σB Inhibits Poly-N-Acetylglucosamine Exopolysaccharide Synthesis and Biofilm Formation in *Staphylococcus aureus*

Jaione Valle,[a]* Maite Echeverz,[a] Iñigo Lasa[a]

[a]Laboratory of Microbial Pathogenesis, Navarrabiomed-Universidad Pública de Navarra (UPNA)-Complejo Hospitalario de Navarra (CHN), Instituto de Investigación Sanitaria de Navarra (IdiSNA), Pamplona, Navarra, Spain

ABSTRACT *Staphylococcus aureus* clinical strains are able to produce at least two distinct types of biofilm matrixes: biofilm matrixes made of the polysaccharide intercellular adhesin (PIA) or poly-*N*-acetylglucosamine (PNAG), whose synthesis is mediated by the *icaADBC* locus, and biofilm matrixes built of proteins (polysaccharide independent). σB is a conserved alternative sigma factor that regulates the expression of more than 100 genes in response to changes in environmental conditions. While numerous studies agree that σB is required for polysaccharide-independent biofilms, controversy persists over the role of σB in the regulation of PIA/PNAG-dependent biofilm development. Here, we show that genetically unrelated *S. aureus* σB-deficient strains produced stronger biofilms under both static and flow conditions and accumulated higher levels of PIA/PNAG exopolysaccharide than their corresponding wild-type strains. The increased accumulation of PIA/PNAG in the σB mutants correlated with a greater accumulation of the IcaC protein showed that it was not due to adjustments in *icaADBC* operon transcription and/or *icaADBC* mRNA stability. Overall, our results reveal that in the presence of active σB, the turnover of Ica proteins is accelerated, reducing the synthesis of PIA/PNAG exopolysaccharide and consequently the PIA/PNAG-dependent biofilm formation capacity.

IMPORTANCE Due to its multifaceted lifestyle, *Staphylococcus aureus* needs a complex regulatory network to connect environmental signals with cellular physiology. One particular transcription factor, named σB (SigB), is involved in the general stress response and the expression of virulence factors. For many years, great confusion has existed about the role of σB in the regulation of the biofilm lifestyle in *S. aureus*. Our study demonstrated that σB is not necessary for exopolysaccharide-dependent biofilms and, even more, that *S. aureus* produces stronger biofilms in the absence of σB. The increased accumulation of exopolysaccharide correlates with higher stability of the proteins responsible for its synthesis. The present findings reveal an additional regulatory layer to control biofilm exopolysaccharide synthesis under stress conditions.

KEYWORDS Biofilm, PNAG, SigB, *Staphylococcus aureus*, *ica* operon

The dangerous community- and hospital-acquired pathogen *Staphylococcus aureus* is the leading cause of a variety of diseases ranging from moderate skin and soft tissue infections to very serious diseases, such as septic shock, toxic shock syndrome, and necrotizing pneumonia (1). The enormous pathogenic competence of *S. aureus* depends on its capacity to grow under a wide variety of environmental conditions, including those encountered in almost every organ of the human body (lung, heart, blood, bone, skin, muscles, eye, joints, and intestinal tract), and to produce a large array of toxins and enzymes in order to evade the immune system. To grow under different environmental conditions, *S. aureus* makes use of an efficient signal transduction system that facilitates the integration of environmental stimuli and adjusts the cellular physiology in response (1). One key factor of such a signal transduction system is the alternative σB

(SigB) transcription factor (2–8). σ^B directly or indirectly regulates processes including cell wall maintenance, intermediary metabolism, membrane transport, and virulence under environmental stresses, such as alkaline and acidic pH, heat shock, hydrogen peroxide, cell wall antibiotics, and entry into stationary phase (5, 7, 9, 10).

One important strategy used by *S. aureus* to grow and survive in host tissues is the adoption of the biofilm lifestyle, where bacteria grow encased in a self-produced extracellular matrix mostly composed of polysaccharides, proteins, and nucleic acids (11–13). Living inside the biofilm provides protection against desiccation, diffusion of antibiotics and other compounds, and the host immune system, among others (14). Given that biofilm development implies changes in the expression levels of hundreds of genes, it is not surprising that σ^B has been considered an optimal candidate for connecting environmental conditions with the development of the biofilm phenotype. However, confusion exists regarding the role of σ^B in regulating *S. aureus* biofilm development. Some studies suggest that σ^B is necessary for *S. aureus* biofilm development (15–17), whereas other studies have found that *S. aureus* is still able to form biofilms in the absence of σ^B (18, 19).

These seemingly contradictory findings might be explained by the capacity of *S. aureus* to produce biofilm matrixes with different compositions. Some *S. aureus* strains produce a proteinaceous biofilm matrix that is composed of a variety of cell wall proteins and extracellular DNA (eDNA) (20–26). In this case, the presence of σ^B is required to build the biofilm matrix (17, 19, 27, 28). This phenotype has been related to increased expression of the regulatory RNA, RNAIII, in the absence of σ^B, which elevates the expression of extracellular proteases and alters the activity of murein hydrolases. Alternatively, *S. aureus* is able to produce a polysaccharide-dependent biofilm that is mediated by the production of the polysaccharide intercellular adhesin (PIA) or poly-*N*-acetylglucosamine (PNAG). PIA/PNAG synthesis depends on the expression of *icaADBC*-encoded enzymes whose expression is mainly repressed at a transcriptional level by the IcaR protein (29–31). Some studies have reported that σ^B positively regulates *icaADBC* expression at a transcriptional level (16, 32). On the other hand, other studies have revealed that the absence of σ^B does not affect PIA/PNAG production and biofilm formation capacity (18). It was therefore of interest to determine the role of σ^B in PNAG-mediated biofilm formation by *S. aureus*.

In this study, we evaluated the consequences of the absence of σ^B in several genetically unrelated *S. aureus* strains that produce PNAG-dependent biofilms by analyzing (i) *icaADBC* transcription, (ii) *ica* mRNA stability, (iii) Ica protein production, and (iv) PIA/PNAG accumulation. The results revealed that *S. aureus* σ^B mutants produce higher levels of PIA/PNAG than wild-type strains by accumulating, at least, higher levels of IcaC protein, thus confirming that σ^B is a repressor of PIA/PNAG synthesis and exopolysaccharide-dependent biofilms.

RESULTS

Impact of σ^B mutation on PNAG-dependent biofilm phenotypes in *S. aureus*. To elucidate the role of σ^B in *S. aureus* PNAG-dependent biofilms, we selected five genetically unrelated clinical isolates that produce biofilms of different intensities depending on the levels of PIA/PNAG exopolysaccharide detected by dot blot analysis using anti-PNAG-specific polyclonal antisera (Fig. 1a). Under the conditions tested, the biofilm formation capacities of these strains were primarily dependent on PIA/PNAG exopolysaccharide, since deletion of the *icaADBC* genes completely inhibited biofilm development (Fig. 1b). Then, we constructed a σ^B mutant of each strain and compared the biofilm formation capacities of the σ^B mutants to those of the corresponding wild-type strains. The results showed that, after 6 h of growth, all the *S. aureus* σ^B mutants displayed a higher capacity to form a biofilm than wild-type strains ($P < 0.05$) (Fig. 1c). Interestingly, these differences remained after 24 h of growth when weak biofilm-forming strains (132, G-6478, and 10833) ($P < 0.05$) were analyzed. In contrast, such differences disappeared in the strong biofilm-forming strains (15981 and ISP479r), probably due to saturation in the quantity of biofilm cells (Fig. 1c). To confirm the role
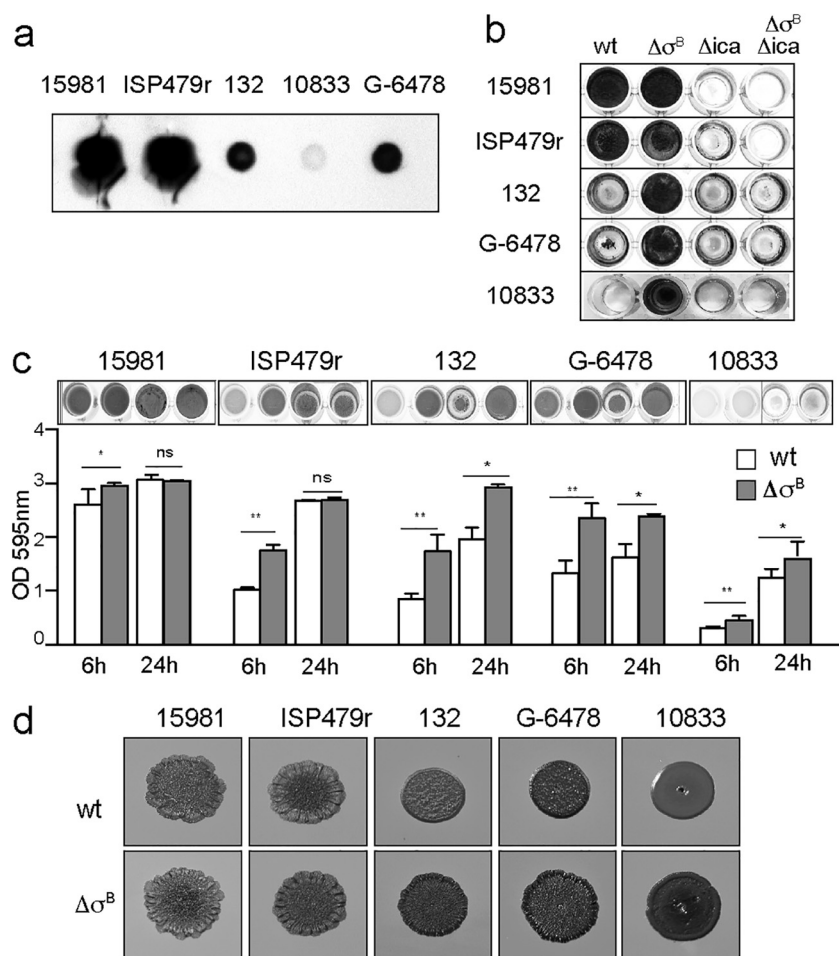
FIG 1 Mutation of σ^B in unrelated biofilm-positive *S. aureus* strains enhances biofilm formation. (a) Dot blot analysis of PNAG accumulation in different *S. aureus* strains. Cell surface extracts of biofilm cultures were spotted onto nitrocellulose filters. PNAG production was detected with anti-*S. aureus* PNAG antiserum. (b) Biofilm formation of the unrelated *S. aureus* strains and their respective mutants in σ^B ($\Delta\sigma^B$), *icaADBC* genes (Δica), and $\Delta\sigma^B$ Δica. Bacteria were grown on polystyrene microtiter plates for 24 h. wt, wild type. (c) Biofilm formation of the wild-type *S. aureus* strains and their respective σ^B mutants on polystyrene microtiter plates after 6 h and 24 h. The bacterial cells were stained with crystal violet, and biofilms were quantified by solubilizing the crystal violet with alcohol-acetone and determining the absorbance at 595 nm. The error bars represent the standard deviations of the results of three independent experiments. *, $P < 0.05$; **, $P < 0.01$. (d) Colony morphologies of biofilm-positive strains and their isogenic σ^B mutants on Congo red agar after 24 h of incubation.

of σ^B mutation in biofilm enhancement, σ^B mutants of the strong biofilm former *S. aureus* 15981 and the weak biofilm former *S. aureus* 132 were complemented with the σ^B gene using the pSK9 plasmid. σ^B complementation led to the restoration of biofilm levels shown by wild-type strains (see Fig. S1 in the supplemental material). Another phenotype associated with biofilm development in *S. aureus* is colony morphology on Congo red agar plates. In agreement with the biofilm formation phenotype, σ^B mutants of the weak biofilm-forming strains produced rougher colonies than their corresponding wild-type strains, which showed a smooth phenotype. In the case of the strong biofilm formers, wild-type strains already showed a rough colony morphology, and thus, the phenotype was not affected by the absence of σ^B (Fig. 1d). Finally, we examined whether the absence of σ^B affects biofilm development under flow culture conditions using microfermentors, where σ^B is expected to be active. After 24 h of incubation, visual inspection of the biofilms that had developed on the surfaces of the slides inside the microfermentors revealed that the majority of *S. aureus* σ^B mutants
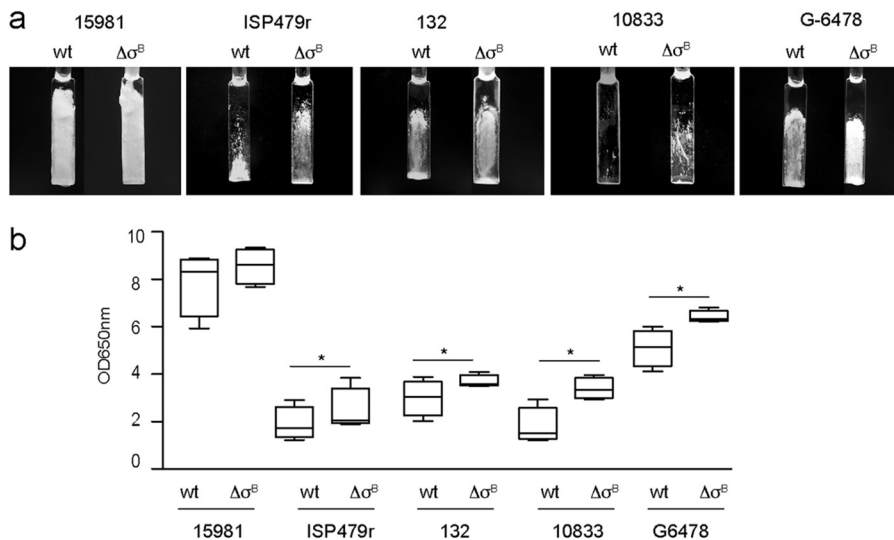
FIG 2 Influence of σ^B deletion on biofilm formation in continuous-flow microfermentors. (a) Biofilm development of the wild-type strain (wt) and its isogenic $\Delta\sigma^B$ mutants on glass slides of microfermentors after 24 h. (b) Quantification of biofilms adhering to glass slides. The glass slides were placed into 10 ml of PBS. Cells were removed from the slides by vigorous vortexing. The optical density of the solution was measured at 650 nm (OD_{650}). The biofilm formation of *S. aureus* 10833 wt and $\Delta\sigma^B$ was represented as 10 times the OD_{650} of the glass slides. The box and whisker plot indicates high and low values, medians, and interquartile ranges. Each group contained 4 or 5 microfermentors. Statistical differences were determined with Mann-Whitney tests. The asterisks indicate differences in competition indexes greater than 1 ($P < 0.05$).

produced a significantly higher biofilm biomass than their corresponding wild-type strains ($P < 0.05$) (Fig. 2). Taken together, these results indicated that deletion of σ^B enhances the PIA/PNAG-dependent biofilm formation capacity of clinical *S. aureus* strains.

σ^B mutants accumulate higher levels of PIA/PNAG exopolysaccharide. We next asked whether the increased biofilm formation capacity shown by *S. aureus* σ^B mutants was due to enhanced accumulation of PIA/PNAG exopolysaccharide. Thus, we compared the levels of PIA/PNAG exopolysaccharide produced by the wild-type and σ^B mutants and found that in all cases, the σ^B mutants produced higher levels of PNAG than the corresponding wild-type strains (Fig. 3a). To unequivocally demonstrate that the enhanced capacity to produce a biofilm was indeed due to greater accumulation of PIA/PNAG, we generated a nonpolar deletion of the *icaADBC* operon in the σ^B mutants. The resulting $\Delta\sigma^B$ Δica double mutants lost the capacity to form a biofilm in microtiter plates (Fig. 1b). To further confirm the positive role of σ^B in PNAG production, we complemented *S. aureus* 15981 Δica and $\Delta\sigma^B$ Δica mutant strains with a plasmid carrying the *icaRADBC* module and found that restoration of PNAG production was even greater in the $\Delta\sigma^B$ Δica double mutant than in the Δica single-mutant strain. Overall, these data indicated that *S. aureus* accumulates higher levels of PIA/PNAG exopolysaccharide in the absence of σ^B.

σ^B does not regulate *icaADBC* expression at a transcriptional level. The discovery that σ^B mutants accumulate higher levels of PNAG than wild-type strains raises the possibility that σ^B negatively regulates *icaADBC* transcription. To analyze this hypothesis, the promoter region of the *ica* operon was fused to *gfpmut2*, generating plasmid pCN52-Pica_gfp (33). The plasmids were introduced into *S. aureus* 15981 and 132 and their corresponding σ^B mutants, and expression of green fluorescent protein (GFP) was determined by Western blotting at the exponential (Exp) and stationary (ON) phases. Despite the fact that σ^B mutants accumulated higher levels of PIA/PNAG during the exponential and stationary phases (Fig. 4a), there were no significant differences in GFP expression between wild-type strains and σ^B-deficient strains (Fig. 4b). These results suggested that regulation of the *icaADBC* operon by σ^B does not occur at the tran-
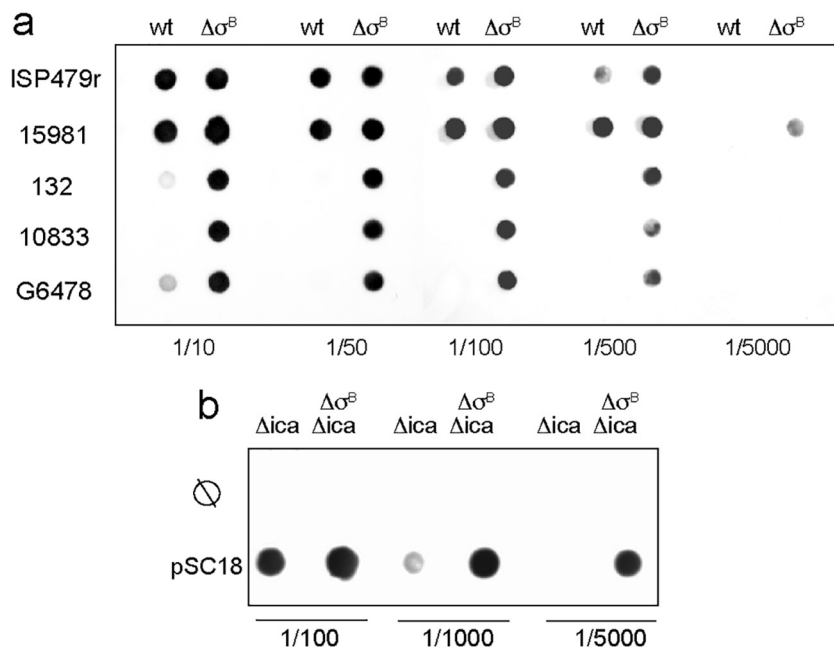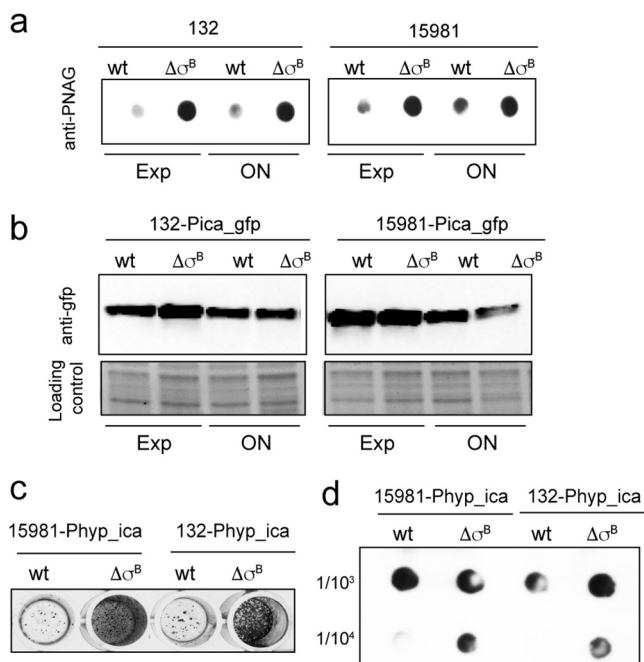
FIG 3 Analysis of PNAG accumulation in σ^B mutants. (a) Dot-blot analysis of PNAG accumulation in *S. aureus* wild-type and $\Delta\sigma^B$ mutant strains. Cell surface extracts of biofilm cultures were treated with proteinase K and spotted onto nitrocellulose filters at different dilutions (1:10 to 1:5,000). PNAG production was detected with anti-*S. aureus* PNAG antiserum. (b) PNAG levels of the *S. aureus* 15981 Δica mutant and the $\Delta\sigma^B$ Δica double mutant complemented with pSC18 plasmid, which carries the *ica* locus. For dot blot analysis, samples diluted 1:100, 1:1,000, and 1:5,000 were spotted onto nitrocellulose membranes, and PNAG production was detected with an anti-PNAG antiserum.

scriptional level. To address this issue, we next replaced, by allelic exchange, the native *ica* promoter (from positions −57 to +1) in the chromosome of *S. aureus* wild-type strains 15981 and 132 and their corresponding σ^B mutant strains using the constitutive Phyper promoter (Phyp-ica) (see Fig. S2 in the supplemental material) (34) and analyzed biofilm formation and PIA/PNAG production by the resulting strains. The results revealed that, even when *ica* operon expression is placed under the control of a constitutive promoter, σ^B-deficient strains still accumulate higher levels of PIA/PNAG and produce stronger biofilms than their corresponding wild-type strains (Fig. 4c and d). Taken together, these data showed that σ^B affects *ica* expression at a posttranscriptional level.

Role of σ^B in posttranscriptional regulation of the *ica* operon. To investigate whether σ^B posttranscriptionally regulates *ica* expression, we used plasmid pHRG to express the 5′ untranslated region (UTR) sequence of the *ica* operon under the control of the constitutive promoter Phyper fused in frame with *gfpmut2* (pHRG_5′UTRica) (Table 1). The plasmid was introduced into *S. aureus* wild-type strains 15981 and 132 and their respective σ^B mutants, and the expression of GFP was determined by Western blotting. Absence of σ^B did not lead to significant changes in GFP expression, suggesting that σ^B does not act on *icaADBC* mRNA translation (Fig. 5a). Then, we investigated whether σ^B affects *icaADBC* mRNA levels. For that, we examined *icaADBC* mRNA in *S. aureus* 15981 and 132 wild-type strains and in their corresponding σ^B mutants by Northern blotting using a riboprobe specific for *icaA* and *icaC* mRNAs. The results showed no differences in *ica* mRNA transcript levels and mRNA processing between wild-type and σ^B mutant strains (Fig. 5b). Collectively, these results suggest that σ^B upregulates PIA/PNAG expression without affecting *icaADBC* mRNA stability.

σ^B controls Ica protein levels. Next, we investigated the possibility that σ^B modulates Ica protein levels. To do so, we tagged the last protein (IcaC) encoded by the *icaADBC* operon in the wild-type strains 15981 and 132 and in their σ^B mutants. The resulting IcaC-3×Flag strains retained the capacity to form a biofilm, indicating that

FIG 4 Analysis of *ica* operon expression in σ^B mutants. (a) PNAG accumulation at different growth stages: exponential (Exp) and stationary (ON). Samples diluted 1:100 or 1:5,000 were spotted onto nitrocellulose membranes. PNAG production was detected with anti-PNAG antiserum. (b) Effects of σ^B deletion on *ica* promoter activity in *S. aureus* strains 15981 and 132 (wt) and their respective $\Delta\sigma^B$ mutants carrying pCN52-Pica_gfp at exponential and stationary phases. Expression of GFP under the control of the *ica* promoter (Pica_gfp) was determined by Western blotting using monoclonal antibodies. (c) Biofilm formation by *S. aureus* 15981 Phyp_ica and 132 Phyp_ica and their respective σ^B mutants on polystyrene microtiter plates after 4 h of incubation. The bacterial cells were stained with crystal violet. (d) PNAG accumulation in cell extracts of *S. aureus* 15981 Phyp_ica and 132 Phyp_ica and their respective σ^B mutants. PNAG production was detected by dot blot analysis using anti-*S. aureus* PNAG antiserum.

addition of the 3×Flag epitope did not impair IcaC functionality (see Fig. S3 in the supplemental material). Then, the IcaC-3×Flag protein was detected by immunoblotting with commercial anti-3×Flag antibodies. Quantification of immunodetected bands showed that levels of IcaC-3×Flag were significantly higher in σ^B mutants than in wild-type strains (Fig. 6a).

To gain insight into the σ^B-mediated accumulation of the IcaC protein, we analyzed IcaC stability by blocking *de novo* protein biosynthesis in exponentially growing wild-type and σ^B mutant cells. Analysis of IcaC levels by Western blotting showed that the protein remained relatively constant after transcriptional inhibition and that gradual degradation occurred after 30 min (Fig. 6b). Densitometry of this progression showed slower decay of the IcaC protein in the σ^B mutant than in the wild-type strain (Fig. 6c). Together, these results suggest that IcaC protein turnover decreases in the absence of σ^B.

DISCUSSION

The transition from a planktonic single-cell lifestyle to a biofilm-associated community in *S. aureus* requires a complex and highly regulated process that needs to be globally coordinated. Increasing evidence indicates that the stress sigma factor σ^B is a key element for biofilm formation in several bacterial species, including *Listeria monocytogenes*, *Bacillus subtilis*, and *Bacillus cereus* (35). However, the contribution of σ^B to regulation of the biofilm formation process of *S. aureus* is still a matter of debate (17, 32, 36–42). There is wide agreement that σ^B is necessary for building PNAG-independent biofilms. However, the role of σ^B in PNAG-dependent biofilms is more arguable. Early reports claimed that disruption of σ^B impaired PNAG synthesis, whereas other reports

**TABLE 1** Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Reference
S. aureus strains		
15981	Biofilm-positive clinical strain	18
ISP479r	ISP479 *rsbU*-positive strain	59
12313	Biofilm-positive clinical strain	This study
132	Biofilm-positive clinical strain; MRSA clinical strain	23
G-6478	Biofilm-positive clinical strain; MRSA clinical strain	23
10833	Clumping factor-positive variant of Newman D2C	29
12313 $\Delta\sigma^B$	12313 with deleted σ^B gene	This study
10833 $\Delta\sigma^B$	10833 with deleted σ^B gene	This study
ISP479r $\Delta\sigma^B$	ISP479r with deleted σ^B gene	18
ISP479r Δica	ISP479r with deleted *icaADBC* operon	This study
ISP479r $\Delta\sigma^B$ Δica	ISP479r σ^B mutant with deleted *icaADBC* operon	This study
G-6478 $\Delta\sigma^B$	G-6478 with deleted σ^B gene	This study
G-6478Δica	G-6478 with deleted *icaADBC* operon	This study
G-6478 $\Delta\sigma^B$ Δica	G-6478 σ^B mutant with deleted *icaADBC* operon	This study
132 $\Delta\sigma^B$	132 with deleted σ^B gene	This study
132 Δica	132 with deleted *icaADBC* operon	23
132 $\Delta\sigma^B\Delta ica$	132 σ^B mutant with deleted *icaADBC* operon	This study
15981 $\Delta\sigma^B$	15981 σ^B mutant	18
15981 Δica	15981 with deleted *icaADBC* operon	56
15981 $\Delta\sigma^B$ Δica	15981 σ^B mutant with deleted *icaADBC* operon	This study
15981 *icaC*-3×*flag*	15981 with a 3×*flag*-tagged *icaC* gene	This study
15981 $\Delta\sigma^B$ *icaC*-3×*flag*	15981 σ^B mutant with a 3×*flag*-tagged *icaC* gene	This study
132 *icaC*-3×*flag*	132 with a 3×*flag*-tagged *icaC* gene	This study
132 $\Delta\sigma^B$ *icaC*-3×*flag*	132 σ^B mutant with a 3×*flag*-tagged *icaC* gene	This study
15981_Pica-gfp	15981 with pCN52-Pica	This study
15981 $\Delta\sigma^B$_Pica-gfp	15981 σ^B mutant with pCN52-Pica	This study
132_Pica-gfp	132 with pCN52-Pica	This study
132 $\Delta\sigma^B$_Pica-gfp	132 σ^B mutant with pCN52-Pica	This study
15981_Phyp-ica	15981 constitutively expressing the *ica* operon	This study
15981 $\Delta\sigma^B$_Phyp-ica	15981 σ^B mutant constitutively expressing the *ica* operon	This study
132_Phyper-ica	132 constitutively expressing the *ica* operon	This study
132 $\Delta\sigma^B$_Phyp-ica	132 σ^B mutant constitutively expressing the *ica* operon	This study
15981 RNAIII	15981 transformed with plasmid pALC2073 RNAIII	This study
15981 $\Delta\sigma^B$ RNAIII	15981 σ^B transformed with plasmid pALC2073 RNAIII	This study
132 RNAIII	132 transformed with plasmid pALC2073 RNAIII	23
132$\Delta\sigma^B$ RNAIII	132 σ^B transformed with plasmid pALC2073 RNAIII	This study
Plasmids		
pMAD	*E. coli-S. aureus* shuttle vector	60
pSK9	Plasmid carrying the σ^B gene	16
pHRG	pCN47 derivative plasmid used for posttranscriptional fusions to GFP	This study
pCN52-Pica_gfp	Plasmid with GFP expressed under the control of the Pica promoter	This study
pCN52-Phyp_gfp	Plasmid with GFP expressed under the control of the Phyper promoter	This study
pHRG-5′UTRica	Plasmid carrying a posttranscriptional fusion of *ica* with GFP	This study

[a]MRSA, methicillin-resistant *Staphylococcus aureus*.

claimed that deletion of σ^B increased aggregation and did not affect PNAG-dependent biofilm development (3, 16, 18, 32). Another source of confusion has been the fact that σ^B participation is crucial for *icaADBC* expression in the closely related species *Staphylococcus epidermidis*, where σ^B downregulates the expression of *icaR* (37). Consequently, *S. epidermidis* σ^B mutants produce high levels of IcaR, which results in the repression of PNAG production (37, 40, 41).

With the aim of clarifying the role of *S. aureus* σ^B in the regulation of PNAG synthesis, we analyzed the consequences of σ^B deletion in five genetically unrelated strains. Deletions were generated by double crossover without the insertion of antibiotic resistance markers to avoid undesirable polar effects. The results unambiguously showed that σ^B is dispensable for PNAG synthesis in *S. aureus* and, indeed, that σ^B mutants displayed a higher capacity to accumulate PNAG on the cell wall and produce a biofilm.

How does σ^B affect PNAG synthesis in *S. aureus*? The simplest and most plausible explanation was that σ^B directly or indirectly regulates *ica* operon transcription. How-
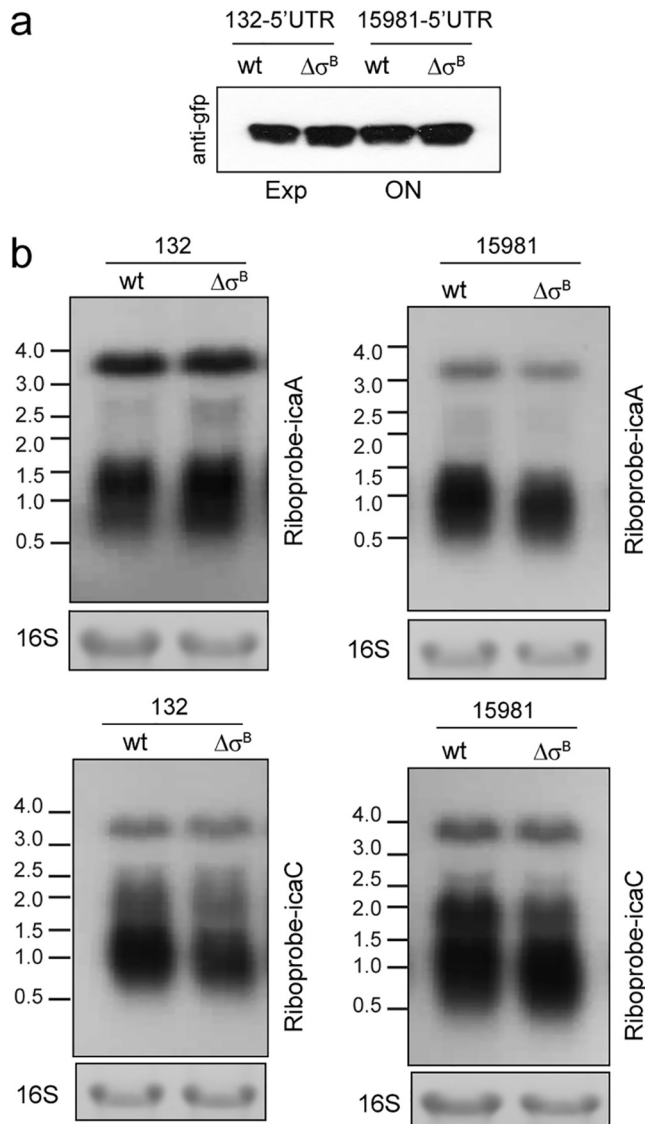
FIG 5 Analysis of the posttranscriptional regulation of the *ica* operon by σ^B. (a) Fusion of the 5′ UTR of the *ica* operon to *gfpmut2* under the control of the Phyper promoter. Levels of GFP were determined in the *S. aureus* 132 and 15981 wild-type strains and their respective σ^B mutants by Western blotting using monoclonal antibodies against GFP. (b) Northern blot analysis of RNA harvested from *S. aureus* 15981 and 132 wild-type strains and their corresponding σ^B mutants. The strains were grown in TSB-gluc at 37°C until exponential phase. The lower gels show 16S ribosome bands stained with ethidium bromide as a loading control. The blots were probed with a riboprobe specific for *icaA* and *icaC* transcripts. The positions of RNA standards in kilobases are indicated.

ever, our results refuted this hypothesis. Evaluation of the *ica* promoter activity in wild-type and σ^B mutant strains showed no differences between the strains. Furthermore, the expression of PNAG remained higher in the absence of σ^B than in the wild type after replacement of the *ica* native promoter by a constitutive Phyper promoter, indicating that σ^B controls *ica* operon expression without affecting *ica* transcriptional levels. These results agreed with previous transcriptome analyses of wild-type *S. aureus* and its corresponding σ^B mutant in which the *ica* genes were never identified as members of the σ^B regulon (9, 43, 44).

Regulation of the expression of the *pgaABCD* operon, homologous to *icaADBC* in *Escherichia coli*, takes place at a posttranscriptional level. The small RNA-binding protein CsrA represses *pga* gene expression and the production of PGA by binding to *pgaA* mRNA. This prevents the 30S ribosome subunit from binding, thus affecting *pgaABCD*
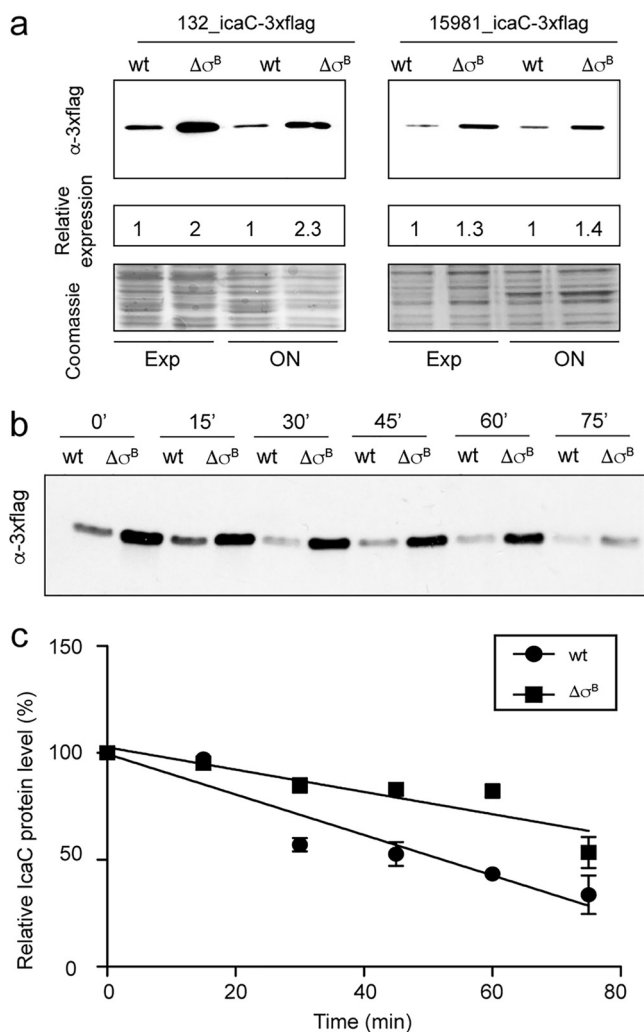
FIG 6 σ^B influence on IcaC protein levels. (a) Immunodetection of IcaC-tagged protein in the wt and the σ^B mutants of *S. aureus* 15981 and 132 at exponential phase. Whole-cell bacterial lysates were subjected to electrophoretic separation in an SDS-12% polyacrylamide gel. The proteins were transferred onto a nitrocellulose membrane and probed with anti-Flag M2 MAb conjugated with peroxidase. Relative quantification of the Flag protein was obtained using the ImageJ program. A Coomassie-stained gel portion is shown as a loading control. (b) Immunodetection of IcaC-tagged protein upon blocking protein biosynthesis in exponentially growing cells. (c) Relative IcaC levels upon blocking protein biosynthesis as an average of the results of two independent experiments; the error bars indicate standard deviations.

mRNA stability and accelerating mRNA degradation (45). Inspired by these findings, we asked whether σ^B might posttranscriptionally regulate *ica* operon expression through the activation of an RNA-binding protein or a small RNA that would bind to the *ica* mRNA, destabilizing the *ica* transcript. However, analysis of GFP expression by the use of a posttranscriptional fusion of the 31-nucleotide (nt) untranslated leader of the *icaA* coding sequence with GFP showed no differences in GFP levels between the wild type and σ^B mutants. Furthermore, Northern blotting analysis with riboprobes specific for *icaA* and *icaC* did not detect any differences in *ica* mRNA levels and mRNA integrity between the wild type and σ^B mutants. Instead, we found that the IcaC protein is somewhat more stable in σ^B mutants than in the corresponding wild-type strains. How does σ^B translationally regulate Ica proteins levels? Analysis of transcriptome sequencing (RNA-seq) data obtained in a previous study (43) that compared *S. aureus* 15981 wild-type and σ^B mutant strains showed the existence of at least 18 small RNAs (sRNAs) whose expression depended on σ^B. We are currently exhaustively studying whether
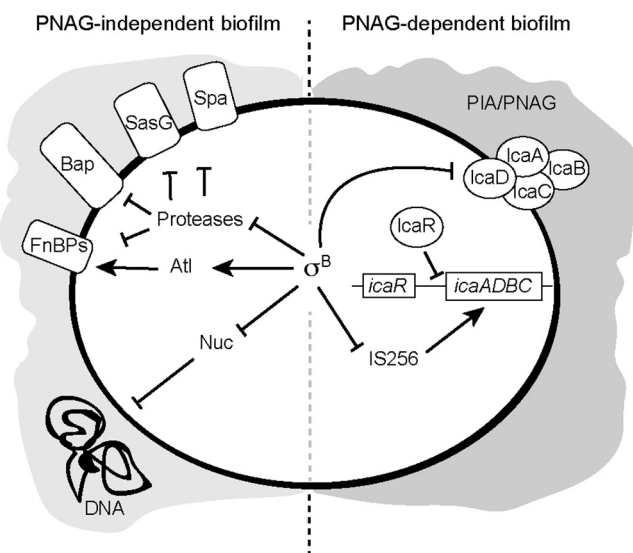
FIG 7 Proposed model of σ^B regulatory effect on *S. aureus* biofilm formation. The transcriptional regulator σ^B induces PNAG-independent biofilm formation by repressing extracellular proteases, by inhibiting nuclease secretion, and/or by the activation of Atl. On the other hand, σ^B regulates PNAG-dependent multicellular behavior by modulating PNAG levels by at least repressing IS*256* and translationally regulating Ica enzymes.

PNAG production depends on any σ^B-regulated sRNAs, which might affect *icaADBC* mRNA translation. Another possible explanation is that specific proteases that may affect Ica protein levels are actually repressed in the σ^B mutant. This possibility seems in principle unlikely because the majority of proteases are in fact induced when σ^B is inactive. However, following the analogy of IcaADBC machinery to PgaABCD machinery in order to synthesize poly-*N*-acetylglucosamine (PIA/PNAG/Pga), we cannot exclude the possibility that the IcaC protein adopts a conformation in the absence of σ^B that makes the protein less susceptible to proteases. In the case of PgaABCD, U. Jenal and colleagues nicely showed that PIA/PNAG synthesis is allosterically controlled through c-di-GMP binding to the membrane-anchored PgaCD complex (46). When the concentration of c-di-GMP decreases, PgaD is no longer able to interact with c-di-GMP and is rapidly removed by proteolysis, and thus, the synthesis of PIA/PNAG is inhibited. *S. aureus* contains only one gene encoding a protein with a conserved GGDEF domain, designated GdpS (47). Different studies have shown that GdpS contributes to staphylococcal biofilm formation (47–49). However, the mechanisms by which GdpS regulates biofilm formation remains unclear. Recombinant GdpS protein is unable to synthesize quantifiable levels of c-di-GMP *in vitro*, and c-di-GMP has not been detected in *S. aureus* extracts, as judged by liquid chromatography-tandem mass spectrometry (LC–MS-MS) (50). Thus, despite the absence of evidence indicating the presence of c-di-GMP in the cytoplasm of *S. aureus*, it is tempting to speculate that c-di-GMP, or another signaling molecule whose level in the bacterial cytoplasm depends on the presence of σ^B, might interact with Ica proteins and affect their accessibility to proteases.

The findings of this study, together with previous results on the effect of σ^B on *S. aureus* multicellular behavior, are summarized in Fig. 7. Under environmental conditions where σ^B is active, it represses the synthesis of proteases and nuclease production and also suppresses PIA/PNAG synthesis, enabling the formation of a polysaccharide-independent biofilm. When σ^B is not active, overexpression of extracellular proteases and nucleases (Nuc) reduces the production of a protein/DNA-mediated biofilm. On the other hand, PIA/PNAG accumulation and the subsequent PIA/PNAG-mediated biofilm development take place through the increase of Ica protein levels controlled at a posttranslational level. Lastly, σ^B can also indirectly affect biofilm production by generating a proper ratio of biofilm-negative variants through regulation of the transpo-

sition activity of the insertion sequence IS*256*, which preferentially inserts within the *icaC* gene (51). Interestingly, in all these regulatory processes, σ^B regulates the synthesis of PIA/PNAG indirectly by affecting the expression of other factors that ultimately alter PIA/PNAG levels. Thus, hierarchical regulation has to exist to coordinate all these confluent mechanisms and efficiently transmit environmental signals to multicellular behavior through the activity of σ^B.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The strains and plasmids used in this study are listed in Table 1. *S. aureus* strains 15981, 132, G-6478, and 10833 were isolated from nosocomial infections at the Microbiology Department of the University Clinics of Navarra (Spain) (Table 1). 132 is a methicillin-resistant *S. aureus* strain. When grown in Trypticase soy broth (TSB) supplemented with 3% NaCl, *S. aureus* 132 produces an exopolysaccharidic biofilm matrix (23). ISP479r is a derivative of ISP479 with a functional *rsbU* gene. *E. coli* XL1-Blue cells were routinely grown in Luria-Bertani (LB) broth or on LB agar (Pronadisa, Spain) with appropriate antibiotics. *S. aureus* strains were cultured on Trypticase soy agar (TSA), in TSB supplemented with 0.25% glucose (TSB-gluc), or in TSB supplemented with 3% NaCl when indicated. Media were supplemented with appropriate antibiotics at the following concentrations: erythromycin (Em), 10 μg ml^{-1} or 1.5 μg ml^{-1}; ampicillin (Am), 100 μg ml^{-1}; and chloramphenicol (Cm), 20 μg ml^{-1}.

Manipulation of DNA. Restriction endonucleases (Thermo Scientific) were used according to the manufacturer's specifications. PCR products were amplified with Phusion high-fidelity DNA polymerase (Thermo Scientific). Oligonucleotides were obtained from Stab Vida Corporation. σ^B and *ica* deletions were performed as previously described (18) using plasmids pMADsigAD and pMADicaAD (23). To construct *S. aureus* strains constitutively expressing *icaADBC*, the *ica* promoter from positions +1 to −50 was replaced by the Phyper promoter (52). For that, 2 PCR fragments of 500 bp that flank this region were amplified using primers IcaR5 (GGATCCAAAAGGATGCTTTCAAATACC)/IcaR3 (GCATGCTTGAAGGATAAGA TTATTGATA) and IcaOp5 (CAACCTAACTAACGAAAGGTAG)/IcaOp3 (GAATTCTAGGATTACCTGTAACT). The IcaOp5 primer contains 55 nt of the Phyper promoter and the SphI restriction enzyme sequence. Then, the two PCR fragments were cloned into the pMAD plasmid, giving pMAD-pHyper-ica. Allelic exchange was performed as previously described (18).

Colonial morphology on Congo red agar. The colony morphology of *S. aureus* was analyzed using Congo red agar plates (53, 54). Congo red agar was prepared as follows: 30 g/liter of Trypticase soy (Pronadisa), 15 g/liter of agar (Pronadisa), 0.8 g/liter of Congo red stain (Sigma), and 20 g/liter of sucrose. The Congo red stain and the sucrose solution were autoclaved separately (121°C for 20 min and 115°C for 15 min, respectively). *S. aureus* strains were streaked on Congo red agar and were incubated at 37°C for 24 h. *S. aureus* biofilm-positive variants display a rough colony morphology when grown on this medium, whereas biofilm-negative variants exhibit a smooth colony morphology.

Biofilm formation assays. A biofilm formation assay in microtiter wells was performed as described previously (18). Briefly, strains were grown overnight at 37°C and diluted 1:40 in growth medium. The cell dilutions were used to inoculate sterile 96-well polystyrene microtiter plates. After 6 or 24 h of incubation at 37°C, the wells were gently rinsed three times with water, dried, and stained with 0.1% crystal violet for 15 min. The wells were rinsed again, and the crystal violet was solubilized in 200 μl of ethanol-acetone (80:20 [vol/vol]). The optical density at 595 nm was determined using a microplate reader (Multiskan EX; Labsystems). All experiments were performed in triplicate. A two-tailed Student *t* test was used to determine the difference in biofilm thickness between the wild type and the σ^B mutants. Biofilm formation under flow conditions was performed using microfermentors (55). Bacteria (10^8) from an overnight preculture were used to inoculate the microfermentors. After 24 h of incubation at 37°C, biofilm development was recorded with a Nikon Coolpix 950 digital camera and quantified by resuspension of the cells attached on the Pyrex slides in 10 ml of TSB. The optical density of the suspension was determined at 650 nm. All experiments were performed in triplicate.

PNAG detection. PNAG production in *S. aureus* strains was detected as described previously (56). Briefly, overnight cultures were diluted 1:100 in growth medium. The cell suspensions were used to inoculate sterile 24-well polystyrene microtiter plates (Costar). After incubation, the cells were centrifuged, and the pellets were resuspended in 0.5 M EDTA (pH 8.0) to obtain the same density for each sample. The cells were incubated for 5 min at 100°C, and 40 μl of the supernatant was incubated with 10 μl of proteinase K (20 mg/ml; Sigma) for 30 min at 37°C. After addition of 10 μl of Tris-buffered saline (20 mM Tris-HCl, 150 mM NaCl [pH 7.4]) containing 0.01% bromophenol blue, 5 μl of sample dilutions was spotted on a nitrocellulose filter using a Bio-Dot microfiltration apparatus (Bio-Rad), blocked overnight, and incubated for 2 h with an anti-*S. aureus* PNAG antibody diluted 1:20,000 (57). Bound antibodies were detected with a peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) antibody.

Generation of Ica transcriptional and posttranscriptional fusions with GFP. To obtain an *ica* transcriptional fusion, we amplified the *ica* promoter using primers AU59 (ATGCCTGCAGGTCGACTTTTT ATAACCCCCTACTGAAAATTAATCACACT)/AU76 (ACGAATTCGAGCTCGGTACCTTTCTTTACCTACCTTTCGTTA GTTAGGTTG) and cloned it using an In-fusion cloning kit (Clontech) in the pCN52 plasmid, giving plasmid pCN52-Pica_gfp. To obtain a posttranscriptional fusion, the 5′ UTR of *ica* was amplified using primers IcaA.Td5.EcoRI (GAATTCCAACCTAACTAACGAAAGGTAG) and IcaA.RBS.3.SpeI (ACTAGTCAATTTCTTTACCT ACCTTTCGT) and cloned into plasmid pHRG. We constructed pHRG, a derivative pCN47 plasmid (33) in which the Phyper promoter and GFP lacking its ribosomal binding site were cloned using SphI and EcoRI

and SpeI and AscI, respectively. The Ica 5′ UTR was fused in frame with *gfpmut2* without its ribosomal binding site and constitutively expressed under the control of the Phyper promoter.

Immunoblot analysis. Overnight cultures of *S. aureus* strains with pCN52-Pica_gfp or pHRG-5′UTRica plasmids were diluted 1:100 and grown in growth medium at 37°C under static conditions. Samples were obtained at the exponential and late stationary growth phases. Cells were resuspended in phosphate-buffered saline (PBS) and lysed using a FastPrep apparatus. Supernatants from total protein extracts were recovered and analyzed by SDS-PAGE and Western blotting, as detailed below. A volume of Laemmli buffer was added to the samples and boiled for 5 min; 4 μg of protein was used for SDS-PAGE analysis with 12% precast gels (Bio-Rad). The gels were stained with 0.25% Coomassie brilliant blue R250 (Sigma) as loading controls. For Western blot analysis, protein extracts were blotted onto Hybond-ECL nitrocellulose membranes (Amersham Biosciences). Anti-GFP antibodies (Living Color A.v. monoclonal antibody JL-8; Clontech) were diluted 1:2,500 with 0.1% PBS-Tween–5% skim milk. Alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (Sigma) diluted 1:5,000 in 0.1% PBS-Tween–5% skim milk was used as a secondary antibody, and the subsequent chemiluminescence reaction was recorded.

RNA extraction and Northern blotting. Bacteria were grown in 100 ml of growth medium at 37°C under shaking conditions for 4 h. The cultures were centrifuged, and the pellets were frozen in liquid nitrogen and stored at −80°C. Total RNA from the bacterial pellets was extracted by using the TRIzol reagent method as described previously (58). Briefly, the bacterial pellets were resuspended in 400 μl of solution A (10% glucose, 12.5 mM Tris [pH 7.6], 10 mM EDTA). The cells were transferred to lysing matrix B tubes (MP Biomedicals) containing 500 μl of acid phenol (Ambion) and mechanically lysed by using a Fastprep apparatus (BIO101). After lysis, the tubes were centrifuged, and the aqueous phase was transferred to 2-ml tubes containing 1 ml of TRIzol, mixed, and incubated for 5 min at room temperature. Chloroform (100 μl) was added, mixed gently, and incubated for 3 min at room temperature. The aqueous phase was transferred into a 2-ml tube containing 200 μl of chloroform, mixed, and incubated for 5 min at room temperature. The tubes were centrifuged, and RNA contained in the aqueous phase was precipitated by addition of isopropanol. Northern blotting was performed as described previously (58). Briefly, 11 μg of total RNA was separated in precast agarose gels (Sigma). RNAs were blotted onto Nytran membranes (0.2-μm pore size; Sigma), UV cross-linked, prehybridized in Ultrahyb solution (Ambion) at 65°C, and labeled with strand-specific riboprobes specific for *icaA*: AU52 (TAAATACGACTCA CTATAGGGTATCCACGTAAATGCAATTTCC)/AU53 (TGGAAGTTCAGATAATACAGC) and *icaC* AU54 (TAATAC GACTCACTATAGGGGTATGATATTGCGTGAATTC)/AU55 (TCACGATACCGTGCTACAC). The membranes were washed, and autoradiography images were registered at different exposure times for each gene.

Protein tagging and immunodetection analysis. Transfer of the 3×Flag sequence into IcaC was performed by recombination using plasmid pMADicaC-3×Flag. To construct pMADicaC-3×Flag, the region of *icaC* corresponding to the C-terminal region of IcaC was amplified using primers CFlag1 (GCAAATGGAGACTATTGG) and CFlag2 (ATAAGCATTAATGTTCAATTTA). The CFlag2 primer contains 66 nt coding for the 3×Flag sequence. Strains containing IcaC with 3×Flag were grown in growth medium for 5 h (Exp) and 24 h (ON). Then, the cells were harvested by centrifugation, and the pellets were resuspended in proportional quantities of PBS buffer containing lysostaphin (12.5 μg/ml; Sigma) and DNase I for 2 h. A volume of Laemmli buffer was added before loading the samples in SDS-12% PAGE. Proteins were transferred onto nitrocellulose membranes (Hybond; Amersham Biosciences), and 3×Flag fusion proteins were immunodetected by the use of anti-Flag M2 monoclonal antibodies (MAbs) conjugated with peroxidase (Sigma). Densitometry analysis of the detected bands was performed using ImageJ (http://rsbweb.nih.gov/ij/).

Translation block experiment. To assess the *in vivo* stability of IcaC-3×Flag, overnight cultures of strains *S. aureus* 132 *icaC*-3×Flag and Δσ^B *icaC*-3×Flag were diluted 1:100 in fresh TSB-NaCl medium, and the cultures were grown to exponential phase at 37°C. Then, protein synthesis was inhibited at time point zero by the addition of 200 μg/ml rifampin and 500 μg/ml lincomycin. Samples were harvested at the indicated times after translation inhibition, and IcaC levels were analyzed by immunoblotting using anti-Flag M2 MAbs conjugated with peroxidase (Sigma). Band intensities were quantified using ImageJ software and normalized to levels present 5 min after translation inhibition for each strain.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB .00098-19.

SUPPLEMENTAL FILE 1, PDF file, 0.04 MB.

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