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Broad-host range expression vectors containing manipulated *meta*-cleavage pathway regulatory elements of the TOL plasmid

Juan L. Ramos, Manuel Gonzalez-Carrero and Kenneth N. Timmis

Department of Medical Biochemistry, University Medical Centre, University of Geneva, Geneva, Switzerland

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The construction of pERD20 and pERD21, two broad-host range expression vectors, is described. The vectors contain the Pm promoter of the *meta*-cleavage pathway operon of the TOL plasmid pWWO; this promoter is present within a polylinker which provides a number of downstream cloning sites close to the transcription initiation site. Transcription from the Pm promoter in these vectors is controlled not by the natural positive regulator of Pm, the XylS protein, but by an XylS mutant analogue, XylS2tr6, which exhibits an altered effector specificity and can mediate a 3–8-fold higher level of transcription than can XylS in a wide range of temperatures. Controlled expression of cloned genes can be achieved in a broad spectrum of Gram negative bacteria grown at a wide range of temperatures.

Catabolic plasmid; Gene cloning; Gram negative bacteria; Positive regulation; Promoter

1. INTRODUCTION

The cloning and expression of genes is a central tool in biomedical research. Although *E. coli* is by far the most useful host for gene cloning, its inability to express some biological properties, on the one hand, and to carry out appropriate modifications and processing of certain gene products, on the other, have encouraged the development of a variety of alternative host-vector systems. Expression of foreign genes, and even overexpression of native genes, can significantly perturb host cell physiology and constitute a strong selective pressure for elimination or inactivation of cloned genes [1]. Vectors in which the expression of cloned genes can be experimentally regulated [2–5] have therefore become increasingly important.

Recently we described the construction of a regulated expression vector, pNM185, for gene cloning in a wide range of Gram negative bacteria [2]. The genetic elements responsible for regulated expression of cloned genes in this vector are derived from the TOL plasmid-carried *meta*-cleavage pathway operon and consist of the operon promoter Pm and the gene of the positive regulator of this promoter, *xylS* [2]. Activation of the XylS protein by benzoate or methylbenzoate results in induction of transcription from Pm [6]. Regulated expression of cloned test genes has been demonstrated in all but three of eighteen different Gram negative bacteria tested [2].

This paper describes several manipulations of the XylS protein which improve the regulatory circuit characteristics, as well as the incorporation of additional cloning sites close to Pm promoter, which thereby confer further desirable properties upon the expression vector.

Correspondence address: J.L. Ramos, CSIC, Estación Experimental del Zaidin, Apto 419, 18080 Granada, Spain

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2. MATERIALS AND METHODS

2.1. *Bacterial strains and plasmids*

These are listed in table 1.

2.2. *Media*

Bacteria were routinely grown in LB medium. Solid medium was AM3 (Difco) or M9 minimal medium [7], except that RM medium was used for *Azotobacter vinelandii* [8]. Antibiotics were added at the following concentrations (μg/ml): 25 Km; 10 Tc; 100 Ap for *E. coli*; 25 Km for *Pseudomonas* strains; 1 Km for *Azotobacter*; and 25 Km for *Shigella, Aeromonas* and *Agrobacterium*.

2.3. *Manipulation of nucleic acids*

Procedures for plasmid isolation, transformation, cleavage by restriction enzymes, analysis by agarose gel and gene cloning have been reported [2,7].

2.4. *Enzyme assays*

β-Galactosidase (β-Gal) activity was determined in permeabilized whole cells as described [6]. Units are as given by Miller [9].

2.5. *Materials*

*Bcl*I linker d(pCTGATCAG) was purchased from New England Nuclear.

3. RESULTS AND DISCUSSION

3.1. *Construction of pERD20 and pERD21*

During the course of a study of XylS protein interaction with effector molecules, we isolated pNM185 derivatives that produced mutant XylS proteins exhibiting new effector specificities [6]. One such protein, XylS2, encoded by the *xyl*S2 allele present on plasmid pERD2, not only was activated by 3-methylsalicylate, which does not activate the XylS protein, but also mediated higher levels of transcription from Pm. 3-Methylsalicylate provoked a 60-fold increase in expression of *lac*Z from Pm in *xyl*S2-carrying bacteria whereas *m*-toluate, the best effector of XylS, provoked only a

Table 1

Strains and plasmids used in this work

Strains or plasmids	Relevant characteristics	Source or reference
Bacteria		
Escherichia coli 5K	*res*K⁻,*thr*,*leu*31,*thi*,*ton*A,*sup*E	Robson, R.L.
E. coli GM272	*dam*3,*dcm*6,*met*B1,*gal*K2,*gal*T22, *lac*Y1,*hsd*21,*stx*78,*mtl*1,*sup*E44,*thi*1, *ton*A31	11
Pseudomonas putida KT2440	*hsd*R1,*hsd*M⁺	2
P. aeruginosa PAO 1162	prototroph	Mermod, N.
Azotobacter vinelandii UW_r	*nif*⁺,rif^r	Kennedy, C.
Aeromonas hydrophila AM133	prototroph	Mermod, N.
Agrobacterium tumefaciens	prototroph	Mermod, N.
Shigella flexneri G108-1-13	*thi*,*pur*E	13
Plasmids		
pEMBL9	Ap^r	14
pJLR100	Ap^r, Pm in pEMBL9	6
pUC19	Ap^r	15
pJLR107	Pm::*lac*Z, Ap^r	6
pJLR200	Pm::*Tet*, Ap^r, pBR322 hybrid	6
pNM185	*Inc*Q, pKT231-based expression vector, *xyl*S⁺,Pm,Km^r	2
pERD2	pNM185 derivative carrying *xyl*S2 allele,Km^r	6

17-fold increase in expression of *lacZ* in *xyl*S-carrying bacteria [6].

The *xyl*S and *xyl*S2 genes are expressed from their natural promoters at low levels at 30°C, but these levels are sufficient to effectively induce expression of genes under the control of Pm [10]. However, the expression of *xyl*S and *xyl*S2 at 37°C is reduced and at 40°C undetectable (Ramos, J.L., unpublished). This in turn leads to reduced and negligible expression from Pm when bacteria bearing *xyl*S and *xyl*S2 are grown at 37°C and 40°C, respectively, in the presence of their effectors (table 2). In order to increase the temperature range of the regulated expression capability of the vector, a desirable property when using the expression vector in thermophilic bacteria, mutants exhibiting temperature-resistant *xyl*S2-mediated activation of the Pm promoter were sought. *xyl*S2tr6 in pERD2tr6 is an *xyl*S2 derivative that is expressed at 40°C and which mediated notable expression from Pm at that temperature when grown in the presence of 3-methylsalicylate (see table 2). pERD2tr6, an EMS-induced pERD2 derivative, was isolated by virtue of it conferring resistance to tetracycline at 40°C in a medium containing 3-methylsalicylate to *E. coli* bearing pERD2 and pJLR200, the latter being a pBR322 derivative in which the tetracycline resistance determinant is expressed from Pm [6].

pNM185 and its derivative pERD2tr6 contain three useful cloning sites, *Eco*RI, *Hpa*I and *Sst*II, approx. 300 bp downstream of Pm [2]. In order to provide additional cloning sites closer to Pm, a polylinker was introduced into pERD2tr6 as follows: a 401 bp *Pst*I fragment carrying Pm was transferred from pJLR100 into the *Pst*I site of pUC19. One resulting plasmid, pMGC1, contained 11 cloning sites in front and adjacent to Pm (fig.1). Plasmids pMGC1 and pERD2tr6 were totally digested with *Eco*RI; pMGC1 was then partially digested with *Pst*I, and pERD2tr6 was totally digested with *Pst*I. The 445 bp *Eco*RI-*Pst*I fragment of pMGC1 which contains Pm in the polylinker, and the 13.5 kb *Eco*RI-*Pst*I fragment of pERD2tr6, were purified by electrophoretic fractionation of endonuclease digested DNA on agarose gels, followed by electroelution of the relevant bands from the gel. The fragments were then ligated to produce plasmid pERD20 (fig.1). This plasmid contains 6 unique sites downstream of Pm: *Eco*RI, *Sst*I, *Kpn*I, *Sal*I, *Hpa*I and *Sst*II, the latter two sites being those originally present in pNM185. The other sites in the polylinker, *Sma*I, *Ava*I, *Pst*I, *Bam*HI, *Xba*I, *Sph*I and *Hin*dIII are not unique in pERD20.

In order to introduce a further unique site that could be used for the cloning of DNA fragments produced by *Sau*3A and other enzymes generating similar termini, a *Bcl*I linker was introduced into the *Sma*I site in the polylinker of pERD20 by partially digesting pERD20 with *Sma*I, isolating linearized plasmid from an agarose gel, and inserting the linker sequence d(pCTGATCAG). The ligation mixture was transformed into *E. coli* LG272 (dcm^-dam^-) [11] and Km^r clones were selected. This selection discriminated against

Table 2

Influence of cultivation temperature upon induction of synthesis of β-galactosidase in bacteria carrying a Pm::*lacZ* fusion and different *xyl*S alleles

Effector	Allele:	*xyl*S			*xyl*S2			*xyl*S2tr6		
	t (°C):	30	37	40	30	37	40	30	37	40
None		126	111	40	142	138	91	194	205	147
Added		2125	882	50	8524	3450	120	12416	10660	1000
Induction ratio		17	8	1	60	25	1	64	52	7

E. coli 5K bacteria containing pJLR107 (Pm::*lacZ*) and pNM185 (*xyl*S), pERD2 (*xyl*S2) or pERD2tr6 (xylS2tr6) were cultured overnight in LB containing Ap and Km. Cells were then diluted 100-fold in the same medium supplemented or not with 3-methylbenzoate (1 mM) for bacteria bearing pNM185, or 3-methylsalicylate (1 mM) for bacteria bearing pERD2 or pERD2tr6. After further cultivation for 5 h at the indicated temperature, bacteria were harvested and their β-galactosidase levels were measured

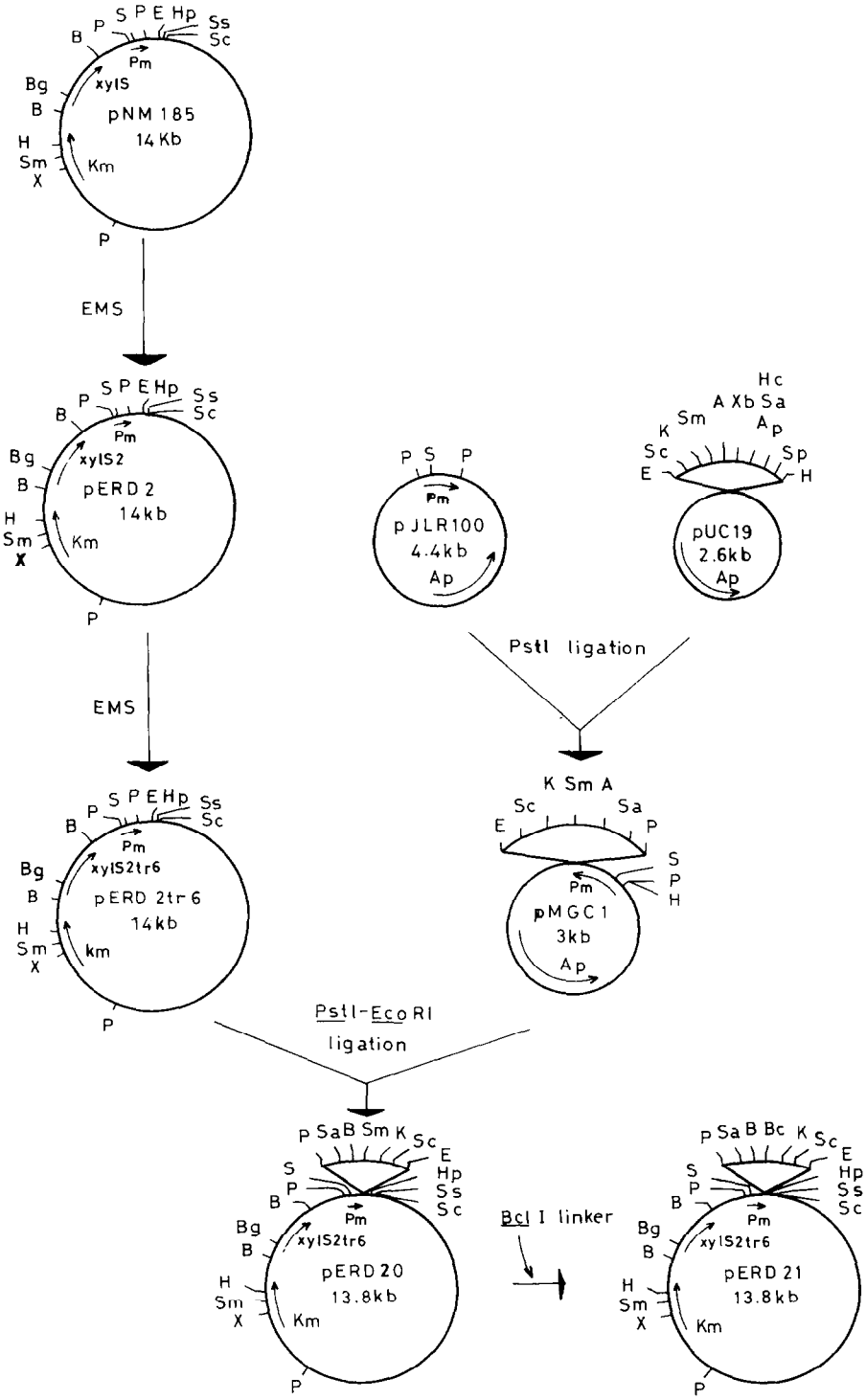

Fig.1. Construction of the pERD20 and pERD21 broad host-range regulated expression vectors. The experimental steps are described in section 3. The following abbreviations are used for restriction endonuclease cleavage sites: A, *Acc*I; B, *Bam*HI; Bc, *Bcl*I; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; Hc, *Hin*cII; Hp, *Hpa*I; K, *Kpn*I; S, *Stu*I; Sa, *Sal*I; Sc, *Sac*I; Sm, *Sma*I; Sp, *Sph*I; Ss, *Sst*II; X, *Xho*I; Xb, *Xba*I. EMS indicates mutagenesis by ethylmethane sulphonate.

clones containing the linker at the *Sma*I site in the Km resistance determinant of pERD20 which are Km^s. The resulting plasmid bearing the *Bcl*I site downstream of Pm was designated pERD21.

3.2. *Expression of genes under Pm control in different bacterial genera at different temperatures*

In order to test the efficacy of pERD20, a 7.7 kb *Eco*RI fragment from pNM188 [2] carrying promoterless *lacZ* and *lacY* genes was cloned into the *Eco*RI site downstream of Pm to produce pERD22. As expected (table 3), with this construction in which the *xyl*S2tr6 allele is in *cis* to the Pm::*lacZ* fusion, the levels of β-galactosidase in *E. coli* grown at different temperatures were similar to those obtained with the genetic elements in *trans* (table 2). Essentially the same levels were obtained in *P. aeruginosa* PAO 1162, a strain that grows at 40°C (table 3). Regulated expression from Pm was also observed in the enteric pathogens *S. flexneri* and *Ae. hydrophila* and in the soil bacteria *P. putida* and *A. vinelandii*. However, no expression was seen in *Ag. tumefaciens* (table 3).

3.3. *Stability of pERD20 and pERD22 in* E. coli *5K and* P. putida *KT2440*

It is known that hybrid plasmids may exhibit instability in their maintenance and structure as a result of the presence of certain cloned sequences and/or the expression of cloned genes [12]. In order to examine these aspects of stability of the new expression vectors, *E. coli* and *P. putida* (pERD20), and *E. coli* and *P. putida* (pERD22) were grown in LB at 30°C for at least 40 generations. Bacteria were then grown on solid medium (i) with and without Km, to determine the stability of the plasmids, and (ii) with Km, 3-methylsalicylate (0.5 mM) and X-Gal to measure the stability of the induced *lacZ* gene in pERD22. In both bacteria pERD20 and pERD22 were stable: more than 98% of the clones retained the plasmids after 40 generations without Km selection. Among Km^r clones, 100% of *E. coli* and about 85% of *P. putida* colonies were blue on plates containing X-Gal and 3-methylsalicylate, suggesting that the *lacZ* gene was also stable. When *E. coli* and *P. putida* (pERD22) were grown in LB plus 3-methylsalicylate (1 mM) for about 40 generations, 100% of *E. coli* and more than 90% of *P. putida* Km^r colonies were blue on X-Gal and 3-methylsalicylate containing plates, suggesting that the *lacZ* gene is stable in pERD22 when host bacteria are grown under conditions that induce high levels of expression of *lacZ*.

4. CONCLUSIONS

The broad-host range expression vector pNM185 contains promoter Pm of the TOL *meta*-cleavage pathway, and the *xyl*S gene, whose product after activation by benzoate effectors stimulates transcription from Pm [2]. Regulated expression of two test genes cloned downstream of Pm in pNM185 occurred in at least 15 different genera of Gram negative bacteria. We have now further increased the usefulness of pNM185 by (i) elevating the induction level obtainable with the vector, (ii) extending the temperature range in which regulated expression of cloned genes can be

Table 3

β-Galactosidase induction levels in various bacteria carrying pERD22 grown at different temperatures

Effector	Strain:	*E. coli*			*P. aeruginosa*			*P. putida*	*S. flexneri*	*Ae. hydrophila*	*A. vinelandii*	*Ag. tumefaciens*
	t (°C):	30	37	40	30	37	40	30	30	30	30	30
None		180	149	164	200	315	275	100	240	210	285	10
Added		11520	8642	1312	14400	8190	2750	5680	9895	13204	3755	10
Induction ratio		64	58	8	72	26	10	57	41	63	13	1

Bacteria bearing pERD22 were grown in LB plus Km or RM plus Km (*Azotobacter*). Overnight cultures were diluted 50- to 100-fold in the same medium with or without 1 mM 3-methylsalicylate (*E. coli, P. aeruginosa* and *P. putida*) or 1 mM salicylate (*S. flexneri, Ae. hydrophila, A. vinelandii* and *Ag. tumefaciens*) and incubated at the indicated temperature for 5–8 h. β-Galactosidase was then measured in permeabilized cells

achieved, and (iii) introducing additional cloning sites close to and downstream of Pm.

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