Plant Physiol. (1990) 91, 368-374
0032-0889/90/91/0368/07/$01.00/0

Received for publication June 14, 1989
and in revised form September 20, 1989

Rhizobium meliloti exopolysaccharide Mutants Elicit Feedback Regulation of Nodule Formation in Alfalfa[1]

Gustavo Caetano-Anollés[2], Antonio Lagares[3], and Wolfgang D. Bauer*
Department of Agronomy, Ohio State University, Columbus, Ohio 43210-1086

ABSTRACT

Nodule formation by wild-type *Rhizobium meliloti* is strongly suppressed in younger parts of alfalfa (*Medicago sativum* L.) root systems as a feedback response to development of the first nodules (G Caetano-Anollés, WD Bauer [1988] Planta 175: 546–557). Mutants of *R. meliloti* deficient in exopolysaccharide synthesis can induce the formation of organized nodular structures (pseudonodules) on alfalfa roots but are defective in their ability to invade and multiply within host tissues. The formation of empty pseudonodules by *exo* mutants was found to elicit a feedback suppression of nodule formation similar to that elicited by the wild-type bacteria. Inoculation of an *exo* mutant onto one side of a split-root system 24 hours before inoculation of the second side with wild-type cells suppressed wild-type nodule formation on the second side in proportion to the extent of pseudonodule formation by the *exo* mutants. The formation of pseudonodules is thus sufficient to elicit systemic feedback control of nodulation in the host root system: infection thread development and internal proliferation of the bacteria are not required for elicitation of feedback. Pseudonodule formation by the *exo* mutants was found to be strongly suppressed in split-root systems by prior inoculation on the opposite side with the wild type. Thus, feedback control elicited by the wild-type inhibits *Rhizobium*-induced redifferentiation of host root cells.

Rhizobia interact with roots of leguminous plants to establish an intimate symbiotic association in which the bacterial partner fixes atmospheric nitrogen for the host in exchange for photosynthetically fixed carbon. The process of nodule formation involves signal exchange between the partners and mutual induction of a series of ordered developmental changes in both organisms (34). Substances from the host root attract the bacteria and induce the expression of genes required for further interactions with the host. In turn, rhizobia induce the deformation of growing root hairs, the initiation of cell divisions within the root cortex, the localized disruption of a host cell wall, the formation of an invaginating infection thread at the site of penetration, and the initiation of a new meristem. The new meristem develops into a complex, vascularized outgrowth called a nodule, which contains differentiated, nitrogen-fixing forms of the bacteria (bacteroids) packaged in membrane-bound vesicles within the plant cells.

Both the formation of new nodules and nitrogen fixation activity within established nodules are substantially inhibited when fixed nitrogen such as nitrate is readily available in the soil (37). Thus, the host legume can regulate these processes in response to environmental circumstances. The excision of functional nodules has been found to stimulate the formation of new nodules, suggesting that levels of symbiotically fixed nitrogen may also regulate new nodule formation (29). Moreover, recent studies indicate that new nodule formation is strongly regulated by host feedback responses which are elicited before the first nodules are capable of any nitrogen fixation (5, 7, 8, 22, 30, 31, 36). In clover, the rate of infection initiation dropped substantially at the time of emergence of the first nodules, several days before these nodules were functional (36). In soybean, the frequency of infection initiation remained approximately constant, but an increased percentage of these infections suffered arrest or abortion very soon after initiation of the first nodules (6). Thus, feedback control can be exerted at either the level of infection initiation or the level of infection development, depending on the host.

Studies with split-root systems have shown that feedback suppression of nodulation is a systemic response in soybean (22, 30), subterranean clover (36), and alfalfa (5). The split-root studies also serve to demonstrate that the total number of nodules formed on a root system is quite constant despite variations in the timing, dosage, location, and strain used for inoculation. This suggests homeostatic control and perhaps optimization of nodule number by the host.

Several mutants of soybean and pea have been isolated which nodulate profusely. These mutants appear to lack both normal feedback regulation of nodule number and normal inhibition of nodulation by exogenous nitrate (7, 8, 15, 20, 21, 30). Grafting experiments with these hyper- and supernodulating host mutants revealed that their nodulation phenotype is generally controlled by the shoot rather than the root (12, 13, 15, 32), indicating that signal transduction in the shoot is an important facet of systemic feedback responses governing nodule formation in the root.

As yet, relatively little is known about the molecular or cellular mechanisms which govern feedback control of nodule formation, either in terms of the events that initially trigger the feedback responses, the role of the shoot in transduction

[1] This work was supported in part by grant 86-CRCR-1-2118 from the U.S. Department of Agriculture Competitive Research Grants Office and by U.S. Department of Energy grant DE-FG02-86ER13522, and is contribution No. 297-89 of the Ohio Agricultural Research and Development Center.

[2] Present address: Plant Molecular Genetics (OHLD), College of Agriculture, The University of Tennessee, Knoxville, TN 37901-1071.

[3] Catedra de Quimica Biologica I, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, 1900-La Plata, Argentina.

of these responses, or the mechanism(s) by which infection initiation and development are suppressed in the root. The present studies seek to contribute to the identification of genes and events required for elicitation of feedback control and the developmental targets of the suppressive response.

Symbiotically defective mutants of rhizobia are potentially useful for these purposes. It has been established that *Rhizobium* strains and mutants that are unable to induce nodule formation are likewise unable to elicit normal feedback suppression of subsequent nodulation by the wild-type (5, 31, 36). *Rhizobium* mutants that generate fewer nodules than the wild-type due to altered host specificity or lower efficiency of nodulation were found to elicit correspondingly reduced systemic feedback responses (5, 36). *R. meliloti* mutants in nodulation region II_a (= *nodIJ*) appear to be an exception in this regard: despite a nodulation phenotype similar to region II_b mutants, the region II_a mutants generated a considerably weaker feedback response than the region II_b mutants (5). Thus, elicitation of feedback control responses may be controlled by specific genetic loci in the bacteria, and this elicitation may be at least partially separable from the ability to induce nodule formation.

In this paper, we examine the ability of various EPS[4] mutants of *R. meliloti* to elicit and to respond to feedback control. *R. meliloti* mutants defective in EPS synthesis generally induce the formation of empty, ineffective nodule-like structures referred to as pseudonodules (9, 14, 16, 17, 24, 25, 28). These pseudonodules lack extensive infection threads and have few, if any, intracellular bacteria; however, they do have well-organized meristems, normal peripheral vascular elements, and a well-defined nodule cortex layer (24). Thus, by examining the effects of pseudonodule formation on subsequent nodulation by the wild-type bacteria, it should be possible to determine whether infection thread development and internal proliferation of the bacteria are required for elicitation of feedback responses. Conversely, by examining the effects of wild-type nodulation on subsequent pseudonodule formation by *exo* mutants, it may be possible to learn whether the redifferentiation of host root cells during pseudonodule formation is suppressed by feedback control mechanisms.

MATERIALS AND METHODS

Plants and Bacteria

Alfalfa (*Medicago sativa* cv Vernal) seeds were provided by R. Van Keuren, Agronomy Department, Ohio State University, Wooster, OH. Seeds were surface-sterilized with ethanol and mercuric chloride and germinated on inverted water-agar Petri dishes (4). Bacterial strains, obtained from J. A. Leigh, University of Washington, Seattle, WA. and J. Denarie, CNRS-INRA, Castanet-Tolosan, France, and some of their relevant characteristics are listed in Table I. The *exoA*, *exoB*, and *exoF* loci are clustered in a 22 kb region of the pRmeSU47b megaplasmid, while the *exoC* and *exoD* loci are present on the chromosome (18, 26). The wild-type strain of *R. meliloti* secretes two EPS relevant to symbiotic nodule formation, EPS I and EPS II (19, 24). EPS I has an octasaccharide repeat unit consisting of seven β-linked glucose residues and one β-linked galactose residue and pyruvate, acetate, and succinate substituents (1). Stock cultures of bacteria could be maintained in yeast extract-mannitol-gluconate semisoft medium (4) for up to 5 months without loss of symbiotic efficiency. Bacteria were grown in yeast extract-mannitol-gluconate liquid medium to late exponential growth phase (OD_{500} = 0.5–0.8) and diluted to the desired bacterial concentration in Hoagland mineral solution (4).

Plant Growth and Inoculation

As described previously (4), 2-d-old seedlings were transferred to ethylene oxide-sterilized plastic growth pouches (Northrup King Seed Co., Minneapolis, MN) containing 10 mL of nitrogen-free Jensen medium. Primary roots were inoculated 3 d later with 100 μL of an appropriate dilution of the bacterial culture by dripping the suspension onto the root surface from the root tip towards the base of the roots. The positions of the RT and EH were marked on the plastic surface of the pouch at inoculation with the aid of a dissecting microscope at ×12 magnification. The plants were cultured in a growth chamber at 80% RH, 26°C in the light, 24°C in the dark, with a photoperiod of 16 h and a photosynthetically active radiation of 250 $\mu E \cdot s^{-1} \cdot m^{-2}$. The number and relative location of individual nodules were determined with a computer-linked graphics tablet 14 d after inoculation.

Split-Root Assays

Systemic suppression of nodule formation was analyzed with split-root systems as described (5). Briefly, the primary root of each alfalfa seedling was severed 3 to 5 mm above the tip with a scalpel 2 d after their transfer to the pouches. Divided root systems were obtained in 5 d, at which time the pouches were cut with scissors and the cut edges sealed with transparent tape so that each half of the root system was separated. Twelve d after imbibition, roots on one side were inoculated with a total of 100 μL of bacterial suspension (or Hoagland solution = sham), and the locations of RT and EH were marked for each individual lateral root. The other side of the split-root was inoculated 24 h later with 100 μL of bacterial suspension. Nodules were counted 26 d after imbibition.

Nodule Occupancy

Nodules were excised from roots, surface sterilized with mercuric chloride, exhaustively rinsed with water, their contents released by crushing in Hoagland solution, and the number and ratio of occupants in each nodule determined on the basis of antibiotic resistance as described earlier (4).

RESULTS AND DISCUSSION

Distribution of Nodules on the Primary Root

Profiles of the distribution of nodules along the primary root following inoculation with the wild-type and *exo* mutant

[4] Abbreviations: EPS, exopolysaccharide; EH, smallest emergent root hair; RT, root tip; kb, kilobase pair.

Table I. *Bacterial Strains and Relevant Characteristics*

Strain	Relevant Characteristics	Source
RCR 2011	Wild-type (=SU47), can synthesize both EPS I and EPS II.[a,b]	(35)
GMI5390	RCR2011 region IIa::Tn5 2412 Sm^{r}Nm^r, (= *nodIJ*), inefficient, slightly delayed nodulation.[c,d]	(11)
GMI5518	RCR2011 region IIb::Tn5 2203 Sm^{r}Nm^r, inefficient, slightly delayed nodulation.[c,d]	(11)
GMI766	RCR2011 Δ (*nod fixA*) 766 spc^r, unable to nodulate	(38)
1021	Sm^r derivative of RCR2011	(27)
Rm7061	1021 *exoA61*::Tn5 Sm^{r}Nm^r, unable to synthesize EPS I.[e]	(24)
Rm7094	1021 *exoB94*::Tn5 Sm^{r}Nm^r, unable to synthesize EPS I or EPS II, makes abnormal LPS.[e,f]	(24)
Rm7020	1021 *exoC20*::Tn5 Sm^{r}Nm^r, unable to synthesize EPS I, β-(1-2)-glucan, makes abnormal LPS.[e,f]	(24)
Rm7017	1021 *exoD17*::Tn5 Sm^{r}Nm^r, makes reduced quantities of EPS I.[e]	(24)
Rm7055	1021 *exoF55*::Tn5 Sm^{r}Nm^r, unable to synthesize EPS I.[e]	(24)
Rm7022	1021 Δ(*exoAHFB*) 22 Sm^{r}Nm^r	(24)

[a] (24). [b] (19). [c] (11). [d] (4). [e] (23). [f] (26).

derivatives are shown in Figure 1. Since alfalfa root cells in the region of emerging root hairs are the only cells susceptible to *Rhizobium* infection and lose their susceptibility to infection by *R. meliloti* within 8 to 12 h as a consequence of acropetal root development (3, 4), the ability of *exo* mutants to generate nodules as far above the RT mark as the wild-type (Fig. 1) implies that they must be able to initiate pseudonodules just as rapidly as the wild-type initiates normal nodules.

In most cases, the *exo* mutants generated fewer pseudonodules in younger regions of the primary root, below the RT mark, than in the initially susceptible region above the RT mark (Fig. 1). For the wild type, this pattern of nodule distribution is a reflection of feedback regulation rather than limiting numbers of bacteria (5, 33). The clustering of pseudonodules in the initially susceptible region provides the first line of evidence that *exo* mutants can elicit feedback responses in alfalfa. This clustering also provides the first indication that pseudonodule formation by *exo* mutants may be susceptible to feedback regulation.

Table II provides a comparison of the ability of different *exo* mutants to form pseudonodules in the initially susceptible region of the primary root, in younger regions of the primary root (below the RT mark), and on lateral roots. Pseudonodule formation on the primary root varied with each mutant. *exoC* formed few pseudonodules anywhere, and *exoD* formed very few pseudonodules below the RT mark. The other *exo* mutants formed roughly as many pseudonodules in the initially susceptible region, above RT, as they did in younger regions of the primary root below RT. This is similar to the distribution of functional nodules formed by the wild type and consistent with the profiles in Figure 1. Except for *exoC*, all of the *exo* mutants formed fewer nodules on the primary root than the wild type and formed more nodules on lateral roots than the wild type. These results are consistent with the notion that pseudonodule formation by *exo* mutants may be governed by feedback regulation. It would be of interest to learn whether increased nodulation of lateral roots by the *exo* mutants is a compensation for limited nodulation of the primary root or an effect of prolonged N starvation in reversing feedback suppression already elicited in the primary root.

Inoculum Dose-Nodulation Response Behavior of *Exo* Mutants

Dose-response curves for nodule initiation by the parent and representative *exo* mutant derivatives are shown in Figure 2. *R. meliloti* 1021 elicited very few nodules in the initially susceptible region above the RT mark at inoculum dosages below 5×10^4 bacteria/plant. A sharp increase in nodulation was observed when the bacterial dose was increased 10-fold, after which a plateau was reached (Fig. 2). Very similar curves have been obtained with strain RCR2011, the streptomycin-sensitive parent of strain 1021 (4). However, compared to RCR2011, strain 1021 requires dosages approximately 10 times higher in order to elicit half-maximal numbers of first nodules, indicating that acquisition of streptomycin resistance by 1021 may have caused a marked decrease in efficiency of nodule initiation.

The *exoB* mutant formed relatively few nodules in the initially susceptible zone, regardless of dosage (Fig. 2). Similar response curves were obtained with *exoA* and *exoC* mutant derivatives. Other *exo* mutants, exemplified by *exoF* in Figure 2, appeared to form somewhat greater numbers of nodules in the initially susceptible region of the root, although there was

no evidence of sharply increased response at high inoculum dosages. The nodulation profiles for the *exo* mutants obtained at different inoculum dosages (data not shown) were similar to those shown in Figure 1. These results serve to eliminate inoculum dosage as a critical variable in our other experiments and indicate that the unexplained ability of wild-type bacteria to help each other in nodule initiation at dosages of about 10^4 cells/plant is not shared by the *exo* mutants.

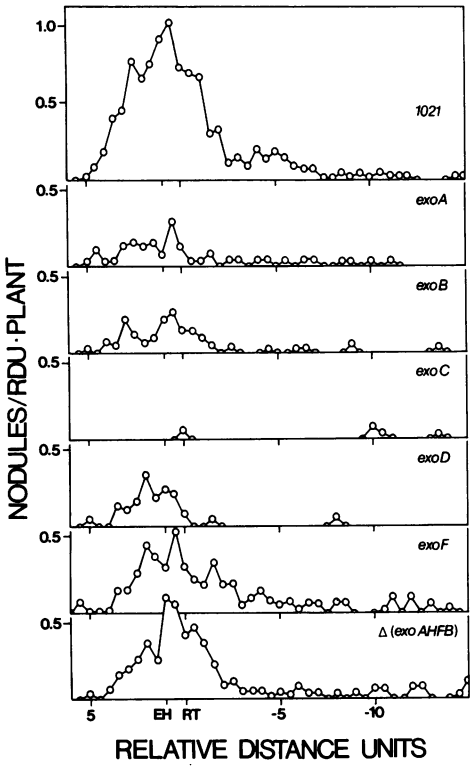

Figure 1. Nodule distribution profiles for alfalfa roots inoculated with *R. meliloti* 1021 and *exo* mutant derivatives. Sets of 72 to 88 plants were inoculated with $1.0 \cdot 10^6$ to $4.0 \cdot 10^6$ bacteria/plant. The relative distance of each nodule (or pseudonodule) on the primary root from the RT mark was expressed as a percentage of the RT to smallest emergent root hair (EH) distance determined for each plant. The average RT-to-EH distance was 3.3 ± 0.4 mm for this experiment. Since the average rate of root elongation is 0.5 ± 0.04 mm·h^{-1}, one relative distance unit (RDU) is equivalent to approximately 7 h of root growth. The direction of root growth is left to right. Some of the mutants nodulated abundantly on secondary roots (see Table II). Results are representative from two independent experiments.

Time of Pseudonodule Emergence

At dosages of 10^6 cells/plant, wild-type nodules and *exo* mutant pseudonodules were first evident 3 to 4 d after inoculation, establishing that rates of nodule and pseudonodule development are comparable after initiation. The subsequent rate of appearance of nodules and pseudonodules was also comparable (data not shown). However, the small, white nodules elicited by *exoF*, for example, required about 1 week longer to reach the same size as nodules formed by the parent. The nodules formed by *exoF*, but not the other *exo* mutants tested, were generally white, multilobed structures at that age, with a few effective, pink nodules.

Elicitation of Systemic Feedback Responses

Prior inoculation of one side of a split-root system with wild-type strain 1021 suppressed subsequent nodulation by 1021 on the opposite side by 75 to 90%, both in the zone above the RT mark and in younger regions of the split-root laterals (Fig. 3). This is consistent with results obtained earlier with strain RCR2011 (5). In similar split-root assays, each of the *exo* mutant derivatives also suppressed nodulation by wild-type 1021. Suppression of wild-type nodulation by the mutants ranged from 30 to 70%. Wild-type nodule formation in regions above the RT mark and nodulation in younger regions of the split-root laterals were suppressed about equally. There was generally a good correlation between the number of pseudonodules formed in the initially susceptible region by the *exo* mutants and their suppressing activity: strains that formed few pseudonodules, like *exoC* and *exoD*, elicited the weakest suppressive response, whereas those forming higher numbers suppressed nodulation almost as well as the parent.

The suppression of wild-type nodulation by the *exo* mutants in these split-root experiments was a rapid and systemic response, similar to nodulation feedback responses generated by the wild-type with respect to the intensity, duration, and developmental timing of suppression (Fig. 3). Thus, it is likely, though not yet proven, that *exo* mutants elicit normal feedback responses in alfalfa. A clear implication of these results

Table II. *Relative Nodulating Ability of R. meliloti 1021 and exo Mutant Derivatives*

Strain	Mutation	Average Number of Nodules (pseudonodules)/Plant[a]		
		Above RT on the primary root	Total on the primary root	Total on lateral roots
1021	Wild type	2.54 ± 0.30	4.46 ± 0.41	0.35 ± 0.15
Rm7061	*exoA*	0.50 ± 0.19	0.82 ± 0.25	4.20 ± 0.97
Rm7094	*exoB*	0.43 ± 0.18	0.79 ± 0.23	6.80 ± 1.03
Rm7020	*exoC*	0.01 ± 0.16	0.19 ± 0.20	0.43 ± 0.19
Rm7017	*exoD*	0.57 ± 0.18	0.68 ± 0.24	2.03 ± 0.76
Rm7055	*exoF*	0.94 ± 0.20	2.03 ± 0.29	3.31 ± 1.03
Rm7022Δ	(*exoAHFB*)	1.26 ± 0.24	3.11 ± 0.39	1.41 ± 0.67

[a] Averages ± SD from duplicate sets of 72 to 88 plants inoculated with $1.4 \cdot 10^6$ to $4.2 \cdot 10^6$ bacteria/plant.

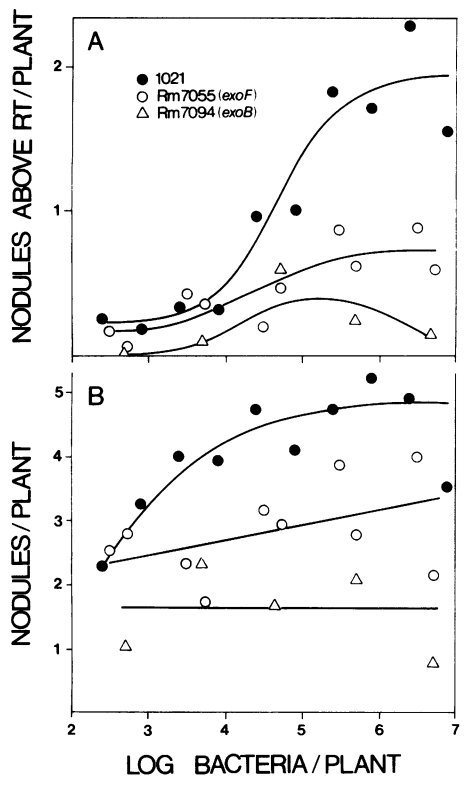

Figure 2. Effect of inoculum dose on nodulation of alfalfa. Sets of 60 to 70 seedlings were inoculated with *R. meliloti* 1021 (●) with *exoF* mutant Rm7055 (○) or with *exoB* mutant Rm7094 (△) after dilution to the indicated dosages. A, Nodulation above the root tip (RT) mark; B, total nodulation on the primary root. Data points are from two independent experiments.

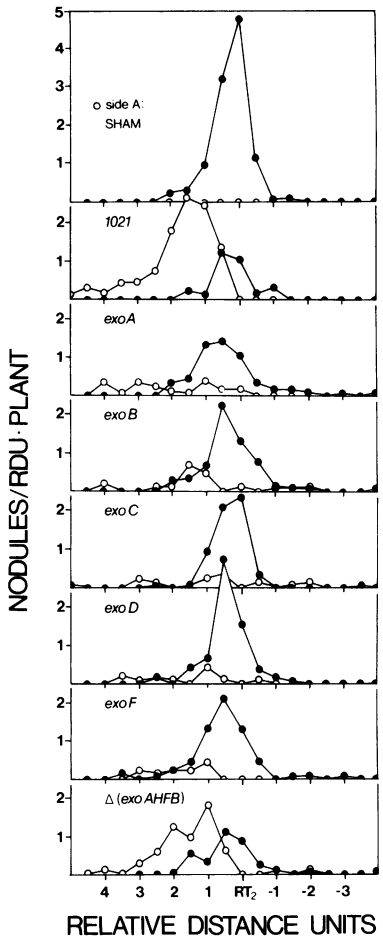

Figure 3. Effect of prior inoculation on one side of an alfalfa split-root system on nodulation on the second side. Nodule distribution profiles were determined for sets of 20 to 30 seedlings. The split-roots were inoculated at the time of marking RT_1 on side A (○) with Hoagland mineral solution (= sham), with $2.7 \cdot 10^6$ to $3.5 \cdot 10^6$ bacteria/plant of wild-type *R. meliloti* 1021, or with *exo* mutants, as indicated on the figure, and then inoculated 24 h later (RT_2) on the opposite side (side B) (●) with $3.3 \cdot 10^6$ bacteria/plant of 1021. The location of nodules on each lateral root from each split root was determined relative to the RT_2 mark and expressed in relative distance units defined by the physical RT_1-to-RT_2 distance determined for each lateral root. The average RT_1-to-RT_2 distance was 8.3 ± 1.5 mm. The average number of lateral roots on each side of the split-root system was 1.18 ± 0.15. Results are representative from two independent experiments.

is that elicitation of nodulation feedback can occur without extensive bacterial infection/proliferation. Hence, we consider it possible that feedback responses are elicited by diffusible substances from the bacteria, as seen for induction of the localized cortical cell divisions that precede nodule meristem formation (2, 10).

Feedback Suppression of Pseudonodule Formation

In reciprocal experiments, it was found that prior inoculation of an alfalfa split-root with the wild-type strain almost completely suppressed subsequent pseudonodule formation by *exoF*, a mutant which makes no EPS I (Fig. 4). Since pseudonodule formation by *exoF* was fully suppressed by normal feedback responses, and since *exoF* forms no infection threads or threads that abort at a very early stage (17, 24, 25), we tentatively conclude that infection thread development is not a crucial target of the mechanisms employed by alfalfa plants to block, arrest, or abort infection development. This leaves host cell division and redifferentiation in developing nodules and pseudonodules as perhaps the most important targets of suppression by feedback control.

Feedback Suppression of Nodulation by Region II Mutants

In similar experiments, *R. meliloti* RCR2011, the streptomycin-sensitive progenitor of 1021, was found strongly and equally to suppress nodule formation by Tn5 mutants in nodulation regions IIa and IIb (Fig. 4). While both region II_a and II_b mutants appear to be equally susceptible to suppressive feedback responses generated by the wild type, they differ significantly in their ability to elicit feedback suppression: region II_b mutants elicit feedback inhibition about as well as the wild type, but region II_a mutants elicit an inhibition averaging only about 20% as great as the wild type (5). These findings indicate that elicitation of feedback inhibition and susceptibility to such inhibition are separate phenomena.

It appears that elicitation of feedback control in alfalfa does not require the synthesis of EPS I. Further, since *exoB* appar-

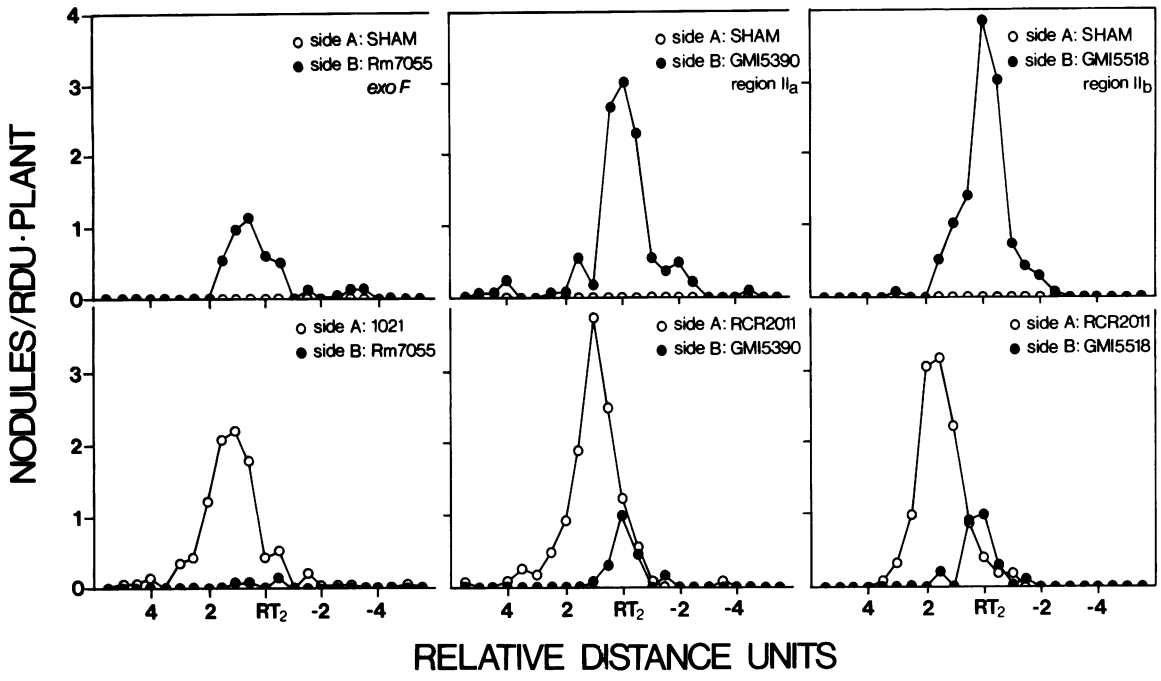

Figure 4. Effect of prior inoculation with wild-type *R. meliloti* strains on one side of a split-root system on nodulation on the second side by *exoF* mutant derivative Rm7055 or region II *nod* mutants. Nodule distribution profiles were determined for sets of 18 to 24 seedlings. The split-roots were inoculated at the time of marking RT_1 (O) with $4.0 \cdot 10^5$ to $4.5 \cdot 10^5$ wild-type bacteria/plant (side A), and inoculated 24 h later (RT_2) ● on the opposite side (side B) with $3.2 \cdot 10^5$ to $5.0 \cdot 10^5$ mutant bacteria/plant. The location of nodules on each lateral root was determined relative to the RT_2 mark and expressed in relative distance units defined by the physical RT_1-to-RT_2 distance determined for each lateral root. The average RT_1-to-RT_2 distance was 8.2 ± 0.7 mm. The average number of lateral roots per split was 1.15 ± 0.08. Results are representative from two independent experiments.

ently lacks the ability to synthesize either EPS I or EPS II (24), but still elicits feedback suppression (Figs. 2 and 3), elicitation probably does not require EPS II. By implication, any steps of nodule development that follow infection thread development (*i.e.* the step that requires EPS I or EPS II) cannot be essential for elicitation of feedback responses. It remains to be determined whether feedback suppression of nodule formation is elicited directly by some specific substance from the bacteria or indirectly as a response to some change in the symbionts during the course of infection development.

Likewise, in regard to the mechanism(s) of feedback suppression, it remains to be established whether the host inhibits infection development directly and/or through some indirect mechanism such as activation of phytoalexin synthesis, lignification, and so forth. The results obtained in this study suggest that infection thread development and subsequent steps are not essential targets of the suppressive mechanism in 'Vernal' alfalfa, since effective suppression is observed in the absence of these events. Cortical cell division and redifferentiation are strongly suppressed by feedback responses in both normal nodules and pseudonodules and may therefore prove to be the principal targets of feedback suppression.

ACKNOWLEDGMENTS

We are indebted to Drs. J. A. Leigh and J. Dénarié for provision of strains, and to Dr. G. Favelukes for supporting A. Lagares during his stay in the laboratory.

LITERATURE CITED

1. **Aman P, McNeil M, Franzen L, Darvill AG, Albersheim P** (1981) Structural elucidation, using HPLC-MS and GLC-MS of the acidic polysaccharide secreted by *Rhizobium meliloti* strain 1021. Carbohydr Res. **95:** 263–282
2. **Bauer WD, Bhuvaneswari TV, Calvert HE, Law IJ, Malik NSA, Vesper SJ** (1985) Recognition and infection by slow-growing rhizobia. *In* HJ Evans, PH Bottomley, WE Newton, eds, Nitrogen Fixation Research Progress. Nijhoff, Dordretch, pp 247–253
3. **Bhuvaneswari TV, Bhagwat AA, Bauer WD** (1981) Transient susceptibility of root cells in four common legumes to nodulation by rhizobia. Plant Physiol **68:** 1141–1149
4. **Caetano-Anollés G, Bauer WD** (1988) Enhanced nodule initiation on alfalfa by wild-type *Rhizobium meliloti* co-inoculated with *nod* gene mutants and other bacteria. Planta **174:** 385–395
5. **Caetano-Anollés G, Bauer WD** (1988) Feedback regulation of nodule formation in alfalfa. Planta **175:** 546–557
6. **Calvert HE, Pence MK, Pierce M, Malik NSA, Bauer WD** (1984) Anatomical analysis of the development and distribution of *Rhizobium* infections in soybean roots. Can J Bot **62:** 2375–2384
7. **Carroll BJ, McNeil DL, Gresshoff PM** (1985) Isolation and properties of soybean (*Glycine max.* (L) Merr.) mutants that nodulate in the presence of high nitrate concentrations. Proc Natl Acad Sci USA **82:** 4162–4166
8. **Carroll BJ, McNeil DL, Gresshoff PM** (1985) A supernodulation and nitrate tolerant symbiotic (nts) soybean mutant. Plant Physiol **78:** 34–40
9. **Chen H, Gray JX, Nayudu MA, Djordjevic MA, Batley M, Redmond JW, Rolfe BG** (1988) Five genetic loci involved in

the synthesis of acidic exopolysaccharide are closely linked in the genome of *Rhizobium* sp. strain NGR234. Mol Gen Genet **212:** 310–316

10. **Cooper JB, Long SR** (1988) Nodule initiation in the alfalfa-*Rhizobium meliloti* symbiosis. *In* NT Keen, T Kosuge, LL Walling, eds, Physiology and Biochemistry of Plant-Microbial Interactions. American Society of Plant Physiologists, Rockville, MD, p 148
11. **Debelle F, Rosenberg C, Vasse J, Maillet F, Martinez E, Dénarié J, Truchet G** (1986) Assignment of symbiotic development phenotypes to common and specific nodulation (*nod*) genetic loci of *Rhizobium meliloti*. J Bacteriol **168:** 1075–1086
12. **Delves AC, Mathews A, Day DA, Carter AS, Carroll BJ, Gresshoff PM** (1986) Regulation of the soybean-*Rhizobium* symbiosis by shoot and root factors. Plant Physiol **82:** 588–590
13. **Delves AC, Higgins A, Gresshoff PM** (1987) A common shoot control of supernodulation in a number of mutant soybeans *Glycine max* (L.) Merr. Aust J Plant Physiol **14:** 689–694
14. **Djordjevic SP, Chen H, Batley M, Redmond JW, Rolfe BG** (1987) Nitrogen fixation ability of exopolysaccharide synthesis mutants of *Rhizobium sp.* strain NGR234 and *Rhizobium trifolii* is restored by the addition of homologous exopolysaccharides. J Bacteriol **169:** 53–60
15. **Duc G, Messager A** (1989) Mutagenesis of pea (*Pisum sativum*, L.) and the isolation of mutants for nodulation and nitrogen fixation. Plant Sci **60:** 207–213
16. **Finan TM** (1988) Genetic and physical analyses of group E Exo$^-$ mutants of *Rhizobium meliloti*. J Bacteriol **170:** 474–477
17. **Finan TM, Hirsch AM, Leigh JA, Johansen E, Kuldau GA, Deegan S, Walker GC, Walker ER** (1985) Symbiotic mutants of *Rhizobium meliloti* that uncouple plant from bacterial differentiation. Cell **40:** 869–877
18. **Finan TM, Kunkel B, DeVos GF, Signer ER** (1986) Second symbiotic megaplasmid in *Rhizobium meliloti* carrying exopolysaccharide and thiamine synthesis genes. J Bacteriol **167:** 66–72
19. **Glazebrook J, Walker GC** (1989) A novel exopolysaccharide can function in place of the Calcofluor-binding exopolysaccharide in nodulation of alfalfa by *Rhizobium meliloti*. Cell **56:** 661–672
20. **Gremaud MF, Harper JE** (1989) Selection and initial characterization of partially nitrate tolerant nodulation mutants of soybean. Plant Physiol **89:** 169–173
21. **Jacobsen E, Feenstra WJ** (1984) A new pea mutant with efficient nodulation in the presence of nitrate. Plant Sci Lett **33:** 337–344
22. **Kosslak RM, Bohlool BB** (1984) Suppression of nodule development of one side of a split-root system of soybeans caused by prior inoculation of the other side. Plant Physiol **75:** 125–130
23. **Leigh JA, Lee CC** (1988) Characterization of polysaccharides of *Rhizobium meliloti exo* mutants that form ineffective nodules. J Bacteriol **170:** 3327–3332
24. **Leigh JA, Signer ER, Walker GC** (1985) Exopolysaccharide-deficient mutants of *Rhizobium meliloti* that form ineffective nodules. Proc Natl Acad Sci USA **82:** 6231–6235
25. **Leigh JA, Reed JW, Hanks JF, Hirsch AM, Walker GC** (1987) *Rhizobium meliloti* mutants that fail to succinylate their Calcofluor-binding exopolysaccharide are defective in nodule invasion. Cell **51:** 579–587
26. **Long S, Reed JW, Himawan J, Walker GC** (1988) Genetic analysis of a cluster of genes required for synthesis of the calcofluor-binding exopolysaccharide of *Rhizobium meliloti*. J Bacteriol **170:** 4239–4248
27. **Meade HM, Long SR, Ruvkun GB, Brown SE, Ausubel FM** (1982) Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon mutagenesis. J Bacteriol **149:** 114–122
28. **Muller P, Hynes M, Kapp D, Niehaus K, Puhler A** (1988) Two classes of *Rhizobium meliloti* infection mutants differ in exopolysaccharide production and in coinoculation properties with nodulation mutants. Mol Gen Genet **211:** 17–26
29. **Nutman PS** (1952) Studies on the physiology of nodule formation. III. Experiments on the excision of root-tips and nodules. Ann Bot **16:** 81–102
30. **Olsson JE, Nakao P, Bohlool BB, Gresshoff PM** (1989) Lack of systemic suppression of nodulation in split-root systems of supernodulating soybean (*Glycine max* [L.] Merr.) mutants. Plant Physiol **90:** 1347–1352
31. **Pierce M, Bauer WD** (1983) A rapid regulatory response governing nodulation in soybean. Plant Physiol **73:** 286–290
32. **Postma JG, Jacobsen E, Feenstra WJ** (1988) Three pea mutants with an altered nodulation studied by genetic analysis and grafting. J Plant Physiol **132:** 424–430
33. **Purchase HF, Nutman PS** (1957) Studies on the physiology of nodule formation. VI. The influence of bacterial numbers in the rhizosphere on nodule initiation. Ann Bot **21:** 439–454
34. **Rolfe BG, Gresshoff PM** (1988) Genetic analysis of legume nodulation. Annu Rev Plant Physiol Plant Mol Biol **39:** 297–319
35. **Rosenberg C, Boistard P, Dénarié J, Casse-Belbart F** (1981) Genes controlling early and late functions in symbiosis are located on a megaplasmid in *Rhizobium meliloti*. Mol Gen Genet **184:** 326–333
36. **Sargent L, Huang SZ, Rolfe BG, Djordjevic MA** (1987) Split-root assays using *Trifolium subterraneum* show that *Rhizobium* infection induces a systemic response that can inhibit nodulation of another invasive *Rhizobium* strain. Appl Environ Microbiol **56:** 1611–1619
37. **Streeter J** (1988) Inhibition of legume nodule formation and nitrogen fixation by nitrate. CRC Crit Rev Plant Sci **7:** 1–23
38. **Truchet G, Debelle F, Vasse J, Terzaghi B, Garnerone AM, Rosenberg C, Batut J, Maillet F, Dénarié J** (1985) Identification of a *Rhizobium meliloti* pSym 2011 region controlling the host specificity of root hair curling and nodulation. J Bacteriol **164:** 1200–1210