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Characterization of the common nodulation genes of the photosynthetic *Bradyrhizobium* sp. ORS285 reveals the presence of a new insertion sequence upstream of *nodA*

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Abstract

We isolated and characterized *nodA* genes from photosynthetic and non-photosynthetic rhizobia nodulating the legume genus *Aeschynomene*, and found that the *nodA* sequence from photosynthetic stem-nodulating bacteria was phylogenetically distant from the other already described *nodA* genes. Characterization of the photosynthetic strain ORS285 common *nod* gene cluster (*nodABC*) showed, upstream of *nodA*, the presence of a new insertion sequence element belonging to the IS3 family and specific to a group of photosynthetic strains from *Aeschynomene*.

Keywords: Nodulation gene; Photosynthetic *Bradyrhizobium*; nodA; Insertion sequence; *Aeschynomene*

1. Introduction

Rhizobia form nitrogen-fixing nodules on the roots of their leguminous host plants. Some species of the genus *Aeschynomene* have the unusual capacity to develop stem nodules, a property shared only with a very few species of the genera *Sesbania*, *Neptunia* and *Discolobium*. A number of stem isolates from *Aeschynomene* are of special interest because of their ability to develop a photosynthetic system [1], which is a rare trait in rhizobia. Three cross-inoculation groups among *Aeschynomene* species have been described [2]. Group I never forms stem nodules and is nodulated by the typical, broad host range, non-photosynthetic *Bradyrhizobium* strains. The two other groups, II and III, develop stem nodulation. Group III is exclusively nodulated by photosynthetic rhizobia, which are highly specific to that group [3]. Group II is intermediary as it is nodulated by both non-photosynthetic *Bradyrhizobium*, and with photosynthetic strains which are also able to nodulate Group III [3].

In the rhizobium–legume interaction, specificity is mainly controlled by Nod factors recognized by the host plant. All Nod factors are chitin oligomers mono *N*-acylated at the non-reducing end and diversely substituted at both ends of the molecules. These various substitutions, which confer plant-specificity, are encoded by host-specific *nod* genes. The synthesis of the *N*-acylated oligosaccharide core of the Nod factors is controlled by the *nodABC* genes, which are present in all rhizobia. The *nodA* gene, involved in the transfer of an acyl chain to the chitin oligosaccharide backbone Nod factor, has been shown to be a good nodulation marker [4].

In this study, we isolated and characterized the *nodA* genes of different photosynthetic and non-photosynthetic *Aeschynomene* rhizobia, and phylogenetically compared their sequence divergence. We found that the *nodA* gene sequence from photosynthetic bacteria was distant from that of the already described rhizobia and, therefore, we sequenced a 6.5-kb fragment containing the common nodulation gene *nodABC*. Here, we report the presence of a new insertion sequence element (IS) upstream of the *nodA* gene specific to Group II photosynthetic strains.

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2. Materials and methods

2.1. Bacterial strains

All the strains used are reported in Table 1. The strains were cultured on YEM medium [5] at 37°C. All strains were stored at −80°C on YEM medium adjusted to 20% (v/v) glycerol.

2.2. Polymerase chain reaction (PCR) amplification, DNA sequencing and sequence analysis

The *nodA* genes from Group I and II non-photosynthetic *Bradyrhizobium* strains were amplified using the degenerated primers nodAf.brad (5'-GTYCAGTGGAGSSTKCGCTGGG-3')/nodAr.brad (5'-TCACARCTCKGGCCCGTTCCG-3') whereas the *nodA* genes from the photosynthetic strains were amplified using the degenerated primers nodA1f (5'-TGCRGTGGAARNTRBVYTGGGAAA-3')/nodAb1r (5'GGNCCGTCRTCRAASGTCARGTA-3'). These four degenerate primers were defined from conserved motifs of NodA sequences available from the databanks. The primers Isf (5'-AGCCCAGCGACCTATTTTAG-3') and Isr (5'- GCCTCGATGAAAGCATTGTC-3') which amplified a 822-bp fragment inside the IS identified in ORS285 were used to check for the presence of this IS element in the different *Aeschynomene* symbionts.

A touchdown PCR was performed for primer pairs nodAf.brad/nodAr.brad and nodA1f/nodAb1r (annealing temperature 60 to 50°C in 20 cycles) as previously described [6]. For the primer pair Isf/Isr (annealing temperature of 55°C), the standard PCR method was used. PCR products were purified with a QIAquick Gel extraction Kit (Qiagen, France). The ABI Prism BigDye Terminator Cycle sequence Kit (Applied Biosystems, Foster City, CA, USA) was used to directly sequence the purified PCR with the primers used for the amplification. Sequencing reactions were analyzed on an Applied Biosystem model 310 DNA sequencer. An alignment was performed, using the program PILEUP [7], with a set of available NodA sequences. A phylogenetic tree was constructed by the neighbor-joining method [8], and a bootstrap confidence analysis was performed on 1000 replicates to determine the reliability of the tree topology so obtained [9].

2.3. Construction and screening of a genomic library of Bradyrhizobium ORS285

Total DNA of *Bradyrhizobium* strain ORS285 was subjected to a partial digestion with *Sau*3AI and dephosphorylated by alkaline phosphatase treatment. Fragments were then ligated to SuperCos I *Xba*I/*Bam*HI arms (Stratagene, La Jolla, CA, USA) as instructed by the manufacturer. Ligated DNA was packaged by using the Gigapack III Gold packaging extract (Stratagene). Screening was performed by PCR using the primers nodA.sp.285.f (5'-ACGCGCTCCCGTTCATGTCG-3')/nodA.sp.285r (5'-GAAATACAAGCACCAACGGC-3') which were designed from the *nodA* sequence of ORS285.

2.4. Nucleotide sequence accession number

The DNA sequences of the common *nod* genes of ORS285 strain have been submitted to the GenBank database under accession number AF284858.

3. Results and discussion

The *nodA* genes of both photosynthetic and non-photosynthetic *Aeschynomene* nodulating rhizobia were first isolated by PCR. By using the degenerated primers nodAf.brad/nodAr.brad, products of the expected size were obtained from all tested non-photosynthetic *Bradyrhizobium* strains isolated from either Group I (e.g. ORS301, ORS302, ORS304, ORS309) or Group II (e.g. ORS336). In contrast, all photosynthetic strains nodulating *Aeschynomene* Group II (e.g. ORS285, ORS287, ORS364) gave a positive amplification signal when using the degenerate primers nodA1f/nodAb1r. In spite of testing numerous primers defined from the consensus region of either NodA, NodB or NodC, we did not succeed in isolating the common *nod* genes from Group III photosynthetic strains. These results indicate a great divergence of the symbiotic genes as compared with the other rhizo-

Table 1
Characteristics of the strains used in this work

Bacterial strain	Original host plant	Group	Photosynthetic character	Reference
ORS301	*Agave americana*	I	−	[2]
ORS302	*A. pfundii*	I	−	[2]
ORS304	*A. elaphroxylon*	I	−	[2]
ORS309	*A. uniflora*	I	−	[3]
ORS285	*A. afraspera*	II	+	[3]
ORS287	*A. afraspera*	II	+	[3]
ORS336	*A. afraspera*	II	−	[3]
ORS364	*Acacia nilotica*	II	+	[3]

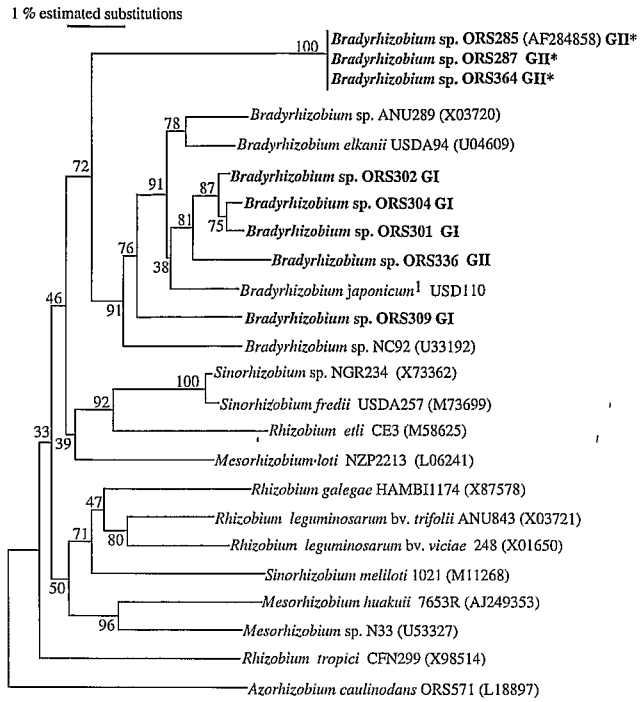

Fig. 1. Phylogenetic tree based on the NodA sequences using the neighbor-joining method. Bootstrap values, expressed as percentages of 1000 replications, are given at the branching points. Numbers, in parentheses, are the accession numbers of the sequences used. The NodA sequence of the *Bradyrhizobia* belonging to Group I and II (presented in bold) are available upon request. The NodA sequence from *B. japonicum* USD110 was kindly provided by Tomasz Stepkowski. The bar represents one estimated substitution per 100 nucleotide positions. *, photosynthetic strain; GI, Group I; GII, Group II.

bia, which could be related to the extreme narrow host-specificity of the Group III photosynthetic strains.

Sequence analysis revealed that the *nodA* gene sequences from Groups I and II non-photosynthetic *Bradyrhizobium*-tested strains are polymorphs with a percentage of similarity ranging from 73.78 to 96.62% at the DNA level. In contrast, the *nodA* gene sequences of the Group II photosynthetic strains were found to be 100% identical, demonstrating that this symbiotic gene is very well conserved between these photosynthetic *Bradyrhizobium*. Phylogenetic NodA sequence analysis between the different rhizobia revealed that the *nodA* gene from the Group II photosynthetic strains constituted a new branch, related but distinct from the other *Bradyrhizobium nodA* gene (Fig. 1). Sequence similarity of NodA from those photosynthetic strains with other NodA *Bradyrhizobium* species was low (63%). These data are in full agreement with previous phylogenetic studies, which showed that photosynthetic *Aeschynomene* strains form a homogeneous and distinct cluster [3], for which a new species should be proposed. Conversely, NodA sequences from the non-photosynthetic Group I and II strains intertwined with the other species of *Bradyrhizobium* (Fig. 1), and the sequence similarity for this genus was high, ranging from 80 to 88%, thus confirming their taxonomic position within the *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* branches [3].

The high level of divergence of the NodA sequence of photosynthetic strains led us to characterize their complete common *nodABC* genes. Two specific primers nodA.sp.285.f/nodA.sp.285r based on *nodA* gene sequence were designed for the PCR screening of a DNA genomic library for strain ORS285 used as a model for Group II photosynthetic *Bradyrhizobium*. A positive clone (pSTM75) which contained an insert of approximately 35 kb was used to characterize the common nodulation genes.

A 6.5-kb region in the inserted DNA fragment of the pSTM75 cosmid, showing a positive hybridization signal to the *nodA* gene used as probe, was sequenced and analyzed, as shown in Fig. 2. This nucleotide sequence had four open reading frames (ORFs) encoding proteins with similarity to known Nod proteins (Fig. 2). Based on sequence similarities, three of these ORFs located from position 3992 to 6407 were assigned to *nodA*, *nodB* and *nodC* genes which presented the same classical organization as already found for other rhizobia. The *nodB* sequence showed 62 to 61% homology with the other *Bradyrhizobium nodB* genes available in databanks, and the *nodC* sequence, 63% homology with that of *Rhizobium* sp. strain N33 and 53% homology with the *nodC* gene of *Bradyrhizobium* sp. strain SNU001. The fourth ORF encoding a putative Nod protein was located from position 1746 to 617 and showed 55% amino acid homology with the *nolL* gene from *Rhizobium etli* (Fig. 2). The *nolL* gene has been shown to be responsible for the acetylation of the fucosyl residue in the nodulation factor [10], which indicates that Nod factors of photosynthetic strains are fucosylated, as already found for all *Bradyrhizobium* studied so far [11–12]. Indeed, we have detected by PCR in strain ORS285 (data not shown) a sequence homologous to the *nodZ* gene which encodes a fucosyl transferase.

Interestingly, we did not find a *nodD* regulatory gene upstream of *nodA*, as usually described for most rhizobia. Instead, we found in the 2-kb region located between *nodA* and *nolL*, an unexpected putative insertion element showing homology to IS sequences belonging to the IS3 family [13]. The analysis of this IS sequence revealed the presence of two ORFs, *ORFA* and *ORFB* (Fig. 2). The *ORFA*, starting with an ATG, encoded a putative protein of 88

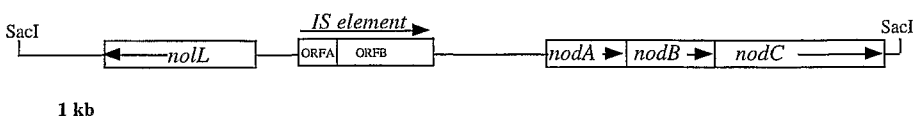

Fig. 2. Physical map of the common nodulation gene region in *Bradyrhizobium* sp. strain ORS285.

amino acids and showed a strong homology to a putative transposase identified in *Yersinia pestis* (59% of identity), the *ORFA* of IS*D*, an insertion element found in *Desulfovibrio vulgaris* (50% of identity) [14], the *ORFA* of IS1222 from *Enterobacter agglomerans* (48% of identity) [15], and also other members of the IS3 family. The *ORFB*, for which the proposed start codon overlapped the stop codon of *ORFA*, encoded a putative protein of 244 amino acids and also showed homology to the same ISs of the IS3 family. The greatest homologies found were 41% identity with the *Yersinia pestis* putative transposase, and 38 and 32% identity with the *ORFBs* of IS1222 [14] and IS*D1* [15], respectively.

The distribution of this IS element among *Aeschynomene* rhizobia was investigated by PCR. All of the photosynthetic strains from Group II, and only those, gave an amplified fragment of the expected size, indicating that this IS element is specific to this group of photosynthetic *Bradyrhizobium*. IS elements have been identified as mobile DNA elements in the genome of a wide range of bacterial genera and species [13]. They have been postulated to play an important role in the evolution and adaptation of bacteria. They have also been identified inside or flanking the symbiotic gene [16–18] where they could play an important role in the generation of nodulation polymorphism and consequently in the evolution of different symbiotic phenotypes [19]. Therefore, we can hypothesize that the transposition events within nodulation genes could have played a role, either by gene rearrangement or gene transfer, in the adaptation of photosynthetic bacteria to *Aeschynomene* stem-nodulation.

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