Construction of pDUO : A bicistronic shuttle vector series for dual expression of recombinant proteins.

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Abstract

Our ability to genetically manipulate microbial systems is often hampered by the availability of genetic tools. Thus, there is a need for the continued expansion of our molecular tool box. In support of this expansion, this study reports the design, construction, and validation of a new bicistronic shuttle vector series, pDUO, for the dual expression of genes in different hosts. Each vector was designed and constructed to contain two *araC-p_{BAD}* inducible promoter systems for tight control over gene expression. Each *araC-p_{BAD}* promoter precedes a ribosomal binding site and a multicloning site (MCS). The 5' end of MCS1 contains a sequence encoding an affinity HIS-tag N-terminus and MCS2 terminates with a sequence encoding an affinity S-tag C-terminus for one-step purification of recombinant proteins encoded by the inserted genes. Both MCS are followed by an *rrnBT1* and T2 transcriptional terminator sequence. Each vector in this series also contains a PBR322 and pRO1600-derived replicon to support replication in different host bacteria along with one of four different selectable markers. The functionality of the pDUO vector series was validated through the dual expression of oxalate biosynthetic component (*obc*) 1 and *mrfp* in *Escherichia coli* and *Pseudomonas fluorescens*. It is anticipated that this new vector series will facilitate functional studies as well as the engineering of bacterial strains for biotechnological purposes.

Keywords: expression system; bicistronic; shuttle vector

1. Introduction

The use of microorganisms for the production of fermented products such as cheese, vinegar, beer and wine are some of the earliest examples of the application of biotechnology for the production of desirable goods. Since then, the use of a variety of different bacteria and other microbes as biotechnological agents has expanded into other fields which include medicine, agriculture, specialty chemicals, as well as other areas of food production (Glazer and Nikaido, 2007; Lee, 2013). The recombinant DNA revolution of the mid 1970's was a primary driving force in this rapid biotechnological evolution.

This technological evolution was dependent on the development of suitable vectors that allowed the engineering of these microbes (Chang and Cohen, 1974; Cohen et al., 1973; Russo, 2003; Sharrer, 2006). Today, wide arrays of *E. coli* vectors exist for purposes of cloning, promoter characterization, gene expression and other recombinant work (Preston, 2003; Rosano

and Ceccarelli, 2014). The majority of these vectors; however, are optimized for expression in *E. coli* and are not suitable for use in other hosts. With the list of bacteria useful for biotechnological applications ever expanding, there is a continuing need for the development of additional vectors for use in these different strains.

Vectors that allow recombinant methodologies to be performed in different species of *Pseudomonas* have been and are continuing to be developed for use in a variety of biotechnological applications (Calero et al., 2016; Nikel et al., 2014; Poblete-Castro et al., 2012). For example, the construction of the pUCP *Escherichia-Pseudomonas* shuttle vector series allowed routine cloning to be conducted in *E. coli* and direct expression of a target gene in *Pseudomonas* (Schweizer, 1991; West et al., 1994). This vector series was based on the well-characterized pUC18/19 vectors (Yanisch-Perron et al., 1985) and the small multicopy plasmid, pRO1600, from *P. aeruginosa* (Olsen et al., 1982). Vectors derived from the pRO1600 plasmid have been shown to replicate in bacterial species which include *P. aeruginosa*, *P. putida*, *P. fluorescens*, *Klebsiella pneumoniae*, *Burkholderia* spp., and *Salmonella* spp. (Choi et al., 2008; Olsen et al., 1982; Qiu et al., 2008; Schweizer et al., 1996; Silva-Rocha et al., 2013). Subsequently, Qiu et al., modified the pUCP20T, -26T, -28T, and -30T *Escherichia-Pseudomonas* shuttle vector series by replacing the lac promoter with an araC-*pBAD* cassette to allow tighter control over the expression of a target gene. This new araC-*pBAD* -based shuttle vector series designated pHERD20T, -26T, -28T, and -30T (Qiu et al., 2008) contains features of the pUCP vector series which include the origin of replication from pBR322, the *oriT* region for conjugation-mediated plasmid transfer (Schweizer, 1992), origin of replication from pRO1600,

and the rep gene encoding the replication-controlling protein (Schweizer, 1991; West et al., 1994).

In this study, a new bicistronic *Escherichia-Pseudomonas* shuttle vector series is constructed based on the pHERD vector series backbone. This new vector series, designated pDUO, allows the simultaneous expression of two genes in a regulated fashion. This vector series should find use both in functional studies and in the engineering of bacteria for biotechnological purposes. This vector set allows recombinant manipulations to be conducted in *E.coli* and direct expression in other biotechnologically important bacterial strains (discussed above). Inducible promoters and affinity tags aid in expression and purification of recombinant proteins including those that may be toxic in other expression systems. The utility of this vector set was shown in *E. coli* and *P. fluorescens*, two bacteria that have been previously shown to be useful for biotechnological applications (Baeshen et al., 2015; Lee, 2009; Montie, 1998; Squires and Talbot, 2004; Zimmer, 2008).

2. Methods

2.1 Bacterial strains and growth conditions

A list of the bacterial strains, plasmids, and primers utilized in this study are listed in Table 1. Bacteria were grown in Luria Broth (Invitrogen Life Technology, Carlsbad, CA). *E.coli* strains were grown at 37°C while *P. fluorescens* was propagated at 30°C unless otherwise noted. Chemicals, antibiotics, and oligonucleotide primers were purchased from Sigma-Aldrich (St. Louis, MO).

2.2 Recombinant DNA methods

Plasmid DNA was isolated using the Wizard miniprep kit (Promega, Madison, WI). DNA ligations, DNA dephosphorylation, and restriction digestions were performed according to standard methods (Sambrook et al., 1989). Restriction enzymes and shrimp alkaline phosphatase were purchased from New England Biolabs (Ipswich, MA) while T4 DNA ligase, Wizard SV gel and PCR clean-up system were bought from Promega. PCR reactions were optimized for each primer pair (Table 1) and amplified utilizing PlatinumTM Taq PCR Supermix High Fidelity (Life Technologies, Carlsbad, CA). PCR products were isolated by gel purification using 1% (w/v) agarose. DNA transformation of *E. coli* and *P. fluorescens* was performed using protocol C as previously described (Roychoudhury et al., 2009). In brief, the bacterial cells were grown to an OD₅₅₀ of 0.5 and made competent utilizing a 2-step treatment. First, the cells were resuspended in an ice-cold solution containing 30 mM potassium acetate, 100 mM RbCl, 10 mM CaCl₂, 50 mM MnCl₂·4H₂O and 15% (v/v) glycerol followed by a second treatment in a solution containing 10 mM MOPS-KOH, pH 6.5, 10 mM RbCl, 75 mM CaCl₂ H₂O and 15% (v/v) glycerol. The subsequent steps in transformation and plating were conducted according to protocol A as previously described (Roychoudhury et al., 2009).

2.3 pDUO shuttle vector construction

Construction of pDUO was initiated utilizing the pHERD cloning vectors (Qiu et al., 2008) that were kindly provided by Dr. Hongwei Yu (Marshall University, West Virginia, U.S.A.). Each pHERD vector (-20T, -26T, -28T, and -30T) was digested sequentially using *Nco*I and *Sfi*I at 37°C and 50°C, respectively. The digested vector backbone then was gel purified

using 1% (w/v) agarose. A DNA cassette (Fig. 1) consisting of an additional *pBAD* promoter, two MCS, and two transcriptional terminators was synthetically synthesized (Genscript, Piscataway, NJ). The 1100 bp cassette, containing the corresponding *NcoI/SfiI* sites, was unidirectionally cloned into each pHERD vector generating a new four vector series differing in marker selection (Fig. 1). This vector series was named pDUO and map construction was created using SnapGene software (GSL Biotech, Chicago, IL; www.snapgene.com).

The GenBank nucleotide sequence accession numbers for the pDUO vector series -20T, -26T, -28T, and -30T are KX806598, KX806599, KX806600, KX806601, respectively.

2.4 pDUO obc1+mrfp co-expression vector construction

To generate the pDUO *obc1+mrfp* expression construct, the *obc1* and *mrfp* coding regions were amplified by PCR using the appropriate primer sets (Table I) and *Burkholderia mallei* genomic DNA (kindly provided by David DeShazer, U.S. Army Medical Research Institute of Infectious Diseases, Frederick, MD) or CD3-976 plasmid DNA (ABRC, Ohio State University, Columbus, OH) as templates, respectively. All hybridization steps were performed on a MJ Research PTC-200 thermal cycler (Watertown, MA).

The *B. mallei obc1* gene (Nakata, 2011) was cloned in two overlapping sections. A 1.4 kb fragment encoding the N-terminus of the gene product was amplified using gene specific primers (Table I). The 5'-primer contained an engineered *EcoRI* site and the 3'-primer contained an internal *BglII* site. A 2 kb fragment encoding the C-terminus was amplified using gene specific primers (Table I) in which the 5'-primer contained an internal *BglII* site and the 3'-primer contained an engineered *HindIII* site. Each PCR reaction was conducted using the PCRx

Enhancer kit (Invitrogen life technologies, Carlsbad, CA) according to the manufacturer's instructions. The amplified fragments were cloned using the Qiagen TA cloning kit (Qiagen Inc., Valencia, CA). The construct encoding the N-terminus was digested with *EcoRI*-*Bgl*II and the construct encoding the C-terminus was digested with *Bgl*II-*Hind*III and the 1.4 and 2 kb fragments were individually purified on a 1% agarose gel. The two isolated fragments were reassembled into a complete ORF by unidirectionally subcloning into the *EcoRI* and *Hind*III sites encoded in MCS1 of the pDUO-26T shuttle vector. The generated construct was amplified in *E. coli* strain DH5 α .

The 750 bp *mrfp* coding region was amplified using primer set (Table I) where the 5'-primer contained an engineered *Nde*I site and the 3'-primer contained an engineered *Kpn*I site. The amplified fragment was cloned using the Qiagen TA cloning kit and amplified in *E. coli* strain DH5 α . The *mrfp* TA construct then was digested with *Nde*I and *Kpn*I and the *Nde*I-*Kpn*I coding fragment inserted into the corresponding restriction sites within MCS2 of the pDUO *obc1* construct generating the pDUO *obc1+mrfp* co-expression construct. This pDUO *obc1+mrfp* construct then was separately transformed in *E. coli* Top 10 cells and *P. fluorescens* ATCC 13525 cells.

2.5 pDUO *obc1+mrfp* dual expression analysis

Cultures of *E. coli* and *P. fluorescens* containing the pDUO *obc1+mrfp* expression construct were grown overnight in LB media supplemented with 10 μ g/mL tetracycline at 30°C. Five hundred microliters of overnight culture was added to 5 mL of individual LB media cultures

containing 0%, 0.002%, 0.02%, 0.2%, or 2% (w/v) L-arabinose. All cultures were grown for 5 h at 30°C before assessment of functional Obc1 and *mrfp* expression.

Functional expression of Obc1 in *E. coli* and *P. fluorescens* was assessed by measuring the biosynthesis of oxalate in liquid cultures. Grown cell cultures were centrifuged at 10,000 x g for 5 minutes. The cell pellet was discarded and the supernatant assayed. Oxalate measurements were made using an oxalate diagnostic kit (Trinity Biotech, St. Louis, MO) according to the manufacturer's instructions. In brief, the oxalate was oxidized by oxalate oxidase to carbon dioxide and hydrogen peroxide. The generated hydrogen peroxide was then allowed to react with 3-methyl-2-benzothiazolinone hydrazone and 3-(dimethylamino) benzoic acid in the presence of peroxidase to give an indamine dye that was read at 590 nm using a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA). The experiment was conducted in triplicate and standard error determined.

The functional expression of mCherry in transformed strains of *E. coli* and *P. fluorescens* was assessed by measuring relative fluorescence using a SpectraMax M2 plate reader. One hundred microliters of each grown bacterial culture was transferred to a black 96-well flat bottom plate (Costar, Corning, New York). Fluorescence was quantified using a 587 nm excitation and 635 nm emission measurement. The experiment was conducted in triplicate and standard error determined.

3. Results and Discussion

The aim of this study was to construct a new shuttle vector series that would allow controlled co-expression of genes and purification of gene products for functional studies and

engineering purposes in *Pseudomonas* and other bacteria. Although a number of vectors have been reported for the expression of an individual gene in *Pseudomonas* and related species, the availability of vectors designed for the controlled expression of two genes simultaneously is, to my knowledge, currently limiting. The usefulness of such dual expression vectors is exemplified by the distribution and use (Aphasizheva and Aphasizhev, 2010; Hussein et al., 2015; Kurumbang et al., 2010; Nakata, 2011; Nakata and He, 2010) of the commercially available co-expression vector pDUET1 (Novagen, EMD Biosciences, Inc. Madison, WI). Although highly useful, the utility of pDUET1 is limited by its narrow host-range (i.e., *E. coli*). It is often desirable to conduct biochemical and biophysical characterizations of proteins produced in their native environment or to express proteins in different bacteria for the production of specialized products. In such instances the availability of suitable vectors with the appropriate selectable markers is often a limitation.

3.1 Construction of the pDUO bicistronic expression shuttle vector series

With the intent of expanding the available collection of bacterial expression vectors, construction of the pDUO co-expression shuttle vector series was initiated utilizing a backbone derived from the pHERD vector set (Qiu et al., 2008). The pHERD vector set was selected as the backbone over other available vector systems because the vector series was readily available and the modifications required to generate a *araC-pBAD* regulated co-expression vector series were relatively straightforward. Although other vector systems, such as those based on the RK2 replicon, have the advantage of conferring the ability to replicate in both *Escherichia* and *Pseudomonas* without the need for two separate replicons, plasmid yields of the pHERD derived

vector set is expected to be several-fold higher than yields from RK2 derived vectors making molecular manipulations easier with the former vector set. It has been reported that the copy number for the pHERD replication system is approximately 13 in *Pseudomonas* and 36 in *Escherichia* (Farinha and Kropinski, 1989; Farinha and Kropinski, 1990) while the copy number for the RK2 replication system is roughly 3 and 5 for *Pseudomonas* and *Escherichia*, respectively (Kües and Stahl, 1989).

As an initial step in converting the pHERD vector series into a dual expression vector series, a synthetic DNA cassette containing MCS1 with the sequence encoding the N-terminal HIS-tag, followed by a transcriptional terminator, *araC-p_{BAD}* promoter, MCS2 with the sequence encoding the C-terminal S-tag, and transcriptional terminator was designed to be inserted just downstream of the *araC-p_{BAD}* promoter encoded by the pHERD vectors. Synthesis of the cassette including the two flanking restriction sites, *NcoI* and *SfiI*, was completed by GenScript (Piscataway, NJ, U.S.A.). This *NcoI/SfiI* cassette was cloned into the corresponding sites of the pHERD-20T, -26T, -28T, and -30T series. This cloning generated the new four vector series pDUO (Fig. 1) which includes the choice of four different antibiotic selectable markers encoding resistance to ampicillin (-20T), tetracycline (-26T), trimethoprim (-28T), or gentamycin (-30T). Previous studies have shown that the vector backbone used to construct the pDUO vector series was functional not only in *E.coli* but also in a number of other bacteria including *P. fluorescens*, *P. aeruginosa*, *P. putida*, *Klebsiella pneumoniae*, *Burkholderia* spp., and *Salmonella* spp. (Choi et al., 2008; Olsen et al., 1982; Qiu et al., 2008; Schweizer et al., 1996).

The *araC-p_{BAD}* inducible promoter system was selected to drive co-expression in the pDUO vector series for a couple of reasons. First, the *araC-p_{BAD}* inducible promoter system

functions in diverse bacterial species (Khlebnikov et al., 2000; Lefebvre and Valvano, 2002; Schleif, 2010; Sukchawalit et al., 1999). Second, the promoter activity can be tightly controlled through the addition of the sugars glucose and arabinose which repress and induce expression, respectively. The ability to tightly regulate the expression of target genes expands the range of protein types that can be produced utilizing this vector series. For example, the ability to turn on and off recombinant protein production as well as the ability to produce the recombinant protein at a slower rate would be beneficial in instances where protein toxicity or the rate of protein folding may be an issue. The bicistronic design of this vector series also enables strict control of the stoichiometry of the co-expressed proteins which is essential in instances requiring protein assembly or protein-protein interactions.

The HIS- and S-affinity tags were included in as part of MCS1 and MCS2, respectively to allow one-step purification of the expressed proteins. An initial step toward purifying, detecting, and/or immunoprecipitating a protein of interest often involves the fusion of an affinity tag, especially if the protein is uncharacterized and/or lacks an available antibody. The two selected tags are both small and rarely affect protein function. Purification procedures as well as commercial kits are readily available for affinity purification and antibody detection of both fusion types. Additional features of the S-tag include an improved solubility. The utility of both these affinity tags has been extensively reported (reviewed by Kimple et al., 2001; Terpe, 2003; Young et al., 2012)

3.2 Validation of the pDUO bicistronic expression shuttle vector series

As a first step toward evaluating the utility of the pDUO vector series, DNA sequences encoding Obc1 and mCherry were cloned into MCS1 and MCS2, respectively. The *obc1* gene encodes an oxalate biosynthetic enzyme that was originally identified in *Burkholderia mallei* (Nakata, 2011) and *mrfp* is a monomeric fluorescent protein that is often used as a marker in cellular studies. Each gene was inserted inframe with the affinity tag encoded within each MCS. This cloning resulted in an N-terminal HIS-tagged Obc1 and a C-terminal S-tagged mCherry. Next, this co-expression construct then was transformed into two different bacteria, *E. coli* and *P. fluorescens*, and the cells selectively grown demonstrating the functionality of the antibiotic markers and replicons of the pDUO vector series in the two bacteria.

Functional co-expression of Obc1 and mCherry was assessed by measuring the biosynthesis of oxalate and relative fluorescence, respectively. Little oxalate was detected in cultures of *E.coli* and *P. fluorescens* when strains were grown on LB without supplementation (Fig. 2). Upon supplementation with differing amounts of L-arabinose, oxalate production was induced in cultures of both *E. coli* (Fig. 2A) and *P. fluorescens* (Fig. 2B) validating the inductive response of the *araC*-*pBAD* promoter located upstream of MCS1. Measurements also revealed that the induction of the pDUO *obc1+mrfp* construct occurred at 10 to 100-fold lower L-arabinose concentration in *E. coli* compared to *P. fluorescens*. As expected, the induction of mCherry expression (Fig. 3) was observed to follow the same general pattern observed in the oxalate biosynthetic profiles with mCherry induction occurring at a much lower L-arabinose concentration in *E. coli* (Fig. 3A) than *P. fluorescens* (Fig. 3B). The more responsive nature of the pDUO *obc1+mrfp* construct to L-arabinose in *E. coli* is most likely attributable to a combination of factors. First, the utilized Top10 strain has been specially engineered to allow

transport of L-arabinose, but not metabolism of this sugar. Thus, the effective concentration does not decrease over time as expected with other bacterial strains that are able to metabolize L-arabinose. Second, as discussed earlier, the pDUO copy number is expected to be approximately 3-fold higher in *E. coli* than *P. fluorescens* (Farinha and Kropinski, 1989; Farinha and Kropinski, 1990). Other factors that may also be influencing the required induction concentrations include differences in *araC*-*pBAD* promoter activity and/or post transcriptional processes such as translational efficiency as well as transcript or protein stability. Overall, this co-expression study validated the arabinose induction of both *araC*-*pBAD* promoters located upstream of MCS1 and MCS2.

4. Conclusion

The pDUO bicistronic shuttle vector series offers a vehicle to co-express two genes in a highly regulated fashion in different bacterial hosts. The utility of this vector series was demonstrated by the co-expression and functional assessment of two recombinant proteins in both *E. coli* and *P. fluorescence*. The ability to make recombinant manipulations in *E. coli* and then directly express the generated construct in different bacterial hosts makes this versatile vector series an attractive addition to our molecular tool box. It is anticipated that this vector series, which includes multiple selectable markers, will find use in functional studies and biotechnological applications.

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Figure Legends

Fig. 1. *Construction of pDUO dual-expression shuttle vector series.* Each pDUO vector contains a *p_{BAD}* L-arabinose inducible promoter (white arrow) upstream of MCS1 and MCS2 to allow inducible co-expression of two different target genes. MCS1 encodes an N-terminus HIS-tag and MCS2 encodes a C-terminus S-tag to allow one-step purification of the target recombinant protein. Each vector in this series contains one of four different selectable markers along with a pBR322 and pRO1600-derived replicon to support replication in different bacterial hosts. Amp, ampicillin; Tet, tetracycline; Tri, trimethoprim; Gen, gentamycin.

Fig. 2. *Functional expression of Obc1.* The *obc1* gene was utilized to validate inducible expression from MCS1 of the pDUO vector. (A) Arabinose-dependent induction of oxalate biosynthesis in *E. coli* expressing pDUO *obc1+mrfp* construct was measured by colorimetric assay. (B) Arabinose-dependent induction of oxalate biosynthesis in *P. fluorescens* containing the construct pDUO *obc1+mrfp*. Data are mean $\pm$ SE from at least three experiments

Fig. 3. *Functional expression of mCherry.* The *mrfp* gene was utilized to validate inducible expression from MCS2 of the pDUO vector. (A) Arabinose-dependent induction of mCherry in *E. coli* expressing the construct pDUO *obc1+mrfp* was assessed by fluorometry. (B) Arabinose-dependent induction of oxalate biosynthesis in *P. fluorescens* containing the construct pDUO *obc1+mrfp* as measured by fluorometry. Data are mean $\pm$ SE from at least three experiments. RFU, relative fluorescence unit.

Fig. 1.

Fig. 2.

Fig. 3.