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Development of a Robust Flow Cytometric Assay for Determining Numbers of Viable Bacteria

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Several fluorescent probes were evaluated as indicators of bacterial viability by flow cytometry. The probes monitor a number of biological factors that are altered during loss of viability. The factors include alterations in membrane permeability, monitored by using fluorogenic substrates and fluorescent intercalating dyes such as propidium iodide, and changes in membrane potential, monitored by using fluorescent cationic and anionic potential-sensitive probes. Of the fluorescent reagents examined, the fluorescent anionic membrane potential probe bis-(1,3-dibutylbarbituric acid)trimethine oxonol [$DiBAC_4(3)$] proved the best candidate for use as a general robust viability marker and is a promising choice for use in high-throughput assays. With this probe, live and dead cells within a population can be identified and counted 10 min after sampling. There was a close correlation between viable counts determined by flow cytometry and by standard CFU assays for samples of untreated cells. The results indicate that flow cytometry is a sensitive analytical technique that can rapidly monitor physiological changes of individual microorganisms as a result of external perturbations. The membrane potential probe $DiBAC_4(3)$ provided a robust flow cytometric indicator for bacterial cell viability.

Enumeration of viable organisms still relies almost exclusively on counting visible colonies after suitable dilutions of bacterial suspensions are spread on agar-solidified media, rather than microscopic counting of individual organisms. The long incubation times (i.e., hours or days) are a major problem. Rapid, direct methods for the detection of viable bacteria are of increasing importance. Assessment of microbial viability is a major requirement in several areas of microbiology, including public health, biotechnology, food technology, the water industry, and the pharmaceutical industry (20).

Flow cytometry makes it possible to perform rapid measurements on individual cells in homogeneous or heterogeneous populations. Flow cytometry permits simultaneous measurements of multiple cellular parameters, both structural and functional (27). These measurements are usually based on light scattering and fluorescence.

A membrane impermeant fluorescent probe that can passively diffuse through the cell wall of a bacterium can act as an indicator of a loss in membrane integrity (27), which, in turn, can often act as an indicator of cell viability. Membrane integrity analysis is based on the capacity of the cells to exclude compounds such as fluorescent intercalating dyes, for example, propidium iodide (PI) (8), ethidium bromide (14), and ethidium monoazide (EMA) (13), which when used at low concentrations do not normally cross intact membranes. Other compounds include fluorogenic substrates, which are lipophilic, nontoxic, uncharged, and nonfluorescent. When taken up by viable cells, fluorogenic substrates are hydrolyzed by nonspecific esterases to polar fluorescent products that are retained by cells with intact membranes. Highly polar products are required for their retention by cells with intact membranes (26). These dyes include the esters of fluorescein (12), such as fluorescein diacetate (FDA). The hydrolysis of FDA produces fluorescein, a relatively impermeant weak acid. Dead or dying cells with compromised membranes rapidly leak the dye, even if they retain some residual esterase activity. Low temperatures and esterase products with high net charges favor retention, although this also depends on the cell type. The fluorescent esterase products leak from cells following membrane perturbation; quantitative assays of this leakage have been used to determine cytotoxicity induced by drugs (15, 17). The staining of viable eukaryotic cells by fluorogenic substrates and their detection by flow cytometry have been reported by several investigators. FDA and flow cytometry have been used to detect viable bacteria (2, 7, 10, 11). Many fluorogenic probes and chromogenic substrates are used in bacterial diagnostics (19).

Mechanisms that are sensitive to changes in membrane potential also provide a means of assessing the viability of a cell. In bacteria, the cellular apparatus for energy metabolism is localized on the cytoplasmic inner membrane. The potential across this membrane shares many characteristics of the mitochondrial membrane potential; it is dependent on energy metabolism and is decreased within a few minutes following removal of energy resources. Membrane potential also decreases when the membrane is perturbed by physical or chemical agents.

The mitochondria of eukaryotic cells have the ability to concentrate lipophilic cations such as rhodamine 123 (4, 6, 7) in an uncoupler-sensitive fashion. The staining of mitochondria with rhodamine 123, in conjunction with flow cytometry, has been used to study their activity (9, 28). Viable bacteria also accumulate rhodamine 123, but nonviable ones do not, and under appropriate conditions, the extent to which individual bacteria take up rhodamine 123 quantitatively reflects the extent of their viability (16). Mason et al. (22) assessed various membrane potential dyes for their ability to discriminate between viable and nonviable bacteria.

The negatively charged oxonol dyes undergo potential-dependent distributions between the cytoplasmic membrane and the extracellular medium. The bis-(1,3-dibutylbarbituric acid) trimethine oxonol [$DiBAC_4(3)$] has a high degree of voltage sensitivity (3) and enters depolarized cells where they bind lipid-rich intracellular components. Their fluorescence is enhanced upon accumulation. It is because oxonols are negatively charged that they are excluded from mitochondria, thus simplifying the measurement of membrane potential in eu-

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karyotes (32). Oxonol has been used to assess the effects of viruses and toxins on the membrane potential of plasma membrane cells (1) and the viability of *Candida albicans* following amphotericin B treatment (5) and bacteria following antibiotic treatment (21).

MATERIALS AND METHODS

Microorganisms and growth medium. Unless stated otherwise, the following strains were routinely used: *Escherichia coli* NCTC 10418, *Staphylococcus aureus* NCTC 6571 (Oxford), and *Pseudomonas aeruginosa* NCTC 10662. They were grown in flasks containing tryptone soya broth (TSB) in a shaking water bath at 37°C. The number of CFU was determined on nutrient agar plates by standard techniques.

Viability probes and staining protocols. The fluorogenic substrates FDA, carboxyfluorescein diacetate (CFDA), and carboxyfluorescein diacetate-acetoxymethylester (CFDA-AM) were obtained from Molecular Probes, Inc. (Eugene, Oreg.). Immediately prior to use, concentrated stock solutions were made up in dimethyl formamide. Unless otherwise stated, the following protocol was used for loading bacteria with the above fluorogenic substrates. Samples (1 ml) of exponentially growing *E. coli*, *P. aeruginosa*, and *S. aureus* were taken from broth cultures. Samples were centrifuged at 11,000 rpm (Biofuge 15; Sepatech GmbH) for 2 min in a microcentrifuge, and the supernatant was discarded. Cell pellets of *S. aureus* were resuspended in 4 ml of filtered (0.22-μm-pore-size filter) phosphate-buffered saline (PBS). Cell pellets of *P. aeruginosa* and *E. coli* were resuspended in 4 ml of filtered (0.22-μm-pore-size filter) 100 mM Tris-HCl (pH 8.0) containing 1 mM EDTA. Samples were diluted to give a final number of approximately 10^7 organisms per ml. Fluorogenic substrates were added to the cell suspensions to give a final concentration of 10 μg/ml. The suspensions were then incubated at 37°C for 30 min. Cells were washed by centrifugation in the appropriate buffers and then analyzed by flow cytometry.

Immediately prior to use, solutions of PI (Sigma) and EMA (Molecular Probes) were made up as stock solutions in ethanol at a concentration of 1 mg/ml. Stock solutions were stored at 4°C in the dark. An exponentially growing culture of *E. coli* (50 μl) was added to 5 ml of PBS containing 5 μg of PI per ml. Samples were allowed to stain for 5 min at room temperature before analysis by flow cytometry.

With EMA, 1-ml samples of an exponentially growing culture of *E. coli* were centrifuged in a microcentrifuge for 2 min at 11,000 rpm (Biofuge). After the supernatant was removed, the pellets were resuspended in PBS. Samples were diluted in buffer to give an optical density at 600 nm of 0.3. The same procedure was repeated with heat-killed cells (65°C for 30 min). Washed cell suspensions (15 ml, each) were added to glass petri dishes. EMA was added to each dish to give a final concentration of 20 μg/ml. Samples were irradiated with a 15-W fluorescent reading lamp at a wavelength of 20 cm for 20 min. Samples were removed, washed twice in PBS, and then fixed by adding buffered formaldehyde (1% [vol/vol], final concentration). Samples were then stored at 4°C in the dark before analysis by flow cytometry.

Stock solutions (1 mg/ml) of the membrane potential probes rhodamine 123 and $DiBAC_4(3)$ were made up in ethanol and stored at −20°C. Fresh stock solutions of rhodamine were made up weekly. $DiBAC_4(3)$ stock solutions remain stable for long periods (≥6 months). Unless otherwise stated, the following protocol was routinely used for staining bacteria with rhodamine 123. Exponentially growing culture samples (1 ml) were removed from flasks and centrifuged at 11,000 rpm (Biofuge 15) for 2 min in a microcentrifuge. After the supernatant was discarded, cell pellets of *E. coli* and *P. aeruginosa* were resuspended in filtered (0.22-μm-pore-size filter) 100 mM Tris buffer (pH 8.0) containing 1 mM EDTA. *S. aureus* was resuspended in filtered (0.22-μm-pore-size filter) 100 mM Tris buffer (pH 7.4). Samples (1 ml) of cell suspensions were added to 5 ml of appropriate buffer containing 5 μg of rhodamine 123 per ml. Samples were incubated at 37°C for 30 min. Samples (1 ml) were removed, washed twice in Tris buffer, and resuspended in a final volume of 5 ml of Tris buffer. Samples were analyzed by flow cytometry.

Unless otherwise stated the following protocol was used for staining bacteria with $DiBAC_4(3)$. Samples of cultures diluted to 10^6 cells per ml were removed from flasks and added to 5 ml of filtered (0.22-μm-pore-size filter) PBS containing 10 μg of $DiBAC_4(3)$ per ml. For dilute suspensions (less than 10^6 organisms per ml), $DiBAC_4(3)$ was added directly to 1-ml samples of the culture. Cells were allowed to stain at room temperature for 10 min before analysis by flow cytometry.

Bacteria killed either by heat treatment (65°C for 30 min) or by treatment with gramicidin S (Sigma) were routinely used as controls. Stained cells were treated with 20 μg of gramicidin S per ml for 2 min. No growth of bacteria was observed on nutrient agar plates following such treatments.

Flow cytometry. Standard bench top BD-FACScan (Becton Dickinson) and Bryte-HS (Bio-Rad) flow cytometers were used for single-cell light scattering and fluorescence measurements. With the FACScan, samples were illuminated with an air-cooled argon ion laser (488 nm), and fluorescence emission was detected at 510 to 530 nm for FDA, CFDA, CFDA-AM, rhodamine 123, and $DiBAC_4(3)$ and at 550 to 640 nm for PI and EMA.

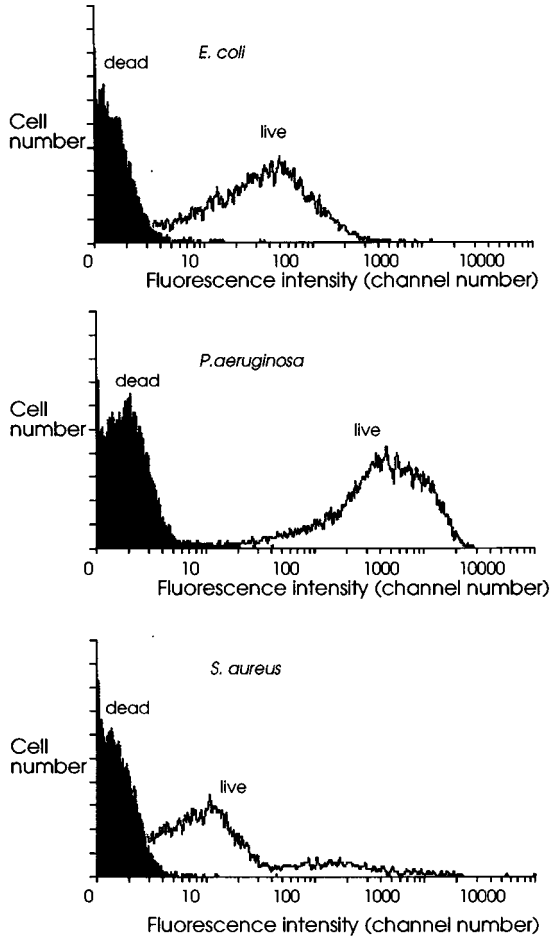

FIG. 1. Live and gramicidin S-treated bacteria examined by using a BD-FACScan flow cytometer after staining with 10 μg of CFDA per ml.

List mode data from approximately 10,000 cells were collected and processed by using Lysis II software (Becton Dickinson). Photomultiplier amplifier gains were set in the linear mode for light scattering and in the logarithmic mode for fluorescence. Light scattering and fluorescence were triggered by forward angle light scattering, with the threshold limit set to the desired value (usually the first 50 channels of 1,024), in order to reduce background noise.

The Bryte-HS flow cytometer (a modified Skatron ARGUS 100 cytometer) is based on the instrument originally designed by Steen and Lindmo (30). In the Bryte-HS, illumination was provided by a 75-W high-pressure mercury-xenon arc lamp (Hammamatsu). Light scattering (in dark-field configuration) and fluorescence from samples delivered by calibrated syringe pump were measured at approximately 1,000 cells per s in a hydrodynamically focused jet of filtered water (Milli-Q, 0.22-μm-pore-size filter; Millipore) at a flow pressure of 7×10^4 Pa across a microscope coverslip, with the bacterial suspension travelling along the jet axis. A standard fluorescein isothiocyanate filter block arrangement (Bio-Rad) was used, providing excitation of 470 to 490 nm, beam splitter of 510 nm, and emission of 520 to 560 nm for $DiBAC_4(3)$. Photomultiplier amplifier gains were set in the linear mode for light scattering and in the logarithmic mode for fluorescence. Forward angle light scattering is defined as the light detected in the angular range of 1° to 18°, and large angle light scattering is the light detected over an angular range of 18° to 85°. Light scattering and fluorescence were triggered by forward angle light scattering, with the threshold limit set to the desired value (usually between the first 10 to 20 channels of 256). Data from approximately 10,000 cells was collected and stored in a list mode by using Windows-based WinBryte software (Bio-Rad).

RESULTS AND DISCUSSION

Membrane integrity as an indicator of viability. Figure 1 depicts overlays of fluorescence histograms from live and gramicidin S-treated bacteria after staining with CFDA. The

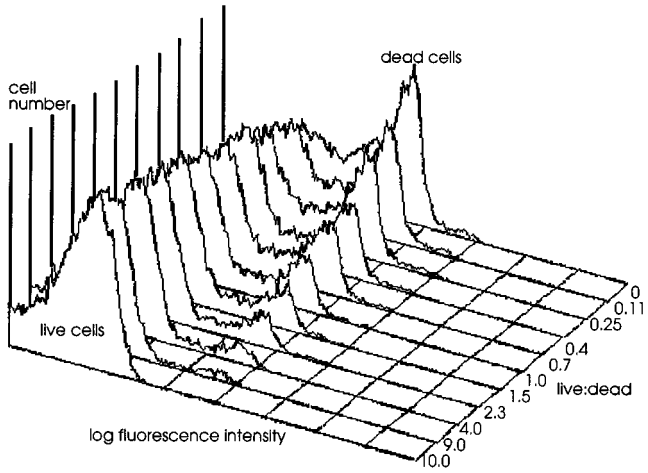

FIG. 2. Live and heat-treated *E. coli* cells mixed at the ratios shown, stained with 5 μg of PI per ml, and analyzed by using a BD-FACScan flow cytometer.

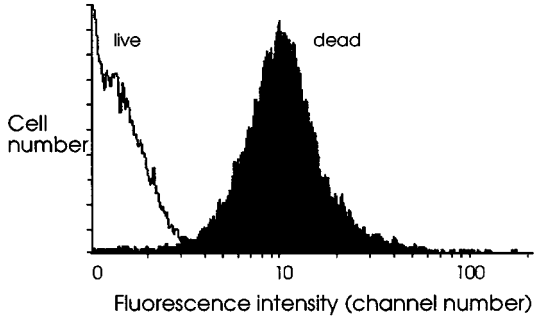

FIG. 3. Fluorescence histogram overlays of live and heat-treated *E. coli* stained with EMA and analyzed by using a BD-FACScan flow cytometer.

untreated cells have a much higher fluorescence intensity than do the corresponding gramicidin-treated cells. In order for the CFDA to penetrate the cell walls of gram-negative bacteria, both *P. aeruginosa* and *E. coli* were treated with EDTA before the fluorogenic enzyme substrate was added. Similar results were usually obtained with FDA. The results were inconsistent, however, with loading and fluorophore retention problems with the use of FDA. Although it has been suggested (12) that CFDA-AM is more effective than both FDA and CFDA as a viability stain for mammalian cells, we encountered some solubility problems that led to inconsistent results.

Although live and dead bacterial cells could be differentiated by using fluorogenic substrates, the staining protocol was not robust enough for the development of a high-throughput viability assay. There are several important disadvantages with the methods required for staining live cells. These include the repeated washing steps, which result in considerable loss of microorganisms. We consistently found that approximately 30% of the cells were lost by washing, although these losses could be considerably reduced by using multiwell filtration plates. Also, there is the requirement for pretreatment of gram-negative bacteria with substances that increase the permeability of their outer membranes. The interaction of EDTA following antibiotic treatment could lead to unpredictable effects. However, once the enzyme substrate is loaded into the bacteria, leakage of fluorophore after antibiotic treatment can provide useful kinetics data on the antimicrobial effects (31).

Figure 2 shows the fluorescence histograms produced from live and heat-killed *E. coli* mixed at various ratios and stained with PI. As the ratio of dead to live cells increases, a second, higher intensity fluorescence peak appears. The number of cells in this second peak increases with the increasing number of added dead cells. Similarly, the number of cells in the live cell populations, that is, lower intensity fluorescence histograms, decreases. In the case of the suspension containing all heat-treated cells, there is still a second, lower intensity fluorescence peak with *E. coli*. However, the mean intensity is higher than that in the cells that have not received any heat treatment. This fluorescence peak may represent cells that have not been fully affected by the heat treatment and may indicate that PI has not fully entered into the *E. coli.*

Figure 3 depicts overlays of fluorescence histograms of live and heat-killed *E. coli* after cells were stained with EMA and later fixed. The live cells show minimal fluorescence, which is probably due to background noise or autofluorescence. However, the heat-treated cells show a marked increase in fluorescence.

EMA, e.g., 3-amino-8-azide-5-ethyl-6-phenyl phenanthridine, has been shown to attach photolytically to nucleic acids in an efficient manner by relatively low-intensity irradiation with a visible or UV light source. EMA binds noncovalently to DNA and can be photolyzed to the covalent complex both in vivo and in vitro (13). It is suggested that EMA forms strong complexes by intercalation between alternating purine-pyrimidine bases in DNA. Photoactivation of EMA generates a highly reactive nitrene, which will then form a covalent bond with DNA (13). EMA has been used in the past with flow cytometry to identify nonviable lymphocytes (25). EMA has several advantages over PI in that cells are stained, washed, and then fixed, thus making it particularly useful when analyzing pathogenic samples such as mycobacteria or infected clinical products. The main disadvantage is that it cannot be used in combination with live cell stains, such as CFDA, or membrane potential probes.

Membrane potential. Figure 4 shows the effects of gramicidin S on *E. coli* after staining with rhodamine 123. The fluorescence intensity of the live cells is approximately 50 times that of the gramicidin-treated cells. Diaper et al. (11) used rhodamine 123 to assess the viability of several different strains of bacteria and found that it could be used for gram-negative bacteria only after they had been treated with EDTA. Matsuyama (23) found that gram-negative bacteria stained with different efficiencies, presumably reflecting the different constitutions of the outer membrane. Our findings also indicated that gram-negative bacteria required EDTA treatment in order for rhodamine 123 to pass through the cell wall. Although

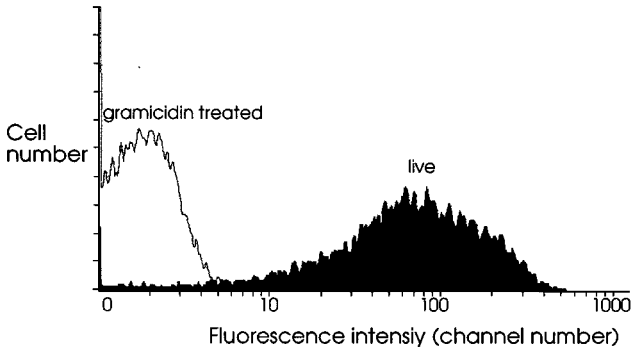

FIG. 4. Effects of gramicidin S on *E. coli* examined by using a BD-FACScan flow cytometer after staining with rhodamine 123.

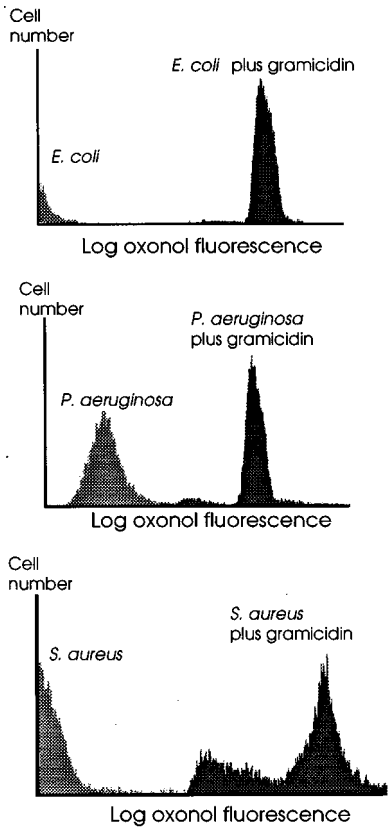

FIG. 5. Fluorescence histogram overlays of oxonol-stained *E. coli*, *P. aeruginosa*, and *S. aureus* before and after treatment with gramicidin S. Samples were analyzed with a BD-FACScan flow cytometer.

the results shown in Fig. 4 demonstrate that live and dead *E. coli* cells could be detected by using rhodamine 123, in general, the results obtained were inconsistent. This inconsistency may be due to a permeability problem and/or may be related to the state of health or age of the culture at the time of rhodamine 123 staining (16). The initial washing step may also significantly affect the *E. coli* membrane potential.

Figure 5 depicts the fluorescence histograms of $DiBAC_4(3)$-stained *E. coli*, *S. aureus*, and *P. aeruginosa* before and after treatment with gramicidin S. The fluorescence intensity increases by approximately 100-fold after gramicidin S treatment. These results were produced with a laser-based BD-FACScan flow cytometer. The intense 488-nm laser light source provides optimum excitation and emission conditions for $DiBAC_4(3)$ and results in excellent separation of the live and dead populations of cells. However, the FACScan cannot easily determine absolute total counts. Therefore, all further work with $DiBAC_4(3)$ was done with the Bryte-HS flow cytometer, which is a purpose-built instrument for analyzing bacteria and is capable of determining absolute counts. It also has an excellent light scattering sensitivity (30).

Comparison of enumeration of viable *E. coli* cells by flow cytometry and standard CFU assays. Figure 6 represents the results of flow cytometric analysis of *E. coli* removed from a 5-h broth culture and stained with oxonol. The dot plot (Fig. 6a) represents forward angle light scattering versus oxonol fluorescence. Only one major population of cells is evident, indicating that the majority of the cells are viable. This is further demonstrated by the single fluorescence histogram shown in

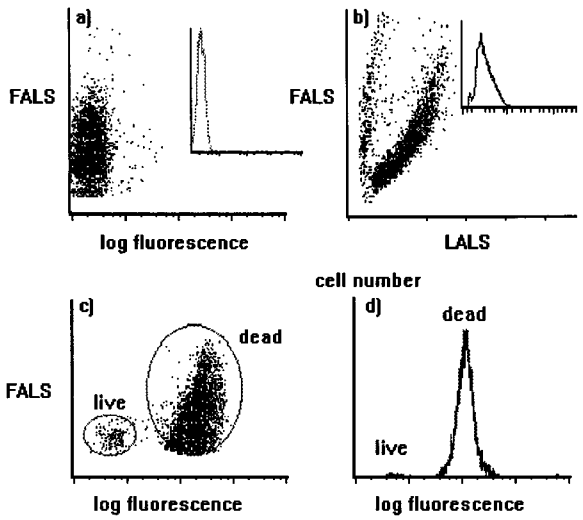

FIG. 6. Light scattering and fluorescence from $DiBAC_4(3)$-stained *E. coli* ATCC 35218 taken after 5 h of growth in broth culture (a and b) and following gramicidin treatment (c and d). (a and c) Dot plots of forward angle light scattering (FALS) versus fluorescence; (b) dot plot of forward angle light scattering versus low angle light scattering (LALS); (d) fluorescence histograms of live and dead cell. The inserts show the fluorescence histogram (a) and FALS (b). Samples were analyzed with a Bio-Rad Bryte-HS flow cytometer.

the insert (Fig. 6a). Figure 6b depicts forward angle versus large angle light scattering, and the insert represents the forward angle light scattering histogram. Forward angle light scattering is generally related to cell size or mass, whereas large angle light scattering is sensitive to cell shape and the internal content (24). The light scattering distribution from this normal unperturbed population of *E. coli* indicates that there is a broad range in cell size, as would be expected from a rapidly dividing population. The second less-dense population (Fig. 6b) probably represents cell debris or other particulate matter in the sheath fluid. No fluorescence is associated with this population, indicating that no intact bacteria are present. Figure 6c and d depict the fluorescence histograms and dot plots of forward angle light scattering versus oxonol fluorescence of *E. coli* following gramicidin treatment. The live and dead populations are clearly distinguishable in both the fluorescence histogram plots and the dot plots. Each population in the dot plots can be defined by drawing regions of interest around them (Fig. 6c). The software allows the calculation of total counts per microliter in the defined regions of interest. By using this simple technique, the numbers of viable and dead

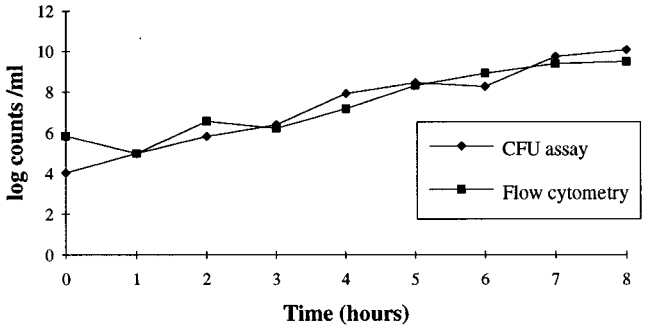

FIG. 7. Growth of *E. coli* ATCC 35218 in tryptose soya broth analyzed by flow cytometry after staining with 10 μg of $DiBAC_4(3)$ per ml and by standard CFU assay. Samples were analyzed with a Bio-Rad Bryte-HS flow cytometer.

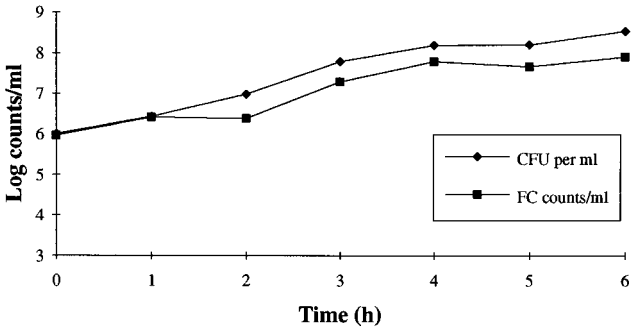

FIG. 8. Growth of *S. aureus* in tryptone soya broth, analyzed by flow cytometry after staining with 10 μg of $DiBAC_4(3)$ per ml and by standard CFU assay. Samples were analyzed with a Bio-Rad Bryte-HS flow cytometer.

cells can be determined. This technique was applied when routinely determining viable counts.

Figure 7 shows viable counts of *E. coli* ATCC 35218 determined by using $DiBAC_4(3)$ staining and flow cytometry compared with standard CFU counts. The graph represents a growth curve of *E. coli* showing an increase in the number of viable bacteria with time. A close correlation between viable counts determined flow cytometrically and by CFU assays is evident.

One major concern is whether the number of viable *S. aureus* cells can be determined by flow cytometry. It was envisaged that the natural clumping of *S. aureus* would significantly affect flow cytometry counts. Figure 8 represents a typical growth curve of *S. aureus*, showing an increase in the number of cells with time. The trend in CFU counts compares well with the flow cytometry viable counts. Similar counts were obtained during the first hour, but thereafter the flow cytometry counts remained at approximately 0.5 log lower than the results by the CFU assay. The CFU counts may be higher because the samples had undergone some dilution steps before being plated onto agar, thus facilitating the breakup of any cell clumps. Evidence for this can perhaps be seen at time zero and 1 h, when similar counts were obtained. Here the CFU sample would have undergone fewer dilution steps. The mean light scattering intensity decreased with growth, indicating that there was no overall increase in cell clumping with time (results not shown).

Figure 9a depicts the light scattering and $DiBAC_4(3)$ fluorescence produced from a 3-h culture of *S. aureus*. The fluorescence intensity profile is broader than that obtained for *E. coli* (Fig. 6a). Also, the light scattering profile indicates that the cell size distribution is also much wider than that obtained for *E. coli*, thus perhaps indicating the presence of some cell aggregates. Figure 9b demonstrates the effect of gramicidin on *S. aureus*. Comparison of the results in Fig. 9a and 9b indicates a large increase in oxonol fluorescence after 2 min of treatment with 20 μg of gramicidin per ml. Very few live cells are present following this treatment. At the same time, there is little effect on the light scattering, indicating that the cells are intact. However, increasing the incubation time with gramicidin by a few minutes or increasing its concentration does lead to significant decreases in light scattering as a result of cell lysis.

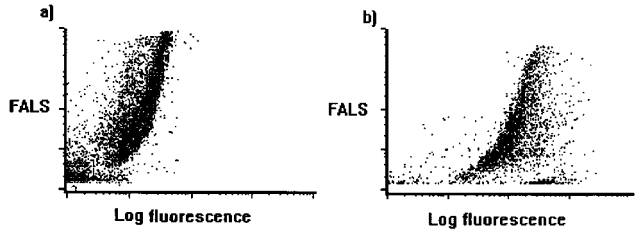

FIG. 9. Forward angle light scattering (FALS) and fluorescence from oxonol [$DiBAC_4(3)$]-stained *S. aureus* Oxford taken after 3 h of growth from broth culture before (a) and after (b) gramicidin treatment. Samples were analyzed with a Bio-Rad Bryte-HS flow cytometer.

Conclusions. The viability of microorganisms can be assessed by using fluorescent probes and flow cytometry. The choice of the fluorescent probe largely depends on the microorganism in question, the experimental conditions under which they are grown, and the type of information required by the experimenter. For example, gram-negative microorganisms are generally impermeant to fluorogenic probes, such as CDFA, and the cationic membrane potential probe rhodamine 123 unless they are first pretreated with an agent that increases membrane permeability, such as 1 mM EDTA. Fluorogenic probes have been used successfully on environmental samples following EDTA treatment (11). We found that there was a general inconsistency in the uptake of fluorogenic probes by bacteria, which appeared to depend on the growth phase of the culture, staining time, and incubation temperature (data not shown). Another major problem was that the staining of both gram-positive and gram-negative bacteria with fluorogenic substrates and rhodamine 123 requires several incubation and cell washing steps. In the laboratory where robust, rapid high-throughput viability assays are required in the presence of antibiotics, fluorogenic probes and rhodamine 123 are not appropriate. However, in situations where the initial effects of antibiotics on membrane integrity (or cellular metabolism) and membrane potential are required, they could be of greater value. Such experiments would involve first loading the viable cells with the probe and then monitoring its release as a decrease in fluorescence as a result of lysis or inhibiting membrane potential.

The intercalating DNA stains PI and EMA gave promising results as viability stains. Further work is required to ascertain their robustness as stains for determining the number of viable bacteria. Although the staining protocol for EMA is too laborious for use in rapid routine assays, it is useful for determining the viability of microorganisms where prefixation may be recommended or convenient, particularly for bacteria in clinical samples. Unlike PI-stained cells, EMA-stained cells are irradiated and then chemically fixed. Thus, it cannot be used in combination with another live cell stain, such as FDA, which precludes dual fluorescence studies.

The anionic membrane potential probe $DiBAC_4(3)$ produced the most robust results as an indicator of viability. It is suitable for both gram-positive and gram-negative bacteria, with no sample pretreatment other than dilution, if required. The results obtained with $DiBAC_4(3)$ are more consistent than those obtained with rhodamine 123.

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