Applied and Environmental Microbiology

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Appl. Environ. Microbiol. **2008, 74(23):7422. DOI: 10.1128/AEM.01369-08.**
Published Ahead of Print 10 October 2008.

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 2008, p. 7422–7426 Vol. 74, No. 23
0099-2240/08/$08.00+0 doi:10.1128/AEM.01369-08

P_{BAD}-Based Shuttle Vectors for Functional Analysis of Toxic and Highly Regulated Genes in *Pseudomonas* and *Burkholderia* spp. and Other Bacteria▽

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Received 18 June 2008/Accepted 6 October 2008

We report the construction of a series of *Escherichia-Pseudomonas* broad-host-range expression vectors utilizing the P_{BAD} promoter and the *araC* regulator for routine cloning, conditional expression, and analysis of tightly controlled and/or toxic genes in pseudomonads.

Gene cloning, disruption, deletion, complementation analysis, and allelic exchange are central to prokaryotic molecular genetics. In *Pseudomonas aeruginosa*, Schweizer and colleagues developed the pUCP family of general-purpose vectors for cloning and gene expression (24, 29) based on the well-characterized pUC18/19 vectors (32) and the cryptic mini-plasmid pRO1614 (19). Other promoters are also in routine use, such as the *tac* (4, 6), T7 (28), and *araBAD* promoter-based (8, 11) vectors for regulated expression in *Escherichia coli* and many other bacterial species (e.g., see references 2, 18, and 25). In *E. coli*, AraC represses the *araBAD* promoter (P_{BAD}) and the expression of a cloned gene is induced by the addition of L-arabinose. *Pseudomonas* researchers have used the inducible properties of the *araC* regulator and the P_{BAD} promoter cassette for the controlled gene expression by integrating the *araC*-P_{BAD}-specific transcription fusion into the chromosome by using a suicide vector or an integration-proficient vector (1, 3, 13, 17, 30, 31). In the present study, we modified the existing *Escherichia-Pseudomonas* shuttle vectors pUCP20T, -26, -28T, and -30T by replacing the *lac* promoter with the *araC*-P_{BAD} cassette to allow conditional expression in pseudomonads and other bacteria, e.g., *Burkholderia* spp.

Construction and features of pHERD vectors. Functional genetic analysis requires vectors capable of conditional expression. The P_{BAD} promoter has been used for gene expression extensively in *E. coli* and some in *P. aeruginosa* and *Burkholderia* spp. (12, 27, 31). We first constructed three shuttle vectors, pHERD20T, -28T, and -30T (Fig. 1), based on *Escherichia-Pseudomonas* shuttle vectors pUCP20T, pUCP28T, and pUCP30T (29) and the commercial expression vector pBAD/Thio-TOPO (Invitrogen). The 368-bp fragment of the pUCP vectors spanning two restriction sites, AflII and EcoRI, was replaced with the *araC*-P_{BAD} fragment (1.3 kb), produced via PCR with pBAD/Thio-TOPO as the template and primers pBAD-F and pBAD-R (Table 1). The PCR product was purified and directly digested with AflII and EcoRI, and the two fragments were ligated into the pUCP vectors, creating pHERD20T (Fig. 1). The EcoRI/AflII regions of these vectors were sequenced to confirm that no mutations were introduced during the cloning pro-

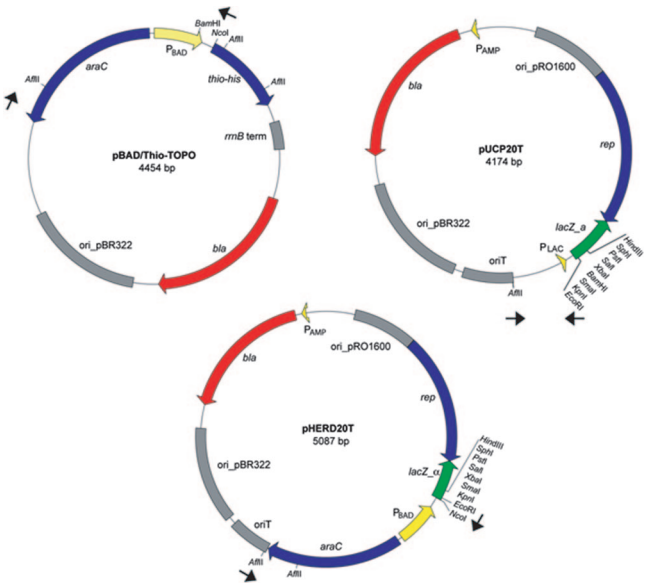

FIG. 1. Construction of an *Escherichia-Pseudomonas* shuttle vector, pHERD20T, an arabinose-inducible expression vector. pHERD20T is a pUCP20T-based, conjugatable vector with pBR322- and pRO1600-derived replicons which support replication in *E. coli*, *P. aeruginosa*, and other bacteria, respectively. The P_{BAD} promoter was derived from the expression vector pBAD/Thio-TOPO (Invitrogen). The P_{lac} promoter in pUCP20T was replaced with the P_{BAD} promoter-containing segment with an EcoRI-AflII fragment generated via PCR containing the *araC* gene and P_{BAD}. Black arrows indicate the region transferred from pBAD/Thio-TOPO into pUCP20T. pHERD20T contains a multiple cloning site within *lacZ*α encoding the β-galactosidase α peptide.

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▽ Published ahead of print on 10 October 2008.

TABLE 1. Bacterial strains and plasmids used in this study

Strain, plasmid, or primer	Genotype, phenotype, or sequence[a]	Source or reference
P. aeruginosa		
PAO1	Alg^{wt} prototroph	P. Phibbs
PAO1VE2Δ*algW*	Alg^+ PAO1 *mucE*$^{+oe}$ (*himar1* Gm^r::P_{GM}::*mucE*) Δ*algW*	20
P. fluorescens Pf-5	Alg^{wt} prototroph	ATCC
E. coli DH5α	F^- φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17* (r_K^- m_K^+) *phoA supE44* λ^- *thi-1 gyrA96 relA1*	Laboratory strain
B. pseudomallei Bp50	Δ(*amrRAB-oprA*) derivative of wild-type strain 1026b	5
Plasmids		
pBAD/Thio-TOPO	*araC*-P_{BAD} ori_{pUC} Ap^r (4,454 bp)	Invitrogen
pHERD20T	pUCP20T P_{lac} replaced with 1.3-kb AflII-EcoRI fragment of *araC*-P_{BAD} cassette (5,087 bp)	This study
pHERD30T	pUCP30T P_{lac} replaced with 1.3-kb AflII-EcoRI fragment of *araC*-P_{BAD} cassette (5,216 bp)	This study
pHERD26T	pUCP26 P_{lac} replaced with 2.4-kb AdhI-EcoRI fragment of *araC*-P_{BAD} cassette and *oriT* (6,166 bp)	This study
pHERD28T	pUCP28T P_{lac} replaced with 1.3-kb AflII-EcoRI fragment of the *araC*-P_{BAD} cassette (4,993 bp)	This study
pHERD30T-*mucE*	*mucE* in pHERD30T EcoRI/HindIII	This study
pHERD20T-*algU*	*algU* in pHERD20T EcoRI/HindIII	This study
pHERD20T-*oprF*	*oprF* (PA1777) in pHERD20T EcoRI/HindIII	This study
pHERD20T-*oprF*-WVF	*oprF* allele encoding OprF ending with the WVF motif cloned in pHERD20T EcoRI/HindIII	This study
Primers		
pBAD-F	AGTATACCTTAAGGAATCCCCAAATTATGACAACTTGACGGCTACATCAT	This study
pBAD-R	AGGATCCCCGGGTACCGAGCTCGAATTCTTATCAGATCCCATGGGTATG TATA	This study
pHERD-SF	ATCGCAACTCTCTACTGTTTCT	This study
pHERD-SR	TGCAAGGCGATTAAGTTGGGT	This study
algU-F	AGAATTCGATGCTAACCCAGGAACAGGA	This study
algU-R	CAAGCTTTCAGGCTTCTCGCAACAAAGGCTGCA	This study
algW-F	AGAATTCGATGCCCAAGGCCCTGCGTTTCCT	This study
algW-R	TGCCAAGCTTTCACTCGCCGCCGTCCTGTTT	This study
mucE-F	AGAATTCGATGGGTTTCCGGCCAGTTA	This study
mucE-R	GAAGCTTCAAAACACCCAGCGCAACTCGTC	This study
oprF-F	AGAATTCGATGAAACTGAAGAACACCTTA	This study
oprF-R	CAAGCTTTACTTGGCTTCAGCTTCTACTTCGGCT	This study
oprF-WVF-R	AAGCTTAAAACACCCAGCGCTTGGCTTCAGCTTCTACTTCGGCT	This study
lacZ-RT-For2	GTCGTGACTGGGAAAACC	This study
lacZ-RT-Rev2	GCCTCTTCGCTATTACGC	This study
Bp23S_F	GTAGACCCGAAACCAGGTGA	This study
Bp23S_R	CACCCCTATCCACAGCTCAT	This study

[a] Alg^{wt}, wild-type nonmucoid phenotype; Alg^-, nonmucoid phenotype; Alg^+, mucoid phenotype. Primers used for cloning carried built-in restriction sites (underlined), with F denoting forward and R denoting reverse primers, respectively.

cess. We next transferred the 2.4-kb AdhI-EcoRI fragment from pHERD20T to pUCP26, generating pHERD26T (Tet^r, 6,166 bp), which includes the *araC*-P_{BAD} cassette and the *oriT* sequence.

The pHERD vectors have the features of the pUCP vector family, including the pBR322 origin, four different antibiotic resistance markers, the *oriT* region for conjugation-mediated plasmid transfer (23), ori_{1600}, and the *rep* gene encoding the replication-controlling protein (24, 29). However, the main advantage for cloning into the pHERD vectors is low expression, which occurs from the P_{BAD} promoter when it is not induced (Fig. 2). α complementation is inducible for blue-white screening, which facilitates the identification of recombinants on a 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal)-containing plate supplemented with arabinose (0.01%). The P_{BAD} promoter responds in a dose-dependent manner (Fig. 2). Two sequencing and PCR primers were designed that anneal to regions on both sides of the multiple cloning site, pHERD-SF 78 bp upstream of the EcoRI site and pHERD-SR 49 bp downstream of the HindIII site. If a gene is cloned in frame into the EcoRI site, a fusion protein with an additional seven NH_2-terminal amino acids (MGSDKNS) derived from thioredoxin of pBAD-TOPO/Thio will result. Thioredoxin acts as a translation leader to facilitate high-level expression and, in some cases, increase solubility in *E. coli* (9). These amino acids at the N terminus of the target protein may

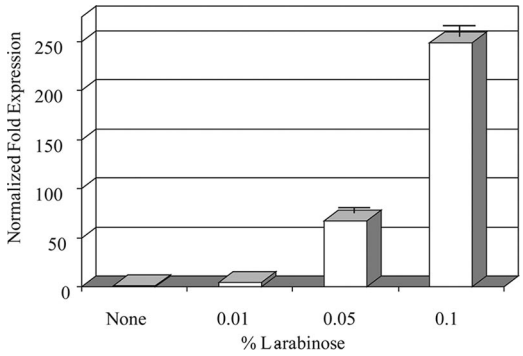

FIG. 2. Arabinose-regulated *lacZ*α expression in *B. pseudomallei*. RNA was extracted from log-phase *B. pseudomallei* Bp50 cells harboring pHERD30T that either had no arabinose added (None) or were induced for 2 h by the addition of the indicated amounts of L-arabinose. Quantitative real-time PCR was performed by using *lacZ*α-specific primers. Data were normalized by using the 23S rRNA gene as the housekeeping control.

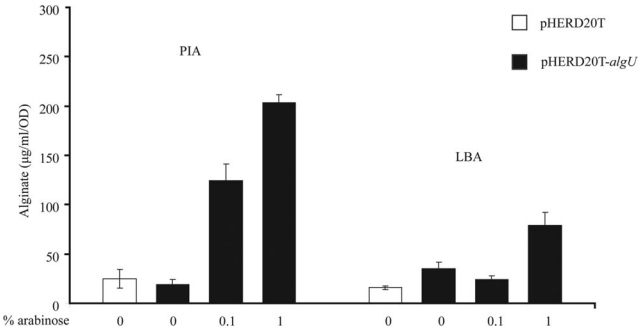

FIG. 3. Arabinose-dependent induction of alginate production in *P. aeruginosa* PAO1 carrying pHERD20T-*algU*. PAO1 with pHERD20T-*algU* was grown at 37°C for 24 h on *Pseudomonas* isolation agar and LB plates supplemented with carbenicillin and 0, 0.1, and 1.0% arabinose, respectively. The empty pHERD20T vector was used as the control (open box). Bars indicate means with standard errors. PAO1/pHERD20T with 0.1 and 1.0% arabinose does not increase alginate production (data not shown). OD, optical density.

also serve as an epitope tag for protein analysis. pHERD vectors can be readily transferred from *E. coli* into *Pseudomonas* species and other bacteria via triparental conjugation (7) or by electroporation. It has been shown that the progenitor plasmid pRO1614 could replicate in a series of bacterial species, including *P. aeruginosa*, *P. putida*, *P. fluorescens*, *Klebsiella pneumoniae* (19), and *Burkholderia* spp. (5, 26). Therefore, the pHERD vectors are most likely functional in these bacteria. Another feature of the P_{BAD} promoter is catabolite repression of expression in the presence of glucose in the growth medium, which reduces intracellular cyclic AMP concentrations in *E. coli* cells, preventing the transcriptional activation of many genes by the cyclic AMP-binding protein (8).

Validation of pHERD20T in *P. aeruginosa* by modulating alginate production. We have observed that pHERD vectors can be used for the high-fidelity cloning and conditional expression of P_{BAD} transcription in the absence of L-arabinose (10). Initial attempts to clone the open reading frame of *P. aeruginosa* alternative sigma factor *algU* into pUCP20T were not successful. All of the *algU* alleles cloned were not functional, and sequence analysis showed that only mutant *algU* alleles were cloned into pUCP20T. This was consistent with the previous observations that *algU*/*T* cannot be cloned into the common expression vectors (16, 21). However, the *algU* gene was readily cloned into pHERD20T. Upon the expression of *algU* from P_{BAD} on pHERD20T, we observed dose-dependent alginate production or mucoidy in *P. aeruginosa* strain PAO1 in response to arabinose in the growth medium (Fig. 3).

Overexpression of the small peptide encoded by *mucE* activates AlgW, inducing alginate production (Fig. 4) in *P. aeruginosa* PAO1 and PA14 (20). Overexpression of *mucE* caused mucoidy in *P. aeruginosa* PAO1 and *P. fluorescens* Pf-5 (Table 2). The C-terminal WVF signal encoded by *mucE* is required for the activation of AlgW. The outer membrane protein OprF does not activate alginate production (Fig. 4); however, addition of the MucE WVF signal motif to the C terminus of OprF did cause alginate production (Table 2). Some genes are not highly expressed, and therefore expression in *trans* for complementation needs to be conditional. Expression of *algW* from P_{BAD} can complement an *algW* mutant back to alginate production due to titratable expression (Table 2). In addition to PAO1, we have used the pHERD vectors in PA14, CF149, environmental *P. fluorescens* isolates, and *P. putida* (data not shown). We have successfully employed pHERD30T for complementation of the Δ(*amrAB-oprA*) efflux pump mutation in *Burkholderia pseudomallei* strain Bp50 (5). In this case, however, complementation was also observed in uninduced cells, presumably because of basal transcription from the P_{BAD} promoter, which could not be overcome by growing cells in the

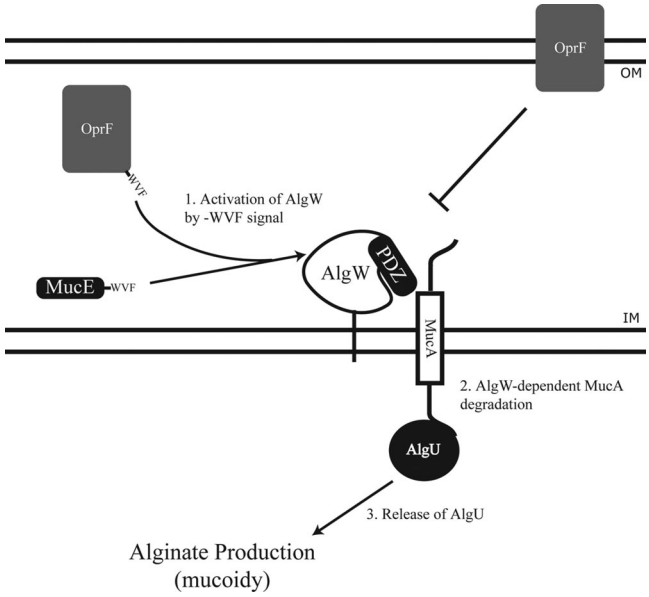

FIG. 4. Regulated alginate production in *P. aeruginosa*. Regulation of alginate production in *P. aeruginosa* involves many genes coding for products with many different functions. Mucoidy or alginate production is directed by the alternative σ^{22} factor AlgU (14). MucA is the cognate anti-sigma factor that negatively regulates AlgU activity by sequestering AlgU to the inner membrane (IM) (22). Sequestering of AlgU by MucA can be relieved by either mutation of *mucA* (15) or proteolytic degradation of MucA by the intramembrane protease AlgW (20). Derepression of MucA causes AlgU activation and alginate production. OM, outer membrane.

TABLE 2. Modulation of mucoidy in *P. aeruginosa* and *P. fluorescens* by pHERD20T-borne alginate regulators

Strain (genotype)	Colony morphology[a]	Plasmid	Colony morphology with plasmid[a]				
			1% Glucose	0% Arabinose	0.1% Arabinose	1% Arabinose	2.5% Arabinose
P. aeruginosa							
PAO1	NM	pHERD20T-*algU*	NM	NM	M	M	NM
		pHERD30T-*mucE*	NM	NM	M	M	M
		pHERD20T-*oprF*		NM	NM		
		pHERD20T-*oprF*-WVF		NM	M		
PAO1VE2Δ*algW* (P_{Gm}-*mucE* Δ*algW*)	NM	pHERD20T-*algW*	NM	M	M	NM	NM
P. fluorescens Pf-5	NM	pHERD30T-*mucE*		NM	M		

[a] NM, nonmucoid; M, mucoid.

presence of 0.2% glucose and was not dependent on the growth medium used for the MIC assays (LB versus Mueller-Hinton broth) (data not shown).

In summary, we constructed a series of small *Escherichia-Pseudomonas* shuttle vectors with the *E. coli araC* and P_{BAD} promoter for highly regulated expression of cloned genes in *Pseudomonas* species and other bacteria and confirmed their utility by modulation of alginate production. Results presented here demonstrate that pHERD vectors are useful tools for bacterial physiological research and gene function studies with pseudomonads, as well as other bacteria, including medically significant *Burkholderia* spp.

Nucleotide sequence accession numbers. The GenBank accession numbers for the nucleotide sequences of pHERD20T, -26T, -28T, and -30T are EU603324, EU603327, EU603325, and EU603326, respectively.

The pHERD vectors described here are dedicated to the memory of the 1970 Marshall University Thundering Herd football team as depicted in the 2006 Warner Bros. film *We Are Marshall*.

This work was supported by a research grant (NNA04CC74G) from the National Aeronautics and Space Administration (NASA) and research grants from the NASA West Virginia Space Grant Consortium to H.D.Y. H.P.S.'s *Burkholderia* research was supported by NIH grant AI065357. F.H.D. was supported by a training grant (NNX06AH20H) from the NASA Graduate Student Researchers Program (GSRP).

We thank N. E. Head for the initial analysis of the mutant *algU* alleles in pUCP20T, V. M. Eisinger for the generation of the VE mutants, and K. D. Dillon for technical assistance with the alginate assay.

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