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A Small Cryptic Plasmid pZMO1 of *Zymomonas mobilis* ATCC10988

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Abstract

The nucleotide sequence of pZMO1, a small cryptic plasmid of *Zymomonas mobilis* ATCC10988 was determined. Analysis of 1,680 bp of sequence revealed 69 % identity with *Shigella sonnei* plasmid, pKYM and 61 % identity with *Nostoc* sp. ss DNA replicating plasmid. Analysis of a deduced amino acid sequence of an orf of pZMO1 revealed 75 % identity and 90 % similarity with the *rep*A gene of *Synechocystis* sp. plasmid pCA2.4. The upstream region of the *rep*A gene of pZMO1 possesses six directed repeat sequences and two inverted repeat sequences at downstream of the IR consensus sequence of nick region of rolling circle replication (RCR) plasmid. A typical terminator hairpin structure was found at the downstream region of *rep*A gene. Degradation of single-stranded plasmid DNA by S1 nuclease was detected by Southern hybridization. It suggests that pZMO1 replicates by a rolling circle mechanism in *Z. mobilis* ATCC10988 cells.

Keywords: *Zymomonas mobilis*, pZMO1, cryptic plasmid, *rep*A

Introduction

Zymomonas is very interesting organisms because of their powerful activity in ethanol fermentation (Skotnicki *et al.*, 1981) by the Entner-Doudoroff (ED) pathway (Swing and De Ley, 1977). *Zymomonas* strains have plasmids that range in size from 1.4 kb to 50 kb, and particularly *Z. mobilis* ATCC10988 has three small size plasmids and three large size plasmids, named pZMO1-pZMO6 (1.7, 1.9, 2.7, 7.3, 16.7, and 31.6kb in size). The small plasmids (pZMO2 and pZMO3) of *Z. mobilis* ATCC 10988 were modified for *E. coli-Z. mobilis* shuttle vectors (Afendra and Drainas, 1987; Scordaki and Drains, 1990). And some shuttle vectors were developed to introduce foreign genes such as a cellulase gene (Brestic-Goachet *et al.*, 1989), a β-carotene degrading gene (Misawa *et al.*, 1991) into *Zymomonas* to overcome their substrate range. The nucleotide sequence of a 2.7 kb plasmid pZMO3 of *Z. mobilis* ATCC10988 was already determined (Misawa *et al.*, 1989). Two orfs were predicted in 2749 bp of pZMO3 and its mobilization function was reported (Scordaki A., and Drainas C., 1990; Afendra *et al.*, 1999).

Results and Discussion

Isolation and cloning of the pZMO1

Plasmids from *Zymomonas* strains have been reported, and the number and the size of *Zymomonas* plasmids were strain-dependent (Skotnicki *et al.*, 1984). Six plasmids of *Z. mobilis* ATCC10988 have been reported and named pZMO1-6 ranging form 1.5kb to 50kb in size. But in this study, five plasmids were identified on 1.0% agarose gel (Fig. 1A). The sizes of the small plasmids were estimated by comparison to the commercial *E. coli* plasmids, whose sizes have already been known. Mobility of the second small plasmid was similar to that of the pNEB193, a 2.7 kb plasmid. The size and the nucleotide sequence of this 2.7 kb plasmid have previously been reported as pZMO3 (Scordaki, and Drains. 1990; Misawa *et al.*, 1989). But pZMO2 was not found in our strain. The smallest plasmid pZMO1 was isolated and digested with restriction enzymes. It has single *Hae*III, *Eco*RI, *Xba*I, *Eco*RV and *Spe*I site. *Eco*RI-digested pZMO1 was cloned into pBC SK+ to give pCZ4. The pCZ4 was mapped by some restriction enzymes (Fig. 1B). Two similar size plasmid pZMO1-D and pZMO2-D (*D; for Drainas C., Arvanitis *et al.*, 2000) of *Z. mobilis* ATCC10988 have been reported. But in this study, only single fragment was detected on agarose gel when the smallest plasmid band(s) was eluted and digested with some restriction enzymes.

Sequence analysis of a plasmid pZMO1

A 1680 bp sequence of the *Z. mobilis* ATCC10988 plasmid

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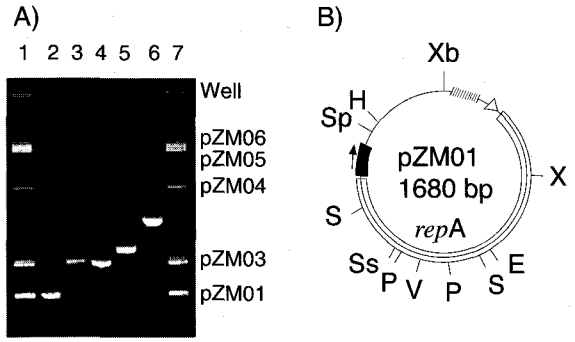

Fig. 1. Analysis of plasmid of *Z. mobilis* ATCC10988. (A) 1, *Z. mobilis* ATCC10988 plasmids pZMO1; 2, pZMO1 eluted; 3, pZMO3 eluted; 4, pNEB193; 5, pBC SK+; 6, pBW3, a modified pWE15 cosmid (4619bp) ; 7, *Z. mobilis* ATCC10988 plasmids. (B) Physical map of the pZMO1. The slashed bar is the seven 12 nt direct repeat sequence. The white arrowhead indicates putative promoter of *rep*A The black bar with an arrow outside is the putative transcription terminator inverted repeat sequence of downstream of *rep*A. Xb, *Xba*I; X, *Xmn*I; E, *Eco*RI; S, *Swa*I; P, *Pvu*II; V, *Eco*RV; Sp, *Spe*I; H, *HaeIII*.

pZMO1 was determined (The nucleotide sequence was numbered from the *Xba*I site) (Fig. 2). The (G+C) content was 38.75 %. This value is very low compared to the (G+C) content of the *Z. mobilis* ZM4 genome (about 46 %, Swing et. al., 1977), while some regions of plasmid sequence (from 1250 to 1680 nt) is relatively high (52.9 %). pZMO1 sequence (pZMO1-K, K for Kang H.L) was 1680bp that was bigger than that of pZMO1-D and pZMO2-D (-D for Drainas; Arvantis *et al.* 2000). Nucleotide homology between pZMO1-K and pZMO2-D was higher (94%) than between pZMO1-K and pZMO1-D (88%). And also the size of pZMO2-D was closer to pZMO1-K (1669) than pZMO1-D (1651bp). The BLASTX program was also used to search the homology of the putative amino acid sequence. The result of the BLASTX search showed that one putative open reading frame (orf) about a 1008 bp (241nt-1238nt) in size was strongly homologous to the

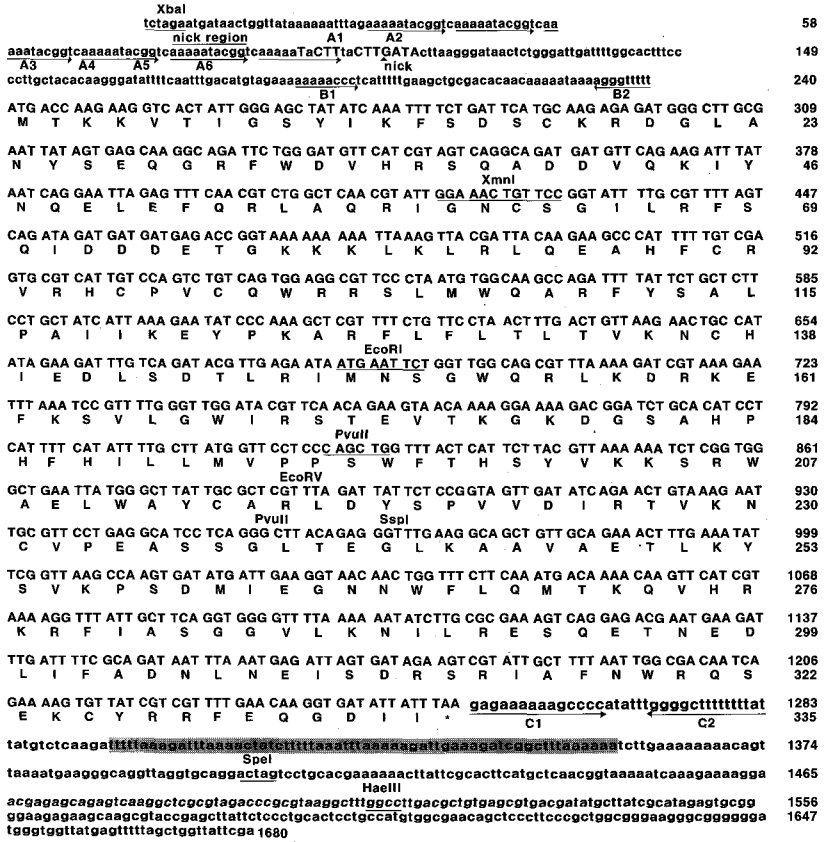

Fig. 2. Complete nucleotide sequence of pZMO1. The orf encoded putative RepA protein is indicated by capital letters (nt 241 to 1248) with the deduced amino acid sequence under the nucleotide sequence. The putative termination codon is indicated by an asterisk. A1-A5; 12 nucleotide direct repeat itrons, A6; similar sequence (AAAAATACTTTAC) to A1-A5 repeats, B1-B2; inverted repeat, C1-C2; inverted repeat at downstream of *rep*A, AT-rich region is shadowed. CTTGATA homologous to the consensus of pC194 are presented in capital letters.

*rep*A gene product (replication initiation protein) of some bacterial plasmid, especially the *Shigella sonnei* plasmid pKYM (74 % identity) (Croft *et al.*, 1983; Kodaira *et al.*, 1995; Sugiura *et al.*, 1984) and *Nostoc* species ss DNA plasmid (61 % identity).

Genebank accession number

The DNA sequence of pZMO1 was directly submitted to Genebank, Accession number AF030624.

Alignment of the pZMO1 open reading frame with other proteins

Many bacterial replication initiation proteins were found to be homologous to the pZMO1-K open reading frame by BLAST analysis (Fig. 3). The *rep*A open reading frame of *Synechocystis* plasmid pCA2.4 (Accession number L13739; identities=45%, similarities=64%), ssDNA plasmid of *Nostoc* (Accession number M81381; identities=43% similarities=65%) and *Shigella sonnei* plasmid pKYM (Accession number M38574; identities =54%, similarity =71%) were very homologous to that of pZMO1-K *rep*A. Although amino acid sequence of pZMO2-D was short (184 residue). It was more homologous to pZMO1-K (334 residue) (identity 96%, similarity 97%). Amino acid sequence homology between pZMO1-D (348 residue) and pZMO1-K (334 residue) was 56% identity and 72% similarity. Therefore, pZMO1-K thought to be a homolog of pZMO2-D. Orf of pZMO1-K has three conserved sequences, region I, II and III. Region II contained motif 2 of pC194 (del Solar *et al.*, 1998) that was thought to be the metal-binding domain of *Rep*A (Fig. 4). Region III also contained motif 3 of pC194 *Rep*A.

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pZMO1-K  ----------MTKKVTIGSYIKFSDSCKRDGLANYSEQGRFWDVHRSQADDVQKIYNQELEFQRLA
pZMO2-D  ----------------------------------------MGLRIIVSKADSGMLHRSQADDVQKIYNQELEFQRLA
pZMO1-D  MRQPKKRGFFMHFSATLGHNIKANFFKCKSNNGFESDKNGLADYSPKDRFWDVHKLQADSVEQIYQTAKEFERYA
pKYM     ----------MSEDKFLSDYSPRDAVWDTQRTLTDSVGGIYQTAGQFERYA
pC2.4    ---------- MSRTLVEDCLQQSDKVVKSNLIDKVLLRQLSPNDAKWDDRRSETDQVRELYRGT-RYDSLA
Nostoc   ----------MSEASEGISLPSIVSFPQQEENSTPALSELSQRDEVWDKHRANSDAVEGYYAGS-EFSKYS

pZMO1-K  QRIGNCSGILRFSQIDDDETGKKKLKLRLQEAHFCRVRHCPVCQWRRSLMWQARFYSALPAIIKEYPKARFLFLT
pZMO2-D  QRIGNCSGILRFSQIDDDETGKKKLKLRLQEAHFCRVRHCPVCQWRRSLMWQARFYSALPAIIKEYPKARFLFLT
pZMO1-D  ERISNCSGVLRF-AVS--EEGAEKN-LKLKEAKFCRVRHCPICQWRRSLMWQARFYTALPEVLKAHMNTNWLFLT
pKYM     LRMASCSGLLRFGWSTIMETGETRL--RLRSAQFCRVRHCPVCQWRRTLMWQARFYQALPKIVVDYPSSRWLFLT
pC2.4    GRISACSGRLEFG-FEIQETGESKL--RLKSARFCRVRHCPVCQWRRSLMWRAKAFKILPQIVEAYPKHRFIFLT
Nostoc   ERISDCAQLLDFRLVPNSETGEYKF--KLSSARFCHVRHCPVWQWRRSLMWKAKAYKILPQLLIDYPKARWLFVT
                                    region I

                                                                  motif 2
pZMO1-K  LTVKNCHIEDLSDTLRIMNSGWQR--LKDRKEFKSVLGWIRSTEVTKGK--DGSAHPHFHILLMVPPSWFTHS-Y
pZMO2-D  LTVKNCHIEDLSDTLRIMNSGWQRLRLKDRKEFKWLLGWIRSTEVTKGK--DGLTSSFSYFAYGS--SQLVCS-F
pZMO1-D  LTVRNCEIGHLRATLQDMAKAWQR--LIKRKAFKPVNGWIRTTEVTRGK--DGSAHPHFHCLLMVRPSYFKKY-Y
pKYM     LTVRNCEIGELGTVLTAMNAAFKR--MEKRKELSPVQGWIRATEVTRGK--DGSAHPHFHCLLMVQPSWFKGKNY
pC2.4    LTVRNCELGQLRETITGMNQAWGR--LVKRAKWP-ADGWIRSLEVTRGK--DGSAHPHFHCLLMVKASYFSHG-Y
Nostoc   LTVKNCAITDLRETLTWMNKSFKR-- FSELKAFP-AEGYIKTVEVTRGKTPDGSAHPHFHVLMMVKPSYFGVG-Y
                                                                region II

                                                        motif 3
pZMO1-K  VKKSRWAELWAYCARLDYSPVVDIRTVKNCVPEASSGLTEGLK AAVAETLKYSVKPSDMIEG-NN-------WFL
pZMO2-D  LR----------------------------------------------------------------------
pZMO1-D  ISQSSWIDLWQQCMKVPYSPNIDVRRVKGKKGEDEN---QALERAVSETLKYAVKPRDMISD-KD-------WFL
pKYM     VKHERWVELWRDCLRVNYEPNIDIRAVKTKTGEVVANVAEQLQSAVAETLKYSVKPEDMAND-PE-------WFL
pC2.4    ISQEVWTKLWEESLRINYRPIVHVQAVRPKNKE--T---LDTLQAILETLKYSVKPDDIIGESEEPTEENKQWLV
Nostoc   LSQAKWVEMWRKSLRVDYKPILDVQSLNPQD---------SLIGLLAEVIKYSVKESDLTSD-RE-------WFL
                                                  region III

pZMO1-K  QMTKQVHRKRFIASGGVLKNILRESQETNEDLIFADNLNEISD-RSRIAFNWRQSEKCYRRFEQGDI--
pZMO2-D  ----------------------------------------------------------------------
pZMO1-D  ELTKQVHQLRFIASGGLLKDILKENLESNQDLISENQDNSFSDDKTRLAFNWQSDESRYRRFSEGDIV-
pKYM     DVTRQLHKRRFISTGGALKNVLQLDRETNEDLVIADDVGDGTDDGKRTAFVWDSGKRRYKRAPEKDKSD
pC2.4    ELTTQLHKTRAVATGGILKEYLKELEKEPEDLIHADDDSEPDNSIASVIFDWEQRIKKYAMNDRI----
Nostoc   ELTRQLHRTKAIAVGGILREYLRDLEQEPEDLVGNDN--EGDVDEGHLYFGWKQKQKKYKLVE------
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Fig. 3. Alignment of the amino acid sequence of the pZMO1-K putative RepA protein with those of other plasmid. pZMO1-K: *Zymomonas mobilis* ATCC10988 pZMO1 (AF030624), pZMO2-D; *Zymomonas mobilis* ATCC10988 pZMO2-D (AJ009976), pZMO1-D: *Zymomonas mobilis* ATCC10988 pZMO1 (AJ009975), pKYM: Shigella sonnei pKYM (M38574), pCA2.4: *Cyanobacterium synechocystis* pCA2.4 (L13739), *Nostoc*: *Nostoc* sp. ssDNA plasmid (M81381)

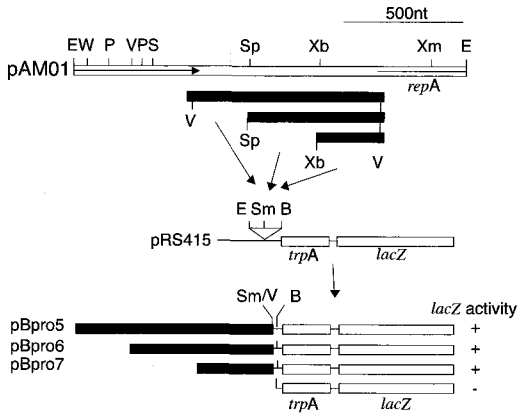

Fig. 4. Test of the putative promoter of repA. Black bars are the PCR products. E, *Eco*RI; W, *Swa*I; P, *Pvu*II; V, *Eco*RV; S, *Ssp*I; Sp, *Spe*I; Xb, *Xba*I; Xm, *Xmn*I; Sm, *Sma*I; B, *BamH*I; *trp*A, *trp*A translation terminator. repA; replication initiation protein A of plasmid pZMO1, *lac*Z; whole orf of *E. coli lac*Z gene, *trp*A; translation termination codon.

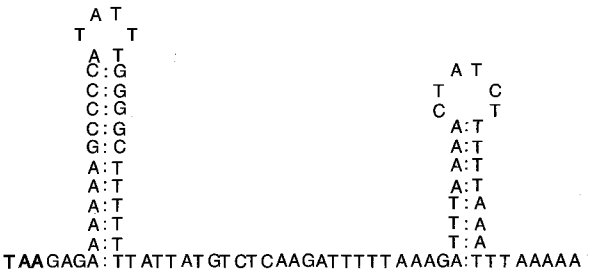

Fig. 5. The secondary structure of the transcription terminator region of repA. The termination codon is indicated with bold latters.

Analysis of the promoter region of *rep*A

In 1680 bp of a complete DNA sequence of the pZMO1, 1008 bp was revealed to be an orf for *rep*A. The nucleotide sequence from 1 to 240 was AT-rich (the G+C content was 31.6%) and was thought to be a promoter region and possible upstream regulation region. Six tandem 12 nt direct repeated sequences (AAAAATAC GGTC repeat) were found from sequence 33 to 104 without intervening sequence. Among these six repeated sequences, five were completely identical (A1-A5), but the last one A6 (AAAAATACTTTAC) was not identical but very similar to A1-A5 repeats. One more inverted repeat (B1 and B2) was found (Fig. 2) right upstream of ATG codon. The repeated sequence of the upstream region of the *rep*A gene of the bacterial plasmid have been reported. In *Erwinia stewartii* plasmid pSW500, these repeated sequences were named *iterons*, which serve as binding sites for the regular A protein and are required for the control of the plasmid copy number and plasmid incompatibility (Fu *et al.*, 1995). In addition, these iterons existed in the origin of replication regions of many plasmids (Chattoraj *et al.*, 1985). Right downstream of A5 sequence, one conserved sequence CTTGATA of pC194 nick region in rolling circle (RC) replicons was found (del Solar *et al.*, 1998).

To prove the promoter sequence, the putative promoter region of the *rep*A gene of pZMO1 was tested by the promoter probing vector pRS415, using the *lac*Z genes as a reporter system. About a 700 bp size upstream region of *rep*A was amplified by the PCR method and cloned *Sma*I site of pRS415. The promoter activity of 700 bp, 630 bp, and 270 bp of insert DNA fragments that were excised from the PCR product were tested (Fig. 4). About one third of the transformants of three plasmid constructs generated blue color. The plasmid DNA that was prepared from the blue colonies generated about 700 bp, 630 bp, and 270 bp fragment, respectively, by *Eco*RI and *Bam*HI double digestion. About half of white colonies also had insert fragments. Those white colonies might resulted from inserting the promoter fragment by reverse orientation into the pRS415. As a result of this test, a minimum of 240 bp fragments of the upstream region of *rep*A worked as a promoter.

Analysis of the downstream region of *rep*A

The (G+C)% of about 130 nt of downstream sequence of *rep*A is very low, 26%. Furthermore, this region revealed that it had an inverted repeat and might form a hairpin structure (Fig. 5). Downstream of the putative hairpin, another AT-rich sequence ($T_{3\text{-}5}A_{3\text{-}5}$)) was found (Fig. 2).

pZMO1 replicates by a rolling circle mechanism

The DNA prepared using the rapid agarose block method (Borges, *et al.*, 1993) was electrophoresed and transferred to an NC membrane directly or after S1 nuclease treatment without denaturation (Yang, *et al.*, 1996). The directly transferred NC membrane could absorb only the ssDNA intermediate of pZMO1, and generated hybridized signal by the pZMO1 probe. But the S1 nuclease-treated sample did not show any hybridized signal at all by the pZMO1 probe. The existence of ssDNA indicates that the pZMO1 replicates by a rolling circle mechanism producing ssDNA intermediate (Fig. 6). The amino acid sequences of many small plasmids which replicate by a rolling circle mechanism exhibit a strong homology with the *Rep*A protein of pZMO1 (Yasukawa, *et al.*, 1991).

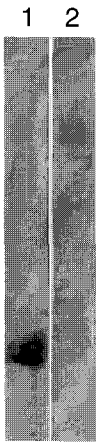

Fig. 6. Detection of the ss plasmid DNA intermediate of pZMO1. 1, DNA was drectly transffered onto NC membrane without S1 nuclease treatment; 2, DNA was transfered onto the NC membrane with S1 nuclease treatment

Methods

Bacterial strains, plasmids and culture conditions

Z. mobilis ATCC10988 (type strain; Km^r Cm^s; contains pZMO1, pZMO3, pZMO4, pZMO5, pZMO6) was grown anaerobically at 30℃ in RM broth(10% glucose, 1% yeast extract) supplemented with $(NH_4)_2SO4$ (0.1%), KH_2PO_4 (0.2%) and $MgSO_4$ (0.1%) (Skotnicki *et al.*, 1981). *E. coli* DH10B (F^-, *mcr*A, (*mrr*$^-$*hsd*RMS$^-$mcrBC) 80*dlac*ZM15 *lacX*74 *deo*R *rec*A1 *end*A1 *ara*D139 (*ara, leu*)7679 *gal*U *gal*K *rps*L *nup*G) *E. coli* was cultured in LB medium containing appropriate antibiotics. Chloramphenicol, ampicillin, streptomycin, and kanamycin were used at a final concentration of 34mg/ml, 50mg/ml, 50mg/ml and 50mg/ml respectively. The cloning vector pNEB193 and pBC SK+ were purchsed from New England Biolabs and Stratagene, respectively. pRS415 was used to prove the promoter region of the *rep*A gene (Simon, *et al*, 1987).

Preparation of plasmids from *Z. mobilis* ATCC 10988 and recombinant techniques

Z. mobilis ATCC10988 cells were grown anaerobically for 1 day in 200 ml of RM broth. The plasmids were extracted by the alkaline lysis method, and then were further purified by CsCl density gradient ultracentrifugation (Sambrook, *et al.*, 1989). DNA were electrophoresed on agarose gel and transferred to the nylon membrane by capillary transfer method. The nylon membranes were fixed by UV crosslinker (Stratagene, USA). Probe labelling and signal detection were carried out by the enhanced chemiluminescence (ECL) method (Amersham, USA). Other recombinant DNA techniques were performed as described by J. Sambrook and D. W. Russeltis (Sambrook, *et al.*, 1989).

Detection of the promoter function

A set of oligomers (REP-1: 5"-ATCGAACAAGGT GATA-TTATTT-3", REP-2: 5"-ATCATAGTGACCTTC TTGGT-CAT-3") were designed to amplify the upstream region of the *rep*A gene. The 700bp PCR product was purified by a PCR purification kit (Qiagen, Germany) and ligated into the *Sma*I site of the pRS415 (Simons RW., 1987) promoter probing vector to give pPro5. The PCR product was also digested with *Spe*I or *Xba*I, filled-in with Klenow fragment. 630bp or 270bp fragments were eluted and also ligated into the *Sma*I site of the pRS415. The ligated DNA was transformed into the *E. coli* DH10B and spread on the LB agar plate containing 50mg/ml ampicillin and 0.002% X-Gal.

Detection of the single stranded plasmid DNA

Single-stranded plasmid DNA was detected by modified method of Yang and McFadden (1993). Well-grown *Z. mobilis* ATCC10988 cells were harvested and lysed in lysis buffer (8% sucrose, 50mM EDTA, 0.1% Triton X-100, 50mM Tris-HCl, pH8.0, 10mg/ml lysozyme, 100㎍/ml RNase A) for 6hr at 37℃ and then treated with 0.5mg/ml proteinase K and 1% sodium N-laurlysarcosine for 6hr at 55℃. Lysates were extracted with phenol and chloroform and then precipitated. Twenty microgram of DNA was treated with 5 unit of S1 nuclease for 1hr at 37℃. Twenty microgram of S1 nuclease-treated or untreated DNA were subjected to 1% agarose gel electrophoresis and then transferred to a nylon membrane in 10X SSC without prior denaturation. Probe was labelled by enhanced chemiluminescence (ECL) method according to the manufacturer' s protocol (Amersham).

Acknowledgments

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