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Mutant Strains of *Rhizobium japonicum* with Increased Ability to Fix Nitrogen for Soybean

Abstract. *A strain of* Rhizobium japonicum *used in commercial inoculants was mutagenized and screened by a rapid effectiveness assay with soybean plants. Two mutant strains nodulated the roots earlier than the wild type and also expressed greater symbiotic nitrogen-fixing activity than the wild type in the presence and absence of fixed nitrogen. In addition, one of the mutants formed more root nodules than the wild type. Plants inoculated with these strains had increased dry weights (~60 percent) and nitrogen content (~100 percent) when grown in growth chambers.*

Mutagenesis is used as a method for obtaining strains of bacteria that synthesize increased levels of microbially produced metabolites. We wished to determine whether mutagenesis of the bacterium *Rhizobium japonicum* would produce strains capable of improving the N_2-fixing root nodule symbiosis between this organism and soybean [*Glycine max* (L.) Merr.]. Current efforts for obtaining more effective *Rhizobium* strains rely on isolating strains from the soil or from nodules of vigorous plants (*1*).

A culture of *R. japonicum* 61A76 (obtained from J. Burton, Nitragin Co., Milwaukee) was selected for this work because it has been used in a commercial inoculum for over a decade. The bacteria were mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and subcultured for many generations in a minimal medium (*2*). The resulting culture was diluted and plated on agar medium. Each colony was tested for symbiotic acetylene reduction by a rapid screening effectiveness assay with 14-day-old vial-grown soybean plants (*3*). Acetylene reduction is an index of N_2 fixation (*4*).

Twenty-five hundred colonies were tested, and two isolates (SM31 and SM35) had significantly greater symbiotic acetylene-reducing activities on 14-day-old plants (cultivar Hodgson) as measured by the effectiveness assay (Table 1). In addition, SM35 formed more nodules than the wild type on soybean roots. The higher activities of SM31 and SM35 (and the greater number of nodules by SM35) also were observed on three other soybean cultivars tested (Corsoy, Chippewa, and Dunn).

Different colony types obtained from a single strain of *Rhizobium* may have different symbiotic properties (*5*). However, SM31 and SM35 had the same colony appearance as the wild type even when dilutions were made in Tween 40 (*5*). Strains SM31 and SM35 had the same growth rate as the wild type in both rich and minimal media and both were lysed by phage specific for the wild type (*2*). The higher activities caused by these strains were not due to resistance to the commercial bleach used for seed sterilization. The plants continued to show higher activities than the wild type when the seeds were sterilized with mercuric chloride (*6*). Three colonies from a diluted culture of the wild type and three colonies from the mutagenized culture were less active than SM 31 and SM35 when tested by the effectiveness assay.

Bacteria were isolated from nodules taken from plants inoculated with either the wild type or one of the two mutant strains. In all cases, the bacteria from the nodules had the same symbiotic properties (tested by the effectiveness assay) as the original strains inoculated on the seeds.

In order to learn more about the nature of this increased acetylene-reducing activity, nodules produced by SM31 and SM35 were picked from the roots and weighed. Total nodule weight per plant was greater with these strains than with the wild type. This wild-type strain of *R. japonicum* is capable of reducing acetylene asymbiotically (*7*) so that it was possible to test for acetylene reduction without the host plant. Using conditions previously described (*2*), we were unable to demonstrate that the two mutants had more asymbiotic activity than the wild type.

Table 1. Comparisons between Hodgson soybeans inoculated with the wild-type and mutant strains. Plants (7, 8, and 14 days old) were grown in plastic bag–covered vials used in the effectiveness assay (*3*). Values are based on a total of 20 plants. The 21-day-old plants had root systems in cellophane pouches (Seed-Pack Growth Pouch; Scientific Products). The remainder of the plant was open to air. Values are the means of four pouches, each containing three plants. The 7-, 8-, 14-, and 21-day-old plants were grown in a plant-growth chamber with an 18-hour photoperiod. Warm and cool fluorescent bulbs and 100-W incandescent bulbs supplied 300 μE m^{-2} sec^{-1}. The plants grown for 38 days were in 7-inch clay pots containing a sterile mixture (equal parts) of vermiculite and perlite with two plants to a pot. These plants were grown in a Biotron growth chamber with a relative humidity of 70 percent, average light intensity of 600 μE m^{-2} sec^{-1} at the top of the pot, and a photoperiod of 16 hours. The day and night temperatures were 28° and 24°C, respectively. The position of the pots in the chamber was changed daily. Each seed was inoculated with approximately 1×10^9 cells at the time of planting. The medium for growth of the plants in the vials was RBN, brought to *p*H 7.0 with NaOH (*3*). This medium, without sucrose, was used in the pouches. The pots were watered with distilled water and an N-free solution described by Evans *et al.* (*14*). The values are means of at least 15 plants.

Measurement	Age of plants (days)	Strain		
		Wild type	SM31	SM35
Active/nodulated plants (No.)	7	0/0	0/9	0/5
	8	0/13	6/18	4/20
C_2H_2 reduced per plant (nmole/hour)	14			
No N		309	530*	623*
10 m*M* KNO_3		44	96*	90*
1m*M* NH_4^+ acetate		66	125*	123*
Mean number of nodules per plant	14			
No N		9.2	12.0	15.6*
1 m*M* NH_4^+ acetate		7.2	9.0	11.7*
Nitrogen increase	21			
Per plant (milligrams)		4.0	4.8	6.3*
Per pouch (micrograms)†		46	59	189‡
Dry weight of plants (grams)	38	1.88	3.26*	2.69‡
Nitrogen per milligram, dry weight (micrograms)	38	22.3	27.5*	28.6*

*With *t*-test analysis values exceed wild type value at 99 percent level of confidence. †Liquid remaining in pouch after the plants were removed. ‡With *t*-test analysis values exceed wild type values at 95 percent level of confidence.

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In many *Rhizobium*-legume symbioses, only 40 to 60 percent of the electron flow to nitrogenase is transferred to N_2, the remainder being lost through evolution of H_2 (*8*). Some of these systems evolve very little H_2 and thus appear to be more efficient in total energy expenditure. It seemed possible, therefore, that SM31 and SM35 were more efficient than the wild type because they evolved less H_2 from the nodule. We found that soybeans inoculated with the mutants and grown for 14 days produced more H_2 than plants inoculated with the wild type. The mutants, therefore, did not produce less H_2 relative to the amount of acetylene reduced. This showed that the mutants were not more efficient because they evolved less H_2.

Addition of fixed N to legumes prevents nodulation and N_2 fixation by *Rhizobium* (*9, 10*). It may be agronomically important to find symbioses that continue to fix N_2 in the presence of high concentrations of fertilizer N (*10, 11*). We tested the mutants and the wild type by the effectiveness assay on plants growing in the presence of ammonium acetate or potassium nitrate. Acetylene reduction was decreased 82 to 86 percent with all three strains in the presence of 10 m*M* KNO_3 (Table 1). However, plants inoculated with SM31 and SM35 and grown in the presence of fixed N still show significantly higher activities than plants inoculated with the wild type. Similar results were seen with plants grown in ammonium acetate. Plants inoculated with SM35 had more nodules than those inoculated with SM31 or the wild type (Table 1). The number of nodules formed by SM35 was decreased in the presence of ammonium acetate; however, this strain still formed more nodules than the wild type under these conditions.

It has been suggested that strains of *Rhizobium* which nodulate early, and thus form the most prominent nodules, should be chosen for use as commercial inoculants (*12*). Because the mutants appeared to have better nodule-forming characteristics than the wild type on 14-day-old plants, we thought that they might be able to produce nodules at an earlier stage of plant growth. Acetylene reduction in the effectiveness assay was performed on 20 plants inoculated with each strain, but the plants were assayed after 7 and 8 days of growth. None of the 7-day-old plants that had been inoculated with the wild type contained nodules (Table 1); however, the mutants were capable of forming nodules at this early stage of plant growth. At 8 days, none of the wild type–inoculated plants showed any activity, whereas some of the plants inoculated with SM31 and SM35 were active. It seems, therefore, that the mutants initiate nodulation earlier than the wild type.

Fig. 1. Hodgson soybean plants inoculated with the wild-type and mutant strains. The plants are 25 days old and were grown under conditions described in Table 1 for 38-day-old plants. The set of pots in the middle were inoculated with the wild type, the pots on the left with SM35, and those on the right with SM31.

The effectiveness assay is performed with vermiculite in the vial and a plastic bag surrounding the plant (*3*). Possibly the higher activities of the mutant-inoculated plants are dependent on these conditions. We grew plants for 21 days in cellophane pouches with the plants emerging open to the air. Kjeldahl nitrogen analyses (*13*) were performed on the dry plant material (including roots). The increase in plant N due to N_2 fixation was then calculated by subtracting from the total plant N, the value obtained from plants inoculated with SM5, a mutant that does not fix N_2 for the plant but does form pink nodules (*2*). The amount of N provided by the symbiosis and incorporated into plant material by the mutants is significantly greater than that found with wild type–inoculated plants (Table 1).

In addition to producing plants containing a greater amount of N, SM35-inoculated plants put more fixed N into the pouch medium surrounding the plant root (Table 1)—the amount in the spent pouch being small compared to that found in the plant. However, this amount is significantly greater for SM35-inoculated plants than for plants inoculated with the wild type or with SM31.

When plants were grown in pots and watered with N-free nutrient solution, we observed that the SM31- and SM35-inoculated plants appeared more vigorous than plants inoculated with the wild type (Fig. 1). The tops of 38-day-old plants were cut off at the base of the stem, and dry weight and Kjeldahl analyses (*13*) were performed on the tops. Table 1 shows that both mutants cause the plant to increase dry weight. A striking increase of 23 percent for SM31 and 28 percent for SM35 in levels of fixed N per milligram of dry plant material also was observed. The mutations causing the phenotypes of SM31 and SM35 seem to be stable, since the effectiveness assay activities of these strains have remained constant even after monthly transfer of stock cultures in rich medium for at least 30 months.

These experiments demonstrate that it may be possible to enhance N_2 fixation in legumes by screening mutagenized cultures of *Rhizobium*. It is hoped that techniques similar to those described here, as well as parallel plant-breeding work, will achieve increased yields of legume crops.

It will be important to determine whether the increased capacity to fix N_2 by plants inoculated with mutants can actually occur under field conditions and whether this increase manifests itself into greater yields of seed (or perhaps, seed with increased protein). Also, the additional problem of competition with indigenous rhizobia must be taken into account.

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Abnormal Auditory Evoked Potentials in Early Infancy Malnutrition

Abstract. *Computer-averaged auditory evoked potentials were found to be abnormal in infants hospitalized because of severe malnutrition (marasmus). They improved as the infants' somatic growth improved during the course of treatment, but were still deviant at the time of discharge from the hospital and at subsequent outpatient follow-up. Abnormalities in evoked potentials may reflect a long-lasting effect of malnutrition on brain function.*

Chronic malnutrition during infancy causes growth failure which is accompanied by many signs and symptoms of developmental delay. There is substantial but still controversial evidence that brain dysfunction caused by early malnutrition may, in part, be irreversible (*1*-*3*). The issue is difficult to resolve because of limitations in the traditional methods for evaluating perceptual and cognitive functions during infancy.

Sensory evoked brain potentials provide measures of cerebral function that are correlated with brain maturation and development (*4*-*6*). They are sensitive to cognitive and perceptual variables (*7*), and they are abnormal in some patients with cerebral dysfunction (*8*). Electroencephalographic (EEG) abnormalities associated with chronic malnutrition have been reported (*9*), and in experimental animals abnormalities of evoked potentials have also been noted (*10*). We have made a systematic study of evoked potentials in human malnutrition, and have found that they are abnormal in malnourished infants. Although the abnormalities improve during nutritional therapy, they continue to be significantly different from the evoked potentials of normal children.

We recorded evoked potentials from 13 boys and 13 girls (3 to 12 months of age) admitted to the nutrition service of a pediatric hospital in Mexico because of severe protein-calorie malnutrition (marasmus). The weight of each infant was less than 60 percent of the expected weight for chronological age. Length was also grossly deviant (*11*). Healthy infants, 25 boys and 21 girls, from the hospital employees' Day Care Center served as control subjects. The mean age and age range for the control subjects matched that of the patients. Children with histories of low birth weight (less than 2500 g) were excluded from both samples (*12*), as were those with neurological dysfunction and congenital abnormalities.

Evoked potentials were obtained from patients (i) shortly after they were admitted to the hospital but after acute metabolic abnormalities and infections had been treated, (ii) at the start of steady weight gain, (iii) when they attained a weight of 5 kg, (iv) just prior to discharge, and (v) at follow-up sessions 2 to 12 months after discharge. Discharge occurred when the patient was in good physical health and had attained a weight-age ratio equal to his or her height-age ratio (*13*). For each control subject we used evoked potentials obtained during one or two recording sessions.

A test session consisted of the presentation of many sets of 100 visual and auditory stimuli during the recording of the EEG (bandpass: 0.7 to 70 Hz) from standard placements (*14*). Averaged evoked potentials for a 1280-msec poststimulus period were derived with the use of a waveform averager (Northern Scientific model 575; sampling rate, one per 5 msec). We report here the results for evoked potentials obtained during unsedated daytime sleep (*15, 16*) in response to 100 clicks (65 dB, sensation level) presented at a rate of one per 2.5 seconds through a loudspeaker and recorded from an electrode at the vertex of the scalp (C_z) referred to joined mastoids. A total of 183 such evoked potentials were collected from the patient group and 68 from the control subjects. Examples are shown in Fig. 1. Latencies and associated amplitudes were measured for the most prominent evoked potential components, that is, N_1, P_2, N_2, and P_3. Approximate latencies of these components were 100, 200, 400, and 700 msec, respectively. In order to quantify the relative normality or abnormality of each evoked potential, an evoked potential index (EPI) was derived. Each EPI was the sum of the absolute values for the deviations (in standard deviations, S.D.'s) from mean normal values of the four peak latencies and corresponding three peak-to-peak amplitudes. Normal means and S.D.'s were available from a previous study of 130 normal children in seven age groups from 0.5 to 36 months of age (*17*). Deviations greater than 2 S.D.'s were scored as 2, resulting in a scale which varied from 0 (all components within 1 S.D. of the normal means) to 14 (all components 2 or more S.D.'s from the means). The evoked potentials in the present study had EPI's that ranged from 0 to 12.

The mean EPI for all evoked potentials from the marasmic subjects was 5.6 ± 2.5. For the control subjects this value was 3.5 ± 1.7. On admission, the mean EPI of the patients was 6.4 (Fig. 2). As a measure after rehabilitation, the

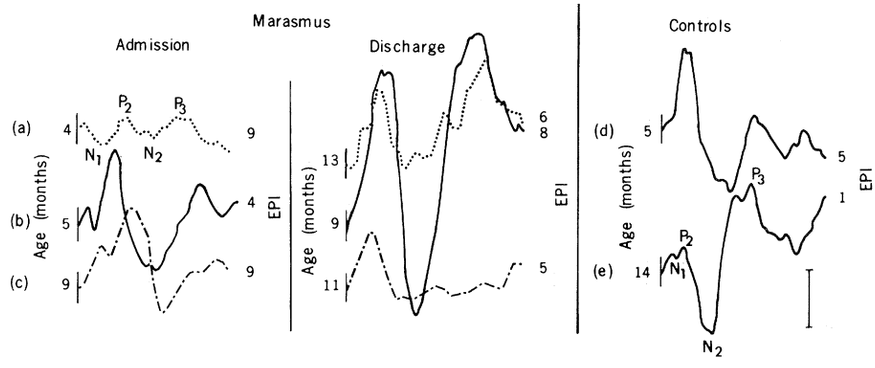

Fig. 1. Averaged evoked potentials to 65-dB clicks (N = 100, interstimulus interval, 2.5 seconds) from three malnourished patients (*a*), (*b*), and (*c*), and two control subjects (*d*) and (*e*). Upward deflection signifies positivity at the vertex (C_z) with respect to a linked mastoid reference. The duration of the tracing is 1 second; the stimulus occurred at the beginning. The calibration, which applies to all the evoked potentials, is 25 μV. The evoked potential index (*EPI*) is a measure of the deviation from normal values (*17*) of the labeled evoked potential components, with a higher score denoting greater deviance.

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