

SarA and not σ^B is essential for biofilm development by *Staphylococcus aureus*

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Summary

Staphylococcus aureus biofilm formation is associated with the production of the polysaccharide intercellular adhesin (PIA/PNAG), the product of the *ica* operon. The staphylococcal accessory regulator, SarA, is a central regulatory element that controls the production of *S. aureus* virulence factors. By screening a library of Tn917 insertions in a clinical *S. aureus* strain, we identified SarA as being essential for biofilm development. Non-polar mutations of *sarA* in four genetically unrelated *S. aureus* strains decreased PIA/PNAG production and completely impaired biofilm development, both in steady state and flow conditions via an *agr*-independent mechanism. Accordingly, real-time PCR showed that the mutation in the *sarA* gene resulted in downregulation of the *ica* operon transcription. We also demonstrated that complete deletion of σ^B did not affect PIA/PNAG production and biofilm formation, although it slightly decreased *ica* operon transcription. Furthermore, the *sarA*- σ^B double mutant showed a significant decrease of *ica* expression but an increase of PIA/PNAG production and biofilm formation compared to the *sarA* single mutant. We propose that SarA activates *S. aureus* development of biofilm by both enhancing the *ica* operon transcription and suppressing the transcription of either a protein involved in the turnover of PIA/PNAG or a repressor of its synthesis, whose expression would be σ^B -dependent.

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Introduction

Staphylococcus aureus is one of the most frequent bacterial causes of community-acquired and hospital-acquired infections. One reason for the ubiquitous nature of infections caused by this pathogen is the easy transmission from the nasal membranes and skin to wounds where bacteria produce numerous extracellular proteins and toxins. In addition, *S. aureus* has the capacity to adhere to catheters and other indwelling devices and form a multicellular community, known as a biofilm, that is then difficult to combat with host defences or antibiotics [for reviews, see Götz and Peters (2000) and Costerton and Stewart (2000)].

Staphylococcus aureus biofilm formation is mediated by the production of the extracellular polysaccharide adhesin PIA/PNAG, whose synthesis depends on the expression of *ica*ADBC-encoded enzymes (Cramton *et al.*, 1999; Mckenney *et al.*, 1999; Gotz, 2002; Maira-Litran *et al.*, 2002). Most of the *S. aureus* strains analysed so far contain the entire *ica* gene cluster (Cramton *et al.*, 1999; Arciola *et al.*, 2001; Martin-Lopez *et al.*, 2002), but only a few express the *ica* operon and produce biofilms in microtitre dish-based assays. These differences could be due to the fact that *ica* expression is subject to environmental regulation. Stimuli such as high osmolarity (3% NaCl), growth in anaerobic conditions, high temperature and subinhibitory concentrations of certain antibiotics are known to enhance *ica* transcription and biofilm formation (Rachid *et al.*, 2000a; Cramton *et al.*, 2001). Moreover, *ica* operon expression in the closely related *S. epidermidis* can be turned on and off by the insertion and excision of the insertion sequence IS256 at specific hot spots of the *icaA* and *icaC* genes (Ziebuhr *et al.*, 1999). By this mechanism, PIA/PNAG production and biofilm formation phenotypes may be phase variable, allowing individual *S. epidermidis* cells to leave the biofilm and colonize new surfaces. However, whether a similar mechanism could explain phase variation in *S. aureus* (Baselga *et al.*, 1993) is unknown.

Furthermore, DtlA and Bap proteins have been identified as contributors to *S. aureus* formation of biofilm. The *dtl*ABCD operon is responsible for the D-alanine incorporation in the teichoic acids. The lack of D-alanine esters causes a strongly negative net charge on the bacterial surface that affects primary attachment to polystyrene or

glass surfaces (Gross *et al.*, 2001). On the other hand, the surface protein Bap (Cucarella *et al.*, 2001) interferes with initial bacterial attachment of other surface molecules (MSCRAMM) to host receptors and cellular internalization (Cucarella *et al.*, 2002). In contrast to the *dtlA* and *ica* operons, all the staphylococcal isolates harbouring *bap* are highly adherent and strong biofilm producers, indicating a strong correlation between the presence of the protein and biofilm formation ability on abiotic surfaces (Cucarella *et al.*, 2001).

The regulatory mechanisms involved in the *S. aureus* biofilm development remain poorly understood. A recent report shows that *icaR*, located adjacent to the *ica* operon, encodes a transcriptional repressor involved in environmental regulation of the *ica* operon expression in *S. epidermidis* (Conlon *et al.*, 2002). In addition, insertional mutagenesis and complementation experiments have demonstrated that transcription of the *ica* operon is at least partially controlled by the alternative transcription factor σ^B (Rachid *et al.*, 2000b). The absence of a nucleotide sequence immediately upstream of the *ica* operon, resembling the consensus sequence recognized by σ^B , suggests the existence of additional regulatory factors. Global quorum sensing regulators have been shown to regulate the biofilm formation of many Gram-negative bacteria, including *Pseudomonas aeruginosa* (Davies *et al.*, 1998), *Burkholderia cepacia* (Huber *et al.*, 2001) and *Pantoea stewartii* (von Bodman *et al.*, 1998). Accordingly, 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*). The *agr* regulator, a two-component quorum sensing system activated during the transition from the exponential to the stationary growth phase, is known to mediate the expression conversion from genes encoding cell-surface proteins to genes encoding exoproteins. The presence of *agr* seems to be negatively correlated with the ability to adhere to polystyrene (Vuong *et al.*, 2000). With respect to the *sar* regulator, the *sar* locus encodes a 14.5 kDa protein that binds to the *agr* promoter to stimulate RNAlII transcription, resulting in the modulation of target genes downstream on the *agr* regulatory cascade (*agr*-dependent pathway). Additionally, SarA can interact directly with target gene promoters to control gene expression (*agr*-independent pathway). Previous work has established that mutation in *sarA* may confer an enhanced adherence to inert surfaces compared to wild-type or *agr* mutant strains, suggesting that SarA might be a negative regulator of biofilm development (Pratten *et al.*, 2001).

In this study, we have carried out a random transposon mutagenesis to further investigate regulatory genes involved in the process of biofilm formation by *S. aureus*. One of the isolated mutants was shown to carry the trans-

poson within the *sarA* gene. Analysis using four genetically non-related *S. aureus* strains revealed that SarA deficiency decreased *ica* operon transcription as well as PIA/PNAG accumulation, and impaired biofilm development both in steady state and flow conditions. We have also demonstrated that neither σ^B nor *agr* are essential for biofilm development. A simple regulatory model on the role of SarA in the *ica* regulatory circuitry is presented and discussed.

Results

Identification of genes required for biofilm formation

Repeated passages of bacterial isolates in liquid culture may select for fast growth and loss of multicellular behaviour attributes. Thus, we decided to investigate biofilm formation processes of a clinical isolate of *S. aureus* 15981. For that, we constructed a collection of approximately 10000 transposon mutations from three independent mutagenesis and screened for mutants that were deficient in biofilm formation on microtitre polystyrene plates in B2 and TSB-gluc media. Sixteen transposon mutants, exhibiting growth rates indistinguishable from the wild type, were found to be deficient in biofilm formation in TSB-gluc, but only five were also unable to produce a biofilm in B2. Interestingly, these five mutants produced smooth colonies in Congo red agar plates and were subsequently selected for further analysis. Sequencing of regions adjacent to the transposon insertion site and homology searches using the BLAST 2.0 program at the NCBI server showed that insertion sites were localized in three loci: one affected the expression of SarA protein (M6); one involved a locus called *pnpA* (M3) and three affected different genes in the *icaADBC* operon already known to be implicated in the biofilm formation process (M1, 4, 5). The finding that three independent mutants affected the *icaADBC* operon suggested that we had performed a saturating mutagenesis screening (see *Supplementary material*, Table S1 for the identification of the mutants deficient in biofilm development). Further analysis of the role of *sarA* in the biofilm formation process is presented below. Analysis of the role of *pnpA* will be presented elsewhere.

Effect of *sarA* deletion among unrelated *S. aureus* isolates

To further support the role of the *sarA* gene in biofilm formation and to exclude spontaneous mutations or polar effects of the transposon insertion on downstream genes, a strain carrying a complete deletion of the *sarA* gene (Δ *sarA*) was constructed by allelic exchange. As shown in Fig. 1, the deletion mutant, akin to the *sarA* transposon mutant, showed a smooth colony morphology phenotype

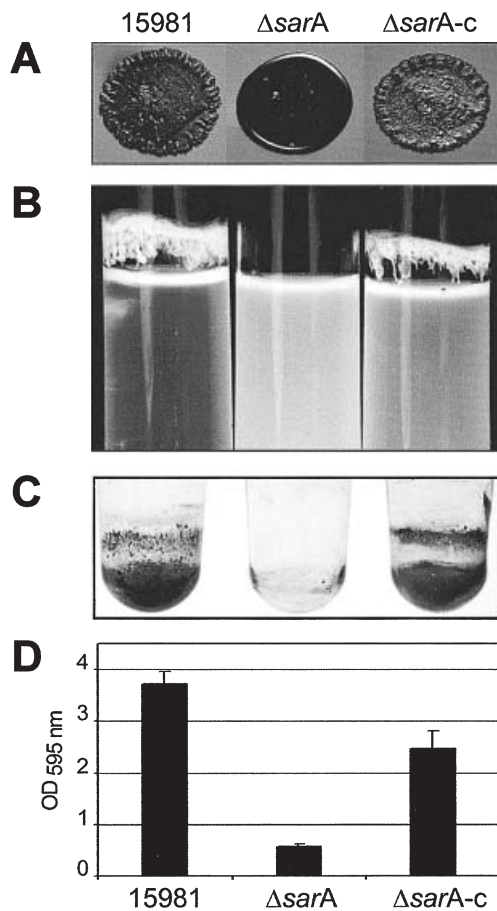


Fig. 1. Characterization of various biofilm phenotypes of *S. aureus* 15981, $\Delta sarA$ and $\Delta sarA$ -c.

A. Comparison of colony morphology on Congo red agar plates at 37°C for 24 h.
 B. Biofilm formation on a glass surface of an overnight culture incubated with shaking.
 C. Biofilm formation in PVC microtitre dishes of cells grown for 24 h in TSB-gluc at 37°C without shaking.
 D. Crystal violet-stained, surface-attached cells were quantified by solubilizing the dye in ethanol/acetone and determining the absorbance at 595 nm.

in Congo Red agar (Fig. 1A), lost the ability to produce a ring of cells adhered to the glass wall at the air-liquid interface (Fig. 1B) and was unable to form a biofilm on polystyrene and PVC microtitre plates (Fig. 1C and D). To confirm that the biofilm-deficient phenotype was due to the deletion of *sarA*, $\Delta sarA$ mutant was complemented with plasmid pCU1 carrying a PCR amplified 1349 bp fragment containing the *sarA* gene under the control of its own promoter. The complemented strain ($\Delta sarA$ -c) displayed a biofilm phenotype similar to that of the wild-type strain (Fig. 1A–D). In order to show the independence of the biofilm deficient phenotype with respect to the strain background, we also produced a non-polar *sarA* deletion in the genetically unrelated *S. aureus* MA12 and V329 strains and an insertional mutation in the ISP479c strain.

The link between the *sarA* mutation and the absence of the biofilm formation ability was confirmed in all the strains (Fig. 2).

Biofilm formation ability of the *sarA* mutant in continuous flow culture microfermenters

In order to rule out the possibility that inhibition of biofilm formation in the $\Delta sarA$ mutant was a result of the accumulation of products during the steady state conditions in the microtitre plates, we analysed the biofilm formation capacity in a continuous flow culture on pyrex slides submerged in microfermenters (Ghigo, 2001). The flow rate of fresh medium (40 ml h⁻¹) imposed in the process was high enough to avoid any significant planktonic growth. As shown in Fig. 3, strains 15981 and the complemented $\Delta sarA$ -c adhered abundantly to the glass substratum and formed a thick biofilm in 8 h. In contrast, after 24 h the $\Delta sarA$ strain only formed microcolonies on the surface of the slide without much biofilm development thereafter. This result demonstrated that SarA affects biofilm formation under flow conditions.

Regulation of biofilm formation by *agr*

SarA is a positive regulator of the *agr* operon and therefore influences the regulation of various virulence factors in an *agr*-dependent pathway. We therefore speculated whether SarA might affect biofilm formation indirectly, by acting through *agr*. To test this hypothesis, we created a complete deletion of the *agr* operon in the 15981 and V329 strains and an insertional mutation in the ISP479c strain. Interestingly, the biofilm formation capacity on

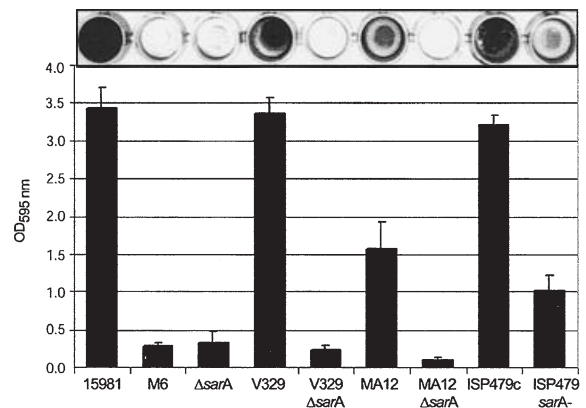


Fig. 2. Biofilm formation phenotype of four genetically unrelated *S. aureus* strains and their respective *sarA* mutants. Biofilm formation capacity of *S. aureus* 15981, V329, MA12 and ISP479c and their corresponding *sarA* mutants on polystyrene microtitre plates after 24 h hours in TSB-gluc medium at 37°C. The bacterial cells were stained with crystal violet and were quantified by solubilizing the dye in ethanol/acetone and determining the absorbance at 595 nm.

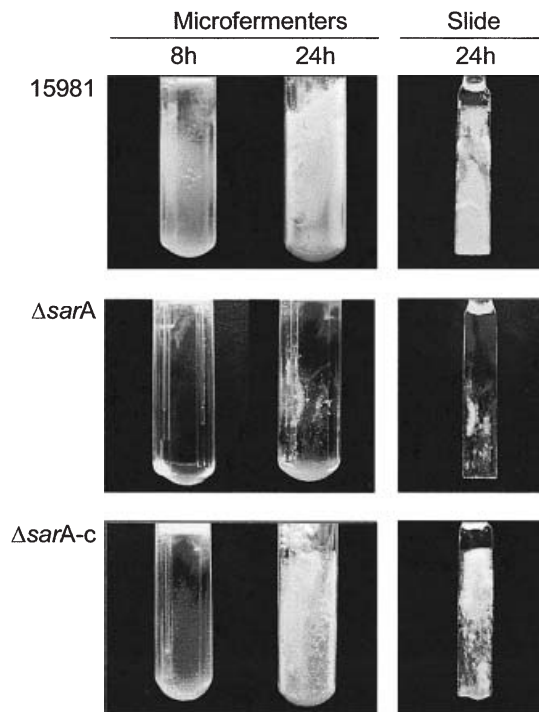


Fig. 3. Biofilm formation phenotype of *S. aureus* 15981, $\Delta sarA$ and $\Delta sarA$ -c in microfermenters. Left, biofilm development of bacteria grown in TSB-gluc at 37°C for 8 h and 24 h in microfermenters. Right, biofilm phenotype on the corresponding Pyrex slides removed from the microfermenters after 24 h of incubation in TSB-gluc at 37°C.

microtitre plates or in continuous flow cultures of the resulting *agr* deficient strains was similar to the corresponding wild-type strains, clearly showing that SarA affects biofilm formation via an *agr*-independent pathway (data not shown).

Relationship between the extracellular proteolytic activity and the biofilm formation capacity of the *sarA* mutant

It is well established that production of proteases is up-regulated in *sarA* mutants (Chan and Foster, 1998). It seems therefore reasonable to suppose that inhibition of biofilm formation in *sarA* mutants could be due to the degradation of a surface protein required for biofilm formation. To investigate this possibility we first verified the increased protease production of *sarA* mutants in milk agar plates. Figure 4A shows that, as expected, *sarA* mutants overproduces proteases. We then performed primary attachment assays on microtitre plates with wild-type and *sarA* mutants grown in the presence of α_2 -macroglobulin, a universal protease inhibitor that inhibits the activity of all major staphylococcal proteases (McGavin *et al.*, 1997; Karlsson *et al.*, 2001) and E64, a cysteine protease inhibitor, that inhibits two of the major *S. aureus*

proteases (SspB and Scp). No significant differences were found on the primary attachment ability of $\Delta sarA$ cells grown in the presence or absence of α_2 -macroglobulin or E64 (Fig. 4B), strongly suggesting that the biofilm formation deficiency of $\Delta sarA$ was not due to the increased production of extracellular proteases. In addition, wild-type 15981 and ISP479c cells were incubated with a concentrated supernatant from a stationary phase culture of their corresponding *sarA* mutant strain. Prolonged incubation of the cells with the supernatant did

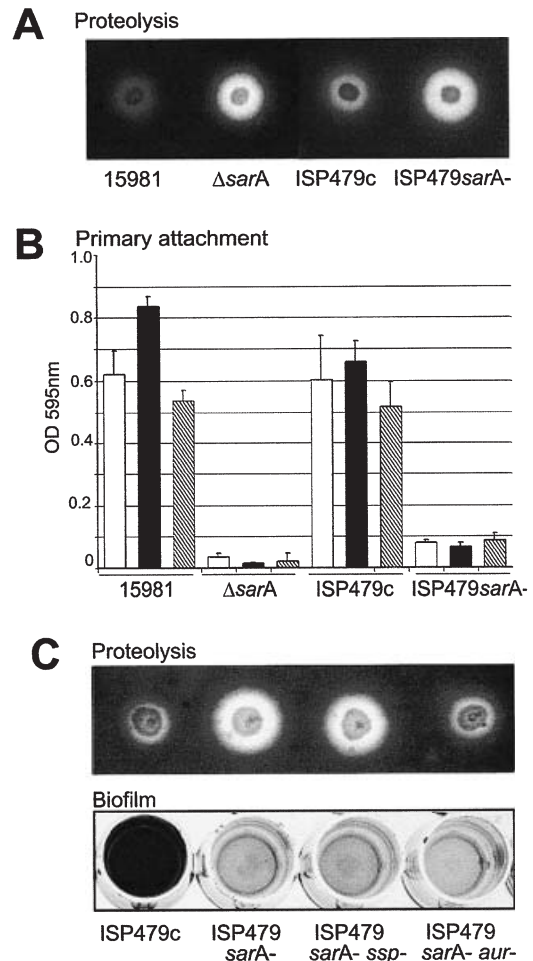


Fig. 4. Effect of protease inhibition on the biofilm formation phenotype.

A. Protease production by *S. aureus* 15981 and ISP479c and their corresponding *sarA* mutants grown in skimmed milk agar plates. B. Comparison of the primary attachment ability of *S. aureus* 15981 and ISP479c and their corresponding *sarA* mutant strains cultured in TSB-gluc (open bar), TSB-gluc with 0.4 U of α_2 -macroglobulin ml⁻¹ (filled bar), or TSB-gluc with 10 μM of E64 (hatched bar). C. Upper part, zones of proteolysis around *S. aureus* ISP479c, *sarA*-, *sarA*-*ssp*- and *sarA*-*aur*- mutant strains grown on skimmed milk agar plates. Lower part, comparison of the ability to produce a biofilm on polystyrene microtitre dishes of *S. aureus* ISP479c, *sarA*-, *sarA*-*ssp*- and *sarA*-*aur*- mutant strains grown in TSB-gluc at 37°C without shaking.

not affect biofilm formation capacity (data not shown). To prove that the major proteases were not responsible for biofilm deficiency, we constructed a *sarA* mutant harbouring a deletion of either aureolysin gene (*aur*) or the serine protease gene (*ssp*) and determined the ability of the double mutants to produce a biofilm. Because of the facility in genetically manipulating ISP479c strain we constructed these mutations only in this strain. The *sarA-aur-* and *sarA-ssp-* double mutants displayed a similar biofilm deficiency to that of the *sarA* mutant (Fig. 4C). Taken together, these data strongly suggest that extracellular proteases were not responsible for the biofilm deficiency of *sarA* mutant cells.

Effect of SarA on production of PIA/PNAG

We hypothesized that the role of SarA in biofilm formation could be the activation of the expression of PIA/PNAG. To investigate whether PIA/PNAG production was altered in the *sarA* mutant strains, the production of PIA was monitored by dot-blot using specific anti-PIA/PNAG polyclonal antisera. Our results showed that whilst significant amounts of PIA/PNAG could be detected in young cultures ($OD_{650nm} = 0.2$) in the wild-type strains, PIA/PNAG production was substantially reduced in the $\Delta sarA$ mutants as compared to the parental strains at all phases of growth (Fig. 5). The reduction level was particularly less pronounced in the ISP479c *sarA* mutant strain. The fact that *sarA* mutants are still able to produce detectable amounts of PIA/PNAG indicates that SarA is relevant but not essential for PIA/PNAG production. Altogether, these results strongly suggest that the biofilm deficient phenotype of the $\Delta sarA$ mutants may be at least partially attributable to a decrease in PIA/PNAG levels.

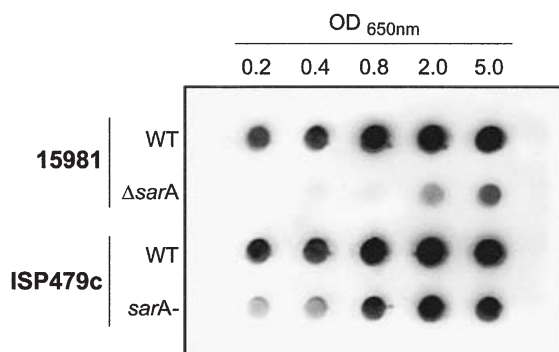


Fig. 5. Dot blot analysis of PIA-PNAG accumulation in *S. aureus* wild-type strains and their corresponding *sarA* mutants at different points of the growth curve. Cell surface extracts at different points of the growth curve of *S. aureus* 15981 and ISP479c and their corresponding *sarA* mutants were treated with proteinase K and spotted onto nitrocellulose filters. PIA/PNAG production was detected with an anti-*S. aureus* PIA/PNAG antiserum. The *sarA* mutant strains produced lower levels of PIA/PNAG product.

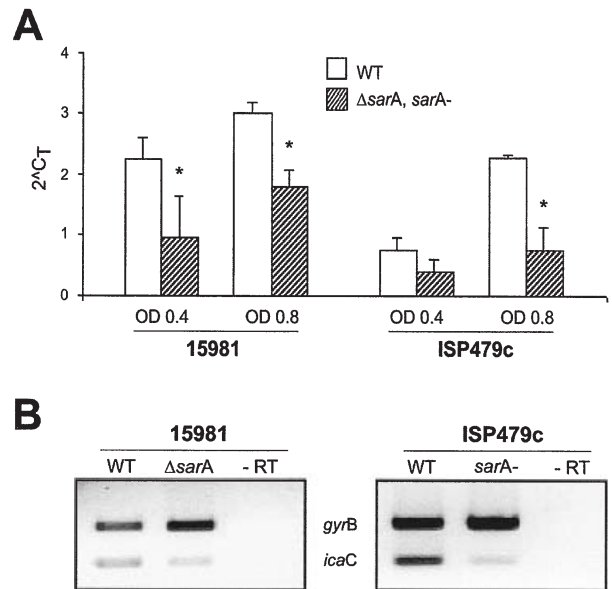


Fig. 6. Analysis of *ica* expression on *S. aureus* wild-type strains and their corresponding *sarA* mutants at different points of the growth curve.

A. Real time quantification of *icaA* expression. The gyrase B transcript was used as endogenous control and results are expressed as n -fold difference relative to the control gene. The figure represents the mean and standard deviation of five independent experiments. Asterisks denote $P < 0.05$.

B. Multiplex PCR of *icaC* and *gyrB* on *S. aureus* wild-type and *sarA* mutant strains at mid-log exponential phase ($OD_{650} = 0.8$). An aliquot of cDNA prepared in the absence of reverse transcriptase (-RT) (see *Experimental procedures*) is included to confirm the absence of genomic DNA.

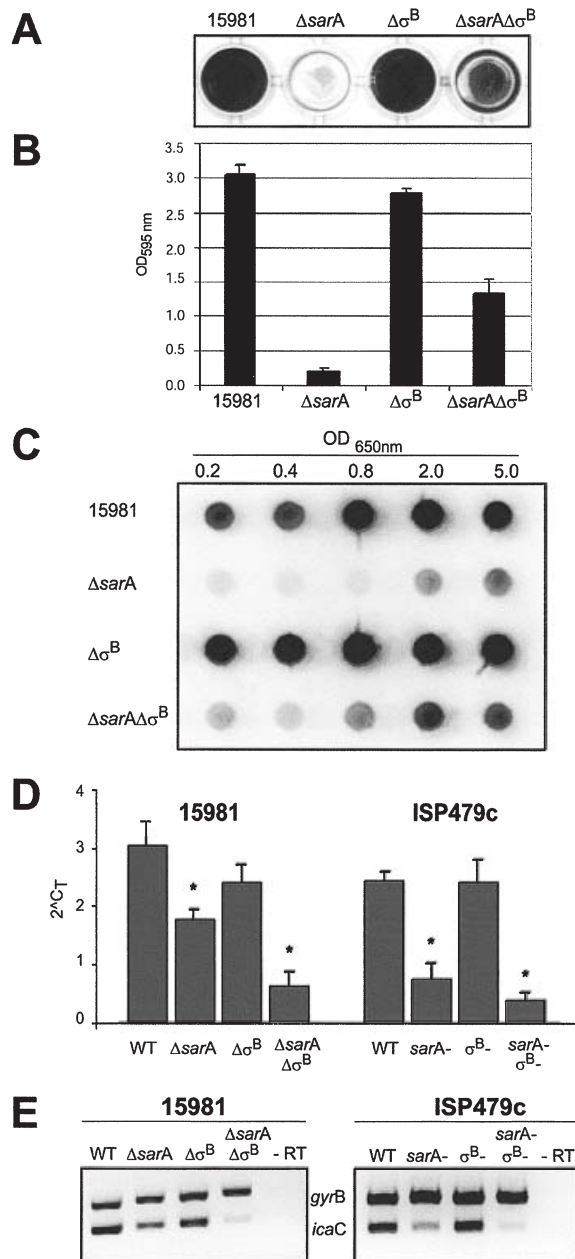
Transcriptional analysis of the *ica* operon expression in *sarA*-deficient strains

We used real-time quantitative PCR to investigate whether the decrease in PIA/PNAG production observed in $\Delta sarA$ mutant was caused by a reduction of the *ica* operon expression. Total RNA of strains 15981 and ISP479c and their corresponding *sarA* mutants was isolated at early exponential and mid-log exponential phases, as the expression of *ica* operon is maximum during this period. After treatment with DNAase to remove contaminant DNA, RNA was reverse transcribed in the presence and absence of reverse transcriptase. The level of expression of *icaA* was normalized on *gyrB* expression (Wolz *et al.*, 2002). Only samples with no amplification of *gyrB* in the minus reverse transcriptase aliquot were included in the study. The results showed that the *sarA* mutation resulted in a significant ($P < 0.05$) decrease of *ica* operon transcription compared to that of the wild-type strains at exponential and mid-log exponential phases (Fig. 6A). Similar results were obtained with RNA purified at early stationary phase ($OD_{650} = 2$) (data not shown). These results were confirmed by multiplex PCR with simulta-

neous amplification of *icaC* and *gyrB* (Fig. 6B). Because *icaADBC* operon is still transcribed, we theorize that SarA is an activator of the *ica* operon expression but that SarA activity alone cannot account for the total *ica* operon expression, thus suggesting that an additional factor(s) may be involved.

Effect of σ^B mutation in a *sarA*-deficient background

It has been reported that a mutation of either σ^B or *rsbU*, an activator of σ^B , results in a complete abolition of salt-induced biofilm formation and a drastical decrease of *ica* transcription (Rachid *et al.*, 2000b; Knobloch *et al.*, 2001).



It has also been proven that one of the *sarA* promoters (P3) is σ^B -dependent (Deora *et al.*, 1997; Manna *et al.*, 1998; Bischoff *et al.*, 2001). We therefore speculated whether σ^B may modulate *sarA* expression and the ensuing *ica* transcription. If such an association existed, double mutants carrying $\Delta sarA$ and $\Delta \sigma^B$ mutations and single mutants in any of these regulators would display the same decrease in *ica* transcription. We therefore produced a complete deletion of σ^B ($\Delta \sigma^B$) by allelic exchange in 15981 and $\Delta sarA$ and insertional mutation in the ISP479c and ISP479 *sarA*-. Remarkably, σ^B mutants: (i) formed large clumps in broth cultures and displayed a rough colony morphology in Congo Red agar plates (data not shown); (ii) retained the ability to form a biofilm on microtitre plates (Fig. 7A and B) and microfermenters; and (iii) produced PIA/PNAG levels similar to those of the wild-type strains (Fig. 7C). Interestingly, the *sarA*- σ^B double mutant displayed a higher capacity to produce a biofilm and an enhanced PIA/PNAG production compared with the *sarA* mutants, though never reached parental strain levels. Analysis of the transcriptional activity of the *ica* operon by real time PCR showed that the level of *icaA* transcripts was slightly reduced in σ^B mutants (Fig. 7D). In contrast to the results of PIA/PNAG production, double *sarA*- σ^B mutants showed a significant reduction ($P < 0.05$) in the *ica* operon transcription compared to that of the *sarA* mutants (Fig. 7D). These results were confirmed by multiplex PCR with simultaneous amplification of *icaC* and *gyrB* (Fig. 7E). Complementation experiments of the $\Delta sarA$ and *sarA*- σ^B mutants with a recombinant shuttle plasmid carrying either *sarA*, σ^B or *icaADBC* operon demonstrated that only SarA was able to restore biofilm formation in both strains. Interestingly, complementation *in trans* with a multicopy plasmid containing the complete *ica* operon slightly increased biofilm development in *sarA*- σ^B mutant and not in $\Delta sarA$ strain (data not shown).

Fig. 7. Analysis of biofilm formation, PIA/PNAG production and *ica* expression on *S. aureus* wild-type, *sarA*, σ^B and *sarA*- σ^B mutant strains.

A. Biofilm formation in polystyrene microtitre dishes of *S. aureus* 15981 and their corresponding $\Delta sarA$, $\Delta \sigma^B$ and $\Delta sarA \Delta \sigma^B$ mutant strains grown for 24 h in TSB-gluc at 37°C without shaking. B. Crystal violet-stained, surface-attached cells were quantified by solubilizing the dye in ethanol/acetone and determining the absorbance at 595 nm. C. Dot blot analysis of PIA-PNAG accumulation on *S. aureus* 15981 and their corresponding $\Delta sarA$, $\Delta \sigma^B$ and $\Delta sarA \Delta \sigma^B$ mutant strains at different points of the growth curve. D. Real time quantification of *icaA* expression on *S. aureus* 15981 and ISP479c strains and their corresponding *sarA*, σ^B and *sarA*- σ^B mutant strains at mid-log exponential phase (OD₆₅₀ = 0.8). E. Quantification of *icaC* expression by multiplex PCR of *icaC* and *gyrB* on *S. aureus* 15981 and ISP479c strains and their corresponding *sarA*, σ^B and *sarA*- σ^B mutant strains at mid-log exponential phase (OD₆₅₀ = 0.8). An aliquot of cDNA prepared in the absence of reverse transcriptase (- RT) (see *Experimental procedures*) is included to confirm the absence of genomic DNA.

Discussion

Bacteria seem to initiate biofilm development in response to a variety of environmental signals, such as nutrient and oxygen availability, osmolarity, temperature or pH. Obviously, the transition from planktonic to multicellular life-style requires the co-ordinated expression of a variety of specialized extracellular and cellular components. With the aim of identifying gene regulators that co-ordinate the biofilm formation process of *S. aureus*, we used the strategy of studying mutants defective in biofilm formation in two laboratory media (TSB-gluc and B2). With a similar strategy we were able to identify in a previous work two classes of genes involved in *Salmonella enteritidis* biofilm formation (Solano *et al.*, 2002). The first class included genes involved in the synthesis of the exopolysaccharidic matrix that was required for biofilm formation in different nutritional conditions. The second class included genes involved in many different pathways that were required to form a biofilm in some media but not in others. Similarly, two classes of genes involved in the biofilm formation process of *E. coli* have been identified using different culture media (Danese *et al.*, 2000). Also in this study, genes associated with *S. aureus* biofilm production were classified in two groups, as 11 of the 16 mutants detected produced a biofilm in B2 but not in TSB-gluc media. The most striking difference between B2 and TSB-gluc media is the NaCl content (2.5% in B2 versus 0.5% in TSB-gluc). Osmotic stress conditions similar to those of B2 (3% NaCl) significantly stimulated *S. aureus* biofilm formation (Rachid *et al.*, 2000b). This suggests that in an environment favourable for biofilm formation, such as B2 for *S. aureus*, only indispensable genes are required for biofilm development. In contrast, bacteria in a less favourable environment would additionally require a specific set of genes for sensing and responding to the particular nutritional characteristics. Therefore, we propose that *sarA* and *ica* genes represent indispensable common genes required for *S. aureus* biofilm formation in different environmental conditions.

Previous work established that *sarA* was a negative regulator of biofilm development, as *sarA* mutation increased adherence to glass (Pratten *et al.*, 2001). In contrast to these results, we have demonstrated that complete deletion of the *sarA* gene in four genetically non-related *S. aureus* strains completely abolished biofilm formation capacity by an *agr*-independent pathway. An explanation to this discrepancy could be that Pratten *et al.* (2001) compared the biofilm formation ability of mutants produced apparently on the same strain, but generated in different laboratories instead of comparing mutants created in the same 8325–4 isolate. Biofilm formation of isolate 8325–4 varies according to the laboratory source (our unpublished results). Therefore, we surmise that their

conclusions could be influenced by the fact that isolates of 8325–4 from different laboratories could have different abilities to produce a biofilm.

In the present work, an examination of *ica* mRNA production in the wild-type strain and the *sarA* mutant at various growth phases by real-time and multiplex PCR showed that the *ica* operon transcription decreased in *sarA* mutant cells. A consensus-directed search strategy for identifying the conserved motif essential for *sarA*-dependent gene regulation (Chien *et al.*, 1999) in the intergenic region between *icaADBC* and *icaR* revealed the presence of a sequence 70 nt upstream the start codon with 58% homology (15 nucleotides identity along 26 overlap) to the predicted *sarA*-like recognition sequence. This finding suggests that SarA may interact directly with the *ica* promoter and induce *ica* transcription. Alternatively, SarA might affect the expression of a regulatory protein that induces the transcription of the *ica* operon downstream of SarA. One such candidate is IcaR (Conlon *et al.*, 2002). IcaR appears to be a member of the *tefR* family of transcriptional regulators and encodes for a repressor of *ica* operon transcription under certain environmental conditions in *S. epidermidis*. Specifically, IcaR is required for ethanol activation of the *ica* operon, but does not affect the activation of *ica* operon expression induced by NaCl-glucose in *S. epidermidis* (Conlon *et al.*, 2002). It seemed reasonable to predict that *sarA* up-regulates *ica* transcription by repressing *icaR* expression. However, preliminary studies on *icaR* expression in the *sarA* mutant strain (our unpublished results) did not support this hypothesis and indicated that other regulatory elements may be involved in the *ica* operon repression. Other candidate regulators are SarH1 (also called SarS) (Tegmark *et al.*, 2000; Cheung *et al.*, 2001), SarT (Schmidt *et al.*, 2001) and Rot (McNamara *et al.*, 2000). Both SarH1 and SarT belong to a family of SarA homologues whose expression is regulated by both *sarA* and *agr*. Thus, *sarA* mutants constitutively express high levels of SarH1 and SarT, which could affect the expression of target genes either as activators or as repressors. However, SarH1 and SarT are also overproduced in an *agr* mutant and therefore we would expect in this mutant a similar decrease in the biofilm phenotype. Because the *agr* mutants that we have produced are not affected in biofilm formation, it seems unlikely that either SarH1 or SarT would be the repressors of the *ica* operon in the *sarA* mutant. The other candidate regulator, *rot*, encodes a repressor that affects the transcription of extracellular virulence factors during the lag and exponential phase of bacterial growth (McNamara *et al.*, 2000). Intriguingly, we have observed that insertional mutation of *rot* impaired biofilm formation in TSB-gluc but did not affect biofilm formation in B2 (Supplementary material, Table S1), strongly suggesting that under certain environmental con-

ditions the Rot protein could be one of the factor(s) involved in the downstream control of biofilm development.

The alternative transcription factor σ^B controls, in conjunction with additional regulators, the differentiation pattern associated with starvation, several stress factors and cell entry into stationary phase. It has been shown that σ^B , among others, transcriptionally regulates *sarA* (Deora *et al.*, 1997; Kullik *et al.*, 1998; Manna *et al.*, 1998; Bischoff *et al.*, 2001) and *ica* operon transcription (Rachid *et al.*, 2000b). In this respect, and based on our results on *ica* operon transcription in *sarA* mutants, we performed additional studies to establish the relationship between σ^B , *sarA* and *ica* operon. Our results showed that *ica* transcription was slightly decreased in the σ^B mutant. However, it did not affect either PIA/PNAG production or biofilm development. This finding apparently contrasts with a previous report (Rachid *et al.*, 2000b) that suggested that σ^B was required for biofilm formation. In the article of Rachid *et al.* (2000b) the strain MA12 and its corresponding σ^B mutant (MA12.2) produced similar low levels of biofilm in TSB media, whereas in the presence of 3% NaCl the biofilm production was only increased in strain MA12. On the other hand, the levels of PIA/PNAG were not measured and nothing was concluded on this matter. The strains used in our study were able to produce biofilm without being subjected to osmotic stress. In this situation σ^B did not significantly affect PIA/PNAG production and biofilm formation although a *sigB* mutation caused a slightly decrease in *ica* operon transcription.

Additionally, we observed that *ica* expression was significantly decreased in the *sarA*- σ^B double mutant compared to $\Delta\sigma^B$ mutant. Remarkably, the levels of PIA/PNAG and biofilm formation capacity increased in this double mutant compared to those of the $\Delta sarA$ strain. Overall, our findings on *ica* transcription (wild-type > σ^B > $\Delta sarA$ > *sarA*- σ^B -), PIA/PNAG production (σ^B \geq wild-type > *sarA*- σ^B - > $\Delta sarA$) and biofilm formation indicate that an increase in *ica* transcription does not necessarily lead to an increase in PIA/PNAG and biofilm production. Both *SarA* and σ^B appear to promote *ica* operon transcription but may have a reverse effect in PIA/PNAG and biofilm production: σ^B mutation leads to a decrease in *ica* transcription but to maintenance or increase of PIA/PNAG and biofilm production. On the other hand, *SarA* mutation leads to a decrease in *ica* transcription and PIA/PNAG production which is at least partially counteracted by a σ^B mutation.

Thus, emerging from this work and aligned with previous findings (Conlon *et al.*, 2002), a possible regulatory mechanism of PIA/PNAG production and biofilm formation could be proposed (Fig. 8). Both *SarA* and σ^B upregulate *ica* operon expression at the transcriptional level; whether this regulation is direct or indirect remains unclear. In addition, *SarA* may affect PIA/PNAG production upon sup-

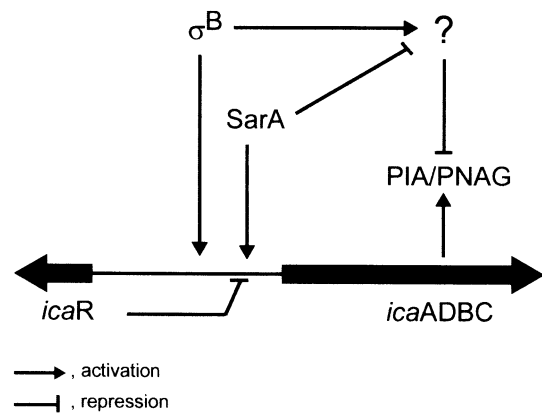


Fig. 8. Summary illustrating the role of *SarA* in the regulation pathway leading to the biofilm formation. *SarA* and σ^B both activate *ica* transcription either directly or indirectly. In addition, *SarA* may affect PIA/PNAG production upon suppression of an unknown element that would either degrade PIA/PNAG product or repress the PIA/PNAG synthesis. σ^B would upregulate the expression of the unknown element. *IcaR* represses *ica* transcription possibly in concert with an additional factor(s) probably located downstream the *sarA* pathway

pression of an unknown element that would either degrade PIA/PNAG product or repress the PIA/PNAG synthesis. σ^B would upregulate the expression of the unknown element. *IcaR* downregulates *ica* transcription in response to specific environmental conditions probably in concert with additional factor(s).

To the well-characterized roles of the *SarA* protein in the regulation of many virulence determinants, we have now added a novel one in the upregulation of biofilm development. The co-regulation of both processes suggests that multicellular behaviour may play a role in several previously identified infection models. *SarA* may thus be a promising target to simultaneously control the biofilm development on indwelling devices and virulence mediated by extracellular toxins.

Experimental procedures

Bacterial strains, culture conditions and plasmids

Staphylococcus aureus 15981, isolated at the Microbiology Department of the University Clinics of Navarra, was selected because of its strong biofilm production phenotype, antibiotic susceptibility profile and availability to accept recombinant DNA by electroporation. The most relevant bacterial strains and plasmids used and constructed in this study are listed in Table 1. *Escherichia coli* XL1-Blue cells were grown in Luria-Bertani (LB) broth or on LB agar (Pronadisa) with appropriate antibiotics. *Staphylococcal* strains were cultured on Trypticase soy agar (TSA), in trypticase soy broth supplemented with glucose (0.25% w/v) when indicated (TSB-gluc), in TSA supplemented with skimmed milk (5% w/v) when indicated (milk agar plates), in B2 broth (Schenk and Laddaga, 1992) and in Congo red agar (Baselga *et al.*, 1993). Media were

Table 1. Strains and plasmids used in this study.

Strain and plasmid	Relevant characteristics	Source and reference
<i>S. aureus</i>		
15981	Clinical strain. Biofilm positive. <i>rsbU</i> +	This study
M6	15981 <i>sarA</i> ::Tn917	This study
M3	15981 <i>prpA</i> ::Tn917	This study
$\Delta sarA$	15981 $\Delta sarA$	This study
$\Delta sarA$ -c	15981 $\Delta sarA$ complemented with <i>sarA</i> gene	This study
$\Delta \sigma^B$	15981 $\Delta \sigma^B$	This study
$\Delta sarA$ - $\Delta \sigma^B$	15981 $\Delta sarA$ - $\Delta \sigma^B$	This study
$\Delta sarA$ - $\Delta \sigma^B$ - <i>sarA</i>	15981 $\Delta sarA$ - $\Delta \sigma^B$ complemented with <i>sarA</i> gene	This study
$\Delta sarA$ - $\Delta \sigma^B$ - σ^B	15981 $\Delta sarA$ - $\Delta \sigma^B$ complemented with pSK9	This study
$\Delta sarA$ - $\Delta \sigma^B$ - <i>ica</i>	15981 $\Delta sarA$ - $\Delta \sigma^B$ complemented with pSC18	This study
Δagr	15981 Δagr	This study
8325-4	Wild-type strain 8325 cured of phages $\phi 11$, $\phi 12$ and $\phi 13$	Novick (1990)
PC1839	8325-4 <i>sarA</i> :: <i>km</i>	Chan and Foster (1998)
RN6911	8325-4 <i>agr</i> :: <i>tet</i>	Novick <i>et al.</i> (1993)
PC400	8325-4 <i>sigB</i> :: <i>tet</i>	Chan and Foster (1998)
RN4220	A mutant of 8325-4 that accepts foreign DNA	Novick (1990)
AK1	8325-4 <i>aur</i> :: <i>ermB</i>	Karlsson <i>et al.</i> (2001)
AK2	8325-4 <i>ssp</i> :: <i>ermB</i>	Karlsson <i>et al.</i> (2001)
ISP479c	ISP479 cured of plasmid. <i>rsbU</i> -	Pattee (1981)
ISP479 <i>sarA</i> -	ISP479c <i>sarA</i> :: <i>km</i>	This study
ISP479 <i>agr</i> -	ISP479c <i>agr</i> :: <i>tet</i>	This study
ISP479 σ^B -	ISP479c <i>sigB</i> :: <i>tet</i>	This study
ISP479 <i>sarA</i> - σ^B -	ISP479c <i>sarA</i> :: <i>km sigB</i> :: <i>tet</i>	This study
ISP479 <i>sarA</i> - <i>aur</i> -	ISP479 <i>sarA</i> :: <i>km aur</i> :: <i>ermB</i>	This study
ISP479 <i>sarA</i> - <i>ssp</i> -	ISP479 <i>sarA</i> :: <i>km ssp</i> :: <i>ermB</i>	This study
V329	Bovine subclinical mastitis isolate. Biofilm positive. <i>rsbU</i> +	Cucarella <i>et al.</i> (2001)
V329 $\Delta sarA$	V329 $\Delta sarA$	This study
V329 Δagr	V329 Δagr	This study
MA12	Mucosal isolate. Biofilm positive. <i>rsbU</i> +	Rachid <i>et al.</i> (2000b)
MA12 $\Delta sarA$	MA12 $\Delta sarA$	This study
Plasmid		
pID408	<i>E. coli</i> – <i>S. aureus</i> shuttle vector with a termosensitive origin of replication for Gram-positive bacteria that contains the transposon Tn917	Mei <i>et al.</i> (1997)
pMAD	<i>E. coli</i> – <i>S. aureus</i> shuttle vector with a termosensitive origin of replication for Gram-positive bacteria	M. Arnaud and M. Debarbouille, unpublished
pCU1	Vector for complementation experiments	Augustin <i>et al.</i> (1992)
pSK9	Plasmid that carries σ^B gene.	Rachid <i>et al.</i> (2000b)
pSC18	Plasmid that carries <i>icaADBC</i> genes	Cramton <i>et al.</i> (1999)

supplemented with appropriate antibiotics at the following concentrations: erythromycin (Er) 20 $\mu\text{g ml}^{-1}$ or 1.5 $\mu\text{g ml}^{-1}$, ampicillin (Am) 100 $\mu\text{g ml}^{-1}$, chloramphenicol (Cm) 20 $\mu\text{g ml}^{-1}$, kanamycin (Km) 50 $\mu\text{g ml}^{-1}$ and tetracycline (Tet) 10 $\mu\text{g ml}^{-1}$. The correspondence of the OD₆₅₀ values with the growth curve is: early exponential phase (OD₆₅₀ = 0.4); mid-log exponential phase (OD₆₅₀ = 0.8); early stationary phase (OD₆₅₀ = 2) and stationary phase (OD₆₅₀ = 5, overnight culture).

DNA manipulations

DNA plasmids were isolated from *E. coli* strain using the Qiagen plasmid miniprep Kit, according to the manufacturer's protocol. Plasmids were transformed into staphylococci by electroporation, using a previously described protocol (Cucarella *et al.*, 2001). Restriction enzymes were purchased from Boehringer Mannheim and used according to the manufacturer's instructions. Oligonucleotides were obtained from Life Technologies (Table 2). *sarA*, *aur*, *ssp*, *agr* and σ^B genes were inactivated in *S. aureus* ISP479c

transferring of the *sar*-, *aur*-, *ssp*-, *agr* and σ^B by phage transduction using $\Phi 85$ (Novick, 1991). For Southern hybridization, chromosomal DNA was purified as previously described (Marmur, 1961), digested and analysed by agarose gel electrophoresis. DNA fragments were transferred by alkaline capillary blotting onto nylon membranes (Schleicher and Schuell) using standard methods (Ausubel *et al.*, 1990). Labelling of the probe and DNA hybridization were performed according to the protocol supplied with PCR-DIG DNA-labelling and chemiluminescence detection kit (Boehringer Mannheim).

Transposon mutagenesis and gene identification

Staphylococcus aureus 15981 was transformed with plasmid pID408 (Mei *et al.*, 1997) by electroporation and random Tn917 insertions were carried out as described (Cucarella *et al.*, 2001). Mutants deficient in biofilm formation were screened on microtitre polystyrene plates using two rich media, B2 and TSB-gluc. B2 broth is a rich medium with high salt content, in which we and others (Lee, 1995) noted that

Table 2. Oligonucleotides used in this study.

Oligonucleotide	
pseq	AGAGAGATGTCACCGTCAAGT
sarB1	GGGGATCCAAAGCGTTGATTTGGGTA
sarB2	GGGGATCCAGTGCCATTAGTGCAAAA
sar-A	GATGATAGTGCAGGTGG
sar-B	GTTTAGCGCAATTTGG
sar-C	TTCACCAAATTGCGCTAAACCCTCCCTATTTGATGCATCTTGCT
sar-D	ACCCGTTATCAATCGG
sigB-A	GCAGTGTAAATACTGCTTC
sigB-B	AGCACATAAATAGAATTTGC
sigB-C	TAATAAGCAAATTTCTATTTATGTGCTGCCATTGGTTAATTTGCTCAG
sigB-D	GTTAATGAAGGAACGGAGG
agr-A	AGCACTGAGTCCAAGG
agr-B	TTTTACACCACTCTCC
agr-C	GTGAGGAGAGTGGTGTAAAAAAGATAATAAAGTCAGTTAACGGC
agr-D	CAGTTATTAGCAGGAT
aur-1	CGATGGTGACAGTAATAAA
aur-2	CGTTAATGCTCGGTAGTA
ssp-1	CCCTTAATGTATTTGTAA
ssp-2	CCATGACGTATACAAATT
IcaAligh-1	CTTGGATGCAGATACTATCG
IcaAligh-2	GCGTTGCTTCCAAAGACCTC
IcaCligh-1	ACACAGCGTTTCACGATACCG
IcaCligh-2	CCAATAGTCTCCATTTGCTAACGC
GyrU	TTATGGTGTCTGGGCAAATACA
GyrL	CACCATGTAAACCACCAGATA

different *S. aureus* strains clumped and formed extremely tenacious biofilm on the surface of the glass container during preparation of electrocompetent cells (Schenk and Laddaga, 1992). To identify genes containing Tn917 insertions, chromosomal DNA of each mutant was digested with *EcoRI* and religated in 200 µl of ligation buffer (Promega) for 12 h at 14°C. The ligation products were transformed into *E. coli* XL1-Blue and selected onto LB agar containing Am. Plasmid DNA was extracted using a Qiagen plasmid miniprep kit. Chromosomal DNA sequences flanking the transposon were obtained using primer pseq (Table 2) which corresponds to the inverted repeat region located 70 bp from the *erm*-proximal end of Tn917. Nucleotide sequences were determined at the DNA Sequencing Service of the University of Navarra (Spain). Homology searches were carried out using BLAST 2.0 program (Altschul *et al.*, 1997) at the NCBI server.

Allelic exchange of chromosomal genes

To construct the deletions, we amplified by PCR two fragments of 500 bp that flanked the left (oligonucleotides A and B) and the right (oligonucleotides C and D) of the sequence targeted for deletion (Table 2). The oligonucleotides B and C have at least a 16-base complementary region (underlined in the oligonucleotide sequence) to allow the products of the first PCR to anneal at their overlapping region. A second PCR was performed with primers A and D to obtain a single fragment. Specifically, 1 µl of each of the first PCR was mixed with 10 pM of the outside primers and PCR amplified. The fusion products were purified and cloned in the pGEM-T easy vector (Promega). The fragment was then cloned into the *EcoRI* site of the shuttle plasmid pMAD (M. Arnaud and M. Debarbouille, unpublished data) and the resulting plasmid was transformed into *S. aureus* by electroporation. pMAD

contains a temperature-sensitive origin of replication and an erythromycin resistance gene. The plasmid was integrated into the chromosome through homologous recombination at non-permissive temperature (43.5°C). From the 43.5°C plate, one to five colonies were picked into 10 ml of TSB-gluc and incubated for 24 h at 30°C. Ten-fold serial dilutions of this culture in sterile TSB-gluc were plated on TSA containing X-gal(5-bromo-4-chloro-3-indolyl-B-D-galactopyranoside) (150 µg ml⁻¹). White colonies, which no longer contained the pMAD plasmid, were tested to confirm the replacement by PCR using the oligonucleotides A and D and Southern blotting.

Complementation of the mutants

The *sarA* gene from *S. aureus* 15981 was amplified with high-fidelity thermophilic DNA polymerase (Dynazyme Ext, Finnzymes) with primers sarB1-sarB2 (Table 2). The PCR products were cloned into pCU1 (Augustin *et al.*, 1992) and the resulting plasmid pCU1 *sar* was transformed by electroporation into *S. aureus* Δ*sarA*. Complementation experiments with wild-type σ^B and *ica* operon were performed using plasmids pSK9 and pSC18 respectively (Table 1).

Biofilm formation and primary attachment assays

Biofilm formation assay in microtitre wells was performed as described (Heilmann *et al.*, 1996). For adherence assays to glass tubes, a single colony was transferred to 5 ml of TSB-gluc and incubated at 37°C in an orbital shaker (250 r.p.m) for 12 h. Primary attachment assays in the presence of protease inhibitors were performed as follows: *S. aureus* strains were grown overnight in TSB-gluc and diluted 1:100 in TSB-

gluc containing either 0.4 U ml⁻¹ of α 2-macroglobulin (Sigma) or 10 μ M of E64 {[L-*trans*-epoxysuccinyl-leucyl-amido-(4-guanidino)butane], Sigma}. Bacteria were incubated in the presence of the inhibitors until mid-log exponential phase (OD₆₅₀ = 0.8). The culture was diluted to OD₆₅₀ = 0.1 and 200 μ l were used to inoculate sterile 96-well polystyrene microtitre plates (Iwaki). After 1 h at 37°C the wells were gently rinsed at least five times with phosphate-buffered saline (PBS), dried in an inverted position and stained with 0.1% of crystal violet for 15 min. The wells were rinsed again, and the crystal violet solubilized in 200 μ l of ethanol-acetone (80 : 20 v/v). The optical density at 595 nm (OD₅₉₅) was determined using a microplate reader (Multiskan EX; Lab-systems). Each assay was performed in triplicate and repeated three times.

To analyse the biofilm formation under flow conditions we used 60-ml microfermenters (Pasteur Institute's Laboratory of Fermentation) with a continuous flow of 40 ml h⁻¹ of TSB-gluc and constant aeration with sterile pressed air (0.3 bar). Submerged pyrex slides served as growth substratum. 10⁸ bacteria from an overnight preculture grown in TSB-gluc of each strain were used to inoculate microfermenters and were cultivated 24 h at 37°C. Biofilm development was recorded with a Nikon Coolpix 950 digital camera.

PIA/PNAG detection

PIA/PNAG production in *S. aureus* 15981 and mutants was detected as described (Cramton *et al.*, 1999). Overnight cultures of *S. aureus* strains were diluted 1:100 and grown to early exponential phase (OD₆₅₀ = 0.4); mid-log exponential phase (OD₆₅₀ = 0.8); early stationary phase (OD₆₅₀ = 2) and stationary phase (OD₆₅₀ = 5, overnight culture). Cells were grown TSB-gluc until the appropriate optical density. The same number of cells of each culture was resuspended in 50 μ l of 0.5 M EDTA (pH 8.0), incubated for 5 min at 100°C and centrifuged to pellet them. 40 μ l of the supernatant was incubated with 10 μ l of proteinase K (20 mg ml⁻¹; Sigma) for 30 min at 37°C. After addition of 10 μ l of Tris-buffered saline [20 mM Tris-HCl, 150 mM NaCl (pH 7.4)] containing 0.01% bromophenol blue, 5 μ l was spotted on a nitrocellulose filter using a Bio-Dot Microfiltration Apparatus (Bio-Rad), blocked overnight with 5% skimmed milk in phosphate-buffered saline (PBS) with 0.1% Tween 20 and incubated for 2 h with an anti-*S. aureus* PIA/PNAG antibody diluted 1:10 000 (Mckenney *et al.*, 1999). Bound antibodies were detected with a peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) antibody (Jackson ImmunoResearch Laboratories) diluted 1:10 000, and the Amersham ECL Western blotting system.

Real-time quantitative PCR

Total *S. aureus* RNA was prepared using the Fast RNA-Blue kit (Bio101) according to the manufacturer's instructions. Two micrograms of each RNA were subjected in duplicate to DNase I (Gibco-BRL) treatment for 30 min at 37°C. The enzyme was inactivated at 65°C in the presence of EDTA. To verify the absence of genomic DNA in every sample, the RNA duplicates were reverse transcribed in the presence and absence of M-MLV Reverse Transcriptase (Gibco-BRL) respectively. All preparations were purified using CentriSep

spin columns (Princeton Separations). One twentieth of each reaction was used for real-time quantitative PCR using a LightCycler and the LC-DNA Master SYBR Green I mix (Roche Diagnostics). The *IcaA* transcripts were amplified using primers *IcaA*Light-1 and *IcaA*Light-2 (Table 2). The *gyrB* transcripts that are constitutively expressed were amplified as endogenous control using primers *GyrU* and *GyrL* (Wolz *et al.*, 2002). To monitor the specificity, final PCR products were analysed by melting curves and electrophoresis. Only samples with no *gyrB* amplification of the minus reverse transcriptase aliquot were considered in the study. The amount of *icaA* transcript was expressed as the n-fold difference relative to the control gene ($2^{-\Delta C_T}$, where ΔC_T represents the difference in threshold cycle between the target and control genes).

Multiplex PCR

One-tenth of each reverse transcriptase reaction was used for the simultaneous amplification of *gyrB* and *icaC* transcripts during 30 cycles at 55°C. The primers used for the amplification of *icaC* were *IcaC*Light-1 and *IcaC*Light-2 (Table 2).

Statistical analysis

The data corresponding to gene expression were compared using the Kruskal–Wallis and the Mann–Whitney tests. All the tests were two-sided and the significance level was 5%. The statistical analysis was performed with the SPSS program.

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Supplementary material

The following material is available from <http://www.blackwellpublishing.com/products/journals/suppmat/mole/mole3493/mmi3493sm.htm>

Table S1. Sequence analysis of transposon-tagged genes of selected biofilm deficient mutants.

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