Regulatory Genes of *Corynebacterium glutamicum* Involved in Stress-Related Response

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A group of 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 *aceB* gene encodes one of the enzymes catalyzing the glyoxylate bypass and is important for the utilization of acetate. *Escherichia coli* cells carrying the isolated clones showed a 50-90% reduction in the β-galactosidase activity. Sequence analysis of the isolated clones identified putative genes involved in carbon catabolite repression and stress response. The *glxR* gene, one of the clones, contained cAMP and DNA binding motifs and the activity of the encoded protein was modulated by cAMP. The *whcB* gene, identified in another clone, was preferentially expressed in the stationary phase and appeared to function under the oxidative stress condition. Additionally, a gene encoding an ECF-type sigma factor was also isolated. These genes might be useful for strain manipulation to enhance the performance of the *C. glutamicum* strains used for amino acid fermentation.

Introduction

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 (*aceA*) and malate synthase (*aceB*), which catalyze the bypass, is tightly regulated by the availability of carbon sources [17]. In *Eschericha coli*, the expression of the *aceA* and *aceB* genes is also related to the stress response. Isocitrate lyase catalyzes the conversion of the TCA intermediate, isocitrate, into glyoxylate and succinate [11, 3]. Malate synthase 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 repressed by glucose and 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.

In this article, we describe the isolation and characterization of regulatory genes from *C. glutamicum* and possible application of the isolated genes to amino acid production.

Results and Discussion

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 *SmaI* site of plasmid pRS415 [15] to generate plasmid pSL130 (Fig. 1) For the amplification, oligonucleotides of 5'CTTAAGTGATTCGCAATGGG3' and 5'GCGTGCTTAGTTTTTTGCTTTGAACTC3' 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'ACCAGTACTAATAGGCGTATCACGAGGCCC3' and 5'TGTAGTACTTGGTGTGTGGGTTAGGTC TGG3', digested with *ScaI*, and then inserted into the *ScaI* site of the pACYC184 vector (New England Biolabs, Beverly, USA) 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

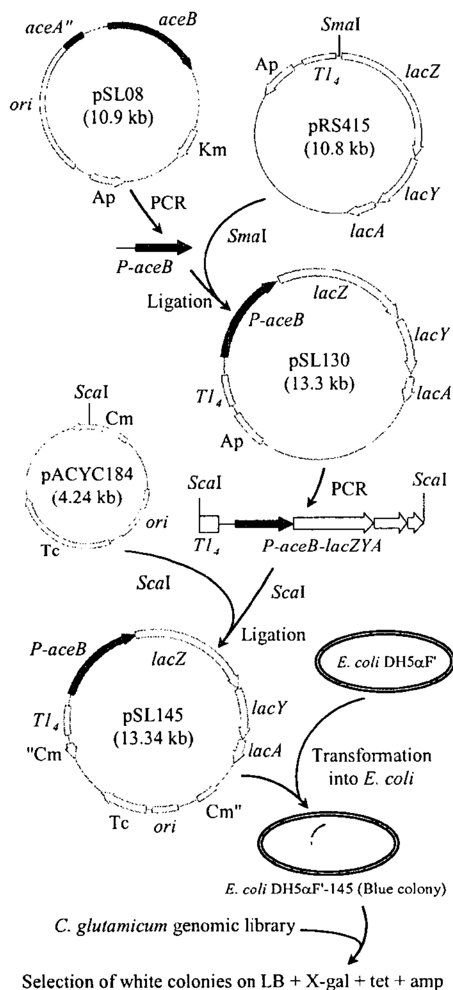

Fig. 1. Construction of plasmid pSL145, the reporter plasmid carrying the *aceB* promoter of *C. glutamicum* and the enteric *lac* operon. *TI_4* represents 4 tandem copies of *TI*, the terminator of the *E. coli rrnB* operon. *P-aceB* represents the region of DNA carrying the *aceB* gene and its upstream 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).

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, USA) 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 and 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 groups A, B, and C, plasmids pSL329, pSL152, and pSL149 were chosen and analyzed further.

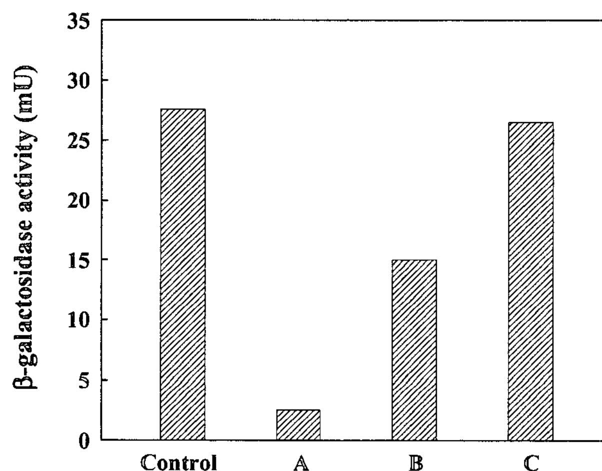

Fig. 2. β-galactosidase activity of *E. coli* DH5αF'-145 cells carrying various clones. The cells were grown to the stationary phase in LB [14], then the cell extracts were prepared as described [2]. An assay for β-galactosidase activity was performed as described in the text. One unit of activity was defined as the amount of enzyme that hydrolyzed 1 μmole of ONPG in 1 minute at 30°C. Plasmid pMT1 carries 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.

Effect of Clones on β-Galactosidase Activity

In addition to the color test on agar plates containing X-gal, β-galactosidase activity [19] in the crude extract of the *E. coli* DH5αF'-145 cells carrying the subclones was also measured. *E. coli* DH5αF'-145 cells carrying plasmid pMT1, an empty vector, showed 28 mU of β-galactosidase activity (Fig. 2). 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. 2). 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].

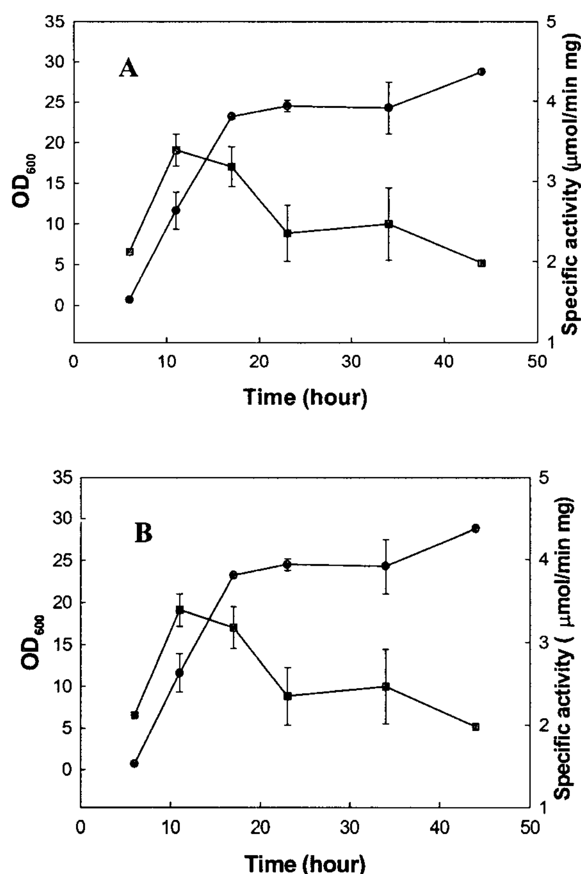

Fig. 3. Expression of *whcB* in medium containing glucose (A) or acetate (B) as the sole carbon source. Cell growth was measured by the optical density at 600 nm, and the *whcB* expression was monitored by CAT (chloramphenicol acetyltransferase) activity. Symbols: ●, growth; ■, *whcB* expression.

Involvement of the *whcB* Gene in Stress Response

Subcloning analysis of plasmid pSL152 identified a region which was responsible for *lacZ*-repressing activity, and the region was found to contain an ORF which is 261 base pairs in length. The predicted polypeptide contained 86 amino acids encoding a protein with molecular weight of 9,586 Da. The putative protein showed similarities of 85% and 75% to the WhiB of *Mycobacterium tuberculosis* and WblE of *Streptomyces coelicolor*, respectively. Thus, the corynebacterial gene was named as *whcB* (*whiB* homolg of

Corynebacterium). The predicted amino acid sequence showed a helix-turn-helix DNA binding motif at the C-terminal region. Upon comparison of the growth pattern of the *whcB* mutant to that of the wild type, the *whcB* mutant showed a longer lag phase on minimal medium containing glucose or acetate as the carbon source. While the strain overexpressing *whcB* showed a 40% reduction in the activity of glyoxylate bypass enzymes, the *whcB* mutant strain showed a 20% increment in the activity. To monitor the expression pattern, a construct carrying CAT (chloramphenicol acetyl transferase) fused to the *whcB* promoter was made. Experiments using the construct indicated increment of CAT activity in nutrient medium containing acetate, and decrement of CAT activity in nutrient medium containing glucose. In addition, the *whcB* mutant showed increased sensitivity to the thiol-specific oxidant, such as diamide as compared to the redox cycling compounds menadione and plumbagin. These results suggest a role of the *whcB* gene in stress response, such as oxidative stress.

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