Genetics and Molecular Biology, 34, 4, 676-680 (2011)

www.sbg.org.br

Short Communication

Isolation of endophytic bacteria from arboreal species of the Amazon and identification by sequencing of the 16S rRNA encoding gene

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Abstract

Endophytic bacteria from three arboreal species native to the Amazon (*Carapa guianenses*, *Ceiba pentandra*, and *Swietenia macrophylla*), were isolated and identified, through partial sequencing of the 16S rRNA encoding gene. From these, 16 isolates were obtained, although, when compared to sequences deposited in GenBank, only seven had produced identifiable fragments. *Bacillus*, *Pantoea* and two non-culturable samples were identified. Results obtained through sequence analysis revealed low genetic diversity across the isolates, even when analyzing different species and plant structures. This is the first report concerning the isolation and identification of endophytic bacteria in these plant species.

Key words: isolation, sequencing, *Bacillus*, *Pantoea*.

Received: February 13, 2011; Accepted: July 17, 2011.

Endophytic microorganisms inhabit the inner organs and tissues of plants, such as leaves, stems, seeds and roots, during at least one period of their life-cycles, without causing diseases or producing visible external manifestation (Azevedo *et al.*, 2000). Endophytic communities are formed mainly by fungi and bacteria. It is estimated that every plant-species constitutes a possible host for endophytic microorganisms, which, in the vast majority and despite their biotechnological potential (Ezra *et al.*, 2004), remain unidentified. Although the interaction between these microorganisms and their respective host-plants is not, as yet, fully understood, over recent years they have been progressively more extensively employed, either in agriculture (Ryan *et al.*, 2008), or in the production of compounds with therapeutic application, such as taxol (Stierle *et al.*, 1993) and leucinostatin A (Strobel and Hess, 1999).

The origin, entrance pathway, colonization and transmission of endophytic bacteria have been the object of considerable research efforts (Azevedo *et al.*, 2002). These bacteria may proliferate in seeds, the rhizosphere, the phylloplane as well as the material that results from vegetative propagation (Stierle *et al.*, 1993; Kuske *et al.*, 1997). Penetration into the host plant may occur via stomata, wounds, or areas of lateral root development, or may even be facilitated by the production of hydrolytic enzymes capable of degrading the cell wall (Souza *et al.*, 2004). Once inside, the endophytic microorganism may lodge in specific tissues, or even systemically colonize the plant, thereby establishing symbiotic, mutualistic, commensal and tropobiotic relationships (Ulrich *et al.*, 2008).

Worldwide, the highest plant diversity is found in the Amazon biome (Strobel and Hess, 1999). Concurrently, it is not surprising that biomes characterized as extremely biodiverse are also believed to harbor significant richness and variety of microorganism populations (Figueiredo *et al.*, 2009). Notwithstanding, there are few reports on endophytic microorganisms isolated from Amazonian plant species. Most studies on native plants have been addressed, either to economically relevant species, as *Euterpe oleracea* (Strobel and Daisy, 2003), *Paullinia cupana* var. *sorbilis* (Hallmann *et al.*, 1997), *Theobroma gradiflorum* (Ribeiro *et al.*, 1999) and *Bactris gasipes* (Downing *et al.*, 2000), or to anthropotoxic forms, such as *Paulicourea longiflora* and *Strychnos cogens* (Souza *et al.*, 2004).

From a conservation view-point, the devastation observed in recent decades in the Amazon Rainforest has very likely caused the extinction of not only plant species, but also the endophytic microorganisms they host (Strobel and Daisy, 2003). Thus, more in-depth knowledge of this microbiota, as well as the interactions it maintains with host-plants and the environment, is an essential variable in the development of conservation strategies directed to sustaining environmental balance, thereby preserving biodiversity as a whole, in efforts that may pave the way for its

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biotechnological application (Azevedo *et al.*, 2002). More specifically, such knowledge gains increased relevance in the context of Amazonian forest plant species, already undergoing intensive exploitation for timber or the production of essential oils.

In this scenario, the present work used the 16S rRNA gene region for identifying endophytic bacteria in three tree-species, native to the Amazon rainforest: *Carapa guianenses* Aublet (andiroba), *Ceiba pentandra* (L.) Gaertn (kapok tree, locally known as sumauma) and *Swietenia macrophylla* King (big-leaf mahogany). The plant species chosen have already been intensively exploited by the timber industry and manufacturers of aromatherapy products, thereby causing a significant decrease in populations in those areas where they are native.

Plant material (leaves, apices, stems and seeds) was collected from five seedlings each of *Carapa guianenses* Aublet (andiroba), *Ceiba pentandra* (L.) Gaertn (sumaúma), and *Swietenia macrophylla* King (big-leaf mahogany), originally from the forest nursery of UniNilton Lins, Manaus, AM, Brazil. The material was first disinfected by treatment with 70% (v/v) ethanol for 5 min, followed by sodium hypochlorite (NaClO) 10% (v/v) for 10 min and then rinsed three times with distilled autoclaved water. To confirm disinfection success, 300-µL aliquots were taken from the final autoclaved water wash-offs, and transferred onto a 2xYT culture medium (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl) in Petri dishes, and incubated for 7 days at 37 ± 2 °C.

After asepsis, 8 x 8 mm leaf fragments, seeds, apices and 50 mm stem sections were inoculated in Petri dishes containing 2xYT medium supplemented with 0.3 g/L Benlate® (DuPont), to inhibit growth of fungal colonies. The material was then incubated for 7 days at 37 ± 2 °C. Plant material presenting bacteria colonies was transferred to liquid culture medium (2xYT), and cultivated for 14 h in the dark at 37 ± 2 °C, with 220 rpm orbital shaking. After three days, 1.5 mL aliquots were separated from the cultures and centrifuged at 12,000 x *g*, for 15 min at 4 °C. The supernatant was disposed of and the pellet stored in glycerol 70% (v/v) at -80 °C, awaiting DNA extraction.

DNA extraction was in accordance with the protocol described in the Wizzard Genomic DNA Purification kit (Promega Co.). Extracted DNA was electrophoresed in 1% (w|v) agarosegels, stained with ethidium bromide, and quantitatively analyzed in a micro-volume spectrometer (NanoDrop 1000, V3.6.0, Thermo Scientific, Waltham, MA, USA). PCR was amplifications were carried out using specific primers for the 16S rRNA encoding gene in a 25-µL final volume containing 200 ng/µL of bacterial DNA, 12.5 µL of Green Master Mix (Promega Co.), 8.5 µL of sterile milli-Q water, and 1 µL of each primer. The primers were the same as those used by Kuske *et al.* (1997): primer 8F (forward, 5'-AGA GTT TGA TCC TGG CTC AG-3'), and primer 1100R (reverse, 5'-GGG TTG CGC TCG TTG-3'). DNA was amplified in a thermal cycler (Techne TC-412, Barloworld Scientific Ltd, UK), according to a 35-cycle program: 30 s at 92 °C; 45 s at 44 °C; 1 min at 72 °C; and a final 5 min extension cycle at 72 °C. PCR products were cloned in a *pGEM-T easy* vector system (Promega,), in accordance with manufacturer's instructions. Plasmid DNA of selected clones was isolated, according to the miniprep procedure.

Sequencing reactions were carried out in microplates using the kit *DNA Sequencing-Big Dye Terminator Cycle Sequencing Ready ABI Prism* version 3. Sequencing reactions were conducted in a 10-µL final volume of a solution prepared with 1 µL *Big Dye*, 1 µL primer, 3 µL plasmid DNA, 1.5 µL buffer, and 3.5 µL sterile milli-Q water. The primer used was M13/pUC 1211 (forward; 5'-GTA AAA CGA CGG CCA GT-3'). The quality of the sequences was assessed based on electropherograms generated with *Sequencing Analysis* 3.5 software, and analyzed with *Phred/Phrap/Consed* software (Guimarães VC, 1998, MSc Dissertação Universidade Federal de São Carlos/ Universidade Federal do Amazonas). Appropriate sequences were selected using Blast2go for automated annotation. Initially, sequences were analyzed for nucleotide similarity in comparison with sequences deposited in GenBank, and then accessed via the National Center for Biotechnology Information (NCBI) website using the BlastN tool (Altschul *et al.*, 1997).

Molecular phylogenetic analysis by Maximum Parsimony (MP) was done using the Close-Neighbor-Interchange Algorithm (Tamura and Nei, 1993) with search level 0. Initial trees were obtained through the random addition of sequences (10 replicates). Analysis involved all the nucleotide sequences. Included codon positions were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated.

For estimating the Maximum Likelihood (ML) values, a user-specified toplogy was used. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise, the BIONJ method with MCL distance matrix was used. Evolutionary analysis was with MEGA5 (Tamura *et al.*, 2011).

As a rule, plants may be simultaneously colonized by a large variety of endophytic bacteria. This bacterial diversity is affected by a number of factors, such as specificity and age of the host-plant, season of the year, ecological niche, and type of tissue (Azevedo *et al.*, 2002). The present work analyzed the diversity of endophytic bacteria isolated from various plant tissues, as caulinar apices, foliar discs, stems and mature seeds, from three arboreal species, native to the Amazon Rainforest, namely, *Carapa guianenses* (andiroba),

Ceiba pentandra (sumauma) and *Swietenia macrophylla* (mahogany). The isolation and growth of bacteria in the culture medium used (2xYT), and the subsequent analysis of partial sequencing in nucleotides of the 16S rRNA encoding gene, lead to the identification of 16 bacterial strains. As to the plant structures used, 18,75% of the isolates were obtained from the apices and stems of *C. guianiensis*, 25% from the seeds and apices of *C. pentandra*, and 56.25% from the stems and leaves of *S. macrophylla*.

The isolation of endophytic bacteria in a culture-medium is considered one of the simplest methods, when assessing bacterial communities (Andreote *et al.*, 2009). However, in terms of diversity, and when considering the total population, this can lead to underestimation, since identification is restricted to those actually capable of growing in the specific culture-medium chosen. Furthermore, differences in growth-rate during *in vitro* incubation, *i.e.*, fast or slow, or, recalcitrance to the cell-lysis process, as observed in Actinobacteria, may also affect the number of isolates obtained (Glare and O'Callaghan, 2000). Most studies based on isolation in a culture medium are faced with such limitations, although the results obtained do indeed shed more light on the way such bacterial populations are structured (Andreote *et al.*, 2009).

In the present work, results were obtained for only seven samples, in which fragments with approximately 1,200 bp were observed (Figure S1). The identification of endophytic bacteria based on the comparison between the sequences obtained and those deposited in GenBank using the BlastN revealed the prevalence of *Bacillus*. Of the total number of samples analyzed, 57.14% belonged to the genus *Bacillus* and 14.28% to *Pantoea*, whereas 28.57% were non-culturable bacteria. A phylogenetic analysis of isolates is shown in Supplementary Figure S2. Gram positive and Gram negative endophytic bacteria have already been isolated from many tissue types in numerous plant-species (Glare and O'Callaghan, 2000). Recently, the endophytic microbiota of several plant-species have been studied, the most prevalent genera isolated including *Pseudomonas*, *Erwinia*, *Bacillus*, *Burkholderia*, *Xanthomonas* and *Enterobacter* (Kuske *et al.*, 1997). Bacteria belonging to the genus *Pantoea* have likewise been observed in citrus-plant species, as well as clover and sugarcane (Araújo *et al.*, 2002; Polanczyk and Alves, 2003).

On identifying isolates at the species level, incongruities were observed. When sequences obtained in the present analysis were compared to those deposited in GenBank using the BlastN, it was observed that one same given sequence was actually similar to those sequences of more than one species (Table 1). The genetically related species were *Bacillus thuringiensis*, *B. cereus*, *B. subtilis*, *B. amyloliquefaciens*, *B. polyfermenticus*, *B. anthracis*, *B. velezensis* and *Pantoea dispersa*. According to Polanczyk and Alves (2003), though the term *Bacillus thuringiensis* is used for one single species (based on taxonomic traits), the bacterium belongs to a complex formed by several species

Table 1 - Identification of endophytic bacteria based on the 16S rRNA region sequence compared to sequences deposited in GenBank, using Blastn.

Isolate	Identity[1]	Plant species	Plant tissue	Max Score	Max ident	Accession number (GenBank)
1b	*Pantoea dispersa*	*Carapa guianensis*	Apex	684	99%	FJ756350.1
5c	Uncultured bacterium	*Carapa guianensis*	Leaf	652	99%	AY838532.1
6b	*Bacillus anthracis*	*Ceiba pentandra*	Seed	1.452e+04	96%	CP001598.1
6c	*Bacillus cereus*	*Ceiba pentandra*	Seed	1.784e+04	95%	CP001186.1
6d	*Bacillus cereus*	*Ceiba pentandra*	Seed	1635	97%	FJ841975.1
6e	*Bacillus thuringiensis serovar konkukian*	*Ceiba pentandra*	Seed	2.007e+04	96%	AE017355.1
6f	*Bacillus cereus*	*Ceiba pentandra*	Seed	1.856e+04	96%	CP001186.1
6g	*Bacillus* sp.	*Ceiba pentandra*	Seed	1443	96%	AB126763.1
6h	*Bacillus cereus*	*Ceiba pentandra*	Seed	1.477e+04	100%	CP001186.1
6i	*Bacillus cereus*	*Ceiba pentandra*	Seed	2.114e+04	98%	CP001407.1
9a	*Bacillus anthracis*	*Swietenia macrophylla*	Stem	1.315e+04	94%	CP001598.1
9b	*Bacillus* sp.	*Swietenia macrophylla*	Stem	1472	98%	EF428972.1
9d	*Bacillus thuringiensis*	*Swietenia macrophylla*	Stem	1249	98%	AM778997.1
9e	*Bacillus cereus*	*Swietenia macrophylla*	Stem	1.907e+04	98%	CP001186.1
9f	*Bacillus cereus*	*Swietenia macrophylla*	Stem	2.036e+04	98%	CP001407.1
9g	*Bacillus cereus*	*Swietenia macrophylla*	Stem	1530	99%	AB508868.1
9h	*Bacillus anthracis*	*Swietenia macrophylla*	Stem	1.639e+04	99%	CP001598.1
9i	*Bacillus cereus*	*Swietenia macrophylla*	Stem	1454	97%	AY224383.1

Table 1 (cont.)

Isolate	Identity[1]	Plant species	Plant tissue	Max Score	Max ident	Accession number (GenBank)
9j	*Bacillus thuringiensis*	*Swietenia macrophylla*	Stem	1341	99%	FJ932761.1
13a	Uncultured *Bacillus* sp.	*Swietenia macrophylla*	Leaf	1158	98%	EU371583.1
13b	*Bacillus subtilis*	*Swietenia macrophylla*	Leaf	970	100%	GQ161967.1
13c	*Bacillus* sp.	*Swietenia macrophylla*	Leaf	515	99%	FJ465166.2
13c	*Bacillus subtilis*	*Swietenia macrophylla*	Leaf	1476	98%	EU257444.1
13d	*Bacillus amyloliquefaciens*	*Swietenia macrophylla*	Leaf	1.352e+04	99%	CP000560.1
13e	*Bacillus velezensis*	*Swietenia macrophylla*	Leaf	1437	98%	EU852930.1
13f	*Bacillus velezensis*	*Swietenia macrophylla*	Leaf	1483	99%	EU852930.1
13g	*Bacillus amyloliquefaciens*	*Swietenia macrophylla*	Leaf	1294	97%	FJ685773.1
13h	*Bacillus amyloliquefaciens*	*Swietenia macrophylla*	Leaf	1391	99%	FJ960508.1
13j	*Bacillus subtilis*	*Swietenia macrophylla*	Leaf	1393	97%	EU862566.1
14a	*Bacillus amyloliquefaciens*	*Swietenia macrophylla*	Leaf	1.342e+04	98%	CP000560.1
14b	*Bacillus polyfermenticus*	*Swietenia macrophylla*	Leaf	1489	98%	AY149473.2
14c	*Bacillus* sp.	*Swietenia macrophylla*	Leaf	453	97%	FJ654441.1
14d	*Bacillus subtilis*	*Swietenia macrophylla*	Leaf	1184	98%	EF428247.2
14e	*Bacillus* sp.	*Swietenia macrophylla*	Leaf	749	99%	FJ463041.1
14f	*Bacillus polyfermenticus*	*Swietenia macrophylla*	Leaf	1520	99%	AY149473.2
14h	*Bacillus polyfermenticus*	*Swietenia macrophylla*	Leaf	1404	98%	AY149473.2
14g	*Bacillus* sp.	*Swietenia macrophylla*	Leaf	1227	98%	FJ465166.2
14i	*Bacillus polyfermenticus*	*Swietenia macrophylla*	Leaf	1415	98%	AY149473.2
16a	Uncultured *bacterium*	*Ceiba pentandra*	Apex	619	99%	GQ096960.1

[1]Seven isolates were used for analysis, four for Bacillus, one for Pantoea and two for uncultured bacteria.

(*B. anthracis*, *B. cereus*, *B. mycoides*, *B. thuringiensis*, and *B. weihenstephanensis*). This complex is called *B. cereus*. Molecular methods, the analysis of fatty acids and phospholipids, comparison of the 16S rRNA sequence, among other analytical approaches, have shown that *B. thuringiensis* and *B. cereus* are, in fact, one and the same species. Therefore, the need for a better distinction between the two has become the central topic of several taxonomy studies (Gordon *et al.*, 1998).

The results obtained on analyzing partial sequences of the 16S rRNA region encoding gene, demonstrate the low genetic divergence between endophytic bacteria isolated from the three arboreal plant-species native to the Amazon. This may be linked to the growth conditions adopted, as well as to the low specificity of the primers used. Notwithstanding, the plant species used in the present study showed good promise as a source of sampling material in studies about the isolation and identification of bacterial genera that may have potential biotechnological applications.

Acknowledgments

The authors are indebted to the Post-Graduation Program *Stricto Sensu*, Academic MSc course in Urban Biology, UniNilton Lins.

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Supplementary Material

The following online material is available for this article:

Figure S1 - Agarose gel showing the PCR products

Figure S2 - Molecular phylogenetic analysis

This material is available as part of the online article at http:www.scielo.br/gmb.

Associate Editor: Luís Carlos de Souza Ferreira

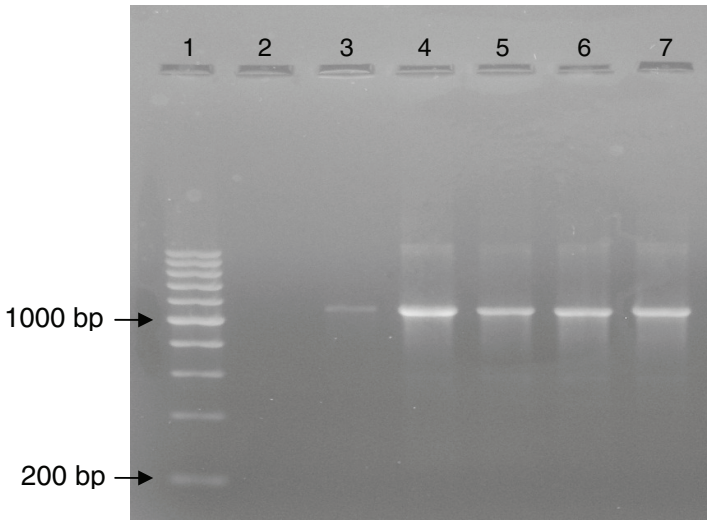

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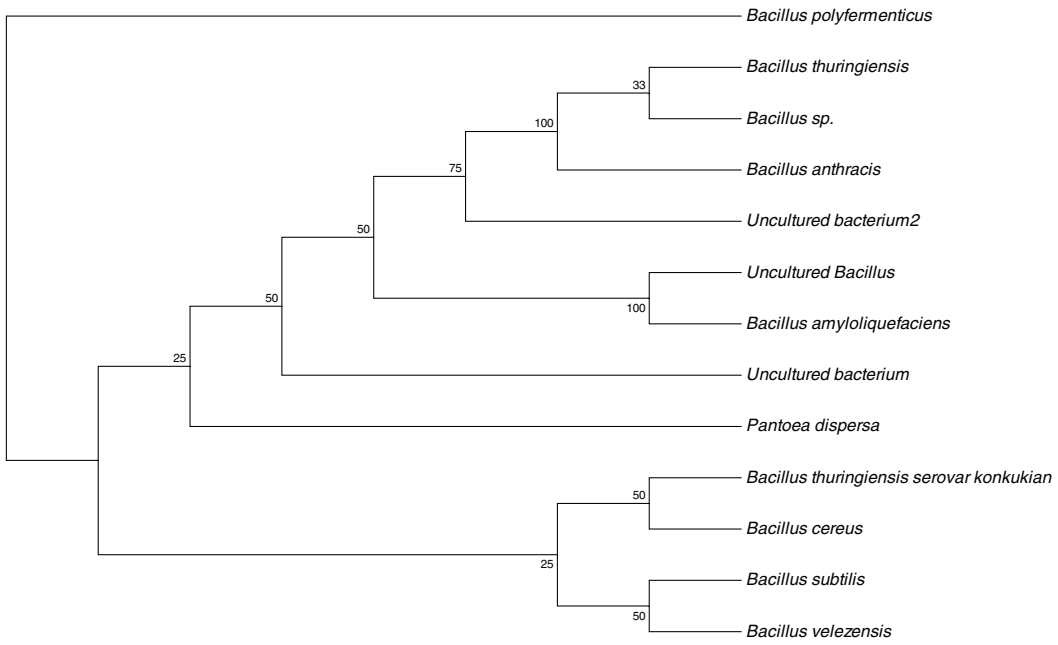
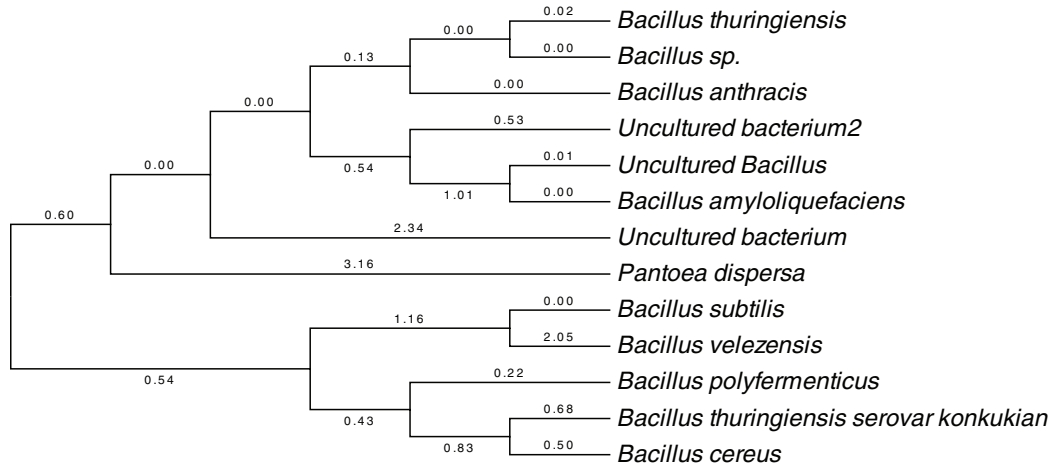
Figure S1 – Agarose gel 1% (w/v) showing the PCR products of the amplification of the 16S rRNA encoding region. 1: 200-bp ladder; 2-7: isolates.



(a)



(b)

Figure S2 – Phylogenetic analysis showing the relationship of the 16S rRNA gene sequences of isolates. The trees were generated using (a) Molecular Phylogenetic anaylsis by Maximum Parsimony (MP) analysis of taxa and (b) Maximum Likelihood (ML) method.