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The Intercellular Adhesion (*ica*) Locus Is Present in *Staphylococcus aureus* and Is Required for Biofilm Formation

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Nosocomial infections that result in the formation of biofilms on the surfaces of biomedical implants are a leading cause of sepsis and are often associated with colonization of the implants by *Staphylococcus epidermidis*. Biofilm formation is thought to require two sequential steps: adhesion of cells to a solid substrate followed by cell-cell adhesion, creating multiple layers of cells. Intercellular adhesion requires the polysaccharide intercellular adhesin (PIA), which is composed of linear β-1,6-linked glucosaminylglycans and can be synthesized in vitro from UDP-*N*-acetylglucosamine by products of the intercellular adhesion (*ica*) locus. We have investigated a variety of *Staphylococcus aureus* strains and find that all strains tested contain the *ica* locus and that several can form biofilms in vitro. Sequence comparison with the *S. epidermidis ica* genes revealed 59 to 78% amino acid identity. Deletion of the *ica* locus results in a loss of the ability to form biofilms, produce PIA, or mediate *N*-acetylglucosaminyltransferase activity in vitro. Cross-species hybridization experiments revealed the presence of *icaA* in several other *Staphylococcus* species, suggesting that cell-cell adhesion and the potential to form biofilms is conserved within this genus.

Chronic nosocomial infections by gram-positive bacteria have become more prevalent in recent years with the increased use of prosthetic biomedical implants. Chronic infection of a prosthetic implant can serve as a septic focus that can lead to osteomyelytis, acute sepsis, and death, particularly in immunocompromised patients (5, 12). Bacteria colonize prosthetic implants as a biofilm, multiple layers of sessile cells that adhere to the implant surface as well as to each other. Once a biofilm has formed, it can be very difficult to treat clinically because the bacteria on the interior of the biofilm are well protected from the host immune response as well as antibiotic agents (16).

Biofilm formation is thought to be a two-step process that requires the adhesion of bacteria to a substrate surface followed by cell-cell adhesion, forming the multiple layers of the biofilm (13, 14, 30). This latter process is associated with the polysaccharide intercellular adhesin (PIA), which is composed of linear β-1,6-linked glucosaminylglycans in *Staphylococcus epidermidis* (22). The intercellular adhesion (*ica*) locus, *icaADB* and *C*, was identified and shown to mediate cell-cell adhesion and PIA production in *S. epidermidis* (15). It was further demonstrated that *icaA* and *icaD* together mediate the synthesis of sugar oligomers in vitro, 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 PIA (8).

The organism most frequently isolated in association with certain types of infections related to biomedical implants, including central venous catheters, cerebrospinal fluid shunts, prosthetic heart valves, and ocular lens implants, is *S. epidermidis* (2, 5, 6, 18, 24). *Staphylococcus aureus* is more frequently isolated in association with peripheral intravascular catheters, endotracheal and tracheotomy tubing, peritoneal dialysis tubing, and corneal infections related to contact lens wear (2, 7, 24, 31, 37, 38). Coagulase-negative staphylococci, primarily *S. epidermidis*, and *S. aureus* are isolated in approximately equal numbers in association with prosthetic joint and vascular graft infections (18, 35); however, the infections that are associated with *S. aureus* represent a more serious clinical hazard due to the higher morbidity and mortality associated with this organism compared to those of *S. epidermidis*.

We set out to investigate whether *S. aureus* can form biofilms in vitro, and if so, whether it is able to mediate cell-cell adhesion and PIA synthesis via the *ica* locus. We found not only that the function of the *ica* locus is conserved between *S. epidermidis* and *S. aureus* but also that the *ica* locus is present in several other *Staphylococcus* species as well, implying that the cell-cell adhesion function mediated by this locus may be conserved within this genus.

MATERIALS AND METHODS

***Staphylococcus* strains.** Most of the strains used for this work are listed in Tables 1 and 2 along with national strain collection reference numbers where applicable. *S. epidermidis* O-47 is a clinical isolate (13), and strain 5179 is a biofilm- and PIA-negative strain (23). *Staphylococcus carnosus* TM300 is a wild-type plasmid and cloning host strain (9, 33). Bacteria were cultured under standard conditions in B medium (1% tryptone [Gibco BRL Life-Technologies GmbH, Eggenstein, Germany], 0.5% yeast extract [Gibco BRL], 0.5% NaCl, 0.1% K_2HPO_4, 0.1% glucose) or Luria-Bertani medium (1% tryptone [Gibco BRL], 0.5% yeast extract, 0.5% NaCl) except as noted below. Media were supplemented when appropriate with chloramphenicol (10 μg/ml), tetracycline (10 μg/ml), or ampicillin (100 μg/ml) except where otherwise noted.

Biofilm assay. Bacteria were grown overnight in tryptic soy broth (TSB; Gibco BRL) supplemented with 0.25% glucose. Cultures were then diluted 1:200 and incubated overnight in stationary U-bottom well polystyrol microtiter plates (Greiner Labortechnik, Frickenhausen, Germany) at 37°C. Microtiter wells were washed twice with phosphate-buffered saline (7 mM Na_2HPO_4, 3 mM NaH_2PO_4, 130 mM NaCl [pH 7.4]), dried in an inverted position, and stained with 0.1% safranin (Serva Feinbiochemica GmbH & Co. KG, Heidelberg, Germany) (13).

PIA detection. PIA production in *S. aureus* was detected, with modifications, as described by Gerke et al. (8). Briefly, cells were grown overnight in TSB supplemented with 0.25% glucose, the optical density was determined, and the same

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TABLE 1. Strains used in this work

Organism	Strain collection no.		Common name	Origin, other information	Well[a]
	ATCC	NCTC, DSM			
S. aureus	ATCC 35556		SA113	Derived from NCTC 8325, restriction deficient	1a
	ATCC 25904	NCTC 10833	Newman D2C	Clumping factor-positive variant of Newman D2C	1b
	ATCC 49832		3A	Produces restriction endonuclease *Sau*3AI	1c
	ATCC 12600	NCTC 8532	Type Strain	Pleural fluid	1d
		DSM 20232	Copenhagen	Cell wall teichoic acid	1e
	ATCC 12601	NCTC 6131			2a
			RN 4220	Derived from 8325-4, restriction deficient	2b
	ATCC 10832		Wood 46	FDA, produces dermatoxin	2c
	ATCC 12598	NCTC 8530	Cowan 1	Septic arthritis	2d
	ATCC 31153	NCTC 8178	Newman	Throat swab, produces clumping factor	2e
S. epidermidis	ATCC 35984		RP62A	Catheter sepsis	3b
			O-47	Clinical isolate	3c
S. carnosus			TM300	Plasmid host	3d

[a] Corresponding well in Fig. 1.

number of cells (2 to 4 ml) from each culture was resuspended in 50 μl 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 μl of the supernatant was incubated with 10 μl of proteinase K (20 mg/ml; Boehringer GmbH, Mannheim, Germany) for 30 min at 37°C. After addition of 10 μl of Tris-buffered saline (20 mM Tris-HCl, 150 mM NaCl [pH 7.4]) containing 0.01% bromphenol blue, 4 μl was spotted on a nitrocellulose filter, dried, blocked with 3% bovine serum albumin, and incubated overnight with an anti-*S. epidermidis* PIA antibody (gift from D. Mack, Hamburg, Germany) (23) absorbed as described by Gerke et al. (8) and diluted 1:5,000. Bound antibodies were detected with an absorbed biotin-conjugated anti-rabbit immunoglobulin G (IgG) antibody (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) diluted 1:5,000, horseradish peroxidase-conjugated streptavidin (Amersham Buchler GmbH & Co. KG, Braunschweig, Germany) diluted 1:3,000, and the Amersham ECL (enhanced chemiluminescence) Western blotting system.

***N*-Acetylglucosaminyltransferase assay.** Crude membranes were prepared by disrupting cells with glass beads as described previously (8). Protein concentrations were determined by the method of Bradford (3). The basis for the *N*-acetylglucosaminyltransferase assays has been described previously (8). Assays with crude membranes from plasmid-bearing *S. carnosus* were performed with 5 mg of protein per ml incubated with 2 mM UDP-*N*-acetylglucosamine (10 μM ^{14}C labeled) and 4 μM dithiothreitol. For assays with crude membranes from *S. epidermidis* and *S. aureus* strains, conditions were modified to 10 μM UDP-*N*-acetylglucosamine (all ^{14}C labeled), 0.4 μM dithiothreitol, and a protein concentration of 1 mg/ml. Products were analyzed by thin-layer chromatography (NH_2-HPTLC plates; Merck, Darmstadt, Germany), 1 μl each, using acetonitrile-water (65:35, vol/vol). *N*-Acetylglucosamine (10 Bq) was used as a reference compound. Fuji HR-E30 film was exposed for 12 weeks.

DNA blots. Chromosomal DNA was prepared, with modifications, by the method of Marmur (25) as follows. Bacteria were grown to an optical density at 578 nm of 1 to 2 and washed in 5× Tris-EDTA. Cells were then resuspended in 100 μl of 5× Tris-EDTA, 50 μl of lysostaphin (0.5 mg/ml; Sigma), and 1 μl of RNase A (20 mg/ml; Boehringer) and incubated at 37°C until viscous. After cell lysis, 150 μl of 2% sodium dodecyl sulfate was added, the mixture incubated for 5 min at 37°C, and 50 μl of proteinase K (20 mg/ml) added for at last 30 min. After incubation for 5 min at room temperature with 150 μl of 5 M $NaClO_4$, the mixture was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) (Merck) and precipitated with an equal volume isopropanol. DNA digests and Southern blotting were performed by standard methods (32). Prehybridization and hybridization were performed with DIG (digoxigenin) Easy Hyb solution (Boehringer) at 60, 50, and 40°C. Washes were performed in 0.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate at 50°C. DIG-labeled probes were made by using PCR, DIG nucleotide labeling mix (Boehringer), and *Taq* polymerase (AGS, Heidelberg, Germany) as recommended by the manufacturers, using a MiniCycler PTC-150 (MJ Research, Inc., Watertown, Mass.).

Operon identification. The existence of an *ica* operon in *S. aureus* was postulated based on the ability to form an in vitro biofilm similar to that of *S. epidermidis*. A search of public nucleotide sequence data libraries using the *S. epidermidis ica* gene sequences (15) revealed no significant homologies to the *ica* locus in *S. aureus*. The genes were detected, however, with Pathoseq, *S. aureus* contig sequence information available from a commercial provider (Incyte Pharmaceuticals, Palo Alto, Calif.). The *S. aureus* clones showing homologies to *S. epidermidis icaA* to *icaC* were SAU1c0610, SAU1c0627, SAU1c0071, SAU1c0377, SAU1c0511, and SAU1c0012. Due to the sequence errors caused by single-read shotgun sequencing and gaps due to missing or incomplete contigs, it was necessary to subclone and resequence the *S. aureus ica* locus. The Pathoseq information was used to design PCR primer SA12 (see below) and some sequencing primers.

PCR amplification, cloning, and sequencing. Chromosomal DNA from *S. aureus* ATCC 35556 was amplified via PCR using primers SA11 and SA12 and the Expand Long Template PCR System (Boehringer) as recommended by the manufacturer and cloned into the *Kpn*I site of shuttle vector pBT5, a derivative of pBT2 lacking the *Eco*RI restriction site in the multiple cloning site (4), creating plasmid pSC18. Primers SA14 and SA15 were used to amplify plasmid pSC18, which deleted nucleotides 2132 to 5862 (mid-*icaR* through *icaC*). The tetracycline resistance cassette from pT181mcs (1) was then ligated by using *Xho*I restriction sites into the deleted region, creating plasmid pSC23. Cloning was performed in *Escherichia coli* DH5α. Portions of pSC18 and chromosomal DNA from *S. aureus* ATCC 35556 were sequenced by using a LI-COR DNA sequencer Long Readir 4200 (Lincoln Corporation, Inc., Lincoln, Neb.). Computer sequence analysis was performed with MacDNASIS Pro (Hitachi Software Engineering, San Bruno, Calif.). Enzymes used for cloning were purchased from Gibco BRL, Boehringer, or New England Biolabs GmbH (Schwalbach, Germa-

TABLE 2. *Staphylococcus* species used in this study and presence of *icaA*

Species	Hybridization	Stock center no.	Name
S. aureus	++++	ATCC 35556	SA113
S. auricularis	+++	ATCC 33753	
S. capitis capitis	+++	CCM 2734	
S. carnosus	−		TM300
S. caseolyticus	−		
S. cohnii cohnii	−	CCM 2736	
S. epidermidis	++++	ATCC 35984	
S. gallinarum	−		Tü3928/47
S. haemolyticus	−	CCM 2737	
S. hominis	−	DSM 20328	
S. hyicus hyicus	−	NCTC 10350	
S. intermedius	++	CCM 5739	
S. lentus	−		
S. lugdunensis	++	ATCC 43809	
S. pasteuri	+	ATCC 51129	
S. piscifermentans	++		SK02
S. saprophyticus	−		NT219
S. sciuri	−		SC116
S. schleiferi schleiferi	−	ATCC 43808	
S. simulans	−	ATCC 27848	
S. warneri	−		
S. xylosus	−		C2a

[a] Hybridization probes were specific to *icaA* from both *S. aureus* and *S. epidermidis*. ++++, signal with hybridization at 60°C; +++, signal with hybridization at 50°C; ++, signal with hybridization at 40°C; +, weak signal with hybridization at 40°C; −, no signal at 40, 50, or 60°C.

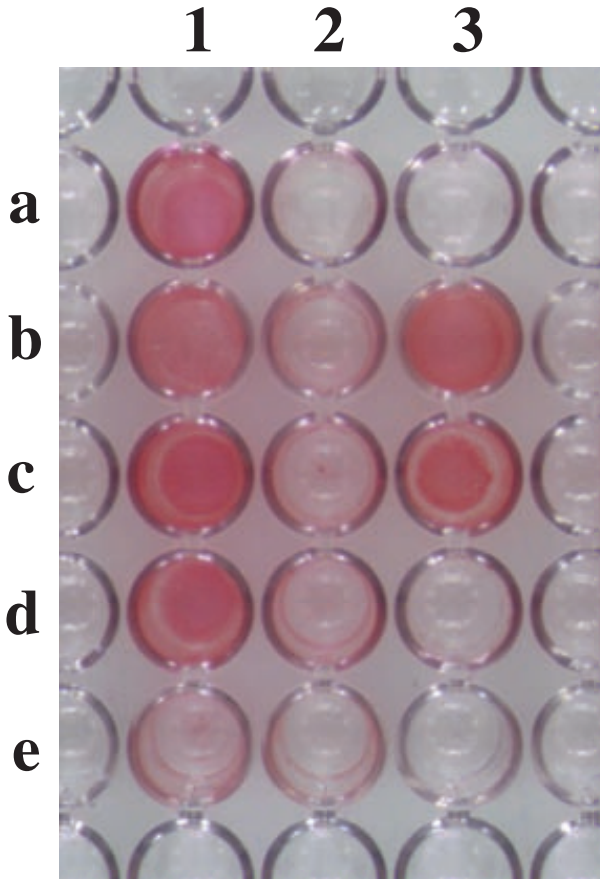

FIG. 1. Biofilm formation in *S. aureus* strains. The strains listed in Table 1 were grown overnight in polystyrol microtiter wells in TSB supplemented with 0.25% glucose. The cells that adhered to the plate after washing were then visualized by staining with safranin.

ny). Primers were obtained from MWG-Biotech (Ebersberg, Germany) or Interactiva (Ulm, Germany). The primers used for DNA amplification in *S. aureus* were SA11 (CGGGGTACCTGCAGGATGGTCATTATGAGTGC), SA12 (AGGGGTACCGAGCTCGCTAATAGGTGACTTTGG), SA14 (ATTTCTC GAGAAGGGGTATGACGGTACAAC), and SA15 (GTAAATGCTCGAGG GAGTGGGACAGAAA). The primers used to amplify the tetracycline resistance cassette from plasmid pT181mcs were tet-2 (GAGCTCGAGTGGCAAA ATGCTAGCCAC) and tet-6 (GCGCTCGAGTTCGCCAGCGATTAACGG A). The *Kpn*I restriction site at the beginning of the sequence indicated by a solid line in Fig. 2A is a cloning artifact introduced via the primer used for PCR amplification and is not found at this position on the chromosome. Plasmids were transformed into staphylococci via protoplast transformation (10, 11) or electroporation (21).

Homologous recombination. Wild-type *S. aureus* ATCC 35556 containing plasmid pSC23 was grown overnight in B medium at 30°C with chloramphenicol (10 μg/ml), diluted 1:1,000 and grown again at 30°C with antibiotic selection, diluted 1:1,000 and grown at 42°C without antibiotic selection twice, diluted 1:100, and plated on TSB plates containing tetracycline (2.5 μg/ml). Homologous recombination and plasmid curing of chloramphenicol-sensitive, tetracycline-resistant colonies were then confirmed by PCR and Southern blotting.

Nucleotide sequence accession number. The sequence indicated by a solid line in Fig. 2A has been submitted to the EMBL/GenBank/DDBJ nucleotide sequence data libraries under accession no. AF086783.

RESULTS

***S. aureus* forms biofilms in vitro and contains the *ica* locus.** Ten commonly studied *S. aureus* strains, listed in Table 1, were tested for the ability to form biofilms in polystyrol microtiter plates (Fig. 1). As is often seen with heterogeneous *S. epidermidis* strains, some strains were able to form a strong biofilm and others formed a weak or no biofilm. Two biofilm-forming *S. epidermidis* strains were included for comparison (Fig. 1, wells 3b and 3c), in addition to the non-biofilm-forming *S. carnosus* (well 3d).

All strains shown in Fig. 1 and listed in Table 1 exception those of *S. carnosus* contain *icaA*, *icaD*, *icaB*, and *icaC* as detected with individual gene probes on Southern blots (data not shown). As is commonly seen with *S. epidermidis* isolates, in vitro biofilm formation is fairly sensitive to growth conditions; for example, the addition of glucose or glucosamine to the media may be required even for "strong" biofilm-forming strains. Since several of the strains fail to form an in vitro biofilm despite the presence of the *ica* genes and growth conditions that allow biofilm formation in other strains, it is possible that these strains contain point mutations within the *ica* locus, that the production of PIA is otherwise negatively regulated, or that biofilm formation is influenced by some other, as yet unidentified factor(s). None of these strains contain insertional elements near the *ica* locus of a size that could be detected by PCR amplification using primers SA11 and SA12 (Fig. 2A) (data not shown) (39).

Sequence of the *ica* locus in *S. aureus* and sequence comparison with *S. epidermidis*. Sequence information available from a commercial provider (Incyte Pharmaceuticals) was used to design a PCR primer, and the *ica* locus from *S. aureus* ATCC 35556 was cloned and sequenced (Fig. 2A). The organization of the locus itself is identical to that of *S. epidermidis*; however, other than the presence of a lipase gene downstream and in the opposite orientation, the surrounding sequence shows no similarity. The predicted gene labeled *icaR*, located upstream and transcribed in the opposite direction from *icaA*, *icaD*, *icaB*, and *icaC* is also present in *S. epidermidis*, but its function is not yet known. A sequence similarity comparison between *S. aureus* ATCC 35556 and *S. epidermidis* ATCC 35984 is shown in Table 3.

Deletion of the *ica* locus in *S. aureus* eliminates biofilm formation and PIA production. To show that the *ica* locus in *S. aureus* is required for biofilm formation, a deletion mutant was constructed in biofilm-forming strain ATCC 35556 (Fig. 1, well 1a). The *ica* genes (*icaADBC* and the beginning of *icaR*) were replaced with the tetracycline resistance cassette from plasmid pT181 as diagrammed in Fig. 2B and described in more detail in Materials and Methods. This temperature-sensitive plasmid construct (pSC23) was used to replace the wild-type *ica* locus via homologous recombination, creating the knockout strain ATCC 35556Δ*ica*::tet.

The deletion mutant was then tested for the ability to form a biofilm in vitro. As shown in Fig. 3, the *ica* knockout mutant is not able to form a strong biofilm compared to the wild-type parent strain; however, when the strain is complemented with plasmid pSC18, carrying the wild-type *ica* locus (see Fig. 2A), biofilm formation is restored. This finding demonstrates that the *ica* locus is required for biofilm formation in *S. aureus*.

The same three strains, wild type, knockout, and complemented knockout, were then assayed for the ability to produce PIA, which is mediated by the *ica* locus. Figure 4 shows the production of PIA as detected with an antibody raised against *S. epidermidis* PIA (gift from D. Mack). Cell surface extracts were treated with proteinase K before spotting on a nitrocellulose filter to eliminate the cross-reaction of *S. aureus* protein A with the IgGs used for detection. The antibody was then able to recognize the remaining sugar polymer in the wild-type *S. aureus* ATCC 35556 (spot A1). The deletion mutant was no longer able to produce PIA, but PIA production was restored in the complemented mutant (spots A2 and A3, respectively). The faint signal remaining in the knockout (spot A2) may

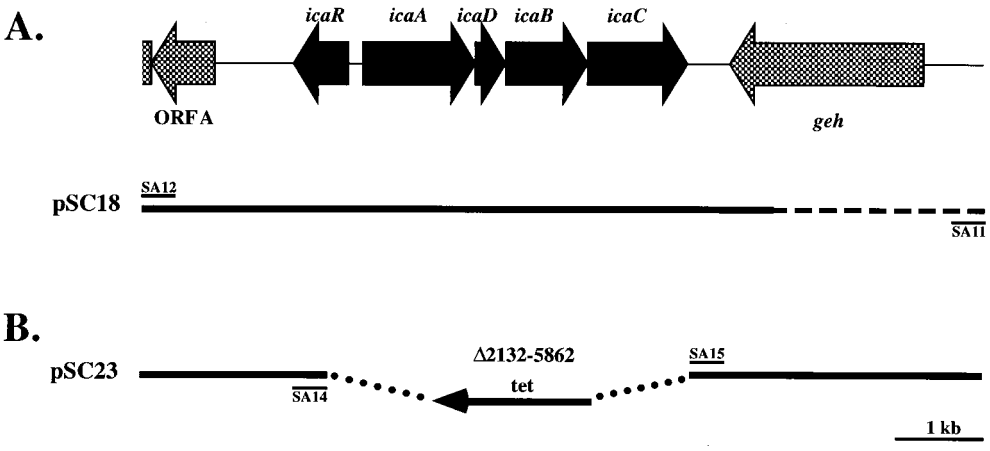

FIG. 2. Map of the *ica* locus and surrounding sequence in *S. aureus* ATCC 35556. (A) Genomic organization of the *ica* locus and surrounding chromosomal region. The region between primers SA12 and SA11 was amplified by PCR and cloned into vector pBT5, creating plasmid pSC18. The solid line indicates the region sequenced and submitted to the EMBL/GenBank/DDBJ nucleotide sequence data libraries; the dashed line indicates previously published sequence (database accession no. M90693). (B) Schematic of pSC23 diagramming the knockout construct. Plasmid pSC18 was amplified by inverse PCR and primers SA14 and SA15, which deleted the sequence between the middle of *icaR* and the end of *icaC*. The tetracycline resistance cassette from pT181 (tet) was then ligated into the deleted *ica* gene locus.

represent undigested protein A or another cross-reaction not affected by deletion of the *ica* locus. PIA-producing *S. epidermidis* ATCC 35984 (RP62A) and O-47 as well as the non-PIA-producing *S. carnosus* strain TM300 were included for comparison (spots B1, B2, and B3, respectively).

To further show that the *ica* genes in *S. aureus* mediate PIA production, we applied crude membrane extracts to an in vitro assay using UDP-*N*-acetylglucosamine as a substrate (8). Figure 5 shows in vitro-synthesized *N*-acetylglucosaminyltransferase products mediated by crude membrane extracts from *S. carnosus* containing a plasmid carrying the *S. epidermidis ica* genes, from the biofilm- and PIA-producing *S. epidermidis* strain ATCC 35984 (RP62A), and from the wild-type *S. aureus* strain ATCC 35556 (lanes 1, 3, and 5, respectively). It is significant that extracts from wild-type *S. epidermidis* and *S. aureus* strains are able to mediate detectable activity in vitro, as had previously been shown for the *S. epidermidis ica* genes on an inducible plasmid expressed in *S. carnosus* (8). The synthesis products were separated on an NH_2-HPTLC plate. Negative controls were crude membrane extracts from *S. carnosus* carrying the vector alone corresponding to the construct in lane 1 (lane 2) and from a non-biofilm- and non-PIA-producing *S. epidermidis* strain, 5179 (lane 4). The *S. aureus* knockout strain (lane 6) did not show *N*-acetylglucosaminyltransferase activity in vitro, demonstrating that the *ica* genes are required for the synthesis of these sugar oligomers in *S. aureus*.

The *ica* locus is also present in other *Staphylococcus* species. Since the sequence of the *ica* locus is well conserved between *S. epidermidis* and *S. aureus* (Table 3), we examined whether

TABLE 3. Sequence comparison of the *ica* locus between *S. aureus* and *S. epidermidis*

Locus[a]	Size of predicted polypeptide (amino acids)	DNA	Protein	
			% Identity	% Similarity
icaA				
S.a.	412	76	78	89
S.e.	412			
icaD				
S.a.	101	72	59	79
S.e.	101			
icaB				
S.a.	290	77	62	82
S.e.	289			
icaC				
S.a.	350	71	67	79
S.e.	355			
icaR				
S.a.	186	89	65	83
S.e.	185			

[a] *S.a., S. aureus* ATCC 35556 database sequence accession no. AF086783.
[b] *S.e., S. epidermidis* ATCC 35984 database sequence accession no. U43366.

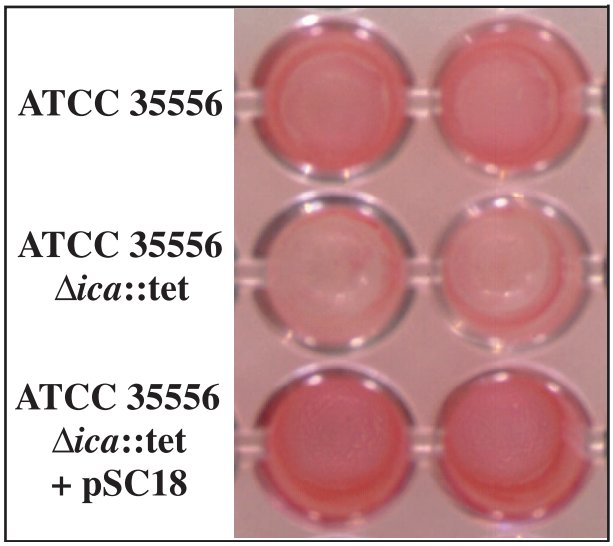

FIG. 3. Loss of biofilm formation in *S. aureus* ATCC 35556Δ*ica*::tet. The *ica* locus in *S. aureus* ATCC 35556 was deleted and replaced with a tetracycline resistance cassette by homologous recombination. The knockout strain, ATCC 35556Δ*ica*::tet, is unable to form a biofilm in vitro; however, the ability to form a biofilm is restored when the knockout strain is complemented with pSC18, carrying the wild type *ica* genes. The assay for each strain is shown in duplicate.

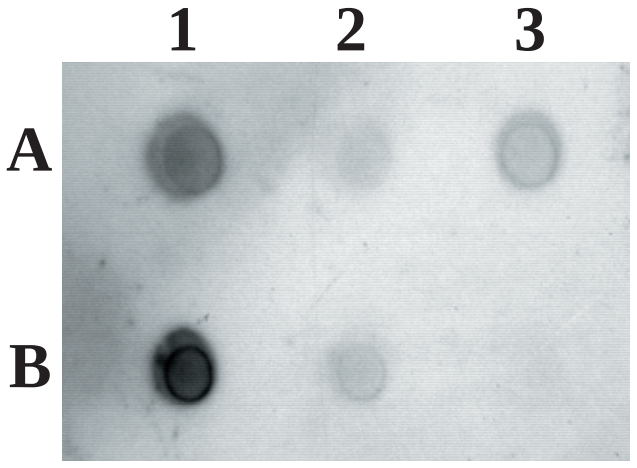

FIG. 4. Loss of PIA production in *S. aureus* ATCC 35556Δ*ica*::tet. Cell surface extracts from overnight cultures of *S. aureus* ATCC 35556 (A1), ATCC 35556Δ*ica*::tet (A2), ATCC 35556Δ*ica*::tet carrying pSC18 (A3), *S. epidermidis* ATCC 35984 (RP62A) (B1), *S. epidermidis* O-47 (B2), and *S. carnosus* TM300 (B3) were treated with proteinase K, and PIA production was detected with an anti-*S. epidermidis* PIA antibody, showing that PIA is no longer produced in the *ica* knockout strain and is restored in the complemented mutant.

other *Staphylococcus* species carry these genes. Curiously, cross-hybridization between *S. aureus* and *S. epidermidis* on DNA blots was weak (data not shown). Therefore, *icaA* DNA probes from both *S. epidermidis* and *S. aureus* were used simultaneously to hybridize cross-species Southern blots of decreasing stringency containing DNA from 22 different *Staphylococcus* species (Table 2). Cross-species hybridization was detected with *S. auricularis* and *S. capitis*, to a lesser extent with *S. intermedius*, *S. lugdunensis*, *S. piscifermentans*, and weakly with *S. pasteuri*. For those species that showed no cross-hybridization with these probes, one can conclude that an *icaA* homologue, however distantly related, is not detectable under these conditions, but one cannot go so far as to say that no homologue is present in the genome of these species. The result does show that the *ica* locus is conserved in some members of this genus, and it suggests that intercellular adhesion mediated by the *ica* locus may be a general phenomenon that is conserved among staphylococci.

In agreement with the presence of an *icaA* homolog, *S. auricularis* and *S. capitis* react weakly with the antibody raised against *S. epidermidis* PIA, as do *S. haemolyticus* and *S. saprophyticus*, though the latter two strains do not cross-hybridize with *icaA* DNA probes, and none of these species form a biofilm in vitro. Conversely, our *S. simulans* representative forms a very strong biofilm in vitro but does not produce a product that cross-reacts with our anti-PIA antibody. *S. gallinarum*, *S. lentus*, and *S. sciuri* were also able to form weak biofilms in vitro, but a PIA-like product was not detectable (data not shown).

DISCUSSION

Intercellular adhesion is conserved between *S. epidermidis* and *S. aureus*. We have shown that there is a functional conservation of intercellular adhesion mediated by the *ica* locus between *S. epidermidis* and *S. aureus*. Although nosocomial infections related to certain types of biomedical implants are commonly associated with *S. epidermidis*, *S. aureus* infections occur at a high frequency in association with other types of prosthetic devices and have more serious clinical consequences due to the expression of various virulence factors and the frequent presence of genes encoding antibiotic resistance. The prevalence of *S. epidermidis* and other coagulase-negative staphylococci in patients with some types of biomedical implant-associated infections may be due to (i) the organisms' increased proximity, and therefore access, to surgical incisions and/or (ii) transmission via skin contact between patients and/or hospital staff. *S. aureus*, in contrast to typical members of the human epidermal flora, resides predominantly in aural and nasal tissues, which are usually remote from surgical implant sites. It is likely that with better access, this species would be found even more frequently in association with all types of biomedical implant-related infections.

The ability to mediate intercellular adhesion and the formation of biofilms in both of these species is unlikely to have arisen recently in conjunction with the invention of prosthetic medical devices. Rather, it must have had a function much earlier in the evolution and survival of these organisms. The presence of the *icaA* gene in other *Staphylococcus* species also supports the notion that the locus has or had a more general function in the survival of this genus in a variety of environments.

Differences in the ability to form biofilms among related *S. aureus* strains. Strains ATCC 35556 (SA113) and RN4220 (Fig. 1, wells 1a and 2b, respectively) are both derivatives of *S. aureus* NCTC 8325 and, in effect, cousins. Strain NCTC 8325

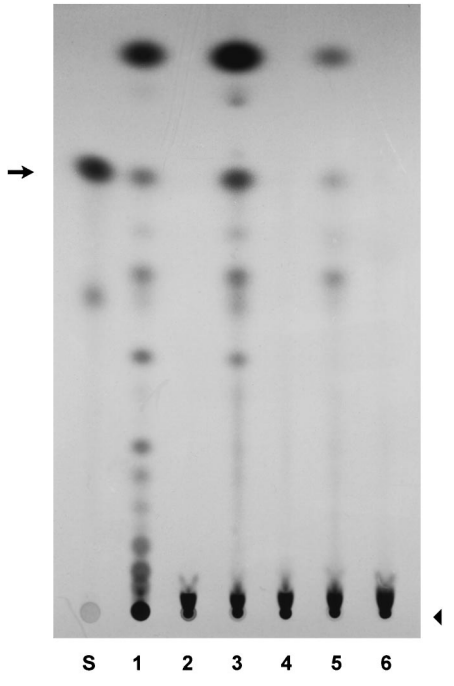

FIG. 5. *N*-Acetylglucosaminyltransferase activity is mediated by the *ica* locus. Crude membrane extracts were incubated with radiolabeled UDP-*N*-acetylglucosamine, and synthesized oligomers were separated on an NH_2-HPTLC plate. Lane S contains the standard, *N*-acetylglucosamine, alone. The remaining lanes contain products synthesized by crude membrane extracts from *S. carnosus* carrying pTX*icaADBC*, an inducible expression plasmid containing *S. epidermidis icaA*, *icaD*, *icaB*, and *icaC* (lane 1), *S. carnosus* harboring the vector, pTX16, alone (lane 2), *S. epidermidis* ATCC 35984 (RP62A), which produces PIA (lane 3), *S. epidermidis* 5179, a strain that does not produce PIA and does not form biofilms (lane 4), *S. aureus* wild-type strain ATCC 35556 (lane 5), and *S. aureus ica* knockout strain ATCC 35556Δ*ica*::tet (lane 6). An arrow indicates the monomer. Oligomers of increasing size are seen as a ladder-like series of spots that descend on the plate toward the origin (arrowhead) at the bottom. The smear just above the origin is unreacted UDP-*N*-acetylglucosamine.

underwent a chemical mutagenesis to produce restriction-negative strain SA113 (ATCC 35556) (17). Strain NCTC 8325 (RN1) was also treated twice with UV light to remove three prophages, producing strain 8325-4 (RN450) (28, 29). This strain was then subjected to a chemical mutagenesis, producing the restriction-negative strain RN4220 (20). While strain SA113 is able to form a strong biofilm, neither strain 8325-4 (not shown) nor strain RN4220 shows this phenotype. Instead, the two latter strains leave a thin layer of cells on the bottom of the microtiter plate well. This same phenotype is also seen in *S. epidermidis* transposon-induced *ica* mutants (13, 15). In each case, cells are able to adhere to the substrate surface, the genetically distinct first step in biofilm formation, but are not able to build a multilayered biofilm due to a defect in cell-cell adhesion. This implies that the multiple mutageneses that separate *S. aureus* SA113 and its cousins, 8325-4 and RN4220, have altered the regulation or expression, either directly or indirectly, of genes required for cell-cell adhesion, and therefore biofilm formation. While RN4220 leaves only a thin layer of cells on the bottom of the microtiter plate well in a biofilm assay (Fig. 1, well 2b), RN4220 carrying pSC18 is able to form a multilayered biofilm (data not shown). In addition, RN4220 carrying plasmid pCN27, containing *icaA* to *icaC* from *S. epidermidis*, was reported to form a strong biofilm (26). This implies that the expression or activity of the *ica* genes or gene products in *S. aureus* RN4220 may be less than for its cousin SA113, but that a plasmid carrying the *ica* region is able to rescue this phenotype. Similarly, the *ica* knockout mutant in *S. aureus* ATCC 35556 described here fails to form cell clusters when grown in culture, unlike the wild-type and complemented mutant strains. Cells from all three strains are able to attach to a polystyrene surface in a primary adhesion assay, however, supporting results for *S. epidermidis* showing that a mutation in the *ica* locus affects cell-cell adhesion but does not affect adhesion to a solid substrate (13).

As can be seen in Fig. 3 and 4, the complementation of the ATCC 35556 *ica* knockout strain is not complete. This phenomenon might be due to an as yet uncharacterized regulatory function in the region that is included on the complementation plasmid. A correlation can, however, be seen between the level of PIA production and the thickness of the biofilm formed (compare wild-type, knockout, and complemented knockout in Fig. 3 and 4).

Presence of the *ica* locus in other *Staphylococcus* species. The *Staphylococcus* species that cross-hybridized with *icaA* DNA probes from *S. aureus* and *S. epidermidis* were the species that are phylogenetically the most closely related. The so-called epidermidis phylogenetic group, based on DNA comparisons as well as some biochemical properties (19, 34), includes *S. auricularis* and *S. capitis*, both of which appear to carry a copy of the *icaA* gene. Other, more distantly related members of this group, *S. haemolyticus*, *S. hominis*, and *S. warneri*, failed to cross-hybridize under the conditions used. Coagulase-positive *S. intermedius* was so named to reflect a sequence composition that places it phylogenetically between *S. aureus* and *S. epidermidis* (27), and accordingly, this species was also able to cross-hybridize with *icaA* probes. The remaining three *icaA*-positive species, *S. lugdunensis*, *S. pasteuri* and *S. piscifermentans* have not been classified into any of the larger phylogenetic groupings based on sequence comparisons (19, 34, 36).

Tests on the 22 different *Staphylococcus* species to detect biofilm formation and PIA production were inconclusive. As seen with both *S. epidermidis* and *S. aureus* (e.g., Fig. 1), strain representatives within the same species behave very differently, and a single tested strain from each species is unlikely to be representative of the species as a whole. In addition, even if these strains produce PIA, there is no guarantee that the available antibody raised against *S. epidermidis* PIA can recognize sugar moieties that may be modified in a different manner, for example, deacetylated or succinated (26), in other, albeit related, species. In addition, nonspecific cross-reactions such as that seen between IgGs and protein A in *S. aureus* cannot be discounted. Those species that appear to carry no *icaA*-like gene yet are able to form a biofilm in vitro or produce a product that reacts with the anti-*S. epidermidis* PIA antibody may well have an entirely different and as yet unidentified mechanism mediating biofilm formation. Studies that include a larger number of representatives for each species are clearly required in order to show that functional intercellular adhesion, and not just the presence of the *ica* locus, occurs in *Staphylococcus* species other than *S. epidermidis* and *S. aureus*.

S. aureus and *S. epidermidis* are the gram-positive bacteria most often associated with medical implant-related infections. We have shown that both species mediate the cell-cell adhesion step of biofilm formation via the *ica* locus and that deletion of the *ica* genes eliminates the ability to produce PIA and form a biofilm in vitro. Due to the high level of morbidity and mortality associated with *S. aureus* infections, as well as the high frequency of infection by both organisms, the *ica* locus represents an important potential clinical target for the prevention of chronic infections associated with prosthetic medical devices.

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