Research Note

Analysis of a Symbiosis-Specific Cytochrome P450 Homolog in *Rhizobium* sp. BR816

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Sequence analysis of the DNA region upstream of *nodO* in *Rhizobium* sp. BR816 revealed an open reading frame in which the deduced amino acid sequence shows homology with cytochrome P450. Because the BR816 P450 homolog shows 73% amino acid similarity with CYP127A1(Y4vG), which is identified on the symbiotic plasmid of *Rhizobium* sp. NGR234, it is named CYP127A2. Transcriptional analysis of *CYP127A2* revealed high expression in bacteroids, whereas no or hardly any expression was observed under free-living conditions. Low-level, free-living expression, however was noticed when cells were grown microaerobically at acid pH levels. A number of possible substrates that may induce P450 gene expression were analyzed, but only the addition of short-chain alcohols to cultures slightly increased *CYP127A2* expression. High levels of *CYP127A2* expression observed in bacteroids of a *nifH* mutant strain, which formed non-fixing nodules on bean, indicated that the genuine substrate for CYP127A2 is not a metabolite resulting from N₂-fixation. Nevertheless, expression analysis pointed to a NifA- and σ^{54} -dependent transcription.

Additional keywords: *gusA*, *Phaseolus vulgaris* cv. Carioca and Negro Jamapa, *Rhizobium etli* CNPAF512.

In addition to the well-characterized genes that are important for nodulation, the synthesis of polysaccharides, and nitrogen fixation (Kijne et al. 1992), there are other rhizobial determinants that influence the overall fate of symbiosis (Luyten and Vanderleyden 2000; Niner and Hirsch 1998; Perret et al. 2000). *Rhizobium* sp. BR816 is a broad-host-range strain isolated from the nodules of the tropical leguminous tree *Leucaena leucocephala* that is able to nodulate a wide range of tropical leguminous plants, including *Phaseolus vulgaris* L. (Hernández-Lucas et al. 1995). The DNA region of *Rhizobium* sp. BR816 under study is part of a megaplasmid region represented by the cosmid clone pBRF2, which was isolated by its capacity to confer a host-range extension of

Rhizobium etli CE3 (only nodulating bean) to *L. leucocephala*. Analysis of this pBRF2 clone revealed the presence of the *nodO* gene, which is sufficient for the Nod⁺ phenotype of the CE3 transconjugants on *Leucaena* spp. (Van Rhijn et al. 1996). Introduction of the complete pBRF2 clone in *R. etli* CE3, however, appeared necessary for the formation of efficiently nitrogen-fixing nodules on *Leucaena* spp. (Vlassak et al. 1998). It seems, therefore, that additional determinants present on pBRF2 are necessary for the formation of an efficient nitrogen-fixing symbiosis between *R. etli* CE3 transconjugants and *L. leucocephala*. Sequence analysis of a 5-kb DNA region upstream of the earlier described *nodO* gene in *Rhizobium* sp. BR816 (Van Rhijn et al. 1996; Vlassak et al. 1998) was conducted by use of overlapping restriction fragments of pBRF2 subcloned in pUC18/19. Double-stranded DNA sequencing of these DNA fragments in pUC18/19 constructs was carried out with the AutoRead sequencing kit (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) based on the dideoxynucleotide chain-termination method (Sanger et al. 1977). Subsequently, the sequencing reaction products were separated with an automated A.L.F. sequencer (Amersham Pharmacia Biotech). Nucleotide sequences were analyzed with the PC/GENE software package (Intelligenetics, Mountain View, CA, U.S.A.), revealing an ORF412 with good conservation to P450 homologs. Immediately downstream of ORF412, four additional open reading frames (ORFs) were identified. Comparison of these ORFs with other entries in the GenBank database are indicated in Table 1. The nucleotide and amino acid sequence data are deposited at the GenBank database, accession no. U26451. The BR816 ORF412-deduced amino acid sequence shows 73% of identical amino acids with CYP127A1 (Y4vG), a P450 homolog identified in NGR234 (Fig. 1). On the basis of sequence similarities, P450s have been classified in different families (CYP). A P450 protein sequence from one gene family usually is defined as having $\leq 40\%$ amino acid identity with a P450 protein from another family (Nelson et al. 1993; Nelson et al. 1996). Therefore, ORF412 is named CYP127A2 (D. R. Nelson, *personal communication*). Whereas the P450 homologous region in NGR234 is part of a *nif* and *fix* cluster (Freiberg et al. 1996; Freiberg et al. 1997), the *CYP127A2* region in BR816 is flanked by *nodO* and *nodD2* (Van Rhijn et al. 1993; Van Rhijn et al. 1996). Cytochrome P450 enzymes are present in almost all living organisms and are involved in the metabolism of a

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wide variety of compounds. The P450 enzyme is a monooxygenase, which means it is able to “activate” molecular oxygen, catalyzing the incorporation of one of the two oxygen atoms into a broad spectrum of substrates, whereas the other oxygen atom is released as H₂O (White and Coon 1980). It is important in the oxidative, peroxidative, and reductive metabolism of numerous endogenous compounds (e.g., steroids, fatty acids, phytoalexins, and plant hormones) as well as that of the xenobiotics in the environment. The most intensively studied bacterial P450 system is P450cam from *Pseudomonas putida* (Mueller et al. 1995), which is able to metabolize the toxic compound camphor. The number of newly identified P450 homologs increases rapidly, and analysis of different identified P450 enzymes indicates that they have individual characteristics of regulation and substrate specificity (Munro and Lindsay 1996). The first bacterial P450 was isolated from the bacteroids of *Bradyrhizobium japonicum* (Appleby 1967). In *B. japonicum*, a cluster of three P450 genes was identified: *CYP112*, *CYP114* and *CYP117* (Tully et al. 1998), of which the deduced amino acid sequences show 83 to 93% similar amino acids to three other P450 homologs, Y4kV, Y4IC, and Y4ID, respectively, which were identified on pNGR234a. The identified CYP112 and CYP114 are present in cells grown under anoxic conditions as well as in bacteroids, but neither protein is present in free-living, oxically grown *B. japonicum* cells (Tully and Keister 1993). Therefore, the authors proposed a possible role during symbiosis, although no symbiotic phenotype was observed after inoculation of the respective mutants on their natural host. A possible biochemical role for the P450 cluster present in *B. japonicum* has been assigned on the basis of a hypothetical substrate specificity of different identified enzymes encoded by four ORFs found downstream of the P450 cluster. The presence of three P450 genes and putative genes encoding a ferredoxin, an alcohol dehydro-

genase, a prenyltransferase, and two possible prenylcyclases allowed the authors to suggest a function in the synthesis of gibberellins (Tully et al. 1998). Gibberellins may have a role during symbiosis because the application of gibberellins on *Lotus japonicum* induces nodule-like structures (Kawaguchi et al. 1996). In the *Rhizobium* sp. BR816 P450 cluster, a putative ferredoxin and short-chain dehydrogenase have been identified.

No other genes similar to the latter ones identified in *B. japonicum* seem to be associated with the region studied in BR816. Unfortunately, we were not able to propose a possible function for the CYP127A2 region on basis of the identified homologies. In order to identify the role of CYP127A2 in *Rhizobium* sp. BR816, *CYP127A2* gene expression was analyzed under different growth conditions. To analyze the expression of *CYP127A2*, a *CYP127A2*–*gusA* fusion was constructed by insertion of a promoterless *gusA*–*aph* cassette into the genomic *CYP127A2* gene, resulting in FAJ1403, a P450 gene mutant of BR816 (Table 2). The 4.2-kb *Sma*I fragment of pBRF2 containing a part of ORF412 was ligated in the suicide vector pJQ200uc (Fig. 1). For mutagenesis, a *gusA*–*aph* interposon of pWM6 (Metcalf and Wanner 1993) was used to inactivate the *CYP127A2* in the *Eco*RI site, resulting in pFAJ1403 and pFAJ1404 with the *gusA*–*aph* cassette oriented in the same or opposite direction. These were introduced in BR816 by triparental mating with pRK2073 as helper. Double homologous recombination could be monitored by resistance to 5% sucrose and kanamycin. The mutation was verified by Southern hybridization with a specific ORF412 gene fragment and the Km resistance cassette as probes (Sambrook et al. 1989). With the use of these mutants, expression of the P450 gene under different growth conditions was tested by measuring β -glucuronidase activity. For analysis of *CYP127A2* gene expression, bacterial strains were grown in acid minimal salt

Table 1. Amino acid sequence similarities between open reading frames (ORFs) found in this study with proteins present in the GenBank database

ORF name	Homology		Reference	Identical ^a	Similar ^a
	ORF or protein	Possible function–organism source			
ORF412 CYP127A2	CYP127A1	Probable oxidoreductase of <i>Rhizobium</i> sp. NGR234	Freiberg et al. 1997	73	82
	P450cam	Camphor monooxygenase of <i>Pseudomonas putida</i>	Unger et al. 1986	33	50
	CYP112	P450 monooxygenase of <i>Bradyrhizobium japonicum</i>	Tully and Keister 1993	27	48
ORF86	Rv1786	Hypothetical protein of <i>Mycobacterium tuberculosis</i>	Cole et al. 1998	38	59
ORF196	FdxD	Probable ferredoxin of <i>M. tuberculosis</i>	Cole et al. 1998	36	54
	Y4vH	Hypothetical protein of <i>Rhizobium</i> sp. NGR234	Freiberg et al. 1997	71	83
	LinA	Gamma-hexachloro-cyclohexane-dehydrochlorinase of <i>Pseudomonas paucimobilis</i>	Nagata et al. 1993	32	44
ORF538	Y4vI	Putative short-chain-type dehydrogenase of <i>Rhizobium</i> sp. NGR234	Freiberg et al. 1997	58	78
	FabG	3-oxoacyl-(ACP) reductase of <i>Thermotoga maritima</i>	Nelson et al. 1999	38	60
	NodG	Nodulation protein of <i>Sinorhizobium meliloti</i>	Debellé and Sharma 1986	36	52
ORF354	Y4vJ	Putative monooxygenase, similar to LuxA/B of <i>Rhizobium</i> sp. NGR234	Freiberg et al. 1997	80	98
	LuxA	Alkanal monooxygenase of <i>Vibrio harveji</i>	N. Ramaiah, J. Ravel, J. Chun, W. L. Straube, R. T. Hill, and R. R. Colwell, unpublished	29	46

^a Percentage of amino acids with identity or similarity on total number of amino acids compared.

(AMS) medium (Poole et al. 1991) supplemented with the appropriate antibiotics.

In a first series of experiments to monitor *CYP127A2* gene expression, an overnight culture of FAJ1403 or FAJ1404 was diluted 10 times in different media and grown overnight under atmospheric O₂ or under 0.3% O₂, eventually adding 500 mM apigenin (data not shown). The β -glucuronidase activity was measured spectrophotometrically with *p*-nitrophenyl- β -D-glucuronide (Jefferson 1987). Units were calculated, as defined by Miller (1972). To evaluate the *CYP127A2*-*gusA* expression during symbiosis, surface-sterilized (Vincent 1970), pregerminated seeds of *L. leucocephala* were planted in polypropylene cups containing a vermiculite-sand mixture (2:1) in 1/2-diluted Norris medium (Waelkens et al. 1995), whereas pregerminated Carioca seeds were planted in bottles containing a Fahreus medium agar slant (Vincent 1970). The seedlings were inoculated with 100 μ l of the overnight rhizobial culture under study. The plants were maintained in a plant growth chamber at 26°C day–22°C night temperatures and a 12-h photoperiod. At harvest, nodules were cut longitudinally and stained for 2 h, as described by Vlassak et al. (1998), or bacteroids were isolated and GusA activity was measured (Michiels et al. 1998). Under free-living conditions, there was only a very low level of GusA activity (15 ± 3 Miller units) after overnight incubation under oxic or microoxic conditions. High expression (1324 ± 94 Miller units) of *CYP127A2*, however, was noticed in FAJ1403 bacteroids isolated from Carioca nodules. Similar results were observed in *B. japonicum*, where a tenfold higher expression of a P450-*gusA* fusion was observed under symbiotic growth conditions in comparison to the level of expression observed under free-living oxic conditions, although a sixfold higher expression level was also observed under free-living anoxic conditions (Tully et al. 1998). In *Rhizobium* sp. NGR234, *CYP127A1* is expressed only at a low level in bacteroids isolated from determinate nodules of *Vigna unguiculata*, whereas no expression was observed in bacteroids isolated from the indeterminate nodules of *Cajanus cajan* (Perret et al. 1999). Analysis of *CYP127A2* gene expres-

sion in nodule sections showed that it is clearly expressed in determinate- and indeterminate-type nodules isolated from *P. vulgaris* and *L. leucocephala*, respectively. Analysis of plant phenotypes 4 (bean) or 8 weeks (*Leucaena*) after inoculation by determination of the nodule number, nodule dry weight, and acetylene reduction activity showed that although high expression of *CYP127A2* occurs in bacteroids, the BR816 *CYP127A2* mutant strain did not show an altered phenotype after inoculation on its natural host, *Leucaena* spp. Likewise, in *B. japonicum*, no discernible phenotype of the P450 mutant was observed. Because the authors assigned a possible role for P450 in gibberellin synthesis in bacteroids, they proposed that plant made gibberellins can compensate for the *B. japonicum* P450 mutation, resulting in an unimpaired phenotype (Tully et al. 1998). Inoculation of FAJ1403 on two different *P. vulgaris* cultivars (Carioca and Negro Jamapa), however, resulted in a decrease of the number of plants that effectively formed nitrogen-fixing nodules, although all plants still formed nodules (Table 3). Moreover, a reduced acetylene reduction activity of nodules induced on the Negro Jamapa cultivar by FAJ1403 in comparison to the wild-type nodules was observed.

Many cytochrome P450 genes are up-regulated following exposure of the cell to an inducing compound. Often this inducer is a substrate of the P450 enzyme and, in the case of xenobiotics, induction of P450 enhances detoxification. Consequently, identification of a possible inducer might lead to the elucidation of the possible function of P450 during symbiosis. In mammals, P450 catalyses the detoxification of drugs and the synthesis of steroid hormones. In plants, the role of P450 in the phenylpropanoid pathway, which results in the synthesis of lignin intermediates, phytoalexins, and flavonoids as well as fatty acid biosynthesis, terpenoid biosynthesis, and detoxification of xenobiotics is well documented (Schuler 1996). Because aromatic compounds can induce P450 genes, several flavonoids were tested for their inducing capacity. Flavonoids differ in the number and position of OH groups and, therefore, different types of flavonoids were tested. For the study of possible substrates, an overnight culture of the

Fig. 1. Genetic and physical map of the P450 region in *Rhizobium* sp. BR816 (top), with the position and orientation of the different open reading frames identified, and comparison with the corresponding region in *Rhizobium* sp. NGR234 (bottom). The percentage of identical amino acids present in two homologous sequences are indicated.

Rhizobium sp. BR816-derived FAJ1403 cells was diluted ten-fold and exposed to 500 nM flavonoids. Tested compounds were flavones, including apigenin, 7,4'-dihydroxyflavone, chrysin, luteolin and quercetin, flavanones such as naringenin and hesperitin, and the isoflavone genistein. These compounds are known to be potent *nod* gene inducers and act as growth regulators for certain rhizobial strains (Vlassak and Vanderleyden 1996). Vanillin, an exudate of wheat, and umbelliferone (7-OH-coumarin) induce *nodABC* gene expression of *Rhizobium* sp. NGR234 and were included in the study (Le Strange et al. 1990). Trigonelline, which is related to betaine compounds and reported to be catabolized by *Rhizobium* spp. strains (Boivin et al. 1990) and coumarin, being a precursor of lignins, also were investigated. In addition, soybean flour and one of its isoflavonoid components, genistein, were included because they are reported to induce the *Streptomyces griseus* P450 gene (Sariaslani and Kunz 1986).

In the search for a multivalent inducer for hydrocarbon-oxidizing microbial P450s, Asperger et al. (1994) proposed the

simple-structured n-hexane with a sterically flexible backbone. Therefore, n-hexane also was tested as an inducer in these experiments (6 g of n-hexane per liter). As positive control, the BR816 *nodO-gusA* fusion strain FAJ1506 was included because it shows expression in oxic and microoxic conditions when induced with apigenin (Vlassak et al. 1998). Strains were grown overnight in the presence of the different potential inducers under microoxic and oxic conditions. Under all of the conditions tested, we could not detect enhanced expression of *CYP127A2*, although clear expression of the *nodO* gene (FAJ1506) in oxic and microoxic conditions in the presence of the appropriate inducer was noticed (results not shown). This is in accordance with spot inoculation of *L. leucocephala* with a *nodA-gusA* fusion, which clearly resulted in blue staining at 6 days postinoculation, whereas no expression was noticed on roots inoculated with the *CYP127A2-gusA* fusion FAJ1403. Therefore, it was concluded that plant phenolic compounds do not seem to induce the BR816 P450 system. In addition, these experiments also exclude a possible role for

Table 2. Bacterial strains and plasmids

Strain-plasmid	Characteristics	Reference
<i>Escherichia coli</i> DH5 α	<i>supE44</i> Δ lacU169 (Φ 80lacZ Δ M15) <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i>	Maniatis et al. 1982
<i>Rhizobium</i> sp. BR816	Wild-type isolate from <i>Leucaena leucocephala</i>	Hungria et al. 1993
FAJ1506	BR816 <i>nodO::gusA-aph</i>	Vlassak et al. 1998
FAJ1403	BR816 P450::gusA-aph	This study
FAJ1404	BR816 P450::gusA-aph with <i>gusA-aph</i> in the opposite orientation	This study
<i>Rhizobium etli</i> CNPAF512	Wild-type strain	Michiels et al. 1994
Rp1000	<i>nifA::aphII</i>	Michiels et al. 1994
FAJ1169	<i>rpoN2::</i> Ω -Km	Michiels et al. 1998
FAJ1170	<i>rpoN1::</i> Ω -Sp <i>rpoN2::</i> Ω -Km	Michiels et al. 1998
FAJ1174	<i>furN::nptII</i>	J. Michiels
CFN42	Wild-type strain	CiFN, Mexico
CFNX249	<i>nifHa::</i> Ω -Sp <i>nifHb</i> Δ Bg/II:: Ω -Km	D. Romero
Plasmids		
pUC18	ColE1 replicon, Ap ^R cloning vector	Vieira and Messing 1982
pVK100	Derivative of pRK290 (incP-1), Km ^R , Tc ^R	Knauf and Nester 1982
pBRF2	pVK100 containing a 23-kb megaplasmid region of <i>Rhizobium</i> sp. BR816, Km ^R , Tc ^R	Van Rhijn et al. 1996
pJQ200uc1	Suicide vector containing the <i>sacB</i> genes of <i>Bacillus subtilis</i> , Gm ^R	Quandt and Hynes 1993
pWM6	Vector containing <i>gusA-aph</i> interposon, Ap ^R , Km ^R	Metcalf and Wanner 1993
pRK2013	ColE1 helper plasmid for triparental mating, Km ^R	Figurski and Helinski 1979
pRK2073	ColE1 helper plasmid for triparental mating, Sm ^R	Figurski and Helinski 1979
pFAJ1403	pJQ200uc containing 4.2-kb <i>Sma</i> I fragment::gusA-aph	This study
pFAJ1404	pJQ200uc containing 4.2kb <i>Sma</i> I fragment::gusA-aph in opposite orientation	This study
pFAJ1407	pVK100 with a <i>Not</i> I fragment of pFAJ1403, containing P450::gusA-aph	This study
pFAJ1408	pVK100 containing <i>gusA-aph</i>	This study

Table 3. *Phaseolus vulgaris* after inoculation with *Rhizobium* sp. BR816 and its P450 mutant strain on two different bean cultivars

Strain	Nodule number/plant ^a	Nodule dry weight (mg/plant) ^a	Acetylene reduction activity (μ mol/h/plant) ^a	Percentage ^b
<i>P. vulgaris</i> cv. Carioca				
BR816	162 (A)	37.8 (AB)	5.586 (A)	100
FAJ1403	162 (A)	41.4 (B)	4.960 (A)	90
FAJ1404	171 (A)	45.9	4.962	100
<i>P. vulgaris</i> cv. Negro Jamapa				
BR816	75.4 (A)	49.9 (A)	4.7 (A)	100
FAJ1403	58.4 (A)	31.9 (B)	2.8 (B)	88

^a Values are the mean of nine independent replicates. Values followed by the same letter are not significantly different ($P \leq 0.05$). Data represented for Carioca cannot be compared with those for Negro Jamapa.

^b Number of nitrogen-fixing plants on total number of plants inoculated.

CYP127A2 in Nod factor modifications. In a second set of experiments, expression of the *CYP127A2* gene was analyzed at low pH in order to mimic nodule environment. The AMS medium pH level was adjusted by the use of different buffers (0.5 M stock solution of 2-[N-morpholine]ethanesulfonic acid, pH 5 to 6.5; *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.5 to 8; and *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid, pH 7 to 8.2; to a final concentration of 30 mM) and in this way expression was analyzed in medium at different pH levels (5 to 7.5) under oxic and microoxic conditions. The highest level of expression under free-living conditions was noticed when cells were grown under microoxic conditions at pH 5.5 (Fig. 2). This condition, however, is not sufficient to induce the high expression levels observed in bacteroids. Possibly, a nodule-specific factor is necessary to induce expression of the P450 gene, as proposed for *B. japonicum*, where the highest levels of CYP112 and CYP114 expression were observed in the bacteroids (Tully and Keister 1993). Therefore, we screened other components that might be related to factors present in the nodule.

In *Rhodococcus fascians*, a P450-type locus is induced during the pathogenic interaction with host plants and seems to play a role in cytokinin biosynthesis (Crespi et al. 1994). In plants, P450 might play a role in auxin biosynthesis. In order to test a possible role in the synthesis of plant-growth regula-

tors, indole-3-acetic acid (IAA), synthetic auxin, naphthyl acetic acid, and the IAA precursor tryptophan were tested. Under none of these conditions was expression of *CYP127A2* observed.

Because plant P450s can catalyze the hydroxylation of oleic acid (Schuler 1996), some fatty acids of different chain lengths also were tested for their inducing capacity (C8, C10, C12, C16, C18:1, C18:2, C20:4, ω -OH-C16, 2-OH-C14 as well as plant-derived organic substrates abscisic acid, chrysanthemic acid, and farnesol [10 μ M]). In addition, Naphenobarbital, inducing mammalian and *Bacillus megaterium* P450 gene, was included (Whitlock and Denison 1995). These additional experiments have been carried out in microoxic conditions at pH 5.5. Because these organic substrates were dissolved in ethanol, a control with FAJ1403 grown in 0.4% ethanol was included. Surprisingly, all samples showed some enhanced *CYP127A2* gene expression but to the same extent as the included control (data not shown). An additional experiment confirmed that the expression of P450 increases after the addition of 0.4 or 0.8% ethanol, 0.4% propanol, and 0.1% butanol (Table 4). Sequence analysis of the putative promoter region of *CYP127A2* suggested the presence of two conserved -24/-12-type promoters (consensus 5'-CTGGCAC-N₃-TTGCA-3') (Barrios et al. 1999), with P₁ (P₁ 5'-ACGGCAT-N5-TTGCA-3') and P₂ (P₂ 5'-GAGGGCG-N5-CCGCA-3'). P₁

Table 4. Influence of different alcohols on expression of the genomic *CYP127A2-gusA* fusion in FAJ1403 (0.3% O₂, pH 5.5) under free-living conditions

Condition	Activity (U) ^a	C ^b
FAJ1403	5.6 (0.4)	
+ Ethanol 0.1%	11.1 (0.6)	+
+ Ethanol 0.4%	13.1 (1.1)	+
+ Ethanol 0.8%	38.1 (2.3)	++
+ Propanol 0.1%	8.7 (1.5)	-
+ Propanol 0.4%	17.2 (2.7)	+
+ Butanol 0.1%	22.4 (2.1)	+
+ Butanol 0.4%	7.1 (1.1)	-

^a β -Glucuronidase activities are expressed as Miller units (U); standard deviations are in parentheses.

^b Comparison +: higher expression than control; -: no difference with control

Fig. 2. Expression of the genomic *CYP127A2-gusA* fusion in FAJ1403 when grown under oxic or microoxic conditions in acid minimal salt medium under different pH conditions.

Table 5. Expression of *CYP127A2* in mutant CNPAF512 backgrounds

Strain	Relevant genotype	Activity (U) ^a		Bacteroid ^b
		Oxic	Microoxic	
CNPAF512	Wild-type	0	0	0
CNPAF512 (pFAJ1408)	Wild-type	26 (1.9)	18 (2.5)	0
CNPAF512 (pFAJ1407)	Wild-type	36 (2.5)	76 (20)	1,508 (90)
Rp1000 (pFAJ1407)	<i>nifA</i> mutant	40 (1.3)	16 (2.8)	0
FAJ1170 (pFAJ1407)	<i>rpoN1 rpoN2</i> mutant	26 (4.2)	17 (1.7)	0
FAJ1169 (pFAJ1407)	<i>rpoN2</i> mutant	nd	nd	376 (55)
FAJ1174 (pFAJ1407)	<i>furN</i> mutant	nd	nd	1,679 (30)
CFN42	Wild-type	0	0	0
CFN42 (pFAJ1407)	Wild-type	14 (2)	5 (2)	746(175)
CFNX249	<i>nifH1/2</i> mutant	0	0	0
CFNX249 (pFAJ1407)	<i>nifH1/2</i> mutant	29 (1.3)	40 (6)	766 (215)
BR816 (pFAJ1407)	Wild-type	33 (0.9)	27 (1.3)	3,204 (441)

^a β -glucuronidase activities are the means of four replicates and are expressed as Miller units. nd: not determined. Standard deviations are in parentheses.

^b β -glucuronidase activity was measured 1/2 h after incubation with *p*-nitrophenyl- β -D-glucoside.

seems to be better conserved than P₂. In addition, a probable upstream activating sequence (UAS), which is the binding site for a specific activator that activates transcription by the alternative sigma factor σ^{54} (RpoN)-dependent RNA polymerase (consensus sequence TGT-N₁₀-ACA) (Morett and Buck 1989), is present. The proposed UAS sequence of *CYP127A2* is TGG-N₁₀-ACA. This UAS sequence also has been reported upstream of the *nifHc* of *R. etli* (Valderrama et al. 1996). To investigate the regulation of *CYP127A2*, a plasmidborne *gusA* fusion with the putative promoter region was constructed (Table 2). An 8.2-kb *NotI* fragment containing the 4.2-kb *SmaI* fragment of pBRF2 with a *gusA*::Km cassette downstream of the promoter region was isolated from the pFAJ1403 clone. This fragment was blunt ligated into pVK100 (*HindIII* digested), resulting in pFAJ1407. As a control, the *gusA*-*aph* cassette was ligated in pVK100 to observe false positive expression of the *gusA* gene, resulting in pFAJ1408. The *CYP127A2*-*gusA* transcriptional fusion (pFAJ1407) was introduced in a *R. etli* CNPAF512 wild-type and several mutant backgrounds. Two *rpoN* copies have been identified in this strain and, therefore, the *CYP127A2*-*gusA* fusion also was introduced in a double *rpoN*₁*rpoN*₂ mutant background (Michiels et al. 1998). In CNPAF512, NifA controls the microoxic and symbiotic expression of *nif* genes (Michiels et al. 1994). In order to verify whether NifA is indeed a transcriptional activator for *CYP127A2* expression, gene expression also was tested in a *nifA* mutant background. Because a *fnr*-type promoter was identified in the P450 promoter region of *B. japonicum*, *CYP127A2* gene expression was analyzed in a CNPAF512 *fnrN* mutant strain (Table 2) (Michiels et al., unpublished). As an additional control, the *CYP127A2*-*gusA* plasmid fusion was introduced into BR816. The different transconjugants were grown under oxic, microoxic, and symbiotic conditions, and *CYP127A2* gene expression was analyzed. In Table 5, the observed levels of expressions in symbiotic, free-living oxic, and microoxic conditions are listed. To determine symbiotic expression of the different *gusA* fusions, bacteroids were isolated from the nodules of 4-week-old plants. All nodules of one plant were collected and treated as described by Michiels et al. (1998). The control fusion plasmid pFAJ1408 shows almost no *CYP127A2* gene expression in the CNPAF512 background, which means that there only is low expression resulting from plasmid transcription. In the CNPAF512 wild-type background, *CYP127A2* was expressed at a low level under free-living conditions, whereas high expression occurs in the bacteroids. This is in accordance with the expression pattern of *CYP127A2* in the wild-type strain BR816. Analyzing expression in the different mutant backgrounds reveals that *CYP127A2* expression during symbiosis seems to be regulated by RpoN and NifA. In addition, it was shown that *CYP127A2* expression occurs independent of FnrN. In order to investigate the possibility that intermediates resulting from N₂ fixation might influence expression, *CYP127A2* expression was analyzed in a Fix⁻ background (Table 5). Comparison of *CYP127A2* expression in a *nifH* mutant background with expression levels observed in the wild-type background indeed indicates no difference.

The precise role of P450 in *Rhizobium* sp. BR816 is not clear. As a result of sequence similarity, P450 is proposed to play a role in mono-oxygenase and oxidoreduction reactions, possibly modifying the biological activity of its substrate. In

free-living conditions, however, no such action could be demonstrated on several possible candidate substrates, although this does not exclude such a modifying role during symbiosis. Therefore, elucidation of the actual function of P450 in symbiosis by identifying its inducer is essential.

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