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Identification of an Antigenic Marker of Slime Production for *Staphylococcus epidermidis*†

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The pathogenic *Staphylococcus epidermidis* strain RP62A (ATCC 35984) adheres to smooth surfaces by forming a tenacious bacterial film known as slime. The mechanism of slime production is not known; however, workers in the laboratory of G. Pier (Harvard Medical School, Boston, Mass.) have isolated from RP62A a galactose-rich capsular polysaccharide adhesin (CPA) which mediates the attachment of the organism to smooth surfaces. We have obtained two daughter strains from RP62A that no longer produce slime. One daughter strain, H4A, was obtained by selection for a spontaneous variant; the other strain, HAM892, was obtained by treating growing cultures of RP62A with acriflavin. Using an antiserum generated against whole cells of RP62A, we have examined lysozyme-lysostaphin digests of RP62A, H4A, and HAM892 by double immunodiffusion. The two strains that no longer produced slime no longer produced a particular antigen, which we refer to as the slime-associated antigen (SAA). SAA was also produced by unrelated strains of slime-producing *S. epidermidis*. SAA was heat and protease stable, had a molecular weight of >50,000, and could be partially purified by chromatographing trypsin-digested material over a Sephadex G-200 column. Chemical analysis of partially purified SAA by gas-liquid chromatography found SAA to be glucose rich (59%) and galactose poor (1.4%). This analysis chemically distinguished SAA from CPA. When tested together by double immunodiffusion with anti-RP62A and anti-CPA antisera, partially purified SAA did not cross-react with CPA. Kinetic studies suggested that SAA is a marker for surface accumulation whereas CPA mediates initial adherence.

When grown in liquid media, many strains of *Staphylococcus epidermidis* coat the walls of the culture vessel with an adhesive film of cells commonly known as slime (8). Clinical and laboratory studies have associated this phenomenon with the selective virulence of *S. epidermidis* for causing infections of indwelling medical devices (2, 8, 10, 11, 13, 19). The in vitro production of slime has been visualized directly on scanning electron micrographs as the gradual accumulation of bacteria on the surface (18) embedded in an amorphous extracellular matrix (8, 12, 19) that stains with the polysaccharide-specific stain Alcian blue (6, 8). Despite these observations, the production of slime remains a biologic phenomenon without specific biochemical or immunologic markers. The precise molecular mediators of slime production are unknown.

Two groups have reported the isolation and characterization of extracellular materials from *S. epidermidis* which may represent the adhesive component of slime. Peters and co-workers have isolated and partially purified a mannose-rich material from the *S. epidermidis* strain KH11 and have named the material extracellular slime substance (ESS) on the basis of the binding of concanavalin A to this bacterial extract (20). The expression of ESS, however, is not equivalent to slime production because most concanavalin A-reactive strains did not produce slime, whereas most strains that produced slime did not react with concanavalin A (20). More recently, workers in the laboratory of Pier (22) have isolated a galactose-rich capsular polysaccharide adhesin (CPA) from strain RP62A. Precoating of a Silastic catheter with CPA inhibited the attachment of RP62A to the catheter surface (22). While it appears that CPA plays a role in the adherence of *S. epidermidis* to smooth surfaces, the expression of CPA, like the expression of ESS, is not equivalent to slime production. In a recent study reported in abstract form by workers in the laboratory of Pier (E. Mullen, Y. Kojima, D. Goldmann, and G. B. Pier, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, B-111, p. 49), only 51% of CPA-positive strains produced slime whereas 20% of CPA-negative strains produced slime.

We have obtained from RP62A two daughter strains that no longer produce slime. The first strain, H4A, was a spontaneous variant (5); the second strain, HAM892, was the product of acriflavin mutagenesis (L. P. Barker, W. A. Simpson, and G. D. Christensen, manuscript submitted for publication). We have examined extracellular extracts of these organisms with antisera against RP62A and have found an antigen, the slime-associated antigen (SAA), which correlates with slime production for RP62A and two other unrelated slime-producing strains of *S. epidermidis*.

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MATERIALS AND METHODS

Microorganisms. We have previously described the bacteriology of strains RP14 (ATCC 35981), SP2 (ATCC 35982), RP12 (ATCC 35983), and RP62A (ATCC 35984) (8, 9). These

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are unrelated organisms originally isolated between 1979 and 1980 from blood cultures of patients in Memphis, Tenn. (7). The organisms RP12 and RP62A are slime-producing *S. epidermidis* sensu stricto strains from patients with intravascular-catheter sepsis. The organisms RP14 and SP2 are *Staphylococcus hominis* strains and do not produce slime in Trypticase soy broth (TSB) (BBL Microbiology Systems, Cockeysville, Md.). RP14 was of uncertain pathogenicity, and SP2 was a blood culture contaminant. We have found that slime production by RP62A is a variable phenomenon (5, 6). Strain H1 is a defined clone of RP62A that produces slime, whereas H4A is a spontaneous derivative of H1 that does not produce slime (5). Strain HAM892 was isolated from acriflavin-treated H1 and, like H4A, does not produce slime (L. P. Barker, G. D. Christensen, and W. A. Simpson, manuscript submitted for publication). We have also described the organism LB, which is a slime-producing *S. epidermidis* sensu stricto strain isolated in 1989 from a patient in Columbia, Mo., who had a cardiac pacemaker electrode infection (1). This organism is represented by two isolates: LB-L, which was recovered from the pacemaker pack, and LB-P, which was recovered from the peripheral blood. The *S. epidermidis* sensu stricto strain KH11 was generously donated by Georg Peters (University of Cologne, Cologne, Federal Republic of Germany), who originally described this organism (19) and has used it for extracting ESS (20).

Adherence. The adherence of these organisms to plastic tissue culture plates was measured by recording the optical density of the adherent bacterial film deposited on the floor of the plate, as previously described (5, 9). Overnight cultures of the test organisms in TSB were diluted with an equal volume of fresh TSB, and 0.2-ml portions were placed in individual wells of tissue culture plates. The plates were washed at 5, 15, and 30 min and at 1, 2, 4, 6, and 24 h, and the optical density of the adherent bacterial film was recorded by an automatic spectrophotometer.

Antisera. Antisera to RP62A were generated in New Zealand White rabbits. The antigen was prepared by boiling a 10-ml overnight TSB culture of RP62A for 5 min. The heat-killed cells were washed twice in phosphate-buffered saline (PBS) and resuspended in 2 ml of PBS. The vaccine was created by emulsifying equal volumes of the cell suspension with complete or incomplete Freund adjuvant. Three rabbits were injected subcutaneously with 0.5 ml of the vaccine suspension (in complete Freund adjuvant [Sigma Chemical Co., St. Louis, Mo.]) and then boosted at 4-week intervals with a similar volume of the suspension (in incomplete Freund adjuvant [Sigma]). Four weeks after the third boost, the animals were exsanguinated and the serum was pooled.

Preparation of SAA. Crude extracts of RP62A were prepared in a manner similar to the method of Pier (22). Bacteria previously plated onto a blood agar plate were swabbed off of the plate after 18 h of incubation at 37°C and suspended in 1 ml of PBS with lysozyme (1 mg/ml) (Sigma) and lysostaphin (1 mg/ml) (Sigma). The suspension was incubated for an additional 24 h at 37°C, the bacterial debris was removed by centrifugation, and the extract was filter sterilized. This solution was the crude extract. The crude extract was concentrated 20- fold on a SpeedVac (equipped with a refrigerated condensation trap) (Savant Instruments, Farmingdale, N.Y.). The concentrated extract was loaded onto a Sephadex G-200 column (Pharmacia Fine Chemicals, Piscataway, N.J.) equilibrated with PBS. The void volume (blue dextran 2000; Pharmacia) was 55 ml, and bovine serum albumin (Sigma) was eluted with 83 ml of PBS. The first peak (50 ml) was collected, pooled, digested with trypsin (1 mg/ml), dialyzed against distilled water with a membrane pore size of 50,000 molecular weight (Spectrum Medical Industries Inc., Los Angeles, Calif.), and lyophilized. This material constituted the semipurified SAA.

Characterization of bacterial extracts. Serologic studies (double immunodiffusion, immunoelectrophoresis, rocket immunoelectrophoresis) were performed as described by Catty and Raykundalia (3). Purified CPA and anti-CPA antisera were generously provided by G. Pier (Boston, Mass.). Crude extract was divided into aliquots and digested at pH 7.2 for 120 min with trypsin (type XIII; Sigma) (1 mg/ml) and protease (type XIV; Sigma). Additional aliquots were boiled for 5 min or were untreated controls.

Chemical analysis. Colorimetric assays for monosaccharide constituents of SAA (phenol-sulfuric acid assay, phenol-boric acid-sulfuric acid assay, carbazole assay, ferric-orcinol assay, and Morgan-Elson assay) were performed as described in detail by Chaplin (4). Colorimetric standards included the appropriate mono- and polysaccharides. Colorimetric assay for protein content of SAA was performed with a commercial kit (BCA Protein Assay Reagent; Pierce Chemical Co., Rockford, Ill.). All carbohydrate analyses were performed as alditol acetates and O-methyloximes as previously described (17). Direct capillary gas-liquid chromatography analysis of carbohydrates was performed with a model 3700 gas-liquid chromatograph (Varian Associates, Park Ridge, Ill.) equipped with dual flame ionization detectors. The chromatographic column employed (30 m by 0.32 mm internal diameter) was a fused silica capillary column (0.25 μm, bonded OV-17) (Supelco, Bellefonte, Pa.). The helium flow rate was 5 ml/min, with an injector and detector temperature of 270°C. After being held initially for 1 min at 180°C, the column was programmed to increase in temperature at 4°C/min to 250°C. Peak areas and retention times were recorded by using a Shimadzu C-R3A Chromatopac integrator (Shimadzu, Columbia, Mass.). The absence or presence of all carbohydrates was verified by mass spectrometry. Mass spectra were obtained on a Kratos MS 50 S mass spectrometer (Kratos, Urmston, Manchester, United Kingdom) interfaced with a Carlo Erba model 4160 gas chromatograph. Mass spectra were recorded at 70 eV with an ionization current of 50 μA, a source temperature of 250°C, and a transfer temperature of 218°C. For amino acid analyses, samples were hydrolyzed in 6.0 N HCl, under nitrogen, for 24 h. Portions were then analyzed on a Beckman model 6300 amino acid analyzer employing norleucine as the internal standard and ninhydrin as the postcolumn derivatization reagent. Total phosphate was analyzed colorimetrically by the procedures of Lowry et al. (15) and by gas-liquid chromatography (16). Lipids were determined by gas-liquid chromatography by the procedure of Korhonen (14).

RESULTS

Serology. Crude extracts of RP62A, a defined clone of RP62A (H1), a spontaneous non-slime-producing variant of H1 (H4A), a non-slime-producing strain derived from acriflavin-treated clone H1 (HAM892), and unrelated strains (RP12, RP14, SP2, KH11, and LB) were reacted against anti-RP62A antiserum by double immunodiffusion. Extracts of strains RP62A, H1, and LB exhibited two precipitin lines which shared identity (Fig. 1), whereas extracts of H4A and HAM892 exhibited only one precipitin line. The common

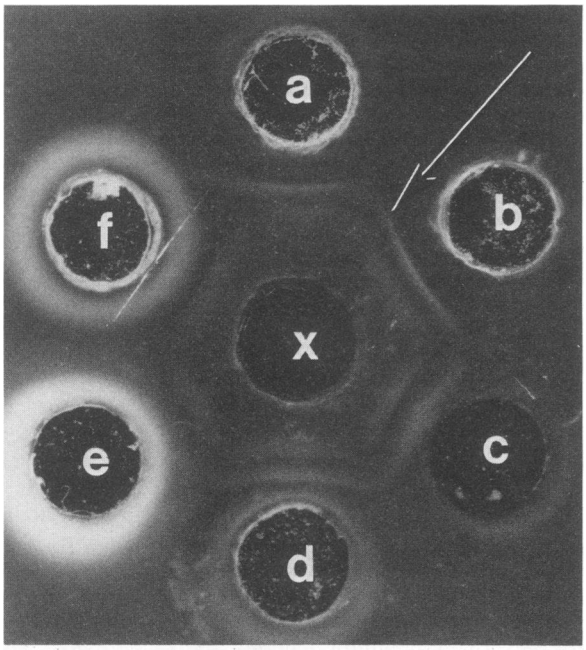

FIG. 1. SAA production by RP62A, RP62A derivative strains, and unrelated strains. Crude extracts of RP62A (a), RP62A derivative strains (H1 [d], H4A [e], and HAM892 [f]), and the unrelated strain LB (represented by isolates LB-L [b] and LB-P [c]) were reacted against anti-RP62A antiserum (whole cell) (x) in the center well by Ouchterlony double immunodiffusion. The experiment demonstrates a common band of identity for all six strains and a second common band (arrow) for the slime-producing strains (RP62A, H1, LB-L, and LB-P) (a to d) but not for the RP62A derivative strains that do not produce slime (H4A and HAM892) (e and f). We refer to the band (arrow) associated with slime production as the SAA.

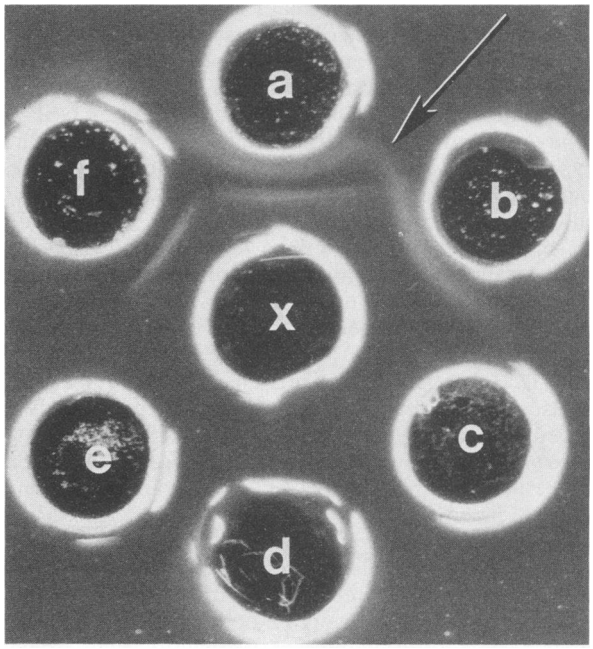

FIG. 2. SAA production by unrelated strains. Crude extracts of selected strains of coagulase-negative staphylococci (a to f) reacted against anti-RP62A antiserum (whole cell) (x) in the center well by Ouchterlony double immunodiffusion. The experiment demonstrates the SAA band (arrow) in extracts from two unrelated slime-producing strains, RP62A (a) and RP12 (b). The strains RP14 (c), KH11 (d), SP2 (e), and H4A (f) do not produce slime in TSB.

antigen for all of these strains was the line closest to the antiserum well (Fig. 1). Extracts from an unrelated strain, RP12, exhibited only one line which shared identity with the RP62A line farthest from the antisera well (Fig. 2). Extracts of SP2, RP14, and KH11 did not react with the antiserum (Fig. 2). Since RP62A (and the RP62A derivative H1), LB, and RP12 produced slime, whereas SP2, RP14, KH11, H4A, and HAM892 did not produce slime, these findings suggested that the RP62A precipitin line farthest from the antiserum well was a marker of slime production. We refer to this precipitin line as the SAA.

Characterization of SAA. RP62A crude extract was digested with trypsin and protease and examined by rocket immunoelectrophoresis with anti-RP62A antiserum. The second precipitin line, which was not associated with slime production, disappeared following digestion with either trypsin or protease, indicating that this antigen was proteinaceous. The height of the SAA rocket, however, was not affected by proteases or by heat treatment (100°C, 5 min). When crude extract was chromatographed over a Sephadex G-200 column, we recovered SAA in the void volume, suggesting a molecular weight for SAA of >100,000. This observation was consistent with the finding that SAA was retained when dialyzed against water with a membrane having a pore size cutoff of molecular weight 50,000. Sephadex-chromatographed, trypsin-digested, dialyzed extract constituted the semipurified product.

Comparison of SAA with CPA. We immunologically compared semipurified SAA with CPA by Ouchterlony double immunodiffusion using both anti-CPA and anti-RP62A antisera. The two preparations were immunologically distinct (Fig. 3), indicating that SAA and CPA were antigenically different compounds.

Chemical analysis. In colorimetric assays, SAA was found

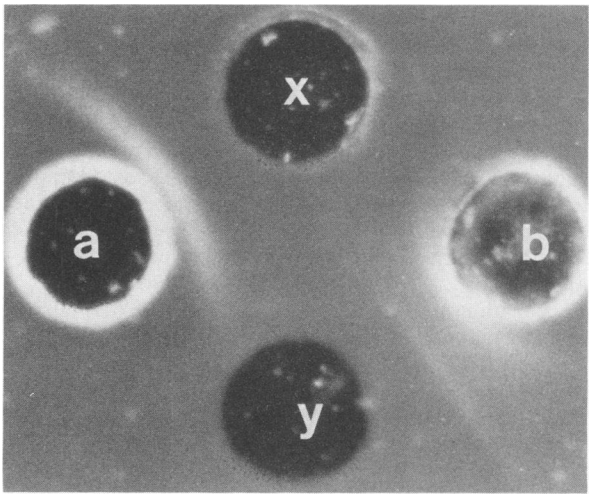

FIG. 3. CPA versus SAA. Trypsin-digested peak 1 material from the Sephadex G-200 column, representing partially purified SAA (a), was reacted by Ouchterlony double immunodiffusion against anti-RP62A antiserum (generated against whole cells) (x) and anti-CPA antiserum (generated against purified CPA; provided by G. Pier) (y). Included on the plate was purified CPA (provided by G. Pier) (b). The experiment demonstrates that CPA and SAA are antigenically distinct.

TABLE 1. Chemical analysis of semipurified SAA

Mode of analysis/component	% (by weight) of sample[a]
Colorimetry	
Protein	7.4
Reducing sugar (phenol-sulfuric acid assay)	64
Ketoses (phenol-boric acid-sulfuric acid assay)	15
Uronic acids (carbazole assay)	9.6
Pentoses (ferric-orcinol assay)	1.4
Amino sugars (Morgan-Elson assay)	0.91
Unidentified	1.7
Gas-liquid chromatography	
Glucose	59
N-Acetylglucosamine	6.4
Glucuronic acid	2.8
Galacturonic acid	1.7
Galactose	1.4
N-Acetylgalactosamine	0.57
Mannose	0.55
Phosphate	ND
Glycol	Tr
Lipid	ND
Protein (automated amino acid analysis)	7.3
Unidentified	20

[a] ND, Not detectable.

to consist primarily of reducing sugars (64%; Table 1) along with significant amounts of ketoses (15%) and uronic acids (9.6%). The protein content was 7.4% by a colorimetric assay and 7.3% by amino acid anlaysis (Table 1). There was no detectable phosphate, indicating that the preparation was free of nucleic and teichoic acids. Lipids were not detectable, and there was only a trace amount of glycerol. By gas-liquid chromatography, SAA was found to consist primarily of glucose (59%; Table 1), suggesting that SAA was a dextran. Small amounts of *N*-acetylglucosamine, glucuronic acid, and galacturonic acid were also found (Table 1). Semipurified SAA was substantially free of galactose (1.4%) and mannose (0.55%), which chemically distinguished SAA from galactose-rich CPA (22) and mannose-rich ESS (20).

Association of SAA with bacterial accumulation. A functional distinction between SAA and CPA was suggested by a comparison of the adherence kinetics of different CPA- and SAA-positive strains. This comparison was made possible by the previous report from the laboratory of Pier of CPA production by many of these strains (22). Kinetic studies were conducted by diluting an overnight culture with an equal volume of fresh TSB. This procedure differs from our usual determination of slime production, which uses a 1:100 dilution of an overnight TSB culture. The larger-than-normal inoculum is required in order to optically measure the initial adherence of the organisms to the plastic plate as well as the subsequent accumulation of bacteria on the plate.

Adherence kinetics for the slime-producing strains RP62A (including H1) and RP12 are depicted in Fig. 4; those for the non-slime-producing strains SP2, RP14, H4A, and HAM892 are depicted in Fig. 5. The CPA- and SAA-positive (CPA^+ SAA^+) strain RP62A adhered in two phases: an initial phase exhibiting measurable adherence at 15 min and a maximum adherence at 2 h, followed by a slower accumulation phase with a maximum at 6 h. Unlike RP62A, RP12 did not exhibit the early adherence phase but did exhibit a late accumulation phase, reaching a maximum at 6 h. We have found RP12 to be SAA^+; RP12 was originally reported to be CPA^- (19) but was subsequently found to be CPA^+, although the positive reaction is difficult to demonstrate (G. Pier, personal communication). Like RP62A, the RP14 strain exhibited the early adherence phase, reaching a maximum at 2 h, but unlike RP62A and RP12 did not demonstrate the late accumulation phase. This strain was CPA^+ but SAA^-. The SP2 strain did not exhibit either early adherence or late accumulation and was likewise both SAA^- and CPA^-. The combination of data from our studies with those from the laboratory of Pier (22) provides circumstantial evidence that SAA was a marker of bacterial accumulation, whereas CPA mediated initial adherence. The adherence kinetics of the non-slime-producing derivatives of RP62A were consistent with this hypothesis. Although we do not know whether these strains still express CPA, neither H4A nor HAM892

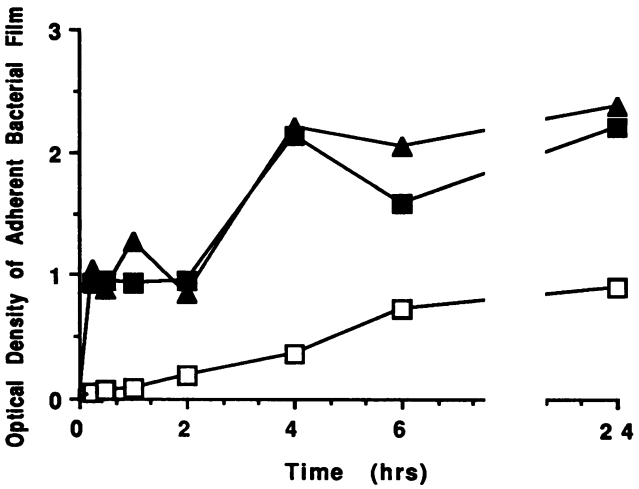

FIG. 4. Adherence kinetics for selected strains of slime-producing coagulase-negative staphylococci. The optical densities of adherent bacterial films deposited on the floor of tissue culture plates as a function of time are reported in this graph. The optical density corresponds to the thickness of the bacterial film and serves as an index of slime formation. RP62A (■) and the slime-producing derivative, H1 (▲), exhibited measurable immediate (early) adherence to the plate as well as a second (late) phase of accumulation on the plate; RP12 (□), however, exhibited only the late accumulation.

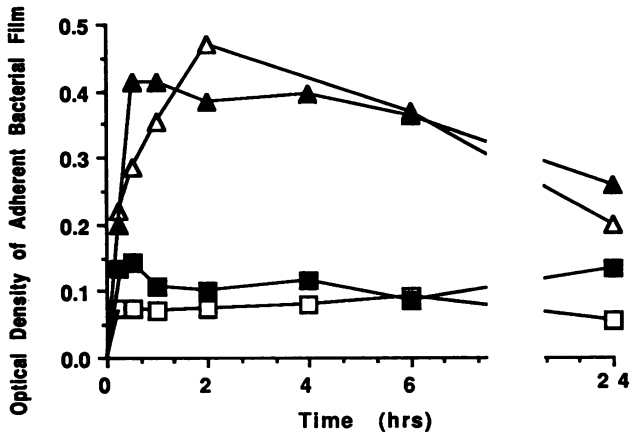

FIG. 5. Adherence kinetics for selected strains of non-slime-producing coagulase-negative staphylococci. For an explanation of adherence kinetics, see the legend to Fig. 4. RP14 (△) and HAM892 (▲) exhibited only early adherence, not late accumulation; SP2 (□) and H4A (■) did not exhibit either early adherence or late accumulation.

demonstrated late accumulation and both were SAA$^-$ (Fig. 5).

DISCUSSION

The SAA was identified on the basis of the loss of this antigen from extracts of two independently derived non-slime-producing daughter strains of the well-characterized slime-producing strain RP62A. The association of this antigen with slime was further strengthened by the finding that the antigen was closely associated with the production of slime by two other well-characterized but unrelated strains of coagulase-negative staphylococci. While more work is required to confirm this association, these data suggest that SAA is a common antigen which might serve as a marker of slime production for coagulase-negative staphylococci.

In kinetic studies of slime production, we found that the colonization of smooth surfaces proceeded through an initial adherence phase followed by an accumulation phase and that SAA was expressed only by those strains exhibiting an accumulation phase. The division of the colonization process into an initial adherence phase and a subsequent accumulation phase could clarify some of the confusion regarding what actually constitutes slime production. We have defined (8) and measured (9) slime production as the elaboration of a tenacious bacterial film on the walls of a culture vessel when a small inoculum of bacteria is allowed to reach stationary growth phase in supportive medium (TSB). This definition emphasizes the accumulation phase of bacterial colonization; consequently, our efforts to determine the molecular mediators of slime production have focused upon an antigenic marker of accumulation. Likewise, the approach followed by workers in the laboratory of Pier has focused upon the immediate adherence of coagulase-negative staphylococci to inanimate surfaces (22), and so it is not unreasonable that their efforts to determine the molecular mediator of this adherence should identify a mediator—CPA—of initial adherence.

The hypothesis that at least two adhesive extracellular materials mediate the colonization of inanimate surfaces by coagulase-negative staphylococci is consistent with other microbial adherence systems which require several different adhesins. It has been proposed that the colonization of dental enamel by *Streptococcus mutans* begins with an initial adherence phase and proceeds through an accumulation phase (21). As noted in the Introduction, electron micrograph studies suggest that a similar process applies to *S. epidermidis* (18).

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