Analyses of enzyme II gene mutants for sugar transport and heterologous expression of fructokinase gene in Corynebacterium glutamicum ATCC 13032

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Abstract

Corynebacterium glutamicum ATCC 13032 has four enzyme II (EII) genes of the phosphotransferase system in its genome encoding transporters for sucrose, glucose, fructose, and an unidentified EII. To analyze the function of these EII genes, they were inactivated via homologous recombination and the resulting mutants characterized for sugar utilization. Whereas the sucrose EII was the only transport system for sucrose in C. glutamicum, fructose and glucose were each transported by a second transporter in addition to their corresponding EII. In addition, the ptsF ptsG double mutant carrying deletions in the EII genes for fructose and glucose accumulated fructose in the culture broth when growing on sucrose. As no fructokinase gene exists in the C. glutamicum genome, the fructokinase gene from Clostridium acetobutylicum was expressed in C. glutamicum and resulted in the direct phosphorylation of fructose without any fructose efflux. Accordingly, since fructokinase could direct fructose flux to the pentose phosphate pathway for the supply of NADPH, fructokinase expression may be a potential strategy for enhancing amino acid production.

Keywords: Corynebacterium glutamicum; Sugar transport; Phosphotransferase system; Enzyme II; Fructokinase

1. Introduction

Corynebacterium glutamicum is widely used for the industrial production of amino acids, especially glutamic acid and lysine, thus extensive genetic improvements have been made to overproduce various amino acids [1]. In addition, since the recent release of the complete genome sequence of C. glutamicum ATCC 13032, extensive genome-wide analyses and strain improvements have also been conducted on this amino acid producer [2,3]. However, despite numerous investigations of sugar metabolism and its regulation in C. glutamicum, relatively few studies have been conducted on the sugar transport systems and their regulation on a molecular level [4]. Since Mori and Shioi [5] first reported the presence of a phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS) in C. glutamicum, several biochemical and physiological studies on sugar transport have revealed that sucrose, fructose and...
glucose are taken up by *C. glutamicum* via the PTS [6–9]. Studies on the PTS for sucrose, fructose and glucose are very important, as the major carbon source for industrial lysine fermentation is molasses, which contains these three sugars. Only three PTS genes encoding enzyme I (EI), glucose enzyme II (EII), and β-glucoside EI have so far been characterized from *C. glutamicum* [10–12]. As one of the strategies for improving amino acid production, the PTS may be a good target because it is the first step of sugar metabolism and PTS-associated global carbon regulation would also seem to play an important role in sugar metabolism [4,13]. Accordingly, in this study, the EII genes identified from the public genome sequence data of *C. glutamicum* were inactivated in order to analyze the sugar transport characteristics of the EII mutants. In addition, the heterologous expression in *C. glutamicum* of a fructokinase gene from *Clostridium acetobutylicum* was performed to phosphorylate intracellular fructose directly without fructose efflux. This would modify the fructose entry point from fructose 1-phosphate (F-1-P) to fructose 6-phosphate (F-6-P) and thus increase the flux through the pentose phosphate pathway (PPP) for the supply of anabolic reducing power.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

The strains and plasmids used in this study are described in Table 1. *C. glutamicum* ATCC 13032 was grown at 30 °C in LB or brain heart infusion (BHI) media. The minimal media contained 5 g l⁻¹ (NH₄)₂SO₄, 5 g l⁻¹ urea, 0.5 g l⁻¹ KH₂PO₄, 0.5 g l⁻¹ K₂HPO₄, 20.9 g l⁻¹ MOPS, 0.25 g l⁻¹ MgSO₄·7H₂O, 10 mg l⁻¹ CaCl₂·H₂O, 10 mg l⁻¹ MnSO₄·H₂O, 10 mg l⁻¹ FeSO₄·7H₂O, 1 mg l⁻¹ ZnSO₄·7H₂O, 0.2 mg l⁻¹ CuSO₄, and 0.2 mg l⁻¹ biotin with various sugars (1%, w/v). If necessary, kanamycin and chloramphenicol were added to 5 and 7 µg ml⁻¹, respectively.

2.2. DNA manipulations and computer analysis

Routine recombinant DNA manipulations were performed according to standard protocols. The electroportation of *C. glutamicum* was performed as described by Liebl et al. [14]. To analyze EII genes of the PTS in the *C. glutamicum* genome, databank searches were performed using the BLAST service of the NCBI (http://www.ncbi.nlm.nih.gov). The DNASTar software package (DNASTar Inc.) was used to process DNA sequence data and the pfam program (http://www.sanger.ac.uk) used for domain analyses.

2.3. Construction of insertion or deletion mutants of EII genes

The defined chromosomal insertions within each EII gene from *C. glutamicum* were constructed using the pSUP301 plasmid [15]. For this approach, the internal regions of each EII gene were amplified via PCR using chromosomal DNA as the template. The three primer sets were constructed as follows: 5'-CTACTGCAAGCTATCACAAATGTTGG-3'/5'-CTACTGCAAGTTGCCAGATCCAGAAAC-3' (ptsS), 5'-TATCTGG-
2.5. Measurement of sugar concentration and enzyme assays

Culture samples (1 ml) were centrifuged at stated intervals and the concentration of sucrose and fructose in the supernatants determined using Sucrose/D-glucose/D-fructose assay Kit (R-Biopharm), as recommended by the supplier. To establish the fructokinase activity in cell-free extracts, C. glutamicum harbouring the recombinant plasmid pXFK1 was washed with 50 mM Tris–HCl (pH 7.0) and lysed by sonication at 4 °C. The cell debris was discarded after centrifugation and the protein concentrations of the resulting supernatant determined. The fructokinase activity was determined at 30 °C by measuring the formation of NADPH in a coupled enzyme reaction [20]. The fructose 1-phosphate kinase was assayed by coupling the formation of fructose 1,6-bisphosphate to the oxidation of NADH [21].

3. Results

3.1. Site-specific disruption of EII genes and analyses of growth phenotypes of the mutants

Based on a genome sequence analysis of C. glutamicum ATCC 13032 (GenBank Accession No. NC_003450), the presence of three genes encoding putative PTS enzyme IIs in addition to the glucose EII gene (ptsG), which was previously characterized by our group, has been suggested [2,12]. Using a similarity search, the two EII homologues, ptsS (NCgl2553) and ptsF (NCgl1861), have been predicted to be connected to sucrose and fructose transport, respectively [2]. Thus, to analyze the function of the three EII genes identified in the C. glutamicum genome, these EII genes were inactivated on the chromosome by the intracellular sucrose 6-phosphate formed by the PTS is hydrolyzed into glucose 6-phosphate and fructose by sucrose 6-phosphate hydrolase [2,22]. The disruption of the putative sucrose 6-phosphate hydrolase
gene (scrB, NCgl2554), which is located upstream of the sucrose EII gene, also resulted in the strain being unable to utilize only sucrose, just like the ptsS mutant (data not shown). When the genes ptsS and scrB were introduced into C. glutamicum after cloning by PCR in E. coli, these genes complemented the growth deficiency of the corresponding mutants on the sucrose minimal medium (data not shown), indicating that sucrose EII is the only transporter for sucrose in C. glutamicum.

Although the ptsF mutant (FR1) was impaired in fructose utilization, it still exhibited very weak growth on the fructose minimal medium (Fig. 1(B)), indicating that even though the gene for fructose EII was disrupted, the ptsF mutant was still able to consume fructose at a reduced rate and retained some capacity to transport fructose. Previously, based on an analysis of a xylitol-resistant mutant lacking fructose PTS activity isolated following treatment with ethylmethanesulfonate, Dominguez and Lindley [6] reported that fructose was also transported by glucose EII in addition to the major fructose EII. Hence, to examine the other transporter responsible for fructose transport a ptsF ptsG double mutant was constructed. In contrast to the ptsF mutant, this mutant did not show any growth on the fructose minimal medium (data not shown). Thus, the weak growth on the fructose minimal medium by the mutant lacking fructose EII was due to the transport of fructose by glucose EII, indicating that glucose EII plays a minor transport role for fructose in addition to transporting glucose and mannose. Also, the ptsG mutant exhibited very weak growth on the minimal medium containing 1% (w/v) glucose, yet its growth rate increased with increasing glucose concentration (2–4%) (data not shown). The current authors previously reported on the presence of a glucokinase gene for phosphorylating intracellular free glucose [23]. The presence of a glucokinase gene and the growth phenotype of the ptsG mutant with a high glucose concentration would seem to suggest the existence of another non-PTS glucose transport system with a low affinity for glucose.

3.2. Characteristics of ptsF mutant on sucrose minimal medium

As previously mentioned, sucrose 6-phosphate hydrolase hydrolyzes the sucrose 6-phosphate formed by the sucrose PTS into glucose 6-phosphate and fructose [22]. Using ethylmethanesulfonate-induced xylitol-resistant mutants lacking fructose PTS activity, Dominguez et al. [6,7] reported that C. glutamicum excreted fructose into the culture broth during growth on sucrose medium, and subsequently transported the extracellular fructose back into the cell by a fructose PTS, resulting in the formation of intracellular fructose 1-phosphate. In the current study, wild-type C. glutamicum accumulated only a small amount of fructose (10 mM) in the culture broth very early in the exponential growth phase, and this was rapidly consumed during the rest of the exponential growth phase (Fig. 2(A)). In contrast to the wild-type strain, the ptsF mutant accumulated fructose in the culture broth during growth on the sucrose minimal medium (Fig. 2(B)). During exponential growth, fructose was released into the external medium at almost the same molar concentration as the sucrose consumed (Fig. 2(B)), indicating that the intracellular fructose derived from sucrose 6-phosphate hydrolysis was excreted into the culture broth. The excreted fructose was not utilized until the sucrose was completely depleted from the medium, after approximately 18 h (Fig. 2(B)). To confirm whether another transporter was responsible for the entry of excreted fructose after sucrose depletion in the ptsF mutant, the ptsF ptsG double mutant was used for growth and fructose efflux experiments on the sucrose medium. As expected, the double mutant was unable to use the excreted fructose at all and accumulated fructose in the culture broth without consumption (Fig. 2(C)). These results corresponded to those of Dominguez and Lindley [6] when using a 2-deoxyglucose and xylitol-resistant double mutant induced by ethylmethanesulfonate that lacked fructose and mannose PTS activity. As shown in Fig. 2(B), when the ptsF mutant was grown on the sucrose minimal medium, it exhibited
poor growth compared with the wild-type. The sucrose consumption of the \textit{ptsF} mutant was also slower compared to that of the wild-type, which coincided with the growth retardation. To examine whether the growth deficiency of the \textit{ptsF} mutant on the sucrose medium was caused by the loss of the fructose EII function, a recombinant plasmid that expressed the fructose EII gene was introduced into the \textit{ptsF} mutant. The introduced fructose EII gene re-established the growth of the \textit{ptsF} mutant on the sucrose minimal medium (data not shown).

3.3. Expression of a heterologous fructokinase gene in \textit{C. glutamicum}

Since the fructose produced by sucrose hydrolysis is converted to fructose 1-phosphate via efflux followed by import through fructose EII, this raised the idea of making a pathway for the direct phosphorylation of intracellular fructose using fructokinase without any fructose efflux. Modifying the fructose metabolic pathway from fructose EII to fructokinase could alter the route of metabolism. As there is no fructokinase gene in \textit{C. glutamicum} [2], the effect of expressing a heterologous fructokinase gene in \textit{C. glutamicum} on carbon metabolism during growth on sucrose medium was investigated. First, the fructokinase gene (\textit{scrK}) o of \textit{C. acetobutylicum} was cloned by PCR [18] using an \textit{E. coli}–\textit{C. glutamicum} shuttle vector [19], then the resulting plasmid pXFK1 was introduced into \textit{C. glutamicum} ATCC 13032 and its \textit{ptsF} mutant. The fructokinase gene was expressed well, apparently from its own promoter, and fructokinase activity was detected in crude extracts of the \textit{C. glutamicum} strains harbouring pXFK1 (data not shown). The recombinant \textit{ptsF} mutant harbouring pXFK1 seemed to phosphorylate the intracellular fructose without any fructose efflux, as no fructose was accumulated in the culture broth in contrast to the \textit{ptsF} mutant containing the control vector pXMJ19 (Figs. 3(A) and (B)). The growth of the recombinant strain was enhanced relative to that of the \textit{ptsF} mutant harbouring the control vector (Figs. 3(A) and (B)), thereby indicating that the fructose was efficiently phosphorylated by the fructokinase and the resulting fructose 6-phosphate was used to enhance growth. In addition, the expression of the fructokinase gene in the wild-type \textit{C. glutamicum} also seemed to result in the direct phosphorylation of the fructose without fructose efflux, as no fructose was detected in the early exponential growth phase in contrast to the strain harbouring the control vector (Figs. 3(C) and (D)). However, interestingly, growth of the wild-type strain harbouring the fructokinase gene was noticeably retarded during the initial stage of growth (Fig. 3(D)). Although the reason for the growth retardation was obscure, the initial growth retardation phenomenon was similar to that of the \textit{ptsF} mutant on the sucrose medium, as shown in Fig. 2(B).

4. Discussion

Recently, Kalinowski et al. [2] reported on the complete genome sequence of \textit{C. glutamicum} and suggested the presence of two additional EII genes for sucrose and fructose besides that for glucose based on a similarity search of the genome database. In this study, we constructed and analyzed three EII-specific knockout mutants. \textit{C. glutamicum} has four EII genes, including
an unidentified EII, in its genome (Fig. 4). Similarly, the closely related Corynebacterium diphtheriae has three EII genes, but lacks a sucrose EII gene. As previously reported through biochemical studies and genome sequence analyses, ATP-dependent fructokinase is not present in C. glutamicum, in contrast to most sucrose operons from other bacteria [6,18]. Meanwhile, the deduced protein of fructose EII consists of 688 amino acids, is a IIABC type, and shares a 50% identity to the putative fructose EII from C. diphtheriae [24], with the only difference being the presence of an additional gene for a fructose 1-phosphate kinase homologue (Ncgl1857) between the genes for the EI and a second homologue of a DeoR-type transcriptional regulator in the C. glutamicum genome. The similarity between the two fructose 1-phosphate kinase homologues is 47%. The deletion mutant of the gene for the fructose 1-phosphate kinase (Ncgl1860) exhibited deficient growth on fructose minimal medium and also did not show enzyme activity, whereas the deletion mutant of the gene for the fructose 1-phosphate kinase homologue (Ncgl1857) did not show any change in the growth phenotype on fructose or enzyme activity of fructose 1-phosphate kinase (data not shown).
Previously, we annotated glucose EII as a mannose EII, because the cloned gene exhibited a substrate specificity for mannose and 2-deoxyglucose as well as glucose [12]. However, based on its high sequence similarity with others in the glucose/sucrose EII family and the growth phenotype of the mutant, its designation as a glucose EII is more appropriate [2,4]. Since glucose EII and sucrose EII belong to the same glucose/sucrose transporter family and are both IIBCA types, they show high similarity (46%). There is a high level of sequence conservation surrounding the putative phosphorylation sites, a histidyl and a cysteyl residue of the IIA and IIB-domains, respectively [12]. In addition, the regulation of the glucose EII gene expression was examined under different growth conditions using Northern blot analysis. Several different carbon sources, including glucose, fructose, maltose, sucrose, and pyruvate, were used. The expression of ptsG was found to be constitutive, regardless of the sugar, in contrast to E. coli or Bacillus subtilis [25,26] (data not shown). To regulate the expression of the ptsG gene, ribonucleic terminator (RAT) sequences in the region upstream of the ptsG gene are present in many bacteria, including C. diphtheria and B. subtilis [18,24]. However, the current study did not find any potential RAT sequences in the region upstream of the ptsG gene in C. glutamicum. In addition, no gene for an antiterminator protein homologue, such as GlcT in C. diphtheria or ScrT in C. acetobutylicum, was found near the ptsG and ptsS genes of C. glutamicum [18,24]. The fourth EII gene (Ncgl2934) encodes a IIAB protein of the fructose/mannitol family (Fig. 4), which shares 76% similarity with a putative IIAB Mtl of C. acetobutylicum R (GenBank Accession No. AF508972) has already been reported by Kotrba et al. [11]. Although no bglF homologue was found in the genome sequence of C. glutamicum ATCC 13032, interestingly, a part of bglG encoding an antiterminator protein was identified as described by Kotrba et al. [11].

Lastly, the heterologous fructokinase gene was successfully expressed in C. glutamicum, thereby modifying the intracellular fructose pool. Kiefer et al. [9] suggested that modifying the fructose flux from the fructose PTS to the mannose PTS could lead to an increased level of flux through the pentose phosphate pathway (PPP), resulting in an increased supply of NADPH for enhanced lysine production. Similarly, the expression of fructokinase could also increase the flow to the PPP on a sucrose medium, as this enzyme can directly phosphorylate intracellular fructose into fructose 6-P instead of fructose 1-P, which is formed by fructose EII. Therefore, changing the route of fructose entry into metabolism via fructokinase could be beneficial for amino acid production during growth on sucrose. In addition, fructokinase uses ATP instead of PEP as a phosphory donor, thus fructokinase expression would seem to be a potential strategy for enhancing amino acid production. Future studies are still required to elucidate the proteins and metabolites that are affected by fructokinase expression, resulting in growth retardation. Also the effect of fructokinase expression on the yield of amino acid production needs to be examined.

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References


