PD-AAW-682

AGENCY FOR INTERNATIONAL DEVELOPMENT
WASHINGTON, D.C. 20523

DATE: 9/9/87

MEMORANDUM

TO: AID/PPC/CDIE/DI, room 209 SA-18

FROM: AID/SCI, Victoria Ose

SUBJECT: Transmittal of AID/SCI Progress Report(s)

Attached for permanent retention/proper disposition is the following:

AID/SCI Progress Report No. 2.A-32
~~Semi~~-annual 1/1/84 - 6/30/84

Attachment

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2. A- 32

SEMI-ANNUAL PROGRESS REPORT

for the Period of Ø1-Ø1-84 to Ø6-3Ø-84

Grant: Aid/Sci 2A-32

Title: "A Genetic Engineering Approach to the Improvement of Rhizobium for Tropical Legumes"

Principal Investigator: B. Ben Bohlool

Grantee Institution: University ofawaii - Manoa
Honolulu, HI 96822

INTRODUCTION AND BACKGROUND: Soil acidity is a well known agricultural stress factor. Although increased hydrogen ion can induce stress per se (at pH values below pH 4.2 where increased [H+] may stop or even reverse cation uptake by roots), acid soils may engender more serious problems. Low pH leads to the increased availability of Mn and Al, and decreased levels of Mg and Ca.

The acid tolerance of legumes varies with their mode of N nutrition; legumes become more sensitive to soil acidity if they depend upon symbiotic nitrogen fixation. Low pH, high levels of soluble aluminum and calcium deficiency inhibit Rhizobium growth, root infection, and nodule activity enough to account for this symbiotic failure. Elite laboratory selected rhizobia capable of fixing increased amounts of nitrogen often fail in the field due to soil stresses such as aluminum. The transfer of the genetic mechanism of aluminum tolerance to elite rhizobia could greatly aid legume production in the tropics.

Mechanisms for resistance/tolerance to metal toxicity in many bacterial systems are often encoded by extrachromasomal genes carried on plasmids. The presence of very large molecular weight plasmids is well documented in the fast-growing rhizobia. As a prelude to our main objective of cloning and transferring the aluminum tolerance gene(s), we undertook a series of experiments to address the physiological basis of Al resistance.

During the first stage of this project we aquired cultures of Rhizobium from various sources. These cultures, as well as many in our own collection were screened for tolerance/sensitivity to acidity (pH 4.45) and acidity plus aluminum (either 50

or 80 uM). These results were tabulated in a previous report. Due to its high level of aluminum tolerance the fast growing R. phaseoli strain CIAT 899 was chosen for further work.

The following sets of experiments were designed to help us focus in on the mechanism of Al tolerance in order to approach the cloning of the appropriate genes more methodically.

PHYSIOLOGICAL STUDIES

(1)ALUMINUM BINDING: Both aluminum tolerant and aluminum sensitive cells were found to bind aluminum and remove it from the medium. Since there was no apparent difference in this Al binding/removal between tolerant and sensitive strains further experiments were designed to try to explain why some strains were tolerant of high levels of Al.

(2)PRE-CONDITIONING OF MEDIA: In this set of experiments Al tolerant strain CIAT 899 and two Al sensitive strains Pre (R. leguminosarum) and USDA 3HOa9 (Rh. for chickpea) were grown in acid media pH (4.45) (control, all strains grew) and acid medium plus 80 uM Al (only 899 grew). The cells were removed from the media by centrifugation and the spent media were resterilized by filtration. The three cultures were freshly inoculated into these spent media conditioned by the other strains. Growth of CIAT 899 in medium + Al did not alleviate the toxic effects of Al for the sensitive strains Pre and USDA 3HOa9.

(3)CO-GROWTH EXPERIMENT: Although CIAT 899 did not detoxify the medium to allow for the growth of Al-sensitive strains we reasoned that growth of CIAT 899 together in the same flask with

an aluminum sensitive strain might alleviate the toxic effects of Al and allow the sensitive strain to grow. For example, a component of the growing cell's surface might be involved in Al tolerance.

Figure 1A illustrates the growth of three strains by viable count (strains grown separately, three replicates) and demonstrates the aluminum sensitivity of strains Pre and USDA 3HOa9. Figures 1B and 1C are the results of the co-growth experiment where population changes were followed by fluorescent antibody filter counts. Although all strains grew well under acid conditions (Ø Al) only CIAT 899 grew when aluminum (8Ø uM) was present. The co-growth of CIAT 899 did not alleviate the toxicity of Al on the sensitive strains.

(4)CELL FREE EXTRACT OF CIAT 899: Since nothing liberated by CIAT 899 into the growth medium, or potentially on the surface of growing cells could alleviate Al toxicity for the sensitive strains, we reasoned that an internal component(s) might be involved in Al tolerance of CIAT 899. To determine if this was the case cell free extracts were made of strain CIAT 899 grown in 6 liters of medium containing 5ØØuM Al. The cells were harvested by centrifugation, and washed three times at pH 4. The washed pellet was passed through a French Press at 4°C. The extract was sterilized by filtration and frozen. Aliquots of this extract (Ø.5ml) were added to flasks containing 5Ø mls of medium with and without 8ØuM AL. There were three replicate flasks per treatment and a total of eight treatments (e.g. two strains in media with and without Al and/or extract). Growth of the strains was followed by viable counts. The results are illustrated in Figure

2. Strain TAL-1472, an Al sensitive R. phaseoli strain was employed in these experiments. Addition of the cell free extract of aluminum grown CIAT 899 did ameliorate the conditions for TAL-1472, and may have even accelerated the die-off.

(5)SUMMARY: CIAT 899 is an extremely aluminum tolerant strain. Nothing liberated by this organism into the growth medium or associated with the growing cell or soluble internal compounds alleviated aluminum toxicity of Al sensitive strains. An abstract of the results of these physiological experiments has been summitted for the March 1984 Annual Meeting of the American Society of Microbiology (see attached).

GENETIC STUDIES

(1)PLASMID SCREENING: Eleven of the fast growing strains were screened for the presence of plasmids. Atleast one plasmid >100 x 10^6 MW was found in each strain. Using the conventional alkaline lysis technique only one megaplasmid was repeatedly observed for Al tolerant CIAT 899. Figure 3 shows an example of a plasmid pattern of six 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 CIAT 899 was screened for plasmids by a direct in well lysis procedure (modified Eckhardt) a second, larger plasmid band was observed indicating the presence of a much larger megaplasmid (FIGURE 4). This plasmid had not been found by the conventional lysis procedures due to its large size and therefore its sensitivity to breakage. Two plasmid bands were also consistantly obtained for strain TAL-1472 when the in

well procedure was employed.

(2)CHEMICAL MUTAGENESIS: In order to generate Al sensitive mutants several chemical mutagens have been employed: NTG (nitrosoguanidine); EMS (ehtylmethanesulfonate); and nitrous acid. The strains employed were found to be resistant to NTG and EMS but CIAT 899 was found to be susceptible to nitrous acid mutagenesis.

(3)TRANSPOSON MUTAGENESIS: The advantage of transposon mutagenesis is that in addition to creating a mutation the transposon also marks the site of the mutation in the genome. This makes finding the segment of DNA, for cloning purposes, relatively easy.

Aluminum tolerant CIAT 899 was mated with E. coli S-17-1 carrying the Tn5 suicide plasmid pSUP2021; 1,200 Tn5 containing (kanamycin resistant) CIAT 899 colonies were patched onto master plates and then replica plated onto minimal media to look for auxotrophs. Out of the 1,200 clones 16 putative auxotrophs were obtained. This low figure is probably due to the high rate of insertion of Tn5 into CIAT 899's two megaplasmids. All of the remaining 1,200 clones grew on media containing 80 uM AL, none became Al sensitive.

(4)PLASMID CURING: To determine whether Al resistance genes reside on plasmids a number of attempts were made to cure CIAT 899 of its plasmids 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 megaplasmids. However, the combination of elevated

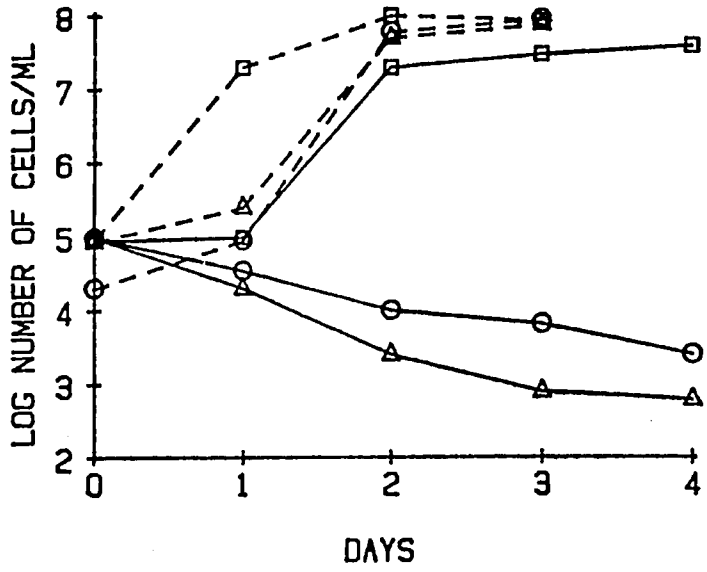
temperature (37°C) and 12 ug/ml acridine orange cured CIAT 899 of one of its two plasmids. The cured mutants fail to nodulate P. vulgaris and are rough when plated onto YEM medium, in contrast to the gummy parental culture. The rough mutants still react with CIAT 899 fluorescent antibody. The mutants have not yet been tested for Al tolerance/sensitivity.

(5)GENE BANK: All materials for constructing a gene bank of strain CIAT 899 have been obtained, and lambda phage packaging extracts have been made. Total DNA from CIAT 899 will be packaged into cosmids to create the clone bank. This bank can then be mated en masse with any of several Al sensitive strains. Any Rhizobium recipient which grows on Al should contain the segment of CIAT 899 DNA responsible for AL tolerance, which can then be further characterized.

Figure 1: Co-Growth of an Aluminum Tolerant Strain with Aluminum Sensitive Strains.

A.

Viable Counts
(single strains)

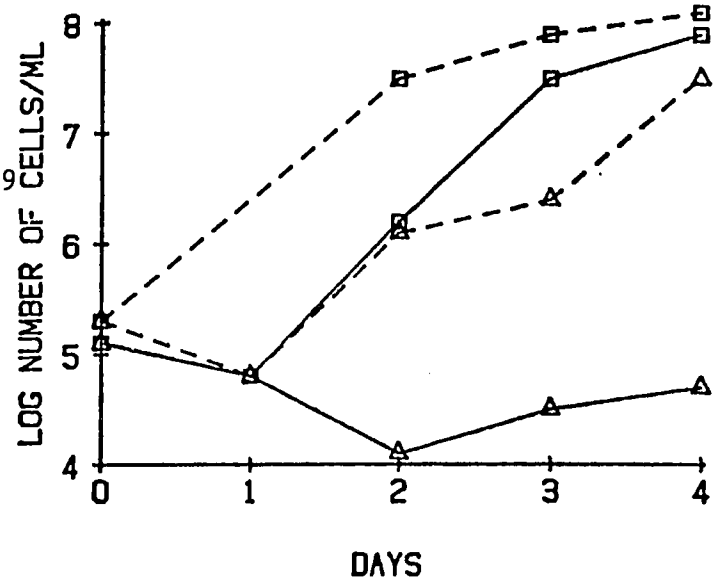


Key:

- □ CIAT 899
- △ USDA 3HOa9
- ○ Pre
- -- H^+
- — 80 μM Al

B.

Co-growth
CIAT 899, USDA 3HOa9
(FA-filter counts)



C.

Co-growth
CIAT 899, USDA 3HOa9
(FA-filter counts)

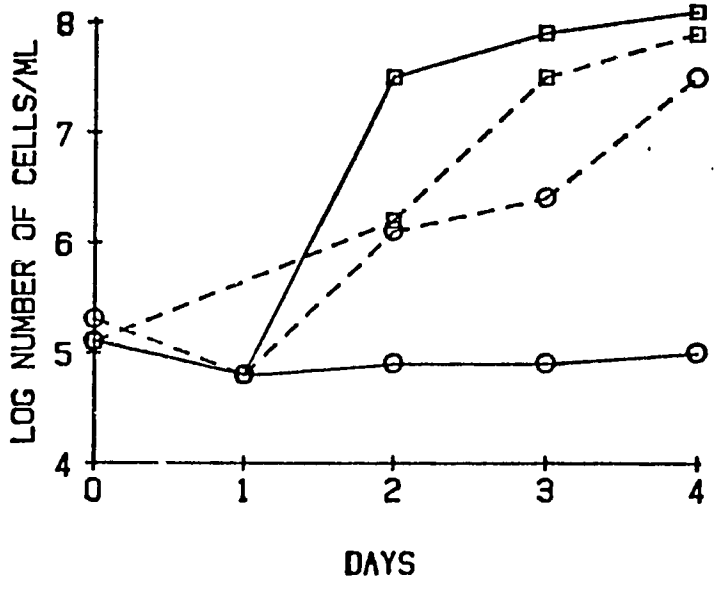
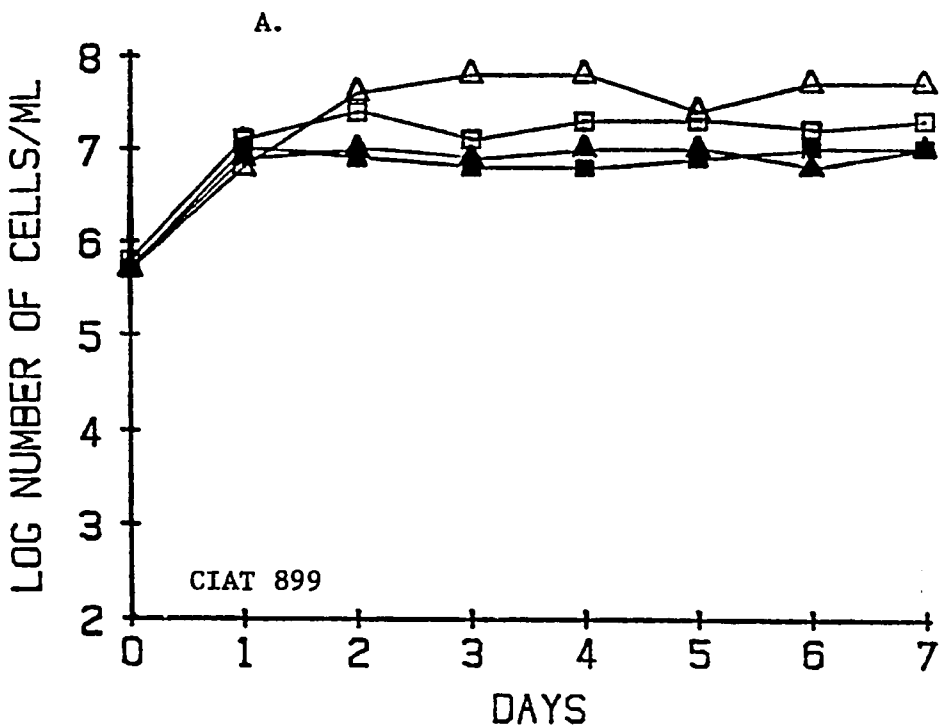


Figure 2: Effect of Cell Free Extract of Aluminum Tolerant CIAT 899 on Growth of CIAT 899 and Sensitive TAL-1472.

Key:
- CIAT 899 Extract
- No Extract
- pH 4.45, no Aluminum
- pH 4.45, Al 80 μM



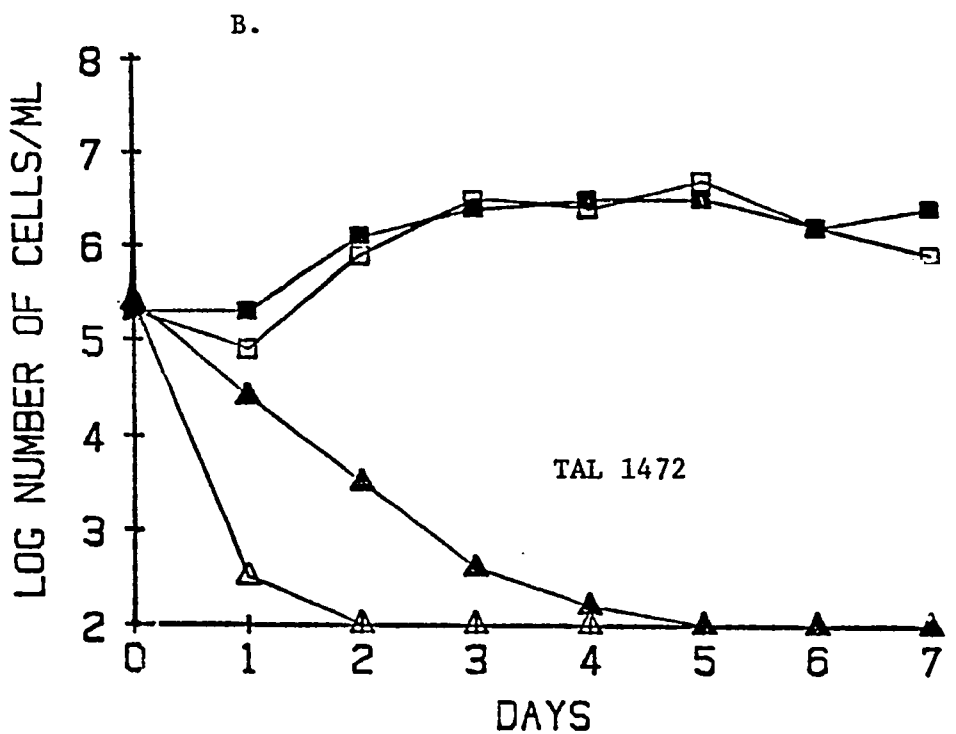


FIGURE 3: Example of plasmid pattern with conventional lysis

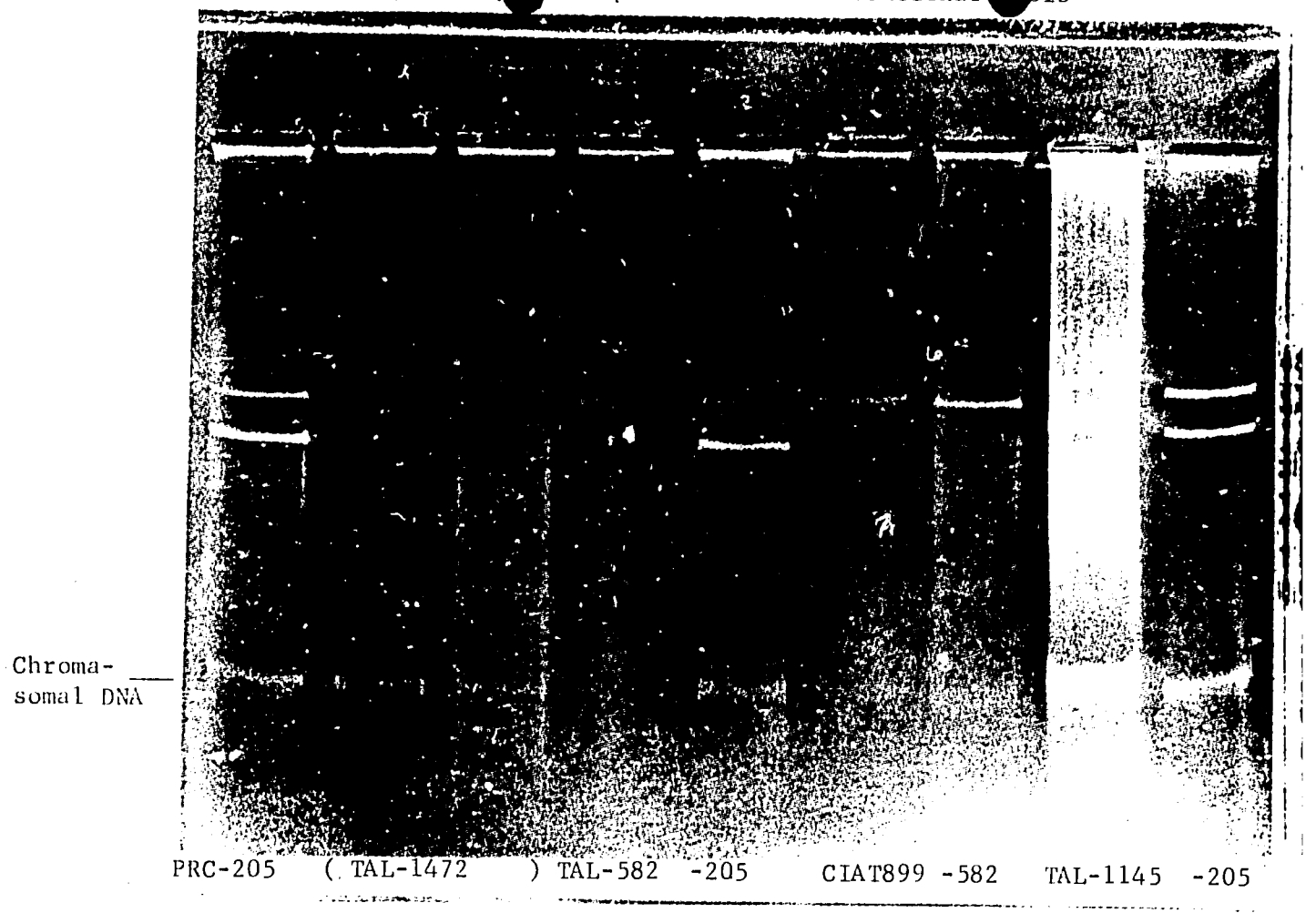
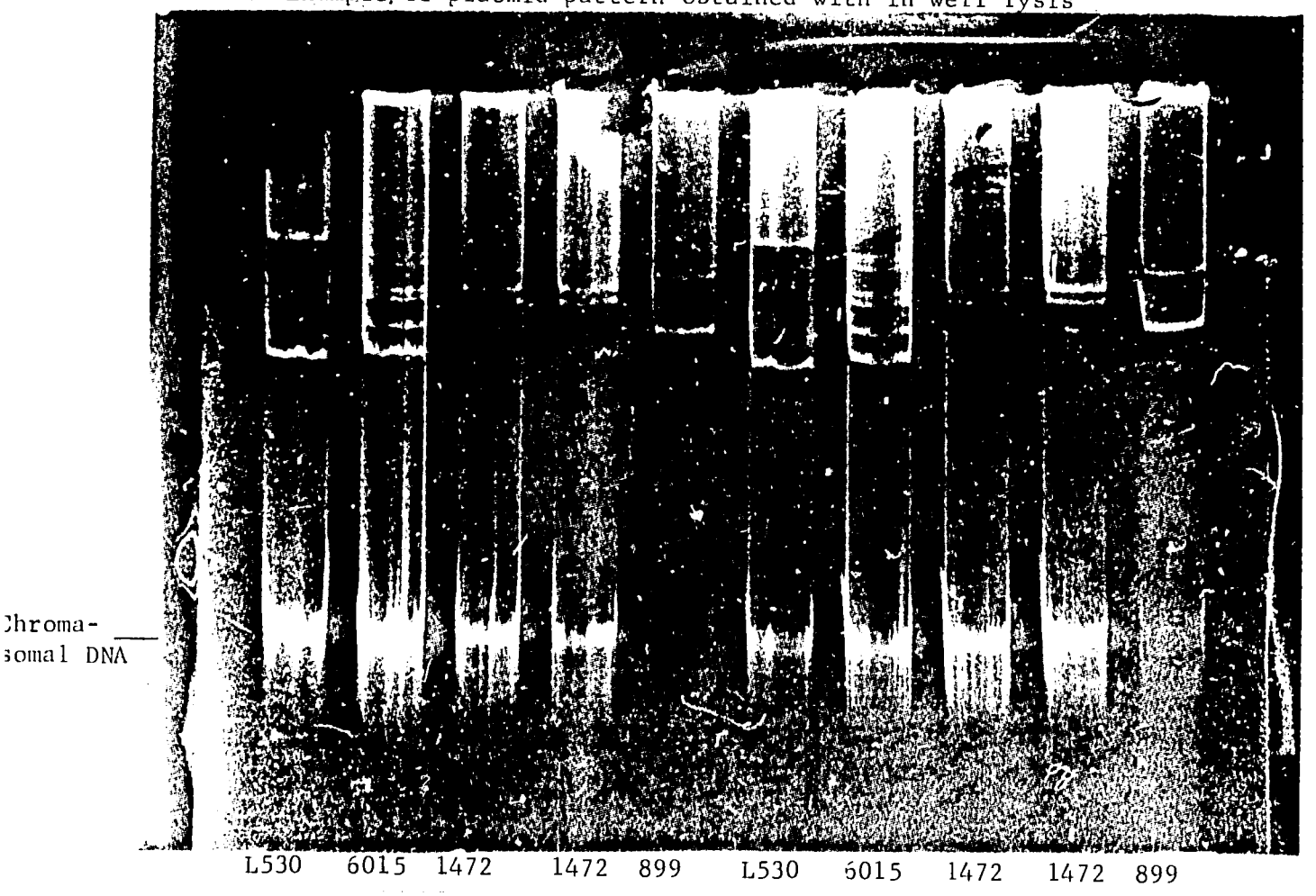


FIGURE 4: Example of plasmid pattern obtained with in well lysis



Official Abstract Form (American Society of Microbiology Annual Meeting; March 1985, Las Vegas

(Read all instructions before typing)

Physiological Investigations of Aluminum Resistance in a Strain of *Rhizobium phaseoli*. M.T. Kingsley, B.B. Bohlool, University of Hawaii, Honolulu, Hawaii

Acid soils in the tropics often contain high levels of soluble aluminum (Al) which may be toxic to microorganisms and plants. Success of the *Rhizobium* inoculum in these soils depends on the ability of the strains to withstand such high levels of Al. Although many Al-resistant strains of *Rhizobium* have been reported, the mechanism of resistance is not understood. In this study we devised three approaches to test whether Al-resistance might be due to: 1) extracellular soluble products -- spent medium from cultures of the resistant *R. phaseoli* (CIAT 899) did not remove Al-toxicity against Al-sensitive strains; 2) cell surface components -- the sensitive and the resistant strains grew well in co-culture in acid media (pH 4.6) in the absence of Al, however, when Al was added to the co-culture the sensitive strain was unable to grow; 3) internal factors -- addition of cell-free extract of the Al-resistant strain did not alleviate aluminum toxicity for the sensitive strain. An understanding of the physiological basis of Al-resistance would be helpful in locating the genes responsible.

☐ AO

☐ AP

☐ R

Instructions

Indicate below the subject category number from the list on p. iv, check your poster or slide session preference, complete the check list on the reverse side of this sheet, and sign your name in the space provided.

Indicate category number from page iv [83]

Category number

Poster/Slide Session Preference

Because of the flexibility in programming afforded by poster sessions, the Program Committee will attempt to schedule *all abstracts* which (i) are considered by elected divisional officers to be of acceptable quality and (ii) conform to rules established by the Program Committee. This has resulted in the elimination of "read-by-title" papers. The decision of whether an abstract is scheduled in a slide or a poster session will be made by the elected Program Committee, which will be guided (but not bound) by the preference of the authors. Approximately 70% of the abstracts will be scheduled in poster sessions. By submitting an abstract, the author agrees that the paper will be presented as scheduled.

Please check one:

☒ Poster session preferred
☐ Slide session preferred
☐ No preference

10