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Identification of a Third Sulfate Activation System in *Sinorhizobium* sp. Strain BR816: the CysDN Sulfate Activation Complex

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***Sinorhizobium* sp. strain BR816 possesses two *nodPQ* copies, providing activated sulfate (3′-phosphoadenosine-5′-phosphosulfate [PAPS]) needed for the biosynthesis of sulfated Nod factors. It was previously shown that the Nod factors synthesized by a *nodPQ* double mutant are not structurally different from those of the wild-type strain. In this study, we describe the characterization of a third sulfate activation locus. Two open reading frames were fully characterized and displayed the highest similarity with the *Sinorhizobium meliloti* housekeeping ATP sulfurylase subunits, encoded by the *cysDN* genes. The growth characteristics as well as the levels of Nod factor sulfation of a *cysD* mutant (FAJ1600) and a *nodP1 nodQ2 cysD* triple mutant (FAJ1604) were determined. FAJ1600 shows a prolonged lag phase only with inorganic sulfate as the sole sulfur source, compared to the wild-type parent. On the other hand, FAJ1604 requires cysteine for growth and produces sulfate-free Nod factors. Apigenin-induced *nod* gene expression for Nod factor synthesis does not influence the growth characteristics of any of the strains studied in the presence of different sulfur sources. In this way, it could be demonstrated that the "household" CysDN sulfate activation complex of *Sinorhizobium* sp. strain BR816 can additionally ensure Nod factor sulfation, whereas the symbiotic PAPS pool, generated by the *nodPQ* sulfate activation loci, can be engaged for sulfation of amino acids. Finally, our results show that rhizobial growth defects are likely the reason for a decreased nitrogen fixation capacity of bean plants inoculated with *cysD* mutant strains, which can be restored by adding methionine to the plant nutrient solution.**

Sulfur is a macronutrient that is required by all organisms. It forms constituents of proteins, lipids, carbohydrates, electron carriers, and numerous cellular metabolites. Sulfate is the most abundant source of utilizable sulfur in the aerobic biosphere. The sulfate assimilation complex, required for the formation of the sulfur-containing amino acid cysteine, has been the subject of intensive study in *Escherichia coli* (21). Cysteine is the central precursor of all organic molecules containing reduced sulfur, ranging from the amino acid methionine to peptides, proteins, vitamins, cofactors such as *S*-adenosylmethionine, and hormones.

Like all inorganic nutrients, sulfate is transported into cells by highly specific membrane transport systems (18). Sulfate assimilation requires its prior activation to adenylate compounds via a pathway that seems to be similar in all organisms. The activation is achieved by the ATP sulfurylase-catalyzed reaction of sulfate with ATP to give adenosine 5′-phosphosulfate (APS), coupled with GTP hydrolysis. Subsequently, APS is phosphorylated by an APS kinase to produce 3′-phosphoadenosine-5′-phosphosulfate (PAPS). In *E. coli*, ATP sulfurylase is encoded by *cysD* and *cysN*, whereas the APS kinase is encoded by *cysC* (27, 28). PAPS is then enzymatically reduced by the *cysH*-encoded PAPS reductase (also known as PAPS sulfotransferase) to sulfite, which enters the cysteine biosynthetic pathway.

PAPS also serves directly as a sulfate donor for the formation of sulfated compounds. For example, *Rhizobium*-legume symbiotic interactions are mediated by a host-specific bacterial signaling molecule (the Nod factor), which can be sulfated. In general, rhizobial species that produce sulfated Nod factors possess at least two sulfate activation systems (6, 12, 24, 25, 40). The three genes that are indispensable for Nod factor sulfation, *nodP*, *nodQ*, and *nodH*, were first isolated from *Sinorhizobium meliloti*. Together, *nodP* and *nodQ* encode both ATP sulfurylase and APS kinase activities (45, 47), whereas the *nodH* gene product, a sulfotransferase, directly transfers the activated sulfate moiety to the Nod factor backbone (8, 44). NodP is homologous to *E. coli* CysD, while the amino- and carboxy-terminal domains of NodQ are homologous to *E. coli* CysN and CysC, respectively. In a recent study, it was reported that the specificity of phytopathogen-host interactions also can be controlled by a sulfated avirulence effector molecule, which is yet to be identified (48). The rice pathogen *Xanthomonas oryzae* pv. oryzae RaxP and RaxQ proteins are responsible for the synthesis of an activated form of sulfate and are similar to the NodP and NodQ host specificity proteins of the bacterial symbiont *S. meliloti*.

In *S. meliloti*, two copies of the *nodPQ* operon are present. Both copies are involved in Nod factor sulfation but are not necessary for cysteine biosynthesis. Recently, in *S. meliloti* and in *Rhizobium tropici* CFN299, homologues of the *cysDN* (ATP sulfurylase) and *cysH* (APS reductase) genes were isolated, but no homologue of the *E. coli cysC* gene (APS kinase) could be identified (1, 23). Consequently, it was demonstrated that in *S. meliloti*, APS rather than PAPS is reduced for sulfite production during cysteine biosynthesis (1). Other members of the

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Reference or source
Sinorhizobium sp. strains		
BR816	Broad-host-range *Sinorhizobium* strain isolated from *Leucaena leucocephala*	16
FAJ1600	*cysD* mutant of BR816; Tc^r	This study
FAJ1604	*nodP1 nodQ2 cysD* triple mutant of BR816; Km^r Sp^r Tc^r	This study
CFNE205	*nodP1* mutant of BR816; Km^r	25
CFNE206	*nodQ2* deletion mutant of BR816; Sp^r	25
CFNE207	*nodP1 nodP2* double mutant of BR816; Km^r Sp^r	25
CFNE208	*nodP1 nodQ2* double mutant of BR816; Km^r Sp^r	T. Laeremans, unpublished results
Plasmids		
pBRE4.8	pUC19 carrying the BR816 *cysDN* genes; Ap^r	This study
pJQ200uc1	*B. subtilis sacB*-containing suicide vector; Gm^r	39
pHP45Ω-Tc	Vector containing Tc^r cassette	38
pUC18/19	Cloning vector; Ap^r	33

Rhizobiaceae, differing in their ability to incorporate sulfate in either a Nod factor or lipopolysaccharide, also preferentially reduce APS instead of PAPS for cysteine biosynthesis. This implies that APS reduction is not necessarily correlated with the presence of PAPS-dependent sulfurylation reactions for symbiosis, which is the case when functional *nodPQ* genes are present (1). Recently, Kopriva et al. (20) have described a phylogenetic classification of APS and PAPS reductase amino acid sequences (both annotated as CysH) from different organisms. The resulting sequence-based prediction of the substrate specificities of these enzymes was confirmed by Williams et al. (58), using genetic complementation experiments.

Sinorhizobium sp. strain BR816 (formerly *Rhizobium* sp. strain BR816) synthesizes Nod factors that are fully sulfated at the reducing terminal residue (50), as is the case for the narrow-host-range *S. meliloti* (26). The sulfate decoration on the Nod factors secreted by *S. meliloti* is essential for nodulation of alfalfa (40). Except for *S. meliloti*, it is still unclear whether rhizobia producing sulfated Nod factors use only the *nodPQ*-dependent PAPS pool as a source of activated sulfate for Nod factor sulfation, the housekeeping PAPS pool, or both (25). Previously, Laeremans et al. (25) demonstrated that *Sinorhizobium* sp. strain BR816 possesses two *nodPQ* copies. Although both copies are functional, as demonstrated by genetic complementation of an *R. tropici nodP* mutant, the double mutants did not show any detectable changes in the amount of sulfated Nod factors produced by this strain (25). It was suggested that in *Sinorhizobium* sp. strain BR816, in contrast to *S. meliloti*, a housekeeping locus as a third PAPS-producing locus could be involved in the sulfation of the Nod factors.

We have isolated the *cysDN* homologues of *Sinorhizobium* sp. strain BR816 and studied the role of this third PAPS-producing locus in relation to Nod factor synthesis. In addition, we were interested to know how the various forms of activated sulfate may be partitioned into the pathways for amino acid biosynthesis and sulfation or methylation of Nod factors and other compounds important during symbiosis. Furthermore, based on the analysis of the phylogenetic relationship among rhizobial ATP sulfurylases, we speculate on the possible origin and functionality of genes for sulfate activation.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were maintained on Luria-Bertani agar at 37°C and grown in Luria-Bertani broth (32). Rhizobial strains were maintained on yeast extract-mannitol medium (55) or on tryptone-yeast medium with added $CaCl_2$ (3) at 30°C. Antibiotics were added to the medium as needed at the following concentrations (micrograms per milliliter): ampicillin, 100; spectinomycin, 50; kanamycin, 50; and nalidixic acid, 31. Tetracycline was added to a final concentration of 1 μg/ml (for *Sinorhizobium* sp. strain BR816) or 10 μg/ml (for *E. coli*). Triparental conjugations and site-directed mutagenesis were done as previously described (31).

Nucleic acid manipulations and analysis. Isolation and cloning of plasmid DNA was performed as described previously (2, 42). Total genomic DNA of *Sinorhizobium* sp. strain BR816 was isolated by using a genomic DNA isolation kit (Gentra Systems) according to the manufacturer's instructions. DNA fragments were recovered from agarose gels by using the Nucleotrap kit (Macherey-Nagel). Southern blotting and hybridizations were carried out as previously described (25). Sequencing of DNA fragments cloned in the pUC18-pUC19 vectors was performed on an automated ALF sequencer with fluorescein-labeled universal and synthetic oligonucleotide primers (Amersham Pharmacia Biotech, Uppsala, Sweden). Database searches for similarity were performed with the BLAST software (National Center for Biotechnology Information, National Institutes of Health).

PCR was performed with *Taq* DNA polymerase (Boehringer, Mannheim, Germany) according to the manufacturer's protocol. For sequencing, the high-fidelity Platinum *Pfx* DNA polymerase (GIBCO-BRL, Life Technologies) was used according to the manufacturer's protocol.

To construct a genomic minilibrary, total genomic DNA from *Sinorhizobium* sp. strain BR816 was digested with *Eco*RI. DNA fragments ranging between 4 and 6 kb were recovered and ligated into the pUC19 cloning vector. Eight hundred Ap^r white colonies were picked up. Plasmid DNA was purified from 15 pools consisting of approximately 50 colonies, and efficient insertion of fragments of the desired size was confirmed. A 450-bp PCR fragment containing an internal part of *cysD* was used as a probe to screen the library.

Phylogenetic analysis of CysD homologues. The amino acid sequences of 19 CysD-like proteins, truncated to the same size as the shortest sequence (position 3 to 299 from the *S. meliloti* NodP1 sequence [gi14523565]) were aligned by using the ClustalW program (http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html). The construction of neighbor-joining trees (41) and bootstrap analysis of 1,000 resamples were performed by using the Treecon for Windows (1.3b) software package (53). In estimating evolutionary distances between amino acid sequences, we used the Poisson correction. Insertions and deletions were not taken into account. For constructing trees by the parsimony method, the PROTPARS program in the PHYLIP package was used (10). Again, bootstrap analysis of 1,000 resamples was performed.

Growth tests. Growth tests of *Sinorhizobium* sp. strain BR816 in sulfate-free liquid medium were carried out in acid minimal salts (AMS) medium (36) containing 1 mM $CaCl_2$ with sulfate salts replaced by equimolar amounts of alternative salts ($MgCl_2$, $ZnCl_2$, $MnCl_2$, and $CuCl_2$). Ammonium chloride (10

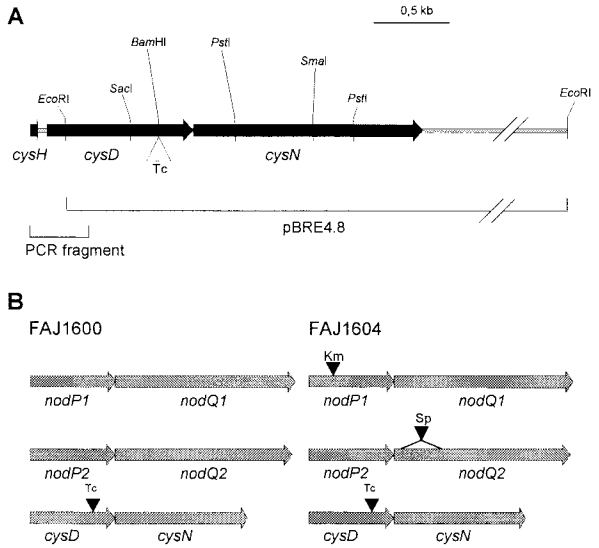

FIG. 1. (A) Physical and genetic maps of the BR816 *cysDN* region. The triangle indicates the position of the inserted Tc^r cassette in the mutants FAJ1600 and FAJ1604. (B) Schematic view of the constructed *Sinorhizobium* sp. strain BR816 mutants with mutations in the *nodPQ* genes and *cysDN* genes (see Table 1). Triangles indicate inserted antibiotic resistance cassettes.

mM) and mannitol (10 mM) were used as nitrogen and carbon sources, respectively. Sulfur compounds (sodium sulfate, sodium sulfite, L-cysteine, and L-methionine) were filter sterilized and added to the autoclaved medium at a concentration of 25 μM. When appropriate, cell cultures were induced with 500 nM apigenin. Cells of the strains tested were grown overnight in tryptone-yeast medium, washed twice in sulfate-free AMS medium, brought to an optical density of 0.4 (measured at 600 nm with a Perkin-Elmer lambda 2 spectrometer), and diluted 6,000-fold in sulfate-free AMS medium with the appropriate concentrations of filter-sterilized antibiotics, apigenin, and sulfur compounds. Bacteria were grown in microtiter plates (final volume, 300 μl) over a 4-day period, and cell growth was monitored automatically by measuring the optical density at 600 nm in BioscreenC (Labsystems) every 30 min. For each time point, the average optical density was calculated from five independent measurements.

Insertion mutagenesis. A *Sinorhizobium* sp. strain BR816 *cysD* single mutant and *nodP1 nodQ2 cysD* triple mutant were constructed as follows. To obtain the *cysD* single mutant, the 1.6-kb *Sma*I fragment of pBRE4.8 was ligated into the *Sma*I site of pJQ200uc1. This vector allows positive selection of double homologous recombinants on sucrose (10%)-containing medium due to the presence of the *Bacillus subtilis sacB* gene. The resulting plasmid was digested with *Bam*HI and then blunt-end ligated to the *Sma*I fragment containing the Ω-Tc^r cassette from pHP45Ω-Tc. This plasmid was conjugated to *Sinorhizobium* sp. strain BR816. Correct insertion of the Tc^r interposon was verified by Southern hybridization with the *cysD* gene and the Tc^r cassette as probes. In this way, the same construct was introduced in CFNE205 (*nodP1*), CFNE206 (*nodQ2*), CFNE207 (*nodP1 nodP2*), and CFNE208 (*nodP1 nodQ2*) (Table 1; Fig. 1). A *cysD* single mutant (FAJ1600) and a *nodP1 nodQ2 cysD* triple mutant (FAJ1604) were obtained and retained for further analysis.

Radioactive labeling of Nod metabolites and thin-layer chromatography (TLC) analysis. Nod factors were labeled by using the isotopes [^{14}C]acetate and [^{35}S]sulfate according to a slightly modified version of the protocol of Mergaert et al. (30), as previously described (25). For this experiment, Nod factors were purified from cells grown in sulfate-free AMS minimal medium supplemented with L-cysteine, as described for the growth tests.

Plant nodulation assay. Seeds of *Phaseolus vulgaris* cv. BAT477 were surface sterilized and germinated as described previously (56). Bean seedlings were planted in 250-ml flasks containing a nitrogen-free Snoeck medium agar slant (C. Snoeck, J. Vanderleyden, and E. Schrevens, submitted for publication) with KH_2PO_4 (7.49 mM), K_2SO_4 (0.43 mM), $CaCl_2$ (2.65 mM), $MgCl_2$ (1.75 mM), $MgSO_4$ (1.2 μM), FeNaEDTA (50.8 μM), $MnSO_4$ (35.2 μM), $CuSO_4$ (0.5 μM), $ZnSO_4$ (1.5 μM), H_3BO_3 (25 μM), and $(NH_4)_6Mo_7O_{24}$ (0.07 μM), with sulfate as the sole sulfur source unless otherwise stated. The seedlings were inoculated with approximately 10^6 bacteria per plant, from a diluted overnight culture that was washed twice with sulfate-free AMS medium. The plants were maintained in a growth chamber at 26°C (day) and 22°C (night) with a 12-h photoperiod. Plants were harvested after 3 weeks. Uninoculated control plants did not show any nodules or nodule-like structures. Ten plants per strain were tested in each experiment. Nitrogenase activity was determined by measuring the acetylene reduction activity of nodulated roots in closed vessels with a Hewlett-Packard 5890A gas chromatograph equipped with a PLOT fused silica column, with propane as an internal standard.

Data analysis. In all experiments, a randomized block design was used with 10 replicate blocks. Nodule number, nodule dry weight, and acetylene reduction activity were analyzed with the means and general linear model procedure (SAS Institute, Cary, N.C.). Comparison among the mean values obtained for each strain was made by Tukey's multiple-range test with a 95% confidence limit.

Nucleotide sequence accession number. Nucleotide sequence data were deposited in the GenBank database under accession number AJ505754.

RESULTS

Cloning and sequencing of a third PAPS-producing locus in *Sinorhizobium* sp. strain BR816. Previous work provided evidence for the presence of a putative third PAPS-producing locus in *Sinorhizobium* sp. strain BR816 on an approximately 4.8-kb *Eco*RI genomic DNA fragment (25). In order to clone this third copy of sulfate activation genes, a genomic minilibrary was constructed (see Materials and Methods), and a single positive clone, pBRE4.8, was obtained. Since the inserted genomic DNA region corresponding to the *cysD* gene was incomplete, the missing part of *cysD* was obtained by PCR with primers that were designed based on existing knowledge of the genomic organizations and DNA sequences of sulfate assimilation genes in other *Rhizobium* spp. (1, 23).

A physical map of the 4.8-kb *Eco*RI fragment and the upstream 442-bp PCR fragment was established (Fig. 1A), and the nucleotide sequence was determined. Similarity with an ATP sulfurylase encoded by the *cysD* and *cysN* genes of *S. meliloti*, *R. tropici* CFN299, and *E. coli* was found. Partial sequence similarity upstream of the *cysD* gene revealed the presence of a *cysH* homologue, encoding an APS or PAPS reductase, whereas no *cysC* homologue was found in the sequenced fragment. The same organization is found in *S. meliloti* and *R. tropici* (1, 23). It is likely that all three open reading frames are in a single operon, since no promoter consensus sequences or transcription termination signals were found in the intergenic *cysH-cysD* sequence of BR816. A similar situation was observed in *S. meliloti*, where two transcriptional start sites were identified, both upstream of the *cysH* homologue (1). In contrast, in *E. coli*, *cysH* does not form an operon with *cysDNC* (21). The *nodP* and *nodQ* homologues have a lower percent G+C content than the *cysD* and *cysN* homologues (data not shown), as observed for the *S. meliloti* genome (13).

The *Sinorhizobium* sp. strain BR816 *cysD* and *cysN* genes encode proteins of 317 and 498 amino acids, respectively. Strong conservation of amino acid residues was found with the respective CysD and CysN proteins of *S. meliloti* (96 and 91% identity, respectively), *R. tropici* (89 and 82% identity), and *E. coli* (68 and 52% identity). CysN contains the characteristic GTP-binding motif (GxxxxGK, DxxG, and NKxD) (7) and also an ITI motif, which is conserved among elongation factors (19). In comparison to the NodQ peptides, the deduced amino acid sequence of *cysN* lacks the carboxy-terminal part that corresponds to *E. coli* CysC. Therefore, no ATP-binding or PAPS-binding motifs were found. Similar observations were

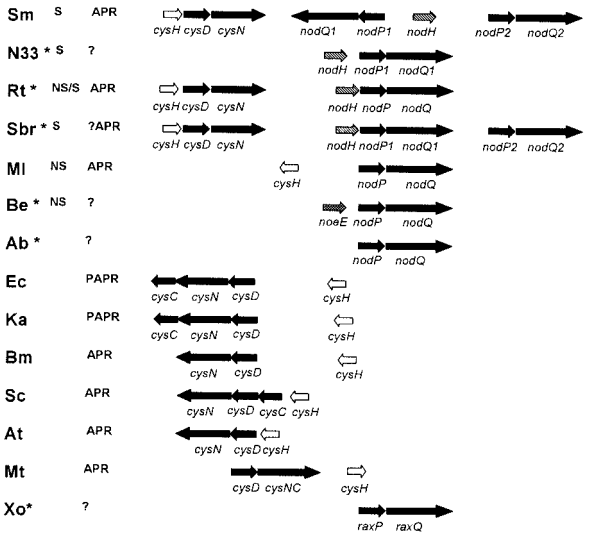

FIG. 2. Schematic representation of sulfate assimilation loci of selected strains for construction of a phylogenetic tree (Fig. 3). Abbreviations: Sm, *S. meliloti* (NodP1, gi14523565; NodP2, gi15140612; CysD, gi5911360); Sbr, *Sinorhizobium* sp. strain BR816 (NodP1, gi2148989; NodP2, gi27125923; CysD, gi24528409); Rt, *R. tropici* CFN299 (NodP, gi1280528; CysD, gi7387610); Bm, *Brucella melitensis* (CysD, gi17988038); N33, *Mesorhizobium* sp. strain N33 (NodP, gi1531624); Ml, *Mesorhizobium loti* (NodP, gi13476292); Be, *Bradyrhizobium elkanii* (NodP, gi14209498); Ab, *Azospirillum brasilense* (NodP, gi142424); Ec, *E. coli* (CysD, gi12517206); Ka, *Klebsiella aerogenes* (CysD, gi11992146); Xo, *X. oryzae* pv. oryzae (NodP, gi21105248); Mt, *Mycobacterium tuberculosis* (CysD, gi15608425); Sc, *Streptomyces coelicolor* (CysD, gi21224427); At, *Agrobacterium tumefaciens* (CysD, gi15155798). S, fully sulfated Nod factors; S/NS, mixture of sulfated and nonsulfated Nod factors; NS, nonsulfated Nod factors; APR, APS-reducing activity; PAPR, PAPS-reducing activity; ?APR, putative APR-reducing activity; ?, APS or PAPS reductase activity unknown; ❋, genome sequence not (fully) determined. Similar open reading frames are shaded identically. Note that *nodP1* of *S. meliloti* is located on megaplasmid 1, *nodP2* is on megaplasmid 2, and *cysHDN* is chromosomally located. *nodP1* of *Sinorhizobium* sp. strain BR816 is located on a megaplasmid, *nodP2* is on the symbiotic plasmid, and *cysHDN* is chromosomally located.

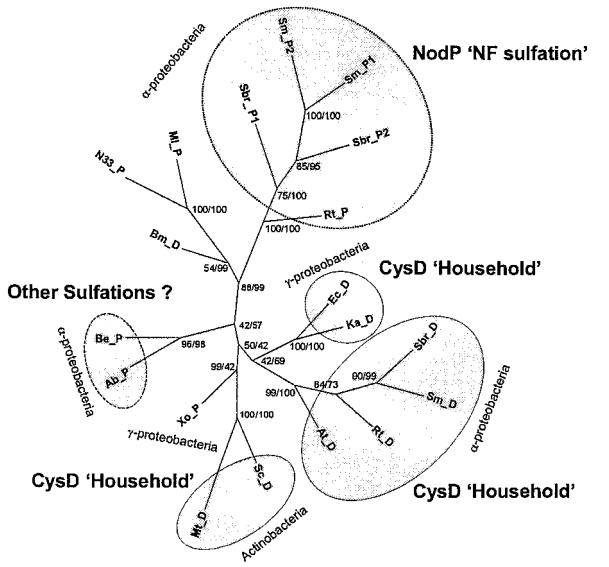

FIG. 3. Phylogenetic relationships among *cysD* gene products. The tree topology was inferred by using the protein parsimony method. Numbers represent the bootstrapping score (9) over 1,000 trials (parsimony/distance). The abbreviations of the species are as for Fig. 2.

made for *S. meliloti* and *R. tropici*. In summary, these data support the ATP sulfurylase activity of the putative proteins encoded by the isolated BR816 *cysDN* genes.

Phylogenetic analysis of CysD and CysN homologues. The BR816 CysD and CysN ATP sulfurylase subunits were compared through multiple-sequence alignment (ClustalW) with homologous ATP sulfurylases subunits retrieved from GenBank. The genomic organizations of the different sulfate assimilation loci of the strains selected for the phylogenetic analysis are schematically drawn in Fig. 2. Phylogenetic analysis of *cysD* and *nodP* gene products by the protein parsimony method resulted in a maximum-parsimony tree, as shown in Fig. 3. An identical tree topology could be inferred by using the neighbor-joining method (data not shown). Similar phylogenetic relationships could be deduced after construction of a phylogenetic dendrogram of CysN and NodQ protein sequences by using either the neighbor-joining method or protein parsimony analysis (data not shown).

It can be observed that the CysD and NodP ATP sulfurylase subunits of *Rhizobium* spp. producing sulfated Nod factors (Fig. 2), which have been shown to be involved in amino acid biosynthesis (1, 23) and Nod factor sulfation (24, 25, 47), respectively, cluster in two different groups (Fig. 3). The CysD protein of *Sinorhizobium* sp. strain BR816 clearly belongs to the protein cluster involved in biosynthesis of sulfur-containing amino acids, supporting its putative function.

Two other "household" clusters could be distinguished, i.e., the γ-*Proteobacteria* clade and the *Actinobacteria* clade. Interestingly, only one gene copy coding for a sulfate activation complex has been described, for *Mycobacterium tuberculosis* (*cysDNC*) (Fig. 2) (58). The sulfate assimilation pathway of *Mycobacterium tuberculosis* proceeds from sulfate through APS (catalyzed by CysDN), which is converted by APS reductase (CysH) in the first step toward cysteine and methionine. APS can also be converted to PAPS, through the action of the APS kinase CysC, and serves as a substrate for sulfotransferases that produce sulfolipids, which putatively function as virulence factors (58). Similarly, APS and PAPS pools are generated through the enzymatic activity of RaxP and RaxQ in *X. oryzae* pv. oryzae and are used for both cysteine synthesis and sulfation of avirulence effector molecules (48).

The CysD-homologous proteins of some members of the *Rhizobiaceae* (among which are *Mesorhizobium loti*, producing nonsulfated Nod factors [29, 34]; *Mesorhizobium* sp. strain N33, producing sulfated Nod factors [35]; and the pathogen *Brucella melitensis*) seem to belong to another cluster. However, these proteins are still more closely related to the NodP Nod factor sulfation cluster than to the CysD household cluster, as defined above. *Brucella melitensis* was previously shown to be genetically closely related to *Rhizobium* spp. (14). Intriguingly, the respective *Bradyrhizobium elkanii* and *Azospirillum brasilense* ATP sulfurylase subunits constitute a separate cluster (Fig. 3). The *nodPQ* genes of *B. elkanii* are situated within a gene cluster comprising genes for symbiotic functions (*fixGHIS* and *noeE*) as well as genes involved in rhizobitoxin

biosynthesis (59). Since the *B. elkanii* Nod factors are not sulfated (4, 43), these genes do not function in Nod factor biosynthesis. The recently finished genome sequencing of the p90 plasmid of *A. brasilense* sheds new light on a possible function of its *nodPQ* copy, which is located within a region carrying genes involved in polysaccharide synthesis (E. Vanbleu and J. Vanderleyden, unpublished results). It was previously shown that *A. brasilense* does not synthesize Nod factors and that deletion of the *nodPQ* copy does not lead to auxotrophy (54). Therefore, it can be speculated that this cluster encompasses proteins belonging to a novel functionality group.

Growth characteristics of *Sinorhizobium* sp. strain BR816 *cysD* mutants under free-living conditions. To investigate the biochemical role of the isolated *cysDN* genes of BR816, the BR816 *cysD* gene was mutated (see Materials and Methods). First, the *cysD* mutants were tested for cysteine auxotrophy. In addition, we were interested to know whether a *cysD* mutation could be complemented by one or both *nodP* copies of *Sinorhizobium* sp. strain BR816. Growth of the wild type and various mutants with mutations in *nodPQ* and/or *cysDN* (FAJ1600, FAJ1604, CFNE205, CFNE206, CFNE207, and CFNE208) was examined in liquid sulfate-free AMS medium supplemented with various sulfur sources (see Materials and Methods). It could be demonstrated that the BR816 *nodPQ* single or double mutants (CFNE205, CFNE206, CFNE207, and CFNE208) exhibit growth patterns similar to that of the wild-type strain in minimal medium with sulfate as the sole sulfur source (data not shown). Therefore, it can be concluded that *nodPQ* mutants are not auxotrophs. Growth of the *cysD* mutant (FAJ1600) with sulfate as the sole sulfur source was clearly affected compared to that of the wild-type strain (Fig. 4A). FAJ1600 showed a prolonged lag phase, although its generation time in exponential growth phase did not markedly differ from that of the wild type. The *nodP1 nodQ2 cysD* triple mutant (FAJ1604) was completely impaired in growth (Fig. 4A). In the presence of sulfite, cysteine, or methionine, the growth of both mutants after 60 h was nearly restored to the wild-type level (Fig. 4B to D). This indicates that the *cysDN* genes are effectively involved in the biosynthesis of sulfur-containing amino acids, more specifically in the step of the sulfate assimilatory pathway just before the reduction of activated sulfate to sulfite. From this experiment we can conclude that knocking out the three sulfate activation systems (FAJ1604) in *Sinorhizobium* sp. strain BR816 leads to cysteine auxotrophy.

Interestingly, the growth characteristics of FAJ1600 showed a course similar to that of the wild type after a certain time interval. This demonstrates that the PAPS pool generated by the NodPQ sulfate activation complex is accessible for reduction by CysH and thus is available for the biosynthesis of sulfur-containing amino acids. The growth delay of FAJ1600 might indicate that CysH of *Sinorhizobium* sp. strain BR816 preferentially shows APS reductase activity rather than PAPS reductase activity toward the formation of sulfite. Moreover, the APS reductase activity of CysH has been recently confirmed in many rhizobial species (1, 20).

One should consider that (i) the growth curves of the wild-type and mutant strains were monitored under conditions in which no Nod factors are produced (no flavonoid induction) and (ii) *nodP2*, which is localized in the nodulation region on

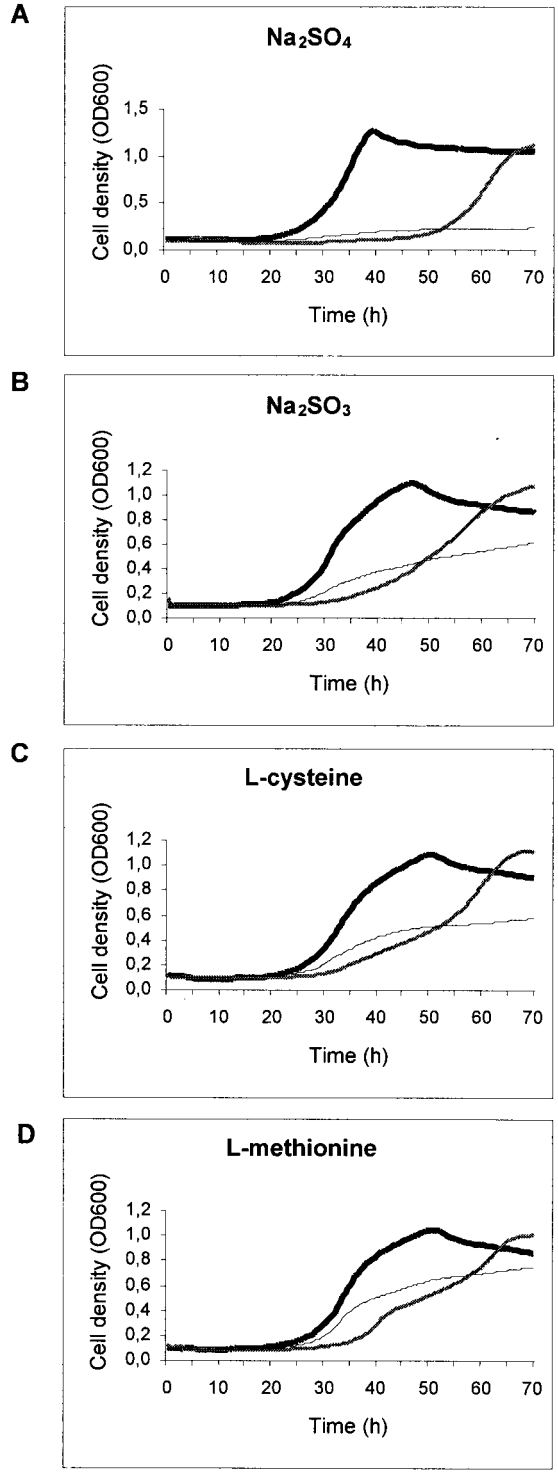

FIG. 4. Effects of various sulfur sources on cell growth of *Sinorhizobium* sp. strain BR816 wild-type and mutant strains determined by measuring optical density at 600 nm (OD600) in a BioscreenC instrument over a 4-day period. Thick black line, BR816; gray line, FAJ1600; thin gray line, FAJ1604). Cultures were grown at 30°C in sulfate-free AMS medium supplemented with sodium sulfate (A), sodium sulfite (B), L-cysteine (C), or L-methionine (D) at a concentration of 25 μM. Each experiment was conducted three times. Results from one experiment are shown.

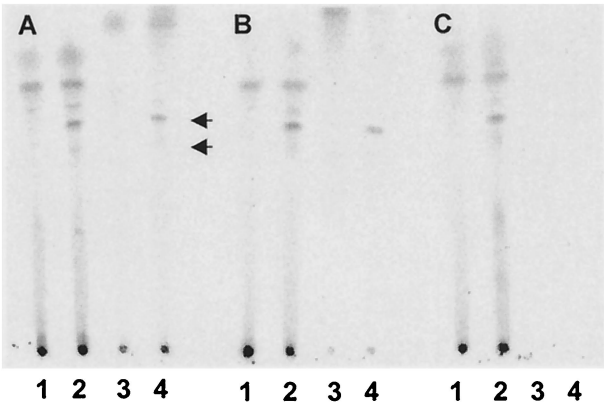

FIG. 5. Autoradiogram of a reverse-phase TLC profile of butanol extracts of radioactively labeled *Sinorhizobium* sp. strain BR816 (A), FAJ1600 (B), and FAJ1604 (C). Lanes 1 and 2, ^{14}C labeling; lanes 3 and 4, ^{35}S labeling. Lanes 1 and 3, noninduced; lanes 2 and 4, apigenin induced. Spots representing sulfated Nod factors are indicated with arrows.

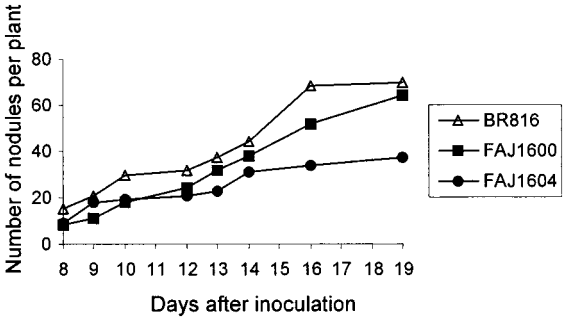

FIG. 6. Nodulation kinetics of *P. vulgaris* BAT477 inoculated with *Sinorhizobium* sp. strain BR816 wild-type and mutant strains. Two independent experiments were set up, and the results of one experiment are shown.

the symbiotic plasmid, probably is *nod* box dependent and thus not expressed (49). Therefore, to investigate whether the simultaneous production of sulfated Nod factors affects growth characteristics of the *cysDN* mutant strains, similar growth tests were performed in the presence of the *nod* gene inducer apigenin and with sulfate as the sole sulfur source. In this case, similar growth courses were obtained for FAJ1600 and FAJ1604 compared to the wild type (data not shown). This implies that at least the expressed *nodPQ* copy can complement and is sufficient for growth of FAJ1600 in minimal medium with sulfate as the sole sulfur source. The use of higher concentrations of inducer did not have a significant effect on the growth curves of the strains tested.

Nod factor sulfation pattern of *Sinorhizobium* sp. strain BR816 *cysD* mutants. Since the available *nodPQ* single and double mutants of *Sinorhizobium* sp. strain BR816 (CFNE205, CFNE206, CFNE207, and CFNE208 [Table 1]) were not auxotrophic and still produced sulfated Nod factors, Laeremans et al. (25) speculated that the housekeeping *cysDN*(*C*) genes can complement mutations in genes responsible for Nod factor sulfation. In order to determine to what level the Nod factors produced by the wild-type strain and the mutant strains FAJ1600 and FAJ1604 were still sulfated, apigenin-induced cell cultures, grown in liquid sulfate-free AMS medium supplemented with cysteine, were labeled with [^{14}C]acetate or [^{35}S]sulfate, and butanol extracts of the cell cultures were analyzed by reverse-phase TLC. Separation of the BR816 Nod factors revealed the presence of apigenin-induced spots on the chromatogram, corresponding to the Nod factors of BR816 (Fig. 5). The triple mutant FAJ1604 no longer produced sulfated Nod factors, which is in clear contrast with the sulfated Nod factor pattern of both the wild-type strain and FAJ1600 (Fig. 5). These results indicate that an activated sulfate source needed for Nod factor sulfation is no longer present. It can be concluded that the *cysDN* sulfate assimilation locus does provide active sulfate for NF sulfation.

Symbiotic phenotype of *cysD* mutants. The *Sinorhizobium* sp. strain BR816 *cysD* mutants were tested for their ability to nodulate common bean (*P. vulgaris* cv. BAT477) and to fix nitrogen. No significant differences in the kinetics of appearance of the first nodules were observed (Fig. 6). However, FAJ1600 (*cysD*) as well as FAJ1604 (*nodP1 nodQ2 cysD*) showed a decreased nodule number per plant over time, but only for FAJ1604 was this difference significant at the 95% level (Tukey's test). Morphologically, the nodules of both mutant strains were generally smaller with apparently less leghemoglobin present (as judged by the absence of pink color).

To study the nitrogen fixation capacity of the nodulated roots, the acetylene reduction activity was measured. The acetylene reduction activity of 21-day-old nodules induced by FAJ1600 or FAJ1604 was significantly lower than that for the wild-type strain ($P < 0.05$; Tukey's test) (data not shown). When methionine was added to the plant nutrient solution, the nitrogen fixation per plant was restored to wild-type levels. Interestingly, supplementation with methionine resulted in an overall higher nitrogen fixation capacity of *P. vulgaris* cv. BAT477 inoculated with *Sinorhizobium* sp. strain BR816 (data not shown).

DISCUSSION

In this study, a third APS-producing locus of the broad-host-range strain *Sinorhizobium* sp. strain BR816 was isolated. The nucleotide sequence of this region was determined, and based on homology searches, *cysD* and *cysN* were identified. Like in *S. meliloti*, no *cysC* homologue could be isolated downstream from *cysDN*. This is an indication that, like in other rhizobia, APS rather than PAPS is reduced to sulfite for cysteine biosynthesis (1). The highest similarity was found with the *cysDN* homologues in *S. meliloti*, supporting the close phylogenetic relationship between *S. meliloti* and *Sinorhizobium* sp. strain BR816 (15). Phylogenetic analysis revealed that CysD does not cluster with NodP1 and NodP2. The two BR816 NodP proteins are closely related and could have originated from a recent gene duplication, as was proposed for the NodP proteins of *S. meliloti* (13). Within the α-*Proteobacteria* clade, two clusters of proteins are clearly functionally distinguished and were designated NodP Nod factor sulfation and CysD household. It has been demonstrated that the *nodPQ* genes are also required for sulfation of *S. meliloti* lipopolysaccharide, proving a dual functionality of members of the NodP Nod factor sulfation cluster

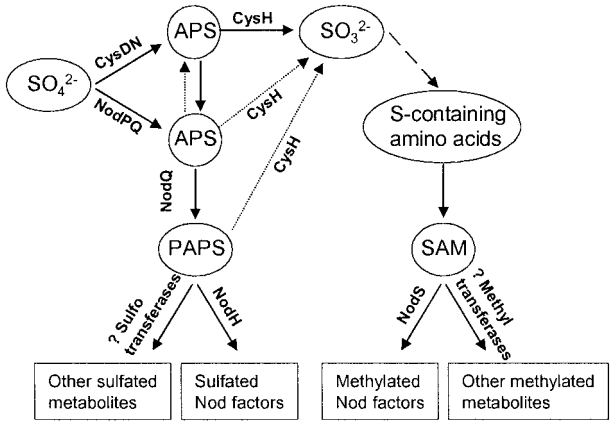

FIG. 7. Schematic representation of the distribution of APS and PAPS for sulfation and methylation processes in *Sinorhizobium* sp. strain BR816. Dotted arrows indicate possible but less favorable enzyme activity.

(5, 17). A potential new NodP-like protein cluster is proposed, comprising proteins involved in sulfate activation for sulfation of compounds that are yet unknown but which could be important during symbiosis. Other closely related CysD and NodP homologous do not fit into a specific functionality group, since these proteins are involved either in sulfation of amino acids (*M. loti* and *B. melitensis*) or in sulfation of Nod factors (*Mesorhizobium* sp. strain N33). It should be noted that within the γ-*Proteobacteria* clade and the *Actinobacteria* clade, only one copy of genes encoding sulfate activating enzymes is present, which seems to be involved in biosynthesis of sulfur-containing amino acids as well as sulfation of other macromolecules.

We examined the effect of a *cysD* mutation under free-living conditions in a wild-type chromosomal background and in a *nodP1 nodQ2* double mutant background. The levels of Nod factor sulfation (Fig. 5) as well as the growth characteristics (Fig. 4) of the different mutants were determined. In this study, we could demonstrate that the household CysDN sulfate activation locus of BR816 can additionally ensure Nod factor sulfation, whereas the symbiotic (P)APS pool, generated by the *nodPQ* sulfate activation complexes, can be engaged for sulfation of amino acids. Figure 7 shows a model of how the various forms of activated sulfate in *Sinorhizobium* sp. strain BR816 may be partitioned into the pathways for amino acid biosynthesis and sulfation of Nod factors and other compounds that might be important during symbiosis. The *cysDN*-dependent APS pool supplies activated sulfate that is subsequently reduced to form sulfite by the CysH APS reductase. Sulfite is further reduced to sulfide, which is then incorporated into the cysteine and methionine biosynthesis pathway. Our data suggest that the symbiotic APS and/or PAPS pool, created by the *nodPQ*-dependent sulfate activation step, can also be used by CysH (in a less efficient manner) for the biosynthesis of sulfur-containing amino acids, when needed. Moreover, both household and symbiotic APS pools can be mutually exchanged. In *S. meliloti*, the *nodPQ*- and *cysDN*-encoded sulfate activation systems cannot substitute for each other (46, 47).

Why would *Sinorhizobium* sp. strain BR816 possess three functional sulfate activation systems for Nod factor sulfation? Besides the use of activated sulfate for the biosynthesis of sulfur-containing amino acids and sulfation of Nod factors, (P)APS is needed for Nod factor methylation (37). Introduction of the *S. meliloti nodPQ* genes into *R. tropici* resulted in a decreased rate of *R. tropici* Nod factor methylation, while all *R. tropici* Nod factor backbones were sulfated. Waelkens et al. (57) showed that methylation of Nod factors is required for nodulation of bean. In *Sinorhizobium* sp. strain BR816, the three operational sulfate-activating systems could play an important role in maintaining substitutions of bacterial determinants for symbiosis.

An *R. tropici nodPQ* mutant (producing drastically reduced amounts of sulfated Nod factors) and an *R. tropici nodH* mutant (producing nonsulfated Nod factors) still activate the signaling cascade for emergence of effective nodules on *P. vulgaris* roots (12, 24). For bean plants, the sulfate moiety of the Nod factor was shown to be involved in the efficiency of nodule formation but appears not to be essential (11, 22). The effects of the *cysD* mutant FAJ1600 and the *nodP1 nodQ2 cysD* triple mutant FAJ1604 on bean symbiosis were seen mainly in the reduction of nodule number per plant. Since under free-living conditions, a *cysDN*-dependent biosynthesis of sulfur-containing amino acids is essential to allow optimal growth of *Sinorhizobium* sp. strain BR816 with sulfate as the sole sulfur source, bacterial growth defects are likely the main reason for the decreased nitrogen fixation of bean plants inoculated with the mutants FAJ1600 and FAJ1604. These defects can be restored by the addition of methionine to the plant nutrient solution. We propose that at the early stages of the nodulation, the plant root exudates of the germinated seedlings provide enough sources of organic sulfur to allow bacterial growth. However, a shortage of an organic sulfur source like methionine impairs bacterial growth inside the plant. Inoculation experiments with a *Rhizobium etli metZ* (*O*-succinylhomoserine sulfhydrylase for methionine biosynthesis) (51) mutant on bean plants resulted in the formation of ineffective (Nod^+ Fix^-) nodules, which suggested that root cells do not supply the inoculant bacteria with enough methionine. The fact that supplemented methionine resulted in an overall higher nitrogen fixation capacity of *P. vulgaris* BAT477 inoculated with BR816 strains supports this hypothesis. In contrast to our observations, an *R. etli cysG* (siroheme synthetase for cysteine biosynthesis) mutant, which is able to induce the formation of effective nodules (Nod^+ Fix^+) on the roots of common bean, seems to dispose of an organic sulfur source like cysteine or glutathione to allow growth inside the plant (52).

How can the strictly separated symbiotic and endogenous (P)APS pools in *S. meliloti* versus the complementary (P)APS pools in *Sinorhizobium* sp. strain BR816 be explained? Presumably, the *nodPQ* genes arose in ancestral rhizobial strains through duplications of the endogenous *cysDNC* genes. Later, these *nodPQ* genes evolved toward more specialized symbiotic genes, whereas the endogenous *cysC* gene, encoding the APS kinase, was apparently lost during evolution. At this stage, complementation between both PAPS pools was still possible (the case of *Sinorhizobium* sp. strain BR816). Then, the genetic separation of the two sulfate-activating systems could have further evolved into two more efficient and energy-saving sep-

arate enzymatic multienzyme complexes (the case of *S. meliloti*).

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