Facilitated diffusion of fructose via the phosphoenolpyruvate/glucose phosphotransferase system of *Escherichia coli*

Hans L. Kornberg*, Linda T. M. Lambourne, and Andrew A. Sproul

Department of Biology, Boston University, 5 Cummington Street, Boston, MA 02215

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From mutants of *Escherichia coli* unable to utilize fructose via the phosphoenolpyruvate/glycose phosphotransferase system (PTS), further mutants were selected that grow on fructose as the sole carbon source, albeit with relatively low affinity for that hexose (K_m for growth ≈8 mM but with V_{max} for generation time ≈1 h 10 min); the fructose thus taken into the cells is phosphorylated to fructose 6-phosphate by ATP and a cytosolic fructo(manno)kinase (Mak). The gene effecting the translocation of fructose was identified by Hfr-mediated conjugations and by phage-mediated transduction as specifying an isoform of the membrane-spanning enzyme II^{Glc} of the PTS, which we designate *ptsG-F*. Exconjugants that had acquired *ptsG*$^+$ from Hfr strains used for mapping (designated *ptsG-I*) grew very poorly on fructose (V_{max} ≈7 h 20 min), even though they were rich in Mak activity. A mutant of *E. coli* also rich in Mak but unable to grow on glucose by virtue of transposon-mediated inactivations both of *ptsG* and of the genes specifying enzyme II^{Man} (*manXYZ*) was restored to growth on glucose by plasmids containing either *ptsG-F* or *ptsG-I*, but only the former restored growth on fructose. Sequence analysis showed that the difference between these two forms of *ptsG*, which was reflected also by differences in the rates at which they translocated mannose and glucose analogs such as methyl α-glucoside and 2-deoxyglucose, resided in a substitution of G in *ptsG-I* by T in *ptsG-F* in the first position of codon 12, with consequent replacement of valine by phenylalanine in the deduced amino acid sequence.

Escherichia coli grow readily on fructose as the sole source of carbon. When this sugar is present in the growth medium at concentrations ≤2 mM, it is taken up and phosphorylated predominantly to fructose 1-phosphate (1) via two membrane-associated proteins specified by *fruA* (2) and *fruB* (3); at concentrations >2 mM, it also is taken up and phosphorylated (but to fructose 6-phosphate) via the membrane-associated uptake system for mannose, specified by *manXYZ* (4, 5). Strains of *E. coli* impaired in both these systems have been shown to mutate further and to take up and phosphorylate fructose to fructose 6-phosphate via derepression of glucitol transport proteins (6). In all of these instances, the simultaneous translocation and phosphorylation of fructose requires the action of the phosphoenolpyruvate/glycose phosphotransferase system (PTS) (7–9).

It has been shown that *Salmonella* mutants lacking one or more of the cytosolic proteins of the PTS, which consequently are unable to use any of the sugars normally taken up via the PTS, can give rise to further mutants that are able to grow on fructose as the sole carbon source (9). Such mutants also had elevated levels of fructo(manno)kinase (Mak) (10), which implies that fructose entered the cells in an unphosphorylated form and only thereafter received a phosphate group from ATP. But it was not known how fructose was translocated across the cell envelope: the available evidence was interpreted (8) as ruling out involvement of any enzyme II of the PTS as well as of the galactose permease, which is known to be able also to transport sugars other than galactose.

We here report the isolation and properties of fructose-positive *E. coli* mutants from strains in which the utilization of fructose by PTS-mediated routes was abolished by deletions in the fructose and glucitol operons, as well as by insertion of a chloramphenicol-resistance transposon into *manXYZ*. That the PTS was not involved in the growth on fructose of such mutants was confirmed by further insertion of a [Δ*ptsH ptsI crr*]KanR cassette (11): this did not alter the fructose-positive phenotype. We show that fructose enters the cells via an isoform of enzyme II^{Glc} specified by a *ptsG* gene in which the G at position 34 in the published *E. coli* nucleotide sequence (12) has been replaced by T; mutants not containing this isoform take up fructose to only a negligible extent. We designate the former as *ptsG-F* and the latter as *ptsG-I*.

Materials and Methods

Strains, Plasmids, and Growth Conditions. The bacterial strains used are listed in Table 1. Bacteria were grown aerobically at 37°, either in liquid culture consisting of LB broth (25 g of LB base per liter of water) or in defined media (13), or on such media solidified with 2% agar.

A 254-bp deletion, which removed amino acids 139–392 from the FruK protein but maintained the reading frame of the gene, (designated Δ*fruk* in this work), was constructed as described in ref. 14; a 504-bp deletion in the *gutA* gene(15) (designated Δ*gutA* in this work) was constructed as described in ref. 16. Procedures used for Hfr-mediated conjugations and phage-mediated transductions are described in ref. 17 and for the uptake of [^{14}C]fructose in ref. 18: this isotopic sugar was obtained from DuPont/NEN.

Manipulations of DNA fragments and agarose gel electrophoresis were performed as described in ref. 19. Genomic DNA was prepared with the aid of a QIAmp Tissue kit (Qiagen, Chatsworth, CA), following the manufacturer's instructions; VENT polymerase (New England Biolabs) was used in PCRs and DNA thus amplified was gel-purified with a QIAquick Gel Extraction kit (Qiagen). Plasmid DNA was isolated and purified with a Wizard Plus SV Miniprep Purification system (Promega); restriction enzymes and T_4 DNA ligase were from New England Biolabs and were used as recommended by the supplier.

Mak activity was measured in extracts of cells that had been disrupted by sonic oscillation and clarified by centrifugation for 5 min at 9,000 × *g* by using the method described in ref. 10.

Sequencing reactions were carried out with the use of the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin–Elmer), as recommended by the manufacturer. Reaction products were purified on Centri-Sep spin columns (Princeton Separations). Samples were analyzed by the Boston University Sequencing Facility on an ABI 377XL DNA sequencer (Perkin–Elmer).

Abbreviations: PTS, phosphoenolpyruvate/glycose phosphotransferase system; αMG, methyl α-glucoside; 2DG, 2-deoxyglucose.

*To whom reprint requests should be addressed. E-mail: hlk@bu.edu.

Table 1. Bacterial strains used in this study

Strain	Relevant genotype	PtsG isoform	Source (ref.)
BW 6160	Hfr (PO 118 of Broda 8) *zdi57*::*Tn10*	I	21
BW 7622	Hfr (PO 4 of KL 96) *trp*::*Tn10*	I	21
HK 1691	*fruA* Δ*fruK manXYZ*::CmR *rpsL*	F	This work
HK 1787	as HK 1691 but Mak$^+$	F	This work
HK 2140	as HK 1691 and Δ*gutA*	F	This work
HK 2148	[P1(2200) × 2140]Fru$^+$	F	This work
HK 2190	as HK 2148 but *ptsG*::CmR	—	This work
HK 2200	as HK 1787 and [Δ*ptsH ptsI crr*] KanR	F	This work
HK 2225	as HK 2240 and [Δ*ptsH ptsI crr*] KanR	I	This work
HK 2237	[P1(2148) × 2190]Glc$^+$	F	This work
HK 2240	[P1(BW 6160) × 2190] Glc$^+$	I	This work

Results

***E. coli* Mutants Unable To Take up Fructose via the PTS Give Rise to Further Mutants that Grow on Fructose.** In the course of studies of the routes by which fructose enters *E. coli* cells and is used for growth, we isolated a mutant (HK 1691) in which the normal PTS-linked modes of fructose uptake were abolished by the introduction of a point mutation in the gene specifying the enzyme II for fructose (*fruA*), a deletion in the gene specifying 1-phosphofructokinase (Δ*fruK*), and insertion of a chloramphenicol-resistance transposon into the genes specifying the uptake and utilization of mannose (*manXYZ*::CmR) (Table 1). When spread on agar plates containing 40 mM fructose as the sole carbon source and incubated at 37° for several days, HK 1691 gave rise to further mutants that grew on this hexose, albeit perceptibly more slowly than did wild-type *E. coli*. One such fructose-positive pseudorevertant (HK 1787) was isolated for further study.

That the general soluble proteins of the PTS (enzyme I, HPr, and enzyme IIA^{Glc}) were not involved in this mode of fructose utilization was confirmed with a derivative (HK 2200) that, through insertion of a [Δ*ptsH ptsI crr*]KanR transposon (11), also lacked these proteins. Like its parent organism, this strain grew in liquid culture with fructose as the sole carbon source at rates strongly dependent on the substrate concentration in the medium: half-maximal rate of growth was not achieved until the fructose concentration exceeded 8 mM, with a doubling time at infinite substrate concentration (V_{max} for generation time) calculated to be ≈1 h 10 m. It had been observed previously (9, 20) that growth on fructose in the absence of PTS function was associated with derepression of Mak: we also found that the specific activity of this enzyme in sonic extracts of HK 1787 and HK 2200 was 15–20 times greater than that in similar extracts of the fructose-negative strain HK 1691. As was expected from the postulated role of Mak, the phage-mediated introduction into

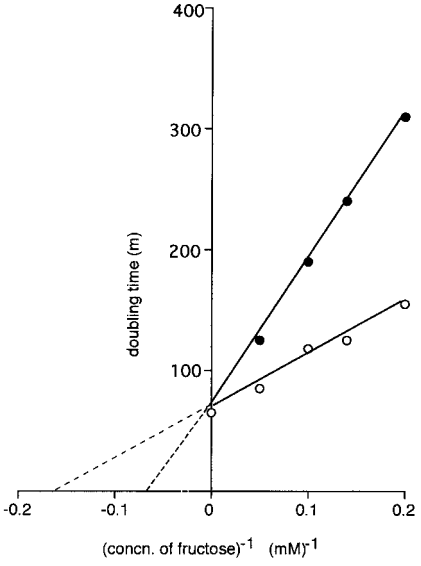

Fig. 2. Growth of strains HK 2200 (○) and HK 2225 (●) on various concentrations of fructose. Both strains carried Δ*gutA* [Δ*ptsH ptsI crr*]KanR *fruA* Δ*fruK* and *manXYZ*::CmR, were rich in Mak activity, and differed only in their ability to effect the facilitated diffusion of fructose. [S]$^{-1}$, Fructose concentration (mM)$^{-1}$; v^{-1}, doubling time (min).

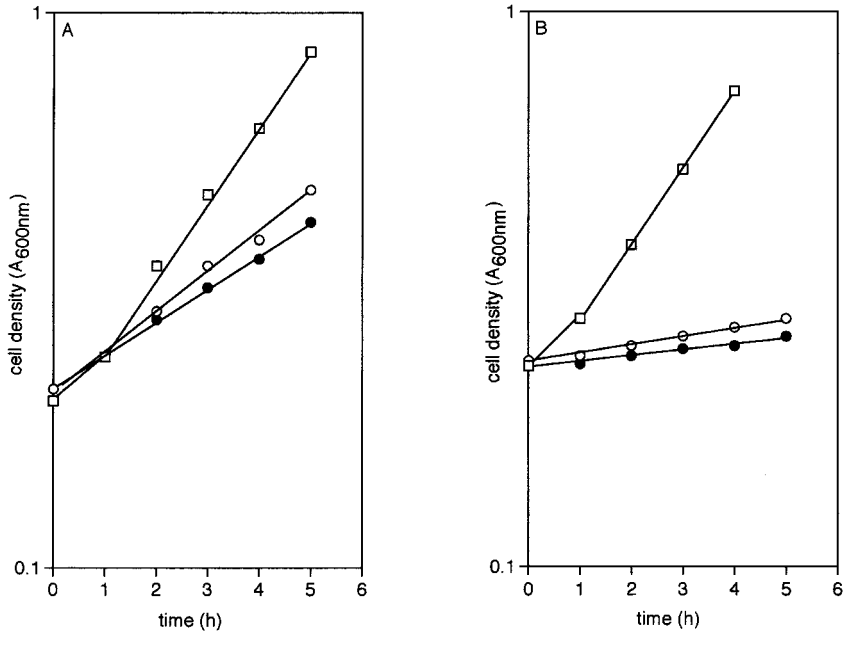

Fig. 1. The growth of strains containing *ptsG–F* (*A*) and *ptsG –I* (*B*), but otherwise isogenic, on 10 mM glucose (□), 10 mM mannose (○), or 20 mM fructose (●).

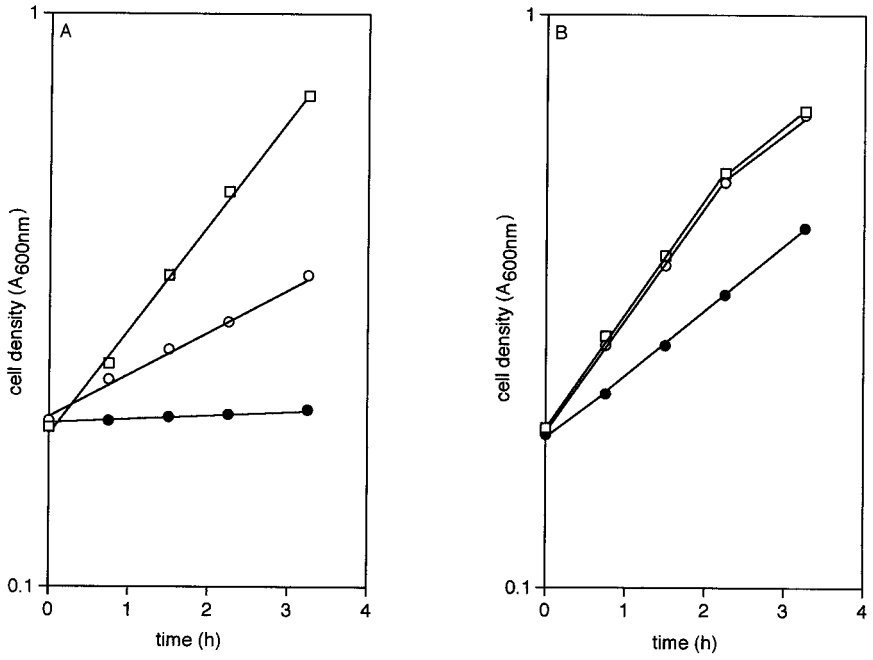

Fig. 3. Effect of glucose analogs on the growth, on glycerol, of strains containing *ptsG-F* (*A*) and *ptsG-I* (*B*) but otherwise isogenic. After overnight growth on 20 mM glycerol, cells were suspended in 20 mM glycerol medium, to which was added water (□) 2DG to 5 mM (○) or αMG to 5 mM (●).

strain HK 1787 of a *pfkA* mutation, which prevented the ATP-dependent conversion of fructose 6-phosphate to fructose 1,6-bisphosphate, abolished growth on fructose.

However, for fructose utilization to occur via this ATP-linked cytosolic enzyme, some agent must facilitate the entry of fructose into the cells. Neither the location of the gene for this translocating agent nor of that for Mak have yet been reported. To locate them, an F^- mutant (HK 2140) was constructed that carried *fruA* Δ*fruK* and *manXYZ*::CmR and in which also the gene specifying the enzyme II for glucitol transport (15) was deleted (Δ*gutA*). A culture of this strain was infected with bacteriophage grown on HK 2200, and transductants were selected on medium containing 40 mM fructose as the sole carbon source. The Mak activity in sonic extracts of such transductants was found to be 17–25 times higher than that of the donor strain, which showed that it was the gene specifying this activity that had been transferred by the phage; it also indicated that the agent that facilitated the entry of fructose was already present in the recipient cells. One such transductant (HK 2148) was used for further study.

Location of the Gene Facilitating Fructose Uptake. To determine where on the chromosome of *E. coli* genes might lie that would affect growth on fructose, the F^- streptomycin-resistant strain HK 2148 was crossed with a variety of Hfr strains, each of which was streptomycin-sensitive and contained the tetracycline-resistance transposon Tn10 integrated at known sites (21) and which therefore enabled exconjugants to be selected, on plates containing tetracycline and streptomycin, after transfer of relatively small regions of the genome. Recombinants that had virtually lost the ability to grow on fructose were found in only two regions of the *E. coli* linkage map (22). Those derived from crosses in which the Hfr strain BW 6160 [point of origin (PO ≈ minute 8, clockwise)] was the donor had only low Mak activity, whereas those that had received DNA from the Hfr strain BW 7622 (PO [≈ minute 45, counterclockwise) had retained the high Mak activity of HK 2148. Analysis of the former type will be reported in a later paper.

The virtual loss of ability to grow on fructose despite high Mak activity suggested that *ptsG*, located at minute 24.9 (22), or a gene close to it, might be involved in fructose translocation. Evidence to this effect was provided by the introduction into HK 2148 of *ptsG*::CmR, a chloramphenicol-resistance transposon integrated into *ptsG*. Because that recipient already contained a chloramphenicol-resistance transposon integrated into *manXYZ,* this introduction required two steps: initially, strain HK 2148 was infected with phage carrying *pyrC*::Tn10, which introduces a requirement for uracil and is known to be ≈10% cotransducible with *ptsG;* $uracil^+$ transductants were selected therefrom after infection with phage carrying *ptsG*::CmR. Whereas 40 of 45 such transductants grew well on glucose and on fructose, five had simultaneously lost the ability to grow on either hexose; one of these (HK 2190) was used for further study. This finding suggested that the isoform of $ptsG^+$ into which the CmR transposon had been introduced was involved in the transport of both sugars.

Confirmation of this hypothesis was obtained in a number of ways. When phage grown on a $ptsG^+$ donor that was also fructose-positive was used to infect HK 2190 and transductants were selected on glucose, all were found to be able to grow also on fructose; in contrast, infection of HK 2190 with phage grown on a $ptsG^+$ donor that was unable to take up fructose yielded glucose-positive transductants that grow only poorly on fructose. Because both types of transductant were also devoid of the PTS-dependent mannose permease specified by *manXYZ,* the growth of the fructose-positive strain on mannose (Fig. 1*A*) and the virtual inability of the fructose-negative transductant to do so (Fig. 1*B*) confirms (23, 24) that *E. coli*, unlike *Salmonella typhimurium* (25), can admit both of these sugars via the PtsG protein but also suggests that only one isoform of PtsG (which we designate *ptsG-F*) does so readily.

After introduction of the [Δ*ptsH ptsI crr*]KanR cassette into such a fructose-negative transductant (yielding strain HK 2225), a direct estimate could be made of the kinetics of facilitated fructose diffusion in the presence of Mak but in the absence of the cytoplasmic proteins of the PTS (Fig. 2). It is evident that, although fructose can diffuse into either strain, the rates at which

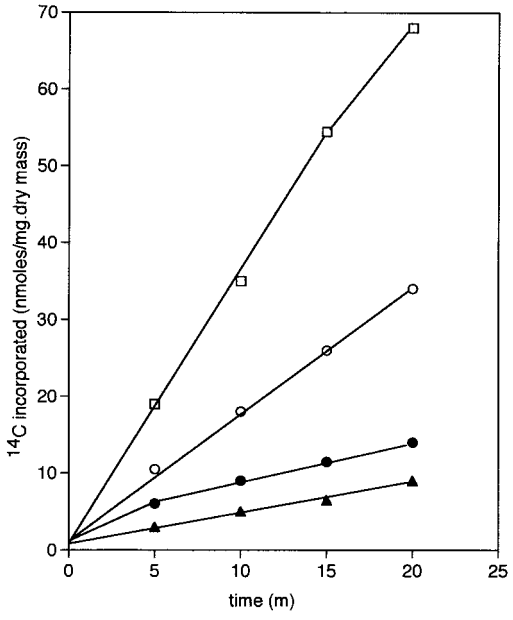

Fig. 4. Incorporation of ^{14}C from [^{14}C]fructose by a mutant carrying *ptsG-F* and [Δ*ptsH ptsI crr*]KanR. Cells were grown overnight on 20 mM fructose, harvested, and washed. Suspensions ($A_{600\ nm}$ = 1) were incubated with 5 mM [^{14}C]fructose to which was added water (□), 1 mM 2DG (○), 1 mM αMG (●), or 5 mM glucose (▲). Samples were taken at known times, the cells were filtered and washed, and their radioactivity was assayed.

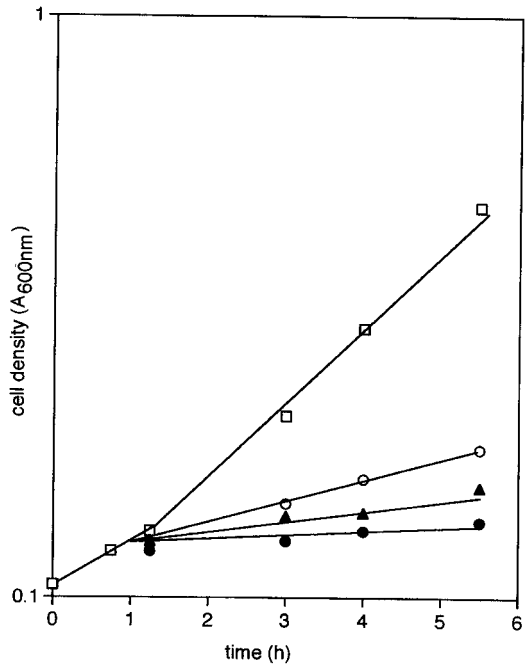

Fig. 5. Effect of glucose analogs on the growth, on fructose, of a mutant carrying *ptsG-F* and [Δ*ptsH ptsI crr*]KanR. Cells were grown overnight on 20 mM fructose and resuspended in four flasks of the same medium (□). After 1 h 15 min, 2DG (○), glucose (▲), or αMG (●) to final concentrations of 1 mM were added.

this process occurs differ greatly. The doubling times at infinite fructose concentration (V_{max} for generation time) are ≈65 min for strain HK 2200 and ≈400 min for strain HK 2225; however, there is no significant difference in the concentrations of fructose at which half-maximal rates (K_m for growth) are achieved.

Although these findings indicate that isoforms of PtsG determine whether fructose enters the cells, it is conceivable that the observed phenotypes are associated not with *ptsG* but with a gene located close to it. Several lines of evidence argue against this view.

Mutants carrying that form of PtsG that is able to effect the diffusion of fructose (*ptsG-F*) and those able to transport glucose but not fructose (*ptsG-I*), but which are otherwise isogenic, differ in their response to analogs of glucose. Addition of 5 mM methyl α-glucoside (αMG) or 5 mM 2-deoxyglucose (2DG) to cultures of *ptsG-F* strains growing on 20 mM glycerol produced rapid inhibition of growth. The former analog caused a virtual cessation of growth, whereas the latter increased the doubling time from 84 min to 230 min (Fig. 3*A*). In contrast, growth on glycerol of cultures of strains containing *ptsG-I* was virtually unaffected by the addition of 2DG and was only slightly inhibited by αMG (Fig. 3*B*), which indicated that, during growth on glycerol, the analogs were not taken up by this isoform of PtsG and consequently did not manifest the known toxic effects of accumulated hexose phosphates (for review, see ref. 1). This hypothesis was confirmed by the behavior of *ptsG-F* and *ptsG-I* mutants in which the PTS had been rendered inoperative by insertion of a [Δ*ptsH ptsI crr*]KanR cassette. Such mutants now were unaffected by the analogs when growing on glycerol, which showed that the PTS was required for the hexose phosphates to be formed and accumulated. However, both the uptake of [^{14}C]fructose by washed suspensions of such fructose-positive PTS$^-$ mutants that had been grown on fructose (Fig. 4), as well as the growth of such mutants on fructose (Fig. 5), were powerfully inhibited by either analog and also by glucose, which supported the view that the analogs and glucose probably entered the cells by the same route as did fructose; in the absence of PTS function, metabolism of glucose did not occur, and this hexose and its analogs acted as competitive inhibitors of fructose transport. Indeed, even as little as 1 μM αMG powerfully inhibited the utilization of 40 mM fructose by strains carrying the [Δ*ptsH ptsI crr*]KanR-transposon (Fig. 6).

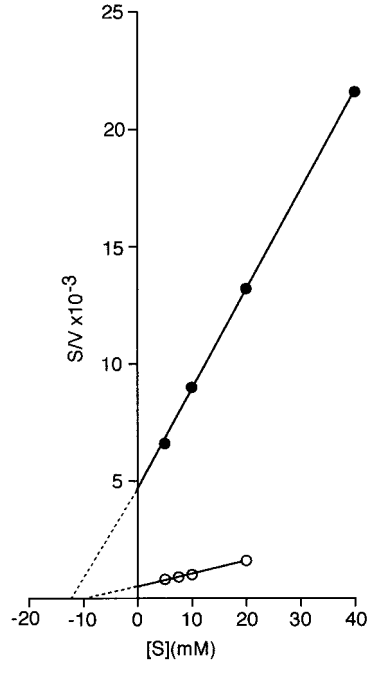

Fig. 6. Hanes plot of the effect of αMG on the growth, on various concentrations of fructose, of a mutant carrying *ptsG-F* and [Δ*ptsH ptsI crr*]KanR. Cells were grown overnight on 20 mM fructose, harvested, washed, and resuspended in flasks of the same medium but containing fructose at 5 mM, 7.2 mM, 10 mM, or 20 mM (○), and 5 mM, 10 mM, 20 mM, or 40 mM (●). αMG to a final concentration of 1 μM was added to flasks indicated by ●.

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Direct evidence that it is PtsG and not a protein specified by an adjacent gene that effects fructose uptake was provided by the introduction into the [*ptsG*::CmR] strain HK 2190 of the *ptsG-F*$^+$ and *ptsG-I*$^+$ genes cloned into a plasmid. These genes had been amplified by PCR from genomic DNA prepared from otherwise isogenic donor strains and the resultant fragments, ≈1.4 kb in length and covering only the structural regions, were ligated into the pGSB7 plasmid (26), replacing the *ptsG* in this plasmid with the two amplified isoforms. Both types of transformant grew readily on glucose but only that containing *ptsG-F* also grew on fructose. Because only the structural genes had been cloned and transferred to the previously PtsG-negative recipient, this shows that the facilitated diffusion of fructose is effected solely by the PtsG-F isoform of the glucose carrier.

Sequence Analysis. DNA prepared from the chromosomal *ptsG* genes of the fructose-positive strain HK 2237 and from the fructose-negative strain HK 2240 and amplified by PCR was sequenced completely. The only difference detected between the sequence of *ptsG-F* and that of *ptsG-I* [the latter of which was identical to the Blattner sequence (12)] was a G/T substitution of the 34th nucleotide, which is the first position of codon 12 and which alters the deduced amino acid sequence of the membrane-spanning protein from valine in PtsG-I to phenylalanine in PtsG-F (V12F). This finding was confirmed by sequencing the DNA that had been cloned into the pGSB7 plasmid: again, the only difference between the two isoforms of *ptsG* that was detected was in nucleotide 34. It was further confirmed that position 34 of the nucleotide sequence of *ptsG* in all of the Hfr strains used for mapping (22) was occupied by G and that these strains therefore contained the *ptsG-I* isoform.

Discussion

Although early work (8) had suggested that enzymes II of the PTS do not effect the transport of their substrates in the absence of phosphorylation, there is a substantial body of evidence that this view is not correct. In particular, mutants in which glucose transport had been uncoupled from phosphorylation can take up glucose in the absence of the cytoplasmic proteins of the PTS (27); moreover, it has been known since 1976 that *galP mgl* strains of *E. coli*, which are devoid of the genes for the active transport of galactose, can still grow on this hexose, which now enters the cells by facilitated diffusion through the PtsG protein (28). This finding has been confirmed with mutants of *S. typhimurium* in which the PTS had been rendered nonfunctional (29). It had been suggested that non-PTS systems with altered activities might be responsible for such a putative PTS-mediated process (8), but this explanation can be ruled out for the mutants of *E. coli* in which altered forms of PtsG have been shown to effect the entry of mannitol (26), ribose (30), and (in the present work) fructose, mannose, αMG, and 2-DG: in each of these, the sites of the mutations were mapped and shown unequivocally to lie within the *ptsG* gene that encodes the membrane-spanning protein of the glucose permease. However, unlike the other mutations in the *ptsG* of *E. coli* that have been shown to be capable of transporting substrates other than glucose or its analogs (26, 27, 30, 31), the isoforms of *ptsG* that affect fructose transport are altered in a deduced amino acid located only 10 residues from the amino terminus of the PtsG protein, which is presumed (31) to be located entirely within the cytosol.

It is likely that this change affects the abundance rather than the specificity of the membrane-spanning protein. The data recorded in Fig. 2 indicate that even strains devoid of PTS activity and containing the PtsG-I isoform can take up fructose, albeit much less readily than similar strains containing PtsG-F; moreover, it has long been known that enzymes II for hexitols with structure analogous to fructose, such as glucitol (6) or mannitol (M. L. Becker and H.L.K., unpublished results), can effect fructose uptake if these permeases are overproduced. It thus may be that the V12F mutation in *ptsG* leads to increased production of the PtsG protein or to production of a PtsG-protein with improved transport activity: this would be in accord with the observation (Fig. 3) that, when growing on glycerol, strains carrying PtsG-F take up glucose analogs to a greater extent than do strains carrying PtsG-I.

Powerful evidence in support of this view also has been provided by the isolation of a variety of different mutants of *E. coli* from continuous cultures subjected simultaneously to glucose and oxygen limitation (32). Remarkably, the predominant type of mutant elicited by this selection procedure was one manifesting the identical V12F change in *ptsG* described in the present work. Furthermore, this mutation was also shown to result in a greatly increased rate of glucose transport compared with that observed with wild-type *ptsG* strains. But it remains to be elucidated why the replacement of valine by phenylalanine so close to the amino terminus of the glucose permease should have such remarkable consequences.

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