M. Tomita
T. Nishioka (Eds.)

Metabolomics
The Frontier of Systems Biology

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Metabolomics

The Frontier of Systems Biology

With 112 Figures, Including 4 in Color

Springer

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Preface

The aim of this book is to review metabolomics research. The information is presented in a way that allows the reader to view the subject of metabolomics from a broad perspective. Creative and progressive research on metabolomes began in Japan and Germany in the 1990s and ranged from the development of specialized chemical analytical techniques to the construction of databases and methods for metabolic simulation. The authors have been directly involved in the development of all the subject areas that are discussed in this book, including research related to capillary electrophoresis, liquid chromatography, mass spectrometry, metabolic databases, and metabolic simulation. As the title suggests, the latest cutting-edge research projects are presented here. In addition, a selected group of applied cases, representative of likely future scenarios, is presented.

It is our hope that this book will generate further metabolomic research across a broad range of life-science disciplines, and that hitherto unforeseen applications and innovative technologies will arise from such efforts. It is especially important that medical institutions and venture enterprises should actively participate in metabolomic research, thus ensuring that it matures into a discipline offering practical medical benefits. To promote the application of metabolomic research, a number of key issues will require breakthroughs; these include the popularization of chemical analytical techniques, the development of simple but stable specialized analyzers, the parallel processing and miniaturization of such devices, and the advancement of metabolic systems biology. Researchers, technicians, and university students are urged to take on these challenges to advance metabolomic research.

We would like to thank the staff of Springer-Verlag, Tokyo, for their help in bringing this book to fruition.

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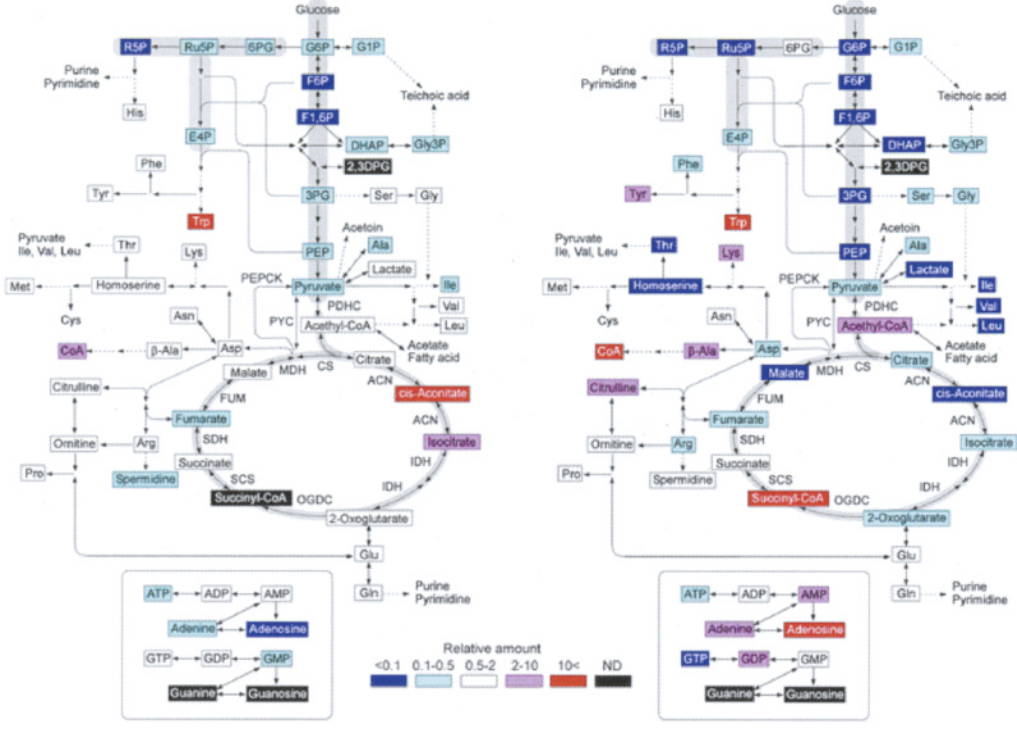

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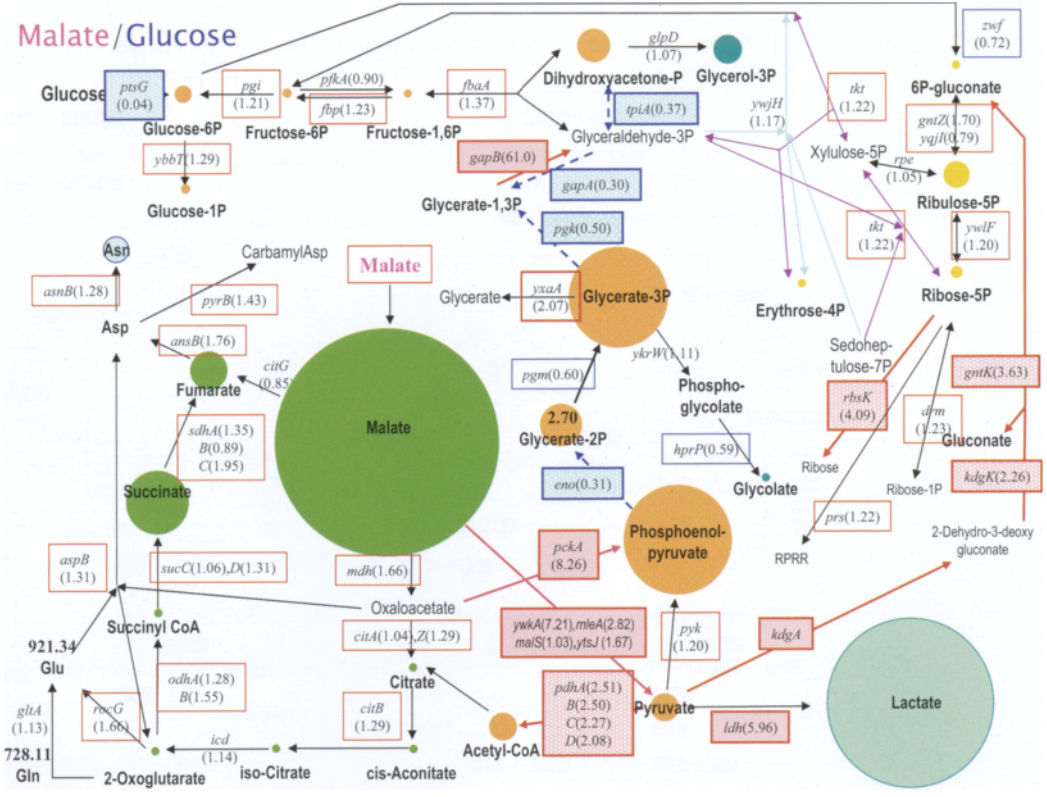

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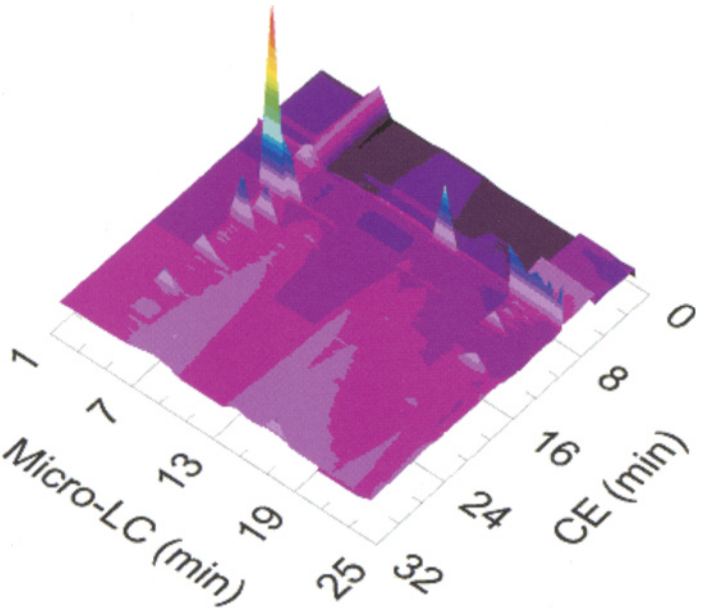

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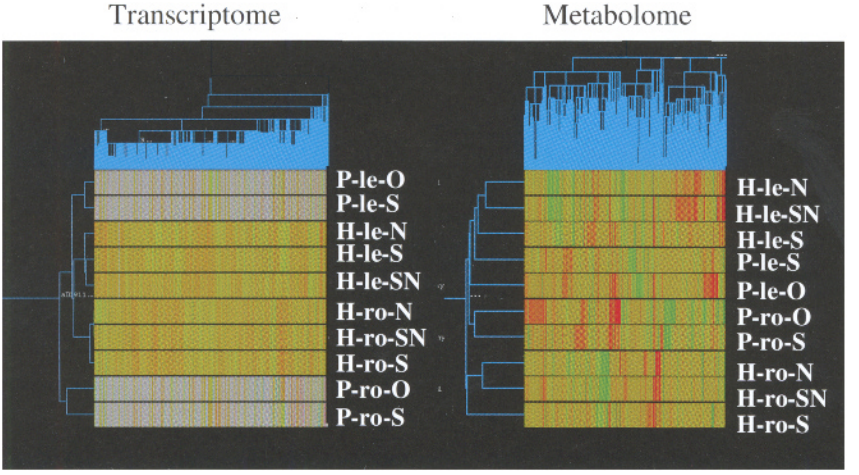

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Part I. Introduction

Chapter 1: Overview

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1. Introduction

A large number of metabolites, including sugars, organic acids, and amino acids, are present in living organisms. Several thousand such molecules, in addition to macromolecules such as nucleic acids and proteins, are involved in the life processes of organisms. Although some of these substances are externally acquired, most are the products and intermediates of metabolic reactions.

Comprehensive analysis of the metabolome, which is the complete set of metabolites in an organism or cell, is crucial to the understanding of cellular function. This large-scale analysis of metabolites is an important addition to extensive studies of DNA sequences (genome) and proteins (proteome). We cannot understand the dynamic behavior of metabolism without in-depth knowledge of the type and quantities of substances that exist, and the conditions under which they are present in living organisms and cells.

Metabolome analysis is applicable to various fields of biotechnology in the postgenomic era. However, the comprehensive analysis of metabolites is a relatively recent concept—even the term "metabolome" is new. There are several reasons as to why this is the case. For example, advanced methodology is needed to measure large numbers of metabolites over a short time period and there are many technical challenges. In the past, studies have therefore tended to pinpoint measurements of the levels of individual preselected substances only. Moreover, even if technology allows the large-scale measurement of metabolites, how can such enormous amounts of data be understood? Previously, metabolic systems were only investigated on a limited scale, and there was no concept of measuring all intracellular metabolites.

However, the recent fusion of biotechnology with information technology has generated "data driven" or "-omics" biosciences, in which large amounts of data can be collected in a comprehensive manner and consolidated for analysis using computers. Metabolomics (metabolome science) is poised to have an important role in the postgenomic era.

This book is a summary of the latest trends in metabolomics and encompasses three major subject areas: metabolome measurement techniques, applications to biosciences, and metabolome informatics. Metabolomics is still an emerging discipline, and this book focuses primarily on research projects in which the editors have taken part.

2. Metabolome Measurement Techniques

Metabolomics requires the simultaneous measurement of a large number of metabolites, and **mass spectrometry** (MS) plays a leading role in this field. A mass spectrometer can rapidly and accurately measure the molecular weights and quantities of many substances. However, although substances within a certain range (for example, molecular weight 70–500 and so on) can be measured in a comprehensive manner using MS, this technique alone cannot distinguish between two or more substances with the same molecular weight. What is therefore required is to separate substances using chromatography, for example, before injecting the sample into the mass spectrometer. In simple terms, chromatography involves placing a sample in a pre-established column and moving it. When this happens, the migration rate will differ depending on the substance, so if the mass spectrometer is connected to an outlet, many substances can be injected into the mass spectrometer at different times. This enables two substances having the same molecular weight to be measured separately. The chromatography migration time is fixed for every substance. Therefore, once the migration time for each substance is known beforehand, the substance can be identified from the molecular weight and migration time, thereby enabling it to be measured.

Liquid chromatography (LC) and **gas chromatography** (GC) are both used as standard methods, and when combined with MS these techniques are known as LC/MS and GC/MS, respectively.

Our research group has recently developed an analytical technique utilizing **capillary electrophoresis** (CE) combined with MS (CE/MS). Most of the metabolites in living organisms are charged substances (anions or cations) and CE/MS is highly suitable with these substances, demonstrating a high resolution with only a small amount of sample. As neutral (noncharged) substances cannot be measured using CE/MS, we aim to identify all of the intracellular metabolites (the complete metabolome) by combining this technique with LC/MS.

3. Applications to Biosciences

Metabolomic information is useful in a wide range of biotechnological fields. For example, samples can be classified on the basis of the metabolic patterns obtained by MS **(metabolome profiling)**. If cancer cells, for instance, are classified and diagnosed using metabolome profiling, it might be possible to develop a specific method of treatment for each different category. Since only the overall pattern is important in metabolome profiling, overlaps of the peaks of two or more substances do not create serious problems; it could be applied even without separation by chromatography.

However, the combined use of MS with chromatography or CE allows the identification of substances, which greatly extends the potential applications of the technique. For example, if the **profile comparison** of two samples reveals a significant difference in a certain peak, the identification of the peak substance allows the clarification of differences in metabolism between the samples. Metabolome comparison between cancer cells and normal cells, for instance, allows the identification of substances that are specific to the former. These might be key substances in the cancer cell and, therefore, potential targets for the treatment of disease. Furthermore, in studies of the effects or toxicity of a drug, the comparison of metabolomes obtained before and after treatment can identify differences in the levels of particular substances. These might be related to metabolic activation or inactivation that is triggered by the drug. Clearly, there is the potential for a wide range of applications of this technique.

Metabolome analysis provides important information that can be used to **model the dynamic behavior** of industrially important metabolic pathways. By measuring quantities of intermediate metabolites in the course of time, dynamics of metabolic pathways can be understood well. In addition, by simultaneously monitoring substances other than the metabolites that are known to be involved in the pathway, it is possible to discover the involvement of unexpected substances.

Finally, one of the major goals of biochemistry and cell biology is to **model intracellular metabolism in its entirety**. Our group is working to meet this challenge by making full use of metabolomic analytical techniques and bioinformatics. Our research comprises three elements:

1. The automatic generation of a "draft" model of the entire metabolic pathway, based on genomic information (top-down approach)
2. The identification of all intracellular metabolites, based on metabolome analysis (bottom-up approach)
3. The integration of resources (1) and (2) using bioinformatics

We have developed a computer program, the **GEM System**, which automatically constructs a model based on genomic information (nucleotide sequences). As the genomic data include a number of genes with unknown functions, the metabolic pathway generated by this top-down approach is clearly incomplete (a draft model). We are therefore producing a list of all the metabolites that exist in the cell using CE/MS and LC/MS (bottom-up approach). We then supplement the draft (incomplete) model with this metabolomic information in order to produce a complete metabolic model.

4. Metabolome Informatics

Informatics (information science) is as indispensable for metabolomics as it is for genomics and proteomics. We discuss four examples of current computer software in this book.

Databases in which large amounts of metabolomic information are consolidated have an essential role in this field, and many can be accessed online. The metabolic pathways are generally displayed in a graphical manner, in which each element is a clickable map that is linked to other databases. The **KEGG database** of Kyoto University, Japan, is the best known metabolic database worldwide.

As mentioned previously, our group has developed the GEM system, which automatically generates a metabolic model from genomic sequence information obtained in a fully automated manner from public resources, such as the Clusters of Orthologous Groups of Proteins (COG), SWISS-PROT, Kyoto Encyclopedia of Genes and Genomes (KEGG), and European Molecular Biology Laboratory (EMBL) databases. As the model generated by the GEM System follows the rule format of the E-CELL system, it is also possible to run the metabolism simulation directly as it is.

When two metabolites are specified, the **ARM system** predicts the metabolic pathways connecting them and displays the pathways in ranked order. This system makes use of molecular structural information of the metabolites. The "draft" pathway models that are automatically generated by the GEM System are incomplete; however, the ARM system can potentially supplement the missing parts of the pathways.

A key goal of metabolomics is to create a comprehensive and accurate simulation of metabolism. The **E-CELL system** is a software package that can simulate the dynamic behavior of metabolisms as a whole, when given the rules for individual metabolic reactions. This system can be used to study the effects of changing the initial value of a metabolite or altering the activity of an enzyme. In addition, this system can be applied in sensitivity

analysis that examines which reactions influence overall metabolism and to what extent. It is also possible to let the system automatically optimize the kinetic parameters, so that the simulation results match the actual experimental data.

5. Conclusions

In April 2001, Keio University established the Institute for Advanced Biosciences, which specializes in Systems Biology, in Tsuruoka City (Yamagata Prefecture), Japan. The ultimate objective of the research at this institute is to construct a computer model of the cellular metabolism, and it has given high priority to metabolome research since its opening. As of 2005, the institute is equipped with the following world-class instrumentation for metabolome analysis: 19 CE systems, six LC systems, two GC/MS systems, nine quadrupole MS systems, four ion-trap MS systems, two Triple QMS/MS systems, six electrospray ionization time-of-flight mass spectrometers (ESI-TOF-MS), one Q-TOF-MS and one nuclear magnetic resonance (NMR) spectrometer.

The major projects being conducted at the Institute include: "*E. coli* Modeling Project" funded by the New Energy and Industrial Technology Development Organization (NEDO) of the Ministry of Economy, Trade and Industry of Japan, with the ultimate aim of designing useful microorganisms; "Leading Project for Biosimulation" funded by the Ministry of Education, Culture, Sports, Science and Technology (Monbusho), which is performing metabolome analysis and simulation of red blood cells; and the Grants for Scientific Research and Scientific Research of Priority Areas (funded also by the Ministry of Education, Culture, Sports, Science and Technology), and 21st Century COE program at Keio University entitled "The Understanding and Control of Life's Function via Systems Biology," which are developing the basic technologies for metabolomics and cell simulation. In addition, the Faculty of Medicine at the University of Tokyo, Japan, established the Center for Metabolome in February 2003. This group is focusing primarily on lipid metabolomics using LC/MS technology.

Finally, Keio University and its partners invested in the establishment of a "bio-venture" company, Human Metabolome Technologies (HMT) Inc., in Tsuruoka City in July 2003. Utilizing the metabolome technology of the Institute for Advanced Biosciences of Keio University, HMT is now conducting joint research with major food companies, to understand bacterial metabolism of fermentation used in the food industry. Their future plan is to

apply this technology to the fields of medical sciences through collaborations with drug companies.

Part II. Analytical Methods for Metabolome Sciences

Chapter 2: Development and Application of Capillary Electrophoresis–Mass Spectrometry Methods to Metabolomics

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1. Introduction

High-throughput and comprehensive analysis of intracellular metabolites can reveal the connection within biochemical networks and provide a systems-level understanding of cell. Metabolomics, or the global analysis of cellular metabolites, has become a powerful new tool for gaining insight into functional biology. Proteins and metabolites are the main effectors of phenotype and thus the functional entities within the cell. Measurement of the level of numerous metabolites within a cell, and tracking metabolite changes under different conditions not only provides direct information on metabolic phenotypes but is also complementary to gene expression and proteomic studies [1,2]. Although metabolome analysis is indispensable, unlike other functional genomic approaches, very few methods for a large-scale metabolite analysis have been developed. Recently, new methods for the comprehensive analysis of charged metabolites by capillary electrophoresis–mass spectrometry (CE/MS) have been developed. Since CE/MS enables direct and quantitative analysis of most charged metabolites, the methods have attracted a great deal of attention. In this chapter, the principles, applications and prospects of this technology will be discussed.

2. Strategy

2.1. Problems in Metabolome Analysis

Despite its importance, only a limited number of methodologies have been developed for metabolome analysis. This is primarily due to the character-

istics of most metabolites that display high polarity, non-volatility, poor detectability and overall similar properties. In addition, the fact that over 1000 different metabolic substrates exist in a cell complicates the analysis.

2.2. Recent Analytical Techniques for Metabolome

With the increasing interest in metabolomics, recently several methods for exhaustive metabolome analysis have been developed. The pioneering work in large-scale metabolite analysis was originally performed by gas chromatography–mass spectrometry (GC/MS) [3,4]. Analysis by GC/MS offers high resolution, selectivity, and sensitivity, and demonstrates outstanding performance. However, it is somewhat limited by the need for multiple derivatization procedures for each chemical moiety. Moreover, even after derivatization, a considerable number of metabolites are still non-volatile, and thus cannot be determined by GC/MS.

High-performance liquid chromatography–mass spectrometry (LC/MS) is also a useful method. However, a large number of charged metabolites are too polar to be significantly retained by the reversed-phase columns that are commonly used in LC/MS. Alternative techniques exist, such as ion-exchange chromatography coupled to mass spectrometry, but are scarcely used due to the lack of appropriate mobile phases compatible with MS detection.

Recently, direct infusion analysis approaches using nuclear magnetic resonance (NMR) and other mass spectrometry methods such as Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) or electrospray ionization–mass spectrometry (ESI-MS) have been developed for metabolome profiling [5–7]. Although these infusion techniques enable the instantaneous acquisition of metabolic snapshots, there are still some important drawbacks. In NMR, sufficient amounts of samples must be prepared, and quantification is difficult. Infusion techniques using MS lack accuracy and precision for quantification due to the ion suppression effect and often cannot separate a number of isomers [8,9]. Therefore, the development of quantitative and high-resolution metabolome analysis methods remains one of the most demanding challenges.

2.3. Principle of CE/MS

A new approach for the comprehensive and quantitative analysis of charged metabolites by capillary electrophoresis electrospray ionization mass spectrometry (CE/MS) has recently been proposed [10]. Metabolites are first separated by CE and selectively detected using MS by monitoring

ions over a large range of *m/z* values. This method enabled the determination of several hundred metabolic standards and its utility was demonstrated in the analysis of 1692 metabolites from *Bacillus subtilis* extracts [10]. The principles of this technique are briefly described below.

Most of intracellular metabolites bear a charge and CE/MS is thus a logical choice because of its power to separate charged species. In this marriage of techniques, CE confers rapid analysis and efficient resolution, and MS provides high selectivity and sensitivity. The major advantages of CE/MS are that this methodology exhibits extremely high resolution and that almost any charged species can be detected by MS.

Figure 1 shows a simple diagram of CE/MS. In CE, ionic species migrate on the basis of their charge and size; therefore, all cations migrate toward the cathode, whereas all anions move in the opposite direction. Therefore, in principle all charged species could be analyzed using only two CE/MS configurations.

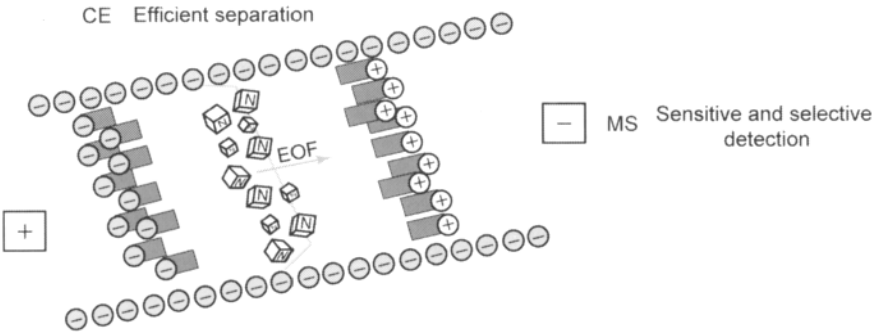

Fig. 1. Basic principle of charged metabolite analysis by capillary electrophoresis–mass spectrometry (CE/MS). When voltage is applied, all cations migrate toward the cathode, whereas all anions move in the opposite direction. All charged species can thus potentially be analyzed using only two CE/MS configurations

3. CE/MS for Metabolome Analysis

3.1. Metabolite Extraction

The development of efficient metabolite extraction procedures from cells must meet several requirements. First, rapid enzyme inhibition and efficient metabolite extraction are necessary to quantify intercellular metabolites since turnover of some metabolites occurs rapidly. Second, samples should be enriched to facilitate detection. Third, simultaneous extraction of both cationic and anionic metabolites is desirable. Finally, metabolites should be dissolved in low conductivity solutions to achieve maximum performance of CE/MS [11]. A method meeting these requirements for *Bacillus subtilis* bacteria is illustrated in Fig. 2. A volume of 10 ml of culture medium is passed through a 0.45 μm pore size filter. Residual *B. subtilis* cells on the filter are washed with Milli-Q water and then plunged into 2 ml of methanol, containing internal standards (methionine sulfone for cations and PIPES for anions), to inactivate enzymes. After a short incubation at room temperature, the methanol solution is withdrawn and then chloroform and Milli-Q water are added to the methanol solution. The mixture is thoroughly mixed to remove phospholipids liberated from cell membranes, which can adsorb on the capillary wall and reduce CE performance. The separated methanol–water layer is then centrifugally filtered through a Millipore 5-kDa-cutoff filter to remove proteins. The filtrate is lyophilized and dissolved in 20 μl of Milli-Q water before CE/MS analysis. Overall, this procedure can result in a 500-fold enrichment of metabolites [10].

3.2. Cationic Metabolite Analysis

In cation analysis, MS is coupled to the cathode as shown in Fig. 1. Separations are carried out on a fused silica capillary. To analyze most of cations simultaneously a low pH solution (1 M formic acid) is used as the electrolyte to confer a positive charge on every cation [12]. In this manner, cationic metabolites can be efficiently separated by CE and then selectively and sensitively detected by MS.

Figure 3 shows the mass spectrum of arginine (molecular weight 174.2), which was acquired by CE/MS in scanning mode from *m/z* 50 to 350. The monoisotopic protonated molecular ion $[M+H]^+$ at *m/z* 175.1 dominates the mass spectrum, and most of other cations show similar results [12]. The smaller peak at 176.1 corresponds to a C13 isotopic peak. Electro-

spray ionization (ESI) is a soft ionization method, so that the protonated molecular ions are dominant for most compounds. Consequently, cationic metabolites were determined as their protonated molecular ions $[M+H]^+$ in the CE/MS method. Figure 4 shows an example of amino acid analysis by CE/MS. Every amino acid can be selectively determined by this method. Since isomers such as leucine and isoleucine cannot be differentiated by mass in MS, they must be separated by CE first. To separate these isomers efficiently, a 100 cm-long capillary was employed [12]. Using this method, a total of 173 cationic metabolite standards such as amino acids, amines, and nucleosides, which are listed in the COMPOUND section in LIGAND database (http://www.genome.ad.jp/kegg/ligand.html), were successfully and simultaneously determined [10].

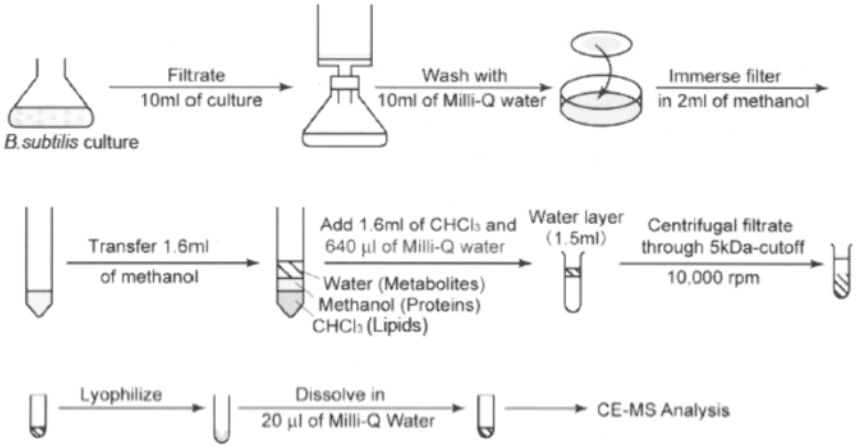

Fig. 2. Metabolite extraction procedure from *Bacillus subtilis* cell. Cells were separated from the culture media using filtration, and then quickly plunged into methanol to inactivate enzymes. After phospholipids and proteins were removed by both liquid-liquid extraction and centrifugal ultrafiltration through a 5-kDa cutoff filter, the filtrate was lyophilized and dissolved in Milli-Q water before CE/MS analysis

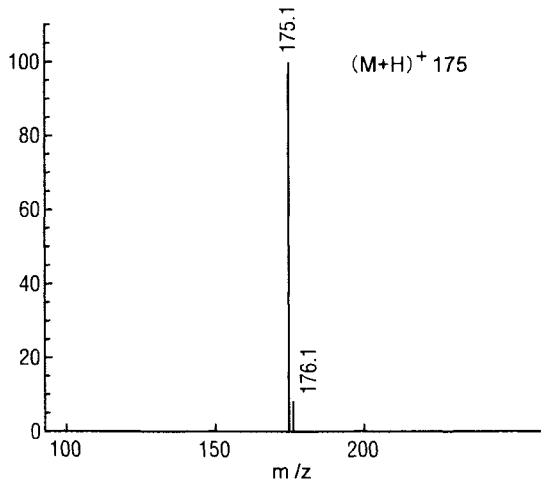

Fig. 3. Positive ion mass spectrum of arginine by CE/MS. The protonated molecular ion $[M+H]^+$ dominates the mass spectrum, and a trace of its ^{13}C isotope ion can also be observed. Very few fragmentation ions were observed in the electrospray ionization mass spectrometry (ESI-MS) (reproduced from Ref. [12] with permission from the American Chemical Society)

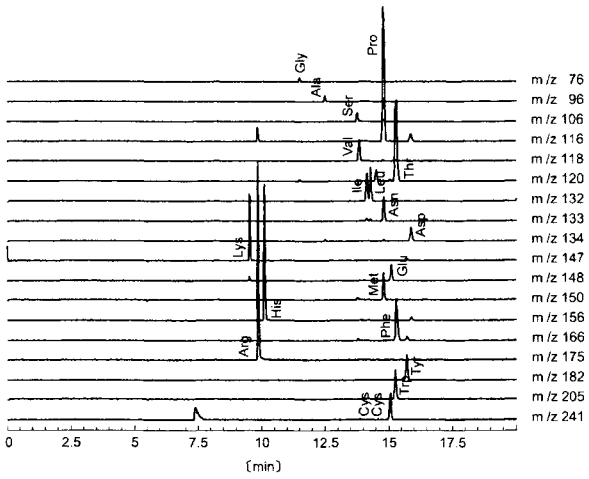

Fig. 4. CE/MS electropherograms for a standard mixture (250 μM each) of 19 amino acids. Each amino acid was selectively detected as its protonated molecular ion. Leucine and isoleucine could be resolved in this method (reproduced from Ref. [12] with permission of the American Chemical Society)

Table 1. Quantification of *Bacillus subtilis* 168 metabolites at $T_{-0.5}$ phase, and overall reproducibility from metabolite extraction through CE/MS analysis

Compound	Mole/cell (amol)[a]	RSD (n=5) (%)	
		Peak area	Relative migration time
Gly	5.8	20	1.5
L-β-Ala	0.14	28	2.7
L-Ala	24	13	1.3
GABA	0.12	27	1.7
L-Ser	4.5	23	0.83
L-Pro	3.8	3.0	0.48
L-Val	4.1	19	0.83
L-Homoserine	2.1	2.0	0.84
L-Thr	13	6.3	0.33
Creatine	2.5	30	1.4
L-Ile	2.2	24	0.61
L-Leu	6.3	21	0.74
L-Hydroxyproline	0.17	29	0.13
L-Ornithine	0.27	60	2.1
L-Asn	0.43	57	1.3
L-Asp	11	11	0.2
Adenine	0.04	63	1.8
Tyramine	0.37	25	1.6
Spermidine	0.12	53	2.9
L-Lys	0.12	33	2.0
L-Gln	190	8.4	0.49
L-Glu	350	4.0	0.47
L-Met	1.4	15	0.51
L-His	0.32	16	2.0
L-Phe	1.2	44	0.37
L-Arg	1.7	32	2.1
L-Citrulline	1.6	8.5	0.55
Tyramine	0.3	50	0.32
L-Carnosine	1.1	50	2.2
Cytidine	0.12	26	0.91
Adenosine	0.06	55	0.84
Pyruvate	3.2	33	0.28
Lactate	23	22	0.18
Fumarate	0.73	25	0.44
Succinate	3.2	6.9	0.31
Malate	0.16	16	0.32
2-Oxoglutarate	1.2	24	0.30
Phosphoenol pyruvate	1.7	30	0.31

Dihydroxyacetone phosphate	2.5	26	0.07
Glycerol 3-phosphate	3.1	22	0.06
3-Phosphoglycerate	9.1	16	0.20
Citrate	0.36	27	0.69
Erythrose 4-phosphate	1.1	12	2.5
Ribulose 5-phosphate	1.7	30	0.10
Ribose 5-phosphate	0.45	25	0.32
Glucose 1-phosphate	1.1	29	0.14
Fructose 6-phosphate	2.6	38	0.17
Glucose 6-phosphate	1.4	49	0.18
6-Phosphogluconate	0.24	28	0.18
Fructose 1,6-diphosphate	1.9	51	0.18
CMP	0.29	23	0.93
AMP	1.3	47	0.78
GMP	0.14	42	0.85
CDP	0.07	52	0.89
ADP	0.72	49	0.43
GDP	0.05	55	0.93
CTP	0.15	37	0.89
ATP	0.83	36	0.61
GTP	0.12	49	0.63
NAD	4.5	8.9	0.72
NADP	0.26	23	0.64
NADPH	0.06	53	0.72
Acetyl CoA	0.67	36	0.59

Reproduced from Ref. [10]
RSD, relative standard deviation
[a]The quantity of each metabolite per cell was calculated using the number of cells per ml culture, which was determined as 1.0×10^8 by colony forming unit on LB plates

3.3. Anionic Metabolite Analysis

The metabolites of key pathways for cellular energy production such as glycolysis, the tricarboxylic acid (TCA) and pentose phosphate cycles are almost entirely anionic species, e.g., carboxylic acids, phosphorylated carboxylic acids, and phosphorylated sugars. An enormous number of anionic metabolites also exist in other pathways. Therefore, a methodology suitable for anionic metabolite analysis is also very important. However, the analysis of anions by CE/MS using a fused silica capillary in negative mode, where the inlet capillary is at the cathode and the outlet at the anode (Fig. 5a), has been difficult. Since the CE/MS system does not contain an outlet solution vial (Fig. 5a), the electro-osmotic flow (EOF) movement toward the cathode (opposite the MS direction) generates a gap in the liq-

uid phase at the capillary exit, resulting in a current drop (Fig. 5b) [13]. For this reason, few reports on anion analysis have been reported by CE/MS such a configuration. To overcome this problem, we have designed a way to reversed the EOF toward the anode by employing a SMILE(+) cationic polymer (Polybrene)-coated capillary (Fig. 5c). This technique of EOF reversal enabled successive anion analysis without the deleterious current drop [14,15].

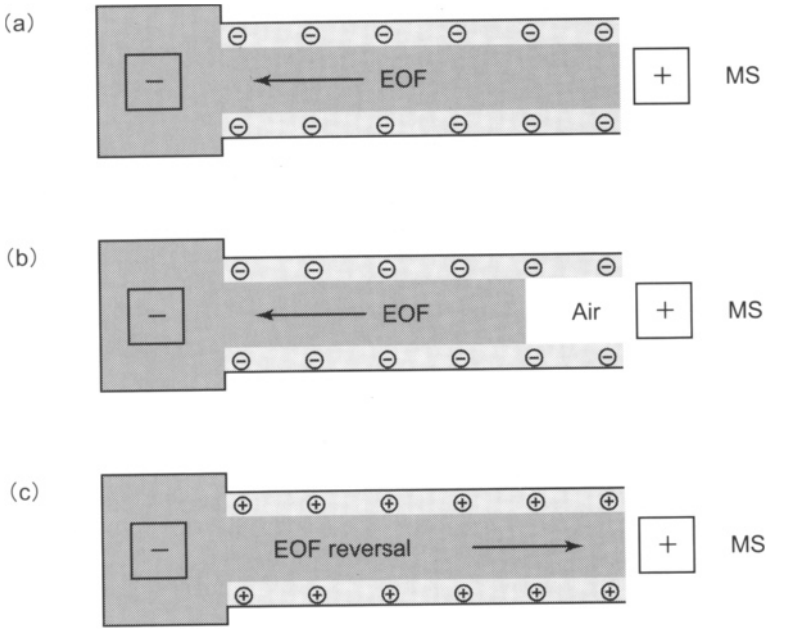

Fig. 5. Schematic of the electro-osmotic flow (EOF) profile in anion analysis by CE/MS in negative mode. a Using a fused silica capillary, the EOF is directed toward the cathode (opposite to MS direction), resulting (b) in a gap in the liquid phase at the capillary outlet, and an associated current drop. c This problem can be overcome by reversing the EOF using a SMILE(+) cationic polymer coated capillary and to enabled successive anion analysis

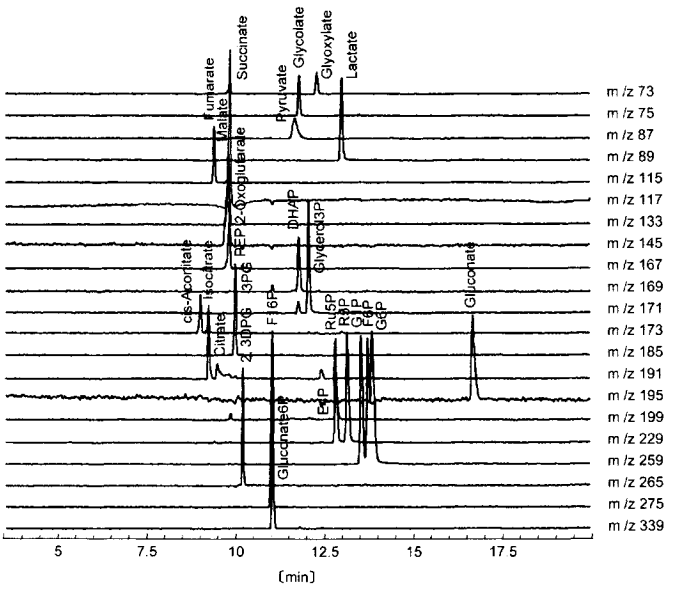

Fig. 6. CE/MS electropherograms for a standard mixture (100 μM each) of 25 metabolites of the glycolytic, TCA, and pentose phosphate pathways. PEP, phosphoenol pyruvate; DHAP, dihydroxyacetone phosphate; 3PG, 3-phosphoglycerate; E4P, erythrose 4-phosphate; Ru5P, ribulose 5-phosphate; R5P, ribose 5-phosphate; G1P, glucose 1-phosphate; F6P, fructose 6-phosphate; G6P, glucose 6-phosphate; 2,3DPG, 2,3-diphosphoglycerate; F16P, fructose 1,6-diphosphate (reproduced from Ref. [15] with permission from the American Chemical Society)

Figure 6 illustrates the electropherograms obtained following the analysis of a 25-anionic standard mixture of glycolytic, TCA, and pentose phosphate pathways obtained by CE/MS. Since the deprotonated molecular ion, $[M-H]^-$, dominated the mass spectrum for each compound, anions were detected at their deprotonated molecular weights [15]. Although the migration times of succinate, malate, 2-oxoglutarate, and phosphoenol pyruvate are very close, they were selectively detected by MS. Even isomers such as ribulose 5-phosphate (Ru5P) and ribose 5-phosphate (R5P), and glucose 1-phosphate (G1P), fructose 6-phosphate (F6P), and glucose 6-phosphate (G6P) were resolved. A total of 124 anionic metabolite standards including carboxylic acids, phosphorylated carboxylic acids, phosphorylated sugars, and other phosphorylated compounds were determined by this method[10].