ACS SyntheticBiology
Research Article
Cite This: ACS Synth. Biol. 2018, 7, 1538−1552
pubs.acs.org/synthbio

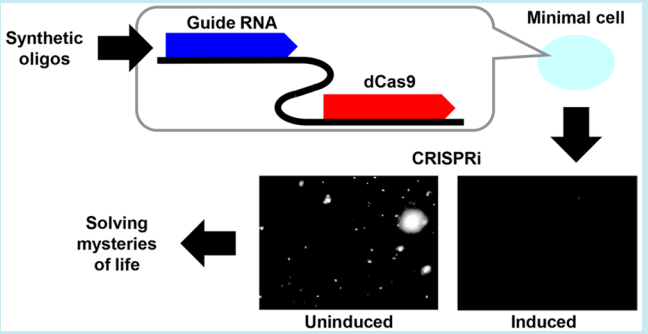
Tuning Gene Activity by Inducible and Targeted Regulation of Gene Expression in Minimal Bacterial Cells

Ana M. Mariscal,[⊗,†] Shigeyuki Kakizawa,[⊗,‡,§] Jonathan Y. Hsu,[‡,∥] Kazuki Tanaka,[‡,⊥,#] Luis González-González,[†] Alicia Broto,[¶,▽] Enrique Querol,[†] Maria Lluch-Senar,[¶,▽] Carlos Piñero-Lambea,[¶,▽] Lijie Sun,[‡] Philip D. Weyman,[‡] Kim S. Wise,[‡] Chuck Merryman,[‡] Gavin Tse,[‡,∥] Adam J. Moore,[‡,∥] Clyde A. Hutchison, III,[‡] Hamilton O. Smith,[‡] Masaru Tomita,[⊥] J. Craig Venter,[‡] John I. Glass,[‡] Jaume Piñol,[†] and Yo Suzuki*[,‡]

[†]Departament de Bioquímica i Biologia Molecular and Institut de Biotecnologia i Biomedicina, Universitat Autònoma de Barcelona, Cerdanyola del Vallès, Barcelona 08193, Spain
[‡]Synthetic Biology Group, J. Craig Venter Institute, La Jolla, California 92037, United States
[§]National Institute of Advanced Industrial Science and Technology, Tsukuba, Ibaraki 305-8560, Japan
[∥]Department of Bioengineering, University of California, San Diego, La Jolla, California 92093, United States
[⊥]Institute for Advanced Biosciences, Keio University, Tsuruoka, Yamagata 997-0035, Japan
[#]Faculty of Environment and Information Studies, Keio University, Fujisawa, Kanagawa 252-0882, Japan
[¶]EMBL/CRG Systems Biology Research Unit, Centre for Genomic Regulation (CRG), The Barcelona Institute of Science and Technology, Barcelona 08036, Spain
[▽]Universitat Pompeu Fabra (UPF), Barcelona 08002, Spain

Supporting Information

ABSTRACT: Functional genomics studies in minimal mycoplasma cells enable unobstructed access to some of the most fundamental processes in biology. Conventional transposon bombardment and gene knockout approaches often fail to reveal functions of genes that are essential for viability, where lethality precludes phenotypic characterization. Conditional inactivation of genes is effective for characterizing functions central to cell growth and division, but tools are limited for this purpose in mycoplasmas. Here we demonstrate systems for inducible repression of gene expression based on clustered regularly interspaced short palindromic repeats-mediated interference (CRISPRi) in *Mycoplasma pneumoniae* and synthetic *Mycoplasma mycoides*, two organisms with reduced genomes actively used in systems biology studies. In the synthetic cell, we also demonstrate inducible gene expression for the first time. Time-course data suggest rapid kinetics and reversible engagement of CRISPRi. Targeting of six selected endogenous genes with this system results in lowered transcript levels or reduced growth rates that agree with lack or shortage of data in previous transposon bombardment studies, and now produces actual cells to analyze. The *ksgA* gene encodes a methylase that modifies 16S rRNA, rendering it vulnerable to inhibition by the antibiotic kasugamycin. Targeting the *ksgA* gene with CRISPRi removes the lethal effect of kasugamycin and enables cell growth, thereby establishing specific and effective gene modulation with our system. The facile methods for conditional gene activation and inactivation in mycoplasmas open the door to systematic dissection of genetic programs at the core of cellular life.



KEYWORDS: *inducible promoters, riboswitch, tetracycline-mediated repression, clustered regularly interspaced short palindromic repeats (CRISPR), mycoplasma, functional genomics*

Genomes of some mycoplasma and related genera contain as few as several hundred genes, yet they are capable of growing in the laboratory without the need for any secondary organism within the culture providing any materials or vital functions. The synthetic bacterium JCVI-syn3.0 has the smallest genome among all known free-living organisms. It operates with only 473 genes derived from *Mycoplasma mycoides*.[1] The natural bacterium *Mycoplasma pneumoniae* contains 733 genes, as well as an additional 117 sequences

Received: January 17, 2018
Published: May 22, 2018

DOI: 10.1021/acssynbio.8b00028
ACS Synth. Biol. 2018, 7, 1538−1552

for which transcripts have been detected.[2] With much of biosynthesis jettisoned, these organisms rely on experimentalists to provide many metabolites and biological building blocks, but they maintain functions that cannot be substituted with supply of materials from outside. Therefore, the genomes in these organisms are expected to be condensed and enriched for genetic codes to support operations that can only be performed by organisms.

Due to extensive genome reduction in nature and in the laboratory, these mycoplasma genomes have high proportions of nonredundant genes essential for cell growth and proliferation. Transposon bombardment studies suggest that 81% of the genes are essential in *Mycoplasma genitalium*, a free-living organism with the smallest natural genome, even in a rich laboratory culture condition.[3] The estimated proportions of essential genes are also high for *M. pneumoniae* (64%) and *M. mycoides* (52%).[4] The synthetic JCVI-syn3.0 cell has a gene set that is almost exclusively comprised of genes that are essential or quasi-essential for growth.[1] In contrast, only 7% of the genes in *Escherichia coli* are essential, as shown in a targeted knockout study.[5] The low frequency of essential genes in *E. coli* can be explained by the prevalence of genes that are required only in specific growth conditions[6,7] and the presence of safeguard mechanisms sometimes involving functionally redundant genes including paralogues that compensate for the loss of a gene with a critical biological function[8,9] in *E. coli*. Therefore, revealing some essential functions in genetic studies in typical bacteria can be challenging with the potential need to identify new culture conditions or new genetic backgrounds that unmask relevant phenotypes. The low complexity of mycoplasma genomes offers opportunities for unobstructed access to the core biological program where many gene functions present themselves as phenotypes resulting from single-gene inactivation in a single growth condition.

Although basic processes underlying cellular life are presumed to be well-understood, a substantial fraction of the essential or quasi-essential genes in the JCVI-syn3.0 cell were genes of unknown function.[1] Sixty-five genes (14%) in the genome had no annotation regarding their functions. Eighty-four genes (18%) were categorized as generic, indicating only a rudimentary understanding of their molecular functions. For example, specific substrates for some enzymes could not be assigned. In *M. pneumoniae*, 25 genes out of 363 genes (7%) that are presumed to be essential are in a category for genes encoding conserved hypothetical proteins with no functional information.[10] Some of these genes may have novel functions, or novel sequences for functions already characterized in other organisms, reflecting mycoplasma-specific modes of life or evolutional history. Alternatively, they may be responsible for fundamental biological functions previously unidentified in other systems due to functional redundancy. Characterization of these functions may have universal implications to cellular life and may be most effectively conducted in minimal cells.

Genetic manipulation of gene activity to link genotype to phenotype has been a standard practice for characterizing gene function. However, complete abrogation of a gene essential for viability would result in lethality. It is challenging to prepare a sample for observation where lethal organisms are well-represented in the population. Also, already dead cells often cannot reveal the primary defects leading to lethality. Conditional gene inactivation is effective for generating a population of lethally destined organisms where a majority of the organisms display a timely phenotype for characterization of genes essential for viability. Traditionally, temperature-sensitive mutants were used for this purpose.[11,12] Inducible repression of gene expression offers a more versatile approach that can be applied to genes encoding various enzymes and proteins, but no tools for this purpose are available for mycoplasmas. Inducible overexpression has been demonstrated in related species, *Spiroplasma citri*,[13] *Mycoplasma agalactiae*,[13] and *M. genitalium*,[14] and serves as a foundation for the current study.

RESULTS AND DISCUSSION

Gene functions can be experimentally determined by creating gain- or loss-of-function conditions for genes and analyzing the resulting phenotypes. However, simple knockout of a gene that is essential for viability in an organism would not result in a population of cells or individuals convenient for phenotypic analysis. Any of several available strategies needs to be implemented for genetic characterization for such a gene. In this study, we establish facile tools for overexpressing and repressing genes in minimal bacterial cells where many genes are essential for viability or rapid proliferation. Specifically, we present data on inducible expression systems based on tetracycline-mediated (Tet) regulation and a riboswitch involving a theophylline aptamer[15] in synthetic *M. mycoides* cells. We then describe results from using the Tet system to drive nuclease-defective Cas9 protein for inducible transcriptional repression[16] in the synthetic cells and in *M. pneumoniae*. Future studies using these tools at a genomic scale are expected to contribute to the precise and complete understanding of chemical reactions underlying these cellular systems.

Inducible Activation of Gene Expression in the Synthetic JCVI-syn1.0 Cells. Inducible gene expression *via* Tet regulation was previously demonstrated in *S. citri*, *M. agalactiae*,[13] and *M. genitalium*.[14] Tet regulation involves the conformational change of tetracycline repressor (TetR) upon binding to tetracycline that releases TetR from its binding site on DNA, tetracycline operator (*tetO*), and enables the transcription of the downstream genes. To establish a system for ectopic gene expression in synthetic *M. mycoides*, we implemented the molecular tools shown to be effective in *M. genitalium*.[14] We initially used the Δ1−6 strain of *M. mycoides* JCVI-syn1.0[17] as a reference strain to develop molecular tools because this strain grows more quickly than strains of the minimal JCVI-syn3.0 organism. Reference strains and transformants with various DNA constructs in each organism are termed strains for the purpose of this publication. The JCVI-syn1.0 Δ1−6 strain lacks all six restriction systems,[17] but still contains all other genes of the JCVI-syn1.0 organism.[18] We generated plasmids (pSD024 and pSD048) containing a Pxyl/tetO_2 promoter,[13,14,19] a codon-optimized gene for the fluorescent protein mCherry[20] (D. Thomas, N. Assad-Garcia, and C. Merryman, unpublished result) as a reporter gene, a gene encoding TetR under the control of the Pspi promoter,[13,21,22] the replication origin *oriC* from *M. mycoides*, and a puromycin selection marker[21] (see Methods), and introduced these plasmids into the strain. When treated with tetracycline, we observed a 40- to 62-fold increase in mCherry expression, as examined using fluorimetry (Figure 1a). Epifluorescence micrographs were consistent with the fluorimeter data (Figure 1b).

In comparing with Tet systems in other mycoplasma species, we found that unlike the example in *M. genitalium*,[14] a Shine−Dalgarno sequence was necessary for observing any expression

DOI: 10.1021/acssynbio.8b00028
ACS Synth. Biol. 2018, 7, 1538−1552

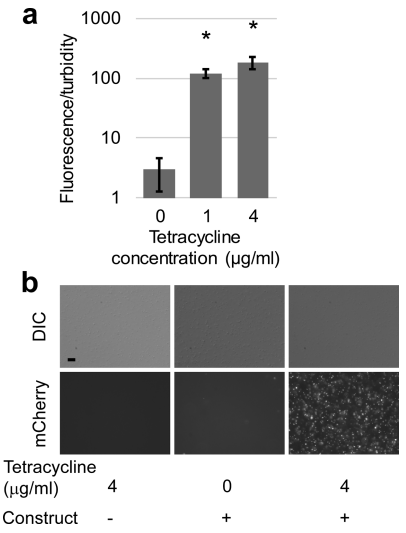


Figure 1. Tetracycline-mediated derepression of gene expression in the synthetic bacterium JCVI-syn1.0. The reporter gene was a Pxyl/tetO$_2$-mCherry gene containing a Shine−Dalgarno sequence for ribosomal binding. (a) Fluorimetry data showing induced expression. The cells contained the plasmid pSD024. Three independent transformants were analyzed for each tetracycline concentration (n = 3). Error bar shows standard deviation. Asterisk indicates statistical significance of difference from data for the uninduced control (P < 0.05). Fluorescence/turbidity values at 1 and 4 μg/mL tetracycline concentrations were 40- and 62-fold higher than the no tetracycline control, respectively. (b) Fluorescent micrographs showing tetracycline-induced expression of mCherry. The brightness was adjusted equally for the three images. DIC denotes images of the identical fields captured with optics for differential interference contrast. The construct was the plasmid pSD048 containing the same mCherry reporter gene as pSD024. Bar indicates 10 μm.

in *M. mycoides* JCVI-syn1.0 Δ1−6 (Figure S1). In addition, a relatively high concentration (>400 ng/mL) of tetracycline was needed for induction (data not shown). This concentration is higher than those required for mycoplasma Tet systems tested so far (~10 ng/mL) and in the range needed for an *E. coli* system (~250 ng/mL or higher).[23] Tetracycline analogues such as anhydrotetracycline have not been tested. The requirement for a high tetracycline concentration is unlikely to be related to the presence of the *tetM* marker in the JCVI-syn1.0 Δ1−6 genome, as the mechanism for resistance for the *tetM* gene product, to bind the ribosome and dislodge tetracycline,[24] would not affect the concentration of tetracycline available to bind TetR. In support of this discussion, when a strain with a variant of the JCVI-syn1.0 Δ1−6 genome not containing the *tetM* marker (V. Paralanov, N. Asada-Garcia, and J.I. Glass, unpublished result) was used, a low tetracycline concentration of 10 ng/mL did not induce gene expression (data not shown). Induction at 100 ng/mL in this strain could not be measured because growth of the cells was inhibited at this level of tetracycline (data not shown). Therefore, it was fortuitous that the *tetM* marker for a different purpose enabled Tet regulation by protecting the cells from tetracycline. It is plausible that membrane composition varies among mycoplasmas, resulting in differing membrane solubility for tetracycline.[25] It may be that mycoplasmas tested previously have a promiscuous transporter that facilitates the influx of tetracycline into the cell.[25] Another possible explanation for the requirement for a higher tetracycline concentration in synthetic *M. mycoides* is that TetR is more abundant, due to the Pspi promoter driving its gene being highly active, in this organism. The need for Shine−Dalgarno sequence in the Pxyl/tetO$_2$ system for detection of gene expression in *M. mycoides*, but not in *M. genitalium*, described above may be consistent with the abundance of TetR and a limited number of transcripts for the reporter protein even in the presence of tetracycline in *M. mycoides*. A testable hypothesis from this idea is whether a lower tetracycline concentration can be used for induction if a weaker promoter drives the expression of TetR.

To compare the Tet system with a second independent inducible system for gene expression, we investigated a riboswitch for translational regulation based on the interaction between a theophylline aptamer and a Shine−Dalgarno sequence.[26] We introduced this riboswitch downstream of a Pspi promoter and upstream of the mCherry gene (see Methods). When tested in JCVI-syn1.0 Δ1−6, we achieved a 4-fold increase in expression upon induction (Figure S2). This level of activation is in line with other examples where the theophylline aptamer was used,[26] confirming that this mechanism can be operational in mycoplasmas. Because this riboswitch controls translation, whereas Tet regulation controls transcription, one advantage of the riboswitch would be fast response time when protein production or activity is the output of interest. However, the difference in expression between the induced state and the uninduced state was greater for the Tet induction system. When induced, the expression levels were similar for the two systems, but the basal level of expression was lower with the Tet system. This greater dynamic range was attractive for creating cleaner distinction between on and off states of gene expression for characterizing gene functions. We thus chose the Tet induction system for subsequent study.

Inducible Attenuation of Gene Expression *via* CRISPRi in the JCVI-syn1.0 Cells. To establish an inducible system for repressing gene expression, we used the Tet system to drive a nuclease-defective variant of Cas9 (dCas9). The dCas9 protein localizes to a DNA site *via* a single guide RNA (sgRNA) and blocks transcription.[16] This mechanism is termed clustered regularly interspaced short palindromic repeats-mediated interference (CRISPRi). When CRISPRi is induced to target a constitutively expressed mCherry gene in the JCVI-syn1.0 Δ1−6 cells, 72−76% repression was observed with the two guide RNA (gRNA) sequences tested (Figure 2), whereas CRISPRi directed by a gRNA sequence that does not exist in the cell (nonmatching gRNA) had no effect on mCherry expression. In these experiments, the cells were transformed using a plasmid containing expression modules for mCherry, dCas9, and sgRNA (see Methods). To gain evidence for tetracycline-induced expression of dCas9, we constructed a gene for a dCas9-mCherry fusion protein and placed it under the same Pxyl/tetO$_2$ promoter used for Tet regulation. The plasmid containing this construct (pSD052) did not contain the constitutively active mCherry gene used as a target in the earlier experiment. When tetracycline was added, the fluorescence of cells increased 2.6-fold (Figure S3).

Kinetics of Inducible Activation and Attenuation of Gene Expression in the JCVI-syn1.0 Cells. To characterize the kinetics of tetracycline-mediated regulation, we first assessed the stability of mCherry protein in the JCVI-syn1.0 Δ1−6 cells. For this purpose, we prepared a culture of a strain carrying a Pxyl/tetO$_2$-mCherry construct (pSD048) that was induced to express mCherry and observed what happened to the mCherry signal after the inducer was removed. When cells in a dense culture with tetracycline were collected, washed to

DOI: 10.1021/acssynbio.8b00028
ACS Synth. Biol. 2018, 7, 1538−1552

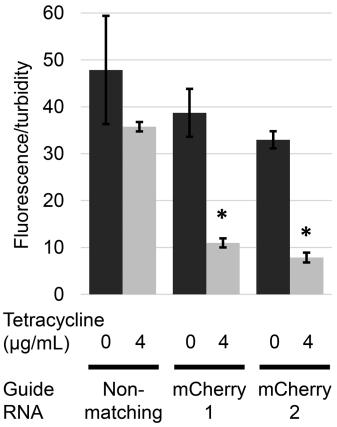


Figure 2. Induced attenuation of gene expression for a mCherry transgene in the JCVI-syn1.0 cells *via* CRISPRi. Guide RNA species designated as nonmatching, mCherry 1, and mCherry 2 were provided by plasmids pSD060, pSD062, and pSD063, respectively. Each plasmid contained a Pxyl/tetO$_2$-dCas9 gene and a constitutively expressed mCherry gene in addition to the sgRNA expression module. Four independent transformants with each plasmid were cultured with or without 4 μg/mL tetracycline for 24 h (n = 4). Tetracycline-mediated induction of dCas9 expression resulted in 72% and 76% decrease in mCherry expression level with pSD062 and pSD063, respectively. Error bar shows standard deviation. Asterisk indicates statistical significance of difference from data for the uninduced control (P < 0.01).

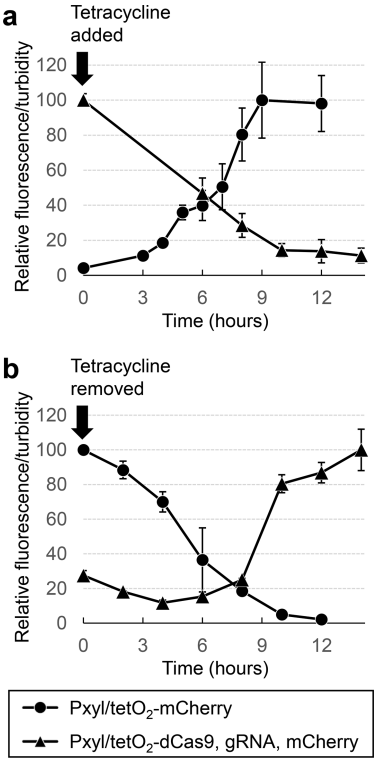


Figure 3. Kinetics of induced derepression and CRISPRi of mCherry gene expression in the JCVI-syn1.0 cells. Tetracycline was added (a) or removed (b) at time 0. Time indicates the duration for which each culture was incubated at a growth-permissive temperature (37 °C). During the rest of the experiment, the culture was kept at a growth-restrictive temperature (4 °C). Two independent transformants were analyzed for each series with addition or removal of tetracycline and the plasmid pSD048 (Pxyl/tetO$_2$-mCherry; circle) or pSD063 (Pxyl/tetO$_2$-dCas9, constitutively expressed mCherry gene, and sgRNA gene 2 for mCherry; triangle). Values for fluorescence/turbidity were normalized to the maximal value in each experiment.

remove tetracycline, suspended with an equal volume of fresh medium without tetracycline, and incubated for 24 h, the fluorescence level was similar to the level for a culture with tetracycline where induction continued (Figure S4, two samples for washed compared), suggesting that mCherry protein is stable over the time frame of the experiment or that the promoter was locked in a derepressed state with old TetR still bound to tetracycline and not much fresh TetR generated. In contrast, when the starting mCherry-expressing cells were diluted 80-fold with fresh medium without tetracycline and incubated for 24 h, mCherry fluorescence diminished by 95% (20-fold) compared to the dense culture without tetracycline when normalized to turbidity or by 98% (51-fold) when not normalized (Figure S4, washed and diluted compared to washed), while the fluorescence level recovered in the presence of tetracycline (washed and diluted). These experiments established that dilution of mCherry protein *via* cell division is necessary to reveal the repression of mCherry transcription. The 20- and 51-fold differences may seem smaller than a difference expected from the 80-fold dilution, but details regarding how growth affects mCherry expression may explain this discrepancy.

We then conducted time-course experiments on engaging and disengaging tetracycline-mediated induction of gene expression and CRISPRi in the JCVI-syn1.0 Δ1−6 cells. We found that Tet regulation initiates readily upon the addition of the tetracycline inducer (Figure 3). Induced expression with a Pxyl/tetO$_2$-mCherry construct (pSD048) reached a maximal level in 9 h. Induced repression *via* CRISPRi with a Pxyl/tetO$_2$-dCas9 construct (pSD063), as assayed by the reduction of the mCherry signal through repression of mCherry gene expression and dilution of the protein *via* cell divisions, was completed in 10 h (Figure 3a). Conversely, when the tetracycline inducer was removed from the medium, mCherry levels from the Pxyl/tetO$_2$-mCherry construct normalized to turbidity fully decreased in 10 h (Figure 3b). The same process fully reversed CRISPRi of a mCherry gene with a Pxyl/tetO$_2$-dCas9 construct in 15 h (Figure 3b). Given that mCherry is stable, it is expected that dCas9 would be diluted similarly to or more quickly than mCherry as the cells are incubated and allowed to divide. Therefore, the longer time needed for recovery of the mCherry signal with Pxyl/tetO$_2$-dCas9 turned off suggests that there is more than a sufficient amount of dCas9 protein to completely repress the mCherry gene in the starting cells.

Although these data with the mCherry reporter can provide a framework for induction experiments, the time required for folding and half-life are dependent on the target protein. For example, for mCherry to be detected, its chromophore would need extra time to mature. Some proteins would have a fast turnover rate, unlike mCherry in our study. These proteins might accumulate more slowly in induced expression and dissipate more rapidly during a CRISPRi process. Tet regulation and CRISPRi target the transcription process, and the turnover rate of the message, as well as the rates of initiation for translation, would also greatly affect the protein abundance. The doubling time of the JCVI-syn1.0 cells used here is approximately 60 min.[1] However, mCherry abundance rarely makes a 2-fold change per hour in any of the three processes in Figure 3 that require the dilution of protein *via* cell division (engagement and disengagement of CRISPRi, and

DOI: 10.1021/acssynbio.8b00028
ACS Synth. Biol. 2018, 7, 1538−1552

engagement of direct transcription regulation with removal of tetracycline), suggesting the need for ramp up of components needed for setting the right condition for Tet regulation or CRISPRi within the cells.

Inducible Attenuation of Gene Expression *via* CRISPRi in *M. pneumoniae*. To establish CRISPRi in a natural mycoplasma of a different phylogenetic lineage, we used the human pathogen *M. pneumoniae*. To introduce a gene for the fluorescent protein Venus to be used as a target, we transformed cells of *M. pneumoniae* strain M129 with the pMTpmp200Venus plasmid harboring a transposon construct including the Venus gene. The transformed cells were selected and pooled to generate a mixed population of cells (termed the V pool), with each cell bearing a copy of the Venus gene at one of many unspecified locations in the genome. Cells from this pool exhibited strong fluorescence along the whole cell body (Figures S5 and S6). Clonal and isogenic populations were not used because (1) it takes a long time to establish these populations due to the long doubling time of *M. pneumoniae* cells (~8 h), and (2) the mixed-population data could effectively show the variability in repression by CRISPRi depending on where in the chromosome the construct is inserted. We then transformed these cells with either TnPac_dCas9cons or TnPac_dCas9ind construct, and transformants from each population were recovered and pooled. Each of these constructs had a sgRNA gene for targeting dCas9 to the Venus gene and the dCas9 gene under the control of either a constitutive promoter[27] (TnPac_dCas9cons) or the Pxyl/tetO_2mod inducible promoter (TnPac_dCas9ind).[14] Therefore, each cell of the new pool (termed the V-dCas9cons or V-dCas9ind pool) contained the Venus construct and a sgRNA/dCas9 construct in two unspecified locations of the genome.

To evaluate CRISPRi-mediated repression of the Venus gene expression, fluorescence from Venus protein was analyzed by epifluorescence microscopy. Because *M. pneumoniae* cells grow on surfaces, unlike JCVI-syn1.0 and JCVI-syn3.0 cells, microscopy with the option to select a focal plane for analysis is more suitable than bulk measurement with fluorimetry. In addition, heterogeneity of fluorescence signal within a cell population can be readily assessed with microscopy. Whereas cells from V pool showed strong fluorescence along the cell body (Figure S5a), only a few cells from V-dCas9cons pool exhibited detectable fluorescence, although those cells that fluoresced in this pool were as bright as cells from V pool (Figure S5b). We then cultured cells from V-dCas9ind pool and V pool with or without tetracycline and examined Venus fluorescence at 24, 48, and 66 h time points. In the absence of tetracycline, the number of fluorescent cells and the fluorescence intensity of the fluorescent cells were very similar between the pools (Figure 4, upper panels for each time point). The data for V pool revealed that Venus expression was not affected with 10 ng/mL tetracycline at any time point (Figure 4). In contrast, Venus fluorescence exhibited a strong decay after 24 h, and no fluorescent cells were detected after 48 and 66 h in V-dCas9ind pool in the presence of tetracycline (Figure 4). Interestingly, induced expression of dCas9 was more effective than constitutive expression in entirely eliminating fluorescing cells from the population. Since the constitutive expression of dCas9 can be detrimental to cell growth in *M. pneumoniae*, it is possible that cells with transposon insertions at sites in the genome allowing for strong expression of dCas9 are negatively selected. Thus, cells bearing transposon

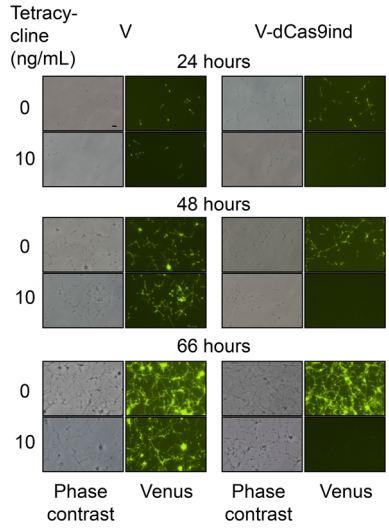


Figure 4. Induced attenuation of gene expression for a Venus transgene in *Mycoplasma pneumoniae* with CRISPRi. A population of cells with an integrated Venus gene (V pool) was first grown for 66 h without tetracycline and then supplemented with 0 or 10 ng/mL tetracycline and incubated for 24, 48, and 66 h. Phase-contrast and epifluorescence micrographs of the population showed that tetracycline itself did not affect Venus expression. The same experiment was performed with cells containing an integrated inducible dCas9 construct with a sgRNA gene for the Venus gene in addition to an integrated Venus gene (V-dCas9ind pool). The induction of CRISPRi resulted in marked reduction of Venus expression. The increase of fluorescence over time for the population in the absence of tetracycline reflects an increase in cell density due to cell divisions that occur during experiments. All images are shown at the same magnification. Scale bar indicates 2 μm.

insertions in genomic regions favoring weak expression of dCas9, for instance by the presence of antisense transcripts, might outcompete cells that are abundantly expressing dCas9. Although alternative explanations are also possible, this finding highlights the importance of using inducible promoters when knocking down gene expression using dCas9.

To quantify CRISPRi-mediated repression and to confirm the effect at the transcription level, the amounts of Venus transcript in the cell pools obtained were analyzed with real-time quantitative PCR (RT-PCR). In the absence of tetracycline, the abundances of Venus transcript in cells showed no significant difference between V-dCas9ind and V pools, but a 79% decrease was observed in V-dCas9cons pool compared to the other pools (Figure 5a). We then tested induction of CRISPRi with tetracycline. When cells from V-dCas9ind pool were grown for 66 h in the presence of 10 ng/mL tetracycline, the Venus transcript level decreased 97% compared to uninduced cells from the same pool (Figure 5b). Previous work has established that certain antibiotics at low, subinhibitory concentrations modulate bacterial transcription patterns.[28] However, no significant differences in Venus transcript level were found in the presence or absence of tetracycline in the V pool cells (Figure 5b), indicating tetracycline itself does not affect Venus expression. Taken together, the data presented here demonstrate that CRISPRi is functional in *M. pneumoniae*.

Induced Activation and Attenuation of Gene Expression in the JCVI-syn3.0 Cells. We then investigated whether dCas9-mediated repression can also be attained in a strain with the minimized JCVI-syn3.0 genome.[1] This organism

DOI: 10.1021/acssynbio.8b00028
ACS Synth. Biol. 2018, 7, 1538−1552

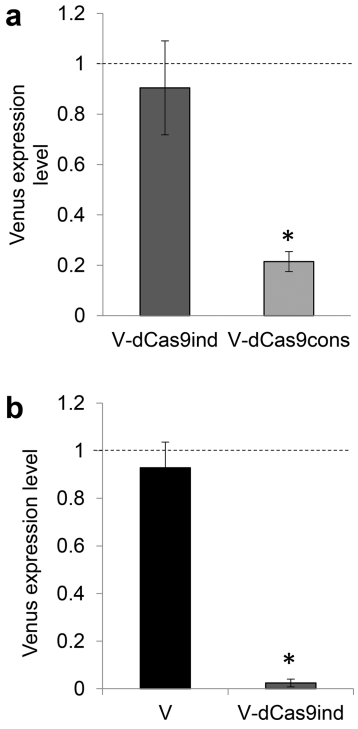


Figure 5. CRISPRi of a Venus gene evaluated with RT-PCR in *M. pneumoniae*. (a) Venus expression levels in V-dCas9ind (inducible dCas9 gene) and V-dCas9cons (constitutive dCas9 gene) pools cultured without tetracycline. Venus expression was normalized in reference to its expression in V-pool. Asterisk indicates statistical significance for the difference from the data of the V pool ($P < 0.05$, $n = 3$). The Venus mRNA level of V-dCas9cons pool decreased 79% from that of V pool. (b) Venus expression levels in cells from V and V-dCas9ind pools grown with 10 ng/mL tetracycline. Venus expression was normalized in reference to its expression in uninduced pools (V and V-dCas9ind, respectively). Asterisk indicates statistical significance for difference from the data for the uninduced control ($P < 0.05$, $n = 3$). Venus mRNA level of V-dCas9ind pool with tetracycline decreased 97% from the uninduced control. Means from three independent biological repeats are plotted with their standard error bars.

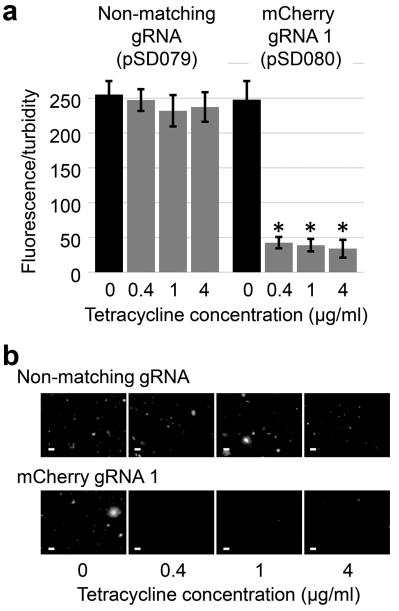


Figure 6. Induced attenuation of gene expression for a mCherry transgene in the JCVI-syn3.0 cells with CRISPRi. Constructs containing a Pxyl-tetO_2-dCas9 gene, a sgRNA gene, a constitutively expressed mCherry gene, and a puromycin resistance gene, as well as *loxP* sites for integration into the landing pad in the JCVI-syn3.0 genome, were provided by plasmids pSD079 (nonmatching gRNA) and pSD080 (gRNA 1 for mCherry). The strains were cultured with indicated concentrations of tetracycline for 48 h. (a) Fluorimetry data. Error bar shows standard deviation. Asterisk indicates statistical significance of difference from data for the uninduced control ($P < 0.01$, $n = 4$). Induction of CRISPRi resulted in 86−83% reduction of mCherry expression compared to the uninduced control. (b) Fluorescent micrographs. Bar indicates 10 μm.

has a region in the genome termed the landing pad for incorporating an incoming DNA construct with a Cre/lox mechanism.[29] We therefore introduced *loxP* sites for recombination in our CRISPRi constructs and targeted them to the landing pad. We first confirmed tetracycline-induced expression of a dCas9-mCherry fusion protein from a gene under the control of the Pxyl/tetO_2 promoter using a construct contained in plasmid pSD083 in the JCVI-syn3.0 cells. Upon induction, the fluorescence of cells increased 7.5-fold, as measured using fluorimetry of bulk populations (Figure S3b), suggesting that Tet regulation operates properly in these cells, as well as in the JCVI-syn1.0 cells.

When a construct containing a constitutively expressed mCherry gene, a Pxyl/tetO_2-dCas9 gene, a *tetR* gene, and a gene encoding sgRNA for mCherry (pSD080) was introduced into JCVI-syn3.0, we observed 86−83% reduction of mCherry fluorescence upon induction with increasing concentrations (0.4, 1.0, and 4.0 μg/mL) of tetracycline (Figure 6a). There was no obvious difference in efficiency among the three concentrations of tetracycline, suggesting that induction of CRISPRi at 0.4 μg/mL of tetracycline already saturates some mechanisms involved in CRISPRi in these cells. When a version of the same construct with a gRNA sequence that does not match any gene in the genome (pSD079) was introduced into JCVI-syn3.0, there was no significant change of mCherry fluorescence upon induction, suggesting that dCas9 when paired with a suitable sgRNA specifically targets the mCherry gene. These results were corroborated by data from analysis with epifluorescence microscopy (Figure 6b).

We also determined the kinetics of the CRISPRi-mediated repression in the JCVI-syn3.0 cells (Figure 7). Upon inducing CRISPRi, mCherry fluorescence started declining after 18 h and reached a lowest level in 33 h. As in JCVI-syn1.0 (Figure S4), it is expected that mCherry is stable and needs to be diluted *via* cell division for the fluorescence to diminish after dCas9 binds the target in JCVI-syn3.0. It is expected that the response time in JCVI-syn3.0 is slower than in JCVI-syn1.0 (Figure 3), partly because the doubling time is longer in JCVI-syn3.0 (~120−180 min)[1] than in JCVI-syn1.0 (~60 min).[30] The initial delay in the decline of fluorescence may be the result of a lag in cell growth potentially experienced by the JCVI-syn3.0 cells.

To see if CRISPRi is applicable to endogenous genes, we selected six genes in JCVI-syn3.0. For this initial study, we selected genes that showed a close-to-average expression level in a transcriptomics analysis of JCVI-syn3.0 (A. Edlund, unpublished result). Three of them (JCVISYN3_0541, JCVISYN3_0654, and JCVISYN3_0835) are presumed to be essential for growth (e genes) based on transposon bombardment.[1] The other three (JCVISYN3_0305, JCVISYN3_0416, and JCVISYN3_0481) are presumed to be quasi-essential (i genes) (Table 1). When CRISPRi is induced for any of the

DOI: 10.1021/acssynbio.8b00028
ACS Synth. Biol. 2018, 7, 1538−1552

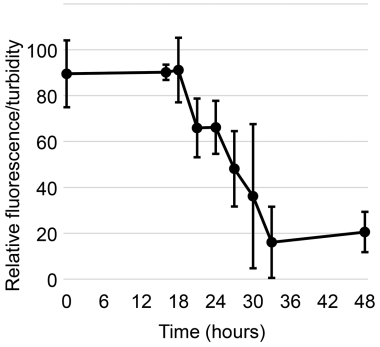


Figure 7. Kinetics of induced CRISPRi of mCherry gene expression in the JCVI-syn3.0 cells. The cells containing a constitutively expressed mCherry gene, a Tet-driven dCas9 gene, and a sgRNA gene with the first gRNA sequence for the mCherry gene derived from the plasmid pSD080 (Figure 6) were collected after 0, 16, 18, 21, 24, 27, 30, 33, and 48 h of induction with 0.4 μg/mL tetracycline. Fluorescence and turbidity were measured using a plate reader. Values for fluorescence/turbidity were normalized to the maximal value in each experiment. Error bar shows standard deviation (n = 2). The decline of mCherry fluorescence started after 18 h and completed after 33 h.

three essential genes, growth of the JCVI-syn3.0 cells was noticeably inhibited, as quantified using the PicoGreen dye to stain the genomic DNA (Figure 8a,b). The effects on growth were inconclusive for the three quasi-essential genes. To see if these targeted genes were repressed by CRISPRi in the cultures, we examined the message levels for two of them using RT-PCR. We found that gene expression for JCVISYN3_0305 and JCVISYN3_0481 were repressed 97% and 96%, respectively, when CRISPRi was induced with a correct sgRNA, whereas little change was observed when a non-matching gRNA was used (Figure 8c). Therefore, the CRISPRi system we developed is suitable for at least some of the genes with average expression levels in the genome.

CRISPRi-Mediated Repression of *ksgA* Gene Expression in the JCVI-syn3.0 Cells. The phenotypes we expected and observed above were defects in growth. To exclude the possibility that CRISPRi simply causes loss of fitness in our organism, we introduced a CRISPRi construct with a gRNA sequence for the *ksgA* gene (JCVISYN3_0004) into the JCVI-syn3.0 cells. The *ksgA* gene encodes a methylase that modifies 16S rRNA (rRNA). This methylation is not essential for the function of 16S rRNA. Rather, it makes 16S rRNA vulnerable to inhibition by an antibiotic kasugamycin.[31] Therefore, when the *ksgA* gene is active, the cells are kasugamycin-sensitive, and when the gene is inactivated, the cells are kasugamycin-resistant. Unlike many resistance mutations for other antibiotics acting on ribosome,[32] loss of a gene function can confer resistance for kasugamycin. We reasoned that the induction of CRISPRi for *ksgA* would enable the cells to grow in the presence of kasugamycin (Figure S6). Because healthy growth would be the opposite of what is expected for any deleterious effect, demonstrating kasugamycin resistance with CRISPRi would showcase the capacity of CRISPRi for specific gene inactivation.

We first examined the sensitivity of the JCVI-syn3.0 cells to kasugamycin. We found that its growth was markedly slowed with 500−1000 μg/mL of kasugamycin (data not shown) similarly to other bacteria.[33] We then performed tetracycline-mediated induction of dCas9 expression with sgRNA for *ksgA* in JCVI-syn3.0 strains using a construct in plasmid pSD090. We found that strains carrying this construct showed stronger resistance to kasugamycin than those with a nonmatching gRNA sequence (Figure 9). However, we observed no difference in growth between induced and uninduced cells carrying this construct (Figure S7).

We thought of a possibility that the Pxyl/tetO$_2$ promoter for dCas9 was deregulated due to some mutations. However, when the whole pSD090 plasmid was sequenced, we did not find any mutation around the expression modules for dCas9 or sgRNA including the tetracycline repressor gene. As shown with a dCas9-mCherry fusion protein, the expression level of a gene under the control of Pxyl/tetO$_2$ in the absence of tetracycline is small, but detectable (Figure S3). Therefore, there would be some leakage of dCas9 expression in an uninduced state. The *ksgA* gene shows one of the lowest expression levels in relative abundance among genes in JCVI-syn3.0 cells (417th among 473 genes) based on a transcriptome analysis of a log-phase culture (A. Edlund, unpublished result). It may be that poorly expressed genes are more susceptible to dCas9-mediated repression, and the uninduced level of dCas9 expression may thus be sufficient to inhibit *ksgA* transcription. Although tetracycline-mediated induction of dCas9 did not affect the outcome of inhibiting *ksgA*, targeting this gene with CRISPRi removed kasugamycin sensitivity and enabled cell growth (Figure S6) without causing any detectable loss of fitness of the cells. This established specific and effective gene inactivation

Table 1. List of Endogenous Genes in the JCVI-syn3.0 Cells Used as Targets for CRISPRi[a]

designation in this study	systematic gene name	predicted protein encoded	essentiality	gRNA sequence	plasmid providing construct
i gene 1	JCVISYN3_0305	Metallopeptidase family protein M24	in/n	GAAAAUCAGUAUCUAUUUUC	pSD084
i gene 2	JCVISYN3_0416	Hypothetical protein	n/in	GCUGAUCUACAGUUCUAUUAUUAAA	pSD085
i gene 3	JCVISYN3_0481	Lipoprotein, putative	n/ie	GUACUACUCAACUCCCCAUUUUC	pSD086
e gene 1	JCVISYN3_0541	DnaJ chaperone protein	e	GAGCAUCUGGAGAUUUAUUUAAAUC	pSD087
e gene 2	JCVISYN3_0654	Ribosomal protein S5	e	GCUAAUUCAAUAACAGCACGAGC	pSD088
e gene 3	JCVISYN3_0835	Lipoprotein, putative	e/ie	GAGCAUGAAAUUGUUGUUAAUGU	pSD089
ksgA	JCVISYN3_0004	KsgA, rRNA adenine dimethyltransferase	in/n	GCUGAAUCAACUUUAGGAAU	pSD090

[a]Essentiality is based on two transposon bombardment experiments, reflecting how many independent transposon insertions presumed to be disruptive to gene function were found in each gene within the populations tested (Hutchison, 2016).[1] Larger numbers of transposon insertions can be expected in nonessential genes than in essential genes in these studies. e, i, and n denote essential, impaired/intermediate, and nonessential, respectively. ie and in indicate a level between i and e and between i and n, respectively. Gene annotations are derived from the genome sequence of JCVI-syn3.0 at accession no. CP014940.1.

DOI: 10.1021/acssynbio.8b00028
ACS Synth. Biol. 2018, 7, 1538−1552

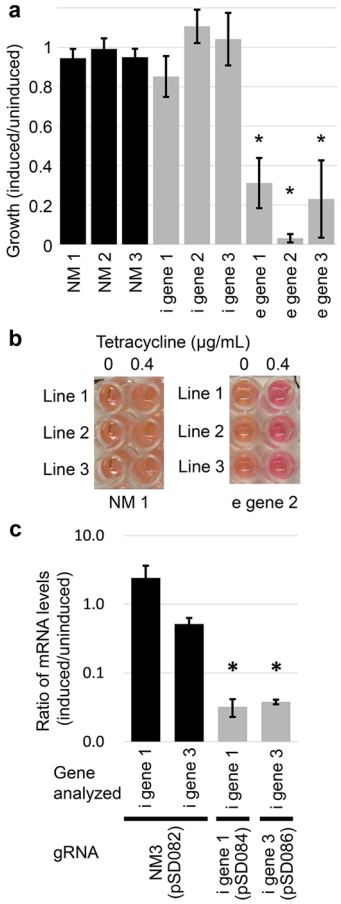


Figure 8. Induced CRISPRi of endogenous genes in the JCVI-syn3.0 cells. Strains were cultured with or without 0.4 μg/mL tetracycline for 48 h. (a) Quantification of genomic DNA as a metric for growth using the PicoGreen dye. Error bar shows standard deviation. Asterisk indicates statistical significance for difference when compared to the nonmatching (NM) sgRNA control ($P < 0.01$, $n = 3$). Strains for impaired (i) genes 1, 2, and 3 had sgRNA for genes JCVISYN3_0305, JCVISYN3_0416, and JCVISYN3_0481, respectively. Those for essential (e) genes 1, 2, and 3 had sgRNA for JCVISYN3_0541, JCVISYN3_0654, and JCVISYN3_0835, respectively. Strains for NM1, 2, and 3 had a nonmatching gRNA (pSD079), gRNA 1 for mCherry (pSD080), and a nonmatching gRNA (pSD082), respectively. CRISPRi of e genes 1, 2, and 3 resulted in 69%, 97%, and 77% reduction of growth, respectively. (b) Colorimetric assay for pH change as a metric for growth. Phenol red in the medium changes colors from red to orange as the cells grow. Lines 1−3 show results with three independent transformants. Growth was inhibited when CRISPRi is induced for e gene 2. (c) Transcriptional repression as revealed with RT-PCR. Total RNA was analyzed to examine the expression levels of three genes, *ldh* (control, L-lactate dehydrogenase gene, JCVISYN3_0475), i gene 1 (JCVISYN3_0305, metallopeptidase family M24, target of pSD084 construct), and i gene 3 (JCVISYN3_0481, putative lipoprotein, target of pSD086 construct). Expression levels of *ldh* gene were used for normalization. Black bars show data with a control nonmatching gRNA (NM3, pSD082), and gray bars show data with constructs targeting one of the two endogenous target genes. Error bar shows standard deviation. Asterisk indicates statistical significance for difference from the corresponding data with the nonmatching control construct ($P < 0.05$, $n = 3$). Upon induction, the expression levels were reduced by 97% and 96% compared to the control for i genes 1 and 3, respectively. i gene 2 was not tested because the primers we selected for i gene 2 did not produce a detectable signal in a pilot RT-PCR experiment where a cDNA sample from an uninduced strain or genomic DNA was used as a template.

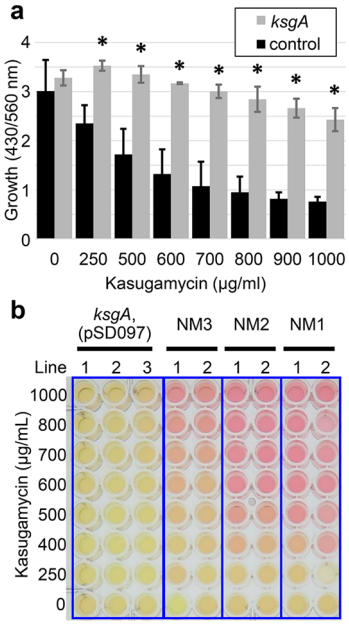


Figure 9. CRISPRi of *ksgA* expression results in kasugamycin resistance in the JCVI-syn3.0 cells. (a) Dose−response analysis with or without CRISPRi of *ksgA* expression. Growth of cells was estimated by measuring absorbance levels for the two forms of the phenol red pH indicator in the medium at 430 and 560 nm. The cells were cultured for 4 days in a medium with tetracycline and 0−1000 μg/mL kasugamycin. Black bars show mean values with control gRNA (nonmatching gRNA with pSD079, gRNA1 for mCherry with pSD080, and nonmatching gRNA with pSD082). Gray bars show mean values of three independent clones with pSD097 (gRNA for the *ksgA* gene). Error bar shows standard deviation. Asterisk indicates statistical significance for difference when compared to the data for the control constructs ($P < 0.01$, $n = 3$). (b) Photograph of cultures of CRISPRi strains. The cells were cultured with tetracycline and a varying concentration of kasugamycin for 4 days. NM1, 2, and 3 denote strains with a nonmatching gRNA (pSD079), gRNA1 for mCherry (pSD080), and a nonmatching gRNA (pSD082), respectively. The lines indicate independent transformants. All three clones with a gRNA for *ksgA* (pSD097) grew faster at high concentrations of kasugamycin than control strains. These data suggest that CRISPRi elicit specific gene inactivation phenotypes rather than general fitness defects when endogenous genes are targeted.

with our system. For restoring inducible CRISPRi for essential genes with low expression levels, the uninduced expression level for dCas9 might need to be further reduced, for example, by deoptimizing the codon-optimized dCas9 gene sequence.

Additional Consideration on CRISPRi Technologies and Inducible Systems in Minimal Cells. As in other bacteria, many genes in mycoplasmas are members of gene clusters resembling operons. It has been reported that CRISPRi of an upstream gene in an operon also leads to the inactivation of the downstream genes.[34,35] Targeting of a downstream gene may not affect the upstream gene, if CRISPRi leads to abortion of transcription alone,[36] but it affects further downstream genes. Therefore, the resolution of CRISPRi analysis is constrained by transcriptional units, and the data need to be interpreted carefully for gene-specific function assignment.

Although the initial CRISPRi study in *E. coli* showed the lack of off-target effects for CRISPRi,[16] the presence of off-target effects when using Cas9 or dCas9 has been widely reported by others,[37,38] and our CRISPRi systems in mycoplasma cells cannot escape this problem. The main source of the off-target

DOI: 10.1021/acssynbio.8b00028
ACS Synth. Biol. 2018, 7, 1538−1552

effects is the partial annealing of gRNA to genes other than the intended target gene. A simple solution is to test multiple gRNA sequences for each gene of interest and regard only the phenotypes shared by all sequences as the target-specific effects. Another cause of off-target effects on transcription may be cellular stress when inducing the expression of dCas9. For dealing with this problem, the comparison of phenotypes should be made only within the cells induced to express dCas9 the same way. The use of a re-engineered version of dCas9 with lower affinity to DNA[39−41] may help in ameliorating the off-target problem. The optimization of the dCas9 expression levels could also be beneficial toward increasing the specificity in targeting.

We have shown that CRISPRi systems are functional in *M. pneumoniae* and synthetic versions of *M. mycoides*, suggesting that these systems can be easily expanded to other mycoplasmas. We also confirmed that both Tet and theophylline inducible systems are functional in mycoplasmas. These systems may be used simultaneously and independently in a single cell. Mycoplasmas are appealing models of minimal cells and their concise genetic repertories make these organisms especially well-suited for systems biology studies and the design-build-test-learn cycles for creating microbial factories and devices.[1,18] However, this group of organisms is uniquely intractable to conventional tools for genetic analysis. Whole genome synthesis and genome activation has been demonstrated in one organism, whereas many mycoplasma species are refractory to any genome engineering. Tools are especially lacking for work with essential genes. Neither conditional mutants nor other common methods to deal with these genes had been previously reported in this group of organisms. The CRISPRi technologies demonstrated in our study are expected to be effective for a majority of genes in mycoplasmas where a suitable sequence exists to serve as a target for gRNA, when the expression levels are not particularly low. Moreover, these technologies are facile and amenable to genome-wide studies of gene functions. Systematic analysis in minimal organisms is expected to lead to a deeper understanding of the cellular life, contribute to improving human health, and enrich an emerging biobased society.

METHODS

Bacterial Strains and Culture Conditions. *Escherichia coli* XL-1 Blue cells were grown at 37 °C in LB medium or on LB agar plates containing 100 μg/mL of ampicillin.[42] *E. coli* NEB 5-alpha cells (New England BioLabs) were grown at 37 °C in low-salt LB medium with 5.0 g/L of NaCl or on its agar version containing 25 μg/mL of zeocin (Thermo Fisher Scientific). *Mycoplasma pneumoniae* M129 wild type strain (ATCC 29342) and its isogenic mutant derivatives were grown at 37 °C under 5% CO_2 in 25 cm^2 and 75 cm^2 tissue culture flasks containing 5 and 20 mL of SP-4 medium, respectively.[43] *M. pneumoniae* was transformed as previously described,[27] and pools of transformant cells were grown for 1 week in SP-4 medium supplemented with 5 μg/mL puromycin or 100 μg/mL gentamycin. JCVI-syn1.0 and JCVI-syn3.0 cells (based on the sequences from *Mycoplasma mycoides*) and their isogenic mutant derivatives were grown at 37 °C in Hayflick medium[44] or SP-4 medium.[43] JCVI-syn1.0 and JCVI-syn3.0 were transformed as previously described.[45] For selection of puromycin resistant strains, puromycin concentrations of 4 and 1 μg/mL were used for JCVI-syn1.0 and JCVI-syn3.0, respectively. For tetracycline induction experiments, tetracycline hydrochloride (Sigma) was used. Tetracycline analogues such as anhydrotetracycline were not used in this study. For kasugamycin resistance assay, kasugamycin hydrochloride (ENZO Life Sciences) was used.

DNA Manipulation. DNA manipulation was performed according to procedures in Sambrook and Russell.[45] Genomic DNA from *M. pneumoniae* was isolated using the E.Z.N.A. Bacterial DNA Kit (Omega BIO-TEK). Plasmid DNA samples were prepared using the Fast Plasmid Mini Eppendorf Kit (VWR) or QIAprep Spin Miniprep Kit (Qiagen). Large amounts of plasmid DNAs were purified using the GenElute HP Plasmid Midiprep Kit (Sigma).

PCR amplification was performed using Phusion High-Fidelity DNA Polymerase (Thermo Scientific) or PrimeStar Max DNA polymerase (Takara) and adjusting the annealing temperature according to the primers used (Table S2). Purification of PCR products and digested DNA fragments from agarose gels was performed using NucleoSpin Gel and PCR Clean-Up (Macherey-Nagel) kits. FastDigest restriction enzymes (Thermo Scientific) and T4 DNA ligase (Roche) were used according to manufacturer instructions.

Sequencing reactions were performed with BigDye v3.1 Cycle Sequencing kit using 100 ng of plasmid DNA following the manufacturer's instructions, or outsourced to Eurofins USA. When sequencing reactions were performed in-house, they were run in an ABI PRISM 3130x1 Genetic Analyzer at Servei de Genòmica i Bioinformàtica (UAB).

Plasmid Construction for *M. pneumoniae* Experiments. Primers used in this work are in Table S1. All the constructs (Figure S8) were screened by restriction analysis and confirmed by Sanger sequencing.

The pMTpmp200Venus vector is based on pMTpmp200-178/mrfp1 vector.[46] First, the promoter from mp200 and a small fragment of mp200 ORF was amplified by PCR from pMTpmp200-178/mrfp1 incorporating the restriction sites *Not*I and *Kpn*I. The Venus gene[47] was amplified by PCR from pMT-ptuf-Venus[48] adding the *Kpn*I and *Eco*NI sites at the ends. Both PCR products were digested with the corresponding restriction enzymes and ligated into a pMT85 vector previously digested with *Not*I and *Kpn*I enzymes.[46] In this way, the new vector is bearing a cassette with an in-frame fusion of the *Pmp200* fragment containing the promoter and a small fragment coding for a 29 amino acid cysteine-rich peptide with the *Venus* ORF followed by *E. coli rrnB* terminator.[46] The 29 amino acid peptide of mp200 ORF was fused with Venus ORF to improve protein stability and provide an ubiquitous cell distribution.[46]

TnPac_dCas9ind was constructed as follows. A synthetic version of Cas9 gene from *Streptococcus pyogenes* codon optimized for expression in *M. pneumoniae* was purchased from GenScript. Nuclease activity of Cas9 was abolished by introducing D10A and H840A mutations (8). First, Cas9 was amplified using 5_BamHI_pxyl_dCas9 and 3_SalI_dCas9 oligonucleotides including *Bam*HI and *Sal*I sites, introducing the D10A mutation. Then, a second Cas9 fragment was amplified using 5_SalI_dCas9_H840A and 3_AatII_Cas9 oligonucleotides including *Sal*I and *Aat*II sites, respectively, to introduce mutation H840A. Both PCR products were purified and digested with the corresponding restriction enzymes. A third DNA fragment including the sequence coding for a sgRNA targeting the Venus gene under the control of the MG_438 constitutive promoter[27] was prepared by GenScript (Figure S8b). This fragment was flanked by *Aat*II and *Mre*I

DOI: 10.1021/acssynbio.8b00028
ACS Synth. Biol. 2018, 7, 1538−1552

sites, and the sgRNA sequence was based on the sgRNA targeting a GFP gene previously described.[16] An additional DNA fragment containing a gene for TetR and the Pxyl/tetO$_2$mod promoter was obtained by digesting plasmid pΔMG_217Cre[14] with *Pst*I and *Bam*HI. All these fragments were finally ligated into a modified TnPac minitransposon[14] previously digested with *Pst*I and *Mre*I.

To obtain the TnPac_dCas9cons construct, the dCas9 gene was first amplified from TnPac_dCas9ind using the primer 5_PstI_p438_Cas9, which includes the MG_438 constitutive promoter and a *Pst*I site, and primer 3_AatII_Cas9, which includes an *Aat*II site. The PCR product was purified and digested with *Pst*I and *Aat*II. A second DNA fragment containing the sgRNA targeting the Venus gene was obtained by digesting plasmid miniTnPac_dCas9ind with *Aat*II and *Mre*I. Both fragments were ligated into a modified TnPac minitransposon[14] previously digested with *Pst*I and *Mre*I.

Plasmid Construction for Experiments in JCVI-syn1.0 and JCVI-syn3.0. Homologous recombination in *E. coli*[49] was used for all plasmid construction unless otherwise indicated. In this approach, PCR fragments with overlapping ends are directly introduced into NEB 5-alpha cells for generating plasmids. The PCR fragments used are described below for each generated plasmid with names of primers and templates for the PCR reactions indicated. The sequences of the primers are found in Table S2. In some cases, a single PCR product with overlapping ends was used to make a circular plasmid (self-closure).[49] For example, gRNA sequences were introduced into a common backbone plasmid using this variant approach. A small modification made to the published protocol[49] was to dilute the *Dpn*I-treated PCR products 10-fold with water, rather than purify the products, before *E. coli* transformation. Guide RNA sequences were designed as previously described.[50] All the construction was confirmed with PCR, restriction analysis, or Sanger sequencing. The lineage of plasmids constructed along with descriptions of the plasmids is found in Figures S9 and S10. The maps of a few representative plasmids are shown in Figure S11.

MmoriC Plasmids for Studies in the JCVI-syn1.0 Cells. The vector backbone amplified from the ZpUC19 plasmid[51] with primers ZpUC_F and ZpUC_R and a DNA fragment containing a Pspi (spiralin promoter from Spiroplasma)-mCherry-terminator gene amplified from the pTF20 plasmid (D. Thomas, N. Assad-Garcia, and C. Merryman, unpublished result) with primers mCh_F and mCh_R were assembled into plasmid pSD001. pSD001 was amplified with primers Riboswitch_F2 and Riboswitch_R2 and recircularized in *E. coli* to make pSD002 with a Shine−Dalgarno (SD) sequence containing a theophylline aptamer sequence replacing the first SD sequence of the Pspi-mCherry-terminator module. To make pSD003 and pSD005, a fragment containing Ptuf-puro[21] (promoter from the EF-Tu gene driving a puromycin resistance gene) and MmoriC (chromosome replication origin sequence *oriC* from *Mycoplasma mycoides*) was amplified from the pBKMyco1 plasmid (B. Karas, unpublished result) with primers Ptuf-puro_MmoriC_F and Ptuf-puro_MmoriC_R and combined with the construct amplified from pSD001 and pSD002, respectively, with primers pSD001_F and pSD001_F. To make pSD006, the second SD of the mCherry gene was deleted by self-closure of a fragment amplified from pSD005 with the Del2_ribo_F and Del2_ribo_R primers. To make pSD009, the second SD and an N-terminal portion of the mCherry open reading frame was deleted in pSD003 by amplifying and recircularizing a fragment amplified from pSD003 with the Del4_reg_F and Del4_reg_R primers. To make pSD020, the sgRNA (single guide RNA) module was amplified from the pgRNA-bacteria plasmid[16] with the AddsgRNA_sgRNA_F and AddsgRNA_sgRNA_R primers and assembled with the construct amplified from pSD006 with the AddsgRNA_Vec_F and AddsgRNA_Vec_R primers. To make pSD021, crossover PCR was first used to combine a Pxyl/tetO$_2$ fragment[13,14,19] (199 bp) amplified from the pMT85-XTST plasmid[13] with the Pxyl-tetO2_R and Mariscal_CO_Pxyl-tetO2_F primers and a Pspi-*tetR* fragment (988 bp) amplified from the same plasmid with the Mariscal_CO_Pspi-tetR_F and tetR_R1 primers and obtain a 1185-bp fragment amplified with the Pxyl-tetO2_R and tetR_R2 + 5_pSD primers. This product was then assembled with a fragment amplified from pSD006 with the pSD_F and pSD_R primers. To make pSD024, self-closure was conducted on a fragment amplified from pSD021 with the Tet-SD-mCherry_2F and Tet-SD-mCherry_2R primers, to delete only the theophylline aptamer sequence in front of the mCherry sequence. To make pSD025, self-closure was performed on a fragment amplified from pSD021 with the Tet-mCherry_2F and Tet-mCherry_2R primers, to delete the theophylline aptamer and the associated SD sequence and generate a Pxyl/tetO$_2$-mCherry gene not containing a SD sequence. To make pSD048, the sgRNA module was amplified from the pgRNA-bacteria plasmid[16] with the AddsgRTet_sgRNA_F and AddsgRTet_sgRNA_R primers and combined with a fragment amplified from the pSD024 plasmid with the AddsgRTet_Vec_F and AddsgRTet_Vec_F primers.

Unlike many organisms, mycoplasmas use the UGA codon to code for tryptophan rather than translational stop, and they preferentially use the UGA codon over UGG for tryptophan. To cope with the toxicity of expression of dCas9 from a high-copy plasmid to *E. coli* (data not shown) hampering our cloning effort and to generate a dCas9 gene suitable for robust expression in mycoplasmas, all seven codons for tryptophan in the *S. pyogenes* dCas9 gene were changed to UGA. The resulting gene would be inactive and harmless in *E. coli* to facilitate the generation of needed dCas9 plasmids in *E. coli*. The first step was to clone the dCas9 gene into a promoter-less vector. For this purpose, the dCas9 gene was amplified from the pdCas9-bacteria plasmid[16] using the Cas9_Z_Ins_F and Cas9_Z_Ins_R primers and combined with the ZpUC19 vector[51] amplified with the Cas9_Z_Vec_F and Cas9_Z_Vec_R primers, to generate pSD030. The ZpUC19 plasmid is derived from the pUC19 plasmid, but the partial *lacZ* gene in pUC19 is missing in ZpUC19. The dCas9 gene was inserted in ZpUC19 in the opposite direction as the *lacZ* gene in pUC19. We then used crossover PCR on a single fragment with overlapping ends sequentially to introduce multiple codon changes into the fragment. The first PCR was done with plasmid pSD030 and primers W1F_dCas9 and W1R_dCas9. The PCR product was purified and diluted 1/60 in the next PCR reaction. The second PCR was performed with the W2F_dCas9 and W2R_dCas9 primers. The third PCR was done with the W3F_dCas9 and W3R_dCas9 primers. All PCR products were used for transformation of *E. coli* for self-closure. Plasmids from colonies were purified and sequenced. The product of the third PCR and self-closure process with a correctly made dCas9 gene with three codons optimized ($W_1W_2W_3$) was named pSD033. The fourth PCR was done from pSD033 with the W4F_dCas9 and W4R_dCas9 primers. The process was repeated on a new product in each round to

DOI: 10.1021/acssynbio.8b00028
ACS Synth. Biol. 2018, 7, 1538−1552

conduct the fifth, sixth, and seventh PCR with the primer sets W5F_dCas9 and W5R_dCas9, W6F_dCas9 and W6R_dCas9, and W7F_dCas9 and W7R_dCas9, respectively. All PCR products were used in transformation of *E. coli* for self-closure. Plasmids obtained from the seventh round (pSD038) had all the recoded codons except the fifth change and had an unexpected 133 bp repeat within the dCas9 gene. Fortuitously, there was a unique *Eco*RI site within the repeat. Digestion of this plasmid with *Eco*RI and self-ligation were used to release the duplicated sequence. The ligated plasmid was directly used in PCR with the W5F_dCas9 and W5R_dCas9 primers to introduce the fifth change (W_5). The resulting plasmid was named pSD040.

To make a Pxyl/tetO_2-dCas9 (tryptophan codon-optimized)-mCherry fusion construct (pSD052), the codon-optimized dCas9 gene was amplified from pSD040 with the Cas9T_F and Cas9_R_mChFus primers and assembled with a fragment amplified from pSD048 with the Vec_F_mChFus and VecT_R primers. To make pSD060, the pSD052 plasmid was amplified with the Unfuse_VecF and Unfuse_VecR primers and combined with a 365-bp fragment amplified from the pSD020 plasmid with the Unfuse_PF and Unfuse_PR primers containing the Pspi promoter with a sequence for a ribosomal binding site regulated by a theophylline aptamer (Pspi-SD_{Theo}), to "unfuse" the dCas9-mCherry fusion protein gene by introducing the Pspi-SD_{Theo} into the gene. The two fragments needed for this engineering were difficult to assemble with homologous recombination in *E. coli*, so we used the Gibson-Assembly method.[52] To make pSD062 and pSD063, sequences for gRNA 1 and gRNA 2 for repressing mCherry gene expression were introduced into pSD060, *via* PCR with the primer sets mCh_gRNA1_F and mCh_gRNA1_R and mCh_gRNA2_F and mCh_gRNA2_R followed by self-closure in *E. coli*, respectively.

Landing Pad-Compatible Constructs for Studies in the JCVI-syn3.0 Cells. Suicide plasmids were used to provide Cre recombinase and a construct flanked by *loxP* sites to be integrated into the landing pad of the JCVI-syn3.0 genome. There were two unwanted *loxP* sites in pSD060, and deleting these sequences was the first step. The pSD060 plasmid was amplified with the Del_loxP_F and Del_loxP_R primers, and recircularized to generate pSD070. The pSD070 plasmid was amplified with the rm_loxP_F and rm_loxP_R primers, and recircularized to generate pSD073.

A 1.5 kb fragment containing a Cre recombinase gene driven by a Pspi promoter (spiralin promoter from *Spiroplasma*) was amplified from the pMod2_loxPuro_Pspi-Cre plasmid with the LP_Cre_F and LP_Cre_R primers and assembled with a CRISPRi construct excluding MmoriC amplified from pSD073 with the LP_Vec_F and LP_Vec_R primers, to generate the plasmid pSD074. This plasmid had one *loxP* site in a suitable region. To introduce another *loxP* site into the plasmid, in a region between the ZpUC19 vector backbone and the sgRNA module, to enable the excision of a functional construct, the pSD074 plasmid was amplified with the ins_loxP_F2 and ins_loxP_R2 primers and recircularized, to generate plasmid pSD075.

The source of the Pspi-Cre fragment, the pMod2_loxPuro_Pspi-Cre plasmid, was originally generated for a separate project. A Ptuf-puro fragment was first amplified from the pLS-Tn5-Puro plasmid[53] with Loxright-rev and Loxleft-for primers using Q5 High-Fidelity 2X Master mix (New England Biolabs). This fragment was cloned into the *Sma*I site of the EZ-Tn5 pMOD2 ⟨MCS⟩ vector (Epicenter) using restriction cloning, to produce the pMod2_loxPuro plasmid. A Pspi-Cre fragment was excised from pLS-Tn5-Puro_Pspi-Cre (J. Dai, C. Basier, R. Chuang, and S. Vashee, unpublished result) by *Pst*I digestion and cloned into the *Pst*I site of the pMod2_loxPuro plasmid, to generate the pMod2_LoxPuroLox_Pspi-Cre plasmid.

To introduce the first gRNA sequence (gRNA 1) for the mCherry gene into the CRISPRi construct in pSD075, self-closure was performed on a pSD075 fragment amplified with the mCh_gRNA1_F and mCh_gRNA1_R primers, generating pSD076. To delete the constitutively expressed mCherry gene from pSD075, the self-closure process was performed on a pSD075 fragment amplified with the Del_mCh_F and Del_mCh_R primers, generating plasmid pSD078.

Sanger sequencing revealed a unintended nonsense mutation within the puromycin resistance gene of pSD070−78. The MmoriC plasmids pSD048, pSD060, and pSD063 did not have the same mutation. This nonsense mutation would inactivate the puromycin resistance gene, so we fixed this mutation in pSD075, pSD076, and pSD078 by amplifying these plasmids with the Puro_Stop_Corr_F1 and Puro_Stop_Corr_R1 primers and recircularizing the products, generating plasmids pSD079 (nonmatching gRNA), pSD080 (gRNA 1 for mCherry) and pSD082, respectively.

To make a dCas9-mCherry fusion gene with tryptophan codon optimization to be expressed in JCVI-syn3.0, the self-closure procedure was performed for a pSD079 fragment amplified with the Del_P_mk_fus_F and Del_P_mk_fus_R primers, generating plasmid pSD083. To introduce gRNA sequences for seven endogenous target genes, fragments amplified from pSD082 with the gR0305F/gR0305R, gR0416F/gR0416R, gR0481F/gR0481R, gR0541F/gR0541R, gR0654F/gR0654R, gR0835F/gR0835R, and gR_0004F/gR_0004R primer pairs were recircularized to make pSD084 (gRNA for JCVISYN3_0305), pSD085 (gRNA for JCVISYN3_0416), pSD086 (gRNA for JCVISYN3_0481), pSD087 (gRNA for JCVISYN3_0541), pSD088 (gRNA for JCVISYN3_0654), pSD089 (gRNA for JCVISYN3_0835), and pSD097 (KsgA, gRNA for JCVISYN3_0004), respectively. After transformation of the JCVI-syn3.0 cells with these landing pad constructs, correct Cre- and *loxP*-mediated recombination was confirmed by junction PCR with primer pairs LP_F1/LP_DG_puro1, LP_R1/LP_DG_sgRNA1, LP_F1/LP_R2, and LP_F2/LP_R1.

RNA Extraction and Real-Time PCR. Mid log phase cultures of *M. pneumoniae* were washed three times with PBS (Sigma) and RNA was extracted using the RNAqueous kit (Life Technologies). Total RNA was treated with Turbo DNase (Life Technologies) following the manufacturer's instructions. cDNA was obtained by reverse transcription using iScript reverse transcriptase (Bio-Rad) and random primers following the manufacturer's instructions.

JCVI-syn3.0 cells were cultured for 48 h in SP-4 medium before total RNA from them was extracted using the RNeasy Mini kit (Qiagen). Reverse transcription was performed using the SuperScript IV VILO kit (Invitrogen) and random primers according to the manufacturer's instructions. For each sample, 200 ng of RNA was used. The procedure included a genomic DNA digestion step with DNase. Three biological and two technical replicates were prepared.

Real-time PCR (RT-PCR) reactions for *M. pneumoniae* were carried out in a final volume of 10 μL containing 4 μL of cDNA, 0.5 μM of each primer (Table S2), and 5 μL of 2x SYBR

DOI: 10.1021/acssynbio.8b00028
ACS Synth. Biol. 2018, 7, 1538−1552

Green Supermix containing iTaq (Bio-Rad). Reactions were run in a CFX384 PCR instrument (Bio-Rad) following a 3-step PCR protocol including an initial denaturation step at 95 °C for 3 min followed by 40 cycles with each cycle comprising a denaturing step at 95 °C for 10 s, an annealing step at 56 °C for 10 s, and an extension step at 72 °C for 10 s. Fluorescence readings were acquired at the end of each extension step, and the specificity of each PCR product was verified with melting curve analysis at the end of each run. Venus expression was normalized by running RT-PCR on the housekeeping genes MPN_208 (30S ribosomal protein S2), MPN_515 (DNA-directed RNA polymerase subunit beta), and MPN_617 (50S ribosomal protein L13).

RT-PCR reactions for JCVI-syn3.0 were carried out in a final volume of 20 μL containing 2 μL of cDNA, 0.6 μM of each 10 μM primer (Table S2), and 10 μL of 2× Master Mix of SYBR Green Kit (Luminaris Color HiGreen High ROX, Thermo Scientific). Reactions were run in an ABI 7900HT Fast Real-Time PCR system (ABI) following a 5-step PCR protocol including two initial treatments at 50 °C for 2 min and denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 50 °C for 30 s, and extension at 72 °C for 30 s. Fluorescence readings were made at the end of each extension step, and the specificity of each PCR product was verified with melting curve analysis at the end of each run. Expression of endogenous target genes was normalized by running RT-PCR on the housekeeping gene *ldh* (L-lactate dehydrogenase gene, JCVISYN3_0475).

Relative gene expression was calculated using the Pfaffl method,[54] which considers the amplification efficiencies of the target and the reference genes, and geometric averaging of the multiple reference genes,[55] which allows for accurate normalization of expression values. Three independent biological replicates and two technical replicates were used for each strain and condition. T-student statistical analyses were carried out using the SPSS or Excel software.

Phase-Contrast and Epifluorescence Microscopy. *M. pneumoniae* cells were passed ten times through a syringe with a 25G needle and incubated in IBIDI chamber slides containing SP-4 medium at 37 °C under 5% CO_2. SP-4 medium was supplemented with tetracycline 10 ng/mL when needed. Cells were incubated for 24, 48, and 66 h before examination under a Nikon Eclipse TE 2000-E microscope. Phase-contrast and YFP epifluorescence (500 nm excitation wavelength and 542/27 emission wavelength) images were captured with a Digital Sight DS-SMC Nikon camera controlled by NIS-Elements BR software. Exposure times were set to 20 ms for phase-contrast and 1500 ms for YFP fluorescence acquisition. Images were analyzed with ImageJ software.

JCVI-syn1.0 and JCVI-syn3.0 cells were cultured in Hayflick and SP-4 media, respectively, and examined on a Zeiss AxioVision microscope. Differential interference contrast and mCherry epifluorescence (587-nm excitation wavelength and 610-nm emission wavelength) images were captured.

Analysis of *Mycoplasma mycoides* Strains. For induction experiments using JCVI-syn1.0 Δ1−6 cells, 10 μL of fresh culture was diluted in 1 mL of Hayflick medium with or without tetracycline. The culture was conducted for 17 h at 37 °C. For induction experiments using JCVI-syn3.0, 20 μL of fresh culture was diluted in 1 mL of SP-4 medium with or without tetracycline. The culture was conducted for 48 h or several days at 37 °C. The cultured cells were separated from the medium using sucrose cushion[53] and examined. A FlexStation 3 plate reader (Molecular Devices) was used to measure cell turbidity (OD_{600}), mCherry fluorescence (575 nm excitation, 620 nm emission, and 610 nm cutoff wavelengths), PicoGreen staining (488 nm excitation, 525 nm emission, and 515 nm cutoff wavelengths), and color change of phenol red in the medium (pH shift; 430/560 nm). The PicoGreen assay was performed as previously reported.[53] After the measurement for mCherry, 90 μL of each cell suspension was transferred from the black plate to a clear-bottom plate (Costar) for cell turbidity (OD_{600}) to be measured.

To evaluate the stability of mCherry protein and fluorescence, we compared dense and dilute cultures. Three independent JCVI-syn1.0 Δ1−6 transformants carrying pSD048 (Pxyl/$tetO_2$ driving just a mCherry gene) were used. Cells in 4 mL of Hayflick medium containing 4 μg/mL puromycin were cultured at 37 °C for 24 h and divided into four parts. Turbidity and mCherry fluorescence of one part was measured immediately to confirm mCherry expression. Another part was stored at 4 °C for 24 h. Two parts were centrifuged at 16 000*g* at room temperature for 10 min, and the medium was aspirated. The cells were washed once with Hayflick medium and resuspended in an equal volume of medium as before (1000 μL). 800 μL of this suspension was cultured with or without 4 μg/mL of tetracycline at 37 °C for 24 h ("washed" sample in Figure S4). Ten μL of the suspension was diluted in 800 μL of Hayflick medium with or without 4 μg/mL of tetracycline and cultured at 37 °C for 24 h ("washed and diluted" sample). Turbidity and mCherry fluorescence were measured for the two washed samples, two washed and diluted samples, and the culture kept at 4 °C.

Time-Course Assay. For experiments with the JCVI-syn1.0 Δ1−6 strain, three independent transformants (n = 3) carrying pSD048 (Pxyl/$tetO_2$ driving a mCherry gene) or pSD063 (a constitutively expressed mCherry gene as a target and Pxyl/$tetO_2$ driving dCas9 with constitutively expressed sgRNA providing gRNA sequence 2 for targeting mCherry gene expression) were cultured and observed at several time points after tetracycline was added to 4 μg/mL or removed. The basal medium was Hayflick medium containing 4 μg/mL puromycin (to maintain the plasmids). To facilitate these experiments, the cultures for each time point were separately set up, aliquoted to 125 μL in PCR tubes, and stored overnight at 4 °C until incubation at 37 °C was performed in a thermocycler. In one series, the 12 h samples were kept at 4 °C overnight in the thermocycler, and the temperature was programmed to change to 37 °C in the morning. The 9, 6, and 3 h samples were placed on the heat block 3, 6, and 9 h later. All cultures completed at the same time, and at that point, cells were harvested and separated from the medium using the sucrose cushion method[53] for measurement of turbidity and fluorescence as described above. To have roughly equal numbers of cells for measurement for all samples, the starting densities of cultures were adjusted, although fluorescent levels were to be normalized to turbidity. For example, in the same series, 1.25, 10, 80, 640, and 5120 μL portions of a log-phase culture were resuspended in 1 mL of medium with or without tetracycline for the 12, 9, 6, 3, and 0 h samples, respectively, assuming a 60 min doubling time. For the exchange of media, the previous culture was centrifuged at 16 000*g* at room temperature for 10 min, and the supernatant was aspirated.

The procedure for CRISPRi-mediated repression of mCherry in the JCVI-syn3.0 cells was similar to the experiments

DOI: 10.1021/acssynbio.8b00028
ACS Synth. Biol. 2018, 7, 1538−1552

described above for the JCVI-syn1.0 cells, except that two independent transformants with the plasmid pSD080 (Pxyl/tetO$_2$ driving dCas9 with a constitutively expressed mCherry gene as a target and constitutively expressed sgRNA providing gRNA sequence 1 for targeting mCherry gene expression) were cultured in SP-4 FBS medium with 1 μg/mL puromycin and without tetracycline for 48 h, then in a medium with 1 μg/mL puromycin and 0.4 μg/mL tetracycline to induce CRISPRi. The induction cultures were prepared at 4 °C, moved to 37 °C for specified lengths of time (0, 16, 18, 21, 24, 27, 30, 33, and 48 h), and moved back to 4 °C until all cultures completed. A regular incubator and a thermocycler were used for the incubation, with the latter used when a temperature shift was needed outside of the experimentalist's working hours. The doubling time was assumed to be 3 h.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.8b00028.

Figure S1: Necessity of a Shine−Dalgarno sequence for inducible expression with the tetracycline system; Figure S2: Riboswitch-mediated inducible expression of an mCherry gene; Figure S3: Tetracycline-mediated induction of a dCas9-mCherry fusion protein in the JCVI-syn1.0 and JCVI-syn3.0 cells; Figure S4: Effect of dilution of mCherry *via* cell division on the fluorescent readout; Figure S5: Effect of constitutively active CRISPRi on Venus expression in *Mycoplasma pneumoniae*; Figure S6: Schematic representation of the mechanism for kasugamycin resistance with CRISPRi of *ksgA* gene expression; Figure S7: Induction-independent effect of CRISPRi of *ksgA* expression on kasugamycin resistance in the JCVI-syn3.0 cells; Table S1: Oligonucleotide primers used for making DNA constructs for *M. pneumoniae* experiments; Figure S8: DNA constructs used in *M. pneumoniae* experiments; Figure S9: Lineage of plasmids constructed for experiments in the JCVI-syn1.0 cells; Figure S10: Lineage of additional plasmids constructed for experiments in the JCVI-syn3.0 cells; Figure S11: Schematic diagrams of plasmids used in experiments in synthetic *M. mycoides* cells; Table S2: Primers used to construct plasmids needed for experiments in the synthetic bacterial cells (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: ysuzuki@jcvi.org.

ORCID

Yo Suzuki: 0000-0002-2797-6922

Author Contributions

⊗A.M.M. and S.K. contributed equally to this work. A.M.M., L.G-.G., A.B., E.Q., M.L.-S., C.P., and J.P. designed and performed experiments in *M. pneumoniae* and analyzed the results. S.K., J.H., K.T., L.S., P.D.W., K.S.W., C.M., and Y.S. designed experiments in synthetic *M. mycoides*. S.K., J.H., K.T., L.S., G.T., A.J.M., and Y.S. performed experiments in synthetic *M. mycoides* and analyzed the results. C.A.H., H.O.S., M.T., J.C.V., and J.I.G. advised the *M. mycoides* team. A.M.M., S.K., J.H., K.T., L.G-.G., A.B., E.Q., M.L.-S., C.P., L.S., K.S.W., J.P., and Y.S. wrote the manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Joël Renaudin for plasmid pMT85-XTST, Stanley Qi for plasmids pdCas9-bacteria and psgRNA-bacteria, and Bogumil Karas for the pBKMyco1 plasmid. We thank Eva Yus and Ronan Bourgeois for construction of the pMTpmp200Venus vector, Luis Serrano and Veronica Lloréns-Rico for fruitful discussions, and Gabrielle Cahill, Shirley Chan, Kevin Huang, Abhijith Chitlur, Hung Nguyen, Branden Hui, Arnav Aggarwal, and Pedro Cabrales for assistance in developing concepts and materials used in parts of this study. We thank Prashant Mali, Luis Serrano, Samyukta Bhat, Rinoka Sato, Shoichiro Ma, Neha Sodhi, Ayesha Khan, and Tharini Siddappa for critical reading of the manuscript. This work was supported by internal funding from the J. Craig Venter Institute to H.O.S. and C.A.H., as well as grants BIO2013-4870R and BIO2013-50176EXP from the Ministerio de Economía y Competitividad to E.Q. and J.P., respectively, and Japan Society for the Promotion of Science KAKENHI grants JP26710015, JP15KK0266, and JP26106004 to S.K. A.M.M. is a recipient of a predoctoral fellowship from the Generalitat de Catalunya (FI-DGR 2014).

■ REFERENCES

(1) Hutchison, C. A., 3rd, Chuang, R. Y., Noskov, V. N., Assad-Garcia, N., Deerinck, T. J., Ellisman, M. H., Gill, J., Kannan, K., Karas, B. J., Ma, L., Pelletier, J. F., Qi, Z. Q., Richter, R. A., Strychalski, E. A., Sun, L., Suzuki, Y., Tsvetanova, B., Wise, K. S., Smith, H. O., Glass, J. I., Merryman, C., Gibson, D. G., and Venter, J. C. (2016) Design and synthesis of a minimal bacterial genome. *Science (Washington, DC, U. S.) 351*, aad6253.

(2) Guell, M., van Noort, V., Yus, E., Chen, W. H., Leigh-Bell, J., Michalodimitrakis, K., Yamada, T., Arumugam, M., Doerks, T., Kuhner, S., Rode, M., Suyama, M., Schmidt, S., Gavin, A. C., Bork, P., and Serrano, L. (2009) Transcriptome complexity in a genome-reduced bacterium. *Science (Washington, DC, U. S.) 326*, 1268−1271.

(3) Glass, J. I., Assad-Garcia, N., Alperovich, N., Yooseph, S., Lewis, M. R., Maruf, M., Hutchison, C. A., 3rd, Smith, H. O., and Venter, J. C. (2006) Essential genes of a minimal bacterium. *Proc. Natl. Acad. Sci. U. S. A. 103*, 425−430.

(4) Glass, J. I., Merryman, C., Wise, K. S., Hutchison, C. A., and Smith, H. O. (2017) Minimal Cells-Real and Imagined. *Cold Spring Harbor Perspect. Biol. 9*, a023861.

(5) Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K. A., Tomita, M., Wanner, B. L., and Mori, H. (2006) Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol. Syst. Biol.*, DOI: 10.1038/msb4100050.

(6) Black, S. L., Dawson, A., Ward, F. B., and Allen, R. J. (2013) Genes required for growth at high hydrostatic pressure in Escherichia coli K-12 identified by genome-wide screening. *PLoS One 8*, e73995.

(7) Mackie, A., Paley, S., Keseler, I. M., Shearer, A., Paulsen, I. T., and Karp, P. D. (2014) Addition of Escherichia coli K-12 growth observation and gene essentiality data to the EcoCyc database. *Journal of bacteriology 196*, 982−988.

(8) Mahadevan, R., and Lovley, D. R. (2008) The degree of redundancy in metabolic genes is linked to mode of metabolism. *Biophys. J. 94*, 1216−1220.

(9) Guell, O., Sagues, F., and Serrano, M. A. (2014) Essential plasticity and redundancy of metabolism unveiled by synthetic lethality analysis. *PLoS Comput. Biol. 10*, e1003637.

(10) Himmelreich, R., Hilbert, H., Plagens, H., Pirkl, E., Li, B. C., and Herrmann, R. (1996) Complete sequence analysis of the genome of

the bacterium Mycoplasma pneumoniae. *Nucleic Acids Res. 24*, 4420−4449.
(11) Edgar, R. S., and Lielausis, I. (1964) Temperature-Sensitive Mutants of Bacteriophage T4d: Their Isolation and Genetic Characterization. *Genetics 49*, 649−662.
(12) Hartwell, L. H. (1967) Macromolecule synthesis in temperature-sensitive mutants of yeast. *J. Bacteriol. 93*, 1662−1670.
(13) Breton, M., Sagne, E., Duret, S., Beven, L., Citti, C., and Renaudin, J. (2010) First report of a tetracycline-inducible gene expression system for mollicutes. *Microbiology (London, U. K.) 156*, 198−205.
(14) Mariscal, A. M., Gonzalez-Gonzalez, L., Querol, E., and Pinol, J. (2016) All-in-one construct for genome engineering using Cre-lox technology. *DNA Res. 23*, 263−270.
(15) Suess, B., Fink, B., Berens, C., Stentz, R., and Hillen, W. (2004) A theophylline responsive riboswitch based on helix slipping controls gene expression in vivo. *Nucleic Acids Res. 32*, 1610−1614.
(16) Qi, L. S., Larson, M. H., Gilbert, L. A., Doudna, J. A., Weissman, J. S., Arkin, A. P., and Lim, W. A. (2013) Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell 152*, 1173−1183.
(17) Karas, B. J., Jablanovic, J., Sun, L., Ma, L., Goldgof, G. M., Stam, J., Ramon, A., Manary, M. J., Winzeler, E. A., Venter, J. C., Weyman, P. D., Gibson, D. G., Glass, J. I., Hutchison, C. A., 3rd, Smith, H. O., and Suzuki, Y. (2013) Direct transfer of whole genomes from bacteria to yeast. *Nat. Methods 10*, 410−412.
(18) Gibson, D. G., Glass, J. I., Lartigue, C., Noskov, V. N., Chuang, R. Y., Algire, M. A., Benders, G. A., Montague, M. G., Ma, L., Moodie, M. M., Merryman, C., Vashee, S., Krishnakumar, R., Assad-Garcia, N., Andrews-Pfannkoch, C., Denisova, E. A., Young, L., Qi, Z. Q., Segall-Shapiro, T. H., Calvey, C. H., Parmar, P. P., Hutchison, C. A., 3rd, Smith, H. O., and Venter, J. C. (2010) Creation of a bacterial cell controlled by a chemically synthesized genome. *Science (Washington, DC, U. S.) 329*, 52−56.
(19) Rygus, T., Scheler, A., Allmansberger, R., and Hillen, W. (1991) Molecular cloning, structure, promoters and regulatory elements for transcription of the Bacillus megaterium encoded regulon for xylose utilization. *Arch. Microbiol. 155*, 535−542.
(20) Shu, X., Shaner, N. C., Yarbrough, C. A., Tsien, R. Y., and Remington, S. J. (2006) Novel chromophores and buried charges control color in mFruits. *Biochemistry 45*, 9639−9647.
(21) Algire, M. A., Lartigue, C., Thomas, D. W., Assad-Garcia, N., Glass, J. I., and Merryman, C. (2009) New selectable marker for manipulating the simple genomes of Mycoplasma species. *Antimicrob. Agents Chemother. 53*, 4429−4432.
(22) Lartigue, C., Duret, S., Garnier, M., and Renaudin, J. (2002) New plasmid vectors for specific gene targeting in Spiroplasma citri. *Plasmid 48*, 149−159.
(23) Bateman, B. T., Donegan, N. P., Jarry, T. M., Palma, M., and Cheung, A. L. (2001) Evaluation of a tetracycline-inducible promoter in Staphylococcus aureus in vitro and in vivo and its application in demonstrating the role of sigB in microcolony formation. *Infection and immunity 69*, 7851−7857.
(24) Donhofer, A., Franckenberg, S., Wickles, S., Berninghausen, O., Beckmann, R., and Wilson, D. N. (2012) Structural basis for TetM-mediated tetracycline resistance. *Proc. Natl. Acad. Sci. U. S. A. 109*, 16900−16905.
(25) Delcour, A. H. (2009) Outer membrane permeability and antibiotic resistance. *Biochim. Biophys. Acta, Proteins Proteomics 1794*, 808−816.
(26) Topp, S., and Gallivan, J. P. (2010) Emerging applications of riboswitches in chemical biology. *ACS Chem. Biol. 5*, 139−148.
(27) Pich, O. Q., Burgos, R., Planell, R., Querol, E., and Pinol, J. (2006) Comparative analysis of antibiotic resistance gene markers in Mycoplasma genitalium: application to studies of the minimal gene complement. *Microbiology 152*, 519−527.
(28) Goh, E.-B., Yim, G., Tsui, W., McClure, J., Surette, M. G., and Davies, J. (2002) Transcriptional modulation of bacterial gene expression by subinhibitory concentrations of antibiotics. *Proc. Natl. Acad. Sci. U. S. A. 99*, 17025−17030.
(29) Krishnakumar, R., Grose, C., Haft, D. H., Zaveri, J., Alperovich, N., Gibson, D. G., Merryman, C., and Glass, J. I. (2014) Simultaneous non-contiguous deletions using large synthetic DNA and site-specific recombinases. *Nucleic Acids Res. 42*, e111.
(30) Suzuki, Y., Assad-Garcia, N., Kostylev, M., Noskov, V. N., Wise, K. S., Karas, B. J., Stam, J., Montague, M. G., Hanly, T. J., Enriquez, N. J., Ramon, A., Goldgof, G. M., Richter, R. A., Vashee, S., Chuang, R. Y., Winzeler, E. A., Hutchison, C. A., 3rd, Gibson, D. G., Smith, H. O., Glass, J. I., and Venter, J. C. (2015) Bacterial genome reduction using the progressive clustering of deletions via yeast sexual cycling. *Genome Res. 25*, 435−444.
(31) van Gemen, B., Koets, H. J., Plooy, C. A., Bodlaender, J., and Van Knippenberg, P. H. (1987) Characterization of the ksgA gene of Escherichia coli determining kasugamycin sensitivity. *Biochimie 69*, 841−848.
(32) Ricaurte, D. E., Martínez-García, E., Nyerges, Á., Pál, C., de Lorenzo, V., and Aparicio, T. (2018) A standardized workflow for surveying recombinases expands bacterial genome-editing capabilities. *Microb. Biotechnol. 11*, 176−188.
(33) Levitan, A. A. (1967) In vitro antibacterial activity of kasugamycin. *Appl. Microbiol. 15*, 750−753.
(34) Choudhary, E., Thakur, P., Pareek, M., and Agarwal, N. (2015) Gene silencing by CRISPR interference in mycobacteria. *Nat. Commun. 6*, 6267.
(35) Hawkins, J. S., Wong, S., Peters, J. M., Almeida, R., and Qi, L. S. (2015) Targeted Transcriptional Repression in Bacteria Using CRISPR Interference (CRISPRi). *Methods Mol. Biol. (N. Y., NY, U. S.) 1311*, 349−362.
(36) Larson, M. H., Gilbert, L. A., Wang, X., Lim, W. A., Weissman, J. S., and Qi, L. S. (2013) CRISPR interference (CRISPRi) for sequence-specific control of gene expression. *Nat. Protoc. 8*, 2180−2196.
(37) Zischewski, J., Fischer, R., and Bortesi, L. (2017) Detection of on-target and off-target mutations generated by CRISPR/Cas9 and other sequence-specific nucleases. *Biotechnol. Adv. 35*, 95−104.
(38) Chen, Y., Liu, X., Zhang, Y., Wang, H., Ying, H., Liu, M., Li, D., Lui, K. O., and Ding, Q. (2016) A Self-restricted CRISPR System to Reduce Off-target Effects. *Mol. Ther. 24*, 1508−1510.
(39) Slaymaker, I. M., Gao, L., Zetsche, B., Scott, D. A., Yan, W. X., and Zhang, F. (2016) Rationally engineered Cas9 nucleases with improved specificity. *Science (Washington, DC, U. S.) 351*, 84−88.
(40) Kleinstiver, B. P., Pattanayak, V., Prew, M. S., Tsai, S. Q., Nguyen, N. T., Zheng, Z., and Joung, J. K. (2016) High-fidelity CRISPR-Cas9 nucleases with no detectable genome-wide off-target effects. *Nature 529*, 490−495.
(41) Chen, J. S., Dagdas, Y. S., Kleinstiver, B. P., Welch, M. M., Sousa, A. A., Harrington, L. B., Sternberg, S. H., Joung, J. K., Yildiz, A., and Doudna, J. A. (2017) Enhanced proofreading governs CRISPR-Cas9 targeting accuracy. *Nature 550*, 407−410.
(42) Bertani, G. (1951) Studies on lysogenesis. I. The mode of phage liberation by lysogenic Escherichia coli. *J. Bacteriol. 62*, 293−300.
(43) Tully, J. G., Rose, D. L., Whitcomb, R. F., and Wenzel, R. P. (1979) Enhanced isolation of Mycoplasma pneumoniae from throat washings with a newly-modified culture medium. *J. Infect. Dis. 139*, 478−482.
(44) Hayflick, L. (1965) Tissue cultures and mycoplasmas. *Texas Rep. Biol. Med. 23*, 285−303.
(45) Sambrook, J., and Russell, D. W. (2001) *Molecular Cloning: A Laboratory Manual*, 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
(46) Zimmerman, C. U., and Herrmann, R. (2005) Synthesis of a small, cysteine-rich, 29 amino acids long peptide in Mycoplasma pneumoniae. *FEMS Microbiol. Lett. 253*, 315−321.
(47) Nagai, T., Ibata, K., Park, E. S., Kubota, M., Mikoshiba, K., and Miyawaki, A. (2002) A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. *Nat. Biotechnol. 20*, 87−90.

DOI: 10.1021/acssynbio.8b00028
ACS Synth. Biol. 2018, 7, 1538−1552

(48) Yus, E., Guell, M., Vivancos, A. P., Chen, W. H., Lluch-Senar, M., Delgado, J., Gavin, A. C., Bork, P., and Serrano, L. (2012) Transcription start site associated RNAs in bacteria. *Mol. Syst. Biol. 8*, 585.
(49) Kostylev, M., Otwell, A. E., Richardson, R. E., and Suzuki, Y. (2015) Cloning Should Be Simple: Escherichia coli DH5alpha-Mediated Assembly of Multiple DNA Fragments with Short End Homologies. *PLoS One 10*, e0137466.
(50) Larson, M. H., Gilbert, L. A., Wang, X., Lim, W. A., Weissman, J. S., and Qi, L. S. (2013) CRISPR interference (CRISPRi) for sequence-specific control of gene expression. *Nat. Protoc. 8*, 2180−2196.
(51) Wright, M. S., Suzuki, Y., Jones, M. B., Marshall, S. H., Rudin, S. D., van Duin, D., Kaye, K., Jacobs, M. R., Bonomo, R. A., and Adams, M. D. (2015) Genomic and transcriptomic analyses of colistin-resistant clinical isolates of Klebsiella pneumoniae reveal multiple pathways of resistance. *Antimicrob. Agents Chemother. 59*, 536−543.
(52) Gibson, D. G., Young, L., Chuang, R. Y., Venter, J. C., Hutchison, C. A., 3rd, and Smith, H. O. (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat. Methods 6*, 343−345.
(53) Karas, B. J., Wise, K. S., Sun, L., Venter, J. C., Glass, J. I., Hutchison, C. A., 3rd, Smith, H. O., and Suzuki, Y. (2014) Rescue of mutant fitness defects using in vitro reconstituted designer transposons in Mycoplasma mycoides. *Front. Microbiol. 5*, 369.
(54) Pfaffl, M. W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic acids research 29*, e45.
(55) Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., and Speleman, F. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome biology*, DOI: 10.1186/gb-2002-3-7-research0034.

DOI: 10.1021/acssynbio.8b00028
ACS Synth. Biol. 2018, 7, 1538−1552