Title
Construction of a novel dual-inducible duet-expression system for gene (over)expression in Pseudomonas putida.

Permalink
https://escholarship.org/uc/item/92s478hs

Authors
Gauttam, Rahul
Mukhopadhyay, Aindrila
Singer, Steven W

Publication Date
2020-07-01

DOI
10.1016/j.plasmid.2020.102514

Peer reviewed
Short Communication

Construction of a novel dual-inducible duet-expression system for gene (over)expression in *Pseudomonas putida*

Rahul Gauttama,b, Aindrila Mukhopadhyaya,b, Steven W. Singerab,c

a The Joint BioEnergy Institute, Emeryville, CA, USA
b Biological Systems and Engineering Division, Lawrence Berkeley National Laboratory, Berkeley, CA, USA

c E-mail address: ssinger@lbl.gov (S.W. Singer).

**ABSTRACT**

*Pseudomonas putida* is a widely used host for metabolic engineering and synthetic biology. However, the use of *P. putida* has been hampered by the availability of a limited set of expression vectors for producing heterologous proteins. To widen the scope of expression vectors for gene co-expression studies, a previously established dual-inducible expression vector pRG_Duet2 developed for *Corynebacterium glutamicum* has been modified for use in *P. putida*. This expression vector, named pRGPDuo2, harbors two origins of replication, *colE1* for replication in *E. coli* and pRO1600 for replication in *P. putida*. Two multiple cloning sites (MCS1 and MCS2) in pRGPDuo2 are individually controlled by inducible promoters *P_{lac}*, or *P_{tetR/tetA}*. Functional validation of pRGPDuo2 was confirmed by the co-expression of genes for the fluorescent proteins namely, superfolder green fluorescent protein (sfGFP), and red fluorescent protein (RFP). Moreover, the strength of the fluorescence signal was dependent on the inducer concentrations present in the culture medium. The expression vector pRGPDuo2 is an attractive addition to the existing repertoire of expression plasmids for expression profiling and adds to the tools available for *P. putida* metabolic engineering.

1. Introduction

*Pseudomonas putida* is a Gram-negative, rod-shaped, soil bacterium that has been widely employed for bio-industrial applications (Tiso et al., 2014). *P. putida* is considered to be a favorable host for the production of heterologous proteins due to its advantageous traits such as low nutritional requirements and diverse aerobic metabolism (Timmis, 2002). Moreover, physiological features like its ability to generate high biomass yield, rapid growth rate, and minimal maintenance requirements allow *P. putida* to be developed as an industrial producer for the production of targeted recombinant proteins (Poblete-Castro et al., 2012). Additionally, compared to other bacterial systems, *P. putida* is known to tolerate various stresses such as toluene, styrene, octanol (Domínguez-Cuevas et al., 2006; Blank et al., 2008) as well as reduced water activity (Hallsworth et al., 2003), adding to its potential as a host to be employed for bioremediation applications and the production of toxic compounds (Kuepper et al., 2015), that would be challenging in bacterial hosts like *E. coli* and *Bacillus* spp.

For *P. putida* to achieve its full potential and to be used as a major microbial host in genetic and biotechnological studies, the key requirement is to develop robust synthetic biology tools for genetic manipulations including plasmid vectors for expression of heterologous proteins and gene knockouts or repression. Over the years, there have been remarkable advances to broaden the existing repertoire of molecular toolbox for *P. putida* by developing improved and more advanced genome engineering tools for functional analysis or gene expression of heterologous pathways (Martínez-García and de Lorenzo, 2017; Nikel and de Lorenzo, 2018; Martínez-García and de Lorenzo, 2019; Calero and Nikel, 2019). For instance, a series of plasmid vectors are available to use in gram-negative bacteria (including *Pseudomonas putida*) from Standard European Vector Architecture Database (SEVA) repository that allows a simple exchange of particular module (eg., origins of replication and antibiotic selection) with another vector element (Silva-Rocha et al., 2013). In a recent advancement, the relatively new CRISPR/(d)Cas9 technology has been adapted to manipulate genome or achieve titrable gene expression in *P. putida* (Tan et al., 2018; Batianis et al., 2020; Wirth et al., 2020). However, it is noteworthy that there is still a limited number of vectors available for this organism that allows the tightly controlled or tunable expression of genes, and that in turn makes rewiring of metabolic pathways difficult (Volke et al., 2020).

**Abbreviations:** IPTG, Isopropyl-β-D-1-thiogalactopyranoside; aTc, anhydrotetracycline; KanR, kanamycin resistance; AmpR, ampicillin resistance; MCSs, multiple cloning sites; RFP, red fluorescent protein; sfGFP, superfolder green fluorescent protein

Corresponding author at: Lawrence Berkeley National Laboratory, 1 Cyclotron Road, Berkeley, CA 94720, USA.

E-mail address: ssinger@lbl.gov (S.W. Singer).

https://doi.org/10.1016/j.plasmid.2020.102514

Received 6 February 2020; Received in revised form 1 April 2020; Accepted 16 April 2020

Available online 03 June 2020

0147-619X/ © 2020 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).
Most of these vectors harbor a single multiple cloning site downstream of either a constitutive or an inducible promoter system such as P_tac, P_aro, P_Zeo. Such vectors are useful but inefficient for cloning large DNA fragments or multiple genes (Radmacher et al., 2002). In these situations, a large number of genes are co-expressed using two or more vectors, each carrying a different antibiotic resistance, therefore alleviating a great deal of stress on bacterial growth (Gauttam et al., 2019b). Especially, for gene co-expression and relieving antibiotic stress, dual-expression vectors have been constructed to allow transcription from individual promoters (Goldbeck and Seibold, 2018; Gauttam et al., 2019a). For instance, the development of a series of T7 promoter based (IPTG-inducible) compatible dual expression vectors in E. coli, P_aro-lac based duo vector in P. putida (Yu et al., 2018) and pDUO dual-expression vector series in Pseudomonas fluorescens (Nakata, 2017) greatly accelerated the co-expression of large number of genes encoding the rate-limiting enzymes in biosynthetic pathways (Tolia and Joshua-Tor, 2006). However, E. coli (and P. putida) duo vectors are IPTG-inducible only, and P. fluorescens duo vectors are arabinose-inducible only, therefore allowing co-expression of two genes but not differential regulation of two genes. Dual-inducible dual-expression vectors (pRG_Duet1, pRG_Duet2 and pRGP_dCas9) have been developed and shown to be functional in Corynebacterium glutamicum (Gauttam et al., 2019a), with each vector harboring two independently inducible promoters, P_aro and Ptet/atek, to regulate the expression of two sets of genes independent of each other (Gauttam et al., 2019a, 2019b). In this study, we have developed a dual-inducible dual expression vector pRGPDuo2 for the expression of heterologous proteins in P. putida. The E. coli – P. putida shuttle expression vector pRGPDuo2 is based on the colE1 replicon of E. coli and the pRO1600 replicon of P. putida, carrying the two multiple cloning sites, MCS1 downstream of P_aro promoter and MCS2 downstream of Ptet/atek promoter.

2. Material and methods

2.1. Bacterial strains, plasmids and culture conditions

Bacterial strains and plasmids used in this study are listed in Table 1. E. coli strains were routinely grown in LB medium (Green and Sambrook, 2012) at 200 rpm and 37 °C. Unless otherwise stated, P. putida (pre-cultures only) were grown in LB medium at 200 rpm and 30 °C. For the cultivation of P. putida, a single colony was picked from a freshly prepared agar plate to inoculate 5 ml of LB medium (first pre-culture) and incubated for 8 h at 200 rpm and 30 °C. The first pre-culture was used as starter culture for second pre-culture, to inoculate M9 minimal medium (6 g l^{−1} (Na2HPO4), 3 g l^{−1} KH2PO4, 1.4 g l^{−1} (NH4)2SO4, 0.5 g l^{−1} NaCl, 0.2 g l^{−1} MgSO4·7H2O) with an added 2.5 ml l^{−1} of a trace elements solution and 0.5% glucose as sole carbon source. The cultivation of all P. putida strains was performed two times in M9 medium before inoculating the main culture for growth and measurement of fluorescence. Bacterial cultures were supplemented with kanamycin (50 μg/ml), carbenicillin (100 μg/ml), IPTG (0 to 1 mM) and/or aTc (0 to 1000 ng/ml), when required. Growth was determined spectrophotometrically by measuring the absorbance at 600 nm (OD600). The relevant information regarding all the strains and plasmids have been deposited in the public instance of the JBEI Registry (http://public-registry.jbei.org/).

2.2. DNA manipulation, preparation and transformation

Standard molecular biology procedures were used for DNA isolation, electrophoresis, gene cloning, E. coli competent cells preparation and transformation (Green and Sambrook, 2012). The recombinant strains were selected on LB agar plates (15 g l^{−1} agar) containing respective antibiotics. Restriction enzymes, T4 DNA Ligase, and alkaline phosphatase employed in this study were obtained from Thermo Fisher Scientific (Waltham MA, USA) and used per instructions from the manufacturer. Oligonucleotides were ordered from Integrated DNA Technologies (IDT, San Diego, California, USA) and are listed in Table 2. The cloned region was sequence-verified by a Sanger Sequencing service (Genewiz, Seattle, WA, USA). Polymerase chain reaction (PCR) conditions were optimized for each primer pair, and DNA fragments were amplified using Q5 High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA, USA). PCR products were separated by electrophoresis on agarose gels (1% w/v) and purified using the NucleoSpin DNA extraction kit from Qiagen (Hilden, Germany). The recombinant plasmids were isolated from E. coli transformants using the NucleoSpin plasmid purification kit from Qiagen, following the instructions of the manufacturer. P. putida transformations were carried out by electroporation. Briefly, the Pseudomonas strains grown overnight on LB agar plates were resuspended in 300 mM sucrose solution and washed twice before resuspension in 500 μl of 300 mM sucrose. Prior to electroporation, 100 μl aliquots were mixed with 500 ng of the plasmid DNA in a 2-mm cuvette and cells were electroporated using a Gene Pulser XCell™ (BioRad Labs GmbH, Munich, Germany) with parameters set to voltage 2.5 kV, the capacitance of 25 μF and resistance of 200 Ω. Cells were then incubated in LB medium for 2 h for recovery before being plated on selection plates. Basic bioinformatics tools and software were used for designing oligonucleotides such as SnapGene software (GSL Biotech; available at snapgene.com), vector map generation (SnapGene) and genome analysis (NCBI Blast).

2.3. Cloning of fluorescent genes (sfGFP and RFP) to construct pRGPDuo2 derived reporter plasmids

Two reporter proteins, a superfoldering variant of GFP (sfGFP), and RFP were chosen to validate the functionality of the expression vector, pRGPDuo2. The gene encoding either sfGFP or RFP was cloned in pRGPDuo2 vector in different combination to create following plasmids: pRGPDuo2-sfGFP_tac, pRGPDuo2-sfGFP_tet, pRGPDuo2-RFP_tac, pRGPDuo2-RFP_tet, and pRGPDuo2-RFP_tac + sfGFP_tet. The gene encoding sfGFP was amplified using pgEM00003 as a template and dualsGFP-fwd/rev as primers with incorporated restriction sites and subcloned into a cloning vector pJE1.2/blunt, to construct donor plasmid pJE1-sfGFP, following the instructions from the manufacturer. Similarly, two variants of RFP donor vectors, namely pJE1-RFP_tac and pJE1-RFP_tet, were created using pBADTetp as a template using duoRFPtet-fwd/rev and duoRFPtat-fwd/rev primers, respectively. These donor vectors were employed to create test plasmids. For instance, plasmid pRGPDuo2-sfGFP_tac was constructed by ligating PslI/BamHI-digested pRGPDuo2 with PslI/BamHI-digested PCR product (containing sfGFP DNA sequence) from pJE1-sfGFP and plasmid pRGPDuo2-sfGFP_tet by ligating NheI/BglII-digested pRGPDuo2 with NheI/BglII-digested product from pJE1-sfGFP. The plasmids expressing gene coding for RFP, plasmid pRGPDuo2-RFP_tac, was constructed by ligating PslI/BamHI-digested pRGPDuo2 with PslI/BamHI-digested PCR product (containing RFP DNA sequence) from pJE1-RFP-tac and plasmid pRGPDuo2-RFP_tet by ligating NheI/BglII-digested pRGPDuo2 with NheI/BglII-digested product from pJE1-RFP_tet. The final plasmid pRGPDuo2-RFP_tac + sfGFP_tet for co-expressing gene for both fluorescent proteins (sfGFP and RFP) was created by ligating NheI/BglII-digested pRGPDuo2-RFP_tac with NheI/BglII-digested product from pJE1-sfGFP.

2.4. Fluorescence spectroscopy

The GFP and RFP fluorescence relative to the optical density (OD600) were measured using TECAN infinite M200 plate reader (Mannedorf, Switzerland) using 48 well plates (Sarstedt, Germany) with each well containing 250 μL of cell culture. Cells were induced at 0 h with the appropriate amount of the inducers IPTG and/or aTc. After induction, cells were grown at 30 °C for 24 h until fluorescence was measured. For sfGFP, λex = 485 nm and λem = 535 nm were used; and for RFP λex = 535 nm and λem = 620 nm were used. The wavelength
2. Results and discussion

While vectors for gene co-expression have been developed in E. coli, C. glutamicum, and P. putida, they have not been developed for P. putida. To overcome the shortcomings of the current P. putida expression vectors and to expand the available repertoire of P. putida expression vectors, a duet-expression vector for P. putida, pRGPDuo2, was designed. Two different inducible repressor systems in a single-plasmid system, lacI-dependent repression system controlled by P_LacUV5 promoter and tetR dependent-repression system controlled by P_TetR/tetA, were combined to establish tight control over targeted gene expression for metabolic engineering purposes. The previously created duet-expression vector pRGPDuo2 (Gauttam et al., 2019a, 2019b) was selected as the backbone to retain all functional elements such as two different multiple cloning sites (MCS1 and MCS2) each controlled by either IPTG-inducible Ptac promoter or atc-inducible P_atc promoters, kanamycin resistance gene (KanR), transcriptional terminators and the origin of replication (oriP_C. glutamicum) was exchanged with the Pseudomonas origin of replication (OriP_P. putida). To achieve this, the DNA fragment containing P. putida PP16 (JPUB_014499) was exchanged in vector pRGPDuo2 (Gauttam et al., 2019a, 2019b) was selected as the backbone to retain all functional elements such as two different multiple cloning sites (MCS1 and MCS2) each controlled by either IPTG-inducible P_LacUV5 promoter or atc-inducible P_atc promoters, kanamycin resistance gene (KanR), transcriptional terminators and the origin of replication (oriP_C. glutamicum) was exchanged with the Pseudomonas origin of replication (OriP_P. putida). To achieve this, the DNA fragment containing P. putida

| Strains or plasmid(1) | Relevant characteristics | Source/reference |
|-----------------------|--------------------------|------------------|
| E. coli DH5α          | F− λφ80lacZAM15 Δ(lacZYA-argF) U169 endA1 recA1 gyrA17 (r−, m−) supE44 thi−1 gcrA96 relA1 phaA | Hanahan, 1983 |
| *Pseudomonas* putida KT2440 | Wild type | ATCC 12633 |
| *Pseudomonas* putida PP1 (JPUB_014489) | P. putida carrying pRGPDuo2; KanR | This study |
| *Pseudomonas* putida PP2 (JPUB_014491) | P. putida carrying pRGPDuo2-stfGFP; KanR | This study |
| *Pseudomonas* putida PP3 (JPUB_014493) | P. putida carrying pRGPDuo2-stGFP; KanR | This study |
| *Pseudomonas* putida PP4 (JPUB_014495) | P. putida carrying pRGPDuo2-RFPtet; KanR | This study |
| *Pseudomonas* putida PP5 (JPUB_014497) | P. putida carrying pRGPDuo2-RFPtet; KanR | This study |
| *Pseudomonas* putida PP6 (JPUB_014499) | P. putida carrying pRGPDuo2-RFPtet+stfGFP; KanR | This study |

Table 1

| Strains and plasmids used in this study. |
|-----------------------------------------|
| **Strains** | **Relevant characteristics** | **Source/reference** |
| E. coli DH5α | F− λφ80lacZAM15 Δ(lacZYA-argF) U169 endA1 recA1 gyrA17 (r−, m−) supE44 thi−1 gcrA96 relA1 phaA | Hanahan, 1983 |

- *Pseudomonas* putida KT2440: Wild type
- *Pseudomonas* putida PP1: P. putida carrying pRGPDuo2; KanR
- *Pseudomonas* putida PP2: P. putida carrying pRGPDuo2-stfGFP; KanR
- *Pseudomonas* putida PP3: P. putida carrying pRGPDuo2-stGFP; KanR
- *Pseudomonas* putida PP4: P. putida carrying pRGPDuo2-RFPtet; KanR
- *Pseudomonas* putida PP5: P. putida carrying pRGPDuo2-RFPtet; KanR
- *Pseudomonas* putida PP6: P. putida carrying pRGPDuo2-RFPtet+stfGFP; KanR

2.5. Accession number

The complete nucleotide sequence of plasmid pRGPDuo2 has been submitted to the NCBI GenBank with the accession number MN913428.

3. Results and discussion

3.1. Construction of a dual-inducible duet-expression shuttle vector pRGPDuo2 in P. putida

While vectors for gene co-expression have been developed in E. coli, C. glutamicum, and P. fluorescens, they have not been developed for P. putida. To overcome the shortcomings of the current P. putida expression vectors and to expand the available repertoire of P. putida expression vectors, a duet-expression vector for P. putida, pRGPDuo2, was designed. Two different inducible repressor systems in a single-plasmid system, lacI-dependent repression system controlled by P_LacUV5 promoter and tetR dependent-repression system controlled by P_TetR/tetA were combined to establish tight control over targeted gene expression for metabolic engineering purposes. The previously created duet-expression vector pRGPDuo2 (Gauttam et al., 2019a, 2019b) was selected as the backbone to retain all functional elements such as two different multiple cloning sites (MCS1 and MCS2) each controlled by either IPTG-inducible P_LacUV5 promoter or atc-inducible P_atc promoters, kanamycin resistance gene (KanR), transcriptional terminators and the E. coli origin of replication colEI. To adapt the C. glutamicum expression vector pRG_Duet2 for use in P. putida, the Corynebacterium origin of replication (oriP_C. glutamicum) was exchanged with the Pseudomonas origin of replication (OriP_P. putida). To achieve this, the DNA fragment containing P. putida

Table 2

| Oligonucleotides used in this study. |
|------------------------------------|
| **Oligo/synthetic fragment** | **Sequence (5’ → 3’)** |
| pRO1600-fwd | ttaagaateattgtgagagagactacatcttc |
| pRO1600-rev | atcgagctaattgactgtgagagagtacatgcag |
| duosfGFP-fwd | gcttttaagagatatacatctctgcttttaagagatatacatctct |
| duosfGFP-rev | gcttttaagaaggagatatacatctctgcttttaagagatatacatctct |
| duorFPPac-fwd | gcttttaagaaggagatatacatctctgcttttaagagatatacatctct |
| duorFPPac-rev | gcttttaagaaggagatatacatctctgcttttaagagatatacatctct |
| duorFPetri-fwd | gcttttaagaaggagatatacatctctgcttttaagagatatacatctct |
| duorFPetri-rev | gcttttaagaaggagatatacatctctgcttttaagagatatacatctct |

Restriction sites are indicated in bold.

for fluorescence excitation and emission were carefully selected to have a little overlap of fluorescence excitation and emission spectra between sfGFP and RFP. Optical density was measured as absorbance at 600 nm. Cell culture fluorescence for each test strain was normalized by its cell density (OD600). All the represented values were mean from at least three independent measurements.

Restriction sites are indicated in bold.

Table 2

| Oligonucleotides used in this study. |
|------------------------------------|
| **Oligo/synthetic fragment** | **Sequence (5’ → 3’)** |
| pRO1600-fwd | ttaagaateattgtgagagagactacatcttc |
| pRO1600-rev | atcgagctaattgactgtgagagagtacatgcag |
| duosfGFP-fwd | gcttttaagagatatacatctctgcttttaagagatatacatctct |
| duosfGFP-rev | gcttttaagaaggagatatacatctctgcttttaagagatatacatctct |
| duorFPPac-fwd | gcttttaagaaggagatatacatctctgcttttaagagatatacatctct |
| duorFPPac-rev | gcttttaagaaggagatatacatctctgcttttaagagatatacatctct |
| duorFPetri-fwd | gcttttaagaaggagatatacatctctgcttttaagagatatacatctct |
| duorFPetri-rev | gcttttaagaaggagatatacatctctgcttttaagagatatacatctct |

Restriction sites are indicated in bold.
a kanamycin resistance marker, origins of replication (colE1 and pRO1600) for stable maintenance in E. coli and P. putida, transcriptional terminators, two multiple cloning sites MCS1 and MCS2, individually controlled by promoters \( P_{lac} \) (IPTG-inducible) and \( P_{tet}/tetA \) (aTc-inducible) respectively (Fig. 1).

3.2. Functional validation of duet-expression vectors pRGPDuo2 in P. putida

To test the expression of cloned reporter genes in P. putida background, the wild type P. putida KT2440 strain was transformed with different plasmids to create the following recombinant strains; PP1 (harboring pRGPDuo2), PP2 (harboring pRGPDuo2-sfGFP\(_{tac}\)), PP3 (harboring pRGPDuo2-sGFP\(_{tet}\)), PP4 (harboring pRGPDuo2-RFP\(_{tet}\)), PP5 (harboring pRGPDuo2-RFP\(_{tac}\)) and PP6 (harboring pRGPDuo2-RFP\(_{tac}\) + sGFP\(_{tet}\)). In order to determine GFP/RFP-fluorescence, all the recombinant strains were cultivated in the presence of inducers IPTG (1 mM) and/or aTc (1000 ng/ml), when required. The P. putida strain PP1 carrying the vector pRGPDuo2 was used as a control for fluorescence experiments. When sGFP fluorescence was measured in recombinant strains, a definite increase in GFP fluorescence was observed in test strains PP2 (1228 ± 52) and PP3 (1285 ± 15) compared to control strain PP1 (176 ± 3) (Fig. 2A). As expected, minimal GFP fluorescence was observed in test strains PP4 (163 ± 18) and PP5 (167 ± 3), which was comparable to the values obtained with control strain PP1 (Fig. 2A).

Similarly, when RFP fluorescence was measured in recombinant strains, fluorescence was found to be increased by approximately 19-fold in P. putida strains PP4 (366 ± 28) and PP5 (347 ± 40) compared to control strain PP1 (19 ± 2) (Fig. 2B). As expected, minimal RFP fluorescence was observed in test strains PP2 (18 ± 1) and PP3 (10 ± 2), which was comparable to the fluorescence observed in control strain PP1 (Fig. 2B). These experiments demonstrated the functionality of both lacI\(_c\) controlled MCS1 as well as tetR controlled MCS2.

When GFP/RFP fluorescence was measured in recombinant strain PP6, the GFP (983 ± 91) and RFP (344 ± 16) fluorescence was found to be significantly increased in this strain compared to the control strain PP1 (Fig. 2A, B). When GFP fluorescence was measured in P. putida strain PP2 supplemented with IPTG concentrations ranging between 0 and 1000 μM (and a constant aTc concentration of 1000 ng/ml), a steady increase in GFP fluorescence was observed from 86 ± 5 to 998 ± 40 (Fig. 3A). Similarly, in P. putida strain PP3, supplemented with aTc concentrations between 0 and 1000 ng/ml (and a constant IPTG concentration of 1000 μM) a steady increase in GFP fluorescence was observed from 344 ± 28 to 1248 ± 55 (Fig. 3B). The experiment also indicated that the presence of opposite inducer (aTc for \( P_{tet} \) and
IPTG for PtetR/tetA) does not influence genes from non-cognate promoter. The fluorescence intensities were determined for P. putida, in the presence of different concentrations (ranging between 0 and 1000 μM or ng/ml) of both the inducers (IPTG and aTc) to show the differential expression of sfGFP and RFP present on the same plasmid (Fig. 3C and D). The gradual increase in both GFP and RFP expression was observed corresponding to a gradual increase in respective inducer(s) concentration (Fig. 3C and D). Our results provide evidence that the expression of two genes can be regulated independently to one another by adding a single inducer, either IPTG or aTc.

4. Conclusion

The presented work successfully demonstrates the functionality of dual-inducible shuttle expression vector pRGPDuo1. The constructed vector has two distinct multiple cloning sites (MCS1 and MCS2) controlled by two different controllable repressor systems (lacI and tetR). The fluorescence expression at different inducer doses was tested that showed gradual expression of a target gene, which in turn correlates to the protein level in cells. Such a system can be a promising tool for providing valuable information regarding the expression profile for designing metabolic engineering strategies. The use of single plasmid pRGPDuo2 to differentially regulate the expression of two genes is also an upgrade to the existing double plasmid-based expression system. The use of pRGPDuo2 is particularly promising in situations where differential expression of genes is the key requirement as in the case of co-chaperone expression to promote expression of targeted genes (Mueller et al., 2018). The vector pRGPDuo2 will be very useful for gene co-expression studies in P. putida and expands the molecular repertoire of molecular tools for genetic manipulation in this organism.

Ethics approval and consent to participate

Not applicable.

Funding

This work was part of the Department of Energy (DOE) Joint BioEnergy Institute (http://www.jbei.org) supported by the U.S. DOE, Office of Science, Office of Biological and Environmental Research, through Contract No. DE-AC02-05CH11231 between Lawrence Berkeley National Laboratory and the U.S. DOE. The United States Government retains and the publisher, by accepting the article for publication, acknowledges that the United States Government retains a nonexclusive, paid-up, irrevocable, worldwide license to publish or reproduce the published form of this manuscript, or allow others to do so, for United States Government purpose.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

We would like to express our gratitude to Prof. Dr. Bernhard J. Eikmanns (Institute for Microbiology and Biotechnology), Ulm University, Germany, for providing the essential plasmids, including pRG_Duet2.
References

Batianis, C., Kozaeva, E., Damalas, S.G., Martín-Pascual, M., Volke, D.C., Nikol, P.I., Martín dos Santos, V.A., 2020. An expanded CRISPRi toolbox for tunable control of gene expression in Pseudomonas putida. Microb. Biotechnol. 13 (2), 368–385.

Bi, C., Su, P., Müller, J., Yeh, Y.C., Chhabra, S.R., Beller, H.R., Singör, S.W., Hillson, N.J., 2013. Development of a broad-host synthetic biology toolbox for Ralstonia eutropha and its application to engineering hydrocarbon biofuel production. Microb. Cell Factories 12 (1), 107.

Blank, L.M., Ionidis, G., Ebert, B.E., Bühler, B., Schmid, A., 2008. Metabolic response of Pseudomonas putida during redox biocatalysis in the presence of a second octanol phase. FEBS J. 275 (20), 5173–5190.

Calero, P., Nikol, P.I., 2019. Chasing bacterial chassis for metabolic engineering: a perspective review from classical to non-traditional microorganisms. Microb. Biotechnol. 12 (1), 98–124.

Dominguez-Cuevas, P., González-Pastor, J.E., Marqués, S., Ramos, J.L., de Lorenzo, V., 2006. Transcriptional tradeoff between metabolic and stress-response programs in Pseudomonas putida KT2440 cells exposed to toluene. J. Biol. Chem. 281 (17), 11981–11991.

Gauttam, R., Desiderato, C., Jung, L., Shah, A., Eikmanns, B.J., 2019a. A step forward: compatible and dual-inducible expression vectors for gene co-expression in Corynebacterium glutamicum. Plasmid 101, 20–27.

Gauttam, R., Seibold, G.M., Mueller, P., Weil, T., Weiß, T., Handrick, R., Eikmanns, B.J., 2019b. A simple dual-inducible CRISPR interference system for multiple gene targeting in Corynebacterium glutamicum. Plasmid 103, 25–35.

Goldbeck, O., Seibold, G.M., 2018. Construction of pOGOduet—an inducible, bicistronic vector for synthesis of recombinant proteins in Corynebacterium glutamicum. Plasmid 95, 11–15.

Green, M.R., Sambrook, J., 2012. Molecular Cloning, a Laboratory Manual, 4th ed. Cold Spring Harbor Laboratory Press.

Hallschmidt, J.E., Heim, S., Timmis, K.N., 2003. Chaotropic solutes cause water stress in Pseudomonas putida. Environ. Microbiol. 5 (12), 1270–1280.

Hanahan, D., 1983. Studies on transformation of Escherichia coli with plasmids. J. Mol. Biol. 166 (4), 557–580.

Kuepper, J., Dickler, J., Biggel, M., Behnken, S., Jäger, G., Wierckx, N., Blank, L.M., 2015. Metabolic engineering of Pseudomonas putida KT2440 to produce anthranilate from glucose. Front. Microbiol. 6, 1310.

Martínez-García, E., de Lorenzo, V., 2017. Molecular tools and emerging strategies for deep genetic/genomic refactoring of Pseudomonas. Curr. Opin. Biotechnol. 47, 120–132.

Martínez-García, E., de Lorenzo, V., 2019. Pseudomonas putida in the quest of programmable chemistry. Curr. Opin. Biotechnol. 59, 111–121.

Mueller, P., Gauttam, R., Raab, N., Handrick, R., Wahl, C., Leptihn, S., Zom, M., Kusmaul, M., Scheffold, M., Eikmanns, B.J., Elling, L., Gaisser, S., 2018. High level in vivo mucin-type glycosylation in Escherichia coli. Microb. Cell Factories 17 (1), 168.

Nakata, P.A., 2017. Construction of pDuo: a bicistronic shuttle vector series for dual expression of recombinant proteins. Plasmids 89, 16–21.

Nikel, P.I., de Lorenzo, V., 2018. Pseudomonas putida as a functional chassis for industrial biocatalysis: from native biochemistry to trans-metabolism. Metabol. Eng. 50, 142–155.

Poblete-Castro, I., Becker, J., Dohnst, K., Dos Santos, V.M., Wittmann, C., 2012. Industrial biotechnology of Pseudomonas putida and related species. Appl. Microbiol. Biotechnol. 93 (6), 2279–2290.

Radmacher, E., Vaitisikova, A., Burger, U., Krumbach, K., Sahm, H., Eggeling, L., 2002. Linking central metabolism with increased pathway flux L-valine accumulation by Corynebacterium glutamicum. Appl. Environ. Microbiol. 68 (5), 2246–2250.

Silva-Rocha, R., Martínez-García, E., Calles, B., Chavarría, M., Arce-Rodríguez, A., de las Heras, A., Durante-Rodriguez, G., Kim, J., Nikol, P.I., Platero, R., 2013. The Standard European Vector Architecture (SEVA): a coherent platform for the analysis and deployment of complex prokaryotic phenotypes. Nuc. Acids Res. 41 (D1), D666–D675.

Tan, S.Z., Reisch, C.R., Prather, K.L., 2018. A robust CRISPR interference gene repression system in Pseudomonas. J. Bacteriol. 200 (7), e00575-17.

Timmis, K.N., 2002. Pseudomonas putida: a cosmopolitan opportunist par excellence. Environ. Microbiol. 4 (12), 779–781.

Tiso, T., Wiercks, N., Blank, L., 2014. Non-pathogenic Pseudomonas as Platform for Industrial Biocatalysis. Industrial Biocatalysis, Pan Stanford, Singapore, pp. 325–372.

Tok, N.H., Joseph-Tor, L., 2006. Strategies for protein coexpression in Escherichia coli. Nat. Methods 3 (1), 55.

Volke, D.C., Turlin, J., Mol, V., Nikol, P.I., 2020. Physical decoupling of XylS/Pm regulatory elements and conditional proteolysis enable precise control of gene expression in Pseudomonas putida. Microb. Biotechnol. 13 (1), 222–232.

Wirth, N.T., Kozaeva, E., Nikol, P.I., 2020. Accelerated genome engineering of Pseudomonas putida by I-SceI-mediated recombination and CRISPR-Cas9 counter-selection. Microb. Biotechnol. 13 (1), 233–249.

Yu, S., Lai, B., Plan, M.R., Hodson, M.P., Lestari, E.A., Song, H., Krömer, J.O., 2018. Improved performance of Pseudomonas putida in a bioelectrochemical system through overexpression of periplasmic glucose dehydrogenase. Biotechnol. Bioeng. 115 (1), 145–155.