1 CRISPR/Cas12a mediated genome engineering in 2 photosynthetic bacteria

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7 ABSTRACT

8 Purple non-sulfur photosynthetic bacteria (PNSB) such as *R. capsulatus* serve as a
9 versatile platform for fundamental studies and various biotechnological applications.
10 In this study, we sought to develop the class II RNA-guided CRISPR/Cas12a system
11 from *Francisella novicida* for both genome editing and gene down-regulation in *R.*
12 *capsulatus*. About 90% editing efficiency was achieved by using CRISPR/Cas12a
13 driven by a strong promoter P_{puc} when targeting *ccoO* or *nifH* gene. When both genes
14 were simultaneously targeted, the multiplex gene editing efficiency reached >63%. In
15 addition, CRISPR interference using deactivated Cas12a was also evaluated using
16 reporter genes *gfp* and *lacZ*, and the repression efficiency reached >80%. In summary,
17 our work represents the first report to develop CRISPR/Cas12a mediated genome
18 editing/transcriptional repression in *R. capsulatus*, which would greatly accelerate
19 PNSB-related researches.

20 **IMPORTANCE** Purple non-sulfur photosynthetic bacteria (PNSB) such as *R.*
21 *capsulatus* serve as a versatile platform for fundamental studies and various

1 biotechnological applications. However, lack of efficient gene editing tools remains a
2 main obstacle for progressing in PNSB-related researches. Here, we developed
3 CRISPR/Cas12a for genome editing via the non-homologous end joining (NHEJ)
4 repair machinery in *R. capsulatus*. In addition, DNase-deactivated Cas12a was found
5 to simultaneously suppress multiple targeted genes. Taken together, our work offers a
6 new set of tools for efficient genome engineering in PNSB such as *R. capsulatus*.

7 **Key words:** CRISPR/Cas12a, genome editing, CRISPR interference, photosynthetic
8 bacteria

9 INTRODUCTION

10 Purple non-sulfur photosynthetic bacteria (PNSB) such as *Rhodobacter capsulatus* are
11 facultative anaerobic Gram-negative bacteria and are generally regarded as safe (GRAS)
12 (1, 2). These bacteria have the versatile metabolic ability to grow under a variety of
13 habitats (3). For instance, *R. capsulatus* could use a wide spectrum of carbon sources
14 including short-chain fatty acids, dicarboxylic acids, sugars, agricultural and food
15 wastes for chemotrophic growth under aerobic to microaerobic conditions (4). It can
16 also utilize the organic or inorganic compounds (such as $S_2O_3^{2-}$, H_2S , and Fe^{2+}) as
17 electron donors for photoheterotrophic growth with energy from light to assimilate CO_2
18 under anaerobic condition (5, 6). Moreover, when the nitrogen source is scarce, *R.*
19 *capsulatus* can perform nitrogen fixation and produce hydrogen via the action of
20 nitrogenases (7). However, due to the lack of photosynthetic system II, the hydrogen
21 donor for photosynthesis is organic or inorganic substrates instead of water, and no
22 oxygen is released during the process (8).

1 To survive under such diverse living conditions, PNSB has evolved a complex
2 metabolic network with highly specialized enzyme complexes and regulatory
3 mechanisms. Recently, PNSB has attracted special attention as a platform
4 microorganism for biotechnological applications such as H₂ production as an
5 alternative renewable energy to fossil fuels (7, 9) and polyhydroxybutyrate as
6 biodegradable plastics (10, 11), bioremediation of wastewater (12) and heavy metal
7 (13), fixation of CO₂ and N₂ (14, 15), expression of membrane proteins (16, 17) and
8 metalloenzymes (18, 19). More importantly, as the synthesis of photopigments in PNSB
9 relies on isoprenoid biosynthetic pathway, it offers a robust and effective isoprenoid
10 metabolism. Therefore, PNSB has been regarded as a promising host for isoprenoid
11 biosynthesis (1, 2, 20).

12 Currently, the synthetic biology tools for engineering PNSB are lacking, which
13 significantly slows the progress in PNSB-related biotechnological applications. The
14 genetic tools in PNSB still heavily rely on traditional homologous recombination using
15 suicide plasmids (9), gene transfer agent (GTA) derived from a phage-like particle
16 capable of transduction (21), and gene mutation using transposon (22). There is a
17 pressing need to develop new and efficient genetic tools to accelerate the researches in
18 PNSB. Recently, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)
19 and CRISPR-associated protein (Cas) systems (23) have been developed as versatile
20 tools for genome editing and transcription regulation in a variety of organisms (24, 25).
21 The class II type II CRISPR/Cas9 system containing a Cas nuclease (Cas9), a trans-
22 activating CRISPR RNA (tracrRNA), and a *cis*-regulatory RNA (crRNA) has been

1 engineered as a powerful genetic tool in various organisms including PNSB such as
2 *Rhodobacter sphaeroides* (26, 27).

3 Cas12a, a class II type V-A endonuclease, is characterized as a dual nuclease referring
4 to crRNA processing, target-site recognition, and DNA cleavage (28). The
5 CRISPR/Cas12a system also has been repurposed as a genetic tool adapted for many
6 species (29, 30). Compared to CRISPR/Cas9 that recognizes the target locus close to a
7 guanine-rich protospacer adjacent motif (PAM) sequence (NGG) to create blunt ends
8 (26), Cas12a assisted by a mature crRNA binds the protospacer segment flanked by a
9 thymidine-rich PAM sequence (TTN) to form staggered ends (28, 31), which might
10 promote the non-homologous end joining (NHEJ) machinery to fix the double strand
11 breaks (DSB). In addition, the compact design of crRNA and self-processing crRNA
12 by Cas12a make CRISPR/Cas12a a more versatile and robust tool than CRISPR/Cas9
13 system. To the best of our knowledge, there is no report on CRISPR/Cas12a mediated
14 genetic tools in PNSB. In this study, we aimed to investigate the feasibility of using
15 CRISPR/Cas12a assisted genetic engineering tools in *R. capsulatus*. In addition, we
16 also attempted to evaluate CRISPR/dCas12a as artificial transcriptional factors for
17 targeted gene knock-down applications.

18 **RESULTS**

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

20 To begin with, we first evaluated the potential toxicity of Cas12a from *Francisella*
21 *novicida* expression in *R. capsulatus*. The *E. coli*-*Rhodobacter* shuttle vector
22 pBBRdMCS, a multicopy vector derived from pBBR1MCS2 (32), was used to express

1 the *sacB* expression cassette and *cas12a* gene under the control of P_{lac} promoter,
 2 resulting the plasmid pBdRCas12a (Figure 1). Notably, P_{lac} promoter has constitutive
 3 transcription activity due to the absence of the *lacI* repressor gene in *R. capsulatus*
 4 genome (33). When the plasmid pBdRCas12a in parallel with the control plasmid
 5 pBdRSacB were transformed into *R. capsulatus*, the conjugation efficiency for
 6 pBdRCas12a was close to that achieved by the control (Figure. 2a). Considering the
 7 size of pBdRCas12a (10876 bp) plasmid was doubled as compared to the control (5144
 8 bp), it was likely that the slight decrease in conjugation efficiency could occur.
 9 Therefore, these findings suggested that Cas12a is non-toxic to *R. capsulatus* under the
 10 tested conditions.

11 To confirm whether Cas12a is functional in *R. capsulatus*, *ccoO* gene encoding a
 12 subunit of *cbb3*-cytochrome oxidase (*cbb3*-Cox) and *nifH* gene encoding a subunit of
 13 nitrogenase were chosen as the disruption targets. The *in vivo* *cbb3*-Cox activity of *R.*
 14 *capsulatus* strains could be visualized using Nadi staining solution. As nitrogenase is
 15 essential for the nitrogen fixation, thus *nifH* mutants would abolish the growth under
 16 nitrogen source depleting conditions. According to the literatures, crRNA to achieve
 17 the sufficient efficiency for genome editing typically comprises a 19-nt direct repeat
 18 (DR) and a 23-nt guide sequence (28). Therefore, the crRNA-expressing module was
 19 assembled into the pBdRCas12a plasmid under the control of the same promoter as
 20 Cas12a. Based on this principle, plasmid pBdRCas12a-ccoO and pBdRCas12a-nifH
 21 were constructed to target genes of *ccoO* and *nifH*, respectively. As shown in Figure 2a,
 22 the conjugation efficiencies declined more than 4 orders of magnitude compared to the

1 control plasmid. Since the generation of DSB at the targeted genes by CRISPR/Cas12a
2 typically results in decreased survival rates (27), these findings suggested that the
3 CRISPR/Cas12a system might be functional in *R. capsulatus*. As shown in Figure 2b
4 and 2c, *ccoO* or *nifH* was successfully mutated by Cas12a mediated by NHEJ
5 mechanism. However, the editing efficiency mediated by CRISPR/Cas12a was only
6 30%~40%, which is not satisfactory when compared to the traditional methods.

7 It was reported that the genome editing efficiency assisted by CRISPR/Cas system was
8 observably impacted by GC content of guide RNA and the target positions (34). In this
9 study, five crRNAs with different GC contents ranging from 39% to 83% were designed
10 for *ccoO* (gccoO-1 to gccoO-5). As shown in Figure 2d, the crRNAs with different GC
11 contents resulted in editing efficiencies ranging from 4% to 33%. In particular, similar
12 efficiencies (25%, 33%, and 30%, respectively) were achieved for GC contents ranging
13 from 39% to 65%, indicating that Cas12a was not as sensitive to the GC content as the
14 Cas9 counterpart. However, higher GC content might affect the crRNA folding and
15 maturity, thereby decreasing the genome editing.

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

17 According to previous reports, efficient expression of Cas12a is crucial to achieve
18 efficient CRISPR/Cas12a assisted genome editing systems (35). As the heterologous
19 promoter *P_{lac}* is a relatively weak promoter, it might limit the CRISPR/Cas12a
20 expression and result in the low editing efficiency. To search alternative strong
21 promoters to drive the expression of CRISPR/Cas12a in *R. capsulatus*, four
22 endogenous promoters including promoters from *puc* operon (encoding light-

1 harvesting complex II), *puf* operon (encoding light-harvesting complex I), *pgk* gene
2 (encoding phosphoglycerate kinase), and *eno* gene (encoding enolase) were chosen.
3 Encouragingly, all promoter candidates exhibited much higher activity than P_{lac} (Figure
4 3a). Among the tested promoters, P_{puc} showed the highest activity, which corresponds
5 to approximately 5.4-fold improvement over that of P_{lac} .
6 On the basis of above findings, the promoter P_{puc} was applied to drive the expression
7 of CRISPR/Cas12a system in the remaining studies. When compared to the first-
8 generation design, the new versions of pBRucCas12a-ccoO and pBRucCas12a-nifH
9 only differed in the promoter sequences (P_{puc} v.s. P_{lac}). These plasmids were
10 transformed into *R. capsulatus* with pBRPucSacB as the control plasmid. As depicted
11 in Figure 3b, the conjugation efficiency of Cas12a expression driven by P_{puc} was close
12 to that of Cas12a driven by P_{lac} , suggesting that higher expression of Cas12a was not
13 toxic to *R. capsulatus*. As shown in Table 1, 10 randomly selected transformants on
14 *ccoO* disruption was verified by Nadi staining and sequencing, and 93% editing
15 efficiency was achieved. The editing efficiency for the disruption of *nifH* reached up to
16 87%. And it was observed that short indels (1 to 4 bp) occurred at the protospacer
17 regions (Figure 3c and 3d). Noteworthily, the editing efficiency using CRISPR/Cas12a
18 driven by a stronger promoter P_{puc} was much higher than that under the control of
19 promoter P_{lac} , suggesting that CRISPR/Cas12a could be an effective genome editing
20 tool in PNSB such as *R. capsulatus*. In addition, approximately 63% positive
21 transformants were observed during simultaneous disruption of *ccoO* and *nifH* as
22 shown in Table 1.

1   **Transcriptional repression by CRISPR interference (CRISPRi) using deactivated** 2   **Cas12a (dCas12a)**

3   As evidenced by CRISPR/Cas12a mediated genome editing, it is possible to further
4   employ CRISPR/dCas12a as an artificial transcriptional factor for targeted gene
5   knockdown. The nuclease activity in the RuvC domain of Cas12a was abolished by
6   introducing the E1006A mutation (36). The dCas12a driven by the promoter P_{puc} was
7   cloned into a low copy *E. coli-Rhodobacter* shuttle vector pRK415 (37), to give plasmid
8   pRKucdCas12a (Figure 4a). The transcriptional repression efficiency of CRISPRi was
9   evaluated by employing the *gfp* and *lacZ* as the report genes, which were preintegrated
10   at the *phbC* and *pucBA* sites, respectively. For each reporter gene, three crRNAs
11   targeting at the template strand were designed (g1, g2, and g3 or z1, z2, and z3, as
12   shown in Figure 4b), and one crRNA targeting at the non-template strand was examined
13   (g4 or z4, Figure 4b). The above plasmids harboring dCas12a with different crRNAs
14   were then transformed into *R. capsulatus* with chromosomal integrated expression
15   cassettes of *gfp* and *lacZ*. As shown in Figure 4c and 4d, all the crRNAs targeting the
16   template strand could lead to different degrees of repression of the *gfp* or *lacZ*
17   expression. Similar to previous findings that crRNA-binding position near the
18   translation initiation site on the template strand is more effective for CRISPRi (38, 39),
19   we found the most efficient repression occurred when the crRNAs were designed near
20   the start codon (*gfp* and *lacZ* repression, 84% and 82%, respectively). However, only
21   ~20% repression was achieved with targeting at the non-template strand for both
22   reporter genes. To test the capability of multiplex gene regulation, dual crRNA

1 expressing plasmid was constructed to simultaneously repress *gfp* and *lacZ* expressions.
 2 The expression levels of *gfp* and *lacZ* were simultaneously reduced by 80% and 77%,
 3 respectively. These findings indicated that the CRISPRi system based on dCas12a has
 4 broad utilities to implement multiplex transcriptional repressions in *R. capsulatus* for
 5 the future metabolic engineering applications.

6 **DISCUSSION**

7 The CRISPR/Cas system is revolutionizing the field of genome editing in various
 8 organisms. The type V-A CRISPR/Cas12a system has the ability to self-process crRNA
 9 and cleave DNA to give staggered ends instead of blunt ends. Due to these
 10 characteristics, CRISPR/Cas12a assisted genome editing is a superior alternative to
 11 CRISPR/Cas9 (28). In this work, we developed an efficient genetic tool using Cas12a
 12 from *F. novicida* in *R. capsulatus*, a promising platform microorganism for PNSB-
 13 related biotechnological applications. Based on previous findings, Cas9 from *S.*
 14 *pyogenes* (SpCas9) is toxic at high expression in several bacteria such as
 15 *Corynebacterium glutamicum* (40) and *Cyanobacteria sp.* (41), which limits the
 16 application of CRISPR/Cas9 in these microorganisms. In this work, we found that
 17 Cas12a expression driven by strong promoters in a multicopy vector did not show
 18 noticeable toxicity to *R. capsulatus* like *C. glutamicum*.
 19 Genome editing with CRISPR/Cas is typically based on DSB repaired by homologous
 20 recombination (HR) or NHEJ. As CRISPR/Cas12a creates a staggered end resulting a
 21 5-nt 5' overhang rather than a blunt cleavage product, which might promote the DSB
 22 repair mediated by NHEJ mechanism. Therefore, CRISPR/Cas12a was applied for

1 genome editing via the NHEJ machinery in *R. capsulatus*. The initial studies revealed
2 that about 30%~40% editing efficiency was obtained when targeting *ccoO* or *nifH* gene
3 based on NHEJ pathway, which was not satisfactory when compared to NHEJ-
4 mediated CRISPR/Cas in *Streptomyces coelicolor* (42) and *E. coli* (43). To further
5 improve the genome editing efficiency in *R. capsulatus*, a strong promoter of pigment
6 operon *puc* (P_{puc}) was identified to drive the CRISPR/Cas12a system, and ~90% editing
7 efficiency was achieved for single gene disruption, whereas 63% editing efficiency was
8 obtained for two gene disruptions. Therefore, the CRISPR/Cas12a system based on the
9 NHEJ mechanism has great potentials for the future multiplex genome editing.

10 To the best of our knowledge, CRISPRi using DNase-deactivated Cas9 (dCas9) or
11 dCas12a has not been reported in *Rhodobacter* species. Previous findings suggested
12 that dCas12a exhibits higher repression efficiency with crRNAs targeting at the
13 template strand over non-template strand, and the closer crRNA near to the translational
14 start sites, the higher repression efficiency is obtained (35, 38, 44). In this study, similar
15 results were also observed in *R. capsulatus* using dCas12a driven by P_{puc} when targeting
16 at *gfp* or *lacZ* gene. More importantly, dual repression of *gfp* and *lacZ* genes reached a
17 similar efficiency to that of single target, indicating that CRISPR/dCas12a might be
18 applicable for multiplex transcription regulation in *R. capsulatus*.

19 **MATERIALS AND METHODS**

20 **Strains and culture conditions**

21 All plasmids were introduced by heat-shock into competent cells of *Escherichia coli*
22 S17-1 or Top 10 cultured with Luria-Bertani (LB) medium containing appropriate

1 antibiotics at 37°C. The wild type of *R. capsulatus* SB1003 was employed for genetic
2 modifications and the strain was cultured in Sistrom's mineral media (MedA) (45) at
3 35°C. Antibiotics were used at the following final concentrations: gentamycin 12
4 mg/mL, tetracycline 12.5 mg/L, kanamycin 50 mg/L for *E. coli*; gentamycin 12 mg/mL,
5 tetracycline 2.5 mg/L, kanamycin 10 mg/L for *R. capsulatus*. All strains used in this
6 study are listed in Supplementary Table S1.

7 **Plasmid constructions**

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

16 **Triparental conjugation procedure**

17 Plasmids were transformed into *R. capsulatus* by triparental conjugation with the helper
18 plasmid of pRK2013 (46). Before the conjugation, *R. capsulatus* was grown with 10
19 mL MedA at 35°C for 48 h. The *R. capsulatus* cells were centrifuged, collected and
20 washed once with fresh MedA, and resuspended with 400 µL MedA. Meanwhile, *E.*
21 *coli* HB101 harboring the helper plasmid pRK2013 and *E. coli* S17-1 carrying the
22 desired plasmid were incubated in 5 mL LB medium supplemented with proper

1 antibiotics at 37°C for overnight. The *E. coli* cells were centrifuged, collected and
 2 washed once with fresh MedA, and resuspended with 1 mL MedA, respectively. *R.*
 3 *capsulatus* of 100 µL as a recipient strain, *E. coli* HB101/pRK2013 of 30 µL as a helper
 4 strain and *E. coli* S17-1 of 30 µL as a donor strain were mixed and spotted into a MedA
 5 agar plate as a ~2 cm diameter spot incubating at 35°C for 24 h. The mixed cells were
 6 centrifuged, collected, washed with fresh MedA and resuspended with 1 mL MedA.
 7 The resuspended cell culture of 100 µL was spread on a MedA agar plate with
 8 appropriate antibiotics and incubated at 35°C for 2-3 days until colony appeared.

9 **CRISPR/Cas12a mediated genome editing and transcriptional repression in *R.*** 10 ***capsulatus***

11 For CRISPR/Cas12a assisted genome editing, pBdRCas12a or pBRucCas12a
 12 derivatives carrying the corresponding crRNAs were transformed into *R. capsulatus* by
 13 triparental conjugation for disrupting the target genes. For CRISPR/dCas12a mediated
 14 transcriptional repression, *gfp* and *lacZ* expression cassette were integrated at *phbC* and
 15 *pucBA* sites of the chromosome via the traditional method mediated by pZJD29c or
 16 pK18mobsacB as previously described (7). Briefly, several recombinants containing
 17 pZJD29c or pK18mobsacB derived plasmids were cultivated in MedA medium for
 18 overnight, and then spread on the MedA agar plate containing 10% sucrose. The
 19 colonies were then subjected to diagnostic PCR analysis to confirm the integration of
 20 *gfp* and *lacZ* cassette into the *phbC* and *pucBA* sites. Next, pRKucdCas12a derived
 21 plasmids carrying the corresponding crRNAs were transformed into *R. capsulatus* and
 22 selected on the MedA agar plate supplemented with tetracycline. The fluorescence

intensities and LacZ activities were measured for comparing the transcriptional repression.

***In vivo cbb₃*-cytochrome oxidase activity**

To test whether the *ccoO* gene encoding for a subunit of *cbb₃*-cytochrome oxidase (*cbb₃*-Cox) was disrupted by CRISPR/Cas12a system, the *in vivo cbb₃*-Cox activity of *R. capsulatus* was visualized qualitatively using Nadi staining solution containing 35 mM α -naphthol and 30 mM N,N,N',N'-dimethyl-p-phenylene diamine (DMPD) dissolved in 1:1 (vol/vol) ethanol and water (47). When the *ccoO* gene was successfully disrupted, the strains showed the natural color (red) due to inactivation of *cbb₃*-Cox, otherwise the colonies appeared blue.

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. Optical density at 630 nm (OD_{630}) and fluorescence intensities were monitored by a microplate reader (Synergy H1, BioTek). The excitation of eGFP was set at 485 nm as well as 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 (48). 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 (μ M ONPG-Hydrolyzed/(min·mg-protein)),

1 OD_{420} is the optical density at 420 nm, t is the reaction time (min) and m is the mass of
2 the protein (mg).

3 **Author contributions**

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

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10 **Competing financial interests**

11 The authors declare no competing financial interests.

12 **Supporting Information**

13 Supplementary materials and methods, list of strains used in the study (Table S1), list
14 of oligonucleotides used in the study (Table S2) and list of plasmids used in the study
15 (Table S3).

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

Target gene	Replicate	Editing efficiency	Average editing efficiency
<i>ccoO</i>	1	100% (10/10)	93%
	2	90% (8/10)	
	3	90% (9/10)	
<i>nifH</i>	1	80% (7/10)	87%
	2	90% (9/10)	
	3	90% (8/10)	
<i>ccoO, nifH</i>	1	70% (7/10)	63%
	2	60% (6/10)	
	3	60% (6/10)	

2

1 **Figure legend**

2 **Figure 1. Schematic diagram of CRISPR-Cas12a system for genome editing in *R.***

3 ***capsulatus*.** Cas12a and crRNA were expressed from a single plasmid with kanamycin
4 marker, shuttle replicon, and mobilization for transferring plasmid from donor strains
5 *E. coli* into recipient strains of *R. capsulatus*. The *sacB* cassette was used as the counter-
6 selection marker for curing plasmid. CRISPR/Cas12a system was first constructed in
7 *E. coli* S-17, then conjugated into *R. capsulatus* via the helper strain of *E. coli* pRK2013.

8 **Figure 2. Genome editing using CRISPR-Cas12a in *R. capsulatus*. (a) Colony**

9 forming unit (c.f.u) of *R. capsulatus* conjugation mixtures containing Cas12a with or
10 without the expression of crRNA target. (b) Verification of *ccoO* disruption by Nadi
11 staining. (c) Verification of *nifH* disruption by growing on the MedA agar plate without
12 nitrogen source. (d) Comparison of the genome editing efficiency of five crRNAs with
13 different GC contents. Experiments were carried out in triplicate, and the data are
14 presented as mean $\pm$ S.D.

15 **Figure 3. Optimization of CRISPR-Cas12a mediated genome editing in *R.***

16 ***capsulatus*.** (a) Transcriptional activities of five promoters in *R. capsulatus* (P_{lac} , P_{puc} ,
17 P_{puf} , P_{pgk} , and P_{eno}) as measured by eGFP fluorescence intensities. (b) Colony forming
18 unit (c.f.u) obtained after conjugation of empty vector and Cas12a with or without the
19 crRNA targets driven by promoter P_{puc} . (c) Confirmation of *ccoO* disruption by Nadi
20 staining and sequencing. (d) Confirmation of *nifH* disruption by growth without
21 nitrogen source and sequencing. WT represents the wild type strain. Experiments were
22 carried out in triplicate, and the data are presented as mean $\pm$ S.D.

Figure 4. Transcriptional repression of *gfp* and *lacZ* by CRISPRi based on dCas12a. (a) dCas12a and crRNA driven by promoter P_{puc} were expressed from a single plasmid with tetracycline marker, shuttle replicon IncC, *tra* combined with *trf* for transferring plasmid into the host. (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 *gfp* or *lacZ* gene. (c) Comparison of *gfp* 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. Experiments were carried out in triplicate, and the data are presented as mean $\pm$ S.D.

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3 **Figure 1**

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2 **Figure 2**

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2 **Figure 3**

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2 **Figure 4**