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NOTE

Construction of an *Escherichia-Pseudomonas* Shuttle Vector Containing an Aminoglycoside Phosphotransferase Gene and a *lacZ'* Gene for α-Complementation

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A new 4.87 kb *Escherichia-Pseudomonas* shuttle vector has been constructed by inserting a 1.27 kb DNA fragment with a replication origin of a *Pseudomonas* plasmid pRO1614 into the 3.6 kb *E. coli* plasmid pBGS18. This vector, designated pJH1, contains an aminoglycoside phosphotransferase gene (*aph*) from Tn*903*, a *lacZ'* gene for α-complementation and a versatile multiple cloning site possessing unique restriction sites for *Eco*RI, *Sac*I, *Kpn*I, *Sma*I, *Bam*HI, *Xba*I, *Sal*I, *Bsp*MI, *Pst*I, *Sph*I, and *Hin*dIII. When pJH1 was transformed into *E. coli* DH5α and into *P. putida* HK-6, it was episomally and stably maintained in both strains. In addition, the enhanced green fluorescent protein (EGFP) gene which was transcriptionally cloned into pJH1 rendered *E. coli* cells fluorescence when its transformants were illuminated at 488 nm.

Keywords: *Pseudomonas*, shuttle vector, kanamycin-resistant gene, cloning

Pseudomonas putida strains exhibit a wide-range of metabolic activities which are of considerable interest in biotechnological applications as well as in pure scientific studies. To facilitate genetic and biotechnological studies of *P. putida*, it is necessary to develop sophisticated molecular tools including specially designed plasmid vectors. So far several *Escherichia-Pseudomonas* shuttle vectors have been constructed and used for *Pseudomonas* species (Davison, 2002). Olsen *et al.* (1982) developed broad-host vectors for the *P. aeruginosa* PAO strain by using a replication origin of pRO1600, a multi-copy plasmid. Later, the nucleotide sequence of the 1.9 kb DNA fragment from pRO1614, a derivative of pRO1600, was identified as a putative replication origin for *Pseudomonas*. This DNA fragment appeared to encode a replication-controlling protein (West *et al.*, 1994). There have been several studies that used this replication origin for shuttle vector construction (Schweizer, 1991; Schweizer, 1992; West *et al.*, 1994; Watson *et al.*, 1996).

Here we report the construction of a new 4.87 kb *Escherichia-Pseudomonas* shuttle vector designated pJH1 (Fig. 1). To construct pJH1, the 1.27 kb DNA fragment containing the replication origin from pRO1614 was amplified by PCR using *pfu* DNA polymerase with two primers whose sequences are 5'-GGAATTCCATATGCCTCTCAGGCGCCGCTGGTG-3' and 5'-GGAATTCCATATGAAAGGCAGGCCGGGCCCTTC-3'. Plasmid pRO1614 was used as the template. The two primers used for PCR both had artificially-added *Nde*I digestion sites at the 5' ends (underlined). The amplified DNA fragment was directly blunt-end ligated into the *Sma*I site of pUC19 and transformed into *E. coli* DH5 α. This recombinant plasmid was purified and its 1.27 kb insert was auto-sequenced to confirm that no mutation had occurred in the replication origin region during PCR. This plasmid was designated pJHori. The 1.27 kb *Nde*I fragment from pJHori, which contains the replication origin for *Pseudomonas,* was isolated and inserted into a unique *Nde*I site of pBGS18 (a kanamycin-resistant vector that is an analogue of plasmid pUC18, which was kindly provided by Professor

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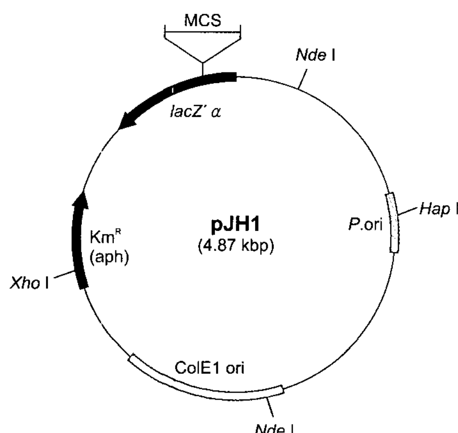

Fig. 1. *Escherichia-Pseudomonas* shuttle vector pJH1. *P.* ori indicates the *Pseudomonas* origin from pRO1614. Restriction sites of the MCS are *Eco*RI, *Sac*I, *Kpn*I, *Sma*I, *Bam*HI, *Xba*I, *Sal*I, *Bsp*MI, *Pst*I, *Sph*I, and *Hin*dIII (counterclockwise) and all unique digestion sites.

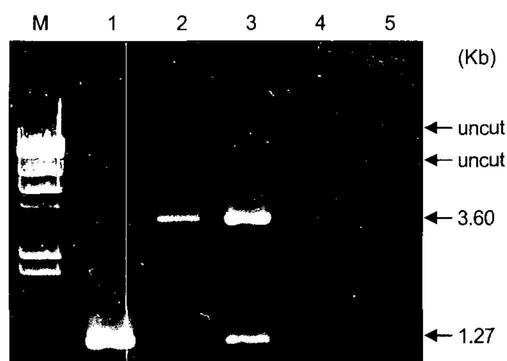

Fig. 2. Agarose gel electrophoresis of DNAs used in this study. M, DNA size marker, *Hin*dIII-digested λ bacteriophage DNA; 1, the 1.27 kb PCR DNA product containing the *Pseudomonas* replication origin; 2, the *Nde*I-digested 3.6 kb pBGS18 plasmid; 3, the *Nde*I-pJH1 shuttle vector; 4, uncut pJH1 purified from HK-6 strain. Low density indicates that pJH1 may exist as low-numbered copies in HK-6; 5, uncut pJH1 containing a EGFP gene purified from HK-6.

B.G. Spratt at Imperial College London). pBGS18, which was employed as a back-born plasmid in this study, has a kanamycin-resistant gene (*aph*) from a transposon *Tn*903, which appeared to induce well in *Pseudomonas* species. *Tn*903 has been used for insertional mutagenesis studies of *Pseudomonas* (Itoh and Haas, 1985; Lam *et al*, 1987). Thus, the newly constructed *Escherichia-Pseudomonas* shuttle vector designated pJH1 possesses not only all convenient features of pUC18 including the multiple cloning site (MCS) and a *lacZ'* gene for the blue/white colony test through α-complementation, but also a kanamycin-resistant gene, a pRO1614 *Pseudomonas* origin and ColE1 *Escherichia* origin (Fig. 1).

The unique restriction sites of the MCS of pJH1 are *Eco*RI, *Sac*I, *Kpn*I, *Sma*I, *Bam*HI, *Xba*I, *Sal*I, *Bsp*MI, *Pst*I, *Sph*I, and *Hin*dIII, which are equivalent to those of a pUC18 plasmid. Kanamycin-resistancy will especially enhance the worth of this vector and facilitate molecular genetic research of *P. putida* because the *P. putida* strains that are currently in use in research are already resistant to various antibiotics in many cases. This means that previously-developed vectors are seldom available for selection.

In order to verify that pJH1 works as a cloning vector for *P. putida*, both electroporation and traditional transformation techniques were used to introduce it into *P. putida* HK-6, a bacterium that degrades explosives such as TNT and RDX (Chang *et al.*, 2004). The electroporation was carried out using Gene Pulser (Bio-Rad, USA), with a 2.5 kV/cm field strength, a 25 μF capacitor, a 200 Ω resistor and a time constant of about 5 ms. The method of Mercer and Loutit (1979) for traditional transformation using 0.15 M $MgCl_2$ and 37°C heat shock was originally designed for *P. aeruginosa* (Mercer and Loutit, 1979), however it has also proved to work well for *P. putida*. After transformation, either LB or *Pseudomonas* selection agar medium containing 20 μg/ml of kanamycin was used to select transformants. Transformation frequency measured by Mercer and Loutit's method was about 2.5×10^{-6} *P. putida* HK-6 cells/μg of pJH1. Electroporation resulted in about 100-fold higher transformation frequency than was achieved using the traditional method.

We next examined whether pJH1 was maintained episomally in the *P. putida* HK-6 strain. Episomal maintenance means that the vector was not integrated into the chromosome and therefore kanamycin-resistancy is expressed from cytoplasmic plasmid and not from the chromosome. pJH1 was purified from *P. putida* HK-6 and successfully re-transformed into *E. coli* cells. In addition, pJH1 plasmid purified from *P. putida* HK-6 was observed through 1.0% (w/v) agarose gel electrophoresis (Fig. 2). These results suggested that pJH1 was indeed episomally maintained in the cytoplasm of *P. putida* HK-6 cells.

Green fluorescent protein (GFP) has been used as a marker of gene expression. To examine the additional use of pJH1 as a potential expression vector, an enhanced GFP gene (EGFP) from the pYEGFP plasmid (Cormack *et al.,* 1997) was amplified and inserted between the *Pst*I and *Hin*dIII sites of pJH1. The inserted gene was transformed into *E. coli* DH5α and *P. putida* HK-6. In this configuration, the EGFP gene is under control of *lac* promoter which originated from pBGS18. This means that it is highly inducible by the addition of isopropyl-ß-D-thiogalactopyranoside (IPTG). After induction with 0.1 mM IPTG, both *E. coli* DH5α and *P. putida* HK-6 cells were observed

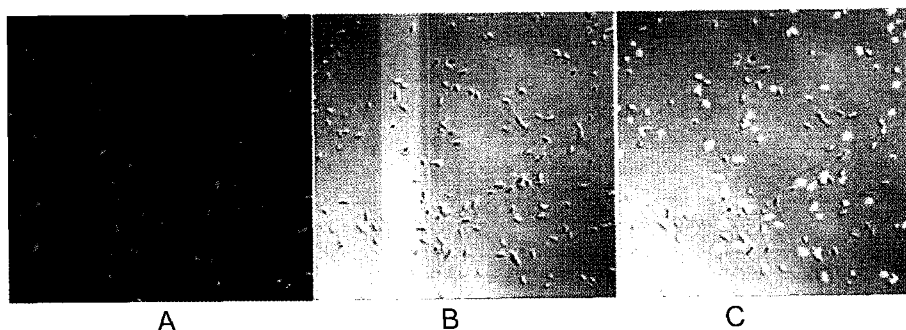

Fig. 3. *E. coli* cells possessing pJH1 inserted with an EGFP gene. A, light micrograph of *E. coli* cells; B, fluorescent micrograph of the same cells illuminated at 488 nm; C, overlapping image of A and B.

under a confocal microscope. *E. coli* cells harboring pJH1 with an EGFP gene were fluorescent when illuminated at 488 nm while *P. putida* cells were not (Fig. 3). At the present, the reason for this lack of fluorescence in *P. putida* cells remains unknown. pJH1 containing the EGFP gene seemed to be maintained well in *P. putida* HK-6 (Fig. 2, lane 5). It may be due to lack of transcription of the *lac* promoter or instability of the EGFP in *P. putida* HK-6 cells. Some of *E. coli* cells were not fluorescent as well (Fig. 3). Although exact reason for this remains unknown, it could also be due to instability of the expressed EGFP or formation of inactive proteins such as inclusion bodies.

There have been several reports regarding the construction of the *Escherichia-Pseudomonas* shuttle vectors. This, however, is the first report to construct a shuttle vector with the pRO1600 replication origin possessing the kanamycin resistant gene (*aph*) of *Tn*903. A series of pUCPs were constructed by West *et al.* (1994). pUCP18 and pUCP26 carried a β-lactamase gene and a tetracycline-resistant gene, respectively. pUCP24 is the most similar to pJH1. It had a 832 bp gentamycin acetyltransferase gene (*aacC1*) instead of a 1.3 kb kanamycin phosphoransferase gene (*aph*). The *aacC1* gene can also confer kanamycin-resistance.

In conclusion, a newly-constructed 4.87 kb shuttle vector with a replication origin of a plasmid pRO1614 was transformed into both *E. coli* and *P. putida* HK-6 and episomally maintained well. Kanamycin-resistance makes pJH1 of greater value since many *Pseudomonas* strains naturally possess ampicillin-resistance. This vector will facilitate molecular genetic studies of the biodegradation of *P. putida* HK-6. It will be especially useful when a genomic library is constructed. If pJH1 is manipulated by inserting an inducible or controllable promoter for *Pseudomonas*, it will be of great importance in *Pseudomonas* research.

Acknowledgements

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