Evidence for Regulation of Gluconeogenesis by the Fructose Phosphotransferase System in *Salmonella typhimurium*

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A genetic locus designated *fruR*, previously mapped to min 3 on the *Salmonella typhimurium* chromosome, gave rise to constitutive expression of the fructose (*fru*) regulon and pleiotropically prevented growth on all Krebs cycle intermediates. Regulatory effects of *fruR* were independent of cyclic AMP and its receptor protein and did not prevent uptake of Krebs cycle intermediates. Instead, the phosphotransferase system appeared to regulate gluconeogenesis by controlling the activities of phosphoenolpyruvate carboxykinase and phosphoenolpyruvate synthase.

The phosphotransferase system (PTS), found in bacteria capable of anaerobic growth, initiates the metabolism of numerous sugars via glycolysis. This enzyme system has evolved to an almost unexcelled degree of complexity (11), functioning in several catalytic and regulatory capacities (9, 13). Results of a recent study in our laboratory suggest that this system may have evolved from a primordial PTS which was specific for a single sugar, fructose (13). This proposal places the fructose system in a central position in the evolutionary scheme, suggesting that the fructose-specific proteins of the PTS might possess some unique and important features. The discovery of a fructose-inducible HPr-like protein (14) supports this contention. In the present study, the effects of the constitutive expression of the fructose (*fru*) regulon are shown to exert pleiotropic effects on the utilization of Krebs cycle intermediates and other metabolites. The locus (*fruR*), which when defective causes constitutive expression of the *fru* genes, was mapped precisely, and its effects on the synthesis of PTS enzymes and gluconeogenic enzymes were measured. The loss of *fruR* function reduced the activities of two key gluconeogenic enzymes, phosphoenolpyruvate (PEP) carboxykinase and PEP synthase. We eliminated the possibilities that the regulatory mechanism involves cyclic AMP, its receptor protein, and the permeases responsible for inducer uptake. Our results suggest that the fructose PTS negatively controls gluconeogenesis.

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Many of the bacterial strains used in this study and the procedures used for their isolation were described in the accompanying study (3). The *fruR51::Tn10* mutation was crossed into other strains by bacteriophage P22 transduction (5) and selection for tetracycline resistance. In vitro assays for the proteins of the PTS (15), fructose-1-phosphate kinase (6), PEP synthase (4), and PEP carboxykinase (7) have been previously described. The procedure used for the measurement of the uptake of radioactive substrates by intact bacteria has also been reported previously (15). Materials used in this study were from commercial sources and were of the highest purity available.

The effects of a single *fruR* mutation on the utilization of a variety of compounds in several different genetic backgrounds are summarized in Table 1. The *fruR51::Tn10* mutation did not prevent the rapid utilization of sugars or PEP for growth. By contrast, growth on minimal-agar plates containing as the sole carbon source acetate, pyruvate, alanine, or any of a variety of Krebs cycle intermediates was completely prevented by the loss of *fruR* function regardless of the genetic background (Table 1). The negative regulatory effect of the *fruR* mutation was not altered by point or deletion mutations in the *pts* operon or by a *crp* mutation which renders catabolite-sensitive operons independent of cyclic AMP (8). Similarly, exogenous cyclic AMP did not allow growth of *fruR* mutants on Krebs cycle intermediates. An additional allele, *dadR18* (16), a promoter mutation which renders D-amino acid dehydrogenase and the utilization of alanine independent of cyclic AMP, was also without effect. Uptake studies revealed that the *fruR51::Tn10* mutants still accumulated [¹⁴C]citrate and [¹⁴C]succinate after growth in a nutrient medium supplemented with either of

TABLE 1. Effect of *fruR* on utilization of a variety of carbon sources in several genetic backgrounds

Carbon source	Carbon source utilization in strains with indicated genetic background ^a							
	LT-2		<i>ptsH15</i>		Δ cysK <i>ptsH141</i>		<i>crp-771</i>	
	LJ709 FruR ⁺	LJ712 FruR ⁻	SB1475 FruR ⁺	LJ711 FruR ⁻	LJ703 FruR ⁺	LJ704 FruR ⁻	PP914 FruR ⁺	LJ713 FruR ⁻
Fructose	+	+	+	+	-	-	+	+
Mannitol	+	+	-	+	-	-	+	+
Glycerol	+	+	-	+	-	-	+	+
Galactose	+	+	+	+	+	+	+	+
PEP	+	+	+	+	+	+	+	+
Acetate	+	-	+	-	+	-	+	-
Pyruvate	+	-	+	-	+	-	+	-
Alanine	+	-	+	-	+	-	+	-
Citrate	+	-	+	-	+	-	+	-
Isocitrate	+	-	+	-	-	-	+	-
Fumarate	+	-	+	-	-	-	+	-
Malate	+	-	+	-	-	-	+	-
Oxaloacetate	+	-	+	-	+	-	+	-

^a Each pair of strains is isogenic except for the presence or absence of the *fruR51::Tn10* mutation. -, No growth after 48 h on solid minimal medium for noncarbohydrates or no fermentation after 24 h on eosin-methylene blue fermentation plates containing the indicated sugar at a concentration of 0.5%.

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TABLE 2. Activities of enzymes of the fructose (*fru*) regulon and of PEP carboxykinase in mutant strains of *S. typhimurium*^a

Strain	Genotype	Relative sp act (per mg of protein) of ^b :				
		Enzyme II ^{fru} (nmol/h)	Enzyme III ^{fru} (nmol/h)	Fructose-1-phosphate kinase (mmol/min)	FPr (nmol/h)	PEP carboxykinase (mmol/min)
LJ715	<i>ptsH15 manA56</i>	3	2	4	2	1,100
LJ716	<i>ptsH15 manA56 fruR51::Tn10</i>	100	100	100	100	100
LJ717	<i>ptsH15 manA56 fruR51::Tn10 fruB53</i>	61	11	96	43	520
LJ718	<i>ptsH15 manA56 fruR51::Tn10 fruA54</i>	2	21	50	50	210

^a All bacteria were grown in nutrient broth, harvested in early stationary phase, washed three times with medium 63, and resuspended in M63 for cell rupture by passage through a French pressure cell. Enzyme assays were conducted as described in the text, except that enzyme II^{fru} was assayed with a high-speed supernatant from LJ705, enzyme III^{fru} was assayed with butanol-urea-extracted membranes supplemented with a high-speed supernatant from LJ144 (12), and FPr was assayed with a crude extract from SB1467 (15).

^b All values are expressed relative to those for strain LJ716. Absolute specific activities for strain LJ716 were as follows (per milligram of protein): enzyme II^{fru}, 56 nmol/h; enzyme III^{fru}, 165 nmol/h; fructose 1-phosphate kinase, 8.1 mmol/min; FPr, 24 nmol/h; and PEP carboxykinase, 0.12 mmol/min.

these compounds. Furthermore, transfer of galactose-grown LJ712 cells to minimal-citrate medium led to immediate growth stasis. These results suggest that the *fruR51::Tn10* mutation may give rise to the negative growth phenotype by preventing gluconeogenesis.

A natural isolate of *Escherichia coli* which expressed the fructose (*fru*) regulon constitutively was shown previously to contain a mutant gene which maps at about 1 min on the *E. coli* chromosome between *ara* and *leu* (10). Phage P22 were therefore grown on strain LJ702 and used to transduce SB1340 (*leuB129 ara-9*; provided by P. E. Hartman) to tetracycline resistance. Leucine prototrophy and arabinose fermentation were scored in 262 transductants. Tet^r and *ara*⁺ exhibited 13% cotransduction, whereas Tet^r and *leuB*⁺ showed 42% cotransduction. These results suggest that the clockwise gene order is *fruR leu ara* and that *fruR* is approximately 0.2 min from *leu* and 0.4 min from *ara*.

fruR mutants of *E. coli* cannot grow in the presence of the toxic fructose analog xylitol (10). The same phenomenon was observed in *Salmonella typhimurium*. Xylitol-resistant mutants usually gave rise to a fructose-negative phenotype. Of those which were mannitol positive, many had gained the ability to grow on some or all of the Krebs cycle intermediates listed in Table 1. When *fruR* strains were mutagenized and the ability to grow on minimal-citrate plates was selected, a significant proportion of the resulting mutants lost the ability to ferment fructose. Mutants selected for either xylitol resistance or growth on minimal citrate retained tetracycline resistance.

Enzyme assays revealed that the *fruR51::Tn10* mutation resulted in the constitutive expression of all known fructose (*fru*) regulon enzymes (Table 2). *fru* mutants which regained the ability to utilize Krebs cycle intermediates exhibited low activities for some or all of these enzymes (Table 2). The results of enzyme analyses reported in Table 2 do not provide unequivocal evidence for or against the involvement of any one enzyme of the fructose PTS in the regulation of the utilization of Krebs cycle intermediates. However, the isolation of fructose-specific mutants by selection for growth on citrate suggests that one or more of the fructose enzymes may play a causal role in the regulation of growth on gluconeogenic substances.

Calvo et al. (2) identified a presumed regulatory locus, likely to be *fruR*, which prevented both the utilization of any Krebs cycle intermediate and the expression of PEP synthase. However, a deficiency in PEP synthase alone cannot account for the growth phenotype (1). Both PEP synthase and PEP carboxykinase exhibited lower activities in strain LJ716 than in the isogenic parental strain LJ715. Data for PEP carboxykinase are included in Table 2. Signif-

icantly, this activity was partially restored by mutations which specifically reduced enzyme III^{fru} activity. This result suggests a possible involvement of enzyme III^{fru} or FPr or both in the regulation of the gluconeogenic enzymes. A complex of enzyme III^{fru} and FPr has been purified to near homogeneity (S. Sutrina and M. H. Saier, Jr., unpublished results). However, inclusion of this purified protein preparation only weakly inhibited the activity of PEP carboxykinase in high-speed supernatants (14) derived from various *Salmonella* strains.

We recently observed that *E. coli* strains which constitutively express the glucitol (*gut*) operon, encoded on a multicopy plasmid, exhibited the same growth properties as the *fruR* mutants described in Table 1. Genetic loss of plasmid-encoded enzyme III^{gut} (but not the enzyme II^{gut}) resulted in restoration of the normal growth phenotype. The negative growth phenotype was found to correlate with low PEP carboxykinase activity (M. Yamada and M. H. Saier, Jr., unpublished results). The involvement of enzyme III^{gut} is therefore suggested.

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