

REVIEW

Current understanding of multi-species biofilms

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Direct observation of a wide range of natural microorganisms has revealed the fact that the majority of microbes persist as surface-attached communities surrounded by matrix materials, called biofilms. Biofilms can be formed by a single bacterial strain. However, most natural biofilms are actually formed by multiple bacterial species. Conventional methods for bacterial cleaning, such as applications of antibiotics and/or disinfectants are often ineffective for biofilm populations due to their special physiology and physical matrix barrier. It has been estimated that billions of dollars are spent every year worldwide to deal with damage to equipment, contaminations of products, energy losses, and infections in human beings resulted from microbial biofilms. Microorganisms compete, cooperate, and communicate with each other in multi-species biofilms. Understanding the mechanisms of multi-species biofilm formation will facilitate the development of methods for combating bacterial biofilms in clinical, environmental, industrial, and agricultural areas. The most recent advances in the understanding of multi-species biofilms are summarized and discussed in the review.

Keywords: biofilms; extracellular polymeric substances; structure development; interactions

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Introduction

Microorganisms tend to form surface-attached biofilm communities as one of the most important survival strategies in different environments. Biofilms consist of microbial cells and a wide range of self-generated extracellular polymeric substances (EPS), including polysaccharides, nucleic acids, and proteins. Biofilm formation is a dynamic process, which is coordinated by the interactions of different microbial species. Biofilm formation plays an important role on our ecological systems. Meanwhile, biofilm formation causes also many problems in our daily lives, from persistent infections to clogging of pipelines. There has been an explosive increase of biofilm knowledge in the last two decades. However, most of the mechanisms regarding biofilm formation are

revealed by means of studying mono-species biofilms. It is a major intention of this short review to summarize the recent advances in the understanding of multi-species biofilms.

Localization and composition of multi-species biofilms

Biofilms are found in natural and industrial aquatic environments, tissues, and medical biomaterials and devices [1]. Multi-species biofilms represent the most important lifestyles of microorganisms in nature. The bacterial species in multi-species biofilms vary a lot, depending on their environment. Table 1 shows some examples of reported multi-species biofilms from nature and infected foci. It is crucial for researchers to identify the species in multi-species biofilms, so that we can better understand and manipulate the functions of biofilms.

Bacterial cells are embedded in EPS in biofilms. EPS are mainly secreted by bacterial cells, which protect bacterial cells from hostile environments such as treatment by antimicrobial agents, UV radiation, and protozoan predation [2]. The chemical composition of EPS is

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very complicated and it changes with the growth stages and environment of the microbes. In general, EPS contain polysaccharides, proteins, lipids, extracellular DNA (eDNA). and metal ions. Disrupting EPS matrix is an

effective approach for biofilm eradication and prevention. For example, eDNA is widely present among multi-species biofilms [3], DNase treatment is now proposed as a way to control biofilm related infections [4-6].

Table 1 Distribution of multiple-species biofilm

Localization	Species	Reference
Marine sediments	<i>Desulfosarcina variabilis</i> , <i>Desulfocapsa sulfoexigens</i> , <i>Nitrospina gracilis</i> , <i>Vibrio splendidus</i> , <i>Pseudoalteromonas</i> sp., <i>Arhodomonas aquaeolei</i> , <i>Anodontia phillipiana</i> , <i>Lucina pectinata</i> , <i>Riftia pachyptila</i> , <i>Alvinella pompejana</i> , <i>Verrucomicrobium</i> sp.	[7]
Chronic wounds	<i>Corynebacterium</i> sp., <i>Bacteroides</i> , <i>Peptoniphilus</i> , <i>Fingoldia</i> , <i>Anaerococcus</i> , <i>Peptostreptococcus</i> sp., <i>Streptococcus</i> , <i>Serratia</i> , <i>Staphylococcus</i> , <i>Enterococcus</i> sp.	[8]
Urinary catheter	<i>Staphylococcus epidermidis</i> , <i>Enterococcus faecalis</i> , <i>Escherichia coli</i> , <i>Proteus mirabilis</i> , <i>Pseudomonas aeruginosa</i> , <i>Klebsiella pneumoniae</i>	[9]
Dental plaque	<i>Streptococcus</i> , <i>Peptostreptococcus</i> , <i>Neisseria</i> , <i>Veillonella</i> , <i>Actinomyces</i> , <i>Bifidobacterium</i> , <i>Corynebacterium</i> , <i>Eubacterium</i> , <i>Lactobacillus</i> , <i>Propionibacterium</i> , <i>Rothia</i> , <i>Campylobacter</i> , <i>Eikenella</i> , <i>Fusobacterium</i> , <i>Haemophilus</i> , <i>Leptotrichia</i> , <i>Prevotella</i> , <i>Porphyromonas</i> , <i>Selenomonas</i> , <i>Treponema</i>	[10]
Industrial bioreactor sludge	<i>Desulfobulbus propionicus</i> , <i>Desulfosarcina variabilis</i> , <i>Desulfovibrio fructosivorans</i> , <i>Desulfovibrio aminophilus</i> , <i>Desulfotomaculum geothermicum</i> , <i>Desulfotomaculum nigrificans</i> , <i>Flavobacterium</i> , <i>Chryseobacterium</i> sp.	[11]

Methods for study of multiple-species biofilms

Various non-cultivation-based and cultivation-based approaches have been developed to identify microbial species and investigate bacterial physiology in the multi-species biofilms.

Ribosomal amplification, cloning and sanger sequencing - based assays are the most routing and high-throughput method to study multi-species biofilms. For example, Noguchi *et al.* have identified 113 biofilm-forming bacterial species on root surfaces outside the apical foramen and associated with refractory periapical periodontitis [12]. More often, the denaturing gradient gel electrophoresis (DGGE) method is employed to separate PCR-amplified community 16S rRNA on the basis of G+C content [13]. This method is widely used by different research groups to describe the microbial diversity and phylogenetic affiliation, and to identify individual species in multi-species biofilms [14-17]. For more details of the application of DGGE in microbial ecology studies, please refer to the review [18]. The traditional PCR-based 16S rRNA assays are difficult to quantify accurately the number of microorganisms because the assays are evaluated after gene amplification is completed. To overcome this drawback, a real-time PCR assay using a TaqMan probe, which is a fluorescent DNA probe based on the 5' to 3' exonuclease activity of

Taq polymerase, has been developed and applied for quantitative analysis of multi-species biofilms [19-20].

Fluorescence *in situ* hybridization (FISH) in combination with epifluorescence and confocal laser scanning microscopy (CLSM) is another standard method to identify and visualize microbial species in the multi-species biofilms. FISH is an accurate and quantitative while relatively low-throughput method to study multi-species biofilms. It can be used to analyze the composition and localization of microbial species biofilms from both natural environments and artificial biofilm models. Al-Ahmad *et al.* have reported using five-colour multiplex FISH to analyze the *in vivo* dynamics of *Streptococcus* sp., *Actinomyces naeslundii* (*A. naeslundii*), *Fusobacterium nucleatum* (*F. nucleatum*) and *Veillonella* sp. in dental plaque biofilm [21]. Malic *et al.* have used peptide nucleic acid fluorescent *in situ* hybridization (PNA FISH), which uses uncharged DNA analogue (pseudopeptide) probes with higher specificity and improved hybridization kinetics [22], to detect and characterize the spatial distribution of *Pseudomonas aeruginosa* (*P. aeruginosa*), *Staphylococcus aureus* (*S. aureus*), *Streptococcus* sp. and *Micrococcus* sp. in biofilms formed at human chronic skin wounds [23]. FISH was also employed by Oosterhof *et al.* to study mixed fungal and bacterial biofilms on tracheoesophageal shunt prostheses [24]. Not only in identification of microbial

species, FISH based methods can also be used to estimate the physiological states of microbial cells. In a study of biofilms from the sputum of cystic fibrosis (CF) patients, Yang *et al.* used FISH to indirectly measure the growth rates of *P. aeruginosa* [25].

Recently, high-throughput system biology tools have been employed to study multi-species biofilms. Random shotgun DNA sequencing has been used to characterize a natural acidophilic biofilm and reconstruct the near-complete genomes of *Leptospirillum* group II and *Ferropasma* type II, and partial genomes of three other species [26]. Yergeau *et al.* have developed anonymous DNA microarrays to perform large-scale metatranscriptomic studies of response of river biofilms to antibiotics [27]. By combining genomic and mass spectrometry-based proteomic methods, Ram *et al.* have examined gene expression and partitioning of metabolic functions in a natural acid mine drainage (AMD) microbial biofilms [28].

Artificial biofilm model systems are used frequently by researchers to perform more specific and reproducible biofilm studies. For example, in the flow-chamber biofilm cultivation system, bacteria tagged by fluorescent proteins are inoculated into small glass chambers and monitored throughout biofilm development by using CLSM [29]. In the flow-chamber system, the physiology of biofilms can be well characterized by using molecular biology, biochemistry, immunology, and other approaches [30-32].

Structure development of multiple-species biofilms

Bacterial species interact extensively with each other and these interactions determine the structure development of multi-species biofilms.

Coaggregation interactions are believed to contribute to multi-species biofilm formation in different environments [33]. Early in the 1970s, coaggregation was already demonstrated to be a common phenomenon between isolates from dental plaque [34-35]. Coaggregation has been detected between hundreds of the culturable oral bacteria, and has been proposed as fundamental process during dental plaque biofilm formation [36]. EPS is accepted as an "intercellular cement" to strengthen adhesion between cells and mediate sequenced coaggregation during multi-species biofilm formation [37].

Bacterial cell surface protein adhesins play important roles for coaggregation during multi-species biofilm formation. In the primary dental plaque biofilm colonizer *Streptococcus oralis* (*S. oralis*) DL1, five distinct adhesins are expressed and responsible for coaggregation with other species in dental plaque [38-41]. Protein adhesins

are widely distributed among bacteria; thus, adhesion mediated coaggregation may be one of the major strategies for multi-species biofilm formation. For example, protein adhesins are also observed in fungi and can mediate fungi-bacteria interactions [42-43]. The *S. oralis* SspB adhesin was reported to interact with cell wall Als3 protein of *Candida albicans* (*C. albicans*) and promote development of fungal-bacterial multi-species communities [44].

Bacterial surface pili, flagella, and their mediated motilities are essential for multi-species biofilm formation. Type IV pili and flagella of *P. aeruginosa* are required for *P. aeruginosa* to bury immature *Agrobacterium tumefaciens* microcolonies and gain growth advantage in multi-species biofilms [45]. Our group recently showed that *P. aeruginosa* type IV pili mediate multi-species microcolony formation with *S. aureus* in multi-species biofilms, and this process is dependent upon the binding of type IV pili to the eDNA. Beside type IV pili, conjugative pili are also found to promote multi-species biofilm formation. Pereira *et al.* showed that F pili expressed by enteroaggregative *Escherichia coli* (*E. coli*) boost biofilm formation when in the presence of aggregative *Citrobacter freundii*, and the formation of this diarrhea-associated multi-species biofilm can be inhibited by zinc, a specific inhibitor of F pili [46]. Reisner *et al.* showed that conjugative plasmid transfer between genetically diverse strains of *E. coli* enhances biofilm formation in their co-cultures, probably through the surface exclusion functions [47]. This study also highlights that horizontal gene transfer may be enhanced during multi-species biofilm formation [48].

Interactions in multi-species biofilms

The structural and functional dynamics of multi-species biofilms are largely due to the interactions between different species of microorganisms. These interactions often change the physiology of biofilm species as well as the functions of the whole community. Jakubovics *et al.* have used DNA microarray to systematic search *Streptococcus gordonii* (*S. gordonii*) genes regulated in response to coaggregation with *A. naeslundii* in multi-species dental plaque biofilm. In this study, they found that 9 *S. gordonii* genes involved in arginine biosynthesis and transport are highly induced in coaggregates, but not in co-cultures with *A. naeslundii*, which enables aerobic *S. gordonii* growth when exogenous arginine is limited [49]. Wen *et al.* reported that expression of *Streptococcus mutans* (*S. mutans*) virulence genes is significantly reduced in multi-species biofilms with *S. oralis* or *Lactobacillus casei* [50]. Interactions in multi-

species biofilms can promote resistance to antimicrobial agents. Harriott *et al.* reported that *C. albicans* induces *S. aureus* vancomycin resistance during multi-species biofilm formation [51]. In another study, Adam *et al.* showed that in multi-species biofilms of *C. albicans* and *S. epidermidis*, extracellular polymer produced by *S. epidermidis* can inhibit penetration of antifungal drug fluconazole while *C. albicans* can protect the slime-negative *S. epidermidis* against vancomycin [52].

One of the most common interactions in multi-species biofilms is competition. Microorganisms compete for nutrients and try to inhibit the growth of other species in biofilms. Toxic substances are secreted by many microbial species to kill or inhibit the growth of other species. For example, *P. aeruginosa* is reported to kill *Candida* in multi-species biofilms by using virulence factors which are well characterized in human infections [53-54]. Tong *et al.* reported that *Streptococcus oligofermentans* uses L-amino acid oxidase to generate hydrogen peroxide (H_2O_2) from peptone and suppress the growth of *S. mutans* in a peptone-rich multi-species biofilms [55]. Rao *et al.* reported that marine bacterium *Pseudoalteromonas tunicate* produces antibacterial protein (AlpP) and inhibits the growth of other marine bacteria isolated from the same environment [56]. Bacteriocins are produced by a wide range of microorganisms [57] and are reported to mediate competitive interactions and proposed to facilitate horizontal gene transfer in oral multi-species biofilms [58-59].

Besides competitive interactions, cooperative interactions also widely exist in multi-species biofilms and are essential for the overall biofilm fitness. Many cooperative interactions are characterized in multi-species biofilms involved in biodegradation and bioremediation processes such as denitrification *via Nitrosomonas* and *Nitrobacter* species [60]. These biodegradation and bioremediation are usually accomplished through sequential biological reactions from various bacterial species in the biofilms. Cooperative interactions typically lead to specific spatial organization of different species in biofilms, which further ensures an efficient diffusion path for organic compounds. Recently, bioenergy production *via* microbial fuel cells (MFCs) is of great interest. In MFCs, electrochemically active bacterial species capture the chemical energy from organic compounds and convert it to electrical energy. Bacteria develop multi-species biofilms on the MFC electrodes, which enable conversion of electricity and opportunities for extracellular electron transfer (EET) [61]. Read *et al.* reported that interactions of Gram-positive *Enterococcus faecium* and other Gram-negative organisms lead to development of different structures in MFC anode biofilms and enhancement of

electricity generation by 30%–70% relative to the cultures of single species [62]. Besides electricity production, MFCs are also used to power desirable reactions in the cathode chamber. Wrighton *et al.* reported that reducing equivalents generated from the anodic oxidation of acetate can stimulate denitrifying bacterial communities and the cathode performance is in accordance with composition and structures of multi-species biofilms in denitrifying cathodes [63].

Diffusible signaling molecules can control the expression of genes involved in a variety of metabolic pathways, production of virulence factors, biosurfactant, EPS and motilities in bacteria [64]. Signaling molecules based interspecies communication plays an important role in interactions in multi-species biofilms. N-acyl homoserine lactone (AHL) autoinducers are the most common signaling molecules in bacteria and can mediate a wide range of cross-species/cross-genus communications [65]. Rickard *et al.* reported that autoinducer 2 produced by *S. oralis* mediates communication between *S. oralis* and *A. naeslundii* and promotes the mutualistic growth of each species in multi-species biofilms in media with saliva as the sole nutrient source, which does not support the growth of either of the species alone [66]. In oral biofilms, the early colonizer *Veillonella* sp. can utilize lactic acid produced from other species and promote mutualistic community development [67]. Eglund *et al.* reported that *Veillonella atypica* (*V. atypica*) can induce the expression of α -amylase-encoding gene *amyB* of *S. gordonii* by a diffusible signal in multi-species biofilm [68]. Johnson *et al.* recently showed that *S. gordonii* transcription factor CcpA is required for the *V. atypica*-induced amylase expression [69]. The respiratory tract of cystic fibrosis (CF) patients is infected with *P. aeruginosa* biofilms, which are nearly impossible to eradicate using conventional antibiotics. Infection is the main cause of morbidity and mortality in these patients [70-72]. Riedel *et al.* reported that CF pathogen *Burkholderia cepacia* is capable of perceiving the AHL signals produced by CF pathogen *P. aeruginosa* [73]. Ryan *et al.* reported that CF pathogen *Stenotrophomonas maltophilia* diffusible signal factor affects biofilm formation and polymyxin tolerance in *P. aeruginosa* through a sensor kinase encoded by *P. aeruginosa* PA1396 gene [74]. These *in vitro* studies suggest that signaling molecules based interspecies communication may mediate multi-species biofilm formation *in vivo*, although multi-species biofilms have not been directly observed in the lungs of CF patients [75]. Using analogues of signaling molecules or enzymes that degrade signaling molecules can significantly repress interspecies communication and interactions in multi-species biofilms and can be an effective

approach to manipulate multi-species biofilm development.

Intensive interactions in multi-species biofilms can serve as driving force of evolution. Hansen *et al.* reported that in multi-species biofilms formed by *Acinetobacter* sp. and *Pseudomonas putida* (*P. putida*), the coexistence of the *P. putida* population is dependent on the benzoate excreted from *Acinetobacter* during the catabolism of benzyl alcohol, the sole carbon source. However, *P. putida* keeps a distance from the *Acinetobacter* micro-colonies since *P. putida* biofilm formation requires oxygen and will disperse under low oxygen conditions. After co-cultured with *Acinetobacter* in mixed biofilms for three days, a rough variant of *P. putida* evolves and can adhere tightly to *Acinetobacter* micro-colonies in mixed biofilms. However, monospecies biofilm formed by the *P. putida* rough variant still disperses in response to oxygen starvation, which indicates that the nondispersal phenotype of the rough variant in the co-culture biofilms is mediated through interactions between the *P. putida* variant with *Acinetobacter*. The authors further showed the derived biofilm is more stable and more productive than the ancestral biofilm [76-77].

Conclusion and future prospects

It is evident that multi-species biofilms are dynamic communities with extensive interactions between different species. Different approaches need to be combined in biofilm research for better understanding of these complex communities. The biological behaviors of different bacterial cells in the multi-species biofilms give us important clinical implications for combating biofilm infections. A current bottleneck in biofilm research is the reproducibility. Researchers from different areas and groups perform the biofilm assays at their own settings, which leads to variations in biofilm studies. A set of standard protocols should be made for biofilm experiments. Robotics workstations should be developed for biofilm research, which can further minimize the experimental variations. Internet-based biofilm databases can help biofilm researchers to share and integrate their biofilm experimental results. These databases will further provide valuable information for biofilm simulation and modeling.

Understanding of biofilms can facilitate development of intelligent biofilm engineering, which designs and controls of biofilm formation. For example, by adding signaling molecules or their analogues, certain specific interactions in biofilms can be induced or repressed [78]. On the one hand, one would like to disperse biofilms which cause problems in hospitals and industrial settings.

On the other hand, one would like to apply stable biofilms in bioremediation of polluted soils and water. More knowledge about biofilms is needed.

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