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Sym Plasmid Genes of *Rhizobium trifolii* Expressed in *Lignobacter* and *Pseudomonas* Strains

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A 14-kilobase (kb) fragment of *Rhizobium trifolii* Sym plasmid containing nodulation (*nod*) genes or the pSym plasmid of *R. trifolii* cointegrated with a broad-host-range vector R68.45 (pPN1) were transferred to *Lignobacter* strain K17 and *Pseudomonas aeruginosa* strain PAO5 by conjugation. *Lignobacter* transconjugants carrying Sym plasmid pPN1 formed nodules on white, red, and subterranean clover plants. *Lignobacter* transconjugants containing a 14-kb fragment of *nod* genes cloned into a multicopy plasmid nodulated only white and subterranean clover plants, whereas transconjugants carrying the same fragment cloned into a low-copy plasmid vector nodulated only white clover plants. All nodules formed by *Lignobacter* transconjugants showed bacterial release from the infection threads into the host cytoplasm. *Pseudomonas* transconjugants with plasmid pPN1 formed nodule-like structures on white clover plants. These structures were not invaded by bacteria; however, a few bacteria were found within the intercellular spaces of the outermost cells of the structures. *Pseudomonas* transconjugants carrying the 14-kb fragment of *R. trifolii nod* genes did not form nodules on tested clover plants. All clover plants inoculated with either *Pseudomonas* or *Lignobacter* transconjugants containing a 14-kb fragment of *nod* genes (but not entire Sym plasmid) showed the "thick-and-short-root" response when compared to the control plants inoculated with the *R. trifolii* wild-type strain.

The ability to fix atmospheric nitrogen through a symbiosis between legumes and soil bacteria is limited only to the members of the bacterial genus *Rhizobium* (25). The genetic basis of this limitation is poorly understood. It is now well established that *Rhizobium* plasmids called Sym(biosis) plasmids control several steps leading to root nodule formation and nitrogen fixation in some leguminous plants (2, 10, 12, 19, 23, 24). One approach to the study of *Rhizobium* nodulation (*nod*) genes is to transfer these genes into other bacteria and look for expression on an appropriate legume host.

It has been reported that *Rhizobium nod* genes can be partially expressed in *Agrobacterium tumefaciens* giving rise to root nodule formation but no nitrogen fixation phenotype on the plant hosts (9, 18, 23, 27). In most cases, *Agrobacterium* strains carrying *Rhizobium nod* genes form pseudonodules which are ineffective and do not contain bacteroid structures (9, 18, 23). Recently, it has been reported that *Escherichia coli* containing *nod* genes from *Rhizobium meliloti* forms pseudonodules on alfalfa (9). However, these structures are completely devoid of bacteria, and only a small percentage of inoculated plants formed nodules.

In this study we report on the expression of *Rhizobium trifolii nod* genes in *Lignobacter* and *Pseudomonas* strains. Neither of these genera belong to the family *Rhizobiaceae*. *Lignobacter* strain K17 is a gram-negative, aerobic, rod-shaped bacterium, originally isolated from decaying wood (16), with the ability to degrade lignin (16) and fix atmospheric nitrogen (5). It also shares some physiological characteristics with *Pseudomonas*, *Achromobacter*, and *Agrobacterium* spp. (5, 16, 21).

We also report on the expression of the Tsr (thick-and-short-root) function in these strains. Up to now, the Tsr function has only been found on the Sym plasmid of *Rhizobium leguminosarum* (24) when this strain had been inoculated onto *Vicia sativa*. In our experiments, the phenotypic expression of the Tsr function on clover plants was only observed when the *R. trifolii* 14-kilobase (kb) nodulation fragment was transferred to *Lignobacter* or *Pseudomonas* strains.

MATERIALS AND METHODS

Organisms and plasmids. A list of strains and plasmids used is given in Table 1.

Culture and mating conditions. *Escherichia coli* strain PN200 (19) was used as a donor in matings with *Lignobacter* and *Pseudomonas* strains. Conjugation between strains PN200 and K17 was done on tryptone-yeast extract (3) plates at 37°C for 18 h. Plasmids pRT170 and pMN3 were transferred from strain RR1 to K17 in the tryptone-yeast extract plate, mating at 37°C for 18 h. *Pseudomonas* strain PAO5 was grown at 43°C before matings. Transfer of plasmids pPN1, pRT170, and pMN3 to strain PAO5 was done on nutrient agar (8) plates at 37°C for 12 h. As a positive control for the nodulation test, the *R. trifolii* strain ANU845 carrying the pPN1 plasmid was used. Strain ANU845(pPN1) was constructed by introducing pPN1 from strain PN200.

Plant assay. Clover seeds *Trifolium repens* (white clover New Zealand 5826), *Trifolium subterranean* (subterranean clover, Mt. Barker variety), and *Trifolium pratense* (red clover) were sterilized and germinated as described previously (6). Plants were inoculated with bacterial colonies onto nitrogen-free medium in rapid plate plant assay (15).

Extraction of bacteria from nodules. Bacteria were isolated from squashed single nodules in osmotically protective media after root surface sterilization (7).

Light and electron microscopy. For light microscopy the roots and nodules were stained in a solution of 0.5% toluidine blue, and for electron microscopy the nodules were prepared and analyzed as described by Trinick (22).

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TABLE 1. Bacterial strains and plasmids used

Bacterial strains and plasmids	Relevant characteristics[a]	Source or reference
R. trifolii		
ANU843	Wild-type strain, Nod$^+$ Fix$^+$ on white, red, and subterranean clovers	15
ANU845	The Sym plasmid-cured derivative of the ANU843 wild-type strain, Nod$^-$ Fix$^-$	17
ANU845 Rif	Spontaneous Rif^r mutant	This study
ANU845(pPN1)	Rif^r, carrying a cointegrate Sym plasmid from *R. trifolii* strain NZP514, Nod$^+$ Fix$^+$ on clover plants	This study
Lignobacter strains		
K17	Gram-negative, Fix$^+$ Sp^r, fast-growing strain on *E. coli* media	This study
K17(pPN1)	Sp^r, carrying a Sym plasmid pPN1	This study
K17(pRT170)	Sp^r, carrying a multicopy, recombinant, plasmid pRT170	This study
K17(pMN3)	Sp^r, carrying a low-copy-number recombinant plasmid pMN3	This study
P. aeruginosa		
PAO5	Trp$^-$ Rif^r, opportunistic human pathogen	M. Nayudu
PAO5(pPN1)	Trp$^-$ Rif^r, carrying pPN1	This study
PAO5(pRT170)	Trp$^-$ Rif^r, carrying pRT170	This study
PAO5(pMN3)	Trp$^-$ Rif^r, carrying pMN3	This study
E. coli		
PN200	HB101, RecA$^-$, carrying pPN1	19
RR1(pRT170)	RecA$^+$, carrying pRT170	This study
RR1(pMN3)	RecA$^+$, carrying pMN3	This study
Plasmids		
pPN1	A self-transmissible cointegrate plasmid composed of *R. trifolii* Sym plasmid pRtr-514a and the broad-host-range plasmid R68.45, Km Tc	19
pRT170	A recombinant multicopy plasmid, a derivative of an *Inc*Q Tra$^+$ plasmid pKT240 (1), carrying a 14-kb *Hin*dIII restriction fragment containing *nod* genes from *R. trifolii* ANU843 and marked with Tn*5* inserted into the vector	M. Djordjevic
pMN3	A recombinant low-copy-number plasmid, Km^s derivative of an *Inc*P Tra$^+$ plasmid R68 (20) carrying the same 14-kb *Hin*dIII fragment as plasmid pRT170	M. Nayudu

[a] Abbreviations: Nod, Nodulation; Fix, fixation; Rif, rifampin; Sp, spectinomycin; Km, kanamycin; Tc, tetracycline; *Inc*Q, incompatibility Q group plasmid; *Inc*P, incompatibility P group plasmid; Tra, transfer function; Tn*5*, transposon 5.

Electrophoresis of plasmid DNA. Plasmid profiles of bacterial strains were visualized according to the agarose gel electrophoresis method described previously (13).

DNA isolation. Total DNAs from PAO5 and PAO5(pPN1) strains were isolated by the method described elsewhere (17).

DNA-DNA hybridization. For the hybridization study a 7.2-kb *Eco*RI restriction fragment encoding early clover nodulation genes (root hair curling and host specificity genes) from *R. trifolii* strain ANU843 (17) was radioactively labeled and used as a probe (18). Transfer of the plasmid DNAs to the nitrocellulose filter and hybridization conditions were described previously (14).

RESULTS

Construction of the hybrid strains. When plasmid pPN1 was transferred into strain K17, Km^r Sp^r *Lignobacter* transconjugants were screened. The presence of the pPN1 in all transconjugants was checked by agarose gel electrophoresis and hybridization analysis with the *R. trifolii*-specific nodulation (*nod*) genes used as a probe (data not shown). Of the 150 transconjugants, 85% contained plasmid pPN1; however, 15% of the transconjugants showed only the presence of plasmid R68.45 and had lost the Sym plasmid (data not shown). The multicopy recombinant plasmid pRT170 was transferred to K17 with a frequency of 10^{-5} to 10^{-6} per recipient cell, whereas the low-copy recombinant plasmid pMN3 was transferred with a frequency of 10^{-3} per recipient cell (Table 2). When plasmid pPN1 was transferred to the *Pseudomonas* strain, only Km^r Tc^r transconjugants were screened. The plasmid content of these transconjugants was tested by the agarose gel electrophoresis technique. In the 100 colonies checked, only a plasmid band corresponding in size to the plasmid R68.45 was found (data not shown). Plasmids pRT170 and pMN3 were transferred into *Pseudomonas aeruginosa* strain PAO5 with frequencies of 10^{-5} and 10^{-3}, respectively (Table 2). The presence of these plasmids in strain PAO5 was also tested by the gel electrophoresis technique (data not shown).

Plant response to inoculation with *Lignobacter-Rhizobium* hybrids. All *Lignobacter* strains carrying an intact plasmid pPN1 were inoculated onto seedlings of white, red, and subterranean clovers by using the plate plant assay. *R. trifolii* strains ANU845 (Sym plasmid cured) and ANU845 carrying pPN1 were used as controls. After 12 days of

TABLE 2. Transfer of pPN1, pRT170, and pMN3 plasmids to *Rhizobium*, *Lignobacter*, and *Pseudomonas* strains

Cross	Selected marker[a]	Frequency of marker transfer per recipient	% of plants nodulated[b]
PN200 × ANU845[c]	Km	10^{-2}	100
RR1(pRT170) × ANU845	Km	10^{-4}	98
RR1(pMN3) × ANU845	Tc	10^{-3}	80
PN200 × K17[d]	Km	10^{-4}–10^{-5}	85
RR1(pRT170) × K17	Km	10^{-5}–10^{-6}	50
RR1(pMN3) × K17	Tc	10^{-3}	50
PN200 × PAO5[c]	Km, Tc	10^{-7}	85
RR1(pRT170) × PAO5	Km	10^{-5}	0
RR1(pMN3) × PAO5	Tc	10^{-3}	0
K17(pPN1) × ANU845	Km	10^{-4}–10^{-5}	100

[a] Km, Kanamycin; Tc, tetracycline.

[b] 100 plants were inoculated for each cross. Data were taken from three independent experimental runs.

[c] Rifampin (60 μg/ml for strain ANU845 and 200 μg/ml for strain PAO5) was used to counterselect donors in these crosses.

[d] Spectinomycin (100 μg/ml) was used to counterselect donors in these crosses.

inoculation, strain ANU845(pPN1) formed effective (Fix^+) nodules on all clover plants, whereas ANU845 showed no plant response. Three clover species showed root hair curling (Hac) 7 days after inoculation with *Lignobacter* strain K17(pPN1) (Fig. 1B). No root hair deformation was observed with the parental *Lignobacter* strain K17 (Fig. 1A). The *Lignobacter* K17(pPN1) transconjugants induced ineffective nodules within 3 weeks on white and red clovers and within 5 weeks on subterranean clover (Table 3). The nodules induced by K17(pPN1) were one-third the size of nodules induced by ANU845(pPN1) on the three clover species (Fig. 2D and E). In contrast to the wild-type indeterminate nodules, ANU845(pPN1) and K17(pPN1) induced spherical structures which were morphologically similar to determinate nodules (Fig. 2F and G).

The bacteria recovered from inside the root nodules formed by K17(pPN1) were identical to those used for inoculation. Moreover, the bacteria isolated from clover nodules were able to transfer plasmid pPN1 into the Sym plasmid-cured *R. trifolii* strain ANU845 (Table 2). These ANU845(pPN1) transconjugants then were able to induce nitrogen-fixing nodules on the three different clover species 12 days after inoculation. Clover root hairs of all three species inoculated with K17(pRT170) transconjugants exhibited poor curling and some distortions 2 weeks after inoculation (Fig. 1C). These transconjugants induced nodule formation on white and red clovers within 4 to 5 weeks but did not nodulate subterranean clover (Table 3). K17(pMN3) transconjugants caused poor hair curling on all three plant species but induced nodules only on white clover plants 5 to 6 weeks after inoculation (Table 3). *Lignobacter* transconjugants carrying either pRT170 or pMN3 plasmids nodulated only 50% of inoculated plants (Table 2). The hybrid strains K17(pPN1), K17(pRT170), and K17(pMN3) were all inoculated onto 100 plants per experimental run for five independent experiments. Nodulated and nonnodulated white and subterranean clover plants inoculated with either K17(pRT170) or K17(pMN3) showed the Tsr effect (Fig.

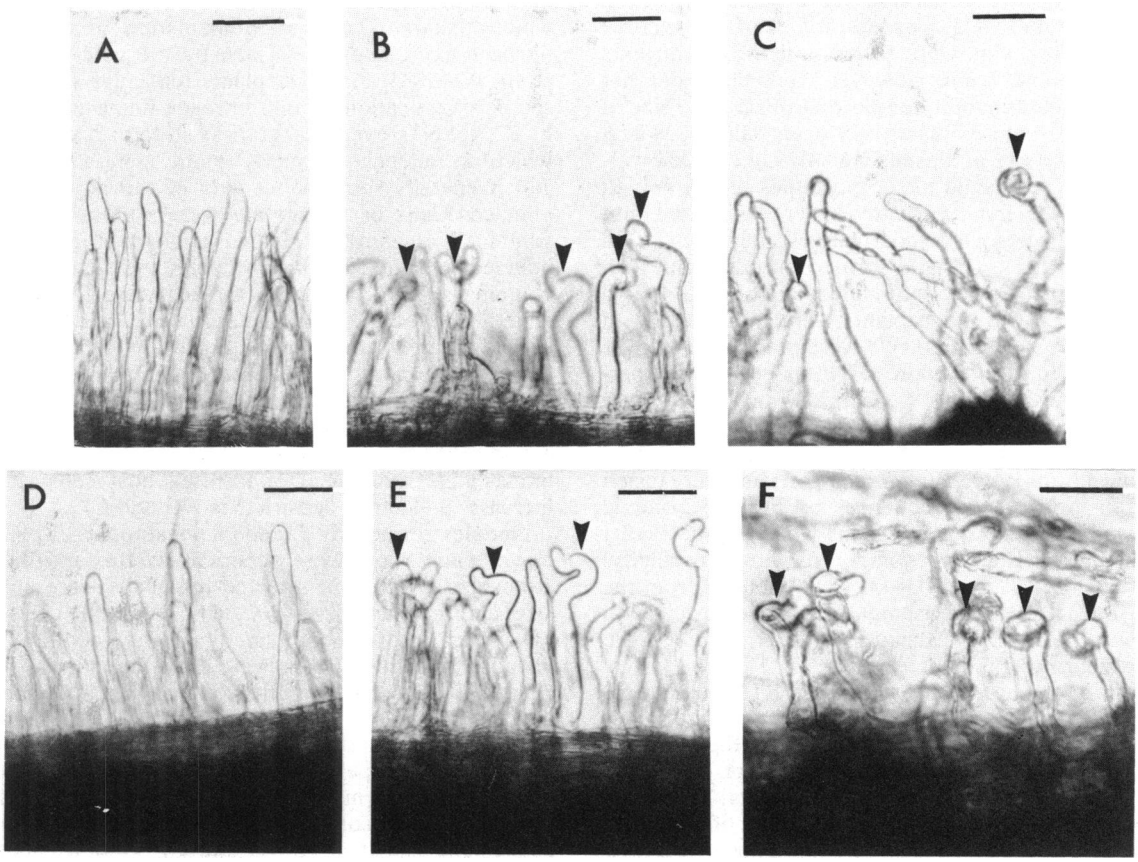

FIG. 1. Root hairs of white clover plants infected by *Lignobacter* strains K17 (A), K17(pPN1) (B), and K17(pRT170) (C), *Pseudomonas* strains PAO5 (D) and PAO5(pPN1) (E) and *R. trifolii* wild-type strain ANU843 (F). Arrows indicate distorted root hairs. Bars, 30 μm.

TABLE 3. Symbiotic properties of constructed *Lignobacter* and *Pseudomonas* strains compared with the control strain ANU845(pPN1)

Strains	Symbiotic properties in[a]:								
	White clover			Red clover			Subterranean clover		
	N	F	H	N	F	H	N	F	H
E. coli PN200	−	−	−	−	−	−	−	−	−
R. trifolii									
ANU845	−	−	−	−	−	−	−	−	−
ANU845(pPN1)	+	+	+	+	+	+	+	+	+
ANU845(pRT170)	+	−	+	+	−	+	+	−	+
ANU845(pMN3)	+	−	±	+	−	±	+	−	±
Lignobacter strains									
K17	−	−	−	−	−	−	−	−	−
K17(pPN1)	+	−	±	+	−	±	+	−	±
K17(pRT170)	+	−	±	+	−	±	−	−	±
K17(pMN3)	+	−	±	−	−	±	−	−	±
P. aeruginosa									
PAO5	−	−	−	−	−	−	−	−	−
PAO5(pPN1)	+	−	±	−	−	−	−	−	−
PAO5(pRT170)	−	−	±	−	−	−	−	−	−
PAO5(pMN3)	−	−	±	−	−	−	−	−	−

[a] Abbreviations: N, Nodulation; F, fixation estimated visually and by an acetylene reduction test; H, root hair curling estimated by light microscopy; +, marked hair curling; ±, poor root hair curling (less than 30% of hairs were curled); −, no root hair curling. White clover, *Trifolium repens*; red clover, *T. pratense*; subterranean clover, *T. subterranean*.

3B). This effect was not observed when plants were inoculated with strain K17(pPN1) (Fig. 3A).

Plant response to inoculation with *Pseudomonas-Rhizobium* hybrids. To check whether plasmid pPN1 had transferred to *Pseudomonas* strain PAO5, plasmid profiles of 100 putative Km^r Tc^r transconjugants were tested. All transconjugants contained a plasmid band; however, this band did not possess the expected mobility of plasmid pPN1. The size of this plasmid was approximately 39 megadaltons, which corresponds to the size of plasmid R68.45 (data not shown). Despite the lack of plasmid pPN1 molecules on the gel, all 100 Km^r Tc^r *Pseudomonas* derivatives were inoculated onto three test plant species. Of 100 derivatives, only one caused root hair curling (Fig. 1E) and formed pseudonodules on white clover plants 6 weeks after inoculation (Fig. 4). No response was observed when plants were inoculated with parental strain PAO5 (Fig. 1D). We were not able to recover bacteria from nodules; however, in five independent experiments nodule-like structures were formed on white clover plants when inoculated with a PAO5 Km^r Tc^r derivative (data not shown). Nodulating PAO5(pPN1) and nonnodulating (parental strain) PAO5 *Pseudomonas* strains were tested for the acquisition of *nod* sequences by probing genomic DNA digests of the two sets of strains with a 7.2-kb *Eco*RI fragment spanning Hac and some Hsp (host specificity) genes of *R. trifolii* strain ANU843. DNA isolated from the nodulating PAO5(pPN1) transconjugant and digested with *Eco*RI showed positive hybridization to the *R. trifolii* 7.2-kb nodulation fragment (Fig. 5).

Pseudomonas strain PAO5 containing pRT170 or pMN3 generally caused hypertrophy and branching on white clover root hairs as well as a pronounced Tsr effect (Fig. 3C). We observed a gradation of plant responses. Some of the Tsr responses took place in the axils of lateral roots. There was a blockage of the lateral root development that often results in the formation of the nodule-like structures (Fig. 6). Moreover, there was abnormal branching of laterals and atypical root hair development (data not shown).

Structure of white clover nodules formed by *Lignobacter-Rhizobium* and *Pseudomonas-Rhizobium* hybrids. The control *R. trifolii* strain ANU845(pPN1) formed nitrogen-fixing nodules on the roots of clover plants (Fig. 2F). The nodules, which appeared 12 days after inoculation, were not similar to nitrogen-fixing nodules formed by the wild-type *R. trifolii* strain ANU843 on clover plants (data not shown). Microscopic examination of non-nitrogen-fixing nodules formed by K17(pPN1) revealed that these structures were nodules in which no independent meristematic zones developed (Fig. 2G). Generally, only about 40% of the nodule cells were infected. Cells of the nodule were uniform in shape and contained a centrally located nucleus (Fig. 7A). In the infected cells the cytoplasm surrounding the nucleus did not contain vacuoles. Infection threads were found in the infected cells (Fig. 7C). Bacteria were released from the infection thread and became enclosed within the peribacteroid membranes as they entered the plant cell cytoplasm (Fig. 7B). Bacteroids were enclosed singularly within the peribacteroid membrane showing a very thick surrounding electron transparent space and dispersion of internal membranes. The cytoplasm of infected host cells showed an increase in electron density (Fig. 7B and C).

Nodules formed by *Lignobacter* strain K17(pRT170) on clover plant roots were spherical, white, and did not fix atmospheric nitrogen. Infected host cells were enlarged in size, and the nucleus was located close to the cell wall (Fig. 7D). The host cell contained a large, centrally located vacuole. Infection threads were often found in the host cells (Fig. 7E). Bacteria were released from the infection threads into the host cytoplasm (Fig. 7D and E). Because bacterial cells released into the host cells did not have the characteristic swollen shape of normal bacteroids, we called them bacteroid-like forms. These forms were enclosed within membranes, and some of them were surrounded by the thin amorphous matrix (Fig. 7E and F). Nodules induced by K17(pMN3) on clover plants had essentially the same morphological structures as nodules induced by K17(pRT170).

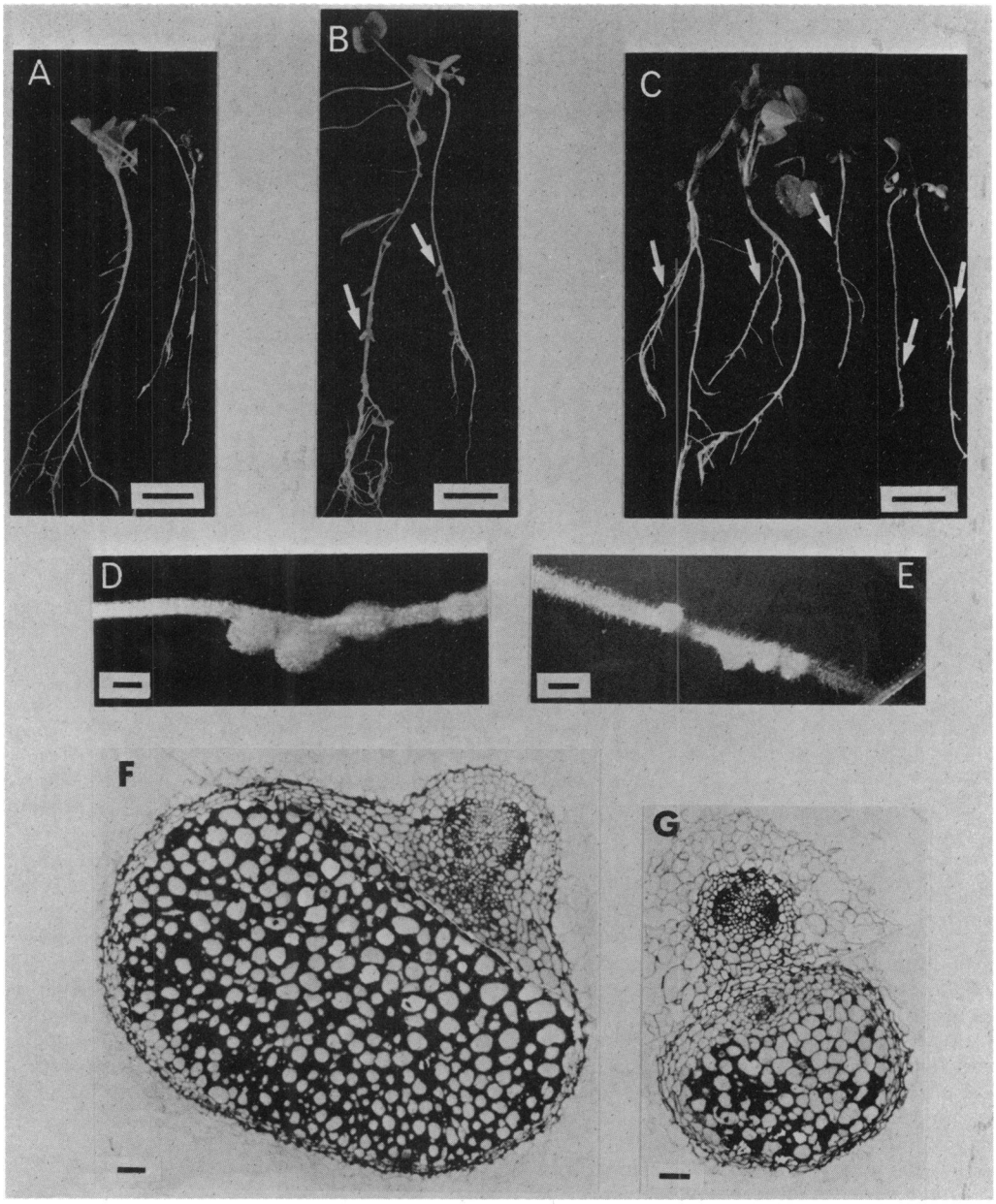

FIG. 2. Light micrographs of clover nodules formed by *Rhizobium* ANU845(pPN1) and *Lignobacter* K17(pPN1) strains. A, Uninoculated subterranean clover (left) and white clover (right) plants. B, Subterranean (left) and white (right) clover plants inoculated with *R. trifolii* strain ANU845(pPN1). C, Two subterranean (left) and three white (right) clover plants inoculated with K17(pPN1). Nodules are indicated by arrows. All plants were photographed 5 weeks after inoculation. Bars (A, B, and C), 1 cm. D and E, Root nodules formed on white clover plant by ANU845(pPN1) and K17(pPN1), respectively. Bars (D and E), 1 mm. F, Section of nodule induced by ANU845(pPN1) on white clover. The nodule has a shape characteristic of a globose, spherical-type structure. It has a symbiotic zone in which cells are filled with bacteroids. Bar, 100 μm; G, Section of nodule induced by K17(pPN1) on white clover. This is a determinate nodule with an infected zone in which relatively few cells contain bacteria. Bar, 100 μm.

Pseudomonas aeruginosa strain PAO5(pPN1) formed pseudonodules on white clover roots (Fig. 4). These root structures were outgrowths from the epidermal tissue and were not infected by bacteria. In the epidermal zone of these root structures we found bacterial cells within intercellular spaces (Fig. 8).

DISCUSSION

Nodulation genes from *R. trifolii* could be expressed in *Lignobacter* strain K17 and *P. aeruginosa* strain PAO5, conferring on these strains the ability to form ineffective nodules and pseudonodules, respectively. The cointegrate *R. trifolii* Sym plasmid pPN1 could be easily transferred into strain K17 where it was expressed. The stability of pPN1 in most *Lignobacter* transconjugants suggests that this bacterium may be naturally inefficient in genetic recombination, since cointegrate plasmids can be easily resolved by *recA*$^+$-mediated homologous recombination between the two copies of their transposon (11).

It has been reported that *Lignobacter* strain K17 can fix

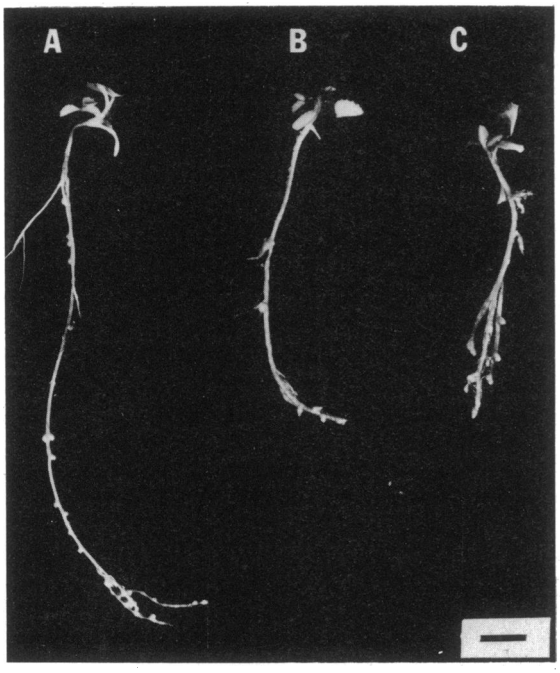

FIG. 3. Appearance of white clover plants inoculated with *Lignobacter* and *Pseudomonas* transconjugants. A, Plant inoculated with the nodulating *Lignobacter* strain K17(pPN1); B, plant inoculated with the nodulating K17(pRT170); C, plant inoculated with the nonnodulating *Pseudomonas* strain PAO5(pRT170). B and C show a Tsr effect. Bar, 1 cm.

atmospheric nitrogen in the free-living state (5). When clover plants were inoculated with K17(pPN1) transconjugants, only non-nitrogen-fixing nodules were formed. Lack of nitrogenase gene expression in these nodules might occur due to the presence of the *Lignobacter* nitrogenase genes interfering with the expression of the clover fixation genes on pPN1. A similar effect, called functional interference, was observed with a *R. leguminosarum* Sym plasmid in a *Rhizobium phaseoli* strain (4). Also, the ineffective nodulation response on clovers of the K17(pPN1) strain may have been caused by the abnormal development of bacteroids.

Since we found that an entire *R. trifolii* Sym plasmid could be transferred to *Lignobacter* sp. where it was expressed,

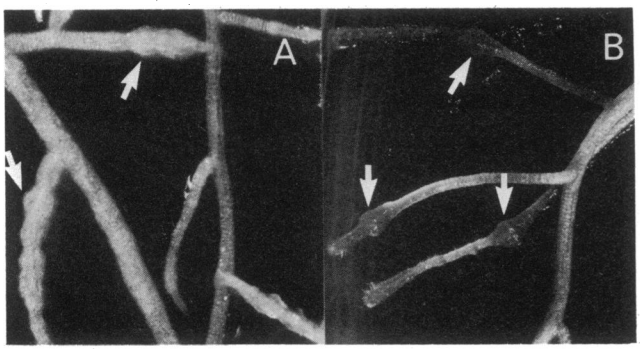

FIG. 4. Pseudonodule structures on white clover plants inoculated with a PAO5(pPN1) transconjugant. A, Arrows indicate clustered structures formed on the lateral roots; B, nodule-like structures formed singularly on the lateral roots (magnification, ×5).

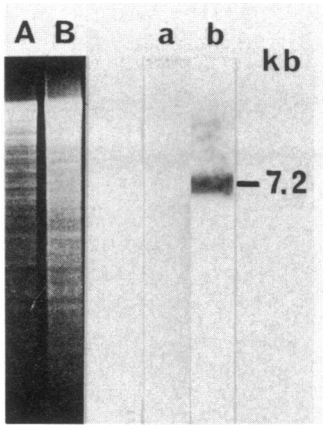

FIG. 5. Southern blots of *Eco*RI-cleaved *P. aeruginosa* DNA. Lanes: A, parental PAO5 DNA; B, PAO5(pPN1) DNA; a and b, corresponding autoradiographs. A 7.2-kb *Eco*RI restriction fragment (spanning some nodulation genes of the *R. trifolii* strain ANU843) used as a probe shows the positive hybridization to the genomic DNA of PAO5(pPN1) strain (lane b).

we wanted to check whether the *R. trifolii* nodulation genes encoded on a 14-kb DNA fragment of the Sym plasmid could also be transferred to and expressed in strain K17. A different nodulation pattern obtained on the tested clover plants inoculated with either K17(pRT170) or K17(pMN3) suggests that the extent of the phenotypic expression of nodulation genes cloned into different vectors depends upon the copy number of the vector used and upon the plant host background.

It has been reported that an introduced plasmid can be integrated into the *Pseudomonas* strain chromosome (26). In the case of PAO5(pPN1) transconjugants we were able to monitor the pPN1 markers in *Pseudomonas* strain PAO5, although the loss of pPN1 as an extrachromosomal element (as detected by agarose gel electrophoresis) was observed. As shown by hybridization studies, the one PAO5 transconjugant which did cause root hair curlihg and formed pseudonodules on white clover plants had the *R. trifolii* Sym plasmid or its fragment integrated into the chromosome.

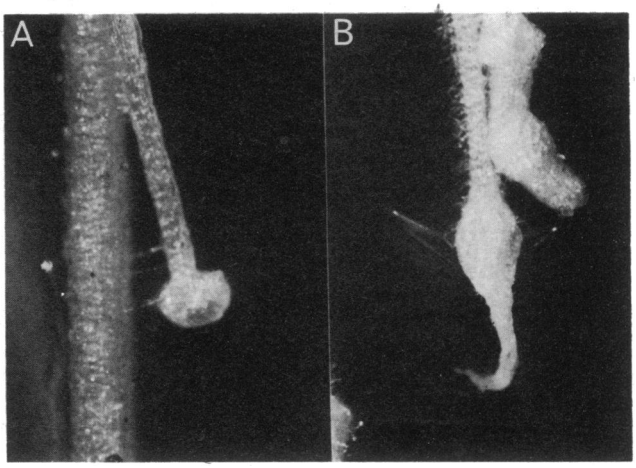

FIG. 6. White clover root Tsr effect induced by K17(pMN3) Nod^- (A) and PAO5(pMN3) Nod^- (B) transconjugants (magnification, ×15).

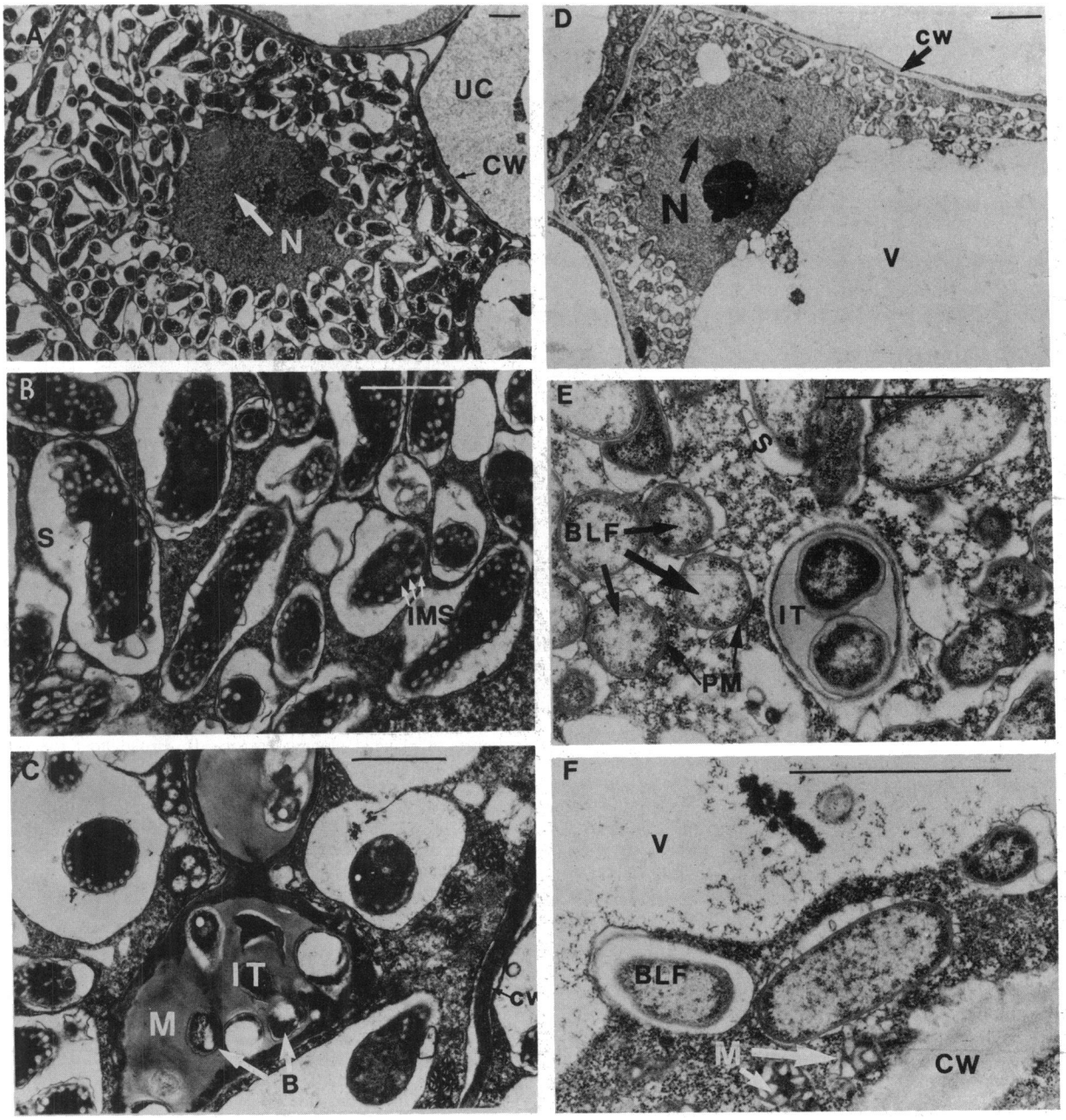

FIG. 7. Electron microscopy of nodules induced on white clover by *Lignobacter* strain K17(pPN1) (A, B, and C) and *Lignobacter* strain K17(pRT170) (D, E, and F). A, Infected nodule cell. Bacteroids are enclosed within the peribacteroid membranes. Bar, 1 μm. B, Enlargement of panel A. There is an electron-transparent space (S) between bacteroids and the peribacteroid membrane. All bacteroids show internal membrane structures (IMS). Bar, 1 μm. C, Micrograph of infection thread showing matrix (M) surrounding the bacteria (B). Bar, 1 μm. D, Micrograph of infected host cell. Bar, 1 μm. E, Bacteroid-like forms (BLF) released into host cytoplasm are surrounded by thin matrix (S). The peribacteroid membrane (PM) is also indicated. Bar, 1 μm. F, Micrograph showing enlargement of the bacteroid-like forms (BLF) and mitochondria (M). Bar, 1 μm. Other abbreviations: V, vacuole; CW, cell wall; N, nucleus; UC, uninfected cell; IT, infection thread.

The Tsr effect has been reported as a function located on a *Rhizobium* Sym plasmid. This was observed on *Vicia sativa* plants when inoculated with different *Rhizobium* strains carrying the Sym plasmid from *R. leguminosarum* (24). Interestingly, this effect was only observed when the *R. leguminosarum nod* genes were expressed in an endogenous genetic background. We found that the 14-kb *R. trifolii* Sym plasmid nodulation fragment also encodes for the Tsr function, which is expressed on clover plants when inoculated with *Lignobacter* and *Pseudomonas* transconjugants carrying the fragment. We observed that, in contrast to *R. leguminosarum*, the *R. trifolii* Tsr function also can be expressed when clover plants inoculated with K17 or PAO5 transconjugants carrying either pRT170 or pMN3 did not show nodule formation. The Tsr effect was most pronounced on white and subterranean clovers. We suggest that the Tsr effect is a result of the ineffective, abnormal infection caused by the bacterium carrying nodulation genes; however, these

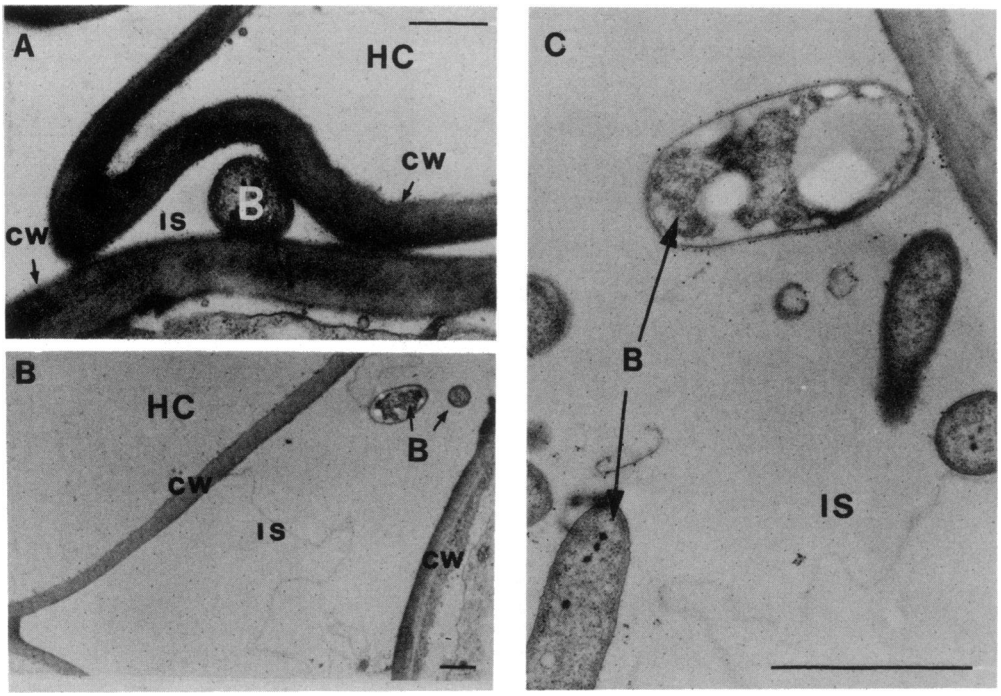

FIG. 8. Electron microscopic view inside root cells of clover plant inoculated with *Pseudomonas* strain PAO5(pPN1). Panels A and B show single bacteria (B) within intercellular spaces (IS). Host cells (HC) were not found to contain the bacteria. CW, Host cell wall. Panel C, Magnification of the bacteria (B) found within the plant root intercellular space (IS). Bar, 1 μm.

genes, due to the foreign genetic background, are not properly expressed.

The nodule induced on clover plants by *Lignobacter* transconjugants carrying pPN1 seemed to be similar to nodules formed by *Agrobacterium tumefaciens* containing an *R. trifolii* Sym plasmid (10, 18) and more developed than those formed by other fast-growing rhizobia while carrying endogenous nodulation genes (9, 10, 23, 27). In the case of nodules formed by K17 containing either pRT170 or pMN3, an increase in electron density of the host cell cytoplasm was observed. These observations indicate that the host cells were degenerated. A similar effect was observed in nodules on alfalfa induced by an *Agrobacterium* strain carrying *R. meliloti* nodulation genes (9). In addition, bacterial cells appeared generally to be in various stages of degeneration.

Our results show that the *R. trifolii* nodulation genes can be expressed in a bacterium such as a *Lignobacter* sp. which is unrelated to *Rhizobium* spp. Construction of a non-*Rhizobium* strain able to nodulate clover plants provides an opportunity to study Sym plasmid genes in the absence of other *Rhizobium* plasmids and the *Rhizobium* chromosome.

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