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September 25, 1990

Dr. Christopher Potter, Ph.D.
AAAs Fellow
Office of Agriculture
Bureau for Science and Technology
Agency for International Development
Washington D.C. 20523

Dear Dr. Potter:

Enclosed with this letter is the final report on research project entitled, "Genetic Engineering of Rhizobia for Nodulation Efficiency" (Grant No. DPE-5542-G-ss-6039-00).

This grant was originally awarded to Dr. T.V. Bhuvaneswari but when she left Battelle, Dr. Vesper became the PI of this project. A year later, Dr. Vesper moved to another company and the project was given to me. In this report I have included contributions from the three PI's at Battelle and also from our LDC Collaborator, Dr. M.H. Ahmad of U.W.I. Jamaica.

On behalf of my predecessors, myself, and Dr. Ahmad, I thank AID for sponsoring this interesting research.

Sincerely,

Nasir S.A. Malik
Principal Research Scientist

G-8445-2201

FINAL REPORT

ON THE COLLABORATIVE RESEARCH PROJECT

on

Study Title

GENETIC ENGINEERING OF RHIZOBIA

FOR NODULATION EFFICIENCY

Sponsor

U. S. Agency for
International Development
Grant No. DPE-G-SS-6039-00

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Final report prepared by Dr. N. Malik

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TABLE OF CONTENTS

TITLE PAGE 1
TABLE OF CONTENTS 2
NODULATION EFFICIENCY 3
THE ROLE OF MEGAPLASMID IN THE REGULATION OF NODULATION EFFICIENCY 4
THE CONSTRUCTION OF A GENOMIC LIBRARY 5
BACTERIAL MATING AND TRANSFORMATION 8
SELECTION OF EFFICIENT CLONES 9
CONCLUSIONS 10
RESEARCH WORK AT THE UNIVERSITY OF THE WEST INDIES 10
- Isolation of local strains of B. japonicum 10
- Isolation and characterization of local rhizobial strains for cowpea 11
- Screening for Effectiveness 11
- Genetic Approaches to Improve Nodulation Efficiency 11
  - Mutation by UV irradiation 11
  - Tn 5 Mutagenesis 11

VISIT TO BATTELLE 12
REFERENCES 12

LIST OF TABLES

Table 1. 13
Table 2. 14
Table 3. 15
Table 4. 16
Table 5. 17
Table 6. 18
Table 7. 19
Table 8. 20

LIST OF FIGURES

Figure 1. 21
Figure 2. 22
Figure 3. 23
Figure 4. 24
Figure 5. 25
Figure 6. 26
Figure 7. 27

Nodulation Efficiency

Nodulation efficiency is defined as the ability of rhizobia to produce optimum nodules with the smallest number of cells in the inoculum. During the course of our studies on general aspects of plant-rhizobia interactions, we observed that different strains of rhizobia differ greatly in nodulation efficiency. For example, B. japonicum strains 138, 123, and 110 were 10-50 times more efficient than strains 61A76 and 83. Interestingly, the efficient strains also appeared to be more competitive, particularly at lower concentrations of inocula. Since a number of workers had shown that the success of a superior rhizobial strain in the field depends largely on its competitive abilities, the aspect of nodulation efficiency appeared very attractive for detailed study.

In addition to finding differences in nodule efficiency among different strains, we also found that any natural population of strain 61A76 consists of at least two types of cells which produce distinct types of colonies on yeast extract mannitol gluconate medium (YEMG) and that the type of cells or substrains differed in nodulation efficiency. One type of cell produced translucent and friable colonies while the other type of cell produced slimy compact and dense colonies. We found that while the translucent type colonies always bred true on subculturing, the slimy colonies always produced both types of colonies on subculturing. In fact, nodules produced by inoculating soybean plants with the slimy type of cells contained both types of cells, but nodules produced by inoculation with translucent type cells contained only translucent cells. These observations indicated that the translucent type colonies breed true, but the slimy type cells always produce both types of cells.

Experiments were conducted to study the nodulation behavior of the two types of colonies of 61A76. The inoculum dose response curve of the two subpopulations on soybean roots is given in Figure 1. The results indicated that the translucent type cells were highly inefficient in comparison to their parent cells in nodulating soybean roots. To obtain comparable nodulation in the infectible region by the translucent type cells, an inoculum of 1×10^7

cells per plant was needed. The most infectible region has previously been shown to be the region on the root approximately 1-2 cm above the root tip (Bhuvaneswari et al. 1979).

The results described above indicated that the translucent type cells do not lose any of the genetic information needed to form nodules. In fact, our preliminary results indicated that the nodules produced by translucent type cells contained leghemoglobin and were able to fix nitrogen. Apparently, the only trait lost in the translucent type cells was the ability to nodulate efficiently. Thus, these cells appeared to provide a genetically "clean" material to identify and analyze the factors that determine the efficiency of nodule formation.

The Role of Megaplasmid in the Regulation of Nodulation Efficiency

The strain 61A76 was one of the few strains of B. japonicum that contained plasmid. Although Haugland and Verma (1981) had shown that the megaplasmids of 61A76 did not contain structural genes for nitrogenase, it was not known whether these plasmids contained any other genes required for nodule formation. Initially, therefore, experiments were conducted to determine whether or not the megaplasmid is present in the true breeding translucent type variant of 61A76.

Isolation of megaplasmids from rhizobia is not an easy task, and difficulties have been reported by a number of workers. After several attempts, however, we were able to show that the megaplasmid is present in both variants; the translucent type cells and the slimy cells (Figure 2). These results indicated that the loss of nodulation efficiency in the translucent type variant cannot be attributed to the loss of megaplasmid. However, the possibility that nodulation efficiency genes may still be present on this plasmid could not be ruled out from these results. In the next set of experiments, therefore, attempts were made to cure the strains of the megaplasmids. Heat treatments and exposure to acridine orange were used to remove plasmids from the parent strains.

For heat curing, the bacterial cells were incubated at 37°C for seven days. At the end of the incubation period the cells were diluted and plated on YEM plates. The plates were incubated for another seven to nine days and then colonies were picked at random for testing the presence of megaplasmid. All of the colonies picked showed the presence of megaplasmids.

Acridine orange is routinely used to eliminate autoreplicating extrachromosomal DNA in bacteria. In our experiment, we tested a range of acridine orange concentrations (from 10 μg/ml to 100 μg/ml) to cure the 61A76 strain of B. japonicum. The cells of the wild type of 61A76 were incubated with acridine orange for six days and then plated on YEM plates. All plates showed bacterial colonies. The plates containing cells treated with 50 μg/ml acridine orange were selected for further tests. Colonies were randomly tested for the presence of megaplasmids. Again, megaplasmid was found in all of the colonies tested.

Interestingly, however, cells treated with acridine orange produced only the slimy type of colonies. On further subculturing a number of slimy colonies turned out to be true-breeding. In the past we had been unable to isolate pure cultures of slimy cells. Ten such colonies were randomly selected and tested for nodulation ability. Seven out of ten colonies could not nodulate soybeans. This was an interesting observation, but could not be used to understand the nodulation efficiency.

Construction of Genomic Library

If the loss of nodulation efficiency in translucent type cells was due to the loss of nodulation efficiency genes (Nef genes) during the multiplication process, one could try to identify the lost genes by complementation experiments. It was, therefore, decided to construct a gene bank of efficient slimy type cells and then introduce the cloned DNA fragments into the inefficient type cells, and select for efficient type transconjugants.

Initially, the broad host range cloning vector, PLAFR1, constructed by Friedman et. al. (1982), was chosen for constructing a gene bank from 61A76.

However, this vector carries tetracycline resistance as the selectable marker. Since 61A76 has a natural resistance to tetracycline at 25 μg/ml, a modified PLAFR1 which contained a kanamycin marker (constructed and supplied by Dr. J. Watson at the Division of Microbiology at CSIRO, Australia), was used to construct a gene library of 61A76. B.japonicum 61A76 is sensitive to kanamycin at 10 μg/ml.

Total DNA from the wild type 61A76 strain was extracted and purified according to the procedure described by Cantrell et. al. (1983). The purified DNA was partially digested with EcoR1 and the fragments produced after digestion were sized by centrifugation on an NaCl gradient. The NaCl gradient (0-1.5 M) was prepared in Tris EDTA buffer (TE buffer) at pH 8.0. Fractions from the gradient were collected and small aliquots were taken from each fraction to determine the size of DAN fragments by agarose gel electrophoresis. Fractions containing 25-30 kb fragments were pooled and precipitated for ligation.

The cloning vector PLAFR1 was digested with EcoR1 and then ligated with the pooled fractions of partially digested genomic DNA, containing 25-30 kb fragments, in the presence of bacteriophage T4 ligase. The ligation mixture was then packaged *in vitro* and then used to infect E. coli cells of strain 803. The transformants were then spread on LB plates containing 30 μg/ml of kanamycin. Colonies obtained from a 2.5 μg of ligated DNA were pooled and stored for subsequent use.

The gene bank produced above was stored at -20°C in DMSO. However, at the time when bacterial mating and transformation experiments were to be conducted, the storage vial had very few viable cells. We were unable to grow cultures by using toothpicks scratched on the surface of the frozen cultures as inoculum. The only way we could grow the culture of the gene bank was by taking very large size inoculum (2-3 ml/10 ml culture). Bacteria from the fresh culture was spread on LB plates containing kanamycin. Single colonies from the plates were picked randomly and then subcultured to isolate cosmids and study the insert size. Cosmids from the cultures produced from single colonies were isolated by the method described by Birnbolm and Doly (1979) as shown in Figure 3. The isolated cosmids were digested with EcoR1 and then the

fragments separated on agarose gel. From the size of the fragments it appeared that the clones had large inserts. However, out of the seven clones tested, two had an identical pattern of fragments and the other two had almost identical patterns (Figure 4). These results led us to believe that our original gene bank was far from being complete. An incomplete bank was considered undesirable because it offered lesser chances of finding a desirable clone. It was therefore decided to make a new gene library. This time, we chose to make the library in cosmid vector PLAFR3 instead of PLAFR1. The choice was based on the knowledge that inserts are usually more stable in PLAFR3 versus PLAFR1. Since we did not have an access to kanamycin resistant PLAFR3, we tested the resistance of cosmid containing E. coli and the wild type 61A76 at higher concentration of tetracycline (tet). Our tests showed that at 100-200 μg/ml concentration of tet cosmid containing bacteria survived but wild type 61A76 could not survive. This test indicated that we could use PLAFR3 with a tet selectable marker for our experiments if we use high concentrations of tetracycline in the medium.

To make a new library in PLAFR3, fresh DNA were isolated from 61A76 cultures. The DNA were purified and then partially digested with EcoR1. However, when 20-40 kb fragments were being purified from the partial digests, Dr. Verma informed us that he had made a gene bank of wild type 61A76 in PLAFR3, and that he was willing to give us the library. Dr. Verma had hundreds of clones kept individually in the microtiter plates. These clones were transferred onto replica microtiter plates. Aliquots from each well were subsequently mixed and grown in LB broth containing 100 μg/ml tet. The culture was spread on individual plates and individual colonies were checked for inserts. Minipreps of cosmids were made and after digestion with EcoR1 the pattern of fragments was checked on agarose gel (Figure 5). The results showed that the cosmids had good size inserts, all with different patterns indicating a more complete representation of the chromosome. This library was therefore suitable for transformation experiments.

Bacterial mating and Transformation

Conjugative transfer of cloned cosmids into the translucent type variant of 61A76 was done by the triparental mating system of Ditta et. al. (1980). In a separate set of experiments, both libraries, one made in PLAFR1 and the other made in PLAFR3, were used for the conjugative transfer of cosmids. In experiments involving PLAFR1, kanamycin (30 μg/ml)was used to select transformed rhizobia, and E. coli containing cosmids were eliminated by adding polymyxin at 100 μg/ml in the media; rhizobia has a natural resistance to polymyxin. In experiments involving PLAFR3, the selection of transconjugants was done on LB plates containing 150 μg/ml tet and 100 μg/ml polymyxin.

For triparental mating, E. coli containing cosmids and the helper plasmid (PRK2013), and the translucent variant of 61A76 were grown in liquid cultures, containing the appropriate type and amount of antibiotic, to early log phase. About 1-5 ml proportions of the cultures were sequentially passed through sterile millipore membranes. After the addition of each culture on the membrane, sterile water was passed through the membrane to remove any residual amounts of antibiotics. After addition of the three cultures the millipore membranes were placed on agar plates containing no antibiotics for mating. Mating was allowed to continue for four days at 28°C. After four days of mating, the membranes were transferred to sterile centrifuge tubes containing sterile water. The membranes were shaken and the bacterial suspensions were spread over agar plates containing selection media with appropriate antibiotics. The plates were incubated at 25°C. After ten days of incubation, the plates containing transconjugants started to grow, while there were no colonies on plates where the unconjugated translucent type variant was plated in the presence of high levels of antibiotics (150 μg/ml tet of 50 μg/ml of kanamycin). Also there were no colonies on plates containing polymyxin over which plasmid/cosmid containing E. coli were spread.

Transconjugant colonies from the selection media plates were individually picked with toothpicks and transferred to YEM plates to clearly distinguish between the translucent and slimy colonies. We were expecting that most of the transconjugants would be translucent and only a few opaque would appear

which would be selected for testing nodulation efficiency. However, most of the transconjugants were opaque looking and very few were true translucent colonies. It was therefore not possible to select colonies simply based on colony morphology.

Although the growth of transconjugants on selection media containing high concentrations of antibiotics (tetracycline or kanamycin) indicated the presence of cosmids, we thought it useful to confirm the presence of cosmids in the transformed variant of 61A76. Just like megaplasmid, the isolation of large cosmids from rhizobia is also not easy. The cosmid isolation method we used for E. coli did not work with rhizobia. A few methods were tested and finally a modified method of Currier and Nester (1976) gave a reasonable preparation. Figure 6 shows the presence of large cosmids and megaplasmids in rhizobial transconjugants confirming that during mating cosmids from the E. coli were transferred to rhizobia.

Selection of Efficient Clones

Originally it was thought that selection of efficient clones would be easily made based on colony morphology. However, as indicated above the colonies to transconjugants did not exhibit such a clear difference. The other alternative was to screen clones by actual nodulation assay. This was a formidable job. Attempts were therefore made to see if we could pick some difference in the pattern of soluble proteins between efficient and inefficient variants of 61A76 which we could use for initial screening of clones. For this purpose soluble proteins from natural variants and clones were separated by SDS gel electrophoresis but no apparent differences could be observed (Figure 7). Single dimension SDS gel has its limitations, but screening with 2-D gel electrophoresis didn't seem an easier task either. Screening by actual nodulation assay therefore had to be done. For this purpose, 100-200 clones were sent to our LDC collaborator for screening, but due to lack of soybean seeds and other materials, the screening at UWI could not be done. However, Mr. I. P. Singh, who is a graduate student working with Dr. Ahmed, visited us in August of 1989 and helped in screening clones.

For the *in vivo* nodulation assay, soybean seedlings were grown on agar for two days and then transferred to plastic growth pouches containing Jensen's nutrient solution. The roots of each seedling were individually inoculated with rhizobia suspended in 400 μl of sterile water and a mark was made on the plastic pouch indicating the position of the root tip at the time of inoculation (RT mark). After inoculation the seedlings were placed in a growth chamber maintained at 29°C for a fourteen hour photoperiod. Seven to nine days after inoculation, seedlings were removed from the growth chamber and the presence of nodules on the roots were determined under the microscope. Usually 40-60 replicate plants were used for each clone to obtain a reasonable estimate of nodulation frequency on the roots. Using this technique we screened a number of clones. Table 1 shows representative data from the screening experiments. As can be seen in this table, clone A6 appeared to produce a higher number of nodules. In addition, the maximum number of plants were nodulated when inoculated with clone A6. This clone was therefore retested to see if it consistently produced a higher number of nodules. Again, as shown in Table 2, this clone produced a higher number of nodules as compared to other clones. In addition, clone C11 also looks promising.

Conclusions

Results from our preliminary screening of transconjugants of the translucent type variant of 61A76 show the presence of some promising clones. More stringent testing of nodulation still needs to be done, however. In order to isolate the nodulation efficiency genes, the cloned inserts will have to be isolated and further digested, and then the smaller fragments will be recloned to obtain subclones with smaller inserts.

RESEARCH WORK AT THE UNIVERSITY OF THE WEST INDIES

Isolation of local strains of *B. japonicum*

It was difficult to isolate soybean nodulating rhizobia from the Jamaican soils because soybean (*Glycine max*) is not cultivated in Jamaica. However, soils from different locations in Jamaica were collected and used for growing

soybeans under greenhouse conditions. A few of the plants in one type of the soil did nodulate. Eventually, five strains of native rhizobia were isolated from the soybean nodules. The isolates were tested for nodulation efficiency and no marked differences were found between different isolates (Table 3).

Isolation and Characterization of Local Rhizobial Strains for Cowpea

Fifty strains of cowpea rhizobia were isolated from the Jamaican soils and tested for their ability to nodulate cowpea and soybean roots using plastic growth pouches. Nearly all of the strains nodulated cowpeas (Table 4) but only two of them nodulated soybean (Table 5).

Screening for Effectiveness

The effectiveness of ten selected strains of cowpea to nodulate and fix nitrogen in cowpea, pigeon pea, and peanut was observed. Two of these strains (JRC 23, and JRC 43) showed higher nodulation counts and total biomass of the cowpea plants (Table 6). However, the effectiveness of these two strains was still much lower than the rhizobial strains found in West Africa (Ahmed, et. al. 1981).

Genetic Approaches to Improve Nodulation Efficiency

Mutation by UV irradiation

The two selected cowpea rhizobial strains (JRC 30, and JRC 18) were irradiated with UV and about fifty mutants were isolated. These mutants were screened for nodulation efficiency. None of the mutants tested showed higher nodulation on cowpeas, and three of the mutants actually lost their ability to nodulate cowpeas (Table 7).

Tn 5 Mutagenesis

The two selected strains of cowpea (JRC 30 and JRC 18) were mutagenized by Tn 5. Donor *E. coli* WA 803/p GS9 and recipient rhizobial strains were grown to

late log phase and mixed in 1:5 ratio on millipore membrane filters. The mating was allowed to continue for forty-eight hours at 30°C. Twenty-five transconjugants were selected and studied for nodulation efficiency. One of the mutants lost nodulation ability but the others displayed a normal nodulation pattern (Table 8).

Visit to Battelle

Mr. I. P. Singh, graduate student of Dr. Ahmed, visited Battelle during July and August of 1989. He had good interactions with members of the Biotechnology Department at Battelle and at The Ohio State University and had a useful learning experience. He helped us screen transconjugants of rhizobia, and learned different molecular techniques. He was particularly interested in learning hybridization techniques and did complete work in this area.

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TABLE 1. RELATIVE ABILITY OF DIFFERENT TRANSCONJUGANTS OF TRANSLUCENT TYPE 61A76 VARIANT TO NODULATE SOYBEAN ROOTS

Transconjugant Number	Average Nodule Number				% Plants Nodulated Above RT
	Above Root Tip		Below Root Tip		
A1	0.35	0.08	0.57	0.14	29
A6	0.82	0.12	1.30	0.15	53
A8	0.46	0.09	1.04	0.13	35
C45	0.48	0.09	0.57	0.10	36
C45	0.36	0.08	0.72	0.11	29
C35	0.27	0.07	0.88	0.13	20
A28	0.68	0.09	0.69	0.09	38
A24	0.65	0.11	0.66	0.12	47

+ A set of 50 to 60 replicate plants was used for testing each clone.

TABLE 2. RELATIVE ABILITY OF DIFFERENT TRANSCONJUGANTS OF TRANSLUCENT TYPE 61A76 VARIANT TO NODULATE SOYBEAN ROOTS

Transconjugate Number	Average Nodule Number				% Plants Nodulated ART
	ART		BRT		
A15	0.27	0.08	0.48	0.08	21
C17	0.52	0.12	0.68	0.14	31
C15	0.53	0.11	0.56	0.33	33
A6	0.7	0.13	1.24	0.17	42
C25	0.67	0.14	0.8	0.14	37
C22	0.56	0.11	0.72	0.14	38
A9	0.32	0.11	0.42	0.11	33/19
A10	0.58	0.12	0.24	0.07	31
A7	0.27	0.06	0.56	0.11	24
C11	1.0	0.19	0.77	0.14	55
C11	0.88	0.15	0.46	0.11	44
C50	0.55	0.11	0.82	0.11	38
Parent	.48	.12	1.2	.6	36

- A set of 50 to 60 replicate plants was used for testing each clone.

TABLE 3. RELATIVE NODULATION ABILITY OF BRADYRIHZOBIUM JAPONICUM STRAINS ISOLATED FROM JAMAICA ON THEIR HOSTS SOYBEAN

Strain	% Nodulation Above RT Mark	Average Number of Nodules			
		Above RT Mark		Below RT Mark and Lateral Root	
JRS 1	57	0.77	0.14	1.21	0.22
JRS 5	70	0.82	0.21	2.85	0.42
JRS 8	52	0.61	0.13	1.50	0.32
JRS 13	71	0.73	0.15	2.07	0.25
JRS 2	60	0.60	0.09	1.85	0.18

- Sets of 30-40 replicate plants were used for each strain.

IV

TABLE 4. RELATIVE NODULATION ABILITY OF BRADYHIZOBIUM SPP. STRAINS ISOLATED FROM JAMAICA ON THEIR HOSTS COWPEA

Strain	% Nodulation Above RT Mark	Average Number of Nodules			
		Above RT Mark		Below RT Mark and Lateral Root	
JRC 1	79	1.643	0.35	11.79	1.19
JRC 3	59	0.90	0.27	14.91	2.66
JRC 4	65	0.95	0.27	10.36	1.42
JRC 5	10	zero		10.60	1.92
JRC 7	5	zero		9.63	2.60
JRC 10	75	1.83	0.50	11.27	1.22
JRC 11	5	zero		11.69	1.12
JRC 12	70	0.89	0.30	10.25	1.62
JRC 14	14	zero		17.15	1.68
JRC 18	59	0.90	0.34	17.18	1.88
JRC 19	76	1.40	0.27	10.78	1.58
JRC 23	92	3.11	0.31	10.63	1.99
JRC 29	90	2.92	0.34	12.36	1.67
JRC 30	85	2.54	0.32	9.72	2.59
JRC 31	10	zero		10.32	2.44
JRC 32	5	zero		10.13	0.73
JRC 35	15	zero		13.58	0.90
JRC 36	80	1.84	0.30	10.20	0.99
JRC 37	10	zero		16.42	1.79
JRC 38	79	1.22	0.32	13.22	1.64
JRC 39	60	0.90	0.31	11.78	1.42
JRC 40	90	2.10	0.39	10.40	1.34
JRC 41	5	zero		12.32	1.78
JRC 43	90	3.67	0.33	18.01	1.84
JRC 44	10	zero		11.20	1.64
JRC 45	56	0.77	0.20	12.36	1.18
JRC 47	52	0.61	0.13	14.40	1.98
JRC 48	10	zero		10.20	1.67
JRC 49	5	zero		12.52	1.43
JRC 50	10	zero		11.65	1.23

- Sets of 30-40 replicate plants were used per strain.

TABLE 5. RELATIVE NODULATION ABILITY OF BRADYRIHIZOBIUM JAPONICUM STRAINS ON COWPEA (V. UNGUICULATA)

Strain	% Nodulation Above RT Mark	Average Number of Nodules			
		Above RT Mark		Below RT Mark and Lateral Root	
JRS 5	60	0.84	0.16	2.56	0.38
JRS 8	45	0.56	0.22	1.34	0.29

- Sets of 50 plants were used for each strain.

TABLE 6. EFFECTIVENESS OF COWPEA RHIZOBIA STRAINS ON DIFFERENT HOSTS

Rhizobia Isolates	Pigeonpea (C. cajan)				Peanut (A. hypogea)				Cowpea (V. unguiculata)			
	Nodule No.	Nodule dry wt.	Shoot dry wt.	$N_2(C_2H_2)$ fixed	Nodule No.	Nodule No.	Shoot dry wt.	$N_2(C_2H_2)$ fixed	Nodule No.	Nodule dry wt.	Shoot dry wt.	$N_2(C_2H_2)$ fixed
JRC 1	13.5	18.8	344	2.3	29.5	16.1	646	1.6	10.5	8.6	300	1.3
JRC 3	14.2	8.9	350	4.2	15.3	11.4	520	1.4	14.5	15.0	320	5.4
JRC 14	12.0	9.0	427	6.1	20.0	12.2	600	1.3	12.4	5.2	350	3.9
JRC 18	15.0	6.5	153	2.9	25.0	16.5	529	1.2	15.5	11.7	400	5.9
JRC 19	11.0	8.2	129	2.6	13.0	10.5	686	1.3	11.3	10.1	301	4.8
JRC 23	14.0	13.7	700	4.8	14.0	10.7	840	1.3	28.0	20.0	470	9.6
JRC 29	32.0	20.1	610	6.1	0	-	780	0	13.0	16.0	450	6.3
JRC 30	21.6	8.0	360	3.0	22.0	18.0	670	1.4	10.2	8.7	400	9.2
JRC 31	14.3	7.5	333	2.4	32.0	16.8	650	1.4	13.3	15.0	600	10.1
JRC 43	22.0	7.8	465	6.3	19.5	13.3	644	2.1	30.0	11.0	210	5.2

* μmol (C_2H_4/g nodule/hr).

TABLE 7. EFFECTIVENESS OF UV-IRRADIATED MUTANTS OF COWPEA RHIZOBIA STRAINS JRC 30 AND JRC 18 ON COWPEA (*V. UNGUICULATA*)

Strain	Nodule Number	Nodule dry wt. (mg)
JRC 18-U1	11.2	9.9
JRC 18-U2	14.4	9.6
JRC 18-U5	15.3	12.6
JRC 18-U10	10.0	6.4
JRC 18-U12	10.5	7.9
JRC 30-U3	8.6	6.8
JRC 30-U6	8.9	9.1
JRC 30-U10	10.3	6.5
JRC 30-U15	11.8	7.9
JRC 30-U16	10.0	8.2

TABLE 8. RELATIVE NODULATION ABILITY OF Tn 5 MUTANTS ISOLATED FROM COWPEA RHIZOBIA STRAIN JRC 30 ON THEIR HOST, COWPEA (V. UNGUICULATA)

Strain	% Nodulation Above RT Mark	Average Number of Nodules			
		Above RT Mark		Below RT Mark	
JRC 30	80	2.42	0.29	11.75	1.30
JRC 30-1	70	1.99	0.24	10.38	1.12
JRC 30-2	5	zero		12.22	0.99
JRC 30-5	90	2.27	0.20	12.36	1.41
JRC 30-6	80	2.30	0.21	10.12	1.20
JRC 30-9	80	1.89	0.19	10.78	1.49
JRC 30-10	75	2.01	0.30	11.60	1.20
JRC 30-11	10	zero		14.20	1.76
JRC 30-15	zero	zero		zero	
JRC 30-17	85	2.20	0.41	13.21	1.50
JRC 30-18	89	2.02	0.23	10.98	1.12
JRC 30-20	5	zero		10.20	0.97
JRC 30-21	77	1.09	0.11	9.66	0.90
JRC 30-23	80	2.12	0.29	9.89	0.95
JRC 30-24	75	2.29	0.31	12.27	1.04
JRC 30-25	76	1.90	0.27	11.68	1.29

- Sets of 40-60 plants were used for each mutant.

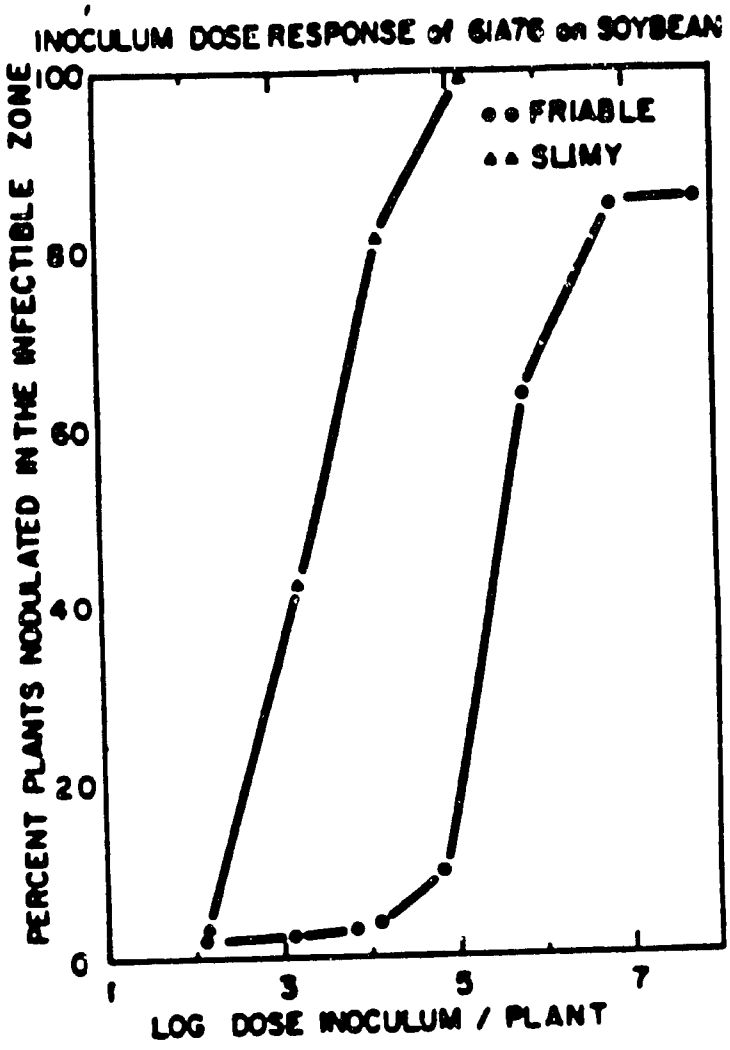

Figure 1. Nodulation Efficiency of R. japonicum Strain 61A76.

Soybean seedlings grown in growth pouches were inoculated with increasing concentration of the inoculum. the region of the root that was infectible at the time of inoculation (approximately 1.5 cm above the growing tip of the root) was marked on the plastic overlaying the root. Nodule development in these marked regions was recorded 1 week after inoculation. Each datum in the figure represents the mean of at least 40-50 plants.

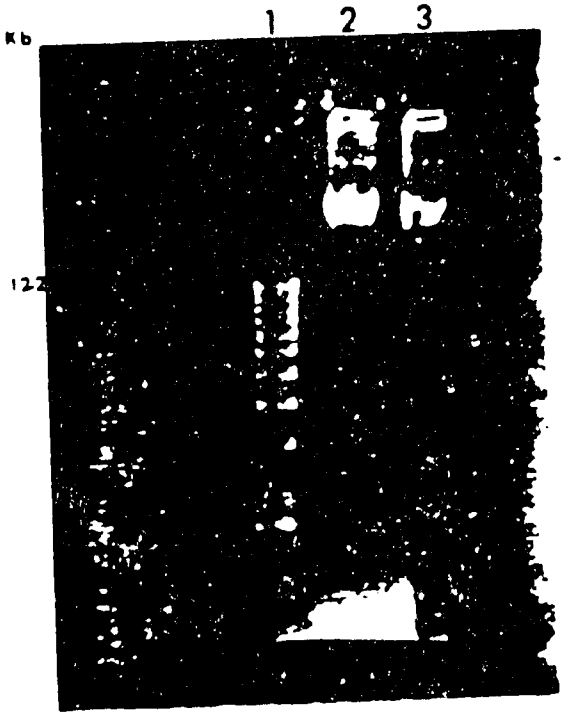

Figure 2. Presence of mega plasmid in both slimy type cells (lane 2), and translucent type cells (lane 3) of 61A76. Lane 1 contains molecular weight standards from 1 kilobase DNA ladder (Life Technologies BRL, Gaithersburg, MD). Mega plasmids are indicated by the arrow.

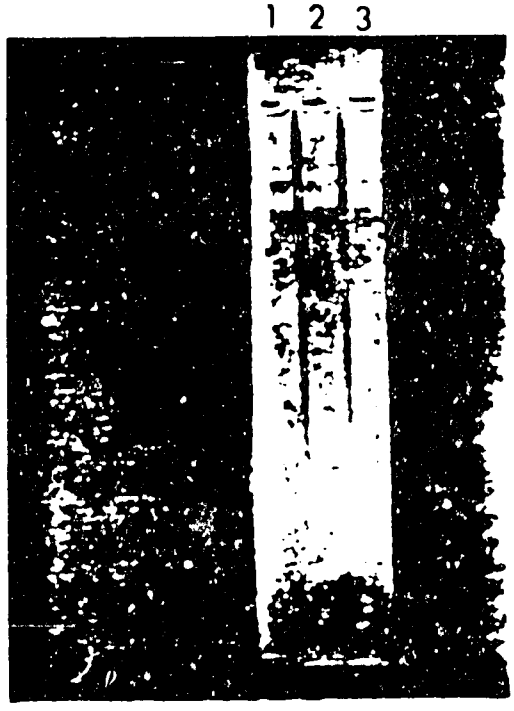

Figure 3. Cosmids from E coli containing gene bank 61A76 in pLAFRI purified by the method described by Birnbolm and Doly*. Samples were run on a 1% agarose gel. Lane – clone #7; Lane 2 – clone #8. Lane 3 – clone #9.

*Birnbolm and Doly. Nucleic Acids Research 7 1515-23 (1979).

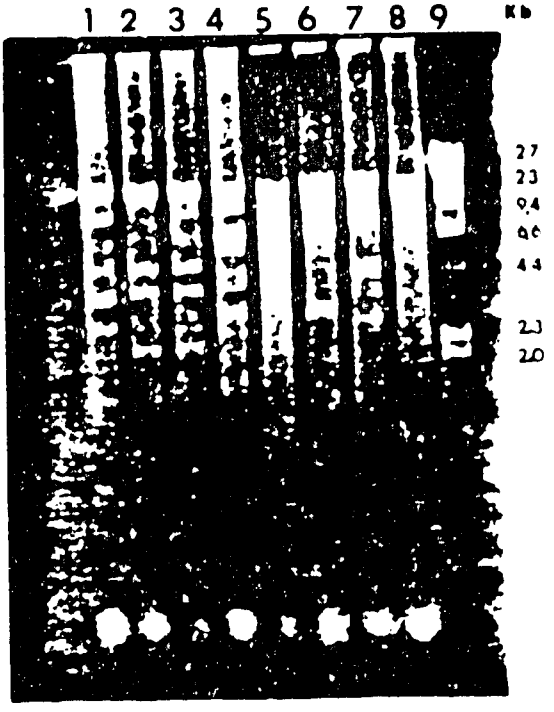

Figure 4. EcoRI digest of the cosmids isolated form E coli containing gene bank 61A76 in pLAFRI. Cosmids were purified by the Birnbolm and Doly method. Restricted DNA was run on a 1% agarose gel. Lanes 1-7 – clone #2, 3, 4, 5, 7, 8, 9, and EcoRI: Lane 8 – pLAFRI + EcoRI: Lane 9 – molecular weight size marker from a Hind III digestion of bacteriophage λ DNA.

25

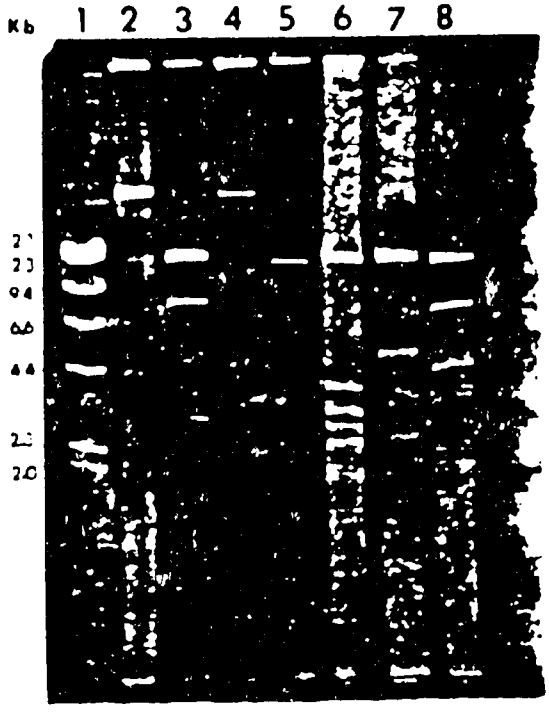

Figure 5. EcoRI digests of the cosmids isolated from randomly selected colonies of E coli containing gene bank 61A76 in pLAFR 3. Cosmids were purified by the Birnbolm and Doly Method. Restricted DNA was run on a 1% agarose gel. Lane 1 – molecular weight size markers from a Hind III digestion of bacteriophage λ DNA; Lane 2 – clone #1, uncut; Lane 3 – clone #1 + EcoRI; Lane 4 – clone #2, uncut; Lane 5 – clone #2 + EcoRI; Lane 6 – clone #3 + EcoRI; Lane 7 – clone #4 + EcoRI; Lane 8 – clone #5 + EcoRI.

Figure 6. The presence of cosmids; arrow B and mega plasmids; arrow A, in transconjugants of 61A76. Arrow C indicates chromosomal DNA.

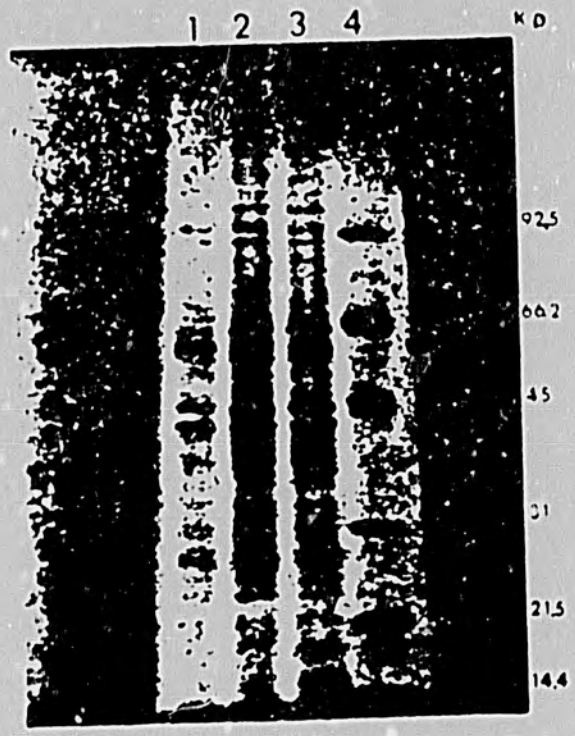

Figure 7. Protein profiles from slimy type cells and translucent type cells were prepared by growing cultures to midlog phase (A_{620} = 0.0. 0.2-0.3), harvesting by centrifugation and sonicating on ice with the microtip probe of a Branson sonicator. Cells were lysed after six 10-second blasts. Lysates were run on a 5%-15% gradient SDS-PAGE with a 3% stack according to Fling*. The gel was stained with Coomassie Blue. Lane 1 and Lane 2 – Translucent type; Lane 3 – slimy type; Lane 4 – low molecular weight markers (BIO-RAD Laboratories, Richmond, CA).

* Steven P. Fling and Dale S. Gregerson, Anal Biochem. 155 83-88 (1986).