

Glucose-Related Dissociation between *icaADBC* Transcription and Biofilm Expression by *Staphylococcus epidermidis*: Evidence for an Additional Factor Required for Polysaccharide Intercellular Adhesin Synthesis

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Biofilm formation in *Staphylococcus epidermidis* depends, in the majority of the strains, on the activity of the *icaADBC* locus. The expression of the operon that encodes the synthetic enzymes of the intercellular polysaccharide adhesin (PIA) depends on a variety of exogenic environmental conditions and is, at least in part, regulated by the alternative sigma factor σ^B . We investigated the transcriptional regulation of the *ica* operon and the respective phenotypes expressed under growth conditions differing in the content of glucose in the growth medium. In the presence of glucose, *S. epidermidis* exhibited a PIA- and biofilm-positive phenotype whereas *ica* transcription was down-regulated in the postexponential and stationary phases of growth. Surprisingly, maximum transcription of *ica* was detectable in the stationary phase of growth in the absence of glucose despite the expression of a PIA- and biofilm-negative phenotype. In vitro enzymatic assays and phenotypic characterization showed that the abundant amount of *ica* mRNA was functionally active because induction of stationary-phase cells with glucose led to immediate PIA synthesis. Induction of biofilm formation could be completely inhibited by chloramphenicol, which, given at a later stage of biofilm accumulation, also inhibited further development of preformed biofilm, indicating that continuous translation of an additional, *icaADBC*-independent factor is required for the expression of a biofilm-positive phenotype.

Coagulase-negative staphylococci, particularly *Staphylococcus epidermidis*, are the leading cause of foreign-body-associated infections and nosocomial bacteremia (3, 4, 51). Despite its low virulence as a commensal member of the human skin and mucous membrane flora, *S. epidermidis* equals or even outnumbers *S. aureus* as the causative organism of medical-device-related infections (3). This pathogenic association has been attributed to the ability of pathogenic strains to form multilayered biofilms on polymer surfaces. The process of biofilm formation has been divided into two distinct phases: initial attachment and biofilm accumulation (32). The genetic and biochemical mechanisms of each phase have been the subject of intense research in recent years. Multiple factors have been found to influence bacterial attachment, including bacterial surface proteins, polysaccharides, and physicochemical interactions (18, 26, 42, 46, 56, 57). The second phase is characterized by bacterial accumulation, which requires cell-to-cell interaction. The intercellular polysaccharide adhesin (PIA) has been found to mediate intercellular adhesion in the majority of clinical *S. epidermidis* isolates (13, 35, 41, 60). This linear glucosaminoglycan is composed of *N*-acetylglucosamine in β -1,6-glycosidic linkages containing deacetylated amino groups and succinate and phosphate substituents (34, 36). PIA is also func-

tional in hemagglutination of erythrocytes (16, 37). The essential role of PIA expression in the pathogenesis of medical-device-associated infections has been shown in several animal models (52–54). An accumulation-associated protein may also be required for biofilm accumulation (22).

PIA synthesis depends on the expression of the *icaADBC* operon, which encodes three membrane proteins (IcaA, IcaD, and IcaC) with enzymatic activity and one extracellular protein (IcaB) (17, 19). The *icaADBC* gene locus has also been detected in *S. aureus* and a range of other coagulase-negative staphylococci (1, 9, 29, 43).

Phenotypically, the amount of biofilm produced by individual *S. epidermidis* strains is highly variable and is influenced by changing environmental conditions (2, 10, 12, 23, 28, 38, 40, 44, 47, 48, 50). Even though our knowledge of the regulation of the *ica* locus is limited, recent data seem to implicate a complex system involving the alternative sigma factor σ^B , *icaR*, and two additional, independent regulatory genetic loci (7, 27, 38, 48). PIA synthesis is also subject to an on-off mode of regulation in phase variants of *S. epidermidis* that, in part, is due to the alternate insertion of an IS element into the *ica* locus (61).

The expression of a biofilm-positive phenotype in *S. epidermidis* depends on the presence of glucose in the growth medium (5, 40, 48). Biofilm-positive strains exhibit a PIA- and biofilm-negative phenotype when cultivated in medium lacking glucose but can be induced to produce PIA by subsequent addition of glucose to the culture. In order to gain further insight into the regulatory mechanisms leading to the differential expression of PIA, we compared the transcriptional ac-

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tivity of *icaADBC* and the respective phenotype expressed under these two physiological growth conditions.

MATERIALS AND METHODS

Bacteria and growth media. Biofilm-producing *S. epidermidis* 1457, 8400, RP62A, and 9142 and the isogenic biofilm-negative transductant 1457-M11 have been described previously (36, 37, 40, 45). Basic T_A medium was assembled from the individual components and contained (wt/vol) 1.7% Tryptone (lot 208080; Oxoid, Basingstoke, England), 0.3% neutralized soya peptone (lot 14782150; Oxoid), 0.5% NaCl, and 0.25% dipotassium phosphate as recommended by the manufacturer. Glucose or fructose from filter-sterilized stock was added to a final concentration of 0.25% as indicated. Glucose-supplemented medium will be referred to as T_{A+glc}.

RNA isolation and Northern blot analysis. For RNA preparation from planktonic bacteria, an overnight culture (OC) of *S. epidermidis* 1457 was diluted 1:100 in fresh, prewarmed T_A or T_{A+glc} except when stated otherwise. The bacteria were incubated at 37°C under constant agitation, and aliquots were removed every hour to measure the optical density at 578 nm (OD₅₇₈). Culture volumes of 20 to 40 ml were harvested at the early-, mid-, postexponential, and stationary growth phases, and total cellular RNA was isolated as described previously (15, 38, 39).

For analysis of static biofilm cultures, *S. epidermidis* was precultured in T_A for 6 to 10 h with shaking at 160 rpm at 37°C. This culture was then diluted 1:100 into T_A or T_{A+glc} in several 9-cm-diameter tissue culture dishes (Nunc, Roskilde, Denmark) with an inoculum of 10 ml per dish. In parallel, aliquots (200 μl) of the prepared dilutions were inoculated in 4 wells of 96-well tissue culture plates (Nunc). After 16 h of incubation at 37°C, biofilm formation was induced by the addition of glucose (0.25% final concentration) at different time points so that the induced cultures could be harvested simultaneously (usually 8 h after induction). The cultures in the 96-well tissue culture plates were induced in parallel to allow monitoring of biofilm formation by the adherence assay (6, 40). Total cellular RNA was then recovered as described previously (15). A 10-μg portion of RNA from each sample was separated on a 1% formaldehyde gel and blotted onto nylon membranes. The amount and quality of the prepared RNA and the efficacy of blotting were controlled by visualization of the respective 16S and 23S rRNAs. Northern blot analysis was performed with a ³²P-labeled *icaC*-specific (5'-GAA ATA GCC ATA CCA TTG TCC-3') oligonucleotide that probed the approximately 3,600-kb length of the *icaADBC* operon (14, 15, 38).

Characterization of culture conditions. One to three colonies of *S. epidermidis* 1457 were inoculated in 200 ml of prewarmed T_A and incubated for 12 h at 37°C under constant shaking at 120 rpm. This culture was referred to as the OC. A subculture (SC) was generated by diluting these stationary-phase cells 1:100 in 200 ml of the same batch of fresh, prewarmed T_A, which was again incubated for 12 h at 37°C to stationary phase. Usually, an OC and an SC of *S. epidermidis* 1457 were inoculated simultaneously so that they would reach the stationary phase of growth at the same time.

Analysis of cell aggregation. Addition of 0.25% glucose to stationary-phase cells grown in T_A led to the induction of PIA and biofilm production and the formation of cell aggregates (40). To monitor the respective cultures for the formation of cell aggregates, 3-ml culture samples were removed and transferred to 1 well of a 24-well plate (Nunc) before and every hour after the addition of glucose to the medium. The 24-well plate was stored at 4°C for approximately 8 h until termination of the experiment and then evaluated macroscopically.

Measurement of biofilm formation. To determine the biofilm formation of *S. epidermidis* under different physiological conditions, a semiquantitative adherence assay with a 96-well tissue culture plate was performed as described earlier (6, 33). Adherent bacterial biofilms were stained with gentian violet. The amount of biofilm formation was quantified by measuring the OD₅₇₀ with an automatic spectrophotometer (Behring, Marburg, Germany). An OD₅₇₀ of >0.1 was defined as a biofilm-positive phenotype. The medium used for preculture was T_A or T_{A+glc}, except when stated otherwise.

Preparation of bacterial extracts and coagglutination assay for detection of PIA. *S. epidermidis* 1457 was grown to stationary phase in T_A or T_{A+glc}. Twenty-milliliter volumes of the cultures were harvested at serial time points, and the cells were washed in phosphate-buffered saline, sedimented by centrifugation (3,000 × g for 10 min), and resuspended in 10 ml of phosphate-buffered saline. Bacterial extracts were prepared by sonication as described previously (36, 40).

To quantitate PIA in bacterial extracts, a specific coagglutination assay was performed as described earlier (33).

Hexosamine assay. The presence of hexosamine in the culture supernatant or in bacterial extracts was determined by the 3-methyl-2-benzothiazolone hydra-

zone hydrochloride method with *N*-acetylglucosamine as the standard as described previously (30, 36).

In vitro synthesis of PIA. An SC of *S. epidermidis* 1457 in T_A was grown to stationary phase, and crude membranes were prepared as described previously (17). Cell disruption was performed by using the FastPrep shaking apparatus (FastPrep System; Bio 101, Inc., Vista, Calif.). For analysis of Ica enzymatic activity, the prepared membranes were incubated with 2 mM UDP-*N*-acetylglucosamine and 50 μg of D-cycloserine per ml for 15 h at 20°C as described previously (17). Membranes incubated without UDP-*N*-acetylglucosamine were used as negative controls, as well as crude membrane preparations of biofilm-negative transposon mutant 1457-M11. As a positive control, we used *S. carnosus* pTXicaADBC, which harbors the cloned *icaADBC* gene locus under the control of a xylose-inducible promoter (8). Crude membranes of *S. carnosus* pTXicaADBC were collected after induction with 0.5% xylose. In vitro synthesis of PIA was verified by coagglutination of the reaction supernatant. Aliquots of the reaction volume were also subjected to periodate oxidation (40).

RESULTS

Analysis of *icaADBC* transcription under different physiological conditions. (i) *S. epidermidis* shaking planktonic culture. The transcriptional activity of *icaADBC* was analyzed with respect to the phenotypes expressed at different times during growth. To this end, *S. epidermidis* 1457 was grown in T_A medium with and without supplementation with 0.25% glucose. In the adherence assay, *S. epidermidis* 1457 was biofilm negative in basic T_A medium (OD₅₇₀, <0.1) but established a strong biofilm in T_{A+glc} (OD₅₇₀, 2.5). An OC of *S. epidermidis* 1457 in T_{A+glc} was diluted 1:100 either in fresh, prewarmed T_A or in T_{A+glc}. The cultures were grown with shaking at 37°C, and aliquots were removed to determine the OD. The cell density (OD₅₇₈) of *S. epidermidis* grown in T_{A+glc} was approximately three times higher than that grown in T_A. However, the time course of the different growth phases seemed to be the same, independent of the medium used (data not shown). Thus, samples of glucose-supplemented medium and medium lacking glucose were taken at the same time points. Total bacterial RNA was extracted at the early-exponential (after 3 to 4 h), mid-exponential (5 to 6 h), postexponential (9 to 10 h), and stationary (16 h) phases of growth.

Northern blot analysis of *S. epidermidis* 1457 in T_{A+glc} revealed a strong *ica*-specific transcript in the early (OD₅₇₈, ~1.3) and mid-exponential (OD₅₇₈, ~4.5) phases of growth, which then faded during the postexponential (OD₅₇₈, ~8.8) and stationary (OD₅₇₈, ~8.5) phases of growth (Fig. 1A). A biofilm assay performed in parallel confirmed strong biofilm formation in the presence of glucose (OD₅₇₀, 2.5).

Northern blot analysis of *S. epidermidis* 1457 grown in basic T_A displayed a very different transcriptional pattern. Despite the expression of a biofilm-negative phenotype under these conditions, an *ica*-specific transcript was detectable in the mid-exponential phase of growth (OD₅₇₈, 1.5) and the amount of *ica*-specific mRNA increased to a maximum level throughout the postexponential (OD₅₇₈, 2.4) and stationary (OD₅₇₈, 2.5) phases of growth (Fig. 1A). To exclude the possibility that the differential *ica* transcription in medium with and without glucose is specific to *S. epidermidis* 1457, we also examined three other biofilm-positive *S. epidermidis* strains (RP62A, 8400, and 9142), which displayed identical transcriptional patterns (data not shown).

To clearly demonstrate the lack of PIA production in medium without glucose supplementation, coagglutination assays with PIA-specific antiserum and hexosamine assays were per-

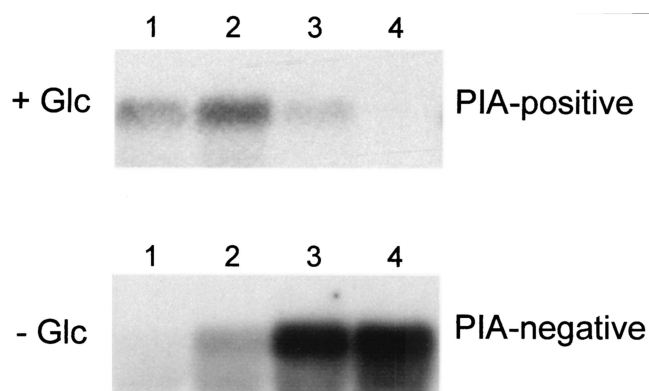


FIG. 1. Northern blot analysis of *icaADBC* in shaking cultures of *S. epidermidis*. An OC of *S. epidermidis* 1457 was diluted 1:100 in fresh, prewarmed T_A (- Glc) and in the same medium supplemented with glucose (+ Glc). The bacteria were incubated at 37°C under constant agitation, and aliquots were removed for RNA extraction at different times during growth. Lanes: 1, early exponential phase of growth (3 to 4 h of incubation); 2, mid-exponential phase of growth (5 to 6 h of incubation); 3, postexponential phase of growth (9 to 10 h of incubation); 4, stationary phase of growth (16 h of incubation). In parallel, a biofilm assay was performed demonstrating a biofilm-negative phenotype in T_A and a biofilm-positive phenotype in T_{A+glc} . Hybridization was performed with an *icaC*-specific oligonucleotide. Data from representative experiments are shown.

formed with crude extracts of stationary-phase cells. In cultures of *S. epidermidis* 1457 grown in basic T_A , no biofilm was produced and no PIA could be detected, whereas in cultures grown in the presence of glucose (T_{A+glc}), a biofilm was generated and PIA was detected with a coagglutination titer of 1:64 (Table 1). Hexosamine assays also demonstrated the absence of hexosamine in extracts prepared from cells grown in the absence of glucose. Biofilm-negative mutant 1457-M11, an isogenic mutant of *S. epidermidis* 1457 with a Tn917 insertion in *icaA*, was used as a negative control (36) (Table 1). Thus, *S. epidermidis* also did not produce any precursor oligosaccharides in the absence of glucose that might have failed to be detected by the PIA-specific antiserum used.

(ii) *S. epidermidis* static biofilm culture. Similar patterns of *ica* transcription were observed when *S. epidermidis* 1457 was grown in 9-cm tissue culture dishes used for generation of *S. epidermidis* biofilms. Northern blot analysis showed a high level of *ica*-specific mRNA in cultures grown in T_A lacking glucose

TABLE 1. Detection of PIA and hexosamine in crude extracts of *S. epidermidis* 1457 and biofilm-negative transposon mutant 1457-M11^a

Strain and medium	Biofilm formation (OD ₅₇₀)	Hexosamine concn (μg/ml) ^b	PIA titer
1457			
T_A + Glc	2.50	9.88	1:64
T_A - Glc	0.06	0	Negative
1457-M11			
T_A + Glc	0.02	0	Negative
T_A - Glc	0.05	0	Negative

^a Both strains were grown in T_A in the presence or absence of glucose.

^b Limit of detection, 1.3 μg/ml.

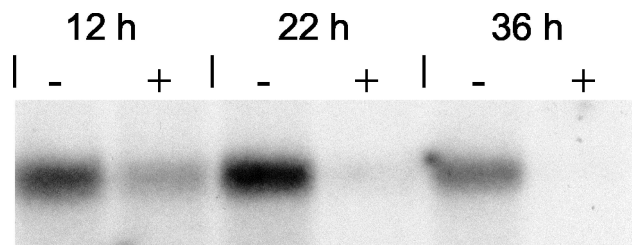


FIG. 2. Northern blot analysis of *icaADBC* in static cultures of *S. epidermidis* in T_A in the presence or absence of glucose. A 6-h preculture of *S. epidermidis* 1457 was diluted 1:100 in fresh T_A (-) and in T_{A+glc} (+) and cultivated in tissue culture plates at 37°C. After 12, 22, and 36 h of incubation, total bacterial RNA was extracted. Hybridization was performed with an *icaC*-specific oligonucleotide. In parallel, a biofilm assay was performed demonstrating a biofilm-positive phenotype in T_{A+glc} . OD₅₇₀s of 1.6, 1.7, and 1.8 were detected at 12, 22, and 36 h, respectively. In T_A , *S. epidermidis* 1457 exhibited a biofilm-negative phenotype.

after 12, 22, and 36 h of incubation (Fig. 2). The OD₅₇₀ was <0.1 at each time point, indicating a biofilm-negative phenotype. In contrast, *ica* transcription in cultures grown in T_{A+glc} was significantly less pronounced at each time point (Fig. 2), even though strong biofilm formation with OD₅₇₀s of 1.6, 1.7, and 1.8 was detected at 12, 22, and 36 h, respectively. These findings indicate that the transcriptional patterns observed in shaking planktonic and static biofilm cultures were similar.

Analysis of Ica enzymatic activity in membranes of biofilm-negative cultures. In order to show that the large amount of *ica*-specific transcripts in stationary-phase cells grown in medium lacking glucose were translated to functionally active proteins, we conducted *in vitro* enzymatic assays with crude membranes of *S. epidermidis* 1457. In membrane preparations incubated with UDP-*N*-acetylglucosamine, coagglutination titers of 1:1,024 were detected, compared to the 1:32 titer of the control membranes (Fig. 3). This result demonstrated *in vitro* synthesis of PIA and confirmed the presence of functionally active Ica proteins in biofilm-negative cells. In contrast, *in vitro* assays conducted with membrane preparations of transposon mutant 1457-M11, which is biofilm negative because of a Tn917 insertion in *icaA*, yielded no PIA because no coagglutination activity could be detected in the reaction supernatant. Membranes prepared from *S. carnosus*/pTX*icaADBC* in which *icaADBC* expression was induced by xylose were used as a positive control. Incubation with UDP-*N*-acetylglucosamine yielded a coagglutination titer of 1:4,096, compared to the 1:32 titer of the control membranes (Fig. 3). The coagglutination activities of both *in vitro* reactions of strain 1457 and the control, *S. carnosus*/pTX*icaADBC*, were completely abolished by periodate oxidation, whereas the coagglutination titers remained unchanged after incubation with water, thus confirming specific detection of PIA (40).

Correlation of *ica* transcription with phenotypic features of PIA production. In order to address the question of whether the amount of *ica*-specific mRNA was relevant with respect to the phenotype expressed after the induction of PIA synthesis by glucose, we compared two simultaneously grown *S. epidermidis* cultures in T_A expressing different basic steady-state levels of *ica* mRNA. To this end, an SC and an OC of *S. epidermidis* 1457 were grown with shaking to stationary phase in T_A

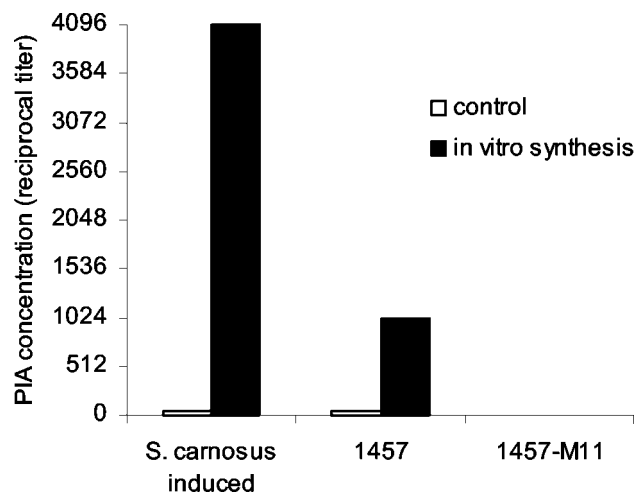


FIG. 3. In vitro synthesis of PIA. Crude membranes of *S. epidermidis* 1457 and biofilm-negative transposon mutant 1457-M11 were prepared from cells grown in T_A SC to the stationary phase of growth. The in vitro assay was performed by incubating the collected membranes with UDP-*N*-acetylglucosamine as described previously (17). The control reaction mixtures did not contain UDP-*N*-acetylglucosamine. Xylose-inducible *S. carnosus* pTXicaADBC was used as a positive control.

as described in Materials and Methods. Northern blot analysis and densitometric measurements revealed that the level of *icaADBC* transcription in the *S. epidermidis* SC was approximately fivefold higher than that of the OC (Fig. 4A). In a kinetic study, both cultures (OC and SC) were analyzed in parallel after induction of PIA synthesis by addition of 0.25% glucose to the medium. Transcriptional analysis revealed an increased level of *ica*-specific mRNA 2 h after glucose induction in both cultures. The level of mRNA was higher in the SC than in the OC, corresponding to its fivefold increase in basal *ica* transcription. Four hours after glucose induction, the level of *ica*-specific mRNA decreased and *ica*-specific mRNA was then undetectable after 6 to 8 h (Fig. 4A). We subsequently compared the phenotypic features of PIA production in these two cultures (OC and SC) to determine if the larger amount of *ica*-specific mRNA in the SC consequently led to a greater amount of PIA produced.

(i) **PIA production.** In T_A , stationary-phase cells of both cultures (OC and SC) did not produce detectable amounts of PIA, as determined by the coagglutination assay (Fig. 4B). Following the addition of glucose, aliquots of the cultures were taken at the same time points as for RNA extraction. Increasing amounts of PIA were detectable after 2, 4, 6, and 8 h of glucose induction in both cultures. In the SC, the coagglutination titer was two- to fourfold higher than that in the OC within the first 6 h after glucose induction. The two cultures reached similar concentrations of PIA after 8 h (Fig. 4B).

(ii) **Cell aggregation.** The addition of glucose to the *S. epidermidis* SC and OC in T_A led to macroscopic aggregation of the cells because of the production of PIA. In the SC, aggregation occurred approximately 1 h earlier and to a higher degree than in the OC (data not shown). Apparently, the higher degree of *ica* expression in the SC enabled the cells to produce PIA more rapidly after induction, indicating that a

larger amount of Ica proteins was present before glucose was added to the culture.

(iii) **Biofilm formation.** To compare the kinetics of biofilm formation, SC and OC cultures of *S. epidermidis* were inoculated as described for shaking culture but were instead dispensed into 96-well tissue culture plates and grown to stationary phase as a static culture. Biofilm formation was induced by addition of glucose to both stationary-phase cultures (40). Corresponding to the increased level of *ica* transcription in the SC, biofilm formation could already be observed 2 h after glucose induction, which was 1 h earlier than in the OC (Fig. 5). Three hours after glucose induction, the OD_{570} of the SC was twice as high as that of the OC but both cultures subsequently reached the same values and both had a maximum OD_{570} of 2.5 6 h after induction.

The cell density, as determined by measuring the OD_{578} , 8 h after glucose induction increased approximately twofold, indicating that the cells did not re-enter exponential growth as a response to glucose induction.

As a negative control, all assays were also performed with transposon mutant 1457-M11 (37) and no biofilm formation, PIA production, or cell aggregation could be detected in this mutant (data not shown).

Inhibition of PIA production and biofilm formation by blocking protein synthesis. The lack of PIA production despite the presence of large amounts of *ica*-specific mRNA in cells grown in the absence of glucose could be due to the lack of a substrate for PIA synthesis or to the absence of an additional, glucose-dependent factor. In order to address this question, we inhibited protein synthesis by adding chloramphenicol to cells grown in T_A before inducing biofilm formation with glucose. To this end, *S. epidermidis* 1457 was grown in T_A for 15 h in 96-well tissue culture plates for the induction experiments and in 9-cm tissue culture dishes for RNA extraction. Northern blot analysis demonstrated a high level of *ica*-specific mRNA before glucose induction, corresponding to the results obtained with a shaking culture (Fig. 6). Therefore, the *icaADBC*-encoded proteins were expected to be present in the cells before glucose induction, as shown by the in vitro experiments described above.

Chloramphenicol was added to the 96-well tissue culture plates in a kinetic study 1 h before induction, at the time of induction, and at different intervals after the addition of glucose. The adherent biofilms were subsequently stained 12 h after induction. Complete inhibition of biofilm formation was observed when chloramphenicol was added before and up to 2 h after glucose induction (Fig. 7). Blocking of protein synthesis 3 to 4 h after glucose induction led to a reduced OD_{570} , but no significant effect was detectable when chloramphenicol was added to an established biofilm at later stages. Apparently, the presence of Ica proteins in *S. epidermidis* was not sufficient for PIA synthesis after the addition of glucose but translation of an additional factor was required at each stage of biofilm maturation. The number of CFU, as determined by plating, was independent of chloramphenicol treatment, indicating that bacterial cells were still viable in the chloramphenicol concentration used.

Similar results were obtained in experiments performed with fructose instead of glucose, a sugar that has also been shown to stimulate biofilm formation in *S. epidermidis* 1457 (data not

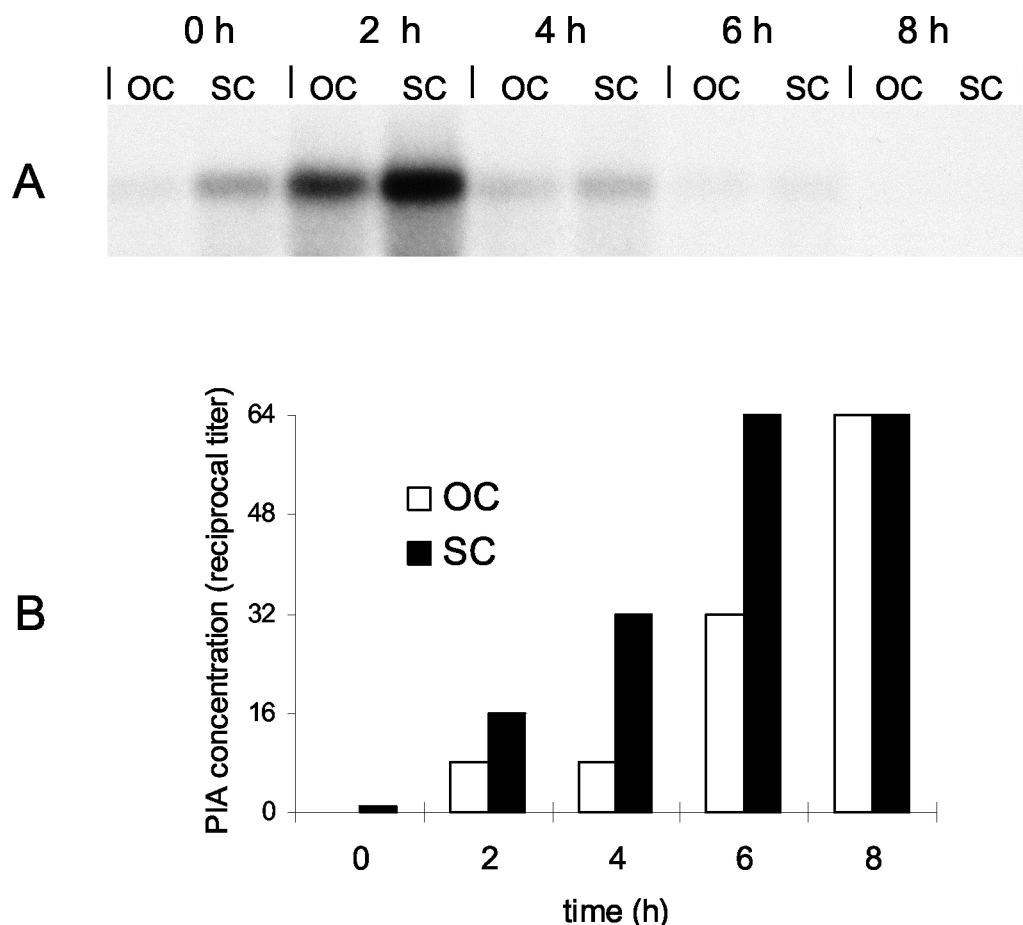


FIG. 4. Correlation of different levels of *ica* transcription in an OC and a repeated SC and phenotypical features of PIA production. (A) Northern blot analysis of *icaADBC* after glucose induction of an OC and a repeated SC of *S. epidermidis*. An OC and an SC of *S. epidermidis* 1457 in T_A were grown to the stationary phase as described in Materials and Methods. Total bacterial RNA was extracted from the cultures before the addition of glucose (0 h) and at 2-h intervals after the addition of glucose. Hybridization was performed with an *icaC*-specific oligonucleotide. (B) PIA production after glucose induction of an OC and a repeated SC of *S. epidermidis*. Twenty-milliliter aliquots were removed from each culture before (0 h) and every 2 h after glucose induction and adjusted to the same OD. Bacterial extracts were prepared by sonication, and the presence of PIA was detected by coagglutination of serial dilutions with PIA-specific antibodies.

shown) (40). We therefore concluded that the additional factor required for biofilm formation was induced not specifically by the addition of glucose but also by other monosaccharides supporting biofilm formation.

DISCUSSION

Expression of the *icaADBC* operon is essential for synthesis of PIA, which mediates cell-to-cell adhesion and promotes a biofilm-positive phenotype of *S. epidermidis* (19, 34). In the present study, we analyzed the transcriptional activity of *ica* under growth conditions supporting a biofilm-positive phenotype of *S. epidermidis* 1457 compared to conditions leading to a biofilm-negative phenotype. By using Northern blot analysis, we demonstrated two different *ica* transcriptional patterns, depending on the presence or absence of glucose, that were in contrast to the respective phenotypes expressed. In biofilm-negative cultures grown in the absence of glucose, transcription of *icaADBC* was highly upregulated in the stationary phase of growth whereas biofilm-positive cultures generated

significantly less *ica* mRNA at the same time point. The level of *ica* mRNA could even be increased by subculturing stationary-phase cells a second time in medium lacking glucose, which might be due to the increased basic transcriptional level expressed at the start of the culture. Growth in the absence of glucose apparently promoted an unusual condition in which high-level steady-state *ica* mRNA accumulated even though PIA synthesis was inhibited at some stage of its biosynthetic pathway. The detected mRNA was functionally active because in vitro synthesis of PIA was demonstrated with membrane preparations of cells grown in the absence of glucose. The steady-state level of *ica* mRNA also correlated with the activity of the encoded Ica proteins. A fivefold higher level of mRNA (SC) in stationary-phase cells led to earlier cell aggregation, PIA production, and biofilm formation following induction with glucose.

Glucose first enhances and subsequently inhibits *ica* transcription. In *Bacillus subtilis* and some other gram-positive bacteria, glucose-mediated regulation is conferred by catabolite control protein A (CcpA) (20). However, there is no evi-

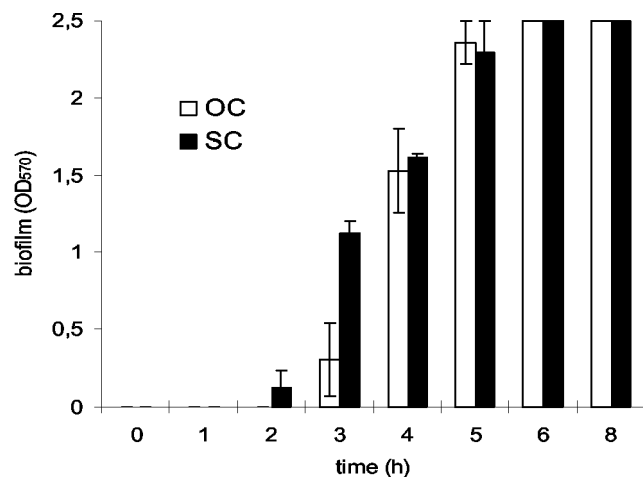


FIG. 5. Biofilm formation after glucose induction of an OC and an SC of *S. epidermidis*. A 12-h OC and an SC of *S. epidermidis* 1457 in T_A were diluted 1:100 in the same medium, and each culture was dispensed to one-half of a 96-well tissue culture plate. After 15 h of incubation at 37°C, both cultures were induced by the addition of glucose (0.25% final concentration) at 1-h intervals until the experiment was finished after 8 h of incubation. Biofilm formation was determined as described in Materials and Methods. Mean values of three independent experiments are shown.

dence of a CcpA binding site termed *cre* (catabolite-responsive element) in the sequence upstream of *icaADBC* (accession no. SEU43366) (19, 21). In *S. aureus*, glucose-mediated repression of gene expression independent of CcpA has been reported, which mainly involves the synthesis of several virulence factors, including enterotoxins A, B, and C (25, 49). However, up- and downregulation has not been described in these cases.

Interestingly, the downregulation of *icaADBC* by glucose did not influence the subsequent production of PIA. Four hours after glucose induction, the level of steady-state *ica* mRNA approximately equaled the level of mRNA before induction; still, a similar increase in the PIA concentration continued to be detected within the next hours. Apparently, the continuous transcription of *ica* was not required for the accumulation of PIA. The activity of the synthetic apparatus remained intact for several hours even though the transcript was no longer detectable in the cells. This finding is in contrast to observations

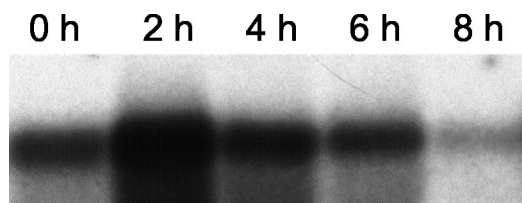


FIG. 6. Northern blot analysis of *icaADBC* after glucose induction of a static *S. epidermidis* culture. A 6-h preculture of *S. epidermidis* 1457 was diluted 1:100 in T_A and grown in tissue culture plates for 15 h at 37°C. The culture was induced by the addition of glucose at 2-h intervals and so that the induced cultures could be harvested simultaneously for RNA extraction after 8 h of incubation. 0 h, time point without glucose induction; 2, 4, 6, and 8 h, respective time after glucose induction. Six hours after induction, a fully established biofilm was detectable.

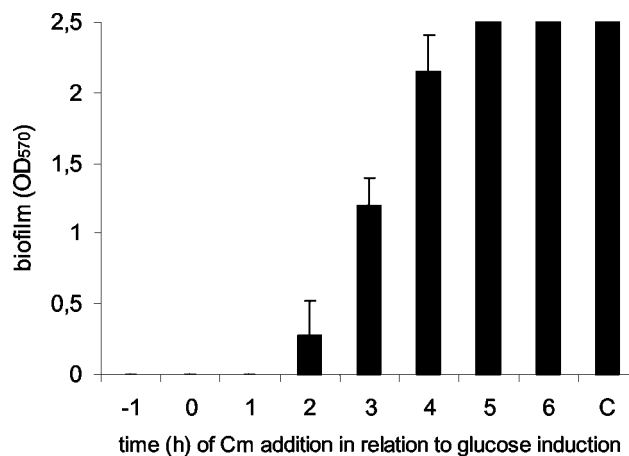


FIG. 7. Inhibition of biofilm formation by blocking of protein synthesis prior to glucose induction. A 6-h preculture of *S. epidermidis* was diluted 1:100 in T_A and incubated in a 96-well tissue culture plate for 15 h at 37°C. Chloramphenicol (Cm) was added (2-mg/ml final concentration) 1 h before (−1), simultaneously with (0), and every hour (1 to 6) after addition of glucose to the culture. The OD₅₇₀s of four wells were determined after 12 h of incubation. The control (C) did not contain chloramphenicol.

made on polysaccharide expression in a variety of other bacteria. In *S. aureus* strain Becker grown in complex Trypticase soy medium, capsular polysaccharide 8 transcription remained at a constant level during the early and late phases of growth, which was compatible with the detected synthesis of CP8 (31). The presence of the *has* operon mRNA of group A streptococci was also closely correlated to the phenotypic expression of the hyaluronate capsule. Encapsulated strains expressed the *has* operon mRNA in the exponential phase of growth, as well as the hyaluronate capsule. As the cells entered the stationary phase of growth, they lost the *has* operon mRNA, which was shown to be concomitant with the loss of their capsule (11).

In staphylococci, knowledge of higher regulatory systems influencing biofilm formation is still limited. The global regulator *agr*, which is activated in the postexponential growth phase, was shown to influence biofilm formation in *S. aureus* even though the expression of PIA is not under the control of *agr* (58). Different environmental conditions were shown to induce biofilm formation by the activation of the alternative sigma factor σ^B (27, 47). It is conceivable that growth in the absence of glucose is a further stimulus for the activation of σ^B , which might explain the strong upregulation of *ica* transcription in the postexponential and stationary phases of growth. Further experimental data are required to evaluate this hypothesis because upregulation of *ica* transcription in the absence of glucose did not result in the stimulation of a biofilm-positive phenotype in *S. epidermidis*. Recently, *icaR* has been identified as a transcriptional repressor of *ica* expression and its presence was required for *ica* activation by ethanol (7). However, induction of *ica* transcription by NaCl-glucose did not require the *icaR* gene, indicating that different regulatory pathways are operative in *ica* expression. Our results provide evidence of the existence of additional factors essential for *icaADBC* activity because even though *icaR* influences *ica* transcription, it is apparently also not the factor required for PIA synthesis (because there is no PIA synthesis) in medium lack-

ing glucose. In *Escherichia coli*, the global regulator CsrA has been shown to affect biofilm formation by negative regulation of intracellular glycogen biosynthesis and catabolism. Biofilm formation was stimulated by disruption of *csrA* in knockout mutants, demonstrating the importance of carbon flux for biofilm formation (24, 59).

The lack of PIA synthesis despite high-level mRNA and its encoded proteins in the absence of glucose suggested the presence of an additional, *icaADBC*-independent factor active in the expression of a PIA- and biofilm-positive phenotype. To gain further insight into the process of PIA production and biofilm accumulation after glucose induction, we inhibited protein synthesis by chloramphenicol before the addition of glucose. Northern blot analysis confirmed a high level of preformed *ica* mRNA before the cells were induced with glucose, so that these cells were already expressing IcaADBC proteins at that time point. Blocking protein synthesis 1 h before and up to 2 h after induction with glucose led to complete inhibition of biofilm formation. These results suggested that translation of an additional, *icaADBC*-independent factor is required for PIA synthesis and biofilm accumulation. Interestingly, preformed biofilm 3 to 5 h after glucose induction failed to develop further after inhibition of protein synthesis, which implies the need for continuous translation of the additional factor. Since this effect could also be observed with fructose as the inducing monosaccharide, the additional factor does not seem to be glucose specific. Potential candidates include enzymes providing energy or UDP precursors for PIA synthesis. Currently, besides *icaADBC*, virtually nothing is known about the biosynthetic pathway of PIA. However, since PIA is composed exclusively of *N*-acetylglucosamine, which is partly de-*N*-acetylated, the synthesis of their respective sugar nucleotide UDP-*N*-acetylglucosamine is required as an immediate precursor (17, 55). UDP-*N*-acetylglucosamine is also used in the formation of cell wall components, so that these two pathways seem to compete for the supply of the same sugar nucleotide. Growth in the absence of glucose might lead to a relative lack of UDP-*N*-acetylglucosamine available for cell wall synthesis, as can be deduced from the reduced cell density in the stationary phase of growth. It is conceivable that factors involved in the distribution of precursors or energy for PIA synthesis might play a role since the addition of the immediate precursor UDP-*N*-acetylglucosamine to crude membranes of cells grown without glucose abolished the blockage of PIA synthesis. Apparently, the enzymatic apparatus for assembly and modification of PIA was already present in the cell membranes under conditions lacking glucose.

Further studies are necessary to elucidate the regulation and biosynthetic pathway of PIA synthesis, which will help to explain the ability of *S. epidermidis* to colonize human niches and foreign medical devices and may open new ways to interfere with this important virulence-associated factor.

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