American Society for Microbiology
Journal of VirologyVolume 94, Issue 23, 9 November 2020
https://doi.org/10.1128/JVI.01630-20

Virus-Cell Interactions

Covalent Modifications of the Bacteriophage Genome Confer a Degree of Resistance to Bacterial CRISPR Systems

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ABSTRACT

The interplay between defense and counterdefense systems of bacteria and bacteriophages has been driving the evolution of both organisms, leading to their great genetic diversity. Restriction-modification systems are well-studied defense mechanisms of bacteria, while phages have evolved covalent modifications as a counterdefense mechanism to protect their genomes against restriction. Here, we present evidence that these genome modifications might also have been selected to counter, broadly, the CRISPR-Cas systems, an adaptive bacterial defense mechanism. We found that the phage T4 genome modified by cytosine hydroxymethylation and glucosylation (ghmC) exhibits various degrees of resistance to the type V CRISPR-Cas12a system, producing orders of magnitude more progeny than the T4(C) mutant, which contains unmodified cytosines. Furthermore, the progeny accumulated CRISPR escape mutations, allowing rapid evolution of mutant phages under CRISPR pressure. A synergistic effect on phage restriction was observed when two CRISPR-Cas12a complexes were targeted to independent sites on the phage genome, another potential countermechanism by bacteria to more effectively defend themselves against modified phages. These studies suggest that the defense-counterdefense mechanisms exhibited by bacteria and phages, while affording protection against one another, also provide evolutionary benefits for both.

IMPORTANCE Restriction-modification (R-M) and CRISPR-Cas systems are two well-known defense mechanisms of bacteria. Both recognize and cleave phage DNA at specific sites while protecting their own genomes. It is well accepted that T4 and other phages have evolved counterdefense mechanisms to protect their genomes from R-M cleavage by covalent modifications, such as the hydroxymethylation and glucosylation of cytosine. However, it is unclear whether such genome modifications also provide broad protection against the CRISPR-Cas systems. Our results suggest that genome modifications indeed afford resistance against CRISPR systems. However, the resistance is not complete, and it is also variable, allowing rapid

evolution of mutant phages that escape CRISPR pressure. Bacteria in turn could target more than one site on the phage genome to more effectively restrict the infection of ghmC-modified phage. Such defense-counterdefense strategies seem to confer survival advantages to both the organisms, one of the possible reasons for their great diversity.
KEYWORDSgenome modification, phage T4, CRISPR-Cas, restriction-modification system, bacteriophage

Received 17 August 2020; Accepted 10 September 2020; Published online 16 September 2020.

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Citation Liu Y, Dai L, Dong J, Chen C, Zhu J, Rao VB, Tao P. 2020. Covalent modifications of the bacteriophage genome confer a degree of resistance to bacterial CRISPR systems. J Virol 94:e01630-20. https://doi.org/10.1128/JVI.01630-20.

INTRODUCTION

Bacteriophages (phages) and their hosts, bacteria, are widely distributed on the planet (1, 2). Phages were discovered in the early 20th century by Frederick Twort and Felix d'Herelle and were later found to be the most abundant organisms in aquatic environments (3). They depend on the host bacteria for reproduction, during which a bacterium infected by a virulent phage lyses to release hundreds of progeny phages in about 20 to 30 min (4). Bacteria evolved multiple strategies, such as restriction-modification (R-M) systems and clustered regularly interspaced short palindromic repeat (CRISPR)-Cas systems, to defend themselves against the onslaught of phage infections (5, 6). Phages in turn evolved counterdefense mechanisms, such as genome modifications, to resist or block bacterial defenses. Such defense-counterdefense battles drive the evolution and ecological balance of phages and bacteria (7–10), which constitute the greatest genetic diversity on Earth.

The CRISPR-Cas system is an acquired immune system of prokaryotes against invaders such as phages (6, 11–13). This system employs an "effector complex" composed of Cas nuclease and CRISPR RNA (crRNA), which guides the complex to bind to the identical target sequence present in a phage genome (called a protospacer) by complementary base pairing (6, 11). The Cas nuclease complex makes a double-stranded break to inactivate the genome. The crRNAs are processed from pre-crRNAs, which are transcribed from the CRISPR arrays containing a number of spacers acquired during previous exposure to phages as a memory of infection (6, 12). Therefore, CRISPR-Cas effector complexes help bacteria prevent infections against the same phage. Currently six types (I to VI) of CRISPR-Cas systems have been defined, and the effector complexes of types I, II, and V CRISPR-Cas mainly target DNA (14). The R-M systems of bacteria first found in 1950s have been well studied (5). They generally consist of two enzymatic activities, a restriction endonuclease that recognizes and cuts the invading phage DNA at specific sites, and a methyltransferase that methylates the same DNA sequence of the bacterial (self) DNA, keeping it safe from its own restriction system (5).

It is well known that phages have evolved strategies to modify their genomes in defense against bacterial restriction systems (15, 16). Phage T4 is particularly notorious because

cytosines in its genome are modified by two covalent chemical reactions, 5-hydroxymethylation and glucosylation (ghmC DNA) (15). These modifications make the T4 genome completely resistant to most restriction endonucleases (15). However, it is unclear if the ghmC modification of the T4 genome has evolved as a common counterdefense mechanism against both the R-M systems and the more recently discovered CRISPR-Cas defense systems. Previous studies using type II CRISPR-Cas reported contradictory results (17–19), while in the case of type V CRISPR-Cas, efficient *in vitro* cleavage of ghmC-modified DNA was reported (19). However, a significant limitation of these studies is that very few, only one to four, protospacer sequences were tested, and no *in vivo* studies were performed in the case of type V CRISPR-Cas (17–19). It is therefore essential to perform thorough analyses using a variety of protospacer targets in more than one CRISPR-Cas system in order to address the fundamental question of ghmC phage resistance against CRISPR defense systems.

Recently, using 25 spacers across the entire phage T4 genome, we showed that the ghmC modifications confer various degrees of resistance to type II CRISPR-Cas9 *in vivo* (10, 20), which clarifies some of the previous contradictory results (17, 18). Here, we analyzed whether this resistance to CRISPR-Cas defenses is broader by determining the susceptibility of the phage T4 ghmC genome to type V CRISPR-Cas cleavage *in vivo*. Our studies show that, unlike the unrestricted cleavage observed *in vitro* using just one spacer (19), the ghmC-modified T4 phage genome showed various degrees of resistance *in vivo* to CRISPR-Cas12a targeted to different protospacer sequences in the T4 genome. However, when two independent sites in phage genome were targeted, a synergistic effect was observed and the ghmC-mediated resistance was nearly abolished. Furthermore, we observed that the type V CRISPR-Cas allowed rapid evolution of CRISPR escape T4 mutants, though it is more restrictive than the type II CRISPR-Cas.

Our studies, using two different types of CRISPR-Cas systems and a wide variety of protospacer targets, demonstrate that the ghmC modification of phage T4 genome confers resistance to bacterial CRISPR-Cas defense mechanisms, although the degree of resistance varies depending on the target. Thus, the ghmC modification may have evolved as a common counterdefense mechanism not only against the R-M systems but also against the CRISPR-Cas defense systems. Furthermore, remarkably, ghmC modification also affords phages rapid evolution as a consequence of CRISPR-Cas cleavages. Bacteria may have further evolved multiple spacers targeting the same phage genome to enhance their restriction capabilities against phages with such modifications. These defense-counterdefense mechanisms, in addition to providing protection against each other, also impart survival benefits to both organisms.

RESULTS

The ghmC modifications differentially resist restriction by type V CRISPR-Cas complexes *in vivo*.

The type V CRISPR-Cas system was introduced into Escherichia coli by transforming the plasmid pLbCas12a, expressing Cas12a of bacteria of the family *Lachnospiraceae* (LbCas12a) and crRNA (Fig. 1A; see Materials and Methods for details). Twenty-two spacers with different GC contents were individually cloned into pLbCas12a under the control of the E. coli J23100 promoter to express the corresponding crRNAs, which then formed CRISPR-Cas complexes with LbCas12a (Fig. 1A and B). The spacers were randomly chosen across the phage T4 genome, and none of them contained the adenine methylation site 5′-GATC-3'or the cytosine methylation site 5′-CCWGG-3′ (Fig. 1B). The plating efficiencies of wild-type (WT) T4 phage and the T4(C) mutant, which contained ghmC and unmodified cytosines, respectively, in the genome, were determined for each spacer by plaque assay. If the function of type V CRISPR-Cas is not affected by ghmC modifications, the crRNA-Cas12a effector complexes will cleave genomes of both the WT T4 and the T4(C) mutant at similar efficiencies. Therefore, similar numbers of plaques will arise for both WT T4 and the T4(C) infections.

FIG 1 Restriction of phage T4 infection by type V CRISPR-Cas. (A) The recombinant plasmid expressing LbCas12a and crRNA was constructed as described in Materials and Methods. Pre-crRNA was transcribed under the control of E. coli promoter J23100 and processed by LbCas12a to form the LbCas12a-crRNA complex. (B) Sequences of spacers to determine the restriction efficiency of type V CRISPR-Cas on phage T4 infection. GC content and the targeted gene of each spacer are shown. (C) E. coli cells containing pLbCas12 and the respective spacer were infected with WT T4 or T4(C), and the efficiency of plating (EOP) was determined by plaque assay. The experiments were performed in triplicate.

Our results show that the WT T4 phage had much higher plating efficiencies, 2 to 5 logs higher, for most of the spacers than T4(C) phage (Fig. 1C). This means that ghmC modifications resisted the genome disruption expected by the type V CRISPR-Cas complex *in vivo*. However, the extent of resistance varied, resulting in various degrees of restriction of WT phage infection. Most spacers showed low restriction, with a plating efficiency of $\sim 10^{-1}$, whereas some showed moderate restriction, with a plating efficiency of $\sim 10^{-2}$ to 10^{-3} (Fig. 1C). A few of the spacers showed high restriction, with a plating efficiency of $\sim 10^{-5}$ to 10^{-6}, but these did not discriminate between WT T4 and T4(C) infections (Fig. 1C). Furthermore, there was no correlation between the plating efficiency and GC or C content of spacers (Fig. 1B and C).

Since the restriction greatly depended on the spacers used, we analyzed the protospacer region of "CRISPR-escaped" plaques produced from WT T4 infection under the pressure of different type V CRISPR-Cas12a complexes. Three spacers representing high (no. 5), medium (no. 16), and low (no. 2) restriction of phage infection were randomly selected, and the protospacer regions of the escaped phages were amplified by PCR and sequenced (Fig. 2A). We found that all the first-generation (G1) plaques from the high-restriction spacer and 4 of 10 plaques from medium-restriction spacer contained escape mutations in the respective protospacer sequences (Fig. 2B and C). However, all the G1 plaques from the low-restriction spacer contained WT sequences (Fig. 2D). These results indicate that different protospacer sequences

are cleaved at different efficiencies *in vivo* by the CRISPR-Cas12a complexes. Consistent with this hypothesis, many genomes escaped cleavage of the low-restriction spacer, no. 2, and retained the WT phenotype in G1, whereas there were fewer escaped WT phages with the medium-restriction spacer, no. 16, and none with the high-restriction spacer, no. 5. Hence, ghmC modifications do restrict Cas12a cleavage to various degrees *in vivo*, unlike the nearly complete cleavage observed *in vitro* (19).

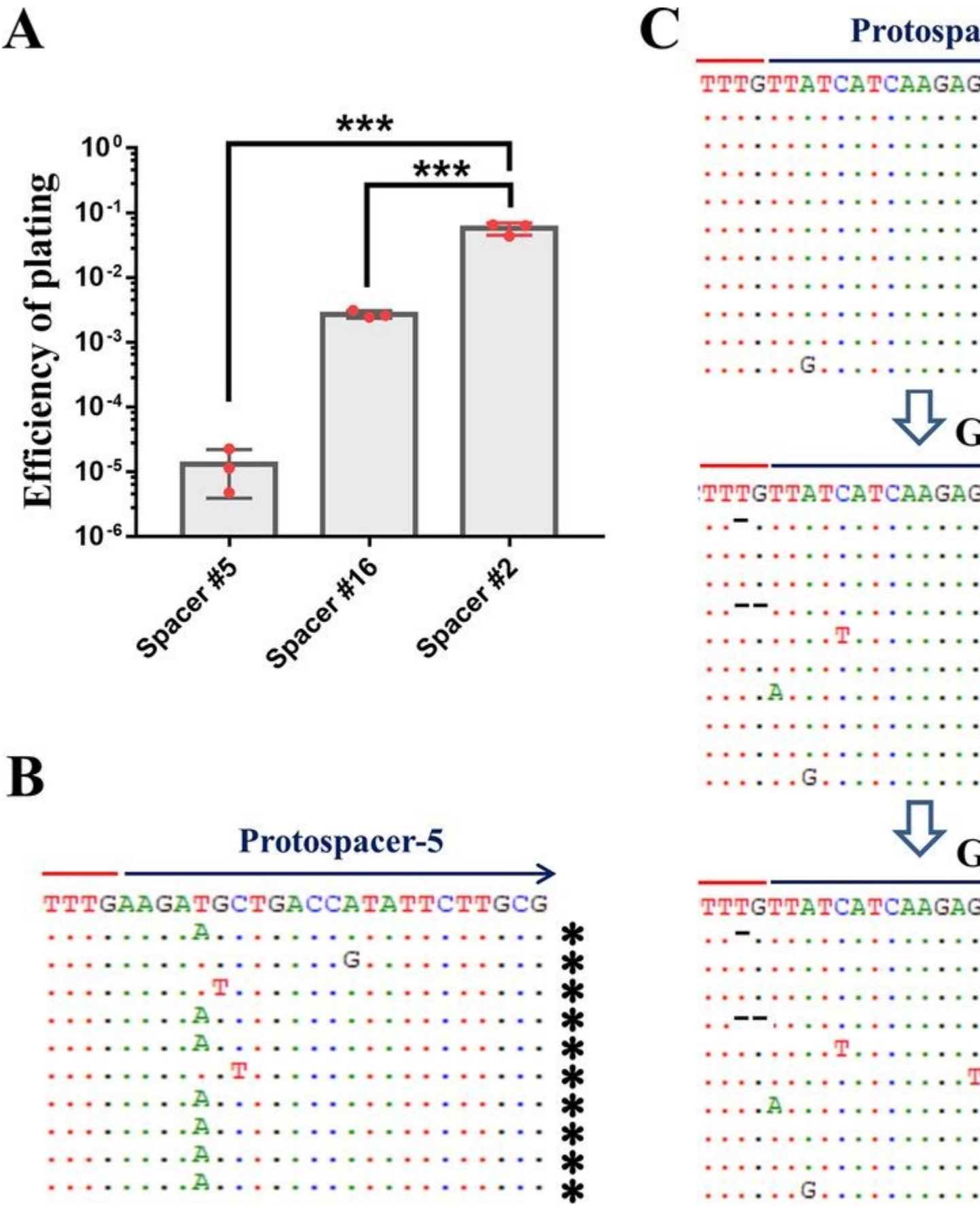
A
***
***
Efficiency of plating
10^0
10^-1
10^-2
10^-3
10^-4
10^-5
10^-6
Spacer #5
Spacer #16
Spacer #2
B
Protospacer-5
TTTGAAGATGCTGACCATATTCTTGCG
C
Protospa
TTTGTTATCATCAAGAG
G
G

FIG 2 CRISPR-Cas12 pressure drives the evolution of escape mutant phages. Three spacers representing high (no. 5), medium (no. 16), and low (no. 2) restriction efficiency (A) were used for analysis of CRISPR escape mutants (B, C, and D). Ten plaques from each infection were randomly picked, and the protospacer region was amplified, sequenced, and aligned with the respective WT sequence. The spacer and PAM sequences are indicated with black arrows and red lines, respectively. The sequences at the top of panels B, C, and D correspond to the WT phage. The CRISPR-escaped plaques containing mutations are marked with asterisks. The data in panel A are means ± standard deviations (SD). ***, $P < 0.001$ (ANOVA).

Some of the Cas12a-cleaved genomes could be rescued *in vivo*, possibly by repair mechanisms that, in the process of repair, introduced mutations that presumably rendered these protospacers resistant to LbCas12a cleavage (Fig. 2B to D). Clearly, by introducing mismatches with the respective spacers, these mutations were probably not tolerated for cleavage by the CRISPR-Cas complexes. Consequently, 40% of plaques arising from the medium-restriction spacer and 100% of plaques from the high-restriction spacer carried mutations in the protospacer sequences (Fig. 2B and C). Some of the latter mutations, since the plating efficiency was on the order of 10^{-5}, might be due to pre-existing mutations that occur to escape Cas12a pressure.

Notably, although ghmC modifications are resistant to cleavage by Cas12a, allowing many WT genomes to escape and form plaques, they are still at a disadvantage in the presence of Cas12a pressure compared to true escape mutants that are no longer targets for Cas12a cleavage. This pressure, therefore, must lead to rapid evolution and selection of mutants, as we observed previously in the type II CRISPR-Cas9 system (10). We therefore transferred CRISPR-escaped plaques from the first generation (G1) to a fresh E. coli plate maintaining the type V CRISPR-Cas12a pressure (Fig. 2C and D). We found that the percentage of CRISPR-escaped plaques containing mutations in protospacer-adjacent motif (PAM) or protospacer sequences increased from 40% in G1 to 90% in G2 and 100% in G3 in the case of the medium-restriction spacer. In the case of the low-restriction spacer, the percentage of escape mutations increased from 0% in G1 to 10% in G2 and 30% in G3. These results show that the continuing pressure of CRISPR-Cas drives the evolution of the ghmC-modified phage genome.

The ghmC-modified phage exhibits variable resistance to type II and type V CRISPR-Cas complexes.

Structural analysis indicated that the type V Cas12a has an intrinsically more open architecture than type II Cas9, which might make it less sensitive to ghmC-modified phages (19). We therefore compared the restriction efficiency of type II and V CRISPR-Cas complexes against WT T4 infection. By scanning the phage T4 genome, we identified six protospacer sequences that can be recognized by both type II and type V CRISPR-Cas complexes (sites 1 to 6) (Fig. 3). Plasmids expressing the corresponding crRNAs were constructed individually and transformed into E. coli cells. The function of type II spacers was confirmed by T4(C) infection, which showed variable restriction with plating efficiencies of 4.14×10^{-6}, 3.70×10^{-6}, 2.35×10^{-1}, 9.21×10^{-4}, 2.89×10^{-6}, and 1.95×10^{-3} for sites 1 to 6, respectively. The E. coli cells expressing each CRISPR-Cas complex were then infected with WT phage T4 to

determine the restriction efficiency. Overall, type V CRISPR-Cas12a complexes showed higher restriction, ~1 to 5 logs higher for all six sites than type II CRISPR-Cas9 (Fig. 3).

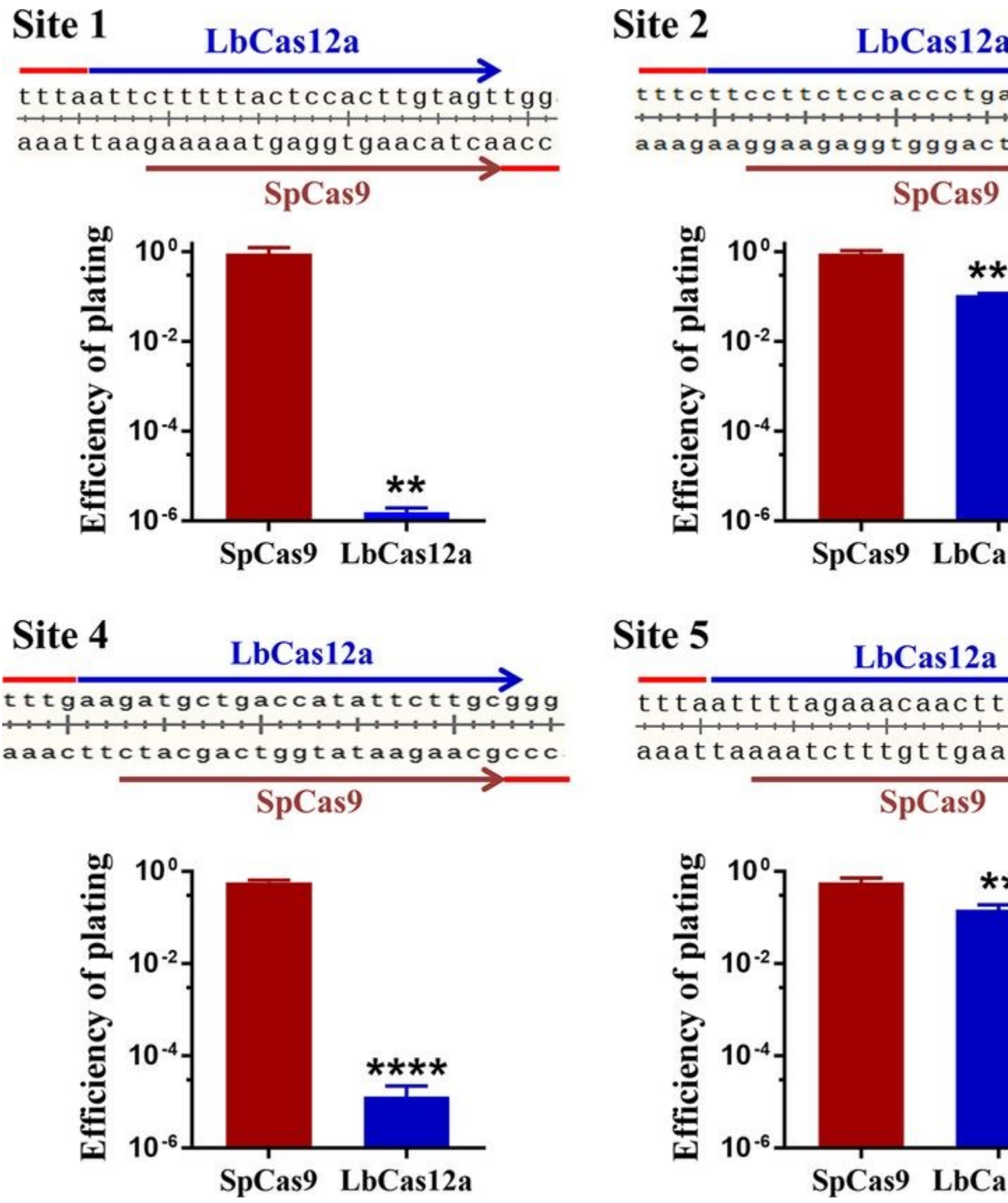
Site 1
LbCas12a
tttaattcttttttactccacttgtagttgg
aaattaagaaaaatgaggtgaacatcaacc
SpCas9
Efficiency of plating
10^0
10^-2
10^-4
10^-6
**
SpCas9
LbCas12a
Site 2
LbCas12a
tttcttccttctccaccctga
aaagaaggaagaggtgggact
SpCas9
Efficiency of plating
10^0
10^-2
10^-4
10^-6
**
SpCas9
LbCa
Site 4
LbCas12a
tttgaagatgctgaccatattcttgcggg
aaacttctacgactggtataagaacgccc
SpCas9
Efficiency of plating
10^0
10^-2
10^-4
10^-6
****
SpCas9
LbCas12a
Site 5
LbCas12a
tttaattttagaaacaactt
aaattaaaatctttgttgaa
SpCas9
Efficiency of plating
10^0
10^-2
10^-4
10^-6
**
SpCas9
LbCa

FIG 3 Restriction efficiencies of type II and V CRISPR-Cas complexes targeting the same protospacer region. Six sites that can be recognized by both LbCas12a (blue arrows) and SpCas9 (dark red arrows) were identified within the T4 genome. The corresponding spacers were cloned into pLbCas12a and pDS-SP plasmids, respectively. The efficiency of plating (bottom of each panel) on E. coli DH5a expressing Cas9 or Cas12a CRISPR systems was determined in triplicate by plaque assay. Data are means and SD. **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ (Student's *t* test).

Similarly, we also identified three sites within the T4 genome where cleavage is expected to occur at the same nucleotide by both type II and V CRISPR-Cas complexes (Fig. 4). As shown in Fig. 4A, the Cas12a-crRNA complexes cleave the target at nucleotides 19 (or 18) and 23 of the protospacer sequence and generate 4-nucleotide (nt) or 5-nt sticky ends (21), whereas Cas9-crRNA complexes cleave 3 bp upstream of the PAM site and generate blunt ends (22). Therefore, for each of the three identified sites, we designed crRNA sequences such that Cas9 cleavages occur precisely at the same cleavage sites as in Cas12a, i.e., nucleotides 18, 19, and 23 (sites 1 to 3) (Fig. 4B). Our results show, once again, that the Cas12a-crRNA complexes gave rise to higher restriction, i.e., lower plating efficiencies, in the range of ~1 to 5 logs for all three protospacer regions compared to Cas9-crRNA complexes (Fig. 4B).

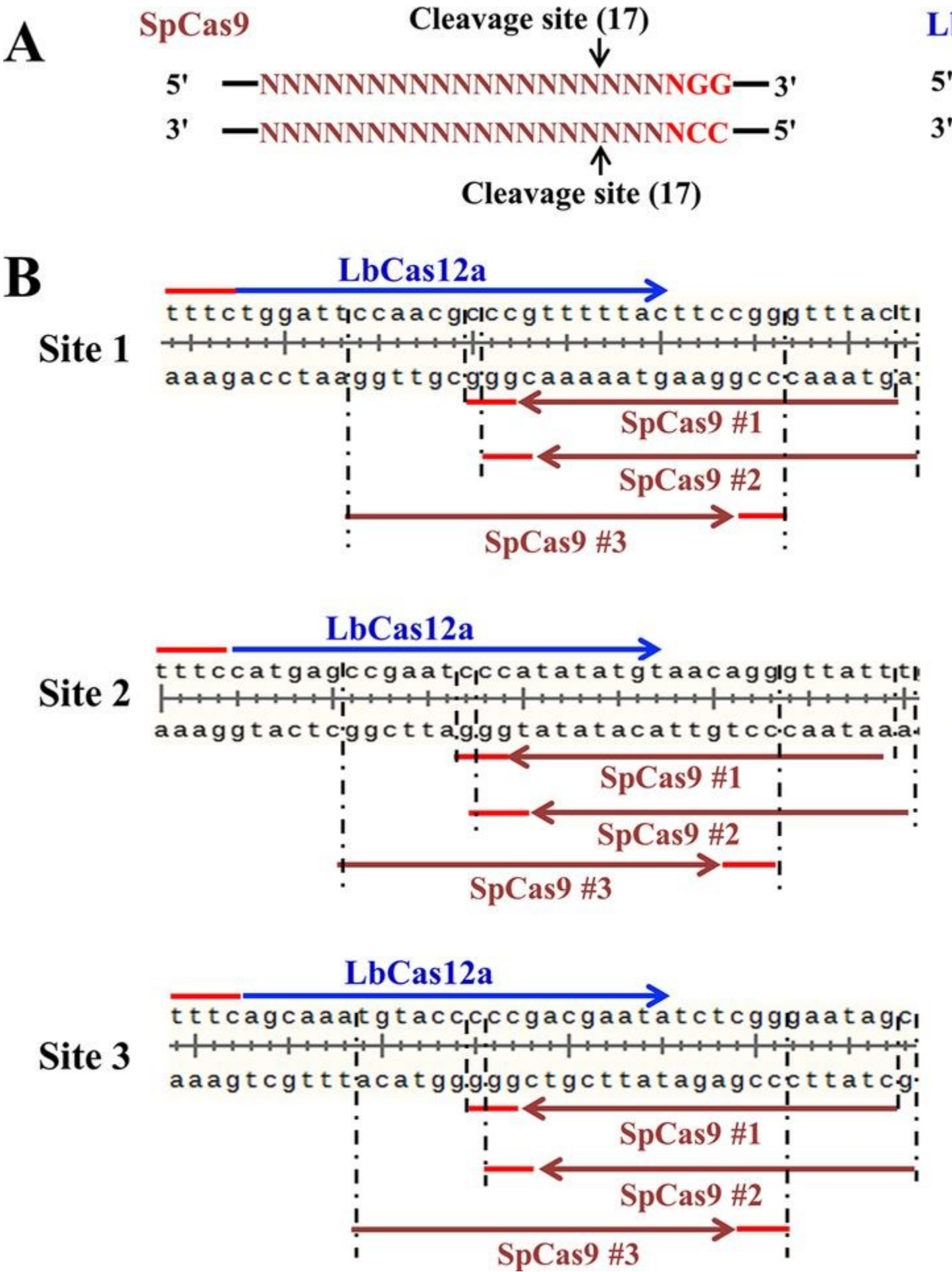
A
SpCas9
Cleavage site (17)
5'
NNNNNNNNNNNNNNNNNNNNGG
3'
3'
NNNNNNNNNNNNNNNNNNNNCC
5'
Cleavage site (17)
B
LbCas12a
Site 1
tttctggatttccaacgtccgttttttacttccgggtttactt
aaagacctaaggttgcgggcaaaaatgaaggcccaaatgaa
SpCas9 #1
SpCas9 #2
SpCas9 #3
LbCas12a
Site 2
tttccatgagccgaatccatatatgtaacagggttatttt
aaaggtactcggcttagggtatatacattgtcccaataaa
SpCas9 #1
SpCas9 #2
SpCas9 #3
LbCas12a
Site 3
tttcagcaaatgtaccccgacgaatatctcgggaatagc
aaagtcgtttacatgggggctgcttatagagcccttatcg
SpCas9 #1
SpCas9 #2
SpCas9 #3

FIG 4 Restriction efficiencies of type II and V CRISPR-Cas complexes with the same cleavage sites. (A) Cleavage pattern of types II (SpCas9 [dark red]) and V (LbCas12a [blue]) CRISPR-Cas complexes. PAM sequences are shown in red, and the cleavage sites are indicated with black arrows. LbCas12a cuts the DNA at nucleotides 19 (or 18) and 23 to generate sticky ends, while SpCas9 cuts the DNA at nucleotide 17 to generates blunt ends. (B) Three specific sites were identified within the T4 genome where the cleavage sites for LbCas12a (blue arrows) and SpCas9 (dark red arrows) overlapped. For each type V spacer, we designed three type II spacers, which guide the SpCas9-crRNA complexes to cut the phage DNA at sites 18, 19, and 23. The corresponding spacers were cloned into pLbCas12a and pDS-SP for comparison of restriction efficiency. The experiments were performed in triplicate. The significance between LbCas12a and SpCas9 no. 1, no. 2, or no. 3 was tested by Student's *t* test (**, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

Taken together, these results demonstrate that the ghmC-modified phage T4 genome exhibits different degrees of resistance to different CRISPR-Cas systems and overall lower resistance to type V CRISPR-Cas complexes than to type II CRISPR-Cas complexes.

Synergistic impact of multiple CRISPR-Cas12a complexes to restrict the ghmC-modified phage genome.

Phage restriction seems to be positively correlated with the number of phage-specific spacers present in bacteria (13, 23). To determine whether bacteria can overcome the CRISPR-Cas resistance mediated by ghmC modification by increasing the number of spacers targeting the same genome, we introduced recombinant plasmids that express two spacer crRNAs targeting different protospacer sites on the phage T4 genome (Fig. 5A). Plasmids expressing individual single crRNAs were used as controls. Six such sets were constructed, and all showed similar results. The bacteria that simultaneously expressed two crRNAs greatly decreased the plating efficiency of WT T4 phage (Fig. 5B). This was true for both the low-restriction spacers (spacers 2 + 1 and spacers 16 + 22) and medium-restriction spacers (spacers 7 + 13, spacers 7 + 8, and spacers 13 + 8). Furthermore, the effect was synergistic; i.e., restriction was ~1 to 3 orders of magnitude greater when combined spacers were used than when individual spacers were used. This is probably because when two double-stranded breaks are made in the same genome, it is harder to repair them and restore the genome integrity without any loss of essential sequence information.

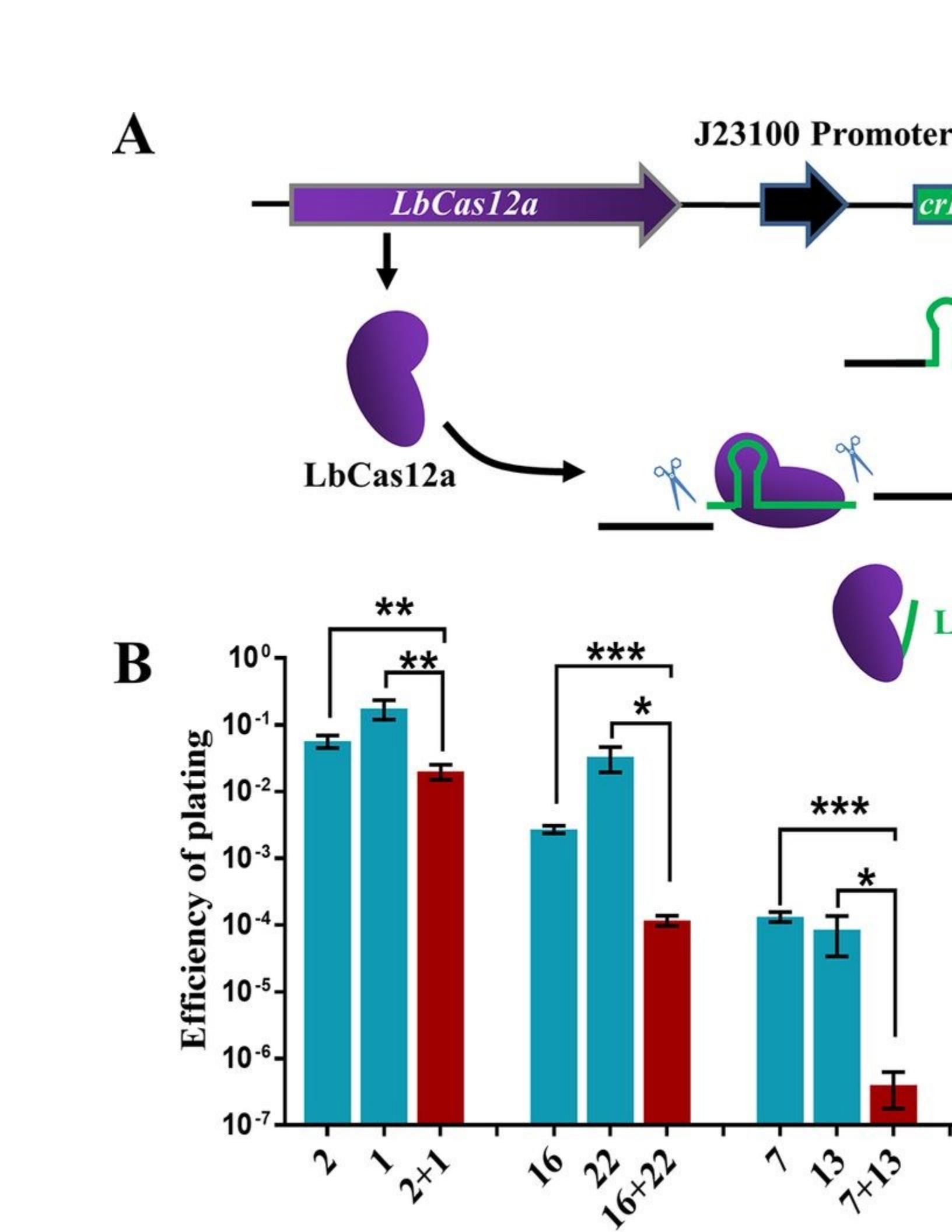
A
J23100 Promoter
LbCas12a
LbCas12a
B
Efficiency of plating
10^0
10^-1
10^-2
10^-3
10^-4
10^-5
10^-6
10^-7
**
**
***
*
***
*
2
1
2+1
16
22
16+22
7
13
7+13

FIG 5 The synergistic effect of two different CRISPR-Cas12a complexes on restricting phage infection. (A) Schematic showing the expression and assembly of two different CRISPR-Cas12a complexes. The spacer array was transcribed into the pre-crRNA precursor, which was then processed by Cas12a to form the mature Cas12a-crRNA complexes along with Cas12a. (B) Restriction efficiency of single or two Cas12a-crRNA complexes on WT T4 phage infection. The EOP was determined in triplicate by plaque assay. Red stars indicate that no plaques were generated when 10^7 PFU phage was used for infection. The significance between single and dual spacers was tested by Student's *t* test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

DISCUSSION

The battle for survival between phages and bacteria has been driving the evolution of both organisms for millions of years (8, 16). The bacterial R-M systems, which cleave the invading phage DNAs at specific sequences, and covalent modification of phage genomes to block these cleavages were the first identified and well-characterized defense-counterdefense mechanisms (5). The CRISPR-Cas defense systems were more recently discovered as an acquired immune defense by bacteria to cleave the genomes of specific phages containing site(s) precisely matching the CRISPR array (6, 13, 24). Not surprisingly, phages have evolved counterdefense mechanisms, such as the phage-encoded anti-CRISPR proteins that can directly interact with CRISPR-Cas complexes and block the cleavages (25–28). However, it is unclear if the covalent genome modifications also provide defense against all CRISPR systems that mainly target DNA. If so, a single phage-encoded mechanism would be able to block or resist multiple bacterial defense mechanisms. Here, we present evidence that the cytosine hydroxymethylation and glucosylation of T4 phage genome might have evolved as a broader evolutionary countermeasure against both the restriction-based and CRISPR-based bacterial defense systems. This is especially relevant for phages, such as T4, that do not seem to encode specific anti-CRISPR proteins.

Six types (I to VI) of CRISPR-Cas systems have been identified so far, of which three, types I, II, and V, were shown to target DNA. Our previous studies as well as others' found that the ghmC modifications confer resistance to type I and type II CRISPR-Cas nucleases *in vivo* (10, 18–20). Our current studies show that the ghmC modification also resists the type V CRISPR-Cas system. Infection efficiency data from 22 spacers spanning across the T4 genome showed that the ghmC-modified WT T4 phage infections produce orders of magnitude more plaques than the unmodified T4(C) phage infections, demonstrating resistance of the modified genome to type V CRISPR-Cas system. However, the resistance is not complete and varies from spacer to spacer, and for a few spacers (e.g., no. 10 and no. 11), minimal resistance is encountered by either ghmC-T4 or T4(C) phages. Similar type of varying resistance was also observed for the type II CRISPR-Cas9 complexes (20). The mechanisms of such variances are unknown, and we found no correlation to GC or C content of the protospacer regions. One explanation is that there might be sequence-dependent differences in the extent of ghmC modifications of protospacer regions (18), which might affect the cleavage efficiency to different extents. This might also explain why our *in vivo* data do not align with the *in vitro* data obtained

with purified Cas12a, where efficient cleavage of ghmC-modified DNA was observed (19). Furthermore, these *in vitro* assays were done using a single spacer in a defined system under controlled conditions, which does not provide a true measure of infection efficiency *in vivo*, as was measured in the current studies. Taken together, the body of evidence strongly suggests that ghmC modifications of phage genomes might work as a common counterdefense mechanism against both the CRISPR-Cas and R-M defense systems.

Notably, consistent with our previous observations with type II CRISPR-Cas system (10), we found rapid evolution of type V CRISPR-escape mutations at the targeted sites. The frequency of such mutations correlated with the plating efficiency; the greater the restriction by Cas12a cleavage, the greater the probability the escape mutants were selected by the time a plaque was generated from a single initial infection, presumably through errors made during the repair of Cas12a-cleaved DNA. The repair processes are quite robust in phages, especially the T4 phage (29, 30). Accordingly, virtually 100% of the plaques generated with high-restriction spacers have mutations in the protospacer sequences, making them resistant to Cas12a cleavage, whereas with low-restriction spacers, it took several generations to accumulate such mutants. Consequently, the fraction of WT plaques diminished, while that of the mutants increased from generation to generation under CRISPR pressure. This counterintuitive mechanism allows rapid phage evolution aided by CRISPR, potentially resulting in additional survival advantages for the phage. Thus, CRISPR defenses benefit not only bacteria, by restricting phage invasion, but also the phage, by accelerating its evolution.

We found synergistic restriction when more than one protospacer site in phage genome was targeted. Spacers that exhibited low or medium restriction (plating efficiency, $\sim 10^{-4}$ to 10^{-3}) showed several orders of magnitude greater restriction (plating efficiency, $\sim 10^{-7}$) when combined with another low- or medium-restriction spacer. This is not entirely unexpected, because it would be much harder to simultaneously repair two double-stranded breaks in order to restore genome integrity. Otherwise, some essential genome might be lost, resulting in loss of infectivity. This might represent a strategy of bacteria to get around the CRISPR-Cas resistance mediated by ghmC modification. Indeed, multiple spacer acquisition is emerging as a coevolutionary mechanism used by bacteria to counter CRISPR-resistant phages (23, 31).

In conclusion, our studies uncovered the possibility that a single counterdefense mechanism by phages such as genome modification could resist or block multiple defense mechanisms by bacteria. Although variable and incomplete, this type of counterdefense mechanism allows accelerated phage evolution aided by the same mechanism that also restricts phage infection. Bacterial evolution of diverse CRISPR systems and multiple targeting mechanisms would then follow ultimately establishing the ecological balance and the great diversity of both bacteria and phage in the biosphere.

MATERIALS AND METHODS

Bacteria and phages.

E. coli strains DH5α [*hsdR17*(r_K^- m_K^+) *sup*^{2}], P301 (*sup*^{0}), and B834 (*hsdR*$_B$ *hsdM*$_B$ *met thi sup*^{0}) were used in this study. Wild-type T4 phage, which contains a ghmC-modified genome, was propagated on E. coli P301. T4(C) is a T4 mutant containing an amber mutation at amino acid 58 of the dCMP hydroxymethylase gene and an amber mutation at amino acid 124 of the dCTPase gene. Both dCMP hydroxymethylase and dCTPase are necessary for ghmC modification of T4 genome. T4(C) was propagated on E. coli B834 as described previously (20) to produce phages with unmodified cytosines in the genome.

Recombinant plasmid construction.

The pLbCas12a plasmid vector was constructed by Gibson assembly of three DNA fragments, the 3,684-bp LbCas12a amplified by PCR from plasmid pET-NLS-LbCpF1, the 221-bp J23100 promoter-driven expression cassette synthesized according to the plasmid DS-SPCas (32) with minor modification, and the 2,302-bp backbone of plasmid DS-SPCas amplified by PCR (Table 1). The CRISPR-LbCas12 spacer plasmids were constructed by Gibson assembly of spacer fragments and EcoRI/XhoI-linearized pLbCas12a vector (Fig. 6). The spacer fragments were prepared by annealing and extension of two synthesized single-stranded DNAs containing 17-bp complementary nucleotides at the 3′ ends (Fig. 6A and Table S1). The sequences of spacers are shown in Fig. 1B. The SpCas9-crRNA plasmids were constructed by cloning spacer sequences into plasmid DS-SPCas as described previously (20). All plasmids were confirmed by sequencing.

Primer	Sequenc
A-F	TACTAG
A-R	CTCAGT
B-F	GACATC
B-R	TCTCAA
C-F	GAATTC
C-R	TTTTGC

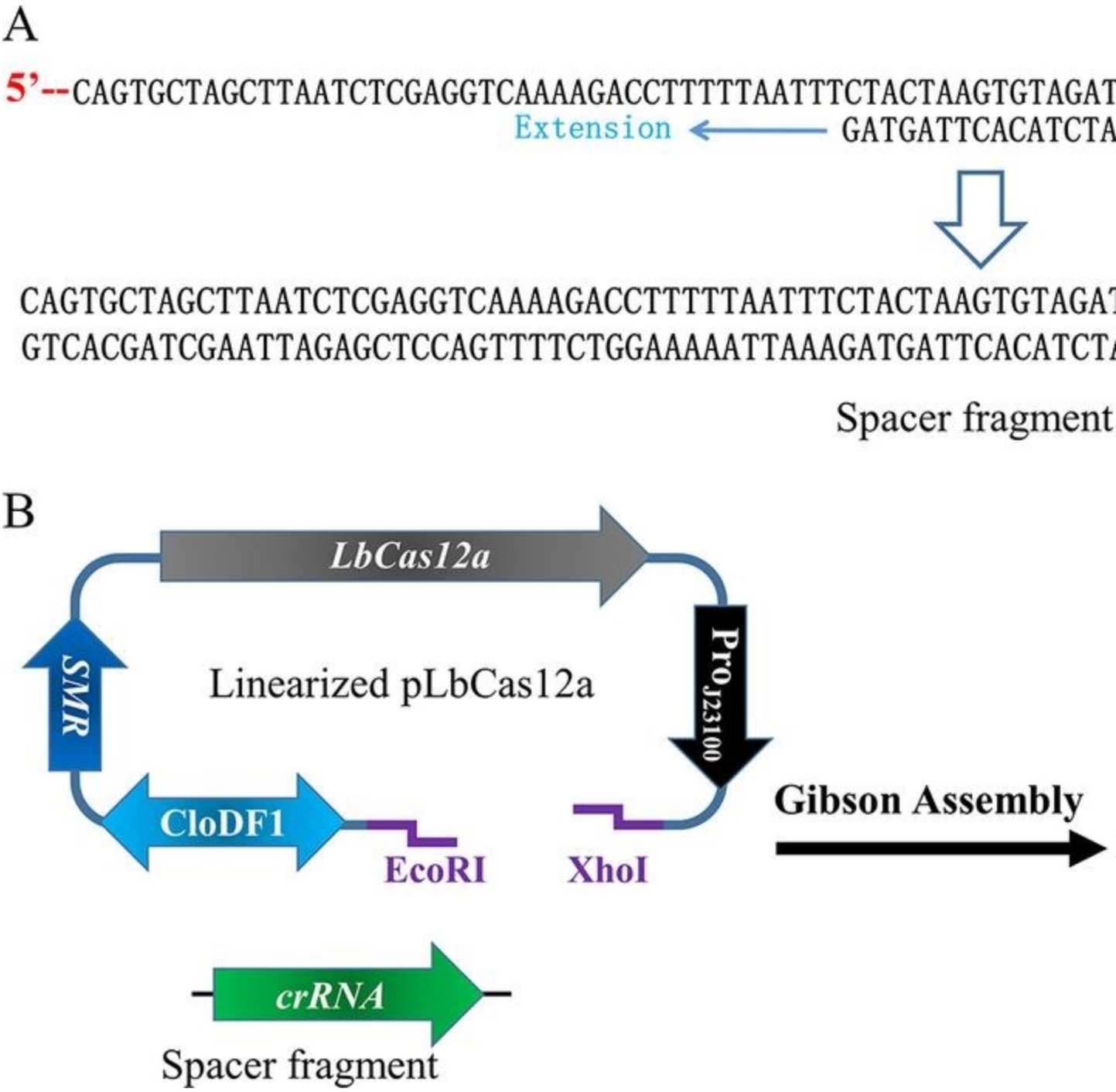

FIG 6 Construction of pLbCas12a-crRNA plasmids. (A) The DNA fragments that encode crRNA were prepared by annealing and extension of two synthesized single-stranded DNAs containing 17-bp complementary nucleotides at the 3′ ends. The "n"s represent the sequence of the spacer. (B) The pLbCas12a-crRNA plasmids were constructed by Gibson assembly of EcoRI/XhoI-linearized pLbCas12a vector and the spacer fragment.

Plaque assays.

E. coli cells transformed with pLbCas12a-crRNA or SpCas9-crRNA plasmid were used for plaque assays to determine the efficiency of individual spacer plasmid in restricting T4 phage

infection. E. coli cells transformed with empty vector, pLbCas12a, were used as controls. An appropriate number of PFU in the range of 10^2 to 10^7 in 100 µl Pi-Mg buffer (26 mM Na_2HPO_4, 68 mM NaCl, 22 mM KH_2PO_4, 1 mM $MgSO_4$ [pH 7.5]) was mixed with 300 µl of E. coli (10^8 cells/ml) and incubated for 7 min at 37°C. After the addition of 3 ml top agar with spectinomycin (50 µg/ml), the infection mixture was poured onto tryptic soy agar (TSA)-spectinomycin plates and incubated overnight at 37°C to produce plaques. The efficiency of plating (EOP) was determined by dividing the PFU generated on the plate by the input PFU. A lower EOP value indicates higher restriction efficiency.

Single-plaque DNA sequencing.

DNA sequencing of single plaques was carried out as described previously (10). Briefly, single plaques were picked from plates and transferred into a 1.5-ml tube containing 200 µl of Pi-Mg buffer. A 4-µl portion of supernatant from each tube was used as a template for PCR. The PCR products were purified and sequenced (Tianyi Huiyanm, Beijing, China).

Statistical analysis.

All data were analyzed by GraphPad Prism software using analysis of variance (ANOVA) or Student's *t* test according to the data. A *P* value of <0.05 was considered statistically significant.

ACKNOWLEDGMENTS

Our research is supported by grants from Fundamental Research Funds for the Central Universities (program no. 2662019PY002 to P.T.), the National Natural Science Foundation of China (grant no. 31870915 to P.T.), the National Institute of Allergy and Infectious Diseases (AI111538 and AI081726 to V.B.R.), and the National Science Foundation (MCB-0923873 to V.B.R.).

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