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A novel phosphate-regulated expression vector in *Escherichia coli*

(Prokaryotic expression vector; recombinant DNA; *ugp* promoter; *pho* regulon; phosphate regulation)

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SUMMARY

The *ugp* promoter (p_{ugp}) responsible for expression of the binding-protein-dependent *sn*-glycerol-3-phosphate transport system in *Escherichia coli* was cloned into a small multicopy plasmid pTER5, a derivative of pBR322, between the transcription terminators *rpoCt* and t_{L1}. The resulting expression vector, pPH3, permits convenient insertion of structural genes containing their own translational-initiation regions, into the multiple-cloning site derived from the pUC19 plasmid. The efficiency and regulatory properties of p_{ugp} were measured using *xylE* and *lacZ* as reporter genes, which code for the corresponding enzymes catechol-2,3-dioxygenase (C23O) and β-galactosidase (βGal), respectively. Enzyme activities were virtually completely repressed in the presence of excess inorganic phosphates (P_i) and high concentrations of glucose. Maximal induction was observed at limiting P_i (<0.1 mM) and normal levels of glucose (0.2–0.4%). The maximum expression of the p_{ugp}-directed βGal synthesis was approx. 80% of that directed by strong p_{tac}. When the *xylE* gene was maximally expressed, the induced enzyme constituted approx. 50% of total cellular protein as judged by laser densitometry following sodium dodecyl sulfate–polyacrylamide-gel electrophoresis. These results suggest the usefulness of the p_{ugp} in expression vectors for strong, but controlled, expression of cloned genes in *E. coli*. This P_i controlled vector can be adapted to large-scale fermentation by using P_i-limiting growth conditions.

INTRODUCTION

Promoter strength and its controllability are two important criteria in the construction of a good expression system. The controllable expression can often be achieved by manipulating the level of regulatory factors, such as specific repressors, co-repressors or inducers (Pouwels et al., 1985). It can also be achieved by control of the copy number of the vector carrying the gene of interest (Pouwels et al., 1985) or by promoter inversion (Hasan and Szybalski, 1987). Many previously described expression systems require inducers such as IPTG which are expensive. For large-scale or repeat experiments such methods may become rather unattractive options. Therefore, it is desirable to have a vector in which

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Abbreviations: A_{600}, absorbance at 600 nm; Ap, ampicillin; βGal, β-galactosidase; *bla*, gene encoding Bla; Bla, β-lactamase; bp, base pair(s); CRP, cAMP receptor protein; C23O, catechol 2,3-dioxygenase; Δ, deletion; *E.*, *Escherichia*; G3P, *sn*-glycerol-3-phosphate; IPTG, isopropyl-β-D-thiogalactopyranoside; kb, kilobase(s) or 1000 bp; MCS, multiple-cloning site; *ori*, origin of DNA replication; PAGE, polyacrylamide-gel electrophoresis; P_i, inorganic phosphate; PolIk, Klenow (large) fragment of *E. coli* DNA polymerase I; p_{tet}, *tet* promoter; p_{ugp}, *ugp* promoter; ^{R}, resistance; RBS, ribosome-binding site; *rpoCt*, *rpoC* terminator; SDS, sodium dodecyl sulfate; *sn*, stereospecific numbering; *tacp/o*, *tac* promoter and operator; TG, Tris(hydroxymethyl)aminomethane and glucose (Echols et al., 1961); t_{L1}, first terminator of the major leftward transcription in phage λ; *tsp*, transcription start point(s); *ugp*, *sn*-glycerol-3-phosphate uptake operon; *xylE*, gene encoding C23O; [ ], designates plasmid-carrier state.

gene expression can be controlled by the levels of nutrients such as P_i.

P_i is an attractive nutrient for purposes of gene regulation for the following reasons: in *E. coli*, the response to P_i starvation is rapid with maximum expression of the genes involved in this stress response achieved in as little as 30–60 min (Schweizer and Boos, 1985). In addition, several genes in the *pho* regulon, e.g. *ugpB* for the G3P binding protein, are expressed at high levels (Schweizer and Boos, 1984). The aim of this study was to develop a novel p_{ugp}-based expression system that is under P_i control.

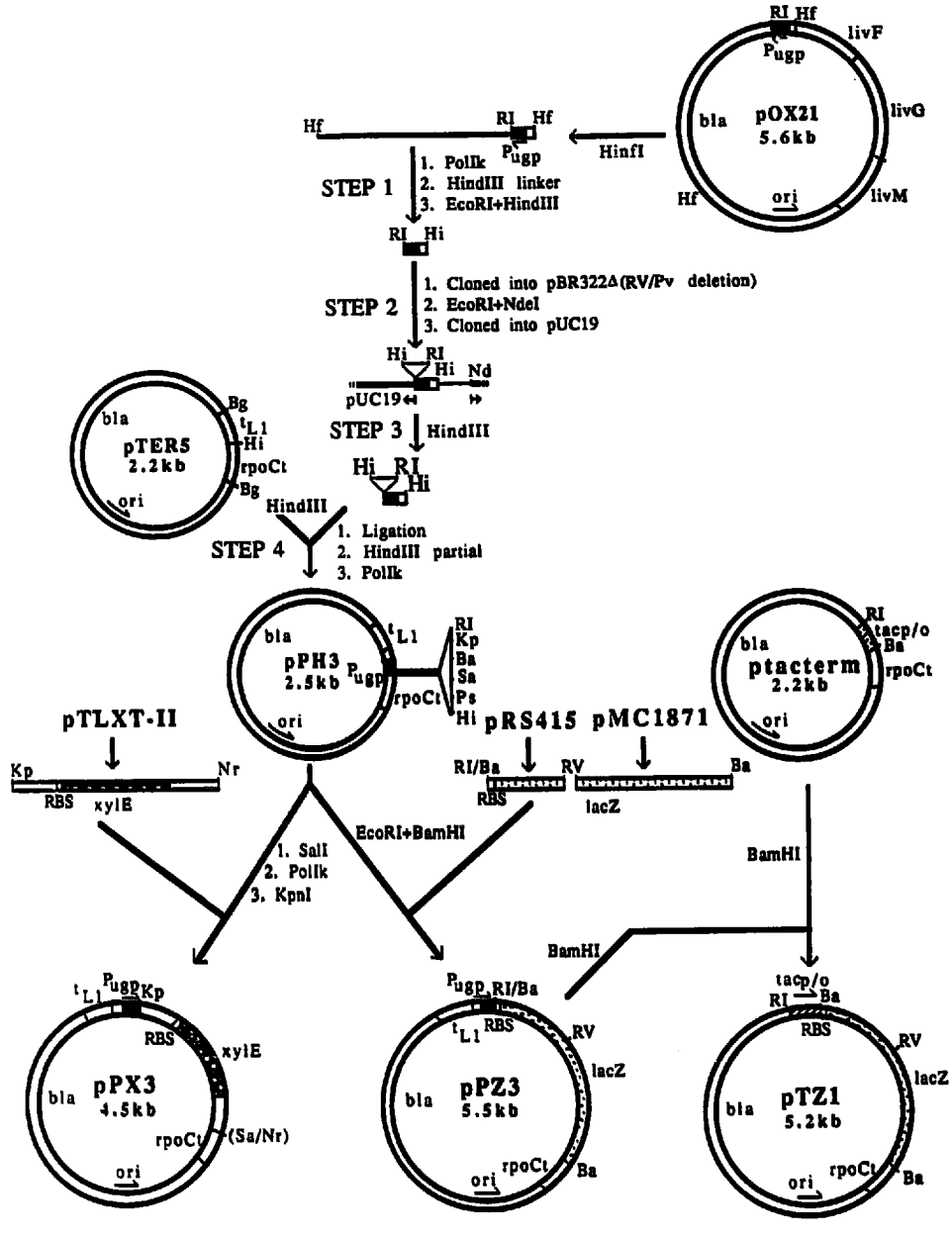

Fig. 1. Construction of plasmids pPH3, pPX3, pPZ3 and pTZ1. A 174-bp fragment from pOX21 (Penelope et al., 1985), which contains the p_{ugp} and its regulatory sequence flanked by *Eco*RI and *Hin*fI, sites, was subcloned into plasmid pTER5 containing the transcription terminators, *rpoCt* and t_{L1} in four steps: (1) replacement of the blunt-ended *Hin*fI site with a *Hin*dIII site by ligating with synthetic *Hin*dIII linkers; (2) positioning of the *Eco*RI-*Hin*dIII fragment next to the *Eco*RI site of the MCS of pUC19; (3) isolation of the *Hin*dIII fragment, which contains the MSC and the p_{ugp}; and (4) insertion of the isolated fragment into the same site of pTER5, followed by elimination of the extra *Hin*dIII site upstream from the p_{ugp} by *Hin*dIII partial digestion and filling in the single-stranded ends. The resulting p_{ugp} expression vector is designated pPH3. To construct pPX3 the *Kpn*I-*Nru*I fragment containing the *xylE* gene was isolated from the promoter probe plasmid pTLXT-11 (Tseng et al., 1988) and inserted into pPH3 treated in succession with *Sal*I, PolIk and *Kpn*I. To construct pPZ3 the *Eco*RI-*Eco*RV 5′ *lacZ* fragment and the *Eco*RV-*Bam*HI 3′*lacZ* fragment were isolated from pRS415 and pMC1871, respectively (Simons et al., 1987; Casadaban et al., 1983), and ligated between the *Eco*RI and *Bam*HI sites on pPH3 plasmid. The pTZ1 plasmid was obtained by inserting the *Bam*HI fragment of the *lacZ* gene from pPZ3 plasmid into the vector ptacterm (Paluh and Yanofsky, 1986). Ba, *Bam*HI; Bg, *Bgl*II; Hf, *Hin*fI; Hi, *Hin*dIII; Kp, *Kpn*I; Nd, *Nde*I; Ps, *Pst*I; Pv, *Pvu*II; RI, *Eco*RI; RV, *Eco*RV. (P = p_{ugp}).

EXPERIMENTAL AND DISCUSSION

(a) Construction of p_{ugp} expression vector

The construction of the p_{ugp}-based expression vector pPH3 (2.5 kb) is outlined in Fig. 1. This high-copy-number plasmid contains: (1) an Ap^R marker; (2) two terminators, *rpoCt* and t_{L1}; (3) the MCS in between the two terminators; and (4) the p_{ugp} immediately upstream from the MCS. The nt sequence from the *tsp* before the MCS is: GCGAGCA-TAAAACGCGT.

(b) Construction of promoter-reporter gene plasmids

Three expression plasmids, pPX3, pPZ3 and pTZ1, were constructed as outlined in Fig. 1. The *xylE* gene for C23O was subcloned from pTLXT-11 (Tseng et al., 1988) into the MCS of the vector pPH3 (pPX3). The *lacZ* structural gene was subcloned from plasmids pRS415 (Simons et al., 1987) and pMC1871 (Casadaban et al., 1983) into vectors pPH3 and ptacterm (Paluh and Yanofsky, 1986), respectively (pPZ3 and pTZ1).

(c) Regulated expression of the p_{ugp}-*xylE* and p_{ugp}-*lacZ* genes

Our recent studies show that, in addition to the dominant *pho* regulation, the p_{ugp} is also subject to cAMP-CRP-mediated control (T.-Z.S. and D.L.O., in preparation). Under high concentrations of both P_i and glucose more than 98% of expression directed by the p_{ugp} could be repressed even after the cells were at steady state of growth for 6 h (Table I), and the basal level of βGal activity from the p_{ugp} was nearly the same as that from the repressed p_{tac} (about 1.7%). The P_i concentration (6.4 mM) as in Table I is in much excess. To adapt this expression vector to large-scale synthesis the concentrations of P_i and other nutrients, such as carbon, can be adjusted in such a way (e.g., 0.5–0.6 mM for P_i) that the P_i becomes limiting when the desired cell mass is achieved. The cells will stop growing and the cloned gene will be expressed. For the purpose of the study reported here the induction of p_{ugp} was achieved by diluting P_i to 0.1 mM and glucose to 0.2–0.4%. Under these conditions the specific activity of the enzymes expressed from the p_{ugp} reached a maximum in approx. 6–8 h after exponential growth.

TABLE I

Effect of glucose on the *pho*-independent expression of p_{ugp}-*lacZ* and p_{ugp}-*xylE* genes

Glucose[a] (%)	βGal[b] (unit/A_{600})	C23O[b] (unit/A_{600})
0.2	5794	645
0.5	782	103
1.0	578	8
2.0	485	2

[a] Cells, JM109[pPZ3] (*Δlac-proAB*, *lacI*^{q}) and DH5αF′ [pPX3], were grown overnight in TG medium supplemented with 6.4 mM K_2HPO_4 (high P_i), harvested, washed twice with P_i- and glucose-free medium, and then inoculated into the same high P_i medium, but with various concentrations of glucose.

[b] The cells were harvested after 6 h at stationary phase and subjected to enzyme assays (Miller, 1972; Tseng et al., 1988). The specific activities shown here are expressed as nmol of products liberated/min in A_{600} = 1 of lysed cells.

(d) Relative strength of the p_{ugp}

Using laser densitometry of the stained bands on the SDS–PAGE gels the induced C23O from the p_{ugp} in plasmid pPX3 was shown to represent approx. 50% of the total cellular proteins (Fig. 2), a level which is much higher than that obtained from p_{tet} in plasmid pTS92 (Inouye et al., 1981). The relative strength of p_{ugp} was further tested by comparing enzyme activities expressed from the p_{ugp}, p_{tet} and p_{tac}. The activity of Bla, encoded by *bla* gene on these

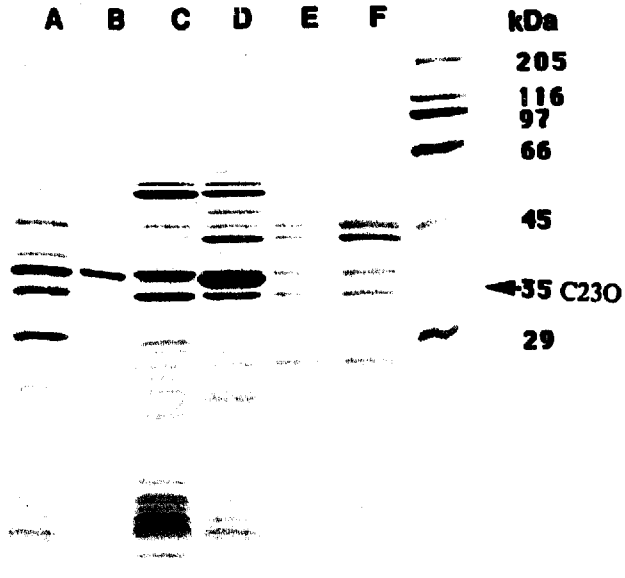

Fig. 2. Comparison of P_{tet} (plasmid pTS92) and P_{ugp} (plasmid pPX3). The overnight culture of DH5αF′ strain (*Δlac*169, *recA*1) harboring plasmids pTS92, pPX3 or pPH3 (control) was grown in TG medium (Echols et al., 1961) supplemented with K_2HPO_4 (1.0 mM), Ap (50 μg/ml) and aa (20 μg/ml each). The cells were harvested, washed twice with P_i-free TG medium, and then resuspended in 10 vols. of high P_i or low P_i TG medium and shaken at 37°C. Samples from low P_i medium (lanes D and F) and high P_i medium (lanes A, C and E) were removed after overnight growth (about 8 h after exponential growth). The whole-cell lysates (corresponding to A_{600} = 0.25) were electrophoresed through a 0.1% SDS–15% PAGE and stained with Coomassie brilliant blue. The C23O was purified according to the procedure by Nozaki (1970). Protein standards (in kDa) are shown on the right margin. Lanes: A, lysate from cells harboring the p_{tet}-*xylE* plasmid pTS92; B, purified C23O; C–D, lysate from cells harboring p_{ugp}-*xylE* plasmid pPX3; E–F, lysate from cells containing the p_{ugp} expression vector pPH3.

TABLE II

Relative strength of the p_{ugp}

Host[a]	Plasmid/promoter	βGal[b] (A)	C23O[b] (B)	Bla[b] (C)	Relative activity (A or B/C)	Relative promoter activity	
						Promoter	Ratio
JM109	pTZ1/p_{tac}	37878		152	249	p_{tac}/p_{ugp}	1/0.80
JM109	pPZ3/p_{ugp}	28300		143	198		
DH5aF'	pPX3/p_{ugp}		11600	152	76.3	p_{ugp}/p_{tet}	7/1
DH5aF'	pTS92/p_{tet}		3200	296	10.8		

[a] Cells were grown overnight and treated as described in Fig. 2. The washed cells were then diluted into 10 vols. TG medium with 0.064 mM K_2HPO_4 (low P_i). For JM109[pTZ1] strain, 1 mM IPTG was added after the inoculation. Aliquots of cells were withdrawn every hour and immediately put on ice. Chloramphenicol was added to the samples to produce 100 μg/ml final concentration.

[b] The ice-cold cells (0.5 ml) were pelleted, washed once, and resuspended in 0.1 M Tris · HCl (pH 8.0) for βGal and Bla assays or in 0.05 M K_2HPO_4 containing 10% acetone (pH 7.5) for C23O assays. For βGal and C23O a differential rate of synthesis was determined, using the slope of the line ($R > 0.99$) generated when enzyme activity is plotted against cell growth (A_{600}). The unit of the differential rate was calculated as nmol of product liberated/min/A_{600}.

promoter-fusion plasmids, was also measured to correct for variable plasmid copy number. The data in Table II indicate that the p_{ugp} appears to be 80% as efficient in promoting expression as the strong p_{tac} and about seven times stronger than the p_{tet}, suggesting that the p_{ugp}-based expression vector can also be used for high-level expression.

(e) Conclusions and discussion

(1) The gene to be expressed using the p_{ugp}-based expression vector pPH3 should contain RBS, however, if translation initiation sequences are inserted at the MCS by site-directed mutagenesis, the resulting vectors will be suitable for expression of genes without initiation signals.

(2) The expression directed by the p_{ugp} was virtually completely repressed at excess P_i (> 1 mM) and high concentrations of glucose (> 1%). The induction appeared when cells were deprived of P_i. For a large-scale fermentor dilution to lower the P_i level is impractical. As an alternative, the starting levels of P_i and glucose in the growth medium can be adjusted, depending on the growth conditions, so that only the P_i becomes limiting when cells reach a predetermined mass. The P_i-starved cells will now produce the desired protein. If the protein to be made is not toxic to the cells, continuous culture can also be an attractive approach to make large quantities of protein of interest by the *pho*-regulated expression system (Pages et al., 1987).

(3) The C23O expressed from the p_{ugp} corresponded to 50% of the total cellular protein. Quite similar results were obtained utilizing p_{ugp}-*glpK* fusion (data not shown). The promoter strength and controllability of the p_{ugp} is close to the strong p_{tac}.

(4) The p_{ugp} is useful for expression of proteins possibly in all *E. coli* strains harboring a functional *pho* regulatory circuit (Wanner, 1987). The p_{ugp} can be used for expression of genes not only when the genes are integrated into a specialized expression vector such as pPH3, but also when integrated into more commonly used cloning vectors, such as pBR322 or pUC19 (data not shown). Therefore, the DNA fragments harboring the portable, P_i-repressible, p_{ugp} may be of general use for expression of genes in already existing replicons.

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