APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1990, p. 533–540
0099-2240/90/020533-08$02.00/0

Vol. 56, No. 2

Determination of Viability within Serotypes of a Soil Population of *Rhizobium leguminosarum* bv. *trifolii*†

PETER J. BOTTOMLEY[1,2]* AND SHARON P. MAGGARD[1]

Departments of Microbiology[1] and Soil Science,[2] Oregon State University, Corvallis, Oregon 97331-3804

Received 25 September 1989/Accepted 20 November 1989

Concern has been raised about the percentage of viable cells within soil rhizobia populations measured by the immunofluorescence direct count method. The purpose of this study was to evaluate a direct viable count technique which is based on the fact that viable bacteria in natural populations undergo cell elongation when they are exposed to a combination of substrate and the inhibitor of DNA gyrase, nalidixic acid. A soil extraction procedure was developed to recover a high proportion of soil bacteria (ca. 10^9/g of soil) in suspensions with an optical clarity suitable for accurate microscopic enumeration. After incubation for 16 to 20 h at 27°C in the presence of yeast extract (200 mg/liter) and nalidixic acid (10 mg/liter), between 65 and 74% of the bacteria in soil suspension became significantly elongated (≥4.2 μm). In contrast, ≤0.5% of the same population could be cultured, regardless of the medium composition, nutrient concentration, or incubation conditions. The direct viable count method was combined with immunofluorescence to compare the percent viability and kinetics of appearance of elongated cells within serotypes of a soil population of *Rhizobium leguminosarum* bv. *trifolii*. Although the majority of these organisms were viable, as observed by immunofluorescence, we obtained evidence that subpopulations within the soil rhizobia community were in different states of competence to respond to substrate. A consistently low percentage (≤30%) of the population of serotype 23 was elongated even after 24 h of incubation and regardless of when the soil was sampled. Although the population densities of serotypes 6 and 36 were similar (0.8×10^6 to 2.0×10^6/g), the appearance of elongated cells of serotype 36 was consistently delayed relative to the appearance of elongated cells of serotype 6, and the maximum percentage of elongated cells of serotype 36 was less than that of serotype 6 when either a low substrate concentration (50 mg/liter) was used or the soil was air-dried before the bacteria were recovered.

Although immunofluorescence has allowed researchers to determine the densities of individual serotypes within indigenous soil populations of *Bradyrhizobium japonicum* (31, 36–38) and *Rhizobium leguminosarum* bv. *trifolii* (1, 7, 45), concern has been expressed about the proportion of cells which are viable within populations of soil rhizobia observed by immunofluorescence (3, 14, 16, 22, 35). Even though there is evidence that nonviable rhizobia cannot persist for significant lengths of time in soil (5, 10), it is a long-established fact that only a small fraction of the total bacterial population in soil enumerated by microscopy can be cultured (13, 28, 29, 34, 40, 42). The identification and viability of the majority of the bacterial population has remained an unresolved enigma. Although a similar phenomenon confounded aquatic microbiologists for many years (18, 20), the development of the nalidixic acid cell elongation assay (direct viable count [DVC] method) provided convincing proof that a much greater percentage of the total direct count of bacteria in aquatic samples was viable and substrate responsive than could be cultured (9, 24–27). Recently, the DVC procedure has been combined with immunofluorescence to show that specific water-borne pathogenic bacteria are often present in a viable state in water samples, even though they are nonculturable by conventional methods (8, 11, 19, 39).

As a result of these persistent controversies in soil microbiology, the following objectives were formulated for this study: (i) evaluation of the suitability of the DVC assay for determining viability within a population of soil bacteria; (ii) use of a combination of the DVC assay with immunofluorescence to determine the percentage of viable cells and the kinetics of cell elongation of specific indigenous serotypes within a naturalized soil population of *R. leguminosarum* bv. *trifolii*.

MATERIALS AND METHODS

Sampling of soil. Soil samples were collected from a depth of 0 to 5 cm from a silty clay loam of the Abiqua series (Cumulic Ultic Haploxeroll) under permanent pasture. The physical and chemical characteristics of the soil have been described elsewhere (1, 7). By using a soil tube auger, 20 soil cores were removed from within each of six grids (1 by 1 m) spaced 20 m apart along a transect running diagonally across the pasture. A composite soil sample was made from the six subsamples and passed through a 2-mm-pore-size screen, and experiments were initiated within 24 h of sampling. The gravimetric moisture content of each composite sample of soil was determined by drying the soil at 105°C for 3 days.

Evaluation of the yeast extract-nalidixic acid cell elongation assay on serotype representatives of *R. leguminosarum* bv. *trifolii*. (i) Preparation of nalidixic acid. Nalidixic acid (150 mg/100 ml) was dissolved in 0.01 M NaOH and filter sterilized. The pH of the medium in which the bacteria were incubated did not significantly change after the addition of portions of the nalidixic acid stock solution sufficient to provide a final concentration of ≤40 μg/ml (pH 6.5 to 7.0). A fresh stock solution of nalidixic acid was prepared at 2-week intervals.

(ii) Comparison of growth response of *R. leguminosarum* bv. *trifolii* to different nutrient sources. Isolates of *R. leguminosarum* bv. *trifolii* representing serotypes 6, 23, and 36 were cultured in large test tubes (20 by 2.5 cm), each of

* Corresponding author.

† Oregon State University Agricultural Experiment Station technical paper 8715.

which contained 30-ml portions of a glutamate-mannitol defined medium. Growth was followed in a temperature-controlled water bath (27°C), and the cultures were continuously aerated with a supply of filter-sterilized air. Mid-log-phase cells were inoculated to a final density of approximately 1×10^6 to 3×10^6 cells per ml into either complete yeast extract-mannitol medium or the mineral salts component of yeast extract-mannitol medium containing filter-sterilized yeast extract (50, 200, or 400 mg/liter) as the sole source of carbon and nitrogen. The optical densities of the cultures were monitored at 3-h intervals under the growth conditions described above.

(iii) Comparison of the kinetics of cell proliferation and cell elongation in the presence of yeast extract and nalidixic acid. Cells grown in yeast extract (200 mg/liter) were used as inocula. Portions of the cultures of each isolate were transferred back into growth tubes containing mineral salts and yeast extract (200 mg/liter) supplemented with or without nalidixic acid (final concentration, 10 mg/liter). At 3-h intervals, portions (1.9 ml) of the cultures were removed from each tube, and Formalin (final concentration, 2% [wt/vol] was added. Samples were maintained at 4°C until they were processed for microscopy.

(iv) Acridine orange staining. Each of the Formalin-fixed samples was incubated in the dark for 10 min after the addition of a portion (0.2 ml) of a solution of acridine orange (0.1 [wt/vol]) in 0.1 M citrate buffer (pH 6.6). Portions (0.25 ml) of the acridine orange-stained samples were added to 8 ml of filter-sterilized (pore size, 0.2 μm) NaCl (0.15 M) to promote uniform dispersion of cells on the filters. The samples were passed through 25-mm-diameter, 0.4-μm-pore-size Sudan black-stained polycarbonate membranes (Nuclepore Corp., Pleasanton, Calif.). Filters were destained with citrate buffers and prepared for epifluorescence microscopy as described elsewhere (7).

(v) Immunofluorescence analysis. Preparation of fluorescein-labeled immunoglobulin conjugates and the methods used for enumerating rhizobia have been described elsewhere (12).

(vi) Procedures for microscopic counting. Enumerations of total cells and elongated cells were made with the aid of a whipple disk (Graticules Ltd., Tonbridge, England) inserted into the eyepiece of an epifluorescence microscope (Zeiss), the optics of which have been described elsewhere (12). At least 50 fields of view per filter were counted at ×1,000 magnification for assessment of the total number of cells. Subsequently, the numbers of elongated bacteria (≥4.2 μm) were counted in another 50 fields. As a result of cell proliferation by the control cultures which did not contain nalidixic acid, variable volumes of cell suspensions were filtered to provide ≤150 bacteria within the calibrated area of the whipple disk.

Extraction from soil. Three-gram portions of field-moist Abiqua soil were dispensed into 160-ml milk dilution bottles, each of which contained a layer of glass beads (diameter, 3 mm) and 27 ml of filter-sterilized 0.15 M NaCl. The bottles were shaken vigorously by hand for 10 min, and the soil suspension was allowed to settle for exactly 5 min. A portion (10 ml) was recovered from the upper layer of suspension in each bottle and was added to 190 ml of filter-sterilized distilled water to give a final soil dilution of approximately 200-fold. For analysis of total bacteria by epifluorescence microscopy, portions (2 ml) of the original soil suspension were added to 98 ml of water, so that the final dilution of soil was approximately 500-fold. Each of the diluted soil suspensions was filtered sequentially through 47-mm-diameter polycarbonate membrane filters with pore sizes of 8.0 and 3.0 μm, respectively. A mineral salt solution was immediately added to the filtered microbial suspension to provide the following (in grams per liter): $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.08; K_2SO_4, 0.17; and K_2HPO_4, 0.05. For enumeration of culturable bacteria, fivefold serial dilutions, commencing with the 1:500 dilution, were made into 1/10 strength mineral salt solution; and portions (0.1 ml) were plated onto the following media solidified with Bacto-Agar (1.5% [wt/vol]; Difco Laboratories, Detroit, Mich.): tryptic soy (0.3 and 3.0 g/liter), yeast extract (0.2 and 2.0 g/liter), yeast extract plus Bacto-Peptone (0.2 g of each per liter; Difco), or brain heart infusion (0.37 g/liter). Plates were incubated at 25°C; and colonies were counted at 4, 9, and 14 days after plating. To evaluate whether filtration removed a significant proportion of culturable bacteria, a similar experiment was conducted, except that the filtration step was omitted.

For cell elongation assays, a filter-sterilized stock solution of yeast extract (final concentration, 200 mg/liter) was added to the filtered suspension of soil microorganisms and mixed thoroughly, and portions (30 ml) were dispensed into the growth tubes as described above. A portion (0.2 ml) of the nalidixic acid stock solution was immediately added to each growth tube to achieve a final concentration of 10 mg/liter. Over a period of 24 h, replicate tubes were sacrificed at 4-h intervals by adding Formalin (final concentration, 2% [wt/vol]) and were then cooled to 4°C and processed for microscopy as described elsewhere (7, 12). Included in every experiment were extra tubes containing filtered soil suspension supplemented with yeast extract and incubated in the presence or absence of nalidixic acid. Growth was followed turbidimetrically in these tubes to provide preliminary evidence that nalidixic acid was preventing cell proliferation and to monitor the kinetics of appearance of visible growth in the absence of nalidixic acid. In preliminary experiments, suspensions of soil microorganisms were incubated with antibiotic alone and without either substrate or antibiotic. Since cell elongation was not observed under either of these two control conditions, these controls were omitted from further experiments.

Efficiency of recovering soil bacteria and *R. leguminosarum* bv. *trifolii*. Triplicate 3-g samples of soil were suspended in centrifuge bottles (250-ml capacity) containing glass beads in portions (27 ml) of 0.15 M NaCl. The suspensions were shaken and allowed to settle, and portions (5 ml) were added to 95-ml volumes of filter-sterilized distilled water and fixed with Formalin. The remainder of each soil suspension was centrifuged at $1,900 \times g$ for 5 min, and the supernatants were carefully removed and discarded. The extraction procedure was repeated three times on each sample of soil with fresh portions of 0.15 M NaCl. The replicates of each of the four extracts were filtered consecutively through 8.0- and 3.0-μm pore-size filters, and *R. leguminosarum* bv. *trifolii* serotype 6 was enumerated by immunofluorescence. Before staining with acridine orange to estimate the total number of soil bacteria, the supernatants were further diluted to a final dilution of 1:500.

Estimation of the total *R. leguminosarum* bv. *trifolii* population by plant infection-soil dilution. By using subclover (*Trifolium subterraneum* L. cv. Mt. Barker) seedlings as test plants, standard procedures were followed (46), with a twofold serial dilution of soil, and six replicate plants per dilution were used to maximize the accuracy of the procedure (95% confidence limits of the population density is equal to the mean value ×/÷ 1.76). Both filtered and

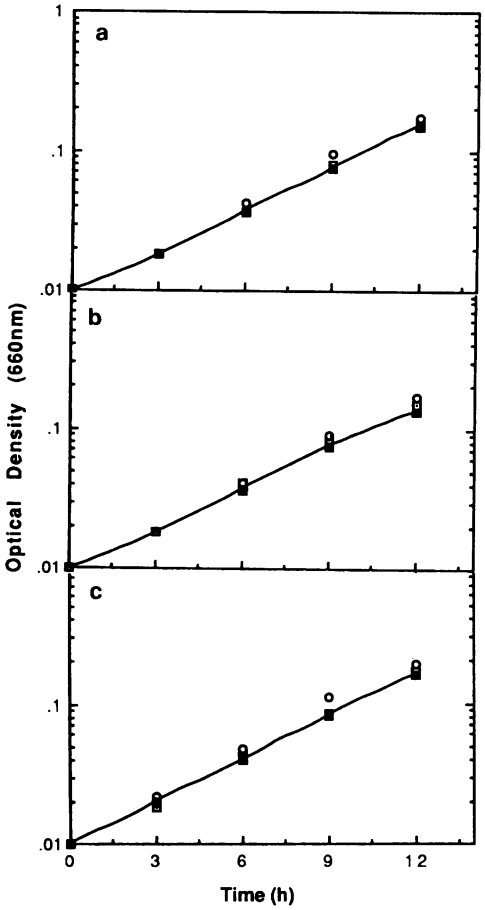

FIG. 1. Growth characteristics of *R. leguminosarum* bv. *trifolii* serotypes 6 (a), 23 (b), and 36 (c) in 200 mg of yeast extract per liter (⊡), 400 mg of yeast extract per liter (○), and complete yeast extract-mannitol medium (■).

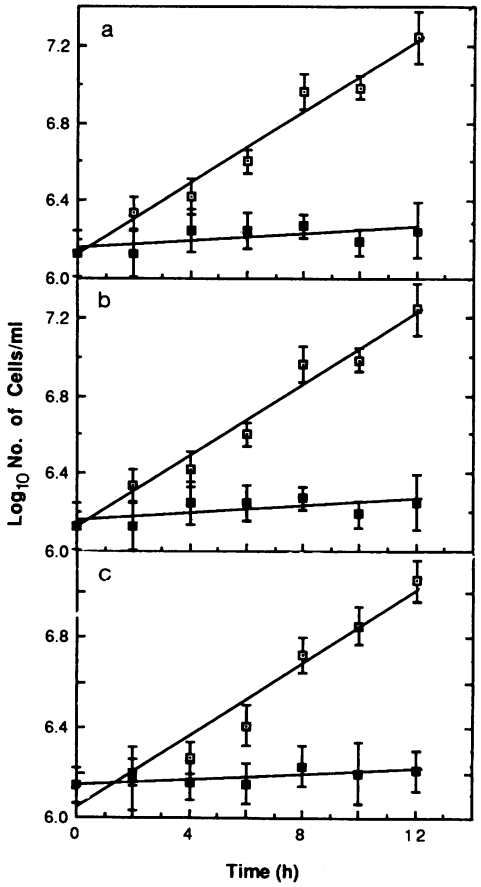

FIG. 2. Growth of representatives of *R. leguminosarum* bv. *trifolii* serotypes 6 (a), 23 (b), and 36 (c) in the presence of yeast extract (200 mg/liter) as the sole source of carbon and nitrogen and with (■) and without (⊡) nalidixic acid. Error bars represent the standard deviation of the mean of three replicates.

nonfiltered soil suspensions (1:500) were diluted in a twofold series, and subclover seedlings were challenged.

RESULTS

Growth and cell elongation response of serotype representatives in pure culture. The results from preliminary experiments showed that representatives of *R. leguminosarum* bv. *trifolii* serotypes, 6, 23, and 36 could grow on yeast extract as the sole source of carbon and nitrogen and that the cells would elongate substantially in the presence of nalidixic acid. Although yeast extract is not a traditional sole nutrient source for *Rhizobium* species, mineral salts supplemented with yeast extract alone supported short-term rates of exponential growth equivalent to generation times of 2.8 ± 0.1 h (Fig. 1). Growth rates supported by 200 mg of yeast extract per liter were not significantly different from those supported by greater concentrations of yeast extract, complete yeast extract-mannitol medium, or a glutamate-mannitol defined medium (data not shown).

At a concentration of 10 mg/liter, nalidixic acid was sufficient to prevent cell proliferation of isolates representing the three serotypes from the soil population (Fig. 2a through c). Furthermore, under these growth conditions the kinetics of appearance of elongated cells in the presence of nalidixic acid were virtually identical for representatives of the different serotypes (Fig. 3). Between 85 and 97% of cells in the cultures were significantly elongated after 6 to 9 h of incubation, which corresponded to a period of time equivalent to two generations. Under these conditions, significant elongation of cells could be accurately determined by immunofluorescence after 4 to 6 h (Fig. 4). Even more dramatic increases in cell length (>6 μm) occurred when the incubation time was extended beyond 8 h.

Recovery of soil bacteria and *R. leguminosarum* bv. *trifolii* from soil. Substantial numbers of both soil bacteria in general and *R. leguminosarum* bv. *trifolii* specifically were recovered from soil samples, shaken, diluted, and passed sequentially through 8.0- and 3.0-μm-pore-size filters, respectively (Table 1). Although significant numbers of cells were recovered by a second extraction, our main goal was to develop a simple method for recovering a substantial portion of the bacteria from field soil and to expose them to the elongation assay without undue delay or excessive manipulation. All remaining data were obtained with populations recovered by one extraction of soil. Consistent numbers of total soil bacteria in general, and of *R. leguminosarum* bv. *trifolii* serotypes specifically, were recovered from samples of soil taken from the field at nine different times of the year. These values (95% confidence limits in parentheses) were as

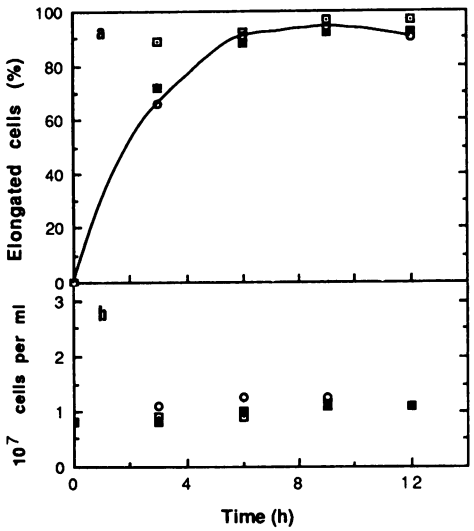

FIG. 3. Percentage of elongated cells (a) and total cell density (b) of representatives of *R. leguminosarum* bv. *trifolii* serotypes 6 (⊡), 23 (■), and 36 (○) incubated in the presence of yeast extract (200 mg/liter) and nalidixic acid (10 mg/liter).

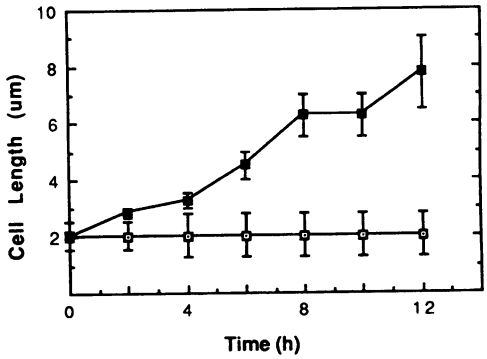

FIG. 4. Mean cell length of *R. leguminosarum* bv. *trifolii* in response to incubation with yeast extract (200 mg/liter) with (■) and without (⊡) nalidixic acid (10 mg/liter). Bars represent the range of the maximum and minimum lengths of cells determined at each time interval.

follows: 1.50 (0.46) × 10^9, 1.13 (0.25) × 10^6, and 1.87 (1.12) × 10^5 per g of oven-dried soil for total soil bacteria and *R. leguminosarum* bv. *trifolii* serotypes 6 and 23, respectively. Furthermore, estimates of both the density of culturable soil bacteria and the most probable number of the nodulating population of *R. leguminosarum* bv. *trifolii* were not significantly different in filtered or nonfiltered soil suspensions (Table 2).

Cell elongation response of total soil bacteria and *R. leguminosarum* bv. *trifolii* in the same soil suspension. A large proportion (65%) of the direct microscopic counts of soil bacteria were significantly elongated (≥4.2 μm) after exposure to yeast extract over a 16-h incubation period (Fig. 5a). Similar experiments, which were carried out on populations of bacteria that were recovered from soil sampled at different times, gave values ranging from 64 to 75% of the total direct count (data not shown). Members of indigenous *R. leguminosarum* bv. *trifolii* serotype 6 responded with similar kinetics of elongation, with 70% of the total population being significantly elongated after 20 h of incubation. Nalidixic acid (10 mg/liter) was sufficient to prevent cell division of the indigenous bacteria, since no significant increase in the density of total cells in either population was observed over the 24-h time course (Fig. 5b). These data show that yeast extract is a substrate to which many soil bacteria are responsive, even though a much lower number showed colony-forming ability on the same substrate (Table 2). Furthermore, a specific serotype of *R. leguminosarum* bv. *trifolii* was competitive for this particular nutrient source, even though it contributed ≤0.1% of the soil bacterial population. Despite the differences between individual serotypes in the total population sizes and in the rates of appearance of elongated cells, variability between replicate determinations of elongated (Fig. 6a) and total (Fig. 6b) cells was found to be small (≤ ∓10%) throughout the time course of incubation.

Cell elongation kinetics reveal differences between the serotypes. Although similar growth and cell elongation responses were given by *R. leguminosarum* bv. *trifolii* serotype representatives when they were exposed to yeast extract under pure culture conditions, several differences were revealed when the cell elongation kinetics of indigenous serotypes were measured. Although the population densities of serotypes 6 and 36 determined by immunofluorescence direct count were similar, the appearance of elongated cells of serotype 36 was consistently delayed relative to that of elongated cells of serotype 6, regardless of the time of year when the soil was sampled (Fig. 7). Nevertheless, similar percentages of elongated cells of both serotypes were measured after 20 to 24 h of incubation in yeast extract (200 mg/liter). Other differences between the two serotypes were revealed either when a limiting quantity of yeast extract (50 mg/liter) was used as substrate or when field soil was air dried to 7% (wt/wt) moisture content (−20 MPa) prior to the recovery of bacteria (Fig. 8). In both cases, a low percentage (30%) of cells of serotype 36 were elongated after 24 h of incubation, whereas the elongation response of serotype 6 cells was not affected by a low substrate concentration and was affected to a lesser degree than serotype 36 was as a consequence of air drying of the soil. Serotype 23 cells maintained a soil population density that was significantly lower (1 × 10^5 to 3 × 10^5/g) than that of cells of either serotype 6 or 36. In the case of serotype 23, the maximum percentage of elongated cells was ≤30% of the total, even

TABLE 1. Influence of consecutive soil extractions on the recovery of soil bacteria

Organism	Population densities/g of bacteria (%) for the following no. of soil extractions[a]:				
	1	2	3	4	Total
R. leguminosarum bv. *trifolii* serotype 6 (10^5)	5.1 ± 0.6 (65.0)	1.7 ± 0.5 (22.0)	0.5 ± 0.07 (6.0)	0.5 ± 0.06 (6.0)	7.8
Total soil bacteria (10^8)	8.3 ± 0.9 (46.0)	3.8 ± 0.2 (21.0)	3.5 ± 0.3 (19.0)	2.6 ± 0.2 (14.0)	18.2

[a] Values are standard deviations of the means of three replicates. Percentages represent the bacteria recovered from each individual extraction as a percentage of the sum of bacteria in all four extractions.

TABLE 2. Influence of filtration of soil suspension, medium type, and concentration on the determinations of culturable soil bacteria and the *R. leguminosarum* bv. *trifolii* population

Soil treatment	Medium	No. of cells (10^6)/g[a]
Filtered	Yeast extract (0.2)[b]	4.4 ± 0.7
	Brain heart infusion (0.4)	4.1 ± 1.7
	Yeast extract (0.2) plus peptone (0.2)	5.2 ± 0.8
	Tryptic soy (0.2)	5.6 ± 2.0
	Most probable number of *R. leguminosarum* bv. *trifolii*	0.1–0.3[c]
Nonfiltered	Yeast extract (0.2)	5.7 ± 2.2
	Yeast extract (2.0)	4.8 ± 2.3
	Tryptic soy (0.2)	5.0 ± 0.9
	Tryptic soy (2.0)	4.7 ± 1.9
	Most probable number of *R. leguminosarum* bv. *trifolii*	0.2–1.1[c]

[a] Values represent the means ± standard deviation of four replicates.
[b] Values in parentheses represent the medium concentration (in grams per liter).
[c] Values represent the 95% confidence limits of the population means determined by the plant infection-soil dilution procedure.

after 24 h of incubation and regardless of the time of year when the soil was sampled (Fig. 9).

DISCUSSION

The yeast extract, nalidixic acid cell elongation method allowed us to confirm in soil the findings of aquatic microbiologists; i.e., a much greater percentage of the bacteria in natural environments are viable and substrate responsive than can be cultured (9, 24–27). Our findings are an extension of many studies that have spanned a period of 40 years, which showed that whereas only a small fraction of the direct microscopic count of soil bacteria could be cultured, the viability of the majority remained an unresolved enigma (2, 7, 13, 17, 28, 29, 34, 42).

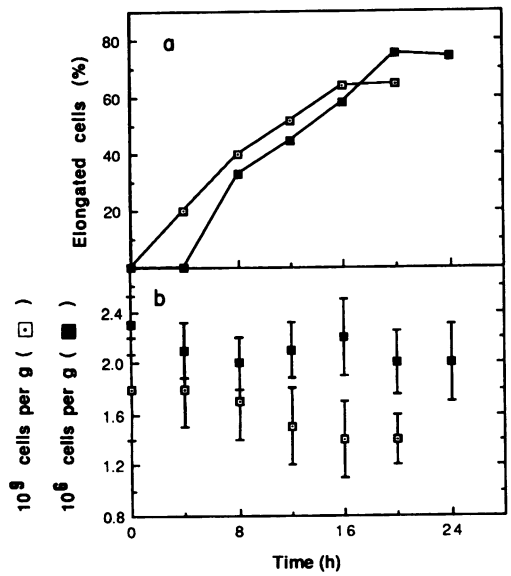

FIG. 5. Percentage of elongated cells (a) and total cell density (b) of soil bacteria (□) and indigenous *R. leguminosarum* bv. *trifolii* serotype 6 (■) incubated in the presence of yeast extract (200 mg/liter) and nalidixic acid (10 mg/liter).

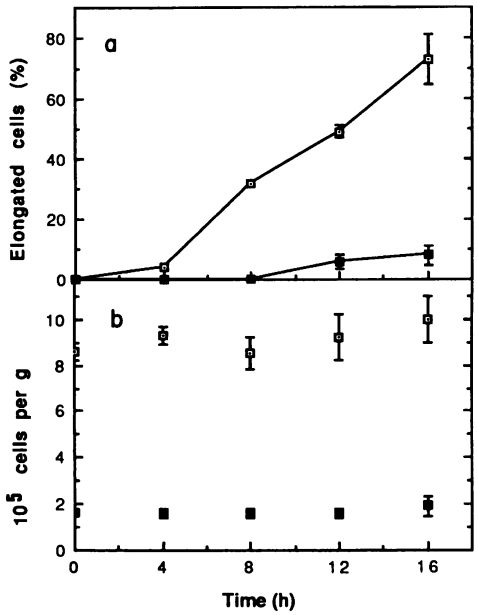

FIG. 6. Variance between replicate samples taken during the time course of incubation to determine the percentage of elongated cells (a) and the total cell density (b) of indigenous serotypes 6 (□) and 23 (■) of *R. leguminosarum* bv. *trifolii*. The absence of error bars indicates that the magnitude of the standard error was less than the size of the symbol.

In recent years, several chemically and physically harsh extraction procedures have been developed to enhance the recovery of soil microorganisms for their accurate microscopic enumeration (2, 6, 12, 13, 23, 30, 41) and for extraction of their DNAs (17, 32, 44). Preliminary experiments carried out in our laboratory showed that several of these extraction procedures are unsatisfactory when evaluation of the physiological responsiveness of the soil population is the major goal. The procedure described in this report works very effectively with Abiqua soil and takes an individual ≤90 min to recover bacteria and initiate incubation in the substrate-antibiotic combination. The reproducibility of the extraction procedure in terms of both the numbers of organisms recovered from different soil samples and the low variance within replicates of a treatment indicates that this method may be promising for other soil microbiological studies.

Since all the published studies in which this method has been used were focused on the aquatic environment, a few comparisons are relevant. Although a wide range of incubation times (6 to 24 h) have been reported, there has been a tendency for a fixed-term incubation period to be used in most studies (8, 24–27). Our data reveal that the incubation time necessary to achieve the maximum percentage of elongated cells can vary between different *Rhizobium* serotypes monitored in the same soil sample and can also vary for the same serotype on different sampling occasions. In general, longer incubation periods (up to 24 h) were required than have been previously reported in the majority of aquatic studies (6 to 8 h). Our findings may reflect on an inherently slow responsiveness of *Rhizobium* species relative to other bacteria or on the influence of the specific soil conditions at the time of recovery on the physiological condition of *Rhizobium* species. However, it is possible that the density of cells in the microbial suspensions undergoing incubation

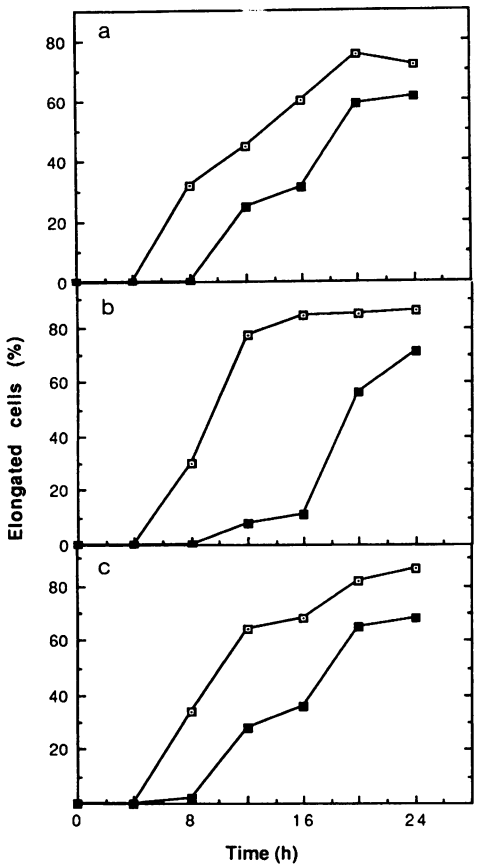

FIG. 7. Kinetics of cell elongation by indigenous serotypes 6 (⊡) and 36 (■) incubated in the presence of yeast extract (200 mg/liter) and nalidixic acid (10 mg/liter) after recovery from soil sampled in January (a), April (b), and May (c).

might influence the response time. In our studies, filtered soil suspensions typically contained 5×10^6 cells per ml, whereas densities of 1×10^5 to 1×10^6/ml were more commonly reported in the aquatic studies.

Since nalidixic acid did not control cell proliferation in seawater samples for greater than 6 h, the use of a mixture of DNA gyrase inhibitors has been recommended to control the proliferation of bacteria (26). In the majority of our studies to date, nalidixic acid (10 mg/liter) alone has been sufficient to prevent statistically significant cell proliferation of total soil bacteria and serotypes of *R. leguminosarum* bv. *trifolii* over a 24-h time course. Nevertheless, there have been notable exceptions. In the case of two indigenous serotypes of *R. leguminosarum* bv. *trifolii*, serotypes 4 and 21, we have found it necessary to use 20 mg of nalidixic acid per liter to control proliferation (unpublished data). Although we are eager to expand these studies to evaluate the response of rhizobia to different concentrations of specific substrates, caution is warranted in lieu of recent findings. The DNA gyrase inhibitors nalidixic acid (≥50 mg/liter) and novobiocin (150 mg/liter) have been shown to prevent derepression of hydrogenase in *B. japonicum* (33) and the expression of bacteroid-associated phenotypes in free-living *Bradyrhizobium* sp. strain 32H1 (15), respectively.

Our data confirm that >50% of rhizobia within the most abundant serotype subpopulations (serotypes 6 and 36) are viable. Although the inability of the majority of rhizobia of

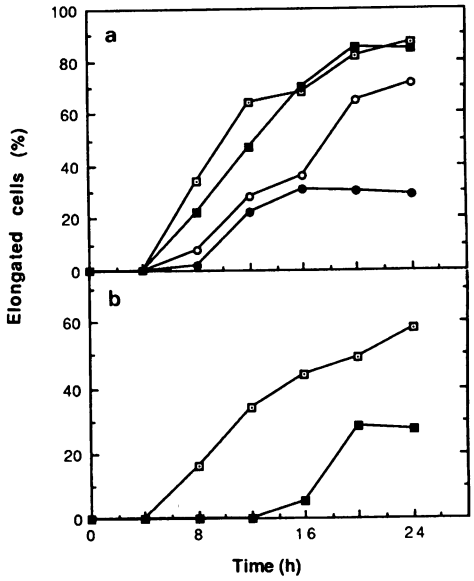

FIG. 8. (a) Kinetics of cell elongation by serotypes 6 (square symbols) and 36 (circle symbols) incubated in the presence of nalidixic acid (10 mg/liter) and yeast extract at 50 (■, ●) or 200 (⊡, ○) mg/liter. (b) Kinetics of cell elongation by serotypes 6 (⊡) and 36 (■) incubated in the presence of yeast extract (200 mg/liter) and nalidixic acid (10 mg/liter) after recovery from air-dried soil (6.9% [wt/wt] moisture content).

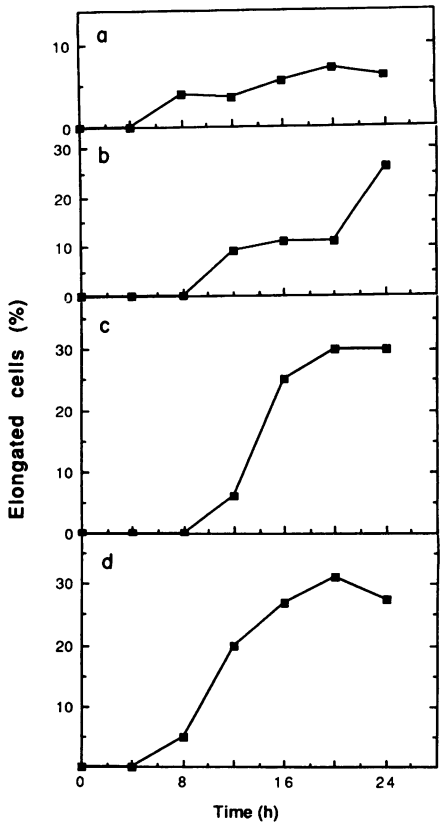

FIG. 9. Kinetics of cell elongation by serotype 23 incubated in the presence of yeast extract (200 mg/liter) and nalidixic acid (10 mg/liter) after recovery from soil sampled in February (a), April (b), May (c), and May (d).

serotype 23 to elongate in the presence of yeast extract is not definitive proof that these cells lack viability, differences in the response of serotypes of yeast extract provide preliminary evidence that subpopulations are in different states of competence to respond to substrate appearance. It remains to be established whether the faster cell elongation response of serotype 6, relative to those of serotypes 23 and 36, is related to the high incidence of serotype 6 and the rare occurrence of serotypes 23 and 36 in subclover root nodules under field conditions (K. Leung and P. J. Bottomley, unpublished data).

In conclusion, the presence of *R. leguminosarum* bv. *trifolii* subpopulations which are viable, and yet which are rarely found in nodules, raises both the interesting and disturbing aspects of studying the ecology of rhizobia by immunofluorescence. In the pioneering studies of Bohlool and Schmidt (4), an indigenous soil-borne actinomycete was found to cross-react strongly with a fluorescent antibody to *B. japonicum*. More recently, nonnodulating bacteria that were either antigenically related or that showed partial DNA homology to symbiotically competent biovars of *R. leguminosarum* have been recovered directly from soil (21, 43). Although the antiserum to serotype 36 of *R. leguminosarum* bv. *trifolii* is one of the most specific in our collection, it remains to be resolved whether the majority of members of serotype 36 are simply poor competitors at nodulating, or are rhizobia which completely lack any ability to nodulate clover, or are fundamentally different soil bacteria that serendipitously carry similar antigenic determinants to serotype 36.

ACKNOWLEDGMENTS

This research was supported by grant 87-CRCR-1-2471 from the Competitive Research Grants Office, U.S. Department of Agriculture, and by funds from the Oregon Agricultural Experiment Station.

We thank C. Pelroy for processing the manuscript and Muktar Dughri for experimentation in the early stages of the study.

LITERATURE CITED

1. **Almendras, A. S., and P. J. Bottomley.** 1987. Influence of lime and phosphate on nodulation of soil-grown *Trifolium subterraneum* L. by indigenous *Rhizobium trifolii*. Appl. Environ. Microbiol. **53**:2090–2097.
2. **Bakken, L. R.** 1985. Separation and purification of bacteria from soil. Appl. Environ. Microbiol. **49**:1482–1487.
3. **Bezdicek, D. F., and M. D. Donaldson.** 1980. Flocculation of *Rhizobium* from soil colloids for enumeration by immunofluorescence, p. 297–309. *In* R. C. W. Berkeley, J. M. Lynch, J. Melling, P. R. Rutter, and B. Vincent (ed.), Microbial adhesion to surfaces. Ellis Horwood Ltd., Chichester, England.
4. **Bohlool, B. B., and E. L. Schmidt.** 1970. Immunofluorescent detection of *Rhizobium japonicum* in soils. Soil Sci. **110**:229–236.
5. **Bohlool, B. B., and E. L. Schmidt.** 1973. Persistence and competition aspects of *Rhizobium japonicum* observed in soil by immunofluorescence microscopy. Soil Sci. Soc. Am. Proc. **37**:561–564.
6. **Bone, T. L., and D. L. Balkwill.** 1986. Improved flotation technique for microscopy of in situ soil and sediment microorganisms. Appl. Environ. Microbiol. **51**:462–468.
7. **Bottomley, P. J., and M. H. Dughri.** 1989. Population size and distribution of *Rhizobium leguminosarum* bv. *trifolii* in relation to total soil bacteria and soil depth. Appl Environ. Microbiol. **55**:959–964.
8. **Brayton, P. R., M. L. Tamplin, A. Huq, and R. R. Colwell.** 1987. Enumeration of *Vibrio cholerae* O1 in Bangladesh waters by fluorescent antibody direct viable count. Appl. Environ. Microbiol. **53**:2862–2865.
9. **Buchanan-Mappin, J. M., P. M. Wallis, and A. G. Buchanan.** 1986. Enumeration and identification of heterotrophic bacteria in groundwater and in a mountain stream. Can. J. Microbiol. **32**:93–98.
10. **Cleyet-Marel, J.-C., and Y. Crozat.** 1982. Etude écologique en immunofluorescence de *Rhizobium japonicum* dans le sol et la rhizosphere. Agronomie **2**:243–248.
11. **Colwell, R. R., P. R. Brayton, D. J. Grimes, D. B. Roszak, S. A. Huq, and L. M. Palmer.** 1985. Viable but nonculturable *Vibrio cholerae* and related pathogens in the environment. Implications for release of genetically-engineered microorganisms. Biotechnology **3**:817–820.
12. **Demezas, D. H., and P. J. Bottomley.** 1986. Autecology in rhizospheres and nodulating behavior of indigenous *Rhizobium trifolii*. Appl. Environ. Microbiol. **52**:1014–1019.
13. **Faegri, A., V. L. Torsvik, and J. Goksoyr.** 1977. Bacterial and fungal activities in soil: separation of bacteria and fungi by a rapid fractionated centrifugation technique. Soil Biol. Biochem. **9**:105–112.
14. **Frederickson, J. K., D. F. Bezdicek, F. J. Brockman, and S. W. Li.** 1988. Enumeration of Tn*5* mutant bacteria in soil by using a most-probable-number-DNA hybridization procedure and antibiotic resistance. Appl. Environ. Microbiol. **54**:446–453.
15. **Gober, J. W., and E. R. Kashket.** 1989. Role of DNA superhelicity in regulation of bacteroid-associated functions of *Bradyrhizobium* sp. strain 32H1. Appl. Environ. Microbiol. **55**:1420–1425.
16. **Heynen, C. E., J. D. Van Elsas, P. J. Kuikman, and J. A. Van Veen.** 1988. Dynamics of *Rhizobium leguminosarum* bv. *trifolii* introduced into soil: the effect of bentonite clay on predation by protozoa. Soil Biol. Biochem. **20**:483–488.
17. **Holben, W. E., J. K. Jansson, B. K. Chelm, and J. M. Tiedje.** 1988. DNA probe method for the detection of specific microorganisms in the soil bacterial community. Appl. Environ. Microbiol. **54**:703–711.
18. **Hoppe, H. G.** 1976. Determination and properties of actively metabolizing heterotrophic bacteria in the sea, investigated by means of microautoradiography. Mar. Biol. **36**:291–302.
19. **Hussong, D., R. R. Colwell, M. O'Brien, E. Weiss, A. D. Pearson, R. M. Weiner, and W. D. Burge.** 1987. Viable *Legionella pneumophila* not detectable by culture on agar media. Biotechnology **5**:947–950.
20. **Jannasch, H. W., and G. E. Jones.** 1959. Bacterial populations in seawater as determined by different methods of enumeration. Limnol. Oceanogr. **4**:128–139.
21. **Jarvis, B. D. W., L. J. H. Ward, and E. A. Slade.** 1989. Expression by soil bacteria of nodulation genes from *Rhizobium leguminosarum* bv. *trifolii*. Appl. Environ. Microbiol. **55**:1426–1434.
22. **Kennedy, A. C., and A. G. Wollum.** 1988. Enumeration of *Bradyrhizobium japonicum* in soil subjected to high temperature: comparison of plate count, most probable number and fluorescent antibody techniques. Soil Biol. Biochem. **20**:933–937.
23. **Kingsley, M. T., and B. B. Bohlool.** 1981. Release of *Rhizobium* spp. from tropical soils and recovery for immunofluorescence enumeration. Appl. Environ. Microbiol. **42**:241–248.
24. **Kogure, K., U. Simidu, and N. Taga.** 1979. A tentative direct microscopic method for counting living marine bacteria. Can. J. Microbiol. **25**:415–420.
25. **Kogure, K., U. Simidu, and N. Taga.** 1980. Distribution of viable marine bacteria in neritic seawater around Japan. Can. J. Microbiol. **26**:318–323.
26. **Kogure, K., U. Simidu, and N. Taga.** 1984. An improved direct viable count method for aquatic bacteria. Arch. Hydrobiol. **102**:117–122.
27. **Kogure, K., U. Simidu, N. Taga, and R. R. Colwell.** 1987. Correlation of direct viable counts with heterotrophic activity for marine bacteria. Appl. Environ. Microbiol. **53**:2332–2337.
28. **Louw, H. A., and D. M. Webley.** 1959. The bacteriology of the root region of the oat plant grown under controlled pot culture conditions. J. Appl. Bacteriol. **2**:216–226.
29. **Lund, V., and J. Goksoyr.** 1980. The effects of water fluctua-

tions on microbial mass and activity in soil. Microb. Ecol. **6**:115–123.
30. **Martin, N. J., and R. M. MacDonald.** 1981. Separation of non-filamentous microorganisms from soil by density gradient centrifugation in percoll. J. Appl. Bacteriol. **51**:243–251.
31. **Moawad, H. A., W. R. Ellis, and E. L. Schmidt.** 1984. Rhizosphere response as a factor in competition among three serogroups of indigenous *Rhizobium japonicum* for nodulation of field-grown soybeans. Appl. Environ. Microbiol. **47**:607–612.
32. **Nannipieri, P., C. Ciardi, and L. Badalucco.** 1986. A method to determine soil DNA and RNA. Soil Biol. Biochem. **18**:275–281.
33. **Novak, P. D., and R. J. Maier.** 1987. Inhibition of hydrogenase synthesis by DNA gyrase inhibitors in *Bradyrhizobium japonicum*. J. Bacteriol. **169**:2708–2712.
34. **Olsen, R. A., and L. R. Bakken.** 1987. Viability of soil bacteria: optimization of plate-counting technique and comparison between total counts and plate counts within different size groups. Microb. Ecol. **13**:59–74.
35. **Postma, J., J. D. Van Elsas, J. M. Govaert, and J. A. van Veen.** 1988. The dynamics of *Rhizobium leguminosarum* biovar *trifolii* introduced into soil as determined by immunofluorescence and selective plating techniques. FEMS Microbiol. Ecol. **53**:251–260.
36. **Reyes, V. G., and E. L. Schmidt.** 1979. Population densities of *Rhizobium japonicum* strain 123 estimated directly in soil and rhizospheres. Appl. Environ. Microbiol. **37**:854–858.
37. **Reyes, V. G., and E. L. Schmidt.** 1981. Populations of *Rhizobium japonicum* associated with the surfaces of soil-grown roots. Plant Soil **61**:71–80.
38. **Robert, F. M., and E. L. Schmidt.** 1985. Response of three indigenous serogroups of *Rhizobium japonicum* to the rhizosphere of pre-emergent seedlings of soybeans. Soil Biol. Biochem. **17**:579–580.
39. **Rollins, D. M., and R. R. Colwell.** 1986. Viable but nonculturable stage of *Campylobacter jejuni* and its role in survival in the natural aquatic environment. Appl. Environ. Microbiol. **52**: 531–538.
40. **Rovira, A. D., E. I. Newman, H. J. Bowen, and R. Campbell.** 1974. Quantitative assessment of the rhizosphere microflora by direct microscopy. Soil Biol. Biochem. **6**:211–216.
41. **Schmidt, E. L.** 1974. Quantitative autecological study of microorganisms in soil by immunofluorescence. Soil Sci. **118**:141–149.
42. **Skinner, F. A., P. C. T. Jones, and J. E. Mollison.** 1952. A comparison of a direct- and a plate-counting technique for the quantitative estimation of soil microorganisms. J. Gen. Microbiol. **6**:261–271.
43. **Soberon-Chavez, G., and R. Najera.** 1989. Isolation from soil of *Rhizobium leguminosarum* lacking symbiotic information. Can. J. Microbiol. **35**:464–468.
44. **Torsvik, V. L., and J. Goksoyr.** 1978. Determination of bacterial DNA in soil. Soil Biol. Biochem. **10**:7–12.
45. **Valdivia, B., M. H. Dughri, and P. J. Bottomley.** 1988. Antigenic and symbiotic characterization of indigenous *Rhizobium leguminosarum* bv. *trifolii* recovered from root nodules of *Trifolium pratense* L. sown into subterranean clover pasture soils. Soil Biol. Biochem. **20**:267–274.
46. **Vincent, J. M.** 1970. A manual for the practical study of root-nodule bacteria. IBP handbook no. 15. Blackwell Scientific Publications, Oxford.