Microbiology (2005), **151**, 749–755 **DOI** 10.1099/mic.0.27460-0

Skew-Laplace distribution in Gram-negative bacterial axenic cultures: new insights into intrinsic cellular heterogeneity

Olga Julià[1] and Josep Vives-Rego[2]

[1]Departament d'Estadística, Facultat de Matemàtiques, Universitat de Barcelona, Gran Via, 585, 08007-Barcelona, Spain

[2]Departament de Microbiologia, Universitat de Barcelona, Av. Diagonal, 645, 08028-Barcelona, Spain

Correspondence
Josep Vives-Rego
jvives@ub.edu

Received 2 July 2004
Revised 18 November 2004
Accepted 24 November 2004

The application of flow cytometry and skew-Laplace statistical analysis to assess cellular heterogeneity in Gram-negative axenic cultures is reported. In particular, fit to the log-skew-Laplace distribution for cellular side scatter or 'granulosity' is reported, and a number of theoretical and applied issues are considered in relation to the biological significance of this fit.

INTRODUCTION

The counting, sizing and distribution analysis of particles is a task common to such diverse fields as archaeology, medicine, geology, biology and technology. Sand grains, crystals, droplets and biological cells represent diverse materials studied in different disciplines that have generated a wide variety of statistical techniques to process particle size and distribution data. It was not until the work of Barndorff-Nielsen and coworkers (Bagnold & Barndorff-Nielsen, 1980; Barndorff-Nielsen, 1977) that a coherent statistical approach was formulated by introducing log-hyperbolics as a suitable model for particle-size distribution. Fieller *et al.* (1992) presented the skew-Laplace distribution as a simple but effective model for particle size. It is easily computed, with the flexibility to handle complex datasets. More recently, several properties, generalizations and applications of the skew-Laplace distribution have been reported (Kotz *et al.*, 1998), demonstrating that it is a natural and sometimes superior alternative to the conventional Gaussian (normal) distribution. Specifically, the unsuitability of the normal distribution to describe bacterial-size distribution in axenic cultures has been reported repeatedly (Koch, 1987; Vives-Rego *et al.*, 1994; Wagensberg *et al.*, 1988). More recently, the intrinsic cellular heterogeneity of bacterial axenic cultures has been substantiated through flow cytometry and advanced statistical analyses (Vives-Rego *et al.*, 2003).

Flow cytometry has become an important tool in microbiology, as it combines direct and rapid assays to determine numbers, cell-size distribution and additional biochemical analysis of individual cells (Robinson, 1999; Shapiro, 2003; Vives-Rego *et al.*, 2000). This makes it particularly attractive in the study of heterogeneous bacterial populations (Davey & Kell, 1996; Vives-Rego *et al.*, 2000). Flow cytometry cell-size estimates are based on the intensity of forward light scatter (FS), which is used in preference to 90° scatter or side light scatter (SS) because of its high signal intensity and its insensitivity to subcellular structure – conventionally described as 'granulosity'. FS is normally assumed to be proportional to bacterial size (Christensen *et al.*, 1995; Julià *et al.*, 2000; Koch *et al.*, 1996; López-Amorós *et al.*, 1994), although the relationship between particle size and FS is not monotonic, as it is also affected by cell structure and chemical composition (Shapiro, 2003).

Studies on the heterogeneity of bacterial axenic cultures are scarce, despite the fact that there is an obvious need to understand its morphological, biochemical and genetic bases. In addition to this, the understanding and monitoring of the internal heterogeneity of axenic cultures is crucial to an understanding of species evolution and diversity. Bacterial macromolecules are constantly synthesized and decomposed at variable rates, due to thermal fluctuations that affect cells. Even in carefully controlled experiments, the quantities and types of bacterial molecules vary from cell to cell, producing cellular-composition fluctuations that may be the origin of the detected spatial inhomogeneity in axenic cultures. Also, a general relationship has been reported between fluctuation and response in *Escherichia coli* clones that might prove useful in predicting evolution rate (Kaneko & Yomo, 1994; Ko *et al.*, 1994). It is assumed that such fluctuations are inevitable in living organisms.

To our knowledge, log-skew-Laplace has never been applied to the size distribution of bacteria, nor as a complement to flow-cytometric statistics. In this paper, we report fit to the log-skew-Laplace distribution for SS values in

Abbreviations: FS, forward light scatter; SS, side light scatter.

Gram-negative axenic cultures and we analyse various theoretical and applied considerations related to their biological properties.

METHODS

Bacterial strains, culture conditions and dispersing methods. Experiments were performed with the following strains: *E. coli* strain 536 (Berger *et al.*, 1982) and strains 31 and 41; the latter two strains are Gram-negative bacteria isolated in our laboratory on brain heart infusion (Oxoid) from the intestinal faeces of laboratory mice (*Mus musculus*). Bacteria were grown overnight in Luria broth medium by incubation at 30 °C and shaking at 300 r.p.m. Cell aggregates were dispersed by adding Triton X-100 at 1 % and sonicated for 1 min at 100 W in a water bath (Ultrasonic Clinic; Funjilab).

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 μm Fluoresbrite carboxylate microspheres; Polysciences Inc. and 4 μm latex fluorosphere beads; Molecular Probes) were used as an internal standard for scatter and fluorescence. The FS detector in the Elite flow cytometer is a photodiode that collects light between 1·5 and 19° from the laser axis and is able to detect particles as small as 0·5 μm in diameter. The SS detector is situated at a 90° angle from the laser axis. Due to the design of the closed flow chamber used, light for both SS and fluorescence is collected at an angle wider than 90°, using a combination of mirror and lens to improve efficiency. Data were analysed with Elitesoft version 4.1 (Coulter Corporation) and WinMDI version 2.5 (Trotter, 1999) software.

Cell-size determination. Cell sizes were determined with an electronic particle-size analyser, Multisizer II (Coulter Corporation), with an aperture tube of 30 μm diameter and processing 100 μl cell suspension in 0·9 % NaCl, previously filtered through 0·2 μm pores. Three types of size measurement were obtained after the transformation of the electric pulses generated by the counter: diameter, volume and revolution surface. Data were analysed by AccuComp software version 1.15 (Coulter Corporation). Files generated by the particle-size analyser (Multisizer II) were exported in an ASCII (tab-delimited) format. Listmode files generated by the flow cytometer were opened with WinMDI software version 2.5 (Trotter, 1999). FS and SS were saved as a single parameter in an FCS ASCII format. The files thus generated were opened and formatted to a single column by using a tailored Microsoft Word macro.

Statistical theory and models. Our goal was to find a satisfactory fit, should one exist, between the measurements yielded by the flow-cytometric scatters and the skew-Laplace distribution. The relationship between Multisizer II measures and the skew-Laplace distribution was also explored. Previous work has shown that size distributions given by the flow cytometer and other methods are not normal (Koch, 1987; Koch *et al.*, 1996; López-Amorós *et al.*, 1994; Vives-Rego *et al.*, 1994, 2000; Wagensberg *et al.*, 1988). Other distributions have been tested in order to measure particle size in various settings (Bagnold & Barndorff-Nielsen, 1980; Barndorff-Nielsen, 1977; Fieller *et al.*, 1992) and the hyperbolic and skew-Laplace distributions stand out from amongst them.

Our data clearly feature asymmetrical tails, explaining why a fit to the normal distribution has not been found. The skew-Laplace distribution allows a different shape for each of its tails, suggesting its adequacy for the present problem. We prefer the skew-Laplace distribution over the hyperbolic one, due to its simpler formula and easy parameter estimation. Estimation of the four parameters of hyperbolic distributions has been shown to be unstable (Fieller *et al.*, 1992), with nearly identical hyperbolic distributions being representable by different parameter–value combinations. An in-depth study on the skew-Laplace distribution was reported by Kotz *et al.* (1998). The skew-Laplace distribution has three parameters, as shown below:

$$f(x;\ \alpha,\ \beta,\ \mu)=\begin{cases}\frac{\alpha\beta}{\alpha+\beta}\exp\{-\alpha(\mu-x)\} & \text{if } x\leqslant\mu\\ \frac{\alpha\beta}{\alpha+\beta}\exp\{-\beta(x-\mu)\} & \text{if } x>\mu\end{cases}$$

where μ is the mean and the parameters α and β describe the left- and right-tail shapes, respectively. A value of α greater than β suggests that the left tail is thinner and, thus, that there is less population to the left side of μ than to the right side; the opposite is of course true if β is greater than α. If $\alpha=\beta$, the distribution is symmetrical. Data yielded by the flow cytometer take the form:

$$\begin{pmatrix}x_1, x_2, \Lambda, x_n\\ f_1, f_2, \Lambda, f_n\end{pmatrix}$$

Λwhere x_i represents channel and f_i represents frequency. The maximum-likelihood estimations of α and β have explicit expression; only the estimation of μ must be obtained numerically through a simple algorithm (Kotz *et al.*, 1998).

Maximum-likelihood estimation of μ. We define the following functions:

$$f(\theta)=\frac{1}{n}\sum_{i=1}^{n}(x_i-\theta)^{+}f_i$$

$$g(\theta)=\frac{1}{n}\sum_{i=1}^{n}(x_i-\theta)^{-}f_i$$

$$h(\theta)=2\log\left[\sqrt{f(\theta)}+\sqrt{g(\theta)}\right]+\sqrt{f(\theta)}\sqrt{g(\theta)}$$

where

$$(x_i-\theta)^{+}=\begin{cases}x_i-\theta & if \quad x_i\geqslant\theta\\ 0 & if \quad x_i>\theta\end{cases}\qquad(x_i-\theta)^{-}=\begin{cases}\theta-x_i & if \quad x_i\leqslant\theta\\ 0 & if \quad x_i>\theta\end{cases}$$

The maximum-likelihood estimation of μ is the value x_i, which minimizes function h evaluated at the points x_1, x_2, Λ, x_n, given that this minimum is not reached at either extreme, i.e.

$$\hat{\mu}=x_i \quad if\ only\ if \quad h(x_i)=\min\{h(x_1),h(x_2),\Lambda,h(x_n)\}$$

$$\text{and} \quad x_i\neq Max\ or\ Min$$

where *Max* and *Min* are the greatest and smallest observed values.

Maximum-likelihood estimations of α and β. Once the maximum-likelihood estimation of μ has been determined, we obtain the estimations of the remaining two parameters by using the expressions:

$$\hat{\alpha}=\left[\left(\sqrt{f(\hat{\mu})}+\sqrt{g(\hat{\mu})}\right)\sqrt{f(\hat{\mu})}\sqrt{g(\hat{\mu})}\right]^{-1}$$

$$\hat{\beta}=\left[\sqrt{f(\hat{\mu})}+\sqrt{g(\hat{\mu})}\right]^{-1}$$

All computations were made by using MatLab (MathWorks) and a tailored program to compute parameter estimations, available on request.

The Laplace distribution can be regarded as a mixture of normal distributions. A symmetrical Laplace ($\alpha=\beta$) random variable X is represented as follows (Kotz *et al.*, 1998):

$$X\overset{d}{=}\mu+\frac{1}{\alpha}\sqrt{2W}Z$$

where $\overset{d}{=}$ means equally distributed and W and Z are independent

random variables quantifying standard exponential and standard normal, respectively. Informally, we can say that a symmetrical Laplace random variable is a normal random variable with mean μ and random variance, distributed exponentially. For any random variable with a skew-Laplace distribution, one can make a similar interpretation, only that the mean is also random, i.e.

$$X \stackrel{d}{=} \mu + \left(\beta^{-1} - \alpha^{-1}\right) W + \sqrt{\frac{2}{\alpha\beta} W} Z \qquad (A)$$

where, as above, W and Z are the independent random variables standard exponential and standard normal, respectively (Kotz *et al.*, 1998).

In order to assess the adequacy of fit, we used two techniques, graphical and numerical. Plots of data quantile versus skew-Laplace quantile (quantile–quantile plot) were obtained. Graphically, the closer this plot is to a straight line, the better the fit. To quantify the suitability of the skew-Laplace distribution, we calculated the critical size, N_{crit}, as proposed by Fieller *et al.* (1992). This statistic can be interpreted as the critical sample size required to just detect a lack of fit at the 5 % level. The critical size, N_{crit}, is a statistic based on the χ^2 goodness-of-fit test, which, as we have shown (Vives-Rego *et al.*, 2003), is more appropriate in our context (flow-cytometer or Multisizer II data) than other tests of goodness of fit, such as Kolmogorov. A comprehensive description of goodness-of-fit tests was described by Conover (1971). N_{crit} is defined as

$$N_{crit} = \frac{\chi^2_{k-m-1;\,0\cdot95}}{\sum_{i=1}^{k} \left\{ r_i - p_i(\hat{\theta}) \right\}^2 / p_i(\hat{\theta})}$$

where k represents the number of intervals, m the number of estimated parameters and r_i and $p_i(\hat{\theta})$ the sample proportion and the estimation skew-Laplace probability of the respective interval. We standardized the procedure so as to ensure that there were 40 identical intervals for every sample.

RESULTS

The skew-Laplace distribution of bacterial sizes

The Multisizer II cell-size measurements of the Gram-negative axenic cultures do not fit with either the skew-Laplace or log-skew-Laplace distribution at any time during incubation (see the low values of N_{crit} in Table 1). A possible reason for this bad fit may have been the existence of irregular cell aggregates. However, sonication treatments in the presence or absence of a mild detergent, such as Triton X-100, did not improve fit (Table 1; Fig. 1), indicating that cell aggregation is not a major cause for poor fit.

Flow-cytometric FS values are related to bacterial size by a second-order function (Julià *et al.*, 2000). As with the Multisizer II data, there was poor fit to both the skew-Laplace and log-skew-Laplace distributions throughout incubation (Fig. 2; Table 1). Also, sonication of the cultures in the presence or absence of detergent did not improve fit. These results indicate strongly that bacterial size does not follow the skew-Laplace distribution.

The skew-Laplace distribution of flow-cytometric SS

The SS values in Gram-negative bacteria fit excellently to the skew-Laplace and also to the log-skew-Laplace

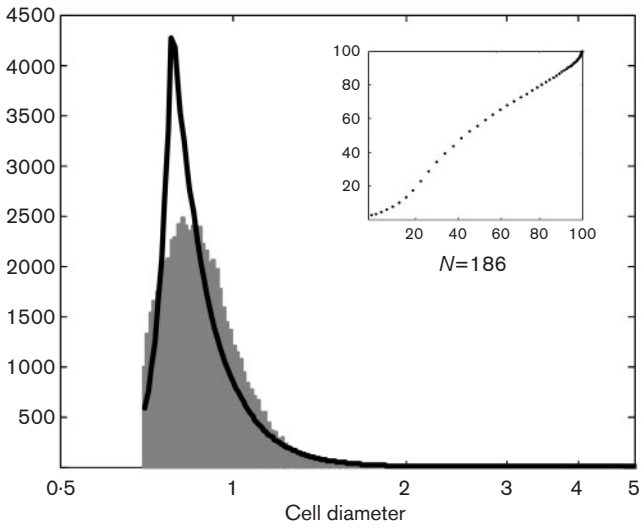

Fig. 1. Log-skew-Laplace fit of cell diameter (logarithmic scale) of an *E. coli* culture after 24 h incubation and after sonication as measured by Multisizer II. The data histogram is in grey shadow and the continuous profile is the estimated log-skew-Laplace fit. Inset, quantile plot validation (*N* is the critical number as defined in Methods and reported in Table 1). On the *y* axis is the log-skew-Laplace quantile and on the *x* axis, the data quantile.

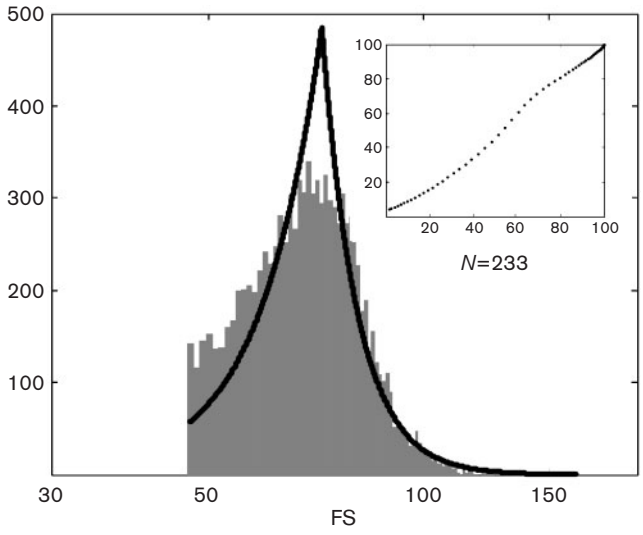

Fig. 2. Log-skew-Laplace fit of values (logarithmic scale) of the cytometric FS of an *E. coli* culture after 24 h incubation. The data histogram is in grey shadow and the continuous profile is the estimated log-skew-Laplace fit. Inset, quantile plot validation (*N* is the critical number as defined in Methods and reported in Table 1). On the *y* axis is the log-skew-Laplace quantile and on the *x* axis, the data quantile.

distribution after 24 h incubation (Fig. 3) and also after only 5 h incubation (Table 1). The fit of the skew-Laplace for SS in strain 31 after 24 h incubation shows a wide gap between the two observed values, which remains to be explained. Sonication does not improve the fit substantially (Table 1). Equation (A) shows the skew-Laplace distribution as a mixture of normal distributions. The good fit between the skew-Laplace distribution and SS values suggests that bacterial axenic cultures may consist of a number of statistically independent subpopulations. In other words, biological processes (e.g. diversity or evolution) take place separately and independently for each subpopulation of the culture.

Such a close mathematical fit suggests an underlying biological explanation, although interpretation is complex due to the facts that SS values are dependent on cell granulosity and that prokaryotic cell morphology is basically associated with ribosome density. As ribosome density in bacteria is associated with metabolic activity, the more metabolic activity, the better the skew-Laplace fit; conversely, the less metabolic activity the culture exhibits, the poorer the skew-Laplace fit. In other words, SS skew-Laplace fit indicates high cell and metabolic activity.

In general, the SS values fit better to the log-skew-Laplace

Table 1. Critical number values (N_{crit}) for the skew-Laplace and log-skew-Laplace fitting of electric sizing (Multisizer II) and cytometric values (FS and SS)

We used strain 31, strain 41 and *E. coli* incubated for 2, 5, 24 and 48 h. Also, the effect on N_{crit} of sonication and sonication in the presence of detergent (sonication + D) is reported. The obtained N_{crit} values range from 0 (impossible to fit) to 1244. We consider that for values >900, the fit is excellent and for values <500, it is unsatisfactory.

	Treatment	Skew-Laplace	Log-skew-Laplace
SS			
E. coli			
2 h	Control	322	640
	Sonication	387	972
	Sonication + D	315	645
5 h	Control	510	805
	Sonication	515	764
	Sonication + D	661	984
24 h	Control	632	587
	Sonication	576	519
	Sonication + D	676	538
48 h	Control	640	534
	Sonication	713	583
	Sonication + D	558	508
Strain 41			
24 h	Control	1182	645
	Control	1182	645
	Control	1105	674
Strain 31			
24 h	Control	344	1137
	Control	1024	1244
FS			
E. coli			
2 h	Control	345	417
	Sonication	167	180
	Sonication + D	246	298
5 h	Control	523	703
	Sonication	401	468
	Sonication + D	496	646
24 h	Control	185	233
	Sonication	197	248
	Sonication + D	209	237
48 h	Control	96	118
	Sonication	0	84
	Sonication + D	112	118
Strain 41			
24 h	Control	199	332
	Control	199	332
	Control	199	338
Strain 31			
24 h	Control	636	998
	Control	632	813
Multisizer II			
E. coli			
2 h	Control	466	296
	Control	378	285
	Sonication	410	373
	Sonication	438	449
	Sonication + D	1001	601
	Sonication + D	832	653
5 h	Control	176	144
	Control	199	157
	Sonication	223	172
	Sonication	192	162
	Sonication + D	177	170
	Sonication + D	171	150
24 h	Control	294	182
	Control	0	181
	Sonication	0	186
	Sonication	309	181
	Sonication + D	317	189
	Sonication + D	218	178
48 h	Control	240	131
	Control	252	138
	Sonication	263	170
	Sonication	261	139
	Sonication + D	265	138
	Sonication + D	266	136

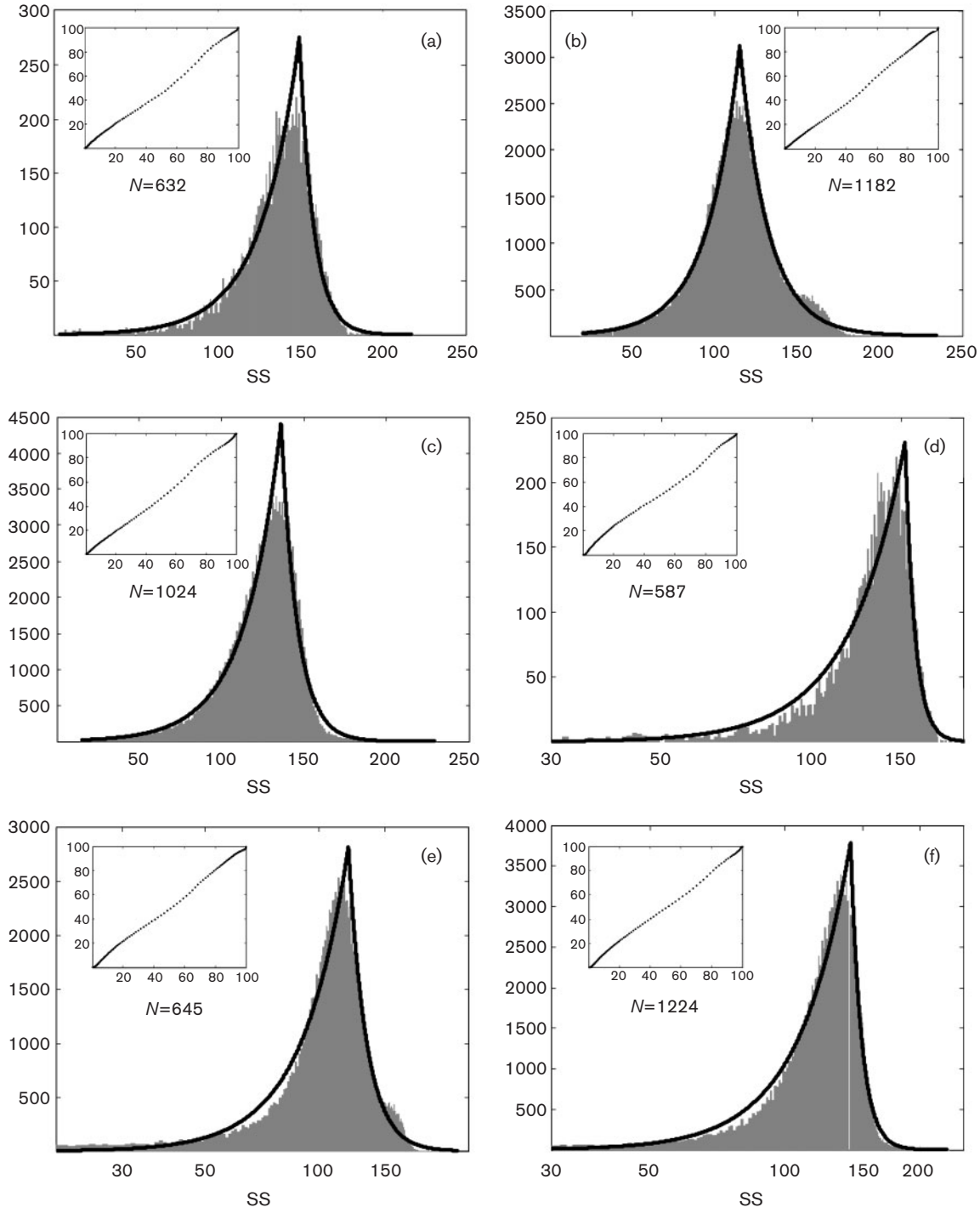

Fig. 3. Skew-Laplace (a–c) and log-skew-Laplace (d–f) fit of cytometric SS values (natural and logarithmic scales, respectively). The data histogram is in grey shadow and the continuous profile is the estimated skew-Laplace or log-skew-Laplace fit. Inset, quantile plot validation (*N* is the critical number as defined in Methods and reported in Table 1). On the *y* axis is the skew-Laplace or log-skew-Laplace quantile and on the *x* axis, the data quantile. (a, d) *E. coli* after 24 h incubation; (b, e) strain 41 after 24 h incubation; (c, f) strain 31 after 24 h incubation.

distribution than to the arithmetic skew-Laplace distribution. Fieller *et al.* (1992) have already commented that it is more appropriate to consider models based on log-size because of the wide range of sizes, but also because of the multiplicative process of breakage underlying particle production in general. These arguments are applicable

specifically to bacterial cultures as, in addition to the variability of sizes that always exists in a culture, the binary transversal division of bacteria involves a breakage process of mother cells, producing smaller daughter cells but preserving ribosome density.

Interpretations of the skew-Laplace distribution in bacterial cultures

Our interpretation of the correlation between skew-Laplace distribution and SS values in Gram-negative bacteria is as follows. SS values reflect cell granulosity, which in turn is an artefact of ribosome density. The more ribosomes, the higher a cell's metabolic activity and cellular performance is. Following this, a good skew-Laplace fit would suggest that an axenic culture is made of two ribosome-density subpopulations that are independent of each other. In other words, the population with high SS (or granulosity, ribosome density or metabolic activity) would be dynamically distinct from the subpopulation with low SS. This situation is present at various points during the incubation period, meaning that, irrespective of the cell-cycle phase, the two subpopulations with different SS coexist in the culture due to their differing biological potential. Consequently, in Gram-negative axenic cultures of any physiological state (young or old), the dominant population is more metabolically active than the non-dominant one, which is an indication that potential cellular changes or reactions towards survival or the mechanisms that generate diversity rely on the existence of two independent populations.

DISCUSSION

It is generally accepted that the life of any cell is determined by its genome and the environment. The genome is duplicated accurately and clonal populations are fully homogeneous if mutation, genetic transfer and internal genetic recombination do not drive the population to heterogeneity. Therefore, mutation, genetic transfer and selection are the main pillars of diversity and evolutionary potential in bacterial populations. A bacterium may express different phenotypes without a difference in genotype, due to differences in the microenvironment. In recent years, such differentiation has been shown to occur in bacteria without spatial information and within the same environment (Kaneko & Yomo, 1994; Ko *et al.*, 1994). Consequently, the higher the population heterogeneity, the more possibilities for genetic and phenotypic diversity a population has. In such contexts, the existence of independent subpopulations in axenic cultures enhances evolutionary and diversification processes.

The skew-Laplace distributions were originally proposed as a pragmatic alternative to the four-parameter hyperbolic family, which had proved notoriously unstable in numerical estimation. This latter family is a natural generalization of the normal distribution and there are theoretical arguments indicating that it might be an appropriate model for size distribution in a wide variety of cases. The skew-Laplace family is a subclass of this more general set. Reliable software is available for fitting hyperbolic distributions, but we have ruled out this family because it is too complex and because similarity of distribution does not necessarily imply that the parameters are similar.

A practical consequence of this newfound property of axenic cultures is that a flow-cytometric definition of high-granulosity subpopulations would be useful in the obtention and selection of mutants (experimentally or naturally). Once the high-granulosity subpopulation is sorted cytometrically, any genetic process affecting the sorted population will be more productive than in cultures containing both high- and low-granulosity populations. Spontaneous and naturally induced mutations would also be more effective in exclusively high-granulosity subpopulations than in conventional mixed cultures containing both high- and low-granulosity populations. Any experiment or natural process within axenic cultures that is intended to obtain high numbers of mutations will be more successful if applied to or occurring in the subpopulation with high granulosity, rather than in that with lower granulosity.

One biological interpretation of the fitting is that bacteria share a general mathematical distribution with small, repetitive biological and non-biological materials. This shared mathematical behaviour probably also reflects a general physical law that applies to all small particles, irrespective of whether they have a biological origin. Another biological interpretation refers to the two independent subpopulations detected by the Laplace distribution. The Laplace fitting confirms that bacterial axenic cultures are made up of two subpopulations, as reported previously by others (Koch, 1987; López-Amorós *et al.*, 1994; Vives-Rego *et al.*, 2003; Wagensberg *et al.*, 1988). In addition, the Laplace fitting shows that the distributions of the two subpopulations are mutually independent. This mathematical independence indicates that the two corresponding biological subpopulations may also be independent. If this is demonstrated, then the concept of axenic culture will need to be re-examined.

Finally, a key question is whether the fit of SS values to the skew-Laplace distribution that we have observed in three Gram-negative small bacilli is applicable to all microorganisms or whether it is only the case for Gram-negative bacteria. To answer this question, more cross-species comparative work is necessary.

ACKNOWLEDGEMENTS

We are grateful to Dr Jaume Comas-Riu (Scientific and Technical Services of the University of Barcelona) for his helpful technical assistance with the flow cytometer, the electronic particle-size analyser and the manipulation of cultures. We express our gratitude to Dr Pedro Puig (Autonomous University of Barcelona) for his suggestion regarding the Laplace distribution. We also thank the anonymous referees for their comments and suggestions.

REFERENCES

Bagnold, R. A. & Barndorff-Nielsen, O. (1980). The pattern of natural size distributions. *Sedimentology* **27**, 199–207.

Barndorff-Nielsen, O. (1977). Exponentially decreasing distributions for the logarithm of particle size. *Proc R Soc Lond A* **353**, 401–419.

Berger, H., Hacker, J., Juarez, A., Hughes, C. & Goebel, W. (1982). Cloning of the chromosomal determinants encoding hemolysin production and mannose-resistant hemagglutination in *Escherichia coli*. *J Bacteriol* **152**, 1241–1247.

Christensen, H., Olsen, R. A. & Bakken, L. R. (1995). Flow cytometric measurements of cell volumes and DNA contents during culture of indigenous soil bacteria. *Microb Ecol* **29**, 49–62.

Conover, W. J. (1971). *Practical Non Parametric Statistics*, 2nd edn. New York: Wiley.

Davey, H. M. & Kell, D. B. (1996). Flow cytometry and cell sorting of heterogeneous microbial populations: the importance of single-cell analyses. *Microbiol Rev* **60**, 641–696.

Fieller, N. R. J., Flenley, E. C. & Olbricht, W. (1992). Statistics of particle size data. *Appl Stat* **41**, 127–146.

Julià, O., Comas, J. & Vives-Rego, J. (2000). Second-order functions are the simplest correlations between flow cytometric light scatter and bacterial diameter. *J Microbiol Methods* **40**, 57–61.

Kaneko, K. & Yomo, T. (1994). Cell division, differentiation and dynamic clustering. *Physica D* **75**, 89–102.

Ko, E. P., Yomo, T. & Urabe, I. (1994). Dynamic clustering of bacterial population. *Physica D* **75**, 81–88.

Koch, A. L. (1987). The variability and individuality of the bacteria. In *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*, 2nd edn, pp. 1606–1614. Edited by F. C. Neidhardt, R. Curtiss, III, J. L. Ingraham & 7 other editors. Washington, DC: American Society for Microbiology.

Koch, A. L., Robertson, B. R. & Button, D. K. (1996). Deduction of the cell volume and mass from forward scatter intensity of bacteria analyzed by flow cytometry. *J Microbiol Methods* **27**, 49–61.

Kotz, S., Kozubowski, T. J. & Podgórski, K. (1998). *The Laplace Distribution and Generalizations*. Berlin: Birkhäuser.

López-Amorós, R., Comas, J., Carulla, C. & Vives-Rego, J. (1994). Variations in flow cytometric forward scatter signals and cell size in batch cultures of *Escherichia coli*. *FEMS Microbiol Lett* **117**, 225–229.

Robinson, J. P. (1999). *Current Protocols in Cytometry*. New York: Wiley.

Shapiro, H. M. (2003). *Practical Flow Cytometry*, 4th edn, pp. 50–52. New York: Wiley.

Trotter, J. (1999). WinMDI version 2.5 software (Windows Multiple Document Interface, a flow cytometry application. Build no. 05 9 March 1999. © 1993–1998 Joseph Trotter, The Scripps Research Institute, CA, USA).

Vives-Rego, J., López-Amorós, R. & Comas, J. (1994). Flow cytometric narrow-angle light scatter and cell size during starvation of *E. coli* in artificial sea water. *Lett Appl Microbiol* **19**, 374–376.

Vives-Rego, J., Lebaron, P. & Nebe-von Caron, G. (2000). Current and future applications of flow cytometry in aquatic microbiology. *FEMS Microbiol Rev* **24**, 429–448.

Vives-Rego, J., Resina, O., Comas, J., Loren, G. & Julià, O. (2003). Statistical analysis and biological interpretation of the flow cytometric heterogeneity observed in bacterial axenic cultures. *J Microbiol Methods* **53**, 43–50.

Wagensberg, J., López, D. & Valls, J. (1988). Statistical aspects of biological organization. *J Phys Chem Solids* **49**, 695–700.