Expression of the *ptsH*$^+$ gene of *Escherichia coli* cloned on plasmid pBR322

A convenient means for obtaining the histidine-containing carrier protein HPr

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The gene *ptsH*$^+$, which specifies HPr on the *E. coli* genome, was cloned on the plasmid pBR322 and was expressed in *recA* cells. HPr was produced in large amounts and was characterized by several criteria.

Plasmid *HPr* E. coli *Phosphoenolpyruvate-dependent phosphotransferase system*

1. INTRODUCTION

The histidine-containing protein HPr plays an essential role in the uptake of many carbohydrates by facultative and obligate anaerobic bacteria [1]. It mediates the transfer of the phosphate group of phospho*enol*pyruvate to the C-1 or C-6 position of the sugars which have been taken up into the cells via proteins in the plasma membrane; the sugar-phosphates thus formed can directly enter metabolic pathways. HPr, of M_r approx. 7700, has been purified from *Staphylococcus aureus* [2] and from *Bacillus subtilis* [3]; a somewhat larger HPr (M_r approx. 9000) has been purified from *Salmonella* [4] and *Escherichia coli* [5]. There are only small amounts of HPr present in wild-type bacteria; in order to obtain yields of HPr adequate for study relatively large masses of organisms (100 g–2 kg) and, therefore, large volumes of bacterial cultures, have to be used [5–7]. Moreover, and for the same reason, the postulated phosphorylation and dephosphorylation of HPr could be seen only with the purified protein and could not be demonstrated in intact or broken cells.

Here, we report the cloning, on the plasmid pBR322, of *ptsH*$^+$ (the gene that specifies HPr on the *E. coli* genome) and its expression; we show that the material thus made is indeed HPr and that it can be phosphorylated by phospho*enol*pyruvate. Growth of cells bearing many copies of this plasmid enables over 100 mg of highly purified HPr to be obtained from only 5 litres of bacterial culture.

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2. MATERIALS AND METHODS

2.1. *Sub-cloning of DNA from phase λJM29 into plasmid pBR322*

We have described the isolation of a specialized transducing phage λJM29 that can complement *ptsH* mutants of *E. coli* [8] and from which a polypeptide of M_r 9000 can be expressed in UV-irradiated cells; we therefore concluded that λJM29 carries the *ptsH*$^+$ gene [9]. To sub-clone the appropriate region of DNA that carries this func-

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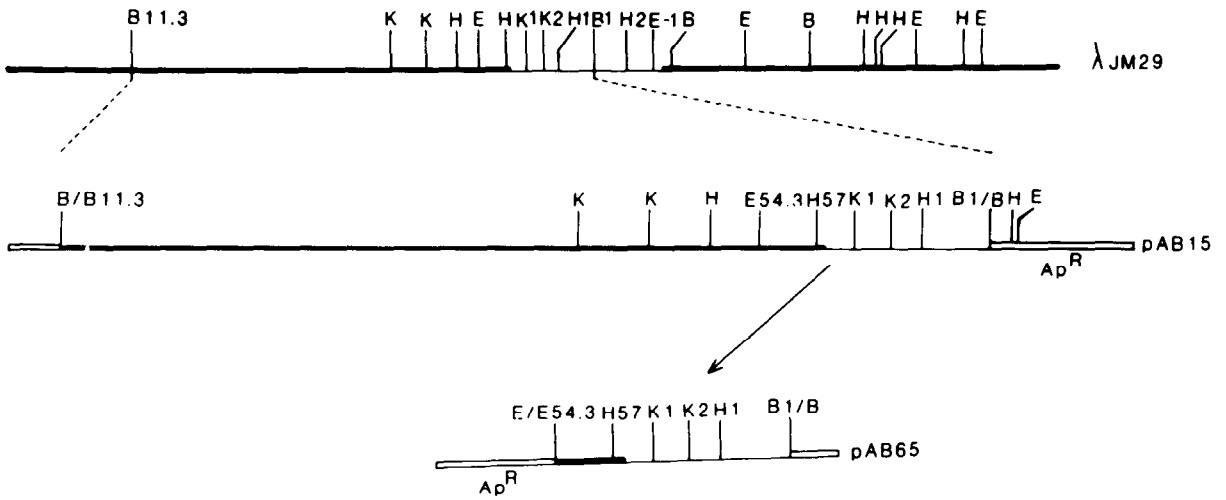

Fig. 1. Construction of plasmids pAB15 and pAB65 from the specialized transducing phage λJM29. The plasmid maps are shown with their ends at the *Aval* restriction site in plasmid pBR322 DNA. The K1–K2 fragment of plasmids pAB15 and pAB65 is 0.8 kb long; λJM29 is drawn to half this scale; (▬) λDNA; (▭) pBR322 DNA; (——) cloned *E. coli* DNA. Ap^R denotes the ampicillin resistance gene of pBR322; the sites at which restriction enzymes act are given as: E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; K, *Kpn*I.

tion into plasmid pBR322, the following procedure was adopted:

(i) DNA isolated from λJM29 was digested with the restriction endonuclease *Bam*HI, and the B11.3-B1 fragment (21.1 kilobases (kb) in length; fig. 1) was ligated into the *Bam*HI site of plasmid pBR322. The resultant plasmid, pAB15, was 25.5 kb long; it could complement *ptsH* mutants (i.e., it enabled such mutants now to grow upon sorbitol [8]) and thus carried $ptsH^+$.

(ii) In order to remove much of the 17 kb of λDNA carried on pAB15, this plasmid was digested with both *Eco*RI and *Bam*HI. This resulted in the release of the 5.34 kb fragment E54.3-B1 (fig. 1) and of a 3.98 kb fragment from the DNA originally in pBR322. Ligation of these two fragments gave rise to the 9.32 kb plasmid pAB65, which still carried the ampicillin resistance marker. Since it complemented *ptsH recA* mutants of *E. coli* and expressed a polypeptide of M_r 9000 in mini-cells, it also carried $ptsH^+$.

2.2. *Other methods*

The methods used for isolation of DNA, treatment with restriction endonucleases and ligases and transformation of competent *E. coli* strains have been described [8,10]. The presence of functional HPr in whole cells was assumed from the ability of such cells to grow upon sorbitol as sole carbon source [8]; it was confirmed by establishing that sonic extracts of such cells restored the ability of similar extracts of a *ptsH*-strain of *E. coli* to catalyse the phospho*enol*pyruvate-dependent phosphorylation of methyl α-glucoside. For this, the procedure used was essentially that in [11]; full details are given in the legend of table 2.

3. RESULTS AND DISCUSSION

3.1. *Purification of HPr from cells carrying the multicopy plasmid pAB65*

The *recA E. coli* strain PB13 transformed with the plasmid pAB65 was used as a source of HPr. A 5 l culture of the organism grown in Luria Broth, supplemented with ampicillin (100 μg.ml^{-1}) to stabilize the plasmid, was grown at 37°C; the cells (10.6 g wet wt) were harvested by centrifugation for 10 min in a Sorvall RC5B centrifuge, GSA rotor, at 10000 × *g*. They were suspended in 70 ml 10 mM Tris–HCl (pH 7.6) and were disrupted by sonic oscillation for 8 × 15 s (6 μm peak-to-peak) at 4°C with an MSE 8-75 MK2 sonicator. The DNA

and RNA were digested by adding 0.5 mg RNase and 0.5 mg DNase and incubating for 30 min. Cell debris was removed by centrifugation in a Sorvall RC5B centrifuge, SS-34 rotor, at 48000 × *g* for 2 h; the supernatant solution from this procedure was dialysed against 10 mM Tris–HCl (pH 7.6) at 4°C. The resultant material was chromatographed on a Whatman DE-52 DEAE column (2.5 × 7 cm) which had been pre-equilibrated with 10 mM Tris–HCl (pH 7.6); a linear concentration gradient (500 ml) of 0–100 mM NaCl in the Tris–HCl buffer was used and 5 ml fractions were collected. Those fractions that contained HPr were pooled and dialysed against 50 mM ammonium bicarbonate (pH 8.0); they were freeze-dried. The material thus obtained was redissolved in 2 ml 10 mM Tris–HCl (pH 7.6) and was further purified by gel permeation chromatography on a Sephadex G-50 column (2 × 57 cm) in 10 mM Tris–HCl (pH 7.6).

HPr was eluted from the DEAE column between 30 and 40 mM NaCl (fig. 2a). Analysis by SDS–PAGE, using 18% gels [9] showed the major polypeptide present to have an M_r of 9000 although some other peptides of higher M_r were also present. However, further purification on Sephadex G-50 (fig. 2b) removed those contaminating peptides. After two-dimensional electrophoresis of the resulting material (ampholines LKB, pH 3.5–10), only one spot was revealed by either silver or

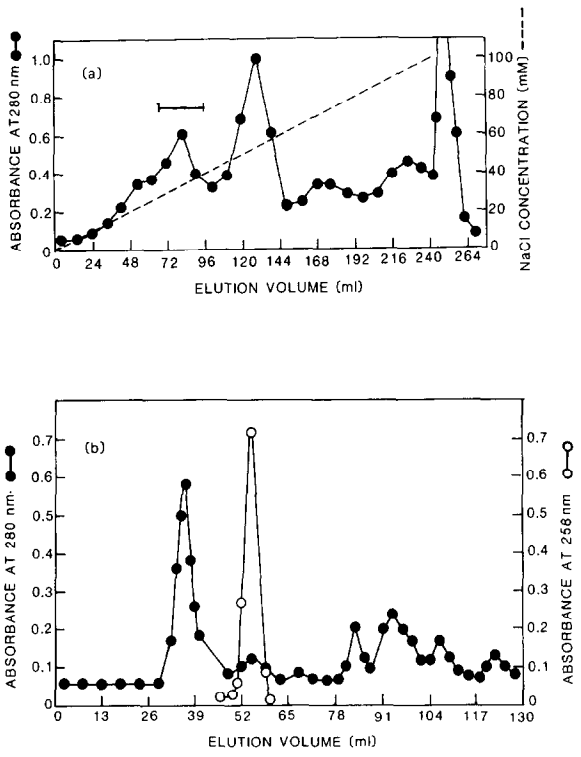

Fig. 2. Purification of HPr. HPr was initially purified by DEAE ion-exchange chromatography (a). The fractions that contained HPr are shown by a bar. After concentrating, by freeze drying, HPr was further purified by gel permeation chromatography on Sephadex G50 (b). The HPr was identified by the characteristic absorbance of phenylalanine at 258 nm.

Table 1

Amino acid composition of HPr

Amino acid	(mol. residue.mol^{-1}) Composition according to:			No. of residues in sequence [4]
	This paper[a]	[5] (24 h hydrolyses)	[12]	
Asp	3.2	3.6	5.2	3.0[c]
Thr	9.8[b]	10.0	8.9	9.0
Ser	6.5[b]	6.9	6.1	6.0
Glu	14.5	13.5	14.8	14.0[d]
Pro	2.6	2.2	2.6	2.0
Gly	6.3	6.7	7.2	6.0
Ala	9.8	9.4	8.7	9.0
Cys	n.d.	<0.05	<0.05	none
Val	7.5	7.1	6.4	7.0
Met	1.9	2.1	1.6	2.0
Ile	3.1	3.4	3.5	3.0
Leu	7.8	8.5	8.0	8.0
Tyr	none	<0.05	0.50	none
Phe	4.0	4.0	4.3	4.0
His	2.3	2.0	2.0	2.0
Lys	7.5	8.1	7.1	8.0
Arg	1.0	0.96	1.6	1.0
Trp	n.d.	<0.1	n.d.	none

[a] Samples (10 nmol) of salt-free HPr, dissolved in 6 M HCl containing 2-mercaptoethanol (0.05%) and phenol (0.05%), were hydrolysed at 105°C for 24, 48 and 72 h [17]. No differences were observed between these times of hydrolysis except for Thr and Ser; only the data for 72 h are shown

[b] Determined after extrapolation to zero time

[c] Consists of 2 Asn and 1 Asp from sequence data

[d] Consists of 7 Gln and 7 Glu from sequence data

n.d. Not done as sequence reported none

Coomassie blue staining, which indicated that the HPr thus prepared was essentially pure. The total yield obtained was 98 ± 3 mg. According to the amino acid analysis of the protein (table 1), the material was devoid of tyrosine; this accorded well with the distribution of amino acids reported [4] for the *Salmonella* HPr sequence. In further agreement with [4] but in contrast with [5,12], spectral analysis revealed no shoulder at 280 nm, which indicated the absence of tyrosine and tryptophan.

That the purified protein was indeed functional HPr was shown in two main ways:

(i) It restored the activity of an extract of the *ptsH*-strain JM1681 of *E. coli* to effect the overall phosphorylation of methyl [^{14}C]glucoside when incubated with it and with phospho*enol*pyruvate (table 2).

(ii) Antibodies raised against it, which did not react with extracts of *ptsH* mutants, were shown to react both with extracts of *ptsH*$^+$ cells and with a sample of HPr purified from a different strain of *E. coli* and kindly given to us by Dr E.B. Waygood (University of Saskatchewan, Saskatoon); antibodies raised against Dr Waygood's purified HPr reacted with the material purified from PB13(pAB65). In agreement with [13] our HPr-antibodies did not

Table 2

Production of methyl α-glucoside 6-phosphate using increasing amounts of purified HPr

nmol HPr added	nmol methyl α-glucoside phosphate produced
0	0
0.018	44.8
0.035	39.8
0.070	58.7
0.350	60.5
0.700	69.6

The incubation mixtures contained in 100 μl final vol. 10 mM KF, 5 mM $MgCl_2$, 5 mM phospho*enol*pyruvate, 1 mM methyl α-[U-^{14}C]glucoside (0.25 μCi: Amersham International plc), 50 mM potassium phosphate (pH 7.2) and a sonic extract of a *ptsH* strain of *E. coli* (1 mg dry mass total). Various amounts of HPr were added as shown. After 30 min at 37°C, the reaction was stopped by adding 900 μl ice-cold water; samples (50 μl) were adsorbed onto Whatman DE81 paper discs (2.3 cm) and unphosphorylated material was washed off with 100 ml water. The radioactivity of the dried discs, suspended in 10 ml of scintillation fluid (0.4% 2,5-diphenyloxazole in 80% (v/v) toluene and 20% (v/v) methoxyethanol), was assayed in a Tri-carb scintillation spectrometer

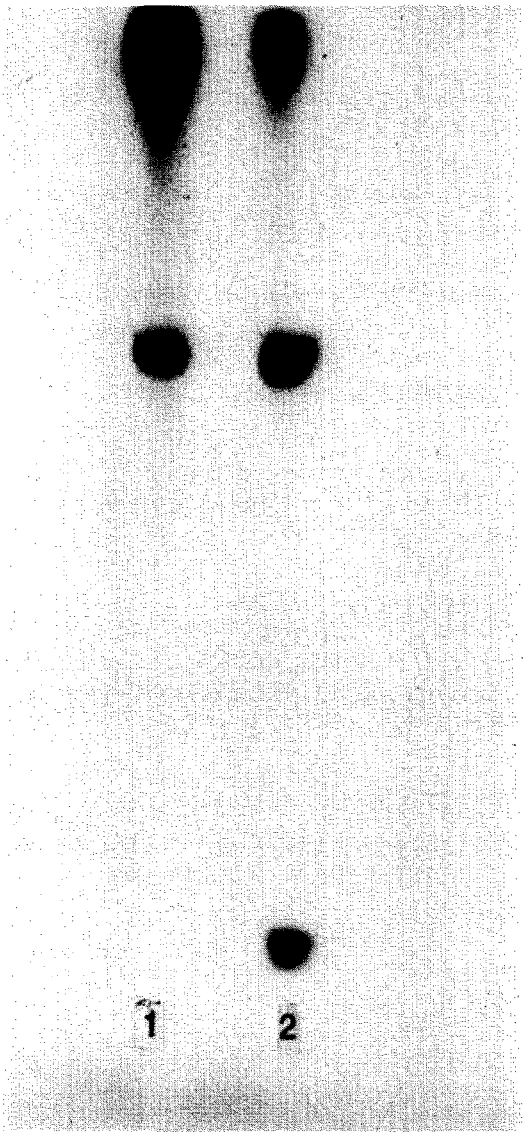

Fig. 3. In vitro phosphorylation of HPr by [^{32}P]phospho*enol*pyruvate. An *E. coli* strain lacking plasmid pAB65 did not produce a detectable phosphorylated HPr (track 1) whereas a strain containing plasmid pAB65 did (track 2). This phosphorylated protein had a M_r of 9000 and electrophoresed with HPr stained by Coomassie blue. The two other phosphorylated proteins observed are of higher M_r and are formed independently of the presence of the plasmid pAB65.

react against the fructose-specific carrier protein FPr [14].

The HPr expressed by the plasmid pAB65 could be phosphorylated by [^{32}P]phospho*enol*pyruvate [15] even in crude extracts of the cells, though no phosphorylated HPr was detected in extracts of cells lacking the plasmid (fig. 3). This may be because the normal levels of HPr are very low, and/or because the phosphorylated form of HPr is very unstable at pH 7.2 [16]; it would be expected that the balance between phosphorylation and dephosphorylation would be tilted towards the accumulation of phospho-HPr only if the concentrations of HPr were raised considerably, as happens in organisms that carry the multicopy plasmid pAB65.

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