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An expanded CRISPRi toolbox for tunable control of gene expression in *Pseudomonas putida*

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We showed that the system enables tunable, tightly controlled gene repression (up to 90%) of chromosomally expressed genes encoding fluorescent proteins, either individually or simultaneously. In addition, we demonstrate that this method allows for suppressing the expression of the essential genes *pyrF* and *ftsZ*, resulting in significantly low growth rates or morphological changes respectively. This versatile system expands the capabilities of the current CRISPRi toolbox for efficient, targeted and controllable manipulation of gene expression in *P. putida*.

Summary

Owing to its wide metabolic versatility and physiological robustness, together with amenability to genetic manipulations and high resistance to stressful conditions, *Pseudomonas putida* is increasingly becoming the organism of choice for a range of applications in both industrial and environmental applications. However, a range of applied synthetic biology and metabolic engineering approaches are still limited by the lack of specific genetic tools to effectively and efficiently regulate the expression of target genes. Here, we present a single-plasmid CRISPR-interference (CRISPRi) system expressing a nuclease-deficient *cas9* gene under the control of the inducible XylS/P_m expression system, along with the option of adopting constitutively expressed guide RNAs (either sgRNA or crRNA and tracrRNA).

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Introduction

Pseudomonas putida KT2440 is Gram-negative soil bacterium and the microbial cell factory of choice for many applications in biotechnology due to a number of unique qualities. It is endowed with considerable metabolic versatility, a remarkable tolerance to various stress conditions as well as rapid growth with simple nutrient requirements (Nelson *et al.*, 2002; Martins dos Santos *et al.*, 2004; Poblete-Castro *et al.*, 2012, 2020; Nikel *et al.*, 2014, 2016; Belda *et al.*, 2016). Moreover, this bacterium is equipped with a unique glycolysis, the *EDEMP cycle*, resulting in catabolic NAD(P)H overproduction that can be used as reducing power for biocatalysis or to counteract oxidative stress (Nikel *et al.*, 2015). All these traits render this bacterium a robust platform for a range of industrial and environmental applications. In connection to this, the available toolbox for manipulating its genome and metabolism is still under extensive development to further enhance the applicability of *P. putida* as a cell platform (Calero and Nikel, 2019; Leprince *et al.*, 2012; Martínez-García and de Lorenzo, 2019).

In an effort to broaden the existing toolbox, many groups have focused on clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas9)-based methods for knocking-out or knocking-down target genes in *P. putida*. Recently, type II CRISPR/Cas systems have been utilized in combination with the λ-Red system, SSR recombinases or the I-*Sce*I meganuclease for precise gene deletion (Martínez-García and de Lorenzo, 2011; Mougiakos *et al.*, 2017; Sun *et al.*, 2018; Aparicio *et al.*, 2019, 2020; Wirth *et al.*,

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2020). Engineered catalytically inactive variants of the Cas9 protein (dead Cas9, dCas9) have been shown to act as a transcription repressor in *Pseudomonas* strains, including *P. putida* KT2440. Tan *et al.* (2018), for instance, used a two-plasmid CRISPR interference (CRISPRi) system, based on the type II dCas9 homologue of *Streptococcus pasteurianus*. In this example, dCas9 from *S. pasteurianus* could be harnessed for efficient CRISPRi-mediated downregulation of genes, requiring specific protospacer adjacent motif (PAM) sequences (5′-NNGTGA-3′ or 5′-NNGCGA-3′, where N represents any nucleotide). These PAM sequences, however, are significantly less abundant in the genome of *P. putida* KT2440 in comparison with the simpler PAM motif 5'-NGG-3', associated with the most commonly used Cas9 from *Streptococcus pyogenes* (SpCas9). Recently, two alternative SpdCas9-based CRISPRi systems were developed and explored in *Pseudomonas* species (Sun *et al.*, 2018; Kim *et al.*, 2020). Both systems were demonstrated to be functional for repression of genes encoding fluorescent proteins, and Kim *et al.* (2020) also employed CRISPRi for metabolic engineering *via* gene repression by depleting the GlpR regulator to enhance the glycerol-dependent synthesis of mevalonate. Other examples on the development of CRISPRi systems have been reported for *P. aeruginosa* (Peters *et al.*, 2019; Qu *et al.*, 2019) and *P. fluorescens* (Noirot-Gros *et al.*, 2019). While the CRISPRi toolbox for *Pseudomonas* species offers alternatives depending on the intended application (ranging from fundamental studies to simple metabolic engineering manipulations), the techniques applied so far are afflicted by either leaky expression of the components or limited ability to titrate repression levels – thus restricting the applicability of the tool in complex engineering approaches. Moreover, the possibility of performing multiple, simultaneous knockdowns in gene expression with minimal cloning efforts is still largely missing.

Here, we present an expanded CRISPRi toolbox allowing for the tunable regulation of one or multiple target genes in *Pseudomonas* species. Specifically, we have developed a set of modular, composable vectors encoding CRISPRi systems using either (i) non-coding *trans*-activating CRISPR RNA (tracrRNA) and the CRISPR locus needed for CRISPR RNA (crRNA) generation, present in the native type II CRISPR/Cas9 system of *S. pyogenes* (Cong and Zhang, 2015), or (ii) a single gRNA (sgRNA) in short fusion form of tracrRNA and crRNA (Jinek *et al.*, 2012). The immense majority of the already developed tools have utilized sgRNAs due to simplicity of working with a single RNA molecule (Zalatan *et al.*, 2015; Deaner and Alper, 2017; Rousset *et al.*, 2018). In general, the efficiency of using just sgRNA or both tracrRNA and crRNA was observed to be comparable – although the architecture of crRNA allows for a rapid and simple cloning strategy when generating multiplex crRNA arrays. Therefore, in this study we tested and employed both sgRNA- and crRNA-based CRISPRi systems in *P. putida* KT2440, and we describe alternative protocols for efficient downregulation of the expression of single or multiple target genes. Furthermore, we compared the efficiency of three different inducible expression systems to control the expression levels of the *SpdCas9* gene, and showed that the XylS/P_m expression system was able to accurately modulate repression levels by adjusting the amount of inducer (3-methylbenzoate, 3-*m*Bz) – thus resulting in a tunable, titrable CRISPRi system. Additionally, we demonstrate that CRISPRi-mediated downregulation of gene expression is more efficient in a *P. putida* strain lacking the main component of the homologous recombination machinery, RecA. Our study expands the currently available CRISPRi toolbox, enabling to gain insights on transcriptional repression in non-model bacteria, and allowing for depletion of one or several proteins of interest to support rational metabolic engineering of *P. putida*.

Overview of the workflow

The overall gene downregulation procedure begins with the construction of the target-specific vector pCRi (Table 1 and extended explanation below), followed by plasmid transformation in the strain of interest and induction of the system for targeted repression (Fig. 1). The whole procedure typically takes around 6 days. All bacterial strains used in this study are listed in Table S1 in the Supporting Information, and the specific steps of the protocol are detailed below.

Overview and construction of derivatives of the pCRi vector

To expand the CRISPRi toolbox, we constructed a set of vectors that harbour *SpdCas9*, along with either the native gRNAs (crRNA and tracrRNA) or its short fusion form (sgRNA). The *SpdCas9* gene and the corresponding gRNAs were expressed from a single vector using the backbone pSEVA441. This approach enabled to construct composable plasmids, the modules of which can be swapped at the user's will (Silva-Rocha *et al.*, 2013; Martínez-García *et al.*,). Both gRNAs were placed under constitutive expression of either the native Sp promoter (crRNA) or the synthetic, constitutive P_{EM7} promoter (Nikel *et al.*, 2013; $P_{EM7} \rightarrow$ sgRNA), while the transcription of *SpdCas9* was driven by inducible expression systems. The level of constitutive expression brought about by the P_{EM7} promoter was shown to be appropriate for expression of the sgRNAs tested in this work, and other versions of the same promoter

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Table 1. Plasmids used in this work.

Name	Relevant features	Source or reference
pSEVA448	Cloning vector; *oriV* (pRO1600/ColE1); XylS, P_m; Sm^R/Sp^R	Silva-Rocha *et al.* (2013)
pSEVA421-Cas9tr	pSEVA421 derivative bearing the *SpCas9* gene and a tracrRNA module; *oriV* (RK2); Sm^R/Sp^R	Aparicio *et al.* (2018)
pSEVA231-CRISPR	pSEVA231 derivative bearing the CRISPR array; *oriV*(pBBR1); Km^R	Aparicio *et al.* (2018)
pCRISPomyces-1	CRISPR array plasmid, *oriT*, *rep*[pSG5(Ts)], *oriV*(ColE1), GC-rich *SpCas9* gene (*GCSpdCas9*), tracrRNA module; Ap^R	Cobb *et al.* (2015)
pSEVA441	Cloning vector; *oriV*(pRO1600/ColE1); Sm^R/Sp^R	Silva-Rocha *et al.* (2013)
pMCRi	Plasmid for CRISPRi; *oriV*(pRO1600/ColE1), *xylS* (cured of *Bsa*I-sites), $P_m \rightarrow$ *SpdCas9,* $P_{EM7} \rightarrow$ sgRNA; Sm^R/Sp^R	This work
pMCRi_*gfp*	Plasmid for CRISPRi; *oriV*(pRO1600/ColE1), *xylS* (cured of *Bsa*I-sites), $P_m \rightarrow$ *SpdCas9,* $P_{EM7} \rightarrow$ *msf-gfp*-specific sgRNA; Sm^R/Sp^R	This work
pMCRi_*pyrF*	Plasmid for CRISPRi; *oriV*(pRO1600/ColE1), *xylS* (cured of *Bsa*I-sites), $P_m \rightarrow$ *SpdCas9,* $P_{EM7} \rightarrow$ *pyrF*-specific sgRNA; Sm^R/Sp^R	This work
pMCRi_*ftsZ*	Plasmid for CRISPRi; *oriV*(pRO1600/ColE1), *xylS* (cured of *Bsa*I-sites), $P_m \rightarrow$ *SpdCas9,* $P_{EM7} \rightarrow$ *ftsZ*-specific sgRNA; Sm^R/Sp^R	This work
pMCRi_*yfp*	Plasmid for CRISPRi; *oriV*(pRO1600/ColE1), *xylS* (cured of *Bsa*I-sites), $P_m \rightarrow$ *SpdCas9,* $P_{EM7} \rightarrow$ *yfp*-specific sgRNA; Sm^R/Sp^R	This work
pCCRi	Plasmid for CRISPRi; *oriV*(pRO1600/ColE1), *chnR*, $P_{chnB} \rightarrow$ *SpdCas9,* $P_{EM7} \rightarrow$ sgRNA; Sm^R/Sp^R	This work
pCCRi_*gfp*	Plasmid for CRISPRi; *oriV*(pRO1600/ColE1), *chnR*, $P_{chnB} \rightarrow$ *SpdCas9,* $P_{EM7} \rightarrow$ *sgRNA*; $P_{EM7} \rightarrow$ *msf-gfp*-specific sgRNA; Sm^R/Sp^R	This work
pDCRi	Plasmid for CRISPRi; *oriV*(pRO1600/ColE1), *cprK1*, $P_{DB3} \rightarrow$ *SpdCas9,* $P_{EM7} \rightarrow$ sgRNA; Sm^R/Sp^R	This work
pDCRi_*gfp*	Plasmid for CRISPRi; *oriV*(pRO1600/ColE1), *cprK1*, $P_{DB3} \rightarrow$ *SpdCas9,* $P_{EM7} \rightarrow$ sgRNA; $P_{EM7} \rightarrow$ *msf-gfp*-specific sgRNA; Sm^R/Sp^R	This work
pGCRi-R	Plasmid for CRISPRi; *oriV*(pRO1600/ColE1), *xylS* (cured of *Bsa*I-sites), $P_m \rightarrow$ *GCSpdCas9,* crRNA cassette with *eforRed*; Sm^R/Sp^R	This work
pGCRi_*yfp*	Plasmid for CRISPRi; *oriV*(pRO1600/ColE1), *xylS* (cured of *Bsa*I-sites), $P_m \rightarrow$ *GCSpdCas9, yfp*-specific crRNA; Sm^R/Sp^R	This work
pGCRi_*ftsZ*	Plasmid for CRISPRi; *oriV*(pRO1600/ColE1), *xylS* (cured of *Bsa*I-sites), $P_m \rightarrow$ *GCSpdCas9, ftsZ*-specific crRNA; Sm^R/Sp^R	This work
pGCRi_*pyrF*	Plasmid for CRISPRi; *oriV*(pRO1600/ColE1), *xylS* (cured of *Bsa*I-sites), $P_m \rightarrow$ *GCSpdCas9, ftsZ*-specific crRNA; Sm^R/Sp^R	This work
pCRiMs	Plasmid for mCRISPRi; *oriV*(pRO1600/ColE1), *xylS* (cured of *Bsa*I-sites), $P_m \rightarrow$ *SpdCas9,* $P_{EM7} \rightarrow$ *ftsZ*-specific sgRNA, $P_{EM7} \rightarrow$ *yfp*-specific sgRNA, $P_{EM7} \rightarrow$ *mCherry*-specific sgRNA; Sm^R/Sp^R	This work
pCRiMc	Plasmid for mCRISPRi; *oriV*(pRO1600/ColE1), *xylS* (cured of *Bsa*I-sites), $P_m \rightarrow$ *GCSpdCas9*, tracrRNA module, *ftsZ*-specific crRNA, *yfp*-specific crRNA, *mCherry*-specific crRNA; Sm^R/Sp^R	This work

Antibiotic markers and abbreviations: Ap, ampicillin; Km, kanamycin; Gm, gentamicin; Sm, streptomycin; and Sp, spectinomycin; Ts, temperature-sensitive replicon; mCRISPRi, multiplex CRISPR interference.

(displaying different strengths) can be implemented if needed (Zobel *et al*., 2015). In this context, the inducible expression of *SpdCas9* would enable tunable repression levels – allowing, in turn, for the interference of essential genes expression as well as controlling the repression timing. First, we created a set of sgRNA-based vectors by using different modules for inducible expression of *SpdCas9*: XylS/P_m, ChnR/P_{chnB} or CprK1/P_{DB3} (Kemp *et al*., 2013; Silva-Rocha *et al*., 2013; Benedetti *et al*., 2016; Martínez-García *et al*.,), which resulted in vectors pMCRi, pCCRi and pDCRi respectively (Fig. 2A). We have selected these three inducible expression systems as they are known to be active in *P. putida*, and they are titrable (i.e. promoter output varies as a consequence of increasing concentrations of the inducer) to different extents (Martínez-García *et al*.,). The well-characterized XylS/P_m expression system was observed to be the most effective mediating the repression of target genes, and we proceeded further in constructing the crRNA-based vector pGCRi by adopting this system. Vector pGCRi consists of three main functional elements: (i) a GC-rich, dead *SpCas9* version [a codon-optimized *SpdCas9* for *Streptomyces* species, termed *GCSpdCas9* (Cobb *et al*., 2015)] under the control of the XylS/P_m expression system, (ii) the tracrRNA under the control of the native, constitutive Sp promoter and (iii) the leader-crRNA. *Streptococcus* species have a relatively low average genomic GC-content (~ 41.2%), while *Pseudomonas* and *Streptomyces* display high average genomic GC-content (~ 60%). We thus reasoned that the *GCSpdCas9* gene (displaying a GC-content ~ 62%) could be suitable for CRISPRi in *P. putida* as compared to the wild-type version of *SpdCas9* (having a GC-content ~ 45%). Additionally, to enable one-step assembly

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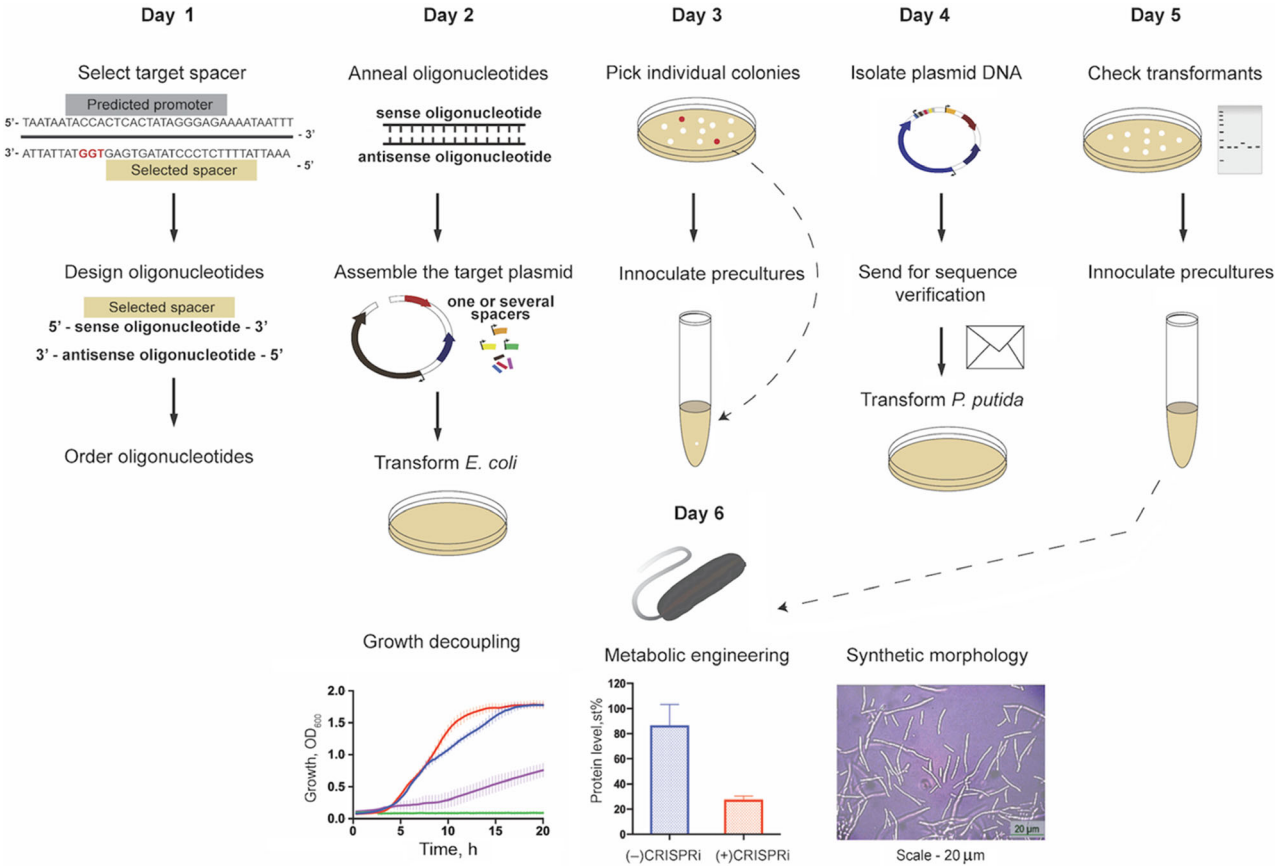

Fig. 1. Overview of the workflow for CRISPRi-mediated gene knock-down in *Pseudomonas putida*. Day 1: Choose a PAM sequence (5′-NGG-3′, where N represents any nucleotide) within the non-template DNA strand sequence of the target gene. For efficient gene repression, we recommend to choose a PAM within the promoter sequence, or, if the promoter is not clearly defined or if it overlaps with a coding sequence, chose the PAM closest to the start *ATG* codon. Design and order two oligonucleotides for cloning of the spacer (upstream the PAM, following the format 5′-*spacer*-NGG-3′) in either sgRNA or crRNA. Day 2: Anneal single-strand DNA oligonucleotides by reverse cooling to form a double-stranded DNA spacer-insert array and clone it into the respective derivative of vector pCRi. Transform a suitable cloning *E. coli* strain and incubate the plates overnight (under streptomycin selection). Day 3: Inoculate three transformants in liquid cultures and grow the cultures overnight. Day 4: Purify three independent plasmids and send them out for sequence verification by DNA sequencing. Transform the target *Pseudomonas* strain with isolated and sequence-verified plasmids, and incubate the plates under streptomycin selection. Day 5: Inoculate a fresh culture in the appropriate medium with verified *Pseudomonas* colonies and the additives needed, and incubate the cultures overnight. Day 6: Perform CRISPRi-mediated gene downregulation in the presence of 1 mM 3-methylbenzoate (or other inducer concentrations as needed) to activate the system

of single or multiple spacers into vector pGCRi *via* Golden Gate cloning and to simplify the selection process, we engineered the crRNA cassette by incorporating an eforRed chromoprotein construct (BBa_K592012; endowed with a constitutive promoter and a ribosome binding site, RBS) flanked by two *Bsa*I recognition sites between the two direct repeats (DRs) (Fig. 2B). When accumulated in cells containing this construct, the eforRed chromoprotein from the coral *Echinopora forskaliana* (Alieva *et al*., 2008) confers a pink or reddish coloration to the colonies. In this way, when ligation of a spacer is successful, the reporter is split and the *E. coli* transformants containing the intended constructs appear white instead of reddish. These operations do not affect the overall efficiency of transformation while they greatly facilitate the screening. The construction of the sgRNA-based vectors was done essentially according to Wirth *et al.* (2020). A detailed description of the pGCRi vector construction, including the list of oligonucleotides used for cloning functional modules into both vectors (Table S2), is given in the Supporting Information.

Protocol for CRISPRi-mediated downregulation of one or multiple targets

Spacer design and selecting a suitable target sequence

The CRISPRi system requires a specific spacer sequence in the gRNA, which determines the binding site of SpdCas9. This sequence has to be selected specifically for each target as a 20-nt spacer for the

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sgRNA or a 30-nt spacer for the crRNA array (Jinek *et al*., 2012; Cong *et al*., 2013). In both cases, it must be immediately followed by a PAM (5'-NGG-3'). DNA strand specificity is relevant for CRISPRi, and targeting the non-template strand is crucial for efficient repression – whereas targeting the template DNA strand of the coding sequence is less effective or not effective at all (Larson *et al*., 2013). It has also been shown that the downregulation effect is the most efficient when dCas9 binds to the promoter sequence (Qi *et al*., 2013; Tan *et al*., 2018). Bacterial promoters can be predicted using freely available *in silico* tools (such as online tool BPROM from Softberry.com). In case that there is no available PAM in the promoter sequence or if the promoter is poorly defined or overlaps with other coding sequences or promoters, the spacer sequence should be chosen on the non-template strand, closest to the beginning of the start *ATG* codon of the target gene. To avoid off-target effects, the spacer sequence should be unique. To ensure that this is the case, a simple BLAST analysis (Ladunga, 2017) against the complete genomic DNA sequence of the target *Pseudomonas* strain (Winsor *et al*., 2016) should reveal no sequence similarities.

Assembly of a target-specific CRISPRi vector in E. coli

The vectors described herein contain two *Bsa*I (a type IIS restriction enzyme targeting the sequence 5′-GGTCTC(N_1)/(N_5)-3′, where N represents any nucleotide) recognition sites that are placed upstream of the sgRNA fusion construct. These recognition targets are placed in either an inverted orientation (to insert a 20-nt spacer) or a forward orientation between the DRs of the crRNA cassette (to insert a 30-nt spacer). Further linearization of the vector by treatment with *Bsa*I allows for the incorporation of a spacer-insert with unique overhangs, resulting in a target-specific vector that expresses *SpCas9*. Below we present two cloning strategies for addition of specific spacers into the corresponding RNAs.

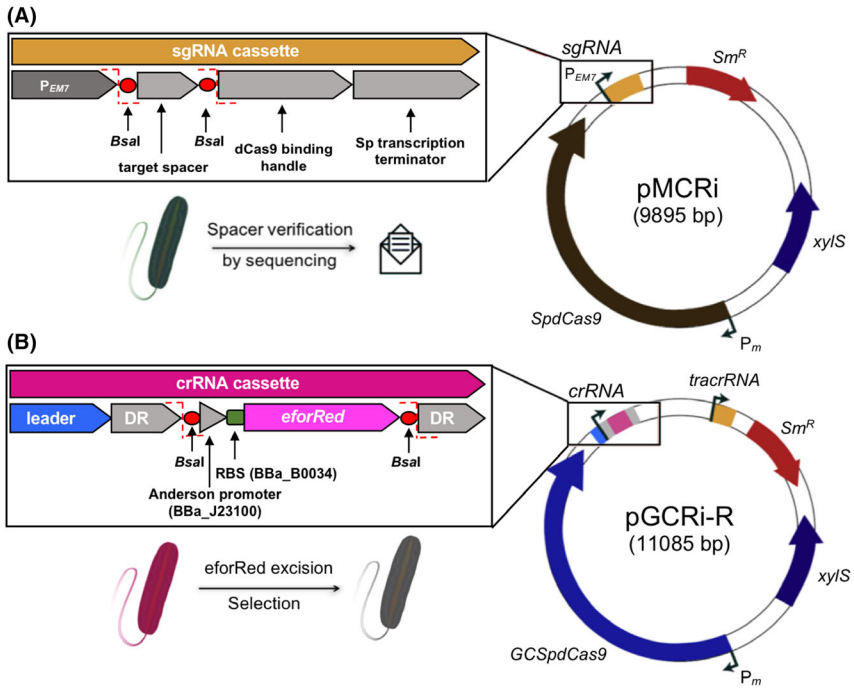

Fig. 2. Overview of key expression vectors constructed for CRISPRi-mediated knock-down of gene expression in *Pseudomonas putida*. A. The sgRNA-based, XylS/P_m-inducible CRISPRi vector pMCRi. Vector pMCRi contains *SpdCas9* under the control of the XylS/P_m expression system and a constitutively expressed sgRNA cassette. The sgRNA cassette is composed by the synthetic, constitutive P_{EM7} promoter followed by the sgRNA chimera, spanning three domains: a 20-nt region for target-specific binding, a 42-nt hairpin for dCas9 binding (dCas9 handle) and a 40-nt transcription terminator (Sp Terminator) derived from *S. pyogenes*. To clone the target spacer, two *Bsa*I recognition sites have been incorporated between the P_{EM7} promoter and the sgRNA cassette. B. The crRNA-based CRISPRi vector pGCRi-R. This vector contains a GC-rich *SpdCas9* gene (the expression of which is placed under control of the XylS/P_m expression system), a constitutively expressed crRNA cassette and the constitutively expressed tracrRNA. The crRNA cassette is formed by an AT-rich sequence (leader) that contains a promoter driving the transcription of the crRNA (Pul *et al*., 2010) and the two direct repeats (DRs) with an intervening gene encoding the eforRed chromoprotein from *Echinopora forskaliana* (expressed from the constitutive BBa_J23100 Anderson promoter and equipped with the BBa_B0034 ribosome binding site). The crRNA cassette is flanked by two *Bsa*I recognition sites to facilitate cloning the target spacer sequence. If the target spacer is successfully assembled by Golden Gate cloning into vector pGCRi, the *eforRed* reporter gene is split (and inactivated), and the resulting *E. coli* transformants will appear white instead of reddish when isolated on solid culture medium plates

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sgRNA design and construction of the pCRi_target vector. Design and order two oligonucleotides that are complementary to each other. The first oligonucleotide contains the 20-nt spacer sequence from non-template strand of target gene and flanked at the 5′-end with 5′-GCGCG-3′. The second oligonucleotide contains a reverse complement 20-nt spacer sequence as mentioned above, with the addition of a 5′-AAAC-3′ motif to its 5′-end and a C nucleotide to its 3'-end.

For example, if the *pyrF* gene (*PP_1815*, encoding orotidine 5′-phosphate decarboxylase, an essential activity for growth of *P. putida* in minimal media) is to be targeted, the resulting sequences are as follows: *oligonucleotide 1 (EK.pyrF-F)*, 5′-**GCG CG**G GAA ATC CAG GGC GAC GAT C-3′; and *oligonucleotide 2 (EK.pyrF-R)*, 5′-**AAA C**GA TCG TCG CCC TGG ATT TCC **C**-3' (Table S2 in the Supporting Information). In these oligonucleotides, the nucleotides in bold represent *Bsa*I-compatible overhangs for efficient ligation of the spacer. To perform a ligation reaction with the linearized pCRi vector, oligonucleotides have to be phosphorylated at the 5′-end, which could be done either by *in situ* phosphorylation using T4 polynucleotide kinase (PNK) or by chemical modification during oligonucleotide synthesis.

Step-by-step procedure (cloning of a construct to target a single gene).

i. Digest vector pCRi with *Bsa*I (or its *Eco*31I isoschizomer; New England Biolabs Inc., Ipswich, MA, USA) according to the manufacturer's recommendations. In order to purify the linearized plasmid after digestion, perform electrophoresis of the digestion mixture followed by gel purification of the isolated fragment. We recommend to purify the amplified pCRi vector fragment (9800 bp) from a gel and to use it as template for further applications.
ii. Dissolve the two spacer oligonucleotides in water at a final concentration of 100 μM. Phosphorylate and anneal the oligonucleotides in a thermocycler. This can be performed in a single 10 μl reaction containing 6 μl of water, 1 μl of each oligonucleotide solution, 1 μl of T4 ligase buffer and 1 μl of T4 PNK (New England Biolabs Inc.). Use the following temperature protocol: 30 min at 37°C, 4 min at 95°C, followed by 70 cycles consisting of 12 s each, starting at 95°C and decreasing the temperature by 1°C in each cycle.
iii. Dilute the annealed and phosphorylated oligonucleotides 1:200 with water, that is to a final concentration of double-stranded DNA (dsDNA) of 50 nM. Ligate the dsDNA encoding the spacer for sgRNA into the linearized pCRi vector in a 10 μl reaction containing 1 μl of diluted insert from the previous step, 10 ng of *Bsa*I-digested and purified pCRi vector or its derivative, 1 μl of T4 ligase buffer, 1 μl of T4 DNA ligase (New England Biolabs Inc.) and water, if needed, to reach the final volume. Other ligases, such as the QuickLigaseTM DNA ligase (New England Biolabs Inc.) can be used as needed.
iv. Ligate 30 min at room temperature, and transform a 100-μl aliquot of chemically competent *E. coli* DH5α cells with the total ligation mixture. Plate the bacterial suspension on LB agar plates supplemented with streptomycin. Purify plasmid DNA from three individual *E. coli* transformants, and verify the sequence integrity by sequencing with primer EK.SEVA_*T0*-F (Table S2 in the Supporting Information).

In order to repress multiple targets, several sgRNAs should be cloned into the vector, each of them placed under its own promoter. Individual P_{EM7} → sgRNA modules are synthesized as ultramers (Integrated DNA Technologies, Leuven, Belgium). Multiple sgRNA ultramers (i.e. containing promoter, target spacer, sgRNA fusion construct and unique overhangs) have to be amplified with the corresponding set of primers (depending on the sequence) and assembled together into the selected pCRiMs vector (Fig. 3), for example with *USER* cloning (Cavaleiro *et al.*, 2015). The *AMUSER* tool (Genee *et al.*, 2015) can be used to design primers with suitable overhangs for the assembly (available online at http://www.cbs.dtu.dk/services/AMUSER/).

Step-by-step procedure (cloning of a construct to target multiple genes).

i. Amplify vector pCRi using 1–5 ng of plasmid as template and primers EK.pCRi-U-F and EK.pCRi-U-R (Table S2 in the Supporting Information) using *Phusion*™ U Hot Start DNA polymerase according to the manufacturer's recommendations (Thermo Fisher Scientific, Waltham, MA, USA), 3 min elongation time, and employing an annealing temperature of 60°C for 30 cycles.
ii. Amplify double-stranded sgRNAs using synthesized ultramer as a template and respective primers (EK.sgRNA-F and EK.sgRNA-R; Table S2 in the Supporting Information) using *Phusion*™ U Hot Start DNA polymerase (according to the manufacturer's recommendations), 20 s elongation time, and annealing temperature of 60°C for 30 cycles.
iii. Combine equimolar amounts of sgRNAs and 100-150 ng of pCRi derivative with 1 μl of 1 U μl^{-1} *USER* enzyme (New England BioLabs Inc.) in a final volume of 10 μl. Incubate 30 min at 37°C or 20 min at room temperature. Transform 50 μl of chemically competent *E. coli* DH5α cells with 10 μl of the

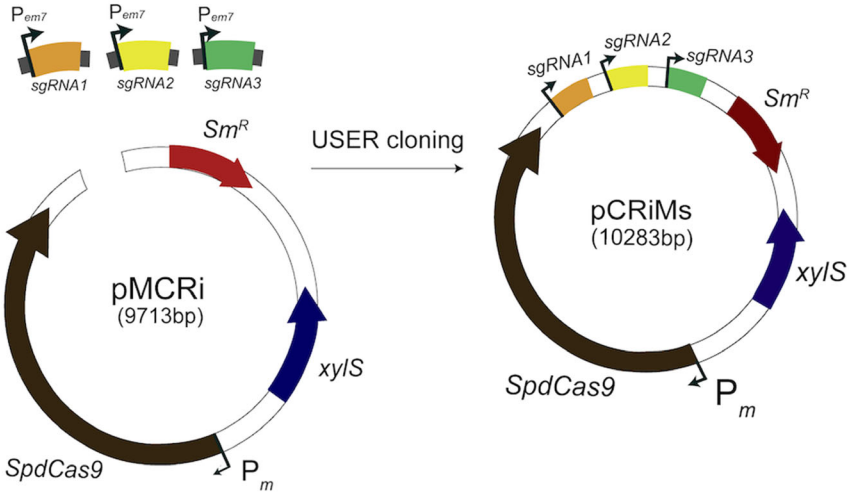

Fig. 3. Assembly of pCRiMs vectors. Double-stranded sgRNA ultramers with unique target spacers are combined with a PCR-amplified pMCRi vector for *USER* cloning reactions, resulting in a suite of vectors tailored for downregulation of several targets (i.e. pCRiMs plasmids)

resulting mixture. Plate the cells on LB medium agar supplemented with streptomycin.

Quickly verify correct constructs *via* PCR of 6-10 colonies with primers EK.SEVA_*T0*-F and EK.sgRNA-R (Table S2 in the Supporting Information). The size of the band for the negative control (i.e. an empty pCRi vector) is 210 bp; each spacer insert will yield a 195-bp longer amplicon. Purify DNA from the remaining volume of the PCR tubes that had the correct insert size and send the samples for DNA sequencing for final verification. Inoculate cultures of two individual *E. coli* clones (having the correct band size and sequence) in LB medium with streptomycin and incubate the cultures overnight at 37°C with shaking for further isolation of the plasmids.

One-step Golden Gate-based cloning for the assembly of single or multiple spacers into the crRNA cassette. To facilitate one-step Golden Gate assembly of a 30-nt spacer into vector pGCRi, two single-stranded (ss) DNA oligonucleotides have to be designed as shown in Fig. 4A. First, the sense (S) and anti-sense (AS) ssDNA oligonucleotides have to be annealed by slowly cooling the mixture to form a double-stranded (ds) DNA spacer unit (see section below) and later assembled with the pGCRi vector by replacing the eforRed chromoprotein sequence (Fig. 4A). This strategy enables ligation without the need of prior linearization of the vector and accelerates the screening process of the colonies that have acquired the correctly assembled plasmid. Construction of the multiplex crRNA array relies on the same design with minor modifications, where two or more dsDNA spacer-repeat units and one or more ds trimmed-repeat units (depending on the number of spacers) are ligated into the pGCRi vector (Fig. 4B). Each oligonucleotide is flanked by *Bsa*I recognition sites, allowing for the complete reconstruction of each DR as all parts will be ligated into the pGCRi vector *via* Golden Gate assembly (Fig. 4B). The 4-bp ssDNA overhangs vary between the different oligo units by altering the length of the trimmed-DR unit as the number of the spacers in the crRNA is further extended. In this way, incompatible 4-bp overhangs are created, allowing for the construction of the multiplex crRNAs with the desired orientation and order. Following this strategy, standard flanks and trimmed-DR units (where needed) were designed for the construction of single, double and triple crRNAs (Table 2). The dsDNA units can be reused in conjunction with others to form new crRNAs with the same number of spacers. For instance, any $spacer_1$-repeat unit of a double crRNA array is compatible with the trimmed-repeat unit as well as with any $spacer_2$-repeat unit.

Step-by-step procedure (cloning of the construct to target single or multiple genes).

i. Select the target spacer sequence (5′→3′) and design the required oligonucleotide using the corresponding standard flanks given in Table 2. Order the designed spacer oligonucleotide and its reverse complement sequence as ssDNA oligonucleotides from a commercial DNA synthesis vendor (e.g. Integrated DNA Technologies).
ii. Dissolve the two ssDNA oligonucleotides at a final concentration of 100 μM and anneal them by setting a single reaction consisting of 1 μl of each oligonucleotide, 2.5 μl NaCl and 45.5 μl water. The reaction

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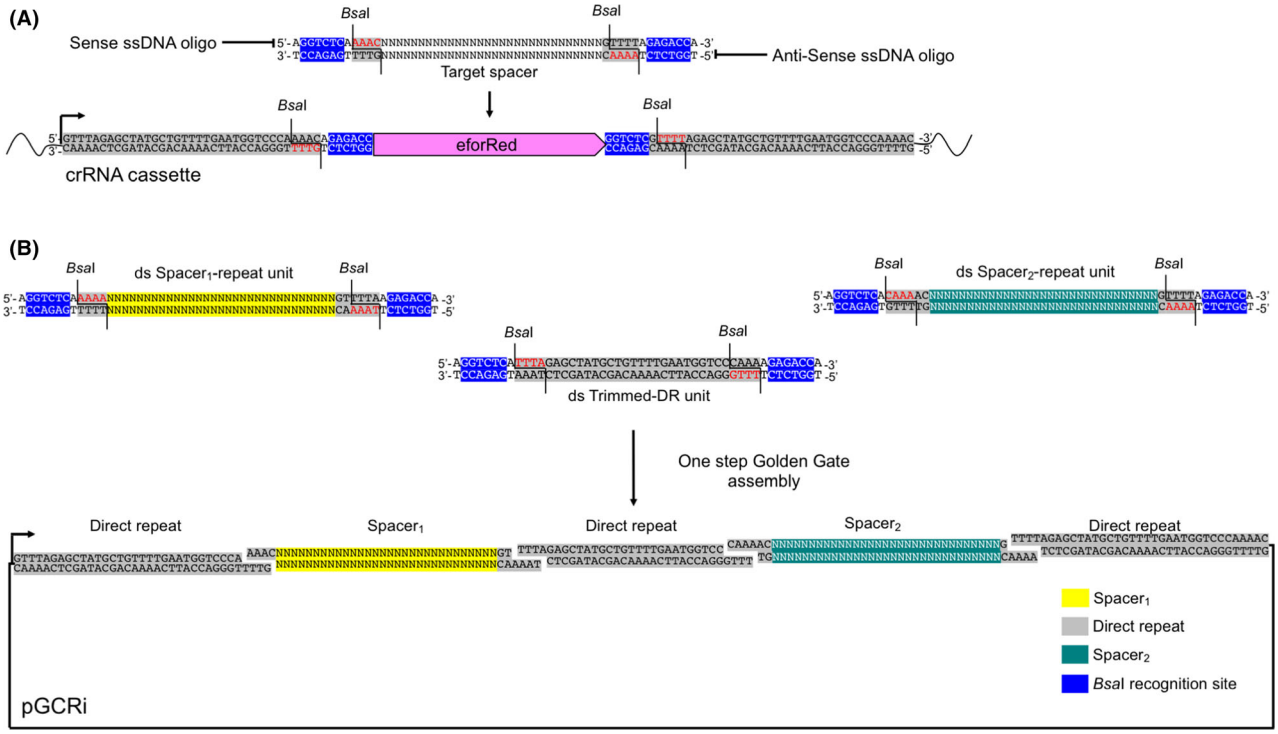

Fig. 4. Schematic representation of dsDNA spacer units and multiplex assembly strategy A. The annealed synthesized oligonucleotides consist of the 30-nt target spacer sequence flanked with four and five nucleotides (indicated in grey) of the upstream and downstream direct repeats (DRs), respectively, and two *Bsa*I recognition sites (highlighted in blue). The crRNA cassette also contains two *Bsa*I recognition sites between the DRs with an intervening eforRed chromoprotein gene to facilitate the cloning of the intended double-stranded DNA (dsDNA) spacer unit. B. Scheme of the dsDNA oligonucleotides required for the construction of a double crRNA array *via* Golden Gate cloning. The double-stranded (ds) spacer$_1$-repeat unit consists of the 30-nt spacer$_1$ sequence (yellow) with additional four and six nucleotides of its upstream and downstream DRs (grey) respectively. The ds spacer$_2$-repeat unit consists of the 30-nt spacer$_2$ sequence (green) with six and five nucleotides of its upstream and downstream DRs (grey) respectively. The trimmed-DR unit consists of the DR sequence lacking the nucleotides that are present in the spacer-repeat units. After digestion with *Bsa*I, all dsDNA oligonucleotides display compatible overhangs that specify their order and directionality as they are ligated to the target pGCRi vector *via* Golden Gate cloning

is incubated at 95°C for 5 min in a thermoblock, followed by a slowly cooling down step of 2 h by leaving the reaction tube inside the thermoblock at room temperature.

iii. Ligate the dsDNA oligonucleotide encoding the spacer into the pGCRi vector *via* a Golden Gate reaction composed by 2 μl of the 1:100-diluted annealed oligonucleotides (in case of multiplexing, add 1 μl of each dsDNA spacer-repeat and trimmed-repeat oligonucleotides), 2 μl of 15 ng μl^{-1} pGCRi plasmid and 2 μl of an in-house Golden Gate assembly master mix [10 μl of non-high fidelity (HF) *Bsa*I restriction enzyme (New England Biolabs Inc.), 15 μl of T4 DNA ligase buffer (New England Biolabs Inc.), 10 μl of T4 DNA ligase (New England Biolabs Inc.), 1 μl of 20 mg ml^{-1} bovine serum albumin (BSA) and 13.5 μl of water]. Incubate the reaction in a thermocycler at 37°C for 5 min; 15 cycles of 16°C for 4 min and 37°C for 3 min; 37°C for 5 min and 85°C for 10 min. Transform the resulting mixture into chemically competent *E. coli* DH5α cells.

iv. Select three white transformants for plasmid purification and verify through DNA sequencing of plasmid DNA.

Plasmid delivery in Pseudomonas by electroporation

i. Inoculate 10 ml of LB medium with the *P. putida* strain to be transformed and grow the cells overnight at 30°C with agitation (170-200 rpm).

ii. Wash the cells three times with 1 ml of 300 mM sucrose (filter-sterilized) and resuspend them in 400 μl of 300 mM sucrose.

iii. Individually electroporate 100 ng of empty pCRi vector and 100 ng of pCRi vector with the target-specific gRNA into 100-μl cell suspension aliquots with a voltage of 2.5 kV, 25 μF capacitance, and 200 Ω resistance (e.g. in a Gene Pulser Xcell™ Electroporation System, Bio-Rad Laboratories Inc., Hercules, CA, USA).

iv. Let the cells recover in LB medium for 2 h at 30°C with agitation and plate them onto LB agar

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Table 2. Standard flanks and trimmed-direct repeats units for one-step Golden Gate spacer assembly

Oligonucleotide	Sequence (5′→3′)[a]
Single array	
Spacer sense	aggtctcaaaac–*spacer*–gtttttgagacca
Double array	
Spacer$_1$ sense	aggtctcaaaac–*spacer$_1$*–gttttaagagacca
Trimmed-repeat sense	aggtctcgtttagagctatgctgttttgaatggtcccaaacgagacc
Spacer$_2$ sense	aggtctcacaaaac–*spacer$_2$*–gtttttgagacca
Triple array	
Spacer$_1$ sense	aggtctcaaaac–*spacer$_1$*–gttttaagagacca
Trimmed-repeat$_1$ sense	aggtctcgtttagagctatgctgttttgaatggtcccaaacgagacca
Spacer$_2$ sense	aggtctcacaaaac–*spacer$_2$*–gttttagtgagacca
Trimmed-repeat$_2$ sense	aggtctcgttagagctatgctgttttgaatggtcccaacgagacca
Spacer$_3$ sense	aggtctcaccaaaac–*spacer$_3$*–gtttttgagacca

[a]The nucleotide sequences indicated in blue and red represent the standard 5′- and 3′-flanks respectively. Both standard flanks incorporate *Bsa*I recognition sites as well as unique sticky ends for directional cloning of the corresponding spacers and direct repeats (DRs) into the crRNA array module. Each sense, single-stranded (ssDNA) oligonucleotide listed in the table has to be annealed with the corresponding anti-sense ssDNA oligonucleotide carrying the exact reverse complement sequence to form the required double-stranded (dsDNA) oligonucleotides.

supplemented with streptomycin (100 μg ml^{-1}). Incubate the plates overnight at 30°C.

Downregulation of the target gene(s) with CRISPRi

The plate with *P. putida* cells harbouring a pCRi plasmid with a functional gRNA should have a repressed gene of interest within 2 h after induction with 3-*m*Bz (for the maximal downregulation we recommend to use the inducer at 1 mM). Inoculate the culture medium of interest with *P. putida* harbouring the derivative of pCRi plasmid with target-specific gRNA(s), and grow the cells overnight at 30°C with agitation as indicated above. We recommend to supplement the culture medium with streptomycin (100 μg ml^{-1}) to select for the pCRi vector derivative.

Application examples

CRISPRi-mediated repression efficiency with different inducible expression systems and tuning of SpdCas9 expression

We first examined the efficiency of the target gene repression with CRISPRi using different expression systems: XylS/P$_m$ on the pMCRi plasmid, ChnR/P$_{chnB}$ on the pCCRi plasmid and CprK1/P$_{DB3}$ on the pDCRi plasmid, which all drive *SpdCas9* expression (Fig. 5A). To determine the capability of the newly established CRISPRi system for regulation of heterologous gene expression, we used *P. putida* strain KT·BG42, harbouring a *msf·gfp* gene (encoding the monomeric super-folder green fluorescent protein, msfGFP) and a gentamicin resistance marker in the unique Tn*7* locus of the bacterial chromosome (Zobel *et al.*, 2015; Volke *et al.*, 2020; see Table S1 in the Supporting Information). Each pCRi vector was transformed into strain KT·BG42, and cells were grown at 30°C on LB medium plates, containing both 100 μg ml^{-1} streptomycin (to select for the plasmid) and 20 μg ml^{-1} gentamicin (to select for the Tn*7* insertion). Three single colonies were then individually inoculated to set overnight pre-cultures into 3 ml of 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} $MgSO_4$, 2.5 ml l^{-1} of a trace elements solution; Nikel and de Lorenzo, 2013, 2014) containing 100 μg ml^{-1} streptomycin and 20 μg ml^{-1} gentamicin. Cultures were incubated for 15 h at 30°C with agitation. Then, 5 μl of the preculture was inoculated into 195 μl of M9 minimal medium containing 100 μg ml^{-1} streptomycin, 20 μg ml^{-1} gentamicin and 1 mM of the corresponding inducer depending on the expression system (XylS/P$_m$, induced by 3-*m*Bz; ChnR/P$_{chnB}$, induced by cyclohexanone; and CprK1/P$_{DB3}$, induced by 3-chloro-4-hydroxyphenylacetic acid). All inducers were directly added to the liquid culture medium from stock, concentrated solutions. The CRISPRi-mediated decrease of msfGFP fluorescence in *P. putida* KT·BG42 transformed with different plasmids varied across conditions depending on the expression system. After 12 h of induction, the msfGFP fluorescence in *P. putida* KT·BG42/pMCRi_*gfp* (where *SpdCas9* expression is placed under control of XylS/P$_m$) decreased by up to 90% compared with control *P. putida* KT·BG42 cells carrying an empty vector. When the ChnR/P$_{chnB}$ or CprK1/P$_{DB3}$ expression systems were used under the same culture and induction conditions, the msfGFP fluorescence decreased by 65% and 80% respectively (Fig. 5B).

In view of the results above, we concluded that the XylS/P$_m$ system outperformed the other expression systems for msfGFP depletion – thus we adopted this system for gene downregulation in further experiments. To analyse its tunability upon induction, we followed the msfGFP fluorescence levels of *P. putida* KT·BG42 cells carrying the corresponding CRISPRi vector in the presence of different concentrations of 3-*m*Bz, ranging from 0 to 1 mM. Expectedly, the level of repression of *msf·gfp* expression increased as a function of the inducer concentration (hence resulting in a graded decrease in the fluorescence levels in the cells;

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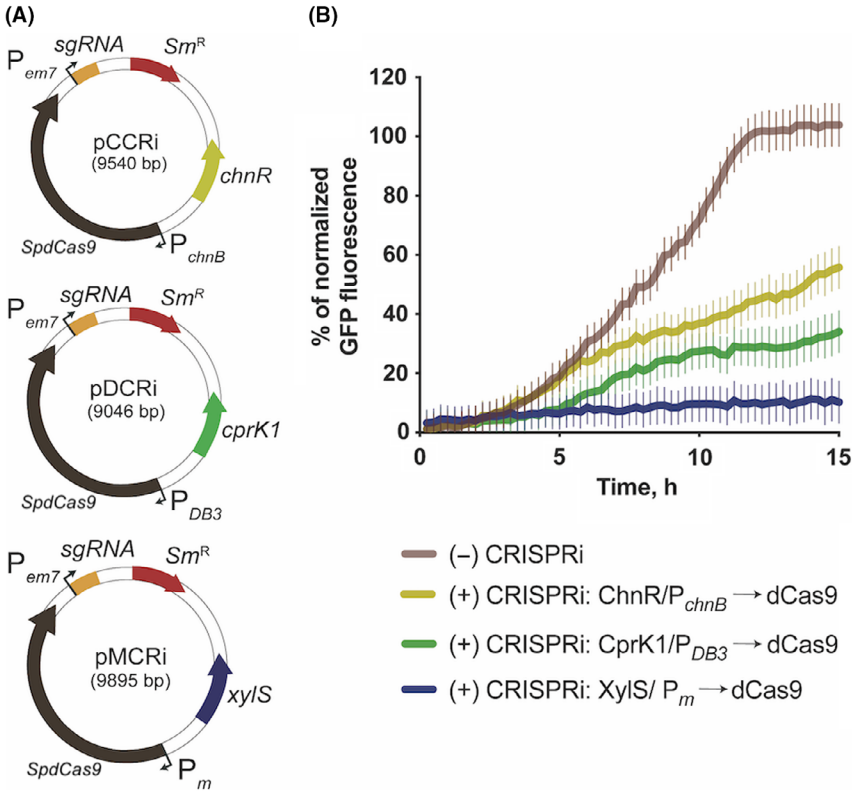

Fig. 5. Titratable downregulation of *msf·gfp* expression with CRISPRi using inducible expression systems to drive *SpdCas9* expression A. Schematic representation of derivatives of plasmid pCRi_*gfp* (plasmids pCCRi, pDCRi and pMCRi). All plasmids were separately introduced into *P. putida* KT·BG42, and three different expression systems were adopted: XylS/P_m (induced by addition of 3-methylbenzoate), ChnR/P_{chnB} (induced by addition of cyclohexanone) and CprK1/P_{DB3} (induced by addition of 3-chloro-4-hydroxyphenylacetic acid). B. CRISPRi experiment on fluorescent proteins. *P. putida* KT·BG42 was used as a control [indicated in the figure as (–) CRISPRi], and grown on M9 minimal medium supplemented with 0.2% (w/v) glucose and 20 μg ml^{-1} gentamicin. *P. putida* KT·BG42 transformants, harbouring the different CRISPRi vectors indicated, were re-grown on M9 minimal medium supplemented with 0.2% (w/v) glucose, 100 μg ml^{-1} streptomycin, 20 μg ml^{-1} gentamicin and 1 mM of the corresponding inducer [indicated in the figure as (+) CRISPRi]. Bacterial growth and msfGFP fluorescence ($\lambda_{excitation}/\lambda_{emission}$ = 485 nm/516 nm) were continuously measured during 15 h in a Synergy HI plate reader (BioTek Instruments, Inc., Winooski, VT, USA) using microtiter 96-well plates incubated at 30°C. Fluorescence readings were normalized to the bacterial growth (estimated as the optical density measured at 600 nm). Each data point represents the mean value of the percentage of normalized fluorescence ± standard deviation from at least three biological replicates

Fig. 6A). In particular, by using various concentrations of 3-*m*Bz (i.e. 0, 0.01, 0.1, 0.5 and 1 mM) we managed to decrease msfGFP fluorescence intensities up to 15%, 55%, 63%, 66% and 88%, respectively, after 15 h of incubation.

In order to demonstrate how the system can be used to modify a physiological property of the cells, we also performed CRISPRi-mediated inhibition of the expression of *ftsZ* (*PP_1342*). This gene encodes the FtsZ protein that plays a key role in septum formation during bacterial cell division (Lutkenhaus and Addinall, 1997; Stricker and Erickson, 2003). Efficient repression of *ftsZ* leads to a filamentous cell phenotype, as previously described by Elhadi *et al.* (2016) and Tan *et al.* (2018). In this case, we showed that, contrary to other reported CRISPRi systems, the very low leakiness of the CRISPRi modules constructed in this study does not result in any visible repression effect in the absence of the inducer. Moreover, we found that gene repression strictly depends on the amount of the inducer (i.e. 3-*m*Bz) added to the culture medium (Fig. 6B). Thus, the present approach is suitable for homogenous and tunable gene repression by adjusting the amounts of SpdCas9 in the cell.

CRISPRi-mediated downregulation of multiple targets (mCRISPRi) in P. putida

The CRISPRi systems described above afford flexibility and modularity features that can be adapted depending on the intended application. We wanted to further extend the range of targets that can be suppressed following the same design principle (mCRISPRi, multiplex CRISPRi). To this end, and in order to express more than one gRNA from a single plasmid, we constructed and adopted two approaches: (i) a CRISPRi system equipped with several

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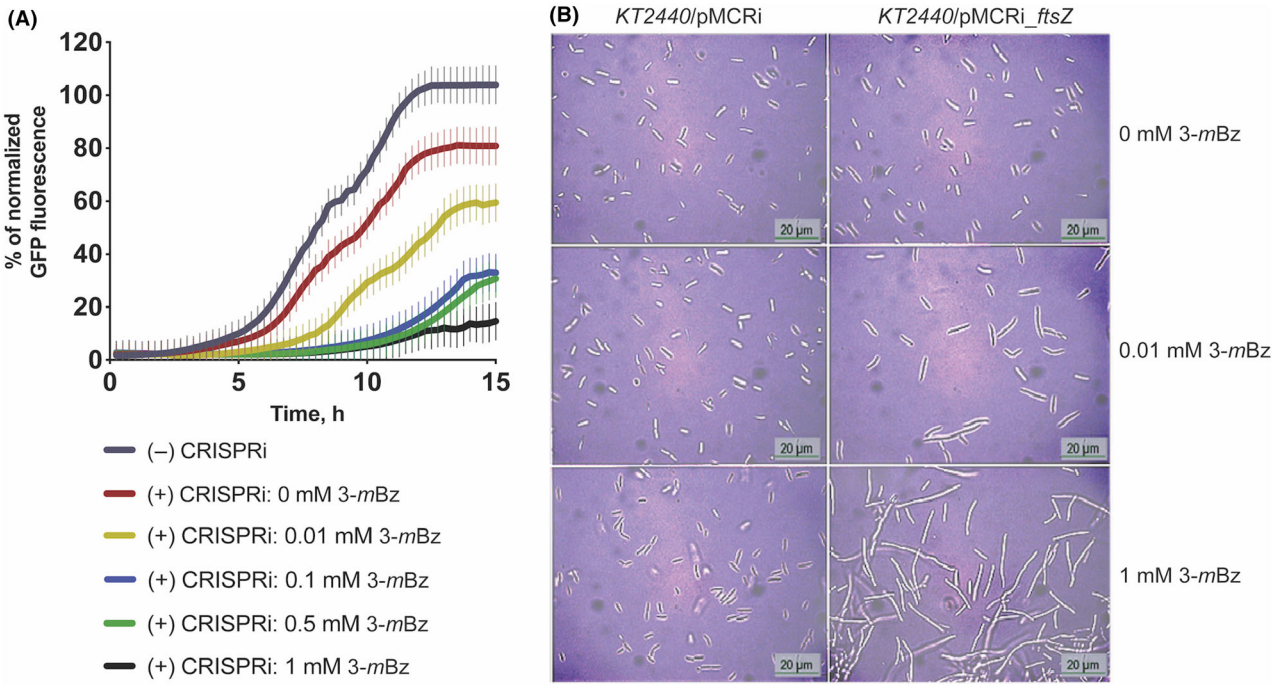

Fig. 6. Tunable effect of the CRISPRi system in *P. putida* KT·BG42 cells harbouring plasmid pMCRi with different inducer concentrations. A. Strain KT·BG42 cells transformed with plasmid pMCRi_*gfp*, harbouring the *msf·gfp*-specific spacer [indicated in the figure as (+) CRISPRi] or with vector pMCRi_*non-target* [harbouring a non-target-specific unique spacer, and indicated in the figure as (–) CRISPRi], were grown on M9 minimal medium with 0.2% (w/v) glucose, 100 μg ml^{-1} streptomycin, 20 μg ml^{-1} gentamicin and different concentrations of 3-methylbenzoate (3-*m*Bz) in the 0 to 1 mM range. Bacterial growth and msfGFP fluorescence ($\lambda_{excitation}/\lambda_{emission}$ = 485 nm/516 nm) were continuously measured during 15 h in a Synergy HI plate reader (BioTek Instruments, Inc., Winooski, VT, USA) using microtiter 96-well plates incubated at 30°C. Fluorescence readings were normalized to the bacterial growth (estimated as the optical density measured at 600 nm). Each data point represents the mean value of the percentage of normalized fluorescence ± standard deviation from at least three biological replicates. B. Microscope pictures showing tunable, inducer-dependent morphology changes of cells during CRISPRi-mediated downregulation of *ftsZ* in wild-type strain KT2440. Pictures were taken after 15 h with a Leica 2000 LED microscopy system (Leica Microsystems GmbH, Germany) at 100 × resolution (F1 type emission oil)

sgRNAs under control of individual P_{EM7} promoters, or (ii) a system harbouring the native crRNA cassette with multiple spacers (Fig. 7A). The detailed structure of the constructs was described in the previous sections. To perform mCRISPRi, we first constructed *P. putida* KT·YFP·mCherry, harbouring constitutively expressed *yfp* and *mCherry* genes – encoding yellow fluorescent protein (YFP) and red fluorescent protein (mCherry), respectively – and a kanamycin resistance marker integrated into the Tn*7* locus of the chromosome (Table S1 in the Supporting Information). The design and construction of *P. putida* KT·YFP·mCherry was done essentially according to Wirth *et al.* (2020). *P. putida* KT·YFP·mCherry is a derivative of wild-type strain KT2440 carrying a P_{100} → *mCherry* and P_{tet} → *yfp* cassette integrated in the Tn*7* locus *via* a synthetic mini-Tn*7* transposon. As such, this strain displays constitutive expression of the two fluorescent protein genes driven by the P_{100} and P_{tet} promoters. We first assessed repression of either *yfp* or *mCherry* expression. *P. putida* KT2440·YFP·mCherry harbouring the empty pGCRi vector was used as a control was grown on M9 minimal medium supplemented with 0.2% (w/v) glucose and streptomycin (100 μg ml^{-1}). *P. putida* KT2440·YFP·mCherry harbouring pGCRi_*yfp or* pMCRi_*mCherry* was grown under the same conditions. The cultures were supplemented with 1 mM 3-m*Bz* to induce the XylS/P_m expression system. In this case, downregulation of single-gene targets (*yfp, mCherry*) resulted in repression up to 65-68 % (Fig. S1A in the Supporting Information).

Furthermore, and in order to simultaneously downregulate the expression of *yfp*, *mCherry* and *ftsZ* (which, as indicated above, affects cell division and thereby results in a filamentous phenotype), *P. putida* KT·YFP·mCherry cells were first transformed with constructs harbouring multiple target-specific spacers (i.e. plasmids pCRiMs and pCRiMc, Fig. 7A). Both systems exhibited similar efficiency in repressing the chosen targets (an expected result, considering that the spacer sequences in plasmids pCRiMs_*yfp/mCherry/ftsZ* and pCRiMc_*yfp/mCherry/ftsZ* were the same; Table S2 in the Supporting Information). Specifically, after 15 h of cultivation, the relative fluorescence was reduced by 55%–65% for mCherry and by 55%–60% for YFP (Fig. 7B). Note that,

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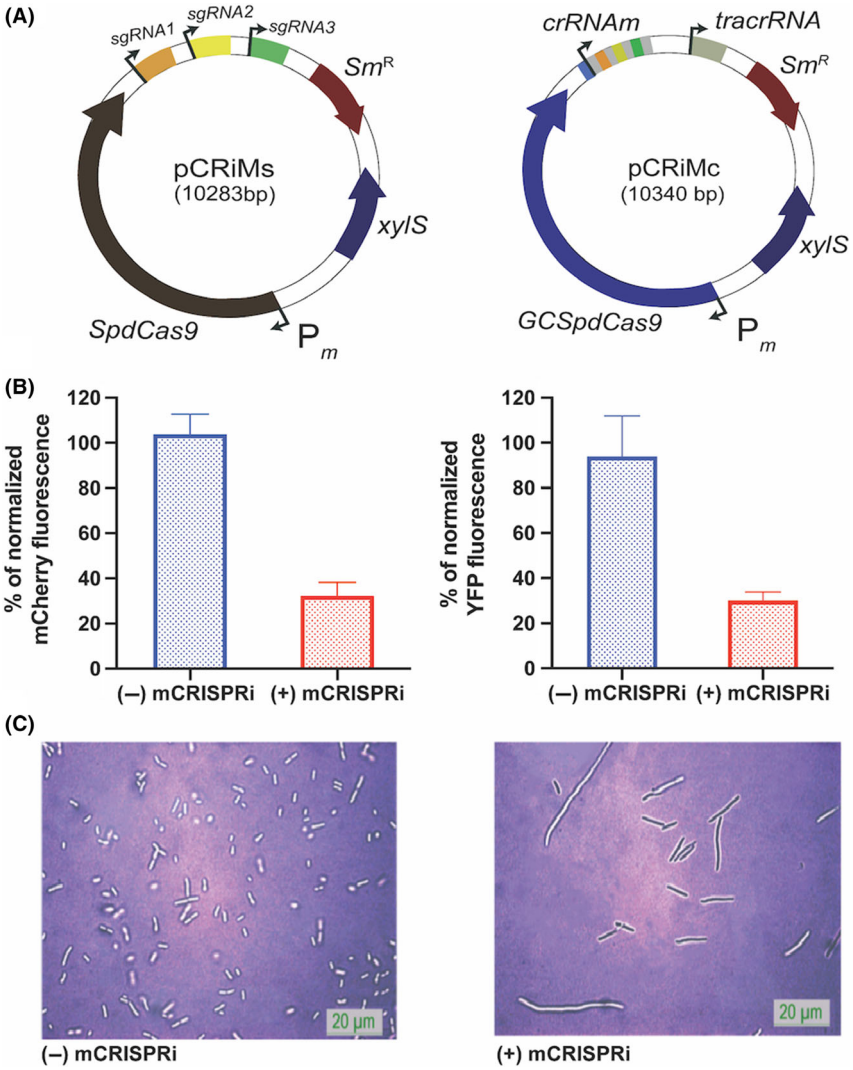

Fig. 7. CRISPRi-mediated downregulation of multiple gene targets (mCRISPRi) in *P. putida* KT·YFP·mCherry. For simultaneous repression of the expression of *mCherry*, *yfp* and *ftsZ*, cells containing the corresponding CRISPRi vector were grown in M9 minimal medium with 0.2% (w/v) glucose, supplemented with 100 μg ml^{-1} streptomycin, 50 μg ml^{-1} kanamycin and induced with 3-methylbenzoate at 1 mM. In the figure, (–) mCRISPRi represents cells harbouring a non-target-specific pMCRi vector, and (+) mCRISPRi represents cells containing pCRiMs vector. A. Schematic representation of vectors pCRiMs and pCRiMc, harbouring target-specific spacers. (B) Bacterial growth and mCherry fluorescence ($\lambda_{excitation}/\lambda_{emission}$ = 567 nm/610 nm) and YFP fluorescence ($\lambda_{excitation}/\lambda_{emission}$ = 495 nm/527 nm) were measured at 15 h in a Synergy HI plate reader (BioTek Instruments, Inc., Winooski, VT, USA) using microtiter 96-well plates incubated at 30°C. Fluorescence readings were normalized to the bacterial growth (estimated as the optical density measured at 600 nm). Basal levels of fluorescence detected in *P. putida* KT2440 were also subtracted from the reading. Each bar represents the mean value of the percentage of normalized fluorescence ± standard deviation from at least three biological replicates. B. Microscope pictures showing morphology changes of cells during mCRISPRi-mediated downregulation of *ftsZ* in wild-type strain KT2440 after 15 h. Pictures were taken with a Leica 2000 LED microscopy system (Leica Microsystems GmbH, Germany) at 100 × resolution (F1 type emission oil)

according to the measurement of fluorescence levels, the repression of the target gene in the absence of the specific spacer was fairly comparable for the two CRISPRi systems. Targeting of *ftsZ* with mCRISPRi resulted in cell morphology changes (detectable under light microscopy), with a significant shift in the cell size from ca. 5 μm rods to filament-like shaped cells with a length up to 60 μm (Fig. 7C). Importantly, repression levels remained within the same range (i.e. around 60%) during multiple or single-gene targeting with CRISPRi (pCRiMc_*yfp/mCherry/ftsZ* or pGCRi_*yfp*, respectively; see Fig. S1B in the Supporting Information). Thus, the single-plasmid mCRISPRi system developed here allows for regulatable downregulation of several genes of interest, which was demonstrated by simultaneous repression of three genes in *P. putida*.

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Characterization of the RecA-dependent genetic stability of the system using CRISPRi-mediated downregulation of pyrF

Several teams have employed CRISPRi-based technologies to arrest cell growth by targeting essential chromosomal loci. In particular, CRISPRi has been successfully deployed in engineered bacteria to redirect carbon fluxes from cell growth to production (Li *et al*., 2016; Shabestary *et al*., 2018; Kent and Dixon, 2019; Tian *et al*., 2019), thereby enhancing yields and titres of target compounds. CRISPRi approaches have also been implemented for high-throughput functional characterization of putative essential genes in bacterial platforms such as *Bacillus subtilis* (Peters *et al*., 2016), *Streptococcus pneumoniae* (Liu *et al*., 2017) and *Vibrio natriegens* (Lee *et al*., 2019). However, it has been shown that long-term repression of essential loci often results in accumulation of deleterious mutations in *dCas9* and/or the gRNAs (or regulatory elements thereof), eventually resulting in their inactivation (Zhao *et al*., 2016).

In order to characterize the stability and long-term efficiency of gene repression mediated by our CRISPRi system in *P. putida*, we downregulated the expression of *pyrF* (*PP_1815*), encoding the key orotidine 5′-phosphate decarboxylase reaction within the pyrimidine biosynthetic pathway. Following the protocol described above, we implemented the *pyrF*-specific CRISPRi vectors pGCRi_*pyrF* and pMCRi_*pyrF* (Table 1). As expected, downregulation of *pyrF* led to significantly low growth rates when the cells were grown in M9 minimal medium with glucose as the only carbon source (Table 3). In particular, and during the first 10 h of cultivation, the growth of *P. putida* KT2440 cells harbouring target-specific pCRi_*pyrF* vectors was repressed up to 90% (Fig. 8A,B). After 10 h, however, bacterial growth resumed, a phenomenon which could be accounted for by the accumulation of mutations in key elements of the CRISPRi system – probably including homologous recombination between the two DRs of the crRNA module and further overgrowth of escapers cells in which the expression of *pyrF* is no longer repressed.

We further analysed the sequence of the constructs in the *pyrF*-downregulated strains harvested at the end of the growth-inhibition experiments, and the results indicated that multiple modifications were accumulated in the constructs, including (i) recombination events between the two DRs of the crRNA cassette that led to the loss of the target spacer (which could be even noticed by PCR amplification of the cassette and separating the amplicons by gel electrophoresis, see Fig. S2 in the Supporting Information) and (ii) point mutations in the promoter sequence and/or the coding sequence of *GC*SpCas9 itself (data not shown). To suppress these deleterious effects, and to further characterize the genetic stability of the target-specific CRISPRi system, we repeated the growth-inhibition experiment using *P. putida* strain EM383, a streamlined, reduced-genome strain derivative of KT2440 lacking several non-adjacent genomic deletions, including the whole flagellar machinery, four prophages, two transposons and three key components of DNA restriction-modification systems (Martínez-García *et al*., 2014). This reduced-genome strain also lacks the gene encoding the main component of the homologous recombination machinery, *recA*, preventing any further recombination (Abella *et al*., 2007; Akkaya *et al*., 2019). We reasoned that such strain would give rise to a limited fraction of escapers upon CRISPRi-mediated downregulation of essential genes. Indeed, the growth arrest in strain EM383, mediated by targeting the essential *pyrF* gene with CRISPRi, was extended from 10 to 20 h (Fig. 8; Table 3). Moreover, wild-type strain KT2440, expressing either the crRNA or sgRNA module targeting *pyrF*, reached a 59% and 56% lower optical density after 20 h compared with the control experiments respectively. At the same time, strain EM383 harbouring the same CRISPRi plasmids showed almost no growth after 20 h in contrast to the corresponding controls. These results indicate a clear role for RecA on the genetic stability of CRISPRi

Table 3. Growth characterization of wild-type *P. putida* KT2440 and the streamlined strain EM383 upon CRISPRi-mediated downregulation of *pyrF* expression

	Growth parameter	
P. putida strain	Specific growth rate[a] (h^{-1})	Final optical density[b]
KT2440	0.27 ± 0.08	1.78 ± 0.09
EM383	0.33 ± 0.05	1.78 ± 0.08
KT2440/pGCRi_no target	0.19 ± 0.02	2.59 ± 0.03
EM383/pGCRi_no target	0.12 ± 0.01	2.32 ± 0.18
KT2440/pGCRi_*pyrF*	N.D.	1.05 ± 0.27
EM383/pGCRi_*pyrF*	N.D.	0.42 ± 0.05
KT2440/pMCRi_no target	0.24 ± 0.02	1.75 ± 0.09
EM383/pMCRi_no target	0.28 ± 0.07	1.73 ± 0.09
KT2440/pMCRi_*pyrF*	N.D.	0.71 ± 0.07
EM383/pMCRi_*pyrF*	N.D.	0.15 ± 0.09

[a]The specific growth rate for each strain was calculated during exponential growth. Cultures were carried out in 96-well microtiter plates, and the optical density at 600 nm was measured every 15 min during 20 h using a Synergy HI Biotek microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). Glucose was added as a carbon source to M9 minimal medium at 0.2% (w/v). 'Results represent the mean and standard deviation of two independent experiments. N.D., not detected.
[b]The final optical density at 600 nm is reported for each strain after 20 h of incubation.

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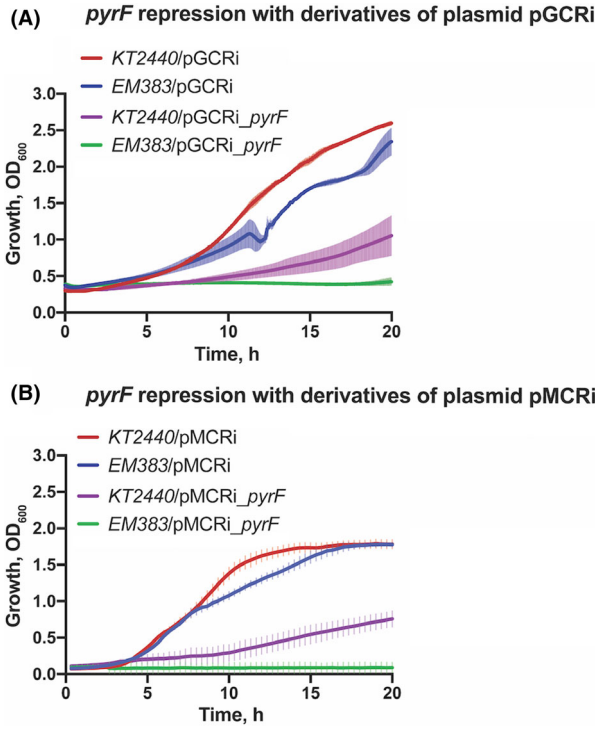

Fig. 8. CRISPRi-based, targeted downregulation of *pyrF* expression in *P. putida*. Wild-type KT2440 and the streamlined *P. putida* strain EM383 were transformed with either non-target-specific vectors pGCRi or pMCRi, or the corresponding target-specific vectors (indicated with the suffix *pyrF*). The resulting strains were grown in 96-well plates in M9 minimal medium supplemented with 0.2% (w/v) glucose, 100 μg ml^{-1} streptomycin and 1 mM 3-methylbenzoate. Bacterial growth was monitored in a Synergy HI plate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA) for 20 h at 30°C with shaking by periodically measuring the optical density at 600 nm (OD_{600}). Gene repression experiments are indicated for cells transformed with (A) plasmid pGCRi (no target and *pyrF*-specific) and (B) plasmid pMCRi (no target and *pyrF*-specific). Each data point represents the mean value of OD_{600} readings ± standard deviation of at least three biological replicates

systems in *P. putida*, which can be circumvented by using strains in which the recombination machinery has been eliminated.

Discussion

CRISPRi technologies have been widely applied for metabolic engineering and synthetic biology approaches in a range of eukaryotic and prokaryotic organisms. CRISPRi systems have been successfully optimized for a range of bacterial *chassis* including *E. coli*, *Bacillus subtilis* and *Corynebacterium glutamicum* (Jakočiūnas *et al.*, 2017; Adli, 2018; Cho *et al.*, 2018; Donohoue *et al.*, 2018). In the present work, we aimed at improving the existing CRISPRi-based genome engineering toolbox for *P. putida*, implementing modularity, robustness and tunability towards the development of this bacterium as an effective, efficient and controllable *chassis* for biotechnological applications. These features are separately discussed in the sections below.

Tunable gene regulation and multiplexing

Two variants of a single-plasmid CRISPRi system (either sgRNA-based or crRNA-based) were developed for the transcriptional control of gene expression in *P. putida*. Tight and tunable downregulation of gene expression was achieved through controlling the expression of *SpdCas9* with the XylS/P_m system. Furthermore, the system displayed low leakiness – up to 15% (when downregulating the expression of *msfgfp, mCherry* or *yfp*) or non-detectable (in the case of targeting *ftsZ*) under non-induced conditions. A linear response of downregulation levels of a chromosomally expressed *msf·gfp* was observed as a function of the inducer (3-*m*Bz) concentration. This feature represents a substantial improvement to previously CRISPRi-based approaches, which did not allow for titratable gene downregulation (Kim *et al.*, 2020), sometimes exhibiting high leakiness (> 50%) in the absence of the corresponding inducers (Tan *et al.*, 2018). Importantly, we also demonstrated the ability to simultaneously downregulate three genes in *P. putida* with this system.

Inducible downregulation through CRISPRi enables conditional control of essential gene expression. Such an approach would be effective for controlling growth (e.g. to establish growth-decoupling switches for bioproduction; Durante-Rodríguez *et al.*, 2018) or for the assessment of fundamental questions in metabolism (e.g. related to gene essentiality; Poulsen *et al.*, 2019). As an example, we applied the versatile CRISPRi system described in this study to control the expression of the conditionally essential gene *pyrF*. The growth of *P. putida* strains harbouring either the sgRNA- or crRNA-based CRISPRi vectors was totally suppressed during the first 10 h in a minimal culture medium supplemented with glucose as sole carbon source. Additionally, we demonstrated a significant increase in the stability of the CRISPRi repression when essential genes are targeted in a strain devoid of the RecA machinery, which points to the importance of RecA-dependent mechanisms for stability of genetic constructs in *P. putida* (Aparicio *et al.*, 2020). Therefore, the expanded, single-plasmid CRISPRi toolbox combines all the essential features desired for precise transcriptional control of single and multiple genes in *P. putida*.

Cloning, standardization and modularity of CRISPRi components and portability

In addition to the examples illustrating the applicability of the tool, we also focused on improving cloning

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procedures and implementing standardization and modularity in the intervening components of the CRISPRi system. Two detailed protocols are provided for the construction of multiplex sgRNA and crRNA arrays. In general, the efficiency of using just sgRNA or both tracrRNA and crRNA was observed to be fairly comparable. The architecture of crRNA allows for a rapid and simple cloning strategy when generating multiplex crRNA arrays. In particular, the construction of multiplex gRNA arrays has been shown to be a laborious and time-consuming process, where often additional PCR or ligation steps are required. The genetic architecture of the sgRNA (promoter–SpCas9 handle–terminator) limits the applicability of several assembly methods that rely on the ligation of homologous sequences – but it is particularly useful and straightforward when targeting a single or dual target (s) for downregulation. To tackle this problem, we constructed the crRNA-based CRISPRi vector (pGCRi), which enables the construction of the array with multiple spacers in a modular and directional single step with all elements in the array regulated by a master promoter. In addition, the pGCRi vector is equipped with the gene encoding an eforRed chromoprotein that gets inactivated if the spacers are correctly integrated into the vector, simplifying and accelerating the selection process. Therefore, vector pGCRi (and derivatives) is suitable for combinatorial, high-throughput genetic screenings where hundreds or thousands of CRISPRi plasmids need to be built (Reis *et al*., 2019). In terms of overall impact of the different CRISPRi systems presented herein on the cell physiology, we would recommend to use plasmids containing *SpdCas9* due to limited effects on bacterial growth parameters as compared with *GCSpdCas9*-bearing vectors.

In conclusion, the present study describes suitable strategies based on CRISPRi to efficiently control transcription levels in *P. putida* KT2440 while implementing modularity, standardization and robustness. Along the line, low leakiness levels and tunable repression of single and multiple genes has been achieved, and detailed protocols for the construction of both sgRNA- and crRNA-based CRISPRi vectors are provided (which can be adapted at the user's will, depending on the intended application and its specific needs). Considering that all the components of the CRISPRi toolbox presented in this study follow the modular and standard formatting brought about by the SEVA platform, the vectors presented in this work can be transferred to other *Pseudomonas* species – and, essentially, to any other Gram-negative bacterium where an appropriate combination of antibiotic resistance markers and origin of replications from the SEVA collection can be used. Preliminary tests in *P. aeruginosa* PAO1 indicate that *SpdCas9*-based CRISPRi on genes encoding fluorescent proteins yield similar levels of repression as those reported herein (data not shown), which sheds a positive light on the system portability across species. As such, our study considerable expands the CRISPRi toolbox of *Pseudomonads* and opens new avenues for functional characterization of genes and advanced metabolic engineering.

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Conflict of interest

The authors declare no conflict of interest.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Down-regulation of single gene targets (*yfp*, *mCherry*) with CRISPRi.

Fig. S2. Recombination events between the two direct repeats of crRNA lead to the loss of the target spacer sequence.

Table S1. Bacterial strains used in this work.

Table S2. Oligonucleotides used in this work.

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