

Chapter 18

Microbial Control of Root-Pathogenic Fungi and Oomycetes

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Abstract The rhizosphere is a complex and dynamic environment in which microbes introduced to control root pathogens must establish and maintain populations of sufficient size and activity to antagonize pathogens directly or by manipulating the host plant's own defenses. Genetic and physiological studies of rhizobacteria with the capacity to control root pathogens have given considerable insight into the microbial side of these interactions, but much remains to be learned about the physical conditions and the chemical and biological activities that take place at the root-microbe interface. This chapter focuses on advances in our understanding of the constraints to the successful introduction of microbial agents for the control of soil-borne root pathogens and the mechanisms involved in pathogen suppression. Chapters elsewhere in this volume address related topics including plant growth promotion, stress control, the activation of the plant's own defense mechanisms by introduced microbes, and powerful new biotechnological advances available to gain insight into rhizosphere processes.

18.1 Colonization: A Necessary Requisite for Biological Control

Root colonization, a challenge to both indigenous microbes and those introduced to control root pathogens, is the process by which bacteria become distributed along the root, propagate, and persist for weeks or longer in the presence of the indigenous rhizosphere microflora. How many bacteria are needed to achieve pathogen control? The minimum population size required will depend in part on the disease pressure,

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the distribution of the bacteria along the root, and the mechanism of control, but several lines of evidence suggest a minimum average population size of 10^5 per gram of root, at least for antibiosis and the induction of systemic resistance (Pieterse et al. 2014).

Bacteria on root surfaces are not uniformly distributed; they reside in discrete mucigel-enclosed aggregates termed biofilms where nutrients are available. Such sites include wounds along the root, root tips, the junctions between epidermal cells, and regions where root hairs and lateral roots emerge. Exudates from these sites are a dominant source of nutrients for rhizobacteria, and there is increasing evidence that the sugars, organic and amino acids, phenolics, and other signal molecules in exudates maintain a complex chemical dialog between the plant and its associated microflora (Zolla et al. 2013). The quality, quantity, and composition of root exudates vary widely with the plant species and the biotic and abiotic stresses acting upon it, and these factors have a significant effect on the structure and composition of the associated microbial communities. There has been considerable effort in recent years to isolate and characterize exudates, but the best methods now available still identify only a fraction of the compounds present (Zolla et al. 2013).

Biofilms The extracellular matrix in which bacterial cells on roots are embedded in biofilms is composed mainly of proteins and exopolysaccharides, of which the latter vary in composition among strains but are key structural components (Martínez-Gil et al. 2013). In nature, biofilms provide microbes with a stable protective barrier against chemical stresses and protozoal grazing. In the model strain *Pseudomonas putida* KT2440, an efficient colonizer of seeds and roots, two very large extracellular proteins, LapA and LapF, have sequential roles in biofilm development. LapA first facilitates a cell-surface interaction resulting in irreversible bacterial attachment, and LapF mediates subsequent cell-cell interactions, providing support for expansion and maturation of the biofilm. Complex interactions modulated by the two-component global regulators GacS/GacA and the bacterial universal second messenger cyclic dimeric guanosine phosphate (c-di-GMP) have integral roles in the balance of protein and polysaccharide constituents within the biofilm (Martínez-Gil et al. 2014) and therefore its structural integrity and characteristics. c-di-GMP also has an important role in the transition of cells in biofilms to the planktonic form associated with motility. Biosurfactants such as cyclic lipopeptides (cLPs) and rhamnolipids also can influence the formation, as well as the stability and dissolution, of biofilms, and it has been postulated that the contrasting roles of this diverse family of compounds may be due to differences in their chemical structures and physicochemical properties, as well the ionic conditions and pH of their environment (Raaijmakers et al. 2010).

Motility Bacterial movement along roots may occur passively with root elongation or redistribution with water. Alternatively, dispersal mediated by flagellar swimming or swarming can be active. Numerous studies have demonstrated that bacterial mutants defective in motility and chemotaxis, the ability to detect and move towards nutrients, also are impaired in rhizosphere colonization (Lugtenberg and Kamilova 2009; Pliego et al. 2011). Recent studies suggest that these mechanisms are likely to require relatively hydrated root surfaces (Dechesne et al. 2010; Dechesne and

Smets 2012) and/or the presence of biosurfactants that can modulate the viscosity and surface tension of the thin water films that are thought to be present on roots under common agricultural conditions. Biosurfactants clearly contribute to microbial surface motility *in vitro*, but whether they have a similar role in the environment remains uncertain (Raaijmakers et al. 1999). Collectively, however, it is apparent that introduced bacteria can and do move along the root, that movement can be facilitated by one or more different mechanisms, and that a greater understanding is needed of the physicochemical and hydrodynamic forces that prevail in the rhizosphere.

18.2 Mechanisms of Biological Control

Biological control of plant root pathogens may be mediated indirectly, by competition for nutrients and niches on the root or by induction of resistance in the host plant. Alternatively, biocontrol agents may directly antagonize the pathogen via the production of biosurfactants, antibiotics, or enzymes that hydrolyze the pathogen cell wall. Biocontrol agents often express more than one mechanism of action, and metabolites such as the antibiotics and iron-sequestering siderophores produced by many biocontrol agents may have multiple activities, affecting not only the target pathogen but also the host plant and other members of the microbial community.

Competition for Niches and Nutrients (CNN) Kamilova et al. (2005) used a classic selective enrichment procedure to recover novel biocontrol agents enhanced in competitive colonization of the root. A mixture of rhizosphere strains was introduced onto surface-sterilized tomato seeds and the seeds were allowed to germinate and grow for one week in a gnotobiotic (germ-free) system. Bacteria that had moved along the root to the tip were then collected, introduced onto sterile seeds, and the cycle was repeated three times. Many of the isolates recovered from root tips after this procedure were found to protect tomato against foot and root rot caused by *Fusarium oxysporum* f. sp. *radicis-lysoopersici*, and studies with mutants showed that both motility and the ability to grow efficiently on nutrients in tomato root exudate were important for biocontrol (Lugtenberg and Kamilova 2009; Pliego et al. 2011). Not all of the recovered isolates were effective against the pathogen, however, and results of another study (Pliego et al. 2011) involving the white root rot pathogen of avocado indicated that not only aggressive colonization of the root, but also colonization of specific target sites of pathogen attack on the root, or even colonization of the surface of the pathogen itself, may be required to achieve disease control.

Induced Systemic Resistance (ISR) In induced systemic resistance, a plant's first line of defense against pathogens, selected beneficial microbe-associated inducers on roots activate and sensitize (*i.e.*, *prime*) the entire plant, including the above-ground parts, for defense against diverse pathogens (Pieterse et al. 2014). Like immunization in animals, *priming* sensitizes the immune system to an enhanced state of readiness such that defenses can be mobilized rapidly and systemically upon

subsequent pathogen attack. However, priming differs from immunization in that immunization targets specific pathogens whereas priming by beneficial root-associated microbes enhances defenses against diverse pathogens and even herbivorous insects. The exposure of roots to certain common microbial structural elements such as flagella, lipopolysaccharides, and chitin, and microbially-produced compounds such as siderophores, certain antibiotics, biosurfactants, and volatiles, can result in priming in a wide variety of plant species.

Because the induction of defense and the enhanced defense reaction in ISR take place in spatially separated locations, plants must employ a complex long-distance signaling pathway that starts at the root-microbe interface. Signaling is modulated by the hormones ethylene and jasmonic acid (and less commonly by salicylic acid). In all cases, however, a complex array of signaling proteins and transcriptional regulators is involved that accumulates after induction of the primed state. This pathway, and the identity of ISR long-distance signals, are currently subjects of active investigation.

Siderophore-Mediated Competition for Iron and Induced Systemic Resistance

Under conditions of low iron availability bacteria produce siderophores, low molecular weight compounds that sequester iron in the environment and facilitate its uptake by bacterial cells (Höfte 1993). Since basically all organisms require iron to grow and function, and iron availability in soil is extremely low, competition for iron between microorganisms in the rhizosphere is expected to be a common phenomenon. Siderophores produced by different microorganisms can belong to different classes and they have different structures (Höfte 1993). Accordingly, specific receptors are required to utilize ferric siderophore complexes (Hartney et al. 2011). Thus it was postulated that bacteria that produce siderophores with a high affinity for iron and that are specific, that is they can be utilized by the producer but not by other microbes, could be effective biological control agents. Indeed competition for iron between microorganisms in the rhizosphere has been demonstrated using reporter gene constructs that respond to bioavailability of iron (Loper and Henkels 1999). In several studies on fluorescent pseudomonads it was demonstrated that mutants unable to produce their fluorescent siderophore also (partially) lost their ability to control disease (see for example De Boer et al. 2003). Based on such studies, siderophore-mediated competition for iron is considered an important mechanism in biological control of soil-borne diseases.

Siderophores also have been implicated as effective elicitors of ISR in plants (Meziane et al. 2005). Thus, when plants are protected from disease by siderophores produced by biological control agents, it is difficult to assess if this is due to competition for iron, to ISR, or to both. In radish, *Pseudomonas putida* WCS358 effectively controls fusarium wilt, caused by *Fusarium oxysporum* f. sp. *raphani*, through the production of its pyoverdine siderophore (De Boer et al. 2003). Strain WCS358 cannot elicit ISR in radish and thus in this case the suppression of disease most likely depends on competition for iron between the biological control agent and the pathogen. Evidence that the fluorescent siderophore produced by strain WCS358 can elicit ISR comes from studies in *Arabidopsis thaliana*, bean and tomato. In all three plant species, application of the purified pyoverdine of WCS358 did elicit ISR.

In *A. thaliana* and bean, a pyoverdinin mutant was as effective as the wild-type, but in tomato the pyoverdinin mutant did not elicit ISR (Meziane et al. 2005). For tomato the role of pyoverdinin in ISR is straightforward but for *A. thaliana* and bean there seems to be redundancy of bacterial elicitors of ISR and next to the pyoverdinin, both lipopolysaccharides and flagella play a role. The observed redundancy may give robustness to biological control, since if one of the factors involved in biocontrol is not produced by the bacterium the additional determinants can still be effective. This situation may however further complicate studies on siderophore-mediated competition for iron. In several studies it was suggested that siderophores do not play a role in disease suppression since knock out mutants were as effective as the wild-type. When we consider that there can be redundancy in biological control traits in a single biological control agent, the mutant approach to study possible involvement of siderophores is not waterproof and in several cases, rejecting their role may have been unjustified. Nevertheless, siderophores have been demonstrated to play a significant role in biocontrol and both competition for iron and triggering ISR can be involved. If both mechanisms were active simultaneously this would first weaken the pathogen in the rhizosphere by iron depletion and then confront it with an enhanced plant defense resulting in effective biocontrol. Such a scenario may explain why siderophores were early on revealed as being important effectors of biological control of soil-borne diseases.

Antibiosis Antibiotics are small organic molecules produced by microorganisms that are deleterious to the growth or metabolic activities of other microorganisms. Most bacteria involved in biocontrol have the capacity to produce antibiotics, and many produce multiple such metabolites with broad-spectrum activity against a wide range of plant pathogens (Raaijmakers and Mazzola 2012). Antibiotic-producing strains typically are detected by their ability to inhibit the growth of target pathogens *in vitro* and then are tested for biocontrol in soil, but this approach often fails due to the producer strain's inability to compete successfully in the rhizosphere or because conditions there do not support the synthesis of inhibitory levels of the active agent in the sites where pathogens attack. While the spatiotemporal and quantitative aspects of antibiotic production in nature are only now beginning to be explored, it has become apparent in recent years that antibiotics play multiple roles in natural systems. At subinhibitory concentrations these molecules can function as molecular signals in such diverse activities as biofilm formation and cellular differentiation, motility and dispersal, and defense against predators and competitors (Raaijmakers and Mazzola 2012), all of which may impact upon biological control, and some antibiotics are produced in the environment in sufficient quantities to inhibit pathogens (Mavrodi et al. 2012). The availability since 2005 of genomic DNA sequences not only for model biocontrol strains, but also for environmental isolates, has greatly facilitated the identification of a repertoire of novel metabolites and gene clusters with the potential to exhibit antibiotic activity. *Pseudomonas* and *Bacillus* spp. are the most widely studied biocontrol agents to date, and *Bacillus* spp. are the most frequently commercialized because they are more readily formulated.

Biosurfactant Antibiotics Produced by *Bacillus* spp Cyclic lipopeptides (cLP) produced by root-associated bacilli are composed of a lipid tail linked to a short cyclic oligopeptide and include members of three main families, the surfactins, iturins and fengycins, which differ in the type, number and sequence of amino acid residues and the nature of peptide cyclization. Given their structural diversity, it is not surprising that these antibiotics also differ in their modes of action and their contributions to the biocontrol activity of the cells that produce them. For example, surfactins are powerful biosurfactants that can interfere with biological membrane integrity, but they have no marked toxicity to fungi. In contrast, both iturins and fengycins exhibit antifungal activity which is thought to be due to their ability to form ion-conducting pores in target cell membranes, leading to an imbalance in transmembrane ion fluxes and cell death. These latter classes of cLPs have been implicated directly in antagonism of soil-borne and foliar fungal phytopathogens including *Fusarium graminearum*, *Rhizoctonia solani*, *Botrytis cinerea*, *Penicillium roqueforti*, and *Aspergillus flavus* (Raaijmakers et al. 2010).

Rhizosphere factors profoundly influence the production of cLPs and the induction of systemic resistance by *Bacillus* spp. Members of the surfactin family, which are inducers of ISR in a broad range of plants, are the main cLPs secreted by *B. amyloliquefaciens* S499 in biofilms and on the roots of tomato, whereas iturins were detected at much lower concentrations and fengycins were not produced in measurable quantities. This is in marked contrast to production *in vitro*, where iturins and fengycins are produced in similar quantities and each was more than half the amount of surfactin present. A combination of elegant electrospray and imaging mass spectrometry-based approaches showed that cLPs, mainly surfactin, were formed by microcolonies directly on tomato roots and root hairs, and that surfactin diffused into the surrounding medium, reaching biologically relevant concentrations within the diffusion zone close to the colonized roots (Nihorimbere et al. 2012). Moreover, a strong correlation was demonstrated between the amount of surfactin produced and the ability to elicit ISR, suggesting that screening for strong producers among members of the *B. subtilis/amyloliquefaciens* complex could facilitate the selection of effective biocontrol agents (Cawoy et al. 2014).

Antibiotics produced by *Pseudomonas* spp Lipopeptide-producing strains of *Pseudomonas* spp., like those of *Bacillus*, are widely distributed in nature. Many have significant impacts on oomycetes such as *Phytophthora* and *Pythium* spp., with members of the viscosin group of compounds including massetolide A of particular note for their effects (ranging with increasing concentration from inducement of encystment to immobilization and lysis) on zoospores. Effects of these cLPs on mycelial morphology and physiology not only of oomycetes, but also of *Rhizoctonia*, typically are less pronounced than on zoospores and include increased hyphal branching and swelling (Raaijmakers et al. 2010; D'ae et al. 2011). cLPs produced by *Pseudomonas* spp. have been implicated in the control of pathogens such as *Pythium ultimum* on sugar beet, *Phytophthora infestans* on tomato, *Pythium* and *Rhizoctonia* spp. on bean, and *R. solani* and *Gaeumannomyces graminis* var. *tritici* on wheat (Yang et al. 2014).

Among the most commonly detected small metabolite antibiotics produced by *Pseudomonas* spp. are phenazine compounds, 2, 4-diacetylphloroglucinol (DAPG), pyrrolnitrin and pyoluteorin. The genetics, regulation of synthesis, and mode(s) of action of these compounds have been studied intensively, and the compounds themselves have, in most cases, been isolated from roots colonized by introduced or indigenous strains of the producing bacteria (Raaijmakers and Mazzola 2012). Of particular note are DAPG, produced by indigenous strains responsible for control of the wheat root disease take-all throughout much if not all of the USA and Europe (Chap. 38) and phenazine-1-carboxylic acid (PCA), present on the roots of wheat grown in dryland regions throughout the American Pacific Northwest (Mavrodi et al. 2012). The polyketide antibiotic DAPG is active against a wide range of phytopathogens, and also can elicit ISR in plants (Weller et al. 2012). The phenazines encompass a large family of pigmented, heterocyclic redox-active compounds that function not only in biological control, but also as microbial fitness determinants, virulence factors in plant and animal disease, and in microbial community dynamics and physiology. The phenazine-producing strain *P. aureofaciens* 1393 is marketed as an active ingredient in the biopesticide “Pseudobacterin-2” in the Russian Federation for the control of a wide range of phytopathogens as well as for the induction of resistance to plant diseases in organic and conventional crops (Thomashow 2013). PCA is the major ingredient in Shenqinmeisu, a microbial pesticide produced in China and applied commercially to protect rice and vegetables against important phytopathogens (Xu 2013).

18.3 Conclusions

In this chapter we have touched briefly upon recent insights towards understanding interactions among microorganisms and their plant hosts, as well as knowledge gaps and needs for future research. Technological advances that enable sensitive detection of metabolites including root exudate components produced *in situ* will continue to be critical to unraveling the complex molecular and organismal interrelationships in the rhizosphere habitat. Better knowledge of the microbe-plant dialogue is essential given the need for increased agricultural productivity to provide food and biofuel feedstocks in the face of climate change, the increasing world population and the loss of arable lands.

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