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Characterization of a Putative *Pseudomonas* UDPglucose Pyrophosphorylase

HWAN-YOU CHANG*, HSIOU-CHEN HUANG**, JEAN-HSIAN LEE***
AND HWEI-LING PENG***,†

**Department of Life Science*
National Tsing Hua University
Hsinchu, Taiwan, R. O. C.
***Graduate Institute of Agricultural Biotechnology*
National Chung Hsing University
Taichung, Taiwan, R. O. C.
****Institute of Biological Science and Technology*
National Chiao-Tung University
Hsinchu, Taiwan, R. O. C.

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ABSTRACT

A UDP-glucose pyrophosphorylase encoding gene was identified through functional complementation screening by using an *Escherichia coli galU* mutant. Sequence analysis of the gene indicated that it is most likely derived from a Pseud monas sp. The gene is located immediately upstream and transcribed in the same direction of the *gor* (glutathione reductase) gene and is capable of encoding a protein 30,943 daltons in size. The gene product synthesized in *Escherichia coli* was purified and its biochemical properties characterized. The recombinant UDP-glucose pyrophosphorylase exhibited a molecular weight of 130 kDa, suggesting a tetrameric organization of the gene product. Two mutant forms of the enzyme were identified. The activity of the mutant enzyme with a tyrosine to histidine (Y26 lH) substitution was found to be greatly reduced. On the other hand, the tyrosine to cysteine (Y84C) substitution resulted in an enzyme that functions normally at 37 °C but rather poorly at temperatures lower than 30 ºC.

Key Words: *galU*, *gor*, *Pseudomonas* sp., UDPglucose pyrophosphorylase

1. Introduction

Uridine diphosphoglucose (UDP-Glc) functions as a glucosyl donor in the biosynthesis of various carbohydrates, including lipopolysaccharides (Fukasawa *et al.*, 1962; Sundararajan *et al.*, 1962), membrane derived oligosaccharides (Shulman and Kennedy, 1977) and capsular polysaccharides (Markovitz, 1977) in bacteria. In organisms which utilize the Leloir pathway, UDP-Glc is also required for the utilization of galactose (Leloir, 1951). UDP-Glc is synthesized from α-D-glucose 1-phosphate and UTP by UDP-Glc pyrophosphorylase (UTP: α-D-glucose 1-phosphate uridylyltransferase) [EC 2.7.7.9]. The enzyme has been isolated and characterized from both eucaryotic (Hansen *et al.*, 1966; Gustafson and Gander, 1972; Pannbacker, 1967; Villar-Palasi and Larner, 1960) and procaryotic (Kamogawa and Kurahashi, 1965; Nikae and Nikaido, 1971a, 1971b) sources. Genes and cDNA clones encoding the enzyme have also been reported by genome sequencing projects for bovine (Konishi *et al.*, 1993), human (Duggleby *et al.*, 1996a), potato tubers (Katsube *et al.*, 1990), slime mold (Ragheb and Dottin, 1987), *Acetobacter xylinum* (Brede *et al.*, 1991), *Escherichia coli* (Weissborn *et al.*, 1994; Hossain *et al.*, 1994) and several other bacterial species. The predicted amino acid sequences of the bacterial enzymes are quite different from those of the eucaryotic enzymes, suggesting that they evolved from two independent ancestors.

In *E. coli*, UDP-Glc pyrophosphorylase is encoded by the *galU* gene. The growth of *E. coli galU* mutants is inhibited by galactose, an effect presumably due to the accumulation of toxic levels of galactose 1-phosphate. The selection against the *galU* bacteria on galactose containing agar provides a convenient way to isolate UDP-Glc pyrophosphorylase encoding genes by complementation of such mutants. The strategy has been successfully applied in our laboratory to isolate a human UDP-Glc pyrophosphorylase cDNA (Duggleby *et al.*, 1996a).

† To whom all correspondence should be addressed.

During the course of screening for different isoforms of human UDP-Glc pyrophosphorylase, we have incidentally identified a *Pseudomonas* gene encoding the enzyme. Since several *Pseudomonas* species, particularly *P. aeruginosa*, are important human pathogens, and UDP-Glc pyrophosphorylase has been known to participate in the pathogenesis of a number of bacteria (Chang *et al.*, 1996; Crater and van de Rijn, 1995), we decided to study the *Pseudomonas* enzyme further. The biochemical properties of the recombinant enzyme and two mutations which affected the enzyme activity were also characterized and are reported herein.

II. Materials and Methods

1. Materials

All restriction endonucleases and DNA modifying enzymes were obtained from either Promega (Madison, WI, U.S.A.) or Boehringer Mannheim (Germany) and used under the conditions recommended by the suppliers. The Sequenase kit and [α-^{35}S] dATP were purchased from Amersham (Buckinghamshire, U.K.). The Econo-Pac DEAE-Blue cartridge was purchased from BioRad Laboratories (Hercules, CA, U.S.A.), and Sephacryl-300 HR was from Pharmacia Biotech (Uppsala, Sweden). Triethanolamine was obtained from Ajax Chemicals (Auburn, Australia). Other chemicals, coenzymes, coupling enzymes and buffers were obtained from the Sigma Chemical Company (St. Louis, MO, U.S.A.).

2. Plasmids, Bacterial Strains, and Growth Conditions

Pseudomonas aeruginosa (ATCC 10145) was obtained from the Culture Collection Research Center, Food Industrial Research Institute, Hsin-Chu, Taiwan. *E. coli galU* mutant FF4001 was a generous gift from Dr. Valla (Brede *et al.*, 1991). *E. coli* HP069, an F′*lac* containing derivative of FF4001, was constructed previously in our laboratory (Peng and Chang ,1993). *E. coli* XL-1 Red (*endA1*, *gyrA96*, *thi-1*, *hsdR17*, *supE44*, *relA1*, *lac*, *mutD5*, *mutS*, *mutT*, Tn10) was obtained from Stratagene (LaJolla, CA. U.S.A.). The human hippocampus cDNA library, constructed in the *Eco*RI site of the lambda ZAP vector, was also a product of Stratagene (Cat.#936205). Luria-Bertani medium (LB) was used to propagate all bacterial species. MacConkey-galactose agar was prepared with MacConkey agar base (Difco Inc., Detroit, MI, U.S.A.) supplemented with 0.4% galactose and 50 μg/ml ampicillin.

The growth rates of bacteria were measured as follows. One ml of bacteria overnight grown was inoculated into a side-armed flask containing 100 ml LB supplemented with 0.4% of either glucose or galactose. The density of the bacterial culture was determined using a Klett-Summerson photoelectric colorimeter (Model 800-3, Klett Mfg., Co., Inc., NY, U.S.A.).

3. Recombinant DNA Techniques and Nucleotide Sequencing

DNA manipulation was performed essentially as described (Sambrook *et al.*, 1989). DNA fragments to be sequenced were subcloned into M13 vectors and their sequences determined using the Sequenase kit with either universal M13 primer or synthetic oligonucleotides. The sequence was confirmed in both strands, and the ambiguities which resulted from banding compression were resolved by sequencing with dITP. The nucleotide sequence was analyzed using the DNASTAR program (DNASTAR Inc. Madison, WI., U.S.A.), on a Macintosh LC-II computer, and the BLAST program (Altschul *et al.*, 1990) at the National Center for Biotechnology Information through the World Wide Web. Phylogenic tree construction was performed using the SeqWeb program of the Genetics Computer Group (GCG), provided by the National Health Research Institute, Taiwan, R.O.C.

4. Overexpression of the UDP-Glc Pyrophosphorylase Gene in *E. coli*

All bacteria for protein analysis were grown in LB at 37 ºC with vigorous shaking until A_{600} = 0.3. Isopropyl-β-D-thio-galactopyranoside (IPTG) was added to a final concentration of 1 mM, and incubation was continued for 90 min. The cells were collected and resuspended into 2 X Laemmli sample buffer, and the proteins were resolved on a 0.5% SDS-12.5% polyacrylamide gel (Laemmli, 1970). The protein profiles were detected by means of staining with Coomassie Blue R250.

5. UDP-Glc Pyrophosphorylase Assay and Data Analysis

The enzyme can be assayed in either the forward or the reverse direction (Hansen *et al.*, 1966). Our routine assay involved quantitative measurement of the pyrophosphorylysis of the nucleotide diphosphate sugars. The reaction mixture contained 100 mM triethanolamine-HCl pH 8.0, 1 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 8 μM glucose 1,6-diphosphate, 1 mM UDP-Glc, 1 mM NADP, 2 mM MgPPi, 5 units each of phosphoglucomutase and glucose 6-phosphate dehydrogenase and the purified enzyme at a final volume of 1 ml. Occasionally, assay towards the synthesis of UDP-Glc was also employed. In this case, the reaction mixture contained 100 mM trietha-

nolamine-HCl, pH 8.0, 3 mM $MgCl_2$, 1 mM DTT, 2 mM UTP, 1 mM glucose 1-phosphate, 2 mM NAD, 0.25 U bovine liver UDP-Glc dehydrogenase and the purified enzyme in a final volume of 1 ml. Reactions were performed at 25 ºC unless otherwise indicated. The reduction of NAD or NADP was monitored spectrophotometrically at 340 nm. One unit of the enzyme was defined as the activity that reduced 1 nmol NAD(P) per min.

Kinetic data were analyzed using the nonlinear regression computer program DNRPEASY as described (Duggleby, 1990).

6. Enzyme Purification

The enzyme synthesized in *E. coli* was purified by traditional column chromatography. All procedures were performed at 4 °C unless otherwise indicated. The IPTG induced cells were harvested and resuspended in ice-cold TM buffer (50 mM Tris HCl, pH 7.8, 3 mM $MgCl_2$ and 1 mM 2-mercaptoethanol). The cells were disrupted by sonication and the debris removed by centrifugation. A 20% streptomycin sulfate solution was added to the supernatant to a final concentration of 1.6% (w/v), and the precipitate was removed by centrifugation at 16,000 *g*. The supernatant was then heated at 50 ºC for 15 min and the denatured proteins clarified by centrifugation at 20,000 g. Ammonium sulfate precipitation followed, and the proteins which salted out at between 30-60% saturation of ammonium sulfate were collected and dialyzed overnight against TM buffer. The dialysate was applied to an Econo-Pac DEAE-Blue cartridge and the enzyme eluted by a linear gradient of 10-500 mM NaCl in TM buffer. The highest levels of enzyme activity were observed in fractions eluted between 100-150 mM NaCl. These fractions were pooled, concentrated on Centricon-10 (Amicon, Beverly, MA, U.S.A.) and applied to a Sephacryl-300 gel filtration column. Fractions with the highest activities were combined, glycerol was then added to 50% (w/w) and the final preparation was stored at –70 ºC.

7. Molecular Weight Determination

The molecular weight of the enzyme subunit was determined by SDS-polyacrylamide gel electrophoresis. The size of the native protein was determined by gel filtration conducted on a column of Sephacryl-300 that was 100 cm in length and had an inner diameter of 1.6 cm. The protein standards used were carbonic anhydrase (29-kDa), bovine serum albumin (66-kDa), alcohol dehydrogenase (150-kDa), β-amylase (200-kDa) and Blue Dextran-2000 (void volume). Samples were eluted in TM buffer at a flow rate of 0.5 ml/min. The molecular weight of the purified protein was estimated using the standard method (Andrews, 1964).

8. Nucleotide Diphosphate Sugar Specificity

The specificity of the enzyme to various nucleotide diphosphate sugars was determined as described (Hansen *et al.*, 1966) with modifications (Duggleby *et al.*, 1996b). Two additional components, 40 μM ADP and 2 U/ml nucleoside diphosphate kinase, were included into the original reaction mixture, which contained 100 mM triethanolamine-HCl, pH 8.0, 1 mM hydrazine sulfate, 2 mM $MgCl_2$, 1 mM sodium pyrophosphate, 1.2 mM 3-phosphoglycerate, 0.15 mM NADH, 1 mM UDP-Glc, 1 mM DTT, 40 U/ml phosphoglycerate kinase and 10 U/ml glyceraldehyde 3-phosphate dehydrogenase. In the presence of ADP, the nucleoside diphosphate kinase converts UTP or CTP into ATP, which is a much better substrate for phosphoglycerate kinase and, hence, improves the sensitivity of the original assay.

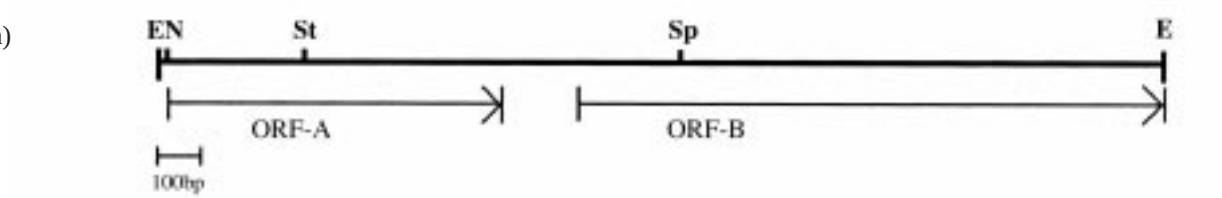
9. Mutagenesis of the UDP-Glc Pyrophosphorylase Gene

Random mutagenesis of the *Pseudomonas galU* gene was performed using the highly mutagenic *E. coli* strain XL-1Red under the conditions recommended by the supplier (Stratagene). Briefly, pHC366 was transformed into *E. coli* XL-1Red, and the transformants were selected on LB agar supplemented with 50 μg/ml ampicillin. After propagating the transformants for several generations, the plasmid DNA which accumulated with spontaneous mutation was made and introduced into *E. coli* HP069 by electroporation using the GenePulsar apparatus (BioRad Laboratories). Duplicates of the ampicillin resistant transformants were made on MacConkey plates containing either glucose or galactose to select for clones defective in galactose utilizing capability.

III. Results

During functional screening of a human hippocampus cDNA library, we identified a phagemid clone that was capable of conferring the *E. coli* HP069 into galactose resistant and fermenting phenotype. The UDP-Glc pyrophosphorylase activity of *E. coli* HP069 carrying this phagemid, designated pHC361, was subsequently determined. Approximately 20 U/mg protein of the enzyme activity was observed in the lysate of *E. coli* HP069 [pHC361], compared to less than 0.1 U/mg protein in the plasmidless host. The nucleotide sequence of the 2.2 kb insert of pHC361 was determined and revealed two major open reading frames (ORF-A and ORF-B), separated by an intervening sequence 105 base pairs in length (Fig. 1). Deduced amino acid sequences of the two ORFs were used to search for homologous files in the GenBank database through the World Wide Web. Although a homology

(a)



(b)

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-28                                                                  GTACGCACAGCCCTTTGTAAGGACTCAT
      M   I   K   K   Y   L   F   P   A   A   G   Y   G   T   R   F   L   P   A   T
  1   ATG ATC AAG AAA TAC TTG TTC CCA GCA GCC GGT TAC GGC ACT CGC TTC TTG CCA GCG ACC
      K   A   M   P   K   E   M   L   P   V   V   N   K   P   L   I   Q   Y   G   V
 61   AAA GCC ATG CCC AAA GAA ATG CTG CCA GTG GTG AAC AAG CCA CTG ATT CAG TAC GGT GTG
      E   E   A   L   D   A   G   L   N   E   I   S   I   V   T   G   R   G   K   R
121   GAA GAG GCA CTG GAT GCC GGT TTG AAC GAG ATC TCC ATC GTC ACG GGT CGT GGT AAG CGC
      A   L   E   D   H   F   D   I   S   Y   E   L   E   N   Q   I   K   G   T   D
181   GCG CTG GAA GAC CAC TTC GAC ATC AGC TAT GAG CTG GAA AAC CAG ATC AAG GGC ACC GAC
      K   E   K   Y   L   V   G   I   R   K   L   L   D   E   C   S   F   S   Y   T
241   AAG GAA AAA TAC CTG GTC GGT ATC CGT AAA CTG CTC GAC GAG TGC TCG TTC TCC TAC ACC
      R   Q   T   Q   M   K   G   L   G   H   A   I   L   T   G   R   P   L   I   G
301   CGT CAG ACT CAG ATG AAA GGC CTC GGC CAC GCC ATC CTC ACC GGC CGC CCG CTG ATC GGT
      D   E   P   F   A   V   V   L   A   D   D   L   C   V   N   L   E   G   D   G
361   GAT GAA CCC TTC GCC GTG GTC CTG GCG GAC GAC CTG TGC GTC AAC CTG GAA GGC GAC GGT
      V   L   T   Q   M   V   K   L   Y   Q   K   Y   R   C   T   I   V   A   V   M
421   GTG CTG ACC CAG ATG GTC AAG CTC TAC CAG AAA TAC CGT TGC ACC ATC GTT GCG GTA ATG
      E   V   N   P   T   E   T   N   K   Y   G   V   I   A   G   D   D   I   G   D
481   GAA GTC AAC CCG ACC GAA ACC AAT AAG TAC GGT GTT ATC GCC GGC GAC GAT ATT GGC GAT
      G   L   I   R   V   R   D   M   V   E   K   P   A   P   E   D   A   P   S   N
541   GGC CTG ATC CGC GTA CGC GAC ATG GTC GAG AAA CCA GCA CCG GAA GAC GCG CCA TCG AAC
      L   A   I   I   G   R   Y   I   L   T   P   D   I   F   K   L   I   E   E   T
601   CTG GCG ATT ATC GGT CGT TAC ATA CTC ACG CCG GAC ATC TTC AAG CTG ATC GAA GAA ACC
      E   P   G   K   G   G   E   I   Q   I   T   D   A   L   L   K   Q   A   K   D
661   GAG CCA GGC AAG GGC GGC GAG ATC CAG ATC ACC GAC GCT CTG CTC AAG CAA GCC AAA GAC
      G   C   V   I   A   Y   K   F   K   G   Q   R   F   D   C   G   G   A   E   G
721   GGC TGC GTG ATT GCC TAC AAG TTC AAG GGC CAG CGT TTC GAC TGC GGC GGC GCT GAG GGC
      Y   I   E   A   T   N   F   C   Y   E   H   F   Y   K   T   G   K   A   Y   *
781   TAC ATC GAA GCG ACC AAC TTC TGC TAC GAG CAT TTC TAC AAG ACG GGC AAG GCT TAC TGA
                                  -------->  <--------
841   TTCACGCTTGCCGGTACGTTTTAAGCGAAAGCCACCTTCGGGTGGCTTTTTCGTTTTTTAGCCCCATGTAAGTCGGTAT
                          gor--> M   A   Y   D   F   D   L   Y   V   I   G   A   G
920   GCTGGTGTCCTGCCGAGGAGAGTGAA  ATG GCC TAC GAT TTT GAC CTG TAT GTA ATT GGC GCC GGT
      S   G   G   V   R   A   A   R   F   A   A   G   F   G   A   K   V   A   V   A
985   TCC GGC GGT GTT CGC GCG GCG CGC TTT GCC GCA GGC TTT GGC GCC AAG GTG GCG GTG GCC
      E   S   R   Y   L   G   G   T   C   V   N   V   G   C   V   P   K   K   L   L
1045  GAG AGC CGC TAC CTG GGT GGC ACC TGT GTG AAC GTC GGC TGC GTG CCA AAG AAG CTG TTG
      V   Y   G   A   H   F   A   E   D
1105  GTG TAC GGC GCG CAT TTT GCC GAA GAC....
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Fig. 1. Restriction map and nucleotide sequence of the putative *Pseudomonas galU* gene. (a) Gene organization of pHC361 insert. The span and direction of the transcription of each open reading frame (ORF) are shown. The ORF-A represents the coding sequence of the UDP-Glc pyrophosphorylase, and the ORF-B is the glutathione reductase encoding gene (*gor*). The relevant restriction enzyme sites are: E, *Eco*RI; N, *Nde*I; St, *Stu*I; Sp, *Sph*I. (b) Nucleotide and deduced amino acid sequences of the UDP-Glc pyrophosphorylase gene. The nucleotide sequence is numbered in the 5' to 3' direction, beginning at the A residue of the putative initiation ATG codon. The potential Shine-Dalgarno sequences for both *galU* and *gor* are underlined. The inverted repeats, which may form a stem-loop structure and may serve as a signal for transcription termination, are indicated by arrows under the sequence. These data have been submitted to the GenBank Data Base and have been assigned accession number U03751.

with UDP-Glc pyrophosphorylase was expected, it was still surprising to find that the predicted amino acid sequence of ORF-A product shares an overall 30-40% amino acid identity with several bacterial UDP-Glc pyrophosphorylases (Fig. 2). This finding was unusual because the primary sequences of all eucaryotic UDP-Glc pyrophosphorylases which have been identified so far are distinct from those of the bacterial enzyme. It was even more striking to observe that the deduced amino acid sequence of the ORF-B product was nearly identical to that of the glutathione reductase of *P. aeruginosa* (Perry *et al.*, 1991). The close linkage of the glutathione reductase gene, *gor*, and the UDP-Glc pyrophosphorylase encoding gene raises the possibility that the pHC361 is actually a bacterial DNA and, most likely, a DNA fragment of a *Pseudomonas* sp.

By using the ^{32}P labeled *Eco*RI-*Sph*I and *Eco*RI-*Stu*I

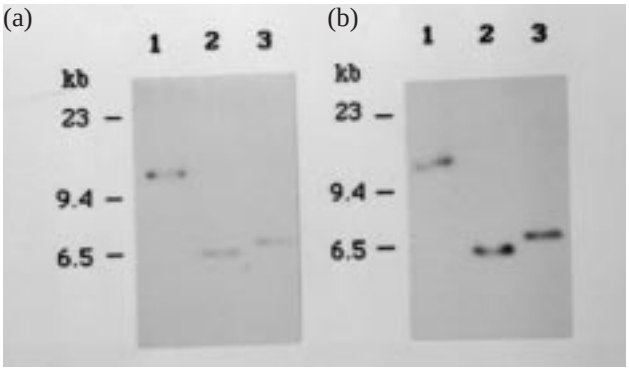

Fig. 2. Southern blot analysis of the *Pseudomonas* UDP-Glc pyrophosphorylase encoding gene and *gor* gene. The chromosomal DNA of *P. aeruginosa* ATCC10145 was digested with restriction endonuclease *Bgl*II (lane 1), *Bam*HI (lane 2) and *Eco*RI (lane 3). The blot was hybridized with probes specific to the *galU* gene (a) and the *gor* gene (b), respectively.

fragments (Fig. 1(a)) of pHC361, respectively, as probes for Southern blotting analysis, strong hybridization signals could be detected in the DNA sample prepared from the type strain of *P. aeruginosa* (ATCC10145). The pattern of the hybridized DNA fragments was also consistent with the notion that the UDP-Glc pyrophosphorylase gene and *gor* gene are closely linked (Fig. 2). In addition, the overall nucleotide sequence and the deduced amino acid sequences of the open reading frames contained in the insert DNA of pHC361 matched very well those of the UDP-Glc pyrophosphorylase encoding genes in *P. syringae* pv. *syringae* and *P. aeruginosa* PAO1 that had been completed earlier in our laboratories. Evolutionary analysis results of several previously identified UDP-Glc pyrophosphorylase encoding gene also suggest that the novel UDP-Glc pyrophosphorylase encoding gene is a *Pseudomonas* gene (Fig. 3). In addition, in all the *Pseudomonas* strains that had thus farbeen analyzed, the UDP-Glc pyrophosphorylase gene was found to link with the *gor* gene as in pHC361. This finding provides another piece of evidence for a close relationship between the novel identified DNA fragment and *Pseudomonas* spp. in evolution. Taken together, these data strongly suggest that the insert DNA of pHC361 was derived from a *Pseudomonas* sp. Since this specific locus in *Pseudomonas* spp. has not been assigned, the gene encoding the UDP-Glc pyrophosphorylase was, therefore, designated as *galU* in accordance with the nomenclature in the *E. coli* genetic system.

A number of bacterial sequences were identified from the GenBank database that displayed a significant homology with the putative *Pseudomonas* UDP-Glc pyrophosphorylase (Fig. 4). These sequences include the products of *A. xylinum celA* (Brede *et al.*, 1991), *Bacillus subtilis gtaB* (Varon *et al.*, 1993), *E. coli galU* (Weissborn *et al.*, 1994; Hossain *et al.*, 1994) *Salmonella typhimurium galF* (Jiang *et al.*, 1991), *Rhizobium melliloti exoN* (Becker *et al.*, 1993), *Streptococcus pneumoniae cps3U* (Dillard *et al.*, 1995), and open reading frames reported based on analysis of the genome of several bacterial species. All the gene products were similar in size, with the putative *Pseudomonas* enzyme being the smallest, that is, 23 amino acid residues shorter than the *E. coli galU* gene product. Comparison of several representative sequences indicates that all members of the bacterial UDP-Glc pyrophosphorylases resemble one another most significantly in the amino terminal 75 amino acid residues. The remaining three-fourths of the enzymes, however, are rather diversified. As shown in Fig. 4, only 20 out of the C-terminal 200 amino acid residues are conserved among all the bacterial UDP-Glc pyrophosphorylases. Again, no similarity was observed between the putative *Pseudomonas* and eucaryotic UDP-Glc pyrophosphorylases.

In order to identify the *Pseudomonas galU* gene product, the *Eco*RI-*Sph*I fragment containing the entire coding sequence was excised from pHC361 and subcloned into the expression vector pPROK1 (Clontech, Palo Alto, CA, U.S.A.) under the control of the *tac* promoter. The resulting plasmid, pHC366, was transformed into *E. col*i HP069, and the transformant was propagated in LB with 1 mM IPTG induction at 37 ºC for 90 min. The total cellular protein profile of the bacteria was analyzed on a 0.5% SDS-12.5% polyacrylamide gel. As shown in Fig. 5, a 32-kDa protein which is not present in *E. coli* HP069 [pHC366] without

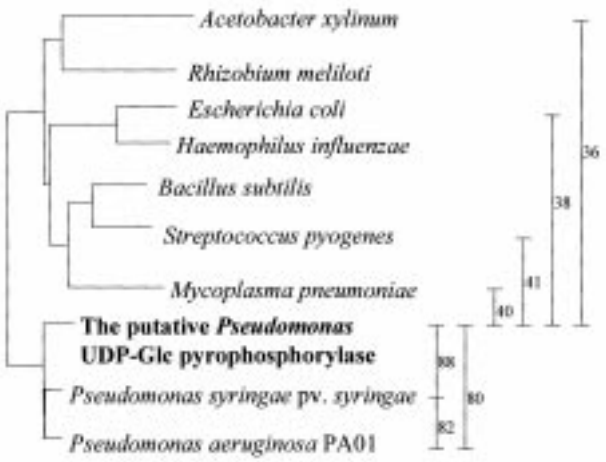

Fig. 3. Evolutionary analysis of the UDP-Glc pyrophosphorylases of eubacteria. The branch length in the dendrogram is directly proportional to the number of amino acid replacements. The numbers on the vertical bars indicate the percentage of amino acid sequence identity of UDP-Glc pyrophosphorylase in the representative species. Species and accession numbers used to construct the tree are: *Acetobacter xylinu*m (P27894), *Rhizobium meliloti* (P33696), *Pseudomonas aeruginosa* PAO1 (AJ010734), *Pseudomonas syringae* pv. *syringae* (submitted), *Escherichia coli* (P25520), *Haemophiluis influenzae* (P44878), *Bacillus subtilis* (Q05852), *Streptococcus pyogenes* (Q54713), and *Mycoplasma pneumoniae* (P75124).

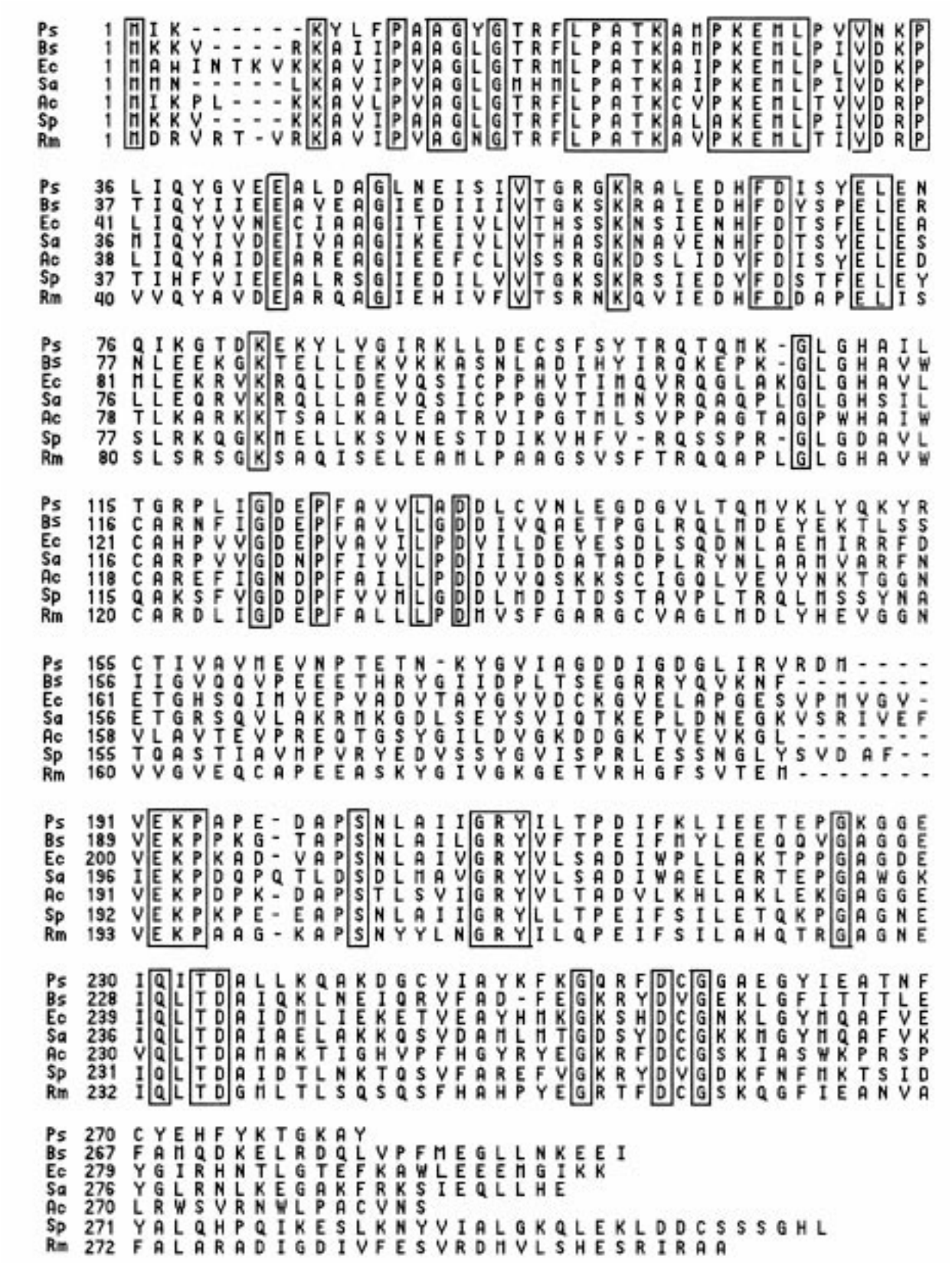

Fig. 4. Sequence comparison of bacterial UDP-Glc pyrophosphorylases. The sequences of the putative *Pseudomonas* (Ps), *A. xylinum* (Ac), *B. subtilis* (Bs), *E. coli* (Ec), *S. typhimurium* (Sa), *Streptococcus pneumoniae* (Sp), and *R. meliloti* (Rm) UDP-Glc pyrophosphorylase are aligned. The positions of the amino acid residues are indicated on the left. Residues conserved among all comparable UDP-Glc pyrophosphorylases are boxed.

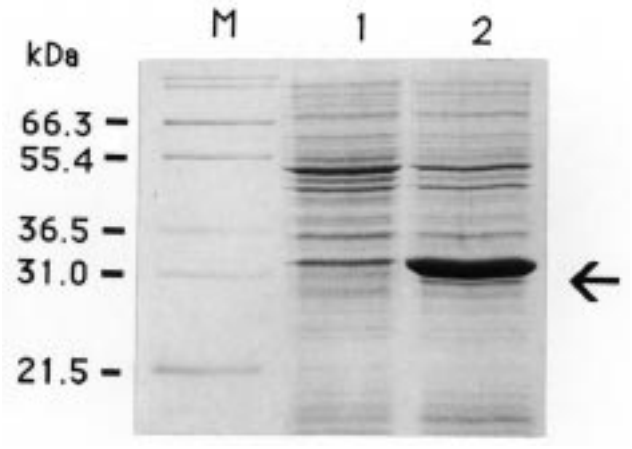

Fig. 5. *In vivo* synthesis in *E. coli* of the putative *Pseudomonas* UDP-Glc pyrophosphorylase. The protein samples were derived from *E. coli* HP069[pHC366] without (lane 1) and with (lane 2) IPTG induction. Protein markers (in kilodaltons, lane M) are shown on the left, and an arrow points to the overproduced 32-kDa enzyme. The protein molecular weight standards were glutamate dehydrogenase (55.4-kDa), lactate dehydrogenase (36.5-kDa), carbonic anhydrase (31.0-kDa), and trypsin inhibitor (21.5-kDa).

IPTG induction is clearly observed in that induced with IPTG. The size of this protein is consistent with that deduced from the nucleotide sequence.

Analysis of *E. coli* HP069 [pHC366] cell lysate has demonstrated that the UDP-Glc pyrophosphorylase activity is greatly increased. Approximately 140 U/mg protein activity can be achieved upon IPTG induction. The clone was, therefore, used as a source for purification of the enzyme. The putative *Pseudomonas* enzyme synthesized in *E. coli* was purified through a series of chromatographic procedures as described in Materials and Methods. The final enzyme preparation was approximately 95% pure as judged by SDS-polyacrylamide gel electrophoresis (Fig. 6 (a)). The purified enzyme remained stable for two weeks at 4 °C . When stored at –70 °C in the presence of 50% glycerol, no significant loss of enzyme activity was detected over a period of two months. The enzyme eluted from a Sephacryl-300 column was approximately 130 kDa (Fig. 6 (b)), suggesting that it is a homotetramer of the *galU*-encoded protein.

The apparent Michaelis constants of the enzyme with respect to its four substrates were measured. The Km values for MgPPi and glucose 1-phosphate were 115 μM and 830 μM, respectively. The double reciprocal plot for UTP and UDP-Glc, however, exhibited major departures from linearity, particularly at lower substrate concentrations (Fig. 7).

The specificity of the enzyme was determined using several nucleotide diphosphate sugars, including UDP-Gal, UDP-GlnA, UDP-Man, UDP-Xyl, ADP-Glc, CDP-Glc and TDP-Glc. None of these nucleotide diphosphate sugars could be utilized by the recombinant enzyme. Among these nucleotide diphosphate sugars, UDP-Gal and ADP-Glc had also been tested previously with the *E. coli* enzyme, and both were shown to be poor substrates as well (Kamogawa and Kurahashi, 1965).

To further explore the amino acid residues critical for enzyme activity, a random mutagenesis experiment was performed using *E. coli* XL-Red, a strain with a high spontaneous mutation rate due to defects at multiple *mut* loci. Nine independent *E. coli* HP069 clones carrying a mutated pHC366 were identified, which displayed slow growth on the MacConkey-galactose plates. Only two out of the nine clones produced a protein with a size equivalent to that of the wild type enzyme on SDS-polyacrylamide gels. One of the two selected clones, *E. coli* HP069 [pHC3005], grew

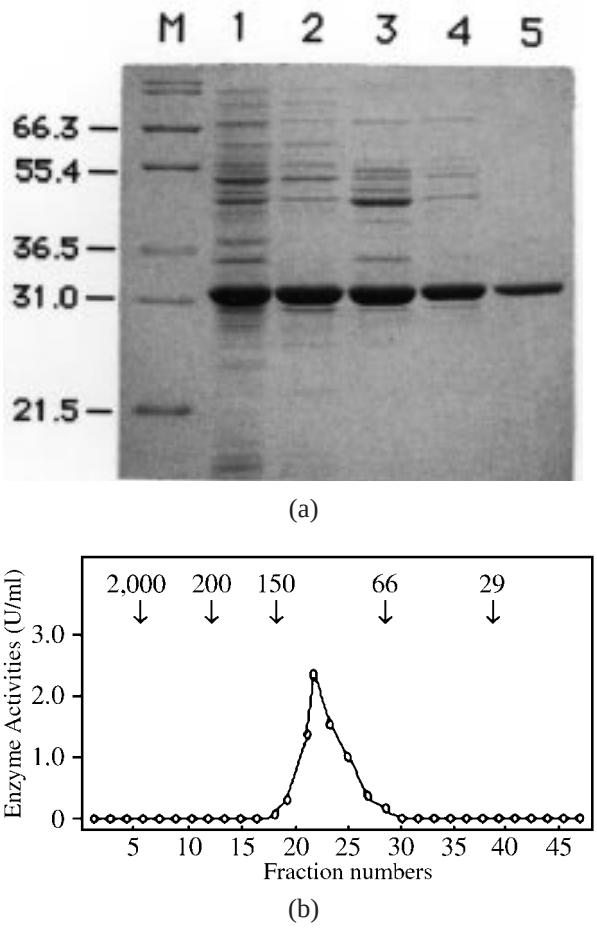

Fig. 6. Purification and molecular mass determination of recombinant UDP-Glc pyrophosphorylase. (a) Analysis of fractions of the purification procedure by SDS-polyacrylamide gel electrophoresis. Lanes M, molecular weight markers; 1, crude cell extract; 2, the enzyme preparation after 50 ºC treatment; 3, after 30-60% ammonium sulfate precipitation; 4, proteins from the pool after EconoPac DEAE-Blue cartridge; 5, proteins eluted from Sephacryl-300. (b) Determination of the molecular mass of the recombinant enzyme by gel permeation with a Sephacryl-300 HR 16/100 column. The sizes of the molecular weight standards are shown in kDa.

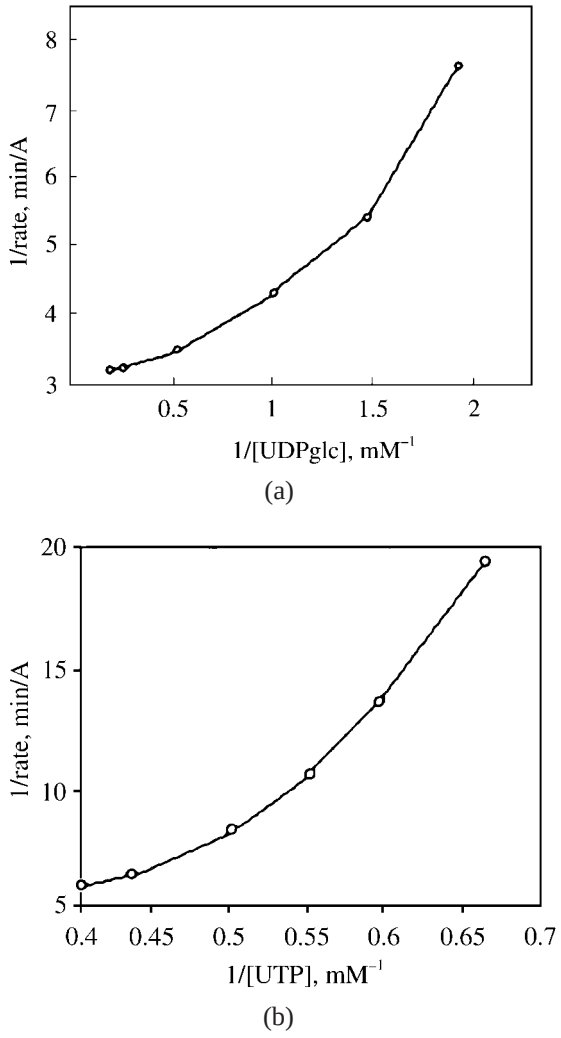

Fig. 7. Kinetic properties of the recombinant UDP-Glc pyrophosphorylase. The reaction was performed under standard conditions using different concentrations of either UDP-Glc (a) or UTP (b), and the enzyme preparation was performed after Econo Pac DEAE cartridge purification. The results are plotted in double reciprocal form using the DNRPEASY program (Duggleby, 1990).

poorly in galactose containing medium at all temperatures tested. The other one, *E. coli* HP069 [pHC3007], grew poorly at 25 °C and was apparently normal at both 37 °C (Fig. 8(a)). Analysis of the cell lysate showed almost no detectable UDP-Glc pyrophosphorylase activity in *E. coli* HP069 [pHC3005] whereas the levels of enzyme activity in *E. coli* HP069 [pHC3007] and *E. coli* HP069 [pHC366] was about equal when assayed at 37 °C . The enzyme was then purified from the *E. coli* HP069 [pHC3007], and its activity at different temperatures was determined. As shown in Fig. 8(b), the activity of the UDP-Glc pyrophosphorylase encoded by pHC3007 was more active at higher temperatures (i.e. 35-45 ºC) than that of the enzyme encoded by pHC366.

The nucleotide sequence of the two mutations affecting enzyme activity was subsequently verified. A point mutation at codon-261 (TAC -> CAC) that resulted in a tyrosine to histidine substitution was identified in pHC3005. The mutation of the *galU* gene on pHC3007 involved codon-84 (TAC -> TGC) and resulted in a tyrosine to cysteine substitution.

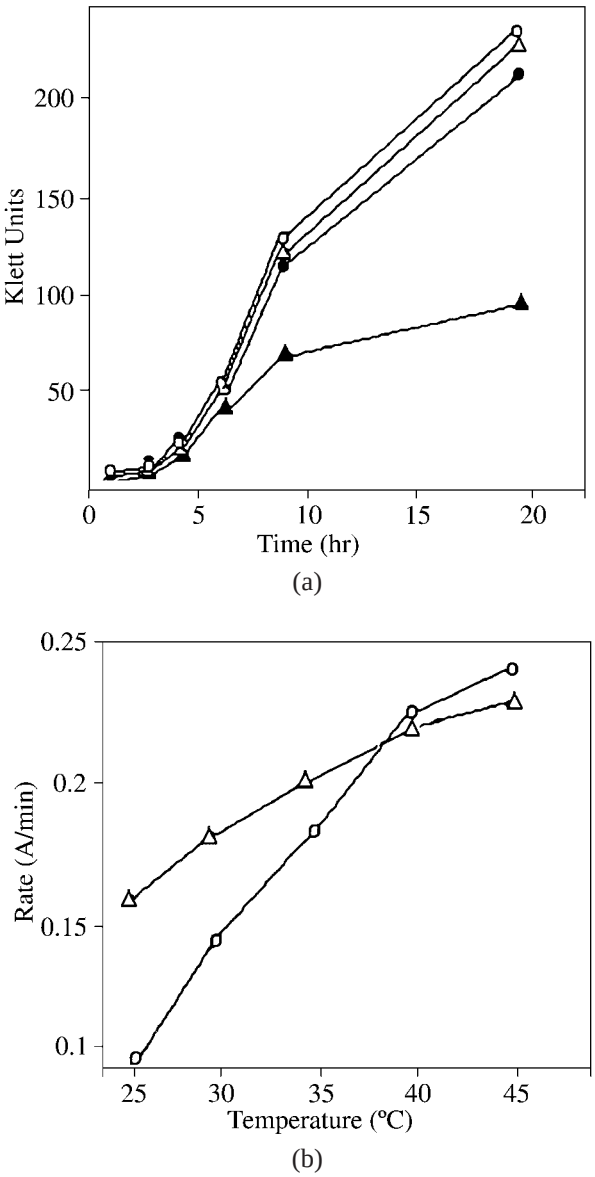

Fig. 8. Temperature dependent growth of *E. coli* HP069 [pHC3007] and *E. coli* HP069 [pHC366] and their UDP-Glc pyrophosphorylase activity. (a) Growth rates of *E. coli* HP069 [pHC3007] and *E. coli* Hp069 [pHC366] in were determined LB containing 0.4% of glucose (-○- and -△-) or galactose (-●- and -▲-) at either 25 ºC or 37 ºC. The circles indicate the density of the bacteria grown at 37 ºC, and the triangles represent those at 25 ºC. (b) UDP-Glc pyrophosphorylase extracted from *E. coli* HP069 [pHC366] (-△-) and *E. coli* HP069 [pHC3007] (-○-) was assayed under standard conditions over a range of temperatures.

IV. Discussion

The origin of the newly identified *galU* gene is somewhat puzzling. The gene is not likely to be a human gene since Southern blotting analysis and database searching of the human genome all turned out to be negative. In contrast, phylogenic analysis of the enzyme sequence (Fig. 3), the similarity in size (Fig. 4), Southern analysis of *Pseudomonas aeruginosa* ATCC 10145 genome (Fig. 2), and its proximity with the *gor* gene (Fig. 1) all suggest that the newly identified *galU* gene is derived from a *Pseudomonas* sp. Since *Pseudomonas* spp. are widely distributed in the environment, it is not surprising that the original hippocampus cDNA pool used for library construction may be contaminated with the bacterial DNA. This contamination would not be noticed if the library was screened by means of hybridization or by using an antibody, but would readily appear if functional screening, such as that performed in this study, was used.

It has been demonstrated that UDP-Glc pyrophosphorylase plays many important physiological roles in bacteria. These include regulation of osmotic strength in the cytoplasm (Giæver *et al.*, 1988), synthesis of virulence determinants (Sundararajan *et al.*, 1962; Markovitz, 1997, Dillard *et al.*, l995), flagellar formation (Komeda *et al.*, 1977), and galactose utilization (Leloir, 1951). In addition, it has been suggested that UDP-Glc is a potential intracellular signal molecule control the expression of σ^s and σ^s-independent genes in *E. coli* (Bohringer *et al.*, 1995). Despite its significance in the normal physiology of bacteria, most available information on UDP-Glc pyrophosphorylase concerns the *E. coli* and *S. typhimurium* enzymes. Among the reported bacterial UDP-Glc pyrophosphorylase encoding genes, only the *A. xylinum celA* and the *E. coli galU* products have been demonstrated to possess such enzyme activity. The other gene products, such as the *S. typhimurium* GalF (Jiang *et al.*, 1991) and the *B. subtilis* GtaB (Varon *et al.*, 1993), as well as many putative UDP-Glc pyrophosphporylase deduced from open reading frames in bacterial genome projects were assumed to be a UDP-Glc pyrophosphorylase on the basis of gene mapping and sequence similarity with the *A. xylinum* CelA. The biochemical properties of these gene products, however, have not been extensively determined. We have shown that the putative *Pseudomonas galU* gene described in this study clearly encodes a UDP-Glc pyrophosphorylase. The gene is capable of complementing a *galU* defect of an *E. coli* strain and greatly increases the UDP-Glc pyrophosphorylase activity in the mutant host. Furthermore, UDP-Glc was found to be the most preferred substrate for the purified enzyme. A *P. aeruginosa* PAO1 mutant with a defect at the *galU gene* is being constructed using the marker rescue strategy to investigate the physiological functions of the enzyme.

It is not clear why substitution of a non-conserved residue (Y261) completely abolished the enzyme activity. This phenomenon is not unique to the putative *Pseudomonas* enzyme, however. Three amino acid residues, Pro-14, Ala-16 and Leu-248, have been shown to be critical for the activity of the *E. coli* enzyme (Weissborn *et al.*, 1994). The first two residues, possibly the allosteric activator-binding sites (Thorson *et al.*, 1994), are conserved in the putative *Pseudomonas* and also in other bacterial UDP-Glc pyrophosphorylases. The Leu-248 of the *E. coli* enzyme and the amino acid residues around it, however, are not conserved among the identified bacterial UDP-Glc pyrophosphorylases. These data suggest that, rather than participating directly in catalysis, these residues most likely play a role in maintaining the proper conformation of the enzyme. How these diversified sequences are organized into a conformation for catalyzing the same reaction is a very interesting question worth pursuing.

Difficulty has been indicated previously in determining the Km for MgPPi in both the bovine and *E. coli* UDP-Glc pyrophosphorylase (Villar-Palasi and Larner, 1960; Kamogawa and Kurahashi, 1965). The problem, however, was not observed for the putative *Pseudomonas* enzyme, presumably due to the use of triethanolamine-HCI to replace Tris-HCI as the buffer system. In contrast, problems involving measurement of the Michaelis constants for UTP and UDP-Glc were encountered. This phenomenon could not be totally explained by a substrate-competing activity contaminating the enzyme preparation since the enzyme engineered with a 6 x His residues tag that was purified by means of affinity chromatography through the His-Bind resin (Novagene) yielded a similar result. In addition, if the reaction was allowed to proceed for a long enough period of time, almost all of the substrates could be used to covert NAD(P) into its reduced form. We speculate that the kinetic property is most likely due to cooperative activation of the enzyme by either UDP-Glc or UTP. Since the major function of UDP-Glc pyrophosphorylase is to provide UDP-Glc for biosynthesis of structural polysaccharides that is not essential for normal growth, the specific kinetic property would prevent the enzyme from competing with RNA polymerase for UTP, particularly when the concentration of UTP is low.

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假單胞桿菌尿嘧啶雙磷酸葡萄糖聚磷酸化E之生化分析

張晃猷[*]　黃秀珍[**]　李靜T[***]　彭慧玲[***]

[*]國立清華大學生命科學系
[**]國立中興大學農業生物科技研究所
[**]國立交通大學生物科技研究所

摘　要

利用大腸桿菌尿嘧啶雙磷酸葡萄糖聚磷酸化E之缺損株，進行功能性互補之篩選，我們得到一株極可能為假單胞桿菌產生此酵素之基因。此基因位於麩胱甘G還原E基因的上游，能合成約31-kDa的蛋白。隨後，我們以重組基因的技術將此基因在大腸桿菌中大量表現，並將基因產物進行純化及各項生化特性之鑑定。結果發現此重組之酵素具高度之受質專一性，分子量約為130-kDa，可能為四個相同之子單元所構成。此外我們也針對此基因進行了隨機突變的實驗，並決定了兩個能影響此酵素活性之點突變。