Journal of Microbiology and Biotechnology
Vol. 3, No. 3, 146-155, 1993

Structure and Regulation of a Complex Promoter Region from an Alkali-tolerent *Bacillus* sp.

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A DNA fragment from an alkali-tolerent *Bacillus* sp., conferring strong promoter activity, was subcloned into the promoter probe plasmid pPL703 and the nucleotide sequence of this promoter region was determined. The sequence analysis suggested that this highly efficient promoter region containing the complex clustered promoters comprised three kinds of promoters (P1, P2 and P3), which are transcribed by σ^B(formerly σ^{37}), σ^E(formerly σ^{29}) and σ^A(formerly σ^{43}) RNA polymerase holoenzymes which play major rules at the onset of endospore formation, during sporulation and at the vegetative phase of growth, respectively. S1 nuclease mapping experiments showed that all three promoters had staggered transcription initiation points. The results of chloramphenicol acetyltransferase assay after the subcloning experiments also indicated that the expression of these clustered promoters was correlated with the programs of growth and endospore development. Promoter P1, P2 and P3 were preceded by 75% AT, 79% AT and 81% AT regions, respectively, and a partial deletion of AT-rich region prevented transcription from promoter P1 *in vivo*. Two sets of 5'-AGTGTT-3' sequences and inverted repeat sequences located around the promoter P1 were speculated as the possible *cis* acting sites for the catabolite repression in *B. subtilis*. *In vivo* transcripts from these sequence regions may be able to form a secondary structure, however, the possibility that a regulatory protein induced by the excess amount of glucose could be bound to such a domain for crucial action remains to be determined.

Sporulation in procaryotes, which is triggered by nutritional deprivation, derives the activation of numerous developmental sets of genes located at many chromosomal positions, and its process involved in drastic changes in the biosynthetic activities within the cell is basically controlled at transcription level by the RNA polymerase sigma subunits (σ) (20). The sigma subunit in *Bacillus subtilis*, which is bound to the catalytic core moiety (αββ') of RNA polymerase, recognizes specifically the corresponding promoter sequence during differentiation of vegetative cells into endospores (6). Since the pioneering works, at least nine different sigma subunits have been known to be produced in *B. subtilis* (13, 16, 19, 42). However, the exact mechanism for molecular switching of sigma subunits remains unclear.

In addition to the primitive example of cellular differentiation, *Bacilli* have played important roles in the industrial fields because of its ability to secrete proteins into the medium (34). One of the elements to improve the productivity of the heterologous protein in *B. subtilis* is the promoter employed to drive heterologous gene expression. In order to express heterologous genes by using the promoter, it is essential to understand the efficiency and regulation of the promoter in *B. subtilis*. Recently, several promoter regions of tandem (30, 41) or overlapping transcription (11, 27, 43) have been found on several genomes of *B. subtilis*. This type of promoter would enable a gene to extend expression periods during the stationary phase of growth.

We had isolated several alkalophiles which produce various useful enzymes and polymers (3, 4, 12, 18, 38, 49, 56), and some of the genes encoding these enzymes were cloned in *E. coli* (15, 29, 31, 48, 53, 54, 57, 58). In order to improve the expression of these genes in *Bacil-*

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Key Words: *Bacillus* sp., complex promoter, regulation

lus host strain, we tried to use the strong promoter of alkali-tolerent *Bacillus* sp. (55). A 2.7 kilobases(kb) DNA fragment with efficient promoter activity in the presence of chloramphenicol was recently cloned by using promoter probe plasmid pPL703, which has a *cat-86* gene coding for chloramphenicol acetyltransferase (CAT) as a reporter gene, and the active promoter region was located through a series of size reductions(14, 32, 33, 50-52). The present paper deals with the nucleotide sequence of the promoter region, and its structure for developmental and nutritional control of the gene expression in *B. subtilis*.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

Escherichia coli JM109(r_k^-, m_k^-, *rec*A1, *end*A1, *gyr*A 96, *thi*, *hsd*R17, *sup*E44, *rel*A1, λ^-, Δ(*lac-pro*AB)/F', *tra*D36, *pro*AB, *lac*I^q ZΔM15) (46) was used as a recipient for bacteriophage M13mp vectors. Alkali-tolerent *Bacillus* sp. YA-14 (Yonsei University stock) (55) was used as the donor of the promoter-containing DNA fragments. *Bacillus subtilis* host strain 207-25(r_k^-, m_k^-, *amy*E07, *hsr*M, *aro*I906, *lys*21, *leu*A8, *rec*E4) (39) was used for routine cloning and RNA isolation experiments. The sporulation-deficient *Bacillus subtilis* strain IS24 (*phe*A1, *trp*C2, *spo*OH81) and strain IS61(*rpo*B2, *spoIIG* 55, *trp*C2) were kindly provided by *Bacillus* Genetic Stock Center(Ohio State University, Columbus, USA).

Plasmid pPL703 (26) was used as the promoter cloning vector and plasmid pPL708 (25) was used as a control for comparing of CAT expression.

Media and Genetic Methods

E. coli cells were cultured on 2YT broth(16 g Bacto-peptone [Difco Laboratories, Detroit, Mich.], 10 g yeast extract [Difco], 5 g NaCl, in 1 liter distilled water, pH 7.5) to produce single-stranded and replicative form(RF) DNA. *E. coli* transformants were selected on the YT agar broth(8 g Bacto-peptone [Difco], 5 g yeast extract [Difco], 5 g NaCl, in 1 liter distilled water, pH 7.5, 15 g Bacto-agar [Difco]) and 3 m*l* of YT top agar containing 10 μ*l* of isopropyl-β-D-thiogalacto-pyranoside (IPTG) (Sigma Chemical Co., St. Louis, Mo), and 50 μ*l* of 2% 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) (Sigma Co.).

Transformation selections for drug resistant *B. subtilis* strains were performed on DM-3 medium (2) containing 500 μg of kanamycin per m*l*. Screening of strains harbouring promoter and the strength of the cloned promoters were detected on Tryptose blood agar base (Scott Laboratories, INC, Fiskeville, Rhode Island) supplemented with 200 μg of chloramphenicol(CM) and 5 to 250 μg of CM per ml, respectively, and scored for visible growth after 24 h of incubation at 37℃. The chloramphenicol acethyltransferase (CAT) activities of *B. subtilis* transformants were determined on 2XSSG (modified Schaeffer's medium) (10) without or with 1.0% (W/V) glucose.

Bacteriophage RF DNA was isolated from *E. coli* by the method of Messing *et al.* (24). The rapid preparation of plasmid DNA from *B. subtilis* was performed by the method of Zaghoul *et al.* (59).

Random Primed DNA Labelling and Hybridization

Probe DNA was labelled with [α-^{32}P]dCTP (Amersham Co.) by using a random primer labelling kit (U.S. Biochemcals Co.). The transfer of DNA to a nitrocellulose filter and DNA-DNA hybridization were performed by the method of Southern (40).

DNA Sequencing and Computer Analysis

The entire nucleotide sequence of the 920 bp promoter fragment was determined by Sanger's dideoxy chain termination procedure (36). The DNA fragments were subcloned into the multi-cloning site of M13mp vectors. Recombinant M13 phage growth, DNA preparation, and DNA chain termination sequencing with sequenase enzyme (U.S. Biochemicals Co.) and [α-^{35}S]dATPαS(Amersham Co.) were performed according to the protocols given by U.S. Biochemicals. Electrophoresis was carried out on a 6% polyacrylamide/8M urea gel. The sequences were read from both strands, and each sequence was read on an average 4 times in each directions. Sequence data were analyzed by the Pustell DNA analysis program (IBI, New Haven, CT).

CAT Assay

B. subtilus cells were grown in 2XSSG medium at 37℃ with vigorous shaking until the culture reached an OD_{550} of 0.5, at which time the culture was diluted sixe fold in 2XSSG medium, and incubation was continued. Samples were harvested by centrifugation at selected intervals during growth and sporulation. CAT activities were determined spectrophotometically by the method of Shaw (37). The protein concentration was determined by the method of Bradford (1) with bovine serum albumin (Sigma Co.) as standard. One unit of enzyme activity was defined as the amount of enzyme which acetylate 1 μmol chloramphenicol per min. per mg protein.

Isolation of RNA

Total cellular RNA was extracted from *B. subtilis* 207-25 cells harbouring promoter clones by the method of Duvall *et al.* (8) with some modifications. The bacteria were cultured in sporulation medium containing kanamycin (10 μg/m*l*) and chloramphenicol(10 μg/m*l*) at 37℃ with vigorous shaking for 12 hr, after three logarithmic transfers as described above. The cells were harvested

by centrifugation (8000 × *g*, 10 min) and then washed three times with cold 0.01 M Tris hydrochloric acid buffer(pH 8.0) containing 10 mM ribonucleoside-vanadyl complex (Sigma Co.). Cells were lysed in the presence of lysozyme (200 μg/m*l*) at 37℃ for 10 min, and further disrupted by adding 0.5 %(W/V) sodium dodecyl sulfate. After 2 min at 95℃, the lysate was treated with hot phenol(65℃) and then extracted twice with phenol-chloroform (1:1) and once with chloroform. The nucleic acids collected by ethanol precipitation were suspended in DNase digestion buffer (200 mM Tris-HCl [pH 8.0], 10 mM $MgCl_2$) and incubated with 50 μg/m*l* of RNase-free DNase I(Sigma Co.) at 37℃ for 60 min. The RNA fraction was then extracted with phenol and chloroform, followed by precipitation with cold ethanol, and the final pellet was dissolved in H_2O. The concentration of total RNA was normalized on the basis of its A_{260} (22).

S1 Nuclease Analysis

For the preparation of the probe, M13mp18-Δf1 RF DNA was digested with *Nsi*I and dephosphorylated with calf intestinal alkaline phosphatase. The fragment(231 bp) was electroeluted from a 0.5% polyacrylamide gel and the 5'-ends were ^{32}P labeled by using [γ-^{32}P]ATP (Amersham Co.) and T4 polynucleotide kinase(Boeringer Manheim Biochemicals) as described by Maniatis *et al.* (22). S1 nuclease mapping was performed by the method of Gilman and Chamberlin (9). The single-stranded probe was annealed to the total RNA (50 μg) isolated from *B. subtilis* 207-25 carrying plasmid p12BSΔf1 at the stage of sporulation, and digested with S1 nuclease. The S1 nuclease resistant DNA fragments were resolved by 6% polyacrylamide gel electrophoresis in 50% urea.

Nucleotide Sequence Acession Number

The 0.9 kb DNA sequence has been submitted to the EMBL Data Library under Acession Number X60 072.

RESULTS

Localization of the YA-14 Promoter Region

Plasmid p12BS derivatives were constructed, based on the restriction sites derived from nucleotide sequence (Fig. 1). First, a 0.5 kb *Mbo*I(the last site from 5' end)-*Sal*I fragment was ligated to the promoter probe plasmid pPL703, linearized by *Bam*HI and *Sal*I digestion , and transformed into protoplasts of *B. subtilis* 207-25. Recombinant plasmid DNA p12BSΔf1 was isolated from the chloramphenicol resistant transformants. To construct plasmid p12BSΔf2, the 0.4 kb fragment resulting from *Eco*RI digestion and *Mbo*I partial digestion was subcloned into plasmid pPL703 cleaved with *Eco*RI and *Bam*HI. Expression of indicator *cat-86* gene of the vector by the original orientation of the inserts indicates that all three promoters lies in plasmid p12BSΔf1 (Fig. 1). The 0.5 kb DNA insert of plasmid p12BSΔf1 was further subcloned into plasmid pPL703 to locate the individual promoters. Plasmid p12BSΔf3 containing both promoter P2 and promoter P3 was constructed as follow. Plasmid p12BS was digested with *Hin*dIII, and the 0.92 kb DNA fragment isolated from agarose gel was inserted into the *Hin*dIII site of M13mp18. From a correct clone identified by DNA sequencing, the 0.58 kb DNA fragment was then released by the *Eco*RI and *Bcl*I double digestion. Purified DNA fragment was ligated to the linearized plasmid DNA pPL703 which is deleted 0.23 kb *Eco*RI-*Bcl*I DNA fragment. Multicloning sites of plasmid p12 BSΔf3 generated from M13mp18 vector was used to construct plasmid p12BSΔf4 containing promoter P3. The 0.5 kb *Eco*RI-*Pst*I DNA fragment carrying the promoter regions of plasmid p12BSΔf1 was purified by electroelution and subsequently digested with *Aha*III. The 0.3 kb *Aha*III-*Pst*I DNA fragment was introduced into the large DNA fragment of plasmid p12BSΔf3 at

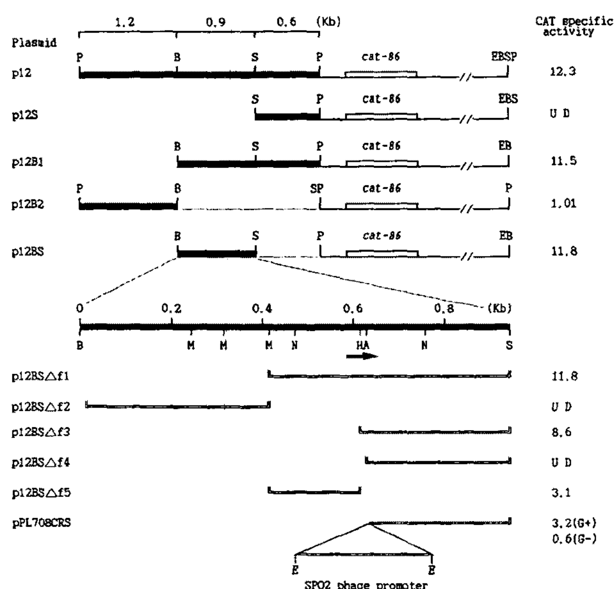

Fig. 1. Structure of plasmid p12 and its derivatives, and localization of the YA-14 promoter region.
Black bars indicate the DNA from alkali-tolerent *Bacillus* sp. Cleavage sites of *Aha*III, *Bam*HI, *Eco*RI, *Hin*dIII, *Mbo*I, *Nsi*I, *Pst*I, and *Sal*I are presented by A, B, E, H, M, N, P, and S, respectively. The position and direction of the YA-14 promoter are indicated by the arrow. CAT specific activities(shown as U/mg of protein in the right column) of plasmids except pPL 708CRS were determined from the cells which were cultured in 2XSSG medium for 12 h at 37℃. CAT specific activity of pPL708CRS was grown in the same medium for 5 h at 37℃ in the presence of 1% glucose (G+) or in the absence of the glucose (G−). UD, undetectable.

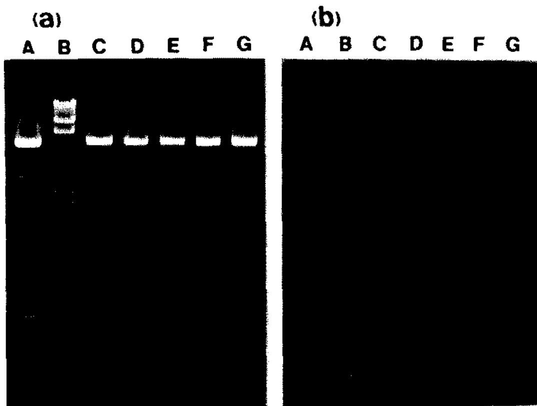

Fig. 2. Southern hybridization of plasmid p12BS and its derivatives.

The DNAs digested with *Eco*RI and *Pst*I were electrophoretically seperated and transfered onto nitrocellulose paper. Probe used for hybridization was the 0.5 kb *Eco*RI-*Pst*I fragment of p12BSΔf1. Panel (a) and (b) indicate the ethidium bromide-stained agarose gel pattern and autoradiogram, respectively. Lanes: A, p12BSΔf2; B, λ DNA digested with *Hin*dIII as markers; C, p12BS as a positive control; D, p12BSΔf1; E, p12BSΔf3; F, p12BSΔf4; G, p12BSΔf5.

*Sma*I and *Pst*I sites. Plasmid p12BSΔf5 containing promoter P1 was constructed by using plasmid pPL718 to which the large DNA fragment of plasmid p12BSΔf3 was religated. The linearized plasmid pPL718 by *Eco*RI and *Sma*I digestion was ligated to the 0.2 kb *Eco*RI-*Aha*III DNA fragment. The plasmid constructed was identified through southern hybridization (Fig. 2) and diagnostic DNA sequencing across (data not shown).

Nucleotide Sequence Analysis of the YA-14 Promoter Region

The map of plasmids constructed from the subcloning of p12 into pPL703 is shown in Fig. 1. CAT levels of the resulting plasmids p12S, p12B1, p12B2 and p12BS indicated that the 0.9 kb *Bam*HI-*Sal*I fragment, designated as YA-14 promoter region, contains functional sequences necessary for the transcriptional activation of *cat-86* gene(Fig. 1), and therefore we sequenced YA-14 promoter region.

The nucleotide sequence of the 920 bp promoter-containing segment is shown in Fig 3. Numerals of the nucleotide sequences in the right column were counted from the *Eco*RI site as +1. An incomplete open reading frame(ORF) for a cryptic protein was identified starting at approximately position 740. The sequence 5′-GGGG-GTG-3′ which occured 11 nucleotides upstream from a possible ATG start codon at position 755, showed high complementarity to the 3′ end of the 16S ribosomal RNA of *Bacillus* (23) and therefore it is presumed to

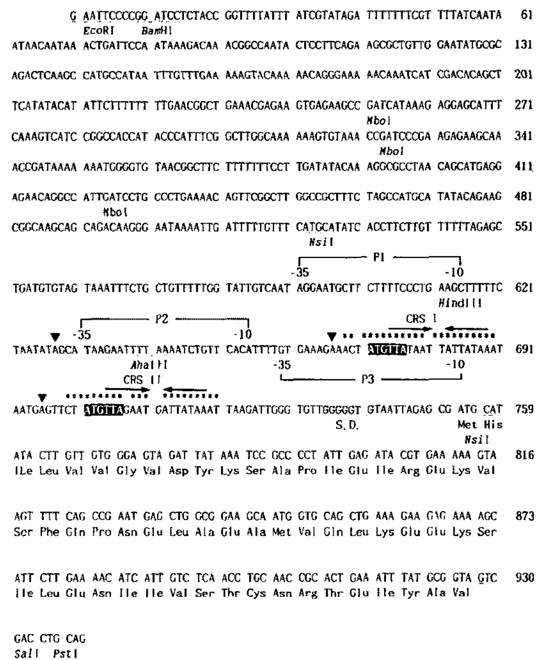

Fig. 3. The nucleotide sequence of non-transcribed strand of the YA-14 promoter region.

Numerals of the nucleotide sequences in the right column counted from the *Eco*RI site. Transcription occurs from left to right with the start-point of transcription indicated as arrow head (▼). The converging arrows above the sequence and reverse fonts identify the IR sequences and conserved sequence that may be subject to the catabolite repression, respectively (CRS I and CRS II). A series of astricks represents 22 bases repeating sequence except 2 bases containing putative catabolite repression sites. The −10 and −35 regions of promoter P1, P2 and P3 which are recognized by $E\sigma^B$, $E\sigma^E$ and $E\sigma^A$ are indicated in the shadowed box, respectively. Restriction enzyme sites and Shine Dalgarno (SD) sequence are underlined.

have the potential to function as a ribosomal binding site. No other likely protein coding sequences were identified. The predicted amino acid sequence of the cryptic protein was compared with the protein data base, Protein Identification Resource(PIR) of National Biomedical Research Foundation(Geogetown University Medical Center, Washington, D.C.). No significant homologies to any listed proteins were detected.

The YA-14 Promoter Region is Composed of Complex Promoters

In order to determine the number and types of promoters actually present in 920 bp DNA fragment, homology comparison with the known conserved region of promoters recognized by the various *B. subtilis* RNA polymerase was carried out using IBI DNA analysis program. Close to the proposed Shine-Dalgarno (SD) sequence, we observed several sets of sequences similar

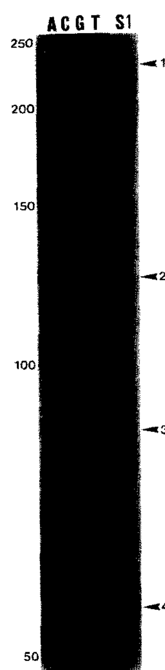

Fig. 4. Transcription initiation sites mapping of the YA-14 promoter by S1 nuclease analysis.
RNA was isolated from stationary phase cultures of *B. subtilis* containing plasmid p12BSΔf1. The protected bands by the transcript of promoter P1, P2 and P3 are shown with arrow head 2, 3 and 4, respectively. The undigested probe is seen near the top of the gel (arrow head 1). The sequence ladder of M13mp18 DNA serves as the size marker for the protected bands.

to the consensus sequence for σ^A-dependent promoter (P3). The sequence 5'-CACATTTT-3' which loosely overlapped with the −35 region of putative promoter P3 and the sequence 5'-GCATAAGA-3' separated by 15 nucleotides upstream from the above sequence showed a high similarity to the consensus sequence of the −10 and −35 region for σ^E-dependent promoter (P2). Another possible promoter region existed tandemly, 10 bp upstream of putative promoter P2. The sequences of 5'-AATAGGAAT-3' (−35 region) and 5'-GAAGCTTT-3' (−10 region) corresponded closely to the conserved recognition sequences for the σ^B-dependent RNA polymerase (P1). No other possible promoter sequences were identified in the YA-14 promoter region. To confirm the presence and the precise location of the promoters, S1 nuclease mapping was carried out using procedure of Gilman and Chamberlin (41). The result is presented in Fig. 4. The 231 bp *NsiI* fragment labelled on the 5' terminus was used in this experiment with RNA isolated from *B. subtilis* 207-25 containing plasmid p12BS, 12 h after initiating the main culture described in Materials and Methods, followed by S1 nuclease digestion,

Table 1. The putative promoter sequences in the YA-14 promoter region.

RPase*	−35 region	Spacing (bp)	−10 region	Reference
$E\sigma^B$	AGGATT	13-18	GAATTNTT	(55)
	AGGAAT	13	GAAGCTTT	
$E\sigma^E$	GAANAANT	13-16	CATATTNT	(51)
	GCATAAGA	14	CACATTTT	
$E\sigma^A$	TTGACA	17-19	TATAAT	(5)
	TTGTGA	18	TATTAT	

*RPase; RNA polymerase holoenzyme. Underlined types represent conserved promoter sequences recognized by the corresponding RPase.

yielded three major protected bands, which corresponded to the transcripts start at approximately position 628 (P1), 667 (P2) and 696 (P3). These +1 initiation sites for transcription indicated that the YA-14 promoter region is regulated by three kinds of promoters, σ^B, σ^E and σ^A promoter. Comparison between the putative promoter sequences and known consensus sequences are summarized in Table 1.

Expression of the *cat-86* gene by the YA-14 Promoter Region During Development

In order to compare the relative promoter strength, the four clones were plated on the TBAB agar plates containing 0.1, 1.0, 5.0, 10, 30, 50, 100, 200 and 250 μg chloramphenicol per m*l*(data not shown). A subclone carrying p12BSΔf1 showed the same chloramphenicol resistance as the clone carrying p12BS up to 250 μg per m*l*, indicating that p12BSΔf1 preserves all sequence nessesary for a full expression of indicator *cat-86* gene. Clones carrying p12BSΔf3 and p12BSΔf5 grew well on plates which were added with up to 200 μg and 30 μg chloramphenicol per ml, respectively. However, a clone carrying p12BSΔf4 failed to grow on plates added with more than a subinhibitory concentration of 0.1 μg chloroamphenicol per m*l*. This differential expression of four clones was studied further by doing a quantative liquid assay of CAT from cultures in the sporulation medium at various growth stages.

B. subtilis 207-25 strains harbouring p12BS derivatives were grown at 37℃ in 2XSSG media in the absence of glucose. Periodically, 0.5 m*l* samples were withdrawn, disrupted and assayed for CAT and total protein. The apperance of refractile spore and heat resistant spore were detected by phase-contrast microscope and heat-treatment method (21), respectively. The expression patterns shown in Fig. 5(a) were obtained. The promoters in plasmid p12BSΔf1 were actively expressed during growth, and their activity increased rapidly when the cell reached at the sporulation phase and reached maxi-

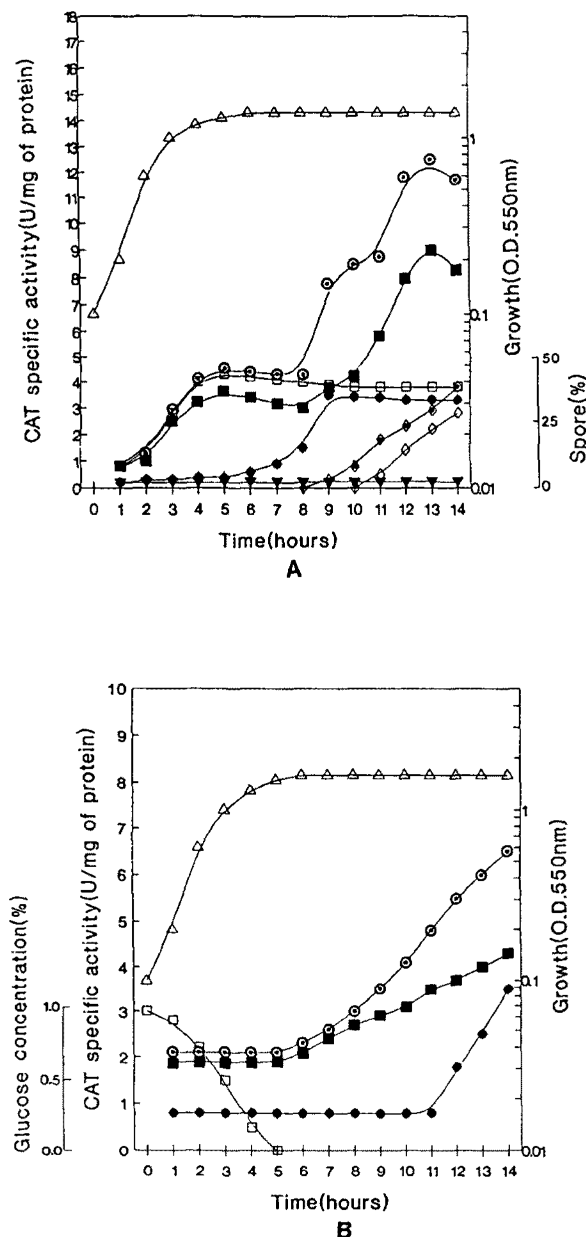

Fig. 5. Regulation of chloramphenicol acetyltransferase (CAT) synthesis during growth and sporulation of *B. subtilis* strains harbouring p12BS derivatives (A) in the absence of the glucose (B) or in the presence of 1% glucose.

Symbol ⊙: p12BSΔf1, ■: p12BSΔf3, ▼: p12BSΔf4, ●: p12 BSΔf5, or □: pPL708. All strains were grown at 37℃ in sporulation medium after three logarithmic growth transfers. Periodically, 0.5 ml samples were withdrown, disrupted, and assayed for CAT and total protein. The appearence of refractile spore (▣) and heat-resistant spore (◇) were determined by phase-contrast microscope and heat-treatment at 80℃ for 15 min, respectively. Growth (△) was measured by absorbance at 550 nm.

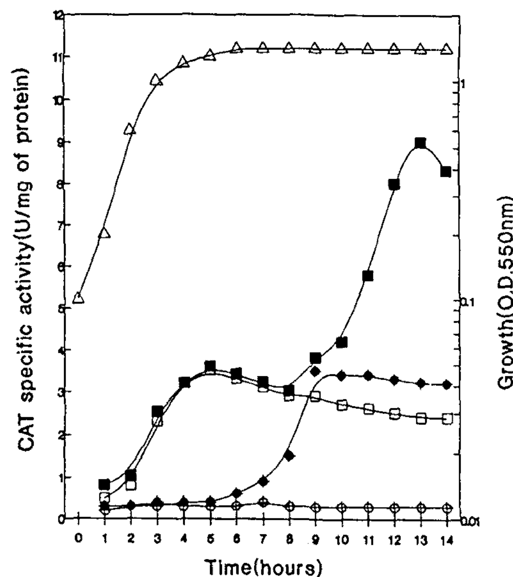

Fig. 6. The effect of *spoIIG* and *spoOH* mutations on expression of *cat*-86 gene in *B. subtilis* strain IS61 and strain IS24 harbouring p12BSΔf3 and p12BSΔf5, respectively.

Strain IS61(*spoIIG*) cells harboring p12BSΔf3(□) and strain IS24(*spoOH*) cells harboring p12BSΔf5(○) were grown in sporulation medium at 37℃. Sample were withdrown at hourly intervals and assayed for CAT and total protein. The spore-positive transformants of p12BSΔf3 (■) or p12BSΔf5 (●) were similarly grown and assayed for CAT.

mum around 9 h after the end of the exponential growth. The rapid increase in the CAT began at approximately the same time when internal spores were first detected in the culture. The time course of CAT expression for p12BSΔf1 indicated that the 0.5 kb gene contains three kinds of promoters(P1, P2 and P3). This result was strongly supported by the expression patterns for p12BSΔf3 and p12BSΔf5. The maximum activities of the P1 and P3 promoter were comparatively similar. In contrast, the P1 promoter in p12BSΔf4 was not expressed at all during growth. During CAT expressions using *spo*OH and *spoII*G mutants, the presence of P1 and P2 promoters were indirectly confirmed, respectively (Fig. 6).

Catabolite Repression of the YA-14 Promoter Region

It has been known that the expression of many developmental processes related genes are subject to catabolite repression in *B. subtilis* (7). To test this phenomenon, regulation of the YA-14 promoter region was investigated from bacterial cultures grown in the presence of a readily metabolized carbon source, glucose, throughout the growth cycle. *B. subtilis* 207-25 harboring plasmid p12BSΔf1, p12BSΔf3 or p12BSΔf5 was grown in 2

XSSG with the addition of 1 % glucose. Culture samples were removed, and the specific CAT activity was measured. The consumption of glucose was determined by Industrial Glucose/Etanol Analyzer (Model 24, YSI Co.).

Promoter activities by the cloned gene on p12BSΔf1 and p12BSΔf3 were repressed in similar manner until the glucose present in the medium was exhausted, and then the activities increased slowly in comparison to the activity in non glucose medium (Fig. 5(b)). The same results were obtained when LB and LB plus 1% glucose were used as nonrepressing and repressing growth media, respectively(data not shown). Laoide *et al.* (17) have confirmed that this effect has nothing to do with plasmid copy number. In the case of promoter expression by plasmid p12BSΔf5, there was a sharp increase in CAT activity 6 h after the glucose was exhausted in the medium, indicating that the delayed expression of promoter P1 arised due to the postponed sporulation caused by the addition of the glucose to the medium.

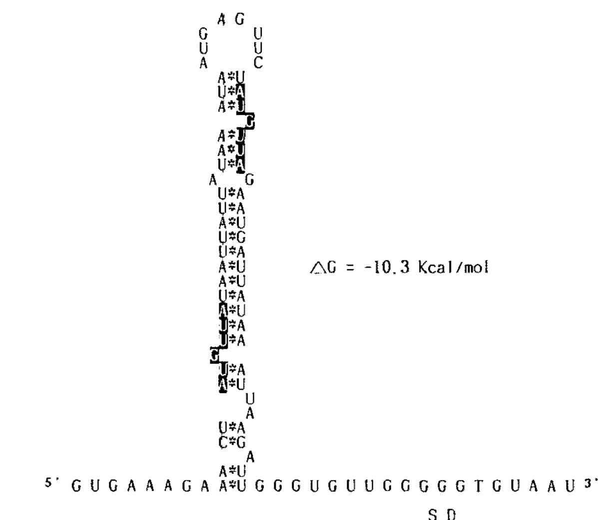

Fig. 7. Potential secondary structure around 5'-end mRNA containing the putative CRS.
The conserved sequence for CRS indicates as reverse font and SD sequence is underlined.

DISCUSSION

DNA sequencing analysis and S1 nuclease mapping showed that the YA-14 promoter region is composed of three kinds of promoters recognized by σ^B, σ^E and σ^A RNA polymerase holoenzyme and their transcription initiated from the different site. Many complex promoters which were organized tandemly (30, 41) or overlappingly (11, 27, 43, 44) have been observed in *B. subtilis* genes. Doi (5) has suggested that these promoters have the possibility of being used temporally, simultaneously or sequencially. When compared with above promoters, the promoters residing in the YA-14 promoter region revealed an interesting feature, that is, the most upstream promoter P1 existed tandemly, at the upstream of loosely overlapped promoters composed of promoter P2 and P3 (Fig. 3). Localization experiments of each promoter and CAT expression of the constructed clones indicate that the 0.5 kb DNA portion was neseccary for full expression of indicator *cat-86* gene, and promoter P1 and P2 were utilized highly at the onset of sporulation and during sporulation, respectively. However, the clone containing only promoter P3 was not expressed at all during growth. A total abolishment of CAT specific mRNA levels of this clone was confirmed by dot blot analysis (data not shown). These data suggest that an important DNA region responsible for the transcription from promoter P3 was deleted. Moran *et al.* (28) have shown that a very strong σ^A promoter for the *veg* gene is preceded by a very AT rich region. They have suggested by the deletion analysis that this region is essential for efficient utilization of *B. subtilis* promoters and considered it as a transcription enhancing region. So far, several promoters which are rich in AT bp upstream from the −35 region have been known (27, 30, 41, 45, 47). Promoter P3 was preceded by AT rich regions of 81% (35 A or T out of 43 bp). Accordingly, it is conceivable that the abolishment of the detectable promoter activity of plasmid p12BSΔf4 may be closely related to the loss of two-thirds of AT rich portion of the upstream member of the −35 region when promoter P3 is subcloned. The DNA upstreams of the −35 regions of promoter P1 and P2 were also characterized by AT contents of 75% and 79%, repectively, that were in excess of the value of 67% for promoter P1s of *rrnA* or *rrnO* (30).

Expression of the YA-14 promoter region was subjected to the catabolite repression by glucose. Laoide *et al.* (17) have elucidated that the catabolite repression of the gene occurred independently of its own promoter and irrespective of the distance between the structural gene and the promoter that is transcribing it. They also have suggested that the putative regulatory protein involved in mediating catabolite repression would be bound to a *cis*-acting site present possibly at or adjacent to the transcriptional start site, and established a candidate hexanucleotide, 5'-A/TTGTNA/T-3', which is contained within an inverted repeat(IR) sequence as a possible cis-acting site. On the basis of this information, we investigated the possible catabolite repression site in the YA-14 promoter region. Interestingly, there were two repeating sequence regions (positions 669 through 691 and positions 699 through 721) with two base differences around the transcriptional initiation site for promoter P1, and each region contained 100% homologous sequence of

5'-AGTGTT-3' and IR sequence (Fig. 3). We designated as CRSI for the upstream site and CRSII for the downstream one. To confirm whether these sites would be able to mediate catabolite repression, we constructed recombinant plasmid pPL708CRS for which the SPO2 phage promoter of pPL708 was inserted into the EcoRI site of plasmid p12BSΔf4 with the original orientation (Fig. 1). CAT activities of the constructed clone were completely repressed when the cells were grown in the presence of the excess amounts of glucose. However, it has yet to be determined whether these putative cis-acting sites act simultaneouly or separately for catabolite repression. The 5'-terminus of the mRNA *in vivo* has the potential to form a secondary structure with a calculated ΔG of -10.4 kcal/mol (Fig. 7). It is conceivable that the putative regulatory protein induced by the glucose may interact in an indirect manner with the RNA polymerase holoenzyme (17). Further detailed studies will be necessary to confirm this assumption.

We concluded that the expression of clustered promoters, YA-14 promoter region, was developmentally controlled and subjected to catabolite repression in *B. subtilis*. The characterization of a strong promoter described in this paper may be useful for the construction of an expression vector.

Acknowledgment

This study was supported by a research grant from the Korea Science and Engineering Foundation.

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(Received June 23, 1993)