Specific Gene Repression by CRISPRi System Transferred through Bacterial Conjugation

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Abstract: In microbial communities, bacterial populations are commonly controlled using indiscriminate, broad range antibiotics. There are few ways to target specific strains effectively without disrupting the entire microbiome and local environment. Here, we use conjugation, a natural DNA horizontal transfer process among bacterial species, to deliver an engineered CRISPR interference (CRISPRi) system for targeting specific genes in recipient Escherichia coli cells. We show that delivery of the CRISPRi system is successful and can specifically repress a reporter gene in recipient cells, thereby establishing a new tool for gene regulation across bacterial cells and potentially for bacterial population control.

Keywords: CRISPR/Cas9, synthetic biology, synthetic gene regulation, horizontal gene transfer, conjugation

The CRISPR (clustered regularly interspaced short palindromic repeats) system, a natural adaptive immunity system found in bacteria, has recently been repurposed as a novel method for sequence-specific gene regulation. A catalytically dead version of the Cas9 nuclease, dCas9, combined with a conjugation engineered target specific has not been repurposed for specific gene regulation using engineered target specificity. This project takes advantage of a natural horizontal gene transfer mechanism in bacteria—conjugation—to deliver an inducible CRISPRi system to repress a specific gene, mRFP, in a target Escherichia coli reporter strain. This work establishes a basic synthetic biology tool for gene regulation between bacterial species that could be elaborated for more complex manipulation of bacterial populations in future applications.

Methods and Results

Design of Conjugative CRISPRi System. For the conjugative donor, we used the E. coli strain S17-1 (ATCC). It contains chromosomal copies of genes from the natural conjugative plasmid RP4 that encode for enzymes (e.g., relaxase), structural proteins (e.g., pil formation), and other regulatory proteins necessary for conjugation. This allows for tighter control of conjugation as the plasmid can only be transferred by the chosen donor. We utilized a compatible 5.5 kilobase pair (kb) plasmid, pARO190 (ATCC), which contains an origin of transfer (oriT) required for conjugation from a donor to a recipient. All E. coli strains are competent to receive conjugative transfer, so we chose a reporter strain containing chromosomal insertions of mRFP and sfGFP to measure CRISPRi gene repression efficiency in our recipient strain.

To transfer the CRISPRi system to the recipient strain, we cloned a previously described ~100 bp chimeric sgRNA specific to mRFP and S. pyogenes dCas9 protein-coding gene into pARO190. The sgRNA was placed under a constitutive promoter (iGEM Parts Registry BBA_J23119), while dCas9 was placed under an anhydrotetracycline (aTc)-inducible promoter (pLtetO-1) (Figure 1B). Once conjugated into a...
recipient strain and induced to produce dCas9, sgRNA and dCas9 form a complex that blocks transcription of mRFP (Figure 1A).

Assay for Conjugative Transfer of CRISPRi System. To test for successful conjugation between E. coli strains, donor and recipient strains were grown to saturation overnight in the appropriate selective media. The cultures were washed three times by pelleting and resuspending in LB without antibiotics. The donor and recipient strains were then each diluted to OD600 0.05 in a 10 mL coculture without antibiotic selection. The cocultures were incubated at 37°C for 8 h to allow for conjugation and then plated and selected for trans-conjugant cells (recipient strain with the conjugated plasmid) by antibiotics specific for both the recipient strain and transferred plasmid. Conjugation efficiency was estimated at 0.44% after 8 h of coculture (Table S2, Supporting Information).

Conjugated CRISPRi System Can Specifically Repress the Target mRFP Gene. Fluorescence was measured by flow cytometry to determine whether the conjugated CRISPRi system specifically repressed mRFP while leaving sgFp unaffected in the recipient strain. After conjugation in coculture and selection for trans-conjugants, liquid cultures were inoculated at OD600 0.05 and dCas9 production was induced by 10 ng/μL aTc (8 h, 37°C). Cultures were washed and resuspended in PBS and run on a LSRII flow cytometer (BD Biosciences) equipped with a high-throughput sampler.

Significant repression of mRFP expression (330-fold reduction compared to that of control cells lacking the CRISPRi system) was observed when the dCas9 and a MFP-specific sgRNA were expressed, but sgFp expression remained high (1.2-fold reduction). Constructs expressing dCas9 alone (i.e., without the sgRNA) showed similar slight reductions in both mRFP and sgFp expression (1.5-fold). This slight reduction correlated with dCas9 expression, potentially by contributing to metabolic burden or nonspecific targeting (Figure 2A). By microscopy, the cells containing the sgRNA against mRFP showed no red fluorescence, while the sgFp signal remained high (Figure 2B). Interestingly, induction of dCas9 did not increase repression, suggesting leaky expression of the dCas9 protein that can be optimized for future applications (data not shown). Taken together, these data demonstrate the transfer of the CRISPRi system by conjugation, and that it can result in repression of a specific reporter gene in the recipient strain.

DISCUSSION

The development of engineered CRISPR/Cas systems has allowed for specific genome-editing capability by introducing DNA double-strand breaks at target sequences; mutants without nuclease function provide further functionality both by

![Figure 1](image1.png)  
**Figure 1.** Design of CRISPRi Conjugative System. (A) Design of CRISPRi conjugation system. The conjugative donor strain S17−1 contains chromosomal copies of genes necessary for conjugation from natural conjugative plasmid RP4, and the recipient strain contains chromosomal insertions of mRFP and sgFp. The conjugative plasmid encodes a CRISPRi system specifically targeting mRFP. Once the CRISPRi plasmid is conjugated from the donor into the recipient and induced to produce dCas9, sgRNA and dCas9 form a complex and block transcription of mRFP. (B) Design of CRISPRi conjugative plasmid. The CRISPRi system was cloned into the pARO190 plasmid, which is competent for conjugative transfer by the presence of an origin of transfer (oriT). S. pyogenes dCas9 was placed under an aTc-inducible promoter (P_LtetO_1) while the sgRNA to mRFP was placed under a medium-level constitutive promoter (P_ON, iGEM Parts Registry BBa_J23119). Plasmid contains ampicillin/carbenicillin resistance and is approximately 10.5 kb.

![Figure 2](image2.png)  
**Figure 2.** Conjugated CRISPRi Causes Specific mRFP repression. (A) Specific repression of mRFP is seen only in the presence of the sgRNA complementary to mRFP, but sgFp is not affected. Fluorescence results represent arithmetic mean ± s.t.d. of three biological replicates after induction by aTc. Control (−) is reporter strain without a conjugated plasmid. Flow cytometry data were analyzed by FlowJo 7.6.1. (B) Microscopic images of mRFP and sgFp expression in target strains. Top panels are mRFP and lower panels are sgFp. mRFP expression is selectively reduced with the presence of the sgRNA, as almost no fluorescence is observed. sgFp expression remains high for all cells. Control shows cells with no fluorescent reporters. Scale bar, 10 μm.
causing gene repression or when used as targeting domains for delivery of other transcriptional regulators. Because the CRISPR system only requires a short sequence of RNA to target nuclease binding, it provides advantages over established genome-editing systems like TALENs and zinc-finger nucleases (ZFNs) which require unique protein domains to achieve binding to the desired sequences. CRISPR sgRNAs are easily produced and can be multiplexed to seek out many targets with a single Cas9 adaptor, resulting in a gene-regulation platform of a compact size that could be transferred between cells.

Here, we demonstrate the ability to deliver a targeted gene silencing system through conjugation between E. coli strains. CRISPR systems have been shown to have highly specific recognition of particular DNA sequences and can distinguish individual strains from mixed populations of bacteria, even between highly similar strains. However, to our knowledge no methods of delivery of the CRISPR system to a natural mixed population of bacteria have been developed.

The technique we describe is the first instance of cell-mediated transfer of the CRISPRi system in bacteria. Our novel design relies upon the engineering of a cell distinct from the target cell for gene knockdown, allowing for downstream manipulation of a target population of cells without direct intervention. Owing to the universality of conjugation among Gram-negative bacteria, the potential scope of targets is vast. While we have not yet demonstrated conjugative transfer in a natural microbiome, as a naturally occurring process we believe it could be optimized for therapeutic application. Alternatively, we see high potential for using bacteriophage as a delivery mechanism.

In addition to gene regulation by CRISPRi (either by repression or activation), we imagine future elaborations on this system such as targeted cell killing by DNA cleavage with catalytically active Cas9, or even transmission of CRISPR circuits that allow for more nuanced cellular responses. Combining multiple guideRNAs to multiple target sites could also provide robustness to the design not currently available with other strategies. This broad range of downstream effects that can be mediated by the CRISPR machinery provides a variety of powerful tools to fine tune the control of bacterial populations.

**REFERENCES**


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**ASSOCIATED CONTENT**

 Supporting Information
 Detailed descriptions of the materials and methods used in this study and supplementary tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

Notes
 The authors declare no competing financial interest.
Supporting Information

Plasmids, Parts, and Strains
The *E. coli* strain S17-1 was purchased from ATCC, and the recipient *E. coli* strain, an MJ1655 strain with chromosomal insertion of sfGFP and mRFP, was obtained from Lei S. Qi. *E. coli* strain Top10 (Life Technologies) was used for cloning purposes.

Plasmid pARO190 was purchased from ATCC. Two additional plasmids, pdCas9_bacteria containing *S. pyogenes* dCas9 protein-coding gene under control of an anhydrotetracycline (aTc)-inducible promoter (pLTetO-1), and pgRNA_RFP containing sgRNA placed under a constitutive promoter (iGEM Parts Registry BBa_J23119), were obtained from Lei S. Qi.

*E. coli* strains transformed with plasmids pARO190, pdCas9_bacteria, and pgRNA_RFP, were maintained in 100 μg/mL carbenicillin, 34 μg/mL chloramphenicol, and 100 μg/mL carbenicillin, respectively. Recipient *E. coli* strain was routinely maintained in 50 μg/mL kanamycin, and strain S17-1 was routinely maintained in 100 μg/mL spectinomycin. All *E. coli* strains were cultured in LB (Luria-Bertani) broth in culture tubes at 37 °C with 300 rpm shaking speed unless otherwise specified.

Supplemental Table 1: Primer Sequences

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Supplementary Methods

Plasmid Construction
To construct plasmid dCas9-pARO190, DNA fragments of dCas9 and pARO190 backbone were amplified by PCR from plasmids pdCas9_bacteria and pARO190, respectively, with a 20 bp overhang for each fragment. Primers 1 and 2 were used to amplify dCas9, and primers 7 and 8 were used to amplify pARO190 backbone. GeneArt® Seamless Cloning and Assembly Enzyme Mix (Life Technologies) was then used for assembly of the two fragments.

To construct plasmid dCas9-sgRNA-pARO190, DNA fragments of dCas9, sgRNA and pARO190 backbone were amplified by PCR from plasmids pdCas9_bacteria, pgRNA_RFP, and pARO190, respectively, with a 20 bp overhang for each fragment. Primers 1 and 3 were used to amplify dCas9, 4 and 5 for sgRNA, and 6 and 8 for pARO190 backbone. GeneArt® Seamless Cloning and Assembly Enzyme Mix (Life Technologies) was then used for assembly of the three fragments.
Primers were chemically synthesized at Integrated DNA Technologies Inc, and PCR amplification was performed with Phusion High-Fidelity DNA Polymerase (New England Biolabs). Chemical transformation was used for cloning purposes, and electroporation was used to transform plasmids into *E. coli* strain S17-1.

**Conjugation**

Overnight cultures of donor and recipient strains were washed three times by centrifuging at 3000 rpm for 2 minutes and then resuspending in fresh LB broth without antibiotics. The resuspended cultures were diluted and co-cultured into 10 mL fresh LB broth without antibiotics, to OD<sub>600</sub> of 0.05 for both strains in the co-culture. The co-culture was then incubated at 37°C with 100 rpm shaking speed to allow for conjugation. After a specific time, the co-culture was vigorously mixed on a vortex mixer to stop conjugation and separate connecting donor and recipient cells.

**Calculation of Conjugation Efficiency**

S17-1 strains transformed with dCas9-sgRNA-pARO190 served as donor strains and the reporter strains served as recipients. After conjugation, co-cultures were plated on LB agar plates containing antibiotics of spectinomycin, kanamycin, and both carbenicillin and kanamycin, for the selection of donor, recipient, and trans-conjugant strains respectively. Serial dilutions (10<sup>3</sup>, 10<sup>4</sup>, 10<sup>5</sup>, 10<sup>6</sup> fold) of the co-cultures were made prior to plating, and 200 μl of each dilutions were plated. After overnight incubation, colonies on each plate were counted, and the results were presented as colony forming units per milliliter culture (cfu/ml). Conjugation efficiencies were calculated by dividing counting results of trans-conjugant cells to that of recipient cells. Time points of 2, 5, and 8 hours of conjugation were tested for estimation of optimal conditions.

**Supplemental Table 2: Conjugation Efficiency**

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**Induction and Flow Cytometry**

The reporter strains were separately co-cultured with S17-1 strains transformed with plasmids dCas9-pARO190 and sgRNA-dCas9-pARO190 (conjugative plasmid) for 8 hours for conjugation. Co-cultures were then diluted 10<sup>4</sup> fold and plated on LB agar plates containing both carbenicillin and chloramphenicol to select for trans-conjugants. After overnight incubation, liquid cultures of trans-conjugant colonies were made and grown overnight for saturation. The overnight cultures were then diluted to OD 0.05 into fresh carbenicillin and chloramphenicol containing LB broth in 2 ml 96-well deep well plates in duplicate, with 10 μg/mL aTc supplemented to one replicate of each conjugation to induce production of the dCas9 protein. Cultures were incubated at 37°C with shaking (1200 rpm) for 8 hours, washed and resuspended in PBS, and run on a LSRII flow cytometer (BD Biosciences) equipped with a high-throughput sampler to determine the levels of fluorescent proteins. Recipient strains without conjugation
were also in parallel tested and served as a negative control experiment. Data were analyzed by FlowJo 7.6.1, and plotted using GraphPad Prism 6. For each experiment, triplicate cultures were measured, and their standard deviation was indicated as error bar.

**Microscopy**

Trans-conjugants were cultured and induced as above for flow cytometry analysis. After 8 hours induction, 5μl of cells were spotted on glass slides under a coverslip and edges sealed with a PAP Pen (Fisher Scientific). Images were acquired in mRFP and sfGFP channels with 5ms exposure on an Olympus BX51 fluorescence microscope with 60x PlanApoN 1.42 NA oil objective equipped with a Hamamatsu Orca-ER CCD camera. Images were processed with ImageJ and presented with identical contrast settings.