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Cell Density and Growth Phase as Factors in the Resistance of a Biofilm of *Pseudomonas aeruginosa* (ATCC 27853) to Iodine

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Previous studies have shown that biofilms exhibit enhanced resistance to iodine. Investigations were conducted to determine the relative importance of growth phase versus cell density on biofilm resistance of *Pseudomonas aeruginosa* (ATCC 27853) to iodine. Cell density is a contributing factor to resistance, whereas growth to the stationary phase is not sufficient to achieve resistance.

Several researchers have noted enhanced resistance to iodinated compounds associated with biofilm formation in both natural and model systems (1–5, 8, 9, 12, 13, 17). *Pseudomonas aeruginosa* has been found in biofilms in water treatment systems (3, 4), on medical equipment (10), and even in polyvinylpyrrolidone-iodine-manufacturing systems and -packaging containers (1, 5). Proposed mechanisms for resistance include cell shielding (10, 14–17), glycocalyx accumulation (1, 3–6, 10, 11, 16, 17), growth-phase-related phenomena (6, 7, 10, 17), and synthesis of protective proteins (6, 10). In this study, experiments were conducted to determine the relative importance of the phase of growth versus cell density on biofilm resistance of *P. aeruginosa* (ATCC 27853) to iodine.

Generation of experimental biofilms. *P. aeruginosa* (ATCC 27853) was grown in nutrient broth supplemented with 0.5% glucose (Nutrient + Glu). The cultures were incubated in 250-ml flasks containing 50 ml of medium at 30°C with shaking at 150 rpm. Various numbers of cells (10^3, 10^5, or 10^7) were harvested from these cultures and filtered onto the surfaces of 47-mm polycarbonate membranes (Nuclepore) with a 0.22-μm pore size. The membranes with associated cells were then transferred (cell-side up) to the surface of Nutrient + Glu agar plates which were then incubated at 30°C to generate biofilms. Cells in these biofilms reached a final density of greater than 10^{10} per membrane.

Determination of biofilm resistance to iodine. Preliminary experiments showed that a 2-min exposure to a 1/16 dilution of Betadine (10% polyvinylpyrrolidone-iodine if undiluted) results in several log units of cell death in planktonic cultures of *P. aeruginosa*. Therefore, cell-laden membranes were removed at 4-h intervals from the agar surfaces and placed (cell-side down) on the surface of 5 ml of a 1/16 dilution of Betadine in sterile beakers. After the 2-min contact time, residual iodine was inactivated by the addition of 5 ml of 5% sodium thiosulfate. Untreated control membranes were exposed to buffer rather than Betadine. After Betadine treatment, the membranes with associated cells were vortexed vigorously for 1 min in phosphate buffer to remove the bacteria. This suspension was serially diluted, and the cells were enumerated by spread-plating dilutions onto Nutrient + Glu agar plates and then incubating them at 30°C.

Figure 1 shows the results of the biofilm resistance assays. For a biofilm inoculum of 10^7 cells, the onset of resistance occurred immediately (Fig. 1A). When the inoculum was decreased to 10^5 cells, 8 h of growth was needed before the onset of Betadine resistance occurred (Fig. 1B). With an inoculum of 10^3 cells, the onset of resistance corresponds to 12 h of growth (Fig. 1C). In each case, the onset of resistance corresponded to a density of about 10^8 cells per membrane which occurred during the late logarithmic phase of growth at each inoculum. Once in the stationary phase, each culture incurred less than 1 log unit of cell death. This is consistent with the findings of Brown et al. (7), who stated that cells in a batch culture begin to undergo phenotypic changes, triggered by reductions in the availability of specific nutrients, several generations before the stationary phase. A biofilm system is similar to a batch system in that there occurs, over time, a steady reduction of nutrients in the medium, which is coupled to cell density and may induce specific phenotypic changes related to survival. Glycocalyx matrix accumulation may represent such a phenotypic change and could be related to cell density.

These experiments demonstrate that biofilms generated as described above exhibit enhanced resistance even when the cells are in the logarithmic phase. However, when 10^9 planktonic cells (in a 5-ml suspension) were exposed to Betadine, 3 log units of cell death was observed. When 10^9 cells, grown in liquid culture, were collected on a filter prior to exposure to Betadine, they exhibited a similar level of cell death. This number of cells was the maximum that could be filtered on a membrane without clogging. Simply having a density of 10^9 cells per membrane was not enough to bring about resistance. Resistance occurs when there is growth in association with a surface and attainment of sufficient cell density.

Induction of stationary-phase biofilms at a final cell density lower than 10^{10} per membrane. To determine whether achieving the stationary phase is sufficient to induce resistance even at a lower final cell density, cells were grown under nutrient-limiting conditions. To prepare nutrient-depleted agar, Nutrient + Glu was diluted 10-fold in a flask and inoculated with *P. aeruginosa*. This flask was incubated as described above. After 24 h of growth, the broth was centrifuged and the cell pellet was discarded. The supernatant was reinoculated with *P. aeruginosa*, incubated, and centrifuged as described above for a total of three complete cycles. After the third cycle, the broth supernatant was filtered through an 0.22-μm-pore-size filter, 1.5% agar was added, and the solution was autoclaved. Biofilms of *P. aeruginosa* were generated on this nutrient-depleted agar from an inoculum of 10^6 cells. The method of biofilm generation was as described above except that distilled

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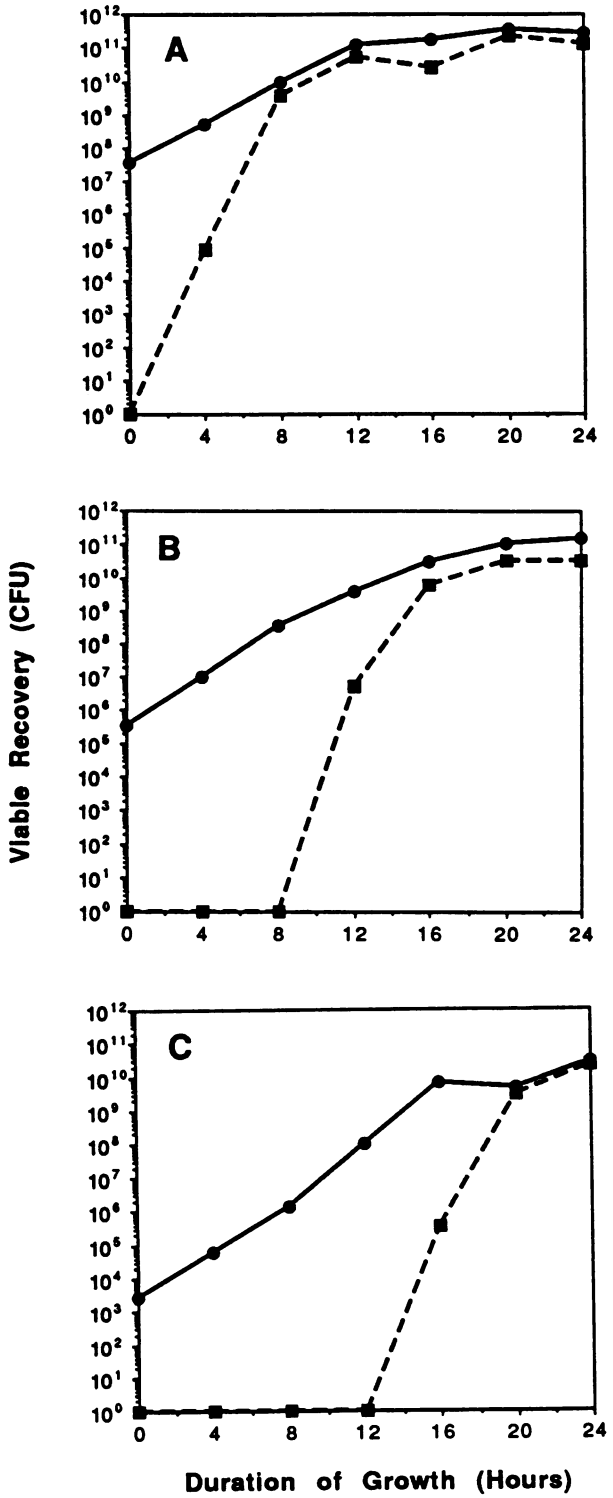

FIG. 1. Relation of cell density and growth phase to the resistance of biofilms of *P. aeruginosa*. The graphs represent the development of resistance to a 1/16 dilution of Betadine over time, with inocula of 10^7 (A), 10^5 (B), and 10^3 (C) cells. The lines represent the mean viable recovery from duplicate experiments with untreated control biofilms (●) and Betadine-treated biofilms (■). Standard deviations were too small to be presented as error bars.

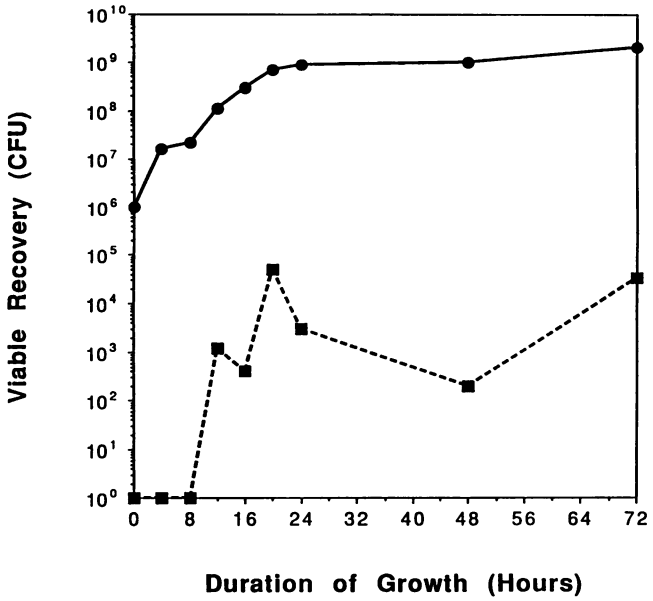

FIG. 2. Effect of induction of stationary phase at a cell density lower than 10^{10} per membrane on the resistance of biofilms of *P. aeruginosa* to a 1/16 dilution of Betadine. The lines represent the mean viable recovery from duplicate experiments with untreated control biofilms (●) and Betadine-treated biofilms (■). Standard deviations were too small to be presented as error bars.

water replaced phosphate buffer at each step to reduce nutrient input from the buffer. Densities in these biofilms reached about 10^9 cells per membrane.

Figure 2 shows that when membrane-grown cells were limited to a density of about 10^9 cells per membrane by growth on depleted media, they exhibited considerably less resistance to Betadine than the Nutrient + Glu biofilms. Under the nutrient-depleted condition, there were reductions in the final cell density and the growth rate in the biofilm. The cells were reaching the stationary phase at slightly less than 10^9 cells per biofilm and had a generation time of 2.3 h compared to 0.9 h in the Nutrient + Glu biofilms. Figure 2 shows that biofilms grown at this lower cell density incurred about 5 log units of cell death, which is greater than that seen with the planktonic cultures and the nonadherent cells mentioned before. At lower cell densities and under conditions of nutrient limitation, the cells in a biofilm may be unable to synthesize protective compounds at a rate or quantity sufficient to provide protection. This is consistent with the theory that de novo biosynthesis from available nutrients (glycocalyx and stress proteins, etc.) is required for biofilm resistance.

The biofilm resistance in these experiments was independent of the time that cell growth on the membrane occurred but dependent on cell density within the biofilm. The emergence of resistance during the late logarithmic phase of growth in these experiments demonstrates that the cells need not be in the stationary phase to begin exhibiting resistance. In fact, biofilms induced to the stationary phase at lower final cell densities demonstrated less-pronounced resistance than those allowed to grow without nutrient limitation. The factors involved in the resistance of these biofilms seem to be related to cell density and possibly to nutrient availability rather than to the growth phase of the cells.

REFERENCES

1. **Anderson, R. L.** 1989. Iodophor antiseptics: intrinsic microbial contamination with resistant bacteria. Infect. Control Hosp. Epidemiol. **10:**443–446.
2. **Anderson, R. L., R. L. Berkelman, and B. W. Holland.** 1983. Microbiologic investigations with iodophor solutions, p. 143–157. *In* Proceedings of the International Symposium on Povidone. University of Kentucky, Lexington.
3. **Anderson, R. L., B. W. Holland, J. K. Carr, W. W. Bond, and M. S. Favero.** 1987. Microbial colonization of PVC water distribution pipes and subsequent resistance to chemical germicides, p. 297–309. *In* Proceedings of the Second International Symposium on Povidone. University of Kentucky, Lexington.
4. **Anderson, R. L., B. W. Holland, J. K. Carr, W. W. Bond, and M. S. Favero.** 1990. Effects of disinfectants on pseudomonads colonized on the interior surface of PVC pipes. Am. J. Public Health **80:**17–21.
5. **Anderson, R. L., R. W. Vess, J. H. Carr, W. W. Bond, A. L. Panlilio, and M. S. Favero.** 1991. Investigations of intrinsic *Pseudomonas cepacia* contamination in commercially manufactured povidone-iodine. Infect. Control Hosp. Epidemiol. **12:**297–302.
6. **Anwar, H., M. K. Dasgupta, and J. W. Costerton.** 1990. Testing the susceptibility of bacteria in biofilms to antibacterial agents. Antimicrob. Agents Chemother. **34:**2043–2046.
7. **Brown, M. R. W., P. J. Collier, and P. Gilbert.** 1990. Influence of growth rate on susceptibility to antimicrobial agents: modification of the cell envelope and batch and continuous culture studies. Antimicrob. Agents Chemother. **34:**1623–1628.
8. **Carson, L. A., N. J. Petersen, M. S. Favero, and S. M. Aguero.** 1978. Growth characteristics of atypical mycobacteria in water and their comparative resistance to disinfectants. Appl. Environ. Microbiol. **36:**839–846.
9. **Chai, T.-J.** 1983. Characteristics of *Escherichia coli* grown in bay water compared with rich medium. Appl. Environ. Microbiol. **45:**1316–1323.
10. **Costerton, J. W., K.-J. Cheng, G. G. Geesey, T. I. Ladd, J. C. Nickel, M. Dasgupta, and T. J. Mattie.** 1987. Bacterial biofilms in nature and disease. Annu. Rev. Microbiol. **41:**435–464.
11. **Costerton, J. W., and R. T. Irwin.** 1981. The bacterial glycocalyx in nature and disease. Annu. Rev. Microbiol. **35:**299–324.
12. **Favero, M. S., and C. H. Drake.** 1966. Factors influencing the occurrence of high numbers of iodine-resistant bacteria in iodinated swimming pools. Appl. Microbiol. **14:**627–635.
13. **Goetz, A., and R. R. Muder.** 1989. *Pseudomonas aeruginosa* infections associated with the use of povidone-iodine in patients receiving continuous peritoneal dialysis. Infect. Control Hosp. Epidemiol. **10:**447–450.
14. **LeChevallier, M. W., C. D. Cawthon, and R. G. Lee.** 1988. Factors promoting survival of bacteria in chlorinated water supplies. Appl. Environ. Microbiol. **54:**649–654.
15. **LeChevallier, M. W., C. D. Cawthon, and R. G. Lee.** 1988. Inactivation of biofilm bacteria. Appl. Environ. Microbiol. **54:**2492–2499.
16. **Nichols, W. W., M. J. Evans, M. P. Slack, and H. L. Walmsley.** 1989. The penetration of antibiotics into aggregates of mucoid and non-mucoid *Pseudomonas aeruginosa*. J. Gen. Microbiol. **135:**1291–1303.
17. **Pyle, B. H., and G. A. McFeters.** 1990. Iodine susceptibility of pseudomonads grown attached to stainless steel surfaces. Biofouling **2:**113–120.