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Construction of a Broad-Host-Range Tn*7*-Based Vector for Single-Copy P_{BAD}-Controlled Gene Expression in Gram-Negative Bacteria

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We describe a mini-Tn*7*-based broad-host-range expression cassette for arabinose-inducible gene expression from the P_{BAD} promoter. This delivery vector, pTJ1, can integrate a single copy of a gene into the chromosome of Gram-negative bacteria for diverse genetic applications, of which several are discussed, using *Pseudomonas aeruginosa* as the model host.

New tools to manipulate gene expression are always needed to facilitate the characterization of genes and pathways. Many plasmid-based vector systems have been developed and extensively used to test hypotheses in bacteria and other organisms. In recent years, the broad-host-range mini-Tn*7* vectors have been developed for single-copy genetic analysis (1). These vectors have been shown to be useful in a number of bacteria (1–7) because the specific site where the vector integrates into the chromosome is highly conserved (1). In this study, our goal was to expand the extensive collection of mini-Tn*7* vectors by adding a P_{BAD} arabinose-inducible promoter for controlled expression of genes integrated into the chromosome.

Incorporation of the *araC* repressor and P_{BAD} promoter into the mini-Tn*7* vector for single-copy arabinose-inducible gene expression. The P_{BAD} promoter is an inducible promoter that is tightly controlled (8). In the presence of arabinose, repression of the P_{BAD} promoter by AraC is lost, resulting in titratable levels of expression of genes under the control of this promoter. The P_{BAD} promoter has proven useful in many hosts and vector constructs, but the pHERD series of plasmid vectors in particular have been useful in both *Pseudomonas* and *Burkholderia* species (9). However, there are several caveats associated with this approach such as the need for antibiotics to maintain plasmids and the possible undesirable effects of having multiple copies of plasmid-borne genes. We addressed these two problems by using a broad-host-range mini-Tn*7* vector to integrate genes in single copies into the bacterial chromosome under the control of the P_{BAD} promoter. To develop a single-copy mini-Tn*7* vector carrying the P_{BAD} expression system, high-fidelity PCR was used to generate amplicons of the pUC18T-mini-Tn*7*T-Tp backbone (4) and the *araC*-P_{BAD} region of pHERD20T (9). The sequences of the primers used to generate the two fragments are listed in Table 1. The two amplicons contained engineered flanking AscI and NotI restriction endonuclease sites. The two PCR fragments were digested with restriction enzymes, purified, and then ligated. For simplicity, the pUC18T-mini-Tn*7*T-Tp-*araC*-P_{BAD}-MCS vector is referred to here as pTJ1 (Fig. 1A; see also Fig. S1 in the supplemental material). The backbone plasmid contains the *dhfRII* gene encoding trimethoprim resistance, which can be used to select for chromosomal integration. We routinely perform quad-parental conjugations into *Pseudomonas aeruginosa* or *Burkholderia cepacia* complex (BCC) species, with *Escherichia coli* containing the helper plasmid pRK2013, *E. coli* containing the plasmid pTNS3, which encodes the Tn*7* transposase (10), and pTJ1 containing the gene of interest. The four strains are grown under selective conditions, mixed, and spotted on Trypticase soy agar (TSA). For *P. aeruginosa*, chromosomal integrants are selected on L-agar (10 g Bacto tryptone [BD-Difco], 5 g Bacto yeast extract [BD-Difco], 5 g NaCl [Fisher Scientific], and 15 g Bacto agar [BD-Difco] per liter of H_2O) containing 1,500 μg/ml trimethoprim and 25 μg/ml spectinomycin to counterselect against the *E. coli* strains. High-efficiency selection can be obtained under these conditions; however, not all strains require the high concentration of trimethoprim used here for chromosomal insertion. Chromosomal integration can be confirmed by PCR amplification with previously described primers (4). The use of the *dhfRII* marker allows compatibility with trimethoprim-sensitive bacteria, such as strains from the *P. aeruginosa* transposon libraries of PAO1 (12) and PA14 (13). For example, if a mutant from these libraries displays a phenotype of interest, pTJ1 can be used to clone the corresponding gene into its multiple-cloning site (MCS), shuttle it to the chromosome in a single copy and in a site- and orientation-specific manner, and express it at various levels by the addition of arabinose to confirm the requirement of the gene for the phenotype. Plasmid pTJ1 also contains Flp recombinase target (*FRT*) sites from the pUC18T-mini-Tn*7*T-Tp parent plasmid, so the *dhfRII* resistance marker can be excised by in *trans* expression of Flp recombinase from vectors such as pFLP2 (14).

To demonstrate the functionality of the vector, we cloned *guaB*, encoding inosine-5′-monophosphate dehydrogenase (IMPDH), from *P. aeruginosa* strain PA14 (15) into pTJ1. IMPDH is an enzyme that is involved in purine nucleotide biosynthesis. A hemagglutinin (HA) epitope tag (YPYDVPDYA) was incorporated into the 3′ end of the *guaB* gene for Western blot detection of expressed IMPDH. We performed high-fidelity PCR with the reverse primer (HindIII-*guaB*-HA-R), which includes the sequence for the HA tag (Table 1), to amplify *guaB*. The mini-Tn*7* element containing the *araC*-P_{BAD}-*guaB*-HA expression cassette was

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TABLE 1 Plasmids and PCR primers used in this study

Plasmid or primer	GenBank accession no. and/or relevant characteristic(s) or sequence	Reference or source
Plasmid		
pUC18T-mini-Tn*7*T-Tp	DQ493875; suicide vector for shuttling single copies of genes directly to the chromosome via a mini-Tn*7* element; *dhfRII* gene encoding trimethoprim resistance marker on Tn*7* element; contains *oriT* for mobilization	4
pHERD20T	EU603324; multicopy shuttle vector; source of the *araC* repressor gene, P_{BAD} promoter, and multiple cloning site (MCS)	9
pTJ1	JX559783; pUC18T-mini-Tn*7*T-Tp-*araC*-P_{BAD}-MCS; a delivery vector containing the mini-Tn*7*T-Tp-*araC*-P_{BAD}-MCS mobile element for cloning and subsequent integration into the chromosome; genes cloned in this vector are under the control of the P_{BAD} arabinose-inducible promoter	This study
pTJ1-*guaB*-HA	pTJ1 with *P. aeruginosa* strain PA14 *guaB*-HA epitope tag; cloned between the EcoRI and HindIII restriction sites	This study
pHERD20T-*guaB*-HA	pHERD20T with *P. aeruginosa* strain PA14 *guaB*-HA epitope tag; cloned between the EcoRI and HindIII restriction sites	This study
pTNS3	Helper plasmid encoding transposase gene necessary for the chromosomal integration of mini-Tn*7*	10
pRK2013	Helper plasmid for conjugation	11
PCR primers		
pH MCS R2 NotI-R	5′-CTAGGCGGCCGCAAGCTTGCATGCCTGCAG	This study
AscI-*araC*-F	5′-CTAGGGCGCGCCCAAATTATGACAACTTGA	This study
AscI-miniTN7-F	5′-CTAGGGCGCGCCACTAGTGAGCTCATGCAT	This study
NotI-miniTN7-R	5′-GATCGCGGCCGCGGGCCCGGTACCTCGCGA	This study
pHERDSF[a]	5′-ATCGCAACTCTCTACTGTTTCT	9
EcoRI-*guaB*-F	5′-CTAGGAATTCCCTGCGAATCAGTCAAGAAGCCC	This study
HindIII-*guaB*-HA-R	5′-CTAGAAGCTTTCAAGCGTAATCTGGAACATCGTATGGGTAACCA ACCCGGTAGTTGGGG	This study

[a] pHERDSF is used for routine PCR screening of clones and sequencing of constructs.

transferred to PA14, and the integration into the chromosome was confirmed as described above. Strains were grown in minimal media lacking or containing arabinose as indicated (Fig. 2). As a control, IMPDH-HA was expressed from the P_{BAD} promoter of pHERD20T and was detected by Western blotting (Fig. 2A, lane 6). In the absence of arabinose, IMPDH-HA was not detected from chromosome (mini-Tn*7*)- or plasmid (pHERD20T)-encoded *guaB*-HA under the control of the P_{BAD} promoter (Fig. 2A, lane 2 and lane 5, respectively), demonstrating the tight control of both vectors by arabinose. Arabinose induction (1% [wt/vol]) resulted in detectable IMPDH-HA signal (Fig. 2A, lane 3) from *guaB*-HA encoded on the chromosome. There is a direct correlation between the amount of arabinose used for induction and the detectable levels of IMPDH-HA (Fig. 2B). IMPDH-HA was not expressed at a detectable level when PA14 containing mini-Tn*7* *guaB*-HA was grown in the presence of 0.01% arabinose (Fig. 2B, lane 1) or in the absence of arabinose (data not shown). IMPDH-HA was detectable in increasing amounts when bacteria were grown in the presence of 0.10%, 0.50%, and 1.00% arabinose (Fig. 2B, lanes 2, 3, and 4, respectively). These results suggest that P_{BAD} carried on mini-Tn*7* is tightly controlled in the same fashion as P_{BAD} in multicopy vectors.

Features, advantages, and applications of pTJ1. The two main features of pTJ1, specifically, the mini-Tn*7*T-Tp-*araC*-P_{BAD} element, are (i) single-copy chromosomal gene integration and (ii) expression of cloned genes from an inducible promoter. Stable integration into the chromosome circumvents the need to use antibiotic markers to maintain the plasmid. Single-copy chromosomal integration also eliminates potentially deleterious effects of having multiple copies of genes in *trans* on a replicative plasmid. The use of an inducible promoter such as P_{BAD} allows for controlled expression of the gene(s) of interest. One useful design feature of the *araC*-P_{BAD}-MCS cassette is that it enables expression of cloned open reading frames from the cassette-encoded protein translation initiation signals, which include a consensus ribosome-binding site (RBS) and ATG initiation codon (Fig. 1B). The MCS and its configuration with respect to the RBS and start codon on mini-Tn*7*T-Tp-*araC*-P_{BAD} are the same as in the pHERD vectors (9), which makes any gene already cloned into a pHERD vector compatible for cloning into pTJ1; restriction fragments of a cloned construct from a pHERD vector can be directly ligated into pTJ1. It is possible that some genes cloned in other vector systems may also be directly compatible. When the EcoRI site is used for cloning, an additional seven N-terminal amino acids (MGSDKNS) are added from the pHERD vector (9). However, when NcoI is used (Fig. S1B), the construct can be designed to start with the native sequence of the gene of interest or other N-terminal sequences, such as an epitope tag, can be added.

Gene complementation with or without in-frame fusion to the vector-provided translation initiation signals is one general application for which pTJ1 is particularly well suited. The vector can also be used for overexpression of genes for applications such as protein purification. Due to the control of the P_{BAD} promoter, pTJ1 is also appropriate for protein stability experiments, similar to how pHERD has been used for examining the degradation kinetics of anti-sigma factors such as MucA from *P. aeruginosa* (16)

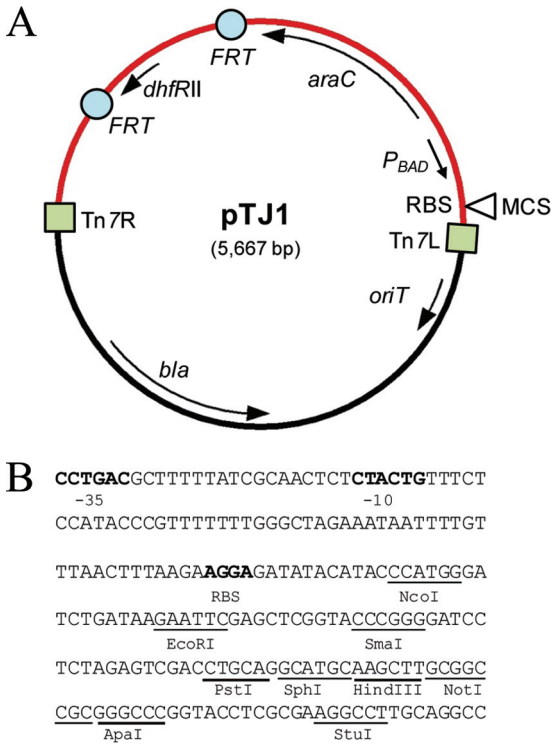

FIG 1 A genetic map of the features of the P_{BAD}-based mini-Tn*7*T-Tp-*araC*-P_{BAD}-MCS delivery vector, pTJ1. (A) The map of pTJ1 was assembled using Gene Construction Kit version 3.04 (Textco). Abbreviations: *araC*, gene encoding the repressor of P_{BAD}; *bla*, β-lactamase-encoding gene; *dhfRII*, gene encoding trimethoprim-resistant dihydrofolate reductase; *FRT*, Flp recombinase target; MCS, multiple-cloning site (see panel B for restriction enzyme cleavage sites); *oriT*, origin of transfer; P_{BAD}, arabinose-inducible promoter; RBS, ribosome-binding site; Tn*7*L and Tn*7*R, Tn*7* left and right ends, respectively. (B) Sequence encompassing P_{BAD} and multiple cloning site of pTJ1. Bold letters indicate the −10 and −35 sequences of the P_{BAD} promoter and a consensus RBS. Unique restriction enzyme cleavage sites suitable for cloning of exogenous DNA fragments are shown.

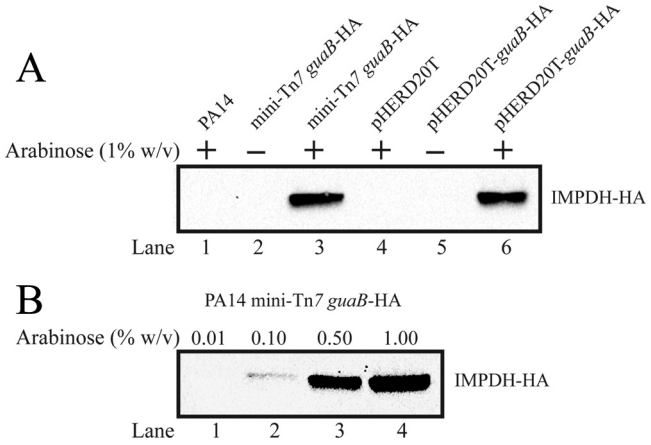

FIG 2 Western blot analysis of IMPDH-HA expressed in *P. aeruginosa*. (A) Detection of IMPDH-HA expressed from the P_{BAD} promoter of mini-Tn*7*T-Tp-*araC*-P_{BAD}-MCS integrated into the *P. aeruginosa* strain PA14 chromosome. The pTJ1-*guaB*-HA expression construct was integrated into the chromosome. Western blot analysis using anti-HA high-affinity antibody (clone 3F10; Roche Applied Science) (16) was used to detect the HA-tagged IMPDH in minimal media lacking (−) or containing (+) 1% (wt/vol) arabinose. The contents of the lanes are as follows: lane 1, *P. aeruginosa* strain PA14 without HA-epitope-tagged *guaB* as a control for antibody cross-reactivity; lanes 2 and 3, PA14 mini-Tn*7*-*guaB*-HA; lane 4, PA14 pHERD20T; lanes 5 and 6, PA14 pHERD20T-*guaB*-HA. IMPDH-HA was detected when *guaB*-HA was expressed from the P_{BAD} promoter from either the autonomously replicating pHERD20T plasmid (lane 6) or following chromosome-encoded *guaB*-HA from pTJ1-*guaB*-HA (lane 3). Samples in lanes 4 to 6 were grown in the presence of 300 μg/ml carbenicillin for maintenance of pHERD20T. (B) Detection of IMPDH-HA expressed from the P_{BAD} promoter from strains grown in minimal media in the presence of different concentrations of arabinose. Western blot analysis was performed as previously described. All lanes contained PA14 mini-Tn*7*-*guaB*-HA. The concentrations of arabinose used were (wt/vol) 0.01%, 0.10%, 0.50%, and 1.00% (lanes 1 to 4, respectively).

and in other unpublished studies from our laboratory. In these pulse-chase-type experiments, the P_{BAD} promoter can be repressed in the presence of glucose and epitope-tagged proteins can be monitored under various conditions by Western blotting. Another potential application of pTJ1 is in the determination of the essentiality of genes, similar to what has been done to assess essentiality of penicillin-binding protein 3 in *Burkholderia pseudomallei* using a mini-Tn*7* element in which genes are under the control of the *E. coli lac-trp* operon P_{tac} hybrid promoter (17). With a gene expressed from mini-Tn*7*T-Tp-*araC*-P_{BAD} integrated into the chromosome, the wild-type chromosomal copy can be deleted or inactivated. If the gene is essential, then growth in the absence of arabinose would not be possible. Experiments using the pTJ1 system for similar essentiality studies are ongoing in the laboratory. Others have used similar rhamnose- or arabinose-inducible promoters to determine essentiality of genes in *B. cenocepacia* (18); however, those experiments were performed with multicopy plasmids.

In this report, we have described the construction of a mini-Tn*7*T-Tp-*araC*-P_{BAD}-MCS vector, pTJ1, for shuttling genes to the chromosome and using the P_{BAD} promoter to control their expression. We predict that this vector will have many potential uses and therefore will expand the arsenal of mini-Tn*7* vectors for genetic analysis in a broad range of Gram-negative bacteria.

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We refer to the delivery vector discussed in this paper as pTJ1 in honor of Thomas Jefferson, prominent author of the Declaration of Independence, third President of the United States, and Founder of the University of Virginia, who was deeply interested in science.

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