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Short Communication

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

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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_{tac} 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; Kan^R, kanamycin resistance; Amp^R, ampicillin resistance; MCSs, multiple cloning sites; RFP, red fluorescent protein; sfGFP, superfolder green fluorescent protein

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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_{tet}, P_{BAD}. 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, duet-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 duet expression vectors in *E. coli*, P_{trc}-lacI based duo vector in *P. putida* (Yu et al., 2018) and pDUO duet-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*) duet vectors are IPTG-inducible only, and *P. fluorescens* duet vectors are arabinose-inducible only, therefore allowing co-expression of two genes but not differential regulation of two genes. Dual-inducible duet-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_{tac} and $P_{tetR/tetA}$, 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 duet 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 *col*E1 replicon of *E. coli* and the pRO1600 replicon of *P. putida*, carrying the two multiple cloning sites, MCS1 downstream of P_{tac} promoter and MCS2 downstream of $P_{tetR/tetA}$ 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} (Na_2HPO_4), 3 g l^{-1} KH_2PO_4, 1.4 g l^{-1} $(NH_4)_2SO_4$, 0.5 g l^{-1} NaCl, 0.2 g l^{-1} $MgSO_4.7H_2O$) 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 (OD_{600}). 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 preparation, manipulation 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 superfolding 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$_{tet}$, pRGPDuo2RFP$_{tac}$, pRGPDuo2RFP$_{tet}$ and pRGPDuo2-RFP$_{tac}$ + sfGFP$_{tet}$. The gene encoding sfGFP was amplified using pGEM00003 as a template and duosfGFP-fwd/rev as primers with incorporated restriction sites and subcloned into a cloning vector pJET1.2/blunt, to construct donor plasmid pJET-sfGFP, following the instructions from the manufacturer. Similarly, two variants of RFP donor vectors, namely pJET-RFP$_{tac}$ and pJET-RFP$_{tet}$, were created using pBADTrfp as a template using duoRFPtac-fwd/rev and duoRFPtet-fwd/rev primers, respectively. These donor vectors were employed to create test plasmids. For instance, plasmid pRGPDuo2-sfGFP$_{tac}$ was constructed by ligating *Pst*I/*Bam*HI-digested pRGPDuo2 with *Pst*I/*Bam*HI-digested PCR product (containing sfGFP DNA sequence) from pJET-sfGFP and plasmid pRGPDuo2-sfGFP$_{tet}$ by ligating *Nhe*I/*Bgl*II-digested pRGPDuo2 with *Nhe*I/*Bgl*II-digested product from pJET-sfGFP. The plasmids expressing gene coding for RFP, plasmid pRGPDuo2-RFP$_{tac}$, was constructed by ligating *Pst*I/*Bam*HI-digested pRGPDuo2 with *Pst*I/*Bam*HI-digested PCR product (containing RFP DNA sequence) from pJET-RFP$_{tac}$ and plasmid pRGPDuo2-RFP$_{tet}$ was constructed by ligating *Nhe*I/*Bgl*II-digested pRGPDuo2 with *Nhe*I/*Bgl*II-digested product from pJET-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 *Nhe*I/*Bgl*II-digested pRGPDuo2-RFP$_{tac}$ with *Nhe*I/*Bgl*II-digested product from pJET-sfGFP.

2.4. Fluorescence spectroscopy

The GFP and RFP fluorescence relative to the optical density (OD_{600}) 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, λ_{ex} = 485 nm and λ_{em} = 535 nm were used; and for RFP λ_{ex} = 535 nm and λ_{em} = 620 nm were used. The wavelength

Table 1
Strains and plasmids used in this study.

Strain or plasmid([a])	Relevant characteristics	Source/reference
Strains		
E. coli DH5α	F^- *φ80lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *endA1 recA1 hsdR17* (r_k^-, m_k^+) *supE44 thi*$^{-1}$ *gyrA996 relA1 phoA*	Hanahan, 1983
Pseudomonas putida KT2440	Wild type	ATCC 12633
P.putida PP1 (JPUB_014489)	*P. putida* carrying pRGPDuo2; Kan^R	This study
P.putida PP2 (JPUB_014491)	*P. putida* carrying pRGPDuo2-sfGFP$_{tac}$; Kan^R	This study
P.putida PP3 (JPUB_014493)	*P. putida* carrying pRGPDuo2-sfGFP$_{tet}$; Kan^R	This study
P.putida PP4 (JPUB_014495)	*P. putida* carrying pRGPDuo2-RFP$_{tac}$; Kan^R	This study
P.putida PP5 (JPUB_014497)	*P. putida* carrying pRGPDuo2-RFP$_{tet}$; Kan^R	This study
P.putida PP6 (JPUB_014499)	*P. putida* carrying pRGPDuo2-RFP$_{tac}$ + sfGFP$_{tet}$; Kan^R	This study
Plasmids		
pRG_Duet2	dual-inducible *E. coli/C. glutamicum* shuttle vector (P_{tac}, $lacI^Q$, $OriV_{C.\ glut}$ (pBL1), $OriV_{E.\ coli}$ (colE1), $P_{tetR/tetA}$, *tetR*); Kan^R	Gauttam et al., 2019a
pFLP3	Carrying yeast Flp recombinase and *sacB* counterselection; $Carb^R$	Tan et al., 2018
pGEM00003	Carrying the gene coding for sfGFP	JBEI registry (unpublished)
pBADTrfp (JPUB_001229)	pBADrfp derivative, P_{BAD}_T7 stem-loop_rfp	Bi et al., 2013
pJET1.2/blunt	Linearized cloning vector for use in *E. coli*; Amp^R	CloneJET PCR Coning Kit (Thermo Scientific)
pJET-RO1600	pJET1.2/blunt carrying gene for RO1600 ($OriV_{P.\ putida}$); Amp^R	This study
pJET-sfGFP	pJET1.2/blunt carrying gene for super folder green fluorescent protein (sfGFP); Amp^R	This study
pJET-RFP$_{tac}$	pJET1.2/blunt carrying gene for red fluorescent protein (RFP), P_{tac}; Amp^R	This study
pJET-RFP$_{tet}$	pJET1.2/blunt vector carrying gene for red fluorescent protein (RFP), $P_{tetR/tetA}$; Amp^R	This study
pRGPDuo2 (JPUB_014490)	dual-inducible *E. coli/P. putida* shuttle vector (P_{tac}, $lacI^Q$, $OriV_{P.\ putida}$ (pRO1600), $OriV_{E.\ coli}$ (colE1), $P_{tetR/tetA}$, *tetR*); Kan^R	This study
pRGPDuo2-sfGFP$_{tac}$ (JPUB_014492)	pRGPDuo2 carrying the gene for super folder green fluorescent protein (sfGFP) under the control of P_{tac}; Kan^R	This study
pRGPDuo2-sfGFP$_{tet}$ (JPUB_014494)	pRGPDuo2 carrying the gene for super folder green fluorescent protein (sfGFP) under the control of $P_{tetR/tetA}$; Kan^R	This study
pRGPDuo2-RFP$_{tac}$ (JPUB_014496)	pRGPDuo2 carrying the gene for red fluorescent protein (RFP) under the control of P_{tac}; Kan^R	This study
pRGPDuo2-RFP$_{tet}$ (JPUB_014498)	pRGPDuo2 carrying the gene for red fluorescent protein (RFP) under the control of $P_{tetR/tetA}$; Kan^R	This study
pRGPDuo2-RFP$_{tac}$ + sfGFP$_{tet}$ (JPUB_014500)	pRGPDuo2 carrying the gene for red fluorescent protein (RFP) under the control of P_{tac} and the gene for super folder green fluorescent protein (sfGFP) under the control of $P_{tetR/tetA}$; Kan^R	This study

[a] Strain name in bracket corresponds to the part ID assigned to each strain for JBEI public registry.

Table 2
Oligonucleotides used in this study.

Oligo/synthetic fragment	Sequence (5′ → 3′)
pRO1600-fwd	ttaagat**caattg**actgagagtgcaccatacctctc
pRO1600-rev	atcgggtaccat**cgtacg**cagttgcgcagcctgaaag
duosfGFP-fwd	ctgcagag**ctgcag**gaattc**agatct**gattaaagaggagaaattaagcatgagcaaagg
duosfGFP-rev	gctagc**gctagc**tagctttt**ggatcc**ttatttgtagagctcatcca
duoRFPtac-fwd	aacgc**ctgcag**gcttttaagaaggagatatacatatggcgagtagcg
duoRFPtac-rev	gga**ggatcc**ttactcgagtttggatcct
duoRFPtet-fwd	gaattcgaattcacg**agatct**aggcttttaagaaggagatatacatatggcgag
duoRFPtet-rev	gctagcacg**gctagc**cttaagcaccggtggagtgac

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 (OD_{600}). All the represented values were mean from at least three independent measurements.

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_{tac} 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_{tac} or by aTc-inducible $P_{tetR/tetA}$ promoters, kanamycin resistance gene (Kan^R), transcriptional terminators and the *E. coli* origin of replication *colE1*. To adapt the *C. glutamicum* expression vector pRG_Duet2 for use in *P. putida*, the *Corynebacterium* origin of replication ($oriV_{C.\ glut}$) was exchanged with the *Pseudomonas* origin of replication ($Ori_{P.\ putida}$). To achieve this, the DNA fragment containing *P. putida*

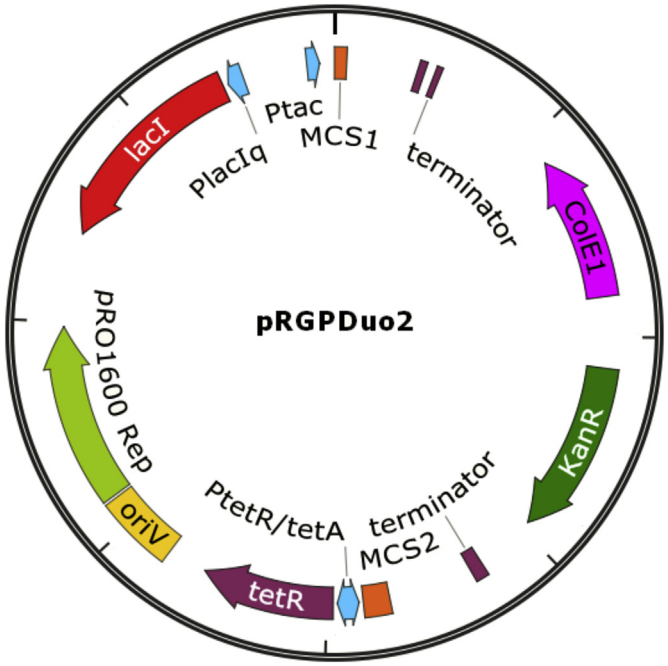

Fig. 1. Plasmid map of shuttle expression vector pRGPDuo2. The dual-inducible duet vector harbors origins of replication for stable maintenance in *E. coli* (*colE1*) and *P.putida* (pRO1600), two multiple cloning sites (MCS1 and MCS2) with sequence for unique restriction enzymes, antibiotic resistance marker (kanamycin), transcriptional terminators, and two independently inducible repression systems, *lacI* and *tetR*, controlling the gene expression through MCS1 and MCS2, respectively. MCS1 contains the recognition sequences for the restriction enzymes *Pst*I, *Sbf*I, *Sal*I, *Bam*HI, *Acc*65I, *Kpn*I, *Eco*53kI, *Sac*I, and *Bsa*I. MCS2 contains the recognition sequence for *Bgl*II, *Bst*Z17I, *Bmt*I, *Nhe*I, *Avr*II, *Swa*I, *Bsr*GI, *Bst*BI, and *Spe*I. Abbreviations are as follows: Kan^R: kanamycin resistance; *tetR*: tetracycline repressor; $P_{tetR/tetA}$: overlapping bacterial promoters of *tetR* and *tetA* (aTc-inducible); P_{tac}: tac promoter (IPTG-inducible); $lacI^q$: repressor $lacI^q$; P_{lacIq}: promoter P_{lacIq}; terminator: transcriptional terminator.

origin of replication (pRO1600) was amplified using plasmid pFLP3 as a template and pRO1600-fwd/rev as primers. The amplified fragment was first cloned in subcloning vector pJET1.2/blunt to construct subcloning vector pJET-RO1600, where the amplified product was sequence-verified. The vector pRGPDuo2 was constructed by ligating *Mfe*I/*Bsi*WI-digested pRGDuet2 with the *Mfe*I/*Bsi*WI-digested PCR product from pJET-RO1600. The resulting plasmid pRGPDuo2 contains 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_{tac} (IPTG-inducible) and $P_{tetR/tetA}$ (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-sfGFP$_{tet}$), PP4 (harboring pRGPDuo2-RFP$_{tac}$), PP5 (harboring pRGPDuo2-RFP$_{tet}$) and PP6 (harboring pRGPDuo2-RFP$_{tac}$ + sfGFP$_{tet}$). 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 sfGFP 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* 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_{tac} and

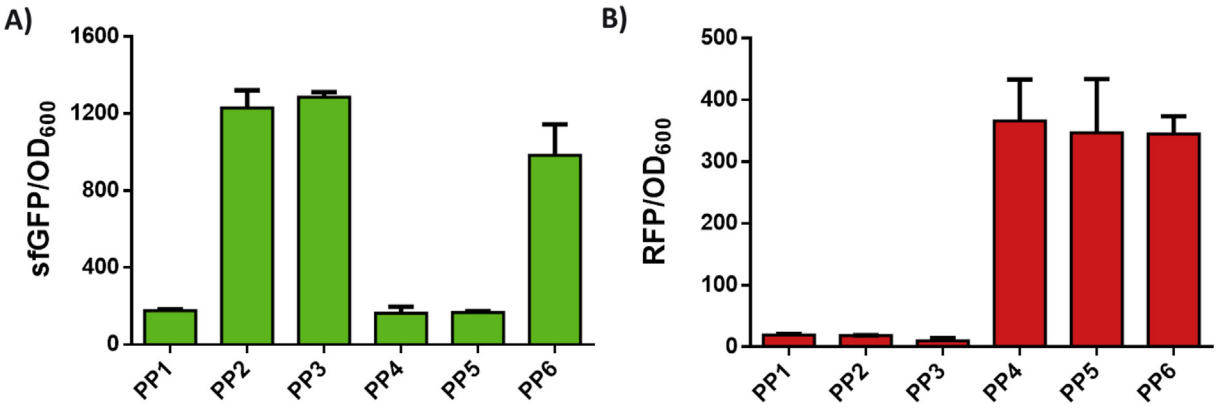

Fig. 2. pRGPDuo2 based expression of GFP and RFP in *P. putida* KT2440. The following recombinant *P. putida* strains were investigated for GFP (A) and RFP (B) fluorescence: PP1 (KT2440 carrying pRGPDuo2), PP2 (KT2440 carrying pRGPDuo2-sfGFP$_{tac}$), PP3 (KT2440 carrying pRGPDuo2-sfGFP$_{tet}$), PP4 (KT2440 carrying pRGPDuo2-RFP$_{tac}$), PP5 (KT2440 carrying pRGPDuo2-RFP$_{tet}$) and PP6 (KT2440 carrying pRGPDuo2-RFP$_{tac}$ + sfGFP$_{tet}$). Data represent mean values of triplicate assays from at least three individual cultivations, and error bars represent standard deviations.

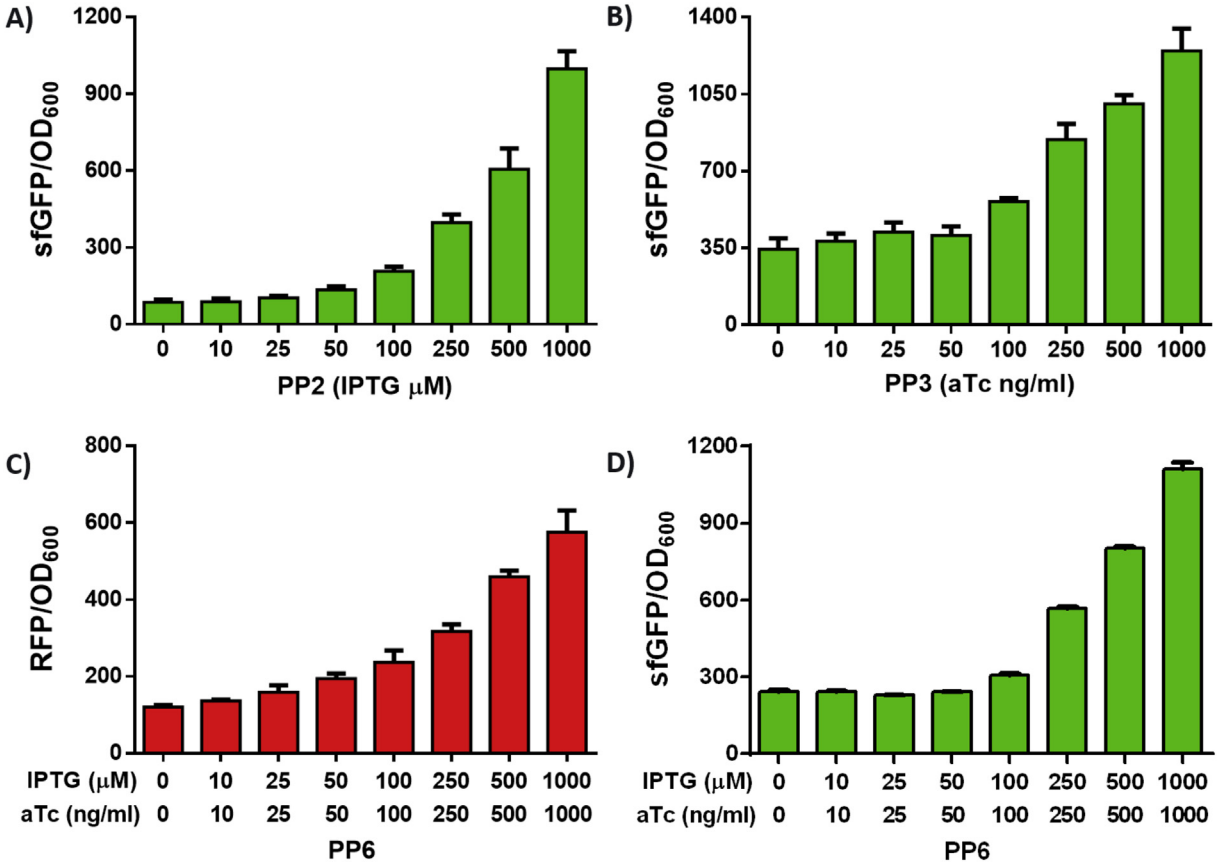

Fig. 3. Expression of fluorescent proteins (sfGFP and RFP) at different concentrations of inducer(s). *P. putida* strains PP2 (KT2440 carrying pRGPDuo2-sfGFP$_{tac}$) and PP3 (KT2440 carrying pRGPDuo2-sfGFP$_{tet}$) and PP6 (KT2440 carrying pRGPDuo2-RFP$_{tac}$ + sfGFP$_{tet}$) were cultivated in M9 minimal medium in presence of different concentrations of inducers, (A) IPTG (0 to 1000 μM and aTc 1000 ng/ml); (B) aTc (0 to 1000 ng/ml and IPTG 1000 μM), (C and D) IPTG (0 to 1000 μM) and aTc (0 to 1000 ng/ml), as indicated. Data represent mean values of triplicate assays from at least three individual cultivations, and error bars represent standard deviations.

IPTG for $P_{tetR/tetA}$) does not influence genes from non-cognate promoter. The fluorescence intensities were determined for *P. putida* PP6, 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.

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Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

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References

Batianis, C., Kozaeva, E., Damalas, S.G., Martín-Pascual, M., Volke, D.C., Nikel, P.I., Martins 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., Singer, 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., Nikel, P.I., 2019. Chasing bacterial chassis for metabolic engineering: a perspective review from classical to non-traditional microorganisms. Microb. Biotechnol. 12 (1), 98–124.

Domínguez-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.

Hallsworth, 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., Zorn, M., Kussmaul, 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., Dohnt, K., Dos Santos, V.M., Wittmann, C., 2012. Industrial biotechnology of *Pseudomonas putida* and related species. Appl. Microbial. Biotechnol. 93 (6), 2279–2290.

Radmacher, E., Vaitsikova, 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., Nikel, 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., Wierckx, N., Blank, L., 2014. Non-pathogenic *Pseudomonas* as Platform for Industrial Biocatalysis. Industrial Biocatalysis, Pan Stanford, Singapore, pp. 323–372.

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

Volke, D.C., Turlin, J., Mol, V., Nikel, 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., Nikel, P.I., 2020. Accelerated genome engineering of *Pseudomonas putida* by I-SceI—mediated recombination and CRISPR-Cas9 counterselection. 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.