Capillary Electrophoresis of Microbes

Byoung Geoun Moon, Yong-Ill Lee,† Seong Ho Kang,‡ and Yongseong Kim*

Division of Chemistry and Chemical Engineering, Kyungnam University, Masan 631-701, Korea
†Department of Chemistry, Changwon University, Changwon 641-773, Korea
‡Department of Chemistry, Chunbuk National University, Jeonju 561-756, Korea
Received October 9, 2002

Direct analysis of microbes such as either gram-positive or gram-negative bacteria without cell lysis was investigated using capillary electrophoresis. Bacteria cells were directly introduced into the microbore fused-silica capillary, then separated under high electric field in less than 15 min. It was found that a proper dispersion of bacteria cells was important for reproducible results. Migration behavior of bacteria at different storage condition was investigated and many unexpected peaks were observed from bacteria stored at room temperature due to the distortion of cells. This phenomenon was attributed to the change of size and shape of the same bacterium and confirmed by the scanning electron microscopic images.

Key Words : Capillary electrophoresis, Bacteria, Microbes, Scanning electron microscopy

Introduction

Direct analysis of bacteria without pretreatment is important in the area of diagnosis and profiling of some diseases, evaluation of soil populations for agriculture, bioremediation, and quality control in fermentation processes.[1]

Conventionally, bacteria cells have been lysed to investigate unique genetic materials or cell components.[2,3] However, many cell components such as lipoproteins, glycoproteins, lipids, lipopolysaccharides, and polynucleotides have similar structures in different bacteria, causing many unwanted peaks or negative identifications of bacteria.[1,4] Fluorescence assay and immunoassay have been introduced and turned out to be efficient. However, the culture of bacteria needs long time and the synthesis of selective antibodies are required before analysis.[5,6] Also, the sample pretreatment process is time consuming and nonspecific absorption of antibody can cause negative results.[7] Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry has been employed for the direct analysis of bacteria using specific biomarker ions in the bacteria cell.[8] However, vast number of m/z peaks was sometimes observed due to many cell constituents, resulting in tough data analysis.

Capillary electrophoresis (CE) would be a prominent analytical technique for direct analysis of bacteria since it is simple and efficient.[9] Capillary electrophoresis has been widely applied to organic and inorganic ions as well as colloidal particles.[10] Lately, capillary electrophoresis has been used for the analysis of deoxyribonucleic acid (DNA), protein, virus, and bacteria.[1,11,12]

In the analysis of intact microbes, capillary electrophoresis showed the successful separation of a tabacco mosaic virus and *Lactobacillus casei*.[13] The analysis of four types of human rhinovirus with a surfactant was reported by capillary isoelectric focusing (CIEF).[14] Yeast cells with different growth phase showed different isoelectric points.[15] The cell viability was determined by capillary electrophoresis with the fluorescence probes.[9] Bacteria from infected urinary tract were separated using a water-soluble polymer solution.[16]

Certain bacteria have strong affinity to the cell surface of other bacteria due to strong hydrophilic and hydrophobic interactions. Therefore, chains or clusters of bacteria cells tend to be easily formed. In our study, we have investigated the formation of chains or clusters of bacteria and demonstrated the successful separation and detection of intact bacteria. Also, the activities of bacteria at different storage conditions for improved diagnosis were evaluated and confirmed by the analysis of scanning electron microscopic images.

Experimental Section

Chemicals. *Aerobacter aerogenes, Pseudomonas fluorescens* and *Micrococcus lysodeikticus* were received as freeze-dried (Sigma-Aldrich Co.). Tris(hydroxymethyl)aminomethane (TRIZMA base), boric acid, etheylenediaminetetra-acetate (EDTA), poy(ethylene)oxide (PEO, Mw = 1,000,000 and 400,000), phosphoric acid, potassium hydroxide were also purchased from Sigma-Aldrich.

Capillary electrophoresis. Buffer (4.5 mM Tris, 4.5 mM boric acid, 0.1 mM EDTA, pH = 8.4) was diluted 8 : 1 by deionzed water. Poly(ethylene)oxide (Mw = 1,000,000 or 400,000) was dissolved in this buffer at the concentration of 0.5%. The polymer solution was stirred for 2 h, then ultrasonicated for 1.5 h for complete dispersion. It was further diluted to 0.0125% and ultrasonicated before the electrophoresis running.

Bacteria samples were prepared by dissolving in the 8 : 1 diluted buffer at the concentration of 1 mg/mL. They were precipitated by ultracentrifuge at 12,000 rpm and the supernatant was decanted. They were redissolved in the 8 : 1 diluted buffer and completely dispersed by ultrasonication for 5 min. For the investigation of migration behavior of bacteria depending on age or growth condition, *Areobacter*

aerogenes was stored at 0 °C or at room temperature for 15 days.

Electrophoresis run was performed by using Beckman PACE 5000. A fused silica capillary (Polymicrotechnology Co.) with 75 micrometer i.d. was employed for the separation. Total length of the capillary was 27 cm with the effective length of 20 cm. The capillary was preconditioned by washing with 0.5 N phosphoric acid for 2 min, deionized water for 3 min, 1 N potassium hydroxide for 2 min, then 0.0125% PEO running buffer for 2 min. Pre-electrophoresis run before bacteria sample injection was underwent for 5 min for the stabilization of baseline. Each bacteria sample was injected by 0.5 psi for 10 s and run at 10 kV. A UV absorption detection at 214 nm was employed.

Scanning electron microscopy (SEM). The images of bacteria stored at 0 °C and at 23 °C were compared by the scanning electron microscopy. Initially, bacteria cells were fixed by glutaraldehyde 20% solution and stored for 24 h. Each sample was then washed twice with 60%, 70%, 80%, 90% and 95% of methanol. Ultracentrifuge with 12,000 rpm were performed between wash steps and the supernatant was decanted. The SEM image was taken by Hitachi S-4200 at 10 kV and magnified by 20000 times. The resolution at 10 kV was around 2.0 nm and a cold-cathode field emission was employed for the generation of the second electron.

Results and Discussion

Table 1 shows the characteristics of bacteria employed in this work. Size and shape are so critical in electrophoresis since the mobility of each bacterium under electric field is determined by those factors. As shown in Table 1, *Pseudomonas fluorescens* and *Aerobacter aerogenes* have flagella, while *Micrococcus lysodeikticus* is non-motile. Most of bacteria are less than 2 micrometer in diameter. The shape of bacteria is unique (rod, sphere spheroidal), and Gram staining is either positive or negative. In general, bacteria are amphoteric and sensitive to pH, ionic strength, and living environment. However, intra- and extracellular components in those bacteria cells are so diverse that the separation of bacteria under electric field has to be carefully controlled. Certain bacteria have strong affinity to organic or inorganic surface, substances from soil, and the cell surface of other kinds of bacteria. Therefore, chains or clusters of bacteria cells tend to be easily formed. The formation of chains or clusters makes the electrophoretic separation of bacteria less feasible since they produce many unwanted peaks in the electropherogram.

Figure 1 shows the electrophoretic separation of *Micrococcus lysodeikticus* and *Pseudomonas fluorescens*. Without proper dispersion, bacteria tend to form many different sizes of chain or cluster resulting in several strong peaks with different migration times as shown in Figure 1a and 1c. When the bacteria samples were well dispersed with ultrasonication for 5 min before injection, *Micrococcus lysodeikticus* and *Pseudomonas fluorescens* showed a strong peak with the great suppression of chain or cluster formation (Figure 1b and 1d). We found that ultrasonication was required before every injection of bacteria samples, meaning that the formation of chains or clusters are favorable under normal buffer condition.[9]

Figure 2 shows the separation of mixed *Micrococcus lysodeikticus* and *Pseudomonas fluorescens* at 8 : 1 diluted running buffer with 0.0125% PEO (Mw = 1,000,000). As shown in Table 1, *Micrococcus lysodeikticus* is non-motile, while *Pseudomonas fluorescens* has flagella. The shape of *Micrococcus lysodeikticus* is close to sphere, while *Pseudomonas fluorescens* is rod-like. Baseline resolution for the mixed *Micrococcus lysodeikticus* and *Pseudomonas fluorescens* sample was obtained with high separation efficiency.

As compared to Figure 1b and 1d, different migration times were observed for *Micrococcus lysodeikticus* and *Pseudomonas fluorescens* in Figure 2 compared to in Figure 1, and this phenomenon can be attributed to a decreased concentration of running buffer. We believe that a lower buffer concentration changed the local environment of bacteria cell surface, generating mobility shifts and improved resolution. Also, many bacteria can secret proteins, enzymes, and other small biomolecules in the buffer. Therefore, the surface properties of mixed bacteria may be altered, resulting in interference of microbe analysis or migration time shifts. Even, the mobility shift at the same running buffer concentration but different sample buffer concentration was reported[16] for the detection of *Escherichia coli* in urinary tract. *Escherichia coli* in concentrated urine had slower mobility than in diluted urine, and the use of internal standard was suggested for the identification of bacteria or comparing electropherograms.

In order to investigate variations of shape or size of microbes caused by the age or changes in growth conditions, *Aerobacter*

Table 1. The classes and properties of bacteria

Bacterium	Gram stain	Diameter (μm)	Form	Flagella	Optimum growth Temperature	Aerobe or anaerobe	Existence in nature
Pseudomonas fluorescens	negative	0.5-1	slightly curved rods	one or several flagella	25-30 °C	strict aerobes	soil and water
Aerobacter aerogenes	negative	0.5-1	motile rods	4-6 peritrichous flagella	$\leq$ 37 °C	facultative aerobes	faces of man other animal, soil, water, sewage
Micrococcus lysodeikticus	positive	1-2	spheres, irregular cluster or regular packets	nonmotile	30 °C	strict aerobes	skin of man and other animal, soil, dust, water

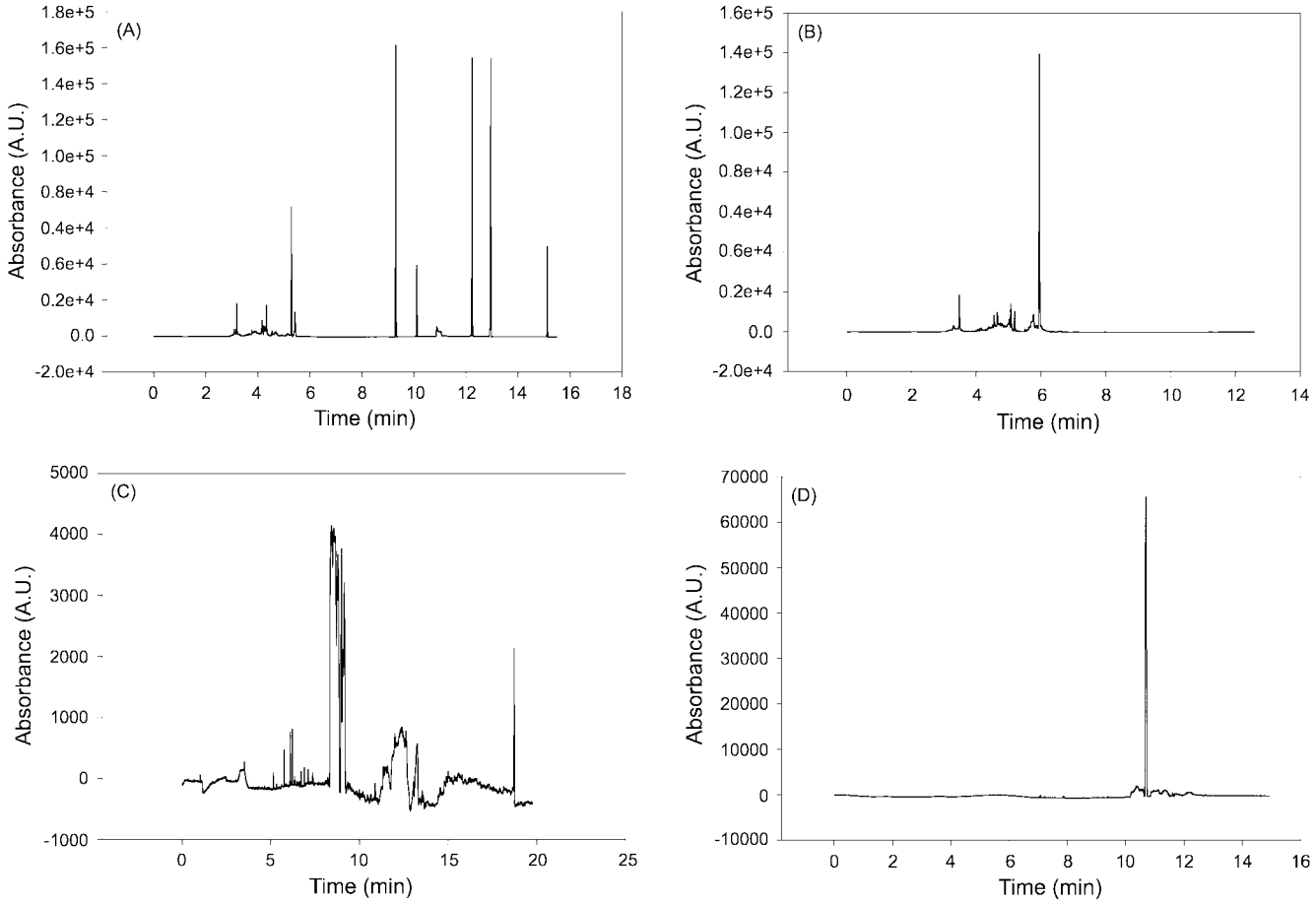

Figure 1. Electropherograms of *Micrococcus lysodeikticus* (Fig. 1a and 1b) and *Pseudonomas fluorescens* (Fig. 1c and 1d). Bacteria samples were dissolved in 8:1 diluted 0.05X TBE buffer at the concentration of 1.0 mg/mL. For figure 1b and 1d, bacteria were dispersed in an ultrasonic bath for 5 min. For figure 1a and 1c, bacteria were injected into the column without the treatment by ultrasound. Running buffer (ph = 8.4) was composed of 4.5 mM Tris, 4.5 mM boric acid, 0.1 mm EDTA, and 0.0125% poly(ethylene)oxide (Mw = 1,000,000). Other condition: fused silica capillary, 75 micrometer i.d., 27 cm total length, 20 cm effective length, + 10 kV running voltage, room temperature, pressure injection at 0.5 psi. for 10 s.

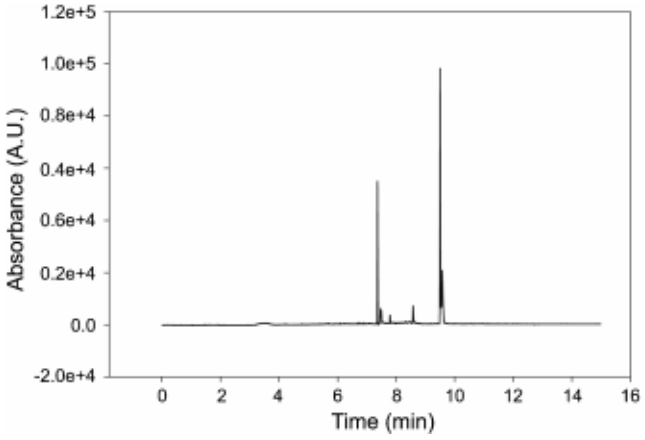

Figure 2. Electropherograms of mixed *Micrococcus lysodeikticus* and *Pseudomonas fluorescens.* Each bacterium concentration was 0.5 mg/mL. Running buffer was further diluted 8 : 1 from that used in figure 1. Other conditions are the same as in figure 1.

aerogenes was stored at two different temperature conditions and compared as shown in Figure 3. The variation may make it difficult to identify microbes, however, the analysis of bacteria at different growth stages may be advantageous in obtaining information regarding the behavior of microbes under high electric field.

When *Aerobacter aerogenes* was stored at 0 ^{o}C (completely dormant), a strong peak with several minor peaks was observed (Figure 3a). However, when it was stored at room temperature (slightly active) for 15 days, many noticeable peaks were obtained (Figure 3b) indicating the formation of chains or clusters. Since bacteria are living organism, they can grow even under unculturable conditions. It was known that the size of bacteria became smaller under those conditions probably due to the secretion of cellular components.[17] The change of size and shape and the formation of chains or clusters would cause the mobility shifts and unwanted peaks. This gives the idea that the identification and separation of microbes should be taken in consideration of growth stage and storage condition. As suggested by Amstrong,[1,16] the use of internal standards would produce the reproducible results for both qualitative and quantitative analysis of microbes.

To confirm the changes of size and shape at different storage conditions, scanning electron microscopic images were taken for *Aerobacter aerogenes* as shown in Figure 4a

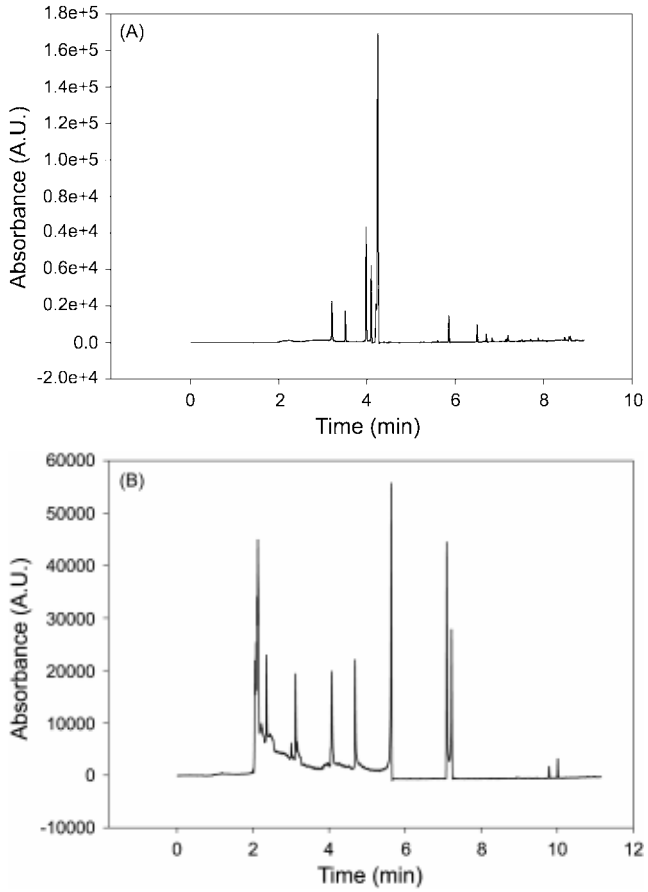

Figure 3. Electropherograms of *Aerobacter aerogenes* stored at 0 °C (Fig. 3a) or stored at room temperature for 15 days (Fig. 3b). Formation of more chains or clusters was observed with *Aerobacter aerogenes* stored at room temperature (Fig. 3b). For figure 3a and 3b, bacterium sample was dispersed by ultrasonication for 5 min before injection.

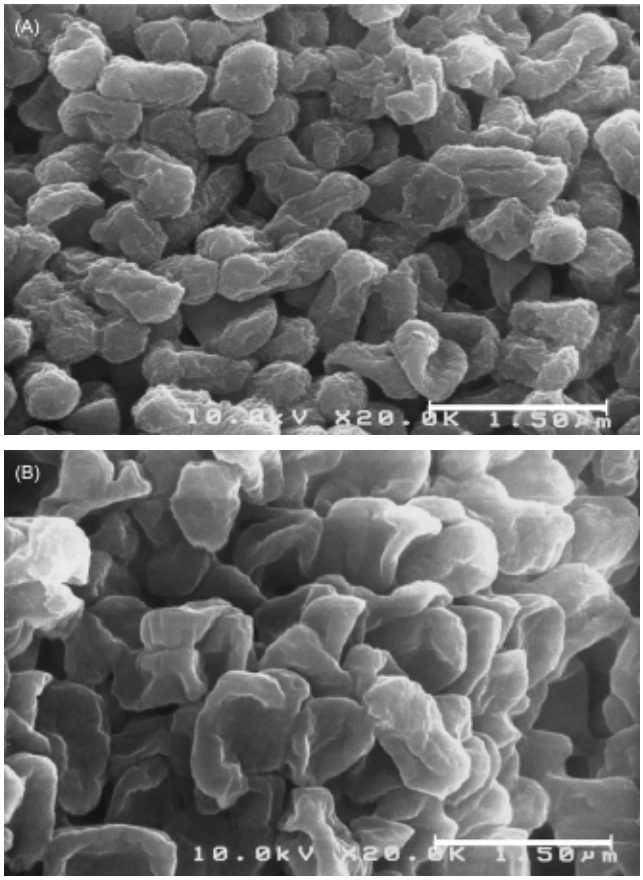

Figure 4. The scanning electron microscopic images of *Aerobacter aerogenes*. Figure 4a and 4b correspond to figure 3a and 3b, respectively. SEM was performed using Hitachi S-4200 at 10 kV and magnified 20,000 times. For the preparation of bacteria, see the experimental section.

and 4b. Figure 4a and 4b correspond to figure 3a and 3b, respectively. Compared to figure 4a, figure 4b (*Aerobacter aerogenes* stored at room temperature for 15 days) shows distorted shapes with uneven sizes. This change would cause many spurious peaks as shown in figure 3b since the electrophoretic mobility is the function of size and shape. SEM images clearly suggest that the growth condition should be carefully considered to separate and identify bacteria.

Conclusions

Microbes with different size and shape were separated using capillary electrophoresis without cell lysis. We found that a proper dispersion of microbes before injection to the capillary inlet was critical for reproducible results. Otherwise, unwanted peaks due to the formation of chains for clusters were observed. Also, the storage condition was turned out to be important for the analysis of bacteria. Electropherograms of bacteria stored at 0 °C or at room temperature showed different migration behavior and it could be attributed to the change of size and shape at different environment. This change was confirmed by analysis of the scanning electron microscopic images.

We believe that direct analysis of pathogenic microbes by capillary electrophoresis would be useful since it shows the great potential as a fast and simple diagnosis tool. Study toward the separation of bacteria with cell fixation and/or using a lab-on-a-chip technique is under progress.

Acknowledgment. This work was supported by grant No. R05-2001-000-00238-0 from the Basic Research Program of the Korea Science & Engineering Foundation.

References

1. Armstrong, D. W.; Schulte, G.; Schneiderheinze, J. M.; Westenberg, D. J. *Anal. Chem.* **1999**, *71*, 5465.
2. Matsheka, M. I.; Elisha, B. G.; Lastovica, A. L.; On, S. L. W. *FEMS Microbiol. Lett.* **2002**, *211*, 17.
3. Tremoulet, F.; Duche, O.; Namane, A.; Martinie, B.; The European *Listeria* Genome Consortium.; Labadie, J. C. *FEMS Microbiol. Lett.* **2002**, *210*, 25.
4. Alexander, P.; Tomas, W. *F. J. Anal. Chem.* **1997**, *359*, 198.
5. Vanderzant, C.; Splittsoesser, D. F. *Compendium of Methods for the Microbiological Examination of Food*, 3rd ed.; American Public Health Association: Washington, DC, 1992.
6. Johnson, J. L.; Rose, B. E.; Sharar, A. K.; Ransom, G. M.;

Lattuada, C. P.; McNamara, A. M. *M. J. Food Prot.* **1995**, *58*, 597.
7. Liu, Y.; Li, Y. *Anal. Chem*. **2001**, *73*, 5180.
8. Yao, Z. P.; Demirev, P. A.; Fenselau, C. *Anal. Chem*. **2002**, *74*, 2529.
9. Schneiderheinze, J. M.; Armstrong, D. W.; Schulte, G.; Westenberg, D. J. *FEMS Microbilo. Lett*. **2000**, *189*, 39.
10. Glynn, J. R.; Belongia, B. M.; Arnold, R. G.; Ogden, K. L.; Baygents, J. C. *Appl. Environ. Microbiol*. **1998**, *64*, 2572.
11. Kim, Y.; Edward, S. Y. *J. Chromatog. A* **1997**, *781*, 315.
12. Simo-alfonso, E.; Conti, M.; Gelfi, C.; Righetti, P. G. *J. Chromatog. A* **1995**, *689*, 85.
13. Okun, V. M.; Ronacher, B.; Blaas, D.; Kenndler, E. *Anal. Chem.* **1999**, *71*, 2028.
14. Schnabel, U.; Groiss, F.; Blaas, D.; Kenndler, E. *Anal. Chem.* **1996**, *68*, 4300.
15. Shen, Y.; Berger, S. J.; Smith, R. D. *Anal. Chem.* **2000**, *72*, 4603.
16. Armstrong, D. W.; He, L. *Anal. Chem.* **2001**, *73*, 4551.
17. Leclerc, H.; Moreau, A. *FEMS Microbilo. Review.* **2002**, *26*, 207.