Region II of *Rhizobium* sp. NGR234 Inhibits Nodulation of *Medicago sativa* by *R. meliloti* *nodIJ* and *nodQ*₁ Mutants

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Rhizobium sp. NGR234 contains a plasmid-borne locus that hybridized strongly to region II of *R. meliloti*. Surprisingly, NGR region II completely inhibited nodulation of *Medicago sativa* when conjugated into either *R. meliloti* *nodIJ* or *nodQ*₁ region II mutants. Further characterization showed that region II of NGR234 contains three putative coding open reading frames (ORFs), which are homologous to, respectively, hypothetical protein A of *R. leguminosarum*, hypothetical protein C of *R. leguminosarum*, and ORF2 of *Agrobacterium tumefaciens* insertion sequence (IS) 66, as well as an ORF of unknown function located downstream of *nodQ*₁ of *R. meliloti*. A site-directed mutation in the IS66 homologue improved nodulation efficiency on some NGR234 hosts, but the structure and composition of the Nod-factor family produced by the mutant were unchanged. The quantity of Nod factors secreted was reduced by two-thirds, however. Insertions or deletions of the genes encoding either the hypothetical protein C or the IS66 homologue abolished the blocking effect observed in the “complementation” tests with *R. meliloti* region II mutants.

Rhizobium-legumes symbioses are among the best-studied interactions between plants and microorganisms. *Rhizobium* sp. NGR234 is a fast-growing, promiscuous isolate that can nodulate more than 110 genera of legumes (S. G. Pueppke and W. J. Broughton, *unpublished*). Physical methods have shown that the symbiotic determinants are dispersed over the 536-kb symbiotic plasmid pNGR234a (Perret et al. 1991; Freiberg et al. 1997). Plant symbiotic signals include flavonoids that are liberated into the rhizosphere and taken up by rhizobia. Under appropriate conditions, these compounds interact with NodD proteins (members of the LysR family of transcriptional regulators) to activate nodulation genes (*nod*, *nol*, and *noe*). In turn, some nodulation genes synthesize lipo-chitoooligosaccharide Nod factors that are responsible for the induction of nodulation (Fellay et al. 1995b). NGR234 produces a family of

about 80 different Nod factors (Price et al. 1992; S. Jabbouri, *personal communication*).

R. meliloti region II is located downstream of *nodC* (Debellé et al. 1986) between the *nodIJ* and *nodQ*₁ genes. Downstream of *nodQ*₁ is an open reading frame (ORF) of unknown function (Schwedock and Long 1994). The *nodIJ* genes encode a transport system (Vásquez et al. 1993; Spaink et al. 1995; Fernández-López et al. 1996) and have been shown to determine the efficiency of Nod-factor secretion (Spaink et al. 1995; Cardenas et al. 1996). Along with *nodH* and *nodP*, the *nodQ*₁ gene is involved in the sulfation of *R. meliloti* Nod factors (Roche et al. 1991). Both the *R. meliloti* *nodIJ* and *nodQ*₁ mutants show a slight delay in nodulation on alfalfa plants when compared with the wild type (Debellé et al. 1986). Presence of a second *nodQ* copy, *nodQ*₂, which is located on the second *R. meliloti* megaplasmid (Schwedock and Long 1992), may explain the partial complementation of the *nodQ*₁ mutant. Experiments with other *Rhizobium* spp. clearly showed that mutations in *nodIJ* gave strains that secrete reduced amounts of Nod factors, resulting in a delayed nodulation phenotype (Spaink et al. 1995; Cardenas et al. 1996). Surprisingly, assays to complement the two *R. meliloti* *nodIJ* and *nodQ*₁ mutants with NGR234 region II resulted in suppression of nodulation, not restoration of the wild-type phenotype. Genetic analysis of the region showed that it is highly homologous to the ORF found downstream of *nodQ*₁. Restriction fragment length polymorphism analysis of NGR234 derivatives showed that this region is duplicated on the chromosome. Here we show that NGR234 region II seems to influence nodulation in both NGR234 and *R. meliloti*.

RESULTS

Sequence and homologies.

A 3.2-kb *Pst*I fragment of *R. meliloti* containing region II was used to probe cosmid clones derived from pNGR234a (Broughton et al. 1986). Only pWA46 reacted positively, and the homologous region was delimited to a 2.85-kb *Eco*RI fragment that we named NGR region II. It is located 5.5 kb upstream of the *nodABC* genes. A reiteration of NGR region II was found on the chromosome of NGR234 by probing restricted genomic DNA of ANU265 (the Sym plasmid cured strain of NGR234) with the 2.85-kb *Eco*RI probe (data not shown). Other hybridizations with the same probe showed

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homologies to DNA of *Agrobacterium tumefaciens*, *R. fredii*, and *R. meliloti* (data not shown).

The sequence of NGR region II was published as part of the entire sequence of the symbiotic plasmid pNGR234a (Freiberg et al. 1997). As expected from the hybridization with *R. meliloti* region II, high homology (77% identity on 800 bp) was found at the DNA level between NGR region II and the *R. meliloti* counterpart. Three complete ORFs are present on the 2.85-kb *EcoRI* fragment: y4hN, y4hO, and y4hP (Fig. 1). The putative products of y4hN and y4hO are homologous to, respectively, *R. leguminosarum* hypothetical proteins A and C (Yeoman et al. 1996). The y4hP putative product is homologous to ORF2 of *A. tumefaciens* insertion sequence (IS) 66 as well as the *R. meliloti* ORF downstream of *nodQ1* (Machida et al. 1984; Schwedock and Long 1994). Competitive RNA hybridizations showed that daidzein induces delayed transcription of the NGR region II (Fellay et al. 1995a). With the same technique, the signal was further delimited to restriction fragments corresponding to the 5' half of region II (Fig. 2).

"Complementation" tests:

Inhibition of nodulation in trans.

As previously mentioned, the *R. meliloti nodIJ* and *nodQ1* Tn5 mutants, named GMI5390 and GMI5392, respectively (Debellé and Sharma 1986; Debellé et al. 1986), cause a slight delay in nodulation of alfalfa: nodules consistently appear 1 to 2 days later than those on plants inoculated with the wild-type bacterium. In addition, the efficiency of nodulation of these mutants is slightly reduced. Accordingly, complementation tests were performed by introducing the 2.85-kb *EcoRI* NGR region II (cloned in pRK7813) into *R. meliloti* region II::Tn5 mutants GMI5390 (*nodIJ*::Tn5) and GMI5392 (*nodQ1*::Tn5). Introduction of pWP14 drastically inhibited the nodulation of the *R. meliloti nodQ1* mutant GMI5392 (Fig. 3A, strain GMI5392c) as well as the *nodIJ* mutant GMI5390 (data not shown). When the vector (pRK7813) or the NGR region II clone (pWP14) were introduced into the wild-type *R. meliloti*, negative effects on nodulation were not observed (Fig. 3A, strains RCR2011v and RCR2011c, respectively), showing that the inhibitory effect was conditional. Mutated NGR region II clones were then introduced into the two *R. meliloti* mutants. These constructs, which contained Tn5 insertions upstream of y4hN or in y4hO (Fig. 1, #2043 and #1952, respectively), yielded transconjugants in

which the blocking effect was completely abolished (Fig. 3A, strains GMI5392T-1 and GMI5392T-2, respectively; identical data for GMI5390 not shown). Complementation tests were also performed with a y4hP mutant in which the internal *XhoI* fragment was deleted (see Figure 1 and Table 1). The resulting GMI5390D-1 strain (as well as GMI5392D-1) regained the ability to nodulate alfalfa (Fig. 3B; data not shown for GMI5390D-1). Clearly, these results show that y4hN, y4hO, and y4hP are involved in the inhibition of nodulation of *M. sativa* by *R. meliloti nodIJ* or *nodQ1* mutants.

Mutagenesis and plant tests.

A Tn5 insertion was made in y4hP (#4235) and the mutated region was homogenized into NGR234, giving MRP4235. The fidelity of marker exchange was verified by hybridizations with both Tn5 and NGR region II as probes against total DNA digests extracted before and after passage through *Macroptilium atropurpureum* nodules (data not shown). MRP4235 was tested on four NGR234 hosts (*Vigna unguiculata* (L.) Walp. 'Red Caloona', *Macroptilium atropurpureum* (Moc. & Sessé ex DC.) Urb. 'Siratro', *Calopogonium caeruleum* (Benth.) Sauv., and *Desmodium uncinatum* (Jacq.) DC.), and two nonhosts (*Medicago sativa* L. 'Gemini' and *Pisum sativum* L. 'Rondo'). MRP4235 had no detrimental effects on nodulation of *C. caeruleum* or *V. unguiculata* nor was an extension of host range to include *M. sativa* or *P. sativum* observed (Table 2). On the other hand, inoculation with the mutant produced about 50% more nodules on *M. atropurpureum* and *D. uncinatum* (Table 2). Kinetic studies showed that MRP4235-induced nodules were more abundant throughout the growth period, demonstrating that the mutation of y4hP increased nodulation efficiency (Rochepeau 1993).

Effect of mutating y4hP on Nod-factor structure and production.

FAB-MS (fast atom bombardment-mass spectrometry) in the positive-ionization mode (see Materials and Methods) was

Fig. 1. Restriction map of NGR region II (2.85-kb *EcoRI* fragment). Restriction enzymes sites are shown below the line. The three open reading frames are shown as black, filled arrows. Positions of the three Tn5 insertions sites are shown as black, filled triangles above the sequence. The 65-bp deletion created between the two *XhoI* sites in y4hP to give pWP-D65 is represented with a delta sign.

Fig. 2. RNA competitive hybridization with NGR region II. **A**, Ethidium bromide-stained gel of NGR234 region II double digested with *EcoRI* (E) and *AccI* (E/A), *PstI* (E/P), *SphI* (E/S), *XhoI* (E/X), and *AccI/SphI* (A/S). **B**, Hybridizations were performed as described by Fellay et al. (1995a) from NGR234 cultures incubated with daidzein for 24 h. Asterisk corresponds to a partial digest.

used to compare the Nod factors produced by NGR234 and by its mutant MRP4235. Molecular ions corresponding to nonsulfated Nod factors produced by the wild-type bacterium are pentamers of *N*-acetyl-D-glucosamine (GlcNAc) with 4-*O*-acetyl, 2-*O*-methylfucose on the reducing GlcNAc residue and *N*-methylated, *N*-acylated on the nonreducing end by *C18:1*, *C18:0*, or *C16:1* (which can be mono- or biscarbamoylated). Ionization in the negative mode produced an identical series of [M+H]⁻ ions from the wild-type Nod factors that corre-

spond to pentameric chitin oligomers possessing 3-*O*-sulfate, 2-*O*-methylfucose at the reducing end, which are in turn *N*-methylated, *N*-(*C18:1* or *C16:1*)-acylated and 3-*O*- and 6-*O*-carbamoylated at the nonreducing terminus. In other words, no differences in Nod-factor structure produced by wild-type NGR234 and its mutant MRP4235 could be observed.

In the tomato-cell suspension assay, the addition of Nod-factor preparations at nanomolar concentrations to the suspen-

Table 1. Bacterial strains and plasmids

Plasmids or strains	Characteristics	Source or reference
pRK7813	IncP1 plasmid, Tc ^r	Jones and Gutterson 1987
pRK2013	Tra ⁺ helper plasmid, replicon ColE1 (Nm ^r)	Figurski and Helinski 1979
pRK600	helper plasmid, pRK2013 <i>npt</i> ::Tn9, (Cm ^r , Nm ^r)	Finan et al. 1986
pPH1J1	IncP1 plasmid (Gm ^r , Spc ^r)	Hirsch and Beringer 1984
pBSM13 ^{+/−}	Sequencing vectors (Ap ^r)	Vector Cloning Systems (San Diego, CA)
pWA46	pNGR234a subclone in pJB8 (Ap ^r)	Broughton et al. 1986
pWP14	NGR234 region 2 (2.85-kb <i>Eco</i> RI fragment cloned into pRK7813)	This work
pWPT1952	Tn5 insertion #1952 in y4hO [pWP14]	This work
pWPT2043	Tn5 insertion #2043 upstream of y4hN [pWP14]	This work
pWP-D65	65-bp deletion between the two <i>Xho</i> I sites in y4hP [pWP14]	This work
<i>Escherichia coli</i>		
FM15	F [−] RecA [−] <i>Dlac-pro Dthi DlacZ</i>	Dowling et al. 1987
S17-1	RP4 2-Tc::Mu-Km::Tn7 on the chromosome mobilization strain	Simon 1984
JvS1	S17-1::Tn5, mutagenesis strain	Stanley et al. 1989
DH5a	<i>supE44</i> , <i>DlacU169</i> (f 80 <i>lacZ</i> DM15) <i>hsdR17</i> <i>recA1</i> , <i>endA1</i> , <i>gyrA96</i> , <i>thi-1</i> , <i>relA1</i>	Hanahan 1983
<i>Rhizobium</i>		
NGR234	NGR234 Rif ^r derivative	Stanley et al. 1987
ANU237	NGR234, wild type	B. G. Rolfe (Australian Natl. Univ., Canberra)
ANU240	NGR234 Sm ^r derivative	Morrison et al. 1983
ANU265	NGR234 cured of pNGR234a	Morrison et al. 1983
RCR2011	<i>Rhizobium meliloti</i> wild type	Rosenberg et al. 1981
GMI5390	Tn5 mutant #2412 (<i>nodII</i>) of RCR2011	Debellé et al. 1986
GMI5392	Tn5 mutant #2402 (<i>nodQ</i>) of RCR2011	Debellé et al. 1986
RCR2011v	RCR2011 [pRK7813]	This work
RCR2011c	RCR2011 [pWP14]	This work
GMI5390c	GMI5390 [pWP14]	This work
GMI5392c	GMI5392 [pWP14]	This work
GMI5390T-1	GMI5390 [pWPT1952]	This work
GMI5390T-2	GMI5390 [pWPT2043]	This work
GMI5390D-1	GMI5390 [pWP-D65]	This work
GMI5392T-1	GMI5392 [pWPT1952]	This work
GMI5392T-2	GMI5392 [pWPT2043]	This work
GMI5392D-1	GMI5392 [pWP-D65]	This work
MRP4235	y4hP::Tn5 mutant (Tn5 #4235) of NGR234	This work

Fig. 3. Complementation test for GMI5292 (*R. meliloti nodQ₁* mutant). **A**, Complementation with NGR region II. Nodulation kinetics of RCR2011 (open triangle; wild type control), RCR2011v (open circle; control with pRK7813), RCR2011c (straight line; control with pWP14), GMI5392 (open square; *nodQ₁* mutant), GMI5392c (line with Xs; *nodQ₁* mutant with pWP14), GMI5392T-1 (black circle; *nodQ₁* mutant with pWPT1952), GMI5392T-2 (black triangle; *nodQ₁* mutant with pWPT2043). **B**, Complementation with pWP-D65. Nodulation kinetics of RCR2011 (open triangle; wild type control), GMI5392 (open square; *nodQ₁* mutant), and GMI5292-D65 (black square; *nodQ₁* mutant with pWP-D65). The *Medicago sativa* cv. Gemini nodules were counted each day over a 50-day period.

sion-cultured tomato cells provoked a rapid and transient alkalization of the culture medium (see Materials and Methods). Measurements of this type clearly show that mutation of y4hP drastically reduces Nod-factor production ($\geq 80\%$, see Figure 4).

DISCUSSION

Ramakrishnan et al. (1986) found homologies between the *A. tumefaciens* IS66 and plasmids of *R. fredii*. More recently, extensive similarities have been found between the products of an *R. meliloti* ORF located downstream of *nodQ*₁ and ORF2 of IS66 (Schwedock and Long 1994). Similarly, NGR region II is strikingly homologous to IS66. In fact, insertion sequences are common in rhizobia: ISR1 in *Bradyrhizobium lupini* (Priefer et al. 1981), *ISRm1* and *ISRm2* in *R. meliloti* (Ruvkun et al. 1982; Dusha et al. 1987), and NGRS-1 in NGR234 (Perret et al. 1991) are just some examples. ISs are usually present in multiple copies (Wheatcroft and Watson 1988; Priefer et al. 1989). This is also true for NGR region II, which is present on both the symbiotic plasmid and the chromosome. Extensive homologies between ISs of *Agrobacterium* and *Rhizobium* raise the question of their origin. Evidence for horizontal transfer from *Rhizobium meliloti* to *Agrobacterium tumefaciens* was recently proposed by Deng et al. (1995), who argued that since two novel insertion sequences, IS1312 and IS1313, of an *Agrobacterium tumefaciens* strain are more related to the *Rhizobium meliloti* ISs *ISRm1* and *ISRm2* than to other *Agrobacterium* IS elements, they must have originated from rhizobia. Our data suggest an *Agrobacterium* origin, as all the homologies found with NGR region II, including rhizobial ones, are related to *Agrobacterium* ISs.

Perhaps because analysis of the plasmid-borne NGR region II genes is complicated by the second chromosomal copy, its role in nodulation is far from clear. Symbiotic responses are controlled by a narrow window of Nod-factor concentrations. With the classic NGR234 host *V. unguiculata* as an example, concentrations between 10^{-9} and 10^{-7} M are the only ones that induce true "shepherd's crook" type curling of the root hairs (Price et al. 1992). Concentrations between 10^{-7} and 10^{-6} M are needed to "complement" Nod-factor-deficient mutants (Relić et al. 1994a). Elevated Nod-factor levels permit NGR234 to nodulate *M. sativa*, a nonhost (Relić et al. 1994b). It is thus possible that site-directed mutation of the IS66

homologue of NGR234, which reduces Nod-factor excretion by more than two-thirds, shifts Nod-factor production down to levels that are more suitable for nodulation of some plants. Only a few NGR234 host plants have been tested. Perhaps extended screening would also reveal legumes on which the nodulation by MRP4235 is impaired by its low level of Nod-factor production. In the *nodII* and *nodQ*₁ mutants of *R. meliloti* (the *nodII* and *nodQ*₁ genes are involved in two separate steps of *R. meliloti* Nod-factor production), we suggest that interference with Nod-factor production may be so extreme as to prohibit nodulation. As mutation of y4hP, which on the one hand unblocks the inhibition seen in the "complementation tests" and on the other drastically reduces the level of Nod factors, we suggest that this gene is a regulator of Nod-factor levels.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media.

All plasmids and strains used in this work are listed in Table 1.

Luria-Bertani medium was used for *Escherichia coli* strains and tryptone-yeast (TY) or RMM (*Rhizobium* minimal medium) for *Rhizobium*. Antibiotics were added to the following concentrations: ampicillin, streptomycin, and neomycin, $100 \mu\text{g} \cdot \text{ml}^{-1}$; rifampicin and spectinomycin, $50 \mu\text{g} \cdot \text{ml}^{-1}$; kanamycin, $30 \mu\text{g} \cdot \text{ml}^{-1}$; gentamicin, $15 \mu\text{g} \cdot \text{ml}^{-1}$; and tetracycline, $10 \mu\text{g} \cdot \text{ml}^{-1}$.

Sequencing.

At first, NGR234 was manually sequenced by dideoxy-based methods (Rochepeau 1993); these were later supplemented with automatic sequencing as described by Freiberg et al. (1997).

Table 2. Nodulation phenotypes of MRP4235 (y4hP::Tn5 mutant of NGR234) on various hosts and non-hosts of NGR234

Host-plants	Nodules per plant (mean no.)	
	NGR234	MRP4235
<i>Vigna unguiculata</i> (L.) Walp. 'Red Caloona'	7.2 ± 2.1^a	7.3 ± 2.3^a
<i>Macroptilium atropurpureum</i> (Moc. & Sessé ex DC.) Urb. 'Siratro'	5.4 ± 1.7^a	8.33 ± 2.4^a
<i>Calopogonium caeruleum</i> (Benth.) Sauv.	31.3 ± 17.3^a	30.8 ± 16.3^a
<i>Medicago sativa</i> L. 'Gemini'	0 ^b	0 ^b
<i>Desmodium uncinatum</i> (Jacq.) DC.	14.5 ± 4.5^a	23.0 ± 8.0^a
<i>Pisum sativum</i> L. 'Rondo'	0	0

^a In Magenta jars, root nodules tend to form in the cotton wick; it was not possible to determine which of the five plants they belonged to.

^b Presence of one to three plurilobal Fix⁻ pseudonodules was found on at most three plants from a total of 20 in the two repetitions.

Fig. 4. Comparison of Nod factors produced by wild-type NGR234, the control NGR234(pA28) (overproducing strain; see Price et al. 1992), and the mutant MRP4235, in cultures with 10^{-6} M apigenin (+) or without apigenin (-).

Strain construction.

To generate random Tn5 insertions in pWP14, a strain of *E. coli* S17-1 that carries Tn5 on the chromosome (strain JvS1) was used (Stanley et al. 1989). JvS1 was transformed with pWP14, and Tc^r transformants were used as donors in crosses with *E. coli* FM15R. Nm^r, Rif^r, and Tc^r transconjugants were analyzed for Tn5 insertions in pWP14. pWP14::Tn5 co-integrates (verified by sequencing) were transferred to NGR234. Homogenization was forced by the subsequent introduction of pPH1JI and selection for its gentamicin resistance as well as the streptomycin resistance of Tn5. Loss of pRK7813 was monitored by sensitivity to tetracycline. Correct integration of the Tn5 was verified by Southern hybridization with Tn5 and region II probes, both before and after passage through *Macroptilium atropurpureum*. RCR2011v, RCR2011c, GMI5390c, and GMI5392c (see Table 1) were constructed by triparental matings with FM15(pRK7813 or pWP14) as donors and pRK2013 as the helper plasmid. Introduction of pWPT1952 and pWPT2043 into GMI5390 and GMI5392 was performed by biparental mating with S17.1(pWPT1952 or pWPT2043) as the donor. Transconjugants GMI5390D-1 and GMI5392D-1 were constructed by triparental matings with DH5 α (pWP-D65) as the donor and pRK600 as the helper plasmid. The 65-bp deletion in y4hP was constructed by digesting pWP14 with *Xho*I and religating. Absence of the *Xho*I fragment was verified by digestion with *Fok*I, as one of the three *Fok*I sites in the region lies within the fragment.

Plant tests.

V. unguiculata cv. Red Caloona, *P. sativum* cv. Rondo, *M. atropurpureum* cv. Siratro, *C. caeruleum*, *M. sativa* cv. Gemini, and *D. uncinatum* seeds were provided by Rawling Seeds, (Orpington, Kent, UK). Seeds of *V. unguiculata*, *P. sativum*, *M. atropurpureum*, *M. sativa*, and *D. uncinatum* were sterilized by soaking for 7 min in 70% ethanol, rinsing in sterile water, soaking in HgCl₂/HCl (1 g per liter and 1%, respectively) for 7 min, and rinsing 6 times in sterile water. *C. caeruleum* seeds were soaked for 50 min in concentrated sulfuric acid and then rinsed 6 times in sterile water. The sterilized seeds were pregerminated for one to two nights on petri dishes containing B+D (Broughton and Dilworth 1971) or water agar medium. For *D. uncinatum* and *C. caeruleum*, five seedlings were then transferred to each Magenta jar (Lewin et al. 1990), two seedlings of *P. sativum* and *V. unguiculata* were placed in each growth pouch (Lewin et al. 1990), and two seedlings of *M. atropurpureum* and *M. sativa* in each glass tube. One day later, each plant root was inoculated with 200 μ l of a 10⁸ rhizobial cells per ml culture. Nodules were counted after 45 days.

RNA hybridizations.

RNA extraction, labeling, and competitive hybridizations were performed as previously described by Perret et al. (1994) and Fellay et al. (1995a).

Quantification of Nod factors.

Nod-factor production was measured by the tomato-cell suspension assay (Staehelin et al. 1994) as described by Relić et al. (1994b): 40 ml of rhizobial culture in RMM was centrifuged (4,000 $\times$ g, 4°C) and applied to 3-ml C₁₈ Sep-pak car-

tridges (Waters, Milford, MA), and the lipophilic content eluted with 5 ml of methanol. Dried samples were dissolved in dimethyl sulfoxide (DMSO)–ethanol (1/1) and appropriate dilution added to suspension culture tomato cells (Staehelin et al. 1994). The alkalization peak was integrated and peak areas translated into nmoles with high-pressure liquid chromatography (HPLC)-purified Nod NGR factors as standards.

Structural analysis of Nod factors produced by the MRP4235 mutant.

Nod factors were purified from culture supernatants of MRP4235 by reverse-phase HPLC as described by Jabbouri et al. (1995). Each fraction was then analyzed by FAB-MS in the positive ionization mode for nonsulfated molecules and both the positive and negative ionization mode for sulfated products, and compared with those produced by the wild type (Price et al. 1992).

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