Functional redundancy of genes for sulphate activation enzymes in Rhizobium sp. BR816

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The broad-host-range, heat-tolerant Rhizobium strain BR816 produces sulphated Nod metabolites. Two ORFs highly homologous to the Sinorhizobium meliloti nodPQ genes were isolated and sequenced. It was found that Rhizobium sp. BR816 contained two copies of these genes; one copy was localized on the symbiotic plasmid, the other on the megaplasmid. Both nodP genes were interrupted by insertion of antibiotic resistance cassettes, thus constructing a double nodP1P2 mutant strain. However, no detectable differences in Nod factor TLC profile from this mutant were observed as compared to the wild-type strain. Additionally, plant inoculation experiments did not reveal differences between the mutant strain and the wild-type. It is proposed that a third, functionally homologous locus complements mutations in the Nod factor sulphation genes. Southern blot analysis suggested that this locus contains genes necessary for the sulphation of amino acids.

Keywords: Nod factor, TLC, sulphate activation, nodPQ, Rhizobium sp. BR816

INTRODUCTION

Symbiotically nitrogen fixation as an economical and environmentally friendly alternative to chemical synthesis of nitrogen fertilizers is an important feature of the symbiosis between legume plants and bacteria of the genera Rhizobium, Bradyrhizobium, Sinorhizobium and Azorhizobium. As a result of the recognition between compatible plant and bacterial symbionts, new plant organs known as nodules emerge. The symbiotic bacterial form, the bacteroid, synthesizes nitrogenase, an enzymic complex that converts atmospheric nitrogen into ammonia, which is directly available for plant nitrogen metabolism (reviewed by Mylona et al., 1995).

During the early stages of the infection, reciprocal signal exchange occurs between the two symbionts. The bacterial NodD sensor protein responds to the presence of flavonoid molecules released by the plant and functions as the main positive regulator of the expression of the structural nodulation genes (nod, nol, noe) (Mulligan & Long, 1985). The products of the nod genes generate the proteins involved in the synthesis and transport of mixtures of bacterial signal molecules, i.e. the Nod factors, also called lipo-chitin oligosaccharides (LCOs). Physiological effects of the LCOs on their leguminous host plants include induction of root hair curling, formation of pre-infection threads, division of cortical root cells and, in some cases, even the formation of nodule-like structures (Truchet et al., 1991; reviewed by Spanik, 1996). The LCOs consist of a backbone of three to six 1,4-β-linked N-acetylglucosamine units linked to a fatty acid group at the non-reducing end, which also contains additional strain-specific substituents.

The enzymes necessary for the production of the LCO backbone are encoded by the nodABC genes found in all nodulating rhizobial strains. These genes are referred to as ‘common’ nod genes (Kondorosi, 1991), although recently Ritsema et al. (1996) found that the replacement of the Rhizobium leguminosarum bv. viciae nodA by its Bradyrhizobium japonicum homologue resulted in the loss of Vicia nodulation. Additionally, Debelle et al. (1996) proved that the ‘common’ NodA protein of Sinorhizobium (previously Rhizobium) meliloti contributes to its host range determination.

The host-specific nodulation genes are responsible for the side groups exclusively encountered on the two extreme glucosamine residues of the LCO core molecule.
On the non-reducing end, the nodS (Geelen et al., 1993), nodL (Downie, 1989) and nodU (Jabbouri et al., 1995) genes have been shown to be responsible for N-methyl (Price et al., 1992), O-acetyl (Carlson et al., 1993) or O-carbamoyl (Price et al., 1992) substitutions, respectively. On the reducing end, sugar modifications such as a fucosyl residue (Price et al., 1992; Sanjuan et al., 1992), an arabinosyl residue (Mergaert et al., 1993) or a mannosyl moiety (Folch-Mallol et al., 1996) can be present (for an overview of Nod factor structures and nodulation genes, see Dénavé et al., 1996). Other reducing end side decorations include an O-acetylation (Price et al., 1992) and an O-sulphation, the latter modification either directly linked to the C6 of the non-reducing sugar (for S. meliloti, see Lerouge et al., 1990) or on a methylfucosyl group (for Rhizobium sp. NGR234, see Price et al., 1992). For sulphate modification of the former type, the involvement of three genes has been reported in S. meliloti. nodP and nodQ together encode an ATP sulphurylase (Schwedock & Long, 1990), whereas nodQ alone additionally encodes an adenosine 5'-phosphosulphosphate (APS) kinase (Schwedock et al., 1994). The NodPQ enzyme complex activates the inorganic sulphate source via APS to 3'-phospho-adenosine 5'-phosphosulphate (PAPS). Finally, NodH catalyses the transfer of the activated sulphate group from its donor PAPS directly onto the 6-O-position of the reducing end glucosamine unit (Schultze et al., 1993; Ehrhardt et al., 1995). Nod factor (NF) sulphation genes were also isolated from Rhizobium tropici type A and B reference strains (Laeremans et al., 1996 and Folch-Mallol et al., 1996, respectively) and from Rhizobium sp. N33 (Cloutier et al., 1996). In these three strains the NF sulphation genes are organized in one nodHPQ operon whereas in S. meliloti, nodH and nodPQ are separated by nodEF and nodG (Debelle & Sharma, 1986; Faucher et al., 1988; Cervantes et al., 1989). For nodulation of alfalfa by S. meliloti, the sulphated NF is indispensable (Roche et al., 1991; Truchet et al., 1991), possibly because the sulphate moiety protects the NF against plant-chitinase degradation (Schultze et al., 1993; Staelin et al., 1994).

Previously, we isolated the nodHPQ operon from R. tropici strain CFN299 and showed that the sulphate substituent was detrimental for nodulation of high-nitrogen-fixing bean cultivars (Laeremans et al., 1996). Since we are interested in the role of the sulphate group for bean nodulation, we isolated the nodPQ genes from Rhizobium sp. BR816, a broad-host-range bacterium related to S. meliloti. BR816 is a heat-tolerant tropical strain originally isolated from Leucaena leucocephala nodules that also nodulates common bean (Hungria et al., 1993). We show that two copies of nodPQ are present, and give evidence that a third locus can provide an activated sulphate source for NF sulphation.

**METHODS**

**Bacterial cultures.** The bacterial strains and plasmids used in this work are listed in Table 1. *Rhizobium* strains were grown on peptone-yeast (PY) medium (Beringer, 1974) at 29°C with nalidixic acid (NaL; 30 mg l−1) and supplemented with the following antibiotics (mg l−1) when necessary: tetracycline (Tc, 5); spectinomycin (Sp, 100); streptomycin (Sm, 100); and kanamycin (Km, 50). Escherichia coli strains were grown in Luria–Bertani medium (Sambrook et al., 1989) at 37°C with the following antibiotics added when required: Tc (10); ampicillin (Ap, 100); Sp (100); Sm (100); gentamycin (Gm, 25); and Km (25).

**DNA manipulations.** Isolation and cloning of plasmid or cosmid DNA was performed using the protocols described by Sambrook et al. (1989). Total genomic DNA of *Rhizobium* strains was isolated using a DNA/RNA Isolation Kit (USB) according to the manufacturer’s instructions. Analysis of plasmid contents of *Rhizobium* sp. BR816 was carried out on horizontal agarose gels as described by Géniaux et al. (1995). PCR using internal nodP primers P1 and P2 was performed as described previously (Laeremans et al., 1996). PCR fragments were directly cloned in the pMOSBlue vector (Amersham). Bacterial triparental matings were done according to van Rhijn et al. (1993).

**Southern hybridization.** Southern-blotted DNA on positively charged nylon membranes (Boehringer Mannheim) was hybridized with non-radioactive digoxigenin-labelles probes using the DIG Labelling and Detection Kit (Boehringer Mannheim) following the manufacturer’s protocol. Hybridizations were always performed under high-stringency conditions unless otherwise stated. For high-stringency conditions we (pre-)hybridized membranes at 68°C and washed them twice for 5 min at room temperature in 2× SSC (a stock solution of 20× SSC contains 2 M NaCl, 0.3 M sodium citrate; pH 7)/0.1% SDS and then at 68°C in 0.1× SSC/0.1% SDS (twice for 15 min). For hybridizations under low-stringency conditions, the (pre-)hybridization temperature was lowered to 60°C and the second washing step was performed at 60°C in 0.5× SSC/0.1% SDS, also twice for 15 min. The detection of hybridizing DNA fragments was performed as indicated by the manufacturer.

**Sequence determination and analysis.** DNA fragments were cloned in the pUC18/19 vectors and the sequence was determined on an ALF DNA sequencer (Pharmacia) using the M13 reverse and universal primers as previously described (Laeremans et al., 1996). Both strands were completely sequenced. We analysed the sequencing data using the PC/Gene software (IntelliGenetics) and the GCG software package (version 8.0.1, 1994; University of Wisconsin).

**Insertion mutagenesis.** For construction of nodP and nodQ insertion mutants, vectors pJQ200SK and pJQ200mp18 were used. These vectors allow positive selection of double homologous recombinants on sucrose (5%)-containing media due to the presence of the *Bacillus subtilis* sacI gene. Firstly, pJQ200SK was adapted by eliminating the unique SacI restriction site had previously been eliminated. For mutagenesis, the Km resistance cassette cloned in this vector was adapted by eliminating the unique SacI restriction site had previously been eliminated. For mutagenesis, the Km resistance cassette of pUC-4K was used. This cassette was isolated as a BglII restriction fragment containing the nodP1′ region (see Fig. 3) was cloned into the pJQ200SK derivative from which the unique SacI restriction site had previously been eliminated. For mutagenesis, the Km resistance cassette cloned in this vector was obtained as a SacI fragment and cloned into the unique SacI site of BR816 nodP1 DNA. The construct obtained was mated into the wild-type strain BR816. We hybridized total genomic DNA from Km-resistant, Gm-sensitive colonies with the BR816 nodP1 gene and the Km resistance cassette to...
**Table 1. Bacterial strains and plasmids**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
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<td><strong>Strains</strong></td>
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<tr>
<td>Rhizobium sp.</td>
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<tr>
<td>BR816</td>
<td>Broad-host-range <em>Rhizobium</em> isolated from <em>Leucaena leucocephala</em></td>
<td>Hungria et al. (1993)</td>
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<tr>
<td>CFNE205</td>
<td>Km' nodP1' mutant of BR816</td>
<td>This study</td>
</tr>
<tr>
<td>CFNE206</td>
<td>Sp'/Sm' nodP2Q2 deletion mutant of BR816</td>
<td>This study</td>
</tr>
<tr>
<td>CFNE207</td>
<td>Km' Sp'/Sm' nodP1P2' double mutant of BR816</td>
<td>This study</td>
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<td><strong>Rhizobium tropici</strong></td>
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<td>CFNE200</td>
<td>Km' nodP' mutant of <em>R. tropici</em> CFN299</td>
<td>Laeremans et al. (1996)</td>
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<tr>
<td><strong>Plasmids</strong></td>
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<td>pVK82</td>
<td>pVK100 carrying the BR816 nodP1Q1 genes</td>
<td>This study</td>
</tr>
<tr>
<td>pVK100</td>
<td>IncP Km' Tc' broad-host-range cosmid</td>
<td>Knauf &amp; Nester (1982)</td>
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<td>pLAFR1 carrying the BR816 nodP2Q2 genes</td>
<td>This study</td>
</tr>
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<td>pRK7813</td>
<td>IncP Tc', mobilizable cosmid vector</td>
<td>Jones &amp; Gutterson (1987)</td>
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<td>8 kb BamHI fragment containing the BR816 nodP1Q1 genes cloned in pRK7813</td>
<td>This study</td>
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<td>pUC18/19</td>
<td>Ap' cloning vector</td>
<td>Norrander et al. (1983)</td>
</tr>
<tr>
<td>pJQ200SK</td>
<td><em>B. subtilis</em> SacB-containing suicide vector, Gm'</td>
<td>Quandt &amp; Hynes (1993)</td>
</tr>
<tr>
<td>pJQ200mp18</td>
<td><em>B. subtilis</em> SacB-containing suicide vector, Gm', with the pUC18 multiple cloning site</td>
<td>Quandt &amp; Hynes (1993)</td>
</tr>
<tr>
<td>pLAFR1</td>
<td>IncP Tc', broad-host-range cosmid</td>
<td>Friedman et al. (1982)</td>
</tr>
<tr>
<td>pUC-4K</td>
<td>Vector containing Km resistance cassette</td>
<td>Pharmacia Biotech</td>
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<tr>
<td>pMOSBlue</td>
<td>Ap' cloning vector, suitable for direct cloning of PCR fragments</td>
<td>Amersham</td>
</tr>
<tr>
<td>pMOSPlQ1C</td>
<td>950 bp internal nodP1Q1 PCR fragment of BR816 cloned in pMOSBlue</td>
<td>This study</td>
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<tr>
<td>pSmUC</td>
<td>Vector carrying an Sm'/Sp resistance cassette</td>
<td>Murillo et al. (1994)</td>
</tr>
<tr>
<td>pIC20::Km</td>
<td>pUC-4K Km resistance cassette cloned in the BamHI site of pIC20-R</td>
<td>Laeremans et al. (1996)</td>
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</table>

confirm the expected hybridization profile of the insertion. One BR816 nodP1 mutant (CFNE205) was retained for TLC characterization.

For the construction of the *Rhizobium* sp. BR816 nodP1P2 double mutant, we first cloned a 950 bp *Rhizobium* sp. BR816 nodP2 PCR fragment, obtained with primers P1 (Laeremans et al., 1996) and the primer Q1C. This study

The single BR816 nodQ2 mutant was constructed as follows. By hybridization of a BR816 genome library, previously cloned in the EcoRI site of cosmid pLAFR1 (P. van Rijn & J. Vanderleyden, unpublished data) with the 1.5 kb PstI fragment of BR816 nodP1Q1 (Fig. 3) one positive clone, BRVII9D9, was isolated that contained a 12 kb EcoRI fragment. A 17 kb Spd1 subfragment containing part of nodP2 and nodQ2 was cloned into pIC20-R and the insert was cut out with restriction enzymes BglII and PstI and subsequently cloned into BamHI/PstI-digested pJQ200mp18. A nodQ2 internal 300 bp SalI fragment, located around 100 bp from the nodQ2 start codon, was then exchanged for a SalI restriction fragment containing the Sp'/Sm resistance cassette. CFNE206 was obtained by conjugating the resulting construct into BR816 followed by sucrose selection for double homologous recombinants. The insertion of the resistance cassette into the nodP2Q2 locus was demonstrated by hybridization of CFNE206 total DNA with the Sp'/Sm resistance cassette and the nodP2Q2 locus.

**Radiolabelling and detection of Nod metabolites by reverse-phase TLC.** We radiolabelled NFs in vivo by a slightly modified version of the protocol of Mergaert et al. (1993). Overnight cultures of *Rhizobium* strains were inoculated in 1 ml liquid PY medium (OD490 of 0.1) and pre-incubated for 1 h before supplementing when necessary with apigenin at a final concentration of 10 μM. Finally, the radioactive label was added 2 h after apigenin induction: 40 μCi (1.5 MBq) [35S]sulphate or 25 μCi (0.9 MBq) [14C]acetate, as sodium salt, and the cells labelled for 1.5 h. The cell suspension was extracted twice with 500 μl n-butanol and washed with ethyl acetate. The solution was vacuum-dried and samples were applied to reverse-phase TLC plates (RP-18F254s, Merck). We used H2O/acetonitrile (1:1, v/v) as the mobile phase. The radioactive compounds were visualized by autoradiography using Hyperfilm max (Amersham) after 4 d of exposure.

**Nodulation assays.** Seeds of the high-nitrogen-fixing *Phaseolus vulgaris* N-8-116 cultivar were surface-sterilized and germinated as described by Martinez et al. (1985). Plant nodulation assays were performed as described by Laeremans et al. (1996). For each inoculation, three different plant
supports were used, mixed with 120 ml Fahreus medium in 250 ml flasks and subsequently sterilized: vermiculite, agar (0.75%) or cotton (7 g). To verify the identity of the bacteria, nodules from each inoculation treatment were surface-sterilized for 3 min in sodium hypochlorite, bacteria were extracted and resistance to antibiotics was determined.

RESULTS AND DISCUSSION

Rhizobium sp. BR816 Nod metabolites are sulphated

To determine whether Rhizobium sp. BR816 produced sulphated NFs, we radiolabelled apigenin-induced cell cultures with $^{14}$C acetate or $^{35}$S sulphate. Two methods of NF isolation and radiolabelling were used. We could not detect any $^{14}$C-labelled metabolites from apigenin-induced Rhizobium sp. BR816 cells following the method described by Laeremans et al. (1996) for the isolation of R. tropici CFN299 NFs from the induced cell supernatants. Rhizobium sp. BR816 probably excretes very small quantities of NFs, as has been reported previously for Rhizobium leguminosarum bv. trifolii by Orgambide et al. (1995). For other rhizobia such as R. tropici (Poupot et al., 1993; Laeremans et al., 1996) and Rhizobium etli (Poupot et al., 1995a) much higher amounts of NFs are excreted and detectable amounts of NF from both strains could be isolated by using only the cell supernatant of flavonoid-induced cultures. Therefore we tried the protocol published by Mergaert et al. (1993), using the total cell cultures instead of the cell supernatants only. After separation of the BR816 NFs on reverse-phase TLC plates (Fig. 1a), we could distinguish at least three apigenin-induced $^{14}$C-labelled metabolites, which were all sulphated. A possible fourth, differently migrating, flavonoid-induced metabolite was only $^{14}$C-labelled. Additional Nod metabolites, not detectable by this protocol due to their low amounts, may also have been present.

Isolation of the Rhizobium sp. BR816 nodP1Q1 genes

Since all bacteria that produce sulphated NFs seem to possess nodPQ genes for sulphate activation, we looked for homology to these genes in Rhizobium sp. BR816. An EcoRI genomic DNA digest of strain BR816, hybridized with an internal S. meliloti nodPQ fragment under low-stringency conditions, revealed the presence of two fragments with estimated sizes of 12 and 2.3 kb, respectively. An additional 4.3 kb EcoRI DNA fragment hybridized but at low intensity (Fig. 2). When we used a 450 bp fragment, obtained by PCR on BR816 total DNA as the template using primers P1 and P2 (Laeremans et al., 1996), an identical hybridization pattern was obtained except that the 4.3 kb EcoRI DNA fragment no longer hybridized (data not shown). Partial sequencing of the cloned 450 bp PCR fragment (P1-P2) revealed high homology to an internal S. meliloti nodP fragment (data not shown). We therefore considered that Rhizobium sp. BR816 contained at least two nodPQ copies.

By hybridization of a Rhizobium sp. BR816 plasmid library (van Rhijn et al., 1996) cloned in the EcoRI site of pVK100, against the internal BR816 nodP PCR fragment, we isolated several cosmids containing the 2.3 kb EcoRI fragment. One cosmid clone, pVK82, was retained for further experiments. It was used to subclone the 8 kb BamHI fragment with the complete nodPQ...
The homologous region and its physical map was determined (Fig. 3).

**Sequence determination and analysis of nodPQ1**

We sequenced a DNA section of 3282 nt from the 8 kb *BamH*I fragment (Fig. 3) and found two putative ORFs showing significant homology to *S. meliloti*, *Azospirillum brasilense*, *R. tropici*, and *Rhizobium* sp. N33 nodP and nodQ genes. We localized the putative start codon of nodP1 at 473 (numbers refer to the submitted GenBank sequence, U59507), preceded by a possible ribosome-binding site, GGGG, at 462. The stop codon of nodP1 (1369) overlaps the start codon of nodQ1 by one base as was found for the nodPQ genes of *S. meliloti* (Cervantes et al., 1989; Schwedock & Long, 1989), *A. brasilense* (Vieille & Elmerich, 1990) and *R. tropici* (Folch-Mallol et al., 1996; Laeremans et al., 1996) strains. The arctic *Rhizobium* sp. N33 is the only exception identified to date in which two cytosine residues separate the stop codon of nodP and the start codon of nodQ (Cloutier et al., 1996). The BR816 nodQ1 stop codon is localized at position 3271. The putative nodQ1 ribosome-binding site (GAGG) and start codon are separated by 10 nt. We found an additional putative 16S rRNA interaction site for nodP (CTCTT) and nodQ1 (TCT) 2 and 5 nt downstream of their start codons, respectively (Petersen et al., 1988). nodP1 and nodQ1 are 900 and 1905 nt long, respectively. The G+C content for nodP1 was 61 mol% and for nodQ1 65 mol%. BR816 NodP1Q1 shows highest homology to *S. meliloti* NodPQ, supporting the close phylogenetic relation between *S. meliloti* and *Rhizobium* sp. BR816 based upon the nucleotide sequence of 16S rRNA gene fragments (Hernández-Lucas et al., 1995). Table 2 shows the homologies between the deduced amino acid sequences of the known nodP and nodQ genes and the cysDNC genes from *E. coli*.

**Fig. 3.** Physical and genetic map of the BR816 nodP1Q1 region. Restriction sites: B, *BamH*I; X, *XbaI*; EV, *EcoRV*; Sa, *SacI*; S, *SalI*; E, *EcoRI*; P, *PstI*; Sp, *SphI*; H, *HindIII*; Sm, *Smal*; Bg, *BglII*. Arrows below the map show positions of primers P1, P2 and Q1C.

BR816 is more distantly related to the tropical broad-host-range strain *R. tropici* CFN299, although both strains have common characteristics such as a sulphated Nod factor and overlapping host ranges (Hernández-Lucas et al., 1995).

We identified a GTP-binding site in the amino-terminal half of BR816 NodQ1 (GxxxxxGK, DxxG and NxxD; Dever et al., 1987) while in the carboxy-terminal region of NodQ1 we localized an ATP-binding motif (GxxxxGK) and a PAPS motif [K(A/G)xGxxxx-(N/E)x(0 or 1)FT] (Satishchandran et al., 1992). These consensus sequences have also been reported for nodQ of *S. meliloti* (Cervantes et al., 1989), *R. tropici* (Folch-Mallol et al., 1996; Laeremans et al., 1996), *Rhizobium* sp. N33 (Cloutier et al., 1996) and *A. brasilense* (Vieille & Elmerich, 1990) and in CysNC of *E. coli* (Leyh et al., 1992). An alignment of nodQ-deduced amino acid sequences with elongation factors, as suggested by Cervantes et al. (1989), reveals for the BR816 NodQ1 sequence alignment conservation of an IT1 motif, conserved among elongation factors (Kohno et al., 1986). Together, these data support the putative ATP sulphurylase and APS kinase activity of the enzymes encoded by the isolated BR816 nodP1Q1 genes. We could not find a putative nod box motif or significant homology to an *E. coli* consensus promoter in the 472 nt sequence determined upstream of nodP1. No significant homology to other genes was found in this region, or in a 500 bp region that was partially sequenced downstream of nodQ2. No stable transcription termination signals were found in the 3282 nt sequenced. nodP1 and nodQ1 probably belong to the same transcription unit. However, the two genes could be translated independently, since both genes possess a putative ribosome-binding site.
Table 2. Matrix showing interbacterial NodP and NodQ similarity/identity

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<th>BR (299)</th>
<th>N33 (301)</th>
<th>Sm (299)</th>
<th>Rt (299)</th>
<th>Ab (301)</th>
<th>Ec (302)</th>
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<td>95/88</td>
<td>91/79</td>
<td>80/67</td>
<td>82/66</td>
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<td>80/67</td>
<td>81/69</td>
<td></td>
</tr>
<tr>
<td>Sm</td>
<td>100/100</td>
<td>92/78</td>
<td>78/64</td>
<td>81/68</td>
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</tr>
<tr>
<td>Rt</td>
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<td>81/62</td>
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<td>Ec</td>
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Mutation analysis of Rhizobium sp. BR816 nodP1

To investigate the biochemical role of the nodP1 gene on NF sulphation, we interrupted the BR816 nodP1 gene by inserting a Km resistance cassette in the SacI site (Fig. 3), and obtained CFNE205. This mutant (data not shown) did not have an altered NF TLC profile compared to that from the wild-type after induction with apigenin (Fig. 1a). Most likely, a second copy of nodPQ is responsible for the complementation of the nodP1 mutation, or the ORFs we characterized as nodPQ could be genes involved in providing precursors for the sulphurylation of amino acids such as cysteine or methionine, encoded by cysDNC in E. coli (Leyh et al., 1988), and are not involved in sulphation of Nod factors. Schwedock & Long (1992) demonstrated that S. meliloti contained, in addition to the two nodPQ copies for NF sulphurylation, an additional sulphur activation locus for amino acid sulphation (called the saa locus). We can exclude the possibility that our identified ORFs are essential for amino acid sulphation since CFNE205 still grows on minimal medium and therefore is not an amino acid auxotroph. To provide evidence that the gene is transcribed, we ligated the promoter region of nodP1 (on a 1 kb XbaI–PstI fragment, Fig. 3) to a promoterless gusA gene from vector pRG960sd (Van den Eede et al., 1992) and introduced the construct into BR816. The reporter gene was expressed but no change in expression level was obtained upon addition of apigenin (data not shown). As for the nodPQ genes of S. meliloti, A. brasilense and R. tropici, BR816 nodP1Q1 expression is probably nod-box-independent and at a low constitutive level. Additional support for the Rhizobium sp. BR816 nodP1Q1 functionality was obtained after the introduction of pRKBRPQ1 into CFNE200, a R. tropici CFN299 NodP- mutant. TLC profiles of the Nod metabolites of the transconjugant strain showed the production of sulphated NFs (data not shown). We assumed that the non-detectable effect of the BR816 nodP1 mutation on NF sulphation was due to complementation by a second copy of nodPQ.

Isolation and localization of Rhizobium sp. BR816 nodP2 and construction of a BR816 NodP1P2- mutant strain

Since we were not able to isolate the 12 kb EcoRI fragment from the BR816 plasmid library by using the P1–P2 PCR probe, we applied another strategy to isolate at least parts of nodP2Q2 from the 12 kb EcoRI fragment. We designed the primer Q1C, based upon the sequence of nodP1Q1, downstream of the unique internal nodP1 SacI restriction site (Fig. 3). When the primer was used for PCR in combination with primer P1, using total DNA of BR816 or CFNE205 as the template, the following fragments were obtained. For PCR on BR816, a fragment of 950 bp (for nodP1 and nodP2) was obtained. For PCR on CFNE205, a fragment of 950 bp (for nodP2) and 2350 bp (for mutated nodP1) was obtained. The 950 bp PCR fragment, obtained using total DNA of CFNE205 as the template, was cloned and its physical map was determined (data not shown). We identified a unique EcoRV restriction site in the middle of the 950 bp fragment. Furthermore, a segment of 450 bp, between primers P1 and P2, was completely sequenced (data not shown). When comparing its deduced amino acid sequence with the GenBank sequences, highest homology was obtained to an internal S. meliloti NodP fragment. Compared to BR816 nodP1, this sequence showed a nucleotide iden-
Fig. 4. Plasmid profile of *Rhizobium* sp. BR816 (a). Autoradiogram of a blotted plasmid profile of *Rhizobium* sp. BR816 CFNE205 (b) or CFNE207 (c) hybridized with the Km and the SpSm resistance cassettes, respectively.

ty of 85%. The deduced amino acid sequence homology and identity compared to the corresponding part of BR816 nodP1 was 94% and 91%, respectively. The G+C content of the 450 bp PCR fragment was 62 mol%, similar to the BR816 nodP1Q1 G+C content but higher than the G+C content of *R. tropici* nodulation genes (Laeremans et al., 1996). BR816 nodP2Q2 also possesses an overlap of 1 bp between the stop codon of nodP and the start codon of nodQ. To make sure that the fragment we isolated was localized on the 12 kb EcoRI DNA segment, we used the 950 bp PCR fragment from CFNE205 as a probe to hybridize against EcoRI-digested BR816 total DNA. Two hybridization bands were obtained identical to those obtained with the 450 bp PCR fragment using primers P1 and P2 with BR816 total DNA as the template (data not shown), but the 12 kb EcoRI fragment hybridized more strongly. Previously, van Rhijn et al. (1993) demonstrated the presence of two plasmids in strain BR816. By using the method described by Géniaux et al. (1995), we detected an additional plasmid (which we here call the megaplasmid) with a higher molecular mass. Using an internal nodP1 or the nodP2Q2 PCR fragment as a probe against a blot of a BR816 Eckhardt profile (Fig. 4a), we localized nodP1 on the megaplasmid and nodP2 on the symbiotic plasmid, pSym (data not shown).

To investigate the effect of a double nodP1P2 mutation in BR816 on the NF sulphation process, we inserted a SpSm resistance cassette into a BR816 nodP2 EcoRV restriction site, positioned a few base pairs downstream of the primer P2 annealing site, and constructed the double nodP1P2 mutant strain (CFNE207) by double homologous recombination in CFNE205. CFNE207 did not require additional cysteine in its growth medium to survive. To confirm the localization of both BR816 nodP copies, we hybridized a Southern blot of an Eckhardt profile against the Km and SpSm resistance cassettes. The Km cassette only hybridized with the megaplasmid (Fig. 4b) whereas the Sp/Sm probe only hybridized with the pSym (Fig. 4c), indisputably showing that, as in *S. meliloti*, the copies are localized on different plasmids. Surprisingly, no differences were found between the CFNE207 and the BR816 NF TLC profiles (Fig. 1).

Subsequent plant inoculation experiments with the common bean cultivar N-8-116 did not reveal statistically significant differences in nodule numbers or in nodule morphology between CFNE207 and the wild-type BR816 strain under any of the three different plant growth conditions tested (data not shown). In *S. meliloti*, mutations in the carboxy-terminal halves of nodP or nodQ still showed wild-type nodulation phenotypes (Schwedock & Long, 1989). A similar situation might have occurred for the *Rhizobium* sp. BR816 nodP2 mutant since the antibiotic resistance cassette is inserted in the 3' region of nodP2. In the event that BR816 nodP2 and nodQ2 do not belong to the same operon and the mutation in nodP2 provokes a truncated but active ATP sulphurylase not affecting a putative nodP internal nodQ promoter sequence, it is possible that the NF sulphur activation complex is still active, although the amount of PAPS for NF sulphation would be expected to be lower (as is the case for *S. meliloti* nodQ1 or nodQ2 single mutants), since the expressed nodP1 is effectively mutated. Roche et al. (1991) demonstrated by TLC and HPLC that in *S. meliloti* both nodPQ copies approximately equally contributed to NF sulphation. Additionally, we constructed a distinct BR816 nodQ2 deletion mutant by exchange of a nodQ2 internal SalI fragment for the SpSm resistance cassette, obtaining CFNE206. As for CFNE205 and CFNE207, the CFNE206 TLC profile did not show any detectable differences to that from the wild-type strain (data not shown). Cosmid BRVIID9, containing the BR816 nodP2Q2 genes, was mated into CFNE200. The transconjugant strain produced sulphated NFs (data not shown). A Southern hybridization of EcoRI-digested BRVIID9 DNA against a heterologous nodH probe revealed the presence of a nodH homologous region on the 12 kb EcoRI fragment that also contains the nodP2Q2 genes (data not shown). On the other hand, if BR816 nodP2 and nodQ2 do belong to the same transcriptional unit, the mutation in nodP2, due to its polarity, should affect nodQ2, assuming that the SpSm resistance cassette does not contain a promoter recognized by BR816, as is the case for some Tn5 insertions in *S. meliloti* genes (Corbin et al., 1983). As a consequence, no activated sulphur source provided by the BR816 nodPQ genes is present, if the two nodPQ copies of BR816 are the only genes responsible for NF sulphation. We therefore concluded that a third locus, encoding enzymes for sulphur activation and at least partially providing an activated sulphate source for NF sulphation, is likely to be present in BR816.

Localization of a third PAPS-producing locus

Previously, we isolated the *R. tropici* CFN299 cysDNC homologues, distinct from the nodHPCO NF sulphation locus (our unpublished results; Laeremans et al., 1996). The cosmid that carries these genes also contains sequences with significant homology to *E. coli* cysH, encoding a PAPS reductase in its amino acid sulphation pathway. This gene does not have a homologous counterpart in the NF sulphation pathway. When we probed Southern blots of EcoRI-digested total DNA of...
strain BR816 under low-stringency conditions against the *R. tropici* CFN299 cysDN, the 4.3 kb EcoRI fragment hybridized, with some signal also observed on the 12 kb EcoRI fragment. In contrast, with the *R. tropici* cysH homologue as a probe only the 4.3 kb EcoRI fragment was revealed (data not shown). From the three hybridizing fragments in Fig. 2, the 12 kb and 2.3 kb fragments showed strongest hybridization with a nodPQ probe, whilst the 4.3 kb fragment showed strongest hybridization with an internal fragment of cysH and cysD genes (T. Laeremans and others, unpublished).

*S. meliloti* also possesses two sulphur activation pathways: a symbiotic pathway (encoded by the nodPQ genes, present in two copies) and a housekeeping pathway. The genes encoding enzymes for the latter pathway are localized at the *saa* locus (Schwedock & Long, 1992), having functional homology to the *E. coli* cysDNC genes, necessary for the production of an activated sulphate donor (PAPS) in the amino acid sulphation pathway (Kredich, 1987). Apparently, both nodPQ and cysDNC (or *saa* in the case of *S. meliloti*) are responsible for the production of PAPS, but the NF sulphotransferase NodH cannot use the sulphate precursor provided by the *saa* locus since a *S. meliloti* double nodQ1Q2 mutant produces non-sulphated NFs (Roche et al., 1991). Why do two sulphate activation systems exist in rhizobia that produce sulphated NFs? Roche et al. (1991) hypothesized that an additional sulphate activation pathway is required for efficient and energy-saving Nod factor sulphation since nodPQ delivers an extra supply of PAPS at the appropriate time and cellular compartment. The ‘housekeeping’ PAPS is probably generated in the cytosol while the ‘symbiotic’ PAPS is produced in the periplasmic space or in the membrane system and no detectable transport of PAPS from the housekeeping to the symbiotic pathway occurs. Otherwise, the in *vivo* formation of the NF sulphurylation complex associating the ATP sulphurylase, the APS kinase and the sulphotransferase, wherein the intermediates are directly transferred to the next enzyme (Schwedock et al., 1994), could explain why in *S. meliloti* a mutation in the NF sulphation locus is not complemented by the amino acid sulphate activation pathway. Similarly, in the *E. coli* sulphur activation pathway, a PAPS synthetase complex is formed in which the intermediate APS is directly transferred between the gene products of cysDNC and cysC (Leyh et al., 1988). The metabolic channelling of substrates and/or a different localization of the enzymes of both sulphur activation pathways, and the inability to transport PAPS from the cytosol to the NF sulphation site, could explain why in *S. meliloti* or in *R. tropici* CFN299 no complementation of mutations in NF sulphurization precursor genes by the *saa* locus occurs. On the contrary, in *Rhizobium* sp. BR816, NodP, NodQ and NodH might not associate into a NF sulphation complex, leaving the possibility that ‘household’ PAPS could enter the NF sulphation pathway. Additionally, the BR816 NF sulphation machinery could be localized in the cytosol. Complementation of mutations in nodulation genes by housekeeping genes is not a new phenomenon. In *S. meliloti* (Baev et al., 1991) and *R. leguminosarum* (Marie et al., 1992), NodM, the enzyme for production of D-glucosamine synthetase, a precursor for the NF backbone, has a householome homologue, GlmS. Both enzymes can provide NF glucosamine precursors. In *Azorhizobium caulinodans*, a second fucosyltransferase, not encoded by *nodZ*, is also present since a mutation in *nodZ* does not completely abolish the existence of fucosylated NFs (Mergaert et al., 1996). Even for nodulation proteins involved in the export of the nodulation factor encoded by *nodI*, secondary proteins may exist with the same function, since *nodI* or *nodJ* mutants of *R. leguminosarum* still export small amounts of NFs (Spank et al., 1995).

In *R. tropici*, Poupout et al. (1993b) suggested a link between the symbiotic sulphate activation pathway and NF methylation. By introducing the *S. meliloti* nodPQ genes into the wild-type *R. tropici*, apart from sulphating all *R. tropici* NFs, the rate of the *R. tropici* NF N-methylation decreased. However, it cannot be excluded that such a link may also exist between the *R. tropici* household sulphate activation and NF methylation pathway. A *R. tropici* strain mutated in nodPQ lacks the symbiotic PAPS form (since its NFs are no longer sulphated) and still nodulates common bean (Laeremans et al., 1996). Since methylation of NFs is required for nodulation of bean (Waeltens et al., 1995), and is dependent on the presence of PAPS, it can be suggested that the PAPS source for NF methylation might come from the household sulphate activation system. Thus, for NF production, nodulation genes are indispensable, but at least in some strains household NFs can take over their functions, possibly even in the Nod factor sulphation pathway. Some important questions still remain unanswered. Why does complementation between the symbiotic and household sulphate activation systems not occur in both directions? When *S. meliloti* nodPQ genes are expressed at a high level, they can restore *E. coli* cysteine auxotrophs (Schwedock et al., 1994). Does a unidirectional cellular PAPS pump system exist in *S. meliloti”? Why do antibodies against *E. coli* CysC only react to NodQ and not to protein products of the *saa* locus from *S. meliloti* (Schwedock et al., 1994)?

In conclusion, this report provides the first evidence that a household locus can complement, at least partially but to a significant extent, mutations in genes responsible for NF sulphation. We are currently investigating the role of the *R. tropici* *saa* locus on NF sulphation and methylation.

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