APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1994, p. 1308–1316
0099-2240/94/$04.00+0
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Vol. 60, No. 4

Luminescence-Based Detection of Activity of Starved and Viable but Nonculturable Bacteria

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Received 12 October 1993/Accepted 21 January 1994

A naturally luminescent bacterium, *Vibrio harveyi*, and two bacteria, *Escherichia coli* and *Pseudomonas fluorescens*, which had been genetically marked with luminescence were starved in liquid medium at 4 and 30°C for 54 days. Total cell concentrations and concentrations of culturable and viable cells were determined by acridine orange staining, dilution plate counting, and direct viable counting, respectively, and population activity was measured by luminometry. *V. harveyi* became nonculturable but maintained viability during starvation at 4°C and maintained both culturability and viability at 30°C. In contrast, *E. coli* became viable but nonculturable during starvation at 30°C but not at 4°C. Luminescence of nonculturable cells of both strains, and culturable cells of *V. harveyi*, decreased to background levels during starvation. Luminescence of starved culturable cells of *E. coli* also fell below background levels but occasionally increased to detectable values. Viable, nonculturable forms of *P. fluorescens* were not detected at either temperature, and cells starved at 4°C showed no decrease in luminescence measured during incubation of samples at 25°C. Following incubation of late-log-phase cells with yeast extract and nalidixic acid, changes in light output directly paralleled changes in cell length, as observed during direct viable counting. Quantification of changes in luminescence following incubation of starved cells with yeast extract enabled measurement of the activity of both culturable and viable but nonculturable cells. Measurement of luminescence was significantly more sensitive, rapid, and convenient in quantifying activity following nutrient amendment than measurement of changes in cell length. Luminescence-based marker systems potentially provide a selective means of detecting the presence and activity of viable but nonculturable cells in the soil and freshwater environments, where indigenous luminescent populations are negligible, and enable assessment of the activity and environmental impact of such populations.

A major problem in assessing microbial numbers in natural environments is the entry of cells into a viable but nonculturable state (18). This is of particular importance for assessment of pathogens such as *Vibrio cholerae* (6, 25) and *Vibrio vulnificus* (13, 16, 24), which may be undetectable by standard techniques that require cultivation on laboratory media but which remain viable and possibly pathogenic (see reference 15). The formation of viable but nonculturable cells is also of significance for risk assessment following environmental release of genetically modified microorganisms, which may be undetectable by traditional enumeration techniques but still capable of activity and gene transfer (6).

In pure culture systems, entry of cells into the viable but nonculturable state is determined by comparing numbers of CFU with those of viable microscopic counts. Culturable cells are determined by dilution plate counts on laboratory media, and total cell counts are determined by using acridine orange or other suitable stains. The number of viable or active cells is determined with activity stains [e.g., 2-(4-iodophenyl)-3-(4-nitrophenyl)-5 phenyltetrazolium chloride] or by the direct viable count (DVC) method of Kogure et al. (12). DVCs are obtained by incubating cells in the presence of yeast extract and nalidixic acid, which prevents cells division, and determining the proportion of cells capable of growth and elongation. In natural samples, DVC can be used in combination with immunofluorescence to detect specific bacteria in mixed communities (25). In addition, molecular techniques, in particular, nucleic acid probing and PCR amplification, have been proposed as alternatives. Although these provide the necessary sensitivity and selectivity, they suffer from a number of disadvantages. Efficiencies of cell and DNA extraction vary for different organisms, quantification of populations using nucleic acid probes is difficult, contamination by humic materials affects the efficiency of DNA probing and PCR reactions, and, significantly, there is evidence of physiological changes in viable but nonculturable cells which affect the ability to amplify DNA by PCR (4). In addition, probing for particular DNA sequences gives information only on their abundance and not on the activity of cells containing such sequences. It is therefore potentially an alternative to total counts but does not provide information obtainable from activity stains or DVC and does not distinguish among culturable, viable, and dead cells. This distinction is of particular importance for risk assessment of genetically modified microorganisms and detection of pathogens in the environment.

Molecule-based marker systems provide an alternative strategy for detecting and tracking microorganisms in the environment. Such techniques enable selective viable cell enumeration of marked organisms and involve introduction of genes for antibiotic or heavy metal resistance (3), *xylE* (23), *lacZY* (8), or luminescence (7, 9, 17, 19, 20). In addition, luminescence-based techniques provide information on the spatial distribution of marked cells (19–21), and in situ activity and potential activity may be determined with luminometry (14). In naturally occurring luminescent organisms (e.g., *V. harveyi*), luciferase

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synthesis is under autoinducible regulation, which may be achieved in marked organisms by incorporation of the complete *lux* cassette. Constitutive production of luciferase requires removal of *luxIR* genes and substitution of a constitutive promoter for expression of the luciferase structural genes, *luxAB*. Removal of the *luxCD* or *-E* gene prevents synthesis and recycling of the aldehyde substrate for luciferase, providing a semiinducible system for luminescence, with maximal light output after exogenous addition of *n*-decyl aldehyde.

The aim of this study was to evaluate the suitability of luminescence-based techniques for detection of nonculturable cells and to assess their activity in three luminescent organisms: a naturally luminescent strain, *V. harveyi*, and strains of *Escherichia coli* and *Pseudomonas fluorescens* which had been genetically marked with luminescence. Traditional enumeration techniques were compared with selective enumeration and luminometry following incubation of each strain in appropriate starvation media at two temperatures, 4 and 30°C.

MATERIALS AND METHODS

Bacterial strains, plasmids, and cultural conditions. *V. harveyi* 1280, obtained from the National Collections of Industrial and Marine Bacteria, is a naturally occurring luminescent strain originally isolated from a luminous amphipod. It contains the complete *lux* cassette, including *luxAB* structural genes for luciferase, the regulatory *luxIR* genes for autoinducible synthesis of luciferase, and *luxCDE* genes for synthesis of the aldehyde substrate. Routine cultivation was carried out in artificial seawater broth (24) containing 3 g of yeast extract and 5 g of peptone (Oxoid) liter^{-1}, with incubation at 30°C. *E. coli* HB101 is an auxotrophic mutant carrying plasmid pFAC510 (9), bearing the *luxAB* genes isolated from *V. harveyi* and under the control of the tetracycline resistance promoter. *P. fluorescens* 10586s/FAC510 is chromosomally marked with the luciferase structural genes, *luxA* and *luxB*, also expressed from the tetracycline resistance promoter (1). It was constructed by triparental mating with *E. coli* HB101(pFAC510). Luciferase production in *E. coli* HB101(pFAC510) and *P. fluorescens* 10586s/FAC510 was constitutive, and light output required exogenous addition of the aldehyde substrate. Light emission characteristics during batch growth of *E. coli* HB101 (pFAC510) and *P. fluorescens* 10586s/FAC510 are described by Grant et al. (9) and Amin-Hanjani et al. (1), respectively. Both strains were routinely cultured at 25°C in LB broth, which was supplemented with 25 μg of kanamycin ml^{-1} for maintenance of pFAC510 in *E. coli* HB101(pFAC510). All parent strains were obtained from the National Collection of Industrial and Marine Bacteria.

Starvation and reactivation studies. Stationary-phase cultures were harvested by centrifugation (11,600 × *g*, 10 min), washed in an equal volume of basal medium, and recentrifuged. The basal medium for *V. harveyi* was 15 mM phosphate buffer (pH 7); for *E. coli* HB101(pFAC510) and *P. fluorescens* 10586s/FAC510, it was artificial seawater with no added organic carbon. Pellets were resuspended in basal medium, and 2 ml of this suspension was used to inoculate 500-ml Erlenmeyer flasks containing 300 ml of basal medium. Initial cell concentrations were 10^6 to 10^7 cells ml^{-1}, and duplicate flasks for each culture were incubated without shaking at either 4 or 30°C for 54 days. Samples were removed at regular intervals from each flask for determination of total cell concentration, dilution plate counting, and viable (metabolically active) cell concentration. The ability of cultures to regain activity following starvation was determined by luminometry and measurement of changes in cell length. For the former, yeast extract (final concentration, 0.025%, wt/vol) was added to duplicate 30-ml samples and luminescence was measured during incubation for several hours. Changes in cell length were determined in separate, duplicate, 30-ml samples following addition of yeast extract and nalidixic acid (final concentration, 0.002%, wt/vol). Each reactivation experiment was carried out in duplicate at either ambient temperature or 30°C. In a preliminary experiment, changes in luminescence and cell length were measured following incubation of late-log-phase cultures of *E. coli* HB101(pFAC510) in basal medium alone, basal medium plus 0.025% yeast extract, and basal medium plus yeast extract and nalidixic acid. Statistical analyses were carried out by comparison of means from duplicate cultures.

Viable cell enumeration. Viable cell enumeration was achieved by construction of a decimal dilution series of samples in the appropriate basal medium and spread plating 10- or 100-μl samples on triplicate plates containing the appropriate medium solidified by addition of 1.5% (wt/vol) Technical No. 3 agar (Oxoid). Plates were incubated for 48 h at 30°C. When counts fell below the level of detection (100 cells ml^{-1}), 2 ml of undiluted samples was filtered through membrane filters (0.2-μm pore size, 25-mm diameter; Nuclepore), which were then placed on solid medium with cells facing upwards. Colonies were counted following incubation as described above. This reduced the lower detection level to 0.5 cell ml^{-1}.

Total cell and direct viable cell enumeration. Total cell concentration was determined by staining cells with 0.1% (wt/vol) acridine orange (22) following filtration of 2-ml samples through membrane filters (0.2-μm pore size) stained with irgalan black (11). Cells were observed with an Olympus BH2 fluorescence microscope and were examined for morphological changes. The proportion of viable cells was determined by the DVC method of Kogure et al. (12). A 10-ml sample was incubated in the presence of yeast extract (final concentration, 0.025%, wt/vol; Oxoid) and nalidixic acid (final concentration, 0.002%, wt/vol; Sigma) for 6 h at ambient temperature. Cells were then fixed with 2% (vol/vol) formalin for 5 min and stained for 2 min with 0.1% (wt/vol) acridine orange. The cell suspension was concentrated by centrifugation (11,600 × *g*, 10 min), and a sample was observed microscopically. Comparison of the total cell concentration and the proportion of cells that had elongated during the incubation period enabled determination of viable cell concentration. The sensitivity of the three strains to nalidixic acid was tested by overnight growth in the presence and absence of the antibiotic. For *E. coli* HB101(pFAC510) and *V. harveyi*, growth, as measured by A_{550}, was completely inhibited, while that of *P. fluorescens* 10586s/FAC510 was reduced by 50%. In reactivation experiments, a video analysis system (10) was used to determine the average length of between 30 and 50 cells in each of two fields of view.

Luminescence measurements. Luminescence was measured in 1-ml samples from each duplicate flask, using an LKB 1251 luminometer with continuous mixing. Triplicate luminescence measurements were carried out for each duplicate sample, with light output integrated over a period of 10 s and expressed as relative light units (RLU). Sterile artificial seawater was used to determine background luminescence. For *E. coli* HB101(pFAC510) and *P. fluorescens* 10586s/FAC510, which lack the genes for synthesis of the luciferase substrate, luminescence was measured following incubation for 4 min in the presence of 1 μl of *n*-decyl aldehyde.

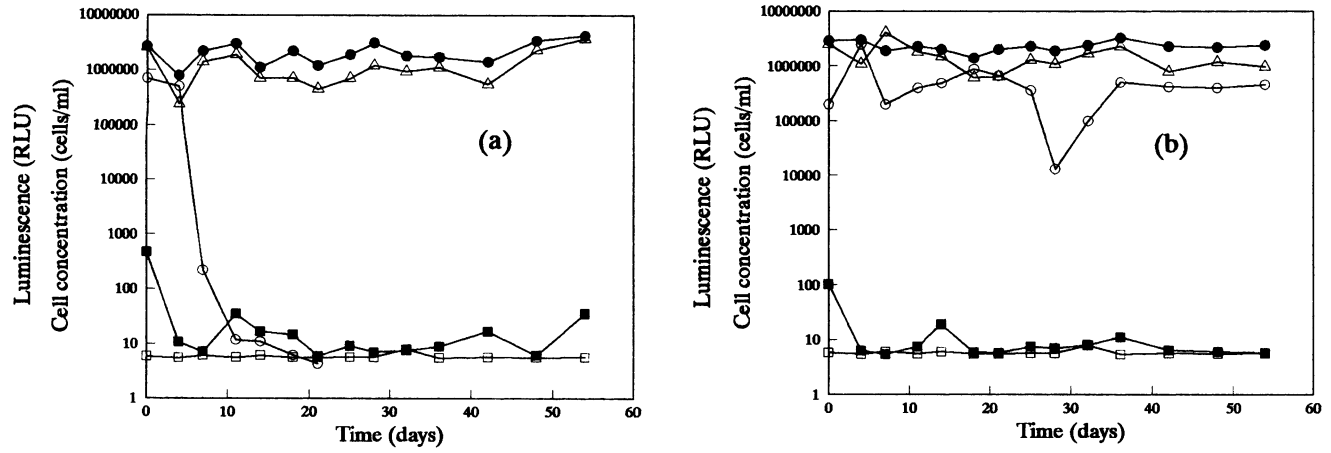

FIG. 1. Changes in concentrations of total cells (●), viable cells (△), culturable cells (○), luminescence (■), and background luminescence (□) in cultures of *V. harveyi* starved at (a) 4°C and (b) 30°C. Each point is the mean of values from duplicate cultures. Standard errors were approximately 23, 14, and 11% of the means for total cell concentration, viable cell concentration, and luminescence, respectively.

RESULTS

Changes in cell concentration during starvation at 4 and 30°C. When starved at 4°C, *V. harveyi* cells entered the viable but nonculturable state within 11 days. Total cell concentration and DVCs were constant until 42 days, when there was a slight increase until 54 days (Fig. 1a). Dilution plate counts decreased to 12 cells ml^{-1} by day 11 and continued to decrease, falling below the level of detection (0.5 cell ml^{-1}) by 25 days. Luminescence decreased to background levels within 7 days but then increased above background between 7 and 19 days and between 42 and 54 days. Entry into the viable but nonculturable state was accompanied by morphological changes, with apparent vacuolation and changes in cytoplasmic structure. *V. harveyi* survived well at 30°C and total counts, DVCs, and dilution plate counts did not vary significantly during incubation for 54 days ($P < 0.05$) (Fig. 1b). Population activity, measured by luminescence, decreased to background levels by the first sampling point (4 days) and subsequently remained at or slightly above this level (Fig. 1b).

In contrast to *V. harveyi*, *E. coli* HB101(pFAC510) cells became viable but nonculturable at 30°C but not at 4°C (Fig. 2). At the latter temperature, starvation resulted in a decline in numbers, measured by all three techniques, of 1 to 2 orders of magnitude by 30 days. At 30°C, total counts and DVCs showed little variation, except for a slight decrease at 21 days, but dilution plate counts decreased from 4×10^6 to approximately 1.4×10^2 cells ml^{-1} by 26 days. At both temperatures, dilution plate counts did not vary significantly after 30 days ($P < 0.05$). Luminescence decreased sharply by 7 days at both temperatures (Fig. 2), with a subsequent decrease to background levels by 21 days. Nonculturable populations also showed morphological changes, with a reduction in cell size and a shift from rod to coccoid cell shape.

P. fluorescens 10586s/FAC510 cells did not become viable but nonculturable at either 4 or 30°C. Cell concentrations measured by all methods increased gradually during incubation at 4°C (Fig. 3a) and did not vary significantly at 30°C (Fig. 3b). At both temperatures, initial luminescence values were less than those for *V. harveyi* and *E. coli* HB101(pFAC510) but remained above background for much of the incubation period

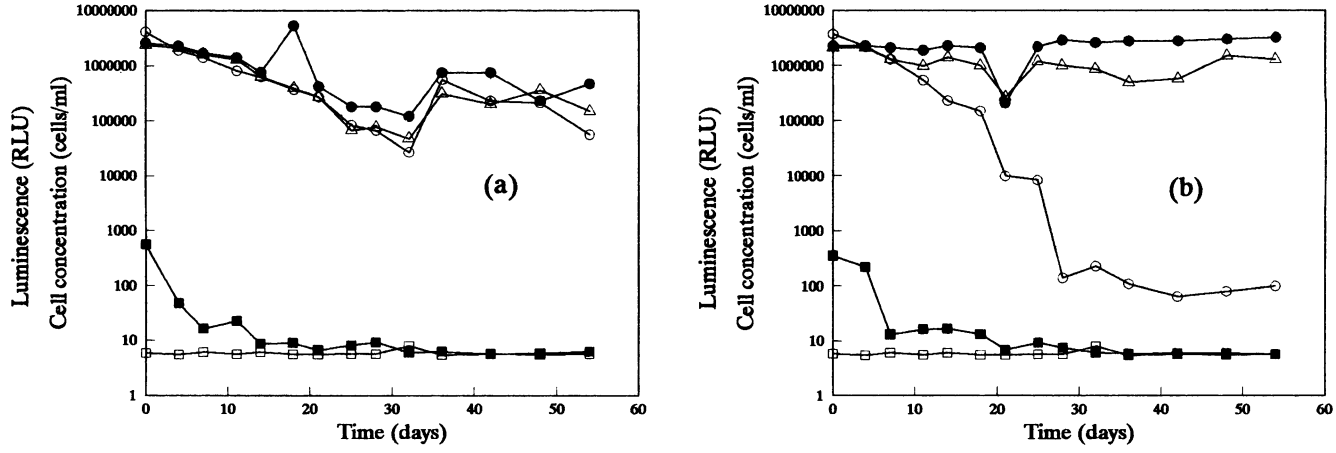

FIG. 2. Changes in concentrations of total cells (●), viable cells (△), culturable cells (○), luminescence (■), and background luminescence (□) in cultures of *E. coli* starved at (a) 4°C and (b) 30°C. Each point is the mean of values from duplicate cultures. Standard errors were as described in the legend to Fig. 1.

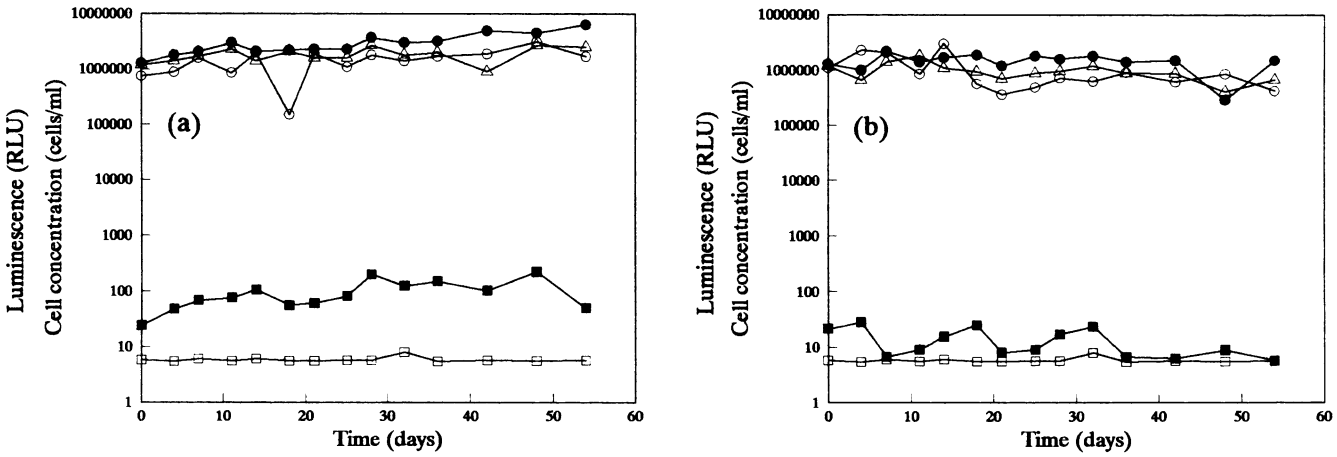

FIG. 3. Changes in concentrations of total cells (●), viable cells (△), culturable cells (○), luminescence (■), and background luminescence (□) in cultures of *P. fluorescens* starved at (a) 4°C and (b) 30°C. Each point is the mean of values from duplicate cultures. Standard errors were as described in the legend to Fig. 1.

at 30°C (Fig. 3b) and increased to 50 to 200 RLU during incubation at 4°C (Fig. 3a).

Reactivation of starved cells. A preliminary experiment was performed to compare the kinetics of reactivation measured by changes in luminescence and changes in cell length as determined in the DVC enumeration technique. Late-log-phase *E. coli* HB101(pFAC510) cells were washed and resuspended in 15 mM phosphate buffer in the presence and absence of yeast extract and nalidixic acid and incubated at room temperature. In the absence of yeast extract, neither luminescence nor cell length varied significantly during incubation for 8.25 h (Fig. 4). Incubation with yeast extract led to an increase in luminescence until approximately 1.5 h, with little subsequent variation in light output. Addition of nalidixic acid did not affect the initial increase in luminescence, but subsequent luminescence was higher than that during incubation with yeast extract alone. Nalidixic acid had little effect on changes in cell length during the first 1.5 h of incubation but subsequently led to increases in cell length due to inhibition of cell division. Maximum changes in cell length in the presence of nalidixic acid were not achieved until approximately 5 to 8 h. The similarities between kinetics for luminescence and increases in cell length indicate the former as an alternative to measurement of cell length for assessment of changes in the activity of viable cells but with significant advantages in terms of convenience and speed. In addition, increases in cell length were approximately twofold, while luminescence increased over 4 orders of magnitude.

Reactivation was measured, using both techniques, for cells of *V. harveyi*, *E. coli* HB101(pFAC510), and *P. fluorescens* 10586s/FAC510 after starvation at 4 or 30°C for two periods. Reactivation of each strain and at each temperature was carried out after different periods of starvation, as simultaneous processing of samples for all experiments was not possible. Results from each of duplicate samples are presented in Fig. 5 to 7. *V. harveyi* entered a viable but nonculturable state after starvation at 4°C but not at 30°C. At the former temperature, the majority of the population was nonculturable after 12 days, and culturable cells were below the detection

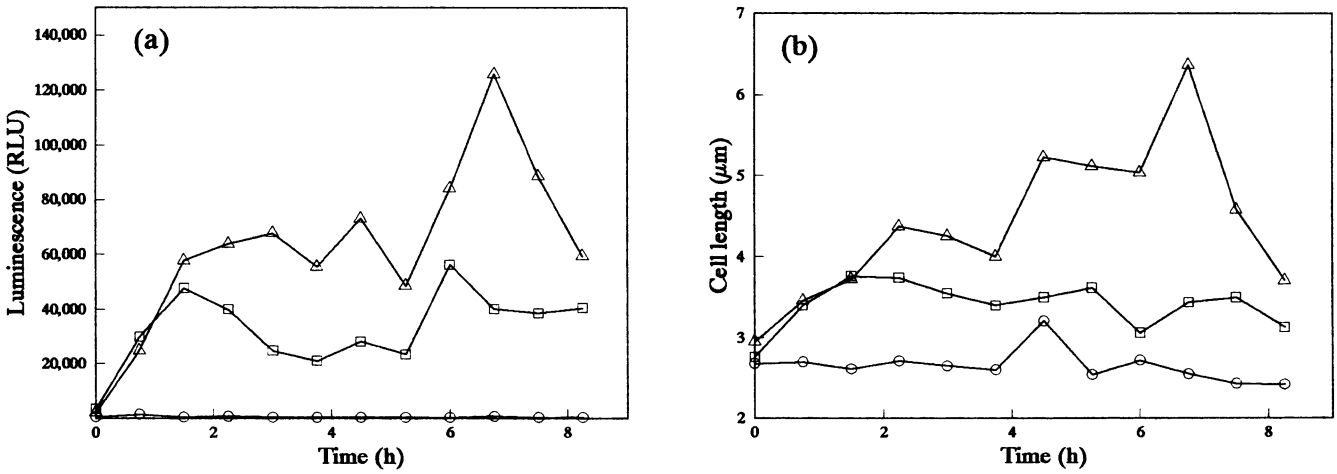

FIG. 4. Changes in (a) luminescence and (b) cell length following incubation of late-log-phase cells of *E. coli* at 30°C in basal medium (○), basal medium plus yeast extract (□), and basal medium plus yeast extract and nalidixic acid (△). Standard errors were approximately 23 and 5% of the means for cell length and luminescence, respectively.

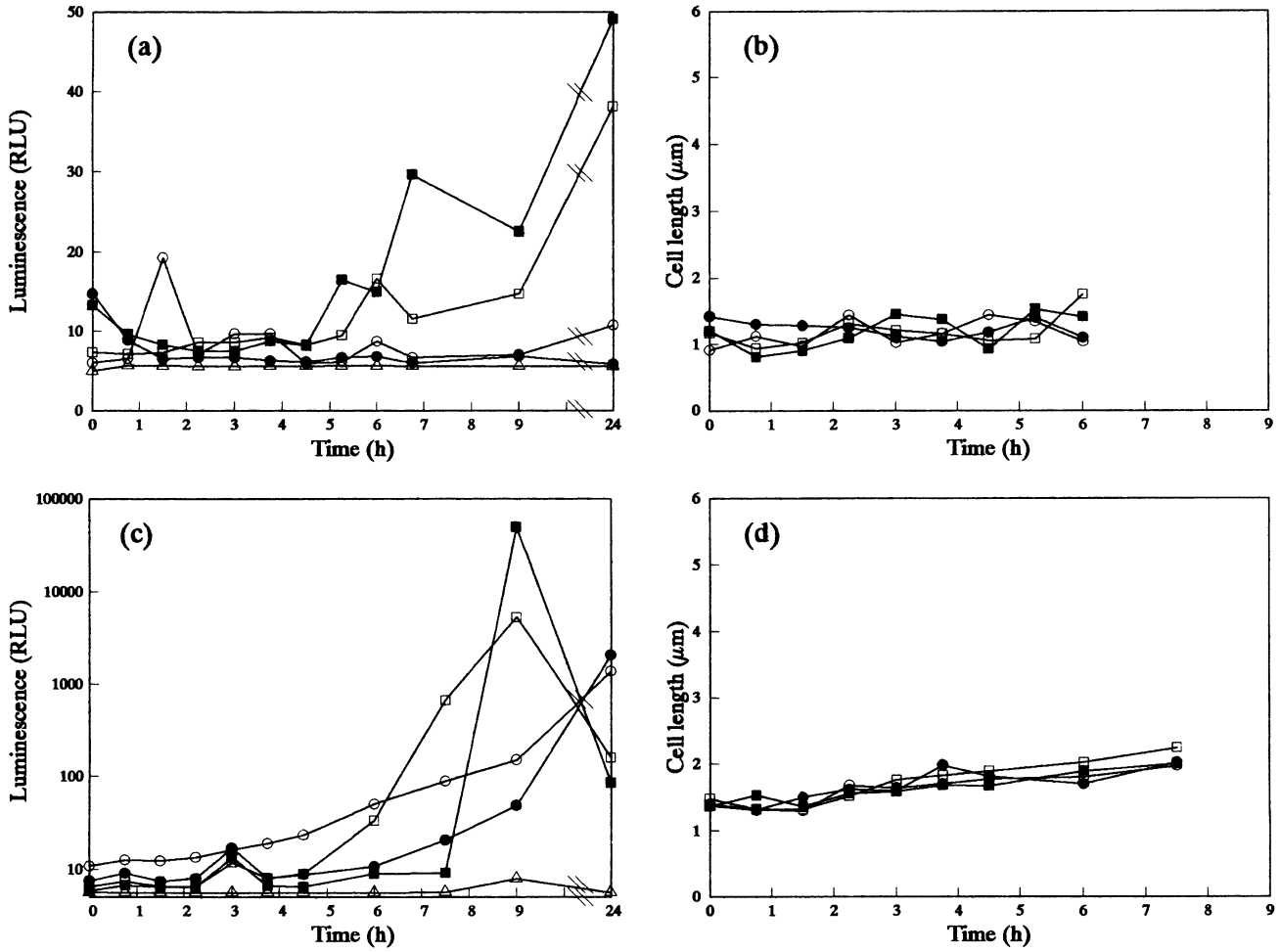

FIG. 5. (a, c) Changes in background luminescence (△) and luminescence and (b, d) changes in cell length in duplicate cultures of *V. harveyi* starved at 4°C (●, ○) and 30°C (■, □) following incubation with yeast extract at room temperature (a and b) and 30°C (c, d). Cells activated at room temperature (a, b) had been starved for 19 days, and those activated at 30°C (c, d) had been starved for 34 days. Standard errors were as described in the legend to Fig. 4.

limit (0.5 cells ml^{-1}) after 24 days (Fig. 1a), although a small number (<15) of culturable cells may have been present in the 30-ml sample. Reactivation was carried out after starvation for 19 and 34 days, by incubating with yeast extract at room temperature and 30°C, respectively. After starvation for 19 days, luminescence from all cultures at all sampling times was detectable above background levels. Standard errors of means from triplicate samples from each flask were always less than 10% of the mean, and all were significantly greater ($P \leq 0.05$) than background levels from uninoculated flasks. Luminescence by culturable cells increased significantly after 4 h of incubation, had doubled by 9 h, and increased further during subsequent incubation up to 24 h (Fig. 5a). Although activity of nonculturable cells was detectable by luminometry, statistically significant increases in luminescence did not occur during incubation for 9 h and increased in one flask only after incubation for 24 h. Pearson correlation coefficients between luminescence and time were significant ($P \leq 0.05$) for culturable (0.98 and 0.91 for duplicate flasks) but not for nonculturable (0.11 and −0.42) cells. Therefore, the activity of nonculturable cells was detectable by luminometry, but their activity was not increased by incubation with yeast extract. Statistically significant differences were not detectable between initial and final average lengths of nonculturable cells following incubation for 6 h at room temperature (Fig. 5b). Lengths of culturable cells showed a slight increase and Pearson correlation coefficients were 0.61 and 0.63 for duplicate flasks, indicating significant correlation ($P \leq 0.05$). Correlation coefficients for nonculturable cells were 0.30 and −0.61. For culturable cells, therefore, proportional increases in lengths were much less than increases in luminescence, and the activity of nonculturable cells was not detectable by cell length measurements.

When cells of *V. harveyi* starved for 34 days were reactivated at 30°C, changes in both luminescence and cell length were detectable. Luminescence of both culturable and nonculturable cells was always significantly greater than background levels. Luminescence increased in all cultures after incubation for 4 h, reaching a maximum for culturable cells after incubation for 9 h, with overall increases of approximately 3 orders of magnitude (Fig. 5c). Luminescence decreased between 9 and 24 h but was still more than 10-fold greater than initial values. Luminescence of nonculturable cells was approximately 1 and 2 orders of magnitude greater than initial values after incuba-

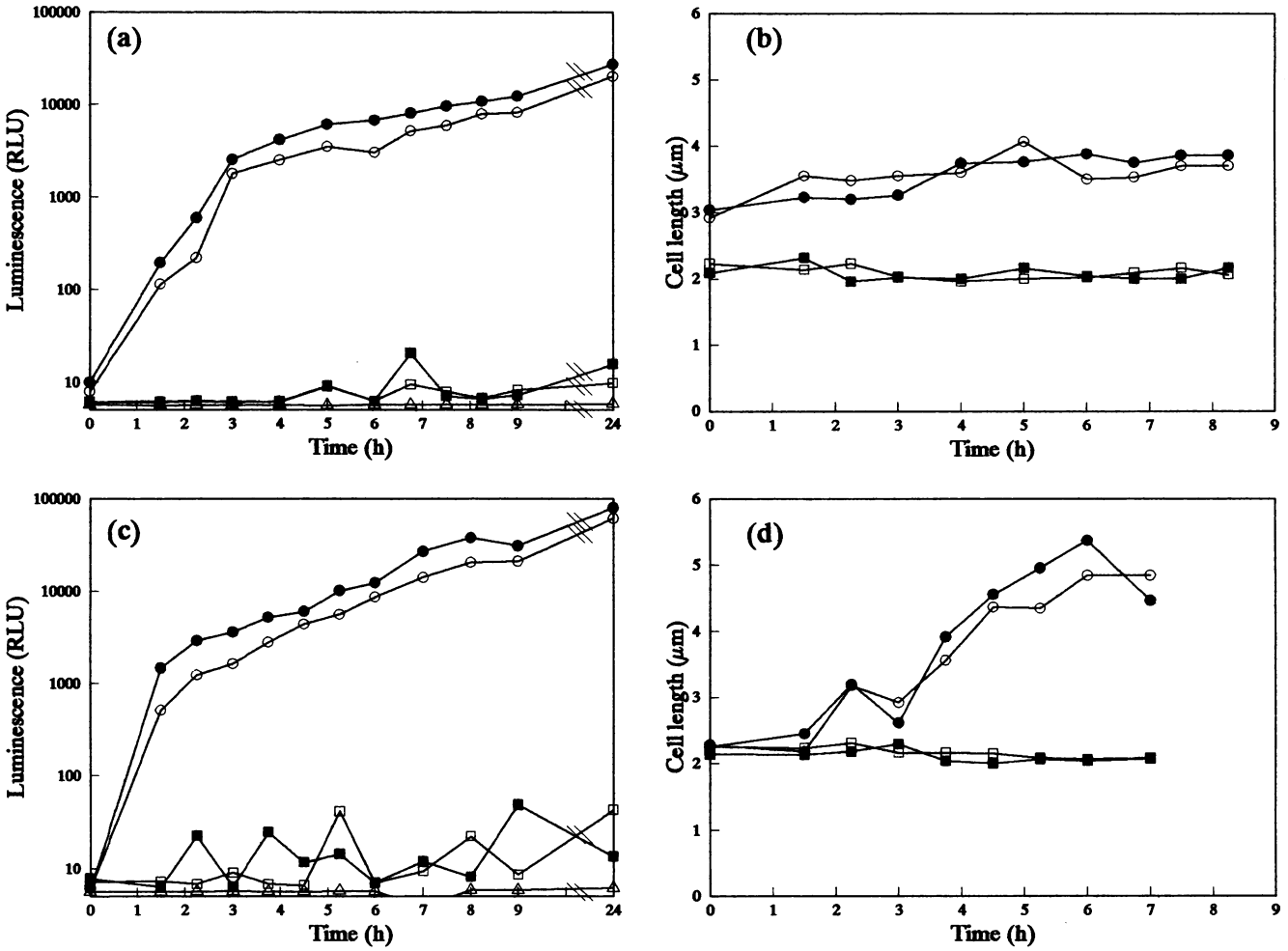

FIG. 6. (a, c) Changes in background luminescence (△) and luminescence and (b, d) changes in cell length in duplicate cultures of *E. coli* starved at 4°C (●, ○) and 30°C (■, □) following incubation with yeast extract at room temperature (a, b) and 30°C (c, d). Cells activated at room temperature (a, b) and at 30°C (c, d) had been starved for 35 and 40 days, respectively. Standard errors were as described in the legend to Fig. 4.

tion for 9 and 24 h, respectively. Lengths of culturable and nonculturable cells did not differ significantly and increased, after a lag period of 1.5 h, by approximately 50% at 7.5 h (Fig. 5d). Pearson correlation coefficients between cell length and time were greater for culturable cells (0.94 and 0.96) than for nonculturable cells (0.88 and 0.87), and all coefficients were significant ($P \leq 0.05$). Following reactivation at 30°C, therefore, luminometry enabled distinction between culturable and nonculturable cells which was not possible on the basis of changes in cell length.

Nonculturable cells of *E. coli* HB101(pFAC510) were formed during starvation at 30°C but not 4°C, reaching a constant proportion (approximately 0.01%) of the total population after 28 days. During reactivation of cells starved for 35 days at room temperature, luminescence of culturable cells increased to approximately 150 RLU within 1.5 h, equivalent to 0.0015 RLU cell^{-1}, and rose to approximately 10^4 RLU after 24 h (Fig. 6a). Luminescence of nonculturable cells was always greater than background levels, reaching values of 7.7 (standard error of the mean, 0.5) RLU and 12.75 (standard error of the mean, 2.95) RLU after 8 and 24 h, respectively. Pearson correlation coefficients were 0.69 and 0.55 and were statistically significant ($P \leq 0.5$). Final values were significantly greater than initial values and were too great to be accounted for by growth or activation of the proportion of the population which had remained culturable. Mean cell length did not double within 8 h, indicating no significant growth. Luminescence by culturable cells (10^2 cells ml^{-1}), calculated using the value of 0.0015 RLU cell^{-1}, would produce 0.15 RLU, approximately 10% of that observed. Although culturable cells may have contributed to luminescence, most resulted from activation of nonculturable cells, and both activity and increases in activity of nonculturable cells were detectable. The mean length of culturable cells increased by 79% during incubation for 8.25 h, but lengths of nonculturable cells did not increase significantly during this period (Fig. 6b). Thus, after starvation for 34 days and incubation with yeast extract at room temperature, the activity of nonculturable cells was detectable by luminometry but not by increases in cell length.

Reactivation at 30°C of cells starved for 40 days showed a pattern similar to that described above. Luminescence of culturable cells was approximately 1,000 RLU after incubation for 1.5 h (Fig. 6c), equivalent to 0.01 RLU cell^{-1}, an order of magnitude greater than after incubation at room temperature,

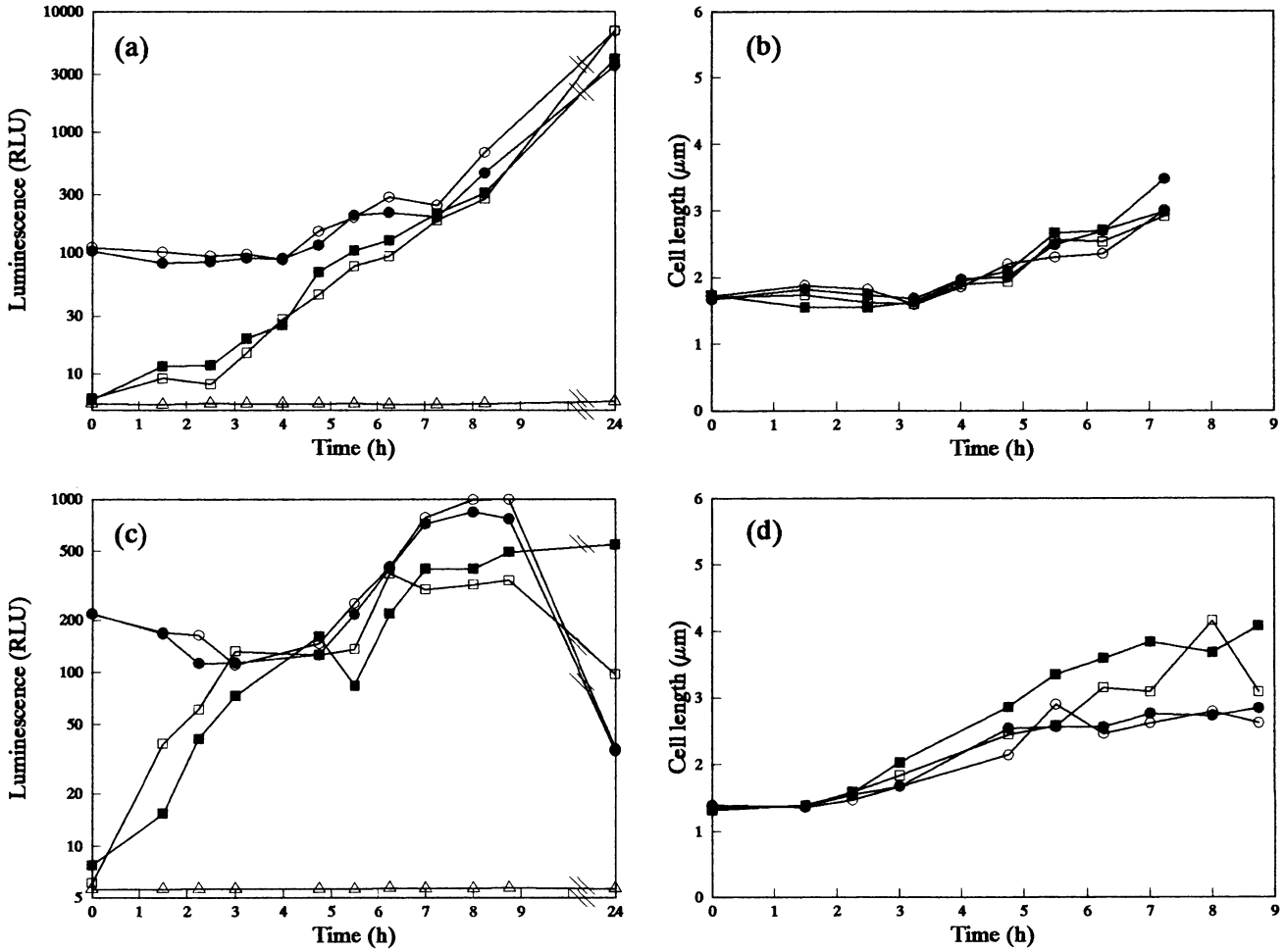

FIG. 7. (a, c) Changes in background luminescence (△) and luminescence and (b, d) changes in cell length in duplicate cultures of *P. fluorescens* starved at 4°C (●, ○) and 30°C (■, □) following incubation with yeast extract at room temperature (a, b) and 30°C (c, d). Cells activated at room temperature (a, b) and at 30°C (c, d) had been starved for 41 and 47 days, respectively. Standard errors were as described in the legend to Fig. 4.

but final luminescence values after 24 h showed no significant difference. Luminescence from nonculturable cells was always significantly above background levels and could not be accounted for solely by activity of culturable cells, which was no greater than 1 RLU. Changes in cell length at 30°C indicate a doubling time of 4 h at 30°C, and growth of 10^2 cells at this rate would lead to a maximum luminescence value of 4 RLU, again significantly less than that observed. Although luminescence was more variable than for culturable cells, activation was easily detectable and by 9 h increased by 1 order of magnitude. Significant changes in lengths of culturable cells were observed after 1.5 h, with 2.5-fold increases after incubation for 7 h (Fig. 6d). No statistically significant changes were observed in lengths of nonculturable cells during this period. Despite starvation for a longer period of time, therefore, incubation at 30°C, rather than room temperature, led to more rapid increases in luminescence and extension of culturable cells and greater increases in luminescence of nonculturable cells, although nonculturable cells were still not detectable by changes in cell length.

Formation of viable but nonculturable cells of *P. fluorescens* 10586s/FAC510 was not observed after starvation for 54 days at either temperature, and activation was carried out at room temperature and 30°C for cells starved for 41 and 47 days, respectively. Luminescence of cells starved at 4°C and activated at room temperature was 10-fold greater than background levels immediately after inoculation and increased after a 4-h lag period by 1.5 orders of magnitude by 8.25 h, reaching approximately 5,500 RLU after 24 h (Fig. 7a). Cells starved at 30°C were initially less active but showed no lag in activation and luminescence increased continuously, reaching values similar to those for cells starved at 4°C after 8.25 and 24 h. No distinction could be made between changes in lengths of cells starved at 4 or 30°C. After a lag period of 4 h, cells in all cultures increased in length to values between 1.5- and 1.9-fold greater than initial lengths (Fig. 7b).

Changes in luminescence for cells starved and activated at 4°C were similar to those after activation at room temperature (Fig. 7c), except for a decrease in luminescence between 8 and 24 h. After incubation for 8 h of cells starved at 30°C, luminescence was similar to that after activation at room temperature, but initial increases in luminescence were greater and there was no increase between 8 and 24 h. Activation at 30°C reduced the lag period before increases in cell length to

2 h for cells starved at both 30 and 4°C, but cells starved at 30°C increased in length at a greater rate (Fig. 7d).

DISCUSSION

The three bacteria investigated showed different responses to starvation and changes in temperature. *V. harveyi* and *E. coli* HB101(pFAC510) entered the viable but nonculturable state at 4 and 30°C, respectively, while *P. fluorescens* 10586s/FAC510 remained viable and culturable at both temperatures throughout the 54-day incubation period. Formation of nonculturable cells by *Vibrio* strains at low temperatures is well documented (16, 24), and the conditions and times required for nonculturable cell formation are similar to those found here for *V. harveyi*. In *V. vulnificus*, nonculturable cells represent an alternative physiological response to that induced by starvation and can occur in the presence of excess nutrients (16). The auxotrophic nature of *E. coli* HB101(pFAC510) may have affected its survival in artificial seawater, but entry of cells into a viable but nonculturable state following temperature upshift is in agreement with the work of Xu et al. (25). They found a greater proportion of viable but nonculturable cells after starvation of *E. coli* for 4 days at 25‰ salinity at 25°C than at 10°C. After longer-term starvation (12 days) at 11‰ and 4 to 6°C, viable cell concentration had decreased by only 0.5 log unit. Anderson et al. (2) also found that the culturability of *E. coli* decreased at a greater rate as temperature was increased. Under the conditions investigated in this study, *E. coli* HB101(pFAC510) took longer than *V. harveyi* to become nonculturable. In addition, a fraction of the population remained culturable even after starvation for 54 days. This may be due to a proportion of the population undergoing a starvation response while the remainder enter the nonculturable state. Nalidixic acid completely inhibited batch growth of *V. harveyi* and *E. coli* HB101(pFAC510) but permitted some growth of *P. fluorescens* 10586s/FAC510, although it significantly reduced biomass yield, suggesting that the DVC method, as employed here, may not be suitable for this organism. However, Byrd et al. (5) found survival of pseudomonads in drinking water to be different from that of other gram-negative organisms, with no decrease in viability after 95 days, and it may be that *P. fluorescens* 10586s/FAC510 responds in a different manner under the stresses imposed.

Metabolic activity of the starved populations was assessed by luminometry. When nonculturable cells were formed, culturable cell concentrations decreased in parallel with luminescence, suggesting that a decrease in metabolic activity may lead to nonculturable cell formation under suitable environmental conditions. Luminescence measurements carried out in the absence of nutrient amendment quantify actual, rather than potential, metabolic activity, and hence did not correlate with DVCs, which involved incubation with yeast extract. Luminescence by starved cultures of *P. fluorescens* 10586s/FAC510 did not follow the same pattern as in *V. harveyi* and *E. coli* HB101(pFAC510) and, at 4°C, increased during the incubation period. This increase in activity is believed to be due to activation of metabolism of readily available endogenous reserves following transfer of cells to 25°C, the temperature at which luminescence was measured. In *E. coli* HB101 (pFAC510) and *P. fluorescens* 10586s/FAC510, luciferase production is constitutive and luminescence is a direct indicator of metabolic activity (14). In *V. harveyi*, however, luciferase is autoinducible and luminometry may be used to assess activity reliably only at high cell concentrations. This explains the lower levels of luminescence following reactivation of *V. harveyi* cultures in comparison with those of the genetically marked organisms.

Luminescence was found to be a better measure of the activity of viable cells than changes in cell length. Experiments with late-log-phase cultures of *E. coli* HB101(pFAC510) indicated direct similarities between changes in cell length and luminescence during incubation with yeast extract and nalidixic acid, but there were significant differences in sensitivity and convenience. For starved cultures of each bacterial strain, and at each incubation temperature, statistically significant increases in light output were detectable before significant changes in cell length occurred, and activation of nonculturable cells detected by luminescence following incubation at room temperature did not give rise to measurable changes in cell length. Thus, although DVCs indicated an increase in cell length for a proportion of the population, these increases did not always significantly affect average cell length. Incubation at 30°C increased the sensitivity of both techniques, but at this temperature, light output by all cells increased over several orders of magnitude, in contrast to changes in cell length, which never increased more than fourfold. Luminescence readings were carried out within 5 min of sampling, and duplicate readings gave less variability than cell length measurements. The latter involved examination of 60 to 100 cells, increasing analysis time even with the use of an automated video analysis system.

Although luminescence measurements were more sensitive and convenient, they are limited to marked organisms, unlike DVC and other activity techniques which may be applied more widely. In addition, luminometry provides information on the bulk activity of a population, rather than the proportion of active cells within a population, although this information may be obtained by simultaneous use of charge-coupled device imaging of single cells (21). The most important advantage of luminescence-based measurement of activity is selectivity. In terrestrial and freshwater environments, background luminescence is negligible. The activity of luminescence-marked organisms may therefore be measured following their introduction into these natural environments, enabling selective assessment of their in situ activity and of changes in activity following nutrient amendment in the presence of the indigenous population. Selective viable cell enumeration can also be used to assess the size of culturable populations. The demonstration that activity of nonculturable cells may be detected by luminometry provides, for the first time, the potential to assess the activity of nonculturable populations of specific, marked, genetically modified microbial inocula and marked pathogens in the environment. This will facilitate quantification of their contribution to microbially mediated processes, compared with culturable organisms, and investigation of the environmental factors involved in entry into, and recovery from, the viable but nonculturable state. This is in contrast to other molecular markers, which have generally been used for viable cell enumeration only, requiring laboratory cultivation, and have not enabled in situ and nonextractive assessment of microbial activity.

ACKNOWLEDGMENTS

We acknowledge the technical assistance of Zena Smith and advice from B. M. Morris.

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