Molecular studies on a new genetic locus linked to the common nodulation genes in *Bradyrhizobium japonicum*

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Abstract

ORFA, an actively transcribed genetic locus linked to the common nodulation genes in *Bradyrhizobium japonicum* USDA110, was sequenced and analysed. The expression of ORFA is neither dependent on the regulatory proteins NifA, NtrC, NtrB and NodD1 nor on either copy of sigma 54, RpoN1 and RpoN2. The transcriptional start site of ORFA was determined and found to overlap the oppositely transcribed nodZ gene by 224 nucleotides. An appropriately located –10 sequence identical to the consensus proposed for rhizobia and a homologous –35 region were identified upstream of the transcriptional start site. ORFA showed no significant homologies to known sequences in gene databases, and its mutational inactivation had no effect on the nodulation of five legume species. Nevertheless, ORFA seems to be conserved among bradyrhizobia, since an ORFA probe hybridised to total DNA extracted from other *Bradyrhizobium* strains.

Keywords: *Bradyrhizobium*; nod gene; Transcriptional control

1. Introduction

The ability of bacteria from the family Rhizobiaceae to produce nodules on the roots of leguminous plants is controlled by the bacterial nodulation (nod/nol) genes (reviewed in [1]). Recently, the nod gene products were shown to be responsible for the production of lipo-chitinsignal molecules (referred to as Nod signals) that stimulate the early developmental events of nodulation [2–9]. The major Nod signal produced by *Bradyrhizobium japonicum* is a pentamer of N-acetylglucosamine, N-acylated with a C18:1 fatty acid on the terminal non-reducing sugar and substituted with a 2-O-methylfucosyl residue at the C6 of the reducing sugar [2,5].

The common nodulation genes nodABC are indispensable for nodulation and for the production of Nod signals. In *B. japonicum*, the nodABC genes are part of the large operon nodYABCSUINolMNO [10–13]. nolO was shown to be required for efficient nodulation and to be involved in Nod signal biosynthesis [10]. nodZ, a host-specific nodulation gene required for the 2-O-methylfucosylation of the *B. japonicum* Nod signal, is located 0.7 kb downstream to nolO [14].
In this work, we describe the molecular characterisation of a new open reading frame, ORFA, located in the intergenic region between *nolO* and *nodZ*.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

*B. japonicum* strains were aerobically grown at 30°C in RDY or HM medium as previously described [12,15] with the appropriate antibiotics (kanamycin (Km), 150 µg ml⁻¹ and 100 µg ml⁻¹ of spectinomycin (Sp), streptomycin (Sm) and/or tetracycline (Tc)). *E. coli* cells were grown at 37°C in LB medium [16] using the appropriate antibiotics (ampicillin (Ap), 100 µg ml⁻¹; Sp, 100 µg ml⁻¹; Sm, 30 µg ml⁻¹; and/or Tc, 20 µg ml⁻¹). *B. japonicum* strains were anaerobically grown in 12 ml Falcon® tubes that were filled to the top with nitrate medium and sealed with parafilm®. The latter medium is a modified RDY medium with a reduced amount of yeast extract (0.25 g l⁻¹) and 10 mM KNO₃. Expression of the anaerobically induced *nifD–lacZ* fusion [17] was used as a control for the effectiveness of anaerobic conditions.

Plasmid pMGS102 (ORFA–*lacZ*) was constructed by random mutagenesis of pMS95 with Tn3-HoHo1 [18]. Tn3-HoHo1 lacks the promoter region of *lacZ* as well as the sequences coding for the first 7 amino acids of β-galactosidase and therefore produces translational fusions in the genes where they are randomly inserted [18]. Plasmid pMS95 contains a 9.5 kb *EcoRI* fragment that includes *nodZ* and the *nodABC* operon. Mapping the position of the fusion was done by Southern hybridisation. pMGS102 was mated into *B. japonicum* strain USDA110 and in mutant backgrounds (see Section 3 and Table 2).

Table 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Size of <em>EcoRI</em> fragment (kb) hybridising to probe for</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ORFA</td>
</tr>
<tr>
<td>USDA 110</td>
<td>9.5</td>
</tr>
<tr>
<td>USDA 6</td>
<td>4</td>
</tr>
<tr>
<td>USDA 46</td>
<td>4.5</td>
</tr>
<tr>
<td>USDA76</td>
<td>–</td>
</tr>
<tr>
<td>USDA 94</td>
<td>–</td>
</tr>
<tr>
<td>USDA122</td>
<td>4</td>
</tr>
<tr>
<td>USDA123</td>
<td>4</td>
</tr>
<tr>
<td>61A101C</td>
<td>–</td>
</tr>
<tr>
<td>IRC:2133A</td>
<td>–</td>
</tr>
<tr>
<td>AN6A</td>
<td>9.5</td>
</tr>
<tr>
<td>32 H1</td>
<td>9.5</td>
</tr>
<tr>
<td>ANU 289</td>
<td>–</td>
</tr>
</tbody>
</table>

*nodZ* hybridisation data was kindly provided by Maria Schell. None of the *Rhizobium* strains hybridized to the ORFA probe (not included in the table).
Strain SL68 (ORFA mutant) was constructed by inserting a Sp Sm resistance cassette [19] into a unique \textit{Bai}I site within ORFA (Fig. 2). The mutated fragment was cloned into the suicide plasmid pSUP202 [20] and mobilised into strain USDA110. Appropriate conditions were provided for the selection of double recombination events. The mutation in strain SL68 was confirmed by Southern hybridisation.

2.2. DNA sequencing of ORFA

The active Tn3-lacZ translational fusion mapped in the intergenic region between \textit{noI} and \textit{nodZ}, suggested the presence of an additional gene in this area. Therefore, the wild-type DNA region, encompassing the site of fusion insertion, was sequenced in both orientations using the dideoxy-nucleotide chain termination method [21]. Each strand was sequenced using the Sequenase kit (United States Biochemicals, USB) as well as by using the heat-stable \textit{Tag} polymerase (USB). DNA sequence analysis was performed using the GCG package (University of Wisconsin genetics computer group).
Table 2
β-Galactosidase activity (in Miller units) of ORFA–lacZ fusions

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Aerobic</th>
<th>Anaerobic</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSL102</td>
<td>USDA110</td>
<td>123</td>
<td>120</td>
</tr>
<tr>
<td>BSL119</td>
<td>Bj101-NifA−</td>
<td>88</td>
<td>110</td>
</tr>
<tr>
<td>BSL121</td>
<td>Bj27147-NtrC−</td>
<td>137*</td>
<td>nd</td>
</tr>
<tr>
<td>BSL120</td>
<td>Bj263-NtBC</td>
<td>151*</td>
<td>nd</td>
</tr>
<tr>
<td>SL105</td>
<td>Bj-N50-RpoN1−</td>
<td>82</td>
<td>102</td>
</tr>
<tr>
<td>SL106</td>
<td>Bj-N63-RpoN2−</td>
<td>87</td>
<td>93</td>
</tr>
<tr>
<td>SL107</td>
<td>BjN50-97-RpoN1;N2−</td>
<td>73</td>
<td>na</td>
</tr>
<tr>
<td>GS300</td>
<td>USDA110</td>
<td>148</td>
<td>3723</td>
</tr>
</tbody>
</table>

Strains USDA110, Bj2101, Bj27147, Bj3263, Bj-N50, Bj-N63 and BjN50-97 all lacking the ORFA–lacZ fusion had less than 1 U of activity. Cultures were grown in nitrate medium.

Each entry is the average of, at least, two experiments.

Standard deviation for all strains except GS300 (anaerobic) was less than 10 U.

nd: not determined; na: not applicable.

*Grown in RDY.

2.3. Southern blot hybridisation

Inter-strain hybridisation was done according to standard procedures [16] with a 32P-labelled 0.1 kb BglII fragment, internal to ORFA as a hybridisation probe (Fig. 1). Total DNA prepared from Bradyrhizobium (Table 1) and Rhizobium (not shown) strains was cut with EcoRI and blotted from a 0.7% agarose gel. Hybridisation and washing were done under conditions of high stringency (hybridisation at 65°C and a final wash with 0.1×SSC at 65°C). Bradyrhizobium strains USDA100, 6, 46 76 94, 122 and 123 and AN6A were obtained from D. Kuykendall, while strains 61A101C, IRC:2133A, 32H1 and ANU289 were obtained from Nitragen, G. Elkan, P. van Berkum and B. Rolfe, respectively.

2.4. β-Galactosidase assays

β-Galactosidase activity was assayed as previously described [22] with chlorophenol red-β-d-galactopyranoside (CPRG) as the chromogenic substrate. B. japonicum strains carrying the ORFA–lacZ translational fusion were grown in RDY or nitrate medium under aerobic or anaerobic conditions.

2.5. Primer extension

The transcriptional start of ORFA was determined by primer extension as previously described [23]. The following primer, P19-2, was used: 5′-ATGG-GAGCTTCGCGGCGGCTC-3′ (Fig. 2). The primer was labelled with [γ-32P]ATP and polynucleotide kinase (USB), co-precipitated with 50 μg of RNA isolated from aerobically grown B. japonicum USDA110, and then resuspended in the following hybridisation buffer: 20 mM Tris-HCl (pH 8.0), 200 mM NaCl, 0.1 mM EDTA. The hybridisation mixture was denatured at 100°C for 3 min and then incubated at 63°C for 90 min. The mixture was adjusted to a final concentration of 6 mM MgCl2, 6 mM DTT, 0.5 mM dNTP then mixed with 30 U of AMV reverse transcriptase (USB) and incubated for 60 min at 37°C. After the completion of the reaction, RNA was digested with RNAse A, and the newly synthesised DNA was precipitated and run on a 6% acrylamide sequencing gel next to a ladder from a sequencing reaction using the same primer P19-2.

2.6. Plant tests

Plants used for nodulation tests were soybean (Glycine max cv. Essex and G. soja PI468397), siratro (Macroptilium atropurpureum), cowpea (Vigna unguiculata cv. Caloona) and mungbean (Vigna radiata cv. King). Seeds were surface-sterilised, germinated and grown in clear plastic pouches as previously described [24]. Seedlings were inoculated with 10^4 cells ml⁻¹ of either B. japonicum strain USDA100 or SL68 (ORFA−), using 1 ml per seedling. The bacterial concentration of each inoculum used was con-
firmed by direct colony count on RDY plates. Nodules/plant were counted daily from days 7 to 20 after inoculation.

3. Results and discussion

3.1. Sequence of ORFA

An active Tn3-lacZ translational fusion was mapped in the intergenic region between nolO and nodZ and found to have an orientation opposite to that of both genes (Fig. 1). The location of this fusion between two nodulation genes suggested its involvement in the nodulation process. Therefore, the wild-type DNA region, encompassing the site of the fusion insertion, was sequenced, a procedure that revealed a 350 bp open reading frame that was given the name ORFA (Fig. 2, bases 140–490). The most probable start codon is an ATG, which is preceded by a possible ribosomal binding site. This start codon is located 13 bp upstream from that of the divergently transcribed nodZ, while the stop codon of ORFA is located 348 bp downstream from that of nolO (Fig. 2). Use of the ‘TestCode’ program (GCG package, University of Wisconsin genetics computer group) strongly suggested that ORFA represents a coding sequence (data not shown). ORFA was predicted to code for a protein of approximately 12 kDa, the sequence of which, when compared to the database, showed no significant similarity to other known genes or recognisable motifs (e.g., membrane spanning or DNA binding).

3.2. Interstrain hybridisation of an ORFA probe

A 32P-labelled 0.1 kb BgIII fragment internal to ORFA (Fig. 1) hybridised to chromosomal DNA of other Bradyrhizobium strains but not to any of the Rhizobium strains tested (Table 1, Rhizobium strains are not listed in the table). Each of the strains that gave a positive signal had only one hybridising band, indicating that ORFA is likely to be present in a single copy in these strains. Interestingly, in all hybridizing strains, the restriction fragments that hybridised to the ORFA probe had the same sizes as those that hybridised to probes from nolO [10] or from nodZ [14] suggesting a possible linkage between the three genes in other bradyrhizobia (Table 1).

3.3. Transcriptional control of ORFA

The transcriptional control of ORFA by regulatory proteins known to be involved in the Rhizobium symbiosis was investigated. The ORFA–lacZ translational fusion plasmid pMGS102 was mated into B. japonicum strains AN314 (NodD1−; [12]), BJ2101 (NifA−; obtained from Chelm/Adams), BJ2147 (NtrC−), BJ3263 (NtrBC; [25]), BJ-N50 (RpoN1−), BJ-N63 (RpoN2−) and BJ-N50-97 (RpoN1N2−; [26]), and the β-galactosidase activity was assayed both under aerobic and anaerobic conditions. The activity of the ORFA–lacZ fusion was comparable in all tested backgrounds (Table 2), indicating that none of the proteins studied was required for ORFA expression under the conditions tested and consistent with the fact that none of the cis-acting elements associated with these proteins were found within the DNA region upstream of ORFA (Fig. 2). This result is also consistent with the lack of flavonoid inducibility of the ORFA–lacZ fusion and with the fact that NodD1 was not required for its activity (data not shown).

3.4. Transcriptional start and promoter region of ORFA

The transcriptional start site of ORFA was determined by primer extension [23] with P19-2 (Fig. 2) as a primer. The site was located 95 bp upstream of the putative GTG translational start site of ORFA (Fig. 2). Lagares et al. have referred to the following consensus for the −10, −35 sequences in rhizobia: TT(AoRG)ANN, 16–17 bp, (AorG)(AorG), 3–5 bp, CA [27]. The −10, −35 sequences for ORFA matched the latter consensus with the exception of one mismatch in the −35 sequence and the larger spacing between the two motifs (TGCTG, 22 bp, AAGAGA, 3 bp, CA, Fig. 2). The transcriptional start site of nodZ has previously been determined [14] and would indicate a 224 nucleotide overlap between the transcripts of the divergently transcribed genes ORFA and nodZ (Figs. 1 and 2), thus, raising the possibility of a regulatory interaction between the two genes.
3.5. Symbiotic phenotype of an ORFA mutant

The location of ORFA within the major symbiosis cluster on the B. japonicum strain USDA110 chromosome (Fig. 1), and its linkage to nodO and nodZ in other Bradyrhizobium strains (Table 1) suggested its involvement in the nodule formation process. To test this hypothesis, a Sp/Sm resistance cassette [19] was inserted into a BalI site internal to ORFA (Fig. 2) resulting in strain SL68 (ORFA−). The latter BalI site is located immediately upstream to the putative transcriptional start site of nodZ (Fig. 2), which would place the antibiotic resistance cassette within the promoter region of nodZ. Nodulation kinetics assays for five legume species (see Section 2) and using a low concentration inoculum showed no significant differences between strains SL68 and USDA110 (data not shown). It is possible, however, that ORFA may be required for the nodule formation of other hosts or for increased competitiveness under field conditions.

The BalI site used for interrupting ORFA is located immediately upstream to the putative transcriptional start site of nodZ (Fig. 2), which would place the antibiotic resistance cassette within the promoter region of nodZ and would raise the possibility that strain SL68 is actually an ORFA-nodZ double mutant. Curiously, however, strain SL68 is fully capable of nodulating siratro, while a nodZ− mutant is unable to nodulate this host [14]. Nevertheless, it is not unlikely that nodZ may be transcribed from a downstream promoting sequence activated by the omission of its native promoter. This phenomenon of promoter supersession by upstream transcription has previously been reported and referred to by Adhya and Gottesman as ‘promoter occlusion’ [28]. Certainly, the possibility of the ORFA mutation suppressing the nodZ phenotype can not be ruled out.

Acknowledgments

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References

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