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Nucleotide sequences of the genes encoding fructosebisphosphatase and phosphoribulokinase from *Xanthobacter flavus* H4-14

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The genes encoding fructosebisphosphatase and phosphoribulokinase present on a 2·5 kb *Sal*I fragment from *Xanthobacter flavus* H4-14 were sequenced. Two large open reading frames (ORFs) were identified, preceded by plausible ribosome-binding sites. The ORFs were transcribed in the same direction and were separated by 39 base pairs. They encoded proteins of 364 and 291 amino acids, with molecular masses of 38739 and 33409 Da, respectively. The ORFs were identified as the genes encoding FBPase and PRK, respectively, on the basis of similarity with FBPase and PRK sequences from other sources.

Introduction

Many organisms, including higher plants, grow autotrophically using the Calvin cycle for CO_2 fixation. Three enzymic steps are considered to be unique to this pathway. Phosphoribulokinase (PRK) catalyses the phosphorylation of ribulose 5-phosphate (RuMP) by ATP, yielding ribulose 1,5-bisphosphate (RuBP) and ADP. The RuBP formed is carboxylated by ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisC/O), leading to the formation of two molecules of 3-phosphoglycerate. RuMP is regenerated by a series of enzymes which, with the exception of sedoheptulosebisphosphatase, also function in other pathways, e.g. during gluconeogenesis. The latter enzyme is bifunctional, also displaying fructosebisphosphatase (FBPase) activity (Tabita, 1988).

The genes encoding FBPase (*cfxF*) and PRK (*cfxP*) have been cloned from the autotrophic bacteria *Alcaligenes eutrophus*, *Rhodobacter sphaeroides* and *Xanthobacter flavus* H4-14. In these organisms the *cfxF* and *cfxP* genes are cotranscribed in the order *cfxF–cfxP* (Gibson & Tabita, 1987, 1988; Hallenbeck & Kaplan, 1987; Klintworth *et al.*, 1988; Meijer *et al.*, 1990*b*). The nucleotide sequences of a number of FBPase-encoding genes have been reported. However, the only Calvin cycle *cfxF* gene that has been sequenced is the one from wheat chloroplast (Raines *et al.*, 1988), and a complete nucleotide sequence of *cfxF* from a prokaryote is not available. Only the sequences of the *cfxP* genes from spinach and *A. eutrophus* have been reported thus far (Kossmann *et al.*, 1990; Roesler & Ogren, 1988).

It thus appears that both PRK and FBPase have received only minor attention despite their important role in the Calvin cycle. We therefore set out to characterize the cloned PRK and FBPase structural genes from *Xanthobacter flavus* H4-14, a bacterium capable of autotrophic growth on methanol and molecular hydrogen (Lidstrom-O'Connor *et al.*, 1983; Meijer *et al.*, 1990*a*). In this paper we report the complete nucleotide sequences of these genes.

Methods

Bacterial strains and plasmids. Escherichia coli JM101 (Yanisch-Perron *et al.*, 1985) was used as a host for pBLUESCRIBE (Vector Cloning Systems), M13mp18 and M13mp19 (Yanisch-Perron *et al.*, 1985) and their derivatives. pCD102 is a pVK100 cosmid, containing a 24 kb chromosomal DNA fragment from *X. flavus* H4-14, encoding FBPase and PRK (Lehmicke & Lidstrom, 1985, Meijer *et al.*, 1990*b*).

Media and growth conditions. E. coli strains were grown on LB medium at 37 °C (Maniatis *et al.*, 1982). Agar was added to 1·5% (w/v) to solidify the medium. When necessary the following supplements

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Abbreviations: FBPase, fructosebisphosphatase, F-2,6-P, fructose 2,6-bisphosphate; LB, Luria broth; ORF, open reading frame; PRK, phosphoribulokinase; RuBP, ribulose bisphosphate; RuBisC/O, ribulose-1,5-bisphosphate carboxylase/oxygenase; RuMP, ribulose monophosphate.

The nucleotide sequence data reported in this paper have been submitted to EMBL and will appear in the EMBL/GenBank/DDBJ Nucleotide Sequence Databases under the accession number X17252.

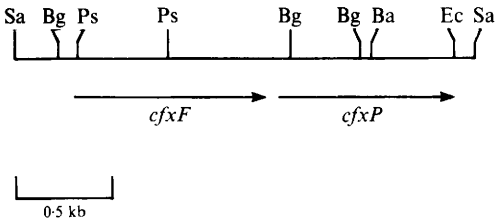

Fig. 1. Restriction map of the 2·5 kb DNA *Sal*I fragment from *X. flavus* H4-14 and the positions of *cfxF* and *cfxP*. Arrows indicate the direction of transcription. Restriction sites: Ba, *Bam*HI; Bg, *Bgl*II; Ec, *Eco*RI; Ps, *Pst*I; Sa, *Sal*I.

were added (in μg ml^{-1}): ampicillin, 50; 5-bromo-4-chloro-3-indolyl β-D-galactoside, 20; tetracycline, 12·5. Isopropyl β-D-thiogalactoside was used at a concentration of 0·1 mM.

DNA manipulations. Plasmid DNA was isolated from *E. coli* by using either the Triton X-100 lysis method of Rodriguez & Tait (1983), in which the column chromatography step was omitted for preparative isolations, or the alkaline lysis method of Birnboim & Doly (1979) for isolations on a smaller scale. Restriction enzymes and T4 DNA ligase were used according to the manufacturer's instructions. Analysis of restriction digests by gel electrophoresis, recovery of DNA fragments from low-melting-point agarose and transformation of *E. coli* were carried out as described by Maniatis *et al.* (1982). Sequence determination was done as described by Sanger *et al.* (1977), except that 7-deaza-dGTP was used instead of dGTP, and Sequenase (United States Biochemical Corporation) was used instead of the Klenow fragment of DNA polymerase. When necessary, custom primers (Eurosequence, Groningen, the Netherlands) were used instead of the universal primer.

Results and Discussion

Nucleotide sequences of cfxF and cfxP

The localization of the *cfxF* and *cfxP* genes in *X. flavus* H4-14, and their cotranscription in the order *cfxFP*, has been established in previous work (Meijer *et al.*, 1990*b*; Fig. 1). A 2·5 kb *Sal*I fragment from pCD102 encoding FBPase and PRK was subcloned in pBLUESCRIBE, yielding plasmid pWL5. This plasmid was digested with the appropriate restriction enzymes, and the restriction fragments were ligated into M13mp18 and M13mp19. The total nucleotide sequence was derived from both DNA strands and fully overlapped. The C + G content of the *Sal*I fragment was 66·4 mol%, which is close to the C + G content of the chromosomal DNA (68 mol%; Meijer *et al.*, 1990*a*).

Two open reading frames (ORFA, ORFB) were identified in the nucleotide sequence, each preceded by plausible ribosome-binding sites. Both were transcribed in the same direction as *cfxF* and *cfxP* (Meijer *et al.*, 1990*b*), and were separated by only 39 bp. ORFA had a length of 1092 bp, and would encode a protein of 38739 Da. ORFB had a length of 873 bp, encoding a protein of 33409 Da. Due to the high C + G content, the codon usage in both ORFA and ORFB displayed a strong bias towards the use of codons having a C or G in the wobble position.

The deduced amino acid sequences derived from both ORFs were compared to 10856 entries in the SWPROT-database (Protein Database, Release 11, June 1989, University of Geneva, Switzerland), using the program FASTP (k-tuple = 2; Lipman & Pearson, 1985). On the basis of this comparison, ORFA and ORFB were identified as *cfxF* and *cfxP*, respectively. The deduced molecular masses of the *cfxF* and *cfxP* gene products corresponded well with the sizes of the *cfxF* gene product, expressed in *E. coli* (40 kDa; W. G. Meijer & L. Dijkhuizen, unpublished observations), and PRK from *X. flavus* H4-14 (33 kDa; Lehmicke & Lidstrom, 1985). The nucleotide sequences and deduced amino acid sequences of *cfxF* and *cfxP* are shown in Fig. 2.

Comparison of FBPase primary structures

A comparison of the primary structures of FBPase from various sources is shown in Fig. 3. The FBPase amino acid sequence from *X. flavus* H4-14 displays 32 to 38% similarity with FBPases from heterotrophic sources, which show 41 to 63% similarity amongst each other (Table 1). Of the two autotrophic FBPase proteins, from wheat chloroplasts and *A. eutrophus*, only the latter shows a high similarity (52%) with the *X. flavus* H4-14 protein, although all three autotrophic FBPase proteins have the same physiological role. In contrast, the RuBisC/O proteins from plants are very similar to those from *A. eutrophus* and *X. flavus* H4-14 (Andersen & Caton, 1987; Meijer *et al.*, 1990*b*). An explanation for this difference in sequence conservation could be that the structural constraints in RuBisC/O, a hexadecamer consisting of two types of subunits, are higher than those in the FBPase protein, allowing less sequence variation. Alternatively, the FBPase proteins from eukaryotic and prokaryotic species may have evolved from different ancestors.

Recently the three-dimensional structure of pig kidney FBPase and its complexes with AMP and fructose 2,6-bisphosphate (F-2,6-P) have become available (Ke *et al.*, 1989). AMP and F-2,6-P inhibit FBPase activity in a number of organisms. AMP inhibition is allosteric, whereas F-2,6-P probably binds to the active site (Ke *et al.*, 1989; Liu *et al.*, 1989). The F-2,6-P-binding site consists of the following residues (numbering for pig kidney FBPase): Asn-212, Tyr-244, Gly-246, Ser-247, Met-248, Tyr-264 and Lys-274. These residues are either identical, or represented by a conservative replacement in the *X. flavus* H4-14 protein. Residues making up a negatively charged pocket, forming one wall of the

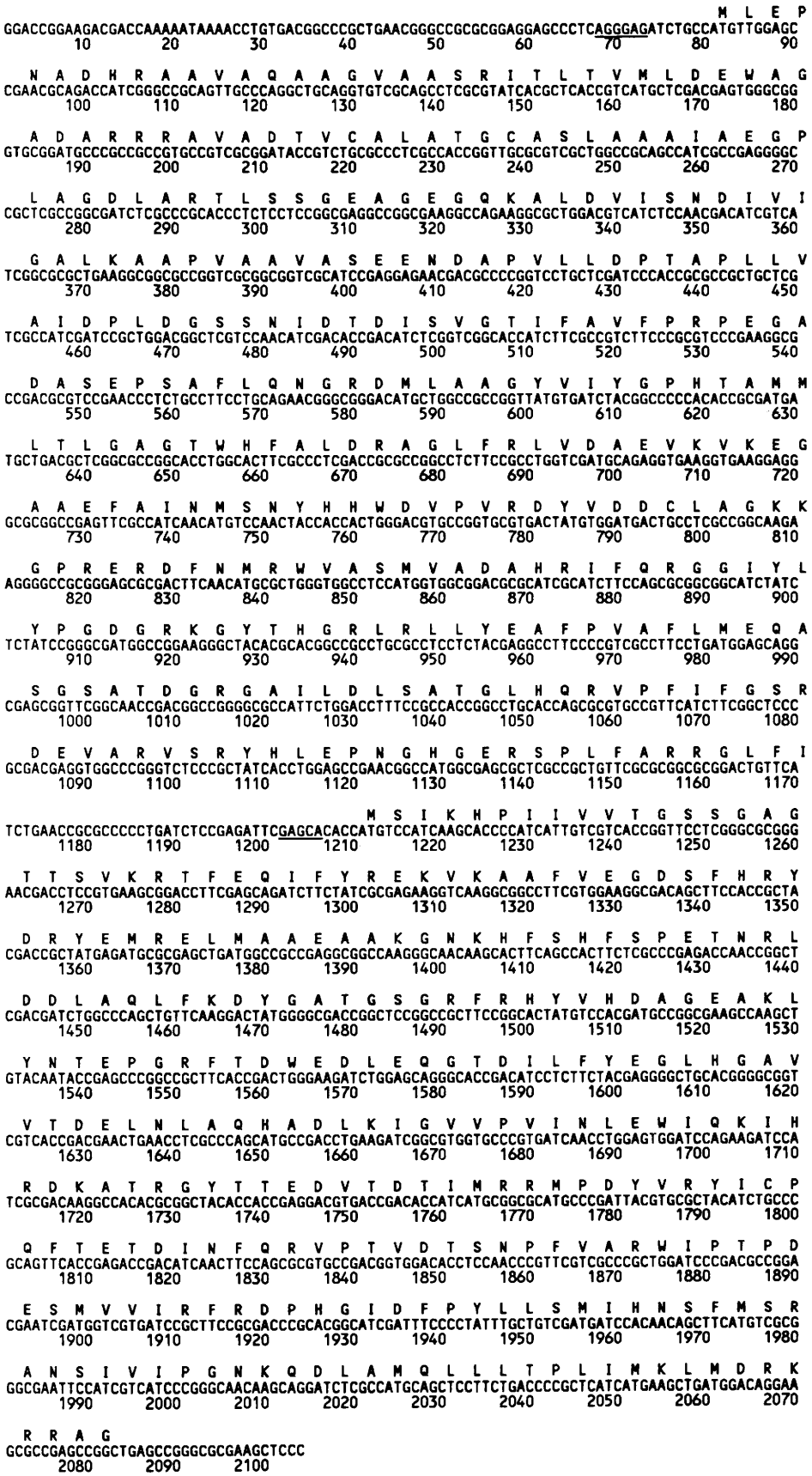

Fig. 2. Nucleotide sequence and deduced amino acid sequence of *cfxF* and *cfxP* from *X. flavus* H4-14. The first open reading frame represents *cfxF* and the second *cfxP*. The amino acids are represented by the one-letter code. The putative ribosome-binding sites are underlined.

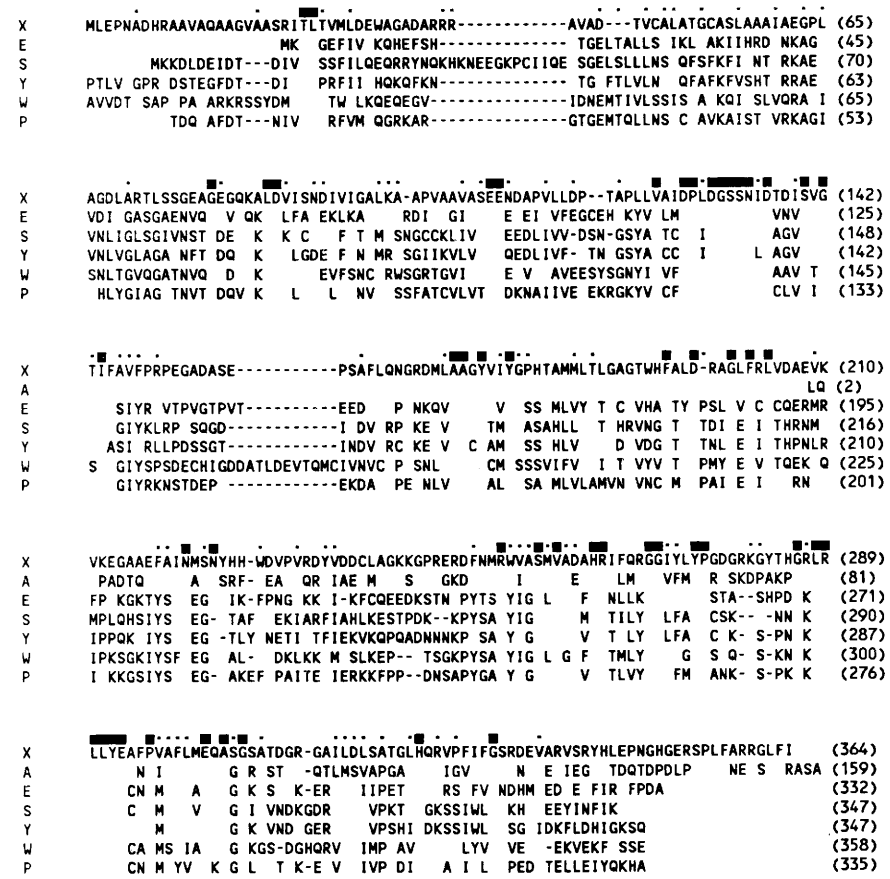

Fig. 3. Alignment of FBPase proteins from: X, *X. flavus* H4-14; A, *Alcaligenes eutrophus* (Kossmann *et al.*, 1990); E, *Escherichia coli* (Hamilton *et al.*, 1988); S, *Schizosaccharomyces pombe* (Rogers *et al.*, 1988); Y, *Saccharomyces cerevisiae* (Rogers *et al.*, 1988); W, wheat chloroplast (Raines *et al.*, 1988); P, pig kidney (Marcus *et al.*, 1982). The mature form of the chloroplast FBPase was used in the alignment. ■, identical residue; ., conservative substitution following the scheme: PAGST, QNED, ILVM, HKR, YFW, C. Only similarities between more than two sequences are indicated. Numbers in parentheses refer to amino acids. Except for the terminal residues, only the residues that differ from the *X. flavus* H4-14 sequence are indicated.

Table 1. *Similarity between FBPase primary structures*

Similarities are in percentages. X, *Xanthobacter flavus* H4-14; A, carboxy terminus from *Alcaligenes eutrophus* (Kossmann *et al.*, 1990); E, *Escherichia coli* (Hamilton *et al.*, 1988); W, wheat chloroplast (Raines *et al.*, 1988); P, pig kidney (Marcus *et al.*, 1982); Y, *Saccharomyces cerevisiae* (Rogers *et al.*, 1988); S, *Schizosaccharomyces pombe* (Rogers *et al.*, 1988).

	X	A	E	W	P	Y	S
X	100						
A	52	100					
E	38	38	100				
W	34	39	46	100			
P	36	36	45	46	100		
Y	35	36	43	41	47	100	
S	32	40	46	43	46	63	100

F-2,6-P-binding site, Asp-118, Asp-121, Glu-280, Glu-97, Glu-98, Lys-71 and Arg-276 (numbering for pig kidney FBPase) are identical in the *X. flavus* H4-14 FBPase sequence. Mg^{2+}, which is essential for FBPase activity, interacts with both the negatively charged pocket and a phosphate group from F-2,6-P. This strongly suggests that F-2,6-P binds to the active site (Ke *et al.*, 1989). Residues close to the AMP-binding site in the pig FBPase sequence are either not conserved or are represented by a conservative substitution in the *X. flavus* H4-14 sequence.

The fact that the F-2,6-P-binding site residues are strongly conserved in the *X. flavus* H4-14 FBPase sequence indicates that this region is indeed essential for FBPase activity, and thus may constitute the active site. Residues close to the AMP-binding site are not conserved. The *Nocardia opaca* FBPase, which is specifically induced during autotrophic growth, is not very sensitive to AMP. In contrast, the FBPase isoenzyme functioning in gluconeogenesis in the same organism is fully inhibited by AMP at concentrations of 100 μM (Amachi & Bowien, 1979). The properties of the enzyme in *X. flavus* H4-14 are currently under investigation.

Characteristics which are specific for a particular FBPase, such as the insertions in the *Schizosaccharomyces pombe* and wheat chloroplast FBPase sequences,

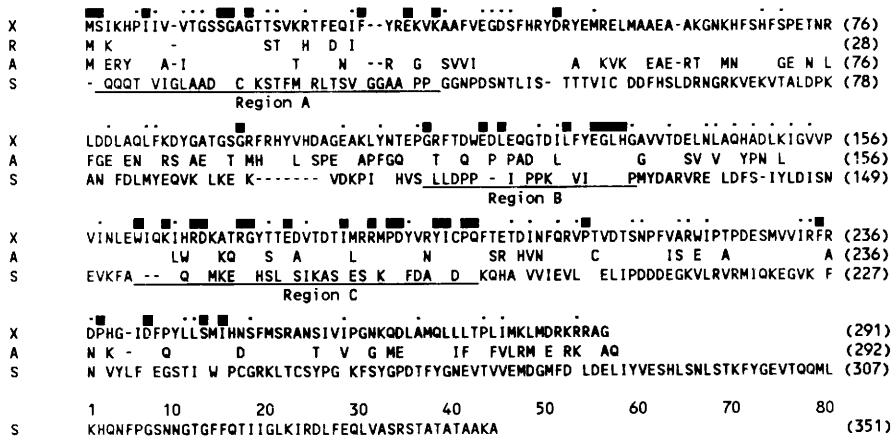

Fig. 4. Alignment of PRK proteins from: X, *X. flavus* H4-14; A, *Alcaligenes eutrophus* (Kossmann *et al.*, 1990); R, *Rhodobacter sphaeroides* (Hallenbeck & Kaplan, 1987); S, spinach (Roesler & Ogren, 1988). ■, identical residue; ., conservative substitution as in Fig. 3. Only similarities between more than two sequences are indicated. Regions of identity are underlined (see text). Numbers in parentheses refer to amino acids. Except for the terminal residues, only the residues that differ from the *X. flavus* H4-14 sequence are indicated.

and the cAMP-dependent protein kinase consensus sequence in the *Saccharomyces cerevisiae* amino terminus, are not conserved in the *X. flavus* H4-14 sequence (Fig. 3; Raines *et al.*, 1988; Rogers *et al.*, 1988).

Comparison of PRK primary structures

As was observed for the FBPase protein, the PRK amino acid sequence from *X. flavus* H4-14 is not very similar (less than 22% identity) to that from a eukaryotic plant, in this case spinach (Fig. 4). In contrast, the *X. flavus* H4-14 and *A. eutrophus* proteins display 65% similarity. This does not come as a surprise, as the quaternary structure of prokaryotic and eukaryotic PRK proteins is very different. In bacteria, PRK is present as an octamer of 33 kDa subunits, whereas in spinach, PRK is a dimer of 45 kDa subunits (Krieger & Miziorko, 1986; Rippel & Bowien, 1984; Siebert & Bowien, 1984). This indicates that residues important in subunit interaction in the prokaryotic holoenzyme are not conserved in the eukaryotic proteins.

In the amino-terminal protein sequence of PRK of both prokaryotic and eukaryotic origin a sequence can be recognized which is supposed to be an ATP-binding site (region A, Fig. 4; Hallenbeck & Kaplan, 1987; Klintworth *et al.*, 1985; Porter *et al.*, 1988). The spinach PRK sequence contains two unique cysteine residues, one at position 16, within the proposed ATP-binding site consensus sequence, the other at position 55. These cysteine residues can be oxidized to form a disulphide bridge, causing inactivation of PRK (Porter *et al.*, 1988). The oxidation and alkylation of Cys-16 by (bromoacetyl) ethanolamine phosphate can be prevented by ATP, strengthening the hypothesis that Cys-16 is at the active site (Porter & Hartman, 1986). The functions of the other conserved regions (B and C: Fig. 4) remain to be established, although they may play a role in the binding of the substrate, RuMP. The identified conserved regions may be suitable targets for mutagenesis to elucidate their function.

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