Nodulation of Soybean by a Transposon-Mutant of Rhizobium fredii USDA257 Is Subject to Competitive Nodulation Blocking by Other Rhizobia

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ABSTRACT
Rhizobium fredii USDA257 fails to nodulate the improved soybean [Glycine max (L.) Merr.] cultivar McCall in plastic growth pouches. Mutant 257DH4, which was derived from USDA257 by transposon mutagenesis, forms nitrogen fixing nodules under these conditions. If USDA257 is present in inocula containing the mutant, most infections are arrested prior to organization of the nodule meristem, and nodule number is reduced by 95%. The improved cultivars Essex, Harosoy, Hodgson 78, and Viçoja, as well as a supernodulating mutant of Williams, respond like McCall to inoculation with such mixtures of bacteria. Nodulation blocking on McCall can be elicited by rhizobia other than USDA257, provided that they meet two criteria: Blocking strains must themselves be able to induce cortical cells of McCall to divide, and such divisions must proceed to the stage of nodule meristem formation. Nodulation by the mutant remains sensitive to a challenge inoculation with USDA257 for only the first 6 to 12 hours after inoculation. Nodulation blocking involving mutant 257DH4 thus appears to be a rapid, generalized process.

Response is sustained and nitrogen fixing nodules result (14). The mutant and parental strain both nodulate primitive soybean cultivars such as Peking, as well as several other legume species, including cowpea (Vigna unguiculata [L.] Walp.) and siratro (Macroptilium atropurpureum [DC] Urb.). The Tn5 insertion thus endows the mutant with the capacity to nodulate agronomically advanced soybean cultivars but does not disrupt its baseline nodulating ability.

Nodulation of McCall soybean by the mutant, however, is acutely sensitive to the presence of the parental strain. Thus, addition of USDA257 to inocula containing 257DH4 can reduce nodule number by 95% or more, for reasons that are not yet clear (4). This nodulation blocking bears close resemblance to that involving European strain PF2 of R. leguminosarum bv. viciae, Asian strain TOM, and the garden pea, Pisum sativum L. (16, 18). In the pea system, nonnodulating strain PF2 functions as the blocker, and nodulation by strain TOM is inhibited. Although the genes that endow strain PF2 with the ability to block nodulation have been isolated (8, 9), the physiological basis of the response remains, as it does in soybean, poorly understood. Here we describe the results of a series of experiments designed to characterize nodulation blocking in soybean. We show that this process is operative with cultivars other than McCall, that other rhizobia can substitute for USDA257 as blockers, and that nodulation by 257DH4 becomes insensitive to USDA257 within 6 to 12 h after inoculation. Blocking activity is associated with the capacity of a strain to induce foci of cortical cell divisions and nodule meristems in the host plant.

MATERIALS AND METHODS

Biological Materials
Soybean seeds were from the following sources: Evans, Hodgson 78, and McCall from D. A. Whited, North Dakota State University; Viçoja from K. Hinson, University of Florida; Harosoy from J. Paxton, University of Illinois; supernodulating Williams from J. Harper, University of Illinois.
Rhizobium fredii strains USDA193, USDA208, and USDA257 were from the Rhizobium collection of the U.S. Department of Agriculture, Beltsville, MD. Rhizobium sp. NGR234 was from W. J. Broughton, University of Geneva, Switzerland. A sym plasmid-cured derivative of USDA193, designated IA728 (23), was from S. Shantharam, Iowa State University. Strain MO728 was constructed by transferring a mobilizable derivative of the sym plasmid of USDA257.

1 Underwritten by grant No. 88-37234-4101 from the U.S. Department of Agriculture. Pedro Balatti was supported by the Consejo Nacional de Investigaciones Científicas y Técnicas de la Republica Argentina. This is Journal Series No. 11293 of the Missouri Agricultural Experiment Station.
assay for competitive nodulation blocking

The basic procedure for measuring nodulation blocking has been described elsewhere (4). Briefly, bacterial cells are harvested by centrifugation from liquid cultures, suspended in sterile PBS, and mixed to achieve concentrations of 10^8 cells of strain USDA257 and 10^6 cells of mutant 257DH4 (or a substitute strain to be tested for blocking activity) per mL. Sets of pregerminated soybean seedlings are transferred to autoclaved plastic growth pouches and inoculated by dispensing 0.25 mL of the mixture onto the root of each plant. Nodulation is assessed visually between 8 and 20 d after inoculation.

For delayed inoculation experiments, the above procedure was modified so that the test bacteria were suspended separately in PBS to the desired cell concentrations. Each root was inoculated with 257DH4 at time 0. Roots were inoculated immediately with mutant 257DH4 (Time 0 treatment) or after intervals of 6, 12, or 18 h. The position of the root tip at the time of the second inoculation was marked on the surface of the pouch. The root tip mark served as a point of reference to differentiate nodules that formed on preexisting tissues (above the mark) or on tissues that formed after inoculation with the blocking strain (below the mark). Sterile PBS replaced the mutant strain in controls. Nodulation was assessed visually as above, and the nodule position relative to that of the root tip mark was noted. In some experiments, the order of addition of the strains was reversed.

Cytological Analysis

Sets of McCall seedlings were inoculated with mixtures of bacteria, and the position of each primary root tip marked at the time of inoculation. Segments of primary root extending from 1 mm above to 9 mm below this mark were harvested after 5 d and processed as described previously (4). Serial, 15-μm longitudinal sections were examined by light microscopy and host cellular responses categorized according to the system of Calvert et al. (3; see legend of Table I for details).

RESULTS

Developmental Analysis of Blocking

Inoculation of McCall soybeans with suspensions containing 10^6 cells of mutant 257DH4 per mL results in 6.2 ± 0.6 nodules per plant after incubation for 20 d in plastic growth pouches. If the 257DH4 inoculum also contains strain USDA257 at 10^6 cells per mL, the average nodule number decreases by 95%, to 0.4 ± 0.2 per plant. This nodulation blocking effect is independent of the absolute number of cells in the inoculum. Thus, blocking of nodulation still is evident when the numbers of mutant: parent cells in the inoculum are reduced to 10^1:10^8 (91% decrease in nodule number) or 10^6:10^8 (88% decrease in nodule number).

Cells of USDA257 block nodulation by arresting development of 257DH4 nodules at an early stage, which corresponds to that at which infections due to USDA257 itself are arrested. This was determined by serially sectioning root segments that had been inoculated with either strain alone or with the mixture (Table I). Inoculation with mutant 257DH4 alone induces distinct centers of cortical cell divisions. As expected, approximately 17% of such foci have well-developed nodule meristems within 5 d of inoculation, and some of the incipient nodules are beginning to protrude from the root. Inoculation with USDA257 alone also stimulates cortical cells to divide,
but development ceases at the stage of appearance of rudimentary nodule meristems, e.g. stage IV as defined by Calvert et al. (3). The response of McCall soybean to the bacterial mixture corresponds quite precisely to that of USDA257 alone—nodule meristems remain unorganized, and most foci consist of fewer than 10 dividing cells (Table I).

**Host and Bacterial Specificity of Blocking**

A series of rhizobia incapable of nodulating McCall were tested for their abilities to substitute for USDA257 in blocking nodulation of McCall by mutant 257DH4 (Table II). Three groupings are evident. The first contains all of the wild-type strains of *R. fredii*, as well as a construct harboring the sym plasmid of USDA257 in the chromosomal background of USDA193. These rhizobia elicit the blocking response, and they all can induce foci of cortical cell divisions that resemble those induced by strain USDA257. Five strains that fail to induce the blocking response constitute a second category. Included in this group are two Nod" mutants of USDA257, including one, 257B3, which lacks nodABC (14). Sym plasmid-cured strains with or without a cosmid containing the DH4 gene, and broad host range strain NGR234, also are in this group. These organisms all fail to induce cortical cell divisions.

Nod" mutant 257F3, tested either alone or as a mixture with the other Nod" mutants of USDA257, triggers a hybrid response. There is little if any blocking activity, but mutant 257F3 nevertheless can stimulate cortical cells to divide. Serial sectioning, however, confirms that the development of these meristematic foci is arrested very early relative to those induced by USDA257. Thus, at 5 d after inoculation with 257F3, 53% of the sites remain at stage I, and the remaining 47% have advanced only to stage II. The corresponding values for McCall seedlings inoculated with a mixture of 257B3, 257F3, and 257M5 are 55% stage I and 45% stage II.

Nodulation blocking involving USDA257 and mutant 257DH4 is not limited to interactions with McCall soybean. This was confirmed by examining four additional, unrelated soybean cultivars, none of which is nodulated in growth pouches by parental strain USDA257 (14). Mutant 257DH4 nodulates each, although the numbers of nodules are not as great as those on McCall (Fig. 1). Nodulation of all four cultivars is virtually abolished by the addition of USDA257. Evans and Harosoy are particularly sensitive to nodulation blocking. At 20 d after inoculation with the mixture, more than 80% of the plants of these two cultivars remain free of nodules.

USDA257 is Nod" with a supernodulating mutant of Williams soybean. Mutant 257DH4, however, abundantly no-

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**Table II. Nodulation of McCall Soybean by Mutant 257DH4 in the Presence of Other Rhizobia**

Sets of pregerminated seedlings were transferred to plastic growth pouches and inoculated with bacterial mixtures containing 10⁵ cells of 257DH4 and 10⁶ cells of a competing strain/mL. In one case, designated 257B3/F3/M5, a combination of 257B3 + 257F3 + 257M5 (3.3 × 10⁶ cells of each/mL) was tested. Controls received 257DH4 alone. Nodule numbers were measured 20 d after inoculation, and each experiment was repeated (n = 24–36). The abilities of the competing strains to induce infection foci in roots of McCall soybean was examined microscopically in separate experiments as described in "Materials and Methods" (n = 5).

<table>
<thead>
<tr>
<th>Competing Strain</th>
<th>Mean No. Nodules (±st) per Plant</th>
<th>Reduction</th>
<th>Infection Foci</th>
</tr>
</thead>
<tbody>
<tr>
<td>USDA193</td>
<td>7.6 ± 0.5</td>
<td>96</td>
<td>+</td>
</tr>
<tr>
<td>USDA208</td>
<td>7.8 ± 1.0</td>
<td>95</td>
<td>+</td>
</tr>
<tr>
<td>USDA257</td>
<td>7.6 ± 0.5</td>
<td>95</td>
<td>+</td>
</tr>
<tr>
<td>MO728</td>
<td>6.4 ± 0.4</td>
<td>91</td>
<td>+</td>
</tr>
<tr>
<td>257B3/F3/M5</td>
<td>7.5 ± 1.0</td>
<td>25</td>
<td>+</td>
</tr>
<tr>
<td>257F3</td>
<td>7.3 ± 0.5</td>
<td>16</td>
<td>+</td>
</tr>
<tr>
<td>257M5</td>
<td>7.3 ± 0.5</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>IA728</td>
<td>5.9 ± 0.5</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>IA728 (pFDH401)</td>
<td>6.7 ± 0.7</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>257B3</td>
<td>7.3 ± 0.5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>NGR234</td>
<td>6.6 ± 0.6</td>
<td>-21</td>
<td></td>
</tr>
</tbody>
</table>

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**Figure 1. Nodulation blocking of mutant 257DH4 by parental strain USDA257 on four soybean cultivars.** Bacterial cells were mixed to achieve final concentrations of 10⁵ cells of 257DH4 and 10⁶ cells of USDA257 per mL of PBS (O—O). Control inocula contained 257DH4 cells only (■■■). Sets of plants were inoculated and incubated in growth pouches as described in "Materials and Methods." Ev, Evans; Ha, Harosoy; Ho, Hodgson; Vi, Vicoja. Each experiment was repeated, and the total number of plants per treatment varied from 20 (Evans) to 36 (Vicoja). Standard errors were less than 10% of the means.
Table III. USDA257 Inhibits Nodulation of Supernodulating Williams Soybean by Mutant 257DH4

Sets of pregerminated seedlings were transferred to plastic growth pouches and inoculated with mixtures of strain USDA257 and mutant 257DH4 (10^8 and 10^6 cells/mL, respectively). Controls received 257DH4 only. Nodule numbers were determined on d 8 and on alternate days thereafter until d 20. A total of n = 60 plants per treatment was examined in replicate experiments.

<table>
<thead>
<tr>
<th>Days after Inoculation</th>
<th>Mean No. Nodules (± se) per plant</th>
<th>Reduction %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Mixture</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>4.8 ± 1.0</td>
<td>96</td>
</tr>
<tr>
<td>10</td>
<td>13.2 ± 1.6</td>
<td>92</td>
</tr>
<tr>
<td>12</td>
<td>18.9 ± 1.8</td>
<td>94</td>
</tr>
<tr>
<td>14</td>
<td>19.4 ± 1.9</td>
<td>93</td>
</tr>
<tr>
<td>16</td>
<td>20.6 ± 2.0</td>
<td>93</td>
</tr>
<tr>
<td>18</td>
<td>20.6 ± 2.0</td>
<td>93</td>
</tr>
<tr>
<td>20</td>
<td>20.6 ± 2.0</td>
<td>93</td>
</tr>
</tbody>
</table>

blocks this mutant, and as is the case with normal cultivars, such nodulation is sensitive to the presence of USDA257 (Table III). Nodulation blocking is readily detectable only 8 d after inoculation and is virtually absolute. Although nodulation of controls already is complete by d 16, no new nodules form after this time on plants inoculated with the mixture. The blocking response thus leads to a sustained, constant 92 to 96% reduction in nodulation.

Kinetics and Spatial Analysis of the Blocking Response

The results of dual inoculation experiments, in which the parental strain was withheld for periods of up to 18 h after inoculation with 257DH4, are given in Figure 2. In the absence of a delay, strain USDA257 attenuates nodule number to nearly zero, and the percentage of nodulated plants is reduced from 100% in controls to only 56%. Nodulation is progressively desensitized to blocking as the interval between the first and the challenge inoculation is lengthened. A delay of only 6 h substantially negates the blocking effect: 100% of the plants nodulate in the presence of USDA257, and the mean nodule number per plant is reduced by only about 50%. If the delay is longer, nodule numbers are only slightly less than those in controls. Reversal of the order of addition of the strains, i.e. USDA257 precedes 257DH4, has no effect on nodulation blocking. Mean nodule number per plant is 0.6 ± 0.2 in simultaneously inoculated controls and ranges from 0.3 ± 0.2 to 0 ± 0.2 when 257DH4 is delayed from 6 to 18 h.

The majority of nodules on the primary roots of soybean (1, 19), including those of cultivar McCall (12, 22), ordinarily cluster near the position occupied by the root tip at the time of inoculation, i.e. the root tip mark on the pouch. Most nodules produced by mutant 257DH4 are similarly positioned (data not shown), and strain USDA257 has little effect on the distribution of nodules above and below the mark. Thus, addition of the blocking strain at time 0 reduces the number of nodules above and below the mark by 94 and 89%, respectively. Delays of 6, 12, and 18 h in addition of the blocking strain result in a progressive 45%, 25%, and 0% reduction in nodulation above the mark and a corresponding 75%, 12%, and 32% reduction below the mark. The average number of lateral root nodules is always less than one per plant, and this number is not influenced by the presence of strain USDA257.

**DISCUSSION**

Transposon-mutant 257DH4 originally was selected for its ability to nodulate the agronomically advanced soybean cultivar, McCall, in plastic growth pouches (12, 14). This attribute distinguishes it from the wild-type parental strain USDA257, which fails to form nodules under these conditions. Nodulation of McCall by 257DH4 appears to proceed in the fashion typical of soybean-Rhizobium interactions (4) but is acutely sensitive to the parental strain. This sort of negative, three-way interaction has been termed competitive nodulation blocking (8). Although it is well known in several Rhizobium-legume systems (5, 16, 18), none involves an isogenic pair of organisms such as USDA257 and 257DH4.

We show here that competitive nodulation blocking involving mutant 257DH4 and soybean is to some extent a gener-

![Figure 2. Response of nodulation of McCall soybean by 257DH4 to delayed addition of blocking strain USDA257. Sets of seedlings were transferred to plastic growth pouches and inoculated with 257DH4 (10^6 cells/mL) at time 0. Cells of USDA257 (10^6/mL) were added at this time or with delays of 6, 12, or 18 h. Controls received PBS at these times. Each experiment was repeated, and the total number of plants per treatment was 30. Standard errors were less than 12% of the means.](http://www.plantphysiol.org)
alized process. Thus, any soybean cultivar that can be nodulated by 257DH4 appears to be an adequate host to support the blocking response. Included in this group is a supernodulating mutant of cultivar Williams (10), which is rapidly and extensively nodulated by 257DH4, but is nod- with USDA257. These observations confirm that competitive nodulation blocking is insensitive to the regulatory abnormalities that condition supernodulation. As a consequence, supernodulating Williams should be especially useful for cytological and quantitative experiments, where insufficient amounts of responding tissue often are limiting factors.

Any of a number of *Rhizobium* strains will substitute for USDA257 as the blocker, provided that one critical prerequisite is fulfilled: The blocking strain must be able to induce cell divisions in the host cortex under the conditions of the experiment, and such divisions must be capable of proceeding to the stage of nodule meristem formation. Calvert *et al.* (3) have shown that cortical cell divisions in soybean ordinarily commence within 12 h after inoculation with *Bradyrhizobium japonicum*. Infection threads first appear 12 h later, and nodule meristems are not visible until 36 h after infection (3, 25). The time frame during which nodulation of McCall by 257DH4 remains subject to disruption by USDA257, however, spans only about the first 10 h after inoculation. This period of sensitivity coincides with the earliest meristematic responses of the host to the mutant and suggests that these initial cell divisions are crucial in determining the fate of the interaction, in a fashion somewhat analogous to the autoregulatory response (19). The ability to block seems nevertheless to be contingent on the strain’s inherent capacity to induce a much later host response—the nodule meristem.

Although we do not yet understand how these nuances of host meristematic activity relate to nodulation blocking, there is increasing evidence that sustained division of host cortical cells in response to rhizobia is generally vulnerable to disruption. Thus, cortical cells of some nonnodulating soybean mutants begin to divide under the influence of rhizobia, but then cease prior to infection and nodule meristem formation (17). The basis for this defect is unknown but has been attributed to rates of meristematic activity (17). LeGal and Hobbs (15) recently have shown that development of genetically incompatible combinations between certain pea cultivars and strains of *R. leguminosarum* bv. *viciae*, too, is arrested at the stage of nodule meristem formation. This is particularly interesting, because meristematic activity in this legume is initiated deep within the cortex and not near the surface, as in soybean.

Although competitive nodulation blocking in soybean can involve combinations of a number of advanced cultivars and blocking strains, mutant 257DH4 appears to be singular in its sensitivity to blocking. Thus, wild-type *R. fredii* strain USDA191 is immune to the inhibitory effects of USDA257 (13), even though it is the symbiotic equivalent of 257DH4 (12, 14, 22). This observation tends to rule out several potential explanations for the blocking phenomenon. For instance, it seems unlikely that USDA257 merely triggers some form of generalized host resistance to further invasion by rhizobia. Similarly doubtful are explanations based on occupancy of nodule initiation sites by blocking strains (2) or saturation of the host’s nodulation capacity with unproductive responses to the blocking strain. Although the actual mechanism of blocking remains obscure, the isogenic USDA257/257DH4 couplet offers advantages that we believe will help to resolve the question.

LITERATURE CITED


18. Ohlendorf H (1986) Symbiotic interactions between the effective *Rhizobium leguminosarum* strains 311d and Tom* with strain-specific resistant (311d) pea lines. Angew Bot 60: 40–47