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Rapid Microbial Detection and Enumeration Using Gel Microdroplets and Colorimetric or Fluorescence Indicator Systems

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A new micromethod employing gel microdroplets (GMDs) and optical measurements can be used for rapid detection and enumeration of viable microorganisms (J. C. Weaver, G. B. Williams, A. M. Klibanov, and A. L. Demain, Bio/Technology 6:1084–1089, 1988) and has several potential applications in clinical microbiology. This method involves entrapping microorganisms in GMDs (10 to 100 μm in diameter) which are surrounded by a hydrophobic (low dielectric) fluid, subsequently distinguishing occupied and unoccupied GMDs with colorimetric or fluorescence indicators, counting both occupied and unoccupied GMDs, and applying Poisson statistical analysis. Acid-producing microorganisms were used to compare colorimetric and fluorescence pH indicator systems. Fluorescence systems were generally superior, particularly for detection before microbial growth occurred. Although colorimetric detection was reasonably fast for fast-growing microorganisms, significantly longer times were needed for slow-growing microorganisms. We investigated the dependence of the detection time on microbial division time, GMD size, and buffering capacity of the medium within GMDs. It was found possible to use a single preparation of GMDs, containing a range of GMD sizes, to simultaneously provide a viable enumeration of growing and nongrowing (e.g., stressed) cells. This was possible because small GMDs responded rapidly to both growing and nongrowing cells, while large GMDs, although slower, responded much more rapidly to growing cells than to nongrowing cells. Separate analysis of small and large GMDs in the same preparation yielded two enumerations, one of nongrowing cells and the other of growing cells. GMDs can also be used with conventional light microscopy to detect and enumerate fast-growing acid-producing bacteria much more quickly than conventional plating methods.

Rapid detection and enumeration of viable microorganisms are extremely important to clinical microbiology and have therefore been the subjects of many investigations and development efforts (7, 10, 11, 14–16, 19). Two major classes of assays can be distinguished. The first class is based on specific cellular composition (e.g., Gram stain) and rapidly detects and identifies specific microorganisms directly from a primary sample, but it cannot differentiate between viable and dead cells. Of these, the specific ligand-binding assays (such as immunoassays and genetic probes) have received the most recent attention (20, 23; P. Zwadyk, Clin. Microbiol. Newsl. **11**:84–86, 1989). These assays are not suitable for enumerating viable microorganisms and are also not likely to provide a basis for antimicrobial susceptibility tests (D. F. Sahm, Clin. Microbiol. Newsl. **11**:9–14, 1989).

The second class of assays is based on cellular behavior rather than cellular composition. A number of recent developments are based on physiochemical measurements of the total activity of a large cell population. These include optical techniques, such as light scattering, which respond to changes in cell size and number (5, 24), or indirect metabolism-based techniques, such as those which measure overall changes in pH (4, 9), carbon dioxide release (3), electrical impedance (7), chemiluminescence (17), or fluorescence (18, 21). All such methods fundamentally respond to the combined effects of all cells present. The indirect total population methods, such as impedance measurement or measurement of carbon dioxide release, are responsive to cell activity with or without cell division but have the fundamental disadvantage of being based on the total or combined effects of a large number of cells and do not, therefore, actually yield a count. One consequence is that the total population methods exhibit detection times which become significantly longer as the cell concentration of the sample decreases. In addition, these less-direct methods often require the use of isolates for inoculation; i.e., cultivation and isolation of the sample by using slow standard plating techniques must precede the rapid method. In summary, although rapid when supplied with a large monoculture inoculum, these methods usually require time-consuming preculture in order to obtain an isolate and do not directly provide an enumeration.

The classic method for obtaining a viable enumeration, i.e., viable plating, is also in the second class. Viable plating involves culturing microorganisms on petri dishes and explicitly involves cellular activity. A basic attribute of this viable plate count, or the most-probable-number method, is that cell division is required, leading eventually to visible colony formation. In most cases, many (e.g., 20 to 30) divisions are required, with the consequence that the method is very slow (11, 22). It is also generally labor- and materials-intensive.

In this paper we describe technical aspects of a recently developed rapid micromethod based on gel microdroplets (GMDs). This application of GMDs can rapidly yield a viable count based on the metabolic activity of individual cells (25–28). This new method has significant advantages, since it can provide viable counts which are (i) based on established, flexible optical measurements which are responsive to both general and specific indicators and/or metabolites; (ii) essentially independent of the concentration of microorganisms present in the sample, because behavior of individual cells is measured; (iii) very rapidly obtained, because determination

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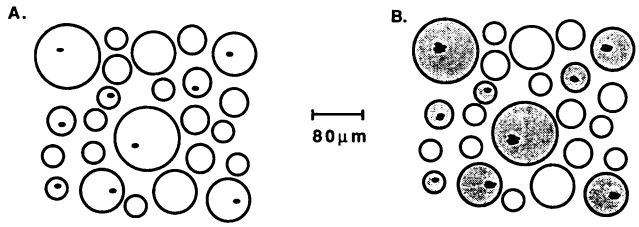

FIG. 1. Schematic representation of detection of occupied GMDs. Drawing of several representative GMDs (circles) which have a high probability of occupation by zero or one cell (dots in circles) in accordance with Poisson statistics. (A) At an initial pH of 6.8, all GMDs are the same color (indicated by an absence of shading) when observed in a microscope. (B) Drawing of the same GMDs after sufficient time has passed to produce a color change (shading) (fluorescence based or absorbance based) in the occupied GMDs. An enumeration is obtained by counting both the number and sizes of all GMDs and comparing this with the number and sizes of all GMDs which exhibit a color change.

of cell viability is based on the localized accumulation of biochemicals in the exceedingly small volume of GMD; (iv) amenable to future automation by current computer-intensive technologies, such as automated microscopy and flow cytometry; and (v) applicable to manual tests through the use of microscopy, either ordinary incandescent illumination microscopy (light microscopy) or fluorescence microscopy. Although some basic features of GMDs have been described previously (25–28), important technical issues relating to the type of optical indicator and the fundamental principles which govern detection time have not been investigated. These issues are important for determining the type of automated optical measurements which should be considered for use with GMDs in clinical microbiology. Further, the conditions under which GMDs can be used with ordinary light microscopy for rapid manual scoring of results and the conditions which require fluorescence microscopy are important for developing applications in clinical microbiology.

GMDs are approximately spherical particles of a gel such as calcium alginate or agarose (Fig. 1). For application to rapid microbial detection, GMDs are generally formed with diameters ranging from 10 to 100 μm, so that the corresponding volumes are 5×10^{-10} to 5×10^{-7} ml, which are exceedingly small assay volumes. During the process of forming GMDs, they are statistically inoculated with cells; that is, large numbers of GMDs are formed in such a way that many have a high probability of containing zero or one initial cell and only a small fraction of the GMDs are multiply occupied. When GMDs are surrounded by hydrophobic fluid, they can be used to rapidly count individual viable cells or microcolonies derived from an initial individual cell, on the basis of the metabolic competence of such cells. This is accomplished by surrounding GMDs with a low dielectric fluid, such as mineral oil, so that dissociable hydrophilic metabolites produced within each GMD are prevented from escaping. Not only does this result in negligible contamination by metabolites from one GMD to another GMD but also the full metabolic activity of a single cell or a resulting microcolony remains associated with that cell or microcolony.

A major advantage of this approach is that an initial single cell in a GMD results in a high effective cell density: $\rho_{eff} = 1$ cell/V_{GMD}, where V_{GMD} is the volume of the GMD. For example, a GMD with a 40-μm diameter has a $V_{GMD} \approx 3 \times 10^{-8}$ ml, so that its occupation by a single cell results in $\rho_{eff} \approx 3 \times 10^{7}$ cells per ml. Such high effective cell densities allow very rapid determinations to be made. A viable count can then be obtained by using optical indicators which allow distinction between occupied and unoccupied GMDs and by counting the number of both occupied and unoccupied GMDs of a given size, e.g., 40 μm in diameter (Fig. 1). The GMD method has potential application to clinical microbiological assays that require rapid (e.g., 30 min to 5 h) enumeration of viable cells directly from primary samples, i.e., without an isolation step, or to assays that require measurement of extracellular product accumulation from single cells or CFUs. Examples of such potential applications include screening clinical samples for the presence of microorganisms and antimicrobial susceptibility testing. We emphasize here investigation of the performance of optical indicator systems that respond to pH, so that retained metabolic acids provide the basis of determinations. Although the GMD assay can be used to enumerate cell viability from primary samples with no prior cultivation (27), it was not the intent of this study to investigate the use of the method with primary samples. Instead, we focus here on a key question regarding the speed of detection and its dependence on the type of optical indicator.

Although GMDs with diameters greater than 100 μm can be analyzed, this study did not include them, since more rapid microbial detection is accomplished with smaller sizes. Instead, in order to investigate basic issues relating to the use of fluorescence and colorimetric indicator systems, a model system using known cell concentrations was used with GMDs ranging from about 20 to about 80 μm in diameter.

Microbial metabolism (except for rare organisms which will not create a pH change upon growth in glucose) results in net acid production and therefore a time-dependent decrease in extracellular pH (4, 9). In the present experiments, the microbially achieved pH endpoint corresponds to an optical endpoint, so that each GMD can be classified as unchanged or changed, compared with the initial optical state. In order to consider the potential application of GMDs to clinical microbiology, it is important to determine what general types of optical detection systems (particularly colorimetric or fluorescence) can be employed to distinguish occupied GMDs from unoccupied GMDs.

For these reasons, GMD detection was studied with emphasis on the comparison of colorimetric and fluorescence detection times in relation to the occurrence or nonoccurrence of cell division, the buffering capacity of detection medium, and GMD size. In addition, the role of a metabolically caused decrease in pH was examined for its effect on inhibiting cellular division, since the effect of pH on growth can be exploited to allow both growing and nongrowing cells to be distinguished and therefore separately enumerated with a single GMD preparation.

MATERIALS AND METHODS

In order to obtain known inocula for the model system study, *Escherichia coli* ATCC 25404, *Proteus mirabilis* ATCC 7002, *Pseudomonas aeruginosa* ATCC 27315, *Staphylococcus aureus* ATCC 25923, *Klebsiella pneumoniae* ATCC 10031, *Saccharomyces cerevisiae* ATCC 2341, *Schizosaccharomyces pombe* ATCC 26189, and *Candida utilis* ATCC 15239 were cultured on 1/10 YPD slants (0.1% yeast extract, 0.2% peptone, 0.2% glucose, 3% agar; Difco Laboratories). Twelve to twenty-four hours prior to an experiment, inoculum from a slant was introduced into full-strength YPD (1% yeast extract, 2% tryptone, 2% glucose)

and incubated at 27°C for yeasts or 37°C for bacteria. To prepare a known concentration of exponentially growing viable cells, 0.5 ml of cell suspension from the tube prepared 12 to 24 h prior to an experiment was diluted into 9.5 ml of fresh YPD medium and incubated for three or four division times prior to an experiment.

Comparison of detection media. The nutrient portions of the different detection media were the same, consisting of 500 μM sodium-potassium phosphate (pH 6.8), 0.05% tryptone, 0.05% yeast extract, and 20 mM glucose. Different indicator dyes, or dye systems, were then used with these nutrients in the following concentrations: (i) 4 mM bromocresol purple (Sigma Chemical Co.), a light absorbance or colorimetric indicator; (ii) 450 μM luminol (Sigma) and 25 μM fluorescein isothiocyanate (FITC)-dextran (Sigma), a fluorescence indicator system based on two pH-sensitive fluorescent dyes (27); and (iii) 300 μM sulforhodamine-640 (Exciton) and 25 μM FITC-dextran (Sigma), a fluorescence indicator system based on one pH-sensitive fluorescent dye and one pH-insensitive (reference) fluorescent dye (28). The color of the dyes in GMDs at different pH values was determined subjectively by using visible light microscopy with incandescent illumination for bromocresol purple and fluorescence microscopy for the fluorescence indicator systems. This involved adjusting the pH of the above detection medial to different values, preparing GMDs, and then visually evaluating the color of the GMDs. The buffering capacity, $B \equiv d[H^+]/d(pH)$, of the medium was determined by measuring the change $\Delta pH \approx d(pH)$ which occurred upon adding a strongly dissociating acid which caused a calculated hydrogen ion activity change $\Delta[H^+] \approx d[H^+]$.

Determination of cell division times. Exponentially growing cells were washed two times in detection medium, resuspended in that medium at a density of about 10^5 cells per ml, and incubated in static cultures. The rationale for the use of static rather than shaken cultures was the desire to closely approximate, within the large-volume (about 10-ml) borosilicate tubes, those conditions expected within GMDs surrounded by mineral oil in a hemacytometer chamber. For bacteria, a small sample of the suspension was serially diluted and plated on YPD–3% agar plates at various times for traditional viable count determinations with petri dishes. After a 24- to 48-h incubation, the number of colonies per plate was determined. The doubling times for yeasts were determined by direct count with a hemacytometer.

GMD preparation. Exponentially growing bacteria or yeasts were centrifuged for 5 min at about 5,000 × *g* for bacteria and 3,000 × *g* for yeasts, and the supernatant fluid was discarded. The cell concentration was determined directly by a cell count for yeasts and by determining the optical density of each of the bacterial suspensions. The cells were washed twice in 3 ml of detection medium with no agarose. Washed cells (100 μl) in detection medium at a density of 5×10^6 cells per ml were then used to inoculate 13- by 100-mm borosilicate tubes (bromocresol purple dye and the FITC-dextran and luminol detection systems) or polypropylene tubes (FITC-dextran and sulforhodamine-640 detection system) containing 400 μl of detection medium and 5% molten agarose (40°C). The cell suspension was mixed into the agarose with a Fisher Vortex-Genie mixer at full speed for 30 s, and 3 ml of mineral oil was added. The tubes were immediately vortexed at full speed for 30 s to prepare an emulsion which was placed in an ice bath for 3 min to cause the molten agarose to gel. This protocol produced GMDs in the size (diameter) range of 5 to 500 μm, but analysis focused on those from 10 to 80 μm. The GMD size distribution is governed by the magnitude of hydrodynamic shear applied to the emulsion but does not significantly depend on the vortexing duration. A small sample of the emulsion (10 μl) was placed in a hemacytometer chamber and incubated for various times, either at 37°C for bacteria or at 27°C for yeasts. The samples were examined by transmission microscopy or fluorescence microscopy or both, with a magnification of 50×. GMDs containing the FITC-dextran and luminol detection system were observed with an Olympus S5-LB 175 fluorescence microscope with a UV excitation filter and a blue pass dichroic filter in combination with a 435-nm barrier filter. The FITC-dextran and sulforhodamine-640 detection system used a blue excitation filter and a green pass dichroic filter. Bromocresol purple was observed by using transmission optics and incandescent illumination in the same microscope.

Determination of single cell occupancy in GMDs. Yeast cells were visualized directly in 40-μm GMDs by comparing the diameter of the GMD with that of a calibrated grid (small squares 50 μm on a side) in the center of the hemacytometer. Because bacterial cells are too small to be visualized at a magnification of 50× and because higher magnifications did not readily allow resolution of individual bacteria in the GMD because of the limited depth of field, single occupancy was determined by applying Poisson statistics to the relative number of occupied GMDs (color change observed) and unoccupied GMDs (no color change observed). This was accomplished by incubating the GMD preparation overnight to allow all GMDs occupied with viable cells to change color. For cells randomly distributed in a sample, the fraction $P(n,\bar{n})$ of GMDs containing exactly n cells is described by the Poisson distribution

$$P(n,\bar{n}) = \frac{\bar{n}^n e^{-\bar{n}}}{n!} \quad (1)$$

where $\bar{n}$ is the average number of cells contained in a GMD of given volume, V_{GMD}. If the cell concentration is ρ, then the average number of cells in a GMD of volume V_{GMD} is $\bar{n} = \rho V_{GMD}$, and the average number of cells in that size GMD is somewhat greater than $1/\rho$. For comparison, the probability of multiple occupation (two or more initial cells) is

$$P(n > 1,\bar{n}) = 1 - [P(0,\bar{n}) + P(1,\bar{n})] \quad (2)$$

We routinely sought inocula giving an average occupancy of 15% ($\bar{n} = 0.15$) in the largest size GMDs used, so that there was a high probability of having zero or one initial cell in essentially all of the GMDs.

Optimization of detection dye concentrations. Two categories of pH indicators were used. The first category consisted of colorimetric indicators, which are single-dye systems whose transmission wavelengths change color in response to changes in pH. A single dye has a molar extinction coefficient, $\varepsilon(\lambda)$, with a dependence on wavelength, λ, that depends on pH. As pH changes, the relative absorption changes differently at different λ regions, which in turn results in a perceived color change. In this case, only a single dye is necessary for visual detection of a color change in GMDs. However, the use of a colorimetric indicator in combination with the very small optical path lengths within a GMD requires a high dye concentration. This results in a large buffering capacity, which slows the detection time (26). The second category is that of fluorescence indicator systems, which consist of dye pairs wherein one or both of the dyes of the pair undergo a change in fluorescence emission intensity in response to pH (27, 28). Unlike the colorimetric

TABLE 1. Comparison of detection media

Detection system	Concn	Color change (pH change)	pH buffer capacity (M per pH)
Bromocresol purple	4 mM	Purple to yellow (6.8 → 6.0)	1.9×10^{-3}
FITC-luminol	25 and 450 μM	Yellow-green to blue (6.8 → 5.9)	5×10^{-4}
FITC-sulforhodamine	25 and 300 μM	Yellow-green to red (6.8 → 5.7)	4×10^{-4}
None (control)			2.5×10^{-4}

indicator, most pH-sensitive fluorescent dyes do not change color significantly with a change in pH. Instead, the fluorescence emission intensity changes with pH; i.e., usually the entire emission spectrum increases or decreases in magnitude, without relative changes within the spectrum (13). In order to create a perceived change in fluorescence color for use with GMDs, two fluorescent dyes with distinct emission wavelengths were employed. As pH changes, either both dyes change emission intensity by significantly different amounts or only one dye changes emission intensity while the other remains constant.

Several criteria governed our choice of dyes. First, a detectable optical change had to occur over a physiologically relevant pH range for the microorganisms used. Second, the dyes had to have a low microbial toxicity. Finally, the dyes had to be partially or totally soluble in the aqueous phase (detection medium) but poorly soluble in the hydrophobic liquid phase (mineral oil). Table 1 lists the salient characteristics of the three pH indicator systems. The concentrations and pH-responding ranges of the dyes chosen for use in GMDs were determined empirically to optimize visual detection by either light or fluorescence microscopy.

RESULTS

The doubling times of the microorganisms in each of the different detection systems are given in Table 2. The doubling times were obtained in early growth, before the cell concentration and pH have changed significantly, since this is relevant to rapid detection. Because high cell concentrations led to very rapid changes in pH, low cell concentrations were used to determine doubling times.

In these experiments, growth was determined in conventional suspension culture. Further, in order to test directly the performance of the different indicator systems, a variety of GMD experiments were carried out by forming GMDs from cell suspensions with a cell density that was expected to give a high probability of zero or one initial cell in 40-μm GMDs. For the yeasts, the high probability of single cell occupation was confirmed by direct microscopic visualization of individually immobilized cells within 40-μm GMDs, whereas for bacteria it was computed with Poisson statistics (see Materials and Methods). In all cases, there was reasonable agreement. The experimental results (Table 2) indicated that acid production by bacteria and yeasts can be rapidly detected with all three indicator systems. Even weak acid producers, such as *P. aeruginosa*, are detected in 4 h (fluorescence indicator system) to 6 h (colorimetric indicator system). In addition, single cells of *S. cerevisiae* and *C. utilis* were detected in 40-μm GMD (before cell division occurred), so that in these cases detection was based on the metabolic activities of individual cells.

The data in Table 2 also show that in many cases, the detection times for the colorimetric detection system were shorter than initially expected, i.e., sometimes only about one and one-half times as long as those for the fluorescence systems (40-μm GMDs). Initially this was surprising, because the buffering capacity of the colorimetric system was about four times greater than that of the fluorescence systems (Table 1). Therefore, we had initially expected a fourfold increase in detection times. However, the time required to detect individual viable cells in GMDs is a function not only of the buffering capacity of the medium but also of the pH range of the indicator system, the acid production per cell, and, of particular significance, the growth rate.

For rapidly growing organisms, a significant increase in biomass can occur while sufficient metabolic acid is produced to cause a color change. This biomass amplification results in microbial activity amplification and can partially offset a lower sensitivity due to greater buffering capacity. The biomass within a GMD is determined by the growth rate of the cell, which in turn is usually dependent on the pH of the medium. Since the pH drops below 6.0 for most bacteria and below much lower values for most yeasts, cells cease to divide and α tends to decrease as cells become increasingly stressed at lower pH values. Detection of GMDs occupied by bacteria occurs over a pH range which influences both cell division and α. In contrast, yeasts have greater acid

TABLE 2. Determination of doubling times and detection times in 40-μm GMDs

Microorganism	Time (min) in the following system:					
	Bromocresol purple		FITC-sulforhodamine		FITC-luminol	
	Doubling	Detection	Doubling	Detection	Doubling	Detection
Escherichia coli	40	150	30	80	30	80
Proteus mirabilis	50	250	50	180	50	180
Pseudomonas aeruginosa[a]	75	360	70	240	70	240
Staphylococcus aureus[b]	40	90	30	40	30	40
Klebsiella pneumoniae	30	150	30	80	40	80
Saccharomyces cerevisiae	90	120	90	45	90	45
Candida utilis	90	120	90	45	90	45

[a] Example of a nonfermenter that can oxidize sugars but not ferment them.
[b] This organism has greater than 1 cell per CFU.

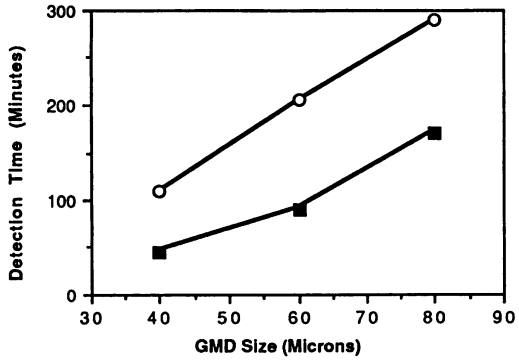

FIG. 2. Detection times observed for GMDs with various sizes by using the yeast *C. utilis*. GMD size (diameter in microns [μm]) is shown as a function of time at which detection occurred for the two fluorescence indicator systems (■) and for the colorimetric indicator (○).

production rates per cell and also have nonoptimal growth within the upper portions of the pH detection ranges of the indicator systems used here. Both phenomena contribute to detection before significant growth occurs (1). In general, because cells with smaller acid production rates require longer times for a measurable change in pH to occur, more growth occurs before a detectable color change is observed.

To illustrate experimentally how buffering capacity, biomass, and GMD volume affect detection time, several sizes of GMDs containing *C. utilis* were examined by using both a colorimetric detection system and a fluorescence detection system. In each case, the number of *C. utilis* cells per GMD was also determined by light microscopy. Figure 2 shows the observed GMD detection times for *C. utilis* for different sizes (diameters) of the GMDs for the colorimetric system and one fluorescence indicator system. In small GMDs (e.g., 10-μm diameter; $V_{GMD} = 4 \times 10^{-9}$ ml), detection occurred before one cell division for both fluorescence and colorimetric indicator systems. In order to understand the relative behavior of these two classes of systems, it is useful to consider the colorimetric-to-fluorescence detection time ratio, R_{CF}, for growth and nongrowth. In the case of nongrowth, R_{CF} should be approximately the ratio of the buffering capacities of the media, i.e., $R_{CF} \approx 4$. That is, colorimetric detection should require four times the number of H^+ to cause a detectable pH change as fluorescence detection, so that without growth about fourfold more time is expected. In the case of growth, however, the increase in acid production associated with a microcolony can reduce R_{CF}. For example, because detection in GMDs 40 μm in diameter is rapid, it can occur before any significant cell growth occurs. In contrast, detection in GMDs 80 μm in diameter requires a longer time, and R_{CF} is smaller. The latter is an example of biological amplification (growth) partially overcoming the larger buffering capacity of colorimetric indicators in large GMDs, an effect not expected or found with detection based on single cells in small GMDs. Thus, the overall result is that the largest colorimetric-to-fluorescence detection time ratio, R_{CF}, occurs for small GMDs and should approach the ratio of buffering capacities.

In order to further elucidate the role of pH in slowing and/or stopping bacterial growth, a conventional cell suspension experiment was performed with *E. coli* (Fig. 3). This type of experiment was undertaken, rather than direct observation as was done with the yeast, because the smallness of the bacteria ruled out direct visual determination of cell numbers in GMDs with the optical system used. In general, it is expected that conventional large-volume cell suspension experiments can provide a useful guide to behavior within the small-volume GMDs, provided that the concentration of cells in the suspension, ρ, corresponds to the effective cell concentration, ρ_{eff}, in a suitable GMD volume. For example, pH changes in a suspension of cells can be used to estimate the effect of a single cell confined in the small volume of a 40-μm GMD ($V_{GMD} = 3 \times 10^{-8}$ ml). In this case, a large-volume suspension should have (1 cell)/(3×10^{-8} ml) = $\rho = 3.3 \times 10^7$ cells per ml. At this cell density, the pH drops from 6.8 to 5.8 in about 80 min for the fluorescence detection systems and from 6.8 to 6.0 in 120 min for the colorimetric system (Fig. 3A). As expected, this was about the same time required for detection of an initial single cell in a 40-μm GMD by these dyes. An important aspect of this type of experiment is that it demonstrates that conventional assays can be used to guide the design of GMD determinations. It also supports the view that essentially all established assays based on metabolic activity profiles (4, 8) can

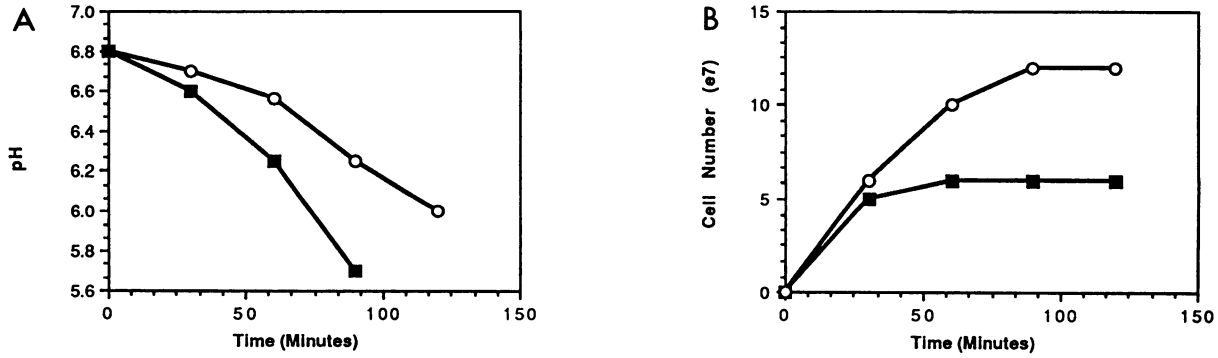

FIG. 3. Large-volume (about 10-ml) cell suspension experiments with *E. coli*. An inoculum of $\rho = 3 \times 10^7$ cells per ml was used to estimate the behavior in 40-μm GMDs which have $V_{GMD} = 3.3 \times 10^{-8}$ ml. Note that one cell within a 40-μm GMD corresponds to an effective cell concentration $\rho_{eff} = 1$ cell/V_{GMD}. These large-volume suspensions were incubated in either the FITC-dextran and luminol detection system or the bromocresol purple detection system at 37°C in standing cultures. (A) At 30-min intervals, the pH of the suspension was determined. (B) As in part A, except that at 30-min intervals, a sample was removed for a viable count determination by conventional plating. Although for early times ($t < 20$ min) the behavior is similar, there is significantly more growth and corresponding amplification of metabolic activity for the colorimetric system (■) than for the fluorescence system (○). This is not surprising, because a colorimetric system has greater buffering capacity and therefore experiences a smaller pH change.

be adopted to the GMD system without significant modification.

DISCUSSION

Within a preparation of GMDs which have a wide range of sizes (e.g., 10 to 100 μm in diameter), a corresponding range of detection performance is expected, since both the probability of occupation, $P(n,\bar{n})$, and the detection time are strongly dependent on V_{GMD}. Fewer small GMDs will contain cells, but those that do will have a high probability of containing one initial cell. As larger GMDs are considered, a greater fraction will be found to be occupied, with a greater probability of multiple occupation as given by the Poisson probability function. In addition, V_{GMD} directly affects detection times because large GMDs need more protons to cause the same pH change; therefore, longer detection times are expected for singly occupied large GMDs than for singly occupied small GMDs. In order to approximately identify their significance, the effects of different parameters on the detection time are estimated below.

We can approximately describe the expected response of GMDs and the associated detection time, t_{det}, by comparing an occupied GMD which contains a nongrowing but metabolically active cell with an occupied GMD which contains a growing cell. In order to identify the parameters which are most significant, we make the following estimate. In the case of the nongrowing cell, we assume that the average metabolic acid production per cell is $\bar{\alpha}$, the minimum detectable pH change in a GMD is $(\Delta pH)_{det}$, and the buffering capacity per volume is B, so that

$$(\Delta pH)_{det} = \int_0^{t_{det}} \frac{d[H^+]dt'}{B} = \int_0^{t_{det}} \frac{\bar{\alpha}ndt'}{BV_{GMD}} \approx \frac{\bar{\alpha}nt_{det}}{\bar{B}V_{GMD}} \quad (3)$$

Assuming further that most GMDs are nearly spherical, it is more convenient to express the GMD volume in terms of the more readily measured diameter, d_{GMD}, so that we substitute $V_{GMD} = \pi d^3_{GMD}/6$. This leads to an estimate for detection time for a GMD containing n nongrowing cell(s):

$$t_{det\text{-}nongrow} \approx \frac{\pi d^3_{GMD}\bar{B}(\Delta pH)_{det}}{6\bar{\alpha}n} \quad (4)$$

From this we see that several parameters are significant. The detection time is expected to decrease with decreasing the average buffering capacity, $\bar{B}$, and with increasing the average cellular acid production rate, $\bar{\alpha}$. In addition, there is a very strong size dependence through d^3_{GMD}. However, there is no dependence on the concentration or density of cells in the sample from which GMDs are formed. Only the probability of occupation is affected by sample density, not the detection time within individual GMDs.

In the case of growing cells, we extend this simple estimate by representing cell growth with the smooth function $n_{cell}(t) \approx n2^{t/t_2}$. That is, $n_{cell}(t)$ represents the changing biomass due to n initial cells within a GMD. For simplicity, no lag time is included and exponential growth with a doubling time, t_2, is used. In this case, the relation between $(\Delta pH)_{det}$ and detection time is

$$(\Delta pH)_{det} \approx \frac{n\alpha t_2}{\ln 2\bar{B}V_{GMD}}[t_{det}e^{t_{det}\ln 2/t_2} - 1] \quad (5)$$

which cannot be solved in closed form for t_{det}. However, by comparison with equation 3, the detection time is shortened if significant growth occurs before detection. This situation can be expected in larger GMDs, for which the combination of $\bar{B}$ and V_{GMD} requires considerable acid production before $(\Delta pH)_{det}$ is reached and the corresponding longer time can allow some growth to occur.

These simple estimates treat α, the metabolic acid production rate, as an average value, $\bar{\alpha}$. However, the rate of acid production per cell or microcolony is a function of the metabolic capacity of the cell (species and medium specific), the physiological state of the cell (pH dependent), and the total biomass present, which is proportional to $n_{cell}(t)$, the number of cells present in a microcolony if growth occurs (1, 6). The pH detection range of a given dye system is defined here as the change, $(\Delta pH)_{det}$, required to cause a visually perceived color change in GMDs when observed in a light or fluorescence microscope. If other means are used to determine indicator changes, the numerical value of $(\Delta pH)_{det}$ will be different. In general, $(\Delta pH)_{det}$ depends on the chemistry of the indicator system and on the optical properties of the dyes (e.g., molar extinction of coefficient of colorimetric dyes and molar extinction coefficient, quantum efficiency, and emission spectra of fluorescent dyes). Finally, Poisson statistics (equation 2) describe the range of GMD volumes which have a high probability of initially containing zero or one cell, and this depends on inoculum cell concentration. Concentrated inocula (e.g., 1.5×10^7 cells per ml) correspond to 70- to 110-μm (diameter) GMDs with a high probability of being multiply occupied, while 40-μm and smaller GMDs simultaneously have a high probability of being individually occupied or unoccupied. Similarly, lower inoculum concentrations (e.g., 1.5×10^6 cells per ml) result in all GMDs ranging from 10 to 100 μm with a high probability of being individually occupied or unoccupied but with significantly more rapid detection in 20-μm than in 80-μm GMDs.

It was noted that the viable count determinations of yeasts occurred within one division time in the case of fluorescence indicator systems and within two division times for the colorimetric indicator system (Table 2). As predicted from equation 3, this observation suggests that the number of divisions that occur before detection can be regulated by either adjusting the buffering capacity of the medium or choosing the size of the analyzed GMD. For example, no cell division need occur in small GMDs, because the pH changes rapidly because of the acid production of even an individual cell. However, as larger GMDs are considered, more divisions will occur before cell division is halted by a lowered pH, because a larger volume has a proportionally greater buffering capacity. Therefore, in tests wherein GMDs with diameters of 20 to 80 μm have a high probability of being individually occupied or unoccupied, a predictable range of microcolony sizes will exist for a given type of microorganism at the conclusion of the assay. Further, note that a similar approach could be used to generate colony size variation by limiting an essential nutrient and maintaining constant pH by providing a highly buffered medium.

As shown, the use of GMDs provides a new way to rapidly enumerate viable cells and combines some of the features of several established methods. The speed of high cell density metabolic methods is combined with the isolation of initial cells that is used in traditional viable counts involving petri dishes. By combining the ability to enumerate and the ability to detect individual cells or CFU on the basis of their metabolic activity, the GMD approach provides the follow-

ing advantages compared with those of established techniques. (i) The present GMD technique allows enumeration of isolated cells or CFUs from primary samples with no prior incubation independent of the initial inoculum concentration. This attribute is similar in principle to plating or most-probable-number procedures but is much faster. (ii) The present GMD method detects cell viability on the basis of measurements of extracellular biochemicals produced by metabolically active cells. Because of the small volumes involved, this technique is very rapid (direct visual determination of single cell or CFU viability within 30 min to 5 h by using acid production as a measure of cell viability). (iii) A direct count of the number of stressed cells, together with the number of actively dividing cells, can be obtained from a single heterogeneous GMD preparation which contains a wide range of GMD sizes (volumes).

For these reasons, the GMD approach is a rapid and flexible technique for determining extracellular changes produced by isolated cells or microcolonies. This approach can be used to determine cell viability with and without cell division. Although acid production was used as an indicator of cell viability is this study, not all microorganisms produce acid. However, it should also be possible to measure other biochemical parameters (12, 13), particularly by employing enzymatic assays for specific cellular metabolites (2). In this way it should be possible to rapidly detect, characterize, and enumerate a wide variety of microorganisms at the fundamental level of individual cells.

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