BIOFILM ANTIMICROBIAL RESISTANCE

Philip S. Stewart, Pranab K. Mukherjee, and Mahmoud A. Ghannoum

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This chapter describes the phenomenon of biofilm-reduced susceptibility to antimicrobial agents, discusses factors that influence biofilm tolerance, and outlines possible protective mechanisms.

REDUCED ANTIMICROBIAL SUSCEPTIBILITY IN BIOFILMS

Microorganisms that band together in biofilms are protected from killing by biocides, disinfectants, and antibiotics. Recognition of this phenomenon can be dated back to at least 1684, when Antonie van Leeuwenhoek recorded his observations of the microbial flora in dental plaque. van Leeuwenhoek noted that microorganisms dispersed from plaque into an antimicrobial solution, such as vinegar, lost all motility. When he rinsed his mouth with vinegar, thereby exposing the intact biofilm, he found that the treated plaque continued to seethe and teem with life. The industrial and medical communities brim with anecdotes that evoke van Leeuwenhoek's simple observation. Industrial microbiologists tell stories about contamination and slime that could not be conquered, even with withering antimicrobial treatments. Likewise, infectious disease specialists have tales of persistent infections that could not be resolved with antibiotics, even when laboratory tests indicated these agents should have been effective.

The phenomenon of biofilm resistance to drugs and antimicrobials is easily reproduced in the laboratory. A few example data sets comparing the time course of killing of free-floating and biofilm cells are graphed in Fig. 1. These data illustrate, in numerical terms, that killing of microorganisms in a biofilm can proceed many times more slowly than killing of the same strain in a planktonic culture.

Hundreds of papers have been published describing comparisons of planktonic and biofilm susceptibility. Some of the microorganisms and antimicrobial agents that have been studied in this way are listed in Tables 1 and 2, respectively. The point of these listings, which are not comprehensive, is to illustrate that resistance in the biofilm state is observed for diverse microbial species and for all sorts of antimicrobial agents. The antimicrobial agents range from brute-force oxidants, such as chlorine, to antibiotics with exquisitely specific cellular tar-

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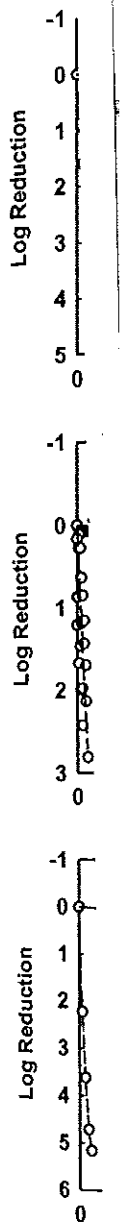

FIGURE 1
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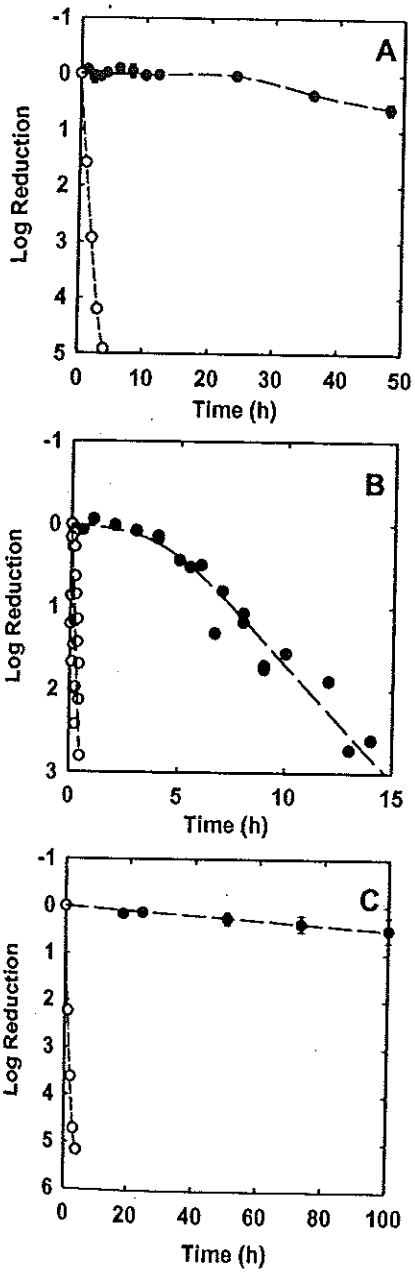


FIGURE 1 Comparison of biofilm (●) and planktonic (○) killing by antimicrobial agents. (A) *S. epidermidis* challenged with 0.1 μg of rifampin per ml (from Zheng and Stewart, 2002). (B) *P. aeruginosa* challenged with 50 mg of glutaraldehyde per liter (reprinted from Grobe et al., 2002, with permission from Springer Verlag). (C) *P. aeruginosa* challenged with 10 μg of tobramycin per ml (from Walters et al., 2003).

TABLE 1 Microorganisms shown to exhibit reduced antimicrobial susceptibility in biofilms

Actinobacillus
Bacillus
Campylobacter
Candida
Citrobacter
Corynebacterium
Desulfovibrio
Enterobacter
Enterococcus
Escherichia
Gardnerella
Lactobacillus
Legionella
Listeria
Mycobacterium
Porphyromonas
Proteus
Pseudomonas
Salmonella
Staphylococcus
Streptococcus
Vibrio

gets. The microorganisms range from bacteria to yeast and from obligate aerobes to sulfate-reducing bacteria and other finicky anaerobes. In other words, biofilm-reduced susceptibility is a robust phenomenon that is widely observed.

One can propose various numerical factors to quantify the degree of protection afforded by biofilm formation. One option is to form the ratio of the concentration of antimicrobial agent required to achieve a certain level of killing in a biofilm with the concentration of agent needed to kill floating cells to the same extent. The treatment duration should be fixed in this comparison. Another option is to compare the killing rate, in response to the same antimicrobial dose, in the planktonic and biofilm states. This can be understood in graphical terms as the ratio of the slopes on plots like those in Fig. 1. For example, the resistance factors calculated in this way for the glutaraldehyde, rifampin, and tobramycin data sets shown in Fig. 1 are 47, 98, and 270, respectively.

When such resistance factors are calculated from literature data and studied, it is difficult to

TABLE 2 Antimicrobial agents shown to exhibit reduced efficacy against microorganisms in biofilms

Biocide	Antibiotic
Amphoteric surfactants	Amikacin
2,2-Dibromo-3-nitrilopropionamide	Amphotericin B
2-Bromo-2-nitro-1,3-propanediol	Ampicillin
Benzalkonium chloride	Aztreonam
Cetylpyridinium chloride	Cefazolin
Chlorhexidine	Ceftazidime
Chlorine	Cefuroxime
Chlorine dioxide	Ciprofloxacin
Chlorosulfamate	Clindamycin
Formaldehyde	Erythromycin
Glutaraldehyde	Fluconazole
Hydrogen peroxide	Fosfomycin
Iodine	Gentamicin
Isothiazolone	Metronidazole
Monochloramine	Novobiocin
Ozone	Ofloxacin
Peracetic acid	Piperacillin
Polyhexamethylene biguanide	Rifampin
Potassium monopersulfate	Tetracycline
Povidone iodine	Tobramycin
Triclosan	Trimethoprim-sulfamethoxazole
	Vancomycin

discern any interesting patterns. This is probably because the susceptibility of microorganisms, in both the biofilm and planktonic states, depends on the specific culture conditions. Resistance factors calculated in one system should not be extrapolated to a different system. One thing that is apparent from this literature is that there are numerous examples of biofilms that are not much different in susceptibility from a planktonic culture. This shows that not all biofilms are protected from every antimicrobial agent. It could also be that reduced susceptibility develops only at a certain stage of biofilm maturation and that younger biofilms are not protected.

When microorganisms are dispersed from a biofilm, their antimicrobial susceptibility is usually rapidly restored. This is exactly the observation that van Leeuwenhoek made. Sometimes the resuspended microbes will exhibit intermediate susceptibility. But if the microorganisms are subcultured, they generally return to a susceptible phenotype. This tells us that the protection afforded in the biofilm mode of growth is probably not the result of mutations or the acquisition of resistance genes.

Several general statements can be made regarding the protection of microorganisms in biofilms from killing by antimicrobial agents. The capacity to form protective biofilms is widely distributed in the microbial world. The resistance mechanisms that are deployed in biofilms constitute a broad-spectrum defense that is effective against many types of antimicrobials. Biofilm resistance can be measured in the laboratory in vitro in a wide variety of reactors and media. This indicates that biofilm protection does not depend on special environmental or host factors. That is, resistance to antimicrobials is not only observed in vivo or in real world settings; it is manifested in even very simple laboratory models. Finally, the fact that dispersed biofilm cells become susceptible again shows that biofilm resistance reflects a reversible physical or phenotypic state.

ANTIFUNGAL SUSCEPTIBILITIES OF FUNGAL BIOFILMS

Fungal biofilms are commonly encountered in cases of invasive catheter-related infections as well as superficial infections like denture stomatitis. Hawser and Douglas (1995) used an in vitro model of *Candida* biofilms formed on discs of polyvinyl chloride (PVC) catheter to determine their susceptibilities against amphotericin B, flucytosine, fluconazole, itraconazole, and ketoconazole. Susceptibilities were determined by (i) [^{3}H]leucine incorporation and (ii) tetrazolium salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) reduction assays (Hawser and Douglas, 1995). Dose-response curve analyses of these data were used to calculate the drug concentration that caused 50% inhibition of [^{3}H]leucine incorporation (IL_{50}) or 50% inhibition of MTT-formazan formation (IF_{50}). All the antifungal agents showed much less activity against mature biofilms than against the planktonic cells, with IL_{50} and IF_{50} values for biofilms being five to eight times higher than those for planktonic cells, and 30 to 2,000 time higher than the relevant MICs (Hawser and Douglas, 1995). These studies clearly showed that *Candida* cells grown as biofilms have dramatically enhanced resistance to antifungal agents compared with their planktonic forms.

A common reservoir of oral microbes is denture biofilms present in oral plaques of patients with denture stomatitis. Among the multiple factors likely to contribute to the development of denture stomatitis, denture plaques are the most common. Ultrastructural studies revealed that denture plaques are complex biofilms composed of bacteria, yeasts, and desquamated epithelial cells (Catalan et al., 1987; Radford and Radford, 1993). A recently developed reproducible model of *Candida albicans* denture biofilms was used to determine the antifungal susceptibility of biofilm- and planktonically grown *C. albicans* (Chandra et al., 2001a, 2001b) (for details of denture biofilm model see chapter 3 in this volume). The MIC of commonly used drugs against biofilms and planktonic *C. albicans* was assayed by the M-27A method (National Committee for Clinical Laboratory Standards, 1997) and by determining the 50% reduction in metabolic activity (RMA_{50}) (Chandra et al., 2001b). As shown in Table 3, *C. albicans* biofilms were resistant to these drugs, while the planktonic cells were highly susceptible. Further investigations of the effect of different drug concentrations showed that metabolic activity of fungal biofilms increases with time, suggesting that the resistance observed in these biofilms is not due to the presence of dormant or dead cells (Fig. 2). These studies demonstrated that *C. albicans* biofilms are significantly more resistant to commonly used antifungals than candidal cells grown in planktonic form.

Silicone elastomer discs are common substrates for in vitro biofilm formation. This material is relevant in invasive biofilm-related infections and is commonly used to make catheters (for details of catheter biofilm model see chapter 3 in this volume). Susceptibility assays performed by Kuhn et al. (2002) revealed that biofilms formed by two *C. albicans* and two *Candida parapsilosis* isolates were resistant against fluconazole (FLC), amphotericin B (AMB), nystatin (NYT), chlorhexidine (CHX), and terbinafine (TERB), compared with planktonic forms (Table 3). Moreover, the two new triazoles, voriconazole (VORI) and ravuconazole (RAVU), also did not inhibit biofilms formed by these strains. In contrast, both liposomal AMB and abelcet (ABLC) exhibited inhibitory activities against *C. albicans* biofilms, with MICs similar to those seen for planktonic cells. *C. parapsilosis* strain P/A71 biofilms were susceptible to ABLC but had a slightly higher MIC_{50} for liposomal AMB. Although lipid complex-NYT showed potent activity against planktonically grown *Candida*, this antifungal failed to inhibit biofilms (Table 3). Inhibition studies of biofilm-grown *C. albicans* (strain M61) in the presence of different concentrations of FLC, AMB, caspofungin, and ABLC revealed a dramatic difference in the susceptibility plots between conventional agents (FLC and AMB) and the novel drugs (caspofungin and ABLC). Micafungin and liposomal AMB

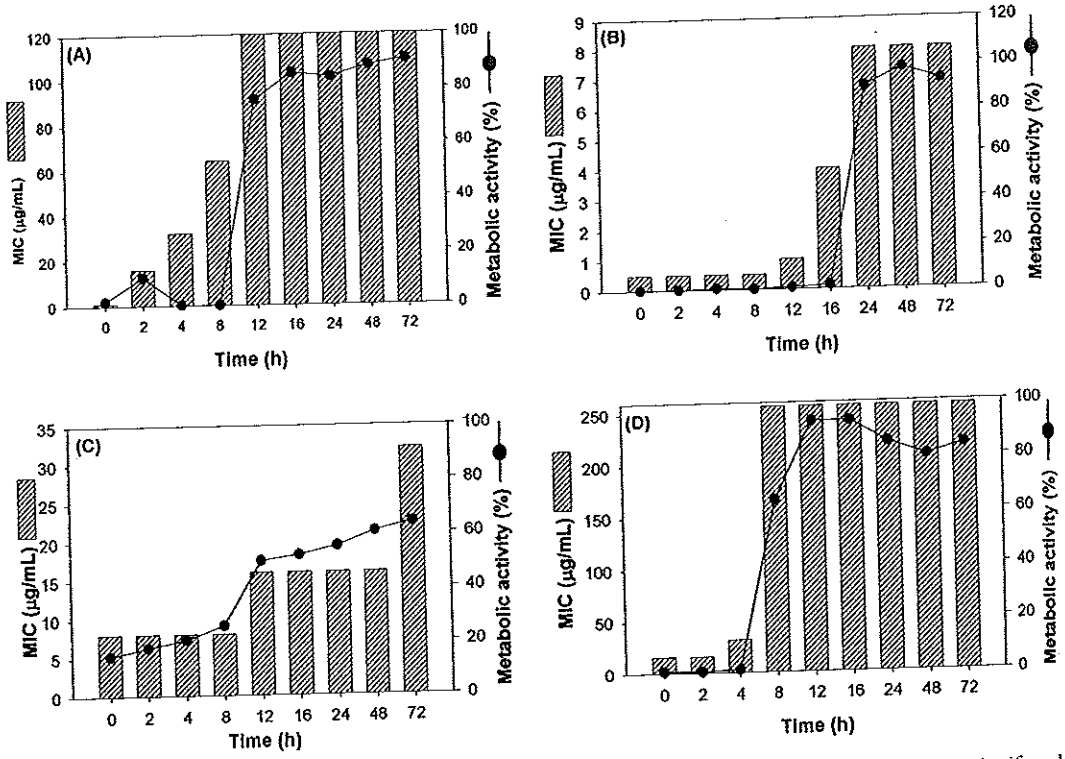


FIGURE 2 Correlation of biofilm development and metabolic activity with antifungal resistance Antifungal susceptibility of *C. albicans* at different stages of biofilm development against FLU (A), AMB (B), NYT (C), CHX (D), respectively, are represented as histograms. The line curves show percent metabolic activity of growing *C. albicans* biofilms exposed to FLU (64 μg/ml), AMB (4 μg/ml), NYT (8 μg/ml) or CHX (64 μg/ml). Metabolic activity was normalized to the control without drugs, which was taken as 100%. Redrawn with permission from *Journal of Bacteriology* (Chandra et al., 2001a).

produced curves similar to the latter agents, while the effects were similar for agents effective with strains GDH, P/A71, and P92 (Kuhn et al., 2002).

FACTORS INFLUENCING BIOFILM SUSCEPTIBILITY

This section reviews the physical and biological factors that govern the degree of resistance mounted in a biofilm. These factors are numerous, and their plentitude is surely one of the reasons that there is so much variability in literature data in this field. Biofilm susceptibility is influenced significantly by such factors as biofilm thickness, biofilm age, biofilm areal cell density, antimicrobial dose concentration, biofilm species composition, and genotype.

Thicker, older, or denser biofilms are generally less susceptible than thin, young, or sparse biofilms. For example, Anwar et al. (1989) reported that 10 μg of tobramycin per ml reduced viable numbers of *Pseudomonas aeruginosa* in 2-day old biofilms (initial cell density, 2×10^7 CFU/cm^2) by a factor of 10^5 within 4 h. The same treatment had no effect whatsoever on 7-day-old biofilms (initial cell density, 2×10^8 CFU/cm^2). Leriche and Carpentier (1995) found that 4-day-old *Salmonella enterica* serovar Typhimurium biofilms were at least three times more resistant to disinfection by hypochlorite than biofilms that were only 1 day old, even though there was little difference in initial cell density between the two biofilms.

TABLE 3 MICs (μg/ml) of antifungal agents against biofilms formed by *Candida albicans*

Antifungal agent[a]	MIC (μg/ml)		Reference
	Planktonic	Biofilm	
AMB	0.25	8	Chandra et al., 2001b
Fluconazole	0.25–1.0	>256	Chandra et al., 2001b
NYT	1	16	Kuhn et al., 2002
Chlorhexidine	8	32–128	Chandra et al., 2001b
			Chandra et al., 2001b
Terbinafine	32	128	Kuhn et al., 2002
Voriconazole	0.5	>256	Kuhn et al., 2002
Ravuconazole	0.1	128	Kuhn et al., 2002
Liposomal AMB	0.5	0.25	Kuhn et al., 2002
Liposomal NYT	0.5	8	Kuhn et al., 2002
Abelcet	0.25	0.25	Kuhn et al., 2002
Caspofungin	0.125	0.25	Kuhn et al., 2002
Micafungin	0.001	0.25	Kuhn et al., 2002

[a]AMB, amphotericin B; NYT, nystatin.

A simple inverse relationship between accumulation of biomass in the biofilm and susceptibility is not universally true, however. Data collected by Srinivasan et al. (1995) show that bacteria in a mixed-species biofilm were more susceptible to disinfection by monochloramine at a middle value of areal cell density than when the biofilm was very sparse or especially thick. One explanation for this behavior is that there are two distinct protective mechanisms, one that is effective for newly attached cells and another that operates in thick biofilms.

Although there are few experimental data, it is likely that the presence of abiotic particles in a biofilm increases resistance to antimicrobial agents (Srinivasan et al., 1995). Most researchers do not encumber their studies with the additional complexity that comes with incorporating abiotic materials. But abiotic particles, such as corrosion products, precipitates, silt, fibers, and dead cells from an animal host, are common constituents of environmental and medical biofilms. These particles can be expected to enhance protection by reducing diffusive transport and increasing sorptive capacity.

The dependence of antimicrobial efficacy on the concentration of the antimicrobial agent is complex. One of the longstanding concepts used in the application of antimicrobial agents is the concentration-time or CT rule. This rule posits that the efficacy of an antimicrobial treatment will be proportional to the dose concentration and also to the dose duration. For example, the CT rule would predict that a 2 h dose at 50 mg/liter would have the same effect as a 10 h dose of 10 mg/liter. While simple and intuitive, the CT rule may mislead, especially when biofilms are involved. Not only is the concentration dependence of killing rarely linear, but the concentration dependence of killing differs between biofilms and planktonic cells. An important insight is that biofilm susceptibility is more strongly dependent on the antimicrobial concentration than is planktonic killing (Gilbert et al., 2001; Grobe et al., 2002). Dosing protocols developed using planktonic tests cannot be expected to yield optimal dosing strategies when targeting a biofilm. For example, Grobe et al. (2002) found that a dose of 200 mg of glutaraldehyde per liter for 34 min was as effective as a 50 mg/liter dose delivered for 650 min in controlling a *P. aeruginosa* biofilm. The dose that was four times more concentrated was able to achieve the same effect as the low-concentration dose in about one-twentieth the time. These results

suggest that biofilms are best controlled by using brief but relatively high concentrations of antimicrobial agents rather than prolonged doses of lower concentrations.

Several studies reveal that the susceptibility of a biofilm is influenced by its species composition (Srinivasan et al., 1995; Budhani and Struthers, 1998; Whiteley et al., 2001b; Adam et al., 2002; Elvers et al., 2002; Lindsay et al., 2002). These studies suggest that coculture can enhance the survival of selected species. For example, the presence of *Vogesella indigofera* protects *Pseudomonas putida* from betadine when these two organisms are grown in a binary-population biofilm (Whiteley et al., 2001b). A β-lactamase-positive *Moraxella catarrhalis* enhanced the resistance of *Streptococcus pneumoniae* to β-lactam antibiotics when the two bacteria were grown in mixed biofilms (Budhani and Struthers, 1998). Interactions like these have also been reported for bacteria and fungi growing together in biofilms (Adam et al., 2002). In general, it can be concluded that mixed-species biofilms are more protected than are single-species biofilms.

Adam et al. (2002) studied the susceptibility of mixed-species biofilms of *C. albicans* and *Staphylococcus epidermidis*, two organisms commonly found in catheter-associated infections. These studies indicated that the extracellular polymer produced by *S. epidermidis* RP62A could protect *C. albicans* cells against azoles by inhibiting their penetration in mixed fungal-bacterial biofilms. Conversely, the *C. albicans* cells in the mixed biofilm appeared to protect the slime-negative staphylococcus against vancomycin, suggesting that fungal cells can modulate the action of antibiotics and that bacteria can affect antifungal activity in mixed fungal-bacterial biofilms (Adam et al., 2002).

Molecular techniques are just beginning to be applied to elucidate the genetic basis of biofilm protection from antimicrobial agents. One type of experiment involves comparing the antimicrobial susceptibility of a wild-type biofilm with a biofilm formed from a defined mutant strain. An interesting phenotype that can be envisioned is a mutant that forms a structurally normal biofilm but fails to mount the usual degree of antimicrobial resistance. There are only a few studies that fit this description cleanly. These involve mutants that are unable to deactivate the antimicrobial agent, namely a β-lactamase-deficient strain challenged with ampicillin (Anderl et al., 2000) and a catalase-deficient strain challenged with hydrogen peroxide (Elkins et al., 1999). In both cases, the mutant biofilm is predictably more sensitive to the antimicrobial agent. Experiments further show that the antimicrobial agent penetrates into the mutant biofilm but not into the wild type.

One can also imagine mutants that alter biofilm susceptibility by causing the biofilm to grow thicker or thinner. Many of the mutations that have been studied for their effect on biofilm resistance fall into this category. For example, Shih and Huang (2002) report that quorum-sensing mutants of *P. aeruginosa* are more susceptible to kanamycin than the parent strain. But they also found that these mutants formed much thinner biofilms than the wild type. Other studies of quorum-sensing mutants of *P. aeruginosa* also found greater susceptibility to the agents hydrogen peroxide (Hassett et al., 1999) and sodium dodecyl sulfate (Davies et al., 1998), but reported concomitant defects in biofilm architecture and accumulation. Mutants that overproduce the extracellular polysaccharide alginate form biofilms that are less susceptible to the antibiotic tobramycin (Hentzer et al., 2001). This mutation also causes a thicker and rougher biofilm to form. *P. aeruginosa* biofilms formed by an *algT* mutant, which is incapable of making much alginate, were not much affected in their susceptibility to hydrogen peroxide and monochloramine (Cochran et al., 2000). The sigma factor *rpoS* has been hypothesized to be important in biofilm protection because of its activity during stationary phase, but *P. aeruginosa rpoS* mutants, when grown in biofilms, are also not much affected in their susceptibility to hydrogen peroxide and monochloramine (Cochran et al., 2000). Other studies of *P. aeruginosa rpoS* mutants have shown that this strain formed biofilms that were thicker than the wild type

(Heydorn et al., 2000) and also less susceptible to tobramycin (Whiteley et al., 2001a). A *P. aeruginosa gacA* mutant was slightly more susceptible to antibiotics, but this mutant did not form mature, wild-type biofilm structures (Parkins et al., 2001). When a mutation affects biofilm structure and thickness it is difficult to determine whether an observed change in susceptibility is a result of the change in architecture or if it is due to a more specific gene function.

Another intriguing class of mutants is those affected in drug efflux pump activity. Efflux pumps are behind much of the antibiotic resistance that has been described in planktonic microorganisms. It is natural to wonder whether pumps contribute to biofilm protection. *Escherichia coli* mutants in the *mar* operon form biofilms that have a similar resistance to ciprofloxacin relative to the wild type (Maira-Litran et al., 2000b). *P. aeruginosa* growing in biofilms also remained resistant to antibiotics even when missing the MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY multidrug resistance pumps (De Kievit et al., 2001). Brooun et al. (2000) found that *P. aeruginosa* biofilm resistance to ciprofloxacin did not depend on the MexAB-OprM pump. These results do not support a role of existing drug efflux pumps in the biofilm defense, but they also do not exclude the possibility that as-yet-uncharacterized efflux pumps might contribute to biofilm resistance.

Another approach to study genetic determinants of biofilm antimicrobial resistance is to identify genes or proteins that are expressed in the biofilm state, either before or after antimicrobial exposure. For example, Elkins et al. (1999) showed the *katB*, encoding an inducible catalase, is expressed in *P. aeruginosa* biofilms treated with high concentrations of hydrogen peroxide. There was no expression from the same reporter in a planktonic culture, probably because free-floating cells were overwhelmed by the antimicrobial effects of the hydrogen peroxide before gene expression could be activated. Maira-Litran et al. (2000a) and De Kievet et al. (2001) used reporter gene constructs to show that drug efflux pumps are not up-expressed in *E. coli* and *P. aeruginosa* biofilms, respectively. Whiteley et al. (2001a) used a DNA microarray to identify a putative efflux pump that is expressed in *P. aeruginosa* biofilm in response to treatment with tobramycin.

What these studies reveal is that the genetic basis of biofilm protection from antimicrobial agents remains obscure. This is clearly an area of opportunity for researchers.

FACTORS CONTRIBUTING TO RESISTANCE IN FUNGAL BIOFILMS

The mechanism(s) contributing to increased antifungal resistance of biofilm-grown *C. albicans* has not been defined, although several contributing factors have been suggested (Douglas, 2003). These include the role of extracellular matrix, physiological state of fungal cells, presence of drug efflux pumps, and developmental phases of biofilm formation.

Role of Extracellular Matrix

In bacterial biofilms, it has been suggested that the thick extracellular matrix (ECM) may contribute to antimicrobial resistance by preventing the diffusion of drugs to target cells. Such prevention can be due either to physical obstruction in drug diffusion or to direct binding of the drug by ECM. Since candidal biofilm formation is characterized by the presence of visible ECM, this factor has been commonly suggested as playing an important role in antifungal resistance of biofilms (Baillie and Douglas, 2000). Hawser et al. (1998) correlated the growth of *C. albicans* biofilms and production of ECM by dry weight estimation, colorimetric and radioisotope assays, and scanning electron microscopy. These investigators showed that ECM production was minimal in biofilms grown under static incubation but increased dramatically when developing biofilms were subjected to a liquid flow (Hawser et al., 1998). The nonstatic biofilms consisted of cells enveloped in ECM, leading the investigators to suggest that production of matrix material could contribute to the resistance of biofilm cells to antifungal agents (Hawser et al., 1998).

To investigate whether ECM plays a role in the resistance of biofilms to antifungal agents, susceptibility profiles of biofilms grown statically (exhibiting minimal ECM) were compared with those grown with shaking (maximal ECM synthesis). The metabolic activity of the biofilms was determined from their [^{3}H]leucine uptake. Uptake of leucine by biofilms was not affected by the presence of most drugs, even at high concentrations (Fig. 3). However, leucine uptake was inhibited by about 50% in biofilms incubated with one time the MIC of amphotericin B (Fig. 3). Biofilms grown with or without shaking did not show significant differences in susceptibility to any of the drugs. Susceptibility to amphotericin B varied remarkably between different catheter types. Thus, biofilms formed on vena cava catheter discs were more susceptible than those grown on Faucher tubes although both types are made of PVC supplied by different manufacturers (Fig. 4). A recent study showed that the resistance phenotype of *C. albicans* biofilms was maintained by sessile cells when resuspended as free-floating cells, thus indicating that biofilm integrity and the presence of ECM are not the sole determinants of biofilm resistance (Ramage et al., 2002). In view of the limited number of studies, the role of ECM in fungal biofilm resistance is yet to be determined unequivocally.

Role of Drug Efflux Pumps in Antifungal Resistance of *Candida* Biofilms

The field of fungal biofilm-associated drug resistance is severely hampered by a very limited number of papers. Unlike the bacterial biofilm field where numerous studies have been performed on resistance (including at the molecular level), in fungal biofilms the resistance field is still in its infancy. One mechanism that has recently been explored is the role of drug efflux pumps (encoded by *CDR* and *MDR* genes) in biofilms and planktonically grown cells. So far only two studies have explored the role of efflux pumps in antifungal resistance of candidal biofilms. The first study (Ramage et al., 2002) used *C. albicans* mutants deficient for one or two efflux pumps and evaluated resistance in biofilms formed by these mutants at intermediate and mature phases (24 and 48 h). A more recent study included a *C. albicans* strain lacking all three major efflux pumps, which used antifungal susceptibility assay, Northern blot analysis, and Western blot analysis to evaluate their roles in biofilm-associated resistance (Mukherjee et al., 2003).

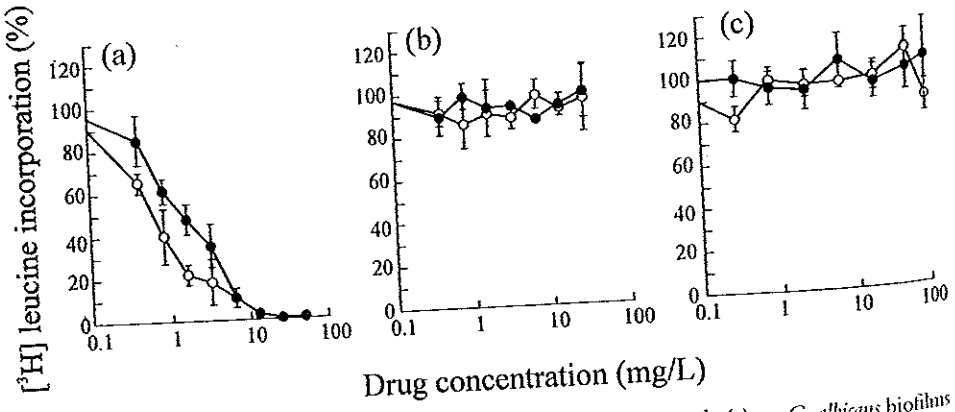


FIGURE 3 Effect of amphotericin B (a), flucytosine (b), and fluconazole (c) on *C. albicans* biofilms grown statically (●) or with gentle shaking (○). [^{3}H]Leucine incorporation by biofilms was determined as a percentage of that for control biofilms incubated in the absence of the antifungal agent. Redrawn from Baillie and Douglas, 2000, by permission of Oxford University Press.

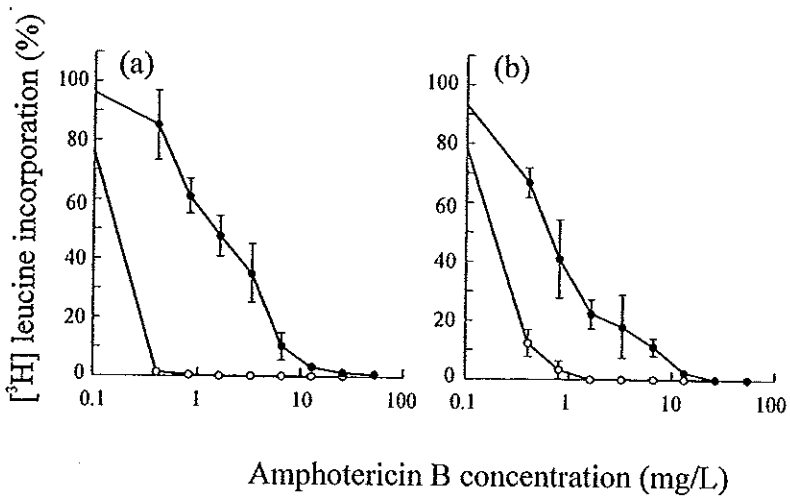
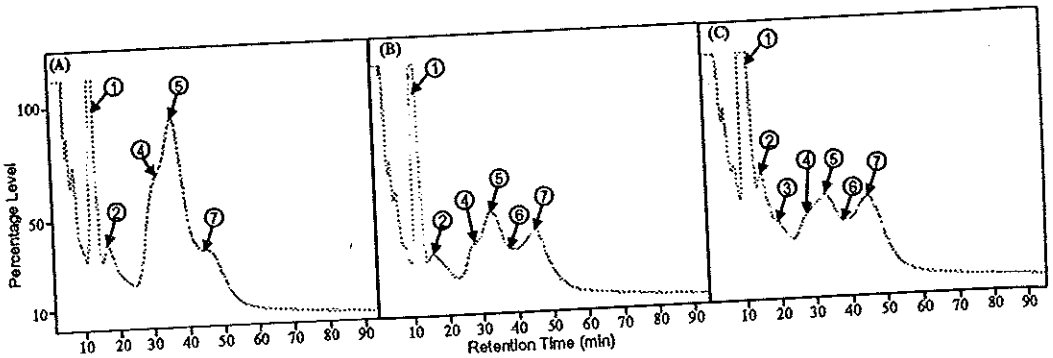


FIGURE 4 Effect of amphotericin B on *C. albicans* biofilms grown statically (a) or with shaking (b) on PVC discs cut from Faucher tubes (Vygon) (●) or vena cava catheters (Jostra) (○). [³H]Leucine incorporation by biofilms was determined as a percentage of that for control biofilms incubated in the absence of the antifungal agent. Redrawn from Baillie and Douglas, 2000, by permission from Oxford University Press.

Ramage et al. (2002) showed that expression of genes encoding efflux pumps was upregulated during the course of biofilm formation and development. Moreover, isogenic *C. albicans* mutants carrying single- and double-deletion mutations (Δ*cdr1*, Δ*cdr2*, Δ*mdr1*, Δ*cdr1*/Δ*cdr2*, and Δ*mdr1*/Δ*cdr1*) were hypersusceptible to FLC when planktonic, but still retained the resistant phenotype during biofilm growth (Ramage et al., 2002). Recent studies using the double- and triple-deletion mutant (Δ*cdr1*/Δ*cdr2*/Δ*mdr1*) strains of *C. albicans* revealed that the azole susceptibilities of biofilm formed by these mutant strains varied with both the number of pumps deleted and the developmental phase of biofilms (Mukherjee et al., 2003). These latter studies revealed that mature-phase biofilms formed by the triple knockout mutant strain had drastically reduced susceptibilities to FLC than planktonic cells, while early-phase biofilms formed by this strain were susceptible to these drugs. Functional studies of efflux pumps using Rhodamine123 (Rh123, a fluorescent substrate for these proteins) (Clark et al., 1996; Chaudhary and Roninson, 1991) was used to demonstrate that, compared with the early-phase biofilm, the level of Rh123 was significantly reduced in intermediate and mature phases, while no significant differences in Rh123 levels were found at 12 and 48 h (Mukherjee et al., 2003). These studies suggested that (i) efflux pumps are differentially expressed during biofilm formation, and (ii) these pumps contribute to biofilm-associated resistance at the early phase of development but not during later stages.

Role of Sterol Composition

The cellular target of fluconazole in *C. albicans* is a cytochrome P-450 hemoprotein involved in the ergosterol biosynthetic pathway (Ghannoum and Rice, 1999). Alterations in sterol composition have previously been linked to antifungal resistance in planktonic cells (Ghannoum et al., 1992; Sanati et al., 1997; Hitchcock et al., 1987). To determine whether this phenotype can be attributed to changes in sterol composition, total membrane sterols were isolated from biofilms and planktonic cells and analyzed by gas-liquid chromatography (Vandenheuvel and Court, 1968; Ibrahim and Ghannoum, 1996). The level of ergosterol



(D) Percentage levels (mean ± SD) of sterols in biofilm and planktonic *C. albicans* grown to 6, 12, and 48h

Peak No.	Sterol	Biofilm			Planktonic		
		6 h	12 h	48 h	6 h	12 h	48 h
1	Squalene	11.40 ± 0.006	33.84 ± 0.064	20.09 ± 0.064	22.44 ± 0.006	9.23 ± 0.006	31.25 ± 0.010
2	Breakdown product 1	8.35 ± 0.012	8.48 ± 0.036	13.32 ± 0.110	10.03 ± 0.006	14.57 ± 0.032	14.26 ± 0.006
3	Breakdown product 2	n.d.*	n.d.*	6.13 ± 0.070	n.d.*	n.d.*	2.03 ± 0.017
4	Zymosterol	27.50 ± 0.422	12.63 ± 0.006	10.53 ± 0.330	n.d.*	n.d.*	4.87 ± 0.012
5	Ergosterol	42.55 ± 0.582	25.10 ± 0.012	21.42 ± 0.789	41.75 ± 0.023	34.00 ± 0.577	31.00 ± 0.029
6	4,14-dimethylzymosterol	n.d.*	10.99 ± 0.047	7.90 ± 0.040	10.13 ± 0.015	16.18 ± 0.017	4.85 ± 0.023
7	Obtusifoliol	10.20 ± 0.056	8.96 ± 0.015	19.86 ± 0.050	15.64 ± 0.012	25.99 ± 0.006	11.33 ± 0.167

*n.d. – not detected

FIGURE 5 Variations of sterol profile of *C. albicans* biofilm at different developmental phases. Sterol pattern for biofilms grown to early (A), intermediate (B), or mature (C) phases were determined by gas-liquid chromatography. (D) Percentage levels of sterols identified in *C. albicans* biofilms and planktonic cells (chromatograph not shown), determined from the corresponding peak areas and retention times relative to ergosterol. Peaks 1 to 7 (panels A to C) represent sterols described in panel D. Redrawn with permission from *Infection and Immunity* (Mukherjee et al., 2003).

decreased by 41% and 50% at intermediate and mature phases, respectively, compared with early-phase biofilm (Fig. 5). These results showed that the level of sterols is modulated during *C. albicans* biofilm formation and suggested that such modulation may contribute to drug resistance.

PROTECTIVE MECHANISMS

The protective mechanisms behind biofilm antimicrobial resistance are still mostly unknown. In this section, five potential mechanisms are presented. These five mechanisms are (i) depletion of the antimicrobial agent in the bulk fluid bathing the biofilm, (ii) slow penetration of the antimicrobial agent into the biofilm, (iii) an altered chemical microenvironment within the biofilm leading to zones of slow or no growth, (iv) adaptive stress responses, and (v) persister cells.

Antimicrobial Depletion

Sometimes an explanation is so obvious, or so wholly unexciting from a scientific standpoint, that it is not given as much attention as it deserves. This would seem to be the case with antimicrobial depletion. This refers to the possibility that neutralizing reactions between the antimicrobial agent and constituents of the biofilm deplete the antimicrobial from the solution bathing the biofilm. It is not difficult to imagine that the antimicrobial concentration could be maintained in a planktonic test conducted against a dilute suspension of cells, but significantly decreased in a test against a heavily fouled biofilm specimen. One could

argue that this is not so much a resistance mechanism as a failure to properly deliver the antimicrobial treatment in the first place. Surprisingly few studies of antimicrobial agent action on biofilms include measurements of the antimicrobial residual concentration at the end of the treatment period. This should be standard practice in biofilm susceptibility testing.

Slow Penetration of Antimicrobial Agents into Biofilms

This is the very intuitive explanation that van Leeuwenhoek offered to explain the failure of vinegar to affect microbes in intact plaque on his teeth. Inside a cell cluster in a biofilm, diffusion is the predominant solute transport mechanism. Nutrients, metabolic products, biocides, and antibiotics move into and out of biofilm according to the physics of diffusion. If an antimicrobial agent does not experience a reaction in the biofilm, it can be predicted to enter over a timescale of seconds or minutes (Stewart, 2003). The delay posed by diffusion alone, therefore, will only be important for very brief antimicrobial doses. For example, diffusion is probably not fast enough for an antimicrobial mouthwash to penetrate to the deeper reaches of dental plaque in a typical rinse time. But in most cases, slow diffusion alone does not appear to be sufficient to explain the heightened resistance of biofilms.

The situation is more interesting if the antimicrobial agent is neutralized by reaction as it diffuses into the biofilm. In this case, its penetration can be profoundly retarded or even indefinitely delayed (Fig. 6). There are a handful of antimicrobial agents for which biofilm penetration limitation due to antimicrobial reaction has been demonstrated. These include chlorine (de Beer et al., 1994a; Stewart et al., 2001), hydrogen peroxide (Liu et al., 1998; Stewart et al., 2000), and β-lactam antibiotics (Anderl et al., 2000). The penetration of aminoglycoside antibiotics into biofilms appears to be retarded by binding of this polycationic molecule to matrix polymers (Gordon et al., 1988; Nichols et al., 1988; Kumon et al., 1994).

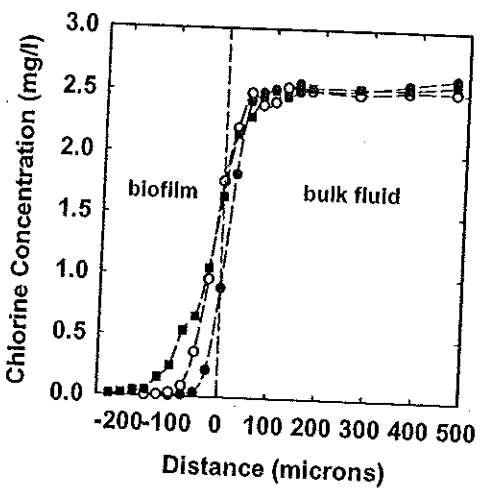


FIGURE 6 Chlorine concentration profiles in a mixed species biofilm. Chlorine at a concentration of approximately 2.5 mg/liter was flowed continuously over a biofilm, which was probed with a chlorine-sensitive microelectrode. At 10 min (●), 30 min (○), and 105 min (■) of exposure, chlorine penetrated only into the surface layers of the biofilm. Redrawn with permission from *Applied and Environmental Microbiology* (de Beer et al., 1994a).

The link between reaction of an antimicrobial and its penetration into biofilm is most elegantly demonstrated by studies with pairs of bacterial strains, the first a wild type carrying an enzyme that deactivates the antimicrobial and the second, a mutant strain that lacks this enzyme. For example, bioactive ampicillin fails to penetrate *Klebsiella pneumoniae* biofilms formed by a β-lactamase-positive strain of this microorganism because the enzyme cleaves and completely deactivates the antibiotic faster than it diffuses in (Anderl et al., 2000). In constrast, a β-lactamase-negative derivative of this same *K. pneumoniae* forms structurally normal biofilms that are readily penetrated by the antibiotic. Hydrogen peroxide cannot penetrate into *P. aeruginosa* biofilms because this organism is catalase positive (Stewart et al., 2000). Biofilms formed by a catalase-deficient mutant (*katA katB*) permit rapid delivery of hydrogen peroxide to the base of the biofilm. These experiments confirm that biocide or antibiotic reactivity in the biofilm controls

the ability of these agents to penetrate. Unfortunately, calculation of the extent of antimicrobial penetration remains difficult since reaction rates of antimicrobial agents are not known in most cases.

Evidence is accumulating that many antimicrobial agents can penetrate biofilms without killing microorganisms. Some agents that penetrate yet do not kill microorganisms at the rate that would be anticipated based on studies with freely suspended cells include ciprofloxacin (Suci et al., 1994; Shigeta et al., 1997; Anderl et al., 2000), chlorosulfamate (Stewart et al., 2001), ampicillin (challenging a β-lactamase-negative biofilm) (Anderl et al., 2000), rifampin (Dunne et al., 1993; Zheng and Stewart, 2002), tetracycline (Stone et al., 2002), and vancomycin (Dunne et al., 1993; Darouiche et al., 1994). There must be mechanisms of protection from antimicrobial agents in biofilm other than slow or incomplete penetration.

Altered Microenvironment and Slow Growth

Another longstanding explanation for the reduced susceptibility of biofilms is that they contain slowly growing or nongrowing cells. Microorganisms that are not growing or that have entered a stationary phase are known to be less susceptible to a wide variety of antimicrobial agents. The slow growth that occurs in the biofilm is presumably a result of local depletion of some required nutrient or electron acceptor. Another version of this protective mechanism is that the altered microenvironment, for example, low pH or anoxia, that is established in regions of the biofilm directly antagonizes the action of the antimicrobial.

There is abundant evidence for nutrient gradients within biofilms. Direct evidence comes from investigations using microelectrodes. These are miniature chemical sensors that can be lowered into the biofilm to directly measure the local concentration of such species as oxygen or ammonia inside a biofilm cluster (de Beer et al., 1994b; Zhang et al., 1995). Indirect evidence of nutrient limitation in biofilm comes from the engineers who study the kinetics of biofilm reactions, as in wastewater treatment processes. They realized long ago that their observations of reduced metabolic activity (on a per biomass basis) could only be understood by invoking substrate concentration gradients within the biofilm. Such concentration gradients arise from a reaction-diffusion interaction. Substrate is being consumed by a metabolic reaction as it diffuses into the biofilm. The balance of reaction and diffusion determines the shape of the concentration profile.

Data on microbial growth rates in biofilms also support the conclusion that microorganisms grow more slowly in biofilms, on average, than they do in the medium in which the biofilm is nourished (Hodgson et al., 1995; Wentland et al., 1996; Walters et al., 2003). Experimental comparisons indicate that average biofilm-specific growth rates can be as low as a few percent of the growth rate of planktonic cells. For example, Hodgson et al. (1995) reported that *Staphylococcus aureus* grew with a specific growth rate of 0.06 h^{-1} in biofilms whereas it grew with a specific growth rate of 0.7 h^{-1} in the planktonic state in the same medium. It should be recognized that a biofilm in which the average specific growth rate is half the growth rate of planktonic cells might actually consist of a population of cells in which half are growing rapidly and half not at all.

It is indeed likely that growth in biofilms is not spatially uniform. This is suggested by experimental investigations using fluorescence microscopy approaches to map indicators of growth such as relative RNA content or protein synthesis. For example, Xu et al. (1998) showed that only bacteria in the top 30 μm of a *P. aeruginosa* biofilm were capable of de novo protein synthesis (Color Plate 13). This region corresponded with the zone of oxygen penetration. The biofilm was 110 μm thick. The bottom 80 μm of the biofilm contained bacteria, but these bacteria lacked oxygen and were metabolically hamstrung in this location. The elegant reporter gene construct described by Sternberg et al. (1995), also in *P. aeruginosa*, reveals a qualitatively similar pattern. Growth

predominated at the surface of cell clusters while the cluster interiors suggested little or no growth.

In summary, the case for growth rate being a critical factor in controlling biofilm antimicrobial resistance is a strong one. Studies show that (i) planktonic susceptiblity is modulated by nutrient status, (ii) biofilms are commonly nutrient limited, (iii) biofilms contain nongrowing or stationary-phase cells, and (iv) biofilm susceptibility is reduced. One weakness of this case as it stands at present is that there are few studies in which all four of these points have been addressed in the same experimental system.

Three scientific reasons come to mind as to why slow growth is not accepted as a sufficient explanation for biofilm protection in general. First, not all antimicrobial agents are understood to be growth rate dependent in their action. Second, even very thin biofilms, in which nutrient limitation and slow growth are difficult to imagine, exhibit some reduced susceptibility. Finally, one would expect that growing cells in the biofilm would be killed, allowing nutrients to penetrate and nourish underlying cells. These previously starved cells would then become susceptible. One explanation for why this progression is not more rapid is that antimicrobial-damaged cells could continue to consume nutrients well past the time when these cells would score as dead in a colony-formation assay. Such damaged cells could shield more deeply embedded cells from seeing the nutrients that would resuscitate them and make them vulnerable.

Even if it is not a universal explanation, there can be no doubt that the altered chemical microenvironment that prevails locally within biofilms contributes to protection of the biofilm in many cases.

Stress Responses

Microorganisms are equipped with numerous genetic and biochemical systems for responding to environmental stresses. These systems include adaptive responses to oxidative stress, low water activity, DNA damage, starvation, and others. Might such stress-response systems be activated in biofilms? One possibility is that the stress response is constitutively turned on in biofilms. That is, the stress-response system is expressed even in the absence of the usual inducing condition. The limited experimental data available do not support this model. For example, bacterial drug efflux pumps are not up-expressed in biofilms prior to antibiotic treatment (Maira-Litran et al., 2000a; De Kievit et al., 2001). Neither is catalase present at elevated levels in biofilms of *P. aeruginosa* prior to hydrogen peroxide treatment (Elkins et al., 1999). Measurements of the expression of the stationary-phase sigma factor *rpoS* in biofilms of the same organism are mixed (Xu et al., 2001; Whiteley et al., 2001a). On the other hand, there is some evidence of efflux pump expression in fungal biofilms in the absence of drugs. An alternative to constitutive expression is that stress responses are implemented more effectively in biofilms than in planktonic cells once an antimicrobial challenge is delivered. This would appear to be the case with catalase induction on hydrogen peroxide treatment. Biofilm cells of *P. aeruginosa* transcribe *katB* and make the enzyme while free-floating cells of the same reporter strain succumb to the antimicrobial effects of the hydrogen peroxide before any induction can be detected (Fig. 7). Evidence for an active response to monochloramine in *P. aeruginosa* biofilms has been presented by Sanderson and Stewart (1997). Perhaps protective mechanisms, such as retarded penetration and slow growth, create enough of a buffer to allow biofilm cells to deploy stress responses in response to antimicrobial treatments that overwhelm planktonic cells.

Persisters

A persister is a hypothetical cell state in which microorganisms are protected from all types of antimicrobial insults. Persisters have alternatively been imagined as spore-like cells that are dormant and incapable of growth and as phenotypic variants that grow as rapidly as the parent strain (Spoering and Lewis, 2001; Drenkard and Ausubel, 2002). In either case, persisters

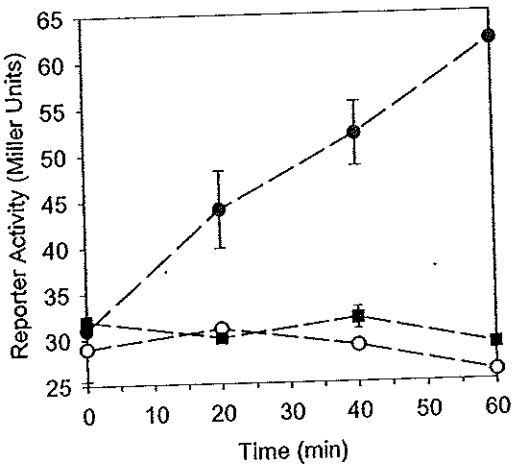


FIGURE 7 Induction of catalase in biofilms (●) and planktonic cells (○) of *P. aeruginosa* in response to hydrogen peroxide treatment. Biofilm cells are able to express this stress response while planktonic cells are not able to respond to the same challenge. Biofilm cells that are not exposed to hydrogen peroxide show no change in activity (■). Redrawn with permission from *Applied and Environmental Microbiology* (Elkins et al., 1999).

may only constitute a percent or less of the biofilm population. Though small in relative numbers, this protected subpopulation is sufficient to reseed the biofilm in the event of catastrophic chemical or physical challenge. The persister state is suggested by kill-versus-time and kill-versus-concentration curves that exhibit tailing. Even when treated for prolonged periods or with elevated antimicrobial concentrations, there is a small fraction of the population that is recalcitrant.

No one suggests that persister cells are unique to the biofilm mode of growth. Persisters presumably also form in suspension cultures. After all, planktonic cells have the same genetic code that would allow for differentiation into the persister state. The difference is either that persister cells are spawned at higher frequency in biofilms or that persister cells are retained in a biofilm better than in a planktonic culture. Spoering and Lewis (2001) hypothesized that persister cells are formed at higher frequency in stationary-phase cultures. Because biofilms experience nutrient limitation, persisters also form in higher numbers in biofilms than they do in a growing planktonic culture. This is a little different than the old slow-growth mechanism of biofilm protection in that the persister cells not only become nutrient limited but also differentiate into an especially resilient state. One can imagine interrupting the pathway for this differentiation to prevent persister formation, even if a culture does enter stationary phase.

The persister hypothesis is the newest explanation for biofilm resistance to antimicrobial agents and it is, understandably, the mechanism with the least experimental evidence available. Persisters are tricky to study experimentally. An assumption implicit to the persister hypothesis is that these protected cells can revert to a susceptible state. In addition to being ephemeral, persisters may be present in low numbers. They might not be detected on gene arrays or by proteomic analysis. Finally, if the persister state is linked to nutrient limitation or stationary-phase existence, then a full understanding of this mechanism will require analysis of nutrient transport and spatial patterns of growth within biofilms.

Multicellular Nature of Protective Mechanisms in Biofilms

Reduced antimicrobial susceptibility of microorganisms in biofilms is thought to be due to a combination of antimicrobial depletion through reactions with biofilm constituents, poor antimicrobial penetration, slow growth or stationary-phase existence in the biofilm, adaptive stress responses, and the formation of protected persister cells. Not one of these mechanisms by itself seems to offer a satisfactory explanation for the tenacious resistance of biofilm microorganisms to so many different types of antimicrobial agents. Rather, it is likely that the biofilms mount a multilayered defense in which two or more of these mechanisms operate in concert. The five protective mechanisms discussed above are summarized in cartoon form in Color Plate 14.

In the balance of this section we discuss the proposition that four of the five resistance mechanisms—slow penetration, slow growth, stress responses, and persisters—are inherently multicellular defenses.

When antimicrobial agents do not penetrate into a biofilm, it is because the antimicrobial is reactively neutralized as it diffuses into a cell cluster. This phenomenon can only be manifested when microbes are aggregated and exert their collective neutralizing activity. A lone cell, floating in a sea of antimicrobial, cannot react the antimicrobial away fast enough to generate concentration gradients. Consider the example of hydrogen peroxide, which is known to penetrate biofilms incompletely because catalase-containing bacteria exert their collective enzymatic action to destroy the antimicrobial molecule. Individual bacterial cells are overwhelmed by the same concentration of hydrogen peroxide, even though they express similar amounts of catalase. In other words, slow penetration is a protective mechanism that can only be implemented by groups of microorganisms. Catalase activity does not depend on the viability of the cell. It is therefore plausible that microorganisms in the surface layers of biofilm could continue to degrade hydrogen peroxide and shield their more deeply embedded neighbors, even after these surface-exposed cells have been killed.

Nutrient limitation and slow growth are also an inherently muticellular protective mechanism. Consider the example of an antibiotic that requires oxygen for effective killing. If microorganisms in the outer layer of the biofilm consume the available oxygen, those bacteria in the depths of the biofilm will be protected. This defense cannot be implemented by planktonic cells because a single cell does not exert sufficient respiratory activity to deplete oxygen from its immediate environment. In the aerobic zone of the biofilm, cells will suffer damage from the antibiotic. But these same cells could continue to consume oxygen, and hence protect their neighbors, long after they have lost the ability to reproduce.

The activation of stress responses can also be understood to depend on multicellular behaviors. It is likely that only a fraction of the cells in a biofilm are able to turn on stress responses. Others, perhaps those near the surface of the biofilm, will be overwhelmed by the antimicrobial agent. Although these cells are sacrificed, they provide enough of a buffer to allow other cells to deploy active responses that would not otherwise be possible.

Finally, the persister hypothesis is another illustration of the inherently multicellular nature of the biofilm defense. Persister cells are suited to surviving harsh challenges, but they must not be evolved for competing under more permissive conditions. If persisters grew rapidly under all conditions and had the ability to withstand antimicrobial treatments, this phenotype would soon dominate any population. In other words, no single cell can be tooled for rapid growth and reproduction and, at the same time, be prepared to withstand an antimicrobial challenge. Microorganisms in a biofilm must accomplish both of these objectives by a division of labor. Most of the cells in a biofilm occupy a relatively susceptible state in which they can, if nutrients are locally present, grow, synthesize matrix, and propagate the genome. A few cells, the persisters, are shunted into a protected state. These cells may not be able to grow or adapt as rapidly, but they will survive and reseed the community in the event of catastrophe. While admittedly this is speculative, it would be a more effective strategy for survival than can be imagined for a homogeneous population.

Biofilm formation is just beginning to be recognized as a multicellular developmental process. New strategies for controlling biofilms will emerge when the multicellular nature of biofilm-protective mechanisms is better understood.

REFERENCES

Adam, B., G. S. Baillie, and L. J. Douglas. 2002. Mixed species biofilms of *Candida albicans* and *Staphylococcus epidermidis*. *J. Med. Microbiol.* **51**:344–349.

Anderl, J. N., M. J. Franklin, and P. S. Stewart. 2000. Role of antibiotic penetration limitation in

Klebsiella pneumoniae biofilm resistance to ampicillin and ciprofloxacin. *Antimicrob. Agents Chemother.* **44**:1818–1824.

Anwar, H., T. van Biesen, M. Dasgupta, K. Lam, and J. W. Costerton. 1989. Interaction of biofilm bacteria with antibiotics in a novel in vitro chemostat system. *Antimicrob. Agents Chemother.* **33**: 1824–1826.

Baillie, G. S., and L. J. Douglas. 2000. Matrix polymers of *Candida* biofilms and their possible role in biofilm resistance to antifungal agents. *J. Antimicrob. Chemother.* **46**:397–403.

Brooun, A., Liu, S., and K. Lewis. 2000. A dose-response study of antibiotic resistance in *Pseudomonas aeruginosa* biofilms. *Antimicrob. Agents Chemother.* **44**: 640–646.

Budhani, R. K., and J. K. Struthers. 1998. Interaction of *Streptococcus pneumoniae* and *Moraxella catarrhalis*: investigation of the indirect pathogenic role of a beta-lactamase-producing Moraxelae by use of a continuous-culture biofilm system. *Antimicrob. Agents Chemother.* **42**:2521–2526.

Catalan, A., R. Herrera, and A. Martinez. 1987. Denture plaque and palatal mucosa in denture stomatitis: scanning electron microscopic and microbiologic study. *J. Prosthet. Dent.* **57**:581–586.

Chandra, J., D. M. Kuhn, P. K. Mukherjee, L. L. Hoyer, T. McCormick, and M. A. Ghannoum. 2001a. Biofilm formation by the fungal pathogen *Candida albicans*: development, architecture and drug resistance. *J. Bacteriol.* **183**: 5385–5394.

Chandra, J., P. K. Mukherjee, S. D. Leidich, F. F. Faddoul, L. L. Hoyer, L. J. Douglas, and M. A. Ghannoum. 2001b. Antifungal resistance of candidal biofilms formed on denture acrylic in vitro. *J. Dent. Res.* **80**:903–908.

Chaudhary, P. M., and I. B. Roninson. 1991. Expression and activity of P-glycoprotein, a multidrug efflux pump, in human hematopoietic stem cells. *Cell* **66**:85–94.

Clark, F. S., T. Parkinson, C. A. Hitchcock, and N. A. Gow. 1996. Correlation between rhodamine 123 accumulation and azole sensitivity in *Candida* species: possible role for drug efflux in drug resistance. *Antimicrob. Agents Chemother.* **40**: 419–425.

Cochran, W. L., S.-J. Suh, G. A. McFeters, and P. S. Stewart. 2000. Role of RpoS and AlgT in *Pseudomonas aeruginosa* biofilm resistance to hydrogen peroxide and monochloramine. *J. Appl. Microbiol.* **88**:546–553.

Darouiche, R. O., A. Dhir, A. J. Miller, G. C. Landon, I. I. Raad, and D. M. Musher. 1994. Vancomycin penetration into biofilm covering infected prostheses and effect on bacteria. *J. Infect. Dis.* **170**:720–723.

Davies, D. G., M. R. Parsek, J. P. Pearson, B. H. Iglewski, J. W. Costerton, and E. P. Greenberg. 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science* **280**:295–298.

de Beer, D., R. Srinivasan, and P. S. Stewart. 1994a. Direct measurement of chlorine penetration into biofilms during disinfection. *Appl. Environ. Microbiol.* **60**:4339–4344.

de Beer, D., P. Stoodley, F. Roe, and Z. Lewandowski. 1994b. Effects of biofilm structure on oxygen distribution and mass transport. *Biotechnol. Bioeng.* **43**:1131–1138.

De Kievit, T. R., M. D. Parkins, R. J. Gillis, R. Srikumar, H. Ceri, K. Poole, B. H. Iglewski, and D. G. Storey. 2001. Multidrug efflux pumps: Expression patterns and contribution to antibiotic resistance in *Pseudomonas aeruginosa* biofilms. *Antimicrob. Agents Chemother.* **45**:1761–1770.

Douglas, L. J. 2003. Candida biofilms and their role in infection. *Trends Microbiol.* **11**:30–36.

Drenkard, E., and F. M. Ausubel. 2002. Pseudomonas biofilm formation and antibiotic resistance are linked to phenotypic variation. *Nature* **416**:740–742.

Dunne, W. M., Jr., E. O. Mason, Jr., and S. L. Kaplan. 1993. Diffusion of rifampin and vancomycin through a *Staphylococcus epidermidis* biofilm. *Antimicrob. Agents Chemother.* **37**:2522–2526.

Elkins, J. G., D. J. Hassett, P. S. Stewart, H. P. Schweizer, and T. R. McDermott. 1999. Protective role of catalase in *Pseudomonas aeruginosa* biofilm resistance to hydrogen peroxide. *Appl. Environ. Microbiol.* **65**:4594–4600.

Elvers, K. T., K. Leeming, and H. M. Lappin-Scott. 2002. Binary and mixed population biofilms: time-lapse image analysis and disinfection with biocides. *J. Ind. Microbiol. Biotechnol.* **29**:331–338.

Ghannoum, M. A., N. M. Moussa, P. Whittaker, I. Swairjo, and K. H. Abu-Elteen. 1992. Subinhibitory concentration of octenidine and pirtenidine: influence on the lipid and sterol contents of *Candida albicans*. *Chemotherapy* **38**: 46–56.

Ghannoum, M. A., and L. B. Rice. 1999. Antifungal agents: mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. *Clin. Microbiol. Rev.* **12**: 501–517.

Gilbert, P., J. R. Das, M. V. Jones, and D. G. Allison. 2001. Assessment of resistance towards biocides following the attachment of micro-organisms to, and growth on, surfaces. *J. Appl. Microbiol.* **91**:248–254.

Gordon, C. A., N. A. Hodges, and C. Marriott. 1988. Antibiotic interaction and diffusion through

alginate and exopolysaccharide of cystic fibrosis-derived *Pseudomonas aeruginosa*. *J. Antimicrob. Chemother.* 22:667–674.

Grobe, K. J., J. Zahller, and P. S. Stewart. 2002. Role of dose concentration in biocide efficacy against *Pseudomonas aeruginosa* biofilms. *J. Ind. Microbiol. Biotechnol.* 29:10–15.

Hassett, D. J., J.-F. Ma, J. G. Elkins, T. R. McDermott, U. A. Ochsner, S. E. H. West, C.-T. Huang, J. Fredericks, S. Burnett, P. S. Stewart, G. A. McFeters, L. Passador, and B. H. Iglewski. 1999. Quorum sensing in *Pseudomonas aeruginosa* controls expression of catalase and superoxide dismutase genes and mediates biofilm susceptibility to hydrogen peroxide. *Mol. Microbiol.* 34:1082–1093.

Hawser, S. P., G. S. Baillie, and L. J. Douglas. 1998. Production of extracellular matrix by *Candida albicans* biofilms. *J. Med. Microbiol.* 47:253–256.

Hawser, S. P., and L. J. Douglas. 1995. Resistance of *Candida albicans* biofilms to antifungal agents in vitro. *Antimicrob. Agents Chemother.* 39:2128–2131.

Hentzer, M., G. M. Teitzel, G. J. Balzer, A. Heydorn, S. Molin, M. Givskov, and M. R. Parsek. 2001. Alginate overproduction affects *Pseudomonas aeruginosa* biofilm structure and function. *J. Bacteriol.* 183:5395–5401.

Heydorn, A., B. K. Ersboll, M. Hentzer, M. R. Parsek, M. Givskov, and S. Molin. 2000. Experimental reproducibility in flow-chamber biofilms. *Microbiology* 146:2409–2415.

Hitchcock, C. A., N. J. Russell, and K. J. Barrett-Bee. 1987. Sterols in *Candida albicans* mutants resistant to polyene or azole antifungals, and of a double mutant *C. albicans* 6.4. *Crit. Rev. Microbiol.* 15:111–115.

Hodgson, A. E., S. M. Nelson, M. R. W. Brown, and P. Gilbert. 1995. A simple in vitro model for growth control of bacterial biofilms. *J. Appl. Bacteriol.* 79:87–93.

Ibrahim, A. S., and M. A. Ghannoum. 1996. Chromatographic analysis of lipids, p. 52–79. *In* R. Prasad (ed.), *Manual on Membrane Lipids*. Springer-Verlag, New York, N.Y.

Kuhn, D. M., T. George, J. Chandra, P. K. Mukherjee, and M. A. Ghannoum. 2002. Antifungal susceptibility of *Candida* biofilms: unique efficacy of amphotericin B lipid formulations and echinocandins. *Antimicrob. Agents Chemother.* 46:1773–1780.

Kumon, H., K. Tomochika, T. Matunaga, M. Ogawa, and H. Ohmori. 1994. A sandwich cup method for the penetration assay of antimicrobial agents through *Pseudomonas* exopolysaccharides. *Microbiol. Immunol.* 38:615–619.

Leriche, V., and B. Carpentier. 1995. Viable but nonculturable *Salmonella typhimurium* in single- and binary-species biofilms in response to chlorine treatment. *J. Food Prot.* 58:1186–1191.

Lindsay, D., V. S. Brozel, J. F. Mostert, and A. Von Holy. 2002. Differential efficacy of a chlorine dioxide-containing sanitizer agasint single species and binary biofilms of a dairy-associated *Bacillus cereus* and a *Pseudomonas fluorescens* isolate. *J. Appl. Microbiol.* 92:352–361.

Liu, X., F. Roe, A. Jesaitis, and Z. Lewandowski. 1998. Resistance of biofilms to the catalase inhibitor 3-amino-1,2,4-triazole. *Biotechnol. Bioeng.* 59:156–162.

Maira-Litran, T., D. G. Allison, and P. Gilbert. 2000a. An evaluation of the potential of the multiple antibiotic resistance operon (*mar*) and the multidrug efflux pump *acrAB* to moderate resistance towards ciprofloxacin in *Escherichia coli* biofilms. *J. Antimicrob. Chemother.* 45:789–795.

Maira-Litran, T., D. G. Allison, and P. Gilbert. 2000b. Expression of the multiple antibiotic resistance operon (*mar*) during growth of *Escherichia coli* as a biofilm. *J. Appl. Microbiol.* 88:243–247.

Mukherjee, P. K., J. Chandra, D. M. Kuhn, and M. A. Ghannoum. 2003. Mechanism of drug resistance in *Candida albicans* biofilms: phase-specific role of efflux pumps and membrane sterols. *Infect. Immun.* 71:4333–4340.

National Committee for Clinical Laboratory Standards. 1997. Reference method for broth dilution antifungal susceptibility testing of yeasts. M-27A. National Committee for Clinical Laboratory Standards, Villanova, Pa.

Neu, T. R., G. J. Verkerke, I. F. Herrmann, H. K. Schutte, H. C. van der Mei, and H. J. Busscher. 1994. Microflora on explanted silicone rubber voice prostheses: taxonomy, hydrophobicity and electrophoretic mobility. *J. Appl. Bacteriol.* 76:521–528.

Nichols, W. W., S. M. Dorrington, M. P. E. Slack, and H. L. Walmsley. 1988. Inhibition of tobramycin diffusion by binding to alginate. *Antimicrob. Agents Chemother.* 32:518–523.

Parkins, M. D., H. Ceri, and D. G. Storey. 2001. *Pseudomonas aeruginosa* GacA, a factor in multihost virulence, is also essential for biofilm formation. *Mol. Microbiol.* 40:1215–1226.

Radford, D. R., and J. R. Radford. 1993. A SEM study of denture plaque and oral mucosa of denture-related stomatitis. *J. Dent.* 21:87–93.

Ramage, G., S. Bachmann, T. F. Patterson, B. L. Wickes, and J. L. Lopez-Ribot. 2002. Investigation of multidrug efflux pumps in relation to fluconazole resistance in *Candida albicans* biofilms. *J. Antimicrob. Chemother.* 49:973–980.

Sanati, H., P. Belanger, R. Fratti, and M. Ghannoum. 1997. A new triazole, voriconazole (UK-109,496), blocks sterol biosynthesis in *Can-*

dida albicans and *Candida krusei*. *Antimicrob. Agents Chemother.* 41:2492–2496.

Sanderson, S. S., and P. S. Stewart. 1997. Evidence of bacterial adaptation to monochloramine in *Pseudomonas aeruginosa* biofilms and evaluation of biocide action model. *Biotechnol. Bioeng.* 56: 201–209.

Shigeta, M., G. Tanaka, H. Komatsuzawa, M. Sugai, H. Suginaka, and T. Usui. 1997. Permeation of antimicrobial agents through *Pseudomonas aeruginosa* biofilms: a simple method. *Chemotherapy* 43:340–345.

Shih, P.-C., and C.-T. Huang. 2002. Effects of quorum-sensing deficiency on *Pseudomonas aeruginosa* biofilm formation and antibiotic resistance. *J. Antimicrob. Chemother.* 49:309–314.

Spoering, A. L., and K. Lewis. 2001. Biofilms and planktonic cells of *Pseudomonas aeruginosa* have similar resistance to killing by antimicrobials. *J. Bacteriol.* 183:6746–6751.

Srinivasan, R., P. S. Stewart, T. Griebe, C.-I. Chen, and X. Xu. 1995. Biofilm parameters influencing biocide efficacy. *Biotechnol. Bioeng.* 46: 553–560.

Sternberg C., B. B. Christensen, T. Johansen, A. T. Nielsen, J. B. Andersen, M. Givskov, and S. Molin. 1999. Distribution of bacterial growth activity in flow-chamber biofilms. *Appl. Environ. Microbiol.* 65:4108–4117.

Stewart, P. S. 2003. Diffusion in biofilms. *J. Bacteriol.* 185:1485–1491.

Stewart, P. S., J. Rayner, F. Roe, and W. M. Rees. 2001 Biofilm penetration and disinfection efficacy of alkaline hypochlorite and chlorosulfamates. *J. Appl. Microbiol.* 91:525–532.

Stewart, P. S., F. Roe, J. Rayner, J. G. Elkins, Z. Lewandowski, U. A. Ochsner, and D. J. Hassett. 2000. Effect of catalase on hydrogen peroxide penetration into *Pseudomonas aeruginosa* biofilms. *Appl. Environ. Microbiol.* 66:836–838.

Stone, G., P. Wood, L. Dixon, M. Keyhan, and A. Matin. 2002. Tetracycline rapidly reaches all the constituent cells of uropathogenic *Escherichia coli* biofilms. *Antimicrob. Agents Chemother.* 46: 2458–2461.

Suci, P., M. W. Mittelman, F. P. Yu, and G. G. Geesey. 1994. Investigation of ciprofloxacin penetration into *Pseudomonas aeruginosa* biofilms. *Antimicrob. Agents Chemother.* 38:2125–2133.

Vandenheuvel, F. A., and A. S. Court. 1968. Reference high-efficiency nonpolar packed columns for the gas-liquid chromatography of nanogram amounts of steroids. I. Retention time data. *J. Chromatogr.* 38:439–459.

Walters, M. C., F. Roe, A. Bugnicourt, M. J. Franklin, and P. S. Stewart. 2003. Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to the tolerance of *Pseudomonas aeruginosa* biofilms to ciprofloxacin and tobramycin. *Antimicrob. Agents Chemother.* 47:317–323.

Wentland, E. J., P. S. Stewart, C.-T. Huang, and G. A. McFeters. 1996. Spatial variations in growth rate within *Klebsiella pneumoniae* colonies and biofilm. *Biotechnol. Prog.* 12:316–321.

Whiteley, M., M. G. Bangera, R. E. Bumgarner, M. R. Parsek, G. M. Teitzel, S. Lory, and E. P. Greenberg. 2001a. Gene expression in *Pseudomonas aeruginosa* biofilms. *Nature* 413:860–864.

Whiteley, M., J. R. Ott, E. A. Weaver, and R. J. C. McLean. 2001b. Effects of community composition and growth rate on aquifer biofilm bacteria and their susceptibility to betadine disinfection. *Environ. Microbiol.* 3:43–52.

Xu, K. D., M. J. Franklin, C.-H. Park, G. A. McFeters, and P. S. Stewart. 2001. Gene expression and protein levels of the stationary phase sigma factor, RpoS, in continuously-fed *Pseudomonas aeruginosa* biofilms. *FEMS Microbiol. Lett.* 199:67–71.

Xu, K. D., P. S. Stewart, F. Xia, C.-T. Huang, and G. A. McFeters. 1998. Spatial physiological heterogeneity in *Pseudomonas aeruginosa* biofilm is determined by oxygen availability. *Appl. Environ. Microbiol.* 64:4035–4039.

Zhang, T. C., Y.-C. Fu, and P. L. Bishop. 1995. Competition for substrate and space in biofilms. *Water Environ. Res.* 67:992–1003.

Zheng, Z., and P. S. Stewart. 2002. Penetration of rifampin through *Staphylococcus epidermidis* biofilms. *Antimicrob. Agents Chemother.* 46:900–903.