Benchmarks

pBBR1-GFP: A Broad-Host-Range Vector for Prokaryotic Promoter Studies

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For the identification of DNA sequences acting as promoters in bacteria and for the determination of environmental conditions under which certain inducible promoters are functioning, it is indispensable to perform genetic analysis in the original bacterial host. However, the number of vectors that are currently available for the isolation and the study of prokaryotic promoters in bacteria other than *Escherichia coli* is limited.

Therefore, we have developed a promoter-selection vector for gram-negative bacteria that allows for the isolation of DNA fragments containing gene promoters and the study of these promoter sequences. To that end, we have chosen the previously described broad-host-range, mobilizable plasmid pBBR1MCS (6) as our tool for modification. In the first step, leading to the construction of the new vector pBBR1MAC2 (not shown), the plasmid selection gene coding for chloramphenicol acetyltransferase (CAT) was replaced by a β -lactamase gene (*bla*) coding for ampicillin resistance, and the β -galactosidase (β -gal) and bacteriophages T3 and T7 promoters were deleted. This eliminates the risk of uncontrolled reporter gene activation by these promoters. We then introduced a promoterless reporter gene encoding CAT into the *Hind*III/*Hinc*II-sites of the pBBR1MCS polylinker. Under chloramphenicol selection, pBBR1MAC2 by itself allows rapid screening of recombinant plasmids for the presence of promoters inserted 5' of the *cat* gene. In a second step, we cloned the promoterless *gfp* cassette *gfp*mut3, encoding the green fluorescent protein (GFP) (7) into the *Pst*I site of pBBR1MAC2, immediately upstream of *cat* and in the same transcriptional orientation as *cat*. The encoded mutated form of GFP fluoresces 21-fold more intensely than the wild-type (WT) form. The lack of a transcriptional ter-

minator at the 3' end of *gfp* results in simultaneous expression of the genes encoding the fluorescent protein and CAT when a promoter is inserted into the cloning sites 5' of *gfp*. In *Brucella* spp. (the bacterial host we used), the repression of the reporter genes was leaky even in the absence of a cloned promoter due to undefined low-level transcriptional activity from the pBBR1MCS vector backbone. Therefore, we inverted the orientation of the artificial *gfp-cat*-operon after digestion of DNA by *Xho*I. This has led to the final construct of 6.64 kb in size, named pBBR1-GFP (Figure 1).

Among the unique cloning sites available upstream from the *gfp-cat* genes, *Bgl*III appears very useful because it allows shotgun cloning of chromosomal DNA fragments partially digested by *Sau*3A. Depending on the

strategy of selection chosen, recombinant bacteria containing cloned promoters can be identified either by acquired resistance to chloramphenicol or by their fluorescence. The gene required for plasmid mobilization when the RK2 transfer function is present in *trans* (1) has been maintained and will allow the introduction of recombinant plasmids into gram-negative bacteria that are difficult to transform by standard techniques.

Elzer et al. have shown that the parent plasmid pBBR1MCS was stably maintained in the six *Brucella* species at a medium copy number (3), and recent publications have demonstrated that it can be used as a cloning vector for genetic complementation in *Brucella* spp. (4,5). Earlier, it has been described that the original plasmid used for pBBR1MCS construction is stable

Figure 1. Physical map of pBBR1-GFP. The β -gal/T3-promoters and the gene encoding chloramphenicol resistance were deleted from the parent plasmid pBBR1MCS following double digestion by *Aat*II and *Kpn*I and replaced by a 1.5-kb *Hae*II/*Ssp*I DNA fragment containing the *bla* gene from pUC18. The T7 promoter was eliminated by double digestion with *Nae*I and *Sac*I (pos. 2594 and 3219 on pBBR1MCS) followed by religation. A promoterless *cat* gene coding for CAT was obtained as a 1.5-kb *Hind*III/*Hinc*II fragment from pA10-CAT2 (2) and cloned into the corresponding sites of the pBBR1MCS-derived vector. The promoterless *gfp* gene was isolated as a 820-bp *Pst*I fragment from pFPV27 (7) and inserted in the same orientation into the *Pst*I site upstream of *cat*. Following digestion by *Xho*I, the orientation of the 2.3-kb *gfp-cat* fragment in the vector was inverted, generating the final construct pBBR1-GFP. For simplification, only the unique cloning sites *Bgl*III and *Kpn*I and both *Xho*I sites are shown. Abbreviations: mob, gene required for plasmid mobilization (1); rep, gene required for plasmid replication (1); bla, beta-lactamase. The complete sequence has been submitted to GenBank® and assigned the Accession No. AF110459.

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in *Bordetella*, *Escherichia*, *Vibrio*, *Pseudomonas* and *Rhizobium*, and that it is compatible with plasmids belonging to the IncP, IncQ and IncW group (1) and with the group of ColE1-containing plasmids. The remarkable advantage of our construct is the simultaneous presence of two promoterless reporter genes arranged as a tandem devoid of any intermediate transcriptional terminator, allowing a positive phenotypic selection either on chloramphenicol-containing media or based on fluorescence. The first approach allows rapid cloning of mainly constitutively expressed genes, whereas the GFP-based screening is of great value for the isolation of transiently expressed genes and for gene expression studies under various environmental conditions, using fluorescence microscopy analysis or flow cytometry.

In our laboratory, we have been able to isolate a constitutively expressed promoter located on a previously cloned 8-kb *EcoRI* fragment of genomic DNA from *Brucella ovis*, following digestion with *Sau3A* and subcloning into pBBR1-GFP. The resulting plasmid pBBR1-GFP-SOG of the recipient strain *B. suis* 1330 has been selected by its resistance to chloramphenicol (25 µg/mL), and it emits green fluorescent light when excited at 450–490 nm, as

visualized by fluorescence microscopy (not shown). The fluorescence was also measured by flow cytometry (Figure 2) after excitation at 488 nm, using a FACSCalibur™ Scanner (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). No chloramphenicol resistance or fluorescence has been detected with the WT and the strain containing pBBR1-GFP with a promoterless *gfp* (Figure 2).

In conclusion, we have developed a new broad-host-range, promoter-selection vector that preserves the advantages of its parent plasmid pBBR1MCS, i.e., to be mobilizable, compatible with various plasmid replicons and present at a medium copy number. The characteristic presence of the two promoterless reporter genes *gfp* and *cat*, described above, distinguishes pBBR1-GFP as a useful tool in molecular biology for a broad range of gram-negative bacteria.

Figure 2. Detection of *B. suis* expressing GFP by flow cytometry. Fluorescence intensity of *gfp*-expressing *B. suis* (pBBR1-GFP-SOG) and non-*gfp*-expressing bacteria (WT, pBBR1-GFP) was examined with a FACSCalibur Scanner, and Cell Quest software (Becton Dickinson Immunocytometry Systems) was used for the analysis and quantitation of fluorescence. $\bar{x}$ = mean fluorescence intensity.

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