Rapid, Automated Separation of Specific Bacteria from Lake Water and Sewage by Flow Cytometry and Cell Sorting

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The use of fluorescence-activated flow cytometric cell sorting to obtain highly enriched populations of viable target bacteria was investigated. Preliminary studies employed mixtures of Staphylococcus aureus and Escherichia coli. Cells of S. aureus, when mixed in different proportions with E. coli, could be selectively recovered at a purity in excess of 90%. This was possible even when S. aureus composed only approximately 0.4% of the total cells. Cell sorting was also tested for the ability to recover E. coli from natural lake water populations and sewage. The environmental samples were challenged with fluorescently labelled antibodies specific for E. coli prior to cell sorting. Final sample purities of greater than 70% were routinely achieved, as determined by CFU. Populations of E. coli released into environmental samples were recovered at greater than 90% purity. The use of flow cytometry and cell sorting to detect and recover viable target bacteria present at levels of less than 1% within an indigenous microflora was also demonstrated.

The potential applications of flow cytometry (FCM) to different areas of microbiology have received much interest (4, 17). Applications for environmental studies, although limited, have demonstrated some of the potential of FCM as a rapid, automated tool in microbial ecology (2, 3, 11, 15). However, the use of the cell sorting facility that is available with more sophisticated machines has yet to be applied to such studies, although it has been used for sorting yeasts according to pigment production (1) and yeast and bacterial colonies trapped in agarose beads according to β-galactosidase activity (10) and for isolation of cells with different fluorescent probe efflux characteristics in pure culture studies of bacteria (9). The application of cell sorting in microbial ecology has not, to our knowledge, been fully demonstrated, although its use has been speculated upon (12) and mentioned in conjunction with preliminary results (15).

Cell sorting involves physically separating cells that can be distinguished by one or more of their cellular characteristics from a mixed sample. In cell sorting operations, the sheath fluid stream is converted into droplets by vibration of the nozzle at a precise frequency. The droplet breaks off from the stream at a defined distance below the stream-laser intersection. The system decides whether to sort a droplet containing a cell on the basis of operator-directed criteria derived from the particle’s characteristic light scatter or fluorescence pattern. If the particle is to be sorted, the sheath fluid is electrically charged when the particle is at the droplet break-off point. The droplets are then deflected by an electrostatic field into a collection vessel. In this way, target cell populations are recovered from samples.

It is often preferable to obtain purified samples of cells from natural environments in order to apply molecular techniques or to extract or enrich samples for detection of low numbers of specific bacteria, such as pathogenic or genetically engineered microorganisms, against a high background of indigenous species and other material. Cell sorting has the potential to achieve this rapidly and with little sample treatment. There may be considerable potential for cell sorting in the detection of contaminating microorganisms in quality control of water or foodstuffs, in which the automation and speed of flow cytometry could be used to reduce microbiological examination of samples. Sorting potential pathogens from such samples for confirmatory microbiological examination would greatly facilitate laboratory throughput as well as permit larger volumes of samples to be examined, thus enhancing detection. The aim of this report is to assess the potential of FCM and cell sorting to isolate and enrich for target bacteria from natural environments by using fluorescent-antibody labelling.

MATERIALS AND METHODS

Environmental sites. Two sampling sites for the environmental studies were selected. Lake water was taken approximately 20 m offshore from a sewage outlet in Windermere South Basin (Windermere, Cumbria, United Kingdom). Effluent was also taken directly from a sewage outlet in Coniston Water (Coniston, Cumbria, United Kingdom). Sampling was carried out during August 1992. All samples were transported to the laboratory and processed within 1 h.

Bacterial maintenance, isolation, and identification. Escherichia coli JM101 and HB101 and one wild-type strain (220), Salmonella pullorum, and Vibrio natriegens were all from the culture collection at the Department of Genetics and Microbiology, University of Liverpool, Liverpool, United Kingdom. A second wild-type E. coli strain was isolated from an environmental sample and identified. E. coli ED8654(pLVI013) was obtained from C. Winstanley (University of Liverpool). Staphylococcus aureus Cowan 1 was obtained from C. Duggleby (Public Health Laboratory Service, Centre for Applied Microbiology and Research, Porton Down, United Kingdom). All E. coli strains, S. aureus Cowan 1, Erwinia herbicola ATCC 21434, Enterobacter cloacae NCIB 8151, Enterobacter aerogenes NCIB 10102, S. pullorum, and V. natriegens were maintained by growth

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at 37°C. Four oxidase-positive and four oxidase-negative nickel-resistant lake water isolates recently isolated from the Windermere sample site (11a) were grown at 30°C. All bacterial species were maintained and grown on nutrient agar or in nutrient broth (Oxoid, Unipath, Ltd., Basingstoke, United Kingdom). V. natriegens media were supplemented with 1% NaCl (BDH, Poole, United Kingdom). Plasmid pLV1013, present in ED8654, encodes a temperature-regulated xylE gene and kanamycin resistance (19). Media for growth and maintenance of ED8654(pLV1013) were supplemented with kanamycin (Sigma) at a concentration of 100 μg/ml.

Samples from the two environmental sites were plated out in duplicate before and after cell sorting. Total plate counts were obtained on R2A agar (13) after incubation at 18°C for 72 h. Total enteric counts were obtained on MacConkey agar (Oxoid) at 37°C for 24 h. All red colonies on MacConkey agar were designated potential E. coli. Confirmation was obtained by testing random selections of these colonies taken before and after sorting. They were subcultured twice on nutrient agar and identified by using API20E strips according to the manufacturer’s instructions.

Differentiation of S. aureus Cowan 1 and E. coli ED8654 (pLV1013) on plates. Cells containing the plasmid (pLV1013) were identified as described by Winstanley et al. (19). After incubation at 42°C for 1 h, the plates were sprayed with a 1% (wt/vol) solution of cefotaxime (Sigma). Colonies containing the plasmid rapidly developed a yellow color and were identified as E. coli ED8654(pLV1013).

AODC. Acidine orange direct counts (AODC) were made as described by Fry (5), using 0.22-μm-pore-size Nuclepore (Pleasanton, Calif.) black membrane filters and a final concentration of acidine orange of 5 μg/ml. Cells were counted by epifluorescence microscopy with a Leitz Orthoplan microscope. The two species, E. coli and S. aureus, could be differentiated by cell morphology (rods versus cocci, respectively).

Antibody labelling of cells. Antibody labelling of laboratory mixtures of bacteria was carried out as described previously (6). Incubations were carried out in the dark at room temperature in phosphate-buffered saline (PBS [16]) containing 3% (wt/vol) bovine serum albumin (BSA [Sigma]). S. aureus Cowan 1 was labelled with fluorescein isothiocyanate (FITC)-labelled human immunoglobulin G (IgG) (Sigma), which is bound by surface-expressed protein A of Cowan 1 (6). Rabbit polyclonal antibody raised against a whole-cell lysate of E. coli K12 C600 was obtained from Dako, Ltd., High Wycombe, United Kingdom. Phycoerythrin-labelled goat antirabbit IgG (Calbiochem Novabiochem [UK], Ltd., Nottingham, United Kingdom) was used as a secondary fluorescent label. The specificity of the E. coli polyclonal antibody was tested against a range of known E. coli strains, other enteric bacteria, and oxidase-positive and -negative nickel-resistant sewage isolates by using immunofluorescence detection by FCM.

Antibody labelling of environmental samples was optimized by testing a range of primary and secondary antibody concentrations (1/10 to 1/1,000 primary, 1/20 to 1/2,000 secondary), different blocking reagents (in PBS), and washing steps to reduce nonspecific binding. E. coli ED8654 was grown to a concentration of 5.0 × 10^7 CFU ml^-1. Fifty microliters of cell suspension was added to 900 μl of blocking reagent, together with 100 μl of sewage, to test labelling. The blocking reagents tested were 3% (wt/vol) BSA, 15% (wt/vol) BSA, 4% (wt/vol) skim milk powder (Oxoid), 20% (wt/vol) skim milk powder, and 4% (wt/vol) casein (BDH).

The washing treatments were (i) none, (ii) washing only after incubation with both antibodies, (iii) washing after each antibody incubation, and (iv) washing by dilution in PBS (1/20 [vol/vol]) and vortexing for 30 s. Centrifugation steps were carried out for 30 s at 8,800 × g before resuspension in PBS.

FCM. (i) Instrumentation. All laboratory solutions used were routinely filtered three times through Millipore membrane filters (0.22-μm pore size) before passage through the flow cytometer. All cytometric analyses and cell sorting were performed with a Becton Dickinson (Oxford, United Kingdom) FACS Star Plus flow cytometer-cell sorter, set to trigger on forward-angle light scatter. In all analyses, the laser output was set at 25 mW at an excitation wavelength of 488 nm. The cytometer was set up and aligned with a 0.5-μm-diameter Fluoresbrite YG fluorescent latex beads (Polysciences, Inc., Warrington, Pa.). The sheath fluid was filtered, autoclaved PBS. Before each cell sorting, the sensing chamber was lightly sprayed with 70% ethanol and absolute ethanol was passed through the sample fluid tubes and nozzle. The nozzle diameter in all cases was 70 μm. Sorting was performed in two different modes, Normal-R and Enrich. In the Normal-R mode, the sort decisions are aimed at rapidly achieving a population of cells with a high degree of purity and a number of target cells are not sorted because of the proximity of nontarget particles. The Enrich mode gives enhanced recovery and sort speed at the cost of purity and count accuracy. Samples were sorted into sterile polystyrene tubes (Falcon; Becton Dickinson). Sheath and sample pressures were kept constant, and an analytical rate of approximately 2,000 events s^-1 was maintained by sample dilution. Fluorescence at 525 nm was detected through fluorescence detector 1, set at a photomultiplier tube voltage of 550 V with logarithmic gain; fluorescence at 575 nm was detected through fluorescence detector 2 (photomultiplier tube voltage of 500 V, log gain). At these settings, the latex calibration beads appeared at mean channels of 360 (forward scatter) and 713 (fluorescence detector 1).

(ii) Cell sorting. Overnight broth cultures (100 μl) of Cowan 1 or ED8654 were added to 900 μl of 3% (wt/vol) BSA in PBS. These suspensions of bacteria were mixed in known proportions (100:0, 75:25, 50:50, 25:75, and 0:100 Cowan 1/ED8654), and 100 μl of each mixture was added to 800 μl of 3% BSA in PBS. To this final suspension was added 100 μl of a 1/10 dilution of FITC-human IgG.

Following a brief analysis of samples, sort windows (i.e., defined parameters) were set by using the FACS Star Plus program around the bacterial population(s) of interest, using pure and environmental samples, some of which were amended with E. coli cells. Samples were collected from both target and waste cell fluid streams. The sort mode used for the two-species laboratory mixtures was Normal-R in all cases. Plate counts were made in duplicate on nutrient agar, before and after sorting, and samples were fixed ready for AODC by the addition of membrane-filtered formalin solution (BDH) to a final concentration of 0.37% (5).

E. coli was selected as a target organism for isolation from the environmental samples. Approximately 100 liters of lake water was collected from Windermere and concentrated 400-fold by tangential flow filtration (Millipore) before sorting. The second environmental sample was effluent from a sewage outlet in Coniston used without any concentration step. A known number of ED8654(pLV1013) cells were released into repeat samples as a control to confirm the effectiveness of the cell sorting procedure. Sort windows were set to include the area in which labelled ED8654 cells were expected.
(pLV1013) cells were easily recognizable against the background. Target populations from the samples (ED8654 and indigenous E. coli) were sorted by using the Normal-R and Enrich modes. Plate counts were made in duplicate on R2A and MacConkey agar before and after sorting.

(iii) Data handling. Sorted samples were immediately reanalyzed by FCM (when possible), and the proportion of targeted cells in each gated area and the total count were enumerated. Percentages were given by the FACS Star Plus computer software package supplied with the cytometer.

Statistical analysis. It was necessary to confirm that cell viability was maintained during the cell sorting procedure. The CFU were expressed as a percentage of the AODC in order to avoid errors due to the dilution effects of cell sorting. These percentages were arc sine transformed to ensure that variance is not a function of the mean preliminary to testing for any significant difference before and after sorting by using one-way analysis of variance (18). All statistical analyses were carried out with Minitab 8.2 software.

RESULTS

Viability assessment during sorting. CFU were expressed as a percentage of the total counts obtained by the AODC. This avoided the errors associated with dilution effects of the sheath fluid. The proportions of the AODC capable of forming colonies on nutrient agar determined before and after sorting were 42.5 and 40.5% for S. aureus and 42.1 and 35.8% for E. coli, respectively. The data obtained before and after cell sorting were not significantly different (P = 0.67 for S. aureus and P = 0.20 for E. coli), confirming that viability was maintained during the sorting procedure.

Antibody specificity. By using FCM immunofluorescence, the E. coli polyclonal antibody was found to bind to all five strains of E. coli tested and to three oxidase-negative lake water isolates. No cross-reactivity was detected against E. herbicola ATCC 2143, E. cloacae NCIB 8151, E. aerogenes NCIB 1010, S. pullorum, or V. natriegens or against four oxidase-positive isolates and one oxidase-negative lake water isolate. No bacterial species tested bound the secondary antibody when used alone.

Antibody labelling of samples. One hundred percent cell labelling was achieved for cells binding the antibody. The addition of the fluorescently labelled secondary antibody alone to cell and environmental samples did not increase fluorescence much above that of control samples. In both cases, control sample fluorescence fell below channel 50 (fluorescence 2 detector).

As cell viability could be reduced by repeated pelleting and resuspension of cells during the washing steps, a number of treatments were tested to minimize physical treatments of cells. Antibody labelling of cells in suspension is routinely carried out with a solution containing protein as a blocking reagent (6). Flow cytometric data of antibody-labelled ED8654 in pure culture or in amended sewage outlet samples were compared after the use of different blocking reagents and different washing protocols. These results showed that an effective blocking reagent and a washing step were important for reducing nonspecific binding in environmental samples and for obtaining meaningful histogram distributions (Fig. 1). Even with these steps, it was sometimes
TABLE 1. Effectiveness of cell sorting for the recovery of FITC-labelled S. aureus from laboratory mixtures

<table>
<thead>
<tr>
<th>Sample</th>
<th>Vol/vol mixture (%)* (before sorting)</th>
<th>Actual % purity (after sorting)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CFU*</td>
</tr>
<tr>
<td>S. aureus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>75</td>
<td>98.2</td>
<td>97.9</td>
</tr>
<tr>
<td>50</td>
<td>95.3</td>
<td>86.7</td>
</tr>
<tr>
<td>25</td>
<td>94.7</td>
<td>100.0</td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>75</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>50</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>25</td>
<td>100.0</td>
<td>100.0</td>
</tr>
</tbody>
</table>

* Taken from vol/vol mixtures of overnight cultures of S. aureus and E. coli.

** Determined by the presence or absence of pLV1013.

* Determined by cell morphology after staining.

+ Determined as percentage of total events in the sorting window. ND, not determined.

were routinely achieved. This was also reflected in the examination of the waste samples which were found to be enriched in E. coli cells because of the removal of S. aureus by cell sorting (Table 1). Appropriate controls confirmed that antibody addition to 3% BSA in PBS resulted in no events being sorted.

The efficacy of cell sorting was also tested with very low numbers of target cells. With S. aureus at levels of 0.32% of the total cell numbers (determined by FCM), it was possible to obtain a sorted-cell purity of greater than 90% (data not shown).

(ii) Environmental samples. The purity of the E. coli cell suspensions obtained after sorting from sewage effluent and lake water, determined by CFU on MacConkey agar, can be seen in Table 2. An example of the effectiveness of the cell sorting can be seen from the FCM fluorescence histograms (Fig. 3). In all cases, the percentage of target organisms in the cell suspension after sorting indicated a high degree of enrichment of target cells from the background population. The exact level of enrichment was found to be dependent on the sort mode employed and the sample origin. However, in all cases, a level of ~70% or greater was achieved from starting levels of less than 1% potential E. coli (Windermere sample, Table 2). The recovery of presumptive E. coli by cell sorting was also greater than 70% (Table 2). The results of cell growth on selective media are given in Table 3. These indicate that populations of potential E. coli were detected and sorted at levels of between 10^2 and 10^6 CFU/ml, against a background of between 10^3 and 10^9 CFU of indigenous bacteria per ml in the lake water concentrate. ED8654 (pLV1013), released at different densities, was easily distinguishable in the samples (Fig. 3a). In all cases, high proportions of both released and indigenous E. coli were recovered. The difficulties in quantifying the efficiency of the cell sorter are discussed below. Comparison of plate counts on MacConkey and R2A agar before and after sorting showed that the putative E. coli colonies occurred as a very low fraction of the total count before sorting but equaled or exceeded the total count after sorting (Table 2). Similar results were obtained from sewage samples although the growth on MacConkey agar made up a greater proportion of total counts on R2A agar than growth from the lake water sample before sorting (Table 3). After sorting, the counts obtained on MacConkey agar equalled or exceeded the total count on R2A agar.

Identification of E. coli with AP120E strips. Random sam-
TABLE 2. Effectiveness of cell sorting for recovery of *E. coli* from environmental samples

<table>
<thead>
<tr>
<th>Target cell type</th>
<th>Sort mode</th>
<th>% Presumptive <em>E. coli</em> &lt;sup&gt;a&lt;/sup&gt; before sorting</th>
<th>% Presumptive <em>E. coli</em> &lt;sup&gt;a&lt;/sup&gt; after sorting</th>
<th>% <em>E. coli</em> in sorted sample&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>R2A agar&lt;sup&gt;c&lt;/sup&gt;</td>
<td>MacConkey agar&lt;sup&gt;d&lt;/sup&gt;</td>
<td>R2A agar</td>
</tr>
<tr>
<td>Windermere</td>
<td>Indigenous</td>
<td>Normal-R</td>
<td>0.8</td>
<td>33.9</td>
</tr>
<tr>
<td></td>
<td>Indigenous</td>
<td>Enrich</td>
<td>0.8</td>
<td>33.9</td>
</tr>
<tr>
<td>ED8654(pLV1013) released at:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10&lt;sup&gt;4&lt;/sup&gt; CFU/ml</td>
<td>Enrich</td>
<td>NA</td>
<td>99.6</td>
<td>NA</td>
</tr>
<tr>
<td>10&lt;sup&gt;5&lt;/sup&gt; CFU/ml</td>
<td>Enrich</td>
<td>NA</td>
<td>96.1</td>
<td>NA</td>
</tr>
<tr>
<td>10&lt;sup&gt;6&lt;/sup&gt; CFU/ml</td>
<td>Enrich</td>
<td>NA</td>
<td>74.4</td>
<td>NA</td>
</tr>
<tr>
<td>Coniston</td>
<td>Indigenous</td>
<td>Enrich</td>
<td>5.9</td>
<td>29.5</td>
</tr>
<tr>
<td>ED8654(pLV1013) released at 10&lt;sup&gt;6&lt;/sup&gt; CFU/ml</td>
<td>Enrich</td>
<td>NA</td>
<td>94.5</td>
<td>NA</td>
</tr>
</tbody>
</table>

<sup>a</sup> All colonies which grew red on MacConkey agar were designated presumptive *E. coli*.

<sup>b</sup> Percent red colonies sorted was calculated from the number of red colonies developed on plates from the sorted samples as a percentage of the total number of red colonies developed (sorted and waste plates).

<sup>c</sup> Growth on R2A agar was used to determine total counts. NA, not applicable. The high numbers of released *E. coli* dominated the background microflora.

<sup>d</sup> Growth on MacConkey agar was used to select for *E. coli*.

ples, each comprising 10 isolates that grew red on MacConkey agar, were taken from samples before and after cell sorting. Of 10 isolates from unsorted Windermere water samples, it was found that only 1 was identified as *E. coli* by using the API20E strip system before sorting. After cell sorting, all 10 were confirmed to be *E. coli*. Similar results were obtained for the Coniston sample, 1 isolate being identified as *E. coli* before sorting and 9 isolates of 10 being identified after sorting. (The majority of the other species isolated were identified as *Aeromonas hydrophila* and *Entrobacter* spp.) It was not possible to show that all red colonies were derived from *E. coli* cells in the sorted sample, as it was not practical to test every colony, but the large enrichment of target cells after sorting was clearly demonstrated.

**DISCUSSION**

The effectiveness of cell sorting as a method for obtaining a suspension, highly enriched in viable cells, from a sample
TABLE 3. CFU from lake water concentrate and from sewage before and after cell sorting

<table>
<thead>
<tr>
<th>Sample</th>
<th>CFU (log_{10} ml^{-1}) before sorting</th>
<th>CFU (log_{10} ml^{-1}) after sortinga</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total count on R2A</td>
<td>E. coli on MacConkey agar</td>
</tr>
<tr>
<td>Windermere</td>
<td>5.85 ± 5.44</td>
<td>3.76 ± 3.39</td>
</tr>
<tr>
<td>Coniston</td>
<td>5.88 ± 4.33</td>
<td>4.65 ± 4.33</td>
</tr>
</tbody>
</table>

a For Windermere samples, the top and bottom values were obtained after sorting by the Enrich and Normal-R modes, respectively; for Coniston samples, the Enrich mode was used.

containing low levels (<1%) of target cells has been demonstrated. This was possible both for laboratory mixtures and for environmental samples. The process of labelling cells with antibodies, followed by passage through the flow cytometer-cell sorter, does not appear to affect viability as determined by colony forming ability. Although maintenance of viability during sorting has been demonstrated previously by Molenaar et al. (9), no effort was made to confirm that all cells survived. Damage during FCM analysis and sorting of phytoplankton (7, 14) due to exposure of photosynthetic pigments to the laser beam has been reported. No evidence that fragile cells distorted or lysed during the process was found.

Comparison of colony counts on R2A agar and MacConkey agar showed that, after sorting, the enteric count equalled or exceeded the total count. This confirms that the target cells were effectively separated from the background organisms and that the purity after sorting was not due to the selectivity of MacConkey agar alone. The results from the identification by the API20E strip method confirm that the target cells which were sorted were E. coli. These results also suggest that the figures found for the numbers of presumptive E. coli before sorting are high and that effective detection and cell sorting from levels of below 0.8% of the total count occurred.

Although differences between the different sort modes were expected, our results showed that there was no clear advantage of one sort mode over the other in terms of final cell purity. However, the Enrich mode produced a much greater volume of cell suspension over equal periods of time than the Normal-R mode and thus was the preferred mode. This is especially important since the accuracy of sorting depends on a reasonable droplet rate to prevent dispersion of infrequently sorted droplets due to environmental effects within the sorting chamber (see below). This is particularly true for very low densities of target cells in the starting sample.

It was not possible to quantify the efficiency of the cell sorter in terms of actual numbers of target cells detected versus cells missed or in terms of sorting events aborted by the system. However, the high percentage of target cells found in the sorted sample in comparison with the waste sample suggests that the majority of the detected cells were sorted. The problems encountered during the sorting centered around the low sort rates resulting from the low concentration of the target organism in relationship to the high indigenous background encountered in the environmental samples. Relatively few droplets were sorted with time, and artifacts due to evaporation, repulsion of like charged droplets, and unstable side streams were introduced. Another problem is that electrostatically repulsed drops are attracted to the opposite charge and could result in a reversal of the sort process. The occurrence of empty and misdirected drops plays a large part in any dilution effect.

The effectiveness of antibody labelling in environmental samples was not exhaustively investigated. Excess antibody was used in this work to ensure labelling of the low numbers of target bacteria present in the environmental samples. This may account in part for the need to use a blocking reagent and a washing step. However, lower concentrations of antibody failed to give a clear signal indicative of E. coli. Possible reasons for this are discussed below.

In order to be detected by FCM, cells must ideally be introduced into the sensing region singly and separately. Minimal sample treatment was employed in this study, and some target cells may have been present as part of larger clumps and thus have no surface area available for effective antibody labelling. Also, it is possible that not all E. coli cells present expressed the necessary surface antigens in the environment. Thus, any target cells with fluorescence properties which meant that they did not fall within the sort windows would obviously not have been sorted. Sorting window parameters were defined around the signal characteristic of a laboratory-cultured population.

The combination of FCM detection and cell sorting may have advantages over some existing methods for specific cell detection. The use of an antibody label obviates the need for culture, as a total count can be rapidly obtained from FCM. This circumvents the possibility of the failure to detect viable but nonculturable bacterial pathogens in a sample (8), assuming that the antigen is expressed in this state. Successful sorting of the target bacteria can be confirmed by traditional culture methods, if required.

Despite possible limitations, this study has demonstrated the effectiveness of FCM and cell sorting for the detection and physical separation of viable target cells occurring at low levels in heterogeneous natural microbial populations. Future work could include the use of this technique to separate organisms from more challenging environments such as soil or sediment. Sample preparation would be of crucial importance in work of this nature, as the effectiveness of primary cell extraction would determine the selectivity and efficiency of the procedure. Once cells are separated from the solid matrix, detection and sorting would become feasible.

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REFERENCES