

## Anaerobic Conditions Induce Expression of Polysaccharide Intercellular Adhesin in *Staphylococcus aureus* and *Staphylococcus epidermidis*

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**Products of the intercellular adhesion (*ica*) operon in *Staphylococcus aureus* and *Staphylococcus epidermidis* synthesize a linear  $\beta$ -1,6-linked glucosaminylglycan. This extracellular polysaccharide mediates bacterial cell-cell adhesion and is required for biofilm formation, which is thought to increase the virulence of both pathogens in association with prosthetic biomedical implants. The environmental signal(s) that triggers *ica* gene product and polysaccharide expression is unknown. Here we demonstrate that anaerobic in vitro growth conditions lead to increased polysaccharide expression in both *S. aureus* and *S. epidermidis*, although the regulation is less stringent in *S. epidermidis*. Anaerobiosis also dramatically stimulates *ica*-specific mRNA expression in *ica*- and polysaccharide-positive strains of both *S. aureus* and *S. epidermidis*. These data suggest a mechanism whereby *ica* gene expression and polysaccharide production may act as a virulence factor in an anaerobic environment in vivo.**

Staphylococci cause a wide range of diseases in animals and humans (17). The virulence of the two major opportunistic pathogens of this genus, *Staphylococcus epidermidis* and *Staphylococcus aureus*, is multifactorial and mediated, particularly in *S. aureus*, by a variety of extracellular toxins and surface structures (2). In general, bacterial virulence is thought to be regulated by environmental signals to which the bacteria respond via global gene regulators, thereby adapting their phenotypes accordingly (21). Thus, typical staphylococcal infections such as endocarditis, meningitis, osteomyelitis, postoperative wound infections, and chronic lung infections may each involve different sets of virulence factors due to different environmental conditions. Similarly, in vitro gene expression by bacterial pathogens may not necessarily reflect in vivo gene expression (13).

Recently, it was shown that *S. aureus* strains expressing capsular polysaccharide type 5 (CP5) in vitro but not in vivo produce another polysaccharide in infected airways of cystic fibrosis (CF) patients, consisting of poly-*N*-succinyl- $\beta$ -1,6-glucosamine (PNSG) (20). PNSG is synthesized by products of the intercellular adhesion (*ica*) gene locus in *S. aureus* (7, 20), which was previously identified in *S. epidermidis* (12) and whose presence in other closely related species such as *Staphylococcus auricularis* and *Staphylococcus capitis* was inferred (7).

Products of the *ica* locus in *S. epidermidis* also produce the polysaccharide intercellular adhesin (PIA). PIA production leads to cell-cell adhesion and is required for biofilm formation (11). This polysaccharide was identified as a linear  $\beta$ -1,6-linked *N*-acetylglucosaminoglycan (18, 19). Antibodies raised against

*S. epidermidis* PIA or *S. aureus* PNSG recognize both antigens. We use a polyclonal antibody raised against *S. epidermidis* PIA in this work; however, where the exact nature of the polysaccharide is not known, we use the term “PIA/PNSG” to refer to the polysaccharide(s) synthesized by gene products of the *ica* locus in either *S. epidermidis* or *S. aureus*.

The *ica* operon (*icaADBC*) consists of four open reading frames, with a putative regulatory gene (*icaR*) located upstream and in the opposite orientation. In an in vitro assay, it was shown that IcaA and IcaD together synthesize sugar oligomers using UDP-*N*-acetylglucosamine as a substrate. This *N*-acetylglucosaminyltransferase activity together with the activity of IcaC produces a product in vitro that is recognized by an antibody raised against *S. epidermidis* PIA (9). Isogenic mutants of wild-type biofilm-forming strains in which the *ica* locus has been mutated or deleted are no longer able to form a biofilm in vitro, demonstrating that the *ica* genes, and therefore PIA/PNSG production, are required for biofilm formation (7, 11, 12).

Biofilm formation is a major concern in nosocomial infections because it protects microorganisms from opsonophagocytosis and antibiotic agents, leading to chronic infection and sepsis, particularly in immunocompromised patients (6). The contribution of PIA/PNSG to the virulence of *S. epidermidis* has been repeatedly demonstrated (24–26, 31); however, the environmental signals which mediate PIA/PNSG expression are at present unknown.

Recently, we provided evidence that the partial pressure of oxygen in airway plugs of CF patients was <4% that of intraluminal air (D. Worlitzsch, K. C. Meyer, P. Birrer, and G. Döring, *Pediatr. Pulmonol.*, abstr. A457, p. 333, 1998). Since we also observed PNSG expression in the *S. aureus*-infected CF lung (20), the objective of the present study was to investigate the impact of anaerobiosis on PIA/PNSG expression in *S. epidermidis* and *S. aureus*. Here we show that PIA/PNSG expression is indeed induced by anaerobic growth conditions and

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TABLE 1. Strains used in this study

Species	Strain collection no.	Common name	Reference or source
<i>S. aureus</i>	ATCC 35556	SA113	16
		ATCC 35556 $\Delta$ <i>ica</i> ::tet	7
	ATCC 49832	3AI	30
	ATCC 10832	Wood 46	U.S. Food and Drug Administration
<i>S. epidermidis</i>		O-47	11
		O-47 $\Delta$ <i>ica</i> ::tet	This study
		O-47 <i>ica</i> ::Tn917 (mut2)	11
	ATCC 35984	RP62A	5
		SE05	This study

that *ica* gene transcription is regulated by environmental oxygen.

#### MATERIALS AND METHODS

**Staphylococcal strains and plasmids.** The strains used in this study are listed in Table 1. The *ica* deletion mutant of *S. aureus* ATCC 35556 was described previously (7). The *S. epidermidis* O-47 *ica* deletion mutant was constructed in a similar manner, replacing the entire *ica* operon (*icaR-icaC*) with a tetracycline resistance cassette. The *S. epidermidis* strain ATCC 35984 (RP62A) *ica* locus and surrounding DNA were amplified using PCR and primers CG-28 (CCGGATCATTGAATAATCATGAAATAATGTC) and SR-1 (CGGGATCCGAGAAA AATTTTCATTTTAAAATAAAC) and cloned into the *Sma*I site of temperature-sensitive shuttle vector pBT5, a derivative of pBT2 lacking the *Eco*RI restriction site in the multiple cloning site (4), creating plasmid pSC1. The *ica* locus was deleted using pSC1, inverse PCR, and primers CG-7 (CTAGAGCTCTAGACC TTTCGTTAGTTAGTTGT) and SR-2 (CGGAATTCACGCGTCACCTGTG ATGTATCTCACTCC) and replaced with the tetracycline resistance cassette from pT181, amplified using primers tet-4 (CTCGAATTCGCCAGTCGATTT AACGGAC) and tet-5 (CTCGAATTCGAGTGGCAAATGCTAGCCAC) and restriction enzyme *Eco*RI, creating plasmid pSC12. Primers were obtained from MWG-Biotech (Ebersberg, Germany). PCR amplifications were performed using a MiniCycler PTC-150 (MJ Research, Inc., Watertown, Mass.). Homologous recombination was performed using wild-type *S. epidermidis* strain O-47 containing plasmid pSC12. Bacteria were grown overnight in B medium (1% tryptone [Gibco BRL-Life Technologies GmbH, Eggenstein, Germany], 0.5% yeast extract [Gibco BRL], 0.5% NaCl, 0.1% K<sub>2</sub>HPO<sub>4</sub>, 0.1% glucose) at 30°C with 10  $\mu$ g of chloramphenicol/ml, diluted 1:1,000, and grown again at 30°C with antibiotic selection; diluted 1:1,000 and grown at 41°C without antibiotic selection twice; and diluted 1:100 and plated on tryptic soy broth (TSB) (Gibco BRL)

plates containing 2.5  $\mu$ g of tetracycline/ml. Homologous recombination and plasmid curing of chloramphenicol-sensitive, tetracycline-resistant colonies were then confirmed with PCR and detection for loss of PIA/PNSG expression. The strain listed in Table 1 as ATCC 10832 is not the same as the strain with the same name used in our previous study (7). Strain SE05 was isolated in association with a central venous catheter removed from a patient in the intensive care unit of the University Hospital, Tübingen, Germany, in January 2000.

**Aerobic and anaerobic growth.** Bacterial strains were cultured in either TSB supplemented with 0.25% glucose (TSB plus Glc) or CYPG medium (23) supplemented with 2% glucose (CYPG plus Glc) and supplemented when appropriate with tetracycline (10  $\mu$ g/ml), chloramphenicol (10  $\mu$ g/ml), or erythromycin (10  $\mu$ g/ml). Bacteria were incubated with shaking under both aerobic and anaerobic conditions in 250-ml Erlenmeyer flasks containing 100 ml of TSB plus Glc. Anaerobic conditions were created by bubbling filtered helium gas through the medium for 10 min following inoculation. Flasks were then immediately sealed such that no air could enter the flask during incubation. The airtight seal consisted of a silicone stopper pierced with two glass pipettes, one of which (gas inlet) was submerged in the medium. Attached to the outside end of each glass pipette was a piece of flexible tubing that could be attached to the helium gas tank and clamped to seal the flask after gassing. Alternatively, overnight cultures of staphylococcal strains grown in CYPG plus Glc were harvested and diluted in CYPG plus Glc to an optical density at 600 nm (OD<sub>600</sub>) of 0.05 and incubated under aerobic conditions (with shaking) and anaerobic conditions (without shaking) in glass tubes or microtiter plates (Becton Dickinson, Heidelberg, Germany) for up to 96 h. For anaerobic growth, glass tubes were placed in an anaerobic jar with Anaerocult A (Merck Eurolab GmbH, Darmstadt, Germany), whereas polystyrol 96-well microtiter plates were sealed with adhesive tape. Anaerobiosis in both of our experimental systems was confirmed using Anaerotest test strips (Merck). PIA/PNSG production and biofilm formation were also tested under controlled (3% O<sub>2</sub>, 1% CO<sub>2</sub>, 96% N<sub>2</sub>) conditions in a shaking gassed BB6060

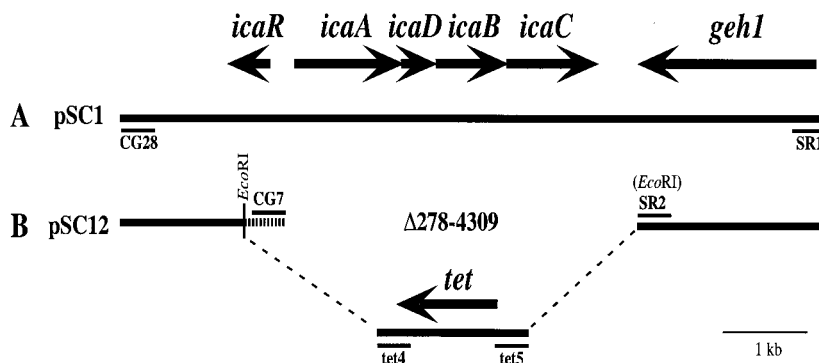


FIG. 1. Schematic representation of cloning strategy used to construct the *ica* deletion-replacement mutant of *S. epidermidis*. A similar strategy was used to construct the *ica* deletion-replacement mutant of *S. aureus*, described in reference 7. (A) Plasmid pSC1. Sequences including and surrounding the *ica* locus are included in database entry U43366. Sequences including and surrounding the *gehI* gene are included in database entry AF053006. PCR primers used to amplify cloned DNA are indicated (CG-28 and SR-1). (B) Plasmid pSC12, the deletion-replacement construct used for homologous recombination. Inverse PCR primers used to delete the *ica* locus (CG-7 and SR-2) and amplify the tetracycline resistance cassette (tet4 and tet5) are indicated. *Eco*RI restriction recognition sites contained within the sequences of primers SR-2, tet-4, and tet-5 and the *Eco*RI restriction site in the middle of *icaR* were used to insert the tetracycline resistance cassette. Deleted sequence numbers refer to database sequence U43366.

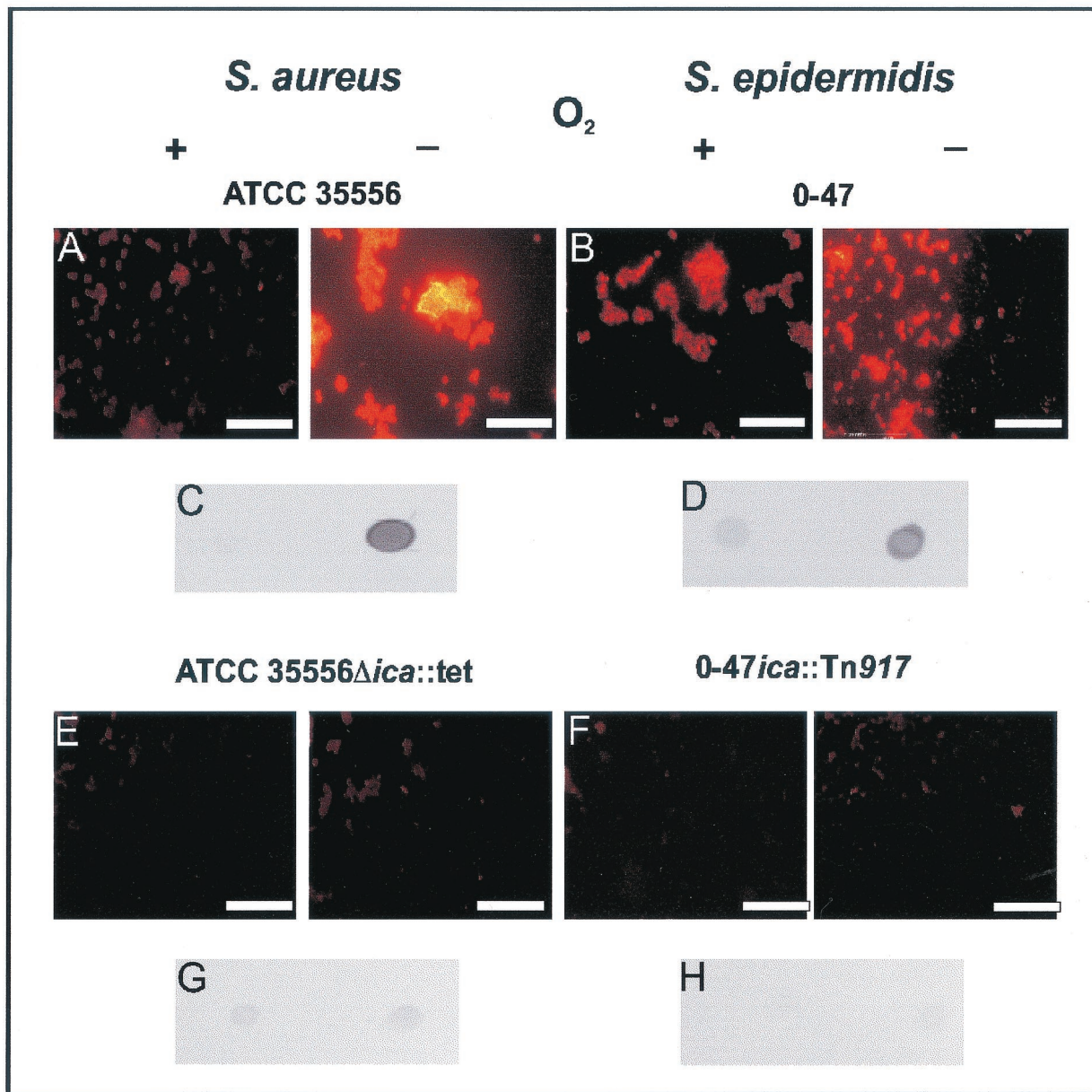


FIG. 2. Increased expression of PIA/PNSG under anaerobic conditions in *S. aureus* and *S. epidermidis* in vitro. The *ica*-positive *S. aureus* strain ATCC 35556 (A), an isogenic *ica*-deletion mutant (ATCC 35556 $\Delta$ *ica::tet*) (E), the *ica*-positive *S. epidermidis* strain O-47 (B), and an isogenic *ica* transposon mutant (O-47*ica::Tn917*) (F) were grown under aerobic (+) and anaerobic (-) conditions for 96 h in CYPG plus Glc. PIA/PNSG expression was detected by indirect immunofluorescence using rabbit antibodies raised against *S. epidermidis* PIA and a Cy3-conjugated goat anti-rabbit IgG antibody. Magnifications for panels A, B, E, and F,  $\times 1,000$ ; bar = 10  $\mu$ m. Cell surface extracts from *S. aureus* (C and G) and *S. epidermidis* (D and H) wild type and isogenic deletion mutants, grown overnight (14 to 18 h) under aerobic or anaerobic conditions in TSB plus Glc, were spotted on nitrocellulose filters, and PIA/PNSG was detected using the same anti-*S. epidermidis* PIA antibody used for panels A, B, E, and F.

incubator (Heraeus, Stuttgart, Germany); however, these conditions were apparently not anaerobic enough, and little difference was seen from cultures incubated in the presence of ambient air. Bacterial growth was determined by measuring CFU on blood agar plates and/or determining OD<sub>578</sub>.

**Detection of PIA/PNSG expression and biofilm formation in vitro.** Biofilm assays and semiquantitative PIA/PNSG detection using cell surface extracts were performed as described previously (7, 11, 12). Briefly, cell surface extracts were prepared by growing cells overnight (14 to 18 h) or to an OD<sub>578</sub> of 2.0 in TSB plus Glc, the optical density was determined, and an equal number of cells (typically 2 to 4 ml) from each culture was resuspended in 50  $\mu$ l of 0.5 M EDTA, pH 8.0. Cells were then incubated for 5 min at 100°C and centrifuged to pellet

the cells, and 40  $\mu$ l of the supernatant was incubated with 10  $\mu$ l of 20-mg/ml proteinase K (Boehringer GmbH, Mannheim, Germany) for 30 min at 37°C to minimize nonspecific background. The extracts were then spotted onto a nitrocellulose membrane, and the membrane was dried, blocked with 3% bovine serum albumin, and incubated with an anti-*S. epidermidis* PIA antibody (diluted 1:5,000) (kind gift of D. Mack, Hamburg, Germany). Anti-PIA antibodies were detected using an alkaline phosphatase-conjugated anti-rabbit immunoglobulin (IgG) antibody (Boehringer) and the 4-nitroblue tetrazolium chloride-5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) color detection system (Boehringer).

Biofilm assays were performed as follows. Bacteria were grown overnight in TSB plus Glc. Cultures were then diluted 1:200 in fresh medium and incubated



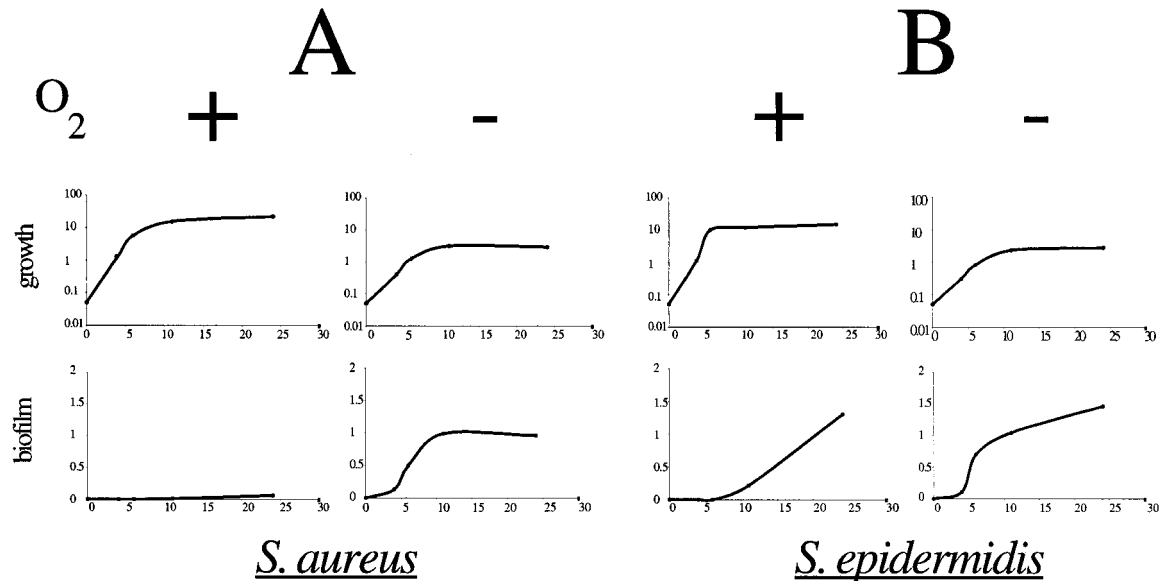


FIG. 3. Biofilm formation under aerobic and anaerobic conditions. The same *S. aureus* (A) and *S. epidermidis* (B) wild-type strains shown in Fig. 2 were grown under aerobic and anaerobic conditions in CYPG plus Glc for up to 24 h in microtiter plates, and biofilm formation was quantified using the *S. epidermidis* anti-PIA antibody. The growth rate of the strains was determined independently by measuring the OD<sub>600</sub> in liquid culture.

overnight in stationary U-bottomed-well polystyrol microtiter plates (Greiner Labortechnik, Frickenhausen, Germany) at 37°C. Microtiter wells were washed twice with phosphate-buffered saline (PBS) (7 mM Na<sub>2</sub>HPO<sub>4</sub>, 3 mM NaH<sub>2</sub>PO<sub>4</sub>, 130 mM NaCl, pH 7.4), dried in an inverted position, and stained with 0.1% safranin (Serva Feinbiochemica GmbH & Co. KG, Heidelberg, Germany).

PIA/PNSG expression in CYPG plus Glc was determined using indirect immunofluorescence microscopy. Briefly, liquid cultures were washed three times with PBS (pH 7.2) containing 0.05% Tween 20. Bacteria were harvested by centrifugation (5,000 × g) and resuspended in 5 ml of PBS. A drop of the suspension was transferred to a glass slide (Becton Dickinson). Samples on glass slides were fixed with 4% formaldehyde for 1 h at 4°C, and nonspecific binding sites were blocked with 5% human IgG (Sigma, Deisenhofen, Germany). Thereafter, slides were incubated with a rabbit antibody raised against *S. epidermidis* PIA (diluted 1:200) or a polyclonal rabbit antibody raised against *S. aureus* PNSG (diluted 1:50) (kind gift of G. Pier, Boston, Mass.) for 1 h at room temperature. After washing with PBS, bound antibody was detected with indocarbocyanin 3 (Cy3)-conjugated goat anti-rabbit IgG (Jackson Dianova, Hamburg, Germany) and analyzed using an Axioplan fluorescence microscope (Zeiss, Oberkochen, Germany) and a Kontron KS 300 imaging system (Kontron Electronic GmbH, Eching, Germany). Results obtained with both antibodies were identical. Data shown were obtained using the anti-PIA antibody only.

In order to observe biofilm formation over time as detected with the anti-PIA antibody, bacteria were grown in CYPG plus Glc in stationary microtiter plates and enzyme-linked immunosorbent assays (ELISAs) were performed. Briefly, microtiter plates were washed gently three times with PBS (pH 7.2), air dried, fixed with formaldehyde, and blocked with human IgG. The plates were incubated with rabbit anti-*S. epidermidis* PIA antibody for 2 h at 37°C, washed, and incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibody (diluted 1:250) (Dako, Hamburg, Germany) for 2 h at 37°C, followed by an incubation with the substrate (Sigma). After 40 min, OD<sub>450</sub>s were measured using an ELISA plate reader (SLT Lab Instruments, Crailsheim, Germany). Each assay was performed in triplicate.

**RNA extraction and detection.** *S. aureus* cultures were grown to an OD<sub>578</sub> of 2.0 in TSB plus Glc. Generally, anaerobic cultures grown overnight (14 to 18 h) reached an OD<sub>578</sub> of approximately 2.0, and aerobic cultures were grown for several hours until an OD<sub>578</sub> of 2.0 was reached. Approximately 10<sup>10</sup> cells were harvested by centrifugation and washed with 0.5 volume of cold 0.5 M EDTA. *S. aureus* cell pellets were then resuspended in 1 ml of 0.5-mg/ml lysostaphin (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) and incubated for 3 min at 37°C. *S. epidermidis* cells were resuspended in cold 0.5 M EDTA and disrupted using glass beads and vortexing. RNA was then prepared using the

RNeasy Midi kit (Qiagen GmbH, Hilden, Germany) as recommended by the manufacturer. RNA yield was measured by OD<sub>260</sub>, and the relative concentration between samples was confirmed by visual inspection of rRNA in samples run on an agarose gel and stained with ethidium bromide. Three micrograms of total RNA from each sample was then applied to a nylon membrane (Boehringer) using a dot blotting apparatus (Bio-Rad Laboratories, Munich, Germany). Filters were hybridized at 50°C using DIG (digoxigenin) Easy Hyb solution (Boehringer) and an *ica*-specific DNA probe. Filters were washed in 0.1 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate at 68°C and exposed to X-ray film after detection using the DIG luminescent detection kit (Boehringer). The DIG-labeled probes were made by using PCR, DIG-labeled nucleotide labeling mix (Boehringer), and the Expand Long Template PCR system (Boehringer) as recommended by the manufacturer. Amplified DNA included sequences within the coding regions from the beginning of *icaA* to the end of *icaC* and was amplified using primers SA9 (GTATTTATGTCTATTACTGGATTGTCGGTTC) and SA6 (GTAGTGATATCGCTAGAAAGCCATTGT) for *S. aureus* and primers SE1 (GTCAATTTACTGGATAGTAGGATCGATTAC) and SE2 (AGTTAGGCTGGTATTGGTCAAATTGTA ACT) for *S. epidermidis*.

## RESULTS

**Construction of an *ica* deletion-replacement mutant in *S. epidermidis*.** An *ica* deletion-replacement mutant was constructed in wild-type biofilm-forming *S. aureus* strain ATCC 35556 and was described previously (7). An *S. epidermidis* *ica* deletion-replacement mutant was constructed on a plasmid in a similar manner, replacing the entire *ica* operon (*icaRADBC*) with a tetracycline resistance cassette. The chromosomal copy of the *ica* operon was replaced by homologous recombination. A schematic representation of the cloning strategy is shown in Fig. 1 and described in more detail in Materials and Methods.

**PIA/PNSG expression is increased in vitro under anaerobic growth conditions.** We tested a number of wild-type strains of *S. aureus* and *S. epidermidis* and isogenic deletion mutants lacking the *ica* gene locus for PIA/PNSG production and biofilm formation under aerobic and anaerobic growth conditions

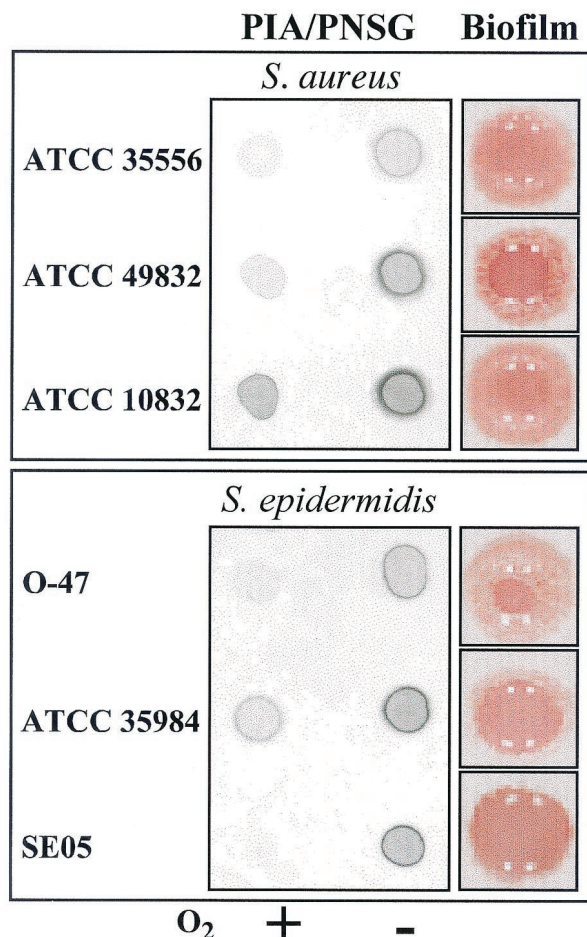


FIG. 4. PIA/PNSG expression is stimulated under anaerobic conditions in six *S. aureus* and *S. epidermidis* biofilm-forming strains. Three *S. aureus* and three *S. epidermidis* biofilm-forming strains were grown under both aerobic (+) and anaerobic (-) conditions to early stationary phase (14 to 18 h) in TSB plus Glc. PIA/PNSG expression (PIA/PNSG) was detected in cell surface extracts isolated from an equal number of cells from each strain using an antibody raised against *S. epidermidis* PIA. Biofilm formation in a microtiter plate well is shown for each strain (Biofilm). Adhering cells are stained with safranin (red color) to facilitate visualization of the biofilm.

in liquid cultures and in polystyrene microtiter plates, respectively, using a polyclonal antibody raised against *S. epidermidis* PIA (7, 12). In contrast to aerobic growth conditions, anaerobiosis strongly stimulated PIA/PNSG production in *ica*-positive wild-type strains of *S. aureus* and *S. epidermidis*, whereas isogenic *ica* deletion mutant strains remained PIA/PNSG negative (Fig. 2). As shown in Fig. 2A, for *S. aureus* ATCC 35556 grown in CYPG medium supplemented with 2% glucose (CYPG plus Glc) for 96 h, PIA/PNSG expression was detectable only under anaerobic conditions. When cell surface extracts from bacteria grown overnight in TSB supplemented with 0.25% glucose (TSB plus Glc) were examined, PIA/PNSG expression was much stronger in cultures grown under anaerobic conditions (Fig. 2C). In contrast to *S. aureus*, *S. epidermidis* O-47 expressed PIA/PNSG under both aerobic and anaerobic conditions in CYPG plus Glc after 96 h; however, the expression was much stronger under anaerobic conditions (Fig.

2B). The difference between aerobic and anaerobic expression of PIA/PNSG in cell surface extracts was more dramatic after overnight growth in TSB plus Glc (Fig. 2D). Since PIA/PNSG is required for biofilm formation, we measured biofilm formation over time using an anti-PIA antibody during *S. aureus* and *S. epidermidis* growth. Biofilm was formed by *S. aureus* only under anaerobic conditions (Fig. 3A). In contrast to *S. aureus*, *S. epidermidis* produced biofilm under both aerobic and anaerobic conditions; however PIA/PNSG was detectable significantly earlier under anaerobic conditions (Fig. 3B). These data suggest that PIA/PNSG production in *S. epidermidis* is less stringently regulated by oxygen than in *S. aureus* and that the amount of PIA/PNSG produced under the aerobic conditions used in this in vitro system is sufficient to mediate biofilm formation.

**Increased PIA/PNSG expression under anaerobic conditions is a general phenomenon.** We examined PIA/PNSG expression in three representative strains from each species under aerobic and anaerobic conditions. Each of the six strains carries the *ica* locus (data not shown), expresses PIA/PNSG, and forms a strong in vitro biofilm. The results confirmed that PIA/PNSG production is stimulated by anaerobic growth conditions in multiple representatives of each species (Fig. 4). PIA/PNSG production seems to be very sensitive to the presence of oxygen. Experiments performed in an incubator with a controlled (3% oxygen) environment compared to parallel cultures grown in an incubator with ambient air showed only minimal differences in PIA/PNSG production. In addition, increased CO<sub>2</sub> concentrations (1%) in the same experiments did not affect PIA/PNSG expression (data not shown).

**Increased PIA/PNSG expression levels reflect increased RNA levels under anaerobic conditions in vitro.** Total RNA was extracted from *S. aureus* strain ATCC 35556, *S. epidermidis* strain O-47, and their respective isogenic *ica* deletion mutants grown under both aerobic and anaerobic conditions. Low levels of *ica*-specific transcripts were detected in wild-type cells of both species grown under aerobic conditions; however, much stronger expression was detected in bacteria grown under anaerobic conditions. This increase was reflected in the expression of PIA/PNSG in cell surface extracts prepared from the same cultures (Fig. 5). The relative level of PIA/PNSG expression between aerobic and anaerobic conditions in this *S. epidermidis* strain was less dramatic under these conditions (both cultures were grown to an OD<sub>578</sub> of 2) than when both cultures were allowed to grow to stationary phase (Fig. 2D). RNA levels, however, were dramatically different, suggesting a further layer of regulation of PIA/PNSG production.

## DISCUSSION

Bacterial biofilm formation is of major concern in association with indwelling medical devices, since the biofilm provides bacteria with considerable resistance to host defenses and antimicrobial agents (6, 10). Single bacterial cells can be easily eliminated by macrophages or neutrophils in the healthy host; however, bacterial colonies enclosed in a self-produced polymeric sugar matrix are far more resistant to oxygen radicals and phagocytosis. Consequently, as has been shown using *S. epidermidis* in animal models, the virulence of *S. epidermidis* strains that produce polysaccharide is greater than that of

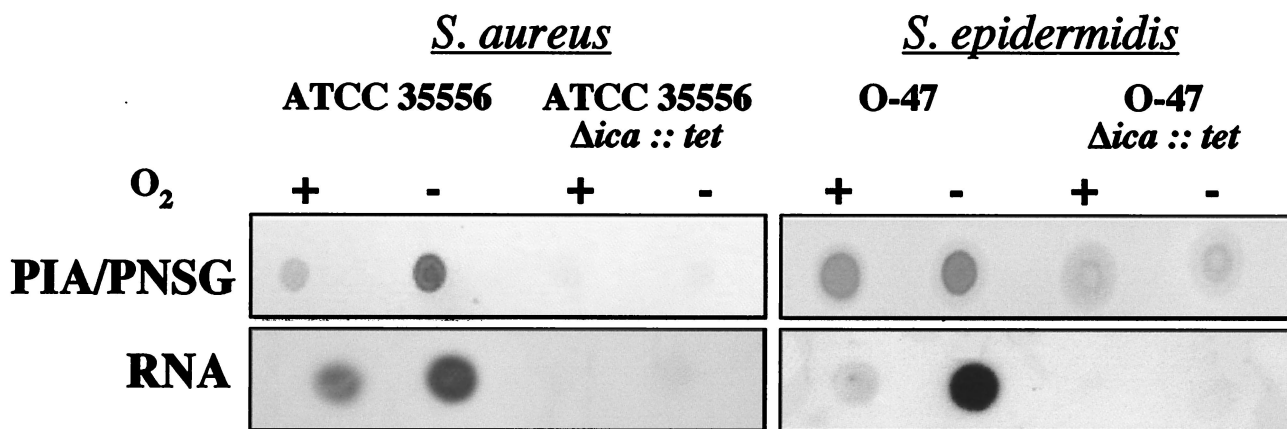


FIG. 5. Increased PIA/PNSG expression under anaerobic conditions correlates with *ica* operon transcript levels in *S. aureus* and *S. epidermidis*. Cell surface extracts from *S. aureus* ATCC 35556, *S. epidermidis* O-47, and their respective isogenic *ica* deletion mutants were grown to an OD<sub>578</sub> of 2.0 under aerobic (+) or anaerobic (-) conditions in TSB plus Glc. Cell surface extracts from an equal number of cells from each strain were spotted onto a nitrocellulose filter, and PIA/PNSG was detected using a polyclonal anti-*S. epidermidis* PIA antibody (PIA/PNSG). RNA was extracted from cells from the same cultures, spotted onto a nylon filter, and hybridized with an *ica*-specific DNA probe (RNA).

polysaccharide-negative *S. epidermidis* strains (25, 26, 28). The self-produced extracellular polysaccharide that forms a matrix around the bacteria may impair the penetration of antimicrobial agents. Once a biofilm has formed, the low metabolic rate of the bacteria may also limit the effectiveness of antibiotics that require active cell division and active metabolism (15).

Until recently, little has been known about the ability of *S. aureus* to form a biofilm. Unlike clinical isolates of *Pseudomonas aeruginosa* from patients with CF (reviewed in reference 8), *S. aureus* produces very little PIA/PNSG in vitro on culture plates but produces predominantly polysaccharide microcapsules of type 5 or 8 (1, 14, 27, 29). Due to this in vitro phenotype, most investigators have thought that *S. aureus* would also express CP5 or CP8 during infections in animals or humans. Only recently was it shown that CP5 expression is in fact reduced in the lungs of CF patients (13) and that PNSG expression is increased (20). Apparently, PNSG production by *S. aureus* is not restricted to the airways of patients with CF, because the polysaccharide was also detected in a mouse renal infection model using immunoelectron microscopy and ELISA (20).

Altogether, although both *S. epidermidis* and *S. aureus* are now known to produce PIA/PNSG in vivo during human and animal infections, little is known about the environmental factor(s) that triggers this expression. Here, we provide evidence that at least one environmental stimulus responsible for increased PIA/PNSG expression is lack of oxygen. This notion is based on our findings showing that in vitro anaerobic growth conditions trigger increased *ica* gene transcription and PIA/PNSG expression by *S. aureus* and *S. epidermidis* strains that carry the *ica* gene locus as detected by RNA blotting and specific antibodies raised against *S. epidermidis* PIA. This does not mean that the strains tested produced no polysaccharide product under aerobic conditions; our data show that production is stimulated under anaerobic conditions, an increase that may be at least partially attributable to transcriptional regulation.

The anaerobic stimulation of extracellular polysaccharide

has been confirmed using two different types of medium and three different methods for generating anaerobic conditions. Our assays measured *ica* transcription, PIA/PNSG production, and biofilm formation. Findings by Barker et al. (3), who showed that undefined coagulase-negative bacteria form biofilms only under aerobic conditions, may be explained by differences in the organisms tested and the assay(s) employed.

While some staphylococcal strains are able to form an in vitro biofilm, others are not (3, 7). We investigated approximately 17 *S. epidermidis* and 41 *S. aureus* strains in the course of this study and found that, for those strains that produce PIA/PNSG, PIA/PNSG production is stimulated by anaerobic conditions. PIA/PNSG production was observed in approximately the same proportion of strains as reported by McKenney et al. to produce PNSG (reference 20 and data not shown). This does not necessarily mean that apparently PIA/PNSG-negative strains produce no PIA/PNSG or PIA/PNSG-like product(s), however. Support for this idea stems from our observation that a few biofilm-positive strains transcribe *ica*-specific RNA but produce no detectable PIA/PNSG (data not shown). Although the *ica* locus appears to be transcribed in these strains, the encoded Ica proteins may not be translated or may contain mutations or truncations that render them unable to produce mature PIA/PNSG. This would imply that these strains are able to form in vitro biofilms in a PIA/PNSG-independent manner. This observation is also supported by the finding of Muller et al. (22) that a few *S. epidermidis* strains that are able to form a biofilm apparently produce no polysaccharide. Alternatively, it is possible that PIA/PNSG is modified, for example, (de)acetylated or (de)succinylated, in a manner such that our antibody(ies) no longer recognizes the antigen(s) produced by these strains. The fact that antibodies raised against *S. epidermidis* PIA or *S. aureus* PNSG detect both antigens implies that the chemical structures of PIA and PNSG are identical or very similar. A direct structural comparison has not been made to date, and the possibility of other, perhaps strain-specific, modifications to the glucosamine backbone cannot be eliminated. Nevertheless, immunological



cross-reactivity was observed in animal experiments where animals actively immunized with the polysaccharide antigen were protected from a challenge with *S. epidermidis* (31) and *S. aureus* (20).

In summary, an anaerobic environment stimulates PIA/PNSG production in both *S. aureus* and *S. epidermidis*. Anaerobic environments may develop in localized infections and stimulate expression of this virulence factor by both pathogens *in vivo*. PIA/PNSG production and biofilm formation pose a considerable risk for the patient. The elucidation of the molecular mechanism(s) of biofilm formation may lead to better tools for clinical intervention in the future.

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