A broad host range reporter plasmid for the analysis of divergent promoter regions

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Although many vectors exist for *Escherichia coli* and closely related species, there are few broad host range vectors that can be conjugated into a large variety of Gram-negative bacteria. We have constructed a broad host range vector, pMJ445, that facilitates the analysis of divergent promoters in Gram-negative bacteria. The vector was validated using two intergenic regions derived from gene clusters involved in hydantoin hydrolysis, from the environmental isolates *Pseudomonas putida* and *Agrobacterium tumefaciens*. The DNA sequences analysed were capable of activating expression of the reporter enzymes, β-glucuronidase and β-galactosidase, present on pMJ445, indicating the presence of divergent promoters in the sequences selected. In addition, we demonstrated that pMJ445 can be applied to gene regulation studies.

Two major difficulties with studying gene regulation in environmental microbial isolates are the lack of stable, broad host range promoter-probe vectors and inefficient methods for introducing recombinant plasmids into these strains. There are even fewer vectors for studying transcriptional activity directed by divergent or bi-directional promoter systems. We have constructed a promoter-probe plasmid, pMJ445 (GenBank Accession No. EU250578), which enables the simultaneous assay of transcriptional activity directed by divergent promoter sequences via β-galactosidase and β-glucuronidase reporter activity in a wide variety of Gram-negative bacteria. pMJ445 is based on the broad host range IncQ replicon derived from pTF-FC2.[1] The pTF-FC2 replicon is small (4.9 kb) and its host range includes *Escherichia coli, Pseudomonas aeruginosa, Acidithiobacillus ferrooxidans* and *Agrobacterium tumefaciens.*[1,2] Furthermore, the pTF-FC2 replicon maintains the plasmid at a low copy number (12–15 copies per cell)[3] and the presence of a poison-antidote system ensures stability of the plasmid in the host cells.[4] To facilitate easy propagation and extraction of plasmid DNA in high concentrations in *E. coli* DH5α, pMJ445 carries the F1 *ori* as well as genes conferring resistance to ampicillin (*E. coli* DH5α) and tetracycline (broad host range). pMJ445 also carries the RK2 *oriT* gene, which allows for efficient mobilization into strains of interest by conjugation in addition to chemical transformation and electroporation. Promoter sequences, including native ribosome binding sites, can be readily inserted into the single *Bgl* II site, located between the divergently orientated *lacZ* and *gus* ORFs (Fig. 1), for analysis.

Microbial hydantoin-hydrolysing enzyme systems have important industrial applications in the biocatalytic production of optically pure D- and L-amino acids, which are used in the synthesis of antibiotics, anti-inflammatory and anti-viral drugs.[5] Hydantoin hydrolysis occurs via two steps. First, the 5-mono-substituted-hydantoin is cleaved by hydantoinase or dihydropyrimidinase to produce the *N*-carbamylamino acid, which is in turn converted to the corresponding amino acid by an *N*-carbamoylase or β-ureidopropionase. In *Pseudomonas putida,* a dihydropyrimidinase and β-ureidopropionase (encoded by *dhp* and *bup* genes, respectively) are responsible for hydantoin hydrolysis.[6] In *Agrobacterium* species, hydantoin hydrolysis is catalysed by a hydantoinase and *N*-carbamoylase (encoded by *hyuH* and *hyuC* genes, respectively).[7,8] In both cases, the *hyuH-hyuC* and *dhp-bup* genes are arranged divergently,[6–8] with an additional gene, encoding a putative permease, located upstream of and in the same orientation as *bup* in *P. putida* strain RU-KM3s.[6] Hydantoin-hydrolysing activity is tightly controlled by a complex regulatory network. The enzymes are expressed in early stationary growth phase and activity is induced when cells are grown in the presence of hydantoin.[9–11] In *Agrobacterium tumefaciens,* hydantoin hydrolysis is also subject to nitrogen catabolite repression,[12] whereas hydantoin hydrolysis in *P. putida* RU-KM3s is regulated by carbon catabolite repression (CCR).[6] The molecular basis for these regulatory pathways is unknown. This provides an opportunity to test the broad host range plasmid pMJ445 in two diverse genera of Gram-negative bacteria and to elucidate the mechanisms involved in the regulation of hydantoin hydrolysis in *A. tumefaciens* RU-AE01 and *P. putida* RU-KM3s.

Materials and methods

Construction of the broad host range vector involved six steps. 1) The RP4 *oriT–tet–*MB1 *oriV* fragment was excised from pTnmodOTc[13] with the restriction enzymes *Pvu* II and *Sal* I and inserted between the *Sma* I and *Sal* I sites of pT7-7.[14] 2) The RP4 *oriT–tet–*MB1 *oriV* fragment was then excised from this intermediate construct with *Csp* 451 and *Kpn* I and inserted between the *Cla* I and *Kpn* I sites of pTV100,[15] generating pJAS13. 3) Plasmid pJAS13 contained a *Bgl* II restriction site that was important in the future cloning strategy. This restriction site was deleted by digesting pJAS13 with *Bgl* II, filling in the termini with Klenow

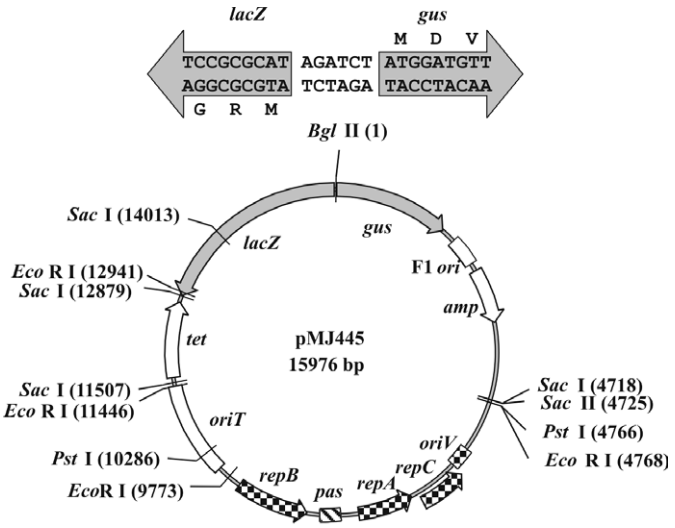

Fig. 1. Schematic map of the broad host range bi-directional promoter-probe vector pMJ445. The plasmid encodes a broad host range origin of replication (*repB, repC, repA* and *oriV*), an *E. coli* origin of replication (F1 *ori*), an origin of transfer (*oriT*), a poison-antidote plasmid stability system (*pas*), genes conferring resistance to the antibiotics ampicillin (*amp*) and tetracycline (*tet*), and divergent genes encoding the reporter enzymes β-galactosidase (*lacZ*) and β-glucuronidase (*gus*).

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enzyme and re-circularizing the plasmid (pMJ417). 4) The *gus*–RU-AE01 promoter–*lacZ* fragment was amplified from pMJ258[19] using the Expand High Fidelity PCR system (Roche) and the primers MJ65 (ggt acc TCA TTG TTT GCC TCC CTG CTG CG) and MJ66 (ccg cgg GGA TTA TTT TTG ACA CCA GAC CAA CTG GTA ATG GTA G), which introduced *Kpn* I and *Sac* II sites, respectively (shown in lower case in the primer sequence), and inserted into pGEM-T-Easy (Promega), generating pMJ383. 5) The *gus*–RU-AE01 promoter–*lacZ* fragment was excised from pMJ383 with the restriction enzymes *Kpn* I and *Sac* II and the termini filled in with Klenow enzyme. Similarly, pMJ417 was digested with C*la* I and *Kpn* I and the termini filled in with Klenow enzyme. These blunt-end DNA fragments were ligated and pMJ441, which encoded *gus*–RU-AE01 promoter–*lacZ*, RP4 *oriT*–*tet* and the broad origin of replication (*repB, repA, repC* and *oriV*), was isolated. 6) Finally, pMJ441 was digested with *Bgl* II, to excise the RU-AE01 promoter fragment, and the DNA backbone re-circularized to produce the broad host range promoter plasmid pMJ445. This broad host range promoter-probe vector was validated using restriction mapping, PCR analysis with primers designed to amplify across the regions where the different fragments were annealed, followed by DNA sequencing.

The divergent promoters and native ribosome binding sites of the *hyuH-hyuC* and *dhp-bup* gene clusters from *A. tumefaciens* RU-AE01 and *P. putida* RU-KM3s were used to validate the application of pMJ445 as a broad host range promoter-probe vector. The region between the *A. tumefaciens hyuH-hyuC* coding sequences was amplified using the Expand High Fidelity PCR system and primers MJ9 (aga tct AAA GCA GCT CTC AGG GTT GAT G) and MJ10 (aga tct GAA CCT TTG CTC CTT CGA TAG TTA AT), which introduced flanking *Bgl* II sites (shown in lower case in the primer sequence), and the PCR product inserted between the reporter genes in pMJ445. Likewise, the *P. putida dhp-bup* promoter region was amplified using primers GFM38 (aga tct GGG GCC TTC TCC AGA TTT TT) and GFM39 (aga tct GCC GTC TTC CTC GCA G) and the PCR product inserted into the *Bgl* II site of pMJ445. pMJ441 carries the *A. tumefaciens hyuH*-promoter upstream of *gus* and the *hyuC*-promoter upstream of *lacZ*, whereas pMJ449 has the *P. putida dhp*-promoter upstream of *gus* and the *bup*-promoter upstream of *lacZ*.

A. tumefaciens RU-AE01 cells were transformed by electroporation[16] with a transformation efficiency of 10^4 colonies per μg plasmid DNA. Plasmids were introduced into *P. putida* RU-KM3s cells by tri-parental mating during which plasmids were mobilized from *E. coli* DH5α cells with the aid of *E. coli* HB101 cells containing the helper plasmid pRK2013.[6] Mating frequencies of 10^{-5}–10^{-4} transconjugants per donor were routinely observed. For enzyme assays, cultures were grown at 28°C in 100 ml Nutrient Broth (NB) (Biolab) with or without inducer (1% hydantoin, Sigma) and 1% succinate (United Scientific) (to detect CCR). *A. tumefaciens* and *P. putida* cells were harvested in late stationary ($OD_{600\ nm}$ = 4.5–5.0) and early stationary growth phase ($OD_{600\ nm}$ = 2.5–3.0), respectively. Cells were collected by centrifugation (5000 rpm for 10 min at 10°C in a Beckman centrifuge in a JA14 rotor), washed in 0.1 M phosphate buffer [pH 8.0 (*P. putida*) or pH 9.0 (*A. tumefaciens*)] and resuspended in 0.1 M phosphate buffer [pH 8.0 (*P. putida*) or pH 9.0 (*A. tumefaciens*)] at 20 mg/ml wet cell mass. The cells were subsequently incubated in the presence of the substrate hydantoin (50 mM) or *N*-carbamylglycine (25 mM) (Sigma) at 40°C, 100 rpm (to ensure constant mixing of the reagents in the assay), for 6 h (*A. tumefaciens*) or 3 h (*P. putida*). The cells in the resting cell reactions were pelleted by centrifugation (13 000 rpm at room temperature in a Heraeus microfuge) and the supernatant analysed for *N*-carbamylamino acids or amino acids by Ehrlich's or Ninhydrin colorimetric assays, respectively.[17,18] Hydantoinase and *N*-carbamoylase enzyme assays in *A. tumefaciens* cells, dihydropyrimidinase and β-ureidopropionase enzyme assays in *P. putida* cells, and β-glucuronidase assays in both genera were conducted as described previously.[6,9,19] *P. putida* cells were disrupted by sonication prior to β-galactosidase assays.[20] Hydantoinase activity is reported as the total (in μmol/ml) *N*-carbamoylglycine and glycine produced from hydantoin as a substrate by resting cells and *N*-carbamoylase activity is reported as the amount of glycine (in μmol/ml) produced from *N*-carbamoylglycine as a substrate. All biocatalytic assays were independently repeated at least three times with freshly cultured cells.

Results

Hydantoinase and *N*-carbamoylase activities were undetectable in *A. tumefaciens* cells grown in NB but there was a 3.5-fold and 2.5-fold increase in hydantoinase and *N*-carbamoylase activity, respectively, when *A. tumefaciens* cells were grown in the presence of hydantoin (Table 1A). No β-glucuronidase or β-galactosidase activity was detected in *A. tumefaciens* cells containing the vector pMJ445 (no promoter), indicating a lack of endogenous reporter enzyme activity under analysis conditions. A 3.5-fold increase in *hyuH*-directed β-glucuronidase activity and a 2.5-fold increase in the *hyuC*-promoter derived β-galactosidase activity were observed in *A. tumefaciens* (pMJ441) cells grown in NB containing hydantoin. The correlation between the hydantoinase and *N*-carbamoylase enzyme activities with those of the reporter enzymes, β-glucuronidase and β-galactosidase, indicated that induction of hydantoin hydrolysis in *A. tumefaciens* cells occurs at the transcriptional level.

In *P. putida* (pMJ445), dihydropyrimidinase activity was induced 7-fold by growth in the presence of hydantoin (Table 1B), while the addition of succinate resulted in a 6.8-fold decrease in dihydropyrimidinase activity. A small increase was observed in *dhp*-directed β-glucuronidase activity in *P. putida* (pMJ449) cells grown in hydantoin, while the addition of succinate resulted in a 2.9-fold decrease of β-glucuronidase activity. As with the dihydropyrimidinase, β-ureidopropionase activity was induced 5.8-fold when *P. putida* (pMJ445) cells were grown in NB containing hydantoin and the presence of succinate resulted in a 9.7-fold reduction in activity. A small but important increase in *bup*-directed β-galactosidase activity was detected in *P. putida* (pMJ449) cells grown in hydantoin with a 3.3-fold decrease in activity in cells grown in both hydantoin and succinate. The results suggested that the changes in dihydropyrimidinase and β-ureidopropionase activities observed in *P. putida* cells grown in the presence of hydantoin or hydantoin and succinate can be attributed, at least in part, to the regulation of transcriptional activation by the *dhp-bup* promoters. The level of induction or repression of the reporter enzymes, β-glucuronidase and β-galactosidase, was not as high as that observed for the native enzymes in *P. putida*. This may be due to the effect of plasmid copy number (12–15 copies per cell versus one chromosomal copy each of *dhp* and *bup*). Alternative explanations may be differences in the half-lives of the reporter enzymes versus the hydantoin-hydrolysing enzymes or the possibility of post-translational modification of the dihydropyrimidinase and β-ureidopropionase enzymes in *P. putida*.

These results demonstrate that pMJ445 can be used to analyse divergent regulatory regions simultaneously. We were able to introduce DNA fragments up to 1 kb between the genes for the reporter enzymes. We also successfully applied the plasmid pMJ445 in deletion analyses and site-directed mutagenesis to

Table 1. Hydantoinase and *N*-carbamoylase enzyme versus reporter enzyme activities in (**A**) *Agrobacterium tumefaciens* RU-AE01 cells and (**B**) *Pseudomonas putida* RU-KM3s cells.

(**A**)

Plasmid	Growth medium supplement	Hydantoinase (Hydantoinase U)	β-glucuronidase (β-Gluc U)	*N*-carbamoylase (*N*-carbamoylase U)	β-galactosidase (Miller U)
		hyuH	*hyuH-gus*	*hyuC*	*hyuC-lacZ*
pMJ445	–	0.02 (± 0.03)	0.0 (± 6.6)	0.08 (± 0.05)	0.0 (± 6.8)
pMJ445	Hyd	3.48 (± 0.17)	0.0 (± 22.4)	2.52 (± 0.22)	0.0 (± 7.7)
pMJ441	–	0.09 (± 0.04)	657.1 (± 81.7)	0.05 (± 0.06)	21.4 (± 4.0)
pMJ441	Hyd	3.59 (± 0.23)	2309.7 (± 183.9)	2.45 (± 0.19)	56.1 (± 1.6)

(**B**)

Plasmid	Growth medium supplement	Dihydropyrimidinase (Dhp U)	β-glucuronidase (β-Gluc U)	β-ureidopropionase (Bup U)	β-galactosidase (Miller U)
		dhp	*dhp-gus*	*bup*	*bup-lacZ*
pMJ445	–	6.39 (± 1.00)	0.0 (± 0.0)	1.26 (± 0.73)	0.0 (± 0.0)
pMJ445	Hyd	43.49 (± 4.55)	0.0 (± 0.0)	9.71 (± 0.51)	0.0 (± 5.6)
pMJ445	Hyd + Suc	6.23 (± 0.63)	0.0 (± 0.0)	1.26 (± 0.11)	0.0 (± 1.1)
pMJ449	–	6.43 (± 0.17)	3964.0 (± 42.5)	1.93 (± 0.16)	41.9 (± 0.1)
pMJ449	Hyd	45.00 (± 1.55)	4803.3 (± 38.0)	11.18 (± 0.78)	50.2 (± 2.6)
pMJ449	Hyd + Suc	6.63 (± 0.65)	1664.6 (± 97.3)	1.15 (± 0.12)	15.1 (± 4.4)

β-Gluc U, β-glucuronidase enzyme activity; Bup U, μmol/ml glycine; Dhp U, μmol/ml NCG and glycine; Hydantoinase U, μmol/ml NCG and glycine; Miller U, β-galactosidase enzyme activity; *N*-carbamoylase U, μmol/ml glycine.

β-Gluc, β-glucuronidase; Bup, β-ureidopropionase; Dhp, dihydropyrimidinase; Hyd, hydantoin; NCG, *N*-carbamylglycine; Suc, succinate; (± s.e.m.), standard error of the mean; U, units of enzyme activity; (n = 3).

identify the regulatory elements in the *dhp-bup* and the *hyuH-hyuC* promoter regions (data not shown). The ability to mobilize pMJ445 and its derivatives efficiently into environmental isolates demonstrates the flexibility of this broad host range plasmid. Finally, the stability of pMJ445 and the ease of use make this vector a valuable tool in the study of regulatory regions in a wide variety of Gram-negative bacterial strains.

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