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Characterization of a cryptic plasmid from endophytic *Enterobacter agglomerans* isolated from Amazon's *Copaifera multijuga*

Alessandra Karisa Costa Lima do Nascimento[1], Lucivana Prata Souza Mourão[2], Enedina Nogueira de Assunção[1], Carlos Gustavo Nunes-Silva[1], Edmar Vaz de Andrade[1], José Odair Pereira[1] and Spartaco Astolfi-Filho[1].

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Resumo

Um pequeno plasmídeo denominado pEA1 foi isolado de *Enterobacter agglomerans*, uma bactéria endofítica que coloniza a *Copaifera multijuga* (copaíba). A sequência do pEA1 foi determinada utilizando a estratégia de *primer walking* e foi depositada no GenBank (número de acesso DQ659147). pEA1 é uma molécula circular com um comprimento de 2545 pb. A análise da sequência mostrou que este plasmídeo tem um teor GC (conteúdo de bases Guanina e Citosina na sequência) de 34% e contém quatro *open reading frames* (ORF) com capacidade de codificar mais de 100 aminoácidos. As ORF1, ORF2 e ORF3 mostraram homologia com proteínas de mobilização. A ORF4 mostrou homologia com proteína envolvida na replicação de um plasmídeo proveniente de *Pectobacterium atrosepticum*.

Palavras-chave: Mobilização, plasmídeo pEA1, *Enterobacter agglomerans*, copaíba.

Characterization of a cryptic plasmid from endophytic *Enterobacter agglomerans* isolated from Amazon's *Copaifera multijuga*. A small plasmid named pEA1 was isolated from *Enterobacter agglomerans*, an endophytic bacterium colonizing *Copaifera multijuga* (copaiba). The pEA1 sequence was determined by using the primer walking strategy and was deposited in GenBank (accession number DQ659147). pEA1 is a circular molecule with a length of 2,545 bp. Sequence analysis showed this plasmid has a GC content of 34% (Guanine and Citosine content in DNA sequence) and contains four open reading frames (ORFs) with capacity of encoding more than 100 amino acids. The ORF1, ORF2, and ORF3 showed homology to mobilization proteins. ORF4 showed homology to protein involved in replication in a plasmid originating from *Pectobacterium atrosepticum*.

Key-words: Mobilization, plasmid pEA1, *Enterobacter agglomerans*, copaiba.

[1] Universidade Federal do Amazonas, Centro de Apoio Multidisciplinar, Laboratório de Tecnologias de DNA. 69077-000. Manaus – AM.
[2] Universidade do Estado do Amazonas, Escola Superior de Ciências da Saúde, Laboratório de Genética. 69065-001. Manaus - AM

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1. Introduction

Bacteria inhabiting the intercellular spaces of plants and establish interactions that can significantly contribute to the viability of the plant are classified as endophytic (HALLMANN *et al*., 1997; AZEVEDO *et al*., 2000). Advantageous interactions occur between host plants and endophytes, with the latter standing out by acting on the natural biological control of insects and phytopathogenic microorganisms (PEIXOTO-NETO *et al*., 2002). The occurrence of endophytic biota in practically all cultivated plants, as well as the diversity of these microorganisms, have been widely reported in recent decades (AZEVEDO *et al*., 2000; BALDANI *et al*., 2002; AZEVEDO AND ARAUJO, 2007; FERREIRA *et al*., 2008; HARDOIM *et al*., 2008; BARROS *et al*., 2010). Among such microorganisms, *Enterobacter agglomerans*, a gram-negative bacterium, has been described in different cultures in Brazil, e.g., citrus (ARAÚJO *et al*., 2001; ARAÚJO *et al*., 2002; LACAVA *et al*., 2006), cassava (TEIXEIRA *et al*., 2007), and eucalyptus (PROCÓPIO *et al*., 2009).

Understanding the peculiar characteristics of endophytic communities can lead to their potential application as candidates forgenetically modified organisms, which could be useful for the biological control of pests and diseases (ANDREOTE *et al*., 2004; BARROS *et al*., 2010). Thus, genes of interest expressed by endophytes can be applied for the benefit of agricultural crops when used, for example, in phytohormones production and nitrogen fixation, along with their contribution to the resistance of plants to stress conditions (CHEN *et al*., 1995; FAHEY *et al*., 1998; BENSALIM *et al*., 1998; MEHNAZ *et al*., 2001; BARROS *et al*., 2010).

In this article, we report the cloning and characterization of a novel plasmid of *E. agglomerans* (synonym: *Pantoea agglomerans*) named pEA1. To date, there are no reports in the literature on the characterization of plasmids originating from this bacterium isolated from *Copaifera multijuga* (copaiba). Copaiba is an important forest resource in the economic scenario of the Amazon region because it produces an oil-resin with excellent healing and anti-inflammatory properties (MEDEIROS AND VIEIRA, 2008).

2. Materials and Methods

2.1. Bacterial strains and culture media

E. agglomerans containing pEA1 was previously isolated from plants of *C. multijuga*. *Escherichia coli* DH5α and TOPO10 strains (Invitrogen) were used as hosts for gene manipulation procedures. LB medium [peptone (1%), NaCl (1%) and yeast extract (0,5%)], pH 7,5 was used for culturing this bacteria at a temperature of 37°C.

2.2. Nucleotide sequencing and analysis

The natural plasmid pEA1 with approximately 2.5 kb from *E. agglomerans* was extracted by alkaline lysis (SAMBROOK *et al*., 1989). Restriction analysis was performed using the enzymes *Acc*I, *Ava*I, *Ava*II, *Bam*HI, *Bgl*II, *Eco*0109, *Eco*RI, *Hae*III, *Hind*III, *Pst*I, *Pvu*II, *Sau*I, *Sau*3AI, *Taq*I, *Xba*I, and *Xho*I, according to the manufacturer's specifications (New England Biolabs-NEB). The enzyme *Pst*I splits the plasmid pEA1 into 2 fragments of an approximate length of 1.0 kb and 1.5 kb, respectively. The 1-kb fragment was purified by agarose gel electrophoresis and cloned into the *Pst*I site of the vector pUC18; the resulting recombinant plasmid was named pEA1.0. Subsequently, this cloned fragment was sequenced by the Sanger-Coulson method using forward and reverse primers specific for pUC18. For this purpose, a DYEnamic™ ETDye terminator cycle sequencing kit (GE Healthcare) and a MegaBACE 1000 DNA sequencing System (GE Healthcare) were used (Figure 1).

From these sequences, a pair of primers was designed, EA3 and EA4 (Table 1), which was able to amplify a DNA fragment of about 2.5 kb, containing all the plasmid pEA1. This

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amplicon was purified by agarose gel electrophoresis and cloned into the pCR2.1-TOPO® vector (Invitrogen), according to the manufacturer's protocol. The cloned fragment was sequenced by the Sanger-Coulson method following primer-walking sequencing using the primers shown in Table 1.

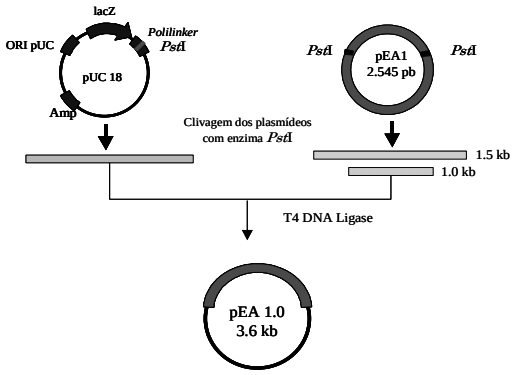

Figure 1. Experimental cloning strategy of derivative fragments of native pEA1.

Table 1. Primers used in the characterization of plasmid pEA1.

Primer	Sequence (5′→ 3′)
EA1	CGAGAAAATATTTAGTTTAGCTC
EA2	CACCTCTCTTTTAGTTTAATTTG
EA3	GAGCTAAACTAAATATTTTCTCG
EA4	CAAATTAAACTAAAAGAGAGGTG
EA5	GGTATACTCACTATACCGAGAAATCTC
EA6	GTGCAAGGTTTGTTAAAGAGATC
EA7	CAAACACCACATATACACGC
EA8	GTTCTGACGGAGATCAGAC
EA9	GTCAACCCTTCACAAGCC
EA10	GAAACAGCAAAGGAAGAAGC

Characterization of the nucleotide sequence of pEA1 was performed using the BLASTN, ORF Finder, GC-Profile (Gao F, Zhang, 2006) and NEBCutter2.0 programs. The complete DNA sequence of plasmid pEA1 was deposited in GenBank with the accession number DQ659147.

3. Results and Discussion

Plasmid pEA1 comprises 2,545 bp and as observed at figure 2 was digest with the enzyme *Pst*I.

It is considered a small plasmid, this feature favors its use for new vectors development, due to, in general, a small size provides a high number of copies and facilitates the manipulation of DNA.

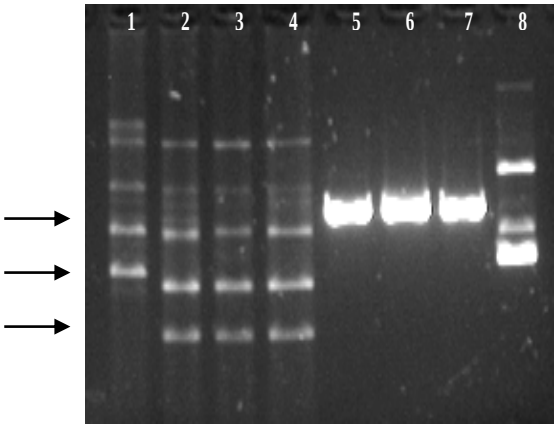

Figure 2. Restricion Profile of pEA1 and pUC18 plasmids. 1- Intact pEA1; 2, 3 e 4- *Pst* I parcially digest pEA1; 5, 6 e 7- *Pst* I digest pUC18; 8- Intact pUC18. The arrows indicate from gel top to botton DNA fragments of 2.5, 1.5 and 1.0 Kb.

Furthermore, recent studies have attributed functions to small plasmids that are mainly related to the process of replication and mobilization (ANDRUP *et al*., 2003; ZHANG *et al*., 2007).

Analysis of pEA1 identified 4 ORFs, of which only 2 encode proteins with more than 100 amino acids (Figure 3).

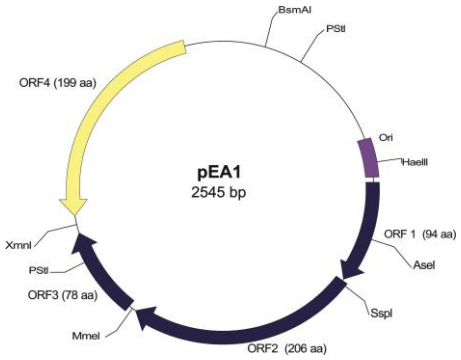

Figure 3. Physical map of natural plasmid pEA1 from *Enterobacter agglomerans*. The location and orientation of the four open reading frames are represented by arrows. At the bottom of the figure, the cleavage sites of the most relevant restriction enzymes are shown.

ORF1 (positions 618–902), ORF2 (positions 910–1530), and ORF3 (positions 1555–1791) possibly encode mobilization (Mob) proteins with 94, 206, and 78 amino

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acids, respectively. The amino acid sequences encoded by these ORFs (1, 2, and 3) showed 100% identity with Mob proteins codified by plasmid pIGMS31 of *Klebsiella pneumoniae* (accession number AAS55465). The ORF4 (positions 1840–2439) encodes a protein with 199 amino acid, which had 51% identity to a plasmid replication protein (ECA2908) of *Pectobacterium atrosepticum* (accession number SCRI1043).

Two functions considered essential do plasmid survive are: DNA replication and horizontal propagation (GARCILLÁN-BARCIA *et al.*, 2009). The last one may occur by three classic mechanisms, transformation, transduction and conjugation and have important evolutionary implications (DE LA CRUZ et al. 2010), since allows homologous recombination between lineages and related species, integrates new genetic information creating functional heels which allow rapid adaptation to new environment or stress conditions, and confer genic mobility, a plasmid ability related to antibiotic resistance (NOGUEIRA et al., 2009).

Among natural plasmids is possible to find conjugative and/or mobilizable (Mob) ones. The absence of the region tra, in which the genes responsible for all stages of conjugation are concentrated, and the presence of mob genes suggest that pEA1 is only a Mob plasmid. Mob plasmids may present genes responsible for their self-cleavage (mob); however, they depend on conjugative plasmids for their transfer to occur. Due to this fact, the mobilization process can be understood as an opportunistic form of transfer that results in widespread diffusion of Mob plasmids, which can be found in gram-positive and gram-negative bacteria (FRANCIA *et al*., 2004; CARYL AND THOMAS, 2006; COELHO *et al*., 2009).

It was observed that Mob and conjugative plasmids may contain a set of genes whose may exhibit low homology with those deposited in GenBank (SMILLIE et al., 2010). Mob plasmids had been frequently reported as small plasmids (around 5Kb) and is predicted a variety of mob genes related to molecular and kinetic of plasmid propagation (RANKIN et al., 2010). Recent studies showed that conjugative plasmids that have mob genes are dependent of them for propagation and this justify to reconsider the classification of these plasmids. New analysis could to group these plasmids in six MOB families based in six different genes of the relaxase protein (WESTRA et al., 2014).

The large distribution of Mob plasmids in different environments suggests that the combination of its characteristics of replication and mobilization make them ecologically very competitive (RAWLINGS AND TIETZE, 2001). Genes that encode Mob proteins are upstream of the operon tra in conjugative plasmids and form the operon mob in mobilizable plasmids (non-conjugative). The group of Mob proteins is formed mainly by MobA (relaxase), MobB, and MobC, which play distinct roles in the formation of the relaxosome. Of these, only relaxase, a site-specific endonuclease, is a well-maintained element in conjugative and mobilizable systems, because it is responsible for cleaving the second essential element for the transfer of DNA, the region oriT, and for generating the T-strand. For the other Mob proteins, MobD and MobE, little information is available (FRANCIA *et al*., 2004; BACKERT AND MEYER, 2006; VARSAKI *et al.*, 2012).

Despite the presence of these ORFs, no similarity was found between the sequence of pEA1 and the sequences reported as plasmid replication origins (ori regions). Meanwhile, in the region preceding mob genes, an AT-rich region was found, an inverted repeat sequence and several adjacent direct repeat sequences, which are described in the literature as traits related to plasmid replication origin and key sites for the control of plasmid replication (IOANNIDIS *et al*., 2007). Multiple dam methylation sites were also found that, although not essential for replication, may act as additional features found in plasmid replication origins (DEL SOLAR *et al*., 1998). Furthermore GC

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analysis revealed a 34% content and two regions assigned with arrows (1 and 2) in the figure 4 where possibly is located the origin of replication of plasmid pEA1. This kind of analysis is useful to localize replication origins in bacterial and Archeas genomes (GAO AND ZHANG, 2006).

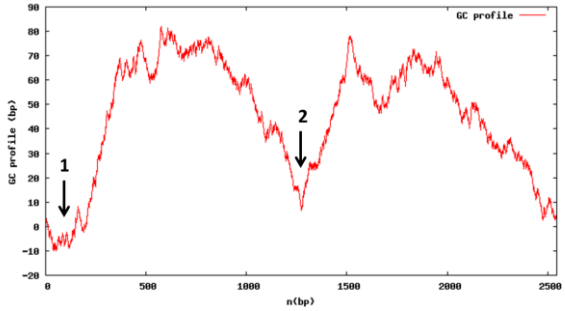

Figure 4. Graphical analysis of pEA1 GC content. Global distribution along 2,545pb pEA1 plasmid sequence.

Some plasmids residing in endophytic strains of *E. agglomerans* have been isolated, characterized, and used for the construction of bifunctional vectors containing a reporter gene. On the basis of previous studies using replication regions of small cryptic plasmids (ANDREOTE *et al*., 2008), efficient genetic tools have been developed to monitor bacterial colonization in different crops (PROCÓPIO *et al*., 2011), as well as bifunctional vectors capable of carrying and expressing exogenous genes within the host plant.

4. Conclusions

In this study, we have described the characterization of the cryptic plasmid pEA1 that was found in *E. agglomerans*, an endophytic bacterium isolated from copaiba tree. The information on the plasmid described above as well as the selection of a reporter gene allow the development of bifunctional vectors in studies of manipulation of endophytic bacteria that can be used for introduction of new genes benefiting host plants.

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Divulgação

Este artigo é inédito e não está sendo considerado para qualquer outra publicação. Os autores e revisores não relataram qualquer conflito de interesse durante a sua avaliação. Logo, a revista *Scientia Amazonia* detém os direitos autorais, tem a aprovação e a permissão dos autores para divulgação, deste artigo, por meio eletrônico.

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