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Gene expression and genome analysis of high pressure-adapted marine bacterium, Piezophilic *Shewanella violacea*

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We have isolated numerous deep sea-adapted using the deep-sea research submersible *SHINKAI 6500*. Many of the isolates are novel bacteria, and these novel piezophiles produce long-chain unsaturated fatty acids, eicosapentaenoic acids and/or docosahexaenoic acids in their cell membrane fractions. These unsaturated fatty acids are a focus of attention in the medical field because of their antioxidant effects. In addition, we completed the sequencing of the entire genome of the piezophile *Shewanella violacea,* which was isolated from the Ryukyu Trench at a depth of 5100 m. This is the first genomic analysis of the piezophiles, and we expect that many biotechnologically useful genes will be identified from the genome information.

Key words *Shewanella violacea*, gene expression, high pressure, low temperature, genome analysis

Introduction

It has been suggested that life may have originated in the deep sea some 3.5 to 4 billion years ago, where it was protected from the damaging effects of ultraviolet light [1]. The deep sea is a particularly high-pressure environment, and hydrostatic pressure would have been a very important stimulus for the early forms of life. Recently, scientists have proposed that life might have originated in deep-sea hydrothermal vents, and thus it appears possible that high-pressure–adapted mechanisms of gene expression could represent a feature present in early forms of life. It has recently been reported that the primary chemical reactions involved in the polymerization of organic materials could have occurred in such an environment. Thus, the study of deep-sea microorganisms may not only enhance our understanding of specific adaptations to abyssal and hadal ocean realms, but may also provide valuable insights into the origin and evolution of life on our planet.

In 1949, Zobell and Johnson began investigating the effect of hydrostatic pressure on microbial activities [2]. The term "barophilic" was first used by them, defined as optimal growth at a pressure higher than 0.1 MPa or a requirement for increased pressure for growth. Recently, the term "piezophilic" has been proposed to replace "barophilic," as the prefixes "baro" and "piezo," derived from Greek, mean "weight" and "pressure," respectively [3]. Thus, the word "piezophilic" is more suitable than "barophilic" to describe bacteria that grow better at high pressure than at atmospheric pressure. Therefore, the authors have opted to use the term "piezophilic" bacteria, meaning high-pressure–loving bacteria.

The model piezophile, *Shewanella violacea*

The moderately piezophilic *S. violacea* strain DSS12 grows optimally at 30 MPa and 8°C, but also at atmospheric pressure (0.1 MPa) and 8°C [4, 5]. Therefore, this piezophilic strain is useful as a model for comparison of various features of bacterial physiology under high and low hydrostatic pressure conditions. An operon identified as a pressure-regulated operon, of which the promoter is activated by growth under high pressure, was cloned and characterized from this strain. This operon, which has five transcription initiation sites, is controlled at the transcriptional level by elevated pressure, as shown in Fig. 1A [6]. Moreover, transcriptional analysis showed that expression of the genes in the pressure-regulated operon is positively controlled at the transcriptional level by elevated pressure, suggesting that most transcripts from the operon at atmospheric pressure coincide with those from initiation site number 2 (Fig. 1A). The sigma 54-containing RNA polymerase has been shown to be responsible for the transcription of several genes e.g., nitrogen metabolic genes such as the *glnA* operon [7]. Glutamine synthetase is one of

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the enzymes involved in nitrogen metabolism. As shown in Fig. 1B, gene expression of the *glnA* operon is also controlled by elevated pressure conditions at the transcriptional level in *S. violacea*, particularly by factor sigma 54 [8]. These results suggest that sigma 54 may play an important role in pressure-regulated transcription in piezophilic bacteria, although the expression of sigma 54 itself is not regulated by pressure conditions [9].

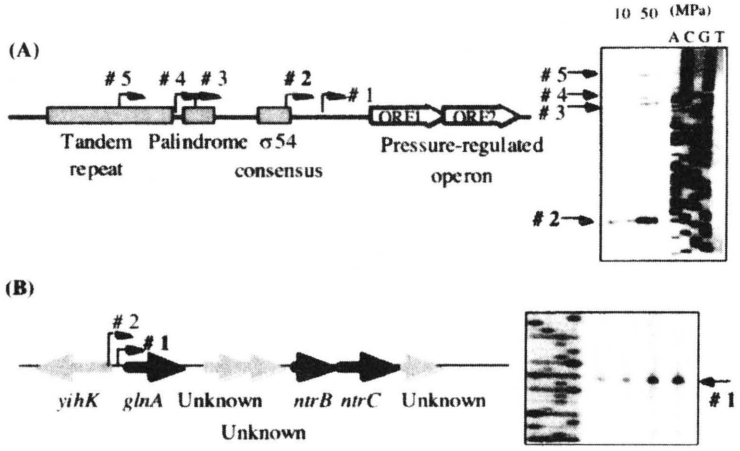

Fig.1 Diagrammatic representation of the pressure-regulated genes in *S. violacea* strain DSS12
(A) Pressure-regulated operon. (B) Glutamine synthetase operon. Red arrows show the transcription controlled by the sigma 54 factor.

In transcription of the sigma 54-dependent promoter, such as the pressure-regulated operon and the *glnA* operon, sigma 54-containing RNA polymerase holoenzyme activates transcription at the promoter assisted by the activity of NtrC, which is controlled by NtrB [10]. As mentioned above, these *trans*-acting factors (sigma 54, NtrC, or NtrB) might play an important role in pressure-regulated transcription at the sigma 54-dependent promoter in this piezophilic bacterium.

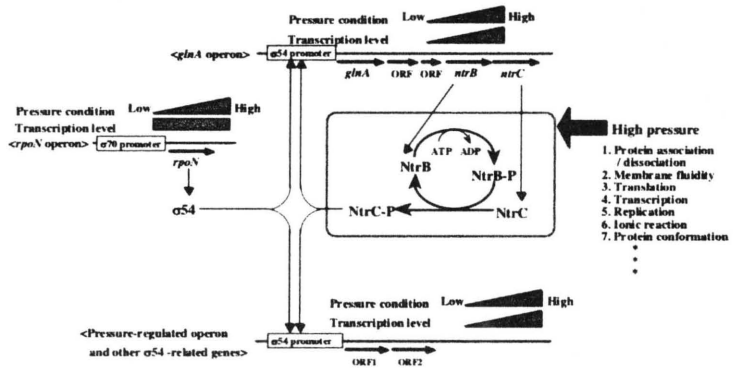

Fig. 2. Model of the transcription mechanisms of pressure-regulated gene expression in piezophilic *S. violacea* strain DSS12.

The sigma 54 in *S. violacea* is expressed at a relatively constant level under both atmospheric and high-pressure conditions, suggesting that the level of functional sigma 54 molecules is possibly regulated by the availability of NtrC. We examined the expression of the NtrC protein in *S. violacea* using Western blot analysis under pressure conditions. The results indicated that the amount of this factor expressed at high pressure is greater than that at atmospheric pressure [11, 12]. Consideration of the results of the pressure regulation by sigma 54 leads us to suggest a possible model for the mechanism of regulated expression of the pressure-regulated operon and the *glnA* operon in the deep-sea piezophilic bacterium *S. violacea*, as shown in Fig. 2. As *S. violacea* sigma 54 is expressed at a consistent level at both atmospheric and high pressure, it is suggested that the intracellular level of sigma 54-containing RNA polymerase holoenzyme under both conditions is constant. This observation also strongly suggests that the transcriptional activity at this sigma 54-dependent promoter is proportional to the amount of NtrC factor and that it regulates the pressure-regulated gene expression under pressure conditions. This model suggests that NtrB might function as a pressure sensor and that this protein would be autophosphorylated under high-pressure

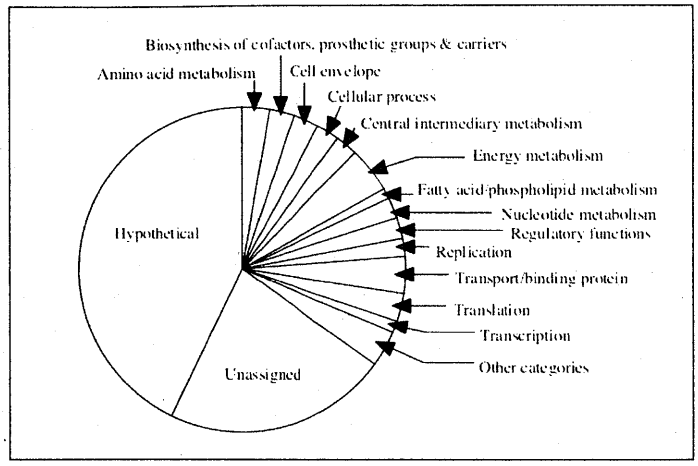

Fig. 3. Annotation of ORFs in the *S. violacea* genome
Total genome size was 4.8 Mbp, and about 4,600 ORFs were predicted.

conditions. Subsequently, the phosphorylated relay affects the NtrC protein, and then the phosphorylated NtrC activates the sigma 54-dependent promoter (Fig. 2). This model explaining explaining gene expression at high pressure should be confirmed using molecular-genetic approaches analyzing several pressure- and low temperature-sensitive mutants in these transcription systems.

Shewanella violacea genome analysis

To understand pressure regulation in deep-sea bacteria, we performed genome analysis of the piezophilic bacterium *S. violacea* strain DSS12 as a model deep-sea bacterium [13]. The genome size of this bacterium is about 4.8 Mbp based on the results of pulse-field electrophoresis analysis, and 12 ribosomal RNA operons were identified. The number of the ribosomal operons might correspond to environmental conditions, and it is likely that the ability of *S. violacea* to grow under high pressure is due to the transcriptional efficiency of mumerous ribosomal proteins under such stress conditions. From the results of genome sequencing, about 4,600 open reading frames (ORFs) were identified. As shown in Figure 4, preliminary analyses of the annotation suggested that almost 70% of the ORFs are hypothetical proteins and unassigned. This result indicates that many genes from deep-sea microbes could be novel and might have also novel functions. Therefore, such marine extremophiles are good materials in which to discover new functions of genes. In the *S. violacea* genome, the following useful genes were identified: haloalkane dehalogenase, extracellular proteases, chitinase, chitobibiase, EPA synthesis clusters, thiamine biosynthesis clusters, lipoprotein, vitamin B6 biosynthesis protein, biotin synthetase, cellulases, organic solvent tolerance proteins, etc. *S. violacea* genome analysis could be completed within 2005 and then we are planning to start a postgenome study called the "PIEZOME project," which will focus on the proteome, transcriptome, and metabolome of piezophiles affected by the pressure environment. We are looking forward to understanding the molecular mechanisms of high-pressure life on our planet based on the results of the PIEZOME project.

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