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Integration-Proficient *Pseudomonas aeruginosa* Vectors for Isolation of Single-Copy Chromosomal *lacZ* and *lux* Gene Fusions

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Transcriptional analyses of many genes of unknown function identified in whole genome sequencing projects or genes whose products are difficult to assay are facilitated by gene fusion technologies (10,12). Although many plasmid-based systems are available for constructing such gene fusions (3,10), they suffer from inherent problems that have been discussed before (3). To facilitate gene integration at a defined neu-

tral site, various phage systems have been designed to integrate exogenous gene sequences at chromosomal phage attachment sites. In *E. coli*, λ -based systems have been developed for single-copy chromosomal integration of gene fusions and other gene cassettes (4,11). We recently developed an integration-proficient fCTX-based system for *Pseudomonas aeruginosa* (3), modeled after an approach that was previously described for mycobacterial integration

vectors (13). This approach included (i) inclusion of the fCTX *int* gene on the same vector containing *attP* and (ii) inclusion of a multiple cloning site (MCS) for facilitated cloning of DNA fragments. We further improved our system by the engineering of *FRT* sites, which allow subsequent *in vivo* removal of unwanted plasmid sequences from the genome (2). In this communication, we describe two new mini-CTX vectors, mini-CTX-*lacZ* and mini-CTX-*lux*, that

Figure 1. Maps of the integration vectors mini-CTX-*lacZ* and mini-CTX-*lux* (A) and illustration of integrase-mediated chromosomal integration of a *lasBlacZ* fusion (B). Plasmids are drawn to scale. The locations of genes and their transcriptional orientations are shown, including the T4 transcription terminators (W). The *R* in mini-CTX-*lacZ* marks the location of an RNase III processing site that is located between the MCS and the *lacZ* ribosome binding site. Other abbreviations: *attB*, chromosomal fCTX attachment site; *attP*, fCTX attachment site; *FRT*, Flp recombinase target sites; *int*, fCTX integrase encoding gene; *lacZ*, *E. coli* b-gal structural gene; *ori*, ColE1 origin of replication; *oriT*, origin of transfer; *P_{lasB}*, elastase structural gene promoter; and *tet*, tetracycline resistance encoding gene. Only important restriction enzyme cleavage sites are shown. The nucleotide sequences of mini-CTX-*lacZ* and mini-CTX-*lux* were deposited in GenBank[®] and assigned the accession nos. AF140579 and AF251497, respectively.

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facilitate the isolation of chromosomally located *lacZ* and *lux* transcriptional fusions, respectively.

The mini-CTX-*lacZ* vector was constructed by subcloning a 3304-bp *EcoRI*-*DraI* fragment from pLPBL (obtained from Dr. Lou Passador, University of Rochester) between the *Bam*HI-*Sac*I (blunt) sites of mini-CTX1. This step preserved many useful cloning sites upstream of the promoterless *lacZ* gene. The resulting mini-CTX-*lacZ* vector (Figure 1A) contains an RNase III processing site between the MCS and *lacZ*, which ensures construction of transcriptional fusions that are translated independently of *lacZ*

(4). The MCS and junction sequences were verified by nucleotide sequencing. To assess its function, a *lasB-lacZ* transcriptional fusion was constructed by ligating a 428-bp *EcoRI*-*Bam*HI fragment from pPS959 (3) containing the cell density-dependent *lasB* promoter between the same sites of mini-CTX-*lacZ*. The *lasB-lacZ* fusion was transferred to the PAO1 chromosome, and unwanted DNA sequences were excised using previously described methods (2,3) and as schematically illustrated in Figure 1B. The resulting strain was named PAO1 (*lasB-lacZ*). As a control, mini-CTX-*lacZ* was integrated into the PAO1 chromosome

yielding strain PAO1 (*lacZ*) containing a chromosomally integrated promoterless *lacZ* cassette. The correct physiological response pattern of the *lasB-lacZ* fusion was assessed by measuring b-galactosidase (b-gal) expression as a function of cell density (Figure 2, A and B). As expected, *lasB-lacZ* was expressed in the typical cell density-dependent manner, and no b-gal activity could be detected in the PAO1 and PAO1 (*lacZ*) strains.

The mini-CTX-*lux* vector contains the *lux* gene cluster from *Xenorhabdus luminescens*, which had been modified to eliminate several restriction sites from the *luxCDABE* operon (14). Advantages of this bioluminescence operon include: (i) it directs the synthesis of a fatty acid reductase complex (encoded by the *luxCDE* genes), which synthesizes tetradecanal, a natural substrate of luciferase (encoded by the *luxAB* genes); thus, no exogenous substrate is required for light emission; and (ii) the *luxAB*-encoded proteins of *X. luminescens* form the most thermostable of the known luciferases (14). The mini-CTX-*lux* vector was derived in several steps. First, the *luxCDABE* operon was subcloned on a 6957-bp blunt-ended *EcoRI* fragment from pLITE201 (14) into the *Sma*I site of pUC19 (15) such that the *lux* operon was transcribed from the plasmid-borne *lac* promoter in the resulting plasmid pUC-*lux*. Second, the *lux* operon was excised from pUC-*lux* on a 6967-bp *Bam*HI-*Kpn*I (blunt) fragment, which allowed directed subcloning between the *Bam*HI-*Sac*I (blunt) sites of mini-CTX1, forming mini-CTX-*lux* (Figure 1A). The MCS and junction sequences were verified by nucleotide sequencing.

To assess the utility of mini-CTX-*lux* for *lux* gene fusion construction, we again subcloned the *lasB* promoter on a 428-bp *EcoRI*-*Bam*HI fragment between the same sites of mini-CTX-*lux*. The *lasB-lux* fusion was transferred to the PAO1 chromosome, and unwanted plasmid sequences were excised; this resulted in strain PAO1 (*lasB-lux*). As a control, strain PAO1 (*lux*) was derived in a similar manner by integrating the promoterless *lux* gene into the PAO1 chromosome via mini-CTX-*lux*, followed by excision of unwanted plasmid

Figure 2. Growth phase-dependent *lasB* expression. Transcriptional activity of the *lasB* promoter was assessed by measuring either b-gal or luciferase activities in strain PAO1 containing chromosomally integrated *lasB-lacZ* transcriptional gene fusions. Control strains used were PAO1 and PAO1 (*lacZ*) or PAO1 (*lux*) containing chromosomally integrated promoterless *lacZ* or *lux* genes, respectively. Cells of the indicated strains were grown at 37°C either in PTSB medium (5% peptone, 0.25% tryptic soy broth) (1) (for b-gal assays) or in LB medium (6) (for Lux assays). Aliquots were withdrawn at the indicated time intervals, and the optical density at 540 nm was recorded (panels A and C). b-gal expression (panel B) was measured in polymyxin B permeabilized cells (7) using a previously described procedure (9) that was adapted for a microplate format (5). Relative luciferase activity (panel D) was determined by measuring light emission in 1-mL samples on a TD-20e luminometer (Turner Designs, Mountain View, CA, USA). Samples were assayed in triplicate, and the *t* distribution was used to establish 95% confidence limits.

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sequences. From the results shown in Figure 2, C and D, it is evident that the *lasB-lux* fusion in PAO1 (*lasB-lux*) was expressed in a cell density-dependent manner, and no or only background *lux* expression was observed in the control strains PAO1 and PAO1 (*lux*), respectively. In contrast to *lasB-lacZ*, *lasB-lux* expression could be measured much earlier in the growth curve, and maximal *lasB* expression was observed within 5 h with a single-copy *lasB-lux* fusion, whereas it took overnight growth to observe high-level expression with a single-copy *lasB-lacZ* fusion. We are not sure why *lasB-lux* expression declines over time, but it may be indicative of competition of the autoinducer synthase LasI and the LuxCDE fatty acid reductase complex for acyl-acyl carrier protein substrates, which would increase over time because LasI expression itself is increased with increasing cell density (8). The observed expression differences were not attributable to media differences because similar results were seen with

cells grown in either peptone-tryptic soy broth medium (PTSB; 5% peptone, 0.25% tryptic soy broth) (1) or LB medium (6) (not shown).

In summary, with the construction of the mini-CTX-*lacZ* and mini-CTX-*lux* vectors, we have generated useful *P. aeruginosa* integration vectors facilitating transcriptional studies involving single-copy, chromosomally located gene fusions. Their usefulness was demonstrated by constructing cell density-regulated *lasB-lacZ* and *lasB-lux* fusions. We have previously shown that a chromosomally integrated *lasB-lacZ* fusion was stably maintained for 100 generations in the absence of selective pressure (3). Though integration of mini-CTX plasmids containing chromosomal DNA fragments could also occur via RecA-mediated homologous recombination leading to merodiploid formation, we and others observed this to be a rare event when keeping homologous sequences to a minimum. Nonetheless, putative integrants are always checked by colony PCR, using a previously described primer pair flanking the chromosomal *attB* site (3) or combinations of these primers with primers designed to the integrated DNA sequences. With appropriate adaptations, similar integration vectors could be developed for other bacteria.

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