Sudan Journal of Science and Technology
Journal homepage:
http://jst.sustech.edu/

Evaluation of Multi-locus Sequence Analysis of different Genes in Rhizobium-Legume incompatibility

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ARTICLE INFO

ARTICLE HISTORY
Received:12/11/2019
Accepted: 23/12/2019
Available online:June2020

KEYWORDS:
inoculation; inoculant; phylogeny; sequence; TAL169

ABSTRACT

This study was performed to characterize TAL169 bacteria which is used widely as bio-inoculants through sequencing of 16SrRNA, nifH, nodC, recA, and glnII genes. DNA of the bacteria was extracted, PCR amplification of different genes and sequencing were done. The results indicated that TAL169 is *Mesorhizobium* with 99% identity according to 16SrRNA, recA and glnII genes sequences analysis. At the same time, nifH gene analysis indicated that it is *Sinorhizobium* with the same identity. The phylogeny analysis of all genes placed TAL169 bacterium in separate categories. The study concluded that there is no genetic relationship between TAL169 and *Bradyrhizobium* which used in inoculation trials in different regions in the world and was named as TAL169. That is why the majority of previous studies findings used this bacterium as *Baradyrhizobium* in bio-inoculation recorded no significant differences in the production of legumes inoculated. So using of this bacterium is a useless process. Accordingly, for successful bio-inoculation and production improvement in agriculture, before preparation of the bacterial inoculants identification of locally isolated Rhizobia by sequencing and analyzing of the above-mentioned genes or more housekeeping genes is recommended.

1ntroduction:

Using chemical fertilizers to improve agricultural yield results in harmful effects on the environment in addition to the high cost. This leads scientists to think about using Rhizobial inoculants as an alternative way to maintain and improve soil fertility and increase yield. However, there are many studies used commercial inoculants incompatible to the leguminous plant such as TAL169 which leads to failure in bio-inoculation trials. This necessitates using

molecular techniques for characterization of the bio-inoculants before using in the bio-fertilization process. Bio-fertilization is an application of nitrogen-fixing bacteria to convert atmospheric nitrogen gas to nitrate absorbed by the leguminous plant and increase soil fertility. This process is used widely in the world by using commercial inoculants prepared by Rhizobia isolated from different legumes. To isolate the bacteria for inoculants preparation, classical isolation methods are used like spread plate, Gram stain, biochemical tests. These methods require time, affected by some environmental factors and there are some nitrogen-fixing bacteria like *Klebsiella* share the same biochemical characters with Rhizobia which lead to wrong characterization and misclassification. Now, it is possible to classify bacteria by using techniques which determine both phenotypic and genotypic characteristics like 16S rRNA gene sequencing and genomic DNA-DNA hybridization (Stackebrandt and Goebel, 1994). 16S rRNA gene sequencing became the most suitable practice to identify and to draw the phylogenetic relationship for all microorganisms in the environment. There are many reasons for using 16SrRNA gene for identification purpose, one of the reasons is presence of this gene in all organisms with the same function, the second reason is that gene sequence is conserved sufficiently, and the last reason is "around 1500 bp of sequence size which is relatively easy to sequence and large enough to contain sufficient information for identification and analysis of phylogeny" (Clarridge, 2004). "16SrRNA gene sequence analysis has demonstrated high resolving power for measuring the degree of relation between organisms above the species level" but " comparative studies clearly revealed the limitations of the sequence analysis of this conserved gene and gene product in the determination of relationships at the strain level " (Stackebrandt and Goebel, 1994). Despite this, many studies recorded that different bacterial species revealed "identical or nearly identical 16S rRNA gene sequences". So the identity of the 16SrRNA gene sequence is not enough to distinguish bacteria below the species level (Fox et al., 1992; Jaspers and Overmann, 2004; Sullivan et al., 1996). Also, it is impossible to identify microbes adequately by using techniques targeting a single gene only like 16S rRNA gene (Jany and Barbier, 2008). Besides that, there is another problem for using 16S rRNA to identify bacteria that is the gene sequence is heterogeneous due to the presence of multiple *rrn* operons within one genome (Acinas et al., 2004). Alternatives technique to 16S rRNA is required for bacterial species identification like multi-locus sequence analysis of housekeeping genes (MLSA). This approach is superior to 16SrRNA genes in species identification and "distinguished by a higher degree of sequence divergence" (Martens, 2008). Because house-keeping genes sequences variation occur slowly during the evaluation process (Das et al., 2014).The genes used by MLSA should be characterized by ubiquity single copy, and not subjected to recombination. MLSA technique "can give sequence clusters at a wide range of taxonomic levels" (Gevers et al., 2005). There are some criteria used by ad hoc committee for the re-evaluation of the species definition. These include “sequencing of a minimum of five well-chosen housekeeping genes, universally distributed, present as single copies and located at distinct chromosomal loci, as a method of great promise for prokaryotic systematic" (Stackebrandt et al., 2002).

Since NifTAL project completed many inoculation trials achieved around the world in which TAL 169 bacterium was used as inoculants with different leguminous plants e.g Somasegaran et al. (1989), in Sudan Elsheikh and Ibrahim (1999) and Ibrahim et al. (2011). Furthermore, there are commercial biofertilizers composed of this bacterium available across the world such as Biozote used by Asad *et al.* (2004). All these studies used this bacterium in the inoculation as *Bradyrhizobium sp*. The question now is that, is TAL169 bacterium is *Bradyrhizobium*? and why

most inoculation trials in which this bacteria used as inoculants were failed to increase legumes yield?
Thus, this study was conducted to answer the above questions through molecular characterization and phylogeny analysis of TAL169 bacterium through amplification and sequencing of 16SrRNA, nifH, nodC and two house-keeping genes (recA and glnII).

Materials and Methods:

Bacterial Strain and DNA Extraction

The bacterial strain (DSM 2729) was purchased from German culture collections. Genomic DNA was isolated by using a modified method of Dhaese *et al*. (1979). Strain of the bacteria was grown in AG (Arabinose Gluconate) medium in incubator shaker (150 rev/minutes) at 28°C for 2 days. About 20 ml of the bacterial culture were collected by centrifugation. The centrifuge was washed with TE buffer (10 mM tris, 1 mM EDTA, pH 8), re-suspended in 300 µl TE buffer. Then 100 µl of 5% SDS (Sodium dodecyl sulfate**)** and 100 µl pronase E were added, mixed and the solution was incubated overnight. After that, the DNA was thoroughly sheared using a syringe. Purification of the DNA was done by two extractions with 300 µl of Tris-buffered phenol and one extraction with methylene chloride. DNA was precipitated with 2.5 volumes of ethanol. The quality and quantity of the DNA were measured by a NanoDrop ND-1000 device (Spectrophotometer, USA) and agarose gel electrophoresis stained with ethidium bromide, using a marker ladder as reference.

Amplification and Sequencing of the Different Genes

Primers used for amplification of 16SrRNA gene were 16Sa (5`-CGCTGGCGGCAGGCTTAACA-3`) and R16Sb (5`-CCAGCCGCAGGTTCCCCT-3`) forward and reverse primers, respectively (van Berkum and Fuhrmann (2000). Primers used for amplification of nifH, recA, glnII and nodC genes were designed *in silico* using Gemi – primer design software developed by Sobhy and Colson (2012).). These latter primers and PCR conditions were illustrated in Tables (1) and (2), respectively. For amplification of 16SrRNA the PCR reaction was set as 5 µl of 10x *pfu* buffer, 1 µl dNTPs, 1 µl forward primer, 1 µl reverse primer, 1 µl template DNA, 1 µl *pfu* DNA polymerase. Then 1.5 µl DMSO (Dimethyl sulfoxide) and 38.5 µl double distilled water were added. The PCR reaction for amplification of nifH, recA, glnII and nodC was 5 µl of 10x Dream *Taq* buffer, 1 µl dNTPs, 1 µl forward primer, 1 µl reverse primer, 1 µl template DNA, 0.25 µl Dream *Taq* DNA polymerase, 1.5 µl DMSO and 39.25 µl double distilled water. The same PCR reaction was done for nodC except 2.5 µl DMSO and 38.25 µl double distilled water were added in state of 1.5 and 39.25 µl DMSO and double distilled water added to the other genes, respectively.

PCR products for all genes were purified by MEGAquick-spin Total fragment DNA purification kit according to manufacturer instructions. For visualization PCR products were separated on 1% agarose gel and stained with ethidium bromide, using a marker ladder as reference. All experiments were done in Molecular Genetics Lab, Institute of Genetics, Faculty of Sciences, Technische Universität Dresden, Germany, and sequencing was done by GATC Biotech (Konstanz, Germany).

Table (1) Primers used for amplification of nifH, recA, glnII and nodC genes

Genes	Primers
nifH	MnifHAI1f (5`-ATCGGCAAGTCCACCACCTCYCAAA-3`) MnifHAI1r (5`-AGCAGCATGTCCTCGAGCTCCTCCA-3`)
recA	MrecAAI1f (5`-TACATGATGTCGAACTCGACCACCT-3`) MrecAAI2r(5`CATTGACGACCAAGGGGTGATGTAT-3`)
glnII	MglnIIAI1f (5`-CGTTYRGTGTTCGGTGAWCAAGCAA-3`) MglnIIAI2 r (5`-TCGTGCTTGCCGGTCARRCGCTTGT-3`)
nodC	MnodCA12f(5`-TTTGCCWCAGCCAGTACKGTTGCCA-3`) MnodCA12r(5`-AGTTGGCGCGCACGAAACGCTGCCA-3`)

Table (2) PCR Reactions for amplification of the different genes

Genes	Conditions
16SrRNA	An initial denaturation at 95°C for 3 minutes, 35 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30seconds, extension at 72°C for 2 minutes and a final extension at 72°C for 15 minutes.
nifH	An initial denaturation at 95°C for 3 minutes, 35 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute, extension at 72°C for 5 seconds and a final extension at 72°C for 5 minutes.
recA	An initial denaturation at 95°C for 3 minutes, 35 cycles of denaturation at 95°C for 1 minute, annealing at 50°C for 1 minute, extension at 72°C for 2 minutes and a final extension at 72°C for 5 minutes.
glnII	An initial denaturation at 95°C for 3 minutes, 35 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute, extension at 72°C for 45 seconds and a final extension at 72°C for 5 minutes.
nodC	An initial denaturation at 95°C for 3 minutes, 35 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute, extension at 72°C for 45 seconds and a final extension at 72°C for 5 minutes.

Data Analysis:

Algorithm BLASTN was used for sequence analyses of the different genes (Altschul *et al*., 1997). Phylogeny trees analyses were done online www.phylogeny.fr (Dereeper *et al*., 2008, 2010; Edgar, 2000; Castresana, 2000; Guindon and Gascuel, 2003; Anisimova and Gascuel, 2006; Chevenet et *al*., 2006).

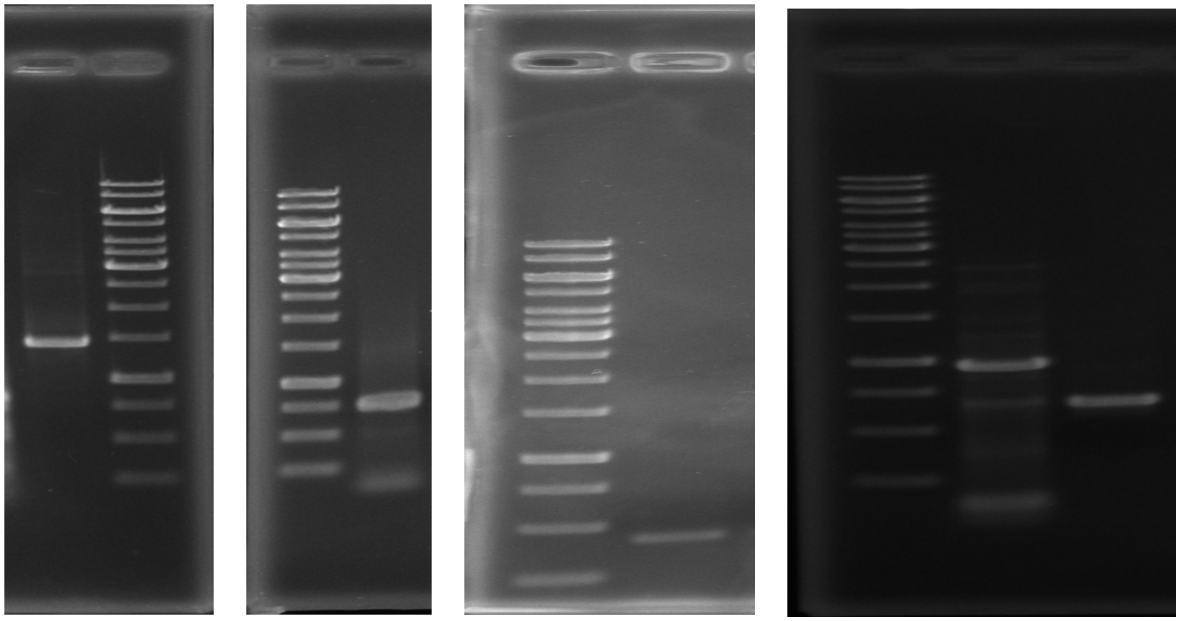
Results and Discussion:

Table (3) shows PCR product concentrations obtained for the different genes. The identification process was started by 16SrRNA sequencing to assigns the genus which defines the genes and primers to be used for MLSA to identify species (Gevers et al., 2005). About 1343, 750, 629 and 427 bp of 16S rRNA, nifH, recA and glnII were obtained, respectively (Figure 1). The analysis of 16SrRNA sequence revealed that TAL169 is *Mesorhizobium* with identity 99%, like that of recA and glnII sequences analyses. The phylogeny analyses of 16SrRNA, recA and glnII genes placed TAL169 in a separate category other than the known *Mesorhizobium spp*. and *Bradyrhizobium* as shown in Figures (2, 3 and 4). This result is in agreement with other studies

which stated that the sequence analysis of 16S rDNA agreed with that from the recA (Dai et al., 2012 and Ghosh and Roy, 2006). The separation of TAL169 in the phylogeny tree analyses indicates that it is another species of *Mesorhizobium*. Although many studies mentioned to 16SrRNA sequence analysis limitation, "16SrRNA sequence characterization is being widely used in evolutionary, taxonomic, and ecological studies not only to define taxa but also to characterize which taxa are present" (Fox et al., 1992). Besides that, it was stated that "a general identification strategy for new isolates could consist of initial partial sequencing of the 16SrRNA gene for genus-level identification" because of the availability of database (Martens et al., 2008). The importance of recA gene sequence analysis in this study represents in its "good capability to identify and classify strains" (Martens et al., 2008).

Table (3) PCR product concentrations of the different genes of TAL 169 bacteria

Genes	16SrRNA	nifH	recA	glnII	nodC
Concentrations ng/ µl	23.8	24.7	43.6	38.6	29.4



nodC and recA glnII nifH 16SrRNA

Figure 1: PCR product of the different genes of TAL169 bacteria

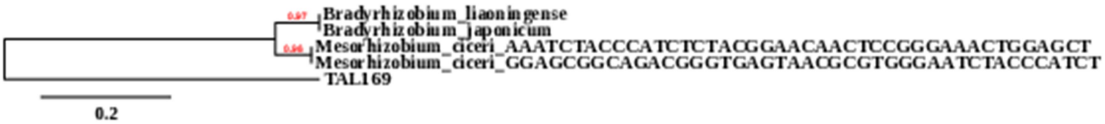


Figure 2: Phylogenetic tree of 16SrRNA gene

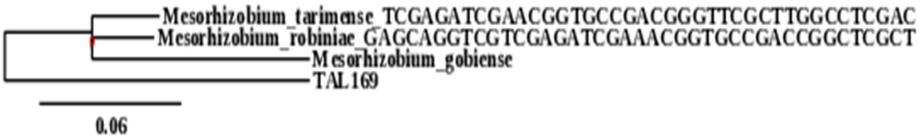


Figure 3: Phylogenetic tree of recA gene

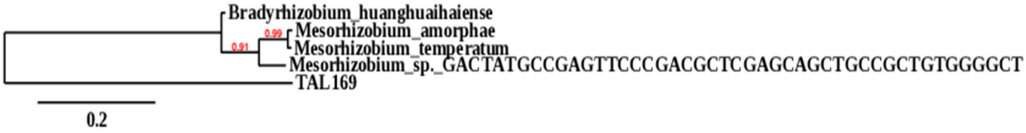


Figure 4: Phylogenetic tree of glnII gene.

Despite the above-mentioned genes proved that TAL169 is *Mesorhizobium*, nifH gene sequence analysis showed it is homology to *Sinorhizobium* with identity of 99% even though the phylogenetic tree analysis of this gene showed that there were genetic differences between TAL169 and the well known *Mesorhizobium* and *Sinorhizobium* spp. (Figure 5). This may be due to that Mesorhizobia evolved according to their geographic distribution, the environmental conditions and host plants (Ji et al., 2015). They were found associated with different plants like chickpea (Zhang et al., 2018) and Sophora root nodules (De Meyer et al., 2016).

The inconsistency between nifH gene analysis results and the other genes may be due to susceptibility of this gene to genetic recombination as it was reported before by Zhang et al., (2008), they isolated rhizobia from *Vigna unguiculata* and *Vigna radiata*, their results showed that 90% of the analyzed strains belonged to or were related to different *Bradyrhizobium and Rhizobium spp.* including *Sinorhizobium fredii*. Their justification of this result is that the symbiotic genes were obtained by vertical transfer from other rhizobia (Zhang et al., 2008; Mousavi et al., 2016).

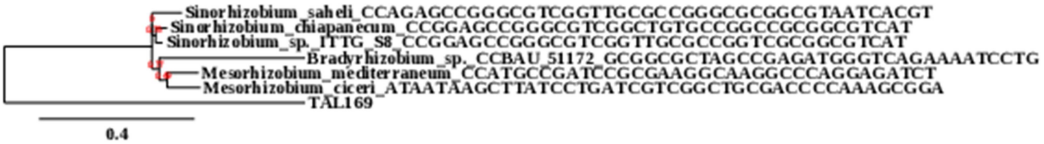


Figure 5: Phylogenetic tree of nifH gene

It is important to mention that, in this study, although sequences for nodC gene were obtained after many trials using different primers, the sequence analysis of this gene revealed that it is unknown bacterium. This supports the claim that TAL169 is another species of *Mesorhizobium* and justifies the inconsistency occurred between nifH gene sequences and the other genes. At the same time it indicates that isolation and amplification of this gene is not easy. These findings verified by that "*nodA* genes of *Mesorhizobium* had different evolutionary histories from their counterparts in other parts of the globe" (Dludlu et al., 2018).

Nod genes are responsible for host specificity and unclearness of genetic information of these genes make understanding of nodulation process in this bacterium so difficult. Thus, more studies on nod genes in this bacterium were required.

It is well known that TAL169 was isolated by NifTAL Project, Paia, Hawaii, USA from *Vigna unguiculata* (Somasegaran et al., 1989). Many studies reported that this plant was infected by different Rhizobia genus and species according to 16SrRNA gene sequence phylogeny.

However, this bacterium has been used as commercial inoculants in different countries around the world as *Bradyrhizobium sp*. with different leguminous plants, and accordingly, there are many studies achieved using this bacterium as reference strain. The inoculation results in some improvement in legumes yield in some studies and failed in others. For example Ibrahim et al., (2011) inoculated two locally isolated strains of *Bradyrhizobium* sp. (ENRRI 16A and ENRRI 16C) provided by Biofertilizers Department, Environment and Natural Resources Institute, National Centre for Research, Khartoum, Sudan, in addition to another two introduced strain of *Bradyrhizobium* sp. (TAL 169 and TAL 1371) offered by NifTAL Project, Paia, Hawaii, USA.

Their results showed significant differences in some parameters. Also, Ibrahim *et al*. (2010) reported that inoculation with the locally isolated strains (ENRRI 16A and ENRRI 16C), as well as the introduced strains (TAL 169 and TAL 1371) of *Bradyrhizobium*, failed to make a significant increase in the mean phosphorus content of guar seeds. *"Bradyrhizobium* strains TAL 169 and TAL 1371 (introduced) and strains ENRRI 16A and ENRRI 16C (local) were used to inoculate five guar cultivars, the locally-isolated strains affected these parameters more than the introduced ones" (Elsheikh and Ibrahim, 1999). Dekunda (1993) inoculated six leguminous trees with *Bradyrhizobium japonicum* TAL169, the result was no respond to inoculation as it was stated. Also, Somasegaran et al. (1989) inoculated *Voandzeia* cultivar No. 12 P.P. Rust in combination with *Bradyrhizobium sp.* TAL 169 showed high potential for growth, nodulation, and nitrogen fixation. In the above inoculation trials, it is noticed that inoculations were done for plants other than the plant from which TAL 169 was isolated. The inoculants were named as *Bradyrhizobium* strain TAL 169. More than that there are many commercial bio-fertilizers manufactured using TAL 169 like Biozote, bio-fertilizer manufactured by Pakistan Agricultural Research Council (PARC) Islamabad. It is composed of living bacteria TAL169 in the carrier material. "Biozote treatment resulted in yield that was slightly lower than Biopower, but was at par with all other treatment" (Asad *et al.*, 2004).

The reason for the failure of some previous studies to improve yield was using incompatible bacteria (TAL169) with different incompatible legumes. So in the future for better inoculation outcome, it is better to identify the inoculants through housekeeping genes sequencing and insure that the inoculants are suitable to the target leguminous plants. This will save time, money and will give assured inoculation results unless other factors play a role and gave converse results.

In conclusion, TAL169 is genetically unrelated to neither known *Mesorhizobium* nor *Bradyrhizobium*, it is also not related to the known *Sinorhizobium*. So using this bacterium as inoculants is useless. Thus, before bio-inoculums preparation, molecular characterization and identification have to be achieved at least by sequencing of 16SrRNA, recA, and glnII or other house-keeping genes to prepare inoculants, to achieve successful inoculation process, and successful compatibility of the bio-inoculants and the host plant which in turn results in production and productivity improvement in the field.

Acknowledgements:

The authors acknowledge Prof. Dr. Michael Gottfrt and his group in Molecular Genetics Lab, Institute of Genetics, Faculty of Sciences, Technische Universität Dresden, Germany for bacterial strain purchasing and providing space and work materials.

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