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Amino acid substitutions in malate dehydrogenases of piezophilic bacteria isolated from intestinal contents of deep-sea fishes retrieved from the abyssal zone

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To examine the occurrence in other deep-sea bacteria of two amino acid substitutions (Ala-180 and His-229) in malate dehydrogenase (MDH) found previously in the deep-sea piezophilic *Moritella* sp. strain 2D2, we cloned and sequenced MDH genes of deep-sea piezophilic *Moritella* and *Shewanella* strains isolated from intestinal contents of deep-sea fishes, as well as other *Moritella* species from deep-sea water and sediments: *M. marina*, *M. japonica*, and *M. yayanosii*. The piezophilic *Moritella* strains had a Val residue or an Ala residue at position 180 and all the *Moritella* strains except for one had a His residue at position 229. However, four piezophilic-strain-specific substitutions at positions 103, 111, 229, and 283 were found to be completely conserved in the MDH of the intestinal *Moritella* strains of deep-sea fishes, indicating the substitutions may be habitat-specific. The piezophilic *Shewanella* strains had a Val residue and a Gln residue at positions 180 and 229, respectively. However, the MDHs of the *Shewanella* strains had five piezophilic-strain-specific substitutions at positions 61, 65, 107, 161, and 202. Therefore, the enzymatic strategies for responding to deep-sea high pressure environments of the MDHs between the genera *Moritella* and *Shewanella* are potentially different. Moreover, homology modeling shows these substitutions found in the MDHs of both genera except for position 229 in the subunit interface are located on the exposed region of the MDH molecules, indicating the substitutions may be related to the hydration state of the molecules.

Key Words—amino acid substitutions; *Coryphaenoides yaquinae*; deep-sea piezophilic bacteria; intestinal contents of deep-sea fishes; malate dehydrogenase (MDH); *Moritella* sp.; *Shewanella* sp.

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Introduction

In a previous paper (Saito and Nakayama, 2004), to elucidate the molecular basis for malate dehydrogenase (MDH) functions at high pressure and low temperature, we cloned, sequenced, and overexpressed the gene encoding MDH from the obligately piezophilic deep-sea bacterium *Moritella* sp. strain 2D2 isolated

from an intestinal content sample of a deep-sea fish (*Coryphaenoides yaquinae*) retrieved from a depth of 6,100 m (Nakayama et al., 1994) and from the psychrophilic *Moritella* sp. strain 5710 isolated from a deep-sea sediment sample from a depth of 2,220 m (Hamamoto et al., 1995). Analyses of sequence-function relationships in the MDHs from these congeneric deep-sea bacteria adapted to different pressures were carried out to determine the amino acid residues important for MDH functions at high pressure. From these experiments, we showed that there are two positions [close to the active site (Ala-180) and in the sub-unit interaction site (His-229)] with 2D2-specific substitutions. Furthermore, we showed that at least the His residue at position 229 of MDH from strain 2D2 may play a role in the thermal stability and the MDH function at high pressure from experiments with wild-type MDHs and mutant MDHs made by site-directed mutageneses of the MDH genes of strains 2D2 and 5710 (Saito and Nakayama, 2004).

In this paper, to examine the occurrence of 2D2-specific substitutions (Ala-180 and His-229) in MDHs of other deep-sea piezophilic bacteria from intestinal contents of deep-sea fishes retrieved from various depths of 3,100 m to 6,100 m, we cloned and sequenced MDH genes of deep-sea piezophilic bacteria. Then, amino acid sequences of MDH deduced from resultant nucleotide sequences of MDH genes were aligned and analyzed by using the Clustal X program (Jeanmougin et al., 1998). To compare with these results, we also cloned and sequenced MDH genes of deep-sea psychrophilic, piezotolerant, and piezophilic *Moritella* strains isolated from deep-sea water (1,200 m), sediment (6,356 m), and sediment (10,898 m) samples, respectively (Kato et al., 1998; Nogi and Kato, 1999; Nogi et al., 1998; Urakawa et al., 1998). Moreover, homology models of both the MDHs were developed by using Swiss Model (Guex and Peitsch, 1997; Schwede et al., 2003) based on the known crystal structures of *Escherichia coli* MDH and were viewed by using Deep View (Swiss PDB-Viewer) (Guex and Peitsch, 1997; Schwede et al., 2003).

Materials and Methods

Deep-sea bacterial strains and growth conditions. Deep-sea bacteria isolated from intestinal contents of deep-sea fishes (*Coryphaenoides yaquinae*, *C. armatus*, and *Ilyophis* sp.) retrieved from depths of 3,100 to

6,100 m in the Northwest Pacific Ocean (Nakayama et al., 1994, 2005; Yano, 1996; Yano et al., 1995, 1997) and *Moritella* sp. strain 5710 from a deep-sea sediment at a depth of 2,220 m (Hamamoto et al., 1995) which had been maintained as glycerol stock cultures at -80°C (Sambrook and Russell, 2001) were used. Table 1 lists the deep-sea bacterial strains used for this study, their sources, depths from which the sources were retrieved, their genera, and their piezophilism.

To harvest cell pellets for extraction of chromosomal DNAs, all the strains shown in Table 1 were incubated as described previously (Nakayama et al., 1994, 2005; Yano, 1996; Yano et al., 1995, 1997). However, the growth pressure used for strains 5710 and T4609 was 0.1 MPa (1 atm). The chromosomal DNAs were prepared as described previously (Saito and Nakayama, 2004).

Table 2 lists the source and piezophilism of *Moritella marina* ATCC15381^T, *Moritella japonica* DSK1, and *Moritella yayanosii* DB21MT-5. These strains were maintained and incubated to harvest cell pellets and the chromosomal DNA of each strain was prepared as described previously (Kato et al., 1998; Nogi and Kato, 1999; Nogi et al., 1998; Urakawa et al., 1998).

Cloning and sequencing of MDH genes of *Moritella* and *Shewanella* species. The chromosomal DNA solutions prepared as described above were also used as templates for PCR amplification of MDH genes of all the strains.

For strains belonging to the genus *Moritella*, a primer set, MDHEXN (5'-AATGGAGAGCTCCATGAAA-GTCGCTGTATTAGG-3') and MDHSTOPC (5'-TAA-TGACTCGAGTTAACCAGCAACGAATTCTT-3') was used for the PCR amplifications which were carried out with Premix Taq (EX Taq version) (TaKaRa) according to the manufacturer's directions. The forward and reverse primer sequences were derived from the nucleotide sequence (DDBJ accession number: AB097559) of MDH gene of strain 2D2 obtained in a previous paper (Saito and Nakayama, 2004). Nucleotides underlined with lines are the starting and ending nucleotide sequences of the gene and nucleotides underlined with dotted lines are SacI and XhoI sites, respectively. Sequencing of the PCR products was determined by MacroGen, Inc. (Seoul, Korea) as described above. Four primers, MDHEXN, MDHSTOPC, MoritellaMDH572F (5'-CCGCTACTTTC-TCAAGTTAAAGG-3'), and MoritellaMDH550R (5'-

Table 1. Source, genus name, and piezophilism of deep-sea bacterial strains isolated from deep-sea fishes for amino acid sequence analysis of their MDHs.

Strain	Source ^a	Depth (m) ^b	Genus name ^c	Piezophilism
47A1 47B1	Sample no. 47 (<i>Coryphaenoides armatus</i>)	3,100	<i>Moritella</i> sp. <i>Moritella</i> sp.	Facultatively piezophilic ¹ Facultatively piezophilic ²
16F1 16H2	Sample no. 16 (<i>Coryphaenoides armatus</i>)	3,200	<i>Moritella</i> sp. <i>Moritella</i> sp.	Facultatively piezophilic ² Facultatively piezophilic ²
T4609	Sample no. 46 (<i>Coryphaenoides yaquinae</i>)	4,300	<i>Shewanella</i> sp.	Facultatively piezophilic ³
36B1 36C1 36G1	Sample no. 36 (<i>Ilyophis</i> sp.)	4,700	<i>Moritella</i> sp. <i>Moritella</i> sp. <i>Moritella</i> sp.	Facultatively piezophilic ¹ Facultatively piezophilic ¹ Obligately piezophilic ¹
33F1 33H2	Sample no. 33 (<i>Coryphaenoides yaquinae</i>)	5,300	<i>Shewanella</i> sp. <i>Shewanella</i> sp.	Facultatively piezophilic ² Facultatively piezophilic ²
56A1	Sample no. 56 (<i>Coryphaenoides yaquinae</i>)	5,600	<i>Moritella</i> sp.	Obligately piezophilic ²
38C1 38F1	Sample no. 38 (<i>Coryphaenoides yaquinae</i>)	5,900	<i>Moritella</i> sp. <i>Moritella</i> sp.	Obligately piezophilic ² Obligately piezophilic ²
2C2	Sample no. 2 (<i>Coryphaenoides yaquinae</i>)	6,100	<i>Moritella</i> sp.	Obligately piezophilic ⁴

Numerical superscripts in this table show references as follows: ¹(Yano, 1996), ²(Nakayama et al., 2005), ³(Yano et al., 1995), and ⁴(Nakayama et al., 1994).

^aThis column shows sample nos. and the scientific names of deep-sea fishes from which the deep-sea bacteria were isolated (Nakayama et al., 1994; Yano et al., 1995).

^bDepths from which the deep-sea fishes were retrieved (Nakayama et al., 1994; Yano et al., 1995).

^cGenus names were determined by a phylogenetic method based on 16S rRNA gene sequences (Nakayama et al., 2005).

Table 2. Source and piezophilism of *Moritella marina* ATCC15381^T, *Moritella japonica* DSK1, and *Moritella yayanosii* DB21MT-5.

Strain	Source	Depth (m)	Piezophilism ^a
<i>Moritella marina</i> ATCC15381 ^T	Water sample in north-western Pacific Ocean	1,200	Piezotolerant
<i>Moritella japonica</i> DSK1	Deep-sea sediment sample from the Japan Trench	6,356	Piezotolerant ^b
<i>Moritella yayanosii</i> DB21MT-5	Deep-sea sediment sample from the Mariana Trench	10,898	Obligately piezophilic

^aPiezophilism was determined when the strain grew at 10°C [*Moritella marina* ATCC15381^T (Nogi et al., 1998; Urakawa et al., 1998), *Moritella japonica* DSK1 (Nogi et al., 1998), and *Moritella yayanosii* DB21MT-5 (Kato et al., 1998; Nogi and Kato, 1999)].

^bPiezophilism became facultatively piezophilic when the strain grew at 15°C (Nogi et al., 1998).

CCTTTAACTTGAGAAAGTAGCGG-3'), were used for sequencing the PCR products.

For strains belonging to the genus *Shewanella*, a primer set, *Shewanella*MDHEXN (5'-ATGAAAGTTGCTGTACTTGGT-3') and *Shewanella*MDHEXC (5'-TTACTTAACGAAATCAACGCCT-3'), was used for the PCR amplifications which were carried out with Premix

Taq (EX *Taq* version) (TaKaRa) according to the manufacturer's directions. The forward and reverse primer sequences were derived from the nucleotide sequence (DDBJ accession number: AE015522) of MDH gene of *Shewanella oneidensis* MR-1 (Heidelberg et al., 2002). Sequencing of the PCR products was determined by MacroGen, Inc. as described above. Four primers,

ShewanellaMDHEXN, ShewanellaMDHEXC, ShewanellaMDH476F (5'-CGTTCTGAGACCTTTATCGC-3'), and ShewanellaMDH457R (5'-GCGATAAAGGTC-TCAGAACG-3') were used for sequencing the PCR products.

Homology modeling of Moritella and Shewanella MDHs. A model of *Moritella* and *Shewanella* MDHs were obtained by homology modeling based on the known crystal structures of *Escherichia coli* MDH using the SWISS-MODEL server and were viewed using Swiss PDB-Viewer (Guex and Peitsch, 1997; Schwede et al., 2003).

Nucleotide sequence accession numbers. The nucleotide sequence data determined in this study have been deposited in the DDBJ nucleotide sequence databases with accession numbers AB185116–AB185129, and AB187216–AB187218.

Results and Discussion

Deduced MDH amino acid sequences of deep-sea bacterial strains

All the MDH genes of *Moritella* strains (strains 47A1, 47B1, 16F1, 16H2, 36B1, 36C1, 36G1, 56A1, 38C1, 38F1, and 2C2) isolated from the intestinal contents of deep-sea fishes as well as other *Moritella* species (*Moritella marina* ATCC15381^T, *Moritella japonica* DSK1, and *Moritella yayanosii* DB21MT-5) isolated from deep-sea water and sediment consisted of 939 bp and were coding 312 amino acid residues, agreeing with the number of amino acid residues in MDH of *Moritella* sp. strain 5710 and *Moritella* sp. strain 2D2 (Saito and Nakayama, 2004), while the MDH genes of strains T4609, 33F1, and 33H2, which belong to the genus *Shewanella*, consisted 936 bp and were coding 311 amino acid residues, agreeing with the number of amino acid residues in MDH of *Shewanella oneidensis* MR-1 (Heidelberg et al., 2002).

First, we'll discuss the strains isolated from the intestinal contents of deep-sea fishes. Although the fourteen strains used in this study and *Moritella* sp. strain 2D2 (Saito and Nakayama, 2004) are different strains based on 16S rRNA gene analysis (Nakayama et al., 2005; Saito and Nakayama, 2004), ten kinds of MDH amino acid sequences were obtained. The MDHs of strains 16F1 and 16H2 that were isolated from sample no. 16 (*Coryphaenoides armatus*), the MDHs of strains 36C1 and 36G1 from sample no. 36 (*Ilyophis* sp.), the MDHs of strains 38C1 and 38F1 from sample no. 38

(*Coryphaenoides yaquinae*), the MDHs of strains 2C2 and 2D2 from sample no. 2 (*Coryphaenoides yaquinae*), and the MDHs of strains 33F1 and 33H1 from sample no. 33 (*Coryphaenoides yaquinae*) showed completely identical amino acid sequences. However, there was one silent mutation at Thr-148 between the MDHs of strain 36C1 and 36G1.

Second, *Moritella marina* ATCC15381^T, *Moritella japonica* DSK1, and *Moritella yayanosii* DB21MT-5 were found to have different amino acid sequences from any of the amino acid sequences described above.

Multiple amino acid sequence alignment of MDHs

Multiple amino acid sequence alignment of the deduced MDH amino acid sequences of the seventeen deep-sea bacterial strains with the reference MDH amino acid sequences of *Moritella* sp. strain 5710 (P48364) (Saito and Nakayama, 2004), *Moritella* sp. strain 2D2 (Q7X3X5) (Saito and Nakayama, 2004), *Shewanella oneidensis* MR-1 (P82177) (Heidelberg et al., 2002), *Photobacterium profundum* SS9 (P37226) (Welch and Bartlett, 1997), *Salmonella typhimurium* (P25077) (Lu and Abdelal, 1993), *Escherichia coli* (P61889) (Blattner et al., 1997; McAlister-Henn et al., 1987), *Vibrio cholerae* (Q9KUT3) (Byun et al., 1999), *Vibrio parahaemolyticus* (Q87SU7) (Makino et al., 2003), and *Haemophilus influenzae* (P44427) (Fleischmann et al., 1995) are shown in Fig. 1. Also in this figure, sixteen strains from *Moritella marina* ATCC15381 to *Moritella yayanosii* DB21MT-5 belonging to the genus *Moritella* were placed in order of sampling depths of the deep-sea fish samples or the deep-sea sediment samples from which these strains were isolated. The number of non-consensus sequence positions in these MDH amino acid sequences was 26 out of 312 amino acid residues. Therefore, the MDH sequence identities between them were very high (more than 91.7%).

Furthermore, four strains from *Shewanella oneidensis* MR-1 to *Shewanella* sp. strain 33H2 were placed in order of sampling depths of the deep-sea fish samples or the lake water sample from which these strains were isolated. The number of non-consensus sequence positions in these MDH amino acid sequences was 40 out of 311 amino acid residues. Therefore, the MDH sequence identities between them were very high (more than 87.1%).

Occurrence of two 2D2-specific amino acid substitutions (Ala-180 and His-229) in other Moritella strains

In a previous paper (Saito and Nakayama, 2004), we reported that there were two positions [close to the active site (Ala-180) and in the subunit interaction site (His-229)] with 2D2-specific substitutions. As shown in Fig. 1, although strains 16F1, 16H2, 36B1, 38C1, and 38F1 were facultatively or obligately piezophilic (Table 1), the MDHs of these strains had a Val residue at position 180 like the piezotolerant strains, *Moritella marina* ATCC15381^T, *Moritella* sp. strain 5710, and *Moritella japonica* DSK1. Moreover, strains 47A1, 47B1, 36C1, 36G1, 56A1, and 2C2, which were also facultatively or obligately piezophilic (Table 1), had an Ala residue at position 180 like the obligately piezophilic strain, *Moritella* sp. strain 2D2. Therefore, the presence of the 2D2-specific Val to Ala substitution at position 180 in the MDH of the *Moritella* strains could not be confirmed. On the other hand, all the strains that were facultatively or obligately piezophilic had a His residue at position 229 like the obligately piezophilic *Moritella* sp. strain 2D2 with only the exception of *Moritella yanosii* DB21MT-5. Although *Moritella yanosii* DB21MT-5 was reported to be obligately piezophilic at 10°C as shown in Table 2 (Kato et al., 1998), the MDH had a Gln residue at position 229 like the piezotolerant strains, *Moritella marina* ATCC15381^T, *Moritella* sp. strain 5710, and *Moritella japonica* DSK1.

These results indicate that the amino acid substitution at position 180, one of the two substitutions which we found in the MDH amino acid sequence of *Moritella* sp. strain 2D2 (Saito and Nakayama, 2004), may not be piezophilic-strain-specific. On the other hand, these results indicate that there was a Gln to His substitution at position 229, the other one of the two 2D2-specific substitutions (Saito and Nakayama, 2004), in the MDH amino acid sequences of almost all the piezophilic *Moritella* strains examined. Moreover, the presence of the substitution supports the previous finding, that the His residue at position 229 of the 2D2 MDH may play a role in the thermal stability and the MDH function at high pressure, induced by site-directed mutageneses of the MDH genes of strains 2D2 and 5710 (Saito and Nakayama, 2004) as the position 229 in MDHs has been shown to be a functionally important residue involved in subunit interaction (Birktoft et al., 1987; Hall et al., 1992; Roderick and Banaszak, 1986; Thompson et al., 1988).

Occurrence of two 2D2-specific amino acid substitutions (Ala-180 and His-229) in other Shewanella strains

As shown in Fig. 1, the two 2D2-specific amino acid substitutions (Ala-180 and His-229) were not found in the MDHs of *Shewanella oneidensis* MR-1, *Shewanella* sp. strain T4609, *Shewanella* sp. strain 33F1, or *Shewanella* sp. strain 33H2. Moreover, all the strains had a Val residue at position 180 and a Gln residue at position 229 like some *Moritella* strains, *Moritella marina* ATCC15381^T, *Moritella* sp. strain 5710, *Moritella japonica* DSK1, and *Moritella yanosii* DB21MT-5, and seven strains belonging to γ -subclass of proteobacteria. These results indicate that the *Moritella* and the *Shewanella* strains may have different enzymatic strategies for responding to deep-sea high pressure environments.

Piezophilic-strain-specificity of the amino acid substitutions in the MDHs of the Moritella strains

We selected four piezophilic-strain-specific positions out of twenty-six non-consensus sequence positions (positions 103, 111, 229, and 283) shown in Fig. 1. Table 3 shows the amino acid residues at these positions of the MDHs from the *Moritella* strains with those of the MDHs from seven strains belonging to the γ -subclass of proteobacteria. The substitutions or the amino acids at positions 103, 111, 229, and 283 were completely conserved only in the MDHs of the deep-sea piezophilic *Moritella* strains isolated from the intestinal contents of deep-sea fishes.

As described above, the MDHs of *Moritella marina* ATCC15381^T, *Moritella* sp. strain 5710, *Moritella japonica* DSK1, *Moritella yanosii* DB21MT-5, and seven strains belonging to the γ -subclass of proteobacteria had a Gln residue at position 229 while the MDHs of the deep-sea piezophilic *Moritella* strains isolated from the intestinal contents of deep-sea fishes had a His residue at the same position. Therefore, it is considered that the substitution at position 229 was piezophilic-strain-specific in these intestinal strains of the deep-sea fishes. Moreover, the fact that the substitution of a Gln residue (non-charged amino acid) with a His residue (charged amino acid) happened at position 229, which is involved in a subunit interaction region, is worthy of note. The Gly or Ala to Ser substitution at position 103 is the exchange of a hydrophobic (non-polar) amino acid with a hydrophilic (polar) amino acid. As the increase of hydrophilic (polar) amino acid

Fig. 1

residues has been observed in psychrophilic proteins (Russell, 2000), the substitution at position 103 may relate to their psychrophilic properties. The Asn to Thr substitution at position 111 and the Glu to Asp substitution at position 283 are the exchange of the same kinds of amino acids (non-charged and charged polar amino acids, respectively). It appears that these substitutions, except for the Ser substitution at position 103 and the His residue at position 229, do not change drastically the characteristics of the amino acids.

As described above, *Moritella marina* ATCC15381^T, *Moritella* sp. strain 5710, *Moritella japonica* DSK1, and *Moritella yayanosii* DB21MT-5 were isolated from deep-sea water or deep-sea sediment samples (Hamamoto et al., 1995; Kato et al., 1998; Nogi and Kato, 1999; Nogi et al., 1998; Urakawa et al., 1998), while the other deep-sea piezophilic *Moritella* strains were isolated from the intestinal contents of deep-sea fishes (Nakayama et al., 1994, 2005; Yano et al., 1995).

Moreover, as reported previously (Nakayama et al., 2005), in a phylogenetic tree based on 16S rRNA gene sequences of these *Moritella* species the strains isolated from deep-sea water or sediment samples and the strains isolated from intestinal contents of deep-sea fishes fell into two different clusters. In deep-sea

environments, the intestines of deep-sea fishes are exceptional habitats with an abundance of organic matter as compared with ambient oligotrophic deep-sea waters and sediments (Jannasch and Taylor, 1984). The significantly different concentrations of organic matters between the two habitats may cause different mutations in the two habitats over a long period. It would appear that the accumulation of the resulting different mutations exhibited itself in the phylogenetic difference between them (Nakayama et al., 2005). Therefore, although there is no data on MDHs of the *Moritella* strains isolated from the intestinal contents of shallow fishes, from a habitat point of view, it appears that these piezophilic-strain-specific substitutions in the MDHs of these intestinal strains of deep-sea fishes may be habitat-specific.

These results indicate that at least four piezophilic-and/or habitat-specific substitutions may occur in the MDHs of the deep-sea piezophilic *Moritella* strains isolated from the intestinal contents of deep-sea fishes.

Piezophilic-strain-specificity of the amino acid substitutions in the MDHs of the Shewanella strains

We selected five piezophilic-strain-specific positions out of forty non-consensus sequence positions (posi-

Fig. 1. Alignment of MDHs between sixteen strains of the genus *Moritella*, four strains of the genus *Shewanella* and six strains belonging to the γ -subclass of proteobacteria.

Deduced amino acid sequences were aligned using the Clustal X program (Jeanmougin et al., 1998). M.maria, 5710, 47A1, 47B1, 16F1, 16H2, 36B1, 36C1, 36G1, 56A1, 38C1, 38F1, 2C2, 2D2, M.japonica, M.yayanosi, S.oneidensis, T4609, 33F1, 33H2, P.profundu, S.typhimur, E.coli, V.cholerae, V.parahaem, and H.influenz indicate *Moritella marina* ATCC15381^T (AB187216) (Urakawa et al., 1998), *Moritella* sp. strain 5710 (P48364) (Saito and Nakayama, 2004), *Moritella* sp. strain 47A1 (AB185116) (Nakayama et al., 2005), *Moritella* sp. strain 47B1 (AB185117) (Nakayama et al., 2005), *Moritella* sp. strain 16F1 (AB185118) (Nakayama et al., 2005), *Moritella* sp. strain 16H2 (AB185119) (Nakayama et al., 2005), *Moritella* sp. strain 36B1 (AB185121) (Nakayama et al., 2005), *Moritella* sp. strain 36C1 (AB185122) (Nakayama et al., 2005), *Moritella* sp. strain 36G1 (AB185123) (Nakayama et al., 2005), *Moritella* sp. strain 56A1 (AB185126) (Nakayama et al., 2005), *Moritella* sp. strain 38C1 (AB185127) (Nakayama et al., 2005), *Moritella* sp. strain 38F1 (AB185128) (Nakayama et al., 2005), *Moritella* sp. strain 2C2 (AB185129) (Nakayama et al., 2005), *Moritella* sp. strain 2D2 (Q7X3X5) (Saito and Nakayama, 2004), *Moritella japonica* DSK1 (AB187218) (Nogi et al., 1998), *Moritella yayanosii* DB21MT-5 (AB187217) (Kato et al., 1998; Nogi and Kato, 1999), *Shewanella oneidensis* MR-1 (P82177) (Heidelberg et al., 2002), *Shewanella* sp. strain T4609 (AB185120) (Nakayama et al., 2005), *Shewanella* sp. strain 33F1 (AB185124) (Nakayama et al., 2005), *Shewanella* sp. strain 33H2 (AB185125) (Nakayama et al., 2005), *Photobacterium profundum* strain SS9 (P37226) (Welch and Bartlett, 1997), *Salmonella typhimurium* (P25077) (Lu and Abdelal, 1993), *Escherichia coli* (P61889) (Blattner et al., 1997; McAlister-Henn et al., 1987), *Vibrio cholerae* (Q9KUT3) (Byun et al., 1999), *Vibrio parahaemolyticus* (Q87SU7) (Makino et al., 2003), and *Haemophilus influenzae* (P44427) (Fleischmann et al., 1995), respectively. Numbers in parentheses are the accession numbers in the Swiss-Prot Database or DDBJ. Mo, Sh, and Pr at the right side of this figure indicate strains of the genus *Moritella*, genus *Shewanella*, and γ -subclass of proteobacteria, respectively. The functions attributed to specific residues on the basis of X-ray crystallographic analyses of the porcine MDH (Birktoft et al., 1987; Roderick and Banaszak, 1986) and *Escherichia coli* MDH (Hall et al., 1992) are indicated by the subscripts (N, substrate binding; Q, subunit interactions; C, catalysis) utilized by Thompson et al. (Thompson et al., 1988; Welch and Bartlett, 1997).

Table 3. Piezophilic-*Moritella*-strain-specific amino acid substitution.

Strain ^a	Amino acid residue no. ^b				Depth ^c (m)	Reference
	103	111	229	283		
<i>Moritella marina</i> ATCC15381 ^T	G	N	Q	E	1,200	This study
<i>Moritella</i> sp. 5710 ¹	G	N	Q	E	2,220	Ref. ¹
47A1	S	T	H	D	3,100	This study
47B1	S	T	H	D	3,100	This study
16F1, 16H2	S	T	H	D	3,200	This study
<i>Moritella</i> sp. 36B1	S	T	H	D	4,700	This study
36C1, 36G1	S	T	H	D	4,700	This study
56A1	S	T	H	D	5,600	This study
38C1, 38F1	S	T	H	D	5,900	This study
2C2	S	T	H	D	6,100	This study
2D2	S	T	H	D	6,100	Ref. ¹
<i>Moritella japonica</i> DSK1	G	N	Q	E	6,356	This study
<i>Moritella yayanosii</i> DB21MT-5	A	N	Q	E	10,898	This study
<i>Photobacterium profundum</i> SS9	E	K	Q	K	2,500	Ref. ²
<i>Shewanella oneidensis</i> MR-1	E	K	Q	E	—	Ref. ³
<i>Salmonella typhimurium</i>	Q	K	Q	T	—	Ref. ⁴
<i>Escherichia coli</i>	Q	K	Q	T	—	Ref. ^{5,6}
<i>Vibrio cholerae</i>	E	K	Q	K	—	Ref. ⁷
<i>Vibrio parahaemolyticus</i>	E	K	Q	E	—	Ref. ⁸
<i>Haemophilus influenzae</i>	E	K	Q	P	—	Ref. ⁹

Numerical superscripts with Refs. show references as follows: ¹ (Saito and Nakayama, 2004); ² (Welch and Bartlett, 1997); ³ (Heidelberg et al., 2002); ⁴ (Lu and Abdelal, 1993); ⁵ (McAlister-Henn et al., 1987); ⁶ (Blattner et al., 1997); ⁷ (Byun et al., 1999); ⁸ (Makino et al., 2003), and ⁹ (Fleischmann et al., 1995).

^a The accession numbers of the Swiss-Prot Database for the MDH amino acid sequences from *Photobacterium profundum* SS9, *Shewanella oneidensis* MR-1, *Salmonella typhimurium*, *Escherichia coli*, *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Haemophilus influenzae* are P37226, P82177, P25077, P61889, Q9KUT3, Q87SU7, and P44427, respectively.

^b Amino acid residue numbers are based on the amino acid sequences published for the *Escherichia coli* MDH (Blattner et al., 1997; McAlister-Henn et al., 1987; Swiss-Prot accession no. P61889).

^c This shows the depths from which deep-sea samples (deep-sea fishes and deep-sea sediment) were retrieved.

tions 61, 65, 107, 161, and 202) shown in Fig. 1. Table 4 shows amino acid residues at these positions of the MDHs from the *Shewanella* strains with those at the same positions of the MDHs from seven strains belonging to the γ -subclass of proteobacteria. The substitutions or the amino acids at positions 61, 65, 107, 161, and 202 were conserved only in the MDHs of the deep-sea piezophilic *Shewanella* strains isolated from the intestinal contents of deep-sea fishes.

The MDHs of *Shewanella oneidensis* MR-1 and seven strains belonging to the γ -subclass of proteobacteria had Leu residues at positions 161 and 202 while the MDHs of *Shewanella* sp. T4609, *Shewanella*

sp. 33F1, and *Shewanella* sp. 33H2 had an Ala residue and a Met residue at positions 161 and 202, respectively. Therefore, although the Leu to Ala substitution at position 161 and the Leu to Met substitution at position 202 are the exchange of the same kind of amino acids (non-polar amino acids), it is considered that these substitutions are highly piezophilic-strain-specific. The Pro to Ala substitution at position 65 is the exchange of rigidity with flexibility and may relate to their psychrophilic properties. The Glu to Gln substitution at position 61 is the exchange of a negatively-charged amino acid with an uncharged amino acid. The Val to Ala substitution at position 107 is the ex-

Table 4. Piezophilic-*Shewanella*-strain-specific amino acid substitution.

Strain ^a	Amino acid residue no. ^b					Depth ^c (m)	Reference
	61	65	107	161	202		
<i>Shewanella oneidensis</i> MR-1	E	P	V	L	L	—	This study
<i>Shewanella</i> sp. T4609 33F1, 33H2	Q	A	A	A	M	4,300	This study
	Q	A	A	A	M	5,300	This study
<i>Photobacterium profundum</i> SS9	V	P	V	L	L	2,500	Ref. ¹
<i>Moritella</i> sp. 5710	M	D	E	L	L	2,220	Ref. ²
<i>Salmonella typhimurium</i>	E	P	K	L	L	—	Ref. ³
<i>Escherichia coli</i>	E	P	K	L	L	—	Ref. ^{4,5}
<i>Vibrio cholerae</i>	E	P	V	L	L	—	Ref. ⁶
<i>Vibrio parahaemolyticus</i>	E	P	V	L	L	—	Ref. ⁷
<i>Haemophilus influenzae</i>	E	P	V	L	L	—	Ref. ⁸

Numerical superscripts with Refs. show references as follows: ¹ (Welch and Bartlett, 1997), ² (Hamamoto et al., 1995), ³ (Lu and Abdelal, 1993), ⁴ (McAlister-Henn et al., 1987), ⁵ (Blattner et al., 1997), ⁶ (Byun et al., 1999), ⁷ (Makino et al., 2003), and ⁸ (Fleischmann et al., 1995).

^aThe accession numbers of the Swiss-Prot Database for the MDH amino acid sequences from *Photobacterium profundum* SS9, *Shewanella oneidensis* MR-1, *Salmonella typhimurium*, *Escherichia coli*, *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Haemophilus influenzae* are P37226, P82177, P25077, P61889, Q9KUT3, Q87SU7, and P44427, respectively.

^bAmino acid residue numbers are based on the amino acid sequences published for the *Escherichia coli* MDH (Blattner et al., 1997; McAlister-Henn et al. 1987; Swiss-Prot accession no. P61889).

^cThis shows the depths from which deep-sea samples (deep-sea fishes and deep-sea sediment) were retrieved.

change of a large hydrophobic (non-polar) amino acid with a small hydrophobic (non-polar) amino acid. However, how these substitutions affect the structure and function of MDHs is still unknown.

Shewanella oneidensis MR-1 was isolated from a sediment sample in Oneida Lake (Venkateswaran et al., 1999), while *Shewanella* sp. strain T4609, *Shewanella* sp. strain 33F1, and *Shewanella* sp. strain 33H2 were isolated from the intestinal contents of deep-sea fishes. According to the discussion as described in the previous section on the MDHs from the *Moritella* strains, although the number of the strains examined belonging to the genus *Shewanella* was only three, it appears that these five amino acid substitutions of the MDH of our deep-sea piezophilic *Shewanella* strains may be piezophilic- and/or habitat-specific.

These results and ones from previous sections indicate that the strategies for responding to deep-sea high pressure environments of the MDHs between the genus *Moritella* and the genus *Shewanella* are potentially different. Moreover, these results were supported by the report on amino acid residues responsible for protein stability or function at low temperature or

high pressure in the MDH amino acid sequence from *Photobacterium profundum* SS9 (Welch and Bartlett, 1997).

Homology modeling of representative MDHs from the deep-sea *Moritella* and *Shewanella* strains

Figure 2 shows models of representative MDHs from *Moritella* sp. strain 2D2 (2D2MDH) and *Shewanella* sp. strain 33F1 (33F1MDH) obtained by homology modeling based on the known crystal structures of *Escherichia coli* MDH using the SWISS-MODEL server and Deep View (Guex and Peitsch, 1997; Schwede et al., 2003). Plate A in Fig. 2 shows the model of the 2D2MDH molecule. The model shows that piezophilic- and habitat-specific substitutions at positions 103, 111, and 283 are located on the exposed region of the 2D2MDH molecule and that position 229 is located in the subunit interface of the molecule. Plate B in Fig. 2 shows the model of the 33F1MDH molecule and all piezophilic- and habitat-specific substitutions at positions 61, 65, 107, 161, and 202 are located on the exposed region of the 33F1MDH molecule. Thus, the substitutions located on the exposed region of the

Fig. 2. Overall three-dimensional structures of 2D2MDH and 33F1MDH, as obtained by homology modeling based on the known crystal structures of *Escherichia coli* MDH using the SWISS-MODEL server and Deep View (Guex and Peitsch, 1997; Schwede et al., 2003).

Plates A and B show 2D2MDH and 33F1MDH, respectively. Ser-103, Thr-111, His-229, and Asp-283 of 2D2MDH in plate A, and Gln-61, Ala-65, Ala-107, Ala-161, and Met-202 in plate B are space-filled to indicate these locations.

MDH molecules may be related to the hydration state of the molecules.

These results described in previous sections are consistent with our suggestion that several amino acid substitutions in the MDH of the deep-sea piezophilic strains isolated from the intestinal contents of deep-sea fishes retrieved from depths of 3,100 m to 6,100 m are piezophilic- and/or habitat-specific. The crystallization and structural determination of the MDH proteins are ongoing, and the determination of the crystal structures should shed more light on the molecular basis of these piezophilic- and/or habitat-specific amino acid substitutions of MDHs. Moreover, methods based on physical chemistry and molecular biology should help to further determine the molecular basis of the relationship between these substitutions and deep-sea envi-

ronments.

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