Production profile of the soluble lytic transglycosylase homologue in *Staphylococcus aureus* during bacterial proliferation

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Abstract

The immunodominant antigen A, IsaA, of *Staphylococcus aureus* is considered to be a sequence homologue of Gram-negative soluble lytic transglycosylase. Recently, it was reported that IsaA production is probably regulated by the essential two-component regulatory system consisting of YycG and YycF. Therefore, we investigated the *isaA* expression profile by quantifying its gene products at different stages of bacterial growth. In the culture supernatant, IsaA was detectable in the early exponential growth phase and its concentration constantly increased until the early stationary phase. Cell surface IsaA was mainly found on growing bacteria, and disappeared proportionately with a decline in cell proliferation. On the other hand, the *isaA* transcript rapidly increased at the beginning of the culture, and then stayed at a constant level until the late exponential growth phase. These findings indicated that *isaA* gene expression was stimulated during the exponential growth phase and repressed in the stationary phase. Thus, IsaA production was associated with active bacterial growth. Our results support the previous report that *isaA* expression is regulated by YycF, and also suggest that IsaA is probably involved in bacterial proliferation by residing on the cell wall. We also discuss the possibility that *isaA* transcription may be controlled by SarA.

Introduction

Immunodominant antigen A (IsaA) is a 29-kDa extracellular protein of *Staphylococcus aureus* (Lorenz *et al.*, 2000). We have previously reported that IsaA includes a possible soluble lytic transglycosylase (SLT) domain and is found on the bacterial cell wall as well as in the culture supernatant (Sakata *et al.*, 2005). The SLT domain is present in a number of prokaryotic, eukaryotic, and phage proteins (Dijkstra & Thunnissen, 1994; Koonin & Rudd, 1994; Mushegian *et al.*, 1996). Among the bacterial proteins, the IsaA SLT motif shows a high similarity to the soluble lytic transglycosylase Slt70 that is one of the *Escherichia coli* peptidoglycan-hydrolysis enzymes (Höltje *et al.*, 1975; Keck *et al.*, 1985; Engel *et al.*, 1991). This type of enzyme is generally found in Gram-negative bacteria and is considered to be an autolytic enzyme involved in the controlled turnover of the bacterial cell wall; it is not known in Gram-positive bacteria (Mushegian *et al.*, 1996; Höltje, 1998).

Recently, it was reported that IsaA might be regulated by the essential two-component regulatory system consisting of YycG and YycF (Dubrac & Msadek, 2004). This system was originally found in *Bacillus subtilis*, and subsequently the homologue has been reported in other low G+C Gram-positive bacteria. This YycG/YycF system is essential for cell growth in *B. subtilis*, *S. aureus* and *Streptococcus pneumoniae* (Fabret & Hoch, 1998; Lange *et al.*, 1999; Martin *et al.*, 1999; Throup *et al.*, 2000), and the inactivation of the *yycF/G* genes is lethal in some bacteria (Fabret & Hoch, 1998; Martin *et al.*, 1999; Fukuchi *et al.*, 2000; Dubrac & Msadek, 2004). The studies using conditional mutants indicated that this system is probably involved in proper regulation of bacterial cell wall and cell membrane composition, as well as bacterial cell division (Martin *et al.*, 1999; Fukuchi *et al.*, 2000; Dubrac & Msadek, 2004). In addition, the consensus sequence for YycF recognition has been defined in a promoter region of the putative regulon (Howell *et al.*, 2003; Dubrac & Msadek, 2004). In *S. aureus*, *isaA* and *lytM*, which codes a staphylococcal glycylglycine endopeptidase, were also reported to include such a target site, and YycF actually bound to their promoter regions (Dubrac & Msadek, 2004). This observation suggests that both staphylococcal genes are probably YycF regulons. It is known that *yycF/G* gene transcripts are mainly found during exponential growth phase (Fabret & Hoch, 1998; Fukuchi *et al.*, 2000). Consequently, the transcripts of proposed YycF

regulons including *lytM* are also increased during the same growth phase (Ramadurai & Jayaswal, 1997; Ramadurai *et al.*, 1999; Howell *et al.*, 2003). However, *isaA* expression profile has not yet been reported. If IsaA production is controlled by this regulatory system, the gene products should be detected in the same manner as those of the other proposed regulon. Moreover, we consider IsaA to be a homologue of the soluble lytic transglycosylase because of its SLT motif and cellular location, though the lytic activity to the staphylococcal whole cells was not yet found in our experiments using a recombinant IsaA protein. This suggests that IsaA production probably accompanies the bacterial growth. Therefore, we have studied the *isaA* expression profile by quantifying its gene products at different stages of bacterial growth.

Materials and methods

Preparation of FITC conjugates

Rabbit IgG was purified from specific antiserum or preimmune serum using MabTrap Kit (Amersham Bioscience) as described before (Sakata *et al.*, 2005). The conjugation of an anti-IsaA IgG or preimmune IgG with fluorescein isothiocyanate (FITC) was performed using FluoroTag^{TM} FITC Conjugation Kit (Sigma) according to the manufacturer's procedure. Briefly, FITC was added at a molar ratio of 10 : 1 to 1 mg of the purified IgG, in 0.1 M sodium carbonate buffer (pH 9.0). The labeling was performed in a final reaction volume of 0.25 mL by incubating for 2 h at room temperature with gentle stirring in the dark. The labeled protein was separated from free FITC by a Sephadex G-25 M PD-10 column (Amersham Bioscience). The fluorescein/protein ratio (F/P) of the conjugate was determined using a spectrophotometer. The anti-IsaA IgG-FITC and preimmune rabbit IgG-FITC were stored at 4 °C in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 0.1% sodium azide.

Comparison of IsaA extraction reagents

A *S. aureus* spontaneous protein A-deficient mutant, strain 2PF-18 (Sugai *et al.*, 1995), was used to extract IsaA from the bacterial cells. The bacterial cells were precultured in Tryptone-soy broth (TSB; Eikenkagaku), and then inoculated into 250 mL of the same fresh medium for additional incubation to the mid-exponential growth phase. The culture was centrifuged and the cells were washed twice with 0.9% NaCl. The cell pellets were resuspended in 4% sodium dodecyl sulphate (SDS) or 3 M LiCl and treated according to Sugai *et al.* (1990). The supernatant was collected and then buffer-exchanged for PBS using the Ultrafree-5 Filter Unit with a 5-kDa molecular weight cutoff (Millipore). All extracts were stored at − 20 °C for further experiments.

Preparation of culture supernatants and cell surface extracts for IsaA quantification

An overnight culture was prepared by inoculating one colony of *S. aureus* strain 2PF-18 into 40 mL of TSB at 37 °C. The cultures were centrifuged at 10 °C for 10 min, and the cells were washed three times with PBS and then suspended in the same buffer so that the absorbance at 420 nm was *c.* 0.5 (i.e. about 2×10^8 cells mL^{-1}). An appropriate volume of the suspension was added to 100 mL of TSB, and the culture was grown at 37 °C with shaking. After a specified incubation period, an aliquot of culture was diluted for cell counting on Tryptone-soy (TS) agar (Eikenkagaku) plates. Another aliquot was also collected to obtain culture supernatant and bacterial cells. The supernatant was passed through a 0.22-μm filter and stored at − 20 °C, and the cell pellets were washed three times with PBS and also stored at − 20 °C. The frozen cells were suspended in 3 M LiCl, with a cell density of about 5×10^9 cells mL^{-1}, and incubated at 4 °C for 60 min. All supernatants were incubated at 60 °C for 30 min and then subjected to a 0.22-μm filter. The filtrates were buffer-exchanged for PBS and concentrated as described above.

Quantification of IsaA protein

EIA plates (Sumitomo) were coated with a capture antibody by adding 100 μL of purified anti-IsaA IgG (20 μg mL^{-1}) in PBS and incubating overnight at 4 °C. The coated plates were washed once with PBS and then treated with Super Block^{TM} Blocking Buffer in PBS (Pierce), according to the manufacturer's procedure.

A collected culture supernatant with 0.1 vol of the blocking buffer was serially 10-fold diluted in TSB containing 0.1 vol of the blocking buffer (TSB-blocking buffer). The diluent (100 μL) was added to each well of the coated plate and incubated at room temperature for 2 h. After washing with PBS containing 0.05% Tween 20 (PBS-Tween), 100 μL of anti-IsaA IgG-FITC (2.5 μg mL^{-1}) diluted with PBS containing 0.1 vol of the blocking buffer (PBS-blocking buffer) was added, and then incubated at room temperature for 1 h. The plate was washed again with PBS-Tween, and treated with 100 μL of rabbit F(ab') anti-FITC alkaline phosphatase conjugate (DakoCytomation), diluted 1 : 400 in PBS-blocking buffer. After 1 h at room temperature, 100 μL of *p*-nitrophenyl phosphate substrate solution (Sigma) was added to the washed plate. To prepare a standard curve for the quantification, a poly histidine-tagged recombinant protein, His-IsaA (Sakata *et al.*, 2005), was also diluted in TSB-blocking buffer and used for the assay system.

Cell surface IsaA was also quantified in LiCl extracts in the same manner as described above, except that the samples and the standards were diluted using PBS-blocking buffer instead of TSB-blocking buffer.

The entire experiment for the IsaA protein quantification, including cell growth and preparation of the samples, was replicated once.

Preparation of a quantitative-PCR standard

Chromosomal DNA isolated from *S. aureus* strain 2PF-18 was utilized as a template for the amplification using 29B-1 and 29B-4 as a primer set (Table 1) (Sakata *et al*., 2005). The reaction was performed using HotStarTaq® DNA polymerase (Qiagen), and the PCR product (628 bp) corresponding to an ORF of *isaA*, excluding the signal peptide coding region, was purified by QIA quick PCR purification kit (Qiagen). The absorbance at 260 nm was measured to evaluate its concentration. The *isaA* PCR product was diluted and subjected to quantitative PCR as a standard.

RNA extraction and cDNA synthesis

Bacteria were grown in TSB, as described above, for RNA extraction. After the specified culture period, an aliquot was plated on TS agar for cell counting. Another aliquot was mixed immediately with 2 vol of RNAprotect Bacteria Reagent (Qiagen) by vortexing and allowed to stand at room temperature for 5 min. The cells were then collected by centrifugation at 5000 ***g*** for 10 min, and stored at − 80 °C. The stored bacterial cells, 1×10^7 to 6×10^8 cells, were resuspended in 200 μL of 10 mM Tris-HCl with 1 mM EDTA (pH 8.0) containing lysostaphin 1 mg mL^{-1} (Wako chemicals) and 10 μL of Proteinase K solution (Qiagen), and then incubated for 4 h at 37 °C. Total RNA was extracted from the suspension using RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Genomic DNA contaminating the extract was digested by RNase-free DNase I (Qiagen) on the RNeasy column membrane during the purification. The RNA was finally dissolved in 50 μL of RNase-free water and stored at − 80 °C.

First-strand cDNA synthesis was performed according to the manufacturer's instructions using SuperScript III Platinum Two-Step qRT-PCR kit (Invitrogen). Briefly, DNase-treated RNA, up to 0.2 μg, was mixed with reverse transcriptase in 20 μL of total volume, and incubated at 25 °C for 10 min and then at 42 °C for 50 min. The reaction was terminated by incubating at 85 °C for 5 min, and the mixture was chilled on ice. It was then treated with 2 U of RNase H by incubating at 37 °C for 20 min, and stored at − 20 °C until use. To test for genomic DNA contamination, the cDNA synthesis reaction without reverse transcriptase was similarly performed for each RNA sample.

Table 1. Primers used in this study

Primer	Nucleotide position	Sequence (5′–3′)
29B-1	84 to 106	CGCTGCTGAAGTAAACGTTGATC
29B-4	711 to 688	ATGAAGGAATTAGAATCCCCAAGC
Isa-RTP1	460 to 481	GCAGGTGCTACTGGTTCATCAG
Isa-RTP2	551 to 532	GATTCACGAGCGATGATTGC
Isa-pro1	− 526 to − 504	ATTGAGCTGCAGTTTATTTGTGC
Isa-pro2	100 to 80	CGTTTACTTCAGCAGCGTGTG

Real-time quantitative PCR

Prior to quantification, each cDNA synthesis reaction mixture was diluted in autoclaved distilled water. Quantification was performed in ABI Prism 7000 Sequence Detection System (Applied Biosystems) with Platinum SYBR Green qPCR SuperMix UDG (Invitrogen), using 10 μL of the diluted cDNA reaction mixture or the purified *isaA* PCR product for the preparation of a standard curve, and 10 pmol of each primer (Isa-RTP1 and Isa-RTP2; Table 1), in a final volume of 50 μL. These primers were newly developed for this work to be at 60 °C of Tm and *c.* 100 bp of the amplified product length. The thermal cycling reaction conditions were as follows: 2 min at 50 °C, 2 min at 95 °C followed by 50 repeats of 15 s at 95 °C, and 30 s at 60 °C. Product specificity was evaluated by melting curve analysis. A negative control (distilled water) and an RNA sample from cDNA synthesis mixture without reverse transcriptase were also subjected to quantitative PCR. The amplification of *isaA* transcript was quantified using a standard curve prepared with a known concentration of the *isaA* 628 bp PCR fragment, and measurements were carried out in triplicate. The standard curve was generated in duplicate using 10-fold serial dilutions (20 to 2×10^6 copies μL^{-1}) of the *isaA* PCR product.

Sequencing of the isaA promoter region

The *isaA* 5′-flanking region of *S. aureus* strain 2PF-18 was amplified using Isa-pro1 and Isa-pro2 as a primer set that was newly developed for this work (Table 1). The PCR product (626 bp), corresponding to − 526–100 nt of the gene, was purified by QIA quick PCR purification kit. Sequencing was performed by ABI PRISM 3100 Genetic Analyzer using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

Protein assay

A protein assay was performed with a BCA (bicinchoninic acid) protein assay reagent (Pierce) using BSA as a standard. Briefly, 0.1 mL of each standard or unknown protein sample was mixed with 2 mL of Working Reagent for the protein assay. For blanks, the diluent was used. After incubation at 60 °C for 30 min, the absorbance of the mixture at 562 nm was measured. All assays were performed in duplicate.

Results

Sensitivity and specificity of the quantification system

To quantify the IsaA protein, we first established a sensitive enzyme-linked immunosorbant assay (ELISA) system. In this system, purified anti-IsaA IgG and its FITC conjugate were used to capture and detect the IsaA protein, respectively. The specificity of the system was evaluated using a recombinant IsaA protein, His-IsaA, as a standard (Fig. 1). Serial diluted His-IsaA was added to the assay plate coated with capture antibody, and then the plate was treated with FITC-labeled anti-IsaA or preimmune rabbit IgG. His-IsaA was detected in direct proportion to its concentration by FITC conjugated anti-IsaA, but no signal was found with the FITC-preimmune IgG, up to a concentration of 100 ng mL^{-1} of His-IsaA. Moreover, higher intensity signals were observed as an increasing dose of the FITC-conjugate was added to the assay system. Accordingly, we concluded that IsaA protein was specifically detectable with this system.

IsaA is found both in the culture supernatant and on the bacterial cell wall (Lorenz *et al.*, 2000; Sakata *et al.*, 2005). To quantify the IsaA protein in each fraction, standard curves were separately prepared using TSB-blocking buffer or PBS-blocking buffer as sample diluents. In both standard curves, the relationship between the absorbances at 405 nm and the IsaA concentrations was consistently linear with a correlation coefficient greater than 0.996 over a concentration range of 0–40 ng mL^{-1} (data not shown). Therefore, this range was used for preparing a standard curve for the quantification.

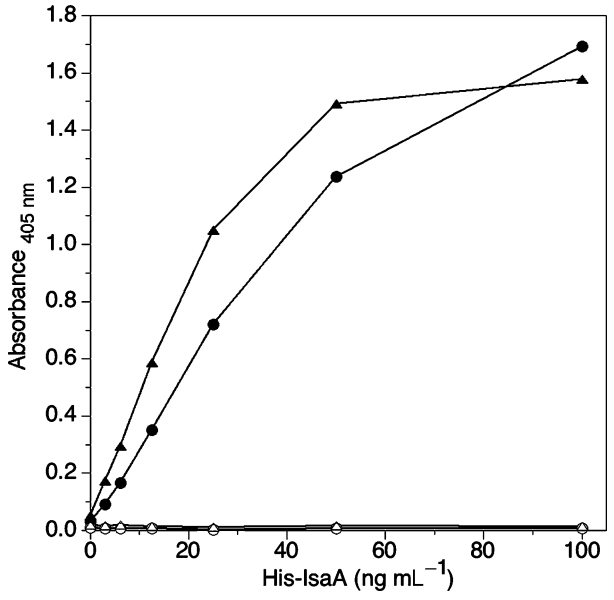

Fig. 1. Specificity of quantitative ELISA for IsaA protein. A poly histidine-tagged recombinant IsaA protein, His-IsaA, was diluted in PBS-blocking buffer and then subjected to the ELISA. The captured His-IsaA on the assay plate was detected with FITC-labeled anti-IsaA (●, 2.5 μg mL^{-1}; ▲, 5.0 μg mL^{-1}) or preimmune rabbit IgG (○, 2.5 μg mL^{-1}; △, 5.0 μg mL^{-1}). The values presented are means of duplicates.

Table 2. IsaA extraction from the bacterial cells with SDS or LiCl

Extraction solvent	Cell weight (g)	Extract vol (μL)	Protein conc. (mg mL^{-1})	IsaA conc. (μg mL^{-1})
SDS	0.312	200	6.2	118.7 ± 3.8
LiCl	0.292	200	1.4	120.4 ± 1.0

Comparison of cell surface IsaA extraction

SDS and LiCl are the most useful reagents for extracting bacterial cell surface proteins, and so both reagents were tested for IsaA isolation from the bacteria cells. The same wet weight of cells from a mid-exponential growth phase was treated with SDS or LiCl as described in the Materials and methods section. The extracts were buffer-exchanged and concentrated using a membrane filter with a 5-kDa molecular weight cutoff, and subjected to quantitative ELISA. SDS treatment was more efficient in total protein extraction than LiCl (Table 2). However, there was no difference in the total amount of IsaA between the two samples (23.7 and 24.0 μg). This indicated that almost all the cell surface IsaA was extracted by LiCl. Therefore, we used LiCl extraction for the following experiments.

Cell growth and IsaA protein production

Using the developed quantitative ELISA system, the IsaA production profile during bacterial growth was studied. *Staphylococcus aureus* strain 2PF-18 cells were grown in TSB, and an aliquot of the culture was taken at different culture periods for preparing a supernatant and a LiCl cell extract. These samples were subjected to the assay system using the standard curve in simultaneous measurements.

When the bacteria were inoculated with 1.1×10^6 CFU mL^{-1} of cell density, the IsaA protein was already detectable in the culture supernatant at a concentration of 14.93 ± 0.29 ng mL^{-1} after 1 h. Subsequently, the value rose exponentially in direct proportion to the increase in the number of bacterial cells (Fig. 2a). Once the growth reached the early stationary phase, 6 h after inoculation, the value declined and the concentration then remained at about 20 μg mL^{-1}. This indicated that IsaA production in the culture medium started in the early growth phase, was sustained during the exponential growth phase, and then stopped in the stationary phase. In other words, IsaA production accompanied bacterial growth.

The amount of cell surface IsaA was about 0.07 ng 10^8 CFU^{-1} before the culture started. This value increased to 1.91 ± 0.03 ng after incubation for 3 h (the mid-exponential phase), and thereafter 1.5–3.6 ng of IsaA was retained on the

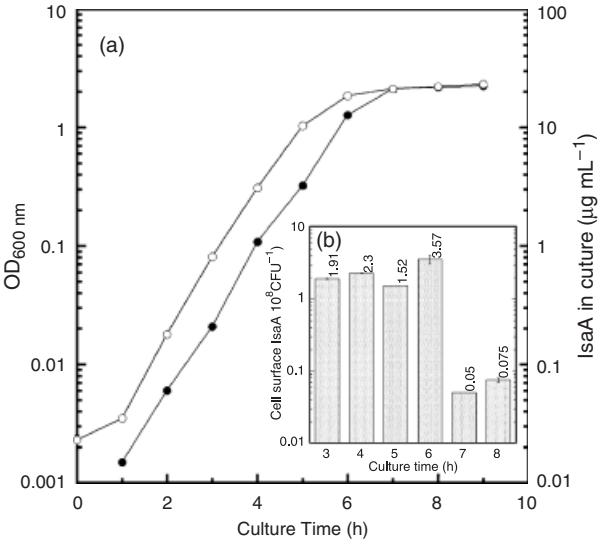

Fig. 2. Bacterial growth and IsaA protein production. The culture supernatant and the cell surface extract were prepared at different culture periods, and then subjected to quantitative ELISA. (a) $OD_{600\,nm}$ of the culture (○) and IsaA concentration in the supernatant (●). (b) IsaA on the cell surface was represented as an amount 10^8 CFU^{-1}. The values presented are means with SEs, and are also given above the bars.

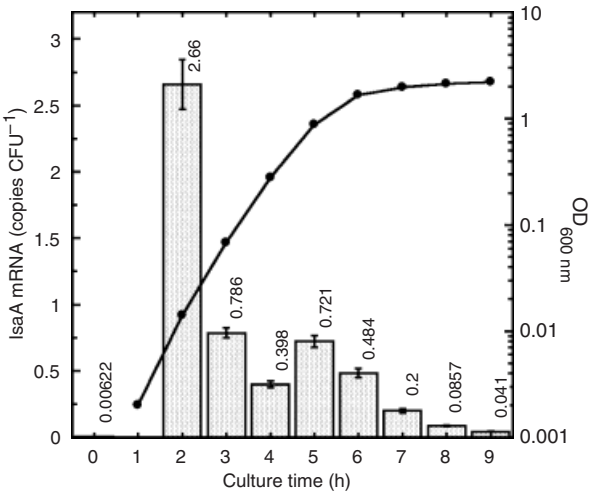

Fig. 3. Expression profile of *isaA* during bacterial growth. Each bar represents the number of copies of *isaA* mRNA CFU^{-1}, and the values are also given above the bars. Filled circles with solid lines represent bacterial growth as $OD_{600\,nm}$. Values are means with SEs from three measurements.

cell surface for up to 6 h (Fig. 2b). As the cells entered the stationary phase, the amount suddenly decreased to the level that existed before starting the culture, about 0.05–0.08 ng 10^8 CFU^{-1}. This suggested that a certain amount of IsaA was ordinarily maintained on the growing bacterial cell surface, and that it was dispersed with the decline in cell proliferation.

A similar production profile in both cell fractions was obtained from another independent experiment for reproducibility.

Cell growth and IsaA gene expression

For the gene expression profile, the *isaA* transcripts were obtained at each culture stage. Cells before inoculation were also used for RNA extraction. Total RNA samples were subjected to first strand cDNA synthesis and quantified as described in Materials and methods. In this experiment, purified *isaA* ORF PCR product was used as a standard for RT-qPCR. Each standard curve was made in duplicate, using 10-fold serial dilution of the standard DNA fragment from 20 to 2×10^6 copies μL^{-1}. The measurements were performed in triplicate. The standard curves for the three measurements gave regression lines with slopes of -3.577, -3.4731 and -3.4416 and correlation coefficients of 0.9997, 0.9999 and 0.9997, respectively. Raw data of the quantifications were reported for each standard curve in simultaneous measurements. The results were then divided by the number of CFU in the same culture (Fig. 3). The number of *isaA* transcripts increased immediately after the start of the culture, and reached 2.66 ± 0.19 copies CFU^{-1} after incubation for 2 h, which was about 400-fold greater than the number in the cells before inoculation. The number of transcripts rapidly declined and then reached the constant level of *c.* 0.4–0.8 copies CFU^{-1}, which persisted until the late exponential stage, although a transient decline was observed after incubation for 4 h. In the stationary phase, the number of transcripts gradually decreased, and finally reached 0.041 ± 0.002 copies CFU^{-1} after 9 h culture. In the samples, contamination with genomic DNA was between 0.2% and 4.4% of a total amount of mRNA. The entire experiment, including mRNA isolation, was replicated once, and a similar expression profile was obtained.

Discussion

IsaA is a staphylococcal extracellular protein that possesses an SLT domain in its C-terminal region (Sakata *et al.*, 2005). This staphylococcal protein is found in the culture broth and also on the bacterial cell surface due to a preferential interaction with a peptidoglycan chain (Sakata *et al.*, 2005). In this study, we defined the *isaA* expression profile by quantifying the gene products, mRNA and IsaA protein in each fraction at different stages of growth (exponential, transitional, and stationary phases).

In the culture supernatant, IsaA was already detected at an early growth stage, and then increased exponentially until the transitional stationary growth phase. As the cells entered the stationary phase, no further increase was found. This

observation indicated that IsaA secretion started at the beginning of growth, and that constant production was maintained until the stationary phase. On the cell surface, a certain amount of IsaA was constantly found during the exponential phase, and this was maintained until the early stationary phase. Once the growth reached the stationary phase, this dropped to the level before inoculation. That is, IsaA is mainly expressed on the growing bacterial cell surface. These results suggest that IsaA protein production is associated with active bacterial cell growth. We consider IsaA to be a homologue of Gram-negative bacterial exomuramidases like *E. coli* SLT70, because the staphylococcal protein possesses an SLT motif that is common in such types of enzymes (Sakata *et al.*, 2005). Therefore, IsaA is expected to work on the bacterial cell surface. It is likely that IsaA is first retained on the cell wall, and then released into the medium. Actually, in our study, cell surface IsaA rapidly diminished between 6 and 7 h of culture, whereas in the culture broth it was still increasing at this time point. This prolonged increase could probably be attributed to a release of cell surface IsaA after suspension of the supply to the cell wall.

We have revealed that IsaA protein production accompanies bacterial proliferation. Such an expression profile was also supported by IsaA mRNA analysis. During early exponential growth, after 2 h of culture, the IsaA mRNA was about 400-fold increased (number of copies CFU^{-1}) compared with its level in the cells before inoculation. Subsequently, the number of transcripts was immediately reduced, and a constant level was maintained until the last stage of exponential growth. In the stationary phase, the mRNA gradually decreased and approached the level that existed before starting the culture. That is, the *isaA* expression was carried out mainly in the exponential growth phase, and not in the stationary phase. The rapid increase at the early stage may suggest that sufficient IsaA has initially to be provided to the bacterial cell surface to start the growth.

Recently, the essential two-component regulatory system, YycG/F, was identified in low G+C Gram-positive bacteria (Fabret & Hoch, 1998; Martin *et al.*, 1999; Throup *et al.*, 2000). YycF, a response regulator of this system, was reported to stimulate the expression of several genes for bacterial cell proliferation (Fukuchi *et al.*, 2000; Martin *et al.*, 2002; Howell *et al.*, 2003). In addition, the consensus sequence for YycF recognition has been defined in a promoter region of the putative regulon (Howell *et al.*, 2003). In *S. aureus*, several genes were also reported to have such a consensus motif. Of these, *isaA*, *lytM* and *ssaA* were actually bound to YycF in their promoter regions (Dubrac & Msadek, 2004). This suggests that these genes are probably controlled by this system. It has been reported that the *yycF/G* operon is transcribed during exponential growth (Fabret & Hoch, 1998; Fukuchi *et al.*, 2000). Therefore, the YycF regulons are probably expressed from the early stage of bacterial growth following the response regulator production. For example, the expression of *lytM* was reported to be highest in the early exponential phase, and its transcript was almost undetectable in the stationary phase (Ramadurai & Jayaswal, 1997; Ramadurai *et al.*, 1999). Also in *B. subtilis*, the *yocH* transcript, a putative YycF regulon encoding a potential autolysin, was highly detectable in the exponential phase, especially at the early stage (Howell *et al.*, 2003). The *isaA* expression profile defined in this report is similar to that of these other putative YycF regulons. *isaA* gene expression is probably also controlled by this two-component regulatory system. Additionally, it was reported that IsaA production might be negatively controlled by another well-known staphylococcal two-component regulatory system, *agr* (Dunman *et al.*, 2001; Kohler *et al.*, 2003). This system is involved in transcriptional regulation of several cell wall associated proteins (e.g. protein A and fibronectin binding protein) and several exoproteins (e.g. α-toxin, β-hemolysin and TSST-1) (Vandenesch *et al.*, 1991; Saravia-Otten *et al.*, 1997; Bronner *et al.*, 2004). Generally, these cell wall proteins are produced during the exponential growth phase, and then repressed from the late exponential stage by the *agr* system. It is likely that IsaA production is stimulated by YycF at the beginning of the growth phase, and then attenuated by the *agr* system from the late exponential phase.

It is known that some cell wall protein production is also controlled by SarA, which is a product from the staphylococcal accessory regulator (*sarA*) locus (Wolz *et al.*, 2000; Dunman *et al.*, 2001; Bronner *et al.*, 2004; Gao & Stewart, 2004). For example, the transcriptions of the *spa* and *fnb* genes are repressed by *agr*, while both genes are also regulated by SarA. We also investigated the *isaA* promoter region of two staphylococcal strains, Cowan I and 2PF-18 (Fig. 4), and consequently found a similar sequence to 'ATTTTAT' that was reported as a consensus for SarA binding by Sterba *et al.* (2003), although the AT-rich recognition motif reported by Chien *et al.* (1999) was not found. Both 7-bp sequences, in which one base substitution was observed between the two staphylococcal genes, were located upstream of the putative −35 promoter site and immediately downstream of the proposed YycF binding region (Fig. 4). At present, we do not know whether the observed sequences in both promoter regions are really recognized by SarA. However, considering the possible SarA box consensus sequence, it is conceivable that SarA binding to the region immediately downstream of the YycF recognition motif may interfere with the stimulatory effect of YycF.

Most of the candidate genes that are regulated by the YycF/G system are expressed from the early growth stage to the late exponential phase, and are probably involved in bacterial cell division directly or indirectly, considering the function of its putative regulon. IsaA possesses an SLT motif, and its production profile is accompanied by bacterial cell

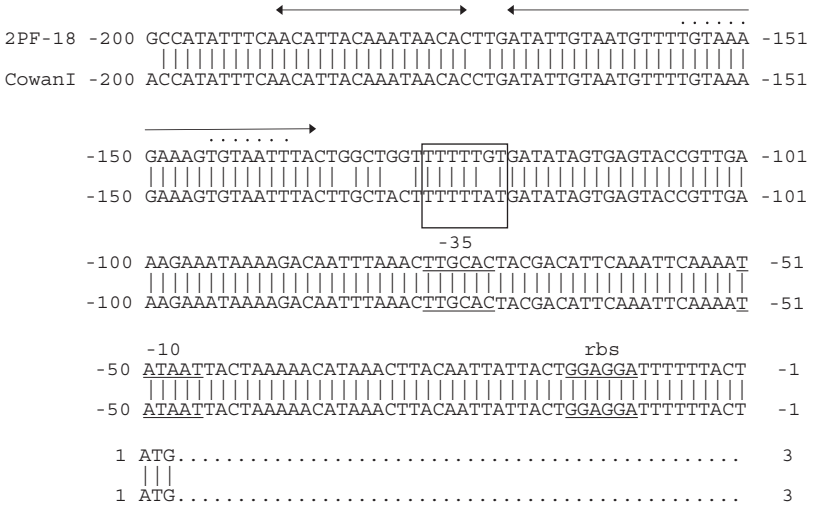

Fig. 4. Nucleotide sequence of the *S. aureus isaA* promoter region. The promoter regions of two strains, 2PF-18 (above) and Cowan I (below), were aligned. The potential ribosome binding sites (rbs) and the −10 and −35 promoter elements are underlined. The regions protected by YycF are and the conserved direct repeats (Dubrac & Msadek, 2004) are indicated by arrows and dots, respectively. Sequences similar to a 7-bp Sar-binding site (Sterba *et al*., 2003) are boxed.

proliferation. Moreover, a certain amount of IsaA is constantly found on the surface of the growing bacterial cell during the exponential phase, and this amount rapidly diminishes with the suspension of cell division. These results may suggest that IsaA is probably involved in bacterial growth on the cell surface, as a regulon of the YycF/G two-component system.

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