Metabolic Activity of Bacterial Cells Enumerated by Direct Viable Count

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The direct viable count (DVC) method was modified by incorporating radiolabeled substrates in microautoradiographic analyses to assess bacterial survival in controlled laboratory microcosms. The DVC method, which permits enumeration of culturable and nonculturable cells, discriminates those cells that are responsive to added nutrients but in which division is inhibited by the addition of nalidixic acid. The resulting elongated cells represent all viable cells; this includes those that are culturable on routine media and those that are not. *Escherichia coli* and *Salmonella enteritidis* were employed in the microcosm studies, and radiolabeled substrates included [*methyl*-^{3}H]thymidine or [U-^{14}C]glutamic acid. Samples taken at selected intervals during the survival experiments were examined by epifluorescence microscopy to enumerate cells by the DVC and acridine orange direct count methods, as well as by culture methods. Good correlation was obtained for cell-associated metabolic activity, measured by microautoradiography and substrate responsiveness (by the DVC method) at various stages of survival. Of the cells responsive to nutrients by the DVC method, ca. 90% were metabolically active by the microautoradiographic method. No significant difference was observed between DVC enumerations with or without added radiolabeled substrate.

The direct viable count (DVC) method (16) has been proposed as a method that can be used to surmount problems of overestimation of the number of viable bacteria when direct microscopic methods are used (10) and of underestimation of the number of viable cells when culture methods of enumeration are used (3). Comparison of results of plate counts, direct microscopic enumeration, and indirect activity measurements indicate that the number of viable bacteria, i.e., those capable of forming colonies on a solid medium, is smaller than the number of bacteria detectable by direct microscopy by several orders of magnitude (8, 11–13, 17, 24, 26). Data obtained by direct microscopic enumeration of bacteria that are present in environmental samples, however, do not provide reliable estimates of viability. They do not indicate whether those cells enumerated by direct microscopy are able to metabolize, grow, respire, and divide (10). Furthermore, indirect measurements of metabolism, including enzymatic activity, substrate uptake, and respiration, are cumbersome and expensive if sampling is frequent and the number of samples to be analyzed is large (3). In addition, indirect methods do not provide an estimation of the portion of the population which is responsive to the given conditions. In fact, the portion of the population of bacterial cells that cannot be cultured but that can be observed by direct observation is often of greatest interest in studies of viable bacteria in the aquatic environment.

Of the many methods employed to identify or examine cells that are viable but that are unable to multiply on standard bacteriological media, radiolabeled substrate uptake, in combination with immunofluorescence, has been used to identify individual metabolically active cells (7, 14, 19, 21, 25). This is accomplished by microscopically observing photographic film emulsions overlaying filter-bound cells in which the radioactive decay of cell-incorporated radio-

labels is allowed to expose the film. Development of the film and staining of the cells in the film preparation reveals the association of developed grains with stained bacterial cells.

In the DVC method, samples are incubated with yeast extract and nalidixic acid for a period of time that is sufficient to induce growth (16, 23). Yeast extract provides nutrients, while nalidixic acid blocks DNA synthesis (5, 9, 15, 18). Thus, cell replication is inhibited, and at the same time, cell growth, i.e., enlargement, is promoted, resulting in filament formation by the substrate-responsive cells. Subsequent acridine orange staining of these preparations, followed by epifluorescence microscopy, allows enumeration of the readily identifiable elongated cells as an estimate of the viable population.

The objective of the study reported here was to demonstrate that those cells responding to, and thus enumerated by, the DVC method are in an active metabolic state and to show that filament formation is a result of metabolism and growth of viable cells.

MATERIALS AND METHODS

Samples. Natural water samples from the Chesapeake Bay were the source of the viable but nonculturable populations employed in this study.

Microorganisms. Bacterial strains employed in this study were *Escherichia coli* H10407, an enterotoxigenic clinical isolate, and *Salmonella enteritidis* S131BB, an environmental isolate.

Microautoradiography. Samples were incubated with 1 μCi of L-[U-^{14}C]glutamic acid or [*methyl*-^{3}H]thymidine per ml for 20 min to 3 h (25). The concentration was determined by preliminary assays and set at that concentration that produced easily resolved, cell-associated silver grains in the film emulsion (2). Determination of the volume to be filtered was based on results of corresponding acridine orange direct count and DVC methods performed on duplicate samples without the addition of radiolabel. Postincubation incorporation was blocked by the addition of formaldehyde to a final

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concentration of 2%, after which the samples were refrigerated overnight (5°C). Controls were prepared for measurement of background radioactivity and detection of background radiation. Appropriate volumes were filtered through Irgalan black (CIBA-GEIGY Corp., Greensboro, N.C.)-stained polycarbonate filters (pore size, 0.2 μm; Nuclepore Corp., Pleasanton, Calif.).

Clean, prewarmed microscope slides were coated with melted and diluted (1:3 with sterile water) nuclear track emulsion (NT2; Eastman Kodak Co., Buffalo, N.Y.); complete darkness was maintained during this step. Preparations were drained, and in a dark room fitted with a dark red filtered safe light, prepared membrane filters were applied to the moist emulsion, with the sample side facing the emulsion. Emulsions were allowed to solidify on a cold tray for several hours in darkness before the slides were transferred to a light-tight black plastic slide box for exposure in a dessicator jar for 3 to 7 days at room temperature.

After exposure, the slide films were developed in darkness in developer (diluted 1:3; D-19; Kodak) for 4 min, rinsed for 30 s in running water, fixed in acid fixer (Kodak) for 4 min, washed in the light for 15 min in running water, and allowed to air dry.

To stain the bacterial cells on the film emulsion, slide preparations were presoaked in citrate buffer (pH 6.6) for 6 min; stained with acridine orange (40 mg/100 ml of citrate buffer; pH 6.6) for 7 min; destained stepwise by soaking for 6 min in pH 6.6 citrate buffer, 6 min in pH 5.0 citrate buffer, and 10 min in pH 4.0 citrate buffer; and rinsed for 1 min in water. Slide preparations were soaked in 1% glycerol for 1 min, and the filter was delicately peeled away from the film emulsion on the slide. Removed filters were also observed by epifluorescence microscopy for complete transfer of bacterial cells to the film emulsion. Slide preparations were observed by epifluorescence microscopy for acridine orange-stained cells and by transmitted light for exposed silver grains in the underlying developed film emulsion. Both stained cells and exposed grains in the film could be seen simultaneously by adjusting the light source. Acridine orange-stained cells were enumerated and scored as plus or minus for associated silver grains.

Enumeration of bacterial cells. (i) Plate count method. Standard plate counts were performed by the spread plate method on standard plate count medium (1). Resuscitation of samples was attempted by using incubation for 2 h at ambient temperature in 1/10 strength nutrient broth prior to plating (22).

(ii) Acridine orange direct count method. Samples were stained with acridine orange and filtered onto Irgalan black (pore size, 0.2 μm; diameter, 22 mm; CIBA-GEIGY)-stained polycarbonate filters (Nuclepore), and the filters were mounted on clean microscope slides (10). All slides employed in the direct enumeration were examined with a microscope (standard model 18; Carl Zeiss Inc., New York, N.Y.) equipped with a ×10 eyepiece, a ×100 neofluor objective, an epifluorescence condenser (IVFL; Zeiss), a 100-W halogen lamp, a band-pass filter (BP 450-490; Zeiss), a beam splitter (FT 510; Zeiss), and a barrier filter (LP 520; Zeiss).

(iii) DVC method. Samples were incubated with yeast extract (0.025%) and nalidixic acid (0.002%) and incubated for 6 to 24 h at 25°C (15). After the samples were incubated for an appropriate period of time, they were stained with acridine orange and processed as described above for the acridine orange direct count method (23). Cells longer than three normal (by the acridine orange direct count method)

cell lengths made up the counts obtained by the DVC method.

(iv) Labeled DVC method. Radiolabeled substrates were used to supplement the DVC incubation mixture by the DVC method to detect the uptake and distribution of the labeled compound by cells and relate the photographic pattern produced in the film emulsion by radioactive decay of the radiolabel to individual cells. From each preparation examined by the labeled DVC method, a count was made of the number of (i) elongated cells, (ii) elongated cells with associated silver grains, (iii) elongated cells without associated silver grains, and (iv) cells with associated silver grains which were not elongated.

Recovery. The recovery protocol used in this study was to harvest viable but nonculturable cells, wash them with sterile salts solution, and inject cells that were concentrated by centrifugation to 10^8 DVC/ml into ligated rabbit loops by the method described by Colwell et al. (4). The protocol was followed to obtain plate counts after standard plate count procedures yielded no colony formation.

RESULTS

Data showing the survival of cells in water from the Chesapeake Bay are provided in Fig. 1. Results of the enumeration methods employed in the microcosm studies are shown in Fig. 2 for *E. coli* and *S. enteritidis*. While the total viable bacterial counts dropped to zero by conventional plate counting methods, numbers obtained by the acridine orange direct count method remained relatively stable. Numbers obtained by the DVC method and cell activity measurements were greater than those obtained by plate counts but less than those obtained by direct counts. As the number of culturable bacteria dropped, an increase was observed in the number of small metabolically active cells by the DVC method. These cells exhibited substrate uptake in microautoradiographic preparations but were not substrate responsive; i.e., elongation was not observed by the DVC method. More than 90% of the bacterial cells enumerated by the direct viable count were also enumerated by the direct viable active count method (Fig. 2). Microscopic observations of microautoradiographic preparations of microcosm samples incubated with radiolabeled substrates, after the cells became nonculturable, demonstrated that such cells were capable of incorporating radiolabeled substrate (Fig. 3).

To demonstrate that in microcosms the somnicell stage occurs, the recovery methods described by Colwell et al. (4)
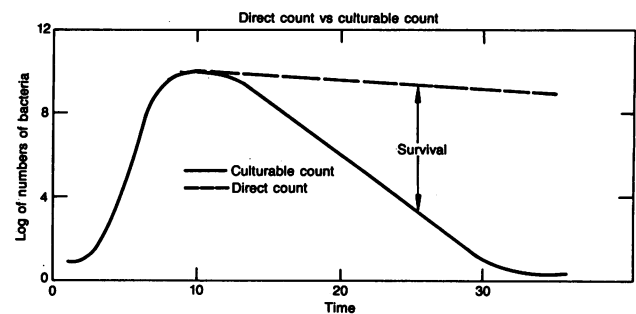

FIG. 1. Survival of the culturable population with time, including the die-off portion of the population. Typical direct count enumeration data are also shown. The difference between direct and culturable counts represents the population that remained viable but not culturable by routine or standard microbiological methods.
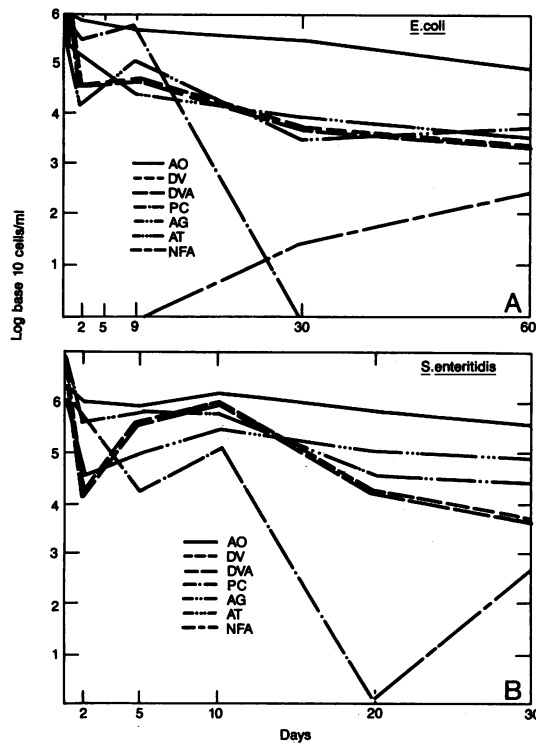
FIG. 2. Comparative counts for *E. coli* (A) and *S. enteritidis* (B) over time under survival conditions by using several methods of enumeration. Abbreviations: AO, acridine orange direct epifluorescence count; DV, direct viable count; DVA, direct viable active (radiolabeled) count; PC, standard plate count; AG, active (radiolabeled) count with [^{14}C]glutamic acid; AT, active (radiolabeled) count with [^{3}H]thymidine; NFA, nonfilamentous; active in DVC with radiolabel.

were employed. Shortly after the bacterial populations became nonculturable, simple resuscitation measures, as employed for injured cells (22), were effective. However, under more extensive exposure to environmental conditions that were suboptimal for growth, in this case prolonged nutrient deprivation, the cells did not respond to resuscitation, yet continued to exhibit substrate responsiveness by the DVC method. That these cells were capable of growth was shown by inoculating the cells into washed, ligated rabbit ileal loops. After an appropriate incubation time cells recovered

from the ileal loop were culturable on standard culture media and showed biochemical characteristics and plasmid profiles identical to those of the strain prior to introduction to the somnicell stage.

Thus, dormant cells can be detected by the acridine orange direct count method and animal passage. The plate count procedure can be used to enumerate culturable, recoverable cells but not dormant cells, unless they have been passaged through animals. Interestingly, as the time that cells remain under survival conditions increases, the growth response, i.e., recoverability by the plate count method, is lost and, eventually, so too is substrate response. The stage of survival of the bacterial population is summarized in Table 1.

DISCUSSION

Since the term *viable* has long been used to refer to plate counts, e.g. total viable count, to avoid further confusion by terminology, we propose the term *somnicell* to encompass bacteria in the viable but nonculturable state which exhibit living attributes other than the ability to reproduce in culture media. The DVC method allows enumeration of viable cells and somnicells; this is the viviform population (Table 1). Nalidixic acid, in addition to preventing DNA synthesis, alters the permeability of living cells (6); thus, in the DVC procedure, the use of nalidixic acid results in organisms that are able to utilize exogenously supplied substrate, while they are temporarily incapable of active transport by passive entry of substrate into the cell, bypassing the necessity for cells to expend energy in capturing exogenous substrate. This sequence of events may be useful in the DVC method by reversing the survival strategy employed by many bacteria in response to adverse conditions which prompts entry into the nonculturable state. The recorded result from the DVC method is elongation, i.e., growth of cells which would otherwise be incapable of growth on standard bacteriological media.

The results presented here confirm that the DVC method provides a better estimate of the total number of viviform bacteria in aquatic samples than do culture methods of enumeration. The DVC method, however, while providing a better estimate than culture methods, still provides only a minimum estimate of the total number of living bacterial cells and is dependent, in part, on the substrate that is employed (20). In later stages of incubation in natural water microcosms, as more bacteria become nonculturable, a larger number of active cells are observed than are detected

TABLE 1. Characterization of cells in survival stages of progressive dormancy

Method of enumeration or detection	Survival at the following stage in the continuum						
	Culturable	Recoverable	Growth responsive	Metabolically active	Dormant	Intact	Dead
Acridine orange direct count	+	+	+	+	+	+	−
Animal-passaged recovery	+	+	+	+	+	−	−
Substrate uptake	+	+	+	+	−	−	−
Direct viable count	+	+	+	−	−	−	−
Acclimation, plate count	+	+	−	−	−	−	−
Standard plate count	+	−	−	−	−	−	−

Viable (Culturable, Recoverable) — Somnicell (Growth responsive, Metabolically active, Dormant, Intact)

Viviform

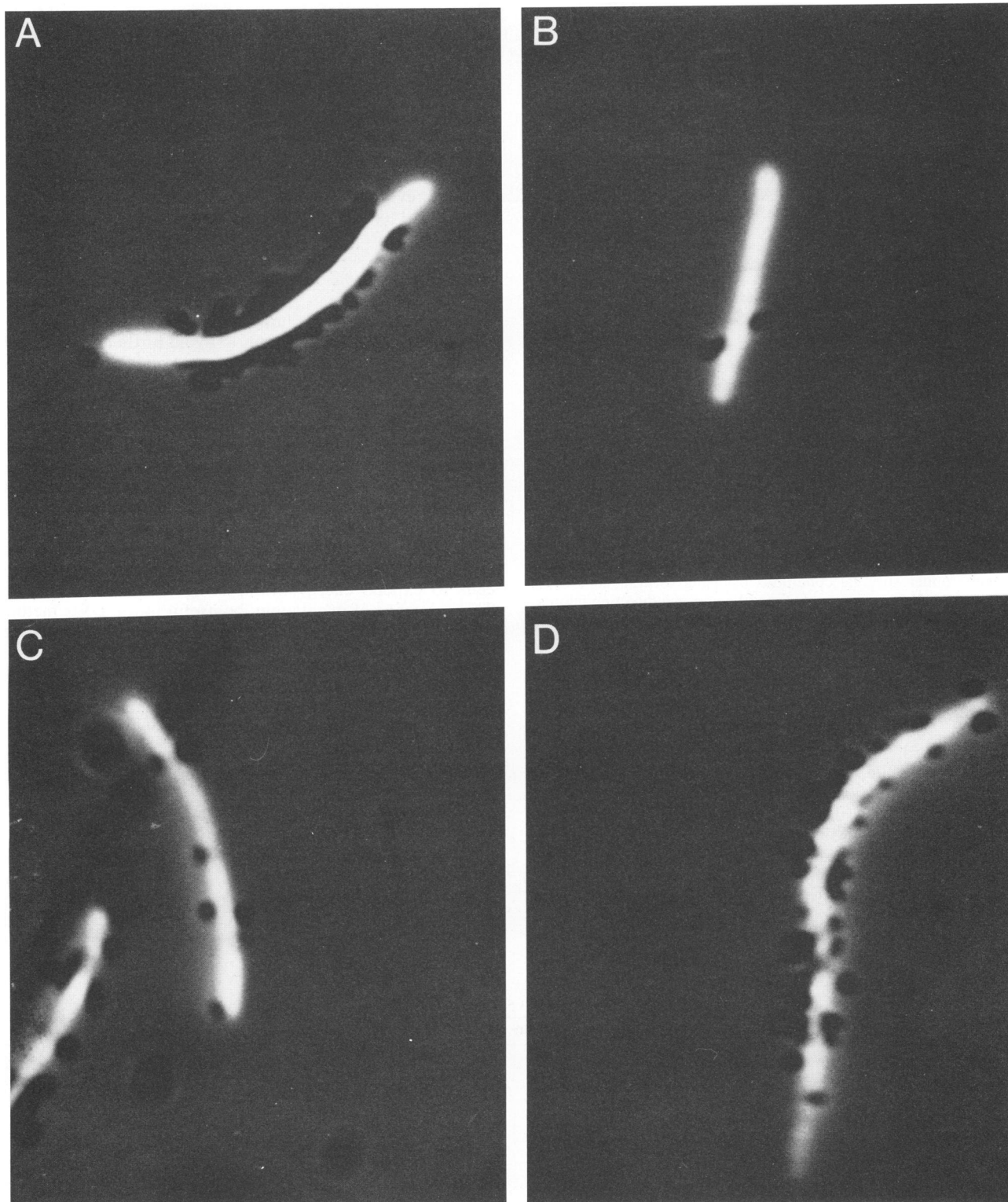Increasing time in survival conditions →

FIG. 3. Acridine orange-stained individual cells in autoradiographic film emulsion preparations of filtered samples for enumeration by the DVC method. (A) ^{3}H label, *E. coli*; (B) ^{14}C label, *E. coli*; (C) ^{14}C label, *S. enteritidis*; (D) ^{3}H label, *S. enteritidis*.

by the DVC method (Fig. 3). These cells are observed in the labeled DVC method as a population of cells which are associated with silver grains (active) but which do not elongate (they are nonfilamentous and are active by the DVC method with radiolabel). It is proposed that such cells incorporate substrate for maintenance functions but not for growth, a further progression in the steps leading to the somnicell stage of survival strategy, which is in accordance with the proposed scheme shown in Table 1. Progression to the somnicell state can be followed by a change in morphology of cells as dormancy progresses. The DVC method is useful in detecting such cells which may otherwise be nondetectable if culture methods are employed.

The functioning of cells in the natural environment when conditions are not optimal for growth and development of a given bacterial population have become a focus of interest in recent years. There is also an increased awareness of the need to monitor genetically engineered organisms released into the environment. The somnicell stage in the bacterial life cycle must be examined in greater detail, especially with respect to the genetic regulation of the successive steps in formation of the somnicell, in view of the increasing need for accuracy in the detection of bacteria in natural ecosystems.

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