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Isolation and Characterization of *Zymomonas mobilis* DNA Fragments Showing Promoter Activity in *Escherichia coli*

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Escherichia coli 에서 Promoter 활성을 보이는 *Zymomonas mobilis* DNA 조각의 분리와 분석

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For the purpose of isolation of the *Zymomonas mobilis* DNA fragments showing promoter activity in *Escherichia coli*, a promoter screening vector, pCMT215 was constructed by transferring a promoterless chloramphenicol acetyltransferase (CAT) gene of pYEJ001 into pMT21 which contains β-lactamase gene and multiple cloning sites. A library of *Z. mobilis Sau*3AI DNA fragments was constructed in *E. coli* using the newly constructed pCMT215. Fourteen clones showing resistance to chloramphenicol ranging in concentration from 30 to 750 μg/m*l* were selected. From five clones of them, the *Z. mobilis* DNA fragments expressing CAT gene of the recombinant plasmids were sequenced and then sites of transcriptional initiation were identified. The nucleotide sequences of the cloned DNA shared AT rich regions, poly A's or T's stretches and palindromic regions. The positions of transcriptional initiation for CAT gene occurred at more than one site spaced over by 4 to 190 base pairs on the cloned fragments in *E. coli*.

Zymomonas mobilis is a facultative anaerobic gram-negative bacterium which can make the rapid and efficient conversion of glucose to ethanol over yeasts (1). However, *Z. mobilis* has a major drawback that the range of utilizable sugars is restricted to glucose, fructose and sucrose (2).

Genetic manipulation using the recombinant DNA techniques makes it possible to construct strains of *Zymomonas* capable of allowing the fermentation of renewable biomass such as starch or cellulose. To achieve this, transformations of *Zymomonas* had been tried with some conjugal plasmids (3, 4) or recombinant plasmids (5, 6) by several groups. It was, recently, reported that the foreign cellulase genes were transferred into *Zymomonas* species (7, 8). In addition, Conway *et al.* (9) reported that β-galactosidase gene was expressed at high level in strain of *Z. mobilis* with several fragments of *Z. mobilis* DNA serving as efficient promoter in both *Z. mobilis* and *E. coli*. Misawa *et al.* (10) also enabled to express a cloned carboxymethyl cellulase gene of *Cellulomonas uda* in *Z. mobilis* by gene fusion between *Zymomonas* promoter fragment and the truncated CMCase gene.

Only a few studies have been, until now, reported on the DNA sequences required for transcription and translation of the *Zymomonas* genes. Recently, the nucleotide sequences of DNA fragment isolated from *Z. mobilis* which contain promoter activity in *E. coli* showed considerable similarity to consensus sequence

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of *E. coli* promoter (9).

In the present work, we have isolated and sequenced the *Z. mobilis* DNA fragments showing promoter activity in *E. coli,* and then identified the positions of transciptional initiation on their DNA sequences.

Materials and Methods

Bacterial strains, plasmids and phages

Z. mobilis ATCC 10988 was used as a source of DNA fragments having promoter activity. pMT21 and pYEJ001 (11) were used to construct a promoter-proving vector pCMT215. Bacteriophage M13 mp8 and mp9 (12) were used as subcloning vectors for DNA sequencing. *E. coli* JM83 and JM103 served as host for transformation.

Media and growth conditions

E. coli cells were grown in LB medium (10g bactotryptone, 5g yeast extract, 5g NaCl, per liter, pH 7.4). *Zymomonas* cells were grown at 30°C without shaking in RM medium prepared by dissolving 20g of dextrose, 10g of yeast extract and 2g of KH_2PO_4 in one liter of H_2O. The pH of RM medium was adjusted to 5.6 by adding concentrated hydrochloric acid solution. If necessary, above media were solidified with 1.5% (w/v) bacto agar

Preparation of DNA and construction of recombinant plasmids

For rapid isolation of plasmids from *E. coli,* the alkaline lysis method described by Birnboim and Doly (13) was employed. Plasmid DNAs were digested with restriction endonucleases according to the supplier's instructions. *Zymomonas* chromosomal DNA was isolated from exponentially growing cells according to preparative method described by Rodriquez and Tait (14). Ligation of linear DNA fragments to construct recombinant plasmids was done with T4 DNA ligase in a total volume of 25 μl at 14°C for cohesive ends. The M13 RF and viral DNAs were isolated from infected culture of *E. coli* JM103.

Construction of a *Z. mobilis* gene bank

Fifty micrograms of the purified *Z. mobilis* chromosomal DNA was partially digested with *Sau*3AI, and DNA fragments ranging from 0.1 to 1.5 kb were isolated by sucrose gradient centrifugation for 20 h at 25,000 rpm in a Beckman SW40 rotor. The *Sau*3AI-generated chromosomal DNA fragments were dephosphorylated and followed by ligation to *Bam*HI-digested pCMT215 which was constructed with pMT21 and pYEJ001 in this work. The ligation mixture was used to transform *E. coli* JM83. Antibiotics used for the selection of transformants were ampicillin (50 μg/m*l*), and chloramphenicol (30 μg/m*l*).

Determination of DNA sequence

All restriction enzyme-generated fragments of the *Z. mobilis* chromosomal DNA were transferred into vector M13 mp8 or mp9 with the appropriate restriction enzymes by forced cloning. Dideoxynucleotide sequencing reaction with a DNA sequencing kit and synthetic oligonucleotide primers obtained from New England Biolabs was used to sequence DNA of *Zymomonas* gene (15).

Analysis of 5' termini of mRNAs

RNA was extracted from *E. coli* by the hot sodium dodecyl sulfate-phenol method (16). The 5' terminus of transcript from *E. coli* harboring recombinant plasmid was mapped by using primer extension analysis (17). The M13 universal primer was used for cDNA synthesis.

Results

Construction of promoter-probing vector pCM215

In order to screen promoter-active DNA fragment of *Z. mobilis* in *E. coli,* the promoter-cloning vector, pCMT215, was constructed as shown in Fig. 1. The plasmid pYEJ001 (11) was digested with *Hin*dIII, and the smaller fragment containing the chloramphenicol acetyltransferase gene (CAT) was eluted on agarose gel. The promoterless plasmid pMT21, which has multiple cloning sites and ampicillin resistant gene, was also digested with *Hin*dIII and mixed with the 0.7-kb eluted DNA framents to ligate by the aid of T4 DNA ligase.

With the ligation mixture, *E. coli* JM83 was transformed. From the *E. coli* transformants showing Amp^r phenotype, plasmids were isolated, digested with *Eco*RI and analyzed by agarose gel electrophoresis confirming that the plasmid pCM215 is a pMT21 derivative containing CAT gene, which was located downstream from multiple cloning sites in

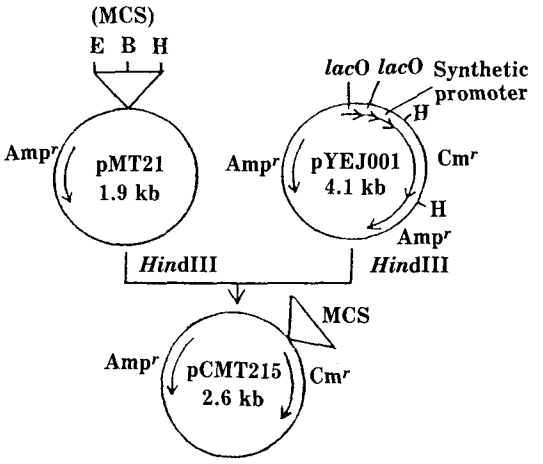

Fig. 1. Schematic representation of the construction of the promoter-probing vector pCMT215.
The plasmids are not drawn to scale but for illustrative purpose only. Restriction enzyme cleavage sites are *Bam*HI (B), *Eco*RI (E), and *Hin*dIII (H). MCS indicates the multiple cloning sites.

the direction of transcription as a promoter-probing marker.

Expression of CAT gene by *Z. mobilis* genomic DNA fragments in *E. coli*

The newly constructed plasmid pCMT215 was used to select promoter activity DNA fragments. It was firstly determined that *E. coli* JM83 carrying pCMT215 was sensitive to chloramphenicol. The *Z. mobilis* genomic library was constructed as described in Materials and Methods.

From fourteen Cm^r clones obtained by transformation of *E. coli* on LB agar plates containing 30 μg of Cm/m*l*, plasmids were isolated and named pCMT series as described in Table 1. The *Z. mobilis* DNA fragments showing promoter activity on pCMT series were estimated in size ranging from 0.1 to 1.5 kb. To determine the promoter activities of the cloned DNAs, chloramphenicol resistant level of *E. coli* clones carrying the recombinant plasmids was measured in the LB broth containing chloramphenicol of various concentration as summarized in Table 1.

Nucleotide sequences of the promoter-active fragments

Among the cloned *Z. mobilis* DNA fragments expressing CAT gene on pCMT series, five fragments were chosen for sequence analysis according to the efficiency of gene expression. Their sequences were determined as shown in Fig. 2. The plasmid pCMT307 and pCMT308 were identified to share a same insert. In addition, the sequenced inserts were identified to be different from the known *Z. mobilis* chromosomal DNA.

Table 1. Expression of CAT gene in *E. coli* by *Z. mobilis* DNA fragments.

E. coli carrying the plasmid	Cm^r range (μg/ml)
pCMT215	<10
pCMT302, pCMT304, pCMT305 pCMT306, pCMT310	30-300
pCMT301, pCMT307, pCMT308 pCMT309, pCMT312, pCMT314	300-500
pCMT311, pCMT313	500-700
pCMT303	750

The sequences were found to have many structural features common to the general promoter regions in bacteria (20, 24, 25). The A+T contents for insert on pCMT301, pCMT303, pCMT307 and pCMT312 were 50.3, 56, 53, 47.9%, respectively. Although these sequences were not rich in purine residues, localized A+T rich regions and poly (A) or (T) stretches were found as shown in Fig. 2. In pCMT303, the nucleotide sequence of about 30 base pairs around -10 region to ATG start codon was composed of up to 80% A+T. It is worth noting that the expression level of CAT gene in *E. coli* harboring this plasmid is particularly higher than others. The pCMT301 contains poly (A) (one with four bases, two with five) and poly (T) (one with four bases, one with five) and poly (T) (one with four bases, one with five). The pCMT303 contains two poly (A) with four bases and one poly (T) with five bases. The pCM307 contains one poly (T) with five bases. The pCMT312 contains two poly (A) with four bases and two poly (T) with four bases.

Analysis of 5' termini of transcripts

The position of transcriptional initiation in four *Zymomonas* fragments sequenced in this work was examined by primer extension analysis in *E. coli*. The small *Eco*RI/*Hin*dIII-digested fragments from the four plasmids pCMT-series were isolated and transferred into pUC18 digested with the same en-

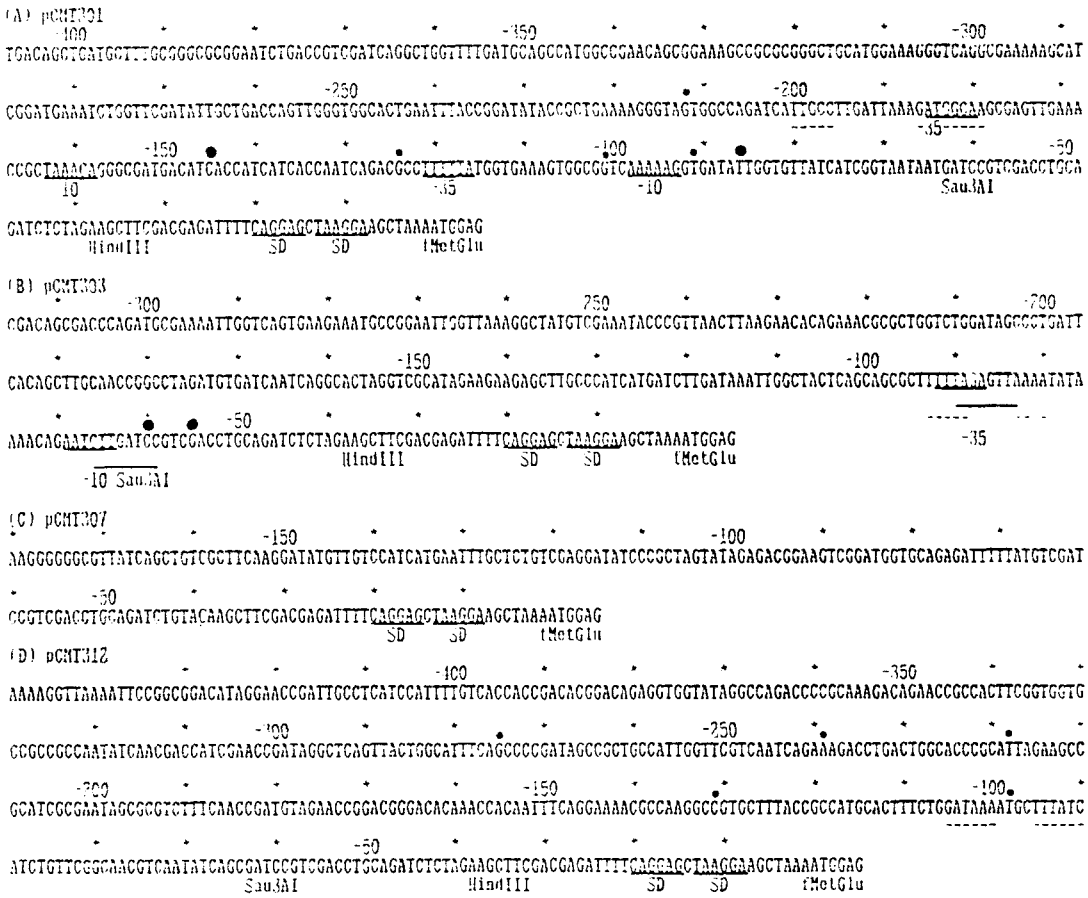

Fig. 2. Nucleotide sequences of inserts from *Z. mobilis* in (A) pCMT301, (B) pCMT303, (C) pCMT307 and (D) pCMT312.

The presumed −35 and −10 regions are presented with underline. The proposed sites for transcriptional starts in *E. coli* are shown as solid circles. The *Sau*3AI sites indicate the points of insertions, and the nucleotide sequence between the *Hin*dIII sites and ATG (+1) was derived from bacterial CAT gene (11). Potential palindromic sequences are shown by dotted lines. Putative ribosomal binding sites are labelled and underlined with SD (Shine-Dalgarno sequence).

zymes. The orientation of promoter was maintained by replacing the multiple restriction sites of pUC18 with the isolated *Eco*RI/*Hin*dIII digested fragment. Total RNAs extacted from *E. coli* cells carrying the recombinant plasmids were annealed with 5' end labelled M13 universal primer (5'-CAGCACTG-ACCCTTTTG-3') showing homology to a region of *lac Z* gene, 23 bases downstream from the *Hin*dIII site of pUC18.

The results of transcriptional analyses are shown in Fig. 3. As shown in Fig 3B, two major and four with moderate frequencies transcriptional start sites spaced over 100 bases pairs were found in the insert of pCMT301. The points of start site are presented in Fig. 2A. There were three G, two T and one C at the point of start. Although 93% of messages start with a purine nucleotide in *E. coli,* C and T are also used in some *E. coli* promoters (18). In the insert of pCMT303 (Fig. 3A), two major transcriptional start sites, separated by four base pairs, were found. The 5' initiation of mRNA was located at C and G. No transcriptional initiation site was found in the insert of pCMT307 suggesting that mRNA was started from the region of 16 base pairs between *Hin*dIII site and Shine Dalgarno sequence of CAT gene. In pCMT312, multiple transcriptional initiations of mRNA at more than 10 different points, which are spaced over by 170 base pairs, were occurred. Major transcriptional

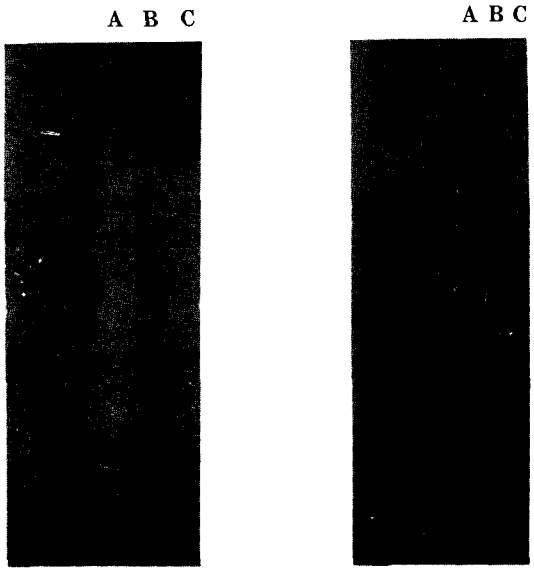

Fig. 3. Primer extension analysis for the determination of transcriptional starts within the *Z. mobilis* DNA fragments.

The products of extension were loaded on a 4% edge-gradient polyacrylamide gel. The sequence ladder was generated by dideoxy sequenceing of the known DNA. Fig. (A), *E. coli* carrying pUC18 derivative fused with; lane A, insert of pCMT307; lane B, insert of pCMT303; lane C, no insert (control). Fig. (B), *E. coli* carrying pUC18 derivative fused with; lane A, insert of pCMT301; lane B, insert of pCMT312; lane C, no insert (control).

initiation was also mapped within the enteric *lac* promoter located in 71 bases upstream from the *Bam*HI insert junction (data not shown) (19). With the exception of pCMT312, the three clones did not show initiation of mRNA in *lac* promoter region, which may accords with the fact that *E. coli* harvoring pCMT312 showed lower expression of CAT gene than others (Table 1).

Possible regions of promoter deduced from the nucleotide sequences and sites of transcriptional initiations are also presented in Fig. 2. The nucleotide sequence of the cloned *Z. mobilis* DNA fragment in pCMT301 included two possible promoter regions of which sequences are ATGGCA (-35), TAAACA (-10) and TTTTTA (-35), AAAAAG (-10) for two major transcriptional initiating points, respectively. In *E. coli* carrying pCMT303, transcription appeared to be initiated from two positions separated by four bases, assuming that both sequences of TTTAGA (-35), AATCTT (-10) and TAGAGT (-35), CTTGAT (-10) correspond to promoter regions. Multiple transcriptional initiations were also proposed on many regions of promoter in the insert of pCMT312.

Discussion

Different levels of chloramphenicol resistance among the transformed *E. coli* cells containing plasmids pCMT series are mainly due to gene expression by inserts but not due to plasmid copy number (data not shown). This was deduced from the similar levels of Amp^r and similar amounts of plasmid DNAs from them. Also, there was no correlation between the level of Cm^r and the DNA insert size.

Construction of promoter probing vector, screening and characterization of promoter-active fragments were performed in *E. coli* as a first step, because there were many difficulties in the direct transfer of plasmids into *Z. mobilis*. However, it is possible for the cloned fragments to be applicable to *Zymomonas* according to the following features. *E. coli* and *Z. mobilis* share considerable similarities in the transcription and translation system. RNA polymerase in *E. coli* and *Z. mobilis* recognize similar regions of DNA. Recent paper reported that both organisms generally recognized common regions of DNA by comparative analysis of the transcriptional initiation sites of fusion genes in *Z. mobilis* and *E. coli* (9). As described by Conway *et al.* (9), the products of gene fusions were also found to have the same properties in both organisms indicating that there is a similarity of requirements for anchoring sequences and translational initiation sites, despite of differences in membrane composition (21, 22).

In addition, β-galactosidase gene was expressed at different levels in *Zymomonas* and *E. coli* cells by DNA fragments from *Z. mobilis* (23). It is, therefore, thought that several ones among the 14 promoter-activity fragments selected in this study (Table 1) may be useful for the expression of CAT gene in *Zymomonas* cells.

요 약

Escherichia coli 내에서 프로모터활성을 보이는 *Zymomonas mobilis* 유래의 유전자 절편을 분리하고 특성을 분석하였다. 프로모터 탐색용 벡터인 pCMT215는 promoter activity가 없는 pMT21의

*Hin*dIII 위치에 pYEJ001의 클로람페니콜 아세틸전이 효소유전자를 함유한 0.7-kb *Hin*dIII 조각을 접합시켜 제조하였다. *Z. mobilis*의 chromosomal DNA를 *Sau*3AI으로 부분절단하여 pCMT215에 도입한 후, 이를 이용하여 대장균을 형질전환시킨 결과 14개의 형질전환주가 선별되었다. 이들은 30-750 $\mu g/ml$ 농도의 chloramphenicol에 내성을 보였으며 클로닝된 유전자조각의 크기는 0.1-1.5 Kb였다. 이 가운데 5개의 염기서열을 분석해 본 결과 일반적인 프로모터의 염기서열과 많은 유사점이 발견되었는데, 대장균의 프로모터인 −35 또는 −10 지역과의 부분적인 일치와 A 또는 T 염기가 풍부한 지역과 연속적인 A 또는 T 염기배열, 그리고 회문형태의 염기서열 등이 발견되었다. 또한 대장균 내에서의 프라이머 연장실험결과 *Z. mobilis*로부터 유래된 DNA 조각에서 전사의 시작이 4-170 염기의 거리를 두고 두 곳 또는 여러 곳에서 일어남을 알 수 있었다.

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