All *nod* Genes of *Rhizobium meliloti* Are Involved in Alfalfa Nodulation by *exo* Mutants

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Nodulation of alfalfa by *exoB* mutants of *Rhizobium meliloti* occurred without root hair curling or infection thread formation. *nod exoB* double mutants had the same nodulation deficiency as single *nod* mutants. Therefore, all the known *nod* genes are involved in nodule induction by *exoB* mutants, which apparently occurs via intercellular invasion.

Rhizobium meliloti induces the formation of root nodules on alfalfa. At least 10 *nod* genes have been identified which affect root hair curling and infection thread formation, the early stages of nodule development (for an overview, see references 6 and 23). Mutations in *nodA*, *nodB*, or *nodC* of the “common *nod*” genes (*nodDABC*) block nodule induction and can be complemented by homologous genes from other *Rhizobium* species (5, 18, 20). In addition to the *nodD* locus in the common *nod* cluster (*nodD1*), two highly homologous copies are present in *R. meliloti* SU47 (*nodD2* and *nodD3*; 22; M. Honma, personal communication). Mutations in *nodGEFH* (also known as *hsn*, for host-specific nodulation) block or delay nodulation and also permit root hair curling on certain legumes not affected by wild-type *R. meliloti*; these mutations cannot be complemented by homologous genes from other *Rhizobium* species (2, 3, 14, 21). In *Rhizobium leguminosarum*, two additional loci (*nodIJ*) are downstream of *nodDABC*, and there is evidence for homologous sequences in *R. meliloti* (7).

Mutations in *R. meliloti exo* genes, including *exoB*, block production of the Calcofluor-white-binding (Cellufluor; Polyscience, Washington, Pa.) acidic extracellular polysaccharide (10, 16). *exoB* mutants induce shepherd crooks at extremely low frequency; some regions along the root show no hair curling, and others show hypercurling. *exoB* mutants do form nodules, but these contain neither infection threads nor intracellular bacteria and do not fix nitrogen (Fix⁻). A few bacteria, which have evidently entered through the epidermis rather than through root hairs, are seen in intercellular spaces (10; A. M. Hirsch, personal communication).

We investigated the involvement of the *nod* genes in nodulation by *exoB* mutants by examining the nodulation response of *nod exoB* double mutants. We found that all the *nod* genes required for normal nodulation by root hair entry, which entails root hair curling and infection thread development, are also required for nodulation by *exoB* mutants, which is apparently independent of these processes.

We first investigated whether the known clusters of *nod* genes on megaplasmid pRmeSU47a were required for nodulation by *exoB* mutants. By transduction with ϕ M12 (9), strains were constructed which carried both the *exoB355* mutation on pRmeSU47b and one of the following deletions of the *nod* region on pRmeSU47a: $\Delta 36a$ or $\Delta 44a$ (M. Honma, personal communication), both extending over the entire *nod-hsn* region; Δ HG0.1, extending over the common *nod* genes only; Δ HG1.4, extending over the common *nod* genes

and the adjacent area covered by plasmid pRmSL26 (17); or $\Delta 42$, a 290-kilobase deletion in pRmeSU47a extending over all the known *nod* genes (24; J. Denarie, personal communication). Nodulation tests were performed on *Medicago sativa* cv. Iroquois, as previously described (12, 25). All of the deletions were Nod⁻ in the *exo* background as well as in the *exo*⁺ background, indicating that the nodules elicited by *exo* mutants require *nod*⁺-encoded functions. Occasionally, white, tumorous protrusions were seen on some of the plants inoculated with these deletion mutants in *exo*⁺ or *exo* backgrounds.

We tested whether both the common *nod* (*nodDABC*) and “*hsn*” (*nodGEFH*) regions were involved in nodulation by *exoB* mutants by introducing into the deletion mutants plasmids which covered one or both of those regions. Plasmid pRmSL26 (17) covers *nodDABC*, and plasmid pRmC36 (M. Honma, personal communication) covers the entire $\Delta 36$ deletion, including both *nodDABC* and *nodGEFH*. Both plasmids were mobilized by plasmid pRK2013 (8) or plasmid pRK600 (11) in triparental matings into isogenic strains containing the $\Delta 36$ or $\Delta 44$ deletions and *exoB355* or *exo*⁺, and plasmid pRmSL26 was introduced into strains carrying the deletions Δ HG0.1, Δ HG1.4, and $\Delta 42$. Plasmid pRmSL26 complemented deletions Δ HG0.1 and Δ HG1.4 (but not $\Delta 42$) to Nod⁺, whether in the *exo* or the *exo*⁺ background, although nodulation by both *exo* and *exo*⁺ Δ HG1.4(pRmSL26) derivatives was slightly delayed. Plasmid pRmC36 complemented deletions $\Delta 36$ and $\Delta 44$ to Nod⁺, whether in the *exo* or the *exo*⁺ background.

Thus, each of the deletions conferred the same Nod⁻ phenotype regardless of whether the strain was *exo*⁺ or *exo*.

We investigated whether individual *nod* genes were required for nodulation by *exoB* mutants by testing a series of double *nod exoB* mutants constructed as described above (Table 1). There was no difference in the nodulation responses of *nod exoB*⁺ and *nod exoB* mutants on alfalfa (Table 2) in terms either of the number of plants nodulated or of the number of total nodules (per 10 plants). Mutations in *nodA*, *nodB*, *nodC*, or *nodH* block nodulation in both *exo*⁺ and *exoB* backgrounds. Mutations in *nodE* or *nodF* delay nodulation (2, 3, 14). The *nodE exoB* and *nodF exoB* mutants were slightly more delayed in nodulating compared with the *nod exoB*⁺ mutants, but the effect may be at least partially ascribed to the delay often seen for single *exo* mutants (S. Klein, unpublished data). We saw no effect from an insert in *nodG* in either an *exo*⁺ or an *exo* background, although Horvath et al. reported a slight delay in nodulation for such mutants (14). For RmD1D2D3 *exo*⁺ (which has inserts in

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TABLE 1. Bacterial strains and plasmids^a

Strain or plasmid	Relevant genotype	Source or reference
<i>R. meliloti</i>		
RCR2011	Wild type (= SU47)	Laboratory collection
Rm1021	SU47 <i>str-21</i>	F. Ausubel
Rm5078	Rm1021 <i>exoB355</i> Ω 5006::Tn5-132	4
Rm5079	Rm1021 Ω 5007 Tn5-132	11
GMI214	SU47 Rf ⁺ Δ 42 (Nod ⁻) Nm ^r	J. Dénarié
GMI357	SU47 Rf ⁺ Δ HG0.1 (Nod ⁻) Nm ^r	J. Dénarié (24)
GMI361	SU47 Rf ⁺ Δ HG1.4 (Nod ⁻) Nm ^r	J. Dénarié (24)
Rm6721	Rm1021 Ω 5007::Tn5-132 Δ HG0.1	ϕ M12:GMI357 to Rm5079
Rm6724	Rm1021 <i>exoB355</i> Ω 5006::Tn5-132 Δ HG0.1	ϕ M12:GMI357 to Rm5078
Rm6722	Rm1021 Ω 5007::Tn5-132 Δ HG4.1	ϕ M12:GMI361 to Rm5079
Rm6725	Rm1021 <i>exoB355</i> Ω 5006::Tn5-132 Δ HG1.4	ϕ M12:GMI361 to Rm5078
Rm6778	GMI214 Ω 5006::Tn5-132 <i>exoB355</i>	ϕ M12:Rm5078 to GMI214
Rm6779	GMI214 Ω 5006::Tn5-132	ϕ M12:Rm5078 to GMI214
Rm3600	Rm1021 Δ 36 (Nod ⁻) Nm ^r	M. Honma
Rm4400	Rm1021 Δ 44 (Nod ⁻) Nm ^r	M. Honma
Rm6837	Rm3600 Ω 5006::Tn5-132	ϕ M12:Rm5078 to Rm3600
Rm6838	Rm3600 Ω 5006::Tn5-132 <i>exoB355</i>	ϕ M12:Rm5078 to Rm3600
Rm6839	Rm4400 Ω 5006::Tn5-132	ϕ M12:Rm5078 to Rm4400
Rm6840	Rm4400 Ω 5006::Tn5-132 <i>exoB355</i>	ϕ M12:Rm5078 to Rm4400
S2A3	Rm1021 <i>fixF</i> ::Tn5	S. R. Long (15)
S159	Rm1021 Ω Tn5 (Nod ⁺)	S. R. Long (15)
S1B2	Rm1021 Ω Tn5 (Nod ⁺)	S. R. Long (15)
S167	Rm1021 Ω Tn5 (Nod ⁺)	S. R. Long (15)
S157	Rm1021 Ω Tn5 (Nod ⁺)	S. R. Long (15)
S1A8	Rm1021 Ω Tn5 (Nod ⁺)	S. R. Long (15)
S9B2	Rm1021 Ω Tn5 (Nod ⁺)	S. R. Long (15)
S161	Rm1021 Ω Tn5 (Nod ⁺)	S. R. Long (15)
S9B8	Rm1021 <i>nodD</i> ::Tn5	S. R. Long (15)
S1A3	Rm1021 <i>nodA</i> ::Tn5	S. R. Long (15)
S2B2	Rm1021 <i>nodB</i> ::Tn5	S. R. Long (15)
S170	Rm1021 <i>nodC</i> ::Tn5	S. R. Long (15)
S8A2	Rm1021 <i>nodC</i> ::Tn5	S. R. Long (15)
S9B6	Rm1021 Ω Tn5	S. R. Long (15)
Rm6764	S2A3 Ω 5006::Tn5-132 <i>exoB355</i>	ϕ M12:Rm5078 to S2A3
Rm6765	S159 Ω 5006::Tn5-132 <i>exoB355</i>	ϕ M12:Rm5078 to S159
Rm6766	S1B2 Ω 5006::Tn5-132 <i>exoB355</i>	ϕ M12:Rm5078 to S1B2
Rm6767	S167 Ω 5006::Tn5-132 <i>exoB355</i>	ϕ M12:Rm5078 to S167
Rm6768	S157 Ω 5006::Tn5-132 <i>exoB355</i>	ϕ M12:Rm5078 to S157
Rm6769	S1A8 Ω 5006::Tn5-132 <i>exoB355</i>	ϕ M12:Rm5078 to S1A8
Rm6770	S9B2 Ω 5006::Tn5-132 <i>exoB355</i>	ϕ M12:Rm5078 to S9B2
Rm6771	S161 Ω 5006::Tn5-132 <i>exoB355</i>	ϕ M12:Rm5078 to S161
Rm6772	S9B8 Ω 5006::Tn5-132 <i>exoB355</i>	ϕ M12:Rm5078 to S9B8
Rm6773	S1A3 Ω 5006::Tn5-132 <i>exoB355</i>	ϕ M12:Rm5078 to S1A3
Rm6774	S2B2 Ω 5006::Tn5-132 <i>exoB355</i>	ϕ M12:Rm5078 to S2B2
Rm6775	S170 Ω 5006::Tn5-132 <i>exoB355</i>	ϕ M12:Rm5078 to S170
Rm6776	S8A2 Ω 5006::Tn5-132 <i>exoB355</i>	ϕ M12:Rm5078 to S8A2
Rm6777	S9B6 Ω 5006::Tn5-132 <i>exoB355</i>	ϕ M12:Rm5078 to S9B6
GMI5393	RCR2011 <i>nodE</i> ::Tn5	J. Dénarié (2)
GMI5117	RCR2011 <i>nodE</i> ::Tn5	J. Dénarié
GMI5140	RCR2011 <i>nodH</i> ::Tn5	J. Dénarié
DEK5	Rm1021 <i>nodF</i> ::Nm ^r	F. Debellé
DEK7	Rm1021 <i>nodG</i> ::Nm ^r	F. Debellé
Rm6870	GMI5393 Ω 5006::Tn5-132 <i>exoB355</i>	ϕ M12:Rm5078 to GMI5393
Rm6871	GMI5393 Ω 5006::Tn5-132	ϕ M12:Rm5078 to GMI5393
Rm6872	GMI5141 Ω 5006::Tn5-132 <i>exoB355</i>	ϕ M12:Rm5078 to GMI5141
Rm6873	GMI5141 Ω 5006::Tn5-132	ϕ M12:Rm5078 to GMI5141
Rm6876	GMI5394 Ω 5006::Tn5-132 <i>exoB355</i>	ϕ M12:Rm5078 to GMI5394
Rm6877	GMI5394 Ω 5006::Tn5-132	ϕ M12:Rm5078 to GMI5394
Rm6921	DEK5 Ω 5006::Tn5-132 <i>exoB355</i>	ϕ M12:Rm5078 to DEK5
Rm6923	DEK7 Ω 5006::Tn5-132 <i>exoB355</i>	ϕ M12:Rm5078 to DEK5
RmD1D2D3	Rm1021 <i>nodD1</i> ::Tn5 <i>nodD2</i> ::Tp ^r <i>nodD3</i> ::Sp ^r	M. Honma
Rm6925	RMD1D2D3 Ω 5006::Tn5-132 <i>exoB355</i>	ϕ M12:Rm5078 to RmD1D2D3
<i>Escherichia coli</i>		
HB101	F ⁻ <i>hsdS20</i> (r _B ⁻ m _B ⁻) <i>recA13</i> <i>ara-14</i> <i>proA2</i> <i>lacY1</i> <i>galK2</i> <i>rpsL20</i> (Sm ^r) <i>xyl-5</i> <i>mtl-1</i> <i>supE44</i>	Laboratory collection
MM294A	<i>pro82</i> <i>thi-1</i> <i>hsdR17</i> <i>supE44</i>	Laboratory collection
YMC9	MM294A Δ (<i>lac-pro</i>)	L. Guarente

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TABLE 1—Continued

Strain or plasmid	Relevant genotype	Source or reference
Plasmids		
pRK2013	Nm ^r , ColE1 replicon, RK2 transfer	8
pRK600	Cm ^r , ColE1 replicon, RK2 transfer	11
pRmC36	Tc ^r , <i>nod-hsn</i> region, RK2 replicon	M. Honma
pRmSL26	Tc ^r , <i>nod</i> region, RK2 replicon	S. Long (17)

^a ϕ M12:(strain) to (strain) indicates ϕ M12-mediated transduction (9) from the first strain as donor to the second strain as recipient. Selection was for neomycin resistance (100 μ g/ml; Nm^r) of Tn5 or oxytetracycline resistance (0.5 μ g/ml) of Tn5-132. Colonies were screened for *exo* by calcofluor-white fluorescence, as previously described (16). When used with trimethoprim (300 μ g/ml; Tp) and spectinomycin (20 μ g/ml; Sp), neomycin was reduced to 40 μ g/ml.

each of the three sequences *nodD1*, *nodD2*, and *nodD3*; M. Honma, personal communication), a few large, white, nodulelike projections were seen as early as 11 days after inoculation, and numerous small, white or brown nodules were seen on several of the plants inoculated with RmD1D2D3 by 7 weeks; only one small nodule was seen on plants inoculated with RmD1D2D3 *exoB* by 7 weeks. In all cases in which nodules occurred, nodules produced by *nod* *exoB* mutants were indistinguishable from nodules produced by *nod*⁺ *exoB* mutants; i.e., they were white or brown, often very small, and failed to fix nitrogen (Fix⁻; 10, 16).

Thus, as with the *nod* deletions, each individual *nod* mutant had the same phenotype in both *exo*⁺ and *exo* backgrounds.

In a search for genetic loci which might be required for nodulation elicited by *exoB* mutants via a separate pathway of infection but not for normal infection-thread-mediated invasion, a number of Tn5 inserts in the vicinity of the common *nod* genes which do not themselves confer a Nod⁻ phenotype (S2A3, S159, S1B2, S167, S157, S1A8, S9B2, S161, and S9B6; 15; Table 1) were also tested in the *exo* background. We were specifically interested in putative additional genes in the common *nod* region, because this region apparently suffices to induce "empty-nodule" morphogenesis on alfalfa by *Agrobacterium tumefaciens* (13). None of the inserts tested had any effect on nodulation in either the *exo*⁺ or the *exo* background.

TABLE 2. Nodulation response of *nod* *exo* mutants

Strain	Relevant genotype	No. of plants (of 10) with nodules at day:		
		7	11	21
S1A3	<i>noda</i>	0	0	0
Rm6773	<i>noda</i> <i>exoB355</i>	0	0	0
S2B2	<i>nodB</i>	0	0	0
Rm6774	<i>nodB</i> <i>exoB355</i>	0	0	0
S8A2	<i>nodC</i>	0	0	0 ^a
Rm6776	<i>nodC</i> <i>exoB355</i>	0	0	0 ^a
RmD1D2D3	<i>nodD1D2D3</i>	0	2	4 ^b
Rm6925	<i>nodD1D2D3</i> <i>exoB355</i>	0	0	0
GMI5092	<i>node</i>	2	3	8
Rm6870	<i>node</i> <i>exoB355</i>	0	0	5
DEK5	<i>nodF</i>	1	1	7
Rm6921	<i>nodF</i> <i>exoB355</i>	0	0	6
DEK7	<i>nodG</i>	4	9	9 ^c
Rm6923	<i>nodG</i> <i>exoB355</i>	0	0	10
GMI5376	<i>nodH</i>	0	0	0
Rm6872	<i>nodH</i> <i>exoB355</i>	0	0	1
Rm1021	Wild type	3	8	10
Rm5078	<i>exoB355</i>	3	6	9

^a Results of a separate experiment.

^b Nodules were large, white and tumorous.

^c Only nine plants survived.

In theory, there might be genetic loci in *R. meliloti* which are required specifically for invasion via infection threads and not for nodule induction which does not involve root-hair-mediated invasion. Mutants with mutations in such genes would be expected to be Nod⁻ for infection-thread-mediated nodulation but Nod⁺ for nodulation which does not require infection thread formation. Therefore, in our system such mutants would be Nod⁻ in the *exo*⁺ background but Nod⁺ in the *exoB* background. We have been unable to identify any loci with those properties. Therefore, either there are no genes (other than *exo*) required specifically for infection thread initiation, or mutants with mutations in such genes do not have a simple Nod⁻ phenotype and have therefore not been found, despite the intensive efforts of several groups (e.g. references 2, 14, 17), possibly due to the presence of multiple gene copies. It is likely that mutations in other genes involved in infection thread morphogenesis induce the formation of aberrant nodules, as indeed do *exo* mutants.

exoB mutants are affected in hair curling and infection thread formation (10, 16; A. M. Hirsch, personal communication). Nodulation of *Neptunia oleracea* by *R. meliloti* does not involve hair curling and infection thread formation and also requires the common *nod* genes *noda*, *nodB*, and *nodC*, although the *hsn* genes (*node*, *nodF*, and *nodH*) are not required (2). Similarly, nodulation of *Arachis hypogaea* by a *Bradyrhizobium* (*Vigna*) sp. proceeds via intercellular invasion and requires *noda*, *nodB*, and *nodC* (19).

Intercellular bacteria are seen in nodules induced by *exoB* mutants (10, 16). Our data imply that both root-hair-mediated nodulation by *exo*⁺ strains and intercellular nodulation by *exo* mutants occur via a common pathway. Intercellular or "apoplastic" invasion is frequently described as "crack entry", suggesting a passive process. Our results suggest rather that infection by *R. meliloti* *exoB* mutants is an active, enzyme-dependent process in which the functions of all of the known *nod* genes participate. One may speculate that the Exo function directs invading rhizobia specifically to root hairs, such that in the absence of Exo function invasion can only occur at other, secondarily susceptible, epidermal sites opposite intercellular junctions.

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