

Corynebacterium glutamicum: a Dissection of the PTS

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Abstract

The high-GC Gram-positive actinomycete *Corynebacterium glutamicum* is commercially exploited as a producer of amino acids that are used as animal feed additives and flavor enhancers. Despite its beneficial role, carbon metabolism and its possible influence on amino acid metabolism is poorly understood. We have addressed this issue by analyzing the phosphotransferase system (PTS), which in many bacteria controls the flux of nutrients and therefore regulates carbon metabolism. The general PTS phosphotransferases enzyme I (EI) and HPr were characterized by demonstration of PEP-dependent phosphotransferase activity. An EI mutant exhibited a pleiotropic negative phenotype in carbon utilization. The role of the PTS as a major sugar uptake system was further demonstrated by the finding that glucose and fructose negative mutants were deficient in the respective enzyme II PTS permease activities. These carbon sources also caused repression of glutamate uptake, which suggests an involvement of the PTS in carbon regulation. The observation that no HPr kinase/phosphatase could be detected suggests that the mechanism of carbon regulation in *C. glutamicum* is different to the one found in low-GC Gram-positive bacteria.

Introduction

The high-GC Gram-positive soil bacterium *Corynebacterium glutamicum* and related species are widely used for the industrial production of amino acids, notably glutamate, aspartate, and lysine (Eikmanns *et al.*, 1993; Jetten and Sinskey, 1995). Amino acid production is influenced by the given carbon source and by certain supplement conditions such as biotin limitation (Shiio *et al.*, 1990). To improve amino acid production, it will be important to understand how carbon sources are metabolized and how their utilization influences cell

metabolism. Such knowledge is at present only fragmentary understood for *C. glutamicum* and related actinomycetes (Parche *et al.*, 1999; Park *et al.*, 2000).

In low-GC Gram-positive and in Gram-negative bacteria the PTS triggers carbohydrate uptake, carbon regulation, and chemotaxis (for review see Postma *et al.*, 1993; Stùlke and Hillen, 1999). The PTS operates by means of phosphoryl group transfer from phosphoenolpyruvate (PEP) to EI and from EI to the second general phosphotransferase HPr. HPr-P, in turn, phosphorylates substrate-specific enzyme II permeases (IIABC^{carbohydrate}), which catalyze the concomitant transport and phosphorylation of more than 20 different carbon sources. PTS transport activity and the resulting levels of metabolic intermediates such as fructose-1,6-diphosphate alter the phosphorylation state of PTS proteins. The intrinsic information is distributed by EI, by HPr, and in Gram-negative bacteria also by enzyme IIA^{Glc} to target proteins by protein phosphorylation or protein-protein interaction to induce carbon regulatory responses (Saier *et al.*, 1995; Stùlke and Hillen, 1999).

In 1987, Mori and Shiio were the first researchers to describe the presence of the PTS in *Corynebacteriaceae* (Mori and Shiio, 1987a). They reported that *Brevibacterium flavum* possesses a constitutive glucose-specific PTS and an inducible fructose-specific PTS. In a subsequent study, mutants were analyzed that had lost either glucose PTS activity, fructose PTS activity or both suggesting that the genes encoding sugar-specific components and general components were mutated (Mori and Shiio, 1987b). PTSs specific for glucose, mannose, fructose, and sucrose have been described in *C. glutamicum* (Malin and Bourd, 1991; Dominguez and Lindley, 1996; Dominguez *et al.*, 1998). A hint as to a regulatory role of the PTS comes from the observation that concomitant utilization of glutamate and glucose results in a diauxic growth curve (Krämer *et al.*, 1990). Although the presence of PTSs has been described in several publications, detailed biochemical and genetic analyses have not been carried out. At present, only one PTS gene sequence has been reported, which was assigned to encode a mannose-specific enzyme II (Lee *et al.*, 1993; Lee *et al.*, 1994).

This communication describes a biochemical and genetic dissection of the PTS of *C. glutamicum*. We demonstrate the presence of the general phosphotransferases EI and HPr. We provide *in vivo* data by analysis of mutants and demonstrate carbon repression exerted by PTS-substrates. Finally, we match these results with the genome data of the closely related *Corynebacterium diphtheriae* (Parche *et al.*, 2001b)

Results

EI and HPr

We first aimed to characterize the general energy coupling proteins EI and HPr that are required for the activity of every PTS permease. To do so, we used heterologous complementation assays as we have described for the

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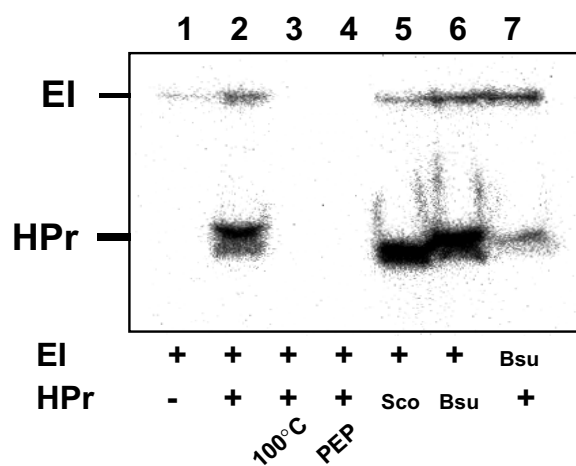


Figure 1. Phosphorylation of EI and HPr. The autoradiography of a 15% SDS polyacrylamide gel shows [³²P]PEP-dependent phosphorylation of native *C. glutamicum* EI and HPr. The following combinations were examined: Lane 1, 5 µl EI of EI-containing gel filtration fraction. Lane 2, 5 µl EI of EI-containing gel filtration fraction plus 10 µl HPr of HPr-containing gel filtration fraction. Lane 3, same as in lane 2 but boiled for 10 minutes prior to protein gel electrophoresis. Lane 4, same as in lane 2 incubated in the presence of an excess of unlabeled PEP (5 mM). Lane 5, 5 µl EI of EI-containing gel filtration fraction plus 15 pmol histidine-tagged HPr of *S. coelicolor*. Lane 6, 5 µl EI of EI-containing gel filtration fraction plus 15 pmol histidine-tagged HPr of *B. subtilis*. Lane 7, 3 pmol histidine-tagged EI of *B. subtilis* plus 10 µl EI of EI-containing gel filtration fraction.

identification of the same proteins in the related actinomycete *Streptomyces coelicolor* (Titgemeyer *et al.*, 1995; Parche *et al.*, 1999). EI was partially purified by gel filtration and an EI activity assay was established that was based on the reconstitution of a fructose PTS. PEP-dependent phosphorylation of fructose (4.3 pmol fructose-1-phosphate min⁻¹) was detected when *C. glutamicum* EI was mixed with *S. coelicolor* HPr and enzyme II^{Fruc}-containing membrane vesicles, while EI protein fraction alone or HPr and II^{Fruc}-containing membranes alone yielded only background activity (< 0.6 pmol fructose-1-phosphate min⁻¹).

In turn, *C. glutamicum* HPr activity was demonstrated in a fraction corresponding to low molecular masses by combining the EI-containing fraction with enzyme II^{Fruc}-containing membrane vesicles. This yielded PEP-dependent fructose phosphorylation of 1.8 pmol fructose-1-phosphate min⁻¹. Thus, activities of EI and HPr were found by their ability to cross communicate with *S. coelicolor* components. It should be noted that cross communication

of *C. glutamicum* HPr with *B. subtilis* PTS components was not detectable with this assay.

We then conducted [³²P]PEP-dependent phosphorylation with fractions containing HPr and EI (Figure 1). EI became phosphorylated upon incubation with radiolabeled PEP (lane 1). In the presence of HPr both proteins were phosphorylated (lane 2). After boiling treatment, no phosphorylated protein was detectable, indicating that the amino-acyl phosphates such as histidine-phosphate were heat-labile (lane 3) (Parche *et al.* 1999). Labeling of EI and HPr was prevented in the presence of an excess of nonlabeled PEP (lane 4). In lanes 5 and 6 of Figure 1, it was demonstrated that *C. glutamicum* EI was capable of transferring its phosphoryl group to HPr of *S. coelicolor* and HPr of *B. subtilis*, respectively. HPr of *C. glutamicum* was poorly phosphorylated by EI of *B. subtilis* (lane 7), which may explain why *C. glutamicum* HPr could not complement a *ptsH* mutant of *B. subtilis* (see above). The proteins exhibiting EI and HPr activity migrated at about the same height as *B. subtilis* histidine-tagged EI and *B. subtilis* histidine-tagged HPr corresponding to molecular masses of about 65 kD for EI and 14 kD for HPr.

Does *C. glutamicum* Have an HPr Kinase?

As many low-GC Gram-positive bacteria possess an ATP-dependent HPr kinase/phosphatase that transfers the γ-phosphoryl group of ATP to serine residue 46 of the HPr protein, we raised the question whether *C. glutamicum* has such an enzyme (Reizer *et al.*, 1998; Kravanja *et al.*, 1999). Therefore, HPr kinase assays were performed with *C. glutamicum* extracts without and with excess of purified *B. subtilis* HPr. In none of the cases, an ATP-dependent phosphorylation of HPr was detected. Control experiments showed that under the same conditions, HPr of *B. subtilis* was ATP-dependently phosphorylated (data not shown).

PTS Mutants Show a Glucose and Fructose Negative Phenotype

To characterize the individual PTS components *in vivo*, mutants were selected that were deficient for utilization of glucose and/or fructose or that were pleiotropically deficient in sugar consumption. Therefore, mutants resistant to xylitol (Xtl) and 2-deoxyglucose (2DG), which are supposed to be toxic analogues of the fructose PTS and of the glucose PTS (Malin and Bourd, 1991), respectively, were isolated. In comparison to the wild-type strain, the Xtl^R strain GSJ171 was deficient in fructose utilization (Table 1). Furthermore, its 2DG^R derivative GSJ271 showed reduced growth on glucose and no growth on mannose. A third mutant class was represented by strain GSJ125 (Xtl^R), which was pleiotropically diminished on all carbon sources tested with the exception of acetate. Although strains GSJ271 and GSJ125 were impaired in fructose and glucose utilization, it should be noted that after prolonged incubation, tiny colonies appeared on agar plates. This indicated that the mutants were still able to consume these carbon sources at a reduced rate.

PTS Mutants Lack Glucose and Fructose PTS Activity

Specific PTS activities were determined to characterize the observed phenotypes of the isolated mutants. Protein extracts of mutant and wild-type strains were used for PEP-

Table 1. Growth phenotype of *C. glutamicum* wild-type (wt) and mutant strains on different carbon sources.

strain/phenotype	glucose	fructose	sucrose	maltose	mannose	acetate
ATCC 13032 / wt	+++	+++	+++	+++	+	++
GSJ125 / EI ⁻	+	+/-	+/-	+/-	-	++
GSJ171 / II ^{Fruc} -	+++	+/-	+++	+++	+	++
GSJ271 / II ^{Glc} - II ^{Fruc} -	+/-	+/-	+++	+++	-	++

Cells were grown for two days on mineral medium plates supplemented with 50 mM sugar or 400 mM acetate. Growth phenotypes are indicated as follows: +++, very good; ++, good; +, fair; +/-, poor; -, no growth.

Table 2. PEP-dependent phosphorylation of [¹⁴C]αMG or [¹⁴C]fructose in extracts of *C. glutamicum* wild-type (wt) and mutant strains.

strain/phenotype	added protein	sugar present during growth	phosphorylation activity	
			[nmol sugar-P 30 min ⁻¹ [¹⁴ C]αMG]	(mg protein ⁻¹) [¹⁴ C]Fru]
ATCC 13032 / wt		-	4.92 ± 0.07	0.97 ± 0.04
		glucose	5.94 ± 0.17	3.98 ± 0.16
		fructose	5.02 ± 0.12	8.41 ± 0.33
GSJ125 / EI ⁻		-	≤ 0.1	≤ 0.1
		glucose	≤ 0.1	≤ 0.1
GSJ271 / II ^{Glc-} II ^{Fru-}		-	≤ 0.1	≤ 0.1
		glucose	≤ 0.1	≤ 0.1
GSJ271 / II ^{Glc-} II ^{Fru-} + GSJ125 / EI ⁻		glucose	3.25 ± 0.07	n. d.
		fructose	n. d.	0.46 ± 0.01
GSJ125 / EI ⁻	+ HPr	fructose	n. d.	≤ 0.1
	+ EI	fructose	n. d.	0.16 ± 0.02
	+ HPr	glucose	≤ 0.1	n. d.
GSJ271 / II ^{Glc-} II ^{Fru-}	+ EI	glucose	4.67 ± 0.33	n. d.
	+ ^a enzyme II ^{Fru-}	-	n. d.	1.36 ± 0.18

Extracts were dialyzed and used at 100 µg protein. Partial purified EI and HPr were added where indicated at 5 µl and 10 µl of EI- and HPr-containing gel filtration fractions, respectively. Membranes of *S. coelicolor* were added at 50 µg protein. Values were determined in triplicate. ^a100 µg *S. coelicolor* membranes bearing EI^{Fru-}; n. d.: not determined; ±: standard deviations.

dependent phosphorylation of methyl α-[¹⁴C]glucoside (αMG) or [¹⁴C]fructose to assay the glucose PTS and the fructose PTS, respectively (Table 2). Wild-type extracts exhibited constitutively high glucose PTS activity, which was slightly elevated when glucose was present in the complex medium. Competition of [¹⁴C]αMG phosphorylation with a tenfold excess of unlabeled glucose abolished PTS activity while a tenfold excess of unlabeled αMG, mannose, or fructose decreased phosphorylation rates to 15%, 75%, and 86%, respectively. This indicates that this enzyme II exhibits the highest specificity for glucose. Fructose PTS activity was eightfold higher when the complex medium was supplemented with fructose.

PEP-dependent phosphorylation of αMG or fructose was not detectable in cell-free extracts of GSJ271 or GSJ125. Both activities were present when mutant extracts were mixed, indicating that both strains carry mutations in different genes. Addition of partially purified EI to protein extract of GSJ125 restored PEP-dependent phosphorylation of αMG and fructose. Thus, GSJ125 carries a mutation involved in EI activity. Combination of GSJ271 protein extract with *S. coelicolor* membranes

bearing active enzyme II^{Fru} led to significant fructose phosphorylation. Thus, GSJ271 carries a mutation involved in enzyme II^{Fru} activity.

Sugar Uptake is Diminished in PTS Mutants

Uptake experiments were conducted to investigate how malfunction of PTS proteins in the different mutant strains affect substrate transport. Therefore, cells were grown in complex medium either in the presence of glucose or in the presence of fructose and velocity of sugar uptake was determined (Table 3). GSJ125 and GSJ271 incorporated glucose and fructose at reduced rates. In the case of GSJ125, lacking detectable EI activity, uptake capacity was about half of what it was for wild-type cells, reflecting the impaired growth response on glucose (Table 1). Transport of GSJ271 was diminished about fivefold for glucose and about eightfold for fructose compared to the wild-type, also reflecting the observed growth phenotype (Table 1). Thus, GSJ125 and GSJ271 retained some capacity to grow on glucose and fructose and to transport PTS substrates.

PTS Substrates Reduce Glutamate Uptake

Previous work has demonstrated that glutamate uptake is susceptible to catabolite repression by glucose (Krämer and Lambert, 1990; Kronemeyer *et al* 1995). To determine this in detail and to see whether this repression could be mediated by further carbohydrates, glutamate transport was measured in the presence of different carbon sources (Figure 2). Among those the PTS sugars fructose and glucose caused the strongest reduction of glutamate uptake (65-80%). In the presence of maltose, mannose, and pyruvate transport activity was decreased between 40% and 50%, while ribose and glycerol caused only marginal or no reduction.

Table 3. Transport of glucose and fructose by *C. glutamicum* wild-type (wt) and mutant strains.

strain/phenotype	sugar present during growth	uptake rate	
		[nmol sugar-P min ⁻¹ (OD cells ⁻¹) [¹⁴ C]Glc]	[¹⁴ C]Fru]
ATCC 13032 / wt	glucose	3.72 ± 0.26	n. d.
	fructose	n. d.	4.86 ± 0.10
GSJ125 / EI ⁻	glucose	1.77 ± 0.04	n. d.
	fructose	n. d.	2.25 ± 0.05
GSJ271 / II ^{Glc-} II ^{Fru-}	glucose	0.81 ± 0.10	n. d.
	fructose	n. d.	0.62 ± 0.03

Washed cells were used at a final OD₆₀₀ of 1.0 for determination of uptake rates of radiolabeled sugars. Values were determined in triplicate.

±: standard deviations; n. d.: not determined.

Discussion

In this study we reported on a dissection of the *C. glutamicum* PTS. We demonstrated the presence of the general phosphotransferases EI and HPr and determined their molecular sizes as well as their PEP-dependent phosphorylation activities. The isolation and characterization of PTS mutants revealed that the system plays a major role in carbon utilization and evidence was provided that indicates that the PTS is intricately involved in carbon regulation.

Biochemical characterization of EI and HPr led to apparent molecular weights of 65 kD for EI and 14 kD for HPr. The protein sizes and the determined activities are in good agreement with the data reported for EI and HPr proteins from other bacteria (Postma *et al.*, 1993; Parche *et al.*, 1999). It should be noted that HPr proteins, which usually comprise 80 to 90 amino acids, often migrate at around 14 kD on SDS-containing polyacrylamide gels (Parche *et al.* 1999). We were able to detect by *in silico* analysis of the closely related pathogen *C. diphtheriae*, a gene encoding an EI (560 aa) and a gene encoding an HPr (88 aa) that could represent the respective homologues (Parche *et al.*, 2001b). However, our approach did not rule out the possibility that *C. glutamicum* may have further paralogues such as fructose-specific EI and HPr protein domains, which are present in Gram-negative bacteria as part of di- or multiphosphoryl transfer proteins (DTP, MTP) (Saier and Reizer, 1994). It is worth noting that a *ptsH* homologue, *ptsF*, was detected within the genome of *C. diphtheriae*, encoding a protein that is composed of an N-terminal domain of unknown function (140 aa) and a C-terminal HPr-like domain (82 aa) (Parche *et al.*, 2001b).

The analysis of sugar fermentation mutants revealed that the PTS plays an important role in sugar catabolism. The conducted PTS complementation assays allowed us in contrast to previous reports to distinguish between the defects in singular PTS components (Mori and Shio, 1987a; Dominguez and Lindley, 1996). Glucose PTS activity was abolished in the Xtl^R/2DG^R strain GSJ271, exhibiting EI and HPr activity (Table 2). This finding suggests that GSJ271 picked up a mutation in the gene encoding the glucose-specific enzyme II, or a mutation that abolishes its expression. Furthermore, since GSJ271 showed a glucose/mannose negative phenotype one might conclude that one single mutation caused both growth defects, and therefore, glucose and mannose are transported by the same PTS. Previous work in *C. glutamicum* led to successful cloning of an enzyme II gene that restored fermentation of mannose and glucose in *E. coli* (Lee *et al.*, 1994). Based on its 2DG sensitivity, it was suggested that the gene encodes the mannose-specific enzyme II. However, these results were based on heterologous complementation of *E. coli* and not by *in vivo* analysis of *C. glutamicum*.

GSJ271 was also abolished in fructose PTS activity. Addition of II^{Fru} from *S. coelicolor* to GSJ271 cell extracts could restore this, suggesting that GSJ271 is mutated in the gene encoding the fructose-specific enzyme II, or a mutation that abolishes its expression (Table 2). Putative fructose operons are present in the genomes of *C. diphtheriae* and *S. coelicolor*, which include genes for an

enzyme IIABC^{Fru} and a fructose 1-phosphate kinase (Parche *et al.*, 2001a; Parche *et al.*, 2001b).

The mutant lacking EI activity showed impaired growth on the four known PTS substrates and also on maltose. Thus, maltose could be transported by a maltose-PTS as it is the case in *B. subtilis*, or by a system that is under PTS control. The latter case is found in *E. coli*, which possesses an ABC (ATP-binding cassette) transporter that is regulated by the PTS via inducer exclusion (Saier *et al.*, 1995; Ehrmann *et al.*, 1998; Reizer *et al.*, 1999).

Beside its function in sugar transport and sugar metabolism we have also looked for regulatory capacities of the PTS. We were able to demonstrate that carbon repression of glutamate uptake is exerted by the PTS substrates fructose and glucose. To see whether this regulatory effect could be mediated by ATP-dependent phosphorylation of HPr, we tested protein extracts of *C. glutamicum* for activity of HPr kinase/phosphatase, an enzyme that together with HPr and the global repressor CcpA constitutes the carbon catabolite repression system of low-GC Gram-positive bacteria (Reizer *et al.*, 1998; Kravanja *et al.*, 1999). Albeit the reaction conditions may not have been optimal, no evidence was obtained for the existence of an HPr kinase in *C. glutamicum*. Similar results were obtained for the related actinomycetes *S. coelicolor* and *Streptomyces lividans* (Butler *et al.*, 1999; Parche *et al.*, 1999). It is also noteworthy that no gene homologues of HPr kinase/phosphatase were detected in the almost completed sequencing projects of the actinomycetes *C. diphtheriae* and *S. coelicolor* (<http://www.sanger.ac.uk>).

In conclusion, our data revealed that i) *C. glutamicum* has the two classical PTS phosphotransferases EI and HPr at its disposal, ii) that glucose and mannose are probably transported by the same enzyme II permease, iii) that fructose is incorporated via a fructose-specific enzyme II, and iv) that PTS substrates exert carbon regulation. The latter finding, together with the fact that HPr kinase/phosphatase activity could not be detected, strengthen the hypothesis that components of the PTS operate in carbon regulation by mechanisms dissimilar to those established for low-GC Gram-positive bacteria. Further studies will be required to elucidate the role of the PTS in carbon regulation and a possible impact this industrially important microorganism has on amino acid production.

Experimental Procedures

Bacterial Strains and Growth Conditions

C. glutamicum ATCC 13032 was used as wild-type strain (Abe *et al.*, 1967). Unless not stated otherwise, cells of *C. glutamicum* were grown in Luria-Bertani broth (LB) at 37°C in a rotary shaker under aerobic conditions. Growth on different carbon sources (50 mM) was tested on CGM mineral medium (Morbach *et al.*, 1996).

Isolation of PTS Mutants

2-deoxyglucose- (2DG^R) and xylitol-resistant (Xtl^R) strains of *C. glutamicum* ATCC 13032 were selected following the method of Malin and Bourd (Malin and Bourd, 1991). Wild-type cells (10³ cells) pregrown on CGM with 50 mM fructose were plated on CGM plates supplemented with 50 mM lactate and 20 mM xylitol. After three days of incubation at 30°C, Xtl^R strains were clonally isolated and tested for growth on different sugars. One strain showing a growth defect on fructose was termed GSJ171. Another mutant that was pleiotropically deficient in sugar fermentation was termed GSJ125. GSJ171 was subjected to a second round of selection on CGM plates in the presence of 100 mM Na-acetate and 6 mM 2-deoxyglucose (2DG). After one week of incubation one 2DG^R strain that exhibited a glucose negative phenotype was termed GSJ271 and was used for further studies.

Protein Purification and Membrane Preparation

Recombinant histidine-tagged HPr of *Streptomyces coelicolor* A3(2) M145, histidine-tagged EI of *B. subtilis*, and enzyme I^{Fr}-containing membranes of *S. coelicolor* were purified as described (Parche *et al.*, 1999). For partial purification of EI and HPr of *C. glutamicum* ATCC 13032, gel filtration was performed on sonified extracts derived from glucose-grown cells. A volume of 0.45 ml corresponding to about 3.7 mg total protein was loaded on an FPLC G200-Superdex column (24-mL bed volume), eluted with 25 mM Tris/HCl pH 7.5, 200 mM KCl, and 2 mM DTT, and fractions of 0.40 ml were collected. Routinely, 5-30 µl of EI- and HPr containing fractions were taken for PTS activity assay. Protein concentrations were determined spectrophotometrically using the Bio-Rad protein assay. Proteins were stored in aliquots at -20°C or -70°C.

Enzyme Assays

PTS activity of *C. glutamicum* was assayed following phosphoenolpyruvate (PEP)-dependent phosphorylation of methyl α -[¹⁴C]glucoside ([¹⁴C]αMG) (12 µM; 40 mCi mmol⁻¹) or [¹⁴C]fructose (12 µM; 42 mCi mmol⁻¹). The assay was carried out for 30 min at 30°C in a reaction volume of 0.1 ml containing 50 mM Tris/HCl pH 7.5, 5 mM MgCl₂, and 3 mM DTT (Parche *et al.*, 1999). Similarly, EI activity of *C. glutamicum* was monitored by complementation of the *S. coelicolor* fructose PTS measuring PEP-dependent phosphorylation in the presence of 20 µg membrane protein of *S. coelicolor* containing fructose-specific enzyme I^{Fr} together with 1.4 µg histidine-tagged HPr of *S. coelicolor*. PTS assays were carried out as described above using 12 µM [¹⁴C]fructose (42 mCi mmol⁻¹). In turn *C. glutamicum* HPr was assayed under the same conditions using 20 µg *S. coelicolor* membrane protein together with partially purified *C. glutamicum* EI. Further experimental details are provided in the legend to Table 2.

[³²P]Phosphoenolpyruvate ([³²P]PEP) was prepared from [γ -³²P]ATP as described by Roossien *et al.* (Roossien *et al.* 1983). Phosphorylation of partially purified proteins was carried out as described by Parche *et al.*, (Parche *et al.*, 1999). Radiolabeled proteins were detected by radioluminography on a phosphorimager (Fuji). Further details are provided in the legend to Figure 1.

Cells of *C. glutamicum* were grown in 100 ml LB supplemented with either 50 mM glucose or 50 mM fructose, harvested in the early exponential growth phase (OD₆₀₀ from 0.5 to 2.0), washed in chilled transport buffer (50 mM Tris/HCl pH 7.5, 50 mM NaCl, and 10 mM KCl), and adjusted to an OD₆₀₀ of 1.0. Cells prepared for transport assays could be stored on ice for several hours. Before uptake was initiated by addition of [¹⁴C]fructose (10 µM; 5 mCi mmol⁻¹) or [¹⁴C]glucose (10 µM; 4.5 mCi mmol⁻¹) cells were pre-equilibrated for 5 min at 37°C. Samples of 1 ml were taken in time intervals of 20 sec, rapidly filtered through nitrocellulose filters (NC45), and washed with 0.1 M LiCl. Radioactivity was determined by scintillation oscilligraphy. For the determination of glutamate uptake rates overnight cultures were diluted in fresh medium and grown to an OD₆₀₀ of about 2.5 as described

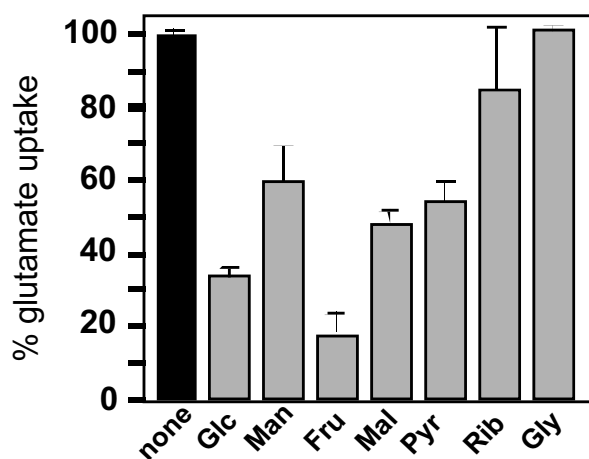


Figure 2. Repression of glutamate uptake. Glutamate uptake was monitored in cells grown in the presence of glutamate (100 mM) and with or without additional carbon source (100 mM) as indicated. Transport activity is expressed relative to the activity detected in cells grown on glutamate as sole carbon source. The data are the mean of at least three independent experiments.

previously. Cells were subsequently washed once with MES/Tris buffer (50 mM pH 8.0, 10 mM NaCl, and 10 mM KCl) and glutamate uptake was determined using L-[¹⁴C]glutamate at a final concentration of 100 µM (6 mCi mmol⁻¹) and a filtration assay as described previously (Kronmeyer *et al.*, 1995).

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