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Bacterial Viability and Antibiotic Susceptibility Testing with SYTOX Green Nucleic Acid Stain

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A fluorescent nucleic acid stain that does not penetrate living cells was used to assess the integrity of the plasma membranes of bacteria. SYTOX Green nucleic acid stain is an unsymmetrical cyanine dye with three positive charges that is completely excluded from live eukaryotic and prokaryotic cells. Binding of SYTOX Green stain to nucleic acids resulted in a >500-fold enhancement in fluorescence emission (absorption and emission maxima at 502 and 523 nm, respectively), rendering bacteria with compromised plasma membranes brightly green fluorescent. SYTOX Green stain is readily excited by the 488-nm line of the argon ion laser. The fluorescence signal from membrane-compromised bacteria labeled with SYTOX Green stain was typically >10-fold brighter than that from intact organisms. Bacterial suspensions labeled with SYTOX Green stain emitted green fluorescence in proportion to the fraction of permeabilized cells in the population, which was quantified by microscopy, fluorometry, or flow cytometry. Flow cytometric and fluorometric approaches were used to quantify the effect of β-lactam antibiotics on the cell membrane integrity of *Escherichia coli*. Detection and discrimination of live and permeabilized cells labeled with SYTOX Green stain by flow cytometry were markedly improved over those by propidium iodide-based tests. These studies showed that bacterial labeling with SYTOX Green stain is an effective alternative to conventional methods for measuring bacterial viability and antibiotic susceptibility.

Bacterial growth and metabolism can be disrupted by a variety of physical treatments, as well as by chemical and biological agents. The effects of these treatments on bacteria have been evaluated by a number of different techniques (8, 19, 39). Viability in bacteria is frequently equated with the ability to form colonies on solid growth medium or to proliferate in nutrient-containing solutions. By these criteria, antimicrobial activity is defined as the ability to limit bacterial growth. Viability tests that require bacterial growth, although conceptually simple, may be time-consuming, costly, and insensitive, particularly for evaluation of slow-growing organisms.

A number of fluorescence-based assays for evaluating bacterial viability have been introduced over the past two decades (33). Metabolic activity may be measured with tetrazolium compounds such as 5-cyano-2,3-ditolyltetrazolium chloride (16, 21, 27, 35, 37), which is reduced by actively respiring microorganisms to yield a fluorescent formazan precipitate. An alternative approach relies on the fluorescence-based detection of the electrical potential gradient across the bacterial plasma membrane monitored by the accumulation of positively charged dyes such as rhodamine 123 (15, 20, 25, 35) or 3,3′-dipentyloxacarbocyanine (25, 30) or by exclusion of negatively charged molecules such as the bis-oxonols from active cells (12, 21, 24, 25, 38). Enzyme activity associated with bacteria may also report on their state of growth and may be detected with a variety of fluorogenic substrates (2, 22). Measurement of enzyme activity, redox potential, or membrane potential can provide very specific and useful information about the physiological state of bacteria.

Several new approaches have been developed to measure specific mechanisms of damage to bacterial cells (12, 14, 20, 25, 26). A decrease in the ability of organisms to exclude polar compounds that are not cell permeant accompanies cell death under many conditions (33). Fluorescent probes are frequently used to detect this loss of plasma membrane integrity (1, 11, 31, 36). For example, the efficacies of antibiotics that affect the structural integrity of the cell wall, ultimately leading to an increase in plasma membrane permeability, have been monitored directly in individual bacteria by fluorescence techniques (7, 17, 24, 30, 33). Also a single-cell technique, flow cytometry is an analytical tool that is being used with increasing frequency to evaluate bacterial viability in clinical and environmental samples (3, 4, 6, 9, 10, 28–30, 32, 34, 38, 40).

The efficacy of fluorescent compounds as labels for membrane-compromised cells is governed by their selectivity, brightness, excitation and emission maxima, and inherent biological toxicity. Ideal indicators of plasma membrane integrity concentrate only in cells with permeabilized membranes and exhibit marked fluorescence enhancement within these cells. DNA and RNA provide large numbers of intracellular binding sites that promote marked fluorescence enhancement of many different stains. The phenanthridium nucleic acid stains such as ethidium bromide, propidium iodide, and ethidium homodimer 1 have been used almost exclusively to evaluate the integrity of the plasma membrane by fluorescence in a variety of animal cells and bacterial species (13, 14, 20, 23, 40). SYTOX Green stain provides several important advantages over these compounds, making it a preferred candidate for a variety of fluorescence-based applications in microbiology (18).

MATERIALS AND METHODS

Bacterial cultures. Two strains of *Escherichia coli* (ampicillin-sensitive ATCC 25922 and ampicillin-resistant ATCC 35218) were obtained from the American Type Culture Collection (Rockville, Md.). Laboratory isolates of *Bacillus cereus*, *E. coli*, and *Staphylococcus aureus* were generously provided by John Fryer, Department of Microbiology, Oregon State University, Corvallis. Bacterial cultures were maintained in Trypticase soy broth or agar medium (Difco Laboratories, Detroit, Mich.). Overnight cultures of all bacteria were grown in 100-ml vessels shaken at 200 rpm in a 30°C temperature-controlled shaking incubator (New Brunswick, Edison, N.J.).

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Reagents and solutions. Ampicillin, amoxicillin, penicillin G, and vancomycin were obtained from Sigma Chemical Company (St. Louis, Mo.). Stock solutions were prepared by dissolving 10 mg of either ampicillin, penicillin G, or vancomycin or 4 mg of amoxicillin in 1 ml of filtered water (0.2-μm-pore-size filter). SYTOX Green stain and propidium iodide are commercially available products of Molecular Probes (Eugene, Oreg.). Stock solutions of SYTOX Green stain and propidium iodide were prepared in dimethyl sulfoxide to a final concentration of 5 mM. Fluorescence spectra of nucleic acids in the presence of SYTOX Green stain were acquired in solutions containing 10 mM Tris and 1 mM EDTA, pH 8.0 (TE).

Permeabilization of bacteria. One milliliter of bacterial culture was pelleted by centrifugation at 8,800 × *g* for 3 min in a 1.7-ml microcentrifuge tube. The cell pellet was resuspended in 1 ml of 70% isopropyl alcohol and allowed to stand at room temperature for 1 h. The bacterial suspension was subsequently pelleted by centrifugation, washed twice in sterile dilute medium consisting of sterile 5% Trypticase soy broth in 0.85% NaCl in water (5% TSB), and stored at 4°C until use. Heat treatment was carried out by pelleting 1 ml of bacterial culture in a 1.7-ml microcentrifuge tube as described above and resuspending it in 1 ml of 5% TSB in water. The tube containing the bacterial suspension was submerged in 100 ml of boiling water for 10 min. The heated cell suspension was then cooled to room temperature, pelleted by centrifugation, washed twice with 5% TSB, and stored at 4°C until use. Both methods of permeabilization resulted in a >99.99% reduction in viable cell number determined by standard plate counting.

Antibiotic susceptibility testing. Bacterial susceptibility to antibiotics was tested in 5% TSB. Media that are highly autofluorescent or contain nucleic acid constituents that could bind the SYTOX Green stain were avoided to reduce background fluorescence. Bacteria, antibiotic, and SYTOX Green stain were combined, and the resulting mixture was incubated for 2 h in a 30°C water bath for fluorometry and 37°C for flow cytometry. A calibrated amount of polystyrene microspheres was added to samples for flow cytometry prior to incubation. For standard plate counts, 0.1-ml samples were spread on 100-mm-diameter Trypticase soy agar plates which were incubated for 1 to 2 days at 30°C. Most-probable-number determinations were carried out by adding 0.5 ml of sample to tubes containing 4.5 ml of 5% TSB, followed by incubation for 3 days at 30°C. Estimation of the density of viable bacteria in the samples was based on eight replicates of eight serial 10-fold dilutions of the bacterial suspensions.

Fluorescence microscopy. Bacteria stained with SYTOX Green stain and other fluorescent compounds were observed with an Axioplan epifluorescence microscope equipped with a 50-W mercury arc lamp, differential interference contrast (DIC) optics, and both a 100×, 1.3-numerical-aperture Plan-Neofluar and a 40×, 0.75-numerical-aperture Plan-Neofluar objective lens (Carl Zeiss, Inc., Thornwood, N.Y.). The epifluorescence filter sets were used as described in individual figure legends. Photomicrographs were acquired with Kodak Ektachrome Elite 400 ASA color slide film.

Spectrofluorometry. Fluorescence spectra were obtained with an Alphascan fluorescence spectrophotometer (Photon Technology International, Inc., South Brunswick, N.J.). Measurement of fluorescence in bacterial suspensions containing 10^6 to 10^8 cells/ml was carried out with 3-ml, 1-cm-path-length acrylic cuvettes that were held at 30°C and stirred continuously with a magnetic stir bar.

Flow cytometry. Flow cytometry was carried out with a FACS Vantage instrument (Becton Dickinson Immunocytochemistry Systems, San Jose, Calif.) equipped with an Enterprise laser (Coherent, Santa Clara, Calif.) in which the 488-nm beam was set to a light-regulated output of 100 mW. Fluorescence emission was collected with interference bandpass filters (Omega Optical, Brattleboro, Vt.): 530 ± 30 nm (FL1 channel) for the SYTOX Green stain and >600 nm (FL3 channel) for propidium iodide. The sheath fluid consisted of 0.9% NaCl, and sample flow rates through a 70-μm nozzle tip were between 200 and 400 particles per s. The standard focusing lens of the flow cytometer was removed prior to measurement of bacterial fluorescence in order to improve the excitation and light collection efficiency. The density of the bacterial suspension was quantified by a modification of the technique described by Obernesser et al. (29) and by Cantinieaux et al. (5). In brief, a suspension of 6-μm nonfluorescent polystyrene beads was diluted in the bacterial suspension to yield a final bead density of 10^6 beads/ml. The number of beads counted in region 1 (R1) of each run was used to determine the volume of suspension sampled, from which the original density of the bacterial suspension was then calculated. The volume of bacterial suspension sampled was determined by dividing the particle counts in R1 (beads) by the known concentration of beads in the sample (10^6 beads/ml). The number of bacteria per milliliter was calculated by dividing the particle counts in the chosen region by the volume of bacterial suspension sampled. Data were stored as list-mode files onto the hard disk of an Apple Macintosh Quadra 650 microcomputer and subsequently displayed with software supplied by the manufacturer using region gates to collect values for fluorescence mean intensity and forward scatter signal.

RESULTS

Enhancement of SYTOX Green stain fluorescence by nucleic acid binding. The fluorescence emission of SYTOX Green stain was measured in solutions with equivalent concentrations of SYTOX Green stain and either double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), a 24-base M13 DNA oligomer, or RNA (Fig. 1). SYTOX Green stain and nucleic acids were added at molar ratios of 50 bp/dye molecule for dsDNA or 50 bases/dye molecule for ssDNA and RNA. Unbound SYTOX Green stain in TE buffer solution was virtually nonfluorescent, whereas association of the dye with different types of nucleic acids, representative of those found in cells, gave rise to various degrees of fluorescence enhancement. The fluorescence emission of SYTOX Green stain was greatest in the presence of dsDNA and least with ssDNA; however, substantial enhancement above intrinsic dye fluorescence was observed in all nucleic acid solutions. No change in the shape of the fluorescence emission spectrum of SYTOX Green stain was observed with any of the nucleic acids tested.

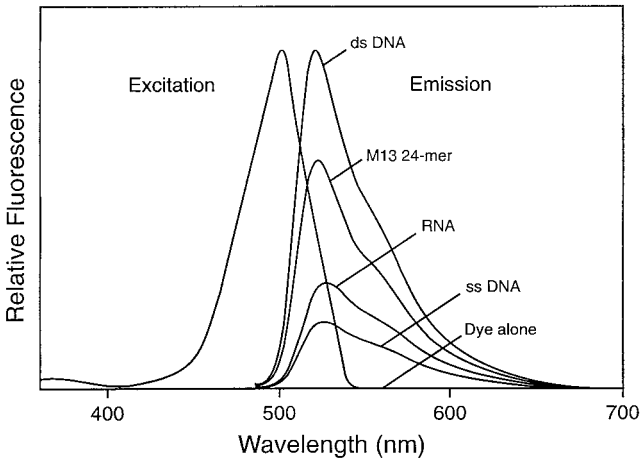

FIG. 1. Fluorescence spectra of SYTOX Green nucleic acid stain. Solutions of dsDNA, ssDNA, RNA, or a 24-base M13 DNA oligomer were mixed with SYTOX Green stain in TE to result in final molar ratios of 50 bp/dye molecule for dsDNA or 50 bases/dye molecule for ssDNA and RNA. SYTOX Green stain in the absence of nucleic acids was virtually nonfluorescent and does not appear on this plot. The single excitation spectrum represents SYTOX Green stain bound to dsDNA.

Staining of bacterial suspensions with SYTOX Green stain. Bacterial suspensions containing either 4.1×10^6 or 3.3×10^7 organisms/ml were stained with either 5 μM SYTOX Green stain or 5 μM propidium iodide (Fig. 2). The fluorescence emissions from live and isopropyl alcohol-permeabilized *E. coli* were compared with the background fluorescence of each stain alone at the optimal excitation wavelength for each nucleic acid stain (propidium iodide, 535 nm; SYTOX Green, 490 nm). At the lower cell density, the total integrated fluorescence emissions of permeabilized suspensions stained with either dye were equivalent (Fig. 2B and D). The fluorescence emission of propidium iodide in the absence of cells, however, was as great as the fluorescence emission of the live-cell suspension and nearly as large as that of the isopropyl alcohol-permeabilized cell suspension. In contrast, although the fluorescence emission of an equivalent SYTOX Green stain-labeled suspension of live cells was only slightly brighter than the signal from SYTOX Green stain without bacteria, the fluorescence emission of the suspension of permeabilized cells was more than three times greater than either signal. A marked enhancement of the fluorescence signal of SYTOX Green stain and propidium iodide was measured in suspensions with eightfold higher cell density (compare Fig. 2A and C). Suspensions of live and permeabilized bacteria labeled with SYTOX Green stain were about two and four times brighter, respectively, than those stained with propidium iodide. More importantly, the

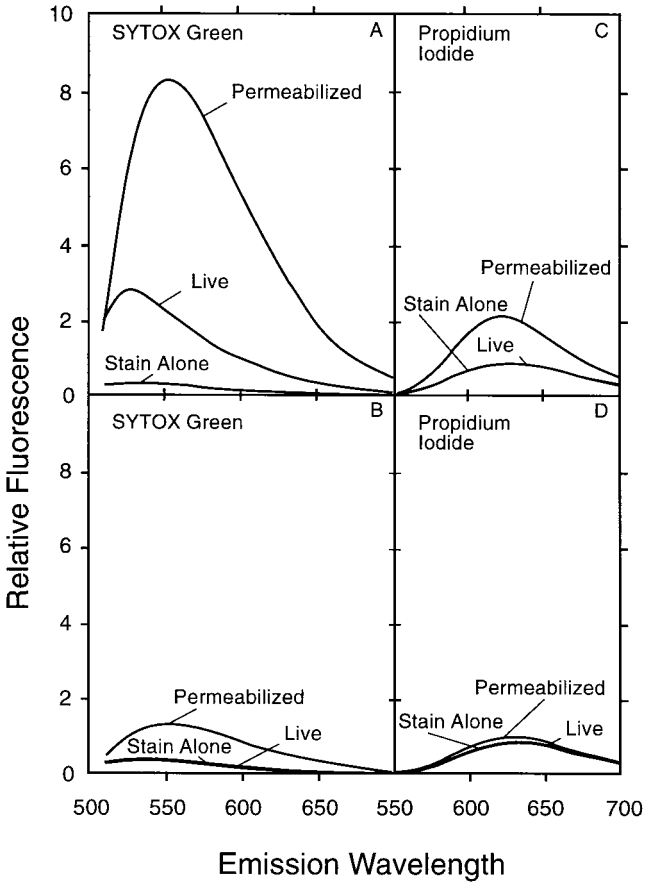

FIG. 2. Enhancement of SYTOX Green stain fluorescence in permeabilized bacteria. Suspensions of stationary-phase live or isopropyl alcohol-permeabilized *E. coli* containing 3.3×10^7 bacteria/ml (A and C) or 4.1×10^6 bacteria/ml (B and D) were stained with either 5 μM SYTOX Green stain (A and B) or with 5 μM propidium iodide (C and D) for 15 min. Fluorescence emission was measured in the fluorometer, where SYTOX Green stain was excited at 480 nm and propidium iodide was excited at 535 nm.

fluorescence emission of the stain alone comprised only about 5% of the total fluorescence measured in permeabilized bacteria labeled with SYTOX Green stain. In cells stained with propidium iodide, the background signal from the dye alone contributed about half of the total fluorescence measured.

SYTOX Green stain labeling of isopropyl alcohol-permeabilized *B. cereus*, *E. coli*, or *S. aureus* occurred rapidly (half maximal at < 30 s with 5 μM SYTOX Green stain [data not shown]). Both live and permeabilized bacteria accumulated surface-bound SYTOX Green stain that underwent very little fluorescence enhancement; however, these individual surface-labeled bacteria were typically bright enough to be detected by microscopy or flow cytometry. Similar surface binding of dye was observed in bacteria stained with propidium iodide, but the fluorescence emission was insufficient to be detected by the flow cytometer.

The large difference in fluorescence emission intensity between intact and permeabilized cells stained with SYTOX Green stain facilitates discrimination of the two populations. Since the optimal concentration of the stain is dependent on the cell density, slightly higher than optimal stain concentrations of 5 to 10 μM were used throughout these studies to avoid suboptimal loading of permeabilized bacteria.

Comparison by flow cytometry of the fluorescence emission intensity of *B. cereus*, *E. coli*, and *S. aureus*, permeabilized by heat or isopropyl alcohol treatment and labeled with either SYTOX Green stain (5 μM) or propidium iodide (5 μM), showed marked differences between the brightnesses of bacteria stained with the two dyes (Table 1). The fluorescence of permeabilized bacteria labeled with SYTOX Green stain was consistently 10- to 25-fold greater than that of bacteria stained with propidium iodide. With the exception of SYTOX Green stain-labeled *S. aureus*, the intensity of fluorescence with either stain was consistently higher in bacteria that were permeabilized by treatment with isopropyl alcohol than in those that were heat treated.

Intact and permeabilized bacteria are differentiated by fluorescence microscopy. After labeling with SYTOX Green stain or propidium iodide, individual bacteria with compromised plasma membranes were easily differentiated in the epifluorescence microscope from organisms with intact membranes. In exponentially growing and stationary-phase cultures of bacteria the majority of organisms incubated with the SYTOX Green stain exhibited low levels of green fluorescent surface labeling; however, a small fraction of the organisms were stained with bright green fluorescence. When portions of a suspension of either *B. cereus* or *E. coli* were stained with either 5 μM propidium iodide or 5 μM SYTOX Green stain, equivalent numbers of bacteria with compromised plasma membranes were labeled (data not shown), reflecting the similarity in specificity of the two dyes.

The fluorescent staining of a stationary-phase culture of *B. cereus* with 5 μM SYTOX Green stain is compared with that of 5 μM propidium iodide in Fig. 3. Photographic exposure times were varied to permit optimal visualization of *B. cereus* cells labeled with either stain and to facilitate direct comparison between the intensity and distribution of the two stains. SYTOX Green stain revealed membrane-compromised *B. cereus* with specificity equivalent to that of propidium iodide, but with greater brightness. Photomicrographs of SYTOX Green stain-labeled cells could be acquired at half the exposure time required for imaging propidium iodide-labeled cells and still appear brighter. When viewed by a combination of epifluorescence and DIC microscopy, the green fluorescence of SYTOX Green stain-labeled bacteria was observed more easily than the red fluorescence of propidium iodide-stained organisms (Fig. 3A, C, and E). This was true when the cells stained with propidium iodide were viewed with a fluorescein long-pass (Fig. 3A and B) or a rhodamine long-pass optical filter set (Fig. 3C and D).

The distribution of SYTOX Green stain-labeling of a bacterial population may be retained by fixation with glutaraldehyde prior to staining. When stationary-phase cultures of *B.*

TABLE 1. Flow cytometric analysis of the relative fluorescence intensity of permeabilized bacteria stained with 5 μM propidium iodide or SYTOX Green stain

Organism	Mean fluorescence intensity of bacteria[a]			
	Heat treated with:		Isopropyl alcohol treated with:	
	Propidium iodide	SYTOX Green	Propidium iodide	SYTOX Green
E. coli	11	273	20	519
B. cereus	35	867	83	1,201
S. aureus	28	461	40	393

[a] Fluorescence (arbitrary units) measured in the FL3 channel (propidium iodide) or in the FL1 channel (SYTOX Green stain).

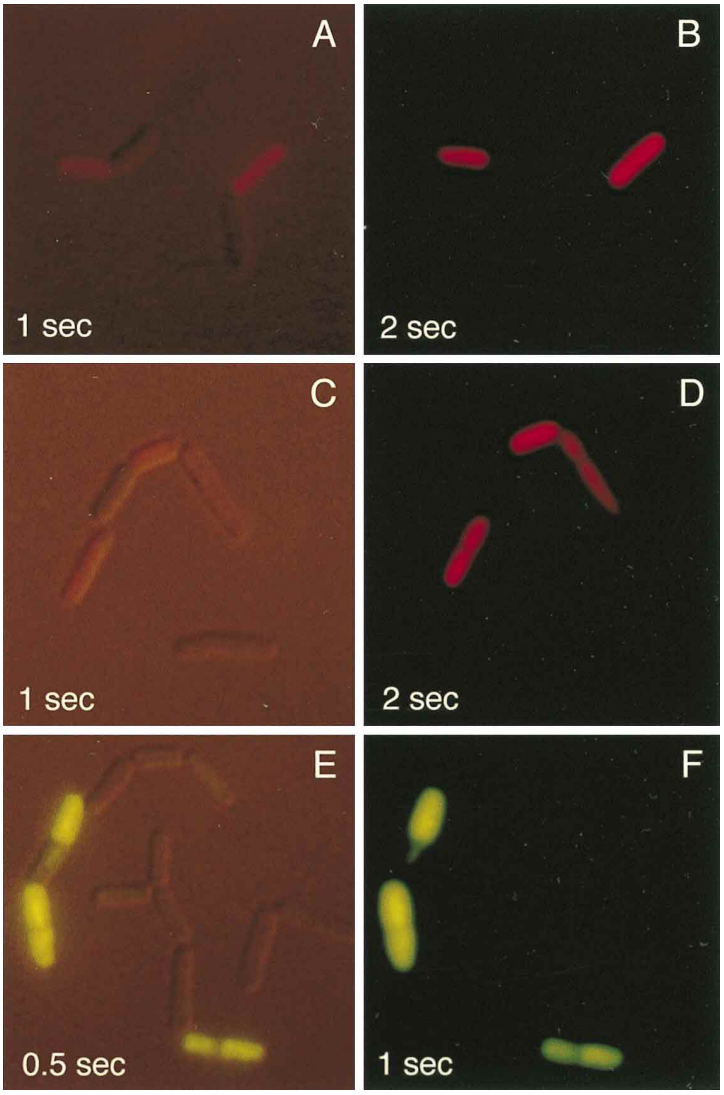

FIG. 3. Fluorescence microscopy of propidium iodide- and SYTOX Green stain-labeled bacteria. The relative brightness and staining patterns of *B. cereus* labeled with 5 μM propidium iodide (A to D) and 5 μM SYTOX Green nucleic acid stain (E and F) were compared by using an epifluorescence microscope. Photographic exposure times (indicated in each panel) were varied to permit optimal visualization of *B. cereus* cells labeled with either stain. Bacteria labeled with propidium iodide were observed by simultaneous DIC and epifluorescence microscopy (A) (excitation, 546 nm; emission, >600 nm) or by epifluorescence alone (B). The intensity of the DIC illumination was kept low to allow photography of propidium iodide staining. Propidium iodide-labeled bacteria were also observed by simultaneous DIC and fluorescence microscopy (C) (excitation, 485 nm; emission, >520 nm) or by epifluorescence alone (D). Bacteria were labeled with 5 μM SYTOX Green stain and observed by simultaneous DIC and epifluorescence microscopy (E) (excitation, 485 nm; emission, >520 nm) or by epifluorescence alone (F).

cereus or *E. coli* were fixed with 4% glutaraldehyde in water for 30 min, washed twice with water, and subsequently stained with the SYTOX Green stain, the same proportion of bacteria was brightly stained in both fixed and unfixed cultures (data not shown). Intact bacteria treated with glutaraldehyde exhibited large amounts of nonspecific fluorescence after staining with propidium iodide, making discrimination from permeabilized cells difficult.

Fluorescence intensity of *E. coli* in suspension is directly related to the proportion of permeabilized cells. Suspensions of intact and isopropyl alcohol-permeabilized bacteria were mixed in various proportions, labeled with 5 μM SYTOX Green stain, and analyzed in a fluorometer (Fig. 4, main plot). The integrated fluorescence emission from 500 to 600 nm of the suspension was proportional to the fraction of permeabilized cells in the mixture (Fig. 4, inset). When identical suspensions of cells were analyzed by flow cytometry, the fluorescence of individual bacteria from mixtures of live and permeabilized *E. coli* fell into two well-separated regions of fluorescence intensity (Fig. 5A) (R1 [high-fluorescence-intensity, permeabilized bacteria] and region 2 [R2] [low-fluorescence-intensity, live bacteria]). As in fluorometric analyses of cell suspensions (typically containing 10^6 to 10^7 organisms/ml), the proportion of permeabilized bacteria measured by flow cytometry of SYTOX Green stain-labeled bacterial populations was linearly related to the fraction of permeabilized bacteria in the sample (Fig. 5B).

Efficacy of cell wall-directed antibiotics determined by fluorescent staining. A marked increase in fluorescence of a suspension of the *E. coli* lab isolate incubated with 60 μg of ampicillin per ml and 5 μM SYTOX Green stain was observed for up to 2 h at 30°C (Fig. 6A). The change in fluorescence of

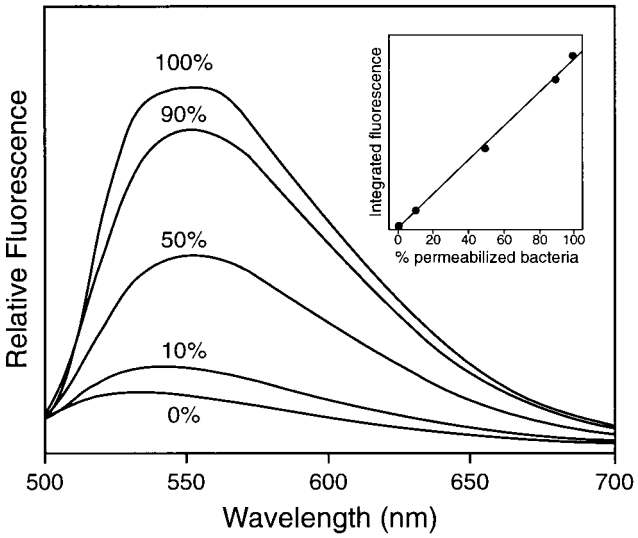

FIG. 4. The 480-nm-excited fluorescence emission spectra of various proportions of live and isopropyl alcohol-permeabilized suspensions of the *E. coli* lab isolate (10^7 bacteria/ml), labeled for 15 min with 5 μM SYTOX Green stain, were acquired in the fluorometer (main plot). (Inset) The known percentage of permeabilized bacteria in each sample was plotted against the total integrated fluorescence from 500 to 600 nm.

the bacterial suspension containing ampicillin and SYTOX Green stain was due neither to the interaction of the nucleic acid stain with ampicillin (Fig. 6B) nor to the dye's association with the nucleic acids of live bacteria in the absence of ampicillin. Exposure of *E. coli* to SYTOX Green stain alone resulted in no measurable increase in fluorescence over 2 h (Fig. 6C), suggesting that the nucleic acid stain alone did not alter the bacterial plasma membrane integrity. Slow proliferation of the bacteria in suspension under these conditions resulted in the association of a larger fraction of the available SYTOX Green stain with the surfaces of cells, but the fluorescence intensity remained unchanged because the stain was excluded from the live bacteria and did not undergo fluorescence enhancement.

Fluorescence emission spectra of SYTOX Green stain-labeled suspensions of the *E. coli* lab isolate illustrate the dose-dependent activity of several antibiotics (Fig. 7), including the β-lactams penicillin G (Fig. 7A), amoxicillin (Fig. 7B), and ampicillin (Fig. 7C), as well as the amphoteric glycopeptide vancomycin (Fig. 7D). Dose-response curves indicating the relative efficacies of the four antibiotics on the *E. coli* lab isolate (Fig. 7E) were generated by plotting the peak fluorescence intensity of the emission spectra in Fig. 7A to D against a range of antibiotic concentrations. Differences in the kinetics of the fluorescence change brought about by the β-lactam antibiotics are shown in Fig. 7A to C. The response of the bacteria to β-lactam antibiotic exposure was characterized by a relatively rapid rise in fluorescence, attributable to permeabilization and lysis of the bacteria, followed by a slow decline, which probably reflects degradation of nucleic acids released from lysed bacteria. The fluorescence of SYTOX Green stain is typically severalfold greater in the presence of completely lysed bacteria than in organisms that are permeabilized yet whole (data not shown). A marked difference was observed between the fluorescence emission of *E. coli* suspensions stained with SYTOX Green stain in the presence of β-lactam antibiotics and that of the same suspension incubated with vancomycin (Fig. 7D and E). *E. coli* suspensions exposed to vancomycin demonstrated a much smaller increase in fluorescence with concentration than did those that were incubated with any of the β-lactam antibiotics tested. Exposure to vancomycin also resulted in a marked increase in the number of brightly fluorescent bacteria observed by fluorescence microscopy but not in the total number of bacteria observed by transmitted light microscopy.

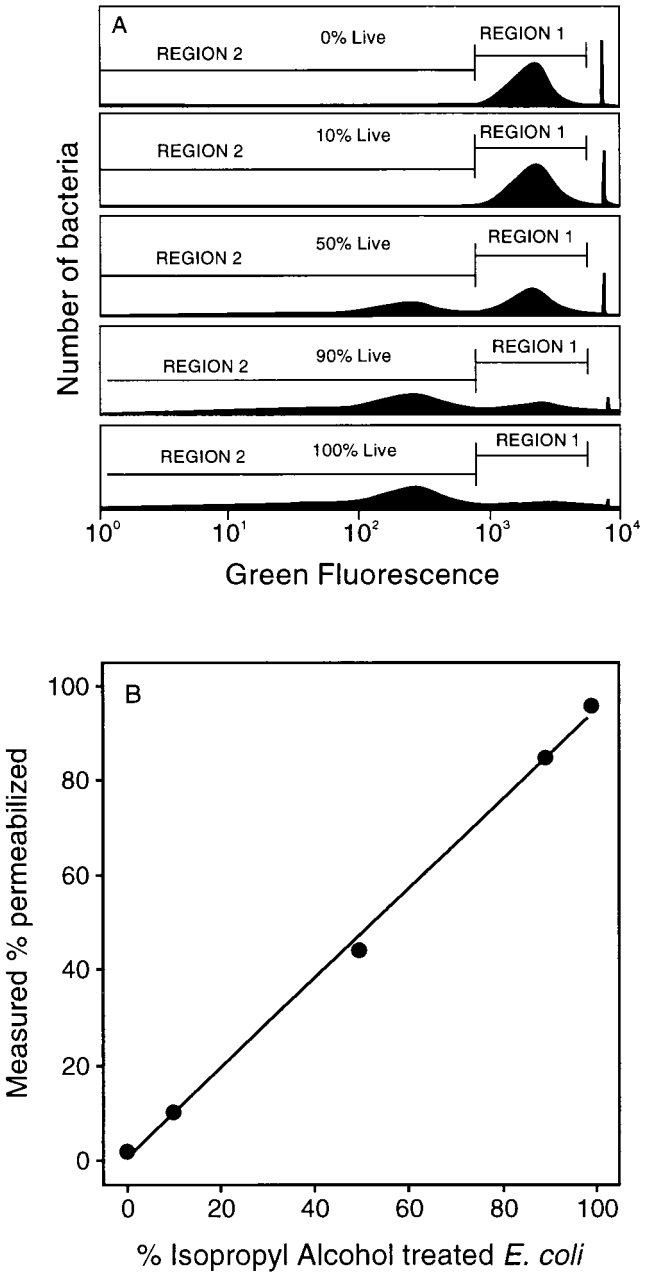

FIG. 5. Flow cytometric discrimination of intact and isopropyl alcohol-permeabilized *E. coli* lab isolate stained with SYTOX Green stain. Live and isopropyl alcohol-permeabilized suspensions of *E. coli* (10^7 bacteria/ml) were mixed in various proportions, labeled for 15 min with 5 μM SYTOX Green stain, and analyzed by flow cytometry. (A) Bacterial fluorescence fell into two well-separated regions of fluorescence intensity (region 1, high-fluorescence-intensity, permeabilized bacteria; region 2, low-fluorescence-intensity, live bacteria). (B) Proportion of permeabilized bacteria (region 1) indicated by flow cytometry of SYTOX Green stain-labeled bacteria plotted against the known percentage of isopropyl alcohol-permeabilized bacteria.

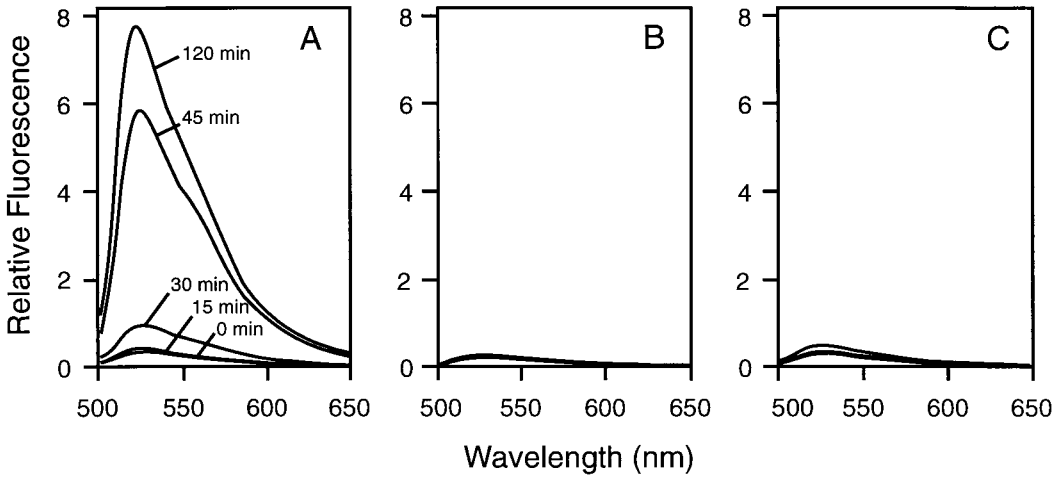

FIG. 6. Fluorometric measurement of SYTOX Green stain labeling of ampicillin-treated *E. coli*. Suspensions of *E. coli* lab isolate (10^7 bacteria/ml) were incubated either with 60 μg of ampicillin per ml and 5 μM SYTOX Green stain (A) or with 5 μM SYTOX Green stain alone (C). (B) In order to monitor potential interaction of the compounds, 60 μg of ampicillin per ml and 5 μM SYTOX Green stain were incubated together without bacteria. All combinations were incubated at 30°C for 2 h, and 480-nm-excited fluorescence emission spectra were acquired at 0, 15, 30, 45, and 120 min.

The relative sensitivities to ampicillin of an ampicillin-resistant (Amp^r) strain (ATCC 35218) and an ampicillin-sensitive (Amp^s) strain (ATCC 25922) of *E. coli* were also characterized fluorometrically with the SYTOX Green stain. Plots of peak fluorescence intensity values over a range of antibiotic concentrations show a much larger fluorescence signal in Amp^s *E. coli*, clearly illustrating the difference in sensitivity to ampicillin of the two strains (Fig. 8).

Ampicillin sensitivity quantified by flow cytometry. Ampicillin susceptibility was evaluated by flow cytometry of SYTOX Green stain-labeled Amp^r (Fig. 9A to E) and Amp^s (Fig. 9F to J) *E. coli*. Both strains of *E. coli* were incubated at 37°C in dilute medium (5% TSB) containing 10^6 unstained 6-μm polystyrene beads/ml and 5 μM SYTOX Green stain in the presence or absence of 60 μg of ampicillin per ml. The intensity of cell-associated SYTOX Green stain fluorescence emission was measured at 30-min intervals for 2 h. Data sampled from four regions of the plots of log green fluorescence versus forward scatter were subsequently used to determine the sample volume, the bacterial density, and the mean fluorescence intensity of the bacteria. R1 encompasses 6-μm underivatized polystyrene beads, which were included in all of the samples for volume calibration. The beads were easily differentiated from bacteria by their large forward scatter, which is indicative of their large size, and their disproportionately low fluorescence. R2 represents live, intact bacteria with low fluorescence and intermediate forward scatter. Region 3 (R3) includes particles with bright fluorescence and intermediate forward scatter, most likely indicative of bacteria that have partially disrupted plasma membranes but have not yet lysed completely. Region 4 (R4) includes both bacteria with a high fluorescence signal and diminished forward scatter and fragments of disrupted cells with low fluorescence and low forward scatter. The mean fluorescence signal associated with Amp^r *E. coli* increased slightly during 2 h of exposure to 60 μg of ampicillin per ml (Fig. 9A to E) but remained within the limits of R2. No change in the numbers of bacterial particles in R3 and R4 was observed. Only 3% of the suspension of Amp^s *E. coli* exposed to ampicillin underwent a profound increase in fluorescence within 60 min (Fig. 9F to H); however, continued incubation resulted in a shift of the population from R2 to R3 and eventually to R4 (Fig. 9H to J). About 33% of the Amp^s bacteria were found in R2 after 2 h. Changes that occurred in the bacterial population paralleled those that were observed in the fluorometer and in the fluorescence microscope. In the flow cytometer, an increase in the number of Amp^s *E. coli* bacteria in R4 was observed at later time points, most likely reflecting the degradation of the bacterial cell walls and partial or complete cellular lysis. This progression was not observed in Amp^s cells in the absence of ampicillin (data not shown).

The number of CFU in the bacterial suspensions was determined by standard plate counting in parallel with flow cytometry. The relative sensitivities to ampicillin of the Amp^r and Amp^s strains of *E. coli*, determined by flow cytometry, were consistent with the colony counts. The number of viable organisms estimated by plate counting was, however, consistently lower than that determined by flow cytometry. For example, in Amp^r *E. coli* at time zero (Fig. 9A), a population found to have 4.9×10^6 CFU/ml by plate count contained 9.1×10^6 particles/ml that fell into R2, indicating the presence of a substantial number of apparently intact, yet nonculturable, organisms. Exposure of Amp^s bacteria to ampicillin brought about a precipitous drop in the number of CFU per milliliter after only 1 h, whereas by flow cytometry the fraction of cells in R2 (live) decreased much less rapidly (Fig. 10). In the 5% TSB without ampicillin, both the Amp^r and Amp^s bacterial cell numbers increased about twofold during the 2-h experiment. To correct for cell growth, the fraction of particle counts remaining in the live region (R2), measured by flow cytometry, is expressed according to the ratio R2/(R2 + R3 + R4) (Fig. 10, method 2). Use of the expression $R2/R2_{initial}$ (Fig. 10, method 1), by not taking into account the populations of cells in R3 and R4, did not correct for cell growth. Calculation method 1 did, however, most closely approximate the plate count data, which was plotted as $(CFU/ml)/(CFU/ml_{initial})$ because only culturable organisms are enumerated by this approach.

DISCUSSION

Development of improved fluorescence methods for detection and evaluation of viability in bacteria has focused to a large extent on the nucleic acid stains. The SYTOX Green stain described here was shown to be effective for measuring the exclusion of compounds that normally do not penetrate the

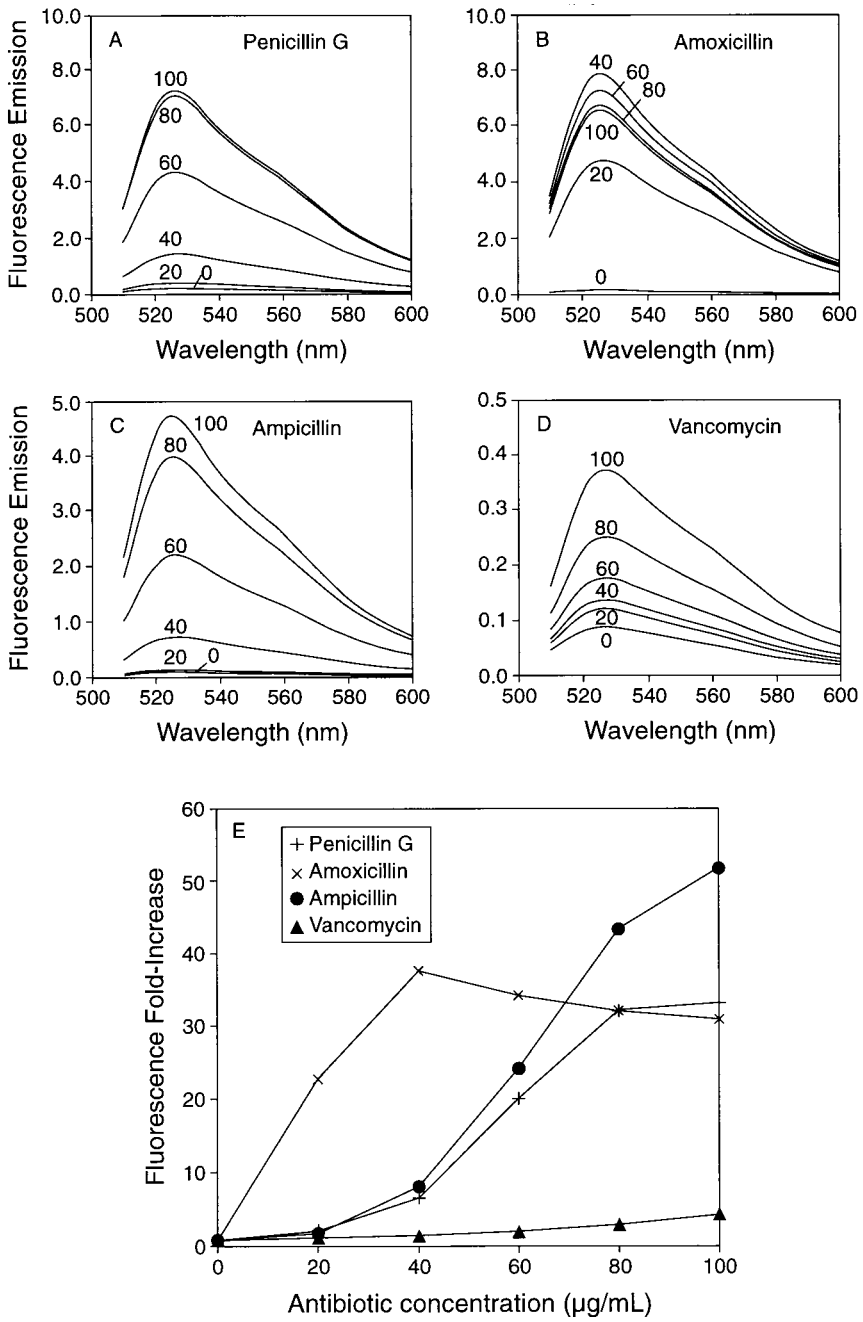

FIG. 7. Fluorometric determination of the relative efficacy of several cell wall-directed antibiotics in suspensions of *E. coli* measured with SYTOX Green stain. Suspensions of an *E. coli* lab isolate (10^7 bacteria/ml) were incubated with 0 to 100 μg of either penicillin G (A), amoxicillin (B), ampicillin (C), or vancomycin (D) per ml in combination with 5 μM SYTOX Green stain in 5% TSB. Fluorescence emission spectra of each sample were acquired after incubation at 30°C for 2 h. The ordinate scales of panels A and B are different from those of panels C and D even though the data were acquired under identical conditions. (E) The peak fluorescence values from each of the emission spectra in panels A to D are plotted against the range of antibiotic concentrations tested.

plasma membrane of bacteria. Staining of bacteria with this compound was analyzed by microscopy, fluorometry, and flow cytometry.

When the plasma membrane barrier of bacteria is compromised, compounds that are typically excluded from the cytoplasm because of size or charge, e.g., SYTOX Green stain, freely enter the cells. Nucleic acids within permeabilized bacteria are subsequently labeled by SYTOX Green stain, giving rise to intense green fluorescence. Surface staining by SYTOX Green stain was typically 10-fold less bright than internal nucleic acid labeling, but it was sufficient to permit detection of intact bacteria by single-cell techniques such as microscopy or flow cytometry. Reproducible separation of live and isopropyl alcohol-permeabilized *B. cereus*, *E. coli*, and *S. aureus* was obtained with between 0.1 and 10.0 μM SYTOX Green stain, depending on the total number of cells and the proportion of permeabilized cells in the population. The concentration of SYTOX Green stain could be varied considerably without af-

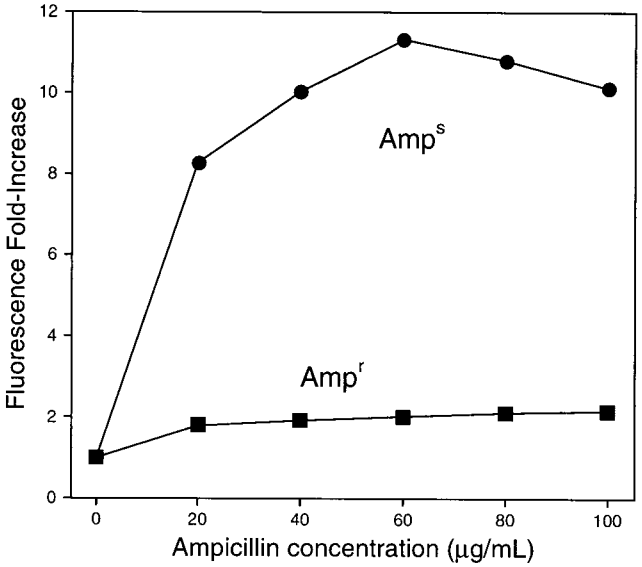

FIG. 8. Fluorometric determination of the relative sensitivity of suspensions of Amp^r and Amp^s *E. coli* strains to ampicillin with SYTOX Green stain. Suspensions (10^7 bacteria/ml) were incubated with 0 to 100 μg of ampicillin per ml at 30°C for 2 h in the presence of the 5 μM SYTOX Green stain in 5% TSB. The fluorescence emission spectrum of each 480-nm-excited sample was acquired following the incubation period, and plots of peak fluorescence intensity values for each spectrum over the range of ampicillin concentrations from 0 to 100 μg/ml are shown.

fecting cell staining intensity; however, insufficient labeling resulted in heterogeneous staining of cell populations. Labeling with excessive concentrations of stain resulted in somewhat reduced cell brightness and in small shifts in the fluorescence emission spectrum (data not shown).

A large quantum yield, large extinction coefficient, 488-nm argon ion laser excitability, low membrane permeability, large fluorescence enhancement with nucleic acid binding, and low but measurable nonspecific binding are properties that contribute to the utility of the SYTOX Green stain. The stain is not toxic to growing bacteria, brightly stains the majority of cells with compromised plasma membranes, and is excluded from bacteria with intact plasma membranes. The SYTOX Green stain is most directly comparable in its range of potential uses to propidium iodide but has distinct advantages for applications involving single cells and cell suspensions. Under conditions that were optimal for staining with propidium iodide, the distributions of live and membrane-compromised bacteria separated by flow cytometry were virtually identical in populations of bacteria labeled with either SYTOX Green stain or propidium iodide. The fluorescence intensity of bacteria stained with SYTOX Green stain was severalfold greater than that of propidium iodide-stained cells, allowing the SYTOX Green stain-labeled bacteria to be measured with greater ease and accuracy. The difference between the fluorescence intensities of bacteria stained with propidium iodide and with SYTOX Green stain is attributable to both the molar extinction coefficients (propidium iodide, ~6,000 cm^{-1} M^{-1}; SYTOX Green stain, ~73,000 cm^{-1} M^{-1}) and the quantum yields (propidium iodide, ~0.2; SYTOX Green stain, ~0.5) of the two stains bound to dsDNA. The peak and integrated fluorescence intensities of permeabilized bacteria stained with propidium iodide were consistently lower than those of bacteria labeled with an equivalent concentration of the SYTOX Green stain. Greater brightness allows discrimination of smaller organisms and requires less sensitive instrumentation.

In the fluorescence microscope SYTOX Green stain-labeled *B. cereus* was much brighter and more easily photographed than bacteria stained with propidium iodide. The intense fluorescence of SYTOX Green stain was especially useful for enumeration of the proportion of permeabilized bacteria in a population by using DIC in combination with epifluorescence microscopy. Increased stain brightness also made possible the detection of individual bacteria with low-magnification-objective lenses, vastly reducing the time required to scan samples for stained cells.

The relatively small enhancement of propidium iodide fluorescence brought about by nucleic acid binding renders it less sensitive than SYTOX Green stain for fluorometric viability determination of bacteria in suspension. The fluorescence of bacterial suspensions stained with propidium iodide can be related to both the concentration of the dye and the density of the bacterial suspension, but a large fraction of the fluorescence emitted originates from free dye in solution. At low cell densities an error of a few percent in either cell or background fluorescence can have a profound influence on assays based on propidium iodide. In contrast, unbound SYTOX Green stain is virtually nonfluorescent and its fluorescence in cell suspensions is directly proportional to the number of permeabilized cells. With SYTOX Green stain, errors of several percent in either measurement of cell fluorescence or background fluorescence will have little effect on the outcome of the assay.

The action of chemical or physical treatments that disrupt the bacterial cell wall and subsequently the plasma membrane can be quantified with SYTOX Green stain. Cell permeabilization and disruption resulting from exposure to ampicillin were measured with SYTOX Green stain, and the stain itself did not promote cell lysis (Fig. 6) or inhibit bacterial growth in liquid or solid growth medium (data not shown). Labeling with SYTOX Green stain provides a rapid, simple alternative method for testing of susceptibility of microorganisms to certain types of antimicrobial compounds.

The fluorescence emission of suspensions of permeabilized, but not lysed, bacteria may be substantially lower than the fluorescence of suspensions of completely lysed bacteria under identical conditions. This difference is likely to be due to increased binding of free dye molecules to the nucleic acids released from the lysed cells. For example, a much smaller increase in fluorescence was observed in *E. coli* suspensions exposed to vancomycin than with the β-lactam antibiotics. The fluorometric measurements alone appear to indicate differential sensitivity of the bacteria to the two types of antibiotics. Exposure to vancomycin increased the number of brightly stained bacteria seen in the epifluorescence microscope, but the total number of bacteria in the sample was unaffected. This indicates that the bacteria were permeabilized but not fully lysed. Furthermore, the fraction of bacteria that were fluorescence labeled with SYTOX Green stain closely paralleled the concentration of vancomycin. Both the β-lactam antibiotics and vancomycin rendered the lab isolate of *E. coli* permeable to the SYTOX Green stain, but a much larger fluorescence signal was obtained in the fluorometer from suspensions in which a substantial fraction of the bacteria were completely lysed. These results stress the idea that fluorescence data acquired with nucleic acid stains such as SYTOX Green stain should be evaluated carefully to avoid misinterpretation.

Flow cytometric analysis of SYTOX Green stain fluorescence in Amp^s *E. coli* exposed to ampicillin clearly showed the time-dependent progression of bacterial permeabilization and lysis. Initially, the fluorescence of a large fraction of cells in-

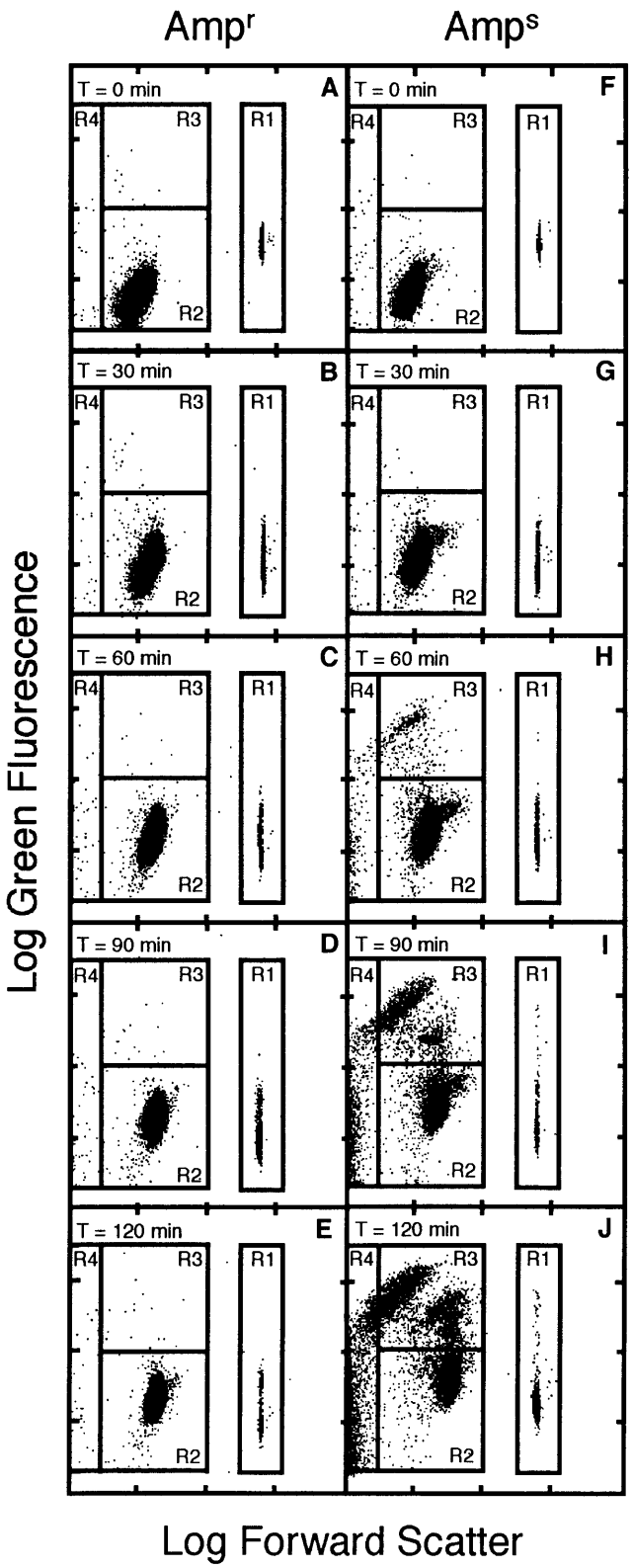

FIG. 9. The relative ampicillin sensitivities of Amp^r (A to E) and Amp^s (F to J) *E. coli* strains were determined by flow cytometry. The bacteria in the experiment were incubated in 5% TSB for 2 h at 37°C in the presence of 10^6 beads/ml and 5 μM SYTOX Green stain with or without 60 μg of ampicillin per ml. Sampling regions of the two-parameter dot plot of green fluorescence versus forward scatter are designated R1, R2, R3, and R4. R1 encompasses only 6-μm unlabeled polystyrene microspheres used for volume calibration; R2 includes cells with low fluorescence and normal forward scatter (live and intact); R3 includes cells with high fluorescence and normal forward scatter (permeabilized yet whole); R4 includes both cells with high fluorescence signal and diminishing forward scatter and cells with low fluorescence and minimal forward scatter (lysed cells and cell debris containing small amounts of bound nucleic acids).

creased nearly 10-fold, followed by the appearance of bacteria that were completely lysed, as suggested by low forward scatter and a range of fluorescence intensities. The loss of intact bacteria suggests that a decrease in the fraction of viable bacteria that results from antibiotic treatment is reflected more accurately by the reduction in numbers of organisms with low fluorescence and normal forward scatter than by an increase in the number of brightly stained bacteria. Even though the efficacy of the antibiotics should be readily apparent from both fluorometry of cell suspensions and cell-by-cell measurements in the flow cytometer, the latter approach provides a more accurate comparison of bacterial sensitivity to antibiotics with different modes of action.

Accurate assessment of the true density of organisms in a sample before and during a treatment is critical. If the bacterial density is not known, a small subpopulation of antibiotic-resistant bacteria present in the suspension may be represented disproportionately once sensitive cells have lysed. The actual number of bacteria in the sample is much more difficult to determine if many cells have undergone lysis because cell fragments may appear as cells in the flow cytometer.

When *E. coli* was stained with SYTOX Green stain and counted in the flow cytometer, the number of particle counts, which is assumed to represent bacterial cells, always exceeded the number of viable bacteria determined by the spread plate technique. Prior to ampicillin treatment, 9.1×10^6 live (faintly fluorescent) bacteria/ml were detected by flow cytometry of the SYTOX Green-stained suspension and 4.9×10^6 CFU/ml were found by plate count; therefore, 4.2×10^6 particles/ml excluded the SYTOX Green stain but did not give rise to colonies on nutrient agar medium. It was not determined whether these particles were intact bacteria or simply debris. However, if the dim fluorescence emitted by these particles indicated that they were organisms with intact plasma membranes, then membrane integrity was not an accurate indicator of growth potential in this system.

Plate counting represents only organisms that are capable of division, whereas cells assumed to be viable because of their ability to exclude SYTOX Green stain could be incapable of reproduction for any one of a variety of reasons. Conversely, some organisms may be unable to proliferate but remain intact and resistant to labeling with stains that do not penetrate intact plasma membranes. Plate counting will not detect these living but nonculturable organisms, whereas labeling with SYTOX Green stain allows detection of intact cells that are incapable of division, fragments of bacteria lacking nucleic acids, and other debris that will not give rise to colonies on solid nutrient media.

Use of the expression $R2/R2_{initial}$ to indicate the proportion of live cells present, by not taking into account the additional cells in R3 and R4, did not correct for cell growth. To best correct for this growth, the fraction of particle counts remaining in the live region (R2), measured by flow cytometry, was expressed according to the ratio R2/(R2 + R3 + R4). This method most closely approximated standard plate counts, which were represented as $(CFU/ml)/(CFU/ml_{initial})$ because only the number of culturable organisms was measured.

For many routine procedures, such as antibiotic sensitivity screening, it may not be critical for new procedures to yield

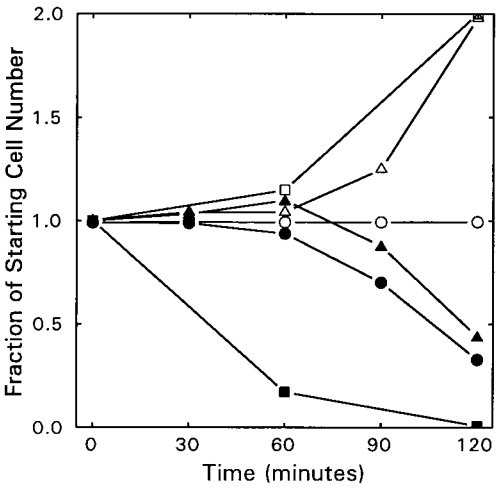

FIG. 10. Comparison of methods for evaluating antibiotic sensitivity of Amp^s and Amp^r strains of *E. coli*. Flow cytometric analysis of SYTOX Green stain fluorescence in *E. coli* exposed to 60 μg of ampicillin per ml (from Fig. 9) was calculated as follows: (i) $R2/R2_{initial}$ (closed triangles, Amp^s; open triangles, Amp^r) and (ii) R2/(R2 + R3 + R4) (closed circles, Amp^s; open circles, Amp^r). Bacteria from samples of the suspensions used for flow cytometry were enumerated by the spread plate technique and are expressed as (CFU/ml)/(CFU/$ml_{initial}$) (closed squares, Amp^s; open squares, Amp^r).

data that are identical to those acquired with conventional techniques. The tests should reliably report the efficacy of the antimicrobial agent on a particular bacterial strain relative to the appropriate sensitive and resistant control organisms. Since each method uses a very different set of criteria to assess viability, each method must be evaluated independently. These results underscore the need to define experimental parameters carefully when comparing bacterial viability, as defined by colony formation or metabolic activity, with the exclusion of dyes from intact cells.

The number and variety of fluorescence-based methods available to microbiologists for assessing the activities of bacteria are growing rapidly. This study demonstrates the use of SYTOX Green nucleic acid stain, a potentially important tool that can be used to assess bacterial membrane integrity with improved sensitivity and accuracy with a wide range of instrumentation. This new stain should be ideal for numerous and diverse applications such as high-throughput screening of antimicrobial compounds or monitoring of microbial contamination by automated imaging microscopy. SYTOX Green stain can also be combined with other spectrally separated fluorescent stains to create a variety of differential and ratiometric tests for prokaryotic and eukaryotic cells.

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