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Interaction of *nod* and *exo Rhizobium meliloti* in Alfalfa Nodulation

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Among the genes of *Rhizobium meliloti* SU47 that affect nitrogen-fixing symbiosis with alfalfa are *nod* genes, in which mutations block nodule induction, and *exo* genes, in which mutations allow nodule formation but block rhizobial exopolysaccharide production as well as nodule invasion and nitrogen fixation. To investigate whether an *exo*$^+$ bacterium can "help" (that is, reverse the symbiotic defect of) an *exo* mutant in *trans*, we have coinoculated alfalfa with pairs of rhizobia of different genotypes. Coinoculant genotypes were chosen so that the *exo*$^+$ helper strain was *nif* while the *exo* "indicator" strain was *nif*$^+$, and thus any fixation observed was carried out by the *exo* coinoculant. We find that a *nod exo*$^+$ coinoculant can help an *exo* mutant both to invade nodules and to fix nitrogen. However, a *nod*$^+$*exo*$^+$ coinoculant cannot help an *exo* mutant: Few *exo* bacteria are recovered from nodules, some bacteroids differentiate into bizarre aberrant forms, and the nodules fail to fix nitrogen. In a triple coinoculation, the effect of *nod*$^+$ helper supersedes that of *nod* helper. Implications of these results for interaction of *nod* and *exo* gene products are discussed.

Additional key words: exopolysaccharide, nitrogen fixation

Rhizobium meliloti SU47 secretes an acidic extracellular heteropolysaccharide (EPS; Zevenhuisen and Scholten-Koerselman 1979; Aman *et al.* 1981; Tolmasky *et al.* 1982), and EPS-deficient mutants (*exo*) give nodules on alfalfa that do not fix nitrogen (Fix$^-$; Finan *et al.* 1985; Leigh *et al.* 1985). Wild-type *R. meliloti* nodulates by inducing a root hair to curl into a "shepherd's crook"; entering the hair within the curl; eliciting a tubular, ramified infection thread down which it travels across cell borders and deep into the root cortex; and finally, after release from the infection thread within a cortical cell, differentiating into a characteristic large elongate "bacteroid" surrounded by a host-derived "peribacteroid membrane." In contrast, *exo* mutants are deficient in curling the root hair, entering the hair, and eliciting the infection thread; within their Fix$^-$ nodules they are found in intercellular spaces only, having invaded in some way that is still uncharacterized. Clearly, Exo function is required for invasion of root hairs. It might also be required for subsequent stages in nodule development. Alternatively, *exo* nodules might be Fix$^-$ simply because the bacteria are in the wrong location in the nodule.

We investigated whether *exo* mutants could induce the formation of Fix$^+$ nodules if the block to invasion were overcome, by coinoculating alfalfa with both a *nif*$^+$*exo* (*nif* = nitrogen fixation) "indicator" strain and a *nif exo*$^+$ "helper." Because coinoculation can overcome the defects of other symbiotic mutants (Rolfe and Gresshoff 1980; Rolfe *et al.* 1980; W. Szeto, personal communication), we thought the coinoculated *nif exo*$^+$ might "help" the *nif*$^+$*exo* in *trans* to invade a root hair and reach an inner cortical cell. There, given the *nif* mutation of the *exo*$^+$ helper, any nitrogen fixation would have to be due to the *nif*$^+$*exo* indicator.

Here we show that *exo* indicator can be helped by coinoculated *exo*$^+$ bacteria to invade inner cortical cells and differentiate there into a nitrogen-fixing bacteroid. However, such helping depends on the *nod* genotype of the helper as well as that of the indicator.

We have given a preliminary report of some of this work (Klein *et al.* 1986).

MATERIALS AND METHODS

Media and growth conditions. The bacterial strains listed in Table 1 were grown as described (Finan *et al.* 1985; Leigh *et al.* 1985). Drugs were supplemented as follows: neomycin (Nm), 100 μg/ml; spectinomycin (Sp), 100 μg/ml; oxytetracycline (Ot), 0.5 μg/ml, all from Sigma Chemical Co. Calcofluor-white (Cellufluor, Polysciences, Inc.) was added to Luria-Bertani (LB) agar to 0.02%. LB-calcofluor plates were buffered with Hepes (10 mM, pH 7.4) from Sigma Chemical Co.

Strain construction. Transduction with ϕM12 has been described (Finan *et al.* 1984). Rm6906 through Rm6910 were constructed by selection for Sp^r of Tn*5-233*. Rm6776 was constructed by selection for Ot^r of Tn*5-132*, which is linked to *exoB* (De Vos *et al.* 1986), and screening of colonies for lack of EPS fluorescence with calcofluor-white (Leigh *et al.* 1985). In strains with multiple insertions, resistance to all relevant antibiotics was confirmed.

Nodulations. Seedlings of alfalfa (*Medicago sativa*) were nodulated in tubes on slants of Jensen's agar (Vincent 1970; Hirsch et al 1983). Colonies from agar plates were resuspended in sterile water to a density of approximately 10^7 bacteria per milliliter, and 0.5 or 1 ml of the suspension (depending on the experiment) was added to each tube. For coinoculations, the individual coinoculants were mixed together in equal amounts before their addition to the seedlings, and the same final volume of bacterial suspension was added per tube as for single inoculant controls. Each sample was inoculated onto a minimum of 10 plants per experiment and was tested in at least two separate experiments. Plants were assayed for acetylene reduction at intervals between 3 and 6 wk (Meade *et al.* 1982).

Recovery of bacteria from nodules. Nodules were surface

Table 1. Bacterial strain

Strain				Source
Rm1021*str*-21*nod*$^+$	*nif*$^+$	*exo*$^+$		F. M. Ausubel
Rm5020*str*-21*nod*$^+$	*nif*$^+$	*exo*B-355	Ω5004::Tn*5*-Nm^{ra}	Finan *et al.* 1985
Rm5078*str*-21*nod*$^+$	*nif*$^+$	*exo*B-355	Ω5006::Tn*5*-132-Ot^{ra}	De Vos *et al.* 1986
Rm7055*str*-21*nod*$^+$	*nif*$^+$	*exo*F::Tn*5*-Nm^r		Leigh *et al.* 1985
Rm1491*str*-21*nod*$^+$	*nif*H::Tn*5*-Nm^r	*exo*$^+$		F. M. Ausubel
Rm6020*str*-21*nod*$^+$	*nif*H::Tn*5*-233-Sp^r	*exo*$^+$		Devos *et al.* 1986
Rm1354*str*-21*nod*$^+$	*nif*A::Tn*5*-Nm^r	*exo*$^+$		F. M. Ausubel
Rm1027*str*-21*nod*C::ISRM1	*nif*$^+$	*exo*$^+$	Tn*5*-Nm^{rb}	Buikema *et al.* 1983
Rm1126*str*-21*nod*C::Tn*5*-Mu-Nm^r	*nif*$^+$	*exo*$^+$		Buikema *et al.* 1983
Rm5610*str*-21*nod*A::Tn*5*-Nm^r	*nif*$^+$	*exo*$^+$		φM12(S9B8^c to 1021)^d M. Williams
Rm5611*str*-21*nod*B::Tn*5*-Nm^r	*nif*$^+$	*exo*$^+$		φM12(S2B2^c to 1021)^d M. Williams
Rm5612*str*-21*nod*C::Tn*5*-Nm^r	*nif*$^+$	*exo*$^+$		φM12(S170^c to 1021)^d M. Williams
Rm5613*str*-21*nod*C::Tn*5*-Nm^r	*nif*$^+$	*exo*$^+$		φM12(S8A2^c to 1021)^d M. Williams
Rm6906*str*-21*nod*C::ISRM1	*nif*H::Tn*5*-233-Sp^r	*exo*$^+$	Tn*5*-Nm^{rb}	φM12(6020 to 1126)^d this work
Rm6907*str*-21*nod*A::Tn*5*-Nm^r	*nif*H::Tn*5*-233-Sp^r	*exo*$^+$		φM12(6020 to 5610)^d this work
Rm6908*str*-21*nod*B::Tn*5*-Nm^r	*nif*H::Tn*5*-233-Sp^r	*exo*$^+$		φM12(6020 to 5611)^d this work
Rm6909*str*-21*nod*C::Tn*5*-Nm^r	*nif*H::Tn*5*-233-Sp^r	*exo*$^+$		φM12(6020 to 5612)^d this work
Rm6910*str*-21*nod*C::Tn*5*-Nm^r	*nif*H::Tn*5*-233-Sp^r	*exo*$^+$		φM12(6020 to 5613)^d this work
Rm6776*str*-21*nod*C::Tn*5*-Nm^r	*nif*$^+$	*exo*B-355	Ω5006::Tn*5*-132-Ot^{ra} (pPH1JI)-Sp^r	φM12(5078 to S8A2^b)^a this work

[a] Insert linked to *exo*B.
[b] Chromosomal insert, linked to *pyr*.
[c] Jacobs *et al.* 1985.
[d] Indicates φM12 transduction, where first strain is donor and second is recipient.

sterilized in 20% sodium hypochlorite and washed once in sterile water and then several times in LB supplemented with Mg^{++} and Ca^{++}. Some nodules were squashed whole and plated as below; other nodules were sliced in two with a sterile razor blade. One half of the nodule was fixed for electron microscopy; the other half of the nodule was squashed in a solution of LB (Mg^{++}, Ca^{++}) containing 0.3 M glucose. Serial dilutions of the squashed nodule mixture in the same medium were plated on LB agar, supplemented with calcofluor-white and/or drugs as appropriate. In some cases, colonies from plates without drugs were replicated onto plates containing drugs for strain confirmation. Colonies on calcofluor-white agar were illuminated with long wavelength ultraviolet and scored as "bright" (*exo*$^+$) or "dark" (*exo*). Because nodules were halved before squashing, numbers probably represent underrecovery of bacteria. We assume that the bacteria that are recovered have not differentiated into bacteroid form.

Microscopy. Nodules were prepared for light and electron microscopy as described by Hirsch *et al.* (1983). Sections were examined from the late symbiotic or bacteroid zone of the nodule.

RESULTS

Single inoculations. The phenotype of each of the strains used is shown in Table 2. By 6 wk after inoculation, there was a clear difference between Fix$^+$ and Fix$^-$ seedlings in the height and color of their tops: Fix$^+$ plants were tall and green, whereas Fix$^-$ plants were stunted and yellow. There was some leakiness in the Exo$^-$ phenotype: In some experiments, up to 10% of plants inoculated with an *exo* strain (Rm5078, Rm5020, or Rm7055) had one or two Fix$^+$ nodules (among a large excess of Fix$^-$ nodules) and such plants were scored as Fix$^+$. The nature of this leakiness is not understood. At 3 wk, wild-type inoculated plants (Rm1021) averaged 4.5 Fix$^+$ nodules per plant (in addition to occasional nodules that failed to reduce acetylene), and *exoB*-inoculated plants (Rm5078) averaged 7.5 nodules per plant, most or all of which were Fix$^-$. When nodules from inoculation with *exoB* (Rm5078) were squashed, no colonies were recovered from a majority (approximately 85%) of nodules (Table 2). In general, few colonies (less than 20) were recovered from the remaining nodules, although occasionally more (1,000–2,000) were obtained.

Table 2. Helping of *exo* bacteria

	Inoculant (s)						Phenotype[a] (% of plants)	
	Helper			Indicator				
	nod	*nif*	*exo*	*nod*	*nif*	*exo*	Nod$^+$	Fix$^+$
Coinoculations								
Helping of *exo*								
1.	+	−	+	+	+	−	100	8
2.	+	−	+	−	+	−	100	0
3.	−	−	+	+	+	−	100	69
Helping of *nod*								
4.	+	−	+	−	+	+	100	69[b]
5.	+	−	−	−	+	+	92	75
6.	+	−	−	−	+	−	100	0
Controls								
7.	+	+	−	−	+	+	100	92[c]
8.	+	+	+	−	+	+	100	100
9.	+	+	+	+	−	+	100	92
10.	+	+	+	+	+	−	100	92
11.	−	−	+	−	+	−	0	
12.	−	−	+	+	−	−	100	0
Single Inoculations								
13.	+	+	+				100	92
14.	−	+	+				0	
15.	+	−	+				100	0
16.	−	−	+				0	
17.	+	+	−				100	0
18.	−	+	−				0	
19.	+	−	−				100	0

[a] Data from a representative experiment. Fix$^+$ plants reduced acetylene at least 50% as well as control plants inoculated with wild type (Rm1021) and were tall and green. Fix$^-$ plants failed to reduce acetylene or reduced acetylene at less than 20% the levels of plants inoculated with wild-type and were yellow and stunted.
[b] Plants reduced acetylene at about half the rate for single inoculation with wild-type.
[c] 30% of plants reduced acetylene at double the rate for single inoculation with wild-type.

Helping of *exo*. Helping was tested in pairwise coinoculations of helper *exo*$^+$*nif* strains, which could provide Exo function but could not fix nitrogen themselves, with indicator *exo nif*$^+$ strains that lacked Exo function. This was done for *nifA* or *nifH* with *exoB* or *exoF*. In some experiments, either helper or indicator was also *nodA*, *B*, or *C*. Surprisingly, *nod*$^+$ and *nod* helpers gave very different results.

The *nod*$^+$*exo*$^+$ helper generally did not give Fix$^+$ nodules with either the *nod*$^+$*exo* or the *nod exo* (Table 2, lines 1 and 2, respectively) indicator (except for occasional Fix$^+$ nodules ascribed to leakiness of the phenotype as above). Both coinoculant genotypes were recovered from a small proportion (5–10%) of these Fix$^-$ nodules (Table 3, lines 5 and 6). In a representative experiment (Rm6020 [*nod*$^+$*nif exo*$^+$] with Rm5078 [*nod*$^+$*nif*$^+$*exo*]), a total of 25 nodules from six plants were squashed, and both *nif exo*$^+$ and *nif*$^+$*exo* colonies were recovered from two of them. Many of the nodules were tiny, with morphology typical of *exo*-induced nodules (Finan *et al.* 1985); among the larger, pinkish nodules. the proportion giving both coinoculant genotypes rose to about 25%.

Ultrastructurally, transmission electron microscopy (TEM) revealed that cells of these Fix nodules were abnormal in two ways (Fig. 1). First, several cells contained two morphologically distinct bacteroid forms, although most cells contained only *nifH*-like bacteroids. (*nifH* bacteroids are elongate with heterogeneous cytoplasm, like bacteroids of wild-type Rm1021, although unlike wild-type, they frequently contain electron-dense deposits of unknown nature [Hirsch *et al.* 1983]). In the Fix$^-$ coinoculant nodules showing two bacteroid morphologies, one form was elongate like wild-type (though perhaps slightly thinner), whereas the other was aberrant. The non-elongate form included a variety of shapes, notably very large almost spherical bacteroids (Fig. 1) not normally observed in alfalfa nodules, and senescent ones with electron-dense cytoplasm and little structural integrity (Fig. 2). Second, occasionally a single peribacteroid membrane enclosed bacteroids of both forms (Fig. 1). Multiply enclosed bacteroids are usually not seen in alfalfa nodules.

In contrast, the *nod* helper did give Fix$^+$ nodules with the corresponding indicator (Table 2, line 3). In a representative experiment (Rm6910 [*nod nif exo*$^+$] and Rm5078 [*nod*$^+$*nif*$^+$*exo*]), both genotypes were recovered from a majority of nodules tested (16 of 20), the remainder yielding no colonies (Table 3, line 7). In a few of the host cells, some bacteroids had the electron-dense bodies characteristic of *nifH* (Hirsch *et al.* 1983; data not shown). However, in general, only one type of bacteroid (elongate and individually surrounded by a peribacteroid membrane) is found in nodules resulting from this coinoculation (Fig. 3).

Helping of *nod*. For comparison. we also checked coinoculation with pairs of strains that were both *exo*$^+$ but carried wild or mutant alleles of *nod* (*A*, *B*, or *C*) and *nif* (*A* or *H*) genes. These generally gave Fix$^+$ nodules, in agreement with Rolfe *et al.* (1980) and W. Szeto (personal communication). For the combination *nod nif*$^+$ with *nod*$^+$*nif* (Table 2, line 4) all plants were nodulated and half to two-thirds of them fixed nitrogen (Fix$^+$), depending upon the experiment. As an example, in coinoculation with Rm1126 (*nod nif*$^+$*exo*$^+$, Nm^r) and Rm6020 (*nod*$^+$*nif exo*$^+$, Sp^r) from Fix$^+$ nodules, roughly three times as many Sp^r

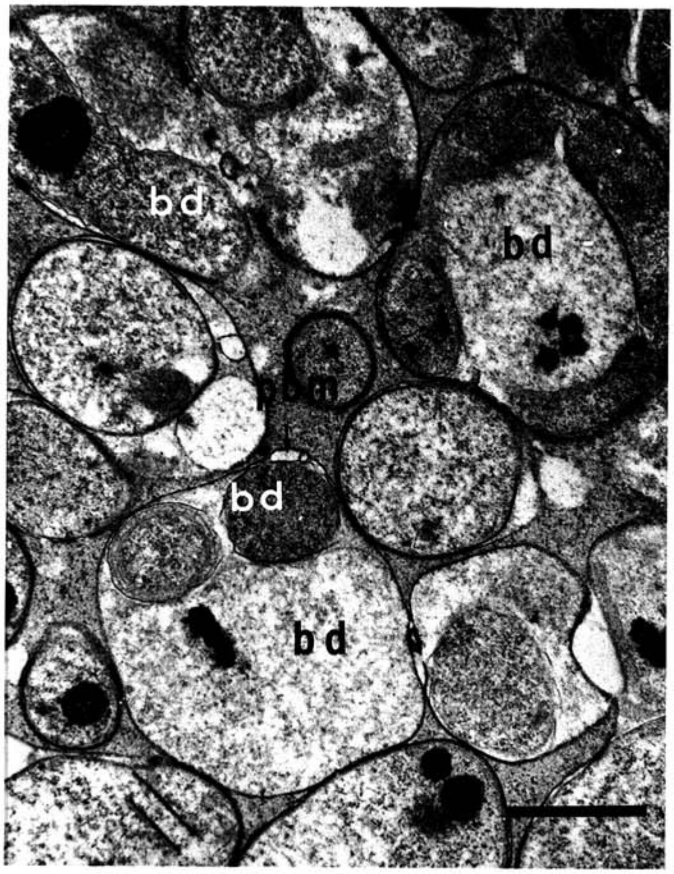

Fig. 1. Bacteroids (bd) in a portion of an alfalfa nodule cell from coinoculation of *nod*$^+$*nif*$^+$*exo* (Rm5078) and *nod*$^+$*nif exo*$^+$ (Rm6020) *Rhizobium meliloti*. Nodule halves were squashed and prepared for transmission electron microscopy (TEM) 3 wk after inoculation. The plant was Fix$^-$. Two types of bacteroids (white and black bd) are present. Occasionally they are both enclosed within a peribacteroid membrane (pbm). Scale bar = 1 μm.

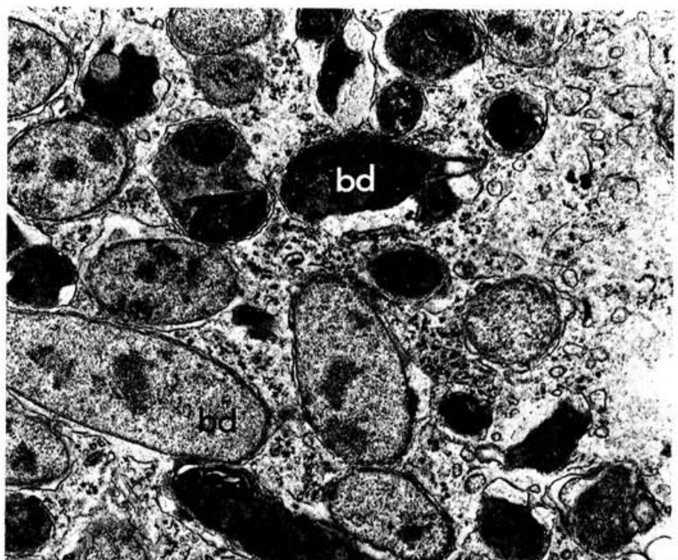

Fig. 2. Bacteroids (bd) from a portion of an alfalfa nodule cell after coinoculation of *nod*$^+$*nif*$^+$*exo* (Rm5020) and *nod*$^+$*nif exo*$^+$ (Rm1354) *R. meliloti*. Nodules were prepared for TEM 3 wk after inoculation. The plant was Fix$^-$. Two types of bacteroids are present but one (black bd) is elongate and the other (white bd) is senescent. Scale bar = 1 μm.

(*nod*$^+$*nif*) as Nm^r (*nod nif*$^+$) colonies were recovered, whereas from Fix$^-$ nodules nearly all colonies were Sp^r and few if any were Nm^r. In a similar experiment, where coinoculants were Rm1027 (*nod nif*$^+$*exo*$^+$) and Rm1491 (*nod*$^+$*nif exo*$^+$), TEM of the nodules showed only one form of bacteroid within alfalfa host cells. These bacteroids were elongate and individually enclosed within the peribacteroid membrane (Fig. 4).

Coinoculation with *nod nif*$^+$*exo*$^+$ and *nod*$^+$*nif exo* (Table 2, line 5) gave mainly Fix$^+$ plants; large pink nodules gave both types of colonies in a ratio of about 3:1 (data not shown). TEM showed elongate bacteroids, some of which contained the electron-dense deposits characteristic of *nifH* mutants (Hirsch *et al.* 1983; Fig. 5).

Controls. Coinoculation with *nod nif*$^+$*exo*$^+$ and *nod*$^+$*nif*$^+$*exo* bacteria (Table 2, line 7) consistently gave mainly Fix$^+$ nodules. This was found for *nodA*, *B*, or *C* with *exoB* or *F* (Rm5610, Rm5611, Rm5612, Rm5613, or Rm1126, with Rm5078; Rm5610 with Rm7055). Plants were green and healthy and had rates of acetylene reduction equal to or better than those for plants inoculated with wild-type.

Coinoculation with wild-type (Rm1021) and a *nod*, *nif*, or *exo* single mutant (Table 2, lines 8 through 10) gave Fix$^+$ plants to the same extent as single inoculation with wild-type (Table 2, line 13). There was no indication of interference by any of the mutants. When nodules from coinoculation of wild-type (Rm1021) and *exoB* (Rm5078) were squashed, only wild-type (*exo*$^+$, Ot^s) colonies were recovered from most (78%) of the nodules. The remaining nodules gave either no colonies or a mixed population with a large excess of wild-type (Table 3, line 3).

At least one of the coinoculating strains had to be *nod*$^+$ for the plants to be nodulated (Table 2, line 11). Similarly, at

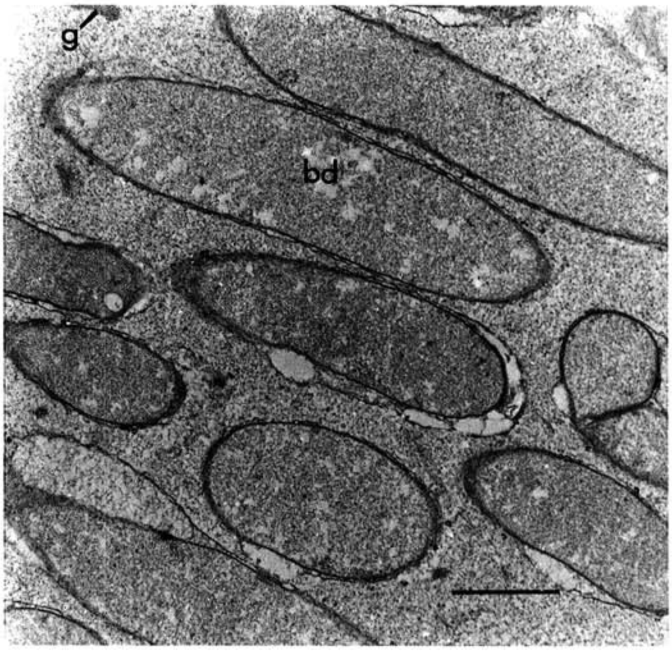

Fig. 3. Bacteroids (bd) from a portion of an alfalfa nodule cell after coinoculation of *nod nif exo*$^+$ (Rm6910) and *nod*$^+$*nif*$^+$*exo* (Rm5078) *R. meliloti*. Nodule halves were squashed and prepared for TEM 3 wk after inoculation. The plant was Fix$^+$. Only one type of bacteroid is evident in nodule cells. A golgi body (g) is in the host cell cytoplasm. Scale bar = 1 μm.

Table 3. Colony recovery

	Inoculant (s)						**Phenotype**	**Percent of nodules giving:**[a]			
	nod	***nif***	***exo***	***nod***	***nif***	***exo***	**Fix**	**No Bacteria**	***exo*$^+$**	***exo*$^-$**	***exo*$^+$ and *exo*[b]**
Controls											
1.	+	+	+				+	21	79	0	0
2.	+	+	−				−	86	0	14	0
3.	+	+	+	+	+	−	+	4	77.5	0	18.5
Helping of *nod*											
4.	+	−	+	−	+	+	+/−	0	100[c]	0	0
Helping of *exo*											
5.	+	−	+	+	+	−	−	33.3	58.3	0	8.3 (>10:1)
6.	+	−	+	−	+	−	−	10	85	0	5 (>10:1)
7.	−	−	+	+	+	−	+	20	0	0	80 (3.3:1)
Triple Inoculations											
8.	+ −	− −	+ +	+	+	−	−	35	52.5[d]	0	12.5[e]
9.	+ −	− −	+ +	−	+	−	−	27.8	72.2[f]	0	0
10.	+ −	− −	− +	+	+	−	+	45.8	0	0	54.2[g]
11.	+ −	− −	− +	−	+	−	−	55.6	5.6	5.6	33.3[h]

[a] Data are from representative experiments.
[b] The ratio of *exo*$^+$ to *exo* is given in brackets.
[c] Fix$^+$ nodules had approximately 3:1 *nif*:*nod* bacteria.
[d] 50% *nif*, 2.5% *nif* + *nod nif*.
[e] 10% all three strains, 2.5% *nif* + *exo*, 0 *nod nif* + *exo*.
[f] 50% *nif*, 22% *nif* + *nod nif*.
[g] 50% *nod nif* + *exo*, 4% *nod nif* + *nif exo* + *exo*.
[h] *nod nif* and *nif exo*.

least one of the coinoculating strains had to be *nif*$^+$ for the nodules to fix nitrogen (Table 2, line 12).

Triple inoculation. The effect of *nod*$^+$ and *nod* helpers was compared by coinoculating both with a single indicator in a triple coinoculation (Table 4). Thus, *nod*$^+$*nif exo*$^+$ (Rm6020) and *nod nif exo*$^+$ (Rm6910) helpers were inoculated together with a *nod*$^+$*nif*$^+$*exo* indicator (Rm5078). The resulting plants were nearly all Fix$^-$. In one experiment, all of the 40 nodules from eight plants were scored for occupancy, with the following results. Four of the nodules (three of them on a single plant) gave all three coinoculant genotypes. One nodule gave both *nod*$^+$*nif exo*$^+$ and *nod*$^+$*nif*$^+$*exo* and another gave both *nod*$^+$*nif exo*$^+$ and *nod nif exo*$^+$, with an excess of *nod*$^+$*nif exo*$^+$ in both cases. Twenty nodules gave only *nod*$^+$*nif exo*$^+$, and the remaining 14 nodules gave no

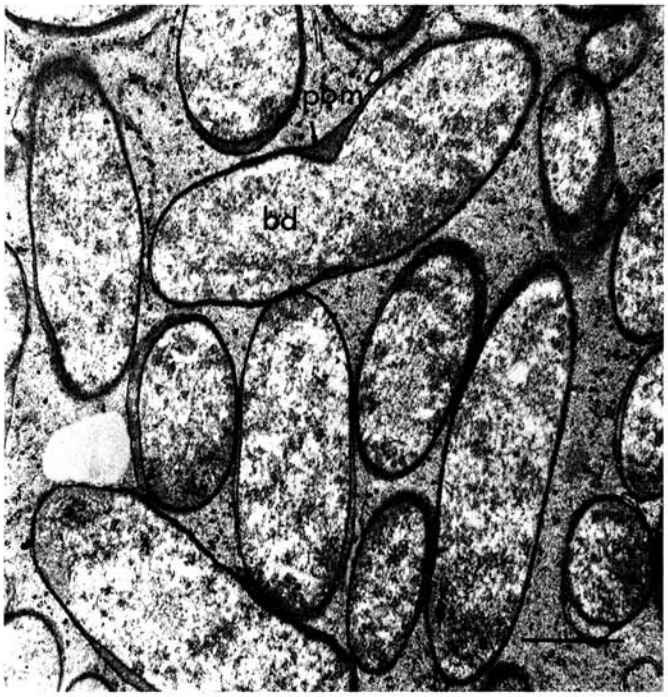

Fig. 4. Bacteroids (bd) in a portion of an alfalfa nodule cell after coinoculation of *nod nif*$^+$*exo*$^+$ (Rm1027) and *nod*$^+$*nif exo*$^+$ (Rm1491) *R. meliloti*. Nodules were prepared for TEM 3 wk after inoculation. The plant was Fix$^+$. Only one morphological type of bacteroid singly enclosed by the peribacteroid membrane (pbm) is present. Scale bar = 1 μm.

Table 4. Triple inoculations

Inoculants						Phenotype[a] (% of plants)	
Helpers			Indicator				
nod	*nif*	*exo*	*nod*	*nif*	*exo*	Nod$^+$	Fix$^+$
+	−	+	+	+	−	100	5
−	−	+					
+	−	+	−	+	−	100	0
−	−	+					
+	−	−	+	+	−	100	55
−	−	+					
+	−	−	−	+	−	100	0
−	−	+					

[a] Fix phenotype was determined by reduction of acetylene, 3 wk after inoculation. As controls, every double pair was also coinoculated. All combinations of helpers were Fix$^-$. The combination of *nod* helper and *nod* indicator was Nod$^-$. In this experiment, for the combination of *nod nif exo*$^+$ and *nod*$^+$*nif*$^+$*exo*, 90% of plants were nodulated and 60% of plants reduced acetylene. All other combinations of helper and indicator were Fix$^-$.

Fig. 5. Bacteroids (bd) in a portion of an alfalfa nodule cell after coinoculation of *nod nif*$^+$*exo*$^+$ (Rm1126) and *nod*$^+$*nif exo* (Rm6905) *R. meliloti*. Nodules were squashed and prepared for TEM 3 wk after inoculation. The plant was Fix$^+$. The bacteroids were elongate and singly enclosed by a peribacteroid membrane (pbm). Some bacteroids contain electron-dense deposits. Scale bar = 1 μm.

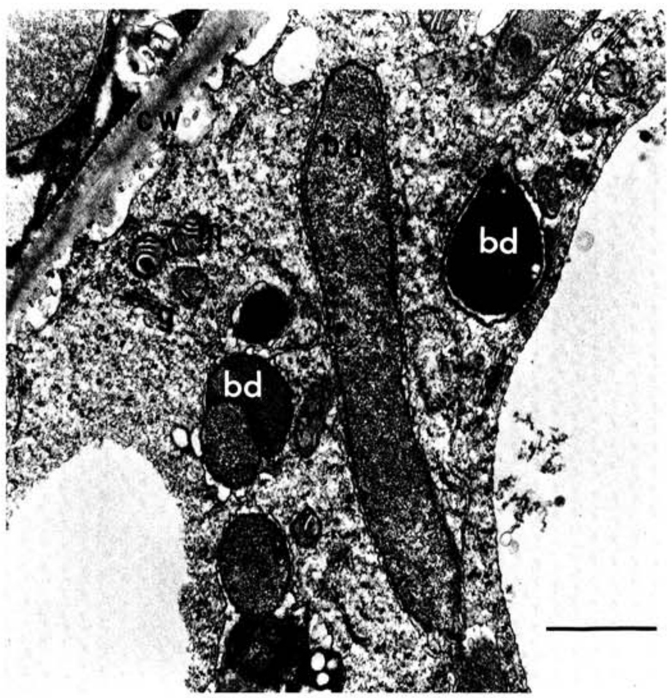

Fig. 6. Bacteroids (bd) in a portion of an alfalfa nodule cell from a triple coinoculation of *nod*$^+$*nif exo*$^+$ (Rm6020), *nod nif exo*$^+$ (Rm6910), and *nod*$^+$*nif*$^+$*exo* (Rm5078) *R. meliloti*. Nodule halves were squashed and prepared for TEM 20 days after inoculation. The plant was Fix$^-$. At least two types of bacteroids (white and black bd) are present. A golgi body (g) and several mitochondria (m) are present in the host cell cytoplasm. A cell wall (cw) separates two alfalfa nodule cells. Scale bar = 1 μm.

bacteria. In other experiments, no $nod^+ nif^+ exo$ indicators were recovered from any nodules. Ultrastructurally, some of these Fix$^-$ nodules (Fig. 6) were similar to Fix$^-$ nodules from ineffective double inoculations, having both elongate and aberrant forms (Figs. 1, 2). (Although we did not see both forms in any of the nodules from which we recovered all three genotypes, we believe this was because most of the triple inoculation nodules were senescent within 3 wk.)

The same $nod^+ nif\ exo^+$ (Rm6020) and $nod\ nif\ exo^+$ (Rm6910) helpers were coinoculated with a $nod\ nif^+ exo$ (Rm6776) indicator, and again, all plants were Fix$^-$. Of 18 nodules squashed, from two plants, four gave both $nod^+ nif\ exo^+$ and $nod\ nif\ exo^+$, nine gave $nod^+ nif\ exo^+$ only, and five gave no bacteria. No $nod\ nif^+ exo$ were recovered.

When $nod^+ nif\ exo$ (Rm6905) and $nod\ nif\ exo^+$ (Rm6910) helpers were inoculated with $nod^+ nif^+ exo$ (Rm5078) indicator, 55% of plants were Fix$^+$ at 3 wk. All 24 nodules from six plants were squashed, with the following results: 12 gave $nod\ nif\ exo^+$ and $nod^+ nif^+ exo$; one gave $nod\ nif\ exo^+$, $nod^+ nif\ exo$, and $nod^+ nif^+ exo$; and 11 gave no bacteria. On the other hand, when the same $nod^+ nif\ exo$ and $nod\ nif\ exo^+$ helpers were coinoculated with $nod\ nif^+ exo$ (Rm6776) indicator, none of the plants was Fix$^+$ at 3 wk. All 36 nodules from six plants were squashed. Twelve gave both $nod\ nif\ exo^+$ and $nod^+ nif\ exo$; two gave only $nod\ nif\ exo^+$; two gave only $nod^+ nif\ exo$; and the remaining 20 gave no bacteria, with the exception of one cluster of tiny nodules that yielded only six colonies, two of each coinoculant. Barring this exception, none of the nodules gave the $nod\ nif^+ exo$.

DISCUSSION

Our results demonstrate that exo^+ bacteria can in principle help *exo* mutants both to reach inner cortical nodule cells and to fix nitrogen there. We do not know what helping involves. The *exo* mutant might respond directly to the molecular species that constitutes Exo function (EPS or some related molecule). (Phenotypic restoration of Fix$^+$ phenotype by addition of EPS or related oligosaccharides to plants infected with EPS-deficient mutants has been reported for *R. trifolii* [Djordjevic *et al.* 1987]. However, repeated attempts to correct the Fix$^-$ phenotype of *R. meliloti exo* mutants by addition of EPS or EPS-derived oligosaccharides have been unsuccessful [B. Kunkel, C. Yang, M. Lopez, and E. R. Signer, unpublished data; J. Leigh, unpublished data].) Alternatively, the *exo* mutants might respond indirectly to other conditions brought about by the exo^+ helper. Clearly, though, there is no intrinsic inability of *exo* bacteria to fix nitrogen.

Our expectation was that, given that helping for Exo could occur, any exo^+ bacterium would be a competent helper. This is clearly not the case. Whether or not helping actually takes place depends on the *nod* genotype of the coinoculated bacteria. In pairwise coinoculation, a $nod\ exo^+$ bacterium is an effective helper, allowing *exo* mutants to differentiate into nitrogen-fixing bacteroids (Fix$^+$); a $nod^+ exo^+$ bacterium is an ineffective helper and does not allow coinoculated *exo* mutants to differentiate into the nitrogen-fixing form (Fix$^-$, Table 2). These results are unexpected, particularly considering the fact that in single inoculation a $nod^+ exo^+$ bacterium (e.g. wild-type) obviously is effective. In other words, $nod^+ exo^+$ is effective for providing Exo function in *cis* but ineffective in *trans*. Moreover, the ineffectiveness of the nod^+ helper supersedes the effectiveness of the *nod* helper: Triple coinoculation of both the ineffective $nod^+ exo^+$ and the effective $nod\ exo^+$ helpers together with a $nod^+ exo$ indicator results in the formation of Fix$^-$ nodules (Table 4). Finally, triple coinoculation also shows that the *exo* indicator must be nod^+ in order to be helped to invade. (The effect of *nod* genotype on the *exo* indicator cannot be tested in pairwise coinoculation, where there is only one helper that must be *nod* to be effective.)

The ineffective combinations examined give poor recovery of the *exo* indicator; few nodules yield *exo* bacteria, and even for those nodules giving mixed recovery of exo^+ and *exo*, the proportion of *exo* is low ($exo^+ : exo \geqslant 10:1$). In the effective combinations, many of the nodules yield mixed recovery, and the proportion of *exo* from some mixed nodules is higher ($exo^+ : exo \simeq 3.3:1$).

Thus the major facts to be accounted for are: 1) $nod\ exo^+$ helps, but $nod^+ exo^+$ does not help; 2) $nod^+ exo^+$ blocks $nod\ exo^+$ helping in triple coinoculation; 3) $nod^+ exo^+$ blocks invasion in *trans* but not in *cis*; 4) $nod^+ exo$ indicator can be helped, but *nod exo* indicator cannot; and 5) bacteria of the indicator genotype are recovered well from Fix$^+$ coinoculations but poorly from nodules with Fix$^-$ coinoculations.

We consider three formal categories of model.

Quantity of Exo. Compared to the $nod\ exo^+$ helper, the $nod^+ exo^+$ helper provides either too little or too much Exo function. Because the $nod^+ exo^+$ helper supersedes the $nod\ exo^+$ helper (fact 2 above), too little Exo function is ruled out. We cannot rule out too much Exo as the explanation, but this seems to us unlikely.

Quality of Exo. The molecular species that constitutes Exo function differs depending upon whether it is provided by the *nod* or the nod^+ helper. For instance, Nod function might modify Exo. The *cis-trans* difference (fact 3 above) might then be explained if invasion requires Exo before its modification by Nod; when Exo is provided in *trans*, it would already be modified and therefore ineffective in helping. To explore this model, we have compared ^{1}H-NMR spectrograms of exopolysaccharide (EPS) produced *in vitro* from *nodC* and nod^+ bacteria under conditions of *nod* gene induction and have found no difference (S. Klein 1987). However, we are unable to compare bacterial EPS made in the nodule itself, nor do we know that EPS itself rather than a related molecule constitutes Exo function. This model does not readily account for the inability of *nod exo* indicator to be helped in triple coinoculation (Table 4), but we might expect the probability of helping a single indicator for two functions (Nod and Exo) from two separate helpers (one $nod^+ exo$, the other $nod\ exo^+$) to be low.

Bacterial competition. Independent of the quantity or quality of Exo, a $nod^+ exo^+$ helper excludes the *exo* indicator from some critical interaction whereas a $nod\ exo^+$ helper does not. For instance, coinoculant bacteria might compete for a site of invasion on the root hair. If so, the facts above suggest a hierarchy of competition:

$$nod^+ exo^+ > nod\ exo^+ > nod^+ exo > nod\ exo,$$

where $>$ indicates "competes better than." In such a hierarchy, $nod^+ exo^+$ would compete only slightly better than $nod\ exo^+$ ($nod^+ nif\ exo^+$ and $nod\ nif^+ exo^+$ cooperate to produce Fix$^+$ nodules; Table 2, line 4), but $nod^+ exo^+$ would compete much better than $nod^+ exo$ ($nod^+ nif\ exo^+$ and $nod^+ nif^+ exo$ fail to cooperate; Table 2, line 1). Similarly, $nod\ exo^+$ would compete only slightly better than $nod^+ exo$ (pairwise $nod\ nif\ exo^+$ and $nod^+ nif^+ exo$ or $nod\ nif^+ exo^+$ and $nod^+ nif\ exo$ produce Fix$^+$ nodules; Table 2, lines 3 and 5), but *nod exo*$^+$ or *nod*$^+$*exo* (or both) would compete much better than *nod exo* (*nod nif exo*$^+$ and *nod*$^+$*nif exo* exclude *nod nif*$^+$*exo* in triple coinoculation; Table 4, line 4). This model is attractive on general grounds, but without a molecular basis for Nod and Exo phenotypes, it is not readily tested.

Two other features of our data, not immediately accounted for by any of the models above, are also worth noting. First is the finding that in triple coinoculation of *nod nif exo*$^+$ and *nod*$^+$*nif exo* with *nod*$^+$*nif*$^+$*exo*, the recovery of *nif exo* is much poorer than that of *nif*$^+$*exo* (Table 3, line 10). It has long been known that the number of nodules is higher for ineffective (Fix$^-$; including *nif*) than for effective (Fix$^+$) bacteria (Zimmerman *et al.* 1983), suggesting that the plant discriminates between Fix$^+$ and Fix$^-$ invasions. Analogously, we may speculate that in coinoculation the plant can somehow discriminate between the incipient Fix$^+$ coinoculation of *nod nif exo*$^+$ and *nod*$^+$*nif*$^+$*exo* and the Fix$^-$ coinoculation of *nod nif exo*$^+$ and *nod*$^+$*nif exo*. (We assume a low probability of coinfection by all three coinoculants.)

Second, and potentially the most interesting aspect of this study although one for which there is no easy explanation, is the demonstration of multiple bacteroid forms. In the ineffective nodules resulting from coinoculation of *nod*$^+$ helper with *exo* indicator, two morphologically distinct bacteroid forms can be distinguished, one of which has differentiated abnormally (Figs. 1, 2, and 6). The simplest interpretation is that the relatively normal-looking bacteroids are *nod*$^+$*nif exo*$^+$ helpers that are unable to fix nitrogen by virtue of being deficient for *nif*, whereas the aberrant bacteroids are *exo*. (We are now exploring bacteroid identification by immunogold labeling.) If so, then *nif*$^+$ rhizobia can reach inner cortical cells within peribacteroid membranes in a differentiated (if aberrant) state and still be unable to fix nitrogen, i.e., differentiation and fixation can be uncoupled. Alternatively, however, the *nod*$^+$*exo*$^+$ might not be able to help the *exo* indicator to invade inner cortical cells (or *a fortiori* to differentiate) so that both bacteroid forms are in fact of the same *nod*$^+$*exo*$^+$ genotype. If so, the *exo* indicator would have to be responsible in some way for the aberrant differentiation of the *nod*$^+$*exo*$^+$ helper, which however would not be fully penetrant. By either interpretation, this aberrant differentiation could reflect either an interaction late in nodule development or the indirect consequence of an early event. Finally, yet another form is seen in the Fix$^+$ nodules resulting from effective coinoculation of *nod exo*$^+$ and *nod*$^+$*exo* bacteria, where the bacteroids appear to be less elongate than normal and to have some abnormality of the peribacteroid membrane. This suggests that even nitrogen-fixing bacteroids can follow an atypical developmental pathway.

These effects on bacteroid morphology suggest that both Nod and Exo functions are required not only for root hair invasion early, but also, directly or indirectly, for correct differentation of bacteroids late in nodulation. In both those processes, invading bacteria interact with the plant cell plasmalemma: Early, at the root hair surface (and then at the middle lamella as well), the plasmalemma invaginates to accommodate the growing infection thread; and later, as rhizobia are released from the infection thread, the plasmalemma surrounds them in the form of the peribacteroid membrane. That similarity may be a clue to what those functions are.

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