Transposon Mutagenesis and Complementation of the Fructokinase Gene in *Rhizobium leguminosarum* biovar *trifolii*

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Transposon Tn5 was used to generate a fructokinase mutation in *Rhizobium leguminosarum* biovar *trifolii* BAL. The section of the genome containing Tn5 was cloned into the *Eco*RI site of the vector pHCT9 and isolated by direct selection on medium containing kanamycin and tetracycline. Total *Eco*RI digestion was used to obtain a single fragment containing Tn5 and flanking DNA sequences. The flanking DNA was used as a probe to isolate an intact fructokinase gene from a pLAFRI cosmid clone bank of the parental strain. A cosmid showing homology to the probe was tri-parentally conjugated into the fructokinase-negative strain, complementing the mutation. The complemented mutant exhibited the wild-type phenotype, with an increase in fructokinase production presumably due to multiple copies of the gene.

INTRODUCTION

Little is known about the central pathways of carbohydrate metabolism in rhizobia. Carbohydrate supply is a major limiting factor in nitrogen fixation by the *Rhizobium*–legume symbiosis (Hardy, 1977; Pate, 1977). Much of the information available about the components of rhizobial pathways has been derived through the use of chemical mutagens. Although highly useful, chemical mutagens may often lead to confusing and conflicting results.

Using transposons to generate mutations eliminates many of the uncertainties associated with chemical mutagens. Many transposons, including Tn5, are very stable once inserted into the genome. This makes them particularly useful because they lead to single site mutations that are easily identified. The isolated segment of DNA can then be used as a specific probe to identify the functional gene from a genomic library of the parental strain (Chakravorty *et al.*, 1982).

Metabolic mutations in *Rhizobium* have been generated by Tn5 mutagenesis (Arwas *et al.*, 1985; Duncan, 1981; Glenn *et al.*, 1984*a, b*), but isolation and complementation of these genes have not previously been reported. In this communication we describe the generation of a fructokinase mutation in *Rhizobium leguminosarum* biovar *trifolii* BAL by Tn5 mutagenesis, isolation of the gene, and complementation of the mutant strain. Fructokinase levels in the parental BAL strain, the mutant and the complemented mutant, were examined, as well as levels of other key metabolic enzymes.

METHODS

Bacterial strains and media. *Rhizobium leguminosarum* biovar *trifolii* BAL and mutant derivative strains were grown on yeast extract mannitol medium (YEM) (Vincent, 1970) or *Rhizobium* defined medium (RDM) containing 0.4% (w/v) carbohydrate (Ronson & Primrose, 1979). BAL strains were maintained on YEM medium supplemented with the appropriate antibiotic (Table 1). *Escherichia coli* strains were grown and maintained on Luria–Bertani (LB) medium (Maniatis *et al.*, 1982) supplemented with the appropriate antibiotic (Table 1). All cultures were incubated at 28 °C and broth cultures were shaken at 200 r.p.m. in an orbital water bath. *Rhizobium* cultures were checked for phenotypic markers (antibiotic and carbohydrate) before and after each experiment. Growth on tryptic soy agar (TSA) was routinely examined as a check for contamination.

Abbreviations: LB, Luria–Bertani; RDM, rhizobium defined medium; YEM, yeast extract mannitol medium.

Table 1. *Bacterial strains and plasmids*

Strain	Phenotype* or genotype	Reference or source
<i>R. leguminosarum</i> biovar <i>trifolii</i>		
BAL	Parental strain	F. B. Dazzo†
BAL 79	Frk ⁻ [<i>frk</i> :: Tn5(Km ^r)]	This report
BAL C79	Frk ⁺ [BAL 79(pLA72)(Tc ^r)]	This report
<i>E. coli</i>		
HB101	F ⁻ <i>hdsS20 recA ara pro</i> Str ^r	Maniatis <i>et al.</i> (1982)
Plasmids		
pHC79	Ap ^r Tc ^r <i>λcos</i>	Hohn & Collins (1980)
pLAFR1	Tc ^r <i>oriT λcos</i>	Friedman <i>et al.</i> (1982)
pSUP1011	Cm ^r Km ^r :: Tn5	Simon <i>et al.</i> (1983)
pLA72	Tc ^r <i>frk</i> ⁺	This report

* Phenotype symbols: Frk⁻/Frk⁺, fructokinase negative/positive; Ap^r ampicillin resistance; Cm^r, chloramphenicol resistance; Tc^r, tetracycline resistance; Km^r, kanamycin resistance on Tn5; Str^r, streptomycin resistance.

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Generation and verification of Tn5 mutants. Tn5 was introduced into the BAL strain using the Mob transfer system (Simon *et al.*, 1983). After incubation for 24 h, cells were washed from filters with 1 ml of a 0.9% (w/v) NaCl solution containing 0.01% (v/v) Tween 20, and were plated on YEM medium containing kanamycin (150 µg ml⁻¹) and streptomycin (75 µg ml⁻¹) to select for Tn5 transconjugants. In addition to antibiotic markers, transposon integration was verified by Southern gel analysis (Southern, 1975) using the internal *Bgl*II fragment of Tn5 as a probe. Probes for colony and Southern hybridizations were labelled with [³²P]dCTP using the Amersham nick translation kit. Hybridizations were done at 55 °C, without formamide, using the solutions and washes recommended by Wahl *et al.* (1981).

DNA isolation. Total DNA was isolated from BAL strains in the following manner. Overnight cultures grown in YEM broth were harvested and resuspended in 0.25 ml alkaline solution I (Maniatis *et al.*, 1982). SDS (10%, w/v) was added to the cell suspension to a final concentration of 1% (v/v) and heated at 65 °C for 10 min. The solution was extracted twice with an equal volume of Tris/EDTA buffer (TE; pH 7.6) (Maniatis *et al.*, 1982), saturated with phenol/chloroform (1:1) and centrifuged for 10 min at approximately 14000 g. DNA was precipitated from the aqueous phase by addition of 3 vols 95% (v/v) ethanol, at room temperature, and further purified by CsCl density centrifugation (Maniatis *et al.*, 1982).

Cosmid vectors and clones were isolated from *E. coli* and BAL C79 using the alkaline extraction method (Birnboim & Doly, 1979) and purified by two successive CsCl density gradients.

Cosmid cloning. Cosmid clone banks of BAL and BAL 79 were constructed as recommended by Maniatis *et al.* (1982). The first was constructed from *Eco*RI restriction endonuclease digested DNA isolated from BAL 79 and ligated into alkaline phosphatase treated vector pHC79. The packaged DNA was transfected into *E. coli* HB101 and plated on LB medium containing kanamycin (50 µg ml⁻¹) and tetracycline (25 µg ml⁻¹) for direct selection of cosmids containing Tn5 inserts. A total of 1000 clones was obtained.

The second clone bank was similarly constructed from the parental strain using cosmid vector pLAFR1. Approximately 700 cosmid clones were selected on LB plus tetracycline (25 µg ml⁻¹) and replica plated to nitrocellulose discs for colony hybridization analysis.

Isolation and complementation of the fructokinase gene. A cosmid containing Tn5 was subcloned to yield a single *Eco*RI fragment, containing the insertionally inactivated fructokinase gene. This fragment was used to probe approximately 450 of the pLAFR1 cosmids by colony hybridization (Grunstein & Hogness, 1975). One of the cosmids showing homology was designated pLA72 and conjugated into BAL 79 by tri-parental mating (Sutton *et al.*, 1984). Transconjugants were selected on RDM-mannitol medium containing kanamycin (150 µg ml⁻¹) and tetracycline (25 µg ml⁻¹).

Cell-free extract preparation. Cultures for enzyme analysis were grown in 100 ml of broth (YEM or RDM) and harvested during exponential phase by centrifugation at 6000 g for 10 min. The cell pellet was washed once with 50 ml of 0.05 M-sodium phosphate buffer (pH 7.2) and resuspended in 2 ml of the same buffer containing 1 mM-dithiothreitol. Cells were then lysed by sonication on ice using the intermediate tip on a Fisher sonic dismembrator, model 300, at 60% power for four 30 s bursts with intermittent 30 s cooling periods. Extracts were clarified by centrifugation at 20000 g for 30 min at 4 °C. Cell free extracts prepared in this manner were used for all enzymic assays.

Uptake of ^{14}C -labelled mannitol. Cells grown in YEM broth were harvested and assayed for the ability to transport [^{14}C]mannitol (Sigma) as described by Ronson & Primrose (1979).

Protein determination. Cell-free extracts from samples were analysed in duplicate for protein content using the dye-binding method (Bradford, 1976), using bovine serum albumin (fraction V, Sigma) as the standard.

Enzyme assays. Glucokinase (EC 2.7.1.2; ATP:D-glucose 6-phosphotransferase), and fructokinase (EC 2.7.1.4; ATP:D-fructose 6-phosphotransferase) activities were determined by following the reduction of NADP^+ by cell-free extracts (Martinez de Drets & Arias, 1970). Excess isomerase and glucose-6-phosphate dehydrogenase (1 unit each) were added to the kinase assays to ensure maximum activity. Mannitol dehydrogenase (EC 1.1.1.14; L-iditol:NAD $^+$ 2-oxido-reductase) was determined by measuring the reduction of NAD^+ (Leissing & McGuinness, 1982). Controls without substrate were run with each assay.

RESULTS AND DISCUSSION

Growth characteristics of *BAL* strains

The wild-type *R. leguminosarum* biovar *trifolii* BAL is a highly efficient and effective nodulating strain which forms double infection threads (F. B. Dazzo, personal communication). However, this strain appears to grow more slowly than others on fructose. The ability of strain BAL to grow efficiently on mannitol and sucrose (Table 2) indicated that internal fructose was metabolized. It was found that the enzyme fructokinase, necessary to initiate metabolism of fructose, was functional (Table 3). This strain is still able to form effective nodules on clover (Dazzo & Hubbell, 1975), which is consistent with data obtained from other fructose negative mutants of *Rhizobium* (Ronson & Primrose, 1979).

BAL 79 is a Tn5 generated mutant of BAL which was unable to grow on mannitol or fructose, and showed impaired growth on sucrose (Table 2); it appeared to nodulate normally. This phenotype has also been described for a fructokinase negative mutant of *R. leguminosarum*

Table 2. Carbohydrate utilization by *R. leguminosarum* biovar *trifolii* BAL

Strain	Growth on RDM medium with:			
	Glucose	Sucrose	Fructose	Mannitol
BAL	+	+	slow	+
BAL 79	+	slow	—	—
BAL C79	+	+	slow	+

+, Growth; —, no growth.

Table 3. Specific activities of fructokinase and mannitol dehydrogenase in *R. leguminosarum* biovar *trifolii* BAL strains

Specific activities are expressed as $\mu\text{mol NADPH or NADH produced min}^{-1} (\text{mg protein})^{-1}$. Each value is the mean of six assays from duplicate cultures.

Enzyme	Strain	Culture medium:			
		RDM-glycerol	RDM-glucose	RDM-glycerol/ mannitol	YEM
Fructokinase	BAL	0.061	0.049	0.172	0.085
	BAL 79	0.021	0.020	0.036	0.023
	BAL C79	0.256	0.313	0.196	0.837
Mannitol dehydrogenase	BAL	0.002	0.002	0.080	0.038
	BAL 79	ND	0.004	0.083	0.019
	BAL C79	0.004	0.004	0.073	0.028

ND, None detected.

Fig. 1. Southern hybridization analysis of *Eco*RI digested DNA from BAL strains using Tn5 with flanking regions as the probe. Lanes 1–4 represent digested DNA and lanes 5–7 represent the hybridization patterns. Lane 1, λ -*Hind*III standard; lanes 2 and 5, BAL 79; lanes 3 and 6, BAL C79; lanes 4 and 7, BAL: A, Tn5-interrupted gene, B, intact gene.

(Glenn *et al.*, 1984a). When grown on YEM medium, BAL 79 had a non-mucoid phenotype due to growth supported solely by yeast extract.

BAL C79 is a cosmid complemented derivative of BAL 79. This complementation allowed normal growth on all carbohydrates tested (Table 2), with increased production of fructokinase (Table 3). BAL C79 also appeared to nodulate normally.

Isolation of the fructokinase mutant

The introduction of Tn5 into BAL resulted in numerous kanamycin/streptomycin resistant colonies. Approximately 1000 of these colonies were replica plated onto RDM containing various single carbohydrates; the majority exhibited normal growth on all carbohydrates tested.

Fig. 2. Uptake of mannitol by strains BAL and BAL 79. Bacteria were grown in YEM and assayed as described in Methods. ■, BAL 79; ○, BAL.

Of the colonies showing growth deficiencies, BAL 79, which was unable to grow on mannitol, was chosen for further study. Southern analysis verified that a single copy of Tn5 was present in the genome.

Isolation and complementation of the fructokinase gene

The fructokinase probe was obtained through *Eco*RI digestion of BAL 79 DNA. Since Tn5 contains no *Eco*RI sites, this allowed for the cloning of Tn5 with flanking regions of the inactivated gene.

One cosmid containing ten *Eco*RI fragments was subcloned to yield a single *Eco*RI fragment. This fragment was used as a probe to isolate the intact fructokinase gene from a pLAFR1 clone bank constructed from the BAL strain. One cosmid (pLA72) showing homology to the probe was used to complement BAL 79. Complementation was verified by the renewed ability to utilize mannitol and fructose, and the presence of pLA72. Southern hybridization analysis of the three BAL strains using the Tn5::flanking DNA probe showed that BAL C79 contained two hybridizing bands (Fig. 1). One band corresponded to the inactive gene seen in BAL 79, and the other band corresponded to the active gene seen in BAL. The band representing the intact gene in BAL C79 had a greater intensity than the other band, presumably due to multiple copies of pLA72. BAL C79 retained the antibiotic markers of Tn5, indicating that the ability to utilize mannitol was not due to Tn5 excision.

Enzyme analysis

BAL strains were grown to mid-exponential phase in four different media (RDM supplemented with glycerol, glucose or glycerol/mannitol; and YEM) and assayed for intracellular glucokinase (not shown), fructokinase and mannitol dehydrogenase (Table 3). The three strains had similar glucokinase activity (data not presented); however, levels of fructokinase differed dramatically. BAL 79 had a low level of fructokinase activity when grown in all media; this may have been due to non-specific glucokinase activity. The BAL and BAL C79 strains showed induction of fructokinase in the presence of mannitol, with BAL C79 showing approximately a sevenfold higher fructokinase level than BAL. Similarly, fructokinase activity was greater for BAL C79 when grown on the other media tested, ranging from three to ten times more. The increased enzyme production may be due to the presence of multiple plasmid copies, as suggested by the more intense hybridization band observed for BAL C79.

Mannitol dehydrogenase activity was measured to establish that internal fructose was available to cells (Table 3). Transport of [¹⁴C]mannitol was determined for BAL and BAL 79. Transport rates were similar for the two strains during the first 5 min. Thereafter rates began to

decrease for BAL 79 (Fig. 2), possibly due to high internal concentrations of fructose accumulated as a result of the fructokinase mutation in this strain. A fructokinase mutant of *R. leguminosarum* has been shown to accumulate levels of fructose to concentrations 300-fold greater than external concentrations (Glenn *et al.*, 1984a).

In summary, a Tn5 fructokinase negative mutant of *R. leguminosarum* biovar *trifolii* BAL was generated. This mutant (BAL 79) was identified by its low fructokinase activity and inability to utilize mannitol as a sole carbon/energy source. The ability of BAL 79 to transport mannitol and the presence of mannitol dehydrogenase further confirmed the location of the mutation. Complementation of the fructokinase mutation in BAL C79 was verified by the renewed utilization of mannitol and increased levels of fructokinase activity. The observed increase in fructokinase activity for BAL C79 was probably due to the presence of multiple plasmid copies. This increase in fructokinase activity does not appear to affect nodulation, probably due to rate limiting enzymes further in the metabolic pathway. Such a rate limiting effect makes it difficult to determine the benefits from multiple copies of metabolic genes.

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