Innate and Induced Resistance Mechanisms of Bacterial Biofilms

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Abstract Bacterial biofilms are highly recalcitrant to antibiotic treatment, which holds serious consequences for therapy of infections that involve biofilms. The genetic mechanisms of this biofilm antibiotic resistance appear to fall into two general classes: innate resistance factors and induced resistance factors. Innate mechanisms are activated as part of the biofilm developmental pathway, the factors being integral parts of biofilm structure and physiology. Innate pathways include decreased diffusion of antibiotics through the biofilm matrix, decreased oxygen and nutrient availability accompanied by altered metabolic activity, formation of persisters, and other specific molecules not fitting into the above groups. Induced resistance factors include those resulting from induction by the antimicrobial agent itself. Biofilm antibiotic resistance is likely manifested as an intricate mixture of innate and induced mechanisms. Many researchers are currently trying to overcome

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this extreme biofilm antibiotic resistance by developing novel therapies aimed at disrupting biofilms and killing the constituent bacteria. These studies have led to the identification of several molecules that effectively disturb biofilm physiology, often by interrupting bacterial quorum sensing. In this manner, manipulation of innate and induced resistance pathways holds much promise for treatment of biofilm infections.

1 Introduction

One of the most confounding aspects of bacterial biofilm formation is the increased resistance of the constituent microbes to antibiotics and other stressors. A biofilm lifestyle affords bacteria a 10- to 1,000-fold increase in antibiotic resistance compared to their planktonic counterparts (Mah and O'Toole 2001). Particularly in the clinic, increased resistance holds serious consequences for infection control, treatment regimes, and disease progression. Biofilms can form on medical implants (Donlan and Costerton 2002), leading to increased morbidity and mortality of affected individuals. Often, removal of the contaminated implant is the only effective treatment. Biofilms that form during specific disease states, such as in the lungs of cystic fibrosis patients, can also be extremely difficult to eliminate (Chernish and Aaron 2003; Gibson et al. 2003; Hoiby et al. 2005).

Despite decades of research, very little is known about the molecular mechanisms of antibiotic resistance in biofilms. Traditional antibiotic resistance (of planktonic bacteria) usually involves inactivation of the antibiotic, modification of targets, and exclusion of the antibiotic (Patel 2005). The actions typically require the acquisition of specific genetic factors, such as genes for β-lactamase or efflux pumps. However, research to date does not support a large role for these mechanisms in biofilm resistance. In this chapter, we define biofilm antibiotic resistance as the ability of biofilm bacteria to survive antibiotic treatment by using its existing complement of genes. This regulation can occur as an innate result of growing in a biofilm or be induced by the antimicrobial agent itself. Indeed, several innate biofilm phenomena and antibiotic-induced factors have been revealed that provide explanations for the ability of bacterial biofilms to survive under antibiotic or other chemical pressures (Costerton et al. 1999; Donlan and Costerton 2002; Dunne 2002; Mah and O'Toole 2001; Patel 2005; Stewart and Costerton 2001). Biofilm antimicrobial resistance is most likely the result of a complex mixture of these innate and induced factors.

In this chapter, we will discuss these innate and induced factors, with particular emphasis on how these pathways influence biofilm antibiotic resistance. First, we will describe innate antibiotic resistance mechanisms of bacterial biofilms: that is, growth in a biofilm resulting in altered genetic regulatory patterns that are an integral part of the biofilm lifestyle. Some of these regulated factors also protect biofilm bacteria from antibiotic killing. Accordingly, we will describe limited

antibiotic diffusion through the biofilm, decreased growth and altered metabolism, and formation of specialized "persisters" as important innate biofilm phenomena that impact antibiotic resistance. Next, we will discuss the evidence for induced resistance factors, or in other words, antibiotic-induced expression of resistance factors. Following this discussion of innate and induced antibiotic resistance factors, we will describe novel therapeutic mechanisms that are being developed to more effectively target biofilm bacteria by disrupting these innate and induced pathways. Finally, we will briefly mention certain industrial applications for which increased biofilm resistance actually benefits the outcome of the application.

2 Innate Mechanisms: Why Wait?

Bacterial biofilm formation, in general, is accompanied by global genetic regulatory changes that occur as planktonic bacteria enter a community lifestyle. Many of these changes render the constituent bacteria resistant to antibiotics. In other words, biofilm antimicrobial resistance, in large part, can be thought of as an innate attribute resulting from conversion to a biofilm lifestyle.

Research has identified the influence of several different innate biofilm factors affecting antibiotic resistance (Costerton et al. 1999; Donlan and Costerton 2002; Dunne 2002; Mah and O'Toole 2001; Patel 2005; Stewart and Costerton 2001). First, the biofilm matrix may act as a diffusion barrier, preventing antibiotics from reaching their targets. Second, establishment of microenvironments within biofilms, such as reduced oxygen zones, leads to slow growth of the bacteria. Third, a small subpopulation of bacteria within the biofilm seems to differentiate into persisters, with greatly reduced susceptibility to antibiotics. Finally, several resistance genes have been identified that are specifically regulated within biofilms. Studies have only recently begun to elucidate the genetic regulation of these innate biofilm antibiotic resistance mechanisms. These molecular details are vital to our understanding of the ability of biofilms to thwart treatment.

2.1 Diffusion Confusion

As antimicrobial agents contact a biofilm, the first obstacle they encounter is the biofilm matrix. Antibiotics must traverse through this thick mixture of exopolysaccharide (EPS), DNA, and protein in order to reach their targets, and it is thought that the matrix acts as a diffusion barrier, limiting access to the biofilm bacteria (Fig. 1) (Donlan and Costerton 2002). A decrease in the levels of antibiotics reaching the bacteria would result in an apparent increase in resistance. Indeed, recent mathematical modeling predicted that while limited antibiotic diffusion may lead to death of the outer layer of bacteria, it provides a chance for a subpopulation of

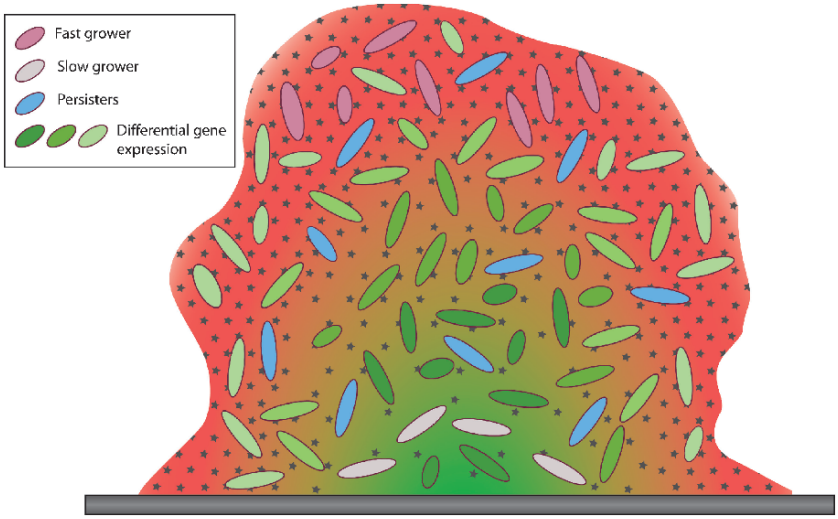

Fig. 1 Innate biofilm antibiotic resistance mechanisms. The single biofilm macrocolony shown here is made up of bacteria (*ovals*) surrounded by an extracellular matrix (*multicolored background*). *Small dark dots* represent the antibiotic molecules to which the biofilm has been exposed. Limited antibiotic diffusion through the matrix (depicted as a decreasing dot density toward the core of the microcolony) might protect bacteria buried deep within the biofilm from antibiotic action. Oxygen and nutrient concentrations also decrease as the deeper parts of the biofilms are approached, symbolized by a color gradient from *red* (aerobic and high nutrient concentrations) to *green* (anaerobic and low nutrient concentrations). These gradients slow the growth of the innermost bacteria (*tan*), and thus facilitate survival in the presence of antibiotic that typically kill only fast growing microorganisms (*magenta*). The *red* to *green* gradient also represents other possible variations within the heterogeneous biofilm, such as pH. Persister cells, also considered nongrowing or slow-growing, are represented by *blue ovals* scattered throughout the biofilm. Finally, the *green ovals* denote biofilm bacteria expressing specific biofilm activated resistance genes, such as *ndvB*. Differential expression of these genes (different shades of *green*) in response to environmental gradients in the community might influence the antibiotic resistance state of individual bacteria within the biofilm

bacteria buried deeper within the biofilm to enact adaptive changes to counter the insult (Szomolay et al. 2005). However, limited antibiotic diffusion does not appear to be a universal trait shared by all biofilms, and, as detailed below, the data conflict over whether the biofilm matrix is a major contributing factor influencing biofilm resistance (Patel 2005).

2.1.1 Antibiotic Trapping

A few studies have found that the biofilm matrix can limit penetration of antimicrobials. Alginate, an EPS produced by *Pseudomonas aeruginosa*, has been intensely studied for its ability to trap antimicrobial agents. This ability appears to be related

to the anionic nature of the exopolymer. Cationic molecules can thus be retained within the matrix and prevented from acting upon the biofilm bacteria. In one study, alginate solutions inhibited disruption of membrane vesicles by cationic antimicrobial peptides, which can spontaneously insert into membranes (Chan et al. 2005). Additionally, incubation of these peptides with alginate led to dissolution of secondary structure and aggregation of the peptides. Alginate has also been shown to afford protection from cationic quaternary ammonium compounds, acting as a hydrophobic shield that decreases activity of these biocides (Campanac et al. 2002). Further, alginate can bind positively charged antibiotics, such as aminoglycosides, and inhibit their activity. Tobramycin, for instance, binds quite well to alginate (Nichols et al. 1988). A recent study found that diffusion of tobramycin through colony biofilms of *P. aeruginosa* was severely delayed; however, antibiotic eventually penetrated through to the distal regions of the biofilm in sufficient concentrations to kill resident microorganisms (Walters et al. 2003).

Anionic extracellular polymeric substances can also bind and sequester toxic cationic heavy metals. Metal chelation has been demonstrated for secreted polymeric molecules from a number of different microorganisms, including *Bacillus licheniformis*, *Xanthomonas campestris*, and freshwater-sediment bacteria (Kaplan et al. 1987; McLean et al. 1990; Mittelman and Geesey 1985; Teitzel and Parsek 2003). Thus, adsorption of positively charged antimicrobial agents by anionic matrix components appears to be an effective survival tool employed by certain bacteria.

2.1.2 Stimulation of EPS by Antibiotics

Intriguingly, a few antibiotics can actually stimulate EPS production. For instance, subinhibitory concentrations of tetracycline, quinupristin-dalfopristin, and erythromycin activated expression of genes encoding for polysaccharide intercellular adhesin in *Staphylococcus epidermidis*, as determined by β-galactosidase transcriptional fusions to the *ica* operon promoter (Rachid et al. 2000). Polysaccharide intercellular adhesin is vital for *S. epidermidis* biofilm formation, and this antibiotic effect corresponded with an increase in biofilm formation on polystyrene microtiter plates. Similarly, subinhibitory concentrations of various β-lactam antibiotics stimulated β-galactosidase expression from a *cps-lacZ* transcriptional fusion in *Escherichia coli* (Sailer et al. 2003). The *cps* genes in *E. coli* encode for enzymes in the production pathway for colanic acid, an EPS important for biofilm formation. In *P. aeruginosa* biofilms, alginate expression was highly upregulated by subinhibitory imipenem treatment (Bagge et al. 2004b). Hoffman et al. have also found that subinhibitory aminoglycoside concentrations enhance biofilm formation in *P. aeruginosa* and *E. coli*, although this effect appears to act by increasing bacterial biomass rather than stimulating matrix formation (Hoffman et al. 2005). These researchers further identified a gene in *P. aeruginosa* (which they named *arr* for aminoglycoside response regulator) that appears to mediate this effect by modulating cyclic-di-GMP levels within the cell. Thus, subinhibitory antibiotic concentrations seem to enhance biofilm formation in certain cases. It is intriguing to speculate that limited antibiotic

diffusion through the biofilm matrix, coupled with a corresponding decrease in antimicrobial concentration, might actually stimulate biofilm formation in some instances by creating a positive feedback loop.

2.1.3 Free Diffusion

However, a large number of studies have shown that many antibiotics can freely diffuse through biofilms. In the case of *Klebsiella pneumoniae* colony biofilms, penetration of ampicillin was severely abrogated (Anderl et al. 2000). However, ampicillin could freely diffuse through β-lactamase-deficient *K. pneumoniae* colony biofilms, demonstrating that the matrix per se does not inhibit ampicillin diffusion, but that β-lactamase secreted by the bacteria inactivated the antibiotic (Anderl et al. 2000). Ciprofloxacin also exhibited unrestrained diffusion through these biofilms, and both ciprofloxacin and ampicillin could reach distal surfaces of biofilms and kill bacteria in these locations (Anderl et al. 2000, 2003; Zahller and Stewart 2002). Likewise, ciprofloxacin diffused relatively uninhibited through *P. aeruginosa* colony biofilms (Walters et al. 2003), rifampin easily penetrated *S. epidermidis* colony biofilms (Zheng and Stewart 2002), and tetracycline reached every bacterial cell within flow-cell grown *E. coli* biofilms (Stone et al. 2002). In most of these cases, the edges of the biofilms experienced small reductions in bacterial numbers, but the presence of antibiotic throughout the community did not drastically impact viability. Thus, while decreased penetration and diffusion of antimicrobials through the biofilm matrix might influence biofilm survival in some cases, this mechanism appears to be far from universal. Additional mechanisms must exist to account for increased biofilm antibiotic resistance.

2.2 Limited Growth Potential

While disagreement remains about the efficacy of the biofilm matrix as a diffusion barrier to antibiotics, altered microenvironments within the biofilm clearly play a role in antibiotic protection. Oxygen limitation in particular has been extensively investigated, and numerous studies have revealed the presence of hypoxic zones deep within biofilms. A recent microarray study of *E. coli* biofilms found an upregulation of the *cydAB* and b2997-*hybABC* gene clusters, which are known to be transcribed in oxygen-limiting conditions (Schembri et al. 2003). Similarly, nutrient diffusion through biofilms is restricted. Oxygen and nutrient deprivation consequently result in a decrease in bacterial metabolic activity and cessation of bacterial growth (Donlan and Costerton 2002; Dunne 2002). Indeed, experimental measurements have revealed a severe reduction in bacterial growth rates within biofilms compared to planktonic cultures (Anderl et al. 2003; Borriello et al. 2004). Even in planktonic cultures of *P. aeruginosa* and *K. pneumoniae*, deprivation of oxygen or nutrients, respectively, has resulted in slow growth and antibiotic resistance

(Anderl et al. 2003; Field et al. 2005). Because antibiotics typically act upon rapidly growing bacteria, slow or nongrowing microorganisms would be protected from killing (Fig. 1) (Brown et al. 1988).

2.2.1 Oxygen Limitation, Metabolism, and Antibiotic Killing

Several studies have shown a correlation between oxygen limitation, metabolic activity, and protection from antibiotic killing in biofilms. Alkaline phosphatase activity and expression of green fluorescent protein (GFP), as measures of general bacterial protein production, have been used to show restriction of bacterial metabolism to the medium-exposed edge of *P. aeruginosa* biofilms (Borriello et al. 2004; Walters et al. 2003; Xu et al. 1998). In these same studies, oxygen microelectrodes were utilized to analyze the dissolved oxygen at various depths within the biofilm. Intriguingly, oxygen penetration was also restricted to the medium-exposed edge, suggesting that decreased oxygen tension throughout the rest of the biofilm inhibited metabolic activity and, consequently, increased antibiotic resistance (Walters et al. 2003; Xu et al. 1998). Similarly, diffusion of glucose and oxygen was inhibited through intact *K. pneumoniae* biofilms, which corresponded to a decrease in bacterial growth and resistance to ampicillin (Anderl et al. 2003). In both of these cases, antibiotics completely permeated the biofilm, yet the drugs only affected the biofilm edge (Anderl et al. 2003; Walters et al. 2003). Thus, limited metabolic activity within these biofilms, created by oxygen and nutrient gradients, protects the constituent bacteria from antibiotic killing.

2.2.2 Anaerobic Metabolic Pathways

Discussion of the metabolic pathways used during anaerobic growth can shed some light on the genetic mechanisms governing the reduced killing of slow-growing bacteria. *P. aeruginosa*, for instance, can utilize NO_{3^-} and NO_{2^-} for anaerobic respiration (Hassett et al. 2002). These processes are carried out by the sequential actions of the *nar*, *nir*, *nor*, and *nos* genetic loci, which reduce the nitrogenous substances to N_2. *P. aeruginosa* tightly regulates these genes in order to prevent buildup of toxic intermediates in the pathway (such as the production of nitric oxide). In fact, altered regulation of these loci in mutants of the quorum sensing gene *rhlR* under anaerobic conditions leads to rapid cell death (Hassett et al. 2002). Consequently, drugs targeting quorum sensing or nitrogen utilization pathways may be efficacious in destroying tenacious biofilms. Intriguingly, treating mature *P. aeruginosa* biofilms under anaerobic conditions with a combination of NO_{3^-} and either ciprofloxacin or tobramycin significantly enhanced killing of the microorganisms compared to antibiotic treatment alone (Borriello et al. 2006). However, these effects were not apparent in younger biofilms (Borriello et al. 2004). Obviously, the age and metabolic state of the biofilm plays a major role in determining its susceptibility to antibiotic treatment.

2.2.3 Stationary Phase and Stress Response Similarities

The slow growth and altered metabolic activity apparent in biofilms have led some researchers to suggest that the biofilm bacteria are in a stationary-phase state (Anderl et al. 2003). One of the hallmark features of stationary-phase bacteria is the activity of *rpoS*, the stationary-phase sigma factor instrumental in regulating expression of stress response factors. Microarray analysis of *E. coli* biofilms revealed the upregulation of nearly 50% of all *rpoS*-regulated genes (Schembri et al. 2003). In the same study, an *rpoS* mutant failed to form a biofilm. On the other hand, a *P. aeruginosa rpoS* mutant formed a much larger and more antibiotic resistant biofilm than wild type (Whiteley et al. 2001).

Additional studies have further implicated stress response factors as integral components of bacterial biofilms. For instance, microarray analysis of tobramycin-treated wild type *P. aeruginosa* biofilms showed upregulation of the stress response chaperones *groES* and *dnaK* (Whiteley et al. 2001). Studies with *K. pneumoniae* demonstrated expression of catalase in stationary-phase planktonic cells and in biofilms, but not in exponentially growing planktonic bacteria (Anderl et al. 2003). Catalase breaks down hydrogen peroxide and consequently protects expressing microorganisms from destruction. In *P. aeruginosa*, the constitutive catalase gene *katA* and the hydrogen peroxide inducible catalase gene *katB* were found to be important in resistance and adaptation to hydrogen peroxide stress in biofilms (Elkins et al. 1999). Thus, stress responses activated within bacterial biofilms may impact bacterial resistance to biocides and potentially to other antimicrobial agents.

In summary, it is clear that altered metabolism within biofilms promotes the creation of a bacterial subpopulation with altered sensitivity to antibiotics (Fig. 1). By decreasing the growth rate and activating vigorous stress responses, biofilms increase their chances of surviving antimicrobial treatment. In this sense, these metabolic changes represent a vital innate biofilm antibiotic resistance mechanism.

2.3 A Persisting Problem

The phenomenon of persistence was recognized in the mid-1940s in experiments in which cultures of penicillin-sensitive bacteria survived treatment with penicillin. The subpopulation of surviving bacteria has been referred to as persisters. Persister cells have been proposed as an additional innate mechanism for biofilm antibiotic resistance (Lewis 2005). In the persister theory, a small subpopulation of bacteria, whether in biofilms or planktonic culture, differentiates into dormant, spore-like cells that survive after extreme antibiotic treatment (Fig. 1). Differentiation into this dormant state has been hypothesized to be the result of phenotypic variation rather than a stable genetic change (Keren et al. 2004a).

2.3.1 Genetic Factors Influencing Persister Formation

Interestingly, the results of recent studies suggest that, while persisters may be phenotypic variants, specific genetic elements are required to form the persister state. Studies by Spoering, Vulíc, and Lewis implicated altered genetic activation of the glycerol-3-phosphate regulated genes *glpD*, *glpABC*, and *plsB* in *E. coli* as a mechanism of persister development (Spoering et al. 2006). The *glpD* gene was initially found to be important for this developmental pathway because plasmid-driven expression of the gene could increase the formation of ampicillin-resistant persisters in the exponential phase by approximately tenfold. Mutating the *glpD* gene or other genes involved in glycerol-3-phosphate metabolism, including *glpABC* or *plsB*, decreased tolerance to ampicillin by greater than 100-fold, indicating a role for glycerol-3-phosphate metabolism in persister formation. However, it was not reported whether these mutations altered the growth rate of the cell or the minimum inhibitory concentration for ampicillin. Further, given glycerol-3-phosphate's central metabolic role, these mutations did not provide any direct mechanistic insight into how persisters might be generated.

One mechanism proposed to explain the ability of persisters to resist the action of antibiotics is similar to a mechanism long hypothesized for biofilm resistance, namely a slowed growth rate. Indeed, persisters exhibit slow or no growth, as observed by microscopy of *E. coli* in a microfluidic device (Balaban et al. 2004). This decreased growth rate may inhibit antimicrobial action, as discussed above in Sect. 2.2. However, persisters can survive even after treatment with ofloxacin, which exerts bactericidal activity against nongrowing microorganisms (Kaldalu et al. 2004; Spoering and Lewis 2001), suggesting that limited growth rate alone cannot account for the increased antibiotic resistance of persisters. Alternatively, global transcriptional profiling by microarray analysis of persister cells revealed activation of numerous stress response pathways (Kaldalu et al. 2004; Keren et al. 2004b), potentially implicating these cells as hardy, stress-resistant microorganisms.

Another major factor influencing formation of persisters appears to be chromosomal toxin/antitoxin (TA) systems (Lewis 2005), which have previously been associated with programmed cell death in bacteria. Several TA modules were upregulated by microarray analysis of persisters in *E. coli*, including *dinJ/yafQ*, *relBE*, and *mazEF* (Keren et al. 2004b; Shah et al. 2006). Overexpression of the *relE* toxin gene, in particular, led to tolerance of high levels of such disparate antibiotics as ofloxacin, cefotaxime, and tobramycin (Keren et al. 2004b). The *hipBA* TA locus has also been found to be important for formation and maintenance of persisters, and mutation of the *hipA* toxin gene can enrich for persisters within in an *E. coli* population (Harrison et al. 2005; Keren et al. 2004a; Keren et al. 2004b; Moyed and Bertrand 1983). It has been suggested that these TA modules actually induce stasis of the bacterial cell by inhibiting the activity of a particular cellular machine, such as the ribosome (Keren et al. 2004b). It was proposed that this inert state then prevents the deleterious functions induced by antibiotics. For instance, an aminoglycoside cannot induce the formation of misfolded proteins if its target ribosome has been rendered static. In this sense, persister bacteria are considered antibiotic-tolerant

rather than antibiotic-resistant (Keren et al. 2004b; Lewis 2005). Evidence for this induced stasis comes from studies demonstrating that, while overexpression of the *relE* or *chpAK* toxin genes in *E. coli* rapidly reduced colony-forming units, subsequent transcription of the *relB* or *chpAI* antitoxins, respectively, led to a restoration of colony formation on agar plates (Pedersen et al. 2002). In other words, the toxin-expressing bacteria were nongrowing, yet non-dead, and addition of antitoxin resuscitated these cells. Thus, random fluctuations of toxin and antitoxin levels may modulate the formation and awakening of dormant persisters.

2.3.2 Persister Controversies

Intriguingly, persister research has led to several claims about biofilm antibiotic resistance in opposition to generally accepted biofilm tenets. Specifically disputed is the widely held, and well-supported, hypothesis that biofilms are more resistant to antimicrobial killing than planktonic bacteria. This argument has led some researchers to solely examine planktonic cultures for phenotypic and genotypic analysis of persisters. For instance, Spoering and Lewis concluded from their studies that stationary-phase *P. aeruginosa* was equally or more resistant than biofilm cultures to several antibiotics (Spoering and Lewis 2001). This effect was quantified as greater bacterial CFU after 6 h of antibiotic challenge and was hypothesized to be the result of equal or greater persister formation in the planktonic stationary-phase bacteria compared to the biofilm population. Similarly, Harrison et al. discovered that planktonic and biofilm populations of *E. coli* required similar levels of amikacin and ceftrioxone to effect complete eradication of the population in 2 h. However, in this latter study, *E. coli* biofilms were more resistant to tobramycin than planktonic phase cells at 2 h. Further, increasing the incubation time to 24 h revealed a much greater antibiotic resistance of biofilms to all three antibiotics compared to planktonic cells. In other words, planktonic bacteria were more sensitive to lower antibiotic concentrations when treated for longer periods of time. This result leads one to wonder whether increased antibiotic incubation periods could have produced a similar effect in the work by Spoering and Lewis and similar studies (Brooun et al. 2000; Spoering and Lewis 2001).

An additional concern in these studies is the variance in bacterial numbers between planktonic and biofilm populations at the start of antibiotic treatment. Thus, while Spoering and Lewis found a greater number of surviving stationary planktonic-phase bacteria compared to biofilm bacteria after antibiotic treatment, they also started with a significantly greater number of stationary planktonic phase bacteria then biofilm bacteria. In effect, in the stationary phase cultures, the units of antibiotic per bacterial cell were markedly decreased relative to biofilm bacteria, and this difference might have led to an apparent increase in antibiotic resistance. In a later study of *E. coli* resistance to metal oxyanions, Harrison et al. equalized planktonic and biofilm bacterial numbers before antibiotic challenge and found that this action did not significantly alter the MIC. However, in these conditions for the planktonic bacteria, they reported that "the proportion of surviving cells was

smaller than the fraction of survivors recovered from biofilms" (Harrison et al. 2005). As with the increased incubation time mentioned above, it would be intriguing to determine the effect of starting with similar bacterial numbers using the system as described by Spoering and Lewis.

Based on the results of these studies, it may be misleading to consider biofilm antibiotic resistance as a stationary-phase persister phenomenon. Alternatively, perhaps planktonic persisters have differentiated into a more biofilm-like phenotype, although there is no data to support this theory at this time. Recent microarray studies of *E. coli* suggested that the persister transcriptional profile represents a unique physiological state, distinct from exponential phase or stationary-phase bacteria (Shah et al. 2006). While no comparison was made to biofilms, it is intriguing to speculate that the persister phenotype is similar to the biofilm state. Indeed, the most highly expressed gene in persisters compared to nonpersisters in this microarray analysis was *ygiU*, which is also induced in biofilms and acts as a global regulator influencing biofilm formation (Shah et al. 2006). Further, mathematical modeling has predicted a steady accumulation of persisters as a biofilm matures and ages (Roberts and Stewart 2005). Thus, despite inconsistencies in persister literature, persister formation remains an intriguing concept as a supporting mechanism of biofilm antibiotic resistance.

In summary, innate formation of persisters might represent a common mechanism used by a wide range of bacteria during biofilm formation. Creation of this tenacious population within the biofilm may drastically inhibit the complete eradication of the biofilm during even prolonged, high-level antibiotic treatment (Fig. 1). However, at this stage, it is unclear what relationship, if any, can be drawn between planktonic persisters and biofilm resistance, and furthermore, the mechanisms(s) by which persisters form and/or confer increased antibiotic tolerance.

2.4 The Specifics

Many biofilm antibiotic resistance factors cannot appropriately be categorized into any previously described overarching phenomena, such as persister formation or decreased diffusion. Instead, certain biofilm-specific gene products may exert smaller, unique functions that enhance the overall antibiotic resistance of the biofilm. These factors have been the most difficult to uncover.

Perhaps the best example of a biofilm-specific factor is the *ndvB* gene of *P. aeruginosa*, identified in a screen for genes important for tobramycin resistance (Mah et al. 2003). The *ndvB* gene appears to encode for an enzyme involved in the synthesis of cyclic glucans. These glucans bind to tobramycin and prevent bacterial cell death, most likely by sequestering the antibiotic. An isogenic mutant in *ndvB* was much more sensitive to tobramycin than wild type (Mah et al. 2003). Interestingly, this effect was seen only in a biofilm and not when the bacteria were grown planktonically. Further, reverse transcriptase PCR on RNA isolated from type cultures demonstrated that *ndvB* was expressed in a biofilm but not expressed when bacteria

were grown planktonically (Mah et al. 2003). Thus, *ndvB* is a factor involved in antibiotic resistance specifically within a biofilm (Fig. 1). Similar studies may reveal biofilm-specific factors in biofilms constructed by other microorganisms.

2.5 A Quick Recap

As discussed above, the process of biofilm formation apparently leads to innate mechanisms of antibiotic resistant bacteria. That is, some mechanisms of resistance appear to be part and parcel of growing in a biofilm. Inhibited diffusion through the matrix, reduced metabolism by nutrient limitation, and formation of dormant persisters all appear to impact the development of a protective environment within the biofilm. Working in combination, these pathways might confer a multilayered network of security for the constituent bacteria. Further exposition of the genetic pathways that lead to these innate phenomena may very well result in improved treatment regimes for disruption and elimination of bacterial biofilms.

3 On Cue: Induced Mechanisms

As with any environmental change, antibiotic treatment can alter regulatory patterns within bacteria. Antibiotic treatment can be a harsh stress, even for bacteria within a biofilm. Consequently, one would predict that there must be some antibiotic-regulated genes that influence antibiotic resistance or sensitivity of biofilm bacteria. As previously mentioned, antibiotics can activate regulatory pathways, leading to a profound effect upon the biofilm matrix and achieved biomass (Bagge et al. 2004b; Hoffman et al. 2005; Rachid et al. 2000; Sailer et al. 2003), and it is likely that numerous genetic loci are activated upon treatment with antibiotics (Fig. 2). These induced factors may work synergistically with innate factors to enhance survival in the face of strong antimicrobial stresses.

Very little work has been done to identify antibiotic-induced factors in biofilms. However, recent microarray analyses have yielded some intriguing clues. Imipenem-treated *P. aeruginosa* biofilms strongly expressed alginate genes and the chromosomal β-galactosidase gene *ampC* (Bagge et al. 2004b). Expression of *ampC* was restricted to the outer edges of microcolonies, as determined by epifluorescence and confocal scanning laser microscopy of an *ampC-GFP* transcriptional fusion (Bagge et al. 2004a). In a separate study, tobramycin treatment of *P. aeruginosa* biofilms resulted in upregulation of *PA1541* and *PA3920*, two possible antibiotic efflux systems (Whiteley et al. 2001). Although no functional data have been generated, both of these studies identified a number of hypothetical genes that were upregulated or repressed upon antibiotic treatment (Bagge et al. 2004b; Whiteley et al. 2001), suggesting that many more factors are potentially involved in biofilm resistance than have currently been identified.

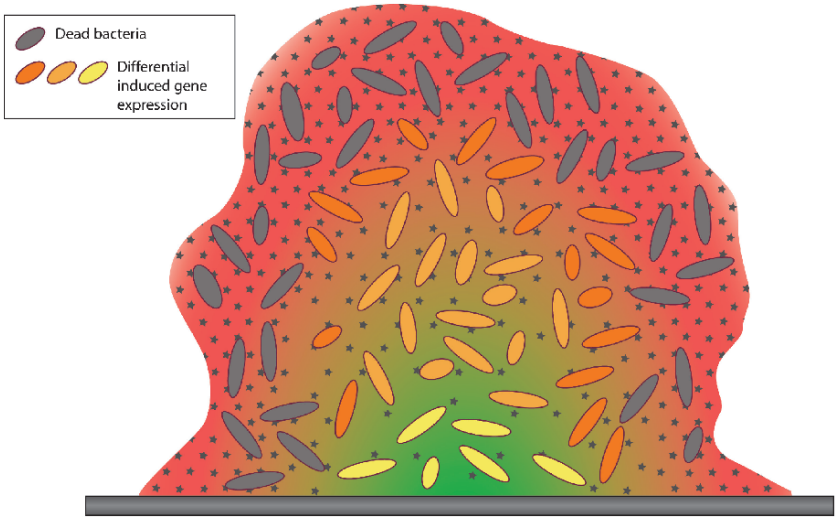

Fig. 2 Induced biofilm antibiotic resistance. Treatment with antibiotics might induce expression of bacterial resistance factors. As in Fig. 1, antibiotic molecules are shown as *dark dots*, and the biofilm bacteria are shown as *multicolored ovals*. Outer bacteria, with limited time to adapt to high antibiotic concentrations, may be rapidly killed (*gray*). Slowed antibiotic diffusion through the microcolony, due to matrix inhibition or other factors, might lead to the establishment of an antimicrobial gradient (*red* to *green* background and decreasing density of stars). This gradient may produce differential gene expression of antibiotic induced factors throughout the biofilm (*orange* to *yellow* bacteria). It is important to note that these induced factors may complement innate biofilm resistance factors, such that biofilm antibiotic resistance is the result of an intricate mixture of innate and induced factors

Efflux pumps and β-lactamases are some of the key mechanisms used by planktonic bacteria to overcome antibiotic challenge. Previous research has generally disregarded these factors and other planktonically associated systems as not important for biofilm antibiotic resistance, and much experimental evidence has supported this view (Patel 2005; Stewart and Costerton 2001). Nevertheless, as mentioned above, β-lactamases and possibly efflux pumps might exert some influence during a biofilm lifestyle (Anderl et al. 2000; Bagge et al. 2004a, 2004b; Whiteley et al. 2001). In reconciling these conflicting observations, it is intriguing to reflect on the numerous hypothetical genes that are differentially regulated during antibiotic exposure. It seems likely that novel orthologs will be discovered that might play a large role in antibiotic resistance specifically within biofilms. For example, *E. coli* alone contains genes for 37 proposed efflux pumps (Pages et al. 2005). It is possible that some of these genes are important for survival in a planktonic state, while others may be biofilm-specific. The putative efflux genes mentioned above, which were discovered by microarray analysis of tobramycin-treated *P. aeruginosa* biofilms (*PA1541* and *PA3920*), may be examples of such biofilm-specific orthologs (Whiteley et al. 2001). It is also possible that redundant

function of similar proteins within the same bacterium may have obscured the activity of previously tested gene products. For instance, in another study, a *P. aeruginosa* mutant with deletions in both the *mexAB-oprM* and the *mexCD-oprJ* efflux pumps could not establish biofilms in the presence of azithromycin, while the single mutation constructs of each behaved as wild type (Gillis et al. 2005).

It is clear that much more research is needed to expose additional and/or novel antibiotic-induced factors in biofilms. The multifactorial nature of biofilm antibiotic resistance has hindered identification of these pathways, and much remains to be elucidated about induced factors in biofilm resistance. Discovery of these unknown factors will lead to new and better treatments for biofilm related infections.

4 Disruptive Behavior: Novel Therapeutics

Considering the extremely robust defense mechanisms of biofilms, designing novel therapeutics may seem like a daunting task. However, some have accepted this challenge and in the process have devised some clever and creative solutions.

4.1 Quorum-Sensing Inhibitors

One area of intense interest is the development of inhibitors of bacterial quorum sensing (Rasmussen and Givskov 2006). Quorum-sensing systems are a vital component in community behavior and biofilm formation for a wide range of diverse bacteria, and treatment with quorum sensing inhibitors could lead to a severe abrogation of biofilm formation. Many large screening projects are currently underway to identity such inhibitors. These endeavors have led to the discovery of three types of molecules: those that block production of the quorum-sensing signal, enzymes or other factors that degrade the signal, and signal analogs that disrupt quorum sensing by blocking binding of the true signal, thus preventing activation of the receptor (Rasmussen and Givskov 2006).

Identification of signal analogs has been a particularly productive endeavor. Many eukaryotes, as a microbial defense mechanism, produce secondary metabolites and other compounds that interfere with quorum sensing and other bacterial processes (Steinberg et al. 1997). The marine alga *Delisea pulchra*, for instance, secretes a class of molecules called furanones (Steinberg et al. 1997). Furanones are structurally quite similar to the acylhomoserine lactone class of quorum-sensing signals, and thus disrupt community behavior of bacteria that utilize this class of autoinducers (Rasmussen and Givskov 2006). The effects of furanones on bacteria and biofilms are many and varied. Treatment of *Serratia liquifaciens* cultures with furanone abrogated swarming motility by inhibiting expression of the quorum-sensing regulated gene *swrA*, involved in production of the swarming surfactant

serrawettin W2 (Rasmussen et al. 2000). Furanone also inhibited quorum-sensing regulated virulence of *Vibrio harvey* and *P. aeruginosa* (Hentzer et al. 2002; Manefield et al. 2000). Furanone compounds penetrated *P. aeruginosa* microcolonies, affected biofilm architecture, and enhanced bacterial detachment from established biofilms. A furanone derivative could even inhibit the growth, swarming, and biofilm formation of the Gram-positive microorganism *Bacillus subtilis* (Ren et al. 2002; Ren et al. 2004). Thus, by interfering with cell–cell communication, furanones can perturb a number of functions of a wide range of different bacteria. The different effects on these several bacterial species most likely relates to differences in regulatory circuitry activated by quorum sensing in these microorganisms. Still, it is clear that furanone compounds inhibit community behaviors.

Several other inhibitors of bacterial quorum sensing have also been discovered. Screens of *Penicillium* extracts revealed two molecules, patulin and penicillic acid, that inhibited quorum-sensing regulation in *P. aeruginosa* (Rasmussen et al. 2005). Patulin also exhibited efficacy as a treatment for *P. aeruginosa* pulmonary infection in a mouse model. Intriguingly, this study found a synergistic effect on in vitro biofilm clearance when patulin and tobramycin were used in combination (Rasmussen et al. 2005). Synergy has also been observed between RNAIII-inhibiting peptide (RIP) and a number of different antibiotics during clearance of device-related *S. epidermidis* infections in vivo (Balaban et al. 2003a). RIP, a modified version of a heptapeptide isolated from cultures of *Staphylococcus xylosus*, prevented phosphorylation of target of RNAIII activating protein (TRAP), which under normal circumstances would activate the *agr* regulatory system of *Staphylococcus* species (Balaban et al. 2003a, 2003b). This hindrance resulted in decreased adherence and biofilm formation of both *S. aureus* and *S. epidermidis* on a variety of abiotic materials as well as mammalian cells in culture. Taken together, these studies point to a profound effect of natural compounds on bacterial quorum sensing. Especially considering antibiotic synergy, quorum-sensing inhibitor molecules have shown great potential for treatment of bacterial biofilms.

4.2 Non-Quorum-Sensing Inhibitors

Additional antibiofilm molecules have been discovered that appear to affect bacterial mechanisms other than quorum sensing. Another molecule that interferes with *S. aureus* biofilm formation is farnesol, produced by *Candida albicans* (Jabra-Rizk et al. 2006). Farnesol compromised membrane integrity of *Staphylococcus aureus* biofilm bacteria and acted synergistically in reducing the minimum inhibitory concentration of gentamicin for both methicillin-sensitive and methicillin-resistant *S. aureus*. In a separate study, Ren et al. screened thousands of natural plant extracts and discovered that ursolic acid disrupts biofilms formed by *E. coli*, *P. aeruginosa*, and *V. harvey* (Ren et al. 2005). It was demonstrated that quorum sensing was not involved in this effect. While the exact mechanism of inhibition remained elusive, microarray profiling implicated motility, heat shock, cysteine synthesis, and sulfur

metabolism as affected by ursolic acid treatment. Finally, subinhibitory concentrations of the macrolide antibiotic clarithromycin inhibited twitching motility of *P. aeruginosa* (Wozniak and Keyser 2004). While macrolides have generally not exhibited activity against *Pseudomonas*, clarithromycin treatment altered *P. aeruginosa* biofilm architecture, raising the possibility of utilizing macrolides in combination with other antibiotics for biofilm eradication.

4.3 Mechanical Means

On the basis of combating biofilm antibiotic resistance by enhanced or more efficient delivery of antimicrobial agents, much research has been focused on engineering better materials and methods for treatment of biofilms (Smith 2005). For instance, electrical, ultrasound, and photodynamic stimulation can disrupt biofilms and enhance the efficiency of certain antimicrobial agents. Further, coating surfaces with antimicrobial agents has shown efficacy in preventing biofilm formation. Similarly, implantable, biodegradable matrices, scaffolds, microparticles, and gels can release high concentrations of drugs in a controlled fashion in vivo. Liposomes have also been used to enhance the concentration and targeting of antimicrobials to biofilms. This strategy has been useful for delivery of drugs to intracellular pathogens as well. Aerosolization of antibiotics has been shown to be quite effective for direct application of these drugs to the respiratory system. In particular, aerosolized tobramycin, and more recently nebulized hypertonic saline, have achieved clinical efficacy in treating *P. aeruginosa* lung infection in patients with cystic fibrosis (Donaldson et al. 2006; Elkins et al. 2006; Gibson et al. 2003). In this manner, higher concentrations of drug can be delivered directly to the site of infection.

Treatment strategies for biofilms are constantly evolving. The synergy between natural compounds and traditional antibiotics seems quite promising for future clinical applications. Coupled with improved delivery mechanisms, these molecules may prove to be a boon to the medical field. Indeed, much progress has already been achieved, as seen with aerosolized delivery of tobramycin. While much research is still needed, novel treatments and biofilm inhibitory molecules are constantly being identified. These potential therapies offer much hope for the future of combating biofilm infections.

5 Beneficial Biofilms

In some instances, formation of highly resistant biofilms has proven to be an advantage. Particularly in industrial settings, chemically resistant bacterial biofilms provide a hardy platform for a number of applications involving high concentrations of toxic metals or other chemicals (Morikawa 2006). Studies have revealed the utility of biofilms in the synthesis of ethanol, poly-3-hydroxybutyrate, benzaldehyde, and

other chemicals (Kunduru and Pometto 1996; Li et al. 2006; Zhang et al. 2004). Biofilms have also assisted wastewater treatment, phenol bioremediation, biodegradation of 2,4- and 2,6-dinitrophenol, and bioremediation of toxic metal contamination of environmental sites (Lendenmann et al. 1998; Luke and Burton 2001; Nicolella et al. 2000; Singh and Cameotra 2004). These applications highlight the usefulness of extremely resistant biofilms in chemical synthesis and breakdown. In these cases, biofilms can be used as a tool for beneficial purposes.

6 Conclusion

The enigma of extreme biofilm resistance has puzzled researchers since the beginnings of biofilm research. Conventional antibiotic resistance mechanisms do not seem to influence biofilm survival, and dispersion of the biofilm bacteria leads to reversion to an antibiotic-sensitive state. These results have led to the identification of several intriguing resistance models, either resulting from an innate property of a biofilm lifestyle (Fig. 1) or an effect induced by the antimicrobial stress itself (Fig. 2). It is tempting to speculate that any one of these models alone (such as persister formation) can fully explain biofilm resistance. However, as we currently understand them, none of these phenomena can adequately account for every aspect of the biofilm resistance phenotype. Further, these models share common features and themes, such as decreased metabolic activity seen in anaerobic microcolony environments and with persisters. Also intriguing is the possibility that the biofilm matrix might slow the progress of an antibiotic through the microcolony such that the bacteria can sufficiently activate expression of protective factors in response to the biocide.

The overlap between these resistance paradigms has led some researchers to propose a layered model of biofilm resistance, wherein the outer layers of the microcolonies provide a first-line defense by inhibiting the diffusion of antimicrobial agents, bacteria deeper within the biofilm can be further protected by altered metabolic states, and development of persisters throughout enhances bacterial survival (Stewart and Costerton 2001). Throughout, innately expressed as well as antibiotic-induced genes might provide additional protection. In short, biofilm antibiotic resistance results from an overlapping mixture of innate and induced microbial activities, intricately woven together with redundant form and function.

Our understanding of the molecular details of resistance mechanisms of bacterial biofilms is still in its infancy. While many genetic factors have been identified, many more questions remain. Even for many known resistance genes, it is uncertain how they interact with each other to establish a resistance phenotype. Much greater knowledge of genetic responses to antimicrobial agents will facilitate the production of new and better drugs to eradicate biofilms. Manipulation of regulatory and expression networks holds much promise for future treatment of biofilm infections. Indeed, quorum-sensing inhibitors have demonstrated an exceptional ability to disrupt biofilm structure and act synergistically with a number of antibiotics.

On the other hand, enhancing and nurturing the impervious nature of beneficial biofilms may lead to improvement, for example, in the production of biologically derived chemicals and bioremediation. In either case, elucidation of the genetic mechanisms of innate and induced biofilm resistance holds the key to solving this great mystery.

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