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Assessment and Interpretation of Bacterial Viability by Using the LIVE/DEAD BacLight Kit in Combination with Flow Cytometry▽

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The commercially available LIVE/DEAD BacLight kit is enjoying increased popularity among researchers in various fields of microbiology. Its use in combination with flow cytometry brought up new questions about how to interpret LIVE/DEAD staining results. Intermediate states, normally difficult to detect with epifluorescence microscopy, are a common phenomenon when the assay is used in flow cytometry and still lack rationale. It is shown here that the application of propidium iodide in combination with a green fluorescent total nucleic acid stain on UVA-irradiated cells of *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, *Shigella flexneri*, and a community of freshwater bacteria resulted in a clear and distinctive flow cytometric staining pattern. In the gram-negative bacterium *E. coli* as well as in the two enteric pathogens, the pattern can be related to the presence of intermediate cellular states characterized by the degree of damage afflicted specifically on the bacterial outer membrane. This hypothesis is supported by the fact that EDTA-treated nonirradiated cells exhibit the same staining properties. On the contrary, this pattern was not observed in gram-positive *Enterococcus faecalis*, which lacks an outer membrane. Our observations add a new aspect to the LIVE/DEAD stain, which so far was believed to be dependent only on cytoplasmic membrane permeability.

The death of a bacterial cell has long been defined as the inability of a cell to grow to a visible colony on bacteriological media. However, with traditional culture methods, one can observe bacterial death only in retrospect (28). Intermediate states like cell injury are difficult to detect with the plating method. Nevertheless, there are several viability indicators that can be assessed at the single-cell level without culturing cells. These indicators are based mostly on fluorescent molecules, which can be detected with epifluorescence microscopy, solid state cytometry, or flow cytometry. Each indicator is based on criteria that reflect different levels of cellular integrity or functionality. Over the last 20 years, multiparameter flow cytometry has become a powerful tool in microbiology, particularly in biotechnological processing, food preservation, and chemical disinfection processes (3, 13, 18, 26, 27), because it is fast and allows single-cell analysis.

The commercially available LIVE/DEAD BacLight kit (Invitrogen) has enjoyed increasing popularity among researchers in various fields since it was released about 10 years ago (6). The kit consists of two stains, propidium iodide (PI) and SYTO9, which both stain nucleic acids. Green fluorescing SYTO9 is able to enter all cells and is used for assessing total cell counts, whereas red fluorescing PI enters only cells with damaged cytoplasmic membranes. The emission properties of the stain mixture bound to DNA change due to the displacement of one stain by the other and quenching by fluorescence resonance energy transfer (31). LIVE/DEAD staining was shown to work not only with (eu)bacteria (6) but also with archaea (21) or eukaryotic cells, such as yeast (*Saccharomyces cerevisiae*) (36). Although this kit enables differentiation only between bacteria with intact and damaged cytoplasmic membranes, it is often used to differentiate between active and dead cells (12, 29). While it seems accurate to assume that membrane-compromised bacterial cells can be considered dead (3, 26), the reverse (that intact cells are active cells) is not necessarily true (18). Microscopic assessment of LIVE/DEAD-stained bacterial cells is usually simplified to either "green"-labeled (live) or "red"-labeled (dead) cells. However, experience with this dye combination and flow cytometry during the last few years by our group and others has shown that the staining of bacterial cells with SYTO9 and PI does not always produce distinct "live" and "dead" populations; intermediate states are also observed (2, 3, 10, 16, 18, 32). In the kit manufacturer's manual, the region of intermediate states is referred to as "unknown" (http://probes.invitrogen.com/media/pis/mp34856.pdf). This can lead to difficulties in the interpretation of results and can be critical when, for example, decisions have to be made about the effectiveness of disinfection methods or the amount of viable bacteria in water distribution systems. Nevertheless, to our knowledge the nature of these intermediate states has not been fully clarified. In the present study, we detected such an intermediate state and linked it to the physiological properties of the bacterial cells. The measurements were conducted with defined pure cultures of gram-negative *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, and *Shigella flexneri*; gram-positive *Enterococcus faecalis*; and for comparison, a community of freshwater bacteria.

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TABLE 1. Relative increase of the geometric mean of green fluorescence intensity (FL1) for UVA-irradiated or EDTA-treated cells

Bacterial strain	Fold green fluorescence increase[a]	
	UVA irradiated	EDTA treated
E. coli	4.4 ± 0.7	4.9 ± 0.3
Salmonella serovar Typhimurium	5.7 ± 0.8	5.0 ± 0.5
S. flexneri	6.6 ± 0.5	6.0 ± 0.8
E. faecalis	ND	ND

[a] Results are shown as GMn_x ± standard deviations and are displayed for bacterial cells going from unstressed to intermediate state (SYTO9-PI staining applied). The effects of UVA irradiation and EDTA treatment are compared. Standard deviations were calculated from three biologically independent experiments. ND, no increase in green fluorescence intensity was detected.

MATERIALS AND METHODS

Bacterial strains. The following strains were used in this study: *Escherichia coli* K-12 MG1655 (ATCC 700926), *Salmonella* serovar Typhimurium ATCC 14028, *Shigella flexneri* ATCC 12022, and *Enterococcus faecalis* ATCC 29212. A community of freshwater bacteria was sampled from the Glatt River (Dübendorf, Switzerland). The river flows out of Lake Greifensee (Uster, Switzerland) and had the following basic composition: pH 8.6 to 8.7; dissolved organic carbon, 3.9 to 4.0 mg/liter; alkalinity, 2.6 mmol/liter; and total bacterial cell concentration, 1.5×10^6 cells/ml. The water was sampled into sterilized glass bottles, kept on ice, and analyzed within 2 h after sampling.

Growth media and cultivation conditions. (i) Pure cultures. Luria-Bertani (LB) broth (10 g tryptone, 5 g yeast extract, and 10 g NaCl per liter) that was filtered with membrane filters (Millex-GP, 0.22 μm; Millipore, Tullagreen, Ireland) and diluted to 33% vol/vol of its original strength (unless indicated otherwise) with ultrapure water (deionized and activated carbon treated) was used for batch cultivation (25). Precultures were prepared for each individual batch experiment from the same stock culture stored at −80°C by streaking the stock solution onto LB agar plates. After 15 to 18 h of incubation at 37°C, one colony was picked, loop inoculated into a 125-ml Erlenmeyer flask containing 20 ml of diluted LB broth, and incubated at 37°C on a rotary shaker at (200 rpm). At an optical density at 546 nm (OD_{546}) of between 0.1 and 0.2 (measured spectrophotometrically in glass cuvettes with a 1-cm light path using a Jasco V550 UV/VIS spectrophotometer; Jasco, Tokyo, Japan), an aliquot of the culture was transferred into 500-ml Erlenmeyer flasks containing 50 ml of prewarmed LB broth to obtain an OD_{546} of 0.002. In this way, no lag phase was observed. These flasks were then shaken at 200 rpm in a temperature-controlled water bath (SBK 25D; Salvis AG, Reussbühl, Switzerland) at 37°C for about 18 h until stationary phase (the specific growth rate [μ] was 0 h^{-1}) was reached. The specific growth rate μ was calculated from five consecutive OD_{546} measurements.

(ii) Community of freshwater bacteria. Before flow cytometric analysis, the river water was filtered through an 8-μm filter to separate bacteria from bigger particles.

Sample preparation and exposure. (i) Pure cultures. Cells were harvested by centrifugation from batch culture (at 13,000 × *g*; Biofuge fresco; Kendro, Zürich, Switzerland), washed three times with filtered (Nuclepore Track-Etch Membrane, 0.22 μm; Sterico AG, Dietikon, Switzerland) commercially available bottled water (Evian, France), and diluted to an OD_{546} of approximately 0.01 (corresponding to 1×10^7 to 5×10^7 cells/ml). For inactivation, samples of 10 ml of bacterial suspension (see above) were exposed to UVA light in 30-ml quartz tubes placed in a carousel reactor (adapted from reference 35) equipped with a Hanau TQ 150 or Hanau TQ 714 Z4 medium-pressure mercury lamp, which was operated at 150 or 700 W, respectively (for wavelength spectra, see reference 4). The lamp with the higher intensity was used for irradiation of *S. flexneri* and *Salmonella* serovar Typhimurium. The lamp was placed in a cooling jacket (Duran 50 borosilicate glass) in the center of the carousel reactor. The light emitted from the lamp passed through the glass jacket and through 35 mm of filter solution before reaching the cells in the quartz tubes. The temperature of the filter solution was maintained at 37°C, and it consisted of 12.75 g/liter sodium nitrate with a cutoff of 320 nm and a half maximum of 340 nm. The transmission property of the filter solution was measured before each experiment. Chemical actinometry with *p*-nitroanisole–pyridine was used to determine the fluence rate at the tube position (35). Bacterial solutions were mixed intermittently with a magnetic stirrer. At each time point, one tube was withdrawn and its contents

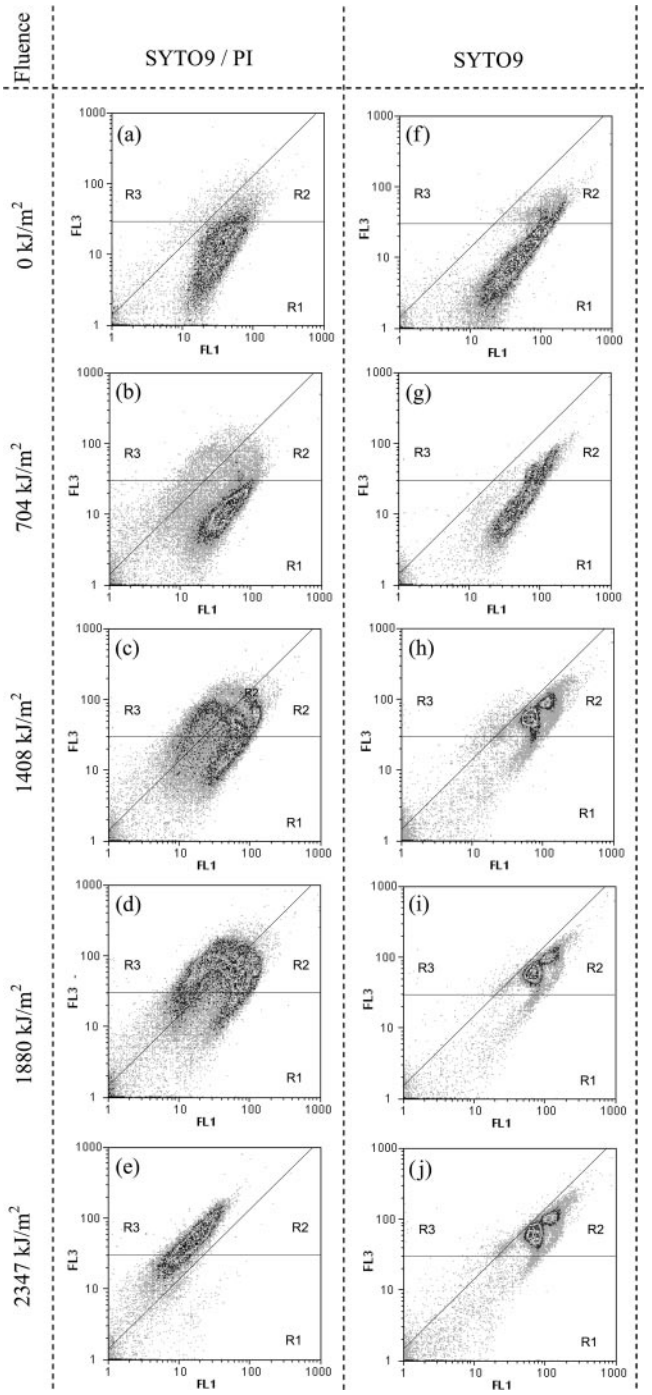

FIG. 1. Flow cytometric analysis of *E. coli* K-12 MG1655 irradiated with artificial UVA light. Bacterial cells were harvested from a stationary-phase LB batch culture, washed, and diluted in mineral water (Evian). After exposure to different fluence levels (irradiation intensity × time) bacterial cell samples were stained either with a mixture of SYTO9 plus PI or with SYTO9 only and analyzed on a flow cytometer. After a fluence level of 1,408 kJ/m^2 was reached, staining with SYTO9 and PI showed intermediate states (c), and both the green and red fluorescence intensity of SYTO9 increased significantly (h). After a fluence level of 2,400 kJ/m^2 was reached, all cells were PI positive (e). FL1, 520 ± 10 nm; FL3, ≥630 nm.

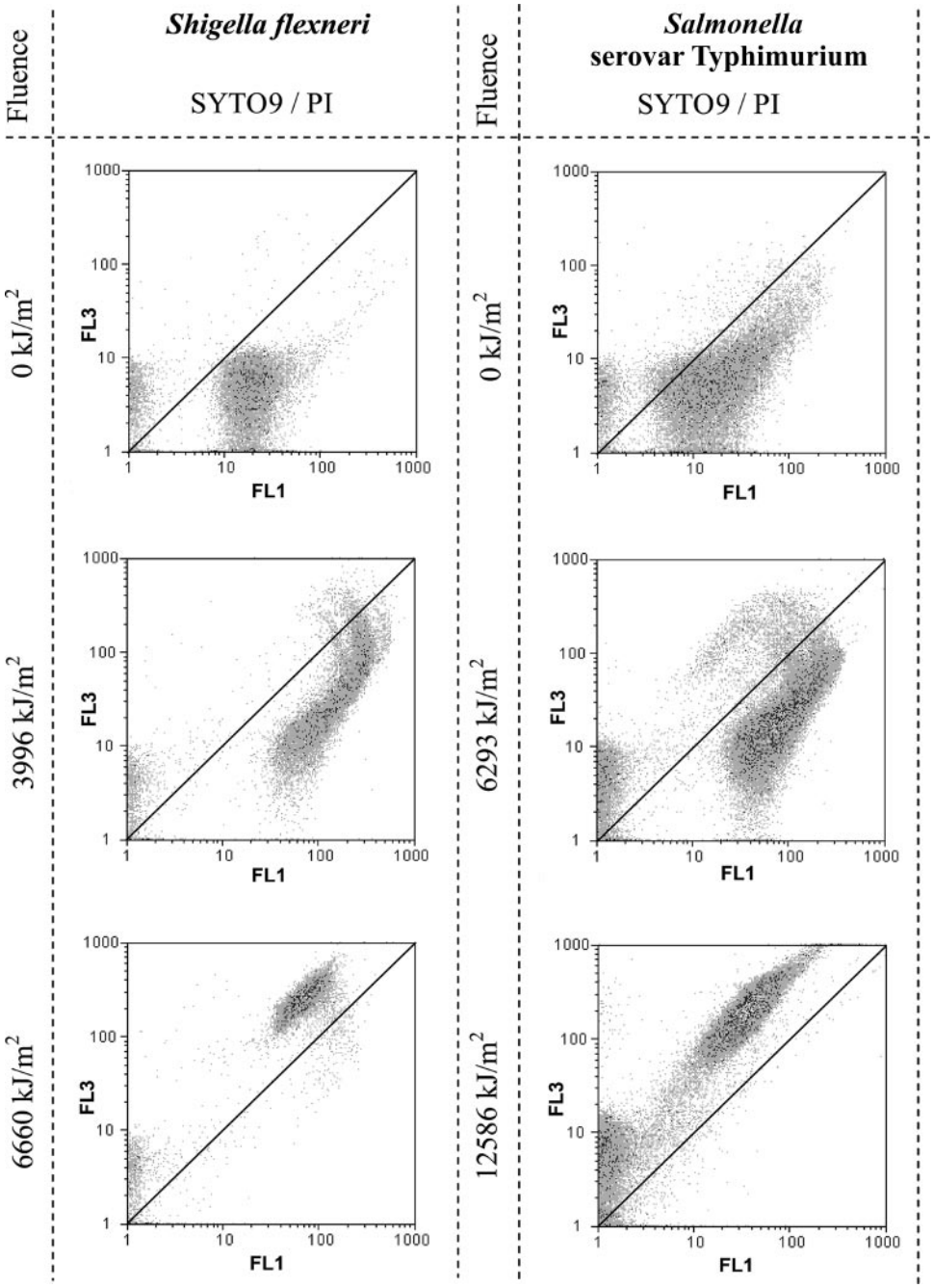

FIG. 2. Flow cytometric analysis of *Salmonella* serovar Typhimurium and *S. flexneri* irradiated with artificial UVA light. Bacterial cells were harvested from a stationary-phase LB batch culture, washed, and diluted in mineral water (Evian). After exposure to different fluence levels (irradiation intensity × time) bacterial cell samples were stained with a mixture of SYTO9 plus PI and analyzed on a flow cytometer (FL1, 520 ± 10 nm; FL3, ≥630 nm).

were processed immediately as described below. As a control for membrane-permeabilized cells, a sample was exposed to 70°C for 3 min and analyzed on the flow cytometer as described below. All samples were stored at room temperature and reanalyzed after 48 h.

(ii) Community of freshwater bacteria. Samples (10 ml) of the community of freshwater bacteria were exposed to UVA light as described above but with a stronger lamp that emits higher wavelength intensities (model no. TQ 718 Z4 operated at 500 W [4]). At different time points, a sample was withdrawn and immediately processed as described below. As a control for membrane-permeabilized cells, a sample was exposed to 90°C for 3 min and analyzed on the flow cytometer as described below. The light intensity was determined by actinometry as described above.

Staining procedure. Three fluorescent dyes were either used alone or used in combination: SYTO9 (Invitrogen AG, Basel, Switzerland), SYBR green (Invitrogen), and PI (Invitrogen). Stock solutions of the dyes were prepared as follows: PI and SYTO9 were used from the LIVE/DEAD BacLight kit (Invitrogen) as proposed by the manufacturer, and SYBR green (10^4 times concentrated in dimethyl sulfoxide [DMSO]) was diluted 100 times in 0.22-μm-filtered DMSO. All stock solutions were stored at −20°C. The molar concentration of SYBR green was not supplied by the manufacturer. The final SYBR green concentra-

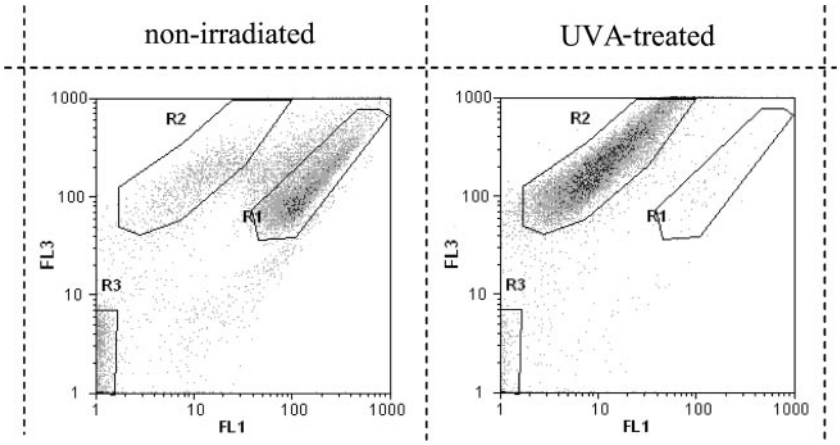

FIG. 3. Flow cytometric analysis of *E. faecalis* irradiated with artificial UVA light. Bacterial cells were harvested from a stationary-phase LB batch culture, washed, and diluted in mineral water (Evian). After exposure to UVA light bacterial cell samples were stained with a mixture of SYTO9 plus PI and analyzed on a flow cytometer. R1, PI-negative cells; R2, PI-positive cells; R3, background signal. FL1, 520 ± 10 nm; FL3, ≥630 nm.

tion used was 10^4 times diluted from the original stock (10^4 times concentrated in DMSO).

Pure cultures. Samples (10^7 cells/ml) taken from irradiation experiments (artificial UVA) were divided into two subsamples and immediately stained with either a mixture of SYTO9 (5 μM final concentration) and PI (30 μM) or with SYTO9 (5 μM) only. Samples were incubated in the dark at room temperature for 20 or 25 min, respectively, before analysis. Prior to flow cytometric analysis, cell samples were diluted with filtered Evian to 1% (vol/vol) of the initial concentration (approximately 1×10^5 cells/ml final concentration). For outer membrane permeabilization, EDTA (pH 8) was added (5 mM final concentration) to the sample, together with the stain (26).

Community of freshwater bacteria. Samples (10^6 cells/ml) taken from UVA exposure were divided into two subsamples and immediately stained with either a mixture of SYBR green (10^4-times diluted from the original stock) and PI (3 μM) or with SYBR green (10^4-times diluted from original stock) only. Samples were incubated in the dark at room temperature for 20 and 25 min), respectively, before analysis. Prior to flow cytometric analysis, cell samples were diluted with 0.22-μm-filtered Evian water to 10% (vol/vol) of the initial concentration (approximately 1×10^5 cells/ml final concentration).

Flow cytometric measurements. Flow cytometric measurements were performed on a Partec CyFlow space flow cytometer (Partec GmbH, Münster, Germany), with 488 nm excitation from a blue solid-state laser at 50 mW. Optical filters were set up such that red fluorescence was measured above 630 nm (FL3) and green fluorescence was measured at 520 nm (FL1). The trigger was set for the green fluorescence channel FL1.

Reproducibility. All experiments were repeated at least three times, and the patterns were reproducible. In Table 1, the results (including standard deviations) from three biologically independent experiments are listed. For the density plots, representative results from one experiment are shown.

RESULTS

Staining of pure cultures exposed to UVA radiation. Membrane integrity staining with red fluorescent PI coupled to a green fluorescent total cell stain (SYTO9 or SYBR green) of bacteria exposed to artificial UVA radiation resulted in a distinctive and reproducible fluorescence pattern as observed with flow cytometry. The pattern can be linked to different cellular states and was shown to occur for three pure cultures of gram-negative bacteria but was observed also for a community of freshwater bacteria. Figure 1 shows the flow cytometric analysis of *E. coli*, which was diluted in bottled mineral water and exposed to different doses of artificial UVA irradiation. As is typical for the LIVE/DEAD stain, the viable bacterial population demonstrated strong green fluorescence and weak red fluorescence (Fig. 1a), while a completely permeabilized population showed weak green fluorescence and strong red fluorescence (Fig. 1e) (3). Membrane integrity was lost after exposure to a UVA fluence of 2,350 kJ/m^2, which concurs with previous work performed by our group on stationary-phase *E. coli* cells (3). However, in the process of cells becoming permeabilized, the bacterial cluster on the two-dimensional dot plot (Fig. 1a to e) moved in a distinctive curve-shaped manner from strong green and weak red fluorescence intensity (region 1 [R1]) (Fig. 1a) to increased green and red fluorescence intensity (R2) (Fig. 1c) and then to weaker green fluorescence intensity (R3) (Fig. 1e). This pattern strongly suggests that intermediate states are occurring, which are characterized by different intracellular concentrations of SYTO9 and PI. It should be noted that the first step in the sequence of this pattern, i.e., an increase in green and red fluorescence intensity, which results in the movement of the bacterial cluster from R1 to R2, could also be observed when cells are stained with SYTO9 only (Fig. 1f to j). Therefore, this initial movement of the bacterial cluster can be attributed solely to higher intracellular SYTO9 concentrations and was not affected by intracellular PI levels. This distinct shift was also observed in heat-treated cells (see Fig. 5). Furthermore, to test our hypothesis, the two pathogenic gram-negative enteric bacterial strains of *Salmonella* serovar Typhimurium and *S. flexneri* were tested as well. For both strains, the same sequence pattern of staining properties as that observed for *E. coli* was found (Fig. 2). The data are summarized in Table 1, which shows the relative increase of green fluorescence intensity when a cell is irradiated with UVA or treated with EDTA. The values for EDTA treatment are in the same range as those for UVA irradiation. When irradiated with UVA light, the gram-positive bacterium *E. faecalis* did not show an initial increase in green fluorescence intensity; only a decrease of green fluorescence intensity, due to the entrance of PI, was observed (Fig. 3; Table 1).

Staining of bacteria treated with EDTA. EDTA permeabilizes the outer membrane of bacteria, thus mimicking the first

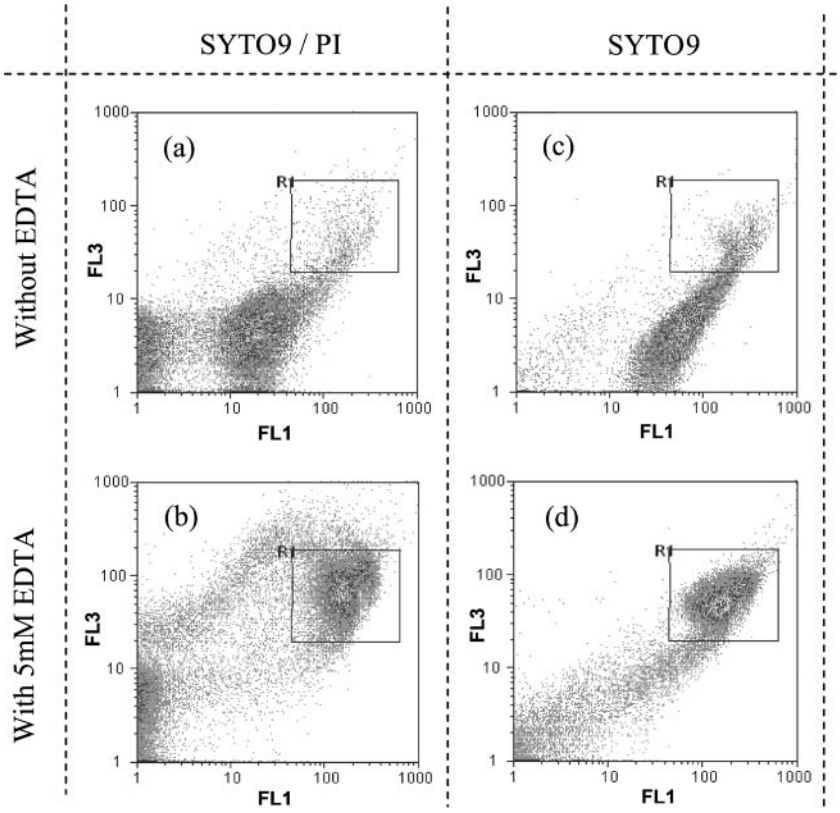

FIG. 4. Flow cytometric analysis of *E. coli* K-12 MG1655 treated with 5 mM EDTA. Bacterial cells were harvested from a stationary-phase LB batch culture, washed, and diluted in mineral water (Evian). Bacterial cell samples were stained either with a mixture of SYTO9 plus PI or with SYTO9 only and analyzed on a flow cytometer. Untreated cells (a and c) and EDTA-treated cells (b and d) are compared. FL1, 520 ± 10 nm; FL3, ≥630 nm.

step of membrane damage. When EDTA was added to live, untreated cells before staining (Fig. 4) the same initial positional fluorescence shift of the bacterial cluster was observed as seen after a UVA dose of about 1,500 kJ/m^2. Again, this shift occurred even when no PI was added to the staining mixture (Fig. 4c and d), and must thus be attributed solely to the presence of increased SYTO9 concentrations in the affected cells. The same results were obtained after EDTA treatment of *Salmonella* serovar Typhimurium and *S. flexneri* cells (Table 1). On the contrary, EDTA treatment of the gram-positive bacterium *E. faecalis* did not show this effect (Table 1).

Occurrence of dual clusters after treatment. A further common observation was that after a UVA dose of 1,400 kJ/m^2 (Fig. 1c and h), two very distinct populations appeared in the

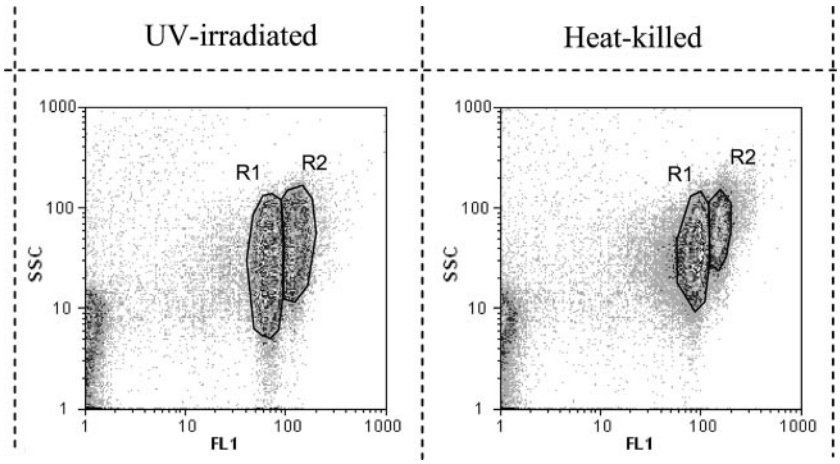

FIG. 5. Comparison of a UVA-irradiated and heat-killed *E. coli* sample stained with SYTO9 and analyzed on the flow cytometer. Green fluorescent signals (FL1, 520 nm) are plotted against SSC signals (SSC, 488 nm). R1, single cells with single DNA content; R2, cells with double DNA content and larger average size.

double-stained sample (Fig. 1c) and the sample stained with SYTO9 only, where it was even more pronounced (Fig. 1 h). The ratio of the geometric mean of green fluorescence intensity (GMn_x) of these two clusters ($GMn_x[R2]/GMn_x[R1]$) is about 1.8, indicating that the cells with increased green fluorescence intensity have about double the amount of nucleic acids. In a two-dimensional dot plot of green fluorescence intensity (FL1) against sideward scatter (SSC) (Fig. 5a), the area of cells with increased green fluorescence intensity (R2) also had an increased SSC signal (geometric mean ratio of 1.8), indicating a larger size. This behavior was reproduced with heat-treated *E. coli* cells (Fig. 5b) and also with cells of *Salmonella* serovar Typhimurium and *S. flexneri* (data not shown). Epifluorescence microscopy revealed that these cells were in an unfinished state of division (data not shown).

Staining of freshwater bacteria exposed to UVA radiation. Different pure cultures tend to show different staining patterns with the LIVE/DEAD stain (manufacturer's manual [http://probes.invitrogen.com/media/pis/mp34856.pdf]). However, a community of freshwater bacteria went through a sequence of staining properties similar to those of *E. coli* (Fig. 6). Although the pattern was similar, the dose needed to permeabilize the majority of the community of freshwater bacteria was about two times higher (5,500 kJ/m^2) than that required for *E. coli* and similar to that required for *S. flexneri*. Freshwater bacterial cells that exhibited high levels of green fluorescence seemed to be permeabilized sooner than the majority of the population, which exhibited lower levels of initial green fluorescence intensity. Interestingly, we found that the use of SYBR green as "total cell stain" in combination with PI gave a more homogeneous and reproducible pattern than that from when a combination of SYTO9 and PI was used (data not shown).

DISCUSSION

Flow cytometry combined with fluorescent markers is emerging as a leading technology for cultivation-free analysis of bacterial viability in disinfection experiments (3), food biotechnology (8), industrial biotechnology (14, 23), and environmental samples (6, 16). The most common of these fluorescent markers is the LIVE/DEAD BacLight stain (SYTO9-PI), which also has been tested extensively with microscopy (1, 21, 22). In this study, we show that for several enteric bacteria and a community of freshwater bacteria, the application of PI staining, in combination with a green fluorescent total nucleic acid stain, results in a clear and distinctive flow cytometric staining pattern. This pattern can be related to the presence of intermediate cellular states characterized by the degree of damage afflicted on the bacterial membrane. Our results indicate that the outer membrane of stationary-phase cells of *E. coli*, *Salmonella* serovar Typhimurium, and *S. flexneri* is, to some extent, a barrier for SYTO9. This phenomenon seems to be specific for gram-negative bacteria due to the presence of an outer membrane. However, it must be mentioned that the three strains tested in this study are physiologically similar.

The LIVE/DEAD BacLight stain kit combines a total nucleic acid stain (SYTO9) and PI, which apparently is excluded from cells with structurally intact cytoplasmic membranes because of the size and charge of the propidium molecule (14). The two stains compete for the same target areas and also

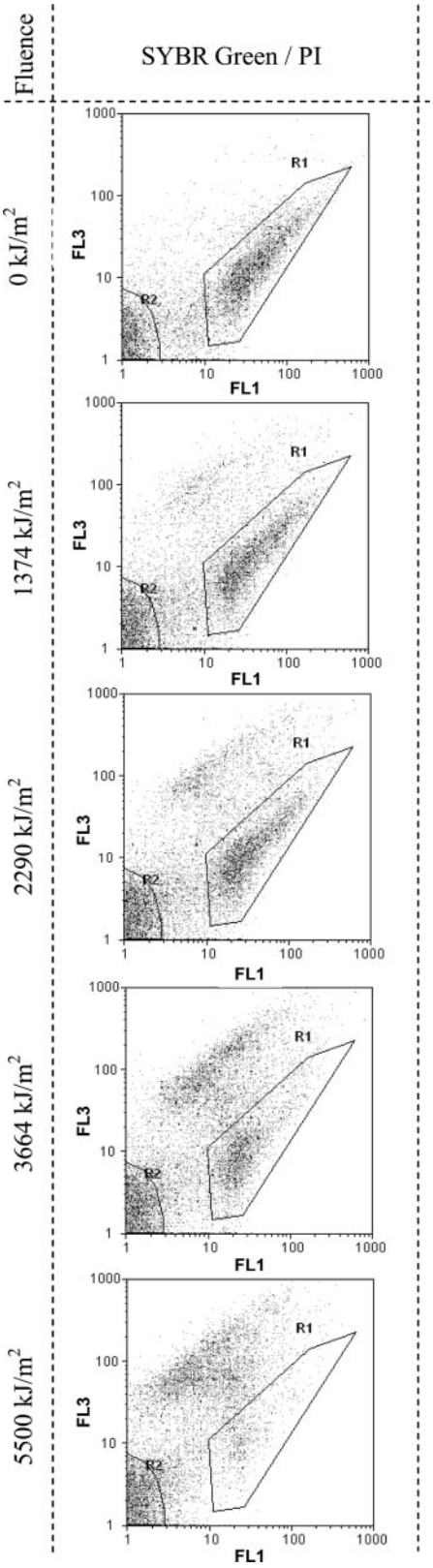

FIG. 6. Flow cytometric analysis of a community of freshwater bacteria during artificial UVA irradiation. After exposure, bacterial cell samples were stained with a mixture of SYBR green and PI and analyzed on a flow cytometer. Cells in gate R1 are PI negative. Gate R2 represents background signal. FL1, 520 ± 10 nm; FL3, ≥630 nm.

interact (fluorescence resonance energy transfer) when present in the same location (31). Microscopically, scientists usually distinguish simply between red or green fluorescent cells. With flow cytometry, intermediate states, with different amounts of both stains, are also detected. The kit manufacturer describes these states as unknown (http://probes.invitrogen.com/media/pis/mp34856.pdf). We have observed a distinctive curve-shaped pattern of fluorescence for *E. coli* cells (and also *S. flexneri* and *Salmonella* serovar Typhimurium cells) that were exposed to UVA irradiation and subsequently stained by using the LIVE/DEAD kit. This pattern was characterized by an initial increase of green and red fluorescence intensity (the increase in red fluorescence intensity is due to SYTO9 emission bands in the red wavelength region [31]). After irradiation, a more homogeneous distribution of fluorescence intensity of cells with different contents of nucleic acids was detected. The same pattern was observed when the three gram-negative strains were pretreated with EDTA. EDTA destabilizes the outer membrane of gram-negative bacteria by chelating divalent cations, which normally tightly cross-link the lipopolysaccharide layer (20). This results in losses of lipopolysaccharide (30 to 50%) and minor amounts of proteins and phospholipids. EDTA is often used in procedures to translocate macromolecules into gram-negative bacterial cells without the loss of viability (15, 24). The numbers in Table 1 suggest that UVA light destabilizes the outer membrane to the same extent as EDTA (5 mM). Furthermore, our results show that the permeabilization of the cytoplasmic membrane of *E. coli* (Fig. 1e) requires almost double the UVA dose as that required for outer membrane permeabilization (Fig. 1 h). Also, for *Salmonella* serovar Typhimurium and *S. flexneri*, we found the same behavior but the fluence levels needed to obtain a similar degree of damage were higher. The differences in inactivation efficacy of UVA treatment have been discussed in a recent paper (5). We show here (and in an earlier paper [3]) that artificial UVA as well as sunlight compromises the outer membrane of stationary-phase *E. coli* cells before the cytoplasmic membrane is disrupted. Also a reanalysis of all samples (exposed but unstained) after 48 h showed identical results, which indicates that the degree of permeabilization was permanent and could not be repaired (data not shown). These damages are in line with earlier reports of lipid peroxidation and other membrane damages caused by near-UV light (9, 33). Therefore, with the dye combination of SYTO9 and PI, one can detect both outer as well as inner membrane permeabilization in gram-negative bacteria. Earlier it was suggested that the outer membrane does not play a role in probe penetration in *Salmonella* serovar Typhimurium LT2 (19). These authors used the same concentration of EDTA and LIVE/DEAD stain as we did, but they did not detect an effect on cell fluorescence. At the same time though, they mentioned that a transfer of the cells from the artificial seawater medium to MOPS (morpholinepropanesulfonic acid) buffer, including a centrifugation step, produced better staining of the cells. This step probably permeabilized the outer membrane so much that EDTA did not produce a detectable effect anymore.

It is known that staining properties are dependent on the physiological state of the bacteria. For example, it was shown earlier that exponential-growth-phase *E. coli* cells stained with a combination of SYBR green and PI displayed higher green fluorescent intensity levels than did stationary-phase bacteria (2). This result might be due to cell envelope alterations (also called cell wall toughening) when nutrients get scarce (e.g., during slow growth or in stationary phase) (11, 30, 34). We have shown recently that *E. coli* cells in this phase are more resistant to stresses like mild heat or sunlight than are cells from exponential phase (4). Additionally, exponential cells are believed to have higher contents of RNA due to increased metabolic activity (7), which can also lead to enhanced green fluorescence intensity. Hence, in the present study, we used only cells from stationary-phase cultures in order to rule out influences on staining properties.

For gram-positive bacteria, controversial data were published. It was shown that intact cells of *Bacillus clausii* in fed-batch cultivation had the same red fluorescence intensity as that of permeabilized cells and differed only in green fluorescence intensity (10). In the manufacturer's manual for the LIVE/DEAD stain kit, a similar shift is shown for *S. aureus* (http://probes.invitrogen.com/media/pis/mp34856.pdf). This resembles our results where *E. coli* cells with outer membrane damage display strong green fluorescence intensity and those cells with cytoplasmic membrane damage display decreased green fluorescence intensity (due to replacement and quenching by PI). Hence, the lacking of outer membrane in gram-positive bacteria enables SYTO9 to enter the cells more easily. Our results with gram-positive *E. faecalis* further support this hypothesis. In another study, intact and permeabilized cells of *Lactococcus lactis* MG1363 exhibited the same green fluorescence intensity but differed in red fluorescence intensity (8). The differences in these studies are probably due to different cell and stain concentration ratios. Stocks (31) demonstrated the importance of validation of stain concentration ratios. For stationary-phase *E. coli* cells (and other gram negatives), we found a concentration ratio of 1:6 (SYTO9 to PI) to work best. Furthermore, in a sample of 10^7 cells/ml, the final concentrations of SYTO9 and PI should be 5 μM and 30 μM (e.g., for 10^6 cells/ml dye concentrations should be 10 times less), respectively.

Our results show that the loss of outer cell membrane integrity also leads to more homogeneous clusters on the flow cytometer. Cells with intact outer membranes appear in a much broader distributed cluster, which probably is due to individual differences in the diffusion properties of the outer membrane for SYTO9. With the destabilization of the outer membrane by either UVA light or EDTA, this effect is leveled out and a differentiation between single and dual cells is possible. Dual cells are probably due to unfinished reductive divisions, an effect often observed in starved cultures (7). Interestingly, PI can mask this effect (Fig. 1e).

For a community of freshwater bacteria, we found SYBR green-PI staining to be well suited for the measurement of cytoplasmic membrane permeability. Also, more homogeneous clusters and intermediate states were observed. Several authors have already proposed LIVE/DEAD staining to be a useful tool for assessing bacterial viability in "natural" water environments, such as drinking water distribution systems (6, 17), river water, or seawater (19). Our study suggests also that for freshwater bacteria, LIVE/DEAD staining does not necessarily give clear-cut results but that intermediate states can occur, which needs further investigation. Therefore, it seems

appropriate to use more than one viability indicator for analysis (see references 3 and 19) and to find the best stain combination for each research question. Fluorescence stains have a large potential for rapid screening of bacterial viability in water but are still a step away from being used in a standardized fashion for water analysis.

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