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Review

Effectiveness and limitation of two-dimensional gel electrophoresis in bacterial membrane protein proteomics and perspectives

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Abstract

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) using isoelectric focusing and SDS–PAGE in the first and second dimensions, respectively, is an established means of simultaneously separating over 1000 proteins and two new types have recently been developed. These procedures have significant shortcomings such as low load ability and poor separation of hydrophobic, acidic and alkaline proteins. We therefore modified the protocols to analyze the *Bacillus subtilis* membrane proteome. The 2D-PAGE techniques effectively separated membrane proteins having one and two transmembrane segments but not those with more than four. Compared with new LC/MS/MS procedures that are independent of electrophoretic separation, 2D-PAGE can globally analyze and quantify proteins at various stages of the cell cycle when labeled with isotopes such as ^{35}S-methionine or the stable isotope, ^{15}N.
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Keywords: ^{15}N-Whole cell labeling; Membrane protein proteomics; *Bacillus subtilis*; SecA

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1. Introduction

High-throughput of DNA sequencing allows the rapid accumulation of abundant sequence data from many organisms. Genome sequences are the essential blueprints of life and proteins expressed from the genome sustain life through indispensable functions in cellular architecture, catalysis, metabolic regulation and contractile processes that are intimately concerned with all cellular physiological events. Therefore, functional proteomic analyses based on genome databases are essential to understand not only cells but also the processes of high order organisms, for example differentiation, development or disease, at the molecular and biochemical levels. Differential proteomic display can comprehensively analyze differences between normal and malignant cells and between normal and stressed cells under pathological conditions, leading to developments in diagnosis and therapy.

Among current methods of protein analysis, two-dimensional gel electrophoresis combined with protein identification using mass spectrometry and continuously evolving analytical software constitute a highly accurate and sensitive detection and identification system. The high-resolution of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was determined by O'Farrell [1] and Klose [2] in 1975, when over 1000 *Escherichia coli* proteins were separated. Using this technique, most proteins synthesized at a specific moment under defined physiological conditions can be analyzed using radiolabeling. Therefore this technique is a powerful and essential tool for investigating global changes in cellular gene expression and protein modification.

However 2D-PAGE has some significant disadvantages. Foremost is low load ability and poor separation of highly hydrophobic proteins. To improve low load ability, we developed a highly reproducible gel system in which essentially identical protein profiles can be recognized in over 10 independent gels, followed by "on membrane digestion" in the presence of 80% acetonitrile for mass spectrometry sample preparation [3]. However, in contrast to the good separation of cellular soluble proteins, 2D-PAGE using isoelectric focusing (IEF) with immobilized pH gradients (IPG) in the first dimension and Tris–glycine SDS–PAGE in the second (IPG-Dalt) might not be suitable for separating hydrophobic membrane proteins. Many of them cannot be resolved by pH gradients during IEF. Membrane proteins are relatively insoluble in non-ionic or zwitterionic detergents, particularly at low ionic strength. Even if they could be solubilized, such proteins often precipitate at pH values close to their isoelectric points. We therefore modified the solution in which dry pre-cast IPG gels for IEF are rehydrated for the first dimension and used Tris–tricine discontinuous SDS–PAGE in the second dimension [3]. These modifications considerably improved protein separation in IPG-Dalt gels.

Macfarlane [4] and Hartinger et al. [5] developed a discontinuous acidic 2D-PAGE system using the cationic detergent, benzyldimethyl-*n*-hexadecylammonium chloride (16-BAC) in the first dimension and Tris–glycine SDS–PAGE in the second. Dreger et al. [6] identified 148 proteins in the nuclear envelope using this method combined with MALDI-TOF MS. Schägger and von Jagow [7] and Schägger and Pfeiffer [8] developed another unique 2D-PAGE system using native PAGE in the first dimension (Blue native PAGE) in which Coomassie Brilliant Blue G induces a charge shift on membrane protein complexes. This method was useful for analyzing the multi-protein complexes involved in mitochondrial oxidative phosphorylation. We further modified these methods to analyze bacterial membrane proteins using Tris–tricine SDS–PAGE in the second dimension. General methods of analyzing the microbial proteome have been summarized by Washburn and Yates III [9].

This review describes the usefulness and limitations of the three types of 2D-PAGE in *Bacillus subtilis* proteome analysis, especially of the cell membrane proteins. *B. subtilis* is a sporulating Gram-positive bacterium. Its 4215 kb genome was sequenced in 1997 [10] and showed that it encodes 4106 genes for proteins and 114 for RNAs. Since *B. subtilis* and its related species produce high levels of extracellular proteins, they are used in industries to produce α-amylase for sweetness, as well as proteases and cellulase for detergents and cyclodextrin glycanotransferase for cyclodextrin production. We also describe the effectiveness of spot quantitation in 2D-PAGE gels that can reveal dynamic and global changes of cellular protein components that adapt to changes in stress, nutrients and metabolism [11] and analyze the function of the protein secretion machinery using ^{15}N-whole cell labeling.

2. Preparation of *B. subtilis* membrane and stepwise extraction of membrane proteins

Protein solubilization by detergents is critical for fine 2D-PAGE separation. However, no universal non-ionic or zwitterionic detergent can yet be applied to IEF for all proteins. Although several powerful solubilization methods for proteins have been developed, comprehensively analyzing membrane proteins using one detergent remains very difficult. To overcome this problem with respect to *B. subtilis* membrane protein analysis, we stepwisely solubilized proteins using several detergents and 1D- and 2D-PAGEs. *B. subtilis* 168 (*trpC2*) cells in the logarithmic phase of growth were harvested, washed and quickly suspended in phosphate buffer containing a protease inhibitor at 4 °C. The cells were disrupted using a chilled French Press, and washed membrane preparations (NDSB insoluble membrane preparations) that are insoluble in Bistris (pH 7.0) buffer including 6-amino-*n*-caproic acid, non-detergent sulfobetaine 256 (NDSB) and protease inhibitor were prepared as shown in Fig. 1. Membrane proteins were extracted stepwise from the NDSB insoluble membrane preparation in the presence of the protease inhibitor. Fraction 4 was the protein fraction solubilized by 1% (w/v) *n*-dodecyl-β-D-maltoside from the membrane preparation. Fraction 5 was the protein fraction extracted from the pellet

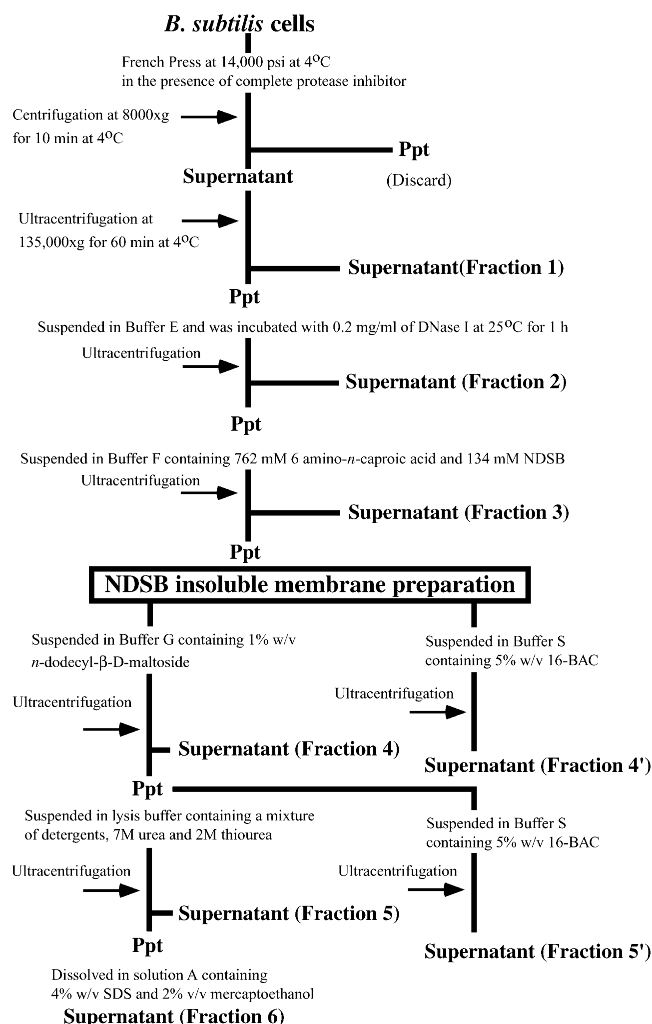


Fig. 1. Preparation of NDSB insoluble membrane of *B. subtilis*, stepwise extraction of membrane proteins into sub-membrane fractions (fractions 4, 4′, 5, 5′ and 6) using mixtures of different detergents (non-ionic, zwitterionic and ionic), chaotropes (urea and thiourea) and reductants (DTT and 2-merchaptoethanol). The chemical composition of Buffers E, F, G, and S, Solution A and lysis buffer are shown elsewhere [12].

into 7 M urea, 2 M thiourea, 1% (w/v) aminosulfobetaine-14 (ASB-14), 4% (w/v) CHAPS, 1% (w/v) NDSB and 1% (w/v) DTT in the presence of protein inhibitor after removing fraction 4. Fraction 6 consisted of proteins remaining in the pellet (after removing fraction 5) that were dissolved in Tris (pH 7.0) buffer including 4% (w/v) SDS, 2% (v/v) 2-mercaptoethanol. No precipitate was obtained from fraction 6 after ultracentrifugation. To solubilize proteins from the NDSB insoluble membrane preparation under cationic conditions, the preparation was resuspended in 4 M urea containing 250 mM NDSB, 5% (w/v) 16-BAC, 75 mM DTT and separated by ultracentrifugation. The supernatant was designated as fraction 4′. The remaining pellet after removing fraction 4 was resuspended under the same cationic conditions and the supernatant after ultracentrifugation was designated as fraction 5′. The procedure for this stepwise membrane protein preparation and the characterization of fractions 1–6 have been published [12]. Immunoblot analysis using several antisera againt cytoplasmic and membrane proteins and comparison of protein constituents among the fractions indicated that the NDSB insoluble membrane preparations were effectively separated the cytoplasm into sub-membrane fractions by the stepwise extraction.

3. 1D- and 2D-PAGEs followed by MALDI-TOF MS to analyze membrane proteins

3.1. Discontinuous Tris–tricine SDS–PAGE

1D-PAGE and the second dimensions of 2D-PAGEs proceeded in 10% gels (13 cm × 16 cm × 0.2 cm) using the discontinuous Tris–tricine buffer system described by Schägger and von Jagow [7] with a slight modification. The proteins were resolved at a constant voltage of 30 V until Coomassie Brilliant Blue G entered the running gel, then 110 V was applied for about 6 h. The gels were fixed in a mixture of 40% ethanol and 10% acetic acid and stained with silver as described by Morrissey [13]. We compared the separation profiles of NDSB insoluble membrane preparation using a regular discontinuous gel and a 4–20% pre-cast gradient gel (Nippon EIDO) in a Tris–glycine SDS–PAGE buffer system and in discontinuous Tris–tricine SDS–PAGE. The protein bands were clearly separated in the 4–20% gradient gels in Tris–glycine and in the discontinuous gels in Tris–tricine and the profiles in the latter were quite reproducible. Fig. 2 shows proteins separated from two sub-membrane fractions (fraction 4) from cells grown on LB and S7 medium using 1D SDS–PAGE in Tris–tricine. Many major bands that differed between the two fractions were excised, digested with protease and analyzed by MALDI-TOF MS. The sub-membrane fractions were rich in the solute-binding proteins of ABC transporters that anchor on the surface of the cell membrane via extended N-terminal hydrophobic lipid in Gram-positive bacteria. The identified solute-binding proteins in the two sub-membrane fractions considerably differed between cells grown on LB and S7 medium in response to the nutrient composition of the two types of media as described [12]. A DNA micro array analysis revealed that the *yclQ* (encoding function unknown protein, similar to ferrichrome ABC transporter) and *yusA* (encoding function unknown protein) genes were expressed at high levels in the cells grown on S7 medium, while the level of *appA* (encoding oligopeptide ABC transporter) was high in LB medium. The *oppA* gene (encoding oligopeptide ABC transporter) was similarly expressed in both culture media (http://genome.ad.jp/kegg/expression/) [12].

3.2. Isoelectric focusing (IEF) in the first dimension

Pre-cast IPG dry gels (Amersham Pharmacia Biotech) for IEF in the first dimension, were rehydrated overnight in 8 M urea containing 1% (w/v) CHAPS, 1% (w/v) Pharmalyte pH

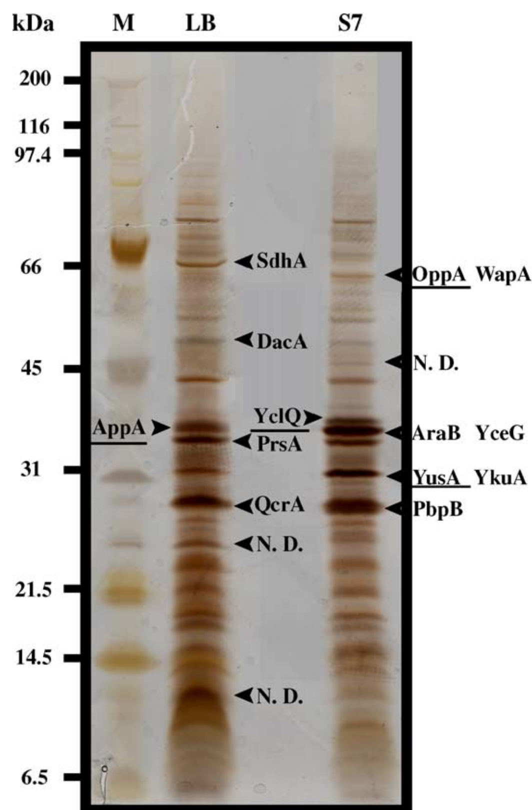


Fig. 2. Comparison of protein components in sub-membrane fraction 4 from cells grown on LB and on S7 synthetic medium. Proteins (100 μg) from each fraction 4 were resolved by 1D Tris–tricine SDS–PAGE and the bands were visualized by silver staining. Major bands that differed between the two fractions were excised and analyzed by MALDI-TOF MS after proteolysis. Identified solute-binding proteins of ABC transporters are underlined. N.D., not determined.

3–10, 0.2% (w/v) DTT, 1% (w/v) Triton X-114, 3% (w/v) NDSB, 1.5% (w/v) ASB14 and protease inhibitor. Samples dissolved in 9 M urea containing 3% (w/v) CHAPS, 0.8% (w/v) Pharmalyte pH 3–10, 1% (w/v) DTT, 1% (w/v) Triton X-114, 1% (w/v) NDSB and a trace of Bromophenol blue were applied to the gels. Proteins were isoelectrically focused on a Multiphore II electrophoresis unit (Amersham Pharmacia Biotech) according to the following program: 6 h at 1000 V, 5 h at 3500 V and 19 h at 3500 V in gradient mode at 20 °C. Focused strips were equilibrated for 10 min in 10 ml of Tris equilibration buffer (pH 6.8) consisting of 6 M urea containing 30% (v/v) glycerol, 2.5% (w/v) SDS and 0.25% (w/v) DTT, and subsequently for 10 min in the same buffer but containing 0.45% (w/v) iodoacetamide instead of DTT. The gels were then embedded in stacking gels for the second dimension.

3.3. Modified colorless native (MCN) 1D-PAGE

Blue native PAGE can analyze the mitochondrial respiratory chain complex that consists of membrane proteins [8]. We optimized this protocol to analyze the membrane proteins of *B. subtilis* (MCN–PAGE). Polyacrylamide linear gradient gels (7–16.5%) in MCN–PAGE Bistris (pH 7.0) buffer containing 6-amino-*n*-caproic acid and NDSB256 were prepared in glass tubes (0.5 cm × 14 cm). Samples (approximately 150 μg proteins) mixed with 20% volumes of MCN–PAGE sample buffer containing glycerol and CBB G, were resolved for 2 h at 30 V and then overnight at 100 V. Thereafter, the gels were incubated in 1% (v/v) 2-mercaptoethanol for 10 min and mounted on stacking gels for the second dimension. This procedure detected more membrane proteins than IPG-Dalt [12].

3.4. 16-BAC polyacrylamide (7.5%) gel electrophoresis for the first dimension

The predicted p*I* values of many membrane proteins of *B. subtilis* are theoretically located in the alkaline region (p*I* 8–14) [14]. Current IEF systems might not be able to separate such proteins. In contrast, proteins separated by 16-BAC/SDS–PAGE as described by Macfarlane [4] and Hartinger et al. [5] are not concerned with the p*I* of individual proteins. We applied 16-BAC/Tris–tricine SDS–PAGE to bacterial membrane protein separation using stacking and separating disc gels containing NDSB256. After electrophoresis, the gels were washed in several changes of isopropanol:acetic acid:water (3.5:1:5.5) for 3 h to remove the 16-BAC. The gels were then incubated for 30 min in three changes of 4% (w/v) SDS and 2% (v/v) 2-mercaptoethanol. The gels were mounted for the second dimension of Tris–tricine SDS–PAGE [12].

3.5. MALDI-TOF MS analysis after PAGE

Proteins separated by PAGE were digested by lysyl endopeptidase (LysC) from *Achromobacter lyticus* M497-1 (Wako Pure Chemicals, Osaka, Japan) using “on membrane” digestion method in the presence of 80% acetnitrile after the separated proteins were electroblotted onto PVDF membrane by a discontinuous buffer system [3], or “in gel” digestion method followed by twice extraction under sonication for 15 min in the presence of 60% acetnitrile and 1% TFA as described by van Montfort et al. [15]. The samples were analyzed by the Axima-CFR MALDI-TOF mass spectrometer (Shimadzu/Kratos, Kyoto, Japan) as described [3].

4. Profiling proteins from NDSB insoluble membrane preparation of *B. subtilis*

Fig. 3A–D shows typical 2D gel separations of sub-membrane fractions prepared from cells grown on LB medium using the three types of 2D-PAGE as described above. The silver stained gels of fractions 4 and 5 using IEF in the first dimension and Tris–tricine SDS–PAGE in the second, are shown in Fig. 3A and B, respectively. More than 300 spots were reproducibly detected in the gels. Three independent gels for each fraction were electroblotted, then

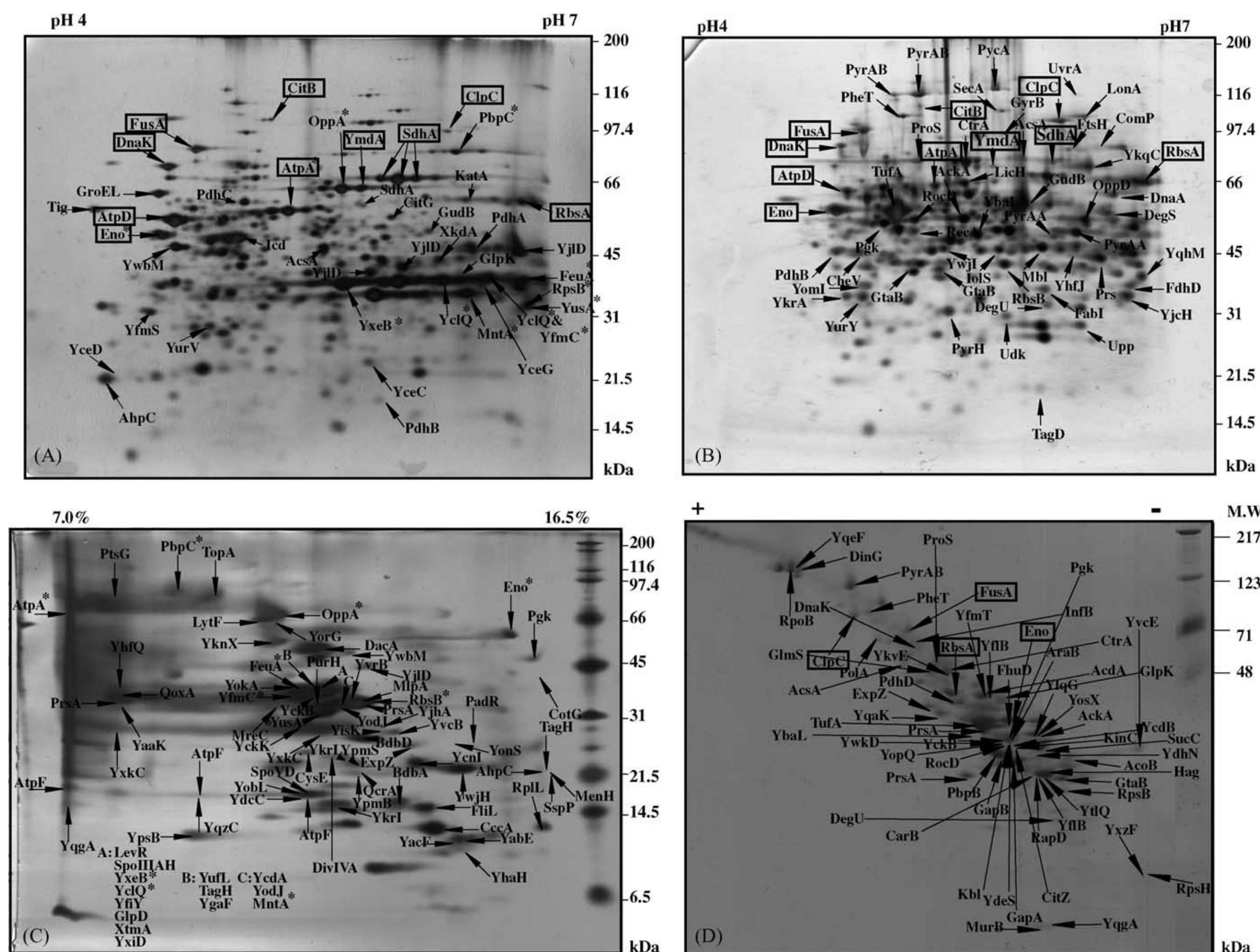


Fig. 3. Separation and identification of membrane related proteins in sub-membrane fractions 4, 5, and 5′ using three 2D-PAGE steps followed by MALDI-TOF MS. (A and C) Fraction 4 prepared from cells grown on LB medium were resolved by IEF (pH 4–7) or MCN in first dimension and Tris–tricine SDS–PAGE in second, respectively. (B and D) Electrophoretic profiles of fractions 5 and 5′ by IEF and 16-BAC–PAGE in first dimension and Tris–tricine SDS–PAGE in second: (A–C) stained with silver; (D) stained with CBB-R; A, B, and C in (C) spots containing many different proteins that are shown on bottom-left of panel. Ten overlapping proteins between fractions 4 (A) and 5 (B) and four in (A), (B) and (D) are boxed. Eleven overlapping proteins in (A) and (C) of fraction 4 are identified by asterisks. pH 4 and 7 at top of (A) and (B) indicate pH gradient of 4–7 used in first dimension IEF. 7% and 16.5% on top of (C), polyacrylamide gradient concentration in MCN–PAGE in first dimension. (+) and (−) on top of (D), indicate direction to anode and cathode, respectively.

approximately 100 obvious spots in each were excised, digested by LysC, and analyzed by MALDI-TOF MS. We identified 39 and 55 proteins in fractions 4 and 5, respectively. Ten overlapping proteins were boxed on the gels at the same positions. This result indicates that the 2D-PAGE separation and MALDI-TOF MS identification were precise. The silver stained gel of fraction 4 using MCN–PAGE in the first dimension and Tris–tricine SDS–PAGE in the second is shown in Fig. 3C. Approximately 200 spots were reproducibly detected. Seventy-eight proteins in five independent gels were identified. The comparison of proteins identified in the gels shown in Fig. 3A and C revealed only eleven common proteins (asterisk). Their positions in the two gels were different each other as expected. The identified proteins between the two gels were complementary. Fig. 3D shows a CBB stained gel of fraction 5′ using 16-BAC-PAGE in the first dimension and Tris–tricine SDS–PAGE in the second. We identified 62 proteins by MALDI-TOF MS. Only 16 of the 62 proteins identified in Fig. 3D overlapped with those shown Fig. 3B. Therefore the proteins of fraction 5 identified by IPG-Dalt and of fraction 5′ identified by 16-BAC/Tris–tricine SDS–PAGE were also complementary. Four proteins that overlapped among Fig. 3A, B and D were boxed. In addition, 219 and 117 spots were identified in fraction 4 from cells grown on LB and S6 medium containing 1% mannose as the sole carbon source that were resolved by 16-BAC/Tris–tricine SDS–PAGE and IPG-Dalt, respectively. In total, 570 spots were identified by the three types of 2D PAGE. Each spot was counted once, yielding 350 proteins. The proteins in fraction 6 that were solubilized in the presence of 4% SDS, were separated by 1D Tris–tricine SDS–PAGE and analyzed. We identified a further 477 proteins using 1D Tris–tricine SDS–PAGE

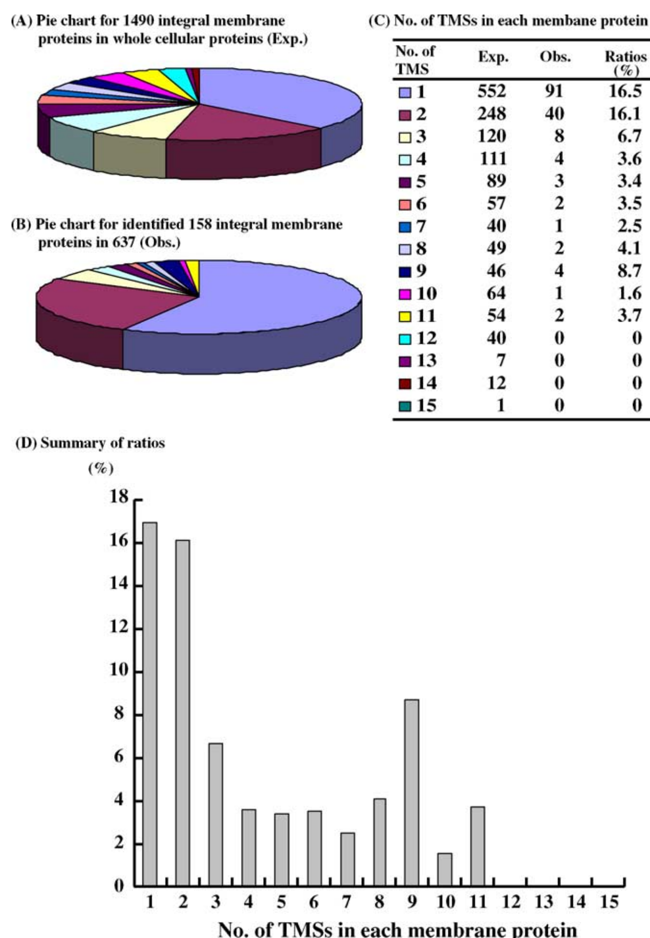
and 1D 16-BAC-PAGE followed by MALDI-TOF MS analysis. Overall we identified 637 proteins corresponding to 16.5% of the total cellular proteins from the NDSB insoluble membrane preparation after subtracting overlapping proteins [12].

We predicted the number of transmembrane segments (TMS) and signal peptides in the amino acid sequences of the identified proteins, using the TopPred II (http://bioweb.pasteur.fr/interfaces.toppred.html) [16] and Signal P [17] algorithms. Of the 637 proteins, we found 158 certain integral and 98 putative membrane proteins as well as 101 lipoproteins/secretory proteins and 280 soluble proteins [12]. The soluble proteins might include many peripheral proteins that function in both the cytoplasm and membrane like SecA and FtsY protein components of the *B. subtilis* protein secretion machinery.

5. Limitation of 2D-PAGE analysis

To predict integral membrane proteins in *B. subtilis* cells, we applied the TopPred II algorithm to 3994 of 4106 total proteins and found that 1490 of them contained more than one definite TMS. We omitted 102 proteins because they were peptides or very small proteins. This indicated that 37.3% of the whole cell proteins would be membrane proteins. To comprehensively analyze the proportions of membrane proteins among all the cellular proteins, Mitaku et al. [18] applied the SOSUI algorithm (http://sosui.proteome.bio.taut.ac.jp/sosuiframe0.html) to 15 microorganisms including mycoplasma (*M. genitalium*, *M. pneumoniae*), bacteria (*Chlamydia trachomatis*, *Treponema pallidum*, *Aquifex aeolicus*, *Helicobacter pylori*, *Borrelia burgdorferi*, *Haemophilus influenzae*, *Bacillus subtilis*, *E. coli*), Synechocystis PCC6803 and Archaea (*Methanococcus jannaschii*, *Metanobacterium thermoautotrophicum*, *Archeoglobus fulgeidus*) as well as to yeast, of which the genome had been published. They found that the predicted proportions were about 30% in all tested microorganisms including yeast.

The proportions (%) of membrane proteins having 1–15 TMS among all *B. subtilis* membrane proteins were then calculated and are shown in pie charts (Fig. 4A) and Fig. 4C. The TopPred II algorithm predicted that 552 (37.0%), 248 (16.6%), 120 (8.1%) and 570 (38.3%) were membrane proteins having one, two, three and over four TMS, respectively. To compare the ratios with those in the recovered membrane proteins having 1–15 TMS, we also calculated the ratios of membrane proteins having 1–15 TMS among the 158 integral membrane proteins of the 637 identified proteins (Fig. 4B and C). We found that 91 (57.6%), 40 (25.3%), 8 (5.1%) and 19 (12.0%) proteins had one, two, three and over four TMS, respectively. These results indicated that the recovery of the membrane proteins having one or two TMS was equally high (16.5% and 16.1%, respectively) but that of proteins containing three (6.7%) or over four (3.5%) was very low (Fig. 4C and D). The recovery (8.7%) of proteins having 9 TMS was exceptionally high. This result indicates that the proteome of membrane proteins containing one or two TMS can be analyzed using 1D- and 2D-PAGE whereas it remains difficult to analyze those with over 4 TMS. This result was also obtained in an analysis of the protein components of ABC transporters. Bioinformatic searching of the sequenced *B. subtilis* genome has estimated 38 importers of ABC transporters [12,19]. A typical importer consists of a functionally differing nucleotide-binding protein (NBP), a membrane-spanning protein (MSP) and a solute-binding protein (SBP). The MSP of each importer generally comprises two proteins. Therefore each importer usually consists of four proteins of which the genes form an operon. Predicted integral and putative membrane proteins, lipoproteins and soluble proteins in the NBPs, MSDs and SBPs constituting the 38 importers were analyzed using the TopPred II and signal P algorithms and Table 1 summarizes the results. Almost all MSPs (60/61)



No. of TMS	Exp.	Obs.	Ratios (%)
1	552	91	16.5
2	248	40	16.1
3	120	8	6.7
4	111	4	3.6
5	89	3	3.4
6	57	2	3.5
7	40	1	2.5
8	49	2	4.1
9	46	4	8.7
10	64	1	1.6
11	54	2	3.7
12	40	0	0
13	7	0	0
14	12	0	0
15	1	0	0

Fig. 4. Prediction and recovery of integral membrane proteins with 1–15 TMS in *B. subtilis.* (A) Pie chart of specific integral membrane proteins having 1–15 TMS among 1490 that were predicted from the whole cellular proteins using TopPred II algorithm. (B) Pie chart of specific integral membrane proteins with 1–15 TMS among 158 that were predicted from 637 identified from the NDSB insoluble membrane preparation. (C) Number of expected (Exp.) and observed (Obs.) integral membrane proteins having 1–15 TMS and recovery (%) of each; (D) summary of recovery ratios.

Table 1
Expected and observed numbers of certain integral membrane proteins, putative membrane proteins, lipoproteins and peripheral soluble proteins in the 38 importers of ABC transporter, which have been predicted from the genome analysis

Protein components of importer proteins	Certain integral membrane proteins			Putative membrane proteins			Lipoproteins			Peripheral soluble proteins			Total		
	Exp.	Obs.	%	Exp.	Obs.	%	Exp.	Obs.	%	Exp.	Obs.	%	Exp.	Obs.	%
NBP	3	0	0	6	1	16.7	0	0	0	26	5	20.8	35	6	17.1
MSP	60	1	1.7	0	0	0	0	0	0	1	0	0	61	1	1.64
SBP	1	1	100	0	0	0	34	27	79.4	3	2	66.7	38	30	78.9

The presence of TMS in the amino acid sequence of each protein in NBP (nucleotide-binding proteins), MSP (membrane spanning proteins) and SBP (sulute-binding proteins) in the 38 importers predicted from the genome analysis (Quentin et al. [19] and functional classification of SubtiList database (http://genolist.pasteur.fr/SubtiList)) was determined by TopPred II algorithm and numbers of the certain integral and putaive membrane proteins as well as lipoproteins and peripheral soluble proteins are shown in Exp. columns. The numbers of lipoproteins having signal peptides were recalculated according to the results of Signal P algorithm [17] and Tjalsma et al. [38]. Numbers of identifed certain integral and putative membrane proteins as well as lipoproteins and peripheral soluble proteins are predicted also and the numbers are shown in Obs. columns. % represents the ratio of identified to expected numbers of proteins.

were predicted as integral membrane proteins having 4 to 10 TMSs whereas most SBPs (34/38) were lipoproteins and NBPs containing many soluble proteins (26/35). Six NBPs (17.1%), 1 MSP (1.6%) and 30 SBPs (78.9%) were located among the 637 proteins identified from the NDSB insoluble membrane preparation. This result indicated that SBPs anchored in the cell membrane as lipoproteins can be precisely identified whereas the recovery of MSPs having over 4 TMSs is very low. Thus our method was seriously limited when analyzing cell membrane proteins. The low recovery of predicted NBPs could be due to their low content in the membrane preparation or they might be washed out during sample preparation.

6. Perspectives of 2D-PAGE in quantitative measurements of separated proteins

Proteomic analysis using 1D- and 2D-PAGE is limited by the inability to analyze very large or very small, acidic, basic or highly hydrophobic proteins. Although 2D-PAGE is hardly new [20], it is highly sensitive and enables the simultaneous separation of a very large number of proteins and their individual quantitation. The first stage of proteomics by 2D-PAGEs will be a comprehensive analysis of all protein components of living cells at specific stages, building databases to functionally classify and determine the subcellular localization of the proteins. Hecker and co-workers organized a 2D protein database for *B. subtilis* named Sub2D (http://microbio2.biologie.uni-greifswald.de:8880/sub2d.htm) that contains around 600 entries [21]. A recent development in bacterial proteomic analysis will provide information about a large collection of proteins. Hecker and co-workers [22,23] analyzed the proteomes of cytoplasmic, alkaline [24], cell-wall associated [25], and extracellular proteins [26], and we also analyzed the proteomes of the extracellular [27] and membrane proteins [12].

To achieve a comprehensive understanding of cellular proteins, the limitations of 2D-PAGE should be overcome by adding other methods such as two-dimensional native and alternative types of chromatography that are independent of gels to separate individual protein components or peptides [28,29] and DNA micro array analysis. The new LC/MS/MS (using Q-TOF, Ion trap or TOF–TOF technology) technologies seem very promising. Wu and Yate III [30] reported that LC/MS/MS can extensively analyze hydrophobic and alkaline proteins. However, all cellular proteins encoded in the genome cannot be visualized at a single stage because a portion of the genome is activated only when environmental stress, starvation stimuli or other extracellular signals are present. Hecker and co-workers used 2D separation to achieve a global view of the stress response, and found a dramatic change in the protein composition of *B. subtilis* in response to stress or starvation [31–33]. We compared 87 proteins in fraction 4 from bacterial cells grown on S6 medium containing 1% mannose as the sole carbon source with 415 proteins in the same fraction from cells grown on LB medium. The results showed that 40 of the 87 proteins were not included among the 415.

The second stage of proteome analysis by 2D-PAGE will be quantitative evaluation of cellular protein components to analyze physiological proteomics [34]. Bernhardt et al. [35] have developed a dual channel imaging technique that can provide considerable information about the relative amount and synthesis rate of individual proteins by silver-staining for accumulated proteins and protein-labeling with ^{35}S-mthionine for proteins synthesized de novo using two digitizable colors such as red and green. This technology allowed them to visualize global changes among proteins in *B. subtilis* cells during entry into the stationary phase triggered by glucose exhaustion. They found that 150 proteins are synthesized de novo while the synthesis of almost 400 proteins is canceled. They also clarified that AcoB, AcoC, BglH and GlvA transiently appeared at the entrance to stationary phase but AcsA and GapB were induced at the same time and persisted into the late stationary phase. Therefore, they suggested that protein synthesis is reprogrammed by glucose starvation. They also analyzed proteomic changes during heat stress and found that over 100 proteins were induced, including many that were functionally unknown. These global and semi-quantitative analyses of protein by the combination of 2D-PAGEs and imaging can elucidate dynamic changes in

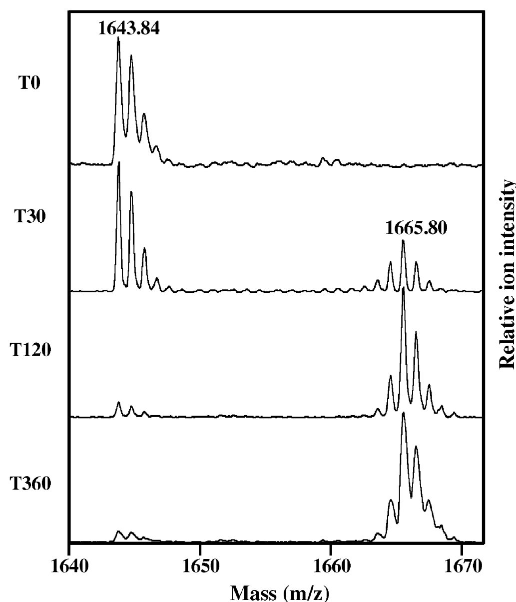


Fig. 5. Accumulation of ^{15}N-labeled peptide ion having amino acid sequence, VVDEAINQVSSQRAK, from Hag (flagella protein) during cultivation in ^{15}N-medium for 0 (T0) to 360 min (T360). Mass spectra at 1643.84 and 1665.80 *m/z* were unlabeled (light) or labeled with ^{15}N- (heavy), respectively. Relative intensity of two peaks indicates relative amount of cellular unlabeled and ^{15}N-labeled Hag protein.

cellular protein networks and how cells adapt to environmental changes at the protein level.

To more directly and accurately analyze changes in cellular protein components, we developed a whole cell-labeling method using the stable isotope, ^{15}N. Mass spectrometry can accurately distinguish the molecular masses of ^{14}N-peptides (light) and ^{15}N-peptides (heavy) peptides after the proteolysis of a preserved and de novo synthesized and labeled mixture of a single protein and it accurately measures the ratios of light and heavy peptides that would be equivalent to the abundance of the corresponding light and heavy proteins [36]. *B. subtilis* 168 was cultured in ^{14}N-medium for 5.5 h at 37 °C to a density of $A_{600} = 0.6$. The medium was changed to that containing ^{15}N to measure proteins synthesized de novo. The cells were harvested at 0 (T0) to 360 min (T360) after changing the medium, washed and then cell-lysates were prepared. Obvious bands of proteins resolved by 1D Tris–tricine SDS PAGE were excised and analyzed by MALDI-TOF MS. Fig. 5 shows that a 1643.84 *m/z* peak of the peptide ion (light peptide), VVDEAINQVSSQRAK derived from Hag (flagella protein), was detected at T0 and that another 1665.80 *m/z* peak corresponded to the ^{15}N-labeled peptide ion (heavy peptide) that accumulated during culture in ^{15}N-medium. The difference in the molecular mass between the two peaks corresponded to the number of N-atoms in the peptide. The ratios (R) of intensity of the 1665.80 *m/z* peak against the total of the two peaks (1643.84 *m/z* + 1665.80 *m/z*) were 0.0 (0%), 0.28 (28%), 0.90 (90%) and 0.92 (92%) at T0, T30, T120, and T360, respectively. The ratios of the intensity of the other ^{15}N-peaks, 1497.71 *m/z* corresponding to the 1476.78 *m/z* ^{14}N-peak for the peptide, MRGQIRGLEMASK and 1903.88 *m/z* to the 1882.88 *m/z* ^{14}N-peak for another peptide, FADNAADTADIGFDAQLK, from Hag were almost identically increased at T30, T120, and T360. Therefore, we assumed that the increased abundance of the proteins synthesized de novo can be monitored by the ratios of the intensity of ^{15}N labeled peptides.

We used this technique to analyze SecA function in protein location into the cell membrane of *B. subtilis*. SecA is a protein translocase ATPase component in the major protein secretion machinery. A SecA temperature-sensitive mutant, *B. subtilis* TB301 (*trpC2*, *secA341ts*) was cultured in the ^{14}N-medium at 30 °C for 5.5 h (the cell density at $A_{600} = 0.6$) and then the cells were cultured at 30 or 45 °C for 15 and 60 min in the ^{15}N-medium. Proteins of the NDSB insoluble membrane preparations from the cells at each culture period were resolved by 16-BAC/Tris–tricine SDS–PAGE and analyzed by MALDI-TOF MS after proteolysis. Fig. 6A shows the appearance and shift of a 1156.66 *m/z* peptide ion having amino acid sequence, YGFIIRYPK, from YodJ to 1169.70 *m/z* at ratios of 3.6% and 49% during 15 and 60 min of cultivation at 30 °C, respectively. YodJ is predicted to be a lipoprotein that is anchored on the cell membrane and its function is unknown but is similar to D-alanyl-D-alanine carboxypeptidase. In contrast, no 1169.70 *m/z* peak was evident in YodJ obtained from the cells cultured at 45 °C for 15 and 60 min (Fig. 6B). No peptides were also labeled with ^{15}N in a group of membrane proteins and lipoproteins during culture at 45 °C for 15 and 60 min. This acute inhibition of the membrane localization of those proteins by the luck of SecA suggest that the proteins interact or bind directly to SecA to export through or insert into the cell membrane. In contrast, the effect of the SecA lesion on another group of membrane proteins but not lipoproteins differed from that on YodJ. These membrane proteins appeared normally in the cell membrane at 45 °C for 15 min like that at 30 °C but the localization was considerably inhibited after an incubation at 45 °C for 60 min. We considered that the localization of those membrane proteins would be related to SecA indirectly. On the other hand, the amount of ^{15}N-labeled hest-shock proteins, DnaK and GroEL, increased in membrane preparations from cells cultured at 45 °C. In addition some ^{15}N-labeled membrane proteins accumulated in the cell membrane during cultivation at 45 °C for 60 min. Therefore, we speculated that SecA also functions in the insertion of some proteins into the cell membrane directly or indirectly and that the localization of some membrane proteins is independent of the SecA function. We ascertained by immunoblotting using anti *B. subtilis* SecA antiserum that SecA341 disappeared from cells cultured at 45 °C for 15 min (Bunai et al., unpublished).

Furthermore, Smalka et al. [37] described an excellent means for the quantitative measurement of proteins using

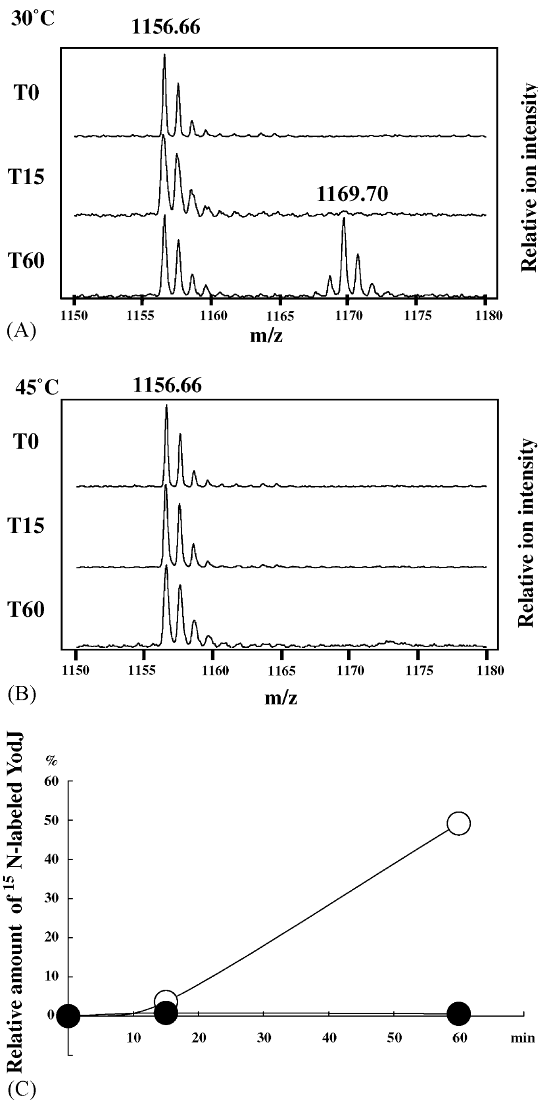


Fig. 6. Effect of SecA on YodJ localization into cell membrane. Submembrane fraction 4 was prepared from *secA* temperature sensitive mutant (*B. subtilis* TB301) that had been cultured at 30 or 45 °C for 0, 15 and 60 min in ^{15}N-medium. Proteins in the fractions were resolved by 16-BAC/Tris–tricine SDS–PAGE and YodJ spots were excised and analyzed by MALDI-TOF MS after proteolysis. (A) Appearance of ^{15}N-labeled 1169.70 *m/z* peptide ion derived from YodJ by LysC-digestion during cultivation at 30 °C for 15 and 60 min. (B) The 1169.70 *m/z* ion did not appear after 45 °C for 15 or 60 min. (C) Time course of ^{15}N-labeled YodJ localization on cell membrane during cultivation at 30 and 45 °C. Relative abundance of ^{15}N-labeled YodJ was calculated from ratios of intensity of ^{15}N-labeled peptide at 1169.70 *m/z* against total intensity of 1156.68 and 1169.70 *m/z* × 100.

in vitro ICAT-labeling for 2D-PAGE. These examples demonstrate that the technological developments in 2D-PAGE combined with mass spectrometry are very important to provide new perspectives from which to achieve a comprehensive understanding of life at the molecular level.

7. Concluding remarks

A large number of proteins can be separated in a single 2D-PAGE gel. Therefore 2D-PAGE coupled with mass spectrometry is a very powerful method not only for identifying protein components in organelles and cells but also for analyzing protein modification, targeting and localization that cannot be addressed using DNA micro arrays. By the introduction of quantitative analyses such as dual channel imaging, in vivo ^{15}N-whole cell-labeling and in vitro ICAT-labeling, 2D-PAGE would provide a powerful tool with which to analyze global regulation of the expression network in the genome that could clarify the physiological states of cells during growth, pathological states and adaptation to stress. However 2D-PAGE cannot analyze total proteins because the cellular content of some proteins can be very high, while that of others can be very low. This problem might be resolved by the combination with DNA micro array analysis. Furthermore, new technologies using types of chromatography that are independent of 2D-PAGE will resolve the problems associated with analyzing hydrophobic and alkaline proteins. Cellular proteomic analyses complemented by DNA micro arrays promise to usher in a new era of understanding of all living organisms.

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