Discoveries of Deep-Sea Piezophiles, and Their Pressure Adapted Enzymes

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Abstract
The psychrophilic, moderately piezophilic bacterium *Shewanella violacea* strain DSS12 is a deep-sea isolate from a sediment sample collected at the Ryukyu Trench (depth: 5,110 m), which grows optimally at 30 MPa and 8°C but also grows at atmospheric pressure (0.1 MPa) and 8°C. We have examined this strain to elucidate the molecular basis for gene and protein regulations at different pressure conditions because this strain is useful as a model bacterium for comparing the various features of bacterial physiology under pressure conditions. Proteins, from such deep-sea adapted piezophiles, could be active under high-pressure conditions in general. Actually atmospheric pressure adapted proteins can be inactive under higher-pressure conditions. For bio-processing under pressure, people are looking for pressure-tolerant enzymes, thus, “piezophilic proteins” would be focus on such industrial applications. In the case of respiratory proteins, cell divisional protein FtsZ, RNA polymerase subunit structure, and dihydrofolate reductase (DHFR), piezophilic proteins were unique for adaptation to high-pressure environment and some of them were more stable and active under higher pressure conditions.

Keywords: piezophiles, high-pressure, deep-sea, respiratory systems, enzymes

1. Introduction

In 1996 March, we have succeeded to obtain a sediment sample from the world deepest point, Mariana Trench Challenger Deep at a depth of 10,898 m, using the unmanned submersible “Kaiko” system [1]. It was a first time to get such deepest sample in the world. Extremely piezophilic bacteria, *Moritella yayanosii* strain DB21MT-5 and *Shewanella benthica* strain DB21MT-2, were isolated from the sediment sample, and those piezophiles could grow only over than 60 MPa pressure conditions [2,3]. This discovery suggested that the functional proteins under high-pressure could be involved in the piezophilic microorganisms, and those proteins might be useful for bio-processing applications under pressure conditions.

Generally, deep-sea piezophilic bacteria have several pressure-sensing gene expression mechanisms. We have analyzed about the pressure regulated gene expressions using the model piezophile, *Shewanella violacea* strain DSS12 isolated from the Ryukyu Trench at a depth of 5,110 m, which could grow at wide range of pressure conditions from atmospheric pressure to 70 MPa, where the optimal pressure condition was at 30 MPa [4]. The gene
expression (transcription level) regulated by sigma 54 promoter was identified as one of pressure-regulated mechanisms [5]. To understand the molecular mechanisms of pressure regulation in general, genome analysis of the model piezophile, *S. violacea*, has performed. There are several unknown genes from the genome annotation result, so it is possible that the piezophiles may keep several undiscovered genes [6]. By the way, we have found several useful genes for bio-remediation and drug discoveries, etc. from the gene homology search. We are now planning to discover the useful enzymes from deep-sea resources.

Proteins, from deep-sea “piezophilic” microorganisms, could be active under high-pressure conditions in general. Actually normal proteins can be inactive under higher-pressure conditions, ca, 500 MPa and more. For bio processing under pressure, people are looking for pressure-tolerant enzymes, thus, “piezophilic proteins” would be focus on such industrial applications. We have done the comparative studies of the same functional proteins and/or enzymes between from *Escherichia coli* and deep-sea piezophiles [7-11]. Generally, piezophilic proteins were much more stable and/or active under higher-pressure conditions than *E. coli*’s proteins. In this article, our current studies for piezophilic proteins were described and concluded.

2. Features of proteins from piezophilic *S. violacea*

2.1 Respiratory proteins in *S. violacea*

As evidence of piezo-adaptation, a pressure-regulated operon was observed in this bacterium as described above. Downstream from this operon, an open reading frame homologous to the *cydD* gene of *E. coli* was found and the significance of the gene in bacterial growth under high-pressure has been suggested [12]. The gene product of *cydD* in *E. coli* is thought to be required for the assembly of respiratory components [13-15]. Further, the expression of a respiratory system was regulated by hydrostatic pressure in *S. violacea* [16-18] and in another piezophilic bacterium, *Shewanella* sp. strain DB-172F [19,20]. These were the first reports concerning respiratory systems in deep-sea bacterium and the first evidence that expression of genes for respiratory components are regulated by physical parameters, such as hydrostatic pressure. Generally, bacteria have branched respiratory chains. Specifically, *Shewanella* strains have many respiratory components for adaptation to environmental changes [21]. These observations and the results of previous studies suggest that pressure-regulation for expression of respiratory systems in *S. violacea* plays an important role in bacterial adaptation to high-pressure.

Cytochrome *bd* is one of the members of the quinol oxidases, distinct from the heme-copper oxidase super family. In *E. coli*, two types of quinol oxidases, cytochrome *bo* and cytochrome *bd* exist and both share roles in respiration. Cytochrome *bo* is expressed in log phase and cytochrome *bd* is expressed in stationary phase [22,23]. Cytochrome *bd* shows higher affinity for O₂ as compared to cytochrome *bo* and the former acts as a terminal oxidase under low oxygen concentration conditions [23]. For the biosynthesis of cytochrome *bd*, structural genes (encoded by *cydAB* operon) and genes for assembly of mature enzyme (encoded by *cydDC* operon) [13,24] are required. Expression of *cydAB* in *E. coli* is regulated by ArcA and Fnr, common O₂-regulated transcriptional regulators [25,26], and that of *cydDC* was regulated by NarL (involved in the two-component regulatory system for nitrate respiration) as well as Fnr [27]. However, in *S. violacea*, no cytochrome *bd* has been detected.
spectrophotometrically under atmospheric pressure even during the stationary phase. Surprisingly, cytochrome bd has been detected only under growth conditions of high hydrostatic pressures (Table 1) [16]. Thus, transcriptional regulation of cytochrome bd in S. violacea may be different from other organisms and this may be important for bacterial adaptation to high-pressure. Furthermore, cytochrome bd-encoding cydAB genes were identified in S. violacea. Transcriptional analysis was carried out for cydAB and cydCD operons, and it was observed that transcription of the cydDC operon was strongly regulated by hydrostatic pressure [28].

Table 1. Composition of cytochromes in S. violacea grown under different pressure conditions.

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<th>Cells grown at 0.1 MPa</th>
<th>Cells grown at 50 MPa</th>
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<tbody>
<tr>
<td>Soluble fraction</td>
<td>c-type (greater amount)</td>
<td>c-type (lesser amount)</td>
</tr>
<tr>
<td>Membrane fraction</td>
<td>b- (and/or o-) type</td>
<td>b- (and/or o-) type</td>
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<tr>
<td></td>
<td>c-type</td>
<td>d-type</td>
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By the way, there are two types of soluble cytochrome c in this bacterium. One of them, cytochrome cA, was constitutively expressed regardless of growth pressure. On the other hand, the expression of another cytochrome c (cytochrome cB) was repressed under high hydrostatic pressure [17]. The respiratory systems concerning cytochrome bd, and c were concluded in Fig. 1. At atmospheric pressure condition, cytochrome c oxidase pathway could be main pathway, however at high-pressure condition, cytochrome bd oxidase pathway could be major. These results suggested that pressure-regulation for expression of the respiratory systems in S. violacea plays an important role in bacterial adaptation to high hydrostatic pressure.

2.2 Cell division protein, FtsZ

Some rod shaped bacteria including E. coli, exhibit cell filamentation without septum formation under high hydrostatic pressure conditions, indicating that the cell division process is affected by hydrostatic pressure [29]. We examined effects of elevated pressure on FtsZ-ring formation in E. coli cells by indirect immunofluorescence microscopy. Elevated pressure completely repressed colony formation of E. coli cell at 40 MPa in our cultivation conditions and the cells exhibited obviously filamentous shapes. In the elongated cells, normal cell division processes appeared to be inhibited, because no FtsZ-rings were observed by indirect immunofluorescence staining. In addition, we also observed that hydrostatic pressure dissociated the E. coli FtsZ (ecFtsZ) polymers in vitro [7]. These results suggest that
the high-pressure directly affects cell survival and morphology through the dissociation of cytoskeletal frameworks.

In the piezophilic *S. violacea* DSS12, the growth was occurred at high-pressure conditions [4]. To analyze the transcription upstream from the *ftsZ* gene, Northern blot and primer extension analyses were performed and the results showed that gene expression was not pressure dependent. Western blot analysis also showed that the *S. violacea* FtsZ protein (svFtsZ) was equally expressed under several pressure conditions in the range of atmospheric (0.1 MPa) to high (50 MPa) pressures [8]. Using immunofluorescence microscopy, the svFtsZ ring was observed in the center of cells at pressure conditions of 0.1 to 50 MPa (Fig. 2). These results imply that the svFtsZ protein function is not affected by elevated pressure in this piezophilic bacterium.

The cell morphology of *E. coli* under high-pressure is, however, quite different from *S. violacea*. The filamentous cells indicate to stop the cell division steps and we expect the hydrostatic pressure affect FtsZ protein function and causes inhibition of cell division. In fact, the C-termini of FtsZ in four bacteria compare are not conserved in each other and the region is essential for polymerization activity [8]. The characteristic property under high-pressure is considered from the variety. Therefore, we expect that characterization of the biochemical features and polymerization activities of the FtsZ and any terrestrial bacteria (eg. *E. coli*) *in vitro* help us to understand the effect of pressure on cell division steps *in vivo*.

### 2.3 RNA polymerase complex

A high-pressure electrophoresis apparatus (HPEA) was developed by a modification of the method previously reported by Erijman and Clegg [9]. The cathode and anode were attached separately above and below the sample chamber. [Pressures of up to 200 MPa can be applied within one minute with silicon oil KF-96-1.5CS (Shin-Etsu Chemical, Tokyo, Japan), using a hand pump.] First, native polyacrylamide gel electrophoresis (PAGE) was carried out in capillary glass tubes 75 mm in length and 0.85 mm in inner diameter under pressure conditions using the HPEA. The sample was kept at the desired pressure for 30 min for equilibration before electrophoresis. Electrophoresis was carried out at the constant voltage of 350 V for 1 h. After decompression, the gel was removed and equilibrated in sodium dodecyl sulfate (SDS) buffer. Next the gel was overlaid onto the SDS gel and subjected to SDS-PAGE using 10% polyacrylamide gels at atmospheric pressure. Proteins were then visualized by silver staining.
RNA polymerase was purified from the piezophile *S. violacea*, and the transcriptional activity after pressure treatment was compared with that of the mesophile *E. coli*. Application of pressure at 100 MPa for 30 min reduced the *E. coli* RNA polymerase (ecRNAP) activity to 60% of the activity at atmospheric pressure, whereas the *S. violacea* RNA polymerase (svRNAP) maintained full activity, indicating that the svRNAP is more stable than ecRNAP [9]. 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 (HPEA), which showed that a pressure of 140 MPa caused dissociation of ecRNAP but not that of svRNAP (Fig. 3). On the other hand, the core enzyme of svRNAP, which lacked the sigma 70 factor, was dissociated at 140 MPa. These results suggest that the sigma 70 factor is required for stabilization of svRNAP under high-pressure conditions. We provide in vitro evidence for piezoadaptation at the transcriptional level, using purified RNA polymerase from cells of *S. violacea* and *E. coli*.

One of our striking results is that the *S. violacea* sigma 70 subunit enhanced the stabilization of RNA polymerase at high-pressure. The sigma subunit is known to change the quaternary structure of ecRNAP [30,31]. It is likely that the *S. violacea* sigma 70 subunit stabilizes the core enzyme through alteration of the quaternary structure of RNA polymerase, resulting in piezotolerance [9]. In this context, the predicted β-sheet domain, which is not observed in the *E. coli* and *S. oneidensis* sigma 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 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 [9] is a powerful tool in combination with techniques based on molecular biology and bioinformatics.

### 2.4 Dihydrofolate reductase (DHFR)

A new dihydrofolate reductase (svDHFR) was purified from a deep-sea bacterium, *S. violacea* DSS12. In contrast with *E. coli* DHFR (ecDHFR), the enzyme activity of svDHFR
increased with increasing hydrostatic pressure up to 100 MPa, suggesting that the enzyme kinetics and structural fluctuation of svDHFR are adapted to a high-pressure environment.

Figure 4 shows the relative activities of the DHFRs as a function of hydrostatic pressure. The enzyme activity of svDHFR increases with pressure, to at most 30% at 100 MPa, and then gradually decreases although maintains a higher activity at 250 MPa than at atmospheric pressure. This is significantly different from the behavior of ecDHFR, which exhibits a monotonous decrease in the activity even under low pressure. Therefore, it can be expected that svDHFR has distinguished characteristics in enzyme kinetics and structural fluctuation to adapt itself to deep-sea conditions. The activation volume of enzyme reaction, $\Delta V^*$, was calculated [10]. The negative $\Delta V^*$ value of svDHFR under pressures below 100 MPa, as expected from the positive slope in Fig. 4, means that the activated state has a smaller volume than the reactant in the catalytic reaction coordinate. At pressures above 100 MPa this is reversed, as shown by the positive $\Delta V^*$ value (also for ecDHFR). These results predict that svDHFR, being highly flexible at pressures below 100 MPa, changes its conformation to be more rigid (like ecDHFR) at higher pressures although the protein may be denatured at pressures above a few hundred MPa. Determining the volume changes in each process and the rate-limiting process, which is in progress, should provide more detailed understanding of the adaptation mechanism of the enzyme reaction.

3. Conclusion

Proteins, from deep-sea adapted microorganisms “piezophiles”, could be active under high-pressure conditions in general. We have done the comparative studies of the same functional proteins and/or enzymes between from *Escherichia coli* and deep-sea piezophiles. In the case of cell divisional protein FtsZ, RNA polymerase subunit structure, and dihydrofolate reductase (DHFR), piezophilic proteins were much more stable and/or active under higher pressure conditions than *E. coli*’s proteins. In the case of respiratory proteins, there were different pathway between under high-pressure and atmospheric pressure conditions in the deep-sea piezophilic strains. These results concluded that piezophilic proteins could be one of valuable source not only for the basic research to understand environmental adaptations, but also for the future biotechnological applications because of their pressure tolerant properties.

4. References


