Chapter 13
Bacterial Biofilms: Role in *Rhizobium*–Legume Symbiosis

Luciana V. Rinaudi and Walter Giordano

Abstract Biofilms are surface-attached communities of bacteria contained within a self-produced extracellular polymeric matrix. They are composed of a single species or, more commonly, several species of bacteria. This multicellular mode of growth provides a protective measure against adverse environmental conditions and promotes survival of organisms. Biofilms can be established on both abiotic and biotic surfaces, typically under stressful conditions. Bacteria that colonize plant surfaces can have either negative (pathogenic) or positive (symbiotic) effects and are therefore important in agriculture. In this chapter, we review the current knowledge of soil bacterial biofilms, various bacterial functions that influence biofilm formation, and the contributions or effects of exopolysaccharides, quorum sensing, rhizobial proteins, and motility on this process.

13.1 Introduction

Plant roots secrete a wide range of compounds into the surrounding soil, the rhizosphere, which creates nutrient-rich conditions for microbial growth. It is reported that up to 40% of the carbon fixed by plants is converted into root exudates (Lynch and Whipps 1990) which contains ions, free oxygen, water, enzymes, and carbon-based compounds (Bais et al. 2006) such as carbohydrates, amino acids, organic acids, mucilage, and proteins. However, root exudates vary with type of soil and nutrient availability, environmental factors such as temperature, light and soil moisture, and physiological stage of the plant. Root-derived compounds create a niche around the roots and mediate positive and negative interactions among microorganisms. Association of plants with beneficial microbes, collectively called

L.V. Rinaudi and W. Giordano (✉)
Departamento de Biología Molecular, Universidad Nacional de Río Cuarto, Ruta 36 Km 601, X5804BYA Río Cuarto, Córdoba, Argentina
e-mail: wgiordano@exa.unrc.edu.ar

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as plant growth-promoting rhizobacteria (PGPR), include nitrogen-fixing bacteria, mycorrhizal fungi, and biocontrol agents, which are grouped in positively interacting organisms. Negative interactions on the other hand include associations of plants with pathogenic bacteria or fungi.

Many microorganisms exist in their natural environment not as free-living bacteria but as sessile multicellular communities called biofilms. Biofilms are defined as an assemblage of microbial cells that are irreversibly associated (not removed by gentle rinsing) with a surface and enclosed in a matrix of primarily polysaccharide material allowing growth and survival in sessile environment (Fig. 13.1). Scientists have recently realized that in the natural world, more than 99% of all bacteria exists as biofilms (Costerton et al. 1987). Inside biofilms, bacteria undergo physiological changes in relation to individual, planktonic cells, leading to special proteomes and metabolic activities (Whiteley et al. 2001; Sauer et al. 2002; Vilain et al. 2004). The extracellular matrix, mostly composed of exopolysaccharides (EPS), is believed to play a key role in biofilm endurance (Stoodley et al. 2002). Other components like proteins, DNA, and products from bacterial lysis provide the matrix for biofilm formation, which allows first, attachment of the cells to a solid surface and to each other and later, colonization of such surface/substrate. This sessile lifestyle confers bacteria within the biofilm resistance against certain environmental stresses (such as desiccation, pH changes, UV radiation) as well as tolerance against antibiotics and defense-related compounds from the host, protection from protozoan predation, enhancement of genetic exchange (through horizontal gene transfer), and production of secondary metabolites and exoenzymes (Danhorn and Fuqua 2007). Such transition from planktonic to sessile state is mediated by numerous environmental signals as well as by accumulation of quorum-sensing signals (called autoinducers), which mediate cell–cell communication in bacteria and coordinate communal behavior by regulating specific genes in response to population density. Biofilm formation may be a result of passive

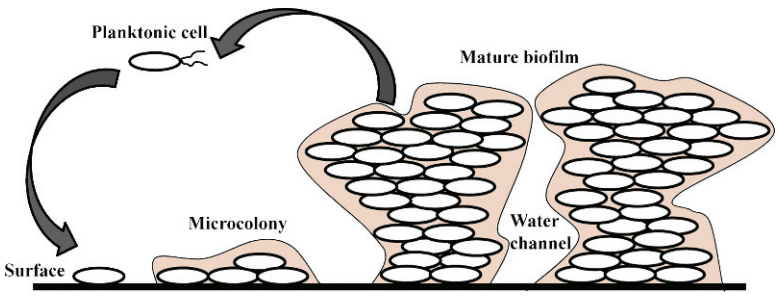

Fig. 13.1 Free-living (planktonic) bacteria are able to attach to a surface and form microcolonies on it. Microorganisms inside such microcolonies start producing the exopolysaccharides that serve as the matrix for biofilms. Finally these communities develop into highly structured communities surrounded by water channels, which allow the exchange of extracellular signals, nutrients and toxic metabolic wastes. Attached bacteria can also return to the swimming mode of life. For this reason biofilm formation and then dispersal of the biofilms are considered as a cycle

deposition and accumulation of bacterial cells on a surface due to, for example, water flow in the rhizosphere or, in other cases, to active processes of bacterial chemotaxis, attachment, and production of exopolymeric substances. No matter the origin of the biofilm, it confers bacteria an adaptive advantage to confront hostile environments, allowing cells within the community to cooperate and realize functions not exhibited by single cells (Morris and Monier 2003).

Root attachment and colonization constitutes the first step during the development of beneficial as well as pathogenic associations between plants and microorganisms. During the *Rhizobium*–legume symbiosis, attachment of rhizobia to legume roots constitutes a requirement for infection and nodulation (Rodríguez-Navarro et al. 2007). Biofilm formation was first reported in rhizobia by Seneviratne and Jayasinghearachchi in 2003, who observed that *Bradyrhizobium* sp. is able to form typical biofilm structures on diverse biotic and abiotic surfaces (Seneviratne and Jayasinghearachchi 2003). Furthermore, roles of EPS (Fujishige et al. 2006b; Russo et al. 2006; Williams et al. 2008; Rinaudi and González 2009), Nod factor (Fujishige et al. 2008) and other mechanisms involved in biofilm formation have been studied in several species of rhizobia as well as the conditioning of biofilm formation by nutrient and osmotic cell status (Rinaudi et al. 2006) (Table 13.1).

Root exudates though provide a carbon-rich environment for microbial growth, yet it initiates cross-talk with soil microbes leading to plant colonization (Bais et al. 2006). Biofilm formation has been reported to be influenced by abiotic factors, as, nutrient availability, osmolarity, temperature, and soil moisture (Stanley and Lazazzera 2004). In addition, biofilm formation is also influenced by nutrient release and exudation at different sites along the roots (Ramey et al. 2004). It has been proposed that more structured biofilms are formed on mature regions of the roots due to high nutrient availability, while lower nutrient availability or the secretion of antimicrobials from the root tip lead to reduced biofilm formation

Table 13.1 Sumary of some of the articles available on biofilm formation by rhizobia

Role described in biofilm formation	Rhizobial species	Source
Exopolysaccharide	*S. meliloti*	Fujishige et al. (2006b), Wells et al. (2007), Rinaudi and González (2009), Rinaudi et al. (2010)
	M. tianshanense	Wang et al. (2008)
	R. leguminosarum	Vanderlinde et al. (2002), Russo et al. (2006), Williams et al. (2008)
	B. japonicum	Pérez-Giménez et al. (2009)
Quorum sensing	*S. meliloti*	Rinaudi and González (2009)
	M. huakii	Wang et al. (2004)
	R. leguminosarum	Edwards et al. (2009)
Rhizobial proteins	*B. japonicum*	Dardanelli et al. (2003)
	R. leguminosarum	Mongiardini et al. (2008)
Motility	*S. meliloti*	Fujishige et al. (2006b)
	R. leguminosarum	Verstraeten et al. (2008)
Nod factors	*S. meliloti*	Fjishige et al. (2008)

(Rudrappa et al. 2008a). In this chapter, the bacterial functions involved in the establishment of beneficial biofilms on the plant root surface as well as the rhizosphere environment are discussed.

13.2 Plant Products Regulating Associations with Microorganisms

Biofilm formation confers some advantages to beneficial and pathogenic bacteria, but how are these interactions regulated is not clear. Interestingly, it has been reported that plants can recognize and attract beneficial organisms, like rhizobia and mycorrizal fungi, by releasing secrete secondary metabolites while prevent the attachment of harmful ones (Ramey et al. 2004). For example, it has recently been shown that *Arabidopsis thaliana* plants infected with *Pseudomonas syringae* can secrete malic acid into rhizosphere, which activates chemotaxis and biofilm formation by *Bacillus subtilis* (Rudrappa et al. 2008b). Consequently, the roots colonized by *B. subtilis* result in protection of *Arabidopsis* plants from infection by foliar pathogen (*P. syringae)* by inducing systemic resistance (ISR) on the host plant (Rudrappa et al. 2008b). Interestingly, biofilm formation by beneficial microorganisms seems to be also regulated by in planta redox potential in the rhizosphere (Rudrappa and Bais 2007). For example, root colonization by *B. subtilis* is suppressed on *A. thaliana NahG* plants through reactive oxygen species (ROS) mediated down-regulation of the *yqxM* and *epsA* operons required for biofilm formation by *Bacillus* (Rudrappa et al. 2007). Plants have, however, evolved several mechanisms to prevent negative interactions. Among these, host plants produce quorum-sensing like signal molecules (plant quorum-sensing mimics) capable of interacting with quorum-sensing systems from different bacterial strains (Teplitski et al. 2000; Mathesius et al. 2003; Keshavan et al. 2005). Such molecules help plants to protect themselves from pathogens and also modify bacterial behavior from pathogens (González and Marketon 2003), such as the formation of biofilms. It has also been reported that plants produce sesquiterpene lactones that are able to inhibit biofilm formation by *Pseudomonas aeruginosa* (Cartagena et al. 2007). Plants also combat bacterial pathogens by secreting antimicrobial compounds through the roots. In this regard, rosmarinic acid secreted by the roots of sweet basil (*Ocymum basilium*) plants upon infection by *P. aeruginosa* showed antibacterial activity against planktonic cells and consequently prevented biofilm formation. However, established biofilms resist microbiocidal effects of rosmarinic acid and ultimately cause plant mortality (Walker et al. 2004). Biofilm formation on *Arabidopsis* roots by the pathogenic *P. aeruginosa* PA14 is affected by the synthesis of salicylic acid, which not only induce plant-defense responses against pathogen attacks but also downregulates the production of several virulence factors on *Pseudomonas* such as the pigment pyocyanin and the exoenzymes protease and elastase (Prithiviraj et al. 2005).

13.3 Mechanisms of Biofilm Formation

13.3.1 Exopolysaccharide Production

During plant–bacterial interactions, EPS are known to be involved in adhesion of bacteria to roots (Michiels et al. 1991), root colonization (Matthysse et al. 2005) and hence, serve as primary factor in the development of biofilms on plant roots (Bianciotto et al. 2001; Ramey et al. 2004; Fujishige et al. 2006a). In the rhizosphere, bacterial EPS contribute further to soil aggregation by cementing particles together (Chenu 1995). Inoculation of plants with EPS-producing rhizobacteria, such as *Rhizobium* sp. YAS34 (Alami et al. 2000) and *Rhizobium* sp. KYGT207 (Kaci et al. 2005), modifies the aggregation of root-adhering soil and eventually improves plant growth. EPS also play an important role in biofilm formation by rhizobia (Fujishige et al. 2006b; Russo et al. 2006; Wells et al. 2007; Wang et al. 2008; Williams et al. 2008; Pérez-Giménez et al. 2009; Rinaudi and González 2009; Rinaudi et al. 2010). For example, *Sinorhizobium meliloti* has the ability to produce two EPS, succinoglycan and EPS II. Biofilm formation by *S. meliloti* Rm1021 (an *expR* mutant) on the contrary seems to be independent of EPS (Rinaudi et al. 2010). However, overproduction of succinoglycan in *exoR* and *exoS* mutants led to an increase in biofilm formation compared to wild type (Fujishige et al. 2006b; Wells et al. 2007). On the other hand, in *S. meliloti* Rm8530 (a strain with an intact copy of the *expR* gene which allows EPS II production) biofilm formation depends on the presence of the low-molecular weight fraction of EPS II, which mediates attachment to abiotic surfaces such as PVC and borosilicate, as well as to roots of the legume host *Medicago sativa* (Rinaudi and González 2009). In this sense, EPS II-producing strains have been found as efficient root-hair colonizers while strains lacking EPS II, or only able to produce HMW fraction of this polymer, form very low levels of biofilm colonizing mostly the principal roots forming patchy colonies (Rinaudi and González 2009). In other studies, *Rhizobium leguminosarum* formed highly structured and organized biofilms on borosilicate when evaluated by Confocal Laser Scanner Microscopy (CLSM) (Russo et al. 2006; Williams et al. 2008). Biofilms formed by cellulose and glucomannan (*celA* and *gmsA*, respectively) are indistinguishable from those of the wild-type strain. However, these mutants were defective in root colonization when incubated with host plant *Vicia hirsuta*, suggesting that interactions between the rhizobia and glass surface are different from those occurring during root cap formation (Williams et al. 2008). A mutant of *R. leguminosarum* bv. *viciae* highly sensitive to desiccation has been isolated (Vanderlinde et al. 2002). This mutant in an ABC transporter shows a reduction in the accumulation of EPS and it is also defective in biofilm formation on polystyrene microplates, which proves the importance of EPS in desiccation tolerance in rhizobia and provided evidence for the role of biofilm formation against environmental stresses. In yet other investigation, an EPS mutant of *B. japonicum*, which lacks UDP-glucose-4′ epimerase activity and produced low levels of a shorter EPS lacking galactose, showed reduced adhesion to soybean (*Glycine*

max) roots compared to wild-type strain, indicating that complete EPS is required for efficient colonization of soybean by *B. japonicum* (Pérez-Giménez et al. 2009). Similarly, EPS-deficient strains of *Mesorhizobium tianshanense* showed low levels of biofilm formation on borosilicate and fail to nodulate *Glycyrrhiza uralensis*, suggesting that EPS are essential for biofilm formation (Wang et al. 2008).

Bacterial attachment by the biocontrol *Pseudomonas fluorescens* CHA0 strain to the external mycelium of *Glomus intraradices,* mycorrhizal, and nonmycorrhizal carrot roots has been evaluated (Bianciotto et al. 2001). In all cases, two mucoid mutants overproducing an alginate-like EPS showed an enhanced attachment to the surfaces compared to that of the wild type (Bianciotto et al. 2001). Biofilm formation and overproduction of the matrix may improve persistence and survival of these mutants in the soil, since it confers resistance to several environmental stresses; however, it has also been proposed that this may not lead to an increased plant protection by biocontrol strains since antifungals or antibiotics may remain trapped within the biofilm and overproduction of EPS may limit diffusion of these compounds to the rhizosphere (Bianciotto et al. 2001). However, overexpression of EPS does not always correlate with an increased biofilm formation capability in all bacteria (Parsek and Fuqua 2004). For instance, *Agrobacterium tumefaciens* is a soil bacterium that forms biofilms on the roots of plants such as tomato, alfalfa, and *A. thaliana* (Matthysse et al. 2005). Cellulose-minus mutants of *A. tumefaciens*, however, fail to attach to tomato roots and showed a reduced colonization of the surface, while overproduction of cellulose resulted in an increased biofilm formation and reduced root colonization when compared to wild type (Matthysse et al. 2005). Although most reports indicate EPS play an important role during biofilm formation this cannot be considered as a rule since EPS production by *Rhizobium* sp. YAS34 is not essential for biofilm formation, either on polypropylene surfaces or on roots of two nonlegume plants, *A. thaliana* and *Brassica napus* (Santaella et al. 2008).

13.3.2 Quorum Sensing

Presence of different quorum-sensing systems has been described in rhizobia, which regulate several phenotypes such as plasmid transfer, nodulation efficiency, nitrogen fixation, EPS production, and swarming motility (Sánchez-Contreras et al. 2007). Recently, quorum sensing has also been shown to play a role in biofilm formation (Wang et al. 2004; Zheng et al. 2006; Edwards et al. 2009; Rinaudi and González 2009). For example, mutant strains in the MrtR-MrtI quorum-sensing system in *M. tianshanense* showed a 60% reduction in root hair attachment efficiency, which may explain the reason why these strains are unable to nodulate the legume host *G. uralensis* (Zheng et al. 2006). While in *R. leguminosarum,* disruption of the CinI/CinR quorum-sensing system led to an increase in biofilm formation (Edwards et al. 2009). This effect seems mediated by the transcriptional regulator ExpR as well as the small protein CinS, coexpressed with the autoinducer

synthase CinI. ExpR and CinS regulate expression of the EPS glycanase PlyB, responsible for the cleavage of the acidic EPS, which has been involved in biofilm formation (Russo et al. 2006; Williams et al. 2008). The presence of an intact ExpR/Sin quorum-sensing system in *S. meliloti* is essential for the formation of large amounts of biofilm on abiotic surfaces and also regulates the structure of mature biofilms (Rinaudi and González 2009). In this way, it has been shown that Rm1021 lacking the *expR* gene forms a flat biofilm with no apparent structure or organization (Fig. 13.2a), while Rm8530, which has an intact ExpR/Sin quorum-sensing system, produces structured and highly organized biofilms (Fig. 13.2b, c).

13.3.3 Rhizobial Proteins

In addition to quorum sensing and EPS, some rhizobial proteins are also involved in biofilm formation. As an example, in *Bradyrhizobium* sp., a rhicadhesin-like protein mediates rhizobial attachment to peanut (*Arachis hypogaea*) roots (Dardanelli et al. 2003). The rhizobial adhesion protein 1 (Rap1), an extracellular calcium-binding protein from *R. leguminosarum* bv. *trifolii* promotes rhizobial autoaggregation through cell poles, and is involved in attachment to the legume host red clover (*Trifolium pratense*) and nonsymbiotic plants such as common bean, alfalfa, and soybean (Mongiardini et al. 2008).

13.3.4 Motility

Various bacterial motility mechanisms, such as swarming, swimming, and twitching, are known to have a profound impact on biofilm formation, including colonization and the subsequent expansion into mature structured surface communities. In a study, nonflagellated and nonchemotactic mutants of *Azospirillum brasilense* showed a strongly reduced colonization of wheat (*Triticum aestivum*) roots as compared to the wild type suggesting that initiation of root colonization requires

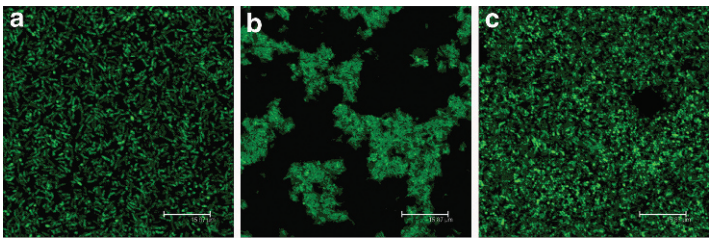

Fig. 13.2 Single-scan images from *Sinorhizobium meliloti* biofilms obtained by confocal laser scanning microscopy. (**a**) "flat" (**b**) "structured", and (**c**) "organized" honeycomb-like biofilms. The size bars indicate 15.8 μm. (Images courtesy of J.E. González)

active bacterial motility (Vande Broek et al. 1998). On the contrary, the motile strains of *P. fluorescens* had a higher rate of survival in soil and attached better to wheat roots than nonmotile strains (Turnbull et al. 2001). Furthermore, the motility allowed movement of bacteria from the roots to the surrounding rhizosphere. This movement was facilitated by signal compounds present in root/seed exudates, known to influence attachment, colonization, and biofilm formation. Similarly, the effect of exudates released by seeds and roots of soybean on chemotaxis and biofilm formation has been studied in the *Bacillus amyloliquefaciens* strain BNM339 with biocontrol activity against several fungi causing crop-related diseases (Yaryura et al. 2008). These and other associated findings thus suggests that chemotaxis, and consequently motility, are regulated by quantitative and qualitative changes in the composition of seed and root exudates. As with other PGPR, Fla^- mutants of *S. meliloti* show a poor biofilm formation capability when compared to wild-type strain Rm1021 (Fujishige et al. 2006b). The *fliP* and *flgH* mutants used in the study showed more than 50% reduction in biofilm formation, being defective in their initial attachment to PVC. Although no assays have been performed on legume roots yet, it may be speculated that such mutants would be impaired as well in biofilm formation on roots, which could explain the delay in nodule development shown by these strains.

13.4 Conclusion

Even though it is yet to establish whether there is a direct relationship between biofilm formation and infectivity, *S. meliloti* succinoglycan-producing strains, though did not colonize alfalfa (*Medicago sativa*) roots as efficiently as EPS II-producing strains, more efficiently invaded the legume (Pellock et al. 2002). This study indicated that biofilm formation may provide rhizobia with an advantageous microenvironment to persist in the soil and eventually colonize root surfaces and establish the symbiosis but it is not essential for legume invasion. On the other hand, plant hosts as well as other soil microorganisms may benefit from the biofilm-forming ability of other plant growth-promoting rhizobacteria since EPS within biofilms improve soil structure and help maintain the soil moisture (Morris and Monier 2003). Additionally, biofilms may also enhance nitrogen and phosphate availability when established on soils, as found with bradyrhizobia and common soil fungi (Sereviratne and Jayasinghearachchi 2005). Moreover, the information strongly suggests that like other bacterial biofilm, rhizobacteria biofilm formation is a complex process and hence, cannot be explained easily at molecular level employing a single mechanism.

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