

Cloning and sequencing of the *phoA* gene encoding alkaline phosphatase from a deep-sea piezophilic bacterium, *Shewanella violacea* strain DSS12

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We have cloned and sequenced the *phoA* gene, encoding alkaline phosphatase, from a λ phage library of the chromosome of the deep-sea bacterium *Shewanella violacea* strain DSS12. This gene, 1,359 bp in length, was found to encode a protein consisting of 452 amino acid residues with a molecular mass of 48,500 Da. Significant homology was evident comparing the *phoA* gene of *S. violacea* with that of *Lactobacillus delbrueckii* (43% identity), *Bacillus subtilis* (type IV) (42% identity), *Bacillus subtilis* (type III) (40% identity), and *Staphylococcus aureus* (37% identity). Phylogenetic analysis of several types of PhoA proteins of several bacteria showed that *S. violacea* is closely related to *L. delbrueckii*.

Keywords : *Shewanella violacea*, *Lactobacillus delbrueckii*, *phoA* gene, phylogenetic tree

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1. Introduction

The moderately piezophilic and psychrophilic bacterium *Shewanella violacea* strain DSS12 from the Ryukyu Trench (depth 5,110 m)¹⁾ grows optimally at 30 MPa and 8°C, but also grows at atmospheric pressure (0.1 MPa) and 8°C²⁾. These growth properties are useful for comparative study of cell physiology under high and low pressure conditions. *S. violacea* shows good growth under high pressure conditions^{1,2)}, whereas *E. coli*, which is closely related to *S. violacea*, shows poor growth under the same conditions³⁾. We have investigated the molecular basis of gene regulation in this strain under high pressure conditions⁴⁾. It is also the candidate (source) for several useful enzymes and bioactive substances, because it was originally isolated as a protease-producing bacterium and produces EPA (eicosapentanoic acid). From these backgrounds, we are trying to clone several useful genes from the strain.

In this study, we isolated and cloned the *phoA* gene from a deep-sea piezophilic bacterium. The results of phylogenetic analyses based on comparison of the PhoA protein will be described.

2. Materials and Methods

2.1. Cloning of the *phoA* gene from *S. violacea*

Two degenerate oligonucleotides were designed and synthesized, based on highly conserved sequences, I(N/T)HATPA and EGSQIDW, in PhoA proteins. The nucleotide sequences of the oligonucleotides were 5'-ATI AII CAI GCI ACI CCI GC-3' (primer 1) and 5'-CCA ITC IAT ITG ICT ICC TTC-3' (primer 2). The PCR product obtained using *S. violacea* chromosomal DNA as the template and the above primers, was cloned into the pCR2.1 vector (Invitrogen Co., Carlsbad, CA) and the nucleotide sequence was determined by the dye terminator method using a DNA sequencer model 377 (Perkin-Elmer/Applied Biosystems Co., Foster, CA). Based on the determined nucleotide sequence, two other PCR primers were designed for use in amplification of the digoxigenin (DIG)-labeled *phoA* fragment. The DIG-labeled fragment was employed as the probe for plaque hybridization in screening by means of the DIG detection system (Boehringer Mannheim Co., Mannheim, Germany).

2.2. Construction of a λ phage library of the chromosome of *S. violacea* and screening of the library for the *phoA* gene region.

Chromosomal DNA isolated from *S. violacea* was partially digested with *Sau3AI*. These fragments were inserted into the *Bam*HI site of lambda DASH II (Stratagene Co., La Jolla, CA). Then, *in vitro* packaging of the ligated DNA was performed using GIGAPACK III XL packaging extracts (Stratagene Co.) according to the manufacturer's instructions. The λ phage library was screened for plaque hybridization with the *phoA* probe and several positive clones were obtained. The positive clones containing the *phoA* gene were each purified by several single plaque isolation steps. Each of the inserts in λ phage were amplified by long and accurate PCR and were subcloned into the pCR-Blunt vector (Invitrogen Co.). For sequencing of these cloned fragments, the random shotgun sequencing method was used with a DNA sequencer model 377 (Perkin-Elmer/Applied Biosystems Co.). Assembling and editing of the determined DNA sequences were performed with AutoAssembler Version 2.0 (Perkin-Elmer/Applied Biosystems Co.). GENETYX-MAC version 10.1 from Software Development (Tokyo, Japan) was used for sequence analysis.

2.3. Phylogenetic analysis of the *phoA* genes and 16S rDNAs

To determine the phylogenetic position of the *S. violacea phoA* gene, evolutionary distance was calculated and a phylogenetic tree was constructed. The PhoA amino acid sequences were aligned and the tree was constructed by the neighbor-joining method. The alignment and calculation were performed using CLASTALX and the PHYLIP package (ver. 3.573; obtained from J. Gelsenstein, University of Washington, Seattle).

3. Results and Discussion

3.1. Isolation of the *phoA* gene

To clone a part of the *phoA* gene from *S. violacea*, the amino acid sequences of the PhoA proteins from several bacteria were aligned and a highly conserved domain was found (data not shown). These conserved sequences were used to design and synthesize degenerate oligonucleotides (primer 1 and primer 2) in order

to amplify a part of the *phoA* gene from *S. violacea*. A fragment containing part of the *phoA* gene, approximately 366 bp, amplified by PCR with the primers, was cloned into the pCR2.1 vector and its nucleotide sequence was determined. The deduced amino acid sequence of the cloned gene was significantly similar to the PhoA of other bacteria. To clone the complete *phoA* gene, the partial *phoA* gene fragment was labeled with digoxigenin by PCR for use as a hybridization probe.

3.2. Structural analysis of the complete *phoA* gene of *S. violacea*

A λ phage library of *S. violacea* chromosomal DNA was constructed and screened using the *phoA* probe. Several positive clones were isolated from among 10^4 plaques and purified by repeated single plaque isolation. These clones were confirmed to contain the *phoA* gene by PCR at each isolation step. As a result of the screening, we obtained five positive clones and the nucleotide sequence of one of these clones containing a DNA insert approximately 18 kb in size was determined by the random shotgun sequencing method. The open reading frame of the *phoA* gene, 1,179 bp in length, was found to encode a polypeptide consisting of 392 amino acid residues with a molecular mass of 40,727 Da (Fig. 1). Analysis of the deduced amino acid sequence confirmed that the cloned gene contained the complete sequence of the PhoA protein (Fig. 2).

3.3. Phylogenetic analyses of the evolutionary distance of *S. violacea* PhoA.

The *phoA* gene is conserved in a wide range of organisms. To confirm the phylogenetic position of the *S. violacea phoA*, the evolutionary distance was calculated based on comparison of 20 organisms and a phylogenetic tree was constructed (Fig. 3). The PhoA amino acid sequences were aligned and the tree was constructed by the neighbor-joining method. The results obtained in calculation of the evolutionary distance comparing the PhoA amino acid sequences of 20 organisms showed that the PhoA protein of *S. violacea* is closely related that of *L. delbrueckii*.

In biotechnological application, construction of a recombinant expression plasmid harboring the *S. violacea phoA* gene is definitely required. In addition, To obtain highly purified recombinant PhoA protein, overexpression of the expression plasmid and detection of the nuclease activity at low temperature after the overproduction and purification are also required.

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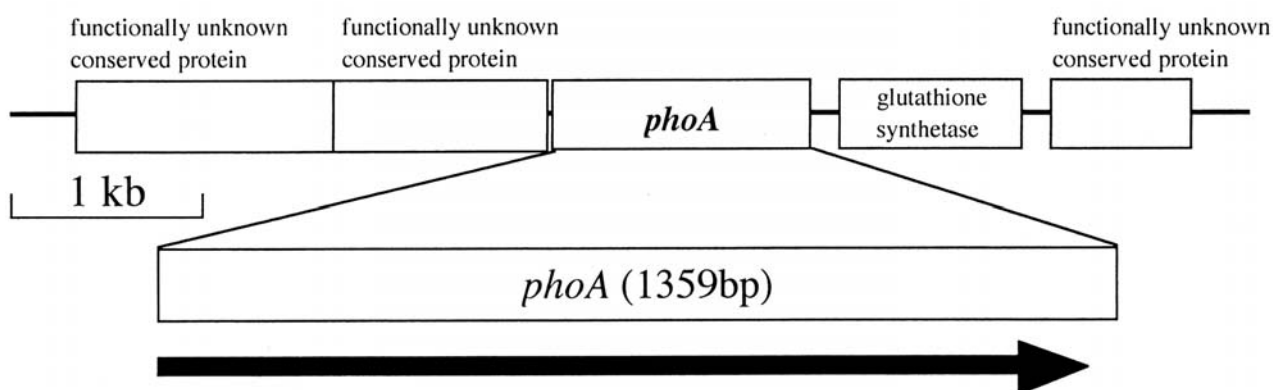


Fig. 1 Location of the *phoA* gene on the chromosome of *S. violacea*.
The coding region and its direction of transcription are indicated by arrows.

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1  ATGGGCGCGACGCAAAAAATGACAATATTTAACTATGTATATTGGCTGGTGCGGCACTA  60
   M G A T Q K M T I F K L C I L A G A A L
61  GTCGGCACCATGAGCGTCGCTGCTCAAGCTATGACTCCGGCGCAGACGATGTTAGGTGAC  120
   V G T M S V A A Q A M T P A Q T M L G D
121 GCACCGAGTGTCCCTAAGAATATAGTGATCATGATAGGTGACGGTATGGGCCCCGCCTAT  180
   A P S V P K N I V I M I G D G M G P A Y
181 ACCAGCGCCTATCGATACTATAAAGACAACCCAGATACACAAGAGATAGAGCAGACGGTA  240
   T S A Y R Y Y K D N P D T Q E I E Q T V
241 TTCGACCGCTTGCTAGTGGGAACCTCGAGTACATACCCAGCTAGAGTGAGTGGTTATGTC  300
   F D R L L V G T S S T Y P A R V S G Y V
301 ACAGATTCCGGCAGCCGCGGCAACGGCACTAGCCACTGGGGTTAAATCCTATAATGGGGCT  360
   T D S A A A A T A L A T G V K S Y N G A
361 ATCTCTGTCGATATCAATAAGCAGACGATTCCTACCATAATGAAAAAGCTAAGGCTCTA  420
   I S V D I N K Q T I P T I M E K A K A L
421 GGACTCGCTACTGGAGTGGCGGTGACATCACAGATTAACCATGCTACTCCGGCCGCATTT  480
   G L A T G V A V T S Q I N H A T P A A F
481 CTCAGCCACAATGAAAGCCGTAGCAATTATGATGCGTTAGCTAAAAGCTACCTTACAAC  540
   L S H N E S R S N Y D A L A K S Y L T T
541 GATACCGATGTGATGTTGGGCGGTGGCAAAAAGTATTTCCCTGTCAGTCTGATAGAAAA  600
   D T D V M L G G G K K Y F P V S L I E K
601 TTCCAGGCTAAGGGATATCAATACCTGTCGGACTTTAACCAGCTGGATTCTATCAAACAA  660
   F Q A K G Y Q Y L S D F N Q L D S I K Q
661 GGTAAGGTCATTGGCCTGTTTGCCAATGTGCAACTGCCATGGGCAATCGATGATAATGAC  720
   G K V I G L F A N V Q L P W A I D D N D
721 AAGAACAAGCTAAGCAAGATGACGGCTAAGGCCTTAGAACTCTTATCTCAACATGACAAA  780
   K N K L S K M T A K A L E L L S Q H D K
781 GGCTTCGTGCTGCTAATCGAGGGCAGTTTGATCGACTGGGCCGGACACAATAATGATATT  840
   G F V L L I E G S L I D W A G H N N D I
841 GTGAGGCAATGGGCGAGATGGAAGAATTTCGCTAATGCAATAGAAGTCGTAGAGCAATAC  900
   V T A M G E M E E F A N A I E V V E Q Y
901 ATCAGGAACAATGACAATACGCTCATGCTAGTAACGGCAGATCACAATACCGGTGGCTTA  960
   I R N N D N T L M L V T A D H N T G G L
961 AGCATAGGTGCCAACGGCGAGTATAGCTGGAATACCAGTATCATCAGGGGTGTACAAGCC  1020
   S I G A N G E Y S W N T S I I R G V Q A
1021 AGCCCGGATTTTATCGCAAACGATGCCCTCATTGATGATGACTGGTTAGCACGAGTCAGC  1080
   S P D F I A N D A L I H D D W L A R V S
1081 TTAAATTTAGGCTTCGAGCCGAATGAAGATGAAGCGTCTCAATTAGCGCGAGCACGTATG  1140
   L N L G F E P N E D E A S Q L A R A R M
1141 CAAGGTAAAGAGGTGATGACCACAAGCATAAAGCACCTCATAGATAGCCGCTCAAACACA  1200
   Q G K E V M T T S I K H L I D S R S N T
1201 GGCTGGACTACAGGCGGACACAGGCGTCGACGTACAAGTATTCGCAACGGGTCCCGCA  1260
   G W T T T G G H T G V D V Q V F A T G P A
1261 GCTAGCCTATTTGCAGGTTATCAGGACAACACAGATATCGCGAATAAACTCATCAGCTTA  1320
   A S L F A G Y Q D N T D I A N K L I S L
1321 CTGCCTAAAGCTAAGTCCAAGTCTCAGTCAAATAAATAA  1359
   L P K A K S K S Q S N K *

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Fig. 2 Nucleotide sequence and deduced amino acid sequence of the *phoA* gene.
The nucleotide and deduced amino acid sequences of the *phoA* gene. The deduced amino acid sequence is given in single-letter code below the nucleotide sequence.

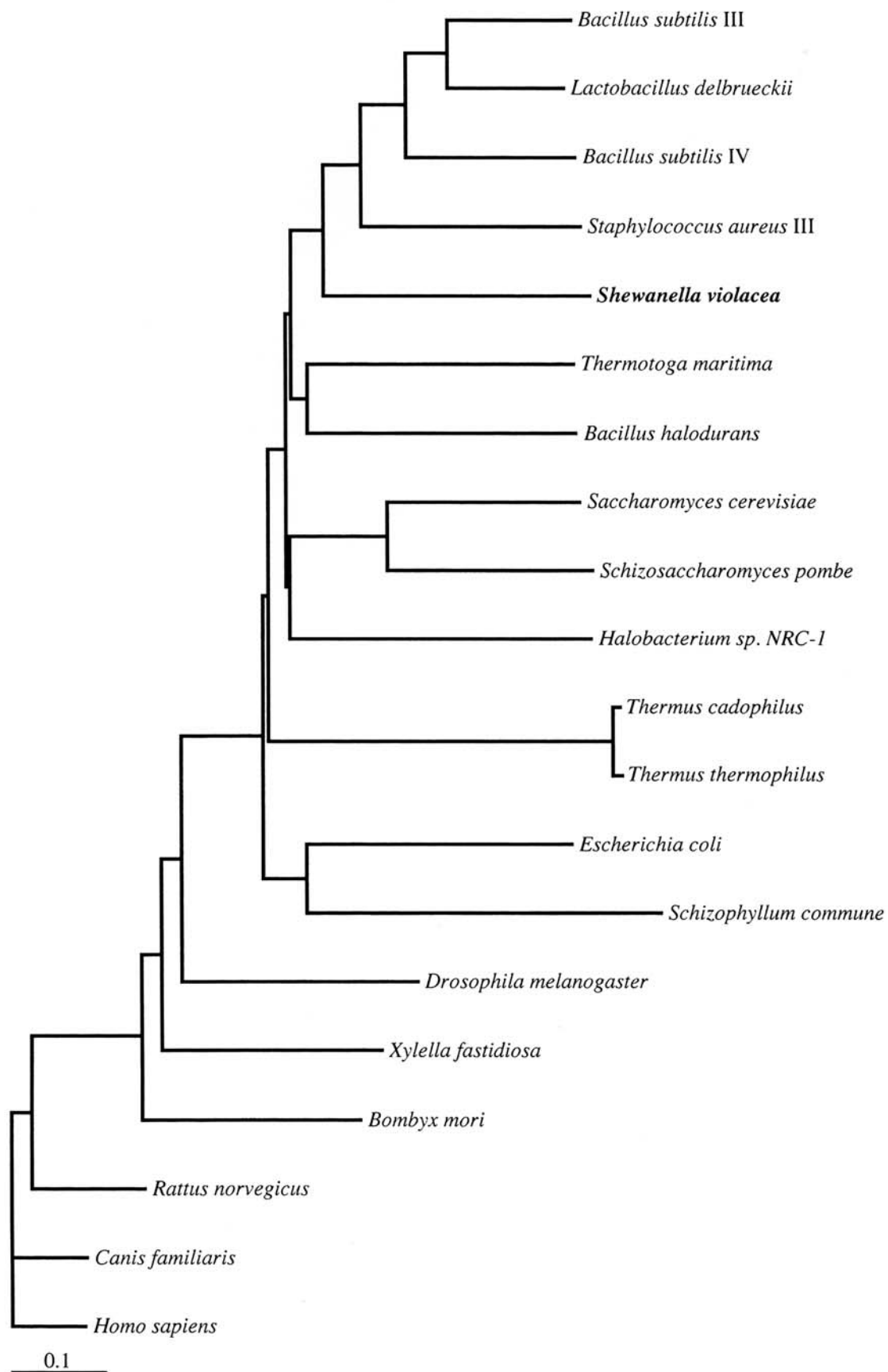


Fig. 3 Phylogenetic tree inferred by neighbor joining analysis of PhoA protein sequences.

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