APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1981, p. 241-248
0099-2240/81/080241-08$02.00/0
Vol. 42, No. 2

Release of *Rhizobium* spp. from Tropical Soils and Recovery for Immunofluorescence Enumeration

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Received 3 February 1981/Accepted 13 May 1981

Limitations associated with immunofluorescence enumeration of bacteria in soil derive largely from the efficiency with which cells can be separated from soil particles and collected on membrane filters for staining. Many tropical soils fix added bacteria tightly, resulting in low recoveries. Eight soils, representative of three of the major soil orders found in the tropics (oxisols, vertisols, and inceptisols), were tested for recovery of added *Rhizobium* strains. All except one Hawaiian andept (Typic Eutrandept) yielded recoveries ranging from <1 to 13%. Recovery from the andept was 100%. In soil-sand mixtures, addition of only a small amount of soil caused a dramatic decrease in recovery of added rhizobia. Increasing the soil content of the mixture from 0% (10 g of sand) to 50% (5 g of soil-5 g of sand) reduced recoveries from >90 to <1%. Varying the ionic strength and pH of the extracting solution did not cause marked increases in recovery. Protein solutions, ethylenediaminetetraacetate, and $NaHCO_3$, on the other hand, improved release of bacteria. We report a modification to the usual membrane filter immunofluorescence procedure which yielded consistently high and reproducible recovery (coefficient of variation, 30%) of rhizobia from several tropical soils. In the modified procedure, partially hydrolyzed gelatin, diluted in ammonium phosphate, was used to suspend the soil. This caused dispersion of the soil and release of the bacteria from soil flocs. The efficiency of recovery of *Rhizobium* spp. from several tropical and two temperate soils remained high as the content of these soils in soil-sand mixtures was increased from 0 to 100%. The modified membrane filter immunofluorescence procedure was used to follow the growth of a strain of chickpea (*Cicer arietinum*) *Rhizobium* in a sterilized oxisol. The results showed a close agreement with viable counts at different stages during the growth cycle. Diluent for the hydrolyzed gelatin also had a marked effect on recovery. The efficiency of release of *Rhizobium* spp. from an oxisol was in the following order for the diluents used: 0.1 M $(NH_4)_2HPO_4$ > 0.1 M Na_2HPO_4 = 0.1 M sodium-phosphate-buffered saline (pH 7.2) > 0.2 M NH_4Cl > 0.2 KCl > NaCl = LiCl > water.

The fluorescent-antibody, or immunofluorescence (IF), technique provides a highly sensitive technique for simultaneous detection and identification of specific microorganisms directly in the natural sample. Various applications of the technique in microbial ecology were reviewed recently (4).

A quantitative membrane filter immunofluorescence (MFIF) procedure has been developed for enumerating specific bacteria directly in soil (3, 13). The essential steps in this method involve dispersion of a diluted soil sample to release bacteria into suspension, flocculation to remove soil colloids from the supernatant, filtration of a portion of the supernatant through an appropriately pretreated nonfluorescent membrane filter, and finally, the microscopic enumeration of the specific bacteria on the fluorescent-antibody-stained membrane filter surface.

Limitations associated with fluorescent-antibody quantification of soil organisms derive largely from the efficiency with which cells can be released from soil particles and retained in suspension after flocculation. Recovery of the desired organism varies with the organism and with the soil. Bohlool and Schmidt (3) reported recoveries for *Rhizobium japonicum* USDA 110 of 25 to 130% relative to viable counts at various growth stages in a sterilized Clarion soil. Recovery of *Escherichia coli* cells from nonsterile soil after 1 and 3 days was reported by Schmidt (13) to be 89 and 64%, respectively, relative to viable counts on selective medium. *R. japonicum* USDA 123 was recovered from a Waukegan soil

by Reyes and Schmidt (11), with an estimated 30% efficiency. Vidor and Miller (17), using the same procedure but 1% $CaCl_2$ as flocculant, recovered *R. japonicum* USDA 110 from a Rossmoyne soil with 80% efficiency, but from a Miami silt loam with only 20% efficiency.

New modifications are needed to enhance recovery from refractory soils, and of refractory organisms, to increase the usefulness of IF as an autecological tool. Wollum and Miller (19) have recently reported a sucrose density gradient centrifugation procedure as a means to achieve high recoveries. Their results indicate recovery rates near 100% with inoculated soils.

We have found that most tropical soils fix added rhizobia rapidly and irreversibly with respect to the usual recovery procedure for quantitative MFIF. In this paper we describe modifications that yield consistently high and reproducible recovery data for *Rhizobium* spp. from several tropical and a few temperate soils. The effectiveness of the procedure is illustrated by comparing IF and viable counts of rhizobia at different stages of growth in sterilized soils.

MATERIALS AND METHODS

Source and maintenance of cultures. Two strains of *Rhizobium* spp., TAL-620 (*Rhizobium* sp. for chickpea, obtained from NifTAL) and Hawaii-5-0 (*R. leguminosarum*, isolated in Hawaii), were used in these experiments. All strains were maintained on YEMS agar medium (2) and grown for inoculum in broth of the same composition. Chickpea strain ICRISAT 3889 (same serotype as TAL-620), obtained from P. Dart, ICRISAT, Hyderabad, India, was used in one experiment.

Chemical reagents. Reagent-grade chemicals were used in all experiments. Antifoam C emulsion, thimerosal (Merthiolate), and Tween 80 were obtained from the Sigma Chemical Co., St. Louis, Mo.; Nonidet P-40 was from Gallard-Schlesinger Chemicals, New York, N.Y. and peptone and gelatin were from Difco Laboratories, Detroit, Mich.

Soils. Surface (0 to 15 cm) samples from eight tropical soils were used in this study. The pH, cation-exchange capacity, percent organic matter, and place of origin of these samples are given in Table 1. Two temperate mollisols were obtained through the courtesy of E. L. Schmidt (University of Minnesota, St. Paul): Clarion (Typic Haploboroll, pH 6.9) and Hubbard (Udorthentic Haploboroll, pH 5.2). All soils were air dried and sieved through a no. 25 mesh (710 μm), and 10-g portions were dispensed in screw-cap tubes (25 by 200 mm). Before inoculation the soils were moistened to 60% of the water-holding capacity (corresponding to 0.08 bar of tension). Soil sterilization was done by autoclaving for 1.5 h at 121°C before moistening.

IF procedures. Fluorescent antibodies were prepared by the method of Schmidt et al. (14). The quantitative MFIF procedure has been described in detail (13). Several modifications to the published procedure should be noted. Polycarbonate membrane filters (Nuclepore Corp., Pleasanton, Calif.) stained overnight with Irgalan Black (5) were substituted for India ink-stained membrane filters (Millipore Corp., Bedford, Mass.); the gelatin-rhodamine isothiocyanate conjugate (1) was allowed to dry completely on filters before staining; and gelatin-coated filters were stored dry in a desiccator or left in the drying oven (60°C) until staining was done. IF observations were made with a Zeiss Universal microscope, equipped with incident illumination from an HBO-200 (OSRAM) light source and a Zeiss fluorescein isothiocyanate filter pack.

Demonstration of the sorptive nature of tropical soils. Exponentially growing cultures of rhizobia were adjusted to 2×10^7 cells per ml with saline (= inoculum). The inoculum size was estimated by

TABLE 1. *Recovery of Rhizobium strain TAL-620 from eight tropical soils by using the usual MFIF procedure*[a]

Soil order/ series	Soil family	Origin	pH	CEC (meq)	% OM	No./g of soil		Mean recovery (%)[b]
						Added	Recovered	
Oxisol								
Wahiawa	Tropeptic Eutrustox	Hawaii	5.4	23	2.3	4×10^6	8×10^2	<1
Molokai	Typic Torrox	Hawaii	6.5	20	3.7	4×10^6	6×10^4	1.5 (1–2)
Vertisol								
Lualualei	Typic Chromustert	Hawaii	7.1	34	0.7	4×10^6	3×10^4	<1
Inceptisol								
PLP[c]	Hydric Dystrandept	Indonesia	4.6	48	5.7	4×10^6	2×10^4	<1
Burabad	Hydric Dystrandept	Philippines	4.8	45	12.9	4×10^6	2×10^4	<1
LPHS[c]	Hydric Dystrandept	Indonesia	5.7	53	6.4	4×10^6	5×10^5	13 (10–16)
Makiki	Andic Humitropept	Hawaii	6.1	NR	NR	4×10^6	5×10^5	13 (10–16)
Waimea	Typic Eutrandept	Hawaii	6.2	50	13	4×10^6	4×10^6	100 (90–110)

[a] Soil physicochemical data were compiled from Ikawa (6) and the Soil Conservation Service (15, 16). CEC, Cation-exchange capacity; OM, organic matter; NR, not reported.

[b] Results are means of duplicate measurements; the range of each of the datum points is given in parentheses.

[c] Soil code, Benchmark Soils Project/University of Hawaii.

Petroff-Hauser and fluorescent-antibody-membrane filter counts. Each tube, containing 10 g of nonsterile soil, was inoculated with 2 ml of inoculum (= 4×10^6 cells per g of soil). Two tubes containing 10 g of silica sand each served as inoculated nonsoil controls. After incubation for 2 h at room temperature, the recovery of the bacteria was determined by using the MFIF procedure (13).

A soil titration experiment was designed to test the effect of increasing concentrations of soil on recovery of rhizobia. Different proportions of soil were mixed with enough sand to give 10 g. To these mixtures were added known numbers of bacteria, and after 2 h each mixture was extracted by the MFIF method (13).

Testing different extractants for improved recovery. The Wahiawa oxisol was chosen as the model problem soil, for it consistently gave low recovery data. Soils were distributed in 10-g portions into test tubes. Known numbers of strain TAL-620 were added to each tube, and their recovery was tested by the MFIF method (13); however, the following extractants were substituted for water: 1 M KCl, 0.4 M HCl, 0.2 M $CuSO_4$–0.002 M Ag_2SO_4 (nitrate extractant), 10% ethanol, 100% methanol, 25% Nonidet P-40 in water, 3 M NaCl, 0.01 and 0.1 M sodium ethylenediaminetetraacetate, 0.5 and 1.0 M $NaHCO_3$ (phosphorus extractant), 1 and 2% peptone, and 0.1% partially hydrolyzed gelatin in various diluents.

Modified quantitative MFIF procedure. (i) Extractant. A 1% solution of gelatin (Difco) in water was adjusted to pH 10.3 and hydrolyzed by autoclaving (121°C, 15 lb/in^2) for 10 min. A 1:10 dilution of this in 0.1 M $(NH_4)_2HPO_4$ was used as the extractant.

(ii) Extraction. A 10-g portion of the soil sample and 95 ml of extractant were placed in a 250-ml screw-cap Erlenmeyer flask and shaken for 5 min on a wrist-action shaker (other means of shaking were unsatisfactory). To the soil suspension was added 0.7 g of flocculant [$Ca(OH)_2$-$MgCO_3$, 2:5], and the contents were mixed and transferred to a 100-ml graduated cylinder. Samples were taken from the supernatant after the flocculated colloids were allowed to settle for 1 h.

(iii) MFIF. Appropriate volumes of the supernatant were filtered through Irgalan Black-treated, 0.4-μm Nucleopore filters. The filter was transferred to a microscope slide, and the effective filtering area was covered with 0.5 ml of gelatin-rhodamine conjugate (1). The filters were dried in an oven at 50 to 60°C and kept there until staining was done. The treated slides were stained (13) with appropriate dilutions of the desired fluorescent antibody.

Growth of *Rhizobium* spp. in sterilized soils. The effectiveness of the modified MFIF procedure for extracting bacteria from soil was tested by comparing IF counts with viable numbers at different stages of growth in sterilized soils. Ten-gram samples of soil, dispensed in screw-cap tubes (20 by 200 mm), were autoclaved for 1.5 h at 121°C and 15 lb/in^2. Known numbers of different *Rhizobium* cultures were added to each tube. Tubes were incubated at 28°C, and four replicates were analyzed at different times for IF and viable counts. Viable counts were done on YEMS medium (2), using the Miles/Misra drop plate method (18).

Statistical analyses. One-way analysis of variance was used to compare differences between different extractants and different diluents for recovery from the Wahiawa oxisol.

The reproducibility of the MFIF technique is expressed as the combined coefficient of variation of all of the datum points (four replicates each) for the growth rate study.

RESULTS

Recovery of *Rhizobium* strain TAL-620 from eight tropical soils. Table 1 illustrates the sorptive nature of most tropical soils in an aqueous extractant. Known numbers of a strain of chickpea (*Cicer arietinum*) *Rhizobium*, TAL-620, were added to eight soils. The MFIF method was used to estimate the recovery of these bacteria after 2 h of incubation in each soil. All soils except a Hawaiian andept (Waimea, Typic Eutrandept) were found to be highly sorptive for the added bacteria: recoveries were <1% in four of the soils, 1.5% in one, and 13% in two, with the Waimea andept releasing 100% of the added cells.

Entrapment of the bacteria in the colloidal flocs is illustrated in Fig. 1A and B, showing strain TAL-620 embedded in a soil floc from a water-Tween 80 suspension of Wahiawa oxisol before (A) and after (B) flocculating with $Ca(OH)_2$-$MgCO_3$.

The results of the soil titration experiment (Fig. 2) illustrate that as the amount of soil is increased relative to a constant number of added bacteria, the recovery of those bacteria is decreased accordingly.

Efficacy of different extractants for recovering *Rhizobium* spp. from soil. Of the 16 different extractants, only 5 improved recovery significantly ($\alpha = 0.05$). These are given in Table 2. Extractants 1 M KCl, 0.4 M HCl, anion extractant ($CuSO_4$-Ag_2SO_4, 0.2:0.002 M), 10% ethanol, and 100% methanol, all containing 0.02% Tween 80 and antifoam, did not improve recovery above the value obtained with water-Tween 80. Nor did substitution of a nonionic detergent, a 25% aqueous solution of Nonidet P-40, for Tween 80 yield greater recoveries.

Partially hydrolyzed gelatin in ammonium phosphate for extracting *Rhizobium* spp. from soils. (i) Added rhizobia. The modified procedure, which uses 0.1% hydrolyzed gelatin in 0.1 M $(NH_4)_2HPO_4$ as an extractant, yielded consistently high and reproducible recoveries of added *Rhizobium* spp. from various field soils. Figure 1C shows that very few bacteria remained on the flocs after treatment with the gelatin extractants. Bacteria that were embedded in soil colloids (Fig. 1A and B) were effectively released into suspension. The results

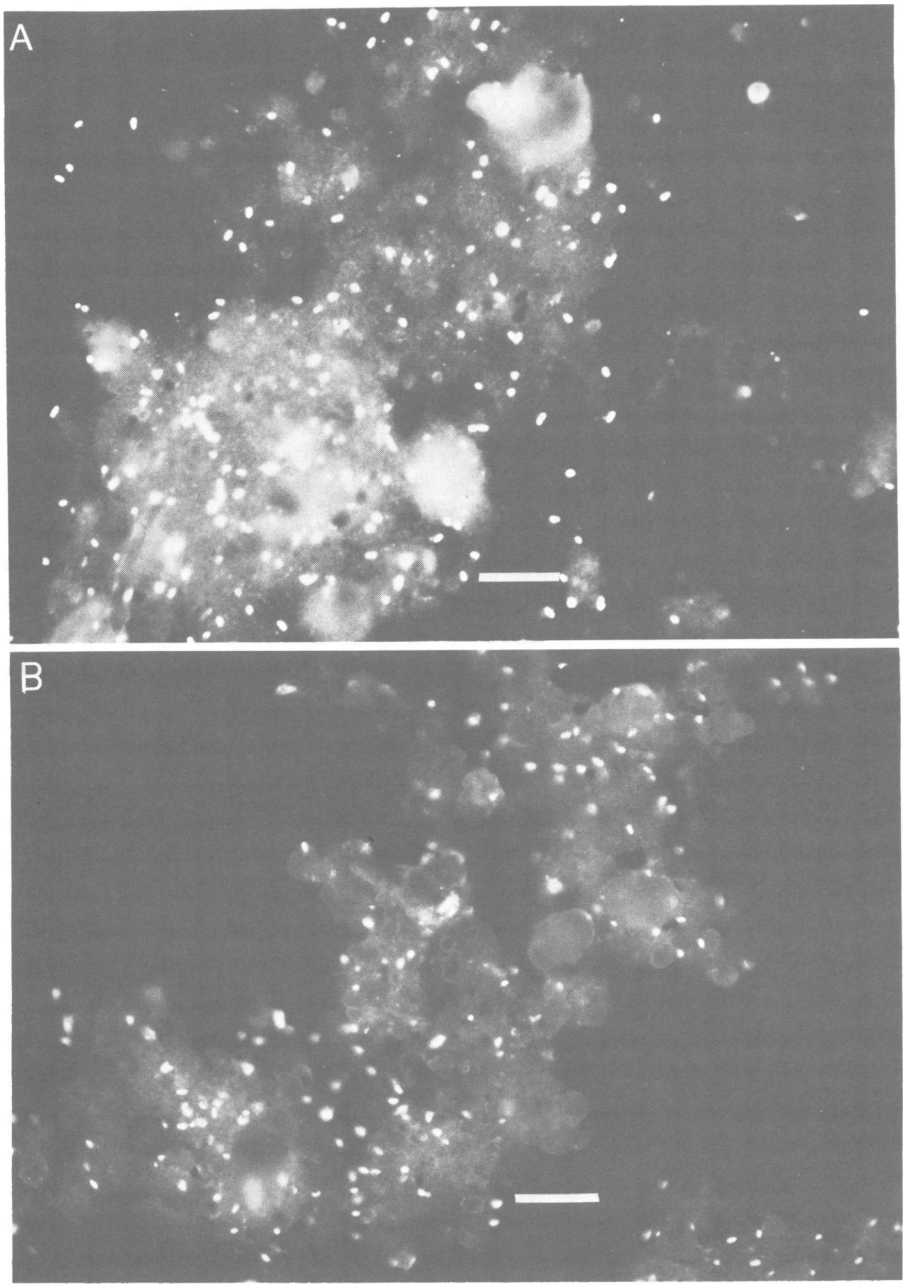

FIG. 1. *Entrapment of Rhizobium strains TAL-620 in flocs of Wahiawa oxisol and their release after treatment with partially hydrolyzed gelatin. IF was used to detect the appropriate strain in the soil flocs: (A) strain TAL-620 in unflocculated colloidal particles; (B) in flocculated [$Ca(OH)_2$-$MgCO_3$, 2:5] particles; and (C) particles after treatment with gelatin. Bar, 10 μm. Soil particles fluoresce orange, due to the rhodamine-gelatin background stain (1), whereas the bacteria shine with an apple-green fluorescence.*

in Fig. 3 illustrate that increasing the amount of soils in a soil-sand mixture did not affect recovery of added rhizobia when the modified procedure was used, whereas recoveries decreased with increasing soil content when water-Tween 80 was used as an extractant.

(ii) Rhizobia growing in soil. In another series of experiments, the modified procedure

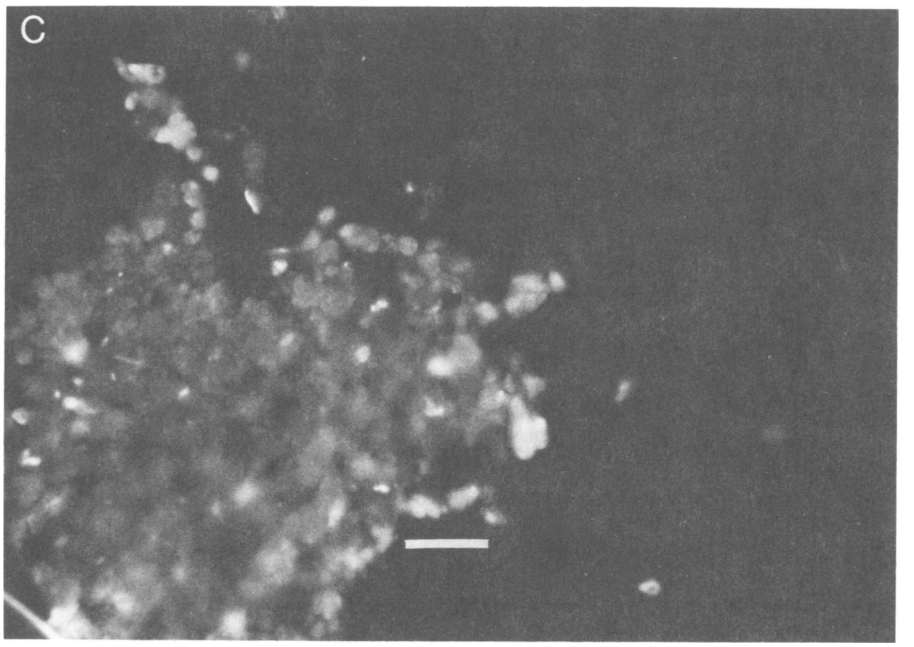

FIG. 1C

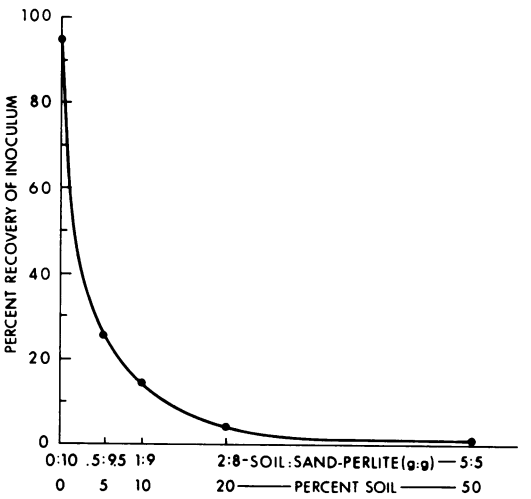

FIG. 2. *Effect of increasing concentrations of Wahiawa oxisol in a soil-sand mixture on recovery of ICRISAT 3889. Each datum point is the mean of duplicate measurements.*

was used to recover rhizobia growing in sterilized soil. Figure 4 shows the growth curve of TAL-620 in sterilized Wahiawa oxisol. The results illustrate the similarity of the growth curves obtained by the modified MFIF and by viable counts on plates.

Effect of different diluents of partially hydrolyzed gelatin on recovery. Table 3 shows that 0.1 M $(NH_4)_2HPO_4$ is the best diluent for hydrolyzed gelatin in releasing strain TAL-620 from Wahiawa oxisol. In general, phosphates were better than chlorides, and ammonium was superior to potassium, sodium, and lithium for removing rhizobia from soil colloids.

TABLE 2. *Extractants that improve recovery of TAL-620 from Wahiawa oxisol*

Extractant	Tween 80	No./g[a]		
		Added	Recovered	% Recovery
3 M NaCl	+	3×10^6	9×10^4	3
0.01 M Na-EDTA[b]	+	1×10^7	1.5×10^6	15
0.1 M Na-EDTA	+	1×10^6	3×10^4	3
0.5 M $NaHCO_3$	−	1×10^7	3×10^6	33
1.0 M $NaHCO_3$[c]	−	1×10^6	8×10^4	8
1% peptone	−	1×10^7	3×10^6	33
2% peptone	−	1×10^6	1.5×10^5	15

[a] Values are means of duplicate measurements.
[b] EDTA, Ethylenediaminetetraacetate.
[c] Phosphorus extractant.

DISCUSSION

The predominance of the solid phase is one major feature that distinguishes soils from other microbial habitats. Even in aquatic environments with a large liquid/solid ratio, it is on surfaces of solids and particulate materials where most microbial activity is found (7, 10).

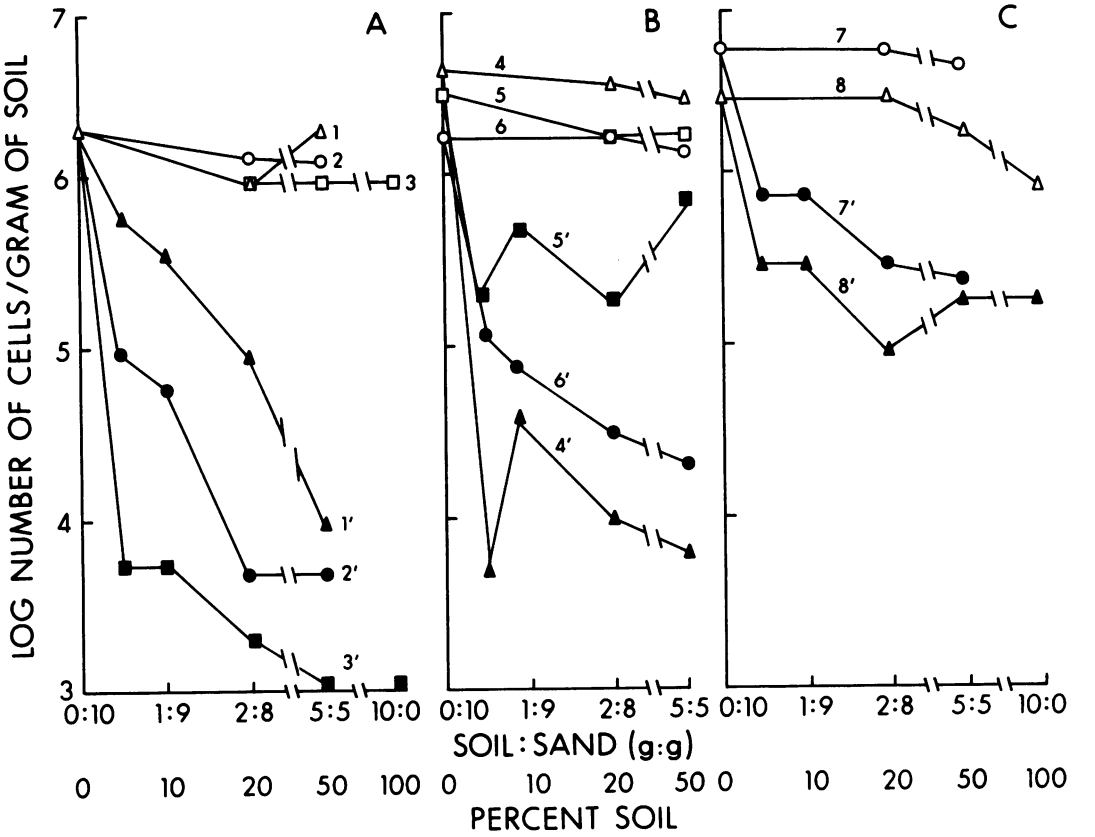

FIG. 3. *Comparison of hydrolyzed gelatin-ammonium phosphate and water-Tween 80 extractants for release and recovery of Rhizobium spp. from soil-sand mixtures. Closed symbols indicate results with MFIF (water extractant); open symbols indicate those with the modified MFIF (gelatin extractant). 1, 1′ = TAL-620 in Wahiawa; 2, 2′ = TAL-620 in Molokai; 3, 3′ = Hawaii-5-0 in Wahiawa; 4, 4′ = TAL-620 in Burabad; 5, 5′ = TAL-620 in Waimea; 6, 6′ = TAL-620 in Lualualei; 7, 7′ = TAL-620 in Clarion; 8, 8′ = TAL-620 in Hubbard (refer to Table 1 for details of soils). Each datum point is the mean of duplicate measurements.*

In soils, microorganisms attach to clay surfaces primarily by electrostatic forces (8). Based on this, Niepold et al. (9) have suggested that detachment of bacteria from soil particles might be influenced by the chemical properties of the extracting solution. They found that recovery of eight strains of hydrogen bacteria with different sizes, morphologies, and slime formation depended more on the bacterial strains than on the type of soil used.

Many tropical soils exhibit a variable charge phenomenon. In these soils clay size minerals and particles may be coated with an additional layer of various oxides whose charges are pH dependent (12).

Problems associated with the application of IF for enumeration of specific bacteria from soil stem largely from our inability to release bacteria from soil particles and retain them in the supernatant after flocculation of those particles (see Fig. 1A and B). This problem is particularly severe in tropical soils with variable charge characteristics.

Eight soils, representative of three of the major soil orders found in the tropics, were tested for recovery of *Rhizobium* spp. for IF enumeration. All soils, except a Hawaiian andept (Typic Eutrandept), yielded unacceptable recoveries (Table 1).

In soil-sand mixtures, addition of only a small amount of a Hawaiian oxisol to the mixture caused a dramatic decrease in recovery of added rhizobia. Increasing the soil content of the mixture from 0 to 50% reduced recoveries from >90 to <1% (Fig. 2).

Varying the ionic strength and pH of the extracting solution did not cause marked increases in recovery. Protein solutions, ethylenediaminetetraacetate, and $NaHCO_3$, on the other hand, improved release of bacteria (Table 2).

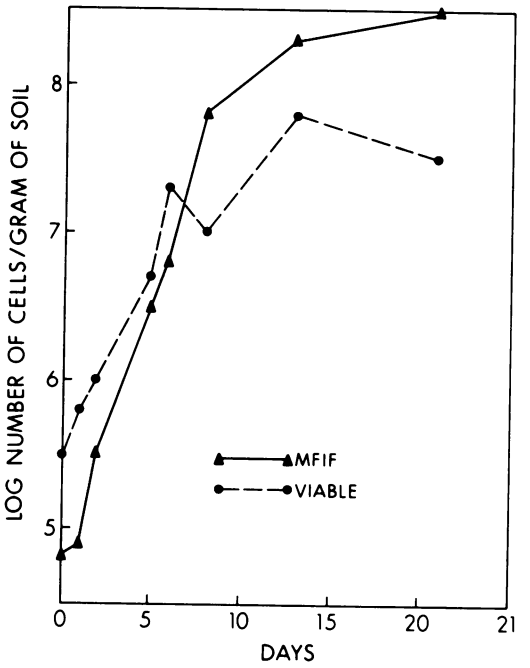

FIG. 4. *Growth of TAL-620 in sterilized Wahiawa oxisol: comparison of results obtained by the modified MFIF and by viable counts. Each datum point is the mean of four replicate measurements. The coefficients of variation are 30% for MFIF and 40% for viable count data.*

TABLE 3. *Effect of different diluents of partially hydrolyzed gelatin (PHG) on recovery of TAL-620 from Wahiawa oxisol*[a]

PHG extractant	No./g		% Recovery
	Added	Recovered	
0.1% PHG-water	7×10^5	7×10^2	<1
0.1% PHG-0.2 M LiCl	3×10^6	1×10^4	1
0.1% PHG-0.2 M NaCl	3×10^6	3×10^4	1
0.1% PHG-0.2 M KCl	3×10^6	6×10^4	2
0.1% PHG—0.2 M NH_4Cl	3×10^6	2×10^5	7
0.1% PHG-PBS[b]	7×10^5	2×10^5	28
0.1% PHG-0.1 M Na_2HPO_4	7×10^5	2×10^5	28
0.1% PHG-0.1 M $(NH_4)_2HPO_4$	7×10^5	5×10^5	71

[a] Values are means of duplicate measurements.

[b] PBS, 0.1 M sodium phosphate-buffered saline (0.9%), pH 7.2.

A preparation of partially hydrolyzed gelatin diluted in ammonium phosphate was found to be the best extractant for *Rhizobium* spp. from the soils tested. The efficiency of recovery of *Rhizobium* spp. from several tropical and two temperate soils remained high as the concentration of these soils in soil-sand mixtures was increased from 0 to 100% (Fig. 3A, B, and C), whereas the efficiencies were greatly reduced by soil content when water was used as extractant.

The modified gelatin technique was used to follow the growth of a strain of chickpea (*C. arietinum*) *Rhizobium* in a sterilized Wahiawa oxisol. The results (Fig. 4) show a close similarity with viable counts obtained at different stages during the growth cycle. Whereas viable count data are also affected by adsorption of bacteria to soil particles, they provide a useful basis for comparison and a necessary check on the flocculation step, because samples are taken before the flocculant is added.

The efficiency of recovery of *Rhizobium* spp. from an oxisol varied significantly when the diluent for the hydrolyzed gelatin was altered. Results (Table 3) show that the efficiency of release was in the following order: 0.1 M $(NH_4)_2HPO_4$ > 0.1 M Na_2HPO_4 = phosphate-buffered saline, pH 7.2 > 0.2 M KCl > 0.2 M NaCl > 0.2 M LiCl > water. This indicates that release of these bacteria from the soil is determined by both the cation and the anion composition of the extracting solution.

In this paper we have described a procedure for releasing bacteria from soil colloids and retaining them in suspension for MFIF. The results given in this paper refer for the most part to recovery of the chickpea *Rhizobium* strain TAL-620 from several tropical soils. We have also obtained recoveries ranging from 60 to 100% with other fast- and slow-growing rhizobia from several other temperate and tropical soils (data not shown). Recently, Moawad and Schmidt (Abstr. Annu. Meet. Am. Soc. Microbiol. 1981 N68, p. 184) have used hydrolyzed gelatin in ammonium phosphate for efficient recovery of three serotypes of *R. japonicum* from the rhizosphere of soybeans growing in a Waukegan soil.

ACKNOWLEDGMENTS

This work was supported in part by grant 701-15-60 from the U.S. Department of Agriculture-Science and Education Administration/CR-AID and grant DSAN-G-0100 from the Agency for International Development.

We thank Gordon Tsuji, Benchmark Soils Project, University of Hawaii, Honolulu, for providing many of the samples from tropical soils, and E. L. Schmidt, University of Minnesota, St. Paul, for the samples of Clarion and Hubbard soils.

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