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Purification and Characterization of the Staphylococcal Slime-Associated Antigen and Its Occurrence among *Staphylococcus epidermidis* Clinical Isolates

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The *Staphylococcus epidermidis* slime-associated antigen (SAA) was purified and characterized. *N*-Acetylglucosamine accounted for 70% of the dry weight of SAA, which was immunolocalized on the ruthenium red-positive material produced by slime-positive strains. A total of 59% of slime-producing *S. epidermidis* clinical isolates expressed SAA, while the phenotype slime$^-$ SAA$^+$ was never recovered.

Coagulase-negative staphylococci are the most common cause of foreign-body-associated infections (2, 5, 9, 16, 17, 21, 30, 34). The pathogenesis of such infections has been correlated with the ability of some strains to produce large amounts of extracellular material termed slime (3, 7, 12, 13, 20, 34).

Several polysaccharide components have been described as chemical markers of slime produced by *Staphylococcus epidermidis* (14, 19, 32). In particular, Mack et al. (24) have indicated hexosamine as the main component of an antigen which may be active in colonization by *S. epidermidis* (25).

Christensen et al. (10) also described a slime-associated antigen (SAA) with an analogous function. In the preliminary analysis of the partially purified SAA, the presence of a high content of reducing sugars (64%) plus small amounts of ketoses, uronic acid, *N*-acetyl-glucosamine, and glucuronic and galacturonic acid was reported (10). Changes in the purification procedure have shown that the composition of the SAA is different from the one previously described. We report here on the chemical characterization of SAA obtained by using the new procedure. Also presented are evidence confirming the identity of SAA with slime and data on its occurrence among clinical isolates of *S. epidermidis.*

Strains used for studies on SAA are listed in Table 1. *S. epidermidis* M187 (28) was kindly provided by E. Muller and G. B. Pier (Harvard University, Cambridge, Mass.); the mitomycin mutant M7 derived from *S. epidermidis* ATCC 35984 (31) was a generous gift of G. Peters (Universitat Munster, Munster, Germany). Contrary to expectations, the non-slime producer M7 was SAA positive, showing a peculiar mode of growth. After overnight incubation, a biofilm was visible along the tube walls but a very delicate shaking caused the detachment of amorphous material, which floated in the medium. Bacterial cells were recoverable after centrifugation at 6,000 rpm (RC5C; Sorvall Instruments, Du Pont) for 15 min, while the loose material did not sediment and remained aggregated in a clot.

Strains were grown in Trypticase soy broth or in a chemically defined medium (HHW) as described by Hussain et al. (18).

Production of slime was measured by a modified version of the plate test (4).

Sera specific to *S. epidermidis* ATCC 35984 were generated as described previously (10). For immunolocalization, whole antiserum was absorbed against slime-negative mutants as described by Baselga et al. (6).

For SAA purification, strains were grown in 50-ml blue-top Falcon tubes (Falcon, Becton Dickinson, Lincoln Park, N.J.) in HHW culture medium. Slime-producing bacteria adhered strongly to the tube walls, so that the medium could be removed together with the nonadherent bacteria and the biofilm could be washed once with phosphate-buffered saline (PBS). Tubes were refilled with PBS and sonicated in ice for a total of 3 min (Soniprep 150, MSE). Bacterial cells and debris were eliminated by centrifugation, and the supernatant was filtered, dialyzed against water, and concentrated. This material was designated crude SAA.

Crude SAA was chromatographed through a BioGel P100 (Bio-Rad Laboratories Inc.) column equilibrated with Tris-HCl (55 mM, pH 7.2). Elution of SAA-positive material was followed by double immunodiffusion with a polyclonal antiserum against whole ATCC 35984 cells. SAA-positive fractions were pooled and treated with trypsin XIII (1 mg/ml; Sigma Chemical Co., St. Louis, Mo.) overnight at 37°C, and the resulting material was rechromatographed. SAA-containing fractions were pooled, dialyzed against water, and dehydrated for

TABLE 1. Characteristics of strains utilized for SAA purification and characterization

Strain	Slime production (OD_{570})[a]	SAA expression	Early adhesion	Late accumulation	Reference
ATCC 35984	2.89 ± 0.02	+	+	+	10
HAM892	0.08 ± 0.01	−	+	−	10
M187	2.77 ± 0.03	+	+	+	28
M7	0.09 ± 0.01	+	+	−	31

[a] OD_{570}, optical density at 570 nm. Values are means ± standard deviations.

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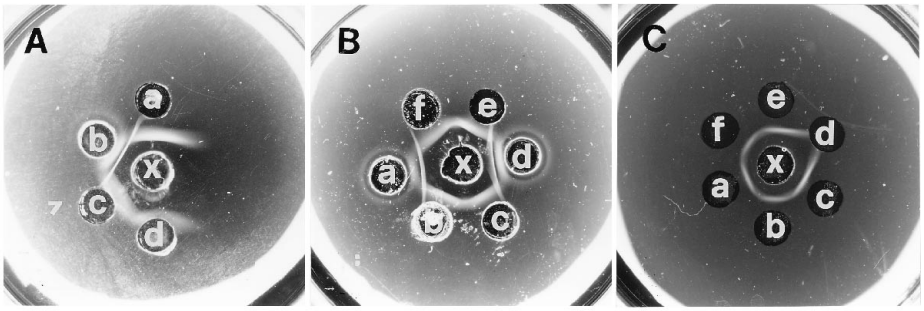


FIG. 1. (A) The formerly utilized procedure to purify SAA yielded extracts showing two precipitin lines (well b) while only one line was visible in samples produced with the new procedure: wells a, c, and d contain three different SAA preparations. (B) Treatment with papain (well b), pronase E (well c), proteinase K (well e), and neuraminidase (well f) (wells a and d are controls) destroyed the additional line present in the material prepared by the old procedure. (C) The antigenicity of crude SAA was unaffected by the enzymatic treatment (enzymes were as in panel B), while it was completely destroyed by sodium meta-periodate treatment (well d).

gravimetric analysis. This material was designated purified SAA.

Peptidoglycan was isolated and partially purified from staphylococcal cells as described by Mattsson et al. (26).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (23) with a 7% resolving gel. Gels were stained with silver stain (Bio-Rad) and with a modified silver stain as described by Tsai and Frasch (33). Samples subjected to SDS-PAGE were transferred onto nitrocellulose, allowed to react against rabbit immune serum, and detected by peroxidase-conjugated goat anti-rabbit immunoglobulins.

Monosaccharide constituents of SAA were evaluated by colorimetric assays as described by Chaplin (8) and by gas chromatography of the alditol acetates and O-methyloxime derivatives. Protein content was estimated by a commercial kit (BCA protein assay reagent; Pierce, Rockford, Ill.). Total phosphorus was measured as described by Ames (1).

The sensitivity of crude and/or purified SAA to enzymatic treatment was assayed with the following enzymes (all from Sigma Chemical Co.): proteinase K (from *Tritirachium album*) and pronase E (from *Streptomyces griseus*) (200 μg/ml in PBS, pH 7.5), papain (from papaya latex; 200 μg/ml in PBS, pH 6.2), and neuraminidase (from *Clostridium perfringens*) (5 U/ml in PBS, pH 5.0). SAA was also treated with 0.2 M sodium *meta*-periodate in water at 4°C overnight as described by Mack et al. (25).

DEAE Sepharose Fast Flow and CM Sepharose Fast Flow (Pharmacia Fine Chemicals) were used for charge determination of SAA.

For electron microscopic studies, cells from overnight cultures were fixed as described by Fassell and coworkers (15). Immunolocalization of SAA was achieved by the method of Molinari et al. (27). No additional staining on sections was done before microscopic observation.

The former preparation procedure for SAA purification (10), which used lysozyme and lysostaphin digestion, led to extracts showing two precipitin lines in the double-immunodiffusion test (Fig. 1A). The characteristics of growth of the biofilm-forming *S. epidermidis* strain ATCC 35984 allowed us to recover the biofilm from the tube walls without any gross contamination from the medium; the crude material thus obtained showed a single precipitin line (Fig. 1A). The additional line present in the crude material obtained with the old procedure was sensitive to protease treatment (Fig. 1B), which in turn did not affect SAA (Fig. 1C). Only sodium periodate completely destroyed SAA antigenicity (Fig. 1C), suggesting its polysaccharide nature.

The novel procedure yielded a mean of 196.5 μg of purified SAA per liter of *S. epidermidis* ATCC 35984 culture.

No bands were visible upon SDS-PAGE of up to 20 μg of the crude material and up to 5 μg of the purified SAA. Even the use of the modified silver stain for lipopolysaccharide de-

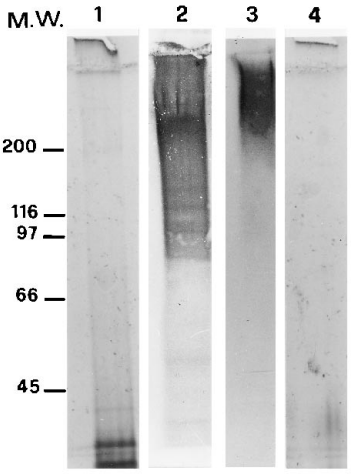


FIG. 2. SDS-PAGE and immunoblot analysis of crude (lanes 1 and 2) and purified (lanes 3 and 4) SAA. Lane 1, SDS-PAGE analysis of crude SAA. Overloading of the well allowed us to observe a few low-molecular-weight bands. Immunoblotting of the same sample revealed instead several bands along the full length of the gel (lane 2). Immunoblotting of purified SAA showed a broad band of high-molecular-weight material (lane 3) which disappeared after treatment with sodium *meta*-periodate (lane 4). M.W., molecular weight (in thousands).

TABLE 2. Sugar composition of extracts from the slime-producing *S. epidermidis* strain ATCC 35984 and its slime-negative mutant HAM892

Component	Amt of carbohydrate in strain[a]:	
	ATCC 35984	HAM892
Hexoses	9.82	12
Hexosamines	137.55	0
Uronic acid	0	0
Ketoses	0	0
Pentoses	0.11	5
Phosphate	<0.03 μmol/mg	0 μmol/mg
Protein	1.57	1.02

[a] Values are in micrograms per liter of bacterial culture except where otherwise noted.

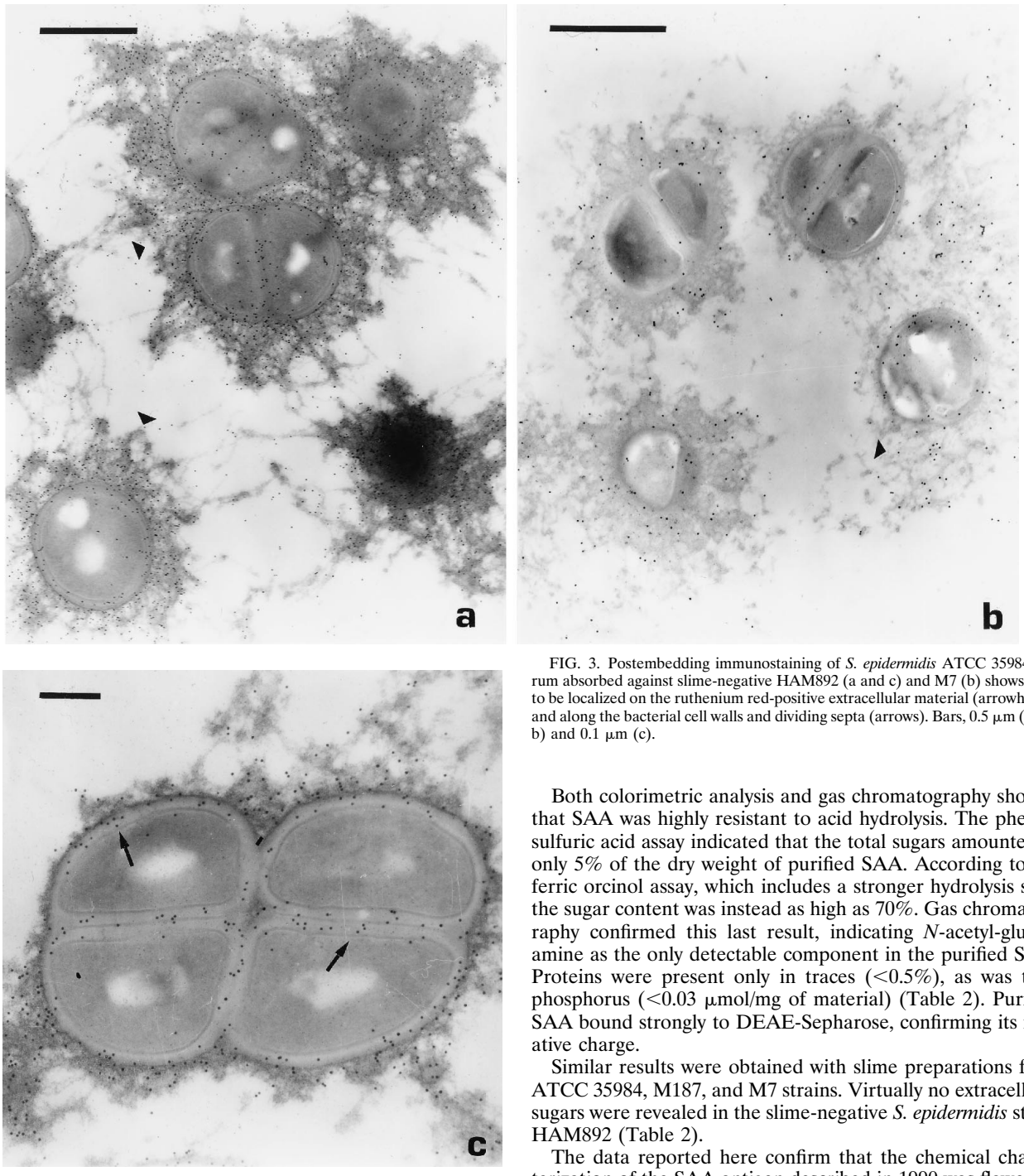


FIG. 3. Postembedding immunostaining of *S. epidermidis* ATCC 35984. Serum absorbed against slime-negative HAM892 (a and c) and M7 (b) shows SAA to be localized on the ruthenium red-positive extracellular material (arrowheads) and along the bacterial cell walls and dividing septa (arrows). Bars, 0.5 μm (a and b) and 0.1 μm (c).

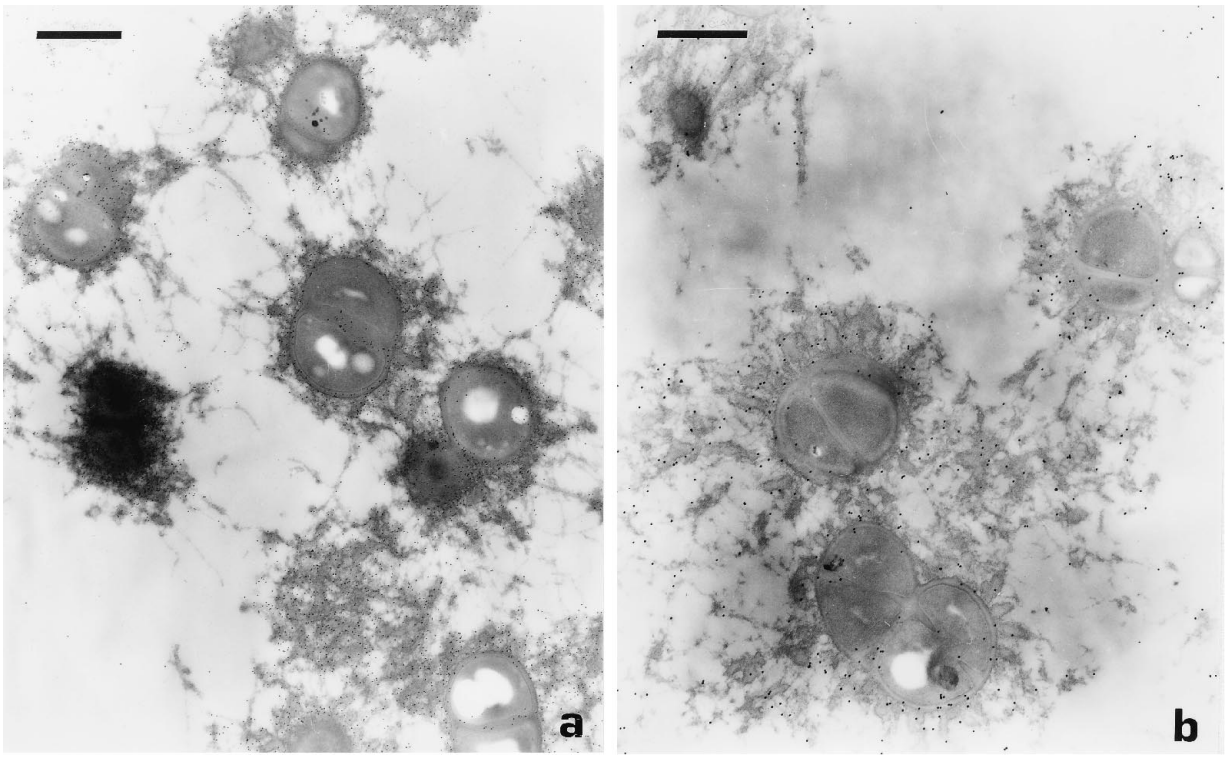
tection did not reveal any major component. A 100-fold concentration (about 2 mg) of the crude material was necessary to observe a series of bands with molecular masses under 45 kDa (Fig. 2, lane 1).

Immunoblotting with rabbit serum raised against whole bacterial cells revealed the presence of several bands with different reactivities in the crude material (Fig. 2, lane 2). With purified SAA, a strong reaction against high-molecular-weight material was observed, and the product appeared as a large band (Fig. 2, lane 3); this disappeared after treatment with sodium *meta*-periodate (Fig. 2, lane 4).

Both colorimetric analysis and gas chromatography showed that SAA was highly resistant to acid hydrolysis. The phenol-sulfuric acid assay indicated that the total sugars amounted to only 5% of the dry weight of purified SAA. According to the ferric orcinol assay, which includes a stronger hydrolysis step, the sugar content was instead as high as 70%. Gas chromatography confirmed this last result, indicating *N*-acetyl-glucosamine as the only detectable component in the purified SAA. Proteins were present only in traces (<0.5%), as was total phosphorus (<0.03 μmol/mg of material) (Table 2). Purified SAA bound strongly to DEAE-Sepharose, confirming its negative charge.

Similar results were obtained with slime preparations from ATCC 35984, M187, and M7 strains. Virtually no extracellular sugars were revealed in the slime-negative *S. epidermidis* strain HAM892 (Table 2).

The data reported here confirm that the chemical characterization of the SAA antigen described in 1990 was flawed by the preparative procedure. In fact, the use of lysostaphin was seen to have affected the integrity of the Sephadex column used in the purification of SAA. Moreover, the bacteria used to be swabbed off blood agar plates, allowing for heavy contamination of the crude preparation with agar and blood components. The composition of SAA indicates that Mack et al. and our group may have been dealing with the same antigenic molecule. Mack and coworkers did not indicate what percentage of their antigen was hexosamine; however, the detection in both cases of a single molecular species as the main component of the slime preparation is certainly significant. While we were not able to find the components accounting for 30% of the dry



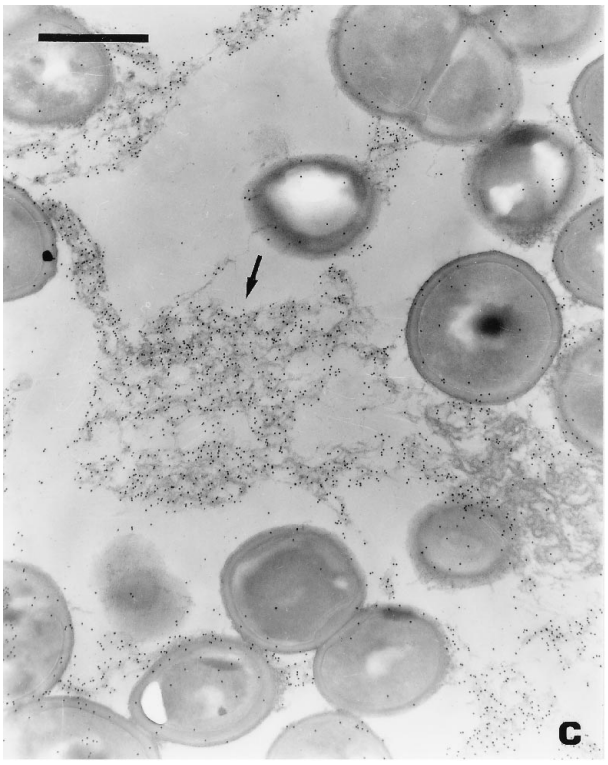


FIG. 4. Postembedding immunostaining of *S. epidermidis* M187 labeled with serum absorbed with either HAM892 (a) or M7 (b). *S. epidermidis* M7 extracellular material also reacted positively with serum absorbed against HAM892 (c) or M7. In this case, however, the SAA-positive material was mostly detached from the bacterial surface (arrow). Bars, 0.5 μm.

weight of the SAA, we could rule out the presence of proteins, DNA, teichoic acid, and long-chain fatty acids (data not shown). The chemical composition indicates that SAA (and slime) is not the same as the cell wall teichoic acid as reported by Hussain et al. (19). We may also exclude peptidoglycan, which did not react in our immunodiffusion assay, and lipoteichoic acid, since SAA did not sensitize erythrocytes when tested by the method of Ofek et al. (29) (data not shown).

For immunolocalization of SAA, antiserum raised against whole slime-positive cells was absorbed against HAM892, which is identical to the parent strain ATCC 35984 except for slime production (10), and M7, which seemed to shed all the SAA-positive material in the medium after shaking. Ultrathin sections of *S. epidermidis* ATCC 35984 showed large amounts of ruthenium red-positive material which reacted strongly with antiserum absorbed either against HAM892 or M7 (Fig. 3). Immunostaining with serum absorbed against M7 was slightly weaker, possibly because of the presence of small amounts of residual SAA during the serum-absorbing procedure. Similar results were obtained with sections of *S. epidermidis* M187 (Fig. 4a and b). When M7 was immunostained with absorbed antiserum, material positive for both ruthenium red and SAA was visible, although such material was very loosely associated with the cell (Fig. 4c). In each case, the localization of the gold particles was strictly specific on the ruthenium red-positive material and the wall and the dividing septa of the cells. The latter finding may indicate a possible means of transport of SAA after its synthesis; we cannot, however, rule out the possibility that antibodies specific for one or more cell wall antigens, absent from the mutant strains together with SAA, were still present after serum absorption.

The apparently discrepant findings regarding the M7 strain, which was negative for slime production by the plate test but produced a visible biofilm on tube walls and was SAA positive, may suggest that this strain, rather than being slime negative, might in fact be mutated in a gene coding for a molecule

TABLE 3. Occurrence of the different phenotypes among clinical *S. epidermidis* isolates

Phenotype	No. (%) of isolates
$Slime^+$ SAA^+	22 (44)
$Slime^+$ SAA^-	15 (30)
$Slime^-$ SAA^+	0 (0)
$Slime^-$ SAA^-	13 (26)
Total	50 (100)

connecting the structure responsible for the initial attachment (polysaccharide adhesin?) (32) and the slime itself.

Fifty *S. epidermidis* strains of clinical origin were examined for their ability to produce slime and/or SAA. For such an evaluation, strains were grown in 5 ml of Trypticase soy broth at 37°C overnight. The culture medium was replaced with PBS, each sample was sonicated and cleared by centrifugation at 3,000 rpm (RC5C; Sorvall Instruments, Du Pont) for 15 min, and the supernatant was concentrated 5 to 10 times. SAA expression was determined by double immunodiffusion. As shown in Table 3, 37 strains (74%) were strong or weak slime producers. This percentage is comparable to that found in other studies among clinical isolates (11, 20, 22, 34). Expression of SAA was always associated with slime production, with 22 of 37 (59.4%) strains possessing the phenotype $slime^+$ SAA^+, underlying the high prevalence of SAA among slime-producing strains. We never recovered the phenotype $slime^-$ SAA^+, suggesting that, while slime may be present in more than one (if less well represented) antigenic form, SAA is strongly connected to this trait. Preliminary results of a larger investigation seem to confirm these data; also, a characterization of the slime produced by SAA-negative strains indicates that at least two different exopolysaccharides, consisting mainly of reducing sugars, are produced (data not shown).

In summary, the data reported here indicate that a majority of slime-producing strains of clinical origin elaborate a polysaccharide whose main constituent is *N*-acetyl-glucosamine. Together with the information obtained by Mack and colleagues, our data indicate a single molecule, namely, the SAA, as one of the principal antigenic determinants of slime. Further studies are in progress to identify other antigenic forms of slime and to evaluate a possible correlation between the production of SAA and the severity of medical device-associated infections.

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