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Expression Vectors for *Acinetobacter baylyi* ADP1

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***Acinetobacter baylyi* ADP1 is naturally competent and proficient at homologous recombination, so it can be transformed without restriction digests or ligation reactions. Expression vectors for this system, however, are not yet widely available. Here we describe the construction and characterization of inducible expression vectors that replicate as plasmids in *A. baylyi* or integrate into a nonessential part of its chromosome. These tools will facilitate the engineering of genes and genomes in this promising model organism.**

Acinetobacter baylyi ADP1 is arguably superior to *Escherichia coli* for the engineering of genes and genomes (4, 10). Mid-log *A. baylyi* cells are naturally competent (11) and catalyze the homologous recombination of DNA efficiently. *A. baylyi* extrudes 1 to 3 μg of chromosomal DNA/ml of liquid culture (16), so sexual recombination occurs under normal growth conditions. The transformation of *A. baylyi* does not require a refrigerated centrifuge, a −80°C freezer, or an electroporator. The synthesis of primers and genes can be outsourced, so a motivated student could potentially engineer and express genes in an inexpensively equipped laboratory (with an Internet-connected computer, a thermocycler, a gel electrophoresis apparatus, and an incubated shaker). Here we describe, and make available, a series of inducible *A. baylyi* expression vectors that either replicate as plasmids or integrate efficiently into the chromosome.

Assembly strategy. We desired a family of *A. baylyi* expression vectors with different inducible promoters, selectable markers, and replication mechanisms (chromosomal integration or plasmid replication) because no single expression vector is ideal for all applications. We did not know *a priori* which promoters and ribosome binding sites would work in *A. baylyi* (particularly in combination with foreign genes and replicons), so we expected some trial and error. We adopted the BioBrick standard because it is well-suited for combinatorial assembly projects, including the construction of vector families (15). A BioBrick is a cloned sequence of DNA (of any length) that is flanked by standard restriction sites (EcoRI-NotI-XbaI-payload-SpeI-NotI-PstI); the compatibility of overhangs created by restriction endonucleases XbaI and SpeI enables the ligation of any two appropriately digested BioBricks to produce a single tandem BioBrick.

BioBrick-accepting plasmid. A multiple cloning site (MCS; SphI-ribosome binding site-NcoI-EcoRV-HindIII) flanked by the BioBrick restriction sites was created by PCR amplification of an intentional primer dimer; the resulting product was purified, restriction digested, and cloned into the DraIII and AflIII sites of pSL1180 (Pharmacia) to create a custom BioBrick-accepting vector (pIM1154 in Table 1, or pIMBB [2]). This plasmid retained its ColE1 origin, which replicates efficiently in *E. coli* but not in *A. baylyi* (7, 8), and beta-lactamase marker. DNA sequences that do not contain internal restriction sites (EcoRI, NotI, XbaI, SpeI, or PstI) become BioBricks when cloned into the MCS of this plasmid. They can subsequently be iteratively combined with other BioBricks for the assembly of the desired vectors.

BioBrick cloning. Markers from *E. coli* plasmids that confer resistance to kanamycin (Kan^r), spectinomycin (Spc^r), and tetracycline (Tet^r) were cloned into pIM1154, thereby converting them into BioBricks (pIM1157, pIM1212, and pIM1265, respectively). A series of expression cassette BioBricks, each containing a regulator gene, a promoter, a ribosome binding site, and the *E. coli gusA* reporter gene (*lacI*-P_{T7}-*gusA*, *lacI*-P_{tac}-*gusA*, *araC*-P_{BAD}-*gusA*, *pobR*-P_{pob}-*gusA*, *lacI*-P_{T5}-*gusA*, P_{T5}-*lacO*^{c}-*gusA*, and P_{T5}-*lacI-gusA*), was constructed by PCR amplification and cloning (yielding pIM1218, pIM1219, pIM1221, pIM1217, pIM1202, pIM1460, and pIM1466, respectively; see the supplemental material). In each case, the reporter gene was preceded by a ribosome binding site and an NcoI site on the 5′ end and an HindIII site on the 3′ end (Fig. 1). The origin of replication of an endogenous *A. baylyi* plasmid, pWH1266 (8), was PCR amplified and cloned into pIMBB-Kan^r (pIM1266) to create pWH1266-pIMBB-Kan^r (pIM1311). Finally, four 1-kb sequences derived from the large prophage region of *A. baylyi* (arbitrarily designated pp2.1, pp2.2, pp4.1, and pp4.2) were cloned into pIM1154 to create pIM1215, pIM1216, pIM1480, and pIM1502, respectively.

Plasmid assembly. We constructed two sets of *A. baylyi* ADP1 expression vectors, one based upon pWH1266, the aforementioned endogenous *Acinetobacter* plasmid (8), and the other upon pBAV1k, a broad-host-range plasmid that is stably maintained at high copy number in Gram-negative and Gram-positive bacteria (3). Several expression cassettes, namely, *lacI*-P_{T7}-*gusA*, *lacI*-P_{tac}-*gusA*, *pobR*-P_{pob}-*gusA*, and *lacI*-P_{T5}-*gusA*, were cloned in parallel into the BioBrick cloning sites of pWH1266-pIMBB-Kan^r (to create pIM1319, pIM1320, pIM1318, and pIM1317, respectively) and pBAV1k (to create pIM1441, pIM1445, pIM1444, and pIM1440, respectively). An arabinose-induced vector, *araC*-P_{BAD}-*gusA*-pBAV1k (pIM1442), was also constructed. *A. baylyi* cells were separately transformed with each of the resulting expression vectors (Table 1) spread onto Luria broth agar plates

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TABLE 1 Plasmids used in this study

Plasmid	Relevant insert(s)	Description, source, and/or reference
Source plasmids	Each source plasmid contains regulators, promoters, and markers (not flanked by BioBrick restriction sites)	Internal EcoRI, NotI, XbaI, SpeI, and PstI sites must also be eliminated
pET28a+	Kan^r	Novagen
pCDF Duet	pCDF-NheI-Spc^r	Novagen
pACYC184	Tet^r	New England Biolabs
lacZ-gfp-pCA24N	*lacI*-P_{T5}	9
pRBL	*lacI*-P_{tac}	14
lacI-pCA24N	*lacI* (no *gfp*)	9
recA-gfp-pCA24N	*recA* (not a BioBrick)	9
gusA-gfp-pCA24N	*gusA* (not a BioBrick)	9
lacY-gfp-pCA24N	*lacY* (not a BioBrick)	9
pBAD myc his a	*araC*-P_{BAD} (not a BioBrick)	Invitrogen
pSL1180	MCS (not a BioBrick)	Pharmacia
pWH1266		8
Non-BioBrick precursors	Plasmids contain internal restriction sites, not BioBrick compatible	All used to create *lacI*-P_{T5}-*gusA* BioBrick (pIM1202)
pIM1117	*lacI*-P_{T5}-pRBL	PCR product of *lacI*-P_{T5} from pCA24N + pRBL
pIM1119	*lacI*-P_{T5}-pCDF Duet	*lacI*-P_{T5} from pIM1117 + pCDF Duet
pIM1155	*lacI*-P_{T5}-*lacI*-pCDF Duet	*lacI* from *lacI*-pCA24N + pIM1119
pIM1159	*lacI*-P_{T5}-*lacI*-pIMBB	*lacI*-P_{T5}-*lacI* from pIM1115 + pIM1154
BioBrick precursors	BioBricks used to assemble regulators, promoters, and selectable markers	All conform to BioBrick standard but are not directly useful for vector assembly
pIM1154	MCS (SphI-ribosome binding site-NcoI-EcoRV-HindIII)	BioBrick cloning vector (pIMBB) (2)
pIM1171	*lacI*-P_{T5}-*recA*-pIMBB	PCR product of *recA* from *recA-gfp*-pCA24N + pIM1179; precursor of pIM1202
pIM1179	*lacI*-P_{T5}-*lacI*-pIMBB	*lacI* from *lacI*-pCA24N + pIM1171; precursor of pIM1202
pIM1278	P_{T5}-*lacI*-pIMBB-3	Upstream *lacI* removed with XhoI and SphI; precursor of pIM1466
pIM1458	P_{T5}-*lacI*-pIMBB-4	pIM1278 without NcoI or HindIII site
pIM1180	*lacI*-P_{T5}-*lacY*-pIMBB	*lacY* from *lacY-gfp*-pCA24N + pIM1171; precursor of pIM1265
pIM1211	Tet^r-*lacY*-pIMBB	Tet^r from pACYC184 + pIM1180; precursor of pIM1265
BioBrick elements	BioBricks used to assemble expression vectors	
pIM1157	Kanamycin phosphotransferase (Kan^r)	Derived from pET28a+ (Novagen)
pIM1212	Spectinomycin adenyltransferase (pCDF-NheI-Spc^r)	Derived from pCDF Duet (Novagen)
pIM1265	Tetracycline/H^+ antiporter (Tet^r)	Derived from pACYC184 (New England Biolabs)
pIM1462	P_{T5}-*lacO^c-tdk*	Derived from ASKA collection
pIM1446	*gusA*	*gusA* from pIM1202 + pIM1154
pIM1218	*lacI*-P_{T7}-*gusA*	Derived from pCDF Duet (Novagen)
pIM1219	*lacI*-P_{tac}-*gusA*	Derived from pRBL (14)
pIM1221	*araC*-P_{BAD}-*gusA*	Derived from pBAD myc his A (Invitrogen)
pIM1217	*pobR*-P_{pob}-*gusA*	Derived from *A. baylyi* genome
pIM1202	*lacI*-P_{T5}-*gusA*	Derived from pCA24N (9)
pIM1460	P_{T5}-*lacO^c-gusA*	Derived by site-directed mutagenesis in pIM1219
pIM1466	P_{T5}-*lacI-gusA*	Derived from pCA24N
pIM1215	ADP1 prophage segment 2.1	PCR product from *A. baylyi* genome + pIM1154
pIM1216	ADP1 prophage segment 2.2	PCR product from *A. baylyi* genome + pIM1154
pIM1480	ADP1 prophage segment 4.1	PCR product from *A. baylyi* genome + pIM1154
pIM1502	ADP1 prophage segment 4.2	PCR product from *A. baylyi* genome + pIM1154
Integration vectors		
pIM1356	pp2.1-*pobR*-P_{pob}-*gusA*-Spc^r-pp2.2	pIM1215 + pIM1217 + pIM1212 + pIM1216
pIM1463	pp2.1-P_{T5}-*lacI-gusA*-Spc^r-pp2.2	pIM1215 + pIM1466 + pIM1212 + pIM1216; available from Addgene
pIM1517	pp4.1-*araC*-P_{BAD}-*gusA*-P_{T5}-*lacO^c-tdk*-Spc^r-pp4.2	pIM1480 + pIM1221 + pIM1462 + pIM1212 + pIM1502
pIM1251	pp2.1-P_{T5}-*lacI-gusA*-Kan^r-pp2.2	pIM1215 + pIM1466 + pIM1157 + pIM1216
pIM1253	pp2.1-P_{T5}-*lacI-gusA*-Tet^r-pp2.2	pIM1215 + pIM1466 + pIM1265 + pIM1216
pBAV1k-based expression vectors		
pIM1522	P_{T5}-*lacO-lacO-gfp*	Broad-host-range plasmid (pBAV1k-T5-*gfp* [GenBank accession no. HQ191434.1]); available from Addgene (3)
pIM440	*lacI*-P_{T5}-*gusA*-pBAV1k	pIM1202 + pIM1522 (GenBank accession no. JF828582); available from Addgene
pIM1441	*lacI*-P_{T7}-*gusA*-pBAV1k	pIM1218 + pIM1522 (GenBank accession no. JF828583); available from Addgene
pIM1445	*lacI*-P_{tac}-*gusA*-pBAV1k	pIM1219 + pIM1522 (GenBank accession no. JF828584); available from Addgene

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TABLE 1 (Continued)

Plasmid	Relevant insert(s)	Description, source, and/or reference
pIM1442	*araC*-P_{BAD}-*gusA*-pBAV1k	pIM1221 + pIM1522 (GenBank accession no. JF828585); available from Addgene
pIM1444	*pobR*-P_{pob}-*gusA*-pBAV1k	pIM1217 + pIM1522 (see Table S2 in the supplemental material)
pWH1266-based plasmid vectors	All Kan^r instead of Amp^r	Expression from these vectors was constitutive
pIM1266	MCS (SphI-ribosome binding site-NcoI-EcoRV-HindIII)	Kan^r from pET28a+ replaces TEM-1 beta-lactamase marker in pIM1154
pIM1272	pWH1266 origin	PCR product of pWH1266 ori + pIM1266
pIM1311	pWH1266 origin	pIM1272 without HindIII site
pIM1317	*lacI*-P_{T5}-*gusA*-pWH1266 ori	pIM1202 + pIM1311
pIM1318	*pobR*-P_{pob}-*gusA*-pWH1266 ori	pIM1217 + pIM1311
pIM1319	*lacI*-P_{T7}-*gusA*-pWH1266 ori	pIM1218 + pIM1311
pIM1320	*lacI*-P_{tac}-*gusA*-pWH1266 ori	pIM1219 + pIM1311

supplemented with 50 μg/ml kanamycin (LB-kan plates) and incubated overnight at 37°C.

Plasmid function. Individual transformed colonies were used to inoculate liquid LB-kan cultures. The cultures were propagated to saturation by overnight agitation at 37°C; saturated cultures were diluted 1:30 in fresh medium, propagated to mid-log phase, and induced with 10 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG; for P_{T7}, P_{tac}, and P_{T5}), 10 mM L-arabinose (for P_{BAD}), 5 mM *p*-hydroxybenzoic acid (for P_{pob}), or sterile water (to provide an uninduced negative control for all expression vectors). The cultures were incubated overnight, harvested by centrifugation, and lysed. The cell extracts were reacted with saturating (1 mM) concentrations of 4-methylumbelliferyl-β-D-glucuronide (BMUG); the formation of the 4-methylumbelliferone product was monitored in a microtiter plate spectrofluorimeter (the Molecular Devices M5 reader). We observed efficient (>100-fold) induction for the phage (P_{T7} and P_{T5}) and *E. coli* (P_{tac} and P_{BAD}) promoters on the pBAV1k-based vectors (Fig. 2, top 8 bars). All four of these plasmids should be useful for the heterologous expression and engineering of other genes in *A. baylyi*.

Native promoter and plasmid origin. Induction of *gusA* from the native *pob* promoter with *p*-hydroxybenzoic acid, however, was far less efficient (<4-fold). Overall expression levels were relatively modest (Fig. 2, compare 9th and 10th bars with top 8 bars). The *pob* promoter is well-characterized (5, 6), but its performance might not scale up at high copy number (58 copies/cell [3]). We were also puzzled by the behavior of the pWH1266-pIMBB-Kan^r-based vectors. All of them produced modest amounts of β-glucuronidase (GUS), with no detectable difference in the presence or absence of an inducer (data not shown). The pWH1266

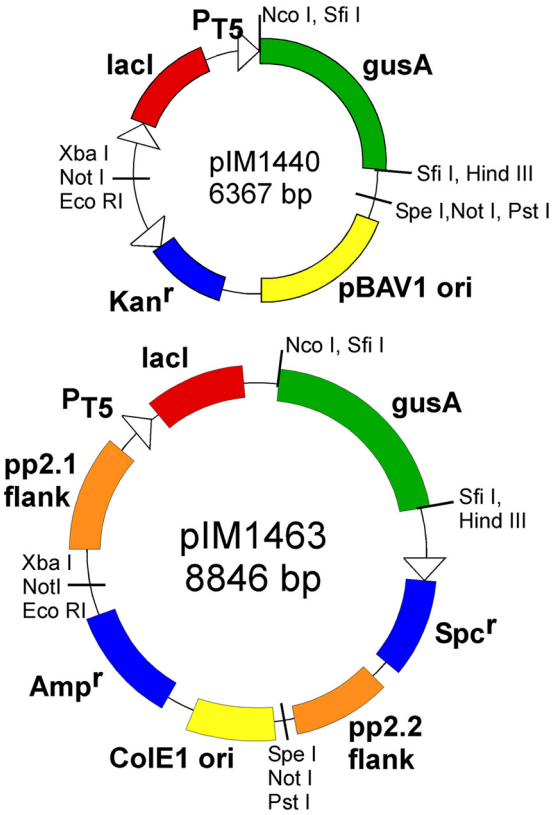

FIG 1 Examples of plasmid (top) and integration (bottom) vectors. Each contains a single BioBrick flanked by restriction sites EcoRI-NotI-XbaI (prefix) and SpeI-NotI-PstI (suffix). The BioBrick in the integration vector (bottom) was assembled from smaller BioBricks carrying the flanks, expression cassette (regulator-promoter-reporter gene), and selectable marker. The vectors rely upon the function of foreign promoters and ribosome binding sites (each pair is indicated by an open triangle) in *A. baylyi* ADP1.

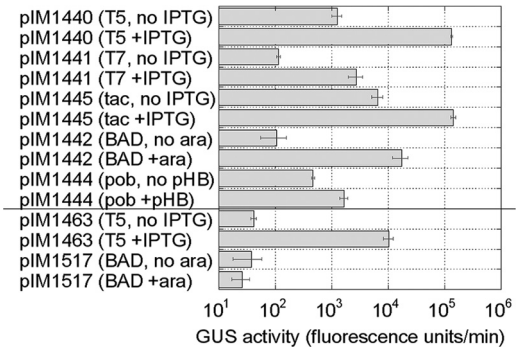

FIG 2 Novel expression vectors function in *A. baylyi* ADP1. Wild-type *A. baylyi* ADP1 cells were transformed with plasmid and integration vectors (described in Table 1). Each transformed strain was separately propagated to mid-log phase and challenged with an inducer (IPTG, arabinose, or *p*-hydroxybenzoate) or water (negative control) for 3 h. The cells were harvested by centrifugation and lysed; the cell extract was reacted with BMUG, and the formation of the fluorescent 4-methylumbelliferone product was monitored in a microtiter plate spectrofluorimeter. The activity was derived from the slope (fluorescence per unit of time) and plotted on the log scale. Error bars represent standard errors from three independent trials.

origin enables replication within *A. baylyi*, but unlike the pBAV1k origin, it apparently affects the function of adjacent foreign promoters.

Integration vector assembly. The BioBricks were also assembled into integration vectors (Fig. 1 and Table 1). Each was constructed in the ColE1-based BioBrick-accepting plasmid pIM1154, which does not replicate efficiently in *A. baylyi* (7, 8). Each was flanked by 1-kb sequences derived from the large prophage of the *A. baylyi* chromosome (nucleotides 2116840 to 2169436 of the chromosomal sequence [1]) to integrate the intervening payload into this nonessential region. *A. baylyi* strains were transformed with integration vectors and spread on LB agar plates supplemented with 50 μg/ml spectinomycin (for pIM1356, pIM1463, and pIM1517) or 50 μg/ml kanamycin (for pIM1251). The tetracycline resistance marker derived from *E. coli* plasmid pBR322 (pIM1253) did not enable the selection of transformed *A. baylyi* in our hands.

Integration vector function. *A. baylyi* transformed with the P_{T5}-*lacI-gusA* integration vector (pIM1463) expressed almost 250-fold more GUS activity in the presence of 1 mM IPTG than in its absence (Fig. 2, 11th and 12th bars). In contrast, the induction of an integrated *araC*-P_{BAD}-*gusA* expression cassette (pIM1517) with 100 mM L-arabinose did not increase GUS activity relative to that in uninduced controls (Fig. 2, 13th and 14th bars), even though the same expression cassette carried by a multicopy plasmid was efficiently induced. Endogenous *A. baylyi* sigma factors apparently do not bind the P_{BAD} promoter very tightly, as high copy numbers are necessary for detectable induction. *A. baylyi* transformed with the *pobR*-P_{pob}-*gusA* integration vector (pIM1356) produces various amounts of GUS activity, either in the presence (1.8 mM) or in the absence of *p*-hydroxybenzoic acid.

Real-time PCR analysis of the *gusA* gene showed that P_{T5}-*lacI-gusA* (pIM1463) *A. baylyi* strains always had a single copy of the *gusA* gene (0.7 ± 0.1/cell, relative to the obligate, single-copy *relA* or *rel*[ACIAD3326] gene). In contrast, different *pobR*-P_{pob}-*gusA* (pIM1356) *A. baylyi* isolates contained highly variable *gusA* copy numbers (23 ± 0.7, 340 ± 35, 114 ± 14, or 531 ± 128 copies/cell). We suspect that the unpredictable copy number variation of *pobR*-P_{pob}-*gusA* (pIM1356) is a function of gene amplification (12, 13), so it is not reliable as an expression vector. We remain confident, however, that the other expression vectors with nonnative promoters, namely, the P_{T5}-*lacI-gusA* integration vector (pIM1463) and the plasmid expression vectors (pIM1440, pIM1441, pIM1445, and pIM1442), will enable the engineering of genes and genomes in *A. baylyi*.

Nucleotide sequence accession numbers. Nucleotide sequences of newly constructed plasmids have been deposited in GenBank and are listed in Table 1 (excluding HQ191434.1).

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