

Isolation of Cold Shock Inducible Gene *cspA* from Deep-sea Bacterium, *Shewanella* sp. Strain DSS12

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Upon the temperature downshift, a subset of proteins called cold shock proteins are transiently induced in many kinds of bacteria. We cloned and sequenced a cold-shock-inducible gene from the psychrophilic and moderately barophilic bacterium, *Shewanella* sp. strain DSS12 by a PCR-based approach using a pair of degenerate primers with sequences corresponding to a highly conserved region within CspA-related proteins. The cloned gene, designated *cspA*, encodes a 70-residue protein which possesses 62% sequence identity with *Escherichia coli* CspA.

Key words: *cspA*; *Shewanella* sp. Strain DSS12; low temperature environment; deep-sea bacterium

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1. Introduction

The cellular response to temperature downshift has not been well understood in contrast to the heat shock response. When an exponentially growing culture of *Escherichia coli* is shifted from 37°C to 15°C, the cessation of growth is accompanied by a severe decrease in synthesis of almost all proteins.

CspA has been identified as the major cold shock protein, which is transiently induced and comprises up to 13% of the total cellular protein upon temperature downshift (Goldstein et al., 1990). A family of CspAs sharing highly conserved sequences (>45% identity) has been recognized in a variety of Gram-positive and Gram-negative bacteria (Graumann and Marahiel, 1996, Willinsky et al., 1992, Yamanaka et al., 1998). In *E. coli*, CspA functions as a RNA chaperone (Graumann et al., 1997) and as a transcriptional regulator for itself and for other cold shock genes (Thieringer et al., 1998). *Shewanella* sp. strain DSS12 is a psychrophilic and moderately barophilic bacterium which was isolated from mud of the Ryukyu Trench (5110m depth) collected by the SHINKAI 6500 System. This bacterium displays optimal growth at a temperature of 8°C and at a pressure of 30 MPa (Kato et al., 1995).

In this study, we used a PCR-based approach and a pair of degenerate primers with sequences corresponding to a highly conserved region in CspAs to clone a *cspA*-like gene from DSS12. This is the first report of a *cspA*-like gene isolated from a barophilic or psychrophilic bacterium.

2. Materials and methods

2.1. PCR amplification and sequencing of a specific fragment of the *cspA*-like gene.

The DNA sequences of the oligonucleotides used for identification of the *cspA*-like gene were based on the amino acid sequences of proteins belonging to the family of the major cold shock protein CspA. We selected amino acid sequences from the alignment of 18 members of the CspA family of proteins. One 20-mer, 5'-GGNMHIGTNAARTGGTTYAA-3' (primer F), matches the *E. coli* CspA sequence from amino acid residues 7 through 13; the other oligomer, a 17-oligonucleotide sequence, 5'-AARTGNACRAANACRTC-3' (complementary, primer R), represents amino acid residues 29 through 34. All oligonucleotides were purchased

from Pharmacia Biotech. The reaction mixture (100 ml) contained 100 ng of DSS12 chromosomal DNA, 5 U of *Ex-Taq* DNA polymerase (TaKaRa, Japan) used with the supplied buffer, 200 pmol of each primer, and the four deoxynucleoside triphosphates at 250 mM each and was submitted to 30 cycles of 15 sec of denaturation at 94°C, 1 min of annealing at 30°C, and 10 sec of elongation at 72°C in the Gene Amp PCR System (Perkin Elmer). A 83-bp amplified fragment was

purified from a 3% agarose gel and cloned in a pT7Blue T-Vector (Novagen). Several *E. coli* DH5a (Toyobo, Japan) transformants containing the plasmid with an insert were isolated, and the cloned DNA was sequenced with an automated DNA sequencer (model 377, Perkin Elmer / Applied Biosystems).

2.2. Isolation and sequencing of the entire *cspA* gene.

Plaque hybridization was performed and some positive phage clones were isolated from a DSS12 genomic DNA library in λ EMBL3 using the DIG-labeled 46-mer oligonucleotide probe described above. The insert DNAs were amplified from positive clones with LA-Taq DNA polymerase (TaKaRa, Japan) by means of a EMBL3/ λ GEM11 LD-insert Screening Amplimer Set (CLONTECH), and sequenced by a direct sequencing procedure with the automated DNA sequencer according to the manual (Model 377, Perkin Elmer / Applied Biosystems).

2.3. Computer analysis

The amino acid sequence of CspA was analyzed using the computer program

GENETIX-MAC 10.0 for calculation of the isoelectric point and molecular weight, and for prediction of the secondary structure of the 5' untranslated region of the *cspA* mRNA.

3. Results and discussion

3.1. PCR amplification of a *cspA*-like gene fragment from *Shewanella* sp. strain DSS12.

To obtain a DNA fragment containing a *cspA*-like gene from DSS12, we designed the pair of primers described in Materials and Methods. The 83-bp amplified fragment encoded a unique sequence (DKGFGE) present in the

amino acid sequence of proteins of the CspA family. We screened a DSS12 genomic library with a DIG-labeled 46-mer oligonucleotide (internal portion of the 83-bp amplified fragment without the probes) probe and obtained six positive phage clones. Sequencing of the entire *cspA* gene, and identification of the *cspA* gene in DSS12 chromosomal DNA. After long and accurate PCR amplification of entire inserts (ca 20 kbp) from the positive phage clones, we sequenced six of the PCR products directly using two primers. The sequences of these primers correspond to bases 356 to 376 (complementary) and bases 378 to 397 in Fig. 1. A region of about 1.6 kbp containing the *cspA* gene was sequenced by walking (data not shown).

Several CspA-like proteins from different sources have been reported for mesophiles but few published data are available concerning CspA-like proteins in cold-adapted bacteria; i.e. psychrotrophic and psychrophilic bacteria. Several sequences of *cspA*-like genes from different sources also have been reported for thermophilic,

mesophilic and psychrotrophic bacteria but not for psychrophilic bacteria. In this study we cloned and sequenced a *cspA*-like gene from a deep-sea psychrophilic bacterium, *Shewanella* sp. strain DSS12.

3.2. Structural analysis of the *cspA* gene

The DNA sequence revealed that the *cspA* gene is 213 bp in length and encodes a protein comprising 70 amino acid residues with a molecular mass of 7,583 Da and a calculated pI of 4.48 (Fig. 1). A potential ribosome binding site (AAGGG) is located 7 nucleotides upstream of the initiation codon in *cspA*. *CspA* has two stop codons followed by a stem loop structure similar to a rho-independent terminator. The deduced amino acid sequence of *cspA* has RNP1 (KGFGL) and RNP2 (V F V H F) motifs, and comparison with sequences in the EMBL database using the program BLAST revealed 62% identity to the major cold shock protein *CspA* of *E. coli*. Putative secondary structure of the 5' untranslated region of *cspA* mRNA, and the alignment with *E. coli* cold-box

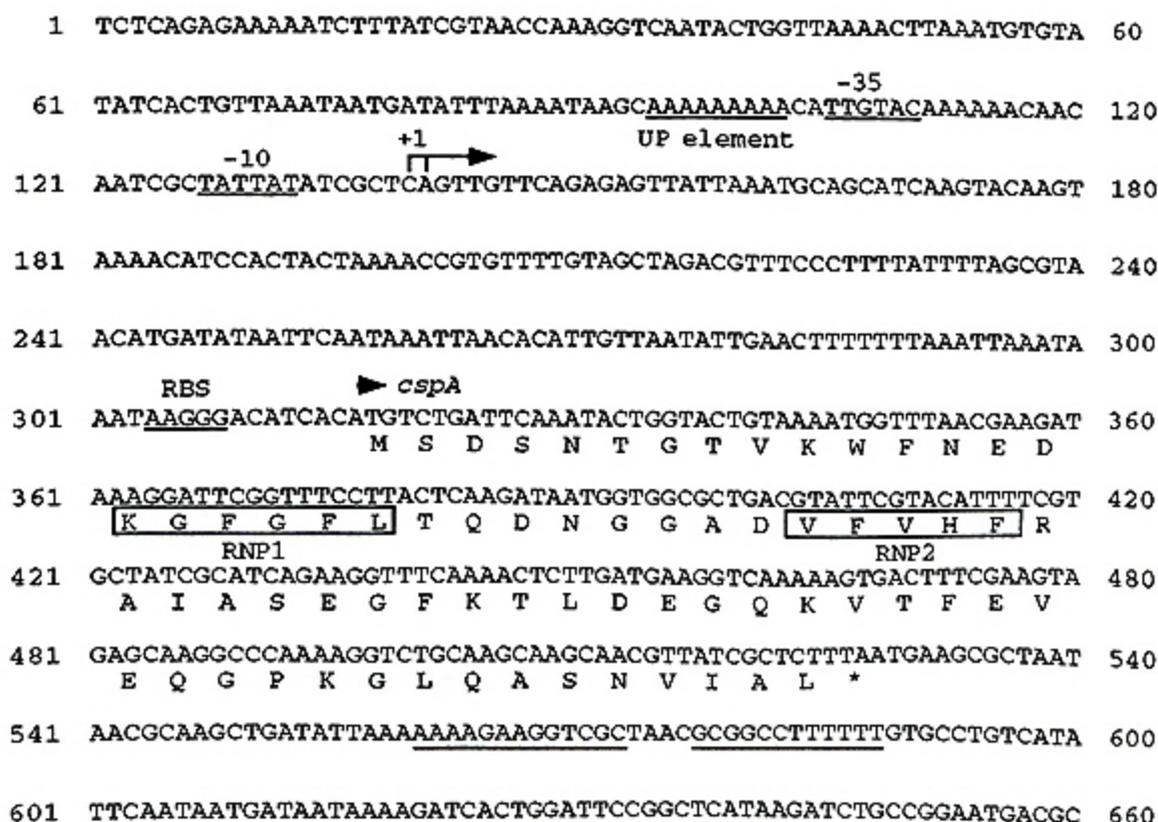


Fig. 1 Nucleotide sequence and deduced amino acid sequence of the *cspA* gene of DSS12. The *cspA* gene is 213 bp in length and encodes an acidic protein of 70 amino acid residues (pI 4.48). RNA binding motifs, RNP1 and RNP2, are boxed. The putative -10 and -35 regions, the UP element, ribosome binding site and the sequence of a potential transcription terminator are underlined. The bent arrow indicates the transcription initiation site.

sequences were shown in Fig. 2, respectively. As indicated by data on the three-dimensional structure of *E. coli* CspA (Newkirk, 1994), this protein forms a b-barrel structure, and eight aromatic residues are arranged on the

surface of the molecule exposed to the solvent. In addition, two lysine residues and two RNA binding motifs exist on the same surface. All of these features are thought to contribute to the role of CspA as an RNA

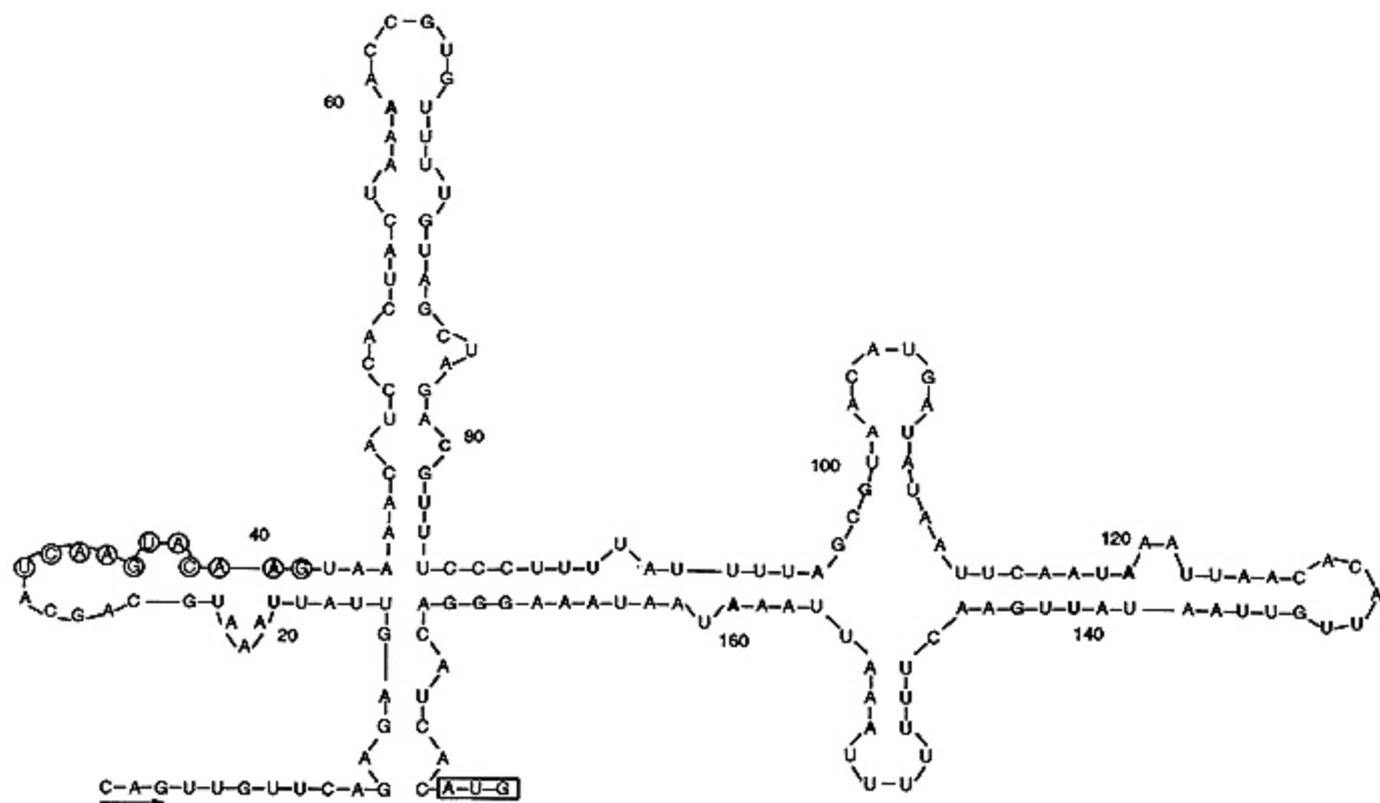


Fig. 2 Putative secondary structure of the 5' untranslated region of *cspA* mRNA, and the alignment with *E. coli* cold-box sequences. DSS12 *cspA* mRNA contains a 5' untranslated region of 177 bases. The initiation codon (AUG) is boxed. The cold-box region (UCAAGUACAAG) are circled.

DSS12	<i>cspA</i> (+31 - +41)	UCAAGUACAAG
<i>E. coli</i>	<i>cspA</i> (+7 - +17)	.G.C.....GA
<i>E. coli</i>	<i>cspB</i> (+8 - +18)	.G...A...GA
<i>E. coli</i>	<i>cspG</i> (+7 - +17)	.G..AG...GA
<i>E. coli</i>	<i>csdA</i> (+48 - +58)	.G.C.....CA
		* * * * *

Fig. 3 Nucleotides identical to DSS12 *cspA* are shown as dots. * indicates a conserved nucleotide.

chaperone (Yamanaka et al., 1998). The high degree of conservation of two RNA binding motifs, RNP1 and RNP2, the eight aromatic residues (W11, F12, F18, F20, F31, F34 and F42) and the two lysine residues (K10 and K16) suggest that DSS12 CspA is an RNA binding protein and functions as an RNA chaperone.

We also found a cold-box sequence (UCAAGUACAAG) in the 5' UTR (Fig. 2). The cold-box region has been reported to be an essential region for the derepression effect of the 5' UTRs of *cspA* in *E. coli* (Fang et al., 1998). There are 4 or 5 mismatches between the DSS12 *cspA* and the *E. coli cspA*, *cspB*, *cspG* or *csdA* in this region (Fig. 3). *CsdA* is not a *cspA* family gene but is cold shock inducible in *E. coli* (Jiang et al., 1996). The cold-box region of *E. coli cspA* and *cspB* is located 7 or 8 bases downstream of the transcription start site, and forms a stem loop structure (Etchegaray et al., 1996). On the other hand, the cold-box region of DSS12 *cspA* is located 31 bases downstream of the transcription initiation site and this region could form a stem loop structure (Fig. 2). These features suggest that the 5' UTRs of *cspA* in DSS12 may function as the derepressor and its cold-box region may be an essential region for the derepression effect as in the case of *E. coli cspA*. mRNA secondary structure or the affinity of a protein(s) for mRNA may be drastically altered under atmospheric pressure or high pressure conditions. It is thought that the affinity of ribosomes for binding to mRNA changes during temperature downshift in the proposed cold adaptation model (Berger et al., 1996). We speculate that DSS12 CspA might function as a RNA chaperone which facilitates the translation of mRNAs under high pressure conditions, and it might be essential for growth under low temperature and high pressure conditions such as in the deep-sea. Obviously, much work needs to be done to elucidate what happens in microorganisms under high pressure conditions, because our understanding of biological processes such as gene regulation and protein function such conditions is still extremely limited.

Acknowledgments

We thank Prof. M. Inouye of Robert Wood Johnson Medical School for his advice and Prof. R. H. Doi of UC

Davis for his advice and critical reading of the manuscript. We also thank Dr. W. R. Bellamy for assistance in editing the manuscript.

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(Manuscript received 10, July 1998)