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A rapid and efficient strategy for combinatorial repression of multiple genes in *Escherichia coli*

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Abstract

Background The regulation of multiple gene expression is pivotal for metabolic engineering. Although CRISPR interference (CRISPRi) has been extensively utilized for multi-gene regulation, the construction of numerous single-guide RNA (sgRNA) expression plasmids for combinatorial regulation remains a significant challenge.

Results In this study, we developed a combinatorial repression system for multiple genes by optimizing the expression of multi-sgRNA with various inducible promoters in *Escherichia coli*. We designed a modified Golden Gate Assembly method to rapidly construct the sgRNA expression plasmid p3gRNA-LTA. By optimizing both the promoter and the sgRNA handle sequence, we substantially mitigated undesired repression caused by the leaky expression of sgRNA. This method facilitates the rapid assessment of the effects of various inhibitory combinations on three genes by simply adding different inducers. Using the biosynthesis of *N*-acetylneuraminic acid (NeuAc) as an example, we found that the optimal combinatorial inhibition of the *pta*, *ptsI*, and *pykA* genes resulted in a 2.4-fold increase in NeuAc yield compared to the control.

Conclusion We anticipate that our combinatorial repression system will greatly simplify the regulation of multiple genes and facilitate the fine-tuning of metabolic flow in the engineered strains.

Keywords CRISPRi, Inducible promoters, Multiple genes, Combinatorial repression, Metabolic flow

Background

Optimizing metabolic flux through the regulation of key gene expression is crucial for metabolic engineering and synthetic biology [1, 2]. However, a multitude of biochemical reactions continuously occurs within cells, forming a complex metabolic network [3, 4]. As a result, the combinatorial regulation of multiple genes is essential for redirecting metabolic flow and biosynthesizing various compounds. The CRISPR/Cas (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein) system has been adapted into an efficient gene regulation tool known as CRISPR interference (CRISPRi). This technique relies on single-guide RNA (sgRNA) to direct a catalytically inactive Cas protein to bind specific DNA loci and repress gene transcription [5–7].

By expressing multiple sgRNAs, CRISPRi has been used to simultaneously regulate multiple key genes in the complex metabolic pathways [8–10]. Yin et al. developed a bifunctional regulation system based on CRISPR/dCpf1, which bidirectionally regulates the expression of

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multiple genes in *Clostridium glutamicum* through the expression of crRNA arrays. By simultaneously inhibiting 4 genes (*arok*, *pyk*, *ptsH*, and *hdpA*), while activating 6 genes (*iolP*, *tkt*, *aroG*, *aroB*, *aroD*, and *aroE*), the strain's shikimic acid production increased by 27-fold. Additionally, simultaneous inhibition of *glyA*, *pyk*, and *gnd*, along with the activation of *serA*, *serB*, and *serC*, resulted in a 10-fold increase in L-serine production in the strain SER-12 [11]. Kim et al. selected 32 genes in *Escherichia coli* DH1 as targets for downregulation that competitively utilize precursors, cofactors, or intermediates of the MVA pathway (i.e., acetyl-CoA, pyruvate precursors, and cofactors). The strategic combination of these targets to enhance titer led to the highest isopentyl glycol titers, achieved through the simultaneous inhibition of *adhE*, *ldhA*, and *fabH* using sgRNA arrays. The strain produced 12.4 ± 1.3 g/L of isopentyl glycol during 2 L fed-batch cultivation [12]. Fang et al. separately inhibited four genes in brewing yeast and subsequently identified three highly efficient inhibition targets: *TYR1*, *AAT2*, and *ALD3*. By constructing a sgRNA array for the simultaneous inhibition of these three targets, the titer of 2-phenylethanol increased by 1.89-fold [13].

From these studies, it is evident that current approaches to multiple inhibition primarily rely on constructing sgRNA arrays to simultaneously express multiple sgRNAs. This requires the construction of a large number of sgRNA expression plasmids to identify the optimal combination for repressing multiple genes, a process that is both time-consuming and labor-intensive. For instance, to optimize the biosynthesis of dicinnamoylmethane, Chu et al. constructed a total of 11 sgRNA plasmids to knock down the genes *sucC*, *fumC*, and *mdh* in the tricarboxylic acid cycle, as well as the genes *fabD* and *fabF* in the fatty acid biosynthesis pathway. This included combinations for 5 single genes, 4 double genes, and 2 triple genes. The strain with CRISPRi repression targeting *fabD*, *fabF*, and *mdh* exhibited the highest production of dicinnamoylmethane, reaching 7.54 μM, which is 5.76-fold higher than that of the wild-type strain [14]. However, to evaluate all possible repression combinations of these four genes, a total of 25 different sgRNA expression plasmids would need to be constructed.

Using multiple inducible promoters to independently control the expression of different genes has become a common approach in synthetic biology, including applications such as synthetic genetic circuits and cascade regulation [15, 16]. Numerous inducible promoters have been developed that feature low background, high dynamic range, increased sensitivity, and minimal crosstalk [17–19]. However, when applying CRISPRi to repress multiple genes, the use of orthogonal inducible promoters to independently control the expression of various sgRNA sequences has not been reported.

In this study, we present an efficient multi-gene combinatorial repression system based on CRISPRi and orthogonal inducible promoters in *E. coli* (Fig. 1). We optimized three inducible promoters with low background leakage and high orthogonality for the expression of different sgRNAs targeting their corresponding genes. The expression levels of these genes can be independently regulated by adding different inducers to express the respective sgRNAs, thereby eliminating the need to construct a large number of sgRNA plasmids. By optimizing the inducible promoters and sgRNA handle sequences, we developed a three-sgRNA combinatorial expression plasmid utilizing the optimized P_{lacO1}, $P_{LtetO-1}$, and P_{araBAD} promoters. Additionally, a modified Golden Gate Assembly method was developed for rapid replacement of the sgRNA targeting sequence on three-sgRNA expression plasmid p3gRNA-LTA. Collectively, we achieved combinatorial regulation of three genes through the addition of various inducers. Taking the production of *N*-acetylneuraminic acid (NeuAc) as an example, we demonstrated the convenience of using inducers instead of constructing a series of sgRNA expression plasmids.

Methods

Bacterial strains, plasmids and media

All bacterial strains and plasmids used in this study are listed in Table S1. *E. coli* DH5α was used for cloning purposes and was propagated in Luria-Bertani (LB) medium at 37 °C under aeration. *E. coli* BW25113 and MG1655 were used for system characterization, and their culture conditions were the same as DH5α. The strains DN5 were used for fermenting to produce NeuAc. Modified terrific broth (MTB) medium (12 g/L tryptone, 24 g/L yeast extract, and 5 g/L NaCl) was used for NeuAc fermentation. Antibiotics were added when necessary (ampicillin 100 μg/mL, kanamycin 25 μg/mL, chloramphenicol 17 μg/mL, or spectinomycin 100 μg/mL).

Plasmid construction

Standard protocols were used for PCR, gel electrophoresis, and transformation experiments [20]. Primers used in this study are listed in Table S2. Polymerases used for PCR reactions were either PrimeSTAR (Takara; Japan) or Phanta Super-Fidelity (Vazyme; Nanjing, China). Restriction endonucleases and Phusion DNA Polymerase were purchased from Thermo Fisher Scientific (Waltham, MA, USA). T4 DNA Ligase, T4 Polynucleotide Kinase (PNK), Type IIS Restriction Endonuclease, Taq DNA Ligase and T5 DNA Exonuclease were purchased from New England Biolabs (Ipswich, MA, USA). The plasmid construction was based on Gibson assembly method [21].

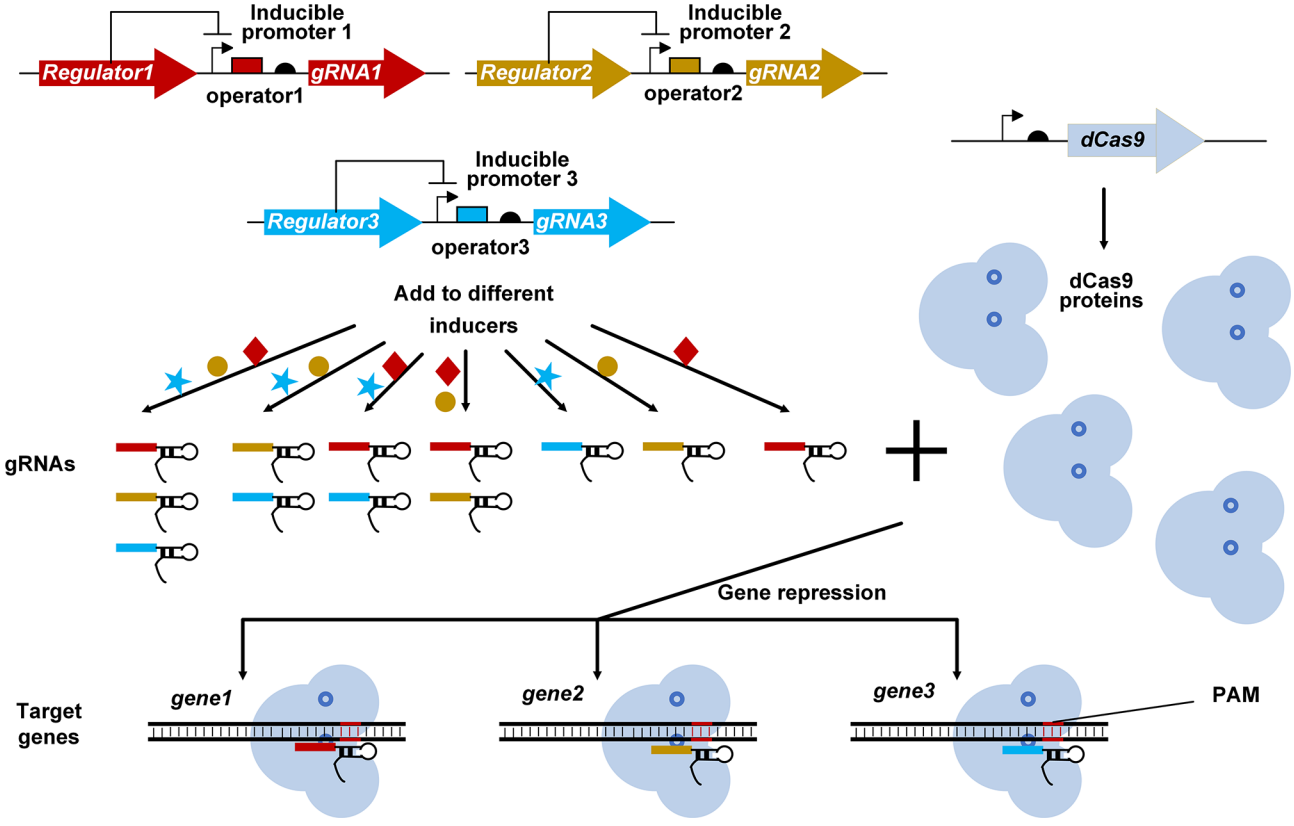

Fig. 1 The schematic of the multi-gene combinatorial repression system

The one-step ligation method according to the golden gate assembly

SgRNA fragments were inserted using a modified Golden Gate Assembly method [22]. The sgRNA expression plasmid, designated p3gRNA-LTA, incorporates three distinct sgRNA insertion sites. Each insertion site is flanked by two identical Type IIS endonuclease recognition sites (BbsI, BsaI, and SapI). Corresponding recognition sites were added to both ends of the sgRNA sequences (Figure S1). The sequence is synthesized by the complementary single-stranded oligonucleotides and annealed to form the double strand sgRNA fragments. The specific spacer sequences are detailed in Table S3.

The first sgRNA fragment was ligated using the conventional Golden Gate Assembly protocol. The 20 μL reaction mixture includes 0.5 μL of the sgRNA fragment, 1 μg of the vector, 1 μL of Type IIS restriction endonuclease, 0.5 μL of T4 DNA ligase, 0.5 μL of T4 polynucleotide kinase, and 2 μL of T4 DNA ligase buffer. The reaction mixture was placed at 37 °C for 5 min and then transferred to 25 °C for 15 min, repeating ten cycles. After the first round of assembly, 1 μL of the second annealed sgRNA fragment, 1 μL of the second Type IIS restriction endonuclease, 0.5 μL of T4 DNA ligase, 0.5 μL of T4 polynucleotide kinase, 2 μL of T4 DNA ligase buffer, and 16 μL of ddH_2O were added to the reaction mixture to ligate the second sgRNA. Upon completion of this reaction, 1 μL of the third annealed spacer fragment, 1 μL of the third Type IIS restriction endonuclease, 0.5 μL of T4 DNA ligase, 0.5 μL of T4 polynucleotide kinase, 2 μL of T4 DNA ligase buffer, and 16 μL of ddH_2O were incorporated to ligate the third sgRNA.

After completion of the ligation reaction, all the ligation products were transformed into *E. coli* competent cells, which were then plated on LB agar plates containing 25 μg/mL spectinomycin. The agar plates were incubated overnight at 37 °C. The resulting colonies were then sequenced to confirm successful insertion of the sgRNA fragments.

Fluorescence intensity detection

The fluorescence intensity of the *mKate* and *rfp* reporter genes was measured with a microplate reader. The overnight cultures were diluted 2% into 2 mL LB medium in 24-well plates. Inducers were added according to the experimental design (Table S4). Plates were incubated at 37 °C with high-speed shaking using the BioTek microplate reader. OD_{600} and the fluorescence intensity (excitation 590 nm, emission 640 nm) were measured every 15 min for 18 h.

β-galactosidase and *β*-glucuronidase assay

The reporter gene *lacZ*, encoding *β*-galactosidase, catalyzes the hydrolysis of *o*-nitrophenyl-*β*-*D*-galactopyranoside (ONPG) to galactose and the yellow-colored *o*-nitrophenol. Under substrate-excess conditions, the *o*-nitrophenol production rate is directly proportional to *β*-galactosidase concentration [23]. Similarly, *gusA*, encoding *β*-glucuronidase, cleaves 4-nitrophenyl-*β*-*D*-glucuronide (4-NPG) to produce the yellow-colored *p*-nitrophenol, which can be quantified at 405 nm.

Enzyme activity assays for *β*-galactosidase and *β*-glucuronidase were adapted from the protocols available on OpenWetWare (Beta-Galactosidase Assay (A better Miller): [https://openwetware.org/wiki/Beta-Galactosidase_Assay_(A_better_Miller)]; Beta-glucuronidase protocols: [https://openwetware.org/wiki/Beta-glucuronidase_protocols]).

Overnight cultures were diluted 2% into 24-well plates (2 mL LB medium per well) and induced according to the experimental design. Following 18 h of incubation at 37 °C and 400 rpm, OD_{600} was measured. For enzyme assays, 120 µL of permeabilization solution (100 mM Na_2HPO_4, 20 mM KCl, 2 mM $MgSO_4$, 0.8 mg/mL CTAB, 0.4 mg/mL sodium deoxycholate, 5.4 µL/mL *β*-mercaptoethanol) was added to 30 µL of cell culture in a 1.5 mL microcentrifuge tube. Following mixing and a 30 min incubation at 30 °C, the lysate was divided into two 100 µL and 50 µL aliquots. For *β*-galactosidase activity, 100 µL of lysate was mixed with 600 µL of substrate solution (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 1 mg/mL ONPG, 2.7 µL/mL *β*-mercaptoethanol) in a 30 °C water bath. The reaction was timed precisely, and terminated by adding 700 µL of stop solution (1 M Na_2CO_3) once sufficient color development was observed. All reactions were timed identically. After centrifugation (13,400 rpm for 10 min), the supernatant absorbance at 420 nm was measured. For *β*-glucuronidase activity, 50 µL of lysate was mixed with 200 µL of gus buffer (50 mM NaH_2PO_4, 10 mM *β*-mercaptoethanol, 1 mM EDTA, 0.1% Triton X-100). Then, 20 µL of 4-NPG stock solution (50 mM NaH_2PO_4, 10 mg/mL 4-NPG) was added, and the reaction was timed precisely. The reaction was terminated with 200 µL of stop solution (200 mM Na_2CO_3) after sufficient color development, ensuring consistent reaction times. Following centrifugation (13,400 rpm for 10 min), the supernatant absorbance at 405 nm was measured.

Batch fermentation of NeuAc

Inoculate a single colony on an agar plate or a glycerol tube into 5 mL LB medium containing ampicillin, and incubate overnight at 37 °C. The culture was transferred to 50 mL LB medium containing ampicillin at a 2% inoculum and cultured for 12 h. Then the culture was inoculated into 50 mL fresh MTB medium containing ampicillin and 30 g/L glucose at an inoculum of 4%, and cultivated to an OD_{600} of 0.4–0.6 under the conditions of 30 °C and 220 rpm. Then 200 µM isopropyl-beta-*D*-thiogalactopyranoside (IPTG) was added for fermentation. Samples were taken every 6–12 h for analysis, and 30% NH_4OH was added to maintain the pH at approximately 7.0. The fermentation was terminated after 48 h.

Analytical methods

OD_{600} was measured using a spectrophotometer (Shimazu, Japan). Fermentation samples were centrifuged at 12,000 rpm for 4 min, and the supernatants were used for extracellular metabolite analysis. Glucose was measured using a Biosensors Analyzer (Jinan, China). Glucose, NeuAc, acetate, ethanol, formate, pyruvate, *N*-acetylglucosamine (GlcNAc), and *N*-acetylmannosamine (ManNAc) were quantitatively determined by high-performance liquid chromatography (HPLC) (Shimadzu, Japan) equipped with a refractive index detector (RID-10 A) (Shimadzu, Japan) and an Aminex HPX-87 H ion exclusion column (Bio-Rad, USA), as described previously [24]. The mobile phase is 5 mM sulfuric acid and the flow rate is 1 mL/min.

Results

Screening of inducible promoters

To achieve combinatorial regulation of multiple genes, we aimed to express different sgRNAs using orthogonal inducible promoters. Eight candidate promoters (Table S5), P_{lacO1}, $P_{LtetO-1}$, P_{araBAD}, P_{rhaBAD}, P_{CymRC}, P_{LuxB}, P_{salTTC}, and P_{Cin}, which were respectively regulated by $LacI^{AM}$, TetR, AraC, RhaS/R, $CymR^{AM}$, LuxR, NahR, and CinR transcriptional regulators, were characterized for the expression of the red fluorescent protein mKate [19, 25–27].

We observed varying induced expression intensities and background leakage among these promoters. The P_{LuxB}, P_{Cin}, P_{salTTC}, and P_{rhaBAD} promoters exhibited the high induced expression intensities, with fluorescence intensities of 34,843, 11,190, 8,982, and 4,760, respectively, after adding the corresponding inducers (Fig. 2A). However, with the exception of P_{rhaBAD}, the other promoters demonstrated significant background leakage. P_{rhaBAD}, P_{araBAD}, $P_{LtetO-1}$, and P_{lacO1} showed minimal background leakage, with relative fluorescence intensities of only 27, 31, 40, and 48, respectively, in the absence of inducers. After induction, the fluorescence intensities increased by 176.3, 107.8, 43.3, and 7.4-fold, respectively (Fig. 2A). To avoid the leaky expression of sgRNA, the four low-leakage inducible promoters P_{rhaBAD}, P_{araBAD}, $P_{LtetO-1}$, and P_{lacO1} were selected for subsequent characterization.

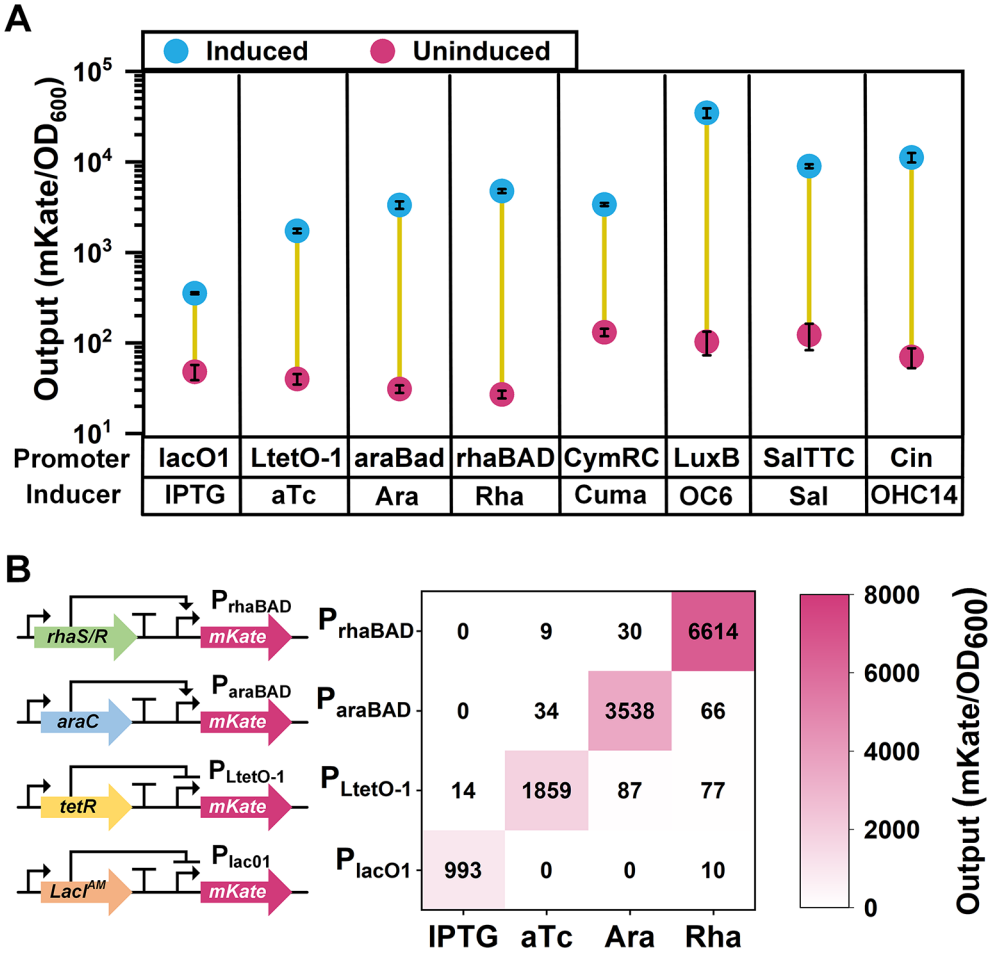

Fig. 2 Screening suitable inducible promoters. (**A**) The expression of eight various inducible promoters under the regulation of their corresponding transcription factor. The red circle indicates the expression intensity of the promoter without adding inducer. The blue circle represents the expression intensity of the promoter when adding the corresponding inducer. The concentration of various inducers is 0.5 mM of IPTG, 4.3 μM of anhydrotetracycline (aTc), 13.3 mM of arabinose (Ara), 121.8 μM of *L*-rhamnose monohydrate (Rha), 100 μM of Cuminic acid (Cuma), 10 μM of 3-oxohexanoyl-homoserine lactone (OC6), 100 μM of sodium salicylate (Sal), and 10 μM 3-hydroxytetradecanoyl-homoserine lactone (OHC14). (**B**) The orthogonality of P_{lacO1}, $P_{LtetO-1}$, P_{araBAD}, and P_{rhaBAD} promoters. The red fluorescent protein gene *mKate* was used as the reporter gene

We assessed the orthogonality of the four selected inducible promoters. The results indicated that each inducer only activated its corresponding promoter (Fig. 2B). The induced expression intensities of these four promoters ranged from 354 to 4,760, with P_{rhaBAD}, P_{araBAD}, $P_{LtetO-1}$ exhibiting expression levels 6.7, 3.6, and 1.9 times higher than that of the P_{lacO1} promoter, respectively.

Furthermore, we found that the presence of glucose in the culture medium affected the expression of P_{rhaBAD} promoters. Rha lost its ability to activate P_{rhaBAD} in the presence of 10 g/L glucose (Figure S2). Consequently, the Rha-induced promoter could not be used for fermentation with glucose as a carbon source and was excluded from subsequent experiments. Thus, the three promoters, P_{lacO1}, $P_{LtetO-1}$, and P_{araBAD}, characterized by low background leakage and high orthogonality, were selected for the expression of sgRNA sequences.

Construction of the combinatorial repression system

To rapidly express multi-sgRNA, we developed an efficient one-step three-sgRNA ligation strategy. Specifically, the initial three-sgRNA expression plasmid p3gRNA-LTA was constructed by inserting pairs of recognition sequences for type II restriction endonucleases BsaI, BbsI and SapI between the three selected inducible promoters and the sgRNA handle sequences (Fig. 3A). Thus, the first sgRNA target sequence was inserted into the first promoter by Golden Gate assembly, and then the target sgRNA sequences of the second and third promoters were sequentially ligated by continuing to add the corresponding restriction endonucleases and target DNA fragments in the same reaction mixture (Fig. 3B).

Using this method, we sequentially assembled three sgRNA target sequences, obtained thousands of transformants in a single transformation, and randomly selected seven clones for sequencing. The results showed that

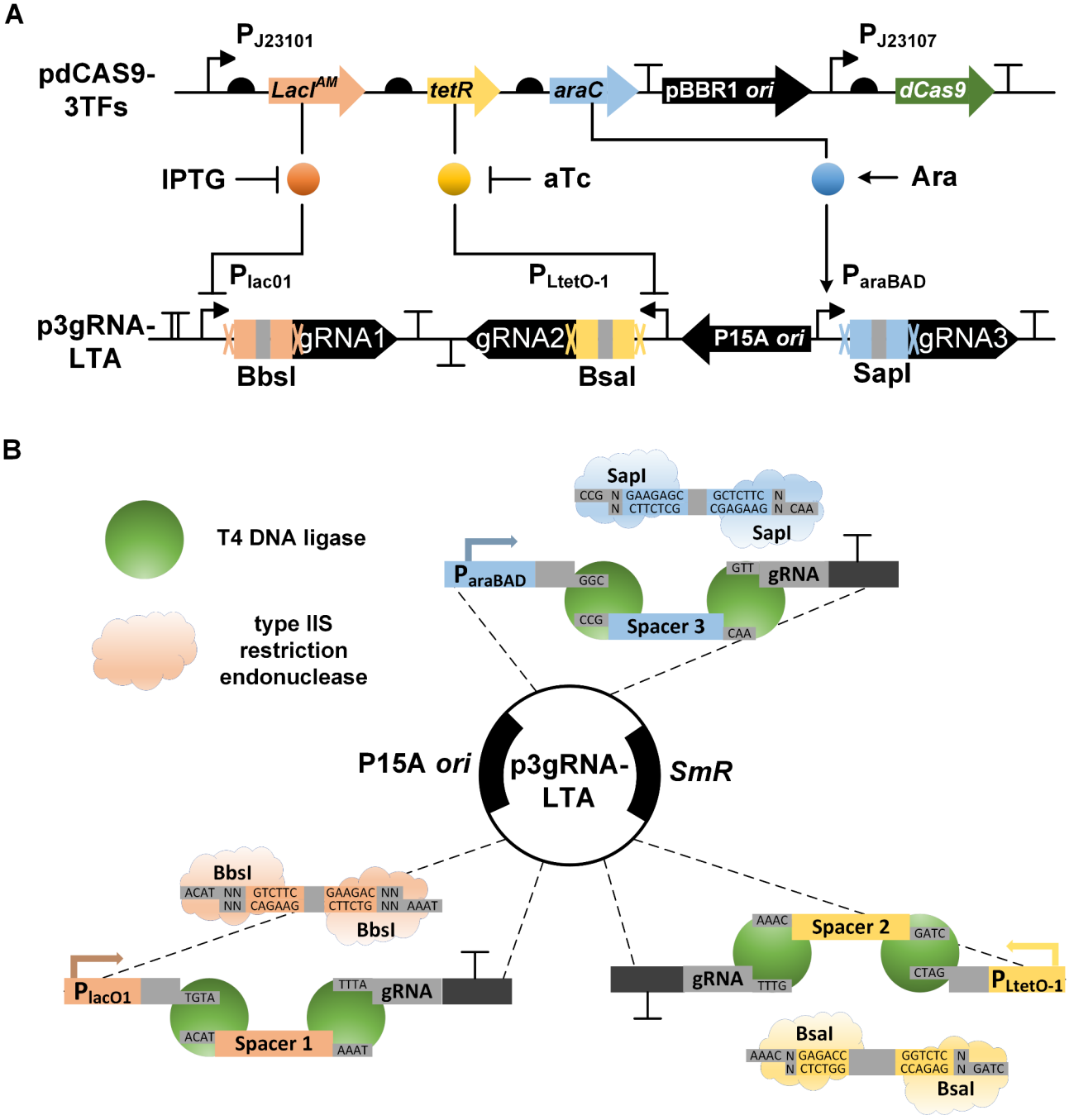

Fig. 3 The components of the multi-gene combinatorial repression system. (**A**) The schematic of three-sgRNA expression plasmid p3gRNA-LTA and TFs expression plasmid pdCas9-3TFs. The TFs $lacI^{AM}$ (red circle), tetR (yellow circle) and araC (red circle) expressed by pdCas9-3TFs regulate the expression of sgRNA from the promoters of P_{lacO1}, $P_{LtetO-1}$ and P_{araBAD}, respectively. The red, yellow and blue squares indicate the recognition sites of BbsI, BsaI and SapI, respectively. The red, yellow, and blue crosses indicate the cleavage sites of BbsI, BsaI and SapI, respectively; (**B**) The schematic of one-step three-sgRNA ligation strategy based on Golden Gate Assembly. Type IIs restriction endonuclease cleaves p3gRNA-LTA to form sticky ends. Spacers with sticky ends are formed by annealing single-stranded oligonucleotides. T4 DNA ligase (green circle) connects spacers to sites with the same sticky ends. The process can be recycled and enables joining of three sgRNA sequences in the same reaction mixture

five clones successfully incorporated all three sgRNA sequences, while the remaining two clones assembled the first two sgRNA sequences, omitting the last one (Figure S3).

Reduction of leaky repression using mutated sgRNA handle sequences

To characterize the effect of gene repression, we separately expressed sgRNA targeting red fluorescent protein

using the three inducible promoters. All tested promoters demonstrated efficient inhibition of the fluorescence. Upon adding the inducer, the fluorescence of the strain with the targeting spacer was significantly lower than that of the control. The red fluorescence decreased by 83.7%, 88.1%, and 89.6% for the sgRNA sequences expressed by the P_{lacO1}, $P_{LtetO-1}$, and P_{araBAD} promoters, respectively (Fig. 4). However, we observed that leaky expression of sgRNA from the three promoters resulted in reductions of red fluorescence by 21.8%, 74.9%, and 37.5%, respectively (Fig. 4).

To mitigate leaky repression, we aimed to adjust the affinity of sgRNA to dCas9 by modifying the sgRNA handle sequence. We found that some sgRNA handle sequence mutations exhibited different intensities of gene repression [28], which may mitigate the leakage effect of CRISPRi. Consequently, we selected seven sgRNA handle mutants that showed a fluorescence intensity decrease of more than 50% upon inducer addition, while maintaining the lowest background leakage. We designated these seven sgRNA handle mutants as #1 to #7 and used *lacZ* encoding *β*-galactosidase as the reporter gene to evaluate their effectiveness in reducing leaky repression (Table S6).

For the P_{lacO1} promoter, sgRNA handle mutants #3 and #5 exhibited a broad regulatory range. The activity of *lacZ* remained nearly intact in the absence of the inducer, while 84.5% and 83% of *lacZ* output were inhibited after inducer addition, respectively (Fig. 4D). We selected mutant #3 for its higher affinity to dCas9. In contrast, for the $P_{LtetO-1}$ and P_{araBAD} promoters, although mutant #3 enabled effective gene repression, significant background repression persisted. Mutant #5, however, exhibited much lower background leakage (Fig. 4D). Therefore, we chose mutant #5, which provides a balance between background leakage and gene repression. The new p3gRNA-LTA was constructed by expressing sgRNA handle mutant #3 with the P_{lacO1} promoter and mutant #5 with the $P_{LtetO-1}$ and P_{araBAD} promoters.

After optimizing the expression of the sgRNA handle sequence, we also assessed the effectiveness of gene repression with different sgRNA targeting sites. We designed and expressed three sgRNA sequences targeting the *lacZ* gene and three targeting the *gusA* gene using the

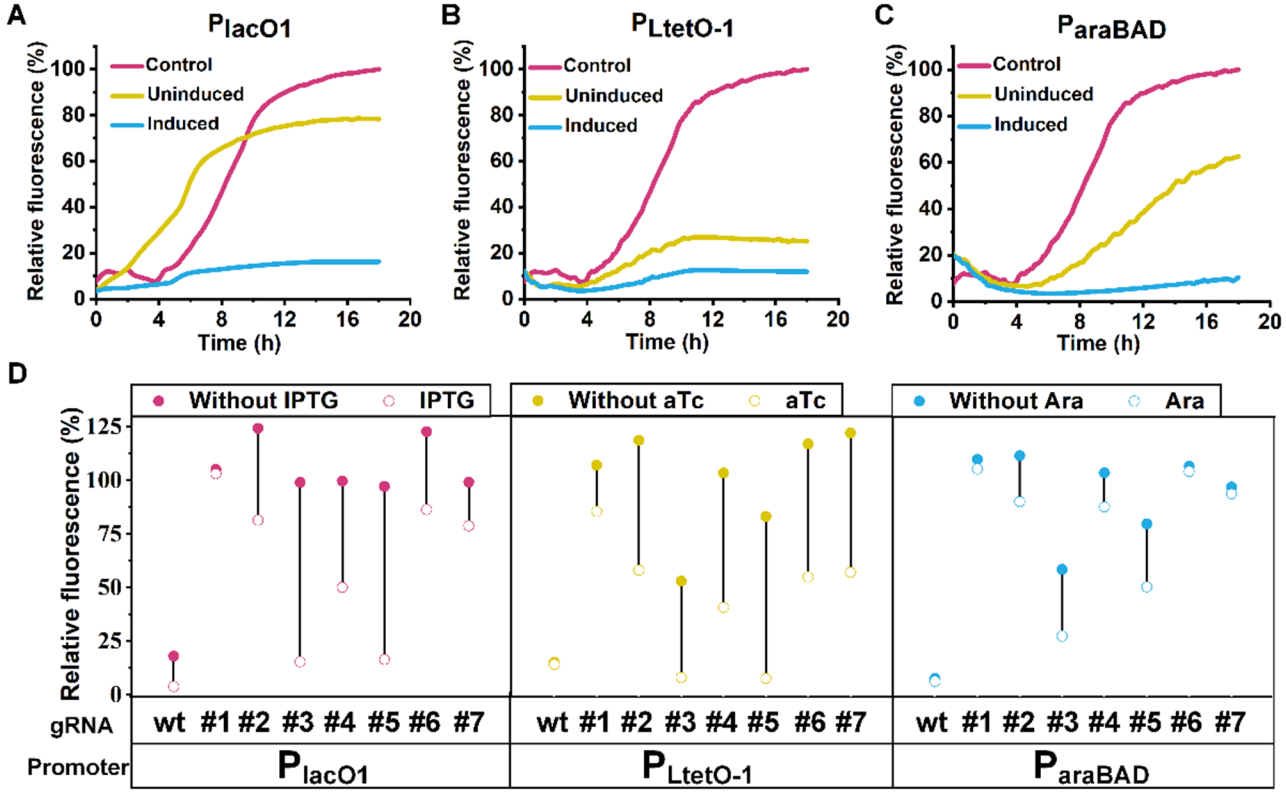

Fig. 4 The system exhibits leaky repression, which can be mitigated by using mutated sgRNA handle sequences. (**A**) The repression of *rfp* using sgRNA sequence expressed by the P_{lacO1} promoter with 0.5 mM IPTG. Control represents a strain that constitutively expresses fluorescent protein and does not contain CRISPRi; (**B**) The repression of *rfp* using sgRNA sequence expressed by the $P_{LtetO-1}$ promoter with 4.3 μM of aTc; (**C**) The repression of *rfp* using sgRNA sequence expressed by the P_{araBAD} promoter with 13.3 mM of Ara. (**D**) Reduction of the leaky repression within the multi-gene combinatorial repression system. The lacZ1 spacer targeting the *lacZ* gene was used to characterize the background leakage of different sgRNA mutants. The red, yellow and blue circles indicate that the P_{lacO1}, $P_{LtetO-1}$ and P_{araBAD} promoters express sgRNA, respectively. The solid circle indicates the enzyme activity of lacZ when no inducer is added, and the hollow circle indicates the enzyme activity of lacZ when the corresponding inducer is added

P_{araBAD} promoter. The results indicated that gene expression was largely unaffected in the absence of the inducer, with varying degrees of repression observed only after inducer addition (Figures S4, S5). Moreover, the intensity of gene repression was influenced by the location of the sgRNA targeting sequences, with closer proximity to the start codon resulting in more pronounced repression. LacZ1 and gusA1, being closest to the start codon, exhibited the most effective suppression.

For the most effective lacZ1 target site, we experimented with various lengths of sgRNA sequences to assess their impact on gene repression. Following inducer addition, lacZ activity decreased by 76.8%, 76.9%, 70.4%, and 79.4% with spacers of 17 bp, 22 bp, 24 bp, and 27 bp, respectively (Figure S6). This suggests that sgRNA target sequences between 17 bp and 27 bp have no significant effect on gene repression.

Characterization of the multi-gene combinatorial repression system

To characterize the optimized combinatorial repression system, we selected two genomic reporter genes, *lacZ* and *gusA*, along with a plasmid reporter gene, *rfp*. We knocked out *lacI* and *gusR*, which regulate the expression of *lacZ* and *gusA*, and replaced the promoters of *lacZ* and *gusA* with constitutive promoters. Simultaneously, we integrated *rfp* into the genome for constitutive expression, resulting in the construction of an *E. coli* MG1655RFP strain designed for detecting the multi-gene combinatorial repression system. The P_{lacO1}, $P_{LtetO-1}$, and P_{araBAD} promoters were used to express sgRNA targeting the *gusA*, *rfp*, and *lacZ* genes, respectively. In the absence of inducers, only $P_{LtetO-1}$ caused slight inhibition, and the intensity of RFP decreased by 22.2% (Fig. 5). When only IPTG was added, the enzyme activity of gusA was reduced by 48.3%. Even after adding the other two inducers, gusA enzyme activity could still be inhibited by 46.5–69.5%. When only aTc was used, the fluorescence intensity of RFP decreased by 74%. The addition of the other two inducers continued to inhibit RFP fluorescence intensity by 43.8–72.1%. Lastly, when only Ara was added, the enzyme activity of lacZ was reduced by 32.4%. After introducing the other two inducers, lacZ enzyme activity remained inhibited by 41.3–45.3% (Fig. 5).

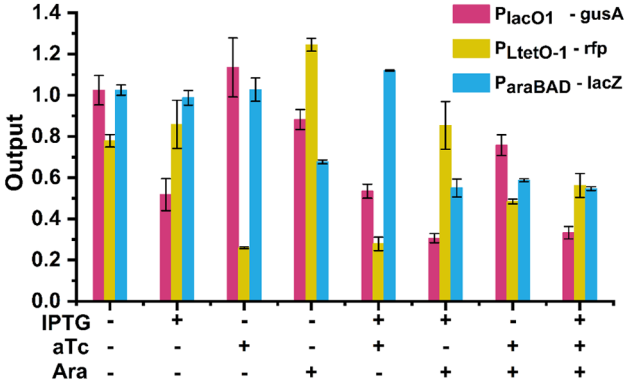

Fig. 5 Characterization of the multi-gene combinatorial repression system using *E. coli* MG1655RFP. Three sgRNA sites respectively targeting *gusA*, *rfp*, and *lacZ* were constructed into the p3gRNA-LTA plasmid. The sgRNA targeting *gusA* is expressed by the P_{lacO1} promoter. The sgRNA targeting *rfp* is expressed by the $P_{LtetO-1}$ promoter. The sgRNA targeting *lacZ* is expressed by the P_{araBAD} promoter. "-" indicates that no inducer is added, and "+" indicates that the inducer is added. Strains without sgRNA targeting any genes were used as controls. The enzyme activity and fluorescence of the control were normalized to 1. Data are expressed as means (± s.d.) from three independent experiments

Optimization of the biosynthesis pathway of NeuAc

The combinatorial repression system was used for optimizing the biosynthesis pathway of NeuAc. We have previously constructed an *E. coli* DH5α engineering strain DN5 in which nan operon (*nanATEK*, NeuAc degradation pathway), nag operon (*nagEBAC*, GlcNAc degradation pathway), gene *ldhA* (lactic acid production pathway), gene *poxB* (acetic acid production pathway), and gene *ackA* (acetic acid production pathway) were deleted [29]. The plasmid pB3 which contained NeuAc synthase *neuB*, GlcNAc 2-epimerase *slr1975*, GlcN-6-P *N*-acetyltransferase *GNA1*, and feedback resistant GlcN-6-P synthase *glms* was transferred into DN5. Strain DN5/pB3 was used for fermentation. After 48 h, the NeuAc titer reached 1.62 g/L [30]. However, this strain accumulated a large amount of ManNAc, which is a direct precursor of NeuAc (Fig. 6A). In contrast, the other precursor, phosphoenolpyruvate (PEP), had no obvious accumulation. This suggested that the intracellular concentration of PEP was insufficient relative to the ManNAc. Therefore, reducing the consumption of intracellular PEP is of great importance for the accumulation of NeuAc.

Intracellular PEP is mainly metabolized to acetyl-CoA or used as an energy source for the glucose phosphotransferase system (PTS) (Fig. 6A). We simultaneously repressed *pykA* (encoding pyruvate carboxykinase), *pta* (encoding phosphotransacetylase) and *ptsI* (encoding PTS-related proteins), the three key genes related to PEP depletion (Fig. 6A), and analyzed the effect on NeuAc accumulation. After repressing the PEP-consuming genes, the titer of NeuAc showed significant diversity. Inhibiting *pta*, *ptsI* and *pykA* resulted in NeuAc titer reaching 126.5%, 240.8%, and 144.2% of the wild-type, respectively, while combined inhibition of *pta*/*ptsI*, *pta*/*pykA* and *ptsI*/*pykA* resulted in the titer reaching 121.2%, 81.6%, and 203.7% respectively, and simultaneous repression of *pta*, *ptsI* and *pykA* obtained 176%. The highest titer was achieved by inhibiting *ptsI* alone, which was 2.62 g/L. The second was the multiple repression of *ptsI*/*pykA* and *pta*/*ptsI*/*pykA*, with titers of 2.22 g/L and

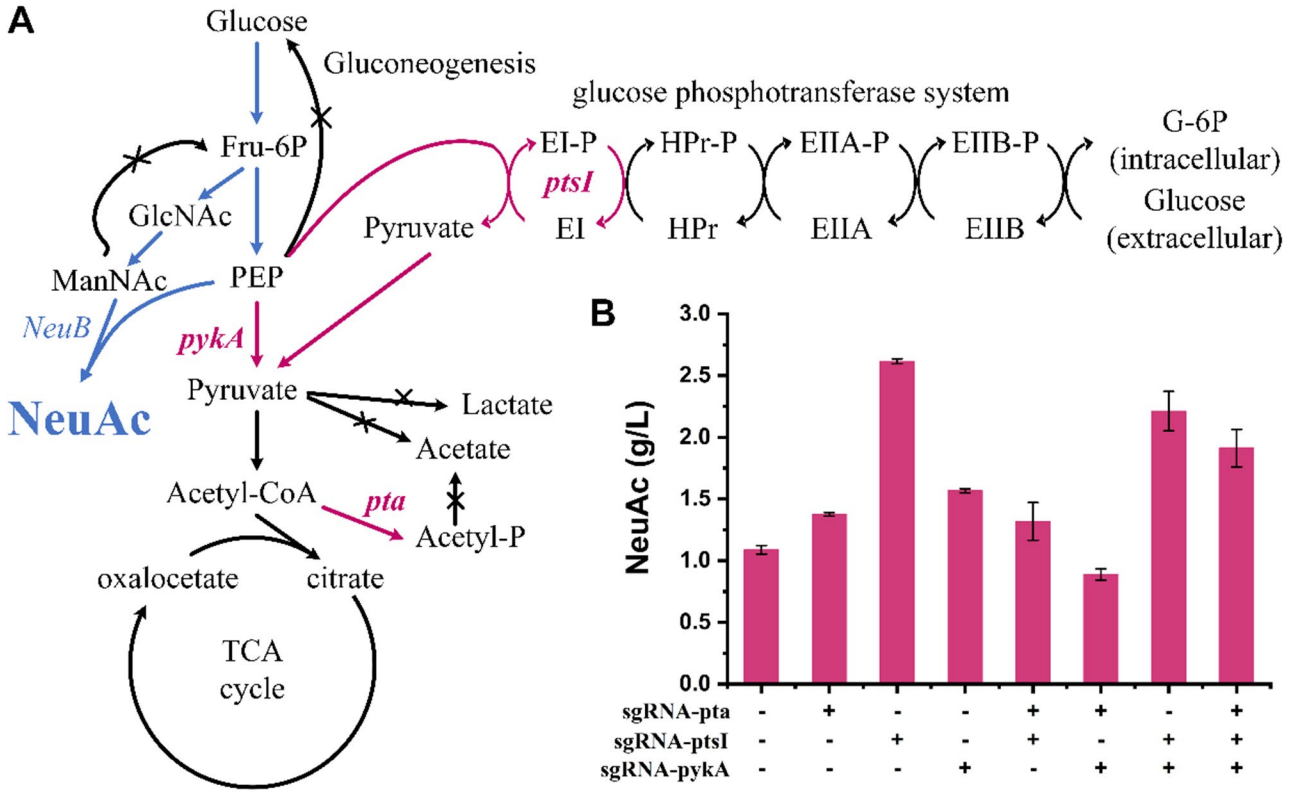

Fig. 6 Combinatorial repression of PEP-consuming genes to optimize the biosynthesis of NeuAc. (**A**) The biosynthesis pathway of NeuAc. The blue arrows indicate the biosynthesis pathway of NeuAc. The "×" indicates the metabolic pathways that have been knocked out by the DN5 strain. The red arrows indicate the CRISPRi targeting genes designed for repression; (**B**) The effect of combinatorial repression of PEP-consuming genes on NeuAc production. P_{lacO1} expresses sgRNA-pta targeting *pta*. $P_{LtetO-1}$ expresses sgRNA-ptsI targeting *ptsI*. P_{araBAD} expresses sgRNA-pykA targeting *pykA*. "-" indicates that no inducer is added, and "+" indicates that the inducer is added. Strains without sgRNA targeting any genes were used as controls. Different combinations of inducers were added when the strain was transferred to the shake flask. After 48 h of fermentation in shake flasks, the titers of NeuAc were measured. Data are expressed as means (± s.d.) from three independent experiments

1.91 g/L, respectively (Fig. 6B). We did not find that the combination of multi-gene repression was superior to *ptsI* single-gene repression for NeuAc accumulation. This result demonstrates the complexity of metabolic pathway regulation. Furthermore, the degree of gene repression may also have an important effect on product accumulation. For studies focusing on the product accumulation, more tests still need to obtain the optimal metabolic fluxes using this system.

The OD_{600} of the strain showed no significant changes following the inhibition of these three genes (Figure S7). Inhibiting *ptsI* and *pykA* reduced the conversion of PEP to pyruvate, thereby directing more PEP toward the synthesis pathway of NeuAc. Inhibition of *ptsI* alone resulted in increases of 83.2% and 63.2% in the concentrations of GlcNAc and ManNAc, respectively, while simultaneously reducing the concentration of pyruvate by 68%. In contrast, inhibiting *pykA* alone increased the concentration of GlcNAc by 11.2% and decreased the concentration of pyruvate by 30.9% (Fig. 7). Unlike single repressions of *ptsI* and *pykA*, *pta* repression did not decrease but rather slightly increased pyruvate concentration. The concentration of acetic acid increased after inhibiting *pta*, which may be attributed to the presence of alternative pathways for acetic acid synthesis (Figure S8).

Discussion

In our previous work, fine-tuning individual key essential genes in the metabolic network using CRISPRi significantly increased the titers of target compounds such as 5-aminolevulinic acid and mevalonic acid (MVA) [31–33]. Redirecting the complex metabolic network and systematically optimizing cell factories often require the regulation of multiple genes, including the regulation of heterologous enzyme expression, redirection of carbon flow, inhibition of competing pathways, and reduction of cytotoxic intermediate accumulation [34]. Numerous examples exist of using CRISPRi for the regulation of multiple genes [35, 36]. However, constructing a large number of sgRNA expression plasmids is typically required for combinatorial repression of multiple genes [14, 37]. For instance, repressing three genes necessitates the use of three single sgRNA expression plasmids, three double sgRNA expression plasmids, and one

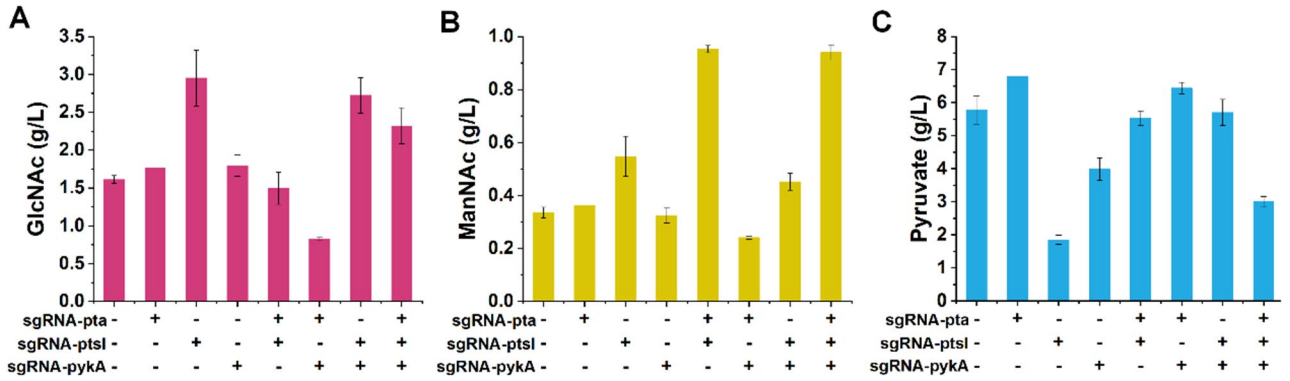

Fig. 7 Detection of fermentation by-products. "-" indicates that no inducer is added, and "+" indicates that the inducer is added. (**A**) GlcNAc; (**B**) ManNAc; (**C**) Pyruvate

triple sgRNA expression plasmid, totaling seven different sgRNA expression plasmids to cover all combinations—an approach that is both labor-intensive and inefficient.

In this study, we constructed an efficient multi-gene combinatorial repression system based on CRISPRi and demonstrated its usefulness with the example of optimizing metabolic flow for NeuAc biosynthesis. This system employs three orthogonal inducible promoters to express three distinct sgRNA sequences. By combining various inducers, we can regulate the expression of these three specific genes, achieving optimal metabolic flow. Although the repression intensity of the combinatorial repression system was generally less robust than that achieved with a single sgRNA target site, it still demonstrated significant repression of target genes compared to conditions without inducers. Most importantly, the use of inducers to express multiple sgRNA targeting sites significantly reduces the number of sgRNA plasmids required for combinatorial repression, yielding considerable savings in both labor and resources. Additionally, the strength of gene repression can be flexibly adjusted according to the bacterial growth stage, allowing for the most effective regulatory outcomes.

To achieve independent regulation of the three genes, it is essential that the three inducible promoters exhibit good orthogonality and low leakage. Despite the availability of many inducible promoters that can express proteins tightly [18, 19], significant leakage often occurs when used for sgRNA expression. This discrepancy arises because RNA expression and protein expression differ fundamentally. Protein expression involves the transcription of mRNA from DNA, translation of mRNA into polypeptide chains, and subsequent folding into the correct conformation [38, 39]. Moreover, the concentration of a protein must reach a certain threshold for detection, such as fluorescence intensity or enzyme activity. In contrast, sgRNA becomes functional immediately upon transcription and does not require additional processing, making the CRISPRi system highly sensitive [6]. In fact, the leaky expression of CRISPRi alone can often suffice for gene repression. For example, Adrian et al. demonstrated that expressing dCpf1 protein using the P_{tet} promoter and sgRNA targeting the *rfp* gene with the P_{lac} promoter resulted in over 60% inhibition of *rfp* in the absence of any inducers [40]. Similarly, when a theophylline aptamer-responsive CRISPRi system was characterized using *rfp*, a more than 50% decrease in fluorescence intensity occurred even without the addition of theophylline [41]. These findings suggest that controlling the rigorous expression of the CRISPRi system using only inducible promoters is challenging without special optimization.

Background leakage of CRISPRi greatly impairs the combinatorial effect of multi-gene repression. Hove et al. proposed three strategies to mitigate the inhibition caused by crRNA leakage expression and to expand the dynamic range of output: introducing mismatches in the reversibility-determining region of crRNA, utilizing decoy crRNA binding sites, and implementing feedback control of crRNA expression [42]. The combination of feedback control and crRNA mismatches can reduce the inhibitory effect of leakage expression from 90 to 30%. Furthermore, it has been reported that some mutations in sgRNA handle sequences exhibit different intensities of gene repression, and they show a slight decrease in affinity towards dCas9 [28]. We hypothesized that it was likely to reduce the background leakage of CRISPRi. Therefore, we opted to mutate the sgRNA handle sequence in the combinatorial repression system. We found that for the sgRNA handle sequences with reduced affinity to dCas9, the background leakage inhibition was almost eliminated. The inhibition of *lacZ* shows that sgRNA handle #3 has effectively eliminated the leakage inhibition of P_{lacO1}, while sgRNA handle #5 can reduce the leakage inhibition of $P_{LtetO-1}$ and P_{araBAD} from 90 to 20%. This method only requires the insertion of a 20 bp targeting sequence and does not require additional design, such as crRNA mismatches or decoy sites, and enables achieving highly

rigorous CRISPRi. This type of highly rigorous inhibition is essential to ensure the successful suppression of multiple gene combinations.

Furthermore, there are potential improvements for the system. The arabinose inducer can be consumed by wild-type *E. coli*, leading to unstable induction. This issue could be mitigated by knocking out the *araBAD* genes involved in arabinose metabolism [43]. In addition to CRISPRi-based gene repression, CRISPRa-based gene activation could also be integrated into this system to achieve combinatorial repression or activation of various genes [44]. Finally, the combinatorial repression system could be combined with metabolite biosensors to establish a genome-wide sgRNA library for screening optimal combinations for multiple gene regulation, thereby optimizing metabolic flow and enhancing the yield of target products.

Conclusions

By optimizing the promoter and sgRNA handle sequence, we eliminated the inhibition caused by the leaky expression of the CRISPRi system. An efficient multi-gene combinatorial inhibition system was constructed using the optimized promoters and sgRNA handle sequences. This system employs a combination of inducers to achieve combinatorial repression of multiple genes, rather than requiring the construction of numerous different sgRNA expression plasmids. We anticipate that our approach will significantly simplify the regulation of multiple genes and facilitate the fine-tuning of metabolic flow.

Abbreviations

CRISPR/Cas	Clustered Regularly Interspaced Short Palindromic Repeats
CRISPRi	CRISPR interference
Cas	CRISPR-associated protein
sgRNA	Single-guide RNA
E. coli	Escherichia coli
MVA	Mevalonic acid
IPTG	Isopropyl-beta-*D*-thiogalactopyranoside
aTc	Anhydrotetracycline
Ara	Arabinose
Rha	*L*-rhamnose monohydrate
NeuAc	*N*-acetylneuraminic acid
ManNAc	*N*-acetylmannosamine
GlcNAc	*N*-acetylglucosamine
PEP	Phosphoenolpyruvate

Supplementary Information

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Supplementary Material 1

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Author contributions

Q.S.Q. and T.Y.S. conceived and directed the research. Y.Z., Y.X.M. and Y.B.Y. performed the experiments. Y.Z. and T.Y.S. analyzed the data, discussed the results and wrote the manuscript. Q.S.Q. supervised the project. All authors reviewed the manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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