REVIEW ARTICLE

Multi-species biofilms: living with friendly neighbors

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Abstract

Our knowledge regarding the nature and development of microbial biofilms has grown significantly since the first report of these communities by Antonie van Leeuwenhoek in the late 1600s. Nevertheless, most biofilm studies examine mono-species cultures, whereas nearly all biofilm communities in nature comprise a variety of microorganisms. The species that constitute a mixed biofilm and the interactions between these microorganisms critically influence the development and shape of the community. In this review, we focus on interactions occurring within a multi-species biofilm and their effects on the nature of the mixed community. In general, interspecies interactions involve communication, typically via quorum sensing, and metabolic cooperation or competition. Interactions among species within a biofilm can be antagonistic, such as competition over nutrients and growth inhibition, or synergistic. The latter can result in the development of several beneficial phenotypes. These include the promotion of biofilm formation by co-aggregation, metabolic cooperation where one species utilizes a metabolite produced by a neighboring species, and increased resistance to antibiotics or host immune responses compared to the mono-species biofilms. These beneficial interactions in mixed biofilms have important environmental, industrial, and clinical implications. The latter, for example, impacts the course and treatment of biofilm-related infections, such as those manifested in the lungs of cystic fibrosis patients.

Introduction

The impact of bacterial biofilms on various aspects of our day-to-day lives has led to an increased number of biofilm-related studies in the past decade. Biofilms are differentiated groups of sessile microorganisms (e.g. bacteria and fungi) arranged as aggregated structures called microcolonies with distinct community properties. Biofilms constitute a unique mode of growth that allows survival in hostile environments. In particular, biofilms exhibit increased resistance to chemical disinfection, antimicrobial therapy, and human immune responses (Costerton *et al.*, 1999; Hall-Stoodley *et al.*, 2004; Hoiby *et al.*, 2010). Despite tremendous research efforts, our current understanding of the physiology and complexity of biofilm communities is still inadequate, especially as it is based mostly on studies of mono-species biofilms (i.e. population of cells). However, interspecies dynamics within mixed biofilms, such as communication and/or competition for nutrients and physical resources, represent those of a community, rather than a single-species population. This distinction is important, as it constitutes a layer of complexity that critically influences the phenotypes of the entire community within the biofilm.

Mixed-species biofilms are undoubtedly the dominant form in nature and are also prominent in the human host, for example in the oral cavity and the lungs of cystic fibrosis (CF) patients. Thus, there is a pressing need for more research directed at delineating interactions within multi-species biofilms and the effects of such interactions on the development, nature and survival of the biofilm community. The present review summarizes current knowledge concerning mixed-species biofilms and aims to understand the processes governing their development. These processes determine the shape and nature of the mixed-species biofilm. One such important process is cell–cell communication (i.e. quorum sensing) which can affect the interactions within the mixed biofilm in a

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number of ways. This includes the influence of neighboring cells on biofilm formation by altering the extracellular concentrations of autoinducers (e.g. degradation or production) or by the expression of quorum-sensing-dependent genes. Overall, the focus of the current review is toward beneficial interactions, which include for example, changes in extracellular polymeric substances (EPS) composition, biofilm resistance to antimicrobial agents and environmental stress conditions, improved utilization of nutritional resources and the spatial distribution within the mixed biofilm community. A companion paper (RenDueles & Ghigo, 2012) addresses competitive and antagonistic interactions in biofilms. It is important to emphasize that because most published work examines clinically relevant bacteria, the present review has a medical focus. It is likely, however, that similar processes occur within biofilms located in natural environments.

Cell–cell communication in mixed-species biofilms

Communication between neighboring bacteria via quorum sensing is a social behavior that enables interactions within mono and mixed bacterial communities. Quorum sensing requires production and release of chemical signal molecules called autoinducers that increase in concentration as a function of cell density but can also depend upon physiological conditions (Ng & Bassler, 2009). The quorum-sensing system allows bacteria to express specific genes in a coordinated fashion (Miller & Bassler, 2001; Antunes & Ferreira, 2009). Quorum sensing has been shown to play an important role in the development of biofilms (Davies *et al.*, 1998; Singh *et al.*, 2000; Zhu *et al.*, 2002; Riedel *et al.*, 2009; Bjarnsholt *et al.*, 2010), although its impact, namely induction vs. repression of biofilm formation, varies depending on the bacterial species and environmental conditions (Hammer & Bassler, 2003; Xu *et al.*, 2006; Sakuragi & Kolter, 2007).

Several quorum-sensing systems have been described to date (an overview of the chemical structures/sequences of autoinducers is shown in Fig. 1). Some are used mainly for intraspecies communication, while others support interspecies communication, enabling bacteria to sense the presence of other species. Specifically, the AI-2 system is considered universal and can mediate interspecies communication (Waters & Bassler, 2005; Federle, 2009). This system was identified in several Gram-negative and Gram-positive bacterial species, many of which are indeed found in intimate contact with one another in the natural environment (McDougald *et al.*, 2003).

An important mixed biofilm in the human host is found within the oral cavity. The oral microbiota comprises hundreds of different bacterial species and their ability to form and reside within biofilms is crucial for survival in the oral cavity (for a recent review see Kolenbrander *et al.*, 2010). Notably, a large number of human oral commensal bacteria have been shown to produce the AI-2 signal and the AI-2 system was shown to be required for mixed biofilm formation and the development of dental plaque (McNab *et al.*, 2003; Kikuchi *et al.*, 2005; Rickard *et al.*, 2008). For example, McNab and colleagues reported that AI-2 mediated the formation of mixed biofilms comprising two common oral bacteria, *Porphyromonas gingivalis* and *Streptococcus gordonii*. Mixed biofilm was not formed on polystyrene surfaces when both species lacked the *luxS* gene required for the

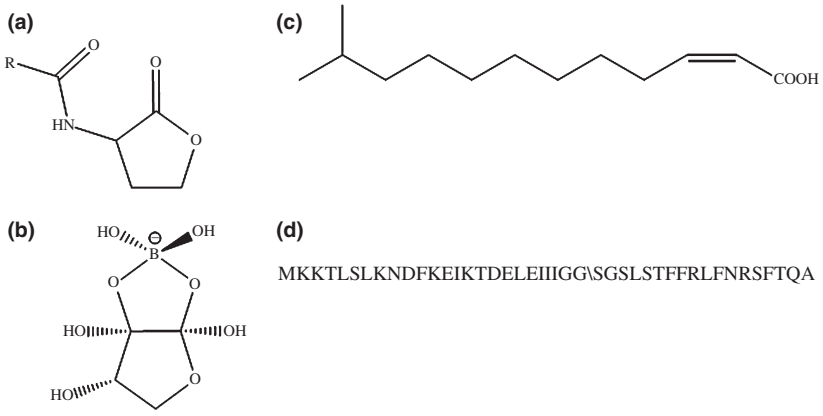

Fig. 1. Structures of quorum-sensing autoinducers discussed in this review. (a) AHL core molecule; (b) AI-2 molecule, here the boronated AI-2 from *Vibrio harveyi* is presented; (c) DSF structure first identified in *Xanthomonas campestris*, as proposed by Ryan & Dow (2011); (d) An example of an autoinducing peptide, in this case the *Streptococcus mutans* CSP amino acid sequence (accession number AM925050; Allan *et al.*, 2007). Predicted cleavage site is indicated by the \ symbol. It is important to note that the CSP amino acid sequence varies within species and an example of one such sequence is presented.

synthesis of the AI-2 signal. However, the ability to form a mixed biofilm was restored when mutants were grown together with a heterologous wild-type strain carrying a functional *luxS* gene (McNab *et al.*, 2003). Similarly, mixed biofilm formation by *Actinomyces naeslundii* and *Streptococcus oralis* in flowing saliva was shown to be dependent on the production of AI-2 by *S. oralis* that produced higher levels than *A. naeslundii*. Generally, the synergistic effect of living as a mixed community is manifested by the higher biovolume exhibited by the mixed biofilm than by either single species grown alone. In line with the specific requirement for AI-2 production by *S. oralis*, a mixed biofilm comprising *A. naeslundii* and the *S. oralis luxS*-deficient strain did not exhibit mutualism and the mixed biofilm was easily dispersed. These results indicate that upon co-aggregation there is a local increase in the AI-2 concentration that triggers mutualism and facilitates biofilm formation (Rickard *et al.*, 2006).

Interspecies quorum sensing involving the AI-2 system is not limited to interactions among different bacterial species, but also occurs between bacteria and fungi. The fungus *Candida albicans* is also found in the human oral cavity (Cannon & Chaffin, 1999). Fungal virulence and the formation of *C. albicans* biofilms are associated with the development of a hyphal fungal form (Mitchell, 1998). In the oral cavity *C. albicans* is found in close proximity to the bacterial microbial communities and is known to co-aggregate with *S. gordonii* (Jenkinson *et al.*, 1990). Mono-species biofilm formation on saliva-coated surfaces by a mutant *S. gordonii* strain lacking the *luxS* gene was found to be only slightly compromised. However, the mixed-species biofilm formed by this mutant and *C. albicans* exhibited significantly reduced biomass compared with the mixed biofilm formed with a wild-type *S. gordonii* strain. Thus, it appears that AI-2 is important for *C. albicans* – *S. gordonii* interactions, perhaps through promoting development of the hyphal *C. albicans* form (Bamford *et al.*, 2009).

Although much research has been focused on the AI-2 signaling molecule it is clearly not the only quorum-sensing signal known to mediate interspecies interactions. *N*-acylhomoserine lactone (AHL)-based quorum-sensing systems are not considered universal and usually support intraspecies communications, however there are several reports describing their involvement in the development of multi-species biofilms. For example two pathogens, *Pseudomonas aeruginosa* and *Burkholderia cepacia*, which are sometimes found in the lungs of CF patients, can form mixed biofilms and each employ AHL-based quorum-sensing systems to control the expression of virulence factors and biofilm formation (Van Delden & Iglewski, 1998; Tummler & Kiewitz, 1999; Huber *et al.*, 2001). Riedel *et al.* utilized both an artificial biofilm flow chamber reactor and alginate bead mouse lung infection model to show that in mixed biofilms *B. cepacia* was capable of recognizing several AHL signals produced by *P. aeruginosa*. Moreover, when *B. cepacia* was grown with AHL-producing *P. aeruginosa* in a biofilm flow chamber, the two species were closely associated and mixed microcolonies formed, however when *B. cepacia* was grown with non-AHL-producing *P. aeruginosa* strains, only separate microcolonies were formed. In addition, it was shown that AHL-mediated communication between these two species occurred during co-infection (Riedel *et al.*, 2001). This finding indicates that AHL-based signaling can influence the architecture of a mixed biofilm community. An *et al.* (2006) reached a similar conclusion after examining interactions between *P. aeruginosa* and *Agrobacterium tumefaciens* in a flow-cell system. The amount of *A. tumefaciens* biomass in the mixed biofilm remained constant when a *P. aeruginosa* quorum-sensing mutant was used that was defective in both the *lasR* and *rhlR* quorum-sensing systems (unable to respond to 3-oxo-C_{12}-HSL and C_4-HSL, respectively), whereas *A. tumefaciens* biomass decreased when co-cultured with wild-type *P. aeruginosa*, compared to the mono-culture biofilm. Notably, in this *P. aeruginosa* mixed biofilm, AHL quorum sensing appeared to impart competitive fitness to *P. aeruginosa* and thus impacted the nature of the multi-species biofilm (An *et al.*, 2006).

It is important to note that mixed-species biofilm phenotypes associated with quorum sensing can be a result of communication between two species or reflect secondary effects of intraspecies communication. A clear example of the latter was observed in model multi-species biofilms comprising *Escherichia coli* and *Serratia plymuthica* grown in flow chambers. When co-cultured, the cell density of *E. coli* was found to depend on the of *S. plymuthica* strain. Specifically, the number of *E. coli* cells in the mixed biofilm with an *S. plymuthica* strain mutated in AHL production was relatively higher than the number in a mixed biofilm with the wild-type *S. plymuthica* strain. Apparently, the *S. plymuthica* quorum-sensing system mediates the production of an antibacterial factor which affects *E. coli* growth and leads to a reduction in the number of competing bacteria (Moons *et al.*, 2006).

Autoinducer peptides are another family of quorum sensing signaling molecules mainly utilized by Gram-positive bacteria. *Streptococcus mutans*, for example, produces a competence-stimulating peptide (CSP) that was found to modulate several virulence factors including biofilm formation (Li *et al.*, 2002). Wang *et al.* (2011) recently demonstrated that when *S. gordonii* was grown with *S. mutans* in polystyrene microtiter plates, the ability of *S. mutants* to form biofilms was impaired. The authors found that *S. gordonii* secreted a protease that degraded

S. mutans CSP and interfered with *S. mutans*' ability to colonize the surface (Wang *et al.*, 2011). Thus peptide signals can mediate interspecies interactions and, more importantly, one species can manipulate quorum-sensing signals to affect the other species' biofilm formation, in this case by decreasing the concentration of the extracellular signaling molecule.

Another quorum-sensing signal recently found to mediate interspecies interactions is a fatty acid signal, termed diffusible signal factor (DSF; Barber *et al.*, 1997). DSF has been identified in several bacterial species including *B. cepacia* (Deng *et al.*, 2010) and *P. aeruginosa* (Davies & Marques, 2009) and was shown to play a role in bacterial virulence, biofilm formation, and antibiotic resistance (Ryan *et al.*, 2009). Moreover, it was demonstrated that DSF molecules influenced the behavior of bacterial species within a mixed biofilm (Ryan & Dow, 2011). For example, in a mixed biofilm comprising *P. aeruginosa* and *Stenotrophomonas maltophilia*, DSF secreted by *S. maltophilia* increased polymyxin resistance and altered biofilm architecture of *P. aeruginosa* grown in a flow-cell system (Ryan *et al.*, 2008). In addition to beneficial effects, DSF can also have an antagonist for impact and inhibit biofilm formation of neighboring species. In bacteria–fungi mixed biofilm, DSF secreted by *Burkholderia cenocepacia* inhibited yeast-to-hyphal transition of *C. albicans* (Boon *et al.*, 2008). This transition is known to be essential for *C. albicans* biofilm formation (Ramage *et al.*, 2002). Although the DSF-based quorum-sensing system is less studied, particularly in the context of mixed biofilms and mixed cultures, the fact that it was identified in numerous species and a variety of niches suggests that it may play a more pivotal role in mediating such interactions than the well-studied AI-2 and AHL signals.

Calling distance and cell aggregation

The spatial heterogeneity and biodiversity in mixed-species biofilms can clearly have a dramatic impact on the ability to communicate as well as on the communication range (i.e. 'calling distance'). Gantner and colleagues determined the calling distance within the rhizosphere (tomato root surface) using *P. putida* AHLs biosensors. Their results showed that colonized bacteria were able to communicate through AHL signals *in situ* (Fig. 2a and b). The effective calling distance was found between cells that were in close proximity of 4–5 μm; however, the maximum calling distance measured was extended to up to 78 μm (Fig. 2d). Interestingly, it was shown that an individual cell produced AHLs sufficient to mediate communication with an adjacent cell (Fig. 2c) even when those two individual cells were separated from a dense population (Fig. 2e; Gantner *et al.*, 2006). In another study that examined the communication range in a dual species model of oral bacteria using a saliva flow-cell biofilm, the authors demonstrated that signaling occurred mainly within, rather than across, cell clusters (Egland *et al.*, 2004). These studies raise the hypothesis that the distance between cells may be more important than the amount of cells present in the environment. Thus, cell aggregation may have a greater effect on the sufficient accumulation of the signal than the population density (Hense *et al.*, 2007).

Synergism vs. antagonism

The close proximity and complex interactions within biofilms underlie both synergistic and antagonistic behaviors. For example, species within a biofilm can compete for nutritional resources or alternatively can coordinate to better utilize nutrients or withstand harsh conditions.

Coexistence and cooperation

Given that mixed biofilms are ubiquitous and found in both ecological and clinical environments, one can assume that synergistic interactions between species predominate over antagonistic ones, particularly synergies that facilitate a robust coexistence (Periasamy & Kolenbrander, 2009). Indeed, there are some species that do not usually form mono-species biofilms, but can participate in mixed-species biofilms. Several studies indicate that oral bacteria rely on interspecies interactions when forming mixed biofilms, with each species playing a distinctive role (Filoche *et al.*, 2004a, b; Sharma *et al.*, 2005; Yamada *et al.*, 2005). *S. mutans* can serve as the initial colonizer of the tooth surface and together with an *Actinomyces* species promote biofilm growth of *Lactobacillus* in an oral mixed-species biofilm (Filoche *et al.*, 2004b). *S. gordonii* is an additional first colonizer on the tooth surface and provides conditions that allow later colonizers including *P. gingivalis*, to adhere. Notably, this co-colonization is not a passive process as *S. gordonii* has been shown to expresses several genes that are required to recruit *P. gingivalis* into the mixed biofilm, including genes involved in biosynthesis of extracellular polymers, cell wall integrity, adhesion, and inter- or intracellular signaling. The genes involved in synthesis of extracellular polymers in *S. gordonii* likely function as co-aggregation receptors for a surface adhesin expressed by *P. gingivalis* (Kuboniwa *et al.*, 2006). Similarly, streptococcal cell wall polysaccharides have been shown to enable other oral bacteria to recognize streptococci, co-aggregate, and ultimately form dental plaque (Cisar *et al.*, 1997; Xu *et al.*, 2003).

The capability of some species to co-aggregate is highly dependent on cell surface components that allow adhesion

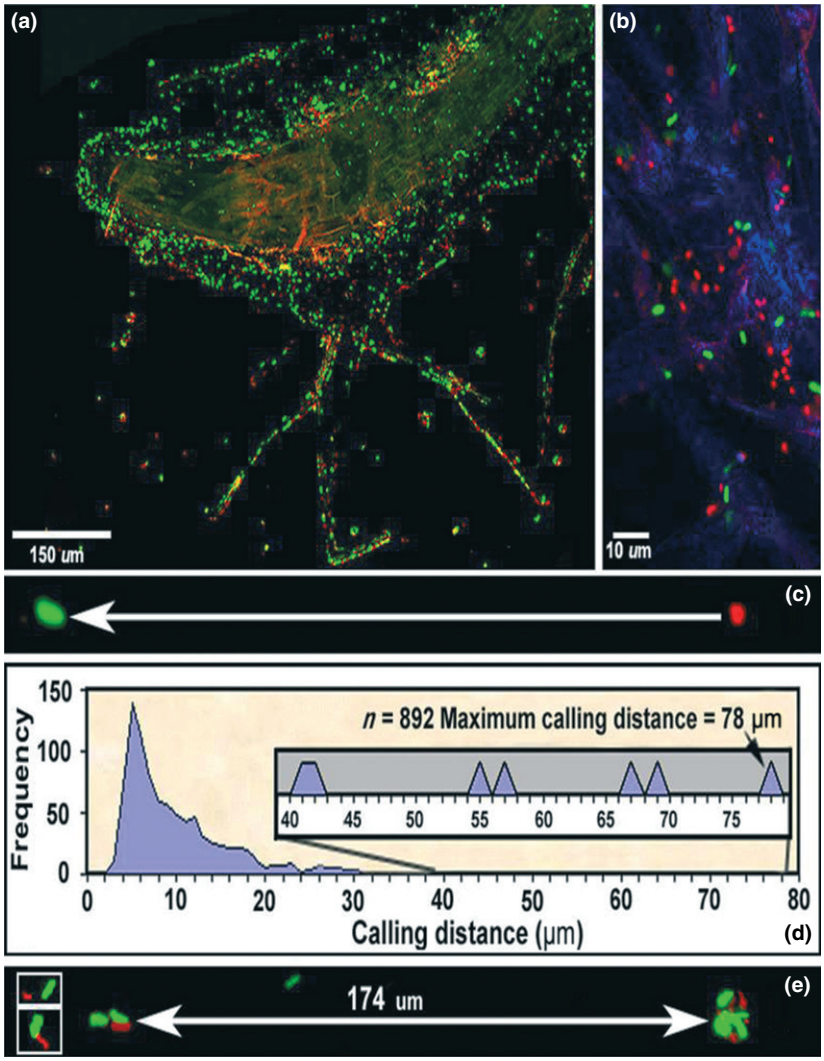

Fig. 2. Calling distance analysis of AHL-mediated communication. (a, b) Confocal laser scanning micrographs of tomato root surface displaying communication between colonized bacteria reporting AHL producers (red) and sensors (green). (c) The distance between two individual communicating bacteria, indicated by an arrow. (d) Frequency histogram displaying the range of calling distances between individual bacteria, longer calling distances are indicated in the insert. (e) AHL-mediated communication between small groups of bacteria separated by long distances from each other and away from a dense population on the root surface. The white-bordered inserts near the left edge of the figure show two examples where the communicating foci consisted of one AHL-source and one AHL-sensor cell taken from areas separated by long distances from dense populations. Distance is indicated by arrow. Figure is reconstructed with permission from Gantner *et al.* (2006).

of one species to the other. Two of the bacterial species that are involved in periodontitis, *P. gingivalis* and *Treponema denticola*, interact synergistically and form a mixed biofilm. In a study carried out by Yamada *et al.* (2005), *P. gingivalis* was the first to colonize the polystyrene substratum and then it co-aggregated with *T. denticola*. The flagella (*flgE*) and cytoplasmic filaments (*cfpA*) in *T. denticola* were found to be essential for this co-aggregation. Similarly, in *P. gingivalis*, the major fimbriae (*fimA*) play an important role in mixed biofilm formation (Yamada *et al.*, 2005). Thus, motility organelles can serve as mediators of multi-species biofilm formation.

The ability of oral bacteria to co-aggregate is a key driving force that shapes the nature of the oral biofilm. However, the phenomenon that one bacterium colonizes the surface, thus providing conditions that allow additional species to form a mixed biofilm is not limited to oral multi-species biofilms (Leung *et al.*, 1998). It was found that mixed biofilm formation by *Enterococcus* relies on the presence of *E. coli*, which has a higher affinity and thus adheres more easily to surfaces. *E. coli* cells were observed to attach initially to a plastic stent surface, facilitating the attachment of *Enterococcus* and subsequent formation of a mixed biofilm (Leung *et al.*, 1998).

Colonization and aggregation represent one instance of cooperation in a mixed biofilm. Another example is metabolic cooperation, such as nutrients that are metabolized by one species and their byproduct is utilized by another. This phenomenon is often referred to as metabolic commensalism and was evidenced using a flow-cell mixed biofilm model composed of *Pseudomonas putida* and *Acinetobacter*. Both of the studied strains can utilize benzyl alcohol as their sole carbon and energy source; thus, an interaction between these two species could entail competition over the same nutrient source. However, when mixed biofilms are grown on benzyl alcohol, *Acinetobacter* produces benzoate that is then metabolized by *P. putida*, and thus, cooperation, and not competition, is the predominant interaction. Furthermore, this characteristic metabolic interaction was found to determine the structure of the mixed biofilm. Namely, *Acinetobacter* resides in the upper layers of the biofilm close to the nutrient source, whereas *P. putida* resides in the lower layers (close to the glass surface), allowing it to benefit from the benzoate secreted from the *Acinetobacter* (Christensen *et al.*, 2002).

Metabolic interactions were also reported for bacteria residing within the human oral cavity. It was demonstrated that *Veillonella* sp. utilized lactic acid produced by *S. oralis* from fermentation of sugars (Periasamy & Kolenbrander, 2010). Two other oral bacteria, *A. naeslundii* and *S. gordonii*, were shown to interact metabolically through arginine. Apparently when these two species co-aggregate, genes involved in arginine biosynthesis were induced in *S. gordonii.* In the absence of a sufficient arginine concentration in the medium, *S. gordonii* could not grow and was only able to grow following co-aggregation with *A. naeslundii* (Jakubovics *et al.*, 2008).

Resistance to host defenses and antimicrobial agents

From a human perspective, one of the most alarming consequences of synergistic interactions between microorganisms is associated with resistance to antimicrobial agents (Bennett, 2008). Within mixed biofilms, such resistance does not necessarily involve direct acquisition or transfer of genes encoding antibiotic resistance, but rather relies on the ability of the community to cooperate in such a way that it can survive exposure to the antimicrobial agent. Several studies have evaluated the antibiotic susceptibility of multi-species vs. mono-species biofilms, and in most cases, the mixed-species biofilm was significantly more resistant to antimicrobial treatment or disinfection (Whiteley *et al.*, 2001; Leriche *et al.*, 2003; Al-Bakri *et al.*, 2005; Burmolle *et al.*, 2006; Kara *et al.*, 2006).

One mechanism of enhancing antibiotic resistance involves change in the composition of the EPS matrix. Although some EPS components are common to most biofilms, the composition of the EPS matrix varies greatly depending on the bacterial species and environmental conditions (Flemming & Wingender, 2010). Accordingly, the composition of the EPS is different in mono- vs. multi-species biofilms and the latter can provide a better defense against antimicrobial treatments. This phenomenon was demonstrated using fungi and bacteria, *C. albicans* and *Staphylococcus epidermidis,* respectively, two pathogens found in catheter-associated infections that form a mixed biofilm (Adam *et al.*, 2002). *S. epidermidis* strains are known to produce extracellular polymers, or slime, which contribute to biofilm formation and antibiotic resistance. Accordingly, the mono-species biofilm formed by a slime-negative strain on polyvinyl chloride catheter disks was considerably more susceptible to vancomycin treatment than the wild-type strain. However, it was found that when a slime-negative *S. epidermidis* strain formed a mixed biofilm with *C. albicans*, it exhibited enhanced resistance to vancomycin. In addition, the EPS produced by wild-type *S. epidermidis* was shown to inhibit penetration of the antifungal drug fluconazole in the mixed *C. albicans-S. epidermidis* biofilm (Adam *et al.*, 2002). Thus, in this mixed biofilm, both the bacterium and the fungus benefit, displaying enhanced survival in the face of antibiotic challenges.

It is important to note that not all community members necessarily benefit from participation in a mixed-species biofilm. For example, Elvers and colleagues examined how a biofilm comprising three bacterial species and three fungal species grown on a glass surface in flow-cell system responded to a particular biocide relative to each mono-species biofilm. While all three bacterial species were observed to exhibit improved biocide resistance when present in the mixed biofilm, the fungal species were less resistant when present in the mixed biofilm. Therefore, in this instance, participation in the mixed biofilm provided protection specifically to bacterial community members (Elvers *et al.*, 2002).

Another mechanism whereby species within a mixed biofilm cooperate to survive in hostile environments involves one member providing conditions that promote survival of other members. For example, anaerobic bacteria are sensitive to oxygen, yet are able to survive and persist under aerobic conditions when grown in the presence of aerobic bacteria in a mixed biofilm (Bradshaw *et al.*, 1996, 1997). Their survival is enabled because the aerobic bacteria consume the oxygen and thus provide anaerobic conditions within the deeper layers of the biofilm in which anaerobic bacteria can multiply (Sbordone & Bortolaia, 2003).

A third way that species within a mixed biofilm cooperate to survive under challenging conditions is speculated to involve one member inducing transient changes in resistance in proximal neighbors. For example, *S. mutans* survives exposure to various antibacterial agents more successfully when present in a mixed biofilm with *Veillonella parvula* than as a mono-species biofilm. This enhanced survival was demonstrated using diverse antibacterial compounds with distinct modes of action including chlorhexidine, hydrogen peroxide, erythromycin, and zinc chloride (Kara *et al.*, 2006). Transcription analysis revealed that *V. parvula* induced changes in gene expression within *S. mutans*. In light of this finding, the authors hypothesized that one species residing within a mixed biofilm can significantly alter the physiology and enhance the antibiotic resistance of neighboring species (Luppens *et al.*, 2008). Considering the troubling increase in antibiotic resistance and the prevalence of mixed-species biofilms, a better understanding of such interspecies interactions is urgently required and will have significant therapeutic impact. Indeed, the suspected impact of such interactions on the resistance of individual community members to antibiotic therapy should be taken into consideration in the clinic even today.

Living in a mixed community can offer protection against threats other than antimicrobial agents. *Pseudoalteromonas tunicata* is able to aggressively replace resident bacteria in established biofilms through production of the broad-range antibacterial protein, AlpP (Rao *et al.*, 2005). However, Burmolle *et al.* (2006) found that a multi-species biofilm composed of four bacterial strains withstood invasion by *P. tunicate* more effectively than mono-species biofilms comprising one of the four member strains. Replacing each of the four members in turn with its supernatant revealed that for two of the members the presence of the bacteria itself was required for this synergistic protective effect, suggesting that physical properties of the cells may be involved. In contrast, the other two strains could be replaced by their corresponding supernatant indicating that secreted compounds likely mediate their synergistic protective effect.

Finally, interspecies interactions can also bolster resistance to host innate immune responses. This phenomenon is observed in the lungs of CF patients, which are often colonized by several bacterial species that reside in biofilms and cause persistent infections (Stewart & Costerton, 2001; Bittar *et al.*, 2008; Armougom *et al.*, 2009). Two of the most common pathogens found in CF lungs are *Staphylococcus aureus* and *P. aeruginosa*; these two species interact synergistically. It appears that an exoproduct of *P. aeruginosa* enhances aminoglycoside resistance by triggering *S. aureus* either to become or to selectively grow as small colony variants (SCVs; Hoffman *et al.*, 2006). In addition to aminoglycoside resistance, the *S. aureus* SCVs also display reduced susceptibility to other antibacterial agents and increased ability to form biofilms (Samuelsen *et al.*, 2005; Singh *et al.*, 2009). Similarly, interactions between two oral bacteria, *S. gordonii* and *Aggregatibacter actinomycetemcomitans*, enhanced the resistance of *A. actinomycetemcomitans* to host innate immune response. Ramsey and Whiteley demonstrated that this resistance is mediated by the metabolite H_2O_2 produced by *S. gordonii*, which induced expression of a complement resistance protein (ApiA) in *A. actinomycetemcomitans* grown in flow-cell biofilm reactors (Ramsey & Whiteley, 2009).

In summary, cooperative interactions between different species can promote survival and biofilm growth of one or more neighboring species under challenging conditions, such as antibiotic agents, hostile environments, invading organisms, and host immune responses. The mechanisms underlying cooperation are diverse and include altering the composition of the EPS matrix, reducing the hostility of the environment, and inducing expression changes in proximal neighbors; the latter is discussed further below.

Competition and growth inhibition

The focus of this review is tended toward beneficial interactions; however, interspecies interactions are not always beneficial and can be competitive (for detailed review of inhibitory interactions see the accompanying paper by RenDueles and Ghigo appearing in this issue). Competition between species is the fundamental driving force underlying evolution and often plays a central role in defining the structure and activity of a multi-species community. When bacterial species are crowded together and resources are limited, members of a biofilm community are more prone to competition. Often, one species will invade a specialized nutritional niche already occupied by another species with similar nutritional requirements. Sometimes competition will involve one species actively inhibiting the growth of others, by producing inhibitory compounds or consuming essential nutrients.

Production of inhibitory agents is an important factor in determining the dominant species within a mixed biofilm and consequently, the architecture of the community. In evolutionary terms, these agents provide a competitive advantage over neighboring microorganisms. Antibiotic-producing bacteria and fungi are found in a variety of ecological niches inhabited by various microorganisms. One such niche is the surface of the marine plant *Ulva lactuca*, where mixed-species biofilms develop. In this particular multi-species biofilm, a marine bacteria called *P. tunicata* outcompetes other species. A detailed

study revealed that this domination of *P. tunicata* was mediated not only by the capability to outgrow competitors during establishment of the biofilm but also by the production of an antibacterial protein called AlpP. Briefly, it was observed that when added to an already formed mixed biofilm (grown in glass flow-cell reactor), a mutant *P. tunicata* strain deficient in AlpP production was less competitive than the wild-type strain and did not remove resident species and, unlike the wild type, allowed formation of mixed biofilms that were stable for extended periods of time. In contrast, when the mixed bacterial species were inoculated simultaneously, the *alpP* mutant *P. tunicata* strain was dominant, in a manner similar to wild type (Rao *et al.*, 2005).

The oral cavity represents another niche where production of an inhibitory protein enables one species to dominate in a mixed biofilm. Specifically, when grown in a 96-well microtiter plate, *Enterococcus faecium* produced an inhibitory protein that restricted biofilm formation by three oral streptococci, *Streptococcus sobrinus*, *Streptococcus sanguinis* and, the leading cause of dental caries, *S. mutans* (Kumada *et al.*, 2009).

Although most multi-species biofilms exhibit an increase in antibiotic resistance relative to the mono-species biofilms, competition within a mixed biofilm can occasionally result in increased sensitivity of the mixed biofilm to antimicrobial agents. For example, a mixed biofilm comprising two enteric bacteria *Enterobacter agglomerans* and *Enterobacter gergoviae* was found to be smaller in size and more sensitive to antimicrobial agents than that formed by each bacteria alone (Tait & Sutherland, 2002). It seems likely that as both species produce bacteriocins that potentially impact the other species, addition of an external antimicrobial agent presents a great burden.

Not surprisingly, competition among species within a mixed biofilm can be influenced by environmental conditions. For example, the oral streptococci *S. sanguinis* and *S. gordonii* inhibit growth of other oral bacteria, including *S. mutans*, by producing hydrogen peroxide (H_2O_2) in mixed biofilms (Kreth *et al.*, 2005). Hydrogen peroxide production is triggered by aerobic conditions; however, the presence of oxygen also stimulates *S. mutans* to release antistreptococcal bacteriocins, allowing the bacteria to initiate a counterattack. Thus, the availability of oxygen affects competition between oral bacteria and impacts the composition of the community (Kreth *et al.*, 2008).

Secretion of antibacterial compounds is not the only approach utilized by bacteria to overcome competitors in mixed biofilms. Surface motility mediated by motility organelles represents another important parameter that not only shapes the spatial distribution within a mixed biofilm, but can also enable one species to outcompete others. Indeed, when *P. aerguinosa* and *A. tumefaciens* were co-cultured in flow-cell reactor system, *P. aeruginosa* dominated by covering the surface-attached *A. tumefaciens* cells, a phenomenon termed 'blanketing'. The ability to 'blanket' depended on the surface motility of *P. aeruginosa*, likely reflecting the capacity to climb on top of clusters of *A. tumefaciens* cells and form a biofilm there. Notably, *A. tumefaciens* was observed to use surface motility to migrate away from the co-culture where it was outcompeted and form its own biofilm elsewhere (An *et al.*, 2006).

In summary, interactions between species within a mixed biofilm are influenced by several factors, including production of antibacterial agents, metabolic requirements, and environmental conditions. Any change in one or more of these factors can dramatically impact the structure and dynamics of the biofilm community.

Spatial distribution

An interesting observation that comes from studies of mixed-species communities is the spatial organizations within the biofilm. It seems that there are three general forms in which the bacteria are organized (Fig. 3). The first is the formation of single-species microcolonies, where each species forms a separate microcolony side by side. For example, it was shown that in a mixed biofilm of *Burkholderia* sp. and *Pseudomonas* sp. grown in flow-cells, the formation of these separate microcolonies was the result of a noncommensal interaction between the two species and that this interaction was dependent on available nutritional resources (Nielsen *et al.*, 2000). The other forms include co-aggregation, where cells of both species are mixed and can be found together throughout the biofilm and a layered structure, where one species can be found in the upper layers and another species can be found in the lower layers of the biofilm. The layered-biofilm structure can be either synergistic, as seen when *Acinetobacter* was present in the upper layers and *P. putida* in the lower layers of the biofilm in the flow chamber, allowing effective metabolic interaction (Christensen *et al.*, 2002), or competitive such as when *P. aeruginosa* outcompeted and covered *A. tumefaciens* in a mixed biofilm (An *et al.*, 2006). In their study, An *et al.* set the term 'blanketing' for this phenomenon; however, considering that this spatial organization is not always used in interspecies competition but can also increase the overall productivity of the community, we suggest the term 'layering'. It is interesting to note that such 'layering' is also found in microbial mats where the microbial species are organized according to their metabolic and energetic properties (Seckbach & Oren, 2010). It should

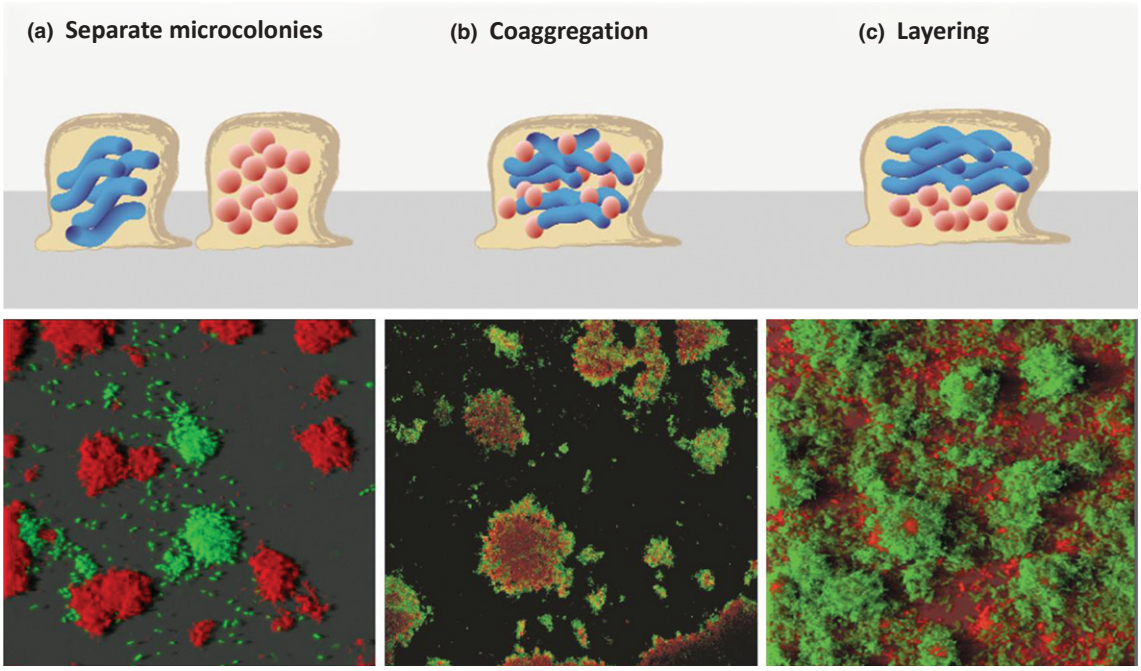

Fig. 3. Spatial distribution within mixed-species biofilms. Species in mixed biofilms can organize in several ways: (a) separate mono-species microcolonies [confocal microscopy image reconstructed with permission from Nielsen *et al.* (2000)]; (b) co-aggregation [confocal microscopy image reconstructed with permission from Rickard *et al.* (2006)]; (c) arranged in layers [confocal microscopy reconstructed with permission from Hansen *et al.* (2007b)]. For details see relevant section in the text.

be emphasized that it is still unclear what processes govern the formation of these three different architectures and whether or not they provide the community with different phenotypic properties. It is reasonable to assume that the interaction within biofilms composed of separate microcolonies (each composed of a different mono-species) can be dramatically different compared to the interaction occurring within co-aggregated biofilms (e.g. calling distance of quorum-sensing molecules, both intra- and interspecies). Indeed, Egland and colleagues have shown that interspecies interactions, in some cases, do require direct contact between cells. Their work demonstrated that in an open system (i.e. flow-cell), where signals do not accumulate, the induction of *S. gordonii* α-amylase gene expression by *Veillonella atypica* occurred only in mixed microcolonies and was not induced in microcolonies that contained only *S. gordonii*, even when these microcolonies were only a few micrometers away from microcolonies of *Veillonella*. This suggests that a threshold signal concentration by *S. gordonii* requires a very close proximity of less than 1 μm which only occurs when the cells are co-aggregated (Egland *et al.*, 2004). Thus, the species spatial distribution adds yet another layer of complexity when examining possible interactions within the community.

Differences in gene expression and cellular response

Changes in gene expression during establishment of mixed biofilms provide a more comprehensive view of interactions occurring between species. Altered gene expression patterns potentially reflect synergistic as well as antagonistic interactions and also environmental cues, which together shape the architecture and function of the multi-species biofilm.

Several studies have monitored changes in gene expression during the transition from a mono-species biofilm to mixed-species biofilm. As described above, when the two oral bacteria *V. parvula* and *S. mutans* were co-cultured, *V. parvula* induced changes in *S. mutans* physiology and *S. mutans* displayed increased resistance to antimicrobial agents. Various *S. mutans* genes were observed to exhibit altered expression upon co-culturing, including those involved in purine metabolism, amino acid metabolism, protein synthesis, and intracellular polysaccharide metabolism (Luppens *et al.*, 2008). As mentioned above, alterations in the expression of metabolism-related genes during bacterial co-aggregation were reported in another dental biofilm model. During co-aggregation with *A. naeslundii*, the expression of genes

involved in arginine biosynthesis was induced in *S. gordonii*. Moreover, *S. gordonii* growth in the absence or in the presence of a low concentration of arginine was dependent upon the formation of co-aggregates with *A. naeslundii* (Jakubovics *et al.*, 2008). Similarly, in the presence of *S. aureus*, *P. aeruginosa* was shown to produce significantly higher amounts of exotoxin A (Goldsworthy, 2008). In this instance, formation of the mixed biofilm triggered alterations in gene expression that resulted in the expression of a virulence gene. Of note, from a clinical perspective, because these latter two microorganisms mediate various infections, the resulting increase in bacterial pathogenicity is a disturbing change in phenotype. It is important to emphasize that *S. aureus* and *P. aeruginosa* do not always mediate synergistic interactions and in some cases, when co-cultured, *P. aeruginosa* outcompetes *S. aureus* (Biswas *et al.*, 2009).

Interactions within mixed biofilms can lead, in some cases, to evolution of species variants. Hansen and colleagues used a two-species community composed of *P. putida* and *Acinetobacter sp.* grown in a flow-cell with benzyl alcohol as the sole carbon source as described earlier. However, the *P. putida* strain used in this study did not utilize benzyl alcohol and was dependent on the presence of *Acinetobacter*. The researchers observed that after several days of intimate contact between *P. putida* and *Acinetobacter* sp., an evolutionary variant of *P. putida* emerged described as 'rough colony variants'. Moreover, this phenotype was heritable and a specific gene was found to be mutated (Hansen *et al.*, 2007b). These mutants covered the *Acinetobacter* biofilm colonies more effectively and thus associated and formed a mixed biofilm more easily with *Acinetobacter* than the ancestral strain. In addition, the rough variants exhibited reduced dispersion in response to oxygen starvation and displayed enhanced production of a cellulose-like polymer that likely mediates both the nondispersal phenotype and their ability to cover *Acinetobacter*. Evolution of these rough *P. putida* variants is clearly an adaptive response to the physical environment, in this instance, the biofilm mode of growth and, more specifically, the presence of *Acinetobacter* that ultimately leads to a more stable and productive community. The observation of species evolution within a biofilm community indicates that spatial structure plays a key role in the determination of species interaction (Hansen *et al.*, 2007a).

Another example of variant species evolution occurs when *S. aureus* and *P. aeruginosa* interact. This interaction was mentioned earlier in the context of synergism that enables survival in the face of biocides. The presence of *P. aeruginosa* induces the emergence of SCVs of *S. aureus*, the induction mediated by secretion of 2-heptyl-4-hydroxyquinoline N-oxide (HQNO) by *P. aeruginosa* (Hoffman *et al.*, 2006), an exoproduct that inhibited the growth of many Gram-positive bacteria including *S. aureus* (Machan *et al.*, 1992). The HQNO activated alternative sigma factor B in *S. aureus*, which altered expression of several virulence factors, including those that regulated the ability to adhere, invade, and persist within host cells, and facilitated emergence of the SCV phenotype (Mitchell *et al.*, 2010). Both HQNO and SCVs of *S. aureus* are found in CF lungs, indicating that evolution of these variants does indeed occur within the human host. Biswas and colleagues have suggested that the formation of *S. aureus* SCVs is a survival strategy to withstand competition by *P. aeruginosa* (Biswas *et al.*, 2009). In any case, this final mixed biofilm example underscores the assertion that interactions between species within biofilms should be taken into account when designing and choosing therapies.

Conclusion

Studies of mixed biofilms are beginning to unravel the complexity of interspecies interactions and their impact in clinical and environmental settings. However, not only the microbial participants but also the environmental conditions in the niche determine the shape and phenotype of a mixed biofilm. It appears from studies carried out to date that the key parameters governing interspecies interactions are the ability to communicate and nutritional requirements. Interactions can be synergistic or antagonist and underlie diverse biofilm phenotypes, including altered gene expression, changes in motility, altered antibiotic resistance, and spatial distribution (these processes are summarized in Fig. 4).

It is clear that microbial life on earth is heavily biased toward multi-species communities, such as mixed biofilms. Microbial ecologists have been addressing this for quite some time, as exemplified by the active research into microbial mats. In contrast, medical microbiologists have, for many years, focused on studying mono-cultures of free-living microorganisms. Only recently has the prevalence of mixed-species biofilms and their involvement in various infections been appreciated. This appreciation has highlighted the need for a better understanding of the interactions and dynamics within these mixed communities, which is necessary to successfully prevent or treat infections involving mixed biofilms. Today, mixed infections are often treated using broad-spectrum antibiotics. In light of current knowledge described here, namely, that interactions between species influence the antibiotic resistance and pathogenicity of a mixed community, the composition of a mixed biofilm should be a key consideration when determining the course of future treatments.

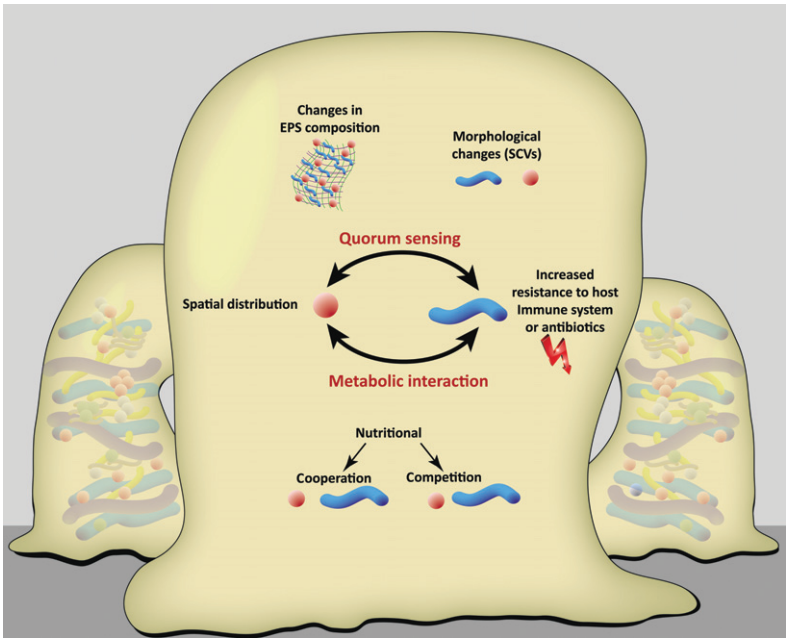

Fig. 4. Individual and social processes occurring within biofilm communities. Microorganisms within a mixed biofilm interact via quorum sensing and/or metabolically. Interactions can be synergistic or antagonistic and result in phenotypic changes, such as increased resistance to antimicrobial agents or to host defense systems, spatial distribution or emergence of variants (SCVs). Nutritional interaction can be either competitive or cooperative. It should be emphasized that in most cases, mixed-species interactions are driven by physiological processes. However, genetic changes have been reported in SCVs and in a few cases of antibiotic resistance and metabolic interactions (see details in the text).

Studies examining mono-species biofilm development have certainly advanced our knowledge of the processes that govern biofilm formation both on the molecular and physiological level. Although to date only a limited number of studies have addressed mixed-species biofilms, they suggest that growing with neighbors is, in most cases, advantageous to the productivity of the community. A major question that still remains unanswered is how relevant are findings based on mono-species cultures to the understanding of mixed-species biofilm communities, when it is clear that crucial driving forces such as competition over nutrients and signal manipulations are usually not an integrated part of the mono-species system. Future work will have to address this important question.

In most of the studies discussed in this review, biofilm cultures were usually grown under artificial conditions using static or flow-cell systems with abiotic surfaces (e.g. glass or polystyrene). Although these models provide controlled and reproducible conditions, different processes and mechanisms may be involved in biofilm formation under natural conditions. For example, it was shown that biofilm formation by *P. fluorescens* on abiotic surfaces and on the rhizoplane involved different genes. Strains mutated in three regulatory genes and a hypermotile isolate from the rhizosphere were defective in biofilm formation on abiotic surfaces but efficiently colonized the rhizosphere (Barahona *et al.*, 2010). Thus, there is a growing need to move studies into more 'real-life' conditions both in terms of the surfaces and growth conditions that are implemented.

Our understanding of the interactions that occur within mixed-species biofilms is still very limited, but these are exciting times as the borders between microbial ecology and medical microbiology begin to fade away. The ability of these two disciplines to join forces is crucial, as clearly one of the main challenges will be to develop new tools that allow us to intimately dissect the complex processes that occur within mixed communities. In fact, it is most likely that the technical difficulties associated with studying mixed-species biofilms are the reason for the fairly limited number of studies in this field. These include the ability to maintain the desired concentration of each member in the consortia to obtain reproducible results, as well as to track and separate between the different members of the mixed biofilm. Breakthroughs in genomics, proteomics and microscopy have certainly advanced our ability to study mixed biofilms, but the field is still awaiting innovative tools that will facilitate molecular and biochemical characterization on a single cell level within the context of mixed communities and allow us to finally decipher exactly who is doing what.

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