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Properties of a Tn5 Insertion Mutant Defective in the Structural Gene (*fruA*) of the Fructose-Specific Phosphotransferase System of *Rhodobacter capsulatus* and Cloning of the *fru* Regulon

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In photosynthetic bacteria such as members of the genera *Rhodospirillum*, *Rhodopseudomonas*, and *Rhodobacter* a single sugar, fructose, is transported by the phosphotransferase system-catalyzed group translocation mechanism. Previous studies indicated that syntheses of the three fructose catabolic enzymes, the integral membrane enzyme II, the peripheral membrane enzyme I, and the soluble fructose-1-phosphate kinase, are coordinately induced. To characterize the genetic apparatus encoding these enzymes, a Tn5 insertion mutation specifically resulting in a fructose-negative, glucose-positive phenotype was isolated in *Rhodobacter capsulatus*. The mutant was totally lacking in fructose fermentation, fructose uptake in vivo, phosphoenolpyruvate-dependent fructose phosphorylation in vitro, and fructose 1-phosphate-dependent fructose transphosphorylation in vitro. Extraction of the membrane fraction of wild-type cells with butanol and urea resulted in the preparation of active enzyme II free of contaminating enzyme I activity. This preparation was used to show that the activity of enzyme I was entirely membrane associated in the parent but largely soluble in the mutant, suggesting the presence of an enzyme I-enzyme II complex in the membranes of wild-type cells. The uninduced mutant exhibited measurable activities of both enzyme I and fructose-1-phosphate kinase, which were increased threefold when it was grown in the presence of fructose. Both activities were about 100-fold inducible in the parental strain. Although the Tn5 insertion mutation was polar on enzyme I expression, fructose-1-phosphate kinase activity was enhanced, relative to the parental strain. ATP-dependent fructokinase activity was low, but twofold inducible and comparable in the two strains. A second *fru*::Tn5 mutant and a chemically induced mutant selected on the basis of xylitol resistance showed pleiotropic loss of enzyme I, enzyme II, and fructose-1-phosphate kinase. These mutants were used to clone the *fru* regulon by complementing the negative phenotype with a wild-type cosmid bank.

The phosphoenolpyruvate (PEP):sugar phosphotransferase system (PTS) is a strictly procaryotic system which, in all but a few organisms possessing the system, consists of two general energy-coupling proteins, enzyme I and HPr, as well as a number of sugar-specific permeases, the enzyme III-independent enzymes II and the enzyme II-III pairs (11, 15). A postulate regarding the evolutionary origin of the PTS suggests that it was once much simpler and may have been specific for fructose (18). This postulate was based in part on the discovery in 1971 of a fructose-specific PTS in *Rhodospirillum rubrum* and *Rhodobacter* species which consisted of only two proteins: a membrane-associated soluble factor that could be released from the membrane and was shown to have a high molecular weight, (hereafter called enzyme I) and an integral membrane particulate factor (hereafter called enzyme II) (17). Figure 1 illustrates schematically the principal structural elements of the system. The two protein components of the PTS in *Rhodospirillum rubrum* and *Rhodobacter sphaeroides* were shown to be 3-fold and 10-fold induced, respectively, by growth in the presence of fructose (14, 17). Subsequent studies in several laboratories revealed that fructose-specific PTSs could also be found in a variety of additional gram-negative bacterial species including *Thiocapsa*, *Thiocystis*, *Pseudomonas*, *Alcaligenes*, *Azospirillum*, and *Fusobacterium* spp. (for reviews, see reference 4 and J. Reizer, J. Deutscher, F. Grenier, J. Thompson, W. Hengstenberg, and M. H. Saier, Jr., Crit. Rev. Microbiol., in press).

Biochemical studies have been reported for the fructose-specific PTSs in *R. sphaeroides* and *Pseudomonas aeruginosa* (2, 7). In the latter study, only one soluble protein, with a molecular weight of 72,000, was discovered in *P. aeruginosa*; it was designated enzyme I. In the former study, an 83-kilodalton protein was identified which could dimerize and exhibited enzymatic cross-reactivity in an *Escherichia coli* extract lacking only *E. coli* enzyme I. Saier et al. (17) had previously reported that *Rhodospirillum rubrum* enzyme I could not substitute for *E. coli* enzyme I. Lolkema et al. (9) reported the identification of a high-molecular-weight phosphorylatable protein, presumed to be *R. sphaeroides* enzyme I. It exhibited no enzymatic cross-reactivity in the *E. coli* system, in agreement with the results of Saier et al. (17). Phosphorylation of an integral membrane dimeric protein, with an apparent monomer molecular weight in sodium dodecyl sulfate-gel electrophoresis of 55,000, believed to be enzyme II, was also demonstrated. Studies on the mechanism of the reaction catalyzed by the system were reported (9). A preliminary description of mutants of *P. aeruginosa* deficient in fructose PTS activity has been reported (13), but identification of the biochemical defects in these mutants was not possible, because these mutants showed reduced activities of both components of the fructose PTS.

In the present study we report the isolation and characterization of a *fruA1*::Tn5 insertion mutant of *Rhodobacter*

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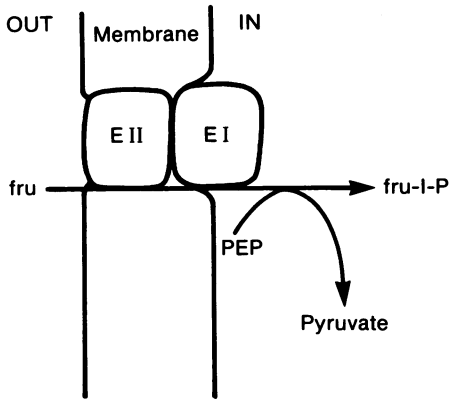

FIG. 1. Diagrammatic representation of the structural constituents of the fructose-specific PTS in *Rhodospirillum rubrum*, *R. sphaeroides*, and *R. capsulatus*. Abbreviations: EII and EI, integral and peripheral membrane protein constituents of the system, respectively; fru, fructose; fru-1-P, fructose 1-phosphate.

capsulatus. The wild-type strain is shown to possess activities of enzyme I, enzyme II, and fructose-1-phosphate kinase, with net PTS activity about 100-fold inducible. The mutant, *fruA1*::Tn*5*, lacks enzyme II activity altogether but still exhibits enzyme I and fructose-1-phosphate kinase activities. Just as in the parental strain, these activities are coordinately induced by the presence of fructose. However, in the mutant, these activities exhibit nearly constitutive activities, with a mere threefold induction upon addition of fructose to the growth medium. The mutation is shown to be polar with respect to enzyme I, but not with respect to fructose-1-phosphate kinase. We also show that although virtually 100% of enzyme I in the wild-type parental strain is membrane associated, the majority of this activity is soluble in the mutant. This result agrees with the suggestion (9, 17) that enzyme I is bound to the membrane by virtue of its high affinity for enzyme II. Finally, the development of a positive mutant selection procedure and the description of other *fru* mutants are presented.

MATERIALS AND METHODS

Media and growth conditions. *R. capsulatus* strains were grown at 32°C under either aerobic conditions in the dark or anaerobic conditions in the light (2,000 lux) in a minimal malate medium lacking yeast extract (6) or in the corresponding minimal medium containing fructose or glucose (0.2%). *E. coli* strains were grown in LB (Luria-Bertani) medium (10). Plates were made by the addition of 1.6% Bacto-Agar (Difco Laboratories) to the media before autoclaving. Antibiotic concentrations of 25 μg of kanamycin per ml and 3 μg of tetracycline per ml were used for *R. capsulatus*, and 50 μg of kanamycin per ml and 15 μg of tetracycline per ml were used for *E. coli*.

Tn*5* mutagenesis. The Tn*5* insertion element was introduced into *R. capsulatus* 37b4 by using *E. coli* S17.1 containing the mobilizable plasmid pSUP201::Tn*5* (21). Approximately 10^8 cells each of the *E. coli* donor and the *R. capsulatus* recipient were taken from exponentially growing cultures, washed twice with 0.9% NaCl, suspended in 50 μl of 0.9% NaCl, and spotted onto membrane filters (pore size 0.45 μm; Millipore Corp.). The filters were placed on LB plates and incubated at 32°C for 5 h. Cells were removed from the filters, plated onto minimal malate agar containing 25 μg of kanamycin per ml, and incubated anaerobically in the light at 32°C for 3 days.

Mutant screening. Eight minimal malate plates, each containing 25 μg of kanamycin per ml and about 50 colonies, were replica plated onto minimal glucose and minimal fructose plates, each containing 25 μg of kanamycin per ml, and cells were grown anaerobically in the light for 4 days. One colony was isolated that had a glucose-positive, fructose-negative phenotype. This mutant colony was subcloned and designated *fruA1*::Tn*5*.

Using the same selection procedure, we isolated a second fructose-negative Tn*5* insertion mutant (*fru-2*::Tn*5*). Additionally, a positive selection procedure for the isolation of *fru* mutants of *R. capsulatus* was developed. This procedure, based on that of Reiner (12) for *E. coli*, was as follows. Cells were grown to the late logarithmic growth phase in minimal malate–0.2% fructose medium, washed once aseptically with minimal malate medium, and plated onto minimal malate plates. A well of 40% xylitol (200 μl) was present on one side of the plate, and a crystal of 1-methyl-3-nitro-1-nitrosoguanidine was placed on the other side. After 2 to 3 days at 32°C, nitrosoguanidine-induced, xylitol-resistant mutants were obtained and clonally isolated. One such mutant, *fru-3*, is described in the Results section.

Preparation of crude extracts, cytoplasmic fractions, and membrane fractions. Cell suspensions were passed three times through a French pressure cell at 10,000 lb/in^2. The resultant broken-cell preparations were then centrifuged in a Sorvall SS34 rotor at 10,000 rpm for 10 min. The supernatants were recovered and stored at −20°C until used. PTS activity was stable indefinitely at −20°C. These preparations are referred to in the text as crude extracts. Further centrifugation of these extracts in a Beckman Ti50 type rotor at 40,000 rpm for 3 h resulted in the separation of cytoplasmic and membrane fractions. Supernatants and resuspended pellets were stored in the same buffer at −20°C. All procedures were performed on ice (at 0°C) or at 4°C.

Butanol-urea-extracted membranes were prepared from membrane fractions derived from 1 liter of fructose-induced cells. Pellets obtained from crude extracts by centrifugation for 2 h at 100,000 × *g* were suspended in 20 ml of 25 mM Tris hydrochloride buffer (pH 7.4) in a 50-ml Erlenmeyer flask. The butanol-urea extraction procedure was the same as described previously (16). Extracted membranes were stable at −20°C for at least 1 month.

Assays. PEP-dependent fructose phosphorylation activity was measured as described previously (20). ATP-dependent fructose phosphorylation was carried out as for the PEP-dependent assay, except for the substitution of ATP for PEP. Fructose-1-phosphate kinase activity was determined by measuring the disappearance of the NADH A_{340} (8). The assay buffer contained 50 mM Tris hydrochloride (pH 8.0), 10 mM KF, 10 mM $MgCl_2$, 2 mM ATP, 30 μM NADH, and 1 mM fructose 1-phosphate. An excess of aldolase and α-glycerophosphate dehydrogenase–triose-phosphate isomerase (Sigma Chemical Co.) was added to the reaction mixture.

Fructose uptake measurement. Cells grown overnight in minimal malate medium containing the appropriate antibiotics were diluted 50-fold in the same medium containing 0.2% fructose and were allowed to grow for an additional 4 to 5 h. The cell suspensions were then chilled to 0°C, harvested, washed three times with growth medium lacking malate and fructose, suspended in the same wash buffer to approximately 0.20 mg (dry weight) per ml, and equilibrated at 32°C for 5 min. Uptake of ^{14}C-labeled sugars was monitored as described previously (20).

Screening of cosmid bank and DNA manipulations. A

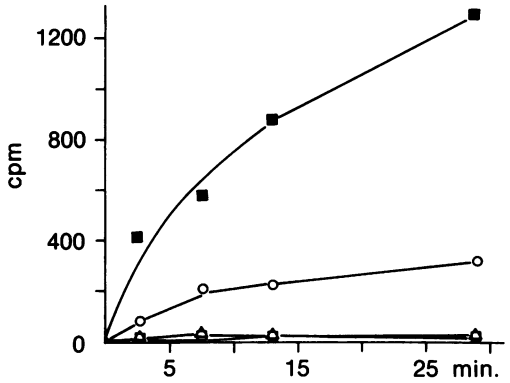

FIG. 2. Uptake of [^{14}C]fructose by wild-type and *fruA1*::Tn*5* mutant strains of *R. capsulatus*. Cells were grown overnight in minimal malate medium plus or minus 0.2% fructose. They were then diluted 1- to 10-fold with the same medium and allowed to grow for 4 to 5 h at 32°C. Cells in exponential growth were chilled to 0°C, harvested by centrifugation, washed twice with minimal salts medium, and suspended in the same medium for the uptake experiments. Cells were preincubated for 5 min at 32°C, and the uptake experiment was performed as described in Materials and Methods with [^{14}C]fructose (20 μCi/μmol) at a final concentration of 4 μM. Symbols: ■, wild type, fructose induced; ○, wild type, uninduced; □, *fruA1*::Tn*5* mutant grown with fructose; △, *fruA1*::Tn*5* mutant grown without fructose.

cosmid bank, obtained from P. M. Vignais, described by Colbeau et al. (3), was used to complement the Tn*5* insertion mutant. The cosmid-containing *E. coli* bank was mated with the *R. capsulatus fruA1*::Tn*5* strain by using an *E. coli* strain containing the helper plasmid pRK2073. Matings were performed under conditions essentially as described for the Tn*5* mutagenesis procedure with approximately 10^8 logarithmically growing cells of the *E. coli* donor, pRK2073, and *R. capsulatus fruA1*::Tn*5* as the recipient. After 3 h at 32°C, the cells were removed from the filters and plated onto minimal fructose plates containing 25 μg of kanamycin per ml and 3 μg of tetracycline per ml. The plates were then incubated at 32°C in the light under anaerobic conditions and checked for growth after 4 days. Several colonies were obtained that were capable of using fructose as a carbon source. Cosmid DNA was isolated from these fructose-positive cells as described by Birnboim and Doly (1) and used to transform *E. coli* HB101 to tetracycline resistance. These *E. coli* cells were mated again with *R. capsulatus fruA1*::Tn*5* and plated onto both minimal fructose or minimal malate plates, both containing 25 μg of kanamycin per ml and 3 μg of tetracycline per ml. Since the number of colonies on the minimal malate plate equaled the number of colonies on the minimal fructose plate, the fructose-positive character could be attributed to the cosmid DNA and not to mutation. A cosmid containing a 23-kilobase (kb) insert and at least part of the *fru* operon was isolated and designated pFRU1. Restriction enzymes and ligase were purchased from Boehringer Mannheim Biochemicals. pRK290 was used to subclone a 9.5-kb fragment containing the fructose regulon (5), and pUC19 was used for further subcloning.

RESULTS

Isolation and characterization of *fruA1*::Tn*5*. Tn*5* was introduced into *R. capsulatus* 37b4 (*fru*$^+$) by using *E. coli* S17.1 containing the mobilized plasmid pSUP201::Tn*5* as described in Materials and Methods. About 400 kanamycin-resistant colonies were isolated and screened for their ability to utilize fructose. A fructose-negative clone (*fruA1*::Tn*5*) was isolated by this procedure. It was shown to be completely negative for fructose utilization both in liquid and on solid minimal medium containing 0.2% fructose as the sole source of carbon. The mutant grew at the normal wild-type rate in complex medium as well as in liquid minimal medium containing either glucose or malate as the sole source of carbon. The lag phase was noted to be longer for the mutant than for the parent when growth was switched from aerobic to anaerobic conditions. The mutation appeared to specifically result in loss of the ability to utilize fructose.

Synthesis of the photosynthetic apparatus in the mutant and parental strains was studied. The bacteria were grown in either minimal malate or complex medium, with or without fructose, under either aerobic conditions in the dark or anaerobic conditions in the light. Cells were harvested both during the exponential and stationary growth phases. Under each set of conditions tested, the spectrum from 350 to 800 nm was the same. Thus, the Tn*5* insertion mutation had no perceptible effect on synthesis of the photosynthetic apparatus.

Fructose uptake in vivo. Figure 2 shows the rates of [^{14}C]fructose uptake by the parental and *fruA1*::Tn*5* mutant strains of *R. capsulatus*. Fructose uptake was about 10-fold inducible in the parental strain. The mutant strain exhibited negligible uptake of [^{14}C]fructose under the conditions used. By contrast, glucose and malate were taken up at approximately the same rates in the parental and mutant strains (data not shown).

Net PTS activities in vitro. Fructose phosphorylation activities, measured by in vitro assays as described in Materials and Methods, are reported in Table 1. It can be seen that PEP-dependent PTS activity in *R. capsulatus* was more than 100-fold inducible by growth in the presence of fructose. No detectable activity was observed in the *fruA1*::Tn*5* mutant extract. Virtually all of the activity was present in the membrane fraction (Table 1; see also Table 3). By contrast, low ATP-dependent fructokinase activity was observed in both the parental and mutant strains. The activity was about twofold induced by the presence of fructose in the growth medium, and the *fruA1*::Tn*5* mutation did not alter either the level of this activity or the inductive response. Kinase activity contrasted with PTS activity in being localized to the soluble fractions. It is interesting that the low fructokinase activity of *R. capsulatus* correlates with that of *Rhodospirillum rubrum* and not with the much higher activity found in *R. sphaeroides* (17).

Quantitative assay for enzyme II. The sugar phosphate: sugar transphosphorylation assay provides a quantitative measure of the various enzymes II of the PTS (16). This assay was used to estimate the activity of enzyme II^{Fru} in the membranes of *R. capsulatus* strains. The parental strain

TABLE 1. Net PTS activity in *R. capsulatus* strains

Cells	Induction	Fructose phosphorylation (nmol/min per mg of protein)			
		With PEP		With ATP	
		Crude extract	Supernatant	Crude extract	Supernatant
Wild type	−	67	<4	88	77
Wild type	+	6,900	<4	173	110
fruA1::Tn*5*	−	78	<4	77	63
fruA1::Tn*5*	+	22	<4	160	156

exhibited fructose-inducible enzyme II activity as expected, but no such activity was detected in the mutant (Table 2). Because of (i) the low activity of the fructose 1-phosphate:[^{14}C]fructose transphosphorylation reaction as compared with the PEP:[^{14}C]fructose activity (the former activity is normally about 1% of the latter [16]) and (ii) the presence of measurable PEP in crude extracts of bacteria, the value obtained for uninduced wild-type bacteria is likely to be artifactually high.

Isolated membranes from fructose-grown wild-type cells were treated with butanol and urea as described in Materials and Methods. This procedure removes virtually all of the peripheral membrane PTS proteins from membranes of enteric bacteria as well as PEP (16). Indeed, background activity in the fructose 1-phosphate:[^{14}C]fructose transphosphorylation reaction (measured in the absence of sugar phosphate and attributed to the PEP-dependent reaction) was reduced to nondetectable levels, but more than 50% of the transphosphorylation activity was retained. This experiment demonstrated that enzyme II in *R. capsulatus*, like those in enteric bacteria, is stable to the conditions of butanol-urea extraction and that the activity measured was in fact due to the transphosphorylation reaction rather than to some side reaction. The availability of butanol-urea-extracted, fructose-induced membranes allowed quantitation of enzyme I activity as described below.

Net activity and subcellular localization of enzyme I in *R. capsulatus* strains. Inclusion of saturating quantities of the butanol-urea-extracted membranes in the assay tubes allowed determination of the enzyme I activities of the various extracts which had been assayed for overall PTS activity (Table 1) and enzyme II-catalyzed transphosphorylation (Table 2). Enzyme I activity in the parental strain was induced over 100-fold by growth in the presence of fructose (Table 3). Activity was also detected in the mutant strain. The activity in the uninduced mutant was clearly greater than that in the uninduced parent and was increased threefold by the inclusion of fructose in the growth medium. This activity was 2.1% of that present in the extract of the induced parental strain.

Table 3 also documents the subcellular localization of this enzyme in the wild-type and mutant strains. Although virtually 100% of the activity was membrane bound in the parent, 61% of the activity in the fructose-induced *fruA1*::Tn*5* mutant was soluble. The Tn*5* insertion in the enzyme II structural gene therefore rendered residual enzyme I activity soluble. Results of assays conducted over a period of days indicated that this activity was stable at 0°C as well as at −20°C.

Fructose-1-phosphate kinase activities in wild-type and mutant extracts. In *E. coli* and *Salmonella typhimurium*, fructose-1-phosphate kinase is encoded within the *fru* regulon. The same appears to be true in *R. capsulatus*. The inducibility of the fructose-1-phosphate kinase activity of the parental and mutant strains (Table 4) paralleled the inducibility of enzyme I (Table 3) and of overall PTS activity (Table 1). Thus, fructose-1-phosphate kinase activity was at least 50-fold higher in the parental strain when cells were grown in the presence of fructose than when they were grown in its absence. By contrast, a much higher constitutive level of expression was observed in the *fruA1*::Tn*5* mutant, and this activity was induced threefold by the inclusion of fructose in the growth medium. The polar effect of the *fruA1*::Tn*5* insertion mutation on enzyme I expression was not observed for fructose-1-phosphate kinase.

Additional mutants (*fru-2*::Tn*5* and *fru-3*), isolated as described in Materials and Methods, were assayed for the fructose-specific enzymes. These two mutants lacked detectable activities of enzyme I, enzyme II, and fructose-1-phosphate kinase.

Cloning of the *fruA* gene from *R. capsulatus*. The *R. capsulatus fruA1*::Tn*5* mutant was used to clone the *fruA* gene (and presumably the entire *fru* regulon) from a cosmid bank of *R. capsulatus* (3). The procedure was as described in Materials and Methods. The cloned genes within plasmid pFRU1 were characterized as follows. DNA was isolated from colonies of the *fru*$^+$ cells grown on solid fructose minimal medium and was used to transform *E. coli* HB101 to tetracycline resistance (1). The same mating procedure described in Materials and Methods was then used to transfer the *fru*$^+$ character back to *R. capsulatus fruA1*::Tn*5*. These strains were shown to be tetracycline resistant and kanamycin resistant, and they exhibited normal rates of fructose utilization. The number of *fru*$^+$ colonies equalled the number of tetracycline-resistant colonies; therefore, the cosmid was responsible for reversion of the phenotype.

The cosmid was isolated, and its size was estimated by agarose gel electrophoresis to be approximately 45 kb. Since the original pLAFR vector contained 22 kb, 23 kb of *R. capsulatus* DNA was incorporated. Treatment with any of a variety of restriction enzymes resulted in the generation of several fragments, revealing the presence of multiple cleav-

TABLE 2. Enzyme II activity in isolated membranes from *R. capsulatus* (fructose-1-phosphate:[^{14}C]fructose transphosphorylation)

Cells	Induction	Fructose phosphorylation (nmol/min per mg of protein)[a]
Wild type	−	12
Wild type	+	87
fruA1::Tn*5*	−	<5
fruA1::Tn*5*	+	<5

[a] No activity was detected in the soluble fractions of any extract.

TABLE 3. Enzyme I activity in *R. capsulatus* strains and its localization to the membrane and cytoplasmic fractions

Cells	Induction	Fructose phosphorylation			
		Total activity (nmol/min per mg of protein)	% of induced wild type	Soluble activity (nmol/min per mg of protein)	% Soluble
Wild type	−	<20	<0.2	<10	
Wild type	+	10,500	100	<10	<1
fruA1::Tn*5*	−	90	0.8	18	20
fruA1::Tn*5*	+	220	2.1	135	61

TABLE 4. Fructose-1-phosphate kinase activity in *R. capsulatus* extracts

Cells	Induction (0.2% fructose)	Amt (%) of fructose 1,6-diphosphate formation (μmol/min per mg of protein)
Wild type	−	0.016 (2)
Wild type	+	0.77 (100)
fruA1	−	0.53 (69)
fruA1	+	1.92 (250)
fruA1(pFRU1)	−	0.085 (11)
fruA1(pFRU1)	+	2.34 (300)

age sites. Complete *Bgl*II digestion yielded four fragments of about 9.5, 7, 4, and 3 kb, in addition to a 20-kb fragment which represented most of the vector. The 9.5-kb fragment was religated into the *Bgl*II site of pRK290, yielding plasmid pFRU2. This plasmid complemented the three mutants, *fruA1*, *fru-2*, and *fru-3*, described in this paper. Subsequent digestion of pFRU2 with *Hin*dIII and *Bgl*II resulted in the generation of two fragments of about 6.7 and 3.0 kb, in addition to the 20-kb fragment which represented the vector. Both fragments were cloned into the high-copy-number plasmid pUC19 and the shuttle vector pRK404. The plasmid containing the 6.7-kb fragment, pFRU3, but not that containing the 3.0-kb fragment, pFRU4, in the shuttle vector was found to complement the three *fru* mutants of *R. capsulatus*. Neither piece cloned into pUC19 complemented a *fru* mutant of *E. coli* (LJ916; HB101 *fruA56*).

DISCUSSION

A previous report identified a novel, two-component PTS with specificity for fructose in photosynthetic bacteria (17). Subsequent work suggested that the soluble factor might represent a complex of enzyme I-like and FPr-like proteins (2) analogous to those found in enteric bacteria (15). More recent results (9), however, render the conclusion of Brouwer et al. (2) questionable. Lolkema et al. (9) used [^{32}P]PEP to identify the phosphorylated PTS proteins of *R. sphaeroides* and were unable to confirm the previous suggestion (2) of the occurrence in this photosynthetic bacterium of FPr-like and enzyme I-like proteins analogous to those in *E. coli*. Instead, a soluble protein (molecular weight, 110,000) and an integral membrane protein of apparent monomeric molecular weight of about 55,000 were detected.

To define the structure of the fructose PTS in photosynthetic bacteria and to establish the evolutionary relationships between this relatively simple system and the more complex systems in other bacteria, we have taken a molecular genetic approach. We first isolated a *fruA*::Tn*5* mutant in *R. capsulatus* and then used this mutant to clone the *fruA* gene by complementation. Because of the size of the cloned fragment, the remainder of the fructose regulon is presumed to be present. Complementation of the *fru-2* and *fru-3* mutations supports this contention. Sequencing of the subcloned *fru* regulon should allow definition of the system and thereby resolve the controversy described above.

Characterization of the *fruA1*::Tn*5* mutant has led to unexpected findings regarding the genetic control and biochemistry of the fructose PTS in *R. capsulatus*. Although the mutation eliminated detectable enzyme II activity, it rendered the activities of enzyme I and fructose-1-phosphate kinase semiconstitutive. Instead of the 100-fold induction observed in the parent, both enzymes exhibited higher basal activities, and both were threefold inducible by the inclusion of fructose in the growth medium. Interestingly, however, the activity of fructose-1-phosphate kinase in the mutant was approximately equal, in the uninduced state, to that of the fully induced parent, and inclusion of fructose in the growth medium caused a three- to fourfold increase over the basal activity. The simplest explanation is that the *fruA*::Tn*5* mutation alters sensitivity of the *fru* regulon to transcriptional regulation and that the low rate of fructose utilization may eliminate the phenomenon of catabolite repression (15). Analogous results have been obtained for enzyme II-negative mutants of *E. coli* and *S. typhimurium*, in which the mutations were clearly shown to be in the structural genes for the enzymes II (15, 19). The results provide evidence for the inclusion of three genes (*fruA*, encoding enzyme II; *fruB*, encoding enzyme I, and *fruK*, encoding fructose-1-phosphate kinase) in a single regulon in *R. capsulatus*. They further suggest a direct or indirect role of enzyme II in transcriptional or translational regulation.

In earlier reports it was shown that the fructose-specific enzyme I in photosynthetic bacteria is bound to the membrane fraction, and it was suggested that this association specifically involves enzyme II (9, 17). However, no direct evidence was available to substantiate this suggestion. In this report we demonstrate that loss of enzyme II (probably by Tn*5* insertion into the structural gene) renders the majority of enzyme I soluble. The most reasonable explanation is that enzyme I is tightly associated with the membrane via enzyme II and that loss of the latter protein therefore renders the former enzyme soluble.

While abolishing enzyme II^{Fru} activity, the Tn*5* insertion mutation had a strongly polar effect on enzyme I expression without exerting a corresponding effect on fructose-1-phosphate kinase. Although more than one explanation is available to account for this observation, possibly the gene order (*fruK-fruA-fruB*) can explain the results. Thus, if this were the gene order of the presumed *fru* operon, or if *fruK* were encoded in an operon separate from that encoding the *fruA* and *fruB* genes, no polar effect on *fruK* would be expected. Similarly, strong polarity of a *fruA*::Tn*5* insertion on *fruB* expression could be expected only if *fruA* preceded the *fruB* gene in a single transcriptional unit. The coordinate fructose inducibility of the *fruK* and *fruB* genes in the *fruA1*::Tn*5* mutant renders less likely the possibility that *fruB* is transcribed from a promoter contained within the insertion element. Moreover, the restoration of wild-type growth rates on fructose by transfer of the cosmid to the *fruA1*::Tn*5* mutant clearly suggests that the cosmid carries both the *fruA* and *fruB* genes. It therefore seems clear that sequencing of the subcloned *fru* genes will lead not only to an understanding of the structures of the PTS proteins, but also to confirmation or refutation of the postulates put forth here, on the basis of the properties of the *fruA1*::Tn*5* mutant, regarding transcriptional regulation of the fructose utilization genes in photosynthetic bacteria.

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