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Authors

Aboulwafa, Mohammad
Zhang, Zhongge
Saier, Milton H

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Protein-Protein Interactions in the Cytoplasmic Membrane of *E. coli:* Influence of the Overexpression of Diverse Transporter-Encoding Genes on the Activities of PTS Sugar Uptake Systems.

Mohammad Aboulwafa[1,2], **Zhongge Zhang**[1], **Milton H. Saier Jr.**[1,*]

[1]Department of Molecular Biology, Division of Biological Sciences, University of California at San Diego, La Jolla, CA 92093-0116

[2]Department of Microbiology and Immunology, Faculty of Pharmacy, Ain Shams University, Abbassia, Cairo, Egypt

Abstract

The prokaryotic phosphoenolpyruvate (PEP):sugar phosphotransferase system (PTS) concomitantly transports and phosphorylates its substrate sugars. In a recent publication, we provided evidence that protein-protein interactions of the fructose-specific integral membrane transporter (FruAB) with other PTS sugar group translocators regulates the activities of the latter systems *in vivo* and sometimes *in vitro*. In this communication, we examine the consequences of the overexpression of several different transport systems on the activities of selected PTS and non-PTS permeases. We report that high levels of these transport systems enhance the *in vivo* activities of several other systems in a fairly specific fashion. Thus, (1) overexpression of *ptsG* (glucose porter) selectively enhanced mannitol, N-acetylglucosamine and 2-deoxyglucose (2DG) uptake rates, (2) overexpression of *mtlA* (mannitol porter) promoted methyl α-glucoside (αMG) and 2DG uptake, (3) *manYZ* (but not *manY* alone) (mannose porter) overexpression enhanced αMG uptake, (4) *galP* (galactose porter) overexpression enhanced mannitol and αMG uptake, and (5) *ansP* (asparagine porter) overexpression preferentially enhanced αMG and 2DG uptake, all presumably as a result of direct protein-protein interactions. Thus, it appears that high level production of several integral membrane permeases enhances sugar uptake rates with the PtsG and ManXYZ systems being most consistently stimulated, but the MtlA and NagE systems being more selectively stimulated and to a lesser extent. Neither enhanced expression nor *in vitro* PEP-

*Corresponding Author: Department of Molecular Biology, Division of Biological Sciences, University of California at San Diego, La Jolla, CA 92093-0116, Tel +1 858 534 4084, Fax: +1 858 534 7108, msaier@ucsd.edu.

Author Contributions

Conceptualization: Mohammad Aboulwafa, Milton H. Saier, Jr.
Data curation: Mohammad Aboulwafa.
Formal analysis: Mohammad Aboulwafa, Milton H. Saier, Jr.
Funding acquisition: Milton H. Saier, Jr.
Investigation: Mohammad Aboulwafa, Zhongge Zhang.
Methodology: Mohammad Aboulwafa, Zhongge Zhang.
Project administration: Milton H. Saier, Jr.
Resources: Milton H. Saier, Jr.
Validation: Mohammad Aboulwafa.
Writing – original draft: Mohammad Aboulwafa, Milton H. Saier, Jr.
Writing – review & editing: Mohammad Aboulwafa, Milton H. Saier, Jr., Zhongge Zhang

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dependent phosphorylation activities of the target PTS systems were appreciably affected. The results are consistent with the suggestion that integral membrane transport proteins form an interacting network *in vivo* with physiological consequences, dependent on specific transporters and their concentrations in the membrane.

Keywords

Sugar transport; Sugar phosphorylation; Phosphotransferase system; PTS; Integral membrane protein:protein interactions; allosteric regulation

Introduction

Bacterial cell envelope protein complexes mediate a wide range of processes, including metabolic coordination, molecular transport, and membrane assembly [Geisinger et al., 2019; Jo et al., 2019; Kudva et al., 2013; Paulsen et al., 1997; Whitfield, 2006]. In a four-way collaborative effort, we identified 1,347 cell envelope proteins in *Escherichia coli*, including 90% of inner and outer membrane proteins, as well as thousands of periplasmic and cytoplasmic proteins [Babu et al., 2018]. After extraction with non-denaturing detergents, we affinity purified 785 endogenously tagged envelope proteins and identified associated protein complexes by precision mass spectrometry (MS). This resulted in the description of a physical interaction network (the *E. coli* interactome). The results suggested interactions that might control molecular transport, macromolecular secretion, assembly of envelope complexes, and maintenance of outer membrane lipid asymmetry [Babu et al., 2018]. In fact, they provided a unique guide for investigating the biochemical and physiological consequences of these interactions [Rodionova et al., 2018a; Rodionova et al., 2018b; Rodionova et al., 2017]. We have used the results of this study as guide for the present work.

As reported in a recent publication [Aboulwafa et al., 2019], we noticed that the *E. coli* interactome data, generated by Babu et al., 2018 [Babu et al., 2018], revealed large numbers of interactions between the fructose-specific Enzyme II complex (FruAB) of the phosphoenolpyruvate (PEP):sugar phosphotransferase system (PTS) and other PTS permeases. This observation led us to study the consequences of these interactions on the *in vivo* and *in vitro* activities of the target Enzyme II (sugar transporter) complexes. Using a variety of genetic, biochemical and physiological approaches, we could show that high level expression of the fructose PTS genes (*fruA* and *fruB*), either by fructose induction in a wild type genetic background, or by overexpression of the *fruAB* genes in the absence of induction, led to enhancement of the activities of some PTS group translocators, but the inhibition of others. Thus, the activities of PtsG (transporter for glucose and α-methylglucoside (αMG)), ManXYZ (transporter for mannose, glucose, 2-deoxyglucose (2DG), and glucosamine), MtlA (transporter for mannitol (Mtl)) and NagE (transporter for N-acetylglucosamine (NAG)) were activated while those of the GatABC (specific for galactitol (Gat)) and TreBC (specific for trehalose (Tre)) were inhibited *in vivo*. Only the MtlA and NagE systems showed enhanced activities *in vitro* in response to high levels of *fruAB* expression. It was shown that expression of the target PTS permease genes using

transcriptional *lacZ* fusions [Hughes and Maloy, 2007] and assays of the transphosphorylation activities of these enzymes [Leonard and Saier, 1983; Saier and Newman, 1976; Saier and Schmidt, 1981] was not influenced by the overexpressed *fruAB* genes (Aboulwafa et al., 2019). These results made physiological sense because the activated systems are catabolite repressing systems while the inhibited systems are catabolite repressed systems [Lengeler, 2015; Saier, 1989; Saier et al., 1996; Westermayer et al., 2016].

In this communication, we extend our previous studies by examining the consequences of the overexpression of several transport protein-encoding genes on the uptake of a variety of relevant substrates in *E. coli.* In addition to several sugar-specific PTS Enzyme II-encoding genes, we examine the *galP* gene [Zheng et al., 2010], encoding the galactose:H^+ symporter and the *ansP* gene [Jennings et al., 1995], encoding the asparagine uptake transporter. While the activities of the non-PTS transporters (GalP and AnsP) were not appreciably activated by overexpression of any of the genes studied, the PTS permeases were selectively activated by high level expression of several of them. Thus, *ptsG* overexpression increased mannitol, N-acetylglucosamine and 2-deoxyglucose uptake rates, while *mtlA* overexpression enhanced N-acetylglucosamine, methyl α-glucoside and 2-deoxyglucose uptake rates. Further, overexpression of *manYZ* (but not *manY* alone) greatly increased the methyl α-glucoside uptake rate, and *galP* overexpression increased the mannitol and N-acetylglucosamine uptake rates, while *ansP* overexpression primarily increased the methyl α-glucoside and 2-deoxyglucose uptake rates. In no case was the *in vitro* PEP-dependent sugar phosphorylation rate increased to comparable extents. *lacZ*-fusion analyses showed that synthesis of target PTS permeases was not increased. The results provide evidence for a network of intramembrane PPIs that influence the activities of select PTS transport systems.

Results

Effects of the overexpression of several genes encoding permeases on PTS sugar uptake rates

We overexpressed genes encoding several transporter systems: *ptsG* (encoding Enzyme $IICB^{G1c}$; TC# 4.A.1.1.1), *mtlA* (encoding Enzyme $IICBA^{Mtl}$; TC# 4.A.2.1.2), *manY* or *manYZ* (encoding Enzyme IIC^{Man} or Enzyme $IICD^{Man}$, respectively; TC# 4.A.6.1.1), *galP* (encoding the galactose permease, TC# 2.A.1.1.1), and *ansP* (encoding the asparagine porter; TC# 2.A.3.1.24). The detailed results are presented in Tables 1–6 while the overall results are presented in Figure 1 and Table 7.

Overexpression of *ptsG* greatly enhanced the uptake of methyl α-glucoside (αMG) as expected since this sugar is a primary substrate of this system. However, it also increases the uptake rate of 2-deoxyglucose (2DG) 10-fold. 2-DG is a poor substrate of PtsG, but the increase could have resulted if *ptsG* overexpression increased the activity of the mannose PTS, ManXYZ. The latter system takes up 2DG with high efficiency and fructose with low efficiency [Rephaeli and Saier, 1980], possibly accounting for the 2-fold increase in fructose uptake. However, *ptsG* overexpression also increased the uptake rates for mannitol (Mtl) and N-acetylglucosamine (NAG) 6-7 fold. This is likely to be due to increases in the activities of their respective Enzyme II complexes (Figure 1) as these sugars are not substrates of PtsG.

The consequences of *mtlA* overexpression are presented in Table 2 and summarized in Figure 1 and Table 7. The MtlA protein transports mannitol but not hexoses. As expected, mannitol uptake increased 9.6-fold, but the uptakes of several other sugars also increased. Fructose uptake increased 3.2x, N-acetylglucosamine uptake increased 3.5x, methyl α-glucoside uptake increased 4.0x, and 2-deoxyglucose uptake increased 7.2x. None of these results can be explained due to overexpression of *mtlA per se,* and therefore probably resulted from stimulatory protein-protein interactions between MtlA and the target PTS group translocators.

The consequences of either *manY* or *manYZ* overexpression were similarly examined. ManY is thought to be unstable in the absence of ManZ [Garcia-Alles et al., 2002], possibly explaining the fact that ManY alone had no effect on the activities of other transporters (Table 3), but ManYZ did (Table 4). The major effects were observed for 2-deoxyglucose uptake (10.8-fold) and methyl α-glucoside (14.0-fold). While the former enhancement can be explained by higher levels of ManYZ, the latter cannot, because methyl α-glucoside is not a substrate of the mannose PTS transporter. High levels of ManYZ presumably stimulate the activity of the PtsG glucose/methyl α-glucoside PTS porter.

The galactose permease, GalP, is known to take up galactose and glucose as well as some of their non-metabolizable derivatives such as 2DG, but not αMG. In addition to large increases in 2DG and galactose uptake, moderate increases in other sugar uptake rates, especially those of mannitol (Mtl) and N-acetylglucosamine (NAG) (3-4 fold) were observed (Tables 5 and 7; Figure 1). These results indicate that the fructose Enzyme II protein constituents [Aboulwafa et al., 2019] and other PTS permeases as noted above, are not the only ones that can promote the activities of other PTS Enzymes II.

When the *ansP* gene was overexpressed, moderate activation of all systems was observed, but surprisingly, large activation was observed when the substrate was αMG (18-fold) or 2DG (8-fold) (Tables 6 and 7; Figure 1). These results confirm the conclusion that overexpression of a gene encoding an integral membrane permease, specific for an amino acid rather than a sugar, can increase the activities of various PTS permeases, even when their substrates and functions are dissimilar. However, they also demonstrate a degree of specificity. Each overexpressed gene differentially affected the activities of the different permeases assayed.

Operon induction properties using *lacZ* fusions

For studies with *lacZ* fusion strains, the wild type strain (BW25113) bearing *mtlA*-, *gatC*- and *manXYZ-lacZ* fusions and overexpressing *ptsG, mtlA, galP* or *ansP* showed essentially no effect on the levels of β-galactosidase activities (Tables 8 and 9). This clearly implies that the *mtl, gat* and *man* operons were not repressed or induced by overexpression of *ptsG, mtlA, galP* or *asnP*. It therefore seems clear that the effects reported on transport activities (Table 7; Figure 1) could not be attributed to altered expression of the mannitol (*mtl*), galactitol (*gat*) or mannose (*man*) PTS transport system-encoding genes.

PEP-dependent phosphorylation of PTS sugars *in vitro* following overexpression of *ptsG* or *mtlA*

Figure 2, Table S1 and Table S2 summarize the consequences of the overexpression of certain genes encoding PTS Enzymes II on the PEP-dependent sugar phosphorylation reactions catalyzed by other PTS Enzymes II *in vitro*. When the *ptsG* gene, encoding the glucose/αMG-selective transporter in a wild type genetic background, was overexpressed, the phosphorylation activities for trehalose, αMG and 2DG increased substantially, although phosphorylation of most other sugars did not change, and that of galactitol decreased. Trehalose, an α,α-disaccharide of glucose, may have been hydrolyzed to glucose, possibly accounting for this result. While the increase in αMG phosphorylation may be fully accounted for by the increase in the *ptsG* gene product (II^{Glc}), this seems unlikely for 2DG which is primarily phosphorylated by the mannose system. Thus, it seems more likely that overexpression of *ptsG* enhances the activity of the ManXYZ Enzyme II complex, but this cannot be claimed with certainty.

Overexpression of the *mtlA* gene enhanced mannitol phosphorylation as expected, but it additionally increased fructose phosphorylation 3-fold (Figure 2 and Table S1). All other Enzyme II complexes assayed showed a decrease in phosphorylation activity, with galactitol showing the largest effect.

When *manY* was overexpressed, none of the other Enzymes II showed a significant change in activity, but when both *manY* and *manZ* were overexpressed together, there was a significant increase of 2-deoxyglucose phosphorylation as expected (Figure 2 and Table S2). In contrast to the other PTS Enzyme II systems assayed, both genes are required to produce the integral membrane complex of the mannose system [Garcia-Alles et al., 2002]. Interestingly, however, it should be noted that the overexpression of any of the genes encoding the integral membrane constituents of a PTS transporter strongly inhibited the in vitro phosphorylation activity of galactitol. This may reflect a novel mechanism of catabolic repression since galactitol is at the bottom of the hierarchy of PTS systems [Lengeler, 1975].

Discussion

The results reported in this communication supplement and extend the data presented in our previous publication on the interactions of the PTS integral membrane fructose permease with other PTS systems in *E. coli* [Aboulwafa et al., 2019]. In that work, it was shown that high level expression of the *fruAB* genes, either by induction or by overexpression, substantially increased the *in vivo* (and sometimes the *in vitro)* activities of other PTS Enzyme II complexes. The *in vivo* data were generated by assaying sugar uptake by intact cells while the *in vitro* data were generated by assaying crude extracts or membrane pellets for sugar phosphorylation using phosphoenolpyruvate (PEP) or sugar-phosphate (sugar-P) as the phosphoryl donor. With PEP as the phosphoryl donor, the entire phosphoryl transfer chain of the PTS is required, but only the IIB and IIC domains of the Enzyme II complex are required when the phosphoryl donor is sugar phosphate [Rephaeli and Saier, 1978; Saier et al., 1977; Saier and Newman, 1976]. Only the PEP-dependent reactions, not the sugar-P-dependent transphosphorylation reactions, showed stimulation, and only two Enzyme II complexes, the mannitol and N-acetylglucosamine Enzyme II complexes, showed elevated

activities when the fructose Enzyme II complex was overproduced. Further, the *in vitro* stimulation was far less than that observed *in vivo.* Since the fructose system interacted with many more systems than any of the other PTS systems [Babu et al., 2018], and because several lines of evidence suggested that the fructose system was the primordial PTS group translocator [Aboulwafa et al., 2019], it was proposed that the stimulatory and inhibitory effects of high-level expression of the fructose PTS permease genes could have both evolutionary and functional significance.

In this paper, we have conducted more global studies by examining the consequences of the overexpression of several transport protein-encoding genes including those encoding three other PTS transporters (the glucose, mannitol and mannose systems) as well as the non-PTS galactose porter, GalP, and the asparagine porter, AnsP. Moreover, the consequences of their overexpression on the uptake of non-PTS substrates as well as several PTS substrates were determined. While stimulation was observed with certain transporters, inhibition was observed for others. The level of stimulation/inhibition was associated with the overexpressed protein level, and because there was often no overlap between the substrate specificities of the overexpressed and target transporters, this interpretation seemed clear, namely that the former stimulated the activity of the latter. The results can therefore be summarized as follows: (1) Overexpression of both the PTS and non-PTS transporter genes specifically enhanced the uptake rates of several PTS substrates, but that of the two non-PTS substrates, galactose and asparagine, were not increased appreciably. (2) The activities of the glucose system, PtsG, and the mannose system, ManXYZ, were stimulated by overexpression of most of the genes examined. (3) The activities of the mannitol (MtlA) and N-acetylglucosamine (NagE) systems were often stimulated, but to a lesser degree than for PtsG and ManXYZ. (4) A certain degree of specificity was observed when each of the permease genes was overexpressed (see Table 7 and Figure 1). (5) The PEP-dependent *in vitro* phosphorylation reactions were not appreciably stimulated. (6) In no case was the expression of a target gene enhanced by overexpression of another permease gene. These observations confirm the results of the previous study [Aboulwafa et al., 2019], which suggested first, that the overexpression of one PTS permease gene influenced the activities of other PTS permeases, and second, that the *in vitro* stimulation of mannitol and N-acetylglucosamine phosphorylation by a high level of expression of the fructose Enzyme II complex genes was specific to these systems.

The results presented here show that overexpression of several PTS and non-PTS transporter encoding genes can positively influence the activities of several PTS transport systems although the non-PTS systems examined were not appreciably affected. These results provide strong evidence for the functional significance of the intramembrane interactions involving specific integral membrane protein levels on other integral membrane transporters present in the same membrane. These interactions may be largely (but not completely) prevented when cells are disrupted for *in vitro* assays. Throughout these studies, whenever a transport protein-encoding gene was overexpressed, the activity of that system was increased. This was expected because an increased level of the transporter should increase the rate of uptake of its substrate, assuming that transport is rate limiting for uptake. However, a quantitative evaluation of the *in vivo* data, summarized in Table 7, leads to the suggestion of an additional effect. Overexpression of *mtlA* or *manYZ* produced a ~10x

increase in mannitol or 2-deoxyglucose uptake, respectively, but overexpression of *ptsG* gave a 66x increase in α-methylglucoside uptake, and *galP* gave a 43x increase in 2-deoxyglucose uptake, while overexpression of *ansP* gave only a 1.9x increase in asparagine uptake. The tremendous divergence in the degrees of increase observed for the different transport systems, acting on their own substrates (i.e., PtsG > GalP > ManYZ = Mtl > AnsP with a range of 66x to only 1.9x) leads to the possibility that high level expression of a transporter stimulates uptake of its substrate(s) not only due to enhanced levels of the transporter, but also due to cooperativity resulting from the generation of higher levels of oligomerization. Thus, PTS Enzymes II (and other permeases such as GalP) allosterically oligomerize, and the higher the degree of oligomerization, the higher the activity may be. Evidence for PTS permease oligomerization has been published [Alguel et al., 2016; Boer et al., 1996; Chen and Amster-Choder, 1998; Friesen et al., 2000; Jacobson, 1992; Roossien and Robillard, 1984; Saraceni-Richards and Jacobson, 1997; Stephan et al., 1989]. Thus, we suggest that the protein-protein interactions resulting in hetero-oligomerizations, promoting transport activities, may also apply to homo-oligomerization. If this is true, then a sigmoidal curve would be expected when transport activity is plotted versus the concentration of the monomeric Enzyme II protein in the membrane. The net result would be auto-stimulation. Further studies will be required to define the detailed mechanisms responsible for the reported phenomena, including the nature of the interactions and the molecular bases for stimulation or inhibition of the target transporter.

Materials and Methods

Construction of overexpression plasmids

pMAL-p2X (Aboulwafa and Saier, 2011) was used to overexpress genes encoding various transporter proteins in *E. coli.* This plasmid carries the *colE1* origin, *lacI*q, and a strong IPTG inducible promoter, *Ptac*, driving expression of the *malE* gene (useful in making N-terminal fusions for protein purification purposes). To make a control plasmid (carrying Ptac but not *malE*), pMAL-p2X was digested by *Bgl*II (+435 to +440 relative to the start site of *malE*) and *Sal*I (located downstream of *malE* in the multiple cloning site region), and then re-ligated, yielding pMAL-empty which is the same as pMAL-p2X except that most of the *malE* gene has been removed.

The structural region of *ptsG* (encoding the glucose-specific PTS enzyme IIBC component), *mtlA* (encoding the mannitol-specific PTS enzyme II), *galP* (encoding the galactose:H^+ symporter), *mtlA* (encoding the mannitol-specific PTS enzyme II), *manY* (encoding the mannose-specific PTS enzyme IIC), *manYZ* (encoding the mannose-specific PTS enzyme IIC and IID), and *ansP* (encoding the L-asparagine transporter) were PCR amplified from BW25113 genomic DNA (using specific pairs of primers as described in Table S4), digested with *Nde*I and *BamH*I, and then ligated into the same sites of pMAL-p2X individually. In each resultant recombinant plasmid, the target structural gene (no promoter and no 5' UTR) is substituted for *malE* in pMAL-p2X, and its expression is exclusively under the control of the IPTG inducible promoter, P*tac.* These plasmids are referred to pMAL-*ptsG*, pMAL-*mtlA*, pMAL-*galP*, pMAL-*manY*, pMAL-*manYZ* and pMAL-*ansP* (Table S3). The levels of the overproduced proteins (with MalE removed) were demonstrated by the levels of the

uptake of their substances (see Table 6), and the results clearly showed that expression of the chromosomal gene did not attenuate expression of the plasmid-encoded gene. The physiological relevance of overexpressing a gene from a plasmid compared to normal induction of the chromosomal gene has been considered in our previous paper [Aboulwafa et al., 2019], as has the effect of low level expression of the chromosomal gene compared to deletion mutants lacking that gene.

Chromosomal P*mtlA-lacZ*, P*man-lacZ* and P*gatY-lacZ* Transcriptional Fusions

The *mtlA* promoter region (−386 bp to +57 bp relative to the *mil A* translational start site), the *man* promoter region (−234 bp to +54 bp relative to the *manX* translational start site) and the *gatY* promoter region (−204 bp to +42 bp relative to the *gatY* translational start site), each plus a stop codon at the 3' end, were amplified from BW25113 genomic DNA. These DNA fragments, referred to as "promoter regions" (each containing a promoter region, the first 14 to 19 codons plus a stop codon at the end) were then inserted between the *Xho*I and *Bam*HI sites of the plasmid pKDT [Klumpp et al., 2009), yielding the plasmids pKDT_P*mtlA*, pKDT_P*man* and pKDT_P*gatY* respectively. In each of these newly constructed plasmids, an *rrnB* terminator (*rrnB*T) is present between the *km* gene and the downstream cloned promoter. The DNA fragments containing the *km* gene, *rrnBT*, and the nucleotides of the promoter regions (plus the first 14 to 19 codons followed by a stop codon) were PCR amplified from the above plasmids and individually integrated into the chromosome of MG1655 carrying the seamless *lacY* deletion (Klummps et al., 2009) to replace *lac*I and P*lacZ* but not the 5' Untranslated Region (UTR) of *lacZ*. All these chromosomal integrations were confirmed by colony PCR and subsequent DNA sequencing analyses. These promoter-*lacZ* fusions were individually transferred to BW25113 by P1 transduction, yielding strains BW_ P*mtlA-lacZ*, BW_P*man-lacZ* and BW_P*gatY-lacZ*, respectively (Table S3). In each of these reporter strains, the promoter of interest drives the first 14 to 19 codons of the target gene followed by the *lacZ*' 5' UTR and structural gene.

β-Galactosidase Assays

E. coli reporter strains were grown in 5 ml of media contained in 18 mm diameter glass test tubes under the same conditions as for the uptake experiments. During incubation, samples were removed for measurements of OD_{600nm} and β-galactosidase activities after being appropriately diluted.

To measure β-galactosidase activities, 0.8 ml of Z-buffer containing β-mercaptoethanol (2.7 μl/ml) and SDS (0.005%) was mixed with 0.2 ml of the sample and 25 μl CHCb in test tubes. The tubes were vortexed twice (each time for 10 seconds) at a constant speed and incubated in a 37 °C water bath till equilibration. A 0.2 ml aliquot of ONPG (4 mg/ml) was then added to each test tube. When sufficient yellow color developed, the reaction was stopped by the addition of 0.5 ml of 1 M Na_2CO_3 followed by vortexing. Then, the reaction mixtures were centrifuged, and the absorbance values of the supernatants were measured at 420 nm and 550 nm. Control tubes were run in parallel using diluted or undiluted LB broth instead of the test sample. β-galactosidase activity was measured in Miller units [(OD420-1.75 X OD550)/(sample volume in ml X time in min X OD_{600nm}] X 1000 X dilution factor (Miller, 1972).

Culture conditions for uptake of radioactive substrates and *in vitro* phosphorylation assays.

A fresh culture (100 µl of the test strain) was used to inoculate 5 ml of LB plus 100 µg/ml ampicillin (pMAL plasmid harboring strains). The 18 mm diameter tubes were incubated in a shaking water bath at 250 rpm and 37°C for 6 h. Aliquots of 100 µg/l of the cultures were used to inoculate 50 ml of LB plus 100 µg/ml ampicillin contained in 250 ml conical flasks, which were incubated in a shaking water bath at 250 rpm and 37°C for 6 h, followed by a 2 h induction period with IPTG at a final concentration of 0.2 mM. During the IPTG induction period, the shaking rate and the incubation temperature were lowered to 200 rpm and 30-32°C, respectively. The cells were harvested by centrifugation in a Sorvall centrifuge at 4°C and 10,000 rpm for 20 min. The cell pellets were washed 3x, each wash with 35 ml of 50 mM Trizma-maleate buffer, pH 7, containing 5 mM $MgCl_2$. The pellets were resuspended in the same buffer to OD_{600nm} values of 0.5, 0.25 and 0.125 for radioactive substrate uptake experiments.

Radioactive substrate uptake

For the uptake assays, each radioactive substrate was used at 5 µCi/pmole for ^{14}C while radioactive tritium was used at 30 µCi/µmole, and in all cases, the substrate concentration of the stock solution was 1 mM. For all uptake assays, the prepared bacterial suspension of the test strain was used within a time period not exceeding 12 h. The assay mixture contained 900 µl of a bacterial cell suspension of specified OD_{600nm}, 50 µl of 1 M arginine (pH 7), and 20 µl of the 1 mM stock radioactive substrate. The volume was brought to 1 ml with 50 mM Trizma-maleate buffer, pH 7, containing 5 mM $MgCl_2$.

The final concentration of the radioactive sugar substrate in the assay mixture was 20 µM for uptake assays. The uptake assays were carried out in a shaking water bath at 37°C for 5-10 min followed by immediate filtration of withdrawn samples (100-250 µl) through 0.45 micron membrane filters under vacuum. The filters containing cells were washed 3x with cold 50 mM Trizma-maleate buffer, pH 7, containing 5 mM MgCh before being dried under infra-red lamps. Each dried filter was mixed with 10 ml Biosafe NA solution in scintillation vials, and the radioactivity, expressed as counts per min (CPM), was measured in a Beckman scintillation counter. For normalization of values among samples for different test strains, the radioactivity was expressed as CPM/0.1 OD_{600nm}/0.1 ml/min. All values reported here, for both *in vivo* and *in vitro* assays, were conducted in duplicate for each of two cell preparations, and the results were averaged ±SD. Procedures were essentially as described previously [Aboulwafa et al., 2019]. A single colony of the test strain carrying or lacking the overexpression plasmid was cultured with shaking (250 RPM) in LB plus ampicillin at 37°C for 6-8 h.

Culture conditions for phosphorylation assays

The culture obtained was diluted (10 to 20-fold) in 50 ml of medium in 250 ml conical flasks or 1 l of medium in 2 l conical flasks of LB plus 100 µg/ml ampicillin. The flasks were incubated at 37°C, either in a gyratory rotating water bath at 250 RPM for the 250 ml flasks, or in a rotating incubator at 275 RPM for the 2 l flasks for 6 h plus a 2 h induction period. Induction was carried out by adding IPTG to a final concentration of 0.2 mM at 32°C

and 200 RPM. The cells were harvested by centrifugation at 4μC, washed 3x with cold modified M63, and then re-suspended in about 7 ml (pellets from 50 ml cultures) or 30 ml (pellets from 1 l cultures) of modified M63 containing 5 mM DTT. The prepared cell suspensions were disintegrated by three passages through a French press at 12,000 PSI. The resultant cell lysates were centrifuged at 10,000 RPM for 10 min at 4°C in a SORVALL centrifuge, and the supernatants produced, termed crude extract, were either used directly for PEP-dependent phosphorylation assays of PTS enzymes II or were centrifuged at 200K x g to prepare membrane pellets (MP). [Aboulwafa and Saier, 2003; Aboulwafa and Saier, 2011; Aboulwafa et al., 2019].

PTS phosphorylation assays

These assays were performed as previously described by Aboulwafa and Saier, 2002 [Aboulwafa and Saier, 2002; Aboulwafa et al., 2019]. For the PEP-dependent reactions, Enzyme II preparations were either membrane pellets (MP), or crude extracts, and the following assay mixtures were used: 50 mM potassium phosphate buffer (pH 7.4), 10 μM [^{14}C]sugar, 5 mM phosphoenolpyruvate (PEP), 12.5 mM MgCh, 25 mM KF and 2.5 mM dithiothreitol (DTT). The resin used to separate [^{14}C]sugar from [^{14}C]sugar-phosphate was Dowex® 1X8, chloride form, 50-100 mesh (Sigma-Aldrich). After 4x washing of columns containing the resin with deionized water, the 1 M lithium chloride eluate (9 ml), containing the radioactive sugar-P, was mixed with 10 ml of Biosafe II solution for radioactivity measurements in a Beckman scintillation counter.

Determination of protein concentrations

The protein concentrations were determined using the Biorad colorimetric protein assay (Cat. #500–0006) with bovine serum albumin as the standard protein.

Statistical analyses of the data

Where indicated the results were represented as average ± SD from data obtained from duplicates or triplicates of determinations using at least two different preparations of cells or extracts [Aboulwafa et al., 2019].

Materials

All radioactive sugars were purchased from New England Nuclear (NEN) Corp. or American Radiolabeled Chemicals (ARC). Nonradioactive compounds were from commercial sources, usually from the Sigma Chem. Corp. unless otherwise noted, and were of the highest purity available commercially.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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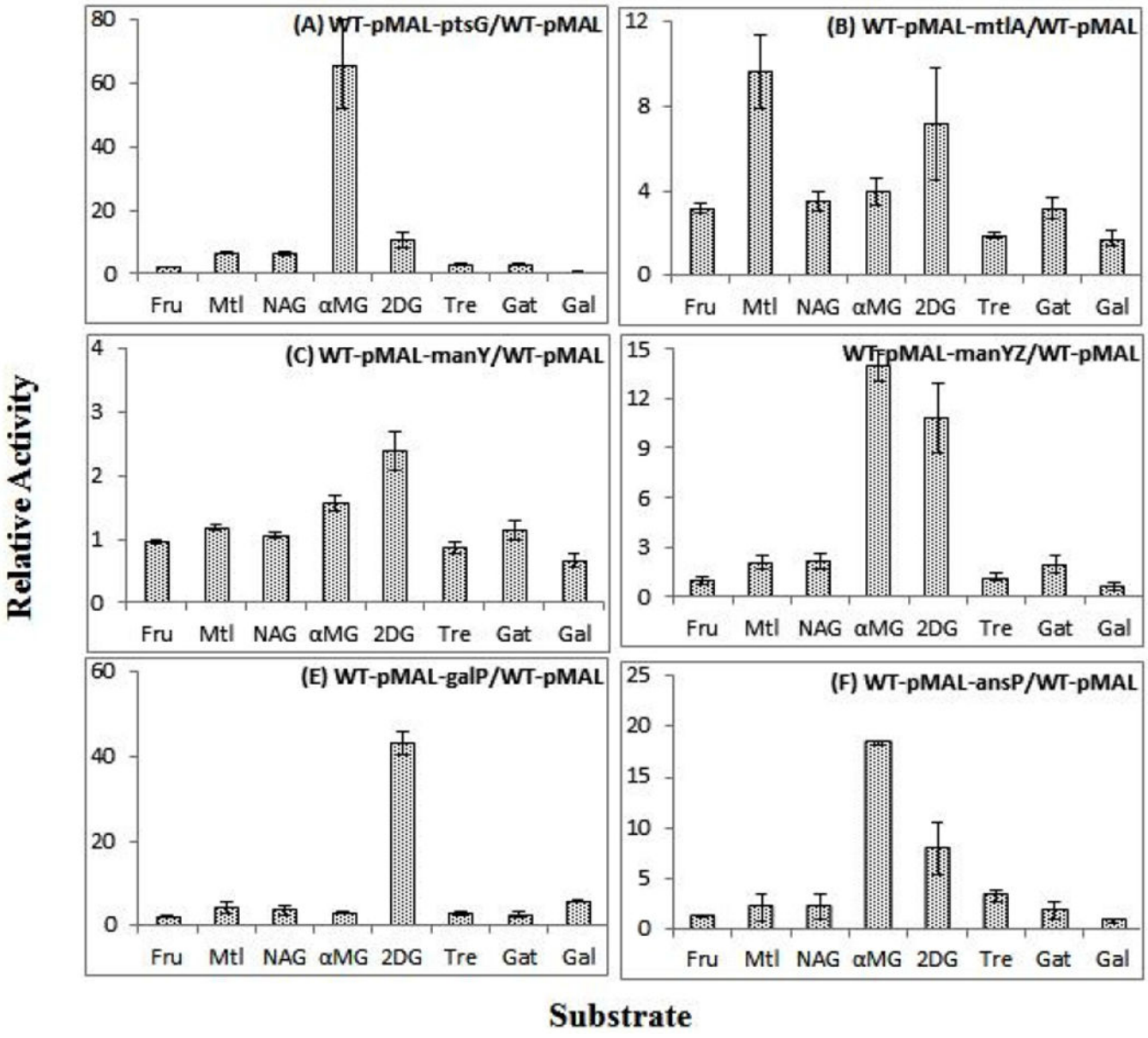

Fig. 1.

Relative activities of transport systems specific for various sugars due to permease protein gene overexpression (OE) compared to controls for *ptsG* (glucose PTS, A); *mtlA* (mannitol PTS, B); *manY* (mannose PTS Y protein, C); *manYZ* (mannose PTS YZ proteins, D); *galP* (galactose permease, E); and *ansP* (asparagine permease, F) in *E. coli* BW25113. Radioactive sugar substrates used in these experiments (x-axis) were: Fru, Fructose; Mtl, Mannitol; NAG, N-acetylglucosamine; αMG, Methyl alpha glucoside; 2DG, 2-Deoxyglucose; Tre, Trehalose; Gat, Galactitol; Gal, Galactose.

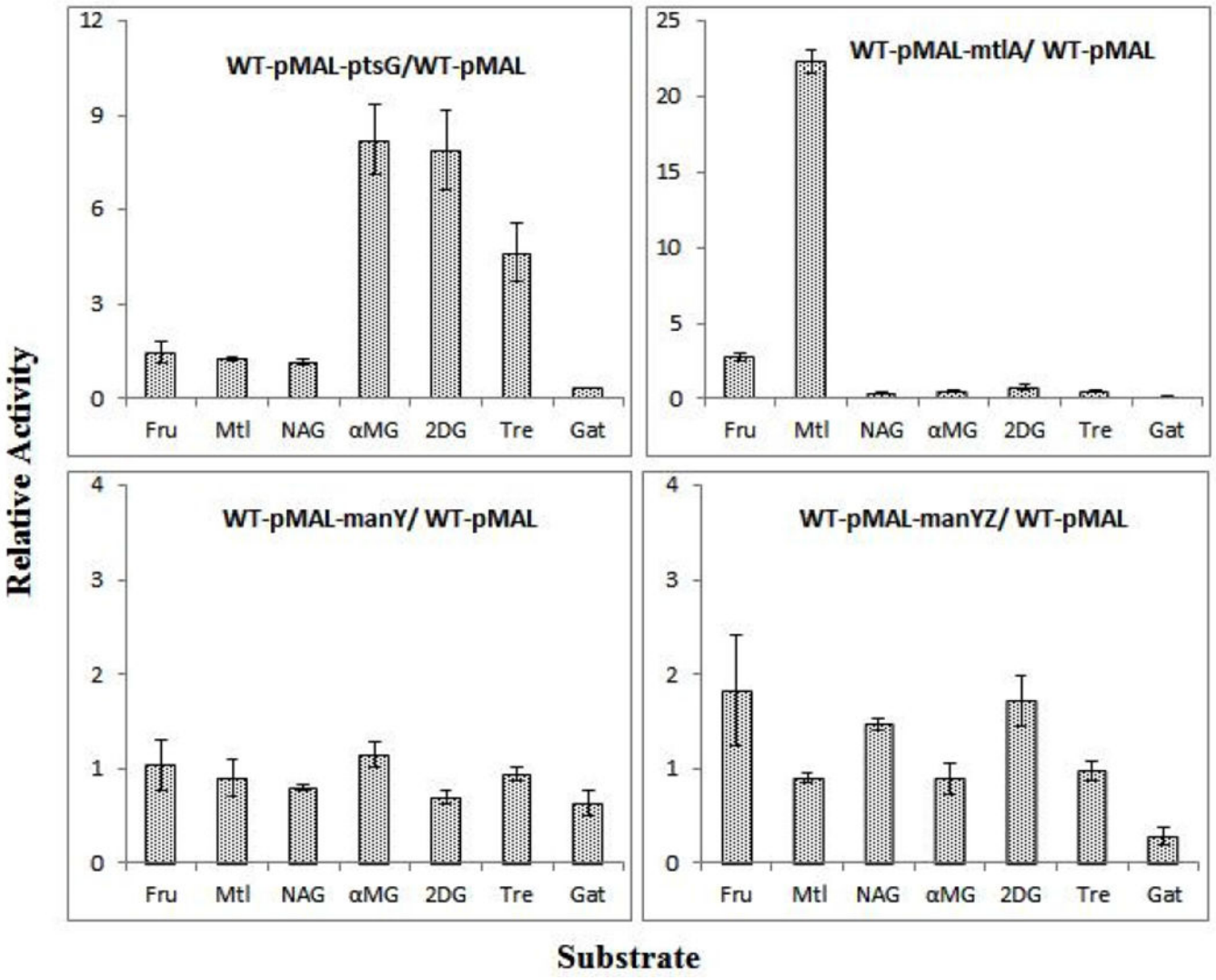

Fig. 2.

Effect of overexpressing *ptsG, mtlA, manY* or *manYZ* on PEP-dependent sugar phosphorylation in vitro by several enzyme II complexes. Sugar substrates: Fru, Fructose; Mtl, Mannitol; NAG, N-acetylglucosamine; αMG, Methyl alpha glucoside; 2DG, 2-Deoxyglucose; Tre, Trehalose; Gat, Galactitol; Gal, Galactose. The raw data are presented in Tables S1 and S2.

Table 1.

Effect of overexpression of *ptsG* on transport of various radioactive substrates as indicated below by the recombinant *E. coli* strain BW25113-pMAL-*PtsG* (WT-pMAL-*ptsG*) as compared to the BW25113-pMAL (WT-pMAL) strain. All radioactive substrates were used at a stock concentration of 1 mM, and each at a specific activity of 5 μCi/μmole except for [3H]galactitol which was used at 30 μCi/μmole. In all cases, the final substrate concentration in the assay mixture was 20 μM.

Radioactive substrate	Transport activity (CPM/min/0.1 OD/0.1 ml)		Relative transport activity (WT-pMAL-*ptsG*/WT-pMAL)		
	WT-pMAL	WT-pMAL-*ptsG*	Value	Average	SD
Fructose	18	36	2.1	2.1	0.0
	16	33	2.1		
Mannitol	24	159	6.5	6.6	0.2
	23	153	6.8		
N-Acetylglucosamine	21	141	6.8	6.3	0.7
	24	142	5.8		
Methyl alpha glucoside	4	245	55.9	65.8	14.0
	4	316	75.8		
2-Deoxyglucose	1	11	12.4	10.7	2.4
	1	12	9.0		
Trehalose	12	36	3.1	2.9	0.3
	13	35	2.7		
Galactitol	23	60	2.6	2.8	0.3
	21	64	3.1		
Galactose	17	17	1.0	1.0	0.0
	15	16	1.1		
Asparagine	79	32	0.4	0.4	0.0
	79	32	0.4		

Table 2.

Effect of overexpression of *mtlA* on transport of radioactive substrates as indicated below by the recombinant *E. coli* strain BW25113-pMAL-*mtlA* (WT-pMAL-*mtlA*) as compared to the BW25113-pMAL (WT-pMAL) strain. The experiment was conducted as described for Table 1.

Radioactive substrate	Transport activity (CPM/min/0.1 OD/0.1 ml)		Relative transport activity (WT-pMAL-*mtlA*/WT-pMAL)		
	WT-pMAL	WT-pMAL-*mtlA*			
	Value	Value	Value	Average	SD
Fructose	13	45	3.4	3.2	0.3
	12	37	3.0		
Mannitol	23	247	10.9	9.6	1.7
	25	214	8.4		
N-Acetylglucosamine	26	98	3.8	3.5	0.4
	28	92	3.2		
Methyl alpha glucoside	4	19	4.4	4.0	0.6
	4	16	3.5		
2-Deoxyglucose	2	9	5.3	7.2	2.6
	1	13	9.0		
Trehalose	14	28	2.0	1.9	0.1
	16	29	1.8		
Galactitol	21	74	3.5	3.2	0.5
	21	59	2.8		
Galactose	13	26	2.0	1.8	0.4
	13	19	1.5		
Asparagine	72	94	1.3	1.3	0.1
	79	94	1.2		

Table 3.

Effect of overexpression of *manY* on transport of radioactive substrates as indicated below by the recombinant *E. coli* strain BW25113-pMAL-*manY* (WT-pMAL-*manY*), as compared to the BW25113-pMAL (WT-pMAL) strain. The experiment was conducted as described for Table 1.

Radioactive substrate	Transport activity (CPM/min/0.1 OD/0.1 ml)		Relative transport activity (WT-pMAL-*manY*/WT-pMAL)		
	WT-pMAL	WT-pMAL-*manY*			
	Value	Value	Value	Average	SD
Fructose	33	31	0.9	1.0	0.03
	38	37	1.0		
Mannitol	83	102	1.2	1.2	0.05
	97	113	1.2		
N-acetylglucosamine	87	89	1.0	1.1	0.05
	106	116	1.1		
Methyl alpha glucoside	5	8	1.5	1.6	0.12
	12	20	1.7		
2-Deoxyglucose	4	11	2.6	2.4	0.30
	7	15	2.2		
Trehalose	28	23	0.8	0.9	0.09
	39	36	0.9		
Galactitol	73	75	1.0	1.1	0.16
	93	117	1.3		
Galactose	21	15	0.7	0.7	0.11
	27	15	0.6		
Asparagine	102	73	0.7	0.7	0.04
	116	89	0.8		

Table 4.

Effect of overexpression of *manYZ* on transport of radioactive substrates as indicated below by the recombinant *E. coli* strain BW25113-pMAL-*manYZ* (WT-pMAL-*manYZ*), as compared to the BW25113-pMAL (WT-pMAL) strain. The experiment was conducted as described for Table 1.

Radioactive substrate	Transport activity (CPM/min/0.1 OD/0.1 ml)		Relative transport activity (WT-pMAL-*manZY*/WT-pMAL)		
	WT-pMAL	WT-pMAL-*manZY*			
	Value	Value	Value	Average	SD
Fructose	26	30	1.1	1.0	0.2
	32	26	0.8		
Mannitol	59	139	2.4	2.1	0.4
	82	144	1.8		
N-acetylglucosamine	64	155	2.4	2.1	0.5
	94	167	1.8		
Methyl alpha glucoside	4	59	13.3	14.0	1.0
	6	81	14.6		
2-Deoxyglucose	3	40	13.7	10.8	4.1
	4	36	7.9		
Trehalose	26	34	1.3	1.2	0.2
	33	33	1.0		
Galactitol	48	112	2.3	2.0	0.6
	67	104	1.6		
Galactose	6	5	0.8	0.6	0.3
	21	10	0.5		
Asparagine	100	38	0.4	0.3	0.2
	109	18	0.2		

Table 5.

Effect of overexpression of *galP* on transport of radioactive substrates as indicated below by recombinant wild type strain *E. coli* strain BW25113-pMAL-*galP* (WT-pMAL-*galP*), as compared to the BW25113-pMAL (WT-pMAL) strain. The experiment was conducted as described in Table 1.

Radioactive substrate	Transport activity (CPM/min/0.1 OD/0.1 ml)		Relative transport activity (WT-pMAL-*glpP*/WT-pMAL)		
	WT-pMAL	WT-pMAL-*galP*			
	Value	Value	Value	Average	SD
Fructose	17	40	2.4	2.3	0.1
	13	28	2.2		
Mannitol	23	121	5.3	4.4	1.3
	24	82	3.5		
N-Acetylglucosamine	21	98	4.6	3.8	1.2
	26	77	3.0		
Methyl alpha glucoside	4	14	3.2	3.1	0.1
	4	13	3.0		
2-Deoxyglucose	1	67	45.0	43.1	2.6
	1	42	41.3		
Trehalose	13	32	2.5	2.9	0.6
	11	38	3.3		
Galactitol	21	70	3.4	2.7	0.9
	38	80	2.1		
Galactose	16	91	5.8	6.0	0.2
	15	90	6.1		
Asparagine	71	73	1.0	1.3	0.4
	43	68	1.6		

Table 6.

Effect of overexpression of *ansP* on transport of radioactive substrates as indicated below by the recombinant *E. coli* strain BW25113-pMAL-*ansP* (WT-pMAL-*ansP*), as compared to the BW25113-pMAL (WT-pMAL) strain. The experiment was conducted as described in Table 1.

Radioactive substrate	Transport activity (CPM/min/0.1 OD/0.1 ml)		Relative transport activity (WT-pMAL-*ansP*/WT-pMAL)		
	WT-pMAL	WT-pMAL-*ansP*			
	Value	Value	Value	Average	SD
Fructose	14	18	1.3	1.3	0.1
	13	16	1.2		
Mannitol	25	57	2.3	2.2	0.1
	23	48	2.1		
N-acetylglucosamine	31	64	2.1	2.2	0.2
	22	52	2.4		
Methyl alpha glucoside	5	93	19.0	18.4	0.8
	4	72	17.8		
2-Deoxyglucose	1	9	7.2	8.0	1.1
	2	13	8.7		
Trehalose	13	44	3.5	3.3	0.3
	14	42	3.1		
Galactitol	17	36	2.0	1.9	0.2
	21	37	1.7		
Galactose	16	13	0.8	0.8	0.0
	14	11	0.8		
Asparagine	45	76	1.7	1.9	0.2
	70	143	2.0		

Table 7.

Summary of the effects of overexpression of various transport proteins on radioactive substrate uptake. These data were derived from Tables 1–6. Only values (ratios of activity +/− overexpression) of at least 2-fold are recorded here. Values in parentheses are those for which the overproduced transporter is likely to have directly increased the substrate transport rate. All other values are attributed to stimulation of the transport activities of other transport systems involving protein-protein interactions.

Radioactive substrate	**Overexpression of**					
	ptsG	***mtlA***	***manY***	***manYZ***	***galP***	***ansP***
Fructose	2.1	3.2	-	-	2.3	-
Mannitol	6.6	(9.6)	-	2.1	4.4	2.2
N-acetylglucosamine	6.3	3.5	-	2.1	3.8	2.2
Methyl alpha glucoside	(65.8)	4.0	-	14.0	3.1	18.4
2-Deoxyglucose	10.7	7.2	2.4	(10.8)	(43.1)	8.0
Trehalose	(2.9)	-	-	-	(2.9)	3.3
Galactitol	2.8	3.2	-	2.0	2.7	-
Galactose	-	-	-	-	(6.0)	-
Asparagine	-	-	-	0.3	-	-

Table 8.

Effect of overexpression of genes encoding several membrane transporters (PtsG, GalP, MtlA, and AsnP) on the expression of three genes encoding PTS transport proteins (MtlA, ManXYZ, and GatY) in *E. coli* using *lacZ* transcriptional fusions as indicated below. In no case was the difference due to gene overexpression significantly different from the control. In the last column, values were calculated relative to controls. A negative sign indicates a decrease in expression level relative to the control.

***E. coli* strain overexpressing membrane transporter genes**	**Tested PTS transporter gene fused to *lacZ***	**LacZ activity Value ± SD**	**(%)* change In expression**
BW25113-*mtlA*-PZ-pMAL (“Wild type” control strain)	*mtlA-PlacZ*	173±1	
BW25113-*mtlA*-PZ-pMAL-ptsG (“Wild type” overexpressing *ptsG)*	*mtlA-PlacZ*	158±8	−8%
BW25113-*mtlA*-PZ-pMAL-*galP* (“Wild type” overexpressing *galP)*	*mtlA-PlacZ*	163±1	−6%
BW25113-*manXYZ*-PZ-pMAL (“Wild type” control strain)	*manXYZ-PlacZ*	141±6	
BW25113-*manXYZ*-PZ-pMAL-*ptsG* (“Wild type” overexpressing *ptsG*)	*manXYZ-PlacZ*	113±2	−20%
BW25113-*manXYZ*-PZ-pMAL-*mtlA* (“Wild type” overexpressing *mtlA*)	*manXYZ-PlacZ*	112±3	−21%
BW25113-*manXYZ*-PZ-pMAL-*galP* (“Wild type” overexpressing *galP*)	*manXYZ-PlacZ*	103±6	−27%
BW25113-*manXYZ*-PZ-pMAL-*ansP* (“Wild type” overexpressing *asnP*)	*manXYZ-PlacZ*	110±11	−22%
BW25113-*gatY*-PZ-pMAL (“Wild type” control strain)	*gatY-PlacZ*	3794±385	
BW25113-*gatY*-PZ-pMAL-*mtlA* (“Wild type” overexpressing *mtlA*)	*gatY-PlacZ*	3475±247	−8%

Table 9.

Summary of responses of *lacZ* fusion bearing strains to various conditions and genetic backgrounds. WT = wild type; OE = overexpression. All strains were grown in LB medium, ND refers to not done.

Strain	*mtlA-lacZ*	*gatC-lacZ*	*manXYZ-lacZ*
WT, *ptsG* OE	0.9	ND	0.8
WT, *mtlA* OE	ND	0.9	0.8
WT, *galP* OE	0.9	ND	0.7
WT, *asnP* OE	ND	ND	0.8