The fructokinase from *Rhizobium leguminosarum* biovar *trifolii* belongs to group I fructokinase enzymes and is encoded separately from other carbohydrate metabolism enzymes

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The *Rhizobium leguminosarum* bv. *trifolii* BAL fructokinase (*frk*) gene was isolated on a 2.4 kb *Bam*HI fragment from the cosmid pLA72 by complementation analysis of the Tn5-induced *frk* mutant BAL79, and confirmed by hybridization analysis. The nucleotide sequence of the *frk* gene was found to contain an open reading frame consisting of 978 bp encoding 326 amino acids, which was then compared to known fructokinase sequences. The fructokinase gene was not contained in an operon and is encoded separately from other enzymes of carbohydrate metabolism. Its product is therefore assigned to the group I fructokinases. A putative promoter (TTGACA-N₁₆-GTTGAT), ribosome-binding site and termination sequence were identified. The Frk protein contained several motifs conserved in other known fructokinase sequences, including an ATP-binding and a substrate-binding motif. The hydropathy plot derived from the *frk* gene sequence data revealed the fructokinase as a hydrophilic protein. The fructokinase protein was purified to electrophoretic homogeneity by a three-step method using chromatofocusing, affinity chromatography and gel filtration. Its purity was confirmed by SDS-PAGE and it was visualized as a single band by silver staining. The N-terminal amino acid sequence of the purified fructokinase confirmed the proposed open reading frame of the *frk* gene. The purified fructokinase had a molecular mass of 36.5 kDa, pI of 4.65, pH activity range of 6.0–9.0 (maximum activity at pH 8.0) and a Mg²⁺ requirement. It had a *K_m* of 0.31 mM and a *V_{max}* of 31 µmol fructose 6-phosphate (mg protein)⁻¹ min⁻¹ with fructose as substrate. The *R. leguminosarum* bv. *trifolii* BAL fructokinase was biochemically and molecularly similar to other bacterial fructokinases.

Keywords: *Rhizobium leguminosarum* bv. *trifolii*, fructokinase, sequence analysis

INTRODUCTION

Carbohydrate supply is a major limiting factor in nitrogen fixation by *Rhizobium*–legume symbioses (Bethlenfalvay & Phillips, 1977; Pate, 1977). Sucrose is the major product of photosynthesis translocated to the nodule (Bach *et al.*, 1958) and fructokinase is a key enzyme in the metabolism of sucrose (as well as mannitol and fructose) in *Rhizobium*, catalysing the phosphorylation of fructose to fructose 6-

phosphate (Martinez deDrets & Arias, 1970). It is induced by the inclusion of these substrates in the growth medium (McLaughlin & Hughes, 1989; Ronson & Primrose, 1979) and repressed by TCA cycle intermediates (Stowers & Elkan, 1985). Transposon-induced mutants defective in fructokinase have been isolated in several *Rhizobium* species (Glenn *et al.*, 1984; Duncan, 1981; McLaughlin & Hughes, 1989), and a *frk* mutant of *R. leguminosarum* bv. *trifolii* BAL was unable to grow on mannitol or fructose and its growth on sucrose was impaired. The *frk* mutant, BAL79, failed to produce polysaccharide capsule on complex or minimal media containing mannitol (McLaughlin & Hughes, 1989).

Abbreviation: TCA, tricarboxylic acid.

The GenBank accession number for the sequence reported in this paper is U08434.

Fructokinase genes have been sequenced from a number of bacteria, including *Escherichia coli*, *Salmonella typhimurium*, *Klebsiella pneumoniae*, *Vibrio alginolyticus*, *Streptococcus mutans* and *Zymomonas mobilis*, but not to our knowledge from any *Rhizobium* species (Aulkemeyer *et al.*, 1991; Blatch *et al.*, 1990; Brockman *et al.*, 1992; Sato *et al.*, 1993; Zembrzusi *et al.*, 1992). These fructokinase genes, designated *scrK*, are commonly found in an operon involved in sucrose utilization. In *K. pneumoniae* and *S. typhimurium* (pUR400), *scrK* is the first gene of a transcriptional unit, *scrKAYBR*, which includes the genes for the membrane porin (*scrY*), sucrose (*scrA*), sucrose-6-phosphate hydrolase (*scrB*) and the repressor, *scrR* (Aulkemeyer *et al.*, 1991; Blatch & Woods, 1991). Jahreis & Lengeler (1993) identified D-fructose and fructose 6-phosphate as the inducer molecules for the sucrose utilization operon and a repressor has been identified, ScrR, which binds directly to a palindromic operator sequence. Previously, the fructokinase gene from *Z. mobilis* was shown to be the only exception, as it is not transcriptionally linked to an operon and is proposed to be involved specifically in fructose and not sucrose utilization. The *Z. mobilis* enzyme is classed as a group I fructokinase; the fructokinases involved in sucrose utilization are classed as group II (Zembrzusi *et al.*, 1993).

Rhizobium leguminosarum bv. *trifolii* BAL produces numerous nodules and infection threads on its host *Trifolium repens*, white clover (Dazzo & Hubble, 1975). McLaughlin & Hughes (1989) isolated the fructokinase gene from *R. leguminosarum* bv. *trifolii* by complementation of the Tn5-induced *frk* mutant BAL79 with the cosmid pLA72. The *frk* mutant was still able to nodulate white clover and fix nitrogen (McLaughlin & Hughes, 1989). Using subcloning and complementation of the *frk* mutant BAL79, the *frk* gene was isolated on a 2.4 kb *Bam*HI fragment and used for nucleotide sequence analysis.

This study reports the nucleotide sequence of the fructokinase (*frk*) gene from *R. leguminosarum* bv. *trifolii* BAL, the deduced amino acid sequence and similarities to other known fructokinases. The fructokinase enzyme was purified to electrophoretic homogeneity and partially characterized. In addition, the N-terminal amino acid sequence of the fructokinase protein was determined, thus confirming the reported gene sequence.

METHODS

Bacterial strains, media and culture conditions. Bacterial strains and plasmids used in this work are listed in Table 1. *Rhizobium leguminosarum* bv. *trifolii* strains were grown on yeast extract mannitol medium (YEM; Vincent, 1970) at 30 °C. *Escherichia coli* strains were grown as described by Maniatis *et al.* (1989) in Luria broth. Mutants and plasmid-containing strains were supplemented with the appropriate antibiotic: tetracycline (12.5 µg ml⁻¹ for *E. coli* and *R. leguminosarum*), and kanamycin (50 µg ml⁻¹ for *E. coli* and 150 µg ml⁻¹ for *R. leguminosarum*) (Table 1). For solid medium 15 g agar l⁻¹ was added.

Subcloning and complementation of the *frk* gene. Cosmid DNA, pLA72, was isolated by the alkaline extraction method and purified by CsCl₂ density-gradient ultracentrifugation (Maniatis *et al.*, 1989). Partial digestion with *Sau*3A was

performed using the dilution method (Maniatis *et al.*, 1989) and fragments were purified by the GeneClean procedure (Bio 101). The *Sau*3A-digested cosmid DNA was ligated into the *Bam*HI site of pRK404 (Ditta *et al.*, 1985) and ligated at 16 °C for 24 h (Maniatis *et al.*, 1989) using T4 ligase. Ligation mixtures were transformed into *E. coli* DH5α using the method of Hanahan (1983). DNA inserts were verified on Luria agar containing 0.1 mM IPTG, 0.04% X-Gal and tetracycline (12.5 µg ml⁻¹). Plasmids were transferred to *R. leguminosarum* bv. *trifolii* by triparental mating (Ditta *et al.*, 1980). Complementation of the *frk* gene was analysed by isolation of the recipient trans-conjugant colonies on YEM-KT agar (YEM plus kanamycin and tetracycline; McLaughlin & Hughes, 1989).

Southern gel analysis and hybridization conditions. Total DNA was isolated and Southern blot analysis done by standard procedures (Maniatis *et al.*, 1989; Southern *et al.*, 1975), using the 2.4 kb fragment containing the *frk* gene as a probe. The probe was labelled with biotin-11-dUTP using the Nick Translation Kit from BRL. Hybridizations were done at 42 °C, with 45% (v/v) formamide, using the solutions and washes as recommended by the manufacturer for biotinylated probes (Leary *et al.*, 1983). Hybridizations were visualized using the BluGene Nonradioactive Nucleic Acid Detection System (BRL).

Subclone analysis and DNA sequencing. The 2.4 kb fragment was subcloned into the *Bam*HI site of pBluescriptSK (Stratagene). Restriction mapping was performed by standard procedures (Maniatis *et al.*, 1989). *E. coli* subclones for sequencing were screened for fructokinase activity (Martinez deDrets & Arias, 1970; Sato *et al.*, 1993) and plasmid DNA for sequencing was prepared using the Plasmid Midi Kit (Qiagen). Sequencing was done by the dideoxy chain-termination method (Sanger *et al.*, 1977), using *Taq* DNA polymerase (Promega) with the DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) and a thermocycler (Perkin Elmer Cetus). Reaction mixtures were purified on a CentraSep spin column (Princeton Separations) and dried in a Speed Vac (Savant Instruments). An automated DNA sequencer (Applied Biosystems, model 373A) was utilized to determine the nucleotide sequence of both strands of the entire 2.4 kb fragment. Analysis of overlapping and complementary sequence data was performed on a Macintosh SE/30 using the Gene Jockey program (Biosoft). Sequence analysis was performed at the NCBI using the BLAST network service (Devereux *et al.*, 1984).

Enzyme assay conditions. Fructokinase activity was determined spectrophotometrically at 25 °C by the method of Martinez deDrets & Arias (1970). The enzyme assay mixture contained (1 ml total volume): 40 mM fructose, 2 mM ATP, 1 mM NADP, 5 mM MgCl₂·H₂O, 1 U phosphoglucose isomerase, 1 U glucose-6-phosphate dehydrogenase, and 75 µl enzyme extract. Fructokinase activity was determined at various pH values using the enzyme assay as described except that the pH of the reaction mixture was adjusted with 0.5 M Tris/HCl to range from 6.0 to 9.0. Divalent cation requirements were determined by substituting the MgCl₂ (1 mM) in the fructokinase assay reaction mixture with an equal molar amount of various other cations (Thompson *et al.*, 1991). The *K_m* and *V_{max}* were determined according to the method of Lineweaver & Burke (1934). Protein was determined by the dye binding method of Bradford (1976).

Enzyme purification

Step 1. Extraction and centrifugation. Cells of *R. leguminosarum* bv. *trifolii* (1 l) were harvested by centrifugation (800 r.p.m. for 15 min) and resuspended in distilled water containing 1 mM DTT. The cells were disrupted by two passages through a

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics*	Reference or source
Strains		
<i>R. leguminosarum</i>		
bv. <i>trifolii</i>		
BAL	Wild-type	Dazzo & Hubbel (1975)
BAL 79	Frk ⁻ [<i>frk</i> ::Tn5(Kan ^r /Str ^r)]	McLaughlin & Hughes (1989)
BAL C79	Frk ⁺ [BAL 79/pLA72(Tet ^r)]	McLaughlin & Hughes (1989)
BAL B30	Frk ⁺ [BAL 79/pB30(Tet ^r)]	This report
<i>E. coli</i>		
HB101	F ⁻ , <i>hsdS20 recA ara pro</i> Str ^r	Maniatis <i>et al.</i> (1982)
DH5 α	F ⁻ , <i>endA1 hsdR17</i> (r _k ⁺ , m _k ⁺) <i>supE44 thi-1 λ^- recA1</i> <i>gyrA96 relA1 ϕ80d lacZDM15</i>	BRL
Plasmids		
pRK404	Tet ^r , <i>oriT oriV lacZ</i>	Ditta <i>et al.</i> (1985)
pRK2013	Kan ^r , <i>oriT tra</i>	Ditta <i>et al.</i> (1980)
pLA72	Tet ^r , <i>frk</i> ⁺	McLaughlin & Hughes (1989)
pBluescriptSK	Amp ^r , <i>lacZ</i> , T3, T7	Stratagene Cloning Systems
pB12	Tet ^r , <i>frk</i> ⁺ <i>oriV</i>	This report
pB30	Tet ^r , <i>frk</i> ⁺ <i>oriV</i>	This report
pB40	Tet ^r , <i>frk</i> ⁺ <i>oriV</i>	This report
pBSK12	Amp ^r , <i>frk</i> ⁺ <i>lacZ</i> , T3, T7	This report

* Frk, fructokinase; Tet^r, tetracycline resistance (12.5 μ g ml⁻¹ for *E. coli* and *R. leguminosarum* bv. *trifolii*); Kan^r, kanamycin resistance (50 μ g ml⁻¹ for *E. coli* and 150 μ g ml⁻¹ for *R. leguminosarum* bv. *trifolii*); Str^r, streptomycin resistance (50 μ g ml⁻¹ for *E. coli* and *R. leguminosarum* bv. *trifolii*); *oriV*, origin of replication from RP4; *oriT*, origin of transfer from RP4; *lacZ*, *lacZ* cloning region; T3 and T7, primer regions.

French pressure cell at 1260 p.s.i. (8.7 MPa) and centrifuged at 16000 r.p.m. for 30 min. The supernatant was used as the cell-free extract.

Step 2. Chromatofocusing. Cell-free extract was dialysed overnight at 4 °C in 25 mM histidine buffer (pH 6.0) containing 1 mM DTT. Sucrose was added to a final concentration of 0.5% and the mixture was loaded onto a column (2.8 × 50 cm) containing polybuffer exchanger PBE94 (Pharmacia) equilibrated with histidine buffer. Samples were eluted using polybuffer 74 (pH 4.0) according to the method of Lallan (1990). Fractions containing enzyme activity were pooled and dialysed against 1 l affinity column buffer (1 mM MOPS pH 7.0 containing 1 mM MgCl₂ and 1 mM DTT). Dialysis was monitored by change in pH.

Step 3. Affinity chromatography. The column matrix was constructed by the method of Vretblad (1976) by mixing 1 g epoxy-activated agarose (Sigma) and 250 mg D-fructose with 100 ml distilled water for 16 h at 25 °C. Excess ligand was removed by filtration on a sintered glass filter using distilled water followed by alternate washings with 0.1 M bicarbonate buffer (pH 8.0) and 0.1 M acetate buffer (pH 4.0). Excess groups were blocked by incubation in 50 ml 1 M ethanolamine overnight. Samples were loaded onto a column (1 × 10 cm) containing the fructose/agarose matrix and washed extensively with column buffer. The fructokinase was eluted with column buffer containing 0.05 M NaCl.

Step 4. Gel filtration. Sephadex G75 (Pharmacia) was used for gel filtration by the method of Curling (1970). Samples were loaded directly onto a column (2 × 50 cm) without dialysis, and

fractions containing fructokinase activity were pooled and concentrated using Centricon 10 microfiltration cells (Amicon) at 6000 r.p.m. and 4 °C.

SDS-PAGE. This was performed by standard procedures (Laemmli, 1970) using the Mini Protean II electrophoresis cell (Bio-Rad). Molecular mass was determined using Dalton Mark VII-L protein mixture (Sigma) as standards. Proteins were detected by staining gels with silver stain (Merril *et al.*, 1981).

IEF. Analytical IEF was performed in IsoGel agarose (FMC Bioproducts) on a Multifor model 2117 flat-bed electrophoresis unit (Pharmacia LKB) using standard procedures (Harper, 1981). Isoelectric point was determined by comparison with IEF standards (Bio-Rad).

N-terminal amino acid sequencing. This was performed by automated Edman degradation using a PI 2090E Integrated Micro-Sequencing System (Porton Instruments). Cysteine residues were detected by the alkylation method of Brune (1992).

RESULTS

Subcloning of the fructokinase gene (*frk*) and hybridization analysis

The cosmid pLA72 was shown to contain the fructokinase gene from *Rhizobium leguminosarum* bv. *trifolii* BAL (McLaughlin & Hughes, 1989). *Sau*3A fragments (1–3 kb) from pLA72 were subcloned into the *Bam*HI site of the conjugal vector pRK404 and transferred into the

Fig. 1. Agarose gel (0.8%) electrophoresis of *Bam*HI digests of recombinant plasmids. Lane 1, pRK404; lane 2, pB12; lane 3, pB30; lane 4, pB40, lane 6, pBluescriptSK; lane 7, pSK12. Lane 8, *Hind*III digest of λ DNA (fragment sizes shown on the right). Plasmid DNA was extracted by the alkaline method of Birnboim and Doly (Maniatis *et al.*, 1982) from *E. coli* DH5 α .

Fig. 2. Southern gel analysis of total genomic and plasmid DNA with the 2.4 kb *frk* encoding fragment as hybridization probe. Lane 1 shows hybridization of *Eco*RI-digested DNA of the *R. leguminosarum* bv. *trifolii* complemented mutant, BAL C79. Lane 2 is C79 *Eco*RI-digested DNA. Lane 3 is *Hind*III-digested λ (fragment sizes shown on the right). A, Hybridization to the Tn5-inserted fragment in the chromosome; B, hybridization to the intact *frk* gene on the cosmid pLA72.

Tn5 mutant, BAL 79, by triparental mating (Ditta *et al.*, 1980). Transconjugants were examined for antibiotic resistance (tetracycline, 12.5 μ g ml⁻¹) indicating the presence of pRK404, and complementation to give the mucoid phenotype on yeast extract mannitol agar (YEM-KT) since *frk* mutants have been shown to lack capsule production when grown on medium containing mannitol (McLaughlin & Hughes, 1989). Kanamycin (150 μ g ml⁻¹) was used as a counter-selection agent against donor strains. Three clones conferring the mucoid phenotype (pB12, pB30 and pB40) contained the plasmid vector, pRK404, and a 2.4 kb *Bam*HI insert (Fig. 1). One clone, pB40, also contained an additional small fragment.

Hybridization analysis of the 2.4 kb fragment with *Eco*RI-digested DNA prepared from the complemented mutant, BAL C79, showed that the probe hybridized to the Tn5-inserted fragment in the BAL C79 chromosome (Fig. 2, A), as well as to the intact *frk* gene located on the cosmid, pLA72 (Fig. 2, B) and confirmed that the *frk* gene isolated was identical to that previously described (McLaughlin & Hughes, 1989). Hybridization analysis also revealed that the *frk* gene was located on the 11 kb *Eco*RI fragment of pLA72 (data not shown).

Fig. 3. Restriction map and sequencing strategy for the *R. leguminosarum* bv. *trifolii* BAL fructokinase gene (*frk*). The arrow above the restriction map indicates the location and the direction of the ORFs encoding Frk and PyrE. The arrows below the map indicate the location and direction in which the sequence data were read. Subclone analysis and fructokinase activity for each plasmid derivative are shown. Ac, *Acc*I; Av, *Ava*I; B, *Bam*HI; C, *Cla*I; H, *Hinc*II; X, *Xho*I.

Gene sequencing

The 2.4 kb fragment containing the *frk* gene was cloned into the *Bam*HI site of pBluescriptSK and was used for restriction analysis and nucleotide sequencing. Restriction enzyme analysis identified internal *Ava*I, *Acc*I, *Cla*I, *Hinc*II and *Xho*I sites (Fig. 3). The fragments resulting from digestion with these enzymes were subcloned into pBluescriptSK and sequenced by the strategy shown in Fig. 3. *E. coli* DH5 α clones containing plasmid derivatives of pB12 were assayed for fructokinase activity. *E. coli* DH5 α clones containing pB12 with the intact 2.4 kb fragment expressed fructokinase activity (0.26 U mg⁻¹); however in *E. coli* DH5 α containing pBluescriptSK minimal activity could also be detected (0.003 U mg⁻¹). *E. coli* has been shown to lack a chromosomally encoded fructokinase (Aulkemeyer *et al.*, 1990) and investigators attributed this minimal activity to the presence of a mannokinase which can phosphorylate fructose in the absence of fructokinase. These low levels of activity did not interfere with the detection of cloned fructokinase activity (Sato *et al.*, 1993). No subclones generated using *Acc*I, *Ava*I, *Hinc*II or *Xho*I from the 2.4 kb fragment and screened in this manner showed fructokinase activity,

Arg Trp Leu Gly Arg Glu Arg Met Val Ala stop
 CGC TGG CTT GGT CGG GAA AGA ATG GTG GCG TAA GTG AGC TTT TCG CTCTACCACT-
 -35 -10 SD
 871 TGACAAAAGCTCGCCTAATCGTTGATGAACGTATAGATGCTGTTGGAGGAATA 17
 Met Ile Leu Cys Cys Gly Glu Ala Leu Ile Asp Met Leu Pro Arg Asp Thr
 926 ATG ATT TTG TGC TGC GGC GAA GCC CTG ATC GAC ATG CTG CCG AGG GAC ACC 34
 Thr Leu Gly Glu Lys Gly Phe Ala Pro Tyr Ala Gly Gly Ala Ile Phe Asn
 978 ACT CTC GGT GAA AAG GGC TTT GCG CCC TAT GCC GCG GCG GCG ATC TTC AAC 51
 Thr Ala Ile Ala Leu Gly Arg Leu Gly Ile Pro Thr Ala Phe Phe Thr Gly
 1029 ACC GCG ATC GCG CTC GGC GCG CTC GGC ATC CCC ACC GCC TTC TTC ACC GGC 68
 Ile Ala Asp Asp Met Met Gly Glu Ile Leu Leu Glu Thr Leu Lys Ala Ser
 1080 ATT GCC GAT GAC ATG ATG GGC GAA ATC CTG CTC GAG ACG CTG AAG GCG AGC 85
 Asn Val Asp Tyr Ser Pro Cys Ala Ile Thr Pro Arg Pro Ser Thr Ile Ala
 1131 AAT GTC GAT TAC AGC CCG TGC GCC ATT ACG CCG CGT CCC TCG ACC ATC GCC 102
 Phe Val Lys Leu Val Asn Gly Gln Ala Thr Tyr Ala Phe Tyr Asp Glu Gly
 1182 TTC GTC AAG CTG GTG AAC GCG CAA GCG ACC TAT GCC TTC TAC GAC GAG GCG 119
 Thr Ala Gly Arg Met Ile Thr Thr Ala Asp Leu Pro Asp Leu Gly Asp Asp
 1233 ACG GCG GCG CCG ATG ATC ACC ACG GCG GAC CTG CCG GAT CTC GCG GAT GAT 136
 Cys Glu Ala Leu His Phe Gly Ala Ile Ser Leu Ile Pro Ser Pro Cys Gly
 1284 TGC GAG GCC TTG CAT TTC GCG GCG ATC AGC CTG ATC CCC AGC CCC TGC GCG 153
 Glu Thr Tyr Glu Ala Leu Leu Asp Arg Glu Ala Ala Ser Arg Val Ile Ser
 1335 GAA ACC TAC GAA GCC CTG CTC GAC GCG GAA GCC GCA AGC GCG GTC ATC TCG 170
 Leu Asp Pro Asn Ile Arg Pro Gly Phe Ile Lys Asp Lys Pro Ser His Met
 1386 CTC GAT CCG AAT ATC CGT CCC GGT TTC ATC AAG GAC AAG CCG TCG CAT ATG 187
 Ala Arg Ile Lys Arg Met Ala Ala Lys Ser Asp Ile Val Lys Phe Ser Asp
 1437 GCC GCG ATC AAG GCG ATG GCC GCC AAA TCC GAC ATC GTC AAG TTC TCC GAC 204
 Glu Asp Leu Asp Trp Phe Gly Leu Gln Gly Asp His Asp Ala Leu Ala Ala
 1488 GAG GAC CTC GAT TGG TTC GGC CTG CAG GCG GAC CAT GAT GCG CTC GCC GCC 221
 His Trp Leu Asn His Gly Ala Lys Leu Val Val Ile Thr Lys Gly Ala Glu
 1539 CAT TGG CTG AAC CAC GCG GCC AAG CTC GTC ATC ACC AAG GCG GCG GAA 238
 Gly Ala Ser Gly Tyr Thr Lys Asp Arg Lys Val Thr Val Pro Ser Glu Arg
 1590 GGC GCT TCC GGT TAT ACC AAG GAT CCG AAG GTC ACG GTG CCG AGT GAA CCG 255
 Val Thr Val Val Asp Thr Val Gly Ala Gly Asp Thr Phe Asp Ala Gly Ile
 1641 GTC ACC GTC GTC GAC ACG GTC GCG GCC GGC GAT ACG TTC GAT GCC GCG ATC 272
 Leu Ala Ser Leu Lys Met Asp Asn Leu Leu Thr Lys Arg Gln Val Ala Ser
 1692 CTG CCG TCG CTG AAG ATG GAC AAT CTG CTG ACC AAG CCG CAA GTC GCC TCG 289
 Leu Asp Glu Gln Ala Leu Arg Asn Gly Pro Asp Pro Arg Arg Gln Thr
 1743 CTG GAT GAG CAG GCG CTG GCG AAC GCG CCT GAC CCT CCG GCG CAA AGC GCG 306
 Arg Arg His Arg Leu Pro Arg Arg Arg Gln Ser Thr Leu Gly Ala Arg Asp
 1794 CCG CGT CAC CGT CTC CCG GCG CCG CCG CAA TCC ACC CTG GCG GCG CGA GAT 323
 Trp Ser Leu Arg Leu Glu Gln Asp Ser Asp Pro His Pro Pro Asp Asp Thr
 1845 TGG TCT TTA AGG CTC GAG CAA GAC TCC GAC CCT CAT CCG CCT GAC GAC ACC 326
 Phe Ser Pro Stop
 1896 TTC TCC CCG TAA ACACGGGGCGAAGGGGATATGCCGCAAACTCTCCGTTCTCTTGG
 transcription terminator
 CGTCAGTATGAACGTCCTCTCTCCGTTCTTACCGGGAGAGGGTTAGGTCATATGCGCTAGGT

Fig. 4. Nucleotide sequence of the *frk* gene. DNA sequencing was performed as described in Methods. The deduced amino acid sequences are shown above the coding sequences. Putative promoter (-35 and -10) and ribosome-binding site (SD) complementary to the 16S rRNA are in boldface and double-underlined. Inverted repeat sequences are single-underlined.

indicating that these sites were located within the *frk* gene. Evaluation of the nucleotide sequence of the 2.4 kb fragment revealed two *Cla*I sites outside of the *frk* open reading frame (Fig. 3). Deletion of this 400 bp fragment yielded a plasmid which still retained fructokinase activity (0.24 U mg⁻¹).

The nucleotide sequence of the *frk* gene and flanking regions are shown in Fig. 4. The sequence was analysed to identify all possible enclosed open reading frames (ORFs). The search revealed two complete ORFs on the reverse strand of the deduced sequence. One (ORF2) was large enough (978 bp) for the Frk protein. The start (ATG) codon was at 926 bp and the stop codon (TAA) was at 1904 bp.

Preceding the start codon of the *frk* gene is a potential ribosome-binding sequence, AGGA, located 6 bp upstream from the ATG initiation codon (Stormo *et al.*, 1982). The sequence contained a putative promoter region (TTGACA-N₁₆-GTTGAT) similar to the *E. coli* consensus promoter (O'Neill, 1989), located 56 bp upstream from the start codon (Fig. 4). A termination sequence was located downstream from the stop codon, from 1971 to

Table 2. Amino acid composition of the *R. leguminosarum* bv. *trifolii* BAL fructokinase

The data were derived from the ORF 2 gene sequence using the Peptidesort program (Devereux *et al.*, 1984).

Amino acid	No.	Mol %
Ala	35	10.736
Cys	5	1.534
Asp	31	9.509
Glu	14	4.294
Phe	12	3.681
Gly	27	8.282
His	7	2.147
Ile	19	5.828
Lys	14	4.294
Leu	32	9.816
Met	8	2.454
Asn	7	2.147
Pro	20	6.135
Gln	7	2.147
Arg	24	7.362
Ser	19	5.828
Thr	22	6.748
Val	14	4.294
Trp	3	0.920
Tyr	6	1.840
Mol. mass = 35469		
Residues = 326		
pI = 5.7		

1999 bp (d'Aubenton *et al.*, 1990). No other ORFs were identified downstream of *frk*.

The ORF upstream of the *frk* gene was identified as the *pyrE* gene. Characterization of this gene will be reported elsewhere.

Sequence analysis

The amino acid sequence derived from the translated *frk* ORF was used to determine the predicted amino acid composition, molecular mass and isoelectric point (Table 2). The *frk* ORF codes for a putative protein consisting of 326 amino acids with an estimated molecular mass of 35469 Da. The predicted isoelectric point is 5.7. The hydropathy plot (Fig. 5) shows that the Frk protein contains mostly hydrophilic amino acid residues, with one area of hydrophobic amino acids (50 residues) at the N-terminus. The predicted amino acid sequence of the Frk protein was assessed for similarity with other known protein sequences. The Frk showed amino acid homology with several kinases, including fructokinases from *S. typhimurium*, *K. pneumoniae*, *Salmonella thompson* and *V. alginolyticus*, and the ribokinase from *E. coli* (Aulkemeyer *et al.*, 1991; Blatch *et al.*, 1990; Hope *et al.*, 1986). The Frk

Fig. 5. Hydrophobicity plot of the Frk sequence from *R. leguminosarum* bv. *trifolii* BAL. The method used was that of Kyte & Doolittle (1982), with a window size of nine amino acids. Plots were generated using sequence data and the Gene Jockey program (Biosoft).

amino acid sequence shared 28.5% identity with the *S. typhimurium* fructokinase (75/263) and 29% identity with the *K. pneumoniae* and *V. alginolyticus* fructokinases (80/263). Fig. 6 shows regions of homology between these sequences including the N-terminus and regions

between residues 158–166, 189–196 and 249–266. These data strongly confirm that the gene identified encodes a fructokinase.

Enzyme purification

The fructokinase was purified 263-fold with a 10% yield using a three-step purification method (Table 3). Inclusion of DTT in all purification buffers was required for enzyme stability. Fructokinases purified from other bacterial sources also required DTT for enzyme stability during purification (Thompson *et al.*, 1991, 1992). The plasmid-complemented mutant, strain B30, expressed higher fructokinase activity (0.27 U mg^{-1}) than the wild-type BAL (0.04 U mg^{-1}) and was used as the source of fructokinase for purification. The cell lysates were subjected to chromatofocusing (Lallan, 1990), and using the narrow-range polybuffer (pH 4–7) the fructokinase eluted at pH 4.65. At this step the fructokinase activity was 1.6 U mg^{-1} , representing a purification of 39-fold.

Affinity chromatography has been shown to be an excellent procedure for purification of kinases (Baysdorfer *et al.*, 1989; Dean & Watson, 1979; Thompson *et al.*,

Fig. 6. Multiple alignment of the Frk sequence of *R. leguminosarum* bv. *trifolii* (RHIZ) with the fructokinases of *S. typhimurium*, *K. pneumoniae* (SAL, KLEB; Aulkemeyer *et al.*, 1991) and *V. alginolyticus* (VIB; Blatch *et al.*, 1990). An asterisk indicates an amino acid residue conserved in the rhizobial fructokinase and other fructokinase sequences. Proposed sequences involved in ATP binding and substrate recognition are boxed.

Table 3. Purification of *R. leguminosarum* bv. *trifolii* BAL fructokinase

Fraction	Activity (U ml ⁻¹)*	Protein (mg ml ⁻¹)	Sp. act. (U mg ⁻¹)*	Purification (-fold)	Yield (%)
Crude extract	0.068	1.67	0.04	1	
Plasmid-enhanced	0.53	2.00	0.27	6.6	100
Chromatofocus	0.80	0.50	1.60	39	30
Affinity chromatography	1.73	0.40	4.33	104.9	13
Gel filtration	0.27	0.025	10.8	263.4	10

* One unit (U) converts 1 mM fructose to fructose 6-phosphate min⁻¹ ml⁻¹ at pH 8.0 at room temperature.

Fig. 7. SDS-PAGE of the purified fructokinase stained with silver. Electrophoresis and gel staining were done by the method of Merril *et al.* (1981). Lane 1, molecular mass standards (bottom to top): α -lactalbumin, trypsin inhibitor, trypsinogen, carbonic anhydrase, glyceraldehyde 3-phosphate, egg albumin, and bovine albumin. Lane 2, purified fructokinase (2 μ g).

1991; Vretblad, 1976). However, commercially available matrices previously used for kinase purification (ATP-agarose, 5'-AMP-agarose or Cibacron Blue 3GA) either failed to bind the fructokinase or inactivated the enzyme. For these reasons a gel matrix was constructed by linking D-fructose to epoxy-activated agarose. The entire amount of bound fructokinase eluted within two or three fractions and allowed 105-fold purification of the enzyme with 13% yield.

Electrophoretic homogeneity was achieved by gel filtration using Sephadex G-75. SDS-PAGE analysis of the samples from gel filtration using silver staining (Merril *et al.*, 1981) identified a single band free of other proteins (Fig. 7). Microsequencing using the alkylation method (Brune, 1992) allowed unambiguous identification of the first 25 N-terminal amino acid residues: Met-Ile-Leu-Cys-Cys-Gly-Glu-Ala-Leu-Ile-Asp-Met-Leu-Pro-Arg-Asp-Thr-Thr-Leu-Gly-Glu-. A comparison of the amino acid sequence from the purified protein with the predicted

amino acid sequence from the *frk* gene indicated that the first 21 amino acids of the fructokinase were identical.

Enzyme characterization

The molecular mass of the purified fructokinase was determined by SDS-PAGE to be 36.5 kDa (Fig. 7). The purified fructokinase was resolved into a single component by IEF; the isoelectric point was 4.65 (data not shown). The K_m obtained with fructose as substrate was 0.31 mM and the V_{max} was 31.6 μ mol fructose 6-phosphate min⁻¹ (mg protein)⁻¹. The pH range for fructokinase activity was 6.0–9.0, with the optimum at pH 8.0. Fructokinase required Mg²⁺ for activity; omission of Mg²⁺ resulted in no fructokinase activity. Activity increased with Co²⁺ (137%) as a divalent cation as compared to Mg²⁺ and activity was also detected with Cd²⁺ (35%) and Mn²⁺ (20%); slight activity was detected with Ca²⁺ (3%). No fructokinase activity was seen with Ba²⁺, Cu²⁺, Fe²⁺ or Zn²⁺.

DISCUSSION

Fructokinase is an important central metabolic enzyme which has been detected in a variety of rhizobia; it is necessary for the metabolism of mannitol, sucrose and fructose (Duncan, 1981; Glenn *et al.*, 1984; McLaughlin & Hughes, 1989). It has been shown to be inducible by the inclusion of these substrates in the growth medium as well as repressible by addition of TCA cycle intermediates (Martinez deDrets & Arias, 1970; Stowers & Elkan, 1985; Ucker & Signer, 1978). Although some biochemical research has been done, the rhizobial fructokinase enzyme has not been characterized and the fructokinase gene has not been examined at the molecular level. To our knowledge, this is the first study to report the characterization of the fructokinase enzyme from a *Rhizobium* species as well as the nucleotide sequence of the fructokinase gene.

Comparison of the fructokinase *R. leguminosarum* bv. *trifolii* (Frk) amino acid sequence with other known fructokinase sequences shows that it contains two conserved regions. It contains the ATP-binding motif, VDTvGAGDtdFAGiLASL, found in the third family of sugar kinases proposed by Wu *et al.* (1991), with the critical duo DG essential for activity and coordination of

the Mg^{2+} (Heinisch *et al.*, 1989) being conserved. Not only does the *R. leguminosarum* bv. *trifolii* Frk contain these highly conserved amino acid residues, but the Mg^{2+} ion has been shown to be essential for the activity of the enzyme. Recently another subfamily of fructokinases has been proposed for the sequences obtained from *S. mutans* (Sato *et al.*, 1993) and *Z. mobilis* (Zembrzuski *et al.*, 1992). The *R. leguminosarum* bv. *trifolii* Frk shows no homology to these two fructokinase sequences, which share N-terminal sequence homology with the protein sequences derived from *Lactococcus lactis* and *Fusobacterium mortiferum* (Sato *et al.*, 1993). The *R. leguminosarum* bv. *trifolii* Frk does not share this N-terminal sequence homology with these fructokinases, however it does share N-terminal homology with the fructokinases from the *pfkB* subfamily of sugar kinases, including ribokinase and phosphofructokinase from *E. coli* (Wu *et al.*, 1991).

In addition to the ATP-binding motif, the *R. leguminosarum* bv. *trifolii* Frk also contained two motifs in the second domain of the protein conserved in fructokinases but not in the other sugar kinase of this subfamily (Wu *et al.*, 1991). This middle domain is the proposed substrate recognition site of the sugar kinases (Aulkemeyer *et al.*, 1991). Although the *R. leguminosarum* bv. *trifolii* Frk is only 29–30% identical to the other fructokinases in this subfamily, the sugar-binding site (residues 110–220) is conserved. These data further confirm this motif as a substrate-specific region.

Hydropathy analysis indicated that the *R. leguminosarum* bv. *trifolii* Frk is a hydrophilic protein (Fig. 5). This plot differs from plots generated by hydrophobic proteins associated with the bacterial membrane (Kyte & Doolittle, 1982; Hardesty *et al.*, 1991), which contain long stretches of hydrophobic aminoacyl residues. However, this plot agreed with hydropathy profiles from other fructokinase sequences and reports that fructokinases are typically hydrophilic proteins found in the cytoplasmic fractions (Aulkemeyer *et al.*, 1991).

To date only one other gene involved in carbon metabolism has been identified and sequenced from *Rhizobium*. Osteras *et al.* (1991) sequenced the *pckA* gene from *Rhizobium* NGR234 that encodes the phosphoenolpyruvate carboxykinase. This kinase was homologous to the subfamily of sugar kinases (Wu *et al.*, 1991) that contain the class I ATP-binding consensus motif; it shows no homology with the *R. leguminosarum* bv. *trifolii* Frk sequence. A potential cAMP receptor protein (CRP)-binding site regulated by catabolite repression (Osteras *et al.*, 1991) was not found in the *frk* gene. Diauxic growth curve studies show that the *R. leguminosarum* bv. *trifolii* fructokinase is not sensitive to glucose repression (McLaughlin, 1989). In pUR400 and *K. pneumoniae*, no cAMP-CAP binding site was found and the *scrK* genes are not subject to catabolite repression by glucose (Aulkemeyer *et al.*, 1991). Catabolic enzymes in *Rhizobium* are repressed by succinate (Stowers & Elkan, 1985; Mandal & Chakrabarty, 1993). In preliminary studies, fructokinase activity in *R. leguminosarum* bv. *trifolii* BAL was repressed by growth on succinate as in other rhizobia (data not shown).

Fructokinase genes (*scrK*) from most bacterial sources are commonly located in an operon adjacent to other genes involved in fructose or sucrose metabolism (Aulkemeyer *et al.*, 1991; Blatch *et al.*, 1990; Cowan *et al.*, 1991; Sato *et al.*, 1993). The enzymes encoded by these genes are included in the group II fructokinases. In *R. leguminosarum* bv. *trifolii* BAL the gene identified immediately upstream from the *frk* gene, *pyrE*, was not involved in hexose metabolism. Since fructokinase genes usually precede or are adjacent to other catabolic genes, this may suggest the absence of a hexose catabolic operon as with the *frk* gene from *Z. mobilis* (Zembrzuski *et al.*, 1993). The *R. leguminosarum* bv. *trifolii* *frk* gene was not identified as part of an operon and its product is assigned to the group I fructokinases.

The *frk* gene sequence contained a putative promoter region located 56 bp upstream from the start codon (TTGACA-N₁₆-GTTGAT) (Fig. 4). The proposed promoter contains a –35 region identical to the *E. coli* consensus region, and the –10 region has three out of six bases in common with the *E. coli* consensus sequence and the *lacZ* promoter (O'Neill, 1989). Surrounding the –35 region is a palindromic sequence capable of stem-loop formation. Palindromic sequences have been implicated as potential binding sites for repressor molecules, as with the repressor of the *scr* operon, ScrR (Jahreis & Lengeler, 1993). However, this sequence was determined to be a terminator of the *pyrE* gene upstream. As in *V. alginolyticus* (Blatch *et al.*, 1990), the terminator of the *scrA* gene upstream encompasses the proposed –35 region of *scrK* gene almost identically to the rhizobial *frk* gene. Lastly, a terminator sequence was found downstream from the stop codon of the *frk* sequence, and was consistent with known strong rho-independent terminators in *E. coli* (d'Aubenton *et al.*, 1990).

The molecular mass of the purified fructokinase was determined to be 36 500 Da and was in agreement with that predicted from the deduced amino acid sequence of 35 473 Da. This represents approximately 3% error, which is lower than usually seen by this type of comparison (Thompson *et al.*, 1991; Wu *et al.*, 1991). The molecular mass was similar to that of other phosphorylating enzymes including fructokinases from *L. lactis* and *F. mortiferum* (Thompson *et al.*, 1991, 1992), and phosphofructokinases PfkA and PfkB from *E. coli* (Wu *et al.*, 1991) and *Bacillus stearothermophilus* (French & Chang, 1987), which range from 32 to 35 kDa.

The purified fructokinase was resolved to a single band by analytical IEF and the isoelectric point was determined to be 4.65. These results are identical to those obtained by chromatofocusing, where the fructokinase eluted at pH 4.65. However, the isoelectric point of the fructokinase predicted by the deduced amino acid sequence was 5.7 (Table 2). These results could differ for a number of reasons. Secondary, tertiary and quaternary structure of the protein contribute significantly to the overall charge of the native protein and this folding of the Frk peptide cannot be easily predicted by computer analysis as can the molecular mass. This emphasizes that all data predicting protein characteristics must be confirmed biochemically.

Microsequencing of the purified fructokinase showed that the initiator methionine is retained on the mature protein, as for the fructokinases from *Z. mobilis* (Zembrzusi *et al.*, 1992) and *F. mortiferum* (Thompson *et al.*, 1992), indicating no post-translational processing of the enzyme as occurs with the fructokinase from *L. lactis* (Thompson *et al.*, 1991).

The fructokinase from *R. leguminosarum* bv. *trifolii* BAL exhibited many similarities to other fructokinases and kinases. Its pH range for activity was similar to that of other fructokinases, and like other fructokinases it required divalent cations (McLaughlin & Hughes, 1989; Sabater & Delafuente, 1975; Thompson *et al.*, 1991, 1992). Fructokinase activity was increased by the substitution of Co^{2+} for Mg^{2+} . Other investigators have reported that replacing Mg^{2+} by Co^{2+} slightly decreased fructokinase activity (Thompson *et al.*, 1991, 1992). However, the substitution of Co^{2+} for Mg^{2+} has been shown to increase phosphoenolpyruvate carboxykinase activity (Podkovyrov & Zeikus, 1993).

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