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Global Gene Expression in *Staphylococcus aureus* Biofilms

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We previously demonstrated that mutation of the staphylococcal accessory regulator (*sarA*) in a clinical isolate of *Staphylococcus aureus* (UAMS-1) results in an impaired capacity to form a biofilm in vitro (K. E. Beenken, J. S. Blevins, and M. S. Smeltzer, Infect. Immun. 71:4206-4211, 2003). In this report, we used a murine model of catheter-based biofilm formation to demonstrate that a UAMS-1 *sarA* mutant also has a reduced capacity to form a biofilm in vivo. Surprisingly, mutation of the UAMS-1 *ica* locus had little impact on biofilm formation in vitro or in vivo. In an effort to identify additional loci that might be relevant to biofilm formation and/or the adaptive response required for persistence of *S. aureus* within a biofilm, we isolated total cellular RNA from UAMS-1 harvested from a biofilm grown in a flow cell and compared the transcriptional profile of this RNA to RNA isolated from both exponential- and stationary-phase planktonic cultures. Comparisons were done using a custom-made Affymetrix GeneChip representing the genomic complement of six strains of *S. aureus* (COL, N315, Mu50, NCTC 8325, EMRSA-16 [strain 252], and MSSA-476). The results confirm that the sessile lifestyle associated with persistence within a biofilm is distinct by comparison to the lifestyles of both the exponential and postexponential phases of planktonic culture. Indeed, we identified 48 genes in which expression was induced at least twofold in biofilms over expression under both planktonic conditions. Similarly, we identified 84 genes in which expression was repressed by a factor of at least 2 compared to expression under both planktonic conditions. A primary theme that emerged from the analysis of these genes is that persistence within a biofilm requires an adaptive response that limits the deleterious effects of the reduced pH associated with anaerobic growth conditions.

Staphylococcus aureus is a prominent human pathogen that causes a wide variety of infections. Of particular interest in our laboratory are musculoskeletal infections including those associated with orthopedic implants. The hallmark characteristic of these infections is formation of a biofilm, which consists of multiple layers of bacteria encased within an exopolysaccharide glycocalyx. The presence of this glycocalyx protects the enclosed bacteria from host defenses and impedes delivery of at least some antibiotics (64). Moreover, bacteria within biofilms adopt a phenotype that confers intrinsic resistance to many antibiotics. For example, the reduced growth rate of biofilm-associated bacteria limits the efficacy of antibiotics that target cell wall biosynthesis, while the reduced oxidative metabolism limits the uptake of aminoglycosides (33, 64, 65). Consequently, biofilm-associated infections are recalcitrant to antimicrobial therapy and often require surgical intervention to debride infected tissues and/or remove colonized implants.

The formation of three-dimensional biofilms is a complex process that can be subdivided into the relatively distinct phases of attachment, accumulation, maturation, and dispersal (10). With respect to staphylococcal biofilms, the primary emphasis so far has been placed on the attachment and accumulation phases, which appear to be mediated by different types of adhesins. More specifically, a group of surface-exposed proteins collectively referred to as MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) (48) appear to be the primary determinants responsible for the initial attachment to both native tissues and biomaterials, while the accumulation phase appears to be dependent on polysaccharide adhesins that promote adhesive interactions between bacterial cells (26). Although a number of candidate polysaccharides have been described, there is an emerging consensus that the primary determinant of the accumulation phase of staphylococcal biofilm formation is the polysaccharide intercellular adhesin (PIA), production of which is dependent upon the genes within the *icaADBC* operon (28). Composition studies have demonstrated that PIA consists of polymeric *N*-acetylglucosamine, and for this reason it has also been referred to as PNAG (40).

The *ica* operon was first identified in *Staphylococcus epidermidis* (28) and has been studied most extensively in that species. However, it is also present and appears to serve the same function in *S. aureus* (14). Most *S. aureus* strains appear to contain the entire *ica* operon (14, 22, 53), although there are reports to the contrary (3), and it is clear that there are strain-dependent differences with respect to the overall capacity to form a biofilm in vitro (5, 14, 53). The *ica* operon is subject to phase variation in *S. epidermidis* (75), and a number of studies have indicated that expression of *ica* in both *S. epidermidis* and *S. aureus* is also subject to environmental regulation. Perhaps most importantly, McKenney et al. (42) demonstrated that

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PNAG production in *S. aureus* is enhanced during in vivo growth. Rachid et al. (52) subsequently demonstrated that expression of *ica* is at least partially controlled by the stress response transcription factor σ^B. In addition, anaerobic growth was found to induce expression of the *ica* operon and PIA production in both *S. epidermidis* and *S. aureus* (15).

Recently, Conlon et al. (12) reported that *icaR*, which is located immediately upstream of the *ica* operon, encodes a repressor that is important for the environmental regulation of *ica* expression in *S. epidermidis*. However, studies done with *S. aureus* have demonstrated that regulation of *ica* expression and the ability to form a biofilm also involve regulatory elements other than σ^B and IcaR (66). Included among these additional regulatory loci are the accessory gene regulator (*agr*) and the staphylococcal accessory regulator (*sarA*). The *agr* locus encodes a two-component quorum-sensing system that modulates production of a regulatory RNA molecule (RNAIII) in a density-dependent manner. Induction of RNAIII synthesis results in reduced production of surface proteins (e.g., MSCRAMMs) and a concomitant increase in production of exotoxins (4, 45). Production of δ-toxin, which is encoded within the RNAIII locus, has been negatively correlated with biofilm formation (69, 70). This suggests that strains expressing *agr* at high levels would have a reduced capacity to form a biofilm, which is consistent both with our results (5) and results from other laboratories (70).

The *sarA* locus encodes a 14.5-kDa DNA-binding protein (SarA) that is required at least under some growth conditions for maximum expression from the *agr* and RNAIII promoters (29). This would imply that mutation of *sarA* would limit production of RNAIII and thereby enhance the ability to form a biofilm. However, recent reports have confirmed that mutation of *sarA* results in a reduced capacity to form a biofilm (5, 66). SarA also regulates expression of other genes in an *agr*-independent manner (6, 19, 72, 74), and Valle et al. (66) recently demonstrated that mutation of *sarA* results in reduced transcription of the *ica* operon and a reduced capacity to produce PNAG. They also suggested that SarA may promote biofilm formation in an indirect manner by suppressing transcription of a repressor of PNAG synthesis or a protein involved in the turnover of PNAG.

The persistence of bacteria within a biofilm also requires an adaptive response appropriate for the sessile lifestyle. The availability of complete bacterial genome sequences has facilitated the use of microarray technologies to identify genes that are differentially expressed by biofilm-encased bacteria. Using an array representing 99% of the *Bacillus subtilis* genome, Stanley et al. (63) identified 519 genes that were differentially expressed in biofilms as opposed to planktonic cultures. Similarly, Schembri et al. (59) found that 5 to 10% of the genes in the *Escherichia coli* genome were differentially expressed in biofilms, depending on which planktonic growth condition was used as a reference. Included among these genes were 30 of the 65 genes previously reported to be under the regulatory control of the general stress response regulator *rpoS* (36). Schembri et al. (59) subsequently demonstrated that an *E. coli rpoS* mutant was incapable of forming a biofilm. However, Whiteley et al. (71) found that expression of *rpoS* was repressed in *Pseudomonas aeruginosa* biofilms and that a *P. aeruginosa rpoS* mutant formed a more extensive biofilm than the corresponding wild-type strain. While these results confirm that biofilms represent a unique growth state by comparison to planktonic cultures, they also suggest the existence of species-specific pathways that contribute to biofilm formation and maintenance of the sessile lifestyle. To date, no comprehensive transcriptional analysis of *S. aureus* biofilms has been reported. However, Prigent-Combaret et al. (51) demonstrated that biofilm-encased *E. coli* encounter high osmolarity, oxygen limitation, and higher cell density than cells grown under planktonic conditions, and all of these factors are known to influence gene expression in *S. aureus* (11, 45).

To further investigate these issues, we generated *sarA* and *ica* mutations in a clinical isolate of *S. aureus* (UAMS-1) and examined their relative capacity to form a biofilm both in vitro and in vivo. We also used a custom-made Affymetrix GeneChip representing the combined genomes of six strains of *S. aureus* (N315, Mu50, COL, NCTC 8325, EMRSA-16 [strain 252], and MSSA-476) to investigate differential gene expression in a mature *S. aureus* biofilm.

MATERIALS AND METHODS

Bacterial strains. The experiments described here focus on the *S. aureus* clinical isolate UAMS-1. This strain was cultured from the bone of a patient suffering from osteomyelitis and was previously shown to form a biofilm both in vitro (5) and in vivo (21). The UAMS-1 *sarA* mutant was generated by transduction as previously described (7). Φ11-mediated transduction from a derivative of *S. aureus* SA113 carrying an *ica*::*tet* mutation (14) was used to generate a UAMS-1 *ica* mutant. Transductants were confirmed by Southern blotting using probes corresponding to the *sarA* and *ica* loci (6, 7).

Detection of PNAG production. To assess the production of PNAG in *S. aureus* clinical isolate UAMS-1 and its *sarA* and *ica* mutants, cultures were grown in tryptic soy broth with the appropriate antibiotic. After overnight incubation, the optical density at 560 nm (OD_{560}) was determined, and an equal number of cells (2 to 5 ml of each culture grown overnight) was harvested by centrifugation. Cells were resuspended in 50 μl of 0.5 M EDTA (pH 8.0) and boiled for 5 min. After cellular debris was removed by centrifugation, a 40-μl aliquot of the supernatant was incubated for 30 min with 10 μl of proteinase K (20 mg/ml) at 37°C to reduce nonspecific background levels. After the addition of 10 μl of Tris-buffered saline (20 mM Tris-HCl, 150 mM NaCl [pH 7.4]), 8 μl of each extract was spotted onto a nitrocellulose membrane using a BIO-dot microfiltration apparatus (Bio-Rad Laboratories, Inc., Hercules, Calif.). After drying, the presence of PNAG in the extract was assessed using the WesternBreeze chemiluminescence immunodetection kit (Invitrogen Corp., Carlsbad, Calif.) and anti-PNAG antiserum (kindly provided by Kimberly Jefferson, Channing Laboratory, Harvard Medical School).

Planktonic culture conditions. Because biofilm formation by strain UAMS-1 in vitro is dependent on supplementation of the medium with 0.5% glucose and 3.0% sodium chloride (5), these supplements were also added to the medium used for planktonic culture. Specifically, 5-ml samples of cultures grown overnight in tryptic soy broth at 37°C with constant aeration were used to inoculate 250 ml of fresh biofilm medium to an OD_{560} of 0.05. Cultures were incubated with aeration at 37°C. Aliquots were then removed at the mid-exponential (OD_{560} = 1.0) and stationary (OD_{560} = 3.5) growth phases. Aliquots were immediately mixed with an equal volume of ice-cold acetone-ethanol (1:1) and stored at −20°C prior to RNA extraction.

Biofilm cultures. Biofilms were generated in disposable flow cells (Stovall Life Science, Greensboro, N.C.) as previously described (5). Briefly, flow cells were precoated overnight at 4°C with 20% human plasma diluted in carbonate buffer (pH 9.6). The inlet side of the flow cell was then connected to a sterile reservoir filled with biofilm medium. The outlet side was connected to a waste reservoir. Tubing upstream of each individual cell was injected with 0.5 ml of the appropriate culture grown overnight. After the flow of medium was started and bacteria were allowed to enter the flow cell, the flow was stopped and the chamber was incubated inverted at 37°C for 1 h. After the flow cell was set upright, nonadherent bacteria were flushed by adjusting the flow rate to 0.5 ml/min, which is sufficient to replace the volume of the flow cell once every minute. Cells harvested after 1 week by aspiration from the downstream side of the flow cell were immediately mixed with acetone-ethanol as described above.

TABLE 1. Sequences of primers and TaqMan probes used in this study

Primer or probe[a]	Oligonucleotide sequence (5′→3′)
cna-F	CAAGCAGTTATTACACCAGACGG
cna-R	CACCTTTTACAGTACCTT
arcA-F	GTGGTTGACTCATACATCTAGGGC
arcA-R	AGACCAGGCGTTGTAGTGACTTA
arcA-P	CCCACGTCCACGTACCAGCTCGCT
pyrR-F	TTGATGATGTGCTGTATACTGG
pyrR-R	CGAATTGGTAACTCACGATGT
pyrR-P	CGGTTCGTGCTTCACTTGATGCT
ureA-F	CATTTTACACAACGAGAGCAAG
ureA-R	ACGTGCTTTACGACGACG
ureA-P	CAACTTCCGCCGCCACTACAATCA
gyrB-F	AGTAACGGATAACGGACGTGGTA
gyrB-R	CCAACACCATGTAAACCACCAGAT
gyrB-P	CCGCCACCGCCGAATTTACCACCA
spa-F	GTAACGGCTTCATTCAAAGTCT
spa-R	TCATAGAAAGCATTTTGTTGTTCT
spa-P	AAAGACGACCCAAGCCAAAGCACT

[a] Forward (F) and reverse (R) primers and the Taqman probe (P) for the ORFs are shown.

Assessment of biofilm formation. Biofilm formation in vitro was assessed using a microtiter plate assay and flow cells as previously described (5). The murine model of catheter-associated biofilm formation described by Kadurugamuwa et al. (31) was used to assess biofilm formation in vivo. Briefly, 20- to 30-g female BALB/c mice (Charles River, Wilmington, Mass.) were anesthetized with ketamine (100 mg/kg of body weight) and xylazine (5 mg/kg), their flanks were shaved, and the skin was cleansed with Betadyne and alcohol. Under aseptic conditions, a 1-cm segment of 14-gauge Teflon intravenous catheter was implanted subcutaneously. The wound was closed with sutures and then cleansed with a Betadyne rinse. Infection was induced approximately 1 h after the implantation procedure by injecting 10^5 CFU of the test strain into the lumen of the catheter. In some cases, mice were coinfected by injection of a mixture containing 10^5 CFU of UAMS-1 and 10^5 CFU of either the *sarA* or *ica* mutant. Mice were euthanized 10 days postinfection. The catheters were removed aseptically and washed with phosphate-buffered saline. Catheters were then placed in 10 ml of sterile phosphate-buffered saline and sonicated for 1 min to remove adherent bacteria. The number of bacteria in the sonicate was then determined by plating on tryptic soy agar. To confirm the identity of recovered bacteria and to determine the proportion of UAMS-1 versus the corresponding *sarA* and *ica* mutants in coinfection experiments, colonies obtained on tryptic soy agar were transferred to the appropriate selective medium and scored for growth.

RNA isolation and cDNA labeling. Aliquots of cells harvested from flow cells and stored as described above were pelleted by centrifugation at 7,500 × g for 10 min at 4°C. Each pellet was washed in an equal volume of TES buffer (150 mM NaCl, 78 mM disodium salt EDTA, 100 mM Tris [pH 7.5]) and resuspended to a concentration of 10^9 CFU per ml in TES buffer containing 100 μg of lysostaphin (Ambicin L; AMBI, Inc., Lawrence, N.Y.) per ml. Samples were incubated at 37°C for 1 h prior to applying the equivalent of 10^{10} CFU to a Qiagen RNeasy Maxi column. Total bacterial RNA was isolated according to the manufacturer's directions (Qiagen, Inc., Valencia, Calif.). After purification, contaminating DNA was removed with RNase-free DNase I (10 U/40 μg of total bacterial RNA at 37°C for 20 min). RNA was then repurified using RNeasy Mini columns (Qiagen, Inc.). The amount of recovered RNA was determined spectrophotometrically, and the absence of DNA was verified by PCR using primers (Table 1) corresponding to the collagen adhesin gene (*cna*). Samples were then stored at −80°C.

RNA was converted to cDNA, and microarray analysis was performed according to the manufacturer's instructions (Affymetrix expression analysis technical manual, Affymetrix, Inc., Santa Clara, Calif.) for antisense prokaryotic arrays. Briefly, 10 μg of total RNA that had been mixed with random hexamer primers (Invitrogen) was denatured at 70°C for 10 min and allowed to anneal at 25°C for 10 min. cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen) in 1× first-strand synthesis buffer, dithiothreitol, deoxynucleoside triphosphates, and SUPERase-In (Ambion, Inc., Austin, Tex.). The mixture was incubated at 25°C for 10 min, 37°C for 60 min, and 42°C for 60 min. The reaction was stopped by incubating for 10 min at 70°C prior to degrading the RNA with 1 N NaOH for 30 min at 65°C and neutralizing with 1 N HCl. The cDNA was purified using a QIAquick PCR purification kit (Qiagen) and fragmented with DNase I in One-Phor-All buffer (Amersham Biosciences, Piscataway, N.J.). DNase I was inactivated by heating the reaction mixture for 10 min at 98°C. The fragmented cDNA products were labeled with biotin on the 3′ terminus using the Enzo BioArray terminal labeling kit with biotin ddUTP (Affymetrix, Inc.).

DNA microarray hybridization and analysis. Labeled cDNA (1.5 μg) was hybridized to custom-made *S. aureus* GeneChips and detected according to the manufacturer's instructions for antisense prokaryotic arrays (Affymetrix, Inc.). The GeneChip used in these experiments included 7,723 qualifiers representing the consensus open reading frame (ORF) sequences identified in the genomes of the *S. aureus* strains N315, Mu50, COL, NCTC 8325, EMRSA-16 (strain 252), and MSSA- 476, as well as novel GenBank entries and N315 intergenic regions greater than 50 bp, After hybridization and staining, the arrays were scanned using the Agilent GeneArray laser scanner (Agilent Technologies, Palo Alto, Calif.). The data from duplicate experiments was normalized and analyzed using GeneSpring version 5.1 gene expression software (Silicon Genetics, Redwood City, Calif.). Genes were considered to be induced in a biofilm if they were determined to be present by Affymetrix algorithms in the biofilm condition and they were transcribed at a level at least twofold higher than the corresponding planktonic growth condition. Genes were considered downregulated in a biofilm if they were determined to be present in either planktonic condition and had an expression level no more than half of that observed in the corresponding planktonic growth condition. Differential expression in biofilms was judged to be significant on the basis of statistical analysis, namely, the *t* test with a *P* value of ≤ 0.05.

Real-time PCR. To confirm the results of our microarray data, the relative expression levels of the *arcA*, *pyrR*, *ureA*, and *spa* genes in each growth condition were also determined by real-time PCR. Briefly, DNase-treated RNA was reverse transcribed using the iScript cDNA synthesis kit as described by the manufacturer (Bio-Rad Laboratories). A portion (1/20th) of each reaction mixture was then used for real-time PCR using an iCycler iQ real-time PCR detection system, gene-specific primers, and TaqMan probes corresponding to each ORF, and the iQ supermix (Bio-Rad). The sequences of the primers and TaqMan probes are shown in Table 1. Relative expression levels were determined by comparison to the level of *gyrB* expression in the same cDNA preparations.

RESULTS

Mutation of *sarA*, but not *ica*, results in a reduced capacity to form a biofilm in vitro. In a previous report from our laboratory, we demonstrated that mutation of *sarA* in clinical isolates of *S. aureus* results in a reduced capacity to form a biofilm (5). Although our experiments did not address the mechanistic basis for this, Valle et al. (66) also observed that mutation of *sarA* results in a reduced capacity to form a biofilm and concluded that this was due to the impact of SarA on production of PIA (also known as PNAG). To more definitively address the role of PNAG in biofilm formation by our clinical isolates, we generated an *S. aureus* UAMS-1 *ica* mutant and assessed its ability to form a biofilm in vitro. Mutation of the *ica* locus was confirmed by Southern blotting (Fig. 1A), and the inability of the *ica* mutant to produce PNAG was confirmed by immunoblotting using PNAG-specific antisera (Fig. 1B). We found that mutation of *ica*, and the resulting inability to produce PNAG, had little impact on biofilm formation (Fig. 2). In contrast, mutation of *sarA* resulted in a reduced capacity to form a biofilm. The relative capacities of the UAMS-1 *sarA* and *ica* mutants to form a biofilm were evident both in our microtiter plate assay (Fig. 2A) and in flow cells (Fig. 2B). Under in vitro growth conditions, mutation of *sarA* resulted in reduced production of PNAG (Fig. 1B), which is consistent with the results of Valle et al. (66). However, the results ob-

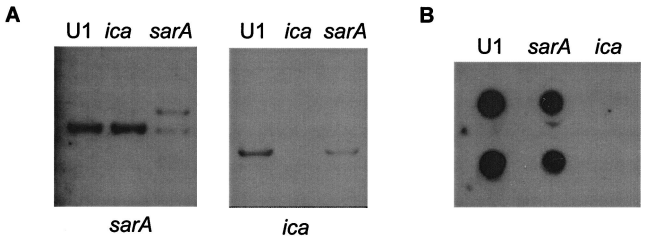

FIG. 1. Confirmation of *S. aureus* UAMS-1 *sarA* and *ica* mutants. (A) Genomic DNA isolated from UAMS-1 (U1) and its corresponding *ica* or *sarA* mutants was digested with HpaI and blotted with probes corresponding to an internal fragment of the *ica* operon or the *sarA* locus. (B) Dot blot analysis of PNAG expression in *S. aureus* strain UAMS-1 and its corresponding *ica* and *sarA* mutants. Duplicate samples prepared as described in Materials and Methods were spotted onto membranes and analyzed using antiserum raised against *S. aureus* PNAG.

served with our *ica* mutant make it difficult to conclude that this would explain the biofilm-deficient phenotype of the UAMS-1 *sarA* mutant.

Mutation of *sarA*, but not *ica*, results in a reduced capacity to form a biofilm in vivo. The results discussed above suggest that *ica* is not required for biofilm formation in at least some strains of *S. aureus* and that the reduced capacity of a UAMS-1 *sarA* mutant to form a biofilm in vitro is not a function of the impact of the *sarA* mutation on expression of the *ica* operon or production of PNAG. However, in *S. aureus*, it is well established that expression of the *ica* locus is tightly regulated and that it is preferentially expressed under in vivo conditions (42). This leaves open the possibility that the results we observed in vitro do not reflect the situation observed in vivo.

To examine this issue directly, we assessed the relative abilities of *S. aureus* UAMS-1 and its *sarA* and *ica* mutants to form a biofilm in vivo using a murine model of catheter-associated biofilm formation (31). The average numbers of bacteria obtained from explanted catheters at 10 days postinfection were 7.1×10^7 CFU per catheter in mice infected with UAMS-1 and 5.9×10^7 CFU per catheter in mice infected with the *ica* mutant (Fig. 3). In contrast, we recovered only 2.3×10^7 CFU per catheter from mice infected with the UAMS-1 *sarA* mutant. Although the *sarA* mutant was capable of colonizing the implanted catheter, the reduced recovery observed with the *sarA* mutant was statistically significant compared to the recovery for both UAMS-1 ($P = 0.001$) and its *ica* mutant ($P = 0.022$).

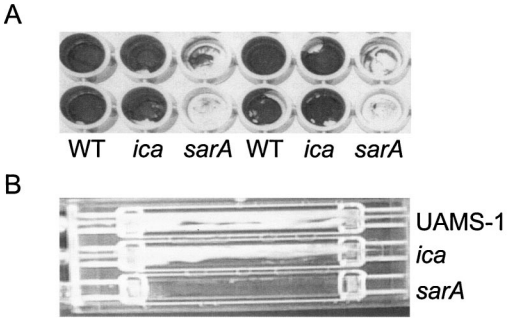

FIG. 2. Biofilm formation in *S. aureus* UAMS-1 *sarA* and *ica* mutants in vitro. Biofilm formation under static (A) and flow (B) conditions was assessed as described in Materials and Methods. WT, wild type.

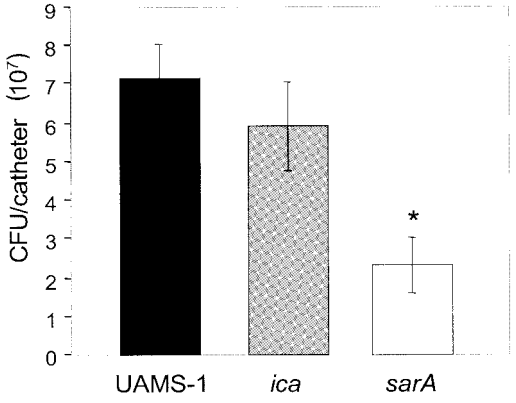

FIG. 3. Biofilm formation in *S. aureus* UAMS-1 *sarA* and *ica* mutants in vivo. Bacteria were recovered from implanted catheters after 10 days in vivo. The number of bacteria recovered from the catheters was determined by plate count as described in Materials and Methods. The value that was statistically significantly different ($P < 0.05$) from the values for UAMS-1 and the *ica* mutant is indicated by the asterisk.

To further investigate the relative capacities of *S. aureus* UAMS-1 and its *ica* and *sarA* mutants to form a biofilm in vivo, we also performed experiments in which catheters were coinfected with equivalent numbers of both UAMS-1 and its *ica* mutant or UAMS-1 and its *sarA* mutant (in both cases, total inoculum of 2×10^5 CFU). In mice coinfected with UAMS-1 and its *ica* mutant, we recovered 3.4×10^7 CFU per catheter of the wild-type strain and 2.3×10^7 CFU per catheter of the *ica* mutant (Fig. 4A). These results confirm our in vitro experiments and demonstrate that UAMS-1 and its corresponding *ica* mutant have an equivalent capacity to form a biofilm not only in vitro but also in vivo. In contrast, when we examined mice coinfected with UAMS-1 and its *sarA* mutant, the number of wild-type cells recovered was similar to the number found in previous experiments (4.9×10^7 CFU), but the number of the UAMS-1 *sarA* mutant we recovered had decreased to an average of only 8.0×10^5 CFU per catheter (Fig. 4B). These results also confirm the results of our in vitro experiments. Moreover, the reduced recovery of the *sarA* mutant in coinfection experiments relative to in vivo experiments in which the *sarA* mutant was introduced without competition from the wild-type strain also indicates that the wild-type strain has a competitive advantage that further limits the capacity of a *sarA* mutant to form a biofilm in vivo.

Transcriptional profiling in *S. aureus* UAMS-1 planktonic cultures. Taken together, the results discussed above confirm that a UAMS-1 *sarA* mutant has a reduced capacity to form a biofilm and that this is not a function of the impact of *sarA* on expression of the *ica* operon or production of PNAG. Moreover, the results observed with our in vitro models were consistent with the results observed in our in vivo model. Because *sarA* is a global regulator of gene expression in *S. aureus*, this suggests that other elements of the *sarA* regulon are also important in biofilm formation both in vitro and in vivo. Presumably, these elements could be identified by defining transcriptional changes observed within *S. aureus* biofilms and correlating these changes with experiments aimed at defining the *sarA* regulon. Because comprehensive transcriptional profiling of an *S. aureus sarA* mutant has been reported (19), we focused our efforts

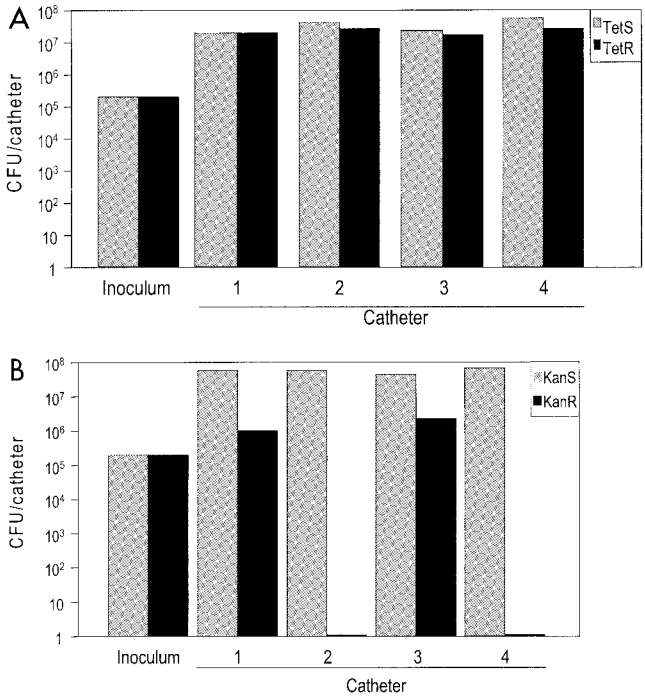

FIG. 4. Biofilm formation in coinfection experiments with *S. aureus* UAMS-1 and its *sarA* and *ica* mutants in vivo. Catheters were coinfected with equal numbers of UAMS-1 and its *ica* mutant (A) or UAMS-1 and its *sarA* mutant (B). The total number of bacteria in the inoculum and the total number recovered from each catheter was determined by plate count on nonselective medium. The number of bacteria resistant to tetracycline (A) or kanamycin (B) was subsequently determined by transferring individual colonies to selective media. Resistance to tetracycline and kanamycin is indicative of the *ica* and *sarA* mutations, respectively. Abbreviations: TetS and TetR, tetracycline sensitive and resistant, respectively; KanS and KanR, kanamycin sensitive and resistant, respectively.

on defining the transcriptional changes that occur when UAMS-1 is grown within a biofilm.

Because biofilm formation by *S. aureus* UAMS-1 requires supplementation of the medium with both glucose and salt (5), we also added these supplements to the medium used for planktonic culture. We first wanted to investigate whether the current paradigm (e.g., preferential expression of surface proteins during the exponential phase, followed by a shift to exoprotein production in the postexponential phase) was altered by supplementation of the medium. As expected, we found that expression of genes encoding protein A, clumping factor B, collagen adhesion, coagulase, and fibronectin-binding protein (*spa*, *clfB*, *cna*, *coa*, and *fnb*, respectively) were upregulated in the exponential versus stationary phase (6, 44, 45) (Table 2). In contrast, expression of the gene (*clfA*) encoding a second fibrinogen-binding protein (ClfA) was upregulated in the postexponential phase. This is consistent with previous reports examining the temporal expression of *clfA* (72, 73). Expression of the secreted proteins was also elevated in stationary phase as expected. Specific examples include the genes encoding several cysteine proteases, the Clp proteinase, alphatoxin, and the genes within the accessory gene regulator (*agr*) operon (*sspA*, *sspB*, *sspC*, *clpC*, *hla*, RNAII, and RNAIII, respectively). Taken together, these results confirm that our growth conditions accurately reflect the transition between the exponential and stationary growth phases and that this transition is not dramatically altered by supplementation of the medium in a manner that promotes biofilm formation.

Transcriptional profiling of *S. aureus* UAMS-1 biofilms. We identified a total of 580 genes that were expressed in an altered fashion when UAMS-1 was harvested from a mature biofilm. The greatest distinction, at least in terms of overall numbers of differentially expressed genes, was between the biofilm mode of growth and the exponential growth phase of planktonic culture (Table 3); however, a significant number of genes were also differentially expressed in comparison to stationary-phase cultures (Table 4). These findings clearly imply that *S. aureus* biofilms represent a unique growth condition by comparison to both exponential- and stationary-phase planktonic cultures. Indeed, we identified 48 genes whose expression was enhanced at least twofold in a biofilm in comparison to both exponential- and stationary-phase planktonic cultures (Table 5). These 48 genes included 30 genes located in six independent clusters as determined by their N315 ORF numbers. Included among these linked genes were the arginine deiminase cluster (*arc*; N315-SA2424-SA2428), a potassium-specific transport system (*kdp*; N315-SA1879-SA1881), the pyrimidine biosynthesis operon (*pyr*; N315-SA1041-SA1049), and the urease operon (*ure*; N315-SA2081-2088). Interestingly, only one gene in the *ica* locus (*icaD*) was found to be significantly upregulated in a biofilm, and this was limited to the comparison between biofilms and the stationary phase of planktonic growth (Table 4). This is consistent with a recent report concluding that *ica* expression is associated with the initial colonization of *S. epidermidis* in a foreign body infection model but not with its persistence (67).

We also identified 84 genes whose expression was reduced by a least a factor of at least 2 by comparison with both planktonic growth conditions (Table 5). Included were 25 genes in eight possible operons including an oligopeptide transport system (*opp*; N315-SA0845-SA0848) and the genes responsible for purine biosynthesis (*pur*; N315-SA0920-SA0925). Most genes in the other six putative operons encode hypothetical or conserved hypothetical proteins with no known function. However, one well-defined gene that was drastically downregulated in biofilms (60 to 139 times higher in the exponential-phase cultures and 12 to 27 times higher in the stationary-phase cultures) was *spa*, the gene that encodes protein A.

Confirmation of transcriptional profiling by real-time PCR. To verify the results of our microarray experiments, we used real-time PCR to examine the relative expression levels of selective target genes. These comparisons were done using RNA isolated from two independent cultures representing each of three growth conditions (biofilm and exponential and stationary growth phases). As observed in our profiling experiments, the *arcA*, *pyrR*, and *ureA* transcripts were present in greater quantities in the biofilm samples than in both exponential- and stationary-phase planktonic cultures (Fig. 5). Indeed, while the patterns of gene expression observed with real-time PCR were consistent with our profiling experiments, the results from the real-time PCR experiments suggest that our profiling experiments may underestimate the actual differences. As with our profiling experiments, we also found that *spa* was significantly downregulated in biofilms on the basis of real-time PCR com-

TABLE 2. Selected genes differentially expressed in exponential-phase versus stationary-phase cells

N315 ORF[a]	Common name[a]	Function[a]	ER[b]
N315-SA0144	*cap5A*	Capsular polysaccharide synthesis enzyme	0.312
N315-SA0145	*cap5B*	Capsular polysaccharide synthesis enzyme	0.306
N315-SA0146	*cap5C*	Capsular polysaccharide synthesis enzyme	0.223
N315-SA0147	*cap5D*	Capsular polysaccharide synthesis enzyme	0.244
N315-SA0148	*cap5E*	Capsular polysaccharide synthesis enzyme	0.206
N315-SA0149	*cap5F*	Capsular polysaccharide synthesis enzyme	0.204
N315-SA0150	*cap5G*	Capsular polysaccharide synthesis enzyme	0.187
N315-SA0155	*cap5L*	Capsular polysaccharide synthesis enzyme	0.277
N315-SA0156	*cap5M*	Capsular polysaccharide synthesis enzyme	0.232
N315-SA0157	*cap5N*	Capsular polysaccharide synthesis enzyme	0.267
N315-SA0158	*cap5O*	Capsular polysaccharide synthesis enzyme	0.262
N315-SA0159	*cap5P*	Capsular polysaccharide synthesis enzyme	0.267
N315-SA0899	*sspC*	Cysteine protease	0.400
N315-SA0900	*sspB*	Cysteine protease precursor	0.393
N315-SA1007		α-Hemolysin precursor	0.327
N315-SA1842	*agrB-c*	Accessory gene regulator B	0.449
N315-SA1843	*agrC-124c*	Accessory gene regulator C	0.269
N315-SA1844	*agrA*	Accessory gene regulator A	0.289
N315-SA2336	*clpC*	ATP-dependent Clp proteinase chain	0.284
	agrB-3	99.5% protein ID[c] to AgrB	0.410
	agrC-3	99.5% protein ID to AgrC	0.364
	agrC-3c	99.5% protein ID to AgrC	0.431
	agrD-3	100% protein ID to AgrD	0.389
	cap8H	100% protein ID to Cap8H capsular polysaccharide synthesis enzyme	0.176
	cap8I	100% protein ID Cap8I capsular polysaccharide synthesis enzyme	0.227
	cap8J	100% protein ID Cap8J capsular polysaccharide synthesis enzyme	0.204
	cap8K	100% protein ID to Cap8K capsular polysaccharide synthesis enzyme	0.226
		86.7% protein ID to ClfA fibrinogen-binding protein A	0.476
	sspA	99.4% protein ID to glutamic acid-specific protease prepropeptide truncated alpha-toxin	0.333
			0.311
N315-SA0107	*spa*	Immunoglobulin G-binding protein A precursor	5.084
N315-SA2423	*clfB*	Clumping factor B	2.744
	clfB	93.7% protein ID to ClfB (clumping factor B)	3.479
	cna	98.7% protein ID to MW2612 collagen adhesin precursor	7.974
	coa	99.8% protein ID to Coa coagulase	3.675
	fnb homolog	87.8% protein ID to Fnb	3.975
	spa		5.007

[a] Based on the published sequence of *S. aureus* strain N315. For genes not present in N315, the gene name and description given are from the *S. aureus* strain COL genome, available from The Institute for Genomic Research website (http://www.tigr.org) or by the putative function.
[b] Normalized values based on the expression ratio (ER), defined as the expression level in exponential-phase cells/expression level in stationary-phase cells.
[c] ID, identity.

parisons (Fig. 5). Collectively, the real-time PCR results provide independent verification of our DNA microarray results.

Roles of genes regulated by *sarA* in biofilm formation. For the reasons discussed above, we are particularly interested in genes that are differentially expressed in biofilms and are part of the *sarA* regulon. Therefore, we compiled a list of genes that were reported by Dunman et al. (19) to be regulated by *sarA* and were either induced or repressed in a biofilm compared to either planktonic condition. This analysis revealed 27 genes that were part of the *sarA* regulon and were differentially expressed in biofilms (Table 6). Because these genes may be genes that are required for biofilm formation, genes that are induced in biofilms and positively regulated by *sarA* would be of particular interest; however, we identified only four genes (*sdhB*, *carA*, an unidentified ORF with similarity to a major histocompatibility complex [MHC] class II analog, and a hypothetical protein) that fell into this category. At the same time, it may be equally important that specific genes be turned off to facilitate biofilm formation, and we identified eight genes (*arc*, *phoP*, *pbp3*, *nuc*, *ndhG*, *spa*, and two hypothetical proteins) that were repressed in biofilms and negatively regulated by *sarA*. The remaining genes were divergently regulated by *sarA* and in biofilms; however, the possibility that the impact of *sarA* is indirect in these cases cannot be ruled out.

DISCUSSION

Valle et al. (66) was the first to demonstrate that mutation of *sarA* results in a reduced capacity to form a biofilm. They concluded that this was due, at least in part, to reduced expression of the *icaADBC* operon. At the same time, Valle et al. (66) also suggested that SarA enhances biofilm formation by suppressing production of a second, unidentified protein that was either a repressor of PNAG synthesis or was involved in the turnover of PNAG. On this basis, they proposed a model in which the impact of *sarA* on biofilm formation was dependent on two pathways, both of which functioned by moderating the production of PNAG.

In this report, we also demonstrated that mutation of *sarA* results in reduced production of PNAG. To further investigate the impact of this on biofilm formation in our clinical isolate, we generated an *ica* mutant and examined its capacity to form a biofilm both in vitro and in vivo. Surprisingly, our *ica* mutant formed a biofilm comparable to that of the parental strain

TABLE 3. Genes differentially expressed in a biofilm versus exponential growth phase

N315 ORF[a]	Common name[a]	Product[a,b]	ER[c]
Cell envelope and cellular processes			
N315-SA1960	*mtlF*	PTS system, mannitol-specific IIBC component	5.46
N315-SA1882	*kdpD*	Sensor protein KdpD	5.13
N315-SA2311		Similar to NAD(P)H-flavin oxidoreductase	2.71
N315-SA1156		ABC transporter (ATP-binding protein) homolog	2.68
N315-SA0724		Similar to cell division inhibitor	0.496
N315-SA2253	*opp-1C*	Oligopeptide transporter putative membrane permease domain	0.490
N315-SA0567		Similar to iron(III) ABC transporter permease protein	0.474
N315-SA2216		Similar to ABC transporter, ATP-binding protein	0.471
N315-SA0980		Similar to ferrichrome ABC transporter	0.471
N315-SA0981		Similar to ferrichrome ABC transporter	0.468
N315-SA0592	*tagA*	Teichoic acid biosynthesis protein	0.462
N315-SA1935	*hmrA*	Similar to amidase	0.442
N315-SA1169		γ-Aminobutyrate permease	0.425
N315-SA0243	*tagB*	Similar to teichoic acid biosynthesis protein B	0.423
N315-SA0110	*sirB*	Lipoprotein	0.399
N315-SA2100		Similar to autolysin E	0.394
N315-SA1458	*lytH*	*N*-Acetylmuramoyl-L-alanine amidase	0.393
N315-SA0109	*sirC*	Lipoprotein	0.378
N315-SA0106	*lctP*	L-Lactate permease homolog	0.376
N315-SA0682		Similar to ditripeptide ABC transporter	0.370
N315-SA2053		Glucose uptake protein homolog	0.331
N315-SA0479	*nupC*	Pyrimidine nucleoside transport protein	0.315
N315-SA0111	*sirA*	Lipoprotein	0.312
N315-SA2339		Similar to antibiotic transport-associated protein	0.291
N315-SA0566		Similar to iron-binding protein	0.287
N315-SA2233		Similar to integral membrane efflux protein	0.281
N315-SA0325	*glpT*	Glycerol-3-phosphate transporter	0.281
N315-SA2112		Similar to sodium-dependent transporter	0.278
N315-SA1025	*mraY*	Phospho-*N*-muramic acid-pentapeptide translocase	0.265
N315-SA0600		Similar to pyrimidine nucleoside transporter	0.255
N315-SA1978		Similar to ferrichrome ABC transporter (permease)	0.203
N315-SA0010	*azlC*	Similar to amino acid permease	0.161
N315-SA2300		Similar to glucarate transporter	0.141
N315-SA0691	*sstD*	Lipoprotein, similar to ferrichrome ABC transporter	0.126
N315-SA0374	*pbuX*	Xanthine permease	0.089
N315-SA0579		Similar to Na^+/H^+ antiporter	0.080
N315-SA0411	*ndhF*	NADH dehydrogenase subunit 5	0.065
N315-SA2302	*stpC*	Similar to ABC transporter	0.046
N315-SA2303	*smpC*	Similar to membrane-spanning protein	0.041
Information pathways			
N315-SA1883	*kdpE*	KDP operon transcriptional regulatory protein	5.42
N315-SA2429	*ArgR*	Similar to arginine repressor	3.92
N315-SA2296		Similar to transcriptional regulator, MerR family	3.72
N315-SA2418		Similar to two-component response regulator	2.13
N315-SA0460	*pth*	Peptidyl-tRNA hydrolase	0.490
N315-SA0652		Similar to transcription regulation protein	0.452
N315-SA1853		Similar to DNA mismatch repair protein MutS	0.445
N315-SA1287	*asnS*	Asparaginyl-tRNA synthetase	0.441
N315-SA0348		Similar to transcription terminator	0.440
N315-SA2358		Similar to transcriptional regulator (TetR/AcrR family)	0.382
N315-SA1697		Similar to protein-tyrosine phosphatase	0.364
N315-SA1120		Similar to transcription regulator GntR family	0.354
N315-SA0298	*pfoR*	Similar to regulatory protein PfoR	0.333
N315-SA1550	*tyrS*	Tyrosyl-tRNA synthetase	0.325
N315-SA2482	*pcp*	Pyrrolidone-carboxylate peptidase	0.297
N315-SA1583	*rot*	Repressor of toxins (Rot)	0.295
N315-SA0653	*fruR*	Similar to transcription repressor of fructose operon	0.229
N315-SA0904		Probable ATL autolysin transcription regulator	0.191
N315-SA1725	*sspB*	Staphopain, cysteine proteinase	0.074
Intermediary metabolism			
N315-SA0328		Similar to NADH-dependent FMN reductase	7.25
N315-SA0122	*butA*	Acetoin (diacetyl)reductase	5.04
N315-SA2297		Similar to GTP-pyrophosphokinase	3.25
N315-SA1142	*glpD*	Aerobic glycerol-3-phosphate dehydrogenase	2.97
N315-SA0016	*purA*	Adenylosuccinate synthase	2.37
N315-SA2397		Similar to pyridoxal-phosphate-dependent aminotransferase	2.04
N315-SA2001		Similar to oxidoreductase, aldo/keto reductase family	2.01
N315-SA1201	*trpD*	Anthranilate phosphoribosyltransferase	0.495
N315-SA1685	*mutY*	Similar to A/G-specific adenine glycosylase	0.481

Continued on following page

TABLE 3—*Continued*

N315 ORF[a]	Common name[a]	Product[a,b]	ER[c]
N315-SA2111		Similar to phosphoglycolate phosphatase	0.475
N315-SA1052	*gmk*	Guanylate kinase homolog	0.462
N315-SA2279		Similar to phosphomannomutase	0.458
N315-SA0902		HisC homolog	0.448
N315-SA0177	*argJ*	Arginine biosynthesis bifunctional protein homolog	0.436
N315-SA1310	*ansA*	Probable L-asparaginase, gene-*ansA*	0.429
N315-SA1309	*cmk*	Cytidylate kinase	0.427
N315-SA1749		Similar to aspartate transaminase protein	0.423
N315-SA2140		Similar to esterase	0.412
N315-SA0507		Similar to *N*-acyl-L-amino acid amidohydrolase	0.407
N315-SA0568		Similar to L-2-haloalkanoic acid dehalogenase	0.374
N315-SA2213	*bioB*	Biotin synthase	0.373
N315-SA0514		Similar to deoxypurine kinase	0.356
N315-SA1121		Similar to processing proteinase homolog	0.340
N315-SA1203	*trpF*	Phosphoribosylanthranilate isomerase	0.323
N315-SA2342	*thgA*	Similar to *O*-acetyltransferase	0.280
N315-SA0511		Similar to UDP-glucose 4-epimerase-related protein	0.256
N315-SA1202	*trpC*	Indole-3-glycerol phosphate synthase	0.246
N315-SA2395	*ldh*	L-Lactate dehydrogenase	0.213
N315-SA0180	*bmQ*	Similar to branched-chain amino acid transport system carrier protein	0.192
N315-SA1200	*trpG*	Anthranilate synthase component II	0.120
N315-SA1199	*trpE*	Similar to anthranilate synthase component I	0.101
N315-SA0373	*xprT*	Xanthine phosphoribosyltransferase	0.077
Other functions			
N315-SA0899	*sspC*	Cysteine protease	7.24
N315-SA0900	*sspB*	Cysteine protease precursor	7.03
N315-SA0150	*cap5G*	Capsular polysaccharide synthesis enzyme	4.80
N315-SA2006		Similar to MHC class II analog	4.69
N315-SA0149	*cap5F*	Capsular polysaccharide synthesis enzyme	4.58
N315-SA0148	*cap5E*	Capsular polysaccharide synthesis enzyme	4.05
N315-SA0146	*cap5C*	Capsular polysaccharide synthesis enzyme	3.31
N315-SA0147	*cap5D*	Capsular polysaccharide synthesis enzyme	3.30
N315-SA0145	*cap5B*	Capsular polysaccharide synthesis enzyme Cap5B	2.43
N315-SA0144	*cap5A*	Capsular polysaccharide synthesis enzyme	2.34
N315-SA0841		Similar to cell surface protein Map-w	2.22
N315-SA1709		Similar to ferritin	2.18
N315-SA0754		Similar to lactococcal prophage ps3 protein 05	0.498
N315-SA1835	*int*	Similar to integrase (pathogenicity island SaPln1), gene = *int*	0.431
N315-SA1559		Similar to smooth muscle caldesmon	0.426
N315-SA0797	*nifU-3*	Similar to nitrogen fixation protein NifU	0.407
N315-SA0780		Similar to hemolysin	0.331
N315-SA0746	*nuc*	Staphylococcal nuclease	0.170
N315-SA1766		HP (bacteriophage ϕN315)	0.069
N315-SA1775		Similar to scaffolding protein (bacteriophage ϕN315)	0.048
N315-SA1765		HP (bacteriophage ϕN315)	0.042
N315-SA1777		HP (bacteriophage ϕN315)	0.038
N315-SA1771		HP (bacteriophage ϕN315)	0.036
N315-SA1762		HP (bacteriophage ϕN315)	0.036
Similar to unknown proteins			
N315-SA0326		CHP (lactoylglutathione lyase and related lyases)	7.94
N315-SA0327		CHP (flavin-dependent oxidoreductases)	7.50
N315-SA2479		CHP	5.04
N315-SA0007		Predicted sugar kinase	4.57
N315-SA0380		CHP (pathogenicity island SaPln2)	3.07
N315-SA0381		CHP (pathogenicity island SaPln2)	2.80
N315-SA1235		CHP	2.04
N315-SA1890		CHP	2.03
N315-SA0230		CHP	2.02
N315-SA0941		CHP	0.497
N315-SA0467		CHP (predicted ATPase of the PP-loop superfamily)	0.489
N315-SA1838		CHP (predicted metal-dependent membrane protease)	0.481
N315-SA2487	*rarD*	Similar to RarD protein	0.462
N315-SA1696		CHP	0.453
N315-SA1448		CHP (TPR repeat-containing proteins)	0.452
N315-SA0329		CHP	0.445
N315-SA2328		CHP (putative effector of murein hydrolase, LrgB)	0.434
N315-SA0979		CHP	0.433
N315-SA1928		HP	0.421
N315-SA1601	*crcB*	CHP (integral membrane protein)	0.404
N315-SA2377		CHP	0.398

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TABLE 3—*Continued*

N315 ORF[a]	Common name[a]	Product[a,b]	ER[c]
N315-SAS081		CHP (ATPase involved in DNA repair)	0.391
N315-SA0334		CHP (Sec-independent protein secretion pathway)	0.378
N315-SA2305		CHP	0.372
N315-SA0840		CHP (phospholipid-binding protein)	0.370
N315-SA1903		CHP	0.366
N315-SA0543		CHP (uncharacterized BCR)	0.353
N315-SA0413		CHP	0.332
N315-SA0341		Similar to low-temperature requirement A protein	0.329
N315-SA0345		CHP (methionine synthase I)	0.324
N315-SA0257		CHP (SAM-dependent methyltransferases)	0.323
N315-SA2096		CHP	0.320
N315-SA0773		CHP (predicted membrane protein)	0.312
N315-SA2212		Similar to 8-amino-7-oxononanoate synthase	0.303
N315-SA1252		CHP (histone acetyltransferase)	0.298
N315-SA2452		CHP (domain typically associated with flavoprotein oxygenases)	0.282
N315-SA0870		CHP (predicted permease)	0.267
N315-SA0335		CHP (Sec-independent protein secretion pathway)	0.265
N315-SA0556		CHP	0.203
N315-SA0753		CHP (lysine efflux permease)	0.179
N315-SA2219		CHP (uncharacterized membrane protein)	0.133
N315-SA0739		CHP	0.129
N315-SAS001		CHP	0.128
N315-SA0412		CHP	0.078
No similarity			
N315-SAS016		HP	12.74
N315-SA0883		HP	5.97
N315-SA1233		HP	2.25
N315-SA0414		HP	0.496
N315-SA2126		HP	0.495
N315-tRNA12	tRNA-Pro		0.491
N315-SA1943		HP	0.491
N315-SA1215		HP	0.486
N315-tRNA11	tRNA-Arg		0.479
N315-SA0613		HP	0.470
N315-SA2485		HP	0.457
N315-SA0088		HP	0.443
N315-tRNA57	tRNA-Lys		0.416
N315-tRNA47	tRNA-Leu		0.409
N315-SA1607		HP	0.402
N315-SA0955		HP	0.372
N315-tRNA07	tRNA-Thr		0.355
N315-SA0105		HP	0.344
N315-SA2118		HP	0.335
N315-SA0336		HP	0.326
N315-SA0363		HP	0.321
N315-SA2055		HP	0.321
N315-SA2224		HP	0.309
N315-SA2249		HP	0.259
N315-SA0749		HP	0.233
N315-tRNA06	tRNA-Val		0.219
N315-SA0748		HP	0.214
N315-SA0889		HP	0.179
N315-SA2488		HP	0.157
N315-SA1778		HP (bacteriophage ϕN315)	0.089
N315-SA1768		HP (bacteriophage ϕN315)	0.050
N315-SA1770		HP (bacteriophage ϕN315)	0.050
N315-SA1774		HP (bacteriophage ϕN315)	0.046
N315-SA1776		HP (bacteriophage ϕN315)	0.044
N315-SAS060		HP (bacteriophage ϕN315)	0.041
N315-SA1769		HP (bacteriophage ϕN315)	0.040
N315-SA1773		HP (bacteriophage ϕN315)	0.032
N315-SA1772		HP (bacteriophage ϕN315)	0.031
N315-SA1767		HP (bacteriophage ϕN315)	0.027
No N315 ORF			
	set5	100% protein ID Set5, exotoxin 5, and HsdM-like protein gene	40.43
	sspA	99.4% protein ID *S. aureus* glutamic acid-specific protease	7.19
	cap8H	100% protein ID to capsular polysaccharide synthase enzyme Cap8H	5.11
	cap8J	100% protein ID to capsular polysaccharide synthesis enzyme Cap8J	4.52
		58.1% protein ID malofactic enzyme, *Oenococcus oeni* bacteria	3.05
		68.1% protein ID SA0329 CHP	2.39

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TABLE 3—*Continued*

N315 ORF[a]	Common name[a]	Product[a,b]	ER[c]
		92.8% protein ID MW1748 HP	2.25
		84.4% protein ID MW0360 HP	2.16
		96.7% protein ID to MW2134 HP	0.500
		95.6% protein ID SA2230	0.473
		58.4% protein ID BH3950 transposase (10), *Bacillus halodurans*	0.467
		49.1% protein ID MW2618	0.463
COL-SA1788		HP	0.434
		87.6% protein ID MW0584	0.423
COL-SA2299		HP	0.421
	Similar to *splE*	62.6% protein ID serine protease SplE	0.420
		97.7% protein ID to SA1559	0.419
		26.9% protein ID MW2498	0.418
COL-SA0866		HP	0.411
	Serine protease	55.4% protein ID serine protease SplB	0.409
		96% protein ID MW2325	0.387
		95.5% protein ID MW0355 HP	0.371
		22.2% protein ID SA0283 HP	0.345
		89.4% protein ID SA0553 CHP	0.338
		78.2% protein ID MW1720 HP	0.315
COL-SA1556		HP	0.311
		99.4% protein ID to MW0053 CHP	0.308
		45.9% protein ID BH3950 transposase (10), *Bacillus halodurans*	0.290
		97.7% protein ID MW0355 HP	0.289
	splB	97.7% protein ID to serine protease SplB	0.280
		79.2% protein ID MW1043 HP	0.270
COL-SA2728		HP	0.237
		99.5% id to SAV1992 HP	0.209
		37.3% protein ID MW1769 HP	0.202
COL-SA1140	*sai-1*	29-kDa cell surface protein	0.164
		85.2% protein ID MW1042 HP	0.141
		99.5% protein ID MWP018	0.138
		99.8% protein ID MWP016 *S. aureus* plasmid pMW2	0.096
		93.4% protein ID SAV1953 ϕPVL ORF 20 and 21 homolog	0.079
Mu50-SAV1953		ϕPVL ORF 20 and 21 homolog	0.071
		100% protein ID to MWP017 HP	0.070
		100% protein ID MW1894 HP	0.060
		78% protein ID MW1892 HP	0.055
		83.2% protein ID SA1763 HP	0.032
		100% protein ID SAP019 HP, *S. aureus* N315 plasmid N315B	0.031

[a] Based on the published sequence of *S. aureus* strain N315. For genes not present in N315, the gene name and description given are from the *S. aureus* strain COL genome, available from The Institute for Genomic Research website (http://www.tigr.org) or by the putative function.
[b] Abbreviations: PTS, phosphotransferase; HP, hypothetical protein; CHP, conserved hypothetical protein; SAM, *S*-adenosylmethionine; ID, identity.
[c] Normalized values based on the expression ratio (ER), which is defined as the expression level in exponential-phase cells/expression level in stationary-phase cells.

under both static and flow conditions. As in our previous experiments (5), the capacity of our UAMS-1 *sarA* mutant to form a biofilm was reduced in comparison to both the wild-type strain and its corresponding *ica* mutant. This clearly indicates that the impact of *sarA* on biofilm formation, at least as defined under in vitro growth conditions, involves a pathway that is independent of the *icaADBC* operon.

The role of *ica* in vivo has been addressed in *S. epidermidis* with contradictory results (10, 23, 41, 47, 49, 55, 56, 57, 62). However, few studies have addressed this issue in *S. aureus*. Cramton et al. (14) demonstrated that *ica* is present in *S. aureus* and that it is required for PIA production and biofilm formation in that species. However, the mutagenesis experiments were limited to a single strain of *S. aureus* (SA113) that was derived from NCTC 8325 by chemical mutagenesis, so these results may not be representative of the situation observed in clinical isolates. More recently, Vandecasteele et al. (67) analyzed expression of biofilm-associated genes, including *icaA* and *icaC*, both in vitro and in vivo. While expression of both genes was induced upon initial exposure to foreign bodies, this induction peaked shortly after the introduction of bacteria and was followed by a slow decrease over time. These results suggest that the *ica* operon is mainly associated with the initial colonization phase of biofilm formation, rather than maturation and persistence. This is consistent with our results and the fact that our analysis was limited to mature biofilms. Additionally, Francois et al. (23) compared an *S. aureus* strain and its corresponding *ica* mutant using a tissue cage model and found that the *ica* mutant retained the capacity to colonize at a level comparable to that of the wild-type strain. They concluded on this basis that biofilms were not an important factor in their model. However, we believe there are two possible alternative explanations. The first is that their model does not accurately reflect the need to form a biofilm. The second is that their *ica* mutant retained the capacity to form a biofilm under in vivo conditions. Our results would support the latter hypothesis. Specifically, we found that while the UAMS-1 *sarA* mutant demonstrated a reduced capacity to colonize catheters in vivo, the UAMS-1 *ica* mutant colonized the same substrates as well as the wild-type strain did. These results also suggest that PNAG is not required for in vivo colonization by *S. aureus* and that *sarA* regulates genes required for biofilm formation inde-

TABLE 4. Genes differentially expressed in a biofilm versus stationary growth phase

N315 ORF[a]	Common name[a]	Product[a,b]	ER[c]
Cell envelope and cellular processes			
N315-SA0655	*fruA*	Fructose-specific permease	14.43
N315-SA0263		Similar to proton antiporter efflux pump	9.55
N315-SA2142	*semB*	Similar to multidrug resistance protein	4.96
N315-SA0293		Similar to formate transporter NirC	4.64
N315-SA1140	*glpF*	Glycerol uptake facilitator	4.40
N315-SA2185	*narG*	Respiratory nitrate reductase alpha chain	4.08
N315-SA2183	*narJ*	Similar to nitrate reductase delta chain	3.87
N315-SA2184	*narH*	Nitrate reductase beta chain NarH	3.56
N315-SA2053		Glucose uptake protein homolog	3.55
N315-SA0166		Similar to nitrate transporter	3.39
N315-SA0167		Similar to membrane lipoprotein SrpL	3.29
N315-SA0702	*llm*	Lipophilic protein affecting bacterial lysis rate and methicillin resistance level	2.83
N315-SA2222		Similar to bicyclomycin resistance protein TcaB	2.75
N315-SA0411	*ndhF*	NADH dehydrogenase subunit 5	2.41
N315-SA2176	*narK*	Nitrite extrusion protein	2.16
N315-SA1960	*mtlF*	PTS system, mannitol-specific IIBC component	0.486
N315-SA1381	*pbp3*	Penicillin-binding protein 3	0.482
N315-SA1219		Similar to phosphate ABC transporter	0.463
N315-SA2311		Similar to NAD(P)H-flavin oxidoreductase	0.460
N315-SA0367		Similar to nitroflavin reductase	0.410
N315-SA1982		Similar to transporter	0.397
N315-SA0260		Similar to ribose transporter RbsU	0.385
N315-SA2074	*modA*	Probable molybdate-binding protein	0.384
N315-SA1848	*amt*	Probable ammonium transporter	0.343
N315-SA0138		Similar to alkylphosphonate ABC transporter	0.323
N315-SA2203	EmrB/QacA subfamily	Similar to multidrug resistance protein	0.304
N315-SA0420		Similar to ABC transporter ATP-binding protein	0.286
N315-SA0422		Similar to lactococcal lipoprotein	0.272
N315-SA0421		Similar to ABC transporter permease protein	0.256
N315-SA0589		Similar to ABC transporter ATP-binding protein	0.163
N315-SA0849		Similar to peptide-binding protein OppA	0.148
Information pathways			
N315-SA0653	*fruR*	Similar to transcription repressor of fructose operon	14.57
N315-SA0476		Similar to transcription regulator GntR family	4.98
N315-SA1058	*def*	Similar to polypeptide deformylase	2.35
N315-SA0460	*pth*	Peptidyl-tRNA hydrolase	2.07
N315-SA1516	*phoP*	Alkaline phosphatase synthesis transcriptional regulatory protein	0.479
N315-SA0130		Similar to trehalose operon transcriptional repressor	0.477
N315-SA1805		Repressor homolog (bacteriophage ϕN315)	0.390
N315-SAS042	*rpmG*	50S ribosomal protein L33	0.362
N315-SA1394	*glyS*	Glycyl-tRNA synthetase	0.351
N315-SA1149	*glnR*	Glutamine synthetase repressor	0.319
N315-SA1360		Xaa-Pro dipeptidase	0.294
Intermediary metabolism			
N315-SA0654	*fruB*	Fructose-1-phosphate kinase	17.08
N315-SA1959	*glmS*	Glucosamine-fructose-6-phosphate aminotransferase	9.27
N315-SA0143	*adhE*	Alcohol-acetaldehyde dehydrogenase	5.79
N315-SA2186	*nasF*	Uroporphyrin III C-methyl transferase	4.56
N315-SA2187	*nasE*	Assimilatory nitrite reductase	4.15
N315-SA1929	*pyrG*	CTP synthase	3.97
N315-SA2188	*nirB*	Nitrite reductase	3.38
N315-SA0973	*kdtB*	Phosphopantetheine adenyltransferase homolog	2.29
N315-SA0572		Similar to esterase or lipase	0.493
N315-SA0528		Similar to hexulose-6-phosphate synthase	0.484
N315-SA1231	*dal*	Similar to alanine racemase	0.471
N315-SA2120		Similar to amino acid amidohydrolase	0.464
N315-SA0008	*hutH*	Histidine ammonia-lyase	0.461
N315-SA1584		Lysophospholipase homolog	0.456
N315-SA1230	*hipO*	Hippurate hydrolase	0.435
N315-SAS044	*dmpI*	4-Oxalocrotonate tautomerase	0.432
N315-SA1225	*lysC*	Aspartokinase II	0.429
N315-SA1229	*dapD*	Tetrahydrodipicolinate acetyltransferase	0.423
N315-SA0258	*rbsK*	Probable ribokinase	0.422
N315-SA0820	*glpQ*	Glycerophosphoryl diester phosphodiesterase	0.419
N315-SA0181	*entB*	Similar to isochorismatase	0.416
N315-SA0512	*ilvE*	Branched-chain amino acid aminotroansferase homolog	0.415
N315-SA2204	*gpm*	Phosphoglycerate mutase	0.408
N315-SA1227	*dapA*	Dihydrodipicolinate synthase	0.406
N315-SA2155	*mqo*	Similar to malate:quinone oxidoreductase	0.404
N315-SA1724	*purB*	Adenylosuccinate lyase	0.394

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TABLE 4—*Continued*

N315 ORF[a]	Common name[a]	Product[a,b]	ER[c]
N315-SA0304	*nanA*	*N*-Acetylneuraminate lyase subunit	0.384
N315-SA1150	*glnA*	Glutamine-ammonia ligase	0.362
N315-SA1531	*ald*	Alanine dehydrogenase	0.357
N315-SA1228	*dapB*	Dihydrodipicolinate reductase	0.354
N315-SA2125	*hutG*	Similar to formiminoglutamase	0.353
N315-SA0098		Similar to aminoacylase	0.346
N315-SA2127	*rpiA*	Similar to ribose 5-phosphate isomerase	0.341
N315-SA1226	*asd*	Aspartate semialdehyde dehydrogenase	0.341
N315-SA1545	*serA*	D-3-Phosphoglycerate dehydrogenase	0.341
N315-SA0679	*hisC*	Similar to histidinol-phosphate aminotransferase	0.337
N315-SA0658		Similar to plant metabolite dehydrogenases	0.335
N315-SA0656	*nagA*	Probable *N*-acetylglucosamine-6-phosphate deacetylase	0.333
N315-SA1184	*citB*	Aconitate hydratase	0.329
N315-SA0915	*folD*	FolD bifunctional protein	0.326
N315-SA1858	*ilvD*	Dihydroxy-acid dehydratase	0.280
N315-SA0430	*gltB*	Glutamate synthase large subunit	0.263
N315-SA1614	*menC*	*o*-Succinylbenzoic acid synthetase	0.234
N315-SA0431	*gltD*	NADH-glutamate synthase small subunit	0.199
N315-SA1553	*fhs*	Formyltetrahydrofolate synthetase	0.168
N315-SA0016	*purA*	Adenylosuccinate synthase	0.117
N315-SA0926	*purD*	Phosphoribosylamine-glycine ligase	0.049
N315-SA0917	*purK*	Phosphoribosylaminoimidazole carboxylase carbon dioxide fixation chain	0.034
N315-SA0916	*purE*	Similar to phosphoribosylaminoimidazole carboxylase	0.030
N315-SA0918	*purC*	Phosphoribosylaminoimidazolesuccinocarboxamide synthetase homolog	0.023
Other functions			
N315-SA2460	*icaD*	Intercellular adhesion protein D	34.06
N315-SA1898		Similar to SceD precursor	26.36
N315-SA2206	*sbi*	IgG-binding protein	26.16
N315-SA1000		Similar to fibrinogen-binding protein	11.51
N315-SA2097	Similar to SsaA	Similar to secretory antigen precursor	4.74
N315-SA2164		Similar to phage infection protein precursor	2.80
N315-SA1382	*sodA*	Superoxide dismutase SodA	0.497
N316-SA1606		Plant metabolite dehydrogenase homolog	0.480
N315-SA0841		Similar to cell surface protein Map-w	0.448
N315-SA1549	*htrA*	Similar to serine proteinase Do, heat shock protein	0.434
N315-SA2406	*gbsA*	Glycine betaine aldehyde dehydrogenase	0.399
N315-SA0659		Similar to CsbB stress response protein	0.390
N315-SA1312	*ebpS*	Elastin-binding protein	0.363
N315-SA0755		Similar to general stress protein 170	0.340
N315-SA1170	*katA*	Catalase	0.309
N315-SA0091	*plc*	1-Phosphatidylinositol phosphodiesterase precursor	0.300
N315-SA2405	*betA*	Choline dehydrogenase	0.252
Similar to unknown proteins			
N315-SA0213		CHP	17.26
N315-SA2256		CHP	4.76
N315-SA0341		HP similar to low-temperature requirement A protein	3.95
N315-SA1176		CHP	3.05
N315-SA0929		CHP	3.04
N315-SA1431		CHP	3.00
N315-SA1340		CHP (lactoylglutathione lyase)	2.53
N315-SAS027		CHP	2.50
N315-SA1932		Similar to HP T13D8.31 *Arabidopsis thaliana*	2.35
N315-SA1464	*yajC*	CHP (preprotein translocase subunit YajC)	2.30
N315-SA1540		CHP (GAF domain-containing protein)	2.24
N315-SA0165		Similar to α-helical coiled-coil protein SrpF	2.16
N315-SA0114		CHP	2.10
N315-SA0529		CHP (predicted sugar phosphate isomerase involved in capsule formation, GutQ)	0.486
N315-SA1019		CHP	0.467
N315-SA1737		CHP (3-carboxymuconate cylase)	0.466
N315-SA0801		CHP (IscA)	0.463
N315-SA1543		CHP (predicted redox protein, regulator of disulfide bond formation)	0.462
N315-SA1380		CHP (5-formyltetrahydrofolate cyclo-ligase)	0.454
N315-SA1129		CHP (predicted HD superfamily hydrolase)	0.447
N315-SA0861		CHP (hemoglobin-like proteins)	0.443
N315-SA0230		CHP	0.438
N315-SA1280		CHP	0.430
N315-SA0957		CHP	0.429
N315-SA1331		CHP (predicted oxidoreductases)	0.393
N315-SA1689		CHP	0.384
N315-SA0513		CHP (predicted phosphatases, Gph)	0.379
N315-SA2367		CHP (predicted hydrolases or acyltransferases)	0.356

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TABLE 4—*Continued*

N315 ORF[a]	Common name[a]	Product[a,b]	ER[c]
N315-SA1167		CHP (predicted hydrolases of the HAD superfamily)	0.337
N315-SA1690		CHP	0.324
N315-SA0089		Similar to DNA helicase	0.315
N315-SA0873		CHP	0.315
N315-SA1544		Similar to soluble hydrogenase 42-kDa subunit	0.314
N315-SA0741		CHP (predicted acetyltransferase)	0.310
N315-SA0362		CHP	0.310
N315-SA1281		CHP	0.310
N315-SA0649		CHP (predicted DNA-binding proteins with PD1-like DNA-binding motif)	0.286
N315-SA0407		CHP (chromosome segregation ATPases)	0.254
N315-SA1611		CHP (dipeptidyl aminopeptidases/acylaminoacyl-peptidases)	0.227
N315-SA0919		CHP (phosphoribosylformylglycinamidine [FGAM] synthase)	0.027
No similarity			
N315-SA0663		HP	7.97
N315-SA2281		HP	5.10
N315-SA0779		HP	3.44
N315-SA2376		HP	3.22
N315-SA0885		HP	3.16
N315-SA1670		HP	2.80
N315-SA2126		HP	2.62
N315-SA0336		HP	2.59
N315-SA0571		HP	2.37
N315-SA2058		HP	2.17
N315-SA0397	*lpl2*	HP (pathogenicity island SaPln2)	0.488
N315-SAS031		HP	0.480
N315-SA1168		HP	0.472
N315-SA0372		HP	0.468
N315-SA0404	*lpl8*	HP (pathogenicity island SaPln2)	0.447
N315-SA1319		HP	0.421
N315-SA0090		HP	0.366
N315-SA1546		HP	0.293
N315-SA0406		HP	0.258
N315-SA2497		HP	0.208
N315-SA0408		HP	0.198
N315-SA2496		HP	0.188
No N315 ORF		72.4% protein ID to MW1041	25.20
COL-SA0674		HP	6.12
COL-SA1165		HP	5.57
		98.1% protein ID to MW2274 CHP	5.33
		98.1% protein ID to NasE assimilatory nitrite reductase	4.25
		57% protein ID to spyM18_1050 HP, *S. pyogenes* MGAS8232	3.55
		88.2% protein ID to MW2323	3.11
		100% protein ID to SAP023 *S. aureus* N315 plasmid pN315B	2.32
		100% protein ID to MW2396	0.497
COL-SA1345		HP	0.491
		100% protein ID to SA1320 HP	0.482
		100% protein ID to lpl11 HP, *S. aureus* MW2	0.465
COL-SA2676	LPXTG	LPXTG-motif cell wall anchor domain protein	0.456
		91.5% protein ID to lpl2 HP, *S. aureus* N315	0.447
COL-SA0293		CHP	0.444
		39.1% protein ID to lin05-11 *Listeria innocua*	0.436
		26.6% protein ID to LigW 5-carboxyvanillate decarboxylase, *Sphingomonas paucimobilis*	0.416
		92.7% protein ID to Lpl7 HP, *S. aureus* N315	0.405
	binL	99.5% protein ID to BinL DNA-invertase, *S. aureus* plasmid pMW2	0.394
		94.8% protein ID to BinL DNA invertase, *S. aureus* plasmid pMW2	0.392
COL-SA0601		HP	0.372
		89.8% protein ID to Lpl10 HP, *S. aureus* MW2	0.364
		25.8% protein ID to MA2121 CHP, *Methanosarcina acetivorans* C2A	0.363
		88% protein ID to MW1374 CHP	0.363
		74.8% protein ID to lpl5 HP, *S. aureus* N315	0.331
COL-SA1343		HP	0.328
		48.6% protein ID to ycnB homolog of multidrug resistance protein B, *B. subtilis*	0.309
		71% protein ID to MW1201 HP	0.282
		93.5% protein ID to MW0402 HP	0.276
		84% protein ID to lpl2 HP, *S. aureus* N315	0.261
		35.9% protein ID to Cgl0945 putative multicopper oxidases, *Corynebacterium glutamicum*	0.259
		57.8% protein ID to CopB ATPase, *Enterococcus hirae*	0.214
		64.2% protein ID to SA0753 CHP	0.191
		33.6% protein ID to RtxC, *Bradyrhizobium elkanii*	0.158

[a] Based on the published sequence of *S. aureus* strain N315. For genes not present in N315, the gene name and description given are from the *S. aureus* strain COL genome, available from The Institute for Genomic Research website (http://www.tigr.org) or by the putative function.

[b] Abbreviations: PTS, phosphotransferase; IgG, immunoglobulin G; CHP, conserved hypothetical protein; HP, hypothetical protein; HAD, haloacid dehalogenase-family protein; ID, identity.

[c] Normalized values based on the expression ratio (ER), which is defined as the expression level in exponential-phase cells/expression level in stationary-phase cells.

TABLE 5. Genes differentially expressed in a biofilm versus exponential and stationary phase

N315 ORF[a]	Common name[a]	Product[a,b]	ER vs EP[c]	ER vs SP[c]
Cell envelope and cellular processes				
N315-SA2426	*arcD*	Arginine/ornithine antiporter	59.91	5.49
N315-SA1881	*kdpA*	Probable potassium-transporting ATPase A chain	51.58	11.30
N315-SA1880	*kdpB*	Probable potassium-transporting ATPase B chain	30.99	9.53
N315-SA1042	*pyrP*	Uracil permease	25.54	7.97
N315-SA1879	*kdpC*	Probable potassium-transporting ATPase C chain	20.82	8.40
N315-SA0417		Similar to sodium-dependent transporter	7.33	16.49
N315-SA2081		Similar to urea transporter	6.49	4.65
N315-SA1688		Similar to teichoic acid translocation ATP-binding protein TagH	0.47	0.42
N315-SA0233		PTS enzyme, maltose and glucose specific, factor II homolog	0.44	0.15
N315-SA0848	*oppF*	Oligopeptide transport system ATP-binding protein homolog	0.42	0.16
N315-SA0847	*oppD*	Oligopeptide transport system ATP-binding protein homolog	0.39	0.15
N315-SA0845	*oppB*	Oligopeptide transport system permease protein	0.38	0.16
N315-SA2242		CHP (predicted permease)	0.37	0.35
N315-SA0846	*oppC*	Similar to oligopeptide transport system permease protein	0.37	0.17
N315-SA0758		Similar to thioredoxin	0.28	0.34
N315-SA2261		Similar to efflux pump	0.25	0.45
N315-SA2132		Similar to ABC transporter (ATP-binding protein)	0.24	0.34
N315-SA0217		Similar to periplasmic iron-binding protein BitC	0.23	0.24
N315-SA1699		Similar to transporter	0.20	0.34
N315-SA1987	*opuD*	Glycine betaine transporter OpuD homolog	0.11	0.21
Information pathways				
N315-SA2424	*acrR*	Similar to transcription regulator Crp/Fnr family protein	48.16	9.87
N315-SA1041	*pyrR*	Pyrimidine operon repressor chain A	16.05	6.82
N315-SA2320		Similar to regulatory protein PfoR	9.87	4.66
N315-SA2502	*rnpA*	RNase P protein component	3.82	5.26
N315-SA2134		Similar to DNA 3-methyladenine glycosidase	0.48	0.40
N315-SA0815		Peptidyl-prolyl *cis-trans* isomerase homolog	0.44	0.49
N315-SA2278		Similar to mutator protein MutT	0.41	0.41
N315-SA1626	*hsdM*	Type I restriction enzyme homolog (SaPln3)	0.41	0.48
N315-SA0097		Similar to transcription regulator AraC/XylS family	0.40	0.49
N315-SA2144		Similar to transcriptional regulator (TetR/AcrR family)	0.39	0.37
N315-SA0189	*hsdR*	Probable type I restriction enzyme restriction chain	0.30	0.36
N315-SA1806		Probable ATP-dependent helicase (bacteriophage ϕN315)	0.29	0.25
Intermediary metabolism				
N315-SA2427	*arcB*	Ornithine transcarbamoylase	124.22	5.83
N315-SA2428	*arcA*	Arginine deiminase	114.59	5.45
N315-SA2425	*arcC*	Carbamate kinase	37.87	5.76
N315-SA1044	*pyrC*	Dihydroorotase	17.84	6.11
N315-SA1045	*carA*	Carbamoyl-phosphate synthase small chain	13.13	6.03
N315-SA1047	*pyrF*	Orotidine-5-phosphate decarboxylase	10.65	4.93
N315-SA1046	*carB*	Carbamoyl-phosphate synthase large chain	10.50	4.97
N315-SA2082	*ureA*	Urease gamma subunit	9.68	3.12
N315-SA2083	*ureAB*	Urease beta subunit	9.29	2.97
N315-SA1048	*pyrE*	Orotate phosphoribosyltransferase	8.94	4.87
N315-SA2319	*sdhB*	Similar to beta-subunit of L-serine dehydratase	8.90	4.84
N315-SA2084	*ureC*	Urease alpha subunit	8.40	3.50
N315-SA2086	*ureF*	Urease accessory protein	8.34	3.33
N315-SA2088	*ureD*	Urease accessory protein	7.72	3.67
N315-SA2085	*ureE*	Urease accessory protein	7.26	2.82
N315-SA2087	*ureG*	Urease accessory protein	6.78	3.16
N315-SA2318	*sdhA*	Similar to L-serine dehydratase	6.49	4.24
N315-SA1043	*pyrB*	Aspartate transcarbamoylase chain A	4.69	6.34
N315-SA2007		Similar to α-acetolactate decarboxylase	4.40	3.09
N315-SA0821	*argH*	Argininosuccinate lyase	3.93	14.65
N315-SA0822	*argG*	Argininosuccinate synthase	3.52	13.48
N315-SA2008	*budB*	α-Acetolactate synthase	3.29	2.53
N315-SA1155	*cls*	Cardiolipin synthetase homolog	2.73	2.28
N315-SA1160	*nuc*	Thermonuclease	2.30	2.47
N315-SA2258		Similar to diaminopimelate epimerase	2.03	2.60
N315-SA1940	*deoD*	Purine nucleoside phosphorylase	0.46	0.49
N315-SA1615	*menE*	*O*-Succinylbenzoic acid-CoA ligase	0.44	0.21
N315-SA0925	*purH*	Bifunctional purine biosynthesis protein	0.43	0.04
N315-SA0241		Similar to 4-diphosphocytidyl-2C-methyl-D-erythritol synthase	0.42	0.46
N315-SA0963	*pyc*	Pyruvate carboxylase	0.41	0.26
N315-SA0011		Similar to homoserine-*o*-acetyltransferase	0.39	0.44
N315-SA0534	*atoB*	Acetyl-CoA c-acetyltransferase	0.38	0.43
N315-SA0920	*purQ*	Phosphoribosylformylglycinamidine synthase I	0.37	0.02
N315-SA0923	*purM*	Phosphoribosylformylglycinamidine cyclo-ligase	0.34	0.03
N315-SA0924	*purN*	Phosphoribosylglycinamide formyltransferase	0.34	0.03
N315-SA0242		Similar to xylitol dehydrogenase	0.34	0.44
N315-SA0921	*purL*	Phosphoribosylformylglycinamidine synthetase	0.33	0.03
N315-SA0922	*purF*	Phosphoribosylpyrophosphate amidotransferase	0.33	0.03

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TABLE 5—*Continued*

N315 ORF[a]	Common name[a]	Product[a,b]	ER vs EP[c]	ER vs SP[c]
N315-SA0344	*metE*	5-Methyltetrahydropteroyltriglutamate-homocysteine Methyltransferase	0.28	0.43
N315-SA0022		Similar to 5′-nucleotidase	0.26	0.29
N315-SA1814		Similar to succinyl-diaminopimelate desuccinylase	0.23	0.20
N315-SA0266		CHP (ABC-type multidrug transport system, ATPase component)	0.15	0.23
Other functions				
N315-SA2353	*ssaA*	Similar to secretory antigen precursor	3.64	5.70
N315-SA0270	*ssaA*	Similar to secretory antigen precursor	0.42	0.39
N315-SA1629	*splC*	Serine protease	0.39	0.49
N315-SA0107	*spa*	Immunoglobulin G-binding protein A precursor	0.01	0.04
Similar to unknown proteins				
N315-SA0023		CHP	0.50	0.35
N315-SA0814	*kapB*	CHP	0.48	0.44
N315-SA1692		CHP (putative intracellular protease/amidase)	0.46	0.37
N315-SA0518		CHP (predicted flavoprotein)	0.46	0.43
N315-SA1612		CHP (NTP pyrophosphohydrolases)	0.34	0.17
N315-SA1133		CHP	0.32	0.44
N315-SA2371		CHP	0.30	0.26
N315-SA0559		CHP (histone acetyltransferase HPA2 and related acetyltransferases)	0.29	0.36
N315-SA0872		CHP (enterochelin esterase and related enzymes)	0.29	0.44
N315-SA2131		CHP (ABC-type Na^+ efflux pump, permease component)	0.27	0.38
N315-SA1733		CHP	0.26	0.29
N315-SA2322		CHP (permeases of the drug/metabolite transporter superfamily)	0.22	0.45
N315-SA0269		HP	0.13	0.10
N315-SA0359		CHP (uncharacterized membrane protein)	0.13	0.37
No similarity				
N315-SA1049		HP	7.42	4.41
N315-SA0575		HP	2.14	2.10
N315-SA1152		HP	0.46	0.46
N315-SA0752		HP	0.41	0.49
N315-SAS025		HP	0.39	0.47
N315-SA2372		HP	0.38	0.28
N315-SA0364		HP	0.36	0.41
N315-SA1332		HP	0.34	0.26
N315-SA1015		HP	0.34	0.23
N315-SA2373		HP	0.32	0.20
N315-SA0740		HP	0.29	0.24
N315-SA0268		HP	0.17	0.17
N315-SA0267		HP	0.17	0.19
N315-SA1726		HP	0.08	0.17
No N315 ORF				
COL-SA2069		HP	42.86	13.21
		87.1% protein ID to Ssp extracellular ECM and plasma-binding protein	33.06	135.82
		No hit in GenPept	16.42	28.08
	mapN	99.7% protein ID to MapN protein	14.76	35.81
		86.9% protein ID to SA1813 (possibly hemolysin)	6.80	12.73
		46.1% protein ID to lin0925 putative membrane protein, *Listeria innocua*	5.73	23.61
		50.7% protein ID to lin0924, *Listeria innocua*	5.52	14.61
		63.2% protein ID to MW0768	4.58	2.71
COL-SA1559		HP	3.68	2.70
		91% protein ID to SA0093 HP	0.37	0.28
		41.4% protein ID to HsdS probable restriction modification system	0.36	0.35
		45.5% protein ID to SA2490	0.33	0.46
COL-SA1043		Glycosyl transferase, group 1	0.33	0.15
		100% ID to SA1814	0.31	0.25
		97.9% protein ID to structure of cassette chromosome (SCC)-like element, stra	0.30	0.21
		61.3% protein ID to SA0553 CHP	0.28	0.50
COL-SA0653		CHP	0.23	0.46
		24.1% protein ID to BdrC3, *Borrelia hermsii*	0.23	0.34
		26.5% protein ID to RSc1168 CHP, *Ralstonia solanacearum*	0.22	0.43
		29.4% protein ID to PF1843 chromosome segregation protein Smc, *Pyrococcus furiosus*	0.22	0.26
COL-SA0654		CHP	0.21	0.48
		50% protein ID to NMB0372 HP, *Neisseria meningitidis*	0.19	0.48
		82.5% protein ID to SA0276	0.17	0.26
		32.9% protein ID to ParA, *B. subtilis*	0.14	0.30
COL-SA0095	*spa*	Immunoglobulin G-binding protein A precursor	0.02	0.08

[a] Based on the published sequence of *S. aureus* strain N315. For genes not present in N315, the gene name and description given are from the *S. aureus* strain COL genome, available from The Institute for Genomic Research website (http://www.tigr.org) or by the putative function.

[b] Abbreviations: PTS, phosphotransferase; CHP, conserved hypothetical protein; CoA, coenzyme A; NTP, nucleoside triphosphate; HP, hypothetical protein; ID, identity; ECM, extracellular matrix.

[c] Normalized values based on the expression ratio (ER), which is defined as the expression level in biofilms/expression level in exponential-phase (EP) or stationary-phase (SP) cells.

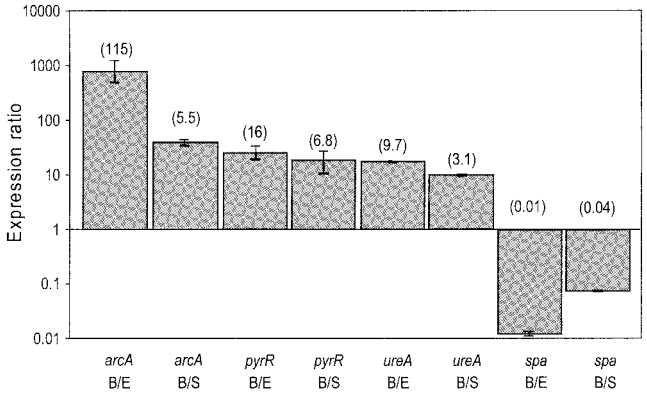

FIG. 5. Relative expression levels as determined by real-time PCR. Expression levels of the *arcA*, *pyrR*, *ureA*, and *spa* genes was determined by real-time PCR. Relative expression levels are illustrated as the ratio of the expression level observed in biofilms (B) versus exponential-phase (E) or stationary-phase (S) planktonic cultures. The numbers in parentheses above the bars indicate the relative expression levels determined by transcriptional profiling.

pendent of its ability to modulate *ica* expression and/or the production of PNAG. We would note, however, that our results are also limited to a single strain, and it is certainly possible that UAMS-1 has an alternative means of promoting intercellular accumulation that attenuates the need for PIA. Whether this is true of other clinical isolates of *S. aureus* remains to be determined, but previous work in our laboratory has confirmed that all other *sarA*-mediated phenotypes are conserved among clinical isolates like UAMS-1 (5, 7).

Dunman et al. (19) recently reported the results of microarray-based transcriptional profiling experiments with *S. aureus sarA* and *agr* mutants. These studies confirmed that *sarA* has global regulatory effects that are mediated through both *agr*-dependent and *agr*-independent pathways. In an effort to determine which of these might be involved in biofilm formation and/or maintenance of the sessile lifestyle, we performed comprehensive transcriptional profiling with RNA isolated from mature *S. aureus* biofilms. Although it would be preferable to do these experiments using RNA derived from biofilms grown in vivo, our attempts to isolate a sufficient quantity of high-quality RNA from in vivo samples have thus far been unsuccessful. However, our studies indicating that mutation of *sarA* results in a reduced capacity to form a biofilm both in vitro and in vivo suggest that biofilms grown in flow cells may also provide a relevant source of RNA for transcriptional profiling. On this basis, we isolated RNA from UAMS-1 grown in flow cells and compared the pattern of gene expression to RNA from the same strain grown in planktonic culture. The results of our comparisons to both exponential- and stationary-phase planktonic cultures clearly indicate that our flow cell biofilm model represents a unique growth environment. Indeed, we identified a total of 580 genes that are differentially expressed in biofilms by comparison to either or both planktonic conditions.

Several of the operons that were induced in biofilms have been found to be important in acid tolerance in other bacterial

TABLE 6. SarA-regulated genes differentially expressed in a biofilm

N315 ORF[a]	Common name[a]	Product[a,b]	B/E[c]	B/S[c]	SarA[d]
N315-SAS016		HP	12.74		Up
N315-SA2425	*arcC*	Carbamate kinase	37.87	5.76	Down
N315-SA2424	*arcR*	Transcription regulator Crp/Fnr family protein	48.16	9.87	Down
N315-SA2321		HP		0.33	Up
N315-SA2319	*sdhB*	Similar to beta: subunit of L-serine dehydratase	8.90	4.84	Up
N315-SA2125	*hutG*	Similar to formiminoglutamase		0.35	Up
N315-SA2006		Similar to MHC class II analog	4.69		Up
N315-SA1928		HP	0.42		Up
N315-SA1611		CHP		0.23	Up
N315-SA1553	*fhs*	Formyltetrahydrofolate synthetase		0.17	Down
N315-SA1516	*phoP*	Alkaline phosphatase synthesis		0.48	Up
N315-SA1381	*pbp3*	Penicillin-binding protein 3		0.48	Down
N315-SA1319		HP		0.42	Up
N315-SA1140	*glpF*	Glycerol uptake facilitator		4.40	Down
N315-SA1120		Similar to transcription regulator GntR family	0.35		Up
N315-SA1045	*carA*	Carbamoyl-phosphate synthase small chain	13.13	6.03	Up
N315-SA0923	*purM*	Phosphoribosylformylglycinamidine cyclo-ligase	0.34	0.03	Down
N315-SA0900	*sspB*	Cysteine protease precursor	7.03		Down
N315-SA0899	*sspC*	Cysteine protease	7.24		Down
N315-SA0754		Similar to lactococcal prophage ps3 protein 05	0.50		Up
N315-SA0746	*nuc*	Staphylococcal nuclease	0.17		Down
N315-SA0412		CHP	0.08		Down
N315-SA0411	*ndhF*	NADH dehydrogenase subunit 5	0.07	2.41	Down
N315-SA0363		HP	0.32		Down
N315-SA0107	*spa*	Immunoglobulin G-binding protein A precursor	0.01	0.04	Down
N315-SA0016	*purA*	Adenylosuccinate synthase	2.37	0.12	Up
	fnbB	Fibronectin-binding protein homolog	0.46		Up

[a] Based on the published sequence of *S. aureus* strain N315. For genes not present in N315, the gene name and description given are from the *S. aureus* strain COL genome, available from The Institute for Genomic Research website (http://www.tigr.org) or by the putative function.
[b] Abbreviations: HP, hypothetical protein; CHP, conserved hypothetical protein.
[c] Normalized values based on expression levels in biofilms versus exponential-phase cells (B/E) or biofilms versus stationary-phase cells (B/S).
[d] Regulation by SarA as reported by Dunman et al. (19).

species. Indeed, maintenance of pH homeostasis within the bacterial cell and buffering of the surrounding microenvironment have been associated with biofilm formation in the oral bacteria *Streptococcus salivarius* (34). One way bacteria combat acidic environments is to produce alkaline compounds, such as ammonia, that can neutralize the acids. Two ways in which bacteria generate ammonia are through the urease and arginine deiminase (ADI) pathways. Interestingly, we found that multiple genes from both of these pathways were induced in *S. aureus* biofilms by comparison to both planktonic conditions.

Under anaerobic conditions, some bacteria are also able to generate ATP as an energy source through catabolism of arginine via the ADI pathway, which is widely distributed in bacteria (16), archaea (54), and eucarya (60). The ADI pathway is comprised of three enzymatic reactions, catalyzed by arginine deiminase (*arcA*), ornithine transcarbamoylase (*arcB*), and carbamate kinase (*arcC*). Collectively, these enzymes convert arginine to ornithine, ammonia, and CO_2, yielding 1 mol of ATP per mol of arginine consumed. ArcD is an arginine-ornithine transporter that catalyzes the uptake of arginine and concomitant export of ornithine, while *arcR* encodes an activator of the ADI operon that is a member of the Crp-Fnr family of regulators. We found that all five of these genes were significantly induced in *S. aureus* biofilms (Table 5). One additional gene that was upregulated 3.9-fold in a biofilm compared to exponential-phase cells is the arginine repressor encoded by *argR* (Table 3). Under anaerobic conditions in the presence of arginine, ArgR represses anabolic ornithine carbamoyltransferase and induces the ADI pathway. In addition to its role in generating ATP anaerobically, the ADI pathway is one of two major ammonia-generating pathways utilized by oral bacteria to maintain pH homeostasis when growing in a biofilm. Ammonia generated by the deimination of arginine can neutralize acids generated by bacterial glycolysis.

The ADI system and its role in acid resistance have also been correlated with virulence in *Streptococcus pyogenes*. ADI in this species was originally called streptococcal acid glycoprotein (SAGP) and was characterized as being an inhibitor of stimulated human peripheral blood mononuclear cell proliferation (17, 18). In addition, it is thought that the acid sensitivity of a SAGP-negative mutant is responsible for its reduced ability to enter and survive in epithelial cells (18).

Also included among the genes induced in biofilms (Table 5) were seven genes that comprise the urease operon (*ureABCE FGD*). Urease (urea amidohydrolase) is a nickel-containing enzyme that catalyzes the hydrolysis of urea to yield two molecules of ammonia and one molecule of CO_2. Ureases of most bacteria are composed of three distinct subunits encoded by three contiguous genes, *ureA*, *ureB*, and *ureC*. Urease gene clusters also encode accessory genes, in addition to these structural genes, that are required for the de novo synthesis of active urease. Urease activity is essential for the colonization of the gastric mucosa by *Helicobacter pylori* and colonization of the urinary tract by both *Proteus mirabilis* and *Staphylococcus saprophyticus* (20, 25, 30). In addition, urease is thought to play a central role in the pathogenesis of *Ureaplasma urealyticum* urinary and respiratory tract infections (27, 35).

Recently, Saïd-Salim et al. (58) found that genes of the urease operon in *S. aureus* are negatively regulated by the SarA homolog Rot (repressor of toxin). Interestingly, expression of *rot* was repressed in biofilms, although this was limited to the comparison with exponential-phase planktonic cultures. The trigger for induced transcription of the urease operon in *S. aureus* has not yet been studied. However, urease synthesis by *Klebsiella aerogenes* is stimulated under conditions of nitrogen starvation, such as when the bacteria are cultured in minimal medium containing a poor nitrogen source, such as proline, arginine, or histidine (24). In *H. pylori*, expression of the urease operon is upregulated by a pH-dependent, posttranscriptional regulatory mechanism. More specifically, Akada et al. (1) showed that a shift to an acidic pH resulted in a significant increase in the level of *ure* operon mRNA, even in the presence of inhibitors of transcription. Until recently, it was believed that urease was associated with the cell surface and that it directly neutralized the microenvironment surrounding the cell (50). However, it is now thought to play a more important role as an intracellular enzyme required for acid resistance (61).

In addition to the production of ammonia, cation transport ATPases, such as the high-affinity K^+-specific transport system encoded by the *kdp* operon, can also contribute to pH homeostasis through the exchange of K^+ for H^+ (13). In this study, we found that three genes of the *kdp* operon (*kdpABC*) were induced in biofilms by comparison to both planktonic growth conditions (Table 5) and two other genes (*kdpDE*) were induced by comparison to the exponential growth phase (Table 3). In *E. coli*, the Kdp system is composed of the ion motive P-type ATPase encoded by the *kdpFABC* operon, and expression of the *kdpFABC* operon is regulated by an adjacent operon, *kdpDE* (2). KdpD is a membrane-spanning sensor kinase, and KdpE is a cytosolic transcriptional activator. In response to an appropriate signal(s) (membrane stretch, alteration in turgor pressure, and external and internal potassium levels), KdpD transphosphorylates KdpE, which in turn upregulates transcription of the *kdpFABC* operon (2, 43). Interestingly, van der Laan et al. (68) recently reported that NH_4^+ ions strongly stimulate the ATPase activity of the KdpFABC complex in *E. coli*.

Several operons of the pyrimidine nucleotide biosynthetic (*pyr*) pathway (*pyrRPBC*, *carAB*, and *pyrFE*) were also induced in biofilms (Table 5). The pathway for the de novo synthesis of pyrimidines consists of six enzymatic steps leading to the formation of UMP. The first step in the pyrimidine biosynthetic pathway is the formation of carbomyl-phosphate (CP) from bicarbonate, glutamine (or ammonia), and ATP by CP synthase, which is encoded by the *carAB* genes. Interestingly, CP is also required for the biosynthesis of arginine. In *B. subtilis*, PyrR regulates transcription of the *pyr* operon by binding in a uridine nucleotide-dependent fashion to *pyr* mRNA and altering the secondary structure of the downstream mRNA (37, 38). Binding of PyrR to the downstream mRNA stabilizes a binding loop and prevents the formation of the antiterminator (39). In the absence of PyrR or when levels of the nucleotides UMP and UTP are low, the antiterminator is a stable secondary structure, and transcription of the downstream genes continues (39). While we do not know if the function of *B. subtilis* and *S. aureus* PyrR is conserved, our results showing that all of the genes in the *pyr* operon were induced in biofilms suggests that the level of UMP in cells growing in a biofilm is severely limited. In addition, upregulation of the *pyr* operon and sub-

sequent CP production may be required for synthesis of sufficient levels of arginine to be used by the ADI pathway during anaerobic growth.

Taken together, the results of our array experiments suggest that mature biofilms are growing anaerobically and that genes of the acid tolerance response are upregulated in response to an acidic environment. While the regulatory pathway for the acid tolerance response in *S. aureus* has not been well characterized, there have been studies suggesting that the global regulators SigB and SarA are involved. Specifically, mutation of *sigB* results in strains with impaired abilities to respond to acid and to induce a stationary-phase acid tolerance response (8, 32). In addition, in the absence of RsbU, expression from the SigB-dependent *sarA* promoter was significantly reduced at pH 5.5 (46).

Our analysis revealed 27 genes that were differentially expressed in biofilms and were part of the *sarA* regulon as defined by Dunman et al. (19) (Table 6). Given the different mechanistic possibilities of how SarA modulates biofilm production, it is difficult to determine which of these genes might be the most relevant candidates for further consideration. For example, because SarA mutants have a reduced capacity to form a biofilm, SarA would presumably be required for production of an activator of biofilm formation or a required effector molecule, in which case transcription of the relevant gene(s) would be upregulated by SarA and in a biofilm. However, it is also possible that SarA represses production of an effector that is deleterious to biofilm formation, in which case the SarA target would be downregulated within a biofilm. One possibility in that regard is that the increased production of proteases observed in *sarA* mutants (7) results in degradation of a required surface protein. However, Valle et al. (66) concluded on the basis of both mutagenesis experiments and experiments employing specific inhibitors that protease production was not responsible for the decreased capacity of a *sarA* mutant to form a biofilm. Nevertheless, it remains possible that a similar scenario in which SarA represses transcription of some factor that has a negative impact on biofilm formation is involved. Moreover, both of these scenarios are based on the assumption that the SarA-dependent regulation of the relevant effector(s) is direct, and it is also possible that *sarA* modulates biofilm formation in an indirect fashion, perhaps via one of the increasing number of SarA homologs (4, 9, 45). It is also possible that *sarA* is required for production of an adhesin or some other effector protein that is required only for initial stages of biofilm formation. In this case, expression of the relevant gene may be transient in a fashion that would not have been apparent in our profiling experiments focusing on mature biofilms.

Finally, it should be noted that biofilms are not homogenous populations of cells and because the experiments we performed did not address this issue, it is certainly possible that we failed to detect genes that are within the *sarA* regulon but are differentially expressed only within certain regions of the biofilm. At the same time, this would imply that the genes we did identify are either differentially expressed throughout the biofilm or that our results actually underestimate the degree of differential gene expression observed within more limited regions. It should also be noted that the profiling experiments of Dunman et al. (19) were done using a first-generation GeneChip that was limited to only 85% of the genes identified in a single strain (COL) of *S. aureus*, and it is certainly possible that this limited the identification of relevant genes that are conserved among clinical isolates like UAMS-1. Moreover, the profiling experiments of Dunman et al. (19) were done using RNA isolated from derivatives of the *S. aureus* strain RN6390, and we have previously demonstrated that the regulatory circuits observed in RN6390 and its corresponding *sarA* and *agr* mutants are different from those of other strains, including UAMS-1 (7). To address these issues, we are currently performing transcriptional profiling experiments using RNA isolated from UAMS-1 *sarA* and *agr* mutants and the more comprehensive chips used for the biofilm profiling experiments described here.

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