

Rapid Assessment of Antibiotic Effects on *Escherichia coli* by bis-(1,3-Dibutylbarbituric Acid) Trimethine Oxonol and Flow Cytometry

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The effects of selected antibiotics on *Escherichia coli* were studied by flow cytometry with the fluorescent anionic membrane potential probe bis-(1,3-dibutylbarbituric acid) trimethine oxonol [DiBAC₄(3)]. The actions of azithromycin, cefuroxime, and ciprofloxacin at five times the MIC on *E. coli* were compared by the traditional CFU assay and flow cytometry. Changes in viable counts of bacteria determined with DiBAC₄(3) and by flow cytometry following treatment with the antibiotics showed trends similar to those found by the CFU assays. However, viable counts determined by flow cytometry following antibiotic treatment were 1 to 2 logs higher than those determined by the corresponding CFU assays. All the results obtained by flow cytometry were provided within 10 min after sampling, whereas the conventional CFU assay results took at least 18 h. The results indicated that flow cytometry is a sensitive analytical technique that can rapidly monitor the physiological changes of individual microorganisms following antibiotic action and can provide information on the mode of action of a drug. The membrane potential probe DiBAC₄(3) provides a robust flow cytometric indicator for bacterial cell viability.

Flow cytometry is an invaluable technique for studying how various agents or diseases affect mammalian cells (15), and lately, its application for studying the modes of action of drugs on bacteria has been reported. For example, flow cytometry was used to study the effects of various protein synthesis inhibitors on *Escherichia coli* DNA initiation of replication, DNA replication, and cell division (2, 13, 14). Flow cytometry was used to study the effects of gentamicin, ampicillin, cefotaxime, mecillinam, and ciprofloxacin on bacterial morphology and membrane permeability (5, 10). Also, the effects of various β -lactam antibiotics on size (reduction, lysis, and filamentation) and DNA ploidy in *E. coli* were examined by flow cytometry (4).

Mechanisms that are sensitive to changes in membrane potential provide a means of assessing the viability of a cell. In bacteria, the cellular apparatus for energy metabolism is localized on the cytoplasmic or inner membrane. The membrane potential is dependent on energy metabolism and is decreased within a few minutes following removal of energy sources. Uncoupling agents eliminate proton gradients across the membrane, thereby decreasing membrane potential in bacteria (7). In addition, the permeability properties of the membrane influence the size of the membrane potential. Thus, only metabolically active bacteria whose membranes have not been damaged are capable of generating and maintaining a normal membrane potential. Consequently, probes that are sensitive to changes in membrane potential provide a means of assessing the viability of a cell. A wide variety of fluorescent probes that can be used to measure membrane potential are now available (8, 12, 17). Many of these probes have been used in single cell measurements of membrane potential by flow cytometry in both eukaryotes and prokaryotes (9, 11, 16).

The negatively charged oxonol dyes undergo a potential-

dependent distribution between the cytoplasm and the extracellular medium in eukaryotic cells. The oxonol bis-(1,3-dibutylbarbituric acid) trimethine oxonol [DiBAC₄(3)] has a high voltage sensitivity (3) and enters depolarized cells, where it binds to lipid-rich intracellular components. Therefore, cells that possess a membrane potential will exclude oxonol and show a weak fluorescence due to the bound dye. Once the membrane potential is lost, the dye enters the cell and fluorescence increases. DiBAC₄(3) was previously shown to be a sensitive, robust bacterial viability probe for use with flow cytometry (6). The findings from that work have been extended in this study to assess antibiotic effects on *E. coli*.

MATERIALS AND METHODS

Culture conditions. *E. coli* ATCC 35218 was routinely grown in flasks containing filtered (pore size, 0.22 μ m; Millipore, Molsheim, France) tryptone soy broth in a shaking water bath at 37°C. To determine the numbers of CFU, cell suspensions were serially diluted in phosphate-buffered saline and plated on nutrient agar. Visible colonies were counted following overnight incubation at 37°C.

Flow cytometry. A Bio-Rad Bryte HS flow cytometer (Bio-Rad, Hemel Hempstead, United Kingdom) was used to measure the light scattering and fluorescence of a single cell. Illumination was provided by a 75-W high-pressure mercury-xenon arc lamp (Hamamatsu). Light scattering (in the dark-field configuration) and fluorescence from samples delivered by a calibrated syringe pump were measured at approximately 1,000 cells per s in a hydrodynamically focused jet of filtered water (pore size, 0.22 μ m; Milli-Q PLUS; Millipore) with a flow pressure of 7×10^4 Pa (velocity, 4.6 m/s) over a microscope cover glass, with the bacterial suspension traveling along the jet axis. The optical characteristics of the relevant filter are as follows: excitation, 470 to 495 nm; band stop, 510 nm; emission, 520 to 550 nm.

Light scattering and fluorescence were converted by photomultiplier detectors (PMTs) into equivalent electrical pulses and were digitized by an analog-digital converter connected to a Compaq Deskpro 66M microcomputer (International Business Machines AT personal computer compatible; 80486 processor). Signals were handled by Bio-Rad WinBryte software, and the results were displayed as single-histogram or dual-parameter isometric density plots (light scattering and fluorescence). PMT voltages were set to a desired value, and unless stated otherwise, the PMT amplifier gains were set in the linear mode for light scattering and the logarithmic mode for fluorescence. Light scattering and fluorescence were triggered by forward-angle light scattering (FALS) with the threshold limit set to the desired value in order to reduce noise arising from background flare, extracellular fluorophore, sample, and sheath fluid debris. FALS is defined

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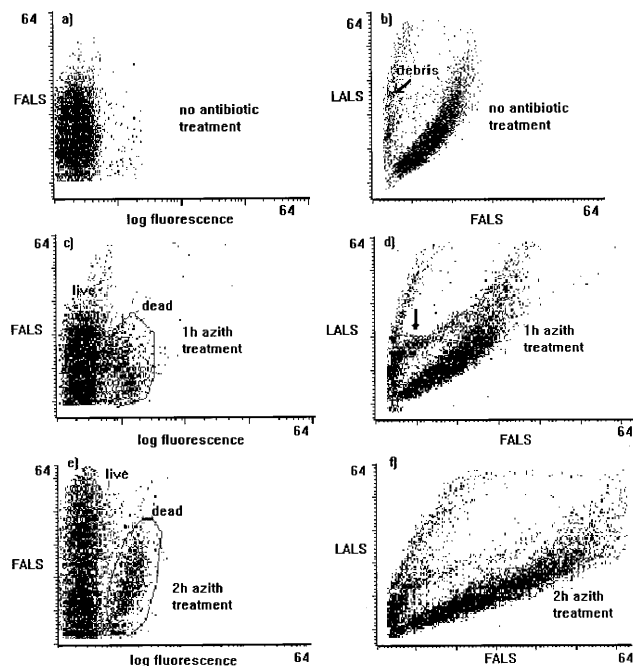


FIG. 1. Light scattering and DiBAC₄(3) fluorescence from cultures of *E. coli* taken after 4 h of growth without antibiotic treatment (a and b) and after 1 h (c and d) and 2 h (e and f) of azithromycin (azith) treatment. (a, c, and e) Dot plots of FALS versus log DiBAC₄(3) fluorescence. (b, d, and f) Dot plots of LALS versus FALS. The arrow in panel d indicates a subpopulation of cells (see text for details).

as the light detected in the angular range of 1 to 18°, and large-angle light scattering (LALS) is the light detected over an angular range of 18 to 85°.

Cell staining. Stock solutions of 1 mg of the oxonol DiBAC₄(3) (Molecular Probes, Inc, Eugene, Oreg.) per ml were made up in ethanol and were stored at -20°C. One-milliliter samples of cultures were removed from flasks and diluted to 10⁶ cells/ml by using filtered phosphate-buffered saline (pore size, 0.22 μm) containing 10 μg of DiBAC₄(3) per ml. For suspensions containing less than 10⁶ bacteria/ml, DiBAC₄(3) was added directly to 1-ml samples of culture. Cells were allowed to stain at room temperature for 2 min before they were analyzed by flow cytometry.

Antibiotic treatments. Filtered (pore size, 0.22 μm) tryptone soy broth was inoculated with *E. coli* taken from an overnight starter culture to give an initial inoculum of approximately 10³ organisms/ml. Growth was allowed to continue for 4 h, after which the culture was divided into equal volumes and placed into flasks. The antibiotics studied were used at five times their MICs for *E. coli* ATCC 35218. The antibiotics were as follows: ciprofloxacin, 0.08 μg/ml (Bayer, Germany); azithromycin, 40 μg/ml (Richborough Pharmaceuticals); and cefuroxime, 20 μg/ml (Sigma Chemical Company Ltd). MICs were determined by the broth microtiter method as described by the National Committee for Clinical Laboratory Standards (10a) before culture and flow cytometric work were carried out. Following the addition of the antibiotics to the separate flasks, samples were taken throughout the experiments at various times for standard viable counts (CFU assays) and for flow cytometry studies. Samples of *E. coli* were removed from 4-h broth cultures, stained with DiBAC₄(3), and treated with 20 μg of gramicidin S (Sigma) per ml for 2 min at room temperature. (The MIC of gramicidin S was not determined.) Such treatments were routinely used as dead cell control treatments to aid in the interpretation of flow cytometry data.

RESULTS AND DISCUSSION

Effects of azithromycin on *E. coli*. Figure 1 shows the effects of the protein synthesis inhibitor azithromycin on DiBAC₄(3) fluorescence, and hence membrane potential and FALS, by flow cytometry. Figure 1a represents a dot plot of FALS versus DiBAC₄(3) fluorescence for *E. coli* cells removed from a culture after 4 h of growth. There is one major population representing viable cells that excluded DiBAC₄(3). There are a few cells with a higher fluorescence intensities, indicating that

some of the *E. coli* cells in the culture have lost membrane potential. Figure 1c depicts FALS versus DiBAC₄(3) fluorescence of *E. coli* cells after 1 h of azithromycin treatment. The population of cells with a high fluorescence intensity lost membrane potential and contained dead or dying bacteria. Each population in the dot plots can be defined by drawing regions of interest (ROIs) around them. The software allows the calculation of total counts per microliter in the defined ROI. By using this simple technique, the number of viable cells can be determined. This technique was applied routinely when determining viable counts by flow cytometry.

Both the live and dead cell populations in the FALS-fluorescence dot plot (Fig. 1c) are associated with the main cluster of cells in the FALS-LALS plot (Fig. 1d). By comparing Fig. 1d (representing cells treated for 1 h) with Fig. 1b (representing untreated cells), the presence of a subpopulation of cells in the FALS-LALS plots is evident (area marked with an arrow). The fluorescence intensity associated with this subpopulation indicates that the cells are morphologically different from both live and dead cells. The reason for the presence of this subpopulation following azithromycin treatment is not known but demonstrates that bacterial populations, even when grown under culture conditions, are heterogeneous with respect to the effects of antibiotics. The effects of azithromycin after 2 h of treatment are shown in Fig. 1e and f. The FALS indicates an increase in mean cell size and size distribution. The relative number of cells in the subpopulation shown in Fig. 1b decreased after 2 h of treatment. Also, the number of dead cells increased following 2 h of treatment, as indicated by the rise in the number of cells with a higher fluorescence intensity.

The light-scattering histograms in Fig. 2 indicate the change in cell size as both the antibiotic-untreated and antibiotic-treated *E. coli* culture ages increase. The mean light-scattering intensity (channel number) decreases between 6 and 8 h, indicating a change in cell size or mass. This decrease in light scattering is typical for *E. coli* because its growth slows down with culture age (13). The mean light-scattering intensity increases between 6 and 7 h for azithromycin-treated bacteria (2 and 3 h after treatment, respectively). However, there is no further increase in light scattering after 4 h of treatment (Fig. 2f). The lower-intensity light scattering peak in the bimodal distribution after 2 h of azithromycin treatment (Fig. 2d) is not present after 3 and 4 h. By selecting ROIs around the live and dead cell populations depicted in the FALS-fluorescence plot (Fig. 1c), it can be shown that the cells represented by this lower-intensity peak (smaller cells) are dead and the cells in the higher-intensity peak are alive.

Figure 3 presents the effects of azithromycin on the numbers of viable *E. coli* cells determined by CFU assay and flow cytometry. The CFU assay shows a rapid decline in the numbers of viable bacteria after the addition of antibiotic, from approximately 10⁸ to less than 100 organisms/ml over the 8-h incubation period. The flow cytometry results indicate a gradual reduction in growth over the first 2 h following azithromycin addition, before decreasing to less than 10⁵ organisms/ml.

Effects of cefuroxime on *E. coli*. Figure 4 indicates the effects of the β-lactam cefuroxime on DiBAC₄(3) fluorescence (membrane potential) and light scattering after 1 and 2 h on a growing culture of *E. coli*. After 1 h there appeared a subpopulation of cells that lost their membrane potential. These dead cells have both lower FALS and lower LALS intensities than the live cells. However, the light scattering from the live cells is significantly higher than that from the untreated control cells taken at the same time point, indicating that there is an increase in mean cell size after the addition of antibiotic. The second subpopulation consists of cells with higher fluorescence

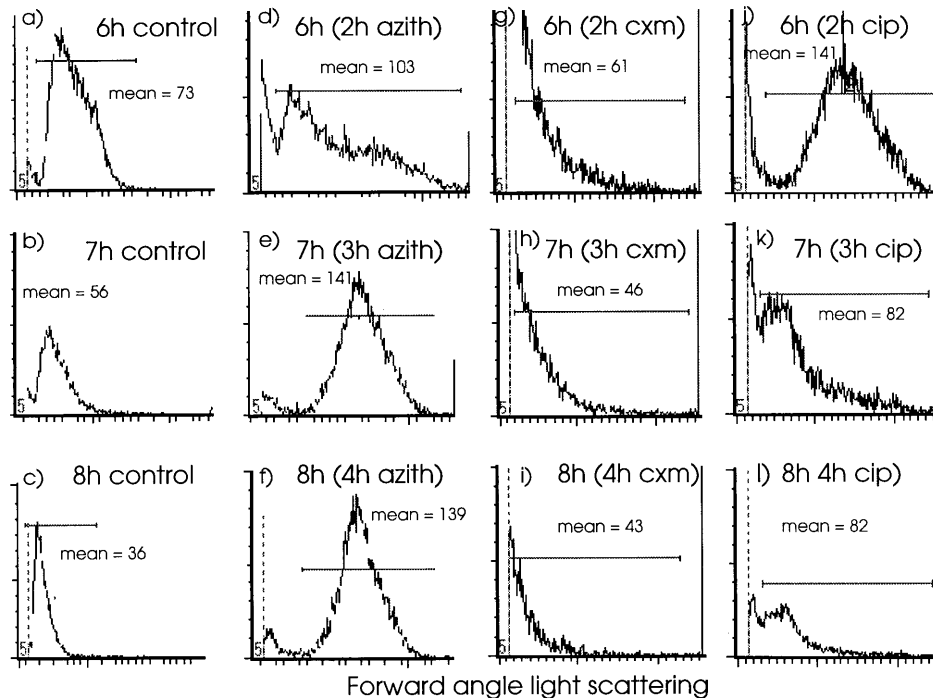


FIG. 2. Comparison of FALS from antibiotic-untreated (a to c) and antibiotic-treated (d to j) *E. coli* cells. The histograms represent samples taken after 6, 7, and 8 h of growth following 2, 3, and 4 h of treatment with 40 µg of azithromycin (azith; d to f) per ml, 20 µg of cefuroxime (cxm; g to i) per ml, and 0.08 µg of ciprofloxacin (cip; j to l) per ml.

and lower light-scattering intensity, indicating that they are smaller and have lost their membrane potential. The effects of cefuroxime after 2 h are shown in Fig. 4c and d. The difference in relative mean fluorescence intensity between live and dead cells increased further following 2 h of treatment. There was also a marked reduction in the number of live cells, whereas the relative number of dead cells increased. There was a further decrease in light-scattering intensity from the treated cells, and the presence of large quantities of debris indicates that cell lysis is occurring. The histograms in Fig. 2g to i indicate that there was a continued decrease in mean light-scattering intensity over the treatment time.

The results indicate that the initial action of cefuroxime causes an increase in light scattering, and hence cell size, with an associated increase in the number of cells that have lost their membrane potential (dead cells). A possible explanation for this is that cells are swelling as a result of osmosis following

damage to the cell wall. Further incubation in the presence of cefuroxime results in an increased loss in membrane potential and a decrease in cell size, probably due to lysis.

Figure 5 compares the effects of cefuroxime on the number of viable *E. coli* cells determined by CFU assay and flow cytometry. The CFU count decreased from approximately 10⁸/ml after 4 h of growth (the time at which antibiotic was added) to approximately 10⁵/ml 2 h later. The CFU count then increased

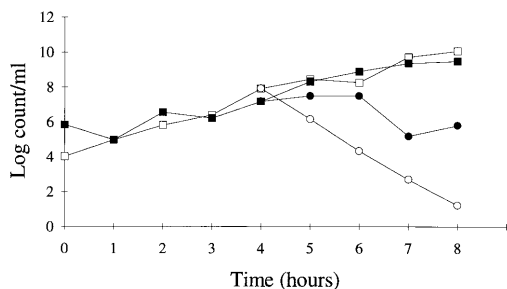


FIG. 3. Effects of 40 µg of azithromycin per ml on *E. coli* cells determined by CFU assays (○) and flow cytometry (●). ■ and □, untreated flow cytometry and CFU assay controls, respectively. Azithromycin was added to the culture after 4 h growth.

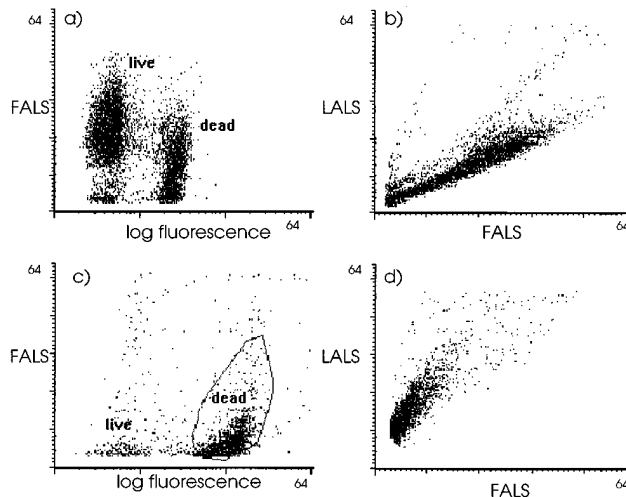


FIG. 4. Light scattering and DiBAC₄(3) fluorescence from cultures of *E. coli* after 1 h (a and b) and 2 h (c and d) of treatment with 20 µg of cefuroxime per ml. (a and c) Dot plots of FALS versus log DiBAC₄(3) fluorescence. (b and d) Dot plots of LALS.

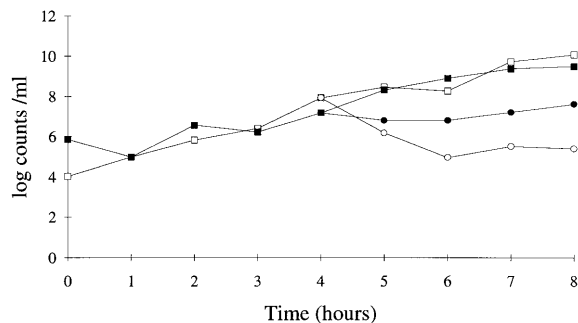


FIG. 5. Effects of 20 μg of cefuroxime per ml on *E. coli* cells determined by CFU assays (\circ) and flow cytometry (\bullet). \blacksquare and \square , untreated flow cytometry and CFU assay controls, respectively. Cefuroxime was added to the culture after 4 h of growth.

to $5 \times 10^5/\text{ml}$ over the remaining incubation time. The reduction in the viable count by flow cytometry was smaller. Here the number of *E. coli* decreased from approximately $2 \times 10^7/\text{ml}$ at 4 h to approximately $5 \times 10^6/\text{ml}$ 2 h later. The viable count then increased to $3 \times 10^7/\text{ml}$ at 8 h. Counts by both CFU assays and flow cytometry indicated signs of regrowth after 2 h in the presence of cefuroxime. However, this regrowth was identified after 10 min of sampling the culture by flow cytometry, whereas overnight incubation was required by the CFU assays.

Effects of ciprofloxacin on *E. coli*. Figure 6 indicates the effects of the DNA gyrase inhibitor ciprofloxacin on DiBAC₄(3) fluorescence (membrane potential) and the light scattering of *E. coli*. After 1 h of treatment a second population of cells with higher levels of fluorescence (dead cells) is evident (Fig. 6a). The light-scattering results in Fig. 6a and b and Fig. 2j indicate that both live and dead cells increased in size compared to the size of untreated control cells (Fig. 1 and 2a). After 2 h of ciprofloxacin treatment the light-scattering intensities from live *E. coli* cells decreased significantly compared to those for the cells treated for 1 h. However, it was still higher

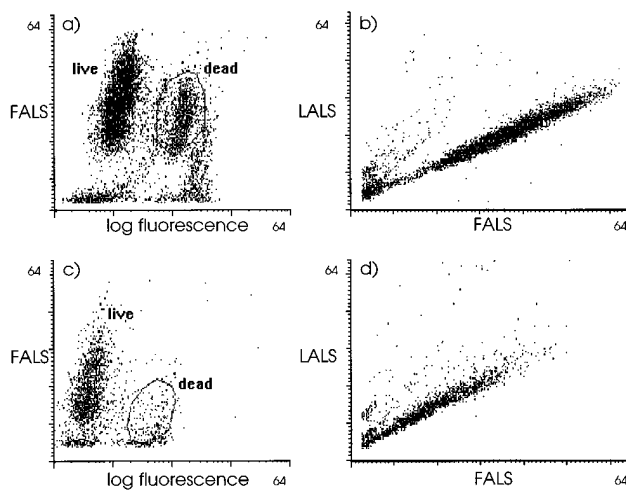


FIG. 6. Light scattering and DiBAC₄(3) fluorescence from cultures of *E. coli* after 1 h (a and b) and 2 h (c and d) of treatment with 0.08 μg of ciprofloxacin per ml. (a and c) Dot plots of FALS versus log DiBAC₄(3) fluorescence. (b and d) Dot plots of LALS versus FALS. The light scatter photomultiplier voltage and amplifier gains were reduced in panels a and b to keep the data on scale. Data for approximately 5,000 cells were collected for panels c and d.

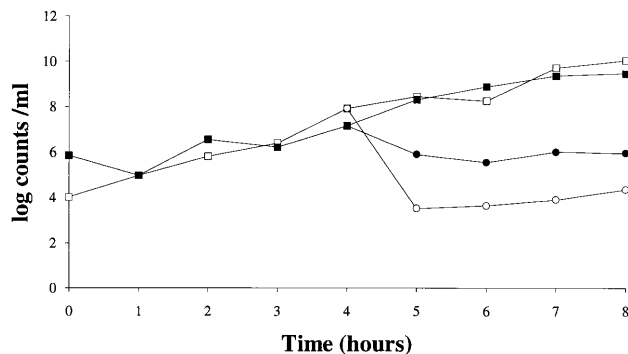


FIG. 7. Effects of 0.08 μg of ciprofloxacin per ml on *E. coli* cells determined by CFU assays (\circ) and flow cytometry (\bullet). \blacksquare and \square , untreated flow cytometry and CFU assay controls, respectively. Ciprofloxacin was added to the culture after 4 h of growth.

compared to those for untreated cells, even after 3 and 4 h of treatment (Fig. 2j to l). Relatively few dead cells were evident after 2 h of treatment, indicating that cells are becoming resistant (Fig. 6c). It appears that ciprofloxacin causes a change in cell morphology or cell size after 1 h of treatment and a significant loss in membrane potential. However, after 2 h of treatment the light-scattering and fluorescence data indicate that the bacteria are returning to normal in terms of morphology and membrane potential. Mason et al. (10) also showed that *E. coli* is capable of maintaining its membrane potential following 2 h of ciprofloxacin treatment at concentrations up to 10 times the MIC.

Figure 7 compares the effects of ciprofloxacin on the numbers of viable *E. coli* cells determined by flow cytometry and the CFU assay. The numbers of CFU decreased from approximately 10^8 to $10^3/\text{ml}$ after 1 h and increased steadily up to $10^4/\text{ml}$ over the next 3 h, demonstrating that regrowth had occurred. The flow cytometry counts also decreased and showed a trend similar to those shown by the CFU assays. However, as with azithromycin and cefuroxime, the flow cytometry counts following antibiotic treatment at equivalent time points were approximately 2 logs higher.

The fluorescent membrane potential-sensitive probe DiBAC₄(3) has been evaluated as an indicator of microbial viability following antibiotic treatment. It provides a sensitive indicator of drug potency within 1 h of drug addition and produces a result in less than 10 min. In no case has the conventional CFU assay subsequently indicated drug activity that was missed by flow cytometry.

Some classes of antibiotics, e.g., β -lactams, might be expected to produce a rapid effect on the membrane potential of bacterial cells. However, antibiotics acting on other biochemical pathways (e.g., protein synthesis inhibitors such as azithromycin) may indirectly prevent the bacterium from maintaining its membrane potential. The final state for either mode of action is a loss of membrane potential, which is indicative of antimicrobial activity.

One of the main findings in this study is that the flow cytometry counts of viable bacteria after antibiotic treatment show trends similar to those shown by the CFU assays, but the counts are higher. CFU assays determine the ability of a cell to divide continuously and to form visible colonies on solidified media, whereas the flow cytometry assay is based on the loss of membrane potential. Part of the systematic difference may be due to the longer incubation times of CFU assays, allowing lethally damaged bacteria time to die during the 18-h incuba-

tion period. It is possible for a cell to maintain an active metabolism but be incapable of dividing. Therefore, when assessing additional antibiotics, a correlation of acute loss of viability, as measured by flow cytometry, with loss of vitality measured by a standard CFU assay should be made. Current work is concentrating on deriving a correlation between flow cytometry data and CFU data, particularly following antibiotic treatment.

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