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Staphylococcal Slime: a Cautionary Tale

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Slime production by *Staphylococcus epidermidis* may be important in the adherence to and colonization of biomedical devices, and slime has been proposed to have various effects on the immune system. Attempts were made to isolate, purify, and chemically characterize slime from *S. epidermidis* cultivated under fluid on tryptic soy broth-agar medium. "Crude slime" from slime-producing strain RP-12 was characterized by a high galactose content. Similar materials in similar yields were isolated from slime-producing strain Kaplan, a non-slime-producing mutant, Kaplan-6A, and sterile medium controls, suggesting that crude slime was derived mainly from the medium. The occurrence of D- and L-galactose and pyruvate and sulfate residues and methylation analysis of these crude slime preparations, monitored by gas-liquid chromatography and mass spectrometry, showed that the agar was the main source of crude slime, suggesting that the preparation was largely an artifact of the growth and isolation procedures. Similar high-galactose-content preparations from both *S. epidermidis* and *Staphylococcus aureus*, assumed to be bacterial products and with a variety of biological activities, have been described by other investigators. Growth attached to a solid surface appears to be important for slime production. An accumulation of turned-over cell surface molecules and released macromolecules such as DNA may contribute to slime production. Avoidance of agar and development of a chemically defined medium for slime production are recommended for further studies.

Extracellular polysaccharides, variously termed capsule, slime, or glycocalyx, appear to be significant virulence factors for some strains of staphylococci. Capsules and microcapsules of *Staphylococcus aureus* are the best defined of the staphylococcal exopolysaccharides. They are relatively firmly attached to the cell wall, have definite external boundaries, are antiphagocytic, and are typically composed of *N*-acetylamino sugars and *N*-acetyl aminohexuronic acids (34, 35). The distribution of capsules in clinical isolates has been studied (1, 31). Capsules and microcapsules have also been observed in coagulase-negative staphylococci (29, 39), but there is little information available on their chemical structures.

The distinction between capsule and slime is not always clear-cut, and glycocalyx may be used as a more general term to refer to exocellular polysaccharides (7). However, slime usually refers to exopolysaccharides loosely associated with the cell and imparting viscosity to the culture medium (36). Much recent interest in *Staphylococcus epidermidis* slime was stimulated by the reports of Christensen et al. (10) and Peters et al. (27) that strains associated with intravascular catheter-related infections formed slimy biofilms on solid surfaces in static cultures or on intravascular catheters. These publications brought to light earlier reports of slime production by *S. epidermidis* (2, 3, 19). Slime production is often assessed by staining with a cationic dye (10, 33). It is generally believed that the elaboration of slime is involved in the adherence to and colonization of biomedical devices. Crude slime preparations have been shown to reduce the lymphoproliferative responses of mononuclear cells to polyclonal stimulators (16) and interfere with granulocyte function (17), although a recent report found no evidence for an antiphagocytic effect of slime (20). Testing for slime production has been claimed to be a useful marker for clinically significant infections with coagulase-negative staphylococci (13).

As yet, there is little information on the chemistry of staphylococcal slimes (35), although some information is available for *S. aureus* slime. Ekstedt and Bernhard (15) isolated and partially purified slime from *S. aureus* grown under fluid on modified, solidified staphylococcus 110 medium. Staphylococcus 110 medium is characterized by relatively high carbohydrate and NaCl contents. Neutral sugars and uronic and amino acids were found, with galactose being the major sugar. Similar findings were noted by Rozgonyi and Seltmann (30) for several methicillin-resistant and -susceptible strains grown on solid 110 medium under fluid. Brock and Reiter (5) reported that slime material from *S. aureus* grown in 110 medium in static liquid cultures contained ribitol teichoic acid. In studies of *S. epidermidis* slime, Ludwicka et al. (22) grew organisms under fluid on N agar supplemented with 3% (wt/vol) Casamino Acids and 1% (wt/vol) glucose. Crude slime was fractionated by DEAE-Sepharose chromatography into four fractions, and galactose was the major sugar in three of the fractions.

At the outset of our investigation, we wanted to isolate, purify, and chemically characterize *S. epidermidis* slime in view of the lack of knowledge of its nature and the interest in slime-producing strains. We found that the cultivation of organisms under fluid on agar yielded a product rich in galactose that was derived largely from the agar.

MATERIALS AND METHODS

Organisms. Slime-producing *S. epidermidis* RP-12 (10) was provided by G. D. Christensen, University of Tennessee College of Medicine, Memphis. Slime-producing *S. epidermidis* Kaplan and a non-slime-producing mutant, Kaplan-6A, created by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis, were provided by G. Archer, Medical College of Virginia, Richmond.

Growth conditions and preparation of "crude slime." The procedure adopted was based on that of Ekstedt and Bern-

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hard (15). Strains were cultured with tryptic soy broth (Difco Laboratories, Detroit, Mich.) or Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) solidified with 2% (wt/vol) no. 3 agar (Oxoid Ltd., Basingstoke, England) in up to 50 flat bottles, each containing 50 ml of medium (surface area of about 100 cm^2 per bottle). Tryptic soy broth and Trypticase soy broth favor slime production (10). Each bottle was inoculated with 10 ml of a Trypticase soy broth culture (shaken for 6 h at 37°C) and incubated for 36 h at 37°C. Cells were harvested with 0.9% (wt/vol) NaCl and recovered by centrifugation at 13,000 × *g* for 5 min at 4°C, and the supernatant was retained. Pelleted cells were resuspended in cold 0.9% (wt/vol) NaCl, and the suspension was blended (five times, 30 s each time) in a domestic blender (Moulinex) and then centrifuged. The two supernatants were combined and concentrated by dialysis against polyethylene glycol compound (P2263; Sigma Chemical Co., St. Louis, Mo.) (24) and then against water and freeze dried. The product was dissolved in water (60 ml), and trichloroacetic acid (final concentration, 5% [wt/vol]) was added. After 15 min at 4°C, the suspension was centrifuged (13,000 × *g*, 30 min, 4°C), and cold ethanol (4 volumes) was added to the supernatant. After 2 days at 4°C, the precipitate, crude slime, was collected, washed with ethanol, and freeze dried (yield, 1.15 g). Similar preparations were obtained from Kaplan and Kaplan-6A. Further experiments involved the use of a smaller inoculum (1 ml per bottle) and controls in which sterile Trypticase soy broth was used as the inoculum, the subsequent steps being essentially those described above.

Further preparations of crude slime from all three strains were obtained from Trypticase soy broth cultures (static or shaken) grown at 37°C for periods between 1 and 8 days. Products derived from the initial supernatant and from the blended cell suspension (combined and dialyzed) were fractionated by using trichloroacetic acid and ethanol essentially as described above. Control products from an equal volume of Trypticase soy broth were isolated and prepared in the same way.

Attempts to purify and fractionate crude slime preparations. Crude slime was subjected to various treatments: (i) further centrifugation (13,000 × *g*, 30 to 60 min) of aqueous solutions, discarding the pellet formed; (ii) fractional precipitation with 33 to 85% (vol/vol) ethanol; (iii) chromatography on a column (55 by 2.5 cm) of Sephacryl S-300 (Pharmacia Ltd., Milton Keynes, England) eluted with pyridine-acetic acid-water (10:4:986, vol/vol/vol; pH 5.4) at a flow rate of 15 ml/h; (iv) chromatography on a column (35 by 2.2 cm) of DEAE-Sepharose CL-6B (Pharmacia) with stepwise elution by 0 to 0.5 M NaCl (0.1 M increments, 300 ml each); and (v) chromatography on a column (55 by 2.5 cm) of Sephadex G-50 (Pharmacia) eluted with pyridine-acetic acid buffer, pH 5.4, at a flow rate of 20 ml/h.

Analytical methods. Spectrophotometric assays for phosphorus, total carbohydrate (expressed as galactose), and hexuronic acid were those listed elsewhere (4, 25). Sulfur analyses were carried out by C.H.N. Analysis Ltd., Leicester, England. D-Galactose was determined by using galactose oxidase (EC 1.1.3.9), and pyruvic acid was determined by using L-lactate dehydrogenase (EC 1.1.1.27). The methods used to release and identify sugars by paper chromatography, paper electrophoresis, and gas-liquid chromatography, as well as the methods used for periodate oxidation and methylation analysis of polymers, were those described previously (4, 25). Amino compounds were determined by autoanalysis after hydrolysis of samples with 4 M HCl at 105°C for 6 h (37).

TABLE 1. Composition of crude slime and related products from *S. epidermidis* strains grown on submerged agar media

Product	%					
	Total carbohydrate	Total galactose	Hexuronic acid	Pyruvic acid	Phosphorus	Sulfur
Crude slime from:						
RP12	57	ND[a]	4	ND	1.2	ND
Kaplan	64	ND	4	ND	0.8	ND
Kaplan-6A	64	ND	3	ND	0.9	ND
Purified slime						
RP-12A10	72	57	1	ND	1.1	2.6
RP-12A1	61	41	ND	0.2	0.7	2.3
CA1[b]	60	42	ND	0.4	0.5	2.3

[a] ND, Not determined.
[b] CA1, Control product from sterile Trypticase soy broth-agar.

RESULTS AND DISCUSSION

Crude slime obtained by the growth of strain RP-12 on submerged tryptic soy broth-agar contained 57% total carbohydrate and 1% phosphorus (Table 1). The major sugar (galactose) was accompanied by small amounts of glucose and xylose and traces of mannose and ribose. Although a spectrophotometric assay indicated the presence of hexuronic acid (about 4%), the color produced differed from that given by glucuronic acid, indicating interference. Attempts to purify the crude slime product by ion-exchange chromatography or fractional precipitation with ethanol were unrewarding. In the former case, the recovery was poor (36%), the major fraction being eluted with 0.5 M NaCl, and in the latter case, most of the material (72%) surprisingly remained soluble in 85% (vol/vol) ethanol. In both cases, the major fraction had a composition similar to that of crude slime. More successful was clarification of an aqueous dispersion by centrifugation (about 81% of the material remained soluble) followed by chromatography on Sephacryl S-300. Although the elution profile for the carbohydrate was very broad, later fractions of eluate were enriched in phosphorus. The earlier fractions, typically containing about 60% of the product applied, were pooled to give "purified slime" (RP-12A10), for which general analyses are shown in Table 1. Analyses for individual sugars showed galactose (57%), glucose (12%), xylose (4%), and ribose and mannose (traces). Autoanalysis showed glutamic acid (2.3%) as the only significant amino component. The presence of sulfur in RP-12A10 (Table 1), together with the contrast between the content of D-galactose (31%) and total galactose (57%), indicated that the product was derived largely from the agar in the growth medium (26). To a lesser extent, this probably applied also to the phosphorus-enriched fractions from the Sephacryl S-300 column, for which the total carbohydrate content was 44%, but with the same neutral sugars in the same proportions.

Support for the inference above came from studies of crude slimes from Trypticase soy broth-agar cultures of strains Kaplan (a producer) and Kaplan-6A (a nonproducer). From equivalent cultures, the yields were 1.0 g (Kaplan), 0.8 g (Kaplan-6A), and 1.15 g (RP-12). Analyses of the products (Table 1) indicated similar compositions, confirmed by the identities and proportions of neutral sugar components (data

not shown). A similar workup of material from a control (sterile) Trypticase soy broth-agar batch gave 0.7 g of crude slime containing 2.1% sulfur and galactose as the dominant neutral sugar. Methylation analyses of all four products gave similar results (see below).

Although submerged agar-based medium is recommended for the promotion of slime production (15, 38), this would seem likely to enhance the possibility of contamination. Accordingly, crude slime was also prepared from cultures in which a smaller volume (1 ml) of inoculum was used. The yield of product from the test culture (466 mg) was smaller than when a larger inoculum (10 ml) was used but not very different from that of a sterile control (392 mg). Also, the composition of the two products (RP-12A1 and CA1) obtained by further purification (clarification of solutions by centrifugation) was almost the same (Table 1). Enzymatic analysis of the products also revealed the presence of a little pyruvic acid, further evidence for an agar origin (26), and the proportions of neutral sugars closely resembled those for RP-12A10.

Methylation analysis of the two bacterial products (RP-12A10 and RP-12A1) and the control, monitored by gas-liquid chromatography and mass spectrometry of the methylated alditol acetates, gave essentially the same results. The origins of the six major components and their relative peak areas (arbitrary units in parentheses) for RP-12A1 (gas-liquid chromatography) were as follows: A, a terminal hexopyranosyl residue (2.6); B, a 3-substituted hexopyranosyl residue (10.0); C, a 3,4-disubstituted hexopyranosyl residue (7.0); D, a 4,6-disubstituted hexopyranosyl residue (4.8); E, a 3,6-disubstituted hexopyranosyl residue (5.9); and F, a 3,4,6-trisubstituted hexopyranosyl residue (6.4). By using available reference materials, components A, B, and C could be identified as derivatives of galactose, and as E and F were also produced by periodate-oxidized RP-12A1 and CA1 (which both contained no glucose), they also were derivatives of galactose. Agar consists of a complex mixture of polysaccharides, the basic units of which are 3-substituted β-D-galactopyranosyl and 4-substituted α-L-galactopyranosyl residues and which may be modified by methylation, glycosylation, sulfation, pyruvylation, or intramolecular bridging (26). In a study of Bacto-Agar (Difco), the presence of a fraction that was enriched in sulfate but depleted in 3,6-anhydro-L-galactose and pyruvic acid and that could be leached out by aqueous extraction at 20°C was demonstrated (14). It appears that such a fraction is largely responsible for the crude slime described here.

The use of Trypticase soy broth in the media used for the above experiments was another potential source of contamination of any slime produced by the strains of *S. epidermidis*. A check in which an equivalent amount of Trypticase soy broth was dialyzed and then subjected to fractionation with trichloroacetic acid and ethanol as described for the Trypticase soy broth-agar products gave 0.18 g of material with the following makeup; total carbohydrate, 5.9%; D-galactose, 3.2%; phosphorus, 3.7%. These data suggested that Trypticase soy broth was not a major contributor to the bulk or the carbohydrate content of crude slime but could be a source of phosphorus. Growth in Trypticase soy broth is known to promote slime formation in static cultures of *S. epidermidis*, including strain RP-12 (10). We confirmed adherent growth by static but not shaken cultures, but all crude slimes prepared from either type of culture by using strains RP-12 and Kaplan were very similar in composition: total carbohydrate, 7 to 11%; phosphorus, 3.4 to 4.2%. Similar data were obtained for the products from sterile broth controls: total carbohydrate, 7 to 12%; phosphorus, 3.5 to 3.6%. However, fractionation of the test and control products on Sephadex G-50 revealed a point of difference. Both gave major fractions of products with low M_rs containing carbohydrate (2%) and phosphorus (4%). The minor, polymeric fractions (6 to 16% of the total) differed in their contents of phosphorus (test, 3 to 4%; control, 0.2%) and carbohydrate (test, 40 to 60%; control, 78%). These results confirm that Trypticase soy broth can contribute phosphorus to crude slime (presumably mainly the material removed by Sephacryl S-300) but also show that an additional phosphorus-containing polymer, possibly (lipo)teichoic acid, is present in the supernatants from bacterial cultures. Further examination of the polymeric fractions (test and control) showed the presence of glucose, galactose, and galactosamine in all cases, together with smaller amounts of arabinose and mannose. These common components are therefore derived from the medium, possibly from glycans or glycoproteins present in medium components. For example, galactose and galactosamine are known components of the phosphoglycoprotein (casein) used in the preparation of Trypticase soy broth (18).

Apart from the small amount of polymeric, phosphorus-containing product present in the supernatants from Trypticase soy broth cultures, no chemical candidate for bacterial slime has been detected in this study, even under conditions in which adherent growth pointed to slime formation. The main message of this paper is that putative slime may be heavily contaminated by medium constituents. This is particularly true for products obtained from submerged solid media.

The reports on the chemistry of *S. aureus* slime by Ekstedt and Bernhard (15) and Rozgonyi and Seltmann (30) and of *S. epidermidis* slime by Ludwicka et al. (22) and Peters et al. (28) must all be considered suspect in that their products, even when subjected to additional purification, were probably heavily contaminated with medium, particularly agar, components. In no case does a sterile medium control appear to have been examined. This casts doubt on the proposed biological properties of slime and their in vivo significance (16, 17). We have shown the occurrence of sulfate in crude slime for the first time, and an agar-derived sulfated galactose polysaccharide is probably responsible for the reported anticoagulant activity of slime (6). Sulfated glycosaminoglycans of mammalian and plant origin, such as heparin, dermatan sulfate, and pentosan sulfate, are known anticoagulants (6).

Substantial quantities of mannose in slime were reported by Rozgonyi and Seltmann (30), who used a modified staphylococcus 110 medium; Ludwicka et al. (22), who used supplemented N medium; and Peters et al. (28), who reported preliminary studies of slime isolated from organisms grown on agar-solidified, chemically defined medium. These last authors also found appreciable amounts of glucose. We did not encounter much mannose in our studies with Trypticase soy broth agar. Thus, a mannose-containing polymer is a candidate for a slime component.

An acidic polysaccharide of equimolar D-glucuronic acid and glucosamine was isolated from the culture supernatant of *S. epidermidis* grown in brain heart infusion broth supplemented with sucrose (24). Brock and Reiter (5) demonstrated the presence of ribitol teichoic acid in slime from *S. aureus* grown in modified staphylococcus 110 medium. Tojo et al. (32) studied one of the slime-producing strains of Christensen, *S. epidermidis* RP-62A, grown in tryptic soy broth. They isolated and purified a fraction from culture

supernatants that they called adhesin because of its abilities to bind to silastic catheter tubing, inhibit strain RP-62A binding, and elicit the production of binding-blocking antibodies. The main components were galactose, glucosamine, and galactosamine. On the basis of immunoelectron microscopy, these authors suggested that the adhesin was part of a capsular polysaccharide in intact organisms.

Thus, the nature of slime remains obscure, no specific, unique macromolecule (exopolysaccharide?) having been identified as a candidate for slime. Probably, growth on a solid surface is important in slime production. There are abundant examples of expression of different phenotypic characteristics in biofilm and planktonic bacteria (11, 21). For example, in *S. aureus*, different cell surface proteins are produced by surface-grown bacteria from those produced by liquid culture-grown organisms (9). For *S. aureus*, staphylococcus 110 medium appears to encourage slime production, whereas most *S. epidermidis* studies have used Trypticase soy broth. The inclusion of substantial amounts of NaCl (>0.5 M) in medium stimulates the autolytic activity of *S. aureus* (12, 23) and leads to the release of an extracellular DNA slime (8, 12). The turnover of cell surface components and release of normally internal macromolecules such as DNA may give cultures a slimy appearance to the naked eye or after staining with cationic dyes. Such materials are less likely to diffuse away from bacteria attached to a solid surface than from those in liquid culture medium (21). Clearly, the use of agar in studies designed to elucidate the chemical nature of slime should be avoided, and the development of a chemically defined medium supporting slime production is needed. Also, incorporation of radioactivity from a small-molecular-weight precursor molecule into a macromolecule would confirm its identification as slime.

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