Chapter 17
Biofilm as a Multicellular Bacterial System

Masanori Toyofuku and Nobuhiko Nomura

Abstract Biofilm is thought to be the primary natural habitat for many bacteria and they are associated with a wide range of human activities, such as wastewater treatment and clinical infections. Current research points out that cells inside biofilms are physiologically distinct from free-floating cells. In biofilms, bacteria are embedded in extracellular polymeric substances (EPS) that have physical and biological functions. One of the main roles of these EPS is to hold the cells together, which leads to the development of multicellular consortia. Once developed, usually the environment inside the biofilm becomes heterogenous which initiates the biofilm to function as a multicellular system. The development of biofilm is well organized following common stages among species. During this process, intercellular and intracellular signaling also takes place. This chapter will give a brief introduction to biofilms to support the understanding of bacterial multicellular systems.

Keywords Biofilm • c-di-GMP • Cell-to-cell communication • Confocal reflection microscopy • Diffusible signal factor (DSF) • EPS • Outer membrane vesicle (OMV)

17.1   Introduction

Although much of the knowledge in microbiology is based on the study of free-floating planktonic cells, it is thought that the primary habitat for many microorganisms is biofilm. Bacterial biofilms are multicellular consortia in which the bacterial cells are embedded in an extracellular matrix or EPS that is often produced by themselves. Microbial mats in the natural environments or plaques that are

---

M. Toyofuku • N. Nomura (✉)
Graduate School of Life and Environmental Sciences, University of Tsukuba,
1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan
e-mail: nomura.nobuhiko.ge@u.tsukuba.ac.jp

developed on the surface of teeth are examples of surface-associated biofilms. Biofilms are not only necessarily attached to the surface but also are present as suspended cell aggregates, also known as flocs and granules (de Beer and Stoodley 2006). Natural biofilms can develop under most conditions were bacteria are present and their relevance in ecosystems has been recognized. Taking advantage of the multicellularity, biofilms are considered as a robust and sustainable system. Currently, biofilms are practically used for biological wastewater treatment. On the other hand, they are also associated with a wide range of problems in industry and also in clinical infections. For instance, biofilms have increased resistance against antimicrobial agents and is hard to eradicate them (Høiby et al. 2010). Given the potential benefits and drawbacks that biofilms can confer, there is high interest in their control.

Accumulating report demonstrates that biofilms are distinct from their free-floating counterparts (López et al. 2010). Cells in biofilms have different gene expression patterns compared to planktonic cells and numerous regulatory mechanisms take place during biofilm formation, suggesting that there is a unique program for biofilm development. Moreover, the gene expressions, phenotypes vary between the cells in a biofilm. This heterogenous nature of biofilm could be a consequence of differential gene expression that is initiated by a different gradient of nutrients, physicochemical factors effecting the cell activates. In addition, emergence of genotypically and phenotypically diverse mutants may lead to the heterogenous nature of biofilm. Remarkably, cells inside biofilms interact physically and chemically with each other to coordinate social behaviors. It is not surprising that the organization and differentiation of biofilm has led some scientists to compare biofilms with higher multicellular organisms.

In this chapter we will give a brief introduction to biofilms and discuss the feature of this multicellular bacterial community.

17.2   Sticking the Cells Together: The EPS Matrix

In order to form a multicellular community, the cells must have a way to connect to each other. EPS is one of the key components in biofilms and it could be simply said that "There is no biofilm without an EPS matrix" (Flemming and Wingender 2010). The EPS consists of different types of biopolymers which are polysaccharides, proteins, lipids, and nucleic acids. Also, polymers from other origin such as humic acid may be embedded into biofilms. It was long believed that polysaccharides are the main constituent of the EPS matrix; however, results from various studies indicate that the main EPS in terms of amounts and their impact on biofilm formation differs among species. The major role of EPS in biofilm is to connect the cells with the substratum or other cells and provide the mechanical stability of the biofilm. Therefore, the maintenance of the structured biofilm depends largely on the production of EPS. Several studies using pure culture have demonstrated that mutants lacking the component of EPS have altered biofilm morphology. For instance, in

Pseudomonas aeruginosa, at least three types of exopolysaccharides that alter biofilm formation are identified. Psl is a mannose- and galactose-rich polysaccharide while Pel is a glucose-rich polysaccharide (Friedman and Kolter 2004a, b). Alginate is the major exopolysaccharide in mucoid strains that are the predominant morphotype of strains isolated from chronic infections (Govan and Deretic 1996); Psl and Pel exopolysaccharides are critical for biofilm formation on abiotic surfaces (Friedman and Kolter 2004a; Jackson et al. 2004; Matsukawa and Greenberg 2004). Further studies have demonstrated extracellular DNA (eDNA) to constitute an important component of the *P. aeruginosa* biofilm matrix (Whitchurch et al. 2002; Allesen-Holm et al. 2006). Although eDNA was initially thought to be a residual material from lysed cells, many groups indicate that it is in fact an integral part of the matrix. In *Rhodovulum* sp., flocculation was disrupted when treated with nucleic acid-degrading enzymes (Watanabe et al. 1998). Similar results are obtained with activated sludge that is composed of multispecies. DNase treatment of the activated sludge resulted in complete dissolution of the flocs in combination with shear force, whereas shear force alone did not (Dominiak et al. 2011). These results demonstrate that eDNA is related to structural integrity. In addition to the role in structural integrity, eDNA in bacteria may retain extracellular proteins as observed in the defensive web of neutrophil extracellular traps (NETs) (Brinkmann et al. 2004).

The presence of proteins in the biofilm matrix has been reported in many samples and they are sometimes even more abundant than polysaccharides in natural cell aggregates (McSwain et al. 2005; Frølund et al. 1996; Urbain et al. 1993). While the functions of these proteins are not fully understood, it is reported that these proteins at least include extracellular enzymes as well as structural proteins. Various extracellular enzymes that are involved in degradation of biopolymers have been detected in the matrix suggesting its role as an external digestion system in addition to the immobilization of the cells (Baty et al. 2001; Flemming and Wingender 2010).

Structural proteins include CdrA which is an extracellular protein in *P. aeruginosa* that is induced upon biofilm formation. This protein cross-links polysaccharides and/or tethers the cells with the polysaccharides providing biofilm stability (Borlee et al. 2010). Carbohydrate-binding proteins, called lectins, are also involved in biofilm formation and stabilization in a wide range of species. Lectins have a very specific recognition of sugars, and therefore, they may be involved in recognizing particular polysaccharides and glycoconjugates on the cell surface of other cells allowing them to coaggregate (Rickard et al. 2003).

Another type of proteins that seems to be ubiquitous among bacterial biofilms are amyloid fibers. Amyloid fibers are defined by their characteristic cross-β-strand structure, where the β-sheets are oriented perpendicular to the fiber axis (Sunde et al. 1997). Amyloids of bacterial origins have been found in various natural biofilms suggesting their importance in the natural habitat (Otzen and Nielsen 2008). In certain species, amyloid proteins such as curli fimbriae in *Escherichia coli* and *Salmonella typhimurium*, TasA in *Bacillus subtilis* have critical roles in biofilm formation together with the polysaccharides (Chapman et al. 2002; Romero et al. 2010; Zogaj et al. 2001).

A group of surface proteins that are named biofilm-associated proteins (Bap) also plays a role in the cell adhesion to biotic and/or abiotic surfaces in Gram-positive and Gram-negative bacteria. These proteins contain core regions of amino acid repeats involved in cellular adhesion (Latasa et al. 2006).

Recent proteomic studies reveal that in addition to these extracellular enzymes, intracellular proteins that are normally localized to the cells are present in the matrix at a certain amount. Examples include membrane proteins, histone-like proteins, ribosomal proteins, and superoxide dismutase (Jiao et al. 2011; Eboigbodin and Biggs 2008). While the functions of the intracellular proteins in the matrix remain to be fully investigated, some reports suggest a role of intracellular proteins outside of the cell. Such proteins that display two distinct functions are known as moonlighting proteins (Jeffery 2003). For example, the elongation factor Tu as well as the chaperonin protein complex GroEL have been identified on the cell surface of lactobacilli and were shown to be involved in adhesion to mucin and human epithelial cells (Bergonzelli et al. 2006; Granato et al. 2004). A recent study suggested that a large amount of these intracellular proteins are delivered to the matrix by membrane vesicles (Toyofuku et al. 2012a).

17.3   The Life Cycle of a Biofilm

Based on the knowledge of a limited number of model organisms, similarity of the biofilm life cycle has been recognized so far. From these observations mainly from mono-species biofilms, it is proposed that the biofilm life cycle consists of initial attachment, production of EPS matrix, maturation to complex colonies, and finally dispersal of cells from the biofilm. Accumulated researches indicate that these steps involve complex regulatory mechanisms that are influenced by environmental conditions and cell interactions. As a consequence, the structure of biofilms becomes highly variable ranging from flat-layered mats to complex mushroomlike structures depending on the nutritional conditions and cell interactions (Davies et al. 1998). Thus, cells undergo flexible and dynamic lifestyles in biofilms.

Biofilm formation begins with attachment to a surface that can be a substratum such as metals, glasses, or a surface of another cell. This step is important for the bacteria so that they settle down in the right environment. Initial attachment to abiotic surfaces is well characterized in the attachment of *Pseudomonas* sp. It was indicated that during the cell attachment, the cells first attach to the surface loosely via adhesins, such as flagellar and pili (O'Toole and Kolter 1998a, b), that may readily detach. Once the cells attach to the surface along their axis, this step is called irreversible attachment, the bacteria commit to their life as a biofilm (Petrova and Sauer 2012). Even this single step in biofilm formation is highly regulated by several factors indicating that biofilm formation is an active biological process, and as discussed later, an intracellular second messenger whose production is controlled by several environmental modulates this step.

While the initial attachment is the beginning of the life as a biofilm, the community comes to an end when cells disperse from the biofilm and seek new environments. The mechanism by which bacteria regulate dispersal is not fully understood and strategies to manipulate biofilm dispersal are of great interest for broad application in controlling biofilm formation. Passive processes for cell dispersal such as the erosion of biofilms by shear stress are observed; however, many bacteria undergo active dispersal processes where the cells convert from sessile to planktonic free-swimming bacteria (Sauer et al. 2002). Several environmental cues such as carbon sources, oxygen depletion, and nitric oxide induce cell dispersal, which allows the cells to respond to changes in environments. As observed in *P. aeruginosa*, not all cells but a subpopulation of cells localized at the center of mature biofilms disperse while the majority of the cells remain in the biofilm. These cells that remain seem to be dead cells which is also an indicative of the heterogenous nature of the cells in the biofilm (Barraud et al. 2006). In the dispersal cells, genes that are related to biofilm formation such as exopolysaccharides are downregulated, whereas genes that are related to motility are induced. In addition to active motility, the cells must disassemble from the EPS matrix. EPS-degrading enzymes such as chitinase and nuclease are also reported that could potentially be involved in the cell dispersion (McDougald et al. 2012).

Several signals that are produced by the cells are also involved in this dispersal event indicating that the cells themselves can initiate cell dispersal in a coordinated fashion. For example, a fatty acid molecule, cis-11-mehyl-2-dodecenoic acid which is known as a diffusible signal factor (DSF), was found to be involved in dispersal in *Xanthomonas campestris* (Dow et al. 2003). DSF in *X. campestris* activate the production of endo-β1, 4-mannanase that disrupts cell aggregates. Further work indicated that DSF reduce the biosynthesis of a putative exopolysaccharide, providing more the detailed mechanism of how DSF regulate dispersal (Tao et al. 2010b). DSF-related molecules are also found in other species implicating its role in inter-species signaling (Zhang and Dong 2004; Ryan and Dow 2011). For instance, DSF produced by *Stenotrophomonas maltophilia* induced stress-related genes in *P. aeruginosa* through a putative sensor kinase whose homologous protein is widespread among pseudomonads (Ryan et al. 2008). *P. aeruginosa* itself also produces a DSF-family signal, *cis*-2-decenoic acid, that induces biofilm dispersion of which the mechanism is not clear (Davies and Marques 2009). Interestingly, DSF-family signals play an interkingdom role where it is recognized by *Candida albicans* (Davies and Marques 2009; Boon et al. 2008).

Signals that induce dispersal are also found from *B. subtilis* biofilms called pellicles that are formed at the air–liquid surface. The cells are held together in the matrix that consists of exopolysaccharide and amyloid fiber which is largely composed of TasA. D-amino acids that are produced during the maturation of biofilm are incorporated into the cell wall peptidoglycan and trigger the release of amyloid fibers from the cell wall. A second biofilm-disassembly factor was found recently from *B. subtilis* that is a polyamine norspermidine. Norspermidine, which is produced in the late life cycle of the pellicle directly, interact directly with the exopolysaccharide component of the matrix (Kolodkin-Gal et al. 2012). Though the

mechanism is not fully understood, both ᴅ-amino acids and norspermidine can induce cell dispersal in other bacteria, including Gram-negative cells.

17.4   c-di-GMP, An Intracellular Second Messenger, Regulates Biofilm Formation

The observations of the stage-specific gene regulation in biofilms indicate that biofilm development is a biologically programmed process. For instance, once the cells find a suitable surface, they should alter their gene expression so that they can settle down to switch their lifestyle from planktonic cells to biofilm cells. Furthermore, cells respond to environmental cues in dispersal. These phenomena observed universally among bacterial suggest that there is a common mechanism that is involved in these processes. Recent studies have found out that a small molecule, bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP), is an (almost) ubiquitous signal that plays a central role in biofilm development of diverse bacteria (Hengge 2009; Römling et al. 2005). C-di-GMP is a second messenger that passes through the information outside the cells into the cytoplasm. In general, high levels of c-di-GMP concentration correlate with the biofilm mode of lifestyle and low concentrations of c-di-GMP correlate with planktonic mode of growth. In other words, a high level of c-di-GMP stimulates the biosynthesis of adhesins and extracellular matrix and inhibits motility, thus allowing the cells to form and maintain biofilms. In dispersal cells, c-di-GMP levels decrease that allow the cells to become motile. The level of c-di-GMP is controlled by the enzymes that produce (diguanylate cyclases) and degrade (phosphodiesterases). Diguanylate cyclases activity is associated with the GGDEF domain, which is named after the highly conserved amino acid sequences they contain, and c-di-GMP-specific phosphodiesterases activity is associated with the EAL or HD-GYP domains. These domains are often found with N-terminal sensory domains, compromising sensor domains as well as phosphoacceptor. The great variety of these sensory domains indicates that c-di-GMP level responds to numerous signals (Hengge 2009). The extracellular signal DSF is also one of the signals that alter c-di-GMP levels by degrading c-di-GMP (Tao et al. 2010a; Deng et al. 2012). Hence, a diverse range of signals are integrated to the c-di-GMP-signaling system enabling the cells to respond to changing environments.

Although the target of c-di-GMP is not fully understood, it has been indicated that c-di-GMP binds to the target component and alter the structure and functions. Several examples demonstrate that the binding components of c-di-GMP are highly diverse that can be proteins as well as riboswitches. For instance, c-di-GMP binding to PilZ proteins is an essential step in c-di-GMP-mediated regulation (Ryjenkov et al. 2006). In riboswitches, c-di-GMP binds to mRNA motifs which alter the expression of the gene (Sudarsan et al. 2008).

17.5 Bacterial Communications that Influence Biofilm Formation

Multicellularity in bacteria can be defined as a group of cells that function as a greater entity. In order to accomplish this, intercellular interactions must play a crucial role to enable cells coordinate their activities (Shapiro 1998). Although it is a still-opening question whether bacteria generally function for the whole in biofilms, scientists have discovered that bacteria can communicate with each other through chemical molecules in order to coordinate the cell behavior. Moreover, cell-to-cell communication has been demonstrated to affect biofilm formation in many species. A typical signaling molecule produced by Gram-negative bacteria is the *N*-acyl homoserine lactones (AHLs). Over 100 species of *Proteobacteria* use AHLs as signal molecules. AHLs usually consist of a fatty chain acid coupled to a homoserine lactone. There are variations in the acyl-group length, substitution at the C3 position (hydrogen, hydroxyl, or oxo group), and the saturation of the fatty acid chain. These variations confer signal specificity that is recognized by their cognate LuxR family transcriptional regulators. AHLs are synthesized by LuxI family proteins. The *luxI* gene is induced in the presence of AHLs, consisting an autoregulatory system.

While the fatty acid side chains were typically found in these homoserine lactone signals, recently, a new class of non-fatty-acid-based AHL signals has been discovered. The phototrophic soil bacterium *Rhodopseudomonas palustris* possesses a pair of LuxIR homologues, RpaIR. RpaI synthesize *p*-coumaroyl-homoserine lactone, from exogenous *p*-coumaric acid, which is thought to be obtained from the plant host. These aryl-homoserine lactone homoserine productions have also been observed in *Bradyrhizobium* sp. BTAi1 and *Silicibacter pomeroyi* DSS-3(Schaefer et al. 2008; Ahlgren et al. 2011).

P. aeruginosa is one of the most studied bacteria in cell-to-cell communication and biofilms. This opportunistic human pathogen produces at least two AHLs that are *N*-butyryl-HSL (C4-HSL) and *N*-(3-oxododecanoyl)-HSL (3-oxo-C12-HSL). Each signal is produced by RhlI and LasI, respectively. The organized structure of biofilms evoked researchers to think that there must be some interactions between cells when forming biofilms. The involvement of AHL on biofilm formation was first reported in 1998, when Davies et al. reported that a *lasI* mutant formed biofilms that were flat compared to the wild type that formed structured microcolonies (Davies et al. 1998). Since then, many groups have confirmed that the effect of cell-to-cell communication on biofilm formation in *P. aeruginosa* depends on strain and experimental conditions (de Kievit 2009).

The mechanism is not clear how cell-to-cell communication influences biofilm formation because AHLs control a wide range of genes. However, several factors that influence biofilm formation are known to be controlled by cell-to-cell communication (de Kievit 2009). These are biofilm matrix, rhamnolipids, and denitrification. In *P. aeruginosa*, at least three types of exopolysaccharide (alginate, Pel, and Psl) that are related to biofilm formation have been confirmed. Thus far, only the

pel-gene cluster has been identified as being regulated by cell-to-cell communication. Pel is a glucose-rich exopolysaccharide which is essential for biofilm formation (Friedman and Kolter 2004a). It was discovered that the Las system is mainly involved in the transcription of *pel* biosynthetic genes (Sakuragi and Kolter 2007). Another important component of the extracellular matrix, extracellular DNA (eDNA) is also suggested to be influenced by QS. The mechanism by which the DNA is exported outside of the cell is not fully understood. Interestingly, spatial analysis of *P. aeruginosa* biofilms revealed distinct patterns of eDNA localization. In the early stages of biofilm formation, eDNA was shown to accumulate on the surface of microcolonies, while in older biofilms eDNA was shown to accumulate at the junction of the stalk and cap of the typical mushroomlike structure of micro-colonies (Allesen-Holm et al. 2006). It was observed that QS-deficient mutants contain less eDNA than the wild type, suggesting that QS induce eDNA (Allesen-Holm et al. 2006).

Rhamnolipids are amphipathic glycolipids that act as biosurfactants. The expression of rhamnolipid synthesis operon *rhlAB* is regulated by the rhl QS system (Pearson et al. 1997). A mutant deficient in rhamnolipid production forms biofilms that are flat and relatively homogenous compared to the structured wild-type biofilms (Davey et al. 2003). From these observations, it is proposed that rhamnolipids are important in forming distinct architecture of the biofilm. In addition to maintaining the biofilm structure, rhamnolipids are necessary for detachment of the cells from the biofilm (Boles et al. 2005). The influence of rhamnolipids on multiple facets of biofilm formation suggests that the production is regulated at particular location and time points during biofilm formation. Supporting this idea, microscopic analysis using *gfp*-fused *rhlAB* genes indicated that rhamnolipid production is maximal in the stalks of a developing microcolony (Lequette and Greenberg 2005).

P. aeruginosa is capable of growing anaerobically by using N-oxides as terminal electron acceptors instead of oxygen (Toyofuku et al. 2012b). Under denitrifying conditions, *P. aeruginosa* forms robust biofilms (Yoon et al. 2002). Inactivation of Rhl QS leads to nitric oxide (NO) accumulation that is toxic to the cells causing cell death. Further study demonstrated that QS repress denitrification activity (Toyofuku et al. 2007, 2008). One of the key factors influencing biofilm formation is NO. While high concentration leads to cell toxicity, sublethal concentrations trigger disperse of the cells from the biofilm (Barraud et al. 2006). Interestingly, the process of biofilm formation under anaerobic condition seems somewhat different from aerobic condition, where cells become filamentous under anaerobic condition (Yawata et al. 2008). The cell elongation is one of the important factors in biofilm formation under anaerobic conditions and it is influenced by the production of NO (Yoon et al. 2011). Hence, fine tune of NO accumulation by QS as well as other factors appears to be important in biofilm formation under anaerobic conditions.

In Gram-positive bacteria amino acid peptides are widely used as signal molecules. Once the precursor signal protein is synthesized within the cell, active signals are generated by cleaving the proteins to short form of peptide chains and these peptides are subject to posttranslational modification in some bacteria resulting in a

great diversity of the signal (Dunny and Leonard 1997). The peptide signals are linear or cyclic usually ranging in the size of 5–26 amino acid residues. Interestingly, some signal peptides also function as bacteriocins. These peptide signals that do not diffuse across the cell membrane are secreted by ABC transporters. Once secreted, the cells then sense the peptide signals by a two-component regulatory system. Most typically, the peptide signal is sensed at the surface of the bacteria by a histidine kinase sensor. This results in the autophosphorylation of the sensor kinase and the phosphoryl group gets transferred to a response regulator that regulates the expression of genes.

In the case of *Staphylococcus aureus*, the autoinducing peptide (AIP) is encoded by *agrD*. The AgrB protein processes and exports AIP. The secreted AIP is detected by a histidine kinase AgrC, which activates the response regulator AgrA by phosphorylation. Activated AgrA then modulates gene expression including the positive control of the *agrBDCA* operon. As expected, the *agr* QS system is involved in biofilm formation. In *S. aureus*, the *agr* QS system mutant forms thicker biofilm than the wild type (Vuong et al. 2000). Furthermore, extracellular proteins such as proteases and nucleases that are involved in dispersal of biofilm are upregulated by the QS system. Hence, the *agr* QS system inhibits biofilm formation.

In addition to AHLs and signal peptides, AI-2 is discovered broadly among Gram-negative and Gram-positive bacteria. AI-2 was originally described in *Vibrio harveyi*, where it was involved in the regulation of bioluminescence (Chen et al. 2002). AI-2 is produced by a spontaneous reaction of 4,5-dihydroxy-2,3-pentanedione which is synthesized by LuxS. The AI-2 signal which is recognized by homologues of the *luxS* gene has been identified in over 350 sequenced bacterial genomes (Shrout and Nerenberg 2012). Thus, AI-2 appears to be an interspecies signal. Whether LuxS is involved in biofilm formation or not is not clear. For instance, in *Staphylococcus epidermidis*, LuxS has negative effects on biofilm formation (Li et al. 2008), while this is not the case in *S. aureus* (Doherty et al. 2006). Taking into account that AI-2 is an interspecies signal, AI-2 may be involved in multispecies biofilm formation.

17.6   Multispecies Biofilm

In the natural environment, bacteria form complex multispecies biofilm. Compared to the mono-species biofilm, our understanding of multispecies biofilm is limited. The common feature of these bacterial communities is that normally the cells are dense and physically close to each other compared to the free-living planktonic cells. As a result of active metabolism of the cells in biofilms, nutrient in the liquid surrounding may be consumed by cells in the outer layers and metabolites may accumulate in the biofilms. These gradients create microenvironments that become niches for other bacteria. One of the techniques that are commonly used for observation of complex natural biofilm is fluorescence in situ hybridization (FISH). With this method, localization of bacterial populations can be visualized by hybridizing a

fluorescently labeled oligonucleotide probe to the target 16S rRNA (Amann et al. 1990; DeLong et al. 1989). Compartmentalized or mixed structures of species within natural biofilms have been observed based on this method. The stratification of species within the biofilm can mostly be explained by the difference of metabolism among the species. For instance, in a nitrifying activated sludge, nitrifying bacteria are split into layers according to their use of different electron acceptors. During nitrification, ammonia is oxidized by ammonia-oxidizing bacteria and the produced nitrite is further oxidized to nitrate by nitrite-oxidizing bacteria. Consequently, ammonia-oxidizing bacteria locate at the outer layer and the nitrite-oxidizing bacteria colonize immediately below the ammonia oxidizers in nitrifying biofilms (Okabe et al. 1999). Compartmentalized is also explained by computational simulations revealing that these structures result from simple diffusion of growth-limiting nutrient (Nadell et al. 2009). Some model experiments using two bacterial species has also revealed that physicochemical conditions determine the localization of bacterial colonies within the biofilm. For example, *Burkholderia xenovorans* LB400 and *Pseudomonas* sp. B13 are able to degrade a pollutant, 3-chlorobiphenyl, to water and carbon dioxide. In this case, *B*. *xenovorans* LB400 degrades 3-chlorobiphenyl only to chlorinated benzoate and no further, while *Pseudomonas* sp. B13 further degrades chlorobenzoate to water and carbon dioxide. Since *Pseudomonas* sp. B13 cannot utilize 3-chlorobiphenyl, it is able to grow only if it is together with *B*. *xenovorans* LB400 when 3-chlorobiphenyl is the sole carbon/energy source. When a mixed-species biofilm was developed by these two strains using 3-chlorobiphenyl as the only available carbon source, *Pseudomonas* sp. B13 was always mixed with *B*. *xenovorans* LB400, whereas separate colonies were observed when another carbon source that can be used by both strains was given (Nielsen et al. 2000). In another case using *Acinetobacter* sp. C6 and *Pseudomonas putida* KT2400, distinct colonies but not mixed-species colonies were formed even under experimental settings that support host-commensal relationship (Hansen et al. 2007). When benzyl alcohol is given as a sole carbon source, the presence of *P*. *putida* KT2400 is dependent on *Acinetobacter* sp. C6. *Acinetobacter* sp. C6 metabolize benzyl alcohol to benzoate that is partly excreted and can be utilized by *P*. *putida* KT2400. Under this condition, *Acinetobacter* sp. C6 and *P*. *putida* KT2400 form distinct colonies where *Acinetobacter* sp. C6 is loosely surrounded by *P*. *putida* KT2400. In this case, competition of oxygen might counteract the host-commensal partnership of carbon source. Hence, the examples show us how the final structure could be affected by different interactions that occur among the consortia. Interestingly, extended incubation of the *Acinetobacter* sp. C6 and *P*. *putida* KT2400 mixed-biofilm under the host-commensal condition led to the emergence of a variant distinct cell from the ancestral type of *P*. *putida* KT2400 (Hansen et al. 2007). The variant was not detected in chemostat culture with *Acinetobacter* sp. C6 or in single-species biofilms, indicating that the variant can evolve in the microenvironment within biofilms in the presence of *Acinetobacter*. The evolved community was more stable and more productive than the ancestral community. Altogether, these examples demonstrate how spatial organization is

important for the function of the community and further leads to the understanding of how a community is optimized in their natural habitat.

In addition to these chemical interactions, direct contact of the cells also plays an important part in mixed-species biofilms. Interspecies electron transfer is known to be important during anaerobic degradation of organic matter to methane. During this process, close physical contact between syntrophs and methanogens is required. Direct observation of granular sludge indicated that these microbes locate close to each other (Harmsen et al. 1996). In *Pelotomaculum thermopropionicum* and *Methanothermobacter thermautotrophicus*, such symbiosis is mediated by a flagellum (Shimoyama et al. 2009). *P. thermopropionicum* can ferment propionate to acetate, bicarbonate, and H_2; however, this conversion is endergonic and the partial pressure of H_2 should be kept low for this process to proceed. In the presence of *M. thermautotrophicus*, H_2 is consumed to CH_4 as an energy source and enable *P. thermopropionicum* to oxidize propionate. When grown together, these two strains form aggregates that are maintained by flagellum of *P. thermopropionicum*. The flagellum not only maintains the symbiosis but also synchronizes their metabolism by inducing gene expression involved in methanogenesis of *M. thermautotrophicus*. More recently, another type of bacterial communication that is based on physical contact was discovered. This communication is mediated by nanotubes that connect neighboring cells in *Bacillus subtilis* (Dubey and Ben-Yehuda 2011). These tubes were not synthesized during planktonic growth but were produced in colonies. These tubes allow bacteria to exchange small molecules including proteins and plasmids even between other species. Although the relevance of this nanotube in the natural environment is yet to be understood, the findings open up a new era of bacterial interaction between species.

Another bacterial-derived structure that potentially has a role in interspecies communication inside biofilms is the outer membrane vesicles (OMVs). OMVs are particles ranging from approximately 20 to 200 nm that are mainly produced in Gram-negative bacteria (Kulp and Kuehn 2010; Tashiro et al. 2012). These vesicles are mainly composed of outer membrane, periplasmic components and also contain DNA. In *P. aeruginosa*, a hydrophobic QS molecule, *Pseudomonas* quinolone signal (PQS) is incorporated in OMVs allowing the PQS to freely diffuse from the cell and shuttle them to the neighboring cells (Mashburn and Whiteley 2005). Consequently, PQS carried by OMVs coordinate social behaviors in *P. aeruginosa*. In addition, PQS are able to induce OMV production in *P. aeruginosa* and other species (Tashiro et al. 2010; Mashburn and Whiteley 2005). Importantly, OMVs have been observed in the EPS fraction of mono-species biofilms such as *P. aeruginosa* and *Myxococcus xanthus* and also mixed-species biofilm of environmental samples (Schooling and Beveridge 2006; Toyofuku et al. 2012a). *M. xanthus* is a bacterium that shows complex social behavior and biofilm (also known as fruiting body) formation. In this bacterium, protein can be exchanged through direct cell contact in which the precise mechanism is not completely understood. As a result of protein exchange, individual cells can endow their neighbors with new characteristics without altering the genome. Direct observation of *M. xanthus* biofilm demonstrated that the intercellular region is packed with OMVs that are tethering on the

cell surface (Palsdottir et al. 2009). From these observations it is hypothesized that these OMVs may play a role in the protein exchange of *M. xanthus* (Remis et al. 2010; Konovalova et al. 2010). OMVs are also found extensively in the EPS of *P. aeruginosa* biofilm. It is well studied that OMVs in *P. aeruginosa* are associated with virulence against the host (Tashiro et al. 2012). Moreover, these OMVs derived from *P. aeruginosa* have the potential to fuse and/or attach to other Gram-negative and Gram-positive cells that may confer new properties to the recipient by transporting proteins or DNA. The role of OMVs in biofilms is yet to be investigated while some report indicates that they are involved in the development of mixed-species biofilm. OMVs produced by *Porphyromonas gingivalis* induce co-aggregation of *S. aureus* and *Prevotella intermedia* via an adhesin localized to the OMVs (Kamaguchi et al. 2003), indicating the role of OMVs in interspecies biofilm formation. Given that there are numerous proteins in the EPS, OMVs could also be contributing to place the proteins in the EPS.

17.7   Recently Developed Methods to Study Biofilms

Since biofilm mode of growth differs from planktonic cells, several methods to cultivate and analyze biofilms have been developed. One of the most popular method used in the assay of biofilm development is the 96-well biofilm assay (O'Toole et al. 1999). In this method, biofilm are developed in a static batch culture and the cells attached to the 96-well plate are stained after washing. This method is very powerful for screening for factors that affect biofilm formation and thus has been used in various species by many scientists. Biofilm structures have been revealed by microscopy techniques such as electron microscopy and the bacterial community structure with their localizations is often analyzed by FISH. FISH can be combined with other useful techniques, for instance, with combination of microautoradiography, uptake of different organic substrate can be monitored in order to demonstrate active cells (Lee et al. 1999; Kindaichi et al. 2004). The limitation of these techniques is that they only give a snapshot of the biofilm images while the biofilm development is highly dynamic. The use of time-lapse microscopy with combination of flow-cell system where the intact biofilm development can be monitored for several days has overcome this limitation for some bacteria. By using the flow-cell system, the lifestyle of biofilms became clear that it consists of several stages. Moreover, the use of confocal laser scanning microscopy (CLSM) with the flow-cell system allows the observation of three-dimensional structure of the biofilm during the time course for fluorescent-labeled bacteria. The fluorescent protein can be expressed constitutively and can be used for mono-species and mixed-species biofilms. In addition, fluorescent protein can be expressed under control of a particular promoter which gene is of interest in order to monitor gene expression in biofilms.

Recently, a technique adapting the confocal reflection microscopy (CRM) was used in biofilm. The advantage of this technique is that intact biofilms can be observed directly by confocal laser scanning microscopy without staining, fixation,
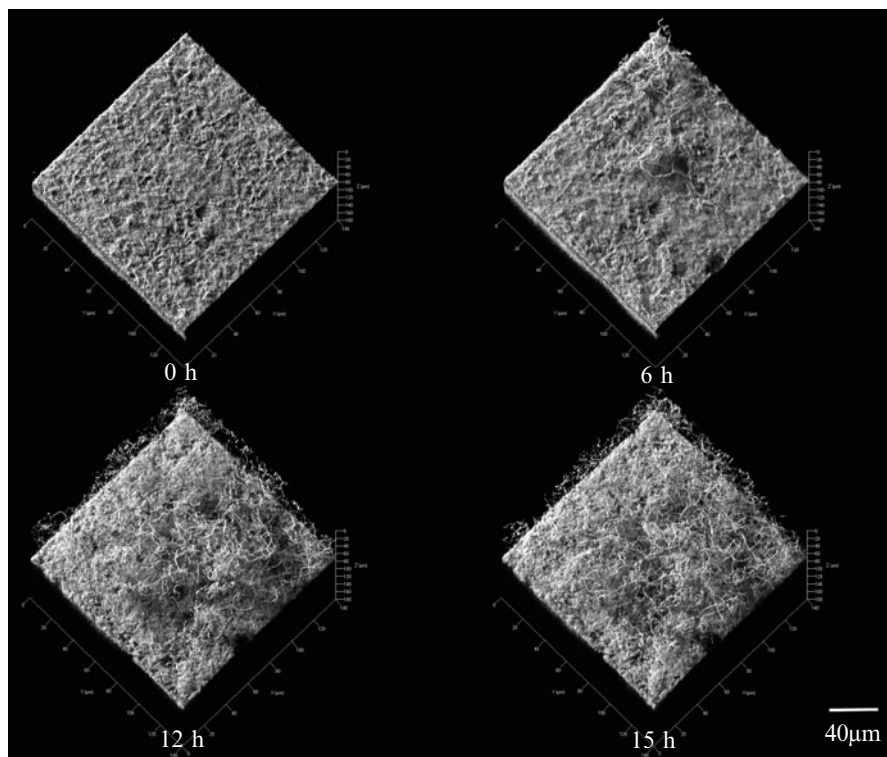
Fig. 17.1 Development of a mixed-species biofilm monitored by COCRM

or labeling with fluorescent proteins. Because CRM relies on the reflected light of the sample, reflection artifact obtained from bright spots such as cover glasses often becomes a problem. To solve this problem, the signal intensity of the cells can be maintained to a constant level along the Z-axis continuously by adjusting the detector gain manually while scanning. This modified mode of CRM called continuous-optimizing-CRM (COCRM) enables direct imaging of mixed-species biofilms and also mono-species biofilms alive during time courses (Yawata et al. 2010) (Fig. 17.1). While microscopy observation enables detailed analysis of the biofilm structure and development, yet there are few nondestructive methods that allow both microscopy analysis and measurement of metabolites. Measurement of metabolites along with the biofilm structure is important in understanding the activity of the biofilm and further understanding how biofilm reacts to the environment as a whole. To this end, flow-cell system was improved that gaseous metabolites can be collected and measured simultaneously with gas chromatograph (Yawata et al. 2008). Another method commonly used to monitor microbial activity is the use of microsensors. Microsensors are used for profiling spatial distributions of specific substrates and products (de Beer and Stoodley 2006). These microsensors can be mounted to chambers that cultivate biofilms and the concentration of certain metabolites can be

monitored real-time. Since microsensors are small with a tip size of 1–20 μm it is feasible for small chambers and was adapted to a micro-fluidic device to monitor NH_4^+ consumption of activated sludge with its structure (Toda et al. 2011). Raman microspectrometer is also a powerful tool in chemically characterizing the biofilms noninvasively without any pretreatment of the sample. Biomolecules along with second metabolites can be identified with its space-resolved information, which will give us a more detailed understanding of the activity and heterogeneity of the cells (Venkata et al. 2011; Patzold et al. 2006). These new techniques with others will provide a new insight into the structure functional correlation of biofilms.

References

Ahlgren NA, Harwood CS, Schaefer AL, Giraud E, Greenberg EP (2011) Aryl-homoserine lactone quorum sensing in stem-nodulating photosynthetic *bradyrhizobia*. Proc Natl Acad Sci USA 108:7183–7188

Allesen-Holm M, Barken KB, Yang L, Klausen M, Webb JS, Kjelleberg S, Molin S, Givskov M, Tolker-Nielsen T (2006) A characterization of DNA release in *Pseudomonas aeruginosa* cultures and biofilms. Mol Microbiol 59:1114–1128

Amann RI, Krumholz L, Stahl DA (1990) Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J Bacteriol 172:762–770

Barraud N, Hassett DJ, Hwang SH, Rice SA, Kjelleberg S, Webb JS (2006) Involvement of nitric oxide in biofilm dispersal of *Pseudomonas aeruginosa*. J Bacteriol 188:7344–7353

Baty AM 3rd, Diwu Z, Dunham G, Eastburn CC, Geesey GG, Goodman AE, Suci PA, Techkarnjanaruk S (2001) Characterization of extracellular chitinolytic activity in biofilms. Methods Enzymol 336:279–301

Bergonzelli GE, Granato D, Pridmore RD, Marvin-Guy LF, Donnicola D, Corthésy-Theulaz IE (2006) GroEL of *Lactobacillus johnsonii* La1 (NCC 533) is cell surface associated: potential role in interactions with the host and the gastric pathogen *Helicobacter pylori*. Infect Immun 74:425–434

Boles BR, Thoendel M, Singh PK (2005) Rhamnolipids mediate detachment of *Pseudomonas aeruginosa* from biofilms. Mol Microbiol 57:1210–1223

Boon C, Deng Y, Wang LH, He Y, Xu JL, Fan Y, Pan SQ, Zhang LH (2008) A novel DSF-like signal from Burkholderia cenocepacia interferes with Candida albicans morphological transition. ISME J 2:27–36

Borlee BR, Goldman AD, Murakami K, Samudrala R, Wozniak DJ, Parsek MR (2010) *Pseudomonas aeruginosa* uses a cyclic-di-GMP-regulated adhesin to reinforce the biofilm extracellular matrix. Mol Microbiol 75:827–842

Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, Weinrauch Y, Zychlinsky A (2004) Neutrophil extracellular traps kill bacteria. Science 303:1532–1535

Chapman MR, Robinson LS, Pinkner JS, Roth R, Heuser J, Hammar M, Normark S, Hultgren SJ (2002) Role of *Escherichia coli* curli operons in directing amyloid fiber formation. Science 295:851–855

Chen X, Schauder S, Potier N, Van Dorsselaer A, Pelczer I, Bassler BL, Hughson FM (2002) Structural identification of a bacterial quorum-sensing signal containing boron. Nature 415:545–549

Davey ME, Caiazza NC, O'Toole GA (2003) Rhamnolipid surfactant production affects biofilm architecture in *Pseudomonas aeruginosa* PAO1. J Bacteriol 185:1027–1236

Davies DG, Marques CN (2009) A fatty acid messenger is responsible for inducing dispersion in microbial biofilms. J Bacteriol 191:1393–1403

Davies DG, Parsek MR, Pearson JP, Iglewski BH, Costerton JW, Greenberg EP (1998) The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science 280:295–298

de Beer D, Stoodley P (2006) Microbial Biofilms. In: Dworkin M, Falkow S, Rosenberg E, Scleifer K, Stackebrandt E (eds) The prokaryotes, vol 1, 3rd edn. Springer, New York

de Kievit TR (2009) Quorum sensing in *Pseudomonas aeruginosa* biofilms. Environ Microbiol 11:279–288

DeLong EF, Wickham GS, Pace NR (1989) Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. Science 243:1360–1363

Deng Y, Schmid N, Wang C, Wang J, Pessi G, Wu D, Lee J, Aguilar C, Ahrens CH, Chang C, Song H, Eberl L, Zhang LH (2012) Cis-2-dodecenoic acid receptor RpfR links quorum-sensing signal perception with regulation of virulence through cyclic dimeric guanosine monophosphate turnover. Proc Natl Acad Sci USA 109:15479–15484

Doherty N, Holden MT, Qazi SN, Williams P, Winzer K (2006) Functional analysis of *luxS* in *Staphylococcus aureus* reveals a role in metabolism but not quorum sensing. J Bacteriol 188:2885–2897

Dominiak DM, Nielsen JL, Nielsen PH (2011) Extracellular DNA is abundant and important for microcolony strength in mixed microbial biofilms. Environ Microbiol 13:710–721

Dow JM, Crossman L, Findlay K, He YQ, Feng JX, Tang JL (2003) Biofilm dispersal in *Xanthomonas campestris* is controlled by cell-cell signaling and is required for full virulence to plants. Proc Natl Acad Sci USA 100:10995–11000

Dubey GP, Ben-Yehuda S (2011) Intercellular nanotubes mediate bacterial communication. Cell 144:590–600

Dunny GM, Leonard BA (1997) Cell-cell communication in gram-positive bacteria. Annu Rev Microbiol 51:527–564

Eboigbodin KE, Biggs CA (2008) Characterization of the extracellular polymeric substances produced by *Escherichia coli* using infrared spectroscopic, proteomic, and aggregation studies. Biomacromolecules 9:686–695

Flemming HC, Wingender J (2010) The biofilm matrix. Natl Rev Microbiol 8:623–633

Friedman L, Kolter R (2004a) Genes involved in matrix formation in *Pseudomonas aeruginosa* PA14 biofilms. Mol Microbiol 51(3):675–690

Friedman L, Kolter R (2004b) Two genetic loci produce distinct carbohydrate-rich structural components of the *Pseudomonas aeruginosa* biofilm matrix. J Bacteriol 186:4457–4465

Frølund B, Palmgren R, Keiding K, Nielsen PH (1996) Extraction of extracellular polymers from activated sludge using a cation exchange resin. Water Res 30:1749–1758

Govan JR, Deretic V (1996) Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. Microbiol Rev 60:539–574

Granato D, Bergonzelli GE, Pridmore RD, Marvin L, Rouvet M, Corthésy-Theulaz IE (2004) Cell surface-associated elongation factor Tu mediates the attachment of *Lactobacillus johnsonii* NCC533 (La1) to human intestinal cells and mucins. Infect Immun 72:2160–2169

Hansen SK, Rainey PB, Haagensen JA, Molin S (2007) Evolution of species interactions in a biofilm community. Nature 445:533–536

Harmsen HJ, Kengen HM, Akkermans AD, Stams AJ, de Vos WM (1996) Detection and localization of syntrophic propionate-oxidizing bacteria in granular sludge by in situ hybridization using 16S rRNA-based oligonucleotide probes. Appl Environ Microbiol 62(5):1656–1663

Hengge R (2009) Principles of c-di-GMP signalling in bacteria. Natl Rev Microbiol 7:263–273

Høiby N, Bjarnsholt T, Givskov M, Molin S, Ciofu O (2010) Antibiotic resistance of bacterial biofilms. Int J Antimicrob Agents 35:322–332

Jackson KD, Starkey M, Kremer S, Parsek MR, Wozniak DJ (2004) Identification of *psl*, a locus encoding a potential exopolysaccharide that is essential for *Pseudomonas aeruginosa* PAO1 biofilm formation. J Bacteriol 186:4466–4475

Jeffery CJ (2003) Moonlighting proteins: old proteins learning new tricks. Trends Genet 19:415–417

Jiao Y, D'Haeseleer P, Dill BD, Shah M, Verberkmoes NC, Hettich RL, Banfield JF, Thelen MP (2011) Identification of biofilm matrix-associated proteins from an acid mine drainage microbial community. Appl Environ Microbiol 77:5230–5237

Kamaguchi A, Nakayama K, Ichiyama S, Nakamura R, Watanabe T, Ohta M, Baba H, Ohyama T (2003) Effect of *Porphyromonas gingivalis* vesicles on coaggregation of *Staphylococcus aureus* to oral microorganisms. Curr Microbiol 47:485–491

Kindaichi T, Ito T, Okabe S (2004) Ecophysiological interaction between nitrifying bacteria and heterotrophic bacteria in autotrophic nitrifying biofilms as determined by microautoradiography-fluorescence in situ hybridization. Appl Environ Microbiol 70:1641–1650

Kolodkin-Gal I, Cao S, Chai L, Bottcher T, Kolter R, Clardy J, Losick R (2012) A self-produced trigger for biofilm disassembly that targets exopolysaccharide. Cell 149:684–692

Konovalova A, Petters T, Søgaard-Andersen L (2010) Extracellular biology of *Myxococcus xanthus*. FEMS Microbiol Rev 34:89–106

Kulp A, Kuehn MJ (2010) Biological functions and biogenesis of secreted bacterial outer membrane vesicles. Annu Rev Microbiol 64:163–184

Latasa C, Solano C, Penades JR, Lasa I (2006) Biofilm-associated proteins. C R Biol 329:849–857

Lee N, Nielsen PH, Andreasen KH, Juretschko S, Nielsen JL, Schleifer KH, Wagner M (1999) Combination of fluorescent in situ hybridization and microautoradiography-a new tool for structure-function analyses in microbial ecology. Appl Environ Microbiol 65:1289–1297

Lequette Y, Greenberg EP (2005) Timing and localization of rhamnolipid synthesis gene expression in *Pseudomonas aeruginosa* biofilms. J Bacteriol 187:37–44

Li M, Villaruz AE, Vadyvaloo V, Sturdevant DE, Otto M (2008) AI-2-dependent gene regulation in *Staphylococcus epidermidis*. BMC Microbiol 8:4

López D, Vlamakis H, Kolter R (2010) Biofilms. Cold Spring Harb Perspect Biol 2:a000398

Mashburn LM, Whiteley M (2005) Membrane vesicles traffic signals and facilitate group activities in a prokaryote. Nature 437(7057):422–425

Matsukawa M, Greenberg EP (2004) Putative exopolysaccharide synthesis genes influence *Pseudomonas aeruginosa* biofilm development. J Bacteriol 186:4449–4456

McDougald D, Rice SA, Barraud N, Steinberg PD, Kjelleberg S (2012) Should we stay or should we go: mechanisms and ecological consequences for biofilm dispersal. Natl Rev Microbiol 10:39–50

McSwain BS, Irvine RL, Hausner M, Wilderer PA (2005) Composition and distribution of extracellular polymeric substances in aerobic flocs and granular sludge. Appl Environ Microbiol 71:1051–1057

Nadell CD, Xavier JB, Foster KR (2009) The sociobiology of biofilms. FEMS Microbiol Rev 33:206–224

Nielsen AT, Tolker-Nielsen T, Barken KB, Molin S (2000) Role of commensal relationships on the spatial structure of a surface-attached microbial consortium. Environ Microbiol 2:59–68

Okabe S, Satoh H, Watanabe Y (1999) In situ analysis of nitrifying biofilms as determined by in situ hybridization and the use of microelectrodes. Appl Environ Microbiol 65:3182–3191

O'Toole GA, Kolter R (1998a) Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. Mol Microbiol 30:295–304

O'Toole GA, Kolter R (1998b) Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. Mol Microbiol 28:449–461

O'Toole GA, Pratt LA, Watnick PI, Newman DK, Weaver VB, Kolter R (1999) Genetic approaches to study of biofilms. Methods Enzymol 310:91–109

Otzen D, Nielsen PH (2008) We find them here, we find them there: functional bacterial amyloid. Cell Mol Life Sci 65:910–927

Palsdottir H, Remis JP, Schaudinn C, O'Toole E, Lux R, Shi W, McDonald KL, Costerton JW, Auer M (2009) Three-dimensional macromolecular organization of cryofixed *Myxococcus xanthus* biofilms as revealed by electron microscopic tomography. J Bacteriol 191:2077–2082

Patzold R, Keuntje M, Anders-von Ahlften A (2006) A new approach to non-destructive analysis of biofilms by confocal Raman microscopy. Anal Bioanal Chem 386:286–292

Pearson JP, Pesci EC, Iglewski BH (1997) Roles of Pseudomonas aeruginosa las and rhl quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes. J Bacteriol 179:5756–5767

Petrova OE, Sauer K (2012) Sticky situations: key components that control bacterial surface attachment. J Bacteriol 194:2413–2425

Remis JP, Costerton JW, Auer M (2010) Biofilms: structures that may facilitate cell-cell interactions. ISME J 4:1085–1087

Rickard AH, Gilbert P, High NJ, Kolenbrander PE, Handley PS (2003) Bacterial coaggregation: an integral process in the development of multi-species biofilms. Trends Microbiol 11:94–100

Romero D, Aguilar C, Losick R, Kolter R (2010) Amyloid fibers provide structural integrity to *Bacillus subtilis* biofilms. Proc Natl Acad Sci USA 107:2230–2234

Römling U, Gomelsky M, Galperin MY (2005) C-di-GMP: the dawning of a novel bacterial signalling system. Mol Microbiol 57:629–639

Ryan RP, Dow JM (2011) Communication with a growing family: diffusible signal factor (DSF) signaling in bacteria. Trends Microbiol 19:145–152

Ryan RP, Fouhy Y, Garcia BF, Watt SA, Niehaus K, Yang L, Tolker-Nielsen T, Dow JM (2008) Interspecies signalling via the Stenotrophomonas maltophilia diffusible signal factor influences biofilm formation and polymyxin tolerance in Pseudomonas aeruginosa. Mol Microbiol 68:75–86

Ryjenkov DA, Simm R, Romling U, Gomelsky M (2006) The PilZ domain is a receptor for the second messenger c-di-GMP: the PilZ domain protein YcgR controls motility in enterobacteria. J Biol Chem 281:30310–30314

Sakuragi Y, Kolter R (2007) Quorum-sensing regulation of the biofilm matrix genes (pel) of *Pseudomonas aeruginosa*. J Bacteriol 189:5383–5386

Sauer K, Camper AK, Ehrlich GD, Costerton JW, Davies DG (2002) *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. J Bacteriol 184:1140–1154

Schaefer AL, Greenberg EP, Oliver CM, Oda Y, Huang JJ, Bittan-Banin G, Peres CM, Schmidt S, Juhaszova K, Sufrin JR, Harwood CS (2008) A new class of homoserine lactone quorum-sensing signals. Nature 454:595–599

Schooling SR, Beveridge TJ (2006) Membrane vesicles: an overlooked component of the matrices of biofilms. J Bacteriol 188:5945–5957

Shapiro JA (1998) Thinking about bacterial populations as multicellular organisms. Annu Rev Microbiol 52:81–104

Shimoyama T, Kato S, Ishii S, Watanabe K (2009) Flagellum mediates symbiosis. Science 323:1574

Shrout JD, Nerenberg R (2012) Monitoring bacterial twitter: does quorum sensing determine the behavior of water and wastewater treatment biofilms? Environ Sci Technol 46:1995–2005

Sudarsan N, Lee ER, Weinberg Z, Moy RH, Kim JN, Link KH, Breaker RR (2008) Riboswitches in eubacteria sense the second messenger cyclic di-GMP. Science 321:411–413

Sunde M, Serpell LC, Bartlam M, Fraser PE, Pepys MB, Blake CC (1997) Common core structure of amyloid fibrils by synchrotron X-ray diffraction. J Mol Biol 273:729–739

Tao F, He YW, Wu DH, Swarup S, Zhang LH (2010a) The cyclic nucleotide monophosphate domain of *Xanthomonas campestris* global regulator Clp defines a new class of cyclic di-GMP effectors. J Bacteriol 192:1020–1029

Tao F, Swarup S, Zhang LH (2010b) Quorum sensing modulation of a putative glycosyltransferase gene cluster essential for *Xanthomonas campestris* biofilm formation. Environ Microbiol 12:3159–3170

Tashiro Y, Ichikawa S, Nakajima-Kambe T, Uchimura H, Nomura N (2010) *Pseudomonas* quinolone signal affects membrane vesicle production in not only Gram-negative but also Gram-positive bacteria. Microbes Environ 25:120–125

Tashiro Y, Uchiyama H, Nomura N (2012) Multifunctional membrane vesicles in *Pseudomonas aeruginosa*. Environ Microbiol 14:1349–1362

Toda K, Yawata Y, Setoyama E, Fukuda J, Nomura N, Suzuki H (2011) Continuous monitoring of ammonia removal activity and observation of morphology of microbial complexes in a microdevice. Appl Environ Microbiol 77:4253–4255

Toyofuku M, Nomura N, Fujii T, Takaya N, Maseda H, Sawada I, Nakajima T, Uchiyama H (2007) Quorum sensing regulates denitrification in *Pseudomonas aeruginosa* PAO1. J Bacteriol 189:4969–4972

Toyofuku M, Nomura N, Kuno E, Tashiro Y, Nakajima T, Uchiyama H (2008) Influence of the *Pseudomonas* quinolone signal on denitrification in *Pseudomonas aeruginosa*. J Bacteriol 190:7947–7956

Toyofuku M, Roschitzki B, Riedel K, Eberl L (2012a) Identification of proteins associated with the *Pseudomonas aeruginosa* Biofilm extracellular matrix. J Proteome Res 11:4906–4915

Toyofuku M, Uchiyama H, Nomura N (2012b) Social behaviours under anaerobic conditions in *Pseudomonas aeruginosa*. Int J Microbiol 2012. doi:10.1155/2012/405191

Urbain V, Block JC, Manem J (1993) Bioflocculation in activated-sludge: an analytic approach. Water Res 27:829–838

Venkata HNN, Nomura N, Shigeto S (2011) Leucine pools in *Escherichia coli* biofilm discovered by Raman imaging. J Raman Spectrosc 42:1913–1915

Vuong C, Saenz HL, Götz F, Otto M (2000) Impact of the agr quorum-sensing system on adherence to polystyrene in *Staphylococcus aureus*. J Infect Dis 182:1688–1693

Watanabe M, Sasaki K, Nakashimada Y, Kakizono T, Noparatnaraporn N, Nishio N (1998) Growth and flocculation of a marine photosynthetic bacterium Rhodovulum sp. Appl Microbiol Biot 50:682–691

Whitchurch CB, Tolker-Nielsen T, Ragas PC, Mattick JS (2002) Extracellular DNA required for bacterial biofilm formation. Science 295:1487

Yawata Y, Nomura N, Uchiyama H (2008) Development of a novel biofilm continuous culture method for simultaneous assessment of architecture and gaseous metabolite production. Appl Environ Microbiol 74:5429–5435

Yawata Y, Toda K, Setoyama E, Fukuda J, Suzuki H, Uchiyama H, Nomura N (2010) Monitoring biofilm development in a microfluidic device using modified confocal reflection microscopy. J Biosci Bioeng 110:377–380

Yoon SS, Hennigan RF, Hilliard GM, Ochsner UA, Parvatiyar K, Kamani MC, Allen HL, DeKievit TR, Gardner PR, Schwab U, Rowe JJ, Iglewski BH, McDermott TR, Mason RP, Wozniak DJ, Hancock RE, Parsek MR, Noah TL, Boucher RC, Hassett DJ (2002) *Pseudomonas aeruginosa* anaerobic respiration in biofilms: relationships to cystic fibrosis pathogenesis. Dev Cell 3:593–603

Yoon MY, Lee KM, Park Y, Yoon SS (2011) Contribution of cell elongation to the biofilm formation of *Pseudomonas aeruginosa* during anaerobic respiration. PLoS One 6:e16105. doi:10.1371/journal.pone.0016105

Zhang LH, Dong YH (2004) Quorum sensing and signal interference: diverse implications. Mol Microbiol 53:1563–1571

Zogaj X, Nimtz M, Rohde M, Bokranz W, Romling U (2001) The multicellular morphotypes of *Salmonella typhimurium* and *Escherichia coli* produce cellulose as the second component of the extracellular matrix. Mol Microbiol 39:1452–1463