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Utilization of *lacZ* to Isolate Regulatory Genes from *Corynebacterium glutamicum*

KIM, HYUNG-JOON[1], JOON-SUNG PARK[1], HEUNG-SHICK LEE[1], AND YOUNHEE KIM[2]*

[1]*Graduate School of Biotechnology, Korea University, Anam-Dong, Sungbuk-Ku, Seoul 136-701, Korea*
[2]*Department of Oriental Medicine, Semyung University, Checheon, Chungbuk 390-230, Korea*

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Abstract A total of 100 Corynebacterial clones exerting a regulatory effect on the *aceB* promoter of *Corynebacterium glutamicum* were isolated by utilizing a reporter carrying the enteric *lacZ* gene fused to the promoter. The isolated clones were classified into 3 groups of A, B, and C, according to their color of colonies. *Escherichia coli* cells carrying clones in groups A and B showed a 90% and 50% reduction in β-galactosidase activity, respectively. The introduction of group A clones into *C. glutamicum* also resulted in an almost complete reduction in the expression of the *aceA* and *aceB* genes, suggesting that the clones express repressor-like proteins for the genes. Although white colonies were formed on plates containing X-gal, *E. coli* cells carrying one of the clones in group C exhibited intact β-galactosidase activity. The result suggests that the clone may encode proteins that prevent the cells from accumulating the chromogenic compound, X-gal.

Key words: Regulation, *aceB*, *Corynebacterium glutamicum*

Corynebacterium glutamicum has been widely used for the industrial production of amino acids [7]. Accordingly, due to its importance in amino acid production, such as glutamic acid and lysine, it has been the target for research to improve amino acid production by genetic engineering. Although significant progress has been made in understanding the biosynthetic pathways of industrially important amino acids [for review see 9 and 13], information on the regulatory mechanisms of gene expression is still very limited.

The glyoxylate bypass of *Corynebacterium glutamicum* is a good candidate for studying the regulatory mechanism of gene expression, because the expression of isocitrate lyase and malate synthase, which catalyze the bypass, is tightly regulated by the availability of carbon sources [17]. Isocitrate lyase, encoded by the *aceA* gene, catalyzes the conversion of the TCA intermediate, isocitrate, into glyoxylate and succinate [11, 3]. Malate synthase, encoded by the *aceB* gene, catalyzes the subsequent aldol-condensation of glyoxylate with acetyl-CoA to produce malate, which in turn enters the TCA cycle [8, 12]. The *aceA* and *aceB* genes are derepressed by two-carbon compounds, such as acetate provided as the sole source of carbon, conserving the acetate carbon for the biosynthesis of cell material by bypassing the CO_2-generating steps of the TCA cycle. Glucose, supplied as a carbon source, represses the *aceA* and *aceB* genes. The expression of the glyoxylate bypass enzymes is regulated at the level of transcription by the available carbon sources [17], yet the mechanism of transcriptional regulation is still unknown. In *E. coli*, an IclR repressor is known to be responsible for the regulation of the *aceBAK* operon [1, 16]. Although the organization of the *aceA* and *aceB* genes in *C. glutamicum* is different from that in *E. coli*, the regulation of gene expression by the available carbon sources appears to have common features.

Construction of *P-aceB-lacZYA* Reporter Plasmid

To isolate genes whose protein products exert regulatory effects on the promoter region of the *C. glutamicum aceB* gene, a reporter plasmid was constructed by utilizing the enteric *lac* operon as follows: A DNA fragment of 2.5 kb carrying the promotor region of the *aceB* gene (*P-aceB*) was amplified by a PCR using plasmid pSL08 [8] as the template, and inserted into the *Sma*I site of plasmid pRS415 [15] to generate plasmid pSL130 (Fig. 1). For the amplification, oligonucleotides of 5'CTTAAGTGATTCG-CAATGGG3' and 5'GCGTGCTTAGTTTTTTGCTTTG-AACTC3' were used as the forward and reverse primers, respectively. Because plasmid pRS415 carries a promoter-less *lacZYA* gene, the expression of the β-galactosidase will depend on the promoter element inserted into the multiple cloning site located upstream of *lacZYA*. As the next step, the region of DNA carrying the Corynebacterial *aceB* promoter and the enteric *lacZYA* genes (9.1 kb, *P-aceB-lacZYA*) was isolated from pSL130 by a PCR using the primers 5'ACCAGTACTAATAGGCGTATCACGAG-

*Corresponding author
Phone: 82-43-649-1346; Fax: 82-43-644-1297;
E-mail: ykim@venus.semyung.ac.kr

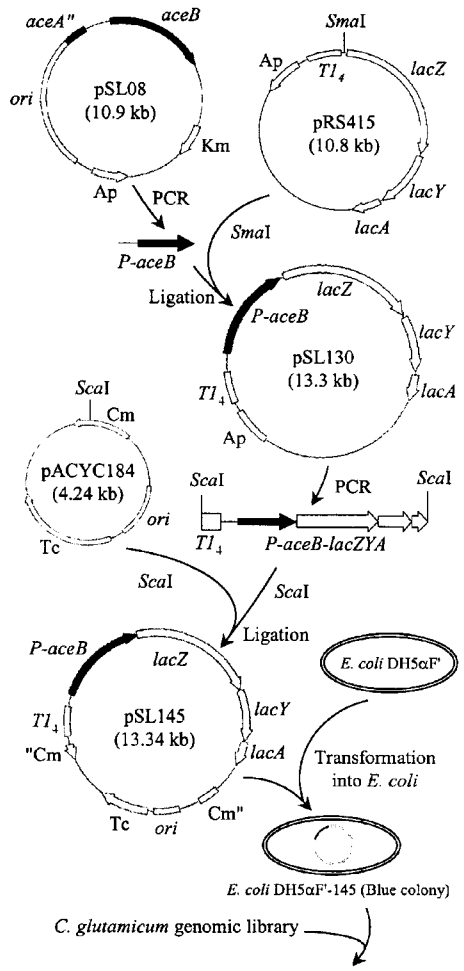

Fig. 1. Construction of plasmid pSL145, a reporter plasmid carrying the *aceB* promoter of *C. glutamicum* and enteric *lac* operon.
$T1_4$ represents 4 tandem copies of *T1*, the terminator of the *E. coli rrnB* operon. *P-aceB* represents the region of DNA carrying the *aceB* gene and its upstream promoter. *aceA"* indicates the truncated 5'-region of the *aceA* gene. Cm" and "Cm represent the 5' and 3' region of the gene conferring chloramphenicol resistance, respectively. The vector is not drawn to scale. *E. coli* DH5αF' harboring pSL145 (*E. coli* DH5αF'-145) was used to screen the Corynebacterial genomic library (see text).

GCCC3' and 5'TGTAGTACTTGGTGTGTGGGTTAGG-TCTGG3', digested with *Sca*I, and then inserted into the *Sca*I site of the pACYC184 vector (New England Biolabs, Beverly, U.S.A.) to generate plasmid pSL145 (Fig. 1). The plasmid pSL145, which was 13 kb in length, was used as the reporter plasmid. The transfer of the 9.1 kb DNA fragment into the pACYC184 vector was necessary, because plasmid pMT1, which was used to construct a Corynebacterial genomic library, was not compatible with pRS415-derived vectors. *E. coli* DH5αF' cells (Gibco BRL, NY, U.S.A.) were transformed with pSL145, and then the resulting *E. coli* DH5αF'-145 strain was used as the host for screening the library. The genomic library of *C. glutamicum* AS019E12 [2, 5], which consisted of 4 to 13 kb *Mbo*I fragments cloned into the *E. coli-Corynebacterium* shuttle vector pMT1, was made as previously described [4].

Screening and Isolation of Putative Corynebacterial Regulatory Genes

E. coli DH5αF' cells carrying the reporter plasmid (*E. coli* DH5αF'-145) formed blue colonies on LB plates [14] containing 40 μg/ml X-gal, 20 μg/ml tetracycline, and 40 μg/ml ampicillin. The cells carrying clones whose protein products had regulatory effects on the promoter region of *aceB*, thus affecting the expression of *lacZ*, were expected to form white colonies on the plate. *E. coli* DH5αF'-145 was transformed with a Corynebacterial genomic library, and the transformed cells were plated onto an LB medium supplemented with X-gal. Among a total of 20,000 colonies screened, 100 white colonies were identified and the restriction maps of the isolated clones were determined. The size of the DNA insert ranged from 3.7 to 12 kb. Four clones, which showed clear white colonies, contained overlapping 1.5 kb inserts. These clones were classified as group A. Eighty-two clones contained 0.2 or 0.8 kb *Eco*RI fragments were classified as group B. Cells carrying the clones formed white colonies. Fourteen clones showed pale blue colonies and were classified as group C. Unlike the clones in groups A and B, the clones belonging to group C did not contain any overlapping fragments, suggesting that each clone in the group may represent a novel gene. Among the clones in group A, plasmid pSL329, which carried a 7.8 kb insert, was chosen and analyzed further. To identify the region responsible for modulating the β-galactosidase activity, various subclones were generated (Fig. 2, Panel A). The β-galactosidase modulating activities of the subclones were determined by patching the cells carrying the subclones on LB media containing X-gal and monitoring the color of the colonies (Fig. 2). Plasmids pSL329-1, pSL329-2, and pSL329-5 were made by ligating the 5.1 kb *Eco*RI-*Xba*I fragment, 5.2 kb *Xho*I fragment, and 1.8 kb *Kpn*I fragment of pSL329 into the pMT1 vector digested with *Sma*I-*Xba*I, *Xho*I, and *Kpn*I, respectively. Among the subclones, plasmid pSL329-5 showed *aceB*-promoter regulating activity. Among the clones belonging to group B, plasmid pSL152, which carried the shortest insert DNA, was chosen and analyzed further. Plasmids pSL152-1 and pSL152-3 were made by ligating the 2.6 kb *Kpn*I-*Xho*I fagment and 1.7 kb *Bam*HI fragment of pSL152 into the pMT1 vector digested with *Kpn*I-*Xho*I and *Bam*HI, respectively. Plasmid pSL152-9 was made by deleting the 0.55 kb *Xho*I-*Mlu*I fragment of pSL152-1. Based on a subcloning analysis, a 2.1 kb fragment responsible for the β-galactosidase modulating activity was identified (Fig. 2, Panel B). Among the clones belonging to group C, plasmid pSL149 was chosen and analyzed further. Plasmids pSL149-1, pSL149-2, and pSL149-4 were made by deleting the 1.3 kb *Kpn*I fragment, 1.1 kb *Xba*I fragment, and 0.85 kb *Xho*I fragment of pSL149,

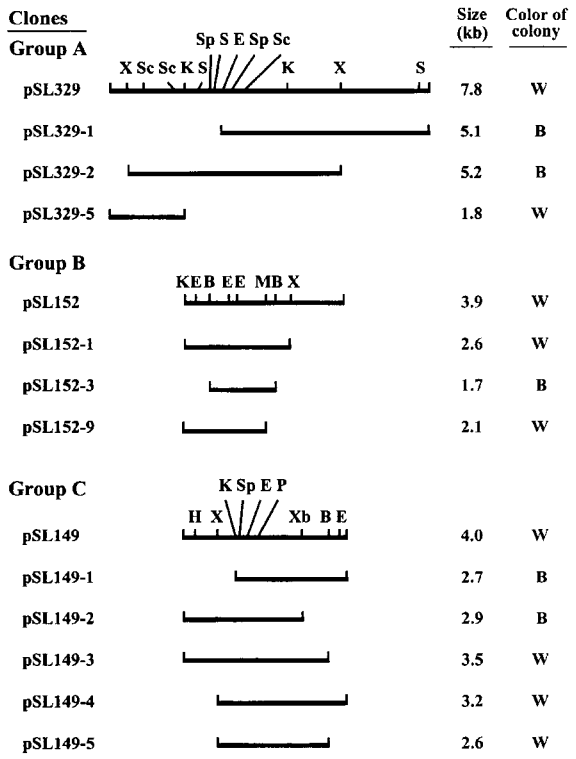

Fig. 2. Schematic diagram of clones and subclones.
Plasmids pSL329, pSL152, and pSL149 are clones isolated from the Corynebacterial genomic library. The cloning vector pMT1 [2] is not shown. The colony color (B, blue; W, white) of the *E. coli* DH5αF'-145 cells carrying each clone was tested on LB plates [14] containing 40 μg/ml X-gal, 20 μg/ml tetracycline, and 40 μg/ml ampicillin. Abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; M, *Mlu*I; P, *Pst*I; S, *Sal*I; Sc, *Sca*I; Sp, *Sph*I; X, *Xho*I; Xb, *Xba*I.

respectively. Plasmid pSL149-5 was made by deleting the 0.9 kb *Xho*I fragment of pSL149-3, which was made by ligating the 3.5 kb *Bam*HI fragment of pSL149 into a *Bam*HI-digested pMT1 vector. A subcloning analysis identified a 2.6 kb fragment as responsible for the β-galactosidase modulating activity (Fig. 2, Panel C).

Effect of Clones on β-Galactosidase Activity

In addition to the color test on agar plates containing X-gal, β-galactosidase activity [19] of the *E. coli* DH5αF'-145 cells carrying the subclones was also measured. To measure the β-galactosidase activity, the cells were grown overnight in LB, washed with 0.1 M potassium phosphate buffer (pH 7.0), and then resuspended with a reaction buffer (pH 7.5) containing 5 mM Tris, 10 mM KCl, and 5% glycerol (v/v). The cells were disrupted by glass-bead vortexing, and then the cell extracts were recovered by centrifugation. The reaction was started by adding ONPG to a final concentration of 2.7 mM. After 10 min of incubation at 30°C, the reaction was stopped by adding Na_2CO_3 to a final concentration of 0.3 mM, then the optical densities were measured at 420 nm. *E. coli* DH5αF'-145 cells carrying plasmid pMT1, an empty vector, showed 28 mU of β-galactosidase activity (Fig. 3). The introduction of plasmid pSL329, which belonged to group A, into the *E. coli* DH5αF'-145 cells showed 2.5 mU, corresponding to a 90% reduction compared to the strain carrying an empty vector (Fig. 3). Cells harboring pSL152, a group B plasmid, showed 15 mU, a 48% reduction compared to the strain carrying an empty vector. Plasmid pSL149, a clone belonging to group C, showed an interesting result. Although the cells carrying plasmid pSL149 formed white colonies, they also exhibited intact β-galactosidase activities, measured at 27 mU. The results suggest that the clones belonging to groups A and B encoded proteins that may have exerted regulatory effects on the *aceB* promoter. The binding of the protein(s) to the promoter region of the *aceB* gene may have interfered with the binding of the RNA polymerase, thereby resulting in a reduced expression of *lacZ*. Although the nature of the clones belonging to Group C is still unclear, it would appear that some clones may have expressed proteins that were involved in interfering with the entry of X-gal into the cell. A possible candidate may be an efflux pump located in the membrane of the cell. Such efflux pumps have been previously reported in diverse organisms [10].

Repressor Activity of Clones in *C. glutamicum*

The isolated clones belonging to groups A and B were introduced into *C. glutamicum* AS019E12 to test for repressor activity at the *aceA* and *aceB* promoters. Crude extracts were prepared from the cells carrying the clones, and the activities of isocitrate lyase and malate synthase were measured

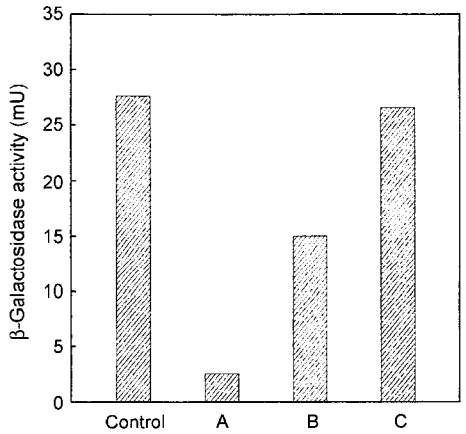

Fig. 3. β-galactosidase activity of *E. coli* DH5αF'-145 cells carrying various clones.
The cells were grown to the stationary phase in LB [14], then the cell extracts were prepared as described [2]. An assay for β-galactosidase activity was performed as described in the text. One unit of activity was defined as the amount of enzyme that hydrolyzed 1 μmole of ONPG in 1 min at 30°C. Plasmid pMT1 carried no insert. Bars: Control, *E. coli* DH5αF'-145/pMT1; A, *E. coli* DH5αF'-145/pSL329; B, *E. coli* DH5αF'-145/pSL152; C, *E. coli* DH5αF'-145/pSL149.

Table 1. Effect of clones on activities of isocitrate lyase (ICL), malate synthase (MS), and isocitrate dehydrogenase (ICDH)[a].

Strain	Plasmids[c]	Description	Specific activity[b], nmol min^{-1} mg^{-1}		
			ICL	MS	ICDH
C. glutamicum AS019E12	pMT1	Empty vector	528	567	973
	pSL329	Group A	21	62	960
	pSL152	Group B	186	173	767

[a]The enzymes were induced by growing the *C. glutamicum* AS019E12 cells to the stationary phase on MB [2, 18] containing 2% sodium acetate.
[b]Activities of malate synthase, isocitrate lyase, and isocitrate dehydrogenase were measured as previously described [8, 11]. The cell extracts were prepared as described [2].
[c]The plasmids were introduced to *Corynebacterium* by electroporation [2].

(Table 1). When compared to the cells carrying an empty vector, the cells carrying plasmid pSL329 showed 90–95% reduction in the activities of the glyoxylate bypass enzymes. The repressing activity appeared to be specific to the glyoxylate bypass enzymes, because no inhibition was observed with isocitrate dehydrogenase, a TCA cycle enzyme (Table 1). Less severe effects were observed with cells harboring pSL152. These results are in agreement with the phenomena observed in *E. coli*.

In conclusion, regulatory genes were successfully isolated from *Corynebacterium* using a reporter plasmid carrying the Corynebacterial *aceB* promoter and enteric *lac* operon. As evidenced by the reduction in the activities of β-galactosidases in *E. coli* and glyoxylate bypass enzymes in *C. glutamicum*, the putative regulatory genes appeared to express repressor-like proteins for the *aceA* and *aceB* genes. However, further investigation is required to elucidate the physiological role of the encoded proteins from the isolated clones.

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