Conjugal vectors for cloning, expression, and insertional mutagenesis in Gram-negative bacteria

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BioTechniques 38:708-712 (May 2005)

The ever increasing pace of genome sequence acquisition, annotation, and transcriptional profiling is providing large data sets of possible open reading frame function. However, detailed gene characterization still requires in vivo genetic manipulation. As a result, there is a need for greater efficiency when applying these procedures to microbial systems. While high-throughput genetic strategies have been described for most members of the family *Enterobacteriaceae* (1,2), *Bacillus subtilis* (3), and the yeast *Saccharomyces cerevisiae* (4), all of which can be easily transformed, there is a need to develop similar approaches for those hosts that cannot be transformed. Here we report the development of a set of conjugal plasmids for insertional mutagenesis, complementation, and inducible expression in a wide variety of Gram-negative bacteria and the test of their efficiency using the psychrotolerant γ-proteobacterium *Photobacterium profundum* strain SS9, for which the whole genome sequence has recently been completed (5).

Site-specific mutagenesis in *P. profundum* has previously been performed using conjugal narrow host-range suicide plasmids for single-crossover insertional mutagenesis (6) or for double-crossover allelic exchange (7). While useful, these methods suffer from the need to first PCR clone fragments of interest into a vector such as pCR2.1 (Invitrogen, Carlsbad, CA, USA) and then subclone into the plasmid to be delivered into *P. profundum*. To overcome this limitation, a TA-cloning (8) conjugal suicide vector (pEE3) was successfully engineered from parental plasmids pBBRI-MCS2 (9,10) and the narrow host-range, mobilizable, ColE1 plasmid pMUT100 (11), which does not replicate in *P. profundum* SS9 (6). Initially, dual *Xcm*I sites were introduced within the *lacZ* gene of pBBRI-MCS2 allowing for the generation 3′ T overhangs upon *Xcm*I restriction. This construct was subsequently subcloned into the *Eco*RI site of the suicide vector pMUT100 yielding pEE3 (Figure 1A). With this, plasmid PCR fragments internal to genes of interest can be amplified with *Taq* DNA polymerase and directly ligated into a *Xcm*I-digested vector for single-crossover insertional mutagenesis. *Escherichia coli* transfor-

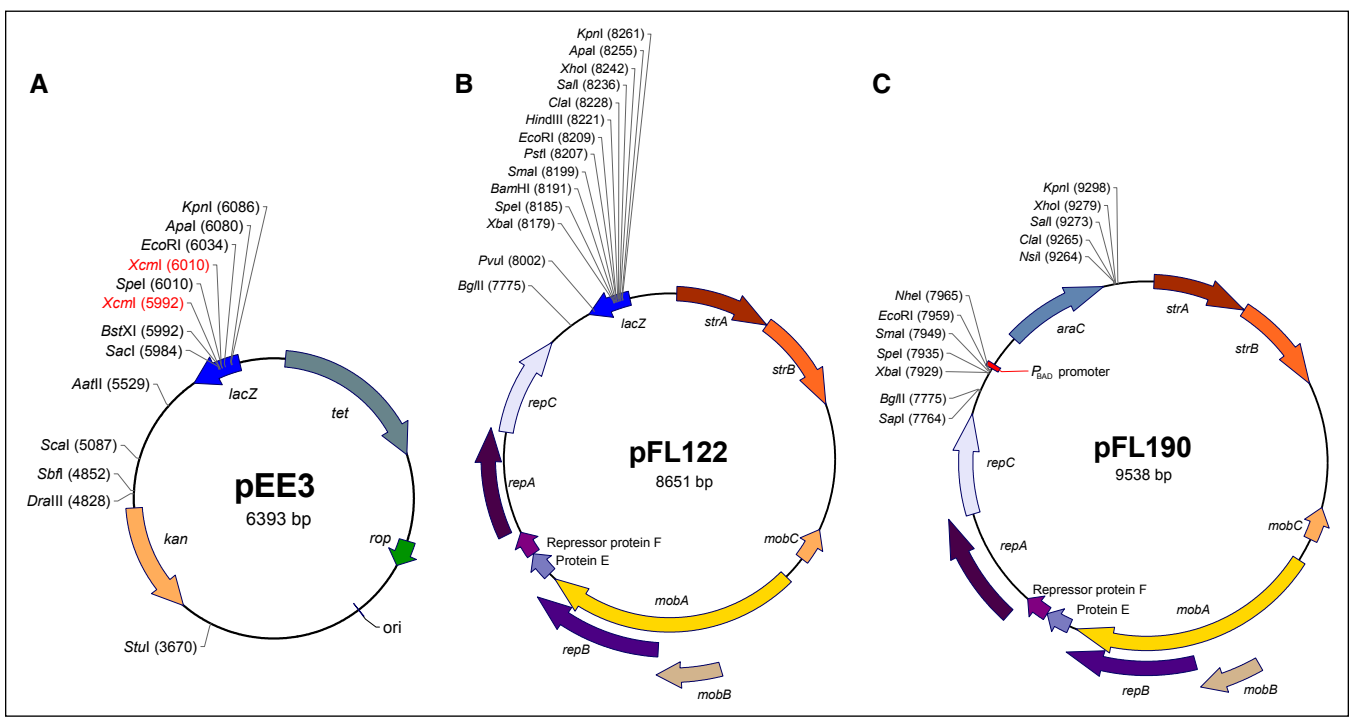

Figure 1. Physical map of the vectors presented in this study. (A) TA-cloning conjugal suicide vector. (B) Broad host-range cloning vector. (C) Broad host-range expression vector. Only the relevant restriction sites are shown. Antibiotic markers: *strA*, streptomycin resistance protein A; *strB*, streptomycin resistance protein B; *tet*, tetracycline resistance protein; *kan*, kanamycin resistance protein. ori, origin of replication.

Table 1. Comparison of the Conjugation Efficiencies of pFL122 with Two Other Broad Host-Range Vectors in *Photobacterium profundum*

Sample	Conjugation Efficiency (exconjugants/ recipients)
pFL122	$1.779 \times 10^{-5} \pm 24.7\%$
pFL190	$1.623 \times 10^{-6} \pm 43.5\%$
pKT231	$4.689 \times 10^{-5} \pm 52.4\%$
pBBR1-MCS2	$<1.2 \times 10^{-9}$

Values are in number of exconjugants per conjugal recipient cells with the relative standard error.

mants containing plasmids with inserts are readily identified on 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal)-containing media as white colonies. The desired recombinant plasmid can then be transferred via conjugation from *E. coli* into the appropriate Gram-negative bacterium by triparental mating using a second *E. coli* strain containing a helper plasmid such as pRK2073 (12). Exconjugants obtained by kanamycin or tetracycline selection are likely to contain an insertion in the gene of interest. As with other gene knockout systems, it is advisable to check that the correct insertion has been obtained by PCR amplification and sequencing of the junction points.

To date, the pEE3 cloning system has been used to generate five knockouts in *P. profundum* with an average efficiency of 2.4×10^{-9} gene disruptions per conjugal recipient. In addition, this vector is also useful for restriction endonuclease-mediated cloning into the multiple cloning sites within *lacZ*.

A second plasmid, designated pFL122, has been designed for complementation studies (Figure 1B). It is an improved version of previous RSF1010-derived (IncQ) vectors, in that it contains a *lacZ* marker with a multiple cloning site for convenient cloning and screening along with streptomycin resistance. The choice of a selectable marker allows pFL122 to be used to complement most types of transposon

Table 2. β-Galactosidase Activity as a Function of Arabinose Concentration in pFL191

L-Arabinose Concentration (%)	pFL191 in *Escherichia coli* DH5α	pFL191 in *Photobacterium profundum* DB110
0	<2	<2
0.01	1153 ± 12.2%	64 ± 38.3%
0.5	1296 ± 9.6%	236 ± 4%

This plasmid is a derivative of pFL190 where the P_{BAD} promoter drives *lacZ* expression. Activity is expressed in Miller units (17) with the relative standard error. *E. coli* DH5α was grown aerobically in Luria-Bertani broth with 100 µg/mL streptomycin at 37°C. *P. profundum* DB110 was grown aerobically in Difco™ Marine Broth 2216 (BD Diagnostic Systems, Sparks, MD, USA) with 150 µg/mL streptomycin at 15°C.

mutants (e.g., see Reference 13) as well as insertional knockouts obtained with pEE3. Conjugation efficiencies of pFL122 into *P. profundum* (Table 1) are comparable to those obtained with the widely used IncQ plasmid pKT231 (14) and are vastly improved over the broad host-range vector pBBR1-MCS2 (9,10). pFL122 has been successfully used to complement the growth defect of four transposon mutants of *P. profundum* (unpublished results).

Finally, we have prepared a new expression vector (Figure 1C). While a wide variety of expression systems are available in *E. coli*, there are only a few reports of successful use of inducible expression systems in other hosts (15). A vector containing the P_{BAD}/*araC* system was prepared by subcloning the relevant portion of pJN105 (15) into an RSF1010 (IncQ) derivative (pFL107) resulting in pFL190. Reporter gene constructs in pFL190 have been successfully used to provide β-galactosidase and green fluorescent protein (GFP) expression in *P. profundum*. Table 2 provides β-galactosidase activity as a function of arabinose concentration in the P_{BAD}:: *lacZ* derivative of pFL190.

Since IncQ plasmids have been shown to replicate in a wide variety of Gram-negative bacteria including, *Agrobacterium*, *Alcaligenes*, *Pseudomonas*, *Rhizobium*, and *Vibrio* (14,16), the above vectors should be applicable to studies in a variety of bacterial hosts. The presence of multiple unique restriction sites outside of the cloning region makes it easy to broaden their applicability by adding other antibiotic resistance markers. The physical map of all these vectors is reported in Figure 1, while nucleotide sequences are available under the GenBank® accession numbers AY785148, AY785149, and AY785150.

ACKNOWLEDGMENTS

This work was supported by National Science Foundation grant no. NSF/MCB 02-37059 to D.H.B.

COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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Received 17 January 2005; accepted 11 February 2005.

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