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Viability of Indigenous Soil Bacteria Assayed by Respiratory Activity and Growth

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The bacterial population in barley field soil was estimated by determining the numbers of (i) cells reducing the artificial electron acceptor 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) to CTC-formazan (respiratory activity), (ii) cells dividing a limited number of times (microcolony formation) on nutrient-poor media, (iii) cells dividing many times (colony formation) on nutrient-poor agar media, and (iv) cells stained with acridine orange (total counts). The CTC reduction assay was used for the first time for populations of indigenous soil bacteria and was further developed for use in this environment. The number of viable cells was highest when estimated by the number of microcolonies developing during 2 months of incubation on filters placed on the surface of nutrient-poor media. The number of bacteria reducing CTC to formazan was slightly lower than the number of bacteria forming microcolonies. Traditional plate counts of CFU (culturable cells) yielded the lowest estimate of viable cell numbers. The microcolony assay gave an estimate of both (i) cells forming true microcolonies (in which growth ceases after a few cell divisions) representing viable but nonculturable cells and (ii) cells forming larger microcolonies (in which growth continues) representing viable, culturable cells. The microcolony assay, allowing single-cell observations, thus seemed to be best suited for estimation of viable cell numbers in soil. The effect on viable and culturable cell numbers of a temperature increase from 4 to 17°C for 5 days was investigated in combination with drying or wetting of the soil. Drying or wetting prior to the temperature increase, rather than the temperature increase per se, affected both the viable and culturable numbers of bacteria; both numbers were reduced in predried soil, while they increased slightly in the prewetted soil.

The total number of indigenous bacteria in soil is routinely determined by staining with acridine orange followed by epifluorescence microscopy, i.e., the acridine orange direct count (AODC) (12). The total count by far exceeds the number of bacteria which are able to form colonies on agar plates, i.e., the culturable bacteria (e.g., see references 1, 2, and 25). Estimates of the culturable fraction of a bacterial community vary and depend on medium composition, as well as incubation conditions (25); typically, the CFU are at least 10- to 100-fold lower than the total number of bacteria. A fraction of cells called the viable but nonculturable bacteria has recently received great attention, as their existence may partly explain the difference between estimates of total and culturable populations in natural communities (36). Viable but nonculturable bacteria are likely to represent bacteria that we are unable to culture by the conditions provided. Techniques which are not based on cell divisions and growth in laboratory media have been developed to detect viable but nonculturable bacteria. One example is the direct viable count assay of Kogure et al. (18), which is based on enlargement of the bacteria without cell division in the presence of nalidixic acid. Microscopic detection of elongated cells is reported to be useful for detection of the viability of specific, known bacteria (26, 46). However, detection of enlarged bacteria in mixed, natural communities is difficult because of the natural variation in cell size (34).

Alternatively, methods for detection of viable bacteria by their first cell divisions in laboratory media have been developed. Detailed studies of early cell divisions have been made after mounting cells in a thin agar layer between a microscope slide and a coverslip (10, 22, 23, 29, 42). Bacteria which are able to complete only one or two cell divisions can thus be detected. Problems associated with the original slide culture technique seem to be potential oxygen and nutrient deficits and accumulation of harmful compounds among the cells in the narrow space between the glass slides. However, the difficulties may be avoided by filtering the bacteria onto a membrane filter and placing the filter on the surface of a growth medium. When this technique was used, aquatic bacteria gave rise to microcolonies when the membrane filters were incubated on absorbent paper soaked with nutrient solutions (14). Binnerup et al. (4) and Rodrigues and Kroll (32, 33) further incubated filters directly on selective and nonselective agar media, while Binnerup and Sørensen (5) incubated filters on liquid media.

Methods used to detect viable bacteria by their respiratory activity include the use of artificial electron acceptors, i.e., tetrazolium salts, which are reduced to formazan. Among a number of different tetrazolium salts (38), the most commonly used in ecological studies is 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT), which is reduced to a red INT-formazan product. The latter forms water-insoluble grains in the cells, detectable by bright-field microscopy, and allows viable, respiring bacterial cells to be seen together with the whole bacterial population (48). The INT-formazan assay

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has been used in several studies of open-water environments (8, 21, 48), groundwater and subsurface sediments (17, 44), and soil (20). Major limitations of the method were the difficulty of detecting the red formazan grains against a black background when using polycarbonate filters for microscopy (12) and dissolution of the INT-formazan grains by microscope immersion oil (48). These problems have been solved by transferring the bacteria from the filter to a gelatin matrix on a glass microscope slide (17, 24, 40). Other improvements in the staining and mounting of specimens, by using different filters and fluorochromes, have been suggested (6, 8, 39). A new tetrazolium salt, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC; Polysciences, Inc., Warrington, Pa.), which is reduced by bacteria to a water-insoluble, red-fluorescent formazan product has lately become available and has been used with both pure cultures of bacteria (15, 16) and environmental water samples (34, 37). By parallel staining of the total number of cells, the fraction of viable bacteria (those accumulating CTC-formazan) could be detected in the same microscopic specimen when switching the filter combinations on the microscope (15, 34, 37).

The objective of this investigation was to compare and improve the methods used to detect viable bacteria (including the nonculturable fraction) extracted from soil. We determined the total number of cells by AODC and evaluated three different methods used to determine the number of viable bacteria, i.e., (i) reduction of CTC to fluorescent CTC-formazan, (ii) formation of microcolonies on membrane filters placed on liquid media, and (iii) formation of conventional colonies on agar plates. We evaluated the effects of different pretreatments of the soil before extraction of the bacteria on the indigenous bacterial population. The pretreatments were a temperature increase and drying or wetting before the temperature increase. Drying prior to the temperature increase was found to limit cell division and microcolony formation and thus decrease the numbers of viable and culturable bacteria, while wetting prior to the temperature increase slightly increased the numbers of viable and culturable bacteria.

MATERIALS AND METHODS

Media. Winogradsky salt solution (W), consisting of 0.4 g of K_2HPO_4, 0.13 g of $MgSO_4 \cdot 7H_2O$, 0.13 g of NaCl, 0.0025 g of $MnSO_4 \cdot 7H_2O$, and 0.5 g of NH_4NO_3 in 1 liter of membrane-filtered (Milli-Q; Millipore), autoclaved water, pH 6.8 to 7.0 (modified form of that described in reference 13), was used for all dilutions and as a growth medium. Cold soil extract (CSE) was also used as a growth medium and was prepared as described by Olsen and Bakken (25). CSE medium was prepared in one batch and stored at −20°C; the medium was used in a 1:9 dilution with W medium. All reagents were analytical grade, and the glassware was thoroughly washed to reduce contamination with organic compounds.

Soil characteristics and extraction of bacteria. Soil was collected in a barley field by using a 5-cm-wide and 30-cm-long corer. Samples were stored in plastic bags (1 month at 4°C), and immediately before use the soil was sieved through a 4-mm (hole size) sieve to remove gravel and larger organic particles, such as plant fragments. The water content was then 19% of the soil dry weight, corresponding to a water-holding capacity of −10 kPa.

Four subsamples of soil were used for the experiment. One of the samples was wetted with membrane-filtered, autoclaved water, which gave a water content of 30% of the soil dry weight (−1 kPa). A second sample was air dried at room temperature for 2 h, which gave a water content of 2% of the soil dry weight (−79 MPa). A third sample retained the original water content of 19% of the soil dry weight (−10 kPa). These three soil samples were all incubated in sterile glass petri dishes sealed with Parafilm for 5 days at 17°C prior to extraction of the bacteria. A fourth sample also retained the original water content, but the bacteria were extracted immediately after sieving.

To extract bacteria, 1 g of soil was mixed with 10 ml of W medium and placed in a water bath sonicator (Metason 200; Struers A/S, Rødoure, Denmark) for 20 min. To remove larger soil particles, the suspension was centrifuged for 5 min at 4,200 × *g*. The supernatant, containing the bacterial extract, was properly diluted and plated on agar media (CFU; see below) or filtered through a 0.2-μm-pore-size polycarbonate filter (Nuclepore; Costar, Cambridge, Mass.). White, unstained filters were used to avoid possible utilization of the staining component as a substrate by the bacteria. Usually, 100 μl of a 10^{-3} dilution was filtered together with 3 ml of prefiltered water. The filters (25 to 30 replicates) for each of the three samples were then placed with the bacterium-containing side up on either W or CSE medium in sterile glass petri dishes for up to 64 days at 17°C. Bacterial extracts from the pretreated soil samples were all incubated on CSE medium. At intervals, filters were removed from the medium surfaces and either used for the CTC reduction assay (CTC-reducing bacteria; see below) or fixed by placement of the filters on a 2% formaldehyde solution at 4°C.

Total counts and micro-CFU. The polycarbonate filters were stained by placement on the surface of an Irgalan Black solution (2 g dissolved in 1 liter of 2% acetic acid as described by Hobbie et al. [12]) for 5 min (bacterium-containing side up), and excess stain was removed in three consecutive transfers to water baths. Acridine orange staining of the bacteria and subsequent mounting of the microscope slides were done as described by Binnerup et al. (4), except that Olympus immersion oil was used instead of paraffin oil. The total numbers of bacteria (AODC) and microcolonies (micro-CFU) in the same specimen were counted by fluorescence microscopy. A microcolony was defined as a tight association of at least two cells. A minimum of 100 single bacteria plus microcolonies was counted in each specimen, which gave a standard deviation of 10%. Additionally, the number and sizes of the microcolonies were estimated in two scans across the entire filter; in these two scans, 1.25% of the total filter area was inspected for microcolonies. The microcolonies were divided into three size classes, comprising 2 or 3 cells, 4 to 11 cells, and 12 or more cells per microcolony. Counting was only rarely disturbed by fungal growth on the filters.

CTC-reducing bacteria. Filters for the CTC reduction assay were placed on 230 μl of 10% tryptic soy broth (Difco) containing 3 mM CTC in a small glass dish for 16 to 24 h at 17°C. We decided on a 3 mM CTC concentration in our assay since 2 to 6 mM CTC was reported to be suitable for CTC-formazan formation with *Pseudomonas putida* (34). In preliminary experiments, the optimal time of incubation was investigated by comparing the number of cells stained by 4′-6-diamidino-2-phenylindole (DAPI; Sigma) and the number of cells with CTC-formazan deposits after 20 min and 1, 3, 6, 16, and 24 h of incubation. The small glass dish was placed in a larger glass petri dish containing a small amount of water to maintain humidity. After incubation, the filters were immediately fixed on 2% formaldehyde solutions (4°C). When the filters were removed from the formaldehyde, water attached to the bottom side of the filters was gently removed by pulling the filters across the surface of a nylon mesh. The filter matrix was stained for 2 min with a 0.1% crystal violet solution rather than an Irgalan Black solution, as the acetic acid in the latter

solution was found to dissolve the CTC-formazan grains. The bacteria on the filters were subsequently stained by placing the filters on the surface of a 5-μg/ml DAPI solution for 5 min (28). Excess DAPI stain was removed by transfer of the filter to a water bath.

The filters were mounted in immersion oil (Olympus) and examined in a fluorescence microscope as described by Jørgensen et al. (15). When counting the number of CTC-reducing cells or microcolonies, 100 randomly chosen microscopic fields were scanned. Microcolonies with CTC-formazan grains were counted as originating from one single cell.

CFU. The numbers of CFU were determined on agar plates with either W or CSE medium with 15 g of Bacto Agar (Difco) per liter. Bacteria extracted from pretreated soil samples were all plated on CSE agar. No fungicide was added to the medium, but 10 replicates of each dilution (10^{-4}, 10^{-5}, and 10^{-6}) were prepared and the plates with fungal growth were discarded. The plates were incubated for a total of 64 days at 17°C, and the same plates were counted several times during incubation.

RESULTS

Assays of viability in soil bacteria. In the CTC reduction assay, incubation time must be long enough to allow visible amounts of fluorescent CTC-formazan to accumulate inside the respiring cells. On the other hand, the time should not be so long as to activate dormant cells or allow cell divisions. In this study, the bacteria were captured on filters before incubation with CTC, so any cell division would thus be detected. Indigenous soil bacteria on the filters were found to deposit fluorescent CTC-formazan within 1 h (17°C) when incubated on the surface of a 10% tryptic soy broth solution containing 3 mM CTC. No cell divisions resulting in microcolonies were observed during the 24 h of incubation, and no further CTC reduction was observed after 1 h of incubation. As a result, we standardized the incubation of bacterium-containing filters to 16 to 24 h (17°C) on a 10% tryptic soy broth solution containing 3 mM CTC. We counted the bacteria on the filters immediately after staining in crystal violet and DAPI solutions and mounting in immersion oil, since the CTC-formazan grains dissolved after storage overnight (4°C). Immersion oil has previously been found to dissolve INT-formazan (8, 40, 48), and as CTC-formazan is soluble in polar organic solvents (27), it is likely that CTC-formazan grains dissolve as well, even though this was not observed by Rodriguez et al. (34).

The first comparison of the four assays to determine total and viable numbers of soil bacteria was made in a mixed population extracted from untreated barley field soil. As shown in Fig. 1, filters with bacteria incubated on W medium for up to 64 days showed a total number of approximately 10^9 cells per g of soil. The total number fluctuated in the beginning because of staining difficulties but later became stable at approximately 10^9 cells per g of soil (day 64). The number of viable, CTC-reducing bacteria varied from 2×10^7 to 5×10^7 cells per g of soil during the 64 days without any significant change with time. When micro-CFU counting was used to estimate the viable population, the number was slightly higher: the first micro-CFU emerged after a few days, and they increased in number with time; after 64 days, we counted approximately 9×10^7 micro-CFU/g of soil. The number of visible CFU on W agar plates also increased during the 64 days and was eventually approximately 5×10^7 CFU/g of soil. The plate counts were obtained from high dilutions, which gave 0 to 10 colonies per plate. All further plate counts were made from lower dilutions, resulting in 10 to 30 colonies per plate.

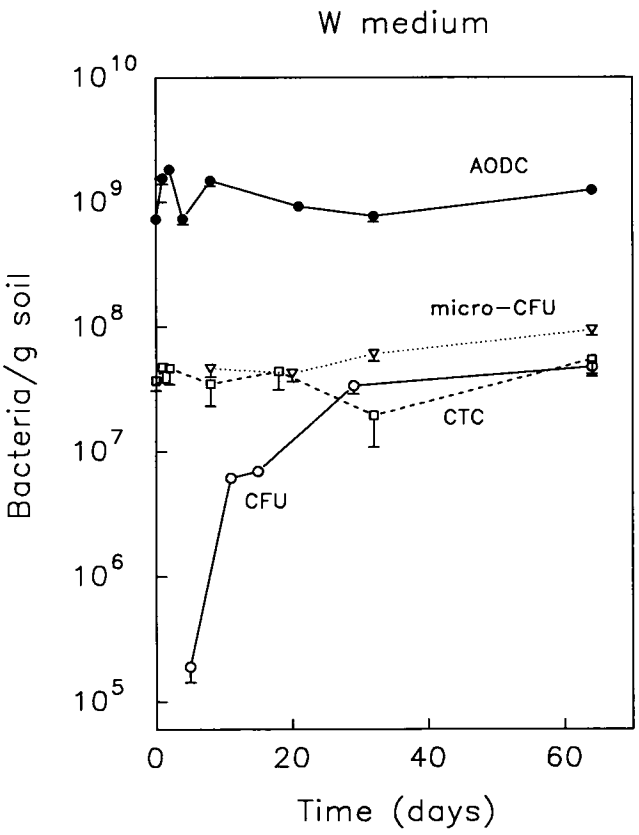

FIG. 1. Numbers of total, viable, and culturable soil bacteria as a function of time when incubated with W and determined by AODC (●) or counting of micro-CFU (▽), bacteria accumulating CTC-formazan (□), or CFU (○). Standard deviations were calculated on the basis of the Poisson distribution ($\sqrt{n}$) and are indicated by vertical bars.

The two growth-dependent assays of viability thus both showed an increase in the number of cells over time, while the CTC assay of electron transport activity resulted in a constant number. The CTC assay, however, had a relatively high standard deviation in the present experiment, as the recorded number of CTC-reducing cells was lower than the number of microcolonies on the filters or colonies on the agar plates.

Effects of water treatments and a temperature increase on the viability of soil bacteria. Total bacteria, CTC-reducing bacteria, micro-CFU, and CFU were compared in four soil samples which were either (i) untreated or subjected to (ii) a temperature increase, (iii) wetting prior to the temperature increase, or (iv) drying prior to the temperature increase. CSE medium was used for incubation, and the results are shown in Fig. 2. The general pattern is the same as that obtained with W medium (Fig. 1). The agar added to the agar plates contains available carbon sources, which may sustain the observed colony development. However, W medium without agar does not include any added carbon substrates, and the bacteria are thus restricted to growth on impurities in the mineral salts, organic substrates in the filtered soil suspension, or organic substances from the air or autotrophic growth. In addition to these sources, CSE medium contains an extract of carbon substrates from the soil. However, this did not affect the recorded number of viable and culturable bacteria detected by the three viability assays, as there was no significant difference

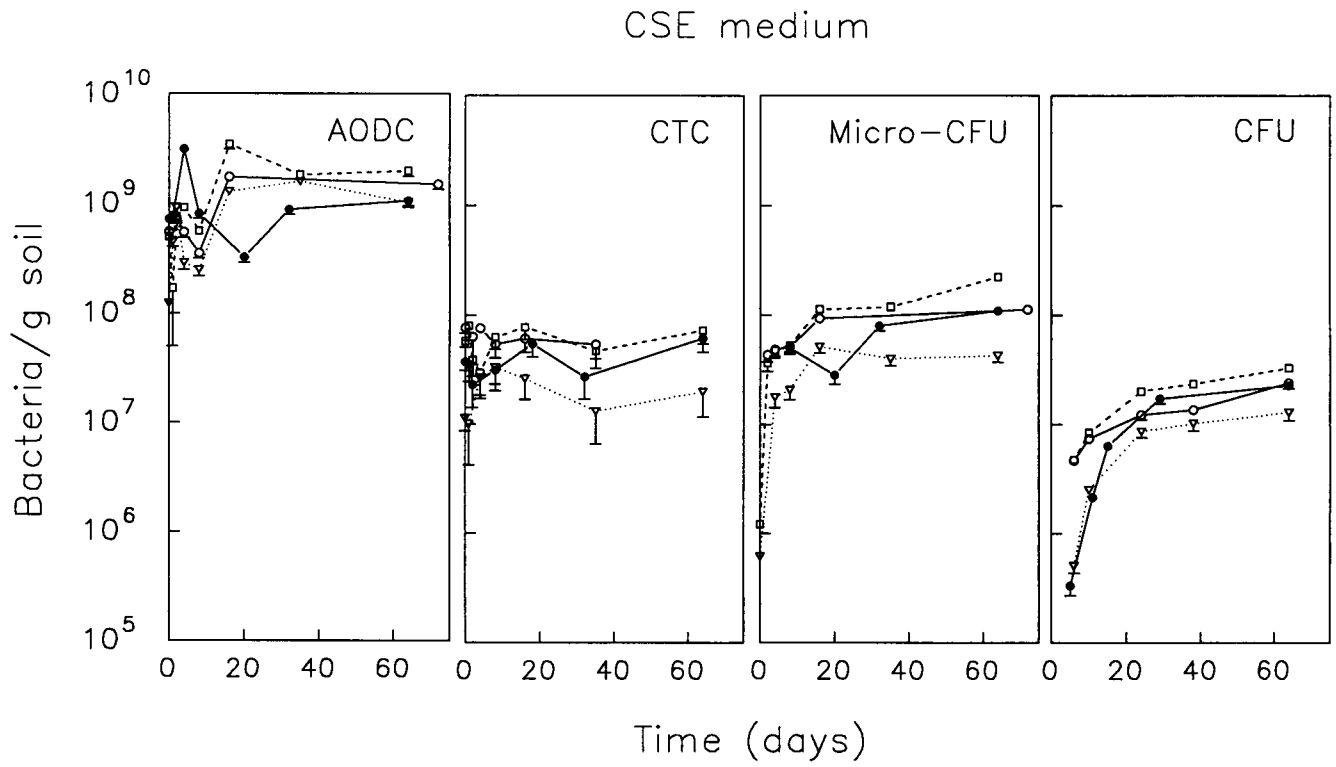

FIG. 2. Total numbers of bacteria and viability and culturability after different treatments of agricultural soil were tested by AODC, counting of micro-CFU on 10% CSE, reduction of CTC to fluorescent CTC-formazan (CTC), and counting of CFU on 10% CSE agar in four soil samples: untreated soil (control) (●), soil stored for 5 days at 17°C before incubation (○), soil wetted and stored at 17°C (□), and soil dried and stored at 17°C (▽). Standard deviations were determined as described in the legend to Fig. 1.

between W medium and CSE medium on the basis of a chi-square test at day 64. This result is in accordance with the observations of Olsen and Bakken (25).

Despite the fluctuating AODC in the beginning of the incubation, the total number of bacteria in all four soils reached a stable level at 1×10^9 to 2×10^9 cells per g of soil after 64 days of incubation (Fig. 2). The fluctuations were not observed in a subsequent repeat of the experiment and were probably due to difficulties in the staining procedures. The fluctuations were not seen in the CTC reduction assay or the microcolony assay, in which the cells fluoresce more brightly and make detection easier. The number of CTC-reducing cells was constant throughout the experiment and reached approximately 5×10^7 to 7×10^7 cells per g of soil after 64 days, except in the soil subjected to drying prior to the temperature increase, in which the number of CTC-reducing bacteria was only approximately 2×10^7 cells per g of soil (Fig. 2). Insufficiently separated cells attached to each other or to organic particles could potentially be counted as microcolonies, but at the beginning of the incubation on Nuclepore filters an insignificant background of 0 to 1.2×10^6 micro-CFU/g of soil was counted. The number of micro-CFU increased from a low level during the first 20 days and eventually reached a constant or slowly increasing level of approximately 10^8 micro-CFU/g of soil. The exception was the soil subjected to drying prior to the temperature increase, in which only 4×10^7 micro-CFU/g of soil were detected (Fig. 2). CFU on CSE plates also increased in number with time; a rapid increase during the first 20 days was followed by a slower increase to a level of 2×10^7 to 3×10^7 CFU/g of soil after 64 days. The exception was the soil subjected to drying prior to temperature increase, which contained only 10^7 CFU/g of soil.

Viable but nonculturable soil bacteria. The microcolony assay (4) was used to demonstrate a fraction of viable but nonculturable bacteria in the four soil samples tested. This was done by observing the numbers of single cells in the developing microcolonies during the 64 days of incubation (Fig. 3). The number of microcolonies containing two or three cells increased during the first 8 days of incubation, which indicates that the cells started dividing. Subsequently, some of the microcolonies with two or three cells counted in the early incubation phase can be expected to continue dividing and pass through the next size class of 4 to 11 cells per microcolony, eventually ending up in the largest size class, containing 12 or more cells per microcolony (Fig. 3). With continued growth, the number of large microcolonies will increase as a function of time. Such a slow but steady increase in the number of microcolonies representing all three size classes was observed in both untreated control soil and soil subjected only to a temperature increase. In contrast, the two soils subjected to wetting or drying prior to the temperature increase demonstrated different patterns of microcolony development. Wetting thus seemed to increase the ability of the bacteria to form microcolonies, while the bacteria in the dried soil show less capacity to divide over time. Actually, the cell divisions eventually came to a complete stop, showing no further increase in size or number of micro-CFU after 16 days. This result is similar to the observation of Binnerup et al. (4), that a fraction of a bacterial population may undergo a limited number of cell

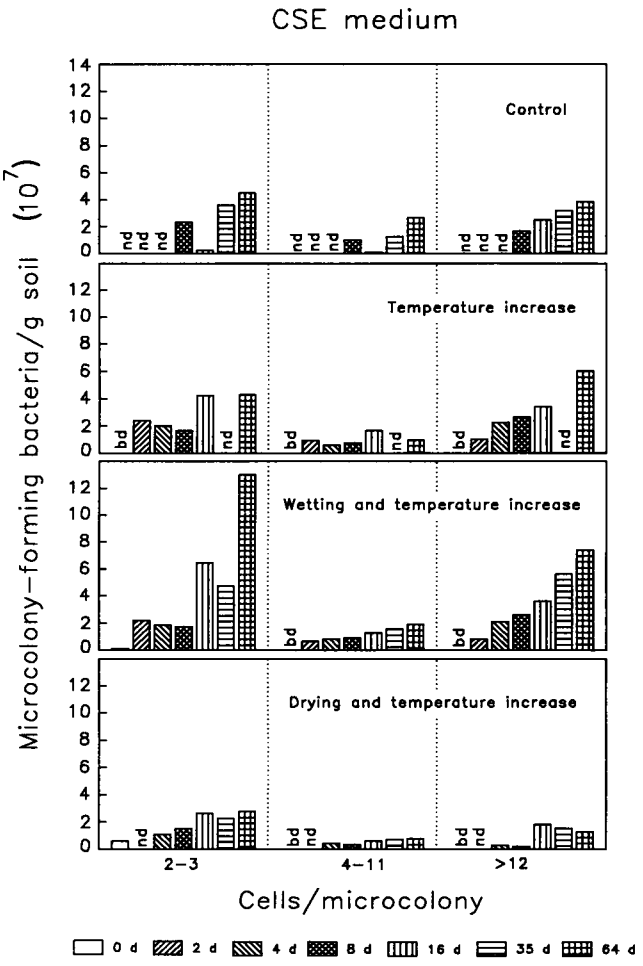

FIG. 3. Number of micro-CFU as a function of the number of cells per microcolony observed at different times during the incubation of four soil samples. bd, below detection limit; nd, not determined; d, days.

divisions before growth ceases. These bacteria thus represent a viable but nonculturable fraction of the population.

DISCUSSION

Viability assays. Respiratory activity measured by reduction of tetrazolium salts has become a popular criterium when determining the viability of bacterial cells. Different incubation times, ranging from 10 min to 4 h, have been used for different tetrazolium salts (21, 34, 37, 40, 48), with generally long incubation times for soil samples (3, 20, 43). As we incubated the cells with CTC after they were immobilized on filters, cell divisions could be detected as microcolony formation at the same time as viability was recorded as the number of bacteria or microcolonies accumulating formazan. Incubation for 16 to 24 h ensures that an adequate amount of CTC-formazan accumulated inside the cells. In some cases, the grains accumulated at the cell surface, indicating either surface-associated dehydrogenase activity or cellular export of the formazan compound (data not shown).

Not all bacteria are able to reduce tetrazolium salts, but the reduction can sometimes be enhanced by addition of intermediate electron carriers, use of nutrient-rich substrates, optimization of oxygen concentrations (41), or inhibition of the cytochrome-dependent electron transport system by cyanide (35). In our study, neither of the intermediate electron carriers methylene blue and NADH gave higher counts of CTC-reducing cells (data not shown). We sometimes noticed that not all of the microcolonies formed had cells with formazan deposits. On the other hand, some CTC-reducing cells remained as single cells throughout the incubation period and thus never showed cell division. Hence, the CTC-reducing cells did not always form microcolonies and vice versa. The coupling between CTC reduction (dehydrogenase) activity and cell division at the initiation of growth in dormant soil bacteria must be investigated further.

Culturability on conventional laboratory media is often taken as a measure of viability of bacterial cells. However, since the concept of viable but nonculturable bacteria (36) has been generally accepted, more attention is being paid to the development of new viability assays. One example is the microcolony assay (4), which detects viable cells by their first couple of cell divisions. In the present study, both the CTC reduction assay and the microcolony assay gave higher cell numbers per gram of soil than did conventional plate counts. Similar results were reported for a specific *P. fluorescens* strain in soil tested with the microcolony assay (4) and for indigenous soil bacteria tested by tetrazolium reduction (21, 34, 37).

Cells giving rise to microcolonies of 12 or more cells per microcolony are likely to represent cells which also form visible colonies on agar plates (Fig. 3). We propose that only a specific fraction of the viable bacteria is culturable, as shown by the ability to form visible colonies on agar plates or relatively large microcolonies on membrane filters. Similarly, it is likely that bacteria which are unable to form colonies on agar plates but are registered as small microcolonies dividing a few times on filters can be regarded as a viable but nonculturable population (4).

As judged from the results obtained with the four soil samples used in this study, culturability, defined by the fraction of the total population able to form visible colonies on agar plates, was 1 to 2%, viability as defined by the bacteria reducing CTC was 2 to 6%, and viability as defined by the bacteria forming microcolonies accounted for the largest fraction of the entire population, 4 to 11%. The same pattern was observed with both W and CSE media (Fig. 1 and 2). Low culturability of soil bacteria may be found on both nutrient-rich media (2, 4, 9) and nutrient-poor media (25) and may be explained partly by insufficient separation of bacterial aggregates and soil particles before plating. Such aggregates may underestimate the real number of culturable bacteria, since they give rise to only one colony on agar plates (30). We therefore believe that viability of soil bacteria should be determined by assays allowing single-cell observations, e.g., the CTC reduction or microcolony formation assay. However, the recorded maximum viability of 11% of the total population raises the question of whether the remaining 89% might be detected by further attention to the assays. Both of the viability assays used in this study are thus based on aerobic incubations, and viable bacteria sensitive to high oxygen concentrations may not be recorded (5). Proper attention to incubation conditions may thus further improve the detection of viable cells.

Effects of wet and dry conditions on viability of soil bacteria. Wetting of the soil prior to the temperature increase resulted in a slightly higher number of both total and viable bacteria than in the control (Fig. 2). West et al. (45) also found that bacterial numbers and biomass increased in soil which was rewetted and stored for 7 days at 25°C. The increased bacterial biomass was explained by soil mixing and release of nutrients

and organic matter. A similar burst of high microbial activity and elevated bacterial numbers after rewetting of dried soil was reported by Lund and Goksøyr (19) and Zelles et al. (47). The increase in microcolonies of two or three cells in wetted soil after 8 days shows that a fraction of the bacteria increased their viability with time (Fig. 3, third set of panels from the top). The long lag phase was probably caused by activation of slowly growing or dormant bacteria during incubation. We suggest that the increase in the number of viable bacteria in the wetted and temperature-treated soil in the present study was caused by increased enzymatic activity making more organic substrates available.

Drying and dry storage of soil have, in several instances, been shown to lower the number and activity of indigenous bacteria (7, 19, 47). Removal of water from larger soil pores may thus lead to a decrease in available space for soil bacteria in capillary soil pores (11). Roberson and Firestone (31) reported that drying of sand inoculated with a pure *Pseudomonas* culture resulted in a decrease of cell biomass, while electron transport activity (INT-formazan production per unit of protein) was unchanged. In the present study, we did not detect any effect of drying on the total number of bacteria (AODC), but reduced viability of the cells was determined by the CTC reduction and microcolony assays and reduced culturability was determined by plate counts (Fig. 2). Among the micro-CFU formed in dried soil, a fraction never developed more than two or three cells per microcolony (Fig. 3, lowest set of panels). This indicated that drying reduced the recruitment of larger microcolonies, which in turn demonstrated that the culturable population of cells decreased.

Comparison of assays used to estimate the number of viable bacteria in soil indicated that (i) plate counts may result in low numbers because of low culturability or clumping of cells and (ii) CTC reduction and microcolony assays result in comparable numbers; while the latter is tedious, the method may provide an estimate of the viable fraction which is not culturable on laboratory media.

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