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深海微生物由来EPA合成遺伝子群の構造解析

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Structural analysis of gene cluster for EPA (eicosapentanoic acid) synthesis in deep-sea bacterium

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Gene cluster for the eicosapentanoic acid (EPA) synthesis from the deep-sea piezophilic bacterium *Shewanella violacea* strain DSS12, was cloned and sequenced by plaque hybridization. The clones designated lepa_1 (18kb) and lepa_2 (17kb) were contained 7ORFs from ORF1 to ORF7. Through database analysis of these ORFs, the frames from ORF3 to OFR6 were found as gene cluster involved in EPA synthesis. The cluster contains the genes for *pfaA* (ORF3), *pfaB* (ORF4), *pfaC* (ORF5), *pfaD* (ORF6) in this order. Comparison of the clusters with same genus *S. oneidensis* which does not produce EPA, showed that *S. oneidensis* lacks the *pfaB* gene, suggesting at least the importance of the gene for EPA synthesis in *S. violacea*.
Keywords *Shewanella violacea* strain DSS12, deep-sea bacterium, eicosapentanoic acid (EPA)

1. Introduction

The piezophilic deep-sea bacterium *Shewanella violacea* strain DSS12, isolated from the Ryukyu Trench (depth: 5,110m), grows optimally at 30 MPa and 8°C, but also grows at atmospheric pressure (0.1 MPa) and 8°C [1, 2]. It is useful as a model for comparison of various features of bacterial physiology under high and low pressure conditions. Recently an operon identified as a pressure-regulated operon, with a promoter activated under high pressure conditions, was cloned and characterized from this strain [3, 4, 5]. We have reported that gene expression from this operon, which has five transcription initiation sites, is controlled at the transcriptional level by elevated pressure [3]. However, the molecular basis of transcription of this operon remains to be elucidated.

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with a promoter activated under high-pressure conditions, was cloned and characterized from this strain [3-5]. We have reported that gene expression from this operon is controlled at the transcriptional level by elevated pressure [3]. However, the mechanisms controlling transcription of this operon at the molecular level remain to be elucidated. One approach to understanding the basis of gene expression under defined condition is via detailed characterization of transcriptional machinery involved.

Thus, cloning of the genes for RNA polymerase subunits from piezophiles and the establishment of an *in vitro* system for reconstitution of the enzyme are essential. Transcription in eubacteria is mediated by an RNA polymerase, which governs the selectivity of the promoter sequences of a gene. It is a mutisubunit complex composed of mainly a, b, b' and one of several s subunits [6]. Among these subunits, we have previously reported piezoresponse of *S. violacea* a operon and some positive regulators may exist and interact with RNA polymerase [7]. It is essential to elucidate and to characterize several factors interact with RNA polymerase and relate to transcriptional regulation at several conditions. In the case of *E. coli*, one of these factors named w protein is present in both highly purified core and holo RNA polymerase, but the roles of this protein is very little known. One of estimated functions of *E. coli* w protein is a regulator of RNA polymerase, to control promoter selectivity at specific stringent promoters, *rpsA* and *rplJ*, mediated by ppGpp [8]. In this study, we report the isolation of the *rpoZ* gene encoding RNA polymerase w protein from *S. violacea*. The results of phylogenetic analyses based on comparison of the RpoZ protein and construction of expression plasmid, overproduction and affinity purification will be described.

2. Materials and Methods

2.1. Cloning of the gene for EPA cluster from *S. violacea*

Two degenerate oligonucleotides were designed and synthesized, based on highly conserved sequences, MARVTV and VIALREI, in RpoZ proteins. The nucleotide sequences of origonucleotides were 5'-ATGGCNCGNGTAACWGTN-3' (primer 1) and 5'-DATYTCRCGYAGNGCDATWAC-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 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 digoxygenin (DIG)-labeled *rpoZ* 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. Southern blotting analysis and mapping of the EPA cluster of *S. violacea*

The *rpoZ* gene was identified from l phage library of *S. violacea* chromosomal DNA and significant homologies to known *rpoZ* gene. Based on the determined nucleotide open reading flame sequence, two other PCR primers were designed for use in amplification of the digoxygenin (DIG)-labeled *rpoZ* fragment and for use in cloning (method of 2.5.). The nucleotide sequences of the oligonucleotides were 5'-ATGGCTCGCGTAACTGTAGAA-3'(primer 1) and 5'-TTTATAATATGTCGTTGCGACC-3' (primer 2). The DIG labeled fragment was employed as the probe for southern blotting analysis. The chromosomal DNA of *S. violacea* was digested with *Bam* HI, *Eco* RI,

Hind III, *Kpn* I, *Sal* I and *Pst* I, digested fragments were separated on 1% agarose gel and blotted onto a PVDF membrane. The fragments were detected by means of the DIG detection system (Boehringer Manheim Co., Mannheim, Germany).

2.3. Construction of a lambda phage library of the chromosome of *S. violacea* and screening of the library for the EPA cluster

Chromosomal DNA isolated from *S. violacea* was partially digested with *Sau*3AI. 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 manufacture's instructions. The 1 phage library was screened for plaque hybridization with the *rpoZ* probe and several positive clones were obtained. The positive clones containing the *rpoZ* gene were each purified by several single plaque isolation steps. Each of the inserts in 1 phage was amplified by long PCR and was 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.

2.4. Phylogenetic analyses of the Pfa proteins

To determine the phylogenetic position of the *S. violacea* RpoZ protein, evolutionary distance was calculated and a phylogenetic tree was constructed. The alignment and calculation were performed using CLASTALX and the PHYLP (ver. 3.573; obtained from J. Gelsenstein, University of Washington, seattle).

2.5. Measuring promoter activity of upstream EPA cluster

To construct plasmids for expression of hexahistidine-tagged derivative of the RpoZ protein from strain DSS12, PCR was performed to amplify the *rpoZ* gene. The PCR product was cloned into the pCR2.1 vector and sequenced. The resulting fragment, digested with both *Bam* HI and *Hind* III, was cloned into expression plasmid pQE80L (QIAGEN) and then it was designated as pQrpoZ (Fig. 4A).

The expression plasmids of pQrpoZ were transformed into *E. coli* JM109. Overexpression of the protein was carried out by the procedure described by Nakasone et al. [7]. Overexpressed protein was fractionated by 15% SDS-PAGE and the molecular mass was determined (Fig. 4B).

3. Results and discussion

3.1. Isolation of the gene for EPA cluster of *S. violacea*

To clone a part of the *rpoZ* gene from *S. violacea*, the amino acid sequences of the RpoZ proteins from several bacteria were aligned and a highly conserved region was found (data not shown). These conserved sequences were used to design and synthesize degenerate oligonucleotides in order to amplify a part of the *rpoZ* gene from *S. violacea*. A fragment containing part of the *rpoZ* gene, approximately 170 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 RpoZ proteins of other bacteria (data not shown). To clone the complete *rpoZ* gene, the partial *rpoZ* gene fragment was labeled with DIG (digoxygenin) by PCR as a hybridization probe.

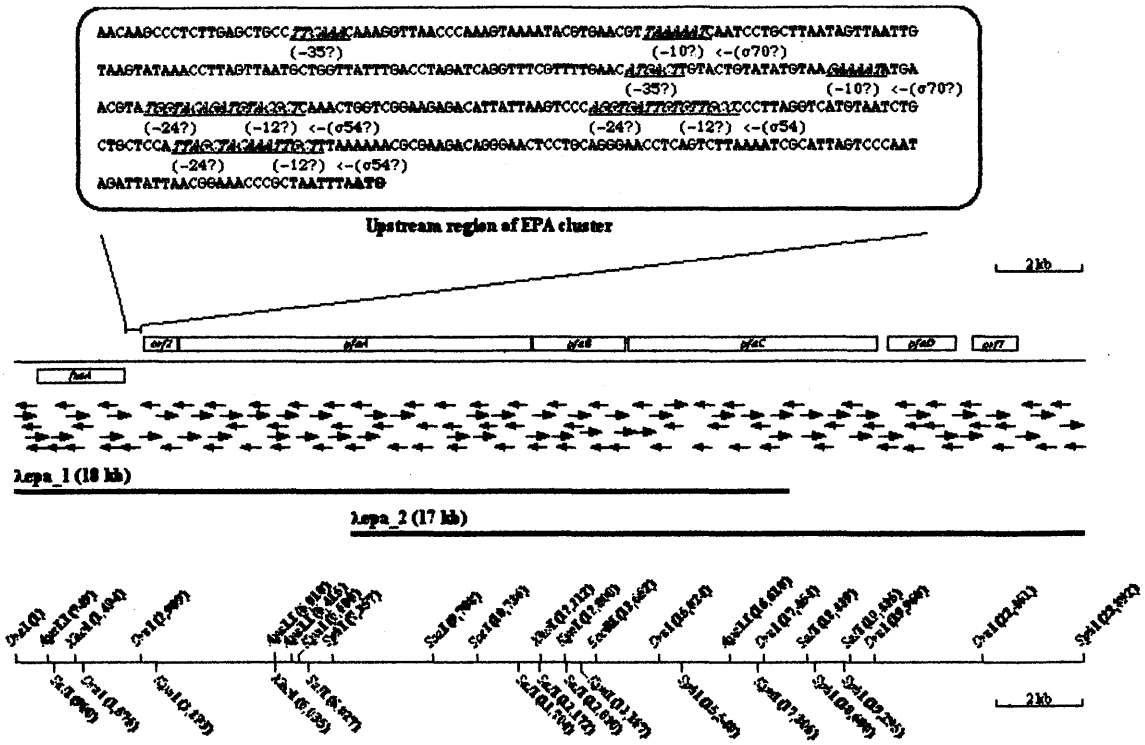

Fig.1 Physical mapping of gene cluster of EPA synthesis

From the Southern blotting analysis using DIG-labeled *S. violacea rpoZ* gene as a probe, a positive band was detected from digested fragments of *S. violacea* Chromosomal DNA with several restriction enzymes (data not shown). As shown in Fig. 1 it showed that the *rpoZ* gene was present in a 35.7 kb (*Bam* HI), 8.1 kb (*Hind* III), 9.6 kb (*Eco* R I), 5.5 kb (*Kpn* I), 2 kb (*Pst* I) and 17.1 kb fragment (*Sal* I).

Several positive clones were isolated from among 10^4 plaques and purified by repeated single plaque isolation.

3.2. Structural analysis of the cluster of *S. violacea*

A l phage library of *S. violacea* chromosomal DNA was constructed and screened using the *rpoZ* probe. These clones were confirmed to contain the *rpoZ* gene by PCR at each isolation step. As a result of screening, we obtained several positive clones and the nucleotide sequence of one of these clones containing a DNA insert approximately 10 kb in size was determined by the random shotgun sequencing method [8]. Based on the results of sequence and Southern blotting analysis, we constructed the restriction map of *rpoZ* gene. As shown in Fig. 1B, the 10 kb DNA fragment, including *rpoZ* gene, had ten open reading frames (ORFs) and each ORFs protein names were determined with Genbank database. Besides of the *rpoZ* gene, the determined 10kb DNA fragment contained nine other genes, *pgtB, mdsC, functionally unknown gene 1, gmkc, spoT, functionally unknown gene 2, spoU , ribD* and *functionally unknown gene 3*, respectively. It was reported that the *rpoZ* gene of *E. coli* is located in an operon that also encodes the *spoT* gene whose product is involved in the metabolism of ppGpp [9-10].

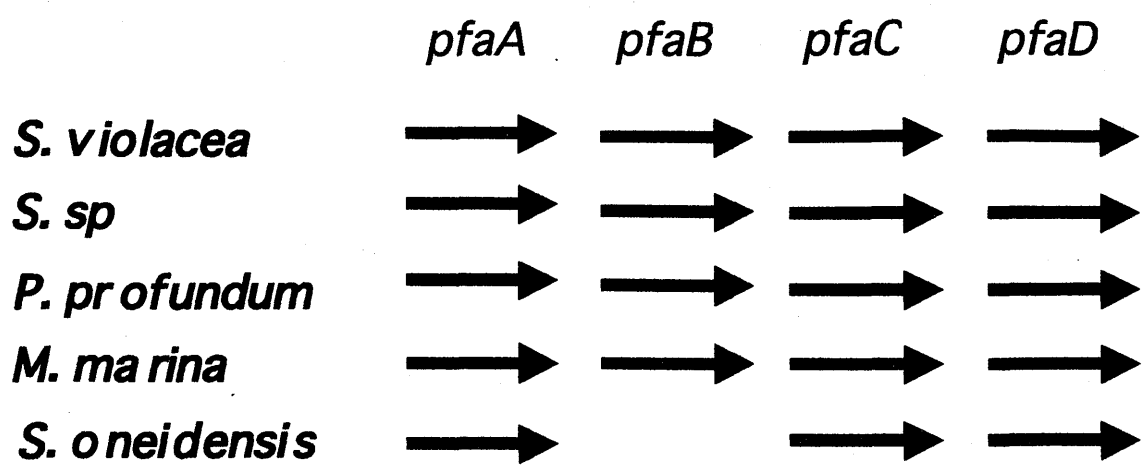

Fig.2 Comparison of operon structure of several organisms

The *rpoZ* gene of *S. violacea* was located at the upstream region of *spoT* gene as well as *E. coli*. From the alignment of the upstream region of *S. violacea* with that of *E. coli*, two promoter like sequences of RNA polymerase were identified (data not shown). From these results, the transcriptional regulation of this region may be conserved in two species and raised possibility of the w is required for transcriptional regulation by ppGpp. The open reading frame of *rpoZ* gene, 285 bp in length, was found to encode a polypeptide consisting of 94 amino acid residues with a molecular mass of 10,227 Da (Fig. 2A). Analysis of the deduced amino acid sequence confirmed that the cloned gene contained the complete sequence of the RpoZ protein (Fig. 1). From the comparison of RpoZ protein with *Escherichia coli* and *Vibrio cholerae*, the amino acid sequences of *S. violacea* RpoZ shows a high degree of similarity to those of RpoZ homologues (Fig. 2B). The N-terminal two-thirds of RpoZ protein is significantly conserved, but the C-terminal region is not so conserved among these organisms. The result suggests that the two-thirds of N-terminal region of RpoZ protein is important to bind with RNA polymerase molecules. As shown in Fig. 2B, it was reported the N-terminal 24 and 25 residues (Arg-Arg) of *E. coli* w protein is efficiently cleavaged by the OmpT protease [11]. This site is observed in the case of w protein of *S. violacea* and *V. cholerae*. This feature indicating the w protein of *S. violacea* is cleavaged if it was overproduced in *E. coli* JM109 as a host. As predicted, one other fragment was observed after overproduced RpoZ protein by SDS-PAGE (described in 3. 3). From the alignment of amino acid residues of the *S. violacea* RpoZ protein with other organisms, the RpoZ protein is conserved in a wide range of organisms (data not shown). To confirm the phylogenetic position of the *S. violacea* RpoZ protein, the evolutionary distance was calculated based on comparison of 17 organisms and a phylogenetic tree was constructed (Fig. 3). The RpoZ amino acid sequences were aligned and the tree was constructed by the neighbor-joining method. The results obtained from calculation of the evolutionary distance comparing the RpoZ amino acid sequences of 17

organisms showed that the RpoZ protein of *S. violacea* is independent of other 17 organism.

3.3. Construction of expression plasmid and overproduction of *S. violacea* w protein

A recombinant expression plasmid harboring the *S. violacea rpoZ* gene was constructed (pQrpoZ) and transformed into *E. coli* JM109 (Fig. 4B). Upon induction, the recombinant RpoZ protein was overexpressed as shown in lane 3 of Fig. 4B.

Table 1 Annotaion of gene cluster of EPA synthesis

ORF No.	Gene	Function	Position		direction	Length of	
			start	stop		nt	aa
ORF1	*fusA*	translation elongation factor G	537 -	2,615	-	2,079	692
ORF2	*orf?*	ptutative transcription regulator	3,004 -	3,864	+	861	286
ORF3	*pfaA*	EPA synthesis	3,864 -	12,089	+	8,226	2,741
ORF4	*pfaB*	EPA synthesis	12,089 -	14,362	+	2,274	757
ORF5	*pfaC*	EPA synthesis	14,362 -	20,178	+	5,817	1,938
ORF6	*pfaD*	EPA synthesis	20,319 -	21,947	+	1,629	542
ORF7	*orf?*	unkwon	22,477 -	23,568	+	1,092	363

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