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Small Broad-Host-Range *lacZ* Operon Fusion Vector with Low Background Activity

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The use of gene fusion technologies for transcriptional analysis of gene expression, especially of those genes whose products are difficult to assay, has been well documented (12–15). Many narrow and broad-host-range plasmid-based systems are available for constructing such gene fusions (6,7, 14). To circumvent some of the inherent problems of plasmid-based systems, which have been discussed before (3), systems have been developed for single-copy, chromosomal insertion of gene fusions (4,9,14). For some applications (e.g., deletion mapping of regulatory regions or use in bacteria for which no integration-proficient vectors are known), plasmid-based fusions are still indispensable. The most useful vectors use a promoterless β -galactosidase (β -gal) gene, *lacZ*, as a reporter. However, most broad-host-range *lacZ*-based vectors available to date exhibit some potentially serious drawbacks, including (i) high levels of background expression due to transcription from plasmid-encoded promoters and/or efficient translation of low levels of *lacZ* message due to a strong consensus Shine-Dalgarno (SD) sequence, (ii) a lack of unique cloning sites, and (iii) large size due to the presence of unknown and usually unnecessary nucleotide sequences. To address these issues, a new small broad-host-range transcriptional *lacZ* operon fusion vector was constructed and tested in *Pseudomonas aeruginosa*.

The new *lacZ* vector, pTZ110 (Figure 1A), was obtained in two steps. First, a 1544-bp blunt-ended *Pst*I-*Stu*I fragment from pSF2 (10), containing an origin of transfer, the *ori*₁₆₀₀ broad-host-range origin of replication and its associated replication protein (16), was ligated into a *Bal*I site located 271 nucleotides downstream of the *lacZ* gene of pGE593 (2) to obtain pTZ100. This operon fusion vector contains unique

Figure 1. Map of the operon fusion vector pTZ110 (A) and GlpR-dependent *glpF-lacZ* expression in *P. aeruginosa* (B). (A) The sequence preceding the promoterless *lacZ* (*lacZ*) gene contains stop codons in all frames and an SD sequence that is close to consensus but not too strong. Other abbreviations: *bla*, β -lactamase-encoding gene; *ori*, pBR322 origin of replication; *ori*₁₆₀₀, pRO1600 origin of replication; *oriT*, origin of transfer; *rep*, *ori*₁₆₀₀ replication protein. The sequence of pTZ110 was submitted to GenBank® and assigned accession no. AF376056. (B) Transcriptional activity of the *glpF-lacZ* operon promoter present on pPFK1 was assessed by measuring either β -gal activities in strains PAO1 (wild-type) or PAO160 (*glpR*) containing the indicated plasmids. The strains were grown aerobically at 37°C in LB medium (Invitrogen, Carlsbad, CA, USA) to log phase (absorbance at 540 nm of approximately 0.7). β -gal expression was measured in chloroform/SDS-permeabilized cells, and activity units were calculated as previously described (5). Samples were assayed in triplicate, and the average values are given above the columns.

*Eco*RI, *Sma*I, and *Bam*HI sites upstream of a promoterless *lacZ* gene. Second, pTZ110 was obtained by ligating a 26-bp oligonucleotide linker with *Eco*RI and *Bam*HI overhangs between the same sites of pTZ100. The resulting operon fusion vector contains a multiple cloning site with six unique restriction enzyme cleavage sites, stop codons in

the three reading frames, and a modified SD sequence, which is close to consensus but not too strong. The multiple cloning site was designed to contain blunt-end generating sites close to each end and compatible sites (e.g., *Bam*HI and *Xho*I allow cloning of fragments generated with *Bgl*II and *Sal*I, respectively). Using the pBR322-based repli-

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con, this vector replicates with an intermediate copy number in *E. coli* and using *ori*₁₆₀₀ with a low copy number in *Pseudomonas* (10–15 copies) (8). These copy numbers allow for easy isolation and manipulation of plasmid DNA from *E. coli* hosts, while they are at the same time desirably low for analysis of gene expression in *Pseudomonas* hosts.

To assess the functionality of pTZ110, a *glpF-lacZ* transcriptional fusion was constructed and analyzed. The *glpF* gene is part of the *P. aeruginosa glpFK* operon, whose expression is negatively regulated by the glycerol-3-phosphate regulon repressor, GlpR (11). The amino-terminal 14 codons of *glpF* and its upstream 219-bp regulatory region containing a single GlpR operator site were PCR amplified from pPS585 (11). The reactions contained the 24-mer universal *lacZ* primer and a *glpF*-specific primer (5'-CTCGGACA-GGCcTTGGCCGAACAG-3'), which introduced a single base change (lower-case letter) and therefore a *StuI* site (underlined) at codon 14 of *glpF*. The resulting PCR fragment was digested with *EcoRI* and *StuI*, and a 282-bp *EcoRI-StuI* fragment was gel-purified and ligated between the *EcoRI* and *SmaI* sites of pTZ110 to form pPFK1. In this construct, translation of an 18-amino-acid GlpF fusion peptide is terminated at the second TAA codon provided by pTZ110. The correct physiological response pattern of the *glpF-lacZ* fusion was assessed by conjugating pPFK1 from *E. coli* strain SM10 into wild-type *P. aeruginosa* PAO1 and the PAO1 *glpR* mutant PAO160 (11). Exconjugants were selected on minimal citrate medium (3) containing 100 µg/mL carbenicillin. Alternatively, plasmids were transferred by electroporation (1) followed by selection on LB medium containing 100 µg/mL carbenicillin. β-gal expression from the *glpF-lacZ* operon fusion was then measured after growth in the appropriate media (Figure 1B). As expected, *glpF-lacZ* was expressed in a GlpR-dependent manner, and very low background β-gal activity was observed in the pTZ110-containing strains. β-gal expression in the wild-type strain is not completely repressed because LB medium contains traces of the inducer glycerol-3-phosphate and *glpFK* operon ex-

pression is easily induced by dislodging GlpR from the single operator site (11).

In summary, with the construction of the pTZ110 *lacZ* operon fusion vector, several problems associated with similar broad-host-range fusion vectors have been resolved. First, background β-gal expression levels are minimized because of the incorporation of an SD sequence that is close to consensus but not too strong. Second, cloning of promoter-containing fragments is facilitated by six unique cloning sites, including two blunt-end-generating restriction sites. Third, the complete nucleotide sequence of the new small 7856-bp vector is known, and it contains only fully functional DNA sequences. Although this broad-host-range vector has thus far only been tested in *P. aeruginosa*, it will undoubtedly be useful with the many *Pseudomonas* and other bacteria in which the *ori*₁₆₀₀ replicon is functional (10).

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Work in the authors' laboratory is supported in part by National Institutes of Health grant no. GM56685. Address correspondence to Dr. Herbert P. Schweizer, Department of Microbiology, Colorado State University, Fort Collins, CO 80523-1677, USA. e-mail: herbert.schweizer@colostate.edu

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**Herbert P. Schweizer and
Rungtip Chuanchuen**
Colorado State University
Fort Collins, CO, USA