, the strains from lane 2 to lane 6 are real variants from the parental strain of lane 1, although there are some differences of DNA band sizes between the strains. These differences imply that genomic rearrangements may come from insertions or deletions of DNA fragments.



**Fig. 3.** Analysis of *Sma*I-digested genomic DNA of non-adhering wild-type strain and biofilm-producing variants by pulsed-field gel electrophoresis. Lane 1, non-adhering wild-type strain; lanes 2–6, biofilm-producing variants.



**Fig. 4.** Southern blot analysis of *ica*, *agr*, *sarA*, and *sigB* genes (A), investigation of *RNAIII* and *asp23* transcription by Northern blot analysis (B), and complementation of *SigB* and the biofilm formation (C).

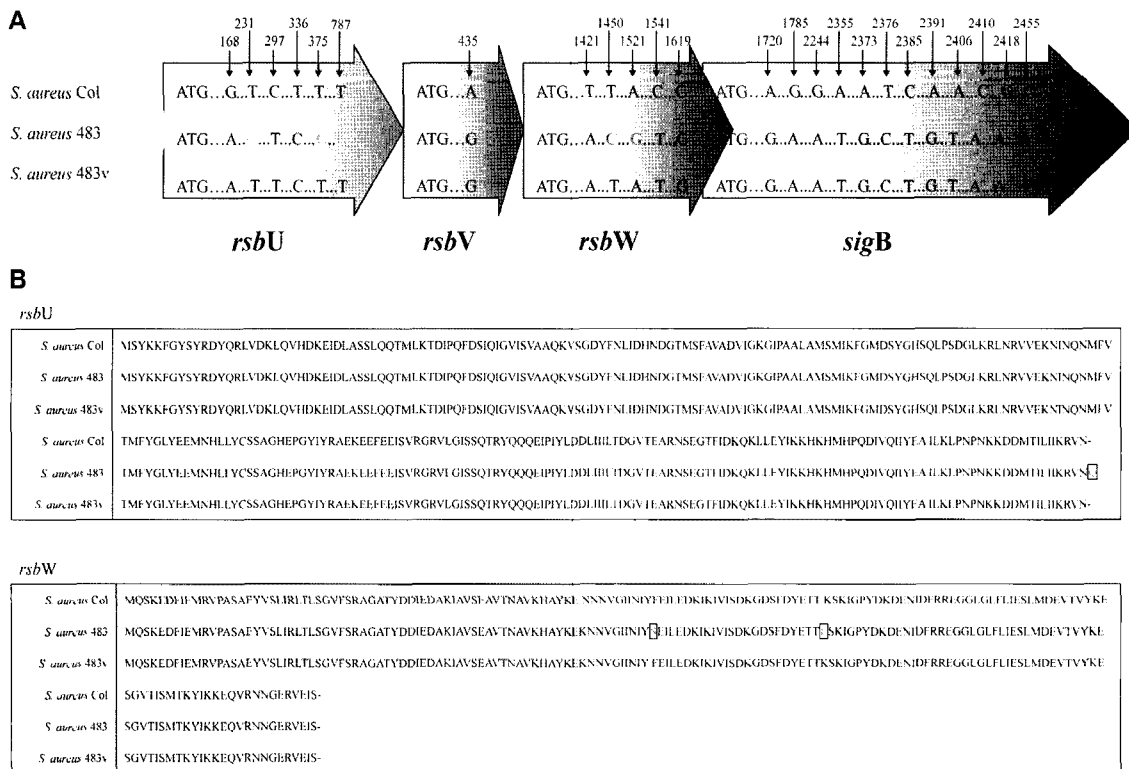
A. Lane 1, non-adhering wild-type strain; lanes 2–6, biofilm-producing variants. B. Lane 1, non-adhering wild-type strain; lanes 2–6, biofilm-producing variants; lane 7, positive control, *S. aureus* MA12. C. Lane 1, non-adhering wild-type strain; lane 2, a biofilm-producing variant (lane 2 of Fig. 4A); lane 3, complementary strain of *SigB* into a biofilm-producing variant of lane 2.

**Relation of the Biofilm Variation with Insertion Sequences, Agr, SarA, and SigB in *S. aureus* 483**

To test whether deficiency of the biofilm formation in the parental strain was influenced by any insertion sequence elements, all strains were hybridized with an *icaC* gene probe on EcoRI-digested Southern blots. It is well known that in *S. epidermidis*, phase variation of biofilm formation is caused by insertional inactivation of the *ica* gene cluster by insertion sequence element IS256 [3, 34]. Recently, the role of IS256 in the mechanism of phase variation in biofilm formation of *S. aureus* was reported [19]. However, it was shown in our data that no insertions and deletions within the *ica* operon were found on the Southern blot. This implies that any of the insertion sequence elements was not present in the *ica* operons of these strains and have not influenced on the *ica* expression. Moreover, the sequence analysis of the *ica* operon indicated that there were no differences of *ica* DNA sequence between the parental strain and variants (data not shown). These data lead us to conclude that the biofilm variation is not caused by a mutation of the *ica* gene cluster. Therefore, we have focused on regulator genes for the demonstration of these variations.

Global quorum sensing regulators have been shown to regulate gene expression of many bacteria [2, 8, 18]. It has been reported that the accessory gene regulator (Agr) and the

staphylococcal accessory regulator (SarA) of *S. aureus* seem to be negatively correlated with the ability to produce biofilm [26, 32]. Recently, it has been demonstrated that the alternative transcription factor  $\sigma^B$  (*sigB*) is required for the biofilm formation by controlling the transcription of the *ica* operon [27]. This demonstration was contradicted in another paper reporting that SarA and not  $\sigma^B$  is essential for biofilm development by *S. aureus* [31]. However, the regulatory mechanisms involved in the *S. aureus* biofilm development remain poorly understood. To gain insight into the molecular mechanisms regulating the biofilm phenotype, *agr*, *sarA*, and *sigB* genes were investigated. Southern hybridization of chromosomal DNA with a *sigB*-specific gene probe revealed four different DNA band shifts (Fig. 4A). To compare the mRNA expression of the strains, the transcription of RNAIII, effector molecule of Agr and alkaline-shock protein (*asp23*) gene, a regulon of SigB, were investigated by Northern blot analysis (Fig. 4B). The differences of lane 3 in Figs. 4A and 4B from other lanes may correlate to the PFGE results (arrow 2) in Fig. 3. This result indicates that a variation occurred in near of *tge agr* genes and it had an effect on the expression of RNAIII. Hybridization of total RNA with an *asp23*-specific probe revealed that the transcription was detectable only in a wild-type strain, *S. aureus* 483. A SigB-negative variant was complemented with the *sigB* gene cloned in



**Fig. 5.** Sequence analysis (A) and the deduced amino acids (B) of the *sigB* operon in the non-adhering wild-type strain *S. aureus* 483 and a biofilm-producing variant *S. aureus* 483v. As a standard strain for the sequence comparison, *S. aureus* Col was used.

vector pRSETc. The complemented strains could not produce the biofilm (Fig. 4C). This result indicates that SigB plays an important role for this biofilm variation in *S. aureus* 483 and the biofilm-producing variants by downregulating the *ica* genes.

### Sequence Analysis of SigB Operon of the Strains

From the result of the Northern blots, we have speculated that mutations are present in near of the *sigB* genes. The regulation and function of *sigB* is well characterized for *Bacillus subtilis* and *S. aureus*. In *S. aureus*, the *sigB* regulates large number of virulence factors [14] and consists of four genes, *rsbUVWsigB*, as an operon [21, 34]. It was demonstrated that RsbW acts as an anti-sigma factor in *S. aureus* [24], and that RsbU and RsbV act as positive regulators of *sigB* activity [25]. The PCR products of the *sigB* operon with 2.8 kb from two different strains, non-adhering wild-type strain *S. aureus* 483 and a biofilm-producing variant *S. aureus* 483v, were compared with each other. As a standard strain for the sequence comparison, *S. aureus* Col was used. The sequence analysis showed that the sequence of *S. aureus* 483 was different, whereas that of other strains possessed the same nucleotides. The important changes of DNA- and amino-acid-sequences were detected in *rsbU* and *rsbW* of *S. aureus* 483. In *S. aureus* 483 in comparison with other strains, three point mutations in *rsbU* and two point mutations in *rsbW* were found (Fig. 5A). The deduced amino acids from these mutations were changed. The changed amino acids are shown in Fig. 5B. One amino acid in *rsbU* and two amino acids in *rsbW* were changed. Interestingly, the changed amino acid in *rsbU* was the stop codon. Owing to the mutation in the stop codon, the protein of *rsbU* in *S. aureus* 483 might be enlarged or not be expressed, or owing to the changes of amino acids in *rsbW*, the function of RsbW might be lost.

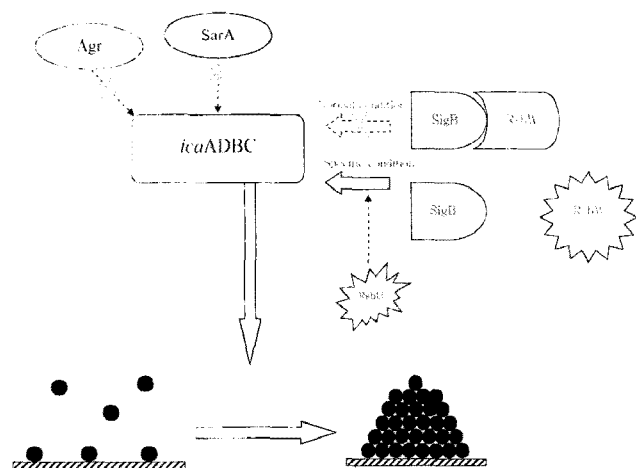


Fig. 6. Summary illustrating the role of SigB in the regulation pathway leading to the biofilm formation in *S. aureus*. SigB downregulates the biofilm formation through *ica* expression.

However, further study on the function of RsbU and RsbW for the SigB expression is necessary.

Our study demonstrates that the heterogeneous expression of the *ica* gene cluster in *S. aureus* for the adaptation to changing environmental conditions is controlled by SigB. The data obtained in this study enables us to draw up a model of regulation of PIA synthesis in *S. aureus* (Fig. 6). The biofilm formation by *ica* expression is blocked in the normal environmental conditions because of the suppression of *sigB* transcription. However, upon the specific environmental conditions, SigB may play a role in the biofilm formation. The restored *sigB* expression due to inactivation of RsbU or RsbW downregulates the *ica* operon expression.

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