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Flow Cytometric Assessment of *Escherichia coli* and *Salmonella typhimurium* Starvation-Survival in Seawater Using Rhodamine 123, Propidium Iodide, and Oxonol

R. LÓPEZ-AMORÓS,[1] J. COMAS,[2] AND J. VIVES-REGO[1]*

Departament de Microbiologia[1] and Servei Científico Tècnic,[2] Universitat de Barcelona, 08028-Barcelona, Spain

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The use of flow cytometry in microbiology allows rapid characterization of cells from a nonhomogeneous population. A method based on flow cytometry to assess the effects of lethal agents and the bacterial survival in starved cultures through the use of membrane potential-sensitive dyes and a nucleic acid marker is presented. The use of propidium iodide, rhodamine, and oxonol has facilitated the differentiation of cells of *Escherichia coli* and *Salmonella typhimurium* of various states of vitality following various treatments (heat, sonication, electroporation, and incubation with gramicidin) and during starvation in artificial seawater. The fluorescence intensity is directly correlated with viable cell counts for rhodamine 123 labelling, whereas oxonol and propidium iodide labelling is inversely correlated with viable counts. The distribution of rhodamine and oxonol uptake during starvation-survival clearly indicates that single-species starved bacteria are heterogeneous populations, and flow cytometry can be a fundamental tool for quantifying this heterogeneity.

Because of the obvious relevance to understanding survival processes, much attention has been devoted to the study of biochemical, metabolical, morphological, and genetic events that occur during the starvation-survival process in bacteria (3, 4, 8, 9, 15, 22, 25, 28). The use of flow cytometry in microbiology allows fast enumeration and physical and biochemical characterization of cells from a nonhomogeneous population (1), and several applications in microbiology have been reported (17).

Rhodamine (Rh) has been used to detect and enumerate viable bacteria under laboratory conditions (7, 13, 14, 17) and in environmental samples (6, 24). Oxonol has been used with mammalian and yeast cells (5) and bacteria (20); since this probe has increased binding affinities for depolarized membranes, cells that have lost the ability to maintain the membrane potential acquire more-intense staining.

Double-staining methods have been used to assess the viability of cells by staining dead cells with one dye and live cells with another dye of a different emission (10, 12). Propidium iodide (PI), a nucleic acid marker excluded by intact cells, has been used to label dead cells (10, 21, 23), although its application to prokaryotes has been limited (11). However, the use of oxonol and its combination with PI and double staining with Rh and PI have not been used previously to study bacterial starvation.

The use of Rh, PI, and oxonol has facilitated the differentiation of cells of various states of vitality following various treatments (heat, sonication, electroporation, French press treatment, and incubation with gramicidin) and during starvation in artificial seawater. The aims of this study were mainly (i) the assessment of membrane potential changes of *Escherichia coli* and *Salmonella typhimurium* during starvation-survival and (ii) determination of the extent to which the starved bacteria are heterogeneous populations, differing mainly in their membrane potential when assessed by flow cytometry.

* Corresponding author. Mailing address: Departament de Microbiologia, Universitat de Barcelona, Av. Diagonal, 645, 08028-Barcelona, Spain. Phone: (343) 402-1485. Fax: (343) 411-0592. Electronic mail address: jvives@porthos.bio.ub.es.

MATERIALS AND METHODS

Strains, cultures, and starvation conditions. Starvation experiments were conducted with *E. coli* 536, an isolate from a human urinary tract infection (2), and *Salmonella typhimurium* ATCC 14028. In starvation experiments, cells were grown overnight in Luria broth medium by incubation at 30°C, harvested, washed twice by centrifugation at 6,000 × *g* for 5 min in artificial seawater (ADSA Micro, Barcelona, Spain), and resuspended in artificial seawater. Appropriate volumes of these resuspended cells were used to inoculate flasks containing 250 ml of sterile artificial seawater to reach an initial population of 10^6 to 10^7 cells per ml. Flasks were maintained at 20°C in the dark for 20 to 25 days in an orbital shaker at 100 rpm. Viable counts were calculated from the CFU on tryptone soya agar plates incubated at 30°C for 5 days.

Staining procedure. Ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) (Sigma) was added to samples to a final concentration of 1 mM before addition of the dyes in order to permeabilize the bacterial outer membrane. Rh 123 (Sigma) was added to a final concentration of 2 μg/ml from a stock solution of 1 mg/ml in ethanol. PI (Molecular Probes, Inc.) was used from a stock solution of 1 mg/ml in water and added to a final concentration of 10 μg/ml. Bis-(1,3-dibutylbarbituric acid) trimethine oxonol ($DiBAC_4$ [3]) was obtained from Molecular Probes, Inc.; 4 μl of a 250 μM solution of oxonol in ethanol was added to 1 ml of sample, and the mixture was incubated for 2 min at room temperature before being passed through the flow cytometer. When dual staining was performed, the same dye concentrations and incubation times were used.

Lethal and sublethal treatments. All the procedures to injure or kill cells were performed on cultures incubated in Luria broth at 30°C overnight. Cells were centrifuged at 6,000 × *g* for 5 min and resuspended in 0.9% NaCl before sonication or electroporation treatments and gramicidin exposure. The heat treatment was performed in two ways. Glass tubes (10 by 150 mm) containing the cells were immersed (i) for 10 min each time in a water bath at 55, 65, and 75°C and (ii) for 2, 5, 10, and 15 min in a water bath at 65°C. Sonication was performed with a Labsonic 1510 (Braun), using the needle probe at 100 W for 30, 60, 120, and 180 s and for 1 min each at 25, 50, 100, and 300 W. Electroporation (2.0 kV, 129 Ω, for 5 s in an Electrocell Manipulator 600 [BTX]) was performed on 70-μl samples. After electroporation, the samples were resuspended up to 1 ml with 0.9% NaCl. Exposure to gramicidin S (Sigma) at room temperature was done in two ways: (i) 20 μg/ml for 2, 5, 10, 15, and 20 min and (ii) 2-min exposures to 10, 20, and 40 μg/ml. The French press (SLA Aminco, SLM Instruments) was filled with 40 ml of a culture, and a pressure of 900 lb/in^2 was applied.

Flow cytometric analysis. A Coulter Epics Elite flow cytometer equipped with an air-cooled 488-nm argon-ion laser at 15-mW power was set up with the standard configuration; fluorescent beads (1-μm-diameter Fluoresbrite carboxylate microspheres; Polysciences Inc., Warrington, Pa.) were used as an internal standard. A band pass filter of 525 (520 to 530) nm was used to collect the

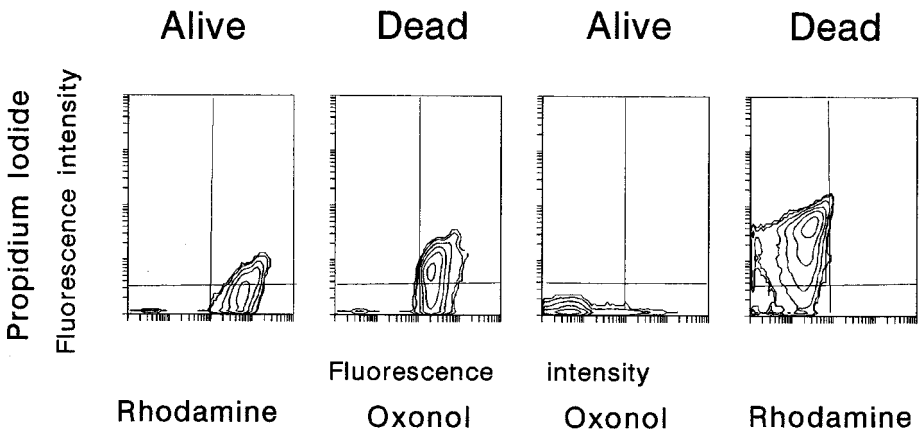

FIG. 1. Dual-parameter histograms of PI-Rh and PI-oxonol fluorescence obtained by staining live and heat-killed cells of *E. coli.*

emission for oxonol and Rh. A band pass filter of 675 (670 to 680) nm was used to collect the emission of the PI.

RESULTS

Validation of the Rh, oxonol, and PI staining. All the cells from overnight cultures took up Rh, but none was stained by PI, nor did they take up oxonol. Conversely, the cells killed by heat (0% viability as determined by plate counts) did not take up Rh, but they were stained by PI and they did take up oxonol (Fig. 1 and 2). The fluorescence intensity is directly correlated with viable cell counts for Rh, whereas the oxonol or PI labelling is inversely correlated with viable counts (Table 1). There is a strong inverse correlation between the Rh and oxonol or PI labelling, indicating that cells taking up Rh take up neither oxonol nor PI (Table 2). The correlation between oxonol and PI is high and direct, indicating that nonviable cells are labelled by neither oxonol nor PI (Table 2). All the PI-labelled cells are also labelled by oxonol, but a subpopulation of the starved, sonicated, and electroporated cells that are oxonol fluorescent are not stained by PI (data not shown).

When all the cells of the population were dead (no colonies determined by plate counts), the distribution of cells according to oxonol fluorescence was high, narrow, and basically symmetric (data not shown). When the treatment killed only a fraction of the population, as is the case for sonication, electroporation, or French press treatment, the distribution of Rh and oxonol fluorescence was in many cases asymmetric (Fig. 2), indicating that the resulting population was more heterogeneous than the population killed by heat.

The relative uptake of Rh and oxonol by cells is considered to be a consequence of the membrane potential. PI cannot enter the cell if the cell membrane is not altered, and consequently, it labels only cells that have suffered damage to the cell membrane. Sonication, electroporation, and French press

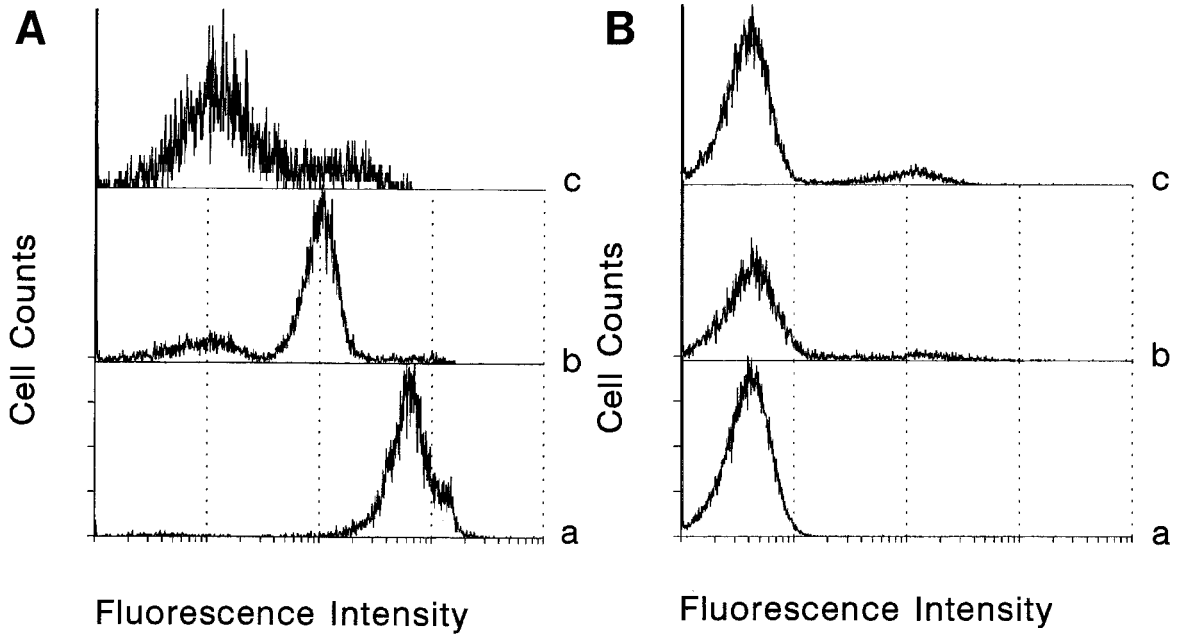

FIG. 2. Single-parameter histograms of cells stained with Rh (A) and oxonol (B). Results for an overnight *E. coli* culture (a), a sonicated *E. coli* culture with 66% viability (b), and a French press-treated *S. typhimurium* culture with 40% viability (c) are shown. The viability percentages are in reference to the untreated population and assessed by plate count.

TABLE 1. Correlation between Rh, oxonol, and PI labelling and viable cell counts after heat, sonication, and gramicidin treatments[a]

Organism	Treatment	Rh		Oxonol		PI	
		RL[b]	r[c]	RL	r	RL	r
E. coli	Heat[d]	$y = -23.2 + 12.4x$	0.99	$y = 89.1 - 7.2x$	−0.90	$y = 90.0 - 3.7x$	−0.92
	Heat[e]	$y = 25.8 + 8.1x$	0.97	$y = 176.7 - 183.4x$	−0.97	$y = 47.1 - 0.7x$	−0.54
	Sonication[f]	$y = -65.0 + 8.6x$	0.87	$y = 139.5 - 143.5x$	−0.87	ND[g]	
	Sonication[h]	$y = 50.6 + 6.6x$	0.60	$y = 189.1 - 249x$	−0.91	ND	
	Gramicidin[i]	$y = -15.8 + 8.1x$	0.94	ND		$y = 92.6 - 1.4x$	−0.83
	Gramicidin[j]	$y = 24.2 + 8.6x$	0.90	ND		$y = 103.2 - 2.9x$	−0.68
S. typhimurium	Heat[d]	$y = -27.9 + 9.7x$	0.99	$y = 89.0 - 4.9x$	−0.91	$y = 95.4 - 2.4x$	−0.96
	Heat[e]	$y = -22.3 + 9.4x$	0.99	$y = 367.1 - 383x$	−0.94	$y = 88.0 - 2.1x$	−0.91
	Sonication[f]	$y = 201.3 + 19.7x$	0.82	$y = 367.4 - 421.2x$	−0.89	ND	
	Sonication[h]	$y = -1{,}292.1 + 96.2x$	0.97	$y = 125.0 - 126.4x$	−0.80	ND	
	Gramicidin[i]	$y = -3.5 + 8.0x$	0.81	ND		$y = 98.2 - 1.3x$	−0.98
	Gramicidin[j]	$y = -1.1 + 33.4x$	0.92	ND		$y = 88.4 - 1.9x$	−0.81

[a] The values for Rh and oxonol used in these correlations are the means of the fluorescence intensity; the PI values are percentages of cells labelled above channel 12. Viable counts used in the calculations of correlations were percentages of the control (100% of viable cells).
[b] RL, regression line.
[c] *r*, correlation index at a significance level of ≤0.05.
[d] To 65°C for 2, 5, 10, and 15 min.
[e] For 10 min each at 55, 65, and 75°C.
[f] At 100 W for 30, 60, 120, and 180 s.
[g] ND, not done.
[h] For 1 min each at 25, 50, 100, and 300 W.
[i] At a concentration of 20 μg/ml for 2, 5, 10, 15, and 20 min.
[j] For 2 min each at 10, 20, and 40 μg/ml.

treatment are less drastic treatments than heat or gramicidin, as shown by the percentages of remaining viable cells and the cytometric histograms (Fig. 1 through 3). The cells with lower levels of Rh uptake were more fluorescent with oxonol and PI. On the other hand, the cells showing high levels of Rh fluorescence presented lower levels of oxonol or PI fluorescence. From Fig. 2, it can be clearly seen that populations with a low percentage of viable cells are more easily detected by the decrease in Rh uptake than by oxonol staining.

Histograms were obtained after 2 min of incubation with the dyes. When cells were incubated with Rh for more than 10 min, the dye drifted unspecifically and a toxic effect of the dye itself was observed. Cells can be incubated with oxonol and PI longer without significant changes in the distribution of the stains. When dual staining was conducted, no significant difference was found in comparison with any of the single-staining results, revealing that the dyes are compatible with each other.

To determine if the results obtained with Rh were due to membrane potential rather than to unspecific factors, cells

TABLE 2. Correlations among Rh, oxonol, and PI labelling after heat, sonication, and gramicidin treatments[a]

Organism	Treatment	Rh-oxonol		Rh-PI		PI-oxonol	
		RL[b]	r[c]	RL	r	RL	r
E. coli	Heat[d]	$y = 14.0 - 1.3x$	−0.87	$y = 81.4 - 8.2x$	−0.90	$y = 2.1 + 4.0x$	0.749
	Heat[e]	$y = 16.5 - 1.5x$	−0.95	$y = 39.2 - 3.7x$	−0.94	$y = 4.3 + 1.0x$	0.78
	Sonication[f]	$y = 1.3 - 5.2x$	−0.87	ND[g]		ND	
	Sonication[h]	$y = 0.9 - 0.02x$	−0.69	ND		ND	
	Gramicidin[i]	ND		$y = 66.7 - 4.3x$	−0.87	ND	
	Gramicidin[j]	ND		$y = 34.9 - 2.0x$	−0.91	ND	
S. typhimurium	Heat[d]	$y = 25.3 - 14.7x$	−0.96	$y = 52.1 - 4.9x$	−0.95	$y = 2.22 + 2.0x$	0.96
	Heat[e]	$y = 15.0 - 1.1x$	−0.87	$y = 30.6 - 2.9x$	−0.87	$y = 1.7 + 2.9x$	0.95
	Sonication[f]	$y = 2.2 - 0.1x$	−0.95	ND		ND	
	Sonication[h]	$y = 1.4 - 0.9x$	−0.93	ND		ND	
	Gramicidin[i]	ND		$y = 76.5 - 5.9x$	−0.79	ND	
	Gramicidin[j]	ND		$y = 40.1 - 3.2x$	−0.85	ND	

[a] The values for Rh and oxonol used in these correlations are the means of the fluorescence intensity; the PI values are percentages of cells labelled above channel 12. Viable counts used in the calculations of correlations were percentages of the control (100% of viable cells).
[b] RL, regression line.
[c] *r*, correlation index at a significance level of ≤0.05.
[d] To 65°C for 2, 5, 10, and 15 min.
[e] For 10 min each at 55, 65, and 75°C.
[f] At 100 W for 30, 60, 120, and 180 s.
[g] ND, not done.
[h] For 1 min each at 25, 50, 100, and 300 W.
[i] At a concentration of 20 μg/ml for 2, 5, 10, 15, and 20 min.
[j] For 2 min each at 10, 20, and 40 μg/ml.

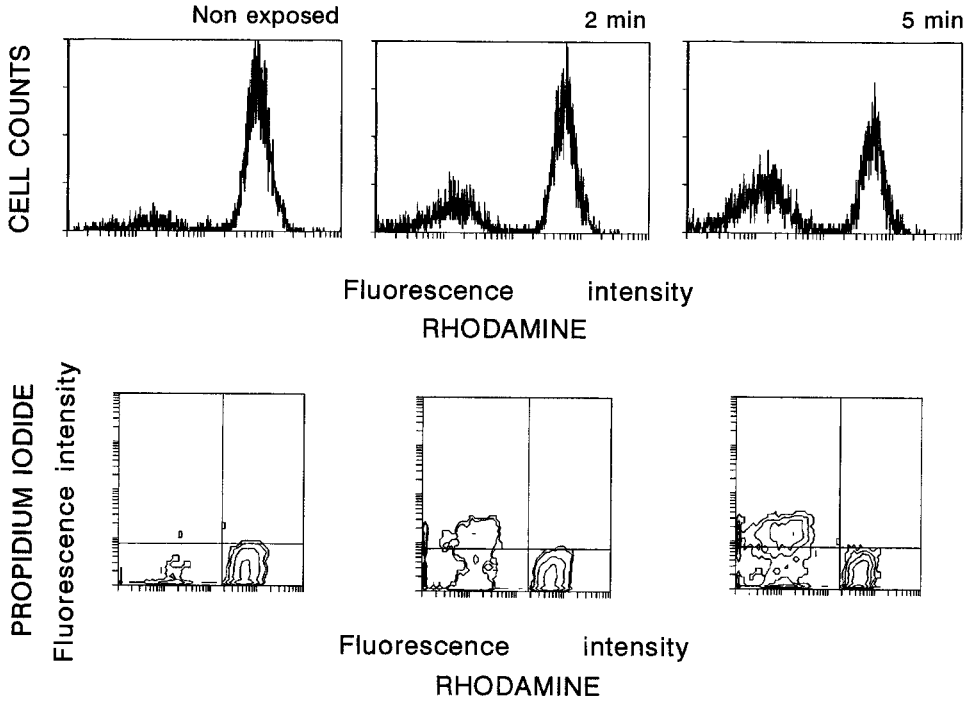

FIG. 3. Single- and dual-parameter histograms of Rh- and PI-stained cells of *E. coli* before and after depolarization (2 and 5 min) with gramicidin. Plate counts were 1.6×10^6 CFU/ml at time zero, 9.5×10^5 CFU/ml after 2 min of gramicidin treatment, and 5.8×10^5 CFU/ml after 5 min of gramicidin treatment.

were exposed to gramicidin to depolarize them by making channels through the membrane, thus rendering them permeable to ions. Figure 3 shows the effect of such treatment, and as expected, there is a decrease in Rh uptake due to the loss of membrane potential, verifying that the Rh uptake depends on the membrane potential. There is also a progressive increase in cell fluorescence from PI due to the greater entry of the dye into the cells. Under our experimental conditions, a 20-min treatment with gramicidin reduced the viable count by more than 97%. The oxonol stain cannot be applied to gramicidin-treated cells because an anomalous and out-of-scale fluorescence is produced, probably because of reactions between oxonol and gramicidin. The effects of sonication, electroporation, French press treatment, and gramicidin exposure as assessed by plate counts and flow cytometry are similar for *E. coli* and *S. typhimurium.*

Assessment of starvation-survival in artificial seawater. Starved populations of *E. coli* and *S. typhimurium* in seawater were studied by flow cytometry using the staining procedure previously tested (Fig. 4 and 5). Samples from flasks with artificial seawater were found to be unable to be stained with Rh because of the salt concentration of the medium itself,

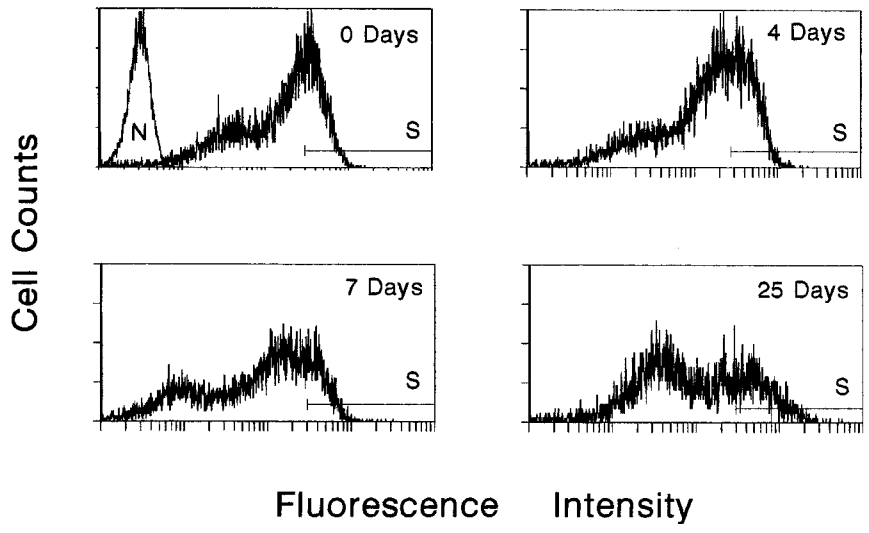

FIG. 4. Rh uptake in *E. coli* cells starved in artificial seawater for 0, 4, 7, and 25 days; plate counts were 4×10^6, 1.3×10^6, 1.4×10^6, and 5.6×10^5 CFU/ml, respectively. N, histogram corresponding to an aliquot control not treated with Rh; S, gate for the significant level of fluorescence (between channels 633 and 1023).

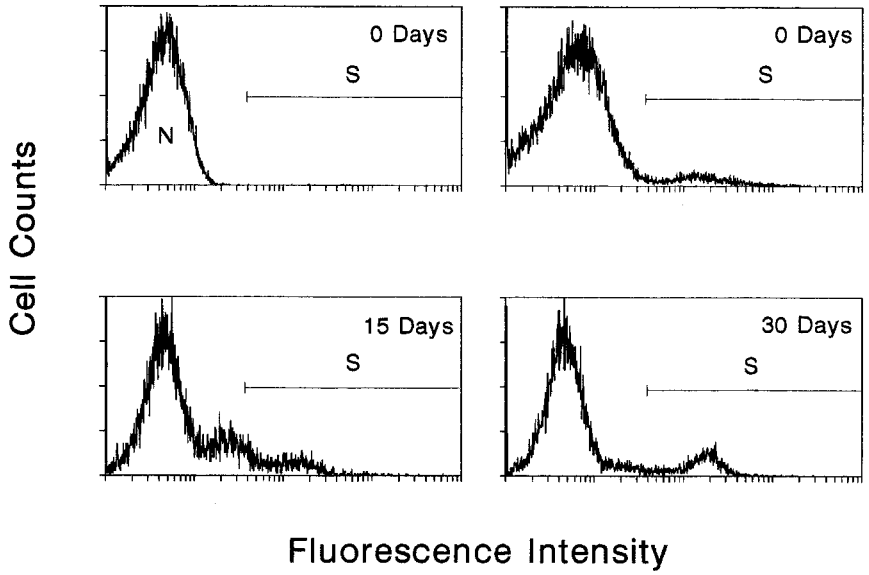

FIG. 5. Oxonol uptake in *S. typhimurium* cells starved for different periods in artificial seawater. Plate counts at 0, 15, and 30 days of starvation were 5.5×10^7, 7.1×10^5, and 6.5×10^5 CFU/ml, respectively. N, histogram corresponding to the aliquot control (not treated with oxonol) at 0 days of starvation; S, gate for the significant level of fluorescence (between channels 415 and 1023).

particularly because of the high concentration of K^+ ions dissolved. To solve this problem, 2-ml samples were filtered through a 0.2-μm-pore-size filter and the filter was resuspended in the same volume of a 0.9% NaCl solution before the staining procedure.

Each diagram (Fig. 3, 4, and 5) displays the distribution of the population among 1,040 channels of fluorescence intensity. The height (*y* axis) indicates the number of cell counts with a particular level of fluorescence, which is related to the total amount of dye per cell. Note that the fluorescence intensity (*x* axis) is shown in log scale, which means that small differences in channel number represent large differences in the amount of dye per cell. PI was not a good marker with which to assess survival during starvation because of the low percentage of the starved population which was stained (data not shown), indicating that the integrity of the cell membrane was not significantly lost in most of the cells. The data clearly illustrate that oxonol (Fig. 5) is a better marker of inactive cells than PI during starvation.

Rh uptake was reduced progressively during starvation (Fig. 4 and Table 3). The histograms show a tendency to be bimodal, heterogeneous, and asymmetric, indicating that the surviving population has a decreasing but heterogeneous membrane potential. At any time during starvation experiments, two subpopulations of cells can be identified according to the Rh uptake: one with a high degree of fluorescence and one with no significant staining. Considering the significant level of fluorescence between channels 633 and 1023, the percentage of starved cells with a fluorescence intensity above channel 633 fluctuated between 32.5 and 19.3% (Table 3) during a 25-day starvation experiment. As was previously done with batch cultures, an ionophor treatment with gramicidin was applied to the starved populations (data not shown). The subpopulation of fluorescent cells lost its fluorescence in a few minutes after addition of the ionophor, indicating that Rh uptake by starved cells is associated with the membrane potential.

The oxonol uptake increases progressively during starvation (Fig. 5 and Table 4) and thus is a more clear-cut and homogeneous marker of the starved cells than Rh. Considering the significant level of fluorescence between channels 415 and 1023, the percentage of starved cells with a fluorescence intensity above channel 633 fluctuated between 5.2 and 12.8% (Table 4) during a 30-day starvation experiment. Another peculiarity of oxonol uptake is that cells labelled with oxonol exhibit a lower level of flow cytometric narrow-angle light scatter than unlabelled cells. During starvation, cell size reductions and chemical changes occur (26–28). A reduction in narrow-angle light scatter is an indication of the period of starvation and can be associated with both cell size reduction and structural and chemical changes (19, 29). Consequently, the oxonol-labelled cells are low-membrane-potential cells whose size may have

TABLE 3. Evolution of the percentage of Rh-stained cells with a fluorescence intensity between channels 633 and 1023 during starvation[a]

Days of starvation	% Labelled cells	% Viable cells
0	32.5	100
4	30.6	43.9
7	24.7	25.5
25	19.3	19.3

[a] Regression line between the percentage of labelled and viable cells: $y = -29.1 + 3.02x$; $r = 0.79$; $P \leq 0.05$.

TABLE 4. Evolution of the percentage of oxonol-stained cells with a fluorescence intensity between channels 415 and 1023 during starvation[a]

Days of starvation	% Labelled cells	% Viable cells
0	5.2	100
15	11.1	12.1
30	12.8	7.2

[a] Regression line between the percentage of labelled and viable cells: $y = 164.9 - 12.9x$; $r = -0.98$; $P \leq 0.05$.

been reduced or whose chemical content may have been modified. The effects of starvation on *E. coli* and *S. typhimurium* as assessed by flow cytometry are similar. During starvation, a direct correlation between viable and Rh-labelled cells and an inverse correlation between viable and oxonol-labelled cells were observed (Tables 3 and 4).

DISCUSSION

The three fluorescent probes used in this study provided clear-cut discrimination and resolution between live and dead *E. coli* and *S. typhimurium* cells after physical treatments if the outer membrane was permeabilized with EGTA. During starvation, Rh and oxonol allowed clear discrimination between subpopulations with different membrane potentials. The use of PI did not provide good discrimination of the subpopulations arising during starvation, indicating that important damage to the cell membrane does not occur during starvation. However, whether the lack of staining is due to PI not entering the cell membrane or to sufficient degradation of the DNA that when PI enters it does not produces fluorescence has yet to be determined.

The heterogeneity of a genetically uniform bacterial population can have three possible sources: differences at different stages of the cell cycles, heterogeneity arising from oscillatory intracellular dynamics, and differences caused by stress or starvation. The degree of heterogeneity of batch cultures (14, 16, 18, 19) and starved populations can be high (27, 29). Flow cytometric data reported in this paper and especially those related to Rh strongly indicate that single-species starved bacteria are heterogeneous populations consisting of several types of subpopulations differing in viability, metabolic activity, and cell integrity. The quantification of heterogeneity in bacterial cultures and during starvation-survival constitutes a fundamental procedure in microbiology that can be studied by flow cytometry.

Rh or oxonol uptake is an indication of the metabolic or vital state of the cell population. The observed correlations between Rh and oxonol labelling and viable cell counts strongly indicate that the proposed cytometric method is useful for assessing the effect of biocidal agents and starvation. However, the results obtained to date do not allow unequivocal association of a specific number of viable cells (assessed by plate counts) with Rh or oxonol staining intensity. At this point, more work is needed in order to establish the specific levels of fluorescence that membrane potential-sensitive dyes confer to viable or nonviable bacterial cells.

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