Seed Bacterial Endophytes: Common Genera, Seed-to-Seed Variability and Their Possible Role in Plants

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Abstract

Seeds contain endophytic bacteria that may be transmitted from generation to generation. Some of these bacteria can benefit plant growth and defense against abiotic and biotic stresses. Little is known however about the mechanisms of bacterial colonization of seeds and their transmission from generation to generation in host plants. In this study we have demonstrated that individual seeds of maize (*Zea mays* L.) taken from the same cob and bean seeds (*Phaseolus vulgaris* L.) from different pods and within individual pods differ in their bacterial content and population diversity. We suggest that this bacterial variability within seed population of individual plants may contribute to the species adaptation to diverse environments and be harnessed in the production of crop plants.

INTRODUCTION

Bacteria and fungi can be vertically transmitted in plants by seeds (Mundt and Hinkle, 1976; Baker and Smith, 1966). Thus one would expect natural selection to favor host plants that tightly control the kind and number of microbes that migrate into the developing seeds. Although diverse bacterial species have been found as endophytes colonizing different plant tissues, some genera are more frequently recovered as plant or seed endophytes than others (Cankar et al., 2005; Rosenblueth and Martinez-Romero, 2006; Compant et al., 2008; Hardoim et al., 2008; Mano and Morisaki, 2008; Ulrich et al., 2008; Mastretta et al., 2009; Puente et al., 2009).

With a novel strategy of stable-isotope-probing of ^{13}C-DNA containing endophytes that were recovered from plants fed with $^{13}CO_2$ Rasche et al. (2009) detected *Acinetobacter*. A new species of *Cohnella* was also obtained from *Phaseolus coccineus* as a nodule endophyte (Garcia-Fraile et al., 2008), and a new species of *Paenibacillus* was isolated from surface-sterilized seeds of peas *Pisum sativum* (Smerda et al., 2005). Similarly, we identified several novel lineages among bacteria in bean seeds corresponding to a number of genera (*Bacillus*, *Enterococcus*, *Nocardioides*, *Knoellia*, *Acinetobacter*, *Rhizobium*, *Phyllobacterium*, *Paracoccus* and *Sphingomonas*) within phyla Firmicutes, Actinobacteria, and alpha, gamma and beta Proteobacteria (López-López et al., 2010). A novel *Rhizobium* species, *R. endophyticum*, was also identified and found not to be symbiotic (López-López et al., 2010). The non-symbiotic rhizobia were also found in the seed interior and symbiotic *R. etli* on the seed coats (Perez-Ramírez et al., 1998). Symbiotic rhizobia were also found as natural maize endophytes (Gutiérrez-Zamora and Martínez-Romero, 2001).

In this study we report endophytic bacteria of individual seeds extracted from maize (*Zea mays*) and bean (*Phaseolus vulgaris* L.) plants. Both bean and maize are suitable for studies on seed endophytes as their seeds are large and can easily be extracted from surface disinfected pods or cobs.

MATERIALS AND METHODS

Seeds from *Phaseolus vulgaris* cultivars DOR 364 and BAT 477 were surface disinfected, placed in 5 lt pots containing sterilized vermiculite, irrigated with free N

Fahraeus solution (Fahraeus, 1957) or water and maintained in the greenhouse for 75 days. Bean pods that were 4 to 7 cm long (average 6 cm) were surface disinfected and immature seeds (6-8 mm) were detached, individually macerated, and seed extract were diluted and plated as described in López-López et al. (2010).

Physiological mature corn-cobs of maize varieties Criollo (creole) San Gregorio and Criollo de Amatlán were supplied by INIFAP (Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias) Campo Experimental Uruapan and by Verónica Pérez Escalante respectively. Cultivar San Gregorio was grown in the central highlands of Michoacán, Mexico (2400 masl and 800-1000mm precipitation) in April 2008 during the rainy season in an Andisol soil fertilized with 40 kg P_2O_5 ha^{-1} as calcium super phosphate and 60 $kgNha^{-1}$ as urea at seeding stage, and 60 kgN ha^{-1} at silking stage. Cultivar Criollo de Amatlán was grown in Amatlán, Morelos (1750 masl and 1200-1400mm precipitation) in June 2008 in an Andisol soil fertilized with 60 kg P_2O_5 ha^{-1} as calcium super phosphate and 60 $kgNha^{-1}$ as urea at seeding stage, and 80 kgN ha^{-1} at silking stage. Maize kernels were detached from corn-cobs and surface disinfected as described (Pereira et al. 2011), germinated in plates with agar 0.7% during two days at 28°C in darkness (five kernels per plate) and subsequently placed in slants in tubes (one kernel per tube) with agar 0.7% with Fahraeus medium (Fahraeus, 1957). They were kept under gnotobiotic conditions (sterile and hermetically closed). Tubes with plantlets were incubated for with 12 h photoperiod and at 28 ± 2°C until sampling. The lower part of the tubes was covered with aluminum foil to protect roots from light.

Maize seedlings were individually macerated with $MgSO_4$ 10mM at two days after germination, as well as roots from seven and fifteen days-old seedlings. Extract dilutions were plated as for beans. Colonies were grown and bacteria identified by 16S rRNA gene sequences as described in López-López et al. (2010); some isolates were only distinguished by their different morphology (morphotypes, Table 2). Additionally, seed-borne maize endophytes were identified using a culture-independent approach. Root macerates from five-day-old plantlets of Criollo de Amatlán were fractionated on a Percoll gradient as described in Pereira et al. (2011). DNA was extracted from the bacterial fractions followed by PCR of 16S rRNA gene. The primers for these gene were design specifically to amplify DNA from bacteria and not from maize chloroplasts or mitochondria (F522 5'-CGTGCCAGCAGCCGCGGTAATA and R1231 5'-CATTGTAGCACGTGTGTAGCCC). Cloning and sequencing of PCR products was performed as described (López-López et al., 2010). A potential inhibition of the growth of bacterial colonies by *Bacillus* spp. on culture plates was seemingly avoided by plating high dilutions (up to 10^{-4}) of seed extracts to obtain few and well- separated colonies.

RESULTS AND DISCUSSION

Seed-to-Seed Variability

By a culture-dependent approach we detected seed-to-seed variability of bacterial endophytes obtained from surface-disinfected bean pods and maze cobs (Table 1). There was not a single bean or maize seed without bacteria, albeit at low numbers. One to 55 CFU were detected in each bean seed. Total endophytes from maize kernels were around 10^{1-2} CFU/g at day two, to 10^{5-8} CFU/g at day seven.

Each seed contained either multiple or single species. Even seeds from a single pod or cob harbored different bacteria (Tables 1 and 2). In maize we found almost as high a variability of bacterial endophytes between kernels from the same cob as between kernels from different cobs (Table 2). This was also true for seeds in a single bean pod (Table 1). It is not known how seed endophytic diversity is determined; is the bacterial transfer process stochastic or selective? Do abundant bacteria compete with others and find their way to seeds more frequently?

Bacteria from the genera *Bacillus* and *Paenibacillus* were abundant in maize kernels two days after germination. Seven days after germination, the variability both in each kernel and between kernels was higher. At this stage we also found *Methylo-*

bacterium, *Alcaligenes*, *Tsukamurella*, *Erwinia*, *Microbacterium*, *and Rhodococcus.* These bacteria came from the seed, but they were not detected in seed earlier because they were either in a non-culturable state or they were present in very low numbers. With a culture-independent approach, we detected *Burkholderia* inside maize seeds and confirmed variability between individual seeds (Fig. 2).

Bacterial variability within seed population of individual plants may contribute to the species adaptation to diverse environments. We speculate that the variable seed content of bacteria or fungi may be advantageous for plants to defend against pathogens or herbivores. Bacteria corresponding to *Paenibacillus* were the most frequently found in the bean seeds analyzed, however *Bacillus* species were the most diverse (larger number of species encountered) in bean and maize seeds. *Bacillus* strains are well known for producing antimicrobials (Ongena et al., 2007), each producing distinct active molecules (Ongena and Jacques, 2008). If these compounds are produced inside plants, they may inhibit different microbes including pathogens, giving plants a selective advantage.

Being Seed-Borne

Endophytic colonizers of plant seeds may derive from seed the host plant originated and/or soil and above ground environments. Some bacterial genera are found recurrently in seeds, suggesting that they are well adapted to seed microenvironment, including desiccation. Seeds and ovules of different plants contained *Bacillus* (Mundt and Hinkle, 1976), which can produce spores that survive desiccation inside seeds and remain viable for long periods of time. *Paenibacillus* was reported in *Eucalyptus* seeds (Ferreira et al., 2008), and *Paenibacillus* and bacteria from other genera inside seeds, while *Rhizobium* was identified only on the surface of rice seeds (Mano et al., 2006).

Roots from plantlets (germinated in vitro from surface-disinfected seeds) contained higher numbers of bacteria than seeds, meaning that after germination, seed bacteria multiply inside plants and colonize their tissues and organs, such as roots (López-López et al., 2010). To grow inside plants, seed endophytes may profit from seed nutrients. Starch, arginine (that may be metabolized to urea) and phytate are abundant seed storage compounds (Coelho et al., 2002). Some endophytes are known to catabolize starch (Schmidt and Michael, 1979). *Methylobacterium*, commonly found as an endophyte, expresses urease in plants (Holland and Polacco, 1990, 1992). Phytate is the most important P reserve compound in bean seeds. We have recently found some maize and bean-seed endophytes (*Bacillus*, *Paenibacillus*, *Acinetobacter* and *Rhizobium endophyticum*) that degrade phytate (López-López et al., 2010). *Bacillus* and *Paenibacillus* secrete phytases (Kerovuo et al., 1998; Idriss et al., 2002; Jorquera et al., 2010), but this capacity was unknown in rhizobia or in *Acinetobacter*. To determine if phytate utilization is necessary for bacterial growth during seed germination, bacterial mutants need to be obtained and tested in inoculation assays in plants.

Common Bacterial Genera as Endophytes

Bacteria belonging to the genera *Bacillus*, *Pseudomonas*, *Enterobacter*, *Klebsiella*, *Paenibacillus*, *Burkholderia*, *Kocuria*, *Lysobacter*, *Janibacter*, *Staphylococcus*, *Micrococcus*, *Methylobacterium*, *Arthrobacter*, *Sphingomonas*, *Streptomyces*, *Paracoccus*, *Cohnella*, *Leptothrix*, *Nocardioides*, *Rhizobium*, and *Rhodococcus* that were isolated from bean (López-López et al., 2010), maize or *M. truncatula* seeds (Martínez Romero, unpublished results) have been reported as endophytes in other plants (Ito and Iizuka, 1971; Holland and Polacco, 1992; Yanni et al., 1997; Chelius and Triplett, 2000; Engelhard et al., 2000; Phillips et al., 2000, Araujo et al., 2001, 2002; Estrada-De los Santos et al., 2001; Gutiérrez-Zamora and Martínez-Romero, 2001; Surette et al., 2003; Kuklinsky- Sobral et al., 2004, 2005; Sessitsch et al., 2004; Sturz and Kimpinski, 2004; Sturz et al., 1997; Smerda et al., 2005; Rasche et al., 2006; Tian et al., 2007, Garcia-Fraile et al., 2008; Mano and Morisaki, 2008; Peng et al., 2008; Thomas et al., 2008; Ulrich et al., 2008; van Overbeek and van Elsas, 2008). Although common genera are

identified, different species or strains may preferentially inhabit a host plant, suggesting that some species or strains may be host-adapted.

To survive inside plants, bacteria must resist the toxic compounds produced by plants, such as MBOA (6-methoxy-2-benzoxazolinone) in maize, an antimicrobial and insecticidal compound found in large quantities during the first days after germination (Park et al., 2004). Some maize-kernel endophytes isolated at days two and seven after germination were able to degrade MBOA (Martínez Romero, unpublished results).

Possible Roles in Plants

Many seed-borne isolated bacteria produce plant hormones such as auxins, cytokinins, and gibberellins; others produce ACC deaminase that decreases ethylene levels (Madhaiyan et al., 2006, 2007). In inoculated plants, evidence of hormonal effects such as a stimulation of root proliferation and stem elongation were observed (reviewed in Nowak and Shulaev, 2003). Plant-growth promotion by isolated bacteria inoculated into plants has been frequently reported and this has attracted research toward their use in phytoremediation or as bioinoculants in agriculture (Estevez de Jensen et al., 2002; Nowak and Shulaev, 2003; Barac et al., 2004).

Seed endophytes may contribute to plant defense (Sessitsch et al., 2004; Sturz and Kimpinski, 2004). Plants that can select and transmit beneficial bacteria to their progeny would be more successful than plants that transmit pathogens (Friesen et al., 2011). Seed pathogens constitute a problem for biocontrol strategies. Seeds have been recognized as reservoirs for pathogens. Beneficial endophytes may compete with plant and human pathogens that are known to inhabit seeds (Brinkerhoff and Hunter, 1963; Elango and Lozano, 1980; Kuan et al., 1985; Schaad et al., 1995; Grum et al., 1998; Mellano and Cooksey, 1988; Berg et al., 2005).

Pending Questions on Seed Endophytes

In comparison to rhizosphere and phyllospere bacteria, seed endophytes have received less attention. Many important questions about seed transmitted bacteria need to be answered. For example: How do bacteria make their way on to seeds? Colonization pathways have been recognized, namely through vascular vessels, pistils or ruptures of seed outer tissues. In pathogens, a Type 3 secretion system and adhesion are required to colonize seeds (Darsonval et al., 2008, 2009). How do bacteria access to seed nutrient reserves (i.e., phytate, found in plant vacuoles)? How do bacteria make their way out of seeds? Does bacterial diversity matter or are different species functionally redundant? Which bacterial products are found in plants? How do bacteria interact among themselves and with plants? Some information may be found in Sturtz et al. (1997). How can we make adequate use of seed endophytes to compete with pathogens or to promote plant growth?

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Tables

Table 1. Endophytic bacteria found in bean seeds within different pods.

Individual seeds	BAT 477							DOR 364					
	Pod 1			Pod 2			Pod 3	Pod 4	Pod 5			Pod 6	
	1	2	3	4	5	6	7	8	9	10	11	12	13
Firmicutes													
Bacillus massilensis		X											
Bacillus sp.													X
Bacillus pumilus													X
Bacillus flexus											X		
Bacillus korlensis				X									
Bacillus silvestris		X											
Paenibacillus			X	X				X	X		X	X	
Enterococcus										X			
Staphylococcus			X	X							X	X	
Actinobacteria													
Arthrobacter					X								
Kocuria						X	X			X			
Micrococcus	X								X	X			
Brachybacterium											X		
Alpha Proteobacteria													
Methylobacterium										X			
Paracoccus									X				
Gamma Proteobacteria													
Acinetobacter					X								

Table 2. Bacteria found in individual maize kernels from different cobs seven days after germination (%).

Cob number	Kernel number	1	2	3	4	5	6	7	8	9	10	11	12
2	2-1						6	6			63		25
	2-2								100				
4	4-1									89		11	
	4-2			100									
5	5-1	100											
	5-2								44	13		43	
	5-4		100										
	5-11	100											
	5-12	100											
8	8-2								50	50			
	8-3								100				
	8-4				71	29							
	8-11	100											

1. *Bacillus*, 2. *Methylobacterium*, 3. *Tukamurella*, 4. *Alcaligenes*, 5. *Erwinia*, 6. *Microbacterium*, 7. *Rhodococcus*, 8-12. different morphotypes.

Figures

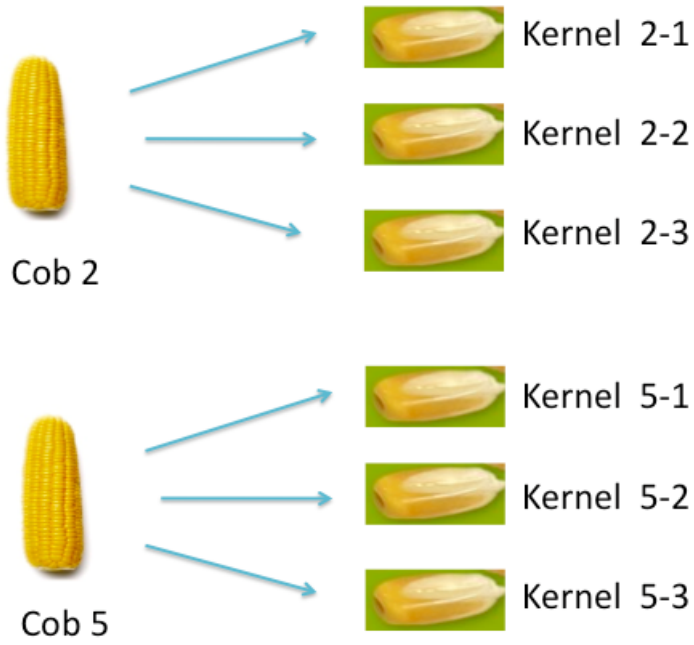

Fig. 1. Example of maize cobs for analysing endophytes. Different kernels from each cob were selected.

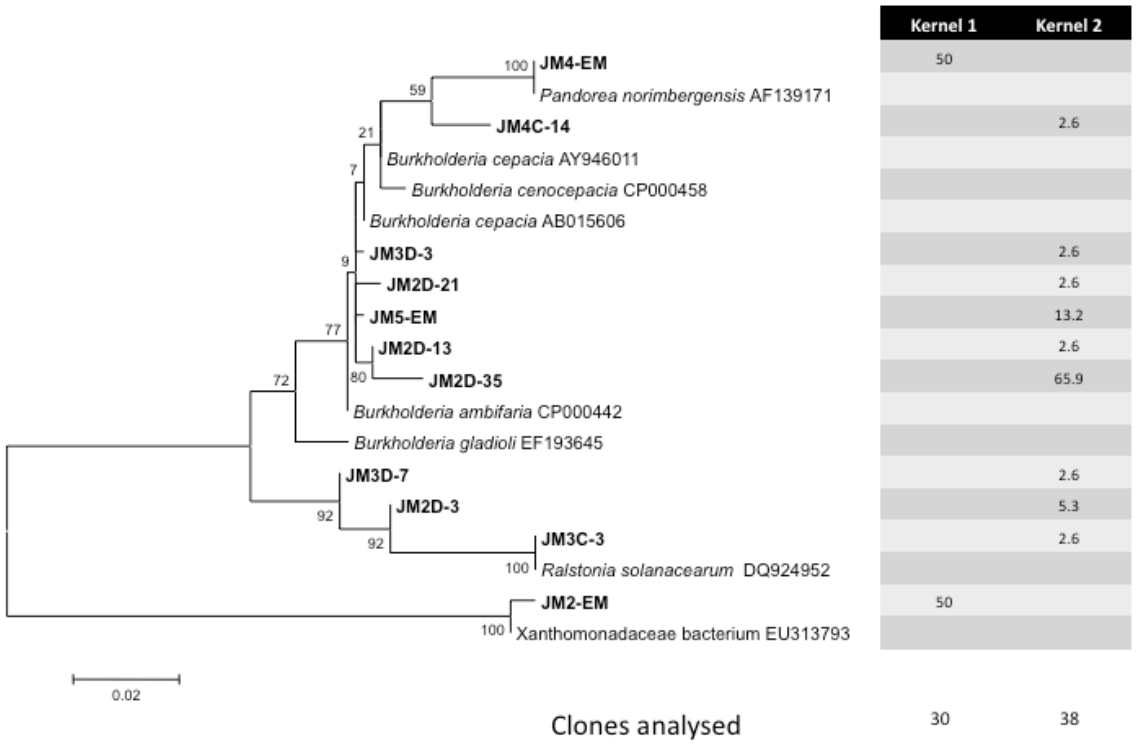

Fig. 2. Percent of clones corresponding to different phylotypes according to the phylogenetic tree of 16S rRNA gene sequences of bacteria obtained from DNA from a Percoll gradient fraction from two different five-day-old maize plants (Criollo de Amatlán). Sequences from this work are in bold. Other sequences from closely related organisms were included. Accession numbers are shown after scientific names. The tree was inferred by Maximum Likelihood under model GTR. Bootstrap values are shown.