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Accelerated Articles

Simultaneous Determination of Anionic Intermediates for *Bacillus subtilis* Metabolic Pathways by Capillary Electrophoresis Electrospray Ionization Mass Spectrometry

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A method for simultaneous determination of anionic metabolites based on capillary electrophoresis (CE) coupled to electrospray ionization mass spectrometry is described. To prevent current drop by the system, electroosmotic flow (EOF) reversal by using a cationic polymer-coated capillary was indispensable. A mixture containing 32 standards including carboxylic acids, phosphorylated carboxylic acids, phosphorylated saccharides, nucleotides, and nicotinamide and flavin adenine coenzymes of glycolysis and the tricarboxylic acid cycle pathways were separated by CE and selectively detected by a quadrupole mass spectrometer with a sheath-flow electrospray ionization interface. Key to the analysis was EOF reversal using a cationic polymer-coated capillary and an electrolyte system consisting of 50 mM ammonium acetate, pH 9.0. The relative standard deviations of the method were better than 0.4% for migration times and between 0.9% and 5.4% for peak areas. The concentration detection limits for these metabolites were between 0.3 and 6.7 μmol/L with pressure injection of 50 mbar for 30 s (30 nL); i.e., mass detection limits ranged from 9 to 200 fmol, at a signal-to-noise ratio of 3. This method was applied to the comprehensive analysis of metabolic intermediates extracted from *Bacillus subtilis,* and 27 anionic metabolites could be directly detected and quantified.

In the postgenomic era, tremendous efforts have been focused on elucidation of biological function. Systematic and high-throughput analyses of mRNA and proteins are central to today's functional genomics initiatives. Recently, metabolomics, i.e., the analysis of all cellular metabolites, has become a powerful new tool for gaining insight into functional biology.[1−4] Measurement of the level of numerous metabolites within a cell, and tracking their change under different conditions, not only provide direct information on metabolic phenotypes but are also complementary to gene expression and proteomic studies. However, only a limited number of methodologies[4−8] have been applied to the analysis of metabolites because of their high polarity, poor detectability, and identical characteristics. In addition, the fact that over 1,000 metabolites exist in the cell complicates the analysis.

Fiehn et al.[4,5] reported that 326 distinct compounds from *Arabidopsis thaliana* leaf extracts were quantified by gas chromatography/mass spectrometry (GC/MS). Although the GC/MS method demonstrated impressive performance, it has some

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drawbacks. For example, different derivatization procedures were necessary for each chemical moiety and every compound could not be analyzed. Bhattacharya et al.[6] applied anion-exchange high-performance liquid chromatography with suppressed conductivity detector in tandem with UV detection to metabolic intermediate analysis and 16 metabolites in the cell extracted from *Escherichia coli* were successfully determined. The method, however, failed to analyze several pairs of metabolites such as succinate and malate, glucose 6-phosphate (G6P) and 2-oxoglutarate, and fructose 6-phosphate (F6P) and fumarate due to poor detection selectivity. Also, nicotinamide coenzymes, biologically important compounds, such as nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH), were not detected.

The metabolites of key pathways for cellular energy production such as glycolysis and the tricarboxylic acid (TCA) cycle are almost entirely anionic species, e.g., carboxylic acids, phosphorylated carboxylic acids, and phosphorylated saccharides. An enormous number of anionic metabolites also exist in other pathways. In addition, many compounds such as nucleotides, nicotinamide coenzymes, and amino acids are negatively charged under neutral or alkaline pH condition. Therefore, methodology for anionic metabolite analysis is required.

Reversed-phase liquid chromatography−mass spectrometry (RPLC−MS) is commonly used for biological sample analysis.[9−11] However, most of intracellular metabolites are too polar to be retained on the reversed-phase column. While anion-exchange liquid chromatography−mass spectrometry (AELC−MS) might be a good candidate to overcome this limitation, appropriate volatile mobile phases for this system are not generally available.

As previously described,[12,13] capillary electrophoresis (CE) has demonstrated outstanding performance for anion analysis. More than 200 anions including inorganic and organic anions, amino acids, nucleotides, carbohydrates, and phosphorylated saccharides have been determined using standardized conditions.[13] Although CE methodology provides superior separation efficiency, its sensitivity and identification capabilities are often insufficient when applied to metabolites in biological samples.

Recently capillary electrophoresis−electrospray ionization mass spectrometry (CE−ESI-MS) has emerged as a powerful analytical tool, and a number of CE−ESI-MS methods have been developed for the analysis of charged species such as carboxylic acids,[14] phenolic compounds,[15] amino acids,[16] metal species,[17] tetramines,[18] and herbicides.[19] In this marriage of techniques, CE confers rapid analysis and efficient resolution, and MS provides high selectivity and sensitivity. The ESI mode has proven to be sensitive, versatile, and relatively easy to use in combination with CE.

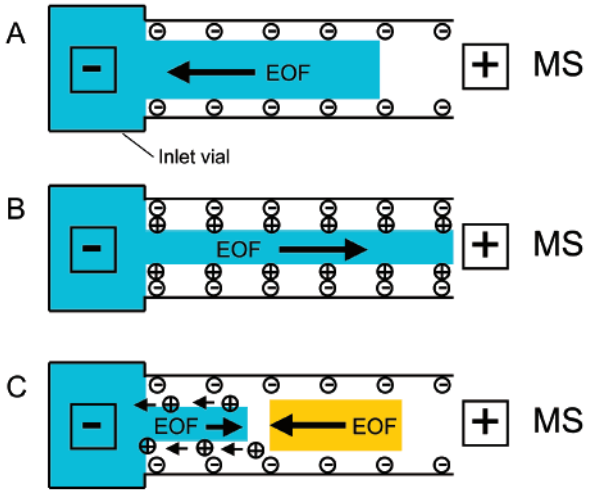

Figure 1. Schematic of the EOF profile in CE−ESI-MS with negative mode. (A) Normal EOF, (B) EOF reversal by addition of cationic surfactant to the electrolyte, and (C) production of no-liquid zone by generating normal EOF.

In this paper, we developed a sensitive, selective, and rapid analytical method for the anionic species by CE−ESI-MS. The method was applied to the comprehensive analysis of intracellular metabolites of glycolysis and the TCA cyclic pathways in bacterial samples.

EXPERIMENTAL SECTION

Chemicals. Dihydroxyacetone phosphate (DHAP), glycerol 3-phosphate (glycerol3P), *cis*-aconitate, 2-phosphoglycerate (2PG), 2,3-diphosphoglycerate (2,3DPG), and fructose 1,6-diphosphate (F1,6P) were purchased from Sigma (St. Louis, MO), 3-phosphoglycerate (3PG) was from Fluka (Buchs, Switzerland), piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) was from Dojindo (Kumamoto, Japan), and DIFCO nutrient broth was from Becton Dickinson (Sparks, MD). All other reagents were obtained from Wako (Osaka, Japan). Individual stock solutions of DHAP and 2,3DPG at a concentration of 10 and 100 mM were prepared in Milli-Q water. The working mixture standard was prepared by diluting these stock solutions with Milli-Q water just before injection. The chemicals used were of analytical or reagent grade. Water was purified with a Milli-Q purification system (Millipore, Bedford, MA).

Instrumentation. All CE−ESI-MS experiments were performed using an Agilent CE capillary electrophoresis system with built-in diode-array detector, an Agilent 1100 series MSD mass spectrometer, an Agilent1100 series isocratic HPLC pump, a G1603A Agilent CE−MS adapter kit, and a G1607A Agilent CE−ESI-MS sprayer kit (all Agilent Technologies, Waldbronn, Germany). All system control, data acquisition, and MSD data evaluation were performed via a G2201AA Agilent ChemStation software for CE-MSD. The CE−MS adapter kit includes a capillary cassette, which facilitates thermostating of the capillary, and the CE−ESI-MS sprayer kit, which simplifies coupling the CE system with MS systems, was equipped with an electrospray source. The sprayer has an orthogonal flow design to reduce the detrimental effects caused by the charged particles or droplets, as described by Voyksner and Lee.[20]

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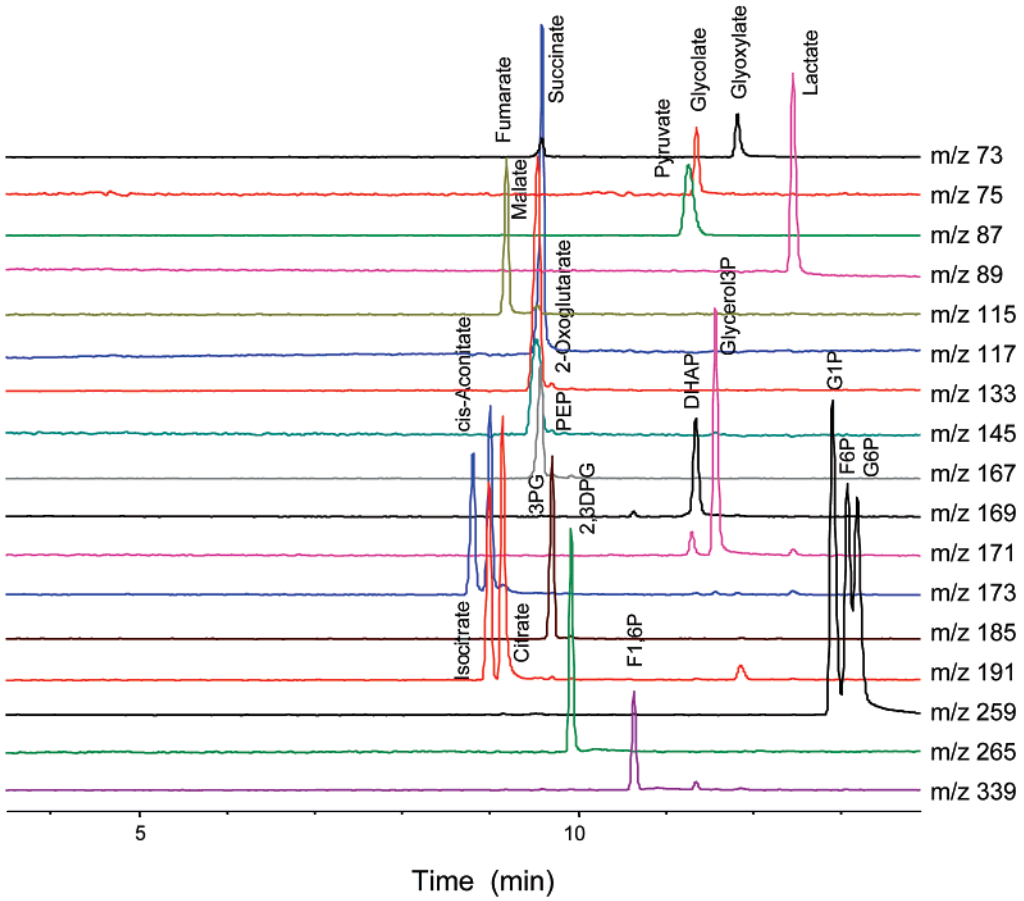

Figure 2. CE−ESI-MS selective ion electropherograms for a standard mixture of 20 metabolites of glycolysis and the TCA cycle. Experimental conditions: sample concentration, 100 μmol/L each; capillary, SMILE(+) 50 μm i.d. × 100 cm; electrolyte, 50 mM ammonium acetate, pH 9.0; applied potential, −30 kV; injection, 30 s at 50 mbar; temperature, 20 °C; sheath liquid, 10 μL/min 5 mM ammonium acetate in 50% (v/v) methanol−water.

Bacterial Strains and Growth Conditions. *Bacillus subtilis* JH642 (laboratory stock) was used in this work and cultured at 37 °C in DS medium (0.8% nutrient broth, 0.1% KCl, 1 mM $MgSO_4$, 1 mM $Ca(NO_3)_2$, 1 μM $FeSO_4$, and 10 μM $MnCl_2$).[21] Growth was monitored by measuring optical density at 660 nm, and the cells were grown to T_0, at the end of exponential growth, and to T_1, 1 h after T_0.

Sample Preparation. Intracellular metabolites in *B. subtilis* JH642 cells were extracted as follows. Cells were grown in DS culture medium, and aliquots of 5 mL were withdrawn at T_0 and T_1. The media were passed through a 0.45-μm pore size filter. The cells were washed with 5 mL of Milli-Q water to prevent contamination of DS medium and plunged into 1 mL of methanol, where enzymes were rapidly deactivated. An internal standard containing of 28 μL of 1 mM PIPES was spiked into the methanol solution. After the incubation for 5 min at room temperature, 1 mL of chloroform and 372 μL of Milli-Q water were added to the solution and then thoroughly mixed. The 200 μL of water layer was removed and centrifugally filtered through a Millipore 5-kDa-cutoff filter to remove proteins. The filtrate was lyophilized and dissolved in 20 μL of Milli-Q water prior to injection.

CE−ESI-MS Conditions. Separations were carried out on commercially available SMILE(+), a cationic capillary coated with successive multiple ionic polymer layers[22] which was purchased from Nacalai Tesque (Kyoto, Japan). The capillary dimension were 50 μm i.d. × 100 cm total length. The electrolyte for the CE

Table 1. Reproducibility, Linearity, and Sensitivity

	RSD. (n = 6) (%)			detection limit	
compound	migration time	peak area	linearity correln	concn (μmol/L)	mass (fmol)
glyoxylate	0.31	1.8	0.999	5.6	170
glycolate	0.29	5.4	0.999	6.0	180
pyruvate	0.27	1.4	0.999	1.1	33
lactate	0.34	1.3	0.999	1.1	33
fumarate	0.17	2.1	0.999	1.2	36
succinate	0.18	1.3	0.998	1.6	48
malate	0.18	2.3	0.998	0.6	18
2-oxoglutarate	0.17	2.7	0.999	2.7	81
PEP	0.19	2.3	0.997	1.8	54
DHAP	0.29	1.9	0.999	1.0	30
glycerol3P	0.30	2.0	0.997	1.0	30
cis-aconitate	0.16	2.6	0.999	0.7	21
3PG	0.19	1.5	0.996	3.0	90
isocitrate	0.17	2.0	0.998	3.3	99
citrate	0.17	3.8	0.998	2.2	66
G1P	0.37	1.4	0.997	0.3	9
F6P	0.39	0.9	0.997	0.3	9
G6P	0.39	3.4	0.997	0.4	12
2,3DPG	0.20	3.2	0.999	6.7	200
F1,6P	0.25	2.1	0.993	6.5	200

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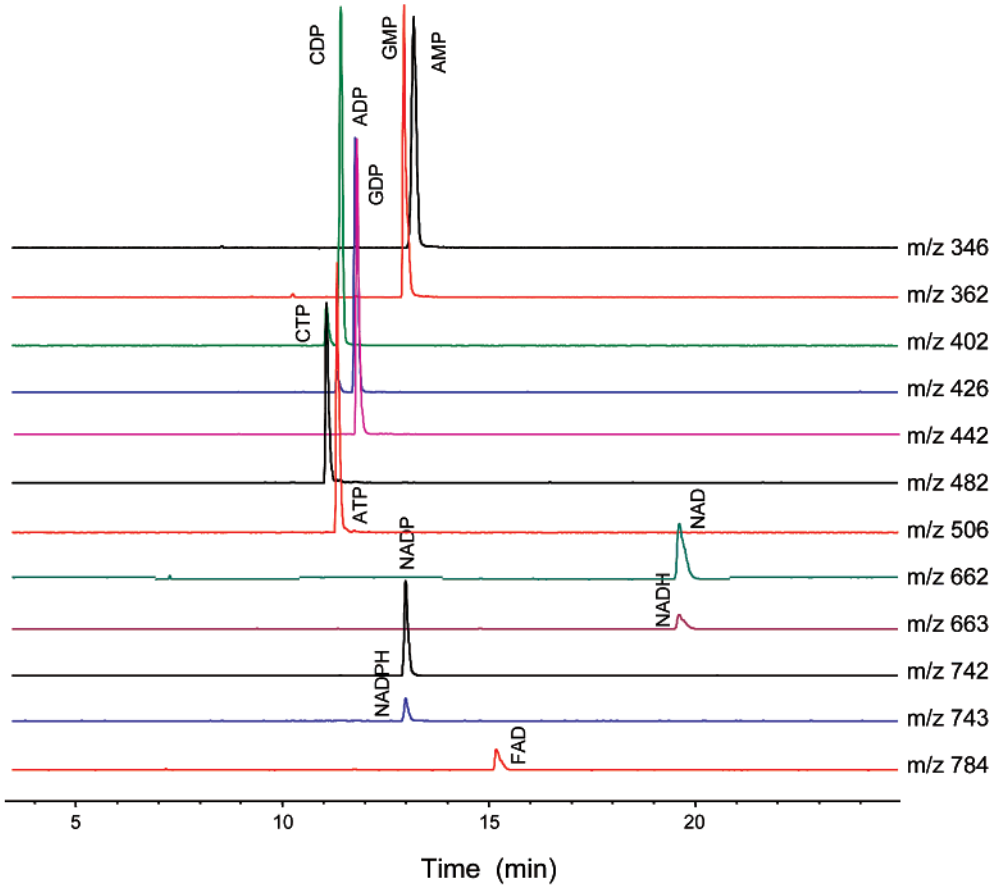

Figure 3. CE−ESI-MS selective ion electropherograms for a standard mixture of nucleotides and nicotinamide and flavin adenine coenzymes. Experimental conditions: sample concentration, 100 μmol/L each; other conditions are the same as in Figure 2.

separation was 50 mM ammonium acetate solution, pH 9.0. Prior to first use, a new capillary was flushed successively with the running electrolyte, 50 mM acetic acid (pH 3.4), and then the electrolyte again for 20 min each. Before each injection, the capillary was equilibrated for 2 min by flushing with 50 mM acetic acid (pH 3.4) and then for 5 min with the running electrolyte. Sample was injected with a pressure injection of 50 mbar for 30 s (~30 nL). The applied voltage was set at −30 kV. The capillary temperature was thermostated to 20 °C, and the sample tray was cooled below 5 °C. The Agilent 1100 series pump equipped with 1:100 splitter was used to deliver 10 μL/min of 5 mM ammonium acetate in 50% (v/v) methanol−water to the CE interface, where it is used as a sheath liquid around the outside of the CE capillary to provide a stable electrical connection between the tip of the capillary and grounded electrospray needle.

ESI-MS was conducted in the negative ion mode, and the capillary voltage was set at 3500 V. A flow of heated dry nitrogen gas (heater temperature 300 °C) was maintained at 10 L/min. The spectrometer was scanned from m/z 70 to 350 at 0.6 s/scan during the separation and detection. In selective ion monitoring mode, deprotonated $[M - H]^-$ ions were monitored for metabolites with 20-ms sampling time for each.

RESULTS AND DISCUSSION

Metabolite Extraction. Rapid enzyme inhibition and efficient metabolite extraction are necessary to accurately quantify metabolites in cells. In this work, organic solvents such as methanol, toluene, and chloroform were investigated because they can easily deactivate enzymes and do not interfere with the CE separation. Cultured *B. subtilis* cells were immersed in each solvent, and denatured proteins were removed using a 5-kDa-cutoff centrifugal filter. After the solvent was evaporated, the residue metabolites were dissolved in Milli-Q water and analyzed by CE. It was found that the glutamate concentration quantified by methanol extraction was 5- and 13-fold higher than that by toluene and chloroform, respectively. For other metabolites, much higher concentrations were also obtained using methanol extraction. Since a previous study[5] utilized hot methanol for metabolite extraction from leaves, the influence of methanol temperature on extraction efficiency was studied at 30, 40, and 50 °C. However, the metabolite concentrations were nearly the same at all temperatures. The plunging time of the cells into methanol was also studied and was found to have no measurable effect on metabolite extraction efficiency. Therefore, metabolites are extracted from cells with room-temperature methanol for 5 min in subsequent experiments.

Choice of Cationic Polymer-Coated Capillary. Analysis of anions by CE is usually performed in negative mode, where the inlet of the capillary is at the cathode and the outlet at the anode. Since the CE−ESI-MS system does not possess the outlet vial, EOF movement toward the cathode (opposite the MS direction) creates a gap in the liquid at the capillary exit, resulting in a current drop (Figure 1A). For this reason, few papers on anion

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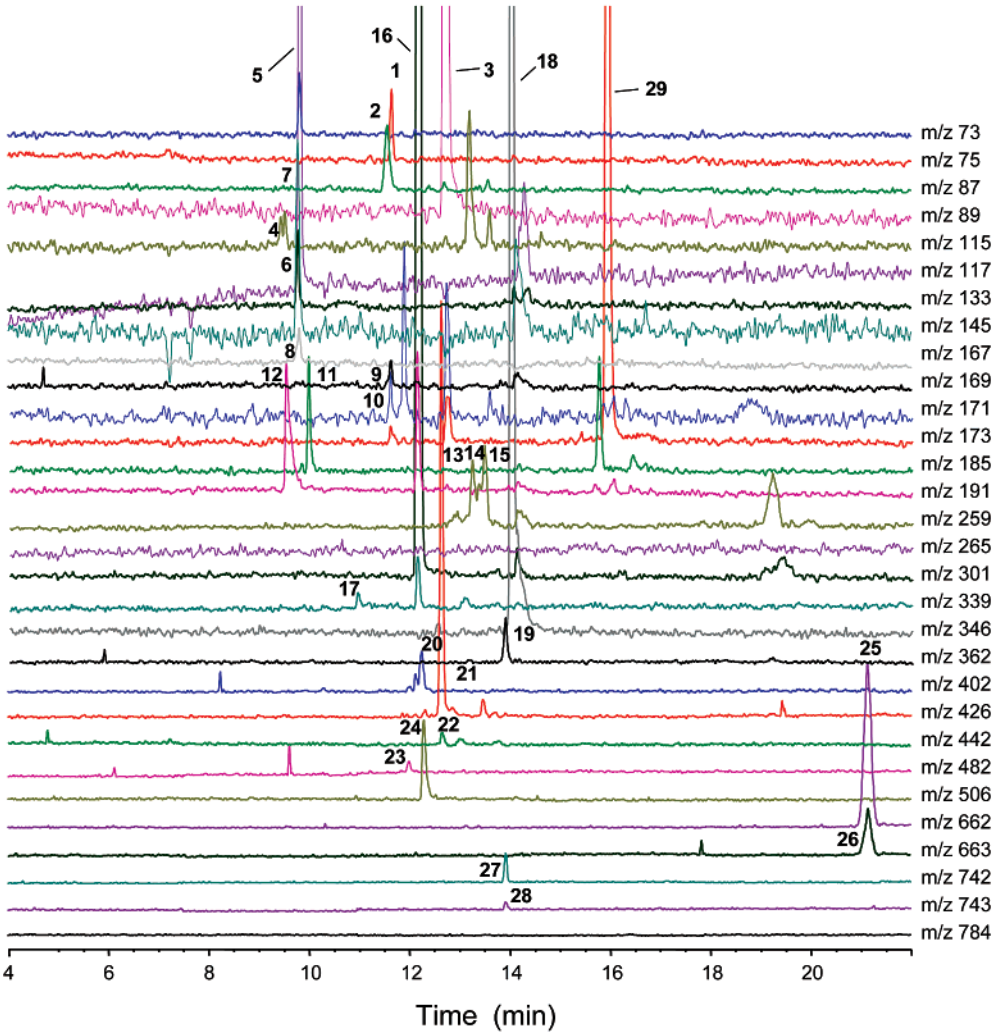

Figure 4. Selected ion electropherograms for metabolic intermediates at T_1 of *B. subtilis* JH642. Peak identification: 1, glycolate; 2, pyruvate; 3, lactate; 4, fumarate; 5, succinate; 6, malate; 7, 2-oxoglutarate; 8, PEP; 9, DHAP; 10, glycerol3P; 11, 3PG; 12, citrate; 13, G1P; 14, F6P; 15, G6P; 16, PIPES (is); 17, F1,6P; 18, AMP; 19, GMP; 20, CDP; 21, ADP; 22, GDP; 23, CTP; 24, ATP; 25, NAD; 26, NADH; 27, NADP; 28, NADPH; 29, unknown. Experimental conditions are the same as in Figure 2.

analysis have been reported by CE−ESI-MS using this configuration.

Johnson et al.[14] applied an EOF reversal technique for carboxylic acid analysis by CE−ESI-MS, which reverses EOF toward the anode by addition of cationic surfactant such as cetyltrimethylammonium bromide[23] to the buffer (Figure 1B). However, in our experience, a current drop was invariably observed within few minutes after the voltage was applied. We assume that cationic surfactants on the capillary wall also migrate toward the inlet vial (cathode), so that silanol groups (SiO^-) are revealed and normal EOF is generated toward the cathode (Figure 1C). Therefore, permanent EOF reversal was necessary, independent of buffer conditions, in CE−ESI-MS with negative mode.

In the present study, this problem was overcome by employing cationic polymer-coated capillary to reverse EOF. As Katayama et al.[22] described the SMILE(+), cationic polymer (Polybrene)-coated, capillary was able to constantly reverse EOF toward the anode without any additives to the buffer and it exhibited strong endurance and chemical stability. Accordingly, we selected the SMILE(+) capillary to reverse EOF for anion analysis by CE−ESI-MS with negative mode, and its utility enabled successive anion analysis without deleterious current drop.

CE−ESI-MS Parameters. Mass spectra of anionic metabolites were acquired in negative ion mode scanning from m/z 100 to 350. The deprotonated molecular ion, $[M - H]^-$, dominated the mass spectrum for each compound; consequently, anions were determined at their deprotonated molecular weights. Commercially available 21-anion standards, which are metabolic intermediates in glycolysis and the TCA cycle pathways, were analyzed with CE−ESI-MS by using 50 mM ammonium acetate electrolyte at pH 6.9. Although all of the components were not electrophoretically separated, most could be selectively detected at their deprotonated molecules by the mass spectrometer except for some isomers. F6P and G6P were not fully resolved, while 2PG and 3PG were overlapped. In this system, isomers must be separated because they exhibited the same mass spectrum, so that they were neither identified nor quantified even by MS.

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In CE, ionic species are separated on the basis of their charge and size. Several reports demonstrated that buffer pH significantly affects the mobilities of analytes.[24−26] Thus, operating pH was optimized in the range from pH 4.7 to 9.0 to achieve satisfactory resolution of the isomers. Resolution of glucose 1-phosphate (G1P), F6P, and G6P isomers did vary with the buffer pH, with better separations at higher pH. The 2PG and 3PG isomers remained completely unresolved at any pH tested. Since the SMILE(+) capillary tended to deteriorate under further alkaline condition, pH 9.0 was selected as the operating pH of the electrolyte.

The choice of the sheath liquid parameters is also very important in developing a method employing CE−ESI-MS. In this work, 5 mM ammonium acetate in 50% (v/v) methanol−water at a flow rate of 10 μL/min was used because it exhibited excellent sensitivity and long-term stability as previously described.[16]

Method Validation. Figure 2 shows selected ion electropherograms of the 20-anionic metabolite standard mixture obtained by CE−ESI-MS. Though the migration times of succinate, malate, 2-oxoglutamate, and phosphoenol pyruvate (PEP) are very close, they could be successfully detected by MS. Using the initial preconditioning of the capillary with the running electrolyte for 5 min, the reproducibility, linearity, and sensitivity of the method were investigated. It was found that the migration times of all the 20 metabolites gradually decreased over time and the relative standard deviation (RSD) values for migration times obtained were not acceptable (1.8%−3.3%, $n = 6$). For reasons that are still unclear, migration times were increased and stabilized when capillary was flushed with low-pH buffer. Therefore, the capillary was flushed with 50 mM acetic acid (pH 3.4) for 2 min before preconditioning with the running electrolyte. Data obtained by using the optimized conditions are listed in Table 1. Satisfactory reproducibility was obtained for both migration times and peak areas, as indicated in the table. The calibration curves for all species were between 0.993 and 0.999 at 5, 10, 20, 50, and 100 μmol/L with correlation coefficients. The concentration detection limits for all components were between 0.3 and 6.7 μmol/L with pressure injection of 50 mbar for 30 s (30 nL) at a signal-to-noise ratio of 3, which resulted in mass detection limits ranging from 9 to 200 fmol. Although the concentration sensitivities by the CE−ESI-MS method were better or equal to that in the HPLC method,[6] mass detection limits were 130−8300-fold better. Thus, the proposed method can be helpful when limited sample volumes are available.

Figure 3 illustrates selected ion electropherograms of other metabolically important compounds including nucleotides and nicotinamide and flavin adenine coenzymes by the present method. It was possible to determine every component in its deprotonated state. In this method, only acetyl- and succinyl-CoA were not detected as well-shaped peaks. Since these CoA compounds exhibit anionic and hydrophobic characteristics, they may be adsorbed on cationic polymer coated on the capillary wall through ion-exchange and hydrophobic interactions.

These results indicate that even though some compounds were not fully measured, the CE−ESI-MS method enabled a single-step, selective metabolic intermediate analysis and can be useful for comprehensive anion analysis of biological and physiological samples.

Table 2. Metabolite Amounts of *B. subtilis* JH642 Cells Grown in 5 mL of DS Medium

	amount (pmol)			amount (pmol)	
compound	T_0	T_1	compound	T_0	T_1
glyoxylate	nd[a]	nd	F6P	26	32
glycolate	121	253	G6P	78	69
pyruvate	113	299	2,3DPG	nd	nd
lactate	7300	6270	F1,6P	5	11
fumarate	90	73	AMP	956	1050
succinate	153	1010	GMP	115	30
malate	15	59	CDP	8	21
2-oxoglutarate	nd	325	ADP	185	195
PEP	60	82	GDP	32	8
DHAP	88	76	CTP	4	10
glycerol3P	83	195	ATP	29	65
cis-aconitate	nd	nd	NAD	505	479
3PG	144	163	NADH	514	485
isocitrate	nd	nd	NADP	48	37
citrate	nd	264	NADPH	53	46
G1P	30	38	FAD	nd	nd.

[a] nd, not detected.

Analysis of Metabolic Intermediates in *Bacillus subtilis*. Nutritional limitation leads *Bacillus* species such as *B. subtilis* and *Bacillus anthracis* to produce a dormant, environmentally resistant spore.[27] This phenomenon is accepted universally as a basic model of bacterial differentiation. The sporulation events are strictly governed by regulatory networks that coordinate nutritional, transcriptional, and morphological signals.[28] One aspect of physiological control of these networks is the metabolic and signaling role of the TCA cycle. A strong linkage between the TCA cycle and sporulation is reflected by the fact that the TCA cycle enzymes are maximally induced.[29,30] It is presumed that induction of the TCA cycle at the end of the exponential growth phase allows stationary-phase cells to fully metabolize byproducts of glycolysis, such as pyruvate, lactate, acetate, and acetoin, yielding energy, reducing power, and biosynthetic intermediates for the synthesis of the RNA, protein, peptidoglycan, and lipid needed to form a spore.[31]

To demonstrate the utility of the developed CE−ESI-MS method, metabolic intermediates of *B. subtilis* cells were analyzed. *B. subtilis* JH642 cells were grown to transition phase, T_0, and to early stage of sporulation, T_1. Subsequently, at the times T_0 and T_1, metabolites were extracted from the cells as described in the Experimental Section. Figure 4 illustrates the results of metabolic intermediate analysis of *B. subtilis* JH642 at T_1. Well-defined selected ion electropherograms were obtained without interference, and 27 metabolites were selectively detected. The amounts of metabolites extracted from the cells grown in 5 mL of the DS medium were quantified using PIPES as an internal standard. The

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results of both sporulation-beginning cells (T_0) and sporulating cells (T_1) are listed in Table 2. Although neither 2-oxoglutarate nor citrate could be observed at T_0, both of these metabolites were detected at the concentrations higher than 200 pmol at T_1. Dingman et al.[32] described that 2-oxoglutarate and citrate were especially implicated in induction of *B. subtilis* sporulation. Also, the amounts of succinate and malate at T_1 were several times higher than those at T_0, while GMP and GDP decreased at T_1. In addition, a large unknown compound with deprotonated molecular weight 173 (peak 29 in Figure 4) appeared only in sporulating cells. In light of these results, we conclude that spore formation of *B. subtilis* is related not only to 2-oxoglutarate and citrate but also to the increased or decreased metabolites, e.g., succinate, malate, GMP, and GDP. The CE−ESI-MS method developed in this study can be a powerful tool for the analysis of intracellular metabolites that involve some biological events such as bacterial differentiation.

CONCLUSIONS

We have developed a simple and reliable CE−ESI-MS method for the simultaneous determination of anionic compounds. Compared with other techniques, this method has several advantages: (1) various types of anions can be analyzed without derivatization, (2) sensitivity and selectivity are relatively high, and (3) the analysis time is fast. In fact, most anions are determined in less than 25 min. Furthermore, the present methodology provides reproducibility, good linearity, and excellent identification capability. Its utility was demonstrated by the simultaneous analysis of metabolic intermediates of glycolysis and the TCA cycle pathways in *B. subtilis*. These results indicate that the method can be useful for the comprehensive analysis of anionic metabolites in bacterial cells, and the method might also be applicable to many types of anions in a wide range of application areas.

(32) Dingman, D. W.; Rosenkrantz, M. S.; Sonenshein, A. L. *J. Bacteriol.* **1987**, *169*, 3068−3075.

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