JOURNAL OF BACTERIOLOGY, Oct. 2008, p. 6530–6533 Vol. 190, No. 19
0021-9193/08/$08.00+0 doi:10.1128/JB.00482-08

Regulation of the Intercellular Adhesin Locus Regulator (*icaR*) by SarA, σ^B, and IcaR in *Staphylococcus aureus*▽

Nuno Cerca, Jamie L. Brooks, and Kimberly K. Jefferson*

Department of Microbiology and Immunology, Virginia Commonwealth University, Richmond Virginia 23928

Received 8 April 2008/Accepted 15 July 2008

The staphylococcal accessory regulator SarA and the alternative sigma factor σ^B have been previously identified as positive regulators, and IcaR as a negative regulator, of *icaADBC* expression. Here, we show that in *Staphylococcus aureus* SarA and σ^B are also required for *icaR* expression and that IcaR does not have a significant effect on its own expression.

The proteins encoded by the intercellular adhesin genes (*icaADBC*) synthesize the polysaccharide poly-*N*-acetylglucosamine (PNAG), which contributes to the formation of a biofilm by *Staphylococcus aureus*. In addition to the proteins that synthesize PNAG, the *ica* locus also encodes the TetR family transcriptional regulator IcaR (23). The *icaR* gene is transcribed divergently from *icaADBC* (10) and is a negative regulator of *icaADBC* expression (9, 14). Biofilm formation by *S. aureus* plays an important role in the pathogenesis of endocarditis, osteomyelitis, and corneal and medical device infections (18). Although the majority of clinical isolates of *S. aureus* contain the *ica* operon, in vitro expression is tightly controlled (19) and the regulation of *ica* has been shown to be a complex and multifactorial process, involving a variety of external environmental factors and internal regulators.

Rachid et al. demonstrated that σ^B was required for biofilm formation under environmental stress conditions in an *S. aureus* mucosal isolate (22). They suggested that the effect of σ^B on *ica* expression could be indirect, as the *ica* promoter does not appear to contain a consensus σ^B binding site. Later, Conlon et al. demonstrated that *icaR* encodes a repressor of *icaADBC* transcription in *Staphylococcus epidermidis*, and we found the same to be true in *S. aureus* (9, 14). Alleviation of IcaR-mediated repression occurs in response to certain *icaADBC*-inducing stimuli such as ethanol but not in response to others (e.g., NaCl) (8), suggesting a role for additional regulatory mechanisms. Valle et al. and Beenken et al. demonstrated a role for SarA in *icaADBC* expression and biofilm formation in *S. aureus* (2, 27), and shortly thereafter Tormo et al. demonstrated a similar role in *S. epidermidis* (26).

While negative regulation of *icaR* by σ^B in *S. epidermidis* has been shown previously (16), its regulation in *S. aureus* has not yet been studied. IcaR belongs to the tetracycline repressor family of proteins, which are involved in gene regulation, acting as either transcriptional activators or repressors. The aim of this work was to elucidate regulation of the *icaR* gene in *S. aureus*.

All strains used in this study were grown at 37°C and 200 rpm in Luria-Bertani (LB) broth or LB agar, except for biofilm formation assays, where tryptic soy broth supplemented with 1% glucose was used. When appropriate, LB broth or LB agar was supplemented with the antibiotics at the following concentrations: 10 μg chloramphenicol/ml, 5 μg tetracycline/ml, and 10 μg erythromycin/ml. After overnight growth, cultures were diluted 1:50 in 5 ml fresh medium and incubated for 6 h before cells were collected for expression studies. Using DNA from strain *S. aureus* MN8 as the template and the primers IcaRpro EcoRI (5′-GAATTCTAGTATTTTAATTTGCAATAGATT GTTGTTATAATTAAACGG-3′) and IcaRproSma (5′-CCCG GGCTTATCCTTCAATTTTTATAACCCC-3′), we amplified the promoter region and the first four codons of the *icaR* gene by PCR, and using the primers icapro1932 (5′-GAATTCGATATA AAGCATCAATTGAATAGTTCG-3′) and icaproREV (5′-CC CGGGGTTAAAAAATTGCAATTTCTTTACCTTTCG-3′), we amplified the *icaADBC* promoter and the first six codons of *icaA*. The DNA fragments were digested with SmaI, fused to the β-galactosidase gene (*bgaB*) from *Bacillus stearothermophilus*, and cloned into the staphylococcal shuttle vector pRB473 (constructed by Reinhold Brückner, Universitat Tubingen, Germany) (4). The reporter construct was sequenced to verify the absence of mutations, electroporated into *S. aureus* RN4220, and transduced to other strains using phage 80 (15, 17). *S. aureus* strain Newman Δ*rsbUVW*, in which the σ^B operon has been replaced with an erythromycin resistance (Erm) cassette, was kindly provided by Kenneth Bayles, University of Nebraska (24). Strain ALC1342, a derivative of strain RN6390 in which the *sarA* gene was replaced with an Erm cassette, was kindly provided by Ambrose Cheung, Dartmouth Medical School (7). RN4220 Δ*icaR*::Erm was constructed previously in our lab (14). Mutations were transduced to strains SA113 and Newman using phage 80. To complement the deletion mutations, *sarA* and 1.2 kb of the region upstream from the gene, which contains the promoters and a 5′ untranslated region; the rsbUVWσ^B locus; or the *icaR* gene was amplified from genomic DNA from strain Newman using the primer pairs attB2SarAFWD (5′-GGGGACCACTTTGTACAAGAAAGCT GGGTCTATATCATTGGTGTCCTAGTTGG-3′) and attB1 SarAREV (5′-GGGGACAAGTTTGTACAAAAAAGCAGGC ATGGATTGGATGGTAATTTAGCTGG-3′); attB2SigBFWD (5′-GGGGACCACTTTGTACAAGAAAGCTGGGTGGAAT CAATTGGAGGTTCTCATATG-3′) and attB1SigBREV (5′-G GGGACAAGTTTGTACAAAAAAGCAGGCCTTTACGTTT

* Corresponding author. Mailing address: Virginia Commonwealth University, Department of Microbiology and Immunology, P.O. Box 980678, Richmond, VA 23928. Phone: (804) 828-9699. Fax: (804) 828-9946. E-mail: kkjefferson@vcu.edu.

▽ Published ahead of print on 25 July 2008.

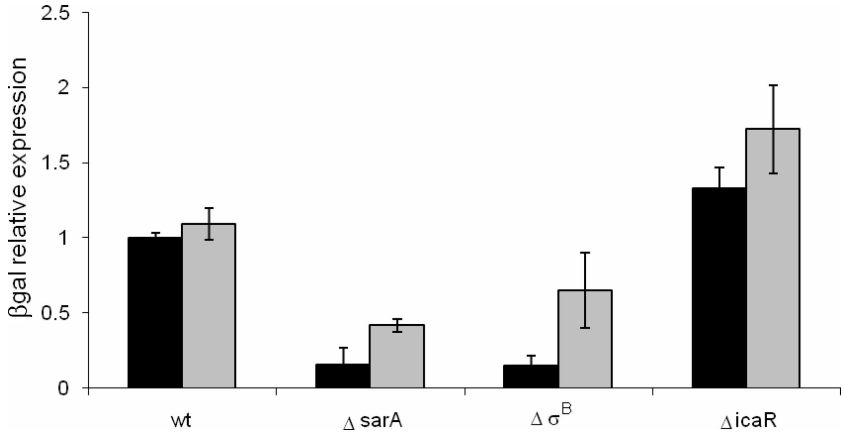

FIG. 1. Effect of SarA, σ^B, and IcaR on the *icaADBC* promoter as determined by β-galactosidase activity in SA113 (black bars) and Newman (gray bars). wt, wild type. Error bars indicate standard deviations.

CGCCTCAGTTCG-3′); or attB2IcaRFWD (5′-GGGGACCAC TTTGTACAAGAAAGCTGGGTGGTTTCCTCCACATAAT CAATCATTG-3′) and attB1IcaRREV (5′-GGGGACAAGTTT GTACAAAAAAGCAGGCTTTCTTTACCTACCTTTCGTT AGTTAGG-3′) and cloned into pKOR1 (1). The plasmids were electroporated into strain RN4220 and transduced to the respective deletion mutants of Newman and SA113 using phage 80 (15). The erythromycin resistance cassettes were then replaced with the intact genetic loci by inducible counterselection.

Regulation of the *icaADBC* promoter was assessed by 2-nitrophenyl β-D-galactopyranoside (ONPG) degradation (as a measurement of β-galactosidase activity) as previously described (6) with minor modifications: bacteria were lysed with lysostaphin, and 50 μl of cell extract and 50 μl of ONPG were combined and incubated for 2 h (for SA113) or 6 h (for Newman). β-Galactosidase activity was normalized to 1.0 (ONPG activity detected in the wild-type strain). Confirming several previous reports, our results showed that in both SA113 and Newman, IcaR repressed *icaADBC* expression, while SarA and σ^B activated this promoter (Fig. 1). Chromosomal complementation of the mutations confirmed that the effects were due to deletion of *icaR*, *sarA*, and the rsbUVWσ^B locus, respectively. Biofilms were formed in 96-well microtiter plates and quantified using the modified microtiter plate method developed by Stepanovic et al. (25). SA113 formed a stronger biofilm than Newman (optical density at 570 nm [OD_{570}] = 1.74 ± 0.05 and 0.44 ± 0.03, respectively). In the absence of SarA or σ^B, the biofilm formation of both strains was impaired (OD_{570} = 0.39 and 0.54 for SA113 and OD_{570} = 0.23 ± 0.02 and 0.33 ± 0.03 for Newman, respectively), whereas chromosomal complementation of the *sarA* and rsbUVWσ^B loci restored biofilm formation (OD_{570} = 1.74 ± 0.02 and 1.75 ± 0.09 for SA113 and OD_{570} = 0.53 ± 0.02 and 0.54 ± 0.05 for Newman, respectively). Conversely, deletion of *icaR* induced a mild positive effect on biofilm formation (OD_{570} = 1.95 ± 0.01 and 0.62 ± 0.04, respectively). Again, complementation of *icaR* restored the biofilms (OD_{570} = 1.80 ± 0.01 for SA113 and 0.49 ± 0.02 for Newman). Previously, in *S. epidermidis*, although *icaR* was shown to repress *icaADBC* expression, biofilm formation was not significantly different in an *icaR* mutant and the wild type in brain heart infusion medium (9), demonstrating that additional factors are also important in the regulation of biofilm formation.

We then repeated the expression assays using the *icaR* promoter fusion. In a previous report it has been shown that in *S. epidermidis*, σ^B represses *icaR* (16). We were expecting a similar result with our constructs, but contrary to the report about *S. epidermidis* strain 1457, σ^B was an important positive regulator of expression of *icaR* in *S. aureus* strains SA113 and Newman (results for SA113 are shown in Fig. 2; results for Newman were similar and are not shown). The strains were fully complemented with an intact rsbuVWσ^B locus. Evidence that biofilm formation is regulated by somewhat different mechanisms within *S. epidermidis* and *S. aureus* comes from the fact that most *icaADBC*-positive strains of *S. epidermidis* produce biofilms in vitro, while most *S. aureus* strains do not (10, 19). While σ^B appears to have an indirect effect on the *icaADBC* promoter, the mechanism of action of its regulation is still unclear, and variability not only between different species of staphylococci but between different strains of *S. aureus* is apparent as well from reports regarding the effect of σ^B on biofilm formation. (22, 27). Furthermore, σ^B also appears to play an important role in the long-term stability of *S. epidermidis* biofilms (13) that is not necessarily related to the *icaADBC*, expression although no similar effect has been described for *S. aureus* biofilms.

Interestingly, SarA was also required for *icaR* expression in both strains (results for SA113 are shown in Fig. 2; results for Newman were similar and are not shown). Complementation of the *sarA* mutation by replacing the Erm cassette with an intact *sarA* gene restored the phenotype. It was previously reported that *icaR* was not repressed by SarA in *S. epidermidis*, but a positive regulatory effect was not noted (26). Positive regulation of both *icaADBC* and its repressor, *icaR*, by SarA could result from unwinding of the promoter and subsequent access for the RNA polymerase complex to initiate transcription in either direction and could serve as a mechanism to prevent overexpression of the polysaccharide.

Valle et al. demonstrated that both SarA and σ^B affected *icaADBC* transcription but that only SarA was relevant for biofilm formation (27), contradicting previous information that attributed to σ^B a relevant role in *S. aureus* biofilm formation

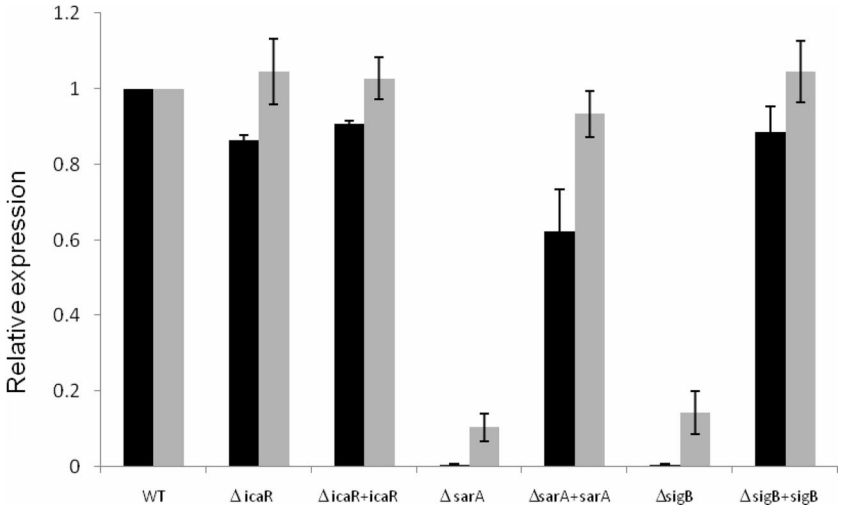

FIG. 2. Effect of SarA, σ^B, and IcaR on the *icaR* promoter on SA113 as determined by β-galactosidase activity (gray) and mRNA levels (black) determined by real-time PCR and normalized to the expression of 16S RNA. WT, wild type. Error bars indicate standard deviations.

(22). Later, Handke et al. showed in an experiment using the *icaADBC* operon controlled by a cadmium-inducible promoter that at least in *S. epidermidis*, both SarA and σ^B were important for biofilm formation (11), although they suggested that while SarA has a direct effect on *ica* transcription, the σ^B effect is indirect. Indeed, microarray-based follow-up studies revealed that σ^B strongly affected SarA expression in *S. aureus*, but the same strong effect was not found for any of the *ica* genes (3, 21). Furthermore, another study showed that *sarA* transcription shifts from the σ^A-dependent promoter during the exponential growth phase to the σ^B-dependent promoter during the late exponential and stationary phases (28), clarifying even more the dependence of SarA on σ^B and suggesting an explanation for the variability of results regarding its role in *icaADBC* expression. Our results showed that for *S. aureus*, deletion of σ^B influences *icaR* expression, *icaADBC* expression, and biofilm formation in two distinct strains.

Although most members of the TetR family of regulatory proteins that are divergently transcribed from the structural gene that they regulate appear to self-regulate their own gene (12), it was described that in *S. epidermidis*, *icaR* had no effect on its own expression (9). Similarly, in *S. aureus* deletion of *icaR* did not have a significant effect on *icaR* promoter activity (results for SA113 are shown in Fig. 2; results for Newman were similar and are not shown).

The reporter constructs were translational fusions, so β-galactosidase activities were a function of both transcription and translation. To assess the regulation of transcription alone, we used real-time PCR. Total cellular RNA was prepared using the FastRNA Pro Blue kit (MP Biomedicals, Solon, OH) accordingly to manufacturer's instructions, and contaminating DNA was removed by two treatments with Turbo DNase (Ambion, Austin, TX). Reverse transcription of 2 μg RNA was performed using AccuScript (Stratagene, La Jolla, CA) and 10 pmol bgaB-RTREV (5′-ATCGATCGGCAAAGAA TCTG-3′) or 16S-RTREV (5′-CCACTTTCCTCTTCTGCA CTCA-3′). SensiMixPlus (Quantace, Norwood, MA) was used for real-time reverse transcription-PCR with the primers bgaB-RTFW (5′-GGGATTTTCAGTTGGAGCAA-3′) and bgaB-RTREV or 16S-RTFW (5′-TCCGGAATTATTGGGC

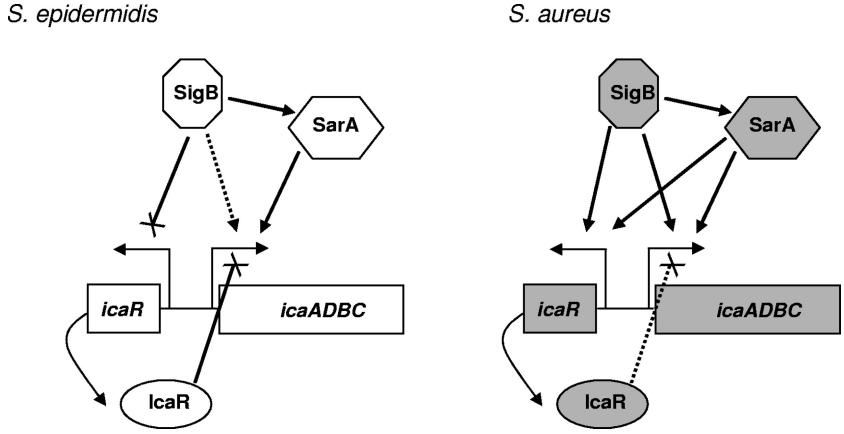

FIG. 3. Diagram of *icaR* and *icaADBC* regulation in *S. epidermidis* (9, 11, 16, 26) versus *S. aureus* (based on work presented here). Solid lines ending in arrowheads represent activation, solid lines ending in "×" represent repression, and dotted lines represent weak activation or repression.

GTAA-3′) and 16S-RTREV. Control reactions lacked reverse transcriptase enzyme to evaluate the level of DNA contamination. Real-time PCR results were similar to ONPG assay results (Fig. 2), suggesting that the observed effects are largely due to transcriptional rather than posttranscriptional control.

Altogether, the results indicate that in contrast to the case in *S. epidermidis*, SarA and σ^B were positive regulators of *icaR* expression in *S. aureus* whereas IcaR had little effect on its own expression and a relatively weak repressive effect on expression of the *icaADBC* operon (Fig. 3). Other regulators of biofilm formation have been described. In a recent study, Pamp et al. demonstrated by Northern blot analysis that in the absence of Spx, *icaR* transcript levels were reduced, while *icaADBC* expression was augmented (20). This illustrates the careful and complex orchestration of this process; biofilm formation is important under certain conditions, but the unregulated expression of PNAG would be metabolically wasteful. This study also revealed distinctions in regulation in different strains. Cafiso et al. found a relationship between Agr type and the relative roles of IcaR and σ^B in biofilm formation, suggesting one potential explanation for strain variability (5).

This work was supported by NIH grant R01 AI068892.

REFERENCES

1. **Bae, T., and O. Schneewind.** 2006. Allelic replacement in *Staphylococcus aureus* with inducible counter-selection. Plasmid **55:**58–63.
2. **Beenken, K. E., J. S. Blevins, and M. S. Smeltzer.** 2003. Mutation of *sarA* in *Staphylococcus aureus* limits biofilm formation. Infect. Immun. **71:**4206–4211.
3. **Bischoff, M., P. Dunman, J. Kormanec, D. Macapagal, E. Murphy, W. Mounts, B. Berger-Bächi, and S. Projan.** 2004. Microarray-based analysis of the *Staphylococcus aureus* σ^B regulon. J. Bacteriol. **186:**4085–4099.
4. **Brückner, R., E. Wagner, and F. Götz.** 1993. Characterization of a sucrase gene from *Staphylococcus xylosus*. J. Bacteriol. **175:**851–857.
5. **Cafiso, V., T. Bertuccio, M. Santagati, V. Demelio, D. Spina, G. Nicoletti, and S. Stefani.** 2007. agr-genotyping and transcriptional analysis of biofilm-producing *Staphylococcus aureus*. FEMS Immunol. Med. Microbiol. **51:**220–227.
6. **Cerca, N., and K. K. Jefferson.** 2008. Effect of growth conditions on poly-N-acetylglucosamine expression and biofilm formation in *Escherichia coli*. FEMS. Microbiol. Lett. **283:**36–41.
7. **Cheung, A. L., and A. C. Manna.** 2005. Role of the distal *sarA* promoters in SarA expression in *Staphylococcus aureus*. Infect. Immun. **73:**4391–4394.
8. **Conlon, K., H. Humphreys, and J. O'Gara.** 2002. Regulation of *icaR* gene expression in *Staphylococcus epidermidis*. FEMS. Microbiol. Lett. **216:**171–177.
9. **Conlon, K. M., H. Humphreys, and J. P. O'Gara.** 2002. *icaR* encodes a transcriptional repressor involved in environmental regulation of *ica* operon expression and biofilm formation in *Staphylococcus epidermidis*. J. Bacteriol. **184:**4400–4408.
10. **Cramton, S. E., C. Gerke, N. F. Schnell, W. W. Nichols, and F. Götz.** 1999. The intercellular adhesion (*ica*) locus is present in *Staphylococcus aureus* and is required for biofilm formation. Infect. Immun. **67:**5427–5433.
11. **Handke, L. D., S. R. Slater, K. M. Conlon, S. T. O'Donnell, M. E. Olson, K. A. Bryant, M. E. Rupp, J. P. O'Gara, and P. D. Fey.** 2007. Sigma B and SarA independently regulate polysaccharide intercellular adhesin production in *Staphylococcus epidermidis*. Can. J. Microbiol. **53:**82–91.
12. **Hillen, W., and C. Berens.** 1994. Mechanisms underlying expression of Tn10 encoded tetracycline resistance. Annu. Rev. Microbiol. **48:**345–369.
13. **Jäger, S., D. Mack, H. Rohde, M. A. Horstkotte, and J. K. Knobloch.** 2005. Disintegration of *Staphylococcus epidermidis* biofilms under glucose-limiting conditions depends on the activity of the alternative sigma factor σ^B. Appl. Environ. Microbiol. **71:**5577–5581.
14. **Jefferson, K., D. Pier, D. Goldmann, and G. Pier.** 2004. The teicoplanin-associated locus regulator (TcaR) and the intercellular adhesin locus regulator (IcaR) are transcriptional inhibitors of the *ica* locus in *Staphylococcus aureus*. J. Bacteriol. **186:**2449–2456.
15. **Kasatiya, S. S., and J. N. Baldwin.** 1967. Nature of the determinant of tetracycline resistance in *Staphylococcus aureus*. Can. J. Microbiol. **13:**1079–1086.
16. **Knobloch, J. K., S. Jäger, M. A. Horstkotte, H. Rohde, and D. Mack.** 2004. RsbU-dependent regulation of *Staphylococcus epidermidis* biofilm formation is mediated via the alternative sigma factor σ^B by repression of the negative regulator gene *icaR*. Infect. Immun. **72:**3838–3848.
17. **Lee, J. C.** 1995. Electrotransformation of staphylococci. Methods Mol. Biol. **47:**209–216.
18. **Lowy, F. D.** 1998. *Staphylococcus aureus* infections. N. Engl. J. Med. **339:**520–532.
19. **O'Gara, J. P.** 2007. *ica* and beyond: biofilm mechanisms and regulation in *Staphylococcus epidermidis* and *Staphylococcus aureus*. FEMS Microbiol. Lett. **270:**179–188.
20. **Pamp, S. J., D. Frees, S. Engelmann, M. Hecker, and H. Ingmer.** 2006. Spx is a global effector impacting stress tolerance and biofilm formation in *Staphylococcus aureus*. J. Bacteriol. **188:**4861–4870.
21. **Pane-Farre, J., B. Jonas, K. Forstner, S. Engelmann, and M. Hecker.** 2006. The sigma B regulon in *Staphylococcus aureus* and its regulation. Int. J. Med. Microbiol. **296:**237–258.
22. **Rachid, S., K. Ohlsen, U. Wallner, J. Hacker, M. Hecker, and W. Ziebuhr.** 2000. Alternative transcription factor σ^B is involved in regulation of biofilm expression in a *Staphylococcus aureus* mucosal isolate. J. Bacteriol. **182:**6824–6826.
23. **Ramos, J. L., M. Martinez-Bueno, A. J. Molina-Henares, W. Teran, K. Watanabe, X. Zhang, M. T. Gallegos, R. Brennan, and R. Tobes.** 2005. The TetR family of transcriptional repressors. Microbiol. Mol. Biol. Rev. **69:**326–356.
24. **Rice, K. C., T. Patton, S. J. Yang, A. Dumoulin, M. Bischoff, and K. W. Bayles.** 2004. Transcription of the *Staphylococcus aureus* Cid and Lrg murein hydrolase regulators is affected by sigma factor B. J. Bacteriol. **186:**3029–3037.
25. **Stepanovic, S., D. Vukovic, I. Dakic, B. Savic, and M. Svabic-Vlahovic.** 2000. A modified microtiter-plate test for quantification of staphylococcal biofilm formation. J. Microbiol. Methods **40:**175–179.
26. **Tormo, M. A., M. Marti, J. Valle, A. C. Manna, A. L. Cheung, I. Lasa, and J. R. Penadés.** 2005. SarA is an essential positive regulator of *Staphylococcus epidermidis* biofilm development. J. Bacteriol. **187:**2348–2356.
27. **Valle, J., A. Toledo-Arana, C. Berasain, J. M. Ghigo, B. Amorena, J. R. Penadés, and I. Lasa.** 2003. SarA and not sigma B is essential for biofilm development by *Staphylococcus aureus*. Mol. Microbiol. **48:**1075–1087.
28. **Ziebandt, A. K., D. Becher, K. Ohlsen, J. Hacker, M. Hecker, and S. Engelmann.** 2004. The influence of agr and sigma B in growth phase dependent regulation of virulence factors in *Staphylococcus aureus*. Proteomics **4:**3034–3047.