Metabolic Engineering 29 (2015) 160–168

Contents lists available at ScienceDirect

Metabolic Engineering

journal homepage: www.elsevier.com/locate/ymben

Application of CRISPRi for prokaryotic metabolic engineering involving multiple genes, a case study: Controllable P(3HB-*co*-4HB) biosynthesis

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ARTICLE INFO

Article history:
Received 26 September 2014
Received in revised form
21 March 2015
Accepted 23 March 2015
Available online 31 March 2015

Keywords:
CRISPRi
Synthetic biology
PHB
4-hydroxybutyrate
P3HB4HB
Metabolic engineering

ABSTRACT

Clustered regularly interspaced short palindromic repeats interference (CRISPRi) is used to edit eukaryotic genomes. Here, we show that CRISPRi can also be used for fine-tuning prokaryotic gene expression while simultaneously regulating multiple essential gene expression with less labor and time consumption. As a case study, CRISPRi was used to control polyhydroxyalkanoate (PHA) biosynthesis pathway flux and to adjust PHA composition. A pathway was constructed in *Escherichia coli* for the production of poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)] from glucose. The native gene *sad* encoding *E. coli* succinate semi-aldehyde dehydrogenase was expressed under the control of CRISPRi using five specially designed single guide RNAs (sgRNAs) for regulating carbon flux to 4-hydroxybutyrate (4HB) biosynthesis. The system allowed formation of P(3HB-*co*-4HB) consisting of 1–9 mol% 4HB. Additionally, succinate, generated by succinyl-coA synthetase and succinate dehydrogenase (respectively encoded by genes *sucC*, *sucD* and *sdhA*, *sdhB*) was channeled preferentially to the 4HB precursor by using selected sgRNAs such as sucC2, sucD2, sdhB2 and sdhA1 via CRISPRi. The resulting 4HB content in P(3HB-*co*-4HB) was found to range from 1.4 to 18.4 mol% depending on the expression levels of down-regulated genes. The results show that CRISPRi is a feasible method to simultaneously manipulate multiple genes in *E. coli*.

1. Introduction

The CRISPR (clustered regularly interspaced short palindromic repeats) system provides a potential platform for targeted gene editing (Barrangou et al., 2007). CRISPR systems use small base-pairing RNAs to target and cleave foreign DNA elements in a sequence-specific manner (Wiedenheft et al., 2012). The optimized CRISPR system requires only a minimal set of two molecules, the Cas9 protein and a single guide RNA (sgRNA) (Jinek et al., 2012); it has therefore been widely used as a host-independent gene-targeting platform for many mammalian systems (Cong et al., 2013; Friedland et al., 2013; Gratz et al., 2013; Hwang et al., 2013; Jinek et al., 2013; Li et al., 2013; Mali et al., 2013; Sakuma et al., 2014).

Recently, it has been demonstrated that CRISPR systems can serve as site-selective RNA-guided genome editing tools (Mali et al., 2013; Cong et al., 2013; Jinek et al., 2013; Jiang et al., 2013; Hwang et al., 2013; Cho et al., 2013; Wang et al., 2013). The Cas9 protein, which features two inactive mutations of D10A and H840A at its RuvC1 and HNH nuclease domains, loses cleaving activity to form the dCas9 protein, yet still exhibits DNA binding capability (Jinek et al., 2012). For the purpose of genome regulation instead of genome editing via flexible CRISPR systems, the catalytically inactive Cas9 (dCas9) can be used as a platform for gRNA-guided transcription regulation; this technique is called CRISPRi (clustered regularly interspaced short palindromic repeats interference) (Qi. et al., 2013).

CRISPRi is a highly favorable tool for prokaryotic metabolic engineering on multiple genes. In this study, microbial metabolic engineering for polyhydroxyalkanoate (PHA) synthesis was investigated to demonstrate CRISPRi for use in applications involving regulation of prokaryotic multiple gene expression.

Polyhydroxyalkanoates (PHAs) are a family of biocompatible and biodegradable materials that participate in an industrial value chain (Martin and Williams, 2003; Park et al., 2005a; Hazer and Steinbüchel, 2007; Chen, 2009). PHAs are synthesized by many bacteria as intracellular carbon and energy reserve materials (Anderson and Dawes, 1990).

Poly-3-hydroxybutyrate (P3HB) is the most common PHA. A disadvantage of P3HB is its brittleness (Antipov et al., 2006; Chen and Wu, 2005), but copolymerization with P3HB can improve

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http://dx.doi.org/10.1016/j.ymben.2015.03.013
1096-7176/

many properties of PHA (Cong et al., 2008; Shi et al., 1997; Sudesh et al., 2000; Vigneswari et al., 2009). Metabolic engineering methods were used for production of various PHA copolymers (Chen et al., 2004; Park and Lee, 2005c; Park et al., 2005b; Jung et al., 2010; Tyo et al., 2009a, Tyo et al., 2009b). Among them, the copolymer P(3HB-*co*-4HB), consisting of 3-hydroxybutyrate (3HB) and 4-hydroxybutyrate (4HB), has been produced on an industrial scale and approved for food contact usage (Chen and Wu, 2005; Martin and Williams, 2003; Volova et al., 2003; Zinn et al., 2001).

P(3HB-*co*-4HB) was first reported to be synthesized by *Ralstonia eutropha* fed with the expensive substrate 4-hydroxybutyrate (4HB) or 1,4-butanediol (BDO) (Doi et al., 1988; Kunioka et al., 1989; Mitomo et al., 2001; Kim et al., 2005; Song and Kim, 2005). Other organisms such as *Hydrogenophaga pseudoflava* (Choi et al., 1999), *Alcaligenes latus* (Kang et al., 1995; Songand Kim, 2005), *Comamonas testosteroni* (Renner et al., 1996) and several recombinant strains (Valentin and Dennis, 1997; Martin and Williams, 2003; Li et al., 2010) were all reported to produce P(3HB-*co*-4HB). Depending on the 3HB/4HB ratio, the physical nature of the copolymer can range from highly elastic rubber to rigid crystalline plastic (Saito et al., 1996; Ishida et al., 2001). The 4HB content of P(3HB-*co*-4HB) was adjustable based on 4-hydroxybutyrate or 1,4-butanediol feeding (Vigneswari et al., 2009). Some engineered *Escherichia coli* strains containing an anaerobic succinate degradation pathway of *Clostridium kluyveri* were reported to produce P(3HB-*co*-4HB) solely from glucose (Fig. 1) (Valentin and Dennis, 1997; Dennis and Valentin, 1998; Park et al., 2007; Li et al., 2010). Li et al. (2010) constructed a metabolically engineered *E. coli* strain able to produce P(3HB-*co*-12 mol% 4HB) from unrelated carbon sources including glucose with limiting 4HB flexibility.

Because the main precursor of 4HB, namely succinyl-CoA, is an intermediate in the TCA cycle, it is difficult to accumulate succinyl-CoA under aerobic conditions (Fig. 1) (Clark, 1989). Instead, most succinyl-CoA is converted to succinate catalyzed by succinyl-CoA synthetase encoded by genes *sucC* and *sucD*. This succinate is turned into fumarate by succinate dehydrogenase encoded by *sdhA* and *sdhB* (Krebs, 1940). It is thus expected that more succinyl-CoA would be channeled to succinate semi-aldehyde (SSA) for 4HB formation if expression of the two enzymes were down-regulated. This work aimed to investigate the use of CRISPRi for controlling the expression of multiple essential genes related to 4HB synthesis so that various PHA materials with flexible properties could be obtained.

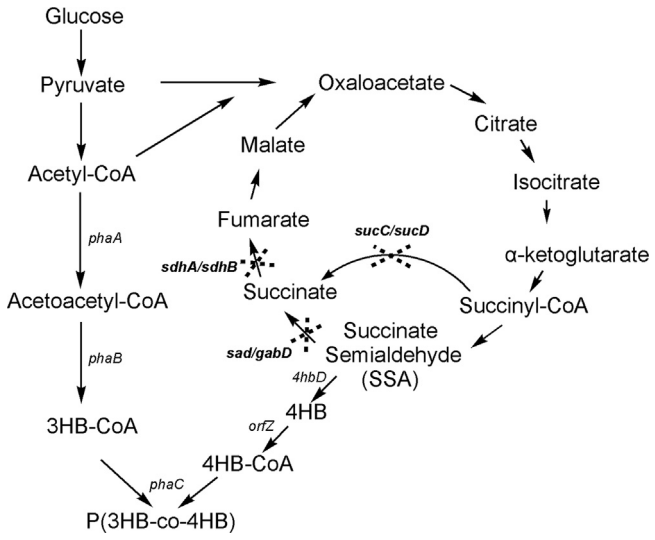

Fig. 1. Engineered pathways for P(3HB-*co*-4HB) synthesis by recombinant *Escherichia coli*. Genes: *phaA*, β-ketothiolase; *phaB*, NADPH-dependent acetoacetyl-CoA reductase; *phaC*, PHA synthase; *sucD*, succinate semi-aldehyde dehydrogenase; *4hbD*, 4-hydroxybutyrate dehydrogenase; *orfZ*, CoA transferase; *sdhA* and *sdhB* encoding succinate dehydrogenase of *E. coli*; *sucC* and *sucD*, the succinyl-CoA synthetase of *E. coli*; *sad* and *gabD*, succinate semi-aldehyde dehydrogenase of *E. coli*.

2. Materials and methods

2.1. Microorganisms and culture medium

The bacterial strains used in this study are listed in Table 1. *E. coli* S17-1 sfGFP (*recA*, *thi-1*, *pro*, *hsdR*, RP4-2-Tc: Mu-Km: Tn7 sfGFP) was kindly donated by Prof. LOU ChunBo, Institute of Microbiology, Chinese Academy of Sciences. The P(3HB-*co*-4HB) production strain was *E. coli* S17-1 co-transformed with plasmids pMCSH5 and pBHR68 encoding 4HB (Li et al., 2010) and P3HB synthesis pathways (donated by Professor Alexander Steinbüchel, Münster University, Germany), respectively. *E. coli* S17-1 (pMCSH5 + pBHR68) was further transformed with a series of plv-dCas9 plasmids for gene expression regulation (Table 1). All of these *E. coli* strains were cultivated in Petri disks with Luria-Bertani medium containing tryptone (10 g/L), yeast extract (5 g/L) and NaCl (10 g/L).

2.2. Construction of a CRISPRi platform suitable for E. coli system

The plasmids used in this study are listed in Table 1. Molecular cloning experiments were carried out according to manufacturers' instructions or standard procedures. Kits for DNA purification and isolation of high quality plasmids were purchased from Qiagen (Shanghai, China). Restriction enzymes and DNA modification enzymes were provided by New England Biolabs (USA). Table S1 lists all primers used for plasmid constructions and tests.

To prepare the plv-dCas9-sgRNA plasmids for multiple gene regulation, the two plasmids plv-dCas9 (mcs) and plv-sgRNA were constructed (Table 1).

Plasmid plv-dCas9 (mcs) was constructed based on the pdCas9-bacterial plasmid (Addgene; Plasmid #44249), which contains a gene encoding dCas9 protein constructed by Qi et al. (2013). Multiple cloning sites (MCS) including *EagI*, *XmaI* and *NgoMIV* were inserted into the pdcas9-bacterial plasmid to form plv-dCas9 (mcs). *XmaI* and *NgoMIV* are isocaudarners that are required for sgRNA biobrick assembly (Fig. S1).

An sgRNA biobrick contains three parts: a 20 bp DNA region complementing the gene sequence of interest called the based-pairing region (BPR), a 42 bp hairpin region for dCas9 protein binding termed the dCas9 handle (DH) and a 40 bp terminator named rrnB (Ter) (Fig. 2A). To construct a platform vector for various sgRNA target sites conveniently, two restriction sites of type-II restriction endonuclease *BspQI* were inserted via inverse PCR into the pgRNA-bacterial plasmid (Addgene; Plasmid #44251), which harbors a sgRNA part including the three regions of BPR, DH and Ter (Qi et al., 2013). Thus, it became more convenient to construct a plv-sgRNA plasmid containing a sgRNA target site without redundant sequences. The 20 – 23 bp target site complementary sequence was designed via primers (Table S1). The forward and reverse primers were subsequently annealed to obtain a double-stranded inserted fragment precisely fitting the plv-sgRNA vector, which could be cleaved by *BspQI* enzyme, resulting in formation of plasmid plv-sgRNA via ligation. This technique allowed convenient changes in the complementary region to suit any interesting gene.

In addition, the same endonucleases were used to construct the plasmid plv-sgRNA (Table 1). The endonuclease sites *EagI* and *XmaI* were introduced upstream of the sgRNA expression cassette, and *NgoMIV* was inserted downstream of it. By digesting the backbone

Table 1
Strains and plasmids used in this study.

Strains/plasmids	Description	Reference/source
E. coli MG1655	F$^-$ λ^- *rph-1* (wild-type)	(Sauer et al., 2004)
E. coli JM109	*rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17 *sup*E44 *rel*A1 Δ(*lac-pro*AB) [F'*tra*D36 *pro*AB *laqI*^{q} *lac*ZΔM15]	Transgene Bio Inc
E. coli S17-1	*recA*,*thi*-1.*pro*,*hsd*R, RP4-2-Tc: Mu-Km: Tn7	This study
E. coli S17-1sfGFP	*recA*,*thi*-1.*pro*,*hsd*R, RP4-2-Tc: Mu-Km: Tn7 sfGFP	This study
E. coli JM109SG	JM109*ΔsadΔgabD*	This study
pdcas9-bacteria	dCas9 protein coding sequence, Plasmid #44249 Cm^R	*Addgene* Bio Inc
pgRNA-bacteria	sgRNA unit sequence, Plasmid #44251 Amp^R	*Addgene* Bio Inc
pBAD2-StaPro68-sfGFP	sfGFP gene reporter plasmid Amp^R	This study
plv-dCas9(mcs)	Multiple cloning sites inserted into pdcas9-bacteria plasmid, Cm^R	This study
plv-sgRNA	*BspQI* enzyme sites inserted into pgRNA-bacteria plasmid, Amp^R	This study
plv-dCas9-sgRNA	sgRNA element inserted into plv-dCas9 (mcs) plasmid, Cm^R	This study
pBHR68	*phaCAB* expression plasmid, Amp^R	(Spiekermann et al., 1999)
p68orfZ	*orfZ* gene inserted into pBHR68,Amp^R	This study
plv-sfGFP	plv-dCas9-sgRNA plasmid target *sfGFP*, Cm^R	This study
plv-sdhA	plv-dCas9-sgRNA plasmid target *sdhA*, Cm^R	This study
plv-sdhB	plv-dCas9-sgRNA plasmid target *sdhB*, Cm^R	This study
plv-sucC	plv-dCas9-sgRNA plasmid target *sucC*, Cm^R	This study
plv-sucD	plv-dCas9-sgRNA plasmid target *sucD*, Cm^R	This study
plv-sad	plv-dCas9-sgRNA plasmid target *sad*, Cm^R	This study
plv-sdhB2sdhA1	plv-dCas9-sgRNA plasmid target *sdhB*, *sdhA*, Cm^R	This study
plv-sad1sucD2	plv-dCas9-sgRNA plasmid target *sad*, *sucD*, Cm^R	This study
plv- sad1sucD2sucC2	plv-dCas9-sgRNA plasmid target *sad*, *sucD*, *sucC*, Cm^R	This study
plv-sad1sucD2 sucC2sdhB2sdhA1	plv-dCas9-sgRNA plasmid target *sad*, *sucD*, *sucC*, *sdhB*, *sdhA*, Cm^R	This study
pEnH5	*sucD-4hbD* inserted into pEn	This study
pMCSH5	P_{pdc}-*sucD-4hbD* inserted into pBBR1MCS-2	This study

plv-dCas9 (mcs) vector and donor plv-sgRNA vector with *EagI*/*XmaI* and *EagI*/*NgoMIV*, respectively, plv-dCas9-sgRNA plasmids were formed. After the ligation, *EagI* and *XmaI* restriction sites were reconstructed for the next round of sgRNA biobrick insertion (Fig. S1). In this way, multiple sgRNA biobricks could be inserted into one vector backbone for manipulating multiple genes simultaneously.

2.3. Determination of growth curves and fluorescence intensities of recombinants containing CRISPRi

A pure single colony was inoculated in LB medium for 12 – 16 h. Samples of 200 μL volume were inoculated into each well of a 96-well plate with three parallel samples for each culture sample. The resulting data thus represents three colony samples inoculated under parallel conditions. Each sample was diluted with the medium to reach an OD600 of 0.001. After treatments with different concentrations of aTc inducer, samples were inoculated again in 96-well plates and cultured for 28 h under continuous rotary shaking (Thermo Scientific Varioskan Flash, Thermo Scientific, USA). Subsequently, OD600 and sfGFP fluorescence intensity were quantified at an appropriate time defined by the computer program. The relationship among bacterial growth, sfGFP fluorescence intensity and inducer concentration was evaluated.

2.4. Real-time PCR

Total RNA was isolated from recombinant *E. coli* S17-1 strains by using the RNA prep pure Cell/Bacteria Kit (Tiangen, Beijing, China). The Fastquant RT Kit (Tiangen, Beijing, China) was used to synthesize the cDNA for mRNA analysis. Using 16S rRNA as the inner standard, real-time PCR (RT-PCR) was carried out for mRNA analysis with SuperReal PreMix (SYBR Green) (Tiangen, Beijing, China). The primers used in this work are listed in Table S1.

The concentration of the total extracted RNA was measured to design a concentration gradient for cDNA synthesis (using random primers according to standard procedures described in the manufacturer's product specification). The resulting cDNA was used immediately for the RT-PCR analysis. The linear interval of total RNA was analyzed as a standard for follow-up experiments to adjust the quantity of the template within its linear range, so that the fluorescence quantitative results could be designed within a rational range. All samples were prepared with three parallel groups to obtain results of ΔCt values from the outputs of RT-PCR.

2.5. Shake flask studies on PHA production

Two control recombinants were constructed as described below: plasmids pMCSH5 and p68orfZ were co-transformed into *E. coli* S17-1 as a control termed *E. coli* S17-1 (pMCSH5 + p68orfZ). Another control was constructed by using *E. coli* S17-1 co-transformed with same plasmids pMCSH5 and p68orfZ together with plv-sgRNA containing the gene of dCas9 protein and sgRNA unit without a targeting sequence; this version is termed *E. coli* S17-1 [(p68orfZ + pMCSH5) + plv-sgRNA]. Subsequently, the recombinants were cultivated for studies of PHA accumulation.

Target strains were similarly constructed: *E. coli* S17-1 was transformed with pMCSH5 and p68orfZ together with plv-sgRNA consisting of the dCas9 and sgRNA targeting sequence(s), resulting in *E. coli* S17-1 [(p68orfZ + pMCSH5) + plv-sad1-5], *E. coli* S17-1 [(p68orfZ + pMCSH5) + plv-sucD2], *E. coli* S17-1 [(p68orfZ + pMCSH5) + plv-sad1sucC2sucD2], *E. coli* S17-1 [(p68orfZ + pMCSH5) + plv-sad1sucC2sucD2sdhB2sdhA1] and so on.

The seed culture was grown at 37 °C in Luria – Bertani medium for 12 h at 200 rpm on a rotary shaker (HZQF160, HDL, Harbin, China). Subsequently, the seed culture was inoculated into 500 mL shake flasks containing 50 mL LB medium supplemented with 20 g/L glucose at an inoculation volume of 4% (v/v). After 4 h cultivation, 2 μM aTc inducer was added to the culture. When necessary, a final concentration of 50 μg/mL kanamycin, 100 μg/ml ampicillin or 25 μg/mL chloramphenicol was added to the culture to maintain the stability of pMCSH5, p68orfZ and the series of CRISPRi system plasmids listed in Tables 2 and 3.

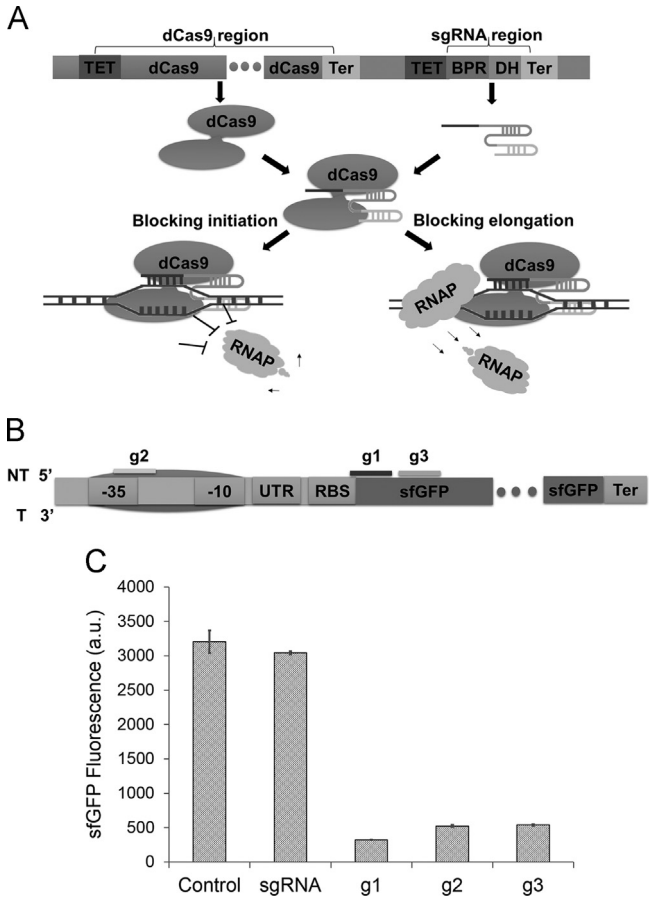

Fig. 2. Schematic description of using CRISPRi to manipulate the P(3HB-*co*-4HB) biosynthesis pathway. (A) Operation of the CRISPRi system to block transcription initiation and elongation. (B) Synthetic sfGFP fluorescence-based reporter system was used to test the function of CRISPRi system. Three sgRNAs that bind to the promoter region or the non-template DNA strand were co-expressed with the dCas9 protein, with their effects on the target sfGFP or its promoter −35 box region measured by in vivo fluorescence assay. (C) The sgRNAs that bind to the different regions showed expression silencing (80–95%). The control showed fluorescence of the cells with neither dCas9 protein nor sgRNA. The sgRNA control showed fluorescence of the cells with dCas9 protein and the non-target sgRNA. All of the samples were treated with 2 μM aTc inducer after culturing for 4 h. Fluorescence results represent average of at least three replicates.

2.6. Analytical methods

Cells were harvested by centrifugation at 12,000 rpm for 8 min and then washed with distilled water. After vacuum lyophilization, the cell dry weight (CDW) was measured. Subsequently, the intracellular PHA polymers were methyl esterified using the lyophilized cells in screw-capped tubes with chloroform heated at 100 °C for 4 h (Li et al., 2010). A gas chromatograph (GC-2014, SHIMADZU, Japan) was used to analyze PHA content and composition (Li et al., 2010). Analytical pure P(3HB) and γ-butyrolactone (Sigma-Aldrich) were used as standards to quantify the 3HB and 4HB monomer contents, respectively.

3. Results

3.1. Bacterial CRISPRi system for regulation of multiple gene expression

A super green fluorescent protein (sfGFP)-based reporter system (Pédelacq et al., 2005) was constructed to study the feasibility of the established bacterial CRISPRi system. Under various concentrations of anhydrotetracycline (aTc) inducer, it was demonstrated that the constructed CRISPRi system and an appropriate inducer concentration did not affect growth of *E. coli* (Fig. S2). To investigate possible repression on gene expression by co-expression of the mutated dCas9 protein and a sgRNA biobrick, three different targeting CRISPRi systems were constructed, each carrying one of the following complementary sgRNAs (Fig. 2B): the g1 sequence complementary to the beginning of the sfGFP coding sequence, the g2 sequence matching the sfGFP −35 box promoter region, and the g3 targeting sequence separated from the sfGFP double-stranded transcription site.

The entire sgRNA biobrick, including g1-3, was able to bind to the non-template DNA strands under a chosen inducer concentration of 2 μM. The sgRNAs were designed to bind to the non-template DNA strands to increase the targeting efficiency based on several previous studies (Gasiunas et al., 2012; Fu et al., 2013; Qi et al., 2013). All of these studies showed that sgRNAs targeting the non-template DNA strands demonstrated effective reduction on gene expression levels, whereas those targeting the template strands revealed only a weaker expression reduction.

The sgRNAs g1, g2 and g3 were able to bind with their respective targets with different efficiencies ranging from 80% to 95% repression due to their different specific targeting locations (Fig. 2C). Real-time PCR results indicated that the CRISPRi system functioned at the mRNA level to regulate sfGFP gene expression, and the effects were consistent with their fluorescence intensity (Fig. S3). Compared with the g3 targeting site, g1 was much closer to the 5′ terminal of the sfGFP gene coding sequence, which was a non-template DNA strand. The g1 sequence thus showed stronger suppressive ability than g3. On the other hand, the g2 sequence that targets the −35 box of the sfGFP promoter region reduced the expression of the targeted sfGFP. Thus, the CRISPRi system was successfully established as a technique for bacterial gene regulation.

Table 2
P(3HB-*co*-4HB) production by controlling single gene expression in recombinant *E. coli* strains grown in shake flasks.

Recombinant *E. coli* S17-1 strains	CDW (g/L)	P(3HB-*co*-4HB) (wt%)	4HB (mol%)
(p68orfZ+pMCSH5)	7.15 ± 0.04	46.37 ± 1.8	1.43 ± 0.11
(p68orfZ+pMCSH5)+plv-sgRNA	11.51 ± 0.62	65.34 ± 3.4	1.62 ± 0.09
(p68orfZ+pMCSH5)+plv-sad1	10.18 ± 0.29	71.63 ± 3.6	8.99 ± 0.42
(p68orfZ+pMCSH5)+plv-sad2	9.33 ± 0.63	53.45 ± 5.56	4.27 ± 0.23
(p68orfZ+pMCSH5)+plv-sad3	9.14 ± 0.08	59.49 ± 1.05	5.56 ± 0.47
(p68orfZ+pMCSH5)+plv-sad4	9.43 ± 0.94	56.45 ± 2.68	2.95 ± 0.19
(p68orfZ+pMCSH5)+plv-sad5	10.55 ± 0.45	62.77 ± 2.00	1.92 ± 0.05

The recombinants harboring p68orfZ and pMCSH5 were cultivated in Luria–Bertani medium containing 2% (w/v) glucose at 37 °C for 48 h as described in Section 2. Data shown are the average and standard deviation of three parallel experiments. Abbreviations: CDW, cell dry weight; p68orfZ, used for 3HB production; pMCSH5, used for 4HB production; plv-sgRNA, plv-dCas9-sgRNA plasmid without target site; plv-sad1, plv-sad2, plv-sad3, plv-sad4, plv-sad5, plv-dCas9-sgRNA plasmids with five different targets of gene *sad*.

3.2. Effect of single gene repression on P(3HB-co-4HB) production

At least six genes were found to be involved in the P(3HB-*co*-4HB) synthesis from glucose as a sole carbon source (Li et al., 2010), including three genes associated with the succinate degradation pathway from *Clostridium kluyveri* and another three genes of P(3HB) synthesis from *Ralstonia eutropha*. The two plasmids p68orfZ and pMCSH5 were used to deliver the above genes (Li et al., 2010). In addition, the CRISPRi system was encoded by another plasmid, plv-dCas9-sgRNA, which can be conveniently manipulated to accommodate any gene targeting site of interest. As described previously (Fig. 1), five *E. coli* chromosomal genes

including *sdhA* and *sdhB* (encoding succinate dehydrogenase), *sucC* and *sucD* (encoding succinyl-CoA synthetase) and *sad* (encoding succinate semi-aldehyde dehydrogenase), were chosen to study the effects of their different expression levels on P(3HB-*co*-4HB) production. For each targeting gene, three to five different complementary sgRNA sequences were designed to exercise various repression levels (Table S1). Three plasmids, namely p68orfZ, pMCSH5 and plv-dCas9-sgRNA, were simultaneously co-transformed into *E. coli* S17-1.

Compared with the control group *E. coli* S17-1 (p68orfZ+pMCSH5) without the CRISPRi system, the strain *E. coli* S17-1 [(p68orfZ +pMCSH5)+plv-sgRNA], which harbored the CRISPRi system and non-target sgRNA, minimally influenced 4HB content in P(3HB-*co*-4HB) (Table 3). However, for unknown reasons, *E. coli* S17-1 [(p68orfZ+pMCSH5)+plv-sgRNA] grew better and accumulated more PHA (Table 3).

Succinate semi-aldehyde dehydrogenase (SSADH), encoded by gene *sad*, can use either $NADP^+$ or NAD^+ as a cofactor to catalyze the degradation of succinate semi-aldehyde (SSA) to succinate. Therefore, it was suggested that 4HB was converted to succinate via SSA catalyzed by SSADH and 4HB dehydrogenase in the P(3HB-*co*-4HB) synthesis pathway (Fig. 1) (Valentin et al., 1995; Lütke-Eversloh and Steinbüchel, 1999). Because succinate biosynthesis competes with the 4HB-CoA pathway, SSADH functions to reduce the carbon flux for 4HB synthesis (Fig. 1). Hence, *sad* repression should lead to increasing carbon flux in 4HB synthesis. Real-time PCR results indicated that the CRISPRi system functioned at the mRNA level to regulate gene expression reduction (Figs. 3A, 4, 5A, S3, S5 and S6).

The gene *sad* was used as an example to demonstrate how five targeting sites were designed (Table S1): sad1 and sad3 were synthesized to target the starting *sad* coding sequence, sad2 maintained some base distance from sad1; and sad2, sad4 and sad5 were designed downstream of the sad3 sequence. Different binding sites resulted in changing repression effects (Fig. 3A). RT-PCR results from using the *sad* gene clearly demonstrated that the target site 1 (plv-sad1) was more efficient than other target sites (Fig. 3 and Table 1) in of the following order: plv-sad1, plv-sad3,

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(p68orfZ+pMCSH5)+plv-sdhA1	10.98 ± 0.72	64.26 ± 3.76	2.92 ± 0.23
(p68orfZ+pMCSH5)+plv-sdhB2	9.24 ± 0.26	61.91 ± 1.15	1.69 ± 0.12
(p68orfZ+pMCSH5)+plv-sdhB2sdhA1	8.74 ± 0.37	67.16 ± 1.06	4.47 ± 0.19
(p68orfZ+pMCSH5)+plv-sucC2	9.98 ± 0.30	64.26 ± 2.59	1.83 ± 0.25
(p68orfZ+pMCSH5)+plv-sucD2	11.72 ± 0.36	62.49 ± 3.46	7.76 ± 0.41
(p68orfZ+pMCSH5)+plv-sad1	10.18 ± 0.29	71.63 ± 3.6	8.99 ± 0.42
(p68orfZ+pMCSH5)+plv-sad1sucD2	10.47 ± 0.27	51.06 ± 3.55	12.12 ± 0.16
(p68orfZ+pMCSH5)+plv-sad1sucD2sucC2	10.53 ± 0.40	61.70 ± 4.00	14.73 ± 0.48
(p68orfZ+pMCSH5)+plv-sad1sucD2sucC2sdhB2sdhA1	7.73 ± 0.14	71.87 ± 1.95	18.43 ± 0.25

The recombinants harboring p68orfZ and pMCSH5 were cultivated in Luria–Bertani medium containing 2% (w/v) glucose at 37 °C for 48 h as described in Section 2. Data shown are the average and standard deviation of three parallel experiments. Abbreviations: CDW, cell dry weight; p68orfZ, used for 3HB production; pMCSH5, used for 4HB production; plv-sgRNA, plv-dCas9-sgRNA plasmid without target site; plv-sdhA1, plv-dCas9-sgRNA plasmid target gene *sdhA*; plv-sdhB2, plv-dCas9-sgRNA plasmid target gene *sdhB*; plv-sdhB2sdhA1, plv-dCas9-sgRNA plasmid target genes *sdhB* and *sdhA*; plv-sucC2, plv-dCas9-sgRNA plasmid target gene *sucC*; plv-sucD2, plv-dCas9-gRNA plasmid target gene *sucD*; plv-sad1, plv-dCas9-sgRNA plasmid target gene *sad*; plv-sad1sucD2, plv-dCas9-sgRNA plasmid target genes *sad* and *sucD*; plv-sad1sucD2-sucC2sdhB2sdhA1, plv-dCas9-sgRNA plasmid target genes *sad*, *sucD*, *sucC*, *sdhB* and *sdhA*.

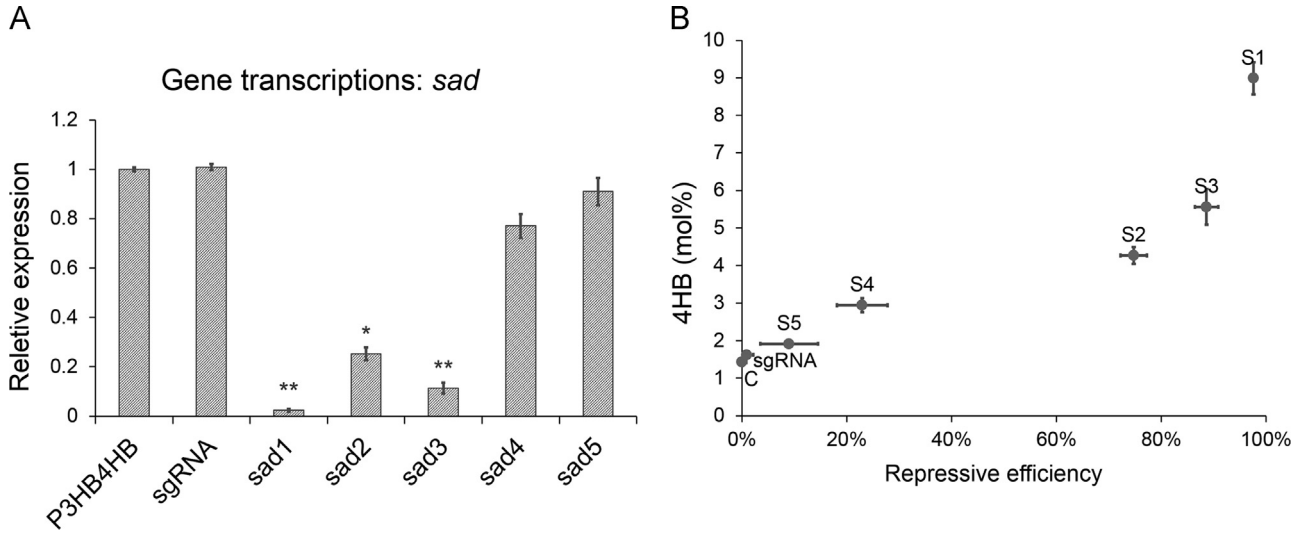

Fig. 3. Manipulating single gene expression in recombinant *E. coli*. (A) The mRNA level of *sad* gene in recombinant *E. coli*. P3HB4HB: *E. coli* S17-1 (p68orfZ+pMCSH5), the strain without CRISPRi system; sgRNA: *E. coli* S17-1 (p68orfZ+pMCSH5)+plv-sgRNA, the strain with non-targeting CRISPRi system; sad1: *E. coli* S17-1 (p68orfZ+pMCSH5)+plv-sad1, the strain with CRISPRi system targeting *sad* gene; sad2:*E. coli* S17-1 (p68orfZ+pMCSH5) +plv-sad2; sad3: *E. coli* S17-1 (p68orfZ+pMCSH5) +plv-sad3; sad4:*E. coli* S17-1 (p68orfZ+pMCSH5)+plv-sad4; sad5: *E. coli* S17-1 (p68orfZ+pMCSH5) +plv-sad5. Mean ± S.E. ($n=3$). * and ** indicate $p<0.05$ and $p<0.01$, respectively. See Section 3.2 for details. (B) Consistency between repressive efficiency and 4HB ratio in P(3HB-*co*-4HB). C: *E. coli* S17-1 (p68orfZ+pMCSH5); sgRNA: *E. coli* S17-1 (p68orfZ+pMCSH5)+plv-sgRNA; S1: *E. coli* S17-1 (p68orfZ+pMCSH5)+plv-sad1; S2: *E. coli* S17-1 (p68orfZ+pMCSH5)+plv-sad2; S3: *E. coli* S17-1 (p68orfZ+pMCSH5)+plv-sad3; S4: *E. coli* S17-1 (p68orfZ+pMCSH5)+plv-sad4; S5: *E. coli* S17-1 (p68orfZ+pMCSH5)+plv-sad5.

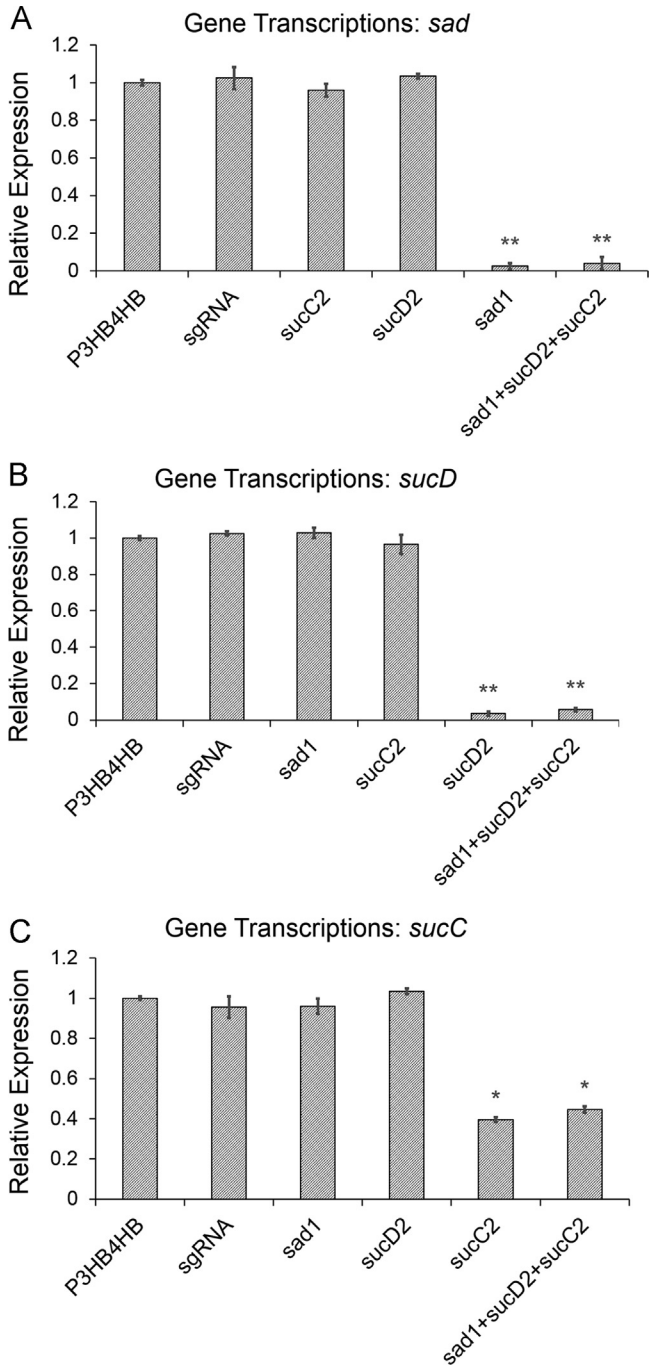

Fig. 4. Manipulation of expression of three genes in recombinant *E. coli*. (A) The mRNA level of *sad* gene in recombinant *E. coli* (B) The mRNA level of *sucD* gene in recombinant *E. coli* (C) The mRNA level of *sucC* gene in recombinant *E. coli* P3HB4HB: *E. coli* S17-1 (p68orfZ+pMCSH5); sgRNA: *E. coli* S17-1 (p68orfZ+pMCSH5)+plv-sgRNA; sad1: *E. coli* S17-1 (p68orfZ+pMCSH5)+plv-sad1; sucC2: *E. coli* S17-1 (p68orfZ+pMCSH5)+plv-sucC2, the strain with CRISPRi system targeting *sucC* gene; sucD2: *E. coli* S17-1 (p68orfZ+pMCSH5)+plv-sucD2, the strain with CRISPRi system targeting *sucD* gene; sad1+sucD2+sucC2: *E. coli* S17-1 (p68orfZ+pMCSH5)+plv-sad1sucD2sucC2, the strain with CRISPRi system targeting *sad*, *sucD* and *sucC* genes. Mean ± S.E. ($n=3$). * and ** indicate $p<0.05$ and $p<0.01$, respectively. See Section 3.3 for details.

plv-sad2, plv-sad4 and plv-sad5. This order was confirmed by western blot (Fig. S4). Therefore, when studying regulation of the remaining genes *sdhA*, *sdhB*, *sucC* and *sucD*, relatively convenient RT-PCR experiments were employed as a guide to design the binding sgRNA(s) sequence instead of the more tedious western blot studies (Fig. S5). All of the sgRNA targets studied (five for *sad*, four for *sdhA*, three for *sdhB*, four for *sucC* and three for *sucD*) were proven effective in reducing gene expression, at different levels (Fig. 3 and S5, Table 1).

Shake flask studies were conducted to investigate how *sad* repression affected 4HB composition in P(3HB-*co*-4HB) (Table 2). The results clearly revealed that the repression levels were consistent with the 4HB composition in the copolymers (Fig. 3B).

3.3. Effect of multiple gene repressions on P(3HB-co-4HB) production

The above study demonstrated that the CRISPRi system was able to control a single gene expression and thus regulate P(3HB-*co*-4HB) composition. By combining the biobrick sgRNAs targeting sad1 and sucD2, the plasmid plv-sad1sucD2 was generated. Furthermore, plv-sad1sucD2sucC2—containing the three sgRNAs sad1, sucD2 and sucC2—was also assembled to investigate effects of multiple gene repression on P(3HB-*co*-4HB) production. RT-PCR was used to investigate regulation of multiple sgRNAs containing plasmids of plv-sad1sucD2sucC2 and plv-sad1sucD2sucC2sdhB2sdhA1 on the expression of three or five genes (Figs. 4, 5A and S6). Except for the five-target strain *E. coli* [(p68orfZ+pMCSH5)+plv-sad1sucD2sucC2sdhB2sdhA1], all strains were found to grow well in shake flasks containing Luria-Bertani medium supplemented with 20 g/L glucose (Table 3), reaching approximately 10 g/L cell dry weight containing over 60% P(3HB-*co*-4HB) after 48 h of incubation (Table 3). The five-target strain *E. coli* [(p68orfZ+pMCSH5)+plv-sad1sucD2sucC2sdhB2sdhA1] could down-regulate five gene expression levels; this strain also accumulated the highest 4HB content of over 18 mol% and the highest PHA content of 72%, although these cells grew relatively slowly. The repression of multiple essential genes may have a negative effect on cell growth (Yu et al., 2006; Cecchini et al., 2002).

When only one gene *sad* was regulated by sgRNA sad1, 9 mol% 4HB was synthesized in the P(3HB-*co*-4HB) copolymer. When regulating genes *sucD* and *sucC* using sgRNAs sucD2 and sucC2 were incorporated, 8 mol% and 2 mol% 4HB was formed, respectively, in the copolymers (Table 3).

Finally, the 4HB content in the P(3HB-*co*-4HB) copolymer was further increased to 18.4 mol% when five sgRNAs including sad1, sucD2, sucC2, sdhA1 and sdhB2 were used simultaneously (Fig. 5B and Table 3). These results demonstrate that multiple gene repression with the CRISPRi system can be used to regulate P(3HB-*co*-4HB) composition.

4. Discussion

As genetic engineering capability has progressed, it has become increasingly important to manipulate several genes involved in a metabolic pathway. Traditionally, to increase flux to the target products, pathways that consume intermediates to the final products are interrupted by deleting the related gene(s). However, cells will die or cell growth will be seriously affected if the deleted gene(s) is/are essential.

The CRISPRi system offers the possibility of repressing expression of multiple genes simultaneously with one plasmid (Figs. 4, 5, S1 and S6), thus avoiding time- and labor-intensive multiple gene deletion, which must be done sequentially. In addition, CRISPRi avoids the disadvantage of the traditional gene knockout method, which is not suitable for manipulating essential gene(s) (Gerdes et al., 2003) such as those encoding the citric acid cycle (TCA) and/or the respiratory chain. CRISPRi can be adjusted and/or optimized for desirable metabolic pathways without affecting the normal

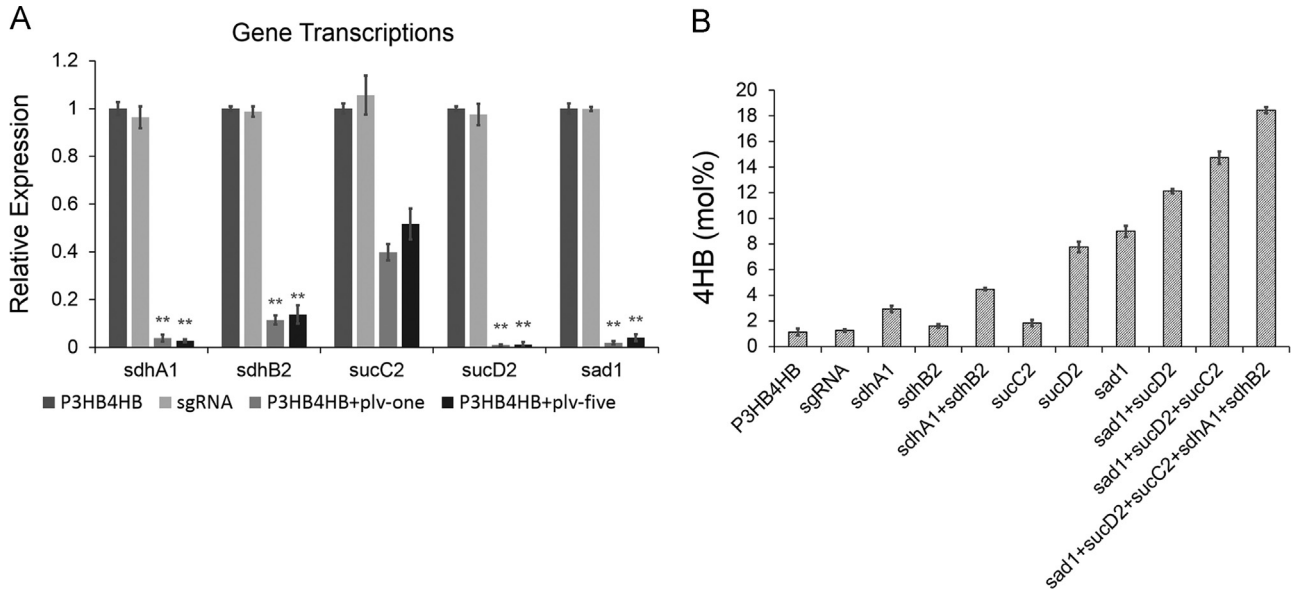

Fig. 5. Manipulation of expression of five genes in recombinant *E. coli*. (A) The mRNA levels of *sdhA*, *sdhB*, *sucC*, *sucD*, and *sad* genes in recombinant *E. coli*. (B) The 4HB ratio in P(3HB-*co*-4HB) synthesis by recombinant *E. coli*. P3HB4HB: *E. coli* S17-1 (p68orfZ+pMCSH5); sgRNA: *E. coli* S17-1 (p68orfZ+pMCSH5)+plv-sgRNA; P3HB4HB+plv-one: *E. coli* S17-1 (p68orfZ+pMCSH5)+plv-sdhA1/sdhB2/sucC2/sucD2/sad1; P3HB4HB+plv-five: *E. coli* S17-1 (p68orfZ+pMCSH5)+plv-sad1sucD2sucC2sdhA1sdhB2; sdhA1: *E. coli* S17-1 (p68orfZ+pMCSH5)+plv-sdhA1, the strain with CRISPRi system targeting *sdhA* gene; sdhB2: *E. coli* S17-1 (p68orfZ+pMCSH5)+plv-sdhB2, the strain with CRISPRi system targeting *sdhB* gene; sdhA1+sdhB2: *E. coli* S17-1 (p68orfZ+pMCSH5)+plv-sdhB2sdhA1, the strain with CRISPRi system targeting *sdhB* and *sdhA* genes; sucC2: *E. coli* S17-1 (p68orfZ+pMCSH5)+plv-sucC2; sucD2: *E. coli* S17-1 (p68orfZ+pMCSH5)+plv-sucD2; sad1: *E. coli* S17-1 (p68orfZ+pMCSH5)+plv-sad1; sad1+sucD2: *E. coli* S17-1 (p68orfZ+pMCSH5)+plv-sad1sucD2, the strain with CRISPRi system targeting *sad* and *sucD* genes; sad1+sucD2+sucC2: *E. coli* S17-1 (p68orfZ+pMCSH5)+plv-sad1sucD2sucC2; sad1+sucD2+sucC2+sdhA1+ sdhB2: *E. coli* S17-1 (p68orfZ+pMCSH5)+plv-sad1sucD2sucC2sdhA1sdhB2, the strain with CRISPRi system targeting *sdhA*, *sdhB*, *sucC*, *sucD*, and *sad* genes. Mean ± S.E. ($n=3$). * and ** indicate $p<0.05$ and $p<0.01$, respectively. See Section 3.3 for details.

growth of the host cells, so that the final product can be obtained with reasonable yield (Fig. 1 and Table 3).

Microbial synthesis of random copolymer P(3HB-*co*-4HB) or poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) demonstrated the capability of the CRISPRi system for achieving simultaneously repressed expression of multiple genes that regulate copolymer synthesis (Fig. 1). Furthermore, CRISPRi allows fine-tuning of single or multiple gene expression with less labor and time consumption (Figs. 2–5, S5 and S6). Such advantages provide a favorable opportunity to optimize multiple genes involving pathways that include the P(3HB-*co*-4HB) synthesis pathway.

To reduce P(3HB-*co*-4HB) production cost, the expense of the 4HB precursor that affects 4HB composition, including 1,4-butanediol, 4-hydroxybutyric acid or γ-butyrolactone, was avoided by constructing a new pathway involving only glucose as the carbon source (Fig. 1) (Kunioka et al., 1989; Mitomo et al., 2001; Lee et al., 2004; Kim et al., 2005; Zhang et al., 2009; Saito et al., 1996; Ishida et al., 2001). However, it was difficult to regulate 4HB composition in the copolymer because several genes affecting metabolic flux to 4HB are essential genes that cannot be deleted (Fig. 1). In this case, CRISPRi serves as a suitable method for controlling flux to 4HB involving multiple genes without deletions.

When expression of a single gene such as *sdhA*, *sdhB*, *sucC*, *sucD* or *sad* in the copolymer synthesis pathway was negatively regulated, recombinant *E. coli* strains grown in shake flasks produced P(3HB-*co*-4HB) consisting of 1–9 mol% 4HB, demonstrating the feasibility of CRISPRi in the *E. coli* system. When two genes involved in 4HB flux were simultaneously regulated, 4HB monomer production was increased to 12 mol% in the copolymer, illustrating the flexibility of the CRISPRi system (Table 3, Fig. 5B). When regulated gene number increased from two to three, P(3HB-*co*-4HB) consisting of 15 mol% 4HB was synthesized (Table 3, Fig. 5B). Remarkably, over 18 mol% 4HB in P(3HB-*co*-4HB) was achieved when all five genes including *sdhA*, *sdhB*, *sucC*, *sucD* and *sad* were repressed by the designed sgRNAs binding to the five genes simultaneously. All of the above results show that CRISPRi allows extensive regulation of gene expression. By designing proper sgRNA(s), it should also be possible to predictably regulate sgRNA(s) in the CRISPRi system.

The CRISPRi system works to regulate expression of various genes in the substrate competitive branches, such as the carbon flux from succinyl-CoA to succinate, succinate semi-aldehyde to succinate and then succinate to fumarate (Fig. 1). All of these reactions consume succinate semi-aldehyde, the 4HB precursor. Therefore, they all need to be restricted to increase the product—namely, the 4HB monomer. Meanwhile, it is also very important to balance the bacterial growth and formation of P(3HB-*co*-4HB) (Fig. S2). Importantly, recombinants harboring CRISPRi seemed to have only minor effect on the cell growth (Table 3). The feasibility of using CRISPRi for prokaryotic systems is thus convincing.

With the success of the CRISPRi system used in prokaryotic metabolic engineering demonstrated in this study, more applications are expected, including multiple gene repressions in β-oxidation pathway for production of PHA homopolymers (Liu et al., 2011), random and block copolymers (Li et al., 2014), as well as chiral hydroxyalkanoates (Chung et al., 2013) by *Pseudomonas* spp. and the manipulation of multiple genes in *Halomonas* spp. for low-cost production of various materials and chemicals (Fu et al., 2014). Very importantly, the manipulation of essential gene (s) becomes possible, as the CRISPRi system does not completely abolish functions of essential genes. With this in mind, we can now better utilize the CRISPRi system for optimizing multiple genes for better growth and larger production.

The recently reported small regulatory RNAs (sRNAs) method appears to be useful for similar functions. The sRNAs are short noncoding RNAs in prokaryotes that can control the expression of target genes in *trans* at the post-transcriptional level. Customized synthetic sRNAs consisting of a scaffold and a variable target-binding sequence can be prepared to target an mRNA to be repressed. This plasmid-based synthetic sRNA system does not require chromosomal modifications (Na et al., 2013; Yoo et al., 2013).

The CRISPRi system and the above-mentioned small regulatory RNAs (sRNAs) can regulate the gene expression level in the genome at different stages: the CRISPRi system influences the gene coding sequence to block transcription initiation or elongation, while the synthetic regulatory small RNAs system controls the expression of target genes in *trans* at the post-transcriptional level. Both systems can be more useful after further modification, such as reducing the PAM sequence (protospacer adjacent motif, sequence: NGG) influence on CRISPRi system binding specificity (Marraffini and Sontheimer, 2010) or enhancing sRNA stability. Very importantly, sequence-specific control of gene expression on a genome scale is an important approach for understanding gene function and for engineering genetic regulatory systems. These aims are becoming increasingly important in the fields of metabolic engineering and synthetic biology, as also evidenced in this study.

In the future, the CRISPRi system encoded by a plasmid should be made independent of antibiotics by using the addictive method or by integrating the plasmid into the host chromosome. The CRISPRi system will definitely play a very important role for metabolic engineering involving multiple gene regulation.

Acknowledgments

We are grateful to Professor LOU ChunBo of the Institute of Microbiology Chinese Academy of Sciences for the generous donation of sfGFP *E. coli* strain and the plasmid pdCas9 (mcs). The wild-type *E. coli* MG1655 was kindly provided by Professor Uwe Sauer of Institute of Molecular Systems Biology, ETH Zurich, Switzerland. We are also very grateful for the assistance of Professor OUYANG Qi of Peking University for many suggestions on this work. Mr. GAO Yan of the Tsinghua Biomed-instrument Center helped conduct the MS – MS analysis. This collaborative research was supported by the State Basic Science Foundation 973 Grant (No. 2012CB725201) to GQC. QW was grateful to 863 projects (No. 2012AA02A702 and No. 2013AA020301) for developing new techniques for controlling PHA compositions. A grant from National Natural Science Foundation of China (Grant no. 31430003 to GQC) also contributed to this study. Tsinghua University Initiative Scientific Research Program 2009THZ01005 partially funded this project.

Appendix. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ymben.2015.03.013.

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