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*REVIEW*

# Bacterial Endophytes and Their Interactions with Hosts

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**Recent molecular studies on endophytic bacterial diversity have revealed a large richness of species. Endophytes promote plant growth and yield, suppress pathogens, may help to remove contaminants, solubilize phosphate, or contribute assimilable nitrogen to plants. Some endophytes are seedborne, but others have mechanisms to colonize the plants that are being studied. Bacterial mutants unable to produce secreted proteins are impaired in the colonization process. Plant genes expressed in the presence of endophytes provide clues as to the effects of endophytes in plants. Molecular analysis showed that plant defense responses limit bacterial populations inside plants. Some human pathogens, such as *Salmonella* spp., have been found as endophytes, and these bacteria are not removed by disinfection procedures that eliminate superficially occurring bacteria. Delivery of endophytes to the environment or agricultural fields should be carefully evaluated to avoid introducing pathogens.**

*Additional keywords*: nitrogen fixation, pathogenic bacteria, plant bacteria, plant colonization.

Plants and animals normally associate with diverse microorganisms. In the gut, bacteria have a remarkable role to stimulate immunity and development (Hooper et al. 2001). Similarly plant bacteria stimulate plant defense responses (de Matos Nogueira et al. 2001). Bacteria on roots and in the rhizosphere benefit from root exudates, but some bacteria and fungi are capable of entering the plant as endophytes that do not cause harm and could establish a mutualistic association (Azevedo et al. 2000; Hallmann et al. 1997; Perotti 1926). Plants constitute vast and diverse niches for endophytic organisms. Endophytic bacteria have been isolated from a large diversity of plants as reviewed by Sturz and associates (2000). Plants reported to harbor endophytes are shown in Table 1, but most likely, there is not a single plant species devoid of endophytes. The few examples of apparent absence of internal populations may be because some microorganisms are not easily isolated or cultured.

In general endophytic bacteria occur at lower population densities than rhizospheric bacteria or bacterial pathogens (Hallmann et al. 1997; Rosenblueth and Martínez-Romero 2004). It has not been resolved whether plants benefit more from an endophyte than from a rhizospheric bacterium or if it is more advantageous for bacteria to become endophytic compared with rhizospheric. It is still not always clear which population of microorganisms (endophytes or rhizospheric bacteria) promotes plant growth; nevertheless, benefits conferred by endophytes are well recognized and will be presented here.

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Endophytic populations, like rhizospheric populations, are conditioned by biotic and abiotic factors (Fuentes Ramírez et al. 1999; Hallmann et al. 1997, 1999; Seghers et al. 2004), but endophytic bacteria could be better protected from biotic and abiotic stresses than rhizospheric bacteria (Hallmann et al. 1997).

Endophytic bacteria in a single plant host are not restricted to a single species but comprise several genera and species. No one knows if communities inside plants interact, and it has been speculated that beneficial effects are the combined effect of their activities. In this review, we will first address the diversity of endophytes. Unfortunately, some of the older papers describing bacteria inside plants have used methods that do not allow an accurate classification of endophytic bacteria.

Criteria to recognize "true" endophytic bacteria have been published (Reinhold-Hurek and Hurek 1998a) and this requires not only the isolation from surface-disinfected tissues but also microscopic evidence to visualize "tagged" bacteria inside plant tissues. The latter criterion is not always fulfilled. Use of the term putative endophytes has been recommended for those not validated microscopically. True endophytes may also be recognized by their capacity to reinfect disinfected seedlings.

Endophytic bacteria have been studied mainly after culturing in laboratory media, but a more complete scheme is emerging, using methods that do not require the bacteria to be cultured and that make use of the analysis of sequences from bacterial genes obtained from DNA isolated from inside plant tissues (Chelius and Triplett 2000a; Engelhard et al. 2000; Miyamoto et al. 2004; Reiter et al. 2003; Sessitsch et al. 2002b). A following molecular approach studying wheat endophytes in Australia revealed a larger diversity of actinobacteria than that obtained by culturing endophytes (Conn and Franco 2004). Evidence that there are endophytic bacteria that have not yet been cultured also comes from the study of citrus endophytes by denaturing gradient gel electrophoresis profiles of 16S rRNA gene fragments amplified from total plant DNA. Some bands did not match any of the isolated bacteria grown in culture media (Araujo et al. 2002). In contrast, no differences were obtained by culturing or culture-independent methods and both revealed similar bacteria from the genera *Pseudomonas* and *Rahnella* in Norway spruce seeds (Cankar et al. 2005).

## Diversity and populations of microorganisms recovered as endophytes.

Mycorrhizal fungi are ancient plant partners (Simon et al. 1993) widespread among plants. Mycorrhiza benefits to plants are well known but agricultural applications in the field have not frequently led to substantial increases in crop yields. Some trees are incapable of growing without their mycorrhiza, and mycorrhizal fungi are fully dependant on the plant for growth. Recently, host specificity has begun to be recognized, using

molecular analysis based on the sequence of ribosomal genes (Chelius and Triplett 1999; Jacquot et al. 2000; Kjoller and Rosendahl 2000) and other genes, such as those for actin or elongation factors (Helgason et al. 2003). Interestingly, some mycorrhizal fungi themselves have endosymbiotic bacteria (*Glomeribacter gigasporarum*; Jargeat et al. 2004). Like mycorrhiza, other endophytic fungi completely depend on the plant and its inside conditions for growth. Some endophytic fungi have been shown to protect plants from herbivores (Schardl et al. 2004) or to be responsible for the synthesis of novel and useful secondary products (Strobel et al. 2004). Rain forest destruction may lead not only to the loss of valuable tree

**Table 1.** Examples of reported bacterial endophytes and plants harboring them

| Endophytes | Plant species | Reference |
|---|---|---|
| α Proteobacteria | | |
| *Azorhizobium caulinodans* | Rice | Engelhard et al. 2000 |
| *Azospirillum brasilense* | Banana | Weber et al. 1999 |
| *Azospirillum amazonense* | Banana, pineapple | Weber et al. 1999 |
| *Bradyrhizobium japonicum* | Rice | Chantreuil et al. 2000 |
| *Gluconacetobacter diazotrophicus* | Sugarcane, coffee | Cavalcante and Döbereiner 1988; Jiménez-Salgado et al. 1997 |
| *Methylobacterium mesophilicum*[a] | Citrus plants | Araujo et al. 2002 |
| *Methylobacterium extorquens* | Scots pine, citrus plants | Araujo et al. 2002; Pirttilä et al. 2004 |
| *Rhizobium leguminosarum* | Rice | Yanni et al. 1997 |
| *Rhizobium* (*Agrobacterium*) *radiobacter* | Carrot, rice | Surette et al. 2003 |
| *Sinorhizobium meliloti* | Sweet potato | Reiter et al. 2003 |
| *Sphingomonas paucimobilis*[a] | Rice | Engelhard et al. 2000 |
| β Proteobacteria | | |
| *Azoarcus* sp. | Kallar grass, rice | Engelhard et al. 2000; Reinhold-Hurek et al. 1993 |
| *Burkholderia pickettii*[a] | Maize | McInroy and Kloepper 1995 |
| *Burkholderia cepacia*[b] | Yellow lupine, citrus plants | Araujo et al. 2001; Barac et al. 2004 |
| *Burkholderia* sp. | Banana, pineapple, rice | Weber et al. 1999; Engelhard et al. 2000 |
| *Chromobacterium violaceum*[a] | Rice | Phillips et al. 2000 |
| *Herbaspirillum seropedicae* | Sugarcane, rice, maize, sorghum, banana | Olivares et al. 1996; Weber et al. 1999 |
| *Herbaspirillum rubrisulbalbicans* | Sugarcane | Olivares et al. 1996 |
| γ Proteobacteria | | |
| *Citrobacter* sp. | Banana | Martínez et al. 2003 |
| *Enterobacter* spp. | Maize | McInroy and Kloepper 1995 |
| *Enterobacter sakazakii*[a] | Soybean | Kuklinsky-Sobral et al. 2004 |
| *Enterobacter cloacae*[a] | Citrus plants, maize | Araujo et al. 2002; Hinton et al. 1995 |
| *Enterobacter agglomerans*[a] | Soybean | Kuklinsky-Sobral et al. 2004 |
| *Enterobacter asburiae* | Sweet potato | Asis and Adachi 2003 |
| *Erwinia* sp. | Soybean | Kuklinsky-Sobral et al. 2004 |
| *Escherichia coli*[b] | Lettuce | Ingham et al. 2005 |
| *Klebsiella* sp. | Wheat, sweet potato, rice | Engelhard et al. 2000; Iniguez et al. 2004; Reiter et al. 2003 |
| *Klebsiella pneumoniae*[b] | Soybean | Kuklinsky-Sobral et al. 2004 |
| *Klebsiella variicola*[b] | Banana, rice, maize, sugarcane | Rosenblueth et al. 2004. |
| *Klebsiella terrigena*[a] | Carrot | Surette et al. 2003 |
| *Klebsiella oxytoca*[b] | Soybean | Kuklinsky-Sobral et al. 2004 |
| *Pantoea* sp. | Rice, soybean | Kuklinsky-Sobral et al. 2004; Verma et al. 2004 |
| *Pantoea agglomerans* | Citrus plants, sweet potato | Araujo et al. 2001, 2002; Asis and Adachi 2003 |
| *Pseudomonas chlororaphis* | Marigold (*Tagetes* spp.), carrot | Sturz and Kimpinski 2004; Surette et al. 2003 |
| *Pseudomonas putida*[a] | Carrot | Surette et al. 2003 |
| *Pseudomonas fluorescens* | Carrot | Surette et al. 2003 |
| *Pseudomonas citronellolis* | Soybean | Kuklinsky-Sobral et al. 2004 |
| *Pseudomonas synxantha* | Scots pine | Prittilä et al. 2004 |
| *Salmonella enterica*[b] | Alfalfa, carrot, radish, tomato | Cooley et al. 2003; Guo et al. 2002; Islam et al. 2004 |
| *Serratia* sp. | Rice | Sandhiya et al. 2005 |
| *Serratia marcescens*[a] | Rice | Gyaneshwar et al. 2001 |
| *Stenotrophomonas*[a] | Dune grasses (*Ammophila arenaria* and *Elymus mollis*) | Dalton et al. 2004 |
| Firmicutes | | |
| *Bacillus spp.* | Citrus plants | Araujo et al. 2001, 2002 |
| *Bacillus megaterium* | Maize, carrot, citrus plants | Araujo et al. 2001; McInroy and Kloepper 1995; Surette et al. 2003 |
| *Clostridium* | Grass *Miscanthus sinensis* | Miyamoto et al. 2004 |
| *Paenibacillus odorifer* | Sweet potato | Reiter et al. 2003 |
| *Staphylococcus saprophyticus*[b] | Carrot, | Surette et al. 2003 |
| Bacteroidetes | | |
| *Sphingobacterium* sp.[a] | Rice | Phillips et al. 2000 |
| Actinobacteria | | |
| *Arthrobacter globiformis* | Maize | Chelius and Triplett 2000a |
| *Curtobacterium flaccumfaciens* | Citrus plants | Araujo et al. 2002 |
| *Kocuria varians* | Marigold | Sturz and Kimpinski 2004 |
| *Microbacterium esteraromaticum* | Marigold | Sturz and Kimpinski 2004 |
| *Microbacterium testaceum* | Maize | Zinniel et al. 2002 |
| *Mycobacterium* sp.[b] | Wheat, Scots pine | Conn and Franco 2004; Prittilä et al. 2005 |
| *Nocardia* sp.[b] | Citrus plants | Araujo et al. 2002 |
| *Streptomyces* | Wheat | Coombs and Franco 2003a |

[a] Opportunistic human pathogenic bacteria.
[b] Common human pathogenic bacteria.

species but also of unknown endophytes, especially fungi (Strobel et al. 2004). Fungal endophytic interactions will not be further reviewed here, as excellent reviews have been recently published on this topic (Hause and Fester 2005; Schardl et al. 2004; Schulz and Boyle 2005; Strobel and Daisy 2003; Strobel et al. 2004).

Methods for the isolation of bacterial endophytes have been reviewed extensively (Hallmann et al. 1997; Reinhold-Hurek and Hurek 1998a). The use of sodium hypochlorite for disinfecting plant surfaces is common. Residual sodium hypochlorite may affect growth or induce mutagenesis and death of microorganisms, thus making it necessary to rinse the tissues with sodium thiosulfate to remove all the residual sodium hypochlorite (Miché and Balandreau 2001), although this is not a common practice.

A review of published endophytic bacteria was reported by Hallmann and associates in 1997, but the list is no longer complete, as there is much interest in this area and new endophytes are continuously being reported (Table 1).

Remarkably, *Salmonella* strains have been detected as endophytes in alfalfa sprouts. Outbreaks with these bacteria in alfalfa sprouts have been recorded in North America, Asia and Europe since 1995 (Ponka et al. 1995). It has been proposed that alfalfa plants and seeds be colonized with safe bacteria to out-compete human pathogens. For example, *Enterobacter absuriae* was found to eliminate *Salmonella enterica* and the enterohemorrhagic *Escherichia coli* from *Arabidopsis thaliana* seeds (Cooley et al. 2003). It is worrisome that there may be human or opportunistic pathogens among plant endophytes.

It seems that the bacteria best adapted for living inside plants are naturally selected. Endophytes are recruited out of a large pool of soil or rhizospheric species and clones. Indeed, in a large study conducted on potato-associated bacterial communities, species richness and diversity was lower for fungal-antagonistic bacteria inside roots than in the rhizosphere of potato (Berg et al. 2005a). Germida and associates (1998) found that the endophytic population was less diverse than the root-surface population and the endophytes appeared to originate from the latter. Mavingui and associates (1992) found that there are different populations of *Bacillus polymyxa* in soil, rhizosphere, and rhizoplane and that wheat roots select specific populations. Rosenblueth and Martínez-Romero (2004) found, both by multilocus enzyme electrophoresis and by plasmid patterns, that *Rhizobium etli* strains that were isolated from inside maize stems were selected subsets of the total pool of *Rhizobium etli* found in rhizosphere, roots, or *Phaseolus vulgaris* nodules. *Rhizobium etli* is found as a natural endophyte of maize plants in traditional agricultural fields in which maize and bean are grown in association (Gutiérrez-Zamora and Martínez-Romero 2001). In planta and ex planta populations of *Pseudomonas* species could be differentiated by biochemical characteristics (van Peer et al. 1990).

Competition experiments with endophytes have shown that some endophytes are more aggressive colonizers and displace others. This was observed with *Pantoea* sp. out-competing *Ochrobactrum* sp. in rice (Verma et al. 2004) and with different *Rhizobium etli* strains in maize (Rosenblueth and Martínez-Romero 2004). However, when the host range of a large diversity of endophytes was analyzed, a seeming lack of strict specificity was observed (Zinniel et al. 2002).

The presence of different endophytic species in soybean depended on the plant genotype, the plant age, the tissue sampled, and also on the season of isolation (Kuklinsky-Sobral et al. 2004). The soil type determined to a large extent the endophytic population in wheat (Conn and Franco 2004). Correlations to growth promotion of tomato plants were observed with inocula levels that promoted endophytic populations but not rhizospheric populations (Pillay and Nowak 1997). On the other hand, the genus *Azospirillum,* one of the best-characterized plant growth-promoting bacteria, exerts its benefits mainly in the rhizosphere (Somers et al. 2004) and rarely colonizes the plant inner cortical tissues (Schloter and Hartmann 1998; Weber et al. 1999).

The addition of the herbicide glyphosphate produced a modification of the endophytic composition of soybean plants (Kuklinsky-Sobral et al. 2005). Similar results were obtained by inoculating a genetically modified *Enterobacter cloacae* strain in citrus seedling (Andreote et al. 2004). The analysis by genomic fingerprinting of the diversity of *Bacillus pumilus* and *Pantoea agglomerans* isolated from surface-disinfected leaves showed that populations inside citrus do not seem to be clones derived from a single genotype (Araujo et al. 2001).

The population density of endophytes is highly variable, depending mainly on the bacterial species and host genotypes but also in the host developmental stage, inoculum density, and environmental conditions (Pillay and Nowak 1997; Tan et al. 2003). Interestingly, this is also the case with epiphytic (on leaf surface) bacteria, which are highly variable in number, varying around 1,000-fold the population size of one individual bacterial species from leaf to leaf. Total bacterial population sizes on inoculated leaves varied by about 30-fold (Mercier and Lindow 2000).

Clostridia were detected in surface-disinfected grass leaves, stems, and roots. A group of clostridia was found exclusively in one of the grass species analyzed and not in the surrounding soil (Miyamoto et al. 2004). Some endophytes are very scarce or absent in soil (Reinhold-Hurek and Hurek 1998a).

Endophytic $N_2$-fixing bacteria seem to constitute only a small proportion of total endophytic bacteria (Barraquio et al. 1997; Ladha et al. 1983; Martínez et al. 2003), and increasing $N_2$-fixing populations in plants has been considered as a possibility to increase nitrogen fixation. Nitrogen-fixing bacteria were identified in sweet potato in N-poor soils with an analysis that consisted of amplifying nitrogenase (*nifH*) genes by polymerase chain reaction (Reiter et al. 2003). The resulting sequences, presumably derived from endophytes, resembled those from rhizobia, including *Sinorhizobium meliloti, Sinorhizobium* sp. strain NGR234, and *Rhizobium etli*. Other detected bacteria were *Klebsiella* spp. and *Paenibacillus odorifer* (Reiter et al. 2003). It is interesting that, in this case and perhaps in relation to the methodology used, a dominance of rhizobia was observed, accounting for around 50% of the sequences obtained. In culture-dependent studies, it seems that fast growing γ-Proteobacteria out-grow slower-growing α-Proteobacteria such as rhizobia.

Endophytic bacteria are found in legume nodules as well. In red clover nodules, some species of rhizobia were found, including *Rhizobium* (*Agrobacterium*) *rhizogenes,* in addition to *R. leguminosarum* bv. *trifolii*, which is the normal clover symbiont (Sturz et al. 1997). Some γ-Proteobacteria are cooccupants with the specific rhizobia in *Hedysarum* plant nodules (Benhizia et al. 2004). In most cases, the endophytic bacteria are unable to form nodules.

**Effects of endophytic bacteria and benefits to the plant.**

The growth stimulation by the microorganisms can be a consequence of nitrogen fixation (Hurek et al. 2002; Iniguez et al. 2004; Sevilla et al. 2001) or the production of phytohormones, biocontrol of phytopathogens in the root zone (through production of antifungal or antibacterial agents, siderophore production, nutrient competition and induction of systematic acquired host resistance, or immunity) or by enhancing availability of minerals (Sessitsch et al. 2002a; Sturz et al. 2000). The elucidation of the mechanisms promoting plant growth

will help to favor species and conditions that lead to greater plant benefits. Volatile substances such as 2-3 butanediol and aceotin produced by bacteria seem to be a newly discovered mechanism responsible for plant-growth promotion (Ryu et al. 2003). It would be interesting to determine if volatiles could be produced inside plants. Endophytes produce adenine ribosides that stimulate growth and mitigate browning of pine tissues (Pirttilä et al. 2004).

Endophytic bacteria of red clover seem to be responsible for the allelopathic effects observed with these plants over maize, causing reduced plant emergence and plant height (Sturz and Christie 1996). For this reason, it was not recommended to grow maize after clover in Canada. Clover endophytic bacteria reproduced the deleterious effects on maize. It would have been more convincing if the authors had tested the effects of clover with and without endophytes on maize germination and development.

A recent review on the mechanisms of biocontrol of plant growth-promoting rhizobacteria includes rhizospheric and endophytic bacteria (Compant et al. 2005). Bacterial endophytes are capable of suppressing nematode proliferation and this may benefit other crops in rotation with the host plants (Sturz and Kimpinski 2004). The frequent isolation of *Curtobacterium flaccumfaciens* as endophytes from asymptomatic citrus plants infected with the pathogen *Xylella fastidiosa* suggested that the endophytic bacteria may help citrus plants to better resist the pathogenic infection (Araujo et al. 2002). Endophytes from potato plants showed antagonistic activity against fungi (Berg et al. 2005a; Sessitsch et al. 2004) and also inhibited bacterial pathogens belonging to the genera *Erwinia* and *Xanthomonas* (Sessitsch et al. 2004). Some of the endophytic isolates produced antibiotics and siderophores in vitro (Sessitsch et al. 2004).

Inhibition of the oak wilt pathogen *Ceratocystis fagacearum* was obtained with 183 endophytic bacteria of 889 isolates tested (Brooks et al. 1994). Of 2,648 bacterial isolates analyzed from the rhizosphere, phyllosphere, endosphere, and endorhiza, only one, a root endophyte corresponding to *Serratia plymuthica*, was a highly effective fungal antagonist (Berg et al. 2005a). Endophytic actinobacteria are effective antagonists of the pathogenic fungus *Gaeumannomyces graminis* in wheat (Coombs et al. 2004), and several endophytes showed antagonism against *Rhizoctonia solani* (Parmeela and Johri 2004). It is worth considering that most of the assays to test antagonism are in vitro and it remains to be established if this correlates to effects in nature.

A near-future application may consider the use of geneti cally engineered endophytes with biological control potential in agricultural crops. The endophytes *Herbaspirillum serope dicae* and *Clavibacter xylii* have been genetically modified to produce and excrete the δ-endotoxin of *Bacillus thuringensis* to control insect pests (Downing et al. 2000; Turner et al. 1991).

Bacteria degrading recalcitrant compounds are more abundant among endophytic populations than in the rhizosphere of plants in contaminated sites (Siciliano et al. 2001), which could mean that endophytes have a role in metabolizing these substances. Engineered endophytic *Burkholderia cepacia* strains improved phytoremediation and promoted plant tolerance to toluene (Barac et al. 2004). There is an increasing interest on genetically modifying endophytes (Andreote et al. 2004). The advantages and obstacles to use bioengineered endophytes have been clearly discussed (Newman and Reynolds 2005; van der Lelie et al. 2004).

Endophytic bacteria possess the capacity to solubilize phosphates, and it was suggested by the authors that the endophytic bacteria from soybean may also participate in phosphate assimilation (Kuklinsky-Sobral et al. 2004).

Brazilian sugarcane plants have been grown for many years with small amounts of fertilizer without showing symptoms of N deficiencies. Out of the many $N_2$-fixing endophytes isolated from sugarcane, it has not been clearly defined which are responsible for fixing N inside the plant. However, there is controversy on the level of N fixed by endophytes and the proportion contributed to the plant (Giller and Merckx 2003). These estimates vary widely in different reports and range from 30 up to 80 kg N/ha/year (Boddey et al. 1995). Under optimal conditions, some plant genotypes seem to obtain part of their N requirements from nitrogen fixation.

Kallar grass grows in N-poor soils in Pakistan and a diver sity of *Azoarcus* spp. have been recovered from it (Reinhold-Hurek et al. 1993). Inside wheat, *Klebsiella* sp. strain Kp342 fixes $N_2$ (Iniguez et al. 2004), and it has been reported that it increases maize yield in the field (Riggs et al 2001). Similarly, nitrogen-fixing endophytes seem to relieve N deficiencies of sweet potato (*Ipomoea batatas*) in N-poor soils (Reiter et al. 2003).

Grasses growing in nutrient-poor sand dunes contain members of genera *Pseudomonas*, *Stenotrophomonas* as well as *Burkholderia*. It seems that the *Burkholderia* endophytes could contribute N to the grasses, because nitrogenase was detected with antibodies in roots within plant cell walls of stems and rhizomas (Dalton et al. 2004).

Some research has been directed to find endophytes that could significantly increase the yields in different crops after their inoculation. To reveal the effects of endophytes, inoculation experiments have been performed, but it has been a problem to eliminate resident or indigenous endophytes from plants in order to have bacteria-free plants or seeds. Functional redundancy of resident endophytes and added inocula may limit the effects observed from inoculation. Very complex microbial community-plant interaction, poor rhizosphere competence with endogenous microorganisms (Sturz et al 2000), and bacterial fluctuations with environmental conditions may also limit the applicability of endophyte inoculation in the field (Sturz and Nowak 2000). Furthermore, in the field, the large abundance and diversity of soil bacteria may be a rich source of endophytes and, for this, inoculation effects may not be observed. Since surface disinfection does not remove endophytes, procedures such as warming and drying seeds have been assayed to diminish bacterial populations inside (Holland and Polacco 1994). Tissue culture has also been used to eliminate or reduce endophytes (Holland and Polacco 1994; Leifert et al. 1994). Inoculants seem to be successful in micropropagated plants, as there are few or no other microorganisms with which to compete. There could be enormous benefits to be gained through the inoculation of microorganisms into soil-less mixes in which plants are transplanted at an early stage in their growth. In such cases, when the plantlets were inoculated, they were more vigorous and had increased drought resistance, an increased resistance to pathogens, less transplanting shock, and lower mortality (Barka et al. 2000; Martínez et al. 2003; Sahay and Varma 1999).

**Plant colonization.**

Methylotrophs are seedborne in soybean (Holland and Pollaco 1994). Many seeds carry a diversity of endophytes (Coombs and Franco 2003b; Hallmann et al. 1997). By being seedborne, endophytes assure their presence in new plants. Plants that propagate vegetatively (such as potatoes or sugarcane) can transmit their endophytes to the next generation and would not require the infection process described below. Some pathogens are also found inside seeds (Berg et al. 2005b; Schaad et al. 1995).

In the rhizosphere there is a selection of the microorganisms that are able to survive in the root exudates and compete with

others. Rosenblueth and Martínez-Romero (2004) found strains that were equally competitive for colonizing the rhizosphere and inside tissues of the root.

In order to colonize the plant, some bacteria must find their way through cracks formed at the emergence of lateral roots or at the zone of elongation and differentiation of the root. Dong and associates (2003) showed that cells of *Klebsiella* sp/ strain Kp342 aggregate at lateral-root junctions of wheat and alfalfa. Similarly, *Gluconacetobacter diazotrophicus* and *Herbaspirillum seropedicae* also colonize lateral-root junctions in high numbers (James and Olivares 1997). Possible infection and colonization sites have been illustrated by Reinhold-Hurek and Hurek (1998b). It has been proposed that cellulolytic and pectinolytic enzymes produced by endophytes are involved in the infection process (Hallmann et al. 1997), as in *Klebsiella* strains, pectate lyase has been implicated to participate during plant colonization (Kovtunovych et al. 1999). The cell wall–degrading enzymes endogluconase and polygalacturonase seem to be required for the infection of *Vitis vinifera* by *Burkholderia* sp. (Compant et al. 2005).

In some assays, early endophytic colonization differed from one cultivar to another, but later endophytes were recovered in approximately similar numbers from the different cultivars (Pillay and Nowak 1997). Fungal colonization could affect colonization by endophytic bacteria (Araujo et al. 2001) or the reverse could be true. Strain to strain variation in colonizing capabilities have been found among *Rhizobium etli* strains (Rosenblueth and Martínez-Romero 2004), and these may be related to differences in genomic content (Rosenblueth et al. *unpublished data*). In general, endophytic isolates were capable of colonizing or recolonizing the inside plant tissues in higher numbers than isolates from the root surface (van Peer et al. 1990; Rosenblueth and Martínez-Romero 2004).

After 1 day of exposure of certain plant roots to bacteria, they can be found in the aerial parts of the plant. Guo and associates (2002) inoculated the roots of hydroponically grown tomato plants with salmonellae at around 4.55 log CFU $ml^{-1}$ and, the next day, found that hypocotyls, cotyledons, and stems had around 3 log CFU $g^{-1}$. The systematic spread of an endophytic *Burkholderia* strain to aerial parts of *Vitis vinifera* seems to be through the transpiration stream (Compant et al. 2005).

Solomon and Matthews (2005) irrigated lettuce plants with *Escherichia coli* 0157:H7 or with fluorescent microspheres (used as bacterial surrogates). After 1, 3, and 5 days, there was no significant difference ($P \leq 0.05$) in populations between days, but there was significant difference in the internalization of bacteria or fluorescent microspheres. It is remarkable that microspheres attain 100-fold higher levels in plants than do *Escherichia coli* cells (Solomon and Matthews 2005). The authors suggested that the entry of *Escherichia coli* into plant roots is not determined by specific bacterial factors but, rather, by the plant.

Endophytes can also play an active role in colonization. *Azoarcus* sp. type IV pili are involved in the adherence to plant surfaces, an essential step towards endophytic colonization (Dörr et al. 1998). Two *Klebsiella* strains differ significantly in their invasion capacity in different plant hosts (*Medicago sativa*, *Medicago truncatula*, *Arabidopsis thaliana*, *Triticum aestivum*, and *Oryza sativa*). One of them (Kp342) was a better colonizer in all hosts and only needed a single cell to colonize the plants substantially a few days after inoculation (Dong et al. 2003). The plant hosts also differed in their ability to be colonized endophytically by the same bacterium, further suggesting an active host role in the colonization process.

Some rhizospheric bacteria can colonize the internal roots and stems, showing that these bacteria are a source for endophytes (Germaine et al. 2004), but also phyllosphere bacteria may be a source of endophytes (Hallmann et al. 1997).

Some flavonoids increased by almost 100% the number of lateral root cracks colonized in *Arabidopsis thaliana* by *Herbaspirillum seropedicae* and *Azorhizobium caulinodans* (Webster et al. 1998). Colonization of wheat by *Azorhizobium caulinodans* and *Azospirillum brasilense* was stimulated by flavonoids (Webster et al. 1998), as was the colonization by *Azorhizobium caulinodans* of two *Brassica napus* (oilseed rape) varieties (O'Callaghan et al. 2000). In *Rhizobium* spp., plant flavonoids were involved in inducing mechanisms to resist plant-defense phytoalexins (González-Pasayo and Martínez-Romero 2000; Parniske et al. 1991). Flavonoids are better known for their role in inducing the expression of *nod* genes that code for enzymes producing Nod factors. Neither Nod genes nor Nod factors are required for the endophytic colonization of *Arabidopsis thaliana* or wheat (Gough et al. 1997; Webster et al. 1998). Therefore, the role of flavonoids in stimulating colonization may be related to regulating other bacterial genes, such as those for phytoalexin resistance, type III secretion (Perret et al. 1999; Viprey et al. 1998), or genes for the synthesis of lipopolysaccharides (Reuhs et al. 2005), participating in the interaction with the plant. Interestingly, a glucosidase enzyme hydrolyzing glucoside isoflavones was purified from an endophytic *Pseudomonas* sp. (Yang et al. 2004). This activity could contribute to produce active (aglycone) flavons inside plants, but this has not been tested.

Changes in plant physiology can lead to the development of a distinct endophytic population (Hallmann et al. 1997). A diminished colonization of sugarcane by *Gluconacetobacter diazotrophicus* was observed in plants under a high nitrogen-fertilization regime as opposed to low N fertilization. It seems that supplying nitrogen to the plants alters its physiology and may cause a decrease in sucrose, which seems to be used for the endophytic growth (Fuentes-Ramírez et al. 1999). Fertilizer effects over endophytic populations were further confirmed. In rice, a rapid change of the nitrogen-fixing population was observed within 15 days after nitrogen fertilization (Tan et al. 2003). Organic amendments to plants also influence the endophytic populations (Hallmann et al. 1997).

Colonization does not depend on the nitrogen-fixing ability of the bacteria, as $Nif^-$ mutants of *Gluconacetobacter diazotrophicus* or *Herbaspirillum seropedicae*, were able to colonize as well as $Nif^+$ strains (Roncato-Maccari et al 2003; Sevilla et al. 2001). In contrast, *Azoarcus* mutants affected in pili were incapable of systemic spread into rice shoots (Dörr et al. 1998); this is also the case with mutants unable to produce a secreted endoglucanase (Reinhold-Hurek et al. 2006). Nonmotile mutants of *Salmonella enterica* were incapable of colonizing or had only a reduced invasion capacity in *Arabidopsis thaliana* (Cooley et al. 2003).

As plants have a determinant role in controlling endophytic colonization, it is important to avoid performing colonization assays in the laboratory with plants under suboptimal growth conditions, as they may show unbalanced interactions with endophytes with occasional overestimation of bacterial colonization by some strains. An increased diversity of bacterial endophytes was found in *Erwinia carotovora*–infected potatoes in comparison with noninfected control plants (Reiter et al. 2002). The colonizing capacity may also be overestimated in vitro, as there is no competition with indigenous soil bacteria (Cooley et al. 2003).

**Plant location.**

The methods used to assess the occurrence and location of endophytic bacteria have been diverse and include immunological detection of bacteria, fluorescence tags, and confocal

laser scanning microscopy (Chelius and Triplett 2000b; Hartmann et al. 2000; Verma et al. 2004). In addition, specific oligonucleotide probes could be of use to analyze bacteria inside plants (Hartmann et al. 2000).

Endophytic bacteria are found in roots, stems, leaves, seeds, fruits, tubers, ovules, and also inside legume nodules (Benhizia et al. 2004; Hallmann et al. 1997; Sturz et al. 1997). In most plants, roots have the higher numbers of endophytes compared with above-ground tissues (Rosenblueth and Martínez-Romero 2004).

It seems that the bacterial endophytes described here do not inhabit living vegetal cells (James and Olivares 1997; Reinhold-Hurek and Hurek 1998a). Intercellular spaces and xylem vessels are the most commonly reported locations for endophytic bacteria (Reinhold-Hurek and Hurek 1998a; Sprent and James 1995). A *Burkholderia* sp. strain was found in xylem vessels and substomatal chambers in *Vitis vinifera* plants (Compant et al. 2005). By inhabiting similar niches as vascular pathogens, endophytes may be used as competing bacteria for disease control (Hallmann et al. 1997).

Surface-washing of sprouts was not an effective way to eliminate *Salmonella enterica* and *Escherichia coli* strains from alfalfa sprouts or seeds, indicating that these bacteria are located in a protected niche (Cooley et al. 2003). By introducing the green fluorescent protein, their location was defined; *Salmonella enterica* colonized seed coats and roots, while *Escherichia coli* colonized only roots (Charkowski et al. 2002).

**Molecular interactions.**

In contrast to the extensive information on the molecular mechanisms of other bacteria-plant interactions (Lugtenberg et al. 2002; Oldroyd and Downie 2004), there is only limited data on the endophyte-host molecular interactions.

*The plant response*. The plant response to endophytes seems to be conditioned, to a large extent, by the plant genotype. Wild races and some plant varieties seem to provide adequate in-plant conditions that stimulate and support nitrogen fixation by endophytes and benefit from it. This has been observed with rice, sugarcane, and maize (Boddey et al. 1995; Engelhard et al. 2000; Gutiérrez-Zamora and Martínez-Romero 2001; Iniguez et al. 2004; Reis et al. 1994; Shrestha and Ladha 1996; Urquiaga et al. 1989).

Plant genes may be modulated by the presence of the bacteria, and the genes so expressed provide clues as to the effects of endophytes in plants. In sugarcane, genes expressed in response to the endophytic colonization of *Gluconacetobacter diazotrophicus* and *Herbaspirillum rubrisubalbicans* are being studied (de Matos Nogueira et al. 2001). The sequence analysis of the cDNA and other libraries derived from messenger RNAs expressed in sugarcane when inoculated with *Gluconacetobacter diazotrophicus* and *Herbaspirillum rubrisubalbicans* revealed that genes for nitrogen assimilation, for carbon metabolism, for plant growth, as well as genes for a limited plant defense were induced (de Matos Nogueira et al. 2001). Similar strategies could be followed to study the effects of other endophytes in plants.

*Arabidopsis thaliana* has been used as a model plant to study its interactions with endophytes like *Azorhizobium caulinodans* and enterobacteria. The advantage of using *Arabidopsis thaliana* is that there are defined mutants that may be tested for their colonization by endophytes. Iniguez and associates (2005) used *Medicago truncatula* and *Arabidopsis thaliana* mutants to determine the role of plant defense-response pathways in regulating the number of endophytic bacteria. They found that ethylene, a signal molecule for induced systemic resistance in plants, decreases endophytic colonization by *Klebsiella* sp. strain Kp342 and *Salmonella enterica* serovar Typhimurium (*Salmonella typhimurium*) strains. An ethylene-insensitive *Medicago truncatula* mutant was hypercolonized by Kp342, while the addition of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid to wild-type *Medicago truncatula* and wheat reduced the amount of endophytic bacteria. By treating the wild-type plants with the ethylene inhibitor 1-methylcyclopropane, a reversion of the reduction of colonization was obtained. Iniguez and associates (2005) also found that the presence of bacterial extracellular components, such as flagella and type III secretion systems (TTSS-SPI1) of *Salmonella* pathogenicity island 1 decrease endophytic colonization, and *Salmonella typhimurium* mutants lacking these components have higher endophytic colonization in *Medicago sativa* and wheat seedlings. *Arabidopsis thaliana* mutants showed that only a salicylic acid (SA)-independent defense response contributes to restriction of the colonization by *Klebsiella* sp. strain Kp342. In the case of colonization by *Salmonella typhimurium*, this is restricted by both SA-dependent and –independent pathways. The study of *Salmonella typhimurium* flagella mutants suggests that flagella are recognized through the SA-independent response and TTSS-SPI1 is recognized by the SA-dependent response that is involved in inducing the promoter of *PR1*, a SA-dependent pathogenesis related gene. Kp342 lacks flagella and TTSS-SPI1 (Dong et al. 2001); thus, when it associates with the plant, it does not induce the SA-dependent responses and may colonize the plant in higher numbers (Iniguez et al. 2005). Kp342 might have lost its flagella during its evolution in association with plants (Iniguez et al. 2005). Other bacteria that interact closely with plants, such as *Rhizobium* and *Agrobacterium* spp., have flagella, but these are not elicitors of plant defense mechanisms (Felix et al. 1999). In plants as well as in mammals and insects, bacterial flagellins are recognized by surface receptors that contain transmembrane proteins with extracellular leucine-rich repeat domains (Gómez-Gómez and Boller 2002). Flagellin acts as an elicitor in whole *Arabidopsis thaliana* plants, inducing an oxidative burst, callose deposition, and ethylene production and leading to the induction of defense-related genes. Plants can detect the presence of molecules from bacteria through chemoperception systems (Boller 1995).

A local host-defense reaction was induced by an endophytic *Burkholderia* strain in *Vitis vinifera* plants (Compant et al. 2005). It remains to be determined if endophytes are affected by innate immunity in plants, as occurs in some animal-bacterial interactions (McPhee et al. 2005). Antimicrobial peptides have been isolated from maize and rice (Duvick et al. 1992) and could have a role in controlling endophytic populations. Limiting carbon sources in the carbon apoplastic fluid may also restrict endophytic growth (Fuentes-Ramírez et al. 1999).

*Bacterial genes expressed in the presence of plants*. Endophytes may differentially express genes that are required to enter and colonize the plant to grow and survive within plant tissues and to stimulate plant growth, compete and suppress pathogens, or produce different substances.

Endophytic bacteria provide useful and rich models to study the genetic expression of bacteria in their natural niches or habitats (inside plants), which are more structured and variable than culture media under controlled laboratory conditions. Nevertheless, very little work has been done on this. Genomic projects are being performed on some endophytic bacteria, such as *Azoarcus* spp. (Battistoni et al. 2005), *Herbaspirillum* sp., *Gluconacetobacter diazotrophicus*, and *Klebsiella* spp., which will certainly be of great help to further understand their molecular interaction with plants. In vivo expression technol ogy used to study gene expression in different niches, (Rediers et al. 2005) including the rhizosphere (Ramos-González et al. 2005), may be used as well to study gene expression during endophytic life.

Roncato-Maccari and associates (2003) examined microscopically a *Herbaspirillum seropedicae* $Nif^+$ strain with a *gus*A cassette in its *nif*H gene after inoculation of maize, sorghum, wheat, and rice. They found expression of *nif* genes in bacterial colonies located in external mucilaginous root material eight days after inoculation, as well as in roots, stems, and leaves. Using a similar approach, Vermeiren and associates (1998) had previously found that *nif*H *gus*A fusions of *Pseudomonas stutzeri* (formerly *Alcaligenes faecalis*) and *Azospirillum irakense* were also expressed in rice roots. It was also observed that the NifH (nitrogenase reductase) of *Klebsiella pneumoniae* occurred in maize roots but not in stems, using an antibody to the purified enzyme (Chelius and Triplett 2000b). In situ hybridization studies demonstrated that *Azoarcus* nitrogenase genes are expressed inside the roots of field-grown Kallar grass (Hurek et al 1997). These results suggest that grass tissues provide a suitable environment for nitrogen-fixation gene expression, but it seems that $N_2$ fixation in the plant is carbon-limited (Christiansen-Weniger et al 1992). Different grasses inoculated with members of genera *Herbaspirillum*, *Azospirillum*, *Klebsiella*, and *Serratia* produced ethylene in acetylene reduction assays only when a carbon source was added (Chelius and Triplett 2000b; Egener et al. 1999; Gyaneshwar et al 2002).

Bacteria can communicate through "quorum sensing," a term applied to the production of diffusible signal molecules that control gene expression in a manner dependent upon bacterial population density (quorum) (Swift et al. 1996). Until now, phytopathogenic bacteria have been reported to respond to quorum to produce antibiotics, virulence factors, and plant cell wall–degrading exo-enzymes (Von Bodman et al. 2003). Interestingly, plants can perceive "quorum sensing" signals from the bacteria and control quorum-regulated bacterial responses (Bauer and Mathesius 2004; Mathesius et al. 2003). It would be interesting to determine if endophytes produce "quorum sensing" molecules inside plants and the effects they cause. Inside the plant, there could be exchange of signal molecules among microorganisms and of bacteria with the host, although this has not yet been reported.

It would also be interesting to address whether some of the well-studied molecular mechanisms used by phytopathogenic bacteria (Van't Slot and Knogge 2002) are to some extent shared with endophytes.

**May endophytes be or become pathogens?**

Most fungal grass endophytes are considered mutualistic with their hosts. The main advantage for the plants is the protection they confer against herbivory by toxic alkaloids (Schardl et al. 2004). These fungal endophytes, like bacteria, may obtain nutrients from the plant and protection from abiotic stresses like desiccation. But some fungal endophytes (Stone et al. 2000) may become plant pathogens, depending on the developmental stage of host and fungus, environmental factors, and host defense responses (Schulz and Boyle 2005). It was frequently found that some bacterial endophytic isolates from healthy plants inhibited the growth of tomato seedlings in reinoculation assays, possibly through the production of certain metabolites (van Peer et al. 1990). Some endophytes seem to be latent pathogens, and infections may proceed under certain conditions. These may be due to changes in environmental conditions such as $CO_2$ accumulation or $O_2$ depletion (Lund and Wyatt 1972), but others could be related to the presence of other microorganisms interacting with the endophyte. There are reports that mixed inoculations of two endophytic bacteria that individually inhibit growth result in plant-growth promotion (Sturz et al. 1997). The order in which endophytic populations are inoculated and become established in the host plant could affect subsequent plant-growth promotion effects (Sturz and Christie 1995). *Herbaspirillum rubrisubalbicans* may cause a mild mottled stripe disease in a few sugarcane varieties but give no symptoms in most hosts (James et al. 1997; Olivares et al. 1997). It seems there is an equilibrium of endophytes and plants that under certain circumstances may be unbalanced to the detriment of one of the partners.

In other cases, endophytes have been found to be closely related to human pathogens or are either human or opportunistic human pathogens. This is the case of endophytic *Salmonella* strains, which have caused outbreaks and constitute a health risk for consumers of raw fruits and vegetables (Guo et al. 2002), and of the *Burkholderia cepacia* strains isolated from plants (Barac et al. 2004). As *Burkholderia cepacia* causes pulmonary infection (even fatal) in human cystic fibrosis patients, a reassessment of the risk and a moratorium on the agricultural use of *Burkholderia* strains have been suggested (Holmes et al. 1998; Parke and Gurian-Sherman 2001). Approaches to reduce raw vegetable contamination produced by pathogens propose strategies to increase the number of safe growth-promoting bacteria in plants (Iniguez et al. 2005).

*Nocardia* endophytes have been isolated from members of genus *Citrus*. Some *Nocardia* species are known to be human pathogens causing nocardiasis (a severe human infection in feet and legs that may lead to amputation) that is transmitted by soil. A fluctuating and prominent population of *Mycobacterium* spp. was found in Scots pine that seemed to be required for bud development. When the tissue was fully developed, the endophyte was no longer detected (Pirttilä et al. 2005). *Mycobacterium* isolates related to those reported as clinical isolates were the most frequently encountered in wheat in Australia (Conn and Franco 2004). It was not analyzed whether these mycobacteria carry some virulence genes.

Parke and Gurian-Sherman (2001) stated "It is not coincidental perhaps that many of the most effective biocontrol agents (*Stenotrophomonas maltophilia*, *Pantoea agglomerans*, … and *Burkholderia cepacia*) of plant diseases are also opportunistic human pathogens. These … are fiercely competitive for nutrients and may produce antimicrobial metabolites and may themselves be resistant to multiple antibiotics." In addition, surviving plant defense reactions may render endophytic bacteria resistant to human defense responses as well. Some endophytes have been found to contain genes that are required for virulence in pathogens (Barak et al. 2005; Dörr et al. 1998). It has been difficult to differentiate environmental isolates and clinical bacteria of *Pseudomonas* spp. (Foght et al. 1996). This is the case with plant endophytes from rice, sugarcane, banana, and maize (Martínez et al. 2003) and clinical isolates from hospitals in Mexico (Rosenblueth et al. 2004) and in Europe (Brisse and Verhoef 2001). All these were classified as *Klebsiella variicola* (Rosenblueth et al. 2004). Although *Klebsiella variicola* seems to be less virulent than *Klebsiella pneumoniae* and has different epidemiological dynamics (Martínez et al. 2004), its use in agriculture was discouraged (Lloret et al. 2004). Even if some plant bacterial lineages may be distinguished from pathogenic lineages, their close genetic relatedness may render the former good recipients to acquire virulence genes by lateral transfer from their pathogenic relatives. Still very little is known of the genetic behavior of bacteria and the frequency of transfer of genes in natural habitats.

**Perspectives.**

The natural condition of plants seems to be in a close interaction with endophytes. Endophytes seem promising to increase crop yields, remove contaminants, inhibit pathogens, and produce fixed nitrogen or novel substances. The repertoire of their

effects and functions in plant has not been comprehensively defined. The challenge and goal is to be able to manage microbial communities to favor plant colonization by beneficial bacteria. This would be amenable when a better knowledge on endophyte ecology and their molecular interactions is attained. The contributions of this research field may have economic and environmental impacts.

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