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Ultrastructural Organization and Regulation of a Biomaterial Adhesin of *Staphylococcus epidermidis*

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Coagulase-negative staphylococci have emerged as important pathogens in infections associated with intravascular devices. Microbial adherence to biomaterial surfaces is a crucial step in the pathogenesis of these infections. Staphylococcal surface proteins (herein referred to as SSP-1 and SSP-2) are involved in the attachment of *Staphylococcus epidermidis* 354 to polystyrene. In the present study we show that the adhesin protrudes from the cell surface as a fimbria-like polymer. Furthermore, in vitro proteolytic cleavage of SSP-1 produces an SSP-2-like protein which coincides with a loss of adhesive function. SSP-1 expression is down-regulated in a phenotypical variant of *S. epidermidis* 354 whereas SSP-2 expression is not. These results could suggest that proteolytic cleavage is a key to the regulation of the adhesive state of *S. epidermidis* in vivo.

Over the last 2 decades coagulase-negative staphylococci (CoNS), particularly strains of *Staphylococcus epidermidis*, have emerged as important pathogens in association with foreign bodies, e.g., intravascular devices. Especially in immunocompromised patients, such as cancer patients and premature neonates, catheter-related sepsis due to CoNS may be a life-threatening complication (1, 2, 5, 12, 13). For many microorganisms, adhesion to host surfaces is a crucial step in the pathogenic process and a prerequisite for colonization of these surfaces (3). It has been demonstrated that CoNS adhere avidly to a variety of biomaterials such as Teflon and polystyrene (6, 8, 16, 19). The initial phase of attachment was found to be correlated with surface hydrophobicity. Both hydrophobicity and adherence were reduced by proteases, such as pepsin, suggesting that hydrophobic cell surface proteins may play an important role in the initial attachment to biomaterials (8, 16). In a previous study (19), we identified a proteinaceous antigen of *S. epidermidis* 354 that apparently is involved in adherence to polystyrene. Evidence was obtained that this antigen, herein referred to as staphylococcal surface proteins (SSP-1 and SSP-2), is present on fimbria-like appendages protruding from the bacterial cell surface. A monoclonal antibody specific for SSP-1 and SSP-2 was shown to be specific and as effective as proteases in preventing this staphylococcal strain from adhering to polystyrene (19). This strain thus provides a model system to study the adherence of CoNS to intravascular devices. In the present study we present data concerning the ultrastructural organization of the staphylococcal fimbria-like appendages, the structural and functional relationship between the antigenically related SSP-1 and SSP-2 proteins, and aspects of adhesion regulation in vivo.

Materials and methods. *S. epidermidis* 354 was isolated from a blood culture of a patient with catheter-related bacteremia. This strain is typical of CoNS with respect to hydrophobicity and adherence to biomaterials.

The monoclonal antibody (designated MAb 36.4 [18]) directed against two proteins (SSP-1 and SSP-2) of *S. epidermidis* 354 was purified from ascitic fluid. Several other strains express antigenically related proteins as assessed with other antibodies (e.g., MAb 36.3 [18]).

For cryo-ultramicrotomy and immunogold-labeling purposes, staphylococci, grown on blood agar plates, were inoculated in nutrient broth (Difco) and cultured overnight at 37°C. After being washed with phosphate-buffered saline (PBS), the bacteria were resuspended and fixed for 1 h in PBS containing 2% paraformaldehyde and 0.25% glutaraldehyde. Subsequent cryo-ultramicrotomy and immunogold labeling were performed as described previously (11, 20), with a goat anti-mouse 1-nm-diameter gold-bead antibody (Aurion, Wageningen, The Netherlands). After the labeling procedure, a Danscher silver enhancement (20 to 25 min) (7), washing with milliQ water (three times for 2 min each), and negative staining with uranyl acetate (saturated, 2 min) were done. Dried preparations were examined with a Philips model CM10 electron microscope at 100 kV. For electron microscopy of soluble proteins, nickel grids with a carbon-Formvar film were exposed for 2 min to the staphylococcal protein suspension, briefly rinsed with PBS, and subsequently left to dry to prevent loss of protein during subsequent incubations. Proteins bound to the grids were labeled as described above.

Cell wall proteins of *S. epidermidis* 354 were isolated as described previously (19) with lysostaphin (Sigma), an endopeptidase which splits glycyl-glycine linkages in the penta- or hexapeptide cross bridge of the peptidoglycan of staphylococci (15). For cell wall protein isolation, *S. epidermidis* was grown overnight in nutrient broth (Difco), with either shaking or rolling at 37°C. Trypsin digestions of cell surface proteins were performed for 15 min at 37°C, with a final concentration of 10 μg of trypsin per ml. Crude staphylococcal protein preparations (supernatants) were purified by using a fast protein liquid

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chromatography gel permeation column (Superose 12; Pharmacia). PBS (pH 7.2) was used as the eluent. Protein samples and fractions were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentrations were determined by using a bicinchoninic acid reagent (Pierce). SDS-PAGE was performed as described previously (10). Gels contained 7.5% (wt/vol) acrylamide. Proteins were either transferred to nitrocellulose paper (100 V, 1 h) or silver stained by subsequent incubations in 50% methanol–12% trichloracetic acid–2% $CuCl_2$ (10 min), 10% ethanol–5% acetic acid (5 min), 0.01% $KMnO_4$ (5 min), 10% ethanol–5% acetic acid (5 min), 10% ethanol (5 min), H_2O (5 min), 0.1% $AgNO_3$ (5 min), H_2O (20 s), 10% K_2CO_3 (1 min), 0.01% formaldehyde–2% K_2CO_3 (as long as necessary), 10% ethanol–5% acetic acid (20 s), and H_2O (15 min). Nitrocellulose blots were stained with MAb 36.4, a goat anti-mouse alkaline phosphatase conjugate (Jackson Immunoresearch Laboratories Inc.), and the phosphatase substrates nitroblue tetrazolium (Sigma) and 5-bromo-4-chloro-3-indolylphosphate (BCIP; Sigma). The phosphatase reaction was carried out at pH 9.5 (4).

Adherence of staphylococci to polystyrene was examined as previously described (19), with minor modifications. Briefly, bacteria were inoculated into Mueller-Hinton broth (Difco) and labeled with [^{3}H]adenine overnight at 37°C. Prior to use, bacteria were washed three times with PBS. Finally, the bacteria were resuspended in PBS to a final concentration of 5×10^8 CFU/ml. Polystyrene spheres (Sanbio) were preincubated for 1 h at 37°C in a 20-well microtiter plate (Abbott) with 150 μl of the various protein solutions. During this preincubation, proteins from the solution were able to block the *S. epidermidis* 354 binding sites of the polystyrene spheres. Then, the preincubation solution was replaced by 200 μl of the bacterial suspension. After 4 h of incubation at 37°C, the spheres were rinsed with PBS and transferred to Beckman biovials. After the addition of scintillation fluid (Safe Fluor S; Lumac/3M), the radioactivity of the adherent label was determined in a Philips scintillation counter. All experiments were performed at least twice in duplicate. The student t test was used to determine whether the adherence inhibition curves were statistically different. To determine t values, the pooled standard deviation was calculated by standard methods (21). Differences were considered to be significant when P was <0.05. As an estimate of adherence inhibition efficiency, the 50% inhibitory doses of the different protein solutions were calculated. The 50% inhibitory dose is an estimate of the protein concentration (in micrograms per milliliter) that causes 50% inhibition of adhesion by using standard conditions.

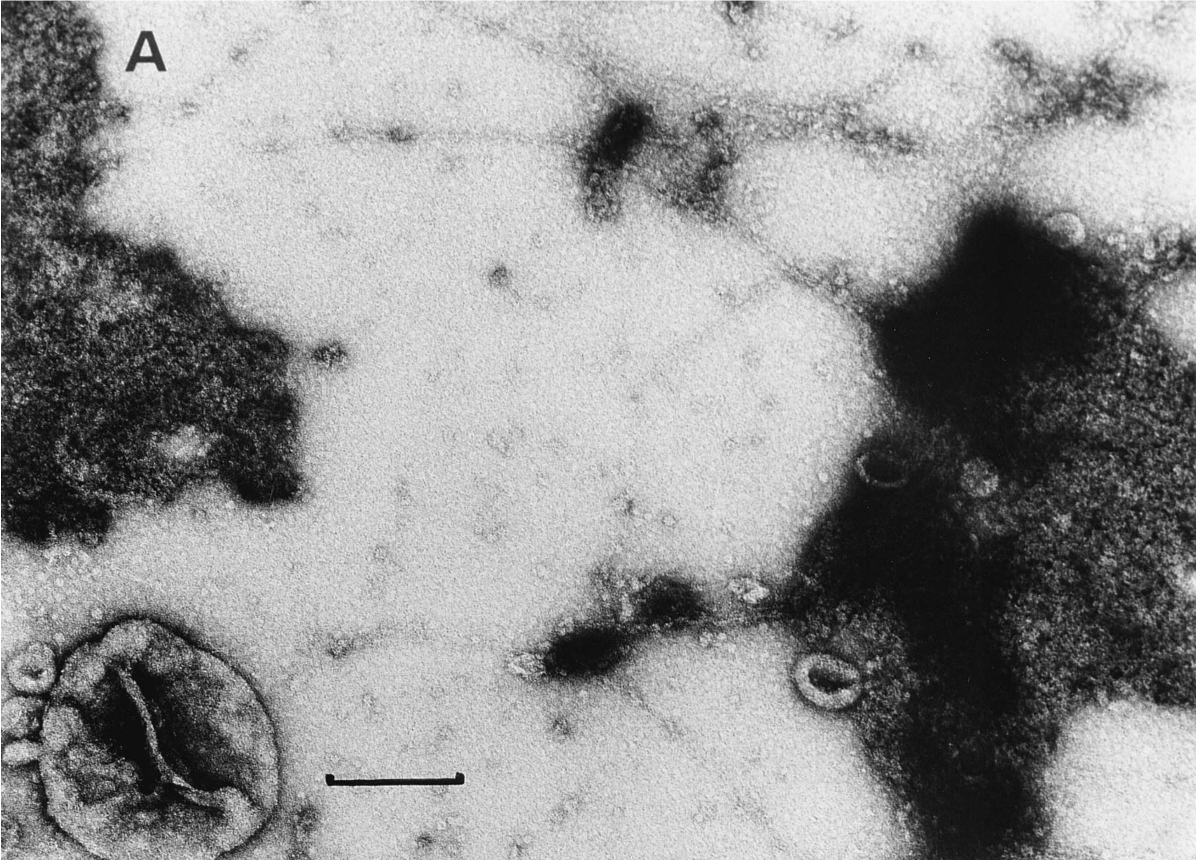
Results and discussion. In a previous study (19), we showed that SSP-1 and SSP-2 mediate adhesion to biomaterials and that these proteins are localized on the cell surface and on fimbria-like structures. We addressed the question of whether the localization of SSP-1 and SSP-2 is restricted to the tip of the fimbria-like appendages or whether the appendages are synthesized as polymers of SSP-1 and SSP-2. We isolated surface proteins (from *S. epidermidis* 354 with the common phenotype [see below]), using lysostaphin digestion of peptidoglycan. As shown in Fig. 1A, fiber-like structures are present in preparations of staphylococcal cell surface proteins, as visualized by electron microscopy. When immunogold labeling followed by electron microscopy is applied to cryosections of staphylococci and to isolated surface proteins, it appears that SSP-1 and SSP-2 are present in clusters, both on the surfaces of cryosectioned staphylococci (Fig. 1B) and in the cell wall protein preparation (Fig. 1C). Frequently, a linear arrangement of labeling was observed, indicating that a single fiber contains multiple molecules of SSP-1 and/or SSP-2. Although additional proteins may be included in the fibers, it appears that SSP-1 and SSP-2 play a structural role in providing an interaction interface for polymerization, in addition to a role in adhesion. This may be similar to the molecular and functional organization of the fimbriae of *Escherichia coli* (reviewed in reference 9).

Analysis by SDS-PAGE and Western blotting (immunoblotting) identifies two distinct molecular forms of the adhesin (Fig. 2A and B, lanes 1). SSP-1 exhibits a molecular mass of approximately 280 kDa, whereas SSP-2 has an estimated molecular mass of 250 kDa. When these proteins were characterized further, it was observed that SSP-1 is trypsin sensitive, whereas SSP-2 is not. Following incubation in the presence of trypsin, all SSP-1 disappears whereas the band of SSP-2 in protein gels and on Western blots is more prominently present (Fig. 2A; Fig. 2B, lanes 1 and 2), indicating that SSP-1 can be converted to SSP-2 or to an SSP-2-like protein in vitro.

Some preparations of *S. epidermidis* 354 surface proteins do not contain SSP-1 but contain only SSP-2 (Fig. 2A, lane 3). This *S. epidermidis* 354 is phenotypically aberrant compared with the *S. epidermidis* 354 characterized in Fig. 2A (lane 1) and that characterized by Timmerman et al. (19). We refer to the phenotype exhibiting either expression of both SSP-1 and SSP-2 or expression of SSP-2 alone as the common or variant phenotype, respectively. The cause of the phenotypic variation in the expression of SSP-1 and SSP-2 is, at present, unknown; further research is required to elucidate the environmental stimuli and the signal transduction pathways involved. It is clear, however, that SSP-1 expression and function are regulated, whereas SSP-2 is present on the surface of *S. epidermidis* 354 with both the common and variant phenotypes. Apparently, *S. epidermidis* is able to down-regulate the expression of SSP-1 if the environmental conditions require that it do so. Other microorganisms have been shown to regulate the expression of adhesins as well (3, 9, 14).

Many proteins possess the property to bind to biomaterials such as polystyrene and Teflon. For example, coating of Teflon catheter segments with bovine serum albumin (BSA) abolishes adherence of *S. epidermidis* in vitro (16). To identify high-affinity protein-biomaterial interactions, the concentration-effect relationship of various protein preparations and fractions was studied in an adhesion inhibition assay. The question was whether staphylococcal surface proteins and especially SSP-1 and SSP-2 are more potent competitors for polystyrene binding sites than other unrelated proteins. This was assayed by determining the extent of binding of labeled bacteria to polystyrene spheres after preincubation of these spheres with different concentrations of protein. In Fig. 3, the concentration-inhibition curves of BSA, unpurified *S. epidermidis* 354 proteins (containing both SSP-1 and SSP-2), and partially purified SSP-1 and SSP-2 are shown. Unpurified staphylococcal proteins inhibit adhesion of *S. epidermidis* 354 to a greater extent than BSA does. Par-

FIG. 1. (A) Fiber-like structures in preparations of staphylococcal proteins visualized by electron microscopy. (B) Immunogold labeling of ultrathin cryosections of *S. epidermidis* 354 with MAb 36.4 and silver-enhanced 1-nm gold goat anti-mouse antibody. The material observed between cells is gelatin (used in the immunohistochemical procedure). Frequently, a linear arrangement of labeling is observed (arrowheads). (C) Immunogold labeling of SSP-1 and SSP-2 derived from common phenotypic variant of *S. epidermidis* 354. The figure shows clustering and a linear arrangement of the label. No labeling was observed in negative controls (no MAb 36.4 was used [data not shown]). Bars, 200 nm.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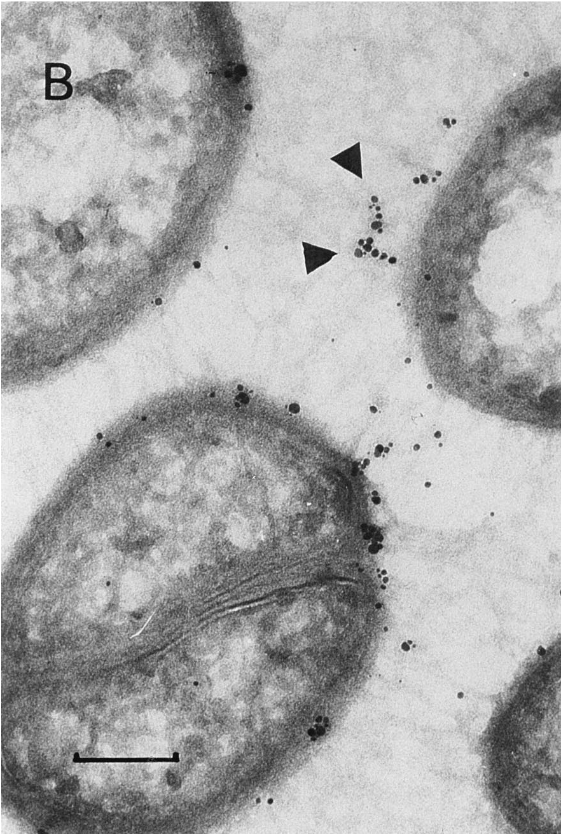
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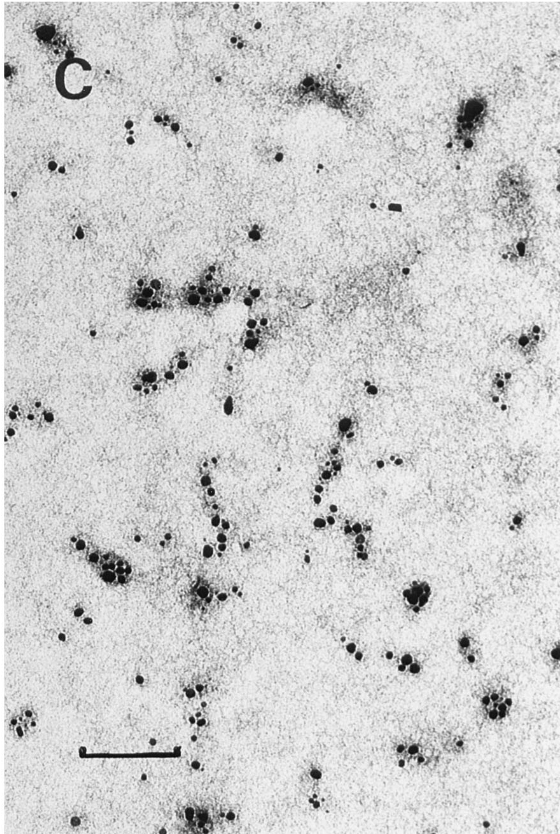
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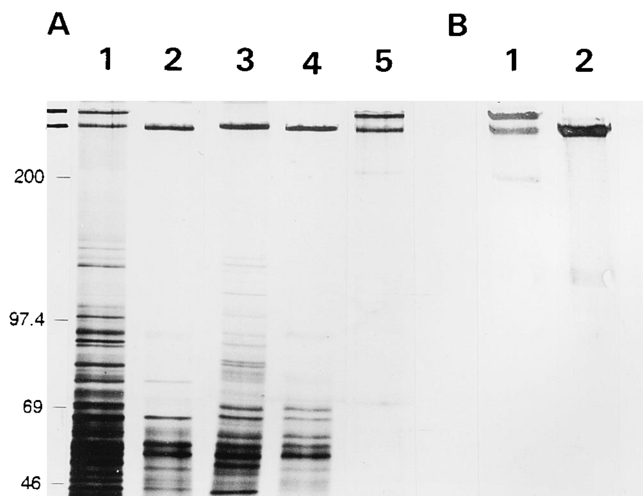

FIG. 2. (A) Silver-stained polyacrylamide gel. Lanes: 1, protein preparation of common phenotype variant of *S. epidermidis* 354, containing both SSP-1 and SSP-2 (see panel B, lane 1); 2, trypsin digestion of preparation used in lane 1 (see panel B, lane 2); 3, protein preparation of phenotypically variant *S. epidermidis* 354, SSP-1 is not present; 4, trypsin digestion of the preparation used in lane 3; 5, SSP fraction, originating from a run in the gel permeation column, contains increased specific activities of SSP-1 and SSP-2. (B) Immunolabeled Western blot. Lanes: 1, control incubation of common phenotype proteins (both SSP-1 and SSP-2 present) at 37°C without trypsin (see panel A, lane 1); 2, trypsin digestion of preparation used in lane 1 (see panel A, lane 2). Molecular size markers (in kilodaltons) are indicated on the left.

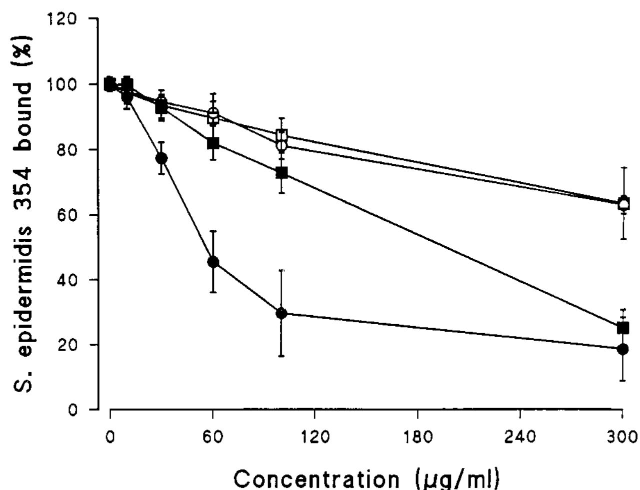

FIG. 4. Concentration-effect curves of protein preparation of phenotypically common *S. epidermidis* 354, containing both SSP-1 and SSP-2, without (●) and with (○) trypsin digestion (Fig. 2A, lanes 1 and 2, respectively) and protein preparation of phenotypically variant *S. epidermidis* 354, containing SSP-2 but not SSP-1 without (■) and with (□) trypsin digestion (Fig. 2A, lanes 3 and 4, respectively). The protein concentrations of trypsin-treated protein preparations are the concentrations prior to trypsin digestion, so differences with the corresponding not-trypsin-treated preparations are fully caused by the disappearance of trypsin-sensitive proteins. Trypsin alone had no effect in the concentrations used.

tially purified SSP-1 and SSP-2, in which the specific concentration of SSP-1 and SSP-2 is 13.8 times higher than that in unpurified staphylococcal proteins, is even more potent, as expected on the basis of previously reported results (19) in which we identified SSP-1 and SSP-2 as the major biomaterial adhesins of *S. epidermidis* 354. The differences between the various protein preparations are statistically significant at concentrations of 30 and 60 μg/ml ($P < 0.025$).

Our observation that SSP-1 is trypsin sensitive but that SSP-2 is not offered the opportunity to discriminate functionally between SSP-1 and SSP-2. In Fig. 2 the SDS-PAGE patterns of the common-phenotype surface protein preparation (both SSP-1 and SSP-2 are present) (Fig. 2A, lane 1), its trypsin digest (Fig. 2A, lane 2), the variant-phenotype protein preparation (no SSP-1 is present) (Fig. 2A, lane 3), and its trypsin digest (Fig. 2A, lane 4) are shown. The concentration-effect curves for these proteins (Fig. 4) show that the protein preparation containing both SSP-1 and SSP-2 inhibits adherence of *S. epidermidis* 354 to polystyrene much more effectively than the protein preparation of the phenotypic variant of *S. epidermidis* 354 (no SSP-1 present) at concentrations of 30, 60, and 100 μg/ml ($P < 0.0005$). The latter preparation exhibits an adherence inhibition capacity similar to that of BSA (Fig. 3); the variant-phenotype surface proteins inhibit adherence of staphylococci to a greater extent only at relatively high protein concentrations (300 μg/ml). This may indicate that some low-affinity interactions are mediated by surface proteins distinct from SSP-1. Trypsin digestion of protein preparations of both phenotypes decreases the capacity to inhibit *S. epidermidis* 354 binding, but the effect of trypsin digestion on the preparation containing both SSP-1 and SSP-2 is much more pronounced than the effect on the preparation containing only SSP-2. For the latter preparation, the effect of trypsin digestion becomes statistically significant only at concentrations of 100 and 300 μg/ml ($P < 0.025$), whereas for the preparation containing both SSP-1 and SSP-2 the effect is already highly significant at a concentration of 30 μg/ml ($P < 0.0005$). The inhibition curves of both trypsin-digested protein preparations are not statistically different at any concentration.

To allow a direct comparison of the various surface protein preparations, 50% inhibition values were calculated from the data shown in Fig. 3 and 4 (Table 1). These values (in micrograms per milliliter) represent a semiquantitative estimate of the inhibitory activity of the various preparations in the adherence inhibition assay. These data clearly demonstrate the superior inhibitory activity of the preparations containing both

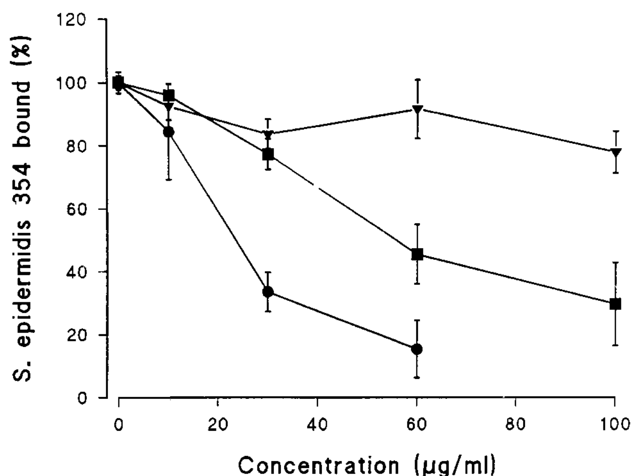

FIG. 3. Concentration-effect curves. *S. epidermidis* 354 binding to polystyrene spheres after preincubation of these spheres with different concentrations of BSA (▼), cell surface proteins of phenotypically normal *S. epidermidis* 354 (■), and partially purified SSP-1 and SSP-2 (●). Error bars indicate standard deviations.

TABLE 1. Inhibition of staphylococcal adherence by various protein preparations

Protein	Concentration causing 50% inhibition (μg/ml)
Purified SSP-1 and SSP-2	23.6
Common phenotype surface proteins (SSP-1 + SSP-2 present)	55.8
Variant phenotype surface proteins (no SSP-1 present)	196
Trypsin-treated common phenotype proteins (no SSP-1 present)	>300
Trypsin-treated variant phenotype proteins (no SSP-1 present)	>300
BSA	>300

SSP-1 and SSP-2 compared with that of the preparations containing only SSP-2, indicating that SSP-1 rather than SSP-2 is the major biomaterial adhesin of *S. epidermidis* 354.

The structural, biochemical, and functional data presented here shed some new light on the molecular structure of the fimbria-like cell surface appendages, the structural and functional differences between SSP-1 and SSP-2, and the regulation of adhesion to biomaterials in vivo.

In addition, the data may have some important implications for the mechanisms that underlie these aspects of adhesion to biomaterials; e.g., if trypsin-digested SSP-1 is distinct from SSP-2, the variant phenotype arises because of down-regulation of SSP-1, which is the functional adhesin (Fig. 4; Table 1), whereas SSP-2 expression is not affected. This down-regulation would most likely be achieved by repression of transcription. We observed that multiple molecules of SSP-1 and SSP-2 are present in a single cell surface fiber (Fig. 1C). Since we cannot discriminate between SSP-1 and SSP-2 by immunohistochemistry, putative functions of SSP-2 would include involvement in the biogenesis of the SSP-1-containing fimbriae and adhesion to other nonbiomaterial surfaces, e.g., eukaryotic cells or staphylococci (intercellular attachment).

Alternatively, trypsin-digested SSP-1 may be identical to SSP-2. This is not unlikely since SSP-2 and trypsin-digested SSP-1 share not only a molecular mass of 250 kDa but also resistance to trypsin and an epitope recognized by MAb 36.4 as well. Sequence analysis is required to prove this. It would imply, however, that SSP-2 originates from proteolytic cleavage of SSP-1, probably mediated by a bacterial protease cleaving the same bond as trypsin (17). The variant phenotype may be generated by an increased proteolytic cleavage of SSP-1, which is under the control of presently unknown environmental conditions. The off switch of staphylococcal adherence may be mediated by an event which removes a functional domain from SSP-1, whereas the on switch results from de novo synthesis of SSP-1.

Whatever mechanism may be involved in down-regulating SSP-1, it is clear that the balance between production and inactivation of SSP-1, which apparently determines the adhesive state of *S. epidermidis* 354, is regulated by environmental conditions. The significance of such a regulation is that when these conditions are unfavorable, SSP-2 predominates over SSP-1, adhesiveness is lost, and the bacteria spread until a more favorable environment is reached. Further biochemical and functional characterizations of regulation of staphylococcal adhesion are required to characterize the molecular mechanisms involved and may be a successful approach to increase the understanding of infections related to intravascular and other prosthetic devices caused by CoNS.

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