Carbohydrate-containing components of biofilms produced *in vitro* by some staphylococcal strains related to orthopaedic prosthesis infections

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Abstract

The capacity of coagulase-negative staphylococci to colonize implanted medical devices is generally attributed to their ability to produce biofilms. Biofilm of the model strain of *Staphylococcus epidermidis* RP62A was shown to contain two carbohydrate-containing moieties, a linear poly-β-(1 → 6)-*N*-acetyl-D-glucosamine (PNAG) and teichoic acid. In the present study, we investigated several biofilm-producing staphylococci isolated from infected orthopaedic implants and characterized the composition of the laboratory-grown biofilms using chemical analysis and ^{1}H nuclear magnetic resonance spectroscopy. Extracellular teichoic acid was produced by all strains studied. Some of the clinical strains were shown to produce biofilms with compositions similar to that of the model strain, containing a varying amount of PNAG. The chemical structure of PNAG of the clinical strains was similar to that previously described for the model strains *S. epidermidis* RP62A and *Staphylococcus aureus* MN8m, differing only in the amount of charged groups. Biofilms of the strains producing a substantial amount of PNAG were detached by dispersin B, a PNAG-degrading enzyme, while being unsusceptible to proteinase K treatment. On the other hand, some strains produced biofilms without any detectable amount of PNAG. The biofilms of these strains were dispersed by proteinase K, but not by dispersin B.

Introduction

Staphylococcus aureus and coagulase-negative staphylococci (CoNS), including *Staphylococcus epidermidis* as a leading species, have emerged as the most frequently isolated pathogens in nosocomial sepsis and cause more infections associated with use of medical devices than any other group of microorganisms (Kloos & Bannerman, 1994). *S. epidermidis*, an inhabitant of skin and mucous membranes, is presently known to be an opportunistic pathogen, causing serious infections in immunocompromised hosts or patients with implanted medical devices, such as peritoneal and intravascular dialysis catheters, prosthetic heart valves, or orthopaedic prostheses (Götz, 2002). The so-called 'implant-related infections', which have been occurring with the increased frequency in the last decades (Printzen, 1996), are characterized by the ability of the causative bacteria to adhere and colonize surfaces in biofilms-multilayered cell clusters, embedded in an extracellular material ('slime'), which holds the cells together and firmly attaches the bacterial mass to the surface of a foreign body (Christensen *et al.*, 1982). This ability to form biofilms is believed to make microorganisms more resistant to antibiotic treatment and the immune host defence system (von Eiff *et al.*, 1999). The knowledge of the exact chemical structure of the constituents of staphylococcal biofilms is essential for a clear understanding of the pathogenic mechanisms of these bacteria and the development of new diagnostic and therapeutic tools against such infections. Extracellular (EC) polymers of staphylococci related to slime or biofilm production have been intensively investigated in the past decades. The most studied component of staphylococcal biofilm is a linear

β-(1 → 6)-*N*-acetyl-D-glucosamine polymer (PNAG), with a part of *N*-acetylglucosamine (Glc*N*Ac) residues deacetylated and partially substituted with *O*-succinyl groups (Mack *et al.*, 1996). This polymer, also referred to as polysaccharide intercellular adhesin (PIA) and synthesized by the enzymes encoded in the intercellular adhesion (*ica*) locus, was considered as mediating cell–cell attachment and subsequent biofilm accumulation. A capsular polysaccharide adhesin (PS/A), also encoded by *ica* operon, was believed to mediate the initial phase of biofilm formation, cell adherence to plastic and similar surfaces (Maira-Litran *et al.*, 2004). An extracellular polysaccharide of *S. aureus* (*S. aureus* exopolysaccharide, hereafter SAE, obtained from a PS/A-overproducing strain *S. aureus* MN8m), was recently reported to be chemically very similar to PIA but with certain differences in the molecular size, physical properties and presence of the charged groups (Maira-Litran *et al.*, 2002; Joyce *et al.*, 2003).

In our recent study, we have shown the structural identity of the extracellular polysaccharides produced by the two model biofilm-forming strains used previously by different authors, *S. epidermidis* RP62A and *S. aureus* MN8m, when bacteria were grown at the same conditions and PNAG from both strains were prepared using the same extraction and purification procedures. Overall, there is now a consensus that 'PIA and PS/A are the same chemical entity – PNAG' (Maira-Litran *et al.*, 2004).

It has been recently found that PGA, also a linear β-(1 → 6)-*N*-acetyl-D-glucosamine polymer, is produced by some strains of *Escherichia coli* (Wang *et al.*, 2004), where it is encoded not by the *ica*, but rather by a *pga* locus. Homologues of the *pga* locus are present in the genomes of a broad range of Gram-negative bacteria, which allows the suggestion that PGA was a polysaccharide involved in biofilm stabilization of these species (Kaplan *et al.*, 2004b; Wang *et al.*, 2004).

The detailed structural elucidation of PNAG has been so far performed only for the model strains *S. epidermidis* 1457, *S. epidermidis* RP62A (Mack *et al.*, 1996; Sadovskaya *et al.*, 2005), *S. aureus* MN8m (Maira-Litran *et al.*, 2002; Joyce *et al.*, 2003) and in a genetically modified *pga*-overexpressing strain of *E. coli* (Wang *et al.*, 2004). As yet, no systematic study has been carried out to investigate the chemical structure of PNAG (or PNAG-like polysaccharides) from the clinical staphylococcal strains.

The second carbohydrate-containing polymer that was characterized in a biofilm of the reference biofilm-positive strain *S. epidermidis* RP62A is an extracellular teichoic acid (EC-TA). In our recent studies, we established the chemical structure of the cell wall (CW) and EC-TAs of *S. epidermidis* RP62A (Sadovskaya *et al.*, 2004) and demonstrated that the EC-TA was an important component of the *in vitro* produced biofilm of this strain (Sadovskaya *et al.*, 2005), as well as of several clinical strains of staphylococci (Kogan *et al.*, 2006).

We have recently characterized 66 strains of CoNS associated with medical implant infections, including several potentially virulent strains of *S. epidermidis*, *Staphylococcus lugdunensis*, *S. hominis, S. capitis*, and *S. warneri*. The strains were screened for the presence of *ica* locus, the ability to form biofilm, and to produce polysaccharide intercellular adhesin (PIA) *in vitro*. Among these medical implant-related biofilm-positive (Bf^+) strains, some either did not contain the *ica* locus (ica^- strains) or did not seem to produce PIA *in vitro*, despite the presence of the *ica* locus (PIA^- strains) (Chokr *et al.*, 2005). These findings indicated that antigens other than PIA (PNAG) were present in the biofilm of these strains. A chemical analysis of the biofilms produced by the four strains from this collection indicated that their biofilms contained predominantly teichoic acid and protein components (Kogan *et al.*, 2006).

The purpose of the present study was to further characterize the composition of the extracellular matrix of a larger number of biofilm-positive staphylococcal strains associated with the infections of orthopaedic implants, and to compare the chemical structure of the PNAG of these strains to that of the PNAG isolated from the model strains. The clinical strains were grown in conditions favourable for biofilm and PNAG production, and the extracellular polymers were extracted and fractionated using the simple protocol developed in our previous study. This moderate purification procedure allowed us to obtain the staphylococcal PNAG close to its native form, with all charged groups in the molecule being conserved because of the absence of any harsh treatment or ion-exchange chromatography purification steps (Sadovskaya *et al.*, 2005). The results of the enzymatic biofilm detachment were in agreement with the chemical composition of the extracellular matrix of these biofilm-forming strains.

Materials and methods

Bacterial strains and culture conditions

The staphylococcal strains used were collected from infected patients hospitalized at the Mignot Hospital of Versailles, France (Table 1). Cells were grown at 37 °C for 24 h statically in 0.7 L of Tryptic Soy Broth (TSB; Becton, Dickinson and Company, Le Pont de Claix, France) in 3 L Erlenmeyer flasks. All cultures were preliminarily inoculated with a 3% (v/v) of 20 h aerobic preculture grown in the same medium.

Preparation of cell-associated polysaccharides

A crude extracellular matrix was isolated and fractionated essentially as previously described (Sadovskaya *et al.*, 2004). Briefly, bacterial cells were collected by centrifugation

Table 1. Bacterial strains used in this study

Strain	Biofilm formation	*icaA*	Source/reference
Staphylococcus epidermidis RP62A	+	+	G. B. Pier
Staphylococcus aureus MN8m	+	+	G. B. Pier
S. epidermidis 5	+	+	Chokr *et al.* (2005)
S. epidermidis 444	+	+	Chokr *et al.* (2005)
S. epidermidis 341	+	+	Chokr *et al.* (2005)
S. epidermidis 455a	+	−	Chokr *et al.* (2005)
S. epidermidis 455b	+	+	Chokr *et al.* (2005)
S. epidermidis 456a	+	+	Chokr *et al.* (2005)
S. epidermidis 521a	−	−	Chokr *et al.* (2005)
S. epidermidis 28	+	+	Chokr *et al.* (2005)
S. aureus 383	+	+	Eleaume & Jabbouri (2004)
S. aureus 343	+	+	Chokr *et al.* (2005)
Staphylococcus lugdunensis 18a	+	−	Chokr *et al.* (2005)

(5000 ***g***, 4 °C, 20 min), suspended in 0.9% NaCl, sonicated on ice in a plastic container (IKASONIC sonicator, IKA Labortechnik, Staufen, Germany) at 50% amplitude, cycle 0.5, 4 × 30 s to extract the cell-associated extracellular material. Cells were removed by centrifugation (5000 ***g***, 15 min at 4 °C). The supernatant was clarified by a short high-speed centrifugation (12 000 ***g***, 15 min at 4 °C) and filter-sterilized to give a crude extract, which was further concentrated using Amicon® Ultrafiltration Cell with a 10 kDa molecular cut-off membrane (Millipore, Bedford, MA). After the proteins were precipitated with the addition of trichloroacetic acid to a final concentration of 5% and then removed by centrifugation, the concentrated crude extract was applied to a Sephacryl S-300 column. Five-milliliter fractions were collected and analysed for the presence of aminosugars using Elson–Morgan assay, with subsequent colorimetric detection at 530 nm (Enghofer & Kress, 1979), and for the presence of neutral sugars using phenol-sulfuric acid assay and colorimetric detection at 485 nm (Dubois *et al.*, 1956). Fractions, corresponding to PNAG, which were positive only at Elson–Morgan assay, were pooled and freeze-dried. Fractions corresponding to EC-TA, which gave colour reaction at both assays, were freeze-dried and, if necessary, purified as described previously (Sadovskaya *et al.*, 2004).

Biofilm detachment assays

Dispersin B, purified as described by Kaplan *et al.* (2003), was a generous gift of J. K. Kaplan, C. Ragunath, and N. Ramasubbu (New Jersey Dental School, Newark, NJ). TSB supplemented with 0.25% glucose was inoculated with 2% (v/v) of the overnight cultures. The wells of a 96-well polystyrene tissue culture treated Nunclon microtiter plate (Nunc, Roskilde, Denmark) were filled with 200 μL aliquots of inoculum, and the plate was incubated statically for 24 h at 37 °C. The biofilms were washed with 200 μL 0.9% NaCl and then treated for 2 h at 37 °C with 100 μL of dispersin B (40 μg mL^{-1}) in PBS Buffer (50 mM sodium phosphate, pH 5.8, 100 mM NaCl) or 100 μL of proteinase K (Sigma, St Louis, MO, 100 μg mL^{-1}) in Tris-buffer (20 mM, pH 7.5, 100 mM NaCl). Control wells were filled with appropriate buffers. After treatment, the biofilms were washed with 200 μL 0.9% NaCl, dried for 45 min at 55 °C, stained with 5% safranine for 5 min, and then washed again with 200 μL of 0.9% NaCl. One hundred microliters of 0.9% NaCl per well was added and the optical density was measured using a microtiter plate reader (Bio-Tek Instruments, Winooski, VT), set to 492 nm. Statistical analysis was performed using a Mann–Whitney *U*-test (http://eatworms.swmed.edu/~leon/stats/utest.cgi).

General and analytical methods

Glycerol and monosaccharides were identified by gas-liquid chromatography (GC) on a Shimadzu GC-14 B gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a Zebron ZB-5 capillary column (30 m × 0.25 mm, Phenomenex, Torrance, CA), with hydrogen as carrier gas, using a temperature program 170 °C (3 min) → 260 °C at 5 °C min^{-1}. Polysaccharide samples were converted to alditol acetates by conventional methods following hydrolysis with 4 M trifluoracetic acid (TFA) at 120 °C for 2 h. Samples containing TA were depolymerized with 48% HF prior to hydrolysis.

Gel permeation chromatography was carried out on a Sephacryl S-300 (1 × 90 cm) and Sephadex G-50 (1.6 × 75 cm, Pharmacia Biotech, Uppsala, Sweden) eluted with water. Dialysis was performed in Spectra/Por membranes (Spectrum Laboratories, Rancho Dominguez, CA) with molecular weight cut-off of 6–8 kDa against de-ionized water.

Nuclear magnetic resonance (NMR) spectra were recorded at 25 °C in D_2O with a Varian UNITY INOVA 500 instrument using acetone as reference (^{1}H, δ 2.225 ppm, ^{13}C, δ 31.5 ppm). Freeze-dried PNAG samples were dissolved in 5 M DCl (30 μL), and the total volume was adjusted to 1 mL with D_2O.

Results

Biofilm production and composition

The selection of the strains was based on the previously performed screening of 66 clinical CoNS strains isolated from the infected orthopaedic prosthesis. These isolates were screened for the biofilm-producing ability, presence of the *ica* locus, and the ability to produce PIA by the immuno dot-blot using a PIA-specific antiserum

(Chokr *et al.*, 2005). Ten biofilm-positive (Bf^+) strains and *S. epidermidis* 521a (Bf^-, PIA^+, *ica*$^-$) have been selected for our study (Table 1). The strain *S. aureus* 383 from the same collection has been previously described by Eleaume & Jabbouri (2004). Bacteria were grown statically in glass Erlenmeyer flasks in TSB medium, a condition which has been previously established as favourable for biofilm and PNAG production for a model strain *S. epidermidis* RP62A (Sadovskaya *et al.*, 2005). It was observed that the biofilms produced by different strains had a varying level of ability to adhere to the glass surface. Some biofilms formed a thick layer on the flask bottom and required to perform its detachment with glass beads (strains *S. epidermidis* 5, *S. epidermidis* 444, and *S. aureus* 343), while other strains formed aggregates suspended in the growth medium or only weakly adhered to the glass surface. Preliminary experiments have shown that the elution profiles of the crude deproteinated extracellular matrices of cells adherent and nonadherent to the bottom of the flask were very similar (data not shown). Extracellular polymers were extracted by a mild sonication procedure with the minimal cell lysis, and proteins were precipitated from the crude concentrated extracts by the addition of TCA up to 5% (Sadovskaya *et al.*, 2005). Extracellular extracts of all strains contained various but consistent amounts of proteins (data not shown). Using the previously established protocol of chromatographic separation and colorimetric detection (Sadovskaya *et al.*, 2005), it was possible to obtain the elution profiles of the extracellular extracts for each strain. Figure 1 presents typical chromatographic elution profiles of the deproteinated extracellular matrix of the investigated staphylococcal strains. The faster eluting carbohydrate fraction that gave positive reaction only in Elson–Morgan aminosugar assay was separated from the later eluting teichoic acid fraction, freeze-dried, and characterized by monosaccharide analysis and ^{1}H-NMR to verify its chemical structure. As can be seen in Figs 1a and b, biofilms produced by some strains had elution profiles very similar to that of the reference strain *S. epidermidis* RP62A (Sadovskaya *et al.*, 2005). Biofilms formed by these strains (*S. epidermidis* 5 and 444, *S. aureus* 343) contained considerable amounts of PNAG, comparable with that found in the reference strain. Other strains (*S. epidermidis* 341 and 521a, *S. lugdunensis* 18a) produced PNAG in much smaller quantities (the typical elution profile is shown in Fig. 1c). The largest group of strains (*S. epidermidis* 455a, 455b, 456a, 28 and *S. aureus* 383), while

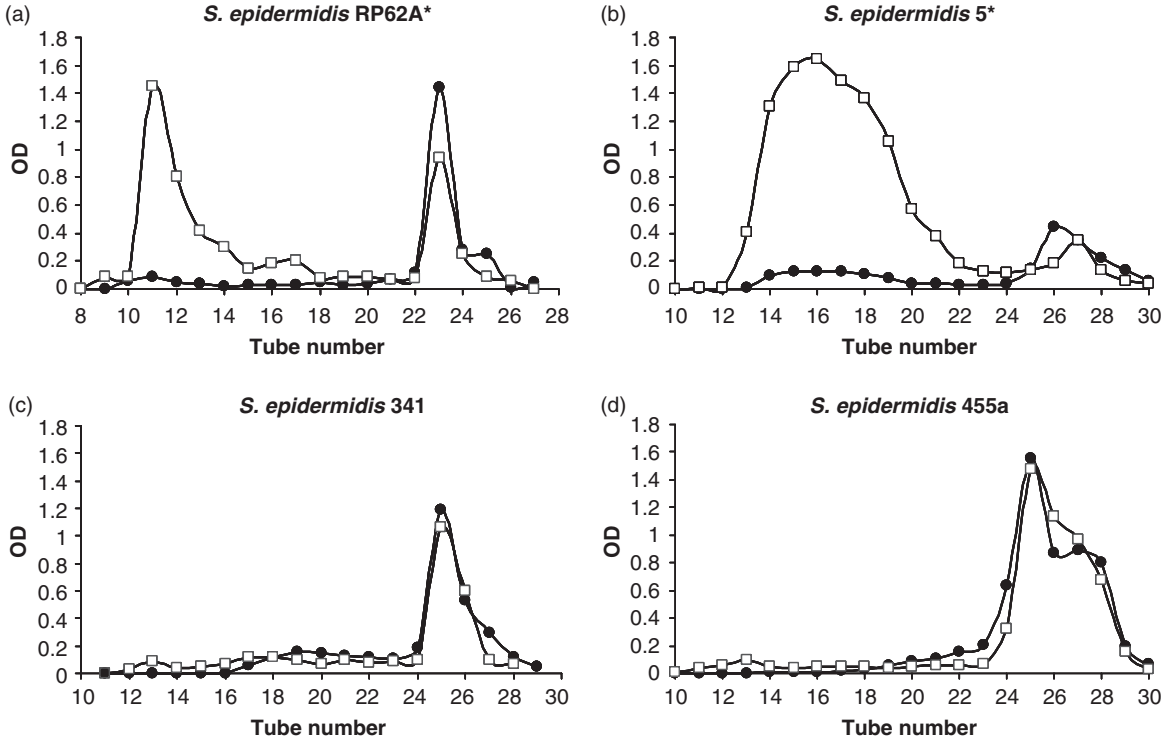

Fig. 1. Typical elution profiles of the extracellular extracts of *Staphylococcus epidermidis* RP62A (a) and some clinical staphylococcal strains (b–d) on a Sephacryl S-300 column. Aliquots (200 μL) of each 5 mL fraction were assessed for neutral sugars (●, $OD_{485\,nm}$) and aminosugars (□, $OD_{530\,nm}$). Data correspond to 3 L of culture for *S. epidermidis* RP62A and 5, and 5 L of culture for *S. epidermidis* 341 and 455a. Strains producing substantial amounts of PNAG are marked with an asterix. Elution profile (b) is typical for the strains *S. epidermidis* 5, 444 and *Staphylococcus aureus* 343; profile (c) for *S. epidermidis* 341 and 521a and *Staphylococcus lugdunensis* 18a), profile (d) for *S. epidermidis* 455a, 455b, 456a, 28 and *S. aureus* 383.

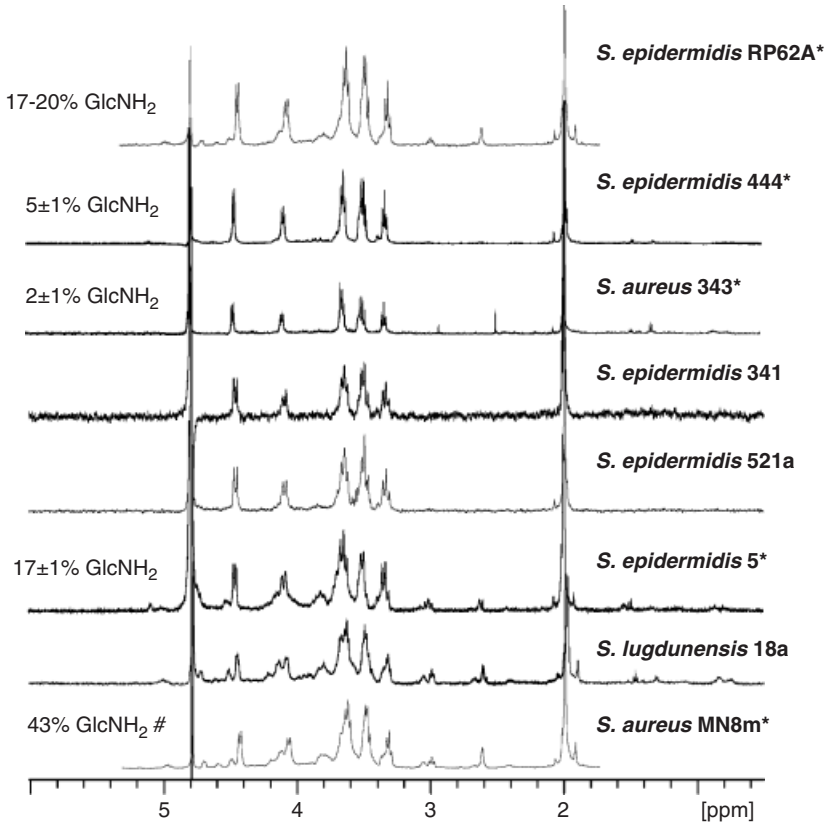

Fig. 2. Proton-nuclear magnetic resonance spectra of the poly-β-(1 → 6)-*N*-acetyl-D-glucosamine (PNAG) fractions isolated from the extracellular extracts of model strains *Staphylococcus epidermidis* RP62A, *Staphylococcus aureus* MN8m, and clinical staphylococcal strains. Strains producing substantial amounts of PNAG are marked with an asterix. #Data of Joyce *et al*. (2003).

producing a thin biofilm on a glass surface, did not seem to produce any detectable amount of PNAG (Fig. 1d). All biofilms contained considerable amounts of the extracellular teichoic acid, the presence of which has been confirmed by the GC analysis of the later-eluting fraction.

Although all strains were grown *in vitro* in the same conditions using the TSB growth medium that is favourable for production of PNAG (Sadovskaya *et al*., 2005), relative amounts of PNAG and EC-TA varied greatly for different strains as can be seen from the elution profiles (Fig. 1). Presence of PNAG in the biofilms of the strains *S. epidermidis* 5, *S. epidermidis* 444 and *S. aureus* 343 made them more adherent to the glass surface, forming a dense, well-structured biofilm, which is in agreement with the previous studies (Jefferson 2004).

Structural characterization of PNAG

The PNAG fraction was collected, freeze-dried and analysed by GC and ^{1}H-NMR spectroscopy. The relative amount of nonacetylated glucosamine residues in PNAG was assayed with a combination of Smith & Gilkerson and Elson–Morgan assays, as described earlier for *S. epidermidis* RP62A (Sadovskaya *et al*., 2005). The spectra corresponding to the material isolated from the biofilms produced by the strains *S. epidermidis* 5, 444, 341, and 521a, *S. lugdunensis* 18a and *S. aureus* 343 are presented in Fig. 2. The ^{1}H-NMR spectra are similar to those reported for the poly-(1 → 6)-*N*-acetyl-β-D-glucosamine backbone of the polysaccharide antigens isolated from the biofilms of the model strains *S. epidermidis* RP62A (Mack *et al*., 1996) and *S. aureus* MN8m (Joyce *et al*., 2003) with the only differences being in the degree of relative charge. ^{1}H-NMR spectra of PNAG from *S. epidermidis* 444, 341, 521a, and *S. aureus* 343 correspond to a regular poly-(1 → 6)-*N*-acetyl-β-D-glucosamine backbone, which is in agreement with the chemically determined relatively low amount of de-*N*-acetylated glucosamine residues: 5 ± 1% for *S. epidermidis* 444 and 2 ± 1% for *S. aureus* 343, which is significantly lower than that found with *S. epidermidis* RP62A [17–20% (Mack *et al*., 1996; Sadovskaya *et al*., 2005)]. The ^{1}H-NMR spectrum of PNAG produced by *S. epidermidis* 5 is more heterogeneous and very similar to that of *S. epidermidis* RP62A, in good agreement with the results of the chemical analysis (*c*. 17 ± 1% free amino groups for *S. epidermidis* 5). The ^{1}H-NMR spectrum of *S. lugdunensis* 18a more closely resembles that of *S. aureus* MN8m (46 ± 9% free NH_2 found in our preparations, 43% according to Joyce *et al*. (2003). Resonances characteristic of the *O*-succinyl substitution (methylene protons centered at 2.6 ppm) were clearly seen in the ^{1}H-NMR spectra of PNAG of *S. epidermidis* 5 and *S. lugdunensis* 18a (Fig. 1). A small sharp signal at *c*. 2.6 ppm, corresponding to the free succinate (Joyce *et al*.,

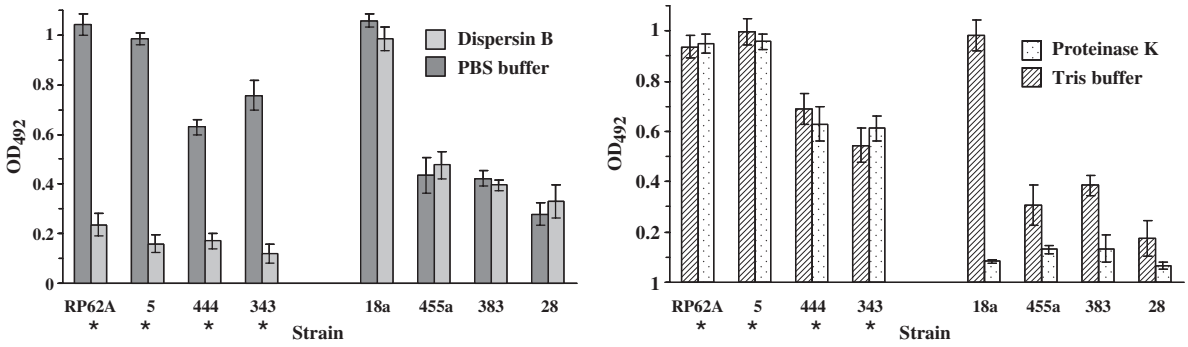

Fig. 3. Biofilm detachment assays of *Staphylococcus epidermidis* RP62A and clinical staphylococcal strains *S. epidermidis* 5, 444, 455a, 28; *Staphylococcus aureus* 343, 383, and *Staphylococcus lugdunensis* 18a by dispersin B. Preformed biofilms were treated with dispersin B (40 μg mL^{-1}) in PBS buffer and with proteinase K (100 μg mL^{-1}) in Tris buffer for 2 h at 37 °C. Control wells were filled with the appropriate buffers. Average results ± standard deviations for eight wells for each strain are shown. The experiments were performed at least twice with similar results. Strains producing substantial amounts of poly-β-(1 → 6)-*N*-acetyl-D-glucosamine (PNAG) are marked with an asterisk.

2003), was present in the sample of PNAG from *S. epidermidis* 444, de-*O*-acylated with ammonium hydroxide (data not shown).

Biofilm detachment

It has been shown that the biofilm of PGA-producing *E. coli* is dispersed by the treatment with sodium periodate $NaIO_4$. Biofilms of *A. actinomycetemcomitans* and *A. pleuropneumonia* are degraded with dispersin B, an enzyme having a PNAG-hydrolysing activity (Kaplan *et al.*, 2003, 2004a). Dispersin B also efficiently removed biofilms produced by the four clinical strains of *S. epidermidis*, isolated from the surfaces of infected intravenous catheters (Kaplan *et al.*, 2004b). We have recently demonstrated that several strains of CoNS formed biofilms that did not contain any detectable amount of PNAG. These biofilms were not sensitive to $NaIO_4$ but were partially degraded with proteinase K (Kogan *et al.*, 2005). In the present study, we tested the sensitivity of the biofilms of the studied staphylococcal strains to proteinase K and dispersin B. As follows from the results presented in Fig. 3, the susceptibility of the biofilms to different enzymatic treatments correlates well with the chemical composition of their extracellular matrix: biofilms of the strains *S. epidermidis* 5, 444 and *S. aureus* 343, which produced a considerable amount of PNAG *in vitro*, were sensible to dispersin B ($P < 0.001$) and insusceptible to proteinase K. On the other hand, biofilms of the strains, which did not produce PNAG, were dispersed with proteinase K ($P < 0.001$) but not with dispersin B (Fig. 3).

Discussion

It is currently generally recognized that biofilms probably comprise the normal environment for most microbial cells in both natural and artificial habitats. Biofilms play an important role in interactions of both nonpathogenic and pathogenic bacteria with the host. Nonpathogenic biofilms in the mammalian gut and on the plant roots provide barriers to invading pathogens (Reid *et al.*, 2001), while pathogens use biofilms to evade host immune defence and antibiotic therapy (Davies, 2003). The major 'cement' for various components of biofilms of different origin is a mixture of polysaccharides secreted by the cells encased in the biofilm (Sutherland, 2001). Therefore, elucidation of the structure of the exopolysaccharides and other carbohydrate-containing moieties composing biofilms, understanding their biosynthesis, and finding the ways of their decomposition present the priority issues in a successful strategy of fighting biofilm-associated microbial infections. In the present study we investigated the chemical composition of carbohydrate-containing polymers of a number of biofilm-positive staphylococcal strains associated with the infections of orthopaedic implants. Of the 11 biofilm-producing clinical staphylococcal strains studied, three (*S. epidermidis* 5 and 444; *S. aureus* 343) produced high amounts of PNAG *in vitro*. The production of PNAG by *S. epidermidis* 5 was higher than that of the model strain S. *epidermidis* RP62A, and therefore this strain may be considered as a PNAG overproducing strain. In agreement with the results of the chemical analysis of the extracellular matrix, the biofilm of these strains was sensitive to dispersin B, thus showing that PNAG was the polymer stabilizing the intercellular structure of their biofilms.

Three strains (*S. epidermidis* 341 and 521a, *S. lugdunensis* 18a) were found to produce small but detectable amount of PNAG. Other strains (455a, 455b, 456a, 28 and 383) did not produce *in vitro* PNAG in an amount that could be detected by direct chemical methods. While the presence of trace amounts of PNAG cannot be excluded, we suggest that biofilms of these strains contain mainly teichoic acid and protein components, which could be easily isolated from

their extracellular extract. These constituents were previously shown to be an important part of the biofilm produced by a reference strain *S. epidermidis* RP62A (Sadovskaya *et al*., 2005) and other clinical staphylococcal strains (Kogan *et al*., 2006). In agreement with this hypothesis, biofilms of these strains were partially disrupted with proteinase K, but not with dispersin B, implying that protein components of the biofilm indeed play an important role in stabilizing its intercellular structure.

Surprisingly, two *ica*$^{-}$ strains, *S. epidermidis* 521a and *S. lugdunensis* 18a, revealed ability to synthesize PNAG. The absence of *ica* locus was assessed by four different couples of primers (Chokr *et al*., 2005), however, some variability in the *ica* locus sequence can not be excluded. On the other hand, having in mind the described involvement of the *pga* gene cluster in biosynthesis of PNAG in *E. coli* and of its homologues in several other bacteria (Wang *et al*., 2004), it is possible that a similar locus is present in the investigated staphylococcal strains and accounts for the synthesis of the detected PNAG in the *in vitro* grown biofilm (Kaplan *et al*., 2004a).

All the PNAG-producing strains synthesized the polysaccharide with the poly-(1 → 6)-*N*-acetyl-β-D-glucosamine backbone with various degrees of *N*-deacetylation and *O*-succinylation. It has been recently shown that the surface-attached protein IcaB is responsible for the deacetylation of PNAG, and that deacetylation is essential for biofilm formation, colonization, and virulence in an animal model of implant infection (Vuong *et al*., 2004). In view of these findings, it would be interesting to evaluate the immunological properties of different staphylococcal PNAG and to assess the differences in the virulence of the studied staphylococcal strains. Such study using an animal model of the implant-associated infection is currently in progress at our laboratory.

Summarizing, in this study we have shown that different biofilm-producing clinical staphylococcal strains isolated from infected orthopaedic prosthesis, produce biofilms that do or do not contain various amounts of PNAG, but that always contain protein components and extracellular teichoic acids. PNAG produced by these strains had different degrees of GlcNAc deacetylation. The susceptibility of the biofilms to different enzymatic treatments correlated well with the chemical composition of their extracellular matrix.

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