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Control of Glucose- and NaCl-Induced Biofilm Formation by *rbf* in *Staphylococcus aureus*

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Both *Staphylococcus aureus* and *S. epidermidis* are capable of forming biofilm on biomaterials. We used Tn*917* mutagenesis to identify a gene, *rbf*, affecting biofilm formation in *S. aureus* NCTC8325-4. Sequencing revealed that Rbf contained a consensus region signature of the AraC/XylS family of regulators, suggesting that Rbf is a transcriptional regulator. Insertional duplication inactivation of the *rbf* gene confirmed that the gene was involved in biofilm formation on polystyrene and glass. Phenotypic analysis of the wild type and the mutant suggested that the *rbf* gene mediates the biofilm formation of *S. aureus* at the multicellular aggregation stage rather than at initial attachment. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis demonstrated that the mutation resulted in the loss of an ~190-kDa protein. Biofilm production by the mutant could be restored by complementation with a 2.5-kb DNA fragment containing the *rbf* gene. The *rbf*-specific mutation affected the induction of biofilm formation by glucose and a high concentration of NaCl but not by ethanol. The mutation did not affect the transcription of the *ica* genes previously shown to be required for biofilm formation. Taken together, our results suggest that the *rbf* gene is involved in the regulation of the multicellular aggregation step of *S. aureus* biofilm formation in response to glucose and salt and that this regulation may be mediated through the 190-kDa protein.

Staphylococci are the most common bacterial pathogens causing foreign-body infections. The ability to form biofilm on biomaterials, which protects the bacteria from the host, is the major contributing factor to such infections. Although not fully understood, the mechanism of biofilm formation appears to involve the attachment of bacteria to solid surfaces followed by the accumulation of multilayered cell clusters surrounded by a slimy matrix (4, 9).

For *Staphylococcus epidermidis*, AtlE has been shown to be involved in the primary attachment required for biofilm formation on hydrophobic polystyrene but not on a hydrophilic glass surface (12), suggesting that the mechanisms of binding of staphylococci to surfaces with different hydrophobicities are different. AtlE is a homologue of the Atl autolysin of *S. aureus* which is required for cell separation after division (30). Two related proteins (SSP1 and SSP2), which may form a fimbria-like structure on the surface of *S. epidermidis*, also have been reported to be involved in attachment to polystyrene (40).

After initial attachment, the accumulation step in biofilm formation depends on the production of polysaccharide intracellular adhesin (PIA), whose synthesis requires genes located in the *icaADBC* operon found in both *S. aureus* and *S. epidermidis* (5, 11, 27). PIA is composed of two closely related polysaccharides with a core polymer of *N*-acetylglucosamine (reviewed in reference 9). The presence of PIA has been correlated with pathogenesis, and mice vaccinated with PIA are protected from subsequent *S. aureus* challenge (28). However, PIA alone is not sufficient for biofilm formation; an additional factor independent of *icaADBC* gene expression in *S. epidermidis* also is required (7). It is likely that this additional factor is the 140-kDa protein, described earlier as the accumulation-association protein, which enhances biofilm formation in *S. epidermidis* (14).

In addition to the factors involved in a distinct step in biofilm formation, a surface protein, Bap, was found to be involved in both primary adhesion and intercellular adhesion. Bap was identified by Tn*917* insertion in an *S. aureus* mastitis isolate that resulted in reduced polystyrene binding. However, Bap could be detected only in some mastitis isolates and not in human clinical isolates (6).

Several environmental factors, such as glucose, osmolarity, ethanol, temperature, and anaerobiosis, have been reported to affect biofilm formation (reviewed in reference 9). Ethanol was shown to induce biofilm formation through *icaR*, which represses the *icaADBC* genes (3). IcaR recently was shown to bind to the promoter region upstream of the *icaA* gene (15). In contrast, the effects of glucose and NaCl are not mediated through *icaR* (3, 7). Environmental stresses are also likely to be mediated through the alternative transcriptional sigma factor σ^B. In fact, several lines of evidence have implied that σ^B modulates biofilm formation (14, 20, 33). However, it is not known how biofilm production is regulated by the alternative sigma factor. The staphylococcal virulence global regulatory gene *agr* has been shown to repress biofilm formation in *S. aureus* (33, 41). How protein factors or polysaccharides involved in biofilm formation are regulated by *agr* is not known.

Thus, the genetic basis of biofilm formation in staphylococci

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TABLE 1. *S. aureus* strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
8325-4	Prophage-free laboratory strain	J. Iandolo
RN4220	8325-4r$^-$ (restriction deficient)	19
CYL193	8325-4 *spa-515*::*ebr*::*kan*	This study
CYL385	CYL193 *rbf*::Tn*917*	This study
CYL1097	8325-4 *rbf*::*tet*	This study
Plasmids		
pGEM-T	Cloning vector	Promega
pLI50	*E. coli-S. aureus* shuttle cloning vector	23
pSL24	XylE fusion vector	36
pTV1	Tn*917* delivery vector	42
pCL53	Cloning vector	This study
pYL8564	724-bp internal fragment of *rbf* in pCL53	This study
pYL8565	3.3-kb fragment containing *rbf* in pLI50	This study

is multifactorial, and much remains to be explored. In this study, we identified and cloned a novel gene, *rbf*, from *S. aureus* and demonstrated that this gene is required for biofilm formation on both polystyrene and glass. Our data suggest that Rbf, which possesses a consensus region of the AraC/XylS family of transcriptional regulators, is a regulator that modulates biofilm formation in response to glucose and salt.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. *Escherichia coli* strain XL1-Blue was used as a host strain for plasmid constructions. The *S. aureus* strains and plasmids used in this study are listed in Table 1. *S. aureus* 8325-4 was used as the parental strain for insertional duplication inactivation of the *rbf* gene. *S. aureus* RN4220 (19) was used as the recipient for electroporation of the constructed plasmids. *S. aureus* strains were cultivated in tryptic soy broth (TSB) or tryptic soy agar (TSA) (Difco Laboratories, Detroit, Mich). *E. coli* strains were cultivated in Luria-Bertani medium (Difco). Electroporation in *S. aureus* was carried out by the procedure of Kraemer and Iandolo (18). Transduction was carried out as described by Shafer and Iandolo (37) with bacteriophage 52A. Plasmid pCL53 was derived from pCL52.2 (36) by *Cla*I digestion to remove the origin of replication of pE194ts. *S. aureus* strain CYL193 was constructed by transducing *spa-515*::*ebr*::*kan* from DU5819 (31) to 8325-4. Concentrations for antibiotic selection were as follows: chloramphenicol, 10 μg/ml; erythromycin (ERY), 10 μg/ml; and tetracycline, 3 μg/ml.

DNA manipulations. Standard DNA manipulations were performed as described by Sambrook et al. (35). Plasmid DNA was purified with a plasmid purification kit (Qiagen, Inc., Chatsworth, Calif.). Rapid small-scale plasmid DNA was purified from *E. coli* by the method of Holmes and Quigley (13). Chromosomal DNA was purified with a kit from Promega (Madison, Wis.). PCR amplification was carried out with an Advantage HF2 PCR kit (Clontech, Palo Alto, Calif.). DNA was transferred to nitrocellulose membranes by the method of Southern (38).

Transposon mutagenesis. *S. aureus* strain CYL193 harboring pTV1 (42) was grown in TSB containing chloramphenicol and ERY at 30°C overnight. The bacterial culture was diluted 1,000-fold in 3 ml of TSB containing ERY, incubated at 42°C for 42 h, and then plated on TSA with ERY. About 20,000 colonies were blotted to nitrocellulose paper and washed with phosphate-buffered saline (PBS). The membranes were incubated in 10 mM Tris-Cl–150 mM NaCl (pH 7.5) (TS) containing 2% goat serum and 1% nonfat dried milk for 1 h at room temperature. Incubation was continued with rabbit antiserum raised against a 200-kDa protein present in biofilm-positive *S. epidermidis* strain RP62A but absent from biofilm-negative strain SP2 (a generous gift from A. Fattom) for an additional 1 h. The membranes were washed with 2 to 4 volumes of TS and then incubated with horseradish peroxidase-conjugated goat anti-rabbit antiserum (Sigma) in TS with nonfat dried milk for 1 h. After being washed with TS, the membranes were developed by using a horseradish peroxidase color development kit (Bio-Rad Laboratories).

Cloning of the Tn*917* flanking sequence by inverse PCR. The chromosomal DNA containing theTn*917* insertion was digested with *Sau*3A and ligated with T4 DNA ligase. The Tn*917* flanking DNA was cloned by inverse PCR (29) as described before (25) and sequenced.

Insertional duplication inactivation of the *rbf* gene. A 724-bp internal fragment of the *rbf* gene from *S. aureus* strain 8325-4 was amplified by PCR with two primers, 5′-TGGTGATTTGCGAGATGAGCV-3′ and 5′-CACTCATAAAAG CTTCTTC-3′, and cloned into pCL53. The resulting plasmid, pYL8564, was transformed into *S. aureus* strain RN4220 with tetracycline selection by electroporation. The insertion was transferred into strain 8325-4 and a clinical isolate by phage 52A transduction and verified by PCR (data not shown).

SDS-PAGE. One-milliliter samples of overnight bacterial cultures grown in TSB at 37°C were harvested and resuspended in 50 μl of 10 mM Tris–0.1 mM EDTA (pH 7.8) containing 400 μg of lysostaphin ml^{-1}. After cell lysis at 37°C for 15 min, 1 μl of DNase I (75.4 U/μl; Gibco BRL) was added, and incubation was continued at 37°C for 15 min. After centrifugation, crude cell lysates were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) analysis by the method of Laemmli (22) and visualized by Coomassie blue staining.

Primary attachment assay on polystyrene. Overnight cultures were diluted in TSB supplemented with 0.25% glucose, and about 300 CFU was added to polystyrene petri dishes (Fisherbrand). After incubation at 37°C for 30 min, the petri dishes were rinsed gently with 5 ml of sterile PBS (pH 7.5) three times and covered with 15 ml of molten 0.8% TSA maintained at 48°C. Primary attachment was expressed as a percentage of CFU remaining on the petri dishes after washing. Each experiment was repeated three times.

Biofilm assay on polystyrene and glass. The biofilm assay on polystyrene was carried out essentially as described by Heilman et al. (10). In brief, overnight cultures were adjusted to an optical density at 660 nm of 3.0 and diluted 250-fold in TSB–0.25% glucose. A 200-μl sample of the cell suspension was inoculated into each well of flat-bottom 96-well polystyrene plates (Corning, Inc., Costar, N.Y.) and incubated at 37°C overnight. After incubation, the wells were rinsed gently with 200 μl of PBS two times, air dried, and stained with 0.1% safranin for 30s. The absorbance at 490 nm was measured with an enzyme-linked immunosorbent assay reader. For assay of the effect of sodium chloride, TSB–0.25% glucose containing sodium chloride at concentrations ranging from 0.1 to 5.6% was used. For assay of the effect of glucose, TSB supplemented with glucose at concentrations ranging from 0 to 2.7% was used.

To carry out the biofilm assay on glass, a 30-μl sample of the cell suspension diluted as described above for the polystyrene assay was placed on sterile glass slides and incubated in a petri dish on a wet paper towel at 30°C overnight. The slides were rinsed gently with distilled water, air dried, and stained with 0.1% safranin for 30s.

Construction of transcriptional fusion plasmids. Transcriptional fusion plasmids were constructed by ligating the PCR-amplified DNA fragments containing the promoter region of *icaR* or *icaA* to the promoterless *xylE* reporter gene in pSL24. The PCR-amplified fragments were verified by sequencing. Each fusion plasmid was electroporated into strain RN4220 and then transduced to strain 8325-4 and its isogenic *rbf* mutant with phage 52A. The catechol 2,3-dioxygenase (the gene product of *xylE*) activities of the strains harboring the fusion plasmids were assayed spectrophotometically as described by Zukowski et al. (43).

Distribution of the *rbf* gene in clinical isolates. Chromosomal DNAs from 27 *S. aureus* isolates (3 methicillin-resistant isolates, 10 clinical isolates from cystic fibrosis patients, and 14 blood isolates) were purified and amplified by PCR with two primers, 5′-GAATTCTAGAAAGAGGTAAAGTTATGGC-3′ and 5′-CAC TCATAAAAGCTTCTTC-3′, within the coding region of the *rbf* gene. The primers amplified a 1.2-kb fragment from strain 8325-4.

RESULTS

Screening of Tn*917*-induced mutants. To identify potential biofilm-negative mutants, we introduced plasmid pTV1 carrying transposon Tn*917* into strain CYL193. Transposon mutagenesis was carried out, and about 20,000 colonies were screened by immunoblotting as described in Materials and Methods. Since strain CYL193 is a protein A-deficient strain, the background due to protein A binding to antibody was avoided. Three putative mutants with much reduced reactivity to the antiserum were identified. Southern hybridization showed that all three mutants had Tn*917* inserted at the same single site (data not shown). The three mutants were backcrossed by phage transduction to the parental strain, and the

resultant transductants showed a phenotype similar to that of the original mutants, indicating that the biofilm-negative phenotype in each mutant is due to the Tn*917* insertion and not to potential secondary mutations during mutagenesis.

Cloning of the *rbf* gene. The Tn*917* insertion site and the flanking chromosomal DNA in one of the mutants were amplified by inverse PCR followed by DNA sequencing. A BLAST search of the sequenced DNA fragment against the genomes of strains 8325 (University of Oklahoma Health Sciences Center) and N315 (21) showed that the insertion was located within a 2,148-bp open reading frame (ORF) which could encode a putative protein of 716 amino acids. We found that the encoded protein contained a region of sequence significantly homologous to the 99-amino-acid consensus sequence of the AraC/XylS family of transcriptional regulators (8), suggesting that the gene that we identified most likely is a regulatory gene (Fig. 1). Accordingly, we named this ORF *rbf*, for "regulator of biofilm formation"; it corresponds to ORF SA0622 of strain N3165 (21). These data suggest that *rbf* is highly conserved among staphylococci. To clone the *rbf* gene, we amplified a 2.5-kb fragment containing the coding region and its potential upstream regulatory region from the chromosome of strain 8325-4. The amplified fragment was cloned into vector pGEM-T and recloned into shuttle vector pLI50 to yield pYL8565. The fragment was confirmed by DNA sequencing.

The *rbf* gene is required for multicellular aggregation of biofilm formation. To confirm that the *rbf* gene was required for biofilm production, we constructed an insertional duplication mutant, CYL1097, as described in Materials and Methods. The wild-type and mutant strains were tested for binding to polystyrene and glass. As shown in Fig. 2, the *rbf* mutation resulted in a drastic reduction in biofilm formation on both polystyrene and glass which could be complemented by pYL8565 containing the wild-type *rbf* gene. These results indicate that the *rbf* gene is indeed involved in biofilm formation. Of note was the larger amount of biofilm formed by the complemented strain than by the wild-type strain, a finding which was most likely due to a gene dosage effect, since complementation was performed with a multicopy plasmid vector. To determine at which step *rbf* affects biofilm formation on polystyrene, a primary attachment experiment was performed with petri dishes as described in Materials and Methods. We found no significant difference between the wild type and CYL1097 (data not shown), suggesting that the *rbf* gene affects multicellular aggregation rather than primary attachment.

As indicated above, *rbf* likely is a regulatory gene. To determine what gene product(s) may be regulated by *rbf*, we performed SDS-PAGE analysis of the *rbf* mutant and the wild-type strain. We found that there was an apparent loss of a ca. 190-kDa protein in the mutant (Fig. 3) which could be complemented by pYL8565. Since the band is much larger than the predicted Rbf protein (~80 kDa), it is likely that the missing band in CYL1097 represents a protein regulated by the *rbf* gene.

Involvement of *rbf* in biofilm induction by glucose. Glucose has been shown to induce the multicellular aggregation step of biofilm formation (26). To investigate whether biofilm induction by glucose is mediated through the *rbf* gene, biofilm assays were performed with the wild type, the isogenic *rbf* mutant, and the *rbf*-complemented strain in the presence of various

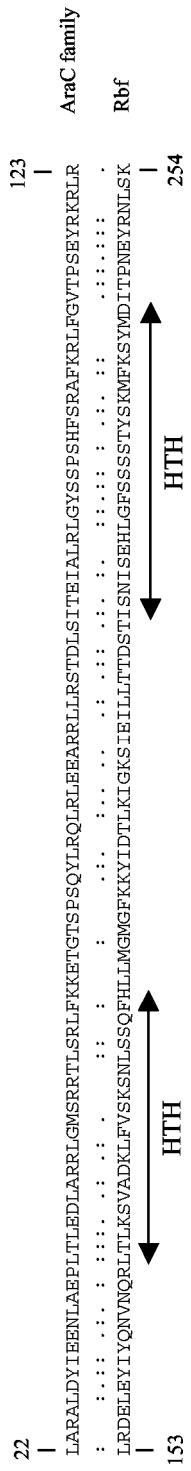

FIG. 1. Comparison of Rbf sequence and AraC family consensus sequence. The helix-turn-helix (HTH) motifs are indicated. Colons and periods indicate identical and similar residues, respectively.

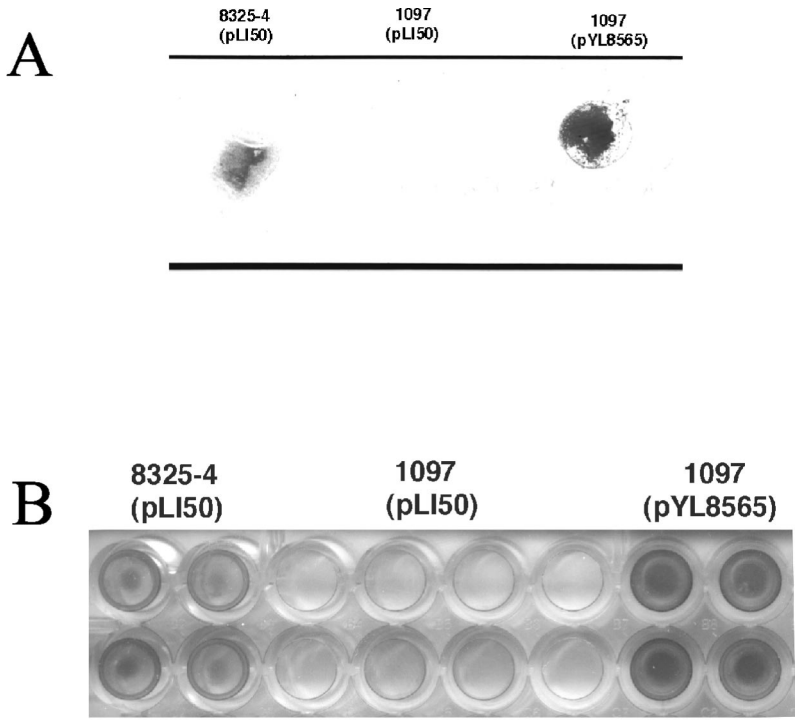

FIG. 2. Biofilm formation of NCTC8325-4, its isogenic *rbf* mutant, and a complemented strain on a glass side (A) and a polystyrene microtiter plate (B).

amounts of glucose. As shown in Fig. 4, the mutation in *rbf* resulted in a loss of glucose-induced biofilm formation at all glucose concentrations tested, indicating that the *rbf* gene mediates biofilm formation on polystyrene in response to glucose. The effect of the *rbf* mutation could be complemented by a plasmid containing the *rbf* gene. Again, the complemented strain produced more biofilm than the wild-type strain.

Involvement of *rbf* in biofilm induction by sodium chloride. High osmolarity, such as a high salt concentration, has been shown to stimulate biofilm formation in *S. aureus* (33). To test whether the *rbf* gene is involved in transduction of the osmolarity signal for biofilm formation, we assayed biofilm formation by the wild-type and mutant strains in the presence of different concentrations of sodium chloride. Figure 5 demonstrates that biofilm production by the wild-type strain increased as the concentration of NaCl increased from 0 to 1.6%. The maximal amount of biofilm was produced at 1.6 to 4.8%, and the amount remained constant until 5.6%, a concentration at which no biofilm was produced. Biofilm production by the *rbf* mutant increased as the concentration of NaCl increased from 0 to 1.6% but decreased to an almost undetectable level as the NaCl concentration reached 2.4% and above. Although the amount of biofilm produced by the mutant was smaller than that produced by the wild type at 0 to 1.6% NaCl, the rate of increase was similar to that of the wild type (the slopes were not significantly different, as revealed by the Student *t* test [$P = 0.0682$]). These results indicate that *rbf* is involved in the signal transduction pathway of NaCl induction for biofilm production when the NaCl concentration is above 1.6% but not when it is below 1.6%. Biofilm production by CYL1097 could be restored

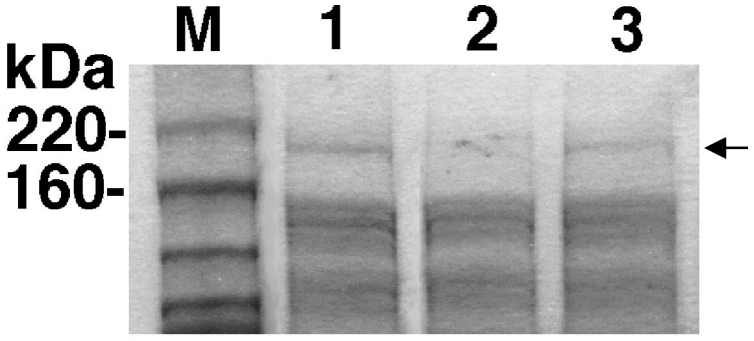

FIG. 3. SDS-PAGE of whole-cell extracts from 8325-4(pLI50) (lane 1), *rbf* mutant CYL1097(pLI50) (lane 2), and CYL1097(pYL8565) (lane 3). Samples were run on a 10% gel. The arrow indicates the 190-kDa protein. Lane M, standards.

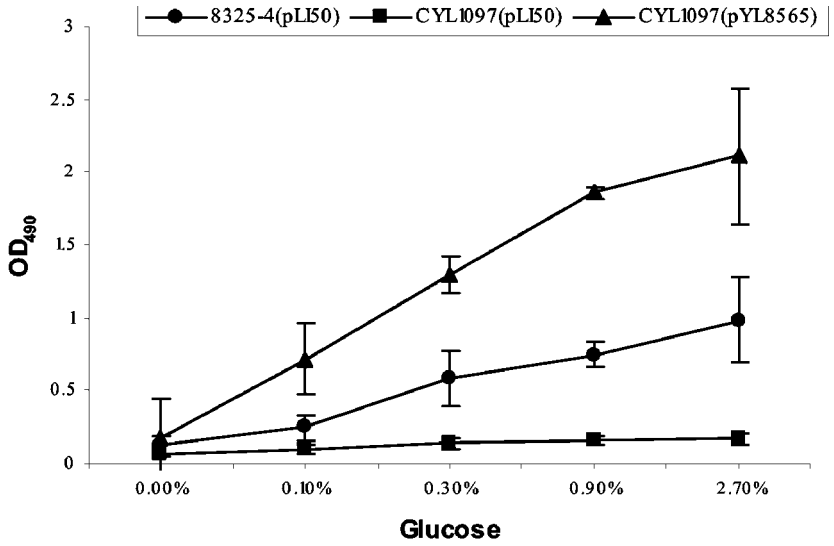

FIG. 4. Biofilm formation in response to glucose. The bacterial strains were tested for the effect of glucose on biofilm formation as described in Materials and Methods. Results represent the averages of at least three independent experiments. Error bars indicate the standard error of the mean. OD_{490}, optical density at 490 nm.

by complementation with the multicopy wild-type *rbf* gene but surprisingly not to the level seen with the wild-type strain. In fact, for the complemented strain, biofilm production remained relatively steady at all NaCl concentrations until 5.6%. The complementation results showed that the overproduction of Rbf impedes rather than enhances biofilm formation.

Induction of biofilm by ethanol is not mediated through *rbf*. Ethanol has been reported to induce biofilm production in *S. epidermidis* (17). Recently, *icaR* was shown to repress *ica* operon transcription, which could be induced by ethanol (3). To determine whether *rbf* also is involved in ethanol induction, we measured biofilm formation by the wild type and the iso-

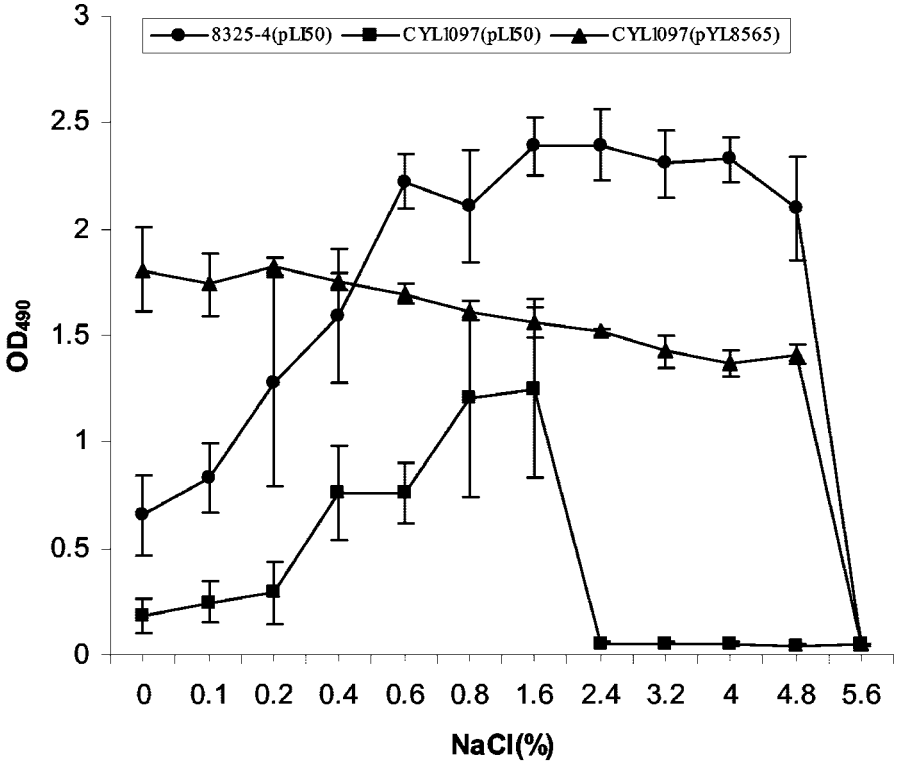

FIG. 5. Biofilm formation in response to NaCl. The bacterial strains were tested for the effect of NaCl on biofilm formation as described in Materials and Methods. Results represent the averages of at least three independent experiments. Error bars indicate the standard error of the mean. OD_{490}, optical density at 490 nm.

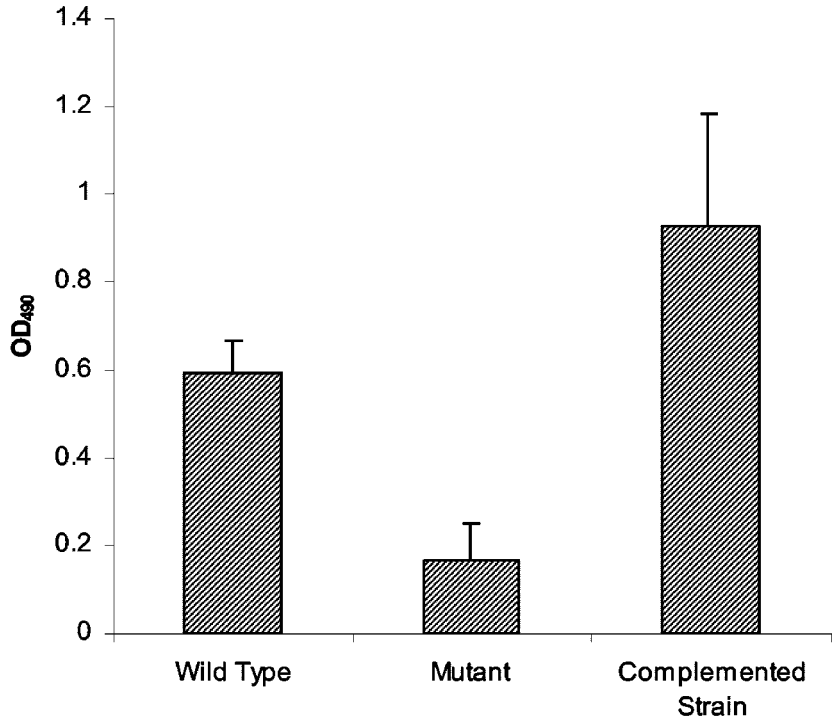

FIG. 6. Effect of the *rbf* mutation on biofilm formation of a clinical isolate. The wild-type strain, its *rbf* mutant, and a complemented strain were tested for polystyrene binding in wells of a microtiter plate. The results represent the averages of three independent experiments. Error bars indicate the standard error of the mean. OD_{490}, optical density at 490 nm.

genic *rbf* mutant in the presence of ethanol up to 4%. The results (not shown) showed that there was no difference among the wild-type, mutant, and complemented strains, indicating that *rbf* is not involved in ethanol induction of biofilm production.

Expression of the *ica* operon does not depend on *rbf*. To examine whether *rbf* regulates biofilm formation through the activation of *ica* operon expression, we transcriptionally fused the *xylE* reporter gene either to the promoter of the *icaADBC* operon or to the promoter of *icaR*. The fusion plasmids were transferred into strain 8325-4 and the isogenic *rbf* mutant CYL1097. Assays of XylE activities showed no difference between the wild type and the mutant, suggesting that the *rbf* gene does not affect *ica* gene expression (data not shown).

Prevalence of *rbf* in clinical isolates. To determine how widespread the *rbf* gene is among the *S. aureus* population, PCR experiments with primers within the coding region of the *rbf* gene were performed with 27 clinical isolates. All isolates showed the expected 1.2-kb band, indicating that the *rbf* gene is widespread among strains of *S. aureus*. However, testing of biofilm formation by these strains showed that 22 of the 27 strains produced a significant amount of biofilm (at least twofold more than the background) on polystyrene plates (data not shown). To show that *rbf* also regulates biofilm formation in clinical isolates, we constructed an *rbf* mutant by transducing the mutation from strain CYL1097 to one of the biofilm-positive clinical isolates. A biofilm assay showed that *rbf* was required for biofilm formation by this clinical isolate. The biofilm-negative phenotype of the mutant was complemented by pYL8565 (Fig. 6). These results suggest that the *rbf* gene is widespread and is involved in biofilm formation by clinical isolates.

DISCUSSION

The process of biofilm formation in *S. aureus* and *S. epidermidis* is understood only partially. To study biofilm formation, we attempted to identify genes involved in this process. In this study, we screened a library of Tn*917* insertion mutants by using an antiserum raised against a ca. 200-kDa protein found in biofilm-positive *S. epidermidis* strain RP62A but not in biofilm-negative strain SP2. Using this method, we identified and cloned a gene that is involved in biofilm formation by *S. aureus*. However, the cloned gene could encode a protein of 716 amino acids with a molecular mass much smaller than the 200 kDa that we expected. Furthermore, the gene product does not possess characteristics of surface or secreted proteins but contains a DNA-binding motif homologous to that of the AraC/XylS family of transcriptional regulators. Thus, it is unlikely that we have obtained the gene that we initially intended to clone. Based on the predicted primary protein sequence and the data presented here, we suggest that the gene is involved in the positive regulation of a surface protein or a secreted protein that reacted to the antiserum used for our initial screening.

The *rbf* gene is present in the genomes of all seven *S. aureus* strains whose sequencing information is available in the public domain. In this study, we also showed that the *rbf* gene was found in all 27 clinical isolates tested. These results imply that *rbf* is widespread among *S. aureus* strains. Furthermore, an *rbf*

homologue also was present in the two sequenced *S. epidermidis* strains. These results suggest therefore that *rbf* is also widespread among other staphylococci The amino acid sequences of the translated Rbf proteins from all seven sequenced strains are almost identical, suggesting that *rbf* is functionally important. Indeed, our results showed that the insertional duplication mutation allele of *rbf* could be transduced to a clinical isolate, resulting in a similar phenotype with respect to biofilm formation (Fig. 6).

Ica proteins are responsible for the biosynthesis of PIA. Although PIA is required for biofilm formation, an additional factor(s) also is required (7). In this study, we showed that *rbf* did not affect the transcription of *ica* gene expression but affected a protein of about 190 kDa. This protein is very similar in size to the protein against which the antiserum was raised and was subsequently used to clone the *rbf* gene. Thus, our results suggest that *rbf* most likely regulates the 190-kDa protein, which may be involved directly in biofilm formation. These results are consistent with the hypothesis that biofilm formation requires at least two factors. Further studies are needed to test whether the *rbf*-regulated protein that we identified is related to proteins previously identified, such as the accumulation-association protein, and whether *rbf* regulates additional proteins. How a protein factor contributes to biofilm formation and how it is regulated have not been studied at the molecular level in staphylococci. Identification of *rbf* and its regulated target genes therefore would provide invaluable information for understanding the underlying mechanism for these processes.

Several environmental factors have been shown to affect the production of biofilm in *S. aureus* and *S. epidermidis* (9). Here we showed that *rbf* regulated biofilm formation in response to glucose and to certain concentrations of NaCl but not to ethanol. In contrast, it has been shown that *icaR* regulation of the *icaADBC* operon is affected by ethanol but not by NaCl or glucose (3). Thus, the *rbf* regulatory pathway controlling biofilm formation responds to environmental stimuli different from those to which the *ica* pathway responds. These results are consistent with the fact that *rbf* affected protein production but not *ica* gene expression, implying that the *rbf* pathway is independent from the *ica* pathway.

It is interesting that the effect of NaCl on biofilm formation is multiphasic (Fig. 5). First, at concentrations below 1.6%, biofilm formation increased proportionally to the concentration of NaCl in both the wild type and the *rbf* mutant, suggesting that a factor different from *rbf* mediates NaCl signal transduction to biofilm formation. Second, at concentrations of 1.6 to 5.6%, biofilm production reached a plateau in the wild type, but no biofilm was detected in the *rbf* mutant. These data suggest that above a threshold of about 1.6% NaCl, biofilm formation in response to NaCl is controlled through *rbf*. Interestingly, in this phase, a further increase in the NaCl concentration does not contribute to an increase in biofilm formation in the wild type, suggesting that Rbf may interact with its inducer or its target DNA in a complicated manner, perhaps through another factor. This notion is further supported by the fact that complementation with *rbf* in a multicopy plasmid resulted in a decreased total amount of biofilm formation compared to that observed with the wild type. Third, no biofilm was detected above 5.6% NaCl in the wild-type strain or even in the complemented strain. Since *S. aureus* can tolerate much higher concentrations of NaCl, it is likely that a specific mechanism halts biofilm formation once the NaCl concentration reaches 5.6%. One possibility is that a regulatory gene is induced and represses biofilm formation either directly or through *rbf*. Further studies are required to test this possibility.

Besides *icaR*, other regulators have been shown to affect biofilm formation. The global regulators *agr* and *sarA* have been shown to affect biofilm formation (1, 32, 34, 39, 41). This finding is not surprising, since both regulators have been shown to regulate cell surface proteins as well as capsular polysaccharide (24). It would be interesting to determine whether *agr* or *sarA* regulates *rbf*. Previous studies also showed that biofilm formation requires σ^B (16, 33). However, at least two reports showed that strains derived from NCTC8325, which lacks σ^B due to an 11-bp deletion in the *rsbU* gene in the *sigB* operon, were also biofilm positive (2, 5). In the study reported here, we used strain 8325-4, which is a derivative of NCTC8325. We showed that 8325-4 was biofilm positive. To ensure the identity of strain 8325-4 from our culture collection, we PCR amplified the *rsbU* gene. Our sequencing results showed that it indeed contained the 11-bp deletion. Thus, our results suggest that σ^B is not required for biofilm formation in strain 8325-4. Most recently, Valle et al. (39) also showed that σ^B was not essential for biofilm formation in a clinical isolate.

Rbf is a member of the AraC/XylS family. Most of the regulators in this family are average-size proteins of 250 to 300 amino acids (8). However, the Rbf protein is more than twice the length of the average AraC family protein, with 716 amino acids. Since our data indicate that *rbf* is involved in glucose and NaCl induction of biofilm formation, it is possible that the Rbf protein contains functional domains to recognize these signals in addition to domains common to members of this family. Whether the unusual length of Rbf correlates with additional specific functional domains remains to be studied.

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