Identification of rpoBC Genes Encoding for β and β' Subunits of RNA Polymerase in a Deep-Sea Piezophilic Bacterium, Shewanella violacea Strain DSS12

Hiroaki Kawano,1,2,* Kaoru Nakasone,3 Fumiyoshi Abe,1 Chiaki Kato,1 Yasuhiro Yoshida,2 Ron Usami,2 and Koki Horikoshi1

1Extremobiosphere Research Center (XBR), Japan Agency for Marine-Earth Science and Technology (JAMSTEC), 2-15 Natsushima-cho, Yokosuka, Kanagawa 237-0061, Japan
2Department of Applied Chemistry, Faculty of Engineering, Toyo University, 2100 Kujirai, Kawagoe, Saitama 350-0852, Japan
3Department of Biotechnology and Chemistry, School of Engineering, Kinki University, 1 Umenobe Takaya, Higashihiroshima, Hiroshima 739-2116, Japan

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RNA polymerase from cells of the deep-sea bacterium Shewanella violacea DSS12 was purified using three chromatographic steps. An in vitro transcription assay indicated that the purified enzyme was σ70 containing RNA polymerase. The enzyme activity was inhibited in the presence of rifampicin when the sensitive domain was targeted. The rpoBC genes encoding for the β and β' subunits of RNA polymerase were cloned and their nucleotide sequences determined. Expression plasmids, designated pQSVB and pQSVC, to overproduce these proteins were constructed, and the proteins were purified using a Ni2+ affinity column. In vitro reconstitution using all proteins for the holoenzyme (α, β, β', σ70) was carried out and the activity of the recombinant RNA polymerase was detected.

Key words: Shewanella violacea; RNA polymerase; enzymatic activity; in vitro transcription; rpoBC genes

The deep sea is an extreme environment characterized by high hydrostatic pressure and low temperatures. Many deep-sea-adapted microorganisms have been successfully isolated and characterized genetically and biochemically. Among those microorganisms, piezophilic bacteria exhibit elevated growth at pressures above 1 atmospheric pressure, and their piezoadaptation mechanisms have been investigated.1–3) These growth properties are unique and the bacterium is thus good material for comparative study of cell physiology under high- and low-pressure conditions. Hence, we targeted this strain to elucidate the molecular basis of gene regulation in piezophilic bacteria.

Recently, several operons regulated by elevated hydrostatic pressure have been identified and characterized from this strain.6–9) We have reported that gene expression from the operon is controlled at the transcriptional level by elevated pressure,6) but the mechanisms controlling transcription of these operons at the molecular level remain to be elucidated. One approach to understanding the basis of gene expression under the defined conditions is via detailed characterization of the components of the transcriptional machinery involved.

Transcription in eubacteria is mediated by RNA polymerase, which governs the selectivity of gene promoter sequences. RNA polymerase is a multisubunit complex composed mainly of α, β, and β' (core enzymes) and one of several σ subunits (holoenzyme).10) One of the adaptation mechanisms to environmental changes in bacteria is performed by alternative RNA polymerase, exchanging multiple species of the σ subunit. Among several σ subunits, σ70 is known to be the principal one, which transcribes most of the growth-related and housekeeping genes important for cell growth in Escherichia coli.11) Hence characterization of σ70-RNA polymerase or the establishment an in vitro method for reconstitution of the enzyme using individually cloned subunit genes from a piezophile is required. We have already cloned subunit genes coding for α, σ70, and ω (a putative chaperone for RNA polymerase) with molecular characterizations.12–14) In a previous study, we

1 To whom correspondence should be addressed. Extremobiosphere Research Center (XBR), Japan Agency for Marine-Earth Science and Technology (JAMSTEC), 2-15 Natsushima-cho, Yokosuka, Kanagawa 237-0061, Japan; Tel: +81-468-67-9695; Fax: +81-468-67-9715; E-mail: kawano@jamstec.go.jp
also found that σ70-RNA polymerase from the cells of S. violacea maintained higher activity than that from E. coli through stabilization of subunit complexes after high-pressure treatment,15 but the basic properties of S. violacea RNA polymerase (SvRNAP) have not been elucidated. In this paper, we report the purification and characterization of the σ70-RNA polymerase from S. violacea. We also isolated the rpoBC genes coding for the RNA polymerase β and β' subunits and constructed expression plasmids for recombinant RNA polymerase.

Materials and Methods

Bacterial strains and culture conditions. S. violacea strain DSS125) and E. coli strain W3110 were used in this study. S. violacea cells were grown at atmospheric pressure and 8°C with vigorous shaking in 15-liter of Marine Broth 2216 medium (Difco, Sparks, MD). Cells of E. coli strain W3110 were grown at 37°C with vigorous shaking in 15-liter of Luria–Bertani medium. The cells were collected in the mid-log phase of growth (approximate optimal density at 660 nm = 0.7), and the cell pellets were stored at −80°C until use.

Purification of RNA polymerases. The holoenzyme of SvRNAP was purified from S. violacea cells as described by Kawano et al.15 Fifteen grams of the cells were used for purification of RNA polymerase. The enzyme fraction obtained after Polymin P precipitation and salt extraction was applied to a High Trap Heparin column (Amersham Pharmacia Biotech, Piscataway, NJ).16 The resulting fractions were collected and applied to a Superose 6 gel filtration column (Amersham Pharmacia Biotech), and the resulting fractions were finally applied to a MonoQ anion-exchange column (Amersham) to separate the holo- and core enzymes of RNA polymerase.17 To obtain core RNA polymerase efficiently, the purified holoenzyme was additionally applied to a phosphocellulose column (P-11 cellulose phosphate, 7.4 mequiv/g, Whatman, Middlesex, U. K.) to remove σ subunits.18

Nonspecific in vitro transcription assay of RNA polymerase activity. Transcriptional activity was measured using a poly(dA-dT)·poly(dA-dT) template according to the method described by Kawano et al.15 Incorporation of radio-labeled UTP into RNA products was measured using a liquid scintillation counter. To determine the optimal temperature for SvRNAP, the reaction mixture was subjected to a temperature gradient of 0°C to 60°C and optimal pH was determined using several Tris–HCl buffers (pH 7–8.5) at 37°C. Thermal stability was measured by subjecting the protein solution to a temperature of 50°C for 5, 10, and 30 min each, and then the heat-treated samples were assayed under the standard conditions (37°C, pH 7.8). Inactivation of enzymatic activity in the presence of rifampicin 10⁻³ to 1μg/ml was also determined. In all measurements, relative activity was calculated based on activity measured under the standard conditions. All measurements were also performed in E. coli RNA polymerase (EcRNAP) purified using the same procedure for comparison as a mesophilic counterpart.

Specific in vitro transcription assay of RNA polymerase activity. The specific in vitro transcriptional activity of SvRNAP and EcRNAP was measured based on the in vitro production of transcripts from the E. coli RNA-I template, which has σ70-dependent promoter sequences.19 The reaction was carried out under single-round reaction conditions.17 The incubation mixture (42.5 μl) contained Tris–HCl (pH 8.0) 20 mM, MgCl₂ 12 mM, NaCl 12 mM, EDTA 24 μM, 12% glycerol, 2-mercaptoethanol 9.5 mM, bovine serum albumin 1.25 μg, template DNA 5 pmol, and RNA polymerase 1 pmol. The mixture was incubated for 10 min at 37°C to obtain the open promoter complex. Transcription was initiated with the addition of 7.5 μl of a mixture of heparin 2 μg, ATP, GTP, and CTP 1.2 mM each, UTP 0.4 mM, and [α-³²P]-UTP 74 KBq, and the mixture was maintained at 37°C for 5 min. The reaction was stopped by the addition of 50 μl of a stopping buffer (EDTA 40 mM and glycerol 0.4 μg). RNA products were precipitated with ethanol and analyzed using electrophoresis in an 8% polyacrylamide gel containing urea 8 M. Incorporation of radio-labeled UTP was detected using a Bio Image Analyzer BAS2000 (Fuji Film, Tokyo, Japan).

Isolation of the rpoBC genes from S. violacea. In E. coli, rpoBC genes coding for the RNA polymerase β and β' subunits have been shown to exist in an operon.20 First, to isolate rpoBC genes, we constructed a hybridization probe for the rpoB gene. Based on the rifampicin sensitivity of SvRNAP and similar electrophoretic mobility on SDS–gel of the β subunit, the genes coding for the rifampicin-sensitive domain in the rpoB gene from several gram-negative bacteria (E. coli, Pseudomonas putida, Vibrio cholerae, and Yersinia pestis) were aligned. Based on these conserved sequences, two synthetic degenerate oligonucleotide primers, primer 1 (5'-GYTRTCYCATTTATGGAYCARA-3') and primer 2 (5'-CTGACGTTGCAAGTTCACCCCA-3'), were designed and synthesized to amplify part of the rpoB gene from S. violacea. A fragment of approximately 500 bp amplified by PCR and expected to contain part of the rpoB gene was cloned into the pCR2.1 vector and its nucleotide sequence was determined. To clone the complete rpoBC genes, the partial rpoB gene fragment was labeled with digoxigenin (DIG) in PCR as a hybridization probe for plaque hybridization. Chromosomal DNA isolated from S. violacea was partially digested with Sau3AI. These fragments were inserted into the BamHI site of lambda DASH II (Stratagene, La Jolla, CA).
GIGAPACK III XL packaging extracts (Stratagene) according to the manufacturer’s instructions. The DSS12 λ phage library was screened for plaque hybridization with the rpoB probe and a positive clone was obtained. The positive clone containing the rpoB gene was purified by several single-plaque isolation steps. The insert in the λ phage was amplified by long PCR and was subcloned into the pCR-Blunt vector (Invitrogen, Carlsbad, CA). For sequencing of these cloned fragments, the random shotgun sequencing method was used with a DNA sequencer model 377 (Perkin-Elmer/Applied Biosystems, Foster City, CA).21) Assembling and editing of the determined DNA sequences were performed with AutoAssembler Version 2.0 (Perkin-Elmer/Applied Biosystems). GENETYX-MAC version 10.1 from Software Development (Tokyo, Japan) was used for sequence analysis. The nucleotide sequence numbers were deposited in the DDBJ (Mishima, Japan), EMBL (Heidelberg, Germany), and GenBank (Mountain View, CA) databases under accession no. AB045725.

Construction of expression plasmids harboring rpoB and rpoC genes. To construct plasmids for the expression of hexahistidine-tagged derivatives of the β and β′ proteins from S. violacea, PCR was performed to amplify the rpoB and rpoC genes respectively. These amplified genes were ligated into the expression vector pQE80L (Invitrogen) to generate pQSVB and pQSVC containing an N-terminal hexahistidine tag fused to these β and β′ proteins. To overproduce β and β′ proteins, these expression plasmids were transformed into E. coli JM109 cells. For reconstitution experiments on σ70-SvRNAP, we also transformed all other expression plasmids, pQESA (for the σ subunit),12) pQESS (for the σ70 subunit),13) and pQrpoZ (for the η chaperone),14) respectively (Table 1). Overexpression and affinity purification of these proteins were carried out using the procedure described by Nakasone et al.15) Overexpressed and purified proteins were fractionated on 10–15% SDS–PAGE.

Table 1. Details of Plasmids Used in This Study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant properties</th>
<th>Source/reference</th>
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<td>pCR 2.1</td>
<td>PCR cloning vector, AmpK</td>
<td>Purchased from Invitrogen</td>
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<tr>
<td>pCR-Blunt</td>
<td>PCR cloning vector for rpoB containing fragment, AmpK</td>
<td>Purchased from Invitrogen</td>
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<tr>
<td>pQESS</td>
<td>PCR fragment containing rpoD gene ligated into pQE80L</td>
<td>13</td>
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<tr>
<td>pQrpoZ</td>
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<td>This study</td>
</tr>
<tr>
<td>pQSVC</td>
<td>PCR fragment containing rpoC gene ligated into pQE80L</td>
<td>This study</td>
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Results and Discussion

Purification of RNA polymerase from S. violacea cells RNA polymerases were purified from both S. violacea strain DSS12 and E. coli strain W3110. Purified holo- and core enzymes were separated on SDS–PAGE (Fig. 1A). SvRNAP consisted of the α, β, β′, and σ70 subunits (Fig. 1A, lane 1), which showed typical eu-bacterial RNA polymerases.10) The molecular masses of the α, β, β′, and σ70 subunits estimated by their mobility on SDS–PAGE were 30,000, 152,000, 162,000, and 82,000 Da respectively, similar to the values for EcRNAP. From the result of purification of SvRNAP, a single nonspecific protein band at 100 kDa was observed on SDS–gel (Fig. 1A, lanes 1, 2). This band is often observed in the purification process for unknown reasons. The protein was not stripped out from holoenzyme by phosphocellulose column chromatography although the σ70 protein was apparently stripped out (Fig. 1A, lane 2), hence this protein is not likely to be one of the σ proteins. To confirm the template selectivity of purified SvRNAP, we carried out an in vitro transcription assay using an RNA-I template recognized by σ70 containing RNA polymerase (Fig. 1B, upper panel). The predicted 105-base transcript from the gel (Fig. 1B, lower panel), suggesting that purified SvRNAP was σ70 containing RNA polymerase. In the case of E. coli, the core enzymes lacking the σ subunit are separated from the holoenzyme upon MonoQ anion-exchange chromatography.17) But we obtained low yields of the relevant core enzymes of SvRNAP, suggesting that the applied sample after Superose 6 gel filtration contained high yields of holoenzyme of SvRNAP. To obtain sufficient core RNA polymerase for several transcription analyses, phosphocellulose column chromatography was performed. As shown in Fig. 1A, the σ subunit was effectively stripped out of the holoenzyme of SvRNAP (Fig. 1A, lane 2). This method can contribute to elucidation of the transcription mechanisms controlled by RNA polymerase containing alternative σ subunits in vitro.

Characterization of S. violacea RNA polymerase

The activity of purified SvRNAP was measured using a nonspecific DNA template to characterize the general properties of the enzyme compared with the activity of EcRNAP. The activity of EcRNAP was also measured as a mesophilic counterpart. The activity of RNA polymerases from both strains showed a maximum at 37°C (Fig. 2A). There was a discrepancy between the optimum temperature for growth and that for transcrip-
Fig. 1. SDS–PAGE Analysis and Results of in Vitro Transcription of Purified S. violacea RNA Polymerase.
(A) SDS–PAGE of purified S. violacea RNA polymerase on 10% polyacrylamide gel. Lane 1, purified S. violacea RNA polymerase holoenzyme; lane 2, purified S. violacea RNA polymerase core enzyme after phosphocellulose column chromatography; lane 3, E. coli RNA polymerase holoenzyme after MonoQ anion-exchange chromatography. Positions of RNA polymerase subunits α, β, β′, and σ70 are indicated by arrows. Molecular sizes are given in kDa to the left. (B) Structure of DNA template and expected RNA product (upper panel). The arrow with the broken line indicates an accurate transcript directed by the truncated DNA template. #1 indicates the transcriptional initiation site, the consensus promoter sequences (−35 and −10) are boxed in black, and core promoter regions are underlined. RNA products of runoff transcription using RNA polymerases from S. violacea (lane 1) and E. coli (lane 2) were analyzed with 8% polyacrylamide gel electrophoresis and autoradiography (lower panel). The sizes of RNA products are indicated to the right.

Fig. 2. Characterization of Basic Properties of S. violacea RNA Polymerase.
(A) Effects of temperature on enzyme activity. (B) Effects of pH on enzyme activity. (C) Effects of temperature on enzyme stability. SvRNAP and EcRNAP were heated for the indicated times at 50°C. (D) Effects of the antibiotic rifampicin on enzyme activity. Relative activity of 100% means the value of activity (cpm μg⁻¹ protein min⁻¹) assayed under standard conditions (37°C, pH 7.8).
tional activity. Therefore, *S. violacea* might utilize the low transcrip-
tional activity of the enzyme under low-
temperature conditions in the deep sea, or factors that activate SvRNAP might be present *in vivo*. The optimum pH for the activity of these enzymes was almost identical (pH 7.8 at 37 °C) (Fig. 2B). As shown in Fig. 2C, SvRNAP was more temperature sensitive than EcRNAP at 50 °C. Based on these results, the thermal stability of the enzymes from the two species likely depends on their habitats, in contrast with the results concerning optimum temperature. In a previous study, we found that SvRNAP maintained higher activity than EcRNAP after high-pressure treatment. A kinetic study of the enzyme under high-pressure conditions is in progress. It has been found that the antibiotic rifampicin inhibits the activity of eubacterial RNA polymerase by interacting with the β subunit in RNA polymerase. As shown in Fig. 2D, rifampicin 0.02 μg/ml inhibited 50% of the activity of SvRNAP, whereas EcRNAP activity was inhibited by 20%. This suggests that the rifampicin-sensitive sites in the β subunit have been evolutionarily conserved. Hence to isolate rpoBC genes from *S. violacea* cells we decided to construct a DIG-labeled probe based on the alignment of rifampicin-sensitive regions in rpoB genes from several gram-negative bacteria.

**Structural analysis of the rpoBC genes of *S. violacea***

By screening the λ phage library, a fragment containing rpoB followed by rpoC was obtained (Fig. 3). Sequence analysis showed that the gene organization and orientation of the fragment containing rpoBC genes are identical with those in *E. coli*. Therefore, rpoBC genes are likely to be transcribed as an operon, as reported for *E. coli*. The open reading frames of the rpoB and rpoC genes consist of 4,032 and 4,230 bp respectively. The rpoB gene encodes a protein consisting of 1,343 amino acid residues with a molecular mass of 150,102 Da (Fig. 4A), whereas the rpoC gene encodes a protein consisting of 1,409 amino acid residues with a molecular mass of 155,749 Da (Fig. 4B). Significant homology was found when comparing the RpoBC proteins of *S. violacea* with those of *E. coli* (rpoB, 79.5%; rpoC, 78.6%), *P. putida* (rpoB, 69.1%; rpoC, 71.0%), and *Bacillus subtilis* (rpoB, 44.2%; rpoC, 46.2%). Evolutionarily conserved regions were also found to exist over the predicted amino acid sequences (Fig. 4A). As predicted by the results of enzymatic analysis, the domains responsible for rifampicin resistance (RifR) in the β subunit, RifR clusters I to III, were highly conserved. In RifR cluster I, a single amino acid difference (residue number 525 [V] with 524 [I]) was found between *S. violacea* and *E. coli*. This single substitution perhaps induced greater sensitivity of SvRNAP to rifampicin than that of *E. coli*. Wild-type *S. violacea* exhibits rifampicin-sensitive growth on Marine Broth 2216 medium plates in the presence of approximately rifampicin 10 μg/ml. Hence the *S. violacea* RifR mutant was screened in our laboratory for genetic studies (data not shown). This mutant may have mutations in RifR clusters in the rpoB gene.

In β proteins, the zinc finger-like motif and DNA polymerase motif were very similar, and amino acid residues in which substitutions resulted in microcin J25 (MccJ25) resistance as reported in *E. coli* were completely conserved between those species (Fig. 4B, lower panel). These results in combination with the molecular characterizations of the σ, σ70, and ω subunits suggest that SvRNAP works similar reaction mechanisms in transcription process with that in *E. coli*.

**Overproduction and affinity purification of all subunit proteins for recombinant RNA polymerase**

Two recombinant expression plasmids harboring the *S. violacea* rpoB and rpoC genes were constructed (pQSVB and pQSVC) and transformed into *E. coli* JM109 cells. Upon induction, the recombinant β and β' proteins were overexpressed, as shown in lane 3 in Fig. 5B and C. Hexahistidine-tagged β and β' proteins were purified from insoluble fractions by a simple chromatographic step, and their molecular masses were determined to be 152 and 162 kDa respectively by SDS-PAGE (lane 4 in Fig. 5B and C). In addition, as shown in Fig. 5A, D, and E, we also overexpressed and purified all other components (α, σ70, and ω subunits) for σ70-RNA polymerase. Using these proteins, we succeeded in in vitro reconstitution of SvRNAP at the mini-scale level, following the procedure described by Borukhov and Goldfarb. The optimal subunit molar ratio for

![Gene Organization of rpoBC Genes in S. violacea](image)

**Fig. 3.** Gene Organization of rpoBC Genes in *S. violacea*.

The similarity of the predicted amino acid sequences encoded by the rpoB and rpoC genes to sequences of polypeptides of *E. coli*, *P. putida*, and *B. subtilis* are given.
reconstitution was also determined to be 2:4:8:1 (αβ:β':α') using a poly(dA-dT)-poly(dA-dT) template (Table 2). Large-scale reconstitution methods for RNA polymerase of this piezophile are currently being investigated. Using this overexpression method, we were able to obtain large amounts of SvRNAP in a single day and could induce mutations in regions where it has been thought difficult to induce them with antibiotic treatment for various forms of functional analysis. Knowledge of the transcription, protein synthesis, membrane lipid composition, and metabolism of deep-sea microbes has slowly accumulated. In combination with the reports in the literature, our approach using reconstituted RNA polymerase should reveal further mechanistic properties of enzymes in piezophiles under low-temperature, high-pressure conditions.

Table 2. Relation of the Subunit Molar Ratio at Reconstitution Process and Relevant RNA Polymerase Activity

<table>
<thead>
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<th>Molar ratio</th>
<th>Activitya (%)</th>
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<tr>
<td>2:1:1:1</td>
<td>20</td>
</tr>
<tr>
<td>2:8:4:1</td>
<td>28</td>
</tr>
<tr>
<td>2:4:8:1</td>
<td>100</td>
</tr>
<tr>
<td>2:8:8:1</td>
<td>95</td>
</tr>
</tbody>
</table>

aActivity of 100% means the value of activity (cpm μg⁻¹ protein min⁻¹) assayed under standard conditions (37°C, pH 7.8).

References

4) Kato, C., Sato, T., and Horikoshi, K., Isolation and properties of barophilic and barotolerant bacteria from...


