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ORIGINAL PAPER

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Pressure-regulated biosynthesis of cytochrome *bd* in piezo- and psychrophilic deep-sea bacterium *Shewanella violacea* DSS12

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Abstract The genes of cytochrome *bd*-encoding *cydAB* were identified from a deep-sea bacterium *Shewanella violacea* DSS12. These showed significant homologies with known *cydAB* gene sequences from various organisms. Additionally, highly conserved regions that are important for the enzymatic function were also conserved in *cydA* of *S. violacea*. Based on the results, transcriptional analysis of *cydAB* operon and *cydDC* operon (required for assembly of cytochrome *bd*) of *S. violacea* in microaerobic condition was performed under the growth condition of various pressures. The gene of *cydA* was expressed even under the condition of atmospheric pressure and its expression was enhanced with pressurization. On the other hand, the expression of *cydC* was strongly depressed under the condition of atmospheric pressure compared with the case under high pressure. It appeared spectrophotometrically that loss of cytochrome *bd* in *S. violacea* under atmospheric pressure shown in previous study is caused mainly by the loss of *cydDC*. Further, under the growth condition of atmospheric pressure, either less amount or no *d*-type cytochrome was expressed compared with the case of high-pressure condition even if the organism was grown under alkaline condition or in the presence of uncoupler, which are the inducible condition of *d*-type cytochrome in *Escherichia coli*. These results suggested that the significant amount of *d*-type cytochrome expression is specific event under the growth condition of high pressure.

Keywords *Shewanella violacea* · Piezophile · Respiratory system · Cytochrome *d*-type terminal oxidase · *cyd* genes · Adaptation to deep-sea environment

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Introduction

The deep-sea environment is different from land with respect to its low temperature and high pressure. Microorganisms living there are expected to have properties for adaptation to live in such extreme conditions. Particularly, all of the deep-sea bacteria should adapt to the environment of high pressure. Piezophilic bacteria were first isolated in 1979 (Yayanos et al. 1979). Many studies have been carried out to date in order to clarify the mechanisms involved in bacterial adaptation to high pressure (Kato et al. 1997). Japan Agency for Marine-Earth Science and Technology (JAMSTEC) has operated manned- and unmanned-submersibles to investigate

the deep-sea environment and organisms living there. Many interesting organisms, including piezophilic and piezotolerant bacteria, have been isolated from the mud of deep-sea collected by these submersibles.

One of these, *Shewanella violacea* DSS12 is a psychrophilic and facultatively piezophilic bacterium which was isolated from the mud of the Ryukyu Trench (5,110-m depth) collected by the manned-submersible SHINKAI 6500 (Kato et al. 1995; Nogi et al. 1998). This bacterium displays optimal growth at a temperature of 8°C and pressure of 30 MPa. *S. violacea* is one of the well-investigated piezophiles (Nakasone et al. 2002), and genome analysis of this organism is underway. Especially, the organism can be an excellent source for the study of bacterial adaptation to the environment of high hydrostatic pressure because it showed significant growth both under a condition of high pressure and atmospheric pressure. As an evidence of piezo-adaptation, a pressure-regulated promoter was found in this bacterium (Kato et al. 1996a). Near this promoter, open reading frame homologous to *cydD* gene of *Escherichia coli* was found, and the significance of the gene in bacterial growth under high pressure has been suggested (Kato et al. 1996b). The gene product of *cydD* in *E. coli* is thought to be required for the assembly of respiratory components (Poole et al. 1989, 1993, 1994). Further, the expression of respiratory system was regulated by hydrostatic pressure in this bacterium (Tamegai et al. 1998; Yamada et al. 2000; Nakasone et al. 2001) and in other piezophilic bacterium, *Shewanella* sp. strain DB-172F (Qureshi et al. 1998a, b). These were the first reports about respiratory system of deep-sea bacterium, and the first evidence that expression of gene for respiratory component is regulated by physical parameter, such as hydrostatic pressure. Generally, bacteria have branched respiratory chain. Especially, Shewanellas have many respiratory components to adapt to environmental change (Heidelberg et al. 2003). These facts and the results of previous studies suggest that pressure-regulation for expression of respiratory system in *S. violacea* plays some important roles in bacterial adaptation to high pressure.

Cytochrome *bd* is one of the members of quinol oxidase, distinguished from heme-copper oxidase super family. In *E. coli*, two types of quinol oxidases, cytochrome *bo* and cytochrome *bd* exist, and share a role in respiratory system. Cytochrome *bo* is expressed in log phase and cytochrome *bd* is expressed in stationary phase (Kita et al. 1984a, b). Cytochrome *bd* shows higher affinity for O_2 as compared to cytochrome *bo*, and it acts as terminal oxidase under low oxygen concentration conditions (Kita et al. 1984b). For the biosynthesis of cytochrome *bd*, structural genes (encoded by *cydAB* operon) and genes for assembly of mature enzyme (encoded by *cydDC* operon) (Georgiou et al. 1987; Poole et al. 1989) are required. Expression of *cydAB* in *E. coli* was regulated by ArcA and Fnr, common O_2-regulated transcriptional regulators (Cotter et al. 1997; Govantes et al. 2000), and that of *cydDC* was regulated by NarL (involved in the two-component regulatory system for nitrate respiration) and Fnr (Cook et al. 1997). However, in *S. violacea*, no cytochrome *bd* has been detected spectrophotometrically under the atmospheric pressure even in the stationary phase. Surprisingly, cytochrome *bd* has been detected only under the growth condition of high hydrostatic pressure (Tamegai et al. 1998). Thus, transcriptional regulation of cytochrome *bd* in *S. violacea* may be different from other organisms, and these facts may be important for bacterial adaptation to high pressure.

In the present study, cytochrome *bd*-encoding *cydAB* genes were identified from *S. violacea* DSS12. Transcriptional analysis was carried out for *cydA* and *cydC*, and it was found that transcription of *cydDC* operon was strongly regulated by hydrostatic pressure. Further, some growth conditions were tested whether *d*-type cytochrome was induced under a condition of atmospheric pressure. Role of cytochrome *bd* under high hydrostatic pressure was discussed.

Materials and methods

Organisms

For the transcriptional analyses, *S. violacea* DSS12 was cultured as described previously (Kato et al. 1995). Marine Broth 2216 (Difco, USA, autoclaved and filtered through a 0.22 μm membrane filter) was used as the medium. The seeded medium was placed into sterilized soft plastic packages, and the packages were tightly packed without gas phase. Cultivation was performed in pressure vessels at 8°C under various pressures for 2 days. Under this growth condition, no more O_2 was dissolved to the medium by pressurization, and absolute O_2 concentration in the medium was not changed with pressurization. *S. violacea* cannot grow by fermentation at least in this time scale. Therefore, the cells of the organism could utilize O_2 which was solubilized to the medium initially and were grown under the microaerobic conditions despite of its pressure. For spectrophotometric analyses, large-scale cultivation (1.2 l of the medium) of the organism under a high pressure and microaerobic condition was performed with DEEP-BATH system in JAMSTEC. This system is also capable for cultivation of the organism under high pressure without dissolving of excess O_2, because culture vessel contains seeded medium without gas phase. Also, for spectrophotometric analyses, cultivation of the organism under atmospheric pressure with various conditions was performed in 500-ml shaking flask (200 ml of the medium). *E. coli* was cultivated on LB medium containing appropriate antibiotics if needed.

Isolation of *cydAB* genes from *S. violacea*

In order to isolate *cydAB* cluster, we prepared a hybridization probe for the gene. Based on the

conserved sequences obtained upon alignment of amino acid sequences of *cydA* gene from several bacteria (*E. coli*, *Salmonella typhimurium*, *Vibrio cholerae*), two synthetic degenerate oligonucleotide primers, 5′-CTNGCNGCNTCNGARGGNGARTGG-3′ (LASEGAEW) and 5′-NCCRTANTCNGCNACRAACCANCC-3′(GWFVAEYG), were designed and synthesized to amplify part of the *cydA* gene from *S. violacea*. A fragment of, approximately, 600 bp was amplified by PCR and expected to contain part of the *cydA* gene was cloned in the pCR2.1 vector and its nucleotide sequence was determined. To clone the complete *cydAB* cluster, the partial *cydA* gene fragment was labeled with digoxygenin (DIG) in PCR as a hybridization probe for plaque hybridization. Chromosomal DNA isolated from *S. violacea* was partially digested with *Sau*3A I. These fragments were inserted into the *Bam*H I site of lambda DASH II (Stratagene Co., La Jolla, CA, USA). Then, in vitro packaging of the ligated DNA was performed using GIGAPACK III XL packaging extracts (Stratagene Co.) according to the manufacturer's instructions. The DSS12 λ phage library was screened for plaque hybridization with the *cydA* probe and a positive clone was obtained. The positive clone containing the *cydAB* cluster was purified by several single-plaque isolation steps. The insert in the λ phage was amplified by long PCR and was subcloned into the pCR-Blunt vector (Invitrogen Co., Carlsbad, CA, USA). For sequencing of these cloned fragments, the random shotgun sequencing method was used with a DNA sequencer model 377 (Perkin-Elmer/Applied Biosystems Co., Foster City, CA, USA).

Northern analysis of *cyd* genes

Purification of total RNA from *S. violacea*, RNA hybridization and detection in Northern blot analysis were described previously (Ishii et al. 2002). As a detection probe, *S. violacea cydA* DIG-labeled gene fragment was amplified with PCR using two oligonucleotides 5′-TGTTGGGGATATTTTCGGCGCACCTTTGGC-3′ and 5′-AGGATATAGTAAGAACTGATAGCCAAGACG-3′. In the same way, *cydC* probe was amplified by these primers; 5′-CTGTTGACTCCAATGGCCGCATCATTCCTG-3′ 5′-GCTTAGCTTCAGACTCTGCCAGCTGGGTCC-3′. 10 μg of total RNA extracted from *S. violacea* was loaded in each lane.

Analysis of cytochrome contents

Fractionation of the bacterial cells was carried out as described previously (Tamegai et al. 1998). Spectrophotometric analyses were performed using a Shimadzu UV-1700 spectrophotometer. Protein concentration was determined by the method of Lowry et al. (1951) with slight modifications (Dulley and Grieve 1975).

Accession number

The nucleotide sequences reported here have been deposited in the DDBJ, GenBank, and EMBL databases (accession no. AB196844).

Results

Identification and characterization of *cyd* genes of *S. violacea*

Cytochrome *bd*-encoding *cydAB* genes in *S. violacea* were identified as described in Materials and Methods. Both *cydA* and *cydB* genes showed significant homologies to known *cyd* genes (Table 1, Fig. 1). Further, by detailed comparison of their sequences, it was clearly indicated that ligands for heme b_{558}-binding, a large hydrophilic domain called Q-loop, and the highly conserved region (GWXXXEXGRQPW) in the sequence of *cydA* were well-conserved in *S. violacea* as shown in Fig. 2 (Jünemann 1997).

Transcriptional regulation of *cydAB* and *cydDC* by hydrostatic pressure

As O_2 concentration in the medium was same (as microaerobic condition) despite of its pressure on the bacterial growth conditions of the present study (see Materials and methods), we could estimate the effects of pressure on the biosynthesis of functional cytochrome *bd* complex without influence of O_2 concentration in the medium. Northern analysis of mRNA from *S. violacea* in microaerobic condition grown under the various hydrostatic pressures showed that the gene of *cydA* (*cydAB* operon) was expressed even at atmospheric pressure to some extent (Fig. 3a), and its expression was enhanced with pressurization. On the other hand, the expression of *cydC* gene (*cydDC* operon) was strongly depressed under the growth condition of atmospheric pressure (Fig. 3b).

Expression of *d*-type cytochrome in various culture conditions

Each membrane fraction of *S. violacea* grown under various conditions was analyzed spectrophotometrically as shown in Fig. 4. The reduced-it minus-oxidized difference spectrum of membrane fraction of the organism grown under a high pressure and microaerobic condition clearly showed a trough at around 650 nm, which is specific spectral property of *d*-type cytochrome (a). On the other hand, each membrane fraction of *S. violacea* grown under atmospheric pressure with O_2-limiting condition (b), with shaking (c), with alkaline condition (d) or in the presence of uncoupler pentachlorophenol (e) showed less (or no) trough.

Table 1 *cydAB* genes of *S. violacea*

Genes	Size (bp)	Proposed function	%:FASTA	Accession number
CydA	1,557	Cytochrome *d* ubiquinol oxidase subunit I	87.3% (*Shewanella oneidensis*) 69.6% (*E. coli*)	AE015765 J03930
CydB	1,140	Cytochrome *d* ubiquinol oxidase subunit II	83.9% (*S. oneidensis*) 64.6% (*E. coli*)	AE015765 J03930

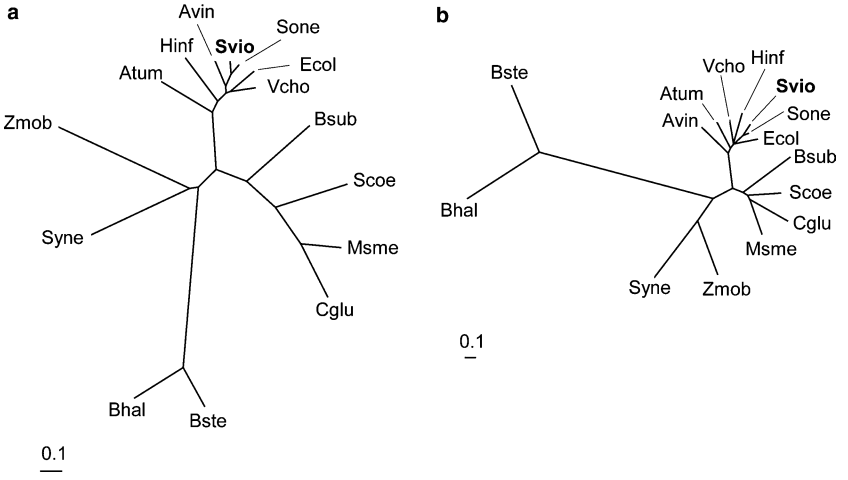

Fig. 1 Phylogenetic trees of CydA (**a**) and CydB (**b**). Svio: *S. violacea*, Sone: *Shewanella oneidensis* (AE015765) Atum; *Agrobacterium tumefaciens* (AF007870), Avin; *Azotobacter vinelandii* (M77787), Bhal; *Bacillus halodurans* (AP001520), Bste; *B. stearothermophilus* (AB016849), Bsub; *B. subtilis* (D83026), Cglu; *Corynebacterium glutamicum* (AB035086), Ecol; *Escherichia coli* (J03939), Hinf; *Haemophilus influenzae* (U32787), Msme; *Mycobacterium smegmatis* (AF196488), Scoe; *Streptomyces coelicolor* (AL034355), Syne; *Synecocystis* sp. PCC6803 (D90904), Vcho; *Vibrio cholerae* (AE004260), Zmob; *Zymomonas mobilis* (AF088897)

Discussion

In the present study, cytochrome *bd*-encoding *cydAB* genes in *S. violacea* were identified. It showed that some well-conserved regions in many *cydA* genes were also conserved in *cydA* of *S. violacea* (Fig. 2). Previous studies (Osborne and Gennis 1999; Zhang et al. 2001) have suggested that highly conserved region (GWXXXEXGRQPW) in the sequence of *cydA* is important for heme b_{595} binding, and located near Q-loop on the periplasm site of the membrane. Q-loop has been suggested to be necessary for quinol oxidation (Dueweke and Gennis 1991). These results indicated that *cydAB* product in *S. violacea* may be functional as quinol oxidase in this organism.

The results of Northern analyses (Fig. 3) showed that hydrostatic pressure was more effective on the regulation of *cydDC* expression than that of *cydAB* expression. These facts suggested that absence of cytochrome *bd* in spectrophotometric analysis (Tamegai et al. 1998) at atmospheric pressure could be caused mainly by absence of *cydDC* products, because *cydDC* has been thought to be required for assembly of functional cytochrome *bd* complex (Poole et al. 1989, 1993, 1994). Further, biosynthesis of some other respiratory component may be controlled by the pressure-regulation of *cydDC* expression in a manner same as in the case of cytochrome *bd*, because *cydDC* product has also been required for the assembly of other respiratory components (Poole et al. 1994). Regulation for expression of *cydDC* may control the biosynthesis of not only the single component but also the high pressure-induced branch of respiratory system in the cells of *S. violacea*.

In the cells of *E. coli*, the expression of both *cydAB* and *cydDC* operon was regulated by O_2 concentration (Cook et al. 1997; Cotter et al. 1997; Govantes et al. 2000). However, in the case of *S. violacea*, *cydDC* was not expressed in microaerobic condition under atmospheric pressure (Fig. 3). These results showed different regulation system rules for the expression of *cydDC* in *S. violacea* and *E. coli*. Experiment using the cells under the aerobic and high-pressure growth conditions may provide us some interesting results. However, such conditions are difficult to maintain due to technical problems.

Cytochrome *bd* is known to be expressed in the cells of *E. coli* grown under the growth conditions of low oxygen concentration (Kita et al. 1984b), alkaline pH (Avetisyan et al. 1992) or presence of protonophore (Bogachev et al. 1993), conditions in which proton-motive-force across the bacterial cytoplasmic membrane is low. However, quite less amount of (or no) cytochrome

Fig. 2 Deduced sequence of CydA in *S. violacea* and comparison with known sequences. *Bold characters* indicate the ligands of heme b_{558}. Underlined region was Q-loop. Highlighted residues show the highly conserved region (GWXXXEXGRQPW). Abbreviations of bacterial names are same as the case of Fig. 1

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Svio    1: MILE-----------EVVELSRFQFAMTAMYHFLFVPLTLGLAFLLAIMESLYVMTGKQI 49
Sone    1: MIVE-----------EVVELSRLQFALTAMYHFLFVPLTLGLAFLLAIMVSLYVMTDKHI 49
Bsub    1: MSELVLA--------------RIQFASTTLFHFLFVPMSIGLVFMVALMETLYLVKKNEL 46
Ecol    1: ------------MLDIVELSRLQFALTAMYHFLFVPLTLGMAFLLAIMETVYVLSGKQI 47
Vcho    1: MSNVSFSRHKGVTMIDVVDLSRLQFALTAMYHFLFVPLTLGMAFLLAIMESLYVMTDKQI 60

Svio   50: YKDMTKFWGKLFGINFALGVTTGLAMEFQFGTNWAYYSHYVGDIFGAPLAIEGLMAFFLE 109
Sone   50: YKDMTKFWGKLFGINFALGVSTGLAMEFQFGTNWSYYSHYVGDIFGAPLAIEGLMAFFLE 109
Bsub   47: YLKMAKFWGHLFLINFAVGVVTGILQEFQFGLNWSDYSRFVGDVFGAPLAIEALLAFFME 106
Ecol   48: YKDMTKFWGKLFGINFALGVATGLTMEFQFGTNWSYYSHYVGDIFGAPLAIEGLMAFFLE 107
Vcho   61: YKDMTKFWGKLFGINFALGVATGLTMEFQFGTNWSYYSHYVGDIFGAPLAIEALVAFFLE 120

Svio  110: STLVGMFFFGWDRFSKRQH-LAVTWLVAFGSNMSALWILIANGWMQHPVGSVFNYETMRM 168
Sone  110: STLVGMFFFGWDRFSKRQH-LAVTWLVALGSNLSALWILVANGWMQNPVGSVFNYETMRM 168
Bsub  107: SIFIGLWIFGWDRLPKKIHALC-IWLVSFGTIMSSFWILTANSFMQEPVG--FTIKNGRA 163
Ecol  108: STFVGLFFFGWDRLGKVQH-MCVTWLVALGSNLSALWILVANGWMQNPIASDFNFETMRM 166
Vcho  121: STFVGLFFFGWDRLSKRQH-LVVTWLVALGSNFSALWILVANGWMQNPVGSEFNFETMRM 179

Svio  169: EMTSWGDVLFNPVAQVKFVHTVASGYVAGAMFVLAISSYYILKKRDLPFARRSFAIAASF 228
Sone  169: EMTSFAEVVFNPVAQVKFVHTVASGYVTGAMFVLAISSYYILKKRDLPFARRSFAIAASF 228
Bsub  164: EMNDFGALITNPQLWVEFPHVIFGALATGAFFIAGVSAFKLLKKKEVPFFKQSFKLAMIV 223
Ecol  167: EMVSFSELVLNPVAQVKFVHTVASGYVTGAMFILGISAWYMLKGRDLAFAKRSFAIAASF 226
Vcho  180: EMVSFADVVLNPVAQVKFVHTVAAGYTTGAMFILGISAYYLLKGRDVAFARRSFAIAASF 239

Svio  229: GLASIISVIVLGDESGYK-VGEVQKVKLAAIEAEWHTEPAPASFTAIGFPNQETMETDYA 287
Sone  229: GMAAILSVIVLGDESGYK-VGEAQRVKLAAIEAEWHTEPAPAAFTAVGFPNQETMETDFA 287
Bsub  224: GLCAGLGVGLSGHMQAEHLM-ESQPMKMAASEGLWEDSGDPAAWTAPATIDTKNEKSSNE 282
Ecol  227: GMAAVLSVIVLGDESGYE-MGDVQKTKLAAIEAEWETQPAPAAFTLFGIPDQEEETNKFA 285
Vcho  240: GMASVLSVIVLGDESGYE-LGEVQRVKLAAIESEWHTEPAPAAFTLFGIPNQEEMRTDYA 298

Svio  288: IKIPFAMGIIATRSLDEQVTGIHDLIADHEIRIRNGIKAYAMLTELRHGNETPELRAAFE 347
Sone  288: VKIPYAMGIVATRSLDEQVTGIHDLISEHEVRIRNGMKAYAMLVKLRAGDTSPELRAEFE 347
Bsub  283: IKVPYALSYLAYQKFSGSVKGMKTLQAEYE-------KIYG-----KGDYIPP-VKTT-- 327
Ecol  286: IQIPYALGIIATRSVDTPVIGLKELMVQHEERIRNGMKAYSLLEQLRSGSTDQAVRDQFN 345
Vcho  299: IKIPFALGIIATRSLDEQVVGLRDLRDEHVERIRTGIYAYDLLERLRAGEKTPENMAAFD 358

Svio  348: EAKVDLGYGFLLKRYTDKVVDATEEQIVAAAKDSIPNVAPMFWTFRIMVGSGMIMLLV-F 406
Sone  348: AAKVDLGYGLLLKRYTNNVVDATEEQIKAAAKDSIPNVAPMFWSFRVMVGLGFVMLFV-F 406
Bsub  328: ----------------------------------------FWSFRIMVGAGVVMILAAL 346
Ecol  346: SMKKDLGYGLLLKRYTPNVADATEAQIQQATKDSIPRVAPLYFAFRIMVACGFLLLAI-I 404
Vcho  359: EVKHDLGYGLLLKRYTDKVTDATEEQIQAAADDSIPTVWPLFWSFRIMVACGFIMLVV-F 417

Svio  407: AAAFWQSTRHRIEEKPWVLKAALYSLPLPWIAIECGWFVSEYGRQPWTISEVLPTFMSAS 466
Sone  407: AAAFWQSTRHQIEENKWVLKAALFSLPLPWIAIECGWFVAEYGRQPWTISEVLPTFMSAS 466
Bsub  347: G-GLWLNRRKKLENSKWYLRIMIALISFPFLANSAGWIMTEIGRQPWTVMGLMTTAQSVS 405
Ecol  405: ALSFWSVIRNRIGEKKWLLRAALYGIPLPWIAVEAGWFVAEYGRQPWAIGEVLPTAVANS 464
Vcho  418: GAAFIQTCRQKIEQKKWVLKAALFSIPLPWIAVETGWFVAEYGRQPWAVGEILPVNVAAS 477

Svio  467: SVSVG-DLWFSIISILTFYTVLLVIEAYLMIKFARMGPSSLKTGRYHFENLDA------- 518
Sone  467: SLTTG-DLWFSIISISLFYTVLLVIEIFLMLKFARLGPSSLKTGRYHFEKLEA------- 518
Bsub  406: PNVTAGSLLFSIIAPGVMYM---ILGALLVFLFIREIKKGAEHDNHHDVPVSTDPFSQEV 462
Ecol  465: SLTAG-DLIFSMVLICGLYTLFLVAELFLMFKFARLGPSSLKTGRYHFEQSSTTTQPAR- 522
Vcho  478: ALSAG-EIITSMLAILALYTIFLIAEVYLMVKFTRKGPSSLKTGRYHFEQGAESVQDQVN 536

Svio  519: ------ 519
Sone  519: ------ 519
Bsub  463: YHGISS 468
Ecol  523: ------ 523
Vcho  537: RQ-VEA 541
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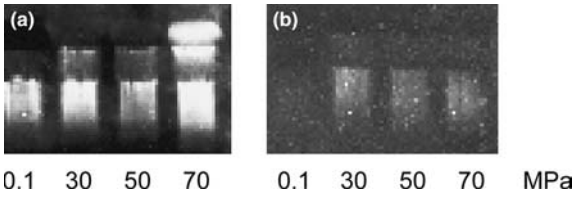

Fig. 3 Northern analysis for *cydA* (**a**) and *cydC* (**b**) in the cells of *S. violacea* grown under the various hydrostatic pressures

bd is detected in the membrane fraction of *S. violacea* grown under atmospheric pressure (Fig. 4). From the spectroscopic analysis (Tamegai et al. 1998), and results of the present study, it is obvious that functional cytochrome *bd* expression in *S. violacea* is the special event only under the growth condition of high hydrostatic pressure, regardless of the intensity of proton-motive-force.

Why is cytochrome *bd* required in high-pressure conditions? There are three positive hypotheses. One is that high pressure may have a direct effect on the respiratory enzyme activity. Bacterial terminal oxidases can be classified into two categories. One is quinol oxidases, and the other is cytochrome *c* oxidases. Nakasone et al. have shown that the transcription of another quinol oxidase-encoding *cyo* operon in *S. violacea* was also enhanced by high pressure (Nakasone et al. 2001). These findings and the results of this study suggests that respiratory system including quinol oxidases may be better system suited to high pressure condition as compared to other systems including cytochrome *c* oxidases. The former system may have a smaller activation volume than the latter system, because small volume of metabolic system is favorable under high hydrostatic pressure (Abe and Horikoshi 2000; Abe and Iida 2003). A second possibility is that there is an indirect relationship between high pressure and expression of the terminal oxidase. That is, high hydrostatic pressure may cause some physiological changes in the bacterial cells, and this change may trigger expression of cytochrome *bd*. High hydrostatic pressure causes many physiological phenomena. For example, Abe and Horikoshi have showed that vacuole of yeasts was acidified by pressurization (Abe and Horikoshi 1995). They have explained that acidification of cytosol of yeasts may occur by pressurization, and to maintain the cytosolic pH, yeast vacuole may serve as a proton sequestrant under high-pressure condition. It is possible to assume that acidification of cytosol by pressurization occurs also in the

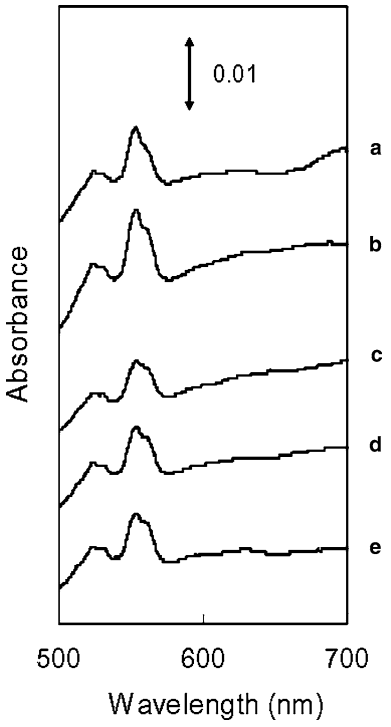

Fig. 4 Reduced-*minus*-oxidized difference spectra of the membrane fractions from *S. violacea*. Each fraction was obtained from the cells grown under a pressure of 50 MPa with O_2-limiting condition (**a**), grown under a pressure of 0.1 MPa with O_2-limiting condition (**b**), grown under a pressure of 0.1 MPa with shaking (**c**) grown under a pressure of 0.1 MPa and alkaline condition (pH 9.0) with shaking (**d**) and grown under a pressure of 0.1 MPa in the presence of uncoupler pentachlorophenol (0.1 mM) with shaking (**e**). The protein concentration in each fraction was 1.0 mg/ml. Reduced forms were prepared by adding a small amount of $Na_2S_2O_4$, and oxidized forms were prepared by adding a small amount of $(NH_4)_2S_2O_8$

cells of *S. violacea*, and change of H^+ concentration in the cells may affect the function and expression of oxidative phosphorylation system. A third hypothesis is the suitability between terminal oxidase and membrane lipids. Usually composition of unsaturated fatty acids in membrane lipid is increased in piezophiles when the cells are grown in high-pressure condition (DeLong and Yayanos 1985). Because terminal oxidases are transmembrane proteins associated with membrane lipids, cytochrome *bd* may be more suitable with such membrane than the other terminal oxidases.

In the present study, we identified *cydAB* gene, structural gene for cytochrome *bd* from deep-sea bacterium *S. violacea* DSS12. Further, we showed that significant amount of cytochrome *bd* expression is specific event for the organism under the growth condition of high pressure, and this pressure-dependent regulation was caused mainly by the pressure-regulation of the expression of *cydDC* at transcriptional level. These results suggested that pressure-regulation for expression of respiratory system in *S. violacea* plays some important roles in bacterial adaptation to high hydrostatic pressure.

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