Selection of Fluorescent Probes for Flow Cytometric Viability Assessment of Listeria monocytogenes Exposed to Membrane-Active and Oxidizing Disinfectants

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ABSTRACT

The aim of this study was to select fluorescence methods for use as alternatives to plate counting to assess the viability of Listeria monocytogenes cells exposed to benzalkonium chloride (BAC) and hydrogen peroxide, two disinfectants with different mechanisms of action. A further aim of this study was to determine whether growth phase influences fluorescence labeling and whether it is possible to predict whether a probe will be a good viability indicator for cells exposed to a certain disinfectant on the basis of the mechanism of action of the disinfectant and the target of the fluorescent probe. The fluorescence methods used were labeling with 5-cyano-2,3-ditolyl tetrazolium chloride (CTC; dehydrogenase activity), labeling with TOTO-1 iodide (TOTO; membrane-impermeant probe), and assessment of pH gradient maintenance in a low-pH buffer after labeling with the pH-sensitive probe 5-(and 6)-carboxyfluorescein succinimidyl ester (CFSE) (the pHin method). Growth phase influenced fluorescent labeling. However, the cutoff value for distinction between viable and nonviable cells was the same for both growth phases. The viability (determined by plate counts) of BAC-exposed cells correlated well with CTC labeling and TOTO exclusion. For both BAC-exposed and hydrogen peroxide-exposed cells, the pHin method gave a good qualitative indication of viability, sublethal damage, and cell death. CTC labeling and TOTO exclusion did not correlate with the viability of hydrogen peroxide-exposed cells. Our results demonstrate that even if the mechanism of action of a disinfectant is known, in some cases it is still difficult to predict whether a certain fluorescent probe is suitable for viability assessment. Thus, the proper selection of fluorescent probes for the assessment of the efficacy of antimicrobial agents is essential.

Listeria monocytogenes is a gram-positive foodborne pathogen that can cause life-threatening illness (15). This organism is able to attach to and form biofilms on the types of surfaces present in food industry settings and in households (16, 23, 30, 38). Attached and biofilm L. monocytogenes cells are more resistant to disinfectants than are free-living cells (16, 38). Consequently, these cells are more difficult to eradicate and thus constitute a food contamination hazard. Therefore, it is necessary to quantitatively assess the viability of these attached and biofilm cells after exposure to disinfectants.

The traditional method for determining the viability of bacteria is plate counting, a method based on the reproductive capacity of cells. However, this method has some disadvantages. The plate count technique requires long incubation times (2 days). Furthermore, for the assessment of the viability of biofilm cells, the cells have to be removed from the surface for analysis. Additionally, several studies indicate that cells can be metabolically active while they are incapable of the cellular division required to form a colony on a plate. This is also known as a viable-but-nonculturable state (11, 13, 26) or better an active-but-nonculturable state (ANC) (21). Such a situation may lead to the overestimation of the efficacy of a disinfectant.

An alternative method for determining viability is labeling with fluorescent probes. These probes indicate whether a cell possesses certain physiological characteristics required for viability, such as membrane integrity, enzyme activity, and energy production (6, 21). Fluorescent probes can be used directly to assess the viability of intact biofilms when their use is combined with fluorescence microscopy (thin layers) (10, 30) or confocal scanning laser microscopy (thick layers) (17, 45). Fluorescent labeling can also be combined with flow cytometry (FCM) (41). FCM is a rapid technique for individual cell analysis. Cells are carried through a fast-flowing fluid stream that passes a focused light beam. Forward angle light scatter (FSC), side angle light scatter (SSC), and fluorescence at selected wavelengths are measured. FCM has previously been applied to analyze heterogeneous populations such as those of starved cells (46) and attached cells (47) and may be used for biofilm cell analysis (28). The advantage of fluorescence methods is that viability can be assessed in 0.5 to 2 h with them.

Cells in biofilms face nutrient gradients (3, 14, 49), and consequently these cells are in various growth phases or have diverse growth rates (43, 49). Since we know that

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fluorescent labeling is influenced by the growth phase of bacteria (4, 13, 20), in this study we decided to test the suitability of fluorescent probes for the assessment of the viability of disinfectant-exposed free-living cells in different growth phases.

The suitability of a fluorescence method for the assessment of viability of disinfectant-exposed cells may be largely influenced by the target of the antimicrobial agent, and therefore a fluorescence method that is suitable for determining the viability of cells exposed to one disinfectant or stress may be unsuitable for determining the viability of cells exposed to another. Therefore, we selected two disinfectants with different modes of action for this study: benzalkonium chloride (BAC; a membrane-active compound) and hydrogen peroxide (an oxidizing agent). These two disinfectants are used in the food industry (36, 48).

In this study, we tested three fluorescent probes. The first, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), is used to measure dehydrogenase activity associated with respiration (22, 42). CTC is not fluorescent but is converted by dehydrogenases into an insoluble red fluorescent compound (CTC-formazan), which accumulates inside the cell. The second fluorescent probe tested, TOTO-1 iodide (TOTO), is green fluorescent and impermeant to cells with an intact membrane. This probe has a high affinity for DNA, and its fluorescence is greatly enhanced (often >1,000-fold) when it binds to DNA (8). Other DNA-binding, membrane-impermeant probes are SYTOX green and propidium iodide (8). The third fluorescent probe, 5-(and 6)-carboxyfluorescein succinimidyl ester (CFSE), is green fluorescent and has been used to measure the internal pH ($pH_{int}$) values of several bacteria, including L. monocytogenes (7, 12, 27). In this study, CFSE was used to assess the ability of the cells to maintain a pH gradient in a low-pH buffer. CFSE is thought to form conjugates with aliphatic amines, preventing CFSE extrusion from the cell (7).

The aim of this study was to select fluorescence methods for use as alternatives to plate counting for the assessment of viability of L. monocytogenes cells exposed to BAC and hydrogen peroxide. Furthermore, we studied whether growth phase influences fluorescence labeling and whether it is possible to predict whether a probe will be a good viability indicator for cells exposed to a certain disinfectant on the basis of the mechanism of action of the disinfectant and the target of the fluorescent probe. This information could assist in the selection of the most suitable probe(s) for the assessment of the viability of, for example, biofilm cells exposed to disinfectants.

**MATERIALS AND METHODS**

**Bacterial strain and growth conditions.** L. monocytogenes Scott A was grown at 30°C in brain heart infusion (BHI) broth with 0.5% (wt/vol) glucose. Stock cultures were kept at -80°C with 25% (wt/vol) glycerol added. A statically grown overnight culture of L. monocytogenes was used to inoculate 150 ml (for exponential-phase cells) or 30 ml (for stationary-phase cells) of fresh medium (2% [vol/vol] inoculum); these different culture volumes were used to ensure that enough cells in both the exponential and the stationary phases were available. The new culture was shaken in a gyrorotatory incubator at 150 rpm in a 1-liter (for exponential-phase cells) or 100-ml (for stationary-phase cells) Erlenmeyer flask. Exponential-phase cells were harvested at an optical density at 620 nm of 0.20 (after ca. 3.5 h). Stationary-phase cells were harvested after 15.5 h of growth. Before cells were used, they were washed once by centrifugation for 10 min at 2,620 x g and resuspended in phosphate-buffered saline (PBS; KCl at 0.2 g/liter, KH$_2$PO$_4$ at 0.2 g/liter, Na$_2$HPO$_4$ at 1.5 g/liter, and NaCl at 8.0 g/liter, adjusted to pH 7.2 with HCl). Cells were resuspended to concentrations ranging from 7 x 10$^6$ to 3 x 10$^7$ CFU/ml in PBS. All experiments in this study were carried out at 30°C unless otherwise noted.

**Chemicals, probes, and disinfectants.** Alkyl-benzyl-dimethylammonium chloride, with an alkyl distribution from C$_8$H$_{17}$ to C$_{16}$H$_{33}$ (BAC), was obtained from Lamers & Pleuger (Den Bosch, The Netherlands), and a 300-g/liter hydrogen peroxide stock solution was obtained from Merck (Darmstadt Germany). BAC was dissolved in demineralized water to a concentration of 50 g/liter and sterilized by passage through a 0.2-µm filter. A 10-fold dilution in demineralized water was prepared from the stock solution before each experiment and used immediately. CTC was obtained from Polysciences Inc. (Warrington, Pa.); TOTO (quinolinium, 1-1'[3-propanediylbisis[(dimethyliminio)-3,1-propanediyl]bis[4-(3-methyl-2(3H)benzothiazolylidene)methyl]-tetraiodide) and CFDA,SE (5- and 6-carboxyfluorescein-diacetate succinimidyl ester) were obtained from Molecular Probes Europe BV (Leiden, The Netherlands). CFDA,SE is a nonfluorescent esterase substrate that yields fluorescent CFSE upon hydrolysis. Stock solutions of 0.68 mmol of CFDA,SE per liter of acetone, 100 µmol of TOTO per liter of dimethyl sulfoxide, and (daily) 5 mmol of CTC per liter of MilliQ water with glucose at 50 mmol/liter were prepared. BHI was obtained from Difco Laboratories (Sparks, Md.), nigericin and valinomycin were obtained from Sigma Chemical Co. (St. Louis, Mo.), and glycerol was obtained from Fluka Chemie AG (Buchs, Switzerland). All other chemicals were obtained from Merck KGaA.

**Exposure to disinfectants.** Cell suspensions were prepared as described above. Killing experiments were carried out with 1 ml of cell suspension. To the cell suspension, 3.5 to 11.5 µl of the 5-g/liter BAC solution was added to attain final concentrations ranging from 17 to 57 mg/liter, or 85 to 154 µl of hydrogen peroxide solution was added to attain final concentrations ranging from 0.68 to 1.2 mol/liter, and the cell suspension was mixed. Five minutes after the addition of the solution, the suspension was diluted 10 times in PBS and centrifuged for 10 min at 2,420 g and then the supernatant was discarded and the pellet was re-suspended in 1 ml of fresh PBS. A control was treated identically except that no disinfectant was added. Hydrogen peroxide exposure experiments were carried out in duplicates and repeated on different days. Most BAC exposure experiments were carried out in duplicate. The statistical test used to analyze the data was a paired Student's t test with a two-tailed distribution and a 0.05 confidence level. The null hypothesis was that there was no significant difference between the viability determined by plate counting on the one hand and that determined by each of the fluorescence methods on the other hand.
Measurement of culturability. Appropriate dilutions of both the untreated and the exposed samples were immediately prepared in PBS, and cells were enumerated by spiral plating in duplicate on BHI agar immediately after dilution. The plates were incubated at 30°C, and the colonies were counted after 2 days.

Fluorescence labeling and flow cytometry. For the measurement of respiratory dehydrogenase activity, 150 μl of sample was added to 1.35 ml of CTC stock solution to obtain a low concentration of phosphate (1.2 mmol/liter) (37) and optimal concentrations of CTC (4.5 mmol/liter) (4, 37) and glucose (45 mmol/liter) (4), and the solution was incubated for 45 min. For the measurement of membrane integrity, both untreated and exposed samples were incubated with TOTO at 1 μmol/liter for 15 min, washed once with PBS, and resuspended in potassium phosphate (KPi) buffer (50 mmol/liter) adjusted to pH 7.2. CTC- and TOTO-labeled cell suspensions were kept on ice for ≈30 min. For the measurement of pH gradient maintenance, both untreated and exposed samples were incubated with CFDA,SE at 17 μmol/liter for 15 min, washed once with PBS, incubated in PBS with glucose (27 mmol/liter) for 15 min, washed once with PBS, resuspended in a 10-fold larger volume of KPi buffer (pH 5.5) with glucose (27 mmol/liter), incubated for 20 min, and measured immediately. In addition, untreated cells were resuspended in KPi buffer (pH 7.2). Other untreated cells were suspended in KPi buffer (pH 5.5 or pH 7.2) to which nigericin (a K+/H+ exchanger; 1 μmol/liter) and valinomycin (a K+ ionophore; 1 μmol/liter) were added to dissipate the pH gradient and the membrane potential of the cells, respectively. These substances were added to make the pHm of the cells equal to the external pH (pHex) so that the fluorescent ratio distribution of cells with pHs of 5.5 and 7.2 could be determined.

FCM analyses were performed with a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, Calif.) equipped with a 15-mW, 488-nm air-cooled argon ion laser. Cell samples were diluted to approximately 5 × 10^6 cells per ml and measured at the low flow rate, corresponding to 750 to 2,500 cells per s. FSC, SSC, and three fluorescent signals were measured. A band pass filter of 530 nm (515 to 545 nm) was used to collect green fluorescence (FL1), a band pass filter of 585 nm (564 to 606 nm) was used to collect yellow-orange fluorescence (FL2), and a long-pass filter of 670 nm was used to collect red fluorescence (FL3). FSC was collected with a diode detector; SSC, FL1, FL2, and FL3 were collected with photomultiplier tubes. All signals were collected with the use of logarithmic amplification. SSC was used to discriminate bacteria from background. For all samples, 5,000 or 10,000 cells were analyzed. FL1 was used for the detection of TOTO, FL3 was used for the detection of CTC, and the CFSE ratio FL1/FL2 was taken as a measure of pHm (12). Data were analyzed with the use of the FCSPress program (Ray Hicks, http://www.fcspress.com/) and the WinMDI program (Version 2.8, Joseph Trotter, John Curtin School of Medical Research, Canberra, Australia, http://jcsnr.anu.edu.au).

Percentage of viable cells. Cell suspensions were prepared as described above. The concentration of viable cells was determined by dilution of the cell suspension in PBS and enumeration by spiral plating on BHI agar immediately after dilution. The plates were incubated for 48 h at 30°C. The total concentration of cells was determined by counting the suspension with the use of a phase-contrast microscope with a Bürker-Türk counting chamber (0.01 mm deep) at ×1,000 times magnification. For each sample, 63 squares of 0.0025 mm^2 were counted. The percentage of viable cells was calculated by dividing the concentration of viable cells by the total concentration of cells and multiplying the resulting number by 100%. The viability level for untreated exponential-phase cells was 125% ± 86% and the viability level for stationary-phase cells was 99% ± 25%. The plate count viability for all untreated cells was assumed to be a 100%.

RESULTS

Viability assessment methods. In this study, three fluorescent labeling methods were compared with plate
FIGURE 3. Distribution of FL1/FL2 ratio classes of *L. monocytogenes* stationary-phase cells stained with CFSE and exposed to a pH of 5.5 with glucose as assessed by FCM (the pH\textsubscript{in} method). Results are shown for untreated cells (black line), cells exposed to 0.68 mol of hydrogen peroxide per liter (dark gray line), and cells exposed to 1.1 mol of hydrogen peroxide per liter (light gray line). In addition, results are shown for valinomycin- and nigericin-treated cells with pH\textsubscript{in} values of 5.5 (thick dotted line) and 7.2 (thin dotted line). Cells to the right of the vertical line are viable.

Counting with respect to their effectiveness in determining viability, Figures 1 through 3 show how viable cells were distinguished from nonviable cells with the different fluorescence methods.

Figure 1 shows results for BAC-exposed and untreated exponential-phase cells labeled with CTC. Two populations could be distinguished (inset). One population had high fluorescence intensity, corresponding to cells with respiratory dehydrogenase activity, and the other population had low fluorescence intensity, corresponding to inactive cells. These two populations were used to set the region of viable cells as shown in the histogram in Figure 1. This histogram of FL3 fluorescence intensity against the number of cells demonstrates that some untreated cells were not labeled. Furthermore, it shows that the population with respiratory activity decreased when the concentration of BAC increased. At the high BAC concentration (40 mg/liter), only a few cells exhibiting respiratory activity remained (3.8%).

Figure 2 demonstrates that TOTO-labeled cells could clearly be distinguished from nonlabeled cells. This is apparent from both the SSC-FL1 dot plot (inset) and the FL1 histogram. The population with the low fluorescence intensity represents the cells with intact membranes. Cells in the population with the high fluorescence have compromised membranes. Practically all untreated cells had intact membranes, and the size of the population with intact membranes decreased when the concentration of BAC increased.

Figure 3 shows the distribution of the FL1/FL2 ratio, which is a measure of pH\textsubscript{in} in hydrogen peroxide–exposed cells and untreated cells incubated for 20 min at pH 5.5. This exposure to a low pH was not lethal for untreated cells (data not shown). In contrast to the experiments involving CTC and TOTO, different fluorescent subpopulations could not be detected with the pH\textsubscript{in} method. We decided to set the region with nonviable cells on the basis of the percentage of nonviable cells initially determined by TOTO labeling. Most of the untreated cells could maintain a pH\textsubscript{in} of 7.2; their fluorescence ratio was similar to that for nigericin- and valinomycin-treated cells with a pH\textsubscript{in} and a pH\textsubscript{ex} of 7.2. The ability of cells exposed to a low concentration of hydrogen peroxide (0.68 mol/liter) to maintain a pH gradient was greatly reduced; their fluorescence ratio dropped considerably. Cells exposed to a high concentration of hydrogen peroxide (1.12 mol/liter) were unable to maintain a pH gradient; their fluorescence ratio was similar to that for nigericin- and valinomycin-treated cells with a pH\textsubscript{in} and a pH\textsubscript{ex} of 5.5.

**Exposure to BAC.** Figure 4 gives an overview of the results obtained with all four methods used to determine the viability of *L. monocytogenes* cells exposed to BAC. Stationary-phase cells were more resistant to BAC than were exponential-phase cells. CTC labeling of cells in both growth phases, except for the labeling of untreated cells, correlated well with plate counts. Especially for stationary-phase cells, labeling of control cells was very low (60%). Furthermore, fluorescence intensity was higher for untreated stationary-phase cells than for untreated exponential-phase cells (data not shown). All cells were labeled with CFSE (confirmed with fluorescence microscopy; results not shown) even after exposure to the high BAC concentration. This finding shows that esterase activity was not influenced by exposure to BAC. The pH\textsubscript{in} method correlated well with plate counts for control cells and exponential-phase cells exposed to the low BAC concentration but not with plate counts for the other BAC-exposed cells.

**Exposure to hydrogen peroxide.** Figure 5 gives an overview of the results for all four methods used to determine the viability of *L. monocytogenes* cells exposed to hydrogen peroxide. Notably, exponential-phase cells were more resistant to hydrogen peroxide than were stationary-phase cells. Not all untreated cells were labeled with CTC; <60% of stationary-phase cells were labeled. Of the exponential-phase cells exposed to hydrogen peroxide, about 35% were labeled with CTC regardless of the hydrogen peroxide concentration. Only at the low hydrogen peroxide concentration was there a significant difference between results obtained with CTC labeling and those obtained with plate counts. When <0.5% of the exponential-phase cells were found to be viable according to plate counts, 29% of the cells were still labeled by CTC (data not shown). Viability levels indicated by CTC labeling of stationary-phase cells were significantly different from those indicated by plate counts for treated and untreated cells. These CTC results may suggest that hydrogen peroxide interfered in some way with CTC la-
bining. In a control experiment, the maximum hydrogen peroxide concentration present in the samples after washing was added to CTC solution and immediately added to untreated cells. The addition of this harmless concentration of hydrogen peroxide had no effect on CTC labeling (data not shown). When TOTO was used, 2 to 8% of the cells (treated and untreated) were labeled. These results suggest that 92 to 98% of the cells were viable. Plate counts indicated that as little as 0.24% of the hydrogen peroxide–exposed cells were viable. For the high hydrogen peroxide concentration, TOTO results completely and significantly disagree with plate count results. The TOTO results may suggest that hydrogen peroxide interfered with TOTO labeling, perhaps by reacting with TOTO. The control experiment, in which cells were first treated with 70% ethanol to disrupt their membranes, washed, exposed to 1.2 mol of hydrogen peroxide per liter, and incubated with TOTO, showed that hydrogen peroxide did not interfere with TOTO labeling. However, in this case, the average fluorescence intensity of the cells was 0.7 log units lower than that for cells exposed to 70% ethanol alone (data not shown), which indicates that hydrogen peroxide induced alterations in the cell DNA. Hydrogen peroxide–exposed cells were labeled with CFSE even after exposure to the high hydrogen peroxide concentration (data not shown). Only for the hydrogen peroxide–exposed stationary-phase cells were the viability levels indicated by the pH<sub>in</sub> method significantly different from those indicated by plate counts.

**DISCUSSION**

In this study, we demonstrate that for the assessment of the viability of disinfectant-exposed cells, the application of FCM in combination with carefully selected fluorescent probes can be a rapid alternative to plate counting in some cases.

**Effects of growth phase on fluorescent labeling.** Our results demonstrate that growth phase influences labeling. First, growth phase influenced the susceptibility of cells to disinfectants. Exponential-phase *L. monocytogenes* cells were more susceptible to BAC than were stationary-phase cells, as was previously demonstrated (27). Stationary-phase cells were more susceptible to hydrogen peroxide than were exponential-phase cells. Similar observations were made in other studies in which bacteria were exposed to oxidative stress (29, 32). Because of these differences in susceptibility to disinfectants, levels of labeling differ for stationary- and exponential-phase cells when the same concentration of disinfectant is used. Second, we found that for TOTO- and CTC-labeled cells the intensity of the fluorescent labeling of some samples depended on the growth phase. Still, we could use the same cutoff value to distinguish between viable and dead cells for both growth phases. Thus, the observed labeling differences do not pose a drawback for the future use of the different probes for cells in different growth phases or heterogeneous populations.

**CTC labeling.** CTC labeling of untreated cells was poor, perhaps because of the toxicity of CTC (40). Poor labeling with CTC (62%) has also been found for *L. monocytogenes* biofilm cells (30). In contrast, Bovill et al. (4) observed 100% labeling of free-living exponential-phase *L. monocytogenes* cells and 90% labeling of stationary-phase cells. After cells were exposed to hydrogen peroxide, CTC labeling was low even when a concentration producing no significant reduction in plate counts was used. This effect was also observed by Huang et al. (19) when *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* biofilms were exposed to the oxidative agent monochloramine. CTC labeling of exponential-phase cells exposed to hydrogen peroxide remained at the same
FIGURE 5. Percentages of viability for L. monocytogenes exponential-phase (A) and stationary-phase (B) cells determined by different methods (PC, plate counting). Results are shown for untreated cells (black bars), cells exposed to a low concentration of hydrogen peroxide (white bars), and cells exposed to a high concentration of hydrogen peroxide (gray bars). For exponential-phase cells, these concentrations were 0.97 and 1.2 mol/liter, respectively, and for stationary-phase cells, these concentrations were 0.68 and 1.1 mol/liter, respectively. Error bars show standard deviations. Viability levels of 100% are $2.7 \times 10^9$ CFU/ml for exponential-phase cells and $2.5 \times 10^9$ CFU/ml for stationary-phase cells.

level even when viability levels indicated by plate counts decreased from 83% to <0.5%. Our results demonstrate that CTC cannot be used to quantify the viability of L. monocytogenes cells after exposure to hydrogen peroxide and that the probe is not very suitable for use as an alternative to plate counting for BAC-exposed L. monocytogenes cells.

TOTO labeling. The percentage of BAC-exposed cells that excluded TOTO correlated well with the percentage of viability determined by plate counts. Several authors have also found the exclusion of DNA-binding membrane-impermeant probes to correlate well with plate counts for gram-positive bacteria killed by membrane-active compounds (44). The exclusion of TOTO by hydrogen peroxide–exposed cells did not correlate with plate counts. Apparently, hydrogen peroxide does not damage the membrane so severely that a large molecule like TOTO (molecular weight, 1,303 g/mol) can enter the cell. Braux et al. (5) observed similar results for the propidium iodide labeling of Escherichia coli exposed to the oxidizing agent peracetic acid. Other authors have observed similar results for other stresses (9). The overall conclusion with regard to TOTO is that it is very suitable for the assessment of the killing of cells by BAC but not for the assessment of hydrogen peroxide efficacy. Some authors have suggested that loss of membrane integrity is a good indicator (8), or even the best general indicator (34), of cell death. In the frequently used LIVE/DEAD BacLight assay from Molecular Probes, membrane integrity is used to assess bacterial viability after stress exposure, since the membrane-impermeant propidium iodide is the dye that is used in this assay to distinguish between live and dead cells. However, our results show that one should be very careful about when to use DNA-binding membrane-impermeant probes.

CFSE labeling and pH$_{in}$ results. The correlation of results obtained with the pH$_{in}$ method with those obtained with plate counts was good for most samples exposed to the low concentrations of disinfectant. For the cells exposed to the high concentrations of disinfectant, results obtained with the pH$_{in}$ method correlated well with results obtained with plate counts only for the exponential-phase cells exposed to hydrogen peroxide. This outcome is mainly a result of the quantitative method that was used to present the results (e.g., compare Figs. 3 and 5). In general, the results for the pH$_{in}$ method indicate that the ability to maintain a pH gradient was largely affected by BAC and hydrogen peroxide before a major loss in viability (according to plate counts) took place. The ability to maintain a pH gradient in a low-pH buffer was completely lost only when no viability was found by plate counts. Thus, the pH$_{in}$ method is good for the determination of complete viability, sublethal damage, and complete loss of viability for hydrogen peroxide– and BAC-exposed cells, but it is not suitable for the quantitative determination of the viability of L. monocytogenes cells. In summary, for BAC-exposed cells, TOTO exclusion was the method whose results correlated best with plate count results. For hydrogen peroxide–exposed cells the best of the three fluorescence methods was the pH$_{in}$ method.

ANC state. Our results did not indicate that BAC-exposed cells were in an ANC state, because results obtained with fluorescent labeling were similar to those obtained with plate counts. For hydrogen peroxide–exposed cells, TOTO results could indicate an ANC state. Although the TOTO results show that the membranes of the hydrogen peroxide–exposed cells in this study were not severely damaged, most other cell activities were not exhibited: cells were not able to maintain a pH gradient, did not contain ATP (data not shown), and exhibited low lev-
bles of respiratory activity or the absence of respiratory activity. Furthermore, our results indicate that the cells had altered DNA, which probably resulted in the impairment of cell replication. Thus, even though the cells had intact membranes, they were not active and were very likely unable to replicate and were consequently not in an ANC state.

**Disinfectant mechanisms of action.** In a previous study (27), we found good correlation between the killing of cells by BAC and the complete inhibition of respiration and the complete leakage of ATP (molecular weight, 503 g/mol) from the cell, which indicates the formation of large holes in the cell membrane. The use of fluorescent probes in the present study confirmed these results: CTC indicated complete inhibition of respiratory dehydrogenase activity when plate counts indicated complete loss of viability and labeling by TOTO (molecular weight, 1,303 g/mol) indicated the formation of large holes in the membrane. These results are consistent with the mechanism of action of BAC: alteration of the barrier function of the cell membrane leads to the leakage of metabolites and coenzymes and the disturbance of the delicate balance of metabolite concentrations within the cell (31). In a previous study, we found that the loss of the ability to maintain a pH gradient in a buffer of pH 7.2 did not correlate with cell death. The pH gradient was dissipated at nonlethal concentrations of BAC (27). In the present study, we only observed a complete loss of the ability of cells to maintain a pH gradient in a buffer with a low pH when plate counts indicated a complete loss of viability. Apparently, the capacity of cells to maintain a pH gradient in a buffer with a low pH is correlated with cell viability.

Hydrogen peroxide is an oxidizing agent that forms free radicals (1, 18) that affect enzymes, DNA, membranes, and lipids, resulting in damage to transport systems and receptors, difficulty in maintaining ionic gradients, impairment of replication, and inactivation of enzyme systems (1, 18, 39). Indeed, CTC results indicate that the enzymes from the respiratory chain (35) were affected. These enzymes may have been directly attacked, or the cellular membrane may have been disturbed. Strikingly, TOTO results demonstrate that even though hydrogen peroxide reacts with membranes according to its mechanism of action, it did not damage the cellular membrane to such an extent that TOTO could enter the cells. The results of the control experiment involving TOTO labeling of cells exposed to ethanol and hydrogen peroxide may indicate that hydrogen peroxide damaged the DNA: the binding of TOTO to the DNA was affected. This assumption is supported by the results of Mortimer et al. (33), who found that antimicrobial agents targeting nucleic acids influence the degree of staining by DNA-binding membrane-impermeant probes. The results for the pHin method showed that cells had difficulty maintaining a pH gradient after exposure to a low hydrogen peroxide concentration and that the complete collapse of the machinery for the maintenance of this gradient coincides with cell death as determined by plate counts. This finding is consistent with the mechanism of action of hydrogen peroxide, discussed above.

**General conclusion.** Our results demonstrate that even if the mechanism of action of a disinfectant is known, in some cases it is still difficult to predict whether a certain fluorescent probe is suitable for the assessment of viability, because it is not known what damage is lethal and how extensive the damage to certain cell constituents is. Lisle et al. (25) proposed the use of multiple indices of physiological activity in viability testing. Such an approach will provide insight into the overall effect of a disinfectant on physiological activity and on the site(s) of sublethal injury (25). In any case, it is very important for a fluorescent probe to be tested under the (stress) conditions it will be used for in order to prevent the overestimation or underestimation of bacterial survival. Furthermore, the use of fluorescent probes in combination with FCM cannot presently completely replace the official method (plate counting (2)) for viability detection after disinfection because the sensitivity of FCM is not yet high enough. For disinfectant testing, a 5-log reduction in the viable cell count has to be detected, and with FCM the lower limit is currently a 2 to 3-log reduction, depending on the signal-to-noise ratio. For now, FCM can be used as a supplement, for instance, for screening strains for possible resistance, for testing new disinfectants, and for studying synergistic effects between disinfectants and additives (24). Thus, when the proper fluorescent probe is selected, FCM can be used as a rapid alternative to plate counting for viability assessment under certain conditions. Our next step is to investigate whether the methods tested in this study can be successfully used for the assessment of the viability of biofilm cells.

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**REFERENCES**


