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深海微生物 RNA ポリメラーゼの精製

仲宗根薫

Purification of RNA polymerase from deep-sea bacterium

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RNA polymerase from deep-sea bacterium *Shewanella violacea* DSS12 was purified using three chromatographic steps. The purified enzyme had the typical composition of a, b, b' and s^{70} subunits of eubacterial counterpart. The molecular masses of these subunits were 30,000, 152,000, 162,000, 82,000 Da, respectively as measured by SDS-PAGE. An *in vitro* transcription assay using the RNAI promoter as a transcription template revealed that the enzyme of *S. violacea* initiated transcription at the same site as that of *Escherichia coli*.

Keywords Piezophilic bacterium, *Shewanella violacea*, Transcription, RNA polymerase

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 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]. However, the molecular basis of transcriptional regulation of this operon remains to be elucidated. Knowledge of the regulation of gene expression and the transcriptional machinery of this piezophile is required for a critical understanding of how these organisms adapt and grow in a high-pressure environment. Transcription in eubacteria is mediated by an RNA polymerase which governs the selectivity of the promoter sequence of gene. Thus, purification of RNA polymerase from piezophiles and the establishment of an *in vitro* transcription method are essential.

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In this paper, we describe the purification of principal form of RNA polymerase from *S. violacea*. We also analyzed promoter recognition of the purified enzyme.

2. Materials and methods

2.1. Bacterial strains, culture conditions

S. violacea DSS12 was the source of RNA polymerase. *S. violacea* was grown at atmospheric pressure (0.1 MPa) and 8°C in 15 liters of Marine Broth 2216 (Difco). When the culture reached A660 of 0.7, cells were harvested by centrifugation at 4°C, and the cell pellet was stored at -80°C until use.

2.2 Chemicals for purification of RNA polymerase

Buffer A; 10 mM Tris-HCl, pH 7.8, containing 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.1 mM pefabloc. Buffer B0; 10 mM Tris-HCl, pH 7.8, containing 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 5% glycerol, 0.1 M NaCl. Buffer B1; 10 mM Tris-HCl, pH 7.8, containing 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 5% glycerol, 0.2 M NaCl. Buffer B2; 10 mM Tris-HCl, pH 7.8, containing 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 5% glycerol, 0.5 M NaCl. Buffer C1; 50 mM Tris-HCl, pH 7.8, containing 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 5% glycerol, 0.5 M NaCl. Buffer D0; 10 mM Tris-HCl, pH 7.8, containing 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 5% glycerol, 0.25 M NaCl. Store buffer; 10 mM Tris-HCl, pH 7.8, containing 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 50% glycerol, 0.2 M KCl.

2.3. Purification of RNA polymerase of *S. violacea*

All procedures were done at 4°C. A sample of 15 g (wet weight) of cells were suspended in 30 ml of buffer A and disrupted by French press (8,000 p.s.i). The extract was centrifuged at 14,000 rpm 15 min and ultra centrifuged at 100,000 x g, 1.5 hr. The supernatant was collected and Polymin P was added to final concentration of 0.25% (w/v) and after this was stirred for 15 min, the precipitate was collected by centrifugation at 14,000 rpm 15 min [6]. The enzyme was extracted from the pellet with 10 ml of buffer B1 and homogenized using homogenizer and was centrifuged at 14,000 rpm 15 min. After discarded of supernatant 10 ml of buffer B2 was added to pellet and homogenized and centrifuged as well and collected this fluid. This fluid was treated with 60% saturation of $(NH_4)_2SO_4$ and precipitate obtained was dissolved in 2 ml of buffer B1 and dialyzed against 2 liters of the buffer B0. The dialysate was put on a Hi-Trap heparin column (5 ml) (Pharmacia Biotech) and eluted with a 0.1-1.0 M NaCl gradient in 70 ml of buffer B [7]. The fractions contained of RNA polymerase observed SDS-PAGE on a 10% gel were collected and treated with 60% saturation of $(NH_4)_2SO_4$ and precipitate obtained was dissolved in 500 µl of buffer C1 and put on Superose 6 column (10 x 30 mm)(Pharmacia Biotech) and eluted in 100 ml of buffer C1. The fractions observed containing of RNA polymerase by SDS-PAGE on a 10% gel were collected and treated with 60% saturation of $(NH_4)_2SO_4$ and precipitate obtained was dissolved in 2 ml of buffer D0 and dialyzed against 2 liters of the buffer D0. The dialysate was put on a Mono Q (1 ml) (Pharmacia Biotech) and eluted with a 0.25-0.5 M NaCl gradient in 80 ml of buffer D [8]. The fractions containing of RNA polymerase by SDS-PAGE on a 10% gel were collected and treated with 60% saturation of $(NH_4)_2SO_4$ and precipitate obtained was dissolved in 500 µl of store buffer and dialyzed against 2 liters of the store buffer and stored at -80°C.

2.4.The assay for promoter recognition

The promoter recognition of *S. violacea* and *E. coli* RNA polymerase was monitored by *in vitro* production of transcripts from *E. coli RNA-I* template, which has *E. coli* s^{70} promoter sequence. The reaction was done under single-round reaction conditions [11]. 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. 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 100 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 followed by autoradiography.

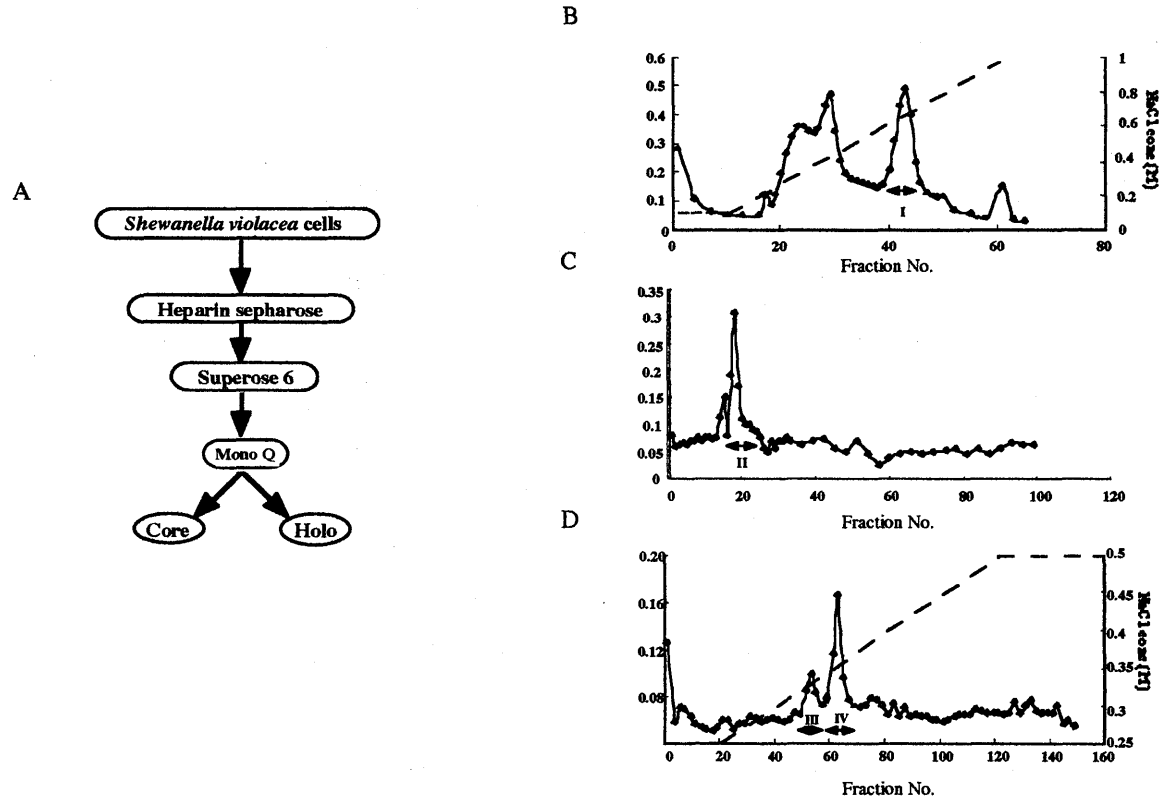

Fig.1 Purification of *S. violacea* RNA polymerase.

(A) The strategy of the purification steps. (B) Heparin sepharose affinity chromatography. (C) Size exclusion chromatography by an FPLC Superose 6HR column. (D) Mono Q anion exchange chromatography.

3. Results and discussion

3.1. Purification of RNA polymerase from *S. violacea* and its subunit structure

RNA polymerase was purified from *S. violacea* as described in the strategic scheme for the purification (Fig. 1 A). During purification of the RNA polymerase, total activity increased. The optical concentration of Polymin P to precipitate the RNA polymerase from a cell extract was 0.25% (w/v), as judged by the disappearance of the b' and b subunits from the supernatant fluid on SDS-PAGE. RNA polymerase was eluted from the Polymin P precipitate most efficiently with 0.5% NaCl (data not shown). Chromatography on a heparin sepharose column provided a nearly homogeneous preparation of the enzyme that eluted at a NaCl concentration of 0.1M – 1.0M for the first column chromatography steps (Fig. 1B). For final steps after purification by size exclusion FPLC on a Superose 6 column (Fig. 1C), we used Mono Q HR column (Fig. 1D)as described under Materials and Methods, yielding two major

peaks of electrophoretically pure core and holo enzyme, eluted at ~0.325 and 0.34 M of NaCl gradient, respectively. The SDS-PAGE pattern of protein fractions at various steps of purification were shown in Fig. 2. The RNA polymerase isolated from S. violacea had the typical subunit structure of eubacterial counterpart with four major subunits of a, b, b' and s^{70}. The molecular masses of the main subunits were estimated by SDS-PAGE in comparison with protein standards including E. coli RNA polymerase subunits, to be 30,000 (a), 152,000 (b) and 162,000 (b')Da, respectively (lane 5 of Fig. 2).

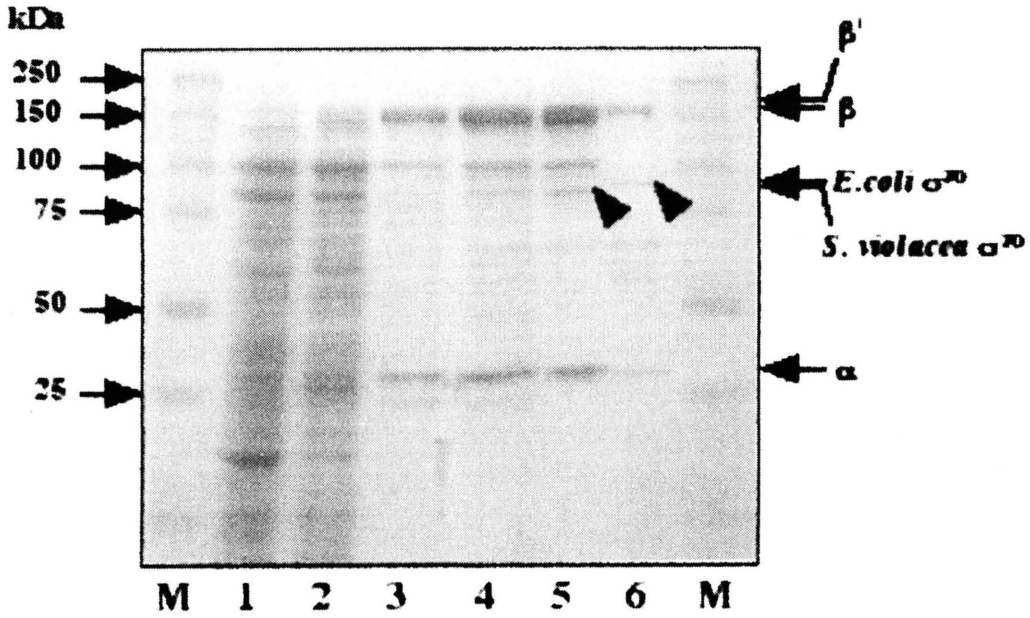

Fig.2 SDS-PAGE analysis of proteins obtained during purification of the *S. violacea* RNA polymerase.
The proteins were separated on 10% acrylamide gel. Lane 1, Polymin P extract; Lane 2, Polymin P extract-salt precipitation; Lane 3, Heparin sepharose pool; Lane 4, Superose 6 pool; Lane 5, purified *S. violacea* RNA polymerase; Lane 6, purified *E. coli* RNA polymerase.

3.2. Comparison of the promoter specificities of *S. violacea* and *E. coli* RNA polymerases

The promoter selectivities of *S. violacea* and *E. coli* RNA polymerases was compared by *in vitro* transcription assay using RNA-I promoter DNA as a template (Fig. 3A). *S. violacea* and *E. coli* RNA polymerases were both able to initiate transcription from the RNA-I promoter and a produce a runoff transcript of 65 bases mRNA (lane 1 of Fig. 3B). These results indicate that the purified major form of RNA polymerase from *S. violacea* is a functional homolog of s^{70} containing RNA polymerase in *E. coli*. The enzyme purified in this study not only will be useful to examine the functional properties of the RNA polymerase itself, but also will facilitate the identification of promoter elements and *trans*-acting factors that control gene expression in this piezophilic strain under both atmospheric and high pressure conditions.

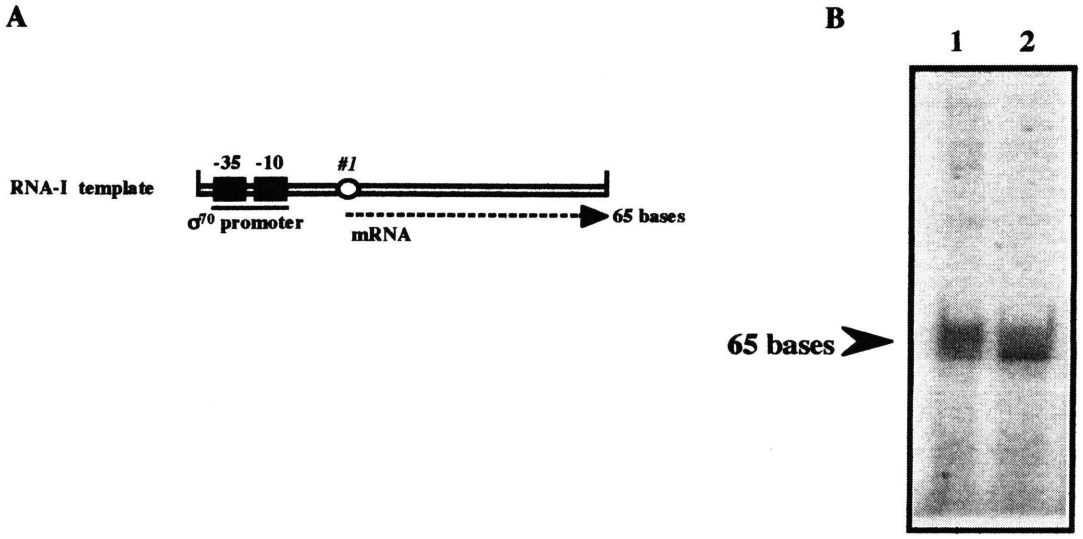

Fig. 3 ***In vitro*** **transcription with RNA polymerase of** ***S. violacea*** **and** ***E. coli.***

(A) Structure of DNA template and expected RNA product. Arrow with broken line indicate accurate transcript directed by the truncated DNA template. Transcriptional initiation site indicated #1, the consensus promoter sequences (-35 and –10) are boxed in black and core promoter regions is underlined. (B) *In vitro* transcription directed by the truncated DNA fragment was done under single–round reaction with RNA polymerase of *S. violacea* and *E. coli*. The RNA products of runoff transcription using RNA polymerase from *S. violacea* (lane 1) and *E. coli* (lane 2) were analyzed by 8% polyacrylamide gel electrophoresis and autoradiography. The arrow shows the sizes of RNA products.

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