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Genetics

Rhizobium meliloti genes required for nodule development are related to chromosomal virulence genes in *Agrobacterium tumefaciens*

(symbiosis/*fix*⁻ mutants/heterologous complementation)

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ABSTRACT **Symbiotically essential genes have been identified in *Rhizobium meliloti* that are structurally and functionally related to chromosomal virulence (*chv*) genes of *Agrobacterium tumefaciens*. Homologous sequences also exist in the genomes of other fast-growing rhizobia including *Rhizobium trifolii*, *Rhizobium leguminosarum*, and *Rhizobium phaseoli*. In *Agrobacterium*, the *chvA* and *chvB* loci are known to be essential for oncogenic transformation of dicotyledonous plants and for attachment to plant cells [Douglas, C. J., Staneloni, R. J., Rubin, R. A. & Nester, E. W. (1985) *J. Bacteriol.* 64, 102–106], and the *chvB* locus has been implicated in the production of (1→2)-β-glucan, a unique exopolysaccharide component [Puvanesarajah, V., Schell, F. M., Stacey, G., Douglas, C. J. & Nester, E. W. (1985) *J. Bacteriol.* 164, 102–106]. Site-directed transposon insertion mutants in the *chvA* and *chvB*-equivalent regions of *R. meliloti* are symbiotically defective. Mutants in the *chvB*-equivalent region have been examined in detail and have been found to induce the formation of nodule-like structures on alfalfa that are devoid of bacteroids, lack infection threads, and cannot fix nitrogen. Such mutants fluoresce normally in the presence of Calcofluor, a histochemical stain for β-linked polysaccharides, and produce normal amounts of total exopolysaccharide. The *Rhizobium* loci have been designated *ndv* because of their requirement for nodule development.**

The family *Rhizobiaceae* has classically been considered to contain only two genera, *Agrobacterium* and *Rhizobium*. *Agrobacterium* species are plant pathogens that induce tumorous growths on a wide variety of dicotyledonous plants, while *Rhizobium* species are agriculturally beneficial plant symbionts that induce nitrogen-fixing nodules on the roots of legumes. We report here what is to our knowledge the first instance in which a group of symbiotically required *Rhizobium* genes has been shown to be structurally and functionally related to the *Agrobacterium* genes that are required for pathogenesis.

Most of the genes necessary for virulence (*vir*) of *Agrobacterium tumefaciens* have been localized to a unique endogenous plasmid called the Ti plasmid (1). Mutations in these *vir* genes prevent oncogenic transformation, presumably by interfering with the successful transfer to the plant of another region on the Ti plasmid called T-DNA, that encodes enzymes involved in phytohormone production (2–4). In addition to plasmid-encoded *vir* genes, two closely linked virulence loci have been found in the chromosome of *Agrobacterium* (5). These loci, designated *chvA* and *chvB*, have the interesting feature that mutations at either locus interfere with the ability of *Agrobacterium* to bind to plant cells. Little is known about how the *chv* gene products function, but *chv* mutants show pleiotropic effects likely to be related to cell envelope changes (6, 7).

The data presented here show that *chvA* and *chvB* are homologous to DNA sequences in the genomes of four different fast-growing *Rhizobium* species and that in the case of *Rhizobium meliloti*, the corresponding genes can functionally complement *Agrobacterium chv* mutants. *R. meliloti* mutants in the *chv*-equivalent loci are still able to induce nodule-like structures on alfalfa, but such nodules do not show normal bacterial invasion and differentiation.

MATERIALS AND METHODS

Strains and Plasmids. The following *Rhizobium* strains were used in this study: *R. meliloti* 102F34 (8), 1021 (9) and 41 (10); *Rhizobium phaseoli* 8002 (11) and its sym plasmid-cured derivative 8400 (11); *Rhizobium leguminosarum* 128C53 (12) and its sym plasmid-cured derivative B151 (12); *Rhizobium trifolii* 162X68, from Nitragin (Milwaukee, WI), and RS 800 (13); *Rhizobium japonicum* USDA 110 (14). *Agrobacterium* strains have previously been described: A348 is *A. tumefaciens* C58 chromosome carrying pTiA6NC (15); Tn*5* and Tn*3*HoHol insertion mutants were used for complementation studies (5). *Escherichia coli* strains were HB101 (*pro, leu, thi, lacY, endoI, recA, hsdR, hsdM, str*^{R}) and HB101::Tn*5*. The following plasmids were used: pPH1JI (16) for marker exchange; pRK290 (8) for subcloning; pLAFR1 (17) cosmid clones pRK290.112 (see Fig. 2, line c), pRK290.51 (see Fig. 2, line d), pRK290.5143 (see Fig. 2, line e).

Hybridization Analysis. Total genomic DNA from *Rhizobium* strains was isolated and used for Southern blotting as previously described (18). Nick-translated (19) DNA probes were hybridized overnight at 37°C in 5× SSC/45% (vol/vol) formamide containing heparin at 100 μg/ml before being washed and processed for autoradiography. (1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7.0.)

Tn*5* Mutagenesis. Tn*5* insertions into plasmid-borne DNAs in *E. coli* were obtained as described (20). These were recombined into the genome of *R. meliloti* 102F34 using the marker exchange procedure of Ruvkun & Ausubel (21) with pPH1JI as the incoming incompatible plasmid. All mutants were verified by Southern blot analysis for fidelity of recombination.

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Abbreviation: kb, kilobase(s).

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Light Microscopy. Nodules were harvested 17 days following inoculation, sliced longitudinally, and fixed in 3% (vol/vol) glutaraldehyde, 2% (wt/vol) paraformaldehyde, 0.1 M cacodylate buffer, pH 7.4, for 2 hr at 0°C, postfixed in 1% OsO_4, 0.1 M cacodylate buffer, pH 7.4, for 2 hr at 0°C, then dehydrated through an ethanol series at 0°C. Fixed nodules were infiltrated with Spurr's low viscosity embedding medium (firm), then flat embedded and baked for 24 hr at 60°C under vacuum.

For bright-field light microscopy, 1-μm thick sections were cut, dried down in drops of water on "subbed" slides, and stained with 0.1% toluidine blue 0 in 1% sodium tetraborate for 1 min on a hot plate, destained with ethanol, dried, and mounted in Permount.

Root Hair Curling Analysis. Plants were surface sterilized with $HgCl_2$ and germinated in the dark on wet filter paper in a Petri dish for 2 days. Three seedlings each were transferred to an agar slant [20 ml of 1% Noble agar in nitrogen-free plant medium (22) in a 25 × 200 mm cotton-stoppered test tube] and grown in a room temperature incubation chamber with 16 hr of light and 8 hr of dark per 24 hr. The root portion of the slant was covered with a black paper sleeve. Twenty-four hours after transfer, the plants were inoculated, and at the indicated times the entire root of each plant was examined by phase–contrast microscopy between a glass slide and coverslip. One-half the plants were scored at 3 days and the remainder at 4 days following inoculation of the roots with 3 ml per tube of 1×10^8 early stationary phase cells per ml in distilled water. All root hairs projecting laterally from the root were examined for shepherd's crooks, which were defined as any root hair that had grown through at least a 180° turn and had made contact, or nearly made contact, with a more medial region of the same hair (i.e., loose "corkscrews" were not scored as shepherd's crooks).

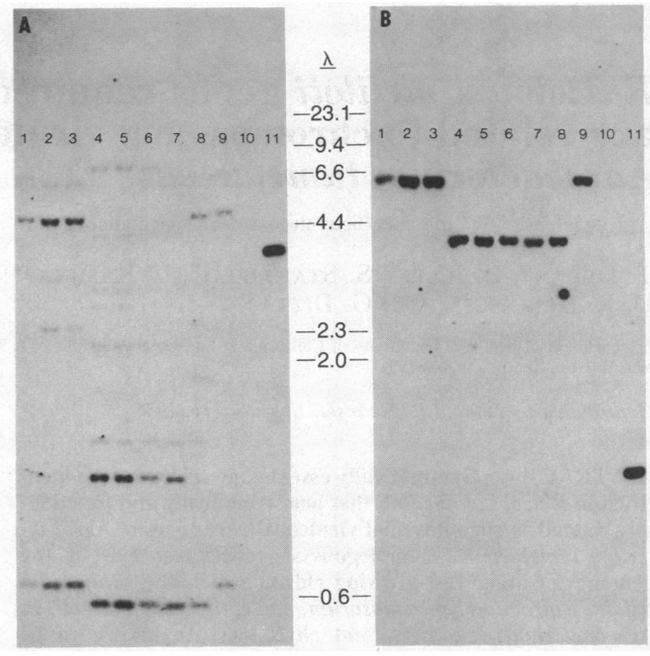

FIG. 1. Southern blot hybridizations using *A. tumefaciens chvA* and *chvB* DNA as probe against total DNA from different *Rhizobium* species. (*A*) *chvA* DNA probe. (*B*) *chvB* DNA probe. Lanes 1–3: *R. meliloti* strains 1021, Rm41, and 102F34, respectively; lanes 4 and 5: *R. phaseoli* and its sym plasmid-cured derivative, respectively; lanes 6 and 7: *R. leguminosarum* and its sym plasmid-cured derivative, respectively; lanes 8 and 9: *R. trifolii* strains 128C53 and RS 800 respectively; lane 10: *R. japonicum* USDA 110; lane 11: *A. tumefaciens* A348. The amount of DNA used in lane 11 was approximately half that used in the other lanes.

RESULTS

Identification of *A. tumefaciens chv* Homologues in *Rhizobium*. Genomic DNA from various *Rhizobium* species was examined for homology to *A. tumefaciens chvA* and *chvB* by Southern blot hybridization as shown in Fig. 1. The DNA probes used to represent the *chvA* and *chvB* loci are shown in Fig. 2*a*. The *chvB* probe was a 1.25-kb *Eco*RI fragment internal to the *chvB* locus; the *chvA* probe was a 2.4-kb *Hin*dIII fragment containing most of *chvA* and up to 1 kb of additional DNA. As can be seen in Fig. 1, DNA fragments homologous to both *chvA* and *chvB* were present in the genomes of all four fast-growing *Rhizobium* species examined: *R. meliloti* (lanes 1–3), *R. phaseoli* (lanes 4 and 5), *R. leguminosarum* (lanes 6 and 7), and *R. trifolii* (lanes 8 and 9). No significant hybridization to either locus was found in DNA from the slow-growing species *R. japonicum* USDA 110 under these conditions (lane 10) or at reduced stringency (data not shown). The three *R. meliloti* strains examined here each contained two *Eco*RI fragments (5.0 kb and 0.6 kb) that were homologous to *chvA* and one *Eco*RI fragment (6.2 kb) that was homologous to *chvB*. Lanes 5 and 7 contain DNA from sym plasmid-cured strains of *R. phaseoli* and *R. leguminosarum*, respectively, demonstrating that the *chv* homologues are not located on these replicons and are thus unlinked to either *nod* genes or nitrogenase genes in these species. No homology was found to either *chvA* or *chvB* in the genomes of *E. coli* or *Pseudomonas putida* (data not shown).

Isolation and Restriction Enzyme Mapping of *R. meliloti* Genes Homologous to *chvA* and *chvB*. Colony blot hybridization was used to identify pLAFR1 cosmid DNA clones in an *R. meliloti* gene bank in *E. coli* that carried homology to either *chvA* or *chvB*. Some of these DNAs are depicted in Fig. 2, lines c, d, and e. Based on restriction enzyme mapping of cosmid and genomic DNAs, the relative positioning of *chvA*- and *chvB*-homologous DNAs in the *R. meliloti* genome is shown in Fig. 2*b* and by the solid bars above it. The latter show maximum limits of hybridization for the *chvA* and *chvB* probes. For reasons discussed below, the *R. meliloti* loci have been designated *ndvA* and *ndvB*. As can be seen, the overall arrangement of *R. meliloti ndv* genes closely parallels that of the *Agrobacterium chv* genes.

Heterologous Complementation of *Agrobacterium chv* Mutants by the *ndv* Genes of *R. meliloti*. To determine whether the *ndv* genes are functionally equivalent to the *chv* genes, various *R. meliloti* cosmid DNA clones and subclones, containing or lacking different Tn*5* insertions, were conjugatively transferred into representative *chvA* and *chvB* mutants of *A. tumefaciens* A348 (5). The exconjugants were used to infect leaves of *Kalanchoë diagremontiana* in a standard test for oncogenic virulence. Strain A348 forms tumors in 10–14 days in this assay. The results of these experiments are summarized in Fig. 2 *c–i*. Both *chvA* and *chvB* mutants of A348 could be complemented to give wild-type levels of virulence on *Kalanchoë* by the cosmid clone shown in Fig. 2*c*. The absence of a 2.3-kb *Eco*RI fragment eliminated *chvA* complementation (Fig. 2*d*), even though this fragment had no homology to the *chvA* probe used, implying that essential functions probably span the junction between the 2.3-kb fragment and the adjacent 0.6-kb fragment. A single 6.2-kb *Eco*RI fragment carried all of the genetic information necessary to complement representative *Agrobacterium chvB* mutants in Fig. 2*e*. Subclones and transposon insertions (Fig. 2 *f–i*) have further narrowed the outermost boundaries of the *chvB*-equivalent DNA to a 4.2-kb stretch of DNA between the left hand boundary of the 6.2-kb *Eco*RI fragment and insertion 12.

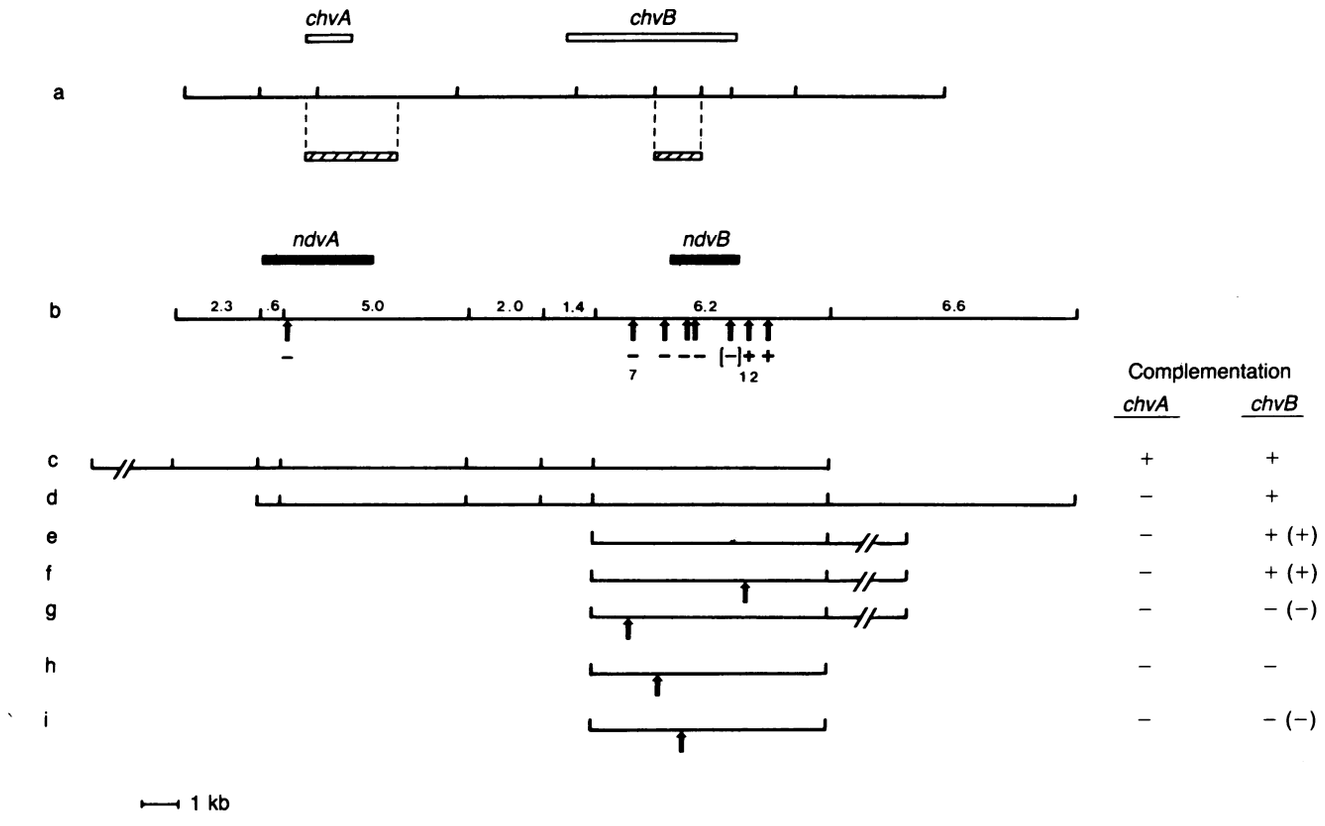

FIG. 2. The organization of the *ndvA* and *ndvB* region of the *R. meliloti* genome, relative to the *chvA* and *chvB* regions of *A. tumefaciens*. Genetic data pertaining to mutations in the *ndvB* region and to heterologous complementation of *Agrobacterium chv* mutants are also shown. (*a*) *Eco*RI restriction enzyme map of the *chvA* and *chvB* region of *A. tumefaciens* (5). The open bars show the limits of *chvA* and *chvB* as determined by transposon mutagenesis. The hatched bars show the DNA fragments used as hybridization probes against *R. meliloti* DNA. (*b*) *Eco*RI restriction enzyme map of the *ndvA* and *ndvB* region of *R. meliloti*. The size of each fragment in kb is shown. The solid bars denote regions within which the *chvA* and *chvB* probes hybridize. The arrows show the locations of transposon Tn*5* insertions that have been recombined into the *R. meliloti* genome with the + or − beneath each arrow indicating whether the insertion resulted in a nod$^+$ fix$^+$ (+) or nod$^+$ fix$^-$ (−) phenotype, respectively. Insertions 7 (mutant TY7) and 12 are discussed in the text. The insertion adjacent to insertion 12 had a partially positive phenotype and is, therefore, denoted (−). (*c–i*) Cloned DNAs representing various portions of the *ndv* region. (*c–e*) Cosmid DNAs isolated from a gene bank maintained in *E. coli*. The broken segments denote additional DNA fragments that are not contiguous with the rest of the region shown. Arrows show the sites of different Tn*5* insertions. To the right of each DNA are indicated the results of virulence assays carried out on *Kalanchoë* leaves, using as inocula *chv* mutants of *A. tumefaciens* carrying the corresponding cloned DNA and a broad host range plasmid. The results in parentheses refer to a single experiment carried out using the assay of Douglas *et al.* (23) to measure the attachment of *Agrobacterium* merodiploids to *Zinnia* cells.

A single experiment was also carried out to test the ability of *A. tumefaciens chvB* mutants carrying the *ndvB* genes of *R. meliloti* to attach to plant cells. Using the assay of Douglas *et al.* (23), it was found that attachment, as well as virulence, could be restored to *chvB* mutant 118 (5) by *ndvB* genes (Fig. 2 *e, f, g,* and *i*).

Creation of *ndv* Mutants of *R. meliloti*. Site-directed transposon mutagenesis (21) was used to mutate the *ndvA* and *ndvB* regions of the *R. meliloti* genome. Insertions into either locus created symbiotically defective mutants that had a nod$^+$fix$^-$phenotype on plants (discussed below). *ndvB* mutants were studied in detail. Introduction of cloned *A. tumefaciens chvB* DNA into *ndvB* mutants restored normal symbiotic capability (data not shown). There were no significant changes in the vegetative growth properties of such mutants. Cells grew on either mannitol or succinate as carbon source and showed no change in their ability to stain with Calcofluor, a fluorescent dye for β-linked polysaccharides. Recovery of total exopolysaccharide was quantitated for one *ndvB* mutant whose symbiotic phenotype is discussed in detail below (*ndvB*-TY7) and found to be comparable to wild type. The boundaries of symbiotically essential DNA in this locus were determined by mutagenesis and complementation of genomic Tn*5* mutations with plasmid-borne Tn*5* insertions (Fig. 2) and are identical to those previously determined for *chvB*-equivalent DNA.

The interaction of one of the *ndvB* mutants, *ndvB*-TY7, with alfalfa plants was further investigated. This mutant induced root hair curling roughly equivalent to that seen in wild type within 4 days after inoculation. Both shepherd's crooks and other deformations were seen. Uninoculated plants gave no reaction. Quantitation of shepherd's crooks is presented in Table 1.

R. meliloti ndvB-TY7 induced the formation of nodule-like structures that differed from those induced by the wild-type parental strain. These nodules were relatively small, more numerous than wild type, round, white to yellowish white, and contained no bacteroids. Nitrogen fixation capability, as

Table 1. Induction of shepherd's crooks on alfalfa cv. Moapa by *R. meliloti* 102F34 and *R. meliloti ndvB*-TY7

Bacterial strain	Plants examined, no.	Plants with SCs	Total SCs	Average number of SCs per responding plant
102F34	23	15	159	10.6
ndvB-TY7	24	12	104	8.7

SC, shepherd's crooks.

measured by acetylene reduction, was completely absent. Fig. 3 shows cross sections of wild-type and mutant nodules. The typical nodule induced by *ndvB*-TY7 is composed of a distal meristematic region and a proximal region of cells containing large starch granules, as determined by iodine staining. No bacteroids or infection threads are present. This was further substantiated by examination under the electron microscope (data not shown). Peripheral vascular bundles were present in most of these nodules although none are visible in Fig. 3*C*. A peripheral endodermis separating the cortical cells from the central region of the nodule is also usually present. These structures, therefore, possess features of genuine nodules as opposed to root-like outgrowths in which a single vascular bundle would be centrally located and surrounded by endodermis. In contrast, cells of the central region of typical wild-type nodules (Fig. 3 *A* and *B*) are filled with bacteroids, have small starch granules, and contain frequent infection threads.

The single *ndvA* mutant generated in this study (Fig. 2*b*) was not as thoroughly examined as *ndvB*-TY7, but was nevertheless found to induce the formation of small, white, *fix*$^-$ nodules on alfalfa.

DISCUSSION

Relatively few *Rhizobium* genes, the *nod* genes, are required for the induction of nodules on the roots of susceptible legume hosts (9, 24). In contrast, a large number of loci have been identified that are required for normal symbiotic development leading to nitrogen fixation (9, 24). These have been referred to either as *fix* or *nif* genes if they appeared to be directly involved in the nitrogen fixation process, or as *sym* genes if no other information was available. In one case, discussed below, a set of loci have been designated *exo* to reflect the fact that they are involved in exopolysaccharide production (25). Here we report on two sets of genes from *R. meliloti* that are required relatively early in the symbiotic process. Because mutations in these genes lead to abnormal nodule development, we have followed the example of Vandenbosch *et al.* (26) and designated these *ndv* genes.

A most interesting feature of the *ndvA* and *ndvB* loci of *R. meliloti* is their structural and functional relatedness to the chromosomal virulence loci *chvA* and *chvB* in *Agrobacterium*. Whereas in *A. tumefaciens* the *chv* genes are required for pathogenicity, in *R. meliloti* they are required for symbiosis. Heterologous complementation of *Agrobacterium* mutants with *Rhizobium* genes and vice versa have shown the *chv* and *ndv* loci to be functionally interchangeable. Based on results from hybridization experiments, *ndv*-type genes probably exist in the other fast-growing *Rhizobium* species. For at least two species, *R. leguminosarum* and *R. phaseoli*, these genes were shown not to be located on the sym plasmid.

Despite the ability to induce nodule-like structures on alfalfa, *ndvB* mutants of *R. meliloti* appear to be noninvasive.

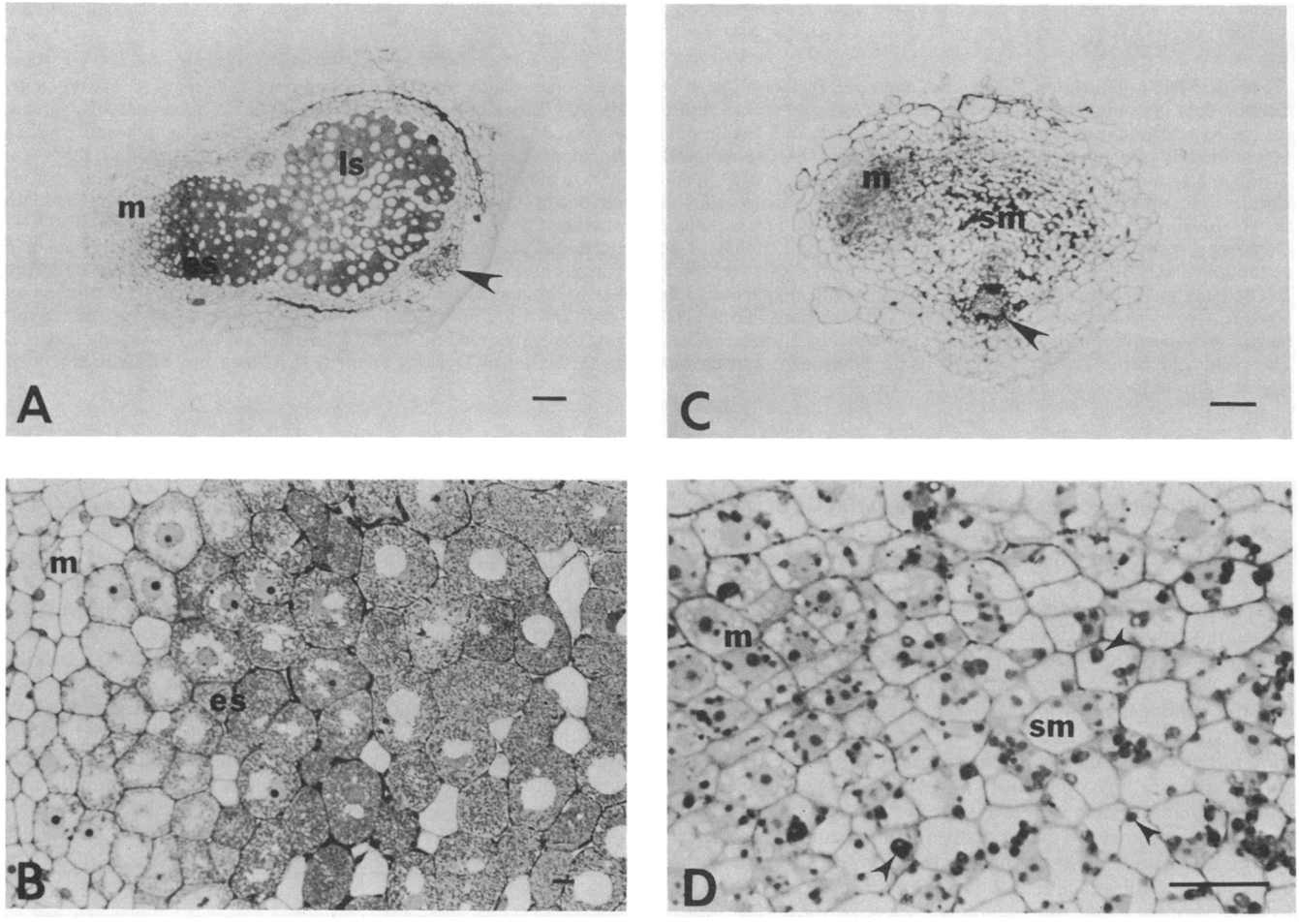

FIG. 3. Light microscopy of nodules induced by wild-type *R. meliloti* 102F34 (*A* and *B*) and a *ndvB* mutant of *R. meliloti* (*C* and *D*). (*A* and *C*) Longitudinal sections (1 μm) through 17-day-old nodules. The arrows indicate the point of attachment to the root. m, meristematic region; es, early symbiotic region; ls, late symbiotic region; sm, submeristematic region. (Bar = 100 μm.) (*B* and *D*) Higher magnification of the meristematic (m) and submeristematic (sm) regions. For *R. meliloti* 102F34-induced nodules, the submeristematic region consists of the early symbiotic (es) region. Arrows indicate nodules. (Bar = 10 μm.)

Mutant nodules contain neither bacteroids nor infection threads, and internal nodule development is incomplete. This basic phenotype has been reported for mutants of *R. meliloti* and *R. phaseoli* (25–27) that cannot produce extracellular acidic heteropolysaccharide. Such mutants do not stain with the fluorescent dye Calcofluor, which detects β-linked polysaccharides. While the *ndvB* mutants reported here produce normal amounts of exopolysaccharide and stain normally with Calcofluor, it is conceivable that these mutants fail to produce a minor but essential exopolysaccharide component. In this regard, the report of Puvanesarajah *et al.* (6) that *chvB* mutants lack (1→2)-β-glucan is significant. The role, if any, of (1→2)-β-glucan in pathogenesis is completely unknown at the present time. While the *ndvB* mutants appear to have a symbiotic phenotype that is very similar to that reported for *exo* mutants, an important difference pertains to root hair curling. *exo* mutants of *R. meliloti* interact infrequently, if at all, with root hairs (25), whereas *ndvB*-TY7, the only mutant examined in detail in this study, is only slightly reduced in its ability to induce root hair curling (Table 1). This suggests that *ndvB* mutants are able to carry out more of the normal infection process than *exo* mutants and that consequently the *ndvB* gene products may be required at a slightly later stage of symbiotic development.

What is the fundamental nature of the defect in these *ndv* mutants? Perhaps the most likely possibility is that recognition between symbiont and host has been interfered with because of cell surface changes in *Rhizobium*. The pleiotropic effects of *chvB* mutations in *Agrobacterium* clearly suggest alterations in the cell envelope (6, 7, 23). This could have far-reaching consequences in terms of the ability of *Rhizobium* to approach plant cells and to initiate and propagate infection threads. Although there was no sign of infection thread proliferation within *ndv* mutant nodules, we did not determine whether limited infection thread growth might be occurring during the earliest stages of infection. Another possible consequence of an altered cell surface is that it might permit rejection of a normally compatible symbiont by the host plant. A negative component has not yet been demonstrated for host-specific interactions involving *Rhizobium*, but the possibility has been noted (28). An intriguing possibility for *ndv* mutants is that proper "signaling" of the host, in advance of direct cell–cell contact, does not occur. Specific exopolysaccharides could conceivably be involved in such a process. Lastly, by analogy with *Agrobacterium*, it is possible that *ndv* mutants are simply unable to attach, or to attach properly, to the surface of their legume host. This would be particularly interesting in light of the fact that *ndv* mutants of *R. meliloti* can nevertheless induce nodule-like structures on alfalfa.

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