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Enumeration, viability and heterogeneity in *Staphylococcus aureus* cultures by flow cytometry

J. Comas[a], J. Vives-Rego[b,*]

[a]*Serveis Científico-Tècnics, Universitat de Barcelona; c/Lluis Solè Sabarís, 1–3, Barcelona, Spain*
[b]*Departament de Microbiologia, Universitat de Barcelona, Av. Diagonal, 645, 08028-Barcelona, Spain*

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Abstract

Several fluorochromes (rhodamine 123, bis-oxonol, propidium iodide, SYTO-13 and calcein) were tested by flow cytometry for their ability to determine cell density, viability and heterogeneity in *Staphylococcus aureus* cultures exposed to heating (60–70–80°C for 2 min), formaldehyde 2% for 20 min and gramicidin-S at 2–5–10 μg/ml for 20 min. Results were validated by viable plate count and counts performed with a particle analyser. Flow cytometry gave quicker results and more accurate information about intermediate states and heterogeneity of *S. aureus* cultures than viable plate counts. Rhodamine 123 and oxonol were found to be efficient dyes for the assessment of bacterial viability. SYTO-13 was an excellent marker for total counts and calcein can be used to assess metabolic activity. © 1998 Elsevier Science B.V.

Keywords: Flow cytometry; Particle size analyser; Calcein; Rhodamine 123; bis-Oxonol; Propidium iodide; SYTO-13; *Staphylococcus aureus*

1. Introduction

Simple methods to enumerate bacteria are an evident need in medical, food and environmental microbiology. Flow cytometry combines direct and rapid assays with additional biochemical analysis of individual cells, and has widespread potential applications in microbiology [1–3]. Although applications of flow cytometry to bacteria are more limited than eukaryotic studies because of its small size, some successful uses have been developed: enumeration of bacteria in pure cultures [4] and aquatic systems [5–9], studies of bacterial cell cycle [10,11], assessment of bacterial starvation [12–15], specific detection of bacterial groups or species [16–18] and biocide assessment [19–21]. In addition, an increasing number of fluorochromes need to be checked for their potential application. Here we evaluate the use of five fluorochromes on *Staphylococcus aureus*. This coccus was chosen because it has received little attention in flow cytometry studies [16] despite its obvious importance in clinical and food microbiology, and because the use of a spherical cell should simplify the interpretation of results in a flow system. We report the application of propidium iodide (PI), $DiBAC_4(3)$ (oxonol), rhodamine 123

*Corresponding author. Fax: +34 3 4110592; e-mail: jvives@porthos.bio.ub.es

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(Rh), calcein and SYTO-13 to assess enumeration, viability and heterogeneity in *S. aureus* cultures.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Experiments were performed with *S. aureus* ATCC 12600. Cells were grown overnight in Luria Broth medium by incubation at 30°C and shaking at 30 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.

2.2. Lethal and sublethal treatments

Overnight *S. aureus* cultures were exposed to heat by immersing microcentrifuge tubes containing 200 μl of cell suspension for 2 min in a water bath at 60, 70 and 80°C. The same type of cultures were also exposed to formaldehyde (2% final concentration, 20 min) and gramicidin-S (Sigma-Aldrich Quimica, S.A. Barcelona, Spain) at final concentrations of 2–5–10 μg/ml for 20 min.

2.3. Staining procedures

Previously described protocols for Rh, PI and oxonol were used [13], consisting of the following steps. Suspensions of an overnight *S. aureus* culture in NaCl (0.9%) at a final concentration of $1–5\times10^6$ cells/ml were used in all assays. PI (Molecular Probes, Eugene, OR, USA) from a stock solution of 1 mg/ml was added to a final concentration of 10 μg/ml.. The oxonol used 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, which gave a final concentration of 1 μM. Rh (Sigma) was added to a final concentration of 0.2 μg/ml from a stock solution of 1 mg/ml in ethanol. Optimal incubation times were: 10–15 min for PI and 2 min for Rh and oxonol. Calcein acetomethyl ester (calcein-AM) (Molecular Probes) from a stock solution of 1 mM in dimethyl sulfoxide was added to a final concentration of 2 mM; cells with dyes were incubated at 30°C for 1 h. Calcein-AM is converted by esterases into calcein, which allows detection of cells by their fluorescence. The final concentration and incubation time for SYTO-13 (Molecular Probes) was estimated to be 1 μM and 60 min.

2.4. Flow cytometric analysis

A Coulter Epics Elite flow cytometer equipped with an air-cooled 488 nm argon-ion laser at 15 mW power for PI, oxonol, Rh, calcein and SYTO-13 excitation was set up with the standard configuration. Fluorescent beads (1 μm Fluoresbrite carboxylate microspheres, Polysciences, Warrington, PA, USA, and 4 μm latex fluorosphere beads, Molecular Probes) were used as an internal standard for scatter and fluorescence. The green emission from oxonol, Rh, calcein and SYTO-13 was collected through a 525 nm band-pass filter. The red emission from PI was collected through a 675 nm band-pass filter. Bacteria were counted by a Cytek Flow Module (Cytek Development, CA, USA) adapted to the flow cytometer. Fluorescence and forward and side scatter signals were plotted in a logarithmic scale. Fluorescence was used rather than scatter for discriminating and counting bacteria when possible (SYTO-13 labelling), thus obtaining a better resolution and decreasing the background. The rest of the analysis were performed using a combination of both side and forward scatter for discriminating bacteria from background. The forward scatter detector in the Elite flow cytometer is a photodiode that collects light between 1.5 and 19° from the laser axis, being 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 closed flow chamber used, light for both side scatter and fluorescence is collected in an angle wider than 90° using a combination of mirror and lens in order to improve efficiency. Data were analysed with Elitesoft version 4.1 (Coulter) and WinMDI version 2.5 software [22].

2.5. Particle analysis

Cell suspensions were adjusted to labelling concentration with a Multisizer II (Coulter) using an aperture tube 30 μm in diameter. A 100 μl volume of the cell suspension in 0.9% NaCl previously

filtered through 0.2 μm was processed keeping the coincidence count below 2% in order to obtain information on the concentration of the bacterial populations. Data were analysed by AccuComp software version 1.15 (Coulter Corporation, Miami, Florida, USA).

2.6. Statistical analysis

Instead using absolute mean values to compare the fluorescence variations produced by the various treatments and markers that differ in their quantum yield, we used the percentage of fluorescence variation calculated by the formula:

$$\%\Delta F = \frac{\bar{X}_T - \bar{X}_C}{\bar{X}_C} \times 100$$

where $\%\Delta F$ is the percentage of variation of the fluorescence; $\bar{X}_T$ is the mean fluorescence of the treated samples, and $\bar{X}_C$ is the mean fluorescence of the control (untreated) samples.

3. Results and discussion

3.1. Technical approach

Parallel counts of 4 μm latex bead suspensions by flow cytometry using the Cytek module and particle analyser gave a good correlation ($r^2 = 0.999$). Bacterial counts after heat treatment shown that total counts detected by flow cytometer and particle size analyser were similar ($r^2 = 0.910$), while viable plate count decreased with the intensity of the treatment (Fig. 1). Bacterial counts by flow cytometry allowed us to assess the biological potential in terms of viability, cell lysis and nucleic acids hydrolysis (Figs. 1,4–6) and the use of the particle size analyser allowed us to study the variations of cell size distributions induced by temperature treatments (Fig. 2). Cell size reduction is probably due to leakage and cell shrinkage that take place when the cell membrane was damaged by heat, as also evidenced by the increase in PI staining (Fig. 4). Total counts obtained by particle analyser showed a slight decrease at higher temperature treatments, while total counts obtained with SYTO-13 labelling and flow cytometry

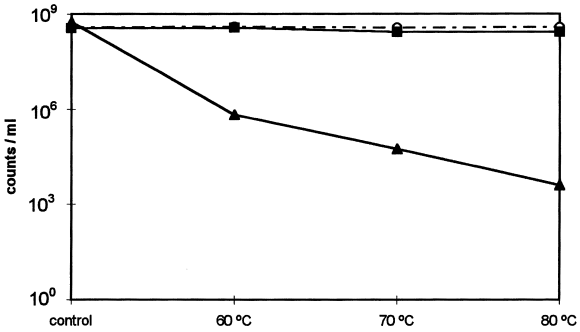

Fig. 1. Evolution of bacterial counts after heat treatment assessed by viable plate count (▲); cytometer counts using SYTO-13 staining (○) and particle size analyser counts (■).

remained constant (Fig. 1). This indicates that a part of the heat-treated population may be not detected by particle size analyser because it is under the detection limit of the used aperture tube.

The five fluorochromes studied were chosen because of their potential ability to distinguish intermediate states between death and alive cells. The changes in intensity of fluorescence gave more information than cell viability assessment by plate count, because fluorescence gives gradual responses, while viable counts do not. Rh fluorescence was associated with active cells and its accumulation inside the bacterial cell was dependant on its membrane potential [14,19,20]. The fluorescence conferred by oxonol or PI was associated with cells who have lost respectively their membrane potential and integrity [14,19]. Oxonol is a negatively charged molecule and its incorporation into cells depends on their depolarisation. SYTO-13 labels nucleic acids, being fully permeable to dead and alive cells [6,19,23] and showed a saturated level of fluorescence after 50 or 60 min of incubation (Fig. 3A). Calcein was chosen as an indicator of esterase activity. Calcein-AM can be passively loaded into cells, as it is a neutral or near-neutral molecule. Once inside, it is converted by intracellular esterases into calcein, a derivative of fluorescein that has about six negative and two positive charges at pH 7. This fluorescent product is retained by the cell if the membrane is intact, thus the detection of calcein increased progressively over time (Fig. 3B). Both calcein-AM and calcein may rapidly leak out from dead or damaged cells with compromised mem-

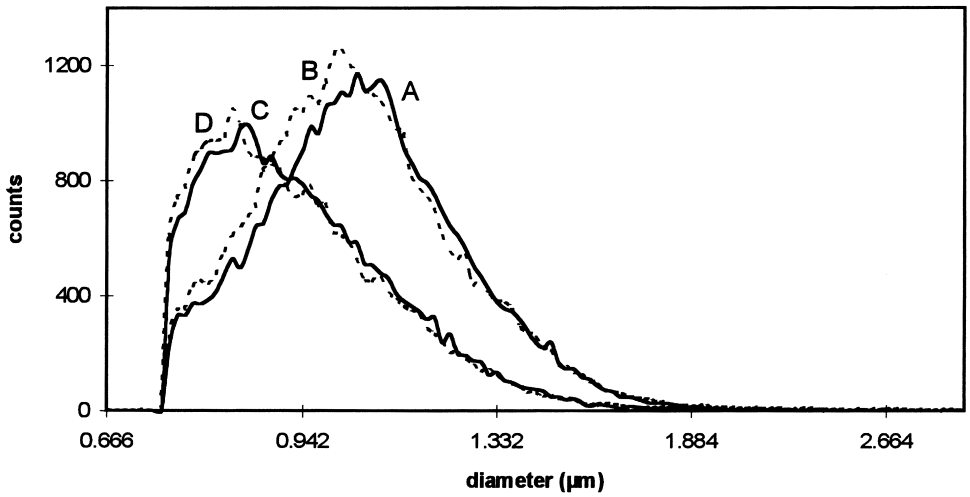

Fig. 2. Cell size distribution after heat treatment by particle size analyser. A (full line), control; B (dotted line), 60°C, 2 min; C (full line), 70°C, 2 min; D (dotted line), 80°C, 2 min.

branes, even if the cell retains some residual esterase activity. Calcein-AM has been used as a viability marker because of its high cell retention and pH-insensitive fluorescence. Addition of Triton X-100 at a final concentration of 0.1% diminished the calcein and SYTO-13 labelling (data not shown).

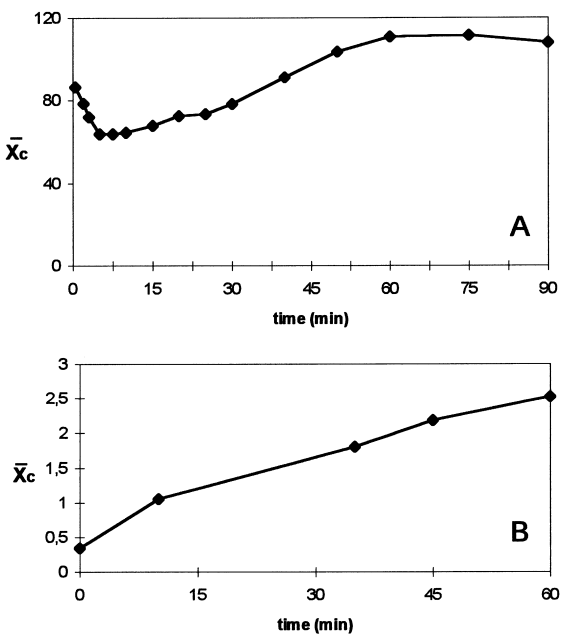

Fig. 3. (A) SYTO-13 incorporation during time at room temperature. (B) Calcein incorporation during time at 30°C.

3.2. Assessment of the lethal effects of temperature, formaldehyde and gramicidin-S

Treatment of cell suspensions ($5–10^8$ cfu)/ml) at 60, 70 and 80°C for 2 min killed 99.88%, 99.99% and more than the 99.99% respectively of the exposed population. Cells after the three heat treatments presented different fluorescence histograms for PI, oxonol, Rh and calcein (Fig. 4). PI histograms indicate a progressive permeabilization of the population as the temperature of the treatment was increased. The PI-labelled population was lower than the viable population detected by viable plate count, indicating that some cells can maintain their membrane integrity to some extent, although they were not able to grow. Oxonol fluorescence increase was proportional to the temperature of the treatment, showing a good resolution among the different states caused by heat treatments. Rh and calcein fluorescence decreased after heat treatments, indicating that membrane potential and esterase activity were respectively lost. The main decrease in calcein fluorescence was observed after the 60°C treatment, indicating that esterase activity was quickly lost. Staining with SYTO-13 did not reflect the viability changes

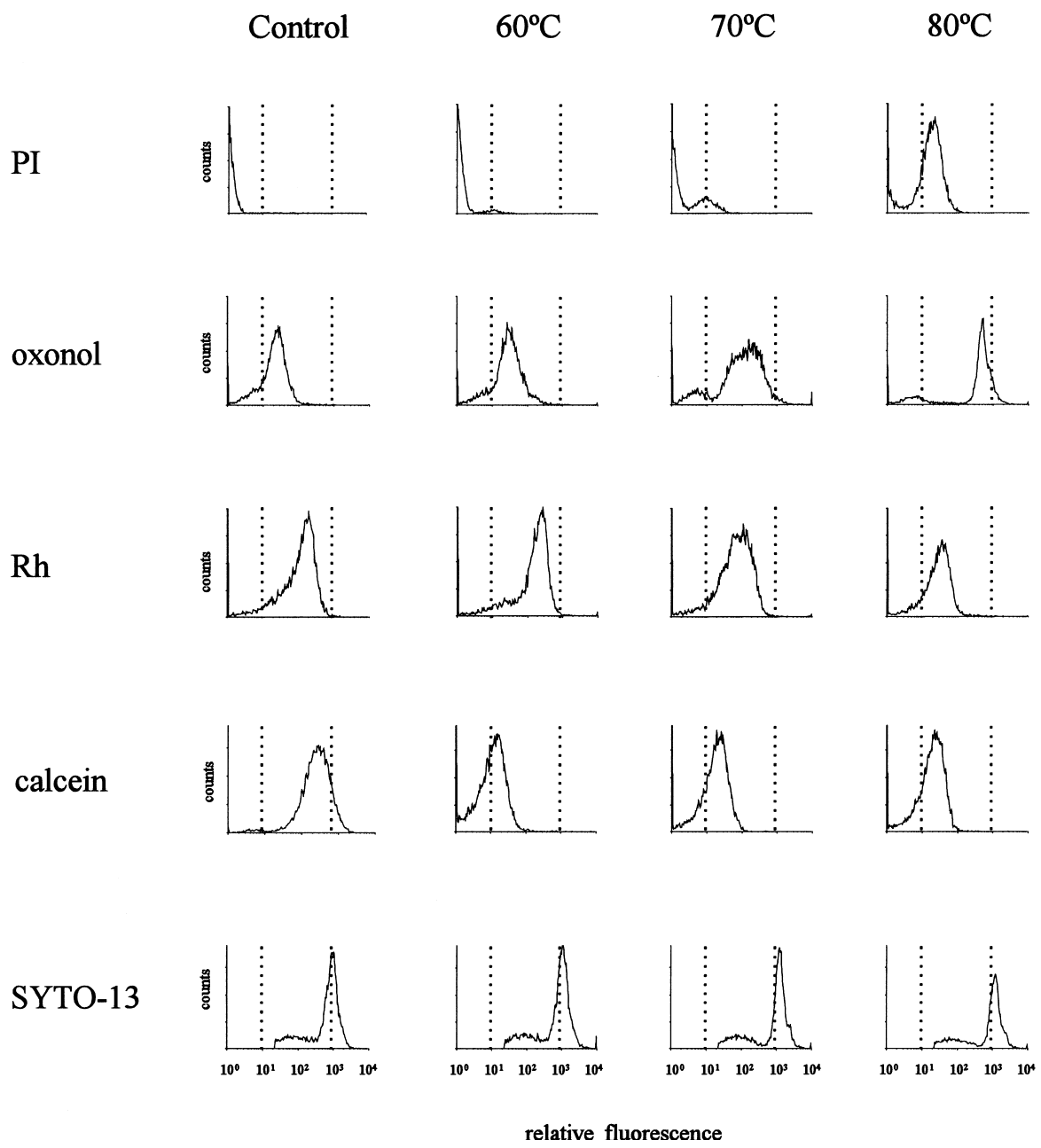

Fig. 4. Histograms obtained for the five studied fluorochromes after heat treatment.

provoked by the temperature treatments, indicating that these temperatures kill cells but do not lyse cells nor hydrolyse nucleic acids in *S. aureus*. The small differences observed among the SYTO-13 histograms corresponding to 60, 70 and 80°C heat treatments, may originate in the melting effect on nucleic acids, thus reducing the binding between dye and DNA. Oxonol, Rh and calcein were useful markers to assess the viability losses after heat treatment, but oxonol was the best to label heat killed cells.

Treatment of cell suspensions with formaldehyde at 2% resulted in a residual viability <0.2%. Fig. 5 shows the labelling obtained with the five studied fluorochromes after formaldehyde exposure. PI was not able to label significantly in these conditions, suggesting that permeabilization was not incremented by formaldehyde. Calcein, Rh and particularly oxonol were the most resolutive markers for viability, oxonol being the best fluorochrome to label dead cells. SYTO-13 labelling gave a lower and

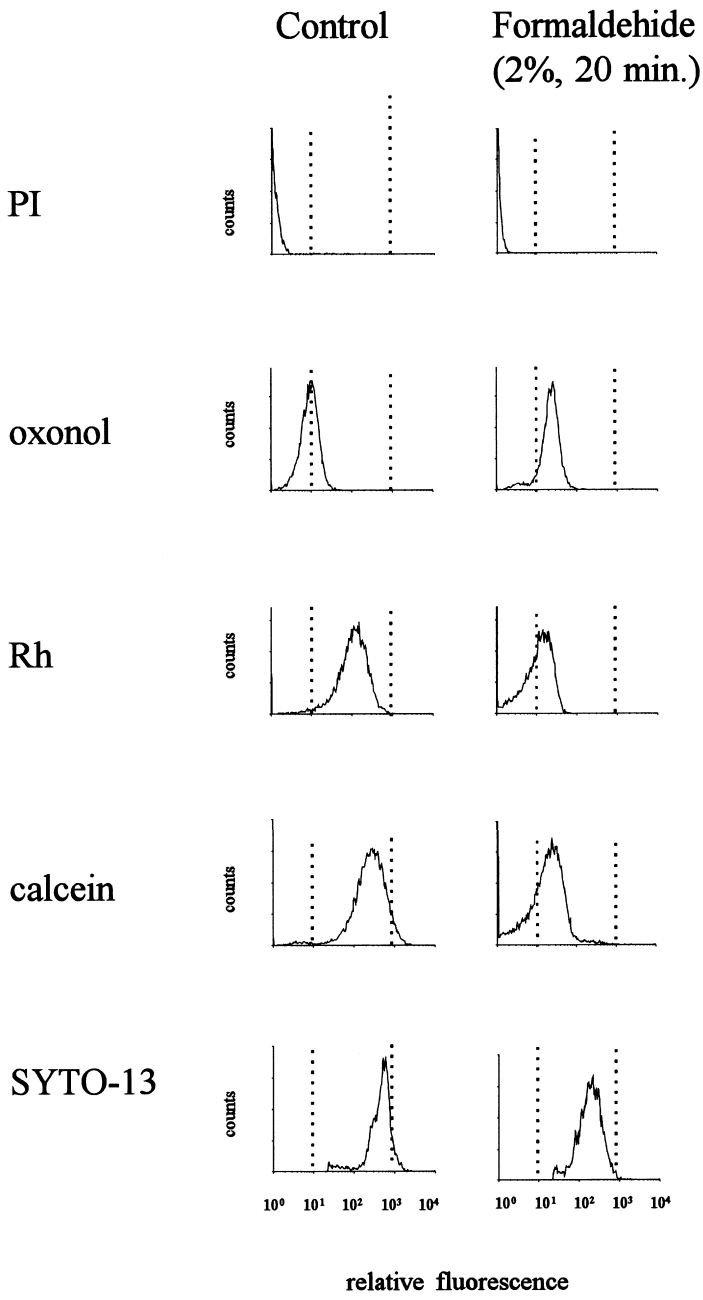

Fig. 5. Histograms obtained for the five studied fluorochromes after formaldehyde treatment.

broader fluorescence distribution, suggesting that formaldehyde treatment changes DNA interaction with this dye.

When cells were treated with gramicidin-S at 2, 5 and 10 μg/ml, the resulting viability assessed by plate counts were 6%, 3% and <0.2% respectively. Fluorescence histograms after gramicidin-S exposure are shown in Fig. 6. Oxonol, Rh and calcein shown

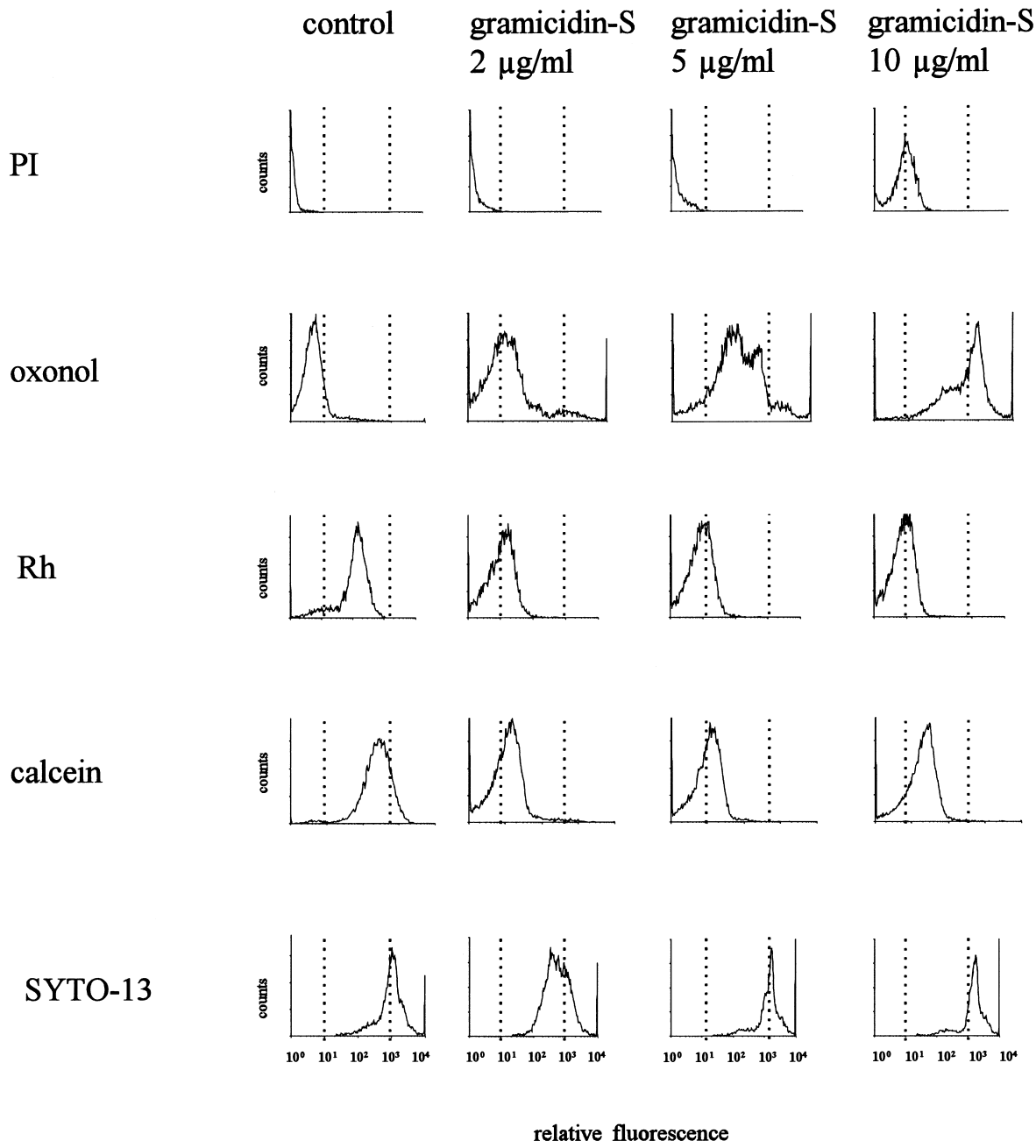

Fig. 6. Histograms obtained for the five studied fluorochromes after gramicidin-S treatment.

clear-cut differences between dead and alive bacteria, although oxonol was again the best marker for dead cells. PI labelled significantly only at the high (10 μg/ml) concentration of gramicidin-S and Rh fluorescence did not label even at low (2 μg/ml) gramicidin-S concentrations. These results are consistent with the fact that gramicidin-S is an ionophore for cell membranes that transport ions non-specifically [2,14]. Esterasic activity assessed by calcein fluorescence was not detected at any of the gramicidin-S concentrations used.

3.3. Comparison of fluorochrome labelling

A comparison among the five studied markers for the three lethal treatments using the percentage of fluorescence variation calculated according Section 2.6, is shown in Table 1. Oxonol gave the highest relative values for all treatments. PI and oxonol fluorescence increases with the strength of the treatment, as they can be accumulated inside the cell when homeostatic conditions are lost. On the other hand, calcein and Rh label viable cells but cells lost their fluorescence when the cell membrane was severely damaged. Changes in Rh or calcein fluorescence can be seen with even mild treatments and did not increase significantly when the strength of the treatment was increased (as happened with viable counts). Calcein fluorescence is rapidly lost in all treatments, showing a regular behaviour. Changes in SYTO-13 did not seem to correlate with the strength of the treatment but with the DNA or RNA accessibility. The high fluorescence of this dye allows a better detection and quantification of *S. aureus* than using viable and particle analyser counts (Figs. 1 and 2).

The main limitation of flow cytometry to assess bacterial viability is that the today available fluorochromes are not of universal use for two reasons: (i) cell permeability for fluorochromes is variable depending on strains and their physiological state; (ii) interaction between fluorochromes and their biological target is also variable depending on strains and their physiological state. The ability to detect and enumerate *S. aureus* by flow cytometry has been previously reported using FITC-immunoglobulin G, Rh, PI and Hoechst 33342 [16]. We provide additional evidence for potential applications of calcein, oxonol and SYTO-13 to count and study *S. aureus*. Although scatter changes often do not correlate with changes in bacterial cell size [13,16,24], the scatter histograms indicated that the cultures we have used are rather homogeneous populations (data not shown). However, the fluorescence histograms shown in Figs. 4–6 indicate that axenic cultures of *S. aureus* may not be as homogenous as expected as per the heterogeneity of its responses to heat, formaldehyde and gramicidin-S. In fact, flow cytometry has been used as a recent tool for assessing heterogeneous bacterial populations [12–14,24,25] and may provide deeper insights into basic and applied bacteriological studies.

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Table 1
Comparison of the results obtained among the five studied fluorochromes and the three lethal treatments

	PI	Oxonol	Rh	Calcein	SYTO-13
Heating 60°C, 2 min	5	21	33	−74	14
Heating 70°C, 2 min	47	300	−34	−59	27
Heating 80°C, 2 min	909	1043	−73	−57	21
Formaldehyde 2%	6	137	−90	−51	−56
Gramicidin-S, 2 μg/ml	11	88	−88	−73	−45
Gramicidin-S, 5 μg/ml	24	2010	−91	−75	35
Gramicidin-S, 10 μg/ml	602	7711	−92	−47	47

Results are expressed as a percentage of variation in the mean fluorescence with respect to an untreated control (see Section 2.6).

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References

[1] D. Lloyd, Flow cytometry in microbiology, Springer–Verlag, London, 1993.
[2] H.M. Shapiro, Practical flow cytometry, third. ed., Willey, New York, 1995, pp. 412–425.
[3] G.J. Vesey, J. Narai, N. Ashbolt, K. Williams, D. Veal, Detection of specific microorganisms in environmental samples using flow cytometry, in: Z. Darynkiewicz, J.P. Robinson, H.A. Crissman, Methods in Cell Biology, vol 42, part B: Flow cytometry, second ed., Academic Press, New York, 1994, pp. 489–522.
[4] A.C. Pinder, P.W. Purdy, S.A.G. Poulter, D.C. Clark, Validation of flow cytometry for rapid enumeration of bacterial concentrations in pure cultures, J. Appl. Bacteriol. 69 (1990) 92–100.
[5] D.K. Button, B.R. Robertson, F. Jüttner, Microflora of a subalpine lake: bacterial populations, size and DNA distributions, and their dependence on phosphate, FEMS Microbiol. Ecol. 21 (1996) 87–101.
[6] P.A. del Giorgio, D.F. Bird, Y.T. Prairie, D. Planas, Flow cytometric determination of bacterial abundance in lake plankton with the the green nucleic acid stain SYTO 13, Limnol. Oceanogr. 41 (1996) 783–789.
[7] B.C. Monger, M.r. Landry, Flow cytometry analysis of marine bacteria with Hoechst 33342, Appl. Environ. Microbiol. 59 (1993) 905–911.
[8] P. Montfort, B. Baleux, Comparison of flow cytometry and epifluorescence microscopy for counting bacteria in aquatic ecosystems, Cytometry 13 (1992) 188–192.
[9] M. Trousselier, C. Courties, S. Zettelmaier, Flow cytometric analysis of coastal lagoon bacterioplankton and picoplankton: fixation and storage effects, Estuarine Coast. Shelf Sci. 40 (1995) 621–633.
[10] J. Parpais, D. Marie, F. Partensky, P. Morin, D. Vaulot, Effect of phosphorus starvation on the cell cycle of the photosynthetic prokaryote *Prochlorococcus* spp. Mar. Ecol. Prog. Ser. 132 (1996) 265–274.
[11] K. Skarstad, H.B. Steen, E. Boye, Cell cycle parameters of slowly growing *Escherichia coli* B/r studied by flow cytometry, J. Bacteriol. 154 (1983) 656–662.
[12] P. Lebaron, F. Joux, Flow cytometry analysis of the cellular DNA content of *Salmonella typhimurium* and *Alteromonas halopanktis* during starvation and recovery in seawater, Appl. Environ. Microbiol. 60 (1994) 4345–4350.
[13] R. López-Amorós, J. Comas, C. Carulla, J. Vives-Rego, Variations in flow cytometric forward scatter signals and cell size in batch cultures of *Escherichia coli*, FEMS Microbiol. Lett. 117 (1994) 225–230.
[14] R. López-Amorós, J. Comas, J. Vives-Rego, Flow cytometric assessment of *Escherichia coli* and *Salmonella typhimurium* starvation-survival in seawater using Rhodamine 123, propidium iodide and oxonol, Appl. Environ. Microbiol. 61 (1995) 2521–2526.
[15] B.K. Thorsen, O. Enger, S. Norland, K. Arne Hoff, Long-term starvation survival of *Yersinia ruckeri* at different salinities studied by microscopical and flow cytometric methods, Appl. Environ. Microbiol. 58 (1992) 1624–1628.
[16] J.P. Diaper, C. Edwards, Survival of *Staphylococcus aureus* in lakewater, Microbiology 140 (1994) 35–42.
[17] R.G. McClelland, A.C. Pinder, Detection of *Salmonella typhimurium* in dairy products with flow cytometry and monoclonal antibodies, Appl. Environ. Microbiol. 60 (1994) 4255–4262.
[18] G. Wallner, R. Amann, W. Beisker, Optimising fluorescent in-situ hybridisation with rRNA-targeted oligonucleotide probes for flow cytometry identification of microorganisms, Cytometry 14 (1993) 136–143.
[19] J. Comas, J. Vives-Rego, Assessment of Gramicidin, formaldehyde and surfactants effect on *Escherichia coli* by flow cytometry using nucleic acid and membrane potential dyes, Cytometry 29 (1997) 58–64.
[20] J.D. Mason, R. López-Amorós, R. Allman, J.M. Stark, D. Lloyd, The ability of membrane potential dyes and calcofluor white to distinguish between viable and non-viable bacteria, J. Appl. Bacteriol. 78 (1995) 309–315.
[21] R.S. Pore, Antibiotic susceptibility by flow cytometry, J. Antimicrob. Chemother. 34 (1994) 613–627.
[22] J. Trotter, WINMDI version 2.5. Flow cytometry application. Build #8. The Scripps Research Institute, California, USA, 1997.
[23] T. Guindulain, J. Comas, J. Vives-Rego, Use of SYTO-13, TOTO-1 and YOYO-1 in the study of *Escherichia coli* and marine prokaryotic populations by flow cytometry, Appl. Environ. Microbiol. 63 (1997) 4608–4611.
[24] J. Vives-Rego, R. López-Amorós, J. Comas, Flow cytometric narrow-angle light scatter and cell size during starvation of *E. coli* in artificial sea water, Lett. Appl. Microbiol. 19 (1994) 374–376.
[25] H.M. Davey, D.B. Kell, Flow cytometry and cell sorting of heterogeneous microbial populations: the importance of single-cell analyses, Microbiol. Rev. 60 (1996) 641–696.