Chapter 46

Rhizobial Genetic Repertoire to Inhabit Legume and Nonlegume Rhizospheres

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46.1 INTRODUCTION

Rhizobia are soil bacteria, best known for forming nitrogen-fixing nodules on legume roots. For a long time, the importance of rhizobial rhizosphere colonization to achieve nodulation has been emphasized (Hossain and Alexander, 1984). As is the case with other bacteria, rhizobia are successful inhabitants of plant rhizospheres. *Rhizobium etli* strains may attain large numbers on plant roots, up to 10^9 cells per gram of maize root (fresh weight) (Gutierrez-Zamora and Martinez-Romero, 2001). *Rhizobium leguminosarum* strains promote rice, canola, and lettuce growth (Noel et al., 1996; Yanni et al., 1997).

Root rhizospheres are rich in nutrients and support the growth and proliferation of diverse microbes (Andrews and Harris, 2000). In contrast to what occurs in nodules, where rhizobia are exclusive or almost exclusive occupants, in the rhizosphere these bacteria share the habitat with many other microorganisms (Barea et al., 2005; see Chapter 4). It is unfortunate that most rhizosphere studies are performed using single strain inoculations and not microbial communities, as proposed (Sørensen et al., 2009).

Besides mucilage and sloughed root cortex tissues, root exudate is an important source of nutrients. Root exudate composition has been analyzed from different species (Rovira, 1969). Exudates contain amino acids, organic acids (Nardi et al., 1997), sugars (Gransee, 2002), and vitamins (Rajamani et al., 2008); while there are common plant exudate molecules, there are others that are plant-specific (reviewed in Walker et al., 2003; see also Chapter 22). Plant growth and root age affect exudate composition (Walker et al., 2003). Exudation is different and more active in some parts of the root (Bringhurst et al., 2001). At the root base the influx of amino acids is greater than in the other regions (Jones and Darrah, 1994). Accordingly, microbial community may change in relation to differences in exudate availability (Baudoin, 2002; Bais et al., 2006). Microbial colonization on roots is not homogeneous (Watt et al., 2006; see Chapter 22).

Among root exudates, flavonoids have attracted great attention for inducing rhizobial *nod* genes (see Chapter 51). Flavonoids induce other genes such as those encoding type III secretion systems (Viprey et al., 1998) or efflux pumps (Parniske et al., 1991; Gonzalez-Pasayo and Martinez-Romero, 2000) or auxin production (our unpublished results). Additionally, there were reports showing that flavonoids stimulated rhizobial growth (Hartwig et al., 1991). Flavonoids stimulate competitiveness in *R. leguminosarum* in the early stages of interaction with clover and vetch (Maj et al., 2010). Induction of rhizobial genes *nif* and *nod* in the presence of host plants or in nodules has been known for quite a number of years. Besides flavonoids, there are other substances in exudates that induce rhizobial *nod* genes,

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such as phenolic compounds (Le Strange et al., 1990), jasmonates (Rosas et al., 1998; Mabood et al., 2006), and xanthones (Yuen et al., 1995).

Rhizobia have large genomes and differential gene expression seems to account for their adaptation to different niches, as occurs in *Pseudomonas* spp. colonizing rhizospheres (Rainey, 1999; Ramos-Gonzalez et al., 2005). Some rhizobial functions expressed in plant rhizospheres are reviewed here and compared to reported results from other rhizospheric bacteria.

46.2 MOTILITY AND ROOT ADHESION

Legume exudates are rhizobial attractants. Flavonoids, sugars, amino acids, and dicarboxylic acids induce chemotaxis (Cooper, 2004). The response toward amino acids and flavonoids has been observed in different rhizobial species (Cooper, 2007). In *Pseudomonas fluorescens*, chemotaxis is required for successful root colonization, as mutants in the *cheA* gene with reduced flagellar-driven chemotaxis have diminished tomato root colonization (see also Chapter 68). Malic and citric acids are attractants in the tomato rhizosphere (de Weert et al., 2002). Using *in vivo* expression technology (IVET) to study genes expressed in the *Pisum sativa* rhizosphere, the *flgG* gene was found to be associated with chemotaxis and motility of *R. leguminosarum* sv. viciae (Barr et al. 2008).

There is compelling evidence that adhesion to roots is an early *sine qua non* step in the root colonization process for many bacteria. For *Rhizobium* and *Bradyrhizobium*, attachment is the first and the most important step in legume plant colonization (Albareda et al., 2006; Downie, 2010; see Chapter 66). In different bacteria, distinct molecules mediate their adhesion to plant roots (Danhorn and Fuqua, 2007).

The *pilAB* gene products are required for the synthesis of a type IV pili needed for the establishment of *Azoarcus* sp. strain BH72 on rice rootlets (Dörr et al., 1998) and is essential for root surface colonization (Böhm et al., 2007). In *Pseudomonas putida* KT244, a secretion system is involved in the release of large proteins implicated in rhizosphere colonization as well as in iron uptake (Molina et al., 2006).

In the rhizosphere or in the presence of exudates, *Rhizobium* produces novel surface polysaccharides and proteins secreted by type I, III, and IV secretion systems, some of which may participate in root adhesion (Cooper, 2007; Krehenbrink and Downie, 2008).

Different phases in *Rhizobium* root attachment are recognized. First, the initial adsorption is the result of the interaction between plant lectins, bacterial surface polysaccharides, and the Ca^{2+} bacterial binding protein rhicadhesin (Dazzo et al., 1984). The subsequent step involves cellulose fibrils secreted by bacteria which are responsible for irreversible binding to root surfaces (Laus et al., 2005; see Chapter 66).

46.3 CATABOLISM OF PLANT SUBSTANCES

Different plants release different metabolites that are largely unknown. Plant metabolite uptake and catabolism confer on rhizobia and other bacteria an adaptative advantage to differentially colonize the rhizosphere. Plants may be engineered to produce particular metabolites to select rhizospheric species (Mansouri et al., 2002; see also Chapters 110, 112, 114).

Induction of catabolism must occur in the presence of the substance or a related substance. Inducible catabolism genes seem to be unevenly distributed among rhizobial species or rhizobial strains. Novel catabolic genes are likely to be found in rhizobia to profit from soil and plant nutrients. The transporters for such substrates may be unknown as well. It is remarkable that hundreds of ABC transporters of unknown substrate have been found in rhizobial genomes (Gonzalez et al., 2006). Adhesion, transport, and catabolism genes seem to constitute a substantial part of the rhizobial genetic repertoire to inhabit legume and nonlegume rhizospheres.

Rhizobia may catabolize rhamnose (Oresnik et al., 1998) and arabinogalactan, which are common in mucilage (Knee et al., 2001), protocatechuate (MacLean et al., 2011), and a diversity of complex carbohydrate molecules and phenolic compounds (Parke et al., 1991). IVET was used to identify genes that are differentially expressed in *R. leguminosarum* bv. viciae A34 in the pea rhizosphere. Induced genes are involved in membrane transport, such as those encoding for a sulfonate ABC transport system, indicating that sulfate-containing compounds are available at the rhizosphere (Barr et al., 2008). Other genes encoding transporters were also expressed. RL0362 (*araJ*) encodes a permease of the major facilitator system (MFS) family transporters. The latter probably forms an operon with RL0363, encoding a glyoxalase, and may be involved in transporting and processing arabinose polymers (Barr et al., 2008).

Homoserine is found in pea exudates and may be used by *R. leguminosarum* (van Egeraat, 1975). Calystegines are secondary metabolites of plants, first found in *Calystegia sepium* (morning glory) that can be poisonous to arthropods and mammals. They are polyhydroxyl nortropame alkaloids that occur in Solanaceae. In a

particular strain of *Sinorhizobium meliloti*, plasmid genes for the catabolism of calystegine contribute to competitive colonization of morning glory (Tepfer et al., 1988) and genetically modified alfalfa plants (Guntli et al., 1999a, b). Other strains of *S. meliloti* do not metabolize calystegine (Tepfer et al., 1988). Mimosine produced by *Leucaena* plants may be catabolized by the *mid* genes in *Rhizobium* strains that nodulate them (Borthakur et al., 2003).

Genes that are involved in bean-exudate uptake (*teu*) are required in *R. etli* and *Rhizobium tropici* for competitive *Phaseolus vulgaris* nodulation. These genes encode an ABC transporter that specifically determines the uptake of a bean-exudate molecule, the chemical structure of which has not been determined yet (Rosenblueth et al., 1998). Proline catabolism genes including *putA* have a role in rhizospheric competitive colonization (van Dillewijn et al., 2002).

In nonsterile conditions, *S. meliloti* is able to use galactosides secreted by alfalfa, clover, and barrel medic seeds. Using a biosensor constructed using the *melA* promoter fused to the *gfp* protein and induced in the presence of galactosides and galactose, it was found that galactosides are present in defined zones at the lateral root initiation and around root hairs but not at the root tip (Bringhurst et al., 2001).

In contrast to the large diversity of carbon and nitrogen sources secreted in the rhizosphere, in nodules only few amino acids and dicarboxylic acids are the main nutrients provided by the plant to rhizobial bacteroids (Lodwig and Poole, 2003).

In *Pseudomonas stutzeri*, the usage of diverse carbon sources seems to be related to rhizospheric colonization capacity (Yan et al., 2008).

46.4 BIOFILM FORMATION AND QUORUM SENSING

S. meliloti cells form biofilms on alfalfa root surfaces (Bringhurst et al., 2001). Exopolysaccharides (EPS) are needed to colonize *Arabidopsis thaliana* and *Brassica napus* roots by an unclassified *Rhizobium* strain, but are not required for biofilm formation *in vitro* (Santaella et al., 2008). Similarly, *R. leguminosarum* biofilm formation on roots does not require the same gene functions as those needed *in vitro* (Williams et al., 2008; see also Chapters 66–69).

Bacteria regulate gene expression in relation to population density by a mechanism called quorum sensing (QS; see also Chapters 70–77). In this process, bacteria produce and secrete an autoinductor that activates a transcriptional regulator that, in turn, regulates the transcription of specific genes. QS controls processes involved in the interaction with eukaryotes, as motility, biofilm formation, as well as the production of toxins, EPS, and virulence factors (González and Keshavan, 2006).

In *P. fluorescens* 2P24, a QS-related system PcoI–PcoR has been identified. A mutant in the *pcoI* gene encoding an *N*-acylhomoserine lactone synthase has been found to have a reduced capacity to form biofilms on nonbiological surfaces and also in the wheat rhizosphere in sterilized and nonsterilized soil. The mutant had a reduced capacity to colonize root tips (Wei and Zhang, 2006). *N*-acylhomoserine lactone has been found to be produced by *P. putida* F117 and *Serratia liquefaciens* MG44 in the tomato rhizosphere (Steidle et al., 2001).

46.5 RHIZOBIAL PROTECTION FROM PLANT DEFENSE

Plants activate defense responses after bacterial colonization. Consequently, rhizobia protect themselves by changing their gene expression in the rhizosphere. Phytoalexin resistance is induced by soybean isoflavonoids in *Bradyrhizobium japonicum* (Parniske et al., 1991). Similarly, genes encoding multidrug efflux systems were identified using IVET in *R. leguminosarum* bv. *viciae* A34 in the rhizosphere (Barr et al., 2008). Promotorless *gusA* genes were introduced as transposons into *R. etli* in a random mutagenesis. The expression of *gusA* in bacteria grown in a minimal medium (MM) or in a MM with *P. vulgaris*-flavonoids was examined. Insertions expressed only in the presence of flavonoids were analyzed. In *R. etli*, genes involved in the production of an efflux pump system were detected that were also induced by phytoalexins (Gonzalez-Pasayo and Martinez-Romero, 2000).

There are surface polysaccharides that are important for the establishment of the rhizobial–plant interaction such as lipopolysaccharides (LPS), EPS, and cyclic β-glucans. They may act as physical barriers to plant defense compounds or as suppressors of plant defense responses (Soto et al., 2009; Downie, 2010). In other bacteria, effector molecules that are exported to plants by type III secretion modulate plant defense responses (Alfano and Collmer, 2004). In *Sinorhizobium* strain NGR234, type III secretion genes are induced by flavonoids (Viprey et al., 1998).

46.6 TRANSCRIPTIONAL REGULATORS EXPRESSED IN THE RHIZOSPHERE

Using IVET, two transcriptional regulators from the LysR and GntR families, one sigma factor and two genes

involved in environmental sensing that use cyclic di-GMP with GGDEF and EAL domains as second messenger were found to be expressed in *R. leguminosarum* in the pea rhizosphere (Barr et al., 2008). *P. putida* bacteria on maize roots express transcriptional regulators belonging to the AraC and TetR families (Matilla et al., 2007).

A proteomic study of the phylospheric *Methylobacterium extorquens* bacterium on *A. thaliana* leaves revealed that a regulatory factor PhyR is required for the expression of a number of stress proteins and is a key regulator in *M. extorquens* for its adaptation to its epiphytic lifestyle (Gourion et al., 2006). Community proteogenomics have revealed insights into the physiology of phyllospheric bacteria (Delmotte et al., 2009). Similar approaches may be used to study rhizobial communities in the rhizosphere.

The transcriptional regulator RpoN has a role in *S. meliloti* (Barnett et al., 2004) and in *R. etli* (Salazar et al., 2010) legume interactions. For another example of the identification of regulatory circuits, see Chapter 82.

46.7 GENOME-WIDE TRANSCRIPTOMIC ANALYSES

A study of gene expression using microarrays of *R. leguminosarum* strain 3841 growing in pea, alfalfa, and sugar beet (nonlegume) rhizospheres allowed the identification of many genes expressed in the rhizosphere. Plant-specific expression was found (Ramachandran et al., 2011). Interestingly, a *R. leguminosarum* plasmid contains many of the genes expressed in the pea rhizosphere.

Genes with unknown function were found to be highly expressed in *Rhizobium phaseoli* Ch24-10 (previously considered as *R. etli*, López-Guerrero et al., 2012b) recovered from maize (nonlegume) rhizosphere by an RNA-Seq procedure using the Illumina sequencing platform (López-Guerrero et al., 2012a). This is similar to what occurs in *R. leguminosarum* in the pea rhizosphere where 66% of the genes expressed are of unknown function (Ramachandran et al., 2011).

In *R. phaseoli* strain Ch24-10, some genes were commonly expressed in maize and bean rhizospheres, but others were plant-specific. Bacteria growing on roots were not physiologically homogeneous, as rhizobial transcripts reflected conflicting bacterial physiological conditions; some genes would correspond to those from bacteria in rich media while others in starvation. All transcripts obtained may not be present in a single cell. Although young roots from 5-day old plants were used, it seems that they are not homogeneous niches for bacterial growth. Reporter gene approaches would allow the spatial detection of gene expression on roots. For other examples of transcriptomics and metatranscriptomics see Chapters 107 and 109.

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