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Gene characterization of *Bradyrhizobium* spp. strains contrasting in biological nitrogen fixation efficiency in soybean

Caracterização dos genes de cepas de *Bradyrhizobium* spp. contrastantes quanto à eficiência de fixação biológica de nitrogênio em soja

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Highlights:
Gene characterization contributes in the soybean BNF process understanding.
Four genes were characterized in *B. japonicum* strains contrasting in BNF efficiency.
Only *blr3208* and *blr4511* genes amplified for most of the strains.
blr3208 and *blr4511* genes showed highly preserved internal regions among strains.
This preservation may be related to bacterial adaptation during evolution.

Abstract

Bacteria from genus *Bradyrhizobium* can establish symbiosis with soybean and supply the plant nitrogen demands via biological nitrogen fixation (BNF). This study aimed to characterize genes related to BNF efficiency in *B. japonicum* strains contrasting in BNF efficiency. These gene sequences were previously identified in *B. japonicum* (strain S370) as probably related to the BNF efficiency in soybean using a DNA subtractive technique. These genes were amplified with primers based on *B. japonicum* USDA110 genome. The PCR products were digested with restriction endonucleases and the RFLP products were analyzed by horizontal electrophoresis. Among the four genes, only *blr3208* and *blr4511* amplified for most of the strains. Neither polymorphism of the restriction profile of *blr3208* and *blr4511* genes nor with endonuclease for PCR-RFLP was observed. The contrasting strains had *blr3208* and *blr4511* genes sequenced and the multiple alignment analysis of nucleotide sequences showed the presence of preserved internal regions, confirming the analysis with PCR-RFLP. The *blr3208* and *blr4511* genes are highly conserved among *B. japonicum* strains, which may be related to adaptive function during the evolutionary process of *Bradyrhizobium* genus.
Key words: *Bradyrhizobium*. Rhizobacteria. PCR-RFLP. Restriction Polymorphisms.

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Resumo

Bactérias do gênero *Bradyrhizobium* podem estabelecer simbiose com a soja e suprir a demanda de nitrogênio pela fixação biológica de nitrogênio (FBN). Este estudo teve como objetivo caracterizar genes relacionados à eficiência na FBN em cepas de *B. japonicum* contrastantes quanto a eficiência na FBN. As sequências gênicas relacionadas à eficiência da FBN em soja foram previamente identificadas na cepa S370 de *B. japonicum* utilizando uma técnica de DNA subtrativo. Os genes foram amplificados com iniciadores construídos a partir do genoma da cepa USDA 110. Os produtos de PCR foram digeridos com endonucleases de restrição e os produtos de RFLP foram analisados por eletroforese horizontal. Dos quatro genes estudados, apenas *blr 3208* e *blr 4511* amplificaram na maioria das cepas. Não foi observado polimorfismo no perfil de restrição dos genes *blr 3208* e *blr 4511* por PCR-RFLP. As cepas contrastantes tiveram os genes *blr 3208* e *blr 4511* sequenciados e a comparação das sequências nucleotídicas por análise de alinhamento múltiplo mostrou a presença de regiões internas preservadas, confirmando a análise realizada por PCR-RFLP. Os genes *blr 3208* e *blr 4511* são altamente conservados entre as cepas de *B. japonicum*, o que pode estar relacionado à função adaptativa durante o processo evolutivo do gênero *Bradyrhizobium*.

Palavras-chave: *Bradyrhizobium*. Rizobactérias. PCR-RFLP. Polimorfismo de restrição.

Introduction

Diazotrophic bacteria, collectively known as rhizobia, have been contributing to intensify soybean (*Glycine max* L.) productivity by using atmospheric nitrogen (N_2). In this process, known as biological nitrogen fixation (BNF), the bacteria fixes the molecular nitrogen that is transferred to the plant as the main nitrogen source. On the other hand, the plant provides photoassimilates necessary for the bacterial development (Göttfert, 1993). BNF based on more responsive bacterial strains enabled soybean to be cultivated in Brazil without using mineral nitrogen and resulted in the decrease of production costs (Bortolan, Barcellos, Marcelino, & Hungria, 2009). The high protein content in soybean grains demands a large amount of nitrogen by the crop. According to Hungria, Campo and Mendes (2007), for a productivity of 3000 kg ha^{-1} of soybean grains, 240 kg of nitrogen are necessary. The provision of nitrogen fertilizer to the plant has a high economical cost and part of the nitrogen is lost by leaching and volatilization (Silva, Freitas, & Stamford, 2002), leading to environmental costs. However, the nitrogen demand of Brazilian soybean can be mainly supplied by BNF, dismissing nitrogen fertilization and making the production process economically and ecologically sustainable (Santos, Nicolás, & Hungria, 2006; Hungria & Mendes, 2015).

The main bacteria capable of acting symbiotically for soybean BNF are *Bradyrhizobium japonicum* Kirchner, 1896 (Jordan, 1982), also reclassified as *Bradyrhizobium diazoefficiens* sp. nov. (Delamuta et al., 2013), and *Bradyrhizobium elkanii* Kuykendall et al., 1993 (Orrell & Nicolson, 2020). The symbiosis between *Bradyrhizobium* spp. and soybean is a complex process that involves the expression of specific genes of the plant and bacterium (Hungria & Stacey, 1997; Wei et al., 2008; Passaglia, 2017) and an exchange of highly coordinated signals between them (Spaink, 2000). Soybean plants secrete the flavonoids genistein and daidzein in the rhizosphere, which are recognized by the bacteria as a signal for the induction of nodulation genes (*nod)* that trigger the production of lipo-chitooligosaccharides (LCO's), known as Nod factors (Liu & Murray, 2016). These signals from the bacteria to the plant start the symbiosis and guide the bacteria to the plant root cortex (Kijne, 1992; Sharma et al., 2020). Next, the plant cells begin a cell division process stimulated by the Nod factor, resulting in a characteristic structure,

the nodule (Caetano-Anollés & Gresshoff, 1991, Bortolan et al., 2009). The formation of root nodules leads to N_2 fixation and assimilation by the plant (Reis, Mendes, Reis, & Hungria, 2011). Two other groups of genes named *nif* and *fix* are involved in the BNF process, specifically in the synthesis of the nitrogenase complex and the electron transport chain, respectively (Hungria & Stacey, 1997; Shamseldin, 2013).

Several studies have been trying to understand the BNF process and identify genes related to rhizobia-legume symbiosis (Barcellos, Batista, Menna, & Hungria, 2009; Wang, Liu, & Zhu, 2018). The nodulation genes (*nod*) and nitrogen-fixation genes (*nif*) are the main drivers of the interaction between rhizobia and host plants (Kaminski, Batut, & Boistard, 1998; Van Hameren, Hayashi, Gresshoff, & Ferguson, 2013). However, other genes also affect the efficiency of nodulation and nitrogen fixation (Mahmud, Makaju, Ibrahim, & Missaoui, 2020). Godoy et al. (2008) reported the partial genome sequencing of *B. japonicum* (strain CPAC15), and identified potential genes related to BNF efficiency and competitiveness.

Barcellos et al. (2009), based on a technique of representational difference analysis (RDA), described by Lisitsyn, Lisitsyn and Wigler (1993), compared two *B. japonicum* strains (S370 and S516) contrasting in their BNF efficiency with soybean. The "tester" strain S370 presented higher capacity (three times more fixed N_2) and efficiency (twice as more N_2 fixed per gram of nodule) of N_2-fixation than the "driver" strain S516. Barcellos et al. (2009) demonstrated the presence of genes related to BNF efficiency only for the strain S370 and identified considerable polymorphism for these genes. On the functional category of transport and binding proteins, six genes were identified such as two putative ABC transporter substrate-binding proteins (*blr4511* and *bll7921* genes), a putative ABC-transporter ATP-binding protein (*bll5893* gene), L-amino-acid ABC-transporter ATP-binding protein (*aapP* gene), maltose-malt-dextrin import ATP-binding protein (*malK* gene), and a multiple sugar-binding periplasmic receptor ChvE precursor (*blr3208* gene). The *malk* and *aapP* genes were previously studied (Batista & Hungria, 2012; Perin et al., 2018). Therefore, in our study we analyzed the genes related to the functional category of transport and binding proteins, as this gene category codes for important membrane transporters in the molecule transport involved in the exchange of signals between bacteria (rhizobia) and leguminous plants (Barcellos et al., 2009). Thus, this study aimed to characterize the blr*4511*, *bll7921*, *bll5893* and *blr3208* genes in *B. japonicum* strains contrasting in BNF efficiency to improve the knowledge on bacterial biodiversity of agroindustrial interest.

Materials and Methods

Biological material

Bradyrhizobium spp. strains from the culture collection of the Brazilian Microbial Resource Center (http://www.bmrc.lncc.br/) at Embrapa Soja were utilized. Fortheen contrasting strains for BNF effectiveness in soybean were studied and coded as CPAC7, S204, S340, S370, S372, S406, S452, S468, S478, S490, S516, SEMIA586, USDA110, and USDA6 (Hungria, Boddey, Santos, & Vargas, 1998; Santos, Vargas, & Hungria, 1999). These strains were also cultivated in culture medium yeast mannitol agar (YMA) or yeast mannitol broth (YMB) as described by Vincent (1970), in the dark, at 28 ºC, and then preserved at -70 ºC in added 30% glycerol, for a long term, or at 4 ºC in YMB or YMA for continuous use (Ribeiro, Barcellos, Thompson, & Hungria, 2009).

DNA extraction and oligonucleotide synthesis

The DNA of each strain was extracted, quantified and diluted in ultrapure water at 5 ng μL^{-1} according to protocol described by Barcellos et al. (2009). The genes listed in Table 1, previously identified by Barcellos et al. (2009) as potentially related to

the BNF efficiency in soybean, were selected for PCR amplification of each strain. The primers were built based on the genome of *B. japonicum* strain USDA110 (NCBI; http://www.ncbi.nlm.nih.gov/nuccore/27375111) whose genome was completely sequenced by Kaneko et al. (2002). The FastPCR software (Kalendar, Lee, & Schulman, 2009) was used for building the primers according to the manufacturer's parameters and recommendations.

Table 1
***Bradyrhizobium japonicum* (USDA 110) genome-based oligonucleotide primers (forward and reverse) and expected size of amplified fragments**

Function	Gene	Annealing temperature (°C)	Forward (5'- 3')	Reverse (5'- 3')	Expected size (bp)
TRAP-transporter substrate-binding protein	*blr4511*	59	TTTTCCCGACGCA CGCTTCTCA	AGAGCTTGCCGAC GGCCTTCT	1009
Sugar ABC-transporter substrate-binding protein	*blr3208* (*chv*E)	58	TGAAGACGACAT TCCTCGCGCT	GTAGTAGCCGCTG TCCACCAG	1043
ABC-transporter substrate-binding protein	*bll7921*	58	CTCCGCAGACGTG CGATCACA	CTACTGCATCGA CACCAGCGAG	1790
ABC-transporter ATP-binding protein	*bll5893*	58	GTGACGATGGCC CAGGAAACC	TATTGCCTCAGCCA GGGATGC	752

PCR amplification

For each reaction, the following was used in a final volume of 50 µL: dNTPs (300 mM of each); PCR buffer (20 mM tris base, pH 8.4, and 50 mM KCl), primers (20 pmol), *Taq* DNA polymerase (1.0 U), and DNA template (20 ng). The amplification cycles were done as described by Ribeiro et al. (2009) with modified annealing temperature of primers, according to Table 1. The initial denaturation cycle was at 95 ºC for 2 min, followed by 35 denaturation cycles at 94 ºC for 45 s, annealing temperature according to each primer for 45 s, and extension at 72 °C for 5 min. All 14 reactions were done in a Master Cycler Gradient thermocycler (Eppendorf®). The strains without amplicons were not considered in the next assays and the genes (Table1) that were more representative in 14 strains were chosen for sequencing.

Restriction analysis (PCR-RFLP) of amplified genes

The restriction enzyme for PCR-RFLP was chosen based on *in silico* analysis of the gene sequences *blr4511* and *blr3208* (also *chv*E) of *B. japonicum* (USDA110) strain with the software Restriction Mapper (http://www.restrictionmapper.org/). AccI with one cutting site for *blr4511* (708 and 301 bp) and three cutting sites for *blr3208* (525, 370, 102, and 46 bp) was used in the analyses.

The PCR products were precipitated by adding to the reaction products 1:10 (volume: volume) 3 M sodium acetate, three volumes of cold ethanol, and kept in ice for 5 min. The ethanol was discarded, the precipitated DNA was re-suspended in 30 µL ultrapure water, and stored at -20 °C for posterior digestion with restriction enzymes.

After the PCR products obtained for each gene had been individually digested with several

restriction endonucleases, the *Acc*I enzyme was chosen to proceed with the analyzes. The digestion conditions were carried out according to the manufacturer's specifications. The RFLP products were analyzed by electrophoresis in 3% agarose gel with ethidium bromide (10 mg mL^{-1}) added to 0.5 µg mL^{-1} final concentration, and photographed under ultraviolet light.

Gene sequencing

PCR amplified products of the genes were purified using the purelink PCR purification kit (Life Technologies) according to the manufacturer's protocols, and sequenced by an ABI-prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocols. The nucleotide sequences were submitted to GenBank (NCBI) to obtain the access numbers.

Multiple alignment

The multiple alignment of nucleotide sequences of genes was carried out using the ClustalW program (https://www.genome.jp/tools-bin/clustalw) according to the protocol described by the program.

Results and Discussion

Among the four analyzed genes potentially related to BNF efficiency in soybean (Table 1), only *blr3208* and *blr4511,* amplified for most of the strains (Table 2), were selected for the next assays. The *bll5893* and the *bll7921* genes amplified for a few strains (Table 2) and were not selected for further assays. Of the 14 strains evaluated, only 12 had their *blr3208* gene and 10 had their *blr4511* gene amplified by PCR (Table 2).

Table 2
PCR amplification of four genes of *Bradyrhizobium* spp. strains

Strains	Genes			
	blr3208	*blr4511*	*bll5893*	*bll7921*
CPAC7	-	-	-	-
S204	+	+	-	-
S340	+	+	-	-
S370	+	+	+	-
S372	+	+	-	-
S406	+	-	+	-
S452	+	+	-	-
S468	+	+	-	-
S478	+	+	-	-
S490	+	+	-	-
S516	+	+	-	+
SEMIA586	+	-	-	-
USDA110	+	+	+	+
USDA6	-	-	-	-

* "+" and "-" indicates with or without amplification, respectively, for each gene in the respective strain.

Only the strains in which *blr3208* and *blr4511* genes were amplified were subjected to PCR-RFLP analysis with the *Acc*I endonuclease and no polymorphism was identified. Some *Acc*I restriction profile examples of *blr4511* gene (Figure 1A) and *blr3208* gene (Figure 1B) are shown.

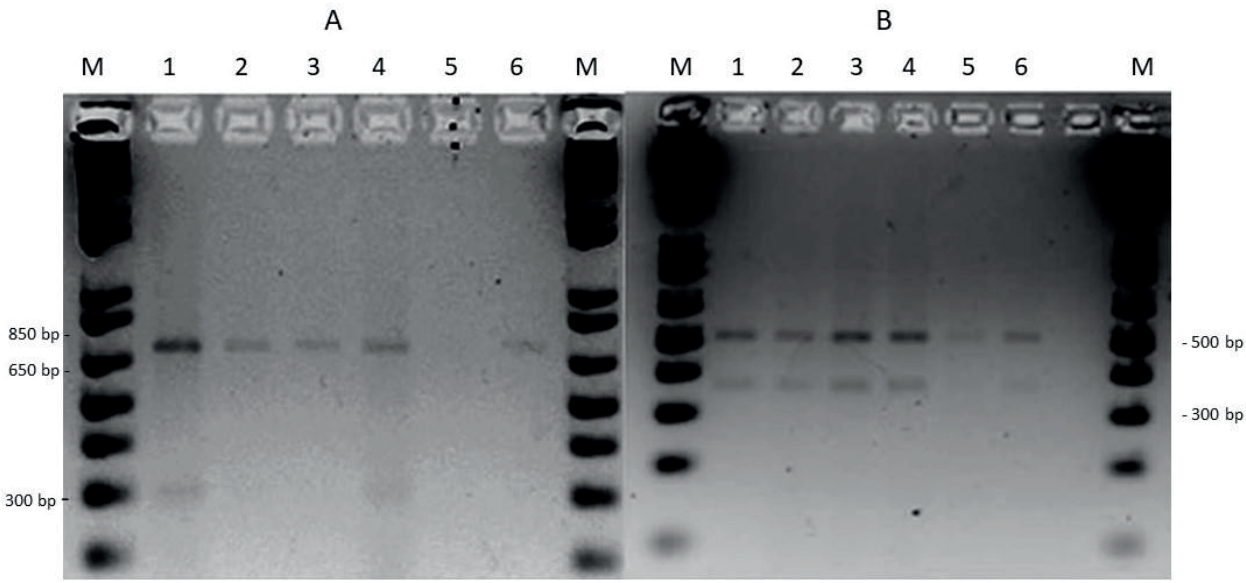

Figure 1. Restriction profiles of *blr4511* (A) and *blr3208* (B) genes with *Acc*I endonuclease after electrophoresis in 3% agarose gel. (M) Molecular mass marker (1 kb plus DNA ladder). (A) 1-6 strains: USDA110, S340, S468, S478, and S452; (B) 1-6 strains: USDA110, S516, S452, S204, S372, and S406.

Bradyrhizobium spp. strains S204, S340, S370, S372, S468, S478, S516, SEMIA586, and USDA110 had the *blr3208* and/or *blr4511* genes sequenced and only the gene sequences with quality were deposited in the GenBank (Table 3).

Table 3
Sequenced amplicons of *blr3208* and *bl4511* genes of *Bradyrhizobium* spp. strains with the respective GenBank access number

Strain	GenBank access number	
	blr3208	*blr4511*
S204	-	MH392304
S340	MH392310	MH392305
S370	MH392311	MH392306
S372	MH392312	MH392307
S468	MH392313	-
S478	MH392314	-
S516	-	MH392308
SEMIA586	MH392315	-
USDA110	MH392309	-

The comparison among nucleotide sequences by multiple alignment analysis (Figures 2 and 3) showed the presence of preserved internal regions of these genes, confirming the results of the restriction analysis that showed no polymorphism (Figure 1A and 1B).

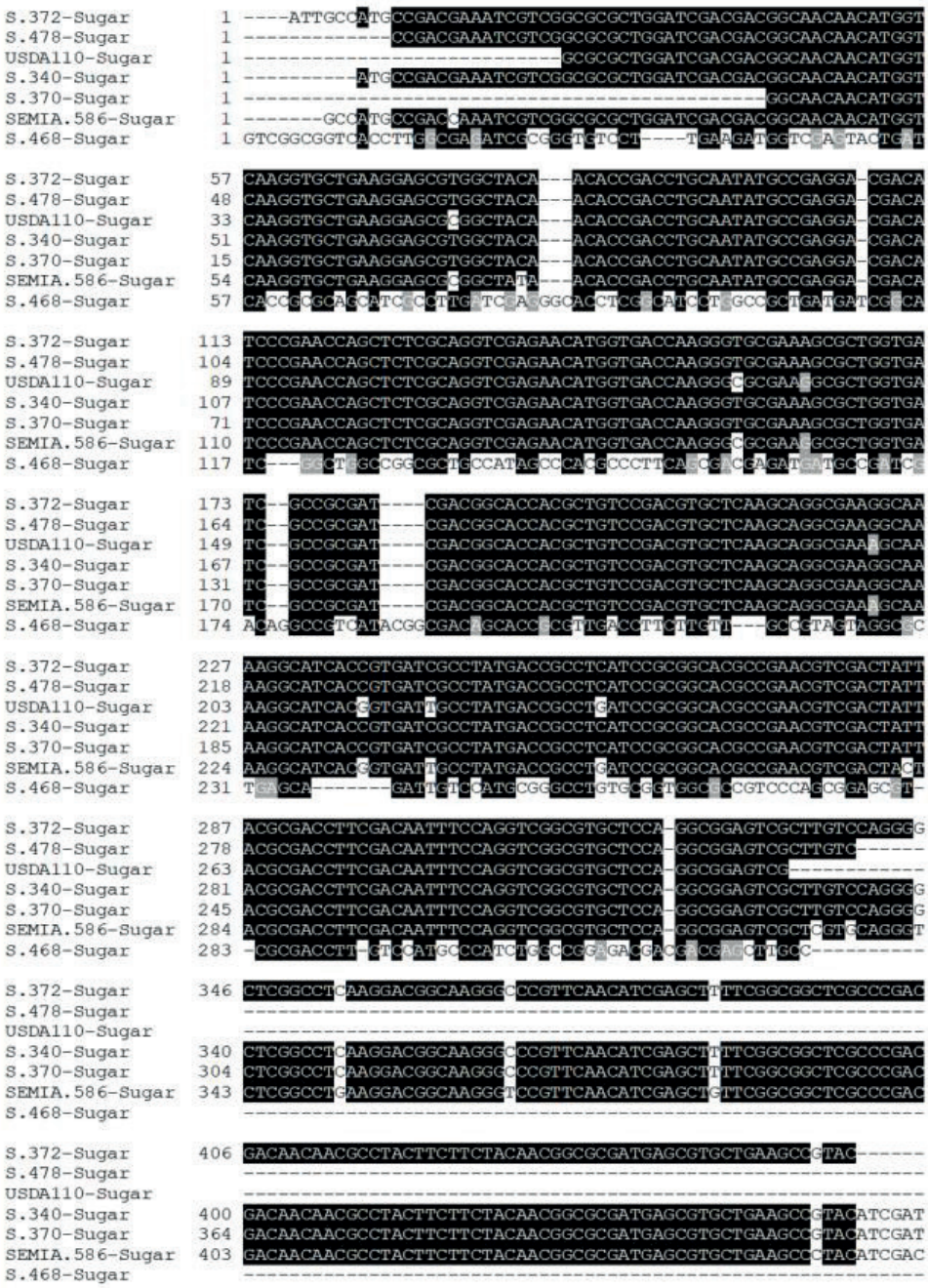

Figure 2. Sequence of multiple alignment of *blr3208* gene (sugar transporter) performed with ClustalW software (https://www.genome.jp/tools-bin/clustalw) for S340, S370, S372, S468, S478, SEMIA586, and USDA110 strains.

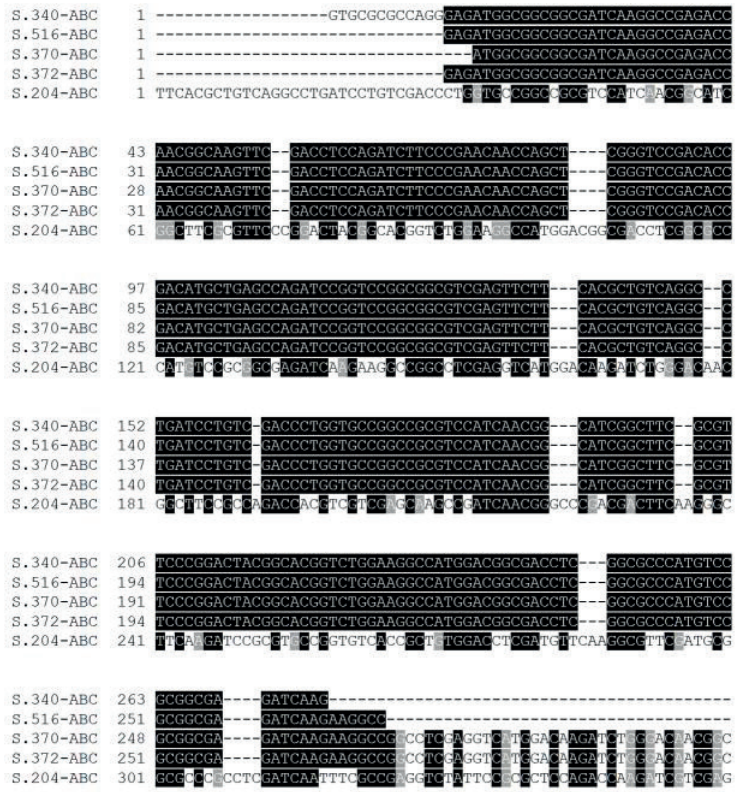

Figure 3. Sequence of multiple alignment of *blr4511* gene (TRAP transporter) performed with ClustalW program (https://www.genome.jp/tools-bin/clustalw) for S204, S340, S370, S372, and S516 strains.

The comparison of the sequence of *blr3208* gene from USDA110 with sequences of strains deposited in the GenBank database (NCBI) with BLAST program (http://blast.ncbi.nlm.nih.gov) is shown in Table 4. Similarity over 90% among sequences of different species and strains of the genus *Bradyrhizobium* (Table 4) was observed. Based on the similarity analysis of the *blr4511* gene with sequences deposited in the GenBank database (NCBI), a similarity over 85% was found with gene sequences of the genus *Bradyrhizobium* (Table 5), known as DctP, which coded a group of proteins named transporter substrate-binding protein (TRAP), more specifically "TRAP-type C4-dicarboxylate transport system, periplasmic component" that transport C4-dicarboxylate acid, as succinate.

Table 4
Similarity among nucleotide sequences of *blr3208* gene from *Bradyrhizobium japonicum* strain USDA110 (also *Bradyrhizobium diazoefficiens*) and sequences of strains deposited in the Genbank database, using the BLAST (NCBI) software with the ten most similar results

Strain	Query cover (%)	E-value	Identity (%)
Bradyrhizobium diazoefficiens strain XF7	100	0.0	100
Bradyrhizobium diazoefficiens strain USDA 122	100	0.0	99.4
Bradyrhizobium diazoefficiens strain NK6	99	0.0	99.4
Bradyrhizobium sp. strain G22	100	0.0	95.2
Bradyrhizobium japonicum strain E109	100	0.0	95.2
Bradyrhizobium japonicum strain USDA 6	100	0.0	95.2
Bradyrhizobium japonicum strain J5	100	0.0	95.1
Bradyrhizobium japonicum strain SEMIA 5079	100	0.0	94.7
Bradyrhizobium sp. strain BF49	100	0.0	94.6
Bradyrhizobium ottawaense strain OO99	99	0.0	94.5

Table 5
Similarity among nucleotide sequences of *blr4511* gene from *Bradyrhizobium japonicum* strain USDA110 (also *Bradyrhizobium diazoefficiens*) and strains deposited in the Genbank database, using the BLAST (NCBI) program with the first ten results obtained

Strain	Query cover (%)	E-value	Identity (%)
Bradyrhizobium diazoefficiens strain XF7	100	0.0	99.90
Bradyrhizobium diazoefficiens strain USDA 122	100	0.0	99.80
Bradyrhizobium diazoefficiens strain NK6	94	0.0	98.45
Bradyrhizobium sp. strain G22	100	0.0	94.80
Bradyrhizobium sp. strain CCBAU 51778	100	0.0	94.41
Bradyrhizobium japonicum strain SEMIA 5079	100	0.0	94.41
Bradyrhizobium japonicum strain J5	100	0.0	94.31
Bradyrhizobium sp. strain ORS 3257	100	0.0	94.39
Bradyrhizobium japonicum strain E109	100	0.0	94.22
Bradyrhizobium japonicum strain USDA 6	100	0.0	94.22

Originally identified in the genome (chromosome DNA) of *Rhizobium* (*Agrobacterium*) *tumefaciens* (Smith and Townsend) Conn (Kemner, Liang, & Nester, 1997), renamed to *Rhizobium radiobacter* (Beijerinck and van Delden) (Young, Kuykendall, Martínez-Romero, Kerr, & Sawada, 2001), the *blr3208* gene encodes a binding protein (ChvE protein) which is part of the ABC-type protein complex, responsible for detection of sugars (monosaccharides) released by plant roots (Kemner et al., 1997; Brencic & Winans, 2005; Eitinger, Rodionov, Grote, & Schneider, 2011). The ChvE protein, chromosomally encoded is located in the periplasmic space and participates in chemotaxis and virulence gene induction in response to monosaccharides occurring in the plant wound environment (Kemner et al., 1997; Hu, Zhao, DeGrado, & Binns, 2013; Nester, 2015). This ABC-type protein complex participates in the bacterial chemotaxis process and interacts with a membrane protein, VirA, activating the virulence process of the bacteria (Kemner et al., 1997; Guo, Huang, & Yang, 2017).

The sequence comparison analysis of *blr3208* gene from USDA110 with sequences deposited at the GenBank database (NCBI) found a great

level of similarity with different sequences of *Bradyrhizobium* spp. and other genera. The high level of similarity found for *blr3208* gene among the different rhizobial strains showed a high level of gene conservation that can be due to a possible adaptive function of ChvE protein in the infection and/or symbiosis process of rhizobia with host plants during the evolution process.

The presence of highly-conserved internal regions and the absence of polymorphism in genes related to BNF efficiency, as *nopP* and *aapP* genes, suggest their potential functions in plant-microbe interactions, as previously reported by Perin et al. (2018) for the same *B. japonicum* strains assessed in this study. The *nopP* gene codifies a secretion protein related to the infection process, the nodulation outer protein P, and the *aapP* gene codifies one of the proteins that compose an amino acid transport system located in the plasmatic membrane. The authors also related the high conservation of these genes to adaptive functions.

Similar to observed for the *blr3208* gene, the sequence comparison analysis of *blr4511* gene of USDA110 strain with sequences deposited in the GenBank database showed a high similarity of gene sequences with other rhizobia. Originally identified in USDA110 strain, the *blr4511* gene was characterized as a hypothetical gene in the functional category of "transport and binding proteins", and coded as a protein of "ABC-transporter substrate-binding" group (https://www.genome.jp/dbget-bin/www_bget?bja:blr4511). Similarity could also be observed with gene sequences known as DctP of *Bradyrhizobium* spp., which coded a group of proteins named TRAP, more specifically "TRAP-type C4-dicarboxylate transport system, periplasmic component". These transporters carry C4-dicarboxylate acid, as succinate, helping the BNF process (Yurgel & Kahn, 2004; Geddes & Oresnik, 2016). Therefore, the similarity identified for the *blr4511* gene among rhizobia may be due to the conservation during the evolution of this function by the transporter protein encoded by this gene. Just like observed for the *blr3208* gene, the evolutionary conservation of *blr4511* gene explains the difficulty to identify polymorphisms. The non-identification of polymorphisms of *blr3208* and *blr4511* genes by PCR-RFLP technique and sequence alignment analysis among the studied strains is probably due to the high level of preservation of these genes in *Bradyrhizobium* spp.

Conclusion

The characterization of genes involved in soybean BNF helps to understand this process. From the four genes related to BNF such as *blr4511*, *bll7921*, *bll5893*, and *blr3208*, characterized in contrasting *B. japonicum* strains regarding BNF efficiency, only *blr3208* and *blr4511* genes amplified for most of the strains. The *blr3208* and *blr4511* genes involved in soybean BNF showed to be highly conserved among *B. japonicum* strains and the conservation of these genes may be related to the adaptive function during the evolutionary process.

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