Extremophiles (2014) 18:617–627
DOI 10.1007/s00792-014-0644-y

ORIGINAL PAPER

Correlation between the optimal growth pressures of four *Shewanella* species and the stabilities of their cytochromes c_5

Misa Masanari · Satoshi Wakai · Manabu Ishida · Chiaki Kato · Yoshihiro Sambongi

Received: 16 January 2014 / Accepted: 23 March 2014 / Published online: 4 April 2014

Abstract *Shewanella* species live widely in deep-sea and shallow-water areas, and thus grow piezophilically and piezosensitively. Piezophilic and psychrophilic *Shewanella benthica* cytochrome c_5 (SB cytc_5) was the most stable against guanidine hydrochloride (GdnHCl) and thermal denaturation, followed by less piezophilic but still psychrophilic *Shewanella violacea* cytochrome c_5 (SV cytc_5). These two were followed, as to stability level, by piezosensitive and mesophilic *Shewanella amazonensis* cytochrome c_5 (SA cytc_5), and piezosensitive and psychrophilic *Shewanella livingstonensis* cytochrome c_5 (SL cytc_5). The midpoint GdnHCl concentrations of SB cytc_5, SV cytc_5, SL cytc_5, and SA cytc_5 correlated with the optimal growth pressures of the species, the correlation coefficient value being 0.93. A similar trend was observed for thermal denaturation. Therefore, the stability of each cytochrome c_5 is related directly to its host's optimal growth pressure. Phylogenetic analysis indicated that Lys-37, Ala-41, and Leu-50 conserved in piezosensitive SL cytc_5 and SA cytc_5 are ancestors of the corresponding residues in piezophilic SB cytc_5 and SV cytc_5, Gln, Thr, and Lys, respectively, which might have been introduced during evolution on adaption to environmental pressure. The monomeric *Shewanella* cytochromes c_5 are suitable tools for examining protein stability with regard to the optimal growth pressures of the source species.

Keywords Correlation · Cytochrome c_5 · Growth pressure · Protein stability · *Shewanella*

Abbreviations

SA cytc_5	*Shewanella amazonensis* cytochrome c_5
SB cytc_5	*Shewanella benthica* cytochrome c_5
SL cytc_5	*Shewanella livingstonensis* cytochrome c_5
SV cytc_5	*Shewanella violacea* cytochrome c_5

Introduction

Shewanella species, gamma-proteobacteria, are distributed in nature from deep-sea to shallow-water areas (Hau and Gralnick 2007). A phylogenetic tree based on the *Shewanella* 16S rRNA gene sequence indicates that the species can be classified into two groups, Group 1 and Group 2 (Kato and Nogi 2001). Group 1 *Shewanella* species are mostly isolated from deep-sea, and Group 2 ones from shallow-water areas. For example, Group 1 *Shewanella violacea* DSS12 and *Shewanella benthica* DB6705 were

Communicated by S. Albers.

M. Masanari · Y. Sambongi (✉)
Graduate School of Biosphere Science, Hiroshima University, 1-4-4 Kagamiyama, Higashi-Hiroshima, Hiroshima 739-8528, Japan
e-mail: sambongi@hiroshima-u.ac.jp

S. Wakai
Organization of Advanced Science and Technology, Kobe University, 1-1 Rokkodai, Nada, Kobe 657-8501, Japan

M. Ishida
Top Runner Incubation Center for Academia-Industry Fusion, Department of Bioengineering, Nagaoka University of Technology, 1603-1 Kamitomioka, Nagaoka, Niigata 940-2188, Japan

C. Kato
Institute of Biogeosciences, Japan Agency for Marine-Earth Science and Technology, 2-15 Natsushima-cho, Yokosuka, Kanagawa 237-0061, Japan

Table 1 *Shewanella* species and cytochromes c_5 used in this study

Species and strain	Isolation depth (m)	Optimal growth pressure (MPa) and piezophilicity	Optimal growth temperature (°C) and psychrophilicity	Reference	Cytochrome c_5 name
S. benthica DB6705	6,356	50 piezophilic	4 psychrophilic	Kato et al. 1995	SB cytc_5
S. violacea DSS12	5,110	30 piezophilic	8 psychrophilic	Nogi et al. 1998	SV cytc_5
S. livingstonensis Ac10	0	0.1[a] piezosensitive	20 psychrophilic	Kulakova et al. 1999	SL cytc_5
S. amazonensis SB2B	0	0.1[a] piezosensitive	37 mesophilic	Venkateswaran et al. 1998	SA cytc_5

[a] The actual value is not available, but it can be speculated to be as it is (see details in Introduction)

obtained from the Ryukyu trench at a depth of 5,110 m (Nogi et al. 1998) and from the Japan Trench at a depth of 6,356 m (Kato et al. 1995), respectively. They show piezophilicity, growing optimally at 30 and 50 MPa, respectively (Table 1). On the other hand, Group 2 *Shewanella livingstonensis* Ac10 (Kulakova et al. 1999) and *Shewanella amazonensis* SB2B (Venkateswaran et al. 1998) were isolated at a depth of 0 m from the Antarctic Sea surface and Amazon River Delta, respectively. Although *S. livingstonensis* and *S. amazonensis* have not been examined as to growth pressure, it is likely that they optimally grow at 0.1 MPa (Table 1), because all the Group 2 *Shewanella* species examined were found to be piezosensitive (Kato and Nogi 2001).

Proteins from piezophilic *Shewanella* species remain active at high pressure, compared with the homologous counterparts from piezosensitive ones (Kato et al. 2008). For example, 3-isopropylmalate dehydrogenase from piezophilic *S. benthica* is more stable and active under high pressure conditions than the homologous enzyme from piezosensitive *Shewanella oneidensis* (De Poorter et al. 2004; Kasahara et al. 2009), indicating a stabilization mechanism against pressure in the former. Another comparative study has been performed on single-stranded DNA-binding proteins from piezophilic and piezosensitive *Shewanella* species (Chilukuri and Bartlett 1997; Chilukuri et al. 2002). However, these proteins are multimeric; thus their protein stability might involve both subunit assembly/dissociation and polypeptide folding/unfolding. In this context, monomeric proteins must provide substantial clues as to protein stability only in terms of folding/unfolding, which is important for understanding of the intrinsic properties of a polypeptide.

Cytochromes *c* are well-characterized monomeric proteins. It is known that pressure affects the expression levels (Yamada et al. 2000) and electron transfer activities (Ohke et al. 2013) of cytochromes *c*. Nevertheless, little study has been carried out on the stability-pressure relationship in terms of biophysics and structural biology. For this purpose, piezophilic *Shewanella* cytochromes c_5 appear to be suitable monomeric proteins. We have found that piezophilic *S. violacea* cytochrome c_5 (SV cytc_5) exhibits higher thermal stability than homologous cytochromes c_5 from piezosensitive *S. livingstonensis* (SL cytc_5) and *S. amazonensis* (SA cytc_5) (Ogawa et al. 2007; Takenaka et al. 2010; Masanari et al. 2011). These results indicate that the pressure adaptation of these *Shewanella* species reflects the differences in the thermal stability of their cytochromes c_5. However, the data obtained so far are too sparse to correlate *Shewanella* optimal growth pressure with cytochrome c_5 stability, because only two growth pressures have been examined (*S. violacea*, 30 MPa; *S. livingstonensis* and *S. amazonensis*, 0.1 MPa).

In this study, we introduced another piezophile, *S. benthica*, which grows optimally at 50 MPa, to our comparative study (Table 1). *S. benthica* cytochrome c_5 (SB cytc_5) exhibits 93, 80, and 84 % amino acid sequence identity with SV cytc_5, SL cytc_5, and SA cytc_5, respectively. Here we measured and compared the denaturant and thermal stabilities of the four *Shewanella* cytochromes c_5 in order to find any correlation between growth pressure and protein stability.

Materials and methods

Cloning and expression of the SB cytc_5 gene

S. benthica DB6705 cells were grown in a liquid medium comprising marine broth 2216 (Difco) at 10 °C and 50 MPa (Kato et al. 1995). Chromosomal DNA was extracted from the cells by the standard method, and used as a template to clone the SB cytc_5 gene through a PCR experiment. PCR primers were designed based on the genome sequence of *S. benthica* KT99 (Forward primer; 5′-GCGAATTCATGAAAAAACTGTTAGCTATG-3′, reverse primer; 5′-GCGTCGACTTACTTAGACATGAACTCG-3′) to amplify the gene encoding mature SB cytc_5 from the chromosomal DNA of *S. benthica* DB6705.

The amplified gene for the mature SB cytc_5 region was fused with the gene for the signal sequence of *Pseudomonas aeruginosa* cytc_{551} (PA cytc_{551}) in the 5′ region, and then the fusion gene was inserted into the pKK223-3 vector (ampicillin resistance) under control of the *tac* promoter, as

described previously (Ogawa et al. 2007). The resulting plasmid was designated as pKK-SB2. This was then used for expression of the SB cytc_5 gene in *Escherichia coli* JCB387. Plasmid pEC86 (chloramphenicol resistance), carrying the *ccmABCDEFGH* genes for cytochrome *c* maturation proteins, was co-transformed into *E. coli* JCB387 together with pKK-SB2.

Protein preparation

E. coli JCB387 cells carrying the pKK-SB2 and pEC86 plasmids were grown in 2 l of liquid Luria–Bertani medium containing 100 μg ml^{-1} ampicillin and 34 μg ml^{-1} chloramphenicol in 5 l flasks, which were then shaken aerobically at 37 °C for 20 h. A periplasmic extract of the resulting *E. coli* cells was obtained by the cold osmotic shock method, as described previously (Sambongi et al. 1996).

The SB cytc_5 protein was first purified by DEAE Toyopearl (3.5 × 10 cm; Tosoh, Tokyo) column chromatography, followed by Hi-Trap Q and SP column (1.6 × 2.5 cm; GE Healthcare, Tokyo), as described previously (Ogawa et al. 2007). Finally, gel-filtration chromatography was performed on a Superdex 75 column (1.6 × 60 cm; GE Healthcare), with equilibration and elution with 10 mM Tris–HCl buffer, pH 8.0, at 4 °C. The purified SB cytc_5 protein solution was subjected directly to N-terminal sequencing with an automatic peptide sequencer (Applied Biosystems, Tokyo). The protein concentrations were determined by the Bradford method using bovine serum albumin as a standard (Bradford 1976).

The SV cytc_5, SL cytc_5, and SA cytc_5 proteins were prepared by the methods described previously (Ogawa et al. 2007; Takenaka et al. 2010; Masanari et al. 2011).

Cyclic voltammetry

The cyclic voltammetry (CV) method was applied to the purified SB cytc_5, SV cytc_5, SL cytc_5, and SA cytc_5 proteins in order to determine their midpoint redox potential (E_m) values relative to the standard hydrogen electrode (SHE) in 10 mM HEPES–NaOH (pH 7.0) at 25 °C. The experimental procedure and data analysis were described in detail previously (Takeda et al. 2009).

Denaturant stability

The stability of air-oxidized SB cytc_5, SV cytc_5, SL cytc_5, and SA cytc_5 against a chemical denaturant was assessed by means of guanidine hydrochloride (GdnHCl)-induced denaturation experiments. Absorption spectra (300 to 500 nm) of the protein samples were obtained using a 1-cm path length cuvette in a V-530 spectrophotometer (Jasco, Tokyo). The proteins (40 μM) were incubated in 20 mM sodium acetate buffer (pH 4.0) containing varying concentrations of GdnHCl at 25 °C for 2 h before the measurements in order to equilibrate the proteins with the denaturant. The absorbance at 396 nm of each protein solution was measured at 25 °C. The ΔG_{H_2O} (free energy change in water during GdnHCl denaturation) value and m (dependence of ΔG on the GdnHCl concentration) value were determined by least-square fitting of the data in the transition region using the equation $\Delta G = \Delta G_{H_2O} - m[\mathrm{GdnHCl}]$ (Pace 1990). The C_m (midpoint GdnHCl concentration of denaturation) value was the GdnHCl concentration at which the ΔG value became zero.

Thermal stability

Thermal denaturation experiments were performed by differential scanning calorimetry (DSC). A solution of an air-oxidized SB cytc_5 protein was dialyzed extensively against 50 mM sodium acetate buffer, pH 4.0, before the measurements. Under this pH conditions, thermal denaturation of the SB cytc_5 occurred in a reversible manner, providing equilibrium thermodynamic parameters. Hence, this pH was used to measure thermal stability. A degassed protein solution (0.5 mg ml^{-1}) was loaded into a calorimeter cell, followed by heating from 10 to 110 °C at about 28 psi, at a heating rate of 1 °C min^{-1}, with a calorimeter VP-DSC (Microcal, Piscataway). Buffer–buffer baselines were recorded at the same heating rate and then subtracted from the sample curves to obtain heat capacity (C_p) curves. After fitting the data with MATHEMATICA 7.0, the transition temperature during thermal denaturation (T_m) and the calorimetric enthalpy change (ΔH) at T_m were obtained.

The heat capacity change accompanying the thermal denaturation, ΔC_p, was estimated as a function of temperature, as described previously (Uchiyama et al. 2002). From these values, thermodynamic parameters (free energy change ΔG, enthalpy change ΔH, and entropy change ΔS) at a given temperature were calculated using the following equations:

$$\Delta H(T) = \Delta H - \Delta C_p(T)(T_m - T)$$
$$\Delta S(T) = \Delta H/T_m - \Delta C_p(T)\ln(T_m/T)$$
$$\Delta G(T) = \Delta H(T) - T\Delta S(T)$$

These calculations facilitated comparison of thermodynamic protein stabilities, as described previously (Uchiyama et al. 2002; Ogawa et al. 2007). The resulting thermodynamic parameters for SB cytc_5 were compared with those for SV cytc_5, SL cytc_5, and SA cytc_5, which had been obtained under the same experimental conditions previously (Ogawa et al. 2007; Takenaka et al. 2010; Masanari et al. 2011).

Phylogenetic analysis of cytochromes c_5

The amino acid sequences of 16 mature cytochromes c_5 from *Shewanella* species and *Pseudomonas aeruginosa*, retrieved from the GenBank, were aligned by means of ClustalW using its default settings before tree building. The alignment was adjusted manually to correct for gaps. A total of 76 residue positions were selected to build a phylogenetic tree using the neighbor-joining method. In order to predict the amino acid sequence at each branch (node), ancestral sequences were inferred from the alignment and the molecular phylogenetic tree of cytochromes c_5 using the CODEML program of the PAML package (Ver. 4.7a) (Yang 1997) for the maximum likelihood method. The WAG substitution model was used as the substitution matrix for amino acid residues.

Results and discussion

Expression of SB cytc_5

In order to clone the SB cytc_5 gene from *S. benthica* DB6705, the PCR primers used in this study were based on the genome sequence of *S. benthica* KT99 that had been deposited in the GenBank. During the gene cloning, we realized that two amino acid residues differed in the mature SB cytc_5 sequences from the two strains; Thr-49 and Phe-56 in the KT99 strain were, respectively, replaced with Ala and Leu in the DB6705 strain. Hereafter, we adopted the DB6705 sequence, because the following experiments were carried out using the SB cytc_5 from this strain.

The recombinant SB cytc_5 expressed in *E. coli* JCB387 cells was recovered in the periplasmic extract after cold osmotic shock of the cells, but not in the membrane or cytoplasmic fractions. The N-terminal amino acid sequence of the recombinant SB cytc_5 expressed in the *E. coli* periplasm was determined to be Gln-Glu-Gly-Lys-Ala, which is identical to the putative sequence for the mature protein. This indicates that the signal peptide of PA cytc_{551} artificially introduced to the present fusion protein was correctly processed in the *E. coli* cells.

Redox potential analysis

Electrochemical properties, i.e., the redox potential (E_m) values of SB cytc_5, SV cytc_5, SL cytc_5, and SA cytc_5, were examined by CV. The E_m values of SB cytc_5, SV cytc_5, SL cytc_5, and SA cytc_5 were 283.7 ± 3.7, 284.3 ± 3.6, 281.0 ± 3.6, and 283.3 ± 4.2 mV, respectively. These values did not differ significantly, indicating that these cytochromes c_5 have similar functions in the host cells. These four *Shewanella* cytochromes c_5 were then examined in order to find any difference in stability.

Denaturant stability

The denaturant stabilities of the four *Shewanella* cytochromes c_5 were compared. Absorption spectra (350–450 nm) of the air-oxidized SB cytc_5, SV cytc_5, SL cytc_5, and SA cytc_5 proteins in the presence of various GdnHCl concentrations at pH 4.0 and 25 °C were obtained (Fig. 1a–d). The Soret absorption peaks, which are specifically derived from the heme group, blue-shifted with an increase in the GdnHCl concentration. Cytochrome *c* usually has a hexacoordinate heme and thus shows a Soret absorption peak at 410 nm. The coordination state changes to a pentacoordinate one with GdnHCl denaturation, and the Soret absorption peak shifts to around 400 nm, as observed for the natively pentacoordinated cytochrome *ć* (Inoue et al. 2011). This also occurred for the four *Shewanella* cytochrome c_5 proteins during GdnHCl denaturation, and the existence of isosbestic points (arrows in Fig. 1a–d) indicated that the observed GdnHCl denaturation can be described as a simple two-state process.

In order to further visualize the effect of GdnHCl, difference spectra were obtained by subtraction of the spectra in the absence of GdnHCl from those in the presence of various concentrations of GdnHCl. There were positive peaks at 396 nm and negative ones at 413 nm (Fig. 1e–h). The GdnHCl-induced denaturation was sharply reflected in the GdnHCl concentration-dependence at the 396-nm peak, which was used to monitor protein denaturation in the following analysis.

The difference spectra data obtained at 396 nm of the SB cytc_5, SV cytc_5, SL cytc_5, and SA cytc_5 proteins as a function of GdnHCl concentration (Fig. 1e–h) were normalized (Fig. 2a). The data adequately fitted to a two-state model for protein denaturation. For further thermodynamic analysis, ΔG values were plotted as a function of GdnHCl concentration (Fig. 2b). The *m* values, i.e., the slopes in Fig. 2b representing the dependence of ΔG on the GdnHCl concentration, for SB cytc_5, SV cytc_5, SL cytc_5, and SA cytc_5 were 9.23 ± 0.97, 9.10 ± 0.99, 9.76 ± 1.12, and 9.58 ± 0.86 kJ mol^{-1} M^{-1}, respectively. Every two sample *t* test revealed that the *m* values of the four proteins were the same, indicating that the GdnHCl denaturation of these proteins occurred in a similar cooperative manner. Therefore, these proteins can be adequately compared with each other.

The C_m values, representing the GdnHCl concentration at which the fraction of the denatured protein is 0.5 in Fig. 2a, for SB cytc_5, SV cytc_5, SL cytc_5, and SA cytc_5 were 1.99 ± 0.08, 1.84 ± 0.06, 1.38 ± 0.06, and 1.54 ± 0.10 M, respectively. These values were

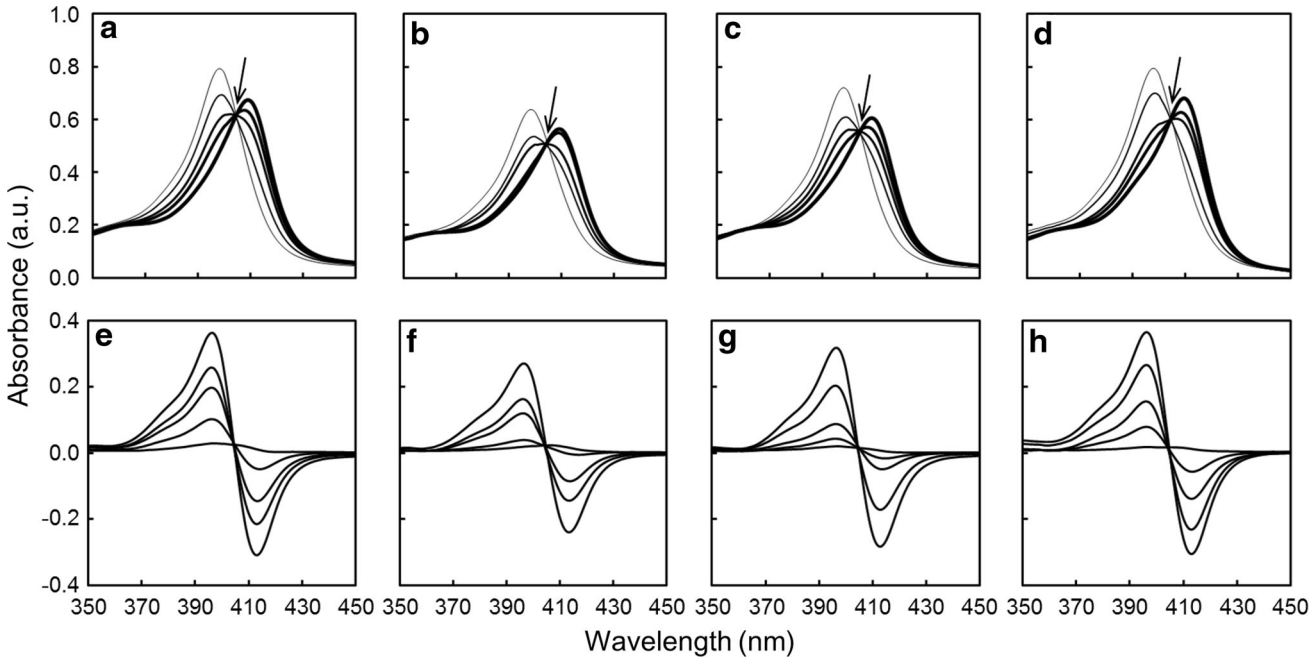

Fig. 1 Representative GdnHCl-induced changes in absorption spectra of the four *Shewanella* cytochrome c_5 proteins. Changes in the absorption spectra of SB cytc_5 (**a**), SV cytc_5 (**b**), SL cytc_5 (**c**), and SA cytc_5 (**d**) are shown in the presence of various GdnHCl concentrations. The line width changes from thickest (0 M GdnHCl) to thinnest (higher concentration of GdnHCl). *Arrows* indicate the isosbestic points. Difference spectra of SB cytc_5 (**e**), SV cytc_5 (**f**), SL cytc_5 (**g**), and SA cytc_5 (**h**) are shown after subtraction of the spectra in the absence of GdnHCl from those in the presence of various GdnHCl concentrations

significantly different; even the closest two, those for SB cytc_5 and SV cytc_5, exhibited $P < 0.05$ with the t test. In addition, these C_m values correlated strongly with the optimal growth pressures of the source species, the correlation coefficient value being 0.93 (Fig. 2c). Previously, dihydrofolate reductases from various piezophilic and piezosensitive *Shewanella* species were examined as monomeric proteins in a similar manner to as in the present study (Murakami et al. 2011). However, their protein stabilities against chemical denaturation did not correlate with the optimal growth pressures of the sources. Therefore, the present results were the first observation of correlation between the protein stability against chemical denaturation and the optimal growth pressures of source organisms. Although both cytochrome c_5 and dihydrofolate reductase are monomeric proteins, their stability mechanisms differ, which might be due to differences in domain number, cofactor, and/or substrate. There is presently no explanation as to why the stability mechanisms are apparently different.

The ΔG_{H_2O} values, representing the conformational stability of a protein in water, obtained in the GdnHCl-induced denaturation experiments for SB cytc_5, SV cytc_5, SL cytc_5, and SA cytc_5 were 19.77 ± 2.62, 17.26 ± 2.09, 14.11 ± 1.05, and 16.05 ± 1.87 kJ mol^{-1}, respectively. Although most similar two, those for SV cytc_5 and SA cytc_5 exhibited $P > 0.18$ with the t test indicating that they are the same, every other pair exhibited the significant difference, i.e., at most $P < 0.05$. Therefore, the resulting ΔG_{H_2O} values correlated with the optimal growth pressures of the source species, the correlation coefficient value being 0.87 (Fig. 2d). A similar trend as for the C_m values was thus observed.

Thermal stability

The thermal stability of SB cytc_5 was measured and compared with those of the three other *Shewanella* cytochromes c_5 that had been published previously (Ogawa et al. 2007; Takenaka et al. 2010; Masanari et al. 2011). An excess molar heat capacity curve for the air-oxidized SB cytc_5 protein was obtained through DSC measurements at pH 4.0 (Fig. 3). At this pH value, equilibrium thermodynamic parameters were obtained because thermal denaturation reversibly occurred, similar to for the other three cytochromes c_5. Under the conditions used, the value for the transition temperature during thermal denaturation (T_m) of SB cytc_5 was 85.3 °C. This was higher than those of SV cytc_5, SL cytc_5, and SA cytc_5 (Table 2), indicating the highest stability of the SB cytc_5 from the most piezophilic *Shewanella* strain among the four examined here.

From the curve for the observed excess molar heat capacity (C_p; Fig. 3), we obtained temperature-dependent

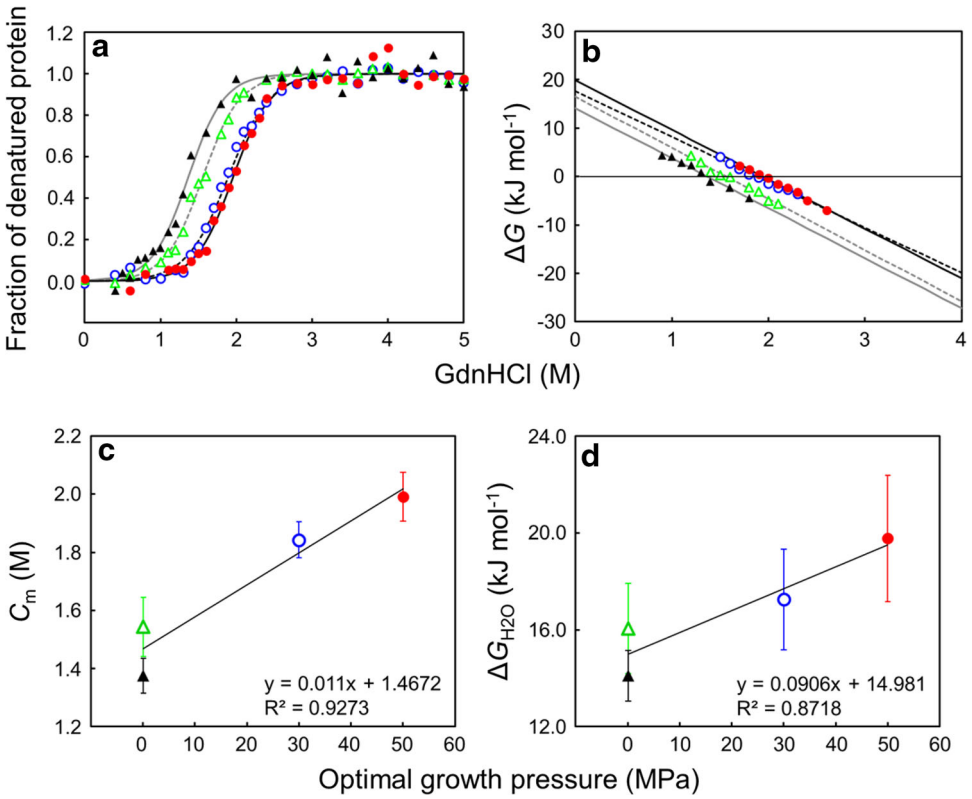

Fig. 2 Analysis of GdnHCl-induced denaturation. Representative normalized denaturation curves (**a**). The fractions of the denatured proteins are plotted as a function of GdnHCl concentration for SB cytc_5 (*red closed circles*), SV cytc_5 (*blue open circles*), SL cytc_5 (*black closed triangles*), and SA cytc_5 (*green open triangles*) with a GdnHCl concentration interval of at least 0.2 M. Fitting curves obtained from the data with a concentration interval of at least 0.1 M for each experiment are also shown. Representative dependence of ΔG values on the GdnHCl concentration (**b**). Least-square fits of the data for the transition regions are shown. Correlations between optimal growth pressures of the *Shewanella* species and the denaturant stabilities of their cytochromes c_5 (**c** for C_m value and panel **d** for ΔG_{H_2O} value). Data symbols used in **b**, **c**, and **d** are same as those in **a**. The data values are the averages for at least three independent measurements with standard deviations. Least-square fitting curves are also shown with the equations and correlation coefficient values (R^2)

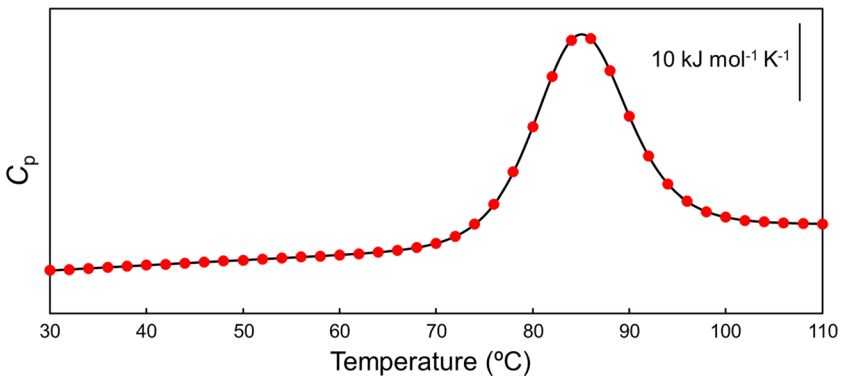

Fig. 3 Thermal denaturation, as monitored by DSC. The heat capacity value (C_p) of SB cytc_5 is shown as a function of temperature with a temperature interval of 2 °C. A fitting curve obtained from the data with a temperature interval of 0.5 °C is also shown

heat capacity changes (ΔC_p) accompanying the thermal denaturation. Using the ΔC_p value at the T_m of the SL cytc_5 protein (72.6 °C, T_m^* at pH 4.0), $\Delta C_p(T_m^*)$, other thermodynamic parameters at 72.6 °C, $\Delta G(T_m^*)$, $\Delta H(T_m^*)$, and $\Delta S(T_m^*)$, could be compared among the four cytochrome c_5 proteins (Table 2). Since the T_m value is defined as being equivalent to the temperature at which ΔG becomes zero, the $\Delta G(T_m^*)$ value of the SL cytc_5 protein was zero. At 72.6 °C, the $\Delta G(T_m^*)$ values of SB cytc_5, SV cytc_5, and SA cytc_5 were 12.6, 10.6, and 6.4 kJ mol^{-1}, respectively

Table 2 Protein stability against thermal denaturation

	T_m (°C)	$\Delta G(T_m^*)$ (kJ mol^{-1})	$\Delta H(T_m^*)$ (kJ mol^{-1})	$\Delta S(T_m^*)$ (kJ mol^{-1} K^{-1})	$\Delta C_p(T_m^*)$ (J mol^{-1} K^{-1})
SB cytc_5	85.3 ± 0.3	12.6 ± 0.5	353.2 ± 13.6	0.99 ± 0.04	1.5 ± 4.4
SV cytc_5[a]	84.5 ± 0.0	10.6 ± 0.1	314.0 ± 2.4	0.88 ± 0.01	2.8 ± 0.6
SL cytc_5[a]	72.6 ± 0.2	0.0 ± 0.0	272.7 ± 1.7	0.79 ± 0.00	7.5 ± 3.5
SA cytc_5[b]	79.8 ± 0.1	6.4 ± 0.1	310.7 ± 2.8	0.88 ± 0.01	3.0 ± 1.1

The values are the averages for at least three independent measurements with standard deviations

[a] The data from Masanari et al. 2011 were calculated

[b] The data from Takenaka et al. 2010 were calculated

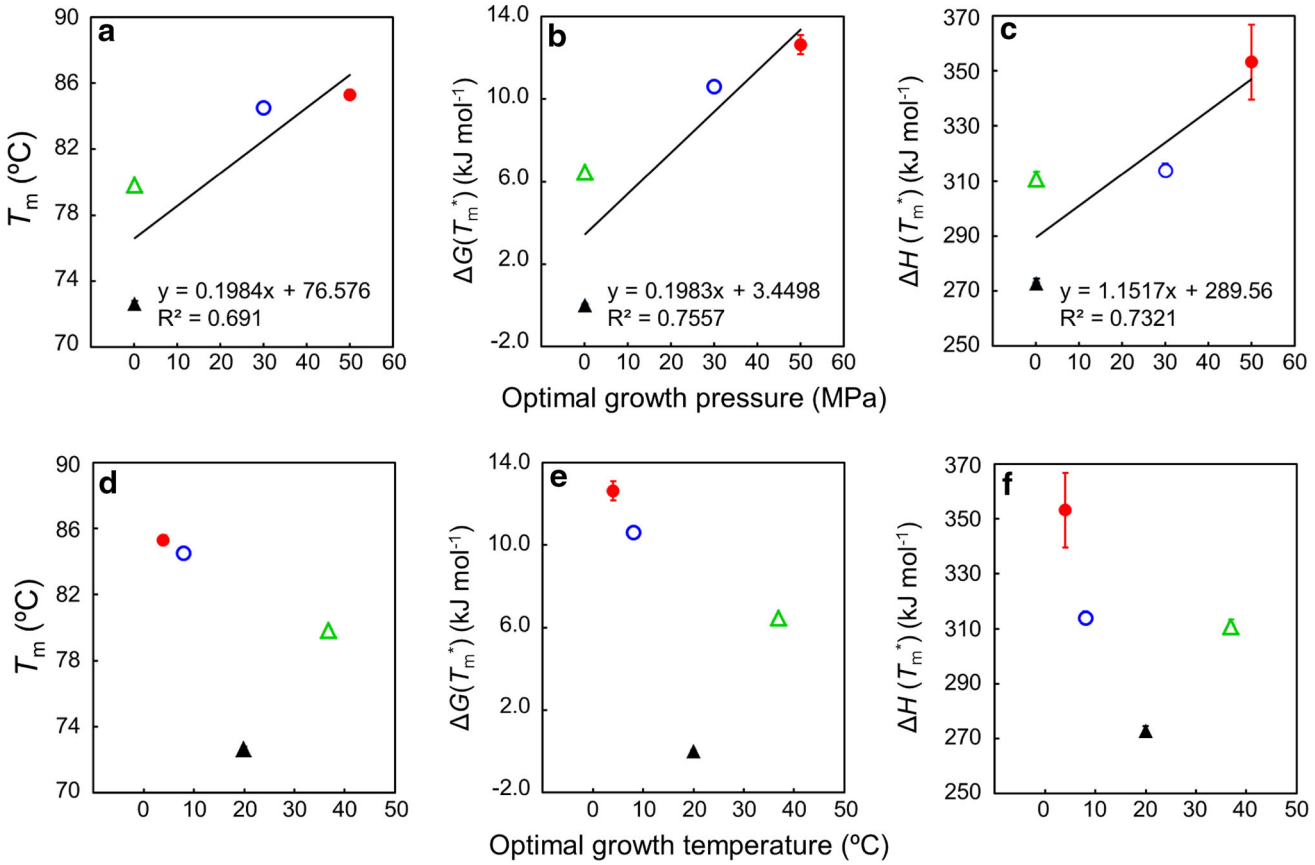

Fig. 4 Relationship between optimal growth conditions of *Shewanella* species and the thermal stability of their cytochromes c_5. Correlation between optimal growth pressure and thermal stability (**a** for T_m, **b** for $\Delta G(T_m^*)$, and **c** for $\Delta H(T_m^*)$ values). Data symbols used in this figure are same as those in **a** of Fig. 2. The data values are the averages for at least three independent measurements with standard deviations. Least-square fitting curves are also shown with the equations and correlation coefficient values (R^2). Relationship between optimal growth temperature and thermal stability (**a** for T_m, **b** for $\Delta G(T_m^*)$, and **c** for $\Delta H(T_m^*)$ values). Data symbols used in this figure are same as those in **a** of Fig. 2

(Table 2). These positive $\Delta G(T_m^*)$ values compared with that of SL cytc_5 indicate that the thermal stabilities of SB cytc_5, SV cytc_5, and SA cytc_5 are higher than that of SL cytc_5. Similar to the C_m values for GdnHCl-induced denaturation, the T_m and $\Delta G(T_m^*)$ values of the four *Shewanella* cytochromes c_5 differed significantly, i.e., at most $P < 0.01$. In addition the T_m and $\Delta G(T_m^*)$ values correlated with the optimal growth pressures of the source species with correlation coefficient values of 0.69 and 0.76, respectively (Fig. 4a–b).

The $\Delta G(T_m^*)$ value can be further dissected into enthalpy (ΔH) and entropy (ΔS) terms, as shown by the equation, $\Delta G = \Delta H - T\Delta S$. The $\Delta H(T_m^*)$ values of SB cytc_5, SV cytc_5, and SA cytc_5 were, respectively, 353.2, 314.0, and

310.7 kJ mol^{-1}, which were significantly higher than that of SL cytc_5, 272.7 kJ mol^{-1} (Table 2). At the same time, the $\Delta S(T_m^*)$ values of SB cytc_5, SV cytc_5, SL cytc_5, and SA cytc_5 were 0.99, 0.88, 0.79, and 0.88 kJ mol^{-1} K^{-1}, respectively. These values show that the higher stabilities of SB cytc_5, SV cytc_5, and SA cytc_5 than that of SL cytc_5 can be attributed to $\Delta H(T_m^*)$ values, indicating an enthalpic stabilization mechanism. Similar to the T_m and $\Delta G(T_m^*)$ values, the significantly different $\Delta H(T_m^*)$ values ($P < 0.03$) also correlated with the growth pressures, the correlation coefficient value being 0.73 (Fig. 4c).

Positive correlations between the optimal growth temperatures of the source organisms and the thermal stabilities of their proteins have already been observed (McFall-Ngai and Horwitz 1990; Gromiha et al. 1999; Akanuma et al. 2013). Moreover, cytochromes *c* from more thermophilic bacteria tend to be more stable than those from mesophiles (Sambongi et al. 2002; Nakamura et al. 2006). We then plotted the present thermal stability parameters, the T_m, $\Delta G(T_m^*)$, and $\Delta H(T_m^*)$ values, of the four *Shewanella* cytochromes c_5 as a function of the optimal growth temperatures of the source species (Fig. 4d–f). Overall, the thermal stability was not dependent on the growth temperature, unlike in the case of the stability-pressure relationship. This was due that the thermal stability of SA cytc_5 from mesophilic and piezosentive *S. amazonensis* was lower than those of SB cytc_5 and SV cytc_5 from psychrophilic and piezophilic *S. benthica* and *S. violacea*, respectively. Although we do not know the pressure effect on the protein stability, SB cytc_5 and SV cytc_5 might be stabilized against high pressure, resulting in a thermal stability increase that exceeds that of mesophilic SA cytc_5.

Taken together, our results emphasize that the thermal stabilities of *Shewanella* cytochromes c_5 correlate with the optimal growth pressures of the source species. A similar conclusion was drawn in the previous study on monomeric G actins from deep-sea fishes, which are heat stable compared with those from mesophilic mammals (Swezey and Somero 1982; Somero 1992). However, these studies including the present one are the few examples, partly because most relevant studies have focused on subunit assembly/dissociation with multimeric proteins, not on polypeptide folding/unfolding with monomeric proteins.

Phylogenetic analysis of cytochromes c_5

In order to determine the lineage of the four *Shewanella* cytochrome c_5 proteins examined here, phylogenetic analysis was carried out. The sequences of 16 cytochromes c_5 from *Shewanella* species, including those of the present four proteins, and mesophilic *Pseudomonas aeruginosa* were inspected through phylogenetic analysis. Neighbor-joining tree topology revealed that the *Shewanella* cytochrome c_5 sequences could be classified into the two groups, piezophilic Group 1 and piezosensitive Group 2 (Fig. 5a), consistent with a tree based on 16S rRNA gene sequences (Kato and Nogi 2001) and with a sequence comparison of dihydrofolate reductases (Murakami et al. 2011). These findings indicate that cytochromes c_5 together with other molecules separated according to the different pressure environments of the source species.

An ancestral sequence is defined as the set of residues in which each residue has the greatest likelihood of existing at its associated position. The ancestral amino acid sequence at each node of the phylogenetic tree of *Shewanella* species was inferred using a statistical model of evolution. Each inferred ancestral sequence has several conserved residues in the two groups mentioned above. For example, Lys-37, Ala-41, and Leu-50 conserved in piezosensitive Group 2 *Shewanella* cytochromes c_5 including SL cytc_5 and SA cytc_5 were found to be ancestral to Gln, Thr, and Lys, respectively, which were conserved in SB cytc_5 and SV cytc_5 among the piezophilic Group 1 proteins (Fig. 5b). It is plausible that these three residues found in SB cytc_5 and SV cytc_5 were introduced during the evolutional time course of adaption to environmental pressure. Consistently, biogeographics and phylogenetics suggest that piezophilic *Shewanella* species might be derived from shallow-water piezosensitive ones (Lauro et al. 2007; Fang et al. 2010), although there is room for further research to verify this hypothesis.

Although the four cytochromes c_5 from *Shewanella piezotolerans, loihica, pealeana*, and *halifaxensis* are classified into piezophilic Group 1 (Fig. 5a), they have ancestral Lys-37, Ala-41, and Leu-50 (Fig. 5b, in which the cytochromes c_5 from *S. piezotolerans* and *S. halifaxensis* are shown, being abbreviated as SP cytc_5 and SH cytc_5, respectively). These four species were isolated from relatively "shallow" deep-sea areas compared with *S. benthica* and *S. violacea*. For example, *S. piezotolerans* was isolated at a depth of 1,914 m, consistently growing optimally at 20 MPa (Xiao et al. 2007), which is lower than the optimal growth pressures of *S. benthica* (50 MPa) and *S. violacea* (30 MPa). Stability experiments on SP cytc_5 would further strengthen the present conclusion of correlation between growth pressure and protein stability, but with only the present results it is possible to speculate that an optimal growth pressure of more than 30 MPa is necessary for the adoption of Gln-37, Thr-41, and Lys-50, as found in SB cytc_5 and SV cytc_5.

Molecular simulation revealed that the Lys-50 residue in SB cytc_5 and SV cytc_5 forms a hydrogen bond with the heme, while the corresponding Leu-50 in SL cytc_5 and SA cytc_5 hydrophobically interacts with amino acid residues

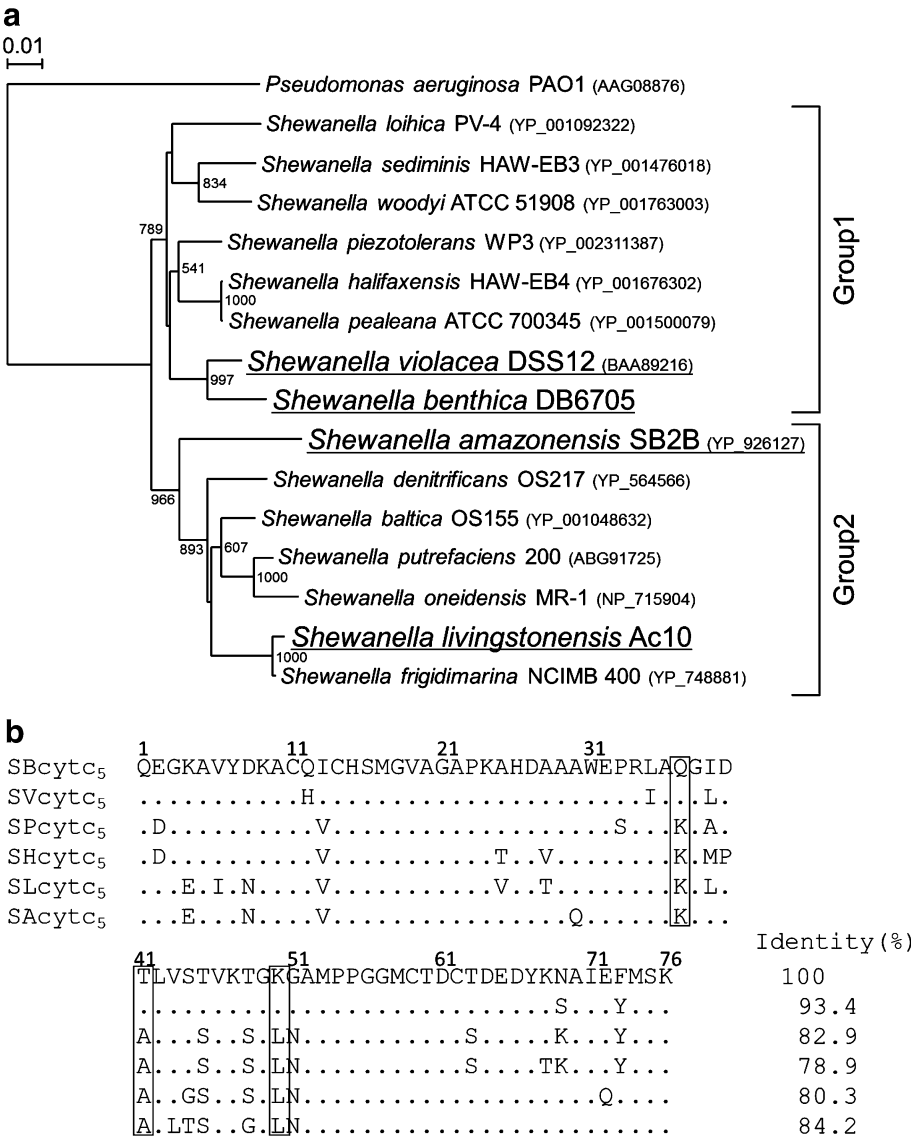

Fig. 5 Sequence analysis of cytochromes c_5. Phylogenetic tree (**a**). Group 1 and Group 2 are indicated on the tree. Accession numbers are shown in parentheses. The numbers on the nodes represent bootstrap values with 1,000 replicates. The *bar* indicates 0.01 substitutions per amino acid position. Comparison of amino acid sequences of cytochromes c_5 (**b**). Identical amino acid residues with those in the SB cytc_5 sequence are indicated by *dots*. Identity to the SB cytc_5 sequence is also indicated. The amino acid numbers are shown above the SB cytc_5 sequence. Relevant amino acid residues mentioned in the text are *boxed*

near the heme propionate (Masanari et al. 2011). The hydrogen-bonding Lys-50 residue in cytochrome c_5 is more likely to contribute to the stability than the ancestral Leu-50 residue responsible for hydrophobic interactions. If this is the case, *S. benthica* and *S. violacea* adapted to deep-sea high pressure environments must have stabilized cytochromes c_5 with Lys at the 50th position.

Conclusion and perspectives

The denaturant and thermal stabilities of the monomeric cytochrome c_5 from most piezophilic *S. benthica* were the highest, followed by those of the other three proteins. The stabilities of the four *Shewanella* cytochromes c_5 correlate with the optimal growth pressures of the source species. In this study, we provide an insight into homologous proteins from piezophilic and piezosensitive organisms; positive correlation between growth pressure and protein stability.

Monomeric cytochromes c_5 from *Shewanella* are suitable tools for examining protein stability with regard to the optimal growth pressures of the source species. Not only between piezophilic proteins (SB cytc_5 and SV cytc_5) and piezosensitive ones (SL cytc_5 and SA cytc_5), as discussed here, but also comparison between piezophilic SB cytc_5 and SV cytc_5 will be of interest, because they exhibit an only five-residue difference, with a significant stability difference, as observed in this study.

Previous studies revealed that pressure has little effect on a protein's three-dimensional structure, but that it can affect its quaternary structure in the pressure range of 0.1–140 MPa (Chilukuri et al. 2002; Kawano et al. 2004). It will be of interest to examine whether piezophilic and piezosensitive *Shewanella* cytochromes c_5 differ in

stability against pressure. Pressure denaturation measurements of wild-type *Shewanella* cytochromes c_5 and a mutation study are now in progress, aiming at elucidation of the pressure stabilization mechanism of a protein, which will be basic for future biotechnology.

Acknowledgments This work was supported by a Grant-in-Aid for Scientific Research on Innovative Areas (No. 20118005) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by the Cooperative Research Program of the "Network of Joint Research Center for Materials and Devices".

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