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Relationship between Glycocalyx and Povidone-Iodine Resistance in *Pseudomonas aeruginosa* (ATCC 27853) Biofilms

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Biofilm-embedded bacteria are generally more resistant to antimicrobial agents than are planktonic bacteria. Two possible mechanisms for biofilm resistance are that the glycocalyx matrix secreted by cells in a biofilm reacts with and neutralizes the antimicrobial agent and that the matrix creates a diffusion barrier to the antimicrobial agent. This study was therefore conducted to examine the relationship between glycocalyx and enhanced povidone-iodine resistance in biofilms of *Pseudomonas aeruginosa* (ATCC 27853). Biofilms were generated by inoculation of polycarbonate membranes with broth-grown cells and incubation of them on the surfaces of nutrient agar plates. The quantities of glycocalyx material per cell were found not to be significantly different between biofilm and planktonic samples. Transmission electron microscopy showed that the distributions of glycocalyx material around cells differed in biofilm and in planktonic samples. Addition of alginic acid to planktonic cell suspensions resulted in a slight increase in resistance to povidone-iodine, suggesting some neutralizing interaction. However, the iodine demands created by biofilm and planktonic samples of equivalent biomass were not significantly different and, therefore, do not explain the contrast in resistance observed between biofilm and planktonic samples. Examination of the relationship between cell death and biomass detachment from the glycocalyx matrix revealed that most cell death occurred in the fraction of biomass that detached from a biofilm during treatment. The overall rate of iodine diffusion through biofilms was not different from that of planktonic cells collected on a polycarbonate membrane. Povidone-iodine resistance seems to be due to the protective layering of cells within the glycocalyx, which increases the time required to detach cells and, therefore, the time required for iodine to contact cells in the deepest layers of the biofilm.

Biofilms are adherent or sessile microbial populations enmeshed in a glycocalyx matrix (18, 21). It is recognized that bacteria in biofilms exhibit enhanced resistance to a variety of antimicrobial agents in natural and model systems (2–4, 9, 19, 25). This resistance in biofilms is not completely understood but has been the subject of many investigations. The glycocalyx matrix around cells within a biofilm has been implicated in resistance (2–5, 14, 15, 21, 23, 25).

Glycocalyx in *Pseudomonas aeruginosa* is composed primarily of uronic acids and carbohydrates (12, 22, 31) and is produced in both biofilm and planktonic cells (15). Therefore, if the mechanism for biofilm resistance is related to glycocalyx there may be differences in the quantity or distribution of the glycocalyx material surrounding cells in resistant biofilm populations and in sensitive planktonic populations. Experiments were conducted to determine if biofilm and planktonic cells of *P. aeruginosa* (ATCC 27853) differ significantly in the quantity or distribution of glycocalyx material present.

The mechanism by which glycocalyx might contribute to biofilm resistance was also examined. It is possible that iodine reacts with glycocalyx compounds, since the interaction of iodine with other organic molecules such as proteins, fatty acids, sulfhydryl compounds, and vitamin C has been documented (1, 22). These interactions involve oxidation of the organic molecule by iodine (1, 22). Characklis and Dydek (10) have demonstrated that chemical interactions between chlorine and biofilm glycocalyx occur and that the glycocalyx material creates a chlorine demand. In such a reaction, the organic material may sequester the available free halogen, limiting its antimicrobial effectiveness (22, 34). Such an interaction could protect a cell surrounded by glycocalyx, whether growing in the biofilm or planktonic mode. Experiments were conducted to determine if the addition of uronic acid, in the form of commercially available alginic acid, would protect planktonic *P. aeruginosa* from povidone-iodine. The iodine demand associated with various concentrations of biofilm and planktonic biomass was also measured to determine if there were qualitative differences in the glycocalyx between such samples.

Another possible mechanism for resistance is that the glycocalyx may create a diffusion barrier to the antimicrobial agent. Diffusion through a biofilm may be affected by charge (ionic) interactions between the glycocalyx and the antimicrobial agent, by an increase in the distance the agent must diffuse, by molecular sieving (size exclusion), and by the viscosity of the glycocalyx. Some researchers suggest that the polyanionic nature of the glycocalyx creates a barrier (charge interactions) to the diffusion of cationic antimicrobial agents (12, 13, 15). Others suggest that cells within a biofilm are protected from antimicrobial agents by the physical thickness of the biofilm material in which the cells are embedded (4). Anderson (2) suggests that a biofilm, with its thick glycocalyx, must be saturated with an antimicrobial agent before bacteria can be killed. The diffusion rates of iodine through biofilms and planktonic cells collected on polycarbonate membranes were investigated

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to determine the relation between diffusion and cell killing. The relationship between cell death and detachment of cell material from the biofilm during treatment with povidone-iodine was also investigated.

MATERIALS AND METHODS

Enumeration of cells. Cells were resuspended from biofilms by vortexing of the polycarbonate membranes vigorously for 1 min as previously described (8). Epifluorescence microscopic examination of these membranes revealed no significant quantity of cells remaining attached. The mean numbers of viable cells in biofilm and planktonic samples were determined by plate counts. Antimicrobial resistance in biofilm and planktonic samples was calculated as log (N_0/N), where N_0 was the viable recovery (cells per milliliter) from the untreated control sample and N was the viable recovery from the treated sample.

A modification of the direct count method of Porter and Feig (24) was employed for comparison with plate counts. Samples were stained with 4′,6-diamidino-2-phenylindole (DAPI) and analyzed with a Leitz Diaplan epifluorescence microscope. A minimum of 8 fields and 150 cells was counted, and the number of cells per milliliter of sample was calculated from the mean number of cells per field.

Growth of *P. aeruginosa* in broth cultures. *P. aeruginosa* (ATCC 27853) was grown in 50 ml of nutrient broth supplemented with 0.5% glucose (N + G) in a 250-ml flask. Cultures were incubated for 24 h at 30°C on a gyratory shaker at 150 rpm. Samples from broth cultures were used for planktonic cell assays and as inocula for experimental biofilms.

Generation of experimental biofilms. To generate each experimental biofilm, an inoculum (10^3 cells) from a planktonic culture was dispersed in 10 ml of sterile buffered water (SBW) (0.63 mM KH_2PO_4 and 0.40 mM $MgCl_2$ in distilled water) and filtered onto the surface of a 47-mm-diameter polycarbonate membrane (0.22-μm pore size; Nuclepore). After inoculation, the cells were washed by filtering SBW through the membrane to remove residual nutrients. The membrane with associated cells was then incubated (cell side up) on the surface of an N + G agar plate to generate a biofilm.

Determination of total carbohydrate and uronic acid in biofilm and planktonic samples. The amounts of carbohydrate and uronic acid in biofilm (96 and 120 h old) and planktonic samples (24 h old) were measured. The planktonic culture was centrifuged at 2,560 × *g* for 15 min at 4°C, and the pellet was washed and resuspended in sterile, distilled water (a volume equal to that of the supernatant liquid removed). Samples (5 ml) from the biofilm and planktonic cell suspensions were then sonicated for 1 min with a cell disrupter (Branson Sonic Power). Total carbohydrate in biofilm and planktonic samples was determined by the phenol-sulfuric acid method reported by Geesey et al. (17). Total uronic acid was determined by the meta-hydroxydiphenyl sulfuric acid method of Blumenkrantz and Asboe-Hansen (6). Data are presented as mean quantities (triplicate experiments) of each compound per unit of cell volume. The means for biofilm (96- and 120-h-old biofilms combined) and planktonic samples were compared by a two-sample *t* test at $\alpha = 0.05$ (35).

TEM examination of glycocalyx distribution. Biofilms (24 to 120 h old) and a planktonic culture (24 h old) were generated for transmission electron microscopy (TEM) examination of glycocalyx distribution around cells in the samples. In addition, a biofilm (72 h old) was exposed to povidone-iodine (1:15 in water) (Betadine; Purdue Frederick) for 2 min prior to primary fixation to allow examination of the effect of iodine on the biofilm. Prior to TEM preparation, all biofilm samples were embedded in 2.5% agar. Planktonic cells were centrifuged at 5,600 × *g* to pellet the cells. All samples were suspended in primary fixative (2.5% glutaraldehyde and 2.5% formaldehyde in 0.2 M cacodylate buffer) for 20 min at room temperature and then washed twice with cacodylate buffer and refrigerated overnight at 4°C. The samples were then suspended in secondary fixative (1% osmium tetroxide) for 30 min at room temperature and washed as described above. Alcian blue (0.05%) was incorporated into the primary fixative, wash buffer, and secondary fixative to stain and preserve the glycocalyx matrix. The samples were dehydrated in a graded ethanol series (25% for 15 min, 50% for 15 min, 75% for 15 min, 95% for 15 min, 100% for 15 min) followed by three 15-min changes of 100% acetone and then stepwise infiltration with 30% Spurr's resin for 2 h, 70% Spurr's resin for 2 h, and 100% Spurr's resin for 2 h. They were then suspended in fresh 100% Spurr's resin and cured at 60°C for 12 h. Thin sections were prepared and poststained with 1% aqueous uranyl acetate followed by Reynolds' lead citrate (28) for 10 min in each stain. Sections were then mounted on Formvar-coated copper grids (100 mesh) and examined with a Zeiss EM10 transmission electron microscope.

Neutralizing interaction of alginic acid with povidone-iodine. Solutions (100 μg/ml) of three types of commercially available alginic acid (types IV, VI, and VII; Sigma) were prepared in distilled water and autoclaved. Types IV, VI, and VII are β-1,4-linked anhydro-β-D-mannuronic acids and have chain lengths of 180,000 to 190,000, 80,000 to 120,000, and 12,000 to 80,000, respectively. Planktonic cultures (24 h old) were generated, and 30-ml samples from each culture were dispensed into four sterile centrifuge tubes and then centrifuged at 8,000 × *g* for 10 min. The supernatant liquids were discarded, and the cell pellets were resuspended by vortexing of the pellets in 30 ml of either alginic acid (100 μg/ml; type IV, VI, or VII) or SBW.

TABLE 1. Concentrations of protein, carbohydrate, and uronic acid in planktonic and biofilm samples of *P. aeruginosa*

Type of sample	Concn (mean ± SD, 10^{-8} μg/μm^3) of[a]:		
	Protein	Carbohydrate	Uronic acid
Planktonic	9.42 ± 1.15	1.04 ± 0.09	1.17 ± 0.66
Biofilm	10.74 ± 2.44	0.85 ± 0.37	0.95 ± 0.17

[a] Samples were stained with DAPI and examined microscopically (as described in the text). Images were captured using a Matrox PIP Video Digitizer Board and Dage-MTI black-and-white camera. Means (n = 100 cells) of cell lengths, breadths, and areas were measured with Bioscan Optimas image analysis software. Cell volumes (V) were calculated from these measurements as $V = \pi r^2 h$, where r is 1/2 of the cell breadth and h is the cell length. All values are means of triplicate samples. Mean values for planktonic and biofilm samples not significantly different at $\alpha = 0.05$.

To determine if added alginic acid increased resistance to povidone-iodine, 5 ml from each resuspended sample was withdrawn and exposed to 5 ml of povidone-iodine (1:15 in water) for 2 min. Following exposure to povidone-iodine, the residual iodine was inactivated by addition of 10 ml of 5% sodium thiosulfate. Five milliliters from each sample was also added to 5 ml of sterile, distilled water, and then 10 ml of 5% sodium thiosulfate was added as an untreated control. Viable cells were enumerated by plate counts, and the antimicrobial resistance was calculated. A single-factor analysis of variance was performed on the calculated mean resistance (from triplicate experiments) for all four types of samples (35). The resistance for each type of added alginic acid was also compared individually to the resistance for the control with no added alginic acid by use of Tukey's test (35).

Determination of iodine demand created by biomass. A planktonic culture (24 h old) was generated and then centrifuged at 2,560 × *g* for 15 min at 4°C. The pellet was washed and resuspended in sterile, distilled water to three concentrations (10^{11}, 10^{10}, and 10^9 cells per ml). Ten milliliters of each concentration of cells or water (as a control) was added to a tube containing 10 ml of povidone-iodine (1:15 in water). Biofilms (24 and 96 h old) were generated, and one biofilm of each age was placed cell side down on 10 ml of povidone-iodine (1:15 in water). The biomass in each sample was determined by weighing of 10 ml of the undiluted culture or a biofilm dried at 105°C for 24 h.

All samples were vortexed and incubated for 60 min at room temperature to allow the I_2 to react with the biomass. The samples were then centrifuged at 2,290 × *g* for 30 min at 4°C. A portion (0.1 ml) of the supernatant liquid was removed from each tube and diluted to a final volume of 1.0 ml (10^{-1}) with sterile, distilled water. Measurements were made in a Beckman DU-8 spectrophotometer at a wavelength of 515 nm (27), and the mean concentration of dissociated I_2 (from triplicate experiments) was calculated from a standard curve. Single-factor analyses of variance and Tukey's tests were performed on the calculated concentrations (moles per liter) from I_2 demand experiments (35).

Povidone-iodine resistance and dependence on biomass detachment from biofilms. Biofilms (72 h old) were generated, and experiments were conducted to examine the relationship between povidone-iodine resistance and detachment of biomass (sloughing) from the biofilm. One membrane with attached biofilm was placed cell side down on 5 ml of povidone-iodine (1:15 in water). Following a 2-min exposure to povidone-iodine, 5 ml of 5% sodium thiosulfate was added to inactivate residual iodine. The membrane with remaining cells was carefully transferred to a sterile test tube containing 10 ml of 5% sodium thiosulfate. This tube was vortexed vigorously and labeled attached-treated. The remaining suspension was transferred to a sterile test tube labeled detached-treated and vortexed. A set of controls was prepared by exact replication of the procedure described above with the exception that sterile, distilled water replaced povidone-iodine. The control fractions generated by this procedure were labeled attached-control and detached-control. All tubes (from triplicate experiments) were vortexed vigorously, and samples were removed for enumeration by plate counts and the direct count method.

To determine the effect of contact time on sloughing and resistance, biofilms (72 h old) were generated and treated for 1, 2, and 4 min with povidone-iodine (1:15 in water). After each treatment, the attached and detached fractions were placed in separate test tubes and labeled attached-treated or detached-treated. These tubes (from triplicate experiments) were vortexed vigorously, and samples were removed for enumeration by plate counts and the direct count method. All assays were conducted in triplicate, and the data were averaged.

Effect of biofilm formation on iodine diffusion. An apparatus was designed to study the diffusion of iodine through polycarbonate membranes, through cells filtered onto polycarbonate membranes, and through biofilms (24 and 96 h old) grown attached to polycarbonate membranes. The apparatus consisted of a Millipore filter funnel and a Plexiglas plate that could be clamped to the filter funnel to hold a membrane in place. A 10-ml reservoir was milled into the plate, and two 14-gauge cannulae were mounted through the plate into the reservoir for sampling.

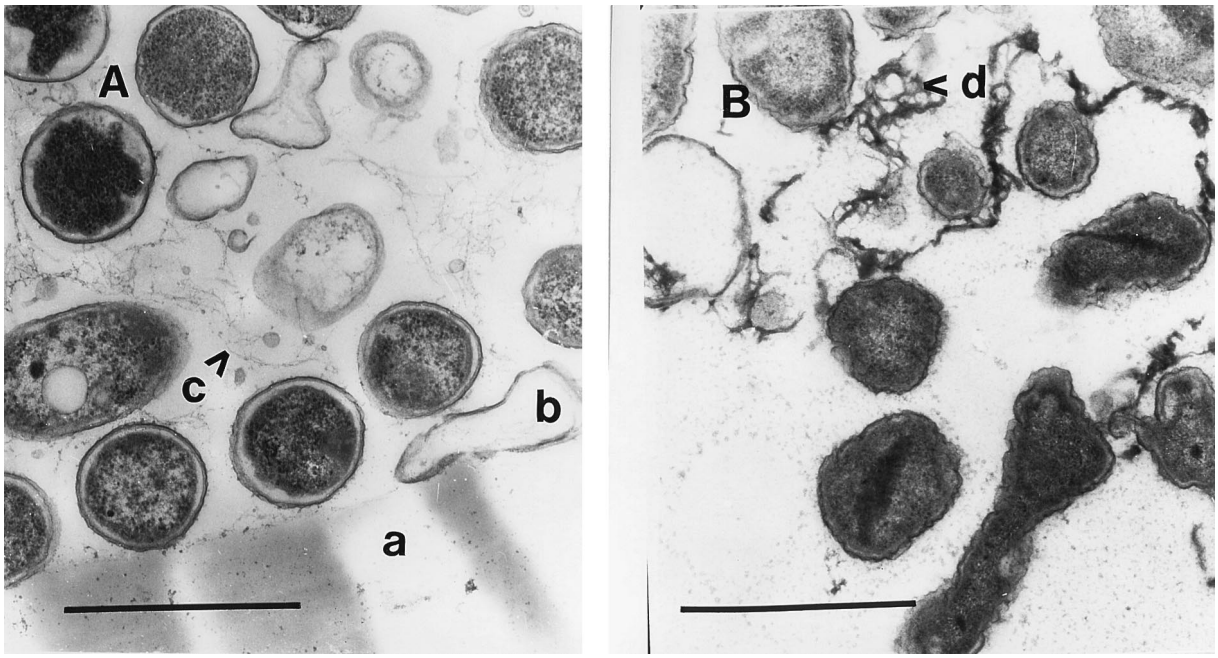

FIG. 1. Transmission electron micrographs of biofilm and planktonic samples of *P. aeruginosa* (ATCC 27853). The glycocalyx was stained with alcian blue. (A) Portion of a biofilm, showing the polycarbonate membrane or substratum (a), an apparently dead (lysed) cell (b), and an area of extended glycocalyx material (c). The biofilm is approximately 160 μm thick; the outer layer of the biofilm is not shown. (B) Planktonic sample, showing an area of condensed glycocalyx material (d). The scale bars represent 1 μm.

Membranes were mounted in the apparatus (cell side up for filtered cells and biofilms), and 10 ml of distilled water was added to the lower chamber through a sampling port. All trapped air was cleared through the sampling ports, and the lower chamber was stirred with a small magnetic stir bar. Ten milliliters of povidone-iodine (1:15 in water) was added to the upper chamber by careful dripping down the side of the funnel. Samples (0.1 ml) were withdrawn at 5-min intervals from the lower reservoir through a sampling port for 45 min. As a sample was withdrawn through one port, 0.1 ml of distilled water was added through the second port. The effect of this slight dilution of the lower chamber was considered less significant than vacuum or hydrostatic effects which might have occurred because of volume changes in the upper and lower chambers. Another membrane was treated as described, except that only distilled water was added to the upper chamber as a control for any absorbance caused by the diffusion of cell material into the lower chamber.

Samples (from triplicate experiments) were diluted to a total volume of 1.0 ml (10^{-1} dilution) with distilled water, and I^- was measured spectrophotometrically at a wavelength of 226 nm (26, 27). The diffusion rate of iodine through planktonic cells (7.35×10^9) filtered onto a polycarbonate membrane was determined and compared with diffusion rates through biofilms (24 and 96 h old). The densities of cells in the 24- and 96-h-old biofilms were 1.39×10^{11} and 1.30×10^{11} cells per biofilm, respectively.

The I^- concentrations (moles per liter) of samples withdrawn from the lower chamber were calculated from a standard curve and plotted versus time. The iodine diffusion rate through each sample type is given as the slope of this curve. A linear regression was performed on each data set, and the slopes were compared for differences with an analysis of variance and Tukey's test (35).

Relationship between diffusion and cell death. A mathematical model was developed to relate the rate of diffusion of iodine through a biofilm with the rate of killing that occurs within a biofilm. If diffusion through a biofilm is thought of as one-dimensional diffusion through a slab, the following equation gives the concentration of iodine at the midpoint of the biofilm (half thickness) over time (29): $C(t) = C_1 \cdot (1/2 - 2/\pi \cdot e^{-\pi^2 Dt/\delta^2})$, where $C(t)$ is the concentration of iodine at time t, C_1 is the concentration of iodine in the povidone-iodine solution prior to any diffusion, D is the diffusion coefficient, and δ is the thickness of the biofilm. This equation is based on the simplifying assumptions that the concentration of iodine above the biofilm is held constant and that there is no iodine on the detection side of the polycarbonate membrane.

The number of iodine molecules required to cause the death of a bacterial cell is not known. However, based on a simple situation in which one iodine molecule contacts a cell and initiates the death of that cell, the following equation gives the proportion of cells surviving at any given concentration of iodine (33): $\ln S = \lambda \cdot C(t)$, where S is the proportion of cells surviving treatment and λ is the average number of deaths per unit of dose. These two equations are related by $C(t)$ and allow the prediction of proportion of cells surviving at any time (t) to be calculated. The predicted curve for survivors determined on the basis of this model was compared with the actual proportion of cells surviving determined on the basis of experimental data.

RESULTS

Analysis of carbohydrate and uronic acid. The concentrations of two components of glycocalyx were measured to determine if the difference in resistance to povidone-iodine in biofilm and planktonic samples correlated with differences in glycocalyx quantities. The concentrations of carbohydrate and uronic acid per unit of cell volume were not significantly different ($\alpha = 0.05$) in the biofilm and planktonic samples (Table 1).

Glycocalyx distribution. Figure 1A demonstrates adherence of cells by glycocalyx matrix to the polycarbonate membrane. Comparison of micrographs of a planktonic sample (Fig. 1B) and of biofilms (Fig. 1A) showed a difference in the distribution of glycocalyx material around the cells. The stained glycocalyx material surrounding the biofilm cells was extended

TABLE 2. Thickness of biofilms

Biofilm age (h)	Biofilm depth (μm)[a]	Biofilm depth (cells)[a]
24	30	27
72	94	73
96	115	133
120	160	172
72 (treated)[b]	54	43

[a] Measurements of biofilm thickness (depth) were made by image analysis of transmission electron micrographs. The image analysis system was the same as that described in the footnote to Table 1, except that the camera was equipped with a Nikon Micro-Nikkor 55-mm lens.

[b] Exposed to povidone-iodine (1:15 in water) for 2 min prior to fixation.

TABLE 3. Effects of added alginic acid on povidone-iodine sensitivity in *P. aeruginosa*

Use of alginic acid in sample	Viable recovery (mean ± SD) with:		Antimicrobial resistance (mean ± SD)[a]
	Control (10^9 cells/ml)	Treatment (10^5 cells/ml)	
None added (control)	2.76 ± 0.06	0.70 ± 0.03	4.60 ± 0.01
Type IV added	2.54 ± 0.08	1.61 ± 0.08	4.20 ± 0.01[b]
Type VI added	3.00 ± 0.10	4.30 ± 0.24	3.84 ± 0.01[b]
Type VII added	1.43 ± 0.04	7.87 ± 0.44	3.26 ± 0.01[b]

[a] Log [viable recovery (control)/viable recovery (treatment)].
[b] Values for antimicrobial resistance of types IV, VI, and VII are significantly different from that for the control (none added) at an α of 0.05.

and more evenly distributed among the cells within the biofilm. In contrast, planktonic cells had a compressed glycocalyx matrix that incompletely surrounded individual cells (Fig. 1B). Examination of micrograph montages of biofilms (figures not shown) revealed that throughout the biofilm, cells were interconnected by a finger-like glycocalyx matrix that extends from the substratum to the outer boundaries of the biofilm. This glycocalyx matrix appears to anchor cells to one another and to the polycarbonate membrane. The montages also demonstrated the presence of two distinct, horizontal layers of cells in each biofilm. The layer of cells proximal to the substratum had a higher cell density than the distal layer. Treatment of 72-h-old biofilms with povidone-iodine (1:15 in water) resulted in biofilms that were about one-half as thick as untreated ones (Table 2), indicating that the outer layer of the biofilm is easily removed during this exposure.

Measurements from transmission electron micrographs showed that the biofilms increase in thickness from 24 to 120 h (Table 2), while previous experiments show that such biofilms enter stationary phase at 24 h (7, 8). Examination of the electron-micrographic montages indicated that the increasing physical thickness of biofilms after stationary phase was due to accumulation of disrupted (nonviable) cells and other cell debris within the glycocalyx matrix.

Neutralizing interaction of alginic acid with povidone-iodine. The antimicrobial resistance of planktonic samples of *P. aeruginosa* without (control) and with added alginic acid (types IV, VI, and VII) was measured to determine if the addition of alginic acid provided protection. The resistance of samples with alginic acid added was greater than that of control samples (significant at an α of 0.05) (Table 3). Resistance increased with decreasing chain length of the added alginic acid.

Iodine (I_2) demand created by biomass. Biomass from biofilm and planktonic samples was mixed with povidone-iodine (1:15 in water) to determine the relationship between biomass concentration and I_2 demand. The concentrations of I_2 in samples were measured after mixing with each concentration of biofilm and of planktonic biomass. Samples (biofilm and planktonic) with 0.050 to 0.500 g of biomass had significantly lower quantities of I_2 than did the water control, while biomass equivalent to 10^{10} cells (0.005 to 0.017 g of biomass) caused no measurable reduction in detectable I_2 (significant at an α of 0.05) (Table 4). The apparent I_2 demand was proportional to the amount of biomass present in the samples and did not vary significantly (α = 0.05) between biofilm and planktonic samples of similar biomass (Table 4).

Povidone-iodine resistance and dependence on biomass detachment from biofilms. The total number of cells and the number of viable cells in the detached and attached fractions of biofilms were determined by direct microscopic counts and plate counts, respectively. The results of enumerations from biofilms treated and control biofilms are summarized in Table 5. There was an 0.79-log-unit reduction in viability in the biofilms following treatment (2 min) with povidone-iodine (1:15 in water). After treatment, about 96% of the detected viable cells were in the attached fraction of the biofilm. The attached and detached fractions had reductions in viability of 0.71 and 1.52 log units, respectively, demonstrating that the detached fraction was less resistant than the attached fraction. The detached-control and attached-control data showed that even without povidone-iodine as much as 15% of the biofilm is mechanically removed (sloughed) into the suspension but that these untreated cells remain viable in the suspension.

Biofilms (72 h old) were treated with povidone-iodine (1:15 in water) for various contact times to determine the relationship between sloughing and biofilm resistance. The numbers of cells detached from the biofilm (determined on the basis of epifluorescence counts) increased slightly as contact time increased (Table 6). The antimicrobial resistance of the attached fraction decreased by about 0.14 log units as the number of cells detached from the biofilm increased (Table 6). The detached fraction exhibited an increase in antimicrobial resistance as the number of cells detached from the biofilm increased, but this increase in resistance did not significantly affect the overall resistance of the biofilm.

Effect of biofilm formation on iodine diffusion. The diffusion rates of iodine through polycarbonate membranes, through cells filtered onto polycarbonate membranes, and through biofilms (24 and 96 h old) grown attached to polycarbonate membranes were measured to determine if there were differences in these rates. Figure 2 shows the rates of diffusion as indicated by the slope of each line. The slopes for filtered cells and biofilms were not significantly different at an α of 0.005. The *x* intercept for each line is the length of time required for initial emergence of iodine in the lower diffusion chamber. The *x* intercepts for the membrane, planktonic cells collected on a membrane, 24-h-old biofilm, and 96-h-old biofilm were 0.95, 0.55, 2.68, and 5.09 min, respectively.

TABLE 4. Iodine demand created by various amounts of biomass

Sample number	Description of sample	No. of viable cells (10^{10}) in treatment (mean ± SD)	Biomass (g) in treatment (mean ± SD)	Iodine concn (10^{-3} mol/liter, mean ± SD)[a]	Significance[b]
1	24-h biofilm	22.33 ± 2.31	0.117 ± 0.012	1.38 ± 1.54	a
2	96-h biofilm	7.67 ± 0.58	0.017 ± 0.001	4.14 ± 0.20	b
3	Undiluted planktonic	102.67 ± 22.30	0.500 ± 0.109	0.19 ± 0.03	a
4	10^{-1} planktonic	10.27 ± 2.23	0.050 ± 0.011	1.54 ± 0.37	a
5	10^{-2} planktonic	1.03 ± 0.22	0.005 ± 0.001	4.38 ± 0.03	b
6	Water control	None		5.94 ± 1.55	b

[a] The mean iodine concentrations for samples 1, 3, and 4 differed significantly from that of sample 6 at an α of 0.05.
[b] Samples with the same letter have mean concentrations not significantly different at an α of 0.05.

TABLE 5. Cells in attached and detached fractions of treated and untreated biofilms

Type of sample	Cell count (10^9/ml, mean ± SD)		Antimicrobial resistance[a]	% Viable[b]
	Plate	Epifluorescence		
Detached-treated	0.05 ± 0.004	1.54 ± 0.63	1.52	2.99
Attached-treated	1.27 ± 0.20	6.55 ± 3.87	0.71	19.39
Total (treated)	1.32	8.09	0.79	16.27
Detached-control	1.33 ± 0.007	1.33 ± 0.33	0.00	100.00
Attached-control	7.53 ± 0.54	7.88 ± 2.05	0.02	95.56
Total (control)	8.86	9.21	0.02	96.20

[a] Log (epifluorescence count/plate count).
[b] (Plate count/epifluorescence count) × 100.

Relationship between diffusion and cell death. Figure 3 shows the proportion of cells surviving in a biofilm as predicted by a model of diffusion and killing kinetics and the actual proportion of cells surviving determined on the basis of experimental data. The correlation between the experimental data and the predicted curve indicates that killing is directly related to diffusion.

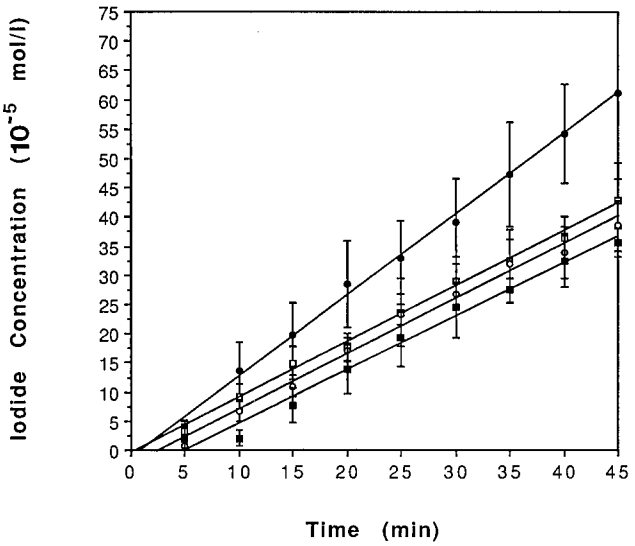

FIG. 2. Effects of biofilm formation on the rate of iodine diffusion. Diffusion rates through polycarbonate membranes (●), cells filtered onto polycarbonate membranes (□), 24-h-old biofilms (○), and 96-h-old biofilms (■) grown attached to polycarbonate membranes were determined. Samples (0.1 ml) were withdrawn at 5-min intervals (45 min total) from the diffusion apparatus and diluted to 1.0 ml in sterile, distilled water. The diluted samples were analyzed for A_{226}. The absorbance values were compared with a standard curve generated for povidone-iodine to determine the concentration of iodide (10^{-5} mol/liter).

DISCUSSION

Natural biofilms vary greatly with regard to oxygen and nutrient availability, as well as microbial composition. In addition, biofilms produced under flow conditions differ from those produced under quiescent conditions, which may be a response to nutritional or physical factors. Consequently, data obtained from a study of a specific natural biofilm may or may not be applicable to other systems. Experimental biofilms have been used to investigate iodine resistance and the effects of nutrient availability, cell density, and biofilm thickness (7, 8) and were used in this study to examine factors which may account for the difference in resistance between biofilms and planktonic samples observed.

This study showed that biofilm and planktonic *P. aeruginosa* (ATCC 27853) did not have significantly different quantities of uronic acid and carbohydrate. Since these two compounds represent the major components of the glycocalyx matrix in *P. aeruginosa*, the resistance observed in experimental biofilms is not the result of major quantitative differences in glycocalyx but may be the result of differences in the distribution of the glycocalyx material around cells. This hypothesis is supported by the electron-microscopic analysis, which showed that planktonic cells had a compressed glycocalyx matrix that incompletely surrounded individual cells. In contrast, examination of montages of biofilm cells demonstrated that the glycocalyx material extended like interconnected fingers between cells throughout the biofilm. The glycocalyx appears to bind cells together within the biofilm and ensures the integrity of the biofilm as a whole. Also, the biofilms appear to be divided into two layers, which is consistent with other models of biofilm development that suggest the presence of two physically and structurally distinct layers within biofilms (11).

Efforts to ensure a fair comparison between biofilm and planktonic samples in the electron microscopy study were made. Biofilm and planktonic cells were grown on the same medium and were in stationary phase at the time of examination. The use of alcian blue is an established histochemical technique for staining and preserving polysaccharides, glycosaminoglycans, and proteoglycans in bacterial samples as well as eukaryotic cells (16). Although the quantities of uronic acids in planktonic cells and biofilms were shown to be similar, other differences in polysaccharide chemistry may account for the different distributions of the polymer around the cells.

Since resistance increased with decreasing chain length of alginic acid added to planktonic cells, there may be some neutralizing interaction between the polymers of uronic acid and the iodine in the povidone-iodine suspension. This is consistent with the work of Characklis and Dydek (10), who demonstrated a chlorine demand created by glycocalyx material in their biofilm system. However, since a 10-fold (100 μg/ml) increase in uronic acid resulted in only a 1.34-log-unit increase in povidone-iodine resistance, the level of demand created by added uronic acid is not sufficient to explain the disparity in resistance observed in biofilm and planktonic samples (8).

TABLE 6. Effects of contact time on sloughing and resistance to povidone-iodine (1:15 in water)

Contact time (min)	Type of sample (all treated)	Cell count (10^9/ml, mean ± SD)		Antimicrobial resistance[a]
		Plate	Epifluorescence	
1	Detached	0.01 ± 0.002	1.36 ± 0.09	2.16
	Attached	3.25 ± 0.64	16.89 ± 2.11	0.72
	Total	3.26	18.25	0.75
2	Detached	0.04 ± 0.005	1.44 ± 0.29	1.58
	Attached	2.65 ± 0.07	15.03 ± 1.29	0.75
	Total	2.69	16.47	0.79
4	Detached	0.11 ± 0.011	1.45 ± 0.19	1.14
	Attached	2.05 ± 0.08	14.79 ± 1.14	0.86
	Total	2.16	16.24	0.88

[a] Log (epifluorescence count/plate count).

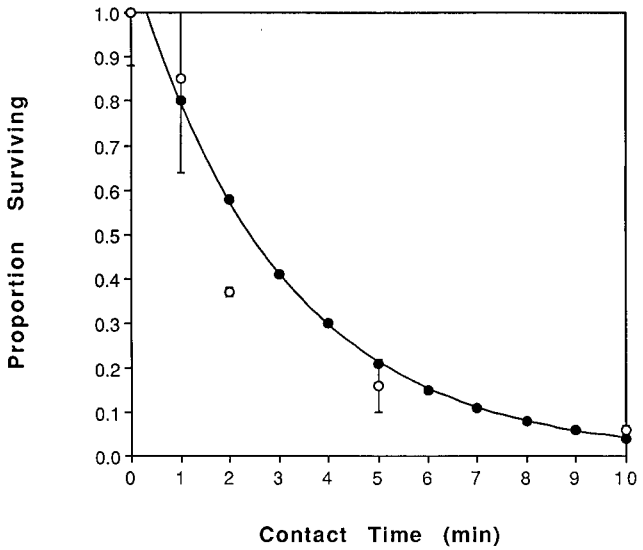

FIG. 3. Relation between biofilm resistance and iodine diffusion. A model was employed to relate the rate of iodine diffusion through a biofilm with the rate of killing over time. The curve (●) represents the proportion of cells surviving treatment with povidone-iodine (1:15 in water) at various contact times as predicted by this model. The open circles (○) represent the actual proportions of cells surviving this treatment, on the basis of experimental data. The experimental data were collected in triplicate, and the data were averaged. The standard deviations of the means are presented as *y* error bars.

A possible demand type interaction with iodine was further tested by a spectrophotometric method of detection of I_2 in the presence of various concentrations of biomass. The povidone-iodine complex in Betadine is known to dissociate in water, yielding several iodine species (20, 30). At the aqueous dilution of Betadine (1:15 in water) used in these experiments, the equilibria should favor I_2 and I^- (30). The I_2 species is responsible for most of the antimicrobial activity of povidone-iodine, with little to no activity associated with I^- (20, 34). For this reason, demand experiments were designed to detect only I_2. The addition of 0.050 to 0.500 g of cell material caused a significant reduction in detectable quantities of I_2 that was inversely proportional to biomass concentration. There was no significant difference between the I_2 demands created by biofilm and by planktonic biomass of similar quantities. This confirms that I_2 demand created by biomass, including glycocalyx, does not explain the disparity in povidone-iodine resistance observed in biofilm and planktonic samples.

Application of povidone-iodine to biofilms prior to electron microscopy appears to cause the outer layers of biomass to slough into the povidone-iodine suspension. The biomass detachment experiments confirm that when exposed to a liquid, the outer layers of the biofilm begin to slough into the liquid. If this liquid contains povidone-iodine, a large portion of the detached population is killed while a smaller portion of cells still attached to the biofilm is killed. If, however, the liquid contains no antimicrobial agent, then the detached cells remained viable and theoretically are capable of recolonizing the biofilm or colonizing new areas.

The resistance of the detached fraction did not significantly affect the resistance of the biofilms as a whole. Sloughing may result in the removal of cell aggregates from the biofilm, which may exhibit some residual resistance related to this aggregation. Previous studies have shown that cells removed from experimental biofilms by vigorous vortexing and then treated with povidone-iodine exhibit sensitivity similar to that of planktonic cultures (7). This lack of resistance after dispersion suggests that the mechanism for resistance in the detached fraction, as well as the attached fraction, is not related to physiological changes in cells within a biofilm. This is consistent with previous results which showed that povidone-iodine resistance was not dependent on the physiological state of cells within a biofilm (7, 8).

As mentioned, chemical equilibria favor I_2 and I^- in an aqueous povidone-iodine system, and it is known that further dilution in an aqueous system causes I_2 to undergo hydrolysis, very rapidly yielding predominantly I^- with an equilibrium that is very rapidly reestablished (20, 30). It would be expected that diffusion of the iodine species present in povidone-iodine into the lower chamber of the diffusion apparatus would result in rapid equilibria favoring I^-. Therefore, measuring the diffusion rate for iodine species in general can be accomplished by measuring the concentration of I^- in the reservoir over time.

While the rates of iodine diffusion (based on regression analysis) for the biofilms and filtered, planktonic cells were not different, the *x* intercepts for the lines were different. Tanaka et al. (32) demonstrated that substances with molecular weights less than 20,000 diffused freely through alginate and that diffusion of such molecules was not affected by increased polymer concentrations (e.g., increased viscosity). Given the molecular weights of I_2 (253.8) and I^- (126.9), the effects of molecular sieving and viscosity are likely to be negligible. Therefore, the differences in *x* intercepts are probably related to differences in the linear distance that the iodine must travel before detection. In the case of diffusion through the membrane, the *x* intercept is the time needed for iodine to travel through the membrane alone. Diffusion through 7.35×10^9 planktonic cells filtered onto a polycarbonate membrane yielded an *x* intercept that was similar to that of the membrane alone, suggesting that the cells in this sample detached rapidly, leaving the thickness of the membrane as the linear distance the iodine must travel. Previous experiments confirm that planktonic cells filtered onto a polycarbonate membrane rapidly (within 15 s) resuspend when treated with povidone-iodine (1:16) (7). The *x* intercepts increased for the biofilms, with the older biofilm having the largest *x* intercept. This is consistent with the electron-microscopic data, which showed that a 96-h-old biofilm was nearly four times as thick as a 24-h-old biofilm and, therefore, would represent a greater linear distance for diffusion. In this way, the layering of attached cells and other biomass within a biofilm may enhance povidone-iodine resistance without affecting the diffusion rate of iodine.

The relation between the rate of iodine diffusion through the biofilm and the rate of killing was examined. If factors other than diffusion limitation contributed significantly to the resistance of biofilms in these experiments, the experimental data for biofilm survival would have plotted above the curve for survival predicted on the basis of diffusion. However, this was not the case, and the level of killing observed in experimental biofilms of *P. aeruginosa* correlated with the level of killing predicted by the diffusion model.

On the basis of these experiments, we conclude that the glycocalyx matrix contributes to biofilm resistance by cementing cells within the biofilm, anchoring them to one another and to the substratum. The binding of cells within this protective matrix increases the time required to suspend cells in the antimicrobial agent and increases the time required for the antimicrobial agent to contact cells that remain attached in deepest portion of the biofilm. We conclude that it is not the quantity of glycocalyx that causes resistance in biofilms but that it is the interaction between the glycocalyx, the cells, the at-

tachment substratum, and the antimicrobial agent that leads to enhanced resistance.

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