A novel repressor of the ica locus discovered from clinically isolated super biofilm-elaborating Staphylococcus aureus

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20 ABSTRACT

21 Staphylococcus aureus TF2758 is a clinical isolate from an atheroma and a super bio-
22 film-elaborating/PIA/PNAG-overproducing strain (S. Looniva et al. Microbiol. Immunol. 60,
23 148-159, 2016). A microarray analysis and DNA genome sequencing were performed to identify
24 the mechanism underlying biofilm overproduction by TF2758. We found high transcriptional
25 expression levels of a 7-gene cluster (satf2580 - satf2586) and the ica operon in TF2758. Within
26 the 7-gene cluster, a putative transcriptional regulator gene designated as rob had a nonsense
27 mutation that caused the truncation of the protein. The complementation of TF2758 with rob
28 from FK300, an rsbU-repaired S. aureus strain NCTC8325-4, significantly decreased biofilm
29 elaboration, suggesting a role for rob in this process. The deletion of rob in bio-
30 film-non-producing FK300 significantly increased biofilm elaboration and PIA/PNAG produc-
31 tion. In the search for gene(s) in the 7-gene cluster for biofilm elaboration controlled by rob, we
32 identified orf SAOUHSC_2898 (satf2584). Our results suggest that orf SAOUHSC_2898
33 (satf2584) and icaADBC are required for enhanced biofilm elaboration and PIA/PNAG produc-
34 tion in the rob deletion mutant. Rob bound to a palindromic sequence within its own promoter
35 region. Furthermore, Rob recognized the TATTT motif within the icaR-icaA intergenic region
36 and bound to a 25-bp DNA stretch containing this motif, which is a critically important short se-
37 quence regulating biofilm elaboration in S. aureus. Our results strongly suggest that Rob is a
38 long-sought repressor that recognizes and binds to the TATTT motif and is an important regula-
tor of biofilm elaboration through its control of SAOUHSC_2898 (SATF2584) and Ica protein expression in *S. aureus*.

**IMPORTANCE**

During the search for molecular mechanism underlying biofilm overproduction of *Staphylococcus aureus* TF2758, we found a putative transcriptional regulator gene designated as *rob* within a 7-gene cluster showing high transcriptional expression level by microarray analysis. The deletion of *rob* in biofilm-non-producing FK300, an *rsbU*-repaired NCTC8325-4, significantly increased biofilm elaboration and PIA/PNAG production. Search for gene(s) in the 7-gene cluster for biofilm elaboration controlled by *rob* identified *orf* SAOUHSC_2898. Besides binding to its own promoter region to control *orf* SAOUHSC_2898 expression, Rob recognized the TATTT motif within the icaR-icaA intergenic region and bound to a 25-bp DNA stretch containing this motif, which is a critically important short sequence regulating biofilm elaboration in *S. aureus*. Our results strongly suggest that Rob is a long-sought repressor that recognizes and binds to the TATTT motif and is a new important regulator of biofilm elaboration through its control of SAOUHSC_2898 and Ica protein expression in *S. aureus*. 
**Staphylococcus aureus** is among the most common human pathogens, causing a wide range of infections, from superficial skin and mucosal infections to bone or lung infections, as well as serious systemic diseases. *S. aureus* colonization has been regarded as a risk factor for developing subsequent infections. Some chronic infections, such as endocarditis, osteomyelitis, and those on implanted medical devices, are characteristically associated with biofilm elaboration (1-3). Development of biofilms has been divided into at least three physiologically different stages: initial attachment, biofilm maturation, and detachment (or dispersal), which involves specific factors (4). The matrix of a staphylococcal biofilm is mainly composed of polysaccharides, cell surface and secreted bacterial proteins, and extracellular DNA (5). Cells encased in the matrix are protected from antibiotic therapy and host immune responses (3, 4, 6). Dispersal of cells from a biofilm may be important for the dissemination of the bacteria (7).

The main exopolysaccharide of the *S. aureus* biofilm matrix is poly-N-acetylglucosamine (PNAG), which is also known as polysaccharide intercellular adhesion (PIA) (8). The synthesis and accumulation of PIA/PNAG on the cell surface is carried out by the products of four genes: *icaA, icaD, icaB, and icaC* (9). These genes are located in one operon and were first identified by Heilmann et al. (10). Recent studies have indicated that the expression of *icaADBC* is affected by a number of regulatory and environmental factors (11-14). The *icaR* gene is located adjacent to *icaADBC*, but is divergently transcribed from this operon (15). The protein encoded by *icaR* belongs to the TetR family of transcriptional regulators and represses *icaADBC* transcription by
binding to a region immediately upstream of the icaA start codon (16). Additionally, environ-
mental factors, including glucose, ethanol, high temperatures, and high osmolarity, have been
reported to affect biofilm elaboration (11-14). Ethanol increases the expression of icaA by re-
pressing icaR transcription (15). In contrast, enhancement of icaA expression by high glucose or
NaCl levels was found to occur independently of icaR.

A 5-nucleotide motif (TATTT) within the icaR-icaA intergenic region was previously shown
to play a key role in the transcription of the ica locus (16). This study also demonstrated that Ic-
aR binds to a 42-bp sequence within the ica promoter region, but not the TATTT sequence.

Hence, the effects of the TATTT motif on icaADBC expression have been suggested to be con-
trolled by other as yet unidentified repressor(s).

We evaluated the biofilm-elaborating ability of clinical isolates in Japan, and found that
TF2758, which was isolated from an atheroma, is an extremely high biofilm producer (17).
Whole-genome sequencing and a microarray analysis of TF2758 discovered a spontaneous mu-
tation in a putative transcriptional regulator gene, within a 7-gene cluster, which was expressed
at markedly higher levels than in a non-biofilm elaborating control strain. We designated this
gene as rob, regulator of biofilm. In the present study, we demonstrate that Rob is a long-sought
repressor that recognizes and binds to the TATTT motif and suggest that Rob is an important
regulator of biofilm elaboration through its control of the expression of an as yet uncharacterized
hypothetical protein SAOUHSC_2898 (SATF2584) and IcaADBC.
RESULTS

Identification of rob from a super biofilm-elaborating strain. As shown in Fig. 1, one of the clinically isolated strains, TF2758, showed a strong biofilm-elaborating ability and hyperproduction of PIA/PNAG (17). In order to elucidate the mechanism underlying the overproduction of biofilms in TF2758, a gene expression analysis using a custom microarray was performed. We used ATCC49775 as a control because it was the strain most closely related to TF2758 by comparative genomic hybridization and a very low biofilm-elaborating strain (Fig. 1A, B). The results obtained showed that there were two strongly up-regulated gene clusters: satf2580 – satf2586 (15-to-40-fold) and the ica operon (satf2686 – satf2689, 2-to-10-fold) (Fig. 1C, Table S1). Sequencing of the TF2758 genome and comparisons with complete genomes of other S. aureus strains indicated that TF2758 possessed a nonsense mutation in the gene satf2583 (Fig. 2A) and a missense mutation in the gene icaR, which resulted in the creation of a stop codon and an alteration in a nucleotide (A to T), respectively (Fig. S1 in the supplemental material). SATF2583 possessed regions homologous to the TetR family and AcrR family of transcriptional regulators, suggesting it acts as a DNA-binding protein (Fig. 2B). In order to clarify the impact of SATF2583 on biofilm elaboration in S. aureus, we transformed TF2758 with the plasmid pC001, pKAT carrying orfSAOUHSC_2897 with a 5’-flanking region cloned from FK300, the rsbU repaired derivative of NCTC8325-4. As shown in Figure 3A, TF2758 carrying pC001 significantly
lost its biofilm-elaborating ability, particularly in the absence of 1% glucose. PIA/PNAG detection by anti-PNAG revealed more clear-cut data (Fig. 3B). In the presence of 1% glucose, TF2758 carrying pC001 still retained its PIA/PNAG-producing ability; however, the amount produced was markedly less than that by the wild type. However, the production of PIA/PNAG was almost completely inhibited in the absence of 1% glucose. These results suggest that SATF2583 is a negative regulator of biofilm elaboration and also that the \textit{satf2583} gene from FK300 is functional.

The \textit{icaR} gene, located adjacent to the \textit{ica} operon, is a member of the TetR family of transcriptional regulators (15, 18). IcaR was previously reported to repress \textit{icaADBC} transcription by binding to a 42-bp region within the \textit{ica} promoter (16). The missense mutation that we identified in \textit{icaR} occurs in the HTH domain (Fig. S1 in the supplemental material) and may affect protein function. Therefore, we complemented TF2758 with pC002, which is pKAT carrying \textit{icaR} from FK300. The resulting strain significantly decreased biofilm elaboration and PIA/PNAG production was inhibited regardless of the presence or absence of glucose (Fig. 3A, B). These results suggest that \textit{icaR} from FK300 is functional and the \textit{satf2583}-involved biofilm elaboration pathway occurs through and upstream of the \textit{ica} operon. We tentatively named this \textit{orf rob} (regulator of biofilm).

\textbf{Effects of Rob on biofilm elaboration, PIA/PNAG production, and ica operon expression in} \textit{S. aureus FK300}. TF2758 was resistant to transformation by the plasmid pKFT and pKOR1,
which are used for allelic exchange in *S. aureus*. Since the *rob* gene in FK300 is functional, we selected strain FK300 for further studies on *rob* function. We introduced the same mutation found in TF2758 into *rob* in FK300 by allelic replacement. As shown in Fig. 4A, this mutation in *rob* resulted in a marked increase in biofilm elaboration in the presence or absence of 1% glucose.

The deletion of the *rob* gene also increased biofilm elaboration in FK300. We complemented the *rob* deletion mutant with plasmids carrying the *rob* gene (SAOUHSC_2897) from FK300 (pC001) or the truncated *rob* gene (*satf2583*) from TF2758 (pC003). We found that the transformant of the *rob* deletion mutant with pC001 exhibited repressed biofilm elaboration, similar to the control, FK300. In contrast, pC003 was unable to complement the *rob* deletion phenotype, which was similar to that of the *rob* deletion mutant carrying the mock vector pKAT (Fig. 4A).

In order to examine whether *rob* regulates biofilm elaboration through the *ica* operon, we measured *icaR* and *icaADBC* expression by qRT-PCR and PIA/PNAG production in wild-type and *rob* mutant strains of *S. aureus* FK300 (Fig. 4B). The results obtained indicated that the *rob* deletion mutant decreased *icaR* expression and increased *icaADBC* expression with a concomitant increase in PIA/PNAG production (Fig. 4C). The deletion of the *ica* operon in the FK300 *rob* deletion mutant abolished biofilm elaboration and PIA/PNAG production (Fig. 4A and C).

Taken together, these results suggest that biofilm elaboration in the *rob* deletion mutant is *ica*-dependent and Rob, at least in part, represses *icaADBC* transcription.
SAOUHSC_2898 (SATF2584) is involved in biofilm elaboration, which is under the control of Rob. The results of a preliminary microarray analysis suggested that Rob suppresses the expression of the surrounding 7-gene cluster (satf2580-satf2586) and the ica operon in TF2758 (Fig. 1C, Supple Table 1). Our RNA-seq data showed that these genes form operons (Fig. S2 in the supplemental material). Therefore, we hypothesized that Rob affects biofilm elaboration by repressing one or more genes in the (satf2580-satf2586) gene cluster. In order to test this possibility, we deleted upstream genes (SAOUHSC_2894, SAOUHSC_2895) and downstream genes (SAOUHSC_2898, SAOUHSC_2899 and SAOUHSC_2900) in the FK300 rob deletion mutant.

The results, shown in Figure 5, revealed that the deletion of the upstream genes had no significant effect, whereas the deletion of the downstream genes significantly reduced biofilm elaboration in the rob deletion mutant to a level similar to that of wild-type FK300.

As described above, there are three adjacent genes located immediately downstream of rob that are under the control of one promoter forming an operon. We deleted each gene individually in the FK300 rob deletion mutant (Fig. 6A). We found that only the SAOUHSC_2898 deletion caused a marked reduction in biofilm elaboration. The SAOUHSC_2899 deletion had a slight effect, whereas the SAOUHSC_2900 deletion had no effect on biofilm elaboration in the rob deletion mutant. Additionally, biofilm elaboration could be restored through complementation of rob, SAOUHSC_2898 double mutant with pC004, which carries the SAOUHSC_2898 gene.
from FK300 (Fig. S3 in the supplemental material). These results suggest that SAOUHSC_2898 is a critical factor mediating biofilm elaboration regulated by Rob.

In order to further confirm the regulation of SAOUHSC_2898 by rob, qRT-PCR was performed with RNA isolated from wild-type FK300 and the rob deletion mutant. The deletion of rob resulted in the increased expression of SAOUHSC_2898 (Fig. 6B). SAOUHSC_2898 is predicted to encode a 2-deoxy-D-gluconate 3-dehydrogenase that belongs to the oxidoreductase family (http://aureowiki.med.uni-greifswald.de/SAOUHSC_02898). Our results suggest that this enzyme is involved in some unknown biosynthetic pathway impacting biofilm elaboration. Rob may repress biofilm elaboration in FK300 by down-regulating the transcription of the SAOUHSC_2898 gene.

**Rob recognizes a palindromic motif in its own promoter.** A microarray analysis showed that the inactivation of rob in TF2758 resulted in the increased expression of surrounding genes. Therefore, using EMSA and DNase I footprint analyses, we investigated whether Rob directly binds to its own promoter and regulates this gene cluster’s transcription. We purified His-tagged Rob from *E. coli* and its binding to an intergenic fragment between SAOUHSC_2896 and rob (Fig. 7A). Rob induced significant dose-dependent shifts in the probe’s mobility. A DNase I footprint analysis clearly demonstrated that Rob bound to a 24-nucleotide DNA (Fig. 7B). We then analyzed the secondary structure of the binding sequence. We found that it contained an almost perfect palindromic sequence (Fig. 7C). Interestingly, the transcription start site (TSS) of
rob was predicted to be within the binding region of Rob by RNA-sequencing (RNA-seq) analysis (Fig. S2 in the supplemental material). These results suggest that Rob represses SAOUHSC_2898 transcription by recognizing the possible palindromic sequence present in the intergenic region of SAOUHSC_2896 and rob.

Recombinant Rob binds to the ica promoter region. As shown in Figure 4, the transcription levels of icaADBC were also significantly increased in the rob mutant. In order to investigate whether Rob directly modulates icaADBC expression by binding to the ica promoter, we used EMSA to analyze the Rob protein binding to a 198-bp probe (FULL) that contained the entire icaR-icaA intergenic region (Fig. 8A). As shown in Figure 8B, the recombinant Rob protein induced several shifts, even with only 0.1 μg of FULL. Rob-DNA complex bands migrated in a ladder-like pattern with increases in Rob protein concentrations. Rob-DNA complexes were outcompeted with a 100-fold excess of unlabeled specific competitor DNA. These results suggest that Rob binds to the icaR-icaA intergenic region in a dose-dependent manner.

Jefferson et al. previously identified a 5-bp (TATT) motif within the icaR-icaA intergenic region that controls the transcriptional regulation of the ica locus (16) (Fig. 8A). They suggested that an unknown repressor(s) utilize(s) the TATTT sequence in order to regulate icaADBC expression. Since Rob represses the ica transcription of the ica locus, we investigated whether Rob recognizes this 5-bp motif. We designed several additional probes for DNA binding assays (Fig. 8A). A 108-bp probe (SHORT-1), the shortest oligonucleotide containing the 5-bp sequence
lacking the 5’ 90-bp sequence of FULL, was dose-dependently shifted by Rob (Fig. 8C). We then
generated an oligonucleotide (SHORT-2) with a 28-bp deletion from the 5’ end of the SHORT-1
probe. As shown in Figure 8D, SHORT-2 had no significant shift in the presence of Rob. In order
to further investigate whether Rob recognizes the 5-bp motif, we made a 193-bp (FULL(- 5-bp))
probe lacking the 5-bp TATTT sequence of FULL. As shown in Figure 8E, FULL(- 5-bp) was
not shifted, as observed in FULL migration in Figure 8B, suggesting that Rob was unable to bind
to the 193-bp (FULL(- 5-bp)) probe. Taken together, these results suggest that Rob recognizes
and binds to the 5-bp motif within the ica promoter region.

We performed a DNase I footprint analysis to identify Rob-binding site(s). As shown in Fig-
ure 9, Rob protected a region of approximately 25 bp that included the 5-bp motif. In order to
further confirm that the 5-bp motif is necessary for the binding of Rob to the icaR-icaA intergen-
ic region, we attempted to screen for proteins bound to the icaR-icaA intergenic DNA fragment
with or without the 5-bp motif using cytosolic proteins of FK300. The cell extract of the
wild-type strain FK300 was mixed with magnetic beads conjugated with either the 198-bp
(FULL) probe or 193-bp (FULL(- 5-bp)) probe, and the bound proteins were then analyzed by
MALDI-TOF-MS. We found that Rob was present in proteins bound to the 198-bp probe, but
was absent in proteins bound to 193-bp (FULL(- 5-bp))(data not shown). Overall, these results
strongly suggest that Rob recognizes and binds to the 5-bp TATTT motif within the promoter re-
gion of the ica locus.
Biofilm elaboration is an important virulence determinant in certain types of *S. aureus* infections, particularly those involving implanted medical devices. Biofilm growth is influenced by a number of regulatory mechanisms. However, it is becoming increasingly apparent that the transcriptional regulation of biofilm-associated genes, such as *icaADBC*, is complex. Staphylococcal regulatory factors, including SarA, SigB, IcaR, TcaR, SrrAB, and Rbf, were previously shown to regulate *icaADBC* expression (11, 15, 19-22). In the present study, we identified a novel TetR/AcrR family regulator, Rob, which is a repressor of biofilm elaboration, by controlling SAOUHSC_2898, within a 7-gene cluster under the control of Rob. Furthermore, we demonstrated that Rob directly binds to the *icaR-icaA* intergenic region and represses *icaADBC*. The binding site in the *icaR-icaA* intergenic region contained the 5-bp motif, which has been suggested to control the transcriptional regulation of *icaADBC* (Fig. S4 in the supplemental material) (16).

The BLAST analysis showed that the *satf2580-satf2586* gene cluster, which was up-regulated in the super biofilm-elaborating strain TF2758, is also present in several other staphylococcal strains, but not in *S. epidermidis*, which is among the most studied and clinically relevant biofilm-elaborating organisms (see Fig. S5 in the supplemental material). Therefore, a novel regulatory pathway appears to be involved in biofilm elaboration in *S. aureus*. 
SAOUHSC_2898 is predicted to encode a 2-deoxy-D-gluconate 3-dehydrogenase, which belongs to the oxidoreductase family. Oxidoreductases specifically act on the CH-OH group of donors with NAD+ or NADP+ as an acceptor. This enzyme participates in pentose and glucuronate interconversions, a metabolic pathway that has recently been shown to be significantly enriched in biofilm elaboration (23). An increase in the expression of oxidoreductase was previously reported to induce staphylococcal biofilm elaboration (24). The detailed characterization of SAOUHC_2898 will provide an insight into ica-dependent biofilm elaboration.

SAOUHSC_2897 and SAOUHSC_2898 were previously reported to be accessory genes of a glucose-induced biofilm designated as gbaAB (25). However, in our assay, the complementation of TF2758 with SAOUHSC_2897 (rob) completely canceled PIA/PNAG production in the absence of 1% glucose. Furthermore, the addition of glucose did not alter the amount of PIA/PNAG produced by the FK300 rob deletion mutant (Fig. 4C). Thus, it is reasonable to assume that an SAOUHSC_2898-catalized pathway controlled by SAOUHSC_2897 (rob) affects biofilm elaboration in a glucose-independent manner (Fig. 10).

A number of regulators, including SigB, SarA, and SarX, and two-component signal transduction systems (TCSs) have been shown to affect staphylococcal biofilm elaboration (26-29). Our genetic analyses showed that the deletion of ica genes or SAOUHSC_2898 resulted in a loss in the ability to elaborate biofilms in the FK300 rob mutant. The rob mutant showed decreased
icaR expression and increased icaADBC transcription, suggesting that rob regulates an ica-dependent pathway for biofilm elaboration, at least in part by activating icaR expression.

Some factors regulate icaADBC expression by binding to the icaR-icaA intergenic region (15, 16, 22, 28). Although Rob is one of the TetR/AcrR family regulators in S. aureus, its role in the regulation of biofilms is not completely clear. Jefferson et al. previously reported that the TATTT sequence has a functional role in the transcriptional regulation of the ica locus (16). The simple deletion of the TATTT motif in S. aureus MN8m markedly increased biofilm elaboration and the transcription of icaADBC. They hypothesized the presence of uncharacterized repressor(s) recognizing and binding to the motif. Most recently, Schwartbeck et al. also showed that the S. aureus isolates carrying the 5-bp deletion exhibited a mucoid phenotype and strong biofilm formation (30). These mucoid isolates were protected against phagocytosis and survived better under starvation conditions. The results of the present study demonstrated that Rob binds to an icaR-icaA intergenic region of approximately 25 bp including the 5-bp TATTT motif, strongly suggesting that Rob is the postulated repressor reported by Jefferson et al. and further support rob regulating biofilm elaboration in an ica-dependent manner. A comparison of the Rob-binding site in the icaR-icaA intergenic region with that in the rob promoter revealed that the right half of the palindrome sequence was also present in the icaR-icaA intergenic region (Fig. 9). This palindrome-like sequence may be recognized by Rob. A previous study showed that the TATTT motif has a functional role in the transcriptional regulation of the ica locus, but not icaR
transcription (16). Ruiz de los Mozos et al. recently demonstrated that the 5’- and 3’- UTR base pairings of icaR mRNA control its transcription in *S. aureus*. The 5-bp motif is located within the 5’-UTR of icaR (Fig. S4 in the supplemental material). The possibility of an interaction between Rob and 5’-UTR to control base pairing remains elusive.

Taken together, the results of the present study suggest that Rob controls the two different pathways of biofilm elaboration in *S. aureus*. The TetR-family transcriptional regulator Rob affects biofilm elaboration through SAOUHSC_2898 and by recognizing/binding the TATTT motif in an ica-dependent manner. These results provide additional insights into the transcriptional regulation of the ica locus. Both Rob-mediated pathways will be investigated in more detail in future studies.

**MATERIALS AND METHODS**

**Bacterial strains and growth media.** The bacterial strains and plasmids used in the present study are listed in Table 1. The *S. aureus* strain, designated TF2758, is a clinical isolate from an atheroma in Japan. *S. aureus ATCC49775* served as a negative control, non-biofilm producer. *S. aureus* FK300, a rsbU-repaired derivative of strain NCTC8325-4, was used in a functional study of the role of rob. *S. aureus* RN4220 (31) was used as the initial recipient for the manipulation of recombinant plasmids. *S. aureus* was routinely grown in tryptic soy broth (TSB, Becton Dickinson Microbiology Systems, Cockeysville, MD) or on tryptic soy agar (TSA) plates. Tetracycline
(Tc, 5 μg/ml) or chloramphenicol (Cp, 10 μg/ml) was added as necessary. *Escherichia coli* strain DH5α was used for the construction and maintenance of plasmids. *E. coli* was grown in lysogeny broth (LB) broth (5 g yeast extract, 10 g polypeptone, and 10 g NaCl per liter; pH 7.2) or on LB agar. When required, ampicillin (Ap, 100 μg/ml), kanamycin (Kn, 30 μg/ml), Tc (10 μg/ml), or Cp (10 μg/ml) was added to the culture medium.

**Plasmid and strain construction.** Routine DNA manipulations were performed as previously described (32). FK300 mutants were constructed by allele replacement using pKFT (33). PCR was performed using KOD-Plus-Neo (Toyobo, Japan) under appropriate cycling conditions. The oligonucleotides used in this study are listed in Table 2. Fragments were cloned into the plasmid pKFT using restriction enzymes and transformed into *E. coli* DH5α. Recombinant plasmids were then introduced into DNA restriction system-deficient *S. aureus* RN4220 by electroporation (34). Modified plasmids were electroporated into *S. aureus* FK300 for allele replacement. Markerless deletion mutants were screened by PCR from tetracycline-sensitive colonies. Fragments were confirmed by DNA sequencing using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA).

In complementation experiments, genes were amplified by PCR using the corresponding primer pairs and then cloned into the HindIII site of pKAT (35). The plasmids pC001, pC002, and pC003 carrying rob-FK300, icaR-FK300, and rob-TF2758 genes, respectively, were constructed.
and transformed into the *S. aureus* strains listed in Table 1 by electroporation. The inserts in all plasmid constructs were verified by PCR and DNA sequencing.

**Biofilm assay.** A biofilm assay using polystyrene plates was performed as described previously (36) with a few modifications. In brief, overnight cultures were diluted 1:100 with TSB. Ten microliters of this dilution was then transferred, in triplicate, into flat-bottom 96-well polystyrene plates (TrueLine, Nippon Genetics Co., Ltd., Japan) containing TSB or TSB plus 1% glucose. After incubation at 37°C for 24 h, the wells were gently washed three times with 300 μl of sterile phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄·12H₂O, and 1.8 mM KH₂PO₄, pH 7.4), and the biofilm was stained with 1% crystal violet for 15 min. Unbound crystal violet was then removed by washing the plate in a container by immersing and agitating gently 10 times in tap water. Biofilm-bound crystal violet was solubilized in 200 μl of 33% glacial acetic acid at room temperature for 15 min. The extracts were diluted 10-fold, and absorbance at 590 nm was measured with an Immuno-Mini NJ-2300 spectrophotometer (Nalge Nunc International K.K., Tokyo, Japan).

**PIA/PNAG detection.** The ability of *S. aureus* strains to produce PIA/PNAG was tested according to a previously described protocol (9). Briefly, *S. aureus* strains were grown at 37°C overnight with shaking in 3 ml of TSB. Cultures were then diluted 1:1,000 in the appropriate medium, and 4 ml of this cell suspension was used to inoculate sterile 12-well polystyrene plates (TrueLine, Nippon Genetics Co., Ltd., Japan). After a 24-h static incubation at 37°C, the cells
were resuspended in 50 μl of 0.5 M EDTA (pH 8.0) and incubated for 5 min at 100°C. Cells were removed by centrifugation, and 40 μl of the supernatant was incubated with 10 μl of protease K (20 mg/ml; Nacalai Tesque, Inc., Kyoto, Japan) at 37°C for 30 min. After the addition of 10 μl of Tris-buffer saline (20 mM Tris-HCl, 150 mM NaCl [pH 7.4]) containing 0.01% bromophenol blue, 5 μl was immobilized on a nitrocellulose membrane (Amersham Protran NC 0.45, GE Healthcare, Buckinghamshire, UK) and dried at room temperature. The membrane was blocked with 5% skimmed milk in PBS with 0.1% Tween 20, and this was followed by a 2-h incubation with rabbit anti-PNAG antiserum (37) diluted at 1:10,000. Bound antibodies were detected with peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) antibodies (MP Biomedicals, LLC-Cappel Products, Ohio, USA) diluted 1:10,000 and developed with Pierce ECL Western Blotting Substrate (Thermo Scientific, Rockford, IL, USA).

RNA isolation, reverse transcription (RT), and real-time PCR. Overnight *S. aureus* cultures were diluted in fresh TSB containing 1% glucose to an initial optical density of 0.02 at 660 nm and harvested after a 6-h incubation with shaking at 37°C. Total RNA was isolated using the FastRNA Pro Blue Kit (MP Biomedicals, LLC, Santa Ana, CA, USA) according to the manufacturer’s instructions. DNA was removed by a treatment with RQ1 RNase-free DNase (Promega, Madison, WI) at 37°C for 30 min. After inactivation of DNase, PCR was performed to confirm the absence of contaminating DNA. RNA was then reverse transcribed with a Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany). The resulting cDNA was diluted
10-fold with Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and then used as a template in the real-time PCR reaction. Quantitative real-time RT-PCR (qRT-PCR) was performed with the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) using a CFX96 Real-Time PCR Detection System (Bio-Rad). The thermal cycling conditions used were as follows: at 95°C for 1 min, followed by 40 cycles of 95°C for 15 s, 60°C (icaR and icaA) or 62°C (gyrB and SAOUHSC_2898) for 15 s, and 72°C for 30 s. All PCR runs were performed in triplicate and data were analyzed using the CFX Manager Software (version 3.0, Bio-Rad) according to the manufacturer’s instructions. The housekeeping gene, gyrase subunit B (gyrB), was used as a reference gene to normalize the expression level of the target gene in each reaction. Real-time PCR primers are listed in Table 2.

Microarray analysis. The design and preparation of probes, which cover more than 98% of the open reading frames (ORFs) of S. aureus MW2, and their immobilization on the glass slide were described elsewhere (38). RNA extraction (after a 2-h incubation) and cDNA synthesis were performed as described above. cDNA was fluorescently labeled with Alexa Fluor 555 (Cy3) and Alexa Fluor 647 (Cy5) (Thermo Fisher Scientific, Oregon, USA). Labeled cDNA samples were mixed and hybridized to the slides. After washing, fluorescent signals were detected using a GenePix 4000B Microarray Scanner (Axon Instruments). Data were then normalized and analyzed using Array Vision 8.0 software (Imaging Research Inc., CT, USA).
non-biofilm-elaborating strain ATCC49775, the genotype of which is the most closely related to
TF2758 in our Japanese clinical isolate collection, was used as the reference strain.

**Transcriptomic analysis of rob operon via RNA-seq.**

Overnight *S. aureus* FK300 (wild-type, Δ*rob*) and TF2758 cultures were diluted in fresh TSB to
an initial density of 0.02 at 660 nm and harvested after a 6-h incubation with shaking at 37°C.

Total RNA was isolated using the FastRNA Pro Blue Kit (MP Biomedicals, LLC, Santa Ana, CA,
USA) according to the manufacturer’s instructions. To eliminate DNA contamination, 1μg total
RNA in each sample was treated with 3 μl (1 U/μl) of RQ1 RNase-free DNase (Promega, Madi-
son, WI) at 37°C for 30 min. After digestion by DNase, PCR of the *gyrB* gene was performed to
confirm the absence of contaminating DNA. The concentration and quality of total RNA were
determined using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific) and Agilent 2200 TapeSta-
tion (Agilent Technogogies), respectively. rRNA was removed using the Ribo-Zero Bacteria Kit
(Epicentre). Removal of rRNA was confirmed by Agilent 2200 TapeStation.

Libraries were generated using the ScriptSeq v2 RNA-Seq (Epicentre) and purified using the
Minelute PCR purification kit (Quiagen) according to the manufacturer’s instructions. Libraries
were sequenced using the index sequences of TruSeq v2 LT Sample Prep Kit on the Illumina
MiSeq platform. Sequence reads were preprocessed for quality, trimmed and mapped to *S. aure-
us* strain NCTC8325 (GenBank accession number NC_007795) as the reference genome using
CLC Genomics Workbench software platform ver.9 (Qiagen) and Integrative Genomics Viewer (IGV) ver.2.

**Sequencing of the TF2758 genome.** Genomic DNA was extracted using the lysostaphin and QIAamp DNA Mini kit (QIAGEN, Germany) according to the manufacturer’s instructions. Libraries were prepared for sequencing with Nextera DNA kits (Illumina, USA) and were sequenced with the Illumina GAIIx system according to Illumina protocols. The raw reads were trimmed and assembled using a SOAPdenovo assembler. The draft genome sequence was automatically annotated using the Microbial Genome Annotation Pipeline (MiGAP) (39) and was manually curated using IMC-GE software (In Silico Biology, Inc., Kanagawa, Japan).

**Protein purification.** To elucidate the DNA-binding properties of Rob, the full-length open reading frame (ORF) of *rob* was amplified from FK300 genomic DNA using primers pET-28a-Rob-F/pET-28a-Rob-R (Table 2) and cloned into the expression vector pET-28a(+) (Novagen) to obtain pET28a-*rob*. The plasmid was then transformed into *E. coli* BL21 (DE3) and bacteria were grown at 37°C in 300 ml LB containing 30 μg/ml kanamycin to an OD of 0.5 at 600 nm. Expression of Rob was induced with 0.5 mM IPTG (isopropyl-β-d-thiogalactopyranoside; Nacalai Tesque, Inc., Kyoto, Japan) and incubation at 37°C for another 6 h. Cells were harvested by centrifugation and frozen at -80°C. Cell pellets were thawed in lysis buffer (50 mM NaH2PO4 and 300 mM NaCl, pH 8.0) and lysed by sonication on ice. Cell debris was removed by centrifugation (10,000 × g at 4°C for 20 min) and the supernatant was
used for isolation of His₆-tagged Rob fusion protein by using TALON Metal Affinity Resins (Clontech Laboratories, Inc.) according to the company’s protocol. The expression and purity of the protein were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 12% gel. Protein concentrations were measured using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA) with bovine serum albumin (BSA) as the standard protein. The recombinant His-tagged IcaR protein was purified as described elsewhere (18).

Electrophoretic mobility shift assays (EMSA). Gel shift assays were performed as described previously (16) with the following modifications. DNA fragments corresponding to the icaR-icaA intergenic region and promoter region of rob were amplified by PCR with the primers listed in Table 2. PCR products were purified using the QIAquick Gel Extraction Kit (QIAGEN). A 20-μl binding reaction containing 0.1 to 2 μg of purified recombinant protein and 1 μg of sonicated salmon sperm DNA as well as 1 μg of poly(dl-dC) in binding buffer (10 mM Hepes [pH 8.0], 60 mM KCl, 4 mM MgCl₂, 0.1 mM EDTA [pH 8.0], 0.1 mg/ml BSA, 0.25 mM dithiothreitol [DTT], and 5% glycerol) was incubated at room temperature for 15 min before the addition of 2 μg of the biotin-labeled probe. The reaction mixtures were incubated for an additional 20 min and then electrophoresed in 5% polyacrylamide gel in prechilled 1× Tris-borate-EDTA (TBE) buffer. DNA was then transferred onto a nylon membrane (BioDyne B, PALL, USA) and band shifts were detected by exposing dried membranes to X-ray films. In order to measure the
binding of Rob to its promoter region, a gel shift assay was performed using an alternative method as described (40).

**DNase I footprint analysis.** Footprinting was performed according to a previously described method (41). DNA fragments were generated by PCR with TaKaRa LA Taq (TaKaRa Bio Inc., Shiga, Japan). PCR products were purified and ligated with pGEM-T Easy (Promega) using Ligation high Ver.2 (Toyobo, Osaka, Japan). The resulting plasmids were then used as a template for the amplification of DNA probes using the primer pair Fp-M13-F and Fp-M13-R (5’-6-FAM-labeled). DNA fragments (0.45 pmol) were mixed with purified proteins in 50 μl of a reaction mixture containing the same buffer used for gel shift assays. After a 20-min incubation at room temperature, the reaction mixtures were treated with 0.3 U of DNase I (Promega, Madison, WI) for 1 min and then purified by phenol/CIAA extraction and ethanol precipitation. After purification, the samples were analyzed using an ABI 3130xl Genetic Analyzer equipped with the Peak Scanner software (Applied Biosystems).

**Isolation and identification of proteins binding to the ica promoter.** A cell-free extract was isolated from strain FK300 as previously described with some modifications (42). Briefly, cultured *S. aureus* cells were pelleted and then washed with buffer A (20 mM Tri-HCl, 5 mM MgCl₂, 0.1 M EDTA, and 5% glycerol, pH 7.8). Cell pellets were resuspended in 10 ml of buffer A and treated with lysostaphin (0.1 mg/ml) at 4°C for 1 h. After freezing at -80°C and thawing at 4°C twice, 6 ml of buffer A (containing KCl at a final concentration of 1.3 M) was added and incu-
bated on ice for 40 min. The cell lysate was treated with DNase I (10 μg/ml) and RNase A (10 μg/ml) at room temperature for 30 min. After centrifugation for 30 min at 40,000 × g, the supernatant was dialyzed against distilled water overnight and stored at -80°C.

Biotinylated DNA was prepared as described above. DNA was immobilized on 2 mg of streptavidin-coated magnetic beads (Dynabeads M-280 Streptavidin; Life Technologies) according to the manufacturer’s protocol. After washing, 100 μl of the cell-free extract was added and incubated at room temperature for 30 min in gel shift binding buffer. The beads were washed twice with buffer B (10 mM Hepes [pH 8.0], 60 mM KCl, 4 mM MgCl₂, 1 mM EDTA [pH 8.0], 1 mM DTT, and 5% glycerol) containing 0.5 μg/ml of salmon sperm DNA, and then washed twice with buffer B. The bound proteins were eluted from immobilized DNA with buffer B containing 0.5 M NaCl. The eluates from two binding reactions were pooled and concentrated by methanol/chloroform precipitation. Proteins were separated by SDS-PAGE, followed by Coo-massie or silver staining. Prior to in-gel trypsin digestion, excised gel pieces were destained and submitted to reduction with DTT and alkylation with iodoacetamide as described previously (43). After being dried, the gel pieces were subjected to trypsin digestion at 35°C overnight with XL-TrypKit (APRO Sci, Japan). Digested peptides were transferred to new tubes and evaporated to < 10 μl in a vacuum centrifuge evaporator, and this was followed by LC-MS/MS analyses for protein identification. LC-MS/MS analyses were performed on nanoflow liquid chromatography coupled with nanoelectrospray MS, a Triple TOF 5600 system (AB SCIEX, CONCORD, ON)
equipped with an Eksigent cHiPLC-nanoflex System (AB SCIEX). The nano HPLC columns used were the cHiPLC trap column (200 μm x 0.5 mm ChromXP C18-CL, 3 μm) and nano cHiPLC analytical capillary column (75 μm x 15 cm ChromXP C18-CL, 3 μm, 120Å). Tryptic peptides (2 μl) were loaded, and trapping and desalting were performed at 2 μl/min for 10 min with 0.1% formic acid. The trapped peptides were separated by a linear gradient at a flow rate of 0.3 μl/min, followed by their introduction into the source of the mass spectrometer online. Mobile phase A (0.1% formic acid in H₂O) and mobile phase B (0.1% formic acid in acetonitrile) were used to establish a 45-min gradient comprising 25 min of 2-32% B, 1 min of 32-90% B, 4 min of 90% B, and finally decreasing to 2% B, which was followed by re-equilibrating at 2% B for 15 min. Eluted peptides from the column were analyzed with a Triple TOF 5600 using an ion spray voltage of 2.2kV. Product ions were scanned in a mass range from 230m/z up to 1500 m/z. MS/MS data acquisition was performed using Analyst 1.5.2 (AB Sciex) and proteins were identified by means of an automated database search using ProteinPilot Software (AS Sciex).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at:

Table S1
Figure S1
Figure S2
ACKNOWLEDGMENTS

We thank Dr. Hidetada Hirakawa, Gunma Univ. for his skillful suggestion on DNase I footprint analysis. This work is supported in part by a grant from the Health and Labor Sciences Research Grants for Research on Allergic Disease and Immunology from the Ministry of Health, Labor, and Welfare of Japan (201322025A), and a grant from the Japan Agency for Medical Research and Development (AMED) (924711).

REFERENCES


(IcaR) are transcriptional inhibitors of the ica locus in *Staphylococcus aureus*. J Bacteriol 186:2449–2456.


Figure legends

FIG. 1 Biofilm elaboration and PIA/PNAG production by Staphylococcus aureus TF2758.

(A) Biofilm elaboration. Bacteria were grown in trypticase soy broth (TSB) in the presence
(Glc+) or absence (Glc-) of 1% glucose. Biofilm elaboration was measured using the polystyrene microtiter plate assay described in the Materials and Methods section. The averages and standard errors from each sample are shown. (B) PIA/PNAG production. Extracts from overnight cultures were spotted on a membrane, and PIA/PNAG was detected by rabbit anti-PNAG, as described in the Materials and Methods section. Non-biofilm-elaborating strain ATCC49775 was used as a control in the comparative microarray analysis. (C) A comparative gene expression analysis of TF2758 and ATCC49775. TF2758 gene expression was represented as fold changes from that of ATCC49775. Two gene clusters exhibiting marked increases in gene expression were colored (red and blue), and these gene clusters were depicted (a, b).

**FIG. 2 Identification of a nonsense mutation in the satf2583 (rob) gene of TF2758 and the predicted domain structure of its transcript.** (A) Comparison of the satf2580-satf2586 region with those of MW2, 8325-4, ATCC49775, and TF2758. A part of the nucleotide sequence of each strain and amino acid sequences are shown. The numbers shown on both sides mean the nucleotide sequence and amino acid sequence positions in the ORF of rob. The nonsense codon created by the mutation (A to T) was indicated by an asterisk. (B) Structural characteristics of Rob. It contained a TetR_N superfamily domain within an AcrR domain.
FIG. 3 Rob and IcaR from FK300 reduce biofilm elaboration and PIA/PNAG synthesis in strain TF2758. (A) Biofilm elaboration and (B) PIA/PNAG production of TF2758 and TF2758 carrying pC001 (pKAT-\textit{rob} (FK300)), pC002 (pKAT-\textit{icaR} (FK300)), or pKAT. Bacteria were grown TSB in the presence (Glc+) or absence (Glc-) of 1% glucose. Biofilm elaboration was measured using the polystyrene microtiter plate assay described in the Materials and Methods section. The averages and standard errors from each sample are shown. Extracts from overnight cultures were spotted on a membrane, and PIA/PNAG was detected by rabbit anti-PIA, as described in the Materials and Methods section.

FIG. 4 Effects of the \textit{rob} deletion on biofilm elaboration and \textit{ica} operon expression in FK300. (A) Biofilm elaboration in wild-type FK300 and its derivatives were assessed using the polystyrene microtiter plate assay described in the Materials and Methods section. The averages and standard errors from each sample are shown. sm, FK300 carrying a stop mutation at adenine nucleotide position 331(A\textsubscript{331} to T\textsubscript{331}); pC001, pKAT with \textit{rob} (FK300); pC003, pKAT with \textit{rob} (TF2758). (B) Quantitative measurements of \textit{icaR} and \textit{icaADB} transcription by qPCR. Total RNA preparation, cDNA synthesis, and then qPCR were performed as described in the Materials and Methods section. Transcript levels in the \textit{rob} deletion mutant compared to those in wild-type strain FK300 were assigned. The expression of the \textit{gyrB} gene was used for sample normalization.
Error bars indicate standard errors. (C) PIA/PNAG production. PIA/PNAG production was measured as described in the legend of Figure 1.

FIG. 5 Biofilm elaboration in the rob deletion mutant requires downstream gene(s), but not upstream genes. Bacteria were grown in TSB in the presence (Glc+) or absence (Glc-) of 1% glucose. Biofilm elaboration was measured using the polystyrene microtiter plate assay described in the Materials and Methods section. The averages and standard errors from each sample are shown. Δrob, FK300 rob deletion mutant; ΔrobΔUpstream, FK300 with deletions of rob and its upstream genes, SAOUHC_2894, SAOUHC_2895, and SAOUHSC_2896; ΔrobΔDownstream, FK300 with the deletion of rob and its downstream genes SAOUHSC_2898, SAOUHSC_2899, and SAOUHSC_2900.

FIG. 6 Contribution of SAOUHSC_2898 to biofilm elaboration by the rob deletion mutant and regulation of SAOUHSC_2898 expression by rob in FK300. Bacteria were grown in TSB in the presence (Glc+) or absence (Glc-) of 1% glucose. Biofilm elaboration was measured using the polystyrene microtiter plate assay described in the Materials and Methods section. (A) Effects of SAOUHSC_2898, SAOUHSC_2899, and SAOUHSC_2900 deletions on biofilm elaboration in the FK300 rob deletion mutant. The averages and standard errors from each sample are shown. (B) Transcription of SAOUHSC_2898 in the FK300 wild-type strain and its rob deletion
mutant. Transcript levels in the rob deletion mutant compared to those in the wild-type strain were assigned. The expression of the gyrB gene was used for sample normalization. Error bars indicate standard errors.

**FIG. 7** Rob binds to a palindromic motif in its own promoter. (A) EMSA for the DNA-binding activity of Rob to the intergenic region between SAOUHSC_2896 and rob. EMSA was performed in the absence (lane 1) or presence (lanes 2 to 5) of the Rob protein. The primers used to amplify the intergenic region for Rob binding are indicated by black arrows. (B) DNase I footprinting assay. The 6-FAM-labeled DNA probe was incubated with or without recombinant Rob (12 pmol) and then subjected to DNase I digestion. The rectangle indicates the region protected by Rob. The palindromic motif is shown in bold. (C) Schematic representation of the secondary structure of the binding region by Rob. The sequence bound by Rob is highlighted by a red frame.

**FIG. 8** Rob binds to the ica promoter region and its binding is TATTT motif-dependent. (A) Schematic representation of the design of DNA probes used in EMSAs. (B-E) EMSAs of Rob. Recombinant Rob was incubated with (B) FULL, (C) SHORT-1, (D) SHORT-2, or (E) FULL (-5-bp) (2 ng/reaction). The amounts (μg/reaction) of Rob were as follows: (B-E) lane 1,
FIG. 9 DNase I footprinting assay of Rob binding to the ica promoter region. (A) A footprint analysis of Rob binding to the ica promoter region. The sequence of the protected region is shown. (B) Comparison of the binding sites of Rob to the rob promoter region and ica promoter region. The palindromic sequence within the Rob binding region is shaded. The bold letters (A) and rectangle (B) indicate the 5-bp TATTT motif.

Fig. 10 Proposed model for the regulation of PIA/PNAG synthesis by Rob in S. aureus FK300. The rob gene product represses the expression of the surrounding 7-gene cluster including rob and SAOUHSC_2988. The gene product of SAOUHSC_2988 may function as an oxidoreductase in a hypothetical pathway through which glucose-independent icaADBC-dependent polysaccharide accumulation occurs. rob also recognizes the TATTT motif in the ica promoter region and binds to this region. The binding of Rob to the ica promoter region may suppress the expression of the icaADBC locus. 5-bp, the TATTT motif important for the expression of ica locus. 42-bp, the IcaR-binding region. Arrows correspond to activation and bars to repression.
FIG. S1 Identification of a missense mutation in the icaR gene of TF2758 and the domain structure of its transcript. (A) Comparison of the nucleotide sequence and amino acid sequence of the icaR gene among MW2, 8325-4, ATCC49775, and TF2758. The numbers shown on both sides mean the nucleotide sequence and amino acid sequence positions in the ORF of icaR. Altered amino acids (A to T) by the mutation at nucleotide position 103 (G to A) were indicated in red. (B) Structural characteristics of IcaR. It contained a TetR_N superfamily domain within an AcrR domain.

FIG. S2 Identification of rob operon and transcription start site of rob using RNA-seq analysis. (A) Visualization of RNA transcript identified by RNA-seq. Total RNA of FK300, FK300 Δrob, and TF2758 were prepared from cultures grown for 6 h at 37°C. After removal of DNA contaminants and rRNA, libraries were generated and purified as described in the Materials and Methods. RNA-seq reads were mapped to S. aureus NCTC8325. Genes with continuous coverage were considered to belong to the same operon. The ORFs of NCTC8325 are shown at the top of the figure. Transcripts identified by RNA-seq are represented as dashed arrows. The sequence from predicted transcription start site (TSS) to the start codon of rob was shown at the bottom of the figure. (B) Diagrammatic representation of the rob promoter region. GENETYXMAC v.15 (Software Development Co., Ltd., Tokyo, Japan) was used for prediction of the -35, -10 sequence. The start codons of genes are indicated by arrows. The Rob-binding site
is indicated by the open rectangle. The transcription start site of rob is highlighted by a bent arrow.

**FIG. S3** Reduced biofilm elaboration in rob SAOUHSC_2898 double mutant was restored through complementation with the SAOUHSC_2898 gene. Bacteria were grown in TSB in the presence (Glc+) or absence (Glc-) of 1% glucose. Biofilm elaboration was measured using the polystyrene microtiter plate assay described in the Materials and Methods section. The averages and standard errors from each sample are shown. pC004, pKAT with SAOUHSC_2898 (FK300).

**FIG. S4** Diagrammatic representation of the icaR-icaA intergenic region. The start sites of icaR and icaA are indicated by arrows. The Rob-binding site is indicated by the open rectangle. The grey shaded rectangle indicates the IcaR-binding site (16). The 5-bp TATTT motif, which has a functional role in the transcriptional regulation of the ica locus, is highlighted by a red frame (16). The Shine-Dalgarno sequence of icaR is underlined. The 5’-UTR of icaR is boxed (dashed line) in the sequence (44). The bent arrow indicates the transcriptional start site of icaA (45).
FIG. S5 Comparison of the satf2580-satf2586 region among different staphylococcus species. The red frame represents the 7-gene cluster highlighted in this study.
**TABLE 1** Strains and plasmids used in the present study

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| Δ*rob* ΔUpstream  | FK300 Δ*rob* ΔSAOUHSC_2894  
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|                  | ΔSAOUHSC_2899 ΔSAOUHSC_2900 | This study |
| Δ*rob* ΔSAOUHSC_2898 | FK300 Δ*rob* ΔSAOUHSC_2898 | This study |
| Δ*rob* ΔSAOUHSC_2899 | FK300 Δ*rob* ΔSAOUHSC_2899 | This study |
| Δ*rob* ΔSAOUHSC_2900 | FK300 Δ*rob* ΔSAOUHSC_2900 | This study |
| **E. coli**       |                             |                     |
| DH5α              | Cloning strain | TaKaRa |
| BL21(DE3)         | Host for recombinant protein production | Novagen |
| **Plasmids**      |                             |                     |
| pGEM-T Easy       | Cloning vector | Promega |
| pKAT              | *E. coli*-*S. aureus* shuttle vector | 35 |
| pC001             | Vector for complementation experiments; containing *rob* from FK300 cloned in pKAT | This study |
pC002 Vector for complementation experiments; containing *icaR* from FK300 cloned in pKAT

This study

pC003 Vector for complementation experiments; containing *rob* from TF2758 cloned in pKAT

This study

pC004 Vector for complementation experiments; containing SAOUHSC_2898 from FK300 cloned in pKAT

This study

pKFT Vector for allele replacement

33

pET-28a(+) *E. coli* expression plasmid

Novagen

pET-22b(+) *E. coli* expression plasmid

Novagen

pET28a-rob His-Rob expression plasmid

This study

pET22b-*icaR* His-IcaR expression plasmid

This study

767

768 **TABLE 2** Primers used in the present study

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SAOUHSC_2900-2  GAATATAACCTAAGTGACCGCCAGGAATAAGATGACAC
SAOUHSC_2900-3  GTGCTCATCTTTATTCCTTGCGGTCACCTTAGTTATATC
SAOUHSC_2900-4  CTATTTGGATCCGTTTCAAAC
icaR-1  TGGTGAAGCTTGATCAACGATAGTATC
icaR-4  TAATAAAGCTTGATACCATCGTACTC
ica-1  AATTGGATCCTCATTGAACAAGAAGCC
ica-2  TAATACTAGTTGTCCCTTCCAGGCCCAC
ica-3  GATGAAACTAGTTATGAAATGCTTATCC
ica-4  AATTGTAACACCTAAGGATCCACCCCTCC

qPCR

gyrB for  AGGTCTTGGAGAAATGAATG
gyrB rev  CAAATGTGTTGGTCCGCTT
icaR for  CGCCTGAGGAATTTTCTG
icaR rev  GGATGCTTTCAATAACCAAC
icaA for  AGTTGTACGACGTTGCTAC
icaA rev  CCAAAGACCTCCCAATG
icaD for  ACCCAACGCTAAAATCATCG
icaD rev  GCGAAAATGCCCATAGTTTC
icaB for  ATACCGGCAACTGGGTTTAT
icaB rev  TGCAATCAGTGGTAGTGTG
icaC for  CTTGGGTATTTGCACGCATT
icaC rev  GCAATATCGTGCCGACACCT
SAOUHSC_2898 for  ATTAGCACCTCGTGACGTG
SAOUHSC_2898 rev  CCACCTGGATACGTTAGC

EMSA and DNase I footprint analysis

ica-p-F  ACAAATATTTCCGTTTAATTATAACAC
ica-p-R (5-Biotin)  TTGCAATTCCTTTACCTACCTTTC
ica-p-R’  TTGCAATTCCTTTACCTACCTTTC
ica-p-F-s1  ACAAAATTTCCGTTTAATTATAACAC
**ica-p-F-s2** AATCTATTGCAAATTAAAATACTATC
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5bp-deletion-3 AATTAAGTTGCAATTACAAACCGTTTAATTATAACAAACAA
**rob-p-F** CGTCTTTGCTCTCTAGTTAAAGAC
**rob-p-R** CTATTCTCTTTGCACTTTTTCGC
T7 promoter-1 cy3\(^a\) TAATACGACTCACTATAGGG
Fp-M13-F GTTTTCCCAGTCACGAC
Fp-M13-R 6-FAM\(^b\) CAGGAAACAGCTATGAC
pET-28a-Rob-F AGGTGGATCCATGCGAAAAGATGC
pET-28a-Rob-R TAACAAGCTTTTAGTCATTACGTCCCACC
pET-22b-IcaR-F GGAATTCCATATGCACCACCACCACCTTGAAGGAT
AAGATTATGATAACGC
pET-22b-IcaR-R CCCAAGCTTTTATTCTTTAAAAATATTTAGTAGCG

\(^a\) cy3 labeled at the 5’ end.

\(^b\) 6-FAM labeled at the 5’ end.

### TABLE S1 List of genes up-regulated in microarray experiments

<table>
<thead>
<tr>
<th>MW2 open reading frame</th>
<th>Name</th>
<th>Product</th>
<th>Fold(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MW2495 (sat2580)</td>
<td>Glyoxalase protein</td>
<td>40.83 16.41 25.94</td>
<td></td>
</tr>
<tr>
<td>MW2496 (sat2581)</td>
<td>NmrA-like family protein</td>
<td>36.14 34.99 40.18</td>
<td></td>
</tr>
<tr>
<td>MW2497 (sat2582)</td>
<td>Conserved hypothetical protein</td>
<td>34.04 18.41 28.12</td>
<td></td>
</tr>
<tr>
<td>MW2498 (sat2583)</td>
<td><strong>rob</strong> Transcriptional regulator</td>
<td>47.86 29.38 20.46</td>
<td></td>
</tr>
<tr>
<td>MW2499 (sat2584)</td>
<td>2-deoxy-D-gluconate 3-dehydrogenase</td>
<td>41.21 32.51 24.43</td>
<td></td>
</tr>
<tr>
<td>MW2500(sat2585)</td>
<td>Amidohydrolase family protein</td>
<td>22.13 34.51 18.32</td>
<td></td>
</tr>
<tr>
<td>MW2501 (sat2586)</td>
<td>Putative hydrolase</td>
<td>10.89 5.27 4.29</td>
<td></td>
</tr>
<tr>
<td>MW2586 (sat2686)</td>
<td><strong>icaA</strong> intercellular adhesion protein A</td>
<td>6.01 2.76 1.75</td>
<td></td>
</tr>
<tr>
<td>MW2587 (sat2687)</td>
<td><strong>icaD</strong> intercellular adhesion protein D</td>
<td>5.59 3.72 5.36</td>
<td></td>
</tr>
<tr>
<td>MW2588 (sat2688)</td>
<td><strong>icaB</strong> intercellular adhesion protein B</td>
<td>8.05 6.35 6.25</td>
<td></td>
</tr>
<tr>
<td>MW2589 (sat2689)</td>
<td><strong>icaC</strong> intercellular adhesion protein C</td>
<td>7.82 1.92 6.12</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Fold changes indicate increases in expression levels in TF2758 from ATCC49775. Experiments were repeated three times.
Figure 5
Figure 9

A

0 pmol Rob

120 pmol Rob

GTTGCAATTACAAAATATTTCCGTTT

B

Binding site of Rob to rob promoter

1 AAACGGAAGTTATCCGTTTGTCA

Binding site of Rob to ica promoter

1 GTTGCAATTACAAAATATTTCCGTTT

** * * * * * * * *
Figure S1

A

[Diagram showing the genetic structure of the ica operon with arrows and 42bp spacing]

B

IcaR conserved domains

[Diagram showing conserved domains TetR_N and AcrR]
Figure S3