

## Isolation and Characterization of Biofilm Formation-Defective Mutants of *Staphylococcus aureus*<sup>∇</sup>

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***Staphylococcus aureus* produces biofilm and this mode of colonization facilitates infections that are often difficult to treat and engender high morbidity and mortality. We have exploited bacteriophage Mu transposition methods to create an insertional mutant library in a highly biofilm-forming *S. aureus* clinical isolate. Our screen identified 38 insertions in 23 distinct genes together with one intergenic region that significantly reduced biofilm formation. Nineteen insertions were mapped in loci not previously known to affect biofilm in this organism. These include insertions in *codY*, *srrA*, *mgrA*, and *fmtA*, a putative DEAD-box helicase, two members of the zinc-metallo- $\beta$  lactamase/ $\beta$ -CASP family, and a hypothetical protein with a GGDEF motif. Fifteen insertions occurred in the *icaADBC* operon, which produces intercellular adhesion antigen (PIA) and is important for biofilm formation in many strains of *S. aureus* and *Staphylococcus epidermidis*. Obtaining a high proportion of independent Em-Mu disruptions in *icaADBC* demonstrated both the importance of PIA for biofilm formation in this clinical strain and the strong validation of the screening procedure that concomitantly uncovered additional mutants. All non-*ica* mutants were further analyzed by immunoblotting and biochemical fractionation for perturbation of PIA and wall teichoic acid. PIA levels were diminished in the majority of non-*ica* insertional mutants. Three mutant strains were chosen and were functionally complemented for restored biofilm formation by transformation with plasmids carrying the cloned wild-type gene under the control of a xylose-inducible promoter. This is a comprehensive collection of biofilm-defective mutants that underscores the multifactorial genetic program underlying the establishment of biofilm in this insidious pathogen.**

The structured lifestyle of bacterial biofilm communities involves a coordinated sequence of events including primary surface attachment, microcolony formation, expansion, and dissemination (21). Microbial biofilms represent an important determinant of human chronic infections (8, 17, 40, 53, 64). Both *Staphylococcus aureus* and *Staphylococcus epidermidis* rank among the clinically most significant gram-positive bacterial pathogens that form biofilm (19, 53). Despite the high incidence of staphylococcal infections and their significant morbidity, an understanding of the underlying mechanisms of biofilm production leading to chronic infection remains limited (19, 53).

The regulation of biofilm formation is poorly understood but is most likely complex and multifactorial. Initial surface attachment of staphylococci to abiotic surfaces is thought to be mediated by multiple factors. Alteration of teichoic acid negative charge, for example, by reducing D-alanyl esterification, affects primary attachment and stable adherence to polystyrene (20). Proteinaceous determinants such as the AtIE autolysin, aggregation-associated protein, and biofilm-associated protein are

also known to modulate surface attachment (11, 24, 28, 37, 41, 60).

Glycocalyx plays a key role in the postattachment phase of biofilm formation by staphylococci (for a review, see references 19; 22, 23, 44, and 45). The glycocalyx, referred to as polysaccharide intercellular adhesion antigen (PIA), is composed of polymeric N-acetylglucosamine, and its biosynthesis is controlled by the *icaADBC* operon (9, 45, 47). The presence of PIA is important for virulence in models of both systemic infection and those promoted by implanted biomaterials (16, 35, 62). It is known that the *icaADBC* operon is repressed by IcaR (7) as well as TcaR, a regulator of the teicoplanin-associated locus (27). Additional global regulators are also involved and include SarA, the staphylococcal accessory regulator (68), and perhaps, the alternative stress sigma factor  $\sigma^B$  that is important for biofilm regulation in *S. epidermidis* but is less important in *S. aureus* (32, 70).

Though prevalent in many laboratory and clinical strains of staphylococci, PIA production is not always correlated with biofilm formation, and indeed, in some model systems, the *ica* locus can be deleted without significantly impacting biofilm formation in vitro or in vivo (2). Several studies have reported biofilm formation in the absence of *ica*, and these have led to proposals that there are both *ica*-dependent and *ica*-independent pathways governing biofilm formation (15, 60, 63, 67).

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Global regulators, including two-component sensors, have been reported to regulate biofilm formation in staphylococci. For example, the accessory gene regulator (*agr*) locus controls production of numerous virulence factors in response to quorum-sensing signals captured by the AgrAC two-component sensory system (49, 72). The effector molecule of the *agr* locus is a regulatory RNA, called RNAPIII. In addition to its effects on virulence factor gene expression, the primary RNAPIII transcript also encodes hemolysin  $\delta$ , whose surfactant properties are thought to modulate biofilm formation (49, 72). A recent study with *S. aureus* also revealed that disruption of the *arlRS* two-component signaling system strongly promoted biofilm by enhancing surface attachment and production of PIA (67). Likewise, inactivation of an alternate quorum-sensing system mediated by LuxS showed increased biofilm formation in vitro and resulted in enhanced virulence in vivo in a rat model of biofilm-mediated infection (77).

Comparison of bacteria in planktonic or biofilm communities by transcriptional profiling has revealed significant alteration in gene expression which suggests that substantial metabolic changes occur between the two conditions (2, 57, 78). In particular, evidence from these studies suggests selective adaptation of biofilms in response to reduced pH and anaerobic growth (2). Microarray studies of biofilm formation also suggest that *ica* transcription peaks around 6 h in biofilm compared to planktonic cultures and then declines thereafter (57). This observation is also reflected in reported post-exponential phase repression of PIA (14). A recent proteomic study which compared differences in expression between biofilm and planktonic cells also revealed extensive changes in protein expression profiles and, in particular, pointed to significant increases in proteins in biofilms associated with cell attachment, peptidoglycan biosynthesis, and formate and pyruvate metabolism (58).

Biofilms protect bacteria from host defense mechanisms, antimicrobial activity, and adverse conditions (17, 19, 46). Numerous environmental factors affect biofilm formation in staphylococci, and those exogenous triggers which are known include salinity and glucose (14, 43), oxygen limitation (10), iron depletion (13, 29), heparin (63), and various stresses such as heat and ethanol (6, 56). Recently, tricarboxylic acid cycle stress has also been shown to induce PIA production and promotion of biofilm, suggesting that endogenous metabolic cues can also be sensed that regulate biofilm formation (73). The sensing systems that respond to signals that affect biofilm formation and development remain largely unknown (15).

In this study, we report the generation of a mini-Mu transposon insertion mutant library in a virulent clinical human *S. aureus* isolate that produces strong biofilm under a variety of conditions. Screening for reduced biofilm formation in an in vitro model on polystyrene led to the isolation of a large collection of novel mutants affecting biofilm formation. Many insertions mapped to regulatory loci, thus shedding new light on factors contributing to biofilm biogenesis. Most importantly, this knowledge expands the range of targets for potential therapeutic intervention and, ultimately, understanding of genetic factors that control of biofilm formation.

## MATERIALS AND METHODS

**Reagents and bacterial strains.** Brain heart infusion (BHI) broth (Difco 256120), tryptic soy broth (TSB) without glucose supplement (Difco 286220), and Mueller-Hinton broth (Difco 212322) were used according to the manufacturer's recommendations. Recombinant lysostaphin was obtained from AMBI Products, LLC (Lawrence, New York). All chemical reagents used were analytical grade or better. *S. aureus* strain S30 is a pediatric isolate from a catheter-related bacteremia from the University of Geneva Hospital collection. It shows a typical methicillin-susceptible *S. aureus* antibiotic profile with resistance only to penicillin. S30 is classed by multilocus typing as 3-3-1-1-4-4-3 type ST8. S30 is fully pigmented, is hemolytic on blood agar, and shows a stable and strong biofilm-forming phenotype on a variety of substrates and various culture conditions. S30 has been typed by a subset of phage of the group III international set (3C, 6, 42E, 47, 53, 54, 75, 77, 81, and HK2). *S. aureus* SA113 (ATCC 15556) and its isogenic derivative SA113 $\Delta$ *ica::tet* have been described previously (9). SA113  $\Delta$ *tagO* does not produce detectable teichoic acid and has been described previously (75). RN4200 is a nonrestricting derivative of NCTC 8325-4, and it was used to shuttle plasmid DNA between *Escherichia coli* and *S. aureus* (34).

**Plasmids and recombinant DNA methods.** Plasmid pWKD56f (W. L. Kelley, unpublished) is based on the *E. coli*-*S. aureus* shuttle plasmid pMK4 and contains a gene encoding emission-enhanced green fluorescent protein (GFPuv4). Translational signals were fitted for optimal expression in *S. aureus* and expressed under the control of the *Bacillus megaterium* xylose-inducible *p<sub>XylR/A'</sub>* promoter (31). Plasmid pWKD56f maintenance was selected in *E. coli* with ampicillin (100  $\mu$ g/ml) and in *S. aureus* with chloramphenicol (15  $\mu$ g/ml). Plasmids used for genetic complementation were constructed as follows. The following primers were designed to incorporate upstream KpnI and downstream PstI restriction sites (underlined) flanking the coding sequence and were used to amplify SA1885 (*bfd-7*), SA0641 (*mgr*), and SA1118 (*bfd-6*) from S30 genomic DNA: SA1885f, 5'-CGGGTACCAAGGAGGAACAATCTTGCAAAATTTTAAAGAACTAGGGTTTC-3'; SA1885r, 5'-AAAACTGCAGTTATTTTGTATGGTCAGCAA TGTG-3'; SA0641f, 5'-CGGGTACCAAGGAGGAACAATCATGTCTGTATC AACATAATTTAAAGAACAGCTATG-3'; SA0641r, 5'-AAAACTGCAGT TATTTTCTTTTGTTCATCAATGCATGAATG-3'; SA1118f, 5'-CGGGTACCAAGGAGGAACAATCTTGAGTTTAAATAAGAAAAAGATAAAG ATATTC-3'; SA1118r, 5'-AAAACTGCAGTTAAATTCAGAAATFACTGG AATAATCATAG-3'. Fragments were digested with KpnI and PstI and first subcloned for sequence verification. Confirmed clones were then transferred to pWKD56f by digestion with KpnI and PstI and simple exchange for the GFPuv4 coding sequence. Plasmids were transferred to RN4220 prior to introduction into the appropriate S30 biofilm mutant by electroporation and selection for chloramphenicol resistance. Electroporation was performed as described previously (39) with minor modifications (0.12  $\mu$ g/ml penicillin G) for enhanced efficiency (52). Complementation assays for biofilm formation were performed with titration of xylose inducer. Negative control experiments were performed using pWKD56f. No effect of the addition of chloramphenicol, or xylose (up to 10% wt/vol), was noted on biofilm formation under conditions of the standard assay.

**Mutant generation and screening.** The erythromycin resistance-encoding Em-Mu transposon and its chromatographic purification scheme from the carrier plasmid pLEB620 have been described previously (50). The assembly, concentration, and electroporation of Mu transposition complexes have been described previously (36, 50). Chromosomal integrations were selected at 37°C on BHI agar plates containing erythromycin (10  $\mu$ g/ml). Erythromycin-resistant colonies were picked with sterile toothpicks, and the bacteria were grown overnight in BHI broth supplemented with 15% (vol/vol) glycerol in ordered 96-deep-well plates and then stored at -80°C as master stocks. For rapid primary screening, mutants were inoculated from frozen stocks and grown for 14 h in 96-well polystyrene plates (catalog no. 167008; Nunc) filled with 200  $\mu$ l of TSB, picked with a plate duplicator, and cultured for a further 6 h in fresh medium in polystyrene plates. The optical density at 600 nm (OD<sub>600</sub>) was then monitored with a Tecan Genios plate reader as an indication for bacterial growth. Supernatants were discarded, and the biofilms were fixed for 20 min at 80°C, followed by staining for 10 min with a solution of 1% (wt/vol) crystal violet solution (CV, Color Gram-2; bioMérieux) freshly diluted twofold in 1% (vol/vol) ethanol-distilled water. After thorough rinsing under running tap water and air drying, residual CV was dissolved for 3.5 h with 300  $\mu$ l dimethyl sulfoxide on a platform shaker with low-speed orbital agitation at 22°C. Half of the dye was transferred to a fresh plate, and the OD<sub>600</sub> was measured. Plots of biofilm formation versus OD were generated together with calculation of a mean biofilm formation for the entire population.

**Standard CV assay and growth curves.** For all biofilm measurements with S30, reference strains, or for stringent screening, overnight cultures (14 h) grown in

TSB were harvested by centrifugation, then washed once in phosphate-buffered saline, and passed through 5- $\mu$ m syringe filters (Sartorius) to reduce aggregation. Optical densities of all samples at 600 nm were equilibrated, and bacterial suspensions were diluted 100-fold into 200  $\mu$ l fresh medium and placed in polystyrene microtiter plates. Plates were incubated for 6 h at 37°C, then fixed, stained with CV, and measured as described above for primary screening. For the determination of growth curves, the Tecan plate reader was set at 37°C and the plates incubated for 6 h without agitation. Measures at 600 nm were taken every 30 min and plotted.

**Genomic DNA extraction.** Overnight cultures (5 ml) were harvested by centrifugation for 2 min at 14,000  $\times$  g. The pellet was resuspended in 300  $\mu$ l phosphate-buffered saline supplemented with lysostaphin 300 ng/ml and incubated at 37°C until lysis. Proteinase K was added to a concentration of 200  $\mu$ g/ml and incubation continued for a further 30 min at 37°C. The solution was extracted three times with 300  $\mu$ l phenol-chloroform-isoamyl alcohol (25:24:1) and separated with Phase Lock Gel heavy columns (Eppendorf). DNA was ethanol precipitated and resuspended in 100  $\mu$ l TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA). RNA was digested with RNase A (500 ng/ $\mu$ l for 30 min at 60°C), and DNA was stored at -20°C.

**Inverse PCR and identification of Em-Mu insertion sites.** Genomic DNA (5  $\mu$ l) was digested either with AseI or DraI for 2 h, diluted 40-fold in sterile water, self-ligated with T4 DNA ligase in ligation buffer overnight at 16°C, ethanol precipitated, washed in 70% (vol/vol) ethanol, and then resuspended in a minimal volume (10  $\mu$ l) of 10 mM Tris-HCl, pH 8.0. Inverse PCRs were then performed on aliquots of the ligation mixtures using primers MuInsert 70-94r, 5'-TTCTCGAGGAATTCTCTAGATGATC-3', and MuInsert 133-152f, 5'-GCATGCCATGGTACCCTTAG-3' (see Fig. 2), and *Pfu* polymerase (Promega). Reactions were 35 cycles using 40°C annealing (1 min) and 72°C (2 min) extension temperatures. The amplicons were gel purified and extracted (QiaQuick; QIAGEN), and the Mu junction flanking sequence was determined by sequencing using an ABI 3100 sequencer (Applied Biosystems) and the primer MuInsert 70-94r. Junction sequences obtained after the Mu repeat were used for BlastN analysis against the *S. aureus* N315 genome (GenBank accession number NC 002745).

**Southern blotting.** DNA (30  $\mu$ l) was digested with AseI and resolved on 1% (wt/vol) agarose gels in TBE (0.5 M Tris-HCl, 0.5 M borate, 5 mM EDTA) at 1 V/cm. DNA was transferred onto Hybond-N<sup>+</sup> membranes (Amersham Biosciences) by downward capillary transfer. A 667-bp Em-Mu-specific probe spanning the AseI restriction site in the transposon (see Fig. 2) was amplified and [ $\alpha$ -<sup>32</sup>P]dCTP radiolabeled by PCR with primers (P1) MuProbe-f (5'-CGGATC GCGGCCGCTG-3') and (P2) MuProbe-r (5'-TATCTTGGTGAATTAAGT GACACGAGTATTACAG-3'). A second 200-bp labeled probe from *dnaN* was also included to control for loading using primers N315-2206f (5'-CACATTA AAGCTATTTACCAAGA-3') and N315-2406r (5'-ACAAAGAATCGTCCA GGAAGTAC-3').

**Dot blot PIA analysis.** Strains were grown overnight in TSB, diluted 1:100 into 2 ml fresh medium, and regrown for 6 h as described for the standard CV assay above. Cultures were harvested by centrifugation, washed once with Tris-buffered saline (20 mM Tris-HCl, pH 7.5, 500 mM NaCl), and then passed through a 5- $\mu$ m syringe filter unit (Sartorius). Following normalization of optical densities at 600 nm, the samples (50  $\mu$ l) were applied on a nitrocellulose membrane (Protran; Schleicher & Schuell) with a BioDot microfiltration apparatus (Bio-Rad) according to the manufacturer's specifications. The membrane was fixed for 30 min at 80°C and then stained with Ponceau S to control for loading. Membranes were blocked for 1 h in TTBS (20 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.02% Tween 20) with 3% (wt/vol) skim milk. Rabbit polyclonal anti-PIA antibody (a kind gift of J. Knobloch) was diluted 3,000-fold in TTBS supplemented with 2% (wt/vol) bovine serum albumin (BSA). Membranes were incubated at room temperature for at least 1 h, washed in TTBS (4 times, 10 min each), and then incubated with secondary goat anti-rabbit immunoglobulin G horseradish peroxidase-conjugated antibody (Amersham Biosciences). Membranes were washed in TTBS (4 times, 10 min each) and then developed with SuperSignal West Pico chemiluminescent substrate (Pierce) according to the manufacturer's recommendations. Control experiments with every strain revealed undetectable secondary antibody binding to *S. aureus* protein A under the conditions of the assay.

**Wall teichoic acid extraction and gel analysis.** Overnight cultures in TSB were harvested by low-speed centrifugation, disrupted with glass beads, and ultracentrifuged to obtain crude membrane extracts essentially as described previously (18). Extracts were sodium dodecyl sulfate purified, and the wall teichoic acids (WTA) were isolated with extraction in 5% (wt/vol) trichloroacetic acid as described previously (54). WTA were resolved on a 15% (wt/vol) polyacrylamide

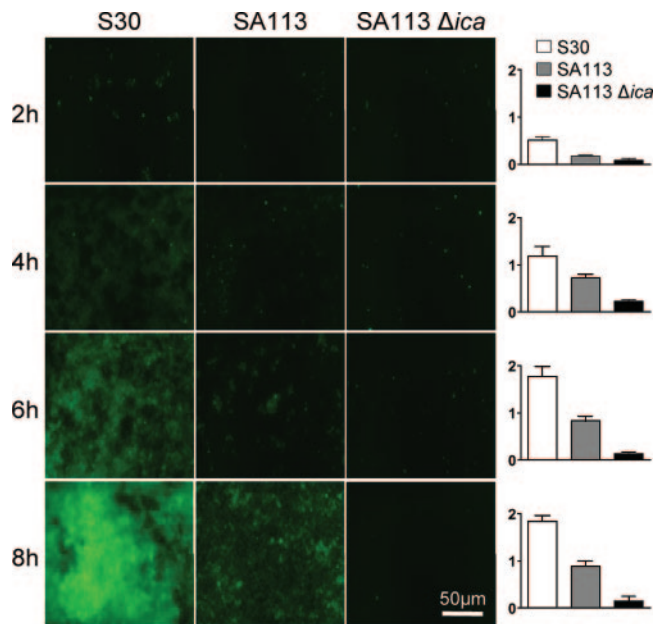


FIG. 1. Kinetics of biofilm formation by *S. aureus* strain S30 compared with two standard reference strains. Imaging of live biofilms on polystyrene surfaces by fluorescence microscopy was obtained using strains expressing GFPuv4 under the control of the pXyl promoter with 2% (wt/vol) xylose. Images were captured using a Zeiss AxioCam charge-coupled device camera and processed with identical magnification and exposure conditions. Quantitation of the three strains was done using a standard CV staining assay ( $n = 12$ ) and the same time points for the images. Note the approximately twofold increase in biofilm formation for S30 compared to SA113 and the strong quantitative correlation of the CV assay data with the optical imaging ( $P < 0.01$  for all pairwise comparisons with Student's one-tailed  $t$  test).

TBE gels and then revealed by a modified Alcian blue silver staining as described previously (76).

**Immunofluorescence microscopy.** Strains harboring GFPuv4-expressing plasmids were grown overnight in the presence of 2% (wt/vol) xylose. Cultures were washed and diluted 1:100 into fresh medium with xylose and incubated at 37°C in polystyrene six-well plates. Growth medium was removed by gentle pipette aspiration, and biofilms were washed with fresh medium. Images of live biofilms were captured directly using a Zeiss Axioskop 2 microscope linked to a Zeiss AxioCam charge-coupled device camera. Data were processed with Zeiss Axiovision 3.0 software with identical magnification and exposure conditions.

**Statistical analysis.** The data for biofilm analysis were obtained from  $n = 12$  determinations (quadruplicate assay of three independent colonies). Significant differences were concluded when  $P$  was  $< 0.01$  for pairwise comparisons with Student's one-tailed  $t$  test. Biofilm formation means for the entire mutant population were computed for the Em-Mu set ( $n = 9,177$ ) using the rapid screen assay and compared with the biofilm formation population mean obtained from 100 independent trials of the S30 wild type. The computed mean biofilm formation value, 2.4, was obtained in each case.

## RESULTS

**The *S. aureus* S30 strain produces strong biofilms.** *Staphylococcus aureus* strain S30 is a virulent clinical isolate from the Geneva University Hospital collection. It forms a strong biofilm, which suggested its use as a model for the isolation of new genes involved in biofilm formation.

To establish experimental parameters, S30 was first initially compared in two biofilm assays to a well-known reference biofilm-forming strain, SA113 (ATCC 15556), together with its

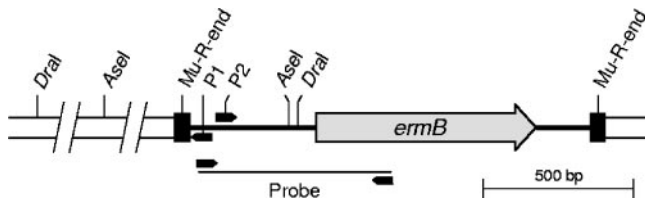


FIG. 2. Diagram of the Em-Mu transposon and the position of probes used for analysis. Em-Mu invert repeat ends are shown as black boxes, and the *ermB* selectable marker orientation is indicated. Oligonucleotide primers used for inverse PCR (P1, P2) or for Southern blot probing are indicated.

isogenic derivative SA113  $\Delta$ *icaADBC::tet*, which produces no PIA and displays significantly reduced biofilm formation (9).

Our results showed that S30 produced biofilm more rapidly than the SA113 control strain at the time points measured (Fig. 1). SA113 $\Delta$ *icaADBC::tet* did not produce significant biofilm, as expected. None of the strains showed significant differences in growth rate that potentially could account for differences in biofilm accumulation (data not shown). The strong ability of S30 to form biofilms was also confirmed at each time point using a quantitative CV staining assay (Fig. 1).

**Construction of an insertion mutant library and screening for biofilm mutants.** The Em-Mu insertion mutant library was constructed in strain S30 by selection for erythromycin resistance (see Materials and Methods). A total of 9,177 erythromycin-resistant clones were obtained and screened for biofilm formation-deficient mutants following 6 h of growth on polystyrene. This time point was chosen for our screen because wild-type S30 produced a substantial dense biofilm (Fig. 1). Many growth medium formulations were tested at the outset of the study, and the conditions chosen for the genetic screen used tryptic soy broth without glucose supplement. We found that this condition reproducibly showed a significant difference between SA113 and SA113 $\Delta$ *icaADBC::tet* over an 8-h interval (Fig. 1 and data not shown) and was therefore a good choice for improving the likelihood of isolating new biofilm-defective mutants in S30.

Candidate biofilm-forming mutants, which were identified in the primary screen (150), were reinoculated from master stocks and retested. The biofilm formation mean (CV assay) of the entire mutant population was experimentally indistinguishable from the biofilm formation mean obtained from 100 independent experimental trials of wild-type S30 using similar growth and assay conditions (data not shown). Forty-one mutants were retained which fell below two standard deviations of the biofilm formation mean of the entire mutant library and exhibited growth rates in liquid medium similar to those of the S30 wild-type control.

**Number of transposon copies and physical mapping of insertion sites.** To analyze the number of transposon copies present within the genomes of mutants, genomic DNA was prepared, digested with *AseI*, and analyzed by Southern blot hybridization using a transposon-specific probe (Fig. 2). We expected to obtain two arbitrary length fragments for each mutant as a unique Em-Mu insertion signature. Three mutants that possessed multiple or ambiguous Em-Mu insertion profiles were rejected by this method (data not shown), leaving 38 mutants for further analysis.

The precise transposon insertion site of each of these 38 mutants was determined by inverse PCR and sequencing. The distribution of inserts on the physical map of the reference *S. aureus* N315 genome is shown in Fig. 3. Details are given in Table 1.

For direct comparison, all 38 mutants obtained were retested in multiple independent trials, normalized to the S30 control (set to 100%), and plotted in decreasing order of their effect on biofilm formation (Fig. 4A).

**Classifying and complementing mutations.** We identified 15 independent insertions in the *icaADBC* operon that produces PIA. The location of each insertion in each of the four *ica* biosynthetic genes is depicted schematically in Fig. 3. All *ica* mutants showed substantially reduced biofilm formation (<70% of S30 wild type) and are highlighted in gray in Fig. 4A. Em-Mu insertion in any of the four genes in the *icaADBC* operon significantly affected biofilm formation. We conclude

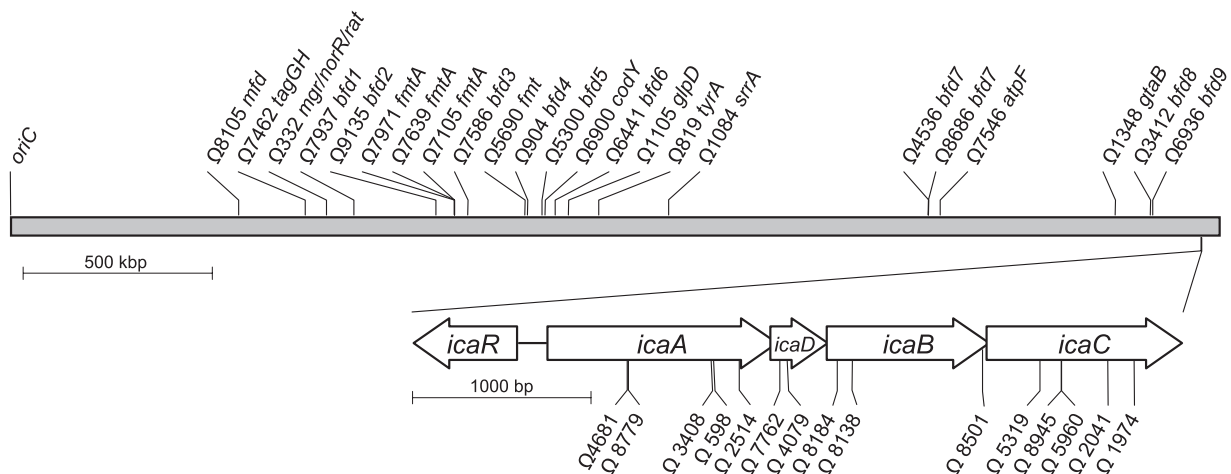


FIG. 3. Distribution of the Em-Mu insertions on the *S. aureus* N315 physical map. Multiple inserts were obtained in *fmtA* and SA1885 as well as the *icaADBC* operon. Though our screen is not saturating, the incidence of multiple, independent hits for three widely separated chromosomal regions suggests an approach to saturation.

TABLE 1. Mini-Mu transposition insertion mutants in *S. aureus* S30 showing significantly defective biofilm formation after 6 h growth on polystyrene plates

S30 Em-Mu mutant	NCBI gene identification	N315 locus tag <sup>a</sup>	Gene name(s)	Gene product(s) or domain similarity(ies)	Insertion site sequence <sup>b</sup>	Em-Mu orientation <sup>c</sup>	PIA <sup>d</sup>	WTA <sup>e</sup>
08105	15926180	SA0461	<i>mfl</i>	Transcription-repair coupling factor	TTTCATACGTTTCTTTCGAATCATTTGGCGCA	→	±	+
07462	15926315	SA0594	<i>tagG</i>	Intergenic region between <i>tagH</i> (teichoic acid translocation ATP-binding protein) and <i>tagG</i> (teichoic acid translocation permease)	ATGATTTGTATACTATATATGATTTTGTAAATA	←	±	+
0332	15926363	SA0641	<i>mgr/hor/ru</i>	Transcriptional regulator, MarK family	GTTAGATTGTACTTCTTAAAAAACTTTGTTA	→	±	+
07937	15926423	SA0701	<i>bfdI</i>	HP <sup>f</sup> , GGDEF domain	CTTAGGTAATGTAATAAAGATTTTGTATAGTAC	←	±	+
09135	15926604	SA0874	<i>bfd2</i>	HP, similar to multidrug resistance protein, major facilitator family	TTGCATGGATATTAATATATATTTATTTGCAATG	←	±	+
07971	15926643	SA0909	<i>fmn4</i>	Autolysis and methicillin resistance-related protein	TCCATGGTTTAAAAACATCTAAGCCTATCCC	←	-	-
07639	15926643	SA0909	<i>fmn4</i>	Autolysis and methicillin resistance-related protein	AACAGTAAGAATCTTGTCGAAAAGATCCCC	→	-	-
07105	15926643	SA0909	<i>fmn4</i>	Autolysis and methicillin resistance-related protein	ACAACATACATCTTATTAATGATGTAACA	→	-	-
07386	15926675	SA0940	<i>bfd3</i>	HP, predicted hydrolase of metallo-β-lactamase/β-CASP superfamily	GCCATTTGAATATCTTTATTTTGGATTTAACT	→	±	+
05690	15926799	SA1059	<i>fmn</i>	Methionyl-tRNA formyltransferase	CCTAATACACTTAATTTATTCATGATCGTGA	→	-	+
0904	15926804	SA1064	<i>bfd4</i>	HP, similar to <i>yecQ</i> ( <i>trgC</i> ) GTPase	ATGGTCGATTCGTAATTTGGCAATATATCCC	→	-	+
05300	15926833	SA1093	<i>bfd5</i>	HP, similar to DNA topoisomerase I <i>topA</i>	GCTACTAAGAAGAACCCGTCGATTTACTTTCT	→	±	+
06900	15926838	SA1098	<i>codY</i>	GTP-sensing pleiotropic transcription repressor	TTCTTCCAACTTTGAAATATTTCTTTGACT	→	-	+
06441	15926858	SA1118	<i>bfd6</i>	HP, predicted hydrolase of metallo-β-lactamase/β-CASP superfamily	GCAATGATTAACCTCCATATTTATAGCAAAAGCC	→	-	+
01105	15926884	SA1142	<i>glpD</i>	Aerobic glycerol-3-phosphate dehydrogenase	GCCGCCCTTTTAGACCTTCTTTTTAACTAA	→	±	+
0819	15926945	SA1197	<i>gvrA</i>	Prephenate dehydrogenase	GTTTTATTACTCAAGGTGATATCTTTCAC	→	-	+
01084	15927073	SA1323	<i>srzA</i>	Staphylococcal respiratory DNA-binding response regulator SrtA	ATAAATAATTCGGTGTGTTTGTATTTGTTCCAGCC	→	++	+
04536	15927656	SA1885	<i>bfd7</i>	HP, predicted ATP-dependent RNA helicase, DEAD box family	GCAGGTAGCTGAACAATTTAAAGAGAATTTAG	→	±	+
08686	15927656	SA1885	<i>bfd7</i>	HP, predicted ATP-dependent RNA helicase, DEAD box family	ATAGGCATACCAACCGAATATACAGTAACAACCT	→	±	+
07546	15927681	SA1909	<i>ampF</i>	ATP synthase β chain	TTCAAGTTTCTGTGCATTTAACTTAGCTTG	→	±	-
01348	15928079	SA2288	<i>gluB</i>	UTP-glucose-1-phosphate uridylyltransferase	TTGGTTATCATTTATTCATACGTTCAATCCG	→	+	+
03412	15928146	SA2354	<i>bfd8</i>	HP, putative membrane acyltransferase	GTGATGAATAATGAAATCACTATCATTAAGCTCC	→	+	+
06936	15928157	SA2364	<i>bfd9</i>	HP, putative DNA binding TelR superfamily transcriptional regulator	TTCAATTCGTTGCCCTAATTTCTTTTGGCATC	→	-	+
04681	15928252	SA2459	<i>ica4</i>	Intercellular adhesion protein A	ACCATATTATATGATTTGAGAAATTTCAAAACA	←	-	+
08779	15928252	SA2459	<i>ica4</i>	Intercellular adhesion protein A	AATATATGTCATCTTGTATCAACGATATAGTAT	→	ND	ND
03408	15928252	SA2459	<i>ica4</i>	Intercellular adhesion protein A	CTCAAAACATCAAAATATATATAAAGAAACCT	→	ND	ND
0598	15928252	SA2459	<i>ica4</i>	Intercellular adhesion protein A	ATCGAGATGATTTGGCTCAAAACATCAAAATA	→	ND	ND
02514	15928252	SA2459	<i>ica4</i>	Intercellular adhesion protein A	TTTATANNAAGTCAATGTAATAATGATAGTAGT	→	ND	ND
04079	15928253	SA2460	<i>icaD</i>	Intercellular adhesion protein D	CAAAAAGACACAAGATATVAGCGATPAAGCTGC	→	ND	+
07762	15928253	SA2460	<i>icaD</i>	Intercellular adhesion protein D	ATTGTAAGCAAAACAGCACTTATCGCTATA	←	ND	ND
08184	15928254	SA2461	<i>icaB</i>	Intercellular adhesion protein B	CAGGCAATATGATCAAGATATCAACCACTA	←	-	+
08138	15928254	SA2461	<i>icaB</i>	Intercellular adhesion protein B	CTGGCATTTAAATTTATCCCGTGTAAAGAAA	←	ND	ND
08501	15928254	SA2461	<i>icaB</i>	Intercellular adhesion protein B	GCTCAAAAAGCATTCATCACTAATTTAATCTAA	→	ND	ND
021974	15928255	SA2462	<i>icaC</i>	Intercellular adhesion protein C	GGCTGCAAAATACCCCAAGATATAACAATAAACA	→	ND	+
05319	15928255	SA2462	<i>icaC</i>	Intercellular adhesion protein C	ACTGTAAAACCAATCCATTAATATGTTAAGG	→	ND	ND
08945	15928255	SA2462	<i>icaC</i>	Intercellular adhesion protein C	CTCAAAAATTAAGAAATTCGATGATTAACAACG	→	ND	ND
05960	15928255	SA2462	<i>icaC</i>	Intercellular adhesion protein C	CTCAAAAATTAAGAAATTCGATGATTAACAACG	→	ND	ND
02041	15928255	SA2462	<i>icaC</i>	Intercellular adhesion protein C	ATAAACAATAAATAAGCTTCTAAGAAATTTAAT	→	ND	ND

<sup>a</sup> Open reading frame numbering (ordered sequence tag) from the N315 genome.  
<sup>b</sup> The first 30 nucleotides after the Em-Mu disruption site in the gene sequence are indicated.  
<sup>c</sup> Transcription sense of Em-Mu cassette relative to the insertion site.  
<sup>d</sup> Immunoreactivity to polysaccharide intercellular adhesion antigen was scored with polyclonal anti-PIA antibodies on whole-cell extracts. +, approximate wild-type expression level (≥80%); ++, above S30 wild-type expression level; ±, reduced expression; -, equivalent Δ 4681 *ica4* mutant expression level.  
<sup>e</sup> Wall teichoic acid extraction, polyacrylamide gel resolution and staining: +, wild-type expression level; -, S30 *tagC* mutant expression level.  
<sup>f</sup> HP, hypothetical protein.

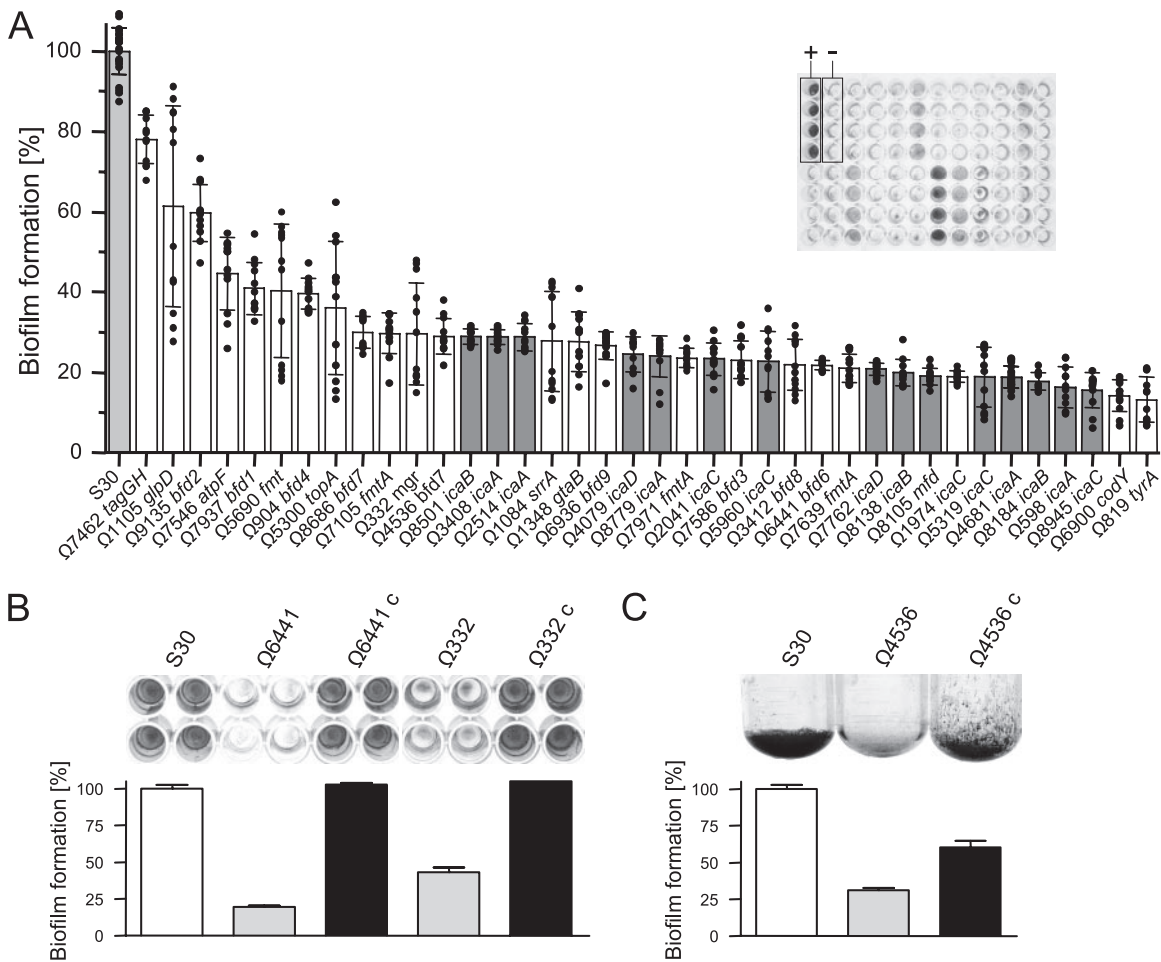


FIG. 4. (A) Biofilm assay showing the repartition of the 38 Em-Mu single-insertion mutants studied. Wild-type S30 (light gray) was set equal to 100%. Mutants mapping in the *icaADBC* locus are indicated in dark gray. The data were obtained from  $n = 12$  determinations of a quadruplicate assay of three independent colonies. Raw data points and standard deviation bars are indicated. The inset shows a typical polystyrene microtiter dish assay plate stained with CV. Typical biofilm-positive (+, S30) wells and biofilm-negative (-, SA113Δ *ica*) wells are boxed and indicated. (B) Restoration of biofilm formation by genetic complementation of the Em-Mu disruption in a quadruplicate microtiter biofilm assay. Mutant strains carrying xylose-inducible complementing plasmids are marked with a “c.” Quantitative measurements are shown graphically. (C) Genetic complementation for disruption of SA1885 in a polystyrene tube assay.

from these results that PIA is important for biofilm formation in S30.

Twenty-three Em-Mu insertions did not map within the *ica* operon. The respective clones showed reduction in biofilm formation comparable to that observed with *ica* insertions (Fig. 3 and 4A). Ten insertion sites were localized within the coding sequences of known genes (*mfd*, *mgrA*, also called *norR* or *rat*, *fntA*, *fnt*, *codY*, *glpD*, *tyrA*, *srrA*, *atpF*, and *gtaB*), and one insertion was mapped in the *tagGH* intergenic region. Nine insertions were mapped in predicted coding sequences that correspond to hypothetical proteins with no previous database annotation and are referred to as *bfd* for biofilm formation defective (Table 1). In two cases, multiple independent Em-Mu insertions were obtained within the same coding sequence. Three insertions mapped in *fntA*, and two insertions mapped in a hypothetical protein coding sequence with ordered locus tag SA1885.

Eleven insertions (Q8105, 9135, 7586, 5690, 904, 5300, 6900, 1105, 1084, 7546, and 6936) were mapped in hypothetical com-

puter-predicted operons in *S. aureus* (74). Thus, it is possible that these insertions exert polar effects on downstream gene expression. Interestingly, at least six disrupted genes (*mfd*, *mgrA*, *codY*, *srrA*, *bfd1*, and *bfd9*) are predicted to be involved in signaling pathways or can be confidently classed as transcription factors, suggesting that we have uncovered additional regulators that modulate biofilm development. None of the coding regions in which we obtained insertions has previously been shown to affect biofilm formation in *S. aureus*.

The Em-Mu insertions that we obtained by our screening procedure suggested, but did not prove, that the disrupted gene affected by each insertion was itself involved in biofilm formation, or alternatively, additional unlinked chromosomal alterations or polar effects were responsible for the phenotype. These issues are usually resolved by backcrossing the mutation with a transducing bacteriophage and/or through genetic complementation with the corresponding wild-type sequence by transformation. Although S30 is susceptible to a few international typing bacteriophage, none of the ones we tried trans-

duced strain S30. Electroporation using high-molecular-weight genomic DNA was also unsuccessful.

We therefore constructed plasmids and attempted genetic complementation of biofilm formation defects in a subset of Em-Mu insertions that did not map in predicted operons. To examine this proof of principle, we first cloned coding sequences for SA0641, SA1885, and SA1118 and placed them under the control of a xylose-inducible promoter in an *E. coli*-*S. aureus* shuttle plasmid. As a negative control, we engineered transformants harboring pWKD56f encoding only enhanced green fluorescent protein (GFPuv4). The results of these experiments are shown in Fig. 4B.

We found complete restoration of biofilm formation for two mutants, *mgrA* and *bfd6*, and partial restoration for a third, *bfd7*. Restoration of biofilm formation was comparable for both independent *bfd7* mutants. Controls with pWKD56f were all negative (data not shown). The concentration of xylose required for successful complementation for biofilm formation varied in each assay. The reason for the variable dependence on xylose induction is unknown but may reflect characteristics of the expression from this promoter, altered gene dosage, or the influence of partial gene products produced by the Em-Mu disruption.

**Effects of Em-Mu insertion on PIA or wall teichoic acid biosynthesis.** In light of the importance of PIA in biofilm development, some S30 Em-Mu biofilm formation-defective mutants could act by altering directly, or indirectly, the regulation of PIA biosynthesis or its surface transport. To address this question, we examined PIA production by semiquantitative dot blot analysis of serial dilutions of fixed whole cells. For PIA reactivity, we assigned the mutants categories designated as PIA positive (+, >80% level compared to S30), PIA intermediate ( $\pm$ , >20% and <80% S30), or PIA negative ( $-$ , <20% S30 and similar to S30 Em-Mu::*icaA*) on the basis of digital scans of at least three independent blots and compared to the S30 wild type. The results are listed in Table 1, and representative data for each assigned category are shown in Fig. 5.

S30 PIA and SA113 PIA immunoreactivity were found to be indistinguishable. In contrast, we observed that the majority of our Em-Mu insertion mutants displayed altered PIA production which may be responsible for some, or even all, observed defective biofilm formation. Specifically, disruption of eight genes (*fmtA*, *fmt*, *codY*, *tyrA*, *bfd4*, *bfd6*, *bfd8*, and *bfd9*) resulted in very low PIA reactivity, equivalent to that measured by complete disruption of the *icaADBC* or Em-Mu insertion in *icaA* alone (category  $-$ ). Disruption of nine genes (*mfd*, *mgrA*, *glpD*, *atpF*, *bfd1*, *bfd2*, *bfd3*, *bfd5*, and *bfd7*) resulted in partial reduction of PIA reactivity (category  $\pm$ ). Interestingly, we observed that disruption of *gtaB*, or the *tagGH* intergenic insertion, resulted in no measurable alteration in PIA immunoreactivity. In contrast, we observed that disruption of *srrA* led to consistently enhanced production of PIA antigen (at least four- to eightfold) compared to S30. Thus, we assigned it to a separate category (++) in Table 1 to reflect this observation.

Each S30 Em-Mu insertion mutant was also biochemically fractionated and tested for production of wall teichoic acid, which is implicated in primary attachment to abiotic surfaces (20). As controls, we used SA113 and its isogenic *tagO* mutant derivative (75), which does not produce detectable teichoic

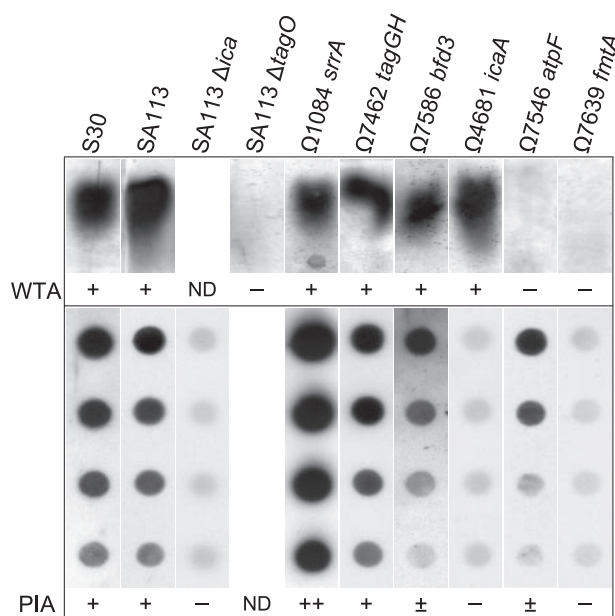


FIG. 5. Representative assay for PIA immunoreactivity (*icaADBC* locus product) or WTA extraction and staining. Six representative S30 mutants corresponding to each category assigned in Table 1 are depicted (WTA/PIA). All immunodot blots show four serial twofold dilutions. Immunoreactivity was scored by digital scans of at least three independent determinations. Wall teichoic acids were extracted, resolved on polyacrylamide gels, and stained with Alcian blue silver. Control experiments for reagent specificity and detection are indicated for SA113  $\Delta$ *tagO* that does not produce teichoic acids (75) and SA113  $\Delta$ *icaADBC::tet* that does not produce PIA (9). ND, not determined. Symbols are as defined for Table 1.

acids and shows reduced biofilm formation (P. Tu Quoc, unpublished). The results are listed in Table 1, and a subset is shown in Fig. 5.

Surprisingly, our results revealed four Em-Mu insertions localized in two genes, *fmtA* and *atpF*, which showed no detectable WTA staining. In contrast, all other mutants tested positive for WTA.

## DISCUSSION

We report the identification of disruptions in 19 genes and one intergenic mutation that negatively affect biofilm formation in *S. aureus*. With the exception of multiple insertions in each gene of the *icaADBC* operon, we uncovered none of the genes previously described for their role in biofilm formation in staphylococci. For example, we did not identify Em-Mu insertions in genes encoding subunits of the Clp ATP-dependent proteases, *rbf*, *sarA*, *dtlA*, *atlE*, *hla*, and *rsbU*. Our screen was large but not saturating, and other biofilm-defective mutants await identification. We do not report a screen for increased biofilm formation with S30. Interestingly, a recent study describes such an increased biofilm screen in another *S. aureus* human clinical isolate and points to the discovery of an *ica*-independent biofilm pathway active in the absence of the ArlRS two-component sensor (67).

Three mutations were functionally complemented for their biofilm defect by reintroducing cloned genes coding for MgrA,

SA1885, and SA1118, respectively. SA1118 and SA0940 are hypothetical proteins that possess similarity to both zinc metallo- $\beta$ -lactamase and  $\beta$ -CASP protein motifs (4). Proteins of this family possess a variety of enzymatic activities, and  $\beta$ -CASP motif proteins are predicted to play important roles in RNA and DNA processing. The family is widespread among the three primary kingdoms, and those found in bacteria remain uncharacterized. To date, no role for either SA1118 or SA0940 has been described for *S. aureus*.

The *mgrA* gene, also called *rat* or *norR*, was identified independently by three laboratories (26, 42, 69). MgrA is a DNA binding protein that acts as an important regulator of autolytic activity (26). Mutants have pleiotropic phenotypes including reduced alpha toxin and protein A production (42), changes in drug sensitivity and aggregation (69), and a partial growth defect that limits stationary-phase culture density (26). How disruption of *mgrA* results in altered biofilm formation is unknown but may be the consequence of altered surface properties or changes in gene expression.

The *bfd7* insertion mutations occur in locus tag SA1885 that has predicted signature domains for both ATPase and DEAD-box helicase. Many DEAD-box helicases have been described that help with RNA metabolism (59). Interestingly, *aggH* in *Lactobacillus reuteri* encodes a helicase with 46% amino acid identity to SA1885, known to mediate aggregation and enhanced genetic exchange in this organism (61). No role, to date, has been described for helicases in biofilm formation, and additional studies will be required to determine whether SA1185 possesses a bona fide helicase activity and to pinpoint its exact mechanism in biofilm regulation.

Three genes uncovered in our screen have links to guanosine-dependent regulation: *codY*, *bfd1*, and *bfd4*. This finding points to the possible involvement of small-molecule metabolic sensing cues that modulate biofilm. CodY has been studied in *Bacillus subtilis*, where it is known to be a GTP-binding pleiotropic transcriptional repressor that regulates genes in the post-exponential growth phase (65). The gene *bfd1* encodes a hypothetical protein that contains a GGDEF motif that is a signature associated with diguanylate cyclase activity. Cyclic diguanylic acid is now known to be a global bacterial regulator of motility, adhesion, and biofilm formation (25, 28). Interestingly, two studies reported that the addition of cyclic diguanylic acid attenuates *S. aureus* biofilm formation both in vitro and in vivo (3, 30). Finally, the *bfd4* gene product is predicted to be 33% identical to the *E. coli yjeQ* (*engC*) GTPase. This enzyme has been shown to have a high hydrolytic rate for GTP coupled with a low catalytic turnover (12). No studies have linked *yjeQ* to biofilm regulation, but a recent report showed that disruption of *yjeQ* led to severely reduced virulence in a mouse kidney abscess infection model (5).

A large proportion of our insertional mutations affect PIA production. This finding underscores the need for a comprehensive analysis of *icaADBC* regulation to fully understand the mechanisms that channel environmental and metabolic inputs to glycocalyx formation. The Em-Mu insertion that we obtained in *srrA* was the only mutant where we observed an increase in PIA production but reduced biofilm formation. SrrAB, also called SrrSR, constitutes a typical histidine kinase two-component signaling pair (55, 66, 79). SrrA is thought to repress virulence factor production by modulating levels of

RNAIII, the major effector of the *agr* locus, under conditions of low oxygen tension. A *srrSR* deletion mutant revealed extensive changes in energy metabolism under different oxygen tensions as well as impaired growth under anaerobic conditions (66). Since biofilm communities are thought to contain anaerobic microenvironments, it is tempting to speculate that the *srrA* mutant is unable to develop biofilm because of a defect linked to oxygen sensing.

Disruption of *gtaB*, as well as disruption of the *tagGH* intergenic region, did not detectably alter WTA or PIA. In *B. subtilis*, loss of *gtaB* affects teichoic acid glycosylation and phage susceptibility (71). The S30 *gtaB*-MuE mutant also shows significantly altered phage susceptibility (W. L. Kelley, unpublished data) and suggests that defects in attachment and/or intercellular interactions may be the consequence of surface modification. Both *tagG* and *tagH* encode putative proteins that belong to the ABC transporter superfamily (38). Thus, it is possible that altered *tagGH* expression modulates teichoic acid in a qualitative manner, undetectable with our techniques.

Two of our mutants in *atpF* and *fntA* showed undetectable formation of wall teichoic acid. How disruption of *atpF* affects biofilm formation is unclear but may be related to altered ATP synthesis or membrane potential. The disruption of *fntA* is associated with alterations in cell wall structure which may impair biofilm formation (33). Interestingly, mutations in either *fntA* or *tagO* (also called *llm*), which produces no teichoic acid (75), are known to modulate drug resistance levels in methicillin-resistant *S. aureus*, potentially providing a link between two major problems confronting the clinical management of staphylococcal infections: biofilm and methicillin-resistant *S. aureus* (33, 48).

Differential gene expression profiles comparing biofilm and planktonic bacteria under a variety of conditions have been reported previously (1, 2, 57, 78). It is noteworthy that several genes uncovered in our study were reported down- or upregulated in these studies. One study revealed significant upregulation of SA2354 (*bfd8*) at 8 h in biofilm compared to planktonic bacteria. In more mature biofilms, these authors also noted an upregulation of other ATP synthase subunits (57). Beenken and coworkers (2) noted significant upregulation of *glpD* in biofilm versus planktonic bacteria, which suggests a link with our finding of reduced biofilm in a *glpD* mutant. Yao and coworkers (78) noted, in contrast, downregulation of the *srrB* membrane sensor together with downregulation of the epsilon and delta subunits of ATP synthase in their biofilm study of *S. epidermidis*. Transcriptomic and proteomic studies also uncovered modulation of *mgrA* (SA0614) when comparing biofilm versus planktonic bacteria (57, 58). The significance of these observations is obscured by wide variation in growth conditions employed in these studies, and refined analysis will be necessary.

Speculation about the possible reason for biofilm defects in other mutants, such as *mfd*, *fnt*, *bfd2*, *bfd5*, *tyrA*, *glpD*, *bfd8*, and *bfd9*, must await further analysis. For instance, the gene *bfd2* encodes a hypothetical protein that shows characteristic features of the major facilitator superfamily (51) that may point to an important role for a small molecule(s) necessary for biofilm formation. Determining the identity of these molecules



will be an important prerequisite for understanding how *bfd2* affects biofilm in S30.

The methodology employed in our study, based on the delivery of Mu transposition complexes via electroporation (36, 50), is highly versatile and circumvents the need for currently used transposition protocols requiring exposure to high temperature for extended periods of time to eliminate thermosensitive plasmid vectors. Mini-Mu transposons can be constructed with a wide range of selectable markers or reporters and easily tailored for expression in a diverse number of bacteria. Our study demonstrates the feasibility of constructing and screening mutant banks in clinical isolates that are poorly defined, cumbersome, or refractory to many standard genetic methods.

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