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Construction of improved plasmid vectors for promoter characterization in *Pseudomonas aeruginosa* and other Gram-negative bacteria

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Abstract

We report the construction of two broad host range promoter-probe plasmid vectors for rapid analysis of promoters in Gram-negative bacteria. The new vectors, pME4507 and pME4510, carry carbenicillin and gentamycin resistance genes, respectively, and are small sized (4 kb) with a flexible multiple cloning site to facilitate directional cloning of putative promoter elements. The vectors allow rapid plate-based screening for promoter activities, using β-galactosidase as the reporter enzyme. In the absence of an inserted promoter fragment, they display very low background activity, making them a useful tool for analysis of low expression level promoters. © 1998 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Reporter gene; Promoter analysis; *Escherichia coli*; Broad host range vector; *lacZ*; β-Galactosidase; *Pseudomonas aeruginosa*

1. Introduction

Pseudomonas aeruginosa is an opportunistic pathogen which plays a critical role in cystic fibrosis, but which is also common in soil environments, and has been used extensively in bioremediation studies. Its broad range of biochemical abilities has been studied in detail, and its genome is currently being sequenced. *P. aeruginosa* promoter structure is quite varied [1,2], and many *P. aeruginosa* promoters are not well recognized in *Escherichia coli*. The identification and characterization of promoter structures will therefore constitute a major challenge during interpretation of data emerging from the *P. aeruginosa* genome sequence project, and the results obtained will be doubly useful, since they will be paradigmatic for promoters throughout the large family of soil pseudomonads. However, there is a limited number of plasmid vectors currently available for promoter analysis studies in non-coliform bacteria, and these are often either quite large (>7 kb), or carry only the β-lactamase gene as a marker. Since many pseudomonads are naturally resistant to high levels of a variety of β-lactam antibiotics, these systems are often of limited use.

To help fill this need for a small, flexible vector for rapid promoter-probe studies, we report here the construction of two small promoter-probe vectors, pME4507 and pME4510, derived from broad host

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range vectors of the pUCP series, for analysis of promoter structures in a range of pseudomonads.

2. Materials and methods

The bacterial strains used were *E. coli* DH5α and *P. aeruginosa* PAO1S, a spontaneously streptomycin-resistant derivative of *P. aeruginosa* PAO1. *P. aeruginosa* PAO1S-Lac was constructed by introduction of the mini-D*lac* element (*lacI*^{q} *lacZΔM15*, Tc^{R}), as previously described [3]. The strains were routinely grown aerobically in Luria–Bertani medium at 37°C. When a defined medium was required, *P. aeruginosa* was grown on a sulfate-free succinate-salts medium, with sulfur sources added as described in the text [4]. Antibiotics were added to the growth medium at the following concentrations (per ml): ampicillin, 100 μg for *E. coli*; tetracycline, 25 μg for *E. coli* and 125 μg for *P. aeruginosa*; gentamycin, 15 μg for *E. coli* and 200 μg for *P. aeruginosa*; streptomycin (500 μg) and carbenicillin (300 μg) for *P. aeruginosa*. Molecular genetic methods (plasmid isolation, restriction enzyme digestion, ligation and transformation) were done by published procedures [5]. Electroporation of *P. aeruginosa* was done with the Gene pulser apparatus (Bio-Rad, Hercules CA). Where required, DNA fragments were isolated from agarose gels with GeneClean (Bio101, La Jolla, CA), or Qiaquick (Qiagen, Basle, Switzerland) spin columns. PCR was carried out in a Trio Block (BioMetra, Göttingen, Germany). Standard PCR reaction mixtures consisted of 50 pmol of primers, 200 nmol of dNTPs, 1 U *Vent* DNA polymerase (New England Biolabs, Bad Schwalbach, Germany) and 1–100 ng of template in a final volume of 50 μl. Nucleotide sequencing was carried out with an ABI 310 capillary sequencer, using fluorescent dye-labeled dideoxynucleotides.

To construct pME4507, the region upstream of the *cat* gene in pKK232-8 [6] was amplified by PCR using primers pKKfor (5′-cccaggcatcaaataaaacg-3′) and pKKrev (5′-gatt<u>caattg</u>tc**cat**tttagcctccttagc-3′; *Mun*I restriction site underlined, *lacZ* translation start on the reverse strand in bold face). The 279-bp fragment was phosphorylated with T4-polynucleotide kinase and then digested with *Mun*I. The broad host range vector pUCP21 [7] was digested with *Sap*I, blunted with T4 DNA polymerase, and digested with *Eco*RI. The PCR fragment was ligated to the vector fragment by standard procedures, to give plasmid pME4507, and the PCR-derived region was sequenced to ensure correct ligation and the absence of PCR-derived errors. Plasmid pME4510 was constructed by a similar procedure, by insertion of the above PCR fragment into plasmid pUCP25 [7].

Expression of putative promoter fragments was tested quantitatively during growth of cells on solid media containing IPTG (1 mM) and X-Gal (40 μg ml^{-1}). β-Galactosidase activity in liquid cultures was measured by the method of Miller [8], in cells grown in liquid medium with 1 mM IPTG, and harvested in the late exponential phase. To increase the sensitivity of the β-galactosidase assay, the cells were washed once with reaction buffer before assay, to remove components of the growth medium.

3. Results and discussion

The two new vectors pME4507 and pME4510 were constructed by deleting the multiple cloning site and *lac* promoter of pUCP21 and pUCP25 by *Sap*I–*Eco*RI digestion, and inserting the multiple

Table 1
β-Galactosidase activity in bacteria carrying the promoter-probe vectors[a]

Plasmid	Inserted promoter	β-Galactosidase activity (Miller units)	
		E. coli DH5α	*P. aeruginosa* PAO1S-Lac
pME4507	none	0.1	0.1
	lac	2400	109
pME4510	none	0.3	0.1
	lac	2130	52

[a]Cells were cultivated in LB medium in the presence of 1 mM IPTG, and β-galactosidase activity was assayed in the late exponential phase according to Miller [8]. The results are representative of four measurements.

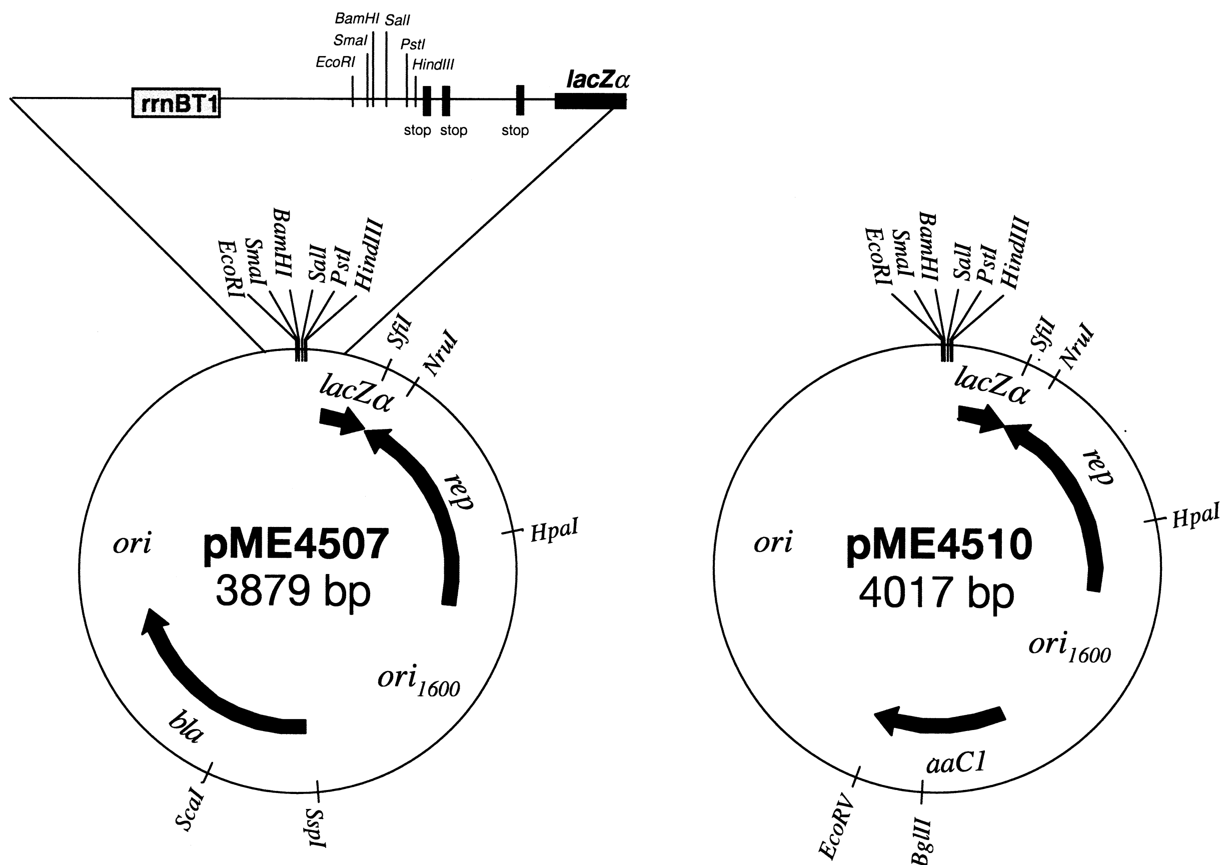

Fig. 1. The structure of plasmids pME4507 (accession no. AJO11791), and pME4510 (accession no. AJO11792) including the *rrnBT1* transcriptional terminator upstream of the MCS, and the stop codons in all three reading frames between the multiple cloning site and the *lacZ*α translational start site.

cloning site from the *E. coli* promoter-probe vector pKK232-8 [6] (Fig. 1). The promoter-probe region of the resulting vectors therefore contains: (a) the strong *rrnBT1* transcriptional terminator, to prevent readthrough from vector sequences; (b) a multiple cloning site; (c) translational stops in all three reading frames; (d) an optimized ribosomal binding site; and (e) the *lacZ*α gene (Fig. 1). The two plasmids are both small sized (3879 and 4017 bp), facilitating cloning procedures in promoter screening studies, and contain a flexible multiple cloning site with unique restriction sites for six enzymes, allowing directional cloning for insertion of putative promoter fragments. Economy in size was achieved by using the *lacZ*α gene as the reporter gene rather than the complete *lacZY* genes, and promoter analysis is therefore carried out by α-complementation in any strain bearing the *lacZ*ΔM15 allele. The *lacZ*ΔM15 allele can easily be introduced into *P. aeruginosa* as a mini-D*lac* element (Tc^R) [3], and into other pseudomonads on the minitransposon miniTn*5Lac4* (Cm^R) [9]. It is also present in many common laboratory strains of *E. coli*. Since the *lacZ* gene is not present in most pseudomonads, it is an optimal choice as a reporter gene in this system. In the absence of the *lacY* gene, full expression of the *lacZ*ΔM15 gene requires higher levels of IPTG in the growth medium than are commonly used ([10], but in our hands, 1 mM IPTG was found to be sufficient. Growth of *P. aeruginosa* PAO1S-Lac was not impaired at this concentration.

The new promoter-probe vectors were tested in

Table 2
Regulation of the *P. aeruginosa cysI* and *cysD* promoters in *P. aeruginosa* PAO1S-Lac[a]

Plasmid	Inserted promoter	β-Galactosidase activity (Miller units)	
		Growth with sulfate	Growth with pentanesulfonate
pME4507	*cysI*	11.5	25.2
	cysD	13.0	17.3

[a]Cells were cultivated in succinate-salts medium with the given sulfur sources (500 μM) in the presence of 1 mM IPTG, and β-galactosidase activity was assayed in the late exponential phase according to Miller [8]. The results are representative of three measurements.

E. coli DH5α and in *P. aeruginosa* PAO1S-Lac. β-Galactosidase activity in the absence of a promoter sequence was found to be extremely low (Table 1), and pure white colonies were observed after growth on plates containing X-Gal/IPTG. The use of the two vectors for qualitative promoter testing was assessed by cloning PCR fragments carrying the promoter regions of two cysteine biosynthesis genes from *P. aeruginosa*, *cysI* (a 457-bp fragment including 173 bp upstream of the *cysI* translation start site) and *cysD* (a 789-bp fragment containing 701 bp upstream of the *cysD* translation start site) [4], into the *Sma*I site of pME4507, and transforming these constructs into *E. coli* or *P. aeruginosa*. These promoters were chosen for testing because genes of sulfur metabolism are often expressed at low levels, and are subject to repression in the presence of inorganic sulfate. Cells containing these constructs were grown on minimal plates with X-Gal/IPTG containing either sulfate or pentanesulfonate as sole sulfur source. In *E. coli* the two promoters were both active (blue colonies) regardless of the sulfur source supplied, whereas in *P. aeruginosa* they were both active during growth with pentanesulfonate (dark blue colonies) but partly repressed (pale blue colonies) in the presence of sulfate, as expected. This confirmed that although the *P. aeruginosa cys* promoters were recognized by the *E. coli* transcription machinery, correct repression by sulfate (mediated in *E. coli* by the CysB protein) did not take place. This corresponds with previous observations that there are significant differences in regulation of cysteine biosynthesis between these two species [4].

The quantitative use of pME4507 and pME4510 as promoter-probe vectors was evaluated with two different approaches. First, a 289-bp fragment carrying the *E. coli lac* promoter was cloned into the *Sma*I sites of the new vectors. In the presence of the *lac* promoter, high levels of β-galactosidase were seen in *E. coli*, whereas a much weaker level of induction was seen in *P. aeruginosa* (Table 1). This agrees well with previous observations that the *lac* promoter is expressed constitutively in pseudomonads, but at a significantly lower level than in *E. coli* [10]. As a second test, β-galactosidase expression from the *P. aeruginosa cysI* and *cysD* promoters described above was measured in *P. aeruginosa* PAO1S-Lac (Table 2). The levels of expression found correlated well with the quantitative data reported above. The results obtained therefore demonstrate the usefulness of these new vectors, and confirm that they may be used for successful analysis of very low levels of promoter activity.

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References

[1] Deretic, V., Konyecsni, W.M., Mohr, C.D., Martin, D.W. and Hibler, N.S., (1989) Common denominators of promoter control in *Pseudomonas* and other bacteria. BioTechnology 7, 1249–1254.

[2] Ronald, S., Farinha, M.A., Allan, B.J. and Kropinski, A.M. (1992) Cloning and physical mapping of transcriptional regulatory (sigma) factors from *Pseudomonas aeruginosa*. In: *Pseudomonas*. Molecular Biology and Biotechnology (Galli, E., Silver, S. and Witholt, B., Eds.), pp. 249–257. ASM Press, Washington, DC.

[3] Karkhoff-Schweizer, R.R. and Schweizer, H.P. (1994) Utilization of a mini-D*lac* transposable element to create an alpha-

complementation and regulated expression system for cloning in *Pseudomonas aeruginosa*. Gene 140, 7–15.

[4] Hummerjohann, J., Kuttel, E., Quadroni, M., Ragaller, J., Leisinger, T. and Kertesz, M.A. (1998) Regulation of the sulfate starvation response in *Pseudomonas aeruginosa*: role of cysteine biosynthetic intermediates. Microbiology 144, 1375–1386.

[5] Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.E., Seidman, J.G., Smith, J.A. and Struhl, K. (1987) Current Protocols in Molecular Biology. Wiley, New York.

[6] Brosius, J. (1984) Plasmid vectors for the selection of promoters. Gene 27, 151–160.

[7] West, S.E.H., Schweizer, H.P., Dall, C., Sample, A.K. and Runyenjanecky, L.J. (1994) Construction of improved *Escherichia–Pseudomonas* shuttle vectors derived from pUC18/19 and sequence of the region required for their replication in *Pseudomonas aeruginosa*. Gene 148, 81–86.

[8] Miller, J.H. (1992) A Short Course in Bacterial Genetics, Cold Spring Harbor Press, Cold Spring Harbor, NY.

[9] Schweizer, H.P. (1994) A method for construction of bacterial hosts for *lac*-based cloning and expression vectors: alpha-complementation and regulated expression. Biotechniques 17, 453–456.

[10] Hansen, L.H., Knudsen, S. and Sorensen, S.J. (1998) The effect of the *lacY* gene on the induction of IPTG inducible promoters, studied in *Escherichia coli* and *Pseudomonas fluorescens*. Curr. Microbiol. 36, 341–347.