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The Intercellular Adhesin Involved in Biofilm Accumulation of *Staphylococcus epidermidis* Is a Linear β-1,6-Linked Glucosaminoglycan: Purification and Structural Analysis

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The primary attachment to polymer surfaces followed by accumulation in multilayered cell clusters leads to biofilm production of *Staphylococcus epidermidis*, which is thought to contribute to virulence in biomaterial-related infections. We purified a specific polysaccharide antigen of biofilm-producing *S. epidermidis* 1457 and RP62A, which was recently shown to have a function in the accumulative phase of biofilm production by mediating intercellular adhesion (D. Mack, M. Nedelmann, A. Krokotsch, A. Schwarzkopf, J. Heesemann, and R. Laufs, Infect. Immun. 62:3244–3253, 1994). Following Sephadex G-200 gel filtration, this antigen was separated by Q-Sepharose chromatography into a major polysaccharide, polysaccharide I (>80%), which did not bind to Q-Sepharose, and a minor polysaccharide, polysaccharide II (<20%), which was moderately anionic. As shown by chemical analyses and nuclear magnetic resonance spectroscopy, polysaccharide I is a linear homoglycan of at least 130 β-1,6-linked 2-deoxy-2-amino-D-glucopyranosyl residues. On average, 80 to 85% of them are N acetylated; the rest are non-N-acetylating and positively charged. Chain cleavage by deamination with HNO_2 revealed a more or less random distribution of the non-N-acetylated glucosaminyl residues, with some prevalence of glucosaminyl-rich sequences. Cation-exchange chromatography separated molecular species whose content of non-N-acetylated glucosaminyl residues varied between 2 and 26%. Polysaccharide II is structurally related to polysaccharide I but has a lower content of non-N-acetylated D-glucosaminyl residues and contains phosphate and ester-linked succinate, rendering it anionic. Enzyme-linked immunosorbent assay inhibition with various monosaccharides revealed the β-anomeric form and the acetylated amino group of the D-glucosaminyl residues as important for reactivity with the specific antiserum. The unbranched polysaccharide structure favors long-range contacts and interactions between polysaccharide strands and the cell wall and/or lectin-like proteins, leading to intercellular adhesion and biofilm accumulation. The structure of the polysaccharide is, so far, considered to be unique and, according to its function, is referred to as *S. epidermidis* polysaccharide intercellular adhesin (PIA).

At present, coagulase-negative staphylococci, mostly *Staphylococcus epidermidis*, represent the most frequent cause by far of nosocomial sepsis and are the most prominent organisms responsible for infections associated with implanted biomaterials like intravascular catheters, peritoneal dialysis catheters, cerebrospinal fluid shunts, prosthetic heart valves, and prosthetic joints, resulting in substantial morbidity and mortality (25, 37, 54, 56).

By scanning electron microscopy, coagulase-negative staphylococci were shown to colonize intravascular catheters in large adherent biofilms composed of multilayered cell clusters embedded in an amorphous extracellular material, which is composed of exopolysaccharides referred to as slime or glycocalyx (13, 16, 28, 45, 51). In vitro, a proportion of coagulase-negative staphylococcal strains are able to produce a macroscopically visible, adherent biofilm on test tubes or tissue culture plates with a morphology in scanning electron micrographs very similar to that of infected intravascular catheters (13, 14, 34, 58). We refer to these strains as biofilm producers, in contrast to biofilm-negative strains (42). The importance of biofilm production for virulence of *S. epidermidis* was supported by several clinical and animal studies (10, 11, 13, 18–20, 24, 35, 50, 69).

Two phases during biofilm production can be kinetically differentiated: rapid primary attachment of *S. epidermidis* cells to the polymer surface is later followed by accumulation of cells in multilayered cell clusters and glycocalyx production (52). A variety of different mechanisms and factors which can contribute to primary attachment of *S. epidermidis* cells to polymer surfaces, including nonspecific van der Waals and hydrophobic interactions (16), specific binding mediated by a capsular polysaccharide adhesin (PS/A [62]) or a 220-kDa cell wall-associated protein (61), specific interaction with plasma proteins adsorbed to the polymer surface (31, 57, 65), binding to activated platelets on the surface (67), and lipoteichoic acid-mediated binding to fibrin-platelet clots (14a), have been described.

However, in addition to the competence for primary attachment to polymer surfaces, which is inherent in most *S. epidermidis* strains (26, 32, 49, 62), it is essential for the *S. epidermidis* cells to express intercellular adhesion to accumulate in an adherent multilayered biofilm, as the majority of *S. epidermidis* cells in the biofilm do not have any direct contact to the polymer surface. We recently described a polysaccharide antigen specific for biofilm-producing *S. epidermidis* strains associated with cell clusters (43, 44). By analysis of isogenic biofilm-

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negative transposon mutants of a biofilm-producing *S. epidermidis* strain, which were impaired in the accumulative phase of biofilm production and were defective in the production of the specific antigen, we obtained direct genetic evidence for a function of this antigen in the accumulative phase of biofilm production by mediating intercellular adhesion (42).

In the present study, we have purified the specific antigen of the biofilm-producing *S. epidermidis* 1457 and RP62A and determined that the structure is, to date, a unique linear glucosaminoglycan. These results will allow the process of intercellular adhesion and biofilm accumulation to be studied at the molecular level. In accordance with its function, we refer to the specific antigen of biofilm-producing *S. epidermidis* as the polysaccharide intercellular adhesin (PIA).

(Part of this work will appear in the doctoral thesis of Andreas Krokotsch, Universitätskrankenhaus Eppendorf, Hamburg, Germany.)

MATERIALS AND METHODS

Materials. Rotiszint Eco Plus was purchased from Roth, Karlsruhe, Germany. A LiChrosphere RP 18e high-performance liquid chromatography (HPLC) column (4 by 250 mm) was from Merck, Darmstadt, Germany, and a TSK-Gel G-Oligo-PW HPLC column (7.8 by 300 mm) and an Oligo guard column (6 by 40 mm) were from Toso Haas GmbH, Stuttgart, Germany. NaB^3H_4 (13.3 GBq/mmol) was obtained from NEN, Du Pont de Nemours, Dreieich, Germany. Dextran 39200 Da, Dextran 9300 Da, and maltohexaose were from Sigma Chemie, Deisenhofen, Germany.

Bacteria. Biofilm-producing *S. epidermidis* 1457, 9142, and RP62A and the biofilm-negative *S. epidermidis* 5179 and 9896 have been described previously (43). *S. epidermidis* 1457-M11 is an isogenic biofilm-negative transductant of biofilm-producing *S. epidermidis* 1457 obtained by transfer of the Tn*917* transposon insertion, leading to the biofilm-negative phenotype of transposon mutant M11 of biofilm-producing *S. epidermidis* 13-1 by phage transduction (42). 1457-M11 expressed a biofilm-negative phenotype and did not produce cell clusters, and production of the specific antigen of biofilm-producing *S. epidermidis* was completely abolished as described previously (42).

Preparation and purification of specific antigen. The specific antigen of *S. epidermidis* 1457 and RP62A was prepared as described previously (42). Briefly, *S. epidermidis* strains were grown for 22 h at 37°C with shaking at 100 rpm/min in 900 ml of dialyzed Trypticase soy broth prepared by dialysis of 100 ml of 10-fold-concentrated TSB (Becton Dickinson, Cockeysville, Md.) against 900 ml of water. Bacterial cells were collected by centrifugation and were suspended in 20 ml of phosphate-buffered saline. The antigen was extracted by sonication. Cells were removed by centrifugation at 6,000 rpm in a Beckman JA 17 rotor at 4°C, and extracts were clarified by centrifuging for 60 min at 12,000 rpm in the same rotor. The extracts were filter sterilized, dialyzed against 2 liters of phosphate-buffered saline or 50 mM Tris-HCl (pH 7.5) overnight, depending on which type of column was chosen for fractionation, and concentrated with Centriprep 10 (Amicon, Witten, Germany).

The polysaccharide in phosphate-buffered saline was fractionated by gel filtration on a Sephadex G-200 column (Pharmacia LKB GmbH, Freiburg, Germany) equilibrated with phosphate-buffered saline as previously described (42). The specific antigen was detected by a specific coagglutination assay, and antigen-containing fractions were concentrated with Centricon 10. Concentrated antigen preparations were dialyzed against 2 liters of 50 mM Tris-HCl, pH 7.5, and fractionated on a Q-Sepharose column (Pharmacia) (1.6 by 11 cm) equilibrated with the same buffer. The column was first washed with 40 ml of 50 mM Tris-HCl, pH 7.5, followed by a 200-ml gradient from 0 to 1,000 mM NaCl in the same buffer. Fractions of 2.4 ml were collected, and the polysaccharide antigen concentration was detected by enzyme-linked immunosorbent assay (ELISA) inhibition. Antigen-containing fractions were pooled, concentrated, and dialyzed against 2 liters of 50 mM sodium acetate, pH 5.0. About 2 to 3 mg of purified polysaccharide was obtained per liter of bacterial culture.

Polysaccharide material eluted from the Q-Sepharose column by starting buffer was further fractionated on an S-Sepharose column (Pharmacia) (1.6 by 11 cm) equilibrated with 50 mM sodium acetate, pH 5.0. The column was first washed with 40 ml of 50 mM sodium acetate, pH 5.0, followed by a 200-ml gradient from 0 to 1,000 mM NaCl in the same buffer. Fractions of 2.4 ml were collected, and the specific antigen was detected by ELISA inhibition or by colorimetric assay for hexosamine.

Detection of specific antigen by coagglutination and ELISA inhibition. Absorbed rabbit antiserum specific for the specific antigen of biofilm-producing *S. epidermidis* strains has been previously described (43); however, absorption of serum was accomplished in this study with the isogenic biofilm-negative transductant 1457-M11 of the biofilm-producing *S. epidermidis* 1457 which was used to raise the antiserum (43). Reactivity of the antiserum was indistinguishable from the original serum absorbed with biofilm-negative *S. epidermidis* 5179 and 9896 grown in TSB and biofilm-producing *S. epidermidis* 1457 grown in TSB lacking glucose (data not shown).

Specific antigen in bacterial extracts was quantitated by a specific coagglutination assay (43). *Staphylococcus aureus* Cowan 1 was sensitized with the absorbed specific antiserum. Aliquots (5 μl) of serial twofold dilutions of bacterial extracts in phosphate-buffered saline were mixed with 25 μl of coagglutination reagent on microscope slides. Agglutination was evaluated after 3 min in bright light against a dark background. The antigen titers were defined as the highest dilution displaying positive coagglutination.

For measuring antigen concentration by ELISA inhibition, microtiter plates (Greiner, Frickenhausen, Germany) were coated with the biofilm-producing *S. epidermidis* 1457 scraped from tissue culture plates (Nunc, Roskilde, Denmark) and suspended in 0.3% methylglyoxal (Sigma), pH 8.0, after growth in TSB for 24 h as previously described (8). The plates were washed and blocked overnight at 4°C with wash buffer (phosphate-buffered saline plus 0.5% Tween 20 [Merck]) containing 0.5% bovine serum albumin (BSA; Serva, Heidelberg, Germany). Antigen-containing samples were diluted with phosphate-buffered saline as appropriate. A calibration curve was established by analyzing twofold dilutions of a crude bacterial extract of biofilm-producing *S. epidermidis* 9142 containing the specific antigen. Samples and controls were analyzed in duplicate. The absorbed antiserum was diluted in wash buffer as required to reach a half-maximal extinction of the ELISA. Samples (20 μl) were mixed with 60 μl of the diluted absorbed antiserum in a microtiter plate and incubated at 37°C for 60 min. The samples were then transferred to ELISA plates (50 μl per well). After 90 min at 37°C, the plates were washed and bound antibodies were detected with alkaline phosphatase-conjugated anti-rabbit monoclonal antibody as previously described (Sigma) (66). Depending on the inhibition of the ELISA signal, the antigen concentration of samples was established from the calibration curve in arbitrary ELISA units.

ELISA inhibition with purified polysaccharides and monosaccharides. Microtiter plates were coated with purified polysaccharides of *S. epidermidis* 1457 or RP62A diluted in 0.3% methylglyoxal, pH 8.0, at a concentration as stated by the respective experiments (see Table 1 and Fig. 4) for 90 min at 37°C followed by further incubation overnight at 4°C (8). After being washed two times with wash buffer, the wells were blocked with wash buffer containing 0.5% BSA for 2 h at 37°C. The absorbed antiserum was diluted in wash buffer as appropriate. The diluted antiserum was then mixed with different concentrations of purified polysaccharide preparations or different monosaccharides, which were diluted in phosphate-buffered saline. The monosaccharides used for ELISA inhibition were D-glucose (Merck) and D-glucosamine, 2-acetamido-2-deoxy-D-glucose, methyl-2-acetamido-2-deoxy-α-D-glucopyranoside, and methyl-2-acetamido-2-deoxy-β-D-glucopyranoside (all from Sigma). The samples were incubated in microtiter plates at 37°C for 60 min. The samples were then transferred to ELISA plates. These were processed further as described above.

Colorimetric assays. Hexose (22), hexosamine (40), ketose (9), uronic acid (6), pentose (9), phosphate (1), and protein (7) contents were determined as described previously (42). To determine the amount of non-N-acetylated amino groups of hexosamines, the initial treatment with 0.5 N HCl for 2 h at 110°C was omitted (40).

Analytical procedures. Glucosamine was quantified after precolumn derivatization with 9-fluorenylmethyl chloroformate by HPLC on a LiChrosphere RP 18e column with taurine as an internal standard (27). Hexosamines and neutral sugars were detected after reductive amination with 2-aminobenzoic acid by HPLC (2) and after conversion into polyolacetates by gas-liquid chromatography (GLC) (4). Amino groups were measured after derivatization with fluorescamine by a fluorometric procedure (64). GLC analyses of alditol acetates, partially methylated alditol acetates (4), and acetylated butyl glycosides (27) were performed as previously described. The presence of sulfate (60) and that of pyruvate acetal (23, 39) were tested after hydrolysis with 1 M HCl at 100°C for 3 h and 0.1 M HCl at 100°C for 4 h, respectively. Succinate (5) and malate (47) were assayed after treatment with 0.2 M NaOH at 60°C for 2 h. Radioactivity was measured in Rotiszint Eco Plus in a Wallac 1410 liquid scintillation counter (Pharmacia LKB GmbH).

2-Butylation. Glucosamine was released from the polysaccharide by hydrolysis in 4 M HCl at 100°C for 8 h, dried in vacuo, and treated with 10 μl of acetic anhydride at room temperature for 3 h. After drying, 200 μl of *R*-(−)-2-butanol, containing 25 μl of acetyl chloride, was added. The mixture was heated at 100°C for 3 h and then taken to dryness in vacuo. For acetylation, acetic anhydride (100 μl) and dry pyridine (25 μl) were added, and after heating at 100°C for 1 h the mixture was used for GLC analysis. Authentic *N*-acetyl-D-glucosamine was derivatized with *R*-(−)-2-butanol and *S*-(+)-2-butanol and peracetylated in the same way.

Periodate oxidation. Polysaccharide (580 nmol of glucosamine) was oxidized in 0.01 M $NaIO_4$ in 0.05 M sodium acetate (pH 4.2) (130 μl) at 4°C, and consumption of periodate was monitored spectrophotometrically (21). Methyl-2-acetamido-2-deoxy-β-D-glucopyranoside was oxidized under the same conditions.

Peracetylation. Polysaccharide (4 μmol of glucosamine) was dissolved in acetic anhydride-formamide-pyridine (5:4:1, vol/vol/vol), and the mixture was kept at room temperature for 86 h. Then 2 ml of H_2O was added on ice, and after the

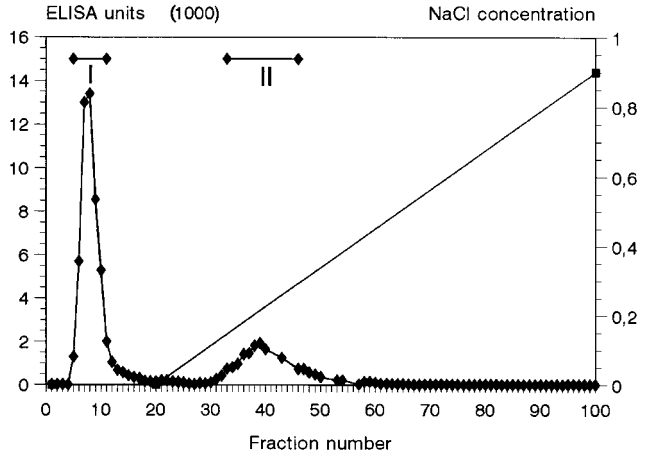

FIG. 1. Fractionation of the specific antigen by anion-exchange chromatography. Antigen-reactive material of *S. epidermidis* 1457 purified on Sephadex G-200 was chromatographed on a Q-Sepharose column as described in Materials and Methods. The effluent was analyzed for antigen by ELISA inhibition with the specific absorbed antiserum. The material of fractions 4 to 10 and that of fractions 30 to 45 were separately pooled and are referred to as polysaccharides I and II, respectively.

mixture stood at room temperature for 30 min, the peracetylated polysaccharide was extracted with $CHCl_3$ and washed several times with water.

Oxidation with CrO_3. Peracetylated polysaccharide was treated with CrO_3 essentially as described by Laine and Renkonen (38). Subsequent hydrolysis was done in 4 M HCl at 100°C for 8 h.

Methylation analysis. Prior to methylation, the polysaccharide was peracetylated as described above. The methylation procedure of Ciucanu and Kerek (15) was followed, except that the reaction time was 90 min with two other additions of CH_3I after 30 and 60 min. After reaction, solid NaOH was removed by centrifugation and washed with dry dimethyl sulfoxide. The supernatants were combined, 2 volumes each of water and $CHCl_3$ were added, and the permethylated polysaccharide was recovered from the $CHCl_3$ layer. Partially methylated monosaccharides were released by hydrolysis in 4 M HCl at 100°C for 2 h and converted into polyolacetates for GLC-mass spectrometry analysis (36).

Deamination. Polysaccharide (1.6 μmol of glucosamine) was dissolved in water (0.5 ml), 33% (vol/vol) acetic acid (0.75 ml) and 5% (wt/vol) $NaNO_2$ (0.75 ml) were added, and the mixture was sonicated in an ultrasonic bath at room temperature for 120 min with two other additions of 5% (wt/vol) $NaNO_2$ (0.25 ml each) after 40 and 80 min. Water (10 ml) was added, and the sample was concentrated to a small volume (0.25 ml) by lyophilization. $NaBH_4$ (1.39 μmol, 18.5 MBq) was added in 0.01 M NaOH (50 μl), and the mixture was sonicated at room temperature for 1 h. After the addition of NaB^3H_4 (13.9 μmol), the reaction was continued for another 45 min. Excess borohydride was oxidized with glucose-6-phosphate (50 μmol). The mixture was then passed through a small column of anion-exchange resin, acetate form, and concentrated to a small volume by lyophilization with several additions of water. Samples were analyzed for amino groups, total glucosamine, and radioactivity. Separation of the fragments was performed by HPLC as detailed in Results.

NMR spectroscopy. Nuclear magnetic resonance (NMR) spectra were determined with a Bruker AMX 500 spectrometer and standard UXNMR acquisition software. Buffer of the polysaccharide solution was exchanged by 50 mM NaCl–0.05% NaN_3 in 96.96% D_2O by seven cycles of concentration and dilution in a Centricon 10 cartridge (Amicon). For measurement, a sample containing 3.07 mg of polysaccharide per ml was used. Spectra were recorded at 25°C. One-dimensional ^{1}H-NMR spectra (500.13 MHz) were recorded in normal acquisition mode, by using 1,024 scans. One-dimensional ^{13}C-NMR spectra (125.77 MHz) were recorded with proton decoupling. HH-correlation spectroscopy spectra were recorded by using standard Bruker software, with 1,024 datum points in f2 and 256 increments each with 128 scans in f1, followed by sine-squared multiplication without phase shifting and finally matrix symmetrization. ^{1}H/^{13}C-heteronuclear-multiple-quantum coherence spectra (HMQC) were recorded by using a pulse sequence with bilinear-rotation-decoupling pulse to suppress signals from protons connected to ^{12}C (46). A block size of 1K in f2 and 512 increments, each with 256 scans in f1, were used in time-proportional-phase-increment mode, followed by zero filling of a 1K × 1K matrix, processing with a π/2 phase-shifted sine filter, and phase correction in f2 and f1 (46).

RESULTS

Purification and characterization of the specific antigen. After fractionation on a Sephadex G-200 column, the antigen-reactive material of biofilm-producing *S. epidermidis* 1457 contained 75% hexosamine, whereas material of the isogenic biofilm-negative transductant 1457-M11 prepared in parallel did not contain hexosamine. Hexose, protein, and phosphate were detected in substantial amounts in the preparations from both strains. This indicated that the specific antigen of biofilm-producing *S. epidermidis* consisted primarily of hexosamine and the remaining components could represent contaminants (42).

Therefore, the antigen-reactive material of *S. epidermidis* 1457 fractionated by Sephadex G-200 was further purified by Q-Sepharose anion-exchange chromatography. The majority of the antigen-reactive material did not bind to the column, whereas a minor fraction was eluted at an NaCl concentration of about 150 mM (Fig. 1). These materials are referred to hereafter as polysaccharides I and II, respectively. When polysaccharides I and II were analyzed by colorimetric assays, only hexosamine was detected, and polysaccharides I and II were present in a ratio of about 7:1. In the concentrated preparations, hexoses, ketoses, uronic acids, pentoses, and protein were below the level of detection. The specific antigen of *S. epidermidis* RP62A exhibited an elution profile from the Q-Sepharose column similar to that of the antigen of *S. epidermidis* 1457 (data not shown). Polysaccharide I of *S. epidermidis* RP62A also exclusively contained hexosamine.

Although both polysaccharides I and II were composed of glucosamine (see below), the presence of different antigens could not be excluded, because the absorbed antiserum used for detection was raised against whole *S. epidermidis* 1457 cells (43). However, equally effective mutual inhibition of the two polysaccharides in binding to the antiserum, as shown in Table 1, indicated identical antigenic determinants.

In later experiments, we found that column chromatography of the crude bacterial extract on Q-Sepharose resulted in an identical elution profile of the antigen, as shown in Fig. 1, and is sufficient to yield polysaccharide I of *S. epidermidis* 1457 and RP62A free of carbohydrate and protein contaminants (data not shown).

Structural analysis of purified polysaccharide I. Purified polysaccharide I of *S. epidermidis* 1457 was used for structural analysis. The polysaccharide was hydrolyzed in 2 M HCl at 100°C for 8 h. HPLC analysis of the hydrolysate for amino sugars (27) revealed glucosamine as the only component. Muramic acid, a characteristic component of peptidoglycans, was

TABLE 1. Competitive inhibition of antibody binding to purified polysaccharides I and II[a]

Amt (μg) of inhibitor polysaccharide	Inhibition of binding to coating polysaccharide[b]:			
	I		II	
	I	II	I	II
0	1.28	1.28	1.56	1.56
1	0.12	0.13	0.26	0.31
4	0.07	0.09	0.12	0.14

[a] ELISA plates were coated with purified polysaccharides I and II (4 μg per well) of *S. epidermidis* 1457, respectively. Specific antiserum was diluted in wash buffer and preincubated for 60 min at 37°C with the indicated quantities of purified polysaccharides per aliquot (50 μl). Samples were transferred to coated ELISA plates and processed as described in Materials and Methods.

[b] Data are optical density values at 405 nm.

not detected. The identity of glucosamine was confirmed and the presence of neutral sugars was excluded when the hydrolysate was analyzed by GLC after treatment with NaB^3H_4 and peracetylation or subjected to HPLC after reductive amination of the monosaccharides with 2-aminobenzoic acid (2). The results of the search for protein by HPLC of amino acids (30) after strong acid hydrolysis (6 M HCl, 150°C, 1.5 h) were negative. The same results were obtained with the purified polysaccharide I of *S. epidermidis* RP62A.

In order to determine the absolute configuration, the glucosamine released by acid hydrolysis was N acetylated and converted into *R*-(−)-2-butyl glycosides, and these were analyzed as acetates by GLC (27). The peaks were identical to those of *R*-(−)-2-butyl-*N*-acetyl-D-glucosaminide but not those of *S*-(+)-2-butyl-*N*-acetyl-D-glucosaminide, which established the D configuration (29).

The native polysaccharide was oxidized with IO_4^- under controlled conditions (pH 4.2, 4°C). The time course paralleled that of methyl-2-acetamido-2-deoxy-β-D-glucopyranoside. After 80 h, the periodate consumption approached final values of 1.2 and 1 mol per mol of glucosaminyl residue of the polysaccharide and the reference compound, respectively. In the polysaccharide-containing sample, excess periodate was reduced by the addition of ethylene glycol, and IO_3^- was removed by passing the mixture through a small column of anion-exchange resin, acetate form. Subsequent hydrolysis in 4 M HCl at 100°C for 8 h revealed that more than 99% of the glucosaminyl residues had been oxidized. These results suggest an unbranched polysaccharide chain with 1,6-interglycosidic linkages.

Prior to methylation, the polysaccharide was peracetylated, and methylation analysis was performed as detailed in Materials and Methods. Optimum conditions for hydrolysis were found by using permethylated chitobiose. Hydrolysis with 4 M HCl at 100°C for 18 h led to a 10 to 20% loss of the methyl groups at O-6 of both sugar residues; the loss decreased with decreasing hydrolysis time and approached zero after hydrolysis for 2 h. Under these conditions, the permethylated polysaccharide yielded 3,4,6-tri-*O*-methyl-*N*-methyl-*N*-acetyl-2-amino-2-deoxyglucose and 3,4-di-*O*-methyl-*N*-methyl-*N*-acetyl-2-amino-2-deoxyglucose in a molar ratio of approximately 1:130. These data confirm 1,6-glycosidic linkages and a linear polysaccharide structure and suggest an average chain length of 130 glucosaminyl residues.

Treatment of the polysaccharide with fluorescamine or ninhydrin revealed the presence of free amino groups in a molar ratio to glucosamine of 0.2. Treatment of the polysaccharide with HNO_2 led to the disappearance of fluorescamine-positive material and a concomitant loss of 20% of glucosamine from the polysaccharide. This indicates that the amino groups represent nonacetylated glucosaminyl residues which are converted by HNO_2 into 2,5-anhydro-mannose under cleavage of the glycosidic bond (68).

NMR analyses of purified polysaccharide I. The one-dimensional ^{1}H-NMR spectra of purified polysaccharide I of *S. epidermidis* 1457 show, together with two-dimensional HH-correlation spectroscopy experiments, broad signals attributable to β-1,6-linked *N*-acetyl-D-glucosaminyl residues ($^3J_{H1\text{-}H2}$ = 8.5 Hz and glycosylation shift for C-6 = 70.32 ppm; Δ = 8.4 ppm). No correlation signal was observed for a small and relatively broad peak at δ = 2.87 ppm in the HH-correlation spectroscopy spectrum. In order to identify this signal, ^{1}H- and ^{13}C-NMR experiments were carried out with authentic α,β-D-glucosamine and *N*-acetyl-α,β-D-glucosamine (Table 2). This allowed us to identify the resonance at 2.87 ppm as the H-2 proton of non-*N*-acetylated β-D-glucosaminyl residues. The

TABLE 2. 500-MHz ^{1}H and 125-MHz ^{13}C chemical shifts of purified polysaccharide I of *S. epidermidis* 1457 and authentic *N*-acetyl-β-D-glucosamine and β-D-glucosamine

Position	Chemical shift of:							
	Polysaccharide I				Authentic sample			
	β-D-GlcNAc		β-D-GlcNH$_2$		β-D-GlcNAc		β-D-GlcNH$_2$	
	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
1	103.45	4.56			95.94	4.73	93.67	5.03
2	57.16	3.75	57.92	2.87	57.73	3.31	57.80	3.08
3	75.58	3.59			74.97		73.00	
4	71.87	3.44			70.75		70.55	
5	76.36	3.62			76.99		77.15	
6	70.32	3.75/4.20			61.72		61.47	
C═O	176.30							
CH_3	24.21	2.09				2.06		

500/125-MHz ^{1}H/^{13}C-HMQC spectrum for the native polysaccharide showed correlation signals for β-1,6-linked *N*-acetyl-D-glucosaminyl residues (Fig. 2). A cross peak at 2.87/57.92 ppm was observed in the HMQC spectrum, but no further evidence for glucosaminyl residues was seen. The final evidence for the nonacetylated glucosaminyl residues was obtained by chemical analysis.

Distribution of non-N-acetylated glucosaminyl residues. In order to find out the distribution of the non-N-acetylated glucosaminyl residues along the polysaccharide chain, we took advantage of the glycosidic bond cleavage which occurs on conversion of the glucosaminyl residues into 2,5-anhydro-mannose by HNO_2 (see above). The deaminated polysaccharide was treated with NaB^3H_4, and the products were separated by HPLC, the effluent being analyzed for glucosamine and radioactivity. The elution profiles in Fig. 3 show that unlabelled polysaccharide was not detectable, most of the radioactivity was associated with low-M_r material and, apart from a shoulder in the ascending part, the radioactive material eluted essentially in three peaks. Peak III appeared at the inclusion volume of the column and consisted of residual radiolabelled water. Peak II was almost free of glucosamine and appeared near the location of authentic 2,5-anhydromannitol.

In order to determine the chain lengths of the fragments in peak I, *N*-acetylglucosamine was radiolabelled with NaB^3H_4 under the same conditions as the deaminated polysaccharide and chromatography was performed with the TSK-Gel column. With the purified compound, the radioactivity per mole of reduced terminus (Bq_t) was determined, and for individual fragments the number of glucosaminyl residues per molecule (n) was calculated by

$$n = \frac{x\text{GlcNAc}}{y\text{Bq}} \cdot \text{Bq}_t$$

where xGlcNAc and yBq are the molar amounts of glucosamine and ^{3}H radioactivity, respectively, in the column fractions of Fig. 3. The relative abundances of the fragments were calculated by $y\text{Bq}/\Sigma y\text{Bq}$. The results, summarized in Table 3, show that 2,5-anhydromannitol, *N*-acetylglucosaminyl-, and tri-*N*-acetylglucosaminyl-2,5-anhydromannitol represent 29, 11, and 46% of the fragments, respectively. Fragments containing up to 64 *N*-acetylglucosaminyl residues were detected, and with increasing chain length the relative abundance gradually decreased. The sum of the chain length (n + 1) multiplied by the relative abundance of the individual fragments (Table 3) yields

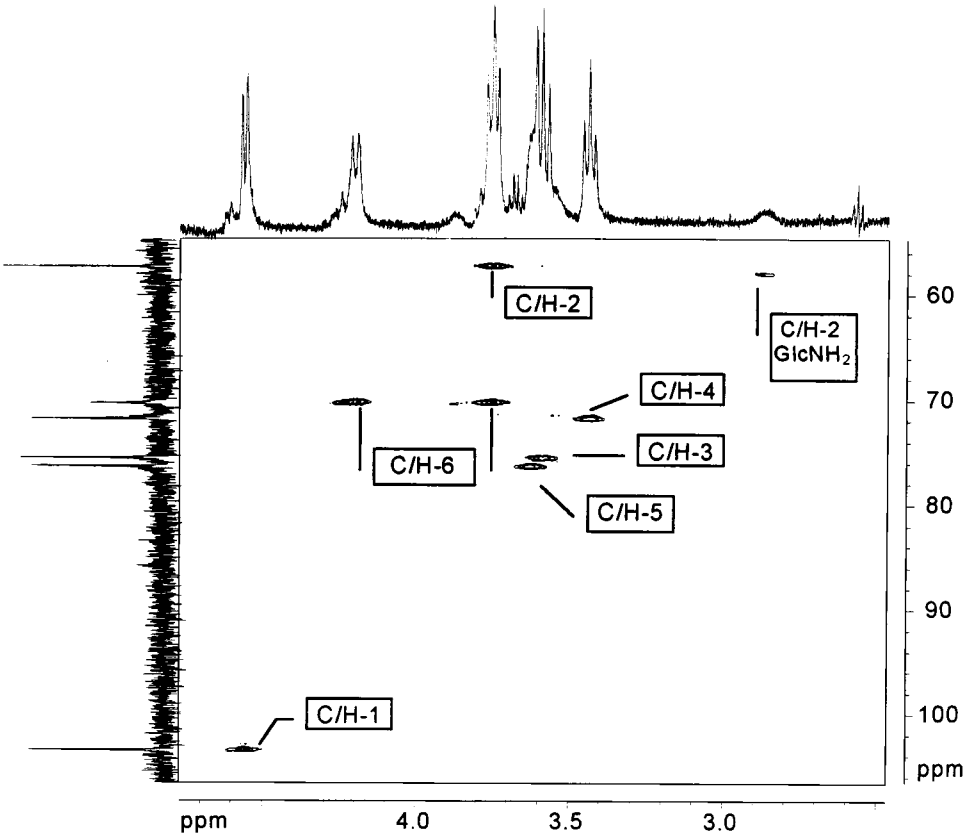

FIG. 2. 500/125-MHz $^{1}H/^{13}C$-HMQC spectrum of native polysaccharide I of *S. epidermidis* 1457.

an average chain length of 4.5, which is close to the theoretical value of 5.0 calculated for 20% of nonacetylated glucosaminyl residues.

The fragmentation pattern obtained by deamination allows us to exclude the occurrence of *N*-acetylglucosaminyl and glucosaminyl residues on separate chains as well as on separate sequences of the same chain, because in both cases only unlabelled polysaccharide and 2,5-anhydromannitol would be found. A regular distribution of the glucosaminyl residues along the chain is also excluded because it would yield predominantly tetra-glucosaminyl-2,5-anhydromannitol. The distribution pattern appears therefore to be more or less random, but the relatively high abundances of 2,5-anhydromannitol and short-chain fragments suggest some prevalence of glucosaminyl-rich sequences.

In a second approach, polysaccharide I was subjected to cation-exchange chromatography on an S-Sepharose column. As shown in Fig. 4, part of the polysaccharide eluted with the starting buffer (50 mM sodium acetate, pH 5.0), whereas the remaining material appeared on elution with an NaCl gradient as a broad peak (Fig. 4). The effluent was collected into four fractions as indicated in Fig. 4, which contained 25.9, 15.2, 38.8, and 20.0% of the recovered hexosamine. Analysis of these fractions revealed non-N-acetylated glucosamine/total glucosamine ratios of 0.02, 0.08, 0.17, and 0.26, respectively, indicating an uneven distribution of the positively charged residues among molecular species.

Search for negatively charged polysaccharide substituents. The anionic properties of polysaccharide II prompted us to search for anionic substituents. Like polysaccharide I, polysaccharide II was composed of glucosamine, and other carbohydrate components could not be detected. As shown in Table 4, polysaccharide II contained less non-N-acetylated glucosaminyl residues and small but reproducibly detectable amounts of phosphate and succinate, which suggests a slight preponderance of negative charges. Accordingly, after mild alkaline treatment (0.2 M NaOH, 37°C, 24 h) which cleaves carboxyl ester bonds, polysaccharide II was no longer retained on the anion-exchange column and appeared at the position of polysaccharide I. In both polysaccharides, other negatively charged substituents, such as alduronic acids, ester-linked malate or sulfate, and pyruvate acetal, could not be detected.

Inhibition of absorbed antiserum reactivity by monosaccharides. The inhibitory activities of selected monosaccharides were tested in an ELISA with purified polysaccharide I of *S. epidermidis* RP62A as the antigen. In accordance with the proposed polysaccharide structure, the inhibitory activity increased in the order D-glucose, D-glucosamine, *N*-acetyl-D-glucosamine, methyl-2-acetamido-2-deoxy-β-D-glucopyranoside (Fig. 5). As expected, the β-anomer of methyl-2-acetamido-2-deoxy-D-glucopyranoside was much more effective than the α-anomer, and the relatively high inhibitory activity of *N*-acetyl-D-glucosamine is explained by the predominance of the β-anomeric form. Non-N-acetylated D-glucosamine was less inhibitory than the N-acetylated derivative, suggesting that the positively charged glucosaminyl residues of the polysaccharide play a subordinate role in the recognition by the antiserum used.

DISCUSSION

In this study, we have characterized the specific antigen of biofilm-producing *S. epidermidis* 1457 by biochemical and

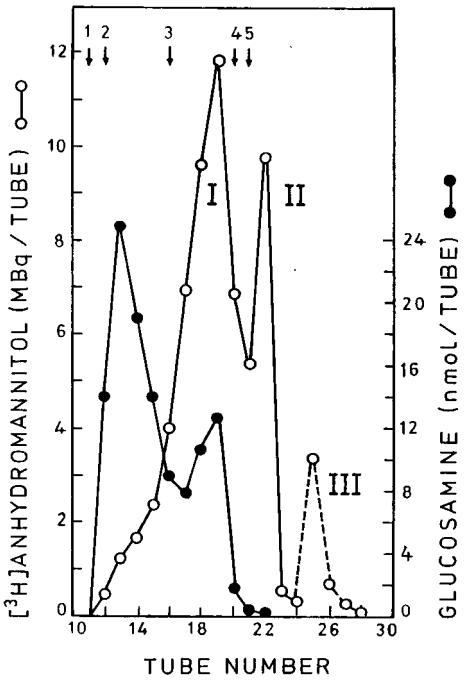

FIG. 3. HPLC of fragments produced from polysaccharide I of *S. epidermidis* 1457 on deamination and subsequent treatment with NaB^3H_4. The products (110 nmol of *N*-acetylglucosamine, 2×10^4 Bq) were applied in H_2O (25 μl) to a TSK-Gel G-Oligo-PW column and were eluted with water at a flow rate of 0.5 ml/min. Fractions of 0.5 ml were collected and analyzed for radioactivity (○) and, after acid hydrolysis, for glucosamine (●). Markers: 1, Dextran 39200 (Glc_{240}); 2, native polysaccharide I ($GlcNAc_{130}$), Dextran 9300 (Glc_{57}); 3, maltohexaose; 4, *N*-acetylglucosaminitol; 5, 2,5-anhydromannitol.

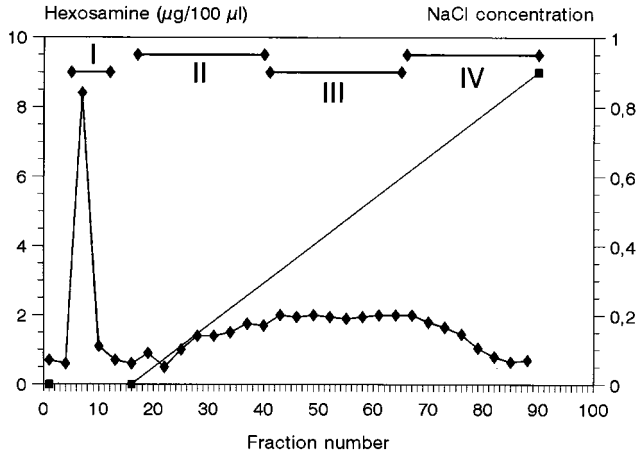

FIG. 4. Fractionation of polysaccharide I by cation-exchange chromatography. Column chromatography on S-Sepharose was performed as described in Materials and Methods. The effluent was analyzed for hexosamine and pooled into four fractions (I to IV) as indicated.

NMR spectroscopical methods, which led for the first time to the elucidation of the structure of a polysaccharide produced by *S. epidermidis* which is functionally involved in biofilm accumulation.

The polysaccharide of *S. epidermidis* exhibits some structural heterogeneity, which leads to separation on a Q-Sepharose column into a major polysaccharide, polysaccharide I, which accounts for at least 80% of the polysaccharide and did not bind to Q-Sepharose, and a minor polysaccharide, polysaccharide II, which was moderately anionic.

The structure of polysaccharide I is so far considered to be unique. It proved to be a homoglycan, made up of 80 to 85% *N*-acetyl-D-glucosaminyl residues, the remainder being non-N-acetylated positively charged D-glucosaminyl residues. Chemical analyses and NMR data led us to propose an unbranched structure with β-1,6 interglycosidic linkages containing at least 130 residues. Fractionation of polysaccharide I by cation-exchange chromatography on S-Sepharose revealed heterogeneity with respect to positive charges, as over the elution profile the content of non-N-acetylated glucosaminyl residues increased from below 2% to 26%. The fragmentation pattern obtained from polysaccharide I by deamination of the non-N-acetylated glucosaminyl residues with HNO_2 suggested a more or less random distribution of the positive charges along the chain overlaid with a slight tendency to accumulation (Fig. 3; Table 3). The unbranched homoglycan structure is reminiscent of other structural polysaccharides such as cellulose and chitin, but the replacement of β-1,4 by β-1,6 linkages renders the overall structure of the polysaccharide of *S. epidermidis* less compact and less rigid. Because of the β-1,6 linkage, the sugar rings are separated by three rather than two bonds, which together with the larger distance of the sugar rings from each other leads to extra freedom of rotation.

Purified polysaccharide II appeared to be structurally very similar to polysaccharide I, as it also consisted exclusively of glucosamine and the absorbed antiserum detected identical antigenic determinants on both polysaccharides (Table 1). As apparent from ELISA inhibition studies, the β-anomeric form of the glycosidic bonds and the acetylation of the amino group were most important for the reactivity of the absorbed antiserum (Fig. 5). Polysaccharide II contained fewer non-N-acetylated glucosaminyl residues than polysaccharide I, prepared from the same extract, and contained small amounts of phosphate and ester-linked succinyl residues. If these prove to be substituents of polysaccharide II, they could be responsible for the moderately anionic character. There was no evidence for other negatively charged substituents such as alduronic acids, ester-linked malate or sulfate, and pyruvate acetal.

Several polysaccharide components of *S. epidermidis* which are major constituents of *S. epidermidis* slime or glycocalyx have been described or have been proposed to be functionally

TABLE 3. Chain lengths and relative abundances of the fragments released from the polysaccharide on deamination[a]

Fragment property	Tube no.												
	12	13	14	15	16	17	18	19	20	21	22	23	24
Chain length	64.9	42.6	26.0	12.5	4.9	2.9	3.0	3.2	1.5	1.1	1.0	1.0	1.0
Relative abundance (× 100)	0.7	1.8	2.3	3.6	6.7	12.4	15.7	17.6	10.9	8.7	16.7	2.6	1.2

[a] The separation by HPLC is shown in Fig. 3. The chain length (n + 1) was defined as the number (n) of *N*-acetylglucosaminyl residues linked to [^{3}H] 2,5-anhydromannitol (see the text).

TABLE 4. Analysis of polysaccharides I and II for charged components

Component	Content of polysaccharide[a]:	
	I	II
Total glucosamine	1.00	1.00
Non-N-acetylated glucosamine	0.15	0.09
Phosphate	<0.01	0.04
Succinate	<0.01	0.06

[a] Values are molar ratios to total glucosamine.

significant for biofilm production. These include an extracellular slime substance, which has mannose and galactose as its major constituents (41, 53); another major slime component with a composition reminiscent of teichoic acid (33); a capsular polysaccharide adhesin (PS/A) consisting mainly of galactose, glucosamine, galactosamine, and alduronic acids (62); a slime-associated antigen containing mainly glucose and small amounts of *N*-acetylglucosamine and *N*acetylgalactosamine (12); and a sulfated slime polysaccharide containing neutral sugars, amino sugars, and minute amounts of alduronic acids (3). Except for PS/A, for which a function in the primary attachment of *S. epidermidis* cells to unmodified silastic catheter tubing was demonstrated (48, 62), no significant function of any of these components for biofilm production has been demonstrated. The structural data reported in this study clearly differentiate the specific antigen of biofilm-producing *S. epidermidis* from these other polysaccharides. In view of the fact that most of them were prepared from *S. epidermidis* RP62A, it should be noted that the specific glucosaminoglycan antigen could also be isolated from this strain.

We recently demonstrated that the observed lack of cell clustering of the isogenic biofilm-negative transposon mutants M10 and M11, which indicated impaired intercellular adhesion, did not result from altered cell surface properties of the mutants leading to significant changes of interaction of the bacterial cell surface with the specific antigen but resulted from completely abolished production of the specific antigen (42). Accordingly, the polysaccharide described in this report acts as an intercellular adhesin and, in accordance with this function in biofilm accumulation, we propose to refer to the specific antigen of biofilm-producing *S. epidermidis* as the polysaccharide intercellular adhesin (PIA).

Both the unbranched polysaccharide structure and the positively charged glucosaminyl residues on polysaccharide I, along with the positively and negatively charged groups on polysaccharide II, might be important for intercellular adhesion and biofilm formation of *S. epidermidis*. The unbranched structure facilitates long-range contacts to adjacent polysaccharide strands and the cell wall and/or lectins, favoring interchain van der Waals interactions and hydrogen bonds. The positively charged glucosaminyl residues distributed along the chain together with the negatively charged groups on some polysaccharide strands may strengthen these contacts by electrostatic interaction.

Whether lectin-like proteins are involved in intercellular adhesion of *S. epidermidis*, as is the case in the aggregation of *Streptococcus mutans* (17, 59), is now of interest. This problem can now be attacked by affinity chromatography with the purified specific polysaccharide as a ligand and extracts of cell wall proteins of the isogenic biofilm-negative transposon mutant M10 or M11. These mutants lack the specific polysaccharide antigen, but, being still competent for interaction with the specific antigen, are efficiently integrated into adherent biofilms on cocultivation with a biofilm-producing *S. epidermidis* strain (42).

Using a rat peritonitis model, it was recently shown that purified capsular polysaccharide of *Bacteroides fragilis*, which contains both positive and negative charges concomitantly on its oligosaccharide repeating unit, was highly abscessogenic as were other unrelated polysaccharides containing concomitantly positive and negative charges (63). Whether polysaccharide II of *S. epidermidis*, which also contains positive and negative charges, exhibits similar properties remains to be investigated. In this context, it is interesting to note that significantly lower 50% infective doses of biofilm-producing *S. epidermidis* strains, including RP62A, were needed to induce abscesses in a subcutaneous foreign-body infection model than with biofilm-negative strains (50).

Non-N-acetylated positively charged hexosaminyl residues are rare in nature. Nucleotide-activated amino sugars used in polymer synthesis usually contain the N-acetylated form such as UDP-*N*-acetylglucosamine or UDP-*N*-acetylgalactosamine. The formation of non-N-acetylated hexosaminyl residues may therefore require deacetylating enzymes, similar to that which is operative in the biosynthesis of lipid A in gram-negative bacteria (55). One might therefore expect that the synthesis of the polyglucosamine in *S. epidermidis* also requires a deacetylase in addition to enzymes and proteins that activate *N*-acetyl-glucosamine, accomplish the polysaccharide synthesis, and transport either activated precursors or the completed polysac-

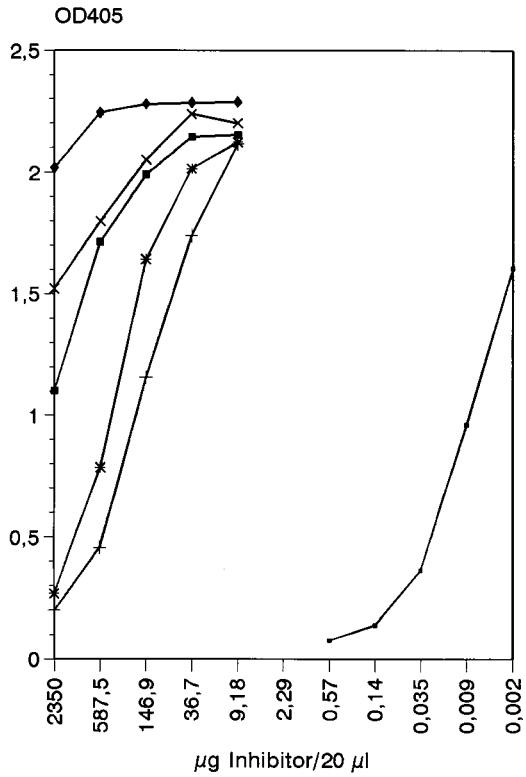

FIG. 5. Competitive inhibition of specific antibody binding to purified polysaccharide I of *S. epidermidis* RP62A. Aliquots (60 µl) of the specific absorbed antiserum diluted 1:3,000 in wash buffer were mixed with serial dilutions (20 µl) of D-glucose (◆), D-glucosamine (■), *N*-acetyl-D-glucosamine (✳), methyl-2-acetamido-2-deoxy-α-D-glucopyranoside (×), methyl-2-acetamido-2-deoxy-β-D-glucopyranoside (+), and purified polysaccharide I of *S. epidermidis* RP62A (■). After incubation at 37°C for 60 min, samples were analyzed for unreacted antibodies by ELISA on plates coated with polysaccharide I (0.125 µg per well) as described in Materials and Methods.

charide through the cytoplasmic membrane. Biofilm-negative transposon mutants such as M10 and M11 may help to elucidate this biosynthetic pathway (42).

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