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RFHR 2-D PAGE装置の安全面における改良とプロテオーム解析の試み

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Improvement in the safety of the RFHR 2-D PAGE device and its application in proteome analysis of extremophile

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Proteomics is a powerful tool to understand the dynamics of proteomes, change in protein components of living cells, with the various environmental stimuli. Two dimensional gel electrophoresis (2-DE) is useful for the analysis of relative protein abundance. Out of several 2-DE, two dimensional electrophoresis of radical-free and highly reducing (RFHR) method developed by Wada is very powerful in analysis especially in dynamics of basic proteins such as proteomes of ribosomes. In this study, we tried to improve the equipment in the safety for use and carried out in order to observe separation patterns of the proteins in deep-sea piezophilic and psychrophilic bacterium, *Shewanella violacea* DSS12. The experimental conditions of the RFHR 2-D PAGE with or without pre-run and reductants in the electrophoretic system, were also tested to compare the patterns of changes in protein components. This method, RFHR 2-D PAGE described here, will be useful for the characterization of the functional proteomics of extremophiles.

Keywords Proteome analysis, two dimensional gel electrophoresis, radical-free and highly reducing (RFHR) method, *Shewanella violacea* strain DSS12, improvement

1. Introduction

The piezophilic deep-sea bacterium *Shewanella violacea* strain DSS12, isolated from the Ryukyu Trench (depth: 5,110m), grows optimally at 30 MPa and 8°C, but also grows at atmospheric pressure (0.1 MPa) and 8°C [1, 2]. It is useful as a model for comparison of various features of bacterial physiology under high and low pressure conditions. Recently an operon identified as a pressure-regulated operon, with a promoter activated under high pressure conditions, was cloned and characterized from this strain [3, 4, 5]. We have reported that gene expression from this operon, which has five transcription initiation sites, is controlled at the transcriptional level by elevated pressure [3].

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with a promoter activated under high-pressure conditions, was cloned and characterized from this strain [3-5]. We have reported that gene expression from this operon is controlled at the transcriptional level by elevated pressure [3]. However, the mechanisms controlling transcription of this operon at the molecular level remain to be elucidated. One approach to understanding the basis of gene expression under defined condition is via detailed characterization of transcriptional machinery involved.

Thus, cloning of the genes for RNA polymerase subunits from piezophiles and the establishment of an *in vitro* system for reconstitution of the enzyme are essential. Transcription in eubacteria is mediated by an RNA polymerase, which governs the selectivity of the promoter sequences of a gene. It is a mutisubunit complex composed of mainly a, b, b' and one of several s subunits [6]. Among these subunits, we have previously reported piezoresponse of *S. violacea* a operon and some positive regulators may exist and interact with RNA polymerase [7]. It is essential to elucidate and to characterize several factors interact with RNA polymerase and relate to transcriptional regulation at several conditions. In the case of *E. coli*, one of these factors named w protein is present in both highly purified core and holo RNA polymerase, but the roles of this protein is very little known. One of estimated functions of *E. coli* w protein is a regulator of RNA polymerase, to control promoter selectivity at specific stringent promoters, *rpsA* and *rplJ*, mediated by ppGpp [8]. In this study, we report the isolation of the *rpoZ* gene encoding RNA polymerase w protein from *S. violacea*. The results of phylogenetic analyses based on comparison of the RpoZ protein and construction of expression plasmid, overproduction and affinity purification will be described.

2. Materials and Methods

2.1. Strains and culture conditions

Shewanella violacea DSS12 was grown in Marine Broth(MB) medium at 8℃,10ml of MB inoculated with single colony of overnight culture was placed in a 500ml flask and incubated on a rotary shaker at a speed of 133 rpm. The cells were harvested at the exponential phase (OD_{660}=1).

2.2. Preparation of cell extract lysate

Frozen cells were thawed and immediately mixed with 5ml of buffer containing 10mM Tris-HCl pH7.5, 6mM _-ME, 2mM p-APMSF by 3g cells. Cells were sonicated into ice for 1-2h.The resulting lysis was centrifuged for 5 min at 12000 rpm in Eppendorf centrifuge to remove unbroken cells. The supernatant was collected and stored at -80 until further analysis.

2.3. 0-D electrophoresis

0-D electrophoresis was introduce to prepare sample gel pieces for 1-D electrophoresis. The apparatus consisted of thee parts, an anode and a cathode buffer vessel which were the same at those of the 1-D electrophoresis, and a gel container. The gel container had 8 gel chambers. It was inserted form the down side into the anode buffer vessel, and the interfaces between them were filled with 0-D gel solution injected by a Pasteur pipette and sealed up by gelating the solution. The bottom of the gel container was immersed into 1-D gel solution in a shallow dish and the bottoms of the gel chambers were sealed up by gelation the gel solution. Thereafter 0-D gel solution was poured into the gel chambers up to the height of 40mm, overlaid with water and gelated. Pre-Run was preformed at 100V for 1h using 1-D Pre-Run buffers. At the end of the Pre-Run, sample solutions were added on the 0-D gels and overlaid gently with anode run buffer. 0-D electrophoresis was performed at 100V for about 1h. pyronine-G and acridine orange in the sample solutions were concentrated rapidly and migrated into the gels. Proteins just follow them. When pyronine-G and

acridine orange had run inside the gels at least about 10 mm, the run was finished. The gels were carefully loosened and taken out of the gel chambers by injecting 0-D anode run buffer through an injecting needle into the interfaces between the gels and the gel cambers. Sample gel pieces containing proteins were obtained by cutting the gels with a razor at position immediately below the band of pyronine-G and within the length of 10mm. The sample gel pieces were immersed in 1-D anode run buffer containing 9mM MEA for exchanging buffer and reduced at room temperature for 15min. Thereafter they were used for 1-D electrophoresis.

2.4. 1-D electrophoresis

1-D gel container had 6 gel chambers. Each gel camber had a window with 10mm length through which a sample gel piece could be inserted. The interface between the gel container and the window cover was sealed using 1-D gel solution as described above. The interface between the gel container and the anode buffer vessel was also sealed similarly. After the bottom of gel cambers were gelated with 1-D gel solution as described above, 1-D gel solution was poured in to fill up the whole length of the gel chambers and gelated. A Pre-Run was performed at 100V 15h with air cooling. Thereafter the window cover was opened and the exposed parts of the gels were cut out with a ground spatula to the same length as the sample gel pieces. The sample gel pieces were inserted into the spaces and a small amount of 1-D anode run buffer was added onto them to remove air bubbles. The window cover was closed again without gelation. 1-D electrophoresis was performed at 150V for about 10h with cooling. At end of the electrophoresis, the sample gel pieces were taken out of the sample gel spaces with a spatula and then the 1-D gels were taken out of the gel cambers with an injector as described above. The excess parts of the 1-D gels were cut off to the same length as the width of the 2-D gels. The length ratio of the cathode side gel to the anode side gel was two.

2.5. 1-D electrophoresis

2-D gel container had 4 gel chambers. All parts of the gel container were firmly assembled using four hatagane and all the interfaces of the parts that enclosed the gel cambers were sealed up by gelating Agarose gel solution. Thereafter the gel container was inserted into the anode buffer vessel, and their interfaces were also sealed up in the same way. The bottoms of gel chambers were made by gelating 50ml of gel solution. Gel chambers and side "ditches" were filled with 2-D gel solution. 1-D gel spacers were inserted into the tops of the gel chambers to leave 5mm depth spaces for setting 1-D and sample gels. After the gelation, the spacers and excess gels were removed and Pre-Run was performed at 100V 24h. Thereafter the 1-D and sample gels were put on the 2-D gels, carefully avoiding bubbles in their interfaces. A piece of cotton gauze wet with anode run buffer was used to cover the 1-D and sample gels. The gauze was pressed by five wedges that were inserted into the five ditches. The gauze was thereby stretched strongly and pressed the 1-D and sample gels against the 2-D gels. In this way, good contact between the 1-D and 2-D gels could be made without using gelation. The 2-D electrophoresis was performed at 100V with air cooling. Pyronine-G remaining in the 1-D gels was useful as a 2-D front marker. When it reached the bottom, the 2-D run was finished. After the 2-D gels were carefully taken out of the gel cambers, they were stained in the same way as K-W's staining procedure. After exposure to running tap water for about 10min, they were put between plastic nets vertically in a destaining vessel and destained electrophoretically in 1% acetic acid at 1A current.

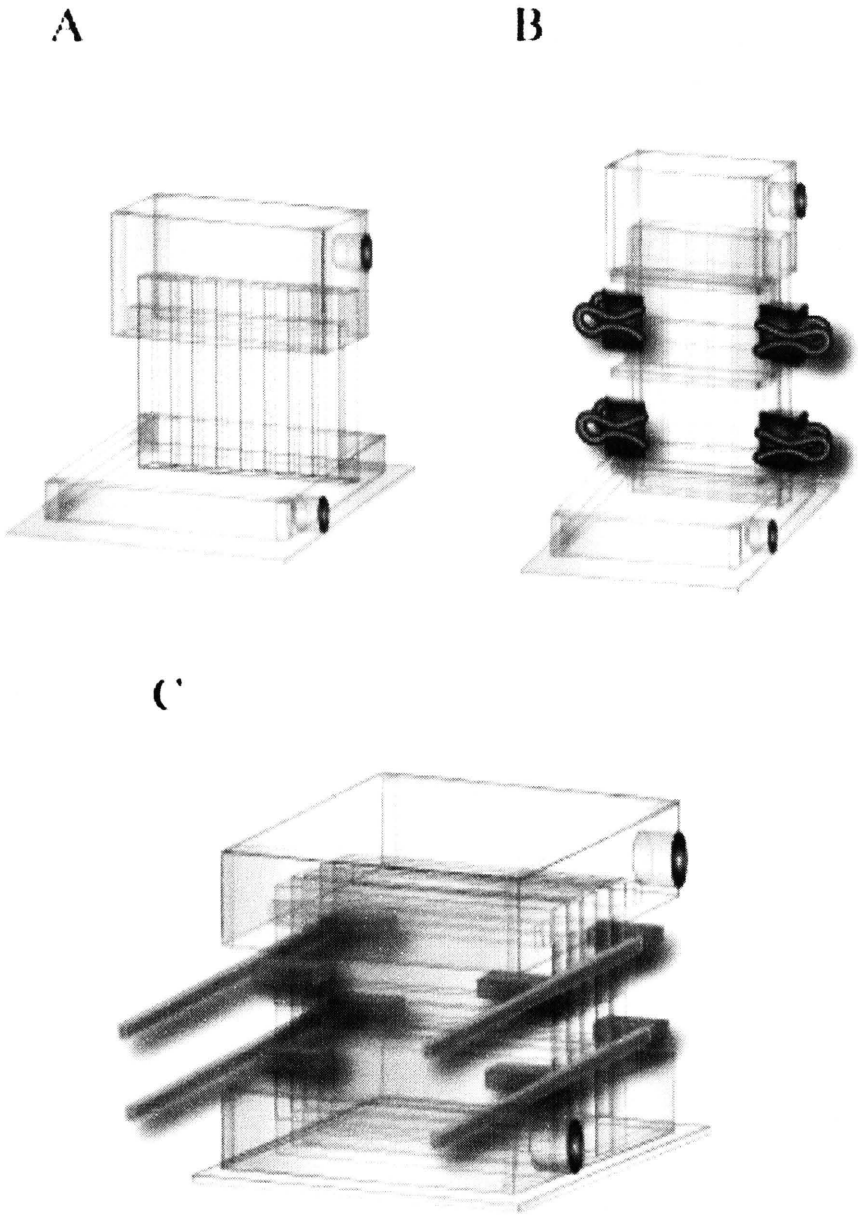

Fig.1 Schematic representation of two dimensional electrophoresis equipment for RFHR method

(A) 0-D electrophoresis apparatus. a: 0-D gel containers apparatus, each of which had 100mm height and 3×5mm^2 cross sectional area. The upper part: (+) anode buffer vessel apparatus (ABV). b: Gal maker bottom dish apparatus. c: (-) cathode buffer vessel apparatus. Fig2-B 0-D electrophoresis unit.

(B) 1-D electrophoresis apparatus. a: (+) anode buffer vessel apparatus. b: top gel cover. c: (-) cathode buffer vessel. d: sample gel cover. e: Gel maker bottom dish. f: bottom gel cover. g: 1-D gel containers apparatus, each of which had 175mm height and 3×5mm^2 cross sectional area. h: clips. Fig3-B 1-D electrophoresis unit.

(C) 2-D electrophoresis apparatus. a: 2-D gel containers apparatus with 140mm height, 160mm width and 3mm thickness. b: (+) anode buffer vessel apparatus. c: Gel spacers apparatus. d: Hatagane, which are a Japanese carpenter's tool. e: (-) cathode buffer vessel apparatus. Fig4-B 2-D electrophoresis unit.

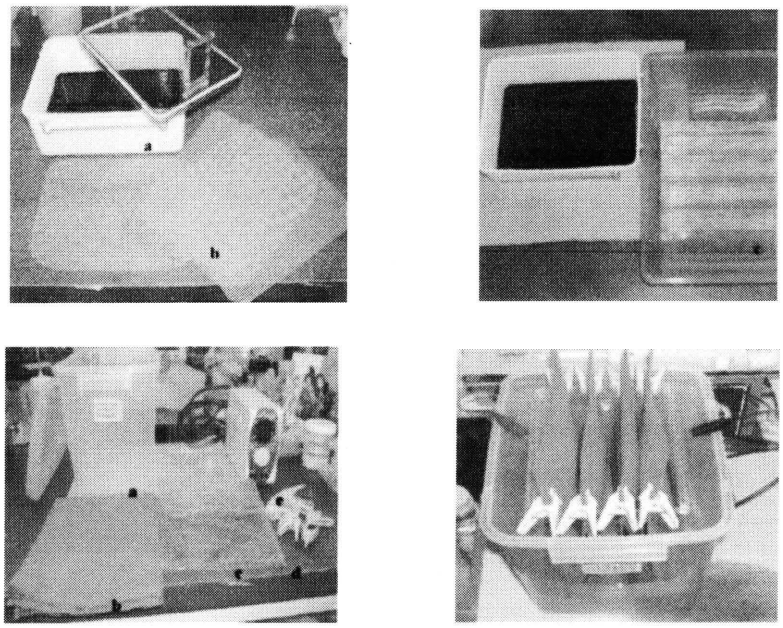

Fig 2. Staining and destaining unit.

(A) Staining unit. a: Plastic bag. b: Plastic nets ($25 \times 20 cm^2$). Fig5-B is a state before staining. c: 2-D gels.

(B) Destaining unit. a: Plastic container. b: Plastic nets ($27 \times 20 cm^2$). c: Steel nets. d: clips. e: Power supply. Fig6-B is a state of electrical destaining in 1% acetic acid at 1A current.

3. Results and discussion

3.1. separation pattern of the proteins of *S. violacea*

To clone a part of the *rpoZ* gene from *S. violacea*, the amino acid sequences of the RpoZ proteins from several bacteria were aligned and a highly conserved region was found (data not shown). These conserved sequences were used to design and synthesize degenerate oligonucleotides in order to amplify a part of the *rpoZ* gene from *S. violacea*. A fragment containing part of the *rpoZ* gene, approximately 170 bp, amplified by PCR with the primers, was cloned into the pCR2.1 vector and its nucleotide sequence was determined. The deduced amino acid sequence of the cloned gene was significantly similar to the RpoZ proteins of other bacteria (data not shown). To clone the complete *rpoZ* gene, the partial *rpoZ* gene fragment was labeled with DIG (digoxygenin) by PCR as a hybridization probe. From the Southern blotting analysis using DIG-labeled *S. violacea rpoZ* gene as a probe, a positive band was detected from digested fragments of *S. violacea* Chromosomal DNA with several restriction enzymes (data not shown). As shown in Fig. 1 it showed that the *rpoZ* gene was present in a 35.7 kb (*Bam* HI), 8.1 kb (*Hind* III), 9.6 kb (*Eco* R I), 5.5 kb (*Kpn* I), 2 kb (*Pst* I) and 17.1 kb fragment (*Sal* I).

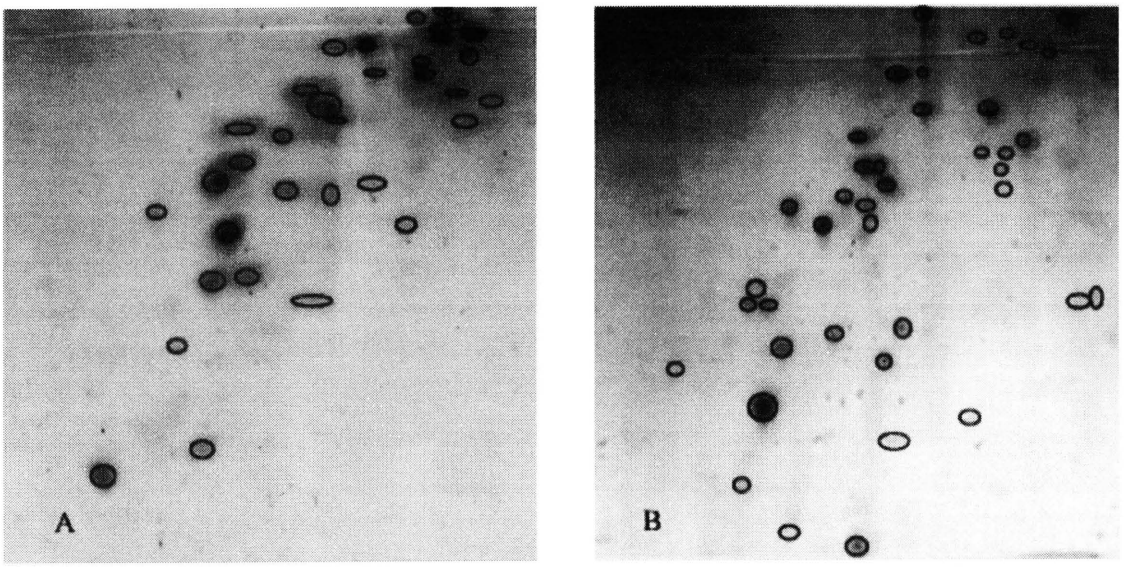

Fig.3 T he RFHR 2-D PAGE with or without pre-run and reductants in the electrophoretic system to compare the patterns of changes in protein component.

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