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試験管内転写反応による深海微生物RNAポリメラーゼの高圧力に対する影響

仲宗根薫

The effect of hydrostatic pressure on activity of RNA polymerase from piezophile by *in vitro* transcription

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We have compared pressure-induced subunit dissociation of RNA polymerase of piezophilic *Shewanella violacea* and mesophilic *Escherichia coli*. The transcriptional activity of the purified pressurized-RNA polymerase from both species was measured by both nonspecific and specific *in vitro* transcription assay, using Poly (dA-dT)·(dA-dT) and the *S. violacea rpoA* promoter as a DNA template. These analyses showed the RNA polymerase from *S. violacea* is stable to pressure-induced inactivation compared with *E. coli*, suggesting molecular adaptation to high pressure environment (piezosphere).
Keywords RNA polymerase, Transcription, High pressure, dissociation In vitro transcription

1. Introduction

The piezophilic deep-sea bacterium *Shewanella violacea* DSS12, isolated from the Ryukyu trench (depth; 5110 m), grows optimally at 30 MPa and 8°C, but also at atmospheric pressure (0.1 MPa) and 8°C [1,2]. It is useful as a model bacterium 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 [3-5]. We have reported that gene expression from this operon is controlled at the transcriptional level by elevated pressure [3]. This piezophilic bacterium, *S. violacea* is one of deep-sea adapted organisms, can grow at the range of 0.1 MPa to 70 MPa, suggesting almost of the protein structures in this piezophle are more stable and retain the activity required to lives under high hydrostatic pressure conditions than that in organisms living under atmospheric conditions.

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Especially, one of the most important proteins for transcription process is RNA polymerase as a transcriptional apparatus, which also governs the selectivity of the promoter sequence of gene. It is a subunit complex protein composed of a, b, b' and one of several s subunits [6].

In this study, we came up with the idea that enzymatic activity of RNA polymerase in the piezophile has a tolerance to high pressure than that in other mesophilic organisms. Here, we report the transcriptional activity of pressurized RNA polymerase from piezophilic *S. violacea* and mesophilic *E. coli* using *in vitro* transcription assays

2. Materials and methods

2.1. Bacterial strains and culture conditions

S. violacea DSS12 and *E. coli* W3110 were used as the strain for purification of RNA polymerase. *S. violacea* was cultivated at 8°C with vigorous shaking in 15 litters of Marine Broth 2216 medium (Difco). *E. coli* W3110 was cultivated at 37°C with vigorous shaking in 15 litters of LB medium. In the mid-log phase of growth, the absorbance of the culture at 660 nm was 0.7 and each of both cells at this stage was harvested and stored at –80°C until use.

2.2. Purification of RNA polymerase

Fifteen grams of strain DSS12 and W3110 cells were used for the enzyme purification. The RNA polymerase of both *S. violacea* and *E. coli* were purified by the modification method described by Burgess et al [8]. The enzyme fraction obtained by Polymin P precipitation and salt extraction was put on a High Trap Heparin column (amersham pharmacia biotech) [9]. The resulted fractions containing RNA polymerase were observed and then it was put on a Superose 6 gel filtration column (amersham pharmacia biotech) and the peak fractions were finally put onto a Mono Q anion exchange column (amersham pharmacia biotech), in order to separate holo and core enzymes [10]. The enzyme was dialyzed against the stock buffer (10 mM Tris-HCl (pH7.8), 10 mM $MgCl_2$, 0.1 mM EDTA, 1mM DTT, 0.2M KCl, 50% Glycerol) and stored at –80°C until use.

2.3. Measurements of the activities of pressurized RNA polymerase by nonspecific in vitro transcription

These purified RNA polymerses were put into the disposable PCR tubes and tubes were closed hard with parafilm. These enzymes were subjected to several pressure conditions using pressure vessel at 25°C for 30 min. After decompression, the activity was measured by following method. The mixture contained Tris-HCl (pH7.8), 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT, 0.2 mM ATP, 0.2 mM [^{3}H]UTP, 1.2 mg Poly (dA-dT)·(dA-dT) and 1 pmol of RNA polymerase [10]. The mixture was incubated at 37°C for 10 min and the RNA product was precipitated by the addition of 3ml of 5% (w/v) trichloroacetic acid followed by incubation for 15 min at 4°C. The acid-precipitable materials was collected on a Whatman GF/C filter, washed with 10 ml of 3% (w/v) trichloroacetic acid and dried. The radioactivity was measured with a liquid scintillation counter. The relative activity was calculated based on the activity at atmospheric pressure.

2.4. Measurement of the activities of pressurized RNA polymerase using specific In vitro transcription

As described in 2. 3, both RNA polymerase from two species were pressurized and decompressed. The specific *in vitro* transcriptional activity of *S. violacea* and *E. coli* RNA polymerase was measured by *in vitro* production of transcripts from *S. violacea rpoA* template, which has s^{70} promoter sequence [11]. The reaction was done under single-round reaction conditions [10]. The incubation mixture (42.5 ml) contained 20 mM Tris-HCl (pH 8.0), 12 mM $MgCl_2$, 12 mM NaCl, 24 mM EDTA, 12% Glycerol, 9.5 mM 2-Mercaptoethanol, 1.25 mg BSA, 0.1 pmol of template DNA, and 1 pmol of RNA polymerase, pressurized 30 min described above. The mixture was incubated for 10 min at 37°C to obtain open promoter complexes. Transcription was started by the addition of

7.5 ml of a mixture of 2 mg heparin, 1.2 mM each of ATP, GTP, CTP, 0.4 mM UTP and 74 KBq of [a-^{32}P] UTP. The RNA synthesis was allowed to proceed for another 5 min, and then the reaction was stopped by the addition of 40 mM EDTA and 20 mg Glycogen. RNA products were precipitated with ethanol and analyzed by electrophoresis in an 8% polyacrylamide gel containing 8 M urea. The resulting gel was exposed to imaging plate and the imaging plate was analyzed with a BioImage Analyzer BAS2000 (Fuji, Tokyo).

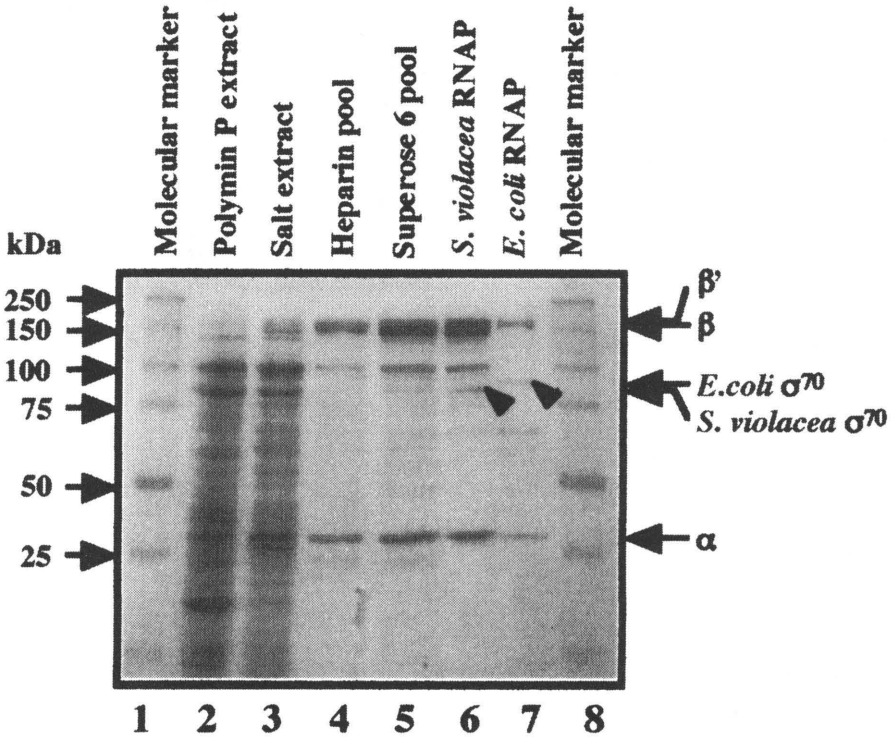

Fig. 1 SDS-PAGE analysis of proteins obtained during purification of the *S. violacea* RNA polymerase. The proteins were separated on 10% acrylamide gel. Lane 1 and 8, standard markers with molecular mass indicated in kDa; lane 2, Polymin P extract; lane 3, salt extract; lane 4, heparin pool; lane 5, Superose 6 pool; lane 6, the *S. violacea* RNA polymerase; lane 7, the *E. coli* RNA polymerase. Each subunits of RNA polymerase, a, b, b' and s^{70} were indicated by arrows.

3. Results and discussion

3.1. Purification of RNA polymerase

The RNA polymerases were purified from piezophilic *S. violacea* and mesophilic *Escherichia coli*. As shown in Fig. 2, we identified that the enzyme had the typical composition of a, b, b' and s subunits of eubacterial RNA polymerase. The molecular mass of the subunits were 30,000, 152,000, 162,000, 82,000 Da, respectively, as measured by SDS polyacryl amide gel electrophoresis. The purified RNA polymerase of *S. violacea* recognized s^{70} promoter in an *in vitro* transcription assay on the *E. coli RNA-I* template (data not shown). From these results, purified enzyme was elucidated s^{70} type RNA polymerase.

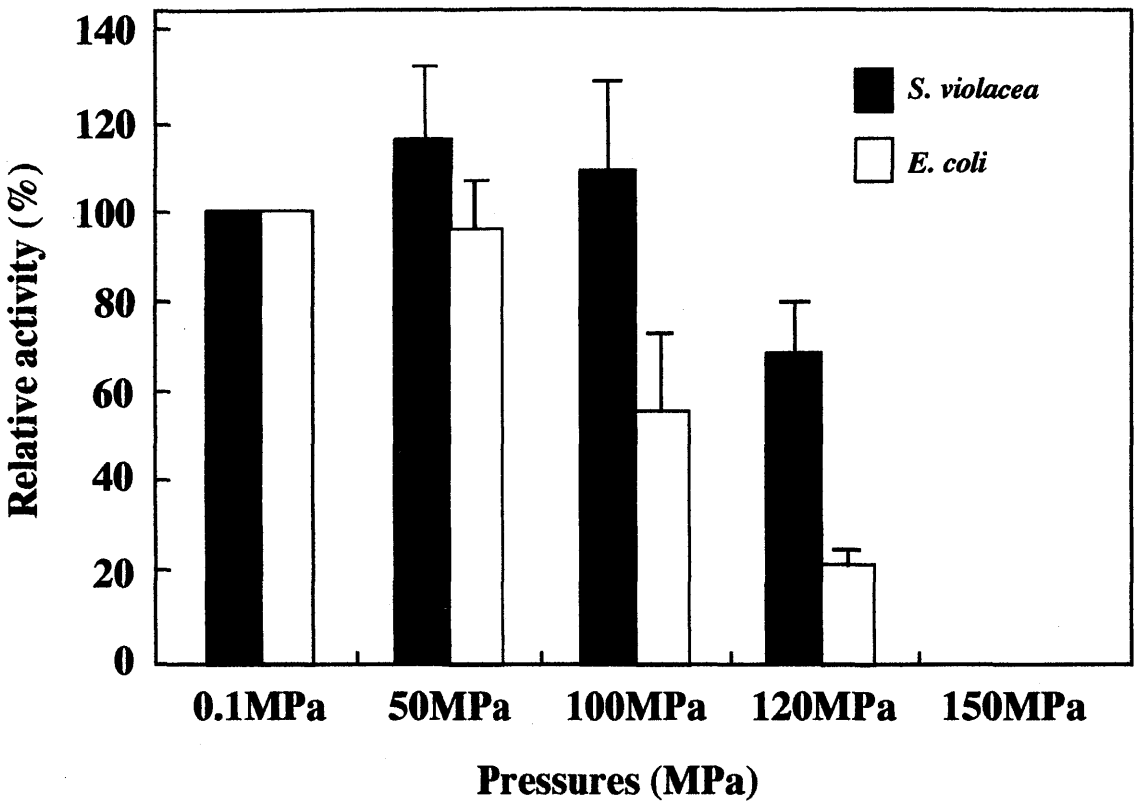

Fig. 2 Comparison of changes in transcriptional activity of RNA polymerase from *S. violacea* and *E. coli.* nonspecific in vitro transcription assay

3.2. Measurements of the activities of pressurized RNA polymerase by in vitro transcription

We measured activity of pressurized purified-RNA polymerase of piezophilic Shewanella violacea and mesophilic Escherichia coli. The transcriptional activity of both species was measured by nonspecific *in vitro* transcription assay, using Poly (dA-dT) · (dA-dT) as a template. As shown in Fig. 3, the both enzymatic activities were almost unchanged at the 50 MPa. In the case of at 100 MPa, the activity of *S. violacea* RNA polymerase was almost unchanged whereas *E. coli* RNA polymerase was decreased its activity about 60% of that at atmospheric pressure (0.1 MPa). Under the pressure of 120 MPa, these enzymes from two species were inactivated but the activity of S. violacea was markedly higher than that of *E. coli*. The enzymatic activity from two species totally disappeared at 150 MPa. These results suggest *S. violacea* RNA polymerase is more stable under high pressure conditions than that of *E. coli*. In this assay, we used nonspecific template composed of only TA sequences for easily recognition wherever on the template by s^{70}RNA polymerase. We also tried specific in vitro transcription to examine whether this result occur again and to study the influence of high pressure for promoter recognition machinery by using determined promoters and detect the transcripts, directly.

3.3. Measurement of the activities of pressurized RNA polymerase using specific in vitro transcription

The specific *in vitro* transcriptional activity of *S. violacea* and *E. coli* RNA polymerase was monitored by *in vitro* production of transcripts from *S. violace rpoA* template, which has two

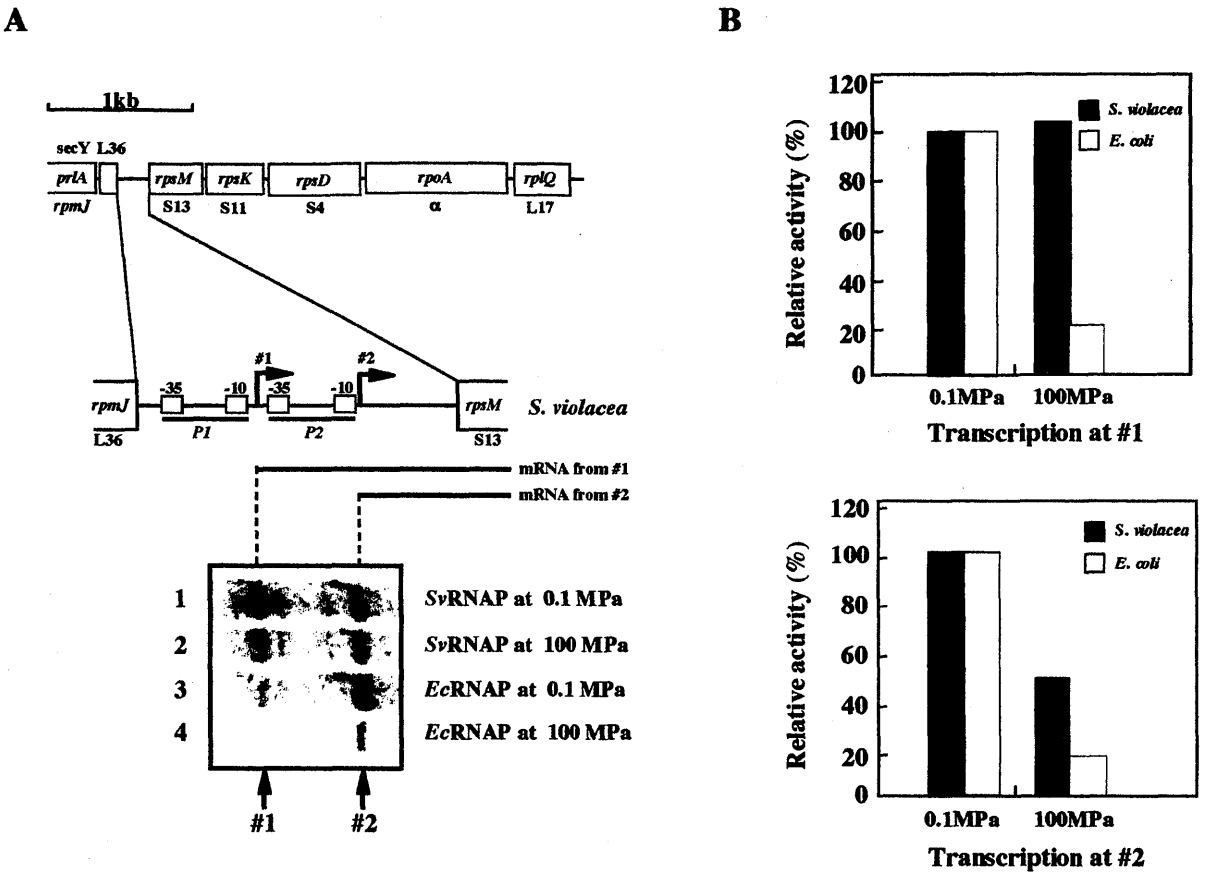

Fig. 3 Comparison of changes in transcriptional activity of RNA polymerase from *S. violacea* and *E. coli*. Nonspecific in vitro transcription assay

s^{70} promoter sequences [11]. The reaction was done under single-round reaction conditions.

Results from comparison of transcriptional activity of RNA polymerase from #1 and #2, were shown in Fig. 3B. As well as result of nonspecific transcription, the transcripts from #1 by *S. violacea* is almost unchanged in spite of the transcripts from #2 was reduced. Surprisingly both transcripts from #1 and #2 transcribed by *E. coli* RNA Polymerase is only 20% of that at atmospheric pressure. The transcripts from #2 of *S. violacea* was approximately 50% compared with that at atmospheric pressure suggesting some of RNAP molecules lost its ability for recognition of promoter 2 or inactivated by high pressure. The activity of pressurized *S. violacea* RNA polymerase from both promoters were higher than that of *E. coli* as well as in the case of Poly (dA-dT)·(dA-dT) as a template. From these results here together with result of 3.2, we raised possibility of RNA polymerase molecules didn't inactivate at higher pressure than *E. coli* and have stable complex against high pressure condition. Based on the findings of these study, the RNA polymerase of piezophilic bacterium has stability to adapt high pressure environment (piezosphere).

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