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Rbf Promotes Biofilm Formation by *Staphylococcus aureus* via Repression of *icaR*, a Negative Regulator of *icaADBC*▽

David Cue,[1] Mei G. Lei,[1] Thanh T. Luong,[1] Lisa Kuechenmeister,[2] Paul M. Dunman,[2] Sinead O'Donnell,[3] Sarah Rowe,[3] James P. O'Gara,[3] and Chia Y. Lee[1]*

Department of Microbiology and Immunology, University of Arkansas for Medical Sciences, Little Rock, Arkansas 72205[1]; Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, Nebraska 68198[2]; and School of Biomolecular and Biomedical Science, Ardmore House, University College Dublin, Belfield, Dublin 4, Ireland[3]

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We previously reported the identification of a gene, *rbf*, involved in the regulation of biofilm formation by *Staphylococcus aureus* 8325-4. In an effort to study the mechanism of regulation, microarrays were used to compare the transcription profiles of the wild-type strain with an *rbf* mutant and an *rbf* overexpression strain of the clinical isolate UAMS-1. Among the genes affected by *rbf* overexpression are those of the intercellular adhesion (*ica*) locus; however, expression of these genes was not affected by an *rbf* deletion in the chromosome. The *icaADBC* genes are responsible for production of poly-*N*-acetylglucosamine (PNAG), a major constituent of biofilm. The *icaR* gene encodes a negative regulator of *icaADBC*. In UAMS-1 carrying an Rbf-encoding plasmid, Rbf was found to repress *icaR* transcription with a concomitant increase in *icaADBC* expression and increased PNAG and biofilm production relative to isogenic strains lacking the plasmid. Sequencing of the *rbf* gene from UAMS-1 showed that there was a 2-bp insertion affecting the 50th codon of the *rbf* open reading frame, suggesting that *rbf* is a pseudogene in UAMS-1. This finding explains why deletion of *rbf* had no effect on biofilm formation in UAMS-1. To further characterize the Rbf regulation on biofilm we compared biofilm formation, *icaA* and *icaR* transcription, and PNAG production in 8325-4 and its isogenic *rbf* and *icaR* single mutants and an *rbf icaR* double mutant. Our results are consistent with a model wherein *rbf* represses synthesis of *icaR*, which in turn results in derepression of *icaADBC* and increased PNAG production. Furthermore, purified *rbf* did not bind to the *icaR* or *icaA* promoter region, suggesting that *rbf* controls expression of an unknown factor(s) that represses *icaR*. The role of *rbf* in controlling the *S. aureus* biofilm phenotype was further demonstrated in a clinical strain, MW2.

Staphylococcus aureus is a major human pathogen capable of causing a broad range of diseases. Some *S. aureus* infections, such as endocarditis and osteomyelitis, are associated with biofilm formation (3, 4, 13, 29). Biofilms are composed of layers of bacteria within a glycocalyx composed of polysaccharides, DNA, and proteins. In addition to aiding bacterial colonization of surfaces, biofilms are believed to increase tolerance to antibiotics and immune defenses (3, 13, 17, 24, 36, 39, 51).

The biofilm-associated polysaccharide of *S. aureus* is referred to as the polysaccharide intercellular adhesin or PIA and has been well characterized (13, 30, 36). It is composed of polymeric *N*-acetylglucosamine (PNAG) that is synthesized by the products of four genes in the *ica* operon, *icaADBC* (10, 20). In some strains, genetic disruption of the *icaADBC* genes results in the loss of biofilm formation (10, 23, 33); however, *ica*-independent biofilm formation has also been described (2, 9, 37, 38, 47). Expression of *icaADBC* is, in part, regulated by *icaR*, a member of the TetR family of regulatory proteins (22). IcaR is encoded at the *ica* locus but is divergently transcribed from *icaADBC*. IcaR binds to the *icaADBC* promoter, 5′ to the *icaA* start codon, and negatively regulates *ica* expression (20). Transcription of *icaADBC* is also subject to positive regulation by the global regulatory factor SarA and in some strains by the stress-induced sigma factor SigB (2, 6, 20, 42). The teicoplanin-associated locus regulator, TcaR, is also a weak negative regulator of *icaADBC* (21).

A number of reports have shown that extracellular DNA is often an important component of biofilms (34, 40, 54), including *S. aureus* biofilms (14, 43, 50). The source of the extracellular DNA is bacterial, with DNA release occurring via lysis of bacterial cells. It has been proposed that DNA influences the early stages of biofilm development (43). Rice et al. (43) reported that inactivation of the *cidA* gene in *S. aureus* strain UAMS-1 inhibits the release of genomic DNA. CidA is a regulatory factor affecting cell lysis and antibiotic resistance. The *cidA* mutant was found to have a decreased capacity to form biofilms both in vitro and in vivo (43). Our laboratory has also found that DNase can disrupt UAMS-1 biofilms (unpublished results), suggesting that DNA is an important structural component.

Biofilm formation by staphylococci is subject to complex regulation that is influenced by a number of environmental factors, including osmolarity, glucose levels, anaerobiosis, temperature, and levels of iron, citrate, ethanol, and nitrites (11, 17, 23, 26, 46, 47). We (26) previously described a novel gene, *rbf*, which regulates biofilm production in response to glucose and NaCl. The *rbf* gene codes for a 716-amino-acid protein

* Corresponding author. Mailing address: Department of Microbiology and Immunology, University of Arkansas for Medical Sciences, 4301 W. Markham Street, Slot 511, Little Rock, AR 72205. Phone: (501) 526-7687. Fax: (501) 686-5359. E-mail: clee2@uams.edu.

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with homology to the AraC/XylS family of transcriptional activators. From this we proposed that *rbf* is involved in positive regulation of a protein or proteins that are important for biofilm formation. A mutant strain with an insertion in *rbf* was impaired in biofilm formation on polystyrene and glass. The mutant strain exhibited no defect in primary attachment to polystyrene, however, suggesting that inactivation of *rbf* affected the multicellular aggregation step rather than the primary attachment step in biofilm formation. In addition, disruption of *rbf* in *S. aureus* 8325-4 had no measurable effect on expression of a P*ica*::*xylE* reporter construct, suggesting that *rbf* may regulate an *ica*-independent pathway for biofilm formation in 8325-4 (26). More recently, we reported that *rbf* enhances bacterial survival in a murine model of foreign body infection (29).

In this report we utilized microarrays and genetic approaches to further investigate the function of *rbf*. Our results suggest that Rbf enhances biofilm formation by activating *icaADBC* expression and that activation is indirect, occurring by *rbf* repression of *icaR*. We also discovered that *rbf* was a pseudogene in UAMS-1. However, the *rbf* defect in UAMS-1 could be complemented with the *rbf* gene from 8325-4, suggesting that *rbf* controls biofilm formation in strains 8325-4 and UAMS-1 via a similar mechanism. The effect of *rbf* on biofilm was also observed in a recently isolated clinical strain, MW2.

MATERIALS AND METHODS

Bacterial strains, culture media, and growth conditions. The bacterial strains used in this study are listed in Table 1. *S. aureus* UAMS-1 is a clinical isolate cultured from the bone of a patient with osteomyelitis (16). *S. aureus* MW2, obtained from the Network on Antimicrobial Resistance in *Staphylococcus aureus*, was originally isolated from a child with fatal septicemia and septic arthritis (5). Staphylococci were cultured in tryptic soy broth (TSB; Difco Laboratories, Detroit, MI) or tryptic soy agar (Difco). In some experiments growth medium was supplemented with glucose and NaCl as described below. Antibiotics were added to culture media, as appropriate and unless otherwise specified, at final concentrations of 10 μg per ml chloramphenicol (Cm) and 100 μg per ml penicillin. *Escherichia coli* strains DH5α and XL1-Blue were used for plasmid construction and maintenance. *E. coli* was cultivated in Luria-Bertani broth or agar (Difco).

Plasmid and strain construction. Allele replacement of *rbf* in strains 8325-4 and MW2 was performed using the same primers and methods as previously described for UAMS-1 Δ*rbf* (29). Allele replacement was confirmed by PCR. Similarly, the *icaADBC* and *icaR* deletion mutants of 8325-4 were constructed by allele replacement using pKOR1 (1). DNA inserts with target region deletions were constructed by overlapping PCR. PCR primers attB1-icaKO1, comp-icaKO2, comp-icaKO3, and attB2-icaKO4 were used in construction of the *icaADBC* mutant (Table 2). Primers attB1-icaRKO1, comp-icaRKO2, comp-icaRKO3, and attB2-icaRKO4 (Table 2) were used in construction of the *icaR* mutant.

The *icaR* gene was amplified by PCR using primers icaR-1 and icaR-3 (Table 2) and cloned into pLI50 at BamHI and EcoRI sites to generate expression plasmid pML3796. The construct was verified by DNA sequencing. Plasmids pLI50, pYL8565, and pML3796 were transduced into the *S. aureus* strains listed in Table 1 by phage 52A.

Biofilm and PNAG assays. Flow cell biofilm assays were performed in flow cells purchased from Stovall Life Science, Greensboro, NC, as previously described (29). UAMS-1 derivatives were cultured in TSB containing 3.5% NaCl, 0.75% glucose, and 10 μg per ml Cm. MW2 derivatives were cultured similarly except that the NaCl concentration was reduced to 1.5% and Cm was used at 5 μg/ml. Bacterial cells were harvested for RNA isolation at the peak biofilm formation. Microtiter biofilm assays were performed in 96-well microtiter plates as described by Lim et al. (26) with minor modifications (29). Assays for PNAG were performed as previously described (10, 55).

RNA methods. RNA was isolated as described by Luong et al. (29). For Northern analysis of the *rbf* transcript in UAMS-1, RNA was isolated from flow cells and separated on a 1% agarose–formaldehyde gel and transferred to a nylon membrane (Roche Diagnostics, Mannheim, Germany) as described previously (7). For hybridization, a 1-kb fragment of *rbf*, amplified using rbf62 and adh14 primers (Table 2), was gel purified and labeled with [^{32}P]dATP by using a High Prime DNA labeling kit from Roche Diagnostics. The membrane was hybridized with the *rbf*-specific probe at 42°C for 22 h in Ultrahyb hybridization buffer (Ambion Inc., Austin, TX). RNA size standards were from Roche. Microarray profiling was performed essentially as described previously (2). Briefly, two RNA samples from each strain were prepared from two separate flow cells and were independently labeled following the manufacturer's recommendations for prokaryotic antisense arrays (Affymetrix, Santa Clara, CA). A 1.5-μg aliquot of a labeled sample was hybridized to a commercially available Affymetrix *S. aureus* GeneChip. Scanned intensity values for each detected RNA species were normalized to the median GeneChip signal value, and biological replicates were averaged using GeneSpring GX 7.3.1 analysis platform software (Agilent Technologies, Redwood City, CA). Genes that were found to be differentially expressed exhibited a ≥2-fold change in expression under the indicated conditions were determined to be above background signal intensity values and considered "present" by Affymetrix algorithms under the induced condition and were considered statistically differentially expressed as determined by Student's *t* test ($P \leq 0.05$). Quantitative real-time reverse transcription-PCR (RT-PCR) was performed as described previously (27).

For isolation of RNA from *S. aureus* strain 8325-4, cultures were grown overnight in TSB containing 3.5% NaCl and 0.75% glucose. Cm was added to cultures of plasmid-bearing strains. The cultures were adjusted to an optical density at 660 nm of 3 and then diluted 1:250 into fresh TSB-glucose-NaCl and cultured for 6 h, with aeration, at 37°C.

TABLE 1. Strains and plasmids

Strain or plasmid	Relevant characteristics	Reference or source
S. aureus strains		
8325-4	Prophage-free laboratory strain	J. Iandolo
UAMS-1	Wild-type clinical isolate	14
MW2	Wild-type clinical isolate	5
CYL6939	UAMS-1(pLI50)	29
CYL6933	UAMS-1(pYL8565)	29
CYL6970	UAMS-1 *rbf*(pLI50)	29
CYL1112	8325-4(pLI50)	This study
CYL6968	8325-4 *rbf*(pLI50)	This study
CYL6973	8325-4(pYL8565)	This study
CYL6974	8325-4 *rbf*(pYL8565)	This study
CYL1097	8325-4 *rbf*::*tet*	26
CYL11688	8325-4 *icaR*(pLI50)	This study
CYL11689	8325-4 *icaR rbf*(pLI50)	This study
CYL11690	8325-4 *icaR rbf*(pYL8565)	This study
CYL11696	8325-4 *icaADBC*	This study
CYL11699	8325-4 *icaR*(pML3796)	This study
CYL11700	8325-4 *icaR rbf*(pML3796)	This study
CYL11683	MW2(pLI50)	This study
CYL11712	MW2 *rbf*(pLI50)	This study
CYL11713	MW2 *rbf* (pYL8565)	This study
E. coli strains		
DH5α	Host strain for plasmids	45
XL1-Blue	Host strain for plasmids	45
Plasmids		
pLI50	*E. coli-S. aureus* shuttle vector	25
pYL8565	Rbf expression plasmid derived from pLI50	26
pML3796	IcaR expression plasmid derived from pLI50	This study
pKOR1	Vector for allele replacement	1

RESULTS

Characterization of *rbf* transcription in wild-type, *rbf*, and *rbf* overexpression strains. To verify expression of *rbf* in

TABLE 2. Oligonucleotide primers used for plasmid and strain construction

Primer	Sequence
adh14	CACTCATAAAAGCTTCTTC
adh15	GGGCCCAAGCGACTTAAATTCGATTCGT
rbf20	ATACCGCGGCGCGTTGTCGCATATTCATT
rbf62	CTTAAATATAGAAAGAGGTA
attB1-icaKO1	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCACTGCTCCAAATTTTTGCG
attB2-icaKO4	GGGGACCACTTTGTACAAGAAAGCTGGGTCGATCTGACGCGTGAGGGTGC
attB1-icaRKO1	GGGGACAAGTTTGTACAAAAAAGCAGGCTGAGCCCATCTCACGCGTTGC
attB2-icaRKO4	GGGGACCACTTTGTACAAGAAAGCTGGGTCACTTACTACAAGACATATTGCCG
comp-icaKO2	TATACATAATCCGCGGCCGCGAGTGCAAGAACATTAGACAACG
comp-icaKO3	GTTCTTGCACTCGCGGCCGCGGATTATGTATAGGTGTCGGC
comp-icaRKO2	TTCCACTGCTCCGCGGCCGCCATCAAGTGTTGTACCGTC
comp-icaRKO3	CAACACTTGATGGCGGCCGCGGAGCAGTGGAAGAAAGTA
icaR-1	GGATCCGAACCGACAATCCAGTAAATAGAC
icaR-3	GAATTCGTAAGTTAATTATTACAAACTAGTAAC

UAMS-1, RNA was isolated from the wild-type strain UAMS-1 (strain 6939), an isogenic *rbf* mutant (strain 6970), and an Rbf overexpression strain (strain 6933). The RNA was subjected to Northern blot analysis using an *rbf*-specific probe. As shown in Fig. 1, the probe hybridized to multiple transcripts in wild-type RNA ranging in size from 1.7 to 4.0 kb. Since *rbf* is 2.2 kb in length, the results suggest that there may be multiple transcriptional start sites and/or processing/degradation of the primary transcript. No transcript was detected in RNA from the *rbf* mutant. As anticipated, a much higher level of hybridizing RNA was detected in the *rbf* overexpression strain. These results were confirmed using real time RT-PCR to measure *rbf* transcription (not shown). These results suggested that our strains and experimental conditions were appropriate to characterize the cellular role of *rbf*.

Identification of *rbf*-regulated genes by microarray. In order to identify what genes are regulated by *rbf*, microarray experiments were performed with RNA isolated from wild-type UAMS-1 (strain 6939) and the *rbf* mutant (strain 6970). In addition, RNA was isolated from an Rbf overexpression strain (strain 6933), as we had previously observed that overexpression of *rbf* enhanced biofilm formation. In these experiments, bacteria were cultured in commercially acquired flow cells. The microarray experiments were performed with RNA from two independent cultures of each strain. Comparing the wild type and the *rbf* mutant, we identified 16 genes upregulated by *rbf* by at least twofold. In the Rbf overexpression strain, 6 genes were upregulated while 35 genes were downregulated compared to the wild-type strain (Tables 4 and 5). Notably, overexpression of *rbf* in strain 6933 resulted in increased expression

TABLE 3. Oligonucleotide primers used in real-time RT-PCR

Primer	Gene	Sequence
SGaldC1	*aldC*	CGTATGATGCCGGCTCAAGAACCACCTTAT
SGaldC2	*aldC*	CGCAAAGTGTACATGAAATCCTGCTGATCCGA
SGbetA1	*betA*	TGCCTGCTGCGTTAATGTTCCCTTCA
SGbetA2	*betA*	ACGACCGCCCATATGTGGTTCTTCAT
SGchp1	*chp*	CAGGAATCAGTACACACCATCATTCAGCGAAAGC
SGchp2	*chp*	AATTTCCTAGCGTTGTAGGAAGACCACTATTT
SGefb1	*efb*	ATGCGAGCGAAGGATACGGTCCAAGAGAAA
SGefb2	*efb*	TGTGGACGTGCACCATATTCGAATGTACCA
SGgyrB3	*gyrB*	GGAATCGGTGGCGACTTTGATCTAGCGAAA
SGgyrB4	*gyrB*	CGCTCCATCCACATCGGCATCAGTCATAAT
SGhlb1	*hlb*	AAACACCTGTACTCGGCCGTTCTCAATCAG
SGhlb2	*hlb*	ACTTACAATCGCTACGCCACCATCTTCTGC
SGicaA1	*icaA*	CTGGCGCAGTCAATACTATTTCGGGTGTCT
SGicaA2	*icaA*	GACCTCCCAATGTTTCTGGAACCAACATCC
icaR for	*icaR*	TACGTTCAATTATCTAATACGCCTGAGGAATTTTCTGGAA
icaR rev	*icaR*	AGGATGCTTTCAAATACCAACTTTCAAGAAACAGCAAATATT
SGlrgB1	*lrgB*	CTCAAGCAGCAACTACAGCGATTGCGTTAC
SGlrgB2	*lrgB*	TTCCAAGTGCTAATCCTCGGGCAATAGGGT
SGnirD1	*nirD*	AAGGACCATTGTCTGAAGGGACAGTGAGTG
SGnirD2	*nirD*	ATATACGTTCCCGTCTGTAACTTCTACCTCA
SGnirK1	*nirK*	TGAAGCGGGATCCGCAAATGGTATCGTATC
SGnirK2	*nirK*	GTGGGAAGAATCCTCCTAAACCACCCATCA
Δrbf for	*rbf*	ACGCGTTGCCAAGATGGCATAGTCTT
Δ rbf rev	*rbf*	AGCCTAATTCCGCAAACCAATCGCTA
SGsarX1	*sarX*	TGAATACTGAGAAATTAGAAACATTGCTTGGCTTCTATAAACA
SGsarX2	*sarX*	TGTCCTACTTAAATCTAGCTCATCCATTGCAGTT

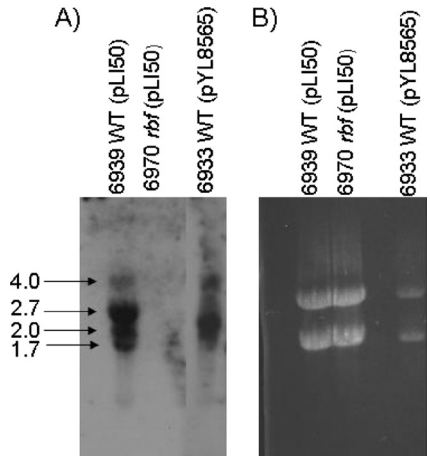

FIG. 1. Northern analysis of *rbf* transcription in UAMS-1. (A) Northern analysis with an *rbf*-specific probe. RNA was isolated from wild-type UAMS-1 (strain 6939), an *rbf* mutant (strain 6970), and an *rbf* overexpression strain (6933). Numbers to the left of the figure are the sizes of the indicated RNA bands (in kb). (B) Ethidium bromide-stained gel of RNAs, showing the relative level of rRNA in each preparation.

of the *icaADBC* operon, the genes required for PNAG synthesis, by five- to sixfold. In addition, two genes known to repress *icaADBC* expression, *icaR* and *tcaR*, were repressed in the Rbf-overproducing strain. These findings could account, at least in part, for the role of *rbf* in biofilm formation.

Overexpression of *rbf* also affected the transcription of at least four other genes likely to impact on biofilm formation. These included *tagB*, encoding teichoic acid biosynthesis protein B, which is upregulated by *rbf*. It has been argued that teichoic acid synthesis is important for biofilm formation (17, 36). Three genes involved in the regulation of cell lysis, *lytS* and *lrgAB*, are repressed in the overproducing strain. It has been shown that cell lysis is the source of extracellular genomic DNA, which is an important constituent of *S. aureus* biofilms (43, 44, 50).

Overexpression of *rbf* has the general effect of decreasing transcription of secreted and surface-associated proteins, including many of which are known or suspected virulence factors (Table 5). These include *chp* (chemotaxis-inhibiting protein), *clfB* (clumping factor B), *fbp* (fibrinogen binding protein), *fnbA* (fibronectin binding protein), *hlb* (β-hemolysin), *map* (major histocompatibility complex class II analogue protein), *set11* (exotoxin 11), SACOL2004 (leukocidin F), SACOL2418 (immunoglobulin G binding protein), and SACOL0468 and SACOL0857, which are a phage-encoded exotoxin and coagulase, respectively. Transcription of *sarX*, which encodes a transcriptional regulator located immediately downstream of the *rbf* gene, was upregulated over 10-fold in the Rbf overexpression strain (strain 6933). Other potential regulatory factors affected by *rbf* are *lytS* and *kdpD*, both encoding sensor histidine kinases, and SACOL1904 (a putative transcription regulator).

Confirmation of microarray results using real-time PCR. In order to verify our microarray results we used real-time RT-PCR to compare expression levels of 12 select UAMS-1 genes (Fig. 2). Bacteria for these experiments were cultured in the same manner as cultures used in the microarray studies. RNA from two independent cultures of each strain was analyzed. Relative expression levels were determined using *gyrB* expression for normalization of data. With some exceptions, the real-time PCR results correlated well with the microarray re-

TABLE 4. Genes upregulated by *rbf*[a]

ORF	Fold change in:		Gene	Description
	*rbf*Δ vs wt	*rbf*$^{++}$ vs wt		
SACOL2641		3.7	*gpxA*	Glutathione peroxidase
SACOL2689		6.1	*icaA*	Intercellular adhesion protein A
SACOL2691		4.5	*icaB*	Intercellular adhesion protein B
SACOL2690		5.9	*icaD*	Intercellular adhesion protein D
SACOL0725	5.4	14.0	*rbf*	Transcriptional regulator, AraC family
SACOL0726		11.5	*sarX*	Transcription factor
		7.1		Intergenic region upstream of *rbf*
SACOL2198	2.1		*aldC*	α-Acetolactate decarboxylase
SACOL1351	2.2		*cls1*	Cardiolipin synthetase
SACOL0357	2.2		*dut*	Prophage L54a, deoxyuridine 5-triphosphate nucleotidohydrolase
SACOL0600	2.1		*ilvE*	Branched-chain amino acid aminotransferase
SACOL2070	2.1		*kdpD*	Sensor histidine kinase
SACOL2397	2.3		*nirD*	Small subunit nitrite reductase
SACOL0696	2.1		*tagB*	Teichoic acid biosynthesis protein B
SACOL0569	2.1			ATP:guanido phosphotransferase family protein
SACOL1591	2.0			Lipoate-protein ligase A family protein
SACOL2138	2.4			Cation efflux family protein
SACOL2357	2.0			ABC transporter, permease protein
SACOL2386	2.0		*narT*/*nirK*	Nitrite extrusion protein
SACOL2396	2.0			Uroporphyrinogen III methylase, SirB, putative
N315-A1617	2.1			Fragment, conserved hypothetical protein
SACOL0568	2.1			Conserved hypothetical protein
SACOL1790	2.1			Conserved hypothetical protein

[a] *rbf*Δ/wt data indicate the increased expression level in the wild type compared to the *rbf* deletion mutant. *rbf*$^{++}$/wt data indicate the increased expression level in the Rbf-overproducing strain compared to the wild type.

TABLE 5. Genes downregulated by *rbf*[a]

ORF	Fold change in *rbf*$^{++}$ vs wt	Gene	Description
SACOL2627	−2.3	*betA*	Choline dehydrogenase
SACOL2628	−2.5	*betB*	Betaine aldehyde dehydrogenase
SACOL0302	−2.1	*brnQ*	Branched-chain amino acid transport system II carrier protein
MSRA252-SAR2036	−3.9	*chp*	Chemotaxis-inhibiting protein
SACOL2652	−2.7	*clfB*	Clumping factor B
SACOL1168	−5.1	*efb*/*fbp*	Fibrinogen binding protein (*efb*)
SACOL2511	−4.3	*fnbA*	Fibronectin binding protein A
SACOL2003	−3.7	*hlb*	Phospholipase C
SACOL2688	−2.5	*icaR*	Intercellular adhesion regulator
SACOL0600	−2.2	*ilvE*	Branched-chain amino acid aminotransferase
SACOL0247	−3.3	*lrgA*	Holin-like protein LrgA
SACOL0248	−4.6	*lrgB*	LrgB protein
SACOL0245	−2.1	*lytS*	Sensor histidine kinase
SACOL2002	−3.3	*map*	Map protein, authentic frameshift
N315-SA0387	−4.0	*set11*	Exotoxin 11
SACOL2353	−2.3	*tcaR*	Transcriptional regulator TcaR
SACOL1364	−2.2	*thrB*	Homoserine kinase
SACOL2004	−4.0		Leukocidin F subunit, putative
SACOL2418	−2.1		Immunoglobulin G binding protein SBI
SACOL0468	−2.6		Exotoxin 3, putative
SACOL0857	−2.0		Staphylocoagulase precursor, putative
SACOL1164	−2.8		Fibrinogen-binding-related protein
SACOL1751	−3.5		Truncated cell surface protein map
SACOL1892	−2.3		Membrane protein, putative
SACOL1904	−2.3		Transcriptional regulator, putative
SACOL2499	−2.1		Helicase, putative
MU50-SAV1941	−4.6		Putative membrane protein
SACOL0199	−3.6		Conserved hypothetical protein
SACOL0480	−2.4		Hypothetical protein
SACOL0767	−3.0		Conserved hypothetical protein
SACOL0768	−2.7		Conserved hypothetical protein
SACOL1777	−4.2		Conserved hypothetical protein
SACOL2492	−2.2		Hypothetical protein
SACOLR1913	−2.1		Hypothetical protein
N315-SAS058	−2.1		Conserved hypothetical protein

[a] The *rbf*$^{++}$ versus wt data indicate the decreased expression level in the *rbf*-overproducing strain compared to the wild type.

sults. Two exceptions were *sarX* and *rbf* when comparing expression levels between the wild type (strain 6939) and the Rbf overexpression strain (6933) (Table 4). For the Rbf overexpression strain, expression levels of *sarX* and *rbf* were 11.5- and 14-fold higher, respectively, in the microarray experiments (Table 4), whereas both genes were expressed approximately 52-fold higher in the real-time PCR experiments (Fig. 2). The underestimation of transcript levels in microarray studies has been reported previously by our laboratory and others (3, 27) and may simply reflect the increased sensitivity of real-time PCR over microarrays. The other exception was *nirD*, encoding the small subunit of nitrite reductase, for which we had anticipated twofold-higher expression in the *rbf* mutant, strain 6970, compared to the wild-type strain, 6939. This result was intriguing, as nitrite has been shown to inhibit biofilm formation by *S. aureus* (46). The real-time PCR results, however, showed an approximate 64% reduction in *nirD* RNA in strain 6933.

Evidence that *rbf* may not be expressed in UAMS-1. The results presented above demonstrate that high-level expression of *rbf* has a profound effect on gene expression by UAMS-1. Moreover, *rbf* overexpression had a dramatic effect on biofilm formation and PNAG synthesis (Fig. 3). In contrast, the *rbf* mutation had only small effects on gene expression, suggesting that *rbf* may be poorly expressed in UAMS-1. Sequencing of the UAMS-1 *rbf* gene revealed the presence of a 2-bp insertion (relative to several sequenced *S. aureus* strains, including NCTC 8325) affecting the 50th codon of the predicted *rbf* open reading frame (ORF). Thus, UAMS-1 appears not to carry an intact *rbf* gene and therefore may not produce a functional protein. It is important to note here that the *rbf* gene carried on pYL8565 was cloned from *S. aureus* 8325-4 (26).

To test whether the Rbf protein is produced by UAMS-1, we used anti-Rbf antibody in Western analyses (data not shown). No detectable Rbf protein was found in UAMS-1, whereas an ~80-kDa band, matching the predicted size of Rbf, was readily detected in 8325-4. This result indicates that Rbf is not produced in UAMS-1, apparently due to the 2-bp insertion in the *rbf* ORF.

***rbf* regulation of *ica* operon expression in *S. aureus* 8325-4.** The microarray and the real-time RT-PCR experiments using UAMS-1 and its derivative strains suggest that *rbf* may regulate biofilm formation by affecting *ica* gene expression. Because we found that the *rbf* gene was functional in 8325-4 but not in UAMS-1, we chose strain 8325-4 for further studies. However, our previous finding using a P*ica*::*xylE* reporter construct showed that an *rbf* mutation in *S. aureus* strain 8325-4 was not associated with altered *ica* expression (26). This inconsistency

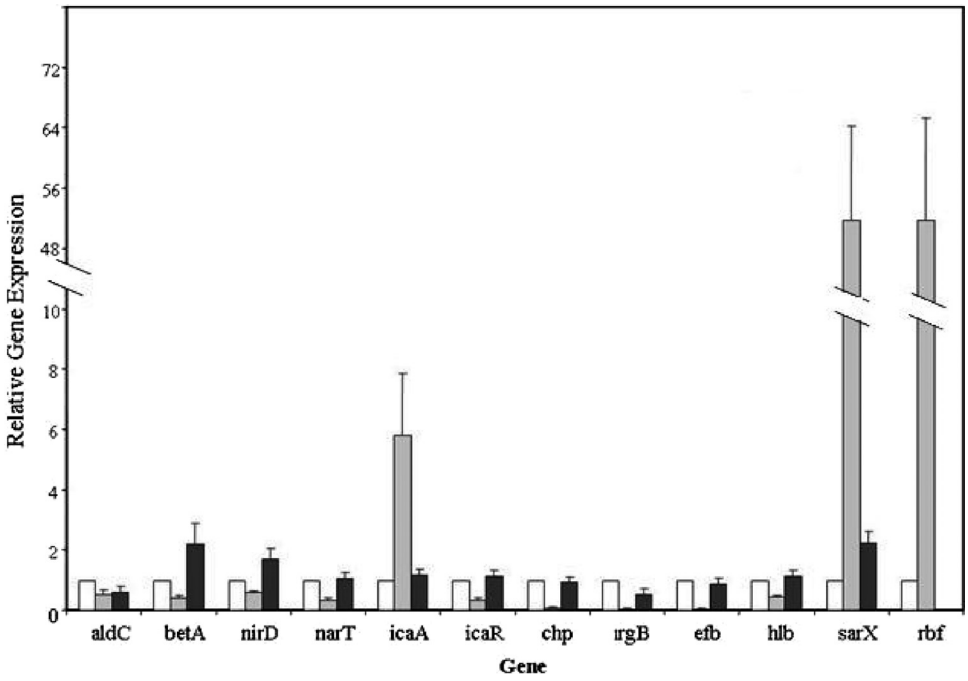

FIG. 2. Confirmation of microarray results using real-time RT-PCR. RNA was isolated from the wild-type strain, 6939 (white bars), an Rbf overexpression strain, 6933 (gray bars), and an *rbf* mutant strain, 6970 (black bars) in flow cell cultures and subjected to real-time RT-PCR using primers specific for the indicated genes (Table 3). Expression levels are expressed relative to that of strain 6939, which was arbitrarily assigned a value of 1. The expression of *gyrB* was used to correct for differences in RNA quantities added to each reaction mixture. Data represent the means ± standard errors from two independent experiments.

may have been due to the relative insensitivity of the *xylE* reporter used in the former study. To investigate this apparent discrepancy we reassessed the role of the strain 8325-4 *rbf* gene in the *ica* operon and PNAG regulation. For these experiments, a stable, internal deletion of *rbf* was constructed in strain 8325-4 by allele replacement. As shown in Fig. 4A, real-time RT-PCR experiments revealed a significant decrease in *icaA* transcript levels in the *rbf* deletion strain (strain 6968)

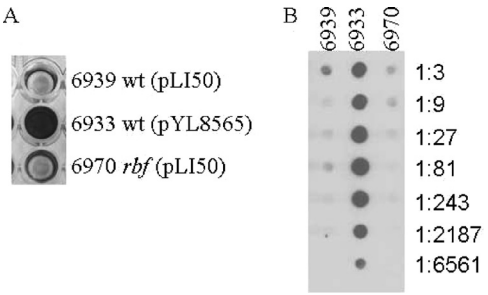

FIG. 3. Rbf enhances PNAG synthesis and biofilm formation in UAMS-1. (A) Biofilm formation by wild-type UAMS-1 (6939), an *rbf* mutant (6970), and a UAMS-1 derivative that overexpresses *rbf* (6933). The 96-well plates were inoculated with each of the indicated strains. Following a 24-h incubation, wells were washed and biofilms were stained with crystal violet. (B) PNAG production. PNAG was extracted from each UAMS-1 derivative, serially diluted, and applied to a membrane. PNAG was detected by incubating the membrane, successively, with rabbit anti-PNAG serum, goat anti-rabbit–horseradish peroxidase, and a chemiluminescent substrate. Numbers to the right of the figure indicate dilutions.

and expression of *rbf* from plasmid pYL8565 effectively complemented biofilm formation in the *rbf* mutant (strain 6974).

As was observed for UAMS-1, overexpression of wild-type *rbf* resulted in repression of *icaR* transcription (Fig. 4A). Carriage of pYL8565 reduced *icaR* expression by approximately twofold in both the *rbf* mutant (strain 6974) and the wild-type strain (strain 6973). These results support the argument that Rbf may promote transcription of *icaADBC* by repression of *icaR*.

To further examine the impact of *rbf* on biofilm regulation in 8325-4, we measured PNAG production by the wild-type strain and its derivatives (Fig. 4B). Consistent with the activation of *ica* operon expression, mutation of *rbf* was associated with decreased production of PNAG and overexpression of *rbf* was associated with increased production (Fig. 4B). Taken together, these data strongly indicate that, similar to our observations in UAMS-1, *rbf* controls biofilm formation in strain 8325-4, at least in part, by controlling expression of the *ica* genes.

Regulation of biofilm formation and *icaADBC* expression by *icaR* and *rbf* in *S. aureus* strain 8325-4. Our results thus far suggest that *rbf* may enhance biofilm formation, at least in part, by promoting transcription of *icaADBC*. The data further suggest that *rbf* activation of the *ica* operon could be indirect, being accomplished by *rbf* repression of *icaR* and/or *tcaR*, both of which are repressors of *icaADBC* (21). It should be noted here that strain 8325-4 contains a mutation in the *tcaR* gene (32); therefore, TcaR may not have an effect on biofilm in the 8325-4 background. To characterize *rbf* regulation of the *ica*

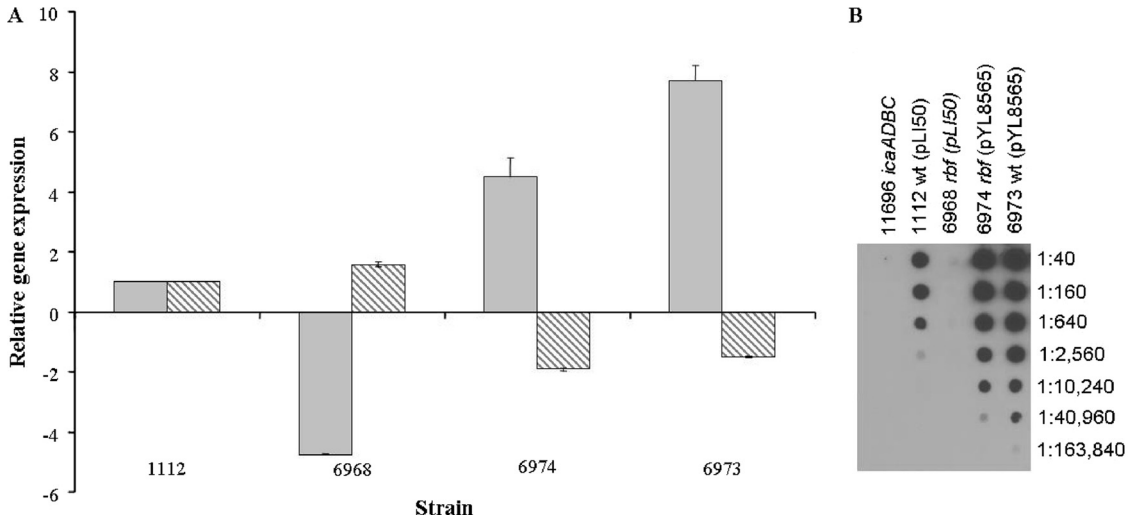

FIG. 4. Effects of Rbf on *icaADBC* and *icaR* expression in strain 8325-4. (A) Comparative measurements of *icaA* (gray bars) and *icaR* (hatched bars) transcription by real-time RT-PCR in the following *S. aureus* strains: 1112, wild type (pLI50); 6968, *rbf*(pLI50); 6974, *rbf*(pYL8565); 6973, wild type (pYL8565). Total RNA was prepared from cultures grown for 6 h at 37°C in TSB containing 3.5% NaCl, 0.75% glucose. Real-time RT-PCR was used to measure the relative expression of *icaA* and *icaR* compared to the *gyrB* gene. Transcript levels in all strains were compared to transcript levels in the wild-type strain, 1112, which was assigned a value of 1. The data presented are the averages of two separate experiments and standard errors are indicated. Plasmid pYL8565 carries the wild-type *rbf* gene, and pLI50 is the plasmid vector. (B) Regulation of PNAG synthesis by *rbf* in 8325-4 and derivatives. Bacterial extracts were prepared from overnight cultures grown at 37°C in TSB containing 3.5% NaCl, 0.75% glucose. PNAG was extracted from cells, serially diluted, and applied to a membrane. PNAG was detected by incubating the membrane, successively, with rabbit anti-PNAG serum, goat anti-rabbit–horseradish peroxidase, and a chemiluminescent substrate. Dilutions are indicated to the right of the figure. Strain numbers and genotypes are listed at the top of the figure.

locus and its potential interaction with *icaR* further, we compared the effects of single and double mutants of *rbf* and *icaR* in strain 8325-4. As shown in Fig. 5, inactivation of *icaR* (strain 11688), in contrast to the *rbf* mutant, promoted biofilm formation which could be complemented with an *icaR*-bearing plasmid, pML3796 (strain 11699). In the *icaR rbf* double mutant, biofilm formation was similar to that of the *icaR* mutant. These results indicate that the effect of *icaR* is epistatic to that of *rbf*, arguing that *icaR* acts downstream of *rbf* in the pathway for biofilm regulation. To further test this possibility, we complemented the *icaR rbf* double mutant with either the *rbf*-bearing plasmid pYL8565 (strain 11690) or the *icaR*-bearing plasmid

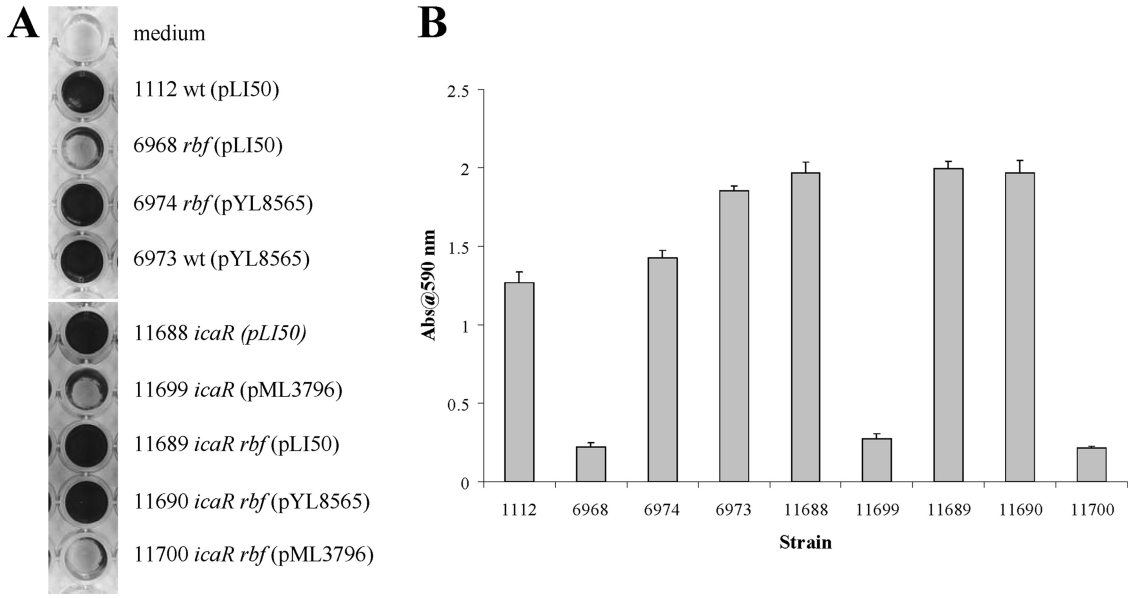

FIG. 5. Contributions of *rbf* and *icaR* to biofilm formation by 8325-4. (A) Biofilm formation under static incubation conditions. Microtiter plate wells were inoculated with each of the indicated 8325-4 derivatives. Following a 24-h incubation, wells were washed and biofilms were stained with crystal violet. Each assay was performed a minimum of two times. (B) Quantitation of biofilms. Crystal violet was extracted from wells by using ethanol-acetic acid and diluted 10-fold, and the absorbance of each extract was measured. Error bars indicate standard errors.

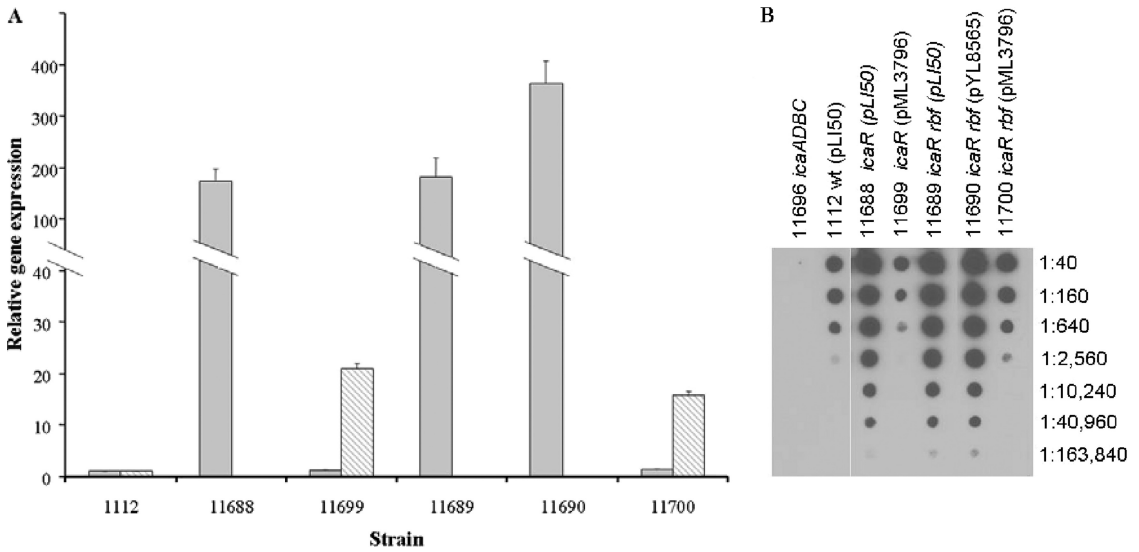

FIG. 6. Regulation of *icaA* expression and PNAG production by *rbf* and *icaR* in 8325-4. (A) Transcription of *icaA* (gray bars) and *icaR* (hatched bars). Experiments were performed as described in the legend to Fig. 4A. (B) PNAG production. PNAG was measured as described in the legend to Fig. 4B. Numbers to the right of the figure indicate dilutions. Strain numbers and genotypes are listed at the top of the figure.

pML3796 (strain 11700). We found that complementation of the double mutant with the *icaR*-bearing plasmid repressed biofilm formation to less than the wild-type level (Fig. 5). In contrast, the presence of the *rbf*-bearing plasmid (strain 11690) did not affect biofilm formation compared to the double mutant strain. These results support the proposal that *icaR* functions downstream of *rbf*.

To confirm that increased biofilm formation was associated with increased *icaADBC* transcription, we compared *icaA* and *icaR* RNA levels in these strains using real-time RT-PCR (Fig. 6A). The results indicated that the *icaR* mutation in both the wild-type background (strain 11688) and the *rbf* mutant (strain 11689) increased *icaA* transcription by nearly 200-fold relative to the wild-type strain, 1112. Carriage of plasmid pYL8565 (strain 6973) increased *icaA* transcription by approximately eightfold with a concomitant reduction in *icaR* expression (Fig. 4A). Thus, measurements of *icaA* and *icaR* mRNA levels in these strains correlated well with the biofilm data, i.e., increased *icaA* expression (Fig. 6) correlated with decreased *icaR* expression and increased biofilm formation (Fig. 5).

Derepression of *icaADBC* was anticipated to result in increased production of PNAG and, in turn, increased biofilm formation. To confirm that PNAG levels correlated with biofilm formation, immunoassays for PNAG were performed (Fig. 6B). The *icaR* mutation increased PNAG production by roughly 64-fold, consistent with robust biofilm formation and increased *icaA* expression. Transformation of *icaR* mutants with pML3796 (strains 11699 and 11700) decreased PNAG synthesis to the wild-type level. Thus, for most strains there is a strong correlation between *rbf* expression, *icaA* transcription, PNAG production, and biofilm formation. The exceptions were the *icaR* single and *icaR rbf* double mutant strains carrying the *icaR*-encoding plasmid pML3796 (strains 11699 and 11700). These strains produced wild-type levels of *icaA* transcript and PNAG, yet neither strain was able to form a stable biofilm. These results suggest that *icaR* may regulate genes other than *icaADBC*, at least when the repressor is overexpressed.

***rbf* regulation of biofilm formation in *S. aureus* MW2.** The MW2 strain was isolated from a child with fatal septicemia and septic arthritis (5). To determine whether *rbf* affected biofilm formation in this clinical isolate, we compared the growth of MW2 (pLI50), an *rbf* mutant [MW2 *rbf*(pLI50)], and a complemented mutant [MW2 *rbf*(pLY8565)] in flow cells. We found, based on three separate experiments, that the time required for initiation of visible biofilm formation was 10.50 ± 1.76 h, 13.33 ± 0.33 h, and 10.33 ± 0.17 h (means ± standard errors) for the wild type, *rbf* mutant, and the complemented mutant, respectively. The results from one of the experiments are shown in Fig. 7A. The average times to peak biofilm formation were 30.50 ± 1.76 h, 39.17 ± 2.68 h, and 31.5 ± 0.29 h for the same three strains. The differences in time to initiation were statistically significant ($P = 0.0076$ for MW2 compared to MW2 *rbf* and $P = 0.0151$ for MW2 *rbf* compared with the complemented strain). The differences for peak biofilm formation were not statistically significant ($P > 0.05$). We also compared PNAG production in flow cell cultures. The results, shown in Fig. 7B, revealed that PNAG production was approximately fourfold lower for MW2 *rbf* than it was for either the wild-type or complemented strains. These results indicate that *rbf* contributes to the regulation of biofilm formation in MW2. It should be noted here that MW2 forms strong biofilms in microtiter plates precoated with human serum but forms very weak biofilms without precoating. In either case, *rbf* had no effect on the biofilm.

DISCUSSION

Many staphylococcal infections are associated with biofilm formation, most notably, infections associated with indwelling medical devices. Therefore, studies of biofilm constituents and the regulatory mechanisms governing their synthesis is an im-

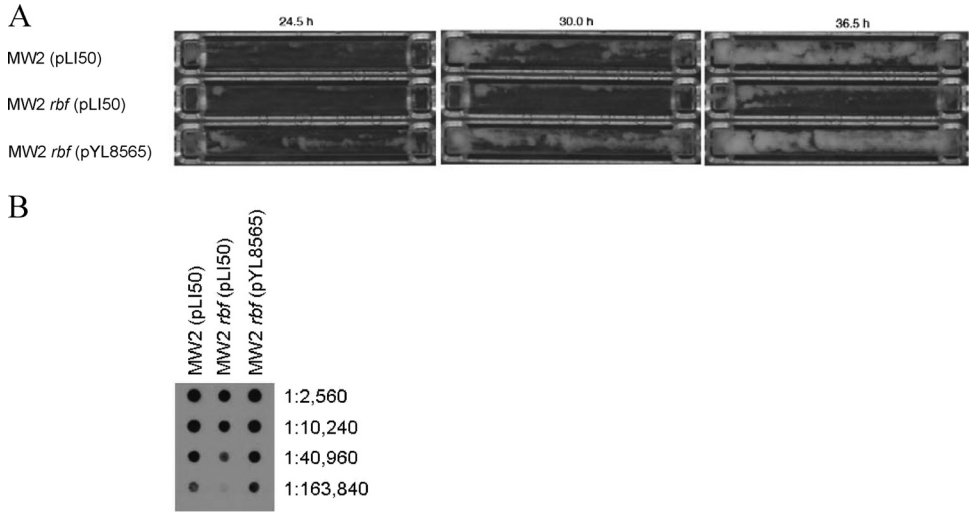

FIG. 7. Regulation of biofilm formation by *rbf* in MW2. (A) Biofilm formation in flow cells after 24.5, 30.0, and 36.5 h of incubation. The results are representative of three separate experiments. Strains grown in each flow cell are indicated to the left of the figure. (B) PNAG production by MW2 and derivatives. Cells were harvested from flow cells after 48 h of incubation. PNAG was extracted and then detected as described in the legend to Fig. 4B. Numbers to the right of the figure indicate dilutions. Strain numbers and genotypes are listed at the top of the figure.

portant area of research that could ultimately lead to therapies for prevention of device-related infections. However, regulation of biofilm formation is complex, being affected by environmental factors such as osmolarity, anaerobiosis, temperature, and levels of glucose, iron, ethanol, citrate, and nitrites (11, 18, 23, 26, 46, 47, 55). Numerous staphylococcal regulatory factors have been implicated in biofilm formation as well, including *agr*, SarA, SigB, IcaR, TcaR, ArlRS, SrrAB, MgrA, and Rbf (2, 26, 42, 48, 50–52). In this study we attempted to define the role of one regulatory factor, Rbf, in biofilm formation by *S. aureus*.

Our microarray studies demonstrated a profound effect of overexpression of *rbf* on gene expression in the clinical isolate UAMS-1, an observation supported by our real-time PCR experiments. Overall, we determined that 6 genes were upregulated and 35 genes were downregulated in an Rbf overexpression strain in comparison to the wild-type parent strain. Several of these genes, including *icaR*, *icaADBC*, *lytS*, and *lrgAB*, have the potential to impact biofilm formation. These results indicate that Rbf could potentially regulate biofilm formation at multiple levels. It was anticipated that genes responsive to overexpression of Rbf would also respond to genetic inactivation of *rbf*. This was generally not the case, however, as comparatively few genes were affected in our UAMS-1 *rbf* deletion mutant and the measured effects were all quite small (typically around twofold). The reason for this is that UAMS-1 is unlikely to produce an active Rbf protein. While we found that *rbf* was actively transcribed, sequencing of the UAMS-1 *rbf* gene revealed a 2-bp insertion within the predicted Rbf coding region. The mutation, which affects codon 50 of Rbf, would result in the production of a truncated protein. Additionally, we were unable to detect Rbf in extracts of UAMS-1 by using Rbf antiserum, whereas we were able to detect Rbf in extracts of strain 8325-4. We concluded that UAMS-1 has acquired a mutation that inactivates *rbf*; therefore, subsequent experiments were performed with *S. aureus* 8325-4. We gathered evidence that *rbf* is functionally expressed in another clinical isolate, MW2. Mutation of *rbf* in this strain reduced both biofilm formation and PNAG production. In a previous study (26) we found that *rbf* was present in 22 of 27 clinical isolates.

Rbf is a member of the AraC/XylS family of transcriptional regulators (26). Although the members of this family are diverse, they all possess a 99-amino-acid conserved region containing a dual helix-turn-helix DNA binding motif (15). The functions of the nonconserved domains of AraC/XylS family members are unknown in most cases, but it is presumed that the nonconserved regions may bind small molecules that could enhance or inhibit transcriptional activation by the protein (15). It is unknown at present whether Rbf binds an inducer or cofactor.

It is also unknown whether Rbf can function as a transcriptional activator, but nearly all other AraC/XylS family members act in this way (15). In this study, we found that Rbf contributed to biofilm formation by activating *icaADBC*. Our genetic analyses further showed that the activation was indirect, occurring via repression of *icaR*. The *ica* operon encodes four genes, *icaA*, *-D*, *-B*, and *-C*, responsible for the synthesis and possible transport of the intercellular adhesin PNAG (18). Numerous studies have demonstrated that *icaADBC* is required for biofilm formation by some strains of *S. aureus* (23, 33, 55); however, recent studies suggested that some *S. aureus* strains can form biofilms independently of *icaADBC* (2, 37, 47) and many strains seem to possess both *ica*-dependent and *ica*-independent biofilm pathways (36).

Several factors that regulate *icaADBC* expression have been described, including SarA, *agr*, TcaR, and IcaR (6, 8, 13, 20–22, 49). IcaR regulation is probably the most studied and best understood. IcaR binds specifically to the *icaADBC* promoter just upstream of the *icaA* start site to block transcription of the *ica* genes (13, 20). When *rbf* is overexpressed in 8325-4, transcription of *icaR* is decreased by 33 to 48%, with a concomitant rise in *icaA* transcription of four- to eightfold. The *rbf* mutation

increases *icaR* transcription, while *icaA* expression is decreased. The *tcaR* gene has been reported to encode a weak repressor of *icaADBC* (21). Our microarray results showed that like *icaR*, *tcaR* is repressed by overproduction of Rbf in UAMS-1. Thus, it is possible that activation of *icaADBC* by *rbf* could be attributed to *rbf* repression of *tcaR*. However, a deletion of the *tcaR* gene in UAMS-1 had no measurable impact on biofilm formation (unpublished data), and 8325-4 harbors a mutation in *tcaR* (32). Therefore, it appears that *tcaR* may not contribute significantly to regulation of *icaADBC* in the strains studied here.

Rbf does not seem to directly repress *icaR* transcription. In fact, our in vitro DNA binding assays revealed that recombinant Rbf protein did not bind directly to a DNA fragment encompassing the *icaR-icaADBC* regulatory region. The same protein did bind to the *S. aureus sdrC* promoter as well as the *Staphylococcus epidermidis sdrF* promoter region, suggesting that it is functional (results not shown). One possible alternative mechanism for *icaR* repression is that Rbf upregulates expression of a gene encoding a repressor of *icaR*. Our experiments did show that *rbf* activated expression of at least one regulatory factor, SarX, along with a putative transcription factor, SACOL1904. SarX is a member of the SarA protein family of gene regulators (31). In our microarray experiments *sarX* transcription was increased 11.5-fold in an Rbf-overproducing strain of UAMS-1. This was confirmed by real-time RT-PCR, wherein we found that *rbf* enhanced *sarX* expression by an average of 52-fold in UAMS-1. SarX has been shown to repress expression of *agr* and *agr*-dependent genes, so it seems possible that SarX is involved in repression of *icaR*. The chromosomal context of *sarX* may also be important, given its location just downstream of *rbf*. Manna and Cheung (31) identified a minor transcript containing sequences transcribed from both *rbf* and *sarX*; thus, it is possible that at least part of *sarX* activation could be due to readthrough transcription of *rbf*. SarX is under positive regulation by the global regulatory protein MgrA (31). It is unlikely that Rbf activates *sarX* via activation of *mgrA* transcription, as MgrA affects the expression of at least 255 genes (28), whereas *rbf* affects only 57 genes.

It remains a possibility that Rbf does bind to the *icaR* regulatory region, but not under the conditions of our in vitro DNA binding assay. DNA binding by some members of the AraC/XylS family, including AraC (15), Rns (35), and MelR (53), involves protein binding to sites up to several hundred base pairs upstream or downstream of a regulated promoter. Thus, it is possible that Rbf binds to DNA sites distal to the *icaR* promoter or requires binding to multiple sites to form a stable complex with DNA. It is also possible that Rbf requires a cofactor protein for regulation of *ica*. At least two AraC/XylS family members, MxiE from *Shigella flexneri* (9) and InvF from *Salmonella enterica* (12, 41), appear to require interaction with a cofactor protein to act as transcriptional activators of virulence genes.

Rbf represses at least three genes, *lytS*, *lrgA*, and *lrgB*, involved in the regulation of cell lysis. This finding is intriguing, as lysis of *S. aureus* would release DNA into biofilms (43). LytS is the sensor component of a two-component regulatory system, LytSR, which positively regulates expression of the *lrgAB* operon. The LrgA protein is a putative antiholin that inhibits cell lysis (19, 43); thus, *rbf* repression of *lytSR* would be predicted to decrease expression of LrgA, thereby increasing cell lysis and DNA release. Extracellular DNA has been proposed to be an important structural component of *S. aureus* biofilms (43, 50).

Although many questions remain, our data suggest a simple working model for how Rbf affects *icaADBC* transcription. Rbf may promote expression of an unknown factor that binds to the regulatory region of *icaR*, thus repressing synthesis of the IcaR repressor. Repression of *icaR* would, in turn, result in derepression of *icaADBC* and PNAG production. Current studies are directed toward testing this model.

ACKNOWLEDGMENTS

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