



# Statistical analysis and biological interpretation of the flow cytometric heterogeneity observed in bacterial axenic cultures

J. Vives-Rego<sup>a,\*</sup>, O. Resina<sup>a</sup>, J. Comas<sup>b</sup>, G. Loren<sup>c</sup>, O. Julià<sup>d</sup>

<sup>a</sup>Departament de Microbiologia, Universitat de Barcelona, Av. Diagonal, 645, 08028 Barcelona, Spain

<sup>b</sup>Serveis Científico-Tècnics, Universitat de Barcelona, c/ Lluís Solè Sabarís, 1-3, 08028 Barcelona, Spain

<sup>c</sup>Departament de Microbiologia i Parasitologia Sanitàries, Facultat de Farmàcia, Universitat de Barcelona, c/ Joan XXIII 1-3, E-08028 Barcelona, Spain

<sup>d</sup>Departament d'Estadística, Facultat de Matemàtiques, Universitat de Barcelona, Gran Via, 585, 08007 Barcelona, Spain

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## Abstract

Histogram comparison and meaningful statistics in flow cytometry is probably the most widely encountered mathematical problem in flow cytometry. Ideally, a test for determining the statistical equality or difference of flow cytometric distributions will identify the significant differences or similarities of the obtained histograms. This situation is of particular interest when flow cytometry is used to study the heterogeneity of axenic bacterial populations. We have statistically measured the heterogeneity of successive cytometric measures, the modifications produced after 20 transfers from the same culture, and the differences between 20 subcultures of identical origin. The heterogeneity of the bacterial populations and the similarity of the obtained 360 histograms were analysed by standard statistical methods. We have studied bacterial axenic cultures in order to detect, quantify and interpret their cytometric heterogeneity, and to assess intrinsic differences and differences produced by laboratory manipulations. We concluded that the standard axenic cultures have a considerable intrinsic cellular and molecular heterogeneity. We suggest that the heterogeneity we have detected basically has two origins: cell size diversity and cell cycle variations.

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## 1. Introduction

Histogram comparison is probably the most widely encountered statistical problem in flow cytometry (Lampariello, 2000; Parikh et al., 1999). Ideally, a

test for determining the statistical equality of flow cytometric distributions must discount differences or similarities caused by noise, chance or random error, but identify the significant differences resulting from the underlying bias. This situation is of considerable interest when using flow cytometry to study the heterogeneity of axenic bacterial populations. Most microbiologists assume that the commonly used overnight cultures are homogeneous or insignificantly

\* Corresponding author. Tel.: +34-93-402-1485; fax: +34-93-411-0592.

E-mail address: [jvives@porthos.bio.ub.es](mailto:jvives@porthos.bio.ub.es) (J. Vives-Rego).

heterogeneous. The present paper analyses and discusses this assumption using flow cytometry and robust statistical methods.

Flow cytometry has become an important tool that combines direct and rapid assays to determine numbers, cell-size distribution and additional biochemical analysis of individual cells (Robinson, 1999; Shapiro, 1995). In fact, flow cytometry is an ideal approach to analyse bacterial heterogeneity (Davey and Kell, 1996; Vives-Rego et al., 2000). Flow cytometric cell sizing is estimated from the intensity of forward light scatter, which is used in preference to 90° scatter because of its high signal intensity and its insensitivity to subcellular structure. Forward scatter is assumed to be proportional to bacterial size (Christensen et al., 1995; Haldal et al., 1994; Button et al., 1996; Bouvier et al., 2001). The Rayleigh–Gans theory has been applied to predict bacterial size from forward scatter values (Koch et al., 1996). It has also been shown that the simplest and most satisfactory mathematical relationship between forward light scatter and bacterial diameter in axenic cultures is a second-order function (Julià et al., 2000).

In this paper, we report that the heterogeneity analysis of bacterial axenic cultures by flow cytometry shows that: (i) the normality and log-normality approach are not satisfactory; (ii) the  $\chi^2$  test is more adequate than Kolmogorov–Smirnov (K–S) and Cramér–von Mises tests when the aim is to compare independent histograms; (iii) flow cytometry reveals that standard axenic cultures have a considerable cellular heterogeneity; (iv) laboratory manipulation of the axenic cultures is the origin of important heterogeneity as detected by flow cytometry; and (v) cell size diversity and cell cycle variations are also the origin of intrinsic heterogeneity.

## 2. Materials and methods

### 2.1. Bacterial strains and culture conditions

Experiments were performed with *Escherichia coli* strain 536 (Berger et al., 1982) and *Staphylococcus aureus* ATCC 12600. Cells were grown overnight in 5 ml of Luria Broth medium after inoculation with a single colony from TSA Petri dish and incubation at 30 °C and shaking at 300 rpm. Three types (A, B and

C) of bacterial suspensions were used to analyse the heterogeneity of bacterial cultures by flow cytometry. These types were obtained according the following protocols: (A) a typical colony from a TSA plate is resuspended in 5 ml of LB and incubated 15–17 hours at 30 °C, 5  $\mu$ l of this culture are resuspended in 5 ml of NaCl 0.9%, then 2 ml is transferred to a cytometric tube and 20 independent runs of 20,000 acquisitions are processed consecutively. (B) After the incubation of a culture identically processed as in protocol A, 5- $\mu$ l aliquots are suspended in 20 independent tubes with 5 ml of NaCl 0.9%; 1 ml from each tube is then transferred to 20 other cytometric tubes, and one run of 20,000 acquisitions is performed from each cytometric tube. (C) Twenty cytometric tubes are prepared from 20 independent cultures prepared according to protocol A and one run of 20,000 acquisitions is performed from each cytometric tube. The initial colonies of protocols A, B and C came from the same Petri dish. Three types of histograms were generated from each run: FSC, SSC and fluorescence after SYTO 13 staining. The three protocols (20 runs each), applied to *E. coli* and *S. aureus* for FSC, SSC and fluorescence produced a total of 360 histograms.

### 2.2. SYTO 13 staining

Samples were stained with 2.5  $\mu$ M (final concentration) of the nucleic acid stain SYTO-13 (Molecular Probes, Eugene, OR), using frozen stock DMSO solutions at 500  $\mu$ M. The green emission from SYTO-13 was collected through a 525-nm band-pass filter. Cultures were diluted 1:100 with 9% sterile NaCl and vigorously vortexed for 10 s after the addition of SYTO 13 and subsequently incubated in the dark at room temperature for 15 min. After that, cell suspensions were kept in an ice bath prior to flow cytometric analysis.

### 2.3. Flow cytometric analysis

A Coulter Epics Elite flow cytometer equipped with an air-cooled 488-nm argon-ion laser at 15 mW power was used. Fluorescent beads (1  $\mu$ m Fluoresbrite carboxylate microspheres, Polysciences, Warrington, PA, and 4  $\mu$ m latex fluorosphere beads, Molecular Probes) were used as an internal standard for scatter

and fluorescence. 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 at a 90° angle from the laser axis. Due to the design of the closed flow chamber used, light for both side scatter and fluorescence is collected at a 90° angle, using a combination of mirror and lens to improve efficiency. Data were analysed with Elitesoft version 4.1 (Coulter Corp.) and WinMDI version 2.5 Software (Windows Multiple Document Interface, a flow cytometry application. Build # 05 03-09-1999, copyright 1993–98 Joseph Trotter, The Scripps Research Institute).

*2.4. Statistical theory and methods*

When we want to decide whether two samples come from the same population, the most well-known nonparametric test is the Kolmogorov–Smirnov (K–S) one, which is implemented in the flow cytometer. Let us consider two flow cytometry data samples,  $(n_1, n_2, \dots, n_{1024})$  and  $(m_1, m_2, \dots, m_{1024})$ , being their respective histogram frequencies, i.e. each vector component,  $n_i$  or  $m_i$ , is the total cell count in the  $i$ th channel for the respective samples. Quite simple calculations allows us to compute the empirical distribution function (Conover, 1980; Brown and Rothery, 1993) for each sample:

$$F(i) = \frac{1}{N} \sum_{k=1}^i n_k$$

$$G(i) = \frac{1}{M} \sum_{k=1}^i m_k \quad \text{for all } i = 1, 2, \dots, 1024$$

where  $N$  and  $M$  are the total count for each sample. The Kolmogorov–Smirnov statistic (K–S) is the largest difference between the two empirical distribution functions, that is,

$$D = \max_i |F(i) - G(i)|$$

This maximum is compared against  $D_{\alpha} = \lambda \sqrt{\frac{N+M}{NM}}$ , where  $\lambda$  depends on the level of significance  $\alpha$  found from statistical tables. When  $D$  is larger than  $D_{\alpha}$ , the K–S test considers that the two samples come from different population with  $1 - \alpha$  confidence level; otherwise, both samples are assumed to belong to the same

population. In particular, at  $\alpha = 0.1, 0.05$  and  $0.01$ , we have  $\lambda = 1.22, 1.36$  and  $1.63$ , respectively.

The first phase of our work was devoted to evaluating the behaviour of the K–S statistics to test whether cellular samples belonged to the same population (null hypothesis) or they do not (alternative hypothesis). As we will show in Section 3, the K–S statistic rejected the null hypothesis far more often than would be expected. The K–S test only takes into account the maximum difference between the two empirical distribution functions, regardless of the relevance of the channel where this difference appears. A same difference seems to be less relevant in a channel with few cells count than in a channel with a lot of cells. In order to cope with this drawback, in the second part of the work, we propose the Cramér–von Mises test (Conover, 1980; D’Agostino and Stephens, 1986), which takes this fact into account. This test is based on Cramér–von Mises statistic:

$$CvM_{NM} = \frac{NM}{(N + M)^2} \sum_{k=1}^{1024} (F(k) - G(k))^2 (n_i + m_i)$$

The null hypothesis is rejected if  $CvM_{NM} > t_{\alpha}$ , where  $t_{\alpha}$  depends on the level of significance (i.e. for  $\alpha = 0.1, 0.05$  and  $0.01$ , we have  $t_{\alpha} = 0.347, 0.461$  and  $0.743$ , respectively). We analysed Cramér–von Mises test behaviour and, as we show below, this test rejects as often as the K–S test does. One of the theoretical assumptions necessary to prove the statistical properties of both tests are violated, and this would be the reason for their bad behaviour. These tests are based on the empirical distribution function and they need a continuous distribution assumption (D’Agostino and Stephens, 1986), that is, one where the measured variable (in our case, the channel) is continuous. Obviously, the cytometer gives a discrete variable (number of channels) with possible values:  $1, 2, \dots, 1024$ . Owing to these considerations, we finally decided to choose an appropriate statistical test to compare two samples coming from discrete distributions. The method chosen was the well-known  $\chi^2$  test. The data of two samples can be summarized as in the following table:

$n_1$	$n_2$	...	$n_{1024}$	$N$
$m_1$	$m_2$	...	$m_{1024}$	$M$
$c_1$	$c_2$	...	$c_{1024}$	$N + M$

where  $c_i = n_i + m_i$  represents the  $i$ th channel total cells count.  $\chi^2$  test does not allow empty table cell, thus numbers less than five are not recommended (Conover, 1980). To solve this, we group together adjacent table cells when necessary. Let  $L$  be the number of table cells obtained, and  $n_i^*$ ,  $m_i^*$ ,  $c_i^*$  the respective counts. The data of the two samples can now be represented as following:

$n_1^*$	$n_2^*$	...	$n_L^*$	$N$
$m_1^*$	$m_2^*$	...	$m_L^*$	$M$
$c_1^*$	$c_2^*$	...	$c_L^*$	$N+M$

The  $\chi^2$  statistic is

$$CH = \frac{(N+M)^2}{N} \sum_{i=1}^L \frac{\left(n_i^* - c_i^* \cdot \frac{N}{N+M}\right)^2}{c_i^*(N+M - c_i^*)}$$

Asymptotically, CH has a  $\chi^2$  distribution with  $L$  degree of freedom (noted by  $\chi_L^2$ ). For a level of significance  $\alpha$ , we find  $t_\alpha$  in any  $\chi^2$  table and we reject the null hypothesis if  $CH > t_\alpha$ . That is, if  $CH > t_\alpha$ , the test considers the samples as coming from a different population.

In order to achieve all proposed comparisons by using the  $\chi^2$ , Kolmogorov–Smirnov (K–S) and Cramér–von Mises tests, the authors have developed a C-language program, available for free to nonprofit institutions upon request. The statistical analysis of normality and log-normality have been performed using the package Splus (MathSoft Engineering and Education, Cambridge, MA 02142-1521, USA).

### 3. Results

#### 3.1. Analysis of the normality and variability of the FSC, SSC and SYTO 13 staining histograms

We obtained a total of 360 histograms distributed in 18 packs of 20 runs each, after the application of the protocols detailed in Section 2.1. None of the 360 flow cytometric histograms fits the normal distribution or log-normal as already reported by Coder et al. (1994) in leukocyte phenotyping. We have compared the 360 obtained histograms (in both linear and logarithmic

scales) with the respective 360 “ideal-normal” histograms built with the mean and variance of the real histogram using the Kolmogorov test. In all cases, the normality was rejected with  $p$ -values between  $3.2 \times 10^{-9}$  and  $2.2 \times 10^{-16}$ . Furthermore, if the pack of 20 consecutive histograms corresponding to the same strain, or to the experimental protocol A, B or C, are grouped as a single sample, the normality is also rejected with even lower  $p$ -values. These extremely low  $p$ -values show that the obtained histograms are far from the normal or log-normal distribution. Thus, a classification of these histograms based on their proximity to the normal and log-normal makes no sense.

In order to assess whether the three protocols A, B and C show the same heterogeneity, we analysed the variability of every 20 consecutive runs in accordance with the conventional ANOVA analysis (Box et al., 1978), as follows:

Total variability is calculated as:

$$S_t = \sum_{i=1}^{20} \sum_{k=1}^{1024} n_k^i (k - \bar{y})^2$$

variability between runs is calculated as:

$$S_b = \sum_{i=1}^{20} N_i (\bar{y}_i - \bar{y})^2$$

and variability inside runs is calculated as:

$$S_r = \sum_{i=1}^{20} \sum_{k=1}^{1024} n_k^i (k - \bar{y}_i)^2$$

where  $(n_1^i, n_2^i, \dots, n_{1024}^i)$  and  $\bar{y}_i$  are, respectively, the histogram frequencies of  $i$ th run and their mean, the total  $i$ th run account is  $N_i = \sum_{k=1}^{1024} n_k^i$ , and  $\bar{y}$  is the mean of the grouped 20 runs (global mean). As is well known,  $S_t = S_r + S_b$ .

After this analysis, we can notice the following (see Fig. 1): (i) The variability inside any pack of 20 runs ( $S_r$ ) is similar for the three protocols A, B and C; (ii) The variability between the runs ( $S_b$ ) is different for each protocol A, B and C. In almost all cases,  $S_b$  have an increasing behavior, that is,  $S_b$  in A  $<$   $S_b$  in B  $<$   $S_b$  in C; and (iii)  $S_r$  is much larger than  $S_b$  for each protocol A, B and C. Thus,  $S_b$  appears to be a good indicator of variability differences between the protocols.

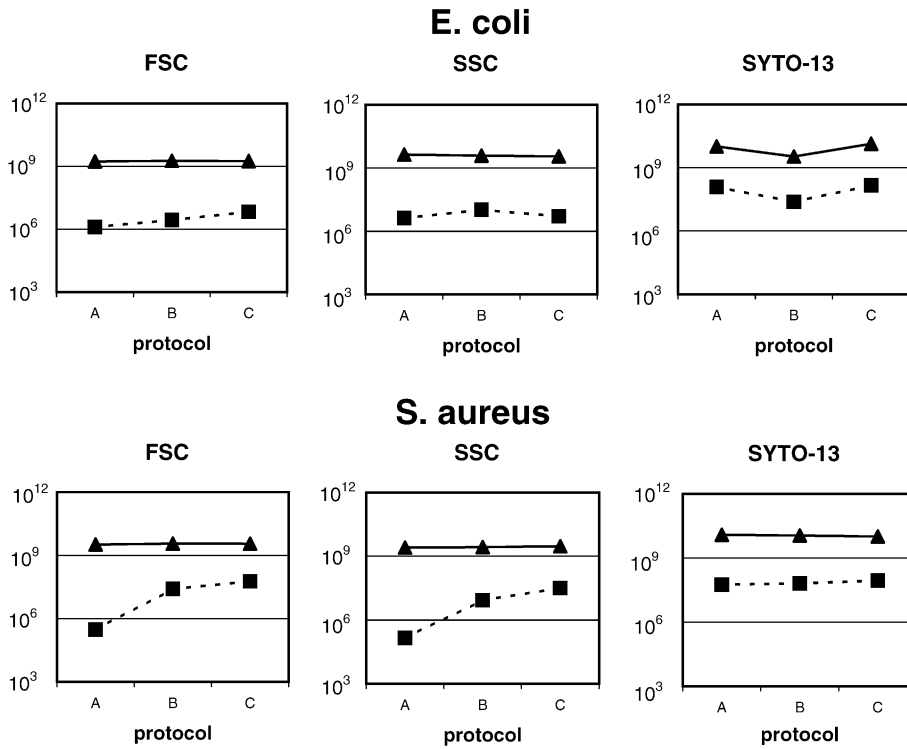


Fig. 1. Behavior of variability inside runs  $S_f$  (triangles) and variability between runs  $S_b$  (squares) for the three protocols and for each cytometric parameter (FSC, SSC and SYTO 13 fluorescence).

The detected histograms variability cannot be attributed to genetic changes that emerged during the experiment because the probability of having and selecting mutations or genetic changes affecting the cell size, cellular granulosity and cell cycle at levels detectable by flow cytometry is not appreciable. Cell size depends on the culture's age and the cell cycle. The heterogeneity of an axenic culture due to cell size and the age of the culture is high and well described (Koch, 1987). On the other hand, the effect of potential contaminants is negligible for two reasons: (i) the probability of sampling a contaminant is very low considering the total amounts of the cells, and (ii) once samples are taken, there is no proliferation in the 0.9% NaCl suspensions nor in the flow cytometry process.

3.2. Comparative analysis of the 360 histograms by Kolmogorov–Smirnov, Cramér–von Mises and  $\chi^2$  tests

For both bacteria *E. coli* and *S. aureus*, for each experimental design (A, B and C) and for each

cytometric determination (FSC, SSC, SYTO-13 fluorescence), we obtained 20 samples. In each group of 20 samples, we made all the possible comparisons, that is, each individual sample was compared vs. the other 19. This was done for three significance levels ( $\alpha=0.1$ ,  $\alpha=0.05$  and  $\alpha=0.01$ ) and for the three statistical tests: K–S, Cramér–von Mises and  $\chi^2$  test. For each case,

Table 1  
Comparison of rejection averages (in %) using the Kolmogorov test, for significance levels  $\alpha=0.1$ ,  $\alpha=0.05$  and  $\alpha=0.01$

		<i>E. coli</i>			<i>S. aureus</i>		
		$\alpha=0.1$	$\alpha=0.05$	$\alpha=0.01$	$\alpha=0.1$	$\alpha=0.05$	$\alpha=0.01$
FSC	A	63	56	42	31	25	12
	B	80	76	68	93	91	87
	C	91	89	86	96	95	92
SSC	A	40	31	16	37	30	19
	B	84	82	76	92	90	86
	C	87	82	69	98	97	96
SYTO-13	A	100	100	98	100	100	100
	B	87	81	73	99	99	99
	C	99	98	98	100	100	100

Table 2

Comparison of rejection averages (in %) using the Cramér–von Mises test, for significance levels  $\alpha=0.1$ ,  $\alpha=0.05$  and  $\alpha=0.01$

		<i>E. coli</i>			<i>S. aureus</i>		
		$\alpha=0.1$	$\alpha=0.05$	$\alpha=0.01$	$\alpha=0.1$	$\alpha=0.05$	$\alpha=0.01$
FSC	A	68	64	53	31	26	13
	B	83	79	73	94	93	89
	C	92	90	87	97	96	92
SSC	A	36	26	15	44	33	16
	B	83	80	77	89	89	86
	C	87	82	68	97	97	95
SYTO-13	A	100	100	99	100	100	100
	B	87	81	74	99	99	99
	C	98	98	97	100	100	100

we computed the number of rejections and their average. The level of significance is the probability to reject the null hypothesis when it should be accepted. In experimental protocols A, B and C, this translates into deciding that the cellular samples do not come from the same population when in fact they do. If we take data samples from the same population, we expect to reject the null hypothesis once every hundred times if  $\alpha=0.01$ , once every 20 times if  $\alpha=0.05$ , and once every 10 times if  $\alpha=0.1$ . Tables 1–3 show the percentage of the rejection average in each case for the three tests.

As can be seen, the three tests show a bad statistical behavior, that is, they reject the null hypotheses more than expected. Among them, however, the K–S and Cramér–von Mises tests give similar results, while  $\chi^2$  test results are closer to those that would be expected on the basis of the above considerations.

Table 3

Comparison of rejection averages (in %) using the  $\chi^2$  test, for significance levels  $\alpha=0.1$ ,  $\alpha=0.05$  and  $\alpha=0.01$

		<i>E. coli</i>			<i>S. aureus</i>		
		$\alpha=0.1$	$\alpha=0.05$	$\alpha=0.01$	$\alpha=0.1$	$\alpha=0.05$	$\alpha=0.01$
FSC	A	22	19	13	11	6	1
	B	49	46	35	69	63	55
	C	74	67	58	82	79	77
SSC	A	24	18	13	19	15	10
	B	70	64	54	77	73	66
	C	51	44	34	92	90	86
SYTO-13	A	92	89	86	94	93	91
	B	90	88	83	99	99	99
	C	98	98	97	100	99	99

Table 4

Mean values of the maximal distances ( $D$ ) between the distribution function for the 18 packs of 20 runs each

Average of distance $D$	Critical value at 95%	Citometric parameter	Experimental protocol	Bacteria
0.010513	< 0.013002	FSC	A	<i>S. aureus</i>
0.011815	< 0.013000	SSC	A	<i>S. aureus</i>
0.014257	> 0.013581	SSC	A	<i>E. coli</i>
0.017208	> 0.013584	FSC	A	<i>E. coli</i>
0.023397	> 0.013600	SSC	C	<i>E. coli</i>
0.025231	> 0.013601	FSC	B	<i>E. coli</i>
0.025978	> 0.013114	SYTO-13	B	<i>E. coli</i>
0.028251	> 0.013600	SSC	B	<i>E. coli</i>
0.037983	> 0.013601	FSC	C	<i>E. coli</i>
0.051186	> 0.013000	SSC	B	<i>S. aureus</i>
0.053629	> 0.013602	FSC	B	<i>S. aureus</i>
0.081364	> 0.013602	FSC	C	<i>S. aureus</i>
0.088747	> 0.013600	SSC	C	<i>S. aureus</i>
0.103433	> 0.013074	SYTO-13	B	<i>S. aureus</i>
0.131843	> 0.013172	SYTO-13	C	<i>E. coli</i>
0.134305	> 0.013394	SYTO-13	A	<i>E. coli</i>
0.141527	> 0.013178	SYTO-13	C	<i>S. aureus</i>
0.172113	> 0.013260	SYTO-13	A	<i>S. aureus</i>

### 3.3. Analysis of the distances ( $D$ ) obtained by the Kolmogorov–Smirnov test

For each pack of 20 runs, there are 190 different binary comparisons; for each comparison, we have computed the maximum distance  $D$  (maximum distance between the empirical distribution functions). We ordered the average of statistic  $D$  for all cases in Table 4. If  $D$  were lower than the critical value for the 95% of these comparisons, we could expect that the mean of these 190 values of  $D$  was less than the critical value too. This is only true, however, for the *S. aureus* in FFS and SSC cytometric determination and protocol A. For the other 16 packs, the mean of  $D$  is greater than the critical value, with  $D$  in some cases exceeding this critical value by 12 times.

## 4. Discussion

The 360 histograms analysed here (one for each run) do not show a normal nor log-normal distribution and are heterogeneous and highly different among each other. The best test to compare them is the  $\chi^2$  test, perhaps because it takes into account the discrete nature of the data. The fact that our case had a lot of data in

each sample (20,000 counts per run) makes it more difficult to choose a good test in order to compare two histograms. In addition, when the sample size is very large, statistics detect small differences. These results are coherent with the fact that the histograms of the studied populations cannot follow normal distributions by definition if we consider that in any bacterial culture the frequency of newborn cells is fourfold the frequency of incipient dividing big cells according to the canonical size distribution model (Koch, 1987).

In fact, a relatively recent new approach to solve comparisons among flow cytometry histograms from human cells, using a probability binning comparison based on a variant of the  $\chi^2$  test, has been reported by Roederer et al. (2001). We have not checked the suitability of this new approach to axenic bacterial cultures for two reasons: (i) the aim of our paper was to check the suitability of the standard and most common tests, and (ii) binning comparison requires more complex calculations.

The effects of mutations on gene products and the cellular characteristics may be silent mutations, inactivation of genes and losses, deletions, modifications or increases of activity. Assuming the mutation rate to be  $1 \times 10^{-9}$  mutations per nucleotide and per generation, and that *E. coli* and *S. aureus* have  $3 \times 10^6$  nucleotides (base pairs), then we will have  $(1 \times 10^{-9})(3 \times 10^6) = 0.0033$  mutations per cell and per generation. This is quite similar to the experimental value reported by Drake et al. (1998) and Ochman et al. (1999). Assuming that a maximum of 5% of the mutations have consequences in the gene product (the amino acid change) and the rest are silent and that the mutations that change the amino acid do not necessarily modify the function of the protein, then there is a negligible probability of having a mutation that affects the cytometric parameters we quantify.

*S. aureus* shows more homogeneous results than *E. coli*, probably because a cocci suspension is more homogeneous than a cocci–bacilli suspension. In the case of the SYTO 13 histograms, we observe a much higher variability than in the FSC and SSC histograms. This may be explained in line with the recent proposal of Guindulain and Vives-Rego (2002), which indicates that the final SYTO 13 staining fluorescence depends not only on the quantitative nucleic acid content but also on its topology and supercoiling, phenomena which are in turn influenced by environmental factors.

The histograms obtained using experimental protocol A are more homogeneous than those obtained using protocol B, and those from B are more homogeneous than the histograms obtained using protocol C. This is shown by the three statistical tests we have performed and could be a clear indication that manipulation and independent cultures are the origin of the detected differences.

The detected lack of homogeneity can only be observed and quantified at present by the high precision and sensitivity of flow cytometry, which has basically two origins: diversity in cell size and cell cycle variations. However, the canonical size distribution of bacterial cultures (Koch, 1987) may be modified by environmental factors like media composition. At present, a sampling protocol that guarantees that sample cell populations are always in the same moment of the cell cycle does not exist.

The biological interpretation of the observed lack of homogeneity in the cell suspensions is that, during growth, the bacterial cell has a tremendous capacity for adaptation and evolution. However, the specific cause of the observed cytometric variability and the precise biological consequence of this variability remain to be determined. The effects of environmental factors on canonical size distributions have not been studied to date. Consequently, and according to Koch (1987), samples removed from balanced growth could have radically different properties if taken at slightly different times.

The possibility of having a tool like flow cytometry for the purpose of rapidly and accurately assessing cell size distribution in a culture is, at least in theory, crucial for genetic, molecular and microbial biotechnology scale-up studies.

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