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Elucidation of the Regulation of Ethanol Catabolic Genes and *ptsG* Using a *glxR* and Adenylate Cyclase Gene (*cyaB*) Deletion Mutants of *Corynebacterium glutamicum* ATCC 13032

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The cyclic AMP receptor protein (CRP) homolog, GlxR, controls the expression of several genes involved in the regulation of diverse physiological processes in *Corynebacterium glutamicum*. *In silico* analysis has revealed the presence of *glxR* binding sites upstream of genes *ptsG*, *adhA*, and *ald*, encoding glucose-specific phosphotransferase system protein, alcohol dehydrogenase (ADH), and acetaldehyde dehydrogenase (ALDH), respectively. However, the involvement of the GlxR-cAMP complex on the expression of these genes has been explored only *in vitro*. In this study, the expressions of *ptsG*, *adhA*, and *ald* were analyzed in detail using an adenylate cyclase gene (*cyaB*) deletion mutant and *glxR* deletion mutant. The specific activities of ADH and ALDH were increased in both the mutants in glucose and glucose plus ethanol media, in contrast to the wild type. In accordance, the promoter activities of *adhA* and *ald* were derepressed in the *cyaB* mutant, indicating that *glxR* acts as a repressor of *adhA*. Similarly, both the mutants exhibited derepression of *ptsG* regardless of the carbon source. These results confirm the involvement of GlxR on the expression of important carbon metabolic genes; *adhA*, *ald*, and *ptsG*.

Keywords: *Corynebacterium glutamicum*, GlxR, *cyaB*, *adhA*, *ald*, *ptsG*

Introduction

Corynebacterium glutamicum is a gram-positive soil bacterium, widely used in the industrial production of amino acids such as lysine and glutamic acid. This organism is able to grow on a variety of carbon and energy sources such as sugars, sugar alcohols, and organic acids [9, 21, 23]. In addition, ethanol can be used as a sole or an additional carbon source, and the major enzymes involved in the breaking down of ethanol in *C. glutamicum* are alcohol dehydrogenase (ADH, encoded by *adhA*) and acetaldehyde dehydrogenase (ALDH, encoded by *ald*) [2, 19]. *C. glutamicum* co-metabolizes glucose with other sugars or with organic acids and exhibits monophasic growth behavior [6-8, 12, 20, 30, 33]. In contrast, the growth of *C. glutamicum* on a mixture of glucose and ethanol shows biphasic growth behavior, which is due to the sequential utilization of glucose before ethanol [1, 19]. It has been reported that this biphasic growth behavior is accompanied by relatively low ADH and ALDH activities in the first and higher activities in the second growth phases, indicating that ethanol catabolism in *C. glutamicum* is subject to carbon catabolite control [1]. In addition, it was revealed that the expressions of *adhA* and *ald* are under the control of transcriptional regulators, RamA and RamB [2]. Whereas RamA is essential for the expression of *adhA* and *ald*, RamB exerts a negative control on *adhA* and *ald* expression in the presence of glucose or acetate in the growth medium. In a RamB deletion mutant, the glucose- and acetate-dependent down-regulation of *adhA* was partially released, indicating the involvement of an additional regulator in the catabolite repression of *adhA* [2]. In addition, they observed a binding motif for the cAMP-dependent regulator, GlxR in the promoter region of *adhA*, 21 bp downstream of the transcriptional start site. In 2008, Kohl *et al*. [17] verified the binding of GlxR upstream of the *adhA* gene by electrophoretic mobility shift assay (EMSA).

In the case of *ald*, putative GlxR binding motifs were detected upstream of the gene [3], and the binding of GlxR was verified by EMSA [18]. However, there are no *in vivo* data showing the direct involvement of GlxR on the expression of ethanol-catabolic genes *adhA* and *ald* in *C. glutamicum*. The *in vivo* evaluation of the regulatory effect of GlxR is limited, as the *glxR* deletion mutant shows severe growth defect [26].

In *C. glutamicum*, the preferred carbon source, glucose, is taken up *via* the phosphoenolpyruvate-dependent phosphotransferase system (PTS) [22, 24]. It has been reported that the expression of *ptsG*, which encodes the glucose-specific PTS enzyme II is repressed by the DeoR-type regulator SugR [11, 31]. In contrast, the transcriptional regulators GntR1 and GntR2 activate the expression of *ptsG* [12]. In addition, a putative binding site for the transcriptional regulator RamB is seen in the promoter region of *ptsG* [13]. The putative binding site of RamB is located downstream of the transcriptional start site of *ptsG*, indicating that RamB might function as a negative regulator of *ptsG* [11]. In 2008, Kohl *et al*. [17] reported the presence of a putative binding site of GlxR in the promoter region of *ptsG*, which was verified by EMSA. Since the binding motif is located downstream of the transcriptional initiation site of *ptsG*, GlxR might act as a repressor of *ptsG* expression. Even though there are *in vitro* data showing the involvement of GlxR and RamB on the expression of *ptsG*, there are no *in vivo* results showing the regulation of *ptsG* by these regulators. Therefore, we have carried out *in vivo* experiments in order to confirm the role of the global regulator GlxR and the master regulator RamB on the expression of *ptsG* in *C. glutamicum*.

As reported earlier, the ethanol metabolic pathway and glucose-uptake system in *C. glutamicum* are under the control of a number of regulators [2, 3, 11-13, 17, 18, 31]. However, the studies revealed that there might be the involvement of additional regulators controlling the expression of ethanol metabolic genes and *ptsG*. In the present study, we investigated the role of GlxR in the regulation of *adhA*, *ald*, and *ptsG*, and of RamB on the expression of *ptsG* in *C. glutamicum*. We present *in vivo* experimental evidence indicating a negative regulation of GlxR on *ptsG*, *adhA*, and *ald*. In addition, the *in vivo* data confirm the repressive effect of RamB on the expression of *ptsG*.

Materials and Methods

Bacterial Strains, Plasmids, Oligonucleotides, and Growth Conditions

The bacterial strains, plasmids, and oligonucleotides used in this study are listed in Table 1. The *E. coli* strain was grown in LB (Luria-Bertani) medium (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl) at 37°C, whereas the *C. glutamicum* ATCC 13032 was grown at 30°C in LB or minimal medium. The minimal medium contained 5 g/l $(NH_4)_2SO_4$, 5 g/l urea, 0.5 g/l KH_2PO_4, 0.5 g/l K_2HPO_4, 21 g/l MOPS, 0.25 g/l $MgSO_4{\cdot}7H_2O$, 10 mg/l $CaCl_2{\cdot}H_2O$, 10 mg/l $MnSO_4{\cdot}H_2O$, 10 mg/l $FeSO_4{\cdot}7H_2O$, 1 mg/l $ZnSO_4{\cdot}7H_2O$, 0.2 mg/l $CuSO_4$, and 0.2 mg/l biotin [5]. As the carbon source, glucose and/or acetate, ethanol and/or glucose were added to the media at a level of 1% (w/v). BHIS (BHI Sorbitol) medium comprised 3.7% (w/v) brain heart infusion (BHI) medium, and 9.1% sorbitol was used for the preparation of competent cells of *C. glutamicum* [10]. *C. glutamicum* strains used in this work were precultured in BHI medium overnight in a rotary shaker at 200 rpm. The cells were harvested and washed with LB or a buffer containing 5 g/l $(NH_4)_2SO_4$, 5 g/l urea, 0.5 g/l KH_2PO_4, 0.5 g/l K_2HPO_4, and 21 g/l MOPS. These cells were used to inoculate main cultures of LB or minimal medium containing apposite carbon sources. When appropriate, kanamycin and chloramphenicol were added at concentrations of 50 and 10 μg/ml, respectively. The oligonucleotides used in this study were purchased from Cosmo Genetech Co., Ltd, Korea.

DNA Manipulation and Transformation

Standard molecular cloning procedures were followed in this study [27]. The chromosomal DNA from the *C. glutamicum* cells was isolated using a genomic DNA purification kit (SolGent Co.), and the DNA fragments from the agarose gel were eluted using a gel extraction kit (SolGent Co.). The plasmids were introduced into *C. glutamicum* by electroporation as described before [32] using MicroPulser (Bio-Rad Laboratories Inc.).

Construction of Transcriptional Fusion Vectors

The promoter probe transcriptional fusion plasmids were constructed using the vector pSK1CAT [25], which harbors a promoterless *cat* (chloramphenicol acetyltransferase) gene encoding chloramphenicol acetyltransferase (CAT) enzyme. The promoter fragments of the *adhA*, *ald*, *ptsG*, and *glxR* genes were generated by PCR using primers tagged with *Bam*HI and cloned in front of the *cat* gene of pSK1CAT. The recombinant plasmid pAD contained the promoter fragment of *adhA*, 383 bp from the translational start site (255 bp from the transcriptional start site) and 18 bp in the *adhA* gene. The promoter fragment, 318 bp upstream of the translational start site (227 bp from the transcriptional start site) and 23 bp in the *ald* gene were taken for constructing the fusion plasmid pAL. DNA fragments containing 657 bp from the translational start site (399 bp from the transcriptional start site) and 11 bp in the *ptsG* gene were used to construct the fusion plasmid pGP. The promoter fragment that includes 520 bp upstream of the translational start site (400 bp from the transcriptional start site) and 14 bp in the *glxR* gene was amplified to construct the recombinant plasmid p*glxR*. The primers used to amplify the promoter sequences are listed in Table 1.

Table 1. Bacterial strains, plasmids, and oligonucleotides used in this study.

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
E. coli DH5α	F⁻ *thi-I endA1 hsdR17* (r^-m^-) *supE44* Δ*lacU169* (Φ80*lacZ*ΔM15) *recA1 gyrA96 relA1*	Bethesda research laboratories / [14]
C. glutamicum ATCC 13032	Type strain	ATCC
Δ*cyaB*	*cyaB* deletion mutant of *C. glutamicum* ATCC 13032	[5]
Δ*glxR*	*glxR* deletion mutant of *C. glutamicum* ATCC 13032	[26]
Δ*ramB*	*ramB* deletion mutant of *C. glutamicum* ATCC 13032	[13]
Plasmids		
pSK1CAT	*Corynebacterium* promoterless *cat* fusion vector, Km^r	[25]
pAD	pSK1CAT with a 0.4 kb *adhA* promoter	This work
pAL	pSK1CAT with a 0.3 kb *ald* promoter	This work
pGP	pSK1CAT with a 0.6 kb *ptsG* promoter	This work
p*glxR*	pSK1CAT with a 0.534 kb *glxR* promoter	This work
Oligonucleotides	Nucleotide sequence (5′-3′)	Purpose
*ptsG*F	ATCGGATCCTAGTTCCTGCACCTCTTAAG	Cloning of promoter region of *ptsG*
*ptsG*R	ATCGGATCCTTGGACGCCATGTCAAACC	Cloning of promoter region of *ptsG*
*adhA*F	ATCGGATCCGTGGCTGGGGGCATCGAA	Cloning of promoter region of *adhA*
*adhA*R	ATCGGATCCGGGTGCAGCAGTGGTCATAA	Cloning of promoter region of *adhA*
*ald*F	ATCGGATCCCATGCTTGTCGACGCCAC	Cloning of promoter region of *ald*
*ald*R	ATCGGATCCCCTGGATTTGCGTAGACAG	Cloning of promoter region of *ald*

Underlined sequences indicate the recognition sites for restriction enzyme.

Enzyme Assays

For all the assays, except for CAT assay with *ptsG* promoter, the strains were cultivated in minimal medium containing acetate or ethanol or/and glucose. To determine the CAT activity with *ptsG* promoter, the strains were cultivated in LB medium containing glucose or/and acetate. The cells were harvested in the exponential phase, washed twice in 50 mM potassium phosphate, pH 7.0 (for ADH assay) or 100 mM Tris-HCl, pH 7.5 (for ALDH assay) or 50 mM Tris–HCl, pH 7.0 (for CAT assay), and resuspended in the same buffer containing 10 mM $MgCl_2$, 1 mM EDTA, and 30% (v/v) glycerol. The cell suspension was mixed with glass beads (Sigma-Aldrich) and subjected to mechanical disruption using a Mini-Beadbeater (Biospec Products, USA) with intermittent cooling on ice for 1 min. After the disruption, the glass beads and cellular debris were removed by centrifugation (13,000 ×*g*, 4°C, 15 min for CAT, ADH, and ALDH assays, and 45,000 ×g, 4°C, 60 min for ADH and ALDH assays), and the supernatant was used for the assay. The protein concentration was measured using the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin as the standard. The ADH activity was assayed following the increase of NADH at 340 nm in 1 ml of 86 mM sodium pyrophosphate buffer (pH 9.0), 19 mM glycin, 6.2 mM semicarbacide-hydrochloride (pH 6.5), 1.8 mM NAD^+, 1 mM glutathione, and 600 mM ethanol, as described previously [4]. One unit of ADH activity corresponds to the production of 1 μmol of NADH per minute at 30°C. ALDH activity was measured according to a modified protocol described previously [4] following the formation of NADH at 340 nm. One unit of ALDH activity is defined as 1 μmol of NADH formed per minute at 30°C. CAT activity was assayed as described by Shaw [29] with modifications outlined by Schreiner *et al*. [28]. Briefly, the assay mixture (1 ml) contained 100 mM Tris-HCl (pH 7.8), 0.1 mM acetyl-CoA, 1 mM 5,5-dithiobis-2-nitrobenzoic acid, 0.25 mM chloramphenicol, and crude extract. The formation of free 5-thio-2-nitrobenzoate was measured photometrically at 412 nm. One unit of CAT activity corresponds to 1 μmol of chloramphenicol acetylated per minute at 37°C.

Results

Involvement of GlxR in the Expression of Alcohol Dehydrogenase and Acetaldehyde Dehydrogenase

In vitro studies have revealed the binding of GlxR to upstream of the *adhA* and *ald* genes [1, 2] and this regulator protein together with RamA and RamB is responsible for carbon catabolite repression in *C. glutamicum*. *In silico* analysis has revealed the presence of two putative GlxR binding sites, -75 and -104 upstream of the translational start site of the *adhA* gene. Two possible GlxR binding

Table 2. Specific ADH activity of wild type (WT), mutant CgYA (Δ*cyaB*), *glxR* deletion mutant (Δ*glxR*), and complemented mutants.

Strains	Specific activity (nmol/mg/min)[a]		
	Ethanol	Glucose	Ethanol/Glucose
WT	600 ± 44	44 ± 5	74 ± 23
Δ*cyaB*	680 ± 1	95 ± 7	173 ± 27
Δ*glxR*	557 ± 3	145 ± 4	297 ± 10
Δ*cyaB* + pXMJ19-*cyaB*	550 ± 40	14 ± 1	17 ± 8
Δ*glxR* + pXMJ19-*glxR*	602 ± 43	39 ± 2	43 ± 4

[a]The strains were grown in minimum medium containing 1% ethanol and/or 1% glucose, and enzyme activity was assayed in cell extracts from early exponential phase. All the values are from at least three independent cultivations and two determinations per experiment with standard deviations.

Table 3. Specific ALDH activity of wild type (WT), mutant CgYA (Δ*cyaB*), *glxR* deletion mutant (Δ*glxR*), and complemented mutants.

Strains	Specific activity (nmol/mg/min)[a]		
	Ethanol	Glucose	Ethanol/Glucose
WT	162 ± 2	24 ± 2	28 ± 1
Δ*cyaB*	211 ± 16	59 ± 8	67 ± 9
Δ*glxR*	329 ± 7	216 ± 8	348 ± 20
Δ*cyaB* + pXMJ19-*cyaB*	102 ± 12	11 ± 1	11 ± 1
Δ*glxR* + pXMJ19-*glxR*	107 ± 11	19 ± 1	17 ± 4

[a]The strains were grown in minimum medium containing 1% ethanol and/or 1% glucose, and enzyme activity was assayed in cell extracts from early exponential phase. All the values are from at least three independent cultivations and two determinations per experiment with standard deviations

motifs, 72 bp and 44 bp upstream of the start codon of the *ald* gene, have also been reported by *in silico* analysis. These observations suggested a direct interaction of GlxR with the *adhA* and *ald* promoters and prompted us to determine specific ADH and ALDH activities in the *glxR* and *cyaB* deletion mutants. It has been reported that GlxR is a cAMP-dependent transcriptional regulator [16], and thus the experiments with *cyaB* deletion mutant could provide additional indirect evidence for the involvement of GlxR on the expression of genes. The *C. glutamicum* wild type, and *glxR* and *cyaB* deletion mutants were grown in minimal medium containing 1% ethanol and/or 1% glucose, and ADH and ALDH activities were measured. As reported earlier [1, 2], the expressions of ADH and ALDH were repressed when the wild-type strain was grown in medium containing glucose, or glucose plus ethanol, when compared with the expression in ethanol-grown wild type (Tables 2 and 3). In contrast, the deletion mutants derepressed the expression of ADH in glucose, or glucose plus ethanol (Table 2). In the case of ALDH, the activity was derepressed in deletion mutants irrespective of the carbon source in the medium. The specific activity of ADH and ALDH in the *glxR* mutant was increased 3-4- and 9-12-folds, respectively, in glucose and glucose plus ethanol medium compared with those in wild-type. In addition, the ALDH activity of *glxR* deletion mutant in ethanol medium was 2-times higher than that of wild type. Similarly, in the *cyaB* mutant, the ADH and ALDH activities were increased 2-fold in glucose and glucose plus ethanol medium compared with those in the wild type. Thus, when taken together, these results confirm that utilization of ethanol is regulated by CCR, and GlxR represses the expression of the genes *adhA* and *ald*.

To investigate the carbon-source-dependent expressions of *adhA* and *ald* in more detail, the wild type and *cyaB* deletion mutant harboring the recombinant plasmids pAD and pAL were cultivated in minimal medium containing 1% ethanol and/or 1% glucose and the CAT activities determined. In accordance with the ADH enzyme assay results, *cyaB* mutant showed increased specific CAT (promoter) activities, with glucose and with glucose plus ethanol,

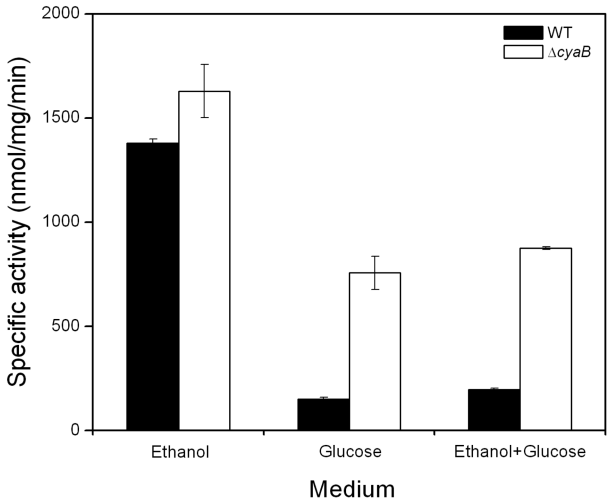

Fig. 1. Specific CAT activities of *C. glutamicum* wild type (WT) and *cyaB* deletion mutant (Δ*cyaB)* carrying the *adhA* promoter fragment in the transcriptional fusion vector pSK1CAT.
The strains were grown in minimal medium containing 1% ethanol and/or 1% glucose. The cells were harvested in the early exponential phase to measure the enzyme activity. All the values are from at least three independent cultivations and two determinations per experiment with standard deviations.

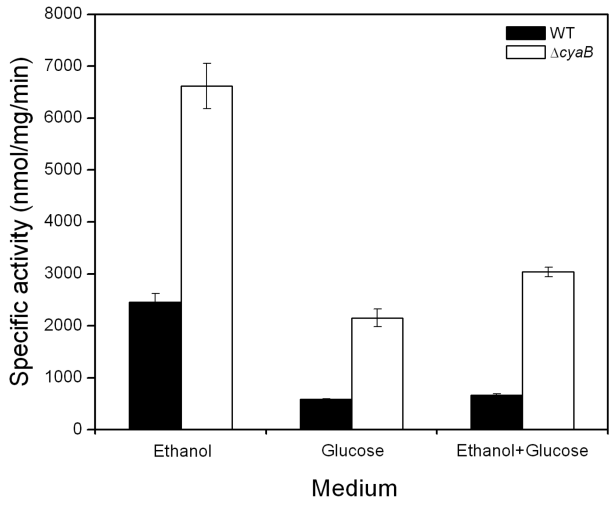

Fig. 2. Specific CAT activities of *C. glutamicum* wild type (WT) and *cyaB* deletion mutant (Δ*cyaB)* carrying the *ald* promoter fragment in the transcriptional fusion vector pSK1CAT.

The cells were harvested in the early exponential phase, after growing in minimal medium containing 1% ethanol and/or 1% glucose. All the values are from at least three independent cultivations and two determinations per experiment with standard deviations.

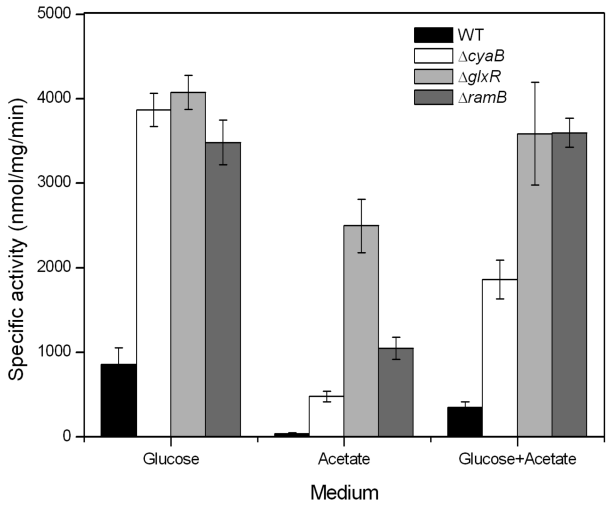

Fig. 3. Specific CAT activities of *C. glutamicum* wild type (WT), mutant CgYA (Δ*cyaB*), *glxR* deletion mutant (Δ*glxR*) and *ramB* deletion mutant (Δ*ramB)* carrying the *ptsG* promoter fragment in the transcriptional fusion vector pSK1CAT.

The cells were grown in LB medium containing 1% glucose and/or 1% acetate, and enzyme activity was assayed in cell extracts from early exponential phase. All the values are from at least three independent cultivations and two determinations per experiment with standard deviations.

when compared with wild type (Fig. 1). The specific CAT activity was 4 times higher in the deletion mutant in glucose and glucose plus ethanol media, compared with those in wild type. In the case of *ald* promoter, the *cyaB* mutant showed increased specific CAT (promoter) activities, when compared with wild type (Fig. 2), irrespective of the carbon source. The specific CAT activity was 2-4 times higher in the deletion mutant in all the media tested, compared with those in wild type. These results conclude that the regulation of *adhA* and *ald* genes occurs by the binding of the cAMP-GlxR complex. Furthermore, we can conclude that GlxR exerts a negative effect on the expression of the *adhA* and *ald* genes.

Expression of *ptsG* Is Controlled by GlxR and RamB

It has previously been reported that a DeoR-type transcriptional regulator, SugR, controls the expression of *ptsG* and other PTS genes during growth on gluconeogenic substrates in *C. glutamicum* [11]. In addition, it has been pointed out that the regulation of *ptsG* in *C. glutamicum* does not depend solely on SugR, but seems to be complex. Another study suggested a putative GlxR binding site upstream of *ptsG*, and this finding was supported by electrophoretic mobility shift assay [17]. To test the transcriptional regulation of *ptsG* by GlxR *in vivo*, the transcriptional fusion plasmid (pGP) carrying the promoter region of *ptsG* was transformed into *C. glutamicum* wild type, *cyaB* deletion mutant, and *glxR* deletion mutant. The strains were cultivated in minimal medium containing glucose (1%), a glucose-acetate substrate mixture (1%), and acetate (1%), and CAT activities were determined. The specific CAT activity was relatively low during growth on acetate compared with glucose-acetate or glucose. With all tested substrates, the specific CAT activities in both the deletion mutants were higher when compared with that of wild type (Fig. 3). The *glxR* deletion mutant showed 67 times higher specific activity in acetate medium compared with that of wild type. In medium containing glucose or glucose plus acetate, the *glxR* deletion mutant showed 5 and 10 times higher levels of expression compared with that in wild type, respectively. In acetate medium, the specific activity in the *cyaB* deletion mutant was 13 times higher than that of wild type *C. glutamicum*. The *cyaB* deletion mutant expressed 5 times higher *ptsG* expression in medium containing glucose or glucose plus acetate, compared with that of wild type. These results conclude that GlxR acts as an essential repressor of the *ptsG* gene in *C. glutamicum*.

In addition to the GlxR binding site, two putative binding sites for the master regulator RamB was reported in the

promoter region of *ptsG* [13]. Since the putative binding sites of RamB (AAATTTTTGCCAA, CAAATTGTGCAAT) are located downstream of the transcriptional start site (+77, +87), it was assumed that RamB might function as a negative regulator of *ptsG* [13]. To test for the effect of RamB on *ptsG* transcription, promoter fusion experiments were performed with the *ramB* deletion mutant and wild-type strain of *C. glutamicum*. For this purpose, plasmid pGP carrying the promoter region of *ptsG* was transformed into the wild type and mutant strain, and after growth in LB containing 1% glucose and/or 1% acetate, the specific CAT activities (*i.e.*, the *ptsG* promoter activities) were determined. As described in Fig. 3, the specific CAT activity was relatively low in wild-type *C. glutamicum* during growth on medium containing acetate. In contrast, the *ramB* deletion mutant showed higher specific CAT activities compared with wild type irrespective of the carbon source (Fig. 3). The promoter activity of *ramB* mutant was found to be 28-fold higher in acetate medium when compared with that of wild type. Four- to 10-fold higher CAT activities were observed in the *ramB* deletion mutant when cultivated with glucose and glucose plus acetate, respectively. These results indicate that RamB is a repressor of the *ptsG* under all the tested carbon sources.

Discussion

GlxR, being the global regulator, controls the expression of over 400 genes in *C. glutamicum* [18]. It was first characterized as a protein that represses the gene *aceB* of the glyoxylate pathway. In *C. glutamicum*, the genes involved in the catabolism of ethanol include *ald*, encoding acetaldehyde dehydrogenase, and *adhA*, encoding alcohol dehydrogenase. Bioinformatic studies have revealed the presence of putative GlxR binding sites upstream of the genes *adhA* and *ald* [18]. Enzyme assay of alcohol dehydrogenase and acetaldehyde dehydrogenase along with the transcriptional fusion experiments of the *adhA* and *ald* promoter regions revealed that GlxR acts as a repressor of these genes. The proteome analysis of wild-type *C. glutamicum* and *glxR* deletion mutant revealed that the alcohol dehydrogenase and acetaldehyde dehydrogenase enzymes are derepressed in the *glxR* deletion mutant compared with that of wild-type (data not shown). Taken together, these results confirm that the ethanol metabolic genes *ald* and *adhA* in *C. glutamicum* are regulated by GlxR, depending on the availability of different carbon sources.

The unpublished data from our laboratory reveals that the cAMP level and *glxR* promoter activity are very high in ethanol medium compared with that in glucose and acetate media [5], thereby suppressing the genes in the glycolytic and gluconeogenic pathways. It has been reported earlier that GlxR represses the master regulator RamB [15]. It could be concluded that the expression of RamB is repressed by the high level of GlxR in ethanol medium, and this might remove the repressive effect of RamB over the genes; *adhA* and *ald* encoding the ethanol catabolic enzymes alcohol dehydrogenase and acetaldehyde dehydrogenase, respectively. In the case of acetate medium, as the *glxR* promoter activity is low, the expressions of glyoxylate and glycolytic pathway enzymes are functional. This might be the reason why *C. glutamicum* is able to utilize glucose along with acetate, in contrast to the case with ethanol, where glucose or acetate cannot be catabolized along with ethanol. In glucose medium, the acetate and ethanol catabolic genes might be repressed because of the high GlxR activity.

In vitro studies have pointed out the involvement of transcriptional regulators, GlxR and RamB, on the expression of *ptsG* in *C. glutamicum*. Gerstmeir *et al*. [13] reported the presence of putative binding sites of RamB, downstream of the transcriptional start site of *ptsG*. Another study revealed the presence of putative GlxR binding sites in the promoter region of *ptsG*, suggesting the involvement of GlxR controlling the expression of *ptsG* [17]. We have carried out *in vivo* experiments to elucidate the role of GlxR and RamB on the expression of *ptsG* in more detail. It has been noticed that in *glxR*, *cyaB,* and *ramB* deletion mutants, the *ptsG* expression was derepressed irrespective of the carbon sources, indicating that GlxR and RamB act as a repressor of *ptsG* in *C. glutamicum*. Earlier studies have revealed the involvement of other regulators, SugR (repressor) and GntR1 and GntR2 (activators), on the expression of *ptsG* [11, 12]. It was noticed that SugR regulates *ptsG* expression in a carbon-source-dependent manner and represses *ptsG* under gluconeogenic carbon sources. GntR1 and GntR2 are functionally redundant transcriptional regulators that activate the expression of *ptsG* in the absence of gluconate. There is a compiled effect of all the five regulators (SugR, GntR1, GntR2, GlxR, and RamB) for the fine-tuning of the expression of *ptsG*. These regulators are the most important players in a complex regulatory network that controls the uptake and metabolism of different carbon sources, allowing the most favorable combination of the available substrates.

As outlined in the introduction, the genes *adhA*, *ald*, and *ptsG* have important roles in the carbon metabolism of *C. glutamicum*. However, there was no complete information on the functional characterization of these genes. In the

present study, we have provided *in vivo* data confirming the binding of different regulators to the promoter region controlling the expression of these genes.

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