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Evidence for *icaADBC*-Independent Biofilm Development Mechanism in Methicillin-Resistant *Staphylococcus aureus* Clinical Isolates

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Synthesis of a polysaccharide adhesin by *icaADBC*-encoded enzymes is currently the best-understood mechanism of staphylococcal biofilm development. In four methicillin-resistant *Staphylococcus aureus* isolates, environmental activation of *icaADBC* did not always correlate with increased biofilm production. Moreover, glucose-mediated biofilm development in these isolates was *icaADBC* independent. Apparently, an environmentally regulated, *ica*-independent mechanism(s) of biofilm development exists in *S. aureus* clinical isolates.

Production of a polysaccharide adhesion, termed polysaccharide intercellular adhesion or polymeric *N*-acetylglucosamine, by *ica* operon-encoded enzymes is currently the best-understood mechanism of staphylococcal biofilm development (18, 19). Although the majority of clinical *Staphylococcus aureus* isolates contain the *ica* operon (2, 7, 10, 11), the expression of the *ica* operon and biofilm production are tightly regulated under in vitro conditions (20). In the laboratory, CO_2 levels, anaerobicity, glucose, and osmotic stress can all influence *ica* operon expression and/or biofilm development (1, 8, 14, 22). Cramton et al. (7) demonstrated that deletion of the *ica* operon in *S. aureus* ATCC 35556 resulted in impaired polysaccharide intercellular adhesion/polymeric *N*-acetylglucosamine production and a biofilm-negative phenotype. However, a recent study demonstrated that mutation of the *ica* locus in *S. aureus* strain UAMS-1 had little impact on biofilm formation in vitro or in vivo (5). In addition, Lim et al. (17) recently identified a new gene, *rbf* (regulator of biofilm formation), which was required for biofilm formation in *S. aureus* but did not regulate *ica* operon expression. It is not clear whether these findings apply to clinical as well as reference isolates.

To investigate the contribution of the *ica* locus to biofilm development in *S. aureus* clinical isolates, the environmental regulation of biofilm development by NaCl and glucose was investigated in four *ica*-positive, methicillin-resistant strains (BH1S, BH1P, BH1Y, and BH1CC) recovered from intensive care unit patients (Table 1). Biofilm assays using Nunclon tissue culture-treated (ΔSurface) 96-well polystyrene plates (Nunc, Denmark) were performed as described previously (5, 15). Biofilm formation was increased four- to eightfold in all four of these isolates when grown in brain heart infusion (BHI) medium supplemented with glucose compared to BHI alone (Fig. 1A). In contrast, growth in BHI supplemented with NaCl failed to induce biofilm in these isolates (Fig. 1A).

To examine the contribution of *ica* operon expression to the biofilm phenotype of these isolates, we used reverse transcriptase PCR (RT-PCR) as described previously (4–6, 12). The methicillin-resistant *S. aureus* (MRSA) isolate BH1CC was grown in BHI medium and in BHI medium supplemented with NaCl, glucose, or both NaCl and glucose (Fig. 1B). The addition of NaCl to the growth medium activated *ica* operon expression in BH1CC even though this *ica* activation was not associated with any increase in biofilm-forming capacity (Fig. 1A and B). In addition, the glucose-mediated induction of biofilm formation was not associated with increased *ica* operon transcription in BH1CC. An examination of *ica* operon expression in BH1S, BH1P, and BH1Y also revealed little correlation between *ica* expression levels and the biofilm phenotype (Fig. 1C). Glucose-mediated induction of biofilm formation correlated with substantially increased *ica* operon expression only in isolate BH1S (Fig. 1C). Similar to results with BH1CC, NaCl activated *ica* operon expression in BH1S but was not associated with a biofilm-positive phenotype (Fig. 1A and C). These findings suggest that in contrast to recent findings with *Staphylococcus epidermidis* (4–6, 9, 16, 21), there appears to be little correlation between *ica* operon expression and biofilm formation in *S. aureus* clinical isolates and that the *ica* operon may not be required for biofilm development in the isolates examined.

In order to more carefully assess the contribution of the *ica* operon to biofilm development in *S. aureus*, an *ica* operon deletion mutant of MRSA isolate BH1CC was constructed. As described above, biofilm development by this strain, which was isolated from a patient with central venous catheter infection, is induced approximately fivefold when grown in BHI-glucose, even though *ica* operon expression was activated only in BHI-

TABLE 1. Strains used in this study[a]

Isolate	Source	Clinical infection	Biofilm phenotype
BH1CC	Line tip	CVCI	Glucose only
BH1S	Blood	CVCI	Glucose only
BH1P	Blood	BSI	Glucose only
BH1Y	Line tip	CVCI	Glucose only

[a] CVCI, central venous catheter infection; BSI, bloodstream infection. Biofilm formation was measured after 24 h of growth at 37°C in BHI broth supplemented with 4% NaCl or 1% glucose or both NaCl and glucose.

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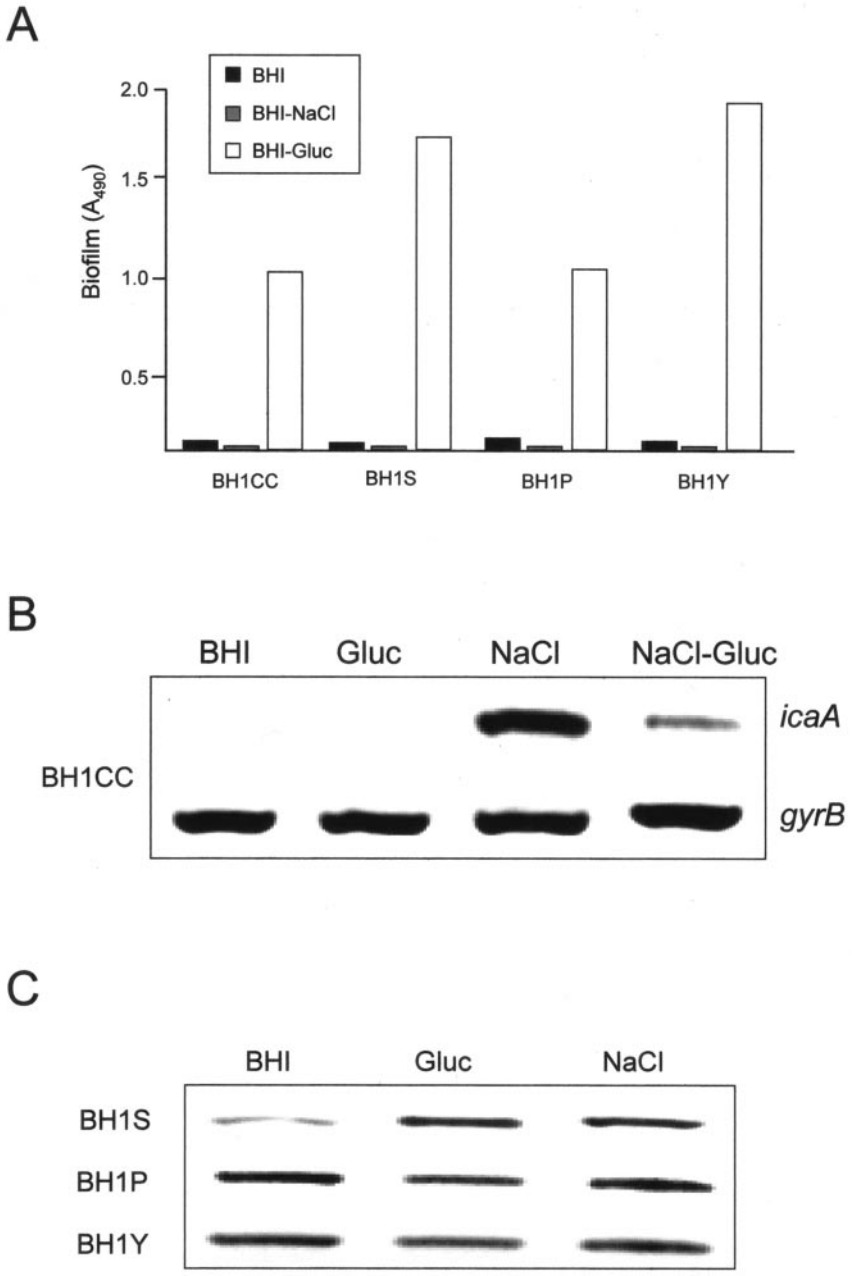

FIG. 1. Comparison of biofilm and *ica* operon expression in *S. aureus* isolates. (A) Biofilm formation in tissue culture-treated 96-well plates by *S. aureus* isolates BH1CC, BH1S, BH1P, BH1Y in BHI medium or in BHI medium supplemented with 4% NaCl or 1% glucose. Biofilm values are the optical densities at 492 nm (OD_{492}) after staining with crystal violet and are the means of at least three independent assays. Standard deviations were less that 25% (data not shown). (B) Comparative measurement of *icaA* and *gyrB* (control) transcription in the *S. aureus* isolate BH1CC. RT-PCR analysis was performed on RNA prepared from cultures grown at 37°C to an OD_{600} of 4.0 in BHI medium or in BHI medium supplemented with 4% NaCl, 1% glucose, or 4% NaCl and 1% glucose. (C) Comparative measurement of *icaA* transcription in the *S. aureus* isolates BH1S, BH1P, and BH1Y. RT-PCR analysis was performed on RNA prepared from cultures grown at 37°C to an OD_{600} of 4.0 in BHI medium or in BHI medium supplemented with 4% NaCl or 1% glucose. Measurement of *gyrB* (control) transcription in BH1S, BH1P, BH1Y was performed to confirm equal loading of RNA (data not shown). Gluc, glucose.

NaCl and not in BHI-glucose. An allele replacement strategy was employed to replace the *ica* operon on the chromosome of BH1CC with the tetracycline resistance cassette (Δ*ica*::*tet*) as described previously (7). PCR with the primers SAdel1 (5′-TGC-AAA-TGC-CCT-TGA-TGT-AA-3′) and SALR2 (5′-GGC-GGA-AAG-TCA-GGT-TAC-AA-3′), which amplify the entire *icaR-icaADBC* locus, was used to confirm the presence of the Δ*ica*::*tet* allele on the chromosome of candidate mutants (data not shown). Interestingly, an analysis of BH1CC and an isogenic Δ*ica*::*tet* mutant grown in BHI, BHI-NaCl, and BHI-glucose revealed no substantial differences in biofilm-forming capacity (Fig. 2A). These data are consistent with our earlier

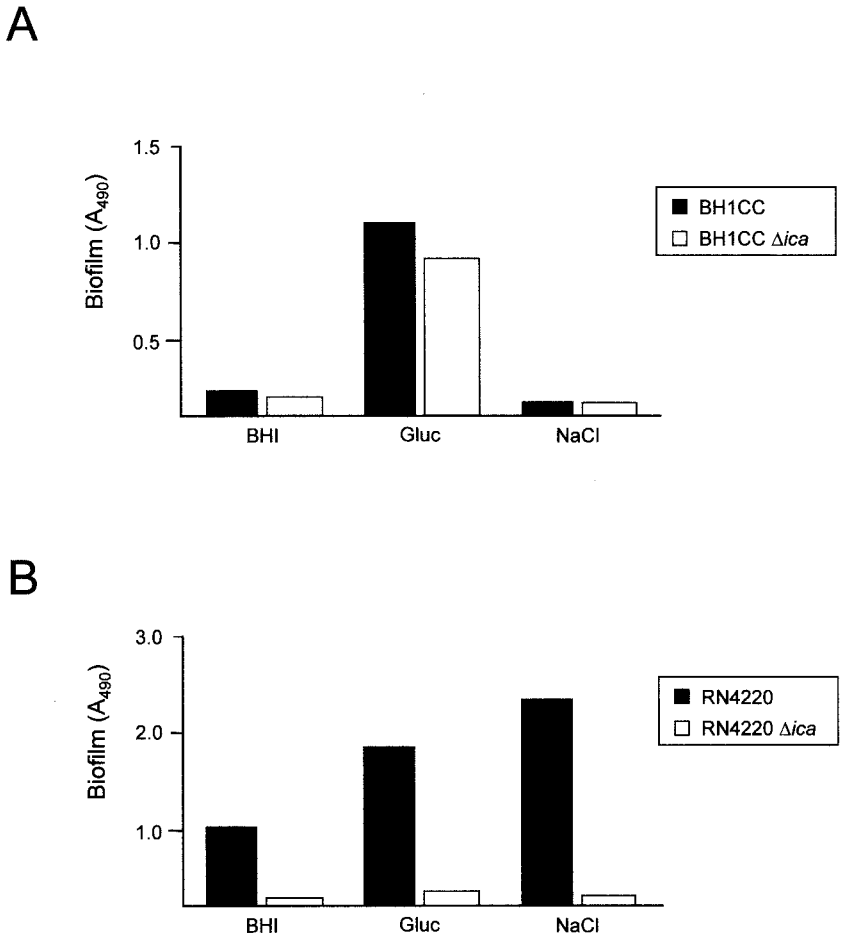

FIG. 2. Comparison of biofilm environmental regulation in *S. aureus* strains BH1CC, BH1CC Δ*ica*::*tet*, RN4220, and RN4220 Δ*ica*::*tet*. (A) Biofilm formation by *S. aureus* BH1CC and BH1CC Δ*ica*::*tet* in BHI medium or in BHI medium supplemented with 4% NaCl or 1% glucose. (B) Biofilm formation by *S. aureus* RN4220 and RN4220 Δ*ica*::*tet* in BHI medium or in BHI medium supplemented with 4% NaCl or 1% glucose. Biofilm values are OD_{492} readings after staining with crystal violet and are the means of at least six independent assays. Standard deviations were less that 25% (data not shown). Gluc, glucose.

findings that *ica* operon expression and regulation do not influence biofilm development in BH1CC and further suggest that the mechanism of glucose-induced biofilm formation in this strain is entirely *ica* independent. Interestingly, Beenken et al. (3) recently reported that in the *S. aureus* clinical isolate UAMS-1, the deletion of the *ica* locus also had no effect on biofilm development in tryptic soy broth media supplemented with both NaCl and glucose. However, it may be significant that the *ica* operon expression by the MRSA isolate BH1CC examined in the present study was induced by glucose alone but inhibited by both NaCl and glucose together (Fig. 1B), perhaps suggesting that one or more *ica*-independent mechanism of biofilm development may exist.

Our evidence that the *ica* operon was not required for biofilm development in BH1CC was inconsistent with the findings of Cramton et al. (7), which revealed that the *ica* locus was required for biofilm development in *S. aureus* ATCC 35556. To further investigate the contribution of the *ica* locus to biofilm development in different *S. aureus* strains, we constructed Δ*ica*::*tet* deletion mutants in the NCTC 8325-derived laboratory strains 8325-4, SH1000, and RN4220. In contrast to results with BH1CC, we have previously observed that biofilm formation in 8325-4, SH1000, and RN4220 was strongly induced by NaCl (15). By using allele replacement, the Δ*ica*::*tet* deletion mutation was constructed in the laboratory strain RN4220, a chemically mutagenized derivative of 8325-4 that can readily accept foreign DNA. Phage 80α was then used to transduce the Δ*ica*::*tet* allele from RN4220 to 8325-4 and SH1000, an *rsbU*-repaired (σ^B-positive) derivative of 8325-4 (13). In contrast to results with *S. aureus* BH1CC, deletion of the *ica* locus in RN4220 (Fig. 2B) resulted in a biofilm-negative phenotype under all environmental conditions. A similar phenotype was associated with *ica* deletion mutations in 8325-4 and SH1000 (data not shown).

The Δ*ica*::*tet* allele was also transduced into the MRSA isolates BH1S, BH1P, and BH1Y, which, like BH1CC, display glucose-induced biofilm formation. Interestingly, similar to our findings with *S. aureus* BH1CC, deletion of the *ica* locus in these isolates did not result in a biofilm-negative phenotype and all three of these isolates displayed a glucose-induced, *ica*-independent biofilm phenotype (data not shown).

It is also relevant to note that all three 8325-4 derivative

strains used in this study produced black, dry, crusty colonies when grown on Congo red agar (CRA), which is indicative of a biofilm-positive phenotype in *S. epidermidis* (6, 12). In contrast, the four clinical isolates produced red, smooth colonies on CRA. Deletion of the *ica* operon in the 8325-4 derivatives resulted in a red, smooth phenotype on CRA and, predictably, had no effect on the CRA phenotype in the clinical isolates. These findings may suggest that the *ica* operon is required for NaCl-induced biofilm in *S. aureus* strains that produce black, crusty colonies on CRA but not in isolates that form red, smooth colonies on CRA. This finding may be significant given that in a survey of 31 *ica*-positive *S. aureus* clinical isolates (F. Fitzpatrick, H. Humphreys, and J. P. O'Gara, unpublished findings), all produced red smooth colonies on CRA, suggesting that the *ica* operon may not play an important role in biofilm development among clinical isolates with this phenotype.

These studies suggest that the regulatory pathways controlling the biofilm phenotype in reference strains may be different than those used by clinical isolates. Such differences may, in turn, be driven by genetic exchange and exposure to environmental stimuli in the complex in vivo infection milieu from which the clinical isolates are recovered. Further studies are under way to elucidate an alternative, *ica*-independent mechanism(s) of biofilm development in clinical isolates of methicillin-resistant *S. aureus*.

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