Biofilms as Refuge against Predation

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Abstract

Bacterial growth and survival in the environment as well as in association with human hosts are constrained by the action of phagocytic eukaryotic cells. Phagocytic predation on bacteria by host immune cells shares a number of cellular mechanisms with free-living protozoa. In and outside the human host, bacteria growing in biofilms appear to be less vulnerable to phagocytic predators than planktonic cells. Widespread resistance against predators is mediated by the interplay of biofilm-specific traits such as substratum adherence, exopolymer production, cellular cooperation, inhibitor secretion, and phenotypic variation. Selective predation is suggested to promote bacterial life in the biofilm niche and to govern structure-function relationships. There is increasing evidence that some of the pathogenicity traits may have their origin specifically in successful antipredator adaptations. Parallel selective pressures in and outside the human host may result in cross-adaptations of bacterial pathogens.

Introduction

Biofilm research today mostly deals with the question how to control problematic biofilms in medical and industrial settings. Particularly, researchers from different backgrounds try to understand biofilms as the preferred life style in persistent human infections and as pathogen reservoirs in the environment. These studies are tightly intertwined with the search for intervention points for prevention and therapy. From a medical perspective, the key characteristic of bacterial biofilms is their tolerance to host defenses and antimicrobials. While antibiotic resistance poses a serious threat to human health in the 21st century, it needs to be stressed that the primary cause for persistent biofilm infections is the fact that intrinsic control mechanisms by the immune system do not unfold fully or efficiently. Similarly, the observation that bacterial biomass and activity in many natural ecosystems preferentially accumulate in association with surfaces raises the question whether the natural loss factors might be compromised by the biofilm mode-of-life.

A key mortality factor in the control of bacterial populations is the uptake and killing of bacteria by phagocytic eukaryotic cells—no matter if we look at the microbial food web of natural ecosystems or at the host immune defense against bacterial pathogens. The antagonistic interaction describing the consumption of prey individuals of another species is generally defined as predation. From an ecological perspective, the control of pathogen
growth at the stage of the non-specific, constitutive host response—as commonly represented by amoeboid phagocytes—is analogous to that of a microbial predator-prey system (Levin and Antia, 2001). Given the importance of predatory control mechanisms in and outside the human host, this chapter attempts to synthesize current knowledge to present a conceptual framework of the role of predator-prey interactions on biofilm formation and persistence.

**Predatory agents in environment and disease**
Before we can dwell on the question how bacterial biofilms cope with phagocytic predation, a brief discourse seems in place on who the predatory agents are that bacteria are confronted with. Interestingly, the major predatory pathway in and outside human hosts is mediated by the same cellular process: phagocytosis. Phagocytosis constitutes not only the primary line of host innate and adaptive defense against incoming microbial pathogens but is also employed by phagotrophic microeukaryotes, the protozoa, which are the primary consumers of bacteria in most soil, freshwater and marine ecosystems.

**Free-living protozoa in environmental biofilms**
The presence of protozoa in close association with environmental bacterial biofilms is well accepted. Surface-associated protozoan habitats range from sediments, rocks, water pipes and filters to dental unit waterlines and the oral cavity. Not only do protozoan numbers associated with biofilm communities often exceed those found in surrounding plankton, the number of protozoan taxa observed in natural biofilms communities also reveals great protozoan diversity (Arndt et al., 2003). Protozoa exhibit a variety of mechanisms to capture and engulf bacterial prey leading to a considerable diversification of protozoan morphologies, commonly grouped into flagellates, ciliates, and amoebae. Biofilm-associated protozoa comprise all three free-living groups, all of which have been shown to efficiently graze on surface-associated bacteria. Flagellates and ciliates contain many feeding types that are specialized on suspended bacteria and some that preferably feed on surface-bound bacterial prey; amoebae almost exclusively feed on biofilm bacteria. For instance, the kinetoplast flagellate *Rhynchomonas nasuta* has been reported to feed on attached *Pseudomonas* spp. at rates between 13 and 120 bacteria per flagellate and hour (Boenigk and Arndt, 2000; Matz et al., 2004a). Ingestion rates by the hypotrich ciliate *Euplotes* sp. feeding on adherent *Vibrio natriegens* and *Pseudomonas fluorescens* were reported to be 120 and 882 bacteria per ciliate and hour, respectively (Lawrence and Snyder, 1998). Amoebae species such as *Hartmanella cantabrigiensis*, *Platyamoeba placida*, *Saccamoeba limax*, *Vahlkampfia avara* were shown to ingest attached *Escherichia coli* at rates of 15 to 440 bacteria per amoeba and hour (Heaton et al., 2001). A thorough review on protozoan grazing rates in freshwater biofilms has recently been published (Parry, 2004).

**Host immune cells and biofilm infection**
The immune response to microbial pathogens relies on both innate and adaptive components. A major mechanism for the destruction of bacteria that have invaded the host is killing by professional phagocytes (macrophages and neutrophil leukocytes). Resident macrophages and neutrophils constitute the primary line of innate defense against most
bacterial pathogens by providing a means for their removal and destruction. Macrophages are found in all body tissues, where they serve as sentinels in wait for pathogens; the invaders shed a variety of chemotactic agents that alert the resident macrophages to the infection. Neutrophils or polymorphonuclear leukocytes (PMNs) are the first cells recruited from the bloodstream to sites of infection and are an essential component of the acute inflammatory response. One of the most classical examples of biofilms in human disease is the chronic lung infection of cystic fibrosis (CF) patients by *P. aeruginosa*. The inflammatory response to chronic *P. aeruginosa* lung infections is mainly characterized by the constant influx of PMNs. Analysis of bronchoalveolar lavage fluid has shown that the number of PMNs recovered from the lungs of patients with CF is 1000 times higher than that recovered from non-infected lungs (Brown and Kelly, 1994). Although PMNs have been described to efficiently feed on adherent bacteria (Lee *et al.*, 1983; Hayashi *et al.*, 1986), their significant phagocytic and secretory arsenal of toxic oxygen species, degrading enzymes, defensins, and lipid inflammatory mediators appears ineffective in combating *P. aeruginosa* infections of the lung. In fact, PMNs contribute with these mediators to the deterioration of lung tissue that is characteristic of inflammatory processes in CF lungs (Koch and Hoiby, 1993). Moreover, PMNs constitute the major leukocytes present in the blood and acutely inflamed tissue and would be expected to respond to growth of biofilms on surfaces implanted in the vasculature or other tissues. Hence, it would be important to understand how the cellular host defense interacts with biofilms under controlled conditions. For the sake of simplicity, this review concentrates mostly on components of the innate immune response; clearly humoral and adaptive immune response need to be included in future considerations.

**Shared predatory mechanisms between protozoa and immune cells**

Phagocytosis is an ancient eukaryotic feature; the first eukaryotic cell was likely to be phagotrophic as phagocytosis is thought to be essential for the uptake of the α-proteobacterial symbiont, from where the mitochondria evolved (Cavalier-Smith, 2002). Hence, bacteria and other prokaryotes should have experienced phagocytic predation since the dawn of eukaryotic life. Phagocytosis can be divided into a series of defined steps (Table 11.1): receptors on the cell membrane recognize and bind bacterial prey; intracellular signals are generated that induce actin polymerization at the site of contact; actin-rich membrane extensions reach out around the particle; the membranes fuse behind the particle, pulling it in toward the center of the cell; and the phagosome matures via a series of membrane fusion and fission events to become a phagolysosome. The phagolysosome is an acidic, hydrolytic compartment in which the bacterium is killed and digested.

Locomotion and the ability to sense and respond to shallow gradients of extracellular bacterial signals are remarkably similar in amoebae such as *Dictyostelium discoideum* and phagocytic immune cells (Devreotes and Zigmond, 1988). Inflammatory signals however appear to be only important for phagocyte chemotaxis. A fundamental step in phagocytosis is the receptor-mediated recognition of an extracellular particle. The recognition mechanisms can be pattern based (non-opsonic phagocytosis) or humoral (opsonic phagocytosis), the latter of which does not apply to amoebae. While scavenger receptors which recognize bacterial surface components (e.g. lipopolysaccharide, lipoteichoic acid) have not yet been described for protozoa, surface lectins such as the mannose receptor are present in both
amoebae and immune phagocytes. However, signals and motifs in prey recognition may differ significantly between protozoa and immune phagocytes as the feeding motivation is somewhat different (recognition of food particles versus recognition of “microbial non-self” particles).

In the phagolysosome the bacteria are killed after exposure to enzymes, antimicrobial peptides and reactive oxygen species (ROS). The arsenal of cytotoxic agents has been traditionally divided into oxygen-dependent and oxygen-independent mechanisms. The latter mechanism employs antimicrobial peptides and proteins such as defensins, lysozyme, elastase and cathepsin. Antimicrobial host defense peptides are widely distributed in animals and plants and are among the most ancient host defense factors (Hoffmann et al., 1999). Most of these peptides have cationic properties that allow interactions with the bacterial cytoplasmic membrane, which usually comprises negatively charged phospholipids. Accordingly, this class of peptides has been termed cationic antimicrobial peptides (CAMPs). Oxygen-dependent killing relies on the generation of oxygen radicals by NADPH oxidase, during the so-called oxidative burst, resulting in the accumulation of reactive oxygen and nitrogen intermediates. The production of both oxygen radicals and membrane-permeabilizing peptides has been described for environmental amoebae (Davies et al., 1991; Herbst et al., 2002). Taken together, the stresses and selective pressures imposed on bacteria by protozoa, specifically by amoebae, and mammalian phagocytes may show significant overlap. To avoid phagocytosis biofilm bacteria need to interfere with at least one of the steps, either with recognition, engulfment, antimicrobial agents or phagocytic activity.

**Table 11.1** Summary of factors commonly involved in phagocyte attack by immune cells and free-living protozoa

<table>
<thead>
<tr>
<th>Step in predator-prey interaction</th>
<th>Factors involved</th>
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<tr>
<td></td>
<td>Macrophages/leukocytes (PMNs)</td>
</tr>
<tr>
<td>Chemotaxis</td>
<td>LPS, formylated peptides, PAMPs, chemokines, IL-8</td>
</tr>
<tr>
<td>Phagocytosis</td>
<td>Non-opsonic, opsonic</td>
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<tr>
<td>Prey recognition</td>
<td>Non-opsonic: Lectins (e.g. mannose-binding receptor), scavenger receptors, Toll-like receptors</td>
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<tr>
<td>Oxygen-dependent killing</td>
<td>Reactive oxygen/nitrogen species</td>
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<td>Oxygen-independent killing</td>
<td>Antimicrobial peptides/proteins</td>
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<td>Signaling</td>
<td>Cytokines</td>
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Are biofilms inherently protected from predation?

The early biofilm concept proclaimed that the gel-like state of the biofilm matrix limits the access of antibacterial agents, such as antibodies and phagocytic eukaryotic cells, and inferred that biofilm bacteria are substantially protected from amoebae and immune cells, similar to the resistance against antibiotics (Costerton et al., 1987). Studies on the antiphagocytic effect of encapsulated streptococci and staphylococci and mucoid P. aeruginosa confirmed this concept and led to the common perception of biofilms being generally protected against predation. The finding of Yersinia pestis forming a biofilm that inhibits feeding by the nematode Caenorhabditis elegans has further added to the view that biofilms may be a bacterial defense against predation (Darby et al., 2002). The observation that biofilm bacteria predominate numerically and metabolically in many aquatic ecosystems may also have led to the current conception that the biofilm mode-of-growth is a universal antipredator strategy.

This view, however, is challenged by the observation that protozoa not only occur widely in close association with natural biofilm communities, but that they are also able to disrupt biofilm structure and cause biofilm sloughing (Pedersen, 1982; Jackson and Jones, 1991; Weitere et al., 2005). Further support for a certain vulnerability of biofilms to predation comes from the simple fact that within the protozoa several feeding types have evolved that specifically feed on biofilms, such as amoebae, and exhibit a great species diversity. In addition, persistent biofilm infections most often occur in immunocompromised patients, which may suggest that a well-functioning immune defense would be capable to counteract biofilm formation and to avert biofilm manifestation.

So, instead of painting a black-and-white picture and regarding biofilms as inherently protective, a more differential view seems to be needed to address the role of biofilms as a refuge against predation. It is important to note that resistance against predation should always be defined in relation to the other bacteria in the prey community. For example, a recent study on Vibrio cholerae demonstrated that biofilm cells resist flagellate grazing while their planktonic counterparts become eliminated (Matz et al., 2005). Findings of lower feeding rates on biofilms compared to suspended bacteria would therefore justify the term “refuge,” as the chances of survival are higher for biofilm bacteria. To understand why biofilms are less efficiently grazed, we need to take a closer look at biofilm-specific traits that are not characteristic for planktonic bacteria.

Biofilm-specific mechanisms of protection

When contrasting planktonic and biofilm bacteria, four hallmarks can be noted that are characteristic for the biofilm mode-of-life: Adherence to a substrate; self-encasement into an extracellular matrix; life at high cell densities; and differentiation resulting in population heterogeneity. In the search for the most successful defensive strategy for biofilms, each of the four features is under suspicion to effectively compromise phagocytic attack (see Figure 11.1). Which impact these biofilm-specific traits have on bacterial survival during predation and on the predator’s fitness will be discussed below.
Adherence effects

The first question is whether adherence to a substratum alone is sufficient to provide effective protection for bacteria facing phagocytic attack (see Figure 11.1A). Bacteria can adhere to almost any surface in any environment by means of cell surface structures, such as fimbriae, pili, and extracellular polymers, or simply by physicochemical interaction forces (see also MacEachran and O’Toole, this volume). The physicochemical basis of adhesion phenomena is the balance of electrostatic and van der Waals’ forces as well as hydrophobic surface interactions that result in either attraction or repulsion between particles. Hydrophobic interactions are strongly attractive and promote adhesion of microorganisms to abiotic surfaces and epithelial cells (Marshall, 1986).

Interestingly, high bacterial surface hydrophobicity and low cell surface charge greatly increase the contact probability and the ingestion efficiency of bacterial cells during non-opsonic and opsonic phagocytosis (van Oss, 1978). For instance, binding of *P. aeruginosa* to phagocytes is inhibited by hydrophobic compounds such as p-nitrophenol, and strains that are hydrophobic and piliated are taken up more readily than hydrophilic, non-piliated...
strains (Speert et al., 1986). While studies on protozoa remain scarce, there are a few findings on planktonic protozoa suggesting similar physicochemical effects: feeding rates of interception-feeding nanoflagellates were lower with decreasing bacterial hydrophobicity (Monger et al., 1999) and increasing bacterial surface charge (Hammer et al., 1999; Matz et al., 2002a).

In many cases, bacterial attachment to surfaces is mediated by cellular structures such as pili and flagella. At the same time these structures serve as ligands in phagocyte recognition. For example, pili and flagella were found to increase the susceptibility of P. aeruginosa to non-opsonic phagocytosis (Kelly et al., 1989; Mahenthiralingam and Speert, 1995). Similarly, E. coli possessing mannose-sensitive fimbriae adhered better to the amoeba Acanthamoeba castellanii and human PMNs than non-fimbriated cells (Lock et al., 1987).

Apparently, the same bacterial cell surface traits that promote adherence to a substratum also increase contact probabilities with eukaryotic cell membranes and thus the probability of bacterial engulfment by phagocytic cells. Instead of finding a refuge from predation, surface-colonizing bacteria may initially face a higher predatory risk than their planktonic counterparts. Predator-prey studies of higher sessile organisms, such as seaweeds, suggest that sessile prey communities face intense grazing pressure due to the lack of avoidance or escape options (Duffy and Hay, 1990). Therefore, high predatory risk and selective grazing pressure on adherent bacterial populations can be expected to drive the necessity to develop protective traits during biofilm formation.

Matrix effects
During surface colonization cell-to-cell contacts become established between bacteria on the substratum, which assist the cells in further development of the biofilm. The close proximity allows cells to interact and cooperate and thus may open up the door to more complex antipredator adaptations in bacterial biofilms compared to planktonic bacteria. In the course of biofilm development the resident bacteria produce extracellular polymeric substances (EPS), which help create and stabilize the biofilm by gluing the cells together (see also Pamp et al., this volume). Historically, it is this EPS matrix that has been made responsible for the protective nature of bacterial biofilms (see Figure 11.1B). Although the composition of the EPS matrix is expected to be highly diverse within and between bacterial species, three principal properties of the matrix can be identified to contribute to the resistance against phagocytic predation.

One of the most obvious mechanisms is that the EPS matrix can form a physical barrier against the attacker. By gluing individual bacterial cells together, EPS allows biofilm bacteria to form defensive units, such as microcolonies, which reach a size beyond the feasible prey size spectrum of size-selective predators. Instead of handling bacterial cells one by one, phagocytic cells are confronted with many times more bacteria. Evidence for such a physical defense mechanisms comes from studies on P. aeruginosa and V. cholerae biofilms co-cultivated with flagellate grazers (Matz et al., 2004a; Matz et al., 2005). The flagellate predators used in these studies handle single bacterial cells, so that two cells glued together lead to longer handling times and reduced feeding efficiencies, which ultimately favor colonial growth in bacteria. As a consequence, an alginate-overproducing strain of P. aeruginosa formed larger microcolonies in response to grazing (Matz et al., 2004a). Size-selective
predation by flagellates stimulates the formation of bacterial cell clusters, so-called microcolonies, in a previously single-celled bacterial population (Matz et al., 2004a). Whether microcolony formation is induced mechanically or chemically remains unanswered at this point in time. Challenging P. aeruginosa biofilms with different protozoan feeding types (flagellate vs. ciliate vs. amoeba), however, provided evidence that microcolonies and alginate production alone do not suffice to allow biofilm persistence in the presence of amoebae and biofilm-browsing ciliates (Weitere et al., 2005).

Alginate has previously been reported to inhibit phagocytosis, thereby decreasing susceptibility of planktonic P. aeruginosa to human leukocytes and macrophages. While there is evidence that P. aeruginosa mutant biofilms lacking the exopolymer alginate become susceptible to leukocyte killing (Leid et al., 2005), experimental data to support the notion of EPS serving as a “physical barrier” against host immune cells remains scarce. Observations of inactive, “paralyzed” or “frustrated” leukocytes settling on biofilms of P. aeruginosa without disrupting or clearing the biofilm (e.g. Jesaitis et al., 2003) do not exclude the possibility that phagocyte inactivation may be mediated chemically by biofilm-secreted effectors. Recent in vitro studies demonstrate that PMNs can penetrate S. aureus biofilms but are unable to engulf the surrounding bacteria which suggests that PMNs become inactive in this process (Leid et al., 2002). In fact, there is mounting evidence now of biofilms interfering chemically with phagocytic activity and cellular processes (see below).

Based on the incomplete data available, one may conclude that the EPS matrix is able to reduce the ability of phagocytic predators to instantly penetrate and phagocytose biofilms. However, it may not offer total protection but forces the attackers to apply extracellular killing mechanisms. PMNs react to intruding foreign organisms either by phagocytosis or secretion; in both cases the PMNs launch a cocktail of antimicrobial agents, in particular free oxygen radicals. The EPS matrix keeps PMNs at a distance and thus provokes an extracellular attack which may not only be imprecise and costly but also less effective as the killing mechanisms in the vacuole.

Another functional property of the EPS matrix is to act as a diffusional barrier and interfere with the powerful antibacterial cocktail (toxic ROS and antibacterial peptides/proteins) released by activated neutrophils. EPS has repeatedly been described to interfere with the oxygen-dependent killing mechanisms of phagocytes by scavenging ROS produced by these cells. In a recent study it was shown that EPS isolated from a mucoid Burkholderia cenocepacia strain could inhibit neutrophil chemotaxis and scavenge neutrophil-derived ROS (Bylund et al., 2006). Similar findings have been presented for alginate from P. aeruginosa (Learn et al., 1987; Simpson et al., 1989). It is interesting to note that despite structural biochemical differences, the EPS material from P. aeruginosa and B. cenocepacia share many properties that could profoundly interfere with the effector functions of immune cells.

Similarly, the EPS matrix can also be considered to function as a chemically reactive barrier to antimicrobial peptides (AMPs) charged during oxygen-independent phagocytic attack. Both P. aeruginosa and S. aureus tolerate considerable amounts of cationic AMPs (CAMPs). It has recently been demonstrated that alginate produced by P. aeruginosa promotes the aggregation and sequestration of CAMPs by inducing conformational changes in the peptide structure (Chan et al., 2004). This mechanism is suggested to result in the removal of the AMP from the cytoplasmic membrane, the prime target of AMP action. In
addition, *P. aeruginosa* CF isolates produce a variant lipopolysaccharide (LPS) that renders the bacterial cells less susceptible to CAMPs (Ernst et al., 1999). Young et al. (2004) found that the major matrix component in staphylococcal biofilms, the polysaccharide intercellular adhesin (PIA), protects against AMPs of human skin and of the neutrophil-specific granules LL-37 and β-defensin. It is suggested that the underlying mechanism is based on the cationic character of PIA which causes electrostatic repulsion of the commonly cationic AMPs. A similar mechanism has recently been described for poly-γ-DL-glutamic acid (PGA) secreted by *S. epidermidis* (Kociánova et al., 2005). Taken together, resistance to AMPs in biofilms appears to be based, at least in part, on the interaction with specific extracellular biofilm polymers.

A fundamental step in phagocytosis is the receptor-mediated recognition of a prey particle. One potential mechanism in biofilm protection which has received very little attention so far is the recognition barrier, which inhibits the binding of phagocyte-receptors, antibodies or complement on their surface. Biofilm bacteria may be less conspicuous to the immune system because antigens and ligands used by phagocytes may be hidden (see section on adherence effects above). It is conceivable that components of the biofilm matrix may function analogously to the protective function of capsular polymeric substances (CPS). Most clinically important mucosal pathogens make carbohydrate capsules that surround the organisms. Although the carbohydrate composition and biosynthetic pathways vary among organisms, it is well established that such structures protect the organism from complement lysis, antibody deposition, and ultimately from phagocytosis (Celli and Finlay, 2002). The pathogens that use this strategy include *Streptococcus pneumoniae*, *E. coli* K1, *Klebsiella pneumoniae*, *Neisseria meningitides* and *S. aureus*. Bacterial S-layers and some types of LPS also can inhibit antibody binding and phagocytosis, presumably by similar mechanisms (Navarre and Schneewind, 1999; van Putten and Robertson, 1995). Another strategy to avoid the immune system surveillance may be to bind host proteins on their surfaces, which disguises the bacterial surface antigens. Many pathogens are capable of binding several host proteins, such as the basement membrane components fibronectin and collagen. It will be interesting to examine the role of the biofilm matrix in averting recognition by phagocytic receptors other components of the host immune response.

General knowledge on receptor-mediated phagocytosis in protozoa is much more limited. For amoebae such as *Acanthamoeba castellanii* and *Dictyostelium discoideum*, the involvement of mannose-sensitive receptors in phagocytosis have been described (see Table 11.1). Wildschutte et al. (2004) reported that *Salmonella enterica* serovars are grazed by intestinal amoebae at different rates depending on O-antigen variability at the bacterial cell surface. Interception-feeding flagellates were also found to differentially feed on synthetic prey items with specific biochemical coatings (proteins, polysaccharides) (Matz et al., 2002a). Although the specific mechanisms remain unclear, these studies suggest that receptor-mediated prey recognition should be included in future studies on biofilm-predator interactions in the environment.

**Cell density effects**

Besides interfering directly with phagocytic attack, the EPS matrix may also indirectly enhance antipredator fitness of biofilm cells as it promotes the formation of localized high
density consortia. The localization of cells in close proximity allows bacterial populations to communicate and cooperate via quorum sensing (QS) (Parsek and Greenberg, 2005). By monitoring bacterial cell density and diffusion, QS is thought to synchronize bacterial behavior and to adjust the population response to changing environmental conditions (see Figure 11.1C), particularly during the transition from logarithmic to stationary growth. A central feature of QS regulated gene expression in pathogenic bacteria is the secretion of virulence factors that interfere with eukaryotic cell activity (Winzer and Williams, 2001). QS controlled exofactors are not strictly biofilm-specific but their production is clearly promoted by the localized high cell densities found in sessile matrix-encased communities (see also Atkinson et al., this volume). The semi-diffusible nature of the EPS matrix may further help to generate a hostile chemo-environment for phagocytic attackers. Potential chemical defense mechanisms may include the inhibition of phagocyte chemotaxis, inactivation of phagocytes, and phagocyte lysis via apoptosis or necrosis.

Bjarnsholt et al. (2005) recently demonstrated that the limited penetration and elimination of P. aeruginosa wild-type biofilms by PMNs is dependent on a functional lasR/rhlR QS system. One mechanism involved in host defense evasion of P. aeruginosa is the production of extracellular factors such as proteases, toxins and phospholipases (Kharazmi, 1991; Smith and Iglewski, 2003). Two proteases, alkaline protease and elastase have been shown to inhibit chemotaxis, oxidative burst, phagocytosis and other microbicidal activities of phagocytes (Kharazmi et al., 1984a; Kharazmi et al., 1984b). In addition, the pigment pyocyanin induces neutrophil apoptosis and impairs neutrophil-mediated host defenses in vitro and in vivo (McClure and Schiller, 1996). QS controlled inhibitors secreted by P. aeruginosa further include cyanide (Pessi and Haas, 2000) with its general activity against most eukaryotic cells as it inhibits mitochondrial cytochrome oxidase c.

Besides regulating the expression of exoenzymes and toxins, QS signal molecules can also directly affect phagocytic activity and inflammatory response. The 3-oxo-dodecanoyl-homoserine lactone (3-oxo-C12-HSL) signal of P. aeruginosa has repeatedly been shown to act as immunomodulator (Telford et al., 1998; Smith et al., 2002; Ritchie et al., 2003). Tateda et al. (2003) reported that 3-oxo-C12-HSL, but not C4-HSL, in concentrations of 12–50 μM accelerate apoptosis in macrophages and neutrophils. Lower concentrations of P. aeruginosa QS signals were found to block the activation of PMNs and their oxidative burst, which may explain the observation that QS mutants cause a faster activation of the host defense system in vivo (Bjarnsholt et al., 2005).

In the interaction between bacteria and protozoan predators, it has been shown in P. aeruginosa, V. cholerae and Chromobacterium violaceum that QS mutants have a significantly reduced antipredator fitness compared to their isogenic wild-type strain (Matz et al., 2004a; Matz et al., 2004b; Matz et al., 2005). Mature biofilms of P. aeruginosa, for example, exhibit acute toxicity to flagellate and amoebal predators (Matz et al., 2004a; Weitere et al., 2005), which is achieved by the upregulation of exofactors mediated by the las/rhl QS system. Studies on the social amoeba D. discoideum also suggest the involvement of the las/rhl QS system in the killing of protozoan predators by P. aeruginosa (Pukatzki et al., 2002; Cosson et al., 2002). Similarly, V. cholerae biofilms were found to induce cell lysis of flagellate predators in a QS dependent fashion via the transcriptional regulator HapR (Matz et
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al., 2005). One factor to be responsible for protozoan cell death has been identified as the previously uncharacterized protease PrtV (Vaitkevicius et al., 2006). Interestingly, the same study shows that PrtV interferes with the production of the interleukin IL-8 from human intestinal epithelial cells.

Diversity effects

Bacteria living in a biofilm at high cell densities generate a complex three-dimensional structure with physicochemical gradients and numerous microenvironments. In biofilms, new niches arise through the actions of the organisms themselves. According to the niche exclusion principle, different types may be favored in each niche. The self-generated heterogeneity thus produces a variety of phenotypes within individual biofilms (Sauer et al., 2002) (see Figure 11.1D). The connection between environmental heterogeneity and diversity has a long history in ecology and population genetics. Rainey and Travisano (1998) documented the emergence of diversity in genetically identical founding populations of P. fluorescens when propagated in spatially heterogeneous environments. Diversity, in the form of niche-specific genotypes, emerged rapidly in spatially structured microcosms, but not in spatially homogeneous microcosms. Another study recently provided evidence that P. aeruginosa undergoes rapid genetic diversification during growth in biofilm communities (Boles et al., 2004). The genetic changes arise via a RecA-dependent mechanism, which likely involves recombination functions and affects multiple biofilm traits. Furthermore, experimental studies of colicin-producing, colicin-resistant, and colicin-sensitive populations of E. coli show that in an environment allowing for localized interactions diversity can be maintained (Kerr et al., 2002). Whereas these experiments show that diversity is rapidly produced in biofilms and/or structured environments, it remains elusive how it is generated. Besides the potentially strong selective pressure in structured environments, biofilm-specific conditions may also promote genetic innovation by higher mutation rates as found in the structured environments of agar surface colonies of E. coli (Taddei et al., 1997; Bjedov et al., 2003) and increased rates of horizontal gene transfer due to the close spatial arrangement of bacteria in biofilms (Molin and Tolkner-Nielsen, 2003). The concerted action of these mechanisms would render biofilms a true “hot spot” for phenotypic innovation. A good example for the innovative potential of biofilm populations may be the finding of a phenotypically diverse subpopulation of dispersal cells generated by biofilms of the marine bacterium Pseudoalteromonas tunicata (Mai-Prochnow et al., 2006).

Ecological theory predicts that diversity renders a community more resistant to environmental challenge than bacteria displaying a single phenotype (McCann, 2000). To date, the only indication that functional diversity actually increases the ability of bacterial populations to resist predation was recently presented in a study on V. cholerae phase variants (Matz et al., 2005). In a variety of bacterial species, stress conditions are known to lead to the generation of phase variants. These include the generation of a rugose variant of V. cholerae and a small colony variant of P. aeruginosa by random on-off switching of phenotypic expression. For example, the rugose variant of V. cholerae is found to occur at high frequencies under starvation conditions and exhibits enhanced biofilm formation and resistance to chlorine (Yildiz and Schoolnik, 1999). Grazing studies on the smooth and rugose variants of V. cholerae revealed that protozoan predation selects for the biofilm-en-
hancing rugose type, which is adapted to the surface-associated niche by the production of exopolymers (Matz et al., 2005). The marked differences in grazing mortality between the two variants specify the fitness conflicts of the “explorer strategy” of the smooth phenotype versus the “persister strategy” of rugose biofilm cells. Similar observations were also made on mucoid and non-mucoid variants of P. fluorescens (Matz et al., 2002b).

Predation represents a strong selective pressure on the prey population to evolve means of escaping. Predators as well as parasites are thought to play a significant role in the diversification of their prey or hosts. Several experiments with bacteria and their phage parasites, for example, have documented the spontaneous evolution of phage-resistant bacteria from a population of sensitive types (Bohannan and Lenski, 2000). Investigations of P. aeruginosa flow-chamber biofilms have indicated that the oxidative stress caused by the host immune response could be one of the factors that generates and/or selects the mucoid mutants. It also appears that the mutations in mucA induced by oxygen stress develop with higher frequencies in biofilm-associated cells than in suspended cells (Mathee et al., 1999; Ciofu et al., 2005). While studies on the direct impact of predation on biofilm diversity remain to be performed, it is well documented in planktonic multi-species communities that protozoan grazing causes shifts in the composition of the bacterial community and leads to the prevalence of grazing-resistant bacterial species (Jürgens and Matz, 2002). Notably, strong grazing activity has led, in most of these studies, to the formation and accumulation of suspended biofilms, so-called flocs or aggregates. Tentative evidence for the potential impact of predation on biofilm diversification comes from a recent study where the functional diversity found in P. aeruginosa biofilms increased the biofilm’s ability to withstand an applied oxidative stress (Boles et al., 2004).

**Inducible responses to predation**
The formation of distinct microcolonies by Pseudomonas spp. and the cell elongation observed in Flectobacillus spp. in response to flagellate grazing have long been discussed as inducible by predator-specific signals (Hahn et al., 1999; Hahn et al., 2000). However, such chemically induced plasticity have not yet been convincingly demonstrated for bacterial grazing responses. Predator-induced plasticity has been described for many organisms from fish to protists and comprises alterations in morphology, chemistry, behavior and life history (Tollrian and Harvell, 1999). One inherent problem in the analysis of such a mechanism in microbial systems is that bacterial predators continuously change the chemical composition of the surrounding medium by their excretions which in turn influence bacterial physiology, growth and thus a number of phenotypic traits (see “physiological benefits” below). Without the identification of specific biologically active molecules it may not be feasible to differentiate between substrate-mediated and signal-mediated effects on predation-relevant bacterial phenotypes.

First insights into inducible cellular responses can be gained from transcriptomic analyses of bacteria growing in co-culture with phagocytic predators. Microarray analysis of S. aureus strains that were resistant to killing by human PMNs and caused greater host cell lysis identified genes comprising a global response to PMN phagocytosis (Voyich et al., 2005). Genes involved in capsule synthesis, oxidative stress, and virulence were upregulated following ingestion of the pathogen. Preliminary data from the laboratory of Mike Givskov
show that upon exposure to PMNs *P. aeruginosa* biofilms can mount either an aggressive or a defensive response (Alhede et al., unpublished data). In the aggressive response, many genes encoding secreted virulence factors, such as *lasB*, *phz*, *rhlAB* and *prpL* were found to be upregulated. The defensive response was characterized by genes involved in general stress responses, such as *lexA*, *katB*, *ohr* and *recN*. Palma et al. (2004) demonstrated by means of transcriptomic analysis that the early response of *P. aeruginosa* to hydrogen peroxide consists of an up regulation of protective mechanisms and a down regulation of primary metabolism.

**Physiological benefits of predation**

Although predator-prey interactions appear severely asymmetric and one-directional (positive for the predator, negative for the prey), it may be worthwhile considering potential benefits for bacteria coexisting with predators in biofilms. Protozoa are known to play an important role in the re-mineralization of nitrogen and phosphorous within planktonic and soil microbial communities (Clarholm, 1985; Caron and Goldman, 1990). Therefore, bacterial populations limited in nitrogen or phosphorous may actually benefit from predation-mediated nutrient recycling. Interestingly, chemostat studies with nitrifying bacteria revealed that predation by a flagellate increased the metabolic activity per bacterial cell (Verhagen and Laanbroek, 1992). Nutrient-limited bacteria may not only benefit directly from substrates excreted by the predators; at the same time predators may also decrease the numbers of bacterial competitors and thus increase the supply of substrates per cell resulting in higher bacterial growth rates and metabolic activity. In one way or another, nutrient-limited biofilms may thus benefit from allowing (limited) predation to increase population maintenance and persistence.

Moreover, predatory eukaryotes themselves offer an attractive nutrient source for biofilm bacteria to be exploited in direct vicinity. Certain pathogenic bacteria, e.g. *Legionella*, are known to avoid the digestive mechanism of protozoa by blocking the digestive processes and multiplying within the food vacuole, on the expense of the host (Greub and Raoult, 2004). As outlined in a previous section, a number of biofilm-forming bacteria are known to kill immune cells and/or protozoa by secreting exoproducts, particularly at the onset of stationary phase. The lysis of predatory cells does not only prevent bacteria from being grazed upon but may provide them with a welcome nutrient boost. Rapid necrosis of human neutrophils in the presence of *P. aeruginosa* was recently found to significantly enhance biofilm formation (Walker et al., 2005). The colonization of cellular debris comprising F-actin and DNA resulted in a 3.5 fold increase in *P. aeruginosa* cell numbers in biofilms. Hence, the biofilm-phagocyte relationship does not appear to be one-sided but rather allow for far more complex interactions ranging from tolerance and defense to aggression and pathogenicity.

**Evolutionary benefits of predation**

Predation represents a strong selective force, so that adaptations that increase the anti-predator fitness of bacteria should be evolutionarily favored. The formation of inedible microcolonies during protozoan grazing is a commonly observed phenomenon in natural bacterioplankton communities (Jürgens and Matz, 2002). Similarly, the distinguishing fea-
ture of biofilm infections is the presence of aggregated, matrix-enclosed microcolonies and the QS-mediated secretion of virulence factors. On the micro-evolutionary scale, Boraas et al. (1998) demonstrated that a size-selective protozoan predator selects for a self-replicating multicellular growth form in the unicellular green alga *Chlorella vulgaris* within less than 100 generations. Taken together, the relative fitness advantage of biofilms over planktonic bacteria in the presence of eukaryotic predators should make a crucial contribution to the evolution of multicellular traits and cooperative behavior found in bacterial biofilms (see also Webb, this volume).

As outlined in the previous section, protozoan cells may offer an attractive nutrient source for bacteria once the predatory threat is overcome. What started as a struggle for life, may climax in the gradual transition of some bacterial species from a grazing resister to an aggressor and facultative pathogen. It is widely believed, for example, that the survival and successful replication of bacteria inside the protozoan niche gave rise to a number of facultative and obligate intracellular pathogens, such as *Listeria*, *Rickettsia*, *Mycobacterium*, *Legionella*, and *Chlamydia*. The fact that (i) these pathogens exhibit intracellular survival within both amoebae and human macrophages by using related mechanisms and that (ii) amoebae and macrophages share similar phagocytic mechanisms (see Table 11.1) support the notion that resistance to amoebae is an important prerequisite and a driving force in the evolution of some bacteria as pathogens (“training ground hypothesis”; Molmeret et al., 2005). Similar to intracellular survival strategies, protective mechanisms acting prior to the internalization by protozoa might be seen as important features for pathogenic bacteria to persist in the environment or to evade eukaryotic immune systems. Specifically, the antipredator mechanisms found for biofilms of *P. aeruginosa* (Matz et al., 2004a) and *V. cholerae* (Matz et al., 2005) suggest a causal link in some pathogenicity aspects between biofilm defense mechanisms against protozoa and professional phagocytes. The recent findings of predation-mediated variation at the *rfb* virulence locus of *Salmonella enterica* illustrate the potential impact of protozoan grazing on the origin of bacterial pathogenicity (Wildschutte et al., 2004). Hence, some of the virulence factors studied in the context of human disease are likely to have an ecological function within natural microbial communities and even have their origin specifically in successful antipredator adaptations.

### Concluding remarks and future directions

Historically, biofilms have been viewed as the bacterial refuge against numerous abiotic and biotic stresses that bacteria encounter in natural and host environments. This causality was derived from the study of mono-culture biofilms of selected bacteria species, such as *P. aeruginosa*, which have dramatically increased our knowledge in recent years on the cellular and molecular mechanisms underlying attachment, migration, recombination, autoregulation, cell–cell signaling, dispersal, and antibiotic resistance. Compared to the rapid progress being made in these areas, our fundamental knowledge of species-species interactions in “real-world” biofilm communities still lags considerably behind. The findings summarized in this chapter support the notion that biofilms provide protection against phagocytic attack in environment and disease. However, our understanding of predator-prey interactions remains fragmentary, in part because the interactions are anticipated to be quite complex; phagocytosis can be viewed as either an obstacle or an opportunity for biofilms.
However, as more focus is directed toward understanding these interactions, interesting patterns and principles may emerge. Biofilm adaptations to predation may be key to how some pathogens persist and diversify in the environment, reach the minimum infectious dose and undermine the first stages of the immune response. There are basic questions that remain to be answered, such as, whether bacteria know that all or parts of their population are under phagocytic attack and whether the attack induces a specific defense program in biofilms. Another emerging area of interest is how predation drives the evolution of virulence factors, the maintenance of their variability, and the emergence of new pathogens. Research in these areas would certainly benefit if some of the ecological thinking characteristic for microbial food web studies was combined with the molecular approaches used by infection microbiologists.

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References


