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icaR Encodes a Transcriptional Repressor Involved in Environmental Regulation of *ica* Operon Expression and Biofilm Formation in *Staphylococcus epidermidis*

Kevin M. Conlon, Hilary Humphreys, and James P. O'Gara*

Department of Microbiology, RCSI Education and Research Centre, Beaumont Hospital, Royal College of Surgeons in Ireland, Dublin 9, Ireland

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Biofilm formation in *Staphylococcus epidermidis* is dependent upon the *ica* operon-encoded polysaccharide intercellular adhesin, which is subject to phase-variable and environmental regulation. The *icaR* gene, located adjacent to the *ica* operon, appears to be a member of the *tetR* family of transcriptional regulators. In the reference strain RP62A, reversible inactivation of the *ica* operon by IS*256* accounts for 25 to 33% of phase variants. In this study, *icaA* and *icaR* regulation were compared in RP62A and a biofilm-forming clinical isolate, CSF41498, in which IS*256* is absent. Predictably, *ica* operon expression was detected only in wild-type CSF41498 and RP62A but not in non-IS*256*-generated phase variants. In contrast, the *icaR* gene was not expressed in RP62A phase variants but was expressed in CSF41498 variants. An *icaR*::Em^r insertion mutation in CSF41498 resulted in an at least a 5.8-fold increase in *ica* operon expression but did not significantly alter regulation of the *icaR* gene itself. Activation of *ica* operon transcription by ethanol in CSF41498 was *icaR* dependent. In contrast, a small but significant induction of *ica* by NaCl and glucose (NaCl-glucose) was observed in the *icaR*::Em^r mutant. In addition, transcription of the *icaR* gene itself was not significantly affected by NaCl-glucose but was repressed by ethanol. Expression of the *ica* operon was induced by ethanol or NaCl-glucose in phase variants of CSF41498 (*icaR*$^+$) but not in RP62A variants (*icaR* deficient). These data indicate that *icaR* encodes a repressor of *ica* operon transcription required for ethanol but not NaCl-glucose activation of *ica* operon expression and biofilm formation.

Biofilm-forming coagulase-negative staphylococci (CoNS), particularly *Staphylococcus epidermidis*, are the etiological agents in a significant proportion of biomaterial-related nosocomial infections. Formation of *S. epidermidis* biofilms is proposed to occur in a two-step manner (20, 24, 26, 39) in which a cellular accumulation process to form the mature biofilm follows a rapid initial attachment to an inert synthetic surface. Initial adherence is mediated by polysaccharide adhesin (PS/A) (24) and/or one of several proteins (including autolysin [19]), and accumulation of cells is due to production of polysaccharide intercellular adhesin (PIA) (20). The PIA is encoded by the *ica* (intercellular adhesin) operon (20); however, it has been reported recently that this operon also encodes PS/A and that PS/A and PIA are chemically closely related (27). Production of PIA, which represents the key virulence factor of *S. epidermidis*, is subject to phase-variable regulation (38, 39). Little is known about the molecular basis of this phase variation process; however, recent evidence has indicated that alternating insertion and excision of an insertion sequence element is responsible for between 25 and 33% of *ica* operon switching in *S. epidermidis* RP62A under laboratory conditions (39). The insertion element IS*257* has also been observed inserting at the *ica* locus of a clinical *S. epidermidis* isolate (32).

The role of the *S. epidermidis* and *Staphylococcus aureus ica* locus in biofilm infections has been examined by a number of investigators. Ziebuhr et al. (38) reported that 85% of *S. epidermidis* blood culture isolates contained the *ica* genes, compared to 6% of saprophytic isolates. A number of studies have indicated the usefulness of targeting the *ica* locus as a diagnostic marker to distinguish between invasive and contaminating isolates of *S. epidermidis* (3, 4, 11, 12). An interesting recent report found no significant difference in terms of *ica* status among CoNS isolated from healthy and sick infants in a neonatal intensive care unit but did note that biofilm-forming capacity was significantly greater in *S. epidermidis* strains isolated from the blood or skin of neonates with bacteremia (10). These authors concluded that regulation of biofilm expression might play a central role in disease caused by CoNS. Using animal models of catheter infection, Rupp et al. demonstrated the essential requirement for an intact *ica* operon and production of PS/A in the pathogenesis of *S. epidermidis* infection (34–36). Finally, the PS/A encoded by the *ica* operon of *S. aureus* was successfully used to immunize mice against *S. aureus* kidney infection (28, 29).

The *ica* gene cluster, which contains all the genes necessary for production of PIA, was identified using transposon mutagenesis to isolate *S. epidermidis* mutants defective in biofilm formation (17, 18, 20, 24). The *ica* locus contains an operon, *icaADBC*, which contains the structural genes required for PIA synthesis. Expression of the *ica* operon is regulated under in vivo conditions in *S. aureus* (29) and by environmental parameters in the laboratory in *S. epidermidis*. Anaerobic growth (9), the presence of subinhibitory concentrations of certain antibiotics, and environmental stresses (22, 31) all result in elevated

* Corresponding author. Mailing address: Department of Microbiology, RCSI Education and Research Centre, Smurfit Building, Beaumont Hospital, Royal College of Surgeons in Ireland, Dublin 9, Ireland. Phone: 353-1-809 3711. Fax: 353-1-809 3709. E-mail: jogara@rcsi.ie.

TABLE 1. Strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strain		
S. epidermidis CSF41498	Biofilm positive, cerebrospinal fluid isolate	This study
S. epidermidis RP62A	Biofilm positive	ATCC 35984[a]
S. epidermidis ICAR1-4	*icaR*::Em^r	This study
S. aureus RN4220	Restriction-negative, intermediate host for plasmid transfer from *E. coli* to *S. epidermidis*	23
E. coli XL-1 Blue	*recA1 endA1* lac [F′ *proAB lacI*^{q} Tn*10*(Tet^r)]	Stratagene
Plasmid		
pEC5	pBluescript KS$^+$ derivative. Source of *ermB* gene (Em^r). Ap^r	5
pBT2	Temperature-sensitive *E. coli-Staphylococcus* shuttle vector. Ap^r (*E. coli*) Cm^r (*Staphylococcus*)	5
pSTBlue	PCR cloning vector. Km^r Ap^r	Novagen
pST*icaR*	786-bp PCR product containing the *icaR* gene amplified from CSF41498 using primers ICAR1 and IPR2 in pSTBlue	This study
pGEM3Z	Cloning vector. Ap^r	Promega
pSER1	810-bp *Bam*HI-*Hin*dIII fragment from pST*icaR* containing *icaR* in pGEM3Z	This study
pSER2	1,227-bp *Eco*RI-*Cla*I *ermB* fragment from pEC5 cloned into *Bpu*10I site of *icaR* gene in pSER1	This study
pSER3	2,037-bp *Bam*HI-*Hin*dIII fragment containing *icaR*::Em^r in pBT2	This study
pSER4	810-bp *Bam*HI-*Hin*dIII fragment from pSER1 containing *icaR* cloned into pBT2	This study

[a] ATCC, American Type Culture Collection.

expression of the *ica* operon or PIA synthesis. Transposon mutations in other loci, including *rsbU*, the first gene in an operon highly homologous to the *sigB* operon of *S. aureus* (22), which alter the expression of PIA synthesis have also been identified (22, 25).

Located upstream of the *icaADBC* operon is the divergently transcribed *icaR* gene. The role of *icaR* in biofilm formation has not been determined; however, amino acid sequence alignments suggest that the *icaR* gene product is a transcriptional regulator (30, 39). In this study we have examined the role of the *icaR* gene in the regulation of *ica* operon expression and biofilm formation in a biofilm-forming clinical isolate of *S. epidermidis*.

MATERIALS AND METHODS

Bacterial strains. The biofilm-forming strain *S. epidermidis* CSF41498 was isolated from cerebrospinal fluid and obtained from the Department of Microbiology, Beaumont Hospital, Dublin, Ireland. The well-characterized reference strain *S. epidermidis* ATCC 35984 (RP62A) is a blood culture isolate originally characterized by Christensen et al. (6, 7) and later by, among others, Ziebuhr et al. (39). The strains and plasmids used in this study are shown in Table 1.

Media and growth conditions. *Escherichia coli* strains were grown at 37°C on Luria-Bertani medium supplemented, when required, with ampicillin (100 μg/ml). *S. epidermidis* and *S. aureus* strains were routinely grown at 37°C on brain heart infusion (BHI) (Oxoid) medium supplemented when required with the following antibiotics: chloramphenicol (10 μg/ml), erythromycin (10 μg/ml). BHI broth was supplemented with NaCl (4%) and glucose (0.5%) (NaCl-glucose) or ethanol (4%) (EtOH) as required. Bacteria were grown on Congo red agar (CRA) plates, which are composed of BHI agar supplemented with 5% sucrose (Sigma) and 0.8 mg of Congo red/ml (Sigma) to distinguish between wild-type (black, dry colony morphology) and variant (red, smooth colony morphology) phenotypes.

Genetic techniques. Genomic and plasmid DNA was prepared using Wizard Genomic DNA and Plasmid Purification kits, respectively (Promega, Madison, Wis.). Prior to DNA extraction cells were pretreated with 50 μg of lysostaphin in 100 μl of 50 mM EDTA to facilitate subsequent lysis. Restriction and DNA-modifying enzymes (Roche, New England Biolabs, and MBI Fermentas) were used throughout according to the manufacturer's instructions. All oligonucleotide primers used for PCR, reverse transcription-PCR (RT-PCR), and DNA sequencing were supplied by MWG Biotech (Ebersberg, Germany). Custom automated DNA sequencing was performed by MWG Biotech (Milton Keynes, United Kingdom).

The primers ICAR1 (5′-CTCGAATTTGTTACATACTAG-3′) and ICAC1 (5′-CCATAGCTTGAATAAGGGAC-3′) were used in long-range PCRs to amplify a 4,204-bp fragment comprising the entire *ica* operon from purified genomic DNA using Expand High-Fidelity *Taq* DNA polymerase (Roche) under the following conditions: 34 cycles of 94°C for 15 s, 45°C for 30 s, and 68°C for 6 min.

Bacterial transformations. Plasmid DNA was introduced into *E. coli* by $CaCl_2$ and heat shock transformation (37). Transformation of staphylococci was achieved by electroporation using a MicroPulser (Bio-Rad) and the following protocol: 25-ml shaking cultures were grown in BHI broth at 37°C to an optical density at 600 nm (OD_{600}) of approximately 2. The cultures were chilled on ice for 15 min before being washed three times with 50 ml of sterile, ice-cold H_2O and being resuspended in 50 μl of sterile H_2O. Plasmid DNA (1 to 5 μg in 25 to 50 μl of H_2O) was mixed with the cells and subjected to one electroporation pulse (1.8 kV, 2.5 ms). The electroporation mixture was then resuspended in 1 ml of BHI containing 0.5 M sucrose and a subinhibitory concentration of chloramphenicol as recommended by Bruckner (5) and incubated at 30°C for 2 to 3 h. Transformed cells were plated out on BHI agar (0.5 M sucrose) supplemented with chloramphenicol (10 μg/ml) and incubated for 24 to 48 h at 30°C.

Construction of plasmids and *icaR*::*ermB* allele replacement. A 784-bp fragment containing the *icaR* gene from *S. epidermidis* CSF41498 was amplified by PCR using the primers ICAR1 (5′-CTCGAATTTGTTACATACTAG-3′) and IPR2 (5′-TTGGATAGAAAAGTAAAAAG-3′), cloned initially into the PCR cloning vector pSTBlue-1 (Novagen) and subsequently into pGEM3Z (Promega) on an 810-bp *Bam*HI-*Hin*dIII fragment to create pSER1. DNA sequencing (data not shown) confirmed the absence of mutations in the PCR-amplified *icaR* gene cloned in pSER1. In order to construct an *icaR* mutant allele, a 1,227-bp *Eco*RI-*Cla*I fragment from pEC5 (5), which contains the *ermB* gene, was treated with T4 DNA polymerase to generate blunt ends and then cloned into a unique *Bpu*10I site in the *icaR* gene. In the resulting plasmid pSER2, the *icaR* gene is disrupted by the *ermB* gene inserted 193 bp from the *icaR* start codon. The orientation of the *ermB* gene in pSER2 was confirmed to ensure that the *ermB* promoter would not influence transcription of the *ica* operon following allele replacement (see Fig. 2B). In order to facilitate delivery of the *icaR*::*ermB* allele onto the chromosome of *S. epidermidis*, a 2,037-bp *Bam*HI-*Hin*dIII fragment from pSER2 was subcloned into the temperature-sensitive *E. coli-Staphylococcus* shuttle vector pBT2 (5), which had also been digested with *Bam*HI and *Hin*dIII. The resulting recombinant plasmid, designated pSER3, was first transformed by electroporation into *S. aureus* RN4220 and finally into *S. epidermidis* CSF41498, and Cm^r (10 μg/ml) colonies were identified on BHI agar (0.5 M sucrose) plates. Plasmid profiles were determined to confirm the presence of intact pSER3 in transformed strains.

Allele replacement of the temperature-sensitive pSER3 in CSF41498 was achieved following two rounds of growth at 42°C for 24 h without antibiotic selection and subsequent selection of erythromycin (10 μg/ml)-resistant colonies on BHI agar plates. Replica plating was then used to identify Em^r Cm^s colonies, and PCR analysis using three different sets of primers to amplify the *icaR* gene confirmed the presence of the *icaR*::*ermB* allele on the chromosome of four independent mutants. Finally, RT-PCR analysis using the KCR1 and KCR2

primers described below (see Fig. 4A) was used to confirm the absence of wild-type *icaR* transcript in the four independent mutants.

For *icaR* overexpression and complementation experiments, an 810-bp *Bam*HI-*Hin*dIII fragment containing the *icaR* gene was cloned from pSER1 into pBT2 and the recombinant plasmid was designated pSER4.

RNA purification and RT-PCR. Bacterial cells were collected and immediately stored in RNA*later* (Ambion) to ensure maintenance of RNA integrity prior to purification. Total RNA was subsequently isolated using the GenElute Total RNA purification kit (Sigma) according to the manufacturer's instructions following a 5- to 10-min pretreatment of the cells with 50 μg of lysostaphin in 100 μl of 50 mM EDTA. Purified RNA was eluted and stored in RNA*secure* resuspension solution (Ambion), and the integrity of the RNA was confirmed by agarose gel electrophoresis. Residual DNA present in RNA preparations following purification was removed using DNA*free* DNase treatment and removal reagents (Ambion).

RT-PCR was performed using the OneStep RT-PCR kit (Qiagen) following the manufacturer's recommended protocol. Master mixes were prepared using primers as follows: for *gyrB* transcripts, 5′-TTATGGTGCTGGACAGATACA-3′ and 5′-CACCGTGAAGACCGCCAGATA-3′; for *icaA* transcripts, 5′-AAC AAGTTGAAGGCATCTCC-3′ and 5′-GATGCTTGTTTGATTCCCT-3′; for *icaR* transcripts, KCR1 5′-GGTAAAGTCCGTCAATGGAA-3′ and KCR2 5′-CGCAATAACCTTATTTTCCG-3′. For all three of these RT-PCRs, RT was performed at 55°C for 30 min followed by 23 amplification cycles of 94°C for 20 s, 50°C for 20 s, and 72°C for 20 s.

RT-PCR analysis of *icaR* expression in the *icaR*::Em^r mutants was carried out using the KCR2 primer together with the primer KCR3 (5′-GCAAAAAATCT ATAAAG-3′) (see Fig. 4A). RT for RT-PCRs using these primers was performed at 45°C for 30 min followed by 23 amplification cycles of 94°C for 20 s, 47°C for 20 s, and 72°C for 20 s.

The *gyrB* gene is constitutively expressed in *S. aureus* (14) and was used as an internal standard in these experiments. The sequences of the primers used for *gyrB* RT-PCR were based on the corresponding *S. aureus gyr* primers (14) but were adjusted for complementarity to the DNA sequence of an *S. epidermidis* genomic clone with homology to the *S. aureus gyrB* gene (GenBank accession number AF269920).

Analysis of RT-PCR data. The intensity of 23S rRNA bands on nondenaturing 1% agarose gels was measured in individual samples prior to RT-PCR to ensure similar RNA loading in RT-PCRs within individual experiments, which are presented below in separate figures. In addition, expression of the constitutively expressed *gyrB* gene was measured in parallel with measurements of *icaA* and *icaR* transcript levels and used to standardize variations in RNA loading between samples in each experiment. All RT-PCRs were optimized to ensure that amplification was terminated in the linear range. For the RT-PCRs described in this study, this was achieved by terminating the PCRs after 23 amplification cycles. Densitometry was performed using the Stratagene Eaglesight software package to compare relative expression levels between samples.

Biofilm assays. Semiquantitative determinations of biofilm formation in 96-well tissue culture plates (Sigma) were performed based on the method of Christensen et al. (8) as described by Ziebuhr et al. (38) with the following modifications. Bacteria were grown at 37°C in BHI and each strain was tested at least eight times before being washed three times with distilled H_2O and dried for 1 h at 56°C as recommended by Gelosia et al. (13) prior to staining with a 0.4% crystal violet solution. The absorbance of the adhered, stained cells was measured at 492 nm using a Multiskan plate reader (Flow Laboratories).

Statistical analysis. Statistical analysis of data was performed using Microsoft Excel or SPSS software packages.

RESULTS

Non-IS*256*-mediated regulation of biofilm formation. Previous data showed that reversible insertion of IS*256* into the *ica* operon could account for between 25 and 33% of phase variants in *S. epidermidis* RP62A (39) and suggested that unpredictable IS*256* insertions at other genetic loci may also contribute to the production of phase variants. In order to investigate non-IS*256*-mediated *ica* operon regulation, we performed a PCR screen for the presence of IS*256* among a collection of biofilm-forming, *ica*-positive cerebrospinal fluid isolates of *S. epidermidis*, using the IS*256* primers described by Ziebuhr et al. (39). One isolate, CSF41498, identified as *ica* positive and IS*256* negative (data not shown) was chosen for further analysis for the following reasons: determination of the *ica* operon nucleotide sequence from CSF41498 and RP62A revealed no DNA sequence variations (data not shown), and in semiquantitative biofilm assays (see Materials and Methods) both were found to have similar biofilm-forming capacities in BHI; the OD_{492} (± standard deviation) of adherent biofilms stained with crystal violet for CSF41498 was 1.69 ± 0.20, compared to 1.65 ± 0.67 for RP62A.

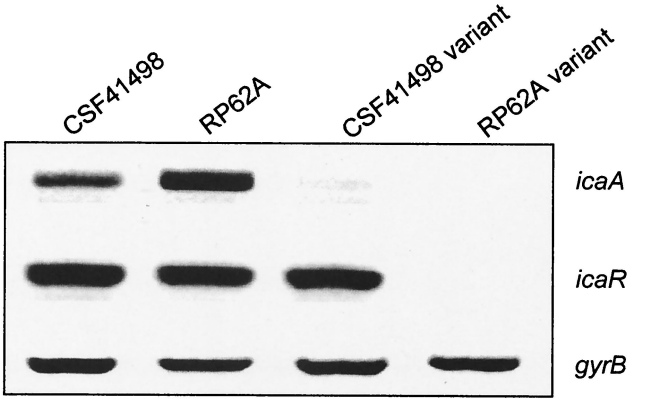

FIG. 1. Comparative measurement of *icaA, icaR*, and *gyrB* (control) transcription in wild-type and phase variants of *S. epidermidis* CSF41498 and RP62A. RNA was prepared from cultures grown in BHI at 37°C to the early exponential phase of the growth curve (OD_{600} = 2). Expression of *gyrB* is constitutive (14) and was used as an internal control in this experiment.

***icaR* gene expression in biofilm-positive and biofilm-negative phase variants of CSF41498.** Two phase variants (red, smooth colony morphology) of CSF41498 were isolated on CRA as described previously (39). PCR amplification of the entire *ica* operon from the two variants generated products of the expected size and confirmed that neither contained an IS*256* insertion in the *ica* operon (data not shown). Plasmid profile analysis of CSF41498 and the two phase variants was performed and revealed that all three strains shared the same plasmid profile (data not shown), thereby confirming their relationship to each other. For comparative analysis two phase variants of RP62A which did not contain IS*256* insertions in the *ica* operon were also isolated.

Semiquantitative biofilm analysis demonstrated that the two RP62A phase variants examined were completely biofilm negative and that the two CSF41498 variants displayed only weak biofilm-forming capacity in BHI broth at 37°C (OD_{492} ± standard deviation, 0.112 ± 0.08).

To examine the genetic basis for the variant phenotypes in CSF41498 and RP62A, we conducted a semiquantitative examination of both *icaA* and *icaR* gene expression by using RT-PCR. This revealed that *icaA* transcription levels were two- to threefold higher in the wild-type RP62A during early logarithmic growth than that in CSF41498, although this increased *ica* operon transcription in RP62A did not correlate with increased biofilm-forming capacity. Predictably, *icaA* transcription levels were significantly reduced or absent in CSF41498 and RP62A variants when compared to their parental strains (Fig. 1). In RP62A, both *icaA* and *icaR* transcription

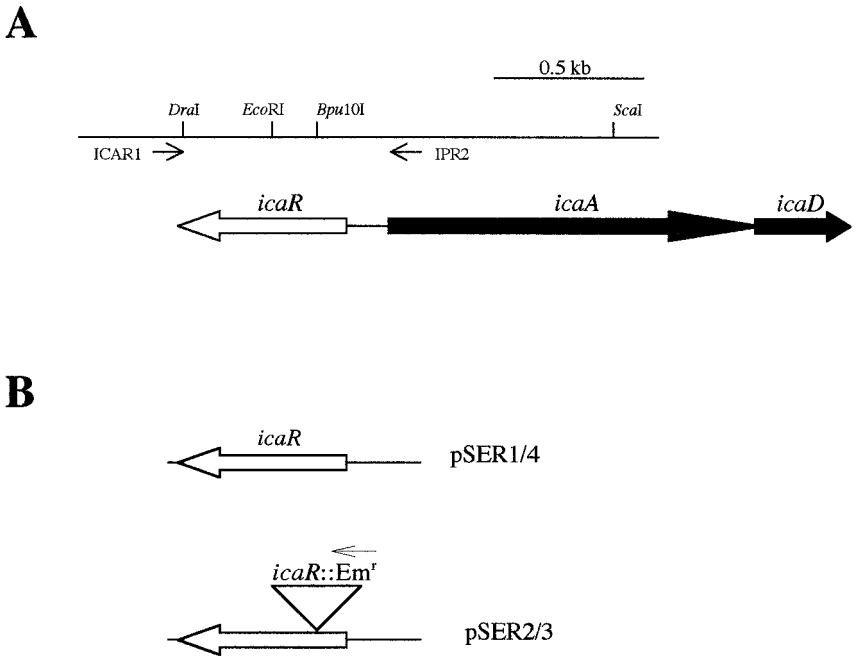

FIG. 2. (A) Physical and restriction maps and primer binding sites of the *S. epidermidis icaR-icaA* region. (B) Plasmids used to construct the *icaR* chromosomal mutation. The position of the *icaR*::*ermB* insertion in pSER3 is indicated

was detected in the wild-type parent, while neither gene was expressed in the phase variants. In contrast, expression of the *icaR* gene was detected in both wild-type and phase variants of CSF41498 (Fig. 1). Thus, there were two major differences between CSF41498 and RP62A variants: the *icaR* gene was expressed only in the CS41498 variants and, unlike RP62A variants, the CSF41498 variants retained the capacity to form a weak biofilm. Nucleotide sequence determination of the *icaR* gene and *ica* operon promoter region from the CSF41498 variants confirmed the absence of regulatory mutations (data not shown). Detection of *icaR* gene expression in wild-type and phase variants of CSF41498 suggested that this gene might play a different regulatory role in the two phenotypic variants of this strain. Alignment of the IcaR predicted amino acid sequence using the Domain Architecture Retrieval Tool on the National Center for Biotechnology Information website suggested that this protein is a member of the *tetR* family of transcriptional regulators.

Construction and characterization of an *icaR* insertion mutation. To investigate the role of the *icaR* gene in the regulation of *ica* operon expression in CSF41498, an IcaR mutant strain was constructed. To achieve this, the *icaR* gene on the chromosome of CSF41498 was replaced with an *icaR*::Em^r allele in which the *icaR* gene was interrupted by the *ermB* gene, which encodes resistance to erythromycin (Fig. 2) (see Materials and Methods). Four independent mutants (ICAR1, ICAR2, ICAR3, and ICAR4) were isolated and characterized. The colony morphology of the ICAR mutant strains grown on CRA at 37°C (black color, smooth regular surface) was different from that of the parental strain (black color, dry irregular surface).

Interestingly, biofilm formation by ICAR1 grown in BHI medium at 37°C for 24 h was unaffected compared to that of CSF41498 (OD_{492}, 1.69 ± 0.20 for CSF41498, compared to 1.57 ± 0.29 for ICAR1), indicating that the *icaR* gene may not be involved in biofilm formation. Similar results were obtained in biofilm assays with all four ICAR mutants (average OD_{492} for ICAR1, ICAR2, ICAR3, and ICAR4 was 1.4 ± 0.18).

To determine the impact of the *icaR*::Em^r mutation on *ica* operon expression, transcriptional regulation of the *icaA* gene was assessed by RT-PCR in all four mutant strains, ICAR1 to -4, and CSF41498 in cultures grown in BHI medium at 37°C. This revealed an at least 5.8-fold increase in *icaA* transcription in the *icaR*::Em^r mutants above that of the wild-type strain (Fig. 3). These data indicate that the *icaR* gene encodes a repressor of *ica* operon expression but that IcaR activity alone does not completely repress *ica* operon expression. Interestingly, our observation that biofilm formation by ICAR1 was not significantly different from that of wild-type CSF41498 in BHI medium was somewhat at odds with these transcriptional data, which indicated that the overall levels of *icaA* transcription are significantly higher in the ICAR1 mutant. One possi-

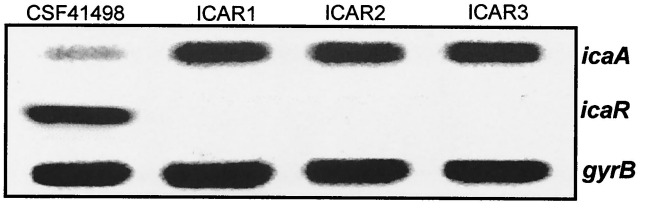

FIG. 3. Comparative measurement of *icaA, icaR* (KCR1 and KCR2 [see Fig. 4A]), and *gyrB* (control) transcription in *S. epidermidis* CSF41498 and three independent *icaR* insertion mutants, ICAR1, ICAR2, and ICAR3. RNA was prepared from cultures grown in BHI at 37°C to the early exponential phase of the growth curve (OD_{600} = 2).

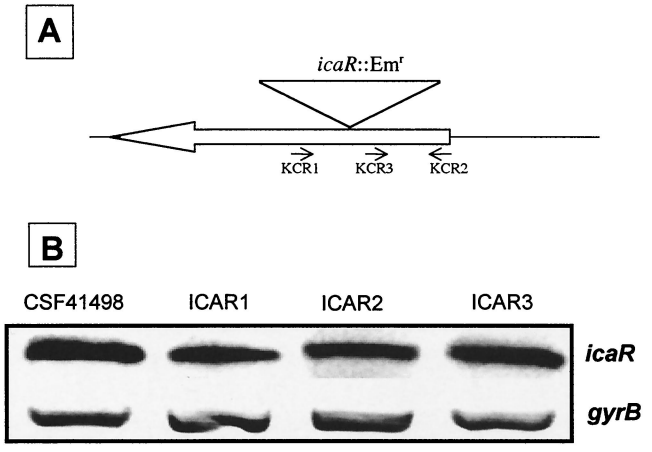

FIG. 4. (A) Physical maps and *icaR* primer binding sites used for RT-PCR analysis in CSF41498 and the *icaR*::Em^r mutants. Primers KCR2 and KCR3 were designed to measure *icaR* transcription in ICAR mutants. (B) Comparative measurement of *icaR* (primers KCR 2 and KCR3 [panel A]) and *gyrB* (control) transcription in *S. epidermidis* CSF41498 and three independent *icaR* insertion mutants, ICAR1, ICAR2, and ICAR3. RNA was prepared from cultures grown in BHI at 37°C to the early exponential phase of the growth curve (OD_{600} = 2).

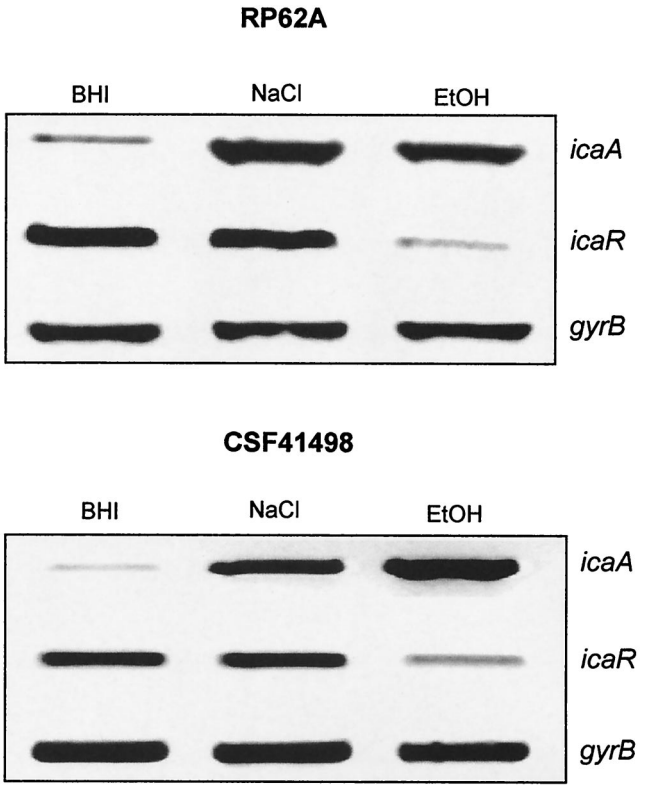

FIG. 5. Comparative measurement of *icaA*, *icaR*, and *gyrB* (control) transcription in *S. epidermidis* RP62A and CSF41498. RNA was prepared from cultures of each strain grown simultaneously to the mid-exponential phase of the growth curve (OD_{600} = 4.5) in BHI, NaCl-glucose (BHI supplemented with 4% NaCl and 0.5% glucose) and EtOH (BHI supplemented with 4% ethanol).

ble explanation may be that the biofilm assays performed in this study using BHI medium are not sensitive enough to reflect phenotypic differences associated with increased *ica* operon expression. This conclusion is supported by our earlier observation that biofilm-forming capacity in RP62A and CSF41498 in BHI medium was similar even though *ica* operon expression was two- to threefold higher in RP62A (Fig. 1).

The affect of the Em^r insertion on the transcription of the *icaR* gene itself was also examined by RT-PCR. Because the primers used to measure *icaR* transcription described earlier flank the *icaR*::Em^r insertion, a different RT primer (KCR3) was used for cDNA synthesis in the ICAR mutants (Fig. 4A). Comparative RT-PCR analysis with wild-type CSF41498 revealed that transcription of the *icaR* gene was not significantly affected in the ICAR mutants (Fig. 4B), which suggested that the *icaR* gene product does not regulate its own transcription.

Role of the *icaR* gene in the environmental modulation of *ica* operon expression. Our finding that the *icaR* gene product is a repressor of *ica* operon transcription prompted us to examine *icaA* and *icaR* expression in the wild-type and ICAR mutant strains under known biofilm-promoting growth conditions by using RT-PCR. Biofilm formation by *S. epidermidis* is subject to environmental regulation: factors such as salt (31) and ethanol (22) can promote biofilm formation. In wild-type CSF41498 we found a strong induction of *ica* operon expression in both NaCl-glucose (16-fold) and ethanol (26-fold) (Fig. 5). In addition, *icaR* transcription displayed a 5.6-fold decrease in the presence of ethanol but was not significantly affected by the presence of NaCl-glucose (Fig. 5). Given that the *icaR* gene product does not appear to regulate its own transcription (Fig. 4), ethanol repression of *icaR* transcription is unlikely to be the result of altered IcaR activity and may involve an additional transcription factor(s). Similar results were obtained for wild-type RP62A, in which growth in the presence of NaCl-glucose or ethanol resulted in 6.6- and 5.2-fold increases in *icaA* transcript levels, respectively. Growth in the presence of ethanol caused a 23-fold reduction in *icaR* transcription, whereas NaCl-glucose had no affect on *icaR* expression (Fig. 5). Interestingly, in the ICAR1 mutant, growth in the presence of ethanol had no significant effect on *ica* operon expression, whereas NaCl-glucose resulted in a small but significant 2.4 ($\pm$ 0.7)-fold ($\pm$ standard error of the mean) induction of *ica* operon expression (P = 0.046) (Fig. 6). These findings suggested that ethanol induction of *ica* operon expression was *icaR* dependent but that activation of *ica* operon expression by NaCl-glucose was *icaR* independent.

To further assess the role of the *icaR* gene in the environmental regulation of *icaA* expression, we tested the ability of ethanol and salt to induce *ica* operon expression in phase variants of RP62A and CSF41498. These experiments revealed that in the CSF41498 variant in which the *icaR* gene is expressed, significant increases in *icaA* expression were detected in the presence of NaCl-glucose and ethanol (Fig. 6), although the effect of the latter was much more potent than the former. Consistent with these data, biofilm assays revealed that ethanol and NaCl-glucose induced biofilm formation in the CSF41498 variants: biofilm-forming capacity (OD_{492} $\pm$ standard deviation) in the standard BHI broth was 0.107 $\pm$ 0.06, compared to 0.410 $\pm$ 0.22 in the presence of ethanol and 0.21 $\pm$ 0.26 in the presence of NaCl-glucose. As in wild-type CSF41498, salt did

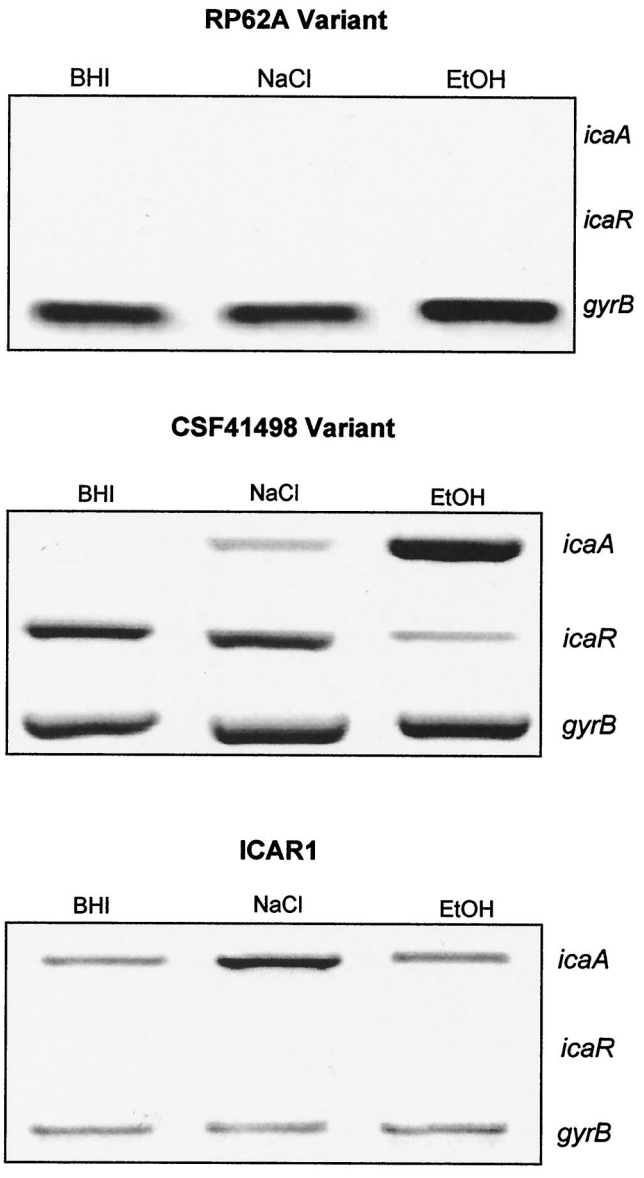

FIG. 6. Comparative measurement of *icaA*, *icaR*, and *gyrB* (control) transcription in phase variants of *S. epidermidis* RP62A and CSF41498 and the *icaR* insertion mutant ICAR1. RNA was prepared from cultures grown to the mid-exponential phase of the growth curve (OD_{600} = 4.5) in BHI, NaCl-glucose (BHI supplemented with 4% NaCl and 0.5% glucose), and EtOH (BHI supplemented with 4% ethanol).

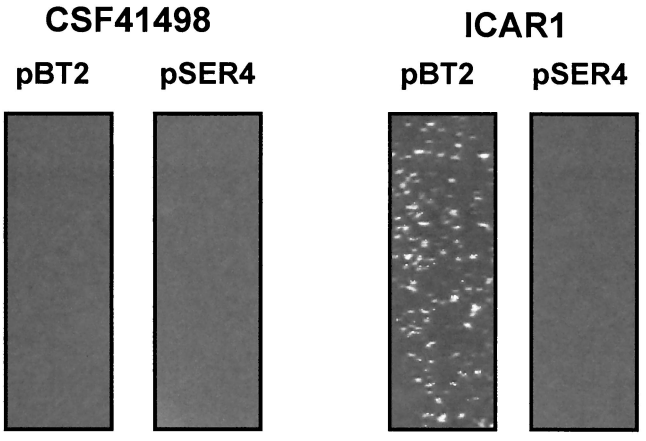

FIG. 7. Cell cluster formation in overnight cultures of *S. epidermidis* CSF41498 (left) and ICAR1 (right) bearing plasmids pBT2 (control) (lanes 1 and 3) and pSER4 (*icaR* gene) (lanes 2 and 4) grown at 30°C in BHI medium supplemented with chloramphenicol (10 μg/ml).

not influence *icaR* expression in the CSF41498 variant, while ethanol resulted in a 5.8-fold decrease in *icaR* transcription (Fig. 6). In contrast, examination of an RP62A variant in which the *icaR* gene was not expressed revealed that neither ethanol nor salt induced *icaA* or *icaR* gene expression (Fig. 6) or biofilm formation (data not shown).

Complementation of ICAR1. Analysis of the *icaR* insertion mutants indicated that IcaR acts as a repressor of *ica* operon expression required for ethanol activation of *ica* operon expression. In order to complement the ICAR1 mutant and to determine the effect of overexpressing the *icaR* gene in *trans*, the *icaR* gene was amplified by PCR from CSF41498 genomic DNA and ultimately cloned into the *E. coli-Staphylococcus* shuttle vector pBT2 as described in Materials and Methods to generate pSER4. Electroporation was used to transform pSER4 and the control vector pBT2 into CSF41498. Because pBT2 contains a temperature-sensitive origin of replication, strains containing pBT2 and derivatives were cultured at 30°C with chloramphenicol (10 μg/ml) selection.

Broth cultures of ICAR1 bearing pBT2 and pSER4 displayed a striking phenotypic difference. Cultures of ICAR1 (pBT2) formed macroscopically visible large cell aggregates or clusters, whereas ICAR1(pSER4) cultures displayed little cell clumping and were similar to wild-type CSF41498(pBT2) (Fig. 7). Growth of CSF41498 and ICAR1 plasmid-bearing strains in the presence of ethanol induced cell cluster formation in all cultures. Enhanced cell cluster formation or intercellular adhesion is consistent with elevated expression of the *ica* operon in the ICAR1 mutant and in CSF41498 grown in the presence of ethanol. Moreover, this was the first indication that the introduction of the *icaR* gene in *trans* was sufficient to complement the *icaR*::Em^r phenotype. It may also be significant that the ICAR1 cell clumping phenotype was more pronounced in cultures of this strain containing pBT2, which were grown at 30°C with chloramphenicol selection.

When the plasmid-bearing CSF41498 and ICAR1 strains were grown at 30°C on CRA containing chloramphenicol (10 μg/ml), strains carrying pSER4 were visibly red compared to the darker color of the control strains containing pBT2. This observation was consistent with the proposed negative regulatory role of the *icaR* gene product. However, we also observed that strains carrying pBT2 grown at 30°C on CRA supplemented with chloramphenicol (10 μg/ml) were not as dark as their plasmid-free parental strains, a phenotypic difference which appeared to be primarily due to the presence of the antibiotic rather than the altered temperature. RT-PCR was used to measure the effect of pBT2 and pSER4 on *icaA* and *icaR* transcript levels in plasmid-bearing CSF41498 cultures grown to an OD_{600} of 1.0 in BHI medium. These experiments confirmed that levels of *icaR* transcript were elevated in the CSF41498 strain containing pSER4 but not in the strain bear-

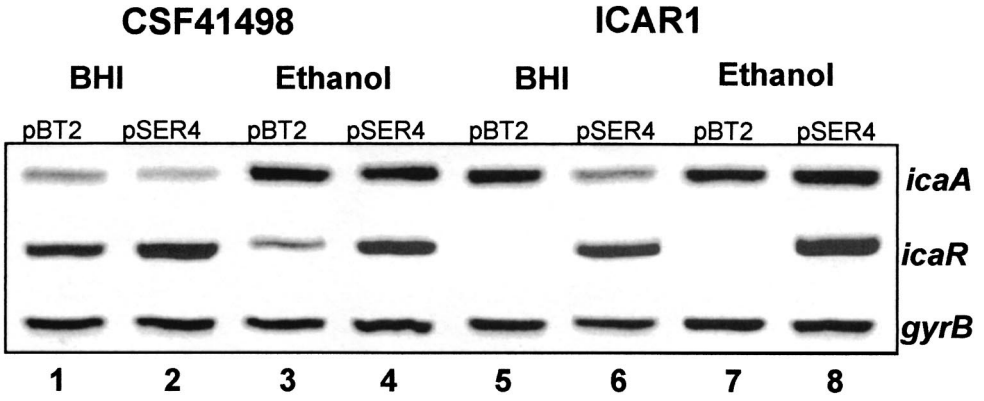

FIG. 8. Comparative measurement of *icaA*, *icaR*, and *gyrB* (control) transcription in *S. epidermidis* CSF41498 and ICAR1 strains complemented with plasmids pBT2 (control) and pSER4 (*icaR*). RNA was prepared from cultures grown to an OD_{600} of 1 at 30°C in BHI and BHI EtOH (4% ethanol), with chloramphenicol (10 μg/ml) selection. Lane 1, CSF41498(pBT2) in BHI; lane 2, CSF41498(pSER4) in BHI; lane 3, CSF41498 (pBT2) in BHI-EtOH; lane 4, CSF41498(pSER4) in BHI-EtOH; lane 5, ICAR1(pBT2) in BHI; lane 6, ICAR1(pSER4) in BHI; lane 7, ICAR1 (pBT2) in BHI-EtOH; lane 8, ICAR1(pSER4) in BHI-EtOH.

ing pBT2 (Fig. 8). Associated with this increased *icaR* expression was an approximately twofold decrease in *icaA* expression (Fig. 8). These findings are consistent with our earlier data indicating that *icaR* encodes a repressor of *ica* operon expression.

In order to determine if the effect of *icaR*::Em^r mutation on *ica* operon transcription could be complemented to wild type, RT-PCR was used to measure *icaA* and *icaR* transcript levels in RNA extracted from plasmid-bearing CSF41498 and ICAR1 cultures. In these experiments, complementation was defined as the restoration of *icaA* transcript levels to those of CSF41498 in complemented strains grown in the presence and absence of ethanol. These experiments revealed that *icaA* expression levels in ICAR1 were restored to wild-type levels observed in CSF41498(pBT2) (Fig. 8). In addition, ethanol failed to induce *icaA* expression in ICAR1(pBT2), but an approximately 2.6-fold *icaA* induction was measured in ICAR1 (pSER 4) grown in ethanol (Fig. 8). These results corroborate our earlier findings that identified the *icaR* gene product as a negative transcriptional regulator which contributes to the environmental modulation of *ica* operon expression and biofilm formation.

DISCUSSION

The persistent and recurrent characteristics of *S. epidermidis* biofilm infections highlight the need to develop new therapeutic strategies targeted at this organism. In this context, understanding the regulation of *ica* operon expression and biofilm formation is of primary importance. This study provides the first experimental evidence to demonstrate that expression of the *ica* operon in a biofilm-forming clinical isolate of *S. epidermidis*, CSF41498, is regulated by a transcriptional repressor encoded by the divergently transcribed *icaR* gene. The function of *icaR* was examined in CSF41498 for a number of reasons. The insertion sequence element IS*256*, which contributes directly (39) and possibly indirectly to *ica* operon regulation, is absent in this strain. In addition, the biofilm-forming capacity of CSF41498 is similar to that of the well-characterized reference strain RP62A, and the nucleotide sequence of the *ica* operons from both strains is identical. Finally, when phase variants of CSF41498 were compared with non-IS*256*-generated RP62A variants, *icaR* gene expression was detected only in the CSF41498 variants (Fig. 1), indicating that there may be a functional role for this gene in both phenotypic variants of this strain.

Differential regulation of *icaR* gene expression suggests that *ica* operon regulation in RP62A and CSF41498 phase variants may be fundamentally different. In CSF41498 variants, it appears that IcaR repression of *ica* operon transcription contributes to impaired biofilm-forming capacity. However, because *icaR* transcript levels are similar in wild-type and phase variants of CSF41498, an additional factor(s) may act in concert with IcaR to repress *ica* operon transcription in these phase variants. In RP62A variants, the absence of *icaR* expression indicates that an alternative regulatory mechanism results in *ica* operon repression.

In CSF41498, a chromosomal *icaR*::Em^r mutation led to increased *ica* operon expression, indicating that the *icaR* gene product is a transcriptional repressor. Consistent with this finding, overexpression of *icaR* on a multicopy plasmid resulted in repression of *ica* operon transcription. Predicted IcaR amino acid sequence alignments suggest that IcaR is a member of the *tetR* family of transcriptional regulators. This family of proteins, which includes the *qacR* repressor of the QacA multidrug efflux pump system in *S. aureus*, are characterized by conserved helix-turn-helix DNA-binding domains at the amino-terminal ends and divergent carboxy-terminal domains which may be involved in interactions with compounds that modulate their regulatory activity (2, 15, 33). Thus, QacR binding to a range of inducing compounds interferes with its ability to repress *qacA* transcription (15). It is tempting to speculate that IcaR activity may also be modulated by interaction with inducing compounds.

Although regulation of the *ica* operon was altered in the *icaR*::Em^r mutants, IcaR did not appear to autoregulate expression of its own gene. Most other members of the *tetR* family of regulatory proteins that are divergently transcribed from the structural gene that they regulate appear to regulate

the expression of their own genes, including TetR (21), *Pseudomonas putida* CamR (1), and *Streptomyces glaucescens* TcmR (16). It therefore appears somewhat anomalous that *icaR* transcription was not altered in the *icaR*::Em^r mutant. However, it is interesting that in *S. aureus* the QacR protein does not autoregulate transcription of its own gene either (15), suggesting that this may be a shared regulatory feature of *tetR* family proteins in staphylococci.

Recent reports have provided evidence that environmental stresses such as high osmolarity (NaCl) or ethanol concentrations can increase *ica* operon expression and promote biofilm formation in *S. epidermidis* (23, 32). In this study we observed that biofilm formation was induced by ethanol in phase variants of CSF41498 which were *icaR*$^+$, but not in RP62A variants which were *icaR* deficient. To investigate the role of the *icaR* gene in the environmental modulation of biofilm formation, we examined the effect of ethanol and NaCl-glucose on the regulation of *ica* operon expression in RP62A, CSF41498, and the ICAR1 mutant. These data revealed that ethanol and NaCl-glucose strongly induced *ica* operon expression in wild-type CSF41498 and phase variants (both *icaR*$^+$). Similarly, ethanol and NaCl-glucose induced *ica* operon expression in the wild-type RP62A (*icaR*$^+$) but not in a phase variant (*icaR* deficient). In contrast, there was no significant induction of *ica* expression by ethanol in the *icaR* insertion mutant, and a small but significant induction was observed in the presence of NaCl-glucose. In both CSF41498 and RP62A, ethanol induction of *icaA* gene expression was associated with decreased *icaR* transcription. In contrast, NaCl-glucose did not significantly affect *icaR* regulation. Apparently, in CSF41498 induction of *ica* operon expression in the presence of ethanol is *icaR* dependent and ethanol directly or indirectly represses *icaR* transcription. In contrast, NaCl-glucose, which also induces *ica* operon expression, does not significantly alter *icaR* regulation and appears to activate the *ica* operon through a separate pathway which does not involve *icaR*. Recently, Knobloch et al. proposed that separate pathways can activate *ica* operon expression in response to different environmental stresses (22). This proposal was based on evidence that a biofilm-negative *rsbU* transposon mutant of *S. epidermidis* 1457 (*rsbU* is a positive regulator of the alternative sigma factor SigB) was enabled to form a biofilm again by ethanol only and not by NaCl (22). Our evidence that NaCl-glucose-induced *ica* operon expression is *icaR* independent but that activation of *ica* operon transcription by ethanol does require the *icaR* gene may also suggest the existence of separate *ica* operon-inducing regulatory pathways. Moreover, these findings may also suggest that *icaR*-dependent ethanol activation of the *ica* operon does not involve SigB but that a separate pathway involving the alternative sigma factor can independently induce *ica* expression. Finally, the possible existence of separate pathways is further supported by our observation that, in CSF41498, ethanol is a more potent activator of *ica* expression than NaCl-glucose.

The evidence presented here suggests that the *icaR* gene product is a transcriptional repressor which plays an adaptive role in *S. epidermidis* biofilm formation by modulating the regulation of *ica* expression in response to specific environmental conditions.

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