Bacterial adaptation to high pressure: a respiratory system in the deep-sea bacterium *Shewanella violacea* DSS12

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Abstract

Shewanella violacea DSS12 is a psychrophilic facultative piezophile isolated from the deep sea. In a previous study, we have shown that the bacterium adapted its respiratory components to alteration in growth pressure. This appears to be one of the bacterial adaptation mechanisms to high pressures. In this study, we measured the respiratory activities of *S. violacea* grown under various pressures. There was no significant difference between the cells grown under atmospheric pressure and a high pressure of 50 MPa relative to oxygen consumption of the cell-free extracts and inhibition patterns in the presence of KCN and antimycin A. Antimycin A did not inhibit the activity completely regardless of growth pressure, suggesting that there were complex III-containing and -eliminating pathways operating in parallel. On the other hand, there was a difference in the terminal oxidase activities. Our results showed that an inhibitor- and pressure-resistant terminal oxidase was expressed in the cells grown under high pressure. This property should contribute to the high-pressure adaptation mechanisms of *S. violacea*.

Introduction

Many deep-sea bacteria have been isolated and identified by the Japan Agency for Marine-Earth Science and Technology (JAMSTEC). One of these, *Shewanella violacea* DSS12, is a psychrophilic facultative piezophilic bacterium that was isolated from the mud of the Ryukyu Trench (5110 m depth) collected by the manned-submersible *SHINKAI* 6500 (Kato *et al.*, 1995; Nogi *et al.*, 1998). This well-investigated facultative piezophile displays optimal growth at a temperature of 8 °C, a pressure of 30 MPa, and can grow from 0.1 to 70 MPa. In a previous study, a pressure-regulated promoter was identified in the genome of *S. violacea* (Kato *et al.*, 1996). Further, it has been reported that the organism expressed a high-pressure-resistant enzyme compared with its counterpart in *Escherichia coli* (Ohmae *et al.*, 2004). These observations suggested that this bacterium has specific mechanisms for high-pressure adaptation and these systems contribute to survival at high pressure.

It is known that many bacteria change their respiratory systems in order to adapt to a particular environment. For example, *Shewanella oneidensis* has many genes coding for respiratory components and the organism can grow under various conditions (Heidelberg *et al.*, 2002). In the case of *S. violacea*, the expression of respiratory components was regulated by pressure (Tamegai *et al.*, 1998). There are two types of soluble cytochromes *c* in *S. violacea*. One of them, cytochrome c_A, was constitutively expressed regardless of growth pressure. On the other hand, the expression of another cytochrome *c* (cytochrome c_B) was repressed under high hydrostatic pressure (Yamada *et al.*, 2000). In the membrane fraction, a *d*-type cytochrome detected by spectrophotometric analysis is expressed when the cells are grown under high pressure (Tamegai *et al.*, 1998).

For the biosynthesis of cytochrome *bd*, structural genes (encoded by the *cydAB* operon) and genes for assembly of the mature enzyme (encoded by the *cydDC* operon) are required in *E. coli* (Jünemann, 1997). Also, in the case of *S. violacea,* both operons were found. The ORFs homologous to *cydD* and *cydC* were observed adjacent to a pressure-regulated promoter (Kato *et al.*, 1996). The expressions of the operons for *cydAB* and *cydDC* were up-regulated by high pressure at the transcriptional level in *S. violacea* (Tamegai *et al.*, 2005). Further, in the case of *E. coli*, the *d*-type cytochrome is expressed under low proton motive force conditions (alkaline conditions, in the presence of uncouplers and under microaerobic conditions) (Kita *et al.*, 1984; Avetisyan *et al.*, 1992; Bogachev *et al.*, 1993). However, no

induction of the *d*-type cytochrome was observed in *S. violacea* under similar conditions (Tamegai *et al.*, 2005). These results suggested that the regulatory system for *d*-type cytochrome expression in *E. coli* differed from that in *S. violacea* and the *d*-type cytochrome might play a significant role in adaptation to deep-sea environments. However, the physiological role of the *d*-type cytochrome in *S. violacea* is still unknown. In this study, we analyzed the respiratory activity of *S. violacea* grown under various pressures and suggest the presence of a branched respiratory chain. The role of the *d*-type cytochrome expressed under high hydrostatic pressure conditions is also discussed.

Materials and methods

Organisms

Shewanella violacea DSS12 was cultured as described previously (Tamegai *et al.*, 1998; Tamegai *et al.*, 2005). Marine Broth 2216 (Difco, autoclaved and filtered through a 0.22 μm membrane filter) was used as the growth medium. For large-scale cultivation (1.2 L of the medium), cells were grown under various pressures (0.1, 30, 50 or 65 MPa) using the JAMSTEC DEEP BATH system. All cultivation was performed under microaerobic conditions. The cells were collected by centrifugation (10 000 ***g***, 10 min), resuspended in 0.25 M potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA and stored at −80 °C until use. Frozen cell suspensions were thawed and subsequently treated with sonication for a total period of 20 min. Unbroken cells were removed by centrifugation (10 000 ***g***, 10 min), and the resultant supernatant was used as the cell-free extract. Further fractionation was carried out to isolate the membrane fraction as described previously (Tamegai *et al.*, 1998).

Measurement of respiratory activity

Oxygen consumption of the cell-free extract was determined using a Clark-type O_2 electrode (Iizima electronics, Aichi, Japan). The measurement conditions were as follows: 1.2 mL of 0.25 M potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA, 0.15 mL of 1 M NADH, and 0.15 mL of cell-free extract at 20 °C. The inhibitor (KCN or antimycin A) was added at a final concentration of 0–0.3 mM and 0–300 μM, respectively.

Terminal oxidase activity of the membrane fraction was measured spectrophotometrically with a Shimadzu UV-1700 spectrophotometer (Kyoto, Japan). The measurement conditions were as follows: 1.2 mL of 0.25 M potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA, 10 μL of 0.1 M $TMPDH_2$ (*N*, *N*, *N*′, *N*′-tetramethyl-*p*-phenyleneamine) and 0.15 μL of each membrane fraction at 20 °C. The activity was monitored by the increase of absorbance at 610 nm ($TMPD\delta\varepsilon_{610\,nm} = 6.7\ mM^{-1}\ cm^{-1}$). The inhibitor (KCN or NaN_3) was added at final concentrations of 0–0.3 mM or 0–4 mM, respectively.

Terminal oxidase activity under high hydrostatic pressures was measured spectrometrically with a Shimadzu UV-1600PC spectrophotometer (Kyoto, Japan) equipped with a high-pressure absorbance cell unit (Teramecs PCR-400, Kyoto, Japan) and a hand pump (Teramecs TP-500). The activity was monitored by the increase of absorbance at 610 nm. The measurement conditions were 1.8 mL of 0.25 M HEPES buffer (pH 7.0) containing 0.1 mM EDTA, 10 μL of 0.1 M $TMPDH_2$ and 10 μL of each membrane fraction at 20 °C. The values for oxygen consumption and terminal oxidase activities represent the averages for at least three independent experiments.

The protein concentration was determined by the method of Lowry *et al.* (1951) with slight modifications (Dulley & Grieve, 1975).

Results

Oxygen consumption activity of cell-free extract

Figure 1 shows the inhibition profiles for oxygen consumption activity with KCN or antimycin A in cell-free extracts obtained from cells grown under atmospheric pressure. No

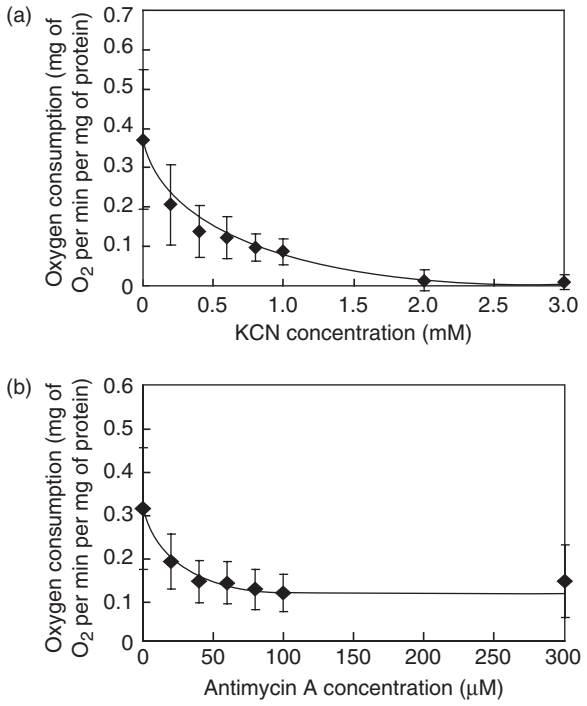

Fig. 1. Oxygen consumption activity of cell-free extracts from the cells of *Shewanella violacea* grown under atmospheric pressure and inhibition of the activity by KCN (a) and antimycin A (b).

significant difference was observed in the case of using the cells grown under 50 MPa (data not shown). The activity was completely inhibited by 2–3 mM KCN. In contrast, even at high concentrations of antimycin A, 40% of the relative activity remained.

Terminal oxidase activity under atmospheric pressure of the membrane fraction from the cells grown under various hydrostatic pressures

The inhibition profiles of terminal oxidase activity of each membrane fraction obtained from the cells grown under various pressures showed growth pressure dependency in contrast to oxygen consumption activity. When the growth pressure was 0.1, 30, 50 or 65 MPa, $TMPDH_2$ oxidase activity of the membrane fraction was completely inhibited at KCN concentrations of 0.01–0.02, 0.05, 0.1 and 0.3 mM, respectively (Fig. 2). Further, as the growth pressure was increased, the inhibition pattern remarkably became more biphasic. Especially in the case of growth pressure at 65 MPa, the activity was dramatically inhibited by 0.05 mM KCN, but low activity remained up to 0.3 mM.

The inhibition pattern by NaN_3 showed the same tendency as with KCN (Fig. 3). When the growth pressure was 0.1, 30, 50 or 65 MPa, $TMPDH_2$ oxidase activity of the membrane fraction was completely inhibited by NaN_3 concentrations of 0.3, 2, 2 and 4 mM, respectively. When the growth pressure was 65 MPa, the activity was

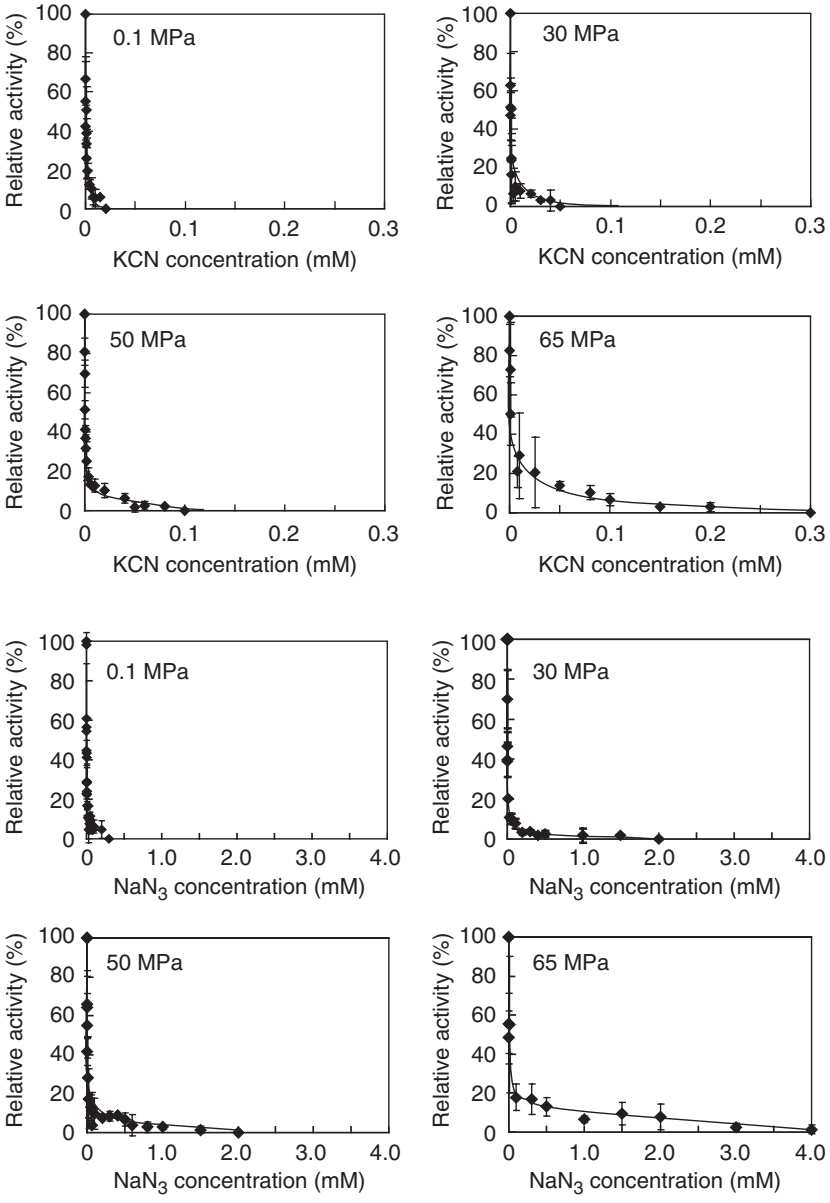

Fig. 2. Terminal oxidase activity of the membrane fraction of *Shewanella violacea* at atmospheric pressure and inhibition of the activity by KCN. The cells of *S. violacea* were grown under each pressure.

Fig. 3. Terminal oxidase activity of the membrane fraction of *Shewanella violacea* at atmospheric pressure and inhibition of the activity by NaN_3. The cells of *S. violacea* were grown under each pressure.

significantly inhibited by 0.1 mM of antimycin A but some activity remained up to 4 mM of the inhibitor. As the growth pressure was increased, the inhibition pattern also became more biphasic.

Terminal oxidase activity under various pressures of the membrane fraction from the cells grown under high hydrostatic and atmospheric pressures

Figure 4 represents the relationship between relative terminal oxidase activity and pressure under the measurement condition. The activity of each membrane fraction under a pressure of 50 MPa was defined as 100%. When the activity was measured at 100 MPa, the activity of the membrane fraction from the cells grown under the pressures of 0.1 and 50 MPa was 70% and 80%, respectively. Further at 150% MPa, 25% and 50% of relative activity was observed, respectively.

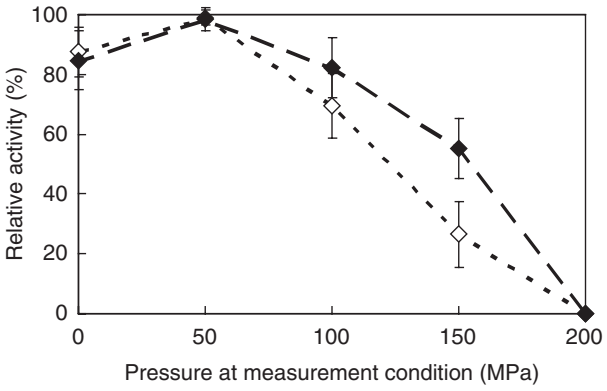

Fig. 4. Terminal oxidase activity of the membrane fraction of *Shewanella violacea* at various pressures. White squares; Profile of the membrane fraction from the cells grown under atmospheric pressure, Black squares; high pressure of 50 MPa, respectively.

Discussion

In the present study, the regulation of the respiratory activity of a deep-sea bacterium was analyzed for the first time. There was no significant difference between the cells grown under atmospheric pressure and those grown under high pressure relative to oxygen consumption activity of cell-free extract and the inhibition produced by KCN and antimycin A (Fig. 1). The respiratory chain of DSS12 consisted of an antimycin-sensitive pathway (probably containing cytochrome bc_1) of *c*. 60% of the total activity and an antimycin-resistant pathway expressing the remaining activity of *c*. 40% (likely independent of cytochrome bc_1) regardless of growth pressure. In a previous report, we showed that there were two soluble cytochromes *c* in *S. violacea* (Yamada *et al.*, 2000). Cytochrome c_A was constitutively expressed and the expression of cytochrome c_B was repressed under high pressure. Our results in this study might suggest that cytochrome c_B is the physiological electron donor for the cytochrome *c* oxidase regardless of growth pressure.

On the other hand, the terminal oxidase activity at atmospheric pressure of membrane fractions from the cells grown under various hydrostatic pressures showed differences that were dependent on the growth pressure. KCN and NaN_3 inhibition patterns became more biphasic when the growth pressures were high (Figs 2 and 3). These results clearly showed that at least two types of terminal oxidases function in cells grown under high pressure. In the cells of DSS12 grown under high pressure, KCN- and NaN_3-resistant terminal oxidase was expressed (Figs 2 and 3). Our previous result that the *d*-type cytochrome was specifically expressed in the cells of *S. violacea* grown under high pressure and the fact that *d*-type cytochrome is comparatively inhibitor-resistant (Jünemann, 1997) suggested that a *d*-type cytochrome is functional in *S. violacea* grown under high pressure. Furthermore, the membranes from high-pressure-grown cells showed greater pressure resistance than that from the cells grown under

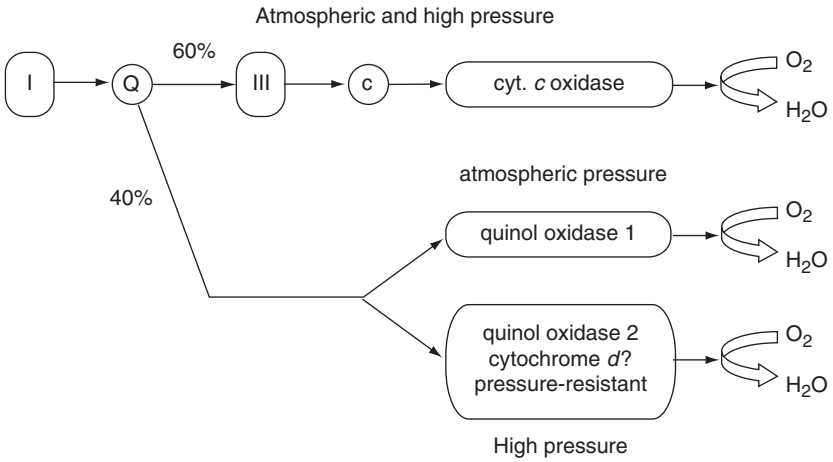

Fig. 5. Deduced respiratory system of *Shewanella violacea*. I, complex I; Q, quinone; III, complex III; c, cytocytome c. Arrows indicated the electron flows in the respiratory system.

atmospheric pressure (Fig. 4). The deduced respiratory system of *S. violacea* was shown in Fig. 5 based on these results.

The results of this study suggest that *S. violacea* expresses a *d*-type terminal oxidase that is pressure resistant. This fact contributes to the maintenance of the respiratory system under high pressure. The pressure-resistant properties may be due to the structure of the enzyme directly, or results from interaction between the enzyme and the membrane. In deep-sea bacteria, the composition of lipids in the membrane changed in a pressure-dependent manner (Kaneko *et al.*, 2000).

Besides our hypothesis, there are alternate possibilities for a role for *d*-type cytochromes in high hydrostatic pressure environments. These may include indirect influences of high pressure on the cells. For example, a high pressure may induce pH changes in the cytosol. In the case of yeast, acidification of vacuoles was also observed under elevated growth pressures. This appeared to result in an elevated cytosolic pH of yeast cells (Abe & Horikoshi, 1995). Acidification of bacterial cytosol also results in a decrease in the proton motive force. In *S. violacea,* conditions of low proton motive force such as alkaline conditions, the presence of uncouplers or low O_2 concentrations did not induce *d*-type cytochromes (Tamegai *et al.*, 2005). However, only an elevation of cytosolic pH may cause such inductions. Further, such changes may produce a change in the redox balance and subsequently the generation of reactive dioxygen species (ROS). It is known that the accumulation of ROS induces the expression of *d*-type cytochrome (Poole & Cook, 2000).

However, it is clear that one of the main roles of *d*-type cytochrome in *S. violacea* under high pressure is the maintenance of the respiratory system. This property should contribute to the adaptation of *S. violacea* to high pressure and should be one of the significant strategies for adaptation to deep-sea environments.

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