

Effects of Deflected Droplet Electrostatic Cell Sorting on the Viability and Exoproteolytic Activity of Bacterial Cultures and Marine Bacterioplankton

OLGA RESINA-PELFORT¹, JAUME COMAS-RIU² and JOSEP VIVES-REGO¹

¹Departament de Microbiologia, Universitat de Barcelona, Barcelona, Spain

²Servei Científic Tècnic, Universitat de Barcelona, Barcelona, Spain

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Summary

The cell-sorting capability of flow cytometers makes it possible to isolate specific populations of cells with pre-defined cytometric characteristics. A better knowledge of the biological effects of the sorting process is necessary for the future cell sorting applications. In this paper we report the effects of flow cytometric sorting on bacterial viability and exoproteolytic activity (EPA) of bacterial cultures and marine bacterioplankton. Sorting bacterial cultures and bacterioplankton samples reduce viability as assessed by plate counts and produce variations in the exoproteolytic activity. These effects indicate that deflected electrostatic sorting may significantly alter the biological properties of the sorted bacteria.

Key words: flow cytometry – sort – Syto-13 – exoproteolytic activity – bacterioplankton – bacterial cultures

Introduction

The addition of cell sorting capability to a flow cytometer makes it possible to isolate specific populations of cells with precisely defined characteristics. Any parameter measurable in a flow cytometer can provide a basis for selection of cells (SHAPIRO, 1995; ROBINSON, 1999; VIVES-REGO et al., 2000). Reported applications in microbiology include the purification of spores by fluorescence activated cell sorting (COHEN et al., 1982), the selection of mutants (BETZ et al., 1984; AN et al., 1991; AZUMA et al., 1992; NIR et al., 1992) and *Cryptosporidium* detection (VESSEY et al., 1993). PORTER et al. (1995) applied sorting technique to separate antibody-stained cells, and WALLNER et al. (1997) sorted cells stained with rRNA oligonucleotide probes. Recently, coupling cell sorting with bacterial assessment activity in order to verify the correlation between bacterial size and activity has been reported (SERVAIS et al., 1999). However, data on the effects of sorting on cultured or aquatic bacteria are lacking.

Cell sorting is a technique that include a droplet generator, a droplet charging and deflecting system, sample supply dispositive with collection components, sensors for measuring cell properties by fluorescence and light scatter, electronic circuitry for processing the analyzer sig-

nals, controlling the droplet formation and for timing and generating the droplet charging pulses. Sorting can be used either to deposit single cells (cloning mode) or to collect large numbers of cells with similar properties (enrichment mode). To better understand how this complex sequence of events affects the sorted cells, we report in this paper data on sorting efficiency, recovery and purity as well as the effects of sorting on viability and exoproteolytic activity (EPA) of axenic cultures and bacterioplankton.

Materials and Methods

Strains, media and growth conditions

The bacterial strains used in this study were the following: *Escherichia coli* strain 536 (BERGER et al., 1982), *Staphylococcus aureus* ATCC 12600, *Micrococcus luteus*, isolated in our Department from a meat product, *Bacillus polymyxa* CECT 155 derived from ATCC 842, *Enterococcus faecalis* CECT 481 derived from ATCC 19433, *Salmonella typhimurium* ATCC 14028 and *Pseudomonas aeruginosa* CECT 110 derived from ATCC 10145. Strains were grown in Luria-Bertani broth (LB) at 30 °C in overnight cultures.

Sample collection and incubation of microcosms

Water from the Mediterranean sea, was collected in Barceloneta, the natural beach of Barcelona city (Spain), an area with more than 2 million people. Mesocosms consisted of 1.5 l of freshly collected sea water distributed in 3 liter-Erlenmeyer flasks and incubated in the dark at 20 °C with orbital shaking (100 rpm) in a Controlled Environment Incubator Shaker (New Brunswick, Edison, USA).

Bacterial counts

Viable counts were calculated from the colony forming units (cfu) on Tryptone and Soya Agar (TSA, ADSA-Micro) plates incubated for 5 days at 30 °C in the case of the axenic cultures and Marine Agar (Difco) plates incubated for 7 days at 20 °C in the case of the marine samples. Total counts were performed by flow cytometry as previously described (GUINDULAIN et al., 1997; VIVES-REGO et al., 1999) and briefly consisted of a staining with 2.5 µM SYTO-13 (Molecular Probes), using frozen stock DMSO (Dimethylsulfoxid) solutions at 500 µM. Samples were vigorously vortexed and incubated in the dark at room temperature for 30 min before flow cytometric analysis.

Flow cytometry

Flow cytometry was performed a Coulter Epics ELITE (Coulter Corporation, Miami, Florida) equipped with a 15 mW air-cooled 488 nm argon-ion laser was set up with the standard configuration. Fluorescent beads (1 µm Fluoresbrite carboxylate microspheres, Polysciences Inc., Warrington, USA) were used as an internal standard for scatter and fluorescence. The green emission from SYTO-13 was collected through a 525 nm band-pass filter. The forward scatter detector in the Elite flow cytometer is a photodiode which collects light between 1.5° and 19° from the laser axis; it is able to discriminate latex particles >0.5 µm in diameter. The side scatter detector is situated at right angle to the laser axis. Flow cytometric acquisitions and cell sorting operations were performed using Isoton II as sheath fluid. The composition of Isoton II (according to the supplier) is, in g per liter: Sodium chloride, 7.9; disodium hydrogen orthophosphate 1.9; EDTA, disodium salt, 0.4; potassium chloride, 0.4; sodium dihydrogen orthophosphate, 0.2; sodium fluoride, 0.3 (this substance is used as preservative of the fluid). Data were analyzed with Elitesoft version 4.1 (Coulter Corporation, Miami, Florida) and WinMDI version 2.8 software. A routine sterilization and subsequent control are needed because cell sorting is done in open compartments and subject to microbial contamination from the environment and the sheath liquids. The sterilization of the flow cytometer was done running a 5% sodium hypochlorite solution as a sample for 5 minutes, followed by a short pulse of 96% ethanol to prevent contamination between samples. The method routinely used to verify the sterility of the flow cytometry circuitry (sheath fluid) and sorting operation (recovery and purity), before assessing the effect of sort on bacterial viability, consisted of sorting a defined number of random-procedure droplets without cells onto plates and subsequent incubation.

Cell sorting recovery, purity and efficiency

The Epics Elite Autoclone sorting option, a programmable single-cell deposition system, was used to sort a defined number of cells per well into multiwell microculture plates and to sort simple bacterial cells directly onto agar plates. With this option, the user only needs to set up the sort criteria and sort delay before pre-setting the desired number of cells to be sorted. Recovery is defined as the percentage of the cells obtained at the end of the sorting process with respect to the total programmed; critically depends on the correct timing for the droplet charging pulse relative to the time point of cell sensing at the optical focus. If sensing and droplet

break-off is out of phase, sorted particles will merge, being sorted as one particle instead two, resulting in a recovery lower than optimal. Purity of the sorted cells is defined as the fraction of sorted cells that belong to the desired category. A recovery rate close to 100% guarantees that the purity will be close to 100%, because in the few cases that the desired cell was missed, the possibility for a deflected droplet to be empty is much larger than the possibility that the droplet contain the unwanted cell (LINDMO et al 1990). We check the sort recovery and purity by counting the number of fluorescent sorted beads with an epifluorescence microscope at 125 diameters, once the sorted particles have been deposited in a microscopic slide. Finally, efficiency is defined as the percentage of sorted droplets that contain only the desired one. The sorting efficiency is a function of the input analysis rate for a number droplet sorting, as well as the frequency and the coincidence of the sorted droplets (LINDMO et al., 1990). To assess the bacterial sorting recovery, purity and efficiency, stained strains with SYTO-13 were sorted on black polycarbonate filters (Millipore) and microscopically counted at a magnification of 400 diameters.

Microscopy

Microscopic examination of sorted calibration beads and bacteria to assess the efficiency of sort recovery was performed using a Jenamed 2 fluorescence microscope (CARL ZEISS, JENA, RDA), with a 12.5× Apochromat objective (0.35 8/0.17 A) and 10× oculars. Fluorescence was produced using a 50 W – Hg arc lamp with a BPF 510-filter combination.

Exoproteolytic activity (EPA)

EPA was assayed spectrofluorimetrically in a Hitachi F-2000 Fluorescence Spectrophotometer (excitation 338 nm, emission 410 nm) by following the generation of fluorescent β-naphthylamine after the addition of 1 mM L-leucyl-β-naphthylamide to the sample (FONTIGNY et al. 1991). The activity was calculated as the nmol l⁻¹ h⁻¹ amount of β-naphthylamine produced, calibrated over the range 2.75–150 mM of β-naphthylamine (FLUKA), ($Y = 0.66 X + 44.9$; $r = 0.999$). To properly compare EPA values in cultures or seawater before and after sorting, the EPA in the non-sorted sample was assayed at the same cell concentration that was tested in the sorted population, after dilution with NaCl (0.9%) or the sheath fluid, Isoton II.

Results

The recovery, purity and efficiency of sorting operations

Repeated sorts of 20 fluorescent beads were done on a slide and verified by epifluorescence microscopy. The mean recovery obtained with beads after 54 calibration operations done before and after the sorting sessions was of 98.47% (SD = 3.17) with a purity of 100%. *M. luteus* gave the best recovery and purity percentages among all the studied strains, being the same that for the beads calibrator, probably because *M.luteus* remain intact after the brutal impact on a glass surface than smaller and less resistant cells as Gram negatives. The efficiency of 3 sorts is 33% in our equipment adjustment (20 kH₃; 1000 cells per second and 98.47% recovery).

Toxicity assessment of SYTO-13, ISOTON and DMSO

When assessing the effects of cell sorting on culturability, the toxicity of the three products that are in contact with the cells during the sorting process, needs to be

evaluated in order to differentiate the effect of the sort process from the toxicity of the accompanying products. The three products that are in contact with bacteria during the sorting process are: 1) Isoton II (the fluid sheath); 2) SYTO-13 (the nucleic acid stain); and 3) DMSO (SYTO-13 solvent). Bacterial staining with SYTO-13 reduced the viability of all strains after 30 min of contact time and in accordance with this, SYTO-13 stained sorted bacteria shown less residual viability than non-stained cells (Table 1). Gram positive bacteria resulted more sensitive than other strains to the toxic effect of SYTO-13, indicating that the outer membrane is a more efficient barrier than glycopeptide as it is generally assumed with heavy metals and organic toxicants (COMAS and VIVES-REGO, 1997). Isoton II which contains sodium fluoride, was non toxic in our assessment (data not shown) and DMSO showed variable toxicity depending on the strain, but Gram positive bacteria were also more sensitive than the other strains to the toxic effect of DMSO, with the exception of *B. polymyxa* (Table 1).

Selection of the method to assess culturability of the sorted bacteria

Three methods were used to assess the culturability of sorted bacteria. One was to sort particles directly in Microtiter wells, which were transferred on agar plates and streaked with a Drigalsky loop. The second was to sort particles directly on a TSA Petri dish on a cushion of 0.1ml NaCl and streaked with a Drigalsky loop. The third system consisted of sorting 42 single cells in a 6×7 matrix directly onto TSA plates, yielding an array of 42 colonies after incubation at 30 °C for 5 days if all sorted cells are viable (Figure 1). The percentages of recovery (SD in brackets) were Microtiter, 78.10% (20.95); TSA plates, 81.20% (13.46) and 6×7 matrix, 88.10% (3.05). In consequence, of the three methods, the single cell sorting in a 6×7matrix was the best probably for four rea-

Table 1. Effect of SYTO-13 (2.5 µM) and DMSO (2,5 µM) after a contact time of 30 min. on the viability of the studied strains and reduction of the viability of the same strains after the sorting of SYTO-13 stained and non-stained cells.

| Strains | SYTO-13 | DMSO | Sorted non stained cells | Sorted cells stained by SYTO-13 |
|--------------------|---------|------|--------------------------|---------------------------------|
| | | | | |
| <i>Salmonella</i> | 92.1 | 92.8 | 52.5 | 50 |
| <i>E.coli 536</i> | 81 | 87.1 | 85.4 | 71.7 |
| <i>Pseudomonas</i> | 62.5 | 63.3 | 67.6 | 28.6 |
| <i>B.polymyxa</i> | 33.3 | 97.5 | 21.9 | 2.4 |
| <i>S.aureus</i> | 9 | 59.1 | 22.4 | 2.14 |
| <i>E.faecalis</i> | 19.2 | 53.7 | 61.9 | 52.1 |
| <i>M.luteus</i> | 1.9 | 27.0 | 41.1 | 0 |

sons: 1) it is simple and the least time consuming; 2) higher percentages of viability recovery; 3) less environmental contamination, probably due to the fact that the 6×7 matrix system requires less time and manipulation; and 4) the sorted cells are accurately positioned in pre-defined location, and so the counts are done quickly and easily.

Cell sorting effects on the viability and EPA of axenic cultures

The viability of sorted bacterial populations was reduced by 21.88–85.40%, being Gram positives more sensitive to the viability losses than Gram negatives (Table 1). If sorted bacteria were previously stained with SYTO-13, the viability losses were greater, according the toxic effect of SYTO-13 described in the precedent section. Sorted bacteria showed a high decrease in the EPA of all studied strains if compared with the EPA in the non-sorted cells used as control (Table 2). Non-sorted

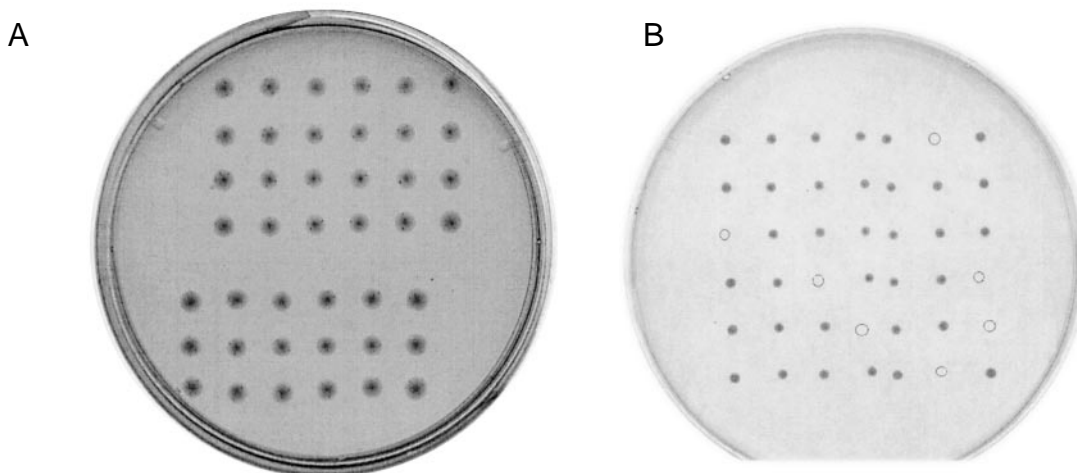


Fig. 1. A) show the 42 colonies in the 6×7 array of sorted cells from an *E.coli* overnight culture. Every sorted particle produce a colony after the incubation (30 °C, 5 days), verifying that all the sorted cells were viable. B) show only 35 colonies from a 6×7 array sorted particles as in plate A. Empty circles are the place where a non viable cell of *E. faecalis* asdeposited. Sorts in A and B are performed at the same conditions.

Table 2. Effect of cell sorting on EPA (numeric values are enzyme units).

| Strains | NaCl | Isoton | Sort |
|----------------------|-------|--------|-------|
| | Total | Total | Total |
| <i>Salmonella</i> | 0.585 | 0.477 | 0.479 |
| <i>E. Coli 536</i> | 2.8 | 1.31 | 0.46 |
| <i>P. aeruginosa</i> | 0.46 | 0.816 | 0.419 |
| <i>B. polymyxa</i> | 0.91 | 28.2 | 0.26 |
| <i>S. aureus</i> | 2.05 | 2.81 | 0.34 |
| <i>E. faecalis</i> | 0.239 | 8.28 | 0.186 |
| <i>M. luteus</i> | 4.82 | 4.28 | 1.64 |
| Sea water (*) | 12.79 | 8.43 | 2.32 |

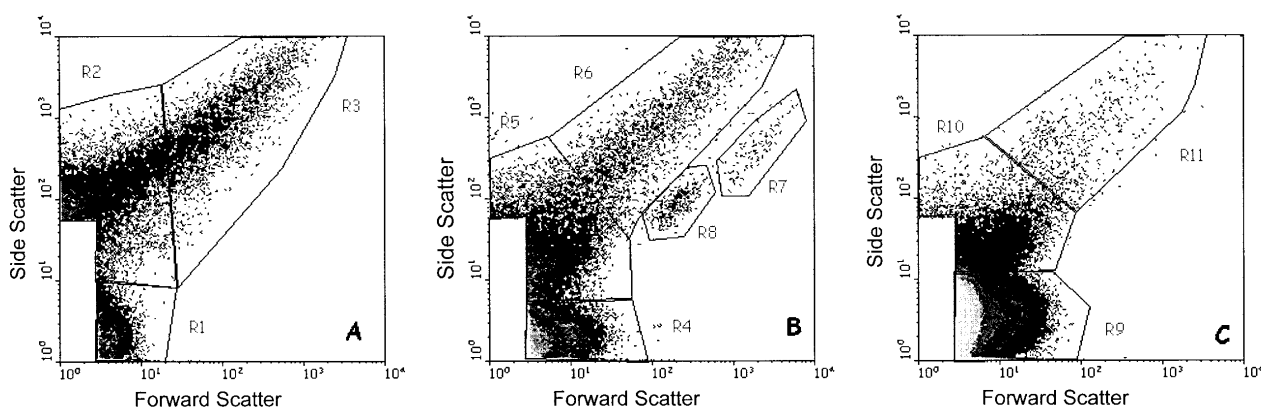
Note: In order to have comparative results, the EPA was assayed on the same number of cells (about 2×10^5 cells) when assessing the NaCl (0.9%) and Isoton effects on non-sorted cells and on sorted cells. The assay for NaCl or Isoton, was done diluting the overnight culture or marine sample (*) of the corresponding strain with NaCl up to obtain the previously sorted number of cells.

cells exhibited variable EPA values for the same strain depending on whether bacteria were suspended in NaCl or Isoton. These variations may be attributable to the effect of the Isoton components (see materials and methods section), and in particular by EDTA or sodium fluoride, which may disrupt or alter the outer membrane and cell membrane resulting in changes in the integrity of cells envelopes and membrane potential (COMAS & VIVES-REGO 1997).

Cell sorting effects on marine bacterioplankton viability and EPA

The viability of the coastal sea water samples we have studied ranged from 0.007% to 1.4%. When the same sample was incubated in the presence of nutrients, the percentage of viable bacteria increased between 0.8 to 16.3% (Figure 2). The percentage of viability in freshly collected (Figure 2, A) or incubated marine samples (Figure 2, B and C), was not the same in all the subpopulations contained in the defined gates (Figure 2, gates from R1 to R11). Since viability losses have also been observed in sorted bacteria from bacterial cultures, it remains to be determined whether the losses of viability in sorted bacterioplankton subpopulations is an intrinsic feature or the consequence of the sorting effect. When bacteria from pre-defined gates (Figure 2, gates from R1 to R11), were sorted and analyzed for viability, the gates with higher scatter showed higher percentage of viability. Such findings strongly suggest that when the bigger is a subpopulation of marine bacteria, the higher is its viability, in accordance with recent flow cytometric findings that correlate bacterioplankton size and activity (SERVAIS et al., 1999).

When total cytometric counts were compared with the sum of the total counts from all the defined gates for the same sample, a sub-estimation ranging between 0.7 and 1.9% was observed (calculations obtained from numeric values in Figure 2). Such small difference show how accurate the sorting recovery is (99.3–98.1%), similar to that obtained with beads and *M. luteus*. This confirms that, if previous adjustment of the recovery with beads and bac-



| Gates | A | B | C | R1 | R2 | R3 | R4 | R5 | R6 | R7 | R8 | R9 | R10 | R11 |
|------------------------|------------------|----------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|----------------|------------------|------------------|------------------|------------------|
| (1) Total count | $1.4 \cdot 10^6$ | $1 \cdot 10^6$ | $2.1 \cdot 10^6$ | $5.3 \cdot 10^5$ | $6.8 \cdot 10^5$ | $1.8 \cdot 10^5$ | $6.5 \cdot 10^5$ | $2.5 \cdot 10^5$ | $6.3 \cdot 10^4$ | $7 \cdot 10^3$ | $1.9 \cdot 10^4$ | $3.8 \cdot 10^5$ | $1.1 \cdot 10^6$ | $5.8 \cdot 10^5$ |
| (2) % of culturability | 1.47 | 16.31 | 0.89 | 17.8 | 0.9 | 6.9 | 1.4 | 4.9 | 20.5 | 5.2 | 5.2 | 3.1 | 15.6 | 30.9 |

Fig. 2. In this figure we illustrate the changes in the percentage of viable bacteria in several subpopulations of marine water, after incubation with nutrients. (A) is the natural sample, (B) is the same sample supplemented with 4 mg/l of Tryptic Soy broth (TSB) and incubated at 20 °C for 2 days with shaking (100 rpm) and (C) is the same as (B) but the incubation was prolonged up to 7 days. Plate counts were obtained from natural (A) or incubated (B and C) samples or from the sorted bacteria from predefined cytometric gates (R from 1 to 11). The percentages of viable or bacteria were calculated according the total counts obtained by flow cytometry and the plate counts in Agar Marine plates incubated 7 days at 20 °C. Side scatter (SSC) = 90°; Forward light scatter (FALS) = 1.5–19°.

teria is controlled, satisfactory sorting in aquatic samples can also be obtained.

EPA in marine samples is also reduced after cell sorting. This conclusion was obtained by comparing the assayed EPA in sorted bacterioplankton samples (about $2 \cdot 10^5$ bacteria) with the EPA in the non-sorted population corrected for the number of sorted cells (Table 2). Calculations based on the EPA value obtained by diluting the natural sample with NaCl (0.9%) or Isoton to obtain the sorted population indicate a large EPA reduction after sorting. The percentages of EPA reduction calculated by these estimations ranged between 73% and 95%. The natural samples diluted with NaCl or Isoton show small variable differences (Table 2), but in no case do this explain the extent of the EPA reduction in the sorted populations. As suggested in the case of the viability and EPA reduction in bacterial cultures due to cell sorting, the most plausible explanation is the effect of the laser and voltage applied at the deflecting plates, although the high pressure and impact that cells are subjected too may also be considered.

Discussion

The mechanisms that affect viability and EPA during cell sorting are unknown at the present, but in absence of other evidence, they are assumed to be associated with the physical conditions (pressure in the fluid sheath and exposure to the laser) and the effects of the voltage applied at the deflecting plates (for details of the sorting process see methods) and the impact on the surface of the collection vessel.

Cells are subjected to the required pressure to produce the desired jet velocity and to overcome friction in the nozzle exit, where the static pressure quickly drops to zero as the jet enters the atmosphere. Cell damage will usually not be caused by this abrupt pressure change, but this might affect fragile cells or bacteria in systems operating at high jet velocity and pressures (high-speed sorting) (LINDMO et al., 1990). It was demonstrated that nonspherical cells tend to become aligned with the long axis parallel to the flow axis, and deformable cells can become considerably elongated in the direction of flow (KACHEL et al., 1990). However, the sheath fluid and sample pressure in our equipment are low (around 12 p.s.i) compared with cytometers that work with fluid sheath pressure up to 50 p.s.i. At higher pressures, membrane instability can occur, resulting in deformation or fragmentation. For moderate stress the instability is time dependent with subsequent molecular creeping in membrane cytoskeleton (ENGSTRÖM and MEISELMAN, 1996). HAUGEN et al. (1987) tested for fluidics stress (125 mm Hg) in four flagellate species and although some physiological damage was found (evidenced by a temporary depression in growth rates) cells recovered after 2 days.

The laser power in our equipment is low (15 mW) compared with cytometers that work with a 100–200mW laser for jet-in-air detection, being these late conditions

more damaging to the sorted cells. RIVKIN et al. (1986) found that exposure to laser light over range of 25 to 500 mW for 2–15 μ s, caused depression in growth rates and photosynthetic rate over the initial 24h post-sorting period. HAUGEN et al. (1987) found no detectable changes in morphology of cell wall, flagella, or chloroplasts resulting from fluidics (125 mm Hg) or laser light effects (50-mW minimum to 300 mW maximum). Although our strains remained microscopically intact under the used hydrostatic pressure and laser light, the viability reduction is a clear evidence for a physiological damage.

Electrodamage induce by electroporation forms small resealable pores in cell membranes or may kill the cells, depending on the application of a strong or low electric field pulse for a few microseconds. It is well known that electric pulses of 10 μ s with field strengths near 3×10^6 v/m may kill cells (SHAPIRO, 1995). In our experiment the charging potential is 75 v and the deflection plate potentials are 2500 v. Although this treatment is not lethal, it may participate in the viability decrease.

Large cells remain intact after the brutal impact on a glass surface at speeds of 30–50 Km/h (high speeds sorters can generate jet stream velocities of up to 180 Km/h, LINDMO et al., 1990). However, in our equipment the distance between the expulsion point and the receiving microscopic slide is about 0.5 cm, being air resistance low or negligible. For these reasons, the cells of interest can be sorted into a sterilized and nutrient-rich agar Petri dishes with a minimal impact effect. The mechanisms that affect viability and EPA during cell sorting are unknown at the present, but in absence of other evidence, seem to be associated to the physical conditions (pressure in the fluid sheath and exposure to the laser), the effects of the voltage applied at the deflecting plates and the impact on the surface of the collection vessel.

We conclude that the efficiency of cell sorting should be determined for beads and bacterial populations independently, since beads and cells do not obviously share the same sorting efficiency and the bacterial species exhibit important physical differences. Bacterial sorting obtained by electric droplet charging and electromagnetic deflection cause variable losses of viability and EPA depending on the strain or environmental sample. These effects indicate that sorted cells are stressed or damaged in a way that may modify its biology and consequently, sorting effects need further study and evaluation for the correct use of the sort applications.

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Corresponding author:

JOSEPH VIVES-REGO, Department de Microbiologia, Universitat de Barcelona, Av. Diagonal, 645, 08028-Barcelona, Spain
 Fax number: (34) 93-411-0592; e-mail: jvives@porthos.bio.ub.es