

Essential Functional Role of the Polysaccharide Intercellular Adhesin of *Staphylococcus epidermidis* in Hemagglutination

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Hemagglutination of erythrocytes is a common property of *Staphylococcus epidermidis* strains, which is related to adherence and biofilm formation and may be essential for the pathogenesis of biomaterial-associated infections caused by *S. epidermidis*. In three independent biofilm-producing, hemagglutination-positive *S. epidermidis* isolates, interruption of the *icaADBC* operon essential for polysaccharide intercellular adhesin (PIA) synthesis by Tn917 insertions led to a hemagglutination-negative phenotype. An immunoglobulin G fraction of antiserum to PIA greatly reduced hemagglutination. Purified PIA led to a 64-fold decrease of hemagglutination titers of these strains; however, it did not mediate hemagglutination by itself. These observations define PIA as the hemagglutinin of *S. epidermidis* or at least as its major functional component.

In recent years, coagulase-negative staphylococci, mostly *Staphylococcus epidermidis*, have emerged as major nosocomial pathogens due to frequent infections associated with implanted medical devices and nosocomial sepsis (3, 11, 20). It is believed that the formation of adherent multilayered biofilms embedded into a glycocalyx composed of exopolysaccharides on implanted devices is essential for the pathogenesis of *S. epidermidis* infections.

Biofilm formation apparently proceeds in two phases (reviewed in reference 12). Most *S. epidermidis* strains are competent for primary attachment, which involves specific surface proteins or a capsular polysaccharide adhesin (PSA) (12). We recently described a polysaccharide intercellular adhesin (PIA), which plays an essential role in the second phase of *S. epidermidis* biofilm accumulation by mediating cell-to-cell adhesion and is expressed by the majority of biofilm-producing clinical *S. epidermidis* isolates (13–15). Two isogenic biofilm-negative Tn917 transposon insertion mutants, M10 and M11, derived from a biofilm-producing *S. epidermidis* strain, did not express detectable amounts of PIA (14). Structural analysis of the purified PIA revealed a linear polysaccharide composed of β -1,6-linked 2-deoxy-2-amino-D-glucopyranosyl residues (16). Of these, 80 to 85% are *N*-acetylated, whereas the rest are non-*N*-acetylated and positively charged. A minor component of PIA in addition contained negative charges introduced by modification with phosphate and succinate groups (16). The *icaADBC* gene locus of *S. epidermidis* contains genes essential for PIA synthesis (5, 9). An accumulation-associated protein was described; however, its function in biofilm accumulation is not yet known (10).

Recently, the ability of *S. epidermidis* to mediate hemagglutination of erythrocytes of different species was shown to be associated with the ability to adhere to plastic and to produce

biofilm and therefore may be important for the pathogenesis of *S. epidermidis* infections (19, 21). The hemagglutinating activity could be extracted from the bacterial cells, and preliminary characterization revealed that the hemagglutinin was a polysaccharide of yet undefined nature (21). As there was a striking similarity in the quantitative relation of the amounts of biofilm formed by individual strains and the hemagglutination titers and the amounts of PIA produced, the functional relation between PIA and the hemagglutinin of *S. epidermidis* was investigated (13, 19, 21).

(Part of this work will appear in the doctoral theses of J.R., H.R., and T.M., Universitätskrankenhaus Eppendorf, Hamburg, Germany.)

Biofilm-producing *S. epidermidis* 1457, 9142, 8400, RP62A, and SE-5 and biofilm-negative *S. epidermidis* 5179 (15, 19) as well as isogenic biofilm-negative Tn917 transposon insertion mutants M10 and M11 (14), which are impaired in the accumulative phase of biofilm production due to abolished intercellular adhesion caused by failure of synthesis of PIA, and isogenic biofilm-negative transductants 9142-M10, 9142-M11, and 1457-M11 (14) have been described. *Staphylococcus carnosus* TM300 (7), kindly provided by Friedrich Götz, University of Tübingen, Tübingen, Germany, and *Escherichia coli* DH5 α (4) were used as hosts in molecular cloning experiments.

Phage transduction by using *S. epidermidis* phage 48, kindly provided by V. T. Rosdahl, Statens Seruminstitut, Copenhagen, Denmark, was performed as described previously (14, 18). Biofilm production by *S. epidermidis* strains grown in Trypticase soy broth (TSB) (Becton Dickinson, Cockeysville, Md.) was determined with a semiquantitative adherence assay by using 96-well tissue culture plates (Nunc, Roskilde, Denmark) (2, 15). Bacterial extracts of *S. epidermidis* strains grown in TSB on plastic tissue culture plates were prepared by sonication (15). Concentration of PIA in bacterial extracts was determined by a specific coagglutination assay (13, 15, 16).

Pulsed-field gel electrophoresis (PFGE) was performed essentially as described (14, 23). Chromosomal and plasmid DNA was isolated and digested with restriction enzymes (Phar-

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macia, Freiburg, Germany) followed by Southern analysis with [³²P]dCTP-labeled plasmid pTV1ts as described previously (14).

Tn917-containing *Eco*RI-fragments of M10 and M11 were cloned directly in *S. carnosus* by protoplast transformation by using pT181mcs as a vector and selecting for erythromycin-resistant transformants (erythromycin concentration, 10 µg/ml) (7, 8). Cloned DNA fragments were subcloned by using pBluescript II SK (Stratagene, La Jolla, Calif.) as a vector in *E. coli* DH5α. Sequences of the transposon insertion sites of M10 and M11 were obtained by using oligonucleotides 5'-GGC CTT GAA ACA TTG GTT TAG TGG G-3' and 5'-CTC ACA ATA GAG AGA TGT CAC CG-3', which are complementary to the 5' and 3' junctions of Tn917 (24), with the Sequenase version 2.0 kit (United States Biochemical, Cleveland, Ohio) (4).

Antisera were raised in rabbits against whole cells of the biofilm-negative, PIA-negative *S. epidermidis* 5179 (anti-5179) and the biofilm-producing, PIA-positive *S. epidermidis* 1457 (anti-1457) grown in TSB on tissue culture plates by serial intravenous injection of formalin-fixed cells (15). In addition, an antiserum which was raised against purified PIA was used (9). Normal rabbit serum was used as a control (Gibco BRL, Eggenstein, Germany). Rabbit immunoglobulin G (IgG) fractions were prepared from the sera by affinity chromatography on a protein A-Sepharose CL-4B column (Pharmacia, Uppsala, Sweden) (6). Protein concentrations were determined by the method of Bradford (1), and IgG preparations were stored at -20°C until used.

Antistaphylococcal antibodies in the respective IgG fractions were titrated by analysis of serial dilutions by an immunofluorescence assay with the biofilm-producing, PIA-positive *S. epidermidis* 1457 and the biofilm-negative, PIA-negative *S. epidermidis* 1457-M11 as antigens (15). In addition, the contents of anti-PIA antibodies in the antisera were determined by a coagglutination assay (15) prepared with *Staphylococcus aureus* Cowan I and the corresponding antisera by using a preparation of purified PIA as antigen.

PIA was purified from *S. epidermidis* 1457 by Q-Sepharose anion exchange chromatography as described, and the major polysaccharide fraction I was used (16). The hexosamine concentration of the purified polysaccharide was determined by colorimetric assay (16).

To assess hemagglutination bacteria were grown in TSB in plastic tissue culture dishes for 22 h at 37°C (15). The medium was aspirated, and the cells were scraped from the surface into 12 ml of phosphate-buffered saline. After passage through a 23-gauge needle the bacterial suspension was adjusted to an optical density at 578 nm (OD₅₇₈) of 1.0. The hemagglutination assay was performed with 96-well (U-shaped) microtiter plates (Greiner, Nürtingen, Germany) by using sheep erythrocytes (Sigma, Deisenhofen, Germany) essentially as described (19). For assessment of the effects of different rabbit IgG fractions on hemagglutination titers, IgG was added to the bacteria in each well of the microtiter plates at a final concentration of 75 µg/ml. After 1 h at room temperature, erythrocytes were added and hemagglutination was assessed as described above. Bacteria incubated in phosphate-buffered saline without added IgG served as a control. To assess the effect of purified PIA on hemagglutination, PIA was added to the bacteria at a concentration of 9 µg/ml of hexosamine. Hemagglutination titers were evaluated as described above. Bacteria incubated in parallel in phosphate-buffered saline without added PIA served as a control.

Hemagglutination of isogenic biofilm-negative *S. epidermidis* transposon insertion mutants. Three biofilm-producing *S. epi-*

TABLE 1. Hemagglutination and PIA production of transductants with transposon insertions of mutants M10 and M11 in different wild-type strains

Strain	(OD ₅₇₀) ^a	PIA ^b (reciprocal titer)	Hemagglutination ^c (reciprocal titer)
1457	2.469	1024	128
1457-M10	0.044	0	0
1457-M11	0.045	0	0
9142	1.836	512	64
9142-M10	0.014	0	0
9142-M11	0.020	0	0
8400	2.080	1024	64
8400-M10	0.056	0	0
8400-M11	0.054	0	0
SE-5	2.224	1024	128
RP62A	1.798	1024	256

^a Strains were grown in TSB in 96-well tissue culture plates, the plates were washed with phosphate-buffered saline, cells were fixed with Bouin's fixative, and adherent bacterial biofilms were stained with gentian violet. Biofilm-producing strains were arbitrarily assigned to have a mean OD₅₇₀ greater than 0.1.

^b PIA expression was determined in extracts of bacteria grown in TSB by coagglutination. A coagglutination reagent specific for PIA was prepared by sensitizing *S. aureus* Cowan I with PIA-specific absorbed antiserum.

^c Hemagglutination was assessed as described in Materials and Methods.

dermidis clinical isolates, 1457, 9142, and 8400, were hemagglutination positive with titers of 1:64 to 1:128 (Table 1). The different banding patterns obtained after *Sma*I digestion in PFGE indicated that these strains were genetically independent clones (data not shown). In addition to the already-obtained transductants 9142-M10, 9142-M11, and 1457-M11 (14), the transposon insertions of the isogenic biofilm-negative transposon mutants M10 and M11 were transferred into *S. epidermidis* 1457 and 8400, respectively. The fragment patterns of these transductants obtained in PFGE were identical to the patterns of the corresponding wild-type strains (data not shown). Southern hybridization by using radioactively labeled plasmid pTV1ts as the probe proved that the Tn917 insertions of mutants M10 and M11 were integrated into a fragment of approximately 60 kb in the respective transductants (data not shown). In contrast to the different biofilm-producing wild-type strains, all transductants exhibited a completely biofilm-negative phenotype, and in bacterial extracts of the transductants, PIA could not be detected at all (Table 1). Comparison in the hemagglutination assay revealed that all biofilm-negative transductants were completely hemagglutination negative (Table 1; see also Fig. 3).

Characterization of Tn917 insertion sites of mutants M10 and M11. To investigate which genes are inactivated by the Tn917 insertions of M10 and M11, chromosomal *Eco*RI fragments of 6.8 and 5.8 kb, respectively, containing insertions of Tn917 in mutants M10 and M11, were cloned in *S. carnosus*. When the cloned DNA fragments of mutants M10 and M11 were used as probes, hybridization with 7.6-kb and 4.3-kb *Xba*I fragments and with 2.2-kb and 0.8-kb *Eco*RI fragments, respectively, was observed with chromosomal DNA of biofilm-producing *S. epidermidis* 9142 (data not shown). In contrast, both probes hybridized with a *Hind*III fragment of identical size (13 kb). Using the cloned probes and two *Ava*I fragments corresponding to the 5' (*erm*) and the 3' junctions of Tn917 (24), mapping of the transposon insertions was performed with restriction enzymes *Xba*I, *Hind*III, and *Bgl*III (Fig. 1). Nucleotide

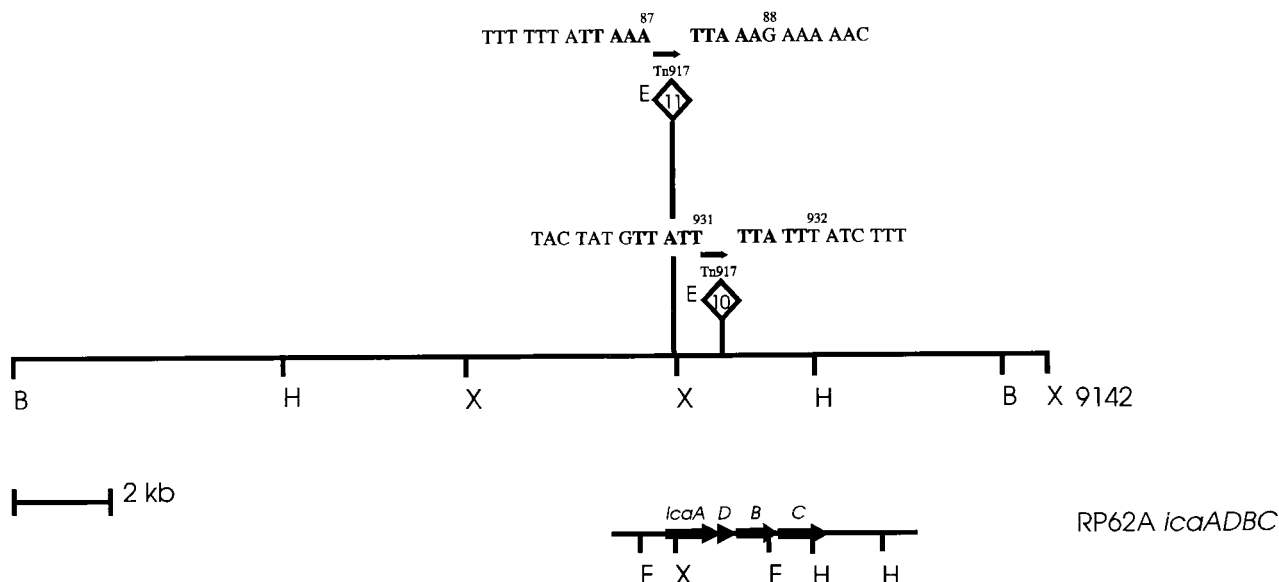


FIG. 1. Mapping of transposon insertions of biofilm-negative mutants M10 and M11. The flanking DNA sequences of Tn917 are indicated; transpositions at nucleotides 931 and 87 of the *icaA* gene were accompanied by 5-bp duplications, indicated in bold letters. E, erythromycin resistance gene *erm*; arrow, direction of transcription of *erm*. Restriction sites: B, *Bgl*II; H, *Hind*III; X, *Xba*I; and E, *Eco*RI. The region containing the transposon insertions in *S. epidermidis* 9142 is aligned to the homologous *icaADBC* operon of *S. epidermidis* RP62A (5, 9).

sequence analysis revealed that mutants M10 and M11 had their Tn917 insertions at nucleotides 931 and 87, respectively, of the coding sequence of the *icaA* gene of *icaADBC* (Fig. 1) (5, 9).

Inhibition of hemagglutination by PIA-specific antibodies.

To investigate whether PIA is specifically involved in hemagglutination of *S. epidermidis*, inhibition of hemagglutination by PIA-specific antibodies was evaluated. Therefore, IgG was prepared from several different antisera. An antiserum raised against purified PIA which specifically reacted in an immunofluorescence assay only with the PIA-positive *S. epidermidis* 1457 and not with the isogenic PIA-negative *S. epidermidis* 1457-M11 was used (Table 2). Anti-5179 reacted with staphylococcal cells irrespective of PIA production and did not contain any PIA-specific antibody (Table 2). Anti-1457 reacted with staphylococcal cells and in addition contained specific anti-PIA antibodies (Table 2). Normal rabbit serum, which did not contain detectable amounts of antistaphylococcal antibodies, was used as a control (Table 2).

With *S. epidermidis* 1457 only marginal differences in hem-

agglutination titers were observed with normal rabbit serum and anti-5179 antiserum as compared to controls incubated in phosphate-buffered saline (Fig. 2). In contrast, a 32-fold-lower hemagglutination titer was observed with the specific anti-PIA antiserum (Fig. 2). Similarly, the anti-1457 antiserum significantly inhibited hemagglutination (Fig. 2). Similar results were

TABLE 2. Antibody titers of different IgG preparations used

Serum	Antigen used in immunofluorescence assay		Coagglutination with purified PIA (titer)
	1457 (PIA positive) (titer)	1457-M11 (PIA negative) (titer)	
Anti-PIA	1:128	<1:32	1:1024
Normal rabbit serum	<1:32	<1:32	Negative
Anti-5179 (PIA-negative)	1:512	1:512	Negative ^a
Anti-1457 (PIA-positive)	1:1024	1:1024	1:256

^a As a coagglutination reagent prepared with anti-5179 displayed autoagglutination, probably by cross-reaction with *S. aureus*, the antiserum was diluted 1:50 in phosphate-buffered saline and absorbed with an overnight culture of the biofilm-negative, PIA-negative *S. epidermidis* 5179 before preparation of the coagglutination reagent. A coagglutination reagent prepared with anti-PIA serum absorbed in parallel displayed a titer of 1:512.

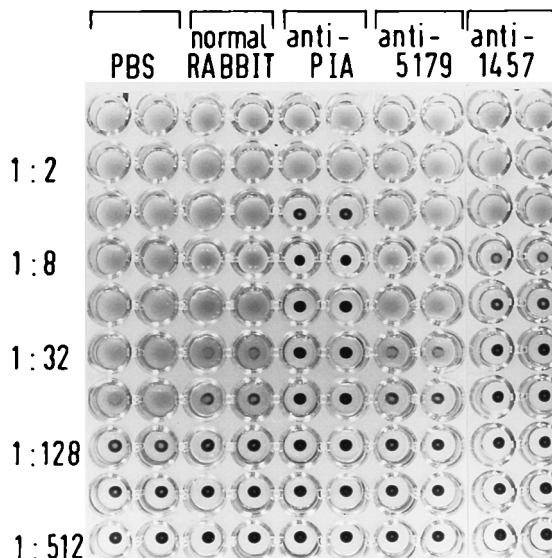


FIG. 2. Inhibition of hemagglutination by different IgG preparations. Serial dilutions of biofilm-producing PIA-positive *S. epidermidis* 1457 were applied to each well of a microtiter plate. Each well contained (at concentrations of 75 µg/ml) the IgG preparation of normal rabbit serum (normal RABBIT), antiserum raised against purified PIA (anti-PIA), antiserum raised against whole cells of the biofilm-negative, PIA-negative *S. epidermidis* 5179 (anti-5179), and antiserum raised against whole cells of the biofilm-producing, PIA-positive *S. epidermidis* 1457 (anti-1457). Control wells contained no IgG and only phosphate-buffered saline (PBS). The hemagglutination titers were assessed as described in Materials and Methods.

TABLE 3. Inhibition of *S. epidermidis* hemagglutination by IgG specific for the PIA

Strain	Hemagglutination (reciprocal titers)				
	Phosphate-buffered saline	Normal IgG	Anti-PIA	Anti-5179	Anti-1457
9142	32	16	1	16	4
8400	64	32	2	32	4
SE-5	128	64	2	64	4
RP62A	256	128	4	128	16

obtained with the biofilm-producing and hemagglutination-positive *S. epidermidis* 9142, 8400, RP62A, and SE-5 (Table 3). Therefore, specific antibody against PIA inhibits hemagglutination of *S. epidermidis*.

Inhibition of hemagglutination by purified PIA. Attempts to reconstitute hemagglutination of erythrocytes by purified PIA were not successful at concentrations up to 18 $\mu\text{g/ml}$ (data not shown). In addition, purified PIA did not lead to hemagglutination of the biofilm-negative, hemagglutination-negative *S. epidermidis* 1457-M11 (Fig. 3). In contrast, purified PIA, at a concentration of 9 $\mu\text{g/ml}$, decreased the hemagglutination titer of *S. epidermidis* 1457 64-fold (Fig. 3). Similar inhibition of hemagglutination by purified PIA was observed with *S. epidermidis* 9142, 8400, RP62A, and SE-5 (Table 4). Apparently, purified PIA significantly interferes with hemagglutination of *S. epidermidis*.

Interruption of the *icaADBC* operon at nucleotides 931 and 87 of the *icaA* gene in mutants M10 and M11 led to a biofilm-negative and hemagglutination-negative phenotype together with complete abolition of PIA synthesis after transfer of the corresponding Tn917 insertions in three independent *S. epi-*

TABLE 4. Inhibition of *S. epidermidis* hemagglutination by purified PIA

Strain	Hemagglutination (reciprocal titer)	
	Phosphate-buffered saline	PIA
9142	64	1
8400	64	2
SE-5	128	2
RP62A	256	4

dermidis wild-type strains. M10 and M11 are the only biofilm-negative *S. epidermidis* transposon insertion mutants yet described that contain Tn917 insertions within the coding sequence of biosynthetic genes responsible for the synthesis of PIA (5, 9). In contrast, the mutant Mut2 described by Heilmann et al. (8, 9) has a Tn917 insertion 72 bp proximal of the ATG start codon of the *icaA* gene, whereas the insertion site of Mut2a is nearly 1 kb proximal to the *icaA* translational start site, which probably leads to polar inhibition of the expression of PIA synthetic genes.

The suggested specific function of PIA in hemagglutination was corroborated by analysis of the effects of anti-PIA antibodies on hemagglutination. Only antibodies specific for PIA significantly inhibited hemagglutination of several different *S. epidermidis* strains (Fig. 2; Table 3). This inhibition of hemagglutination is specifically caused by interaction of antibody with PIA and not merely by steric hindrance by binding of antibody to the bacterial cell surface leading to interference with a still undefined hemagglutinin, because anti-5179, which contains high titers of antistaphylococcal antibodies but no anti-PIA antibodies, only marginally inhibited hemagglutination, similarly to normal rabbit serum. To further ascertain the specific function of PIA in hemagglutination of *S. epidermidis*, we investigated the effect of the purified PIA on hemagglutination. Purified PIA decreased the hemagglutination titers of five independent hemagglutination-positive *S. epidermidis* strains. The amount of PIA used corresponds to an approximately 10- to 100-fold excess of exogenously added purified PIA compared to the amount of PIA produced by the *S. epidermidis* cells used in the hemagglutination assay. This indicates that purified PIA as used in our study competitively inhibits hemagglutination.

Published evidence regarding the properties of the crude hemagglutinin extracted from *S. epidermidis*, including its sensitivity to periodate oxidation and degradation by glycosidases containing β -*N*-acetylglucosaminidase activity, molecular size as determined by ultrafiltration, and dependence of expression on the glucose concentration of the growth medium, is consistent with the idea that PIA is the active compound (15, 19, 21). In addition, the identity of PIA with the hemagglutinin clearly explains the similar quantitative relation of the amounts of biofilm formed by individual strains and the hemagglutination titers and the amounts of PIA produced by different clinical *S. epidermidis* isolates (13, 19, 21).

In the present study we could not reconstitute hemagglutination using purified PIA. Several reasons could account for that observation. First, extraction of PIA from *S. epidermidis* by sonication may alter the configuration of the polysaccharide chains, rendering them inactive as a hemagglutinin but still functional as a competitive inhibitor. Second, in hemagglutination inhibition experiments, we used polysaccharide I of PIA, which contains exclusively positive charges due to non-*N*-acetylated glucosamine residues in the polysaccharide chain (16). Polysaccharide II, which is a minor component of PIA

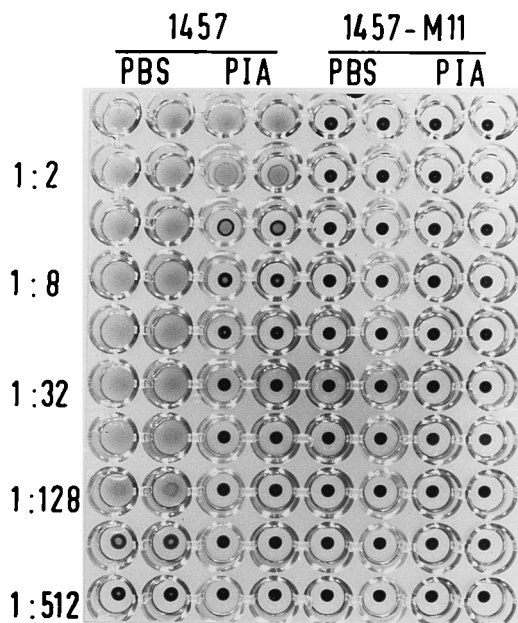


FIG. 3. Inhibition of hemagglutination by purified PIA. Serial dilutions of the biofilm-producing, PIA-positive *S. epidermidis* 1457 and the isogenic biofilm-negative PIA-negative transductant 1457-M11 were applied to the wells of a microtiter plate. To each well purified PIA was added at a concentration of 9 $\mu\text{g/ml}$ (PIA). Control wells did not contain PIA and contained only phosphate-buffered saline (PBS). The hemagglutination titers were assessed as described in Materials and Methods.

accounting for approximately 15% of the polysaccharide, contains negative charges introduced by modification of the polysaccharide by phosphate and succinate residues (16). The mechanism of hemagglutination mediated by PIA could be related to synergistic interactions of the differentially charged polysaccharide species with the negatively charged surface of the erythrocyte. In contrast, as purified PIA inhibits hemagglutination, PIA might interact with a putative specific receptor for the hemagglutinin on the erythrocyte surface. At present we cannot completely exclude the possibility that a component in addition to PIA is necessary for the functional activity of the hemagglutinin of *S. epidermidis*. However, it seems highly unlikely that polysaccharides completely unrelated to PIA are involved in hemagglutination, as the *icaADBC* operon contains only a single gene homologous to a glycosyltransferase and no additional synthetic genes related to sugar precursor biosynthesis (5, 9) whose interruption could lead to impaired synthesis of a polysaccharide different from PIA. It is interesting that in a recent report it was proposed that PSA, which in its purified form was reported to contain 54% hexoses, 20% amino sugars, and 10% uronic acids and as specific sugars 22% galactose, 15% glucosamine, and 5% galactosamine (25), is in fact an acidic polysaccharide that is very similar to PIA and composed exclusively from β -1,6-linked glucosamine residues containing succinate and acetate and that its production is dependent on the *icaADBC* locus (17). As detailed data for the structural analysis of this potential variant of PIA have not yet been reported, differentiation of this polysaccharide from polysaccharide II of PIA awaits further studies.

Taken together, the observations that *S. epidermidis* mutants that are impaired in PIA synthesis by a defined genetic manipulation are hemagglutination negative and that specific anti-PIA antibodies and purified PIA inhibit hemagglutination define PIA as the hemagglutinin of *S. epidermidis* or at least as its major functional component. As these results were corroborated by results obtained with several independent clinical *S. epidermidis* isolates, including the reference strains RP62A and SE-5, it seems reasonable to generalize this conclusion to other hemagglutination-positive *S. epidermidis* strains.

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