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APPLICATION OF REPRESENTATIONAL DIFFERENCE ANALYSIS TO IDENTIFY GENOMIC DIFFERENCES BETWEEN *BRADYRHIZOBIUM ELKANII* AND *B. JAPONICUM* SPECIES

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ABSTRACT

Bradyrhizobium elkanii is successfully used in the formulation of commercial inoculants and, together with *B. japonicum*, it fully supplies the plant nitrogen demands. Despite the similarity between *B. japonicum* and *B. elkanii* species, several works demonstrated genetic and physiological differences between them. In this work Representational Difference Analysis (RDA) was used for genomic comparison between *B. elkanii* SEMIA 587, a crop inoculant strain, and *B. japonicum* USDA 110, a reference strain. Two hundred sequences were obtained. From these, 46 sequences belonged exclusively to the genome of *B. elkanii* strain, and 154 showed similarity to sequences from *B. japonicum* genome. From the 46 sequences with no similarity to sequences from *B. japonicum*, 39 showed no similarity to sequences in public databases and seven showed similarity to sequences of genes coding for known proteins. These seven sequences were divided in three groups: similar to sequences from other *Bradyrhizobium* strains, similar to sequences from other nitrogen-fixing bacteria, and similar to sequences from non nitrogen-fixing bacteria. These new sequences could be used as DNA markers in order to investigate the rates of genetic material gain and loss in natural *Bradyrhizobium* strains.

Key words: *Bradyrhizobium;* Genome comparison; Nitrogen fixation; Representational difference analysis.

INTRODUCTION

Soybean [*Glycine max* (L.) Merrill] can establish effective biological nitrogen fixation (BNF) symbiosis with species of fast-growing rhizobia (13, 23, 25) as well with species of slow growing bradyrhizobia, like *Bradyrhizobium japonicum* (27), *B. elkanii* (30), and *B. liaoningense* (50). In theory, the nitrogen sources available for soybean cultures are nitrogen fertilizers and BNF (23). In Brazil, for example, the use of nitrogen as fertilizer is not recommended for soybean, as the inoculation with nitrogen-fixing bacteria fully supplies the plant nitrogen demands. Besides the saving generated annually by the inoculation - about US$ 3 billion per year (24), the practice avoids environmental pollution, very common when nitrogen fertilizers are employed. Since soybean Brazilian's fields lacked native bacteria able to nodulate this leguminous, the production of inoculants in this country started in the 1960s with North American strains (12). Nowadays, only four *B. japonicum* and *B. elkanii* strains have been used in the formulation of Brazilian's commercial inoculants and they have resulted in an established population in most soils cropped with soybean (15). Of those recommended strains, SEMIA 587 and SEMIA 5019 belonged to the *B. elkanii* species (42), while SEMIA 5080 and SEMIA 5079 belonged to the *B. japonicum* species (32).

In the 1980s the genus *Bradyrhizobium* was originated

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from the slow-growing specie *Rhizobium japonicum* (27). In the beginning the soybean-nodulating *B. japonicum* was the only specie described. To date, five additional species have been validly named in this genus, and besides *B. japonicum* other two species are able to nodulate *Glycine max*, *B. elkanii* and *B. liaoningense* (49). Despite the similarity between *B. japonicum* and *B. elkanii*, years of research demonstrated genetic and physiological differences among them. Boddey and Hungria (7) when studying 40 soybean *Bradyrhizobium* strains showed remarkable differences between these two species according to the resistance to antibiotics, where *B. elkanii* SEMIA 587 was resistant to kanamycin, rifampicin, spectinomycin, streptomycin, carbenicillin, chloramphenicol, nalidixic acid, tetracycline and erythromycin, while *B. japonicum* USDA 110 was resistant only to low levels of rifampicin and spectinomycin. These authors also found differences concerning to the production of indole acetic acid (IAA): *B. japonicum* strains accumulated between 4.88 to 7.08 µM of IAA ml^{-1}, while *B. elkanii* strains were able to accumulate about six times more (44.36 µM of IAA ml^{-1}). Genetics studies like the sequencing of *nifDK* and *nifE* genes (34) and the 16S rRNA gene (51), together with hybridization analysis with the *hup* gene (35), *nifDH* genes, and homologous sequences of *nodD* gene (44) also helped in the final division of *B. japonicum* and *B. elkanii* made by Kuykendall and coworkers (30). However, up to now the majority of published works about the genus *Bradyrhizobium* are related to *B. japonicum* strains. Nowadays, *B. japonicum* USDA 110 (28) and *Bradyrhizobium* sp. BTAi1 (17) are the only *Bradyrhizobium* species to have the whole genome sequenced. Concerning *B. elkanii* species little is published and known about it, especially in the field of genetics and genomics. Basically *nif*, *nod*, *recA*, *glnII*, and ribosomal genes sequences are found in the public databases.

The original Representational Difference Analysis (RDA) protocol was developed for detection of genomic differences between two genomes (33). The methodology has been modified and employed as a sensitive method for cloning of differentially expressed genes (1, 4, 8, 31). Microbiologists also succeeded to reveal genomic differences by comparing bacterial strains belonging to the same species with contrasting properties (6, 18).

Due to the ecological and environmental role of the BNF, the economy and efficiency of inoculants and their massive usage over soybean crops, it is urgent a better understanding of the *B. elkanii* genetics. To obtain such understanding *B. elkanii* SEMIA 587 and the reference strain *B. japonicum* USDA 110 were submitted to RDA experiment.

MATERIALS AND METHODS

Bacterial strains, growth conditions and DNA extraction

The rhizobial strains used in this study were the reference strain *Bradyrhizobium japonicum* USDA 110, and the crop inoculant strain *Bradyrhizobium elkanii* SEMIA 587; both strains were supplied by Fundação Estadual de Pesquisa Agropecuária (FEPAGRO, RS, Brazil). Each strain was grown on Yeast Mannitol Broth (10 g mannitol, 0.5 g potassium phosphate, 0.2 g magnesium sulphate, 0.1 g sodium chloride, 0.5 g yeast extract, water to one liter, pH 6.8) for seven days at 28ºC in an orbital shaker. Genomic DNA from bacteria was extracted according to basic molecular biology protocols (43).

RDA

The technique was modified from that described by Tinsley and Nassif (46). DNA from *B. japonicum* strain USDA 110 was used as driver DNA and DNA from *B. elkanii* strain SEMIA 587 was used as tester DNA. Fifteen µg of driver DNA were used for the whole RDA experiment. One µg of tester gDNA was completely digested with *Sau*3AI, resulting in a population of fragments with 200-300 pb, that were purified by column (Wizard® SV Gel and PCR Clean-Up System - Promega) and ligated to 500 pmol of each adapters NBam 12 (GATCCTCCCTCG) and NBam 24 (AGGCAACTGTGCTAT CCGAGGGAG). The Oscilating Phenol Emulsion Reassociation Technique [OsPERT, (10)] was used for the subtractive hybridization. In the first round 50 ng of tester DNA were mixed with 5 µg of driver DNA, resulting in a rate

of 1:100, and held for 15 min at room temperature to denature in a final volume of 34.6 µl of 0.3 N NaOH. Solution was neutralized with addition of 5.4 µl of 3 M MOPS, 50 µl of 1.5 M NaCl, 2 mM EDTA. Ten µl of buffer-saturated phenol pH 8.0 was added and mixed. The sample was heated at 65ºC for 1 minute and put on ice to generate a phenol emulsion. Then it was placed in a thermal cycler (PCR Express ThermoHybaid Thermal Cycler) programmed for 99 cycles of 15 seconds at 25 ºC and 15 seconds at 65 ºC. The phenol was removed with chloroform and the supernatant was purified by column. Five µl of the hybridization was added to 100 µl of reaction mix without oligonucleotides (1X buffer, 0.25 mM of each dNTP, 25 mM of $MgCl_2$, 2 U *Taq* Polymerase - Invitrogen) and incubated for 3 min at 72ºC to fill in the ends of the reannealed fragments. After denaturation at 94 °C for 5 min and addition of the oligonucleotide NBam 24 (100 pmol) the hybridized fragments were amplified by PCR (30 cycles of 1 min at 94 °C, 1 min at 62 °C, and 1 min at 72 °C followed by 5 min at 72 °C - PCR Express ThermoHybaid Thermal Cycler). The PCR products were again purified by column. An aliquot of these purified fragments was separated for a 1:10 dilution. The rest of them were treated with 1 U of Mung Bean Nuclease (Promega) for 15 min and column purified. Two PCR were performed, one reaction used 2 µl of the 1:10 dilution as template and the other reaction used 10 µl of the Mung Bean treated fragments as template. These PCR were performed as above described without the filling step. Next, the adapters of the differential products 1 (DP1) were removed by *Sau*3AI cleavage and a new set of adapters was ligated to DP1 [JBam 12 (GATCCGTTCATG) and JBam 24 (ACCGACGTCGACT ATCCATGAACG)]. The new adapter ligated DP1 (5 ng) was used in the second round of subtractive hybridization with 5 µg of driver DNA in a rate of 1:1000, and selective amplification was performed as in the first round. The third and final round was performed as described for the previous rounds but with adapters RBam 12 (GATCCTCGGTGA) and RBam 24 (AGCACTCTCCAGCCTCTCTCACCGAG); 500 pg of DP2 was hybridized with 5 µg of driver DNA resulting in a rate of 1:10000.

Construction of subtractive libraries and sequencing

An aliquot of the final RDA products (DP3 fragments) was filled in with Klenow fragment and phosphorylated with T4 polynucleotide kinase (Promega). Upon heat inactivation, the samples were column purified and ligated to *Sma*I-digested and dephosphorylated pUC18 vector. *Escherichia coli* XL1 Blue competent cells were heat-shock transformed with the ligation products. White *Escherichia coli* colonies were selected and extracted plasmids were sequenced in both directions in the ACT Gene Laboratory (Centro de Biotecnologia, UFRGS, Brazil) using the automatic sequencer ABI-PRISM 3100 Genetic Analyzer and M13 universal primers. Labeling reactions were performed in a Gene Amp PCR System 9700 (Applied Biosystems) thermocycler. The sequences were analyzed with the Staden Package software (v. 1.7.0, http://www.sourceforge.net). The final resulting sequences were compared to the GenBank database using the BLASTN program.

RESULTS

RDA procedure

Three rounds of subtractive hybridization and kinetic enrichment were performed using DNA from *B. elkanii* SEMIA 587 as tester and DNA from *B. japonicum* USDA110 as driver. As expected, exponential amplification of fragments specific to the tester DNA was obtained (see below). Linear amplification of fragments that were present on either DNA populations (tester and driver) was also observed. Several works had suggested the use of Mung bean nuclease to eliminate or reduce the unwanted linear amplification during the amplification step (38, 45) while others suggested that a 1:10 dilution is enough for the achievement of this proposal (2). Both alternatives were used in the present work during the PCR step. As similar amplifications results were achieved using either Mung bean or 1:10 dilution as template, both amplifications products were mixed and used as one in the following RDA rounds.

BLAST Results

Several genetic differences between DNAs from *B. japonicum* USDA110 and *B. elkanii* SEMIA 587 strains were successfully isolated by RDA methodology. After the three rounds of subtractive hybridization and kinetic enrichment the resultant products were used to construct a RDA library. The pUC18 cloned fragments were sequenced, analyzed by Staden Package and screened for similarity with those available on the GenBank database using BLASTN program. As the complete genome sequence of *B. japonicum* USDA110 is also available on GenBank database the RDA library sequences could be screened directly against it.

From the 234 total sequences obtained, 52 were redundant. One consensus sequence grouped 11 redundant sequences (this consensus sequence showed no obvious similarity with any sequence available in the public database), five consensus sequences grouped three redundant sequences each, and 13 consensus sequences grouped two redundant sequences each. After grouping the redundancy 200 RDA unique sequences were obtained. Forty six sequences among these 200 belonged exclusively to the genome of the tester strain *B. elkanii* SEMIA 587, and 154 showed similarities to sequences belonged to the genome of the driver strain *B. japonicum* USDA110. The 154 sequences with similarity to the genome of *B. japonicum* USDA110 were divided in five groups according to their identity (Table 1).

Table 1. Grouping of 154 sequences of *B. elkanii* SEMIA 587 with similarity to *B. japonicum* USDA 110 genome.

Group	Number of sequences	Percentage of total analyzed sequences [a]	Similarity [b]
I	2	1	60 to 69%
II	9	4.5	70 to 79%
III	19	9.5	80 to 89%
IV	68	34	90 to 99%
V	56	28	100%

[a] Percentage based on the 200 consensus sequences. Sequences with no similarity to *B. japonicum* (46) genome represents 23%.
[b] Similarity of the clone sequence related to their similar sequence in *B. japonicum* USDA110.

From the 46 sequences with no similarity to *B. japonicum* USDA 110 genome, 39 showed no similarity with sequences in public databases and seven sequences showed similarity with sequences of genes that coding for known proteins. These seven sequences were divided in three groups: similar to sequences from other *Bradyrhizobium* strains, similar to sequences from other nitrogen-fixing bacteria, and similar to sequences from non nitrogen-fixing bacteria. The group with similarity to sequences from other *Bradyrhizobium* strains was composed by two clones: clone i5 presented similarity to the gene encoding a putative toxin secretion ABC transporter from *Bradyrhizobium sp*. ORS278, and clone i29 was similar to the gene encoding a putative carboxylase like RuBisCO small subunit from the photoorganotroph *Bradyrhizobium sp*. BTA1. The group with similarity to sequences from other nitrogen-fixing bacteria was composed by three clones: clone i150 presented similarity to the gene encoding a 4-hydroxybenzoyl-CoA reductase alpha-subunit of *Mesorhizobium loti,* clone i170 to the gene encoding a conserved hypothetical protein of *Rhodopseudomonas palustris*, and clone ii23 to the gene encoding a virulence factor MVIN family protein of *Xanthobacter autotrophicus* Py2. The group with similarity to sequences from other non nitrogen-fixing bacteria was also composed by two clones: clone i65 presented similarity to the gene encoding a peptidase M19 of *Sphingopyxis alaskensis* RB2256, and clone i157 to the gene encoding a conserved hypothetical protein of *Nitrobacter winogradsky*. Table 2 shows the complete information about these data.

Table 2. List of *B. elkanii* SEMIA 587 clones with similarity to know genes obtained through BLASTN search.

Group	Clone	Gene[a]	Species	Similarities %	Expected value	GenBank accession numbers
Similars to other *Bradyrhizobium* strains	i5	Putative toxin secretion ABC transporter	*Bradyrhizobium sp.* ORS278	80	$2e^{-40}$	FJ624080
	i29	Putative carboxylase, like RuBisCO, small subunit	*Bradyrhizobium sp.* BTAi1	76	$2e^{-11}$	FJ624081
Similars to other nitrogen-fixing bacteria	i150	4-Hydroxybenzoyl-CoA reductase alpha-subunit	*Mesorhizobium loti*	75	$1e^{-15}$	FJ624082
	i170	Conserved hypothetical protein	*Rhodopseudomonas palustris*	70	$5e^{-07}$	FJ624083
	ii23	Virulence factor MVIN family protein	*Xanthobacter autotrophicus* Py2	69	$9e^{-12}$	FJ624084
Similars to other non nitrogen-fixing bacteria	i65	Peptidase M19	*Sphingopyxis alaskensis* RB2256	76	$3e^{-16}$	FJ624085
	i157	Conserved hypothetical protein	*Nitrobacter winogradsky*	76	$1e^{-22}$	FJ624086

[a] In this case each gene identified means the best hit that was obtained through BLASTN search.

Sequences belonging exclusively to both *B. japonicum* and *B. elkanii* strains

Besides the exclusive *B. elkanii* SEMIA 587 sequences, three sequences of *B. elkanii* SEMIA 587 that matched only sequences from *B. japonicum* USDA 110 and no other organism were found. They were: clone i184 (107 pb) and clone i92 (196 pb) that showed 77% and 94% of similarity, respectively, with two genes for different transcriptional regulatory proteins, and clone i176 (91 pb) that showed 74% of similarity with an intergenic region (74 bp at 3' side of *bsl6114* and 162 bp at 5' side of *blr6113*, respectively, a hypothetical protein and an unknown protein).

Sequence acquisition numbers

GenBank accession numbers of described unique tester sequences are: clone i5, FJ624080; clone i29, FJ624081; clone i150, FJ624082; clone i170, FJ624083; clone ii23, FJ624084; clone i65, FJ624085, and clone i157, FJ624086.

DISCUSSION

RDA strategy

There are two basic RDA protocols usually performed to find genomic unique sequences: The original protocol described by Lisitsyn and coworkers (33) and the modification of the RDA procedure described by Tinsley and Nassif (46). In the standard RDA approach, chromosomal DNAs of both tester and driver DNAs are digested to completion with *Bgl*II and complementary oligonucleotides are ligated to them. A 'representation' of both sets of fragments is generated using PCR. Following amplification, oligonucleotides from the tester are digested off and the tester is ligated to a new ones. Both 'representations' are used in the hybridization step. In the Tinsley and Nassif (46) procedure tester DNA is digested to completion with *Sau*3AI to generate a pool of smaller fragments than those generated by *Bgl*II digestion. Chromosomal DNA from the driver strain is sheared mechanically by 60 passes through a 30 gauge needle to generate fragments in the 3 kb to 4 kb range. *Sau*3AI digested tester DNA is ligated directly to oligonucleotides. Tester and sheared driver are mixed and hybridized. Calia and coworkers (11) made a comparison of these two protocols while studying two strains of *Vibrio cholerae*. Although the two methods can be used, the second was suggested to yield a more complete set of different products for analyzing genomic differences. In the

present work the Tinsley and Nassif (46) RDA protocol was used with some modifications. Instead shearing mechanically the driver genome by passes through a 30 gauge needle the driver DNA was partially digested with the same *Sau*3AI restriction enzyme (43). Fragments ranging from 1 kb to 10 kb were chosen to perform the RDA experiment.

From the 200 unique sequences, out of 234 sequences that were obtained, 46 had no similarity to the sequences from the driver DNA, indicating that the RDA library constructed was not extinguished and more unique sequences could be obtained through more sequencing. The method applied in this work yielded a proportion of approximately 20% of unique tester fragments.

The option to compare *Bradyrhizobium elkanii* SEMIA 587 and *Bradyrhizobium japonicum* USDA 110, which has its whole genome sequenced, had proved to be a valuable choice. Since the complete genome sequence of *B. japonicum* USDA 110 is available in public databases, a direct comparison of the data obtained from tester DNA through sequencing could be easily performed, avoiding the time and labor consuming Southern blot experiment to the validation of the RDA procedure. Additional information were also obtained like those regarding the tester sequences that have some similarity with driver sequences (see Table 1), and the identification of sequences that belong exclusively to the genomes of *Bradyrhizobium elkanii* SEMIA 587 and *B. japonicum* USDA 110 strains.

***B. elkanii* SEMIA 587 unique sequences**

The RDA approach revealed 46 sequences without similarity to *B. japonicum* USDA 110 genome. From these, 39 showed no similarity with sequences in public databases and seven showed similarity with sequences of genes that codify proteins, some of them with known function.

The sequence carried by clone i29 was similar to a sequence of the gene that codes for a putative carboxylase (small subunit) like Rubisco of *Bradyrhizobium sp*. BTAi1. This enzyme catalyzes the biological reduction and assimilation of carbon dioxide gas to organic carbon and it is the key enzyme responsible for the bulk of organic matter found on earth. Its biochemical role is chiefly as the central part of the Calvin–Benson–Basham (CBB) reductive pentose phosphate pathway where it combines CO_2 with ribulose 1,5-bisphosphate to form two molecules of 3-phosphoglycerate (5). Rubisco forms I and II are directly involved in carbon metabolism and have a well recognized autotrophic CO_2 fixing function. These enzymes are found in a wide range of chemo, organo, and phototrophs, occurring in bacteria, algae, and plants. Form III Rubisco has only been found in archaea and form IV enzymes have been shown to be functional in the methionine salvage pathway in many bacteria. (3). *Bradyrhizobium japonicum* USDA 110 strain, which DNA was used as a driver, has an organotroph metabolism; it means no photosynthesis is made. The putative Rubisco-like gene found in *B. elkanii* SEMIA 587 genome showed a high level of similarity with genes that coding for proteins that contain the AHS2 superfamily conserved region, which includes the Rubisco class I of *Bradyrhizobium sp.* BTAi1, a facultative photoorganomixotroph bacterium (5). As this is the first evidence of a Rubisco-like gene in the strain of study the necessity to evaluate if this gene is functional in *B. elkanii* SEMIA 587 will be very important.

The sequence carried by clone i5 was similar to the gene that codes for the toxin secretion ABC transporter (*hlyB* like protein) of *Bradyrhizobium sp*. ORS278. ABC (ATP Binding Cassette) proteins constitute a specific class of ATPases that are involved with a wide variety of transport processes, including both inward (import) and outward (export or secretion), the latter being conserved from bacteria to humans (20, 21). The most studied of these transporters are key components of the Type I secretion pathway in Gram-negative bacteria. The hemolysin B (*hlyB*) protein of *E. coli* is required in conjunction with hemolysin D (*hlyD*) and the extern membrane protein TolC for the secretion of hemolysin-A (*hlAy*) protein (19, 48). The Type I secretion pathway does not only export *hlyA* but also a great range of substrate like, proteases, lipases and different toxins. The substrate can be small as 19 kDa or even protein complexes of 800 kDa (22).

The systems constitute a complex of three proteins, a transporter located in the cytoplasmic membrane, a periplasmic membrane fusion protein, and an outer membrane channel protein. When the complex is assembled a periplasm crossing channel is formed (16).

Clone i150 contained a DNA fragment which sequence had showed similarity to the sequence of the gene that codes for *Mesorhizobium loti* 4-Hydroxybenzoyl-CoA reductase alpha-subunit. This enzyme catalyzes an important reaction in the anaerobic metabolism of phenolic compounds, i.e. the reductive removal of an aromatic hydroxyl group. Anaerobic metabolism is based on a reductive chemistry, by which groups at the ring are often reductively removed and dearomatization of the aromatic ring structures proceeds by reductions. One central intermediate is benzoyl-CoA, which is dearomatized by reduction. Most natural aromatic substrates are hydroxylated compounds that require reductive dehydroxylation of phenolic hydroxyl groups if benzoyl-CoA is to be formed (9).

Homolog to *Xanthobacter autotrophicus* Py2 virulence factor MVIN family protein was first described in *Salmonella typhimurium.* This protein has been shown to be an important virulence factor for this organism when infecting mouse (29) and it is part of a *Sinorhizobium meliloti* essential operon (41). Moreover, the *mviN* gene in *Rhizobium tropici* was found to be involved in motility (37) and a mutation in *mviN* gene in the soft rot-causing bacterium *Pseudomonas viridiflava* was shown to influence motility as well as virulence (14). However, the biochemical function of the product encoded by *mviN* and similar genes is not fully understood (26). A 69% of similarity was found between the sequence present at clone ii23 and the gene coding for the Py2 virulence factor MVIN from *X. autotrophicus.* It will be interesting to perform mutagenesis studies in *B. elkanii* in order to verify if this gene is also involved in the motility or other process in this bacterium.

The sequence carried by clone i65 was similar to the sequence of the gene that codes for *Sphingopyxis alaskensis* RB2256 peptidase M19 or renal dipeptidase, which is an enzyme that in humans is a membrane bound glycoprotein involved in the hydrolysis of dipeptides and is well-known as the sole metabolic enzyme for penem and carbapenem β-lactam antibiotics in mammals. These antibiotics are presently prescribed in clinical practice with the dipeptidyl inhibitor cilastatin (36). Peptidases are grouped into clans and families. Clans are groups of families for which there is evidence of common ancestry. Families are grouped by their catalytic type: A, aspartic; C, cysteine; G, glutamic acid; M, metallo; S, serine; T, threonine; and U, unknown. In metalloproteases a divalent cation, usually zinc, activates the water molecule. The metal ion is held in place by amino acid ligands, usually three in number (40). As already mentioned, *B. elkanii* SEMIA 587 was found to be more resistant to antibiotics than *B. japonicum* USDA 110 (7). Bacteria in general has a wide range of mechanisms to resist the actions of noxious agents: enzymatic reactions like β-lactamase, which hydrolyzes the β-lactam ring of antibiotics, the mutational alteration of the drug target to reduce the target's affinity for the drug or to reduce the permeability of the cell envelope. One example of the last one is the use of active transporters that belong to the ATP binding cassette (ABC) superfamily and utilize the free energy of ATP hydrolysis to expel drugs from the cell (39, 47). Two unique tester sequences that could codify proteins involved in the antibiotic resistance of *B. elkanii* were found in this work. They were the sequence of clone i65 that was similar to the gene that codes for the peptidase M19, which has the ability to cleave the β-lactam ring of penem and carbapenem antibiotics like carbenicilin, and the sequence of clone i5 that was similar to the gene that codes for toxin secretion ABC transporter (*hlyB* like protein) of *Bradyrhizobium sp*. ORS278, which could be involved in the exporting of noxious molecules from the bacterial cell.

The two remaining unique *B. elkanii* sequences identified by the RDA approach were both classified as genes that coding for conserved hypothetical proteins with no function predicted yet. One of them (clone i170) was similar to a DNA region from *Rhodopseudomonas palustris* genome and the other (clone i157) had showed a similarity of 76% to a genomic DNA region of *Nitrobacter winogradskyi.*

The possibility of RDA technique to identify unique

sequences had been confirmed in this work, since several DNA fragments unique to the tester DNA were obtained. These fragments included a large number of sequences that were not present in the genome of *B. japonicum* USDA 110. Some of the genes identified could be involved in mechanisms related to antibiotics resistance, motility, photosynthesis and transport, and could be the responsible for the phenotypic differences found between these two bacterial species. Further studies will be necessary to confirm the expression and functionalities of the predicted gene products. Nevertheless, the information provided will contribute to our knowledge concerning the molecular differences between these two species as well as the *B. elkanii* unique sequences identified could be used as DNA markers in order to investigate the rates of genetic material gain and loss in natural *Bradyrhizobium* strains.

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REFERENCES

1. Aiello, L.P.; Robinson, G.S.; Lin, Y.W.; Nishio, Y.; King, G.L. (1994). Identification of multiple genes in bovine retinal pericytes altered by exposure to elevated levels of glucose by using mRNA differential display. *Proc. Natl. Acad. Sci.* 91, 6231-6235.
2. Allen, N.L.; Penn, C.W.; Hilton, A.C. (2003). Representational difference analysis: critical appraisal and method development for the identification of unique DNA sequences from prokaryotes. *J. Microbiol. Meth.* 55, 73-81.
3. Ashida, H.; Saito, Y.; Kojima, C.; Kobayashi, K.; Ogasawara, N.; Yokota, A. (2003). A functional link between RuBisCO-like protein of *Bacillus* and photosynthetic RuBisCO. *Science* 302, 286–290.
4. Baeza, L.C.; Bailao, A.M.; Borges, C.L.; Pereira, M.; Soares, C.M.A.; Giannini, M.J.S.M. (2007). cDNA representational difference analysis used in the identification of genes expressed by *Trichophyton rubrum* during contact with keratin. *Microbes Infect.* 9, 1415-1421.
5. Badger, M.R.; Bek, E.J. (2008). Multiple Rubisco forms in proteobacteria: their functional significance in relation to CO_2 acquisition by the CBB cycle. *J. Exp. Bot*. 59, 1525–1541.
6. Barcellos, F.G. Batista, J.S.S.; Menna, P.; Hungria, M. (2009). Genetic differences between *Bradyrhizobium japonicum* variant strains contrasting in N_2-fixation efficiency revealed by representational difference analysis. *Arch. Microbiol*. 191, 113-122.
7. Boddey, L.H.; Hungria, M. (1997). Phenotypic grouping of Brazilian *Bradyrhizobium* strains which nodulate soybean. *Biol. Fertil. Soils* 25, 407–415.
8. Bowler, L.D.; Hubank, M.; Spratt, B.G. (1999). Representational difference analysis of cDNA for the detection of differential gene expression in bacteria: development using a model of iron-regulated gene expression in *Neisseria meningitidis*. *Microbiology* 145, 3529–3537.
9. Breese, K.; Fuchs, G. (1998). 4-Hydroxybenzoyl-CoA reductase (dehydroxylating) from the denitrifying bacterium *Thauera aromatica* Prosthetic groups, electron donor, and genes of a member of the molybdenum-flavin-iron-sulfur proteins. *Eur. J. Biochem*. 251, 916-923.
10. Bruzel, A.; Cheung, V.G. (2006). DNA reassociation using oscillating phenol emulsions. *Genomics* 87, 286-289.
11. Calia, K.; Waldor, M.; Calderwood, S. (1998). Use of representational difference analysis to identify genomic differences between pathogenic strains of *Vibrio cholerae*. *Infect. Immun.* 66, 849-852.
12. Cattelan, A.J.; Hungria, M. (1994). Nitrogen nutrition and inoculation. In: Embrapa CNPSo. (Org.). *Tropical soybean: Improvement and Production*. 1st ed. Rome, Italy: Food and Agriculture Organization of the United Nations (FAO), pp 201-215.
13. Chen, L.S.; Figueredo, A.; Pedrosa, F.O.; Hungria, M. (2000). Genetic characterization of soybean rhizobia in Paraguay. *Appl. Environ. Microbiol.* 66, 5099–5103.
14. Czelleng, A.; Bozso, Z.; Ott, P.G.; Besenyei, E.; Varga, G.J.; Szatmári, A.; Király, L.; Klement, Z. (2006). Identification of Virulence-Associated Genes of *Pseudomonas viridiflava* Activated During Infection by Use of a Novel IVET Promoter Probing Plasmid. *Curr. Microbiol*. 52, 282–286.
15. Ferreira, M.C.; Andrade, D.S.; Chueire, L.M.O.; Takemura, S.M.; Hungria, M. (2000). Tillage method and crop rotation effects on the population sizes and diversity of bradyrhizobia nodulating soybean. *Soil Biol. Biochem*. 32, 627–637.
16. Gentschev, I.; Dietrich, G.; Goebel, W. (2002). The *Escherichia coli* alphahemolysin secretion system and its use in vaccine development. *Trends Microbiol.* 1, 39-45.
17. Giraud, E.; Moulin, L.; Vallenet, D.; Barbe, V.; Cytryn, E.; Avarre, J.C.; Jaubert, M.; Simon, D.; Cartieaux, F.; Prin, Y.; Bena, G.; Hannibal, L.; Fardoux, J.; Kojadinovic, M.; Vuillet, L.; Lajus, A.; Cruveiller, S.; Rouy, Z.; Mangenot, S.; Segurens, B.; Dossat, C.; Franck, W.L.; Chang, W.S.; Saunders, E.; Bruce, D.; Richardson, P.; Normand, P.; Dreyfus, B.; Pignol, D.; Stacey, G.; Emerich, D.; Vermeglio, A.; Medigue, C.; Sadowsky, M. (2007). Legumes symbioses: absence of Nod genes in photosynthetic bradyrhizobia. *Science* 316, 1307-1312.

18. Guo, H.; Sun, S.; Finan, T.M.; Xu, J. (2005). Novel DNA Sequences from Natural Strains of the Nitrogen-Fixing Symbiotic Bacterium *Sinorhizobium meliloti*. *Appl. Environ. Microbiol*. 71, 7130-7138.
19. Higgins, C.F. (1992). ABC transporters: from microorganisms to man. *Ann. Rev. Cell Biol.* 8, 67–113.
20. Holland, I.B.; Blight, M.A. (1999). ABC-ATPases, adaptable energy generators fuelling transmembrane movement of a variety of molecules in organisms from bacteria to humans. *J. Mol. Biol*. 293, 381–399.
21. Holland, I.B.; Benabdelhak, H.; Young, J. (2003). *ABC Proteins: From Bacteria to Man*. Academic Press, London, UK.
22. Holland, I.B.; Schmitt, L.; Young, J. (2005). Type 1 protein secretion in bacteria, the ABC-transporter dependent pathway. *Mol. Memb. Biol*. 22, 29-39.
23. Hungria, M.; Chueire, L.M.O.; Coca, R.G.; Megías, M. (2001). Preliminary characterization of fast growing rhizobial strains isolated from soybean nodules in Brazil. *Soil Biol. Biochem.* 33, 1349–1361.
24. Hungria, M.; Franchini, J.C.; Campo, R.J.; Graham, P.H. (2005). The importance of nitrogen fixation to soybean cropping in South America. In: Werner, D., Newton, W.E. (eds). *Nitrogen fixation in agriculture, forestry, ecology, and the environment*. Springer, NY, USA.
25. Hungria, M.; Chueire, L.M.O.; Megías, M.; Lamrabet, Y.; Probanza, A.; Guttierrez-Manero, F.J.; Campo, R.J. (2006). Genetic diversity of indigenous tropical fast-growing rhizobia isolated from soybean nodules. *Plant Soil* 288, 343– 356.
26. Hvorup, R.N.; Winnen, B.; Chang, A.B.; Jiang, Y.; Zhou, X.F.; Saier, M.H.Jr. (2003). The multidrug/oligosaccharidyl-lipid/polysaccharide (MOP) exporter superfamily. *Eur. J. Biochem.* 270, 799–813.
27. Jordan, D.C. (1982). Transfer of *Rhizobium japonicum* Buchanan 1980 to *Bradyrhizobium* gen. nov., a genus of slow growing root nodule bacteria from leguminous plants. *Int. J. Syst. Bacteriol*. 32, 136–139.
28. Kaneko, T.; Nakamura, Y.; Sato, S.; Minamisawa, K.; Uchiumi, T.; Sasamoto, S.; Watanabe, A.; Idesawa, K.; Iriguchi, M.; Kawashima, K.; Kohara, M.; Matsumoto, M.; Shimpo, S.; Tsuruoka, H.; Wada, T.; Yamada, M.; Tabata, S. (2002). Complete genomic sequence of nitrogen-fixing symbiotic bacterium *Bradyrhizobium japonicum* USDA110. *DNA Res*. 9, 225-256.
29. Kutsukake, K.; Okada, T.; Yokoseki, T.; Iino, T. (1994). Sequence analysis of the *flgA* gene and its adjacent region in *Salmonella typhimurium*, and identification of another flagellar gene, *flgN*. *Gene* 143, 49-54.
30. Kuykendall, L.D.; Saxena, B.; Devine, T.E.; Udell, S.E. (1992). Genetic diversity in *Bradyrhizobium japonicum* Jordan (1982) and a proposal for *Bradyrhizobium elkanii* sp. nov. *Can. J. Microbiol*. 38, 501–505.
31. Lei, L.; Sun, C.; Lu, S.; Feng, X.; Wang, J.; Han, W. (2008). Selection of serotype-specific vaccine candidate genes in *Actinobacillus pleuropneumoniae* and heterologous immunization with *Propionibacterium acnes*. *Vaccine* 26, 6274-6280.
32. Lemos, E.G.M. (1994). Classificação e identificação de bradirrizóbios que nodulam soja por análise de padrões isoenzimáticos, sorologia, morfologia de colônias e atividade de hidrogenase. São Paulo: UNESP (PhD Tesis).
33. Lisitsyn, N.; Lisitsyn, N.; Wigler, M. (1993). Cloning the differences between two complex genomes. *Science* 259, 946-51.
34. Minamisawa, K. (1990). Division of rhizobitoxine-producing and hydrogen-uptake positive strains of *Bradyrhizobium japonicum* by *nifDKE* sequence divergence. *Plant Cell Physiol.* 31, 81–89.
35. Minamisawa, K.; Seki, T.; Onodera, S.; Kubota, M.; Asami, T. (1992). Genetic relatedness of *Bradyrhizobium japonicum* field isolates as revealed by repeated sequences and various other characteristics. *Appl. Environ. Microbiol.* 58, 2832–2839.
36. Nitanai, Y.; Satow, Y.; Adachi, H.; Tsujimoto, M. (2002). Crystal structure of human renal dipeptidase involved in β-lactam hydrolysis. *J. Mol. Biol.* 321, 177–184.
37. O'Connell, K.P.; Raffel, S.J.; Saville, B.J.; Handelsman, J. (1998). Mutants of *Rhizobium tropici* strain CIAT899 that do not induce chlorosis in plants. *Microbiology* 144, 2607-2617.
38. Pastorian, K.; Hawel, L.; Byus, C.V. (2000). Optimization of cDNA Representational Difference Analysis for the Identification of Differentially Expressed mRNAs. *Anal. Biochem.* 283, 89–98.
39. Putman, M.; van Veen, H.W.; Konings, W.N. (2000). Molecular properties of bacterial multidrug transporters. *Microbiol. Mol. Biol. Rev*. 64, 672–693.
40. Rawlings, N.D.; Barrett, A.J. (1995). Evolutionary families of metallopeptidases. *Meth. Enzymol*. 248, 183-228.
41. Rudnick, P.A.; Arcondeguy, T.; Kennedy, C.K.; Kahn, D. (2001). *glnD* and *mviN a*re genes of an essential operon in *Sinorhizobium meliloti. J. Bacteriol*. 183, 2682-2685.
42. Rumjanek, N.G.; Dobert, R.C.; van Berkum, P.; Triplett, E.W. (1993). Common soybean inoculant strains in Brazil are members of *Bradyrhizobium elkanii*. *Appl. Environ. Microbiol,* 59, 4371–4373.
43. Sambrook, J.; Russell, D.W. (2001). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, NY, USA.
44. Stanley, J.S.; Brown, G.G.; Verma, D.P.S. (1985). Slow-growing *Rhizobium japonicum* comprises two highly divergent symbiotic types. *J. Bacteriol*. 163, 148–154.
45. Taylor, D.L.; Ward, P.N.; Rapier, C.D.; Leigh, J.A.; Bowler, L.D. (2003). Identification of a Differentially Expressed Oligopeptide Binding Protein (OppA2) in *Streptococcus uberis* by Representational Difference Analysis of cDNA. *J. Bacteriol.* 185, 5210–5219.
46. Tinsley, C.R.; Nassif, X. (1996). Analysis of the genetic differences between *Neisseria meningitidis* and *Neisseria gonorrhoeae*: two closely related bacteria expressing two different pathogenicities. *Proc. Natl. Acad. Sci.* 93, 11109–11114.
47. Walsh, C. (2000). Molecular mechanisms that confer antibacterial drug resistance. *Nature* 406, 775–781.
48. Wandersman, C.; Delepelaire, P. (1990). TolC, an *Escherichia coli* outer

membrane protein required for hemolysin secretion. *Proc. Natl. Acad. Sci.* 87, 4776-4780.

49. Willems, A. (2006). The taxonomy of rhizobia: an overview. *Plant Soil* 287, 3–14.
50. Xu, L.M.; Ge, C.; Cui, Z.; Li, J.; Fan, H. (1995). *Bradyrhizobium liaoningense* sp. nov. isolated from the root nodules of soybean. *Int. J. Syst. Bacteriol*. 45, 706–711.
51. Young, J.P.W.; Downer, H.L.; Eardly, B.D. (1991). Phylogeny of the phototropic *Rhizobium* strain BTAi1 by polymerase chain reaction based sequencing of a 16S rRNA gene segment. *J. Bacteriol*. 173, 2271–2277.