Protein:Protein Interactions in the Cytoplasmic Membrane Influencing Sugar Transport and Phosphorylation Activities of the *E. coli* Phosphotransferase System.

Mohammad Aboulwafa\textsuperscript{1,2}, Zhongge Zhang\textsuperscript{1} and Milton H. Saier, Jr.\textsuperscript{1*}

\textsuperscript{1}Department of Molecular Biology, Division of Biological Sciences, University of California at San Diego, La Jolla, CA 92093-0116.

\textsuperscript{2}Department of Microbiology and Immunology, Faculty of Pharmacy, Ain Shams University, Abbassia, Cairo, Egypt.

\textsuperscript{*}Corresponding Author: Tel +1 858 534 4084
Fax: +1 858 534 7108
Email: msaier@ucsd.edu

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Running title:
Interacting PTS integral membrane proteins
Abstract

The multicomponent phosphoenolpyruvate-dependent sugar-transporting phosphotransferase system (PTS) in *Escherichia coli* takes up sugar substrates and concomitantly phosphorylates them. We have recently provided evidence that many of the integral membrane PTS permeases interact with the fructose PTS (FruA/FruB) [1]. However, the biochemical and physiological significance of this finding was not known. We have carried out molecular genetic/biochemical/physiological studies that show that interactions of the fructose PTS often enhance, but sometimes inhibit the activities of other PTS transporters many fold, depending on the target PTS system under study. Thus, the glucose, mannose, mannitol and N-acetylglucosamine permeases exhibit enhanced *in vivo* sugar transport and sometimes *in vitro* PEP-dependent sugar phosphorylation activities while the galactitol and trehalose systems show inhibited activities. This is observed when the fructose system is induced to high levels and prevented when the *fruA/fruB* genes are deleted. Overexpression of the *fruA* and/or *fruB* genes in the absence of fructose induction during growth also enhances the rates of uptake of other hexoses. The β-galactosidase activities of *man, mtl*, and *gat-lacZ* transcriptional fusions and the sugar-specific transphosphorylation activities of these enzyme transporters were not affected either by fructose induction or *fruAB* overexpression, showing that the rates of synthesis and protein levels in the membrane of the target PTS permeases were not altered. We thus suggest that specific protein-protein interactions within the cytoplasmic membrane regulate transport *in vivo* (and sometimes the PEP-dependent phosphorylation activities *in vitro* of PTS permeases) in a physiologically meaningful way that may help to provide a hierarchy of preferred PTS sugars. These observations appear to be applicable in principle to other types of transport systems as well.
Introduction

The prokaryotic phosphotransferase system (PTS) consists of two general energy coupling proteins, Enzyme I, (EI, PtsI) and HPr (HPr, PtsH), as well as the sugar-specific Enzyme II (EII) complexes [1-5]. The EII complexes usually consist of three proteins or protein domains, IIA, IIB and IIC, although one family, the mannose EII family, consists of systems with four proteins, IIA, IIB, IIC and IID [6, 7]. The IIA and IIB proteins are cytoplasmic phosphoryl carrier proteins or protein domains while the IIC proteins/domains are integral membrane transporters that catalyze sugar phosphorylation concomitantly with uptake [8, 9]. In these coupled sugar transport/phosphorylation reactions, the phosphoryl moiety of IIB-P is transferred to the incoming sugar to yield a cytoplasmic sugar phosphate (Sugar-P) [10]. The overall phosphoryl transfer reactions are therefore:

\[
\begin{align*}
IIA^{\text{Mtl}} & \rightarrow II B^{\text{Mtl}} \rightarrow I I C^{\text{Mtl}} & \text{Mannitol (out)} \\
\text{PEP} & \rightarrow EI \rightarrow H Pr \rightarrow I I A^{\text{Fru}} \rightarrow II B^{\text{Fru}} \rightarrow I I C^{\text{Fru}} & \text{Fructose (out)} \\
IIA^{\text{NAG}} & \rightarrow II B^{\text{NAG}} \rightarrow I I C^{\text{NAG}} & \text{N-acetylglucosamine (out)} \\
\text{Mtl = mannitol; Fru = fructose; NAG = N-acetylglucosamine} \\
\end{align*}
\]

In addition to these three D-sugars, mannitol (Mtl), fructose (Fru) and N-acetylglucosamine (NAG), the PTS transports and phosphorylates many other sugars, and each bacterial or archaeal species possessing the PTS has a different complement of PTS Enzyme II
complexes. Sugars transported via the PTS in various organisms include aldo- and keto-hexoses, amino sugars and their N-acetylated derivatives, hexitols, pentoses, pentitols and a variety of disaccharides, oligosaccharides and glycosides [8]. In Escherichia coli, there are many Enzyme II (EII) complexes, some of which are still not functionally characterized [11]. The EIIs we will be concerned with in this report, in addition to the three described above, are specific for sugars such as galactitol (Gat), glucose (Glc) and the non-metabolizable glucose analogue, methyl α-glucoside (αMG), trehalose (Tre), and mannose (Man) [this system also transports Glc, glucosamine (Glm) and 2-deoxyglucose (2DG)]. These systems are tabulated in Table 1 with their protein abbreviations, protein domain orders and TC numbers (TC #s) in the Transporter Classification Database (TCDB) [12-15].

Table 1. Proteins of the PTS in E. coli, relevant to the study reported here. References for these systems can be found in TCDB.

| PTS Complex     | Protein Constituents (Domains)                                    | TC #   |
|-----------------|------------------------------------------------------------------|--------|
| Fructose (Fru)  | FruA: IIC\textsubscript{Fru}-IIB\textsubscript{Fru}-II\textsubscript{B}\textsubscript{Fru} \  
|                 | FruB: IIA-FPr                                                    | 4.A.2.11 |
| Mannitol (Mtl)  | MtlA: IIC\textsubscript{Mtl}-IIB\textsubscript{Mtl}-IIA\textsubscript{Mtl} \  
| Galactitol (Gat)| GatA: IIA\textsubscript{Gat}; GatB: IIB\textsubscript{Gat}; \  
|                 | GatC: IIC\textsubscript{Gat}                                    | 4.A.5.1.1 |
| N-Acetylglucosamine (NAG) | NagE: IIC\textsubscript{NAG}-IIB\textsubscript{NAG} \  
| Glucose (Glc)   | PtsG: IIC\textsubscript{Glc}-II\textsubscript{B}\textsubscript{Glc}; \  
|                 | Crr: IIA\textsubscript{Glc}                                     | 4.A.1.1.1 |
| Mannose (Man)   | ManX: IIAB\textsubscript{Man}; ManY: IIC\textsubscript{Man}; \  
|                 | ManZ: IID\textsubscript{Man}                                    | 4.A.6.1.1 |
| Trehalose (Tre) | TreB: IIB\textsubscript{Tre}-II\textsubscript{C}\textsubscript{Tre} \  
|                 | (Uses IIA\textsubscript{Glc}; there is no IIA\textsubscript{Tre}) | 4.A.1.2.4 |

In addition to the transport reaction that can be studied in vivo, there are two in vitro sugar phosphorylation reactions, the PEP-dependent sugar phosphorylation reaction that depends on EI, HPr and the complete EII complex, and a sugar-P:sugar transphosphorylation (TP)
reaction that depends only on IIB and IIC [16-19]. All of these reactions have been used to investigate the consequences of integral membrane protein interactions in the current study.

Recently, Babu et al published global interactome data for *E. coli*, including cytoplasmic proteins, integral inner and outer membrane proteins and periplasmic proteins [1]. These studies revealed that many Enzyme II components interact with other soluble and integral membrane proteins of the PTS as well as with other non-PTS proteins. A representative selection of these PTS protein interactions is reproduced in Table 2. Of all the Enzyme II constituents, the fructose PTS proteins, FruA and FruB, appeared to form the most extensive PTS interactome network.

The observation that the Fru PTS might serve to coordinate the activities of other PTS EII complexes correlated with the previously published suggestion that the fructose PTS was the primordial PEP-dependent PTS Enzyme II complex [20]. This suggestion resulted from several observations. (1) The fructose PTS, of all the sugar-specific Enzyme II complexes, is most widely distributed in the prokaryotic world. (2) The fructose system in *E. coli* has more fructose-like Enzyme II complex homologs than any other. (3) Fructose is the only sugar that feeds directly into glycolysis without interconversion to another sugar derivative. (4) Only fructose can feed into glycolysis via two distinct PTS-mediated pathways: Fru → Fru-1-P → Fru-1,6-bis-P (II\textsubscript{Fru}), and Fru → Fru 6-P → Fru-1,6-bis P (II\textsubscript{Man}). (5) Only the fructose PTS of functionally characterized systems in *E. coli* has its own HPr-like protein, FPr. (6) Only the fructose Enzyme II complex of *E. coli* and many other bacteria have extra IIB domains that function in protein-protein interactions rather than phosphoryl transfer [21].

More recent studies revealed that there are at least three evolutionarily distinct superfamilies of PTS Enzyme IIC components [22]. The PTS Glucose-Glucoside (Glc) family, the Fructose-Mannitol (Fru) family, (3) the Lactose-N, N′-Diacytethylchitobiose-β-Glucoside (Lac)
family, and (4) probably the Glucitol (Gut) family comprise one large sequence-divergent
superfamily with all IIC constituents being homologous (TC# 4.A.1-4; see TCDB,
www.tcdb.org) [12, 13, 23, 24]. Second, the Galactitol (Gat) family (TC# 4.A.5) and the L-
Ascorbate (L-Asc) family (TC# 4.A.7) comprise a distinct superfamily [11, 24, 25], and finally,
the functionally diverse Mannose family (TC# 4.A.6) comprises the third PTS superfamily [26].
Each of these three superfamilies of IIC permeases are believed to have evolved independently
of each other [22, 27]. However, it should be noted that the IIA and IIB phospho-carrier
constituents, which also probably evolved at least 3 times independently of each other, did not
coevolve with the IIC permease constituents, suggesting that there has been extensive shuffling
of the IIA, IIB and IIC constituents/domains during the evolutionary divergence of these protein
complexes [22, 28-30].

Bacterial cytoplasmic membranes are crowded with integral membrane proteins that form
complexes, inhibiting their lateral mobilities, constraining their flexibilities and probably altering
their activities [31-34]. Our integral membrane interactome data suggested that many membrane
proteins interact with many other membrane proteins, with a wide range of affinities [1]. Some
of the high scoring interactions are likely to be permanent, high affinity interactions, while lower
scoring interactions are more likely to be transient in nature [35, 36]. Among the observed
interactions were many involving Enzyme IIC constituents of the PTS, particularly the fructose
IIC protein, FruA, which also bears two additional domains, IIB and IIB’, where IIB’ is not
involved in phosphoryl transfer, but plays a role in protein-protein interactions [21] (Table 2).
Table 2. Selected high scoring PTS transporter (Enzyme II complex) interactions. The data were derived from Babu et al., 2018 [1]. The higher the score, the stronger the interaction should be, although these scores are also dependent on the protein concentrations.

| Protein 1 | Protein 2 | Score |
|-----------|-----------|-------|
| FruA      | FruB      | 12.9  |
| FruA      | MtlA      | 11.4  |
| FruA      | GatC      | 10.3  |
| FruA      | NagE      | 9.4   |
| FruA      | TreB      | 7.4   |
| FruA      | MngA      | 7.1   |
| FruA      | DhaL      | 6.9   |
| MtlA      | GatC      | 9.7   |
| MtlA      | FruB      | 8.4   |
| ManY      | GatA      | 7.5   |
| ManY      | GatB      | 6.9   |
| ManY      | GatC      | 7.4   |
| ManY      | MngA      | 7.7   |
| NagE      | PtsG      | 7.0   |
| NagE      | FruB      | 5.4   |

As noted above, the PTS offers technical advantages over other transport systems for its characterization. It can be assayed *in vivo* by measuring its transport activities, and it can be assayed *in vitro* by measuring its sugar phosphorylation reactions. These include its PEP-dependent sugar phosphorylating activities as well as its sugar-P-dependent transphosphorylating (TP) activities [19, 37]. The latter reactions require only the IIB and IIC domains which are usually, but not always, fused together (see Table 1). Thus, while the PEP:sugar activity requires protein:protein interactions involving EI, HPr, IIA and IIBC, the sugar-P:sugar TP reaction does not depend on these interactions (except for IIP\(^{\text{Man}}\)) since only the IIC and IIB domains are directly involved.

The results of the studies reported here suggest that in the cytoplasmic membrane of *E. coli*, the transport of various PTS sugars is influenced by the presence of other PTS permeases in general, and the degree of activation or inhibition depends on the specific systems under study as
well as their concentrations in the membrane. Thus, high level expression of the fructose EII complex activates the mannitol, N-acetyl glucosamine, glucose and mannose systems, but it inhibits the galactitol and trehalose systems in wild type cells. Although these protein-protein interactions do not appreciably affect the sugar-P:sugar TP reactions or synthesis of the target EIIs, we did observe a highly specific activation of the in vitro PEP-dependent phosphorylation of mannitol and N-acetylglucosamine, dependent on both FruA and FruB. In this case, we propose that FruB activates MtlA, but that this activation depends on the presence of FruA which could anchor FruB to the membrane, adjacent to MtlA. The more general conclusion is that integral membrane transport proteins in the cytoplasmic membranes of \textit{E. coli} are in close contact and influence each other’s activities in the intact cell, either in one direction (activation) or the other (inhibition). In some cases, these effects appear to be highly specific, while in others they may be more general. This is, to the best of our knowledge, the first example where global proteomic analyses based on a complete interactome data set, have led to the discovery of regulating interactions influencing the enzymatic and transport activities of some of the interacting integral membrane proteins.

\textbf{Results}

The \textit{E. coli} integral membrane protein interactome, which includes putative interactions with other integral membrane proteins as well as other cellular proteins, has been published \cite{1}. Table 2 summarizes some of the high scoring interactions observed between the constituents of different Enzyme II complexes of the PTS. Not surprisingly, the fructose-1-P-forming fructose-specific membrane constituent of the PTS, FruA (the enzyme \textit{IICBB}^{Fru}), interacts with its own FruB protein, the fructose-specific enzyme II\textit{A-FPr} protein, the immediate phosphoryl donor for fructose transport energization and phosphorylation, thus providing the energy for fructose
uptake via FruA (Table 1; [21]). The interaction score is high (12.9), indicating that this
interaction occurs with high affinity since the conditions used to measure these interactions
involved growth in LB medium without induction of PTS gene expression [1]. In addition, FruA
appeared to interact with several other Enzyme II constituents of the PTS with large scores, far
more than for any other PTS Enzyme IIC. These interactions include those with MtlA (the
IICBA specific for mannitol; score of 11.4), GatC (The galactitol Enzyme IIC; score of 10.3),
NagE (The N-acetylglucosamine Enzyme IICBA; score of 9.4), TreB (The trehalose Enzyme
IIBC; score of 7.4), and MngA (the 2-0-α-mannosyl-D-glycerate Enzyme IIABC; score of 7.1)
(Tables 1 and 2). Other Enzymes II appeared to interact with FruA with lower scores. MtlA
additionally interacts with FruB (score, 8.4) and GatC (score, 9.7), while the broad specificity
glucose/mannose/fructose/glucosamine Enzyme IIC, ManY, interacts with all three constituents
of the galactitol Enzyme II complex, GatA (7.5), GatB (6.9) and GatC (7.4) as well as MngA
(7.7) (Table 2).

Many other integral membrane proteins, several of which proved to be transporters, also
appeared to interact with FruA. These include the MDR pumps, AcrB (score of 12.9) and MdtF
(17.9), the dicarboxylate transporter, DcuA (17.2), the iron porters, FeoB (11.4) and Fet (YbbM;
14.3), the FocA formate channel (9.5), the NupC nucleoside uptake porter (19.6), the MgtA Mg2+
uptake ATPase (12.1) and others [1]. These results suggest that numerous integral membrane
transport systems interact, possibly within the hydrophobic matrix of the cytoplasmic membrane,
but their biochemical and physiological significance could not be determined from these data
alone. Therefore, the transport studies and biochemical analyses reported here were conducted.
Confirmation of the interactome results using a bacterial two hybrid system

As reported by Babu et al. [1], FruA interacts with other membrane proteins as well as its cognate soluble partner FruB (Table 2). A bacterial Adenyl Cyclase two hybrid (BACTH) system was used to confirm these interactions, conducted according to the manufacturers’ instructions (Euromedex bacterial two-hybrid system, Cat # EUK001). These interactions of FruA were demonstrated for the GatC, NagE, TreB and MtlA membrane proteins as well as the cytoplasmic FruB protein. Also, interactions of FruB were observed for MtlA and NagE (S2 Fig). These observations confirm the interactions reported by Babu et al., 2018 and suggest that many integral membrane proteins interact with each other in the plane of the membrane, many of them most likely in a transient fashion.

Transport studies involving PTS group translocators

Because the FruA protein seemed to interact with many other Enzyme II protein complexes with high scores, more than for any of the other PTS proteins, we initially decided to examine the effect of growth with and without fructose to induce synthesis of the fructose-specific Enzyme II complex, FruA/FruB, on the rates of uptake of other PTS sugars in wild type (WT) *E. coli* cells. The results are summarized in Fig 1A, which gives the relative rates of sugar uptake by the fructose-induced cells relative to the uninduced cells. The raw data for this Figure is presented in S1, S2, S3, S3’, S4, S4’, S5, and S5’ Tables. As a control, the same experiments were conducted with the isogenic strain deleted for the *fruBKA* operon (*ΔfruBKA*; triple mutant (TM)), Fig 1B.

**Figure 1. Uptake ratios for various sugars (PTS substrates and galactose) by wild type *E. coli* BW25113 (WT) and its *fruBKA* isogenic mutants (BW25113*ΔfruA*, BW25113*ΔfruB* and BW25113-*fruBKA:kn* (TM)) using cells grown in LB with and without fructose.**

(A) Relative sugar uptake by wild type *E. coli* BW25113 (WT) grown in the presence and absence of fructose. (B) Relative sugar uptake by the *fruBKA* triple *E. coli* BW25113 mutant (TM) grown in the presence and absence of fructose. (C&D) Sugar uptake by the triple mutant (TM) relative to that of the wild
The results presented in Fig 1A show that in a wild type E. coli strain, growth in the presence of fructose enhanced the rates of uptake of some sugars while decreasing the rates of uptake of others. Thus, the mannitol (Mtl) uptake rate increased 7-fold, N-acetylglucosamine (NAG) uptake increased 6.4-fold, 2-deoxyglucose (2DG) uptake increased 8.7-fold, and methyl α-glucoside (αMG) uptake increased 2.8-fold, due to the presence of fructose in the growth medium. Interestingly, trehalose (Tre) and galactitol (Gat) rates of uptake decreased while the galactose (Gal, a non-PTS sugar) uptake rate did not change appreciably.

The fructose operon was completely deleted, and this ΔfruBKA deletion strain (triple mutant; TM) was used in parallel experiments (Fig 1B). With the fruBKA operon absent (triple mutant; TM), the fructose uptake rate was reduced to 5% of the wild type rate. None of the other PTS sugar uptake rates, except for that of 2-deoxyglucose, taken up by the mannose system, were affected by the inclusion of fructose in the growth medium. When the TM was examined relative to the wild type (WT) strain, with only LB in the growth medium, none of the sugar uptake rates were affected (Fig 1C), but when fructose was present during growth, the uptake of those sugars that had been stimulated by fructose induction in the WT showed low ratios of uptake, while those that were inhibited in the WT showed increased ratios (Fig 1D), in agreement with those presented in Fig 1A. When either fruA or fruB was deleted, all regulatory effects were abolished (Figs 1E and 1G), as was true for the ΔfruBKA strain (Fig 1C). However, the uptake ratios, ΔfruA/WT and ΔfruB/WT, showed depressed values for those sugars whose uptake was increased by fru operon induction in the WT (Mtl, NAG, αMG and 2DG), and
increased values for those sugars whose uptake was depressed by \textit{fru} operon induction (Tre and Gat) (compare Figs 1F and 1H with Figs 1A and 1D).

In summary, it appears that high level expression of the \textit{fruBKA} operon has the greatest effect on the transport of other PTS sugars, with Mtl, NAG and 2DG uptake rates consistently showing the greatest activation while those for the Gat and Tre systems showed the greatest inhibition. In \textit{ΔfruA} and \textit{ΔfruB} mutants, the opposite effects were observed relative to the wild type strain. These results suggested that either FruA, FruB, or more likely, both, serve(s) regulatory roles, influencing the rates of uptake of other PTS sugars, positively or negatively, depending on the sugar-specific transport system assayed.

Fig 2 presents corresponding results for strains in which the \textit{fruA}, \textit{fruB}, or \textit{fruA} and \textit{fruB} genes were overexpressed. Overexpression of \textit{fruA} in the TM genetic background increased Mtl and NAG uptake rates 4-5-fold, but increased αMG and 2DG uptake rates 7 and 11-fold, respectively, compared to the control strain bearing the empty pMAL plasmid (Fig 2A). When the same experiment was conducted in the wild type genetic background, qualitatively similar results were obtained, but enhancement of the uptake rates were diminished except for the mannose system, which was increased (Fig 2B). Only in the WT background did overexpression of \textit{fruA} or \textit{fruB} increase the 2DG uptake rate. Surprisingly, in both cases, Tre and Gat uptake rates were not appreciably depressed. When \textit{fruB} was overexpressed in either the triple mutant (TM; Fig 2C) or the WT genetic background (WT; Fig 2D), there was a minor (~2x) increase in activities except that \textit{fruB} overexpression did seem to stimulate 2DG uptake. Finally, when both \textit{fruA} and \textit{fruB} were simultaneously overexpressed using two different compatible plasmids, in either the TM or WT genetic background, the stimulatory effects on Mtl, NAG, αMG and 2DG uptake rates were similar to those observe when only \textit{fruA} was overexpressed. Again, in this
experiment, overexpression of \textit{fruA}, \textit{fruB}, or \textit{fruA} and \textit{fruB} did not cause the Tre and Gat uptake rates to decrease. Here, we have to realize that the overexpression of FruA and FruB caused by fructose induction was dissimilar from that obtained using the two compatible plasmids, pMAL and pZA31-PtetM2, with different copy numbers. Thus, balanced and equimolar expression for FruA and FruB was achieved in the former case but not in the latter one. Additionally, both FruA and FruB interact with many proteins (see Introduction) which may influence the results. Nevertheless, the results confirm the suggestion that FruA is primarily responsible for the stimulation of Mtl, NAG, 2DG and \( \alpha \)MG uptake, although FruB may play a lesser role.

\textbf{Figure 2. Uptake ratios for various sugars (PTS substrates and galactose) by wild type \textit{E. coli} BW25113 (WT) and its \textit{fruBKA} triple mutant (BW25113-\textit{fruBKA}:kn} (TM) over-expressing individual or combined \textit{fruBKA} operon genes.}

Ratios of transport rates for [\(^{14}\text{C}\)]sugar uptake in wild type (WT) and \( \Delta \text{fruBKA} \) mutant backgrounds over-expressing individual or combined \textit{fruBKA} operon genes as presented at the tops of the figures. A. \textit{fruA} in the TM strain. B. \textit{fruA} in the WT parental strain. C. \textit{fruB} in the TM strain. D. \textit{fruB} in the WT parental strain. E. \textit{fruA} and \textit{fruB} in the TM strain. F. \textit{fruA} and \textit{fruB} in the WT parental strain. 1, Fructose; 2, Mannitol; 3, N-acetylglucosamine; 4, Methyl alpha glucoside; 5, 2-Deoxyglucose; 6, Trehalose; 7, Galactitol and 8, Galactose.

The raw data for these plots are provided in supplementary materials (S6-S12 Tables). ND, not determined.

\textbf{Operon induction properties using lacZ fusions}

The three transport systems showing largest responses to fructose induction in wild type cells were the systems encoded by the mannitol (\textit{mtl}), galactitol (\textit{gat}) and mannose (\textit{man}) operons (see Fig 1). Therefore, in order to determine if these effects reflected changes in operon transcription or EII activities, transcriptional \textit{lacZ} fusions were constructed to operons encoding these three PTS systems (\textit{mtlA-lacZ}; \textit{gatY-lacZ} and \textit{manXYZ-lacZ}). These were used in studies to determine the effects of fructose in the growth medium prior to transport rate determinations. The results are presented in Table 3. It can be seen that the presence of fructose in the growth medium, or the overexpression of specific \textit{fru} operon genes, had no apparent effect on the
induction of mtl, gat, and man operon expression. These results imply that the effects observed in Figs 1 and 2 reflect the enzyme II activities and not their syntheses. This conclusion is substantiated by the results obtained when the transphosphorylation reactions were studied (see Table 5 below).

**Table 3.** Ratios of the response of lacZ fusion-bearing strains to various conditions and genetic backgrounds (S13-S15 Tables). WT = wild type, BW25113; OE = overexpression; ΔfruBKA = deletion of the entire fruBKA operon, also called triple mutant, TM. All strains were grown in LB medium to which fructose was added (0.2%) only for the first two entries as indicated. The remaining entries reveal the consequences of the overexpression of specific fru genes or gene combinations on lacZ-fusion gene expression. In no case were the changes significant, suggesting that the presence of fructose in the growth medium or the overexpression (OE) of specific fru genes, or the entire fru operon, did not influence expression of the mtl, gat, or man operons.

|                         | mtlA-lacZ | gatY-lacZ | manXYZ-lacZ |
|-------------------------|-----------|-----------|-------------|
| WT, ± 0.2% fructose/WT (S13 Table) | 0.9       | 0.4       | 0.7         |
| ΔfruBKA, ± 0.2% fructose/ΔfruBKA (S13 Table) | 0.9       | 1.2       | 1.6         |
| WT fruA OE/WT (S14 Table) | 1.2       | 1.0       | 1.3         |
| WT fruB OE/WT (S14 Table) | 1.1       | 1.1       | 1.1         |
| ΔfruBKA, fruA OE/ΔfruBKA (S14 Table) | 1.1       | 1.1       | 1.0         |
| ΔfruBKA, fruB OE/ΔfruBKA (S14 Table) | 1.1       | 1.1       | 1.0         |
| ΔfruBKA, fruA/fruB OE/ΔfruBKA (S15 Table) | 1.0       | 0.9       | 1.1         |

Summarizing, growth with or without fructose, or after overexpressing fruA and/or fruB had essentially no effect on the levels of the β-galactosidase activities of the mtlA-, gatY- and manXYZ-lacZ-bearing strains. This clearly implied that the mtl, gat and man operons were not repressed or induced by the presence of fructose in a wild type or ΔfruBKA strain or by single or dual overexpression of fruA and fruB in the triple mutant, ΔfruBKA, background.

**PEP-dependent phosphorylation of PTS sugars in vitro**

Fig 3A presents the consequences of growth in the presence of fructose on the PEP-dependent phosphorylation of various PTS sugars. As expected, fructose phosphorylation activity increased dramatically, but mannitol and N-acetylglucosamine phosphorylation activities
increased as well. Interestingly, galactitol phosphorylation decreased dramatically although phosphorylation of other PTS sugars were apparently not affected. Upon overexpression of the complete fruBKA operon (Fig 3B), or of the fruA and fruB genes (Figs 3C and 3D), co-expressed on compatible plasmids, similar results were obtained with increased phosphorylation activities of Fru > Mtl > NAG. FruA or FruB overproduction alone had little or no effect, and the phosphorylation of other PTS sugars also showed little effect. Mannitol phosphorylation increased 4 to 6-fold, while phosphorylation of N-acetylglucosamine increased 2 to 3-fold, and phosphorylation of galactitol decreased to about one eighth. Inclusion of fructose, fructose 1-P, fructose 6-P or fructose 1,6-bisphosphate during the in vitro assay had essentially no effect on mannitol, N-acetylglucosamine or galactitol phosphorylation (data not shown).

The mtlA gene was inactivated with a kanamycin resistance gene insertion or was deleted (ΔmtlA), and the crude extracts were compared with the wild type. Crude extracts were assayed for [14C]mannitol phosphorylation after growth with or without fructose, and the activity was reduced to a few percent of the wild type level in the insertion or deletion mutant (S22 Table), showing that mannitol cannot be phosphorylated at an appreciable rate by other PTS Enzyme II complexes under these conditions (data not shown).

Figure 3. PEP-dependent phosphorylation of various radioactive PTS sugars by the crude extract preparations of wild type and ΔfruBKA strains of E. coli following either induction with fructose or overexpression of fruBKA or fruA and fruB. Ratios of the PEP-dependent phosphorylation rates for various radioactive PTS sugars by crude extracts of wild type and ΔfruBKA strains of E. coli. A. wild type cells induced by growth in LB + fructose (0.2%) compared to LB grown cells. B. Effect of the overexpression of the entire fruBKA operon on in vitro PEP-dependent sugar phosphorylation rates when cells were grown in LB medium. C. The consequences of the simultaneous overexpression of fruA and fruB in the WT background. D. The same as C except that the ΔfruBKA strain was used. 1, Fructose; 2, Mannitol; 3, N-acetylglucosamine; 4, Methyl alpha glucoside; 5, 2-Deoxyglucose; 6, Trehalose and 7, Galactitol. The raw data for these plots are presented in S16-21 Tables. Other conditions and combinations of gene overexpression did not result in appreciable changes in activities (see S16-21 Tables).
To further examine the effects of the FruA and FruB proteins on activation of other PTS Enzyme II complexes, recombinant FruB was purified to near homogeneity (S1 Fig), and it was added to crude extracts of the recombinant ΔfruBKA mutant ± overexpression of fruA. The results, presented in Table 4, show that purified FruB had a FruA-dependent activating effect on [14C]mannitol phosphorylation in vitro when PEP was the phosphoryl donor, regardless of whether a membrane pellet was used from the WT or ΔfruBKA strains, or whether a crude extract was used from the mutant lacking the fru operon. Purified HPr did not stimulate mannitol phosphorylation.

Moreover, while mannitol phosphorylation responded dramatically to the inclusion of FruB in the assay mixture, a lesser activation was observed when N-acetylglucosamine was the sugar substrate (Fig 4). Activation by FruB but not HPr showed that FruB activation is not due to the activity of the FPr domain of FruB but is dependent on the presence of FruA (Table 4).

**Table 4.** Effect of purified FruB or HPr on the PEP-dependent phosphorylation of [14C]mannitol in membrane pellets (MP) of *E. coli* strain BW25113-pMAL-fruA and BW25113-ΔfruBKA:kn-pMAL-fruA or crude extracts (Cr.Ext.) of strain BW25113-ΔfruBKA:kn-pMAL-fruA as compared to their corresponding control strains BW25113-pMAL and BW25113-ΔfruBKA:kn-pMAL

| Purified protein added | Activity ratio (Enzyme II plus FruB or HPr /Enzyme II alone) |
|------------------------|---------------------------------------------------------------|
|                        | MP-WT-pMAL       | MP-WT-pMAL-fruA | MP-ΔfruBKA-pMAL | MP-ΔfruBKA-pMAL-fruA | Cr.Ext-ΔfruBKA-pMAL | Cr.Ext-ΔfruBKA-pMAL-fruA |
| FruB (0.23 μM)          | 1.5              | 8.6             | 1.2             | 8.1             | 1.2              | 7.5                       |
| HPr (0.55 μM)           | 2.0              | 2.3             | 1.7             | 2.3             | 1.5              | 1.5                       |
The crude extract of *E. coli* strain BW25113-\textit{mtlA:kn} or BW25113\textit{ΔmtlA} grown in LB+0.2% fructose was prepared and used to demonstrate the absence of mannitol phosphorylation by FruA (S22 Table). The results eliminate the possibility that stimulation of mannitol phosphorylation was due to cross substrate specificity causing mannitol phosphorylation by EII\textsubscript{Fru}.

**Effect of a high-speed supernatant (HSS), derived from *E. coli* cells with fruBKA overexpressed, on the in vitro PEP-dependent phosphorylation activities of membrane pellet preparations with overexpressed fruA or galP.**

In view of the in vitro phosphorylation results described above and presented in Table 4 and Fig 4, the effects of a high-speed supernatant [(HSS), supplying all of the soluble proteins of the PTS (Enzyme I, HPr and FruB)] from a strain overexpressing the \textit{fruBKA} operon, were examined for the effects on phosphorylation activities of membrane pellet preparations overexpressing either \textit{fruA} or \textit{galP} (Fig 5 and S24 Table).

Increased expression of \textit{fruB} in high speed supernatants (HSSs) from cells overexpressing \textit{fruBKA}, gave a large increase (about 56-fold) of fructose phosphorylation as expected, but mannitol phosphorylation increased 9.1-fold, while N-acetylglucosamine phosphorylation increased 2.7-fold for the overexpressed \textit{fruA} membrane pellets (Fig 5A). Overexpressed \textit{galP} membrane pellets did not show activation (Fig 5B; S25 Table). Interestingly, inhibition of \textsuperscript{[14]C}galactitol phosphorylation observed in \textit{fruA} overexpressed membrane pellets was similarly observed in \textit{galP} overexpressed membrane pellets. These results suggest that while the activation of mannitol (and to a lesser extent, N-acetylglucosamine) phosphorylation in vitro is specific to the fructose PTS enzymes, FruA and FruB, the inhibition of galactitol phosphorylation is not as specific.

**Fig 5.** Effects of using an HSS from lysed *E. coli* BW25113 \textit{chs kn:T\textsubscript{Ptet-fruBKA}} cells, overexpressing the \textit{fruBKA} operon, on the PEP-dependent phosphorylation activities of enzymes II in membrane pellets (MP) of *E. coli* BW25113 overexpressing \textit{fruA} (A) or \textit{galP} (B) for PTS sugars: 1, Fructose; 2, Mannitol; 3, N-acetylglucosamine; 4, Galactitol. An aliquot of 8 h HSS...
from *E. coli* BW25113 *chs kn:T:Ptet-fruBKA* (WT OE fruBKA operon) was used as a source of soluble PTS enzymes for PEP-dependent phosphorylation activities. The raw data are presented in S24 Table.

**Effects of fruA overexpression and purified fruB on sugar-P:sugar transphosphorylation reactions**

In contrast to the PEP-dependent phosphorylation reactions catalyzed by the PTS, the sugar-phosphate:sugar transphosphorylation reactions depend only on the Enzyme IIB-IIC complex (as well as IID for the mannose system), not on Enzyme I, HPr or the Enzyme IIA proteins. Therefore, these reactions were studied as controls. The phosphorylation of several sugars (mannitol, N-acetylglucosamine, trehalose, methyl α-glucoside and 2-deoxyglucose) were assayed in the presence of either FruA (due to the overexpression of the *fruA* gene) alone or FruA and variable quantities of purified FruB (0.24, 0.37, 1.48 and 1.85 µg per reaction mixture). As shown in Table 5, in no case was there a significant effect (>50%) on the transphosphorylation reactions examined (see S25 & S26 Tables for the raw data).

**Table 5.** Effects of overexpressing *fruA* and varying amounts of purified FruB on transphosphorylation activities of tested enzyme II complexes. Results are expressed as the ratios of enzyme activities in the presence relative to the absence of purified FruB for the different preparations. The raw data are presented in S25 and 26 Tables. In all cases, neither FruA nor FruB influenced the transphosphorylation activities for the 5 sugar-specific Enzyme II complexes assayed.

| Radioactive sugar | Relative enzyme activity (OE FruA/TM) | Relative enzyme activity (EII plus purified FruB /EII alone) |
|-------------------|--------------------------------------|---------------------------------------------------------------|
|                   |                                       | TM-pMAL-fruA | Purified FruB (µg) | TM-pMAL-fruB (µg) |
|                   |                                       | Value±SD | 0.24 | 0.37 | 1.48 | 1.85 | Value±SD | 0.24 | 0.37 | 1.48 | 1.85 |
| Mannitol          | 1±0.14                                | 1.01±0.1 | 0.98±0.11 | 0.87±0.05 | 0.8±0.08 | 1.07±0.13 |
| N-Acetylglucosamine | 0.8±0.14                             | 1.05±0.09 | 0.98±0.08 | 1.05±0.24 | 1.15±0.15 | 1.1±0.17 |
| Trehalose         | 0.5±0.02                              | 1.06±0.09 | 0.92±0.01 | 0.94±0.06 | 0.96±0.06 | 1.07±0.08 |
These transphosphorylation reactions do not depend on protein:protein interactions, except for the mannose system, in which the IIAB\textsubscript{man} protein must interact with the IIC\textsubscript{man}-IID\textsubscript{man} complex, and in this case, it appears that all constituents of the system are highly associated in the membrane. In all other cases examined, the IIB and IIC domains are present in a single polypeptide chain (see Table 1). Thus, it appears likely that the effects of varying the expression level(s) of one or more PTS IIC/IIB component(s) on the \textit{in vivo} transport and \textit{in vitro} phosphorylation activities of other Enzyme II complexes of different specificities is due to facilitation or inhibition of protein-protein interactions within and between the Enzyme II complexes of the PTS. Thus, the results confirm the results obtained using the \textit{lacZ} fusions. FruA and FruB do not promote or inhibit synthesis of other PTS Enzymes. The effects appear to be on the transport and PEP-dependent phosphorylation \textit{activities} of these enzyme transporters, not on their \textit{syntheses} or sugar-P:sugar transphosphorylation activities.

\textbf{Discussion}

As noted in the introductory section of this paper, the PTS, found only in prokaryotes, both bacteria and archaea, but not in eukaryotes, is complex, both in structure and function [2-11]. It consists of energy-coupling phosphoryl transfer proteins (Enzyme I, HPr, and the sugar-specific IIA and IIB proteins or protein domains) as well as the PTS sugar transporters, the IIC constituents of the system that catalyze transfer of the phosphoryl moiety of the IIB–P proteins to the incoming sugars [3, 4, 6] (see Introduction and Table 1). However, it has also been shown

|                  | 1.2±0.04 | 0.97±0.04 | 1.01±0.02 | 1.04±0.01 | 0.97±0.09 | 1.02±0.08 |
|------------------|----------|-----------|-----------|-----------|-----------|-----------|
| Methyl alpha glucoside |          |           |           |           |           |           |
| 2-Deoxyglucose   | 1.2±0.01 | 1.13±0.13 | 1.12±0.2  | 1±0.05    | 1.02±0.16 | 1.03±0.13 |
that the PTS serves as a chemoreception system [38-41] and regulates carbon, nitrogen and energy metabolism [42-44]. Prior to the present study, the enzyme IIC proteins had not been shown to function as parts of regulatory networks involving direct protein-protein interactions within the membrane.

In a global interactome study involving both soluble and integral membrane proteins in *E. coli* K12 [1], we reported that many integral membrane proteins interact with each other, and particularly prominent, were those involving the fructose-specific Enzyme IIC protein of the PTS (see Table 2). Because these PTS enzyme-transporters can be assayed *in vitro* as well as *in vivo*, we initially attempted to investigate the consequences of these interactions before moving on to examine the consequences of interactions between other PTS enzymes, and finally, between PTS and non-PTS integral membrane proteins. Most striking was the observation that many of the PTS permeases interact with the fructose PTS (FruA/FruB), more than with any other PTS Enzyme II complex (Table 2). However, the biochemical and physiological significance of these interactions was not known. As a result, we carried out the molecular genetic, biochemical and physiological studies reported here, revealing that the activities of the Enzyme IICs of several different PTS sugar-specific transporters are enhanced by their interactions with FruA (the fructose Enzyme IIBC), but that others are inhibited by the corresponding interactions. The results also showed that regulation of transport of PTS sugars *in vivo* does not correlate quantitatively with regulation of sugar phosphorylation *in vitro*. This confirms conclusions of previous studies suggesting that although the two processes, transport and phosphorylation, are coupled under normal conditions *in vivo*, the rate limiting steps for phosphorylation *in vitro* are different from those for sugar uptake *in vivo* [45-47].
The level of the fructose Enzyme II complex in the membrane proved to determine the extent of activation or inhibition of the target Enzyme II systems. Moreover, the magnitude and direction of regulation depended on the specific PTS transport system under study. Thus, the glucose, mannose, mannitol and N-acetylglucosamine permeases exhibited enhanced *in vivo* sugar transport activities, and sometimes enhanced *in vitro* PEP-dependent sugar phosphorylation activities (but to differing extents), while the galactitol and trehalose systems showed inhibited activities (also to differing extents) when the fructose system was present at a high level. Most of these effects were shown to occur either due to *fruBKA* operon induction by the presence of fructose during growth of the wild type cells used for the assay, or to *fruA* and/or *fruB* gene overexpression in the absence of fructose in the growth medium. We found that the presence of fructose during assay *in vivo* or *in vitro* was unimportant for this regulation except, for induction of the regulating system as noted above. In fact, exhaustive studies failed to reveal a significant role for the presence of the sugar substrate or sugar-phosphate product of the activating or inhibiting Enzyme II complex on the extent of regulation, either during assay or during growth, except for induction of the regulating system (data not shown).

As controls, first, the sugar-specific *transphosphorylation* activities of these PTS enzyme/transporters (dependent only on the presence of the IIBC domains, not on the presence of Enzyme I, HPr or the Enzymes IIA) [16-19, 48], and second, the rates of *synthesis* of the target PTS permeases, as measured using *lacZ* transcriptional fusions, were shown not to be affected. These observations clearly suggested that the activating or inhibiting effects observed were due to the consequences of direct protein-protein interactions within the membrane, only on the *activities*, not the synthesis or turnover, of the target enzymes II. It seemed reasonable, but
was not proven, that regulation depended on interactions of the target PTS permeases with the energy coupling proteins of the PTS as well as the regulating Enzyme II. We thus suggest that specific protein-protein interactions (PPIs) within the cytoplasmic membrane regulate the activities of PTS permeases in a physiologically meaningful way that may contribute to or enhance the importance of the hierarchy of preferred PTS sugars as previously demonstrated when studying catabolite repression [2, 49, 50]. Since these effects could be demonstrated in the absence of a functional fructose-transporting system, we suggest that it is the direct PPIs, and not the activities of the regulating proteins, that determine the regulatory effects. Thus, it is the levels of the regulating system that seem to determine the degrees of activation or inhibition of the target Enzyme II complexes.

It is interesting to note that in a previous publication, we suggested that the fructose Enzyme IIABC complex was the primordial system, and that all other PTS Enzymes II arose later during evolution of the more complex PTS [20, 51]. The evidence for this suggestion is as follows: (1) Of all the sugars transported by the PTS, only fructose feeds directly into glycolysis without conversion to another sugar. (2) When the genomes of different bacteria are screened for the presence of different Enzymes II of the PTS, there prove to be larger numbers of fructose-like systems compared to homologues of any of the other sugar-specific Enzymes II [25]. (3) Several bacteria and archaea have only a fructose-specific system, lacking Enzyme II complexes specific for other sugars, but this is rare for any other PTS Enzyme II complex [51]. (4) Several bacteria lack fructose 6-phosphate kinase (PFK), which acts on the product of the mannose PTS, fructose 6-P, but possess fructose 1-phosphate kinase (FPK), which acts on the product of the fructose PTS, fructose 1-P, and in these bacteria, the only sugar metabolized via the glycolytic pathway is fructose [51]. (5) The fact that only fructose is metabolized directly
via glycolysis without conversion to another sugar supports the postulate that glycolysis evolved initially just for fructose catabolism. (6) Only the fructose PTS, in *E. coli* and many other bacteria, have their own HPr protein (called FPr) [52]. (7) Only the fructose Enzyme II has a duplicated IIB domain (IIB’) that functions in protein-protein interactions and not in phosphoryl transfer [21]. It is possible that this “extra” domain plays a role in the regulatory interactions documented in this report. (8) We now find and demonstrate the biochemical significance of the larger number of protein-protein interactions for the fructose PTS, more than for any other sugar-specific Enzyme II complex, again consistent with the suggestion that the fructose PTS was the primordial system (this paper).

The observations cited above for the interactions of the fructose PTS with other Enzyme II complexes appear to be applicable in principle to other Enzymes II as well as to other (non-PTS) types of transport systems. Thus, in preliminary studies, we found that several Enzymes II, in addition to the fructose Enzyme II, interact with other Enzymes II, having activating or inhibiting effects that depend both on the level of the inhibiting Enzyme II complex and apparently, on the hierarchical position of the target systems under study. Moreover, we found that high level expression of genes encoding the Enzymes II of the PTS influence the activities of certain non-PTS integral membrane transporters in vivo, and vice versa. These observations lead to the suggestion that interactions of many integral membrane transport proteins (and possibly a variety of integral membrane enzymes) occur in the plane of the membrane, influencing their activities, possibly in a physiologically significant and relevant way. The experiments reported here therefore serve as guides to direct further experimentation aimed at defining the consequences of intra-membrane protein-protein interactions. Future studies will be required to
determine the extent to which these interactions occur and prove to be physiologically important.

Experimental Procedures

Constructions of bacterial two-hybrid plasmids

The Euromedex bacterial two-hybrid system (Euromedex, Cat # EUK001), consisting of two compatible plasmids pUT18 and pKNT25, were designed to detect and characterize protein-protein interactions in vivo. pUT18 encodes Ap resistance and carries ColE1 origin and Plac driven T18 domain of cAMP synthase of Bordetella pertussis while pKNT25 encodes Kn resistance and carries p15A origin and Plac driven T25 domain of cAMP synthase. To determine any possible interactions between two target proteins, one gene was fused to N-terminus of T18 in pUT18 while the other was fused to N-terminus of T25 of pKNT25. These two plasmids were co-transformed to an E. coli strain lacking its own cAMP synthase gene cyaA. In the case that two target proteins were closely interacted thereby bringing together T18 and T25 domains, the E. coli cells enabled to synthesize cAMP, thereby exhibiting unique phenotypes that could be assessed.

fruA, fruB, gatC, nagE, treB, mtlA, galP, lacY and pheP were amplified by PCR from BW25113 genomic DNA using carefully designed oligonucleotides (SY Table), digested by appropriate restriction enzymes, then ligated into the same sites of pUT18 individually. In each of these resultant plasmids, the target structural gene with no stop codon is inserted immediately upstream of the N-terminus of T18 in pUT18, creating a single hybrid gene that encodes the target protein at N-terminus and T18 domain at C-terminus. These plasmids are denoted as
pUT18-fruA, pUT18-fruB, pUT18-gatC, pUT18-nagE, pUT18-treB, pUT18-mltA, pUT18-galP, 

pUT18-lacY and pUT18-pheP (SX Table).

Similarly fruA, fruB, mtlA and nagE were fused to the N-terminus of T25 domain in pKNT25 individually. Each of these resultant plasmids carries a single hybrid gene encoding the target protein at N-terminus and T25 domain at C-terminus. These plasmids are denoted as pKNT25-fruA, pKNT25-fruB, pKNT25-mltA and pKNT25-nagE, respectively (SX Table).

To test the possible interaction between two proteins, a recombinant pUT18 plasmid and a recombinant pKNT25 plasmid were co-transformed by electroporation into E. coli BTH101 strain deleted for cyaA. The transformants were inoculated onto MacConkey agar plates supplemented with maltose, Kn, Ap and IPTG. The plates were incubated at 30°C for up to 48 h. The appearance of red colonies shows positive interactions between the target proteins. The intensity of red color is proportionally correlated to the level of protein-protein interactions.

Constructions of fruA, fruB, fruBKA and mtlA deletion mutants

Strains (and plasmids) and DNA oligonucleotides used in this study are described in Supplementary Tables SX and SY, respectively. The deletion mutants of fruA, fruB, fruBKA and mtlA were generated from the parental strain (E. coli K-12 strain BW25113) using a standard method as described in [53]. Briefly, to construct each mutant, a kanamycin resistance gene (kn), flanked by the FLP recognition site (FRT), amplified from the template plasmid pKD4 using a pair of specific mutation oligos (SY Table), was first substituted for the target gene or operon. Where indicated, the kn gene was subsequently eliminated (leaving an 85-bp FRT sequence) using plasmid pCP20 that bears the FLP recombinase. The replacements of the target genes/operons by the FRT-flanking kn gene and the subsequent removal of the kn gene were confirmed by colony
PCR and DNA sequencing, yielding deletion mutants ΔfruA, ΔfruB, ΔfruBKA and ΔmltA, respectively (SX Table).

**Construction of the Ptet driven fruBKA strain**

Using plasmid pKDT:Ptet (Klumpp et al, 2009) as template, the DNA region containing the kn gene and the Ptet promoter was PCR amplified using a specific primer pair, PtetfruB-P1/PtetfruB-P2 (SY Table). The PCR product from this pair of primers was integrated into the BW25113 chromosome to replace the fruBKA promoter (PfruBKA between the 123\textsuperscript{th} nucleotide and the 1\textsuperscript{st} nucleotide relative to the translational start point of fruB). This chromosomal integration led to Ptet-driven fruBKA expression (strain BW_Ptet-fruBKA) (SX Table).

**Construction of overexpression plasmids**

pMAL-p2X [54] was used to overexpress various cytoplasmic and transport proteins in *E. coli*. This plasmid carries a *colE1* origin, *lacIq* and a strong IPTG inducible promoter Ptac driving malE gene expression (useful in making N-terminal fusions for protein purification purposes). To make a control plasmid (carrying Ptac but not malE), pMAL-p2X was digested with BglII (+435 to +440 relative to the start site of malE) and Sall (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 malE has been removed.

The structural regions of fruA (encoding the fructose uptake transporter), fruB (encoding the fructose-specific PTS multiphosphoryl transfer protein) and galP (encoding the galactose:H\textsuperscript{+} symporter), were PCR amplified from BW25113 genomic DNA (using specific pairs of primers as indicated in SY Table), digested with Ndel and BamHI, 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 Ptac. These plasmids were referred to pMAL-fruA, pMAL-fruB and pMAL-galP (SX Table).

**Chromosomal PmtlA-lacZ, Pman-lacZ and PgatY-lacZ Transcriptional Fusions**

The mtlA promoter region (-386 bp to +57 bp relative to the mtlA translational start point), the man promoter region (-234 bp to +54 bp relative to the manX translational start point) and the gatY promoter region (-204 bp to +42 bp relative to the gatY translational start point), 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 XhoI and BamHI sites of the plasmid pKDT (Klumpp et al., 2009), yielding the plasmids pKDT_PmtlA, pKDT_Pman and pKDT_PgatY, respectively. In each of these newly made plasmids, an rrnB terminator (rrnBT) is present between the kn gene and the downstream cloned promoter. The DNA fragments containing the kn gene, rrnBT and those 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 lacI and PlacZ but not the 5’ 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_PmtlA-lacZ, BW_Pman-lacZ and BW_PgatY-lacZ, respectively. Similarly, these reporters were transferred to ΔfruBKA, yielding strains ΔfruBKA_PmtlA-lacZ, ΔfruBKA_Pman-lacZ and ΔfruBKA_PgatY-lacZ, respectively (SX Table). 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**
The *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%) were mixed with 0.2 ml of sample and 25 μl of CHCl$_3$ in test tubes [55]. The tubes were vortexed twice (each for 10 seconds) at a constant speed and incubated in a 37 °C water bath until equilibration. A 0.2 ml aliquot of ONPG substrate (4 mg/ml) was then added to each test tube. When yellow color developed, the reaction was stopped by adding 0.5 ml 1 M Na$_2$CO$_3$ followed by vortexing. After that, the reaction mixtures were centrifuged, and the absorbance values of the supernatants were measured at 420 nm and 550 nm. A control tube was run in parallel using diluted or undiluted LB broth instead of the test sample. β-galactosidase activity (Miller units) = \[(OD_{420}-1.75\times OD_{550})/(sample\ volume\ in\ ml) \times \text{time}\ in\ min \times OD_{600nm}]\times 1000 \times \text{dilution factor} (Miller, 1997).

**Culture conditions for uptake of radioactive substrates**

A fresh culture (100 μl of the test strain) was used to inoculate 5 ml of LB (in the case of wild type or mutant strains) or LB plus either 100 μg/ml ampicillin for pMAL plasmid harboring strains, or 100 μg/ml ampicillin plus 25 μg/ml chloramphenicol for strains harboring the pMAL/pZA31P$_{tet}$-M2-GFM plasmids. The 18 mm diameter tubes were incubated in a shaking water bath at 250 rpm and 37°C for about 6 h. Aliquots of 100 μl of the cultures were used to inoculate 50 ml of LB/5 mM MgSO$_4$ with or without 0.2% fructose (in the cases of wild type or mutant strains, and strains containing the P$_{tet}$ promoter in their chromosomes), or 50 ml of LB plus either 100 μg/ml ampicillin or 100 μg/ml ampicillin plus 25 μg/ml chloramphenicol (in the cases
of the dual plasmid-harboring strains mentioned above) contained in 250 ml conical flasks, which were incubated in a shaking water bath at 250 rpm and 37°C for 8 h (in the cases of wild type and mutant strains and strains containing the P_{tet} promoter in their chromosomes) or 6 h, followed by a 2 h induction period with IPTG at a final concentration of 0.2 mM (recombinant plasmid harboring strains). During the IPTG induction period, fructose (0.2% or 0.4%) was included in certain experiments, and 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 (in the absence of added sugar in the growth medium) or 4x (when a sugar was present in the growth medium). Each wash was with 35 ml of 50 mM Trizma-maleate buffer, pH 7, containing 5 mM MgCl₂. The pellets were resuspended in the same buffer to OD_{600nm} values of 0.5, 0.25 or 0.125 for radioactive substrate uptake experiments.

**Radioactive substrate uptake**

For the uptake assays, the stock solution of each radioactive substrate was used at 5 μCi/μmole for ¹⁴C while radioactive tritium was used at 30 μCi/μmole, and in all cases, the substrate concentration of the stock solution was 1 mM. Whenever stated, uptake assays were performed in the presence and absence of certain added non-radioactive sugars at the specified test concentrations and conditions. 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 a cold sugar solution and/or 50 mM Trizma-maleate buffer, pH 7, containing 5 mM MgCl₂.
The final concentration of the radioactive sugar substrate in the assay mixture was 20 µM for uptake assays and 10 µM for phosphorylation assays unless otherwise stated. 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 MgCl₂ 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 OD₆₀₀nm/0.1 ml/min.

Culture conditions for phosphorylation assays

A fresh culture of the test strain was used as an inoculum which was prepared by inoculating 5 ml contained in an 18 mm diameter test tube or 50 ml contained in 250 ml conical flasks of LB medium without or with the appropriate sugar (e.g., fructose at 0.2%) and antibiotic(s) (100 µg/ml ampicillin for pMAL plasmid harboring strains, or 100 µg/ml ampicillin plus 25 µg/ml chloramphenicol for strains harboring the pMAL/pZA31P_{tet}M2-GFM plasmids). The flasks were incubated at 37°C, either in a gyratory shaking water bath at 250 RPM for the 250 ml flasks, or in a shaking incubator at 275 RPM for the 2 l flasks for 8 h (wild type, mutant strains and strains containing the chromosomal P_{tet} promoter), or for 6 h plus a 2 h induction period (recombinant strains). 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 4°C, 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 extracts, were either used directly for PEP-dependent phosphorylation assays of PTS enzymes II or subjected to ultracentrifugation in a Beckman centrifuge, Ti-70 titanium rotor, for 2 h at 40,000 RPM. The pellets produced were re-suspended in an appropriate volume of modified M63/5 mM DTT to give the membrane pellets (MP) that were used for PEP-dependent and transphosphorylation assays of PTS enzymes. The 2 h high speed supernatants (HSS) produced were kept overnight on ice at 4°C and then subjected to one or more 2 h centrifugation cycles under the previously mentioned conditions. This was repeated either once, twice or thrice to get 4, 6 or 8 h HSSs, which were used as sources of soluble PTS enzymes for PEP-dependent phosphorylation assays. The repeated centrifugations were necessary to remove the last traces of the membranous Enzymes II [16, 54].

**PTS phosphorylation assays**

These assays were performed as previously described by Aboulwafa and Saier, 2002 [56]. For the PEP-dependent reactions, Enzyme II preparations were either membrane pellets (MP), or crude extracts (CE), and the following assay mixtures were used: 50 mM potassium phosphate buffer (pH 7.4), 10 μM [14C]sugar, or [3H]sugar (in the case of galactitol), 5 mM phosphoenolpyruvate (PEP), 12.5 mM MgCl2, 25 mM KF and 2.5 mM dithiothreitol (DTT). For the sugar-P-dependent reactions (transphosphorylation), the same was applied except that membrane pellets only were used as the enzyme II source, PEP was replaced by a sugar-P (usually at a 1000-fold higher concentration than that of the radioactive sugar unless otherwise stated), and the pH of the potassium phosphate buffer was 6.0. The sugar phosphates used were: glucose-6-phosphate with [14C]methyl α-glucoside or [14C]2-deoxyglucose; fructose-1-phosphate with
[14C]fructose, N-acetylglucosamine-6-phosphate with [14C]N-acetylglucosamine, trehalose-6-phosphate with [14C]trehalose, and mannitol-1-phosphate with [14C]mannitol. The radioactive sugar/sugar phosphate concentrations were 10 µM/10 mM except that they were 50 µM/5 mM and 25 µM/5 mM for the [14C]methyl α-glucoside and [14C]2-deoxyglucose phosphorylation assays, respectively. The resin used to separate [14C]sugar from [14C]sugar-phosphate was Dowex® 1X8, chloride form, 50-100 mesh (Sigma-Aldrich). After 4x washing of columns containing 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. Cold sugar, sugar phosphate, the soluble purified FruB enzyme or the purified HPr protein was incorporated in the reaction mixtures at the specified concentrations.

**Protein purification**

The His-tagged FruB from an E. coli strain BW25113-fruBKA:kn-pMAL-fruB culture, overexpressing the fruB gene or the equivalent strain overexpressing the ptsH (HPr) gene, was purified by nickel affinity chromatography. The cells were grown in LB and induced for 2 h with 0.2 mM IPTG as stated before for the preparation of cells for the transport assays. The cells were harvested by centrifugation, washed 3x with modified M63 and subjected to 3 cycles of freezing at -20°C and thawing before being lysed with B-PER Complete Bacterial Protein Extraction Reagent (from Thermo Scientific, Cat # 89821) following the manufacturer’s instructions. His-FruB or His-HPr in the cell extract was purified using HisPurTM-Ni-NTA Superflow Agarose (from Thermo Scientific, Cat # 25214) following the manufacturer’s instructions. The eluates, produced from the agarose columns, containing purified His-FruB or His-HPr, were desalted by passage through pre-equilibrated (with modified M63/5 mM DTT) ZebaTM Spin Desalting columns (Thermo Scientific, Cat # 89894). Each Zeba column contained 10 ml of size exclusion resin
MWCO 7000 Da. The desalted preparations were concentrated using Amicon ultra centrifugal filter devices, MWCO 5000 Da, Cat # UFC 900502. The purity of the resultant preparation was checked by SDS-PAGE as described by Aboulwafa and Saier, 2011 [54]. Phenylmethylsulfonyl fluoride (PMSF) was incorporated into the purified preparation at 1 mM before being aliquoted and stored at -80°C.

**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.

**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.

**Conflict of Interest**

The authors declare that they have no conflict of interest with the contents of this article.

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Fig 2
Fig 3
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