The Teicoplanin-Associated Locus Regulator (TcaR) and the Intercellular Adhesin Locus Regulator (IcaR) Are Transcriptional Inhibitors of the *ica* Locus in *Staphylococcus aureus*

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Infections involving *Staphylococcus aureus* are often more severe and difficult to treat when the organism assumes a biofilm mode of growth. The polysaccharide poly-*N*-acetylglucosamine (PNAG), also known as polysaccharide intercellular adhesin, is synthesized by the products of the intercellular adhesin (*ica*) locus and plays a key role in biofilm formation. Numerous conditions and exogenous factors influence *ica* transcription and PNAG synthesis, but the regulatory factors and pathways through which these environmental stimuli act have been only partially characterized. We developed a DNA affinity chromatography system to purify potential regulatory proteins that bind to the *ica* promoter region. Using this technique, we isolated four proteins, including the staphylococcal gene regulator SarA, a MarR family transcriptional regulator of the teicoplanin-associated locus TcaR, DNA-binding protein II, and topoisomerase IV, that bound to the *ica* promoter. Site-directed deletion mutagenesis of *tcaR* indicated that TcaR was a negative regulator of *ica* transcription, but deletion of *tcaR* alone did not induce any changes in PNAG production or in adherence to polystyrene. We also investigated the role of IcaR, encoded within the *ica* locus but divergently transcribed from the biosynthetic genes. As has been shown previously in *Staphylococcus epidermidis*, we found that IcaR was also a negative regulator of *ica* transcription in *S. aureus*. We also demonstrate that mutation of *icaR* augmented PNAG production and adherence to polystyrene. Transcription of the *ica* locus, PNAG production, and adherence to polystyrene were further increased in a *tcaR icaR* double mutant. In summary, TcaR appeared to be a weak negative regulator of transcription of the *ica* locus, whereas IcaR was a strong negative regulator, and in their absence PNAG production and biofilm formation were enhanced.

Staphylococcus aureus is an exceptionally adaptable organism and responds rapidly to changes in its environment by modulating the expression of appropriate genes. For example, molecular regulatory processes, which at present are not well understood, enable *S. aureus* to switch from a free-floating and relatively independent existence to an adherent, community-based biofilm mode of growth. Biofilm formation is an important aspect of endocarditis, osteomyelitis, and corneal and medical device infections related *to S. aureus* (10, 16, 17).

Critical to *S. aureus* biofilm formation is the production of a poly-*N*-acetylglucosamine (PNAG) polysaccharide, of which a low-molecular-weight variant referred to as the polysaccharide intercellular adhesin has been studied by some groups (7, 18). The intercellular adhesin (*ica*) locus, originally identified in *Staphylococcus epidermidis* (11) and later found in *S. aureus* (5, 19), encodes the genes involved in PNAG production. The four proteins implicated in PNAG synthesis, IcaA, IcaD, IcaB, and IcaC, are translated from a single transcript. The *icaR* gene is transcribed divergently from the other genes and encodes a regulatory protein that binds to the *ica* promoter just upstream of the initiation codon for the IcaA protein (4, 5, 12), although the exact mechanism by which it regulates *icaADCB* transcription and PNAG synthesis is not known.

In a previous report, we described the use of an isogenic pair of *S. aureus* strains, MN8 and MN8m, for an initial characterization of transcriptional regulation of the *ica* locus (12). *S. aureus* strain MN8 is a clinical isolate that normally produces only low levels of PNAG and a weak biofilm in vitro, which is augmented in the presence of glucose. This strain underwent a spontaneous mutation during a chemostat culture which led it to constitutively hyperproduce PNAG (19). A 5-nucleotide deletion within the *ica* promoter region of the mutant strain (MN8m) is responsible for the phenotype and confers the PNAG-overproducing phenotype upon other *S. aureus* strains (12). This report also demonstrated that IcaR binds to the *ica* promoter but not in the region of the deletion mutation.

In an effort to isolate potential regulatory factors with high affinity for the region of the *ica* promoter in which the deletion occurs, we developed a DNA affinity chromatography system. This technique revealed a number of proteins that bind this region of the *ica* promoter, including two nonspecific DNA binding proteins, DNA-binding protein II and topoisomerase IV, the global regulatory factor SarA, previously identified as a regulator of *ica* (1, 27), and TcaR, a MarR family protein originally described as a putative transcriptional regulator of the teicoplanin-associated locus (*tca*) (2). Site-directed mutagenesis was used to delete *tcaR* from MN8, MN8m, and an unrelated strain, and the effects on *ica* transcription, PNAG production, and adherence to polystyrene were analyzed. We found that *ica* transcription was augmented approximately fivefold in the absence of TcaR but that PNAG production and adherence were not significantly increased.

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In order to compare the role of TcaR with that of IcaR, we deleted *icaR* and found that deletion of *icaR* augmented *ica* transcription approximately 100-fold, and even though it had only a moderate impact on adherence, it increased PNAG production by more than 10-fold. When both *tcaR* and *icaR* were deleted, *ica* transcription increased approximately 500-fold with respect to the parent strains, the amount of PNAG elaborated was 100-fold greater, and adherence was significantly increased with respect to both the parent strains and the *icaR* single knockouts.

MATERIALS AND METHODS

Staphylococcal strains and media. *S. aureus* MN8 is a clinical isolate provided by Patrick Schlievert, Minneapolis, Minn. Strain MN8m is a spontaneous mutant isolated from a chemostat culture of strain MN8 (19). Strain NCTC 10833 (ATCC 25904) is a clumping factor-positive variant of a throat swab isolate. Partial deletion of the *ica* locus to produce strains 10833 Δ*ica*::*tet* and MN8 Δ*ica*::*tet* was performed as described by Cramton et al. (5). The strains were grown at 37°C on tryptic soy agar plates containing the appropriate antibiotic. Liquid cultures were either in tryptic soy broth (TSB) lacking glucose and containing (per liter) 17 g of peptone from casein, 3 g of peptone from soymeal, 5 g of NaCl, and 2.5 g of K_2HPO_4 or in TSB–1% glucose.

Plasmids, primers, and cloning. Plasmid purifications were performed with the QIAprep spin miniprep kit (Qiagen, Valencia, Calif.). Primers were custom synthesized by Qiagen Operon (Alameda, Calif.). Restriction enzymes and DNA-modifying enzymes were purchased from New England Biolabs (Beverly, Mass.). The *tcaR* gene was amplified with primer pair tcaRF (TTTCTTCAAA AATATATTTAGTAGCGAATACAC) and tcaRR (AAGGATAAGATTATT GATAACGCAATAAC) and ligated to the expression plasmid pCRT7-NT (Invitrogen, Carlsbad, Calif.). The recombinant proteins were expressed in *Escherichia coli* BL21/pLysS cells according to the manufacturer's instructions. The pCRT7-NT vector adds a tag of six histidine residues and an Xpress epitope to the amino terminus of the protein. The IcaR and TcaR proteins and the vector expression control (the histidine tag and Xpress epitope alone) were sequentially purified with Probond nickel affinity chromatography resin (Invitrogen) and the strong cation exchange resin HiTrap SP XL (Amersham). The *lac* operon from *Staphylococcus xylosus* was amplified by PCR with lacHfwd (GAGTGAGTGCT CATTGCTTG) and lacHrev (GCCCTAGTTGACTATCATTAG) and cloned into the EcoRV site of the temperature-sensitive plasmid pBT9 (3). The resulting plasmid was amplified with the primer pair lacHmutfwd (GGACATGTTC CGACACTCGG) and lacHmutrev (TGATCTTCAAGATAGGTTCCATC) to delete the KpnI site within the *lacH* gene and religated to create pLacH.

All plasmid constructs were initially transformed into the restriction-deficient *S. aureus* strain RN4220 according to the method of Lee (15). Constructs were transferred to other strains of *S. aureus* by transduction with phage 80 (13). In order to create *icaR* and *tcaR* deletion mutants, the region surrounding the gene was amplified by PCR from the total DNA obtained from strain 10833 with one of the primer pairs icaRdelfwd and icardelrev (GGGGGTACCGGAAACCTT TTCGTTTTCATTGTGC) or tcaRdelfwd (GGGGGTACCCTTCAGTAACAT CTACCGTTTCAGAATTAC) and tcaRdelrev (GGGGGTACCGTGATATGG TGTATGACTTCCGACACCATC) and cloned into pCR-TOPO (Invitrogen). The resulting plasmid was amplified with icaR-xhofwd (CTCGAGCATCAAGT GTTGTACCGTCATACC) and icaR-xhorev (CTCGAGTGGAGCAGTGGAA GAAAGTAAAAGTC) or tcaR-xhofwd (CTCGAGGTAAATGTTTTACCAT AATTATTTCTCCC) and tcaR-xhorev (CTCGAGGAAGATATTGAAAATG TAAGGCAAGTATTAGAAG), phosphorylated with T4 kinase, and religated to produce a construct (pIcaRdel or pTcaRdel) with an XhoI restriction site in place of the coding region.

The erythromycin resistance cassette was amplified from Tn*917* by PCR with ermfwd (GGGGGTACCCTCGAGGCCTACGAGAAATTTGTATCG) and ermrev (GGGGGTACCCGTGTAACTTTCCAAATTTACAAAAGCG), digested with XhoI, and ligated into the XhoI site of pIcaRdel-TOPO and pTcaRdel-TOPO. The entire insert was then subcloned into the KpnI site of pLacH to produce pIcaR::erm-placH and pTcaR::erm-placH, which were transformed into RN4220 by electroporation.

Transformants were cultured overnight at 30°C in the presence of 10 µg of erythromycin/ml and 10 µg of chloramphenicol/ml, diluted 1:1,000, and subcultured overnight at 42°C without antibiotics. The cultures were diluted 1:10, plated on Luria-Bertani (LB) agar plates containing 10 µg of erythromycin/ml and 200 µg of Bluo-gal (Invitrogen)/ml, and grown for 48 h at 42°C. White

colonies were picked and screened for the double-crossover events, initially by PCR and then by DNA sequencing, which was carried out by the Microbiology Core Facility at Harvard Medical School (Boston, Mass.). In order to create the *icaR tcaR* double knockout, the tetracycline resistance cassette was excised from plasmid pTX15 (22) with NheI and ClaI. The ends were filled in with Klenow, and the fragment was ligated to XhoI-digested, blunt-ended pTcaR::erm-placH to produce pTcaR::tet-pLacH. This plasmid was used to replace *tcaR* in RN4220 with the tetracycline resistance marker, and the mutation was transduced to MN8 Δ*ica*::*erm* and 10833 Δ*ica*::*erm*.

DNA affinity chromatography. Four liters of TSB was inoculated with 40 ml of an overnight culture of *S. aureus* MN8. Cultures were well aerated and grown at 37°C for 5 h (A_{600} = 0.9). Cells were lysed essentially as described by Fournier et al. (8); they were first washed in 200 ml of buffer A (20 mM Tris-HCl, 50 mM $MgCl_2$, 1 mM dithiothreitol [DTT], 0.1 mM EDTA, 5% glycerol), frozen for 2 h at −80°C, and lysed in 50 ml of buffer A containing 100-µg/ml lysostaphin and EDTA-free complete protease inhibitors (Boehringer Mannheim, Indianapolis, Ind.) for 3.5 h on ice; 30 ml of buffer A–1.3 M KCl was added, and the incubation was continued on ice for 30 min. Lysates were cleared by centrifugation, precipitated with 50% ammonium sulfate at 4°C for 1 h, resuspended in 2 ml of phosphate-buffered saline (PBS), and dialyzed through a 3,500-molecular-weight-cutoff membrane (Pierce, Rockford, Ill.) overnight against 4 liters of 10 mM HEPES–1 mM $MgCl_2$–0.5 mM DTT at 4°C. Then 2 nmol of double-stranded MID-ICA probe (CTATGTTACAGGAAAATTAAGTTGCAATTAC AAATATTTCCGTTTAATTATAA annealed to its reverse complement) was biotinylated at one end and immobilized on 10 mg of streptavidin-coated M-280 Dynabeads (Dynal, Oslo, Norway) according to the manufacturer's instructions. The beads were equilibrated with binding buffer 1 (BB1; 10 mM HEPES, 60 mM KCl, 4 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mg of bovine serum albumin per ml, and 0.25 mM DTT); 1.5 ml of cell-free lysate (approximately 20 mg of protein) was combined with 6 ml of BB1 and 1.5 mg of sonicated salmon sperm DNA and chilled on ice for 10 min. The lysate mixture was added to the beads and incubated on ice for 10 min. The beads were washed once with BB1–200 µg of sonicated salmon sperm DNA per ml and twice with BB1 without bovine serum albumin or sonicated salmon sperm DNA. DNA-binding proteins were eluted with 1 ml of 10 mM HEPES–0.25 M KCl. The eluate was concentrated and desalted with Microcon YM-3.5 centrifugal concentrators (Millipore). The concentrated sample was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with NuPAGE 4 to 15% gradient gels and MOPS (morpholineethanesulfonic acid) buffer (Invitrogen). The gels were stained with Coomassie blue, and protein bands were excised and submitted to the Molecular Biology Core Facility (Dana-Farber Cancer Institute, Boston, Mass.) for sequencing by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectral analysis.

Electrophoretic mobility shift. The 198-bp probe was generated by PCR with genomic DNA from strain MN8 as a template, obtained from either *S. aureus* MN8 to yield the wild-type probe or *S. aureus* MN8m to yield the mutant probe with the primer pair icaFWD (ATTGCGTTATCAATAATCTTATCCTTC) and icaREV (TTGCAATTTCTTTACCTACCTTTC). The nonspecific competitor used in gel shift assays was also a 198-bp PCR product representing a sequence from the *icaA* gene and was amplified from MN8 total DNA with primer pair icaA-FWD (CCTGTATTTATGTCTATTTACTGG) and icaA-Rev (CTTCTC GTATTTGAGTGCAAG). The PCR products were purified with the Qiagen PCR purification kit. For gel shift analysis, the double-stranded probes were labeled with [γ-^{32}P]ATP with T4 kinase. Gel shift assays were performed essentially as described by Fournier et al. (8). A 20-µl binding reaction containing 5 to 25 ng (14 to 70 nM) of purified recombinant TcaR, 1 µg of sonicated salmon sperm DNA, and 1 µg of poly(dI-dC) in BB1 was incubated at 21°C for 10 min before 20,000 cpm of radiolabeled probe (final concentration of probe, 5 nM) was added. The reaction was incubated for an additional 15 min, loaded onto a 5% nondenaturing polyacrylamide gel, and electrophoresed in chilled 1× Tris-borate-EDTA (TBE) at 360 V for 1 h. Gels were dried and exposed to radiographic film overnight at −80°C.

DNase I footprint analysis. Footprinting was carried out essentially as described by Sandaltzopoulos and Becker, with the modifications described by Jefferson et al. (12, 26); 10 pmol of icaREV was end labeled with [γ-^{32}P]ATP with T4 kinase. The labeled oligonucleotide was precipitated with ethanol and used in a 50-µl PCR with 10 pmol of 5′-biotinylated icaFWD and genomic DNA from MN8 or MN8m as a template for the amplification of the 198-bp probe representing the *ica* promoter region. The biotinylated, radiolabeled PCR product was immobilized on 500 µg of Dynabeads according to the manufacturer's instructions. The magnetic beads were resuspended in 100 µl of binding buffer 2 [BB2; 10 mM Tris-Cl, 5 mM $MgCl_2$, 2 mM DTT, 50 µg of bovine serum albumin per ml, 2 µg of poly(dI-dC) per ml, 0.5 µg of sonicated salmon sperm DNA per

ml, 100 mM KCl]; 10 μl of oligonucleotide-coated beads, 100 ng of purified recombinant TcaR (112 nM) in Tris buffer, and 25 μl of BB2 were combined and incubated for 10 min at 21°C. Then 5 μl of a DNase I solution (10 mM Tris-Cl, 5 mM MgCl$_2$, 2 mM DTT, 10 mM CaCl$_2$, 100 mM KCl, 2.5 U of DNase I per ml) was added, and the reaction was carried out for 2 min at 21°C before the addition of 50 μl of 2× stop buffer (4 M NaCl, 100 mM EDTA). The beads were washed in 1× stop buffer, resuspended in 95% formamide–6 μg of bromophenol blue per ml, heated to 76°C for 5 min, and loaded on a 6% prerun sequencing gel at 55 W for 25 min. The gel was dried at 80°C and analyzed by autoradiography.

RNA slot blot analysis. *S. aureus* cultures were grown in TSB–1% glucose at 37°C for 16 h. RNA was extracted from 10^9 cells with the RNeasy Protect miniprep kit and RNase-free DNase kit (Qiagen) as described in the manufacturer's instructions except that 0.1 mg of lysostaphin per ml was used in place of lysozyme to lyse the cells. RNA concentrations were determined by absorbance at 260 nm, and 10 μg of each sample was immobilized on a nylon membrane. The single *icaADBC* transcript was detected with a DNA probe specific for *icaAD* that was generated by PCR (icaAfwd, GTATTTATGTCTATTTACTGGATTG TCGGTTC, and icaBrev, TCCGGCAATATGATCAAGATACTCAACA) and labeled with the ECL direct nucleic acid labeling and detection system (Amersham). Blots were reprobed with a probe specific for the housekeeping gene for DNA gyrase B, which was also generated by PCR (gyrBFwd, TTATGGTGCT GGGCAAATACAAG, and gyrBRev, ACCTTCGAATTGAGGATCACC) to ensure equal loading.

Adherence assay. Microtiter well adherence assays for PNAG production were performed essentially as described by Valle et al. with minor modifications (27). Cultures were grown overnight in tryptic soy broth (TSB)–1% glucose, diluted to an optical density at 650 nm (OD$_{650}$) of 0.2, and aliquoted into 96-well polystyrene flat-bottomed microtiter plates (Corning). The plates were centrifuged at 3,000 rpm for 10 min and incubated at 37°C for 1 h. The wells were emptied, washed twice with phosphate-buffered saline (PBS), dried at ambient temperature, stained for 30 s with safranin, and washed under gently running tap water. The stained bacteria were assessed qualitatively for relative adherence by visual inspection and quantitatively by resuspending the adherent cells in 200 μl of PBS by gentle sonication and measuring the OD$_{405}$ with an enzyme-linked immunosorbent assay reader. Statistical analysis was performed on the OD$_{405}$ values with Tukey's multiple-comparison test. Calculations were carried out with Prism software.

PNAG slot blot analysis. PNAG slot blots were performed essentially as described previously (6) with minor modifications. Bacteria were grown overnight in TSB–1% glucose, and 10^9 cells were pelleted, washed once with PBS, resuspended in 50 μl of 5 N NaOH, and vortexed for 30 s. The extracts were cleared by centrifugation, and diluted 1:5 in Tris-buffered saline (TBS), and 100 μl of extract from *S. aureus* strain MN8 or 10833 or 1 μl from strain MN8m was serially diluted in TBS and immobilized on nitrocellulose with a vacuum manifold. Blots were blocked for 1 h in 5% skim milk, probed with 1:50,000-diluted rabbit antiserum specific for PNAG (18) for 2 h at 21°C, washed, and probed with 1:10,000-diluted goat anti-rabbit immunoglobulin-horseradish peroxidase conjugate for 1 h at 21°C. Bands were visualized with the enhanced chemiluminescence kit (Amersham).

RESULTS

DNA affinity chromatography. *S. aureus* strain MN8m was originally isolated as a spontaneous PNAG-hyperproducing mutant of strain MN8. A deletion of a 5-nucleotide TATTT sequence located in the center of the *ica* promoter is sufficient for the PNAG-overproducing phenotype associated with strain MN8m, but the molecular basis for this effect has not been elucidated (12). IcaR binds to a distinct region of the *ica* promoter, and binding is unaffected by the deletion mutation. We therefore sought to isolate potential regulatory factors that bind to the region of the *ica* promoter in which the deletion mutation occurs. We designed a DNA affinity chromatography system to isolate proteins with high affinity for a 53-bp oligonucleotide representing the region of the *ica* promoter surrounding the TATTT motif. The oligonucleotide, MID-ICA, spans the *ica* promoter sequence from 131 to 79 bases upstream from the *icaA* start site. MID-ICA was biotinylated at one end, immobilized on streptavidin-coated magnetic parti-
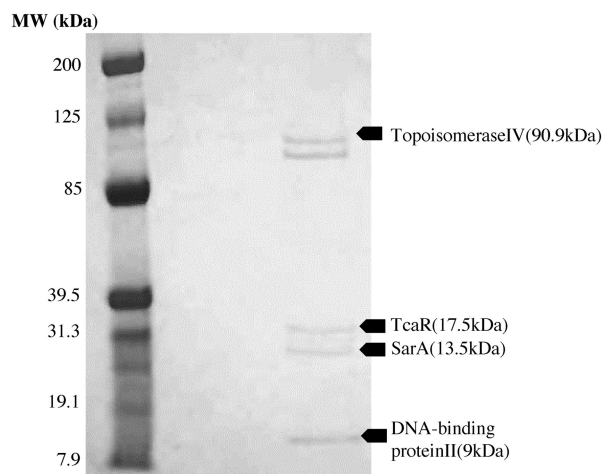

FIG. 1. SDS-PAGE analysis of proteins isolated by DNA affinity chromatography. The left lane contains standard proteins, and the right lane contains proteins isolated by their affinity for an oligonucleotide representing the sequence from the center of the *ica* promoter (MID-ICA). The gel was stained with Coomassie blue. The band intensities in this figure were digitally enhanced by using Adobe PhotoShop for the purpose of clarity. The indicated bands were excised and sequenced by MALDI-TOF mass spectral analysis. The proteins predicted by the sequence obtained are indicated.

cles, and used to purify specific DNA-binding proteins from an *S. aureus* lysate. The purified proteins were separated by SDS-PAGE and stained with Coomassie blue, as shown in Fig. 1. Bands were carefully excised and analyzed by MALDI-TOF mass spectral analysis.

Mass spectral analysis identified two nonspecific DNA binding proteins, DNA-binding protein II and topoisomerase IV, and SarA, which has been described previously as an *ica* regulator (1, 27). Studies with these proteins were not pursued further. The approximately 90-kDa protein that migrated slightly faster than topoisomerase IV was not successfully sequenced. Mass spectral analysis also indicated that an approximately 20-kDa protein isolated from the DNA affinity beads was most likely TcaR, a protein that displays significant homology to the MarR family of regulatory factors. The *tcaR* gene is part of the three-cistron teicoplanin-associated locus (*tca*), so named for the discovery that teicoplanin resistance increases in *S. aureus* when the locus is deleted or interrupted (2).

Affinity of TcaR for the *ica* promoter. In order to determine whether or not TcaR binds specifically to the *ica* promoter, we expressed histidine-tagged TcaR in *E. coli* and combined purified recombinant TcaR with a 198-bp probe representing the sequence of the entire wild-type *ica* promoter from strain MN8 or the mutant *ica* promoter from strain MN8m in electrophoretic mobility shift assays. The recombinant TcaR protein induced shifts in both the wild-type (lanes 2 and 3) and mutant (lanes 7 and 8) probes, producing a ladder-like pattern of migration (Fig. 2). The TcaR-induced shifts were lost when a 100-fold excess of unlabeled specific probe was added to the mixture but were unaffected by a 100-fold excess of a nonspecific, control probe (a 198-bp fragment of the *icaA* gene). An expression control (purified lysate from *E. coli* containing the empty expression vector) failed to shift either probe (data not
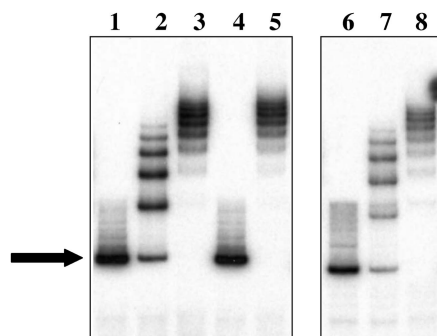
FIG. 2. Electrophoretic mobility shift assay indicates that TcaR binds to the *ica* promoter. Lanes 1 to 5 show mobility shift analysis of the probe representing the wild-type strain MN8 *ica* promoter. Additions to this probe were as follows: lane 1, nothing (free probe only); lane 2, 5 ng (14 nM) of purified recombinant TcaR; lane 3, 25 ng (70 nM) of TcaR; lane 4, a 100-fold excess of specific competitor (unlabeled wild-type probe) and 25 ng (70 nM) of TcaR; and lane 5, 100-fold excess nonspecific competitor (198 bp sequence from *icaA*) and 25 ng (70 nM) of TcaR. Lanes 6 to 8 show mobility shift analyses of the probe representing the *ica* promoter of the mucoid strain MN8m (MUC). Additions to this probe were as follows: lane 6, nothing (free probe only); lane 7, 5 ng (14 nM) of TcaR; and lane 8, 25 ng (70 nM) of TcaR. The solid arrow indicates the free probe. TcaR induced a number of shifts in both the wild-type and mutant probes.

shown). These results indicate that TcaR binds to the *ica* promoter specifically and that binding is not dependent upon the 5-bp TATTT motif.

In order to localize the TcaR binding site(s) within the *ica* promoter, we performed DNase I footprint analysis. DNase I footprinting resulted in a diffuse pattern of protection over approximately 120 bases in the center of the *ica* promoter, indicating that TcaR protects multiple sites within the *ica* promoter (data not shown). The pattern of protection was similar within the wild-type and mutant *ica* promoter sequences. This result, coupled with the observation of a ladder effect in the gel shift assays, could indicate the presence of multiple TcaR recognition sites within the *ica* promoter sequence or that TcaR oligomerizes once it is bound to the promoter region.

Effect of TcaR on *ica* transcription. The specificity with which TcaR bound to the *ica* promoter suggested that the protein may have a regulatory function in *ica* transcription. In order to characterize the effect of TcaR on *ica* transcription and PNAG synthesis, we replaced the *tcaR* gene in the RN4220 chromosome by allelic replacement with an erythromycin resistance cassette and transduced the mutation to *S. aureus* strains MN8, MN8m, and 10833. The transcriptional activity of the *ica* locus was characterized in the resulting strains, MN8 Δ*tcaR*::*erm*, MN8m Δ*tcaR*::*erm*, and 10833 Δ*tcaR*::*erm*. We constructed a probe that binds to *icaAD* mRNA and used it to detect *ica* transcript levels in bacterial extracts by Northern slot blot analysis. The *icaADBC* genes are transcribed as a single transcript, so *icaAD* mRNA is representative of the transcription of all four genes. All blots were reprobed with a probe specific for the housekeeping gene DNA gyrase B to ensure equal loading of RNA (data not shown). Transcription of the *ica* locus was previously shown to be elevated in the PNAG hyperproducing strain MN8m, so only 2 μg of total RNA from the MN8m strains was blotted onto nylon membranes, whereas
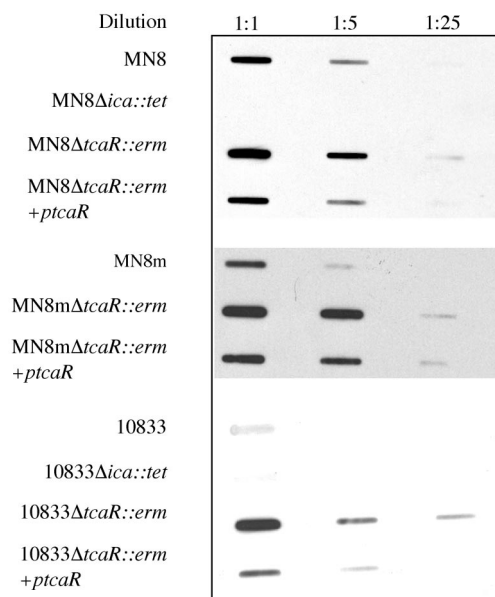

FIG. 3. Slot blot analysis of RNA indicates that deletion of *tcaR* augments *icaADBC* transcription in strains MN8, MN8m, and 10833. Total RNA was isolated from the indicated strains, serial dilutions of the RNA were made, and 10, 2, or 0.4 μg of MN8 and 10833 RNA or 2, 0.4, or 0.08 μg of MN8m RNA was immobilized on nylon membranes with a slot blot apparatus. The blots were probed with a single-stranded DNA probe specific for the *icaAD* transcript labeled with horseradish peroxidase. A chemiluminescent substrate was added, and the blots were exposed to autoradiographic film. Blots representative of three separate experiments are shown.

10 μg of total RNA from the weakly PNAG-producing strains 10833 and MN8 was analyzed. Northern analysis indicated an approximately fivefold increase in *icaAD* transcript level in all three strains in the absence of *tcaR* (Fig. 3). This increase was lost when the mutants were complemented with a plasmid carrying *tcaR* under the control of an inducible promoter (p*tcaR*), indicating that the phenotype was due to the lack of *tcaR*. Thus, TcaR appears to be a negative regulator of *ica* transcription.

Effect of TcaR on PNAG production. We hypothesized that because *ica* transcription was elevated in the *tcaR* deletion mutants, PNAG synthesis and biofilm formation would also be augmented in these strains. The ability of these strains to form a biofilm was measured by a microtiter well adherence assay. The strains were grown in TSB–1% glucose overnight and then diluted and allowed to adhere to polystyrene microtiter wells. Adherence was assessed by visual inspection of the dry, stained biofilms and by optical density measurement of resuspended, stained bacteria. The MN8 Δ*ica*::*tet* and 10833 Δ*ica*::*tet* mutants, which lack the *icaADBC* genes and are therefore unable to synthesize PNAG, were used as controls in the adherence assay, and both produced very little biofilm. As shown in Fig. 4A and B, deletion of *tcaR* did not have a significant effect on the ability of the strains to adhere ($P > 0.05$, Tukey's multiple comparison test).

Adherence to plastic is a good relative measure of PNAG production, but other surface proteins and polysaccharides can also play a role in adherence. In order to measure PNAG production more directly, we performed a PNAG slot blot
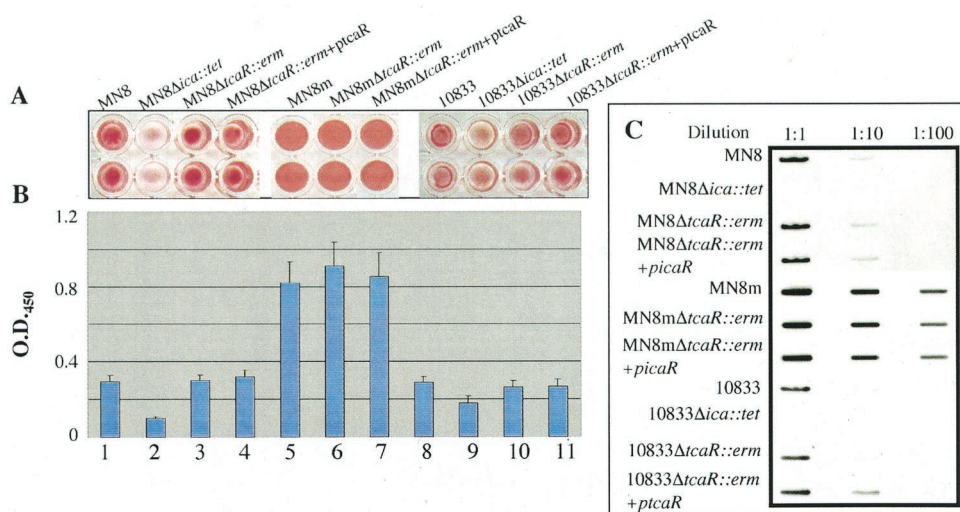
FIG. 4. Adherence assays and slot blot analysis of cell surface extracts indicate that the *tcaR* gene does not have a significant effect on PNAG production or adherence in *S. aureus*. (A) Bacteria were grown in TSB–1% glucose overnight. Strains were diluted to an OD_{650} of 0.2, and the cells in 200 µl of each culture were centrifuged to the bottom of wells in a polystyrene microtiter plate. The wells were washed with PBS, and adherent cells were stained with safranin. (B) After staining, 200 µl of PBS was added to each microtiter well, and the bacteria were homogenized by sonication. Bars reflect the mean OD_{405} of eight samples, and error bars show the standard deviations. (C) Bacteria were grown in 5 ml of TSB–1% glucose for 16 h. Cultures were centrifuged to pellet the cells. Cell surface extracts were made by resuspending bacteria in 50 µl of 5 N NaOH. Extracts were cleared by centrifugation and diluted with 250 µl of TBS, and serial 10-fold dilutions of 100 µl of MN8 and 10833 extracts or of 1 µl of MN8m extract were immobilized on nitrocellulose membranes. The blots were blocked with 5% milk and probed with PNAG-specific rabbit antiserum and polyclonal goat anti-rabbit immunoglobulin-horseradish peroxidase conjugate. A chemiluminescent substrate was added to the blots, and the blots were exposed to autoradiographic film.

assay with polyclonal antiserum specific for PNAG. Cell surface extracts were prepared from stationary-phase cultures grown in TSB–1% glucose, blotted onto nitrocellulose, and probed with a PNAG-specific polyclonal antiserum (18). As MN8m is a PNAG hyperproducer, extracts from this strain and MN8m Δ*tcaR*::*erm* were diluted 100-fold before they were blotted onto nitrocellulose. Again, MN8 Δ*ica*::*tet* and 10833 Δ*ica*::*tet* were used as negative controls and did not react with the PNAG-specific antiserum. There was no obvious difference in the amount of PNAG elaborated by the *tcaR* mutants with respect to the parent strains (Fig. 4).

We were surprised that despite the increase in *ica* transcription in the absence of TcaR, there was no discernible effect on PNAG synthesis, and we reasoned that the effects of the *tcaR* deletion might be manifested under different growth conditions. To test this, cultures were grown under various conditions that have been shown previously to affect biofilm formation, including high osmolarity (4% NaCl), elevated temperature (45°C), and the presence of a subinhibitory concentration of tetracycline (0.05 µg/ml) (24). The *tcaR* deletant did not exhibit significantly greater PNAG expression than the wild-type strain under any conditions (data not shown). These findings suggests that while TcaR appears to be a negative regulator of *ica* transcription, an effect on PNAG expression in the *tcaR* deletant strains was not detected by our methods. The effect of the *tcaR* deletion might be masked under normal growth conditions either by a posttranscriptional regulatory mechanism or possibly by an additional negative regulator of transcription. We therefore hypothesized that IcaR, a known regulator of *ica* transcription in *S. epidermidis*, might obscure

the effect of TcaR, and we next sought to compare the phenotype of an *icaR* deletant with that of the *tcaR* deletant.

Effect of IcaR on *icaADBC* transcript levels. It has been reported that IcaR is a repressor of *icaADBC* transcription in *S. epidermidis*, although in this report, deletion of *icaR* did not affect biofilm formation (4). We previously demonstrated, by DNase I protection and electrophoretic mobility shift analysis, that IcaR binds to the *S. aureus ica* promoter, but direct evidence that it is a repressor of *ica* transcription in *S. aureus* has not yet been presented (12). In order to confirm that IcaR acts as a repressor of *ica* transcription in *S. aureus*, we inactivated the *icaR* gene within three *S. aureus* strains, MN8, MN8m, and 10833, by allelic replacement with an erythromycin resistance cassette. Total RNA from 16-h TSB–1% glucose cultures of the resulting strains, MN8 Δ*icaR*::*erm*, MN8m Δ*icaR*::*erm*, and 10833 Δ*icaR*::*erm,* was subjected to Northern slot blot analysis with the *icaAD*-specific probe. All blots were also analyzed with a DNA gyrase-specific probe to ensure equal loading (data not shown). As explained above, only 2 µg of RNA from the MN8m and MN8m Δ*icaR*::*erm* strains was analyzed, whereas 10 µg of RNA from the MN8 and 10833 strains was analyzed.

In all three strains, inactivation of *icaR* increased the level of the *icaAD* transcript (Fig. 5). This increase was estimated to be at least 100-fold, as measured by densitometric analysis with NIH Image software (data not shown). These results demonstrate that, in *S. aureus*, IcaR is a repressor of *ica* transcription and that it is functional even in the PNAG-overproducing strain MN8m. The finding that the negative regulatory effects of both IcaR and TcaR are unaffected by the 5-bp deletion
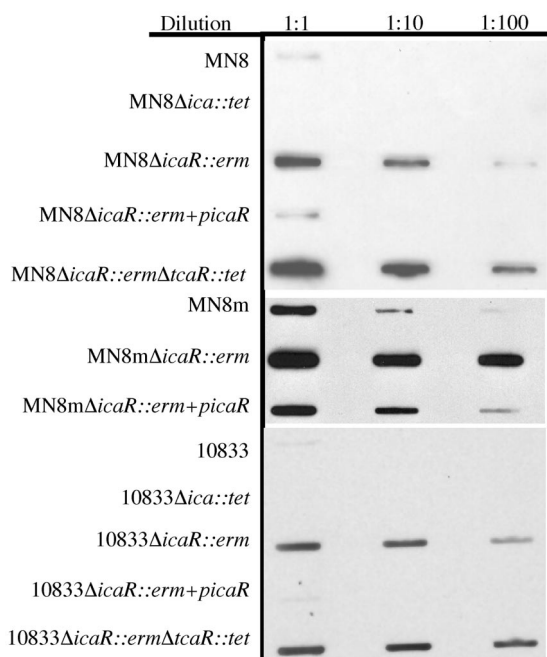
FIG. 5. Slot blot analysis of RNA indicates that deletion of *icaR* augments *icaADBC* transcription in strains MN8, MN8m, and 10833. Total RNA was isolated from the indicated strains, serial dilutions of the RNA were made, and 10, 1, or 0.1 μg of MN8 and 10833 RNA or 2, 0.2, or 2 ng of MN8m RNA was immobilized onto nylon membranes with a slot blot apparatus. The blots were probed with a single-stranded DNA probe specific for the *icaAD* transcript labeled with horseradish peroxidase. A chemiluminescent substrate was added, and the blots were exposed to autoradiographic film. Blots representative of three separate experiments are shown.

within the *ica* promoter of MN8m is somewhat surprising, considering that PNAG expression is so high in this strain. This suggests that MN8m may have lost a requirement for a positive regulator or that the 5-bp deletion somehow increases the efficiency of transcriptional initiation by the RNA polymerase complex at this promoter.

Mutation of *icaR* augmented *ica* transcription 100-fold, whereas mutation of *tcaR* induced only a 5-fold increase in transcription, suggesting that IcaR is a stronger repressor of *ica* transcription. We therefore reasoned that functional IcaR likely prevents any increase in PNAG production in the *tcaR* mutant strains. We replaced *tcaR* with a tetracycline resistance cassette in the *icaR* knockout strains to produce MN8 Δ*icaR*::*erm* Δ*tcaR*::*tet* and 10833 Δ*icaR*::*erm* Δ*tcaR*::*tet*. The double-knockout strains exhibited an approximately 500-fold increase in *ica* transcription with respect to the wild-type strain, suggesting a synergistic effect of deleting both regulators.

Effect of *icaR* on PNAG production. To assess PNAG production in the *icaR* mutants, the microtiter adherence assay was again used. As shown in Fig. 6A and B, the *icaR* knockout strains exhibited increased adherence to the polystyrene microtiter wells with respect to their parent strains ($P < 0.001$, Tukey's multiple-comparison test). There was an approximately twofold increase in the OD_{405} of the MN8 Δ*icaR*::*erm*,

MN8m Δ*icaR*::*erm*, and 10833 Δ*icaR*::*erm* constructs compared to strains MN8, MN8m, and 10833, respectively.

To confirm that the increase in adherence was a direct result of inactivating *icaR*, we complemented 10833 Δ*icaR*::*erm*, MN8 Δ*icaR*::*erm*, and MN8m Δ*icaR*::*erm* with a plasmid carrying an inducible copy of the *icaR* gene (picaR). Complementation of the *icaR* deletions reduced adherence, confirming that the increase in adherence was a result of deleting *icaR* (Fig. 6A and B). It is notable that strain MN8m, which normally produces copious quantities of PNAG, exhibited even greater adherence and was more aggressive in the absence of IcaR. On TSA plates, MN8m Δ*icaR*::*erm* formed small tight colonies that were even stickier that MN8m colonies, and when grown in TSB, the bacteria were so aggressive that they formed a single compact pellet (data not shown). This suggests that despite the PNAG-hyperproducing phenotype of strain MN8m, IcaR-mediated repression of *ica* is still effective in this strain.

Adherence of the *icaR tcaR* double knockout strains MN8 Δ*icaR*::*erm* Δ*tcaR*::*tet* and 10833 Δ*icaR*::*erm* Δ*tcaR*::*tet* was significantly greater than that of the single *icaR* mutant strains ($P < 0.001$), suggesting that, as would be expected from its role in regulation of *ica* transcription, TcaR can affect biofilm formation.

We also measured PNAG production in the *icaR* mutant strains. Figure 6C shows that PNAG synthesis was approximately 10-fold higher in the *icaR* deletion mutants than in the respective parent strains, substantiating the findings in the adherence assays. Complementation of the *icaR* gene reduced PNAG expression to wild-type levels (Fig. 6C). Interestingly, deletion of both *icaR* and *tcaR* resulted in a 100-fold increase in PNAG elaboration, 10-fold greater than the increase observed in the *icaR* single knockout. Overall, these results suggest that both IcaR and TcaR repress *ica* transcription and subsequent PNAG production but that IcaR is a more potent repressor and can override the effects of deleting *tcaR*. Deleting both *tcaR* and *icaR* resulted in a great increase in PNAG production and adherence, but the level of PNAG synthesis in the double knockouts of the wild-type strains was not as high as that associated with strain MN8m, suggesting that other regulatory factors are involved in *ica* transcriptional control.

When working with the MN8m strains, we noted that while this strain was not highly aggregative in TSB made without glucose, MN8m Δ*icaR*::*erm* grew as an aggregated cellular mass even in the absence of glucose. We therefore performed PNAG blots on the strains and *icaR* deletants grown in TSB without any glucose. The strains were first grown overnight in the absence of glucose, diluted the following day 1:100 in TSB without glucose, and grown to late log phase. Figure 6D shows that deletion of *icaR* significantly augmented PNAG production in the absence of glucose. In fact, in strains MN8 and 10833, PNAG production was undetectable by our methods in the absence of glucose but was strong in the *icaR* knockout strains. This suggests that IcaR plays a role in the suppression of PNAG production in the absence of glucose.

DISCUSSION

A thorough comprehension of the mechanism by which PNAG elaboration is regulated in *S. aureus* is an important
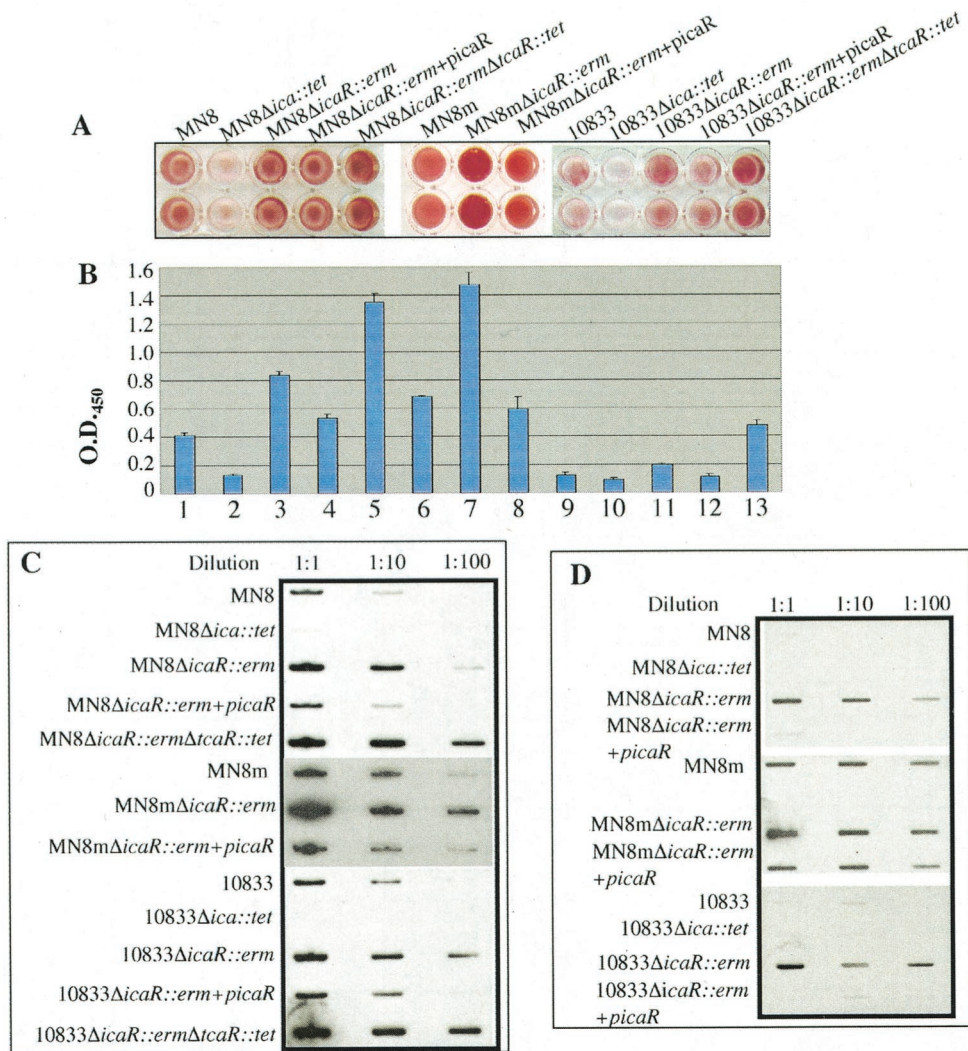
FIG. 6. Slot blot analysis of cell surface extracts indicates that deletion of the *icaR* gene augments PNAG production and adherence in *S. aureus*. (A) Bacteria were grown in TSB–1% glucose overnight. Strains were diluted to an OD_{650} of 0.2, and the cells in 200 μl of each culture were centrifuged to the bottom of the wells in a polystyrene microtiter plate. The wells were washed with PBS, and adherent cells were stained with safranin. (B) After staining, 200 μl of PBS was added to each microtiter well, and the bacteria were homogenized by sonication. Bars reflect the mean OD_{405} of eight samples, and error bars show the standard deviations. (C) Bacteria were grown in TSB–1% glucose overnight. Cultures were diluted to an OD_{650} of 1.0, and 1 ml was centrifuged to pellet the cells. Cell surface extracts were made by resuspending bacteria in 50 μl of 5 N NaOH. Extracts were cleared by centrifugation and diluted with 250 μl of TBS, and serial 10-fold dilutions of 100 μl of MN8 and 10833 extracts or of 1 μl of MN8m extract were immobilized on nitrocellulose membranes. The blots were blocked with 5% milk and probed with PNAG-specific rabbit antiserum and polyclonal goat anti-rabbit immunoglobulin-horseradish peroxidase conjugate. A chemiluminescent substrate was added to the blots, and they were exposed to autoradiographic film.

prerequisite to understanding biofilm formation and could ultimately lead to the development of methods to repress the expression of this important virulence factor. It is becoming increasingly apparent, however, that transcriptional regulation of the genes involved in PNAG synthesis, the *ica* genes, is quite complex. It has previously been reported that IcaR is a repressor of *ica* transcription in *S. epidermidis*, although an influence of IcaR on PNAG production and bacterial adherence or biofilm formation was not demonstrated (4). We have shown here that IcaR is also a repressor of *ica* transcription in *S. aureus* and that deletion of *icaR* results in a substantial increase in PNAG elaboration and bacterial adherence to polystyrene. We

also present evidence that glucose impacts *ica* transcription and PNAG production by counteracting the negative regulatory effect of IcaR. Dobinsky et al. reported that despite elevated transcription of the *icaADBC* genes, a single strain of *S. epidermidis* did not form a biofilm or produce detectable amounts of the low-molecular-weight form of PNAG in the absence of glucose (7). In the presence of glucose, the level of *icaADBC* transcript was very low, but biofilms and the low-molecular-weight form of PNAG were produced. The most intuitive explanation for this dissociation between *ica* transcription and PNAG production is that glucose is required as a substrate for factors involved in PNAG production. The results

presented here indicate that in *S. aureus*, the glucose-mediated increase in PNAG is due, at least in part, to alleviation of IcaR-mediated repression and a subsequent increase in *ica* transcription. When *icaR* was deleted in *S. aureus* strains MN8, 10833, and MN8m, *ica* transcription was elevated and PNAG production was greatly increased even in the absence of any exogenous glucose source. This strongly suggests that the positive effect that glucose has on PNAG expression is at least partially attributable to alleviation of IcaR-mediated repression and transcriptional activation of *ica*. Further studies will be required to determine whether glucose interacts directly with IcaR to alleviate repression of *ica* transcription or if glucose exerts its effect on IcaR indirectly, through some other regulatory factor.

Interestingly, the MN8m ΔicaR::erm strain, which produced copious quantities of PNAG, was quite growth deficient. Colonies were small, and in broth the bacteria formed a small compact pellet. Presumably the bacteria were so aggregative that they were deprived of the space and nutrients required for replication. This helps to explain why the *ica* locus is so tightly regulated. Limited PNAG production likely allows the bacteria to form a protective biofilm and to localize in a nutrient-rich environment, but when PNAG is produced unchecked, growth of the cell population is retarded. In addition to its role in biofilm formation, PNAG may act as a "sink" in which excess glucose can be removed from the cell and possibly stored for future use. Alternately, unregulated PNAG production may divert glucose and other substrates into polysaccharide production and deprive the bacterial cells of sufficient nutrients to grow and divide. Overall, if a bacterium synthesizes too much PNAG, it is likely using excessive energy and resources for the production of one factor at the expense of reproduction, indicating one reason why staphylococci need an effective means of tightly regulating PNAG elaboration.

TcaR proved to have a strong interaction with the *ica* promoter in vitro. Although Northern analysis of *tcaR* knockout strains suggested that TcaR is a negative regulator of *ica* transcription, deletion of *tcaR* did not have a significant impact on PNAG production in strains that were also able to make IcaR. IcaR appeared to be a more potent repressor of *ica* transcription and was able to mask any phenotypic effects due to the deletion of *tcaR*. The effects of knocking out both *icaR* and *tcaR* on *ica* transcription, PNAG production, and adherence were synergistic. Overall, both the *icaR* and *tcaR* genes encode proteins regulating *ica* transcription and PNAG production, which, along with *sarA*, can now be considered part of the regulatory network affecting biofilm formation by *S. aureus*.

REFERENCES

1. **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.
2. **Brandenberger, M., M. Tschierske, P. Giachino, A. Wada, and B. Berger-Bachi.** 2000. Inactivation of a novel three-cistronic operon *tcaR-tcaA-tcaB* increases teicoplanin resistance in *Staphylococcus aureus.* Biochim. Biophys. Acta **1523:**135–139.
3. **Brückner, R.** 1997. Gene replacement in *Staphylococcus carnosus* and *Staphylococcus xylosus.* FEMS Microbiol. Lett. **151:**1–8.
4. **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.
5. **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.
6. **Cramton, S. E., M. Ulrich, F. Götz, and G. Döring.** 2001. Anaerobic conditions induce expression of polysaccharide intercellular adhesin in *Staphylococcus aureus* and *Staphylococcus epidermidis.* Infect. Immun. **69:**4079–4085.
7. **Dobinsky, S., K. Kiel, H. Rohde, K. Bartscht, J. K. Knobloch, M. A. Horstkotte, and D. Mack.** 2003. Glucose-related dissociation between *icaADBC* transcription and biofilm expression by *Staphylococcus epidermidis*: evidence for an additional factor required for polysaccharide intercellular adhesin synthesis. J. Bacteriol. **185:**2879–2886.
8. **Fournier, B., R. Aras, and D. C. Hooper.** 2000. Expression of the multidrug resistance transporter NorA from *Staphylococcus aureus* is modified by a two-component regulatory system. J. Bacteriol. **182:**664–671.
9. **Galdbart, J. O., J. Allignet, H. S. Tung, C. Ryden, and N. El Solh.** 2000. Screening for *Staphylococcus epidermidis* markers discriminating between skin-flora strains and those responsible for infections of joint prostheses. J. Infect. Dis. **182:**351–355.
10. **Götz, F., C. Heilmann, and S. E. Cramton.** 2000. Molecular basis of catheter associated infections by staphylococci. Adv. Exp. Med. Biol. **485:**103–111.
11. **Heilmann, C., O. Schweitzer, C. Gerke, N. Vanittanakom, D. Mack, and F. Götz.** 1996. Molecular basis of intercellular adhesion in the biofilm-forming *Staphylococcus epidermidis.* Mol. Microbiol. **20:**1083–1091.
12. **Jefferson, K. K., S. E. Cramton, F. Götz, and G. B. Pier.** 2003. Identification of a 5-nucleotide sequence that controls expression of the *ica* locus in *Staphylococcus aureus* and characterization of the DNA-binding properties of IcaR. Mol. Microbiol. **48:**889–899.
13. **Kasatiya, S. S., and J. N. Baldwin.** 1967. Nature of the determinant of tetracycline resistance in *Staphylococcus aureus.* Can. J. Microbiol. **13:**1079–1086.
14. **Knobloch, J. K., K. Bartscht, A. Sabottke, H. Rohde, H. H. Feucht, and D. Mack.** 2001. Biofilm formation by *Staphylococcus epidermidis* depends on functional RsbU, an activator of the *sigB* operon: differential activation mechanisms due to ethanol and salt stress. J. Bacteriol. **183:**2624–2633.
15. **Lee, J. C.** 1993. Methods in molecular biology, vol. 47. Electrotransformation of staphylococci. Humana Press Inc., Totowa, N.J.
16. **Leid, J. G., J. W. Costerton, M. E. Shirtliff, M. S. Gilmore, and M. Engelbert.** 2002. Immunology of Staphylococcal biofilm infections in the eye: new tools to study biofilm endophthalmitis. DNA Cell Biol. **21:**405–413.
17. **Lowy, F. D.** 1998. *Staphylococcus aureus* infections. N. Engl. J. Med. **339:**520–532.
18. **Maira-Litran, T., A. Kropec, C. Abeygunawardana, J. Joyce, G. Mark III, D. A. Goldmann, and G. B. Pier.** 2002. Immunochemical properties of the staphylococcal poly-*N*-acetylglucosamine surface polysaccharide. Infect. Immun. **70:**4433–4440.
19. **McKenney, D., J. Hubner, E. Muller, Y. Wang, D. A. Goldmann, and G. B. Pier.** 1998. The *ica* locus of *Staphylococcus epidermidis* encodes production of the capsular polysaccharide/adhesin. Infect. Immun. **66:**4711–4720.
20. **McKenney, D., K. L. Pouliot, Y. Wang, V. Murthy, M. Ulrich, G. Döring, J. C. Lee, D. A. Goldmann, and G. B. Pier.** 1999. Broadly protective vaccine for *Staphylococcus aureus* based on an in vivo-expressed antigen. Science **284:**1523–1527.
21. **Peacock, S. J., C. E. Moore, A. Justice, M. Kantzanou, L. Story, K. Mackie, G. O'Neill, and N. P. Day.** 2002. Virulent combinations of adhesin and toxin genes in natural populations of *Staphylococcus aureus.* Infect. Immun. **70:**4987–4996.
22. **Peschel, A., B. Ottenwalder, and F. Götz.** 1996. Inducible production and cellular location of the epidermin biosynthetic enzyme EpiB using an improved staphylococcal expression system. FEMS Microbiol. Lett. **137:**279–284.
23. **Rachid, S., K. Ohlsen, U. Wallner, J. Hacker, M. Hecker, and W. Ziebuhr.** 2000. Alternative transcription factor 32 σ^B is involved in regulation of biofilm expression in a *Staphylococcus aureus* mucosal isolate. J. Bacteriol. **182:**6824–6826.
24. **Rachid, S., K. Ohlsen, W. Witte, J. Hacker, and W. Ziebuhr.** 2000. Effect of subinhibitory antibiotic concentrations on polysaccharide intercellular adhesin expression in biofilm-forming *Staphylococcus epidermidis.* Antimicrob. Agents Chemother. **44:**3357–3363.
25. **Reese, M. G.** 2001. Application of a time-delay neural network to promoter annotation in the *Drosophila melanogaster* genome. Comput. Chem. **26:**51–56.
26. **Sandaltzopoulos, R., and P. B. Becker.** 1994. Solid phase DNase I footprinting: quick and versatile. Nucleic Acids Res. **22:**1511–1512.
27. **Valle, J., A. Toledo-Arana, C. Berasain, J. M. Ghigo, B. Amorena, J. R. Penades, and I. Lasa.** 2003. SarA and not sigma(B) is essential for biofilm development by *Staphylococcus aureus.* Mol. Microbiol. **48:**1075–1087.