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Assessment of the Effects of Gramicidin, Formaldehyde, and Surfactants on *Escherichia coli* by Flow Cytometry Using Nucleic Acid and Membrane Potential Dyes

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Two membrane potential sensitive dyes (Rhodamine 123 and bis-oxonol) and three nucleic acid dyes (propidium iodide, SYTO-13, and SYTO-17) were used to assess the effect of surfactants on *Escherichia coli.* The ability of *E. coli* to be stained by these probes was validated at different physiological states. Propidium iodide was used to assess the integrity of cell envelopes. Two double staining methods based on propidium iodide with SYTO-13 and bis-oxonol with SYTO-17 were used to improve the discrimination between bacteria and micelles or aggregated particles generated by the presence of surfactants. A rapid (1 h contact time between cells and surfactants, and less than 5 min for staining and obtaining data) Rhodamine 123 flow cytometric assay was developed to assess the bactericidal effect of surfactants. Cytometry 29:58–64, 1997.

Key terms: *E. coli;* flow cytometry; particle size analyzer; surfactants; Rhodamine 123; bis-oxonol; propidium iodide; SYTO-13; SYTO-17

Traditional assessment of antimicrobial action involves the incubation of microorganisms in liquid or solid media for 18–48 h in the presence of the biocide. The biocidal effect is quantified by zones of inhibition on plates or inhibition of growth in liquid media. In the last few years, diverse studies of prokaryotes (13,18,19) for the rapid assessment of bacterial viability and survival (4,5,11,12,15) and analyses of eukaryote and prokaryote drug susceptibilities (6,14,16,17,18,19) using flow cytometry have been reported. However, studies on the biocidal effect of surfactants estimated by flow cytometry are lacking.

One of the main points of interest in the use of flow cytometry in the assessment of biocidal drugs is the possibility it offers for rapid assays, in contrast to the conventional techniques which need at least 24–48 h. Promising dyes for assessing biocidal drug effects using flow cytometry are Rhodamine 123 (Rh), oxonol, and propidium iodide (PI). The signals from these fluorochromes detect changes in membrane potential or envelope integrity of the challenged cells (3,7,11,12,15,16). The level of accumulation of Rh and oxonol inside the cell depends on its membrane potential or the difference in charge between both sides of the plasma-membrane.

The aim of this study was to find a rapid method to assess bacterial surfactant susceptibility using membrane potential probes in flow cytometry. This was based on the hypothesis that surfactants disrupt or alter the outer membrane and cell membrane in gram–negative bacteria, resulting in changes in the integrity of cell envelopes and membrane potential. Rh is a cationic lipophilic dye (accumulated cytosolically by cells with an inside negative transmembrane electrochemical potential) that has been used extensively to study bacterial viability (3,5,7,11,15). Oxonols are anionic lipophilic dyes which, unlike Rh, are not extensively accumulated cytosolically by cells with an inside negative transmembrane potential, and which have also been used to assess bacterial viability (11,15,16). Therefore, their fluorescence response is opposite that of cationic Rh. When membrane potential increases, the fluorescence response of Rh increases, but the fluorescence response of oxonol decreases. Conversely, when membrane potential decreases, Rh fluorescence decreases but oxonol fluorescence increases. In this paper, we report on the use of stains based on Rh, bis-oxonol $DiBaC(4)_3$, PI (a nucleic acid stain not taken up by intact cells) and two nucleic acid dyes (SYTO-13 and SYTO-17). Use of these dyes allows a quick and accurate assessment of the surfactant effect on *E. coli.*

MATERIALS AND METHODS

Strains and Culture Conditions

Experiments were conducted with *Escherichia coli* 536, an isolate from a human urinary tract infection (2), *E. coli*

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ATCC 11303, and *Staphylococcus aureus* ATCC 12600. Cells were grown overnight in Luria Broth medium by incubation at 37°C in an orbital shaker at 60 r.p.m. Viable counts were calculated from the colony forming units (cfu) on tryptone soy agar plates incubated at 30°C for 2 days.

Assay of the Surfactant Effect

The surfactants were added at final concentrations of 5, 20, and 100 µg/ml to an overnight culture diluted 1/1000 in NaCl 0.9% (pH 7.0). After 1 h incubation at room temperature, samples were stained with the fluorochromes and processed for flow cytometry and the particle analyzer. The six surfactants used in this study were: sodium dodecyl sulfate (SDS) from Boehringer (Germany); linear alkyl benzene sulfonate (LAS) from (Petresa, Spain); Empilan KB10, a commercial nonionic surfactant consisting of a fatty alcohol with alkyl chain lengths from C_{12} to C_{14} and with mainly 10 oxyethylenated units (Albright & Wilson, England); and four quaternary ammonium compounds with different biological properties: Quartamin D86-PI (Kao), dialkyl dimethyl ammonium chloride; Tetranyl BC-80 (Kao), a dimethyl lauryl benzyl ammonium chloride; Tetranyl AT-75 (Kao), a triethanolamine dimethyl sulfate-quaternized; and benzalkonium chloride (Fluka 13400), a C_{16} hexadecyl benzyl dimethyl ammonium chloride. Quartamin D86-PI and Tetranyl AT-75 are cationic surfactants with null biocide activity used as raw material for fabric softeners, and Tetranyl BC80 and benzalkonium chloride are classic cationics used as an algicide and bactericide, respectively.

Staining Procedure

Previously described protocols for Rh, PI, and oxonol were used (11), consisting of the following steps. EGTA (Sigma) was added to the overnight culture previously diluted 1/1000 in NaCl 0.9% at a final concentration of 1 mM in order to permeabilize the bacterial outer membrane. Rhodamine 123 (Sigma) was added to a final concentration of 0.2 µg/ml from a stock solution of 1 mg/ml in ethanol. Propidium iodide (Molecular Probes) was used from a stock solution of 1 mg/ml in water and added to a final concentration of 10 µg/ml. The oxonol used in this work was bis-(1,3-dibutylbarbituric acid) trimethine oxonol ($DiBAC_4(3)$) (Molecular Probes); 4 µl of a 250 µM solution of oxonol in ethanol was added to 1 ml of sample and incubated for 2 min at room temperature before passing through the flow cytometer. The optimal dye concentration and incubation time for SYTO-13 and SYTO-17 (Molecular Probes) were estimated to be 1 µM and 60 min. EGTA was not used when staining with SYTO-13 or PI. Optimal incubation times for the other dyes were 2 min for Rh and oxonol, 15–20 min for PI.

Validation Assays

E. coli cultures incubated in Luria Broth at 30°C overnight were exposed for 1 h at room temperature to Gramicidin S (Sigma) at a final concentration of 20 µg/ml and formaldehyde at 2% final concentration.

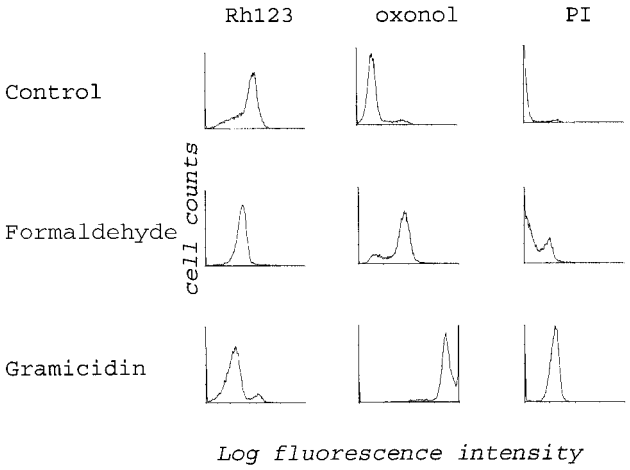

FIG. 1. Validation of Rh, oxonol, and PI single stain for formaldehyde and Gramicidin-S against *E. coli*. Plate counts in TSA, 30°C, 48 h after formaldehyde and Gramicidin-S treatments were <0.3% of the non-treated cell suspensions.

Table 1
Biocidal Effect of Surfactants Assessed by Plate Counts

	Viability, %*		
Surfactant	5 ppm	20 ppm	100 ppm
Tetranyl BC80	<0.02	<0.02	<0.02
Benzalkonium chloride	1	<0.04	<0.04
Tetranyl AT75	100	100	80
Quartamin PI86	100	100	76
LAS	100	100	100
SDS	100	100	100
Empilan KB10	100	100	100

*Calculated as the percentage of the population that survives in relation with the control without surfactant by plate count.

Flow Cytometric Analysis

A Coulter Epics Elite flow cytometer equipped with a 15 mW air-cooled 488 nm argon-ion laser (for Rh, oxonol, PI, and SYTO-13 excitation) and a 10 mW 633 nm helium-neon laser (for SYTO-17 excitation) was set up with the standard configuration. Fluorescent beads (1 µm Fluoresbrite carboxylate microspheres, Polysciences, Warrington, PA) were used as an internal standard for scatter and fluorescence. The green emission from Rh, oxonol, and SYTO-13 was collected through a 525 nm band-pass filter. The red emission from PI and SYTO-17 was collected with a 675 nm band-pass filter. Bacteria were counted using a Cytek Flow Module (Cytek Development, CA) adapted to the flow cytometer and corrected to measure bacterial populations detected by the fluorescence signal due to SYTO-13 or SYTO-17. Forward- and side-scatter and fluorescence signals were collected in a logarithmic scale. A significant percentage of the bacterial population that can be detected by its SYTO fluorescence appears in the first channel of the scatter. Consequently, fluorescence is used to discriminate bacteria rather than scatter, thus obtaining a better resolution and decreasing the background. The forward-scatter detector in the Elite flow cytometer is a

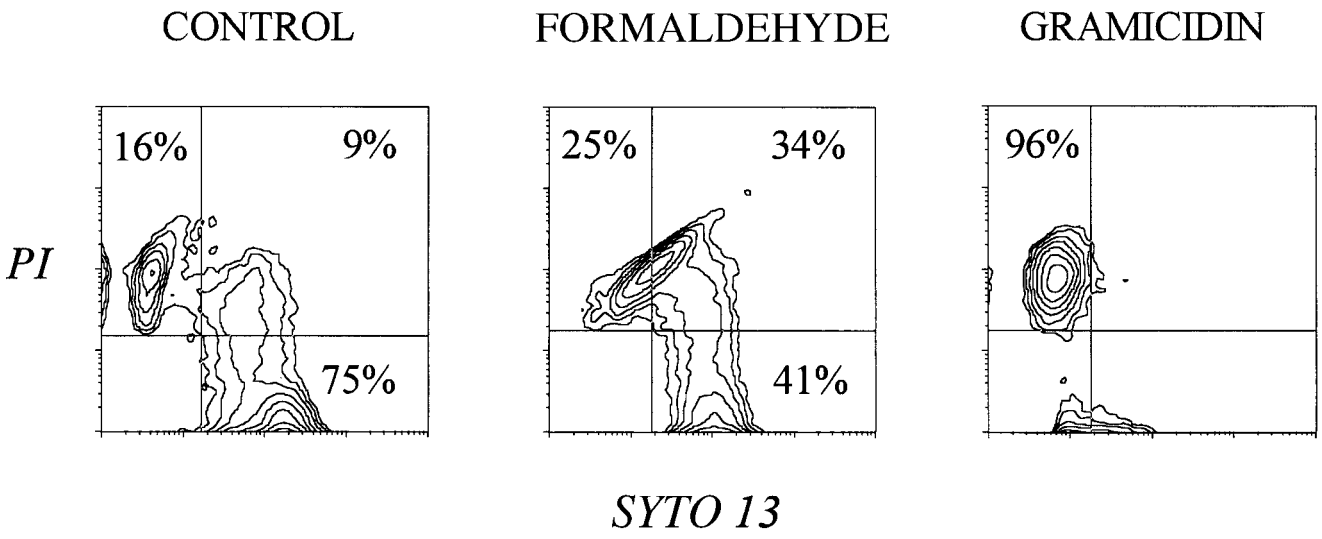

FIG. 2. Validation of the formaldehyde and Gramicidin-S treatment against *E. coli* using a dual PI with SYTO-13 stain. Plate counts in TSA, 30°C, 48 h after formaldehyde and Gramicidin-S treatments were <0.3% of the non-treated cell suspensions.

photodiode which collects light between 1.5 and 19° from the laser axis; it is able to discriminate particles >0.5 µm in diameter. The side-scatter detector is situated in a 90° position from the laser axis. Due to the design of the flow-in quartz flow chamber used, light for both side-scatter and fluorescence is collected in an angle wider than 90° using a combination of a mirror and a lens in order to improve efficiency. Data were analyzed with Elitesoft version 4.1 (Coulter) and WinMDI version 2.1.3 software (Joseph Trotter, 1996).

Particle Size Analysis

Cell suspensions were analyzed with a Multisizer II (Coulter) using a 30 µm aperture. Cell suspensions were diluted (1/1000) in 0.9% NaCl previously passed through a 0.2 µm filter, and processed maintaining the coincidence level below 2%. Data were analysed by AccuComp software version 1.15 (Coulter Corporation).

RESULTS AND DISCUSSION

Intercalibration Between Multisizer II and the Flow Cytometer

Parallel counts of either 4 µm or 1 µm bead suspensions by flow cytometer and particle analyzer gave good correlations ($R^2 = 0.999$). Parallel counts using E. coli and S. aureus suspensions at different cell concentrations also resulted in good correlations ($R^2 = 0.910$) and no tendency to count more particles either by the flow cytometer or the particle analyzer was observed.

Validation of Rhodamine 123, Oxonol, and Propidium Iodide Staining

Validation of Rh, PI, and oxonol staining have already been done for E. coli for treatments with heat, sonication, French press, and Gramicidin (11). Now we compare the effects of Gramicidin and formaldehyde using Rh, PI, and oxonol staining. Figure 1 shows the effect of such treatments. As expected, there is a decrease in Rh uptake and an increase in oxonol and PI uptake after killing the bacteria with formaldehyde or Gramicidin. The Rh, PI, and oxonol staining levels are correlated with cell viability (data in Table 1 and legends of Figures 1, 3, and 5). Oxonol and PI give better discrimination than Rh between live and dead cells after treatment with Gramicidin or formaldehyde. It is interesting that the oxonol, Rh, and PI histograms for Gramicidin are more conclusive than those for formaldehyde. The observed differences in cells killed by Gramicidin or by formaldehyde indicate that differences in their biocide action mechanisms that are missed by plate counts can be detected by flow cytometry. Gramicidin produces pores in the membrane, thus rendering cells more permeable to ions and probably increasing the uptake of oxonol or PI. These dyes enter the organism more easily when killed by Gramicidin than when killed by formaldehyde, probably because in E. coli the outer membrane is the primary barrier that formaldehyde encounters when it interacts with bacteria, and is of prime importance in conferring formaldehyde tolerance or sensitivity (1).

The differences between cells killed by Gramicidin or by formaldehyde became even more evident when using a dual PI/SYTO-13 stain (Fig. 2); Gramicidin treatment yields a more homogeneous effect. Detecting the homogeneity of the biocidal effect is of particular interest in cases of heterogeneous initial populations, inherent to growing cultures (8,9,10,11), or starved populations (11,20), or when mixed populations are studied.

Effect of Surfactants on Particle Analyzer and Flow Cytometer Scatter Signals

Surfactant solutions in NaCl (with no cells) generate particles detectable by the cytometer scatter signals and by

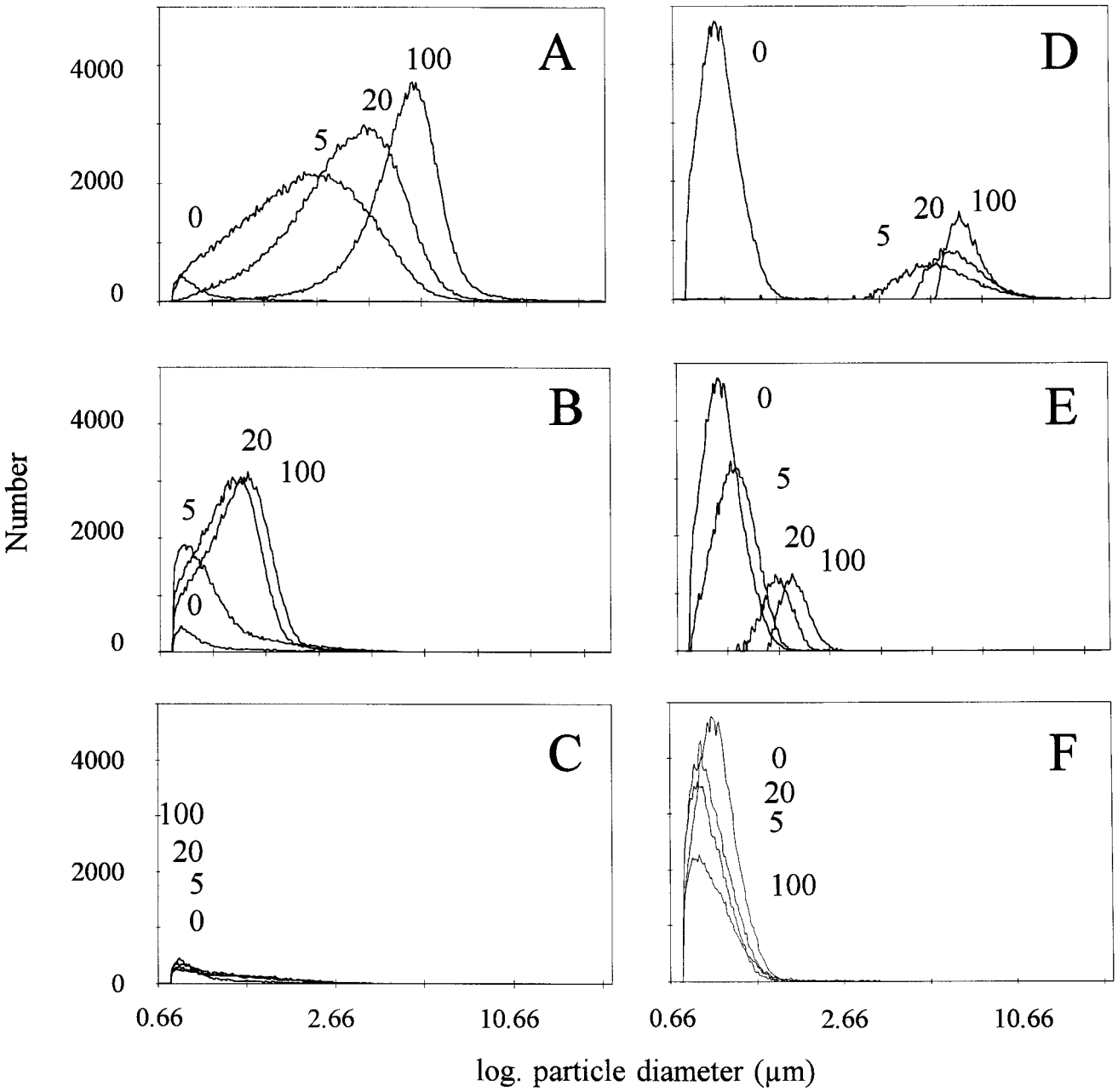

FIG. 3. Assessment of the surfactants' effect on *E. coli* by particle analyzer. **A** and **D** for Quartamin; **B** and **E** for LAS; **C** and **F** for Tetranyl BC80. A, B, and C are the background noise produced by cell-free surfactant solutions; D, E, and F are histograms of cell suspensions after the subtraction of the relevant background. Numbers above or at the peak of the histogram are the concentrations of surfactants in μg/ml. The resulting viable plate counts for Quartamin at 5, 20, and 100 μg/ml were 100%, 100%, and 76% of the control, respectively; for LAS at 5, 20, and 100 ppm they were 100% of the control for the three concentrations and for Tetranyl BC80 at 5, 20, and 100 μg/ml they were less than 0.02% of the control for the three concentrations. Benzalkonium chloride produces results similar to those of Tetranyl BC80.

the particle analyzer; the electric signal is generally proportional to the surfactant concentration. These particles detected by the two cytometry methods can in principle be attributed to micelle formation and cellular aggregation, especially when the critical micelle point is exceeded (Fig. 3). For Tetranyl AT75 and Quartamin, the number of detected particles is independent of the surfactant concentration, but the size of the detected particles increases with surfactant concentration, indicating that formation of micelles or cellular aggregation takes place. In the case of benzalkonium chloride, Tetranyl BC80, SDS, LAS, and Empilan KB10, the particle counter signal does not exhibit significant numbers of particles and their size is smaller than for Tetranyl AT75 and Quartamin. Consequently, particle counter signals generated by the presence of surfactants have to be considered as background to the

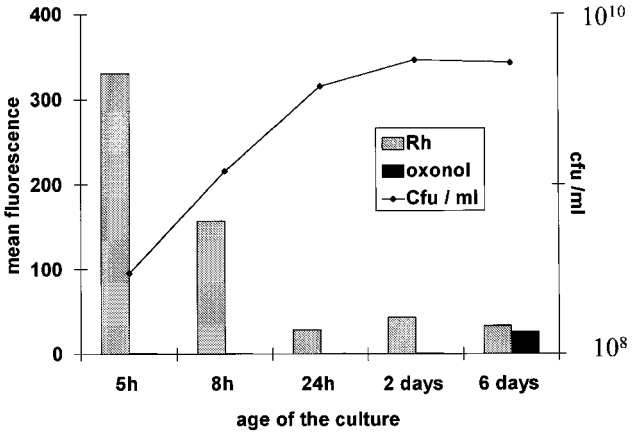

FIG. 4. Variations of Rh and oxonol uptake during exponential and stationary phases of *E. coli*.

analysis with bacterial cells to give an accurate reading of its biological effect, especially for Tetranyl AT75 and Quartamin, which generate very high counts and large particles.

SYTO-13 and SYTO-17 label DNA and RNA and are used to discriminate and count the bacterial population (if not lysed by the addition of surfactants), thus improving the discrimination between lysed cells, micelles, or aggregates from the intact microorganisms containing DNA and RNA. When surfactant solution with no cells and SYTO-13 or SYTO-17 are processed by the cytometer in order to assess the background, more noise is observed with SYTO-17 than with SYTO-13, indicating that SYTO-13 would be more convenient for counting the residual bacterial population. Although the fluorescence detected by the cytometer is attributed to nucleic acids when using SYTO-13 and SYTO-17, a fluorescence background proportional to surfactant concentration is also detected.

Oxonol and Rh Uptake at Exponential and Stationary Phases

Oxonol and Rh staining at different culture times have been determined in order to check whether Rh and oxonol are taken up at different rates during the exponential and stationary phases. A progressive decline in Rh uptake is observed during the growth phase (Fig. 4). Cells showing high rhodamine fluorescence exhibit little or no fluorescence with oxonol. Exponential or stationary populations do not incorporate oxonol, which is only incorporated late in the death phase.

Because the fluorescence intensity of Rh-stained populations is highly dependent upon their physiological state, standardization of the inoculum is an important factor in these cytometric assays. We have used stationary populations instead of exponential phase cells mainly for two reasons: 1) stationary inocula are used in conventional antimicrobial assays, and 2) stationary cells can be stable for a longer time than exponential or dead phase cells.

Effect of Surfactants on *E. coli*

Tetranyl BC80 and benzalkonium chloride (which are currently used as bactericides) were the only surfactants we tested in this report which exhibited significant bactericidal activity in our plate counts (Table 1), particle analysis (Fig. 3), and flow cytometry analysis (Figs. 5 and 6). The other surfactants studied are used as cleaning or washing softener agents and do not kill *E. coli* (Table 1). SDS, LAS, Empilan KB10, Tetranyl AT75, and Quartamin PI86 do not work as potent biocides, but they produce effects on bacterial envelopes that can be detected by flow cytometry using dual staining (Fig. 5) or single stain (Fig. 6). However, these effects cannot be measured, or even detected, by plate counts.

The lack of a biocidal effect of SDS, LAS, Empilan KB10, Tetranyl AT75, and Quartamin PI86 are confirmed by the particle analyzer and plate counts, but good correlation was difficult to obtain, probably because the formation of micelles and their aggregates generate signals which hamper bacterial discrimination. Particle analysis can be of help in assessing biocidal surfactant effects only as a rapid qualitative assessment, when micelles and cell aggregates are not produced, but is not applicable to quantitative and accurate biocidal studies.

The non-cell particles generated by the presence of surfactants can be better discriminated from bacteria in the cytometer by using SYTO-13 and SYTO-17. Dual staining with PI/SYTO-13 or oxonol/SYTO-17 resulted in accurate bacterial discrimination (Fig. 5). Dead and viable bacterial populations are stained by SYTO dyes; however, killed populations are labeled by both oxonol and SYTO-17 or by PI and SYTO-13. The combination of PI and SYTO-13 is superior in resolution to the combination of oxonol and SYTO-17, probably because SYTO-13 produces higher fluorescence and less background than SYTO-17. The dual stain (using PI and SYTO-13) gives additional information on the heterogeneity of cultures (9,10) and on biocidal effects that cannot be observed by plate counts.

The populations that lost viability after benzalkonium chloride treatment show a progressive labeling by PI, together with a progressive decrease in SYTO-13 labeling. As more benzalkonium chloride concentration is applied, competitive effects produce more PI labeling, and less SYTO-13 staining. Possible explanations for this may be that when both PI and SYTO-13 enter the cell together: 1) SYTO-13 is displaced by PI; 2) PI exhibits higher affinity for DNA; and 3) an energy transfer from SYTO-13 to PI can also be expected.

The simplest and quickest method (about 5 min, once the contact time between cells and surfactant have elapsed) to assess the biocidal surfactant effect by evaluating the surviving population is by staining with Rh (Fig. 6). Oxonol is a good and rapid (about 5 min) complement for this type of analysis, since it allows us to verify the proportion of killed cells, especially as Rh staining is not always reliable. The dual staining procedure using PI with SYTO-13, although less rapid and more expensive, also provides a rapid method (about 25 min) which is excellent

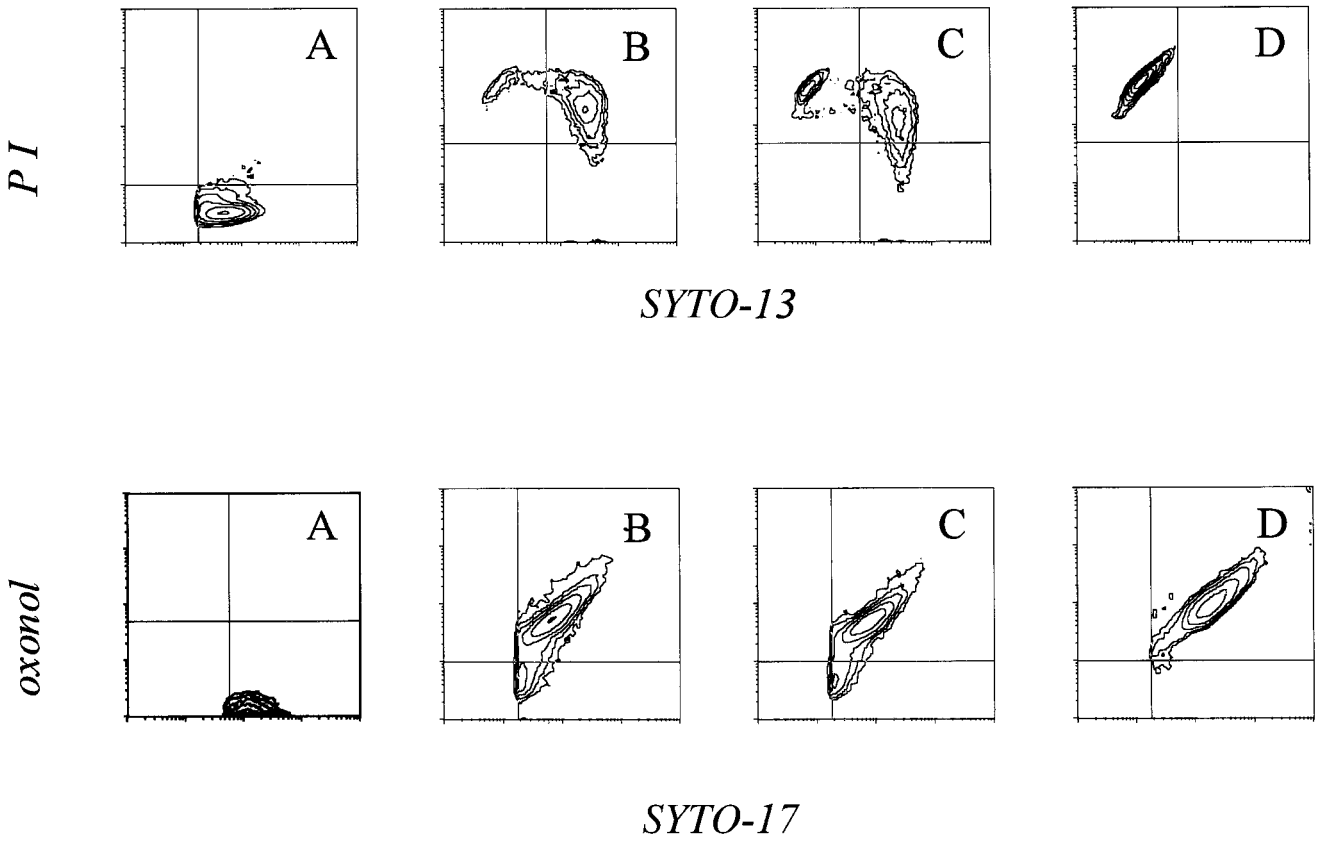

FIG. 5. Assessment of the benzalkonium chloride effect on *E. coli* after a dual stain with PI and SYTO-13 or oxonol and SYTO-17. (**A**) Control (no benzalkonium chloride treatment). (**B**) Benzalkonium chloride at 5 µg/ml and 1% viability. (**C**) Benzalkonium chloride at 20 µg/ml and less than 0.1% viability. (**D**) Benzalkonium chloride at 100 µg/ml and less than 0.1% viability. Benzalkonium chloride produces results similar to those of Tetranyl BC80.

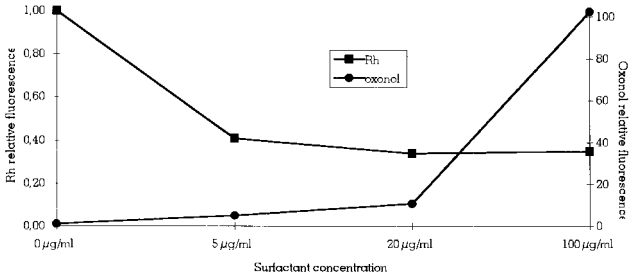

FIG. 6. Assessment of the Tetranyl BC80 effect at several concentrations on *E. coli* for Rh and oxonol staining. Benzalkonium chloride produces results similar to those of Tetranyl BC80.

for the study of intermediate or subtle interactions between gram–negatives and surfactants that cannot be detected by plate counts. The dual staining procedure with PI and SYTO-13 is also the best way to minimize the background problems associated with the micelles or cellular aggregation produced by some surfactants.

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