Fructose phosphotransferase system of *Xanthomonas campestris* pv. *campestris*: characterization of the *fruB* gene

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In the plant pathogen *Xanthomonas campestris* pv. *campestris*, fructose is transported by a specific phosphotransferase system (PTS). This PTS involves a multiphosphoryl transfer protein (MTP) encoded by the *fruB* gene, which was cloned and sequenced. *fruB* is part of a transcriptional unit together with the *fruK* gene, coding for 1-phosphofructokinase, which is located upstream from the *fruA* gene, coding for the fructose-specific permease (EIIB^{BC^{fru}}). The amino acid sequence of the *X. campestris* MTP deduced from the *fruB* sequence shared 46% identical residues with an MTP identified in *Rhodobacter capsulatus*. The *X. campestris* MTP (837 amino acid residues) consists of three moieties: a fructose-specific enzyme-IIA-like N-terminal moiety (residues 1–148), followed by an HPr-like moiety (161–251) and an enzyme-I-like C-terminal moiety (274–837). The three domains are separated by two flexible hinge regions rich in proline and alanine residues. The construction of a *fruB* mutant confirmed the role of the MTP in fructose transport and phosphorylation in *X. campestris*.

Keywords: *Xanthomonas campestris*, fructose transport, PTS, multiphosphoryl transfer protein, 1-phosphofructokinase

INTRODUCTION

The phytopathogenic Gram-negative bacterium *Xanthomonas campestris* pv. *campestris* has the property of secreting large quantities of an exopolysaccharide, xanthan gum (Jeanes *et al.*, 1961), used in a variety of industrial and food applications (Sutherland & Ellwood, 1979). The study of carbohydrate metabolism in this organism is of great interest, for the understanding of both xanthan gum biosynthesis and the mechanisms of plant colonization by the pathogen. We have previously shown that fructose is transported in this bacterium by a phosphoenolpyruvate-(PEP)-dependent phosphotransferase system (PTS) which, using PEP as the energy source, catalyses the transport and phosphorylation of fructose to yield intracellular fructose 1-phosphate (De

Crécy-Lagard *et al.*, 1991b). Bacterial PTSs are complex, including membrane-bound-sugar specific components called Enzymes II. Enzymes II consist of three domains that can be either fused together or free: one integral membrane carbohydrate recognition component, EIIC, and two cytoplasmic components, EIIB and EIIA (Saier *et al.*, 1988; Saier & Reizer, 1992). The phosphoryl group is transferred from PEP to the EIIs by two general components, Enzyme I (EI) and HPr. The phosphate transfer cascade from PEP to the PTS sugar can be summarized as follows:

The *X. campestris fruA* gene, encoding the fructose-specific permease (EIIB^{BC^{fru}}), and the *fruK* gene, encoding the 1-phosphofructokinase (1-PFK) which contributes to further metabolism of fructose, have been cloned and sequenced (De Crécy-Lagard *et al.*, 1991a, b). The nucleotide sequence of complete *fru* operons has now been determined in three organisms: *Escherichia coli*, *Salmonella typhimurium* (Prior & Kornberg, 1988; Geerse *et al.*, 1989; Orchard & Kornberg, 1990; Reizer *et al.*,

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Abbreviations: DTP, diphosphoryl transfer protein; MTP, multiphosphoryl transfer protein; PEP, phosphoenolpyruvate; 1-PFK, 1-phosphofructokinase; PTS, phosphotransferase system.

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1994b) and *Rhodobacter capsulatus* (Wu *et al.*, 1990, 1991; Wu & Saier, 1990). In enteric bacteria, the *fru* operon consists of the genes *fruBKA*; *fruB* encodes the diphosphoryl transfer protein (DTP) which combines the functions of an EIIA^{Fru} domain and an HPr-like protein. In *R. capsulatus*, the *fru* operon consists of the genes *fruBKA*; *fruB* encodes a protein named MTP (for multiphosphoryl transfer protein) which combines the EIIA^{Fru}, HPr and EI domains. In this paper, we establish the nucleotide sequence of the general PTS genes of *X. campestris* and discuss the organization of the fructose PTS genes in this organism.

METHODS

Bacterial strains, plasmids, media and growth conditions.

The *X. campestris* strains used were the wild-type strain NRRL B1459 (Jeanes *et al.*, 1961) and XC1504 (*fru-2::Omegon-Km*) (De Crécy-Lagard *et al.*, 1991b). *E. coli* XL1-Blue [*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 λ⁻ (lac)*] {F' *proAB lacI^qΔM15 Tn10* (Tet^R)}; Stratagene] was used as host for transformation of different plasmids. pBluescript was purchased from Stratagene, and pUCK4 (Kan^R Amp^R) from Pharmacia. All strains were grown in Luria Broth at 37 °C for plasmid manipulation. *X. campestris* growth media were either complete Luria Broth, containing fructose (0.2%) when required, or synthetic MM1 medium supplemented with 0.2% of the appropriate carbon source (Roy *et al.*, 1988). *X. campestris* was grown at 30 °C. Ampicillin and kanamycin were used when required at the respective final concentrations of 100 µg ml⁻¹ and 50 µg ml⁻¹.

Recombinant DNA techniques. Isolation of plasmid DNA, restriction endonuclease digestion, ligation, agarose electrophoresis and Southern experiments were performed as described in Sambrook *et al.* (1989). *X. campestris* was transformed by electroporation as previously reported (De Crécy-Lagard *et al.*, 1990). *X. campestris* chromosomal DNA was prepared following Silhavy *et al.* (1984). Standard methods for DNA sequencing using dideoxynucleotides (Sanger *et al.*, 1977) and single-stranded DNA from M13 phage (Messing & Vieira, 1982) were employed. DNA sequencing was conducted using the T7 sequencing kit from Pharmacia. Universal primers were first used and subsequently primers were synthesized according to the sequence obtained in previous sequencing reactions. The synthetic oligonucleotides were furnished by the Service de Chimie organique, Institut Pasteur.

Construction of mutant *X. campestris* strains XC1509 and XC1510.

A *Sma*I site situated 842 bp downstream from the putative start codon of the *fruB* gene was used for the inactivation of the gene by insertional mutagenesis. A 1.2 kb *Bam*HI–*Hinc*II fragment containing an internal portion of the *fruB* gene was cloned in the corresponding sites of pBSK⁺, yielding plasmid pDIA4838 (Fig. 1b). The kanamycin resistance gene of pUCK4 is entirely contained in a 1.3 kb *Hinc*II fragment. This fragment was cloned into the unique *Sma*I site of pDIA4838, yielding pDIA4840. *X. campestris* was then electrotransformed by pDIA4840. Kan^R transformants were selected and screened for sensitivity to ampicillin indicating that the vector had been lost and gene replacement had occurred corresponding to double crossovers. Previous experiments had shown that such events occur at high frequency in *X. campestris* (De Crécy-Lagard *et al.*, 1990). Nineteen Kan^R clones were obtained, of which 12 (63%) were Amp^S. Correct gene replacement at *fruB* was verified by Southern hybridization experiments. Chromosomal DNA isolated from strain XC1509,

one of the pDIA4840 Kan^R Amp^S transformants, was digested with *Bam*HI and *Hinc*II and Southern hybridized with a 3 kb probe covering the *fruB*–*fruK* region. The hybridization profile showed that the *Bam*HI–*Hinc*II fragment was 1.3 kb longer in the mutant than in the wild-type strain, indicating the correct insertion of the Kan^R gene (data not shown). In similar attempts to obtain a *X. campestris fruK* mutant, the insertion by a single crossing-over event of a pBSK⁺ derivative led to the construction of strain XC1510. As shown in Fig. 1(c), XC1510 contains an intact copy of the *fruK* gene and a copy in which a Kan^R gene has been inserted upstream from the *fruA* gene.

Computer analyses. Sequence analysis was performed with various programs included in the Genetics Computer Group package (1991).

Uptake and phosphorylation of [¹⁴C]fructose. Uptake of [¹⁴C]fructose by intact *X. campestris* cells and [¹⁴C]fructose phosphorylation in toluene-treated *X. campestris* cells were monitored as described previously (De Crécy-Lagard *et al.*, 1991b), PEP being the phosphate donor at a final concentration of 5 mM. The final concentration of [¹⁴C]fructose was 50 µM and its specific activity was 0.29 mCi mmol⁻¹ (10.7 MBq mmol⁻¹). Uptake and phosphorylation measurements were performed at least three times each. The conditions of the PTS activity test were not optimized to measure PTS specific activities but to measure the presence or absence of a PEP-dependent phosphorylation activity. The results are therefore presented as the percentage of labelled fructose converted to phosphorylated [¹⁴C]fructose in the presence of PEP at 30 °C.

Preparation of cell-free extracts and spectrophotometric enzyme assays. Crude extracts of *X. campestris* cultures were prepared, and 1-PFK monitored, as previously described (De Crécy-Lagard *et al.*, 1991a). Specific activity was defined as µmol substrate utilized min⁻¹ (mg protein)⁻¹.

RESULTS AND DISCUSSION

Identification of a *fruB* gene upstream from the *fruK* gene in *X. campestris*

We previously reported the cloning of a 18 kb *Eco*RI fragment of *X. campestris* chromosomal DNA containing the two adjacent *fruK* and *fruA* genes encoding the 1-PFK and the domains IIB'BC^{Fru} of the fructose PTS respectively (De Crécy-Lagard *et al.*, 1991a, b) (Fig. 1a). This organization was similar to that observed for the *E. coli*, *S. typhimurium* and *R. capsulatus* fructose operons. In these organisms, genes coding for the fructose PTS proteins, DTP or MTP, were adjacent to *fruK* (Prior & Kornberg, 1988; Geerse *et al.*, 1989; Orchard & Kornberg, 1990; Reizer *et al.*, 1994b; Wu *et al.*, 1990, 1991).

The sequence of a 2623 bp DNA fragment located upstream from the *fruK* gene was therefore determined on both strands following the sequencing strategy outlined in Fig. 1(b). The nucleotide sequence is shown in Fig. 2. A possible coding sequence was identified on one strand, following the rules described by Sheperd (1981), from position 102 to position 2615, starting with a putative TTG codon at position 102–104. This start codon is preceded by a potential ribosome-binding site, GGAG, located 7 bp upstream from the TTG initiation codon. The end of this coding sequence overlaps the initiation codon of the *fruK* gene, suggesting translational coupling.

Fig. 1. (a) Restriction map of an 18 kb *EcoRI* fragment containing the *fruB*, *fruK* and *fruA* genes. The upper arrows represent the direction of transcription of the three genes. (b) Map of a 3.1 kb *SmaI*-*PstI* fragment upstream from the *fruK* gene. The 1.2 kb *Bam*HI-*Hinc*II fragment was ligated into the corresponding sites of the Bluescript vector pBSK⁺, yielding pDIA4838. The sequencing strategy of the 2.6 kb fragment upstream from the *fruK* gene is shown underneath. The arrows indicate sequencing direction of the clones used to obtain the nucleotide sequence of this DNA fragment. The location of the gene identified is shown by the upper arrow. (c) Map of the *fruK*-*fruA* region in mutant strain XC1510, showing the insertion of the pBSK⁺ vector (dark shading) between a wild-type *fruK* gene and a *fruK* gene disrupted by a Kan^R gene (light shading). Restriction sites: E, *EcoRI*; S, *SmaI*; B, *Bam*HI; Sm, *SmaI*; H, *Hinc*II; P, *PstI*.

Preceding the initiation codon, potential -10 and -35 regions were identified according to the *E. coli* standard promoter sequences. The two potential promoter sequences are separated by a 16 bp palindrome that could act as a regulator site. Since in *X. campestris* fructose PTS activity is induced by fructose (De Crécy-Lagard *et al.*, 1991b), regulation of the expression of the *fru* genes through this site is probable. It is interesting that the sequence of this palindrome differs from that of the 12 bp palindrome which was shown to serve as the FruR binding site in the *fru* operon of *S. typhimurium* and *E. coli* (Ramseier *et al.*, 1993). FruR is a pleiotropic transcriptional regulatory protein which negatively controls the expression of the *fru* operon in enteric bacteria (for a review see Postma *et al.*, 1993). At the beginning of the sequenced fragment a short ORF of 40 nucleotides was identified following the rules described by Sheperd (1981), suggesting the presence of a coding sequence upstream from the first one.

The deduced amino acid sequence of the first coding sequence is shown in Fig. 2. A striking similarity (46%

identical residues) was found between the sequence of this 837 amino acid protein and the sequence of the *R. capsulatus* MTP protein encoded by the *fruB* gene (Wu *et al.*, 1990). These results indicated that in *X. campestris*, like in *R. capsulatus*, a gene encoding an MTP is located upstream from the *fruK* gene.

Comparison of the *X. campestris* MTP domains to known PTS enzymes

Amino acid sequence comparisons indicate that the *X. campestris* MTP consists of three moieties (Fig. 3). The N-terminal moiety (residues 1-148) is homologous to the EIIA^{Fru} domains of the *R. capsulatus* MTP (Wu *et al.*, 1990) and of the DTP proteins of *E. coli* (Reizer *et al.*, 1994b) and *S. typhimurium* (Geerse *et al.*, 1989). The central moiety (residues 161-251) is homologous to the HPr-like domains of the *R. capsulatus* MTP and of the DTP proteins of enteric bacteria. The C-terminal domain (residues 274-837) is homologous to the Enzyme I domain of the *R. capsulatus* MTP and to the Enzyme I of *E. coli* and *S. typhimurium* (Saffen *et al.*, 1987; De Reuse & Danchin, 1988; LiCalsi *et al.*, 1991).

The PTS Enzymes II have been classified into four major groups (Lengeler *et al.*, 1990; Postma *et al.*, 1993). As shown in Fig. 4, the EIIA^{Fru} domain of the *X. campestris* MTP clearly belongs to the EIIA^{Mtl} subclass. The homology found between the N-terminal sequences of the *E. coli* EIIA^{Mtl} and the *X. campestris* MTP confirms the choice of the *fruB* starting codon. Nine members of this EIIA subclass have now been sequenced, and sequence comparisons can therefore provide evidence concerning potentially important structural and catalytic residues. We did not include the sequences of the recently discovered EIIF^{FruA} and EIIF^{Ani} domains (Reizer *et al.*, 1994a; Saier & Reizer, 1994) as it is not known if these proteins have a role in sugar transport in *E. coli*. The main conserved motif of the EIIA^{Mtl}, A-PH, is found around His⁶⁹ (referring to residue numbers of the protein alignment presented in Fig. 4), which has been shown to be phosphorylated in the *E. coli* enzyme (Van Weeghel *et al.*, 1991). The role of the other conserved residues (Fig. 4) may become clear once the three-dimensional structure of the *E. coli* EIIA^{Mtl} domain is resolved (Kroon *et al.*, 1993). EIIF^{Fru} domains from four organisms have now been sequenced. Only four residues are conserved in all EIIF^{Fru} domains and absent in the EIIF^{Mtl} domains (Gln²⁹, Arg⁷⁵, Gly⁹⁹ and Ser¹¹⁴) whereas two residues are conserved in all EIIF^{Mtl} domains and are absent in the EIIF^{Fru} domains (Ala⁴⁷ and Asp/Glu⁹⁹). These residues could be involved in specific interactions with the EIIBC^{Fru} and EIIBC^{Mtl} domains respectively.

A number of HPr proteins have now been sequenced and it is interesting to note that the three-dimensional structural data and mutagenesis studies have confirmed the role of the residues that are highly conserved amongst these sequences. Four possible catalytic residues are conserved in all the compared sequences including the MTP sequence of *X. campestris*: His¹⁷⁵, Arg¹⁷⁷, Ser²⁰⁶ and

T R S T T G F L C T A H *
 AACCCGAAGCACTACAGGGTTTTTGTGCACCGCAATTGACCTGGACCTGGGAAGCTTC 60
 -35
 M S S P S I A
 CCACTATACTCCAGCGGTGCCACCCCGGAGCCCGCTTGTCTCTCCGTGATTG 120
 -10 50
 P V T P D L V R L R A T A R D K D D A I
 CCCCCTGACCCCCGACCTGGTGCCTGCGGCCACTGCGCGGACAAGGACGACGCCA 180
 A Q A A Q L L V A A G C V A P G Y D A S
 TTGCCAGGCGCCAGCTGCTGGTGGCAGCGGATGCTGGCGCGGGCTACGACGCCA 240
 M R R R E G L A N T F L G H G L A I P H
 GCATGGCGGCGCGCAAGGCTGGGGAATACTTCTGGGCGAGCGCTGGCGATTCCGC 300
 G V G E D R H L V R R D G I A V L Q L P
 ACGGCTGGGCGAAGACCGGACCTGGTGCCTGCGCGGACGCGATCGCGGTGCTGACGTGC 360
 E G V E W N P G Q T T R L V V G I A A Q
 CCGAGGCGGTGGAATGGAACCCCGGCGAGACACCCGCTGGTGGTGGCATCGCCGCC 420
 S D T H I T L V R R L T R L I Q D P A Q
 AGTCCGACACCATCATCCCTGTCGCGCGGTGACCGGCTGATCAGGATCGGGCC 480
 L E A L F T T D D P A V I V A A L T G D
 AGCTGGAAGCGCTGTTACACACGACGACCGCGCTGATCGCGCGCGTGCACGGCG 540
 R A P D T S A A P A T D L A E R F E W T
 ACCGCGCACCCGACACGAGCGCGCCCGCGGCGACGCTGGCGAAGCGTTCGAATGGA 600
 I A Y P S G L H A R P A T R W A E T A R
 CCATCTGCTACCCAGCGGCTGACCGCGCGCGGCGCTGCTGGCGGAGACCGCGC 660
 G F S A R A Q V R A G D Q A A D A K S L
 GCGGCTTTTGGCGCGCGCGGCTGCGCGCGGTGACGAGCGCGGATGCCAAGAGCC 720
 V G L L Q L G L R A G D S I T V S A K G
 TGGTGGCGCTGCTCAACTGGGCTGCGCGCGGCGACGACATTACGGTCTCAGCGAAG 780
 S D A P A L L K R L R A V M D S L T A Q
 GCAGCGATGCCCGCGCTGCTCAAGCGCTGCGCGCGGTGATGGACAGCTGACCGCGC 840
 E K A D A E R A A Q R R A A P V I G W T
 AGGAAAGCGCTGATGCCGAGCTGCGCGCGCAAGCGCGCGCGGCGCTGATCGGGTGA 900
 P P Q A Q P A I V G I G A S P G V A I G
 CCCCCCGGAGCGGCGAGCGCGCATGCTGGCATCGCGCGCGCGGCGCTGCAATCG 960
 I V H R L R A A Q T E V A D Q P I G L G
 GCATCGTGACCGCTGCTGCGCGCGGCGAGCGAGGTGGCGACGCGATCGCGCTGG 1020
 D G G V L L H D A L T R T R Q Q L A A I
 GCGAGCGGCGGTGCTGCTGATGACGCGCTCACCCGACCGCGGCGAGCTGCGCGCA 1080
 Q D D T Q R R L G A S D A A I F K A Q A
 TCCAGGACGACCGCGCGCGCTGCGCGCGCTGCGAGCGCGGATCTCAAGCGCCAGG 1140
 C E G L L N D T L I T R T C Q L M V E G H
 CCGAGCTGCTCAACGACACCGGCTGATCAGCGCGCTGCGAGCTGATGGTGAAGGCC 1200
 G V A W S W H Q A V E Q I A S G L A A L
 ACGGTGTGGCGTGTGCTGGCAGCGCGGTGGAACAGATCGCTCCGGCTCGCGGCGC 1260
 G N P V L A G R A A D L R D V G R R V L
 TGGGCAACCGGTGCTGGCGCGCGCGCGCTGACTGCGGAGCTGGCGCGCGCTGTC 1320
 A Q L D A A A A G A G G L T D L P E Q P C
 TGGCCCAACTGACGCGCGGCGCGCGCGCTGCGGAGCTGCGCGAGCGGCGCT 1380
 I L L A G D L S P S D T A N L D T D C V
 GCATCTGCTGCGCGGACCTGTCGCGCTGCGGACCGCGCAACCTGACACCGATTGCG 1440
 L G L A T A Q G G P T S H T A I L S R T
 TGCTTGGCTGGCCACCGCGCGGCTGGGCGGCTGCGACACCGGATCTGTCGCGCA 1500
 L G L P A L V A A G G Q L L D I E D G V
 CGCTCGGCTGCGCGCGCTGCTGCGGCTGGCGGCGAGTGTGATATCGAAGCGGG 1560
 T A I I D G S S G R L Y I N P S E Q D L
 TCACCGCATCATCGCGGACGCGCGGCGCTCTACATCAACCGTCAGAACGAGTAT 1620
 D A A R T H I A E Q Q A I R E R E A A Q
 TGGAGCGCGCGCGACGATATCGCGGAGCGAGGCGATCGCGGAGCGCAAGCGCGC 1680
 R A L P A E T T D G H H I D I G A N V N
 AGCGCGCGCTGCGCGCGGAAACACCGGCGGCGGCGGCGGCGGCGGCGGCGGCG 1740
 L P E Q V A M A L T Q G A N V G L M R T
 ACCTGCGCGAGCGGTGGCGATGGCTTGACCGAGGCGCGCAACGTGGCGCTGATCGCG 1800
 E F L F L E R G S T P T E D E Q Y H T Y
 CAGAGTCTGCTTCTGAGGCGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGG 1860

L A M A Q A L N G R P L I V R A L D I G
 ACCTGGCAATGGCACAGGCGCTGAATGGCCGCCGCTGATCGTGGTGGCGTGGACATCG 1920
 G D K Q V A H L E S P H E E N P F L G V
 GCGCGCAAGCAGGTGGCGACCTGGAGTGGCGCGCAAGAAACCCGCTCTCTGGCGG 1980
 R G A R L S S R R P D L L E P Q S R A L
 TGCTGGCGCGCGCTGCTGCTGCGCGCGCGCGGACCTGCTGAGCCACAGTCCGCGGCGC 2040
 Y R A A K D G A R L S I M F P M I T S V
 TGTATCGCGCGGCAAGGACGCGGCGGCTGCTGATCATGTTCCCGATGATCCTCGG 2100
 P E L I S L R E I C A A I R A E L H A P
 TGCCGAGCTGATCAGCTGCGCGAGATCTGCGCAGCCATCGCGCGGAACTGCACGCAC 2160
 E L P I G I M I E V P A A A A Q A D V L
 CGGAGCTGCCGATCGCATCATGATGAAGTGGCGCGCGCGCGCGCGCGCGCGCGGATGTC 2220
 A R H A D F F S I G T N D L T Q Y V L A
 TGGCGCGCATCGCGGATTTCTTCTGATCGCGCAACGACCTCACCAATACGTGCTGG 2280
 I D R Q N P E L A A E A S S L H P A V L
 CAATGACCGCGAAGCCGGAATGGCGCGGAGCGAGCGCTGCATCGCGCGGTGC 2340
 R M I R S T I D G A R K H D R W V G V G T
 TGCGCATGATCGCGACGATGAGCGCGCGCGCAAGCAGCGGCTGGTGGTGGTGGT 2400
 G G L A G D P F G A S L L A G L G V Q E
 GCGCGGTGCTGGCGCGGCGCGGCTGCGCGCGGCGCGGCTGCGCGCGGTGGCGTGCAGG 2460
 L S M T P N D I P A V K A R L R G R A L
 AATGTCGATGACGCGCAACGACATTCGCGCGGTCAAGGCGCGGCTGCTGGCGCGGCGC 2520
 S A L Q Q L A E Q A L H C E T A E Q V R
 TGAGCGCTTGCGACGCTGGCGGAGCGGCGCTGCTGCGGAGCGCGGAGCGAGCGGTC 2580
 A L E A Q R E G Q A *
 GTGCGCTTGAAGCAGCGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCG 2623
 M S L Q

Fig. 2. Nucleotide sequence of the 2623 bp fragment located upstream of the *fruK* gene. The putative sequence corresponding to the -35 and -10 regions of a promoter and the SD region are underlined. The 16 bp palindromic sequence is indicated by arrows above the sequence. The linker regions in the coding sequence are underlined.

Asp²²⁹ (numbers refer to the position in the *X. campestris* MTP sequence) (data not shown). His¹⁷⁵, the phosphorylation site (Beyreuther *et al.*, 1977), and Arg¹⁷⁷ are part of the active site (Herzberg *et al.*, 1992). Ser²⁰⁶ is phosphorylated in an ATP-dependent manner only in Gram-positive bacteria (Reizer *et al.*, 1989). Mutating Asp²²⁹ significantly reduces PTS activity (Sharma *et al.*, 1991) and as this residue is located on an outer flexible loop (Herzberg *et al.*, 1992), it could be involved in interactions with the EIIA domains. The HPr domain of DTP can substitute for HPr in *E. coli* and *S. typhimurium* (Geerse *et al.*, 1986; Waygood, 1980). No sequence specificity could be detected in the fructose HPr-like proteins since no residues conserved only in these proteins and not in the general HPr proteins could be identified. However, whereas in general HPr proteins are acidic in nature (pI 4.0–5.6) it is noteworthy that the *X. campestris* HPr domain has a very high isoelectric point (pI 10.6) like the *R. capsulatus* and *E. coli* fructose-inducible HPr domains (pI 9.9–10.2) and the *Mycoplasma capricolum* HPr (pI 9.18) (Zhu *et al.*, 1993).

Eight Enzyme I proteins have been sequenced and a remarkable degree of conservation between enzymes of very diverse origins can be noticed. Local similarities of Enzyme I proteins with the pyruvate:orthophosphate dikinase (PPDK) of maize (Pocalyko *et al.*, 1990) and the PEP synthase (PPS) of *E. coli* (Niersbach *et al.*, 1992)

[illegible]

Several authors have suggested that fusion and dissociation of protein domains must have occurred frequently during the evolution of PTS proteins (Wu *et al.*, 1990; Postma *et al.*, 1993). The PTS protein domains are often separated by general linkers, Q linkers and Ala/Pro-rich hinges (Erni *et al.*, 1987; Wu *et al.*, 1990; Postma *et al.*, 1993). We observed that the three *X. campestris* MTP moieties are separated by two linkers (Fig. 2). The nucleotide sequences of these linkers are GC-rich (80 mol%) compared to the surrounding sequenced region (70 mol%), and rich in Ala, Pro and charged residues. The two linkers of the *X. campestris* MTP are not homologous and differ from the *R. capsulatus* MTP linkers. We propose that the absence of structural constraint in these regions explains the incorporation of a higher percentage of G and C residues in first and second codon positions in organisms of high GC content. The consequence is a high number of Ala and Pro residues which, by their flexibility, happen to be good linker region residues.

	51									100
X. c. Fru	REGLANTF..	LGHG	LP	PHG	VGED	HLVR	DGI	AVL	QLPE	GVENN..PG
R. c. Fru	REAVANTF..	LGNIA	PHG		LPD	LDI	LD	TAIA	VQLPA	GVENR..PG
S. t. Fru	REQQTSTF..	LGNIA	PHG		TTD	TD	QVLK	TGV	QVFQFP	GVITG..EG
E. c. Fru	REQQTSTF..	LGNIA	PHG		TTD	TD	QVLK	TGV	QVFQFP	GVITG..DG
E. c. Mtl	REKLTPSY..	LGESIA	PHG		TEAAK	DRVLK	TG	GVF	QCYPE	GVNFGEEDY
E. f. Mtl	RDQLLSAH..	MGNFIA	PHG		TVAK	KLVRK	SG	VCVQ	VP	GVNFGEDE
S. m. Mtl	RDQLLSVY..	MGNFIA	PHG		TDDAK	QDVLK	SG	ITV	VQVPD	GVNFGEDEP
S. c. Mtl	REAVSTF..	MGNGLA	PHG		TDEAK	SALV	TG	SLT	LQIPE	GVING..DD
S. c. Cmt	STINGPYII	LAPGV	PHG		RPE..	CGALK	QSM	TLST	LTLEO	GVNFPNGED

	101				150
X. c. Fru	QTARLVVGI.	AASDDELITL	VRRLTRIQQ	PAQLEALFTT	DDPAVIAAALTGDR
R. c. Fru	DTARLVVAI.	AASDDELQV	LSNLTDLVG	EAEAEERLATT	LDAAVIAVRLT...
S. t. Fru	QVAVYVIGI.	AASDDELGL	LRQLTHVLSD	DSVAEQKSA	TTAEELRALLMGEK
E. c. Fru	QVAVYVIGI.	AASDDELGL	LRQLTHVLSD	DSVAEQKSA	TTAEELRALLMGEK
E. c. Mtl	DIARLVIGI.	AARNNEITQV	ITSLTNALDD	ESVIERLAHT	TSKVEELLELAGRK
E. f. Mtl	KIATLVFVGI.	AGVGEELQV	QQVQALYCSQ	MDNVVLQADA	LSKEEITENLAIA.
S. m. Mtl	QVATVLVFGI.	AGIGDEHLQI	IQNISIFCAD	VDNVVKLADA	QTEDEVVKLLSQVN
S. c. Mtl	.VAKVVVGI.	AGKGDGHLIL	LSKIAITFSE	EENVDRIVNT	KSPKEIKAVFEED
E. c. Cmt	.IKLLIGLS	AADADSLTA	QIALSEELCE	EELLEQLLTA	SEKQLADITISRG

Uptake of [^{14}C]fructose was monitored in the wild-type strain and in the *fruB* mutant strain. As shown in Fig. 5, the rate of [^{14}C]fructose uptake was 20 times slower in the mutant strain XC1509. PEP-dependent phosphorylation of [^{14}C]fructose in toluenized mutant cells was four times

Fig. 5. Uptake of [^{14}C]fructose by intact *X. campestris* cells. $\circ$, Wild-type; $\square$, XC1510; $\bullet$, XC1509. Uptake of [^{14}C]fructose was monitored as described in Methods.

Table 1. [^{14}C]Fructose phosphorylation in toluene-treated *X. campestris* cells

PEP-dependent phosphorylation was monitored as described in Methods, with assay times of 5 or 15 min at 30 °C. The PEP concentration was 5 mM.

	Phosphorylated [^{14}C]fructose (%)			
	5 min		15 min	
	– PEP	+ PEP	– PEP	+ PEP
Wild-type	3.7	32.4	6.7	44
XC1504 (<i>fruA</i>)	1.5	8	1.8	9
XC1509 (<i>fruB</i>)	1.8	11.5	3.9	10
XC1510 (<i>FruK</i> [–])	3	23	3.4	29.4

less efficient than that in wild-type cells (Table 1). Since *fruK* is immediately adjacent to *fruB*, the insertion of a Kan^R gene in *fruB* was expected to exert a polar effect on *fruK*. 1-PFK activity was therefore measured in strain XC1509. No 1-PFK activity could be detected in XC1509 cells when an activity of 0.234 $\mu\text{mol min}^{-1} (\text{mg protein})^{-1}$ was found in the wild-type cells. The Kan^R gene insertion in the *fruB* gene was thus polar on the downstream *fruK* gene. It is highly probable that the two genes are in the same transcriptional unit and subject to translational coupling, as seems to be the case for the equivalent genes in *E. coli* and *R. capsulatus* (Jones-Mortimer & Kornberg, 1974; Wu *et al.*, 1991). However, it seems unlikely that the *pts* phenotype of the *fruB* insertional mutant is due to a polar effect on the downstream *fruK* gene.

We also do not favour the hypothesis that the phenotype of the *fruB* insertional mutant is due to a polar effect on the distal *fruA* gene, for the following reasons. We have shown previously that an *X. campestris fruA* null mutant could be complemented *in trans* by a DNA fragment containing the 3'-end of the *fruK* gene, the 120 bp intergenic region and the *fruA* gene cloned in a broad-

host-range plasmid (De Crécy-Lagard *et al.*, 1991b). This strongly suggested that the *fruA* gene was transcribed from its own promoter located in the intergenic region separating *fruK* from *fruA* and not from the *fruB* promoter region. In addition, unlike the situation in the other sequenced *fru* operons, where the *fruK* and *fruA* genes are only separated by approximately 20 bp (Prior & Kornberg, 1988; Wu *et al.*, 1991), the *fruK* and *fruA* genes of *X. campestris* are separated by 120 bp. Moreover a potential terminator sequence and consensus promoter sequences were identified between these two genes (De Crécy-Lagard *et al.*, 1991a), again suggesting that *fruA* is not part of the *fruB–fruK* transcriptional unit. This hypothesis was confirmed by the phenotype of strain XC1510 (Fig. 1c). This strain, described in Methods, has an unexpected *FruK*[–] phenotype which could be due to a transdominant negative phenotype of the truncated copy of the *fruK* gene as 1-PFK enzymes are most likely multimeric (Von Hugo & Gottschalk, 1974). XC1510 does not grow on fructose, fructose uptake is impaired (Fig. 5) and no 1-PFK activity can be detected in cell extracts. However, PTS activity in toluenized cells of XC1510 is comparable to that in cells of the wild-type (Table 1), showing that an insertion in *fruK* has no polar effect on the *fruA* gene. These arguments make it highly probable that the *Pts*[–] phenotype of the *fruB::Kan*^R mutant is due to the inactivation of the *fruB* gene itself and confirms the role of the MTP in the *X. campestris* fructose PTS.

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