Large-Scale Transposon Mutagenesis of Photosynthetic *Bradyrhizobium* Sp. Strain ORS278 Reveals New Genetic Loci Putatively Important for Nod-Independent Symbiosis with *Aeschynomene indica*

Katia Bonaldi,^{1,2} Benjamin Gourion,¹ Joel Fardoux,¹ Laure Hannibal,¹ Fabienne Cartieaux,¹ Marc Boursot,¹ David Vallenet,³ Clémence Chaintreuil,¹ Yves Prin,⁴ Nico Nouwen,¹ and Eric Giraud¹

¹Laboratoire des Symbioses Tropicales et Méditerranéennes, IRD, UMR-IRD/SupAgro/INRA/UM2/CIRAD, F-34398 Montpellier, France; ²UM2, F-34398 Montpellier, France; ³Genoscope, CNRS-UMR 8030, Atelier de Génomique Comparative, F-91057 Evry, France; ⁴Laboratoire des Symbioses Tropicales et Méditerranéennes, CIRAD, UMR-IRD/SupAgro/INRA/UM2/CIRAD, F-34398 Montpellier, France

Submitted 30 October 2009. Accepted 23 February 2010.

Photosynthetic *Bradyrhizobium* strains possess the unusual ability to form nitrogen-fixing nodules on a specific group of legumes in the absence of Nod factors. To obtain insight into the bacterial genes involved in this Nod-independent symbiosis, we screened 15,648 Tn5 mutants of *Bradyrhizobium* sp. strain ORS278 for clones affected in root symbiosis with *Aeschynomene indica*. From the 268 isolated mutants, 120 mutants were altered in nodule development (Ndv⁻) and 148 mutants were found to be deficient in nitrogen fixation (Fix⁻). More than 50% of the Ndv⁻ mutants were found to be altered in purine biosynthesis, strengthening the previous hypothesis of a symbiotic role of a bacterial purine derivative during the Nod-independent symbiosis. The other Ndv⁻ mutants were auxotrophic for pyrimidines and amino acids (leucine, glutamate, and lysine) or impaired in genes encoding proteins of unknown function. The Fix⁻ mutants were found to be affected in a wide variety of cellular processes, including both novel ($n = 56$) and previously identified ($n = 31$) genes important in symbiosis. Among the novel genes identified, several were involved in the Calvin cycle, suggesting that CO₂ fixation could play an important role during this symbiosis.

Nitrogen-fixing symbiosis between rhizobia and legume plants plays an important role in agriculture and natural ecosystems. This interaction typically results in the formation of a specialized root organ, the nodule, which houses the bacteria. The initiation and development of nodules are mediated by an exchange of diffusible signal molecules between both partners that controls the specificity of recognition, the nodule organogenesis, and the infection process (Perret et al. 2000; Jones et al. 2007; Gibson et al. 2008). Initially, flavonoids exuded by the host plant interact with rhizobial NodD regulatory proteins and induce the expression of nodulation genes (*nod*, *nol*, and *noe*). This leads to the synthesis and secretion of rhizobial lipochito-oligosaccharide signal molecules called Nod factors

(NF). The perception of NF by plant kinases containing LysM domains triggers, in turn, a number of early responses in the plant root (ion fluxes, rhythmic calcium oscillations, root hair curling, and cortical cell divisions) that lead to the formation of a nodule primordium and the penetration of bacteria (Geurts et al. 2005; Stacey et al. 2006; Oldroyd and Downie 2008). In general, rhizobia penetrate the plant via a sophisticated infection process involving root hair curling and the formation of a host-derived infection thread that guides the bacteria to the emerging nodule primordium. After release from the infection thread, the rhizobia invade the plant cells, differentiate into bacteroids, and reduce atmospheric nitrogen to ammonia to the benefit of the plant. In exchange, the plant supplies the rhizobia with carbon sources.

Three *nod* genes are essential for the synthesis of the NF core structure: i) *nodC* encodes an *N*-acetyl-glucosaminyltransferase that polymerizes UDP-*N*-acetyl-D-glucosamine into oligosaccharide chains, ii) *nodB* encodes a deacetylase that removes the *N*-acetyl moiety from the nonreducing terminus of these oligosaccharides, and iii) *nodA* encodes an acyltransferase that links an acyl chain to the nonreducing end of the oligosaccharides. In addition to these three canonical *nodABC* genes, each rhizobium species contains several additional nodulation genes (*nod*, *nol*, or *noe*) encoding proteins that modify the basic NF core structure and that are major determinants of the bacterium host specificity. The canonical *nodABC* genes were identified in all characterized rhizobia, including the β -proteobacteria that nodulate legumes (Moulin et al. 2001; Amadou et al. 2008); therefore, it was supposed that NF were absolutely required for the nodule development in legumes. However, it has been recently shown that the genome of two photosynthetic *Bradyrhizobium* strains (BTai1 and ORS278) that elicit nitrogen-fixing nodules on roots and stems of some legumes belonging to the *Aeschynomene* genus do not contain the *nodABC* genes (Giraud et al. 2007). This finding overturned the universality of the NF paradigm and indicated that some rhizobia use an alternative Nod-independent process to establish a symbiotic interaction with a legume plant.

Photosynthetic bradyrhizobia enter *Aeschynomene* spp. via cracks in the epidermis that result from lateral or adventitious root protrusion as described for the symbiotic couple *Sesbania rostrata*/*Azorhizobium caulinodans* (Goormachtig et al. 2004). The formation of infection threads is required to guide Azo-

Corresponding author: Eric Giraud; Telephone: 33 467593783; Fax: 33 467593802; E-mail: eric.giraud@ird.fr

*The e-Xtra logo stands for “electronic extra” and indicates that Figures 1 and 2 appear in color online.

rhizobium caulinodans whereas it is assumed that photosynthetic bradyrhizobia progress intercellularly (Arora 1954). This mode of invasion by “crack-entry” in the absence of infection threads has been well described in *Arachis hypogea* (Chandler 1978) or *Stylosanthes* spp. (Chandler et al. 1982) and is likely to be common among legumes belonging to the Dalbergiaceae and Genisteae tribes (Sprent 2007).

In contrast to the well-characterized NF-dependent symbiosis, little is known about the Nod-independent process. In a previous study, 9,600 Tn5 mutants of *Bradyrhizobium* sp. strain ORS278 were screened for clones affected in the induction of nodules on the roots of *Aeschynomene sensitiva* (Giraud et al. 2007). However, none of the selected mutants was found to be completely deficient in nodulation. Because *Bradyrhizobium* sp. strain ORS278 has a large genome (7.5 Mb), the number of tested mutants might not have been sufficient to cover the whole genome, and essential genes for the Nod-independent nodule organogenesis could have been missed. In addition, during this previous study, mutants that formed nodules with a normal shape but that were nitrogen deficient (Fix⁻) were not considered whereas the later steps of this new symbiotic system could also differ.

This study had two goals: first, extend the knowledge of the bacterial genes involved during the early steps of this Nod-independent symbiosis and, second, identify the bacterial genes involved during the later steps in order to understand the physiology of the bacteria in this new symbiotic context. For this purpose, we screened 15,648 additional Tn5 mutants of strain ORS278 for clones deficient in their ability to induce nodules or to fix nitrogen. With this genome-wide mutational analysis, we were able to identify important metabolite routes and regulatory circuits employed by photosynthetic bradyrhizobia during symbiosis with *Aeschynomene* spp.

RESULTS

Isolation of Tn5 mutants of *Bradyrhizobium* sp. strain ORS278 affected in symbiosis.

To isolate new bacterial genes involved in the Nod-independent nodulation process, a large-scale screening was carried out using 15,648 additional Tn5 mutants of *Bradyrhizobium* sp. strain ORS278. This screening was basically performed in 48 wells plates as described by Giraud and associates (2007) but, because of a lack of seed availability and handling facilities, *A. indica*, a species of the same cross inoculation group as *A. sensitiva*, was used. In this plant culture system, *A. indica* inoculated with the wild-type (WT) strain displayed first nodules after 1 week and the root system harbored 5 to 10 nodules after 3 weeks. The nodules were always located at the emergences of lateral roots; they displayed a spherical shape with a diameter of 1 to 2 mm and exhibited a dark-green color due to the formation of chloroplasts in the nodule parenchyma and cortex upon light exposure of the root system (Fig. 1A). In the first round of screening, 1,992 mutants eliciting nodules with an unusual phenotype in shape, size, or color were selected, of which 268 could be confirmed (Fig. 1A). Among them, 120 were altered in their ability to induce nodules with a normal shape and were termed nodule-development-defective (Ndv⁻) mutants. However, no completely deficient mutant (Nod⁻) could be found because the formation of bumps was observed on a small number of plants, even for the most affected mutants. The 148 other mutants elicited WT-shaped nodules with a yellow color indicative of nitrogen fixation deficiency (Noel et al. 1988) and were termed Fix⁻. Phenotypic characteristics (symbiotic property, growth rate, and nitrogenase activity) of the selected Ndv⁻ and Fix⁻ mutants are displayed in Tables 1 and 2, respectively. For each mutant, additional data, including

pictures of the corresponding nodules and the genomic context of the altered genes, can be found on online (GenoScope MaGe NewNodBrady database). This web resource is integrated into the Rhizoscope MaGe project that offers many connections to other databases or systems, especially the metabolic or genomic databases built for *Rhizobium* spp. (Vallenet et al. 2006).

A significant variation in the severity of the nodulation deficiency was observed in the Ndv⁻ class, ranging from mutants that only induced bumps or very rare pseudonodules on a small fraction of the plants to mutants that induced protuberant pseudonodules on all the plant tested (Fig. 1). Examination of bacterial growth on rich and minimal media indicated that the Ndv⁻ mutants were affected in their general metabolism, except for the mutants in BRADO0213, BRADO4535, and BRADO6039 (Table 1). As expected, none of the plants inoculated with Ndv⁻ mutants fixed nitrogen.

Within the class of Fix⁻ mutants, two types could be distinguished. Type 1 mutants induced only yellow nodules whereas type 2 mutants induced light-green nodules or a mixture of yellow and light-green nodules on the same plants (Fig. 1A). Measurements of nitrogenase activity confirmed that all the mutants initially selected on the basis of a difference in nodule color were actually affected in their ability to fix nitrogen in symbiosis with *A. indica* and, among them, the type I mutants were affected most drastically.

Determination of Tn5 insertion sites.

Tn5 insertion sites in the selected mutants were mapped using a two-stage semidegenerate polymerase chain reaction (PCR) and sequencing of the 5' transposon boundary (Jacobs et al 2003). This approach was successful for 91% of the tested mutants. Although all clones corresponded to different insertion events, for some coding sequences (CDS), multiple Tn5 insertions were found (up to 19 insertions). For the 120 Ndv⁻ mutants, the Tn5 was inserted into 29 different CDS and, for the 148 Fix⁻ mutants, the Tn5 insertion affected 87 different CDS (Tables 1 and 2). These mutants were classified according to the metabolic pathways or cellular processes that were altered. It appeared that Ndv⁻ mutants were mainly affected in the purine and leucine biosynthesis pathways whereas Fix⁻ mutants were affected in a wide variety of cellular processes (nitrogenase activity, energetic and carbon metabolism, gluconeogenesis, transport, CO₂ fixation, regulation, chemotaxis, and so on).

In rhizobial genomes, symbiosis-specific genes are usually clustered on plasmids or in chromosomal genomic regions acquired by horizontal transfer and characterized by a low GC content (Barnett et al. 2001; Kaneko et al. 2002; Young et al. 2006). Mapping of the Tn5 insertions of Ndv⁻ and Fix⁻ mutants on the *Bradyrhizobium* ORS278 genome did not permit identification of a clear hot spot integration site in such regions (Fig. 1B), suggesting the absence of a genomic region specifically dedicated to the symbiotic process. Similarly, no clear region could be identified by omitting the mutants affected in central metabolism in this mapping (data not shown).

DISCUSSION

The largest genetic screen in the rhizobia/legumes field.

In this study, to better understand the molecular basis of the Nod-independent symbiosis between photosynthetic bradyrhizobia and *Aeschynomene* spp. we screened 15,648 Tn5 mutants of strain ORS278 for their inability to induce nodules or to fix nitrogen in association with plants. Considering the 9,600 mutants previously tested on *A. sensitiva*, 25,248 mutants, in total, were analyzed, making this the most extensive

Fig. 1. Screening of the Tn5 mutant library. **A**, Screening strategy used to select symbiotically deficient clones. The plant culture system in microplate, the typical appearance of nodules elicited by the wild-type (WT) strain, and nodule development-defective (Ndv⁻) and nitrogen-deficient (Fix⁻) mutants are shown. Scale bars = 1 mm. White arrowheads show bumps or pseudonodules elicited by the Ndv⁻ mutants. The final number of mutants selected corresponds to the mutants for which the symbiotic deficiency was confirmed for all the plants tested and for which the Tn5 insertion site was mapped. **B**, Genomic localization of the Tn5 insertions of the selected clones. Circles displayed from the outside show i) GC percent deviation (GC window – mean GC) in a 1,000-bp window, ii) Fix⁻ transposon insertions in genes (green), iii) Ndv⁻ transposon insertions in genes (blue), and iv) putative horizontally acquired genomic island (purple) and the nif/fix region (red) (Giraud et al. 2007).

screening of a Tn5 mutant library reported to date for rhizobia. Below, genes identified during our study will be discussed. It is important to keep in mind that they remain candidate genes because the symbiotic defect of the corresponding mutants is not necessarily caused by the observed Tn5 insertion but could result from polar effect or from a secondary unidentified mutation. Therefore, further investigations are necessary to confirm their symbiotic role.

Absence of a complete Nod⁻ mutant in the Tn5 collection.

In all, 120 mutants altered in 29 different CDS were identified as severely impaired in nodulation. Surprisingly, none of them was completely nodulation deficient. This might be due to a high degree of redundancy in the nodulation determinants, either through the presence of homologous genes (more than 20% of ORS278 genes have paralogues) or of different genes that are functionally interchangeable. Alternatively, it is possible that

nodule organogenesis in *A. indica* is not triggered by a specific signal as NF but by a synergistic action of multiple factors that could partially complement each other. Finally, two other hypotheses explaining the absence of the isolation of a strict Nod⁻ mutant should be considered: i) the genes necessary for symbiosis are essential for bacterial survival and ii) key genes necessary to induce nodule organogenesis have been missed in our screen. Although our mutant collection provides almost 4x coverage of all predicted CDS (6,752), we cannot completely exclude the latter hypothesis because a nonrandom distribution of the Tn5 insertions is likely and the chance to disrupt small genes is low (Boyd et al. 1993; Jacobs et al. 2003).

Most of the Ndv⁻ mutants were auxotrophic for nucleotides (purines or pyrimidines) or amino acids (Glu, Leu, and Lys). Two classes of auxotrophs emerged, purine and leucine mutants that represented 54 and 29% of the Ndv⁻ mutants, respectively. Purine and leucine auxotrophs were among the first mu-

Table 1. Phenotypes and Tn5 insertion sites of nodule development-deficient mutants

Functional class, CDS ^b	Collection ^c	Gene annotation ^d	Mutant no. ^e	Growth rate (%) ^a		
				YMm	MM	N ₂ rate ^f
Nucleotides biosynthesis	...	...	64	...	...	...
Purines	...	...	58	...	...	...
BRADO0338	STM6333	<i>purH</i>	14	65	10	ND
BRADO1206	STM6338	<i>purE</i>	3	93	13	ND
BRADO2779	STM5423	<i>purQ</i>	1	83	12	ND
BRADO2785	STM6410	<i>purL</i>	10	85	12	ND
BRADO2830	STM5308	<i>purB</i>	14	94	4	ND
BRADO3165	STM5295	<i>guaB</i>	7	54	12	ND
BRADO3169	STM5326	<i>guaA</i>	2	51	14	ND
BRADO3280	STM5291	<i>purF</i>	1	91	14	ND
BRADO3344	STM5402	<i>purM</i>	1	86	13	ND
BRADO3345	STM6326	<i>purN</i>	2	92	11	ND
BRADO6088	STM6408	<i>purD</i>	3	95	11	ND
Pyrimidines	...	...	6	...	...	...
BRADO4502	STM6358	<i>pyrB</i>	1	63	6	ND
BRADO4503	STM6396	<i>pyrC</i>	4	59	6	ND
BRADO6742	STM5443	<i>pyrD</i>	1	52	4	ND
Amino acids biosynthesis	...	...	45	...	...	...
Leucine	...	...	38	...	...	...
BRADO0353	STM5281	<i>leuB</i>	2	68	11	ND
BRADO0365	STM5333	<i>leuD</i>	7	88	7	ND
BRADO0374	STM5264	<i>leuC</i>	19	66	8	ND
BRADO5528	STM5266	<i>leuA</i>	6	63	5	ND
<i>BRADO6039</i>	STM5360	<i>put. IlvE-like protein</i>	1	86	70	ND
Glutamate	...	...	8	...	...	...
BRADO6238	STM5257	<i>gltB</i>	7	91	3*	ND
BRADO6240	STM5258	<i>gltD</i>	1	95	6*	ND
Lysine	...	...	2	...	...	...
BRADO0158	STM5328	<i>dapB</i>	2	53	54	ND
Others	...	...	3	...	...	...
BRADO0277^g	STM6339	<i>folD</i>	1	74	12	ND
BRADO3272^h	STM6376	<i>put. transcriptional regulatory protein TetR/AcrR family</i>	1	92	13	ND
BRADO4272ⁱ	STM6378	<i>ssuA</i>	1	89	12	ND
Proteins of unknown function	...	...	8	...	...	...
BRADO0213	STM5312	<i>CHP</i>	4	100	78	ND
BRADO2778^j	STM6304	<i>CHP</i>	2	92	38	ND
BRADO4535	STM5302	<i>CHP</i>	1	86	95	ND
BRADO5309	STM6553	<i>CHP</i>	1	78	10	ND

^a Relative growth rate (percentage of wild type [WT]) estimated on rich medium (yeast extract mannitol [YMm]) or minimal medium (MM); * indicates relative growth rate (percentage of WT) estimated on MM supplemented by 15 mM KNO₃ instead of glutamate.

^b CDS names are indicated according to the published genome sequence (Giraud et al. 2007). Coding sequences (CDS) in bold correspond to genes that have not been isolated during the first screening performed on *Aeschynomene sensitiva* and CDS in italic correspond to mutants for which the Tn5 insertion is located upstream of the CDS.

^c Representative mutant strain used to estimate growth and nitrogen fixation rate.

^d Abbreviations: put., putative; CHP, conserved hypothetical protein.

^e Number of independent mutants identified in the corresponding CDS.

^f Fixation rate: relative acetylene reduction activity estimated on whole plants; ND = not detected (percentage of WT).

^g 10-Formyl tetrahydrofolate biosynthesis.

^h Specific to photosynthetic *Bradyrhizobium*.

ⁱ Sulfonate ABC transporter.

^j Upstream of *purQ*.

tants described as Ndv⁻ in previous genetic screens on classical rhizobium (Dénarié and Bergeron 1976; Pain 1979; Long 1989). It is still not clear whether their phenotype is due to growth impairment or to an alteration of the symbiotic process. It has been proposed that a purine precursor (AICAR) could play a symbiotic role (Noel et al. 1988; Newman et al. 1994); however, the selection of several mutants altered in genes involved in downstream steps of the purine pathway suggests

that AICAR does not play such a role in the Nod-independent symbiosis. The production by rhizobia of a cytokinin-like compound from adenine was also proposed to stimulate host cell division (Pain 1979). This old hypothesis, which had been ruled out with the discovery of Nod factors, resurfaces today with the finding that a *Lotus japonicus* gain-of-function mutant in a cytokinin receptor (LHK1) develops nodules spontaneously in the absence of rhizobia (Tirichine et al. 2007). It has

Table 2. Phenotypes and Tn5 insertion sites of nitrogen deficient (Fix⁻) mutants

Functional class, CDS ^b	Collection ^c	Gene annotation ^d	Mutant ^e	Growth rate(%) ^a		N ₂ (%) ^f	Fix ^{-g}	Comments
				YMm	MM			
Nitrogenase activity	...	...	6	...	...	...	...	...
BRADO5394	STM6439	<i>nifH</i>	1	95	115	80	t2	...
BRADO5436	STM6466	<i>nifN</i>	2	100	121	ND	t1	...
BRADO5437	STM6469	<i>nifE</i>	1	100	120	ND	t1	...
BRADO5438	STM6453	<i>nifK</i>	2	79	128	ND	t1	...
Nif genes regulators	...	...	16	...	...	...	...	...
BRADO0113	STM6420	<i>rpoN</i>	1	60	119	ND	t1	...
BRADO0530	STM6479	<i>glnD</i>	9	91	74	42	t2	...
BRADO3866	STM6448	<i>ntrC</i>	1	98	102	ND	t1	...
BRADO3867	STM6309	<i>ntrB</i>	2	109	108	ND	t1	...
BRADO3868	STM6409	<i>put. NifR3-like protein</i>	1	93	38	ND	t1	...
BRADO5449	STM5456	<i>nifA</i>	2	102	95	ND	t1	...
Electron transfers	...	...	10	...	...	...	...	...
BRADO2020	STM6351	<i>put. electron transfer flavoprotein dehydrogenase</i>	5	54	62	19	t2	...
BRADO2734	STM6375	<i>cycK</i>	1	76	76	27	t2	...
BRADO4317	STM6330	<i>put. pyruvate ferredoxin/ flavodoxin oxidoreductase</i>	1	84	106	ND	t1	...
BRADO6491	STM5341	<i>etfA</i>	2	45	45	24	t2	...
BRADO6492	STM5415	<i>etfB</i>	1	44	47	26	t2	...
Central metabolism	...	...	37	...	...	...	...	...
BRADO0157	STM6303	<i>gpmA</i>	1	80	31	5	t1	...
BRADO0234	STM6321	<i>sdhA</i>	1	82	43	ND	t1	...
BRADO0404	STM6313	<i>sucC</i>	3	32	24	ND	t1	...
BRADO0405	STM6319	<i>sucD</i>	4	32	25	ND	t1	...
BRADO0409	STM6359	<i>lpD</i>	5	79	61	85	t2	...
BRADO2034	STM6550	<i>ppdK</i>	1	108	125	34	t2	...
BRADO2682	STM6364	<i>mutA</i>	1	59	119	13	t2	...
BRADO3367	STM6328	<i>dme</i>	1	103	115	86	t2	...
BRADO4083	STM5393	<i>lpD</i>	1	86	46	ND	t1	...
BRADO4086	STM5303	<i>pdhB</i>	6	43	37	ND	t1	...
BRADO4087	STM5346	<i>pdhA</i>	7	46	32	6	t1	...
BRADO4094	STM5353	<i>eno</i>	1	51	57	ND	t1	...
BRADO5016	STM5332	<i>fumA</i>	4	82	115	4	t1	...
BRADO5815	STM6317	<i>put. amyloamylase</i>	1	100	134	6	t1	...
Calvin cycle	...	...	4	...	...	...	...	...
BRADO1122	STM6457	<i>cbbK</i>	1	30	91	80	t2	...
BRADO2274	STM5345	<i>cbbL</i>	1	78	118	30	t2	...
BRADO2842	STM5301	<i>cbbE</i>	2	84	13	ND	t1	...
Amino acids biosynthesis	...	...	3	...	...	...	...	...
BRADO0212	STM5385	<i>hisB</i>	2	40	13	54	t2	...
BRADO5973	STM6361	<i>aata</i>	1	75	96	50	t2	...
Transporters	...	...	9	...	...	...	...	...
BRADO1675	STM5361	<i>pstC</i>	2	83	97	3	t1	...
BRADO1677	STM5389	<i>pstB</i>	1	89	107	ND	t1	...
BRADO3096	STM6397	<i>put. ABC transporter</i>	1	108	102	22	t2	...
BRADO4782	STM5366	<i>put. ABC transporter</i>	1	86	86	5	t1	...
BRADO5117	STM6373	<i>put. permease</i>	1	94	107	ND	t1	...
BRADO6119	STM5363	<i>put. ABC transporter</i>	3	97	106	4	t1	...
Chemotaxis	...	...	2	...	...	...	...	...
BRADO1477	STM5383	<i>cheR</i>	1	89	94	3	t1	...
BRADO3225	STM5381	<i>put. methyl-accepting chemotaxis protein</i>	1	90	85	11	t1	...

(continued on next page)

^a Relative growth rate (percentage of wild type [WT]) estimated on rich medium (yeast extract mannitol [YMm]) or minimal medium (MM).

^b CDS names are indicated according to the published genome sequence (Giraud et al. 2007). CDS in bold correspond to novel symbiotic genes and coding sequences (CDS) in italic correspond to mutants for which the Tn5 insertion is located upstream of the CDS.

^c Representative mutant strain used to estimate growth and nitrogen fixation rate.

^d Abbreviations : put., putative; HP, hypothetical protein; CHP, conserved hypothetical protein.

^e Number of independent mutants identified in the corresponding CDS.

^f Fixation rate: relative acetylene reduction activity (percentage of WT) estimated on whole plants; ND = not detected.

^g Fix⁻ phenotype.

now become clear that cytokinins act downstream of Nod factors to mediate primordium formation (Gonzalez-Rizzo et al. 2006; Murray et al. 2007; Oldroyd 2007; Frugier et al. 2008). In a symbiotic system in which NF are not required, we can ask whether bacterial cytokinins could constitute the first signals triggering nodulation. Experiments at the plant and bacterial levels are currently in progress to test this hypothesis.

Among the 19 new genes identified as potentially involved in nodule development in this study (Table 1), three are of spe-

cial interest: BRADO3272, BRADO0213, and BRADO4535. BRADO3272 encodes a transcriptional regulatory protein of the TetR family. Intriguingly, this CDS as well as the two upstream CDS encoding proteins of unknown function appear specific to photosynthetic bradyrhizobia. Nevertheless, the mutation also causes significant growth impairment in minimal medium (MM), which might be responsible for the observed symbiotic deficiency of this mutant. BRADO0213 and BRADO4535 encode proteins of unknown function. In contrast to the other

Table 2. (continued from preceding page)

Functional class, CDS ^b	Collection ^c	Gene annotation ^d	Mutant ^e	Growth rate(%) ^a		N ₂ (%) ^f	Fix ^g	Comments
				YMm	MM			
Cofactors biosynthesis	...	...	4	...	...	...	...	...
BRADO2036	STM5364	<i>nadB</i>	1	85	104	59	t2	...
BRADO2133	STM6315	<i>put. RibBA-like protein</i>	3	84	95	4	t1	...
Nucleotides biosynthesis	...	...	8	...	...	...	...	...
BRADO1205	STM6316	<i>purK</i>	7	94	23	58	t2	...
BRADO2776	STM6324	<i>purC</i>	1	90	35	32	t2	...
Others	...	...	9	...	...	...	...	...
BRADO0045	STM6447	<i>Int</i>	1	50	120	69	t2	...
BRADO0589	STM6394	<i>put. peptidylpropyl isomerase</i>	1	102	67	17	t2	...
BRADO2179	STM6459	<i>put. acylCoA dehydrogenase</i>	1	87	91	6	t1	...
BRADO2938	STM6437	<i>put. hydrolase</i>	1	52	94	18	t2	...
BRADO3673	STM6418	<i>put. cold shock DNA binding protein</i>	1	65	90	90	t2	...
BRADO3794	STM6349	<i>put. sulfite reductase</i>	1	61	6	74	t2	...
BRADO4549	STM5430	<i>put. D-alanyl-D-alanine carboxypeptidase</i>	1	105	114	10	t2	...
BRADO5368	STM5351	<i>put. PhnM protein</i>	1	88	96	3	t1	Putative metal-dependant hydrolase
BRADO6079	STM5427	<i>mutL</i>	1	112	109	26	t2	DNA mismatch repair
Various regulators	...	...	6	...	...	...	...	...
BRADO0560	STM5434	<i>put. GGDEF/EAL protein</i>	1	98	119	ND	t2	...
BRADO1049	STM5387	<i>put. PhyR-like protein</i>	1	89	102	75	t2	...
BRADO3946	STM5395	<i>put. sensor HK</i>	1	111	98	3	t1	...
BRADO4470	STM6334	<i>put. LabA-like protein</i>	1	90	112	3	t1	...
BRADO4636	STM6398	<i>put. transcriptional regulator</i>	1	91	103	ND	t1	...
BRADO6832	STM6461	<i>two-component system regulator</i>	1	96	102	56	t2	...
Translational processes	...	...	16	...	...	...	...	...
BRADO0134	STM5347	<i>infC</i>	1	67	73	23	t2	Translation initiation factor IF3
BRADO0340	STM6450	<i>put. RsmB-like protein</i>	1	61	110	5	t2	16S RNA methyltransferase
BRADO0376	STM5386	<i>trmD</i>	1	103	108	28	t2	tRNA methyltransferase
BRADO0377	STM5399	<i>rimM</i>	1	105	86	30	t2	Ribosome maturation factor
BRADO0571	STM6399	<i>put. HemK-like protein</i>	5	65	103	6	t2	...
BRADO3306	STM6363	<i>50s ribosomal protein L9</i>	2	70	57	75	t2	...
BRADO3308	STM5412	<i>rpsR</i>	1	99	115	68	t2	30S ribosomal protein
BRADO3359	STM6485	<i>aspS</i>	1	88	113	16	t2	Aspartyl-tRNA synthetase
BRADO3881	STM6480	<i>valS</i>	1	72	117	67	t2	Valine-tRNA ligase
BRADO6137	STM5452	<i>rpmJ</i>	1	84	112	74	t2	50S ribosomal protein
Proteins of unknown function	...	...	19	...	...	...	...	...
BRADO0575	STM5349	<i>CHP</i>	2	91	85	ND	t1	...
BRADO0665	STM6381	<i>HP</i>	1	82	93	76	t2	Specific to photosynthetic <i>Bradyrhizobium</i>
BRADO1364	STM6464	<i>CHP</i>	1	31	98	37	t2	...
BRADO1426	STM6393	<i>HP</i>	1	118	18	4	t1	Specific to photosynthetic <i>Bradyrhizobium</i>
BRADO2671	STM6320	<i>CHP</i>	2	27	51	55	t2	...
BRADO2937	STM6314	<i>CHP</i>	1	97	20	56	t2	...
BRADO3770	STM5331	<i>CHP</i>	2	97	95	ND	t1	...
BRADO4085	STM5344	<i>CHP</i>	1	52	40	ND	t1	...
BRADO4683	STM6438	<i>CHP</i>	1	45	119	64	t2	...
BRADO4701	STM6452	<i>CHP</i>	1	60	116	10	t2	...
BRADO5022	STM5356	<i>CHP</i>	1	92	76	6	t2	...
BRADO5546	STM6386	<i>CHP</i>	1	109	83	6	t1	...
BRADO5734	STM6360	<i>CHP</i>	1	83	15	ND	t1	...
BRADO5803	STM5377	<i>HP</i>	1	92	97	ND	t1	...
BRADO6852	STM5340	<i>CHP</i>	1	86	114	ND	t1	...
BRADO6923	STM6417	<i>HP</i>	1	90	80	11	t1	...

Ndv⁻ mutants, their growth in MM medium was hardly affected, suggesting that their symbiotic phenotype results from a direct modification of the symbiotic process. However, *Bradyrhizobium japonicum* USDA110 has homologues of both CDS in a genomic context similar to that found in the ORS278 genome. Therefore, it seems unlikely that BRADO0213 and BRADO4535 are specific determinants of the Nod-independent symbiosis.

Novel insights in bradyrhizobial physiology from known nitrogen fixation-related genes.

To get insights into the bacterial physiology during symbiosis, mutants impaired in nitrogen fixation were selected. Among them were 74 mutants altered in 31 genes known to be involved in bacteroid functioning (Table 2; Fig. 2). These genes participate in i) nitrogenase complex formation (*nifK*, *nifE*, *nifN*, and *nifH*), ii) *nif* genes regulation (*nifA*, *rpoN*, *glnD*, *ntrC*, *ntrB*, and *nifR3*), iii) tricarboxylic acid cycle (TCA) (*sdhA*, *sucC*, *sucB*, *lpD*, *fumA*, *pdhA*, *pdhB*, and *dme*), iv) gluconeogenesis (*ppdK*, *gpmA*, and *eno*), v) transport (*pstB* and *pstC*), vi) amino-acid biosynthesis (*hisB* and *aata*), and vii) in the general stress response recently described in *B. japonicum* (*phyR*) (Gourion et al. 2009).

Although these genes were already reported to play a role in symbiosis, new insights into the physiology of photosynthetic bradyrhizobia emerged.

i) In contrast to *B. japonicum* and *Sinorhizobium meliloti*, mutants in the two-component system NtrB/NtrC were drastically impaired in nitrogen fixation (Szeto et al. 1987; Martin et al. 1988). Regulation of *nif* genes in rhizobia depends directly on the NifA protein, expression of which is under the control of additional regulatory circuits that differ markedly between *Rhizobium* spp. (Fischer 1994). Therefore, it is possible that, in photosynthetic bradyrhizobia, *nifA* is under the control of the nitrogen regulatory (*ntr*) system.

ii) We isolated several mutants in three key gluconeogenic enzymes: enolase (Eno), phosphoglyceromutase (GpmA), and pyruvate phosphate dikinase (PPDK). In contrast to *eno* and *gpmA* mutants, which were completely deficient for nitrogen fixation, the *ppdK* mutant retained 34% of the WT nitrogenase activity. The partial activity of the latter most likely results from the presence of an alternative route for phosphoenol pyruvate (PEP) formation involving the PEP carboxykinase (PCK) (Fig. 2).

iii) We finally isolated two Fix⁻ mutants putatively involved in the degradation of the major storage compounds commonly found in rhizobia, poly-β-hydroxybutyrate (PHB) and glycogen (Lodwig et al. 2005; Trainer and Charles 2006). BRADO2682 encodes the methylmalonyl-CoA mutase (MutA), an enzymatic activity that is required for PHB degradation but which is dispensable during symbiosis in *S. meliloti* (Charles and Aneja 1999). BRADO5815 encodes 4-α-glucanotransferase (amylomaltase), implicated in glycogen catabolism. It has been suggested that PHB and glycogen accumulated by the bacteria prior to infection serve to fuel bacteroid

formation (Lodwig et al. 2005). Therefore, it is possible that the weak nitrogenase activity measured for both mutants (13 and 6%, respectively) results from an alteration in bacteroid formation. Accordingly, preliminary microscopic observations revealed that, in mature nodules elicited by both mutants, bacteria were undifferentiated (data not shown).

The Fix⁻ mutants reveal novel symbiotic genes required for nitrogen fixation.

In addition to known symbiotic genes, we identified 56 new genes potentially involved in symbiotic nitrogen fixation. Some of these genes are grouped by physiological function and are described below.

A putative role for CO₂ assimilation in symbiosis. Four mutants impaired in symbiotic nitrogen fixation were affected in three genes involved in CO₂ assimilation via the Calvin Benson Bassham (CBB) cycle (*cbbL*, BRADO2274; *cbbE*, BRADO2842; and *cbbK*, BRADO1122). Genome annotation predicts that strain ORS278 possesses all the genes required for a functional CBB cycle.

The key step of the CBB cycle is the carboxylation of ribulose bis phosphate catalyzed by Rubisco (ribulose biphosphate carboxylase/oxygenase), which is encoded by the *cbbL* and *cbbS* genes. Interestingly, as in strain BTAi1, ORS278 has two distantly related Rubisco gene sets (*cbbL* and *cbbS*; 57% amino acid identity between the two CbbL). BRADO1659/BRADO1660 is found in other rhizobia (including *B. japonicum*) and is associated with other *cbb* genes. BRADO2274/BRADO2275, on the other hand, seems to be restricted to photosynthetic bradyrhizobia. It is located in a genomic island acquired by lateral transfer (Giraud et al. 2007) and is linked to genes involved in a CO₂ concentration mechanism (carboxysome encoding genes). The fact that the BRADO1659/BRADO1660 gene set does not complement the mutation in BRADO2274 strongly suggests that they play different physiological roles.

Other rhizobia (BTAi1, *B. japonicum*, and *S. meliloti*) have been reported to fix CO₂ via the CBB cycle under free-living conditions (Hungria et al. 1993; Manian et al. 1984, Pickering and Oresnik 2008) but, to our knowledge, bacterial CO₂ fixation has never been reported to be required for effective nitrogen fixation in a rhizobium-legume symbiosis. Our aim is now to determine the exact role of the bacterial CBB cycle during symbiosis. Possibly, photosynthetic bradyrhizobia use the CBB cycle as an electron sink to equilibrate the red/ox balance or the bacteroids use it as an important carbon supply.

Novel regulatory and signaling proteins involved in bacteroid functioning. A second class of novel symbiosis genes is related to regulation and signaling and is represented by five mutants that exhibit a strong deficiency in their nitrogen fixation activity.

i) BRADO3946 encodes a large sensory histidine kinase of 2,117 amino acids. Interestingly, a previous screening of a mutant library for clones altered in the photosystem synthesis identified mutants in this CDS (Jaubert et al. 2009). It was

Fig. 2. Main metabolic routes found to be important during the symbiosis between ORS278 and *Aeschynomene indica*. The nodule development-deficient (Ndv⁻) mutants are in red underlined, whereas the nitrogen fixation-deficient (Fix⁻) mutants are in blue bold. The mutants identified during the previous screening are in green and highlighted with an asterisk (Giraud et al. 2007). For clarity, some biosynthesis pathways are not given in detail but are represented by dotted arrows and enzymatic steps for which symbiotically defective mutants were isolated. R5P = ribose-5-phosphate, G3P = glyceraldehyde-3-phosphate, F6P = fructose-6-phosphate, PBH = polyhydroxybutyrate, PRPP = 5-phosphoribosylpyrophosphate, GAR = 5'-phosphoribosylglycinamide, FGAR = 5'-phosphoribosylformylglycinamide, FGAM = 5'-phosphoribosylformylglycinamide, AIR = 5'-phosphoribosyl-5-aminoimidazole, CAIR = 5'-phosphoribosyl-4-carboxy-5-aminoimidazole, SAICAR = 5'-phosphoribosyl-4-(N-succinocarboxamide)-5-aminoimidazole, AICAR = 5'-phosphoribosyl-5-amino-4-imidazole carboxamide, FAICAR = 5'-phosphoribosyl-5-formamido-4-imidazole carboxamide, IMP = inosine 5'-monophosphate, XMP = xanthosine 5'-monophosphate, GMP = guanosine 5'-monophosphate, AMP =, adenosine 5'-monophosphate, UMP = uridine 5'-monophosphate, AMP = adenosine 5'-monophosphate, UMP = uridine 5'-monophosphate, CMP = cytidine-5'-monophosphate, and dTMP = deoxythymidine 5'-monophosphate.

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postulated that this gene would control not only photosynthesis but also other metabolic pathways because a homologue was found in the nonphotosynthetic *B. japonicum* USDA110. The drastic nitrogen fixation deficiency of the mutant isolated during this study strengthens the hypothesis that BRADO3946 could act as a global regulator.

ii) BRADO4470 encodes a homologue of LabA (36% identity and 57% similarity), identified in cyanobacteria and required for feedback control of KaiC, which is a central component of the circadian clock system (Taniguchi et al. 2007). Although a photoperiodicity is necessary to induce photosystem formation under white light conditions (Giraud and Fleischman 2004), and two kaiBC operons are present in the ORS278 and BTAi1 genomes, circadian gene expression in photosynthetic bradyrhizobia has never been reported. Nevertheless, a circadian control of the *nif* genes would be particularly beneficial to optimize the symbiotic interaction in the green stem nodules. Indeed, by restricting the synthesis of the nitrogenase to the night time, the deleterious effect of O₂ production by the photosynthetic activity of the chloroplasts during the day could be prevented.

iii) BRADO4636 encodes a putative transcriptional regulatory protein related to the MarR family. This CDS is found close to a putative oxidoreductase (BRADO4637) and such genetic organization is well conserved in bacteria, including several rhizobium strains (*B. japonicum*, *S. meliloti*, *Mesorhizobium loti*, and others), suggesting that BRADO4636 could target this oxidoreductase.

iv and v) BRADO3225 and BRADO1477 encode putative chemotaxis genes, a methyl-accepting chemotaxis protein (MCP) and a CheR homologue, respectively. Chemotaxis and motility are known in rhizobia to confer a competitive advantage in the early infection events (Ames and Bergman 1981; Miller et al. 2007). However, once the infection has begun, motility is not required anymore (Caetano-Anollés et al. 1988). Unexpectedly, the mutants in BRADO1477 and BRADO3225 appeared to be drastically impaired in their efficiency to fix N₂. It has been shown that some *che* operons are implicated in controlling cellular functions other than motility, including biofilm formation, initiation of developmental programs, EPS biosynthesis, and so on (Hickman et al. 2005; Kirby and Zusman 2003; Bible et al. 2008). Considering that 4 *che* operons and 49 MCP are identified in the ORS278 genome, mutants in BRADO1477 and BRADO3225 might be affected in biological processes other than chemotaxis.

Critical role of membrane integrity for efficient nitrogen fixation. Two mutants affected in CDS putatively involved in cell-wall biogenesis were isolated: BRADO4549 encodes a putative D-alanyl-D-alanine carboxypeptidase involved in peptidoglycan biosynthesis and BRADO0045 encodes an apolipoprotein N-acyltransferase that transfers a fatty acyl group on membrane lipoproteins. Possibly, BRADO0045 and BRADO4549 may be needed for membrane integrity, which is likely to be critically important during the early stages of bacterial differentiation within plant cells.

Conclusions and perspectives.

From this study, we gained insights into the metabolism of photosynthetic bradyrhizobia during root symbiosis with *Aeschynomene* spp. The main routes (TCA cycle, gluconeogenesis, PHB, and glycogen degradation) already known to play a central role in bacteroid metabolism were revealed to be also required by photosynthetic bradyrhizobia for an efficient symbiosis. Furthermore, we identified unexpected biological processes, such as CO₂ fixation and chemotaxis, as well as a large number of novel genes, including regulators, which appear to be important for symbiosis. Some of these determinants are

conserved among rhizobia, suggesting that they could also be required in other rhizobium-legume symbioses. Further investigations on these newly identified genes are needed to confirm their role during nodulation and, if confirmed, to unravel their precise function in the symbiotic process.

To better characterize the molecular mechanisms involved during the early steps of this Nod-independent symbiotic process, other approaches besides the screening of additional mutants are required, mainly because of the likely redundancy of functions involved in these steps. Therefore, we are currently developing i) a positive screen by transferring a cosmid library of ORS278 in a conventional *Rhizobium* sp. and selecting transconjugants that elicit nodules or nodule-like structures on *A. indica* and ii) transcriptomic and proteomic analyses in the presence or absence of root exudates.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

Escherichia coli strains were grown at 37°C in Luria-Bertani medium (Sambrook et al. 1989). The WT strain *Bradyrhizobium* ORS278 and its derivatives were cultivated at 37°C in modified yeast extract mannitol (YMm) medium (Giraud et al. 2000) or in MM. The MM (pH 6.8) contained K₂HPO₄ (1.6 g liter⁻¹), KH₂PO₄ (0.8 g liter⁻¹), sodium glutamate (0.56 g liter⁻¹), xylose (3 g liter⁻¹), NaCl (50 mg liter⁻¹), MgSO₄ (50 mg liter⁻¹), CaCl₂ (40 mg liter⁻¹), ZnSO₄ · 7H₂O (22 mg liter⁻¹), H₃BO₃ (11 mg liter⁻¹), MnCl₂ · 4H₂O (5 mg liter⁻¹), FeSO₄ · 7H₂O (5 mg liter⁻¹), CoCl₂ · 6H₂O (1.6 mg liter⁻¹), CuSO₄ · 5H₂O (1.6 mg liter⁻¹), (NH₄)₆Mo₇O₂₄ · 4H₂O (1.1 mg liter⁻¹), Na₄EDTA · 4H₂O (60 mg liter⁻¹), riboflavin (200 µg liter⁻¹), biotin (120 µg liter⁻¹), thiamine-HCl (800 µg liter⁻¹), myo-inositol (48 µg liter⁻¹), p-aminobenzoic acid (80 µg liter⁻¹), nicotinic acid (500 µg liter⁻¹), calcium pantothenate (800 µg liter⁻¹), and cyanocobalamin (1 µg liter⁻¹). When required, the media were supplemented with kanamycin (50 µg/ml) for *E. coli* and with a mixture of kanamycin (50 µg/ml) and nalidixic acid (35 µg liter⁻¹) for ORS278 mutants.

Transposon mutagenesis and colony selection.

Transposon insertions in the ORS278 genome were generated by a biparental mating with *Bradyrhizobium* sp. strain ORS278 and *E. coli* sp. strain BW20767 containing the plasmid pCRS487 harboring the minitransposon mTn5-GNm (Reeve et al. 1999) according to the protocol previously described (Jaubert et al. 2009). The clones were picked and arrayed into 96-well plates, each plates containing 120 µl of YMm medium with selective antibiotics. Plates were incubated for 7 days at 37°C under agitation (100 rpm). After addition of glycerol (50%) at 40 µl/well, the plates were stored at -80°C.

In planta screening.

A. indica seed were surface sterilized with concentrated (96%) sulfuric acid for 30 min, abundantly rinsed with sterile water, and soaked overnight in sterile water. The seed were transferred onto sterile 0.8% water agar plates and germinated upside-down overnight at 37°C in the dark. One-day-old seedlings were transferred to 48-well plates (6.0-ml storage plate, ref: AB-0988; Thermoscientific) previously filled with 5.6 ml of nitrogen-free Jensen's medium (Vincent 1970), inoculated individually with 100 µl of 7-day-old mutant cultures, and covered by sterile adhesive aluminum foil. To allow passage of the radicle, a small hole in the aluminum cover was made using a sterile toothpick. The 48-well plates containing the seedlings were placed in a small greenhouse containing 1 liter of sterile water and placed for 3 weeks in a 28°C growth chamber with a 16-h light regime and 70% humidity. Mutants impaired in their

ability to form nodules (Ndv⁻) or to fix nitrogen by eliciting yellow nodules (Fix⁻) when tested on single plants were then reinoculated onto two plants each. Symbiotic phenotypes were determined as above and the symbiotically defective mutants were reinoculated onto six plants each and their phenotype was reexamined as above. Mutants showing a symbiotic deficiency and for which the Tn5 insertion site determination was successful were retained for further characterization.

Nitrogenase activity.

Acetylene reduction assays were performed 3 weeks after inoculation. Two whole plants were placed into 125-ml glass vials sealed with rubber septa. Air (12.5 ml) was removed from each vial, to which 12.5 ml of acetylene was then injected. Gas samples (1 ml) were withdrawn after 3 h of incubation at 25°C and the ethylene produced was measured by gas chromatography. The assay was done in triplicate and compared with plants that had been inoculated with the WT strain.

Bacterial growth rate.

Cultures grown for 5 days in YMm medium were pelleted by centrifugation, washed twice with NaCl (9 g liter⁻¹) and inoculated in MM or YMm medium to obtain an initial optical density at 600 nm (OD₆₀₀) of 0.02. After 4 days of incubation at 37°C, the OD₆₀₀ of each culture was measured and compared with the WT strain.

Transposon insertion location.

Transposon insertion location was determined by a two-stage semidegenerative PCR and sequencing protocol according to Jacobs and associates (2003). The precise location of the Tn5 insertions in the ORS278 genome was determined via a BLASTN search using the obtained sequences as query.

ACKNOWLEDGMENTS

This work was supported by grants from the French national research agency (ANR-06-NLAN-0095) and the "Ministère de l'Éducation Nationale, de l'Enseignement Supérieur et de la Recherche" (fellowship to K. Bonaldi) and the European Molecular Biology Organization (fellowship to B. Gourion). We thank A. Verméglio (CEA, Cadarache, France) for critical reading of the manuscript, D. Gourion (Department of Mathematics, University of Avignon, France) for stimulating discussions, and our anonymous reviewers for their useful comments on the manuscript.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

GenoScope MaGe NewNodBrady database:
www.genoscope.cns.fr/agc/newnodbrady