microbial biotechnology

Open Access

Brief Report

CRISPR/Cas12a-mediated genome engineering in the photosynthetic bacterium *Rhodobacter capsulatus*

Yang Zhang and Jifeng Yuan*

State Key Laboratory of Cellular Stress Biology, Innovation Center for Cell Signaling Network, School of Life Sciences, Xiamen University, Fujian, 361102, China.

Summary

Purple non-sulfur photosynthetic bacteria (PNSB) such as *Rhodobacter capsulatus* serve as a versatile platform for fundamental studies and various biotechnological applications. In this study, we sought to develop the class II RNA-guided CRISPR/Cas12a system from *Francisella novicida* for genome editing and transcriptional regulation in *R. capsulatus*. Template-free disruption method mediated by CRISPR/Cas12a reached ~ 90% editing efficiency when targeting *ccoO* or *nifH* gene. When both genes were simultaneously edited, the multiplex editing efficiency reached > 63%. In addition, CRISPR interference (CRISPRi) using deactivated Cas12a was also evaluated using reporter genes *egfp* and *lacZ*, and the transcriptional repression efficiency reached ~ 80%. In summary, our work represents the first report to develop CRISPR/Cas12a-mediated genome editing and transcriptional regulation in *R. capsulatus*, which would greatly accelerate PNSB-related researches.

Introduction

Purple non-sulfur photosynthetic bacteria (PNSB) such as *Rhodobacter capsulatus* are facultative anaerobic gram-negative bacteria and are non-pathogenic (Heck and Drepper, 2017; Troost *et al.,* 2019). These bacteria have the versatile metabolic abilities to grow in a variety of habitats (Zannoni, 1995). For instance, *R. capsulatus* could use a wide spectrum of carbon sources including short-chain fatty acids, dicarboxylic acids, sugars, agricultural and food wastes for chemotrophic growth under aerobic to microaerobic conditions (Adessi *et al.,* 2017). It can also utilize the organic and inorganic compounds (such as $S_2O_3^{2-}$, H_2S and Fe^{2+}) as electron donors for photoheterotrophic growth (Romagnoli and Tabita, 2009; Jaschke *et al.,* 2011). Moreover, when the nitrogen source is scarce, *R. capsulatus* can perform the nitrogen fixation and hydrogen production via the action of nitrogenases (Zhang *et al.,* 2016).

To survive under such diverse living conditions, PNSB have evolved a complex metabolic network with highly specialized enzyme complexes and regulatory mechanisms. Recently, PNSB have attracted special attention as a platform microorganism for biotechnological applications such as H_2 production as an alternative renewable energy to fossil fuels (Zhang *et al*., 2016, 2017a,b,c) and polyhydroxybutyrate as a biodegradable plastic (Kranz *et al.,* 1997; Merugu *et al.,* 2012), bioremediation of wastewater (Idi *et al.,* 2014) and heavy metal (Feng *et al.,* 2007), fixation of CO_2 and N_2 (Wahlund *et al.,* 1996; Atsumi *et al.,* 2009), expression of membrane proteins (Berry *et al.,* 2004; Han and Perner, 2016) and metalloenzymes (Vignais *et al.,* 2000; Kappler and McEwan, 2002). As the synthesis of photopigments in PNSB relies on the isoprenoid biosynthetic pathway, it provides a robust isoprenoid metabolism that makes PNSB as a promising host for isoprenoid biosynthesis (Heck and Drepper, 2017; Loeschcke *et al.,* 2017; Troost *et al.,* 2019).

Currently, the genetic tools in PNSB heavily rely on traditional homologous recombination using suicide plasmids (Zhang *et al*., 2017a,b,c), interposon-mutagenesis using gene transfer agent (GTA) that leads to gene knockout via the insertion of a kanamycin cassette (Daldal *et al.,* 1986), and gene mutation using transposon (Zhang *et al.,* 2016). The synthetic biology tools for engineering PNSB are still limited, which significantly slows the progress in PNSB-related biotechnological applications. Recently, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated protein (Cas) systems

Received 7 December, 2020; revised 11 March, 2021; accepted 12 March, 2021.
*For correspondence. E-mail jfyuan@xmu.edu.cn; Tel. 86-592-2181602 ; Fax 86-592-2181015.
Microbial Biotechnology (2021) **14**(6), 2700–2710
doi:10.1111/1751-7915.13805
Funding information
This work was supported by Xiamen University (No. 0660-X2123310), the Natural Science Foundation of Fujian Province of China (No. 2020J05011), XMU Training Program of Innovation and Entrepreneurship for Undergraduates (No. 2020Y1000 and 202010384194), and ZhenSheng Biotech, China.

(Marraffini, 2015) have been developed as versatile tools for genome editing in a variety of organisms (Savitskaya *et al.,* 2016; Pickar-Oliver and Gersbach, 2019). For instance, the class II type II CRISPR/Cas9-mediated genome editing was recently established in PNSB such as *R. sphaeroides* (Mougiakos *et al.,* 2019; Luo *et al.,* 2020).

Cas12a, a class II type V-A endonuclease, is characterized as a dual nuclease referring to CRISPR RNA (crRNA) processing, target-site recognition and DNA cleavage (Zetsche *et al.,* 2015). To date, the CRISPR/Cas12a system has also been engineered into a genome editing tool in many species such as *Escherichia coli*, *Mycobacterium smegmatis* and mammalian cells (Yan *et al.,* 2017; Zetsche *et al.,* 2017). Compared with CRISPR/Cas9 that recognizes the target locus close to a guanine-rich protospacer adjacent motif (PAM) sequence to create blunt ends (Hsu *et al.,* 2014), Cas12a assisted by a mature crRNA binds the protospacer segment flanked by a thymidine-rich PAM to form staggered ends (Zetsche *et al.,* 2015; Swarts *et al.,* 2017). Considering PNSB normally has high GC content (~70%), the chance of off-target is expected to be higher for CRISPR/Cas9 than that of CRISPR/Cas12a. The compact design of crRNA and self-processing ability by Cas12a make CRISPR/Cas12a a more versatile and robust tool for multiplex genome engineering. To the best of our knowledge, there is no report on CRISPR/Cas12a-mediated genetic tools in PNSB. In this study, we sought to assess the feasibility of CRISPR/Cas12a-assisted genome editing in *R. capsulatus* (Fig. 1). In addition, we also attempted to evaluate CRISPR/dCas12a as an artificial transcriptional factor for CRISPR interference (CRISPRi) applications.

Results

Evaluation of CRISPR/Cas12a-mediated genome editing in R. capsulatus

We first evaluated the potential toxicity of Cas12a from *Francisella novicida* in *R. capsulatus*. The *Escherichia coli-Rhodobacter* shuttle vector pBBRdMCS, a multicopy vector derived from pBBR1MCS2 (Kovach *et al.,* 1995), was used to express *cas12a* gene under the control of P_{lac} promoter, and the resulting plasmid was designated as pBdRCas12a. In particular, we also included a *sacB* counter-selection marker from *Bacillus subtilis* for plasmid curing (Fig. 1) (Link *et al.,* 1997), whereas its expression is lethal when exposed to sucrose. According to the literature, P_{lac} promoter from *E. coli* was reported to confer constitutive activity in *R. capsulatus* due to the absence of the *lacI* repressor gene (Khan *et al.,* 2015). When the plasmid pBdRCas12a in parallel with the control plasmid pBdRSacB was transformed into *R. capsulatus*, the conjugation efficiency for pBdRCas12a was close to that achieved by the control (Fig. S1a). In addition, we did not observe noticeable growth differences between strains harbouring pBdRCas12a and pBdRSacB, suggesting that Cas12a is non-toxic to *R. capsulatus* under the tested condition.

To confirm that Cas12a is functional in *R. capsulatus*, *ccoO* gene encoding a subunit of cbb_3-cytochrome

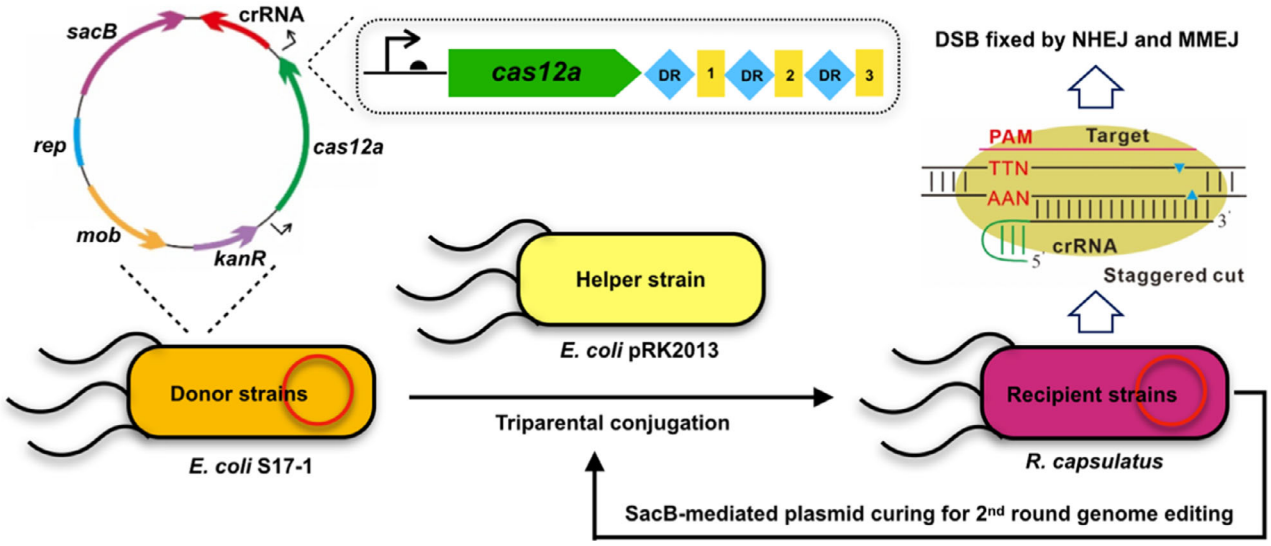

Fig. 1. Schematic diagram of CRISPR/Cas12a system for genome editing in *R. capsulatus*. Both Cas12a and crRNA were expressed from a low copy plasmid with kanamycin marker (*kanR*), shuttle replicon (*rep*) and mobilization element (*mob*) for transferring plasmid from the donor strains *E. coli* into the recipient strains of *R. capsulatus*. DSB, double-strand breaks; NHEJ, non-homologous end joining; MMEJ, microhomology-mediated end joining. The *sacB* cassette was used as the counter-selection marker for curing plasmid. The CRISPR/Cas12a system was first constructed in *E. coli* S17-1, then conjugated into *R. capsulatus* via the helper strain of *E. coli* pRK2013. The use of helper strain of *E. coli* pRK2013 is for the purpose of increasing the conjugation efficiency.

oxidase (cbb_3-Cox) and *nifH* gene encoding a subunit of nitrogenase were chosen as the disruption targets. The visualization of cbb_3-Cox activity using colorimetric assay was reported in *R. capsulatus* (Ekici *et al.,* 2012). As nitrogenase is essential for the nitrogen fixation, thus, *nifH* mutants would abolish the diazotrophic growth under nitrogen depleting conditions in a similar way to a previous report (Ungerer and Pakrasi, 2016). According to the literature, crRNA comprising of a 19-nt direct repeat and a 23-nt guide sequence is typically required to achieve the efficient genome editing (Zetsche *et al.,* 2015), and all the crRNAs were designed according to this criteria. The crRNA-expressing module was assembled into the pBdRCas12a plasmid under the control of the same promoter as Cas12a. We found the conjugation efficiencies declined more than 4 orders of magnitude compared with the control plasmid (Fig. S1a). The generation of double-strand breaks at the targeted genes by CRISPR/Cas12a typically results in decreased survival rates (Mougiakos *et al.,* 2019). As shown in Fig. 2A and B, *ccoO* or *nifH* was successfully mutated by CRISPR/Cas12a-mediated genome editing. However, the editing efficiency mediated by CRISPR/Cas12a was only 30%~40%.

It was reported that the genome editing efficiency assisted by CRISPR/Cas9 system was observably impacted by GC content of guide RNA and the target positions (Wang *et al.,* 2014). In this study, five crRNAs with different GC contents ranging from 39% to 83% were designed for *ccoO*. As shown in Fig. 2C, the crRNAs with different GC contents resulted in editing efficiencies ranging from 4% to 33%. In particular, similar efficiencies (25%, 33% and 30% respectively) were achieved for GC contents ranging from 39% to 65%, indicating that Cas12a might be not as sensitive to the GC content as the Cas9 counterpart.

Optimization of CRISPR/Cas12a-mediated genome editing in R. capsulatus

According to previous reports, efficient expression of Cas12a is also crucial to achieve efficient CRISPR/Cas12a-assisted genome editing system (Zhao *et al.,* 2019). As the heterologous promoter P_{lac} is a relatively weak promoter, the low editing efficiency might be attributed to the insufficient CRISPR/Cas12a expression. To validate this hypothesis, we sought to search alternative promoters to drive the expression of CRISPR/Cas12a in *R. capsulatus*. It was reported that promoters of *puc* operon (encoding light-harvesting complex II) and *puf* operon (encoding light-harvesting complex I) possess relatively high levels of expression under aerobic conditions (Hu *et al.,* 2010). As glycolytic enzymes are typically expressed at high levels in the presence of glucose, promoters of phosphoglycerate kinase gene (*pgk*) (Piper *et al.,* 1988) and enolase gene (*eno*) (Toda *et al.,* 2001) were also included in this study. The upstream ~ 400 bp noncoding regions from *puc*, *puf*, *pgk* and *eno* genes were cloned for measuring the promoter strengths using eGFP as the reporter. As shown in Fig. 3A, all native promoters exhibited much higher activity than P_{lac} under the tested condition. Among them, P_{puc} showed the highest activity, which corresponds to approximately 5.4-fold improvement over that of P_{lac}.

Next, the promoter P_{puc} was applied to drive the expression of CRISPR/Cas12a system in the remaining studies. When compared to the first-generation design, the new version of CRISPR/Cas12a system only differed in the promoter sequences (P_{puc} vs P_{lac}). These plasmids were transformed into *R. capsulatus* with pBRPpucSacB as the control plasmid. As depicted in Fig. S1b, the conjugation efficiency of Cas12a expression driven by P_{puc} was close to that of Cas12a driven by P_{lac}, suggesting that higher expression level of Cas12a was not toxic to *R. capsulatus*. As shown in Table 1, 10 randomly selected transformants on *ccoO* disruption conferred 93% editing efficiency as verified by α-naphthol + *N*′ *N*′-dimethyl-*p*-phenylenediamine (NADI) staining. The editing efficiency for the disruption of *nifH* reached up to 87% (Table 1). And it was observed that short indels (1 to 4 bp) occurred at the protospacer regions by further sequencing validation (Fig. 3B and C). In addition, approximately 63% positive transformants were observed during simultaneous disruption of *ccoO* and *nifH* as shown in Table 1. To further investigate the ability to simultaneously edit multiple genes, uracil-phosphoribosyltransferase (*upp*) gene was employed as the third target. Since *upp* disruption results in mutant strains resistant to 5-fuorouracil, *upp* mutants could be screened under 5-fuorouracil-containing medium in a similar way to a previous report (Mougiakos *et al.,* 2019). It was found that approximately 37% positive transformants were obtained when simultaneously editing *ccoO*, *nifH* and *upp* (Table 1, Figure S2). Taken together, these findings suggested that CRISPR/Cas12a could be an effective genome editing tool in PNSB such as *R. capsulatus*.

Transcriptional repression by CRISPRi using deactivated Cas12a (dCas12a)

Next, we proceeded to develop CRISPR/dCas12a as an artificial transcriptional factor for targeted gene knockdown. The nuclease activity in the RuvC domain of Cas12a was abolished by introducing the E1006A mutation (Tak *et al.,* 2017). The dCas12a driven by the promoter P_{puc} was cloned into a low copy *E. coli-Rhodobacter* shuttle vector pRK415 (Ditta *et al.,* 1985),

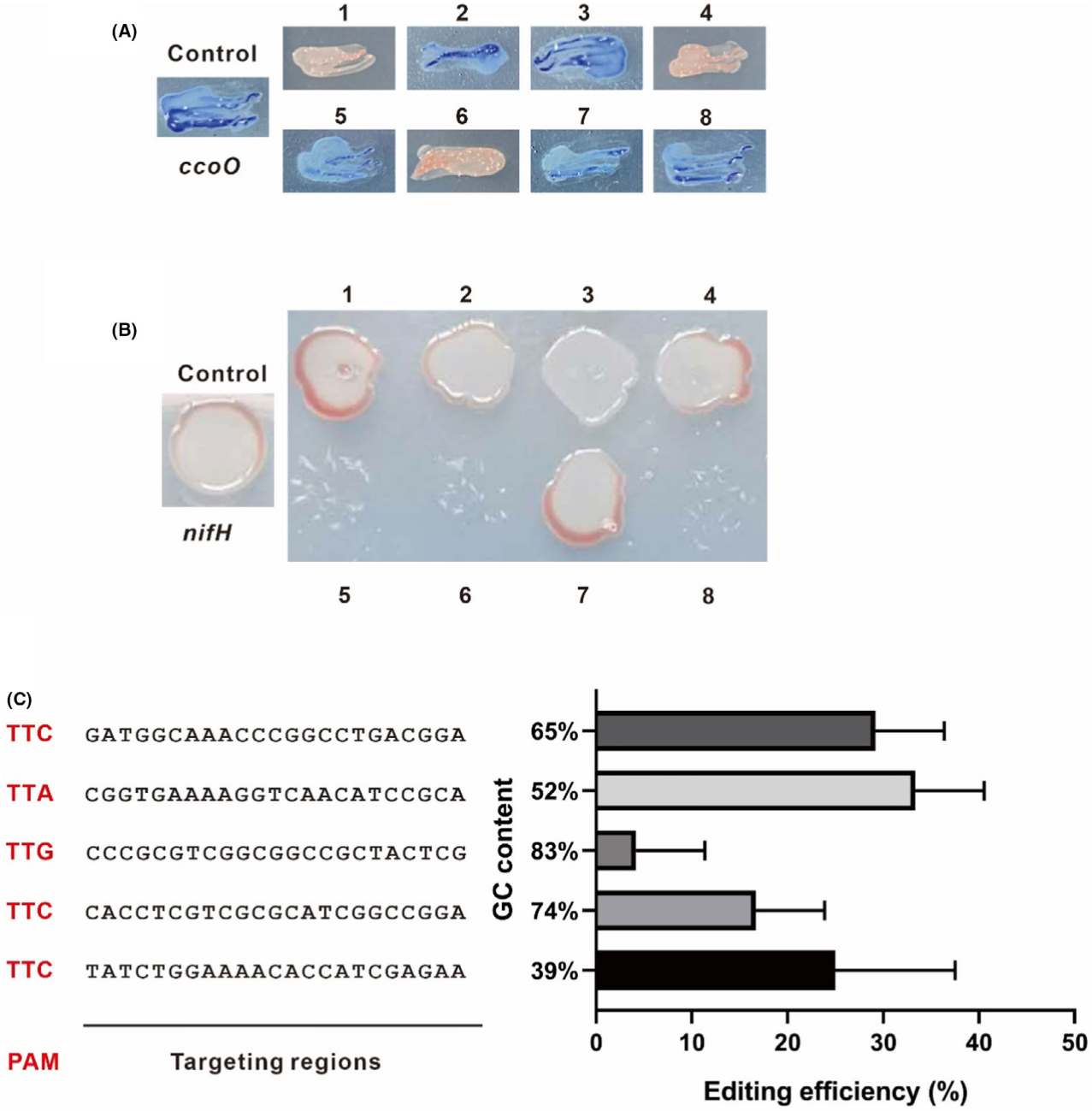

Fig. 2. Genome editing using CRISPR/Cas12a in *R. capsulatus*.
A. Verification of *ccoO* disruption by NADI staining. The parental *R. capsulatus* strain containing pBdRCas12a was employed as the control. The disruption of *ccoO* that abolishes the cbb_3-Cox activity would appear background colour instead of blue staining. Eight randomly selected colonies were tested for *ccoO* disruption.
B. Verification of *nifH* disruption by the diazotrophic growth under nitrogen depleting conditions. The parental *R. capsulatus* strain containing pBdRCas12a was employed as the control. The disruption of *nifH* is expected to abolish the diazotrophic growth under nitrogen depleting conditions. Eight randomly selected colonies were tested for *nifH* disruption.
C. Comparison of the genome editing efficiency of five crRNAs with different GC contents. Experiments were carried out in triplicate, and the data are presented as mean ± SD.

to obtain plasmid pRKucdCas12a (Fig. 4A). The transcriptional repression efficiency of CRISPRi was evaluated by employing the *egfp* and *lacZ* as the reporter genes, which were pre-integrated at the *phbC* encoding for poly-β-hydroxybutyrate (PHB) synthase and *pucBA* from the *pucBACDE* operon respectively. For each reporter gene, three crRNAs targeting at the template strand were designed (g1, g2 and g3 or z1, z2 and z3,

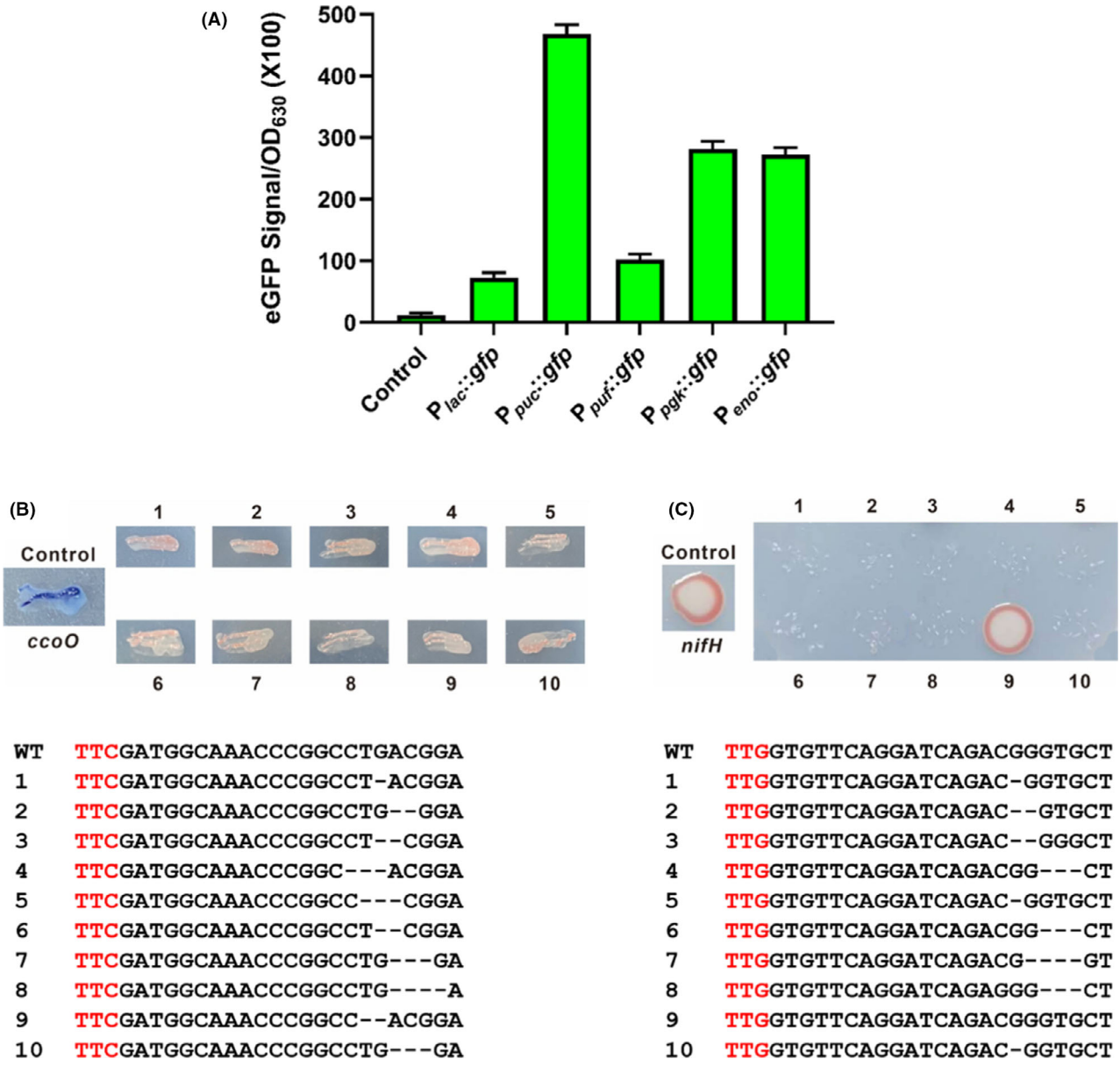

Fig. 3. Optimization of CRISPR/Cas12a-mediated genome editing in *R. capsulatus*.
A. Transcriptional activities of five promoters in *R. capsulatus* (P_{lac}, P_{puc}, P_{puf}, P_{pgk} and P_{eno}) as measured by eGFP fluorescence intensities. The control indicates the strain with the empty plasmid pBBR1MCS2. Experiments were carried out in triplicate, and the data are presented as mean ± SD.
B. NADI staining results of *ccoO* disruption and sequencing verification. The parental *R. capsulatus* strain containing pBRucCas12a was employed as the control. Ten randomly picked colonies were tested for *ccoO* disruption.
C. Results of the diazotrophic growth on *nifH* disruption and sequencing verification. The parental *R. capsulatus* strain containing pBRucCas12a was employed as the control. Ten randomly picked colonies were tested for *nifH* disruption.

as shown in Fig. 4B), and one crRNA targeting at the non-template strand was examined (g4 or z4, Fig. 4B). The above plasmids harbouring dCas12a with different crRNAs were then transformed into *R. capsulatus* with chromosomal integrated expression cassettes of *egfp* and *lacZ*. As shown in Fig. 4C and D, all the crRNAs targeting the template strand could lead to different degrees of repression of the *egfp* or *lacZ* expression. Similar to previous findings that crRNA-binding position near the translation initiation site on the template strand is more effective for CRISPRi (Kim *et al.,* 2017; Liu *et al.,* 2019), we found the most efficient repression occurred when the crRNAs were designed near the start codon (*egfp* and *lacZ* repression, 84% and 82%

Table 1. Editing efficiency using CRISPR/Cas12a driven by P_{puc} promoter.

Target gene	Replicate	Editing efficiency	Average editing efficiency
ccoO	1	100% (10/10)	93%[a]
	2	90% (9/10)	
	3	90% (9/10)	
nifH	1	90% (9/10)	87%[a]
	2	80% (8/10)	
	3	90% (9/10)	
ccoO, nifH	1	70% (7/10)	63%[a]
	2	60% (6/10)	
	3	60% (6/10)	
ccoO, nifH, upp	1	40% (4/10)	37%[b]
	2	40% (4/10)	
	3	30% (3/10)	

a. $P < 0.005$.
b. $P < 0.01$ using *t* test.

respectively). However, only ~ 20% repression was achieved when the non-template strand was targeted for both reporter genes. To test the capability of multiplex gene regulation, dual crRNA-expressing plasmid was constructed to simultaneously repress *egfp* and *lacZ* expressions. The expression levels of *egfp* and *lacZ* were reduced by 80% and 77% respectively. These findings indicated that the CRISPRi system based on dCas12a would have broad utilities to implement multiplex transcriptional repression in *R. capsulatus* for the future metabolic engineering applications.

Discussion

The CRISPR/Cas system is revolutionizing the field of genome editing in various organisms. The type V-A CRISPR/Cas12a system has the ability to self-process crRNA, recognize the thymidine-rich PAM and cleave DNA to give staggered ends. Due to these characteristics, CRISPR/Cas12a-assisted genome editing is a superior alternative to CRISPR/Cas9 in PNSB. In this work, we developed an efficient genetic tool using Cas12a from *F. novicida* in *R. capsulatus*, a promising platform microorganism for PNSB-related biotechnological applications. Based on previous findings, Cas9 from *S. pyogenes* (SpCas9) is toxic at high expression in several bacteria such as *Corynebacterium glutamicum* (Jiang *et al.,* 2017) and *Cyanobacteria* sp. (Ungerer and Pakrasi, 2016), which limits the application of CRISPR/Cas9 in these microorganisms. In this work, we found that Cas12a expression did not show any noticeable toxicity in *R. capsulatus*.

As CRISPR/Cas12a creates a staggered end resulting a 5-nt 5′ overhang rather than a blunt cleavage product, which might promote the double-strand break repair mediated by non-homologous end joining (NHEJ) and/or microhomology-mediated end joining (MMEJ) mechanism (Finger-Bou *et al.,* 2020). The initial studies revealed that about 30%~40% editing efficiency was obtained when targeting *ccoO* or *nifH* gene, which was not satisfactory when compared to NHEJ-mediated CRISPR/Cas in *Streptomyces coelicolor* (Li *et al.,* 2018) and *E. coli* (Zheng *et al.,* 2017). To further improve the genome editing efficiency in *R. capsulatus*, a strong promoter of photosynthetic operon *puc* (P_{puc}) was identified to drive the CRISPR/Cas12a system, and ~ 90% editing efficiency was achieved for single gene disruption, whereas 63% editing efficiency was obtained for two gene disruptions. Moreover, the ability of this system to simultaneous multiple genes editing was further investigated, 37% editing efficiency was observed for triple gene disruption. Recently, CRISPR/Cas9 and CRISPR/Cas9-deaminase have been successfully implemented into *R. sphaeroides* (Mougiakos *et al.,* 2019; Luo *et al.,* 2020). Considering *R. capsulatus* has relatively high GC content (~ 70%), the chance of off-target is expected to be higher for CRISPR/Cas9 than that of CRISPR/Cas12a.

To the best of our knowledge, CRISPRi using DNase-deactivated Cas9 (dCas9) or dCas12a has not been reported in any *Rhodobacter*-related species. Previous findings suggested that dCas12a exhibits better repression efficiency with crRNAs targeting at the template strand over the non-template strand, and the closer crRNA near to the translation start site, the higher repression efficiency is obtained (Zhang *et al*., 2017a,b, c; Liu *et al.,* 2019; Zhao *et al.,* 2019). In this study, similar results were observed when targeting at *egfp* or *lacZ* reporter genes in *R. capsulatus* using CRISPR/dCas12a. More importantly, dual repression of *egfp* and *lacZ* genes reached a similar efficiency to that of single target, indicating that CRISPR/dCas12a might be applicable for multiplex transcription regulation in *R. capsulatus*.

Experimental procedures

Strains and culture conditions

All plasmids were introduced by heat-shock into competent cells of *Escherichia coli* S17-1 or Top 10 cultured with Luria-Bertani (LB) medium containing appropriate antibiotics at 37°C. *R. capsulatus* SB1003 was employed for genetic modifications, and the strain was cultured in Sistrom's mineral media supplemented with 10 g l^{-1} glucose (MedA) (Sistrom, 1960) at 35°C under aerobic conditions. Antibiotics were used at the following final concentrations: gentamycin 12 mg ml^{-1}, tetracycline 12.5 mg l^{-1} and kanamycin 50 mg l^{-1} for *E. coli*; gentamycin 12 mg ml^{-1}, tetracycline 2.5 mg l^{-1} and kanamycin 10 mg l^{-1} for *R. capsulatus*. All strains used in this study are listed in Table S1.

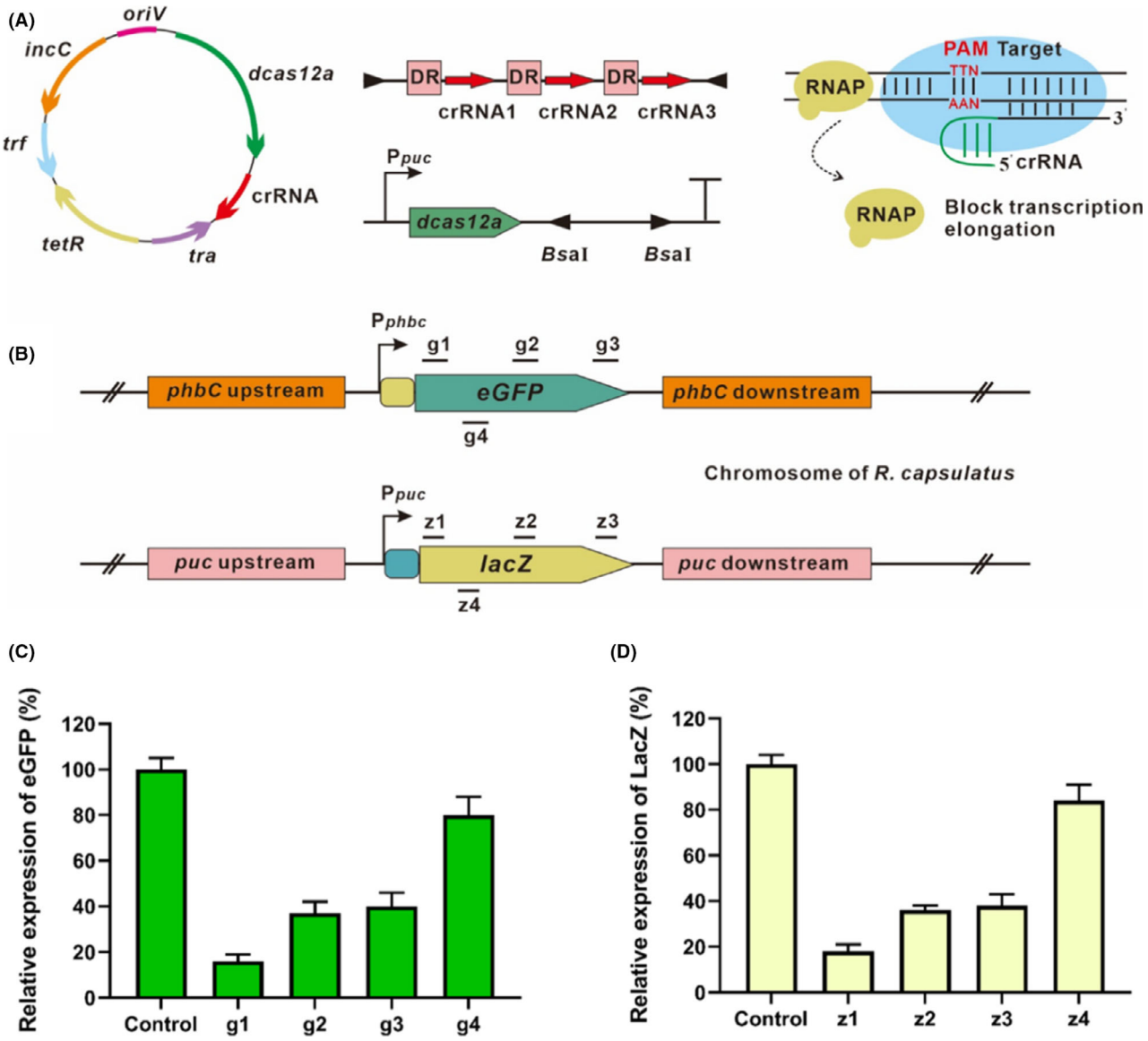

Fig. 4. Transcriptional repression of *egfp* and *lacZ* by CRISPRi based on dCas12a.
A. dCas12a and crRNA driven by promoter P_{puc} were expressed from a low copy plasmid with tetracycline marker (*tetR*), shuttle replicon *incC*, origin of replication *oriV*, *tra* combined with *trf* for transferring plasmid from *E. coli* to *R. capsulatus*.
B. crRNAs targeting different positions on the template strand (g1, g2 and g3 or z1, z2 and z3) and the non-template strand (g4 or z4) of *egfp* or *lacZ* gene, which were pre-integrated into the *R. capsulatus* chromosome.
C. Comparison of *egfp* expression level suppressed by dCas12a when targeting at different positions of the template and non-template strand.
D. Comparison of *lacZ* expression level suppressed by dCas12a when targeting different positions of the template and non-template strand. All the above experiments were carried out in triplicate, and the control indicates strains expressing dCas12a without crRNA. The data are presented as mean ± SD.

Plasmid constructions

All oligonucleotides utilized in this work are listed in Table S2. Plasmids and genomic DNA were extracted by Biospin kits from Bioer Technology (Shanghai, China). PCR amplification was conducted by High Fidelity Phusion DNA polymerase or Taq polymerase from New England Biolab (Ipswich, MA, USA). Plasmids were constructed by standard restriction endonucleases and ligation approach followed by digestion analysis and DNA sequencing verification. The detailed procedures were described in Supplementary Materials and Methods. All the plasmid constructs utilized in this study are listed in Table S3.

Triparental conjugation procedure

Plasmids were transformed into *R. capsulatus* by triparental conjugation with the helper plasmid of pRK2013

(Ditta *et al.,* 1980). Before the conjugation, *R. capsulatus* was grown with 10 ml MedA at 35°C and 250 r.p.m. for 48 h under aerobic conditions until optical density at 630 nm (OD_{630}) reached approximately 1.8. The *R. capsulatus* cells were centrifuged, collected and washed once with fresh MedA and re-suspended with 400 μl MedA. Meanwhile, *E. coli* HB101 harbouring the helper plasmid pRK2013 and *E. coli* S17-1 carrying the desired plasmid were incubated in 5 ml LB medium supplemented with proper antibiotics at 37°C and 250 r.p.m. for overnight until the respective OD at 600 nm reached approximately 2.8. The *E. coli* cells were centrifuged, collected and washed once with fresh MedA and re-suspended with 1 ml MedA respectively. *R. capsulatus* of 100 μl as a recipient strain, *E. coli* HB101/pRK2013 of 30 μl as a helper strain and *E. coli* S17-1 of 30 μl as a donor strain were mixed and spotted into a MedA agar plate as a ~ 2 cm diameter spot incubating at 35°C for 24 h. The mixed cells were centrifuged, collected, washed with fresh MedA and re-suspended with 1 ml MedA. The re-suspended cell culture of 100 μl was spread on a MedA agar plate with appropriate antibiotic and incubated at 35°C for 2–3 days until colonies appeared.

CRISPR/Cas12a-mediated genome editing and transcriptional repression in R. capsulatus

For CRISPR/Cas12a-assisted genome editing, pBdRCas12a or pBRucCas12a derivatives carrying the corresponding crRNAs were transformed into *R. capsulatus* by triparental conjugation for disrupting the target genes. In order to assess the editing efficiency, single colonies from each plate were randomly selected. Each colony was re-suspended in 10 μl sterile H_2O, which was used as the template for PCR amplification of about 500 bp flanking the region. Then, the PCR products of the tested colonies were sent for sequencing, whereas the parental strain was used as the negative control. Meanwhile, the remaining bacterial mixture was streaked or spread onto the plates for phenotype observation, whereas the parental strain carrying pBdRCas12a or pBRucCas12a was used as the negative control. For curing the CRISPR/Cas12a plasmid, the *R. capsulatus* mutants were re-streaked on MedA plates containing 10% sucrose at 35°C for 48 h. Only, these colonies that are kanamycin sensitive were considered to have the CRISPR/Cas12a plasmid removed, and these strains would be used for the subsequent rounds of genetic modifications.

For CRISPR/dCas12a mediated transcriptional repression, *egfp* and *lacZ* expression cassettes were integrated at *phbC* and *pucBA* sites of the chromosome via the traditional homologous recombination mediated by suicide plasmids, pZJD29c and pRK18mobSacB as previously described (Zhang *et al.,* 2016). Briefly, several recombinants containing pZJ-ΔphbC::eGFP or pRK18-ΔpucBA::lacZ were obtained by triparental conjugation, which generated single-crossover chromosomal integrants. The colonies were then cultivated in MedA medium for overnight so that there was a second homologous recombination event to excise the plasmid DNA. The culture was diluted and spread on the MedA agar plates containing 10% sucrose. Colonies grown on these plates were subjected to diagnostic PCR analysis to confirm the integration of *egfp* and *lacZ* cassette into the *phbC* and *pucBA* sites. Next, pRKucdCas12a-derived plasmids carrying the corresponding crRNAs were transformed into *R. capsulatus* and selected on the MedA agar plate supplemented with tetracycline. The fluorescence intensities and LacZ activities were measured for comparing the transcriptional repression.

In vivo cbb_3-*cytochrome oxidase activity assay*

To test whether the *ccoO* gene encoding for a subunit of cbb_3-cytochrome oxidase (cbb_3-Cox) was disrupted by CRISPR/Cas12a system, the *in vivo* cbb_3-Cox activity of *R. capsulatus* was visualized qualitatively using the NADI reaction (α-naphthol + *N*′ *N*′-dimethyl-*p*-phenylenediamine → indophenol blue + H_2O) by staining the plates with the mixture of 35 mM α-naphthol and 30 mM *N*′ *N*′-dimethyl-*p*-phenylenediamine dissolved in 1:1 (vol/vol) ethanol and water (Ekici *et al.,* 2012). The randomly selected colonies of *R. capsulatus* were streaked on MedA plates with kanamycin grown at 35°C for 48 h. When the *ccoO* gene was successfully disrupted, the colonies lack of cbb_3-Cox activity showed no staining ($NADI^-$) up to 15 min, otherwise, those with cbb_3-Cox activity exhibited dark blue staining ($NADI^+$) within 30 s to 1 min.

Nitrogenase activity assay

To test whether the *nifH* gene encoding for a structural gene of nitrogenase was disrupted by CRISPR/Cas12a system, the nitrogenase activity was visualized qualitatively by the diazotrophic growth with MedA in the absence of nitrogen source (MedA-N). The randomly selected colonies of *R. capsulatus* were incubated in MedA with kanamycin for 24 h. Then, 1 ml of cell culture was pelleted by centrifugation, washed once with MedA-N and re-suspended in 1 ml MedA-N. 10 μl of re-suspended culture was spotted onto MedA-N agar plates with kanamycin and cultivated for 48 h at 35°C. When the *nifH* gene was disrupted, the strains would abolish the diazotrophic growth on the MedA-N plates, while those with intact *nifH* would grow normally.

5-Fuorouracil screening for upp *disruption*

To test whether the *upp* gene encoding for uracil-phosphoribosyltransferase was disrupted by CRISPR/Cas12a system, the mutants were visualized qualitatively by the growth with MedA in the presence of 100 mg l^{-1} 5-fuorouracil (MedAF) using a 50 g l^{-1} stock solution prepared in dimethyl sulfoxide. The randomly selected colonies of *R. capsulatus* were incubated in MedA with kanamycin for 24 h. Then, 1 ml of cell culture was pelleted by centrifugation, washed once with MedAF and re-suspended in 1 ml MedAF. 10 μl of re-suspended culture was spotted onto MedAF agar plates with kanamycin and cultivated for 48 h at 35°C. When the *upp* gene was disrupted, the strains grow normally on the MedAF plates, while those with intact *upp* would not survive.

Fluorescence intensities and β-galactosidase activity assays

To measure the eGFP fluorescence, *R. capsulatus* recombinants were cultured in 5 ml MedA medium for 48 h under aerobic conditions. Optical density at 630 nm and fluorescence intensities was monitored by a microplate reader Synergy H1 from BioTek (Winooski, VT, USA). The excitation of eGFP was set at 485 nm and emission at 510 nm. Fluorescence intensities were normalized to culture OD_{630} for comparing the relative expression level of eGFP. The β-galactosidase activity assay was performed as previously described (Zhang *et al*., 2017a,b,c). In brief, the active protein exacted from *R. capsulatus* reacted with *o*-nitrophenyl-β-D-galactopyranoside (ONPG) at 35°C. When the reaction is over, the mixture was employed to detect β-galactosidase activity using the following equation:

$$GA = \frac{10^6 \times OD_{420}}{4860 \times tm}$$

where GA is the β-galactosidase activity (μM ONPG-Hydrolyzed/(min·mg-protein)), OD_{420} is the optical density at 420 nm, t is the reaction time (min) and m is the mass of the protein (mg).

Acknowledgements

This work was supported by Xiamen University (No. 0660-X2123310), the Natural Science Foundation of Fujian Province of China (No. 2020J05011), XMU Training Program of Innovation and Entrepreneurship for Undergraduates (No. 2020Y1000 and 202010384194), and ZhenSheng Biotech, China.

Conflict of interests

The authors declare no competing financial interests.

Author contributions

J.Y. conceived the project. Y.Z. performed the experiments and collected the data. J.Y. and Y.Z. interpreted the data and wrote the manuscript.

REFERENCES

Adessi, A., Corneli, E., and De Philippis, R. (2017) Photosynthetic purple non sulfur bacteria in hydrogen producing systems: New approaches in the use of well known and innovative substrates. In *Modern Topics in the Phototrophic Prokaryotes: Environmental and Applied Aspects*. Hallenbeck, P.C. (ed). Cham: Springer International Publishing, pp. 321–350.

Atsumi, S., Higashide, W., and Liao, J.C. (2009) Direct photosynthetic recycling of carbon dioxide to isobutyraldehyde. *Nat Biotechnol* **27:** 1177–1180.

Berry, E.A., Huang, L.S., Saechao, L.K., Pon, N.G., Valkova-Valchanova, M., and Daldal, F. (2004) X-ray structure of Rhodobacter capsulatus cytochrome bc_1: comparison with its mitochondrial and chloroplast counterparts. *Photosynth Res* **81:** 251–275.

Daldal, F., Cheng, S., Applebaum, J., Davidson, E., and Prince, R.C. (1986) Cytochrome c_2 is not essential for photosynthetic growth of *Rhodopseudomonas capsulata*. *Proc Natl Acad Sci USA* **83:** 2012–2016.

Ditta, G., Schmidhauser, T., Yakobson, E., Lu, P., Liang, X.W., Finlay, D.R., *et al*. (1985) Plasmids related to the broad host range vector, pRK290, useful for gene cloning and for monitoring gene expression. *Plasmid* **13:** 149–153.

Ditta, G., Stanfield, S., Corbin, D., and Helinski, D.R. (1980) Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. *Proc Natl Acad Sci USA* **77:** 7347–7351.

Ekici, S., Yang, H., Koch, H.G., and Daldal, F. (2012) Novel transporter required for biogenesis of cbb3-type cytochrome c oxidase in *Rhodobacter capsulatus*. *MBio* **3:** e00293-11.

Feng, Y., Yu, Y., Wang, Y., and Lin, X. (2007) Biosorption and bioreduction of trivalent aurum by photosynthetic bacteria *Rhodobacter capsulatus*. *Curr Microbiol* **55:** 402–408.

Finger-Bou, M., Orsi, E., van der Oost, J., and Staals, R.H.J. (2020) CRISPR with a happy ending: Non-templated DNA repair for prokaryotic genome engineering. *Biotechnol J* **15:** e1900404.

Han, Y.C., and Perner, M. (2016) Sulfide consumption in sulfurimonas denitrificans and heterologous expression of its three sulfide-quinone reductase homologs. *J Bacteriol* **198:** 1260–1267.

Heck, A., and Drepper, T. (2017) Engineering photosynthetic α-proteobacteria for the production of recombinant proteins and terpenoids. In *Modern Topics in the Phototrophic Prokaryotes: Environmental and Applied Aspects*. Hallenbeck, P.C. (ed). Cham: Springer International Publishing, pp. 395–425.

Hsu, P.D., Lander, E.S., and Zhang, F. (2014) Development and applications of CRISPR-Cas9 for genome engineering. *Cell* **157:** 1262–1278.

Hu, Z.L., Zhao, Z.P., Pan, Y., Tu, Y., and Chen, G.P. (2010) A powerful hybrid puc operon promoter tightly regulated by both IPTG and low oxygen level. *Biochemistry* **75:** 519–525.

Idi, A., Md Nor, M.H., Abdul Wahab, M.F., and Ibrahim, Z. (2014) Photosynthetic bacteria: an eco-friendly and cheap tool for bioremediation. *Rev Environ Sci BioTechnol* **14:** 271–285.

Jaschke, P.R., Saer, R.G., Noll, S., and Beatty, J.T. (2011) Chapter twenty-three - Modification of the genome of *Rhodobacter sphaeroides* and construction of synthetic operons. In *Methods in Enzymology*. Voigt, C. (ed). Cambridge, MA: Academic Press, pp. 519–538.

Jiang, Y., Qian, F.H., Yang, J.J., Liu, Y.M., Dong, F., Xu, C.M., *et al*. (2017) CRISPR-Cpf1 assisted genome editing of *Corynebacterium glutamicum*. *Nat Commun* **8:** 15179.

Kappler, U., and McEwan, A.G. (2002) A system for the heterologous expression of complex redox proteins in *Rhodobacter capsulatus*: characterisation of recombinant sulphite:cytochrome c oxidoreductase from *Starkeya novella*. *FEBS Lett* **529:** 208–214.

Khan, N.E., Nybo, S.E., Chappell, J., and Curtis, W.R. (2015) Triterpene hydrocarbon production engineered into a metabolically versatile host-*Rhodobacter capsulatus*. *Biotechnol Bioeng* **112:** 1523–1532.

Kim, S.K., Kim, H., Ahn, W.C., Park, K.H., Woo, E.J., Lee, D.H., and Lee, S.G. (2017) Efficient transcriptional gene repression by Type V-A CRISPR-Cpf1 from *Eubacterium eligens*. *ACS Synth Biol* **6:** 1273–1282.

Kovach, M.E., Elzer, P.H., Hill, D.S., Robertson, G.T., Farris, M.A., Roop, R.M. 2nd, and Peterson, K.M. (1995) Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* **166:** 175–176.

Kranz, R.G., Gabbert, K.K., Locke, T.A., and Madigan, M.T. (1997) Polyhydroxyalkanoate production in *Rhodobacter capsulatus*: genes, mutants, expression, and physiology. *Appl Environ Microb* **63:** 3003–3009.

Li, L., Wei, K., Zheng, G., Liu, X., Chen, S., Jiang, W., and Lu, Y. (2018) CRISPR-Cpf1-assisted multiplex genome editing and transcriptional repression in Streptomyces. *Appl Environ Microb* **84:** e00827-18.

Link, A.J., Phillips, D., and Church, G.M. (1997) Methods for generating precise deletions and insertions in the genome of wild-type *Escherichia coli*: application to open reading frame characterization. *J Bacteriol* **179:** 6228–6237.

Liu, W., Tang, D., Wang, H., Lian, J., Huang, L., and Xu, Z. (2019) Combined genome editing and transcriptional repression for metabolic pathway engineering in Corynebacterium glutamicum using a catalytically active Cas12a. *Appl Environ Microb* **103:** 8911–8922.

Loeschcke, A., Dienst, D., Wewer, V., Hage-Hulsmann, J., Dietsch, M., Kranz-Finger, S., *et al*. (2017) The photosynthetic bacteria *Rhodobacter capsulatus* and *Synechocystis sp*. PCC 6803 as new hosts for cyclic plant triterpene biosynthesis. *PLoS One* **12:** e0189816.

Luo, Y., Ge, M., Wang, B., Sun, C., Wang, J., Dong, Y., and Xi, J.J. (2020) CRISPR/Cas9-deaminase enables robust base editing in *Rhodobacter sphaeroides* 2.4.1. *Microb Cell Fact* **19:** 93.

Marraffini, L.A. (2015) CRISPR-Cas immunity in prokaryotes. *Nature* **526:** 55–61.

Merugu, R., Rudra, M., Sivadevuni, G., and Reddy, S. (2012) PHB (Polyhydroxy butyrate) production under nitrogen limitation by *Rhodobacter capsulatus* KU002 isolated from tannery effluent. *Int J Chem Tech Res* **4:** 1099–1102.

Mougiakos, I., Orsi, E., Ghiffary, M.R., Post, W., de Maria, A., Adiego-Perez, B., *et al*. (2019) Efficient Cas9-based genome editing of *Rhodobacter sphaeroides* for metabolic engineering. *Microb Cell Fact* **18:** 204.

Pickar-Oliver, A., and Gersbach, C.A. (2019) The next generation of CRISPR-Cas technologies and applications. *Nat Rev Mol Cell Biol* **20:** 490–507.

Piper, P.W., Curran, B., Davies, M.W., Hirst, K., Lockheart, A., Ogden, J.E., *et al*. (1988) A heat shock element in the phosphoglycerate kinase gene promoter of yeast. *Nucleic Acids Res* **16:** 1333–1348.

Romagnoli, S., and Tabita, F.R. (2009) Carbon dioxide metabolism and its regulation in nonsulfur purple photosynthetic bacteria. In *The Purple Phototrophic Bacteria*. Hunter, C.N., Daldal, F., Thurnauer, M.C., and Beatty, J.T. (eds). Dordrecht, The Netherlands: Springer, pp. 563–576.

Savitskaya, E.E., Musharova, O.S., and Severinov, K.V. (2016) Diversity of CRISPR-Cas-mediated mechanisms of adaptive immunity in prokaryotes and their application in biotechnology. *Biochemistry* **81:** 653–661.

Sistrom, W.R. (1960) A requirement for sodium in the growth of *Rhodopseudomonas spheroides*. *J Gen Microbiol* **22:** 778–785.

Swarts, D.C., van der Oost, J., and Jinek, M. (2017) Structural basis for guide RNA processing and seed-dependent DNA targeting by CRISPR-Cas12a. *Mol Cell* **66:** 221–233.

Tak, Y.E., Kleinstiver, B.P., Nunez, J.K., Hsu, J.Y., Horng, J.E., Gong, J.Y., *et al*. (2017) Inducible and multiplex gene regulation using CRISPR-Cpf1-based transcription factors. *Nat Methods* **14:** 1163–1166.

Toda, T., Sano, M., Honda, M., Rimoldi, O.J., Yang, Y., Yamamoto, M., *et al*. (2001) Deletion analysis of the enolase gene (enoA) promoter from the filamentous fungus *Aspegillus oryzae*. *Curr Genet* **40:** 260–267.

Troost, K., Loeschcke, A., Hilgers, F., Ozgur, A.Y., Weber, T.M., Santiago-Schubel, B., *et al*. (2019) Engineered *Rhodobacter capsulatus* as a phototrophic platform organism for the synthesis of plant sesquiterpenoids. *Front Microbiol* **10:** 1998.

Ungerer, J., and Pakrasi, H.B. (2016) Cpf1 is a versatile tool for CRISPR genome editing across diverse species of Cyanobacteria. *Sci Rep* **6:** 39681.

Vignais, P.M., Dimon, B., Zorin, N.A., Tomiyama, M., and Colbeau, A. (2000) Characterization of the hydrogen-deuterium exchange activities of the energy-transducing HupSL hydrogenase and H_2-signaling HupUV hydrogenase in *Rhodobacter capsulatus*. *J Bacteriol* **182:** 5997–6004.

Wahlund, T.M., Conway, T., and Tabita, F.R. (1996) Bioconversion of CO_2 to ethanol and other compounds. Abstr Pap Am Chem S 212: 120-Fuel.

Wang, T., Wei, J.J., Sabatini, D.M., and Lander, E.S. (2014) Genetic screens in human cells using the CRISPR-Cas9 system. *Science* **343:** 80–84.

Yan, M.Y., Yan, H.Q., Ren, G.X., Zhao, J.P., Guo, X.P., and Sun, Y.C. (2017) CRISPR-Cas12a-assisted recombineering in bacteria. *Appl Environ Microb* **83:** e00947-17.

Zannoni, D. (1995) Aerobic and anaerobic electron transport chains in anoxygenic phototrophic bacteria. In *Anoxygenic Photosynthetic Bacteria*. Blankenship, R.E., Madigan, M.T., and Bauer, C.E. (eds). Dordrecht, The Netherlands: Springer, pp. 949–971.

Zetsche, B., Gootenberg, J.S., Abudayyeh, O.O., Slaymaker, I.M., Makarova, K.S., Essletzbichler, P., *et al*. (2015) Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. *Cell* **163:** 759–771.

Zetsche, B., Heidenreich, M., Mohanraju, P., Fedorova, I., Kneppers, J., DeGennaro, E.M., *et al*. (2017) Multiplex gene editing by CRISPR-Cpf1 using a single crRNA array. *Nat Biotechnol* **35:** 31–34.

Zhang, X.C., Wang, J.M., Cheng, Q.X., Zheng, X., Zhao, G.P., and Wang, J. (2017a) Multiplex gene regulation by CRISPR-ddCpf1. *Cell Discov* **3:** 17018.

Zhang, Y., Hu, J., Ma, H., Yang, H., and Guo, L. (2017b) Overexpressing *atpXF* enhanced photo-fermentative hydrogen production performance of *Rhodobacter sphaeroides*. *Int J Hydrog Energy* **42:** 9641–9649.

Zhang, Y., Li, Q., Wang, X., Yang, H., and Guo, L. (2017c) Enhanced biohydrogen production from cornstalk through a two-step fermentation: Dark fermentation and photofermentation. *Int J Energy Res* **41:** 2491–2501.

Zhang, Y., Yang, H., and Guo, L. (2016) Enhancing photofermentative hydrogen production performance of *Rhodobacter capsulatus* by disrupting methylmalonate-semialdehyde dehydrogenase gene. *Int J Hydrogen Energy* **41:** 190–197.

Zhao, R., Liu, Y., Zhang, H., Chai, C., Wang, J., Jiang, W., and Gu, Y. (2019) CRISPR-Cas12a-mediated gene deletion and regulation in *Clostridium ljungdahlii* and its application in carbon flux redirection in synthesis gas fermentation. *ACS Syn Biol* **8:** 2270–2279.

Zheng, X., Li, S.Y., Zhao, G.P., and Wang, J. (2017) An efficient system for deletion of large DNA fragments in *Escherichia coli* via introduction of both Cas9 and the non-homologous end joining system from *Mycobacterium smegmatis*. *Biochem Biophys Res Commun* **485:** 768–774.

Supporting information

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

Table S1. Strains used in this study.

Table S2. Oligonucleotides used in this study.

Table S3. Plasmids used in this study.

Fig. S1. The conjugation efficiency of *R. capsulatus* using CRISPR/Cas12a system. (a) Colony forming unit (c.f.u) of conjugation mixtures containing Cas12a driven by promoter P_{lac}. The control indicates the conjugation efficiency of *R. capsulatus* with plasmid pBdRSacB. (b) Colony forming unit obtained after conjugation of empty vector and Cas12a by promoter P_{puc}. The control indicates the conjugation efficiency of *R. capsulatus* with plasmid pBRPpucSacB. Experiments were carried out in triplicate, and the data are presented as mean $\pm$ SD.

Fig. S2. Triple gene deletion in *R. capsulatus* using CRISPR/Cas12a. (a) NADI staining results of *ccoO* disruption and sequencing verification. (b) Results of the diazotrophic growth on *nifH* disruption and sequencing verification. (c) 5-Fuorouracil screening results of *upp* disruption and sequencing verification. The parental *R. capsulatus* strain containing pBRucCas12a was employed as the control. Ten randomly picked colonies were tested for *ccoO, nifH, upp* disruption.

