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Lectin–biotin assay for slime present in *in situ* biofilm produced by *Staphylococcus epidermidis* using transmission electron microscopy (TEM)

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A lectin–biotin assay was developed for use in the specific detection of slime produced by *Staphylococcus epidermidis* RP62A and M187sp11 grown in a chemically defined medium. Mature biofilm was formed on polyvinylchloride (PVC) disks using a combined chemostat-modified Robbins device (MRD) model system. Specimens fixed *in situ* were: 1) stained with ruthenium red; 2) reacted overnight with biotin-labeled lectins (WGA, succinyl-WGA, Con A, or APA) followed by treatment with gold-labeled extravidin; or 3) reacted with antibodies against *S. epidermidis* RP62A capsular polysaccharide/adhesin (PS/A) using an immunogold procedure. WGA and succinyl-WGA (S-WGA), which specifically bind *N*-acetylglucosamine, were shown by TEM to react only with slime, both cell-associated and exocellular. In contrast, Con A, APA and anti-PS/A reacted with the bacterial cell surface but did not react with slime. These results indicate the usefulness of WGA lectin as a specific marker for detection of the presence and distribution of slime matrix material in *S. epidermidis* biofilm.

Keywords: *S. epidermidis*; biofilm; slime; lectin marker

Introduction

The increased use of medical devices and prosthetic implants in humans has resulted in a concomitant rise in bacterial infections, and *Staphylococcus epidermidis* has emerged as the most common cause [8]. In early studies, these cocci were shown to form microcolonies embedded in a mucoid slime matrix (biofilm) on infected Spitz–Holter valves [3] and on intravascular catheters exposed *in vitro* [4,21]. Wilkinson [28] defined slime as an anionic exopolysaccharide material, distinct from capsule, that loosely surrounds the cell in a diffuse layer; it is generally harvested by washing the cells or by extracting material released into growth medium. When a slime matrix is formed *in situ* by sessile cells, it apparently 'glues' cells to the surface of biopolymers and protects them from antimicrobials and the host's immune system [7]. While methods are available for quantifying the number of cells colonizing the surface of a biopolymer [7], comparable tests *specific* for slime, which may or may not be produced, are not available. In the drive to develop medical grade biopolymers with biofilm-resistant surfaces, a slime test would be very useful in determining the effect, if any, of surface modifications on the production, accumulation, distribution, and persistence of slime. In the present work, we developed a lectin–biotin assay system for testing the capacity of certain lectins to bind to the slime matrix material present in mature (intact) biofilm formed *in situ* by *S. epidermidis* grown on polyvinylchloride. Previous studies on the chemical characterization of slime produced by planktonic cells, particularly those grown in chemically defined media [11], have shown that mannose [22], glucose [1,6,14,22], galactose [6,22,26] and glucosamine [1,6,14,22,26] are major or minor components of solubilized slime. For this reason, lectins selected for testing had binding specificities for these particular carbohydrates.

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Materials and methods

Bacteria

Two slime-producing strains of *S. epidermidis* were used: RP62A (ATCC 35984) [5] and M187sp11 [18]. Strain RP62A was kindly supplied by Gordon Christensen (Harry S Truman Memorial Veterans Hospital, Columbia, MO, USA); strain M187sp11 was kindly supplied by Eugene Muller (Channing Laboratory, Department of Medicine, Brigham and Women's Hospital, Boston, MA, USA).

Biomaterials

Injection-molded polyvinylchloride (PVC) test polymer disks (7.8 mm diameter × 2 mm thick) were supplied by Duane Horton (Mallinckrodt Medical, Inc, St Louis, MO, USA). Disks were cleaned with 0.1% Count-Off (NEN Research Products, Boston, MA, USA) in distilled H_2O, washed exhaustively in distilled H_2O, drained and air-dried. Disks were placed in a modified Robbins flow-through device (MRD) [20] (University of Exeter, Department of Biological Sciences, Hatherly Laboratories, Exeter, UK) which is designed to hold 25 samples in a fixed position so that the surface of a disk is flush with the top of a trough that runs the length of the device. This design prevents settling out of bacterial cells on the disks' surfaces. The device, with disks, was sterilized by ethylene oxide, and degassed for 7 days before use.

Combined chemostat-modified Robbins device model system for biofilm formation

This procedure was described previously [24]. Briefly, 750 ml of sterile FMC (an enriched chemically defined medium [17], pH 7.2) was placed in the culture vessel of a Bioflo bench top chemostat (model C30, New Brunswick Scientific Co, Edison, NJ, USA) and inoculated with ~10^6 colony forming units (CFU) of bacteria. The culture was aerated at 0.2 L min^{-1} at 37° C and agitated at 200 rpm for 24 h; fresh medium was added at a rate of ~30 ml h^{-1}. The culture suspension (2×10^9 CFU ml^{-1}, T_D [generation time] of 14 h) was then pumped at the same rate through the MRD which was placed in an incubator at 37° C. Disks removed from the MRD were drained, agitated for 5 s in sterile phosphate-buffered saline (PBS), pH 7.2 (FTA haemagglutination buffer; BBL, Cockeysville, MD, USA), drained, and agitated in two fresh changes of PBS to remove unattached bacteria. Specimens were removed from the stubs using a sterile needle and processed for quantification of bound bacteria or for electron microscopy.

Quantitation of adherent staphylococci

Multiple samples were processed for quantification of adherent staphylococci: a disk was placed in a 10-ml vol of PBS that contained 0.1% Tween-80 (Fisher Scientific Co, Pittsburgh, PA, USA) and sonicated in a Branson 1200 ultrasonic cleaner waterbath (Branson Ultrasonics Corp, Danbury, CT, USA) for 30 min at room temperature to detach adherent bacteria [2]. The bacterial suspension was vortexed vigorously for 15 s to break up clumps. Ten-fold serial dilutions of each suspension were plated on trypticase soy agar (TSA; Difco Laboratories, Detroit, MI, USA) using the agar-overlay technique, incubated at 37° C for 18 h and the mean number of CFU was determined.

Transmission electron microscopy

Disks were rinsed 3× in PBS, prefixed overnight in 2% glutaraldehyde (Eastman Kodak Co, Rochester, NY, USA) in 0.2 M cacodylic acid (sodium salt; Ernest F Fullam Inc, Schenectady, NY, USA) -HCl buffer at 4° C, rinsed with 0.1 M PO_4 buffer, then fixed for 30 min in either 1% OsO_4 (Electron Microscopy Sciences, Ft Washington, PA, USA) in 1% sodium veronal (barbital; Sigma Chemical Co, St Louis, MO, USA) -sodium acetate (Sigma) buffer [13] or 0.25% ruthenium red stain (EMS) in aqueous 1% OsO_4 [16]. Specimens were rinsed with veronal-acetate buffer; disks stained with ruthenium red were also rinsed in distilled H_2O. All specimens were dehydrated through a graded series of ethanol, placed in 1 : 1 Polybed 812 (Polysciences, Inc, Warrington, PA, USA) : ethanol for 1 h then in 100% PolyBed 812 under vacuum for 1 h. Disks were embedded in flat molds in fresh PolyBed 812 at 70° C for 18 h, 80° C for 3 h, then −20° C until cool. Sections, either unstained or stained with 7% aqueous uranyl acetate (EMS) -Reynolds' lead citrate [13] using a microwave staining procedure [12], were examined with a Philips 301 transmission electron microscope (Phillips Electronic Instruments Co, Mahwah, NJ, USA). Treatment of the specimens with propylene oxide, a standard step in most procedures, was omitted to prevent dissolution of the polymer disks.

Surface binding of lectins

Disks were prefixed as described, rinsed 3× with 0.2 M cacodylate-HCl buffer (1 min each), then covered with a 1-ml vol of CT buffer (0.5% casein [acid hydrolysate of casein, amicase, from bovine milk; Sigma], 0.05% Tween 20, 0.1 mM Ca^{2+} ($CaCl_2$); and 0.1 mM Mn^{2+} ($MnCl_2$) in PBS, pH 7.4) for 60 min at ambient temperature to quench free aldehyde groups and to minimize non-specific binding. Specimens were rinsed with PBS and covered with a 1-ml vol of CT buffer containing biotin-labeled lectin as follows: 31 μg of wheat germ agglutinin (WGA; from *Triticum vulgaris*; Sigma) or succinyl wheat germ agglutinin (S-WGA; E-Y Laboratories, Inc, San Mateo, CA, USA), 16 μg of concanavalin A (Con A; from *Canavalia ensiformis* [jack bean]; Sigma), or 1.88 μg of jequirity bean agglutinin (APA; from *Abrus precatorius*; Sigma). Specimens were held at 4° C for 18 h then rinsed repeatedly with PBS to remove unbound lectin. Each specimen was covered with a 1-ml vol of a 1 : 20 dilution of colloidal gold (10 nm)-labeled extravidin (Sigma) in CT buffer for 4 h at ambient temperature then rinsed repeatedly with PBS. Specimens were postfixed with 2% glutaraldehyde in 0.2 M cacodylate-HCl buffer then dehydrated and embedded as described for transmission electron microscopy. Controls included gold-labeled extravidin only, and unlabeled lectin plus labeled extravidin.

Surface binding of antibodies

Disks were prefixed, rinsed, and incubated in CT buffer as described. Specimens were rinsed with PBS, covered with a 1-ml vol of a 1 : 200 dilution of antiserum in CT buffer, and held at 4° C for 18 h. The antiserum contained antibodies raised in rabbits against a purified polysaccharide adhesin (PS/A) from RP62A [26] and was kindly supplied by Eugene Muller (Channing Laboratory). Disks were rinsed 3× with PBS to remove unbound antibodies and covered with a 1-ml vol of a 1 : 20 dilution colloidal gold (20 nm)-labeled protein A (Sigma) in CT for 4 h at ambient temperature. Disks were rinsed repeatedly with PBS, postfixed with 2% glutaraldehyde in 0.2 M cacodylate-HCl buffer and dehydrated and embedded as described. Controls included gold-labeled protein A only, and nonimmune rabbit serum plus gold-labeled protein A.

Results and discussion

Extended exposure of PVC disks to *S. epidermidis* showed that both test strains colonized the surface of the disks to a comparable level. By day 6, the mean CFU × 10^6 ± SEM was 7 ± 1.4 for strain RP62A and 10.7 ± 1.2 for strain M187sp11. Disk specimens were subsequently reacted, *in situ*, with each of the lectins and observed by transmission electron microscopy. WGA (Figures 1 and 2), specific for GlcNAcβ-1,4_n and sialic acid, and S-WGA (not shown), specific just for GlcNAcβ-1,4_n [10] were the only lectins reactive with slime produced by both strains; the results obtained with these two lectins in the lectin–biotin assay were identical. Figure 1 is representative of a mature biofilm that shows both a multilayered microcolony of staphylococci and the WGA-reactive exopolymer matrix; slime embedded the cocci and formed an interface between

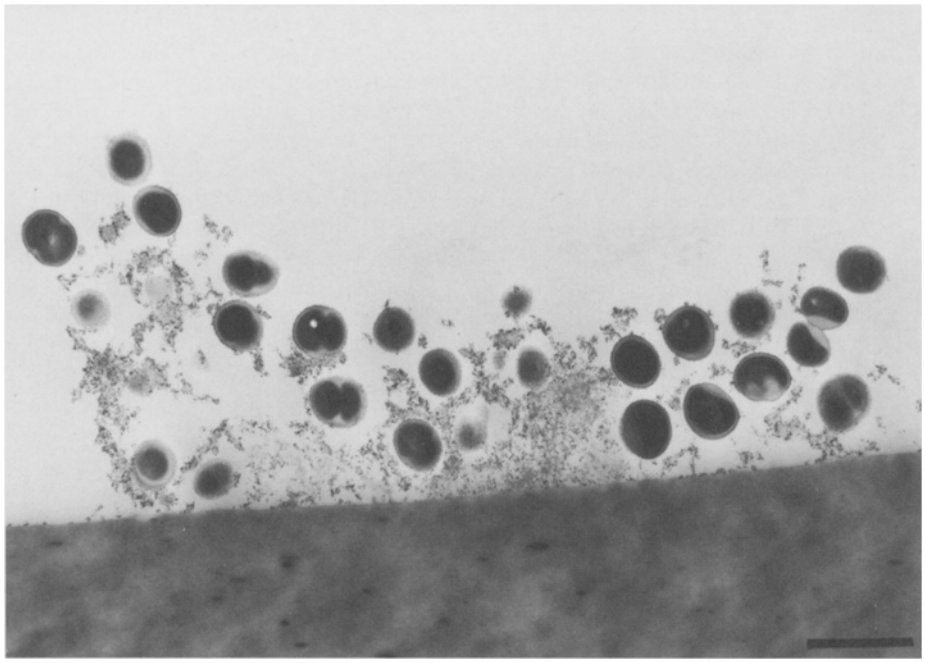

Figure 1 Transmission electron micrograph of a biofilm formed on the surface of a PVC disk exposed to a free-flow of *S. epidermidis* RP62A using a combined chemostat-MRD model system. The specimen was reacted *in situ* with biotin-labeled WGA followed by gold-labeled extravidin. The multilayered microcolony was embedded in a slime matrix material which was detected by bound WGA (10 nm gold particles). Bar = 1 μm

the cocci and the surface of the PVC disk. At cell surfaces, the slime reacted specifically with WGA (Figures 1, 2b, 2c, and 2e) and nonspecifically with the cationic electron stain, ruthenium red (Figures 2a and 2d). The cell-associated slime appeared as formed globules, of various sizes, which were located in discrete areas on the surface of the cocci and which seemed to coalesce when released from the cell surface to form the matrix of the biofilm. Even though the specimens were dehydrated in processing for TEM, the slime did not appear to shrink and collapse into an evenly distributed layer around the cell wall as might be expected if the exopolymer simply formed a loose unattached layer around the cells. Also, the cell- and matrix-associated slime was obviously not solubilized and washed away in the constant free-flow of medium through the MRD. Controls, which included WGA without the biotin label + gold-labeled extravidin and labeled extravidin alone, were all negative (no matrix material was visualized due to the absence of gold particles). Combined results with WGA and S-WGA suggested that GlcNAcβ-1,4$_n$ was one of the components in the mature slime matrix present probably as a terminal residue and accessible to binding with lectin.

Surprisingly, the remaining two lectins—Con A, which is specific for manα-1, glcα-1, and glcNAcα-1, and APA, which is specific for β-D-gal [10], did not react with slime globules on the surface of the cocci and did not bind to the exocellular slime matrix material. Representative results with Con A and APA are shown in Figure 3. Con A, and to a lesser extent APA, bound directly to the surface of cells embedded in the matrix. Again, controls (lectin without the biotin label + gold-labeled extravidin) were negative (not shown). Similarly, Slusher *et al* [25] used TEM to demonstrate that Con A localized on the surface of *S. epidermidis* cells colonizing a contact lens. In fact, Davidson *et al* [9] showed that Con A-binding carbohydrates are commonly present on the surface of *S. epidermidis* strains, grown on blood agar, while WGA-binding carbohydrates are usually not present. Con A-reactivity has been used as a marker for slime [14,15,22]. However, Wilcox *et al* [27] reported that the agglutination of *S. epidermidis* with Con A was still positive after a washing procedure sufficient to remove water-soluble slime from the cells, which implies that Con A may have bound to cell wall carbohydrates as opposed to slime material. One possibility is that the Con A binds to the anionic cell wall teichoic acid. Reeder and Ekstedt [23] showed that Con A reacts with α-glucosylated, but not β-glucosylated, teichoic acids from *S. epidermidis*. Hussain *et al* [14] suggested that teichoic acid is present in solubilized slime preparations isolated from several strains of *S. epidermidis*, including strain RP62A. They also found that slime was agglutinated by both Con A and a potato lectin which has the same binding specificity as S-WGA. In the present study, slime matrix material produced by sessile cells in an *in situ* mature biofilm on PVC, under controlled free-flow conditions, may have different phenotypic characteristics when compared to solubilized slime isolated from cells grown on agar or released by planktonic cells grown in batch culture [7]. In any case, WGA and S-WGA, but not Con A nor APA, demonstrated a specificity for slime.

Muller *et al* [19] reported that expression of a capsular polysaccharide adhesin (PS/A) is common among slime-producing clinical isolates of coagulase-negative staphylo-

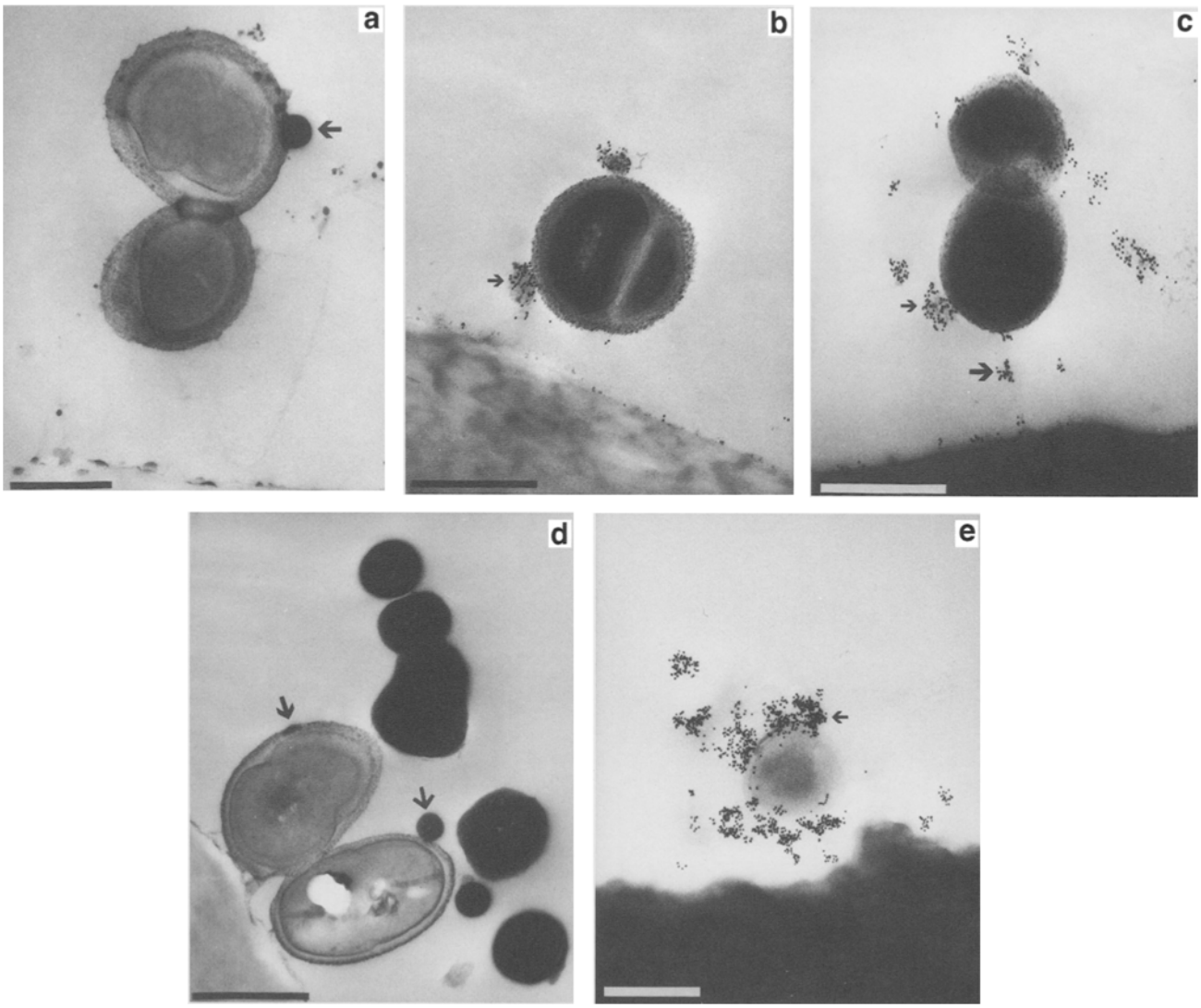

Figure 2 Transmission electron micrographs of the surface of PVC disks exposed to *S. epidermidis* RP62A (a,b,c) and M187sp11 (d,e) for 3 or 6 days. Specimens (a,d) were postfixed with osmium and stained with ruthenium red. Exopolymer material, detected by staining with ruthenium red, was present as slime globules (large arrows) that appeared to form on the cell surface as shown in (a) and (d) before being released into the surrounding milieu (d). Specimens (b,c,e) were reacted *in situ* with biotin-labeled WGA followed by gold-labeled extravidin. WGA reacted with the slime globules present in multiple, discrete areas of the cell surface (small arrow) of M187sp11 (e) (unstained) and RP62A (b,c) (stained with uranyl acetate-lead citrate). In addition, WGA reacted with slime material bound to a filamentous structure (slime?) (large arrow) which appeared to anchor the cells to the surface of the PVC disk (c) but was not observed to react with anchoring filaments alone. Bar = 0.5 μm

cocci (predominantly *S. epidermidis*), and Tojo *et al* [26] identified PS/A as a component of slime. In the present study, an immunogold procedure was used to determine the capacity of anti-PS/A antibody to react with slime. The antibody reacted primarily with surface antigens on both test strains, rather than with slime globules or matrix material. Figure 4 is representative of the distribution pattern of the antibody and WGA when cells were treated simultaneously, *in situ*, with both reagents. WGA reacted with slime while antibody did not. Controls, which included nonimmune rabbit serum + gold-labeled protein A and gold-labeled protein A only, were negative. In this assay system, anti-PS/A antibody could not be used as a specific marker of slime in *in situ* mature biofilm.

The lectin–biotin assay procedure for TEM offers direct proof that WGA and S-WGA can be used as specific markers of *in situ* slime. These lectins are potentially potent tools for use in enzyme-linked lectinsorbent assays for quantitating slime produced *in situ* on the surface of biomaterials, as well as for screening clinical isolates of coagulase-negative staphylococci for slime production. In addition, fluorescent WGA can be used as a molecular probe in studying the architecture of biofilm with laser scanning confocal microscopy. These studies are in progress.

Acknowledgements

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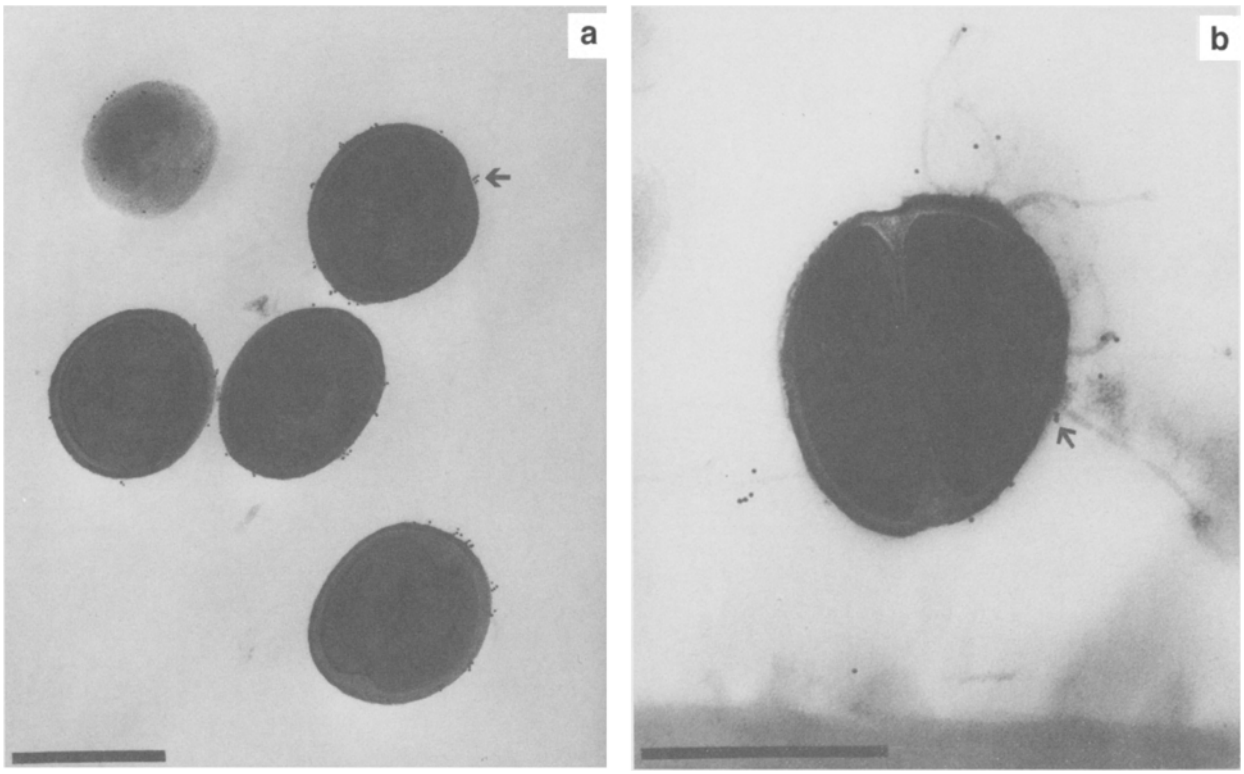

Figure 3 Transmission electron micrographs of the surface of PVC disks exposed to *S. epidermidis* RP62A for 3 days. Specimens were reacted *in situ* with biotin-labeled lectins followed by gold-labeled extravidin. All specimens were postfixed with osmium and sections were poststained with uranyl acetate-lead citrate. ConA (a) and APA (b) were detected on the cell surface (small arrows), but neither lectin reacted with slime globules. Bar = 0.5 μm

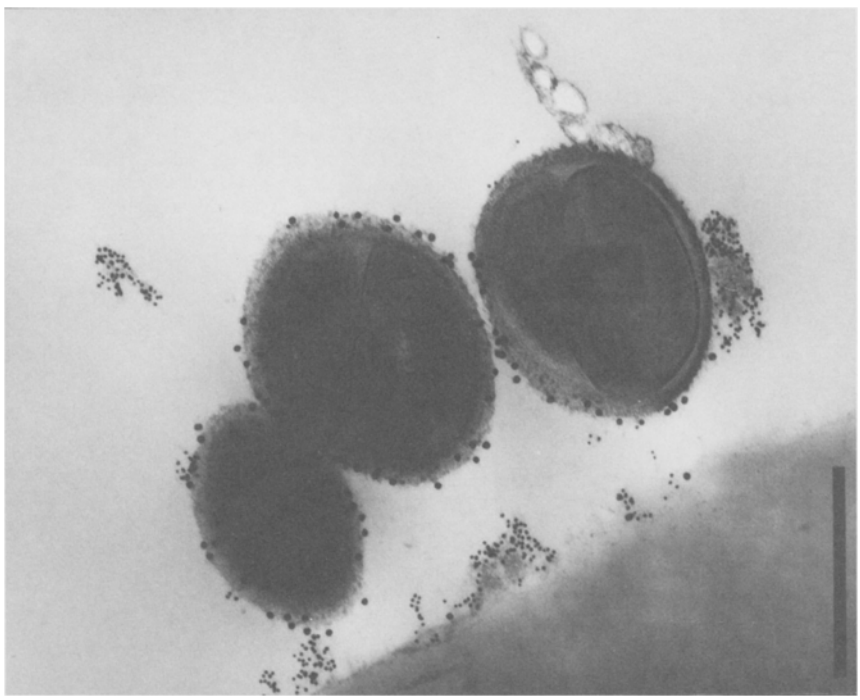

Figure 4 Transmission electron micrograph of the surface of a PVC disk exposed to *S. epidermidis* RP62A for 3 days. Specimens were reacted simultaneously *in situ* with biotin-labeled WGA (detected by gold labeled-extravidin) and antiserum containing unlabeled antibodies against PS/A (detected by gold-labeled protein A). The section was stained with uranyl acetate-lead citrate. Antibodies (20 nm gold particles) were localized on the cell surface and did not react with slime globules, while WGA (10 nm gold particles) reacted with slime globules. Bar = 0.5 μm

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