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Cloning and Expression of the Gene Encoding Mannose Enzyme II of the *Corynebacterium glutamicum* Phosphoenolpyruvate-Dependent Phosphotransferase System in *Escherichia coli*

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The gene for mannose enzyme II of phosphoenolpyruvate-dependent phosphotransferase system from *Corynebacterium glutamicum* KCTC 1445 was cloned into *Escherichia coli* ZSC113 using plasmid pBR 322. The recombinant plasmid, designated pCTS3, contained 2.2 kb DNA fragment, and the physical map of the cloned DNA fragment was determined. The *E. coli ptsM ptsG* mutant transformed with pCTS3 restored glucose and mannose fermentation ability, and grew well on these sugars as the sole carbon source in the minimal medium. The transformant harboring pCTS3 showed a PTS-mediated repression of growth on maltose by mannose analogue, 2-deoxyglucose. The specificity of the response to 2DG therefore indicates that the cloned DNA fragment carries mannose enzyme II gene.

The bacterial phosphoenolpyruvate (PEP) dependent: carbohydrate phosphotransferase system (PTS) plays a central role in the uptake of a number of carbohydrate in both Gram-positive and Gram-negative bacteria (12, 14, 17). The PTS catalyzes the translocation of carbohydrate across the bacterial membrane and their concomitant phosphorylation. The PTS consists of two cytoplasmic phosphorylated carrier protein, enzyme I and HPr (which are not sugar-specific) and a number of sugar specific membrane bound enzyme II. For certain carbohydrate, a third cytoplasmic intermediates in the phosphorylating cascade exists, designated enzyme III. Enzyme I and HPr are required as general phosphocarrier proteins for the transport and phosphorylation of all PTS sugars. Sugar specificity is determined by enzyme II complex (8, 9, 15). Individual sugar permeases of the bacterial PTS consist either of a single polypeptide chain (an Enzyme II; Molecular weight≈ 68,000) or of two polypeptide chains (an Enzyme II-III pair; total molecular weight≈ 68,000) (18). It has been suggested that PTS permease posseses soluble enzyme III as a general rule in Gram-positive bacteria (13). In *E. coli* enzyme II^{Man} complex consists of at least two integral membrane protein, EII-P^{Man} and EII-B^{Man}, and phosphorylates mannose, glucose, N-acetylglucosasmine and their analogues (3, 4, 5). The phosphate group from intracellular PEP is sequencially transferred to enzyme I, to HPr, to a set of sugar-specific enzyme II or EII-EIII pair, and finally to an incoming specific carbohydrate molecule (20). In addition to sugar transport and phosphorylation function, the PTS is involved in metabolic regulation (12, 19, 20). In enterobacteria, enzyme III^{Glc}($EIII^{Glc}$) serves as a central regulatory protein, controlling the activity of adenylate cyclase, a variety of non-PTS carbohydrate permeases, and catabolic enzymes (7, 19, 20).

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Abbreviation: PTS, phosphoenolpyruvate:glycose phosphotransferase system; PEP, phosphoenolpyruvate; EI, enzyme I of the PTS; HPr, histidine-containing phosphocarrier protein; EII, enzyme II of the PTS; EII^{Man}, mannose enzyme II of the PTS; EIII, enzyme III of the PTS; $EIII^{Glc}$, membrane-associated subunit of the glucose permease; EII-P^{Man} and EII-B^{Man}, membrane-linked subunit of the mannose permease; Glc^+ Man^+, capable of glucose and mannose fermentation; αMG, methyl α-D-glucoside; 2DG, 2-deoxyglucose.

Corynebacterium glutamicum has long been used industrially for the large scale production of amino acids, mainly L-glutamic acid and L-lysine. But not many studies about glucose or mannose uptake system of *C. glutamicum* have been done. The present experiments are concerned with mannose receptor, enzyme II^{Man}, among the PTS proteins of *C. glutamicum*. We describe the molecular cloning of the mannose enzyme II gene and phenotypic characterization of *E. coli* transformant carrying *C. glutamicum* mannose enzyme II gene.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

The genotypes of bacterial strains and plasmids used in these studies are listed in Table 1. *C. glutamicum* KCTC 1445 was used as a source of PTS EII gene. *E. coli* ZSC113 served as a host for transformation. Plasmid pBR322 was used as a vector for the cloning of genes.

Media and Culture Condition

C. glutamicum KCTC 1445 and *E. coli* were cultured at 37℃ in a LB broth (10 g of trypton, 5 g of yeast extract, and 10 g of NaCl per liter of distilled water, pH 7.0). *E. coli* transformants harboring EII gene were selected on MacConkey agar base supplemented with 30 μg of ampicillin/ml and 1% glucose. *E. coli* ZSC113 harboring various plasmids was grown in medium A (1 g of $(NH_4)_2SO_4$, 10.5 g of K_2HPO_4, 4.5 g of KH_2PO_4, and 1 g of $MgSO_4$ per liter of distilled water) supplemented with 50 μg of thiamine/ml, 30 μg of ampicillin/ml and 0.2% glucose or maltose as a sole carbon source (2).

Preparation of DNA and Plasmids Construction

The plasmid DNA from *E. coli* was prepared by cesium chloride-ethidium bromide centrifugation of cleared lysate (11). For rapid isolation of plasmids from the *E. coli* transformants, the alkaline lysis method described by Birnboim and Doly (19) was employed. Chromosomal DNA of *C. glutamicum* was isolated from cells of mid-exponential phase cultured in LB broth supplemented with 1% glycine according to the preparative method described by Rodriguez and Tait (16). Plasmid DNA and their restriction digests were analyzed by electrophoresis using 0.7% to 1% agarose gel.

Construction of Gene Library

50 μg of chromosomal DNA purified from *C. glutamicum* KCTC 1445 was partially digested with *Sau*3AI, and DNA fragment ranging from 2 to 10 kb were isolated by sucrose gradient centrifugation for 20 hrs at 25, 000 rpm in a Beckman SW40 roter. The *Sau*3AI-generated chromosomal DNA fragments (3 μg) were ligated to pBR322 (1 μg) which was digested with *Bam*HI and dephosphorylated using calf intestinal alkaline phosphatase(Boeringer Mannheim). The ligation mixture was used to transform *E. coli* ZSC113.

Test for Fermentation Ability of Transformants

The ability to ferment sugars was demonstrated by streaking the transformants on MacConkey agar base containing 1% sugars. MacConkey indicator plates were used to moniter sugar fermentation. The various *E. coli* PTS EII mutants strains formed white, nonfermenting colonies on this medium, in contrast to transformants which ferment sugar formed a red colony, indicating efficient transport and metabolism of sugar.

Phenotypic Properties of Transformed Cells

Several *E. coli ptsM ptsG* mutants harboring various plasmids were tested for growth in medium A supplemented with ampicillin (30 μg/ml), thiamine (50 μg/ml), and 0.2% glucose or mannose. *E. coli ptsM ptsG* mutants harboring various plasmids were tested for repression of growth on non-PTS sugars in the presence of methyl α-glucoside or 2-deoxyglucose. The cultures were grown at 37℃ in a medium A containing 0.2% maltose as the sole carbon source, and the optical density was measured at 600 nm. When the cultures reached an optical density of ≈ 0.15, αMG (Sigma) or 2DG (Sigma, grade III) was added to a final concentration of 10 mM.

RESULTS AND DISCUSSION

Table 1. Bacterial strains and plasmids

Strains/plasmids	Relevant properties	Reference or source
C. glutamicum KCTC 1445		KCTC
E. coli		
ZSC113	*ptsM ptsG glK lacZ rha rpsL relA HFr leu136 thi*	2
236Sr	lacZ4 mtlA9 rpsL8 *SupE44*Δ*gut recA*	10
JF201	Δ*lac*(X74)Δ(*pho-bgl*)*ara*B1$^-$	6
pBR322	Apr, Tcr	
pCTS10-pCTS13	Man$^+$, Glc$^+$	This study
pCTS3	Man$^+$, Glc$^+$	This study

Table 2. Restriction enzyme analysis of the different cloned EII genes

Recombinant plasmids	Fragments size of plasmids digested with		Total size of insert(Kb)
	*Pst*I	*Hin*dIII	
pCTS10	4.2, 3.0, 2.2, 0.4	4.6, 3.0, 2.2	5.5
pCTS11	4.2, 2.8, 2.2, 0.4	4.6, 2.4, 2.2, 0.4	5.3
pCTS12	5.8, 2.2, 1.7	7.1, 2.2, 0.4	5.4
pCTS13	4.4, 2.8, 2.2, 0.4	4.8, 2.4, 2.2, 0.4	5.5

Cloning of the DNA Fragments Carrying EII^{Man} Gene

The genomic library of *C. glutamicum* KCTC 1445 was constructed in *E. coli* ZSC113 using pBR322 as a vector. Since *E. coli* ZSC113 lacks both glucose permease and mannose permease activity, it could not ferment glucose nor mannose. In order to detect *E. coli* clones exhibiting mannose or glucose EII activity, *E. coli* transformants were screened for glucose fermentation on MacConkey agar base supplemented with 1% glucose as a carbon source. Glucose fermenting colonies showed red color. Among approximately 5,000 transformants, 4 positive clones showing red color on MacConkey fermentation indicator plate were obtained. In order to investigate the cloned genes, plasmid DNAs were isolated from the *E. coli* clones, and named as pCTS10, 11, 12, and 13, respectively. After digesting them with *Pst*I and *Hin*dIII, the digested plasmids were analyzed by agarose gel electrophoresis. It was identified that these plasmids share a common EII gene although they are different in length of the chromosomal DNA franking the gene on both side (Table 2).

Subcloning and Physical map of EII^{Man} Gene

As shown in Table 2, all isolated hybrid plasmids contained 2.2 kb *Hin*dIII fragment in the insert. In order to reduce the size of cloned gene, the common 2.2 kb *Hin*dIII insert was prepared from hybrid plasmid pCTS 13 by electroelution. The hybrid plasmid, designated pCTS3, was constructed by subcloning the 2.2 kb *Hin*dIII fragment into pBR322. The *E. coli ptsM ptsG* mutants transformed with recombinant plasmid pCTS3, could ferment glucose and mannose, indicating that the 2.2 kb *Hin*dIII fragment carried the EII gene (Fig. 1). It was also found that the transformant grew well in the minimal medium using these substrates as the sole carbon source. A restriction endonuclease map of hybrid plasmid pCTS 3 is shown in Fig. 2.

Substrate Specificity of EII^{Man} Coded by pCTS3

To determine the sugar specificity of the cloned gene, *E. coli* transformant harboring pCTS3 was examined for fermentation ability on several sugars. In enteric bacteria, two PTSs acting on glucose, the glucose- PTS and mannose-PTS, have been reported(12). The former is

Fig. 1. Photograph of mannose fermentation of *E. coli* transformants on MacConkey agar base supplemented with 1% mannose.
A; *E. coli* ZSC113(pBR322), B; *E. coli* ZSC113 (pCTS3)

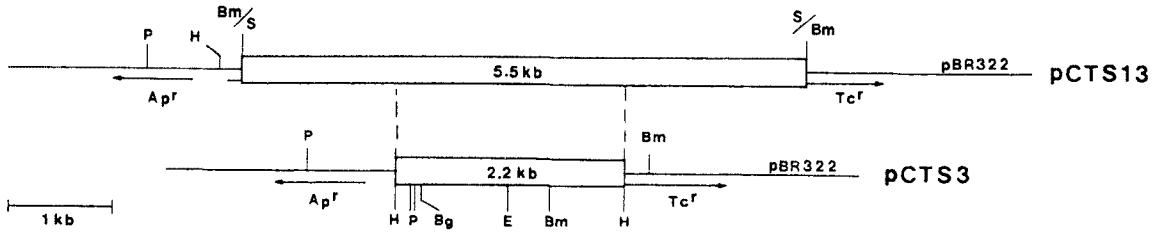

Fig. 2. Restriction endonuclease map of pCTS3.
The thin line represents the plasmid vector pBR322 and rectangle represents chromosomal DNA corresponding to mannose Enzyme II gene derived from *C. glutamicum*. Restriction enzymes used are: Bg; *Bgl*II, Bm; *Bam*HI, E; *Eco*RI, H; *Hin*dIII, P; *Pst*I, S; *Sau*3AI.

Table 3. Sugar fermentation ability of *E. coli* clones carrying recombinant plasmids

Substrates	*E. coli* EII mutants harboring the plasmid	
	pCTS3	pBR322
Glucose	+	−
Mannose	+	−
Salicine(or Arbutin)	−	−
Mannitol	−	−
Glucitol	−	−

All fermentation responses were recorded on MacConkey fermentation plates containg 1% carbohydrate. +; fermentation (red colony), −; little or no fermentation(white colony).

inducible and more active on glucose and αMG than on mannose, and 2DG, while the latter is constitutive and active on glucose, mannose, glucosamine and 2DG but only a little on αMG, and N-acethylglucosamine. As shown in Table 3, EII^{Man} of *C. glutamicum* had substrate specificity for glucose and mannose, but not for β-glucoside(salicine or arbutin), mannitol, and glucitol. The sugar substrate specificities of EII^{Man} of *C. glutamicum* were similar to those of enteric bacteria. In fact, several species of Gram-positive bacteria have constitutive PTSs whose substrate specificities correspond to those of the glucose (Glc, and αMG) or mannose (Glc, Man, 2DG) system in enteric bacteria (12).

PTS-mediated Repression by 2-DG

PTS-mediated regulation of uptake and utilization of non-PTS sugars have been reported in Gram-negative and Gram-positive bacteria (2, 12, 19, 20). It is expected that characteristic of Man^+ cell is a PTS mediated repression by mannose analogue, 2DG. The 2DG inhibits the cells from utilizing non-PTS sugars such as glycerol, or maltose. In addition, 2DG can be transferred into the cell and be phosphorylated by the PTS, but the phosphorylated 2DG cannot be metabolized and it is accumulated in the cell. We tested the repression of cell growth on maltose in the presence of 2-DG and glucose analogue, methyl α-glucoside. As shown in Fig. 3, *E. coli* ZSC113 containing pBR322 and transformant harboring recombinant plasmid pCTS3 grew well on non-PTS sugar, maltose, as a sole carbon source. When 2DG or αMG was added to the medium, the control strains did not show repression and continued to grow normally. The control strains containing pBR322 did not show PTS-mediated repression in the presence of αMG or 2DG. But the transformant harboring pCTS3 showed an immediate repression of growth by 2DG, but it did not show significant repression by αMG. Similar results were obtained when non-PTS sugar, glycerol, was used instead of maltose (date not shown). The specificity

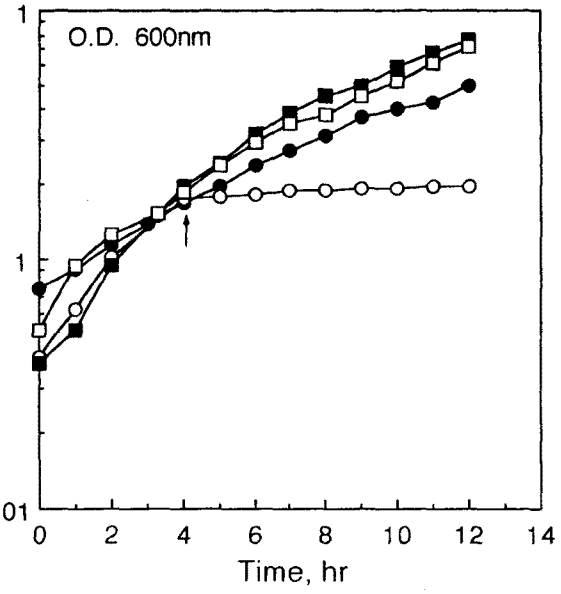

Fig. 3. Repression of growth on non-PTS sugar by non-metabolizable sugar analogue.
αMG was added to be a final concentration of 10 mM at the point indicated by the arrow.
●−●; pCTS3/αMG, ○−○; pCTS3/2DG, ■−■; pBR322/αMG, □−□; pBR322/2DG.

of the response to 2DG therefore indicates that the cloned DNA fragment carries the gene encoding Ezyme II^{Man}.

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REFEREMCES

1. **Birnboim, H.C. and J. Doly.** 1979. A rapid alkaline extraction procedure of screening recombinant plasmid DNA. *Nucleic Acid Res.* **7**: 1513-1523.
2. **Bouma, C.L., N.D. Meadow, E.W. Stover, and S. Roseman.** 1987. II-B^{Glc}, a glucose receptor of the bacterial phophotransferase system : Molecular cloning of *ptsG* and purification of the receptor from an overproducing system of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA.* **84**: 930-934.
3. **Erni, B. and B. Zanolari.** 1985. The mannose-permeases of the bacterial phophotransferase system. *J. Biol. Chem.* **260**: 15495-15503.
4. **Erni. B., B. Zanolari and H.P. Kocher.** 1987. The mannose permease of *Escherichia coli* consists of three different proteins. *J. Biol. Chem.* **262**: 5238-5247.
5. **Erni. B., B. Zanolari, P. Graff and H.P. Kocher.** 1989. Mannose permease of *Escherichia coli*. *J. Biol. Chem.* **264**:

18733-18741.

6. **Karin schnetz, Christian toloczyki, and Bodo Rak.** 1987. β-glucoside (*bgl*) operon of *Escherichia coli* K-12: Nuclotide sequence, genetic organization, and possible evolutionary relationship to regulatory components of two *Bacillus subtilis* genes. *J. Bacteriol.* **169**: 2579-2590.
7. **Kathleen A. Presper, Cing-Yuen Wong, Ninda Liu, and Norman D. Meadow.** 1989. Site-directed mutagenesis of the phosphocarrier protein, III^{Glc}, a major signal-transducing protein in *Escherichia coli*. *Proc. Natl. Acad.Sci. USA.* **86**: 4052-405510.
8. **Lengeler, J.W.** 1990. Molecular analysis of the Enzyme II-complexes of the bacterial phosphotransferase system (PTS) as carbohydrate transport systems. *Biochimica et Biophysica Acta.* **1018**: 155-159.
9. **Lengeler, J.W., F. Titgemeyer, A.P. Voger and B.M. Wohrl.** 1990. Structures and homologies of carbohydrate : phosphotransferase system (PTS) proteins. *Phil. Trans. R. Soc. Lond. B.* **326**: 489-504.
10. **Mamoru Yamada and M.H. Saier Jr.** 1988. Positive and negative regulations for glucitol (*gut*) operon expression in *Eschericha coli*. *J. Mol.Biol.* **203**: 569-583.
11. **Maniatis, T., E.F. Fritsh and J. Sambrook.** 1982. *Molecular cloning-A Laboratiry Manual.* p.93-94. Cold Spring Harbor.
12. **Meadow, N.D., D.K. Fox and S. Roseman.** 1990. The bacterial phosphoenolpyruvate glycose phosphotransferase system. *Annu. Rev. Biochem.* **59**: 497-542.
13. **Postma, P.W. and J.W. Lengeler.** 1985. Phosphoenolpyruvate:carbohydrate phosphotransferase system of bacteria. *Microbiol. Rev.* **49**: 232-269.
14. **Reizer J., M.H. Saier, Jr., J. Deutcher, F. Grenier, J. Thompson and W. Hengstenberg.** 1988. The phosphoenolpyruvate: sugar phosphotransferase system in Gram-positive bacteria: properties, mechanism, and regulation. *Crit. Rev. Microbiol.* **15**: 297-338.
15. **Robillard, G.T. and J.S. Lolkema.** 1988. Enzymes II of the Phosphoenolpyruvate-dependent sugar transport systems: a review of their structure and mechanism of sugar transport. *Biochimica et Biophysica Acta.* **947**: 493-519.
16. **Rodriguez, R.L. and R.C. Tait.** 1983. *Recombinant DNA Techniques-An Introduction.* p.45-46. Addison-Weslley Pub. Co.
17. **Saier, M.H., Jr.** 1985. *Mechanisms and regulation of carbohydrate transport in bacteria*, Academic press, Orlando, FL.
18. **Saier, M.H., Jr., M. Yamada, B. Erni, K. Suda, J. Lengeler, R. Ebner, P. Argos, B. Rak, K. Schnetz, C.A. Lee, G.C. Stewart, F. Breidt, Jr., E.B. Waygood, K.G. Peri and R.F. Doolittle.** 1988. Sugar Permeases of the bacterial phosphoenolpyruvate-dependent phosphotransferase system: sequence comparisons. *FASEB. J.* **2**: 199-208.
19. **Saier, M.H., Jr.** 1989. Protein phosphorylation and allosteric control of inducer exclusion and catabolite repression by the bacterial phosphoenolpyruvate:sugar phosphotransferase system. *Microbiol. Rev.* **53**: 109-120.
20. **Saul Roseman and N.D. Meadow.** 1990. Signal transduction by the bacterial phosphotransferase system. *J. Biol. Chem.* **265**: 2993-2996.

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