

Pressure Response in Deep-sea Piezophilic Bacteria

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Abstract

Several piezophilic bacteria have been isolated from deep-sea environments under high hydrostatic pressure. Taxonomic studies of the isolates showed that the piezophilic bacteria are not widely distributed in terms of taxonomic positions, and all were assigned to particular branches of the Proteobacteria gamma-subgroup. A pressure-regulated operon from piezophilic bacteria of the genus *Shewanella*, *S. benthica* and *S. violacea*, was cloned and sequenced, and downstream of this operon another pressure regulated operon, *cydD-C*, was found. The *cydD* gene was found to be essential for the bacterial growth under high-pressure conditions, and the product of this gene was found to play a role in their respiratory system. Results obtained later indicated that the respiratory system in piezophilic bacteria may be important for survival in a high-pressure environment, and more studies focusing on other components of the respiratory chain have been conducted. These studies suggested that piezophilic bacteria are capable of changing their respiratory system in response to pressure conditions, and a proposed respiratory chain model has been suggested in this regard.

Introduction

The deep-sea is regarded as an extreme environment with conditions of high hydrostatic pressure [up to 110 megapascals (MPa)], and predominantly low temperature (1-2 °C), but with occasional regions of extremely high temperature (up to 375 °C) at sites of hydrothermal vents, darkness and low nutrient availability. It is accepted that deep-sea microbiology as a definable field did not exist before the middle of this century, and little attention was paid to this field except for the efforts of Certes and Portier (Jannasch and Taylor, 1984). Certes, during the Travaillier and Talisman Expeditions (1882-1883), examined sediment and water collected from depths to 5,000 m and found bacteria in almost every sample. He noted that bacteria survived at great pressure and might live in a state of suspended animation (Certes, 1884). In 1904, Portier used a sealed and autoclaved glass tube as a bacteriological sampling device and reported colony counts of bacteria from various depths and locations

(Richard, 1907). In 1949, ZoBell and Johnson (1949) started work on the effect of hydrostatic pressure on microbial activities. The term "barophilic" was first used, defined as optimal growth at a pressure higher than 0.1 MPa or a requirement for increased pressure for growth. Recently, the term "piezophilic" was proposed to replace "barophilic" as the prefixes "baro" and "piezo", derived from Greek, mean "weight" and "pressure", respectively (Yayanos, 1995). Thus, the word piezophilic may be more suitable than barophilic to indicate bacteria that grow better at high pressure than at atmospheric pressure.

In this review, the authors have opted to use the term "piezophilic bacteria" which means high-pressure loving bacteria, and this review focuses on the taxonomy of deep-sea piezophiles and the features of their respiratory systems related to high pressure adaptation.

Taxonomic Positions of Deep-sea Piezophilic Bacteria

Bacteria living in the deep-sea display several unusual features that allow them to thrive in their extreme environment. The first pure culture of a piezophilic isolate, strain CNPT-3, was reported in 1979 (Yayanos *et al.*, 1979). This spirillum-like bacterium had a rapid doubling rate at 50 MPa, but no colonies were formed at atmospheric pressure even after incubation for several weeks. Numerous piezophilic and piezotolerant bacteria have since been isolated and characterized by the DEEPSTAR group at JAMSTEC, from deep-sea sediments at depths ranging from 2,500 m-11,000 m (Kato *et al.*, 1995a; 1996a; 1996b; 1998). Most of the isolated strains are not only piezophilic, but also psychrophilic, and they cannot be cultured at temperatures above 20 °C. The effects of pressure and temperature on cell growth, comparing the deep-sea piezophilic strains isolated by Yayanos and the DEEPSTAR group, are similar, in that all strains become more piezophilic at higher temperatures (Kato *et al.*, 1995a; Yayanos, 1986). These studies indicate that all piezophilic isolates are obligately piezophilic above the temperature at which growth occurs at atmospheric pressure. This means that the upper temperature limit for growth can be extended by high pressure. Likewise, piezophilic bacteria reproduce more rapidly at a lower temperature (such as 2 °C) when the pressure is less than that at its capture depth. It also appears to be true as a general rule that the pressure at which the rate of reproduction at 2 °C is maximal may reflect the true habitat depth of an isolate (Yayanos *et al.*, 1982).

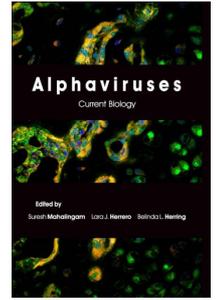
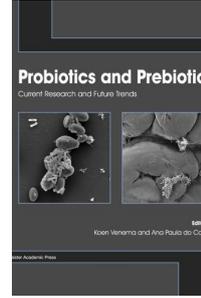
Many deep-sea piezophilic bacteria have been shown to belong to the gamma-Proteobacteria through comparison of 5S and 16S rDNA sequences. As a result of a taxonomic study based on 5S rDNA sequences, Deming reported that the obligate piezophilic bacterium *Colwellia hadaliensis* belongs to the Proteobacteria gamma-subgroup (Deming *et al.*, 1988). DeLong *et al.* (1997) have also documented the existence of piezophilic and psychrophilic deep-sea bacteria that belong to this

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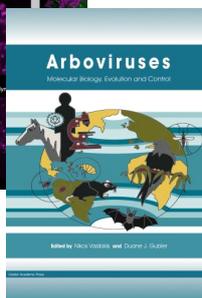
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subgroup, as indicated by 16S rDNA sequences. The piezophilic bacteria reported by Liesack *et al.* (1991) are included in the same branch of the gamma-Proteobacteria as are the strains isolated by the DEEPSTAR group (Kato *et al.*, 1995a). These data suggest that most of the deep-sea high-pressure adapted piezophilic bacteria that can be readily cultured belong to the Proteobacteria gamma-subgroup, and these may not be widely distributed within the domain Bacteria.

DeLong *et al.* (1997) reported that eleven cultivated psychrophilic and piezophilic deep-sea bacteria are affiliated with one of five genera within the gamma-subgroup: *Shewanella*, *Photobacterium*, *Colwellia*, *Moritella*, and an unidentified genus. The only deep-sea piezophilic species of two of these genera were reported to be *S. benthica* in the genus *Shewanella* (Deming *et al.*, 1984; MacDonell and Colwell, 1985) and *C. hadaliensis* in the genus *Colwellia* (Deming *et al.*, 1988) prior to the reports from the DEEPSTAR group. We have identified four new piezophilic species within those genera based on the results of chromosomal DNA-DNA hybridization studies and several other taxonomic properties. Both previously described and new species of bacteria have been identified among the piezophilic bacterial isolates.

Photobacterium profundum, a new species, was identified through studies of the moderately piezophilic strains DSJ4 and SS9 (Nogi *et al.*, 1998a). *P. profundum* strain SS9 has been extensively studied with regard to the molecular mechanisms of pressure-regulation (Bartlett *et al.*, 1989; Bartlett *et al.*, 1996; Welch and Bartlett, 1998). *P. profundum* is the only species within the genus *Photobacterium* known to display piezophily, and the only one known to produce the long-chain polyunsaturated fatty acid (PUFA), eicosapentaenoic acid (EPA). No other species of *Photobacterium* produces EPA (Nogi *et al.*, 1998a).

The moderately piezophilic strain DSS12 isolated from the Ryukyu Trench at a depth of 5,110 m was identified as *Shewanella violacea* (Nogi *et al.*, 1998b), a novel piezophilic species belonging within the *Shewanella* piezophile branch (Li *et al.*, 1998). Other *Shewanella* piezophilic strains, PT-99 (DeLong and Yayanos, 1986), DB5501, DB6101, DB6705, DB6906 (Kato *et al.*, 1995a), DB172F, DB172R (Kato *et al.*, 1996b) and DB21MT-2 (Kato *et al.*, 1998) were all identified as members of the same species, *S. benthica* (Li *et al.*, 1998; Nogi *et al.*, 1998b;

Nogi and Kato, 1999). The piezophilic and psychrophilic *Shewanella* strains, including *S. violacea* and *S. benthica*, also produce EPA (DeLong *et al.*, 1997; Kato *et al.*, 1998; Nogi *et al.*, 1998b), thus the occurrence of this PUFA is a property shared by many deep-sea bacteria. *Shewanella violacea* strain DSS12 has been well studied at JAMSTEC, particularly with respect to its molecular mechanisms of adaptation to high pressure. This strain is moderately piezophilic, with a fairly constant doubling time at pressures between 0.1 MPa and 70 MPa, whereas the doubling times of most of the piezophilic *S. benthica* strains change substantially with increasing pressure (Kato *et al.*, 1995a; 1996a; 1996b; 1998). Because there are few differences in the growth characteristics of strain DSS12 under different pressure conditions, this strain is a very convenient deep-sea bacterium for use in studies on the mechanisms of adaptation to high pressure environments. Studies using this strain include analyses of the pressure-regulation of gene expression (Kato *et al.*, 1997a; 1997b) and of the role of *d*-type cytochromes in the growth of cells under high pressure (Kato *et al.*, 1996c; Tamegai *et al.*, 1998). The molecular mechanisms of gene expression have been analyzed focusing on a cloned pressure-regulated promoter and more detailed studies are in progress (Nakasone *et al.*, 1998).

Strain DSK1, which is a moderately piezophilic bacterium isolated from the Japan Trench, was identified as *Moritella japonica*. This is the first piezophilic species identified in the genus *Moritella* (Nogi *et al.*, 1998c). The type strain of the genus *Moritella* is *M. marina*, previously known as *Vibrio marinus* (Urakawa *et al.*, 1998), one of the most common psychrophilic organisms isolated from marine environments (Colwell and Morita, 1964). *M. marina* is closely related to the genus *Shewanella* on the basis of 16S rDNA data, and is not a piezophilic bacterium (Nogi *et al.*, 1998c). The extremely piezophilic bacterium strain DB21MT-5 isolated from the world deepest sea bottom, the Mariana Trench Challenger Deep, at a depth of 10,898 m (Kato *et al.*, 1998), was identified as a *Moritella* species and designated *M. yayanosii* (Nogi and Kato, 1999). The optimal pressure for growth of *M. yayanosii* strain DB21MT-5 is 80 MPa, and this strain is not able to grow at pressures below 50 MPa, but it is able to grow well at higher pressures, even as high as 100 MPa (Kato *et al.*, 1998). Production of the long chain PUFA, docosahexaenoic acid (DHA), is one of the characteristic properties of the genus

Table 1. List of Identified Deep-sea Piezophilic Bacterial Species

Genus	Species	Strain	Properties	Reference
<i>Colwellia</i>	<i>C. hadaliensis</i>	BNL-1	Extremely piezophilic ¹	Deming <i>et al.</i> , 1988
<i>Photobacterium</i>	<i>P. profundum</i> *	SS9, DSJ4	Moderately piezophilic ²	Nogi <i>et al.</i> , 1998a
<i>Shewanella</i>	<i>S. benthica</i>	PT-99, DB-series	Moderately, obligately and extremely piezophilic	Kato <i>et al.</i> , 1998, Deming <i>et al.</i> , 1984, Li <i>et al.</i> , 1998, Macdonell and Colwell, 1985, Nogi <i>et al.</i> , 1998b
<i>Moritella</i>	<i>S. violacea</i> *	DSS12	Moderately piezophile	Nogi <i>et al.</i> , 1998b
	<i>M. japonica</i> *	DSK1	Moderately piezophile	Nogi <i>et al.</i> , 1998c
	<i>M. yayanosii</i> *	DB21MT-5	Extremely piezophile	Kato <i>et al.</i> , 1998, Nogi and Kato, 1999

¹Extremely piezophilic bacteria are defined as bacteria that are unable to grow at pressures below 50 MPa but able to grow well at 100 MPa.

²Moderately piezophilic bacteria are defined as bacteria displaying optimal growth at a pressure of less than 40 MPa, and which are able to grow well at atmospheric pressure.

*Novel deep-sea species reported by the DEEPSTAR group.

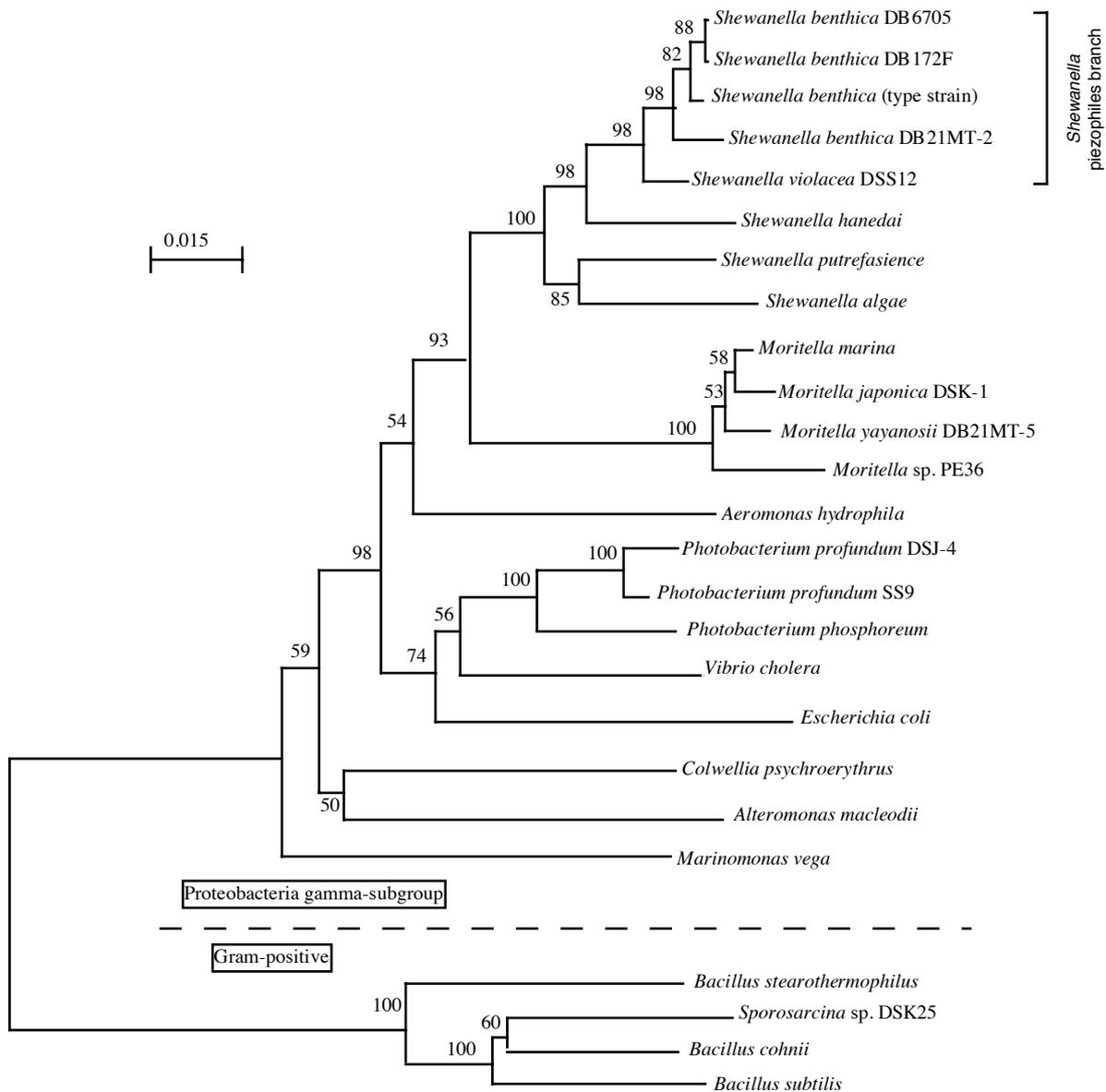


Figure 1. Phylogenetic Tree

Phylogenetic tree showing the relationships between isolated deep-sea barophilic bacteria within the gamma-subgroup of Proteobacteria and the genus *Bacillus* as determined by comparing 16S rDNA sequences using the neighbor-joining method. The scale represents the average number of nucleotide substitutions per site. Bootstrap values (%) are shown for frequencies above the threshold of 50%.

Moritella (DeLong *et al.*, 1997; Kato *et al.*, 1998; Nogi *et al.*, 1998c). DeLong and Yayanos (1985; 1986) reported that the fatty acid composition of the piezophilic strains changed as a function of pressure, and in general, greater amounts of PUFAs were synthesized at higher growth pressures. Approximately 70 % of the membrane lipids in *M. yayanosii* are PUFA, which is a finding consistent with its adaptation to very high pressures.

From the above studies, four more piezophilic species could be added to the list of identified deep-sea piezophiles shown in Table 1, and their phylogenetic positions, based on comparisons of 16 rDNA sequences, are also shown in Figure 1.

Pressure-regulation of Gene Expression and Protein Expression in Piezophilic Bacteria of the Genus *Shewanella*

A promoter, activated by growth at high pressure, was cloned from the piezophilic *S. benthica* strain DB6705 into *E. coli* (Kato *et al.*, 1995b). Downstream from this promoter, two open reading frames (ORF1 and 2) were identified as one operon, designated as a pressure-regulated operon (Kato *et al.*, 1996d). The highly conserved pressure-regulated operon from the moderately piezophilic *S. violacea* strain DSS12 was also cloned and sequenced (Kato *et al.*, 1997a). Its sequence is almost identical to the operon from *S. benthica* strain DB6705. Downstream from

this operon, another pressure-regulated operon was discovered whose first ORF was designated ORF3 and whose gene expression was also enhanced by high pressure (Kato *et al.*, 1997a). According to the results of transcriptional analyses, the pressure-regulated operons are expressed at elevated pressure, and at 70 MPa the transcripts are present in largest amount. Based upon the deduced amino acid sequence and the results of heterologous complementation studies, ORF3 appears to encode the CydD protein (Kato *et al.*, 1996c). In *E. coli*, CydD is required for the assembly of the cytochrome *bd* complex, one of the components of the aerobic respiratory chain (Poole *et al.*, 1994). *E. coli cydD* mutants display increased sensitivity to high pressure, but can be converted to cells which display wild type levels of high pressure

sensitivity if the DSS12 ORF3 gene is introduced into the cells on a plasmid. In fact, the cytochrome *bd* protein complex of strain DSS12 was observed spectrophotometrically only in the case of cells grown under high pressure conditions (Tamegai *et al.*, 1998). It seems likely that the pressure regulation of this respiratory system in piezophilic bacteria plays a significant role in cell growth under high pressure conditions.

To study the pressure-regulated respiratory system in piezophilic *Shewanella* sp. in more detail, *c*-types cytochromes and a terminal quinol oxidase were purified and characterized from *S. benthica* strain DB172F (Qureshi *et al.*, 1998a; 1998b). Two kinds of cytochromes *c* from the membrane and the cytoplasm were purified, and named cytochrome *c*-551 and cytochrome *c*-552, respectively

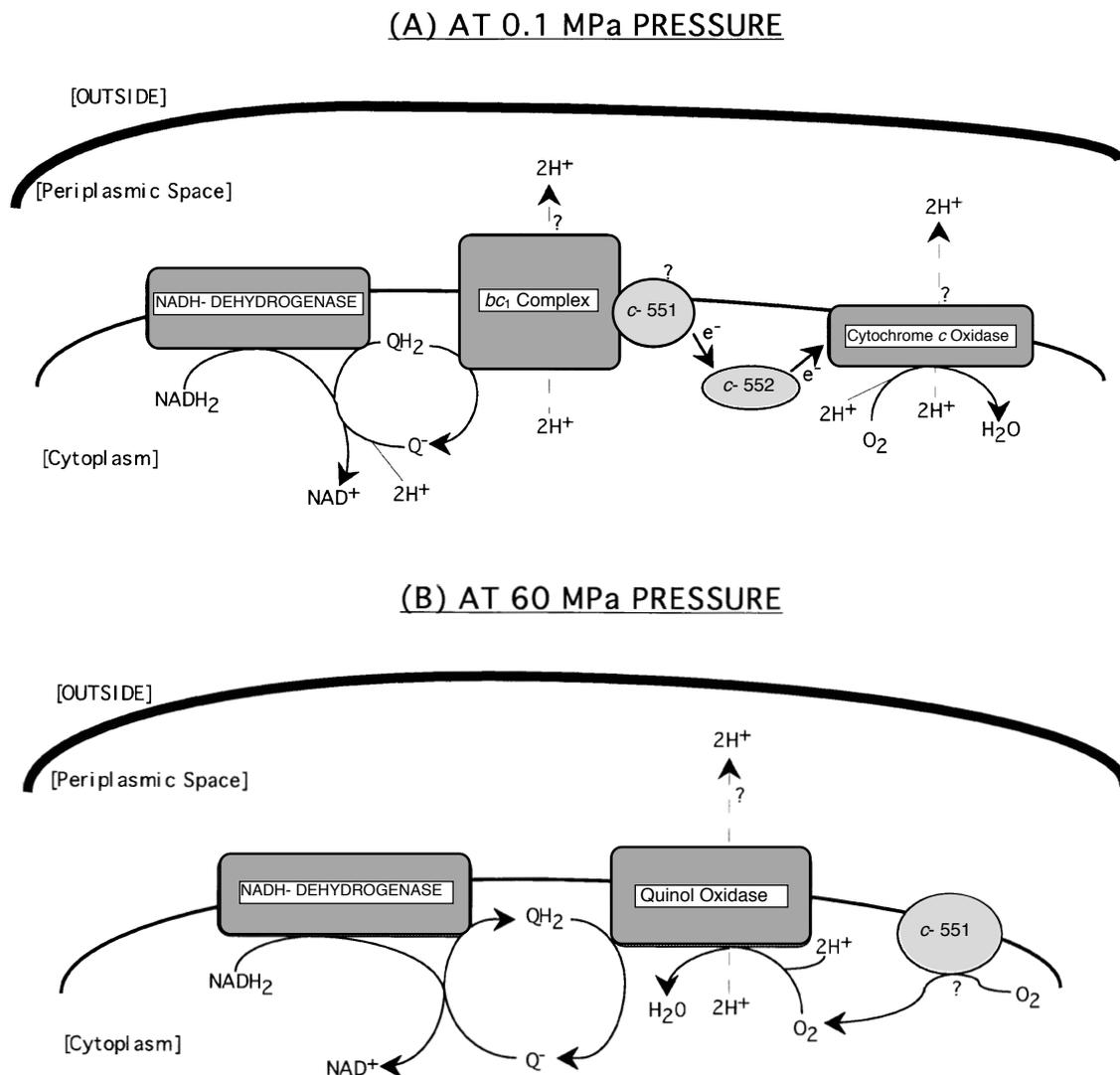


Figure 2. Electron Transport System in Piezophilic *Shewanella benthica*
Proposed electron transport system in piezophilic *Shewanella benthica* strain DB172F grown at 0.1 MPa (A) and 60 MPa (B). Abbreviations used are: Q, quinone; QH₂, quinol; C-551, cytochrome *c*-551; C-552, cytochrome *c*-552.

(Qureshi *et al.*, 1998a). Cytochrome *c*-551 was found to contain 44.2 nmol of heme *c* per mg of protein and cytochrome *c*-552 was found to contain 31.3 nmol of heme *c* per mg of protein. The CO difference spectrum of cytochrome *c*-551 showed a peak at 413.7 nm and troughs at 423.2, 522 and 552 nm which showed that this cytochrome combined with CO. Cytochrome *c*-551 was found to consist of two subunits with molecular masses of 29.1 kDa and 14.7 kDa, and each subunit contains a heme *c* molecule. Cytochrome *c*-552 was found to consist also of two subunits with molecular masses of 16.9 kDa and 14.7 kDa, and contains only one heme *c*. Cytochrome *c*-551 is constantly expressed during growth either 0.1 MPa or 60 MPa, whereas cytochrome *c*-552 is expressed only during growth at 0.1 MPa (Qureshi *et al.*, 1998a).

A novel membrane bound *ccb*-type quinol oxidase, from cells grown at 60 MPa, was purified to an electrophoretically homogenous state from the same strain (Qureshi *et al.*, 1998b). The purified enzyme complex was found to consist of four kinds of subunits with molecular masses of 98, 66, 18.5 and 15 kDa, and the complex contained 0.96 mol of protoheme and 1.95 mol of covalently bound heme *c* per mol of enzyme. Only the protoheme in the enzyme reacted with CO and CN⁻ and the catalytic activity of the enzyme was 50 % inhibited by 4 μM CN⁻. The isoelectric point of the native enzyme complex was determined to be 5.0. This enzyme was found to be specifically induced only under elevated hydrostatic pressure conditions and it is expressed at high levels in cells grown at 60 MPa. In contrast, the membranes of cells grown at atmospheric pressure (0.1 MPa) exhibit high levels of cytochrome *c* oxidase activity and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPDH₂)-oxidase activity (Qureshi *et al.*, 1998b).

These interesting observations suggest that the external growth pressure significantly alters the respiratory chain components, and furthermore, two kinds of respiratory chains regulated in response to pressure in the deep-sea *S. benthica* strain DB172F are present. A possible model of the respiratory system regulated by pressure is shown in Figure 2.

Description of the Respiratory System Model

Proposed Respiratory System Functioning at 0.1 MPa (Figure 2A)

In this model, three respiratory chain enzyme complexes are present, NADH-dehydrogenase (also called complex I), *bc*₁- complex (also called complex III) and terminal cytochrome *c* oxidase (also called complex IV). Apparently, this is the typical respiratory chain found normally proposed in mitochondria and mesophilic bacteria. In this model, the flux of respiratory electrons occurs passing from complex I to complex IV. Pairs of high energy electrons derived from the metabolism of glucose are stored in the form of NADH and FADH₂. Complex I oxidizes NADH₂ to NAD and the two electrons released are transferred to quinone Q (oxidized form), which is then converted to the reduced state, quinol QH₂. Complex III then transfers the two electrons from quinol to membrane-bound cytochrome *c*-551. The soluble cytochrome *c*-552 then takes up the two electrons from *c*-551 and transfers them to the terminal oxidase. The terminal oxidase then reduces oxygen and pumps protons into the periplasmic space. The proton

pump function of complex III is driven by the flow of electrons. Under normal physiological conditions, the donor of electrons to terminal oxidase is *c*-552, we suppose that *c*-551 might perform this function to involve with the complex III as an electron acceptor. However, further studies are needed in this regard.

Proposed Respiratory System Functioning at 60 MPa (Figure 2B)

At high pressure, the terminal oxidase enzyme in strain DB172F is quinol oxidase. The membrane-bound cytochrome *c*-551 is thought to have the ability to bind oxygen and supply it to the terminal quinol oxidase. This function is suggested by the finding that cytochrome *c*-551 is capable of binding with ligands such as carbon monoxide and cyanide. Hence, under elevated pressure conditions, the respiratory chain seems to be more compact and "short-cut" as compared to that under normal atmospheric pressure conditions. This may intimately related to the bacterial piezophilic property. Being a new field of study, this area need further exploration for the novel bioenergetic characteristics of the deep-sea bacteria.

Acknowledgements

We are very grateful to Prof. Koki Horikoshi for providing the opportunity to conduct much of the research described in this review, as well as for providing encouragement. We also thank other colleagues at JAMSTEC for providing deep-sea samples and for additional scientific cooperation, and Dr. W. R. Bellamy for assistance in editing the manuscript.

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