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'BCFrU). 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:

\[
\text{PEP} \rightarrow \text{EI} \sim \text{P} \rightarrow \text{HPr} \sim \text{P} \rightarrow \text{EIIA} \sim \text{P} \rightarrow \text{EIIB} \sim \text{P} \rightarrow \text{FrU} \sim \text{P} \rightarrow \text{sugar} \sim \text{P}
\]

The *X. campestris fruA* gene, encoding the fructose-specific permease (EIIB'BCFrU), 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.*, 1994).
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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 EIITA 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 EIITA, 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 (Jeanel et al., 1961) and XC1504 (fru-2::Omeqon-Km) (De Cre`cy-Lagard et al., 1991b). E. coli XL1-Blue [recA1 endAl 4, lac, F'(proAB lacIqZAM15 Tn10 (TetR)]; Stratagene was used as host for transformation. pBluescript was purchased from Stratagene and pUCK4 (KanR AmpR) 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 Cre`cy-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 Smal site situated 842 bp downstream from the putative start codon of the fruB gene was used for the inactivation of the gene by insertion mutagenesis. A 1.2 kb BamHI-HincII 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 HincII fragment. This fragment was cloned into the unique Smal site of pDIA4838, yielding pDIA4840. X. campestris was then electrotransformed by pDIA4840. KanR transformans 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 occurs at high frequency in X. campestris (De Cre`cy-Lagard et al., 1990). Nineteen KanR clones were obtained, of which 12 (63%) were AmpR. Correct gene replacement at fruB was verified by Southern hybridization experiments. Chromosomal DNA isolated from strain XC1509, one of the pDIA4840 KanR AmpR transformants, was digested with BamHI and HincII and Southern hybridized with a 3 kb probe covering the fruB-fruK region. The hybridization profile showed that the BamHI-HincII fragment was 1.3 kb longer in the mutant than in the wild-type strain, indicating the correct insertion of the KanR 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 KanR 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 Cre`cy-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 Cre`cy-Lagard et al., 1991a). Specific activity was defined as µmol substrate utilized min-1 (mg protein)-1.

RESULTS AND DISCUSSION

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

We previously reported the cloning of a 18 kb EcoRI fragment of X. campestris chromosomal DNA containing the two adjacent fruK and fruA genes encoding the 1-PFK and the domains IIIB'C' of the fructose PTS respectively (De Cre`cy-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; Geere 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.
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$^{Mu}$ subclass. The homology found between the N-terminal sequences of the E. coli EIIA$^{Mu}$ 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 EII$^{PyA}$ and EIIA$^{Am}$ 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$^{Mu}$, A-PH, is found around His$^{69}$ (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$^{Mu}$ domain is resolved (Kroon et al., 1993). EIIA$^{FrU}$ domains from four organisms have now been sequenced. Only four residues are conserved in all EIIA$^{FrU}$ domains and absent in the EIIA$^{Mu}$ domains (Gln$^{69}$, Arg$^{72}$, Gly$^{74}$ and Ser$^{75}$) whereas two residues are conserved in all EIIA$^{Mu}$ domains and are absent in the EIIA$^{FrU}$ domains (Ala$^{47}$ and Asp/Glu$^{88}$). These residues could be involved in specific interactions with the EIIBC$^{FrU}$ and EIIBC$^{Mu}$ 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: His$^{171}$, Arg$^{177}$, Ser$^{196}$ and
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 palindrome is indicated by arrows above the sequence. The linker regions in the coding sequence are underlined.

Asp^229 (numbers refer to the position in the X. campestris MTP sequence) (data not shown). His^176, the phosphorylation site (Beyreuther et al., 1977), and Asp^77 are part of the active site (Herzberg et al., 1992). Ser^206 is phosphorylated in an ATP-dependent manner only in Gram-positive bacteria (Reizer 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 has a very high isoelectric point (PI 10.6) like Enzyme I proteins with the pyruvate orthophosphate dikinase (PPDK) of maize (Pocalsky et al., 1990) and the PEP synthase (PPS) of E. coli (Niersbach et al., 1992).
Fig. 3. Comparison of the X. campestris MTP with the R. capsulatus MTP and with the DTP and EI proteins of E. coli and S. typhimurium. The percentage of identical residues between the X. campestris protein and the corresponding domains from the three other bacteria are indicated. Abbreviations: EI, EI, EI' intermediate; M, central domain of the DTP of enteric bacteria (Wu et al., 1990).

Fig. 4. Alignments of amino acid sequences of four sequenced Elia' domains and five sequenced Elia' domains of the bacterial PTS. Residues conserved in the nine proteins are boxed. Residues conserved only in the Elia' domains and five sequenced Elia' domains of the three other bacteria are indicated. Abbreviations: Elia', Elia', Elia'; M, central domain of the DTP of enteric bacteria (Wu et al., 1990).
Methods, with assay times of 5 or 15 min at 30 °C. The PEP-dependent phosphorylation was monitored as described in Methods. 

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

<table>
<thead>
<tr>
<th>Phosphorylated [14C]fructose (%)</th>
<th>5 min</th>
<th>15 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−PEP</td>
<td>+PEP</td>
</tr>
<tr>
<td>Wild-type</td>
<td>3-7</td>
<td>32-4</td>
</tr>
<tr>
<td>XC1504 (fruA)</td>
<td>1-5</td>
<td>8</td>
</tr>
<tr>
<td>XC1509 (fruB)</td>
<td>1-8</td>
<td>11-5</td>
</tr>
<tr>
<td>XC1510 (fruK⁻)</td>
<td>3</td>
<td>23</td>
</tr>
</tbody>
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less efficient than that in wild-type cells (Table 1). Since fruK is immediately adjacent to fruB, the insertion of a KanR 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 μmol min⁻¹ (mg protein)⁻¹ was found in the wild-type cells. The KanR 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 tolunized cells of X1510 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::KanR 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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