Enzymatic Detachment of *Staphylococcus epidermidis* Biofilms

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The gram-positive bacterium *Staphylococcus epidermidis* is the most common cause of infections associated with catheters and other indwelling medical devices. *S. epidermidis* produces an extracellular slime that enables it to form adherent biofilms on plastic surfaces. We found that a biofilm-releasing enzyme produced by the gram-negative periodontal pathogen *Actinobacillus actinomycetemcomitans* rapidly and efficiently removed *S. epidermidis* biofilms from plastic surfaces. The enzyme worked by releasing extracellular slime from *S. epidermidis* cells. Precoating surfaces with the enzyme prevented *S. epidermidis* biofilm formation. Our findings demonstrate that biofilm-releasing enzymes can exhibit broad-spectrum activity and that these enzymes may be useful as antibiofilm agents.

*S. epidermidis* slime is also a polysaccharide that contains primarily N-acetylglucosamine residues (12), we decided to test whether *A. actinomycetemcomitans* dispersin B could cause the detachment of *S. epidermidis* biofilms from plastic surfaces. In this report we show that *A. actinomycetemcomitans* dispersin B exhibits potent biofilm-releasing activity against slime-producing, clinical strains of *S. epidermidis*.

### MATERIALS AND METHODS

**Bacterial strains, media, and growth conditions.** Four *S. epidermidis* strains (designated NJ9709, NJ9710, NJ9711, and NJ9712) were isolated from the surfaces of infected intravenous catheters removed from patients at University Hospital, Newark, N.J. Strains were identified by using the Api-Staph biochemical identification kit (bioMérieux, Durham, N.C.). All four strains contained the ica genetic locus (1) and produced black colonies on Congo red agar (2), both of which are indicative of slime production. Strains were streaked onto blood agar plates and incubated for 24 h at 37°C in air. Plates were stored at 4°C, and bacteria were passaged weekly. Biofilms were grown in Trypticase soy broth (Becton-Dickinson) supplemented with 6 g of yeast extract and 8 g of glucose per liter. Inoculated culture vessels were incubated statically in air at 37°C.

**Preparation of inocula.** A loopful of colonies scraped from the surface of an agar plate was transferred to a microcentrifuge tube containing 200 μl of fresh medium. The tube was vortexed for 30 s at high speed, and the cells were allowed to settle for 5 min. One hundred microliters of the upper layer was transferred to a 100-mm-diameter polystyrene petri dish (model 3003; Falcon) containing 20 ml of fresh medium, and the dish was incubated for 16 h. The biofilm that formed on the surface of the dish was rinsed with phosphate-buffered saline (PBS) and then scraped from the surface of the dish into 3 ml of PBS by using a cell scraper. The cell aggregate was transferred to a 15-ml conical centrifuge tube, vortexed for 30 s, and allowed to settle to the bottom of the tube for 10 min. A 0.5-ml aliquot of the top layer was transferred to a tube containing 5 ml of fresh broth, and the tube was vortexed briefly. The resulting inoculum contained 10³ to 10⁶ CFU ml⁻¹. Serial decimal dilutions were made with fresh broth.

**Enzymes.** *A. actinomycetemcomitans* dispersin B (formerly DspB) was purified as previously described (10). Protein concentration was determined by using a Bio-Rad protein assay kit. The purified enzyme had a specific activity of 970 units per mg of protein, where 1 unit of enzyme activity was defined as the amount of enzyme needed to hydrolyze 1 μmol of 4-nitrophenyl-N-acetylglucosaminide to 4-nitrophenol and N-acetylglucosamine per min at pH 4.5 at 25°C in 50 mM sodium phosphate buffer at 100°C for 3 min. *Serratia marcescens* chitinase and Jack bean N-acetylglucosaminidase were purchased from Sigma Chemical Company.

**Growth of biofilms in 96-well polystyrene microtiter plates.** The wells of a 96-well polystyrene microtiter plate (model 3959; Corning) were filled with 100-μl aliquots of inoculum, and the plate was incubated for 16 h. The wells were rinsed with either three 200-μl aliquots of PBS or by submerging the entire plate in a tube of cold, running tap water. Biofilms were stained with crystal violet as...
previously described (8). The optical densities (OD) of the wells were determined by using a Bio-Rad Benchmark microplate reader set to 590 nm. To assay biofilm detachment, 1 \( \mu l \) of enzyme was added directly into the well of the microtiter plate 30 min prior to rinsing. Unless otherwise indicated, all enzyme treatments were carried out for 30 min at 30°C with a final enzyme concentration of 40 \( \mu g\) ml\(^{-1}\). In some experiments, the wells were washed with water and then filled with 100 \( \mu l \) of PBS prior to addition of the enzyme.

**Growth of biofilms on polystyrene rods.** Polystyrene rods (1.5-mm-diameter; Plastruct Corp., City of Industry, Calif.) were cut to 35-mm lengths, sterilized in 70% ethanol for 30 min, and air dried in a biological safety cabinet. Rods were placed into 1.5-ml microcentrifuge tubes containing 0.5 ml of a 10\(^{-3}\) dilution of \( S.\) epidermidis strain N39709 and incubated for 16 h. The rods were then rinsed with water and placed into fresh microcentrifuge tubes containing 0.75 ml of PBS or PBS containing 40 \( \mu g\) ml\(^{-1}\) of dispersin B. After 15 min, the rods were rinsed with water and stained with crystal violet as previously described (8). For sonication, rods were placed in 15-ml conical centrifuge tubes containing 3 ml of fresh broth and then sonicated for 30 s at 40% duty cycle and 70% capacity in a Branson model 200 sonicator equipped with a cup horn. For quantitation of detached cells, sonicates were serially diluted in fresh broth and spread on agar medium. Colonies were enumerated after 24 h.

**Growth of biofilms on intravenous catheters.** Polyurethane catheters (20 gauge, 1.1-mm diameter; model 381434; Becton-Dickinson) and Te (model 340165; Corning), and the dish was incubated for 16 h. The biofilm that formed on the surface of the dish was rinsed with water, scraped from the surface into 1 ml of PBS, and transferred to a 1.5-ml microcentrifuge tube. Biofilm cells were treated with 40 \( \mu g\) ml\(^{-1}\) of dispersin B or mock treated for 5 min at room temperature. Coated catheters were dried at 37°C for 1 h prior to use. Polyurethane catheters were stained in a solution of 1% methylene blue in water for 2 min and then rinsed with water.

**Carbohydrate techniques.** Four milliliters of a 10\(^{-3}\) dilution of \( S.\) epidermidis strain N39709 was transferred to a 35-mm-diameter polystyrene petri dish (model 340165; Corning), and the dish was incubated for 16 h. The biofilm that formed on the surface of the dish was rinsed with water, scraped from the surface into 1 ml of PBS, and transferred to a 1.5-ml microcentrifuge tube. Biofilm cells were treated with 40 \( \mu g\) ml\(^{-1}\) of dispersin B or mock treated for 5 min at room temperature with gentle rocking. The tube was then centrifuged for 2 min at 15,000 rpm in a Sorvall MC12V microcentrifuge, and the supernatant was transferred to a new tube.

Supernatants were analyzed for the presence of glycosaminoglycans by using the quantitative measurement of Alcian Blue-glycosaminoglycan complexes described by Whitteman (20). Total hexosamine was determined after acid hydrolysis (4 M HCl, 16 h, 100°C) by using the Morgan-Elson assay (17). Total hexosamine was determined by using the Morgan-Elson assay (17). Total hexosamine was determined after acid hydrolysis (4 M HCl, 16 h, 100°C) by using the Morgan-Elson assay (17). Total hexosamine was determined after acid hydrolysis (4 M HCl, 16 h, 100°C) by using the Morgan-Elson assay (17).

**RESULTS**

**Detachment of** \( S.\) epidermidis **biofilms by** \( A.\) actinomyctecomitans **dispersin B.** Four strains of \( S.\) epidermidis isolated from infected intravenous catheters were tested for their ability to form biofilms by growing serial dilutions of overnight cultures in the wells of a 96-well polystyrene microtiter plate. After 16 h of incubation, the wells were washed to remove loosely adherent cells and the bacteria remaining attached to the bottoms of the wells were stained with crystal violet (Fig. 1A). All four strains produced adherent biofilms as indicated by the presence of dark-staining material on the bottoms of the wells. The amount of biofilm could be quantitated by measuring the OD of the wells at 590 nm with a microtiter plate reader (Fig. 1B). When dispersin B was added to the wells 30 min prior to washing (final concentration, 40 \( \mu g\) ml\(^{-1}\)), little or no biofilm was evident (Fig. 1A and B). Heat-inactivated dispersin B had no effect on \( S.\) epidermidis biofilms (Fig. 1C). Two other family 20 glycosid hydrolases, \( S.\) marcescens chitinase and Jack bean N-acetylglucosaminidase, also had no effect on \( S.\) epidermidis biofilms (Fig. 1D). Dispersin B had no bactericidal effect on \( S.\) epidermidis cells (data not shown). These results indicate that the enzymatic activity of \( A.\) actinomyctecomitans dispersin B caused the detachment of \( S.\) epidermidis biofilm cells from the surfaces of the wells.

**Kinetics of biofilm detachment.** \( S.\) epidermidis biofilms were grown for 16 h in microtiter plate wells and then treated with increasing amounts of dispersin B (final concentrations, 200 pg to 120 \( \mu g\) ml\(^{-1}\)) for 0 to 9 min (Fig. 2A and B). A concentration of 4.8 \( \mu g\) ml\(^{-1}\) of dispersin B resulted in a decrease in OD to background levels (ca. 0.09 OD units) after 2 min, and 40 ng ml\(^{-1}\) of dispersin B resulted in a >50% reduction in OD (from 3.63 to 1.74 OD units) after 9 min. These data demonstrate that dispersin B-mediated detachment of \( S.\) epidermidis biofilms was rapid and efficient and occurred at clinically achievable concentrations of enzyme.

**Quantitation of biofilm detachment.** \( S.\) epidermidis biofilms were grown attached to the surfaces of polystyrene rods and then treated with 40 \( \mu g\) ml\(^{-1}\) of dispersin B for 15 min. The bacteria remaining attached to the rods after treatment were removed by sonication and then quantitated by plating on agar. Figure 3A shows mock-treated and dispersin B-treated rods after staining with crystal violet. The mock-treated control rod (rod 1) contained a layer of dark-staining material corresponding to the thick biofilm that formed on its surface. The dispersin B-treated rod showed no trace of dark-staining material and was similar in appearance to a rod which had been soni-
cated prior to staining (rod 3) and to an uninoculated rod (rod 4). Quantitation of cells remaining attached to the rods revealed that dispersin B treatment resulted in a 5.8 log reduction in the number of surface-associated bacteria (Fig. 3B).

Dispersin B displayed similar biofilm-releasing activity against *S. epidermidis* biofilms grown attached to the surfaces of polyurethane and Teflon catheters (Fig. 3C and D). Precoating catheters with dispersin B prevented *S. epidermidis* biofilm formation (Fig. 3E and F). Precoated polyurethane catheters retained enzyme activity for at least 30 days when stored at room temperature (data not shown).

**DISPERSIN B degrades *S. epidermidis* slime.** To demonstrate that dispersin B degraded the *S. epidermidis* intercellular slime matrix, we scraped biofilm cells from the surface of a culture vessel and transferred them to a tube. Under these conditions, the cells formed a sticky aggregate that rapidly settled to the bottom of the tube (Fig. 4A, left). Treatment of cell aggregates with dispersin B resulted in uniformly turbid cell suspensions (Fig. 4A, right), indicating that treatment with dispersin B interfered with intercellular adhesion. Treatment with dispersin B also resulted in increased amounts of glycosaminoglycans (Fig. 4B) and total hexosamine (Fig. 4C) in cell supernatants. When polysaccharide was purified from supernatants of mock-treated and dispersin B-treated cells and then analyzed by polyacrylamide gel electrophoresis, a single band with an apparent molecular mass of 20 kDa was observed in the supernatant of dispersin B-treated cells (Fig. 4D). This band may correspond to a 20-kDa species of low-sulfated polysaccharide that has been shown to be a component of *S. epidermidis* exopolysaccharide slime (11). These data are consistent with the hypothesis that dispersin B is disrupting the *S. epidermidis* slime matrix, either by degrading it or by detaching it from the cell surface.

**DISCUSSION**

Our findings demonstrate that *A. actinomycetemcomitans* dispersin B exhibits high biofilm-releasing activity against *S. epidermidis* biofilms. The enzyme was active at physiologically achievable concentrations and prevented biofilm formation when used to coat plastic biomaterials. These properties suggest that dispersin B could be used as an agent to prevent or treat *S. epidermidis* infections of catheters and other medical devices.

We previously showed that purified dispersin B hydrolyzes...
FIG. 4. Dispersin B releases extracellular slime from S. epidermidis biofilm cells. Minus sign, mock-treated cells; plus sign, dispersin B-treated cells. (A) Biofilm cells that were rinsed and scraped from the surface of a culture vessel formed an aggregate that settled to the bottom of the tube (left). Treatment of the cell aggregate with dispersin B for 5 min resulted in complete dispersion of the aggregate (right). (B) Quantitation of glycosaminoglycans in cell culture supernatants. Values show means (± standard error) for triplicate samples. (C) Quantitation of total hexosamine in cell culture supernatants. Values show means (± standard error) for triplicate samples. (D) Polysaccharides purified from cell culture supernatants were analyzed by polyacrylamide gel electrophoresis and stained with silver.

β-substituted N-acetylglucosamine (10). S. epidermidis slime consists of a polymer of β(1→6)-linked N-acetyl-D-glucosamine residues (12), suggesting that the natural substrate for dispersin B is a β(1→6)-linked glycosamine polymer.

Biological role of dispersin B. We originally proposed that the function of dispersin B was to cause the detachment of cells from biofilm colonies during the dispersal phase of the A. actinomycetemcomitans life cycle (8–10). Our present findings raise the possibility that dispersin B may act as a colonization factor that is capable of detaching biofilms produced by other species of bacteria, thereby clearing a surface which can subsequently be colonized by A. actinomycetemcomitans. It is unlikely that S. epidermidis is a natural target for dispersin B, because S. epidermidis and A. actinomycetemcomitans colonize different surfaces of the human body and are not likely to compete in nature. It is possible that dispersin B can detach biofilms produced by other bacterial species that are present in the oral cavity. It is also possible that the observed interspecific biofilm-detaching activity of dispersin B plays no ecological role but instead reflects a common evolutionary origin of the

expopolysaccharide genes of S. epidermidis and A. actinomycetemcomitans.

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