CRISPR interference-mediated gene regulation in Pseudomonas putida KT2440

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Introduction

Pseudomonas putida KT2440 is a Gram-negative soil bacterium that is classified as a generally regarded as safe (GRAS)-certified strain (Loeschcke and Thies, 2015; Nikel and de Lorenzo, 2018). This strain has attracted substantial attention for industrial applications owing to its fast growth, native metabolic versatility and high robustness in harsh environmental conditions, which are particularly suitable traits for bio-remediation of environmental contamination and metabolic engineering (Nikel et al., 2016; Nikel and de Lorenzo, 2018). In addition, genome-scale metabolic models of P. putida KT2440 were developed to better understand the versatile metabolism of the strain at the systems level, which were applied to reprogram the phenotype for biotechnological implementations (Nogales et al., 2008; Sohn et al., 2010; Hintermayer and Weuster-Botz, 2017). Indeed, well-established molecular biological tools, including systems for gene expression and genome editing tools for diverse organisms, including bacteria (Cho et al., 2018), mammalian cells (Komor et al., 2019), mammalian cells (Komor et al., 2019).
CRISPRi-mediated gene regulation in Pseudomonas

CRISPRi-based gene regulation has also been reported for some *Pseudomonas* spp. Tan et al. (2018) developed a tunable CRISPRi system for realizing dynamic gene repression in *Pseudomonas aeruginosa* using the type II dCas9 homologue of *Streptococcus pneumoniae*. Specifically, they constructed two plasmids to express the dCas9 gene and sgRNA from a Ptet and Plac promoter respectively. However, the developed two-plasmid CRISPRi system showed leaky expression of the dCas9 gene in *P. aeruginosa*, which resulted in repression of the target gene in the absence of the inducer. Moreover, the type I CRISPR-dCas system was adopted as a transcriptional repressor in *P. aeruginosa* PA14, which requires deletion of the Cas3 gene from *P. aeruginosa* or expression of anti-CRISPR proteins from a prophage (Bondy-Denomy et al., 2015). More recently, a CRISPRi technique was developed using dCas9 of *Streptococcus pyogenes* (SpdCas9), which demonstrated functionality using enhanced green fluorescent protein (eGFP) in *P. putida* KT2440 (Sun et al., 2018). However, this study lacked CRISPRi applications to metabolic engineering or synthetic biology of *P. putida* KT2440.

Here, we present an L-rhamnose-inducible single-plasmid CRISPRi system for achieving the simple and efficient regulation of target genes in *P. putida* KT2440. This regulatable CRISPRi system was able to control the expression of exogenous and endogenous genes in *P. putida* KT2440. We further provide examples of its application for metabolic flux alteration to enhance the production of mevalonate (MVA), a key intermediate metabolite for the biosynthesis of a myriad of terpenoids. *Pseudomonas putida* KT2440 shows a prolonged lag phase on glycerol as the sole carbon source; thus, strategies to reduce this lag phase could help to overcome this limitation of the strain in using a cost-effective renewable resource for the microbial production of terpenoids. Using a key regulator involved in glycerol metabolism of *P. putida* KT2440 as a target gene for the single-plasmid CRISPRi system proved to be a robust platform for modulation of endogenous gene expression. This system can therefore accelerate the metabolic engineering of *P. putida* KT2440 for the development of microbial cell factories that can produce industrially valuable products in the future.

**Protocol**

**Overview of the CRISPRi system**

To develop a CRISPRi system in *P. putida* KT2440, we first created a single pSECRi plasmid that expresses catalytically inactive SpdCas9 and a target-specific sgRNA (Fig. 1A). By designing a 20 bp spacer sequence to bind the target sequence (N20 spacer sequence, Fig. 1B), the SpdCas9-sgRNA complex can be recruited to the gene of interest, leading to transcriptional repression. For expression of the SpdCas9 protein and sgRNA, the low copy number pSEVA221 plasmid (Kues and Stahl, 1989) was used as the backbone plasmid for generating pSECRi, which minimized metabolic burden and allowed other antibiotic-resistance genes and replication origins to be swapped for use in applications with other bacterial hosts (Silva-Rocha et al., 2013). To enable tunable control of gene repression in *P. putida* KT2440, the SpdCas9 gene was first placed under control of the L-rhamnose-inducible promoter (P_{rhaBAD}) including RhaR and RhaS regulators, whereas sgRNA expression was driven by a constitutive BBA_J23119 promoter (http://parts.igem.org/Part:BBA_J23119, hereafter named as J23119) on a pSEVA221-derived plasmid. P_{rhaBAD} is a tightly regulated promoter among various inducible promoters including isopropyl-β-D-1-thiogalactopyranoside (IPTG)-inducible (P_{lacUV5}, P_{TT}) and XylS/P_{m} promoters that have been widely used in *P. putida* KT2440 for the expression of heterologous genes in metabolic engineering efforts (Calero et al., 2016). In *E. coli*, the RhaS and RhaR activators of the P_{rhaBAD} promoter in the CRISPRi plasmid can be removed to reduce the plasmid size without changing functionality (Wegerer et al., 2008). However, RhaS and RhaR activators are essential to the functionality of the P_{rhaBAD} promoter in *P. putida* KT2440 (Jeske and Altenbuchner, 2010). Therefore, we chose the L-rhamnose-inducible and J23119 promoters as an orthogonal control for transcription of the SpdCas9 gene and sgRNA, respectively, in the present CRISPRi system.

**General workflow for CRISPRi**

To implement CRISPRi for repression of target genes, sgRNA design, cloning and expression are carried out. The general CRISPRi steps for *P. putida* KT2440 using the pSECRi harbouring the SpdCas9 are summarized as...
SGRNA design for CRISPRi

For the application of CRISPRi, the first step is designing the spacer sequence of sgRNA to regulate the gene of interest. If information regarding the promoter location is available, it is possible to design the sgRNA-binding site to prevent transcription initiation, but in general, it is easier to select the sgRNA-binding site in the coding sequence (CDS) of the target gene to block transcriptional elongation. For SpdCas9, the 3' end of the target region should contain a PAM sequence (5'-NGG-3') and a 20 bp spacer is adequate for efficient repression of the target gene expression (Qi et al., 2013). For CRISPRi, a critical point is that the sgRNA should bind to the non-template DNA strand proximal to the ATG translational start codon of the target gene for high repression efficiency (Qi et al., 2013; Kim et al., 2017). Although off-target effects are less concerning for CRISPRi, a genome-wide search for matching sequences with the PAM-proximal 12 bp 'seed' region including 5'-NGG-3' PAM might be helpful for improving the specific repression of target genes (Bikard et al., 2013). For this purpose, we used ARTEMIS Software to analyse potential off-target sites against the P. putida KT2440 genome sequence (NCBI number: NC_002947) by searching genes that contain the 15 bp sequence (i.e. 12 bp seed sequence).
sequence of the sgRNA and 3 bp of the 5′-NGG-3′ PAM sequence).

Bacterial strains, media and culture conditions

_Escherichia coli_ DH5α was used for cloning experiments and gene expression analysis at the single-cell level of the P_{rhaBAD} promoter. _Pseudomonas putida_ KT2440 was used for all CRISPRi experiments. Bacteria were cultured in lysogeny broth (LB, 10 g l\(^{-1}\) tryptone, 5 g l\(^{-1}\) yeast extract and 10 g l\(^{-1}\) NaCl) at 30°C. For _gfpR_ gene repression, _P. putida_ KT2440 was grown on M9 minimal medium (6.78 g l\(^{-1}\) Na₂HPO₄, 3 g l\(^{-1}\) KH₂PO₄, 0.5 g l\(^{-1}\) NaCl, 1 g l\(^{-1}\) NH₄Cl, 0.241 g l\(^{-1}\) MgSO₄ and 2.5 ml l\(^{-1}\) A9 solution) (Abril et al., 1989) with 4 g l\(^{-1}\) glycerol as the carbon source and appropriate antibiotics at 30°C. For CRISPRi-based gene repression, 1 mM L-rhamnose was added to the culture medium. IPTG was added to the culture medium at 0.1 mM unless otherwise noted. All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA).

Construction of reporter and MVA plasmids

The plasmids and primers used in this study are listed in Table 1 and Table 2 respectively. All restriction enzymes, T4 DNA ligase, T4 polynucleotide kinase and KOD-Plus-Gibson Assembly Master Mix were purchased from New England Biolabs (Ipswich, MA, USA). KOD-Plus-Neo polymerase (Toyobo, Osaka, Japan) was used for high-fidelity polymerase chain reaction (PCR), and colony PCR was performed using AccuPower® PCR Pre-Mix (Bioneer, Daejeon, Korea). The plasmid miniprep and DNA purification kits were obtained from Promega (Madison, WI, USA). Oligonucleotides were synthesized by Macrogen (Seoul, Korea). All experiments were conducted according to the manufacturer’s instructions.

**pSR-GFP plasmid.** To construct the L-rhamnose-inducible GFP expression plasmid, the _sfgfp_ gene was amplified from the pK7-sfGFP plasmid using the GFP-IF and GFP-IR primers (Lee et al., 2016). The vector backbone was amplified from the pSECRI plasmid using the GFP-VF and GFP-VR primers (Kim et al., 2016; Lee et al., 2016). The two fragments were assembled by the Gibson assembly method, resulting in a _pSR-GFP_ plasmid.

**pST-GFP plasmid.** To generate the IPTG-inducible GFP expression plasmid, _pST-GFP_, we first created a _pT-GFP_ plasmid as follows. The vector backbone was amplified from the pSNA-MrBBS-IspA plasmid using the primers TG-VF and TG-VR (Han et al., 2016; Kim et al., 2016), and the _sfgfp_ gene was amplified from the _pK7-sfGFP_ plasmid using the primers TG-IF and TG-IR (Lee et al., 2016). The two fragments were assembled by the Gibson assembly method, resulting in the _pT-GFP_ plasmid. Using this plasmid as a template, the _gfp_ cassette, including the _lacR_, _P_{lac} promoter, _gfp_ gene and _rmb_ terminator, was amplified using the primers STG-IF and STG-VR. The amplified fragment was assembled into the _pSEVA631_ plasmid prepared by digestion with EcoRI and HindIII using the Gibson assembly method, which resulted in the _pST-GFP_ plasmid.

**pST-MVA plasmid.** To construct the MVA production plasmid, all MVA pathway genes (mvaK1-mvaD-mvaK2-idi-mvaE-mvaS) were amplified from the pSNA-MrBBS-IspA plasmid using the primers STB-IF and STB-VR (Kim et al., 2016). The vector backbone was amplified from the _pSEVA231_ plasmid using the primers STB-VF and STB-VR. Two fragments were assembled by the Gibson assembly method.
assembly method, resulting in the pST-BISA plasmid. To remove unnecessary genes (mvaK1-mvaD-mvaK2-idi) from the pST-BISA plasmid, we amplified the DNA fragment using the pST-BISA plasmid and primers (MVA-FYI and MVA-R). The amplified linear DNA was gel-purified, 5'-phosphorylated with T4 polynucleotide kinase, and ligated with T4 DNA ligase, which yielded the pST-MVA plasmid.

Construction of the CRISPRi plasmid

To determine the capability of the established CRISPRi system for the regulation of heterologous genes in *P. putida* KT2440, we first selected the *gfp* gene, which is controlled by the IPTG-inducible Ptrc promoter from the multicopy plasmid. Next, we designed a 20-nt sgRNA spacer sequence (5’-CCATCCAGTTCCACCAGAAT-3’) targeting the non-template strand of the *gfp* gene and a 5’-CGG-3’ PAM sequence that was located only 33 bp away from the start codon of the gene (Fig. 3A). Then, we constructed a CRISPRi plasmid targeting the *gfp* gene of the pST-GFP plasmid with Primer 1 (5’-CCATCCAGTTCCACCAGAATGTTTTAGAGCTAGAAATAGC-3’) and Primer 2 (5’-ACTAGTATTATACCTAGGAC-3’) synthesized from a commercial vendor. Using these primers, inverse PCR from the pSECRi plasmid template was performed using high-fidelity KOD-Plus-Neo polymerase under the following thermal cycling conditions: 94°C for 2 min; 35 cycles of 98°C for 10 s, 55°C for 30 s and 68°C for 10 min; and 68°C for 5 min. After PCR amplification, the amplified DNA fragment of 10.6 kb was gel-purified using the Wizard/Genomic II Gel and PCR Clean-Up System and the eluate was treated with DpnI at 37°C for 1 h to remove any trace of the template plasmid. The reaction mixture was further treated with T4 polynucleotide kinase and T4 DNA ligase to phosphorylate and ligate the PCR product respectively. The ligated plasmid was transformed into highly competent *E. coli* DH5α cells, and transformants were selected on LB plates containing 25 μg ml⁻¹ kanamycin. The colonies were used for PCR analysis using the primers CRIout-F and CRIout-R with AccuPower PCR PreMix to identify positive colonies showing the 435 bp amplicon. Plasmids of the positive colonies were then prepared with the Wizard/Genome II SV Minipreps DNA Purification System, and Sanger sequencing was performed using the Seq-R primer to confirm the sgRNA sequence. The pSECRi(GfpR) plasmid was constructed in the same manner using the primers CRI(GfpR)-F and CRI-R.

Electroporation of plasmids into *Pseudomonas putida* KT2440

Preparation of electro-competent cells and transformation of plasmids into *P. putida* KT2440 was performed according to a previous method with some modification.
In order to prepare competent cells, a single colony of *P. putida* KT2440 was inoculated into 3 ml of LB media and grown overnight at 30°C and 200 rpm. A total of 1 ml of cultured cells was transferred to fresh 50 ml of LB media and cultured to OD600 ~0.6. The cells were harvested by centrifugation at 4°C and 5000 × g for 10 min. After washing the cells three times with ice-cold 3 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), they were resuspended in 250 μl of 3 mM HEPES. Electroporation was carried out in a 2 mm cuvette after adding plasmids to a 50 μl aliquot of the electro-competent cells using the Bio-Rad Gene Pulser Xcell™ (Bio-Rad, Hercules, CA, USA) with settings at 2.5 kV and 200 Ω. The transformants were cultured in LB media at 30°C and 200 rpm for 2 h and then spread on LB plates with appropriate antibiotics.

**FACS-based assay**

The cultured cells were diluted with phosphate-buffered saline (PBS) to approximately 5 × 10⁶ cells ml⁻¹. Then, single-cell fluorescence was measured using a FACScalibur™ flow cytometer (BD Biosciences, San Jose, CA, USA) equipped with a blue laser (excitation: 488 nm) and FL1 (emission: 530/30 nm) photomultiplier tube detector. To exclude debris and dead cells, correct cell populations were acquired using an FSC-A/SSC-A gate with 10,000 gated events recorded. BD CELLQUEST™ PRO software (BD Biosciences, San Jose, CA, USA) was used for acquisition flow cytometry, and the acquired data were analysed using the FLOWJO software package (FlowJo, Ashland, OR, USA).

**Examples**

**Evaluation of *P._rhamBAD* promoter function**

We first examined the functionality of the *l*-rhamnose-inducible promoter *P._rhamBAD* that drives *SpdCas9* expression for the controlled regulation of the target gene. For this purpose, we replaced the *SpdCas9* gene on the pSECRi plasmid with *gfp*, which created the reporter plasmid pSR-GFP to analyse the response of the *P._rhamBAD* promoter at the single-cell level (Fig. 2A). The pSR-GFP plasmid was introduced into *P. putida* KT2440 or *E. coli* DH5α, and the transformants were grown on LB medium supplemented with various concentrations of *l*-rhamnose ranging from 0–10 mM. Single-cell fluorescence was measured by flow cytometry after growth for 12 h at 30°C. As expected, the *P._rhamBAD* promoter showed tight regulation, with no leaky expression of GFP observed in the absence of *l*-rhamnose in both *P. putida* KT2440 and *E. coli* DH5α (Fig. 2B). However, the *P. putida* KT2440 cells were mainly divided into two populations: non-fluorescent (37%) or fluorescent (54%) cells in the presence of 0.05 mM *l*-rhamnose (Fig. 2B). In addition, the majority of the *P. putida* KT2440 cells showed maximal green fluorescence in the presence of more than 0.25 mM *l*-rhamnose, whereas the GFP fluorescence of the *E. coli* DH5α cells increased gradually and uniformly with increasing concentrations of *l*-rhamnose (Fig. 2B). Based on these results, we concluded that the *P._rhamBAD* promoter showed an all-or-none induction of the target gene.
phenomenon in P. putida KT2440. In E. coli, it was reported that the inability to degrade l-rhamnose leads to the disappearance of the dose-dependent response for P_{rhaBAD} promoter-controlled GFP expression (Hjelm et al., 2017). Similarly, the response of the P_{rhaBAD} promoter was not homogenous on intermediate l-rhamnose concentrations in P. putida KT2440 because l-rhamnose might not be consumed after being imported by non-specific sugar transporters (Jeske and Altenbuchner, 2010; Calero et al., 2016). Thus, the P_{rhaBAD} promoter is considered to be optimal for the on/off regulation of target genes, but not suitable for homogenous and tunable gene repression by adjusting the amount of SpdCas9 through l-rhamnose concentrations, as observed for E. coli (Lee et al., 2016). However, design of the sgRNA-binding target region far from the ATG start codon or mismatches in the 5' region of the spacer sequence can be employed to achieve CRISPRi-based controllable repression (Bikard et al., 2013; Qi et al., 2013).

CRISPRi-mediated repression of a heterologous gene on a multicopy plasmid

To determine the capability of the established CRISPRi system for regulation of heterologous genes encoded by a multicopy plasmid in P. putida KT2440, we constructed the GFP reporter plasmid (pST-GFP) that was derived from the pBBR1-based pSEVA plasmid. We chose the pBBR1 replicon because it is a broad-host-range plasmid and has been widely used for the production of various biochemicals in P. putida (Wang et al., 2011; Nikel and de Lorenzo, 2014; Kuepper et al., 2015; Yu et al., 2016). Then, the pST-GFP and pSECRi(GFP) plasmids (Fig. 3A) were co-transformed into P. putida KT2440, and the transformants were selected on LB plates containing both 50 µg ml⁻¹ kanamycin and 20 µg ml⁻¹ gentamicin at 30°C. Three single colonies were then individually inoculated into 3 ml of LB medium containing 50 µg ml⁻¹ kanamycin, 20 µg ml⁻¹ gentamicin and 1 mM l-rhamnose (for SpdCas9 induction), and incubated for 18 h at 30°C. Finally, 2 µL of the culture broth was inoculated into 200 µL of LB medium containing 50 µg ml⁻¹ kanamycin, 20 µg ml⁻¹ gentamicin, 1 mM l-rhamnose and 0.1 mM IPTG (for GFP induction). Cell growth and GFP fluorescence were monitored simultaneously using an Infinite 200 PRO reader for 23 h at 30°C. In the absence of IPTG, GFP fluorescence slightly increased after 10 h of growth due to the leaky expression of GFP by the P_{tet} promoter. This GFP fluorescence was decreased by up to 1.6-fold between the l-rhamnose-induced and uninduced CRISPRi (Fig. 3B). In the presence of 0.1 mM IPTG, the GFP fluorescence decreased by 11-fold, nearly reaching the basal level of GFP expression (Fig. 3C).

CRISPRi-mediated repression of an endogenous gene

The GlpR is a transcriptional regulator that represses the expression of glycerol kinase (GlpK) and glycerol 3-phosphate dehydrogenase (GlpD) that are responsible for the utilization of glycerol as a carbon source in P. putida KT2440 (Nikel et al., 2015). For this reason, P. putida KT2440 shows a prolonged lag phase on glycerol as the sole carbon source unless the glpR gene is deleted. To rewire the regulation of glycerol metabolism by the GlpR regulator in P. putida KT2440, a 20-nt spacer sequence targeting glpR (5'-GGCGGTCTCTTTG GGGCTGC-3') was designed and the pSECRi(GlpR) plasmid was constructed as described above using Primer 3 (5'-GGCGGTCTCTTTG GGGCTGCCTTTTGAG CTAGAAATAGC-3') and Primer 4 (5'-ACTAGTAGTTATA CCTAGGAC-3') (Fig. 4A). The constructed pSECRi (GlpR) plasmid was transformed into P. putida KT2440, and the transformants were selected on LB plates containing 50 µg ml⁻¹ kanamycin at 30°C. Three single colonies were separately inoculated into 3 ml of LB medium containing 50 µg ml⁻¹ kanamycin and 1 mM l-rhamnose, and incubated for 18 h at 30°C. The cultured cells were washed twice with M9 minimal medium without carbon source by centrifugation and resuspended. Two microlitres of the resuspended cells were inoculated into 200 µL of M9 minimal medium containing 4 g l⁻¹ glycerol, 50 µg ml⁻¹ kanamycin and 1 mM l-rhamnose, and cell growth was monitored by an Infinite 200 PRO microplate reader for 40 h at 30°C. Similar to previous reports (Escapa et al., 2012; Nikel et al., 2015), a prolonged lag phase (19 h) was observed in P. putida KT2440 harbouring the pSEVA221 plasmid (uninduced CRISPRi) on glycerol-M9 minimal medium. However, the lag phase was significantly reduced from 19 h to 9 h and cell growth was accelerated when the glpR gene was repressed by CRISPRi (induced CRISPRi, Fig. 4B).

Enhanced production of MVA under CRISPRi repression of glpR

Inspired by the success of glpR repression with our system, we next attempted to produce MVA, a precursor for sustainable biopolymers (Xiong et al., 2014) and terpenoids (Liao et al., 2016), from glycerol in P. putida KT2440. To this end, we constructed an MVA production plasmid (pST-MVA), which encodes mvaE and mvaS to convert acetyl-coA into MVA (Fig. 5A). We also generated the pSECRi(GlpR)-Gen plasmid containing a gentamicin-resistance gene instead of a kanamycin-resistance gene. Both the pST-MVA and pSECRi(GlpR)-Gen plasmids were co-transformed into P. putida KT2440, and the transformants were selected on LB plates containing 50 µg ml⁻¹ kanamycin and 20 µg ml⁻¹ kanamycin and 20
gentamicin at 30°C. Three single colonies were separately inoculated into 3 ml of LB medium containing 50 μg ml⁻¹ kanamycin, 20 μg ml⁻¹ gentamicin and 1 mM L-rhamnose, and the inoculated cells were cultivated for 18 h at 30°C. The cultured cells were washed twice in M9 minimal medium without carbon source, and 250 μL of the resuspended cells were inoculated into 25 ml M9 minimal medium containing 4 g l⁻¹ glycerol, 50 μg ml⁻¹ kanamycin, 20 μg ml⁻¹ gentamicin and 1 mM L-rhamnose in a 250-ml baffled Erlenmeyer flask and cultivated for 72 h at 30°C. The culture broth was centrifuged at 3,000 rpm for 20 min at 4°C, and the supernatant was filtered through a 0.45-μm filter. The filtrate was then used for quantifying the glycerol and MVA.

Fig. 3. The CRISPRi-mediated repression of a heterologous gene in Pseudomonas putida KT2440. (A) Schematic representation of CRISPRi targeting the green fluorescent protein (GFP) of pST-GFP plasmid. The pST-GFP plasmid expresses GFP under the control of IPTG-inducible Pinc promoter. A 20 bp sgRNA spacer sequence targeting the gfp gene of pST-GFP was designed to bind the non-template DNA strand 33 bp away from the ATG translational start codon of the gfp gene. (B, C) CRISPRi-mediated repression of plasmid-borne gfp gene in P. putida KT2440. The pST-GFP and pSECri(GFP) plasmids were co-transformed into P. putida KT2440, and the transformants were grown on LB medium containing 1 mM L-rhamnose in the presence (0.1 mM) or absence of IPTG. Cell growth and GFP fluorescence were monitored simultaneously using an Infinite 200 PRO reader for 23 h at 30°C. Each graph represents the mean value of the corresponding optical density at 600 nm (OD600) or green fluorescence ± standard deviation of duplicate measurements from at least three independent experiments.
Fig. 4. The CRISPRi-mediated repression of an endogenous gene in *Pseudomonas putida* KT2440. (A) Schematic representation of CRISPRi targeting the endogenous GlpR regulator in *P. putida* KT2440. The endogenous GlpR regulator represses *glpFKRD* gene cluster involved in glycerol catabolism of *P. putida* KT2440. (B) CRISPRi-mediated repression of endogenous *glpR* gene in *P. putida* KT2440. The pSECRi(GlpR) plasmid was transformed into *P. putida* KT2440, and the transformants were cultured on M9 minimal medium containing 4 g l\(^{-1}\) glycerol in the presence 1 mM l-rhamnose. Cell growth was monitored by an Infinite 200 PRO microplate reader for 40 h at 30°C. As a control ((-) CRISPRi), *P. putida* KT2440 harbouring the pSEVA221 plasmid was used. Each graph represents the mean value of the corresponding optical density at 600 nm (OD\(_{600}\)) ± standard deviation of duplicate measurements from at least three independent experiments.

Fig. 5. Application of the CRISPRi system for enhancing MVA production in *Pseudomonas putida* KT2440. (A) Schematic representation of the MVA production pathway and plasmid (pST-MVA). The engineered MVA pathway encoded by pST-MVA plasmid consists of two enzymes: MvaE, a dual function enzyme, acetoacetyl-CoA thiolase and 3-hydroxy-3-methylglutaryl-CoA reductase of *Enterococcus faecalis*; MvaS, 3-hydroxy-3-methylglutaryl-CoA synthase of *E. faecalis*. MVA is produced by the heterologous MVA pathway from glycerol. (B) Enhanced cell growth and MVA production in *P. putida* KT2440. Both pST-MVA and pSECRi(GlpR)-Gen plasmids were co-transformed into *P. putida* KT2440, and the transformants were cultured in M9 minimal medium containing 4 g l\(^{-1}\) glycerol in a 250-ml baffled Erlenmeyer flask for 72 h at 30°C. Cell growth and mevalonate concentration were determined by spectrophotometer and HPLC respectively. As a control ((-) CRISPRi), *P. putida* KT2440 harbouring the pSEVA221 plasmid was used. Each bar represents the mean value of the corresponding OD\(_{600}\) or MVA concentration ± standard deviation of duplicate measurements from at least three independent experiments.
concentrations by high-performance liquid chromatography (HPLC; Shimadzu, Kyoto, Japan) equipped with a refractive index detector at 454 nm using an Aminex HPX-87H column (1300 mm × 7.8 mm, Bio-Rad, Hercules, CA, USA). The mobile phase was sulphuric acid (0.4 mM) at a flow rate of 0.3 ml min⁻¹ at 50°C. Cell growth was determined by spectrophotometry according to the optical density at 600 nm (OD₆₀₀). Control P. putida KT2440 harbouring the pSEVA221 plasmid reached an OD₆₀₀ value of 1.14, and the MVA titre was 72 mg l⁻¹ on glycerol-M9 minimal medium (Fig. 5B). However, CRISPRi-mediated GlpR repression enhanced the cell growth by 1.9-fold (OD₆₀₀, 2.22) and the MVA production by 3.3-fold (237 mg l⁻¹). In addition, glycerol consumption increased from 0.96 g l⁻¹ to 3.14 g l⁻¹.

Discussion

In E. coli, the PₙBAD promoter is capable of homogenous and L-rhamnose-dependent control of the transcription of heterologous genes and shows undetectable background expression in the absence of L-rhamnose (Giacalone et al., 2006; Wegerer et al., 2008). Although the PₙBAD promoter was previously used for gene expression in P. putida KT2440 (Jeske and Altenbuchner, 2010), there was no report on whether or not the PₙBAD promoter is subject to a dose-dependent homogenous expression or all-or-none induction phenotype under various concentrations of L-rhamnose in P. putida KT2440. We found an all-or-none induction mode of the promoter in P. putida KT2440 in contrast to its effects in E. coli, indicating that it is not suitable for the tunable regulation of a target gene by adjusting L-rhamnose concentrations.

In a previous report, basal expression of dCas9 due to leakiness from the inducible promoters (Pₜet, Pₜac, Pₜara) caused up to 50% repression in Pseudomonas spp. in the absence of inducers, which limited the basal repression of target genes (Tan et al., 2018). In this study, we employed the L-rhamnose-inducible promoter to control SpdCas9 expression, which showed no leaky expression of SpdCas9 gene (Fig. 2B). Therefore, our single-plasmid CRISPRi system may be more effective and controllable to repress target genes without CRISPRi basal repression (Fig. 3C).

Glycerol has been used as a cost-effective renewable resource for the production of biofuels and biochemicals, including terpenoids, because it is produced as a major by-product of the biodiesel industry. However, the application of P. putida KT2440 for glycerol utilization has been limited due to the endogenous GlpR regulator that represses metabolic enzymes involved in glycerol catabolism (Nikel et al., 2015). Therefore, we chose the glpR regulator gene to be repressed by our newly developed CRISPRi system. In a previous report, P. putida KT2440 grown on glycerol showed a bistable growth phenotype (non-growing and growing population), which resulted in an unexpectedly long lag phase on glycerol. GlpR knockout eliminated this bistable growth phenotype, leading to unimodal behaviour (i.e. single growing population) and reduced the lag time significantly (Nikel et al., 2015). In the current study, CRISPRi-mediated glpR repression also reduced the lag time on glycerol remarkably, indicating that repression of an endogenous regulator gene by CRISPRi reduced phenotypic cell-to-cell variations, allowing P. putida KT2440 to better utilize glycerol as a carbon source. Compared with the conventional methods for gene knockout, the CRISPRi system has advantages as follows: (i) it is simple and easy to repress the glpR gene because it requires only coexpression of a SpdCas9 protein and an sgRNA; (ii) it is able to repress multiple genes simultaneously including the glpR gene, even genes that are essential for cell growth; (iii) knockdown effects of the glpR gene in various P. putida strains can be simultaneously examined by simply introducing the single CRISPRi plasmid; and (iv) a metabolic flux towards glycerol metabolism is temporally controlled by CRISPRi-based glpR repression. Thus, this system could overcome the hurdle of using P. putida KT2440 as microbial cell factories to produce valuable products from glycerol.

Taken together, our single-plasmid-based CRISPRi system developed for P. putida KT2440 demonstrates simplicity and efficiency for regulation of exogenous and endogenous genes. Using this system, enhanced MVA production was achieved by rewiring glycerol metabolism through CRISPRi-mediated repression of the P. putida KT2440 glpR gene. Therefore, the CRISPRi system is a robust tool for expanding the metabolic engineering capabilities of P. putida KT2440, which can lead to the development of microbial cell factories.

Acknowledgements

This work was supported by the Bio & Medical Technology Development Program [grant number: 2018M3A 9H3024746] of the National Research Foundation (NRF) funded by the Ministry of Science and ICT of the Republic of Korea. This research was also supported by the KRIIB Research Initiative Program.

Conflict of interest

The authors do not have any conflict of interest.

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