Reversible Gene Expression Control in *Yersinia pestis* by Using an Optimized CRISPR Interference System

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ABSTRACT Many genes in the bacterial pathogen *Yersinia pestis*, the causative agent of three plague pandemics, remain uncharacterized, greatly hampering the development of measures for plague prevention and control. Clustered regularly interspaced short palindromic repeat interference (CRISPRi) has been shown to be an effective tool for gene knockdown in model bacteria. In this system, a catalytically dead Cas9 (dCas9) and a small guide RNA (sgRNA) form a complex, binding to the specific DNA target through base pairing, thereby impeding RNA polymerase binding and causing target gene repression. Here, we introduce an optimized CRISPRi system using *Streptococcus pyogenes* Cas9-derived dCas9 for gene knockdown in *Y. pestis*. Multiple genes harbored on either the chromosome or plasmids of *Y. pestis* were efficiently knocked down (up to 380-fold) in a strictly anhydrotetracycline-inducible manner using this CRISPRi approach. Knockdown of *hmsH* (responsible for biofilm formation) or *cspB* (encoding a cold shock protein) resulted in greatly decreased biofilm formation or impaired cold tolerance in *in vitro* phenotypic assays. Furthermore, silencing of the virulence-associated genes *yscB* or *ail* using this CRISPRi system resulted in attenuation of virulence in HeLa cells and mice similar to that previously reported for *yscB* and *ail* null mutants. Taken together, our results confirm that this optimized CRISPRi system can reversibly and efficiently repress the expression of target genes in *Y. pestis*, providing an alternative to conventional gene knockdown techniques, as well as a strategy for high-throughput phenotypic screening of *Y. pestis* genes with unknown functions.

IMPORTANCE *Yersinia pestis* is a lethal pathogen responsible for millions of human deaths in history. It has also attracted much attention for potential uses as a bioweapon or bioterrorism agent, against which new vaccines are desperately needed. However, many *Y. pestis* genes remain uncharacterized, greatly hampering the development of measures for plague prevention and control. Clustered regularly interspaced short palindromic repeat interference (CRISPRi) has been successfully used in a variety of bacteria in functional genomic studies, but no such genetic tool has been reported in *Y. pestis*. Here, we systematically optimized the CRISPRi approach for use in *Y. pestis*, which ultimately repressed target gene expression with high efficiency in a reversible manner. Knockdown of functional genes using this method produced phenotypes that were readily detected by *in vitro* assays, cell infection assays, and mouse infection experiments. This is a report of a CRISPRi approach in *Y. pestis* and highlights the potential use of this approach in high-throughput functional genomics studies of this pathogen.

KEYWORDS CRISPRi, *Yersinia pestis*, gene knockdown

Yersinia pestis, the causative agent of plague, has caused millions of deaths in three worldwide pandemics in history (1, 2). Plague is predominately a flea-borne zoonotic disease transmitted via rodents, and humans are only the accidental hosts (2),

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usually infected by fleabite or intimate contact with plague victims (3). While sporadic human plague outbreaks occur in various regions around the world each year, the 2017 plague epidemic in Madagascar was unprecedented in Africa, resulting in 2,417 cases and 209 deaths (4). *Y. pestis* has also attracted much attention for its potential uses as a bioweapon or bioterrorism agent (5, 6), against which new vaccines are desperately needed. The genome of *Y. pestis* harbors over 4,000 genes with many remaining functionally uncharacterized (7–9), greatly hampering in-depth functional genomic studies. Thus, characterization of genes with unknown functions is urgently required for better understanding of plague to aid in the development of measures for plague prevention and control.

Prokaryotic clustered regularly interspaced short palindromic repeats/associated endonuclease (CRISPR-Cas) systems widely distributed in archaea and bacteria provide a sophisticated adaptive immune system against invasive genetic elements, such as bacteriophages and plasmids (10–13). CRISPR utilizes Cas proteins and noncoding CRISPR RNA (crRNA) elements to cleave the foreign DNA elements (14, 15). In type II CRISPR systems, the complex of a single endonuclease protein, Cas9, with crRNA-transacting RNA (tracrRNA) can specifically bind to the target DNA and induce a double-stranded break (16). We have witnessed in recent years the significant progress in genome editing in both eukaryotes and prokaryotes using CRISPR systems, especially those derived from type II CRISPR-Cas9 system from *Streptococcus pyogenes* (17–24). CRISPR interference (CRISPRi) repurposes the CRISPR system to regulate target gene transcription (25, 26). In CRISPRi, a catalytically dead Cas9 (dCas9) without endonucleolytic activity binds to small guide RNAs (sgRNAs) complementary to the target DNA sequence; the resulting dCas9-sgRNA complex sterically impedes the binding of RNA polymerase, thereby repressing gene expression without disturbing the genome content (23, 26, 27). Highly efficient CRISPRi-based gene knockdown systems, in which silencing of target genes is reversibly regulated via an introduced inducible promoter, have been established in several bacteria (26, 28–30). Because of the advantages of reversible gene knockdown without genome editing, CRISPRi not only provides an alternative strategy for genetic manipulation of bacterial genomes but also makes it possible to study essential genes (24, 28, 31).

Although a CRISPR-Cas assisted recombineering system has been set up in *Y. pestis* (32), the CRISPRi approach in this species is not available yet. Here, we established an optimized CRISPRi system to repress gene expression in *Y. pestis*. Surveillance of the *Y. pestis* genome (strain CO92) reveals the existence of a Cas locus comprised of 6 genes, YPO2462 to YPO2465, YPO2467, and YPO2468, which encode proteins of the type I-F system showing homology with Cas6, Csy3, Csy2, Csy1, Cas2/Cas3, and Cas1, respectively. A type II CRISPR-Cas locus is absent in the genome of *Y. pestis*. We started by introducing a CRISPRi system shown to be effective in *Escherichia coli* (26) into *Y. pestis*. While this system was efficient in gene repression, serious leaky repression of the target gene exists in the absence of the inducer. After systematic optimization process, expression of the sgRNA and dCas9 can be tightly controlled using a tetracycline-inducible promoter consisting of the Tet repressor protein TetR under the control of P_{L2tetO} , a derivative of the $P_{LtetO-1}$ promoter (33, 34). Multiple genes on both the chromosome and plasmids of *Y. pestis* were successfully silenced following induction with anhydrotetracycline (ATC), and no significant repression occurred in the absence of the inducer. We showed that knockdown of functional genes resulted in significant phenotypic characteristics that were readily detected using various *in vivo* and *in vitro* phenotypic assays, highlighting the potential of this CRISPRi system in future functional genomics studies of *Y. pestis*.

RESULTS

Construction and optimization of CRISPRi systems suitable for *Y. pestis*. The *S. pyogenes* dCas9 CRISPRi system developed by Qi et al. (26) has successfully been used for target gene knockdown in *Escherichia coli*; thus, we first assessed its efficacy in *Y. pestis*. The system, designated CRISPRi-I, is composed of a p dCas9 vector that produces

FIG 1 Optimization of the CRISPRi system. (a) Schematic illustrations of the CRISPRi system tested in this study. A 20-bp sequence targeting *phoP* was cloned into the individual sgRNA-expressing vectors and cotransformed into *Y. pestis* with the corresponding dCas9-expressing vectors, and the different combinations of the sgRNA- and dCas9-expressing plasmids were designated CRISPRi-I, -II, -III, and -IV as shown. Bacterial strains were cultured at 26°C in LB broth with or without ATc, as indicated, to the stationary phase. Bacterial cells were then collected and subjected to total RNA isolation, which was used to generate cDNA. The mRNA levels of *sgRNA-phoP* (b), *dcas9* (c), and *phoP* (d) were determined by qRT-PCR analysis. Quantitative analysis of *sgRNA-phoP* and *dcas9* mRNA was normalized to levels expressed in the CRISPRi-I system, and their induction fold changes are labeled at the top of the bars. Quantitative analysis of *phoP* mRNA in each of the different strains was normalized to levels expressed in the wild-type strain, and the repression fold changes are labeled at the top of the bars. All qRT-PCR measurements were performed in triplicate and the results are shown as means $\pm$ standard deviation (SD).

an enzymatically dead mutant of SpCas9 driven by $P_{L2tetO-1}$, along with a pgRNA vector that produces sgRNA under the control of the constitutive promoter J23119 (Fig. 1a). A 20-bp sequence targeting *phoP* from *Y. pestis* was cloned into the pgRNA plasmid, generating plasmid *pgRNA-phoP* (Table 1), which was then coelectroporated into *Y. pestis* with plasmid *pdCas9*. *phoP* expression was then determined by quantitative real-time PCR (qRT-PCR) analysis (Table 2). We found that *phoP* expression was repressed by ~ 100 -fold; however, repression occurred even when ATc was absent (Fig. 1d). Immunoblotting analysis confirmed that production of PhoP was strongly repressed in the absence of ATc, although the leaky expression of dCas9 was very limited (Fig. S1), in line with results of the qRT-PCR analysis of *dcas9* mRNA (Fig. 1c). We predicted that constitutive *sgRNA-phoP* expression driven by J23119 might be the major cause of the gene knockdown in the absence of ATc. Surprisingly, *sgRNA-phoP* expression increased dramatically after the addition of ATc (Fig. 1b). qRT-PCR analysis showed that, when induced by ATc, the expression of *sgRNA-phoP* in the *Y. pestis* strain harboring both *pgRNA* and *pdCas9* vectors was much higher than that in the strain harboring only *pgRNA-phoP*, while there was no detectable difference without ATc treatment (Fig. S2). We speculated that over 2,100-fold expression of *dcas9* following ATc treatment (Fig. 1c, Fig. S1a) might deplete free *sgRNA-phoP* molecules by binding to them, which in turn would promote their synthesis.

To remove the gene silencing effects in the absence of the inducer, we introduced the ATc-inducible promoter P_{L2tetO} which contains two *tet* operators (*tetO*) (33), to replace J23119 in the pgRNA vector. The resulting plasmid, *pgRNA-tetO*, produced sgRNA under the control of P_{L2tetO} (Fig. 1b). In addition, the multicloning sites flanking

TABLE 1 Plasmids used in this study

Plasmid	Relevant characteristics	Source
pdCas9	Expressing dCas9 protein under the control of an ATc-inducible promoter with a TetR cassette; Cm ^r	Addgene plasmid no. 44249
pgRNA	Expressing small guide RNA; Ap ^r	Addgene plasmid no. 44251
pgRNA- <i>tetO</i>	Expressing small guide RNA driven by p _{L2tetO} ; Ap ^r	This study
pdCas9- <i>tetO</i>	Expressing dCas9 protein under the control of p _{L2tetO} ; Cm ^r	This study
pdCas9- <i>tetO</i> -JTetR	Expressing TetR driven by J23119 and dCas9 protein driven p _{L2tetO} ; Cm ^r	This study
pgRNA- <i>tetO</i> -JTetR	Expressing TetR driven by promoter J23119 and small guide RNA driven by p _{L2tetO} ; Ap ^r	This study
pgRNA- <i>phoP</i>	20-bp targeting sequence for <i>phoP</i> gene was inserted into pgRNA; Ap ^r	This study
pgRNA- <i>tetO</i> - <i>phoP</i>	20-bp targeting sequence for <i>phoP</i> gene was inserted into pgRNA- <i>tetO</i> ; Ap ^r	This study
pgRNA- <i>tetO</i> -JTetR- <i>phoP</i>	20-bp targeting sequence for <i>phoP</i> gene was inserted into pgRNA- <i>tetO</i> -JTetR; Ap ^r	This study
pgRNA- <i>tetO</i> -JTetR- <i>ail</i>	20-bp targeting sequence for <i>ail</i> gene was inserted into pgRNA- <i>tetO</i> -JTetR; Ap ^r	This study
pgRNA- <i>tetO</i> -JTetR- <i>cobB</i>	20-bp targeting sequence for <i>cobB</i> gene was inserted into pgRNA- <i>tetO</i> -JTetR; Ap ^r	This study
pgRNA- <i>tetO</i> -JTetR- <i>cspB</i>	20-bp targeting sequence for <i>cspB</i> gene was inserted into pgRNA- <i>tetO</i> -JTetR; Ap ^r	This study
pgRNA- <i>tetO</i> -JTetR- <i>hdeD</i>	20-bp targeting sequence for <i>hdeD</i> gene was inserted into pgRNA- <i>tetO</i> -JTetR; Ap ^r	This study
pgRNA- <i>tetO</i> -JTetR- <i>hmsH</i>	20-bp targeting sequence for <i>hmsH</i> gene was inserted into pgRNA- <i>tetO</i> -JTetR; Ap ^r	This study
pgRNA- <i>tetO</i> -JTetR- <i>ibpB</i>	20-bp targeting sequence for <i>ibpB</i> gene was inserted into pgRNA- <i>tetO</i> -JTetR; Ap ^r	This study
pgRNA- <i>tetO</i> -JTetR- <i>pla</i>	20-bp targeting sequence for <i>pla</i> gene was inserted into pgRNA- <i>tetO</i> -JTetR; Ap ^r	This study
pgRNA- <i>tetO</i> -JTetR- <i>slyA</i>	20-bp targeting sequence for <i>slyA</i> gene was inserted into pgRNA- <i>tetO</i> -JTetR; Ap ^r	This study
pgRNA- <i>tetO</i> -JTetR- <i>yscB</i>	20-bp targeting sequence for <i>yscB</i> gene was inserted into pgRNA- <i>tetO</i> -JTetR; Ap ^r	This study

the 20-bp targeting region were replaced with two BbsI recognition sites. As a result, vectors expressing sgRNA can easily be constructed by ligating synthesized targeting oligonucleotides with BbsI-digested plasmid (35, 36). Using the same *phoP*-targeting sequence, *phoP* repression by the CRISPRi-II system was reduced from 100-fold to ~20- to 30-fold in the absence of ATc, but it was obviously still present (Fig. 1d). Next, an additional *tetO* was added to the P_{LtetO-1} promoter in pdCas9, while J23119 was used to drive *tetR* expression to increase the concentration of TetR and reduce the leaky expression of dCas9. The resulting plasmid was designated pdCas9-*tetO*-JTetR (Fig. 1a). The combined use of pdCas9-*tetO*-JTetR and pgRNA-*tetO*-*phoP* further decreased *phoP* repression to less than 20-fold in the absence of ATc (Fig. 1d). To more strictly repress sgRNA and dCas9 expression driven by P_{L2tetO}, the J23119 promoter-driven *tetR* was removed from the pdCas9 expression vector and introduced into the high-copy-number sgRNA-expressing vector to increase TetR concentrations inside the bacterial cells (34). The results showed that the optimized CRISPRi system did not repress *phoP* expression in the absence of ATc and demonstrated highly efficient *phoP* silencing in an ATc concentration-dependent manner (Fig. 1d).

In summary, we optimized the CRISPRi system for use in *Y. pestis* in a stepwise manner, using different combinations of the J23119 and TetR/*tetO* regulatory elements (Fig. 1a). The maximum repression for *phoP* was over 100-fold, slightly lower than the repression effects on red fluorescent protein (RFP; 300-fold) found by Qi et al. (26) and was comparable to or much higher than those reported in *Mycobacterium*, *Clostridium*, and *Cyanobacteria* (28–30, 37). For controlling the leaky expression of sgRNA, the CRISPRi-IV system performed best (Fig. 1d); in this system, about 39.2- ± 8.3-fold lower expression of sgRNA targeting *phoP* and 1.9- ± 0.1-fold higher expression of *dcas9* were observed compared with those in the original system (Fig. 1b and c). The significantly decreased level of sgRNA targeting *phoP* seemingly contributed greatly to the better tightness of the CRISPRi system under noninducing conditions, which allows the system to repress the target gene expression only in the presence of ATc.

Gene knockdown in *Y. pestis* using the optimized CRISPRi system. First, we determined the most suitable ATc concentration for gene knockdown by the optimized CRISPRi system. The *Y. pestis* strain containing the pgRNA-*tetO*-JTetR-*phoP* and pdCas9-*tetO* plasmids was cultured in Luria-Bertani (LB) medium with increasing concentrations of ATc. qRT-PCR analysis showed that among the tested concentrations, an ATc concentration of 1.0 µg/ml induced the greatest repression of *phoP* (Fig. 2a). Growth curves for *Y. pestis* in LB broth with or without 1.0 µg/ml ATc showed that there was no

TABLE 2 Primers used in this study

Primer name ^a	Primer sequence (5'–3')	Usage
phoP-1-F	ACTAGTATCCTTGATAAAACGTTAACGTTTTAGAGCTAGAAATAGC	Construction of pgRNA- <i>phoP</i>
phoP-1-R	AAGCTTCAAAAAAGCACCGACTCG	Construction of pgRNA- <i>phoP</i>
gRNA-v-R	CCCAAGCTTCAAAAAAGCACCGACTCG	PCR identification of constructed pgRNA vector
gRNA-s-F	AAAGGGCAAAAGTGAGTATGG	Sequencing of constructed pgRNA vector
pg-tetR-v-F	GACAGCTAGCTCAGTCTAGGTATA	PCR identification of pgRNA- <i>tetO</i> -JTetR
pg-tetR-v-R	CATCTCAATGGCTAAGGCGTC	PCR identification of pgRNA- <i>tetO</i> -JTetR
pg-tetR-s-F	AAAGGGCAAAAGTGAGTATGG	Sequencing of pgRNA- <i>tetO</i> -JTetR
pg-tetO-F	AATTCTTAAGACCCACTA	Construction of pgRNA- <i>tetO</i>
pg-tetO-R	CTAGTAGTGGGTCTTAAG	Construction of pgRNA- <i>tetO</i>
pg-tetO-v-F	AAAATAAACAAATAGGGG	PCR identification of pgRNA- <i>tetO</i>
pg-tetO-v-R	TTTCAAGTTGATAACGGA	PCR identification of pgRNA- <i>tetO</i>
phoP-1n-F	GCACATCTTGATATAAACGTTAAC	Construction of pgRNA- <i>tetO</i> -JTetR- <i>phoP</i> and pgRNA- <i>tetO</i> - <i>phoP</i>
phoP-1n-R	AAACGTTAACGTTTTATCAAGGAT	Construction of pgRNA- <i>tetO</i> -JTetR- <i>phoP</i> and pgRNA- <i>tetO</i> - <i>phoP</i>
phoP-sg-F	CCTTGATAAAACGTTAACG	qPCR assay of sgRNA of <i>phoP</i>
phoP-sg-R	GTTGATAACGGACTAGCC	qPCR assay of sgRNA of <i>phoP</i>
dCas9-F	AGCTCTATCTCTATTATCTCC	qPCR assay of mRNA of <i>dcas9</i>
dCas9-R	CATCTTTTGGACTACTTCTTC	qPCR assay of mRNA of <i>dcas9</i>
16S-F	GCCCACTGGAAGTGAACACG	qPCR assay of 16S rRNA
16S-R	CGCTGAAAGTGCTTACAACCC	qPCR assay of 16S rRNA
pdCas-tetR-F	GGACGCTCTTAAGACCCACTTTCAC	Construction of pdCas9- <i>tetO</i> -JTetR
pdCas-tetR-R	GAAGATCTGTGCTCAGTATCTCTATCAC	Construction of pdCas9- <i>tetO</i> -JTetR
pdCas-tetR-v-F	GACAGCTAGCTCAGTCTAGGTATA	PCR identification of pdCas9- <i>tetO</i> -JTetR
pdCas-tetR-v-R	CATCTCAATGGCTAAGGCGTC	PCR identification of pdCas9- <i>tetO</i> -JTetR
pdCas-tetO-F	GGACGCTCCCTATCAGTGATAGAGATTGACATCCCTATCAGT GATAGAGATACTGAGCACAGATCTTC	Construction of pdCas9- <i>tetO</i>
pdCas-tetO-R	GAAGATCTGTGCTCAGTATCTCTATCACTGATAGGGATGTCAA TCTCTATCACTGATAGGAGACGTCC	Construction of pdCas9- <i>tetO</i>
pdCas-tetO-v-F	TTTCTCATTTTAGCTTCTT	PCR identification of pdCas9- <i>tetO</i>
pdCas-tetO-v-R	TTTTTGATACTGTGGCGGCT	PCR identification of pdCas9- <i>tetO</i>
slyA-1n-F	GCACGTTCCAACGGTTTCAGCCGA	Construction of pgRNA- <i>tetO</i> -JTetR- <i>slyA</i>
slyA-1n-R	AAACTCGGCTGAAACCGTTGGAAC	Construction of pgRNA- <i>tetO</i> -JTetR- <i>slyA</i>
hmsH-1n-F	GCACAGCAACTTGTGTGATGATCA	Construction of pgRNA- <i>tetO</i> -JTetR- <i>hmsH</i>
hmsH-1n-R	AAACTGATCATCAAAACAGTTGCT	Construction of pgRNA- <i>tetO</i> -JTetR- <i>hmsH</i>
cobB-1n-F	GCACCGCTGCACCACGAACGCC	Construction of pgRNA- <i>tetO</i> -JTetR- <i>cobB</i>
cobB-1n-R	AAACGGGGCGTTCGTGGTGCAGCG	Construction of pgRNA- <i>tetO</i> -JTetR- <i>cobB</i>
pla-1n-F	GCACTGATGCTGCATTAGCACTCC	Construction of pgRNA- <i>tetO</i> -JTetR- <i>pla</i>
pla-1n-R	AAACGAGATGCTAATGCAGCATCA	Construction of pgRNA- <i>tetO</i> -JTetR- <i>pla</i>
ail-1n-F	GCACACAGTAACACAGACTAAAG	Construction of pgRNA- <i>tetO</i> -JTetR- <i>ail</i>
ail-1n-R	AAACCTTTAGTCTGTGGTACTGT	Construction of pgRNA- <i>tetO</i> -JTetR- <i>ail</i>
cspB-1n-F	GCACGTGCCACTACATGGCAATTG	Construction of pgRNA- <i>tetO</i> -JTetR- <i>cspB</i>
cspB-1n-R	AAACCAATTGCCATGTAGTGGCAC	Construction of pgRNA- <i>tetO</i> -JTetR- <i>cspB</i>
ibpB-1n-F	GCACAGCGAGATAATAGGTAGCCA	Construction of pgRNA- <i>tetO</i> -JTetR- <i>ibpB</i>
ibpB-1n-R	AAACTGGCTACCTATTATCTCGCT	Construction of pgRNA- <i>tetO</i> -JTetR- <i>ibpB</i>
hdeD-1n-F	GCACCTTTTAAACGCGCTTTCATCG	Construction of pgRNA- <i>tetO</i> -JTetR- <i>hdeD</i>
hdeD-1n-R	AAACCGATGAAAGCGCGTTAAAAA	Construction of pgRNA- <i>tetO</i> -JTetR- <i>hdeD</i>
yscB-1n-F	GCACAGAAATATGCAAAATTTAC	Construction of pgRNA- <i>tetO</i> -JTetR- <i>yscB</i>
yscB-1n-R	AAACGTAAATTTTGCATTATTTCT	Construction of pgRNA- <i>tetO</i> -JTetR- <i>yscB</i>
PhoP-F	AGATAAAGTCGCTGTACTGGA	qPCR assay of mRNA of <i>phoP</i>
phoP-R	TTGGTTAACACATAACTCACG	qPCR assay of mRNA of <i>phoP</i>
hmsH-F	AGAATGGCATCAGATGGAGTT	qPCR assay of mRNA of <i>hmsH</i>
hmsH-R	AATAGGTGGGCTGTAAAGGAG	qPCR assay of mRNA of <i>hmsH</i>
slyA-F	TTACCACCAGAGCAATCACAG	qPCR assay of mRNA of <i>slyA</i>
slyA-R	AACAAATCACGCCATCAACCT	qPCR assay of mRNA of <i>slyA</i>
cobB-F	TTTCGCTCCCGTATTTTTCAT	qPCR assay of mRNA of <i>cobB</i>
cobB-R	CAGGTGTCGCCACATCTTCTA	qPCR assay of mRNA of <i>cobB</i>
pla-F	GGGTGGACGCTCTCTGGCTTCC	qPCR assay of mRNA of <i>pla</i>
pla-R	TCCTGTGTTATACCTGCTTT	qPCR assay of mRNA of <i>pla</i>
ail-F	GTATGGATTATTGGGGGCAGG	qPCR assay of mRNA of <i>ail</i>
ail-R	TTCACATCATCGAGTTTGGAG	qPCR assay of mRNA of <i>ail</i>
ibpB-F	AACCACTATCGCATCTCACTG	qPCR assay of mRNA of <i>ibpB</i>
ibpB-R	GAACCTTTACGAACCAAGCCC	qPCR assay of mRNA of <i>ibpB</i>
cspB-F	GGTTTTGGTTTTATTTCTCT	qPCR assay of mRNA of <i>cspB</i>
cspB-R	ACTCAACGTTCTGGCCTTCAT	qPCR assay of mRNA of <i>cspB</i>
yscB-F	GGAAGAAAACCGTTTGTT	qPCR assay of mRNA of <i>yscB</i>
yscB-R	CTTGTTGCATTAGGGAGC	qPCR assay of mRNA of <i>yscB</i>
hdeD-F	TAAGGATCTAAAAAGACCGGG	qPCR assay of mRNA of <i>hdeD</i>
hdeD-R	CTAGCAACCATAAAGCAACTG	qPCR assay of mRNA of <i>hdeD</i>

^aF, forward; R, reverse.

FIG 2 Determination of the most suitable concentrations of ATc inducer. The wild-type *Y. pestis* strain and the strain containing the pdCas9-*tetO* and psgRNA-*tetO*-J*TetR-phoP* plasmids were cultured at 26°C in LB medium supplemented with ATc at concentrations of 0, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, or 2.0 μg/ml until the stationary phase. The mRNA levels of *phoP* in the various strains were determined by qRT-PCR analysis, and the fold repression of *phoP* was calculated (a). The results indicated that 1 μg/ml was the most suitable concentration for knockdown of the target gene. qRT-PCR analyses were performed in triplicate, and the fold repression values are shown as the mean ± SD. Wild-type *Y. pestis* was cultured at 26°C in LB medium with or without 1 μg/ml ATc, and the OD₆₂₀ values were measured every 2 h (b). Results showed that 1 μg/ml of ATc did not influence the growth of *Y. pestis*. Growth curves were performed in biological triplicates, and results are shown as mean ± SD.

detectable difference in growth between the two conditions (Fig. 2b), indicating that ATc at this concentration did not affect bacterial growth. Therefore, an ATc concentration of 1.0 μg/ml was used in all further assays.

Next, 10 genes, including those harbored in the chromosome or the indigenous plasmids of *Y. pestis* (Table 2), were selected as target genes to evaluate the performance of the optimized CRISPRi system. For each of the genes, at least two 20-nucleotide (nt) targeting sequences were designed, and the most efficient one was chosen for further assays, based on qRT-PCR analysis of the gene knockdown efficiency (Table 2). Repression of the target genes varied from 2.5- to 375-fold, and no significant repression was detected under noninducing conditions (Fig. 3a to j). In line with the observed >100-fold repression of mRNA levels, immunoblotting analysis showed that production of SlyA, HmsH, and PhoP was almost completely eliminated following ATc induction but remained comparable to that of the wild-type strain in cultures grown without ATc (Fig. 3k to m). The abundance of Pla decreased significantly in the presence of ATc but remained strong enough to be clearly detected, which was consistent with the moderate (~14-fold) repression of mRNA levels (Fig. 3j and n). Taken together, these results indicated that *Y. pestis* genes can be inducibly and efficiently silenced using our optimized CRISPRi system.

Reversibility of gene silencing by the optimized CRISPRi system. The TetR/*tet*-regulated CRISPRi system should allow target gene expression to return to normal levels after the removal of ATc induction. To verify that this occurred and to determine the interval between removal of induction and restoration of wild-type-level gene expression, bacterial cells cultured in LB broth with ATc were collected at the mid-log phase, thoroughly washed to remove residual inducer, resuspended in the same volume of fresh LB medium without ATc, and then incubated at 26°C. The abundance of PhoP was monitored by immunoblotting of samples collected at various time points. PhoP expression increased gradually after the removal of ATc repression but was not fully restored to wild-type levels, even by day 4 (Fig. 4a). Given that the bacteria likely entered the stationary phase and stopped replicating soon after the culture medium was replaced, which might lead to slow restoration of the silenced gene, we then tried inoculating the bacterial cultures into fresh LB broth (1:10 dilution) at days 4 and 5. PhoP abundance was quickly restored to wild-type levels after the first inoculation, which suggested that restoring expression of the repressed genes might only occur in actively replicating bacteria. Thus, we next inoculated the bacterial cultures into fresh LB medium immediately after the removal of ATc and repeated the inoculation procedure after 12 h. PhoP abundance increased much faster and was restored to

FIG 3 The optimized CRISPRi system can be used to repress target genes with high efficiency. The wild-type *Y. pestis* strain and strains containing pdCas9-*tetO* and pgRNA-*tetO*-Jtetr plasmids expressing sgRNA specific for *ail*, *cspB*, *hdeD*, *cob*, *yscB*, *hmsH*, *slyA*, *phoP*, or *pla* were cultured in LB medium with or without ATc, as indicated (a to j). mRNA levels of each of the individual genes were then determined by qRT-PCR analysis, as described in Fig. 1d, and the number at the top of each bar indicates the repression fold of the target gene. The production of HmsH, SlyA, PhoP, and Pla in the various *Y. pestis* strains was detected by immunoblotting analysis using rabbit antibodies against the corresponding proteins and IRDye 800CW-conjugated goat anti-rabbit secondary antibody (k to n). The expression of GroEL in each sample was also detected by immunoblotting and used as an equal-loading control. Images of the immunoblots were acquired using an Odyssey SA imaging system.

~50% of the wild-type level at 12 h, and was fully restored by 24 h post-removal of ATc (Fig. 4b). The abundance of dCas9 decreased slowly and did not return to background levels until 24 h after the removal of ATc. These results indicated that the CRISPRi-mediated gene knockdown was reversible; however, restoration of expression of the silenced genes requires active bacterial replication.

In vitro phenotypic analysis of *Y. pestis* strains with CRISPRi-mediated gene knockdown. Biofilm production by *Y. pestis* requires the *hmsHFRS* operon (38–40). Biofilm-producing *Y. pestis* strains form rugose colonies on LB agar plates and red colonies on Congo red agar plates at $\leq 28^\circ\text{C}$ (40–43). We demonstrated that a *Y. pestis*

FIG 4 Gene knockdown mediated by the CRISPRi system was reversible after removal of the inducer. The wild-type *Y. pestis* strain and the strain containing the *pdCas9-tetO* and *pgRNA-tetO-JTetR-phoP* plasmids were cultured at 26°C in LB broth supplemented with ATc to induce the expression of *dcas9* and *sgRNA-phoP*. Upon reaching the stationary phase, ATc induction was removed by resuspending cultures in fresh LB medium. Cultures were then incubated at 26°C. PhoP and dCas9 expression by each of the strains was then examined at various time points between 0 and 6 days after the removal of ATc by immunoblotting analysis using antibodies against PhoP or Cas9 (a). In a second round of experiments, the expression of PhoP and dCas9 by the *phoP* knockdown strain was examined using the same method at 0, 4, 8, 12, 24, 36, and 48 h after the removal of ATc induction (b). Arrows denote that the bacterial cultures were inoculated once in a 1:10 dilution at the indicated time point.

CRISPRi-mediated *hmsH* knockdown strain formed pink colonies on Congo red plates in the presence of ATc but formed dark red colonies similar to those of the wild-type strain when grown without ATc (Fig. 5a). Similarly, the *hmsH* knockdown strain formed relatively smooth colonies under ATc induction but rough colonies like those of the wild-type strain in the absence of ATc (Fig. 5b). The biofilm formation capabilities of the various *Y. pestis* strains were then further analyzed by crystal violet staining (40, 43), with a strong correlation observed between biofilm formation and ATc concentration (Fig. 5c). This result indicated that the phenotype of the knockdown strain could be controlled via subtle adjustments of the inducer concentration.

cspB is important for the cold shock response of *Y. pestis* (44). When *cspB* expression was repressed by our CRISPRi system, the cold tolerance of *Y. pestis* was significantly hampered; however, no difference in cold tolerance was observed between the knockdown strain and the wild-type in the absence of ATc (Fig. 5d). These results confirmed that the cold tolerance of *Y. pestis* decreased dramatically when the functionally related gene was silenced by CRISPRi, even when repression was as low as ~2.5-fold (Fig. 3b). This finding differs from that of a previous study in mycobacteria, which suggested that many genes require high-level knockdown before a phenotype can be observed (37). Therefore, we hypothesized that the degree of repression required to reveal an unambiguous phenotype is likely gene and organism dependent.

These results confirmed that knockdown of a functional gene in *Y. pestis* using our CRISPRi approach can produce a stable phenotype that is readily detected by *in vitro* assays.

Phenotypic analysis of *Y. pestis* strains with CRISPRi-mediated gene knockdown in a cell infection model. We next attempted to determine whether attenuated virulence of *Y. pestis* strains with CRISPRi-silenced virulence factors could be detected using a cell infection model. The gene *ail* encodes the major adhesin Ail, which is essential for the full virulence of *Y. pestis* (45–47). In the current study, we repressed *ail* using CRISPRi and then measured bacterial adherence to HeLa cells by immunofluo-

FIG 5 Knockdown of functional genes using the optimized CRISPRi system produced stable phenotypes that are easily detected by *in vitro* assays. The wild-type *Y. pestis* strain and the strain containing pdCas9-*tetO* and psgrNA-*tetO*-JTetR-*hmsH* were streaked onto Congo red agar plates with or without ATc and incubated at 26°C for 4 days. The addition of ATc led to the knockdown of *hmsH*, accompanied by the formation of pink colonies, in contrast to the red colonies of the wild-type strain (a). On LB agar plates, the *hmsH* knockdown strain displayed a smooth morphology in the presence of ATc compared with the rugose colonies of the wild-type strain (b). Bacterial strains were grown in 24-well tissue culture plates at 26°C in LB medium without or with ATc at 0.25, 0.5, 0.75, or 1 μg/ml for 48 h. Biofilm formation was determined for three biological replicates using crystal violet staining, with data shown as means ± SD of the three wells (c). Knockdown of *cspB* by CRISPRi resulted in a significant decrease in cold tolerance. The experiments were performed in triplicate, and the results are shown as mean ± SD. Statistical significance was calculated by unpaired *t* test (**, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$). n.s., not significant.

rescence staining. The number of bacterial cells attached to the surface of HeLa cells was substantially decreased when repression was induced with ATc but was similar to that of the wild-type strain in the absence of ATc (Fig. 6a). YscB is indispensable for the delivery of virulent effectors into host cells via the *Yersinia* type III secretion system (T3SS) (48, 49), with the cytotoxicity of a *yscB* mutant becoming highly attenuated as a result of a defective T3SS (50). Real-time cell analysis system (RTCA) systems can monitor infected HeLa cells seeded onto the gold matrix by measuring the cell index (CI), a value that reflects cellular changes, including cell number and cell adhesion. Our results showed that the CI readouts of HeLa cells infected with the wild-type *Y. pestis* strain dropped rapidly within 3 h of infection but did not decrease within 7 h of infection for cells infected with the *ail* knockdown strain. In addition, CI values remained stable for 5 h after infection for cells infected with the *yscB*-knockdown strain (Fig. 6b and c). These results clearly demonstrated that knockdown of genes involved in virulence using our CRISPRi system can result in distinct phenotypes that are readily detected using a cell infection model.

Determination of the virulence of CRISPRi-mediated gene knockdown strains in mice. Inducible CRISPRi gene silencing has been successfully applied in an increasing number of bacteria (51–55). However, as far as we know, the virulence phenotype in animal models has not been determined for any of the strains with virulence genes silenced by CRISPRi. ATc, the inducer of TetR used in this study, has little cytotoxicity to mammalian cells, as well as low animal toxicity (56), making it an ideal inducer when the CRISPRi gene knockdown strains are used to challenge the animals. Here, we sought to determine whether *Y. pestis* strains with *ail* or *yscB* knockdowns generated using our CRISPRi system showed attenuated virulence in mice, similar to results

FIG 6 Cytotoxicity analysis of *Y. pestis* strains with CRISPRi-mediated gene knockdown in a cell infection model. HeLa cells were infected with wild-type *Y. pestis* or the strain containing pdCas9-*tetO* and pgRNA-*tetO*-JTetR-*ail* at an MOI of 100. ATc at 1 μ g/ml was added as indicated to repress *ail* expression. Following infection for 2 h, unattached bacteria were removed, and HeLa cells were fixed and stained using anti-F1 antigen antibody and Alexa Fluor 555-conjugated donkey anti-mouse antibody. Images were acquired using an LSM 880 confocal microscope (a). HeLa cells were infected with wild-type *Y. pestis* or the strains containing pdCas9-*tetO* and pgRNA-*tetO*-JTetR-*ail* (b) or pgRNA-*tetO*-JTetR-*yscB* (c) at an MOI of 20, and monitored by RTCA at 5-min intervals. ATc at 1 μ g/ml was added as indicated. Arrows indicate the time points at which the HeLa cells were infected with the bacterial strains. The experiments were performed in triplicate and the results are shown as means $\pm$ SD. BF, bright field.

previously obtained for *ail* and *yscB* null mutants (45, 50, 57). Groups of BALB/c mice were challenged subcutaneously with 10^3 CFU of the different strains cultured in LB with or without ATc. To induce sgRNA and dCas9 expression in the infected mice, ATc was intraperitoneally injected into the mice twice daily along with the antibiotics to sustain selection pressure on the CRISPRi plasmids. Survival percentages showed no significant difference between mice infected with the wild-type *Y. pestis* strain and those infected with the strains carrying CRISPRi plasmids targeting *ail* or *yscB* in the absence of ATc (Fig. 7). In sharp contrast, when infected mice received twice-daily ATc injections, the *ail* knockdown strain produced no mortality within the 2-week experimental period (Fig. 7a), while the *yscB* knockdown strain was highly attenuated, with only half of the infected mice dying after 14 days (Fig. 7a). These findings were consistent with previous studies on the *ail* and *yscB* mutants (45, 50). Together, these results showed that our optimized CRISPRi system can be used with a mouse infection

FIG 7 Determination of the virulence of CRISPRi-mediated gene knockdown strains in a mouse infection model. Groups of mice ($n = 10$) were infected with the wild-type *Y. pestis* strain, the strain containing pdCas9-*tetO* and pgRNA-*tetO*-JTetR-*ail* (a), or the strain containing pdCas9-*tetO* and pgRNA-*tetO*-JTetR-*yscB* (b) by subcutaneous injection of $\sim 10^3$ CFU of each strain. To induce the expression of *dcas9* and the sgRNAs targeting *ail* or *yscB*, mice infected with the corresponding strains were intraperitoneally injected with ATc at 4 mg/kg, ampicillin at 100 mg/kg, and chloramphenicol at 34 mg/kg twice daily, while mice in the control group were only treated with antibiotics. Infected mice were observed continuously for 2 weeks, and percent survival for each group was calculated and analyzed using GraphPad Prism 6 software (log rank [Mantel-Cox] test, ****, $P < 0.0001$). n.s., not significant.

model to evaluate the contribution of genes with unknown functions to the virulence of *Y. pestis*.

DISCUSSION

Gene knockout approaches widely used in the study of *Yersinia* species include λ -Red homologous recombination and suicide plasmid-based scarless gene deletion methods (58–60). Both methods have their own limitations, with the former method requiring the insertion of an antibiotic resistance gene into the target gene and the latter method requiring multiple rounds of cloning and selection. Here, we took advantage of the rapid progress in CRISPR-Cas9-based gene silencing technologies to establish an optimized CRISPRi system that can be used for the reversible and inducible knockout of target genes in *Y. pestis*. Although CRISPRi systems have been successful in some bacteria, including *Escherichia coli* and *Mycobacterium tuberculosis*, this approach requires a significant amount of optimization and assessment to make it feasible for gene silencing in *Y. pestis*. The CRISPRi system used in *E. coli* (26) is unsuitable for use in *Y. pestis* because strong inhibition of the target gene occurs even in the absence of the inducer (Fig. 1d). Therefore, we improved the system by introducing $P_{L2tetOr}$, a derivative of the ATc-inducible $P_{LtetO-1}$ promoter, to drive the expression of sgRNA, and by adding TetR to the high copy number sgRNA-expressing plasmid along with the addition of multiple *tetO* elements to the promoter region of *dcas9*. The optimized CRISPRi system ultimately repressed target gene expression in *Y. pestis* with high efficiency in a reversible manner.

We also introduced BbsI recognition sites into the sgRNA vectors so that plasmids expressing sgRNAs targeting different genes can be conveniently constructed through the ligation of synthesized oligonucleotides with the BbsI-linearized vectors. This feature is especially useful when constructing a large number of sgRNA-expressing vectors. The limitations of the CRISPRi method noted in other organisms, such as the polar effects on downstream genes within polycistronic mRNA, also exist for its application in *Y. pestis*. This disadvantage is insurmountable when knockdown of a single specific gene is required; however, in high-throughput studies aimed at revealing associations between unknown genes and specific phenotypes, the weakness becomes an advantage, as a reduced number of sgRNAs are needed under such circumstances. The repression of the targeted genes varied in the range of 2.5- to 350-fold, and we assume that several factors can lead to this variation. sgRNA sequences that form secondary structures impeding their binding to the target sites will fail to base pair with the target DNA. Some structures of sgRNA disrupt the dCas9 binding handle, resulting in poor binding of sgRNA to dCas9. Both of these conditions can cause decreased repression efficiency, and optimized sgRNA design, such as exclusion of sgRNAs with the potential secondary structure, will help to improve transcription repression in the future use of this approach.

We showed that knockdown of *hmsH* and *cspB*, which are essential for biofilm formation and cold resistance, respectively, resulted in greatly decreased biofilm formation capabilities and impaired tolerance of cold stimulus, both of which could be readily detected in phenotypic assays (Fig. 5). Similarly, knockdown of *ail*, which encodes the major adhesin of *Y. pestis*, drastically decreased the adherence of bacteria to HeLa cells, which was easily detected in cell infection experiments (Fig. 6). These results indicate that the CRISPRi system developed here shows promise for use in high-throughput studies of functionally uncharacterized genes in *Y. pestis* when coupled with appropriate *in vitro* phenotypic assays. Furthermore, we showed that silencing of the virulence-associated genes *yscB* and *ail* using the CRISPRi system resulted in virulence attenuation in mice similar to that of the previously reported *yscB* and *ail* null mutants (Fig. 7), demonstrating that the CRISPRi system can be used to study the mechanisms of pathogenesis in *Y. pestis*.

In conclusion, the CRISPRi system established here can be used to efficiently and reversibly inhibit the expression of genes on both the chromosome and plasmids of *Y. pestis*, providing an alternative to traditional engineering approaches and a powerful solution for high-throughput characterization of gene function.

MATERIALS AND METHODS

Bacterial strains. Wild-type *Y. pestis* biovar Microtus strain 201 is highly virulent in mice but avirulent in humans (7). *Y. pestis* strains were inoculated into Luria-Bertani (LB) broth, and antibiotics were added to the growth medium of all *Y. pestis* strains carrying CRISPRi-associated plasmids at the following concentrations: ampicillin, 50 mg/ml, and chloramphenicol, 34 mg/ml. ATc was added at 1 μ g/ml, except where stated otherwise, to induce the expression of the TetR-*tetO*-driven genes.

Cell cultures, reagents, and antibodies. HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS) and 2 mM L-glutamine at 37°C in a 5% CO₂ incubator. TRIzol reagent, RNase-free DNase I, and SuperScript II reverse transcriptase were purchased from Thermo Fisher Scientific. Rabbit antibodies specific for HmsH, PhoP, SlyA, and Pla were prepared in our laboratory as previously described (61). Rabbit anti-GroEL antibody, rabbit anti-Cas9 antibody, and ATc were purchased from Abcam (Cambridge, MA, USA). IRDye 800CW-conjugated goat anti-rabbit antibody was purchased from LI-COR Biosciences (Lincoln, NE, USA). Immobilion-P transfer membrane was purchased from Millipore (Bedford, MA, USA).

Construction of plasmids. The pgRNA-bacteria (Addgene plasmid no. 44251) and pdCas9-bacteria (Addgene plasmid no. 44249) that can express dCas9 of *S. pyogenes* (SpdCas9) were gifts from Stanley Qi (26). An 855-bp DNA fragment containing *tetR*, constitutive promoter J23119, ATc-inducible promoter P_{L2tetO}, an 18-bp nontargeting control sgRNA sequence flanked by BbsI sites, a Cas9-binding hairpin, and a sgRNA transcriptional terminator, in sequential order, was synthesized with EcoRI and HindIII recognition sites at the 5' and 3' ends, respectively. The synthesized DNA fragment (Table S1) and pgRNA vector were digested with EcoRI/HindIII and ligated, generating the pgRNA-*tetO*-JTetR plasmid. The TetR coding sequence was removed by EcoRI/SpeI double-digestion of pgRNA-*tetO*-JTetR, and the linearized DNA fragments obtained were ligated to the two synthesized complementary oligonucleotides containing EcoRI/SpeI adhesive ends, generating plasmid pgRNA-*tetO*. To construct a dCas9-expressing plasmid, the promoter region of *tetR*, along with P_{L2tetO}, was amplified from pgRNA-*tetO*-JTetR using primers containing AatII or BglII sites at the 5' ends. The amplicons and the pdCas9 vector were digested with AatII/BglII and ligated, generating plasmid pdCas9-*tetO*-JTetR. The pdCas9-*tetO* plasmid was adapted from the pdCas9-*tetO*-JTetR plasmid using a similar method to that used for pgRNA-*tetO* construction. Plasmids described here are listed in Table 1. Primer sequences and the target regions of all sgRNAs used in this study are listed in Table 2.

The dCas9-sgRNA complex requires a protospacer-adjacent motif (PAM), identified as 5'-NGG or 5'-NAG (where N indicates any nucleotide) for SpdCas9 (62), to bind to the target DNA sequence. The 20-nt base pairing region of the sgRNAs was designed by searching for the 20-nt regions 5' to NGG/NAG at the template DNA strand and choosing a target closer to the 5' end of the gene in order to get a greater repression efficiency (26). Two oligonucleotides consisting of 20-nt targeting regions with BbsI cohesive ends were chemically synthesized and annealed before being cloned into pgRNA-*tetO*-JTetR by Golden Gate assembly (63). All recombinant plasmids were confirmed by DNA sequencing.

Growth curves. *Y. pestis* strains were cultured in LB broth at 26°C to an optical density at 620 nm (OD₆₂₀) of 1.0. Bacterial cultures were then diluted 1:20 in fresh LB with or without ATc before being incubated at 26°C with shaking at 230 rpm. Bacterial growth was monitored by measuring the OD₆₂₀ at regular intervals.

Quantitative real-time PCR analysis. Total RNA was extracted from *Y. pestis* samples using TRIzol reagent and then treated with RNase-free DNase I to remove DNA contaminants. Resulting RNA concentrations were measured using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific), and 2 μ g of each sample were reverse-transcribed to cDNA using SuperScript II reverse transcriptase. PCR amplifications were performed in a LightCycler 480 instrument (Roche, Basel, Switzerland) using the primers listed in Table 2. Relative expression levels of the target genes were then calculated and normalized to the expression of the 16S rRNA gene.

Immunoblotting analysis. Various *Y. pestis* strains were cultured in LB broth with or without ATc at 26°C to an OD₆₂₀ of 1.0. Bacterial cells were harvested by centrifugation, mixed with Laemmli loading buffer, and boiled for 5 min before being electrophoresed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels, followed by transfer onto Immobilon-P membrane. Specific proteins were detected using specific rabbit antibodies against the SlyA, PhoP, HmsH, and Pla proteins from *Y. pestis*, a rabbit anti-GroEL antibody, or anti-CRISPR-Cas9 antibodies, followed by incubation with an IRDye 800CW-conjugated goat anti-rabbit secondary antibody. Images of the immunoblotting results were captured using an Odyssey SA imaging system (LI-COR Biosciences, Lincoln, NE, USA).

Crystal violet staining of biofilms. The biofilm formation capabilities of the *Y. pestis* strains were determined using a crystal violet staining assay as described previously (64). In brief, bacterial strains were inoculated into 24-well tissue culture plates in 1 ml of LB broth with or without ATc and cultured at 26°C for 24 h. Bacterial cultures were then aspirated, and the OD₆₂₀ value for each sample was determined. The wells of the 24-well tissue culture plates were then washed moderately three times with H₂O and heated at 80°C for 10 min. Adherent cells were stained with 0.1% crystal violet and the bound dye was dissolved in ethanol. The OD₅₇₀ values were measured, and the OD₅₇₀/OD₆₂₀ ratio was then calculated for each sample to indicate relative biofilm formation ability.

Pigmentation and colony morphology assays. Bacterial strains were cultured in LB broth at 26°C to the mid-log phase and inoculated by streaking onto Congo red agar plates (supplemented with 5 mg/ml Congo red), followed by incubation for 4 days at 26°C. Aliquots (10 µl) of glycerol stocks of the various *Y. pestis* strains were dropped onto LB agar plates and incubated for 7 days at 26°C. Ampicillin and chloramphenicol were added to Congo red and LB agar plates for all strains except for the wild-type, and ATc was added to induce the expression of sgRNA-*hmsH* and dCas9. Images of the Congo red pigmentation and morphologies of all colonies were acquired using a Canon 60D DSLR camera (Canon, Inc., Tokyo, Japan).

Cold resistance assay. Bacterial strains were inoculated into fresh LB broth and grown at 26°C to an OD₆₂₀ of ~1.0. Bacterial cells were collected by centrifugation, resuspended in the same volume of LB, and inoculated into fresh LB broth at a 1:100 dilution, followed by incubation for 24 h at 4°C (65). Bacterial suspensions that did not undergo the cold treatment served as untreated controls. All bacterial suspensions were then serially diluted and plated onto LB agar plates to determine viable bacterial counts. All strains were analyzed in triplicate, and percent bacterial survival was calculated by dividing the number of viable cells in the cold-treated samples by that of the controls. Two-way analysis of variance (ANOVA) with Bonferroni's multiple-comparison test was performed using GraphPad Prism 6 software (GraphPad Software, La Jolla, CA, USA) to analyze the significance of differences between the ATc-treated and untreated bacteria.

Cell cytotoxicity analysis using a real-time cell analysis system. Aliquots (150 µl) of DMEM supplemented with 10% FBS were pipetted into each well of the E-plate, which was connected to the RTCA iCELLigence system (Acea Biosciences, San Diego, CA USA), to obtain baseline measurements. Approximately 1.5×10^6 HeLa cells were then seeded into each well, and the plates were incubated in a 5% CO₂ incubator at 37°C for 18 to 20 h until the cell index (CI) was stable. Various *Y. pestis* strains were then cultured in LB medium at 26°C to an OD₆₂₀ of ~1.0. Bacterial cells were harvested, resuspended in DMEM, and used to infect HeLa cells at a multiplicity of infection (MOI) of 5. HeLa cells were incubated at 37°C, and the CI was measured at 5-min intervals.

Immunofluorescence staining and confocal microscopy. HeLa cells were seeded into glass-bottom culture dishes (Nest Scientific, San Diego, CA, USA) the day prior to infection, while cytochalasin D was added at a concentration of 1 µM immediately prior to infection. HeLa cells were infected at an MOI of 100 and centrifuged at $300 \times g$ for 5 min to promote contact between bacteria and HeLa cells. After 2 h of infection, cells were thoroughly washed for 5 min to remove the free bacteria and fixed in 3.8% paraformaldehyde for 10 min. *Y. pestis* cells were visualized with a monoclonal antibody against F1 antigen and Alexa Fluor 555-conjugated donkey anti-mouse antibody. Nuclei were counterstained with Hoechst 33342. Images were acquired using an LSM 880 confocal microscope (Carl Zeiss, Oberkochen, Germany).

Virulence assays. Overnight cultures of *Y. pestis* strains were diluted 1:20 in LB broth and then incubated at 26°C to the mid-log phase. Bacterial suspensions were then prepared at a concentration of 10⁴ CFU/ml. Five groups of female BALB/c mice (6 to 8 weeks old, $n = 10$ per group) were subcutaneously inoculated in the inguinal region with 10³ CFU of each of the different strains. Antibiotics (ampicillin at 100 mg/kg and chloramphenicol at 34 mg/kg) and ATc at 4 mg/kg were administered intraperitoneally twice daily to maintain the plasmids or induce the CRISPRi system. All animal experiments were carried out in accordance with the Guidelines for the Welfare and Ethics of Laboratory Animals of China, approved by the Committee of Laboratory Animal Welfare and Ethics of the Institute for Endemic Disease Prevention and Control of Qinghai Province, China (approval no. 201704). The infected mice were observed continuously for 2 weeks, and survival percentages for each group were calculated and analyzed using GraphPad Prism 6 software.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.00097-19>.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.05 MB.

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REFERENCES

- Perry R, Fetherston J. 1997. *Yersinia pestis*—etiologic agent of plague. Clin Microbiol Rev 10:35–66. <https://doi.org/10.1128/CMR.10.1.35>.
- Stenseth NC, Atshabar BB, Begon M, Belmain SR, Bertherat E, Carniel E, Gage KL, Leirs H, Rahalison L. 2008. Plague: past, present, and future. PLoS Med 5:e3. <https://doi.org/10.1371/journal.pmed.0050003>.
- Hinnebusch BJ. 2005. The evolution of flea-borne transmission in *Yersinia pestis*. Curr Issues Mol Biol 7:197–212.
- Nguyen VK, Parra-Rojas C, Hernandez-Vargas EA. 2018. The 2017 plague outbreak in Madagascar: data descriptions and epidemic modelling. Epidemics 25:20–25. <https://doi.org/10.1016/j.epidem.2018.05.001>.
- Butler T. 2009. Plague into the 21st century. Clin Infect Dis 49:736–742. <https://doi.org/10.1086/604718>.
- Riedel S. 2005. Plague: from natural disease to bioterrorism. Proc (Bayl Univ Med Cent) 18:116–124. <https://doi.org/10.1080/08998280.2005.11928049>.
- Song Y, Tong Z, Wang J, Wang L, Guo Z, Han Y, Zhang J, Pei D, Zhou D, Qin H, Pang X, Han Y, Zhai J, Li M, Cui B, Qi Z, Jin L, Dai R, Chen F, Li S, Ye C, Du Z, Lin W, Wang J, Yu J, Yang H, Wang J, Huang P, Yang R. 2004. Complete genome sequence of *Yersinia pestis* strain 91001, an isolate avirulent to humans. DNA Res 11:179–197. <https://doi.org/10.1093/dnares/11.3.179>.
- Deng W, Burland V, Plunkett G, 3rd, Boutin A, Mayhew GF, Liss P, Perna NT, Rose DJ, Mau B, Zhou S, Schwartz DC, Fetherston JD, Lindler LE, Brubaker RR, Plano GV, Straley SC, McDonough KA, Nilles ML, Matson JS, Blattner FR, Perry RD. 2002. Genome sequence of *Yersinia pestis* KIM. J Bacteriol 184:4601–4611. <https://doi.org/10.1128/JB.184.16.4601-4611.2002>.
- Parkhill J, Wren BW, Thomson NR, Titball RW, Holden MT, Prentice MB, Sebaihia M, James KD, Churcher C, Mungall KL, Baker S, Basham D, Bentley SD, Brooks K, Cerdano-Tarraga AM, Chillingworth T, Cronin A, Davies RM, Davis P, Dougan G, Feltham T, Hamlin N, Holroyd S, Jagels K, Karlyshev AV, Leather S, Moule S, Oyston PC, Quail M, Rutherford K, Simmonds M, Skelton J, Stevens K, Whitehead S, Barrall BG. 2001. Genome sequence of *Yersinia pestis*, the causative agent of plague. Nature 413:523–527. <https://doi.org/10.1038/35097083>.
- Garneau JE, Dupuis ME, Villion M, Romero DA, Barrangou R, Boyaval P, Fremaux C, Horvath P, Magadan AH, Moineau S. 2010. The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. Nature 468:67–71. <https://doi.org/10.1038/nature09523>.
- Datsenko KA, Pougach K, Tikhonov A, Wanner BL, Severinov K, Semenova E. 2012. Molecular memory of prior infections activates the CRISPR/Cas adaptive bacterial immunity system. Nat Commun 3:945. <https://doi.org/10.1038/ncomms1937>.
- Goren M, Yosef I, Edgar R, Qimron U. 2012. The bacterial CRISPR/Cas system as analog of the mammalian adaptive immune system. RNA Biol 9:549–554. <https://doi.org/10.4161/rna.20177>.
- Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S, Romero DA, Horvath P. 2007. CRISPR provides acquired resistance against viruses in prokaryotes. Science 315:1709–1712. <https://doi.org/10.1126/science.1138140>.
- Wiedenheft B, Lander GC, Zhou K, Jore MM, Brouns SJJ, van der Oost J, Doudna JA, Nogales E. 2011. Structures of the RNA-guided surveillance complex from a bacterial immune system. Nature 477:486–489. <https://doi.org/10.1038/nature10402>.
- Brouns SJ, Jore MM, Lundgren M, Westra ER, Slijkhuys RJ, Snijders AP, Dickman MJ, Makarova KS, Koonin EV, van der Oost J. 2008. Small CRISPR RNAs guide antiviral defense in prokaryotes. Science 321:960–964. <https://doi.org/10.1126/science.1159689>.
- Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. 2012. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 337:816–821. <https://doi.org/10.1126/science.1225829>.
- Cong L, Zhang F. 2015. Genome engineering using CRISPR-Cas9 system. Methods Mol Biol 1239:197–217. https://doi.org/10.1007/978-1-4939-1862-1_10.
- Hsu PD, Scott DA, Weinstein JA, Ran FA, Konermann S, Agarwala V, Li Y, Fine EJ, Wu X, Shalem O, Cradick TJ, Marraffini LA, Bao G, Zhang F. 2013. DNA targeting specificity of RNA-guided Cas9 nucleases. Nat Biotechnol 31:827–832. <https://doi.org/10.1038/nbt.2647>.
- Ran FA, Hsu PD, Lin CY, Gootenberg JS, Konermann S, Trevino AE, Scott DA, Inoue A, Matoba S, Zhang Y, Zhang F. 2013. Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. Cell 154:1380–1389. <https://doi.org/10.1016/j.cell.2013.08.021>.
- Platt RJ, Chen S, Zhou Y, Yim MJ, Swiech L, Kempton HR, Dahlman JE, Parnas O, Eisenhaure TM, Jovanovic M, Graham DB, Jhunjhunwala S, Heidenreich M, Xavier RJ, Langer R, Anderson DG, Hacohen N, Regev A, Feng G, Sharp PA, Zhang F. 2014. CRISPR-Cas9 knockin mice for genome editing and cancer modeling. Cell 159:440–455. <https://doi.org/10.1016/j.cell.2014.09.014>.
- Bikard D, Jiang W, Samai P, Hochschild A, Zhang F, Marraffini LA. 2013. Programmable repression and activation of bacterial gene expression using an engineered CRISPR-Cas system. Nucleic Acids Res 41:7429–7437. <https://doi.org/10.1093/nar/gkt520>.
- Jiang W, Bikard D, Cox D, Zhang F, Marraffini LA. 2013. RNA-guided editing of bacterial genomes using CRISPR-Cas systems. Nat Biotechnol 31:233–239. <https://doi.org/10.1038/nbt.2508>.
- Hawkins JS, Wong S, Peters JM, Almeida R, Qi LS. 2015. Targeted transcriptional repression in bacteria using CRISPR interference (CRISPRi). Methods Mol Biol 1311:349–362. https://doi.org/10.1007/978-1-4939-2687-9_23.
- Peters JM, Colavin A, Shi H, Czarny TL, Larson MH, Wong S, Hawkins JS, Lu CHS, Koo BM, Marta E, Shiver AL, Whitehead EH, Weissman JS, Brown ED, Qi LS, Huang KC, Gross CA. 2016. A comprehensive, CRISPR-based functional analysis of essential genes in bacteria. Cell 165:1493–1506. <https://doi.org/10.1016/j.cell.2016.05.003>.
- Larson MH, Gilbert LA, Wang X, Lim WA, Weissman JS, Qi LS. 2013. CRISPR interference (CRISPRi) for sequence-specific control of gene expression. Nat Protoc 8:2180–2196. <https://doi.org/10.1038/nprot.2013.132>.
- Qi LS, Larson MH, Gilbert LA, Doudna JA, Weissman JS, Arkin AP, Lim WA. 2013. Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. Cell 152:1173–1183. <https://doi.org/10.1016/j.cell.2013.02.022>.
- Plagens A, Richter H, Charpentier E, Randau L. 2015. DNA and RNA interference mechanisms by CRISPR-Cas surveillance complexes. FEMS Microbiol Rev 39:442–463. <https://doi.org/10.1093/femsre/fuv019>.
- Choudhary E, Thakur P, Pareek M, Agarwal N. 2015. Gene silencing by CRISPR interference in mycobacteria. Nat Commun 6:6267. <https://doi.org/10.1038/ncomms7267>.
- Wang Y, Zhang ZT, Seo SO, Lynn P, Lu T, Jin YS, Blaschek HP. 2016. Gene transcription repression in *Clostridium beijerinckii* using CRISPR-dCas9. Biotechnol Bioeng 113:2739–2743. <https://doi.org/10.1002/bit.26020>.
- Yao L, Cengic I, Anfelt J, Hudson EP. 2016. Multiple gene repression in cyanobacteria using CRISPRi. ACS Synth Biol 5:207–212. <https://doi.org/10.1021/acssynbio.5b00264>.
- Evers B, Jastrzebski K, Heijmans JP, Grenrum W, Beijersbergen RL, Bernards R. 2016. CRISPR knockout screening outperforms shRNA and CRISPRi in identifying essential genes. Nat Biotechnol 34:631–633. <https://doi.org/10.1038/nbt.3536>.
- Yan MY, Yan HQ, Ren GX, Zhao JP, Guo XP, Sun YC. 2017. CRISPR-Cas12a-assisted recombineering in bacteria. Appl Environ Microbiol 83:e00947-17. <https://doi.org/10.1128/AEM.00947-17>.
- Lutz R, Bujard H. 1997. Independent and tight regulation of transcriptional units in *Escherichia coli* via the LacR/O, the TetR/O and AraC/I1-2 regulatory elements. Nucleic Acids Res 25:1203–1210. <https://doi.org/10.1093/nar/25.6.1203>.
- Gossen M, Bujard H. 1992. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc Natl Acad Sci U S A 89:5547–5551. <https://doi.org/10.1073/pnas.89.12.5547>.

35. Engler C, Kandzia R, Marillonnet S. 2008. A one pot, one step, precision cloning method with high throughput capability. *PLoS One* 3:e3647. <https://doi.org/10.1371/journal.pone.0003647>.
36. Cobb RE, Wang Y, Zhao H. 2015. High-efficiency multiplex genome editing of *Streptomyces* species using an engineered CRISPR/Cas system. *ACS Synth Biol* 4:723–728. <https://doi.org/10.1021/sb500351f>.
37. Singh AK, Carette X, Potluri LP, Sharp JD, Xu R, Priscis S, Husson RN. 2016. Investigating essential gene function in *Mycobacterium tuberculosis* using an efficient CRISPR interference system. *Nucleic Acids Res* 44:e143. <https://doi.org/10.1093/nar/gkw625>.
38. Stoodley P, Sauer K, Davies DG, Costerton JW. 2002. Biofilms as complex differentiated communities. *Annu Rev Microbiol* 56:187–209. <https://doi.org/10.1146/annurev.micro.56.012302.160705>.
39. Jefferson KK. 2004. What drives bacteria to produce a biofilm? *FEMS Microbiol Lett* 236:163–173. <https://doi.org/10.1016/j.femsle.2004.06.005>.
40. Sun YC, Koumoutsis A, Jarrett C, Lawrence K, Gherardini FC, Darby C, Hinnebusch BJ. 2011. Differential control of *Yersinia pestis* biofilm formation in vitro and in the flea vector by two c-di-GMP diguanylate cyclases. *PLoS One* 6:e19267. <https://doi.org/10.1371/journal.pone.0019267>.
41. Sargalla M, Beesley E. 1969. Congo red-agar plating medium for detecting pigmentation in *Pasteurella pestis*. *Appl Microbiol* 18:834–837.
42. Sun Y, Hinnebusch B, Darby C. 2008. Experimental evidence for negative selection in the evolution of a *Yersinia pestis* pseudogene. *Proc Natl Acad Sci U S A* 105:8097–8101. <https://doi.org/10.1073/pnas.0803525105>.
43. Sun F, Gao H, Zhang Y, Wang L, Fang N, Tan Y, Guo Z, Xia P, Zhou D, Yang R. 2012. Fur is a repressor of biofilm formation in *Yersinia pestis*. *PLoS One* 7:e52392. <https://doi.org/10.1371/journal.pone.0052392>.
44. Chapot-Chartier M, Schouler C, Lepeuple A, Gripon J, Chopin M. 1997. Characterization of *cspB*, a cold-shock-inducible gene from *Lactococcus lactis*, and evidence for a family of genes homologous to the *Escherichia coli cspA* major cold shock gene. *J Bacteriol* 179:5589–5593. <https://doi.org/10.1128/jb.179.17.5589-5593.1997>.
45. Miller V, Beer K, Heussipp G, Young B, Wachtel M. 2001. Identification of regions of Ail required for the invasion and serum resistance phenotypes. *Mol Microbiol* 41:1053–1062.
46. Mikula KM, Kolodziejczyk R, Goldman A. 2012. *Yersinia* infection tools—characterization of structure and function of adhesins. *Front Cell Infect Microbiol* 2:169. <https://doi.org/10.3389/fcimb.2012.00169>.
47. Felek S, Tsang TM, Krukonis ES. 2010. Three *Yersinia pestis* adhesins facilitate Yop delivery to eukaryotic cells and contribute to plague virulence. *Infect Immun* 78:4134–4150. <https://doi.org/10.1128/IAI.00167-10>.
48. Day J, Plano G. 1998. A complex composed of SycN and YscB functions as a specific chaperone for YopN in *Yersinia pestis*. *Mol Microbiol* 30:777–788. <https://doi.org/10.1046/j.1365-2958.1998.01110.x>.
49. Cornelius GR, Van Gijsegem F. 2000. Assembly and function of type III secretory systems. *Annu Rev Microbiol* 54:735–774. <https://doi.org/10.1146/annurev.micro.54.1.735>.
50. Zhang X, Qi Z, Du Z, Bi Y, Zhang Q, Tan Y, Yang H, Xin Y, Yang R, Wang X. 2013. A live attenuated strain of *Yersinia pestis* DeltayscB provides protection against bubonic and pneumonic plagues in mouse model. *Vaccine* 31:2539–2542. <https://doi.org/10.1016/j.vaccine.2013.03.054>.
51. Zhang B, Liu ZQ, Liu C, Zheng YG. 2016. Application of CRISPRi in *Corynebacterium glutamicum* for shikimic acid production. *Biotechnol Lett* 38:2153–2161. <https://doi.org/10.1007/s10529-016-2207-z>.
52. Li D, Lv L, Chen JC, Chen GQ. 2017. Controlling microbial PHB synthesis via CRISPRi. *Appl Microbiol Biotechnol* 101:5861–5867. <https://doi.org/10.1007/s00253-017-8374-6>.
53. Liu X, Gallay C, Kjos M, Domenech A, Slager J, van Kessel SP, Knoops K, Sorg RA, Zhang JR, Veening JW. 2017. High-throughput CRISPRi phenotyping identifies new essential genes in *Streptococcus pneumoniae*. *Mol Syst Biol* 13:931. <https://doi.org/10.15252/msb.20167449>.
54. Rock JM, Hopkins FF, Chavez A, Diallo M, Chase MR, Gerrick ER, Pritchard JR, Church GM, Rubin EJ, Sassetti CM, Schnappinger D, Fortune SM. 2017. Programmable transcriptional repression in mycobacteria using an orthogonal CRISPR interference platform. *Nat Microbiol* 2:16274. <https://doi.org/10.1038/nmicrobiol.2016.274>.
55. Wang T, Guan C, Guo J, Liu B, Wu Y, Xie Z, Zhang C, Xing XH. 2018. Pooled CRISPR interference screening enables genome-scale functional genomics study in bacteria with superior performance. *Nat Commun* 9:2475. <https://doi.org/10.1038/s41467-018-04899-x>.
56. Gossen M, Bujard H. 1993. Anhydrotetracycline, a novel effector for tetracycline controlled gene expression systems in eukaryotic cells. *Nucleic Acids Res* 21:4411–4412. <https://doi.org/10.1093/nar/21.18.4411>.
57. Hinnebusch BJ, Jarrett CO, Callison JA, Gardner D, Buchanan SK, Plano GV. 2011. Role of the *Yersinia pestis* Ail protein in preventing a protective polymorphonuclear leukocyte response during bubonic plague. *Infect Immun* 79:4984–4989. <https://doi.org/10.1128/IAI.05307-11>.
58. Derbise A, Lesic B, Dacheux D, Ghigo JM, Carniel E. 2003. A rapid and simple method for inactivating chromosomal genes in *Yersinia*. *FEMS Immunol Med Microbiol* 38:113–116. [https://doi.org/10.1016/S0928-8244\(03\)00181-0](https://doi.org/10.1016/S0928-8244(03)00181-0).
59. Datsenko KA, Wanner BL. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A* 97:6640–6645. <https://doi.org/10.1073/pnas.120163297>.
60. Iriarte M, Cornelis GR. 1999. Identification of SycN, YscX, and YscY, three new elements of the *Yersinia* Yop virulon. *J Bacteriol* 181:675–680.
61. Yang F, Ke Y, Tan Y, Bi Y, Shi Q, Yang H, Qiu J, Wang X, Guo Z, Ling H, Yang R, Du Z. 2010. Cell membrane is impaired, accompanied by enhanced type III secretion system expression in *Yersinia pestis* deficient in RovA regulator. *PLoS One* 5:e12840. <https://doi.org/10.1371/journal.pone.0012840>.
62. Hsu PD, Lander ES, Zhang F. 2014. Development and applications of CRISPR-Cas9 for genome engineering. *Cell* 157:1262–1278. <https://doi.org/10.1016/j.cell.2014.05.010>.
63. Engler C, Gruetzner R, Kandzia R, Marillonnet S. 2009. Golden gate shuffling: a one-pot DNA shuffling method based on type IIs restriction enzymes. *PLoS One* 4:e5553. <https://doi.org/10.1371/journal.pone.0005553>.
64. Fang N, Gao H, Wang L, Qu S, Zhang YQ, Yang RF, Zhou DS. 2013. Optimized methods for biofilm analysis in *Yersinia pestis*. *Biomed Environ Sci* 26:408–411. <https://doi.org/10.3967/0895-3988.2013.05.012>.
65. Liu W, Tan Y, Cao S, Zhao H, Fang H, Yang X, Wang T, Zhou Y, Yan Y, Han Y, Song Y, Bi Y, Wang X, Yang R, Du Z. 2018. Protein acetylation mediated by YfiQ and CobB is involved in the virulence and stress response of *Yersinia pestis*. *Infect Immun* 86:e00224-18. <https://doi.org/10.1128/IAI.00224-18>.