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Mechanisms of antibiotic resistance in bacterial biofilms

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Abstract

Bacteria that attach to a surface and grow as a biofilm are protected from killing by antibiotics. Reduced antibiotic susceptibility contributes to the persistence of biofilm infections such as those associated with implanted devices. The protective mechanisms at work in biofilms appear to be distinct from those that are responsible for conventional antibiotic resistance. In biofilms, poor antibiotic penetration, nutrient limitation and slow growth, adaptive stress responses, and formation of persister cells are hypothesized to constitute a multi-layered defense. The genetic and biochemical details of these biofilm defenses are only now beginning to emerge. Each gene and gene product contributing to this resistance may be a target for the development of new chemotherapeutic agents. Disabling biofilm resistance may enhance the ability of existing antibiotics to clear infections involving biofilms that are refractory to current treatments.

Key words: biofilm – penetration – growth rate – stress response – persister

Introduction

When bacteria attach to a surface and grow as a biofilm they are protected from killing by antibiotics, biocides, and other chemical or physical challenges (Lewis, 2001; Mah and O'Toole, 2001; Stewart and Costerton, 2001). This phenomenon is increasingly recognized as a key factor in the persistence of varied infections (Costerton et al., 1999). Chronic infections in which biofilms have been demonstrated to be involved include periodontitis, cystic fibrosis pneumonia, and numerous infections associated with indwelling devices such as catheters, heart valves, and prostheses.

Antibiotic resistance of bacteria in biofilms is easily reproduced in vitro, showing that host factors are not required for this manifestation of biofilm defense. While biofilms of the common opportunistic pathogens *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* are well known for their antibiotic resistance, diverse other microorganisms form biofilms which confer protection against many antibiotics. For example, when grown in biofilms, the yeast *Candida albicans* (Hawser and Douglas, 1994) and the obligate anaerobe *Porphyromonas gingivalis* (Hansen et al., 2000) have been shown to be less susceptible to antibiotics than free-floating cells. Many different types of antibiotics have been tested against bacterial biofilms, and in almost every case bacteria in the biofilm mode of growth are found to be less sensitive to killing than the same strain when grown in free aqueous suspension. These observations tell us that the capacity to form protective biofilms is widely distributed among microbes and that the resistance mechanisms that operate in biofilms constitute a broad spectrum defense.

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Biofilm resistance mechanisms

A great deal is known about the genetic and molecular basis of antibiotic resistance in bacteria. These now familiar protective mechanisms – target mutations, low cell permeability, efflux pumps, and modifying enzymes – do not, however, appear to be at the root of the reduced antimicrobial susceptibility in biofilms. For example, bacteria that lack protective mutations or that lack plasmids or other mobile genetic elements carrying resistance genes, nevertheless become less susceptible when grown in the biofilm state (Anderl et al., 2000). Antibiotic sensitivity is usually quickly restored when bacteria are dispersed from a biofilm. The rapid reversal of resistance upon dispersion from a biofilm suggests an adaptive resistance mechanism rather than a genetic alteration.

There is an interesting, and relatively unexplored intersection between conventional antibiotic resistance and biofilm reduced susceptibility. The natural protection afforded by a biofilm may provide a breeding ground for spontaneous mutants. Further, the close spatial proximity of bacterial cells within a biofilm has been speculated to accelerate plasmid transfer (Hausner and Wuertz, 1999). Does the biofilm mode of growth facilitate the emergence of resistant mutants or speed the process of gene transfer? Investigations addressing these questions are just beginning to appear.
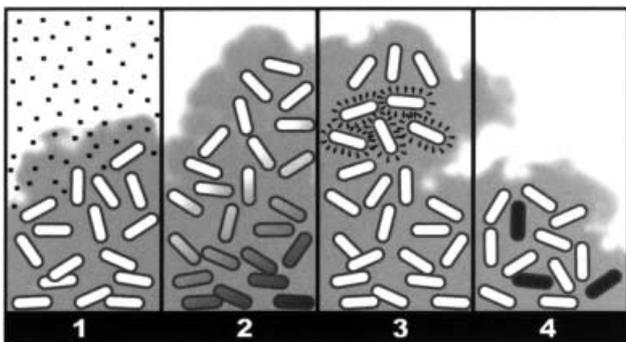
In the following sections of this article, I review the principal hypotheses addressing reduced antibiotic susceptibility of bacteria in biofilms (Figure 1). Though the genetic and molecular basis of each of these mechanisms are still largely unexplored, I offer

Fig. 1. Four hypothesized biofilm resistance mechanisms. *1* – The antibiotic (squares) penetrates slowly or incompletely; *2* – a concentration gradient of a metabolic substrate or product leads to zones of slow or non-growing bacteria (shaded cells); *3* – an adaptive stress response is expressed by some of the cells (marked cells); *4* – a small fraction of the cells differentiate into a highly protected persister state (dark cells).

some speculation about the types of genes or molecules that might be involved given these hypothetical mechanisms.

Antibiotic penetration of biofilms

Biofilms are mostly water, and solutes the size of antibiotics diffuse readily in the biofilm matrix. Measurements in biofilms of effective diffusion coefficients of solutes, with molecular weights of 100 to 1000, average about 40 percent of the respective diffusion coefficient in pure water (Stewart, 1998). Consistent with this are measurements of antibiotic diffusion coefficients in glycoprotein or polysaccharide gels, which range from 36 to 76 percent of their values in pure water (Cheema et al., 1986; Gordon et al., 1988). The reduction in antibiotic mobility by a factor of perhaps two to three in biofilms is insufficient to explain the level of resistance of aggregated bacteria to killing. However, the physical mobility of antibiotics in biofilm does not ensure that the antibiotic will penetrate the biofilm. If the antibiotic is inactivated by reaction, or sequestered by binding as it diffuses into the biofilm, its delivery to the depths of the film can be profoundly retarded. Such a reaction-diffusion interaction is sufficient to prevent a penicillin antibiotic from penetrating a biofilm formed by a beta-lactamase-positive bacterium (Anderl et al., 2000). In this same study, ampicillin was shown to readily penetrate a biofilm formed by a beta-lactamase-negative mutant. There is some evidence suggesting that binding of the positively charged aminoglycosides to negatively charged biofilm matrix polymers retards penetration of these agents (Gordon et al., 1988; Nichols et al., 1988; Kumon et al., 1994). In most cases, however, no sufficiently rapid neutralization or sorption of the antibiotic occurs, and antibiotics have been shown to penetrate biofilms in experimental test systems in vitro (Stewart, 1996) (Table 1). Significant limitations to biofilm penetration have only been reported for beta-lactams and aminoglycosides, and such limitation has not been found in every study (Table 1).

If an antibiotic slowly penetrates the biofilm, then enzymes that inactivate or modify the antibiotic are critical, otherwise saturation is eventually reached unless the biofilm is completely impervious to the agent. Such enzymes include beta-lactamases, aminoglycoside-modifying enzymes, or chloramphenicol acetyltransferases, for example. In most of the examples in which effective antibiotic penetration has been demonstrated experimentally, the investi-

Table 1. Experimental measurements of antibiotic penetration into biofilms. The criterion for penetration was attainment of 30% of the applied antibiotic concentration (or 30% of the antibiotic concentration determined in a sterile control) during the test duration.

Antibiotic	Microorganism	Penetration?	Reference
Piperacillin	*P. aeruginosa*	No	Hoyle et al., 1992
Rifampin	*S. epidermidis*	Yes	Dunne et al., 1993
Vancomycin		Yes	
Gentamicin	*P. aeruginosa*	No	Yasuda et al., 1993
Ofloxacin		Yes	
Vancomycin	*S. epidermidis*	Yes	Darouiche et al., 1994
Latamoxef	*E. coli*	Yes	Jouenne et al., 1994
Ciprofloxacin	*P. aeruginosa*	Yes	Suci et al., 1994; Vrany et al., 1997
Levofloxacin		Yes	
Ofloxacin	*S. epidermidis*	Yes	Yasuda et al., 1994
Cefotiam		Yes	
Amikacin	*P. aeruginosa*	No	Shigeta et al., 1997
Ciprofloxacin		Yes	
Gentamicin		No	
Imipenem		Yes	
Levofloxacin		Yes	
Ofloxacin		Yes	
Piperacillin		Yes	
Sparfloxacin		Yes	
Ampicillin	*K. pneumoniae*	No	Anderl et al., 2000
Ciprofloxacin		Yes	
Ciprofloxacin	*P. aeruginosa*	Yes	Unpublished data of Walters and Stewart
Tobramycin		No	
Rifampin	*S. epidermidis*	Yes	Zheng and Stewart, 2002

gators report survival of the test microorganism (Dunne et al., 1993; Darouiche et al., 1994; Anderl et al., 2000). This proves that protective mechanisms other than penetration failure must be at work in biofilms.

Altered microenvironment and slow growth

Killing by many antibiotics is growth-dependent (Gilbert and Brown, 1995). Penicillins, for example, only kill growing bacteria (Tuomanen et al., 1986). Since most antibiotics target some type of macromolecular synthesis, these agents would not be expected to have much effect on bacteria in which macromolecular synthesis is arrested. Over the past decade, researchers have begun to directly visualize patterns of bacterial growth and activity in biofilms using fluorescent probes and reporter genes (Wentland et al., 1996; Xu et al., 1998; Sternberg et al., 1999). It is now clear that within biofilms, microgradients occur in the concentration of key metabolic substrates and products (Wimpenny and Kinniment, 1995). Because of these chemical gradients, biofilms include slow-growing or stationary-phase cells (Molin et al., 2000). Even in single-species biofilms, the bacterial population is hetero-

geneous with respect to growth states, which cover the spectrum from rapidly growing to metabolically inactive. Bacteria in non-growing zones of a biofilm are uniquely well positioned to survive antimicrobial challenge (Brown et al., 1988; Gilbert and Brown, 1995), and are less susceptible than a biofilm in which all of the bacteria grow at a uniform intermediate rate (Xu et al., 2000).

Factors other than slow growth may contribute to antibiotic resistance in biofilms. The same chemical gradients that lead to growth limitation in biofilms may be sufficient in themselves to alter antibiotic potency. For example, oxygen availability alone is known to modulate action of the aminoglycosides (Tack and Sabath, 1985). Bacteria in an anaerobic region of a biofilm may be differentially protected from these antibiotics, even if they are capable of fermentative growth. Gradients in pH may similarly impact antibiotic efficacy negatively (Venglarcik et al., 1983; Retsema et al., 1991).

If reduced antibiotic susceptibility in biofilms depends on metabolically inactive or slow-growing bacteria, then genes, the products of which are involved in switching bacterial metabolism pathways, or in the pathways themselves, would be predicted to be essential for the biofilm defense. These may include genes required for the formation of multicellular structures. The establishment of nutrient-limited zones in biofilms depends on cell

aggregates reaching a certain critical dimension. Three *P. aeruginosa* mutants have been described with reduced susceptibility to antibiotics. The first mutant overproduces the extracellular polymer alginate, which makes biofilms thicker than the wild type (Hentzer et al., 2001). Biofilms formed by this mucoid mutant are less susceptible to tobramycin than the wild type. The second mutant is affected in the stationary-phase sigma factor *rpoS*. This mutant also makes biofilms that are thicker than those formed by the wild-type strain (Heydorn et al., 2000), and these biofilms are also less sensitive to tobramycin (Whiteley et al., 2001). The third mutant has a lesion in *gacA*, part of a two-component regulatory system required for normal biofilm development (Parkins et al., 2001). Biofilms of the *gacA* mutant fail to form mature structures and are slightly more susceptible to several antibiotics.

Adaptive responses

Bacteria are equipped with a host of stress responses that allow them to cope with environmental fluctuations, such as abrupt temperature changes, oxidative stress, low water activity, DNA damage, starvation, and others. Many of these stress responses have been characterized in molecular and genetic detail using planktonic bacteria (Matin, 1991; Poolman and Glaasker, 1998; Storz and Imlay, 1999). These protective responses may be deployed in biofilms. RpoS, a sigma factor expressed in Gram-negative bacteria as they enter stationary phase, has been detected in continuously-fed biofilms of *P. aeruginosa* (Xu et al., 2001). The *rpoS* transcript has also been detected in the sputa of cystic fibrosis patients (Foley et al., 1999). Studies of antimicrobial susceptibility of biofilms formed by *rpoS* mutants fail to support any role for this gene in protecting biofilms (Cochran et al., 2000b; Whiteley et al., 2001). The constitutive expression of multi-drug efflux pumps in biofilms may contribute to resistance. So far there is no evidence for elevated expression of efflux pumps in biofilms prior to antibiotic challenge (Maira-Litran, 2000a, b; De Kievet et al., 2001). However, using DNA microarrays, it has recently been reported that biofilms of *P. aeruginosa* challenged with tobramycin were able to transcribe the gene for an efflux pump (Whiteley et al., 2001).

Stress responses may be induced in biofilm bacteria by environmental challenge, just as they are in suspended bacteria. The difference between a free-floating and biofilm-embedded cell may be that biofilm cells are afforded a greater opportunity to express these traits as the result of retarded antibiotic penetration or slow growth, enabling biofilm cells to respond to an antimicrobial challenge that overwhelms planktonic cells. For example, *P. aeruginosa* in a biofilm are able to activate *katB*, an inducible catalase gene, in response to treatment with 50 mM hydrogen peroxide (Elkins et al., 1999). Peroxide treatment of the same strain of bacteria in the planktonic state resulted in no catalase expression, presumably because free-floating cells were overwhelmed by the antimicrobial effects of the hydrogen peroxide before the stress response could be activated. A similar mechanism may be behind the induction of beta-lactamase activity in *P. aeruginosa* biofilms (Bagge et al., 2000).

Persisters

Bacteria in biofilms not only evade killing by antibiotics, they also resist chemical disinfectants, such as chlorine bleach and glutaraldehyde. The presence of a subpopulation of persisters in the biofilm may account for the observed broad resistance. Persisters may constitute a relatively small fraction of the population, but these few cells have entered a highly protected, perhaps spore-like, state (Lewis, 2001; Stewart and Costerton, 2001). The difference between planktonic and biofilm communities is that the frequency of persisters is much higher in the biofilm population.

Data in support of the persister hypothesis include measurements of biphasic biofilm killing in which most of the population is rapidly killed but a fraction of the cells are unaffected even by prolonged antibiotic treatment (Goto et al., 1999; Brooun et al., 2000). The fact that bacteria can develop reduced susceptibility even in very thin biofilms can be explained by persisters but not alternative hypotheses (Das et al., 1998; Cochran et al., 2000a). Genes that contribute to the persister state may include those encoding regulatory circuits that determine the entry and exit from this state as well as specific protective responses. Genes for high-level persistence (*hip*) have been described in *E. coli* (see (Lewis, 2001)).

Conclusions

Reduced antibiotic susceptibility of bacteria in biofilms is thought to be due to a combination of

poor antibiotic penetration, an altered microenvironment, adaptive responses, and the presence of bacterial persister cells. However, the genetic and biochemical details of the biofilm defenses are only now beginning to emerge. Each gene and gene product contributing to this resistance may be a target for the development of new chemotherapeutic agents. Disabling biofilm resistance may enhance the ability of existing antibiotics to clear infections involving biofilms that are refractory to current treatments.

References

Anderl, J. N., Franklin, M. J., Stewart, P. S.: Role of antibiotic penetration limitation in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin. Antimicrob. Agents Chemother. 44, 1818–1824 (2000).

Bagge, N., Ciofu, O., Skovgaard, L. T., Høiby, N.: Rapid development in vitro and in vivo of resistance to ceftazidime in biofilm-growing *Pseudomonas aeruginosa* due to chromosomal beta-lactamase. APMIS 108, 589–600 (2000).

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

Brown, M. R. W., Allison, D. G., Gilbert, P.: Resistance of bacterial biofilms to antibiotics: a growth-rate related effect? J. Antimicrob. Chemother. 22, 777–783 (1988).

Cheema, M. S., Rassing, J. E., Marriott, C.: The diffusion characteristics of antibiotics in mucus glycoprotein gels. J. Pharm. Pharmacol., Suppl. 38, 53P (1986).

Cochran, W. L., McFeters, G. A., Stewart, P. S.: Reduced susceptibility of thin *Pseudomonas aeruginosa* biofilms to hydrogen peroxide and monochloramine. J. Appl. Microbiol. 88, 22–30 (2000a).

Cochran, W. L., Suh, S. J., McFeters, G. A., Stewart, P. S.: Role of RpoS and AlgT in *Pseudomonas aeruginosa* biofilm resistance to hydrogen peroxide and monochloramine. J. Appl. Microbiol. 68, 546–553 (2000b).

Costerton, J. W., Stewart, P. S., Greenberg, E. P.: Bacterial biofilms: A common cause of persistent infections. Science 284, 1318–1322 (1999).

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

Das, J. R., Bhakoo, M., Jones, M. V., Gilbert, P.: Changes in the biocide susceptibility of *Staphylococcus epidermidis* and *Escherichia coli* cells associated with rapid attachment to plastic surfaces. J. Appl. Microbiol. 84, 852–858 (1998).

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

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

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

Foley, I., Marsh, P., Wellington, E. M. H., Smith, A. W., Brown, M. R. W.: General stress response master regulator *rpoS* is expressed in human infection: a possible role in chronicity. J. Antimicrob. Chemother. 43, 164–165 (1999).

Gilbert, P., Brown, M. R. W.: Mechanisms of the protection of bacterial biofilms from antimicrobial agents. In: Microbial biofilms (H. M. Lappin-Scott, J. W. Costerton, eds.), pp. 118–130. Cambridge University Press, Cambridge 1995.

Gordon, C. A., Hodges, N. A., Marriott, C.: Antibiotic interaction and diffusion through alginate and exopolysaccharide of cystic fibrosis-derived *Pseudomonas aeruginosa*. J. Antimicrob. Chemother. 22, 667–674 (1988).

Goto, T., Nakame, Y., Nishida, M., Ohi, Y.: In vitro bactericidal activities of beta-lactamases, amikacin, and fluoroquinolones against *Pseudomonas aeruginosa* biofilm in artificial urine. Urology 53, 1058–1062 (1999).

Hansen, M. C., Palmer, R. J., White, D. C.: Flowcell culture of *Porphyromonas gingivalis* biofilms under anaerobic conditions. J. Microbiol. Methods 40, 233–239 (2000).

Hausner, M., Wuertz, S.: High rates of conjugation in bacterial biofilms as determined by quantitative in situ analysis. Appl. Environ. Microbiol. 65, 3710–3713 (1999).

Hawser, S. P., Douglas, L. J.: Biofilm formation by *Candida* species on the surface of catheter materials in vitro. Infect. Immun. 62, 915–921 (1994).

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

Heydorn, A., Ersboll, B. K., Hentzer, M., Parsek, M. R., Givskov, M., Molin, S. Experimental reproducibility

in flow-chamber biofilms. Microbiology 146, 2409 – 2415 (2000).

Hoyle, B. D., Alcantara, J., Costerton, J. W.: *Pseudomonas aeruginosa* biofilm as a diffusion barrier to piperacillin. Antimicrob. Agents Chemother. 36, 2054 – 2056 (1992).

Jouenne, T., Tresse, O., Junter, G. A.: Agar-entrapped bacteria as an in vitro model of biofilms and their susceptibility to antibiotics. FEMS Microbiol. Lett. 119, 237 – 242 (1994).

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

Lewis, K.: Riddle of biofilm resistance. Antimicrob. Agents Chemother. 45, 999 – 1007 (2001).

Mah, T.-F., O'Toole, G. A.: Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol. 9, 34 – 39 (2001).

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

Maira-Litran, T., Allison, D. G., Gilbert, P.: 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 (2000b).

Matin, A.: The molecular basis of carbon-starvation-induced general resistance in *Escherichia coli*. Mol. Microbiol. 5, 3 – 10 (1991).

Molin, S., Nielsen, A. T., Christensen, B. B., Andersen, J. B., Licht, T. R., Tolker-Nielsen, T., Sternberg, C., Hansen, M. C., Ramos, C., Givskov, M.: Molecular ecology of biofilms. In: Biofilms II (J. D Bryers, ed.), pp. 89 – 120. John Wiley and Sons, New York 2000.

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

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

Poolman, B., Glaasker, E.: Regulation of compatible solute accumulation in bacteria. Mol. Microbiol. 29, 397 – 407 (1998).

Retsema, J. A., Brennan, L. A., Girard, A. E.: Effects of environmental factors on the in vitro potency of azithromycin. Eur. J. Clin. Microbiol. 10, 834 – 842 (1991).

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

Sternberg, C., Christensen, B. B., Johansen, T., Nielsen, A. T., Andersen, J. B., Givskov, M., Molin, S.: Distribution of bacterial growth activity in flow-

chamber biofilms. Appl. Environ. Microbiol. 65, 4108 – 4117 (1999).

Stewart, P. S.: Theoretical aspects of antibiotic diffusion into microbial biofilms. Antimicrob. Agents Chemother. 40, 2517 – 2522 (1996).

Stewart, P. S.: A review of experimental measurements of effective diffusive permeabilities and effective diffusion coefficients in biofilms. Biotechnol. Bioeng. 59, 261 – 272 (1998).

Stewart, P. S., Costerton, J. W.: Antibiotic resistance of bacteria in biofilms. Lancet 358, 135 – 138 (2001).

Storz, G., Imlay, J. A.: Oxidative stress. Curr. Opin. Microbiol. 2, 188 – 194 (1999).

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

Tack, K. J., Sabath, L. D.: Increased minimum inhibitory concentrations with anaerobiasis for tobramycin, gentamicin, and amikacin, compared to latamoxef, piperacillin, chloramphenicol, and clindamycin. Chemotherapy 31, 204 – 210 (1985).

Tuomanen, E., Cozens, R., Tosch, W., Zak, O., Tomasz, A.: The rate of killing of *Escherichia coli* by beta-lactam antibiotics is strictly proportional to the rate of bacterial growth. J. Gen. Microbiol. 132, 1297 – 1304 (1986).

Venglarcik, J. S., Blair, L. L., Dunkle, L. M.: pH-dependent oxacillin tolerance of *Staphylococcus aureus*. Antimicrob. Agents Chemother. 23, 232 – 235 (1983).

Vrany, J. D., Stewart, P. S., Suci, P. A.: Comparison of recalcitrance to ciprofloxacin and levofloxacin exhibited by *Pseudomonas aeruginosa* biofilms displaying rapid-transport characteristics. Antimicrob. Agents Chemother. 41, 1352 – 1358 (1997).

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

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

Wimpenny, J. W. T., Kinniment, S. L.: Biochemical reactions and the establishment of gradients within biofilms. In: Microbial biofilms (H. M. Lappin-Scott, J. W. Costerton, eds.), pp. 99 – 117. Cambridge University Press, Cambridge 1995.

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

Xu, K. D., McFeters, G. A., Stewart, P. S.: Biofilm resistance to antimicrobial agents. Microbiology 146, 547 – 549 (2000).

Xu, K. D., Franklin, M. J., Park, C.-H., McFeters, G. A., Stewart, P. S.: Gene expression and protein levels of the stationary phase sigma factor, RpoS, in continu-

ously-fed *Pseudomonas aeruginosa* biofilms. FEMS Microbiol. Lett. 199, 67 – 71 (2001).

Yasuda, H., Ajiki, Y., Koga, T., Kawada, H., Yokota, T.: Interaction between biofilms formed by *Pseudomonas aeruginosa* and clarithromycin. Antimicrob. Agents Chemother. 37, 1749 – 1755 (1993).

Yasuda, H., Ajiki, Y., Koga, T., Yokota, T.: Interaction between clarithromycin and biofilms formed by *Staphylococcus epidermidis*. Antimicrob. Agents Chemother. 38, 138 – 141 (1994).

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