A CRISPR-Cas9 tool to explore the genetics of Bacillus subtilis phages

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Significance and Impact of the Study: This study presents a new CRISPR-Cas9 vector specifically designed for the use with Bacillus phages. It is more efficient than existing systems, and in combination with the established vector-specific construction of artificial CRISPR arrays, it will contribute to better exploitation and understanding of viral diversity.

Keywords
Bacillus subtilis, Cas9, CRISPR array, cloning, engineering, isolation, resistance, phage.

Introduction
Burkholder and Giles (1947) exposed native Bacillus subtilis to a sub-lethal dose of X-ray and thus created the legendary tryptophan auxotrophic strain 168. This strain spread worldwide as a result of the experiments by John Spizizen, who discovered its ability to develop genetic competence (Spizizen 1958). In this way, B. subtilis 168 became a model organism for many aspects of bacterial molecular biology, and through this popularity one of the most frequently used hosts for B. subtilis-related viruses also called phages (Hemphill and Whiteley 1975). Today B. subtilis 168 is still of enormous interest for phage biology with its various mutants and its wide range of methods, which are constantly further developed.

Current sequencing technology leads to the acquisition of numerous phage genomes. However, their detailed bioinformatical analysis frequently results in large numbers of putative genes with unknown functions or homologs (Cresawn et al. 2011). To disclose their function, the generation of deletion mutants and phenotype investigation could be an option. However, the ability to modify phage genomes was limited until the discovery of CRISPR-Cas (Pires et al. 2016).

CRISPR-Cas systems naturally serve as a prokaryotic immune system to protect the organism from invading DNA, such as plasmids or viral genomes (Terns and Terns 2011; Koonin et al. 2017). The CRISPR (clustered regularly interspaced short palindromic repeats) array provides a memory of previous confrontations by encoding short sequences (spacers) of invader DNA separated by repeats. After transcription of the CRISPR array, the pre-crRNA is maturated to active crRNAs. Those align to a corresponding protospacer DNA sequence on a potential invader genome, which subsequently leads to its cleavage mediated by Cas proteins (Garneau et al. 2010; Koonin et al. 2017). The association of a specific protospacer adjacent motif (PAM) with the protospacer is essential for the initiation of this process (Sternberg et al. 2014). In the case of a type II CRISPR-Cas system, a
trans-acting RNA (tracrRNA) is needed for the correct processing of pre-crRNA (Deltcheva et al. 2011) and the formation of a functional crRNA Cas9 complex (Jinek et al. 2012).

For the genetic engineering of B. subtilis two systems were published, which are based on the Cas9 of Streptococcus pyogenes (Altenbuchner 2016; Westbrook et al. 2016). The single-vector system pJOE8999 developed by Josef Altenbuchner (2016) has already been successfully employed for the genetic modification of B. subtilis phages (Schilling et al. 2018). In contrast to the vectors used for E. coli (Tao et al. 2017) and L. lactis (Lemay et al. 2017) which use a micro CRISPR array with only one spacer and a separate tracrRNA, plasmid pJOE8999 recruits a gRNA which is a functional fusion of a crRNA with a tracrRNA (Jinek et al. 2012). Recently, plasmid pJOE8999 was further developed and made ready for application in other Bacillus species via conjugative variants and universally inducible promoters (Toymentseva and Altenbuchner 2019).

Thus far, none of these systems were specifically developed to facilitate genetic modification of B. subtilis phages. All rely on inducible promoters, which is essential for setting up the system and its transfer to Bacillus. In E. coli, a regulated promoter prevents the overexpression of cas9, and reduces unpredictable off-target interactions. Such can result in inefficient cloning due to random mutagenesis of essential components of the host. In Bacillus, a regulated promoter ensures an efficient transfer as it allows the establishment of the vector system without Cas9 already attacking its genomic targets. However, if phages are aimed, the mentioned concerns are not as serious, because target sequences are only present during infection, which in turn is applied on a ready set strain. Thus, an inducible promoter is not necessary for work with phages and just generates additional working steps and costs. Furthermore, individual inductions of several experiments can lead to deviations and impact final results. Additionally, currently available vector systems do not allow the use of Cas9 in combination with several gRNAs in one vector system. Usage of multiple spacer sequences could be of interest when employing recombination cassettes with reduced size (Tao et al. 2017), or on Cas9-mediated resistance against phages. Thus, we designed and tested an improved CRISPR/Cas9-based Bacillus phage modification system to address these demands.

Results and Discussion

A Cas9 vector for Bacillus phage engineering

Before replacing the inducible promoter of pJOE8999 with a constitutive one, the pUC19 (Norlander et al. 1983)-derived E. coli origin (modified pBR322), present in pJOE8999, was replaced with the pACYC184 (p15A) low-copy origin of replication (Chang and Cohen 1978). In this way, vector pTS021 was created, a low-copy equivalent to pJOE8999. It served as the recipient of the native constitutive promoter of S. pyogenes cas9 with the tracrRNA, derived from the vector pCas9 (Jiang et al. 2013), resulting in vector pRH030 (Fig. 1a).

No differences between pRH030 and pJOE8999 were observed in transformation efficiencies, cell growth or density, and stability during construction in E. coli and cloning to Bacillus.

To verify the overall functionality of pRH030, both pRH030 (pRH035) and pJOE8999 (pTS007) were equipped with a gRNA targeting the pre-neck appendage protein gene of Goe1 (Goe1_c00220). The new plasmids pRH035 and pTS007 served only the purpose of proving Cas9 activity through the reduction in viable phages and thus did not hold a recombination cassette. They were transferred into B. subtilis TS01, and the resulting strains used for experiments to quantify reduction in plaque-forming units (PFU) (Table 1).

Plaque-forming unit counts obtained with a plasmid-free strain and a strain with pRH030 were comparable. Thus, in the absence of a gRNA, this vector did not affect replication of the invading phage. However, supplied with a functional gRNA but lacking a homologous DNA sequence for the repair of the dsDNA break introduced by Cas9, the numbers of recovered plaques dropped significantly. Six surviving phages were Sanger sequenced on the respective region. Four revealed two types of deviations in the PAM. The remaining two showed individual mutations in the target sequence itself, allowing these clones to escape Cas9 activity.

Compared to the PFU numbers obtained with the pJOE8999 plasmid system, the pRH030 based system was about two orders of magnitude more efficient. This is likely due to the experimental set-up itself. Using the pJOE8999 plasmid, the induction of cas9 with D-mannitol only takes place on the agar plate as no D-mannitol is present before or during infection. Consequently, phages infect unprotected cells without Cas9 present, as long as the cells are not on D-mannitol containing plates (Schilling et al. 2018). During this time, phages can initiate genome replication, and thereby even create new genetic variants, which in turn can escape the Cas9 and manifest in a PFU.

To investigate how pRH030 performs in phage mutagenesis, it was used to build the vector pRH045 harbouring a gRNA and recombination cassette for the generation of a ΔaimP mutant of vB_BsuS_Goe11 (Goe11). In fact, after mutagenesis, all twenty investigated phage mutants revealed correct recombination with the deletion cassette.
Thus, the previously observed efficiency rate of 40%, obtained using pJOE8999 (Schilling et al. 2018), was increased to 100%. In this context, pRH030 outperformed the original system.

Two spacers on pRH030

However, in future, limitations might still occur when using pRH030 with short recombination cassettes for phage mutagenesis (Tao et al. 2017). The frequency of spontaneous sequence mutations on a phage genome, leading to resistance to a particular gRNA, may exceed the rate of the recombination events. The deletion of genes Goe1_Δc00030 from the phage Goe1 (Flank A 320 bp, Flank B 671 bp) revealed only 10% positive clones, whereas the deletion of Goe1_Δc00180 (Flank A 712 bp, Flank B 709 bp) about 40% (Schilling et al. 2018). This effect might be more pronounced if

Figure 1 pRH030, a Cas9 vector for Bacillus phage engineering. (a) Vector pRH030 and its relevant genetic elements. (b) gRNA precursor (0) in its original state, (1) with a gRNA, (2) with a crRNA–gRNA fusion, (3) an artificial CRISPR array consisting of two crRNAs and a gRNA, (4) an artificial CRISPR array consisting of three crRNAs and a gRNA, and (5) an artificial CRISPR array consisting of four crRNAs and one gRNA. Oligo names are depicted in green. Oligo pairs used for individual hybridization before the final array hybridization are marked with coloured dots. Red primer areas represent CRISPR repeat sequence regions and blue spacer regions. Black triangles indicate not yet ligated dsDNA nicks, which are repaired by Escherichia coli post-transformation. [Colour figure can be viewed at wileyonlinelibrary.com]
Table 1 Efficiency of phage plating Bacillus subtilis strains harbouring diverse Cas9 vector systems

| Vector | cas9 promoter | Target | Exp. 1  | Exp. 2 |
|--------|--------------|--------|--------|--------|
| pRH030 | Constitutive  | None   | $1 \times 10^{11}$ | $1.4 \times 10^{11}$ |
| pRH035 | Constitutive  | Goe1   | $3 \times 10^{6}$  | $3 \times 10^{6}$  |
| pTS007 | Inducible    | Goe1   | $1.2 \times 10^{6}$ | $0.7 \times 10^{6}$ |

The titre of the applied Goe1 phage suspension was $1.7 \times 10^{11}$ PFU per ml.

recombination cassettes became even shorter. A high number of phages can be expected to represent a variety of mutants, which cannot be addressed by a single gRNA, thus leaving a viable fraction without recombination. Employing a second gRNA for the same target can be a solution to sort out even rare recombination events, as two spontaneous mutations on the same target gene are more unlikely. Several concepts exist to realize multiple gRNAs (McCarty et al. 2020), like sequential cloning (Cress et al. 2015) or additional vectors (Zuckermann et al. 2018). However, the exact transfer of such strategies was not directly applicable to pRH030. Therefore, we developed a new specific strategy to set up multiple gRNAs.

Currently, target specification is achieved via a gRNA, representing a fusion of a crRNA and a tracrRNA on a single RNA transcript (Deltcheva et al. 2011). The advantage of the gRNA is its immediate operational readiness without any need for further processing. However, the presence of tracrRNA, RNaseIII and Cas9 allows the processing of CRISPR arrays and thereby the creation of multiple crRNA–tracrRNA units, each directing Cas9 to a specific target (Deltcheva et al. 2011). To take advantage of this set-up, a dimeric CRISPR array was constructed, with no repeat region at its beginning and a gRNA at its end. Experimentally, this dimeric array was realized analogously to the creation of a gRNA. Two overlapping oligonucleotides (oligos), containing two spacers separated by a CRISPR repeat sequence (Fig. 1b2), were hybridized and cloned into the BsaI restriction sites of plasmid pRH030. The correct processing of the artificial CRISPR array was experimentally verified with pRH044, containing a crRNA targeting the head fibre gene (Goe1_c00180) of Goe1 and a gRNA targeting the bgaB gene of the Goe1 Act00180::bgaB mutant. For both phage strains, a reduced PFU count was observed (Goe1 wild-type $10^{10}$ phage to $10^{6}$ and Goe1 Goe_Ac00180::bgaB mutant $10^{5}$ phage to $10^{3}$), thereby verifying crRNA and gRNA as functional, both originating from the dimeric array. If that had not been the case, one of the phage strains could reproduce without restraint. Furthermore, vector pRH073, supplied with two spacers addressing the same phage (Goe11), provided B. subtilis with comprehensive resistance, and thus the ability to eradicate an infection load of $10^{8}$ phages, a phage inactivation efficiency not observed with only one gRNA (Table 1).

Multiple spacers on pRH030

In the context of industrial fermentation, it is of great interest that pRH030 with two spacers provide B. subtilis with full resistance to a phage. However, production strains usually face more than one phage strain, which raises the question of whether more than two spacers can be cloned simultaneously to pRH030 to extend resistance further.

Cloning two target sequences turned out as easy as cloning a single gRNA, achieved by simply using elongated oligos with an additional target sequence separated from the first by a CRISPR repeat sequence. However, oligos cannot be extended indefinitely and must not contain repeat regions. Thus, adding further spacer-CRISPR-repeat units requires their separation through the use of additional separate oligo pairs (Fig. 1b).

Two strategies were pursued to explore the limits of simultaneously cloneable spacers. In the first procedure, all primers were hybridized directly in one reaction leading to a spontaneous concatenation of the artificial array. In the second, primer pairs were specifically pre-hybridized and subsequently specifically chained in a follow-up reaction. The second procedure required an additional work hour but held the potential to reduce possible misassemblies, which are feasible in the direct first procedure (Fig. 1b5, green and blue dots), as it ensures controlled chaining of the primers.

The number of spacer-CRISPR-array units was gradually increased to explore the limits of both strategies. Array assemblies were ligated with T4 DNA ligase into the BsaI-digested pRH030 and transferred into E. coli. The used oligonucleotides were not 5’-phosphorylated, leading to single-strand breaks in the array, which were finally closed in E. coli. The highest observed number of chained gRNAs was seven, albeit with mutations (Table 2). With an increased number of spacers in one array, both cloning efficiency and frequency of a correct construct reduced. That was expected, as the combination of more than four spacers can result in incorrect assembly possibilities (Fig. 1b5, green and blue dots). Surprisingly, pre-hybridized oligo pairs did not lead to meaningfully better results. Although clones with six and seven spacers could be identified, they
Table 2 Efficiency of simultaneous cloning of multiple spacers into plasmid pRH030

| Number of spacers | Number of used oligos for array assembly | Expected PCR product size (bp) | 1. PCR-verified clones after direct hybridization | 1. Fraction with correct sequence | 2. PCR-verified clones after pair-wise pre-hybridization | 2. Fraction with correct sequence |
|------------------|----------------------------------------|-------------------------------|-----------------------------------------------|---------------------------------|-------------------------------------------------|---------------------------------|
| 0 (lacZ)         | -                                      | -                             | -                                             | -                               | -                                               | -                               |
| 1                | 2                                      | 863                           | 87.5%                                         | 100%                            | no exp.                                         | no exp.                          |
| 2                | 2                                      | 939                           | 64.29%                                        | 78.26%                          | no exp.                                         | no exp.                          |
| 3                | 4                                      | 1005                          | 62.5%                                         | 75%                             | 87.5%                                           | 25%                             |
| 4                | 6                                      | 1071                          | 25%                                           | 50%                             | 12.5%                                          | 100%                            |
| 5                | 8                                      | 1137                          | 25%                                           | 50%                             | 12.5%                                          | 0%                              |
| 6                | 10                                     | 1203                          | 41.7%                                         | 0%                              | 62.5%                                          | 33.34%*                         |
| 7                | 12                                     | 1269                          | 25%                                           | 0%                              | 43.75%                                         | 0%                              |

CRISPR arrays were verified by colony PCR with the oligonucleotides RH002/RH039. No PCR product, amplification of the gRNA precursor resembling the original empty vector situation, or PCR products with divergent sizes were considered as a negative result. Numbers are based on at least eight investigated clones per experiment and cloning but were increased up to 27 in case of rare positive events. This was particularly the case for constructs with 6 and 7 spacers. no-exp. = no experiment present. * = Clones encountered mutation, located in the 10 bp of the spacer not relevant for Cas9 activity.

were rare and frequently contained mutations. Thus, we conclude that the direct cloning of five spacers in pRH030 represents the upper limit for an efficient and reliable workflow leading to the desired outcome, without consuming more time and effort as the cloning of a single gRNA.

Conclusion and Outlook

A new Cas9 vector was created, ideally adapted for genetic work on Bacillus phages or for mediating phage resistance to protect Bacillus strains from phage invasions. The ability to quickly set up the system with multiple target sequences could also make it attractive for research on Bacillus phage diversity, or the isolation of less common phage strains by efficiently excluding abundant representatives.

Materials and Methods

Media and solutions

All media and aqueous solutions were prepared with deionized water processed with an arium® pro device (Sartorius AG, Göttingen, Germany). If not otherwise stated, E. coli and B. subtilis strains were cultivated in LB medium (10 g l⁻¹ tryptone, 10 g l⁻¹ NaCl and 5 g l⁻¹ yeast extract) (Bertani 1951). LB medium was supplemented with 1.5% (weight per volume) Agar–Agar Kobe I (Carl Roth GmbH+Co KG, Karlsruhe, Germany) to prepare solid LB agar plates. Overlay agar for plaque assays was prepared from LB medium supplementation with 0.4% (weight per volume) Biozym LE Agarose (Biozym Scientific GmbH, Hessisch Oldendorf, Germany).

Oligonucleotides

Oligonucleotides used in this investigation are listed in Table 3.

DNA modification and cloning

All E. coli–concerning molecular methods were performed according to Sambrook and Russell (2001). Vectors were selected with kanamycin (25 µg ml⁻¹) in solid and liquid media for E. coli and B. subtilis. Endonucleases, T4-DNA ligase and buffer, FastAP thermosensitive alkaline phosphatase, and Phusion DNA polymerase were applied as recommended by the manufacturer (Thermo Fisher Scientific, Darmstadt, Germany). NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany) was used to extract dsDNA from PCR-reaction and agarose gels following the instructions of the manufacturer. NucleoSpin Plasmid (Macherey-Nagel) was used to extract plasmids from E. coli. Sanger sequencing was performed by SeqLab-Microsynth (Göttingen, Germany). Escherichia coli DH10B served as cloning-strain. Transformation of B. subtilis TS01 was realized as described elsewhere (Schilling et al. 2018). Competent E. coli and B. subtilis cells were preserved at −80°C after adding glycerol to a final concentration of 15% (weight per volume).

Construction of pRH030

pTS021 was generated by amplifying the p15A origin of replication from the pACYC184 vector (Chang and Cohen 1978) and cloning the origin into pOE8999 via the BsrGI/XhoI restriction sites, thereby replacing the pUC origin of replication (Norrander et al. 1983). Vector
### Table 3 Oligonucleotides

| Oligonucleotides | 5’ Sequence | Purpose |
|------------------|-------------|---------|
| RH001            | AGCTTAGGC   | Cas9 sequencing |
| RH002            | CAGCTAGGAGTGCAGAG | Array PCR and sequencing |
| RH037            | TGCCACCTGAGGCTTTC | Cas9 sequencing |
| RH039            | GCACTGACTGCTGACATC | Array PCR and sequencing |
| RH229            | ttcgTCTCTGTTTAAG | pRH044 |
| RH230            | aaactTTCTGAAAGCAGGAG | pRH043 |
| TS053            | TAAGCTACCTCACTCGA | Cas9 sequencing |
| TS054            | GAGCTAGGAGGTCCTTTC | Cas9 sequencing and PCR on pCas9 |
| TS055            | TAAGCTACCTCACTCGA | Cas9 sequencing |
| TS056            | AGAGCTAGGCTGATAAAAGCCTT | pRH078, pRH079 |
| TS057            | GGCTCACTACCTTGGTTT | pRH070 |
| RH196            | atatgtacaGGGTC | pRH070 |
| PP107            | tgcGAGCTAGGCTTTC | GoE1_c00220 sequencing |
| PP108            | aaactGGACAGTCAATG | pRH071 |
| PP115            | GCGGACTACCTACTAGAAAGCTG | pRH071 |
| PP116            | TGGTAGGCTGAGACCTGAA | pRH071 |
| PP130            | aaacGCTATTATATATATGAAGCTTGGTC | pRH078, pRH079 |
| PP131            | tgcGAGCTAGGCTTTC | pRH070 |
| PP132            | aaatGCTAGGCTTTC | pRH071 |
| PP133            | tgcGAGCTAGGCTTTC | pRH070 |
| PP134            | GCGGACTACCTTGGTT | pRH071 |
| PP135            | TGGTACCTGCAGCTTGA | pRH071 |
| PP136            | aaacGCTATTATATATGAAGCTTGGTC | pRH078, pRH079 |
| PP137            | tgcGAGCTAGGCTTTC | pRH070 |
| PP139            | tgcGAGCTAGGCTTTC | pRH073 |
| PP140            | aaacGCTATTATATATGAAGCTTGGTC | pRH078, pRH079 |
| PP149            | GCGGACTACCTTGGTTT | pRH070 |
| PP150            | TGGTAGGCTGAGACCTGAA | pRH079 |
| PP151            | tgcGAGCTAGGCTTTC | pRH079 |
| PP152            | GCGGACTACCTTGGTTT | pRH079 |
| PP153            | GCGGACTACCTTGGTTT | pRH079 |
| PP154            | GCGGACTACCTTGGTTT | pRH079 |

Priming areas are presented in capital letters. Overhangs required for cloning are presented in lower case letters. *Streptococcus pyogenes* CRISPR array repeat is presented in red capital letters.
pRH030 was constructed via amplification of tracrRNA-P<sub>native-cas9</sub>' from the vector pCas9 (Jiang et al. 2013) with oligonucleotides TS055/RH196 and cloned into the pTS021 via the BsrGI/KpnI restriction sites (5840 bp fragment purified via gel extraction). Desired constructs were selected on LB agar plates with kanamycin. The resulting construct was verified via Sanger sequencing with oligonucleotides RH001, RH037, TS053, TS054, TS055, TS056, TS057. The ability to replicate in <i>Bacillus</i> was verified via the successful transformation of <i>B. subtilis</i> TS01. The activity of the native <i>S. pyogenes</i> cas9 promoter in <i>B. subtilis</i> was previously reported (Westbrook et al. 2016).

Identification and cloning of spacer sequences

Target sequences and oligonucleotides for gRNA design were identified with CutSPR (Schilling et al. 2018). Construction of the deletion vectors was done as described previously (Schilling et al. 2018). Cloning of three and more spacers was realized with two approaches. In the direct hybridization, 5 pmol of each oligonucleotide was combined in a final volume of 20 µl in 1xT4 ligase buffer. The buffered oligonucleotide mix was placed in a preheated block at 95°C for 2 min. Subsequently, the block was switching off and allowed to passively cool down to room temperature (~1 h). For the pair-wise hybridization, only two oligonucleotides were hybridized as described above and subsequently combined in equal amounts for chaining hybridization at room temperature (~1 h). Of each hybridization, 1 µl was used for ligation with the T4-DNA ligase, and ~40 ng of BsaI-digested and gel-purified pRH030 vector. The design and cloning of a recombination cassette were realized as described elsewhere (Schilling et al. 2018). Blue-white screening was not used since the gel-purified cloning vector rarely led to blue religands, and white potentially positive clones still had to be verified by PCR. Resulting vectors are listed in Table 4.

### Colony PCR

Chromosomal deletions as well as correct vector constructs were verified with colony PCR. <i>E. coli</i> colonies were picked with a sterile toothpick and dissolved in 100 µl sterile LB medium. One µl of this cell suspension served as PCR template in a regular 50-µl PCR reaction, using Phusion DNA polymerase (Thermo Fisher Scientific), HF-Buffer, 35 cycles and the elongation of the initial denaturation step to 2 min. <i>Bacillus</i> colonies were picked and used to inoculate 2 ml of LB medium in a reaction tube, which was incubated for 3 h at 30°C. One µl of this culture served as PCR template, as described for <i>E. coli</i>. For phages, 1 µl of sterile-filtered lysate was directly used as a template in a PCR reaction.

### Plaque assay

The host strain was adjusted to an OD<sub>600</sub> of 0.1 in 4ml of LB in a test tube from a starting culture and incubated under vigorous shaking at 30°C until OD<sub>600</sub> of 1 was reached. For infection, 100 µl of these cells were mixed with 100 µl of phage suspension in a sterile test tube and incubated for 5 min without agitation. The infected cells were mixed with 2.3 ml of overlay agar and spread on a pre-warmed LB plate. After solidification of the overlay agar, the plates were incubated at 30°C for ~16 h. Plaques were manually counted.

### PFU reduction efficiency determination

PFU reduction efficiency was determined by a plaque assay with a defined titre of phage serving as a reference to detect Cas9-gRNA activity through the deviation from the defined titre.

### Phages

<i>Bacillus subtilis</i> phages used in this investigation are Goel = vB_BsuP-Goe1 (KU831549), Goe2 = vB_BsuM-

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**Table 4** Vectors used in this investigation

| Vector | Backbone | Target | Purpose |
|--------|----------|--------|---------|
| pTS021 | pJOE8999, pACYC184 | Non | Empty vector |
| pRH030 | pTS021 | Non | Empty vector |
| pTS007 | pJOE8999 | Goe1 (Goe1_c00180) | gRNA |
| pRH035 | pRH030 | Goe1 (Goe1_c00180) | gRNA |
| pRH043 | pRH030 | Goe11 | 1× gRNA |
| pRH044 | pRH030 | Goe1, Goe11; c00180; bgaB (bgaB) | 2× crRNA-gRNA |
| pRH045 | pRH030 | Goe11 (aimP) | ΔaimP mutant |
| pRH070 | pRH030 | Goe2, Goe3 | 2× spacer |
| pRH071 | pRH030 | Goe2, Goe3 | 3× spacer |
| pRH073 | pRH030 | Goe11 (aimP) | 2× spacer |
| pRH078 | pRH030 | Goe1 (2x), Goe11 (2x) | 4× spacer |
| pRH079 | pRH030 | Goe1 (2x), Goe2 (1x), Goe11 (2x) | 5× spacer |
Goe2 (KY368639), Goe3 = vB_BsuM-Goe3 (KY368640) and Goe11 = vB_BsuS-Goe11 (MT601272).

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Conflict of Interest

The authors have no conflict of interest to declare.

Statement of Contribution

R.H. designed and administrated the study. T.S. and N.M.K. conducted the vectors. A.D.F. and L.P.B.L. performed PFU reduction experiments and mutagenesis experiments. K.O. established the CRISPR-array cloning. V.T.L. and R.H. drafted the manuscript. All authors critically revised the final manuscript and approved it for submission.

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