CRISPR-Cas9-mediated Large Cluster Deletion and Multiplex Genome Editing in *Paenibacillus polymyxa*

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Abstract

Clustered regularly interspaced short palindromic repeats (CRISPR) system has rapidly advanced genetic engineering research. The system has been applied for different genetic engineering purposes in multiple organisms including the quite rarely explored *Paenibacillus polymyxa*. Only limited studies on CRISPR-based system have been described for this highly interesting and versatile bacterium. Here, we demonstrated the utilization of a Cas9-based system to realize 32.8 kb deletion of genomic region by using a single targeting sgRNA. Large cluster deletion was successfully performed with remarkable efficiency of 97 %. Furthermore, we also exploited the system for multiplexing by editing of two distantly located genes at once. We investigated double gene knockouts as well as simultaneous gene integrations and reached editing efficiencies of 78 % and 50 %, respectively. The findings reported in this study are anticipated to accelerate future research in *P. polymyxa* and related species.

Keywords: CRISPR, Cas9, multiplexing, *Paenibacillus polymyxa*, genetic engineering

Introduction

Paenibacillus polymyxa is a Gram-positive, facultative anaerobic, spore forming, non-pathogenic soil bacterium. It is known for its nitrogen fixation capability which makes it attractive for future utilization in modern agriculture. Furthermore, *P. polymyxa* naturally produces a wide range of valuable compounds like exopolysaccharides (EPS), 2,3-butanediol, as well as antibiotics and antimicrobial peptides such as polymyxin and fusaricidin.[1] *P. polymyxa* produces different types of EPS depending on the carbon source. The EPS produced have unique characteristics and physiochemical properties which could be explored for different applications, such as cosmetics, coatings or paper industries.[2]

As *P. polymyxa* gains more and more interests as biotechnological workhorse, the availability of stable and robust genetic tools is essential to further study and engineer the strain toward a versatile platform organism. While several molecular biology tools have been described for *Paenibacillus* species in the past,[3,4] the efficiencies were quite low and further development is of highest importance. Genome editing by means of a suicide plasmid system has been described, which relies on the double crossover of the conditional plasmid that is able to replicate in *Escherichia coli* but not in *P. polymyxa*.[5] This particular method is applicable for gene disruption but markerless deletion could not be achieved as a selection marker has to be integrated. As a consequence, disruption of other genes at different loci in the genome is complicated due to the needs of different selection markers.

The discovery of CRISPR-Cas has revolutionized biotechnology research in unprecedented ways. This system is based on an RNA-guided adaptive defense mechanism which is naturally present in many bacteria and archaea.[6] Today, it has been widely adapted in different research

fields and has been applied for genetic engineering in various organisms. Several CRISPR-associated proteins (Cas) have been described but Cas9 is the most studied and well-characterized thus far. Cas9 belongs to the class 2 type II CRISPR system as it has a single large effector protein.[7] To realize a functional CRISPR-Cas system, expression of Cas9 needs to be accompanied by gRNA which consists of crRNA-tracrRNA and contains a spacer sequence homologous to the target region. A shorter and synthetic crRNA-tracrRNA, called single guide RNA (sgRNA), is commonly used today. The gRNA acts as a guide to lead the Cas9 to the targeted sequence and Cas9 itself is an endonuclease which recognizes the corresponding protospacer adjacent motive (PAM) sequence of 5′-NGG downstream of the spacer. Upon binding to the target region, Cas9 will perform the cleavage thus generating the dsDNA break. This ds-break will trigger the native DNA repair system which, depending on the organisms, could be realized by homology-directed repair (HDR), non-homologous end joining, or alternative end-joining repair mechanisms.[8–10] Versatility of the CRISPR-Cas system makes it very appealing for different purposes from genome editing to gene regulation.

In 2017, the implementation of a CRISPR-based system to facilitate efficient genome editing in *P. polymyxa* has been reported for the first time.[11] The single vector-based system was used to mediate single gene deletions as well as the deletion of a genomic region of 18 kb by utilizing two targeting sgRNAs. Furthermore, a system for single and multiple gene regulation has been developed by employing the DNase-inactive variant of an optimized Cas12a (dCas12a) from *Acidaminococcus sp*.[12] In addition, Kim *et al*.[13] have recently developed a multiplex base editing tool for *P. polymyxa* by fusing dCas9 with cytidine deaminase. However, these base editing tool works on a certain editing window and could only mediate C to T substitutions. No studies have reported multiplex gene deletions or integrations in *P. polymyxa* thus far. As modification of various genes are often needed for metabolic engineering and strain engineering purposes, the possibility to perform targeted multiple gene editing will definitely speed up the process and reduce laborious cloning and screening efforts. In this study, we scope on the exploitation of multiplex genome editing to realize simultaneous gene deletions and integrations in *P. polymyxa*. The genes and clusters which are essential for EPS production were selected as suitable targets to allow eased screening. In addition, we demonstrated the functionality of a single sgRNA-guided CRISPR-Cas9 approach to facilitate large cluster deletion which will also be important to realize minimized genome variants towards the development of a robust and versatile chassis organism.

Results and Discussion

As target for the cluster deletion we have chosen the EPS cluster which is responsible for the biosynthesis of paenan, the glucose-derived EPS produced by *P. polymyxa*. The region is 32.8 kb in size and comprises 28 genes which support the EPS biosynthesis such as sugar precursor genes, glycosyltransferases, polymerases, and export machineries (Figure 1A). Deletion of this cluster will lead to diminishment of paenan biosynthesis and therefore the mutants will not show a slimy phenotype when grown on EPS-inducing plates with glucose as carbon source. The pCasPP plasmid was used as base to realize the different genomic modifications.[11] In brief, this plasmid contains the Cas9 from *Streptococcus pyogenes* which is codon-optimized for expression in *Streptomyces*. The Cas9 is under *sgsE* constitutive promoter from *Geobacillus stearothermophilus* while the sgRNA is controlled by the *gapdh* promoter from *Eggerthella lenta*.[14,15] Homologous flanks of ~1 kb from upstream and downstream regions of the cluster were provided as the template for HDR. The spacer sequence was 20-nt upstream of 5’-NGG PAM sites. Two sgRNAs were tested to realize the cluster knockout by targeting different positions. Only one of the sgRNAs was used for each approach, targeting either the *pepC* or *pepQ* gene (Figure 1B). Both genes encode for initial

glycosyltransferases and are located at different position in the cluster. The *pepC* is located quite in the beginning of 5' region with a distance of 2.5 kb from the upstream and 30.3 kb from the downstream sequence, which were used as homologous flanks. Whereas, *pepQ* is 24 kb and 8.8 kb away from the 5' end and 3' end homologous flanks, respectively. Interestingly, the cluster deletion could be achieved when *pepQ* was targeted what was not the case if *pepC was targeted.* In average, ~100 exconjugants were obtained from *pepQ* targeting while no colonies were obtained by targeting *pepC*. In total, 38 colonies of the *pepQ* targeting approach were screened and showed a remarkable editing efficiency of 97 % as confirmed by the plate-based assay, PCR amplification, and fragment sequencing (Figure 1B). Colonies for which the EPS-cluster was deleted resulted in a 3 kb fragment when screened with primers binding outside of the homologous flanks. No amplicon was obtained for the wild type in which the 32.8 kb cluster is still present. As expected, no slimy appearance was observed for the mutants on EPS-glucose plates (Figure 1C). This result indicates that there is a correlation between the distance of the cut site to the homologous flanks concerning the editing success. In the case of targeting *pepC*, distance between the cut site and downstream homologous flanks might be too far away for a successful homologous recombination. Previous studies have demonstrated large cluster deletion in bacteria by means of CRISPR-based systems. Commonly, dual targeting system with two sgRNAs are used. Each of the sgRNA targets the beginning and the end of the cluster, allowing a close proximity between the cut site and the homologous flanks.[16] A study by So *et al*.[17] showed that two sgRNAs were required to delete an 38 kb operon in *Bacillus subtilis* as utilization of one sgRNA failed to do so. Studies by using only one sgRNA-guided large cluster deletion in bacteria are scarce. Recently, it was reported that a chromosomally integrated Cas12a was able to facilitate large cluster deletion in *B. subtilis* by use of only one sgRNA.[18] Here, we demonstrated highly efficient Cas9-mediated large cluster deletion by using a one plasmid system with only one sgRNA, which has to be chosen carefully within the targeted sequence. In addition to the distance between cut site and homologous flanks, accessibility of the targeted region as well as the three-dimensional structure of the genome might also need to be considered especially when aiming for large genomic region deletions. Overall, the system described in this study allows simple cloning and transformation methods without the needs of preliminary engineering of the host strain, as it is not necessary to integrate the *cas*-gene.

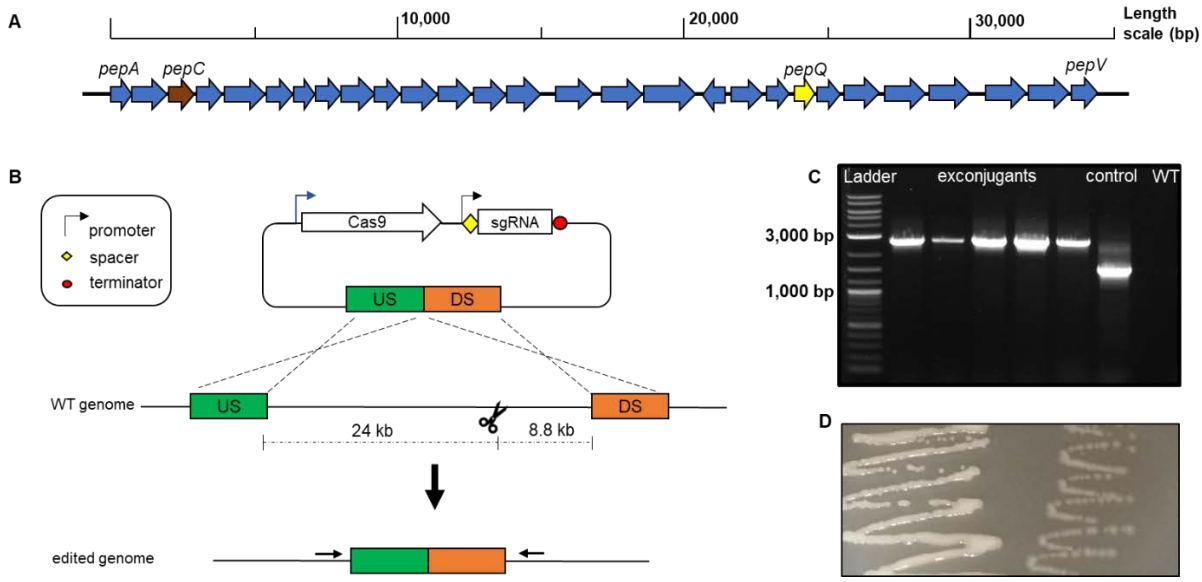

Figure 1. CRISPR-Cas9-mediated large cluster deletion. (A) EPS cluster responsible for paenan biosynthesis in *P. polymyxa*. The targeted genes *pepC* and *pepQ* are highlighted. (B) Schematic overview for cluster deletion using one sgRNA targeting *pepQ*. Approximately 1 kb upstream and downstream homologous flanks were provided as

repair template. Primers which bind outside of the homologous flanks were used to screen for the cluster deletion. (C) PCR screening from randomly picked exconjugants, wild type (WT), and a positive control of the colony PCR by amplification of the 16S rRNA (control). The DNA ladder used was 1 kb Plus DNA Ladder from NEB. (D) Growth of WT (left) and mutant strains (right) on EPS-glucose plate.

Next to Cas9, Cas12a is one of the most studied Cas proteins and has been gaining interests in recent years. One of the most attractive features of Cas12a is its simplicity to facilitate multiplexing as it has also RNase activity in addition to DNase, which turns it to be able to process the maturation of the crRNA itself.[19] Therefore, it would be intriguing to investigate the ability of Cas12a to facilitate multiplex gene editing in *P. polymyxa*. For this, we constructed the pCREDIT plasmid by activating the dCas12a of the pCRai plasmid, which has been shown in previous studies to be functional in facilitating multiplex gene regulation in *P. polymyxa*.[12] The activation was done by introduction the A908D mutation in the dCas12a protein. The Cas12a used in pCRai is an engineered variant of *Acidaminococcus sp*. with several mutations for improved activity.[20] However, after several trials and fine-tuning we were not able to obtain the desired knockout mutants by using the activated Cas12a construct (data not shown). It is still not clear why the deletion could not be achieved and further investigations are currently ongoing. By that, we decided to investigate the ability of Cas9 to facilitate multiplex gene editing instead of using the Cas12a. Different strategies can be employed to realize multiplexing, either by using single sgRNA expression cassettes or polycistronic sgRNAs. In the latter case, additional approach is needed to allow the processing of the gRNAs for example by co-expression of Csy4 or fusing the gRNA with tRNA sequences.[21]

For investigation of the multiplexing efficiency, *pepC* and *sacB* were selected as target genes (Table 1). Both are essential genes of the different EPS biosynthesis pathways of *P. polymyxa*. The *pepC* gene encodes for initial glycosyltransferase required for paenan production. On the other hand, *sacB* encodes for a levansucrase which represents the single enzyme required for biosynthesis of levan, a sucrose-derived fructanosyl polymer.[22] Disruption of these two genes will lead to a complete non-EPS producing strain of *P. polymyxa*. In this study, we used the one plasmid system containing Cas9 and two sgRNA expression cassettes, each of them containing a promoter, the sgRNA, and a terminator (Figure 2A).

Table 1. Overview of the targeted genes and the cluster investigated in this study.

Target(s)	Size (bp)	Multiplexing	Purpose
pepC	699	No	Gene deletion
sacB	1,500	No	Gene deletion
EPS cluster	32,846	No	Cluster deletion
pepC and *sacB*	699 and 1,500	Yes	Multiplex gene deletion
pepC and *sacB* replacement with *mRFP*	773 (*mRFP*)	Yes	Multiplex gene integration

When targeting a single gene, the number of conjugants was 100-fold lower compared to the non-targeting plasmid (Figure 2B). Typically, around 200 to 300 colonies were obtained for single gene knockouts, although this number seems to vary for different target genes. Deletion of the large cluster resulted in a moderate decrease of the number of exconjugants. Overall, the results demonstrate that the system shows an editing efficiency of nearly 100 % for single gene or large cluster deletions (Figure 2C and S1). When targeting the simultaneous deletion of *pepC* and *sacB*, the number of exconjugants dropped significantly by more than 30-fold compared to the single gene deletions. As expected, deletion of *pepC* leads to no EPS production on EPS-glucose plates. Additional deletion of *sacB* leads to no EPS production on EPS-sucrose plates which indicated successful double knockout mutants. Furthermore, the colonies were checked with PCR and sequencing to prove the desired deletions. The results

obtained from PCR and sequencing perfectly matched the plate-based screening, which showed the reliability of the screening method. Despite the significant decrease in the number of colonies, the editing efficiency of simultaneous genes deletion remained high with around 78 %. However, some escapers were observed, either with only one or none of the targeted gene deletions. Among the escapers which showed only one of the targeted gene deletions, no preference could be observed for either *pepC* or *sacB* deletion.

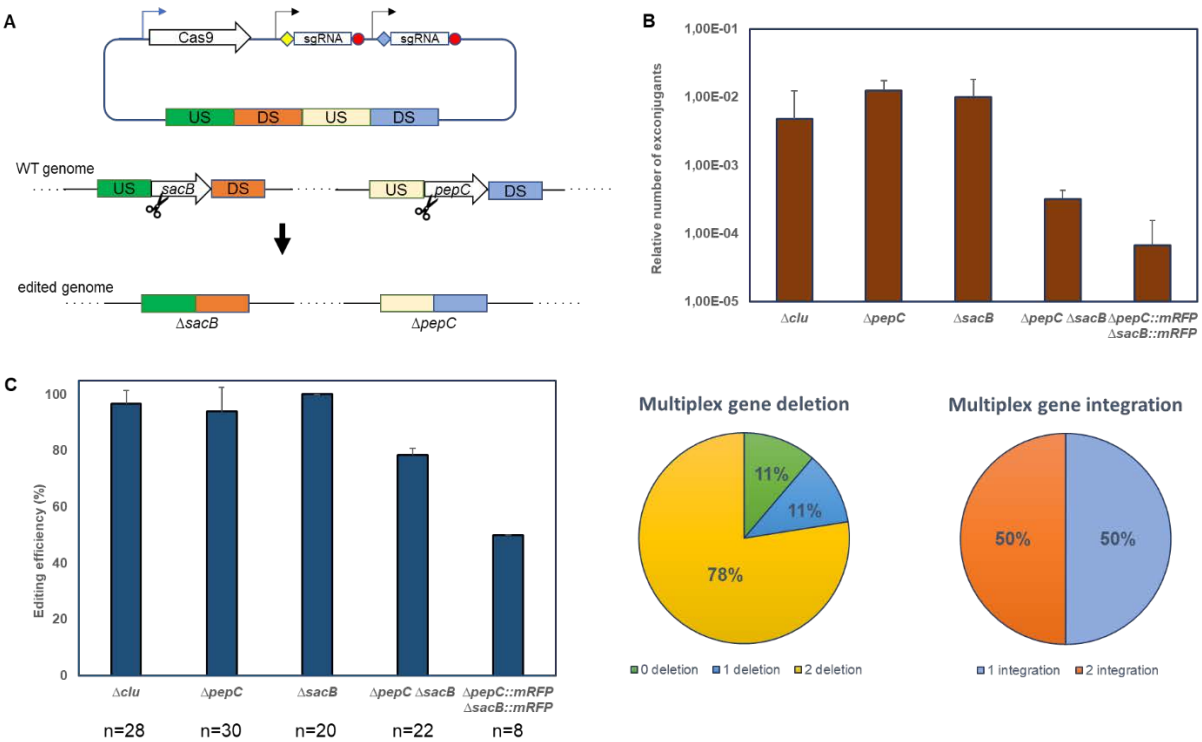

Figure 2. CRISPR-Cas9-based multiplex genome editing. (A) Schematic overview of the multiplex gene deletion approach. Two sgRNAs cassettes were used which targeted *pepC* and *sacB*. For multiplex gene integration, *mRFP* was placed in between the homologous flanks of each gene. (B) The number of exconjugants from the different experiments relative to the exconjugants from the non-targeting plasmid. (C) Editing efficiency for single gene deletion, large cluster deletion, multiplex deletions as well as integrations. Total number of exconjugants screened are indicated (n).

In addition to multiplex gene deletions, we also exploited the system to investigate whether multiplex gene integrations could be achieved. For this, *pepC* and *sacB* were targeted by using the same sgRNAs cassettes as used for the deletion experiments. The *mRFP* was placed in between the homologous flanks of each targeted gene and represented the repair template for the HDR. By that approach the efficiency decreased significantly, and finally, 8 colonies were obtained and screened, for which in total 4 colonies showed the correct integration of the *mRFP* in the targeted positions of *pepC* and *sacB*, as confirmed by plate-assay, PCR, and sequencing (Figure S2). The other 4 colonies only showed that *mRFP* replaced *sacB* while *pepC* was still intact at the original location. Interestingly, the *mRFP* was not functionally expressed despite the sequencing confirmed that it was successfully integrated in the desired positions. No red coloration of the colonies nor a fluorescence signal was observed in *P. polymyxa* although the coloration was readily visible in *E. coli* Turbo and *E. coli* S17-1. It remains unclear why the *mRFP* was not expressed in *P. polymyxa* and therefore further investigations are necessary, for example, by optimization of the promoter and RBS sequence. Nevertheless, simultaneous gene integrations were confirmed with an editing efficiency of 50 %. As the number of exconjugants was very low, optimization of transformation efficiency might be able to improve the overall system described in this study.[23] Previous studies have

reported CRISPR-Cas-based multiplex genetic engineering in different microorganisms.[24–26] However, only a few successfully demonstrated multiplex gene integrations, which was mainly performed in yeast.[27,28] While multiplex gene insertion has been reported in *E. coli*,[29] to the best of our knowledge, our study is the first to utilize a Cas9-based system to achieve multiplex gene integrations in Gram-positive bacteria.

The use of CRISPR-Cas-based tools has greatly accelerated genetic engineering approaches. In this study, we demonstrated the utilization of our Cas9-based system for multiple purposes in *P. polymyxa*. The system showed high efficiency and thus is expected to be a substantial genetic tool in strain development, especially when aiming for iterative genome editing. The ability to perform large cluster knockouts and simultaneous modifications would even speed up the process in designing the desired strain variants. With our findings, we anticipate a wider utilization of our Cas9-based system for future research in *P. polymyxa* and related species.

Methods

Strains and cultivation

P. polymyxa DSM 365 was obtained from the German Collection of Microorganisms and Cell Culture (DSMZ, Germany). *E. coli* Turbo (New England Biolabs) was used for plasmid cloning and storage. *E. coli* S17-1 was used to facilitate the conjugation of *P. polymyxa*. The strains were cultivated in LB media (10 g/L peptone, 5 g/L yeast extract, 5 g/L NaCl). For plate media, 1.5 % agar was added. If necessary, the media was supplemented with 50 µg/mL neomycin and/or 40 µg/mL polymyxin. *P. polymyxa* was cultivated at 30 °C and *E. coli* strains at 37 °C, unless stated otherwise. Liquid cultures were prepared in 3 mL media in 13 mL tubes and incubated at 250 rpm. To check for EPS formation, *P. polymyxa* was grown on EPS-inducing plates containing 30 g/L glucose or 30 g/L sucrose, 5 g/L peptone, 1.33 g/L $MgSO_4.7H_2O$, 0.05 g/L $CaCl_2$, 1.67 g/L KH_2PO_4, 2 mL/L RPMI 1640 vitamin solution (Sigma-Aldrich), and 1 mL/L trace elements (2.5 g/L $FeSO_4.7H_2O$, 2.1 g/L $C_4H_4Na_2O_6.2H_2O$, 1.8 g/L $MnCl_2.4H_2O$, 0.075 g/L $CoCl_2.6H_2O$, 0.031 g/L $CuSO_4.7H_2O$, 0.258 g/L H_3BO_3, 0.023 g/L Na_2MoO_4, 0.021 g/L $ZnCl_2$).[11] For longer storage, the strains were stored in 24 % glycerol and kept at -80 °C. All strains used in this study are listed in Table S1.

Plasmid construction

For cloning purposes, PCR fragments were amplified using Accuzyme polymerase (Bioline). Gel purification was performed by using the Monarch Gel Purification Kit (NEB). Isolation of genomic DNA (gDNA) was performed by use of the DNeasy Blood & Tissue Kit (Qiagen). The pCasPP plasmid was used as vector for the different experiments in this study.[11] The plasmid itself is 9.7 kb in size, containing Cas9 of *S. pyogenes* which was codon optimized for *Streptomyces* as well as sgRNA expression cassette.[14] In addition, pCasPP also has the elements for replication in *E. coli* and *P. polymyxa* as well as origin of transfer for conjugation purpose. All the plasmids were assembled by isothermal assembly. Linear fragments were obtained from PCR amplifications using primers with suitable overlapping sequences. The spacer sequences were identified within the targeted genes and located directly upstream of the 5'-NGG PAM sites. In this study, 20-nt spacer sequences were used. Approximately 1 kb homologous flanks upstream and downstream of the target region were amplified from *P. polymyxa* gDNA and provided for the HDR template. For integration purpose, the *mRFP* was amplified from the pLux01 plasmid, which was a gift from Tom Ellis (Addgene plasmid #78281),[30] and placed in between the homologous flanks. Finally, all fragments were brought together in the isothermal assembly master mix and incubated at 50 °C for 1 hour followed by transformation of *E. coli* Turbo via the heat shock method. Transformed cells were plated on

LB agar containing 50 µg/mL of neomycin. The transformants were initially screened with colony PCR using GoTaq polymerase (Promega) then plasmid isolation was performed by using the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific). Subsequently, the plasmid was checked by sequencing to confirm that the correct plasmid was assembled. Upon confirmation, *E. coli* S17-1 was transformed with the plasmid by using the heat shock method. All primers synthesis and sequencing analysis were performed by Microsynth AG (Switzerland). In silico primer design, cloning, and sequence alignment were performed using SnapGene software. The plasmids, oligonucleotides, and spacer sequences used in this study are listed in Table S1-S3.

Conjugation

P. polymyxa transformation was performed via conjugation by use of *E. coli* S17-1 as the donor strain. Single colonies of the strains were inoculated in 3 mL LB media, with or without 50 µg/mL of neomycin, and cultivated overnight. The overnight culture was diluted 1:100 in fresh media by following cultivation for 4 h at 37 °C and 250 rpm. Subsequently, 900 µL of *P. polymyxa* culture was incubated at 42 °C for 15 min then mixed with 300 µl of the *E. coli* S17-1 harboring the plasmid of interest. The mixture was centrifuged at 8,000 rpm for 3 min and the pellet was resuspended in 100 µL of fresh LB media. The solution was dropped on a LB plate and incubated overnight at 30 °C. Afterwards, the culture was scrapped off the plate and resuspended in 500 µL of fresh LB media, Subsequently, 100 µL of the culture was plated on LB plates containing 50 µg/mL of neomycin and 40 µg/mL of polymyxin followed by incubation for 48 h at 30 °C. All experiments were performed in three biological replicates.

Screening of *P. polymyxa* exconjugants

Screening of the exconjugants was performed by plate-based assays as well as PCR and sequencing. First, the colonies were streaked on EPS-inducing media to check for the loss of slimy phenotype which indicated elimination of EPS formation. An EPS-glucose plate was used for initial screening of the EPS cluster and *pepC* knockout. In addition, an EPS-sucrose plate was used to screen for the Δ*sacB* Δ*pepC* double knockout mutants. The colonies were incubated at 30 °C overnight. Furthermore, colonies were checked via colony PCR to verify the accuracy of the plate-based assay. Colony PCR was performed using external primers which bind outside of the homologous flanks provided as repair template. The resulting fragment was purified from the gel and verified by sequencing to proof the correct modifications. Finally, gDNA was isolated from some of the colonies to reconfirm the colony PCR results.

Associated Content

Supporting Information

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Author Contributions

MM and JS designed the experiments and wrote the manuscript. MM and CT performed the experiments and analyzed the data. All authors read and approved the final manuscript.

Notes

The authors declare no competing financial interests.

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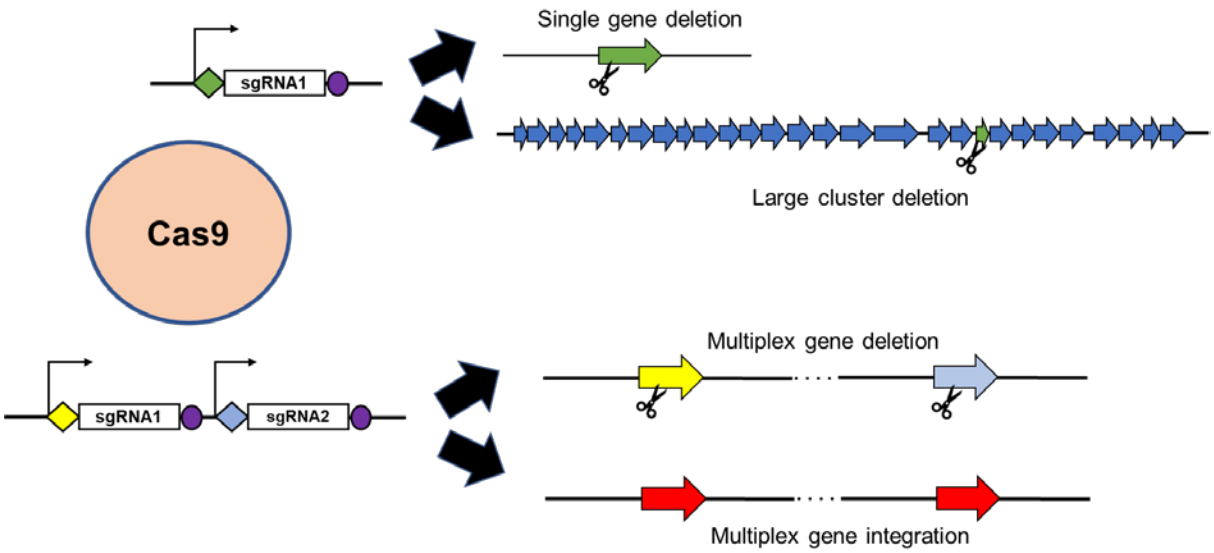

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