# Promoter/repressor system of Lactobacillus plantarum phage øg1e: characterization of the promoters pR49–pR–pL and overproduction of the Cro-like protein Cng in Escherichia coli

# Promoter/repressor system of *Lactobacillus plantarum* phage øg1e: characterization of the promoters *pR49–pR–pL* and overproduction of the Cro-like protein Cng in *Escherichia coli*

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**Abstract**

The *Lactobacillus plantarum* phage øg1e (42 259 bp) has two repressor-like genes *cng* and *cpg* oriented oppositely, accompanied by three potential promoters *pR*, *pL* and *pR49*, and seven operator-like sequences (GATAC-boxes) (Kodaira et al., 1997). In this study, the øg1e putative promoters were introduced into the *Escherichia coli* promoter-detecting plasmid pKK232-8. In *E. coli* CK111, *pR* (pKPR1), *pL* (pKPL1) and *pR49* (pKPR49) exhibited distinct CAT activities. When pKPR1 or pKPL1 was coexistent with a compatible plasmid pACYC184 carrying *pR-cng* (pA4PRCN1), the CAT activity was decreased significantly. On the other hand, *cng* directed a protein (Cng) of 10.1 kDa in *E. coli* under the control of T7 promoter. Gel mobility-shift assays demonstrated that Cng binds specifically to a DNA region containing the GATAC-boxes. In addition, primer extension analyses demonstrated that the two sequences *pR* and *pL* act as a promoter in *L. plantarum* as well as in *E. coli*. These results suggested that the potential promoters *pR* and *pL* probably function for the lytic and lysogenic pathways, respectively, and Cng may act as a repressor presumably through the GATAC-boxes as operators.

*Keywords:* DNA-binding protein; Gel-shift assay; Operator; Primer extension

## 1. Introduction

Prevalence of lysogeny in various Gram-positive and -negative bacteria has been reported (Davidson et al., 1990; Campbell, 1994). For their propagation in two different ways (lysis or lysogeny), it has been thought that the phages have a set of regulatory proteins, as well as their recognizable DNA domains.

In *Escherichia coli* phages such as lambda, P2 and 186, the lysis–lysogeny decision depends upon phage-encoded proteins, known as repressors, which regulate gene expression by binding to specific DNA sequences called operators (Campbell, 1994; Neufing et al., 1996). Contrary to the coliphages, molecular details on the lysis–lysogeny switch of the phages from lactic acid bacteria are scanty, although several putative repressor/operator systems have been reported, e.g., the *Lactococcus* phages r1t (Nauta et al., 1996), BK5-T (Boyce et al., 1995) and Tuc2009 (van de Guchte et al., 1994).

Recently, we isolated a new *Lactobacillus plantarum* temperate phage øg1e (Kakikawa et al., 1996), and determined the total genome sequence of 42 259 bp DNA (Kodaira et al., 1997). The sequencing result revealed that a region of approx. 850 bp of the øg1e

Abbreviations: Amp, ampicillin; Cm, chloramphenicol; *cat*, Cm acetyltransferase (CAT) gene; *cng*, gene encoding repressor (Cng); *cpg*, gene encoding repressor (Cpg); DTT, dithiothreitol; Em, erythromycin; Km, kanamycin; LB, Luria–Bertani (medium); *Lorf143*, ORF encoding 143-amino acids protein; *Lorf166*, ORF encoding 166-amino acids protein; *Lorf304*, ORF encoding 304-amino acids protein; MMC, Mitomycin C; ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; *pL*, promoter on the L-strand; p*lac*, promoter of lacZ′ gene; *pR*, promoter on the R-strand; *pR49*, promoter on the R-strand within *cng*; $^{r}$, resistant; *Rorf49*, ORF encoding 49-amino acids protein; *Rorf58*, ORF encoding 58-amino acids protein; $^{s}$, sensitive; SDS, sodium dodecyl sulfate; Tet, tetracycline; UV, ultra violet light; $^{+}$, utilizable.

Table 1
Bacteria, phages and plasmids used in this study

| Strain | Genotype or construct | Source or reference |
|---|---|---|
| Bacteria | | |
| *Escherichia coli* | | |
| CK111 | *recA* | Lab. stock |
| XL1-Blue | *supE*44, *hsdR*17, *recA*1, *endA*1, *gyrA*46, *thi*, *relA*1, *lac*$^-$, F′[*proAB*$^+$, *lacI*$^q$, *lacZ*δM15, Tn*10*(*Tet*$^r$)] | Lab. stock |
| BL21(DE3) | *hsdE*44, *gal*(λ cI *ts*857, *ind*1, *Sam*7, *nin*5, *lac*UV5-T7 gene*1*) | Studier et al. (1990) |
| BL21(DE3)p*lysS* | BL21(DE3) containing p*lysS* | Studier et al. (1990) |
| *Lactobacillus plantarum* | | |
| G1e | *Tet*$^r$, *Km*$^r$, *lac*$^+$, *suc*$^+$, øg1e, pG1e | Lab. stock |
| Phages | | |
| øg1e | 42259 bp template phage of *L. plantarum* G1e | Lab. stock |
| Plasmids | | |
| pUC118 | 3162 bp *E. coli* vector, *Amp*$^r$, *plac*, *lacZ*′ | Lab. stock |
| pKK232-8 | 5096 bp *E. coli* promoter-detecting vector, *Amp*$^r$, containing promoterless *cat* | Brosius (1984) |
| pACYC184 | 3.97 kbp *E. coli* vector, *Tc*$^r$, *Cm*$^r$ | Chang and Cohen (1978) |
| pET-3c | Cloning vector containing T7 promoter | Studier et al. (1990) |
| p118PR1 | pUC118::454 bp *Alu*I–*Rsa*I fragment from øg1e (*pR* under *plac*) | This study |
| p118PL1 | pUC118::454 bp *Alu*I–*Rsa*I fragment from øg1e (*pL* under *plac*) | This study |
| pKPR1 | pKK232-8::230 bp fragment from p118PR1 (*cat* under *pR*) | This study |
| pKPL1 | pKK232-8::227 bp fragment from p118PL1 (*cat* under *pL*) | This study |
| pKPR49 | pKK232-8::141 bp fragment from p118PR1 (*cat* under *pR49*) | This study |
| pKPRCN1 | pKK232-8::471 bp fragment from p118PR1 (*cat* under *pR–pR49*) | This study |
| pA4PRCN1 | pACYC184::454 bp fragment from p118PR1 | This study |
| pE3CNG1 | pET-3c::267 bp fragment from p118R1 containing *cng* under control of T7 promoter | This study |

Table 2
Synthetic oligonucleotides used in this study

| Primers | Nucleotide sequence | Source |
|---|---|---|
| CNGI | 5′-aacatATGAAGCGTGAGCGACTTATT-3′ | in *cng* (578–559) |
| CNGII | 5′-aaaggatccttATTTTGCTGCCTCCTTTGT-3′ | in *cng* (315–333) |
| CNGVI | 5′-GCAATAAGTC(a/c)CTCACGCTTC-3′ | in *cng* (557–577) |
| PL-PE1 | 5′-CTCATCTTGTGTCTTGCCTTGC-3′ | in *cng* (814–793) |
| PR49-PE1 | 5′-ACTACTAGGTTAGTAACTGATTG-3′ | in *Lorf49* (252–274) |
| CAT-N | 5′-CATTTTAGCTTCCTTAGCTCCT-3′ | in PKK232-8 (271–250) |
| M13RV | 5′-CAGGAAACAGCTATGAC-3′ | in *lacZ*′ |
| M13–20 | 5′-GTAAAACGACGGCCAGT-3′ | in *lacZ*′ |
| M13–47 | 5′-CGCCAGGGTTTTCCCAGTCACGAC-3′ | in *lacZ*′ |

Oligonucleotides used in this study (for PCR, DNA sequencing and primer extension) were obtained from KURABO (in *cng*, *Lorf49* and pK232-8) and Takara (in *lacZ*′). Sequence positions given in øg1e (*cng* and *Lorf49*) and pKK232-8 were from Kodaira et al. (1997) and Brosius (1984), respectively. Artificial nucleotides shown in lower-case letters in CNGI, CNGII, or CNGVI are described in Section 3.1.

Table 3
CAT activity of *E. coli* CK111 carrying øg1e-promoter-containing plasmids

| Plasmids[a] | Promoter | CAT activity[b] with[c] | |
|---|---|---|---|
| | | No plasmid | pA4PRCN1 |
| pKK232-8 | None | <0.5 | <0.5 |
| pKPR1 | *pR* | 626.8 | 106.7 |
| pKRR49 | *pR49* | 12.8 | 10.1 |
| pKPL1 | *pL* | 37.8 | 0.8 |

CAT activity was assayed as described in Section 2.3.
[a]Plasmids containing øg1e promoters (*pR*, *pR49* and *pL*) upstream of *cat* are indicated in Fig. 2 (Table 1).
[b]Values, average of 3–5 experiments.
[c]Plasmid pA4PRCN1, coresident with promoter-containing plasmid.

genome contains two repressor-like genes, *cng* and *cpg*, oppositely oriented at a distance of 166 bp away, accompanied by seven 15 bp inverted repeats (named GATAC-box) and three potential promoter sequences for mRNA transcription. The predicted *cng* and *cpg* proteins (Cng and Cpg, respectively) contain a potential helix-turn-helix DNA binding motif in the N-termini, like other repressor proteins from coliphages. øg1e Cng (88 amino acids) somewhat resembles lambda Cro (Albright et al., 1996), whereas Cpg (132 amino acids) bears resemblance to lambda CI (Pabo and Sauer, 1984). In addition, the øg1e genes for lytic pathway (e.g. virion proteins and cell lysis) are located downstream of *cng*, and the øg1e integrase gene is encoded downstream of *cpg* (Kakikawa et al., 1996, 1997; Oki et al., 1996, 1997; Kodaira et al.,

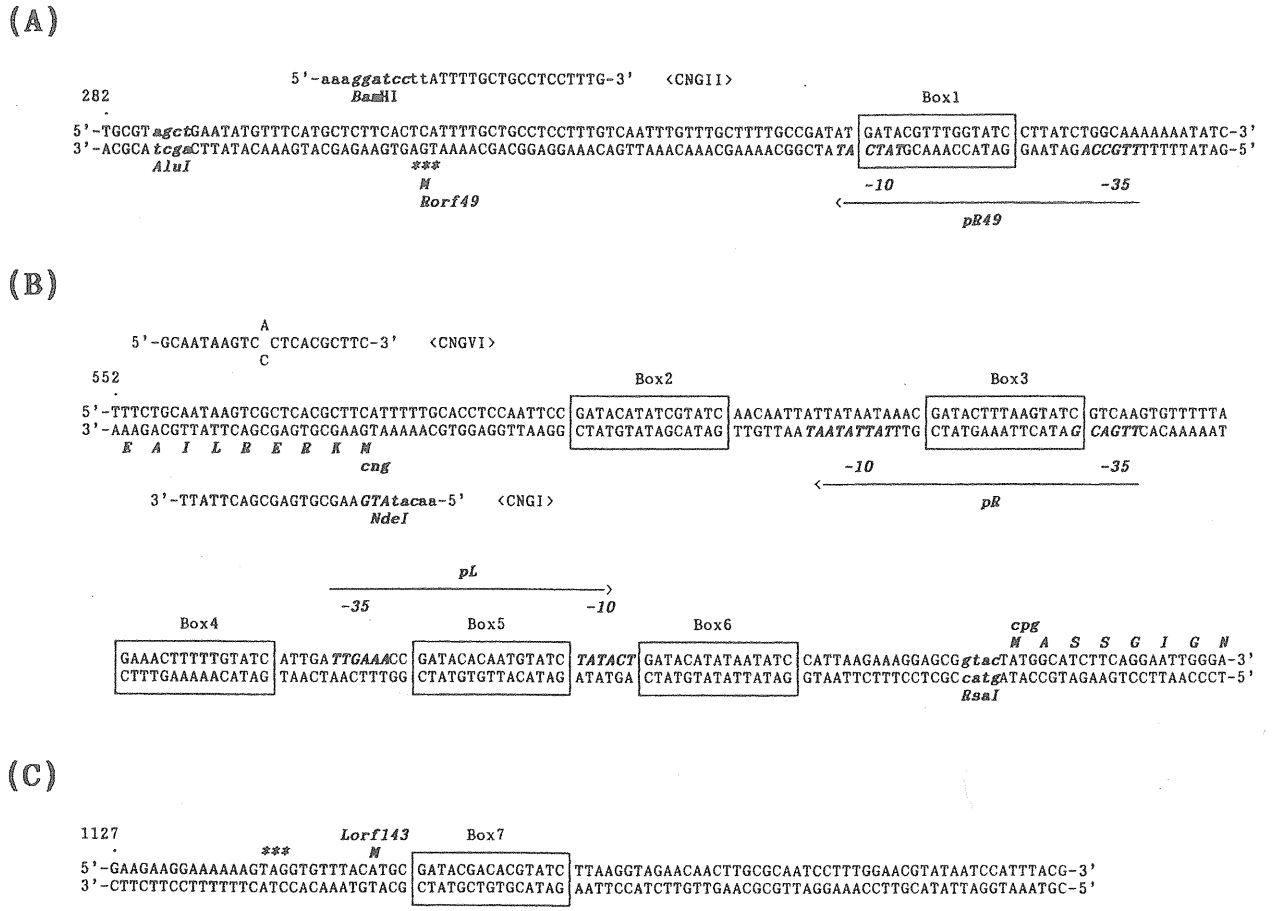



Fig. 1. DNA sequences of the øg1e promoter/operators. The DNA sequences, nucleotide numbers, and putative genes and ORFs were from Kodaira et al. (1997) (EMBL accession number, X98106). The predicted promoters, *pL*, *pR* and *pR49* are indicated by long arrows. The −35 and −10 sequences are shown by bold italic letters. The seven GATAC-boxes are boxed. (A) *pR49* and box 1. ***, stop codon for *cng*; *M*, start of *Rorf49*. The *Alu*I site (lower-case bold letters) and the primer CNGII (Table 2) are also shown. (B) *pR–pL* and boxes 2–6. The N-terminal amino acids of *cng* and *cpg* are shown. The *Rsa*I site (lower-case bold letters) and the primers CNGI and CNGVI (Table 2) are shown. (C) Box 7. ***, stop codon for *cpg*; *M*, start of *Lorf143*.

1997; Yasukawa et al., 1997). These previous results suggest that the *cng–cpg* region functions as a regulatory domain for the lysis–lysogeny decision.

In the present study, function of the predicted øg1e promoters was investigated in *E. coli* using *cat* as a reporter gene, and primer extension analyses were performed in *L. plantarum* as well as in *E. coli*. Moreover, Cng, a DNA-binding protein overproduced in *E. coli*, was characterized in vitro.

## 2. Materials and methods

### 2.1. Bacteria, phage and plasmids

The *L. plantarum* G1e and its temperate phage øg1e were from our laboratory stock, and were propagated in M17 broth as described previously (Kakikawa et al., 1996). The *E. coli* vector plasmids (pUC118, pKK232-8, pACYC184, pET-3c) and their host XL1-Blue were propagated in LB broth (Oki et al., 1996). The recombinant plasmids constructed in this study (see Sections 3.1 and 3.3) are summarized in Table 1 (see also Fig. 2).

### 2.2. Analysis of DNA, RNA and protein

Cloning and sequencing analyses of øg1e DNA were carried out essentially as described by Kakikawa et al. (1996, 1997). Gel-shift assays were performed as described by Yasukawa et al. (1997): cell extracts of *E. coli* BL21(DE3)p*lysS* carrying a plasmid were prepared by the lysozyme-freeze-thawing method, and the cell extract (1 μg/μl of protein) was incubated with DNA fragment at 0°C for 20 min in 10 μl of reaction buffer [10 mM Tris (pH 7.5), 1 mM EDTA, 100 mM KCl, 0.1 mM DTT, 5% glycerol, 50 mg/ml bovine serum albumin, 1 μg/ml sheared salmon sperm DNA], and electrophoresed on a non-denaturing 6% polyacrylamide

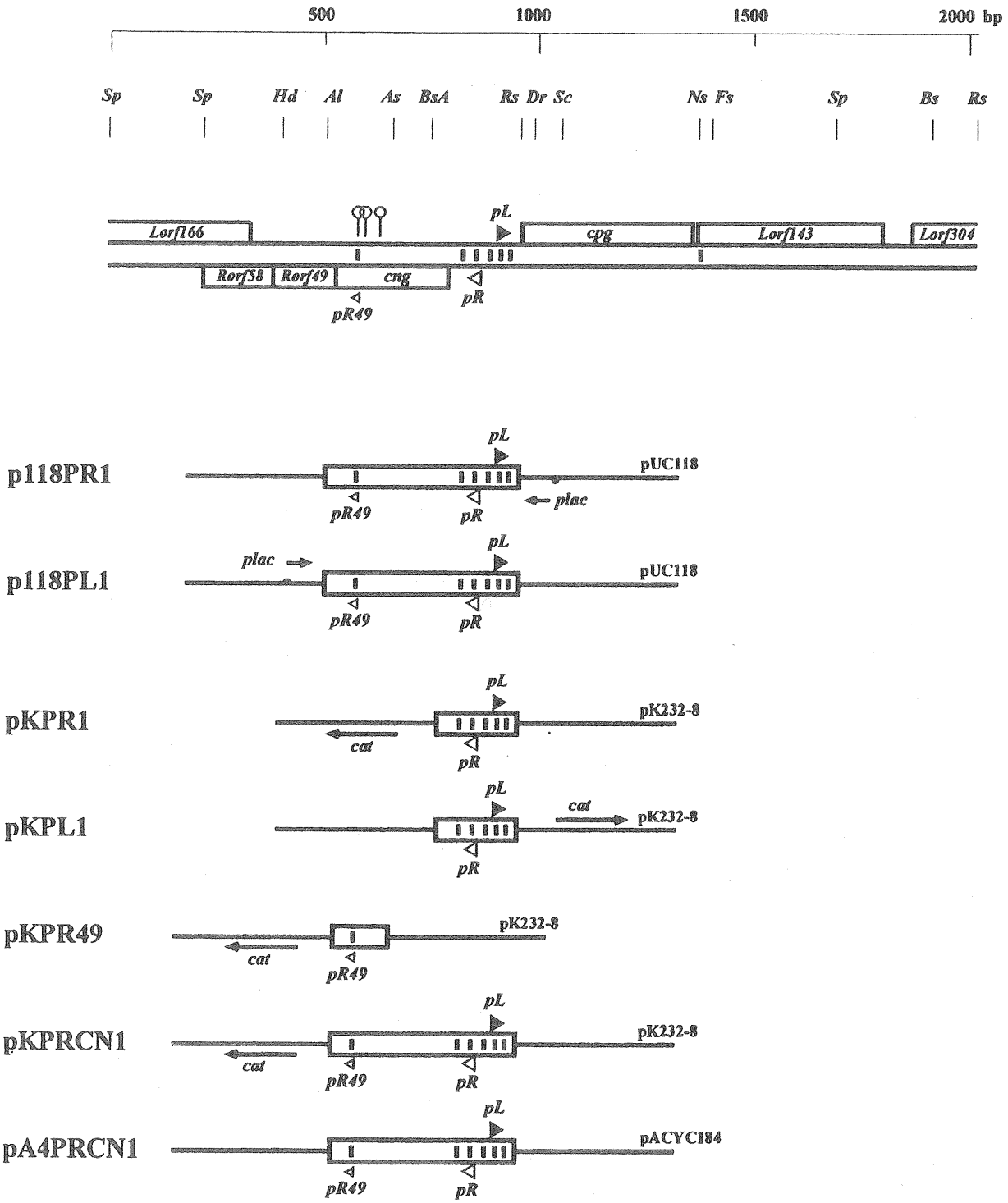



Fig. 2. Construction of the øgle promoter-containing plasmids. A 2030 bp physical map of the øgle genome is represented. øgle DNA is shown by two thick lines. The putative genes and ORFs (see Fig. 1) are indicated by open boxes with their directions. The seven GATAC-boxes (see Fig. 1) are indicated by small closed boxes. The three promoters are indicated by *pL* (closed arrowhead), *pR* (open arrowhead) and *pR49* (small open arrowhead) above or below the øgle DNA, depending on their direction. The possible secondary structures (Kodaira et al., 1997) are shown by open circles with a bar. Restriction enzyme sites are shown in the top: *Al*, *Alu*I; *Bs*, *Bsm*I; *BsA*, *Bsm*AI; *Dr*, *Dra*I; *Fs*, *Fsp*I; *Hd*, *Hin*dIII; *Ns*, *Nsp*I; *Rsa*, *Rsa*I; *Sc*, *Sac*I; *Sp*, *Sph*I. Under the physical map, the recombinant plasmids constructed (Section 3.1) are shown. øgle DNA regions are indicated by boxes, and vector plasmids are shown by thick lines. The directions of p*lac* and *cat* are shown by arrows.

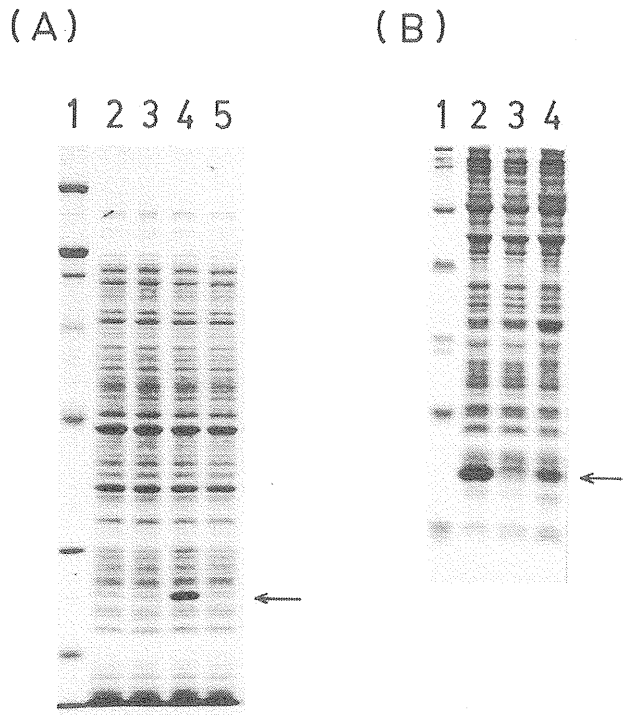



Fig. 3. Protein profiles. Cell extracts of *E. coli* XL1-Blue (A) or BL21(DE3) p*lysS* (B) carrying a plasmid were subjected to SDS–PAGE (Section 2.2). Proteins were stained with Coomassie brilliant blue R-250. (A) The samples were electrophoresed on a 12.5% SDS gel. Lane 1, markers; lane 2, pKK232-8; lane 3, pKPL1; lane 4, pKPR1; lane 5, pKPR49. Arrow: an induced band (CAT). (B) The samples were electrophoresed on a 16.5% Tricine–SDS gel. Lane 1, markers; lane 2, pE3CNG1 (after 2 h induction with IPTG); lane 3, pET-3c (2 h); lane 4, pE3CNG1 (1 h). Arrow: an induced band (Cng).

gel containing 10% glycerol at 4°C. For analysis of mRNA transcript, RNA was prepared as described by Oki et al. (1997): primer-extension analysis was followed by the method of Sambrook et al. (1989). For analysis of protein, SDS–PAGE was carried out as described by Kakikawa et al. (1996): proteins were visualized by staining with Coomassie Brilliant Blue (CBB) R-250. Tricine-SDS–PAGE was performed by the method of Ploug et al. (1989). The N-terminal amino acid sequence of the protein was determined as described by Kakikawa et al. (1996), using 490 Procise protein sequencing system (Applied Biosystems).

### 2.3. *CAT assay*

CAT activity was assayed by the method of Shaw (1975). *E. coli* carrying a plasmid(s) was grown in LB broth at 37°C. When $A_{660}$ had reached 0.5, the cells were harvested by centrifugation, and suspended in 50 mM Tris–HCl buffer (pH 7.8) containing 30 μM DTT. The cells were frozen by dry ice–ethanol bath, and thawed at 0°C for 6 min. 1/50 volume of lysozyme (15 mg/ml)/EDTA (50 mM) was added, and incubated at 0°C for 30 min. After freeze-thawing once more, cell debris was removed by centrifugation. The extracts were subjected to CAT assays.

### 2.4. *Enzymes and biochemicals*

Restriction enzymes, phage T4 DNA ligase, and reverse transcriptase (RAV-2) were purchased from Takara Shuzo (Kyoto). Buffers for each enzyme were as recommended by the manufacturer. Synthetic oligonucleotide primers for PCR and DNA sequencing are summarized in Table 2. [α-$^{32}$P]dCTP was from NEN.

## 3. Results

### 3.1. *Construction of øg1e promoter-containing plasmids*

Figs. 1 and 2 show the øg1e *cng–cpg* region (850 bp) containing the seven GATAC-boxes. Box 1 is located within *cng* (Fig. 1A), the five boxes 2–6 are between *cng* and *cpg* (Fig. 1B), and box 7 is downstream of *cpg* (Fig. 1C). The three boxes 1, 3 and 5 overlap potential promoter sequences (*pR49*, *pR* and *pL*, respectively), which share high similarity (71.6, 63.9 and 62.7%, respectively) with the *E. coli* promoter consensus (−35 and −10 sequences: Harley and Reynolds, 1987).

As summarized in Table 1 (see Fig. 2), the øg1e promoter-sequences were cloned into *E. coli* plasmids. A 456 bp *Alu*I/*Rsa*I fragment containing *cng/pR49–pR–pL* was introduced into a *Sma*I site of an *E. coli* vector pUC118, yielding p118PR1(*pR* under *plac*) and p118PL1(*pL* under *plac*). Using p118PR1, a 231 bp DNA region of *pR–pL* (containing truncated *cng* and *cpg*) was amplified by PCR using two synthetic oligonucleotide primers, CNGVI and M13-RV (Table 2). CNGV1 was arranged to convert fifth codon of *cng* from CGA(Arg) to TGA(stop codon) or GGA (Gly: changing to an uncharged residue) (Fig. 1B). The PCR product was cloned into a *Sma*I site upstream of *cat* in *E. coli* promoter-detecting plasmid pKK232-8, resulting in pKPR1(*pL–pR–cat*) and pKPL1 (*pR–pL–cat*). The cloned regions were confirmed by DNA sequencing. Expectedly, the fifth codon of *cng* in pKPR1 was changed from CGA(Arg) to TGA(stop codon), whereas that in pKPL1 was changed to GGA(Gly), consequently yielding a truncated *cng*; fortuitously, both plasmids lacked one nucleotide at the 3′-end of M13-RV, and pKPL1 (but not pKPR1) was deleted in three nucleotides (5′-GCA-3′) from nucleotide position 557–559 in øg1e DNA (Fig. 1B). To place *pR49* upstream *cat*, a 141 bp fragment of p118PR1, containing *pR49*, was cut with *Ase*I (Fig. 2) and introduced into a *Sma*I site of pKK232-8, to yield pKPR49. In addition, a 484 bp fragment containing *pL–pR–cng/pR49* was amplified with two primers M13-RV and CNGII

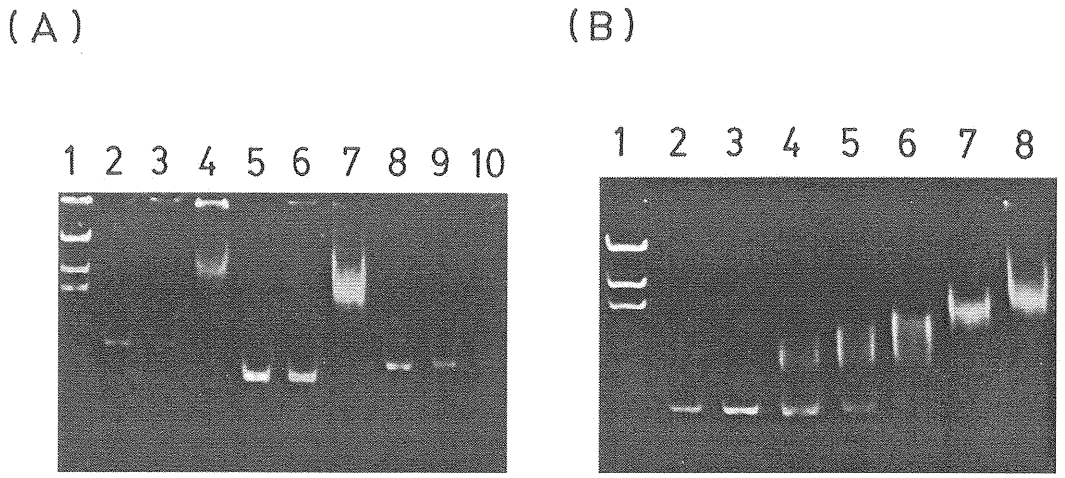



Fig. 4. Gel shift assays. Cell extracts of *E. coli* BL21(DE3)p*lysS* carrying a plasmid was incubated with DNA fragment at 0°C for 20 min, and electrophoresed on a non-denaturing 6% polyacrylamide gel (Section 2.2). The DNA bands were visualized by ethidium bromide staining. (A) Three DNA fragments used (each 1 μg) were as follows: a 300 bp *Ase*I/*Rsa*I fragment (Fig. 2), lanes 2–4; a PCR- produced 231 bp fragment using CNGVI and M13-RV (Table 2), lanes 5–7; a 269 bp *Rsa*I fragment from pUC118, lanes 8–10. Cell extracts (3 μl of 1 μg/μl of protein) used were as follows: *E. coli* BL21(DE3)p*lysS* (lanes 2, 5 and 8); *E. coli* BL21(DE3)p*lysS*/pET-3c (lanes 3, 6 and 9); *E. coli* BL21(DE3)p*lysS*/pE3CNG1 (lanes 4, 7 and 10). Lane 1, *Rsa*I fragments of pUC118 as markers. (B) A PCR-derived 231 bp fragment as in (A) was incubated with various amounts of cell extract from *E. coli* BL21(DE3)p*lysS*/pE3CNG1. Lane 1, *Rsa*I fragments of pUC118 as markers. The ratios of DNA (μg) and cell extract (μl) were as follows: lane 2, 1 μg/containing no cell extract; lane 3, 1 μg/0.5 μl; lane 4, 2 μg/1 μl; lane 5, 3 μg/2 μl; lane 6, 3 μg/3 μl; lane 7, 3 μg/4 μl; lane 8, 3 μg/5 μl).

(Table 2 and Fig. 1A) using p118PR1 DNA, and cloned into a *Sma*I site of pKK232-8, yielding pKPRCN1. As shown in Fig. 1A, the stop codon of *cng* (5′-ATGA-*3′) overlaps a start codon of the putative ORF Rorf49* (5′-ATGA-3′). Therefore, the primer CNGII was arranged to change the stop-codon of *cng* from TGA to TAA, so that this recombinant plasmid is no longer truncated in *Rorf49*. By DNA sequencing, pKPRCN1 was found to be lacking in 2 bp (5′-CA-3′) within M13-RV region.

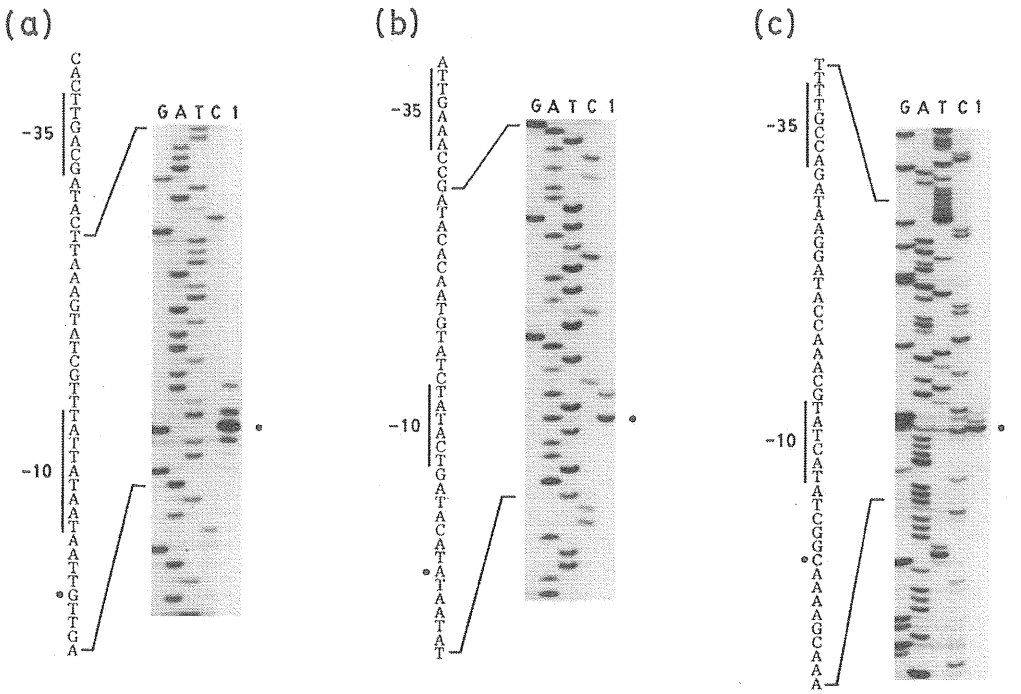
### 3.2. *Promoter activities of* pR, pR49 *and* pL

*E. coli* cells (XL1-Blue or CK111) carrying pKPR1 grew in the presence of up to 400 μg/ml of Cm. On the other hand, the upper limit of Cm resistance in *E. coli* cells containing pKPL1 or pKPR49 was about 200 μg/ml. Subsequently, CAT activities associated with pKPR1, pKPL1, or pKPR49 were assayed (Section 2.3). In *E. coli* CK111, the mean value of pKPR1 was 626.8 (units/mg), and those of pKPL1 and pKPR49 were 37.8 and 12.8, respectively, whereas that of pKK232-8 was 0.5 (Table 3). These results indicate that *pR, pR49* and *pL* function as a promoter, and *pR* has the strongest activity, at least in *E. coli*. In addition, total proteins were extracted from XL1-Blue carrying pKPR1, pKPL1, or pKPR49, and subjected to SDS–PAGE (Fig. 3A). In pKPR1, steadily increasing synthesis of a protein was detected; this overproduced protein (apparent molecular mass 26.5 kDa) was probably chloramphenicol acetyltransferase (25.7 kDa estimated from DNA sequence: Brosius, 1984).

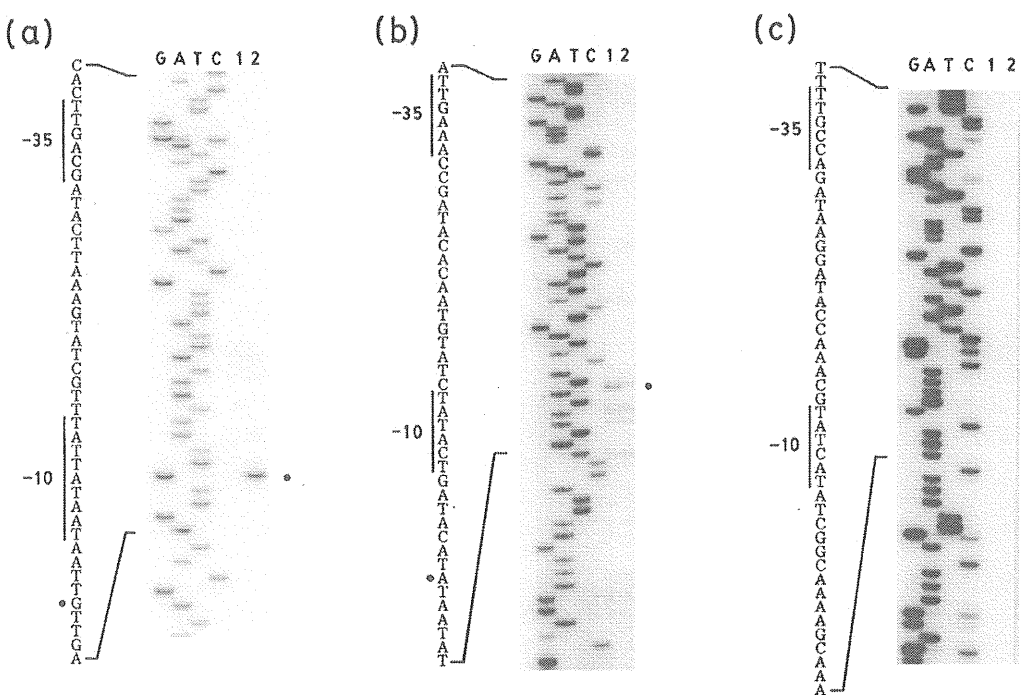
On the other hand, pKPRCN1 containing *pL–pR–cng/pR49* (Section 3.1) exhibited lower CAT activity (one-sixth of pKPR1), suggesting that *cng* probably functions as a repressor. To investigate this repressive activity of Cng, a 454 bp *Rsa*I/*Hin*cII fragment containing *pL–pR–cng/pR49* was cut from pKPRCN1, and then introduced into pACYC184 at a blunted *Eco*RI site, yielding pA4PRCN1 (*pR*: opposite to the CM gene of pACYC184). When pKPR1, pKPL1, or pKPR49 was coexistent with pA4PRCN1 in *E. coli* CK111, the CAT activities of pKPR1 and pKPL1 (but not pKPR49) were reduced significantly (Table 3). These results sug-

Fig. 5. Primer extension analyses. Total RNA was isolated from cells of *E. coli* carrying a plasmid or *L. plantarum*, and cDNA synthesized by reverse transcriptase was analyzed, along with DNA sequencing ladders, on 6% polyacrylamide gels (Section 2.2). The relevant nucleotide sequences are indicated in the left-hand margins. The major transctiption start points are represented by closed circles. The putative mRNA promoters are shown by −35 and −10. (A) Mapping of the transcription start sites from *pR* (a: lane 1), *pL* (b: lane 1) and *pR49* (c: lane 1) in *E. coli*. RNA was from *E. coli* XL1-Blue/ pKPR1 (for *pR*), XL1-Blue/pKPL1 (for *pL*), or XL1-Blue/pKPRCN1 (for *pR49*), and cDNA was synthesized with a primer (Table 2), CNGVI (for *pR*), M13RV (*pL*), or CAT-N (for *pR49*). (B) Mapping of the transcription start sites from *pR* (a), *pL* (b) and *pR49* (c) in *L. plantarum*. RNA was from *L. plantarum* G1e untreated with MMC (lane 1) or treated with MMC (final concentration: 5 μg/ml) for 2 h (lane 2). cDNA was synthesized with a primer (Table 2), CNGVI (for *pR*), PL-PE1 (for *pL*), or PR49-PE1 (for *pR49*).

(A)




(B)



gested that *cng* can repress *pR* as well as *pL*, although there remains a possibility that the 5′-end and the 5′-untranslated region of the pACYC184 *cat* gene may interfere with *cat* expression from the pKK232-8 derivatives.

### 3.3. Overproduction and DNA-binding of Cng

To determine whether *cng* actually directs synthesis of the protein Cng, a 267 bp region including *cng* was amplified by PCR using primers CNG I and II (Table 2), which carry additional *Nde*I and *Bam*HI sites, respectively. The PCR fragment was introduced into *E. coli* vector pET-3c at the *Nde*I/*Bam*HI sites, yielding pE3CNG1. Primary structure of the cloned region was confirmed by DNA sequencing. *E. coli* BL21(DE3)-p*lysS* carrying pE3CNG1 grew almost normally at 37°C in LB broth, whereas BL21(DE3)/pE3CNG1 could not multiply (Cng might be toxic in *E. coli*).

After induction by IPTG (1 mM) at 37°C, total proteins were extracted from *E. coli* BL21(DE3)-p*lysS*/pE3CNG1, and subjected to Tricine–SDS–PAGE (Section 2.2). pE3CNG1 directed steadily increasing synthesis of a protein (Fig. 3B); its apparent molecular mass of about 10.5 kDa corresponds well with that of Cng (10.1 kDa) predicted from DNA sequence (Kodaira et al., 1997). This protein was not detected in BL21(DE3)p*lysS*/pET-3c. N-Terminal amino acid sequence of the overproduced protein (Section 2.2) showed that the amino acid alignments, MKRERLIAE, completely coincided with that predicted from DNA sequence (Fig. 1B).

To clarify whether Cng is able to bind the GATAC-boxes specifically, gel-shift assays (Section 2.1) were performed. When cell-free extract of *E. coli* BL21(DE3)p*lysS*/pE3CNG1 was added to a 300 bp *Ase*I/*Rsa*I DNA fragment (Fig. 4A: lanes 2–4) or a 231 bp PCR-produced DNA fragment (Fig. 4A: lanes 5–6) containing five GATAC-boxes (2–6), mobility of these DNA fragments was lowered. In addition, the mobility of the 231 bp fragment shifted along with increase in Cng content (Fig. 4B).

### 3.4. Transcription initiation sites from pR, pL and pR49

The transcription start points from *pR, pL* and *pR49* were determined by primer extension analysis in *E. coli* as well as *L. plantarum*. Fig. 5A shows the results in *E. coli*. From *pR* (Fig. 5Aa), four 5′-ends of the transcripts were detected: the major start point was mapped at position 616(G), and the other three minors were at positions 615(T), 617(T) and 619(A). From *pL* (Fig. 5Ab), two initiation sites were mapped: the major one at position 718(A) and the minor at 716(A). On the other hand, *pR49* (Fig. 5Ac) exhibited three start points: the major was at position 351(C), and the two minors were at 352(G) and 353(G).

As reported previously (Kodaira et al., 1997), øg1e is a lysogenic phage of *L. plantarum* G1e, and is inducible with MMC or UV. Primer extension analyses were thus performed in the G1e cells. Without MMC treatment, one major transcript from *pL* was detected, and no substantial signals from *pR* or *pR49* were obtained (Fig. 5B). The start point from *pL* was mapped at position 718(A) (Fig. 5Bb: lane 1), corresponding with that (A) in *E. coli* (Fig. 5Ab). When the G1e cells were treated with MMC for 2 h, a unique transcript from *pR* emerged, but no transcripts were detected from *pL* or *pR49* (Fig. 5B). The start site from *pR* was mapped at position 616(G) (Fig. 5Ba: lane 2), coincident with that (G) in *E. coli* (Fig. 5Aa).

## 4. Discussion

In the present study, the putative promoters *pR, pL* and *pR49* of *L. plantarum* phage øg1e were investigated. In the *E. coli* system: (i) the øg1e sequences *pR, pL* and *pR49* probably functioned as promoters, (ii) *cng* directed a protein Cng with an apparent molecular mass of 10.5 kDa on a SDS gel, and (iii) Cng specifically bound to a DNA region containing the GATAC-boxes. In addition, primer extension analyses demonstrated that the two potential promoters *pR* and *pL* work well in *L. plantarum* as well as in *E. coli*, suggesting that they function for the lytic and lysogenic pathways, respectively. Although *pR49* indicated a promoter-like function in *E. coli*, it may not play an important role in *L. plantarum*. Thus, these results suggest that the *cng–cpg* region of øg1e is involved in lysis–lysogeny decision, and Cng (and Cpg) may act as a repressor, presumably on the GATAC-boxes.

A 15 bp consensus of the GATAC-boxes, 5′-GATAC$(N)_5$GTATC-3′, shows no resemblance to those of other predicted operators of the *Lactococcus* phages r1t (Nauta et al., 1996) and BK5-T (Boyce et al., 1995). In general arrangement, øg1e boxes 1 and 7 somewhat resemble operators of coliphage 186 (Neufing et al., 1996), FR and FL, respectively; FR is located downstream of a repressor gene *apl* directing the lysis pathway, whereas FL is within another repressor gene *cI* directing the lysogenic pathway (Dodd and Egan, 1996). Additionally, *pL* of øg1e resembles promoters controlling lysogenic life-cycles of the coliphage 186 (Neufing et al., 1996) and Mu (van Ulsen et al., 1996), which are weaker than their promoters for the lytic cycle.

Preliminary experiments have shown that, like *cng*, *cpg* directs a protein, whose apparent molecular mass on a SDS-gel is about 16.0 kDa, corresponding well with that (15.1 kDa) estimated from DNA sequencing (unpublished data). As in coliphages such as lambda

and 186, the øg1e lysis–lysogeny switch might be under a complicated control. Further studies on structure, expression and function of the øg1e regulatory region, *cng–cpg*, are now in progress.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