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Genomic panorama of *Bradyrhizobium japonicum* CPAC 15, a commercial inoculant strain largely established in Brazilian soils and belonging to the same serogroup as USDA 123

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ABSTRACT

Of the many genomes of prokaryotes that have been sequenced, most are pathogenic organisms and very few of agriculturally beneficial bacteria. Soybean, the most important cash crop in Brazil, can provide its need for nitrogen through a symbiosis with exotic strains of bradyrhizobia. *Bradyrhizobium japonicum* strain CPAC 15 (equates to SEMIA 5079, the same serogroup as USDA 123), which is a highly competitive commercial strain applied to soybean crops since the early 1990s, is now established on several millions of hectares. As financial resources for sequencing genomes are still very limited in developing countries, a panoramic genomic view of CPAC 15 was generated. A total of 4328 shotgun reads resulted in 2,046,740 bp with a phred score ≥ 20; the assemblage resulted in 1106 phrap contigs scattered by 69 scaffolds and 966 isolated contigs, with an average of 2.5 reads per contig, covering approximately 13% of the genome. Annotation identified 1371 coding DNA sequences (CDSs), 53% with putative known functions, 23% encoding conserved hypothetical and 24% hypothetical genes, representing about 16% of the estimated putative genes. Several comparisons – on COG and KEGG databases, tRNAs, transposases, G + C content of CPAC 15 with the complete genome of *B. japonicum* strain USDA 110 indicated a successful coverage of the whole genome. However, the two strains were surprisingly different, as at least 35% of the CDS of CPAC 15 shows higher similarity to microorganisms other than strain USDA 110. Several new putative genes and others with low similarity to USDA 110, were identified. These were related to nodulation, interaction with the host plant and adaptation, e.g., *nodB*, *nodW*, *ndvA*, effector *nopP*, genes of secretion systems, transporters and environmentally related genes.

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1. Introduction

Prokaryotes were established on Earth billions of years before the first single-celled eukaryotes evolved, and the long period of adaptation to a variety of environments explains their enormous diversity and versatility. The genomes of almost 700 prokaryotes have been sequenced – with several others in progress or accessible as drafts (GOLD, 2008) – Genomes OnLine Database). Variability in size, number, density and organization of genes in operons has been demonstrated, and each new genome provides unexpected and sometimes intriguing information. For example, small genomes such as that of the thermophile *Nanoarchaeum equitans* strain Kin4-M (490,885 bp; Waters et al., 2003) may reveal the minimum set of essential genes and metabolic processes necessary to guarantee survival (Ochman, 2005). Large genomes such as that of *Burkholderia xenovorans* strain LB400 (9,731,138 bp; Chain et al., 2006) may provide understanding of events such as horizontal gene transfer and reveal functions of large sets of genes (Kaneko et al., 2002; Chain et al., 2006). Even within the same species, remarkable differences have been reported; for example, the genome of *Escherichia coli* strain K12 was estimated at 4,646,332 bp coding for 4337 genes (Riley et al., 2006), whereas a larger genome was reported for strain O157:H7 – 5,498,450 bp coding for 5449

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genes (Perna et al., 2001). Furthermore, the photosynthetic *Bradyrhizobium* sp. strain BTAi1 has more than 1 million bp than the ORFS278 strain (Giraud et al., 2007). However, the large majority of sequenced genomes are of pathogenic microorganisms, with emphasis on agents of human disease (GOLD – Genomes OnLine Database).

Soybean was introduced to Brazil 125 years ago and is now grown on over 22 million hectares, about 45% of the country's cropped land (Hungria et al., 2006). This legume's need for N may be supplied by a symbiosis in which bacteria belonging mainly to the species *Bradyrhizobium japonicum* and *B. elkanii* penetrate the roots resulting in the formation of nodules in which atmospheric N_2 is fixed and passed on to the host plant. Research on N_2 fixation, with emphasis on identification of superior strains of bradyrhizobia, has been a chief contributor to the success of the crop in Brazil, fostering high yields with low input costs (Hungria et al., 2006).

Strain-selection programs for soybean were established in Brazil with the first commercial plantings in the early 1960s, and *B. japonicum* SEMIA 566 – isolated from the nodules of a vigorous soybean plant inoculated with a North American inoculant – was one of the first "selected" strains. It belongs to the same serogroup as USDA 123 – recognized as the most competitive in the USA (Ham et al., 1971; Weber et al., 1989) – and was employed in commercial inoculants from 1966 to 1978, greatly contributing to the successful establishment of the crop in southern Brazil (Hungria et al., 2006). A decade later, higher demands for N by newer, more-productive soybean genotypes necessitated identification of strains with greater capacity for N_2 fixation and tolerance to the more stressful environmental conditions of newly cropped tropical regions in the Brazilian Cerrados. Selection emphasis was then on identification of adapted naturalized strains with relatively high efficiency of N_2 fixation, and CPAC 15 (=SEMIA 5079) was recognized as a very effective variant strain derived from SEMIA 566 (Hungria and Vargas, 2000; Hungria et al., 2006). CPAC 15 has been extensively used in Brazilian commercial inoculants since 1992, and the high competitiveness of both SEMIA 566 and CPAC 15 explain the dominance of this serogroup in practically all areas cropped to soybean, as well as its dispersion and establishment in non-inoculated areas (Freire et al., 1983; Vargas et al., 1993; Ferreira and Hungria, 2002; Mendes et al., 2004; Hungria et al., 2006; Batista et al., 2007).

When considering genome sequencing of prokaryotes, three main points should be taken into account. Firstly, despite the importance of the symbiotic N_2 fixation to the global N cycle (Newton, 2000), only a few complete genomes of rhizobia have been published so far: *Mesorhizobium loti* strain MAFF303099 (Kaneko et al., 2000), *Sinorhizobium meliloti* strain 1021 (Galibert et al., 2001), *B. japonicum* strain USDA 110 (Kaneko et al., 2002), *Rhizobium etli* bv. phaseoli strain CFN 42 (González et al., 2006), *R. leguminosarum* biovar *viciae* strain 3841 (Young et al., 2006), *Bradyrhizobium* sp. strains ORS278 and BTAi1 (Giraud et al., 2007), and *Azorhizobium caulinodans* strain ORS571 (Lee et al., 2008). Secondly, regardless of the economic and environmental importance of biological N_2 fixation to Brazilian agriculture, research funding for genomic studies in the country remains low, as in the great majority of developing countries. Finally, contrary to initial predictions (Chothia, 1992), the number of new protein families grows with each new genome sequenced, particularly among prokaryotes (Kunin et al., 2003), and mathematical models predict that, even with hundreds of genomes per species, new protein families with important and novel biochemical properties may yet be discovered (Tettelin et al., 2005). Therefore, creative, low-cost initiatives are needed to allow prospecting of genes of important microorganisms such as strain CPAC 15. A possibility is the partial sequencing of the genome, as proposed by (Viprey et al., 2000), allowing understanding of the main classes of genes and their distribution on the genome. Therefore, this study aimed at partially covering the genome of *B. japonicum* strain CPAC 15, to detect the main classes of genes and their similarities to those of other rhizobial genomes.

2. Material and methods

2.1. Rhizobial strain and growth conditions

B. japonicum strain CPAC 15 [CPAC refers to Embrapa-Centro de Pesquisa Agropecuária dos Cerrados, Planaltina, Distrito Federal, Brazil; the designation of the strain at the National collection of rhizobia, at FEPAGRO (Fundação Estadual de Pesquisa Agropecuária, Porto Alegre, Rio Grande do Sul, Brazil) is SEMIA 5079; other designations for the strain are 566a and DF 24] was obtained from the "Diazotrophic and Plant Growth Promoting Bacteria Culture Collection" of Embrapa Soja. The strain was grown on Luria–Bertani (LB) medium (Sambrook et al., 1989) for five days at 28 °C, pellets were obtained after centrifugation at 10,000*g* for 20 min and were stored at −70 °C.

2.2. Library construction

Shotgun libraries of strain CPAC 15 were prepared as described before (Vasconcelos et al., 2003) and involved DNA purification and random mechanical shearing by nebulization. Total DNA mixed with glycerol at 50% and NaOAc 3 M was nebulized by 30 s at 2 kgf cm^{-2} to obtain fragments ranging from 1 to 3 kb, that were repaired using T4 DNA polymerase, polynucleotide kinase (PNK), and Klenow polymerase of *E. coli*, and size-fractionated by low melting agarose (Promega) gel electrophoresis. After the extraction from the gel using the "Gel Extraction Kit" from Qiagen, fragments were cloned into the vector pUC18. The vector pUC18 used to clone the DNA fragments was previously digested with *Sma*I and dephosphorylated with the enzyme BAP (*b*acterial *a*lkaline *p*hosphatase), and then the DNA fragments were ligated to pUC18 with the use of T4 DNA ligase (Invitrogen™). For the transformation *E. coli* strain DH10B was used. Transformants were plated on LB medium containing ampicillin (250 μg mL^{-1}), 5-bromo-4-chloro-indoyl-β-D-galactoside (20 μL of an stock of 50 μg μL^{-1}) and isopropyl-β-thiogalactopyranoside (100 μL of 0.1 M IPTG) and grown overnight at 37 °C. Recombinant clones were identified and transferred to 96-well plates containing "Terrific Grow – TB" (Invitrogen™) medium with ampicillin (250 μg mL^{-1}) and after growing were maintained in 80% glycerol (Sigma, >99.5%) at −70 °C.

2.3. Sequencing of shotgun clones

Individual colonies of the libraries were inoculated in 96-well microplates containing "Terrific Grow – TB" medium with ampicillin (250 μg mL^{-1}) and grown at 150 rpm for 16 h at 37 °C. DNA was extracted by the usual method of alkaline lysis (Sambrook et al., 1989), with a modification in the final procedure, passing the supernatant by multiple filters (MultiScreen, Millipore) before the DNA precipitation (Vasconcelos et al., 2003). Purified DNA was resuspended in water and verified in 0.8% (1%) gel agarose, as described by Sambrook et al. (1989). DNA was precipitated with 3 M KOAc and then sequenced utilizing the DYEnamic™ ET dye terminator cycle sequencing (MegaBACE™) kit (Amersham Pharmacia Biotech). The PCR reactions were performed using universal and reverse (Invitrogen™ or RW genes) primers. PCR-products were analyzed on a MegaBACE 1000 capillary sequencer (Amersham Pharmacia Biotech). The quality of a library was checked by sequencing a small number of plasmids, which were assembled with the phrap program (Ewing et al., 1998) and aligned by BLAST (www.ncbi.nlm.nih.gov) to validate the randomness of the library and the proportion of vector sequences.

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