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Short Communication

A fruiting body-specific novel cDNA, *mfbB*c, containing continuous 5′-CCA(A/C)CA direct repeats within the coding region, derived from the basidiomycete *Lentinula edodes*

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Screening for cDNA(s) homologous to *Leras* cDNA (Hori et al. 1991. Gene 105: 91–96.) in the basidiomycete *Lentinula edodes* resulted in the isolation of a cDNA (designated *mfbB*c) encoding 394 amino acids, in place of isolation of a second *ras* cDNA. The *mfbB* gene showed developmental regulation, with mature fruiting bodies having much higher levels of *mfbB* transcript. Preprimordial mycelia and primordia contained low levels of *mfbB* transcript. *mfbB*c was found to contain eight continuous 5′-CCA(A/C)CA direct repeats and a 5′-CCACCG sequence in the proximity of the 5′ end on the coding strand. When cloned into a plasmid and propagated in *Escherichia coli*, the CA/TG direct repeats region was shown to decrease the superhelical density of the plasmid, suggesting the formation of an altered DNA structure.

Key Words——cDNA; development; direct repeat; *Lentinula edodes;* superhelical density.

The molecular mechanisms of fruiting in the basidiomycetes are an attractive topic for scientific investigation. Our previous study with the edible basidiomycete *Lentinula edodes* (Berk.) Pegler FMC2 (Katayose et al., 1986; Shishido, 1992) suggested that a high level of intracellular cAMP is closely related to the onset of fruiting and/or primordium formation (Takagi et al., 1988). In the yeast *Saccharomyces cerevisiae*, *ras* gene products have been shown to stimulate adenylate cyclase activity and participate in cell cycle regulation by influencing cAMP level (Toda et al., 1985; Field et al., 1988). Thus we have isolated a *ras* gene homologue (named *Leras*) (Hori et al., 1991) from *L. edodes* using the v-Ha-*ras* gene (Barbacid, 1987) as a hybridization probe. Northern-blot analysis, however, showed that the *Leras* gene is transcribed at similar levels during mycelial development in fruiting-body formation, suggesting no direct correlation of *Leras* expression with intracellular cAMP levels in the fungus. Thus we attempted to isolate the second *ras* gene using *Leras* as a hybridization probe, resulting in the isolation of a cDNA clone from a primordial cDNA library of *L. edodes* that weakly hybridizes to *Leras* cDNA (named *Leras*c) (Hori et al., 1991). Although, unfortunately, it was not *ras* itself, the cloned cDNA was found to be developmentally regulated and carried the unique direct repeat sequences which were suggested to be involved in formation of an altered structure in supercoiled plasmid DNA. We present here the experimental data about the novel cDNA, although our initial purpose was not attained.

A *L. edodes* cDNA library of 2×10^6 recombinants was constructed by insertion of cDNA (Gubler and Hoffman, 1983) synthesized on poly(A)$^+$RNA (Gilham, 1964; Han et al., 1987) prepared from primordia into dephosphorylated *Eco*RI-cut λgt10 vector (Huynh et al., 1985). The library was screened by plaque hybridization (Benton and Davis, 1977) using the ^{32}P-labeled *Leras*c as a probe. Out of 2×10^5 cDNA clones, one candidate was obtained which contained an approx. 1.5-kb DNA insert. This cDNA was designated *mfbB*c. *mfbB* denotes the second clone that is most actively expressed in mature fruiting bodies (see Fig. 2).

Sequencing revealed that the 1.5-kb *mfbB*c consisted of 1493 bp encoding 394 amino acids (Fig. 1). Judging from the size of its transcript (see Fig. 2), the cloned *mfbB*c is considered to contain the entire coding sequence. Homology search of the deduced amino acid sequence showed that *mfbB*c is not *ras* homologue. Instead, *mfbB*c contained a nucleotide sequence (approx. 200 bp) partially homologous (approx. 40% identical) to the *Leras*c sequence.

The molecular weight of the predicted polypeptide of MfbB was calculated to be 42,383. We searched the NBRF-PIR protein sequence database for homologous protein(s) using the IDEAS program (Kanehisa, 1982) but obtained a negative result. The deduced MfbB protein (394 amino acids) was rich in Leu (52 residues), Ser (45 residues), Thr (41 residues) and Pro (41 residues).

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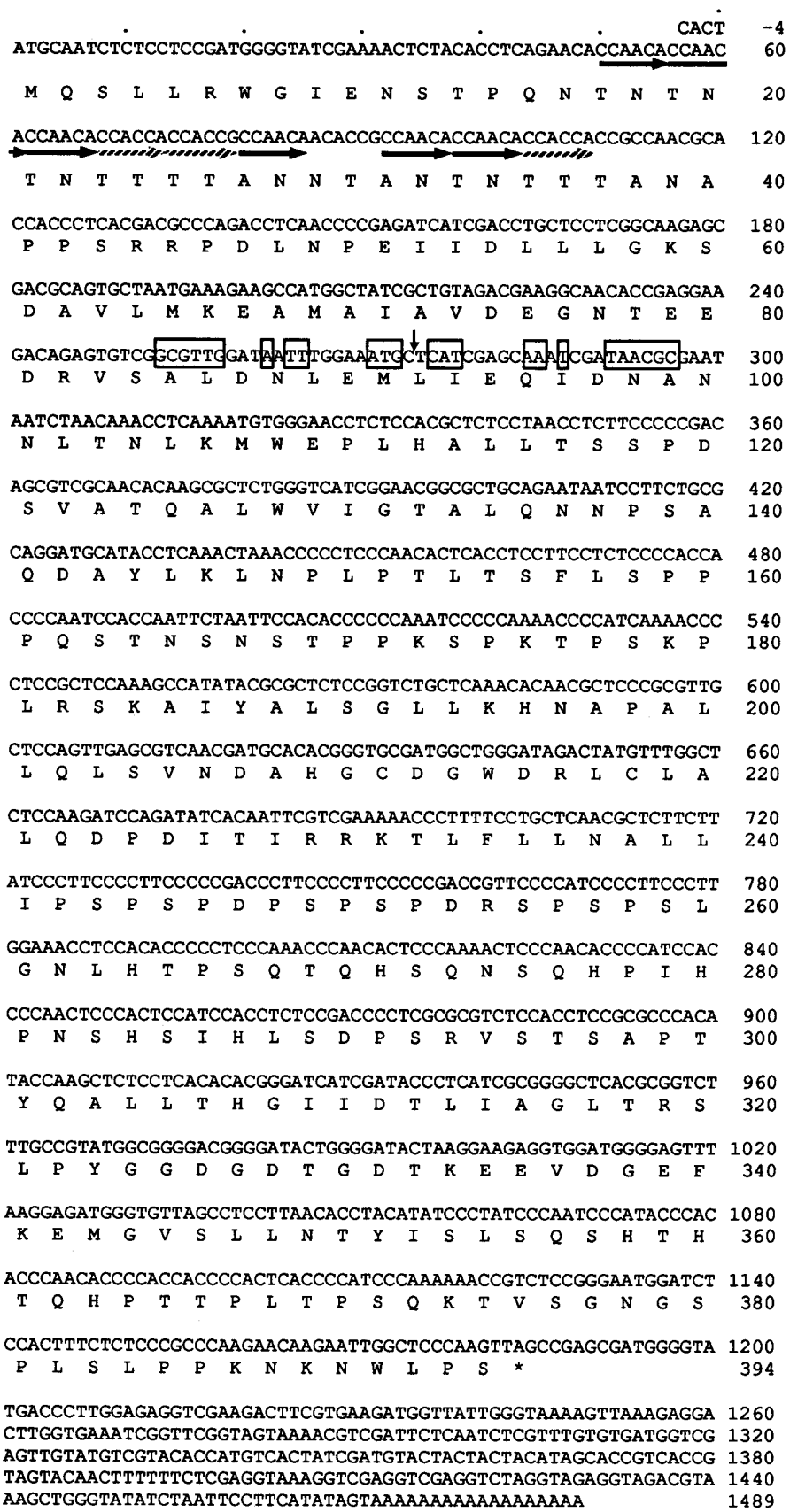
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T N T T T T A N N T A N T N T T T A N A 40
CCACCCTCACGACGCCCAGACCTCAACCCCGAGATCATCGACCTGCTCCTCGGCAAGAGC 180
P P S R R P D L N P E I I D L L L G K S 60
GACGCAGTGCTAATGAAAGAAGCCATGGCTATCGCTGTAGACGAAGGCAACACCGAGGAA 240
D A V L M K E A M A I A V D E G N T E E 80
GACAGAGTGTCGGCGTTCGATAATTTGGAAATGCTCATCGAGCAAATCGATAACGCGAAT 300
D R V S A L D N L E M L I E Q I D N A N 100
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N L T N L K M W E P L H A L L T S S P D 120
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S V A T Q A L W V I G T A L Q N N P S A 140
CAGGATGCATACCTCAAACTAAACCCCCTCCCAACACTCACCTCCTTCCTCTCCCCACCA 480
Q D A Y L K L N P L P T L T S F L S P P 160
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P Q S T N S N S T P P K S P K T P S K P 180
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L R S K A I Y A L S G L L K H N A P A L 200
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L Q L S V N D A H G C D G W D R L C L A 220
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L Q D P D I T I R R K T L F L L N A L L 240
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I P S P S P D P S P S P D R S P S P S L 260
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G N L H T P S Q T Q H S Q N S Q H P I H 280
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Y Q A L L T H G I I D T L I A G L T R S 320
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L P Y G G D G D T G D T K E E V D G E F 340
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K E M G V S L L N T Y I S L S Q S H T H 360
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T Q H P T T T P L T P S Q K T V S G N G S 380
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P L S L P P K N K N W L P S * 394
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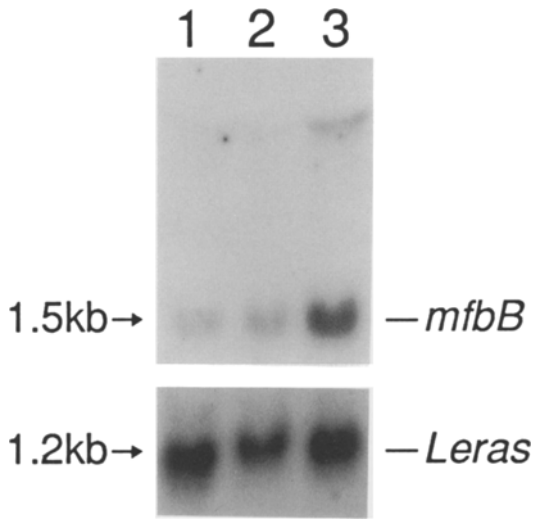

Fig. 2. Transcriptional expression of the *mfbB* gene during mycelial development in fruiting-body formation.
In *L. edodes,* mycelial development is as follows. Mycelial aggregation on solid sawdust-rice bran medium (Takagi et al., 1988) at 17°C under a continuous illumination with a fluorescent lamp results in the formation of thick membrane-like mycelia (designated preprimordial mycelia). Primordia are formed on the preprimordial mycelia and they grow into mature fruiting bodies. Total cellular RNA samples (20 μg each) isolated from the preprimordial mycelia (lane 1), primordia (lane 2), and mature fruiting bodies (lane 3) were size-fractionated, transferred to a nylon filter, hybridized with ^{32}P-labeled *mfbB*c probe under stringent conditions according to the method previously described (Hori et al., 1991), and autoradiographed. The Northern blots of *mfbB* were put into boiling 0.1% SDS and left there to cool to room temperature. After confirming the complete removal of the *mfbB*c probe by autoradiography, the resulting Northern blots were rehybridized with ^{32}P-labeled *Leras*c (Hori et al., 1991) under stringent conditions, and autoradiographed. The sizes of *mfbB* and *Leras* transcripts are indicated on the left.

Northern-blot analysis was used to investigate expression of the *mfbB* gene during fruiting-body development. Total cellular RNA was isolated from preprimordial mycelia, primordia and mature fruiting bodies. Probes used were *mfbB*c (1.5 kb) and the constitutively expressed *Leras*c (Fig. 2). A strong 1.5-kb signal (*mfbB* band) was detected in the RNA blot of the mature fruiting bodies (lane 3), although the signal was of lower intensity than the *ras* signal. Preprimordial mycelia (lane 1) and primordia (lane 2) had low levels of the transcript. The *mfbB* gene may play a role mainly in maturation of fruiting body.

Of interest was the fact that *mfbB*c contains six 5′-CCAACA and two 5′-CCACCA direct repeats, and one 5′-CCACCG sequence within 60 bp in the proximity of the 5′ end on the coding strand (Fig. 1). These direct repeats were similar to the repeating units 5′-C_{1-8} $(A/T)_{1-4}$ found in telomeres which define the ends of chromosomes in eukaryotes (Blackburn, 1984). So far the direct repeats region like this has not been reported, as far as we know. Lyamichev et al. (1989) have reported that the DNA sequence 5′-$(C_4A_2)_9C_3(C_4A_2)_7C_3$ derived from *Tetrahymena* telomeric motif does adopt a novel DNA conformation under superhelical stress and low pH, exhibiting hypersensitivity to S1 nuclease (Shishido and Ando, 1982). In this structure, the C-rich strand forms a hairpin stabilized by non-Watson-Crick protonated base pairs $C \cdot C^+$ and $A \cdot A^+$, whereas the G-rich strand remains unstructured (Lyamichev et al., 1989). The 60-bp CA/TG direct repeats region of *mfbB*c has the potential to form an imperfect CA-hairpin made by fourteen $C \cdot C^+$ and eight $A \cdot A^+$ base pairs.

To investigate the possibility whether the *mfbB*c direct repeats region does form an S1-cleavable, unbasepaired structure under superhelical stress and low pH, we inserted the 61-bp fragment containing the CA/TG direct repeats between *Pst*I and *Eco*RI on pBR322 (Figs. 3A, 3B). The resulting recombinant plasmid (named pBR-CA) was propagated in *Escherichia coli* DNA topoisomerase I deletion mutant DM800 (Sternglanz et al., 1981; DiNardo et al., 1982) and subjected to analysis of S1-sensitivity at pH 4.5. The results showed that the CA/TG direct repeats region does not form a structure sensitive to S1 action, although an imperfect inverted complementary repeat sequence 144 bp downstream from the CA/TG direct repeats (see Fig. 1) formed an S1-cleavable structure (data not shown).

We attempted to analyze the superhelical density of the plasmid pBR-CA using two-dimensional chloroquine-agarose gel electrophoresis (see Fig. 3). pBR322 DNA isolated from DM800 has been shown to be extremely heterogeneous in linking number and highly negatively supercoiled (Pruss and Drlica, 1986). The high levels of negative supercoiling are attributed to the generation of twin supercoiled domains during transcription of the tetracycline-resistance gene (*tet*): in the absence of the topoisomerase I, the positively supercoiled domain is effectively relaxed by gyrase, resulting in a net accumulation of negative supercoils (Liu and Wang, 1987; Wu et al., 1988; Tsao et al., 1989). The presence of the β-lactamase gene (*bla*) is not required for high levels of negative plasmid supercoiling (Pruss and Drlica, 1986; Shishi-

Fig. 1. The nucleotide sequence of *mfbB*c and the deduced amino acid sequence.
The restriction fragments of the 1.5-kb *mfbB*c to be sequenced were subcloned into pUC18, pUC19, M13mp18 and M13mp19 (Yanisch-Perron et al., 1985). Sequencing was carried out by the dideoxy chain-termination method of Sanger et al. (1977). Both strands were sequenced. The nucleotide sequence coordinates and amino acid sequence coordinates are presented on the right-hand side. Start codon is assigned the +1 coordinate. The 5′-CCAACA and 5′-CCACC(A/G) direct repeats are shown by closed and striped horizontal bold arrows, respectively. The imperfect inverted complementary repeat sequence is indicated by boxes, with a short vertical arrow at its center. The nucleotide sequence data reported in this paper will appear in DDBJ/EMBL/GenBank Data Libraries under the accession number D01210.

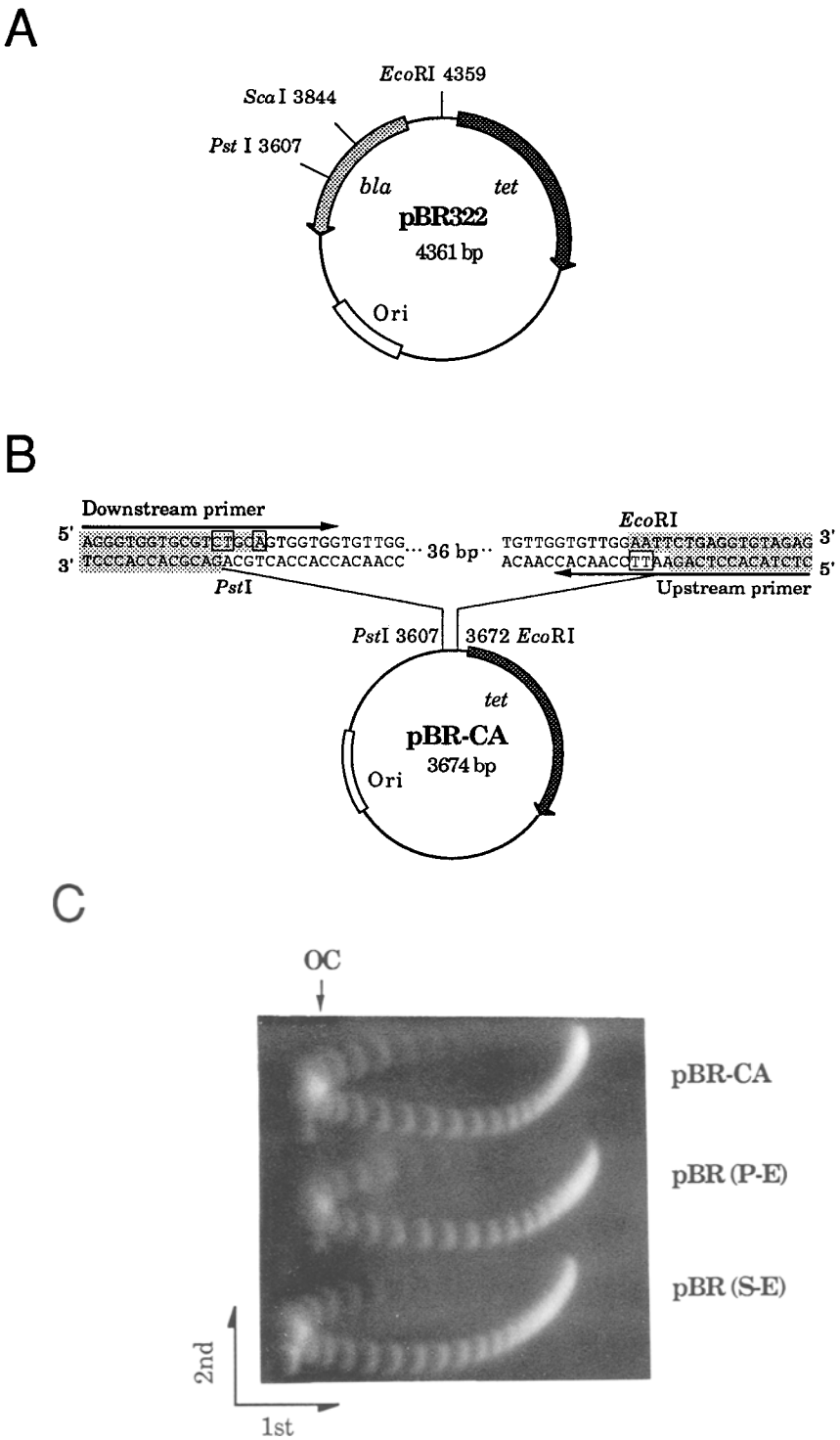

Fig. 3. Two-dimensional chloroquine-agarose gel electrophoretic analysis of pBR322 derivatives isolated from DM800.
(A) Simplified restriction and genetic maps of pBR322. The numbers indicate the positions of restriction sites. (B) The PCR primers (horizontal arrows) for preparing 61-bp fragment containing the CA/TG direct repeats. The primers were designed to create *Eco*RI and *Pst*I sites: five nucleotide residues (boxed) were changed. (C) The electrophoretic patterns of pBR322 derivatives. For all three plasmids, DNA from similar amounts of cells was loaded on gel. Chloroquine was present at 25 μg/ml in the first (horizontal) dimension and at 100 μg/ml in the second (vertical) dimension. During electrophoresis in the first dimension, topoisomers in the lower arcs of the three distributions were negatively supercoiled, while those in the upper arcs were positively supercoiled, causing the two arcs of each distribution to be superimposed. Electrophoresis in the second dimension resolved the superimposed topoisomers. The position of open circular (OC) DNA is indicated.

do et al., 1989). As transcription proceeds, DNA in front of the transcription complex becomes positively supercoiled, and DNA behind the complex becomes negatively supercoiled (Tsao et al., 1989; Shishido et al., 1989). The results of the two-dimensional chloroquine-agarose gel electrophoresis of pBR-CA isolated from DM800 are shown in Fig. 3C. As a control for the experiment two deletion plasmids were constructed by deleting the *Sca*I-*Eco*RI fragment and the *Pst*I-*Eco*RI fragment (see Fig. 3A) and named pBR(S-E) and pBR(P-E), respectively. Compared with pBR(S-E) and pBR(P-E), pBR-CA exhibited supercoiling distribution containing slightly more positively supercoiled DNA molecules (in the upper arc), which were derived from less negatively supercoiled DNA species propagated in DM800. These results show that the CA/TG direct repeats region decrease the superhelical density of the plasmid DNA.

The levels of tetracycline resistance conferred by the three plasmids tested were almost the same (data not shown), suggesting very similar levels of transcription of the *tet* gene. The above results may suggest that the inserted CA/TG direct repeats region present in the negative domain of the plasmid adopts an altered structure such that some gyrase molecules do remove negative turns. Our previous study showed that a pBR322 derivative having the *par* sequence of plasmid pSC101 (Wahle and Kornberg, 1988), which shows a DNA bendability and a high affinity for gyrase, at the *Eco*RI site (in the negative domain) contains less highly negatively supercoiled DNA species than does pBR322 (Ishii et al., 1992). The CA/TG direct repeats region, however, displayed no DNA bendability. To clarify its structure, more detailed analysis is necessary. The biological significance of *mfbB* gene and function of the CA/TG direct repeats region totally remains to be determined.

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