Extremophiles (2004) 8:367–375
DOI 10.1007/s00792-004-0397-0

ORIGINAL PAPER

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Differential pressure resistance in the activity of RNA polymerase isolated from *Shewanella violacea* and *Escherichia coli*

Received: 17 February 2004 / Accepted: 20 April 2004 / Published online: 7 July 2004
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Abstract RNA polymerase was purified from the piezophile *Shewanella violacea* DSS12, and the transcriptional activity after pressure treatment was compared with that of the mesophile *Escherichia coli*. Application of pressure at 100 MPa for 30 min reduced the *E. coli* RNA polymerase activity to 60% of the activity at atmospheric pressure, whereas the *S. violacea* RNA polymerase maintained full activity, indicating that the *S. violacea* RNA polymerase is more stable than its *E. coli* counterpart. This result was supported by the analysis of the strength of subunit interactions of the enzyme from both species, using a high-pressure electrophoresis apparatus, which showed that a pressure of 140 MPa caused dissociation of *E. coli* RNA polymerase but not that of *S. violacea* RNA polymerase. On the other hand, the core enzyme of *S. violacea* RNA polymerase, which lacked the σ^{70} factor, was dissociated at 140 MPa. These results suggest that the σ^{70} factor is required for stabilization of *S. violacea* RNA polymerase under high-pressure conditions. In this paper, we provide in vitro evidence for piezoadaptation at the transcriptional level, using purified RNA polymerase from cells of *S. violacea* and *E. coli*.

Keywords High-pressure electrophoresis apparatus · High-pressure resistance · Piezophile · RNA polymerase · *Shewanella violacea*

Communicated by K. Horikoshi

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Introduction

High hydrostatic pressure is known to reduce microbial growth and viability, depending on the magnitude of applied pressure. Deep-sea microorganisms have developed the ability to grow under extreme pressure conditions. Numerous deep-sea microorganisms have been isolated and identified taxonomically, although only a few species have been investigated to elucidate piezoadaptation at the molecular level (reviewed in Nakasone et al. 1998; Abe et al. 1999; Bartlett 2002; Nakasone et al. 2002). The first reported study of molecular adaptation at the transcriptional level was in the moderately piezophilic bacterium *Photobacterium profundum* strain SS9, for which the optimal growth pressure is 28 MPa (Bartlett et al. 1989). It has been shown that the *ompH* and *ompL* genes encoding putative nutrient transporters in the outer membrane are regulated under high-pressure conditions. *ompH* is induced by increasing hydrostatic pressure, whereas *ompL* is reduced by increasing pressure in a manner dependent on ToxR-ToxS, putative piezosensor proteins (Welch and Bartlett 1998).

The piezophilic deep-sea bacterium *Shewanella violacea* strain DSS12, isolated from the Ryukyu Trench (depth: 5,110 m), grows in a wide range of pressures from 0.1–70 MPa, with optimal growth at 30 MPa

(Kato et al. 1995; Nogi et al. 1998). Pressure-regulated operons, such as the open reading frame (ORF)1-ORF2, *glnA*, and *rpoA* have been identified in this strain, and the promoter was activated under high-pressure conditions (Kato et al. 1997; Ikegami et al. 2000; Nakasone et al. 2000a, 2000b). RNA polymerase is one of the most important oligomeric enzymes in transcription, is composed of the α, β, β', and one of several σ subunits, and governs the selectivity of the promoter gene sequence (Yura and Ishihama 1979). The subunits of *Escherichia coli* RNA polymerase (EcRNAP) have been shown to be dissociated by high pressure (Erijman and Clegg 1996). This led us to investigate the stability of the RNA polymerase of *S. violacea* (SvRNAP) under high-pressure conditions because this organism is capable of growth at high pressure. In the present investigation, we analyzed the transcriptional activity of RNA polymerases after high-pressure treatment and compared the strength of subunit associations between EcRNAP and SvRNAP, using a high-pressure electrophoresis apparatus (HPEA).

Fig. 1A, B Diagram of the high-pressure electrophoresis apparatus (HPEA). **A** High-pressure electrophoresis chamber for HPEA. *a* Connection to the power supply (anode), *b* connection to the power supply (cathode), *c* buffers, *d* silicone oil KF-96-1.5CS, *e* glass microcapillary tube, *f* o-ring to partition a space into upper and lower spaces, *g* connection to a high-pressure pump. **B** Photograph of the HPEA. *1* High-pressure electrophoresis chamber, *2* high-pressure hand pump, *3* pressure gauge

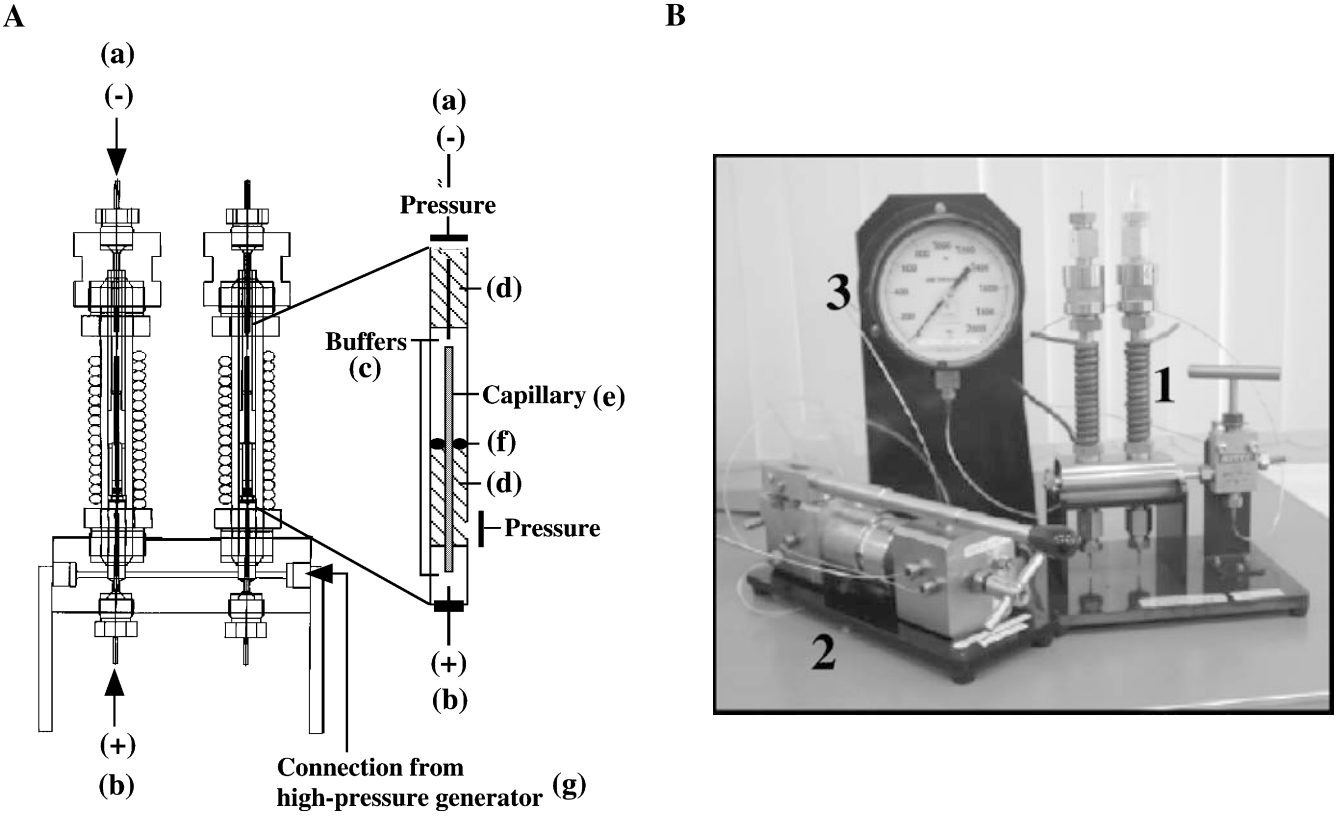
Materials and methods

Bacterial strains and culture conditions

Shewanella violacea strain DSS12 (Nogi et al. 1998) and *Escherichia coli* strain W3110 were used in this study. *S. violacea* cells were grown at atmospheric pressure and 8°C with vigorous shaking in 15 l of Marine Broth 2216 medium (Difco, Sparks, Md., USA). Cells of *E. coli* strain W3110 were grown at 37°C with vigorous shaking in 15 l 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

SvRNAP and EcRNAP were purified from cells of *S. violacea* and *E. coli*, respectively, as described for that from *E. coli* by Burgess and Jendrisak (1975), with some modification. Fifteen grams of the cells were used for the purification of RNA polymerase. The enzyme fraction obtained by Polymin P precipitation and salt extraction was applied to a HiTrap Heparin column (Amersham Pharmacia Biotech, Piscataway, N.J., USA) (Kumar and Chatterji 1988). The resulting fractions containing RNA polymerase were 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 Pharmacia Biotech) to separate the holo- and core enzymes of RNA polymerase (Hager et al. 1990). The holoenzymes were dialyzed against a stock buffer [10 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 0.2 M KCl, 50% glycerol] and stored at −80°C until use.

Nonspecific in vitro transcription assay of RNA polymerase activity

The holoenzyme was placed in a tube, and the tube was sealed with parafilm. The tube was placed in a high-pressure vessel and subjected to the desired pressures for 30 min at 25°C. After decompression, the remaining activity was measured with the transcriptional assay at atmospheric pressure, using a nonspecific DNA template. The assay solution (250 µl) consisted of 40 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT, 0.2 mM ATP, 0.2 mM [^{3}H]-UTP (Amersham Pharmacia Biotech), 1.2 µg of poly (dA-dT)·poly (dA-dT) (Amersham Pharmacia Biotech), and 1 pmol of RNA polymerase (Fujita and Amemura 1992). The mixture was incubated at 37°C for 10 min, and RNA products were precipitated by the addition of 3 ml of 5% (w/v) trichloroacetic acid, followed by incubation for 15 min at 4°C. The acid-precipitable material was filtered through a Whatman GF/C filter, washed with 10 ml cold 3% (w/v) trichloroacetic acid, and dried. The radioactivity was measured using a liquid scintillation counter. The relative activity was calculated with respect to the activity in samples maintained at atmospheric pressure.

Measurement of activities of pressurized purified RNA polymerase using specific in vitro transcription

RNA polymerases of the two strains were pressurized for 30 min at 25°C. After decompression, the specific in vitro transcriptional activity of SvRNAP and EcRNAP was measured based on the in vitro production of transcripts from the *S. violacea rpoA* template, which has two σ^{70}-dependent promoter sequences (Nakasone et al. 2000b). The reaction was carried out under single-round reaction conditions (Hager et al. 1990). The incubation mixture (42.5 µl) contained 20 mM Tris-HCl (pH 8.0), 12 mM $MgCl_2$, 12 mM NaCl, 24 µM EDTA, 12% glycerol, 9.5 mM 2-mercaptoethanol, 1.25 µg bovine serum albumin, 5 pmol template DNA, and 1 pmol pressurized RNA polymerase. 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 2 µg heparin; 1.2 mM each ATP, GTP, and CTP; 0.4 mM UTP; and 74 KBq [α–^{32}P]-UTP, 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 (40 mM EDTA and 0.4 µg glycogen; Fujita and Amemura 1992). RNA products were precipitated with ethanol and analyzed by electrophoresis in an 8% polyacrylamide gel containing 8 M urea. Incorporation of radiolabeled UTP was detected using the Bio Image Analyzer BAS2000 (Fuji Film, Tokyo, Japan).

2-D electrophoresis using the HPEA

The HPEA was developed with a modification of the method previously reported by Erijman and Clegg (1996) (Fig. 1A, B). The cathode and anode were attached separately above and below the sample chamber. [Pressure of up to 200 MPa can be applied with silicon oil KF-96-1.5CS (Shin-Etsu Chemical, Tokyo, Japan), using a hand pump within 1 min.] First, native polyacrylamide gel electrophoresis (PAGE) was carried out in capillary glass tubes 75 mm in length and 0.85 mm in inner diameter (Nichiden Rika Glass Co., Ltd., Kobe, Japan) under pressure conditions, using the HPEA. The composition of native acrylamide gel was 7.5% acrylamide (3.3% *N*,*N*′-methylenbisacrylamid), 375 mM Tris-HCl (pH 8.8). Two micrograms of RNA polymerase sample were mixed with an equal volume of sample buffer (10 mM Tris-HCl, pH 6.8, 20% glycerol) and loaded onto the top of the gel with a microsyringe. The sample was kept at the desired pressure for 30 min for equilibration. Electrophoresis was carried out at the constant voltage of 350 V for 1 h, using running buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine). After decompression, the gel was removed and equilibrated in sodium dodecyl sulfate (SDS) buffer (1% SDS, 1% 2-mercaptethanol, 10 mM Tris-HCl, pH 6.8, 20% glycerol) for 10 min. The gel was overlaid on the SDS gel and subjected to SDS-PAGE, using 10% polyacrylamide gel at atmospheric pressure. Proteins were visualized by silver staining using a kit (Silver Stain II Kit, WAKO, Osaka, Japan).

Prediction of the 3-D structure of the σ^{70} subunit of *S. violacea*

The 3-D structures of the σ^{70} subunit of *S. violacea* and *S. oneidensis* MR-1 (Heidelberg et al. 2002) were predicted using the 3D-JIGSAW program (version 2.0, Cancer Research, London, UK). The crystal structure of the *E. coli* σ^{70} subunit was used as a template (Malhotra et al. 1996).

Results

Purification of RNA polymerases

RNA polymerases were purified from *Shewanella violacea* strain DSS12 and *Escherichia coli* strain W3110. Proteins obtained at each step of purification were separated on SDS-PAGE (Fig. 2). SvRNAP consists of the α, β, β', and σ^{70} subunits (Fig. 2, lane 5; Kawano et al.

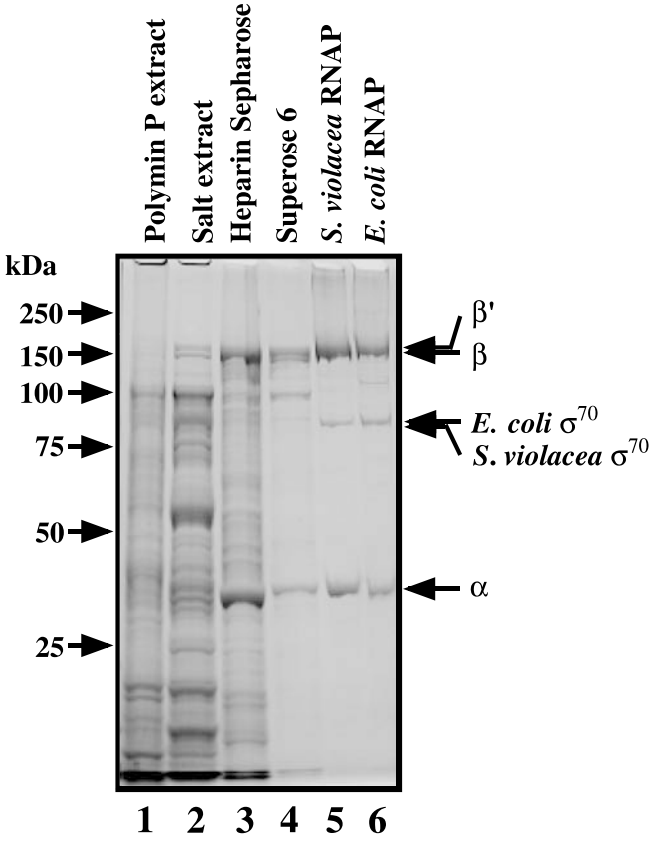


Fig. 2 Purification of *Shewanella violacea* RNA polymerase (SvRNAP). Proteins during purification were separated on sodium dodecyl sulfate (SDS)-PAGE (10% polyacrylamide gel). *Lane 1* Polymin P extract, *lane 2* salt extract, *lane 3* after heparin Sepharose, *lane 4* after Superose 6, *lane 5* SvRNAP after MonoQ, *lane 6 Escherichia coli* RNAP. Positions of RNA polymerase subunits α, β, β′, and σ^{70} are indicated by *arrows*

2002), like typical eubacterial RNA polymerases (Yura and Ishihama 1979). The molecular masses of the α, β, β′, and σ^{70} subunits estimated by mobility on SDS-PAGE were 30,000, 152,000, 162,000, and 82,000 Da, respectively, which were similar to EcRNAP. Since the RNA polymerases from both strains were maximally active at 37°C (data not shown), subsequent transcription assays were carried out at this temperature. We have confirmed that SvRNAP can recognize the σ^{70} promoter in the *E. coli* RNA I template by in vitro transcription assay (Kawano et al. 2002), suggesting that SvRNAP is a homologue of σ^{70}-type EcRNAP.

Effects of high hydrostatic pressure on RNA polymerase activity

We measured the activity of RNA polymerases remaining after high-pressure treatment. The transcriptional activity was analyzed by measuring the incorporation of [^{3}H]-UTP into transcripts, using poly (dA-dT)·poly (dA-dT) as a template DNA. As shown in Fig. 3, both enzymes were stable in the pressure range from 0.1–50 MPa. After application of pressure of

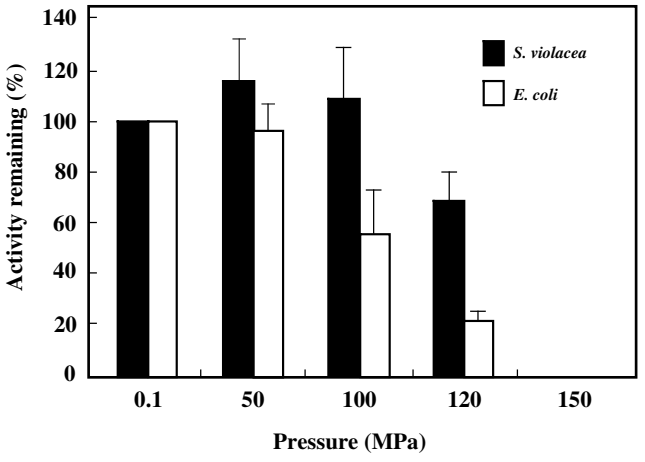


Fig. 3 Effects of high hydrostatic pressure on transcriptional activity of RNA polymerase of *S. violacea* and *E. coli* using a nonspecific DNA template. The nonspecific in vitro transcription assay was carried out using poly (dA-dT)·poly (dA-dT) as a template. The relative activity of RNA polymerase is indicated with respect to that obtained from a sample maintained at the respective pressures. Data are expressed as mean values ± SD from three independent experiments

100 MPa, SvRNAP maintained full activity, although EcRNAP had only 60% of the activity at 0.1 MPa. After application of pressure of 120 MPa, the activities of the two enzymes decreased, but SvRNAP maintained a higher level of activity compared with EcRNAP. Both enzymes lost all activity at 150 MPa. The pressure dependency on inactivation of EcRNAP was very similar to that reported by Erijman and Clegg (1995). These results indicate that SvRNAP is more stable under high-pressure conditions than EcRNAP.

We next carried out the specific in vitro transcription assay, using the *S. violacea rpoA* promoter. This promoter has two σ^{70} recognition sites, and thereby two mRNAs are transcribed initiating at positions 1 and 2 (Fig. 4A; Nakasone et al. 2000b). Pressure of 100 MPa did not affect the transcriptional activity initiated at position 1 in *S. violacea*, although this pressure significantly decreased the activity of EcRNAP. In contrast, pressure of 100 MPa reduced transcriptional activity initiated at position 2 in both strains, but SvRNAP was more stable than EcRNAP. The activity of transcription from position 2 of SvRNAP decreased to approximately 50% with high-pressure treatment at 100 MPa, indicating that the RNA polymerase molecules lost the ability to recognize the promoter initiating at position 2 at high pressure. These results suggest that SvRNAP may have adapted to a high-pressure environment through the stabilization of its subunit associations.

Comparison of strength of subunit–subunit interactions in the HPEA

To investigate the effect of increasing hydrostatic pressure on the stability of subunit formation of RNA polymerases, we performed electrophoresis under high-

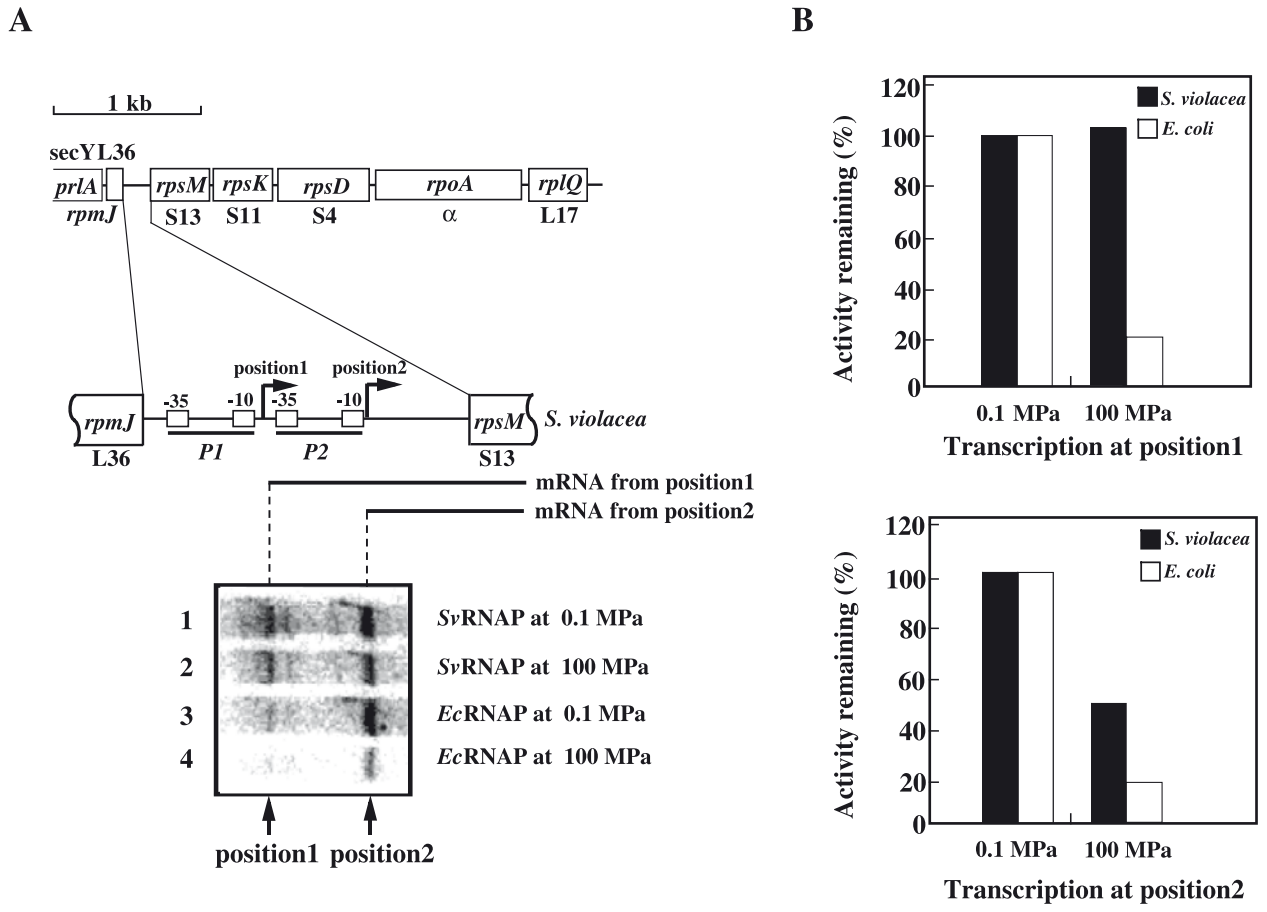


Fig. 4A, B Effects of high hydrostatic pressure on transcriptional activity of RNA polymerase of *S. violacea* and *E. coli*, using the specific DNA template. **A** Structure of *S. violacea rpoA* operon (*upper panel*). Transcription is initiated at positions 1 and 2. Consensus promoter sequences (−35 and −10, *boxes*) and core promoter regions (P1 and P2, *overlined*). Visualization of transcripts using the Bio Image Analyzer BAS2000 (*lower panel*). Transcription assay was carried out after pressure treatment at 0.1 or 100 MPa for 30 min, followed by electrophoresis. **B** Quantitative representation of activity of RNA polymerase remaining after high-pressure treatment initiated at positions 1 (*upper panel*) and 2 (*lower panel*). Data are shown as values relative to the activity obtained without high-pressure treatment

pressure conditions, using the HPEA. The holoenzymes of *E. coli* and *S. violacea* were stable during electrophoresis at atmospheric pressure (Fig. 5A, B, upper panels). Although pressure of 100 MPa reduced the transcriptional activity of EcRNAP (Figs. 3, 4), EcRNAP was not dissociated at this pressure (data not shown), indicating that high pressure impairs the enzymatic activity without a loss of subunit association as far as analyzed using the HPEA. However, at pressure of 140 MPa, the holoenzyme of EcRNAP became dissociated (Fig. 5A, lower panel). In contrast, the holoenzyme of SvRNAP was stable and still formed a complex under the same pressure conditions (Fig. 5B, lower panel). These results clearly indicate that SvRNAP is more stable than EcRNAP at the level of the association of the protein molecules at high pressure.

We next analyzed the role of the σ^{70} subunit in stabilization of the holoenzyme of SvRNAP at high pressure. At atmospheric pressure, both the holoenzyme (consisting of the α, β, β', and σ^{70} subunits) and the core enzyme (consisting of the α, β and β' subunits) were stable during electrophoresis (Fig. 6A, B, upper panels). Although the holoenzyme was stable at high pressure of 140 MPa (Fig. 6A, lower panel), the core enzyme was dissociated under the same pressure conditions (Fig. 6B, lower panel). This result suggests that the σ^{70} subunit of *S. violacea* plays a role in stabilization of the protein under high-pressure conditions.

We next predicted the 3-D structure of the σ^{70} subunit of *S. violacea*, using the method of homology modeling based on the crystal structure of the σ^{70} subunit from *E. coli*. The predicted structure of the σ^{70} subunit of a mesophilic counterpart, *S. oneidensis*, was used for comparison. The sequence identity of the σ^{70} subunit is 78% (*S. violacea* vs *S. oneidensis*), 74% (*S. violacea* vs *E. coli*) and 74% (*S. oneidensis* vs *E. coli*), so the predicted structures are very similar each other (Fig. 7). In particular, the domains for DNA melting, DNA recognition, and the core-binding site appeared to be highly conserved. Interestingly, the loop-structure domain was unique in each. The loop structure of the *E. coli* σ^{70} subunit (residue number 192–211) is rich in aspartates and glutamic acids. The relevant domain of the *S. violacea* σ^{70}, composed of two β sheets starting at

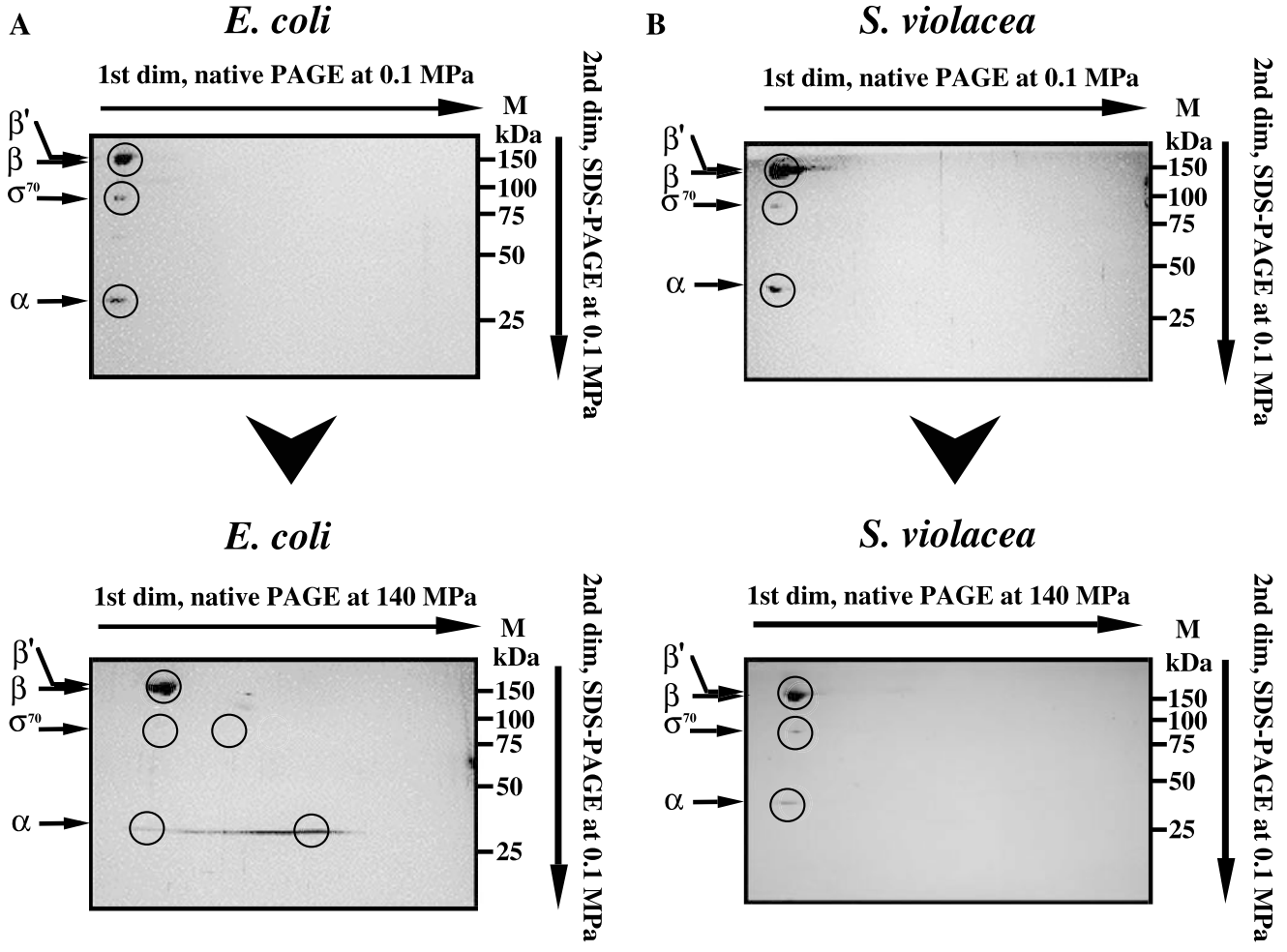


Fig. 5A, B Effects of high hydrostatic pressure on subunit association in RNA polymerase in *E. coli* (**A**) and *S. violacea* (**B**). Native PAGE was performed at 0.1 MPa (*upper panels*) or 140 MPa (*lower panels*), followed by SDS-PAGE at 0.1 MPa. Proteins were visualized by silver staining. Each subunit of RNA polymerase is shown by an *open circle*. *M* Size markers of molecular weight, *1st dim* 1-D separation, *2nd dim* 2-D separation

residue number 191, and that of the *S. oneidensis* σ^{70} was a loop, followed by an α helix starting at residue number 191. The acidic loop in the *E. coli* σ^{70} subunit within or near the DNA-binding cleft is assumed to help in the inhibition of the interaction with DNA and also to repel the negatively charged DNA electrostatically. σ^{70} subunits in several bacteria have lost the loop structure (Malhotra et al. 1996), and thus, this domain could not be essential under normal growth conditions. It is likely that the β-sheet domain may be required for stabilization of the holoenzyme of SvRNAP under high-pressure conditions.

Discussion

We have been investigating gene expression in the piezophile *Shewanella violacea* under high-pressure conditions (Kato et al. 1997; Ikegami et al. 2000; Nakasone et al. 2000a, b). In this study, we compared the stability of subunit association of RNA polymerase between *S. violacea* and *Escherichia coli*. SvRNAP was more stable than EcRNAP in terms of activity remaining after pressure treatment and subunit association at high pressure as analyzed using the HPEA.

As has been well documented, denaturation of monomeric proteins usually requires pressure of a few hundred megaPascals, whereas pressure-induced dissociation of multimeric proteins is observed within the biologically relevant pressure range of less than 100 MPa, raising questions on the possible mechanism underlying growth inhibition of microorganisms under high-pressure conditions (Silva and Weber 1993; Gross and Jaenicke 1994; Abe et al. 1999). The effects of hydrostatic pressure on protein quaternary structure were compared in the recombinant single-stranded DNA-binding (SSB) protein derived from piezophilic and piezosensitive *Shewanella* strains (Chilukuri and Bartlett 1997; Chilukuri et al. 2002). Pressure-induced dissociation of SSB proteins analyzed using fluorescence anisotropy revealed that SSB protein from a piezophilic *Shewanella* strain was stable compared with those from its piezosensitive counterparts in terms of volume

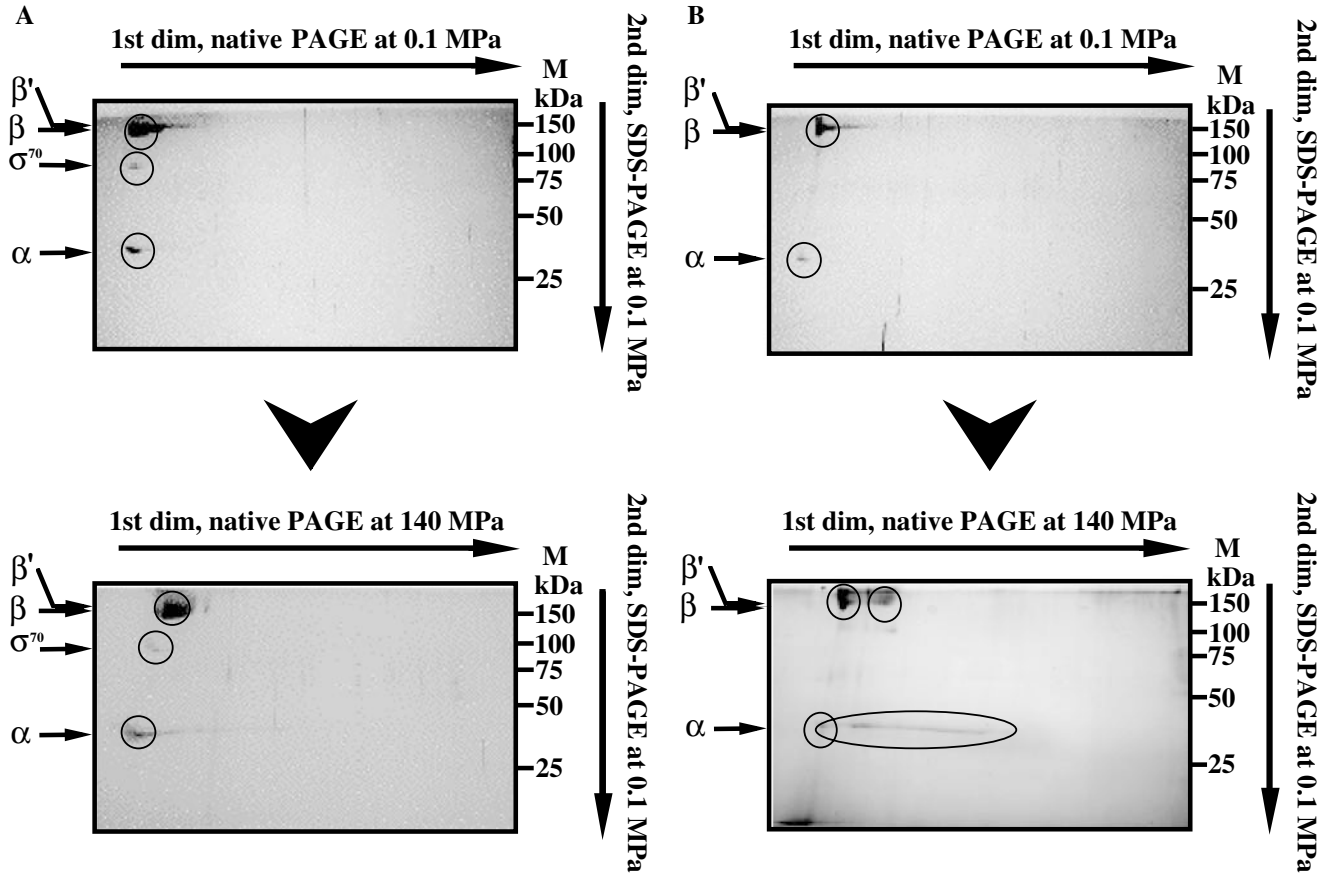


Fig. 6A, B Effects of high hydrostatic pressure on subunit association in the holoenzyme (**A**) and the core enzyme (**B**) in SvRNAP. Native PAGE was performed at 0.1 MPa (*upper panels*) or 140 MPa (*lower panels*), followed by SDS-PAGE at 0.1 MPa. Proteins were visualized by silver staining. Each subunit of RNA polymerase is shown by an *open circle*. *M* Size markers of molecular weight, *1st dim* 1-D separation, *2nd dim* 2-D separation

changes of association, dissociation constants at atmospheric pressure, and standard free energies of association for SSB proteins. Within the N- and C-terminal regions, *Shewanella* SSB proteins display a remarkable similarity in amino acid composition, whereas striking differences between the primary structures are observed within the central variable region (Chilukuri and Bartlett 1997). A reduction in helix-breaking (proline) and helix-destabilizing (glycine) residues is assumed to reduce the flexibility of SSB protein of the piezophile, causing low compressibility and thereby stabilizing the protein under high-pressure conditions. According to the sequences of RNA polymerase subunits between *S. violacea* and *E. coli*, the identity between each subunit was 81% (α), 79% (β), 74% (β'), and 74% (σ^{70}). In contrast to the observation in SSB proteins, SvRNAP contains slightly more abundant glycine and proline compared with EcRNAP, suggesting that protein flexibility may not be a determining factor in the piezotolerance of SvRNAP, but rather that some other critical amino acid residues could be responsible for the piezotolerance.

As evidenced by the presented data, both EcRNAP and SvRNAP still maintained the transcriptional activity at 50 and 100 MPa, respectively (Fig. 3), at which the growth was significantly impaired (Kato et al. 1995). In addition, SvRNAP was highly stable at a pressure of 140 MPa, at which the growth of *S. violacea* completely arrested. We have no evidence to explain the discrepancy observed between intact cells and in vitro transcriptional assay. As indicated in the case of the SSB protein by Chilukuri et al. (2002), concentration of the RNA polymerase protein during in vitro assay is likely to affect the sensitivity to increasing hydrostatic pressure. It is also likely that there may be another factor that limits growth at high pressure in the living cell.

One of our striking results is that the *S. violacea* σ^{70} subunit enhanced the stabilization of RNA polymerase at high pressure. The σ subunit is known to change the quaternary structure of EcRNAP (Wu et al. 1976; Greiner et al. 1996). It is likely that the *S. violacea* σ^{70} subunit stabilizes the core enzyme through alteration of the quaternary structure of RNA polymerase, resulting in piezotolerance. In this context, the predicted β-sheet domain, which is not observed in the *E. coli* and *S. oneidensis* σ^{70} subunits, may have a role in stabilization of RNA polymerase at high pressure. Further experimentation is required to determine the significance of the β-sheet domain by comparing the structure with that of other mesophilic *Shewanella* strains and by analysis of the effects of mutations within the domain on the

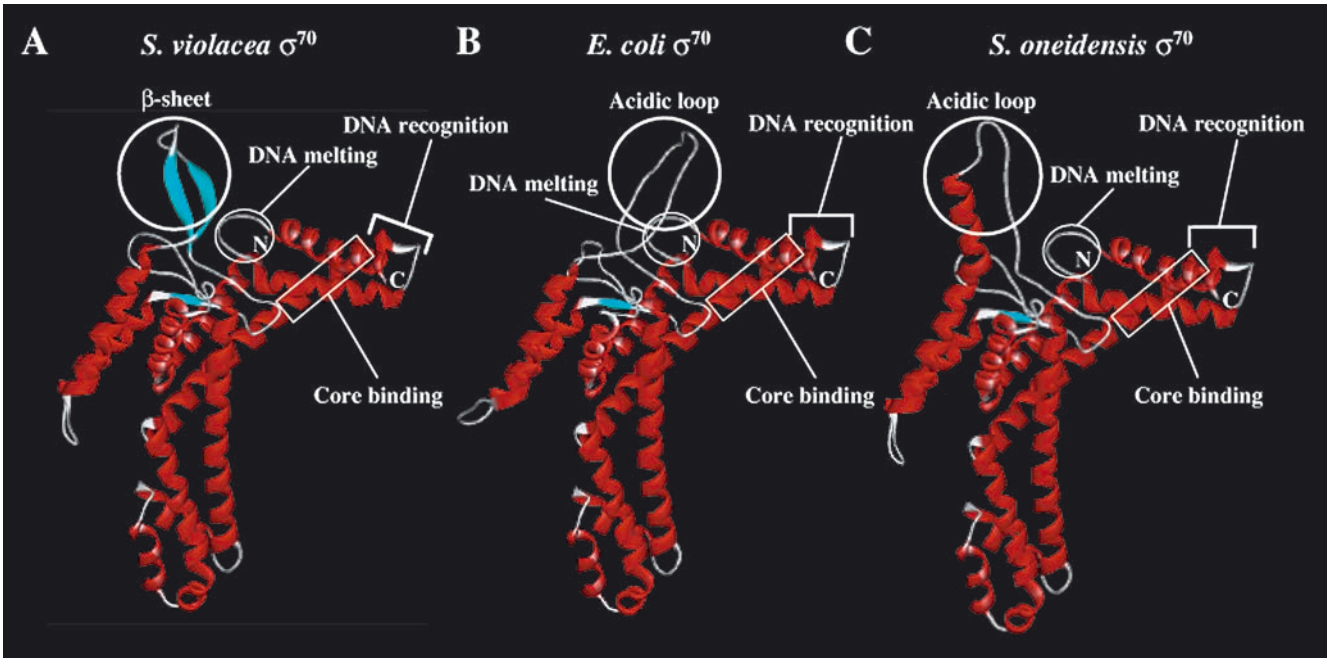


Fig. 7A–C Predicted 3-D structures of the σ^{70} subunit of *S. violacea* and *S. oneidensis*. **A** 3-D structure of the σ^{70} subunit of *S. violacea* (residues 114–447) predicted by the 3D-JIGSAW program (version 2.0), using the crystal structure of *E. coli* σ^{70} subunit as a template. **B** The structure of the *E. coli* σ^{70} subunit (residues 114–448) was also visualized using the same program as a comparison. **C** The structure of the *S. oneidensis* σ^{70} subunit (residues 114–449) was predicted using the same program as a mesophilic *Shewanella*. The domains for DNA melting, DNA recognition, core binding, loop structure, and two β-sheet structures are indicated

piezotolerance of RNA polymerase in terms of transcriptional activity and subunit association. For investigations of the molecular adaptation of proteins to high hydrostatic pressure, the HPEA is a powerful tool in combination with techniques based on molecular biology and bioinformatics.

Acknowledgements We thank Tetsuya Miwa of JAMSTEC for suggestions on the construction of the HPEA. This work was supported in part by a Grant-in-Aid for Scientific Research, Scientific Research on Priority Areas, Single-Cell Molecular Technology (area number: 736) and in part by a Grant-in-Aid for Young Scientists B-15780065 from the Japan Society for the Promotion of Science (F. Abe).

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