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Satawat Saenton[a]; Hookeun Lee[b]; Yu-Shu Gao[c]; James F. Ranville[b]; S. Kim Ratanathanawongs Williams[b]
[a] DIVISION OF ENVIRONMENTAL SCIENCE AND ENGINEERING, COLORADO SCHOOL OF MINES, GOLDEN, COLORADO, USA [b] DEPARTMENT OF CHEMISTRY AND GEOCHEMISTRY, COLORADO SCHOOL OF MINES, GOLDEN, COLORADO, USA [c] COLLEGE OF PHARMACY, XAVIER UNIVERSITY OF LOUISIANA, NEW ORLEANS, LOUISIANA, USA

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Evaluation of Different Field-Flow Fractionation Techniques for Separating Bacteria

SATAWAT SAENTON
DIVISION OF ENVIRONMENTAL SCIENCE AND ENGINEERING
COLORADO SCHOOL OF MINES
GOLDEN, COLORADO 80401, USA

HOOKEUN LEE
DEPARTMENT OF CHEMISTRY AND GEOCHEMISTRY
COLORADO SCHOOL OF MINES
GOLDEN, COLORADO 80401, USA

YU-SHU GAO
COLLEGE OF PHARMACY
XAVIER UNIVERSITY OF LOUISIANA
NEW ORLEANS, LOUISIANA 70125, USA

JAMES F. RANVILLE and S. KIM RATANATHANAWONGS WILLIAMS*
DEPARTMENT OF CHEMISTRY AND GEOCHEMISTRY
COLORADO SCHOOL OF MINES
GOLDEN, COLORADO 80401, USA

ABSTRACT

Field-flow fractionation (FFF) techniques were used to separate various strains of bacteria and differentiate live from dead bacteria. The sedimentation, flow, and electrical FFF separations were accomplished in less than 15 minutes using the rapid hyperlayer mode. The bacteria used in these studies include *Pseudomonas putida*, *Escherichia coli*, and *Staphylococcus epidermidis*. Sedimentation FFF gives the highest resolution separations of a mixture of spherical and rod-shaped bacteria, and a mixture of two rod-shaped bacteria.

Key Words. Field-flow fractionation; Bacteria; Separation; Viability; *Pseudomonas putida*; *Escherichia coli*; *Staphylococcus epidermidis*

* To whom correspondence should be addressed. Telephone: (303) 273-3245. E-mail: krwillia@mines.edu

INTRODUCTION

Bacteria analyses have received a great deal of interest because of their use in such diverse fields as bioremediation, biotechnology, and biological warfare. When present in complex sample matrices such as soil, food, and blood, it is difficult to differentiate bacteria from other particulate components. In addition, the ability to distinguish live and dead bacteria from a mixture is important for a number of reasons. First, pathogenicity is usually exhibited by live microorganisms. However, some gram-negative strains can be more toxic because the dead bacteria can disintegrate and liberate endotoxins (1). A rapid test for bacteria viability is necessary so that appropriate and immediate precautions can be taken. Second, the removal of dead bacteria from a mixture prior to cell culturing results in a purer colony and unhindered growth rate. Lastly, the separation of bacteria from other components in the mixture prior to detection and identification minimizes interferences and provides more reliable results.

Techniques that have been used to isolate or separate bacteria include flow cytometry (2–4), capillary zone electrophoresis (5), dielectrophoresis (6), and cell sorting using immunomagnetic beads (7–10). Flow cytometry and dielectrophoresis have also been used to assess bacteria viability (2, 3) and to separate viable and nonviable yeast cells (11). Flow cytometry offers considerable potential in the separation and enumeration of viable bacteria. However, isolation is often limited to a few (typically 3 or 4) populations. Although it provides accurate and precise separation and quantification, the instrument is costly. In addition, flow cytometry has a lower size limit of 0.2–0.5 μm that consequently excludes the detection of small size bacteria (12, 13). Capillary zone electrophoresis offers the possibility for separating bacteria on the basis of differences in electrophoretic mobilities μ (5). Since μ strongly depends on the charge-to-size ratio and ionic strength, this technique requires careful control of the buffer concentration. Low or high ionic strengths can also cause cell burst or plasmolysis (cell shrinkage), respectively (1). As a result, capillary zone electrophoresis is not applicable to the separation of aquatic and marine bacteria samples in their natural environments which have ionic strengths of 0.2–0.8 M. Dielectrophoresis is not commonly used because it is not commercially available. Although immunology-based methods are selective, fast, and sensitive, they are expensive and prone to interferences from the matrix. Culturing remains the traditional method for differentiating live and dead bacteria but results require as long as 3 to 7 days (1–3). It is evident that there is a need for an inexpensive and rapid technique capable of separating and differentiating live and dead bacteria and bacteria strains. Field-flow fractionation (FFF) is a family of techniques devised specifically for separation and characterization of macromolecules, colloids, and particles (14).

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The three commercially available techniques, sedimentation (Sd), flow (F1), and electrical (E1) have been applied to the analysis of numerous biological particles (15–21). Sedimentation FFF has been used to determine bacteria biomass and density (22–24) and to differentiate bacteria with different mobilities (25). These works involve mainly submicron size bacteria and a normal mode separation mechanism. In this paper a different FFF separation mechanism, hyperlayer mode, is exploited to yield fast and high-resolution separations. The different separation mechanisms will be discussed in the next section.

It is the object of this paper to assess the ability of three FFF techniques, sedimentation, flow, and electrical, in differentiating live from dead bacteria. In addition, this paper demonstrates the capability of FFF techniques to separate different bacteria strains. Bacteria used in this study are *Pseudomonas putida*, *Escherichia coli*, and *Staphylococcus epidermidis*. These three bacteria were selected because of their different morphologies. *S. epidermidis* is spherical, whereas *E. coli* and *P. putida* are rod-shaped with different length-to-width ratios. In addition, the selected bacteria strains are related to pathogenic strains and are ubiquitous in the environment. It is important to have rapid analytical methods capable of separating the bacteria and assessing their viabilities.

BASIC PRINCIPLES OF FIELD-FLOW FRACTIONATION

Field-flow fractionation (FFF) is a family of separation methods based on elution from a thin, ribbonlike, open channel in which the separation is induced and controlled by an external field acting in a direction perpendicular to laminar flow in the channel (14). There are two essential major components in achieving separation: the parabolic flow profile in the channel and the field that is applied perpendicular to the parabolic flow. The field positions particles of varying physicochemical properties in different velocity streamlines of the parabolic flow profile. Particles at different elevations in the flow profile will elute at different times and hence be fractionated.

The separation mechanisms of the three modes of FFF are illustrated in Fig. 1. In normal mode FFF a steady-state zone with an exponential concentration distribution is formed according to the balance between field-induced and diffusive forces. The average distance of the sample zone from the accumulation wall determines its velocity and thus its elution time. Smaller particles with a larger diffusion coefficient will form a zone that extends a greater distance into the center of the channel as shown in Fig. 1(a). Consequently, small particles elute first in the normal mode.

As the particle size approaches 1 μm, its diffusion coefficient becomes negligible and consequently the particle remains adjacent to the accumulation

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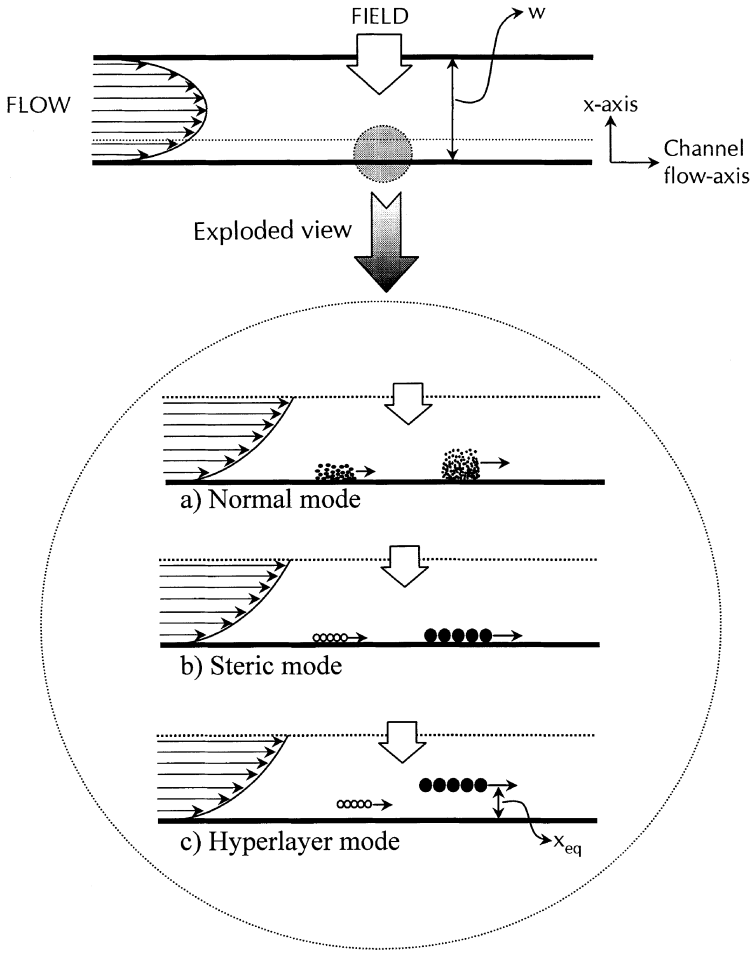

FIG. 1 Schematic view of particle positions in different modes of separation.

wall (26). In this case the protrusion of particles into the flowing stream is determined by their finite size instead of by diffusion. This size-based elution is termed steric FFF (27–30). In the steric mode large particles have centers of mass further from the accumulation wall and are thus carried toward the channel outlet faster than the small particles (Fig. 1b). As a result the elution order is opposite to that observed in normal mode FFF.

As the average velocity of the carrier fluid in the channel increases, particles begin to experience hydrodynamic lift forces (31). With sufficiently strong lift forces, particles are elevated from their position at or near the wall and focused in a confined region (called a hyperlayer) as shown in Fig. 1(c). Since the particles in hyperlayer FFF occupy faster streamlines, the elution order is the same as that of the steric mode but the retention time is significantly reduced (32, 33). In addition, narrower peaks are usually obtained (34). These advantages make hyperlayer the preferred mode of operation for the work described here.

The retention ratio R, defined as the ratio of the migration velocity v of any given particle relative to mean velocity of the channel flow $\langle v \rangle$, can be written as (35)

$$R = \frac{v}{\langle v \rangle} = 6 \frac{x_{eq}}{w} \left(1 - \frac{x_{eq}}{w} \right) \quad (1)$$

where x_{eq} is the equilibrium position or the distance of the particle from the accumulation wall and w is the channel thickness. The retention ratio R can be obtained from the fractogram by using the relationship

$$R = t^0/t_r \quad (2)$$

where t^0 is the void time and t_r is the retention time. In steric and hyperlayer FFF the retention ratio is approximated by the equation (36, 37)

$$R \approx 6\gamma a/w \quad (3)$$

where γ is the steric correction factor accounting for hydrodynamic effects and a is the particle radius. The value of γ depends on the field strength and flow rate (26, 36, 37). Ratanathanawongs and Giddings (26) proposed that separations where $\gamma < 2$ be designated as steric mode and $\gamma \geq 2$ as hyperlayer mode.

The three FFF techniques evaluated for the separation of bacteria are sedimentation, flow, and electrical. For SdFFF, the channel is inserted into a centrifuge basket and spun to generate the centrifugal field that forces sample to the accumulation wall. In F1FFF, a second stream of liquid is introduced perpendicular to the main channel flow using a second pump. This crossflow sweeps everything in its path toward a semipermeable accumulation wall. In ElFFF, the two major walls of the channel are electrodes. Application of a potential between these electrodes causes migration of the charged species to the oppositely charged electrode wall. Detailed instrumentation descriptions are given elsewhere (26, 38, 39).

MATERIALS AND METHODS

The three FFF systems described in this section were used to conduct two sets of experiments. The first set involved separation of different species of bacteria, and the second set focused on differentiating live and dead bacteria.

Bacteria Samples

Bacteria samples were prepared from culture grown for 16 hours in Difco tryptic soy broth (TSB) (Difco Laboratories, Detroit, MI, USA). Initially, 1.5 mL of cells in TSB was centrifuged at 15,000 rpm (19,115g) for 1 minute to pellet the cells. The supernatant was removed and the cells were resuspended

in sterile 2% ammonium chloride (NH_4Cl) (J. T. Baker, Inc., Phillipsburg, NJ, USA) and centrifuged again. This process was repeated twice to remove growth media. The cleaned pellet was diluted in 100 μL 2% NH_4Cl and used for FFF experiments. Dead bacteria were prepared by suspending viable cells in 1% ethanol aqueous solution containing 0.15% 2-methylthiazalone and 0.15% bromo-nitro-dioxane.

Sedimentation FFF

The sedimentation FFF system used in this study is similar to the Model S101 Fractionator from FFFractionation, Inc. (Salt Lake City, UT, USA). The breadth of the channel was 1.0 cm, the thickness was 0.0127 cm, and the tip-to-tip length was 90.0 cm. The rotor radius of the channel was 15.1 cm. A Kontron Analytic LC pump model 410 (Kontron Electrolab, London, UK) was used to deliver the carrier liquid. An FMI Lab pump model QD-2 (Fluid Metering, Inc., Oysterbay, NY, USA) was used to clean the channel by flushing at 25–30 $mL \cdot min^{-1}$ after each sample injection. The Spectra 100 UV-Vis detector (Spectro-Physics, Chicago, IL, USA), set at 254 nm, was used to monitor the turbidity of the eluting materials. The carrier solution was deionized water containing 0.1% of the surfactant FL-70 (Fisher Scientific, Fair Lawn, NJ, USA), and 0.02% of the bactericide sodium azide (NaN_3) (Sigma Chemical Co., St. Louis, MO, USA). FL-70 is a routinely used carrier liquid. Polystyrene latex standards (Duke Scientific, Palo Alto, CA, USA) with nominal diameters of 9.87, 5.01, 2.98, and 2.01 μm were used to determine the size selectivity S_d of the systems. Approximately 10 to 25 μL of the polystyrene and bacteria samples suspensions were injected in each experiment. The amounts of sample injected were based on the results of overloading studies. Retention times were constant when less than ~10^6 bacteria cells were used. The injection volume was varied to account for the differences in cell concentration of each bacteria culture.

Flow FFF

A model 1000-FIFO Universal Fractionator (FFFractionation, Inc., Salt Lake City, UT, USA) was used in this study. The flow FFF channel had dimensions of 29.4 cm tip-to-tip length, 2.0 cm breadth, and 0.022 cm thickness, and a 30 kDa cut-off regenerated cellulose membrane (Millipore Corporation, Bedford, MA, USA) as the accumulation wall. The carrier liquid contained 0.01% Triton X-100 (J. T. Baker, Inc., Phillipsburg, NJ, USA) and 10^{-4} M NH_4Cl. The pH was adjusted to 7.00 with aqueous ammonia. A Hewlett-Packard series 1050 pump (Agilent Technologies, Inc., Palo Alto, CA, USA) and Pharmacia Biotech Pump P-500 (Amersham Pharmacia Biotech AB, Uppsala, Sweden) were used to drive the channel flow and crossflow, respec-

tively. Detection was achieved using a Linear UV-Vis 200 (Linear Instruments Corporation, Reno, NV, USA) set at 254 nm.

Electrical FFF

The electrical FFF system consisted of two platinum-plated titanium electrodes that sandwich a Mylar spacer containing the silhouette of the FFF channel. The channel was of 35.0 cm tip-to-tip length, 2.0 cm width, and 0.0127 cm thickness. The power supply model EC 135 (E-C Apparatus Corporation, St. Petersburg, FL, USA) was used to generate the electric field. The deionized water carrier liquid was filtered through a 0.02 μm pore size Whatman filter (Whatman International Limited, Maidstone, UK) and delivered to the channel by a metering pump Series 3 (Scientific System, Inc., State College, PA, USA). The eluent was detected by a Dawn DSP-F multiangle light scattering instrument and data analysis was done using ASTRA 4.5 software (Wyatt Technology, Santa Barbara, CA, USA).

RESULTS AND DISCUSSION

The performance of different FFF techniques in the steric or hyperlayer modes can be represented in terms of a diameter-based selectivity S_d (40). The S_d is defined as $|d \log(t_r)/d \log(d)|$ where t_r and d are the retention time and diameter, respectively. The value of S_d represents the intrinsic resolving power of the system. The higher the S_d value, the larger the difference in retention times between particles of different sizes. Spherical polystyrene (PS) latex standards of known diameters are usually employed to establish the $\log(t_r)$ versus $\log(d)$ calibration plots. The slope, which corresponds to S_d, is specific to the experimental conditions and other factors that will be discussed in subsequent sections. The S_d values that were empirically determined for ElFFF, FlFFF, and SdFFF are 0.528, 0.823, and 0.844, respectively. These numbers provide a basis for comparing the performance of each FFF system and the selected experimental conditions with previous and future results. The S_d value for SdFFF is similar to those reported by Giddings et al. (29) whereas S_d for ElFFF is slightly lower than the 0.6 reported by Schimpf et al. (41) for the steric mode. Previously reported S_d for FlFFF range from 0.74 to 1.56 depending on the type of membrane and the experimental conditions used (26, 34, 42). Aside from establishing a performance baseline, the S_d values indicate that SdFFF and FlFFF possess a similar ability to differentiate PS latex beads of different sizes whereas ElFFF will yield lower resolution separations.

One main objective of this study was to establish experimental conditions for rapid separation of bacteria (<15 minutes). Optimum flow rates and field strengths were empirically determined for flow and sedimentation FFF. The

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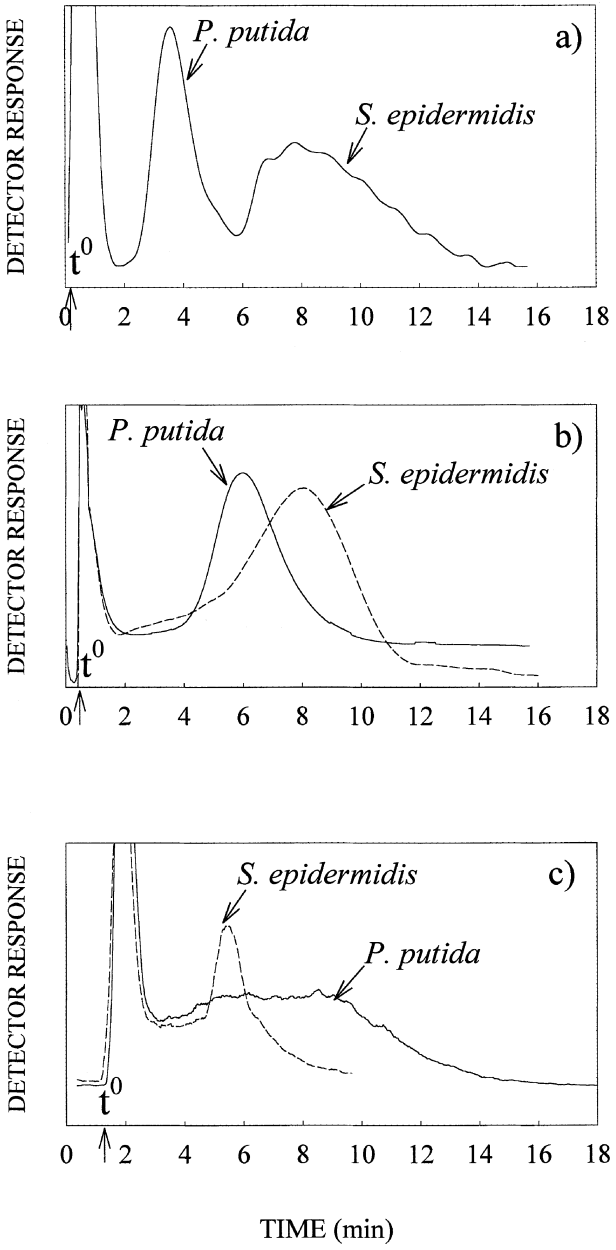

FIG. 2 Fractograms of dead *S. epidermidis* and dead *P. putida*. (a) Sedimentation FFF (flow rate = 3.84 mL·min^{-1}, 1000 rpm, stopflow time = 2 minutes, carrier liquid is deionized water containing 0.1% FL-70, 0.02% NaN_3). (b) Flow FFF (flow rate = 2.0 mL·min^{-1}, crossflow rate = 0.7 mL·min^{-1}, stopflow time = 4 minutes, carrier liquid is deionized water containing 10^{-4} M NH_4Cl and 0.01% Triton X-100, pH 7.0). (c) Electrical FFF (flow rate = 0.5 mL·min^{-1}, Pt electrodes, 1.12 V, 0.57 mA, carrier liquid is deionized water, no stopflow relaxation).

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S_d values listed above were measured at these optimum conditions as were all subsequent flow and sedimentation FFF bacteria separations.

Cultured *P. putida* and *S. epidermidis* bacteria were injected separately into the flow and electrical FFF channels, and as a mixture into the sedimentation FFF channel. Unless otherwise noted, dead bacteria were injected. The fractograms superimposed in Fig. 2(a–b) show that *P. putida* elutes before *S. epidermidis* for flow and sedimentation FFF. The SdFFF result, shown in Fig. 2(a) demonstrates a high resolution separation of *P. putida* and *S. epidermidis*. Retention in sedimentation/hyperlayer FFF is based principally on differences in size and to a lesser extent on effective mass. Figure 2(a) reflects the size difference in the two bacteria. Flow FFF is capable of differentiating the two bacteria species, but with a high degree of overlap between the two peaks. These results are not surprising since microscopic examination reveals that a fraction of the rod-shaped *P. putida* is of similar size to the spherical *S. epidermidis*.

Markedly different elution profiles are observed for each bacteria species in the electrical FFF work (Fig. 2c). These analyses were done under unoptimized experimental conditions. Stopflow relaxation, a procedure whereby sample is allowed to form equilibrium layers before initiating the elution processes (21), was not used. The results demonstrate that bacteria can be retained using ElFFF and that different elution profiles can be obtained. Since retention in ElFFF is based on a combination of size and charge, the broadness of the *P. putida* peak indicates a large array of size–charge combinations.

Sedimentation FFF is also capable of separating two rod-shaped bacteria (*E. coli* and *P. putida*) as demonstrated in Fig. 3.

Bacteria cell dimensions were determined by microscopy (43). *S. epidermidis* has an average diameter of 0.9 μm whereas *E. coli* and *P. putida* have average dimensions of 2.1 μm × 0.61 μm and 3.3 μm × 0.77 μm (average

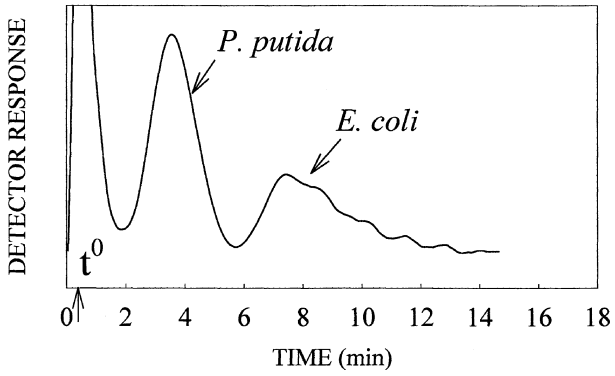

FIG. 3 Sedimentation FFF fractograms of dead *E. coli* and dead *P. putida* (flow rate = 3.84 mL·min^{-1}, 1000 rpm, stopflow time = 2 minutes, carrier liquid is deionized water containing 0.1% FL-70, 0.02% NaN_3).

length × average width), respectively. Both rod-shaped bacteria are of the typical size reported in literature (1). Size information can also be estimated from the S_d calibration plot by interpolating a hydrodynamic diameter from a measured t_r. This procedure assumes equal lift force effects on the standard and the sample of the same diameter. Since flow FFF separations are independent of density and charge, it is possible to obtain the diameters of spherical sample particles directly from a calibration plot established using spherical PS latex beads. Based on the FFF retention time, the spherical *S. epidermidis* has a diameter of 1.1 μm. This is approximately 20% larger than the microscopic results which are based on fewer measurements (127 cells by microscopy versus ~10^6 cells by FFF). Surprisingly the calibration curve also gives a reasonable size estimate for the rod-shaped *P. putida* despite the high aspect (length-to-width) ratio of 4.3. The FlFFF t_r for *P. putida* corresponds to a hydrodynamic diameter of 1.8 μm which is ~28% higher than the equivalent spherical diameter calculated from microscopically determined dimensions. The effects of various particle and solvent parameters, e.g., particle size and shape and solvent viscosity, on lift forces have not yet been fully characterized (31). Attempts to use the S_d calibration plot to obtain bacteria size information from ElFFF and SdFFF retention times are further complicated by the additional dependency of t_r on charge and density, respectively.

For SdFFF, cell size information can be obtained by employing the density-compensation method developed by Giddings et al. (29). However, this method is not easily applicable to bacteria because of the lack of accurate density information.

Hyperlayer mode separations require the use of particle size standards whose properties match those of the sample. It is possible to create standards from the sample itself. Fractions of a size-separated sample eluting from a FlFFF channel can be collected and the particle dimensions measured by microscopy. These "standards" of known dimensions can then be reinjected into the FFF channel and the resulting t_rs used to establish a calibration curve.

Table 1 lists the equilibrium positions x_{eq} of bacteria as they are transported in the FFF channel. The equilibrium positions are calculated using measured retention times, known channel dimensions, and Eqs. (1) and (2). The x_{eq} value is used to assess whether the desired hyperlayer mode of separation has been achieved. As mentioned previously, the main advantages of hyperlayer mode are speed and narrow peaks. In the steric or hyperlayer modes a particle migrates with the same velocity as the streamline that coincides with its center of gravity. If the particle's center of gravity is located at a distance greater than approximately two radii from the accumulation wall, i.e., $\gamma \geq 2$, it is considered to have undergone a hyperlayer mode separation. The x_{eq} value for *S. epidermidis* is located 1 μm from the accumulation wall. Since the radius of this spherical bacteria is ~0.5 μm, the particle's center of gravity is two radii

TABLE 1
Equilibrium Positions x_{eq} and Calculated Dimensionless Steric Correction Factor γ[a]

	Equilibrium position, x_{eq} (μm)			Steric correction factor, γ		
Bacteria	SdFFF	F1FFF	E1FFF	SdFFF	F1FFF	E1FFF
Dead *P. putida*	3.8	4.8	4.4	2.3	2.9	2.6
Live *P. putida*	—	6.1	3.0	—	—	—
Dead *S. epidermidis*	1.0	2.7	—	2.2	5.9	—
Dead *E. coli*	1.1	—	—	1.0	—	—

[a] The calculations of x_{eq} and γ for *P. putida* are based on the fractograms shown in Fig. 4 (a–c).

away from the wall, i.e., $\gamma \approx 2$, and hyperlayer mode separation has been achieved (see Table 1). For rod-shaped bacteria, the cell motion and dimensions that would generate the smallest γ value are used. If the *P. putida* cell tumbles end-on-end, the maximum distance of the cell's center of gravity is equal to half of the cell length ($L/2$). For *P. putida*, $L/2$ corresponds to 1.65 μm. The x_{eq} values listed in Table 1 indicate hyperlayer mode separations for all analyses with the exception of *E. coli* ($L/2 = 1.1$ μm) which elutes in the steric mode.

The previous fractograms (Fig. 2a–c) are of bacteria that have been killed using chemical treatments. Live bacteria have also been injected into the three FFF channels and their elution profiles and retention times compared to their dead counterparts. Figure 4(a–c) shows the elution profiles of live and dead *P. putida* with the live cells always eluting first. (Stopflow relaxation was used in these E1FFF runs.) The results in Fig. 4 can be attributed to the presence of polar flagella on the *P. putida* which propel the live bacteria toward the center of the channel and cause the observed early elution (1). Jiang (25) found that *E. coli* with intact flagella have higher mobilities than cells with their flagella removed. In addition, the motion of the bacteria in the centrifugal field of an SdFFF channel was no longer a random walk. Rather, the bacteria "swim" against the direction of the centrifugal force. An alternative explanation for the earlier elution of the live bacteria is that the chemical process used to kill bacteria causes changes in the cell deformability and density. The deformability or rigidity of the cell wall will lead to different magnitudes of hydrodynamic lift forces (17) and subsequently different retention times. Further experiments are planned to investigate this facet more thoroughly.

Many aspects need to be taken into consideration in the evaluation of E1FFF, F1FFF, and SdFFF as techniques for bacteria separations. The series of fractograms in Fig. 2 show that SdFFF yields the best separation of dead *P.*

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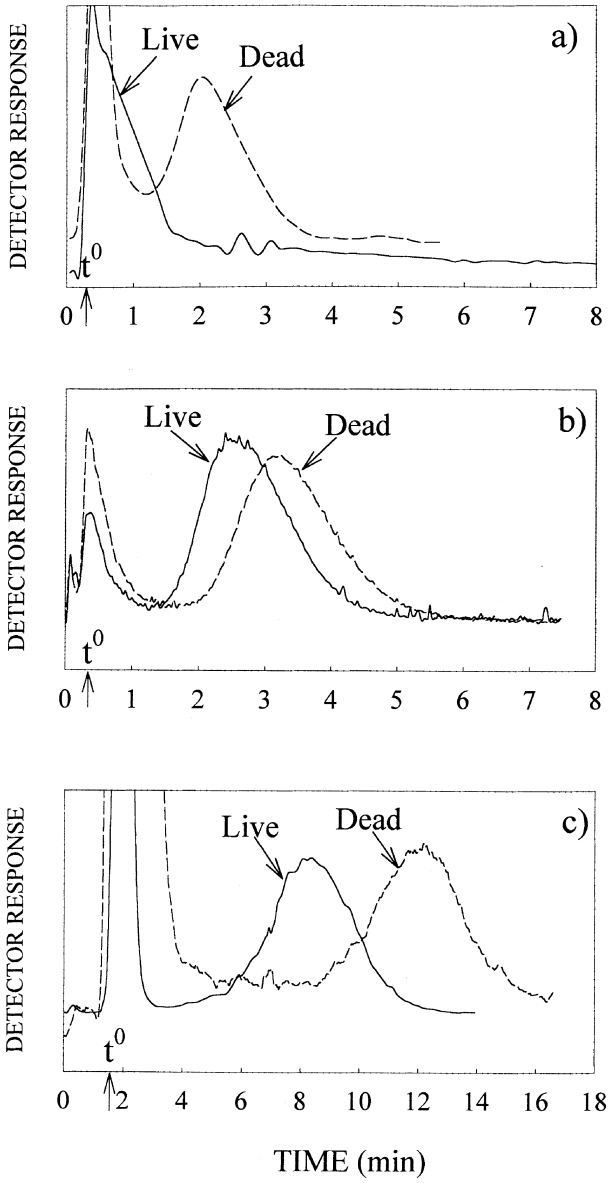

FIG. 4 Fractograms of live and dead *P. putida*. (a) Sedimentation FFF (flow rate = 3.84 mL·min^{-1}, 1000 rpm, stopflow time = 2 minutes, carrier liquid is deionized water containing 0.1% FL-70, 0.02% NaN_3). (b) Flow FFF (flow rate = 3.0 mL·min^{-1}, crossflow rate = 0.5 mL·min^{-1}, stopflow time = 4 minutes, carrier liquid is deionized water containing 10^{-4} M NH_4Cl and 0.01% Triton X-100, pH 7.0). (c) Electrical FFF (flow rate = 0.5 mL·min^{-1}, Pt electrode, 0.93 V, 0.28 mA, stopflow time = 2 minutes, carrier liquid is deionized water).

putida from dead *S. epidermidis*. In addition, the ability of SdFFF to separate dead bacteria of similar shape is confirmed in Fig. 3. In this case the rod-shaped *E. coli* and *P. putida* are also baseline resolved. However, these results do not predict that SdFFF will provide the best separation for all combinations

of bacteria. Size, density, and shape are all important sample properties that affect retention times. The effect of shape can be eliminated if standards of the same shape as the sample are available for establishing the S_d calibration plot. Such standards may be obtained by collecting fractions of the bacteria eluting from the FlFFF channel, using microscopy to measure their dimensions, and reinjecting each "standard" into the FFF channel. The dependence of retention time (and hydrodynamic lift forces) on size and density complicates the interpretation of the resulting SdFFF fractograms. In addition, the dramatic decrease in γ with increasing particle diameter (42) can lead to dual mode elution in a single analysis with small particles separated by the hyperlayer mode and large particles by the steric mode. Electrical FFF separations are governed by charge as well as size. Like SdFFF, the fractograms can be difficult to interpret. However, in situations where sample properties such as size and density are identical, ElFFF may provide a means of separating on the basis of differences in charge. Assuming the availability of a same shape standard, retention in hyperlayer mode FlFFF is based only on size. Interpretation of the fractograms is straightforward with large particles eluting first. Thus, separations of complex samples containing particles of different size, density, charge, etc. may be best done using flow FFF. Unlike SdFFF, the crossflow "field" does not strongly suppress hydrodynamic lift forces. Hence, hyperlayer flow FFF is readily achievable and sustainable over the entire size range of the separation.

Other aspects to take into consideration include cost, availability, ruggedness, and limitations of the different FFF systems. The SdFFF system is costly and has moving parts. The ElFFF system is not currently readily available and the choice of carrier liquids is limited. Significant retention of bacteria was observed only when deionized water was used as the carrier liquid. No retention was observed in buffered solutions due to a build-up of polarization layers at the electrode–carrier liquid interface which significantly reduces the effective potential gradient (41). The flow FFF channel uses a semipermeable membrane as the accumulation wall. This membrane is not as rugged as the solid metallic walls of the other two systems. The flow and electrical FFF systems are more portable for use in field work. Since all three FFF methods are capable of differentiating between strains and viability, preference for a particular technique will depend on the specific objectives of the project.

CONCLUSIONS

This work demonstrates that FFF can be used to rapidly differentiate bacteria strains and viability. Analyses were completed within 15 minutes. Sedimentation FFF has been successfully used to baseline resolve a mixture of spherical and rod-shaped bacteria and a mixture of two rod-shaped bacteria of different aspect ratios. The desired high-speed hyperlayer mode separation

was confirmed by calculating the equilibrium distances from the accumulation wall. The three FFF techniques have various strengths and weaknesses making each suitable for different sets of objectives.

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