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Isolation and Characterization of Transcriptional Elements from *Corynebacterium glutamicum*

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Abstract A promoter-probe shuttle vector pSK1Cat was constructed for the isolation of transcriptional signal sequences from *Corynebacterium glutamicum*. Besides conferring resistance to kanamycin in *Escherichia coli* and *C. glutamicum*, the vector carried a promoterless *cat* gene to confer resistance to chloramphenicol upon insertion of the appropriate transcriptional signals in the multiple cloning site. By utilizing the vector, a series of transcriptionally active fragments were isolated from the genome of *C. glutamicum*. The clones, ranging from 200 bp to 1 kb in size, were grouped into 3 classes of strong, medium, and weak, based on the chloramphenicol acetyltransferase (CAT) activity and sensitivity to the chloramphenicol of the clone-carrying *C. glutamicum* cells. *C. glutamicum* cells carrying the P_{19} clone, a representative in the strong class, were able to grow on minimal agar plates containing over 40 mg/ml chloramphenicol, and showed CAT activity of 10 mmol/mg·min, performing slightly better than the cells carrying P_{tac}, a strong *E. coli* promoter. Subcloning analysis of the P_{19} clone identified a 180 bp intergenic fragment (P_{180}), which was located upstream of a gene encoding a hypothetical membrane protein. The expression conferred by P_{180} was not affected by either the kinds of carbon sources or changes in temperature. These properties make the P_{180} clone useful for the deregulated expression of biosynthetic genes in *C. glutamicum* during amino acid fermentation.

Key words: *Corynebacterium glutamicum*, promoter, promoter-probe vector, *tac*

C. glutamicum is a Gram-positive organism and has been known as a producer of amino acids such as glutamic acid and lysine. Owing to its role in amino acid production, the organism has been the target for research to improve amino acid production by genetic engineering, which involves the introduction of heterologous genes, amplification of biosynthetic genes, and deregulation of the biosynthetic enzymes [2, 9, 11, 12, 22, 23]. The ideal approach to control gene expression is by manipulation at the level of transcription. This can be accomplished by replacing the native promoters of the target gene with promoters of appropriate strength and regulatory properties. Therefore, knowledge of and availability of promoters in a particular organism are prerequisites for the elaborate manipulation of gene expression.

Bacterial promoters consist of at least three elements of -10, -35, and UP regions [13, 21]. The -10 and -35 elements are recognized by the σ subunit of the RNA polymerase holoenzyme, while the UP element is recognized by the C-terminal domain of the a subunit of the core enzyme [14]. The -10 and -35 regions of *E. coli* are characterized by the consensus sequences of TATAAT and TTGACA, respectively, while the consensus UP element is characterized by two AT-rich subsites located between positions -40 and -60 relative to the transcription start site +1. An additional regulatory element includes a TG motif located upstream of the -10 hexamer [8]. The length of the spacer region connecting the two hexamers at -10 and -35 is also conserved (17±1 bp). The structure of *C. glutamicum* promoters shows similarities and differences when compared to the structure of *E. coli*, although the promoters appear to conform to the common bacterial promoter scheme in functionality. Pátek *et al.* [19] proposed a conserved extended -10 region tgngnTA(c/t)aaTgg and less well-conserved -35 region for *C. glutamicum*. In addition, a number of corynebacterial promoters contain AT-rich tracts within the -40 to -55 region [16, 20].

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Although general properties of *C. glutamicum* promoters have emerged in recent years [17, 18, 19], knowledge of the utility of the promoters is still very limited. In this study, transcriptionally active fragments were directly isolated from *C. glutamicum* and the transcriptional activity of the clones was compared to the *tac* promoter of *E. coli.*

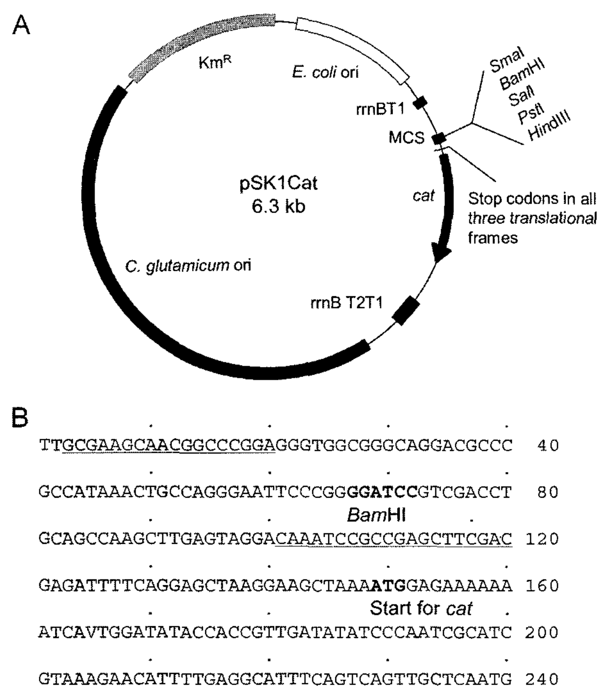

Fig. 1. Map of the pSK1Cat (Panel A) and nucleotide sequences near the *Bam*HI cloning site (Panel B).
The underlined sequences in Panel B indicate regions used for the preparation of sequencing primers. The start site for the *cat* gene and the *Bam*HI cloning site are indicated. See text for construction and detailed structural features.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions

Bacterial strains, plasmids, and primers are listed in Table 1. *C. glutamicum* AS019E12 was the source of chromosomal DNA for the construction of a promoter library. *E. coli* and *C. glutamicum* cells were cultured at 37°C in LB [24] and at 30°C in MB [7], respectively. Minimal media for *E. coli* and *C. glutamicum* were M9 [24] and MCGC [26], respectively. Glucose was added to a final concentration of 1%. Ampicillin and kanamycin were added to final concentrations of 50 and 25 mg/l, respectively. Chloramphenicol was added as indicated. All amino acids were added to a final concentration of 40 mg/l.

DNA Technology

Routine DNA manipulations were performed as described by Sambrook *et al.* [22]. Plasmid DNA from *C. glutamicum* was purified as described by Yoshihama *et al.* [25]. Chromosomal DNA of *C. glutamicum* was prepared from cells growing at the late exponential phase by following the method outlined by Eikmanns *et al.* [5]. *C. glutamicum* cells were transformed as described by

Table 1. Bacterial strains, plasmids, and primers.

Strains or plasmids	Relevant genotypes or phenotypes[a]	References
E. coli		
DH5αF'	φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1*	Bethesda Research Laboratory
C. glutamicum		
AS019E12	Restriction-deficient variant of AS019	6
Plasmids		
pTn*Mod*-OKm	Plasposon	3
pKK232-8	*E. coli* promoter analysis vector, promoterless CAT	Pharmacia Biotech.
pKK223-3	*E. coli* expression vector carrying P_{tac}, Km^R	Pharmacia Biotech.
pMT1	*E. coli-C. glutamicum* shuttle vector, Ap^R Km^R	7
pSK1Cat	*E. coli-C. glutamicum* shuttle promoter-probe vector carrying a promoterless *cat* gene, Km^R	This study
pSK1CatP_{tac}	pSK1Cat carrying P_{tac}	This study
pSK1CatP_{180}	pSK1Cat carrying P_{180}	This study
Oligonucleotides[b]		
A	5'-GGAAGATCTTTCAAGAATTCCCAGGCA-3' (*Bgl*II)	This study
B	5'-GGGGTACCTACCGTATCTGTGGGGGG-3' (*Kpn*I)	This study
E	5'-GCGGATCCTAATAAAGGTGGAGAA3-3' (*Bam*HI)	This study
F	5'-GTCGAAGCTCGGCGGATTTG-3'	This study

[a]R superscripts indicate resistance. Ap, ampicillin; Km, kanamycin.
[b]Underlined sequences indicate recognition sites for restriction enzymes as shown in parentheses.

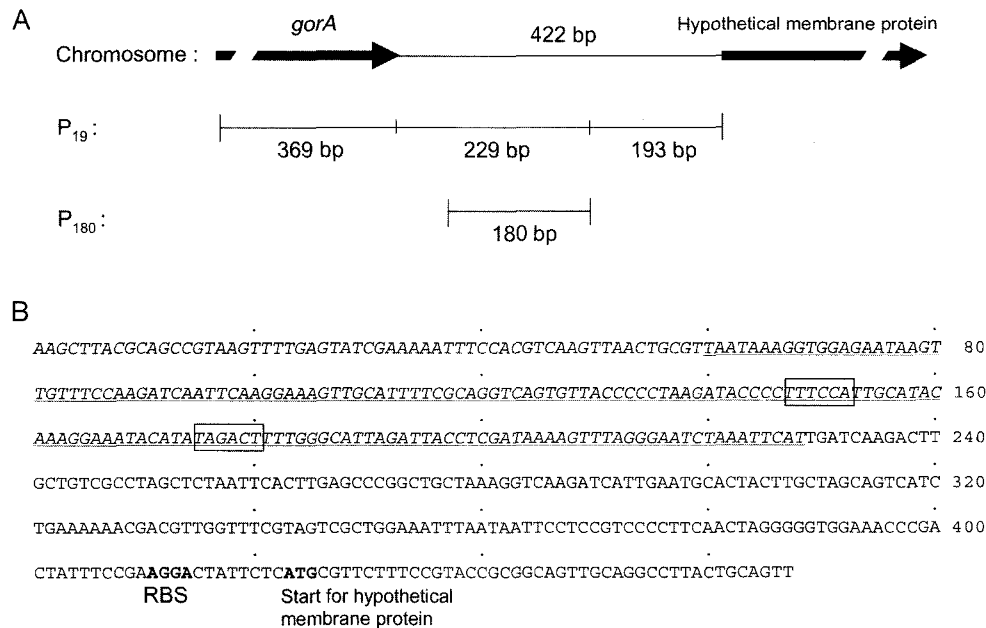

Fig. 2. Map of the P_{19} clone and its subclone P_{180} (Panel A).
Sequence information near the cloned fragments was from the *C. glutamicum* ATCC13032 genome sequence. The sequences in italics (Panel B) represent the 229 bp sequence in P_{19}. The underlined sequences represent the 180 bp sequence in P_{180}. Additional sequences are from the *C. glutamicum* ATCC13032 genome sequence. The ribosome binding site (RBS) and the start site for translation for the hypothetical membrane protein are shown. The boxes indicate the putative -10 and -35 regions.

Follettie *et al.* [7]. *E. coli* DH5αF' and *C. glutamicum* AS019E12 were used as hosts for typical transformation. The nucleotide sequences were determined commercially at the Korea Research Center for Basic Sciences (Taejon, Korea) using the primer sequences shown in Fig. 1. Relevant sequence information near the cloned fragments was retrieved from the National Center for Biotechnology Information at http://www.ncbi.nlm.nih.

Construction of Plasmids

Plasmids used for the construction of promoter probe vector pSK1Cat were plasposon pTn*Mod*-OKm, pKK232-8, and the *C. glutamicum*/*E. coli* shuttle vector pMT1. To remove the restriction sites trpA, *Sac*I, *Kpn*I, *Sac*I, and *Bam*HI, the shuttle vector pMT1 was digested with *Xho*I - *Bam*HI, treated with the Klenow fragments, and rejoined to generate pMT1-del. The 2.7 kb *Bgl*II-*Xba*I fragment of the pMT1-del vector carrying the pSR1 *ori* for *C. glutamicum* was isolated and ligated with the 2.0 kb *Bgl*II-*Xba*I fragment from the plasposon pTn*Mod*-OKm to generate pSK1. The fragment from the plasposon pTn*Mod*-OKm carried the pMB1 replication origin for *E. coli* and a kanamycin resistance marker that originated from Tn903. A promoterless *cat* gene of 1.6 kb was amplified from pKK232-8 by utilizing primers A and B (Table 1), and the amplified fragments were inserted into the pSK1 vector after digesting the inserts and the vector with *Bgl*II and *Kpn*I. The product was designated pSK1Cat (Fig. 1). Plasmid pSK1CatP_{tac} was constructed by inserting the 267 bp *Bam*HI-fragment of pKK223-3 carrying the *tac* promoter (P_{tac}) into the *Bam*HI-digested pSK1Cat. Plasmid pSK1CatP_{180} was constructed as follows. The ca. 180 bp fragment (Fig. 2, underlined sequence) of P_{19} was amplified using primers E and F, digested with *Bam*HI and *Pst*I, and inserted into the pSK1Cat, previously digested with *Bam*HI and *Pst*I, to generate pSK1CatP_{180}.

Cloning of Transcriptionally Active Fragments

The chromosomal DNA from *C. glutamicum* was partially digested with *Sau*3AI and the resulting fragments of 0.4–1.0 kb in size were isolated and ligated into the *Bam*HI-digested pSK1Cat. The ligation mixture was introduced into *C. glutamicum* AS019E12 by electroporation and the transformed cells were spread on plates containing 5 µg/ml of chloramphenicol. Plasmids were isolated from individual colonies and analyzed.

Biochemical Assays

Crude extracts were prepared as previously described [10]. Chloramphenicol acetyltransferase (CAT) was measured by the methods of Shaw [25]. The reaction mixture contained 100 mM Tris·HCl, pH7.8, 1 mM DTNB, 0.1 mM acetyl-CoA, 0.25 mM chloramphenicol, and an appropriate amount of enzyme. The changes in the optical density were measured at 412 nm. Proteins were measured as described previously [1].

RESULTS

Construction and Analysis of Promoter-Probe Vector pSK1Cat

To isolate transcriptionally active fragments from *C. glutamicum*, a promoter-probe vector, pSK1Cat, was constructed (Fig. 1). The vector carries a unique *Bam*HI cloning site in the upstream of the *cat* reporter gene, replicates in *C. glutamicum* and *E. coli*, and confers kanamycin resistance in both strains. In the upstream of the cloning site, a transcriptional terminator *rrnB*T1 was placed to prevent read-through from the vector sequence. In addition, the region between the multiple cloning site and the reporter gene contained stop codons in all three reading frames to prevent possible translational read-through from the upstream vector sequence. Transcription terminators T_1 and T_2 of the *rrnB* gene were placed downstream of the *cat* gene to prevent transcriptional read-through towards the vector sequence.

C. glutamicum cells carrying the pSK1Cat vector could not grow on MB or MCGC plates containing 5 µg/ml of chloramphenicol, suggesting that the *cat* gene was not expressed in the absence of an appropriate promoter. To evaluate the functionality of the vector, an *E. coli tac* promoter (P_{tac}) was inserted into the vector and the expression of the *cat* gene was monitored. *C. glutamicum* cells carrying the vector pSK1CatP_{tac} formed clearly visible single colonies on MB and MCGC plates containing 40 µg/ml of chloramphenicol. However, almost no growth was observed on plates containing 50 µg/ml of chloramphenicol, i.e., colonies were too small to be visible. No growth was possible on plates containing 60 µg/ml of chloramphenicol. *E. coli* cells carrying the pSK1CatP_{tac} vector formed clearly visible single colonies on LB plates containing 400 µg/ml of chloramphenicol. No growth was observed on plates

Table 2. Characteristics of the isolated promoter clones from *C. glutamicum.*

Class	Resistance to chloramphenicol[a]	Clones	*E. coli*[b]	Inserts (kb)	CAT activity (µmol/mg·min)	Putative downstream genes	Location on *C. glutamicum* map[c]
P_{tac}	40 µg/ml	P_{tac}	600	0.3	8.8	-	
Strong	Over 40 µg/ml	P_{2-10}	600	0.2	7.4	Sugar phosphate epimerase	Cgl2554 (NCgl2466) 2709459 - 2709665
		P_{19}	400	0.2	10	Hypothetical membrane protein	Cgl2004 (NCgl1929) 2118361 - 2118542
Medium	40 µg/ml	P_{1-35}	600	0.3	9.5	ABC type transporter	Cgl2141 (NCgl2060) 2262565 - 2262844
		P_{2-6}	300	0.3	5.1	Glucose-6-phosphate isomerase	Cgl0851 (NCgl0817) 909580 - 909319
		P_{43}	400	0.6	3.1	Hypothetical membrane protein	Cgl2557 - (NCgl2469) 2718778 - 2718197 -
Weak	Under 40 µg/ml	P_{1-9}	300	1.0	0.1	Two-component system sensory transduction histidine kinase	Cgl0068 (NCgl0067) 69669-71525
		P_{1-34}	600	0.7	5.1	Predicted permease	Cgl0138 (NCgl0137) 151456 - 152190
		P_{1-50}	600	0.8	2.4	Nucleoside diphosphate sugar epimerase	Cgl1372 (NCgl1318) 1437072 - 1437787
		P_5	300	0.7	1.4	Superoxide dismutase	Cgl2927 (NCgl2826) 3125818 - 3126572
		P_{12}	400	0.4	3.6	AraC-type DNA-binding domain-containing protein	Cgl0982 (NCgl0943) 1040726 - 1041141
		P_{14}	300	0.3	1.5	Hypothetical membrane protein	Cgl2017 (NCgl1941) 2131055 - 2131357
		P_{20}	300	0.1	1.5	Internal region of ABC-type transport protein	Cgl0676 (NCgl0646) 691467 - 691321
		P_{30}	400	0.4	1.9	Putative helicase	Cgl1156 (Ncgl1109) 1209679 - 1210110

[a]Ability of the *C. glutamicum* cells carrying the clones in pSK1Cat to form single colonies on MCGC agar plates containing the indicated amount of chloramphenicol.
[b]Ability of the *E. coli* cells carrying the clones in pSK1Cat to form patches on LB agar plates containing the indicated amount of chloramphenicol.
[c]The Cgl and Ncgl designations are from the *C. glutamicum* ATCC13032 genome sequence. Numbers given below denote the location of the cloned inserts on the genome sequence.

containing 600 μg/ml of chloramphenicol. These data indicate that the pSK1Cat vector is suitable for use in isolating and analyzing transcriptionally active fragments from *C. glutamicum*.

Cloning of Promoter-Containing Fragments from *C. glutamicum*

Chromosomal DNA isolated from *C. glutamicum* AS019E12 was partially digested with *Sau*3AI and ligated into the *Bam*HI-digested pSK1Cat vector. The ligation mixtures were introduced into *C. glutamicum* and the transformed cells were plated on plates containing 5 μg/ml chloramphenicol. A total of 86 clones were isolated and 13 of them were analyzed further. As shown in Table 2, by analyzing the ability of the clone-carrying *C. glutamicum* cells to form single colonies on minimal MCGC plates containing chloramphenicol, clones were classified into groups of three. Group designation of strong, medium, and weak indicates the ability of the clone-carrying *C. glutamicum* cells to grow on plates containing over, near, and under 40 μg/ml chloramphenicol, respectively. The P_{tac} promoter was closest to the strong promoter in our classification scheme (Table 2).

Analysis of the Promoter Strength by CAT Activity

The CAT activities of the clone-carrying *C. glutamicum* cells were measured to assess the relative strength of the cloned fragments (Table 2). Cells carrying pSK1CatP_{tac} showed 8.8 units of CAT activity. Among the clones isolated, P_{19} and P_{2-10} showed high activities of 10 and 7.4, respectively. Although cells carrying the P_{2-10} clone were classified as strong due to their ability to grow on plates containing 40 μg/ml chloramphenicol, the CAT activity (7.4) of the cells was weaker than that of the cells carrying pSK1CatP_{tac}. To define the active region of the P_{19} clone, a subcloning analysis was performed, and the region responsible for the activity was determined. As shown in Fig. 2 (Panel A), the active region resided in the ca. 180 bp fragment (P_{180}). The activity conferred by the fragment was identical to the parental P_{19} (data not shown). The CAT activities of the cells carrying the clones classified as medium and weak were distributed from 0.1 to 9.5 and are listed in Table 2. Except for a few clones, such as P_{1-35} and P_{1-34}, the CAT activities generally correlated with the level of resistance to chloramphenicol.

Activities in *E. coli*

To test whether the cloned *C. glutamicum* clones function in *E. coli*, the chloramphenicol resistance was also determined in *E. coli* harboring various clones (Table 2). Most plasmids carrying the cloned fragments conferred much higher chloramphenicol resistance in *E. coli* than *C. glutamicum*. Although higher by more than 10-fold, the relative pattern of chloramphenicol resistance obtained in *E. coli* roughly correlated to the pattern observed in *C. glutamicum*. The clones P_{2-10} and P_{1-35} conferred relatively high resistance to chloramphenicol in both bacteria, whereas the clones P_5, P_{14}, and P_{20} led to relatively low levels of resistance in both bacteria. However, although the clone P_{19} showed strong activity in *C. glutamicum*, the activity observed in *E. coli* was relatively weak. In addition, several clones were not functional at all in *E. coli* (data not shown).

Characteristics of the Promoter Clones

Restriction analyses of the clones revealed insert sizes ranging from 0.1 kb to 1.0 kb. The nucleotide sequences of the isolated clones were determined and the sequences were compared with the genome data of *C. glutamicum* ATCC13032 which were available at http://www.ncbi.nlm.nih.gov. As shown in Table 2, the clones appeared to carry promoter regions of various genes. Among the 13 clones, 5 of them contained promoter regions for genes with membrane-associated functions. The sequences and possible features of the -10 and -35 regions, ribosome binding sites, and the ATG start site for P_{2-10} and P_{180}, which is a subclone of P_{19}, are shown in Fig. 3 and Fig. 2, respectively. As shown in Fig. 2, P_{19} carried the 3' end of 369 bases of the putative *gorA* gene encoding dihydrolipoamide dehydrogenase/glutathione oxidoreductase. The 3'-end of the P_{19} clone carried an intergenic region which was located upstream of a hypothetical membrane protein. Scrutiny of the sequences revealed a putative -10 and -35 region of TAGACT and TTTCCA, respectively (Fig. 2, Panel B), however, the functionality of the sequences requires further investigation. As shown in Fig 3, clone P_{2-10} also carried the intergenic region located between genes encoding putative 16s rRNA and a hypothetical protein (putative sugar phosphate epimerase) (Fig. 3). It is likely that the promoter region for the P_{2-10} clone is from the gene encoding sugar phosphate epimerase, as judged by the location of the cloned fragment. Putative -10 and -35 region sequences of TGTGAT and ATAAGC were identified (Fig. 3).

The effects of various carbon sources on the expression of the clones were tested. *C. glutamicum* strain carrying each clone was grown on MCGC minimal media containing glucose, fructose, sucrose, glycerol, lactose, or maltose as a carbon source, and the resistance of the cell to chloramphenicol conferred by the clones was monitored. The expression from the clones was not affected by the kind of carbon sources, and the pattern of expression was similar to that observed in the complex medium MB (data not shown). The effects of temperature on the expression from the clones were tested at 24, 30, and 37°C. The relative temperature-sensitivity of the cells carrying the clones to chloramphenicol was not affected (data not shown).

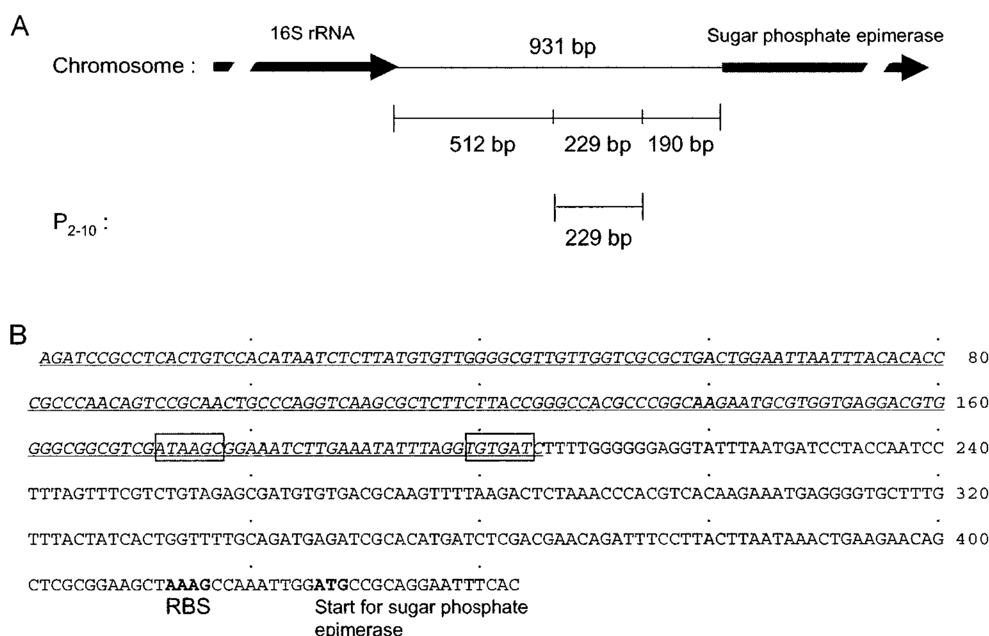

Fig. 3. Map of the P_{2-10} clone (Panel A).
Sequence information near the cloned fragments was from the *C. glutamicum* ATCC13032 genome sequence. The underlined sequences (Panel B) represent the 229 bp sequence in P_{2-10}. Additional sequences are from the *C. glutamicum* ATCC13032 genome sequence. The ribosome binding site (RBS) and the start site for translation of the hypothetical membrane protein are shown. The boxes indicate the putative -10 and -35 regions.

DISCUSSION

In this study, transcriptionally active fragments were isolated from *C. glutamicum* using the promoter-probe vector pSK1Cat and characterized. The general features of the vector are similar to the pEKplCm vector reported by Eikmanns *et al.* [4]. In addition to its smaller size, the pSK1Cat vector carries corynebacterial pRS1 *ori*, while the pEKplCm vector carries the pBL1 *ori*, therefore, the pSK1Cat vector provides additional flexibility in vector compatibility. Using the vector, we aimed at isolating transcriptionally active fragments with strong activities. However, the isolated clones with strong activities performed slightly better than the *tac* promoter of *E. coli* as measured by CAT activities. Eikmanns *et al.* [4] reported that the activities conferred by the *tac* promoter in *C. glutamicum* are almost comparable to those in *E. coli.* Therefore, activities observed by a strong clone such as P_{180} may represent a generally strong activity in *C. glutamicum.* In accordance with that assumption, promoters isolated from *C. ammoniagenes* also showed activities no stronger than those of the *tac* promoter [14].

The relative level of chloramphenicol resistance obtained in *C. glutamicum* roughly correlated to the level observed in *E. coli.* As reported by Pátek *et al.* [17], these results indicate that the structural features of some promoters isolated from *C. glutamicum* are probably similar to those of *E. coli* promoters. However, as observed with a few clones, the activity in *C. glutamicum* did not correlate with the activity observed in *E. coli.* In addition, several clones were not functional at all in *E. coli.* The differences in promoter activities of the cloned DNA fragments in both bacteria could be the result of gene regulation in *C. glutamicum* which does not function in *E. coli,* and/or the structural differences in the promoters of both bacteria. In addition, for some clones, the CAT activity did not correlate with the sensitivity of the clone-carrying cells to chloramphenicol. The phenomenon was also observed by Pátek *et al.* [15]. Although the mechanism is uncertain, the phenomenon could be due to the presence of other genes in the cloned fragment, that is genes encoding membrane proteins may lead to such a phenomenon.

As shown in this study, the promoter region of the P_{180} clone was relatively far apart from the downstream gene encoding a hypothetical membrane protein. Optimization of spacing between the proposed -10 region of the P_{180} fragment and the ATG start site of a target gene may even increase the transcriptional activity of the clone. In addition, the possibility that the promoter activity by the P_{180} fragment is due to promoter-like sequences present in the clone which do not function *in vivo* can not be ruled out. Further analysis is underway to address that question.

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REFERENCES

1. Bradford, M. M. 1976. A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248-254.
2. de Graaf, A. A., L. Eggeling, and H. Sahm. 2001. Metabolic engineering for L-lysine production by *Corynebacterium glutamicum. Adv. Biochem. Eng. Biotechnol.* **73:** 9-29.
3. Dennis, J. J. and G. J. Zylstra. 1998. Plasposons: Modular self-cloning minitransposon derivatives for rapid genetic analysis of gram-negative bacterial genomes. *Appl. Environ. Microbiol.* **64:** 2710-2715.
4. Eikmanns, B. J., E. Kleinertz, W. Liebl, and H. Sahm. 1991. A family of *Corynebacterium glutamicum/Escherichia coli* shuttle vectors for cloning, controlled gene expression, and promoter probing. *Gene* **102:** 93-98.
5. Eikmanns, B. J., N. Thum-Schmitz, L. Eggeling, K. U. Lüdtke, and H. Sahm. 1994. Nucleotide sequence, expression and transcriptional analysis of the *Corynebacterium glutamicum gltA* gene encoding citrate synthase. *Microbiology* **140:** 1817-1828.
6. Follettie, M. T. and A. J. Sinskey. 1986. Recombinant DNA technology for *Corynebacterium glutamicum. Food Technol.* **40:** 88-94.
7. Follettie, M. T., O. P. Peoples, C. Agoropoulou, and A. J. Sinskey. 1993. Gene structure and expression of the *Corynebacterium flavum* N13 *ask-asd* operon. *J. Bacteriol.* **175:** 4096-4103.
8. Harley, C. B. and R. P. Reynolds. 1987. Analysis of *E. coli* promoter sequences. *Nucleic Acids Res.* **15:** 2343-2361.
9. Herrman, T. 2003. Industrial production of amino acids by coryneform bacteria. *J. Biotechnol.* **104:** 155-172.
10. Jetten, M. S. M. and A. J. Sinskey. 1993. Characterization of phosphoenolpyruvate carboxykinase from *Corynebacterium glutamicum. FEMS Microbiol. Lett.* **111:** 183-188.
11. Kim, H.-J., J.-S. Park, H.-S. Lee, and Y. Kim. 2002. Utilization of *lacZ* to isolate regulatory genes from *Corynebacterium glutamicum. J. Microbiol. Biotechnol.* **12:** 336-339.
12. Ko, S.-Y., S.-H. Yim, H.-S. Lee, and M.-S. Lee. 2003. Isolation and analysis of the *argG* gene encoding argininosuccinate synthetase from *Corynebacterium glutamicum. J. Microbiol. Biotechnol.* **13:** 949-954.
13. Lee, J. H. 2002. Importance of nucleotides adjacent to the core region of diphtheria *tox* promoter/operator. *J. Microbiol. Biotechnol.* **12:** 622-627.
14. Meng, W., T. Belyaeva, N. J. Savery, S. J. Busby, W. E. Ross, T. Gaal, R. L. Gourse, and M. S. Thomas. 2001. UP element dependent transcription at the *Escherichia coli rrnB* P1 promoter: Positional requirements and role of the RNA polymerase alpha subunit linker. *Nucleic Acids Res.* **29:** 4166-4178.
15. Paik, J. E. and B. R. Baek. 2003. Isolation of transcription initiation signals from *Corynebacterium ammoniagenes* and comparison of their gene expression levels in *C. ammoniagenes* and *Escherichia coli. Biotechnol. Lett.* **25:** 1311-1316.
16. Park, S.-H., D.-H. Lee, K.-H. Oh, K. Lee, and C.-K. Kim. 2002. Detection of aromatic pollutants by bacterial biosensors bearing gene fusions constructed with the *dnaK* promoter of *Pseudomonas* sp. DJ-12. *J. Microbiol. Biotechnol.* **12:** 417-422.
17. Pátek, M., B. J. Eikmanns, J. Pátek, and H. Sahm. 1996. Promoters from *Corynebacterium glutamicum*, molecular analysis and search for a consensus motif. *Microbiology* **142:** 1297-1309.
18. Pátek, M., G. Muth, and W. Wohlleben. 2003. Function of *Corynebacterium glutamicum* promoters in *Escherichia coli, Streptomyces lividans*, and *Bacillus subtilis. J. Biotechnol.* **104:** 325-334.
19. Pátek, M., J. Nešvera, A. Guyonvarch, O. Reyes, and G. Leblon. 2003. Promoters of *Corynebacterium glutamicum. J. Biotechnol.* **104:** 311-323.
20. Perez-Martin, J., F. Rojo, and V. de Lorenzo, 1994. Promoters responsive to DNA bending: A common theme in prokaryotic gene expression. *Microbiol. Rev.* **58:** 268-290.
21. Ross, W., K. K. Gosink, J. Salomon, K. Igarashi, C. Zou, A. Ishihama, K. Severinov, and R. L. Gourse. 1993. A third recognition element in bacterial promoters: DNA binding by the alpha subunit of RNA polymerase. *Science* **262:** 1407-1413.
22. Sahm, H., L. Eggeling, and A. A. de Graaf. 2000. Pathway analysis and metabolic engineering in *Corynebacterium glutamicum. Biol. Chem.* **381:** 899-910.
23. Sahm, H., L. Eggeling, B. Eikmanns, and R. Krämer. 1995. Metabolic design in amino acid producing bacterium *Corynebacterium glutamicum. FEMS Microbiol. Rev.* **16:** 243-252.
24. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning-A Laboratory Manual.*, 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., U.S.A.
25. Shaw, W. V. 1975. Chloramphenicol acetyltransferase from chloramphenicol-resistant bacteria. *Methods Enzymol.* **43:** 737-755.
26. von der Osten, C. H., C. K. Gionnetti, and A. J. Sinskey. 1989. Design of defined medium for growth of *Corynebacterium glutamicum* in which citrate facilitates iron uptake. *Biotechnol. Lett.* **11:** 11-16.
27. Yoshihama, M., K. Higashiro, E. A. Rao, M. Akedo, W. G. Shanabruch, M. T. Follettie, G. C. Walker, and A. J. Sinskey. 1985. Cloning vector system for *Corynebacterium glutamicum. J. Bacteriol.* **162:** 591-597.