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Comparison of the Gene Expression of Aspartate β-D-Semialdehyde Dehydrogenase at Elevated Hydrostatic Pressure in Deep-Sea Bacteria[1]

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Aspartate β-D-semialdehyde dehydrogenase genes (*asd*) were cloned and sequenced from a deep-sea-adapted strictly barophilic bacterium, *Shewanella* sp. strain DB6705, and a moderately barophilic bacterium, *Shewanella* sp. strain DSS12. The determined *asd* sequences of these two strains were very similar, and the identity of the deduced amino acids sequences was 96.2%. The 5′-ends of the *asd* mRNA from both strains were localized at corresponding sites by primer extension analysis, and two transcriptional starting points, which differed by only 1 base, were detected. In strain DB6705, a pressure-regulated transcript was mainly observed, whereas in strain DSS12, a pressure-tolerant transcript was observed together with the pressure-regulated transcript. Western-blotting analysis showed that the ASD protein was expressed under higher pressure conditions in DB6705, and under all pressure conditions tested in DSS12, as reflected in the primer extension results. Our findings suggest that *asd* expression controlled by pressure is one of the important mechanisms involved in the adaptation of microorganisms to the deep-sea environment.

Key words: aspartate β-D-semialdehyde dehydrogenase, deep-sea barophilic bacteria, gene expression, high pressure, *Shewanella* sp.

The deep-sea environment is a unique habitat characterized by extremely high pressure and low temperature. Microorganisms living there have adapted to this extreme environment. To investigate the mechanisms of adaptation to high pressure in the deep-sea, we isolated several barophilic and barotolerant bacteria from deep-sea mud samples (*1-3*). The isolated strains are Gram-negative, and they belong to the Proteobacteria γ-subgroup as indicated by phylogenetic analysis of 16S rRNA sequences. The ability of these bacteria to grow at high hydrostatic pressure indicates that they are probably specifically adapted to the deep-sea environment. Therefore, some gene expression systems of these bacteria may be regulated by high pressure. Investigation of the gene expression systems under high pressure is important to understand how deep-sea bacteria grow in their natural environment.

A pressure-regulated promoter fragment from the strictly barophilic bacterium, *Shewanella* sp. strain DB6705, has been cloned and analyzed (*4*). Gene expression from this promoter was found to be controlled positively at the level of transcription under conditions of elevated hydrostatic pressure, in both the barophilic strain and in an *Escherichia coli* transformant. A pressure-regulated operon was identified just downstream of this promoter region in strain DB6705 (*5*), and it seems that these sequences may be common in high-pressure adapted bacteria living in the deep-sea environment (*2, 4*).

Filament formation in *E. coli* adapted to growth at 1 atmosphere pressure has been reported when the organisms are grown at increased hydrostatic pressure (*6, 7*). We have observed filament formation when strictly barophilic bacteria were grown at low hydrostatic pressure, though no change in cell shape indicative of a pressure effect was detected in moderately barophilic or barotolerant bacteria. It is possible that high pressure affects cell division and influences the composition of the bacterial cell wall. Aspartate β-D-semialdehyde dehydrogenase (ASD) is a key enzyme in the biosynthetic pathway of lysine, threonine, methionine, diaminopimelic acid (DAP), and isoleucine. DAP is an essential component present in the peptidoglycan of all Gram-negative and some Gram-positive bacteria (*8*). Therefore, expression of ASD in strictly and moderately barophilic deep-sea bacteria, and in *E. coli*, may be influenced by hydrostatic pressure.

In this paper, we describe the results of a comparison of *asd* gene expression under high pressure in the strictly barophilic bacterium strain DB6705, the moderately barophilic bacterium strain DSS12, and atmospheric pressure-adapted *E. coli*. Our findings suggest that *asd* expression controlled by pressure is one of the important mechanisms of high pressure adaptation in microorganisms.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Recombinant DNA Techniques—The strictly barophilic bacterium *Shewanella* sp. strain DB6705, isolated from the Japan trench (depth, 6,356 m; 40°06.84′N, 144°11.04′E), and the moderately barophilic bacterium *Shewanella* sp. strain DSS12, isolated

[1] The nucleotide sequence data reported in this paper appear in DDBJ/EMBL/GenBank DNA databases with the accession numbers D49539 and D49540.

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from the Ryukyu trench (depth, 5,110 m, 24°15.23'N, 126°47.30'E), were used as the donors of DNA encoding the *asd* genes (*1*). The *asd*-deficient mutant, *E. coli* strain χ6097 (*9*), was used for cloning of the genes. The phage plasmid pUC119 and the host strain *E. coli* strain JM109 were used for cloning and DNA sequencing studies (*10*). The expression plasmid pTrcHisB and the host strain *E. coli* strain Top10 (Xpress™ System Protein TrcHis; Invitrogen, San Diego, CA, USA) were used for over-expression and purification of the recombinant proteins. Recombinant DNA work was carried out as described by Sambrook *et al.* (*11*). The cloned DNA was sequenced using an automated DNA sequencer, model 373A (Perkin Elmer/Applied Biosystems Div., Foster City, CA, USA), by the dideoxy terminator procedure (*12*), as described in the manual. The GENETYX-MAC program (ver 8.0; Software, Tokyo) was used to analyze DNA sequences.

Preparation of Chromosomal DNA and the Cloning Procedure—Strains DB6705 and DSS12 were grown in marine broth 2216 (Difco, Detroit, MI, USA) at 50 MPa and 10°C for 3 days, and at 0.1 MPa and 8°C for 2 days, respectively, as described before (*1*). Cells were collected by centrifugation (6,000 rpm for 15 min.) and chromosomal DNA was extracted by the method of Saito and Miura (*13*). Chromosomal DNA was digested with *Hin*dIII, and the DNA fragments of 1-8 kb in size were isolated from an agarose gel using glassmilk (Gene Clean II kit, Bio 101, La Jolla, CA, USA). The isolated DNA fragments were ligated into the vector pUC119 digested with the same restriction endonuclease. The ligated DNA was introduced into *E. coli* strain χ6097, and transformants were selected by culture overnight at 37°C on LB agar medium (1% w/v Bacto-tryptone, 0.5% w/v Bacto-yeast extract, 1% w/v NaCl, and 2% w/v agar, without DAP), containing ampicillin (50 μg/ml), because the *asd* deficient mutant strain χ6097 can grow only on LB agar medium with DAP (*9*). Colonies (*asd*$^+$) on the plates were examined for the presence of recombinant plasmids by a simple plasmid screening procedure (*14*). The recombinant plasmids, named pMSDB1 and pMSDS1 containing *asd* from strain DB6705 and strain DSS12, respectively, were isolated from among the *asd*$^+$ transformants (Fig. 1).

Identification of the Gene Products—The *asd* gene products encoded by these plasmids were analyzed using an *in vitro* transcription and translation system (SE30 system; Promega, Madison, WI, USA) and [^{35}S]methionine (Amersham, Amersham, UK). In order to determine the molecular weights of the *asd* gene products, proteins were separated on SDS-PAGE (15% gel), according to the method of Laemmli (*15*). Gene products from the plasmid pBR329 were used as molecular weight markers: Tet^r, 35 kDa; Amp^r, 31 kDa; Cam^r, 23 kDa.

RNA Preparation and the Procedure of Genetic Analysis—RNA preparation from the deep-sea strains, DB6705 and DSS12, was carried out as described previously (*4*). Total RNA from *E. coli* strain JM109 (*asd*$^+$) was prepared as described by Ausubel *et al.* (*16*). The RNA pellet was dissolved in diethyl pyrocarbonate-treated water, quantified by spectrophotometry, and stored at −80°C. The transcriptional start point was determined by primer extension analysis with the biotinylated oligonucleotides 5'-AATGGTTTGACCCACTGCGCCCGATGCACC-3' for strain DB6705, 5'-CATGGTTTGACCCACTGCGCCCGATGCACC-3' for strain DSS12, and 5'-GAACGGAGCCGACCATACCGCGCCAGCCGA-3' for *E. coli*, synthesized on an Applied Biosystems Model 392 DNA/RNA Synthesizer. The sequences of these primers are complementary to the nucleotides 858 to 887 of DB6705 *asd* (Fig. 2A), the

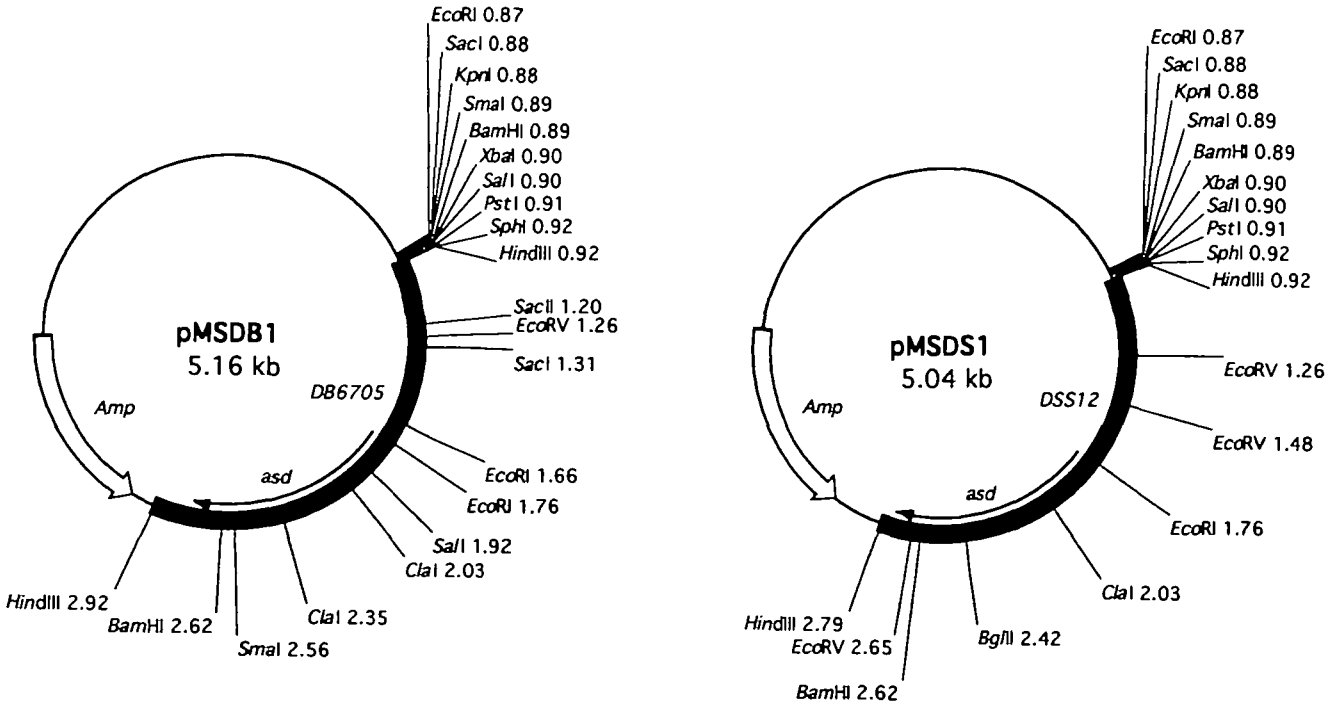

Fig. 1. **Restriction maps of the recombinant plasmids pMSDB1 and pMSDS1, which encode the *asd* gene from strains DB6705 and DSS12, respectively.** The thin line indicates the vector pUC119, and thick lines indicate the DNA of the deep-sea bacteria.

nucleotides 858 to 887 of DSS12 *asd* (Fig. 2B), and the nucleotides 259 to 288 of *E. coli asd* according to the sequence reported by Haziza *et al.* (*17*). The transcripts expressed by these strains at several pressures were detected by chemiluminescence as described previously (*4*). To see how high pressure regulated the expression of RNA transcripts from the *asd* genes, the effect of increasing pressure on the abundance of the mRNA transcript was also examined using total RNA from these strains grown at several pressures at the same time as the primer extension study.

Western-Blotting Analysis—To analyze the gene products of the strains DB6705 and DSS12, grown under several pressure conditions, a Western-blotting experiment was performed using an immunological assay kit (Bio-Rad Lab., Hercules, CA, USA) as described by Towbin *et al.* (*18*). At first, the recombinant ASD protein was over-expressed using the Xpress™ System (Invitrogen) and purified by the following procedure. pMSDB1 was digested with *Eco*RI and *Hin*dIII, and the 1.16 kb fragment including the *asd* gene of strain DB6705 was purified and ligated into pTrcHisB digested with the same enzymes. The recombinant plasmid was introduced into *E. coli* strain TOP10, and the fusion protein (hexahistidine-tagged ASD) was over-expressed and purified to homogeneity by affinity chromatography using an Ni-NTA spin column according to the instructions of the manufacturer. Secondly, rabbit antiserum against the purified fusion ASD protein was prepared for use in the Western-blotting analysis. This antiserum was found to react not only with ASD from strain DB6705, but also with ASD from strain DSS12.

Nucleotide Sequence Accession Numbers—The DNA sequences reported in this paper have been deposited in the DDBJ (Mishima), EMBL (Heidelberg, Germany), and

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HindIII 10        20        30        40        50        60        70        80        90        100       110       120
AAGCTTAAAGACAAGACAGTGGGTATAGTGGGGGCGGGTAACACAGGATCGGCGGTGGCTAAGTGTCTTCAGGCCTATGGCGTGACTGTGCTGCTGCATGATCCAGTTATTCAAGATAGC

      130       140       150       160       170       180       190       200       210       220       230       240
GATCCCCGTGATTTTATCTCATTAGATGAACTGATTGCTTGCTGTGATGTGATAAGTCTTCATGTGCCTATCACTAAGACTGGCGAGCATAAGACCTGGTATCTGTTCGATGAGGCCAGG

      250       260       270       280 SacII 290       300       310       320       330       EcoRV     350       360
CTCAATAGCTTAAAACAAGGCACCTGGTTGCTTAACTGCTGCCGCGGTGAGGTGATAGATAATCAGGCGCTGATTAAGGTTAAGCTGGAAAGGCCCGGATATCAAGTTGGTACTCGATGTG

      370       380       SacI      400       410       420       430       440       450       460       470       480
TGGGAAGGGGAGCCTAATCCTATGCATGAGCTCATCCCACTGGTCGAATTGGCGACGCCTCATATTGCAGGTTATAGCCTTGAAGGTAAGGCTCGCGGCACTTTTATGCTGTATCAGAAG

      490       500       510       520       530       540       550       560       570       580       590       600
TTGATGCAGGTACTGGGCAAAGATGCCGATAAGTCCATGACTGCGCTATTACCGTCTCTATGGAGTGTTCAGTTGGATGTCGAGTCGATACCTGATCAAAAATCCCTGCTACAACTCGCC

      610       620       630       640       650       660       670       680       690       700       710       720
CGTTTTATCTATGATCTTCGCGATGACGATGAATTATTTAGAAAAACCATCCTTGATGATTCATCAAAAAATCCCCAAGTTAATTCACTTAATAACAATGGGTTTGACCTTATGAGAAAA

      730       EcoRI     750       760       770       780       790 ++    800       810       820       830       EcoRI
AATCATCTGCATAGACGTGAATTCAGTGCCTTAAGGCTAGTAAACACTGGGCATTCTGATGTAAATTGGCTGACTAATTTAGGTTTTTCAGGAATCGGACAGTAATTATGTCGCAAGAAT
                                -35                 -10                                                   M  S  Q  E  F

      850       860       870       880       890       900       910       920       930       940       950       960
TCAATGTTGTCGTATTAGGTGCATCGGGCGCAGTGGGTCAAACCATTATAGAGATCCTCCAAGAGCGCAATTTTCCCATTGCGAAATTATTTCCTCTAGCCAGTAGTCGTAGCGCTGGTG
  N  V  V  V  L  G  A  S  G  A  V  G  Q  T  I  I  E  I  L  Q  E  R  N  F  P  I  A  K  L  F  P  L  A  S  S  R  S  A  G  G

      970       980       990       1000 SalI 1010      1020      1030      1040      1050      1060      1070      1080
GTACGGTCAGCTTTAACGGTAAACAGGTAGAAATTCTTGACGTCGACGACTTCGATTGGTCTCAGGCGCAGATAGGCTTCTTCTCTGCCGGTGGCGATGTTTCTGAAAAGTGGGCGCCTA
  T  V  S  F  N  G  K  Q  V  E  I  L  D  V  D  D  F  D  W  S  Q  A  Q  I  G  F  F  S  A  G  G  D  V  S  E  K  W  A  P  I

      1090      1100      ClaI      1120      1130      1140      1150      1160      1170      1180      1190      1200
TCGCCGCAGAGAGCGGCTGTGTTGTCATCGATAACACCTCTCATTTCAGATACGATAACGATGTACCTTTGGTTATACCTGAGGTCAACCCGGAAGCGATTGCCGATTTCCGTAACCGTA
  A  A  E  S  G  C  V  V  I  D  N  T  S  H  F  R  Y  D  N  D  V  P  L  V  I  P  E  V  N  P  E  A  I  A  D  F  R  N  R  N

      1210      1220      1230      1240      1250      1260      1270      1280      1290      1300      1310      1320
ATATCATCGCTAACCCAAACTGTTCGACGATTCAGATGTTGGTTGCGCTTAAACCCATCTATGATGCATTCGGTATTTCACGTATCAATGTCGCCACCTATCAGTCGGTATCGGGCTCAG
  I  I  A  N  P  N  C  S  T  I  Q  M  L  V  A  L  K  P  I  Y  D  A  F  G  I  S  R  I  N  V  A  T  Y  Q  S  V  S  G  S  G

      1330      1340      1350      1360      1370      1380      1390      1400      1410      1420      1430 ClaI 1440
GTAAAGAGGCTATCGCAGAGCTTGCTGGCCAATGCTCTAAGCTACTTCAAGGTCTTCCTATAGAGCCTAAGGTTTATTCAAAACAGATCGCCTTCAACGTGTTACCGCAAATCGATACGT
  K  E  A  I  A  E  L  A  G  Q  C  S  K  L  L  Q  G  L  P  I  E  P  K  V  Y  S  K  Q  I  A  F  N  V  L  P  Q  I  D  T  F

      1450      1460      1470      1480      1490      1500      1510      1520      1530      1540      1550      1560
TTATGGAAAATGGTTACACCAAGGAAGAGATGAAGATGGTCTGGGAAACTCAGAAAATCTTCGGTGATGACAACATTGTCGTCAATCCTACTGCGGTTCGAGTGCCGGTGTTTTTCGGTC
  M  E  N  G  Y  T  K  E  E  M  K  M  V  W  E  T  Q  K  I  F  G  D  D  N  I  V  V  N  P  T  A  V  R  V  P  V  F  F  G  H

      1570      1580      1590      1600      1610      1620      1630      SmaI      1650      1660      1670      1680
ACTCGGAAGCGATACATCTGGAGACCATCCAACCCGCCGAAGCTGAAGATGTTAAGGCTGTGTTGCGTGATGCTCCCGGGATTGAATTATTTGAATCCAATGAAGAGTATCCTACGGCTG
  S  E  A  I  H  L  E  T  I  Q  P  A  E  A  E  D  V  K  A  V  L  R  D  A  P  G  I  E  L  F  E  S  N  E  E  Y  P  T  A  V

      1690      BamHI     1710      1720      1730      1740      1750      1760      1770      1780      1790      1800
TGACAGAATCTGCTGGCACGGATCCTGTCTATGTGGGACGTGTACGCAAAGACATCTCTCATTCACACGGTATCAACCTTTGGGTTGTATCTGATAATATTAGAAAAGGTGCGGCACTTA
  T  E  S  A  G  T  D  P  V  Y  V  G  R  V  R  K  D  I  S  H  S  H  G  I  N  L  W  V  V  S  D  N  I  R  K  G  A  A  L  N

      1810      1820      1830      1840      1850      1860      1870      1880      1890      1900      1910      1920
ACAGTGTTCAAATAGCCGAGGTGTTGATTCGCGATTATTATTAATTTCGAATTTTATAATAGGTTAAATACCTAGTTTGATGAAAAAGTCTGCAATGTGCAGGCTTTTTTGTTTTTGCA
  S  V  Q  I  A  E  V  L  I  R  D  Y  Y  *

      1930      1940      1950      1960      1970      1980      1990      HindIII 2001
GTTGTTTTTGAATTGTGAACAGTAGCAGACCTGGGATTTCTGGTTTATAGTAATCTCAATAAATACAATTGCCTAAAGCTT
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Fig. 2A

GenBank (Mountain View, CA) nucleotide sequence data bases. The accession numbers of the DNA sequences containing *asd* from the strains DB6705 and DSS12 are D49539 and D49540, respectively.

RESULTS

Sequencing of asd Genes from Shewanella sp. Strains DB6705 and DSS12—The *asd* genes of the strictly barophilic bacterium strain DB6705 and the moderately barophilic bacterium strain DSS12 were cloned as a 2.0 kb *Hin*dIII fragment in the plasmid pMSDB1 and as a 1.9 kb *Hin*dIII fragment in the plasmid pMSDS1, respectively, detected in the *asd*$^+$ *E. coli* χ6097 transformants (Fig. 1). The DNA sequence of each of these fragments was determined and the amino acid sequences of the ASDs, which were found to consist of 338 amino acids in both strains, were deduced to be as shown in Fig. 2. The DNA sequences were very similar, and the identity of the deduced amino acids sequences of the two ASDs was 96.2%.

Identification of the asd Products—The molecular weights of the products of the cloned *asd* gene from strain DB6705 (plasmid, pMSDB1) and strain DSS12 (plasmid, pMSDS1) were both determined to be about 37 kDa by analyzing the *in vitro*-translated products by SDS-PAGE in each instance (Fig. 3). This is consistent with the predicted size of the polypeptides based on the deduced amino acid sequences as determined by DNA sequence analysis.

Phylogenetic Relationship between the ASD Proteins—The phylogenetic relationship between the ASDs of strains DB6705 and DSS12, and those of other bacteria as indicated by amino acid sequence comparison was determined by

B

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HindIII 10        20        30        40        50        60        70        80        90        100       110       120
AAGCTTAAAGACAAGACTGTGGGTATCGTAGGGGCGGGTAACACTGGCTCGGCGGTGGCTAAGTGTCTTCAGGCCTATGGCGTGACTGTGCTGCTACACGATCCGGTTATTCAAGATAGC

        130       140       150       160       170       180       190       200       210       220       230       240
GACCCCCGCGACTTTATCTCATTAGATGAATTGATAGCTCGCTGTGATGTGATAAGTCTTCATGTGCCAATCATTAAGACTGGCGAGCATAAGACCTGGTATCTGTTCGATGAGACCAGA

        250       260       270       280       290       300       310       320       330       EcoRV     350       360
CTCAATAGCTTAAAGCCTGGGACCTGGCTACTTAACTGTTGTCGTGGTGAGGTGATAGATAATCGCGCACTTATTAAGGTTAAGCAGCAAAGGCCGGATATCAAGCTGGTACTCGATGTG

        370       380       390       400       410       420       430       440       450       460       470       480
TGGGAAGGGGAGCCTAATCCTATGCATGAGCTTATTCCTTTGGTCGAATTGGCGACGCCTCATATCGCAGGTTACAGCCTAGAAGGTAAGGCTCGTGGCACTTATATGCTGTATCAGAAG

        490       500       510       520       530       540       550       EcoRV     570       580       590       600
CTAATGCAGGTACTGGGCAGAGATGCCGATAAGTCCATGACTACACTATTACCGTCGCTATGGAGCGTGCAATTGGATATCGAGCCGATACCCAATGAGAAGTCCTTGTTGAAACTGGCT

        610       620       630       640       650       660       670       680       690       700       710       720
CGTTTTATCTACGACCTTCGTGATGGCGATGAATTATTTAGAGAAACAATCCTCGATGATTCATCAAAAAATGACCAAGTTAATTGTGTTAATAACAATGGATTTGATCTTATGAGAAAA

        730       740       750       760       770       780       790 +‡    800       810       820       830       EcoRI
AATCACCAGCATAGACGTGAATTTCGTGCCTTAAGGCTAGTAAACACTGGGCATTCTGATGTAAATTGGTTGACCAATTTAGGTTTTTCAGGAGTCGGACAGTAATTATGTCTCAAGAAT
                                  -35                 -10                                                       M  S  Q  E  F

        850       860       870       880       890       900       910       920       930       940       950       960
TCAATGTTGTCGTATTAGGTGCATCGGGCGCAGTGGGTCAAACCATGATAGAGATCCTCGAAGAGCGCAATTTCCCCGTCGCGAAATTATTTCCTCTAGCCAGCAGCCGCAGTGCTGGTG
  N  V  V  V  L  G  A  S  G  A  V  G  Q  T  M  I  E  I  L  E  E  R  N  F  P  V  A  K  L  P  P  L  A  S  S  R  S  A  G  G

        970       980       990       1000      1010      1020      1030      1040      1050      1060      1070      1080
GTACGGTTAGTTTCAACGGTAAACAGGTAGAAATTCTTGACGTAGACGACTTCGATTGGTCTCAGGCGCAGATTGGCTTCTTCTCTGCCGGTGGTGATGTCTCTGAAAAGTGGGCGCCAA
  T  V  S  F  N  G  K  Q  V  E  I  L  D  V  D  D  F  D  W  S  Q  A  Q  I  G  F  F  S  A  G  G  D  V  S  E  K  W  A  P  I

        1090      1100      ClaI      1120      1130      1140      1150      1160      1170      1180      1190      1200
TCGCCGCTGAGAATGGTTGTGTTGTCATCGATAACACCTCTCAATTCAGATACGATAACGATGTACCTTTGGTTATTCCAGAGGTTAATCCGGAAGCCATTGCTGATTTCCGTAATCGTA
  A  A  E  N  G  C  V  V  I  D  N  T  S  Q  F  R  Y  D  N  D  V  P  L  V  I  P  E  V  N  P  E  A  I  A  D  F  R  N  R  N

        1210      1220      1230      1240      1250      1260      1270      1280      1290      1300      1310      1320
ATATCATAGCGAATCCAAACTGTTCGACAATTCAGATGTTGGTTGCCCTTAAACCTATTTATGATGCATTTGGTATTTCACGTATCAATGTCGCCACATACCAGTCGGTATCGGGTTCAG
  I  I  A  N  P  N  C  S  T  I  Q  M  L  V  A  L  K  P  I  Y  D  A  F  G  I  S  R  I  N  V  A  T  Y  Q  S  V  S  G  S  G

        1330      1340      1350      1360      1370      1380      1390      1400      1410      1420      1430      1440
GTAAAGAGGCTATTACAGAGCTTGCTGGCCAATGTTCTAAATTACTTCACGGCCTTCCTGCTGAGTCTAAGGTTTATCCAAAACAGATCGCCTTCAACGTGTTGCCGCAAATTGATAAGT
  K  E  A  I  T  E  L  A  G  Q  C  S  K  L  L  H  G  L  P  A  E  S  K  V  Y  P  K  Q  I  A  F  N  V  L  P  Q  I  D  K  F

        1450      1460      1470      1480      1490      BglII     1510      1520      1530      1540      1550      1560
TTATGGAAAACGGTTACACCAAGGAAGAGATGAAGATGGTTTGGGAAACCCAGAAGATCTTCGGTGATGACAATATTGTCGTCAATCCAACTGCGGTTCGAGTGCCGGTATTTTATGGTC
  M  E  N  G  Y  T  K  E  E  M  K  M  V  W  E  T  Q  K  I  F  G  D  D  N  I  V  V  N  P  T  A  V  R  V  P  V  F  Y  G  H

        1570      1580      1590      1600      1610      1620      1630      1640      1650      1660      1670      1680
ACTCGGAAGCAATACATCTTGAGACTATTCAACCGCCGAAGCTGAAGATGTTAAAGCTGTGTTGCGTGAAGCTCCGGGAATTGAATTATTTGAGTCCAATGAAGAGTATCCTACGGCTG
  S  E  A  I  H  L  E  T  I  Q  P  A  E  A  E  D  V  K  A  V  L  R  E  A  P  G  I  E  L  F  E  S  N  E  E  Y  P  T  A  V

        1690      1700      1710      1720      1730 EcoRV 1740    1750      1760      1770      1780      1790      1800
TGACAGAATCAGCGGGCACGGATCCTGTTTATGTGGGACGTGTACGCAAAGATATCTCTCATTCACATGGTATAAACCTTTGGGTTGTCTCTGATAACATTAGAAAAGGTGCGGCACTCA
  T  E  S  A  G  T  D  P  V  Y  V  G  R  V  R  K  D  I  S  H  S  H  G  I  N  L  W  V  V  S  D  N  I  R  K  G  A  A  L  N

        1810      1820      1830      1840      1850      1860      1870 HindIII 1875
ATAGTGTTCAGATAGCCGAAGTTTTGATTAGAGATTATTATTAATTTATAACGACTTTGAATCGGTTTAAAGCTT
  S  V  Q  I  A  E  V  L  I  R  D  Y  Y  *
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Fig. 2. **Nucleotide and deduced amino acid sequences of the cloned fragments from strain DB6705 (A) and from strain DSS12 (B).** The −35 and −10 sequences are located upstream from the transcriptional start point, which was determined by primer extension (marked as + and ‡). The putative −35 and −10 regions upstream of the *asd* gene and the putative transcriptional termination sequence downstream of the gene are underlined.

the UPGMA method (*19*) as shown in Fig. 4. The results suggest that the ASDs from the deep-sea bacteria were more similar to those of the genus *Vibrio*, and those of Gram-positive bacteria, than to the ASD of *E. coli*. Nevertheless, the deep-sea bacteria described in this paper (genus *Shewanella*), as well as the genus *Vibrio*, and *E. coli*, belong to a common group of Gram-negative bacteria categorized as the γ-subgroup of Proteobacteria as indicated by taxonomic studies of 16S rRNA sequences (*2, 3*).

Analysis of Transcription by Primer Extension and ASD Expression—The 5′-ends of the mRNA were identified as nucleotide 792 and 793 respectively in strains DB6705 and DSS12, as indicated by "*" and "**" in Fig. 5. The 1st transcription product beginning at nucleotide 792 (*) was designated as transcript #1, and the 2nd transcription product beginning at nucleotide 793 (**) was designated as transcript #2. In the strictly barophilic strain DB6705, transcript #1 was produced in minor amounts, and transcript #2 was the major transcript, while the converse was seen in the moderately barophilic strain DSS12. In both strains, the expression of transcript #2 was clearly enhanced at elevated hydrostatic pressures. However, the expression of transcript #1 appeared to be unaffected by pressure. Therefore, the total amount of *asd* transcript in strain DSS12 also increased as the pressure increased.

Western-blotting analysis was performed using anti-ASD antiserum and a crude extract prepared from each of the strains cultivated in the same way as for the primer extension analysis. In the case of strain DB6705 cultivated at 10 MPa, a faint band representing a very small amount of ASD protein was detectable, but when the organism was cultivated at pressures over 30 MPa, this ASD band was more evident. In the case of strain DSS12, the ASD band was clearly evident under all of the pressure conditions employed for cultivation (0.1-70 MPa), as shown in Fig. 6. This result is not exactly consistent with the result of the primer extension analysis (Fig. 5), so the quantity of ASD product may be controlled by proteolytic enzymes when the protein is produced in excess in those bacteria under high pressure.

The transcripts of the *E. coli asd* gene grown at elevated hydrostatic pressures were also analyzed by primer extension (Fig. 7). Three different sizes of transcripts were found, *i.e.*, transcripts #1, #2, and #3, as shown in Fig. 7. Interestingly, the expression of transcripts #1 and #2 was found to decrease at high pressure, whereas the expression of transcript #3 increased with elevated pressure up to 30 MPa, as seen in the case of transcript #2 of the *asd* gene of the deep-sea bacteria. But at 50 MPa, the *asd* transcripts were almost undetectable. This might indicate that very little ASD enzyme was synthesized in the bacterial cells, and this may be one of the causes of filament formation and growth inhibition at high pressure in *E. coli*.

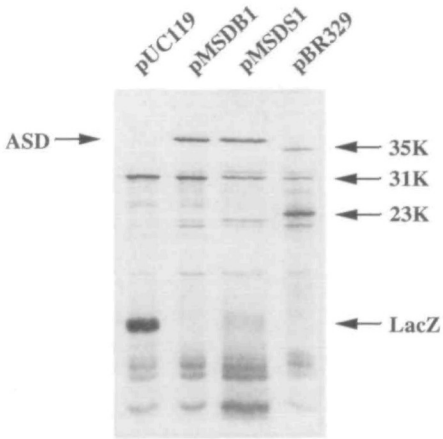

Fig. 3. **Proteins synthesized in an *in vitro* transcription/translation system from recombinant and vector plasmids.** Synthesized polypeptides were resolved by 15% SDS-PAGE.

DISCUSSION

We have cloned *asd* genes from the deep-sea adapted barophilic strains, DB6705 (strictly barophilic) and DSS12 (moderately barophilic), and determined their nucleotide sequences (Fig. 2). Phylogenetic relationships between the ASDs as indicated by amino acid sequence comparison showed that the ASDs of the barophilic strains and the genus *Vibrio* were closer to the ASDs of Gram-positive

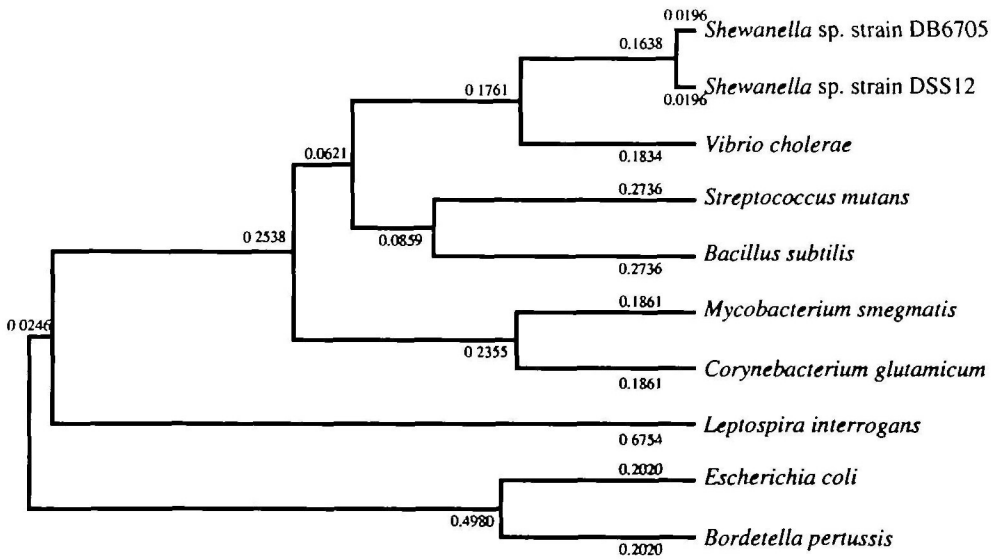

Fig. 4. **Phylogenetic tree showing the relationship between the ASD amino acid sequences of the deep-sea strains, and those of the bacterial strains reported previously as determined using the UPGMA method.** The accession numbers of the ASD of *Vibrio cholerae, Streptococcus mutans, Bacillus subtilis, Mycobacterium smegmatis, Corynebacterium glutamicum, Leptospira interrogans, E. coli*, and *Bordetella pertussis* are S14523, A29137, S34599, S31803, S12251, A44846, DEECDA, and S38611, respectively in the data base of Swiss Prot. The numbers represent the average number of nucleotide substitutions per site.

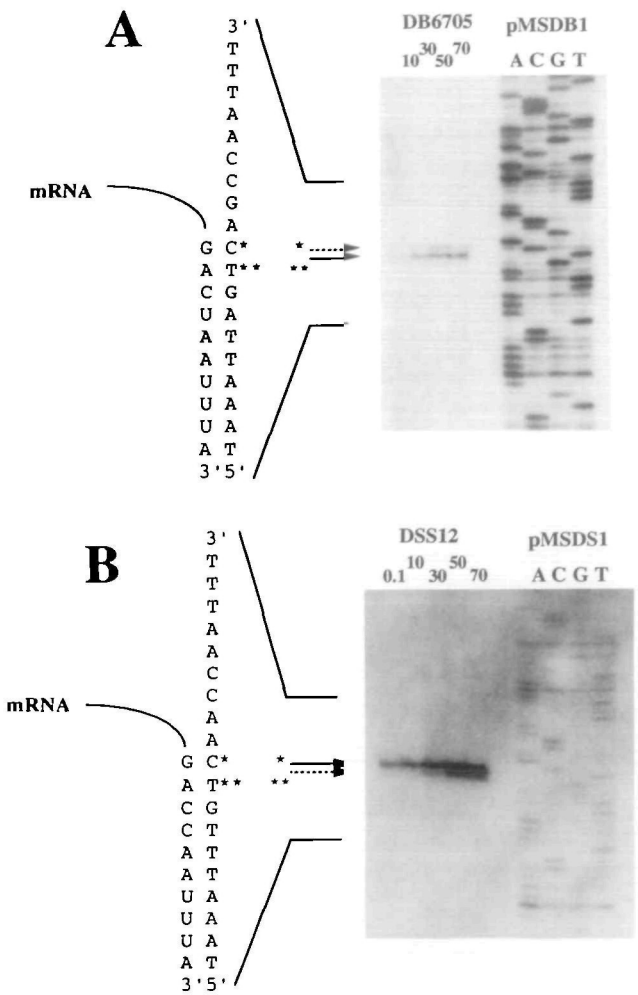

Fig. 5. **Primer extension analysis to determine the transcriptional start point of the *asd* genes in the strains DB6705 (A) and DSS12 (B), and level of mRNA derived from the promoter in response to elevated pressure.** The DNA sequence ladders of the DB6705 *asd* gene (from pMSDB1) and the DSS12 *asd* gene (from pMSDS1) were obtained by the method of Sanger *et al.* (*12*), employing the same primers. The nucleotide sequence corresponding to the ladder is shown on the left. The transcriptional start points are indicated by arrows and asterisks, and the 5′-end of the mRNA is also shown. Lined arrows show the major transcript, and dotted arrows show the minor transcript. Asterisks "*" and "**" show the transcripts #1 and #2, respectively, as described in the text. Lanes: 0.1, RNA from the strain DSS12 (B) grown at 0.1 MPa; 10, RNA from the strain DB6705 (A) or DSS12 (B) grown at 10 MPa; 30, at 30 MPa; 50, at 50 MPa; and 70, at 70 MPa.

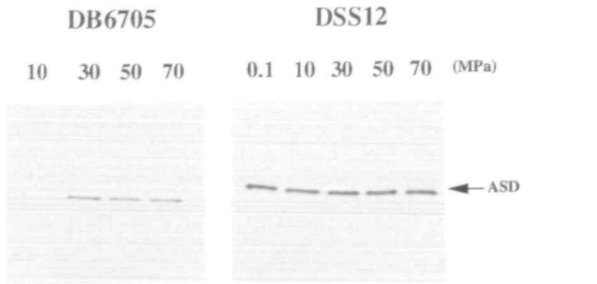

Fig. 6. **Western-blotting analysis of the quantities of ASD protein expressed in strains DB6705 and DSS12 in response to elevated pressures.** Numbers mean the same as in Fig. 5.

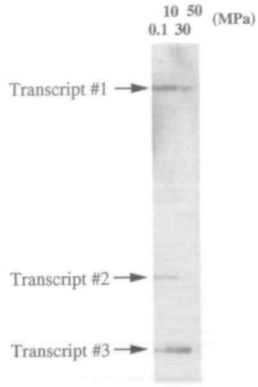

Fig. 7. **Primer extension analysis of the *E. coli asd* gene.** Arrows show the transcripts #1, #2, and #3. Lanes: 0.1, RNA from *E. coli* JM109 grown at 0.1 MPa; 10, at 10 MPa; 30, at 30 MPa; and 50, at 50 MPa.

bacteria than to the ASD of *E. coli* (Fig. 4). Interestingly, these relationships are very different from the phylogenetic relationships based on comparison of 16S rRNA sequences.

The transcription of each *asd* was analyzed by primer extension, and the results suggest that the gene expression of one of the transcripts (#2) is regulated by elevated hydrostatic pressure in both the strictly and moderately barophilic strains (Fig. 5). In these strains, the transcriptional starting points were at similar locations, and putative −10 [TAAATT] and −35 [TAAACA] sequences of the promoter region, which are different from the *E. coli* σ^{70} consensus sequence (*20*), were present. However, the promoter consensus of the genus *Shewanella* is not yet established. The nucleotide sequences around the *asd* promoter regions of both strains were very similar, so the difference in *asd* transcription in these strains may depend on differences in transcription factors in strictly barophilic and moderately barophilic bacteria. The quantities of ASD protein in the crude extracts of cells of these strains cultivated under several pressure conditions were consistent with the results of primer extension analysis (Fig. 6). These findings suggested that the gene expression systems of strictly barophilic bacteria are activated to express ASD under high pressure, whereas those of moderately barophilic bacteria function to express ASD under any pressure condition. This may be one of the reasons why strictly barophilic bacteria cannot grow at atmospheric pressure, whereas moderately barophilic bacteria can grow over a wide range of pressures. It is very interesting that in *E. coli* adapted to growth at 1 atmosphere pressure, the expression of several *asd* transcripts is modulated by high pressure, such that expression of two of them is pressure-sensitive and expression of one of them is pressure-dependent up to 30 MPa (Fig. 7). We have reported that the expression of some genes in *E. coli* is influenced by high hydrostatic pressure (*21-23*), and *asd* expression in *E. coli* may be similar. It seems possible that the mechanisms of gene expression functioning in high pressure-adapted microorganisms are conserved in *E. coli*. The results of analysis of bacterial *asd* gene expression suggest that the morphological changes observed under high pressure conditions

correspond to the changes in bacterial growth and morphology. Thus, *asd* gene expression controlled by pressure in deep-sea-adapted bacteria appears to be one of the important mechanisms for survival in the deep-sea environment.

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