PN-ABT-165
91205
2. A. 32

7

FINAL REPORT

GRANT: USAID #DAN-5542-G-SS-2095

TITLE: "A GENETIC ENGINEERING APPROACH TO THE IMPROVEMENT OF RHIZOBIUM FOR TROPICAL LEGUMES

PI: B. BEN BOHLOOL

UNIVERSITY OF HAWAII
COLLEGE OF TROPICAL AGRICULTURE AND HUMAN RESOURCES
HONOLULU, HI 96822

BEST AVAILABLE

FINAL REPORT

GRANT: USAID #DAN-5542-G-SS-2095

TITLE: "A GENETIC ENGINEERING APPROACH TO THE IMPROVEMENT OF RHIZOBIUM FOR TROPICAL LEGUMES"

PI: B. BEN BOHLOOL

GRANTEE INSTITUTION: UNIVERSITY OF HAWAII
COLLEGE OF TROPICAL AGRICULTURE AND HUMAN RESOURCES
HONOLULU, HI 96822

PROJECT OVERVIEW

One of the most pressing agronomic concerns with legume cultivation is the inability of selected inoculant strains to establish in the soil and compete successfully with indigenous rhizobia. This problem is confounded in the tropics where stressful soil conditions, such as high acidity and the presence of toxic levels of aluminum and manganese, affect survival of the inoculum. Alternatively, the indigenous Rhizobium strains are well-adapted to the prevailing stresses found in these soils.

In Rhizobium, tolerance of soil acidity and acidity- related factors (aluminum, manganese, etc.), is a trait that varies markedly from strain to strain. Some strains within the same species cannot withstand the stress, while others have been found to function at levels equivalent, if not higher than those found in most acid tropical soils (Keyser and Munns, 1979a,b,; Date and Halliday, 1979). These highly tolerant strains represent a valuable germplasm to be exploited for genetic manipulation.

In many bacteria, the ability to tolerate high levels of toxic metals and antibiotics is known to be directed by genes carried on extrachromasomal elements known as plasmids (Davies and Smith, 1978; Summers and Silver 1978; Reanny, 1976; Davey and Reanny, 1980). It is also well established that many of these traits can be easily transferred within and between species, even genera, of bacteria (Reanny, 1976; Summers and Silver, 1978). In the fast-growing rhizobia many of the genes encoding host specificity, nodulation and nitrogen fixation are found on large extrachromasomal elements of DNA called Sym(biosis) plasmids -- pSym. Some of these plasmids are self-transmissible and are transferable to other strains of Rhizobium and alter their host range and possibly, even improve their symbiotic properties.

A majority of the DNA of pSyms and the other plasmids found in fast-growing rhizobia is cryptic. That is, what the DNA encodes is unknown. However, as with genes for heavy metal resistance which are located on plasmids, in stress tolerant rhizobia plasmids may harbor genes for aluminum resistance, in addition to those for the symbiosis.

The improvement of the tropical legume/Rhizobium symbiosis will require a multifaceted approach: A) host varieties must be selected that can better withstand the prevailing stresses; B) better symbiotic bacteria must be developed to nodulate the appropriate host and improve its nitrogen nutrition. The goal of this project is to improve the symbiotic performance of the microsymbiont partner -- Rhizobium. I propose to (I) determine the role of the pSym in aluminum resistance and to investigate physiological mechanisms involved in aluminum resistance which could lead to identification of the gene(s) involved (II) to construct stress-resistant rhizobia for alternate host legumes via Sym-plasmid transfer.

INTRODUCTION AND BACKGROUND

The vast majority of the land potentially useful for food and biomass production in the tropics is acidic and therefore subject to the stresses associated with acid soils (Sanchez, 1976; Haug, 1984). Among these stresses are poor air-water relationships, low availability of phosphorus, increased solubilities of aluminum and manganese leading to toxicity, and deficiencies of macro- (Ca^{2+}, Mg^{2+}) and micronutrients (Baker, 1976).

High soil acidity can induce nitrogen deficiency in legumes by preventing normal nodulation (Munns et al., 1981). The severity of the stress depends upon such factors as the concentration of aluminum in solution, the species of legume, and the level of calcium (Carvalho et al., 1981; Sanchez, 1976). Acidity stress may affect any stage of the legume-*Rhizobium* symbiosis, for example the survival of inoculant rhizobia in the soil, nodule formation, and growth of the host plant (Cooper et al., 1983). Low pH per se is not the factor limiting growth of a number of legumes although strains are pH sensitive (Cooper et al., 1987).

Several tropical legumes are well adapted to acid soils: *Stylosanthes*, *Desmodium*, *Centrosema*, *Calopogonium*, and tropical kudzu (*Pueraria phaseoloides*) are the primary ones. Among the grain legumes, cowpeas (*Vigna*) and pigeon peas (*Cajanus*) seem to be more tolerant of soil acidity than beans (*Phaseolus vulgaris*) or soybeans (*Glycine max*) (Sanchez, 1976). *Medicago sativa*, *Glycine wightii*, and some *Trifolium*

species can grow well at pH 4.0 provided they are supplied with combined nitrogen and adequate levels of calcium (Cooper et al. 1981). In summary, legumes become more sensitive to acid soils and high levels of aluminum if they depend upon symbiotic nitrogen fixation than on combined (fertilizer) nitrogen.

Though few reports exist in the literature specifically dealing with the physiological effects of aluminum in bacteria, aluminum can be toxic to bacteria. Both the rate of growth as well as the dry matter yield of a strain of Bacillus megaterium were reduced by the addition of between 10uM (0.3ppm) to 100uM (2.7ppm) aluminum to the growth medium (Lewis et al., 1971). The authors attributed the effect of aluminum to interference with iron uptake by this strain. Many strains of cowpea rhizobia are sensitive to aluminum, as a component separate from pH (Weaver and Munns, 1979). Kingsley and Bohlool (unpublished) obtained similar results when examining both fast- and slow-growing rhizobia in aluminum-containing media (see below).

Many species of gram positive (g+) and gram negative (g-) bacteria have genes determining resistances to a wide range of toxic inorganic cations and anions, including some organic (and organomercurials), cadmium, arsenic, antimony, bismuth, chromium, silver and tellurium (Silver, 1981). The resistance to mercury and organomercurials is due to enzymatic transformation of the materials by mercuric reductases and organomercurial lyases respectively, which liberates volatile

compounds of lesser toxicity that escape from the growth medium.

In fast-growing rhizobia many of the genes encoding host specificity of nodulation (Hsn) and nitrogen fixation (Nif, Fix) are located on large plasmids called pSyms (for review see Prakash and Atherly, 1986). Determinants of bacteriocin production/resistance may also reside on these plasmids as well (Hirsch et al., 1980). However, since much of the DNA on these large plasmids still remains cryptic, genes encoding stress resistance mechanisms might reside on these pSyms. No one has investigated the role Sym plasmids might play in stress resistance in fast-growing rhizobia, or investigated the physiological mechanisms for aluminum resistance sensitivity.

I. Role of Symbiosis Plasmids in Aluminum Resistance

During the first stage of this project I acquired cultures of *Rhizobium* from various sources. I screened these cultures, as well as many in our own collection for resistance to acidity (pH 4.45) and acidity plus aluminum (various concentrations ranging from 5uM to 1. m). During this screening stage several different media formulations were evaluated (Keyser and Munns, 1979; [illegible] and Halliday 1979; [illegible], Weckman and Munns, preprint). These media were not satisfactory for several reasons, such as inhibitory when autoclaved, and inability to support the consistent growth of both fast- and slow-growing rhizobia. I therefore formulated

my own medium, MAM. This medium contained approximately the same inorganic salts as Keyser and Munns (1979b), used Na-glutamate as a nitrogen source (Keyser and Munns, 1979b), Fe-EDTA as an iron source (Keyser and Munns, 1979b), the B vitamins niacin, thiamine and pantothenate, and employed glycerol as a carbon source. Both fast and slow growing rhizobia were able to grow in this medium. The pH of the medium remained stable when autoclaved.

The results of some of the strain screenings are listed in Table 1. Due to its high levels of pH and aluminum resistance I chose the fast-growing R. phaseoli strain CIAT 899 for further work. For some experiments the strains Pra (R. leguminosarum) and USDA TH0a9 Rhizobium spp. for chickpea were used as pH tolerant but aluminum sensitive strains. However, the pH tolerance of these two strains was limited (pH 4.7) and additionally, it was necessary to add 0.01% yeast extract to the MAM basal medium to obtain consistent growth. Therefore in most experiments I employed TAL-1472 as the aluminum sensitive strain. This strain is tolerant down to pH 4.2 and grows in standard MAM (i.e. without any yeast extract).

1. Genetic Studies

Determining the role of pSym in stress resistance should be a straight forward process since cent-curing is usually possible simply by incubating the culture at an elevated temperature. A correlation of the loss of stress resistance with the loss of the pSym would indicate that the pSym-encoded

Table 1: Response of some fast- and slow-growing rhizobia subjected to low pH 50 µM Al.

CULTURE	SOURCE/ORIGIN	GROWTH	
FAST-GROWERS		pH4.5	pH4.5 + 50 µM Al
Rhizobium sp. (*C. arietinum*)			
TAL-263		-[a]	-
TAL-480	NifTAL	-	-
TAL-620		>[b]	>
Nitragin 27A3	J. Burton, Nitragin	-	-
Nitrogin 27A16		+[c]	+
USDA 3HOa9	D. Weber, USDA	+	-
R. leguminosarum			
Nitragin 92A3 (NZP 5400)		+	+
Nitragin 175P1	J. Burton, Nitragin	-	-
Nitragin 128C53		-	-
C 4202	D. Bezdicek	-	-
LK 3005		>	>
I2		+	+
I41		-	-
I42		-	-
F24	Morocco	-	-
Allen 311	NifTAL	+	+
Allen 344		+	+
Pre	Rothamsted	-	-
TA101	Australia	-	-
NZP 2089		-	-
NZP 5262	New Zealand	-	-
R. phaseoli			
CNPAF 23		-	-
CNPAF 26	Brazil	+	+
CNPAF 43		+	+
Semia 4002		>	>
CIAT 899	CIAT	+	+
CIAT 2545		+	+
Bel 7.1	E. Schmidt	>	>
NZP 5097		+	+
NZP 5232	New Zealand	+	+
NZP 5260		-	-
R. meliloti			
NZP 4013	New Zealand	-	-
L-530	B. Rolfe	-	-
R. trifolii			
T-12	B. Rolfe	+	-
R. lupini			
CC 814	Australia	-	-
WU 425		-	-

(cont'd.)

CULTURE	SOURCE/ORIGIN	GROWTH	
FAST-GROWERS		pH4.5	pH4.5 + 50μM Al
Rhizobium sp. (*L. leucocephala*)			
TAL-582		+	+
TAL-1145	NifTAL	>	>
NGR-234	Australia	-	-
RL-18		-	-
RL-52	Hawaii	-	-
RL-72		>	>
SLOW-GROWERS			
R. japonicum			
USDA 31		+	+
USDA 110	D. Weber, USDA	>	>
USDA 138		+	-
Nitragin 61A118	J. Burton, Nitragin	+	-
Nitragin 61A148		+	-
TAL-183	NifTAL	>	>
CB1809 (TAL-379)		+	-
WB64		+	-
WB68		-	-
2859	New Zealand	+	+
2860		+	+
2861		+	-
2864		+	-
Rhizobium sp. (cowpea misc.)			
CB756	Australia	+	-
CB1024		+	+
TAL-420	NifTAL	+	+
32H1	G. Elkan	+	-
PDD3722	New Zealand	+	-

[a] - no growth

[b] > "cheater" reaised pH of medium

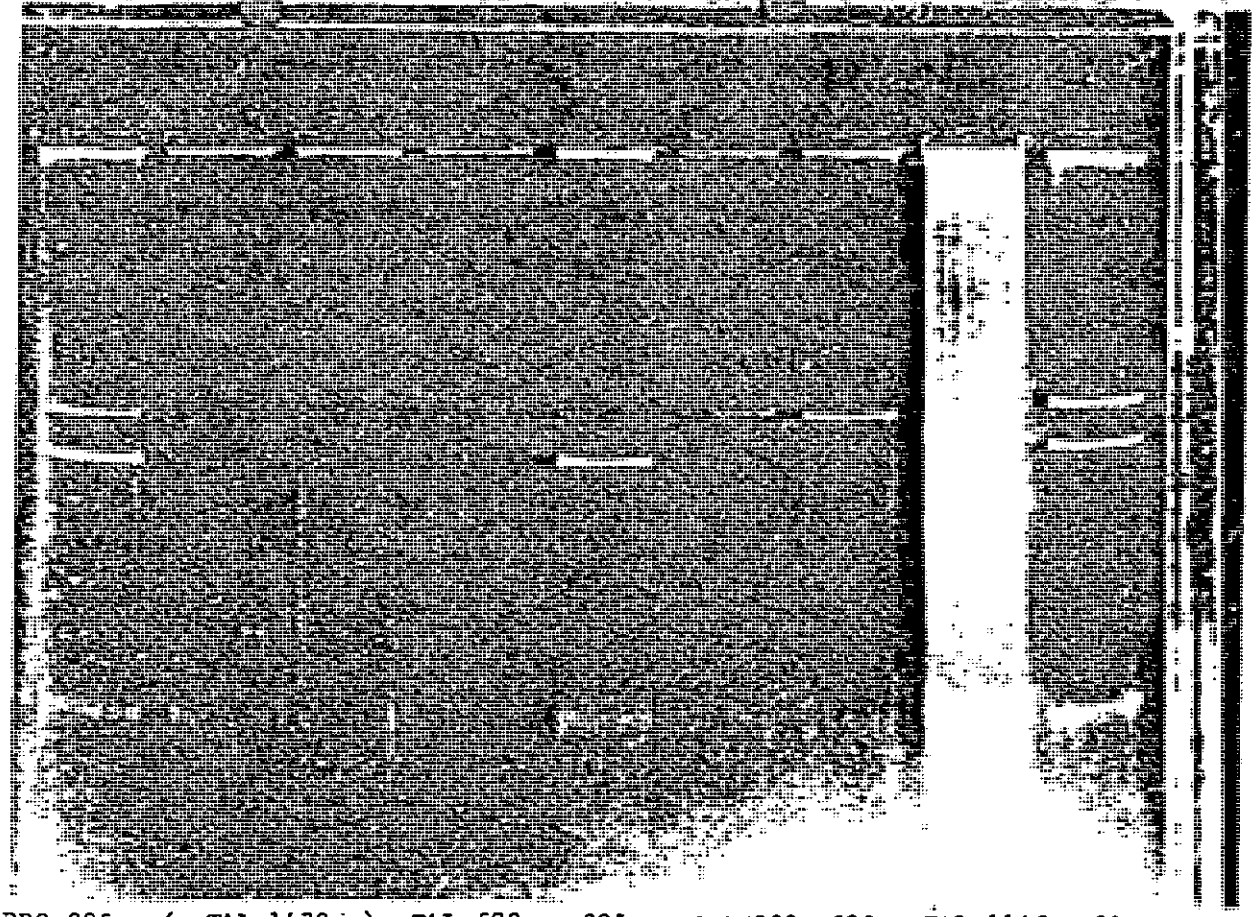
[c] + growth

genes are necessary for the resistant phenotype. If stress resistance genes are located on pSyms transfer of such pSyms to other related rhizobia could create new stress resistant transconjugants that are more effective than the donor strain. Phenotypic expression of pSym-encoded symbiotic functions (i.e., Nif) can be affected by the host Rhizobium strain with the transconjugants being more effective than the donor (Sadowsky and Schloot, 1985; Brewin et al. 1983).

(A) Plasmid Screening. Eleven of the fast-growing strains were screened for the presence of plasmids. Each strain contained at least one plasmid of 1.7 x 10^6 MW. Using the conventional alkaline lysis procedure (Hirsch et al., 1980), I observed repeatedly only one plasmid band for aluminum resistant CIAT 899. Figure 1 shows an enlargement of a plasmid pattern of all strains obtained with the conventional lysis procedure. Note that only one plasmid band is present for CIAT 899, and no bands are visible in the two lanes containing TAL-1472. However, when I screened these strains for plasmids via a direct in-well lysis procedure (modified Eckhardt Rosenberg et al., 1982) I a second, larger plasmid band appeared (approx. 12.0 x 10^6 MW) (see Figure 2). This plasmid was not found with the conventional lysis procedures due to its large size and therefore its sensitivity to shearing. Two plasmid bands were also consistently obtained for strain TAL-1472 when I used the in-well procedure.

(B) Transposon Mutagenesis. The advantage of transposon Tn5 mutagenesis is that in addition to creating a mutation, the

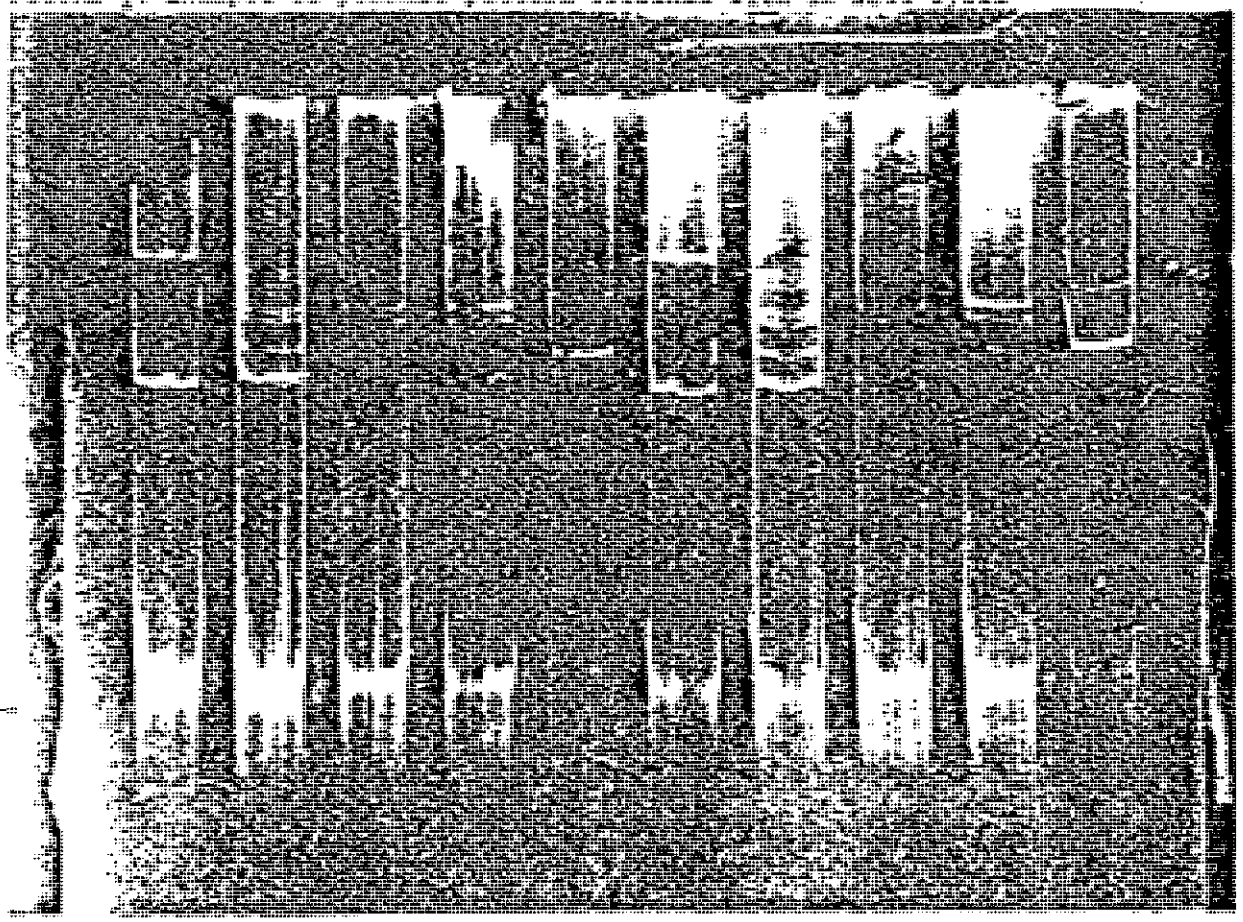
FIGURE 1. Example of plasmid pattern with conventional lysis



Chromasomal DNA

PRC-205 (TAL-1472) TAL-582 -205 CIAT899 -582 TAL-1145 -205

FIGURE 2. Example of plasmid pattern obtained with in well lysis



Chromasomal DNA

L530 6015 1472 1472 899 L530 6015 1472 1472 899

transposon also marks the site of the mutation in the genome (for a review on Tn5 see deBruijin and Lupski, 1981). This makes finding the segment of DNA, for cloning purposes, relatively easy.

Aluminum-resistant CIAT 899 was mated with E. coli S-17-1 carrying the Tn5 suicide vector pSUP2021 (Simon et al, 1983), kanamycin resistant clones arose at a frequency of 5 X 10^{-5} per recipient. 1,200 Tn5 containing (Km^r) CIAT 899 colonies were patched onto master plates of rich medium and then replica plated onto minimal media to screen for auxotrophs. Out of the 1,200 clones, no auxotrophic mutants were obtained. This was probably due to insertion of Tn5 into CIAT 899's two plasmids. All of the 1,200 colonies grew on media containing 80uM aluminum, none became aluminum sensitive.

(C) Plasmid Curing. To determine whether aluminum resistance gene(s) reside on the Sym plasmid in CIAT 899, a number of attempts were made to cure the CIAT 899 pSym using various agents or techniques. Several concentrations of acridine orange, ethidium bromide, novobiocin at ambient temperature (24°C), as well as growth at elevated temperature (40°C) [no chemical agent present] failed to cure CIAT 899 of its plasmids. However, the combination of elevated temperature (37°C) and 12ug/ml acridine orange cured CIAT 899 of one of its two plasmids. The cured mutants were Nod^- on beans, and are rough (Exo^-) when plated onto yeast extract mannitol medium, in contrast to the gummy parental culture (Exo^+). The

rough mutants continued to react with CIAT 899 fluorescent antibody.

One of the cured mutants (UHM-5) [= CIAT 899(pSym$^-$), Exo$^-$], and the parental culture, were grown in liquid media containing 0, 80 150, 250, 300, 400 and 500uM aluminum at pH 4.45. TAL-1472 was employed as an aluminum sensitive control (0 and 80uM Al treatments only). The results of these growth experiments are illustrated in Figure 3a to 3g. Strain UHM-5 grows equally well as strain CIAT 899 at all concentrations of aluminum. This implies that the pSym carries no determinants for aluminum resistance (or there are additional copies located elsewhere). In addition, the loss of extracellular polysaccharide production had no apparent effect upon the growth of UHM-5 in aluminum containing media.

The pSym of another aluminum resistant strain ANU1173 (R. trifolii, resistant to 25uM Al), was cured by incubation at elevated temperature. Unlike UHM-5 the cured mutant remained as gummy as the parental culture when grown on plates of YEM. However, as with CIAT 899 the pSym of ANU1173 was not responsible for determining aluminum resistance. Figures 4A to 4D illustrate the growth of ANU1173, two pSym-cured isolates of 1173, and the aluminum-sensitive control strain TAL-1472 in 0uM Al, pH4.5 (A), 25uM Al (B), 50uM Al (C), and 80um Al (D). All ANU1173 cultures behaved identically in all four levels of aluminum. Therefore the pSym does not encode stress resistance mechanisms.

Figure 3. Comparison of aluminum resistance between CIAT 899 and a pSym-cured, Exo⁻ derivative (M5) in seven levels of aluminum.

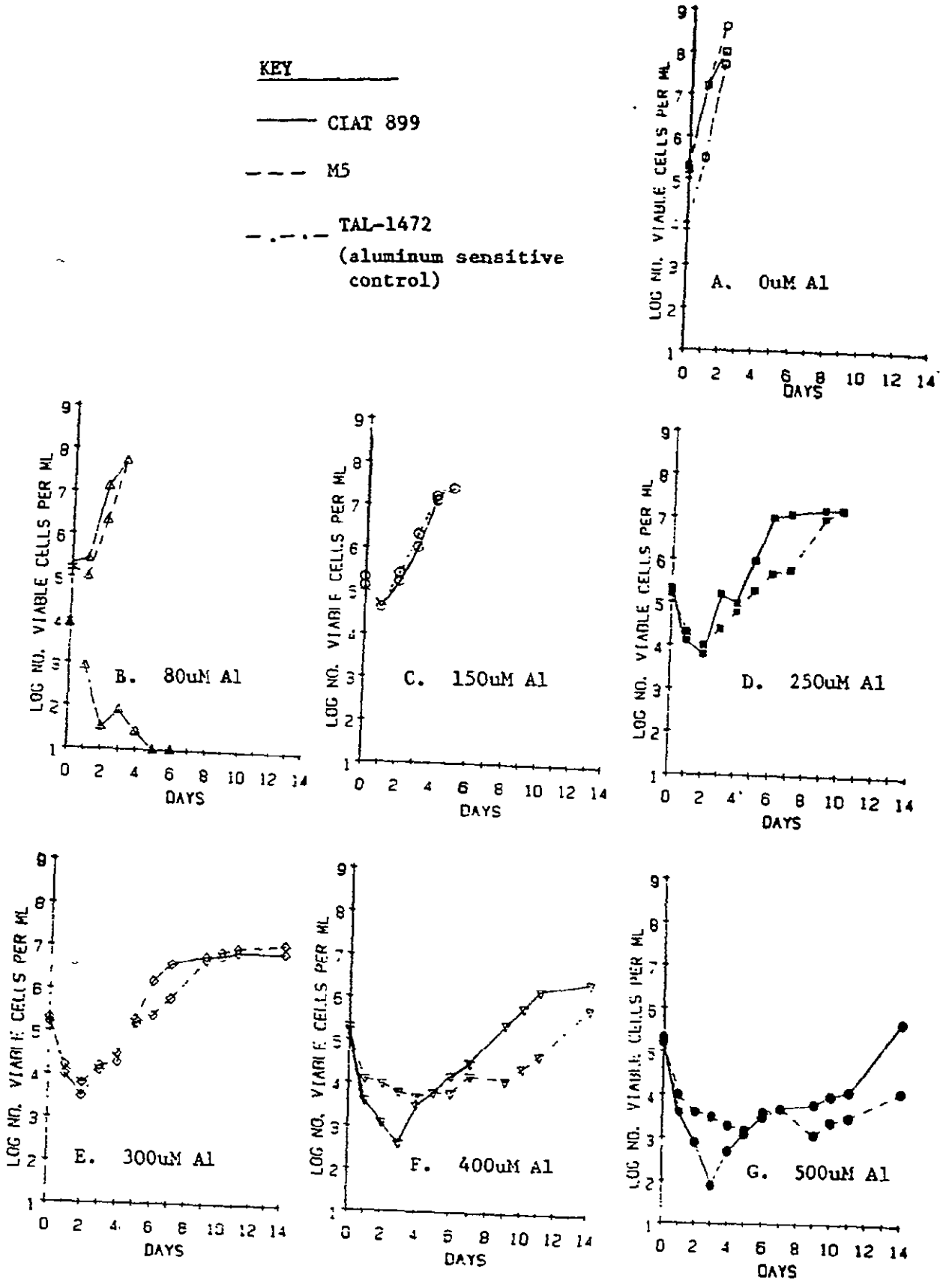
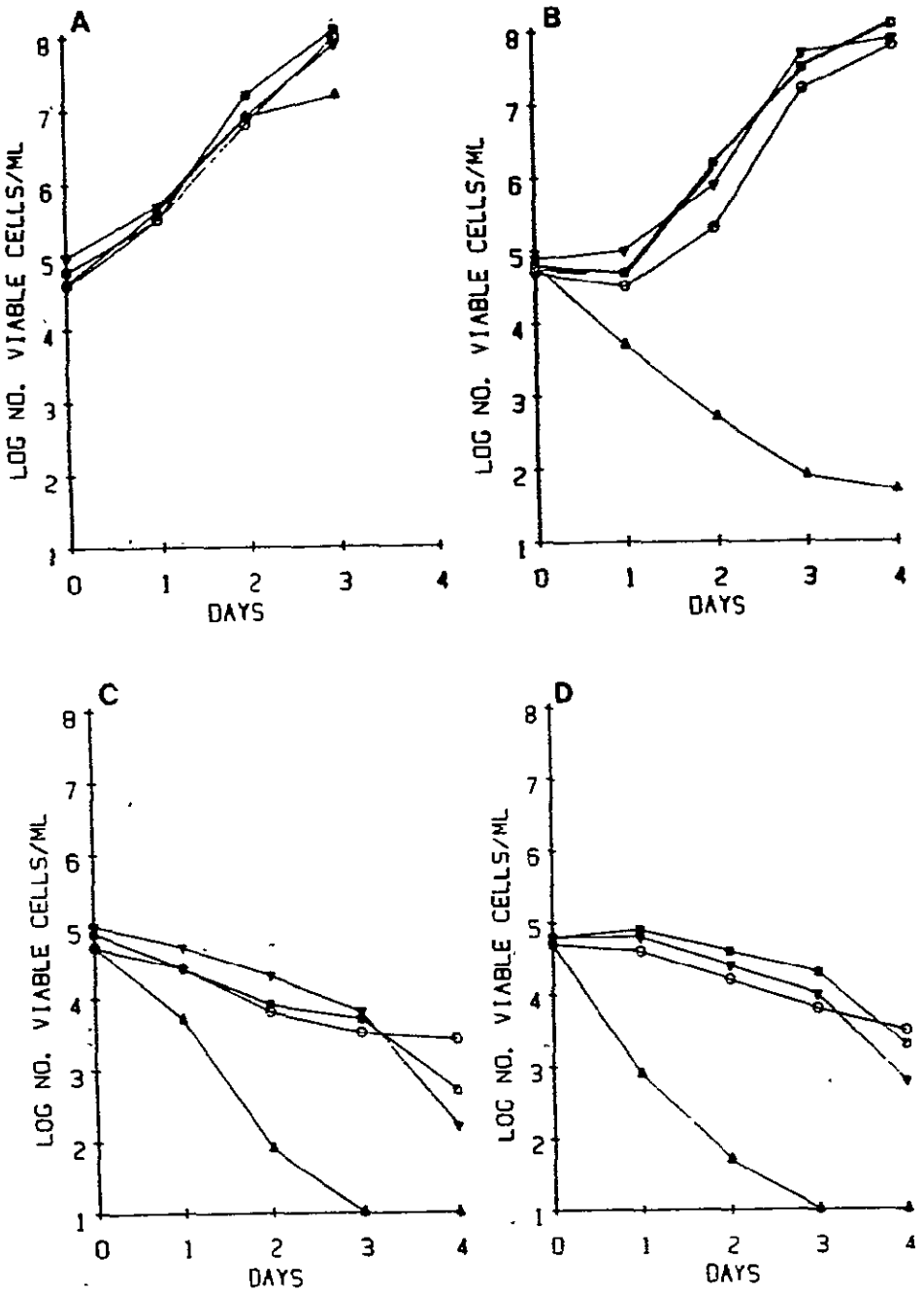


Figure 4. Comparison of aluminum resistance between ANU1173 and two pSym⁻ derivatives at four levels of aluminum.

KEY

4A 0uM Al, pH4.5
4B 25uM Al, "
4C 50uM Al, "
4D 80uM Al, "

ANU1173 ○
" pSym⁻1 ▽
" pSym⁻2 □
TAL-1472 △
(aluminum sensitive)



Summary. Transposon mutagenesis and Sym plasmid curing of CIAT 899 failed to produce any aluminum sensitive mutants. The fact that no auxotrophs were obtained from among the 1,200 Km^r clones indicated that Tn5 most likely entered the plasmids, especially the pSym. Symbiosis related genes are unexpressed in the free-living state, unless induced by host plant root and/or seed extracts (NodD is an exception; it is expressed in both states) (Innes et al.,1985; Long et al, 1985; Mulligan and Long, 1985; van Brussel et al, 1986), therefore a Tn5 insertion would not produce an altered phenotype. The curing of the pSyms of CIAT 899 and ANU1173 indicates strongly that no aluminum-related determinants reside on these plasmids. The lack of extracellular polysaccharide in the cured strain UHM-5 had no detectable effect on the aluminum resistant phenotype.

2.Physiological Studies

Since the pSym does not carry the genetic information encoding aluminum resistance (1), and transposon mutagenesis was not successful in creating aluminum-sensitive mutants (2), the following sets of experiments were designed to help focus in on the mechanism of aluminum resistance in CIAT 899 as an aid in locating the genes involved. For example, if a protein were to be induced in response to aluminum, this protein could be isolated and either: (1) antibodies produced against it, the antibodies then used to precipitate the protein as it is transcribed, mRNA recovered, a cDNA copy of the mRNA produced and used as a probe of a gene bank of CIAT 899 to find the

gene; or (2) the protein could be partially sequenced, a synthetic oligonucleotide corresponding to this protein sequence produced and used as a probe.

(A) Aluminum Binding. Both aluminum-resistant and aluminum-sensitive cells were found to bind aluminum and remove it from the medium. The change in the aluminum concentration in the medium was followed by using a colorimetric assay employing eriochrome cyanine, which forms colored complexes with aluminum under acid conditions. Since there was no apparent difference in this aluminum binding/removal between resistant and sensitive strains further experiments were designed to try to explain why some strains were tolerant of high levels of aluminum.

(B) Pre-Conditioning of Media. In this set of experiments aluminum-tolerant strain CIAT 899 and two aluminum-sensitive strains Pre (R. leguminosarum) and USDA 3HQa9 (Rh. for chickpea) were grown in acid media at pH 4.7, supplemented with 0.001% yeast extract (control, all strains grew) and acid medium plus 80uM aluminum (only CIAT 899 grew). The cells were removed from the media by centrifugation and the spent media were resterilized by filtration, the pH was readjusted to pH 4.7 and the levels of aluminum were readjusted to 80uM. The three cultures were freshly inoculated into these spent media conditioned by the other strains. The results are summarized in Table 2.

Table 2. Extracellular Soluble Products: Strains were inoculated into media (Part A) at 1×10^6 cells/ml and later removed by centrifugation and filtration. In Part B aliquots of filter-sterilized, pre-conditioned media (from Part A) were re-inoculated and strains allowed to grow.

Part A

Strains	Media: 80uM Al pH 4.7	pH 4.7
CIAT 899 (Al-tol.)	+	+
Pre (Al-sens.)	-	+
USDA 3H0a9 (Al-sens.)	-	+

+ indicates turbidity/growth
- indicates no growth

Part B

1st Inoculum/ 2nd Inoculum[a]	80uM al pH 4.7	pH 4.7
899/Pre	-	+
899/3H0a9	-	+
899/899	+	+
Pre/899	+	+
3H0a9/899	+	+

[a]1st inoculum removed by centrifugation and filtration, media was re adjusted to original concentrations of Al and pH (i.e., 80uM Al, pH 4.7). Media were resterilized by aseptic filtration. All strains were inoculated at 1×10^6 cells/ml.

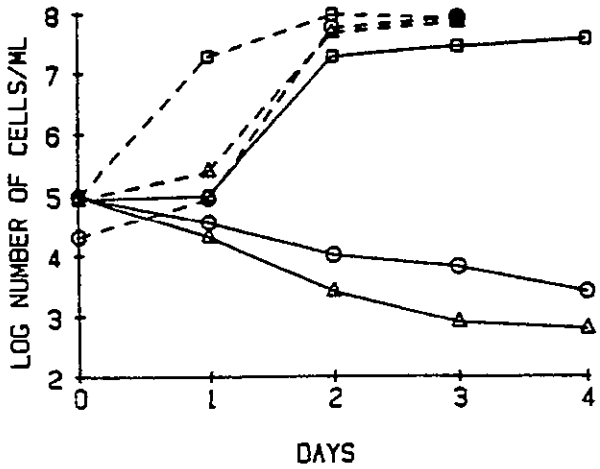
Growth of CIAT 899 in medium plus aluminum did not alleviate the toxic effects of aluminum for the sensitive strains Pre and USDA 3H0a9.

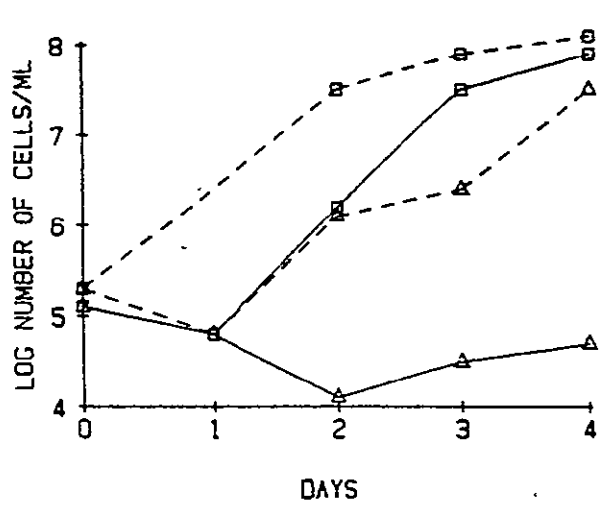
(C) Co-Culture Experiment. Although CIAT 899 did not detoxify the medium and allow for the growth of the aluminum sensitive strains, growth of CIAT 899 together in the same flask with an aluminum sensitive strain might alleviate the toxic effects of aluminum and allow the sensitive strains to grow. For example, a component of the growing cell's surface might be involved in aluminum resistance. Also, readjusting the level of aluminum in the medium to 80uM might have potentially overwhelmed any excreted product.

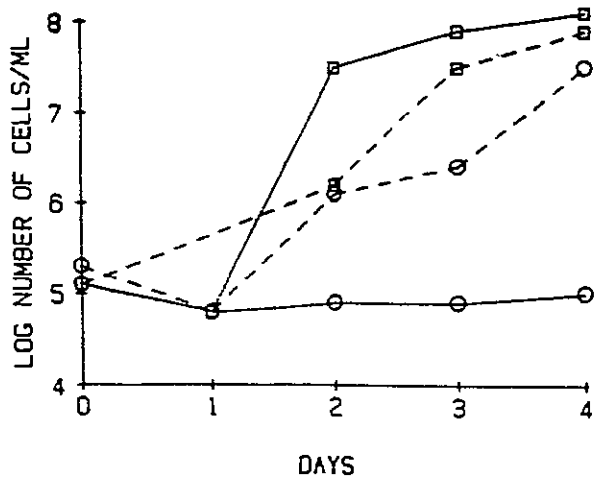
Figure 5a illustrates the growth of the strains by viable counts (strains grown separately, three replicates) and demonstrates the aluminum-sensitivity of strains Pre and USDA 3H0a9. Figures 5b and 5c are the results of the co-culture experiment where populations were followed by membrane-filter immunofluorescence enumeration. In co-culture all strains grew under acid conditions (0uM Al, pH 4.7), however, only CIAT 899 grew when media contained 80uM aluminum.

(D) Cell Free Extract of CIAT 899. Since nothing liberated by CIAT 899 into the growth medium (1), or potentially on the surface of growing cells (2) could alleviate aluminum toxicity for the sensitive strains, an internal component(s) might be involved in aluminum resistance of CIAT 899. Such (a)

Figure 5. Cell Surface Components: Growth of an Aluminum Tolerant Strain with Aluminum Sensitive Strains







component(s) could potentially eliminate aluminum toxicity for the sensitive strain TAL-1472 if added to the growth medium.

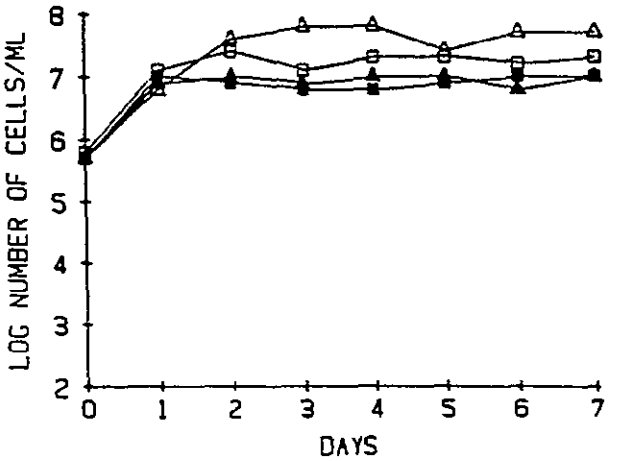
To determine if this was the case, a cell-free extract was made from strain CIAT 899 grown in six liters of medium containing 500uM aluminum. The cells, harvested by centrifugation, washed 3X at pH 4, were passed through a French Press at 4°C. The extract was sterilized by filtration and frozen. Aliquots of this extract (0.5ml) were added to 50mls of MAM with and without aluminum. There were three replicate flasks per treatment and a total of eight treatments (e.g., two strains in media with or without aluminum and/or extract). Growth of the strains was followed by viable counts. The results are illustrated in Figures 6a and 6b.

Addition of the cell-free extract of aluminum-grown CIAT 899 did not decrease the toxic condition of MAM containing 80uM aluminum for TAL-1472, and may have even accelerated the die-off.

(E) Growth of CIAT 899 -- Influence on Aluminum Concentration in Liquid Media.

Figure 7a illustrates the effect of CIAT 899 on the pH level in 50mls of liquid medium (in triplicate). The pH level of the control flasks (no cells) remained constant for the eight-day duration of the experiment. Growth of CIAT 899 did not alter the pH of the medium until after stationary phase was reached. Maximum cell numbers were reached on Day 3 and the pH did not change during log growth (compare 7a with 7c). The 0.2 pH unit increase is still well within the solubility range of aluminum [aluminum solubility drastically decreases

Figure 6. Internal Factors: Effect of Cell Free Extract of Aluminum-Tolerant CIAT 899 on Growth of CIAT 899 and Al-sensitive TAL-1472 (Growth Followed by Viable Counts).



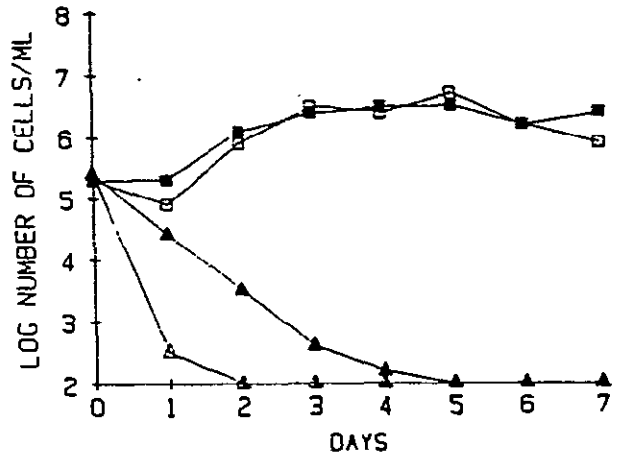
A. CIAT 899 (Aluminum tolerant)

open symb. 1% CIAT 899 cell-free extract

solid symb. No extract

□ ■ H^+ (pH 4.45)

△ ▲ 80 uM Al (pH 4.45)



B. TAL-1472 (Alum. sensitive)

open symb. 1% CIAT 899 cell-free extract

solid symb. No extract

□ ■ H^+ (pH 4.45)

△ ▲ 80 uM Al (pH 4.45)

above pH 5]. Although the level of available aluminum in the medium decreased from approximately 80uM at Day 0 to approximately 60uM at Day 8, the decrease is purely a chemical phenomenon. The control flasks, without cells, decreased at the same rate as those containing CIAT 899.

From these experiments I concluded that CIAT 899 did not take up aluminum from the medium, at least at levels detectable by the assay used, and only binds aluminum at detectable levels when high cell numbers are used (experiment 2A).

(F) Summary. CIAT 899 is an extremely aluminum resistant strain. Nothing liberated by this organism into the growth medium, or associated with the growing cell, or soluble internal components alleviated aluminum toxicity for the sensitive strains growing in media containing 80uM aluminum. [The results of some of these physiological experiments were presented at the 1985 Annual Meeting of the American Society for Microbiology (Abstract N107, Proceedings of the Annual Meeting of the American Society for Microbiology. 1985.).]

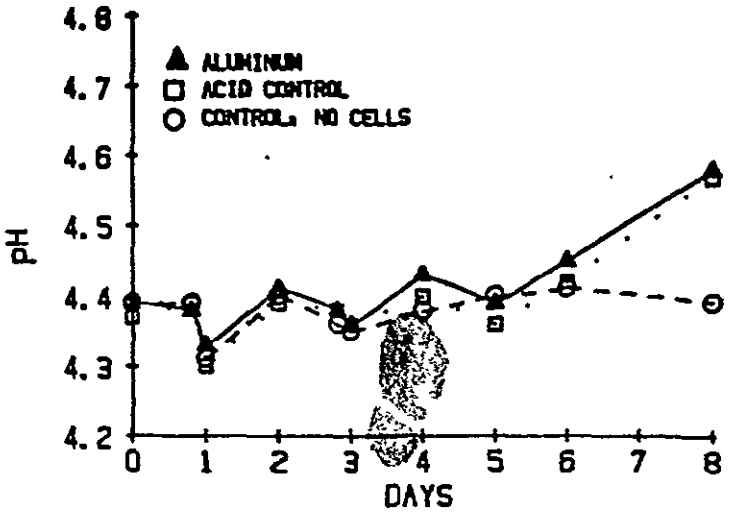
3. Physiological aspects of Aluminum Sensitivity

Since the preceding sets of experiments provided no readily apparent explanation for a mechanism of aluminum resistance in CIAT 899, I decided to take the opposite approach and investigate the physiological basis for aluminum sensitivity in R. phaseoli strain TAL-1472.

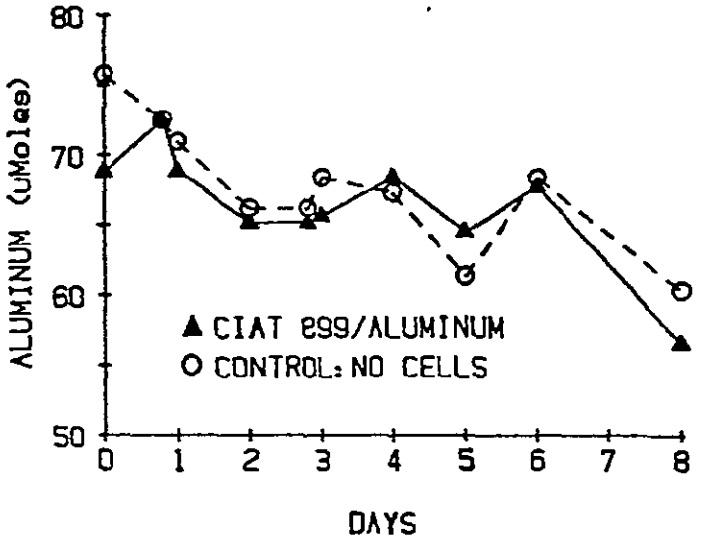
(A) Concentration of Aluminum. Figure 8 illustrates a set of experiments to determine the level of aluminum sensitivity of

Figure 7. Correlation of growth of CIAT 899, in media with and without aluminum (Fig. 3C), with pH (Fig. 3A) and level of aluminum (Fig. 3B).

A. pH



B. Aluminum



C. Growth

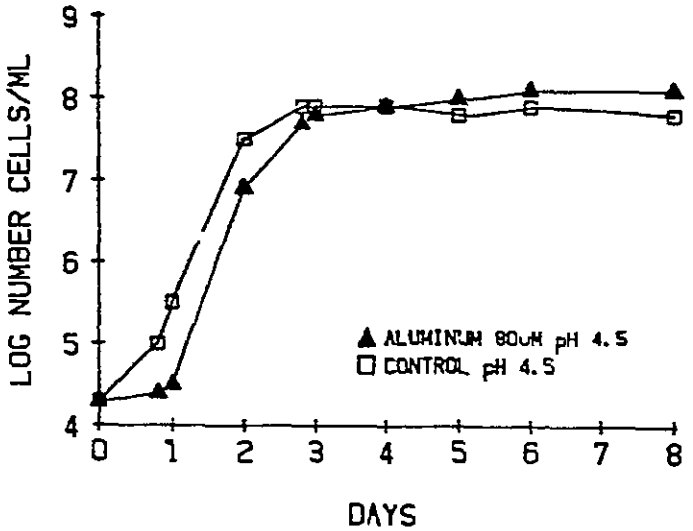
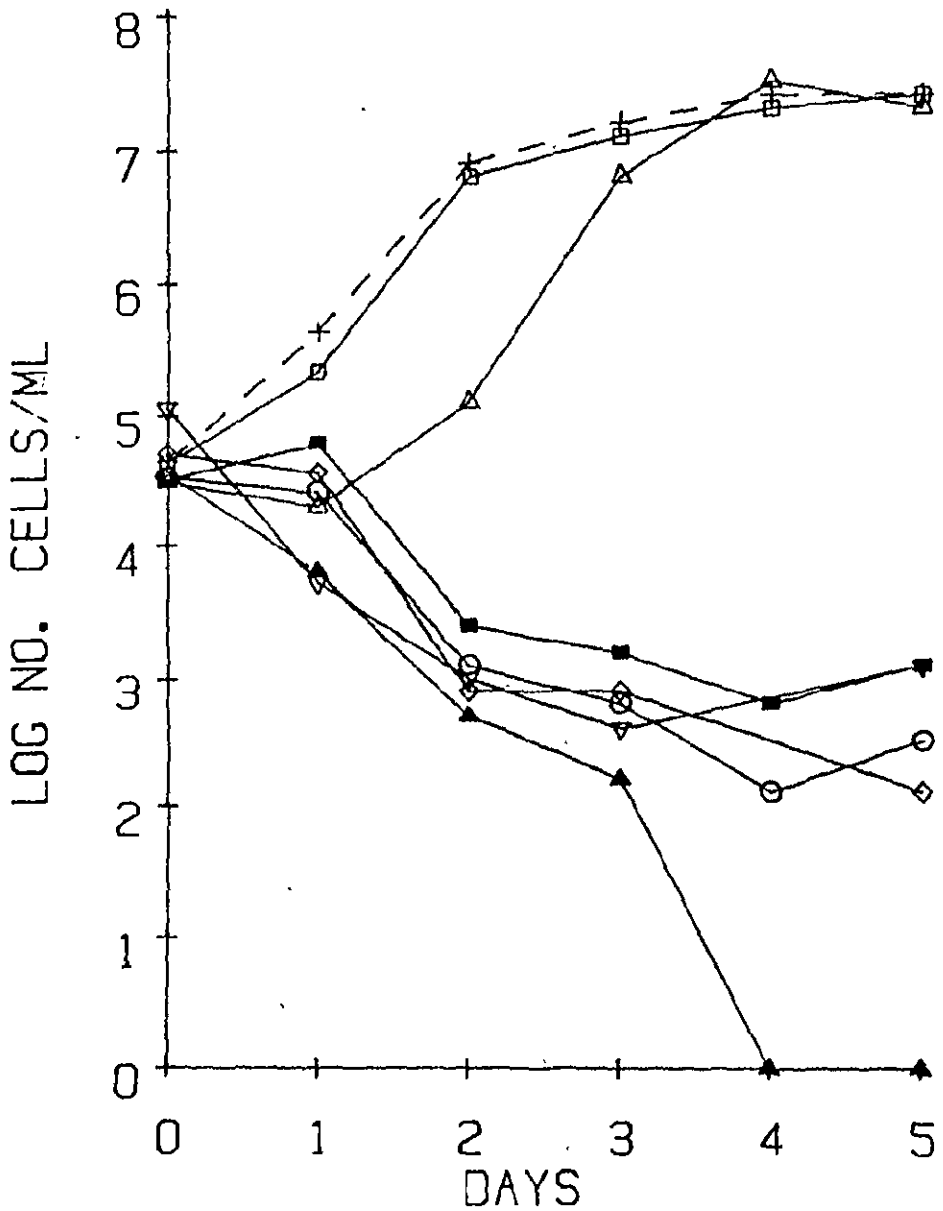


Figure 8. Growth of TAL-1472 in MAM containing 5uM phosphate at eight concentrations of aluminum.



KEY		Al:P Ratio
+	0uM Al	
□	5uM Al	1:1
△	10uM Al	2:1
▽	15uM Al	3:1
◇	20uM Al	4:1
■	25uM Al	5:1
○	50uM Al	10:1
▲	80uM Al	16:1

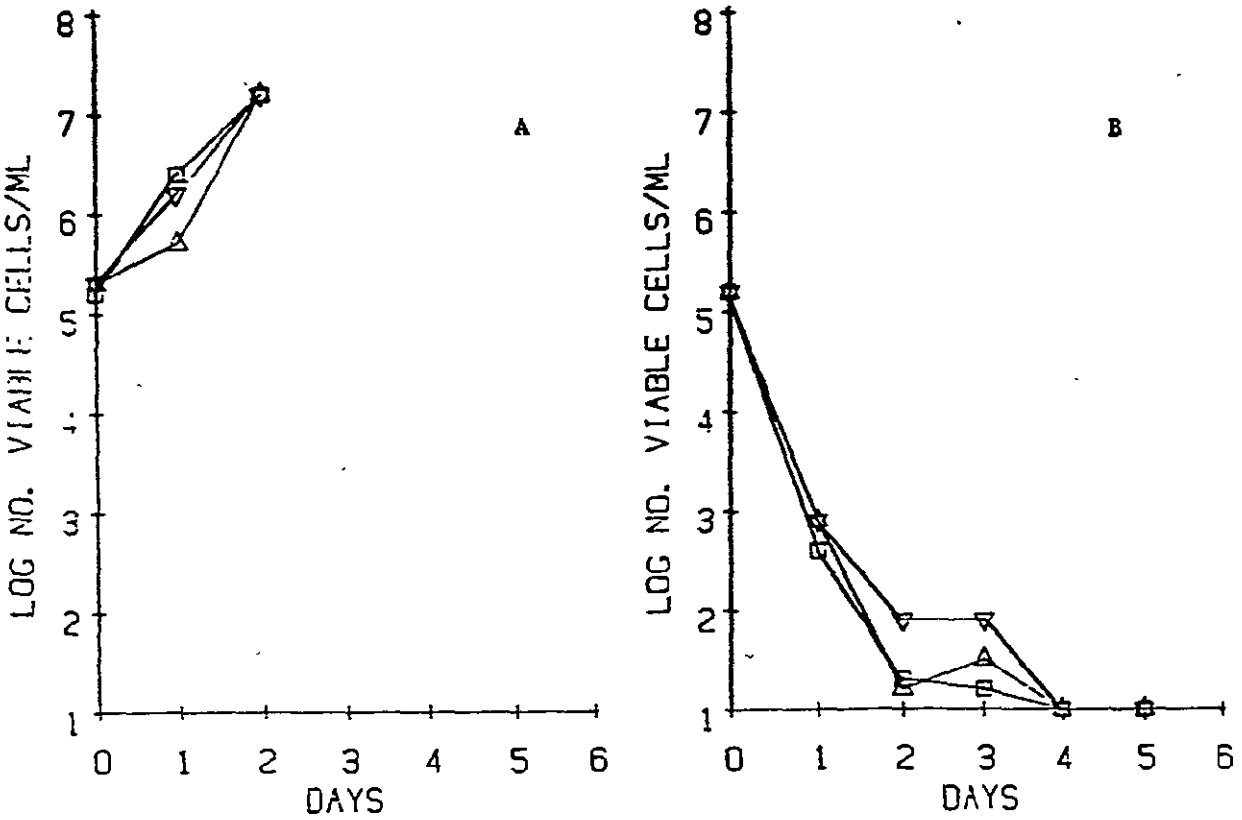
TAL-1472. TAL-1472 grew in the presence of 0, 5 and 10uM aluminum (pH4.5). However, the strain was sensitive to aluminum concentrations $\geq$ 15uM. Therefore, unlike strain CIAT 899 or its derivatives, which grew at concentrations of aluminum $\geq$ 300uM, TAL-1472 was sensitive to low levels of aluminum.

(B) Concentration of Fe-EDTA. Davies et al. (1971) indicated that aluminum interfered with iron uptake in a strain of Bacillus megaterium; and Keyser and Munns (1979b) as well, found a strain of cowpea Bradyrhizobium spp. (TAL-174N) to be more sensitive to 50uM aluminum when $FeCl_3$ was used as an iron source, than when Fe-EDTA was employed. Although the poor growth effect was due to a lack of iron, they attributed this effect to Fe-phosphate precipitates ($Fe(OH)_2H_2PO_4$) rather than to a direct competition of aluminum with iron, as was the case with the former report. Keyser and Munns state that the stability constant of Fe^{III}EDTA greatly exceeds that of Al^{III}EDTA, such that essentially no Al-EDTA complex should form at pH 4.5 (Keyser and Munns, 1979b; Novell, 1972). Their data (Keyser and Munns, 1979b) indicate essentially no lag phase when TAL-174N grows in the presence of 50uM aluminum when using Fe-EDTA, and starting from 10^4 cells/ml reaches a final cell number of 10^7/ml in 15 days. When they employed $FeCl_3$, a lag phase of four days occurred prior to log phase and the final cell number was reduced to 10^6/ml after 13 days growth.

Since iron might play an important role in aluminum sensitivity in TAL-1472, I designed an experiment to explore the effect of iron on strain TAL-1472 when grown in the presence of 80uM aluminum. Figure 9A illustrates the growth of TAL-1472 employing three levels of Fe-EDTA under acidic conditions (control, pH 4.45). There was no influence on the growth rate of the cells with either a low level of iron [10uM Fe-EDTA, = 1/3 amount present in standard medium (i.e., 30uM)] or at an elevated concentration of Fe-EDTA [80uM Fe-EDTA]. I chose this quantity since it was equimolar with the level of aluminum (in those treatments containing aluminum, Figure 9B). As illustrated in Figure 9B altering the level of Fe-EDTA had no significant effect upon the response of TAL-1472 in the presence of 80uM aluminum (pH 4.45). The die-off rate between the three treatments was essentially identical; after four days of growth the cultures reached the lower limits of cell detection ($\leq 1 \times 10^{-1}$ cells/ml). From this experiment I concluded that the level of iron in the medium was not a factor in aluminum sensitivity of R. phaseoli strain TAL-1472.

(C) Concentration of Phosphate. Acid tropical soils, in addition to their soil acidity-related stresses such as increased solubilities of aluminum and manganese, usually have low levels of available phosphorus, generally <1uM (Resienauer, 1966). As Keyser and Munns (1979b) showed, low levels of phosphorus in rhizobial culture media alone, without added aluminum, can be a severe stress for some strains. Also, when formulating media the phosphorus concentrations are

Figure 9. Influence of three concentrations of Fe-EDTA on the growth of TAL-1472 in media without (A) and with (B) aluminum.



Key

□ 10uM Fe-EDTA

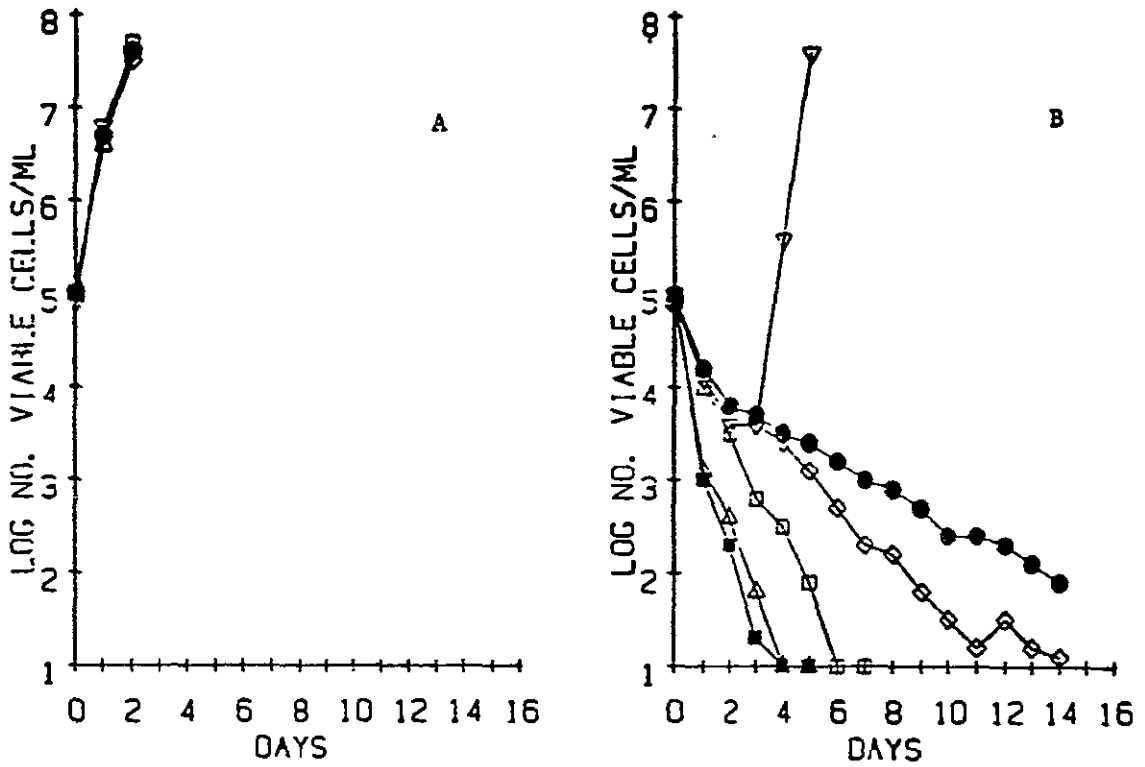
△ 30uM Fe-EDTA (Std. level)

▽ 80uM Fe-EDTA

important since a variety of aluminum-phosphate precipitates form. The type of precipitate formed depends upon the aluminum:phosphate (Al:P) ratio, the nature and ratio of other components of the solution, the temperature and the pH (White et al, 1976; Cooper et al, 1983). Since TAL-1472 grew well at aluminum:phosphate ratios of 1:1 (5uM Al:5uM P) and 2:1 (10uM Al:5um P) but not when the ratio was 3:1 or greater (Figure 8, in Experiment 3A), I decided to investigate this phenomenon.

In the experiments summarized in Figure 8 (Experiment 3A) the concentration of phosphate was held constant at 5uM while the level of aluminum ranged from 5um to 80uM. Since an aluminum-phosphate interaction might have influenced growth I decided to investigate the effect of concentrations of phosphate ranging from 5uM (standard concentration, = control) to 80uM phosphate while keeping the aluminum concentration constant at 80uM. Under acidic conditions (pH 4.45, 0uM Al) increasing the level of phosphate from 5uM to 80uM did not influence the growth of TAL-1472 (Figure 10A). The cells in all treatments grew at essentially the same rate reaching maximum cell numbers on Day 2. In contrast, the level of phosphate in media containing 80uM aluminum (Figure 10B) markedly affected the growth response of TAL-1472. When the Al:P ratio was 16:1 (either 80uM Al:5uM P, or 1280uM Al: 80uM P) the cells were killed within four days. As the ratio of Al:P decreased from 8:1 to 2:1 the cell die-off abated. However, unlike the results illustrated in Figure 8, where

Figure 10. Growth of TAL-1472 at five levels of phosphate in MAM without aluminum (A) and with 80uM aluminum (B).



Key		Al:P Ratio
△	5uM P	16:1
□	10uM P	8:1
◇	20uM P	4:1
●	40uM P	2:1
▽	80uM P	1:1
■	1280uM Al, 80uM P*	16:1

*(Figure B only)

TAL-1472 grew at a 2:1 ratio of Al:P (10uM Al:5uM P), in this experiment (three replicate flasks) TAL-1472 continued to die-off in media with a 2:1 ratio of Al:P (80uM Al:40uM P), although at a slower rate than for higher ratios of Al:P. As with experiment 3A, when the Al:P ratio was 1:1 (5uM Al:5uM P in Experiment 3A; 80uM Al:80uM P in experiment 3C) the cultures reached the same final cell number as the acid control flasks. However, a three day die-off preceded cell growth indicating that the aluminum level in the medium, at least initially, was toxic.

Although the results of experiment 3C clearly indicated an aluminum-phosphate interaction an exact interpretation is complicated due to the solution chemistry of aluminum and phosphate. In practice, 50uM aluminum should remain in solution in the presence of phosphate up to 30uM at pH 4.35, 10uM at pH 4.5 and 5uM at pH 4.65 (Cooper et al., 1983). Above these levels precipitates will form which, although they decrease the available levels of aluminum, lower the phosphate concentration as well. I did not observe any obvious precipitates in any of the treatments except for the last treatment, a 16:1 ratio of aluminum:phosphate (1280uM Al:80uM P). Theorectically, no precipitates should form at phosphate levels from 5uM (standard phosphate concentration employed) to perhaps 10 - 20uM, in the presence of 80uM aluminum at pH 4.45.

In both experiments 3A and 3C, 1:1 ratios of Al:P (regardless of concentrations employed) allowed TAL-1472 to

grow. With a 2:1 Al:P ratio in Experiment 3C, where a higher level of both aluminum and phosphate were employed, growth of TAL-1472 did not occur. This is in contrast to experiment 3A where growth occurred readily in the presence of 10uM Al:5uM P (2:1 Al:P). (These concentrations are well within the solubility ranges predicted by Cooper et al (1983)). If precipitation of Al and P occurred in Experiment 3C, the level of aluminum was potentially too great for TAL-1472 to grow.

Summary. These experiments indicated that TAL-1472 was sensitive to low levels of aluminum in the presence of 5uM phosphate. If a higher level of aluminum was maintained (80uM), an increased level of phosphate counteracted the toxicity (i.e., die-off took longer). However, this latter effect was most likely due to a complexation/precipitation of aluminum, thereby lowering the overall level present in the medium. At this juncture the significance of the aluminum-phosphate interaction remains unclear. Whether aluminum sensitivity in strain TAL-1472 is directly attributable to a competition for phosphate or whether other constituents are involved remains unkown. Iron, however, does not seem to be involved directly.

<u>CONCLUSIONS</u>: The investigations of the physiological mechanisms of aluminum resistance/sensitivity did not indicate any definite mechanism(s). Electron microscopic examination of thin sections of aluminum-resistant and aluminum-senstive cells, stained to reveal aluminum (Wen and Wisniewski, 1985), could potentially indicate the fate of aluminum in these cells

(i.e., internal, external, etc.) as well as indicating any association of aluminum with particular cellular structures. This avenue is not currently being pursued. The Sym(biosis) plasmids of fast-growing rhizobia do not contain aluminum-resistance genes, since pSym-cured derivatives grew as well as parental cultures in the presence of aluminum.

II. Construction of Stress Resistant Rhizobia for Alternate Host Legumes via Sym Plasmid Transfer.

Planned Approach

Construction of superior strains of Rhizobium species by genetic manipulations has been limited by available information on the genetics of the genus. Genetics of the Rhizobiaceae is essentially that of the fast-growing species R. leguminosarum, R. meliloti R. phaseoli and R. trifolii (Schwinghamer, 1977; Beringer et al., 1980; Prakash and Atherly, 1986). With these species, the nitrogen fixation (Nif, Fix) genes, as well as many of the essential genes for infection of the host (Nod), are located on large indigenous plasmids (pSym) (for reviews see: Nuti et al., 1979; Stanley and Dunican, 1979; Ruvkun and Ausubel, 1980; Prakash and Atherly, 1986). Furthermore, among the symbiosis-related genes, those that confer host specificity (Hsn) can be mobilized from one species to another (Johnston et al., 1978; reviewed by Prakash and Atherly 1986). The genes responsible for nodule formation have also been transferred to the plant tumor-forming bacterium Agrobacterium tumefaciens (Hooykaas et al., 1981; reviewed by Prakash and Atherly, 1986).

It is clear from the findings described in section I(1C) that pSyms are not involved in determining stress resistance, since the strains (CIAT 899 and ANU1173) remained aluminum resistant following curing. The pSym cured derivatives are a valuble resource to be exploited for the construction of stress resistant rhizobia for alternate host legumes. For example, CIAT 899 is an aluminum resistant bean Rhizobium, by inserting a pea pSym or a clover pSym into the cured derivative it should be possible to construct rhizobia for peas and clovers which are not only highly effective but are stress resistant as well. The benefits of this approach, towards improving legume symbioses in tropical areas containing acid soils, could be great.

Sadowsky and Bohlool (1985) constructed a highly effective strain for peas, screened under controlled conditions, by transferring the R. leguminosarum pSym pJB5JI to a Nod⁻ strain of clover Rhizobium (#2). The primary transconjugants remained Nod⁻ on clover; however, pea nodule reisolates gained the ability to nodulate clovers ineffectively. This result would imply a genetic modification of the transconjugant during plant passage. Their results also indicate the importance of the genotypic background of the microsymbiont. The donor strain 6015(pJB5JI) is only partially effective on peas, while the #2(pJB5JI) transconjugant was highly effective. DeJong et al. (1982) improved, under controlled conditions, the symbiotic properties of a strain of R. leguminosarum (pea microsymbiont)

by plasmid transfer. The constructed strain reduced significantly more nitrogen than the parent; and it also proved superior to the field isolate from which the plasmids were obtained.

Brewin et al. (1983) showed, again under controlled conditions, that pSym content affects the percent nodule occupancy by the host strain. pSym content had no significant effect on the rhizosphere populations of the constructed strains; the population levels were essentially equal [same Rhizobium background genotype (three strains) containing one of four pSyms, all four transconjugants within the same background inoculated simultaneously, in approximately equal numbers, on peas (see Tables 2 and 3 Brewin et al., below)]. Therefore determinants on the pSym, in conjunction with the genotypic background of the microsymbiont host, ultimately dictated nodule occupancy. What the phenotypic property of the transconjugants is, that results in successful nodule occupancy, remains unknown. The authors did not report the nitrogen fixation efficiency of the individual Rhizobium/pSym combinations, so it is difficult to draw any strong conclusion as to why the host plant preferred particular combinations to the exclusion of others. One of the pSyms (pIJ1016) always formed the lowest percentage of nodules regardless of the genotypic background, while the percent nodule occupancy by the other strain/pSym combinations varied [see Tables 2&3 (Brewin et al., 1983)].

Table 2. *Percentage of each synthetic strain (streptomycin-resistant) present in mixtures with the standard strain 7,26 (rifampicin-resistant) in the initial inoculum, in the final root surface population and in the contents of individual nodules*

Synthetic strain		Initial inoculum			Final rhizosphere			Final nodules*		
Background genotype	sym plasmid	Mean	S.E.M.	n†	Mean	S.E.M.	n†	Mean	S.E.M.	n†
B151	pIJ1008	54·9	3·7	5	44·5	10·3	5	91·3	4·2	9
B151	pJB5	37·4	2·2	5	43·4	7·7	5	86·7	9·3	10
B151	pIJ1019	52·7	3·7	5	47·6	3·7	5	45·2	7·1	10
B151	pIJ1016	52·9	1·8	5	46·5	4·5	5	11·0	4·7	9
3617	pIJ1008	49·8	1·2	5	61·4	3·1	5	77·0	7·8	9
3617	pJB5	56·1	2·2	4	63·6	3·1	5	95·1	3·5	9
3617	pIJ1019	48·2	3·0	5	61·3	6·6	5	87·6	6·0	8
3617	pIJ1016	47·4	1·9	5	62·1	8·6	5	38·7	13·4	8
8401	pIJ1008	51·2	3·7	5	78·5	2·8	5	99·5	0·5	10
8401	pJB5	59·3	2·2	5	84·7	3·4	5	97·9	1·6	9
8401	pIJ1019	56·6	2·6	5	77·1	2·1	5	100·0	0·0	8
8401	pIJ1016	59·4	1·4	5	85·8	4·2	5	88·1	4·0	9

* The minority of mixed nodules (containing both Str-r and Rif-r bacteria) have not been included in this analysis.
† *n*, number of replicates.

Table 3. *Analyses of variance for percentage of streptomycin-resistant strain (after angular transformation)*

The angular transformation is given by arcsin $\sqrt{\text{percent}/100}$ (in radians). DF, degrees of freedom; MS, mean square value.

	Inoculum		Rhizosphere		Nodules	
Source	DF	MS	DF	MS	DF	MS
Plasmid	3	0·0014	3	0·0075	3	2·6798***
Strain	2	0·0308***	2	0·7915***	2	2·9256***
Interaction	6	0·0229***	6	0·0069	6	0·5567***
Error	47	0·0028	48	0·0151	96	0·0633

*** Highly significant effects ($P < 0·0005$).

(Brewin et al., 1983)

Screening System. Ineffective strains, though capable of forming nodules on the plant, do so without contributing much nitrogen to the economy of the host. Consequently, plants nodulated with these rhizobia turn yellow within two to three weeks of growth in nitrogen-free cultures. Any improvements in the effectiveness of a strain will show up in the gross morphology and coloration of the plant nodulated by it. All of the genetically-manipulated strains will be screened on the

appropriate host growing in sterile vermiculite moistened with nitrogen-free nutrient solution (for peas and beans); clover plants will be screened using an agar plate assay (Rolfe et al., 1980). All plants showing growth differences compared with the uninoculated controls will be confirmed by acetylene reduction. Fresh isolation of the bacteria will be made from nodules of plants that are significantly healthier than the controls. The stability of these strains will then be ascertained by further growth chamber tests.

Work Plan

Strains And Sym Plasmids. The relevant characteristics of the strains and plasmids to be used are listed in Table 3.

Plasmid Curing. The standard methods of curing plasmids in *Rhizobium* (heat curing, acridine orange) will be employed.

Bacterial Matings. Standard membrane-filter mating techniques will be employed (Hirsch et al., 1980). Recipients for pSym transfer will be marked with antibiotic resistance markers via standard microbiological practices (spontaneous mutation) to counter-select against the donor strains.

Screening of Transconjugants. Fluorescent antibodies prepared against recipient strains will be used to screen the resulting transconjugants and assure their purity. To demonstrate the presence of pSyms in the recipient strains I will use gel electrophoresis. Backcrosses of the

Table 3. Bacterial Stains and Plasmids used.

Strains and plasmids	Relevant Characteristics	Source
STRAINS		
R. phaseoli		
CIAT 899	Wild-type, Nod$^+$ Fix$^+$ on beans Al^r(80uM)	D. Munns
UHM-5	CIAT 899 pSym$^-$, Ap^r Cm^r Pm^r Al^r(80um)	This study
UHM-11	CIAT 899, Ap^r Cm^r Pm^r, Al^r(80uM) Nod$^+$ Fix$^+$on beans	This study
R. leguminosarum		
6015	pSym$^-$,phe$^-$ trp$^-$	B. Rolfe
R. trifolii		
ANU 1173	Wild-type, Nod$^+$ Fix$^-$ on white clover, Al^r(25uM)	B. Rolfe
UHM-1173	ANU 1173 pSym$^-$, Al^r(25um)	This study
A. tumefaciens		
GM19023	contains R.p.CFN 42 pSym, p42d::Tn5mob, Km^r	R. Palacios
E. coli		
1830(pJB3JI)	pJB3JI is a km^s derivative of RP4 (Km^r Ap^r Tc^r), used for mobilizing Tn5mob containing plasmids	R. Palacios
PN200	=HB101(pRtr514a::R68.45) (R. trifolii pSym cointegrate with the wide host-range self-transmissible plasmid R68.45)	C. Pankhurst
Plasmids		
pJB5JI	R. leguminosarum pSym marked with Tn5, self-transmissible, Nod$^+$ on peas, Nod$^-$ on clover	B. Rolfe
pBR1AN	pJB5JI derivative with altered host-specificity for clovers Nod$^-$ on peas, Nod$^+$ on clover	B. Rolfe

transconjugants, to strains cured of their pSym, will ensure the integrity of the transferred Sym plasmid in its new host.

1. Construction of CIAT 899 Transconjugants.

The symbiotic properties of several transconjugants of CIAT 899, UHM-5 (M5) and the relevant backcrosses to R. leguminosarum 6015 are listed in Table 4. None of the transconjugants of the plasmid-cured strain UHM-5 were Fix$^+$ on any host tested. Transfer of the plasmids pJB5JI and pBR1AN to the wild-type parent CIAT 899 resulted in transconjugants that could generally still nodulate beans effectively but were ineffective on the host for the introduced pSym. When the various transconjugants and backcrosses were screened by gel electrophoresis their plasmid profiles (data not shown) were very different. CIAT 899 has an apparent plasmid incompatibility with both pJB5JI and pBR1AN since the transfer frequency is low and there is extensive rearrangement of plasmid profiles. Other evidence for plasmid rearragement is the phenotypic data of the various backcrosses, many of which are Nod$^-$ or Fix$^-$.

Construction of successful transconjugants requires strains which do not restrict or rearrange the introduced plasmids. Since CIAT 899 is not a strain of choice for these types of constructions another pSym-cured aluminum resistant strain will be employed - UHM-117C.

Table 4. Symbiotic properties of control strains, transconjugants and backcrosses on three host legumes

STRAIN	HOST LEGUME		
	BEANS	CLOVER	PEAS
controls			
CIAT 899	(+/+) *	-	-
M5	-	-	-
6015(pJB5JI)	-	-	(+/±)
6015(pBR1AN)	-	(+/+)	-
transconjugants			
M5(pJB5JI)1	-	ND	-
M5(pJB5JI)5	+/-	ND	-
M5(pJB5JI)10	+/-	ND	-
M5(pJB5JI)15	+/-	ND	-
backcross			
6015(pJB5JI)BC1C	-	ND	+/±
6015(pJB5JI)BC5B	+/±	ND	-
6015(pJB5JI)BC10C	-	ND	-
6015(pJB5JI)BC15B	±	ND	+/+
6015(pJB5JI)BC15C	±	ND	-
6015(pJB5JI)BC15E	+/-	ND	+/-
transconjugants			
M5(pBR1AN)3A	-	-	?
M5(pBR1AN)3C	±/-	+/-	?
M5(pBR1AN)3G	-	-	?
M5(pBR1AN)3J	-	-	?
backcross			
6015(pBR1AN)3G2	-	-	?
6015(pBR1AN)3G3	-	-	?
6015(pBR1AN)3J2	-	-	?
transconjugants			
CIAT 899(pJB5JI)1A	+/+	-	+
CIAT 899(pJB5JI)1B	+/+	-	?
CIAT 899(pBR1AN)2A	+/+	+/-	-
CIAT 899(pBR1AN)2B	-	+/-	-
CIAT 899(pBR1AN)2J	+/+	+/-	+/?
backcross			
6015(pBR1AN)2A2	-	+/-	?
6015(pBR1AN)2B5	-	+/-	?
6015(pBR1AN)2B4	-	+/-	?
6015(pBR1AN)2J1	±	+/-	?

*(Nodulation/Fixation)

LITERATURE CITED

Ayanaba, A., Asanuma, S., and Munns, D.N. 19 . An agar plate method for rapid screening of Rhizobium for tolerance to acid-aluminum stress. (preprint)

Baker, D.E. 1976. Acid soils. pp. 127-140. In M. J. Wright (ed.) Plant Adaptation to Mineral Stress in Problem Soils. Cornell University Agricultural Experiment Station, Ithaca.

Beringer, J, Brewin, N.J., and Johnston, A.W.B. 1980. The genetic analysis of Rhizobium in relation to symbiotic nitrogen fixation. Heredity. 45:161-186.

Brewin, N.J., Wood, E.A., and Young , J.P.W. 1983. Contribution of the symbiotic plasmid in competitiveness of Rhizobium leguminosarum. J. Gen. Microbiol. 129:2973-2977.

Cooper, J.E., Wood, M., and Holding, A.J. 1983. The influence of soil acidity factors on rhizobia. p. 319-335. In D.G. Jones and D. R. Davies (eds.) Temperate Legumes: Physiology, Genetics and Nodulation. Pitman Advanced Publishing Program, Boston.

Date, R.A. and Halliday, J. 1979. Selecting Rhizobium for acid infertile soils of the tropics. Nature. 277:62-64.

Davey, R.B. and Reanny, D.C. 1980. Extrachromasomal genetic elements and the adaptive evolution of bacteria. Evolution. Biol. 13:113-147.

Davies, J. and Smith, D.I. 1978. Plasmid determined resistance to antimicrobial agents. Ann. Rev. Microbiol. 32:469-518.

Davis, W.B., McCauley, M.J., and Byers, B.R. 1971. Iron requirements and aluminum sensitivity of an hydroxamic acid-requiring strain of Bacillus megaterium. J. Bacteriol. 105:589-594.

de Bruijin, F.J. and Lupski, J.R. 1984. The use of transposon Tn5 mutagenesis in the rapid generation of correlated physical and genetic maps of DNA segments cloned into multicopy plasmids - a review. Gene. 27:131-149.

De Carvalho, M.M., Bushby, H.V.A., and, Edwards, D.G. 1981. Survival of Rhizobium in nutrient solutions containing aluminum. Soil Biol. Biochem. 13:542-542.

DeJong, T.M., Brewin, N.J., Johnston, A.W.B. and Phillips, D.A. 1982. Improvement of symbiotic propterties in Rhizobium leguminosarum by plasmid transfer. J. Gen. Microbiol. 128:1829-1839.

Haug, A. 1984. Molecular aspects of aluminum toxicity. CRC Crit. Revs. Plant Sci. 1:345 373.

Hirsch. P.R., van Montagu, M., Johnston, A.W.B., Brewin, N.J., and Schell, J. 1980. Physical identification of bacteriocinogenic, nodulation, and other plasmids of Rhizobium leguminosarum. J. Gen. Microbiol. 120:403-412.

Hooykaas, P.J.J., van Brussel, A.A.N., den Dulk-Ras, H., van Slogteren, G.M.S., and Schilperoort. R.A. 1981. Sym plasmid of Rhizobium trifolii expressed in different rhizobial species and Agrobacterium tumefaciens. Nature. 291:351-353.

Innes, R.W., Kuempel, P.L., Plazinski, J., Canter Cremers, H., Rolfe, B.G., and Djordjevic, M.A. 1985. Plant factors induce expression of nodulation and host-range genes in Rhizobium trifolii Mol. Gen. Genet. 201:426_432.

Johnston, A.W.B., Beynon, J.L., Buchanan-Wollaston, A.V., Setchell, S.M., Hirsch, P.R., and Beringer, J.E. 1978. High frequency transfer of nodulating ability between strains and species of Rhizobium. Nature. 276:635-636.

Keyser, H.H. and Munns, D. 1979a. Effects of calcium, manganese, and aluminum on growth of rhizobia in acid media. Soils Sci. Soc. Am. J. 43:500-503.

Keyser, H.H. and Munns, D. 1979b. Tolerance of rhizobia to acidity aluminum and phosphate. Soil Sci. Soc. Am. J. 43:519-523.

Long, S. R., Egelhoff, T.T., Fisher, R.F., Jacobs, T.W., and Mulligan. J.T. 1985. Fine structure studies of R. meliloti nodDABC genes. p.87-93. In H.J. Evans, P.J. Bottomley, and W.E. Newton (eds.) Nitrogen Fixation Research Progress. Martinus Nijhoff, Dordrecht.

Mulligan, J.T., and Long, S.R. 1986. Induction of Rhizobium meliloti nodC expression by plant exudate requires nodD. Proc. Natl. Acad. Sci. USA. 82:6609-6613.

Munns, D.N., Hohenberg, J.S., Righetti, T.L., and Lauter, D.J. 1981. Soil acidity tolerance of symbiotic and nitrogen-fertilized soybeans. Agron. J. 73:407-410.

Novell, W.A. 1972. Equilibria of metal chelates in soil solution. p. 115-138 In J.J. Mortvedt, F.M. Giordana, and W.L. Lindsey (eds.) Micronutrients in Agriculture. Soil Sci Soc. Am., Madison.

Nuti, M.P., Lepidi, A.A., Prakash, R.K., Schilperoort, R.A., and Cannon, F.C. 1979. Evidence for nitrogen fixation (nif) genes on indigenous Rhizobium plasmids. Nature. 282:533-535.

Prakash, R.K., and Atherly, A.G. 1986. Plasmids of Rhizobium and their role in symbiotic nitrogen fixation. Int. Rev. Cytol.

Reanny, D. 1976. Extrachromasomal elements a possible agents of adaptation and development. Bacteriol. Rev. 40:552-590.

Reisenauer, H.M. 1966. Mineral nutrients in soil solution. p. 507-508. In P.L. Altman and D.S. Dittman (eds.) Environmental Biology. Fe. Amer. Societies for Experimental Biology.

Rolfe, B.G., Gresshoff, P.M., and Shine, J. 1980 Rapid screening for symbiotic mutants of Rhizobium and white clover. Plant Sci Lett. 19:277-264.

Rosenberg, C., Casse-Delbart, F., Dusha, I., David, M., and Boucher,C. 1982. Megaplasmids in the plant-associated bacteria Rhizobium meliloti and Pseudomonas solanacearum. J. Bacteriol. 150:402-406.

Ruvkun, G.B. and Ausubel, F.M. 1980. Interspecies homology of nitrogenase genes. Proc. Natl. Acad. Sci. 77:191-195.

Sanchez, P.A. 1976. Properties and Management of Soils in the Tropics. John Wiley and Sons, New York.

Sadowsky, M.J. and Bohlool, B.B. 1985. Differential expression of the pea symbiotic plasmid pJB5J1 in genetically dissimilar backgrounds. Symbiosis. 1:125:138.

Schwinghamer, E.A. 1977. Genetic aspects of nodulation and dinitrogen fixation by legumes: the microsymbiont. p. 577-622 In R.W.F. Hardy and W.S. Silver (eds.) Treatise on Dinitrogen Fixation. Section 3. Biology. J. Wiley and Sons, New York.

Silver, S. 1981. Mechanisms of plasmid-determined heavy metal resistances. p. 179-189. In S.B. Levy, R.B Clowes, and E.L. Koenig (eds.) Molecular Biology, Pathogenicity, and Ecology of Bacterial Plasmids. Plenum Press, New York.

Simon, R., Priefer, U., and Puhler, A. 1983. Vector plasmids for in-vivo and in-vitro manipulations of gram-negative bacteria. p.98-106. In A. Puhler (ed.) Molecular Genetics of the Bacteria-Plant Interaction. Springer-Verlag, Berlin.

Stanley, J., and Dunican, L.K. 1979. Intergeneric mobilization of Rhizobium nif genes to Agrobacterium and Klebsiella. Molec. Gen Genet. 175:211-220.

Summers, A.O. and Silver, S. 1978. Microbial transformations of metals. Ann. Rev. Microbiol. 32:637-672.

van Brussel, A.A.A., Zaat, S.A.J., Canter Cremers, H.C.J., Wijffelman, C.A., Pees, E., Tak, T., and Lugtenberg, B.J.J. 1986. Role of plant root exudate and sym plasmid-localized nodulation genes in the synthesis by Rhizobium leguminosarum of Tsr factor, which causes thick and short roots on common vetch. J. Bacteriol. 165:517-522.

Wen, G.Y., and Wisniewski, H.M. 1985. Histochemical localization of aluminum in the rabbit CNS. Acta Neuropathol. 68:175-184.

White, R.E., Tiffin, L.O., and Taylor, A.W. 1976. The existence of poymeric complexes in dilute solutions of aluminum and orthophosphate. Plant and Soil. 45:521-529.