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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

Symbiotic 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 Spaink, 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, Debellé *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.

Abbreviations: APS, adenosine 5′-phosphosulphate; LCO, lipo-chitin oligosaccharide; NF, Nod factor; PAPS, 3′-phosphoadenosine 5′-phosphosulphate; Ap, ampicillin; Gm, gentamycin; Km, kanamycin; Nal, nalidixic acid; Sm, streptomycin; Sp, spectinomycin; Tc, tetracycline.

The GenBank accession number for the sequence reported in this paper is U59507.

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 Dénarié *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′-phosphosulphate (APS) kinase (Schwedock *et al.*, 1994). The NodPQ enzyme complex activates the inorganic sulphate source via APS to 3′-phosphoadenosine 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.*, 1995; 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* (Debellé & 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; Staehelin *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); ampillicin (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 pMOS*Blue* 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-labelled 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 3 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 sacB* gene. Firstly, pJQ200SK was adapted by eliminating the unique *Sac*I restriction site (Laeremans *et al.*, 1996). To obtain the *Rhizobium* sp. BR816 *nodP1* mutant, the 1063 bp *Xba*I–*Pst*I fragment containing the *nodP1* 5′ region (see Fig. 3) was cloned into the pJQ200SK derivative from which the unique *Sac*I restriction site had previously been eliminated. For mutagenesis, the Km resistance cassette of pUC-4K was used. This cassette was isolated as a *Bam*HI restriction fragment and cloned into the *Bam*HI site of pIC20-R (Laeremans *et al.*, 1996). Subsequently, the Km resistance cassette cloned in this vector was obtained as a *Sac*I fragment and cloned into the unique *Sac*I 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

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
Rhizobium sp.		
BR816	Broad-host-range *Rhizobium* isolated from *Leucaena leucocephala*	Hungria *et al.* (1993)
CFNE205	Km^r NodP1$^-$ mutant of BR816	This study
CFNE206	Sp/Sm^r NodP2Q2 deletion mutant of BR816	This study
CFNE207	Km^r Sp/Sm^r NodP1P2$^-$ double mutant of BR816	This study
Rhizobium tropici		
CFNE200	Km^r NodP$^-$ mutant of *R. tropici* CFN299	Laeremans *et al.* (1996)
Plasmids		
pVK82	pVK100 carrying the BR816 *nodP1Q1* genes	This study
pVK100	IncP Km^r Tc^r broad-host-range cosmid	Knauf & Nester (1982)
BRVIID9	pLAFR1 carrying the BR816 *nodP2Q2* genes	This study
pRK7813	IncP Tc^r, mobilizable cosmid vector	Jones & Gutterson (1987)
pRKBRP1Q1	8 kb *Bam*H1 fragment containing the BR816 *nodP1Q1* genes cloned in pRK7813	This study
pUC18/19	Ap^r cloning vector	Norrander *et al.* (1983)
pJQ200SK	*B. subtilis SacB*-containing suicide vector, Gm^r	Quandt & Hynes (1993)
pJQ200mp18	*B. subtilis SacB*-containing suicide vector, Gm^r, with the pUC18 multiple cloning site	Quandt & Hynes (1993)
pLAFR1	IncP Tc^r, broad-host-range cosmid	Friedman *et al.* (1982)
pUC-4K	Vector containing Km resistance cassette	Pharmacia Biotech
pIC20-R	Ap^r cloning vector derived from pUC19	Marsh *et al.* (1984)
pMOS*Blue*	Ap^r cloning vector, suitable for direct cloning of PCR fragments	Amersham
pMOS*P1Q1C*	950 bp internal *nodP1Q1* PCR fragment of BR816 cloned in pMOS*Blue*	This study
pSmUC	Vector carrying an Sm/Sp resistance cassette	Murillo *et al.* (1994)
pIC20::Km	pUC-4K Km resistance cassette cloned in the *Bam*HI site of pIC20-R	Laeremans *et al.* (1996)

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, 5′ ACAGTCGCCCGATC-ARGGTCGATTTGCCGTC 3′ (derived from the sequenced BR816 *nodQ1* copy) into the pMOS*Blue* vector (Amersham). We then ligated the 2·2 kb *Hin*dIII fragment from pSmUC containing the Sp/Sm resistance cassette, previously blunted by using Klenow polymerase, into the unique *Eco*RV restriction site of the *nodP2* PCR fragment. Finally, we ligated the resulting 3·1 kb *Xba*I–*Bam*HI *nodP2*::Sp/Sm fragment in pJQ200SK. This construct was conjugated into CFNE205 to obtain the double *Rhizobium* sp. BR816 *nodP1P2* mutant (CFNE207). By hybridization with the *nodP2* gene and the Sp/Sm resistance cassette, the insertion of the cassette was demonstrated.

The single BR816 *nodQ2* mutant was constructed as follows. By hybridization of a BR816 genome library, previously cloned in the *Eco*RI site of cosmid pLAFR1 (P. van Rhijn & J. Vanderleyden, unpublished data) with the 1·5 kb *Pst*I fragment of BR816 *nodP1Q1* (Fig. 3) one positive clone, BRVIID9, was isolated that contained a 12 kb *Eco*RI fragment. A 1·7 kb *Sph*I subfragment containing part of *nodP2* and *nodQ2* was cloned into pIC20-R and the insert was cut out with restriction enzymes *Bgl*II and *Pst*I and subsequently cloned into *Bam*HI/*Pst*I-digested pJQ200mp18. A *nodQ2* internal 300 bp *Sal*I fragment, located around 100 bp from the *nodQ2* start codon, was then exchanged for a *Sal*I 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 (OD_{600} 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) [^{35}S]sulphate or 25 μCi (0·9 MBq) [2-^{14}C]acetic acid, as sodium salt, and the cells labelled for 15 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-18F_{254}s, Merck). We used H_2O/acetonitrile (1:1, v/v) as the mobile phase. The radioactive compounds were visualized by autoradiography using Hyperfilm βmax (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 Martínez *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.

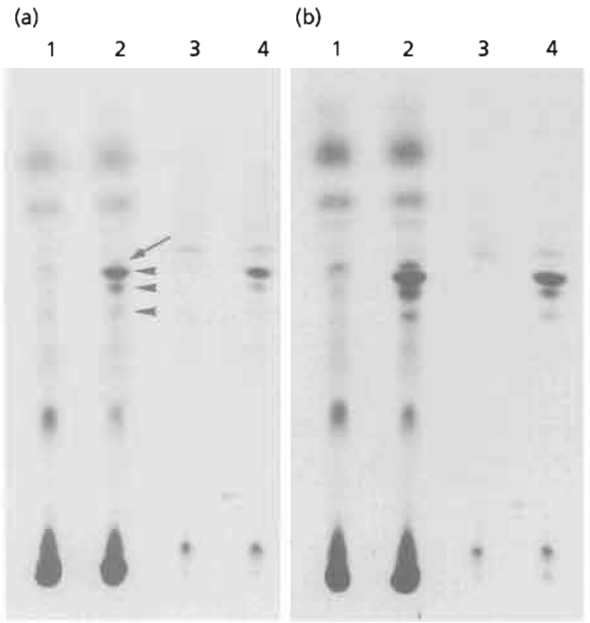

Fig. 1. Autoradiogram of a reverse-phase TLC profile of butanol extracts of radioactively labelled *Rhizobium* sp. BR816 (a) and CFNE207 (b). Lanes 1 and 2, ^{14}C-labelled; lanes 3 and 4, ^{35}S-labelled. Lanes 1 and 3, non-induced; lanes 2 and 4, apigenin-induced. Putative sulphated and non-sulphated NFs are indicated with arrowheads and an arrow, respectively.

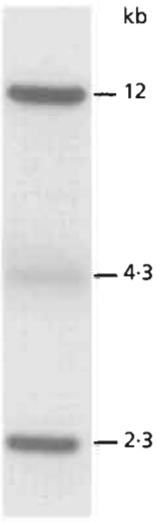

Fig. 2. Autoradiogram of Southern-blotted *Eco*RI-digested genomic DNA of *Rhizobium* sp. BR816 probed against a *S. meliloti* internal *nodPQ* gene fragment.

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 *Eco*RI 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 *Eco*RI 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 *Eco*RI 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 *Eco*RI site of pVK100, against the internal BR816 *nodP* PCR fragment, we isolated several cosmids containing the 2·3 kb *Eco*RI fragment. One cosmid clone, pVK82, was retained for further experiments. It was used to subclone the 8 kb *Bam*HI fragment with the complete *nodPQ*

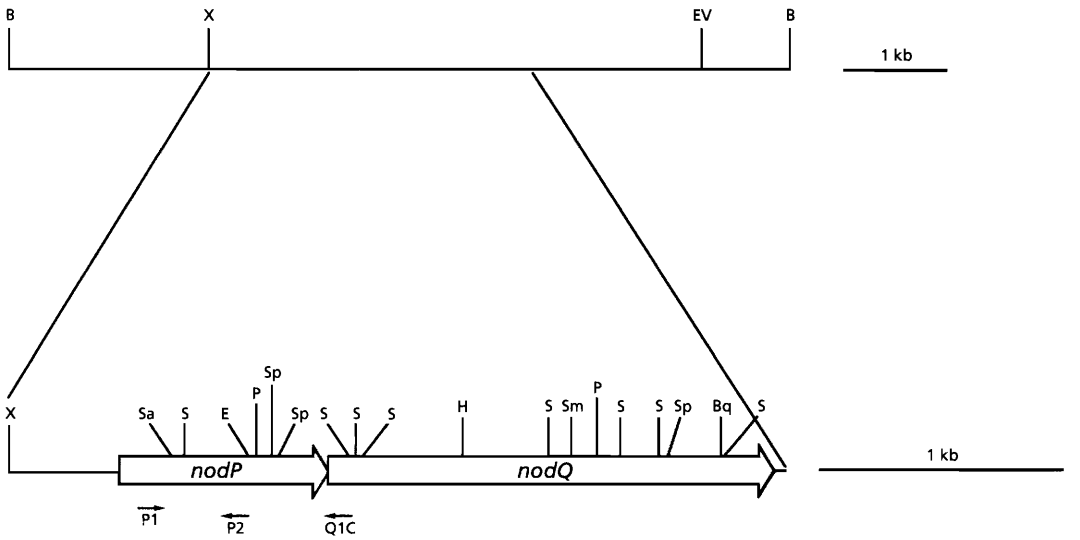

Fig. 3. Physical and genetic map of the BR816 *nodP1Q1* region. Restriction sites: B, *Bam*HI; X, *Xba*I; EV, *Eco*RV; Sa, *Sac*I; S, *Sal*I; E, *Eco*RI; P, *Pst*I; Sp, *Sph*I; H, *Hin*dIII; Sm, *Sma*I; Bg, *Bgl*II. Arrows below the map show positions of primers P1, P2 and Q1C.

homologous region and its physical map was determined (Fig. 3).

Sequence determination and analysis of *nodP1Q1*

We sequenced a DNA section of 3282 nt from the 8 kb *Bam*HI 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 *nodP1* (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*. *Rhizobium* sp. 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 (GxxxxGK, DxxG and NKxD; 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)xxGxxx-(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 ITI 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

Bacterial strains: BR, *Rhizobium* sp. BR816; N33, *Rhizobium* sp. N33; Sm, *Sinorhizobium meliloti*; Rt, *Rhizobium tropici*; Ab, *Azospirillum brasilense*; Ec, *Escherichia coli*; see text for references. Numbers in parentheses refer to the number of amino acid residues of the respective NodP or NodQ proteins. In *E. coli*, the NodP and NodQ homologues are named CysD and CysNC, respectively.

NodP:	BR (299)	N33 (301)	Sm (299)	Rt (299)	Ab (301)	Ec (302)
BR	100/100	86/76	95/88	91/79	80/67	82/66
N33		100/100	85/75	84/69	80/67	81/69
Sm			100/100	92/78	78/64	81/68
Rt				100/100	81/66	81/62
Ab					100/100	78/63
Ec						100/100
NodQ:	**BR (633)**	**N33 (646)**	**Sm (641)**	**Rt (632)**	**Ab (620)**	**Ec (677)**
BR	100/100	80/67	93/86	88/78	74/58	69/52
N33		100/100	79/65	79/63	77/59	72/55
Sm			100/100	87/77	74/57	68/51
Rt				100/100	74/57	69/50
Ab					100/100	70/53
Ec						100/100

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 *Sac*I 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 *Xba*I–*Pst*I 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 pRKBRP1Q1 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 *Eco*RI 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 *Eco*RI fragment. We designed the primer Q1C, based upon the sequence of *nodP1Q1*, downstream of the unique internal *nodP1 Sac*I 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 *Eco*RV 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-

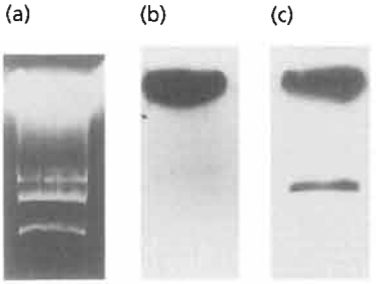

Fig. 4. Plasmid profile of *Rhizobium* sp. BR816 (a). Autoradiogram of a blotted plasmid profile of *Rhizobium* sp. BR816 CFNE205 (b) or CFNE207 (c) hybridized with the Km and the Sp/Sm resistance cassettes, respectively.

tity 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 *Eco*RI DNA segment, we used the 950 bp PCR fragment from CFNE205 as a probe to hybridize against *Eco*RI-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 *Eco*RI 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 Sp/Sm resistance cassette into a BR816 *nodP2 Eco*RV 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 Sp/Sm 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 *Sal*I fragment for the Sp/Sm 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 *Eco*RI-digested BRVIID9 DNA against a heterologous *nodH* probe revealed the presence of a *nodH* homologous region on the 12 kb *Eco*RI 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 Sp/Sm 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 *nodHPQ* 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 *Eco*RI-digested total DNA of

strain BR816 under low-stringency conditions against the *R. tropici* CFN299 *cysDN*, the 4·3 kb *Eco*RI fragment hybridized, with some signal also observed on the 12 kb *Eco*RI fragment. In contrast, with the *R. tropici cysH* homologue as a probe only the 4·3 kb *Eco*RI 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 household 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 *cysDN* 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 sulphation 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 household 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 *nodIJ*, secondary proteins may exist with the same function, since *nodI* or *nodJ* mutants of *R. leguminosarum* still export small amounts of NFs (Spaink *et al.*, 1995).

In *R. tropici*, Poupot *et al.* (1995b) 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 *nodP* 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 (Waelkens *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 genes 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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