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 metabolism, 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 Furthermore, the pTF-FC2 replicon maintains the plasmid at a low copy number (12–15 copies per cell) and its host range includes Escherichia coli, Pseudomonas aeruginosa, Acidithiobacillus ferroxidans and Agrobacterium tumefaciens. The pTF-FC2 replicon maintained the plasmid at a low copy number (12–15 copies per cell) and the presence of a poison-antidote system ensures stability of the plasmid in the host cells. 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 BglII site, located between the divergently oriented 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 metabolism occurs via two steps. First, the 5-mono-substituted-hydantoin is cleaved by hydantoinase or dihydopyrimidinase to produce the N-carbamylaminoo acid, which is in turn converted to the corresponding amino acid by an N-carbamoylase or β-ureidopropionase. In Pseudomonas putida, a dihydopyrimidinase and β-ureidopropionase (encoded by dhp and rep genes, respectively) are responsible for hydantoin hydrolysis.6 In Agrobacterium species, hydantoin hydrolysis is catalysed by a hydantoinase and N-carbamoylase (encoded by huyH and huyC genes, respectively).7,8 In both cases, the huyH-huyC and dhp-rep genes are arranged divergently,8 with an additional gene, encoding a putative permease, located upstream of and in the same orientation as rep in P. putida strain RU-KM3s. 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,11 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 pTnmodOTc13 with the restriction enzymes PvuII and SalI and inserted between the Smal and SalI sites of pT7-7.12 2) The RP4 oriT–tet–MB1 oriV fragment was then excised from this intermediate construct with SpI 451 and KpmI and inserted between the ClaI and KpmI sites of pTV100,13 generating pJAS13. 3) Plasmid pJAS13 contained a BglII restriction site that was important in the future cloning strategy. This restriction site was deleted by digesting pJAS13 with BglII, filling in the termini with Klenow

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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).
enzyme and re-circularizing the plasmid (pMJ417). 4) The gus–RU-AE01 promoter–lacZ fragment was amplified from pMJ258\(^1\) using the Expand High Fidelity PCR system (Roche) and the primers MJ65 (ggc att cta ttt tgt gcc tcc ctg ctg cg) and MJ66 (ccg cgg gga tta ttt tgt gag cca gcc caa ctg gta gtg tgt ga), 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 Cla 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–let and the broad origin of replication (repB, repA, repC and oriV), was isolated. 6) Finally, pMJ441 was digested with BgII 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 divergent regulatory regions simultaneously. We were able to confirm the presence of the substrate hydantoin (50 mM) or glycine produced from hydantoin as a substrate by 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.\(^{7,10}\) Hydantoinase and \(N\)-carbamoylase enzyme assays in \(A.\) \(tumefaciens\) cells, dihydropyrimidinase and \(\beta\)-ureidopropionase enzyme assays in \(P.\) \(putida\) cells, and \(\beta\)-glucuronidase assays in both genera were conducted as described previously.\(^{6,9}\) \(P.\) \(putida\) cells were disrupted by sonication prior to \(\beta\)-galactosidase assays.\(^{20}\) Hydantoinase activity is reported as the total (in \(\mu\)mol/ml) \(N\)-carbamoylglucuronic acid and glycine produced from hydantoin as a substrate by resting cells and \(N\)-carbamoylase activity is reported as the amount of glycine (in \(\mu\)mol/ml) produced from \(N\)-carbamoylglucuronic acid as a substrate. All biocatalytic assays were independently repeated at least three times with freshly cultivated 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 \(\beta\)-glucuronidase or \(\beta\)-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 \(\beta\)-glucuronidase activity and a 2.5-fold increase in the \(hyuC\)-promoter derived \(\beta\)-galactosidase activity were observed in \(A.\) \(tumefaciens\) cells grown in NB containing hydantoin. The correlation between the hydantoinase and \(N\)-carbamoylase enzyme activities with those of the reporter enzymes, \(\beta\)-glucuronidase and \(\beta\)-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 \(\beta\)-glucuronidase activity in \(P.\) \(putida\) (pMJ449) cells grown in hydantoin, while the addition of succinate resulted in a 2.9-fold decrease of \(\beta\)-glucuronidase activity. As with the dihydropyrimidinase, \(\beta\)-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 \(\beta\)-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 \(\beta\)-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, \(\beta\)-glucuronidase and \(\beta\)-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 \(\beta\)-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
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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