Nucleotide sequence and expression analysis of the *Acetobacter xylinum* phosphoglucomutase gene

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The *Acetobacter xylinum* gene (*celB*) encoding phosphoglucomutase (EC 5.4.2.2) has previously been cloned by complementation of cellulose-negative mutants. In the present report the nucleotide sequence of a 2.0 kb DNA fragment containing *celB* is described. Expression analysis using the bacteriophage T7 RNA polymerase promoter $\phi 10$ resulted in identification of a probable translational start codon of *celB*, and this conclusion was confirmed by N-terminal amino acid sequencing of the recombinant protein. From the nucleotide sequence data it was deduced that *celB* encodes a protein with a calculated molecular mass of 59.6 kDa. A protein of similar size was visualized after *in vitro* transcription and translation, using the cloned 2.0 kb fragment as template. The results of an amino acid sequence comparison and a biochemical analysis indicated that the CelB protein is structurally and functionally related to the previously characterized human and rabbit phosphoglucomutases.

Keywords: *Acetobacter xylinum*, phosphoglucomutase, *celB*, cellulose

INTRODUCTION

Phosphoglucomutase catalyses the interconversion of D-glucose 6-phosphate and D-glucose 1-phosphate, which represents a branch point in carbohydrate metabolism. Biochemical studies indicate that phosphoglucomutases from a variety of organisms convert glucose 1-phosphate much more efficiently than the corresponding 6-phosphate isomer (Lowry & Passonneau, 1969; Ray & Peck, 1972). Glucose 6-phosphate enters catabolic processes to yield energy and reducing power, whereas glucose 1-phosphate is the precursor of sugar nucleotides that are used by the cells in the synthesis of various glucose-containing polysaccharides. In a previous report we showed that phosphoglucomutase is essential for the formation of extracellular cellulose in *Acetobacter xylinum*, as mutants deficient in the corresponding gene are unable to produce cellulose (Fjærvik *et al.*, 1991). A similar observation has also recently been reported for xanthan production in *Xanthomonas campestris* (Köplin *et al.*, 1992). In *Escherichia coli* it has been shown that mutants deficient in phosphoglucomutase accumulate intracellular amylose when grown in the presence of maltose (Adhya & Schwartz, 1971). The probable reason for this is that maltose metabolism results in the formation of glucose 1-

phosphate which cannot be channelled into catabolism due to the phosphoglucomutase deficiency.

In the active form of phosphoglucomutase, a divalent metal ion is bound to the enzyme, and a serine residue at the catalytic site is phosphorylated (Ray & Peck, 1972). This phosphate group is initially transferred to the substrate, and the serine residue is rephosphorylated concomitant with the release of either of the two phosphoglucose isomers (Rhyu *et al.*, 1984). Recently the crystal structure of rabbit muscle phosphoglucomutase was described, and it was found that three loops are closely spaced in the active-site cleft (Dai *et al.*, 1992). One loop contains the active-site serine, one is a metal-ion-binding loop, and the third is suggested to be involved in substrate-binding specificity.

Phosphomannomutase catalyses a reaction similar to the phosphoglucomutase reaction, and it has been reported that the phosphomannomutase (AlgC) of *Pseudomonas aeruginosa* contains both the active-site and the metal-binding-loop sequence motifs typical of phosphoglucosomutases (Zielinsky *et al.*, 1991). Interestingly, it has been shown that an enzyme from *X. campestris* can efficiently convert both phosphorylated glucose and mannose (Köplin *et al.*, 1992).

In this paper the nucleotide sequencing and expression analysis of the *A. xylinum* phosphoglucomutase gene *celB*

The GenBank accession number for the sequence data reported in this paper is L24077.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics* and references
<i>E. coli</i>	
Hfr3000	<i>pgm</i> ⁺ (Adhya & Schwartz, 1971)
PGM1	<i>pgm</i> derivative of Hfr3000 (Adhya & Schwartz, 1971)
Plasmids	
pUC18,8.6B.dE	pUC18 with a 3.8 kb <i>Eco</i> RI- <i>Bam</i> HI insert; <i>celB</i> ⁺ Ap ^r (Fjærvik <i>et al.</i> , 1991)
pUC128	Inducible <i>lac</i> promoter upstream of a polylinker; Ap ^r (Keen <i>et al.</i> , 1988)
pT7-4	Bacteriophage T7 ϕ 10 promoter upstream of a polylinker; Ap ^r (Tabor & Richardson, 1985)
pGP1-2	Thermo-inducible T7 RNA polymerase gene; Km ^r (Tabor & Richardson, 1985)
pT7-4S	pT7-4 with an <i>Sph</i> I linker ligated into the polylinker <i>Sma</i> I site (this study)
pTB2A/B	pUC128 with a 2.0 kb <i>Sph</i> I insert from pUC18,8.6B.dE in orientation A and B, respectively; <i>celB</i> ⁺ (this study)
pTB3A/B	pT7-4S with a 2.0 kb <i>Sph</i> I insert from pUC18,8.6B.dE in orientation A and B, respectively; <i>celB</i> ⁺ (this study)
pTB1	pTB3A carrying nt 99 through 2058 from <i>celB</i> (this study)
pTB8	pTB3A carrying nt 110 through 2058 from <i>celB</i> (this study)
pTB16	pTB3A carrying nt 161 through 2058 from <i>celB</i> (this study)

* Ap^r, ampicillin resistance; Km^r, kanamycin resistance.

is reported, and some biological properties of the gene product are described.

METHODS

Bacterial strains, plasmids and growth media. Strains and plasmids used in this study are listed in Table 1. *E. coli* strains were, unless otherwise stated, grown at 37 °C in L-broth or on L-agar (Valla *et al.*, 1989) supplemented when appropriate with ampicillin (100 µg ml⁻¹) or kanamycin (50 µg ml⁻¹).

Preparation of DNA, molecular cloning, construction of deletion mutants, DNA sequencing, and computer analysis.

Plasmid isolations, agarose gel electrophoresis, restriction endonuclease digestions, ligations and other routine DNA manipulations were performed according to standard protocols (Sambrook *et al.*, 1989). Transformations were performed according to Chung *et al.* (1989). Unidirectional exonuclease degradations (Henikoff, 1987) of plasmids pTB3A and pTB3B were done with the Erase-a-base system (Promega). The resulting deletion derivatives were used for sequencing of both DNA strands by the dideoxy chain-termination method of Sanger *et al.* (1977). Homology searches with the deduced amino acid sequence of CelB were done using the BLAST network service at NCBI and the non-redundant DNA and protein sequence databases (Altschul *et al.*, 1990).

In vivo expression studies of *celB*. The 2.0 kb *celB*-containing *Sph*I fragment from pUC18,8.6B.dE was cloned in both orientations into the corresponding site of the pUC128 polylinker, generating plasmids pTB2A and pTB2B. These plasmids were transformed into *E. coli* PGM1 (deficient in phosphoglucomutase), and the phosphoglucomutase activities in the

transformants were measured in extracts prepared from cells grown overnight. Expression analysis of the three pTB3A deletion derivatives pTB1, pTB8 and pTB16 were performed in strain PGM1 and PGM1(pGP1-2). Cells were grown at 30 °C to a density of approximately 5×10^8 cells ml⁻¹, and T7 RNA polymerase-mediated transcription from the ϕ 10 promoter was induced by heat as described by Tabor & Richardson (1985). Preparation of cell-free extracts and measurements of phosphoglucomutase activities were performed as described previously (Fjærvik *et al.*, 1991). Phosphomannomutase activity of CelB was assayed in extracts prepared from cells of PGM1(pTB2A) according to Sa'-Correia *et al.* (1987). Addition of mannose 6-phosphate (1 mM final concentration) instead of 1 mM mannose 1-phosphate was used as a control in the assay system. All enzyme assays were done at least twice. The results presented in Table 2 are representative values.

In vitro expression of *celB*. The 2.0 kb *Sph*I fragment (2 µg) containing *celB* was electroeluted from an agarose gel and expressed *in vitro* using an *E. coli* S30 coupled transcription-translation system (Promega). The translation product was labelled with [³⁵S]methionine (1200 Ci/nmol⁻¹; 44.4 TBq nmol⁻¹) according to the manufacturer's instructions. Aliquots (5–15 µl), together with ¹⁴C-labelled protein molecular mass markers, were subjected to SDS-PAGE (8%, w/v, acrylamide). The gel was fixed and soaked in Amplify (Amersham), dried, and exposed to X-ray film.

Determination of the N-terminal amino acid sequence of CelB. Expression of *celB* was induced by heat in PGM1 cells (1.0 l culture) containing plasmids pTB1 and pGP1-2. Cells were harvested by centrifugation, washed twice in 60 ml buffer 1 (20 mM Tris/HCl, 1 mM Na₂EDTA, 1 mM dithiothreitol, 10%, v/v, glycerol, and 100 mM KCl, pH 7.4) and resuspended

sonication, and cell debris was removed by centrifugation (10 000 *g*, 1 min). Aliquots (1 ml) of the supernatants were used directly for amylose analysis by adding 0.1 ml staining solution (0.1% I_2 , 1.0% KI). The presence of amylose was detected as appearance of a deep blue colour. The experiments were done three times. Quantitative measurements were done in one of these experiments by measuring the absorbance at 610 nm. Solutions containing pure amylose and the staining mixture were used as standards.

RESULTS AND DISCUSSION

Localization of *celB* within the cloned fragment

Plasmid pUC18,8.6B.dE was previously shown to express the *A. xylinum celB* gene in *E. coli* (Fjærvik *et al.*, 1991). Deletion derivatives of the 3.8 kb DNA insert in this plasmid were constructed, and enzyme assays of PGM1 cells containing such plasmid derivatives indicated that a

Table 2. Expression analysis of *celB* using the T7 RNA polymerase promoter system

The numbers represent nmol substrate transformed min^{-1} (mg total protein) $^{-1}$. PGM1 was used as host in the experiments, and all measurements were performed under induced conditions.

Plasmid	Phosphoglucosyltransferase activity	
	+ pGP1-2	- pGP1-2
pTB1	18000	700
pTB16	4400	400
pTB8	20	0

Fig. 2. Autoradiogram of an SDS-PAGE gel showing the *in vitro*-translated and [^{35}S]methionine-labelled *celB* gene product. Lanes: 1, protein molecular mass standards (sizes in kDa in left margin); 2, CelB.

2.0 kb *SphI* fragment encoded the phosphoglucosyltransferase activity. This was confirmed by enzyme assays of PGM1 cells containing pTB2A and pTB2B, which demonstrated that *celB* was expressed from both plasmids (data not shown). pTB2A expressed a 10-fold higher phosphoglucosyltransferase activity than pTB2B, indicating that the orientation of the *SphI* fragment in pTB2A allowed *celB* to be transcribed from the *lac* promoter in the vector.

Identification of the *celB* coding sequence

The molecular masses of phosphoglucosyltransferases from several organisms have been determined; they range from 60 to 65 kDa (Ray & Peck, 1972). This indicates that the coding sequences for the corresponding genes are between 1.6 and 1.8 kb, and we therefore decided to sequence the entire 2.0 kb *SphI* fragment. Prior to sequencing, the fragment was subcloned in both orientations into the corresponding site in the plasmid expression vector pT7-4S, generating plasmids pTB3A and pTB3B. The orientation of the *SphI* fragment relative to the *lac*/ ϕ 10 promoters was found to be the same in pTB2A and pTB3A. This indicates that the 5' end of *celB* in pTB3A is localized proximal to the ϕ 10 promoter. The nucleotide sequence of the *SphI* fragment was determined, and the sequence data revealed an open reading frame (ORF1) starting with ATG at nucleotides (nt) 145–147 and terminating with TGA at nt 1810–1812 (Fig. 1).

Inserts cloned in the polylinker of pT7-4S can be transcribed from the ϕ 10 promoter, which is specifically recognized by the bacteriophage T7 RNA polymerase (encoded by pGP1-2). In order to identify the biologically active start codon we performed an expression analysis of three deletion derivatives of pTB3A (pTB1, pTB8 and pTB16). The results of this analysis (Table 2) demonstrated that pTB1 and pTB16 expressed *celB*, and that the expression was strongly stimulated by T7 RNA polymerase. Very little or no activity was detected in cells containing pTB8. There is no Shine–Dalgarno (SD) sequence in the ϕ 10-polylinker region in pT7-4S, and the results thus indicated the presence of a biologically active SD sequence in the inserts of pTB1 and pTB16. A putative SD sequence (GGAG) was identified upstream of ORF1 at nt 135–138 (Fig. 1). These experiments thus indicated that the ATG at nt 145–147 in ORF1 represents the biologically active start codon of *celB*. ORF1 corresponds to a polypeptide consisting of 555 amino acids and the calculated molecular mass of the protein is 59.6 kDa. This is consistent with the results of an *in vitro* expression analysis of *celB*, which demonstrated that the 2.0 kb *SphI* fragment directed the synthesis of a protein with a molecular mass of approximately 60 kDa (Fig. 2).

The purified CelB protein obtained by *in vivo* expression in *E. coli* was sequenced at the amino-terminal end; in eight of the eleven first sequencing steps the amino acids could be definitely identified. All these amino acids are consistent with the deduced sequence of ORF1 (Fig. 1), with the exception that they were all assigned a position one residue number lower than predicted from the nucleotide sequence. The results thus indicated that the initiating

Fig. 3. Comparison between the deduced amino acid sequences of CelB and other related enzymes. **Top:** Schematic representation of individual amino acid sequences depicting the localization of conserved regions (boxes I, II, and III). The numbers to the right refer to the number of amino acid residues in the different proteins. **Bottom:** Alignment of boxes I, II and III. The number after each sequence is the number of the last residue in each motif in its corresponding sequence. The abbreviations used for the enzymes, and the sources of the sequences, are as follows: CelB, *A. xylinum* phosphoglucosyltransferase (this study); PGM1, human phosphoglucosyltransferase (Whitehouse *et al.*, 1992); PGM, rabbit muscle phosphoglucosyltransferase (Ray *et al.*, 1983); AlgC, *P. aeruginosa* phosphomannomutase (Zielinski *et al.*, 1991); XanA, *X. campestris* phosphoglucosyltransferase (Köplin *et al.*, 1992); CpsG (Se), *Salmonella enterica* phosphomannomutase (Stevenson *et al.*, 1991); RfbK (St), *Salmonella typhimurium* phosphomannomutase (Jiang *et al.*, 1991); RfbK (Ec), *E. coli* phosphomannomutase (Marolda & Valvano, 1993).

formylmethionine is removed from CelB when expressed in *E. coli*.

Effect of CelB on intracellular amylose synthesis in *E. coli*

The *in vivo* effect of CelB on intracellular amylose synthesis in *E. coli* was analysed by growing cells in the presence of glucose or maltose as carbon sources. In agreement with previous observations (Adhya & Schwartz, 1971), it was found that PGM1 accumulated amylose [0.14 mg (mg dry wt)⁻¹] when grown on maltose, while no amylose was detected when glucose was used as carbon source. PGM1 cells containing a *celB*-expressing plasmid (pUC18.8.6.dE) did not accumulate amylose when grown under these conditions, and the same was true for the isogenic wild-type strain of PGM1 (Hfr3000). These experiments thus indicated that CelB could substitute for the *E. coli* enzyme in transforming the glucose 1-phosphate formed in the cells during growth on maltose to glucose 6-phosphate. In the biosynthesis of cellulose in *A. xylinum*, CelB is

essential for the formation of glucose 1-phosphate. The ability of CelB to catalyse both directions of the glucose phosphate isomerization *in vivo* is thus demonstrated.

Structural and biochemical comparisons between CelB and other related enzymes

The amino acid sequence of CelB was compared with the primary structures of previously reported phosphoglucosyltransferases and phosphomannomutases (Fig. 3). The polypeptide chains of CelB and the human and rabbit phosphoglucosyltransferases are about 100 amino acids longer than the phosphomannomutases. Although there was no overall sequence similarity, the analysis revealed two highly conserved regions (Box I and Box II). The Box I motif contains the catalytic site of the enzymes, which was originally identified in rabbit muscle phosphoglucosyltransferase (Ray & Peck, 1972). In rabbit muscle phosphoglucosyltransferase the Box II motif contains a metal-ion-binding loop (DGDGD) which is exposed in the active-site cavity (Dai *et al.*, 1992).

The third region of homology (Box III, not reported previously) was found only in CelB and the two eukaryotic (human and rabbit) phosphoglucosyltransferases. This motif consists of 39 amino acids, and 38 of these are the same in the rabbit and human enzymes, while there are 16 matches (41 %) between CelB and the two eukaryotic sequences. In the rabbit muscle enzyme three amino acids (ESF) at the end of this motif are exposed in the active-site cavity, and it is suggested that they interact with the substrate glucose ring (Dai *et al.*, 1992).

Among all the phosphoglucosyltransferases previously reported, the *X. campestris* enzyme (XanA) is unique in that it can efficiently use both phosphorylated mannose and glucose as substrates. We have analysed CelB with respect to this property, and the results of these experiments showed that the activity with mannose 1-phosphate was less than 0.2 % of the activity with glucose 1-phosphate. CelB is in this respect similar to the rabbit and human phosphoglucosyltransferases, which have been reported to be specific for glucose phosphates (Lowry & Passonneau, 1969). The presence of the Box III motif only in these three enzymes suggests the possibility that this motif is responsible for their phosphoglucose specificity, and that the enzymes lacking this motif may have a broader substrate specificity. Except for XanA, the ability to convert phosphoglucose has to our knowledge not been determined for the phosphomannosyltransferases included in this comparison.

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