Biofilm resistance to antimicrobial agents

The vexing persistence of infections caused by microbial biofilms (4), even in the face of active immune defences and aggressive antibiotic therapy, continues to motivate the search for the fundamental mechanisms of biofilm reduced susceptibility. One of the most attractive hypotheses to explain the resistance of attached micro-organisms to antimicrobial agents suggests that free-floating and sessile cells inhabit distinct physiological states even when grown in the same medium (2, 3). We outline here a fresh version of this hypothesis that hinges on the spatial heterogeneity of physiological activity within the biofilm.

It has long been recognized that gradients in nutrient concentrations inside biofilms probably lead to corresponding gradients in growth rate or other physiological activities within the biofilm. Now fluorescent probe and reporter gene technologies have advanced to the point that these hypothesized physiological gradients can be directly visualized. Such experimental approaches demonstrate, often vividly, that cells of the same microbial species can inhabit extremely different physiological states, even though they may be separated by as little as 10 µm.

Examples of experimentally measured gradients in physiological activity within biofilms include adenylate charge (8), growth rate (16, 12), culturability (17) and the capacity for protein synthesis (7, 18). These reports paint a picture of the biofilm in which activity and viability are maximal near the biofilm/bulk fluid interface and decrease with depth into the interior of the film or microcolony. Similar physiological gradients have been measured in immobilized cell or ‘artificial biofilm’ systems (9, 11, 13, 14). All of these studies were performed with single species systems.

We have recently investigated physiological gradients within biofilms of Pseudomonas aeruginosa. Biofilms were grown on a glucose minimal medium in aerated, continuous flow reactors by methods described elsewhere (18). Patterns of protein synthesis within the biofilm were visualized by subjecting biofilms to phosphate starvation to induce alkaline phosphatase, then staining for the enzyme activity (7, 18). Respiratory activity was determined by staining with 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) and DNA distribution by staining with 4',6-diamidino-2-phenylindole (DAPI) as described by Huang et al. (6). Relative RNA content, which reflects growth rate, was visualized by acridine orange staining performed according to Wentland (16) and by hybridization to rRNA oligonucleotide probe Eub338 (1). Hybridization was performed on frozen biofilm sections that had been fixed in 4% paraformaldehyde and washed with buffered saline. Sections were dehydrated in an ethanol series, then air-dried. Ten microlitres of a hybridization solution containing 5 ng probe, 20% formamide, 0-9 M NaCl and 0-01% SDS in 20 mM Tris/HCl were blotted onto the section. Slides were incubated in a humidified environment at 46°C for 1.5 h. Specimens were then washed with buffer (20 mM Tris/HCl, 0-01% SDS, 0-225 M NaCl) and incubated in 50 ml of the same buffer at 48°C for 20 min. Finally, the slides were briefly rinsed with distilled water, air-dried and mounted. A probe whose sequence was complementary to Eub338 exhibited negligible staining, showing that the binding observed with Eub338 was specific.

Our measurements using P. aeruginosa biofilms reveal a sharply heterogeneous distribution of physiological activity (Fig. 1) that is qualitatively consistent with the studies mentioned above. A DNA-binding fluorochrome (DAPI) stained the entire 110 µm thickness of the biofilm relatively uniformly (Fig. 1a). Visualization techniques that indicated protein synthesis, respiratory activity and relative RNA content showed that activity was limited in each case to a narrow zone located immediately adjacent to the biofilm/bulk fluid interface. The dimensions of the active zones, as measured by image analysis, were 30 ± 4 µm for induced alkaline phosphatase (Fig. 1b), 24 ± 3 µm for CTC staining (Fig. 1c), 20 ± 2 µm for acridine orange staining (Fig. 1d), and 21 ± 2 µm for in situ hybridization (Fig. 1e). These measurements demonstrate that only the top one-fifth of the biofilm was metabolically active in this model system.

Recognizing the intrinsic physiological heterogeneity of biofilm populations is critical to understanding the remarkable resistance of biofilm micro-organisms to many types of antimicrobial agents. The probable alteration of physiological status in biofilm and consequent reduced susceptibility have been discussed and investigated (2, 5). For example, this literature supports a general, though not universal, correlation between decreased growth rate in biofilms and increased resistance to killing. The
physiological activity within Fig. 1. Visualization of spatial heterogeneity of susceptibility of the biofilm cells as determined by they will all eventually be killed. The susceptibility of the biofilm would be measured for suspended cells grown in the medium that bathes the biofilm. In the second biofilm, the specific growth rate is one-third the growth rate that would be measured for rapidly growing planktonic cells. Since all of the cells are growing, the specific growth rate in both cases would be one-third of the planktonic cell growth rate. The response of these two biofilms to an antimicrobial agent whose action is growth-rate-dependent is expected to be quite different. In the homogeneous biofilm, microbial cells will be killed at a uniform rate that is somewhat less than the rate that would be measured for the rapidly growing planktonic cells. Since all of the cells are growing, they will all eventually be killed. The susceptibility of the biofilm cells as determined by the overall rate of killing would be decreased by perhaps a factor of three compared to free-floating cells. In the heterogeneous biofilm, killing will follow a very different profile. Rapidly growing cells in the outer one-third of the biofilm will be quickly killed. Cells in the interior two-thirds of the biofilm will not be killed by virtue of their residence in a non-growing state. Because the majority of the biofilm escapes killing indefinitely, this scenario could easily give rise to biofilm resistance factors of a hundred or a thousand. Spatial heterogeneity thus greatly amplifies the effects of physiological variations in biofilms on the susceptibility of sessile microorganisms to antimicrobial challenge. When spatial heterogeneity is accounted for, the altered physiological mechanism of biofilm resistance realizes its full power and is capable of explaining the magnitude of resistance that is experimentally observed.

The spatial heterogeneity of physiological status that may be a common feature of biofilm growth could arise from nutrient limitation or product inhibition. If it does, then we are faced with a paradox in the hypothesis articulated above. This problem is best illustrated by returning to the thought experiment. Suppose that growth is limited to the outer one-third of the heterogeneous biofilm because an essential nutrient only penetrates one-third of the way into the biofilm. When the biofilm is exposed to a growth-dependent antimicrobial agent, cells in the outer third will be killed. Once cells in the growing zone have been killed they will cease to consume nutrients. The limiting nutrient will now penetrate and feed cells in the middle third of the biofilm. Thus, the middle third of the biofilm will be made susceptible to the antimicrobial agent. Layer by layer, all the biofilm microorganisms will be alternately nourished and killed. The entire biofilm would eventually succumb. So it would seem by this reasoning that the spatial heterogeneity hypothesis of biofilm resistance would lose its explanatory power when the heterogeneity derives from a nutrient limitation.

We propose two possible resolutions to this paradox. It may be that spatial heterogeneity of activity within a biofilm does not derive simply from nutrient limitation. Perhaps cell-cell signalling or other regulatory mechanisms switch cells into a dormant, and protected phenotypic state. Cells might not be awakened from this state by the replenishment of nutrients alone. A second explanation depends on the continued utilization of nutrients by cells that are no longer viable by the usual colony formation tests. We speculate that it is quite likely that some antimicrobial-treated cells, though irreparably compromised in terms of their ability to reproduce, would eventually succumb. So it would seem to consume nutrients for hours or even days beyond the time that they will have been judged to have expired based on an assay for culturability. A couple of examples of this
phenomenon are illustrated here. Biofilms treated for 1 h with monochloramine suffered a 3 log reduction in c.f.u. but were scarcely affected in their utilization of glucose and oxygen (15). Escherichia coli exposed to ciprofloxacin for 1 h experienced a 3 log decrease in c.f.u. but continued to reduce a tetrazolium dye, maintain a membrane potential and synthesize protein for up to 5 h post-exposure (10). If such damaged cells appear in the outer layers of a biofilm, they will shield underlying cells from nutrient exposure that would stir them from their non-growing state and render them susceptible to killing.

The resolution of these and other important biofilm questions depends on the continued application of methods to detect microscale spatial patterns within biofilms. Karen D. Xu,† Gordon A. McFeters1 and Philip S. Stewart*

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