Introduction

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Abstract This introductory chapter discusses the problem of drug resistance and persistent medical biofilm infections, emphasizing the need for alternative approaches to the prevention and treatment of biofilm infections. Such alternative approaches are described in subsequent chapters, culminating with clinical studies that describe treating otherwise untreatable wound infections with the aid of antibiofilm approaches.

1 The Problem: Untreatable Bacterial Infections[1–4]

The discovery of penicillin by Fleming in 1929 opened the era of antimicrobial chemotherapy, which has saved millions of lives by bringing many serious bacterial infections under control (Drews 2000; Fleming 1929). However, this medical miracle is being eroded by the emergence and spread of bacterial drug resistance. This problem has become a serious global issue. For instance, *Staphylococcus aureus* and *S. epidermidis* are leading causes of hospital-acquired infections, and the mortality associated with *S. aureus* bacteremia remains approximately 20–40% despite the availability of effective antimicrobials (Lowy 2003). Of the 2 million nosocomial infections each year, staphylococci cause over 90 000 deaths a year in the United

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States alone (Lowy 2003). The first effective antibiotic against *S. aureus*, penicillin, became available in the 1940s. Soon after, the bacteria evolved resistance to penicillin, and by the late 1950s, 50% of all *S. aureus* strains were resistant. Today, fewer than 10% of *S. aureus* infections can be cured with penicillin. The next weapons against *S. aureus*, methicillin and cephalosporins, became available in the 1960s and 1970s. By the late 1970s, some strains (2%) of *S. aureus* had evolved resistance to these drugs. Today, as much as 70% of *S. aureus* isolated from U.S. hospitals are resistant to methicillin (Fig. 1). The last effective defense against methicillin-resistant *S. aureus* (MRSA) is vancomycin. However, the increasing use of vancomycin has set the stage for the evolution of vancomycin-resistant *S. aureus* (VRSA) (Lowy 2003; Appelbaum et al. 2006). Over the past 20 years, MRSA infections have been limited primarily to patients in hospitals or long-term-care facilities. However, recent reports of "community-acquired" MRSA infections are alarming.

The same trend is observed for *S. epidermidis*. A study of hundreds of clinical *S. epidermidis* isolates derived from clinical orthopedic infections associated with prosthetic devices indicated that 37–38% were resistant to beta-lactams such as oxacillin and imipenem, while resistance to penicillin, ampicillin, cefazolin, and cefamandole was consistently observed in over 80% of the strains. Forty-one percent were resistant to erythromycin, 16% to clindamycin, 10% to chloramphenicol, 23% to sulfamethoxazole, and 26% to ciprofloxacin (Arciola et al. 2005).

Another example is *Pseudomonas aeruginosa* infections. *P. aeruginosa* is the fourth most commonly isolated nosocomial pathogen, accounting for 10% of all hospital-acquired infections. The gram-negative bacterium *P. aeruginosa* is adept at infecting many different organs and tissues. Because it causes disease primarily in persons whose health is compromised in some manner, it is considered an opportunistic pathogen. Mechanical ventilation, for instance, predisposes patients to pneumonia caused by *P. aeruginosa*. Likewise, the presence of a urinary catheter is associated with an increased risk of urinary tract infections. Patients with cancer who have neutropenia resulting from chemotherapy or hematologic malignan-

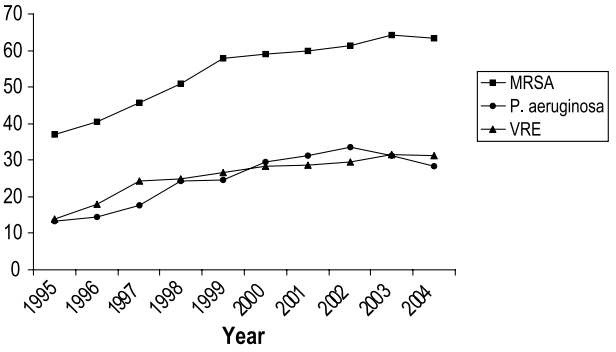

Fig. 1 Percentage of nosocomial infections caused by methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant enterococci (VRE), and fluoroquinolone-resistant *P. aeruginosa* in intensive-care patients in the United States in 1995–2004 (data source: National Nosocomial Infections Surveillance)

cies are prone to bacteremia, and burn patients often experience wound infections. Although each of these infections is most often categorized as hospital-acquired, *P. aeruginosa* frequently causes community-acquired infections in patients with cystic fibrosis (Hauser and Sriram 2005). *P. aeruginosa* is frequently resistant to many commonly used antibiotics. Although many strains are susceptible to gentamicin, tobramycin, colistin, and amikacin, resistant forms have developed; for example, fluoroquinolone-resistant *P. aeruginosa* strains have risen from 14% to 25% in the last 10 years (Fig. 1).

The rapid development of antimicrobial resistance could eventually lead to failure of most, if not all, of the currently available antibiotics. Hence, it poses a great threat to the economy and public health. While the problem is partially caused by overuse of antibiotics, it is also due to the inhibitory mechanisms of presently available antimicrobials. Most of these drugs were discovered for growth inhibition of individual cells in growing cultures—that is, in planktonic conditions (Stewart and Costerton 2001). However, the vast majority of bacteria exist within bacterial communities, otherwise known as biofilms (see below). The biofilm mode of growth plays an important role in antimicrobial resistance: Biofilm cells are up to 1000 times less susceptible to environmental stresses and disinfection treatments than planktonic (free-swimming) cells (Hoyle and Costerton 1991; LeChevallier et al. 1988). Whereas the planktonic cells are easily eliminated, the biofilm cells can survive and therefore provide a source of recontamination in both medical and engineering environments.

In clinical settings, biofilms are believed to be a common cause of persistent infections. The ability of biofilm-forming bacteria, such as *S. aureus*, *S. epidermidis*, and *P. aeruginosa*, to establish sessile communities on inert surfaces of medical devices or on dead as well as living tissue is now being recognized as a major problem (Costerton et al. 1999). Growing in biofilms, bacteria are protected against antibodies, leukocytes, and antibiotics. In addition, biofilms may spawn systemic infections by sloughing of planktonic bacteria, leading to dissemination, bacteremia, sepsis, and death.

2 Biofilm[3,4]

Costerton et al. (1999) proposed a basic definition of biofilm as "a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface." The matrix components can be exopolysaccharides, proteins, nucleic acids, or other substances (referred to as extrapolymeric substances, or EPS) that are believed to provide the cells with an array of advantages as compared to planktonic cells (Costerton et al. 1987, 1999; Anwar et al. 1990; Matz et al. 2004). This is important, especially in the clinical context, where it is estimated that about 60% of all microbial infections involve bacterial biofilms (Lewis 2001). (Refer to the case studies in the chapter *Clinical Wound Healing Using Signal Inhibitors*.)

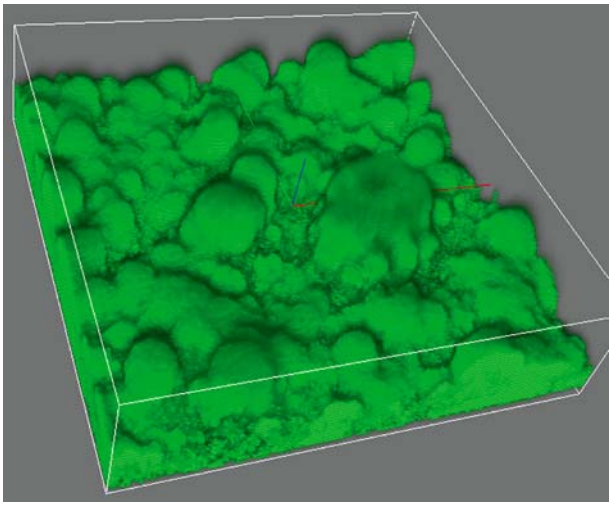

Fig. 2 Biofilm formed by Gfp-tagged *P. aeruginosa* in a continuous flow cell

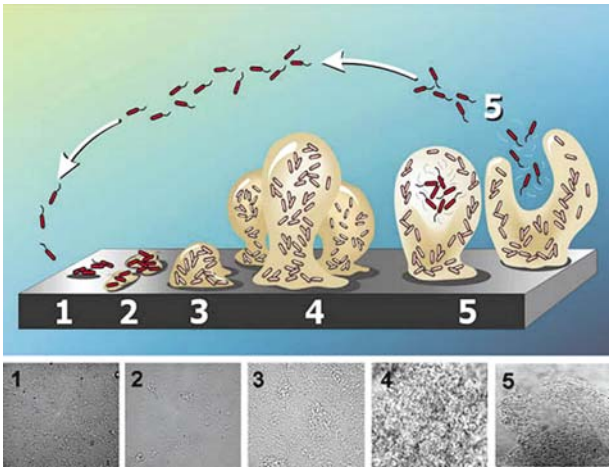

Fig. 3 Development of a *P. aeruginosa* biofilm. (1) Initial attachment. (2) Bacterial adherence. (3) Microcolony formation. (4) Biofilm maturation and development of three-dimensional structures. (5) Release/sloughing of cells able to form new biofilms. Reprinted, with permission, from the Annual Review of Microbiology, Volume 56 © 2002 by Annual Reviews www.annualreviews.org

Biofilms are not homogenous layers of cells; they are highly heterogeneous because they are comprised of patches of cells that are interspersed in the EPS matrix, which itself varies in density. This creates open areas where water channels are formed, allowing nutrients to enter the lower layers of the biofilm and, in addition, allowing waste products to be removed (Davey and O'Toole 2000; Dunne 2002). The bacteria found in a biofilm can either be of one species or it can, depending on the environment, be composed of multiple species.

In vitro biofilm formation by *P. aeruginosa* is one of the most intensively studied cases. After initial attachment of *P. aeruginosa* to a surface, microcolonies are formed, which in turn can grow to larger structures such as towers and mushrooms (Figs. 2 and 3). Recent analysis based on transcriptomics revealed that biofilm cells express their genes in a pattern that differs from that expressed by most stages of growth of planktonic bacteria, and the bulk of biofilm cells, even in the early stages, express genes in a pattern that is reminiscent of gene expression seen in the early stationary phase of planktonic cells (Hentzer et al. 2005). Although the experimental conditions would differ in the various experiments, the existence of a specific biofilm program would always require a core set of genes to be expressed, regardless of the experimental conditions. To date, transcriptomic studies such as of *P. aeruginosa* biofilms have not delivered such an outcome, and it strongly suggests that multiple pathways exist by which a biofilm can be built. Regardless, what is becoming evident is that bacterial cell-to-cell communication is required for a successful biofilm to form in vivo; this is discussed in subsequent chapters.

3 Resistance to Antibiotics[1–4]

3.1 Inherent Bacterial Resistance to Antibiotics

P. aeruginosa and *S. aureus* will be used here as examples of antibiotic resistance. Several factors contribute to the antibiotic resistance of *P. aeruginosa*. It appears that the bacterium has an intrinsic resistance conferred by lowered permeability of the outer membrane as well as efflux pumps that rapidly shuttle many different compounds out of the cell (Hancock 1998; Lee et al. 2000). Five different efflux systems have been identified in *P. aeruginosa*, but the sequence analysis by Stover et al. (2000) suggests that there may be up to 30. The identified systems include the MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexJK-OprM, and MexXY-OprM systems (Adewoye et al. 2002). The highly homologous efflux pump proteins consist of a cytoplasmic-membrane-associated drug-proton antiporter, a membrane channel-forming protein, and a periplasmic fusion protein. The pumps have broad specificity and transport varying molecules, including dyes, detergents, antibiotics, organic solvents, and secondary metabolites and signaling molecules such as *N*-acyl homoserine lactone (AHLs) (Poole and Srikumar 2001). The antibiotics to which the multidrug efflux pumps confer resistance include chloramphenicol, gentamicin, trimethoprim, imipenem, and tetracycline as well as other quinolones, macrolides, and beta-lactams (Kohler et al. 1997, 1999; Yoneyama et al. 1997; Pumbwe and Piddock 2000). Other compounds also affected by the action of the pumps include the heavy metal vanadium (Aendekerk et al. 2002). In addition, *P. aeruginosa* (and staphylococci; see below) produce beta-lactamases encoded on the chromosome, conferring enhanced resistance to beta-lactam-based antibiotics such as imipenem (Bagge et al. 2002).

Staphylococcal antibiotic resistance has been extensively reviewed (Lowy 2003). As described, staphylococcal resistance to penicillin is mediated by *blaZ*, the chromosomal gene that encodes beta-lactamase. Methicillin resistance (leading to MRSA strains) requires the presence of the chromosomally localized *mecA* gene Chambers 1997). *mecA* is responsible for synthesis of penicillin-binding protein 2a (PBP2a) (Hartman and Tomasz 1984; Utsui and Yokota 1985; Song et al. 1987). PBPs are membrane-bound enzymes that catalyze the transpeptidation reaction that is necessary for cross-linkage of peptidoglycan chains (Ghuysen 1994). PBP2a substitutes for the other PBPs and, because of its low affinity for all beta-lactam antibiotics, enables staphylococci to survive exposure to high concentrations of these agents. Thus, resistance to methicillin confers resistance to all beta-lactam agents, including cephalosporins. Resistance to quinolones results from the stepwise acquisition of chromosomal mutations. The quinolones act on DNA gyrase, which relieves DNA supercoiling, and topoisomerase IV, which separates concatenated DNA strands. Amino acid changes in critical regions of the enzyme DNA complex (quinolone resistance-determining region) reduce quinolone affinity for both of its targets. The ParC subunit (GrlA in *S. aureus*) of topoisomerase IV and the GyrA subunit in gyrase are the most common sites of resistance mutations; topoisomerase IV mutations are the most critical because they are the primary drug targets in staphylococci (Hooper 2002; Ng et al. 1996).

The reduced susceptibility to vancomycin appears to result from changes in peptidoglycan biosynthesis. The VISA (vancomycin-intermediate *S. aureus*) strains are notable for the additional quantities of synthesized peptidoglycan that result in irregularly shaped, thickened cell walls. There is also decreased cross-linking of peptidoglycan strands, which leads to the exposure of more D-Ala-D-Ala residues (Hanaki et al. 1998a, b). As a result, more D-Ala-D-Ala residues are available to bind and trap vancomycin. The bound vancomycin then acts as a further impediment to drug molecules reaching their target on the cytoplasmic membrane. The second form of vancomycin resistance results from the probable conjugal transfer of the *vanA* operon from a vancomycin-resistant *E. faecalis*. Resistance in these VRSA isolates is caused by alteration of the terminal peptide to D-Ala-D-Lac instead of D-Ala-D-Ala. Synthesis of D-Ala-D-Lac occurs only with exposure to low concentrations of vancomycin (Lowy 2003).

3.2 Resistance of Biofilms to Antibiotics

Generally, resistance to a drug or a heavy metal means that a bacterium can grow and form a culture or colony in the presence of that particular drug or heavy metal. Tolerance, on the other hand, refers to the situation in which a bacterial culture is not eradicated by treatments with that particular drug. Whether it is resistance or tolerance, both may contribute to the fact that the biofilm mode of growth enables the bacteria to survive the exposure to 1000-fold higher concentrations of a number of antibiotics compared with their growing counterparts (Anwar et al. 1990; Allison and Gilbert 1995; Teitzel and Parsek 2003). In other words, the underlying mechan-

ism responsible for biofilm resistance is multifactorial, and in the literature (as in the present book), authors usually do not discriminate between resistance and tolerance, because the underlying mechanism in the context of a biofilm is unknown.

Restricted penetration of antimicrobial compounds into the biofilm accounts for some of the resistance. This is especially true for some compounds such as aminoglycosides but not as much for others, such as fluoroquinolones. Because restricted penetration is based on binding of the molecules to, most probably, the EPS matrix, it is believed that at some point, the matrix becomes saturated, and penetration will eventually occur without delay. On the other hand, EPS is probably constantly being produced, creating new spots for antimicrobial binding (Lewis 2001; Campanac et al. 2002; Drenkard 2003; Teitzel and Parsek 2003).

Another factor adding greatly to biofilm tolerance is the very heterogeneous metabolic activity of the biofilm cells. In a biofilm, there exist gradients of nutrients and oxygen, which limit the growth rate of most of the cells (except for the cells on the surface of the film). Because antimicrobials mostly target metabolically active cells, the large slow or nongrowing parts of the biofilms are very difficult to target. Some antibiotics have reduced activity in oxygen-deprived environments, which also contributes to biofilm resistance as availability of oxygen is reduced in deeper levels of a biofilm (Lewis 2001; Drenkard 2003). Another option is the expression of certain genes in a biofilm, conferring enhanced resistance to antibiotics. The exact nature of these genes remains to be elucidated (Lewis 2001; Drenkard 2003; Sauer et al. 2002; Whiteley et al. 2001). A glimpse into this is provided by Drenkard and Ausubel (2002), who identified a gene, *pvrR* (phenotypic variant regulator), that is involved in conversion of wild type *P. aeruginosa* into a rough-colony phenotypic variant. The rough-colony variant has a highly elevated resistance to antibiotics (Drenkard and Ausubel 2002; Drenkard 2003). Mah et al. (2003) recently reported on the presence of a specific gene product that inactivates tobramycin by binding to the drug in *P. aeruginosa* PA14 is upregulated in biofilms. The locus identified, *ndvB*, is required for the synthesis of periplasmic glucans. These periplasmic glucans interact physically with drugs and therefore might prevent antibiotics such as gentamicin, ciprofloxacin, chloramphenicol, and ofloxacin from reaching their sites of action by sequestering these antimicrobial agents in the periplasm.

In staphylococci, persistence within a biofilm requires an adaptive response that limits the deleterious effects of the reduced pH associated with anaerobic growth conditions (Beenken et al. 2004). Several of the operons that were induced in biofilms have also been found to be important in acid tolerance in other bacterial species, including the oral bacteria *Streptococcus salivarius* (Li et al. 2000), and have been correlated with virulence in *Streptococcus pyogenes*. Bacteria can combat acidic environments by producing alkaline compounds such as ammonia. Two ways in which bacteria generate ammonia are through the urease and arginine deiminase (ADI) pathways. Multiple genes from both of these pathways [arginine deiminase (*arcA*), ornithine transcarbamylase (*arcB*), and carbamate kinase (*arcC*)] were indeed induced in *S. aureus* biofilms in comparison to both planktonic conditions (Beenken et al. 2004).

An additional gene upregulated in *S. aureus* biofilm is the arginine repressor encoded by *argR*. Under anaerobic conditions in the presence of arginine, ArgR represses anabolic ornithine carbamoyltransferase and induces the ADI pathway. Ammonia generated by the deimination of arginine can neutralize acids generated by bacterial glycolysis (Beenken et al. 2004).

Also included among the genes induced in biofilms are seven genes that comprise the urease operon (Beenken et al. 2004). Urease (urea amidohydrolase) is a nickel-containing enzyme that catalyzes the hydrolysis of urea to yield two molecules of ammonia and one molecule of CO_2. Ureases of most bacteria are composed of three distinct subunits encoded by three contiguous genes, *ureA*, *ureB*, and *ureC*. Urease activity is essential for colonization of the gastric mucosa by *Helicobacter pylori* and colonization of the urinary tract by both *Proteus mirabilis* and *Staphylococcus saprophyticus* (Eaton et al. 2002; Gatermann and Marre 1989; Jones et al. 1990). In addition, urease is thought to play a central role in the pathogenesis of *Ureaplasma urealyticum* urinary and respiratory tract infections (Hedelin et al. 1984; Ligon and Kenny 1991).

Several operons of the pyrimidine nucleotide biosynthetic (*pyr*) pathway (*pyrRPBC, carAB*, and *pyrFE*) are also induced in biofilms. The pathway for the de novo synthesis of pyrimidines consists of six enzymatic steps leading to the formation of UMP. This is important because the level of UMP in cells growing in a biofilm is severely limited. In addition, upregulation of the *pyr* operon may be required for synthesis of sufficient levels of arginine to be used by the ADI pathway during anaerobic growth (Beenken et al. 2004).

Taken together, as indicated by microarray studies (Beenken et al. 2004; Hentzer et al. 2005), mature biofilms grow anaerobically, and genes of the acid tolerance response are upregulated in response to an acidic environment. Global regulators such as the quorum-sensing sensor TRAP (Korem et al. 2005; see also below), sigma factor B (SigB), and staphylococcal accessory regulator (SarA) are involved (Beenken et al. 2004).

Although more work is needed to fully understand antimicrobial resistance in biofilms, it is clearly not caused by a single mechanism but by several factors acting in concert. First, as mentioned above, bacterial cells in biofilms produce a matrix of polysaccharide, which may retard or block antimicrobial agents from reaching the cell (Elvers and Lappin-Scott 2000). In addition, it was found that 40% of the cell wall proteins in biofilm cells are different from those of planktonic cells; therefore, the permeability of the cell membrane may change (Potera 1999), making it difficult for antimicrobials or immune factors to reach their targets. Recent studies have also shown that biofilm cells have profound changes in gene expression and cell physiology compared with planktonic cells, even though they have identical genotypes (Hall-Stoodley et al. 2004; Prigent-Combaret et al. 1999).

Most antimicrobials inhibit growth-related cellular activities, such as protein, DNA, and cell wall synthesis. Hence, they are not efficient against biofilm cells that have slow or even no growth (Potera 1999; Xu et al. 2000). Furthermore, the close cell-to-cell contact in biofilms provides a favorable environment for horizontal gene transfer (Li et al. 2001b), which results in easy spread of antimicrobial resistance.

Although several factors are responsible for antimicrobial resistance in biofilms, they are all related to the multicellular nature of biofilm communities (Stewart and Costerton 2001) because it has been shown that the biofilm cells lose their resistance rapidly after resuspension and planktonic growth (Anwar et al. 1989).

The next chapters will address how understanding bacterial cell-to-cell communication and bacterial response to the environment can lead to the development of novel therapeutics.

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