

Induction of Pathogenic-Like Responses in the Legume *Macroptilium atropurpureum* by a Transposon-Induced Mutant of the Fast-Growing, Broad-Host-Range *Rhizobium* Strain NGR234

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Mutant strain ANU2861, a transposon Tn5 mutant of the fast-growing, broad-host-range *Rhizobium* strain ANU280 (NGR234 Sm^r Rf^r) overproduces polysaccharide, is an *ade* auxotroph, and induces poorly developed nodules on *Leucaena leucocephala* and *Lablab purpureus* (H. C. Chen, M. Batley, J. W. Redmond, and B. G. Rolfe, J. Plant Physiol. 120:331-349, 1985). Strain ANU2861 cannot form nodules on *Macroptilium atropurpureum* Urb. (siratro) or on *Desmodium intortum* and *D. uncinatum* and the nonlegume *Parasponia*. The parent strain, ANU280, effectively nodulates all these legume species except *Parasponia*, on which it forms ineffective nodules. Ultrastructural examination of infection sites on the legume siratro showed that mutant strain ANU2861 caused root hair curling (Hac⁺ phenotype), some cortical cell division (Noi⁺), but no infection threads (Inf⁻). Localized cellular responses, known to occur in phytopathological interactions, were observed in electron micrographs of the epidermal tissue at or near the infection zone after inoculation with strain ANU2861 but not the wild-type parental strain. These include (i) the rapid (within 20 h) accumulation of osmiophilic droplets attached to membranes at potential sites of strain ANU2861 penetration and (after 48 h) in the epidermal cells in the immediate region of the curled root hairs, and (ii) localized cell death of the epidermal cells. In addition, strain ANU2861 can initiate a systemic response in split-root siratro plants which prevents the successful nodulation of strain ANU280. A 6.3-kilobase fragment of wild-type genomic DNA, which includes the site of Tn5 insertion in strain ANU2861, was cloned and introduced to strain ANU2861. All the phenotypic defects of the mutant strain were corrected by the introduction of this DNA fragment. This indicates that the original Tn5 insertion is responsible for the phenotype.

Although the precise role of exopolysaccharide (EPS) in bacterium-plant interactions remains unknown, its production is considered an essential feature in plant-pathogenic interactions, since most mutants with a dry colony morphology are avirulent (1, 2, 7, 29, 39, 40). Wild-type *Rhizobium* cells produce large quantities of EPS, and the colonies they form are mucoid (Muc⁺) in appearance. Mutants defective in EPS production (Exo⁻) fail to successfully invade host nodule tissue. Exo⁻ mutants of *Rhizobium meliloti* induce ineffective nodules that do not contain intracellular bacteria or bacteroids and that form without root hair curling or infection threads (19, 27). Although Exo⁻ mutants of *R. trifolii* and *Rhizobium* strain NGR234 also induce ineffective nodules, these strains initiate nodule invasion with normal root hair curling and infection thread formation (4, 6, 13).

Predominantly, *Rhizobium* mutants auxotrophic for a wide range of nutritional requirements effectively nodulate their host plant(s) (9, 31). However, in much of this earlier work, *Rhizobium* mutants were induced by chemical mutagens and were subject to reversion rates that complicated symbiotic studies. In some legume-*Rhizobium* associations, the host plant can supply the auxotrophic symbiont with the required nutrient(s), although this ability varies according to the host plant and, to a lesser extent, the *Rhizobium* mutant (B. J. Bassam, H. K. Mahanty, and P. M. Gresshoff, Endocytob. Cell Res., in press).

Previous studies have shown that purine and pyrimidine auxotrophs are unable to effectively nodulate their host plants (31, 32, 38). Strain ANU2861, a Tn5-induced mutant of the broad-host-range *Rhizobium* strain NGR234, produces excessive amounts of EPS, is an *ade* auxotroph, and induces root hair curling (Hac⁺) on *Macroptilium atropurpureum* Urb. (siratro) and *Desmodium* plants, but fails to form nodules (Nod⁻ phenotype) (5). Although minimal medium supplemented with high concentrations of adenine (up to 25 µg ml⁻¹) improves the growth of strain ANU2861, the addition of various concentrations of adenine in plant nodulation assays fails to correct the Nod⁻ phenotype or the overproduction of EPS (5).

This paper presents both ultrastructural and physiological details of the infection events of strain ANU2861 on siratro plants. During the infection of siratro, strain ANU2861 induces both a localized response, which leads to cell death at and around the infection site, and a delayed systemic response, which affects nodulation ability elsewhere on the plant. Molecular complementation experiments showed that the single Tn5 insertion in strain ANU2861 was responsible for the phenotypic defects seen. The introduction of a 6.3-kilobase (kb) *Eco*RI fragment of wild-type DNA restores a wild-type phenotype to strain ANU2861. Chemical analysis of cell surface EPSs showed that the EPS synthesized in large amounts by strain ANU2861 was identical to that made by the parent strain.

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MATERIALS AND METHODS

Bacterial strains and plasmids. Strain ANU280 (5) is a streptomycin-resistant, rifampin-resistant derivative of the fast-growing, broad-host-range *Rhizobium* strain NGR234 (42). Strain ANU2861 (5) is a transposon-induced mutant of strain ANU280. The broad-host-range IncP1 vector pWB5a was a gift from W. Buikema. Plasmid pWB5a carries a tetracycline resistance marker and was used to introduce DNA fragments into *Rhizobium* backgrounds. Bluescribe (Ap^r) vector (Vector Cloning Systems, San Diego, Calif.) was used for the cloning of DNA fragments and subsequent transformation into *Escherichia coli* backgrounds. Plasmid pSD1 was derived from the cloning of a 6.3-kb *Eco*RI fragment from strain ANU280 into the *Eco*RI site of pWB5a. Plasmid pSD2 was derived from the cloning of an internal 1.3-kb *Hind*III-*Cla*I fragment (spanning the region of Tn5 insertion in the mutant strain ANU2861) into the *Hind*III-*Cla*I restriction sites of pWB5a. Cosmid pCD523 (15) contains approximately 20 kb of *Agrobacterium tumefaciens* DNA spanning both *chvA* and *chvB* chromosomal virulence loci. This construct was a gift from E. Nester.

Media. All liquid and solid media (BMM, L broth, and F) were prepared as described previously (35). Strain ANU2861 is an *ade* auxotroph and grows poorly on minimal media (5). Strain ANU280 and mutant strain ANU2861 could be easily distinguished on the basis of colony morphology when plated onto minimal mannitol (MM) medium. MM medium (pH 6.8) contains the following components (in milligrams per liter): FeCl₃, 15; MgSO₄ · 7H₂O, 100; CaCl₂ · 2H₂O, 150; K₂HPO₄, 220; sodium glutamate, 500; biotin, 0.75; thiamine, 0.75; pantothenate, 0.75; and mannitol, 3 × 10³.

Recombinant DNA techniques. DNA was isolated, detected, and manipulated by standard procedures (24). Restriction endonuclease reactions took place exclusively in TA buffer (30). Nitrocellulose and Hybond-N sheets were hybridized overnight at 65°C, thoroughly washed in 2 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and exposed to Kodak type RP5 X-ray film at -80°C for 4 days with a Cronex Lightning-Plus (E. I. du Pont de Nemours & Co., Inc.) intensifying screen.

Molecular cloning and plasmid construction. Total DNA (100 to 200 µg) isolated from strains ANU280 and ANU2861 was digested to completion with *Eco*RI and loaded onto 10 to 40% layered sucrose gradients (11 ml). Gradients were spun in an SW41 rotor (Beckman Instruments, Inc.) at 34,000 rpm for 16 to 18 h at 20°C. DNA fractions (200 to 500 µl) from mutant strain ANU2861 which hybridized to a Tn5-specific probe were ligated into the *Eco*RI site of Bluescribe. Ligated molecules were transformed into *E. coli* RRI cells, and transformants were selected on LB agar containing kanamycin (selection for a Tn5-containing fragment) and ampicillin (vector selection). A *Rhizobium* DNA fragment approximately 12 kb in size and containing transposon Tn5 was isolated in this manner. This cloned fragment was then used as a hybridization probe to select *Eco*RI size fractions from strain ANU280. The hybridizing fractions were then cloned into the *Eco*RI site of pWB5a and transformed into *E. coli* RRI cells as described above. Transformants were selected on LB agar containing tetracycline, and colonies showing hybridization to the 12-kb *Eco*RI probe were isolated. This permitted the isolation of an *Eco*RI fragment of approximately 6.3 kb containing *Rhizobium* DNA sequences spanning the site of the ANU2861 Tn5 insertion. A 1.3-kb internal *Hind*III-*Cla*I fragment was subcloned from this 6.3-kb fragment into pWB5a. These constructs, designated pSD1 and

pSD2, respectively, were transferred into strain ANU2861 for complementation analysis. *E. coli* ANU1073 supplied the necessary *tra* functions in triparental matings (10). *Rhizobium* transconjugants were selected on BMM and MM agar medium containing kanamycin (Tn5) and tetracycline (pWB5a). To determine whether complementation or recombination had occurred, plasmid DNA was isolated from transconjugants and transformed into *E. coli*. Transformants were selected for tetracycline resistance and then screened for the presence of kanamycin resistance (Tn5). The presence of Tn5 was confirmed by hybridization analysis.

Isolation and purification of *Rhizobium* EPSs. Strains ANU280 and ANU2861 were grown for 5 days in a glutamic acid-D-mannitol-salts medium (12). EPS and oligosaccharide repeat unit was isolated from strains ANU280 and ANU2861 by using an Amicon DC10L hollow fiber filtration system (12, 13). The acidic polysaccharide was readily removed from cells by washing with water; it is considered to be extracellular rather than capsular. The polysaccharide was purified by precipitation as the cetyltrimethylammonium salt, enabling removal of glucans and polyhydroxybutyrate. The resulting polymer had a sugar composition identical to that of the related oligosaccharide isolated by ion-exchange and gel chromatography (12, 13).

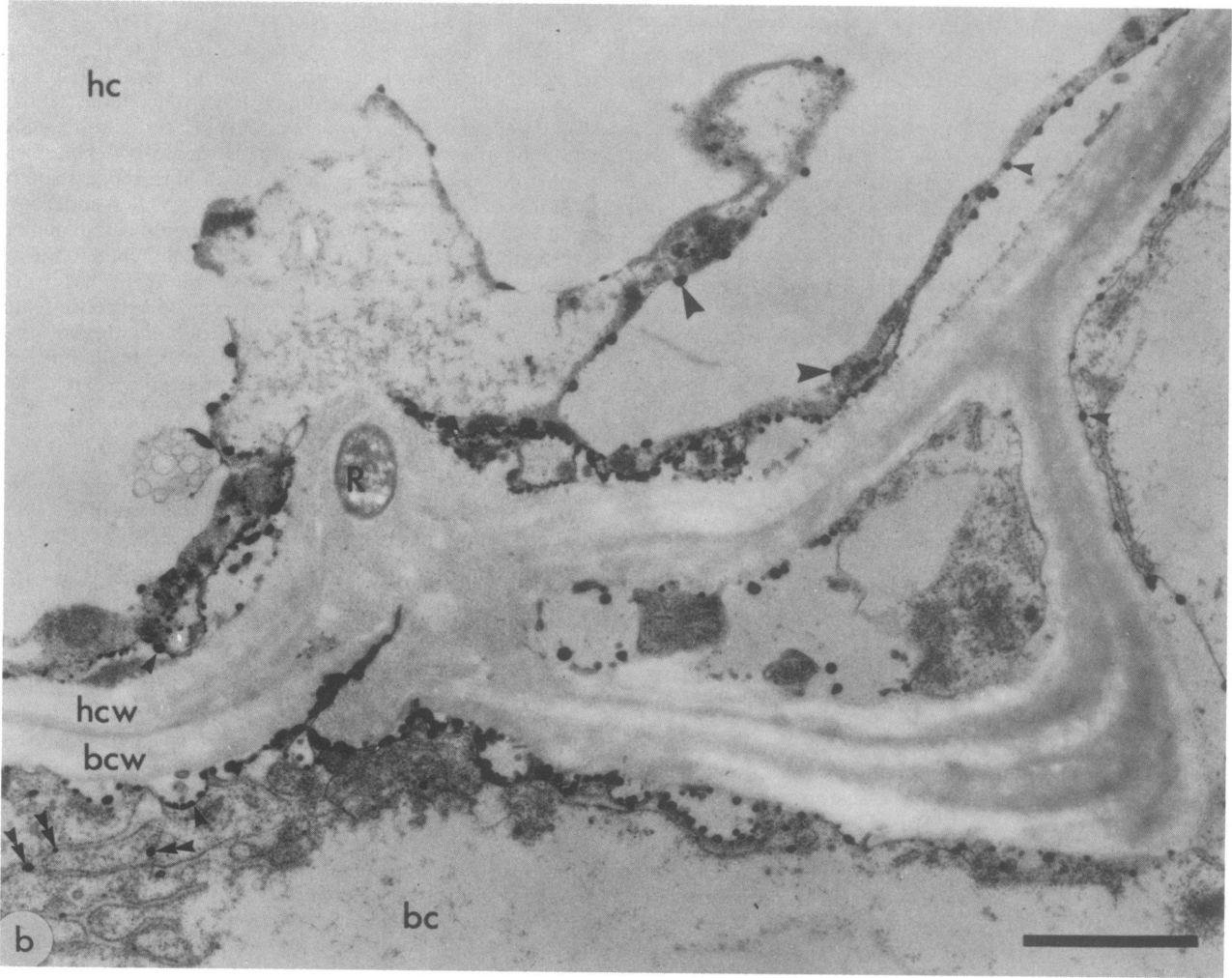
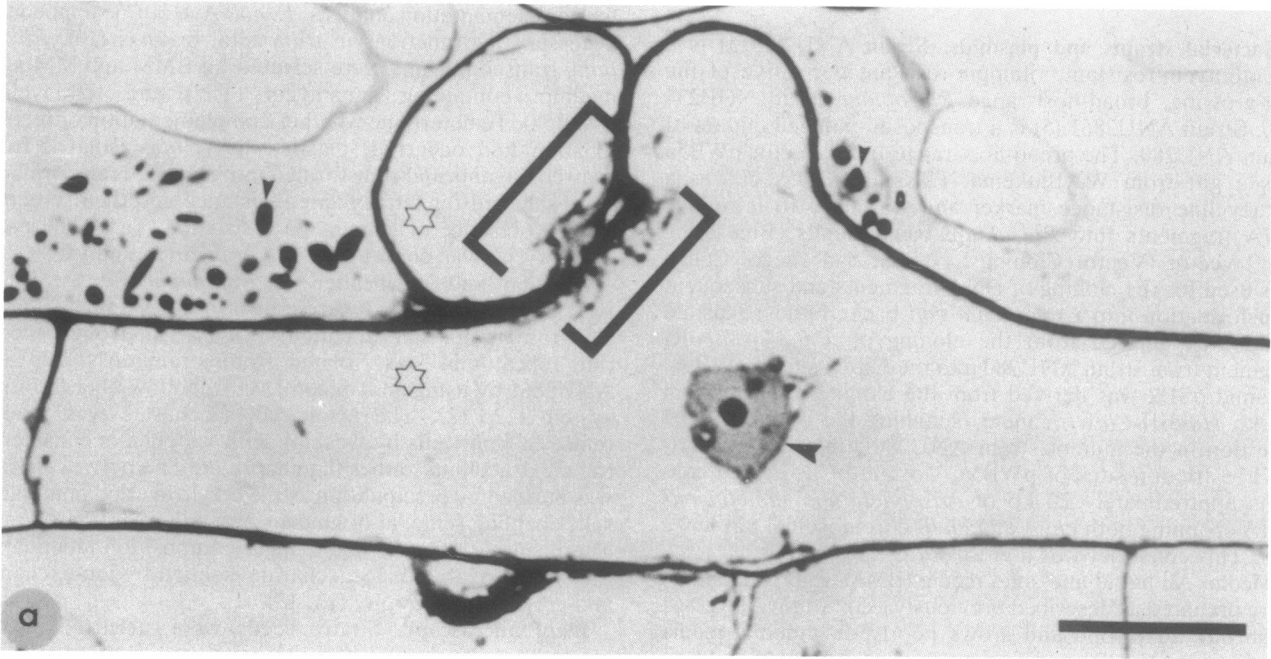
Plant microscopy. Siratro seeds were sterilized as described previously (35). Growth conditions for siratro seedlings, spot inoculation procedures, and transmission electron microscopy were as described previously (34).

Split-root assays. Split-root siratro plants were generated by the method published previously for clover plants (37), with the following modifications. Siratro seedlings showing well-developed lateral root systems of comparable length and robustness were placed onto large (15-cm) plates containing F agar medium. Plants containing more than two lateral roots were trimmed by excising smaller underdeveloped laterals. Split-root plants were then allowed to incubate overnight to ensure that they were firmly attached to the agar surface. Inoculum strains ANU280 and ANU2861 freshly grown on BMM-agar plates containing the appropriate antibiotics were streaked just ahead of the advancing root tips to avoid damage to the root. The plants were maintained in a growth cabinet for up to 5 weeks as described previously (3).

RESULTS

Analysis of the aborted infection of root hairs by mutant strain ANU2861. Although strain NGR234 is normally able to induce a nitrogen-fixing response on the tropical legumes siratro, *Desmodium uncinatum*, *D. intortum*, *Lablab purpureum*, and *Leucaena leucocephala* (42), the mutant strain ANU2861 is Nod⁻ on siratro, *Desmodium* spp., and the nonlegume *Parasponia* sp. and induces a few poor nodules on *Lablab* and *Leucaena* spp. (5).

After spot inoculation of strain ANU2861 onto siratro plants, a detailed microscopic analysis of the infectivity of this strain was undertaken to determine at what stage the infection of siratro plants failed. Strain ANU2861 was able to curl siratro root hairs in a similar manner and rate as during infection by the original parent strain NGR234 (34). However, in 28 of 32 infection sites examined 24 h post inoculation, root hairs infected by strain ANU2861 showed the nucleus to be in the basal part of the hair cell, away from the infection site (Fig. 1a). This is in contrast to successful infections with strain NGR234, in which the nucleus was always found to be very close to the penetration site induced by the invading *Rhizobium* cells (34). At the site of penetra-



tion of strain ANU2861 (Fig. 1b) an accumulation of osmophilic droplets (OPDs) occurred. OPDs can be distinguished from lipid droplets by their greater electron density (Fig. 1b). These OPDs accumulated especially at the plasmalemma, the tonoplast, and the outer membranes of mitochondria. They also accumulated next to the nuclear envelope, although at much reduced levels, and also at lower levels on the plasmalemma throughout the infected cells. The cytoplasm of the root hair cell infected by strain ANU2861 had none of the density and granularity found in strain NGR234 infections (34).

Although strain ANU2861 showed an inability to infect siratro farther than the root hair cells, evidence of cortical cell division could be seen after 48 to 72 h directly below the curled root hair (Fig. 2). Serial sections through the sites of cortical cell division showed no evidence of infection threads or bacterial proliferation in or around plant cells. A novel feature of ANU2861 infections was that 48 h after inoculation, the epidermal cells surrounding the infected root hair cells appeared to be necrotic, with their cytoplasm and nucleus considerably increased in electron density compared with the tissue below (Fig. 3a). The number of cells and the prominence of the nucleus and nucleolus in each of these necrotic cells indicated that they had undergone cell division before dying. Examination of the necrotic epidermal cells shows an accumulation of OPDs along the plasmalemma and in the cell walls (Fig. 3b). No bacterial invasion of the tissue was detected through serial sections of five samples.

Genetic analysis of strain ANU2861. Hybridization analysis showed that a single copy of Tn5 is inserted into a 12-kb *EcoRI* fragment in strain ANU2861. This 12-kb DNA fragment was cloned and subsequently used as a hybridization probe to clone the corresponding wild-type 6.3-kb fragment into plasmid pWB5a (pSD1). Both orientations of the cloned fragment were obtained, and both were used in all manipulations of pSD1. In all of the transconjugants tested, the introduction of pSD1 into strain ANU2861 corrected the *ade* auxotrophy and restored both the wild-type colony morphology (Fig. 4) and effective nodulation ability on siratro (Fig. 5). This demonstrates that all the coding and regulatory sequences which impart the ANU2861 phenotype are encoded on this fragment. All bacteria reisolated from nitrogen-fixing nodules were tetracycline and kanamycin resistant, indicating that the introduced plasmid (pSD1) was stable even after a passage through nodules. A 1.3-kb *HindIII*-*ClaI* fragment spanning the region of Tn5 insertion (pSD2), cloned from the parental strain ANU280, was introduced into strain ANU2861. Replacement by marker exchange of the internal 1.3-kb *HindIII*-*ClaI* fragment with the fragment from the parent strain restored all the phenotypic characteristics of the mutant strain (results not shown). These experiments clearly demonstrated that the Tn5 insertion in strain ANU2861 was responsible for all the phenotypic defects of this strain.

EPS production by mutant ANU2861. Strain ANU2861

forms highly mucoid, translucent colonies on rich media, unlike those formed by the parent strain ANU280, which are white and less mucoid (Fig. 6). Different media were used to contrast the colony morphologies of the parent strain ANU280 and the mutant strain ANU2861. First, strain ANU2861 (which requires *ade* for growth) cannot grow on liquid minimal medium, but produces slow-forming, small translucent colonies on MM medium. Strain ANU280 forms white, mucoid colonies on MM medium. Mannitol was used as a carbon source in MM medium to promote polysaccharide production in the parent strain ANU280, thus enhancing the contrast between the parental and mutant strain colony morphologies. Since a role for EPS and associated capsular material has been inferred in several pathogenic bacterial-plant interactions (1, 2, 7, 18, 29, 36), we investigated the possibility that strain ANU2861 produces an altered EPS structure compared with that produced by the parent strain ANU280. A thorough structural analysis of the EPSs and the oligosaccharide repeat units produced by both parent strain ANU280 and mutant strain ANU2861 showed that these molecules were identical to those previously published for ANU280 (12, 13) under the conditions used (data not shown).

Although strain ANU2861 produces an identical acidic EPS to that of the parent strain, ANU280, further analysis has shown that it produces reduced levels of β -D-1,2-glucans (J. Redmond, personal communication). Since mutants of *A. tumefaciens* and *R. meliloti* defective in the production of β -D-1,2-glucans are, respectively, avirulent and noninvasive (16, 21, 33), we investigated the possibility that the gene mutated in strain ANU2861 was homologous to *chvA* or *chvB* of *A. tumefaciens*. The 6.3-kb *EcoRI* fragment containing the insertion site of Tn5 in strain ANU2861 was used as a hybridization probe to plasmid pCD523 (which codes for the *A. tumefaciens chvA* and *chvB* genes). No hybridization was observed (hybridization at 55°C in 3× SSC) to pCD523 DNA. The introduction of pCD523 into strain ANU2861 failed to restore a wild-type colony morphology or the ability to nodulate siratro, suggesting that either little or no homology exists between these loci.

Strain ANU2861 induces a systemic plant response which affects nodulation by the wild-type strain. By using split-root plants, it has been shown recently for clovers that infectivity of a rootlet inoculated with one strain can be affected by inoculation elsewhere on the plant (37). A split-root assay for siratro was developed to see whether the localized necrotic response generated at, and around, the site of infection of strain ANU2861 could prevent or inhibit the successful nodulation of the wild-type strain ANU280 on the second rootlet. A marked decrease was observed in the ability of the wild-type strain ANU280 to successfully nodulate siratro (Fig. 7c) 24 to 48 h following the inoculation of strain ANU2861. The nodules formed were small, lacked pigmentation, and were reduced in number (approximately 50% fewer than on rootlets inoculated with strain ANU280).

FIG. 1. (a) Light micrograph (longitudinal section) of a representative infection site of strain ANU2861 on *M. atropurpureum* 24 h postinoculation. The bracket outline is the approximate area of panel b. In this particular infection, the hair has curled parallel to the root surface. Stars mark the curled and the basal part of the infected hair cell. Rhizobia on the surface of the root are indicated by small arrowheads, and the nucleus is indicated by a large arrowhead. Magnification, $\times 900$; bar, 35 μ m. (b) Transmission electron micrograph of the penetration site shown in panel a. The apparent three cells are all part of the single infected cell. Note the presence of OPDs on the outer surface of the plasma membrane (small arrowheads) and tonoplast (large arrowheads) and occasionally parts of the endoplasmic reticulum (double arrowheads). The OPDs accumulate particularly around the site of the penetrating *Rhizobium* cell (R), which appears to have been pushing into the cell at the time of fixation. Infected cells such as this example had small amounts of cytoplasm at the periphery of the cell and around the nucleus. The lack of clearly defined membrane within the cell suggests that the cytoplasm is in a state of degeneration. Abbreviations: hcw, hair cell wall; bcw, wall of basal part of hair cell. Magnification, $\times 12,000$; bar, 2 μ m.

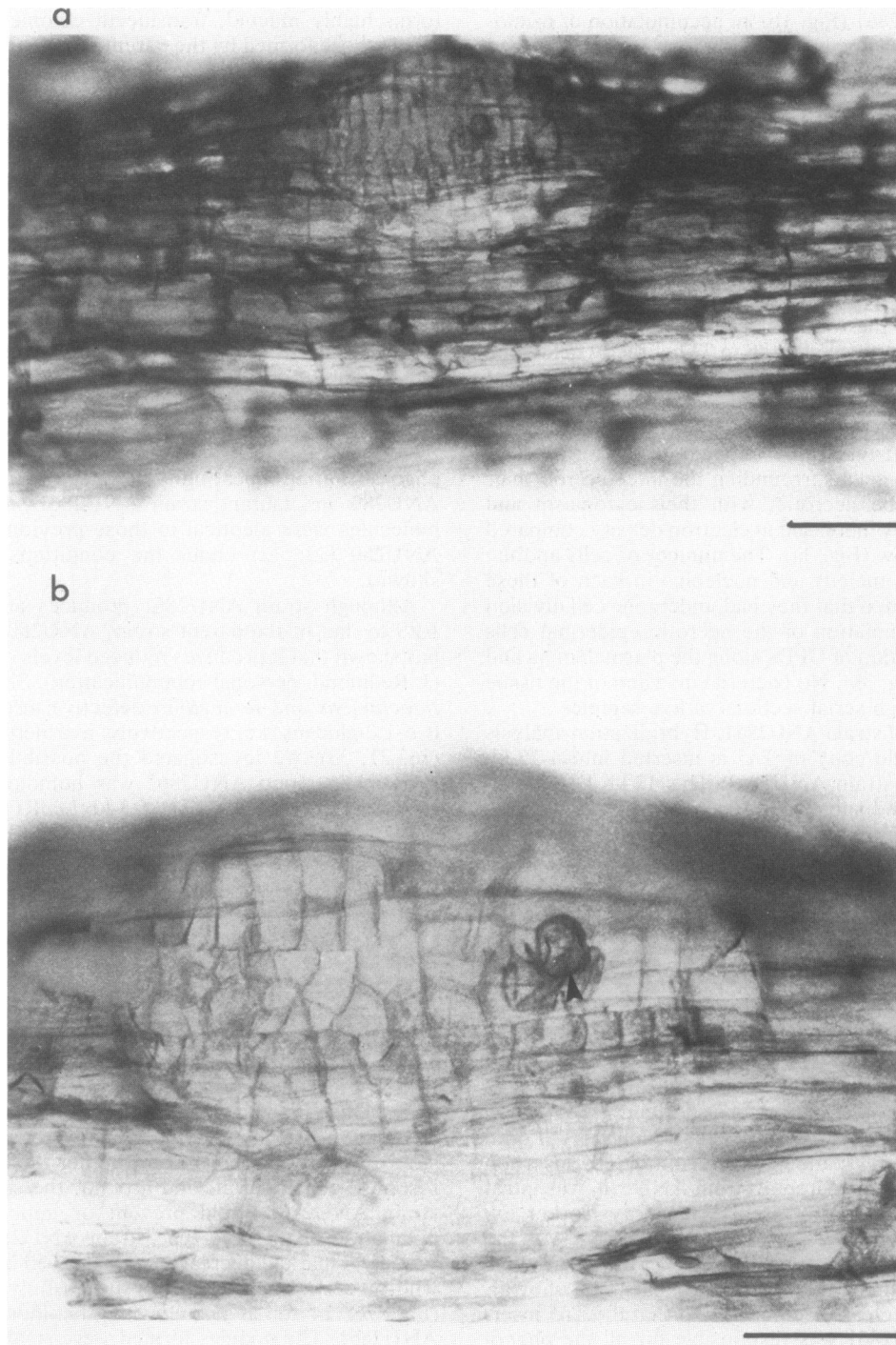
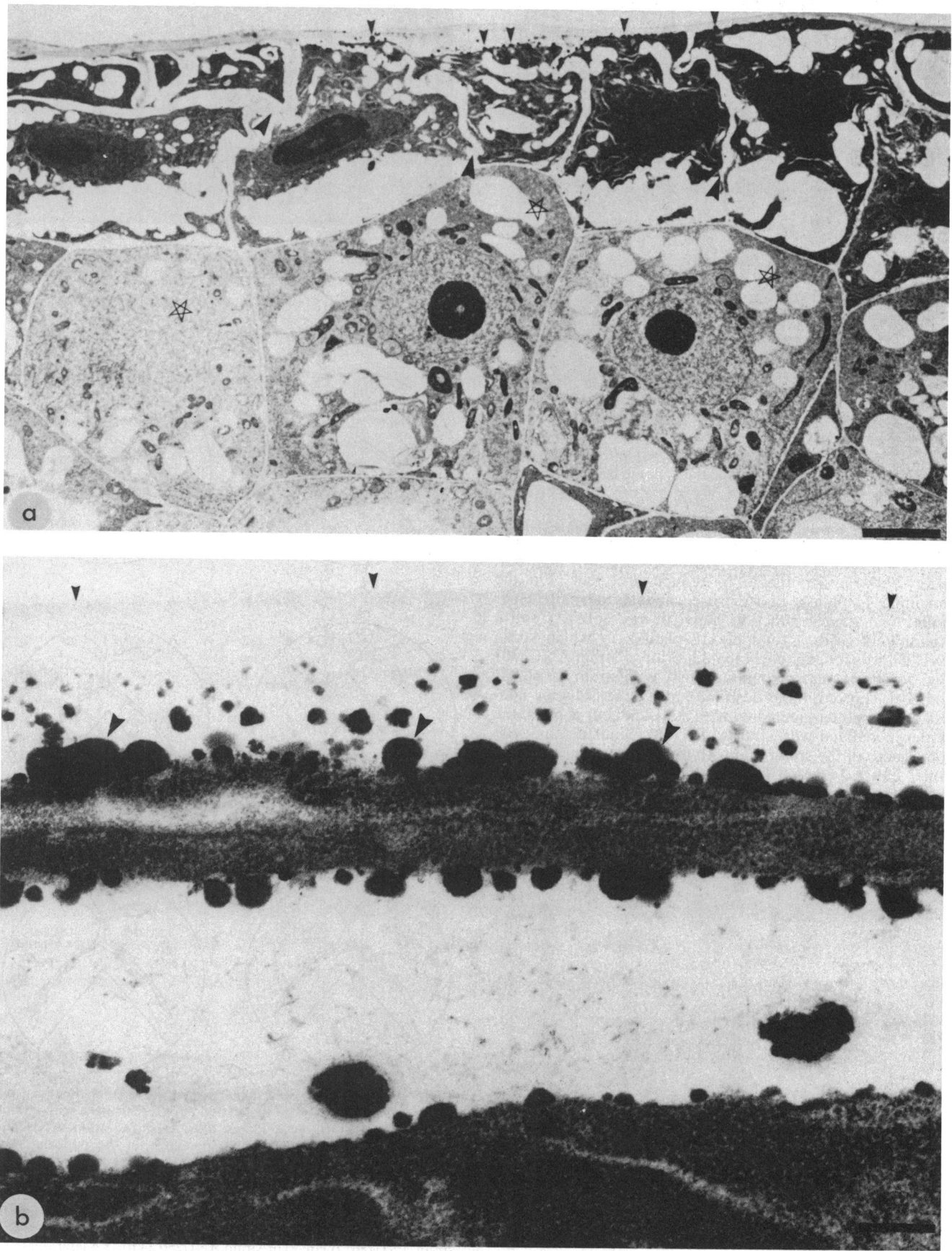


FIG. 2. Example of an area of cell division caused by strain ANU2861 48 to 72 h postinoculation. The infected root hair is indicated by an arrowhead. (a) Magnification, $\times 100$; bar, 200 μm ; (b) magnification, $\times 260$; bar, 100 μm .

FIG. 3. (a) Low-magnification transmission electron microscope view of the central part of cell divisions induced by strain ANU2861 48 h postinoculation. The epidermal cells (uppermost cells in micrograph) appear to have undergone cell division, but were in the process of necrosis at the time of fixation, as judged by the densely staining nature of the cytoplasm. This could be due to the pressure of cortical cell division within the root, since the epidermal walls are quite distorted (large arrowheads). OPDs accumulated mostly in the outer walls of the epidermis (small arrowheads). The cortical cells (stars) appear as normal (dividing) tissue. Magnification, $\times 3,750$; bar, 4 μm . (b) High-magnification transmission electron microscope view of the epidermal wall near the infected root hair. OPDs (large arrowheads) accumulated in the cell wall, mostly next to the plasma membrane. The root surface is indicated by small arrow heads. Magnification, $\times 70,000$; bar, 200 μm .



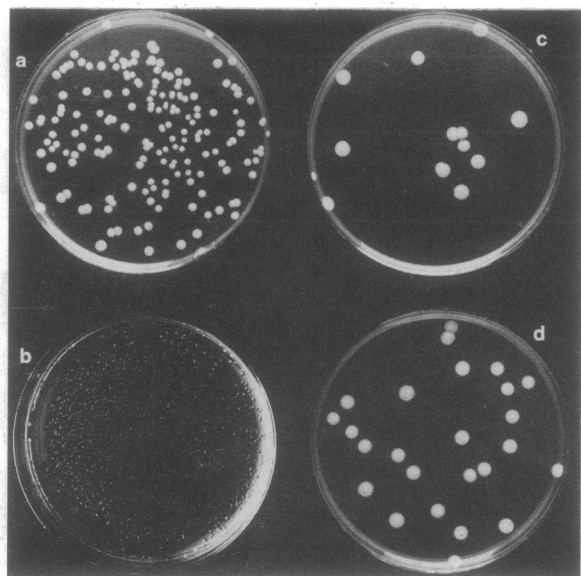


FIG. 4. Colony morphologies of strain NGR234 on MM medium. (a) Parent strain ANU280. (b) Mutant strain ANU2861. (c) Strain ANU2861 carrying pSD1. (d) Strain ANU2861 carrying pSD1 with the 6.3-kb *EcoRI* fragment cloned in the opposite orientation to that in panel C.

These experiments were carried out as three separate trials involving a total of 51 plants. Of these, 37 plants (approximately 70%) were unable to reduce acetylene. On the remaining 14 plants, two or three nodules developed per rootlet and some reduction activity was noted. Control experiments (with a total of 118 plants), in which strain ANU280 was inoculated 24 to 48 h prior to strain ANU2861 (Fig. 7e) or inoculated simultaneously with strain ANU2861 (Fig. 7b), showed numerous large, acetylene-reducing nodules on the rootlet inoculated with ANU280, but none on the rootlet inoculated with strain ANU2861. Similarly, inoculation of both of the split roots with the parent strain ANU280 alone, either 24 or 48 h apart, resulted in nitrogen-fixing nodules on both root systems (Fig. 7f).

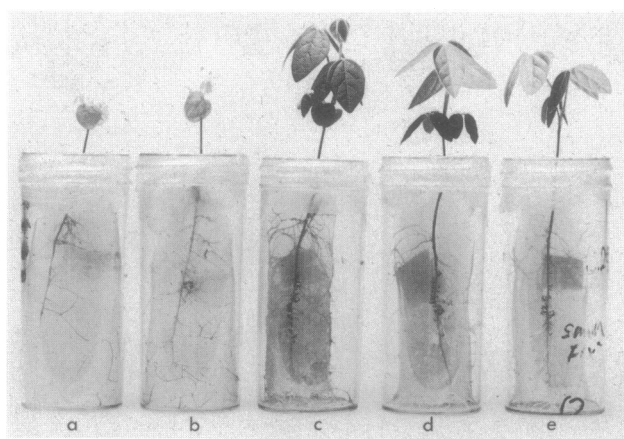


FIG. 5. Siratro nodulation assay. (a) Uninoculated siratro plant. (b) Siratro inoculated with mutant strain ANU2861. (c and d) Siratro inoculated with strain ANU2861 carrying pSD1. The 6.3-kb fragment is cloned in both orientations. (e) Siratro inoculated with wild-type strain ANU280.

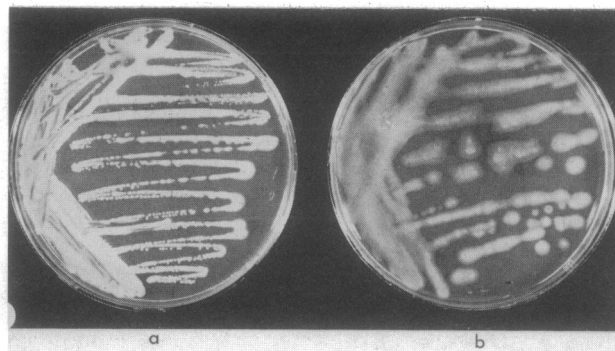


FIG. 6. Colony morphologies of parent strain ANU280 (a) and mutant ANU2861 (b) grown for 5 days on BMM-agar medium.

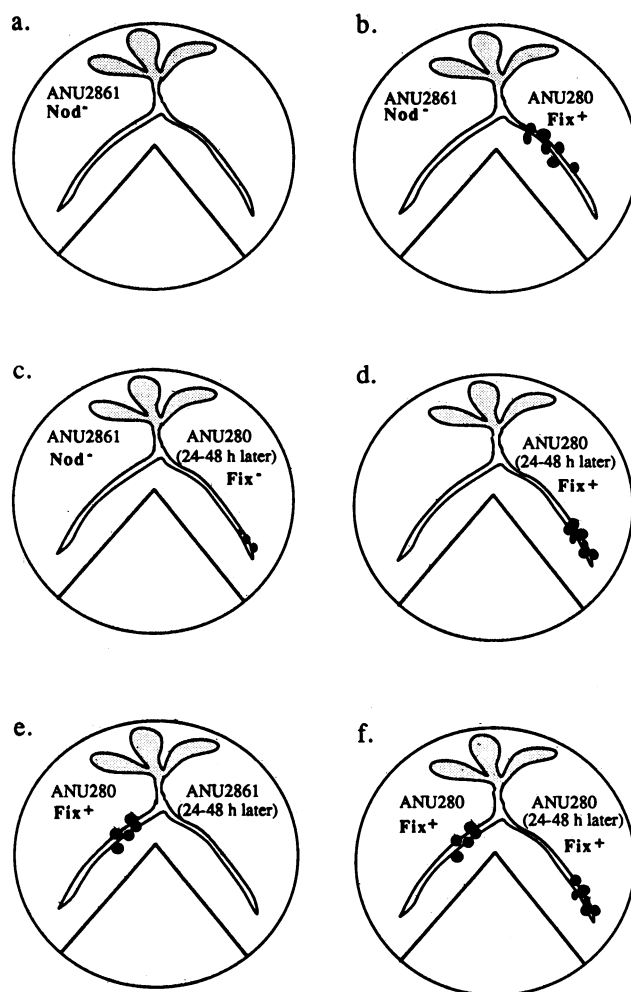


FIG. 7. Split-root experiments with siratro plants. Plates a to f represent split-root siratro inoculated at different times with strains ANU280 and ANU2861. Plates a to c show strain ANU2861 inoculated at zero time with no inoculum on the opposite root system (plate a), strain ANU280 inoculated simultaneously (plate b), and strain ANU280 inoculated 24 to 48 h later (plate c). Plate d shows strain ANU280 inoculated with a 24 to 48 h delay with no inoculum on the the opposite rootlet. Plates e and f show strain ANU280 inoculated at zero time with subsequent inoculation with strain ANU2861 (plate e) or strain ANU280 24 to 48 h later (plate f).

DISCUSSION

The effect of the ANU2861 mutation on nodulation ability has been shown to vary for a range of legume hosts (5). Similar results were recently reported for a *Bradyrhizobium* sp. (*Arachis*) strain NC92 Tn5 mutant (45). A more detailed analysis of the Nod⁻ phenotype on siratro plants showed that mutant strain ANU2861 caused root hair curling (Hac⁺ phenotype) and appeared to degrade the root hair wall at a region in the curled hair where penetration would normally occur with the parent strain NGR234. However, penetration of root hairs did not occur, and the mutant strain failed to pass through the wall to the plasma membrane. As a result, mutant strain ANU2861 failed to induce an infection thread (Inf⁻ phenotype). A careful examination of electron micrographs of the root hairs showed that strain ANU2861 elicits a response from the plant similar to that seen with incompatible host-pathogen interactions. Evidence for this was the rapid formation of OPDs in the infected cell (but not those one cell layer deeper in the plant tissue) and the apparently rapid death of the infected cell and eventually those adjacent to the infected epidermal cell. This plant reaction occurs between 15 and 24 h after inoculation. The response induced by strain ANU2861 on siratro plants is microscopically similar to the hypersensitive reaction (HR) induced by avirulent strains of *Pseudomonas solanacearum* on tobacco plants (40). These OPDs had already accumulated after 24 h of exposure of strain ANU2861 to siratro plants. Osmium tetroxide reacts primarily with unsaturated lipids, but also reacts with phenolic, hydroxyl, carboxyl, amino, and heterocyclic groups (20). Phenolic compounds and phytoalexins are commonly induced in pathogen-plant relationships (22, 25) and may be the compounds present in these OPDs, although the possibility of other osmiophilic compounds cannot be excluded. Since the nucleus failed to migrate to the infection site, this may preclude any of the host cell-bacterial cell communication that is necessary for continued infection. Stimulation of cortical cell division, induced by mutant strain ANU2861, without any obvious indication of entry into a root hair and with no infection thread formation, suggests that the bacterial signals responsible can be transmitted into the root cortex without invasion. After 48 h of exposure to strain ANU2861, the presence of OPDs was confined largely to the epidermal layers surrounding the infected root hair cell, and the localization of this response is similar to the HR reported for other systems (14, 23, 41). These responses by the plant are interesting in view of the hypothesis that the *Rhizobium*-legume symbiosis is a controlled disease (11, 43).

Recently, a group of genes that are required for elicitation of hypersensitive necrosis by *Pseudomonas syringae* pv. *phaseolicola* on nonhost plants and on resistant cultivars of the susceptible host have been reported (28). These genes are also required for pathogenicity on the susceptible cultivar, suggesting that the generation of an avirulent response and pathogenicity share one or more steps or components controlled by the bacterial genome. The insertion of a single copy of the transposon Tn5 into the chromosome of NGR234 results in a Nod⁻ phenotype on four species of plants and also results in poor nodulation on several other legumes. It is clear that mutation of the ANU2861 gene results in the induction of an HR-like response in the host plant and that this leads to a loss in virulence (successful nodulation). This is precisely the opposite effect observed with mutations in race specificity genes identified in *P. syringae* pv. *glycinea*. The avirulence genes in these cases are not required for

pathogenicity, but determine avirulence on host cultivars carrying complementary resistance genes (17). Avirulence in these cases is expressed as an HR, and mutations in these avirulence determinants result in the abolition of an HR and compatible infections. Hence, the ANU2861 gene is required for successful penetration and colonization of the plant host, and it is clear that secretions from the plant are unable to correct the defects of this strain. The ANU2861 locus clearly does not fall into the two classes of avirulence loci present in *Pseudomonas* strains.

Resistance to virulent pathogens in many plant species can be induced by preexposing susceptible plants to avirulent or nonpathogenic strains of fungi, viruses, and bacteria (26). Strain ANU2861 appears to be able not only to initiate a rapid HR at, and near, the site of infection, but also to initiate a systemic response in siratro which can prevent the successful infection of the parent strain, ANU280, inoculated at a separate but equally infectible site (the second rootlet). The development of the systemic phenomena requires a preexposure of strain ANU2861 to the plant of between 24 and 48 h, since simultaneous inoculations of strains ANU280 and ANU2861 onto separate rootlets did not result in the repression of the nodulation response of the wild-type strain. This observation is consistent with data obtained in other systems in which the induction of a systemic protection response requires a preexposure period between induction and challenge (26). It has been suggested that the ability of a plant to recognize an infectious agent and evoke a successful resistance response depends upon the speed and magnitude at which the genetic information of the plant is expressed (8, 26). The lack of repression of the nodulation response of ANU280 by ANU2861 when inoculated simultaneously may suggest that ANU280 represses the induction of the HR-like response normally induced by strain ANU2861 infections. Alternatively, this result could indicate that it is only the initiation of *Rhizobium* infection which is blocked by the plant response to strain ANU2861 and that this response is mounted only after infection has begun.

The insertion of Tn5 in strain ANU2861 has clear pleiotropic effects including *ade* auxotrophy. Adenine mutants of *R. leguminosarum* and *R. phaseoli* are phenotypically similar to ANU2861 in that they are all symbiotically defective and are affected in the early stages of nodule development (31, 44) and the addition of nutritional requirements did not restore nodulation (A. J. Diebold and K. D. Noel, personal communication).

Mutants defective in the early stages of the infection process are valuable biological tools for studying the interactions between invading rhizobia and legume root cells. The molecular analysis of strain ANU2861 and the restoration of the wild-type nodulation ability to mutant ANU2861 by the introduction of cloned wild-type DNA sequences clearly demonstrate that the defect induced by the original Tn5 insertion was responsible for the nodulation-defective phenotype of mutant ANU2861. However, elicitation of the deposition of electron-dense substances by the plant in response to infection by strain ANU2861 suggests that a wild-type *Rhizobium* strain is normally able to override or avoid stimulation of such a plant response. A simple explanation of the mutant phenotype on siratro would be that the ability of strain ANU2861 to initiate an infection thread is blocked. Alternatively, the gene(s) encoded on the 6.3-kb *EcoRI* fragment isolated from wild-type strain ANU280 could be involved (directly or indirectly) in the ability of *Rhizobium* strains to avoid recognition by the plant host,

since the introduction of this region into mutant strain ANU2861 restores the ability to effectively nodulate siratro plants. Mutation of the *chvA* and *chvB* loci of *A. tumefaciens* and the *ndv* loci of *Rhizobium* strains yield phenotypic responses similar to that seen with strain ANU2861. However, the failure of the 6.3-kb region to hybridize to pCD523 shows that the genes are different. It is possible that the pSD1 genes act together with, or regulate, *ndv* loci or that the molecular bases for the two phenotypes are dissimilar. Another possibility is that the mutation of these genes results in a range of pleiotropic effects (including adenine auxotrophy) which then result in the inability of the mutant to elaborate factor(s) which normally repress the ability of the plant to initiate an HR. Whatever the real explanation is, the use of such bacterial mutants will clearly assist investigation of the host plant recognition and defense systems.

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