J. Microbiol. Biotechnol. (2017), 27(2), 335–341
https://doi.org/10.4014/jmb.1608.08019

jmb

High-Frequency Targeted Mutagenesis in *Pseudomonas stutzeri* Using a Vector-Free Allele-Exchange Protocol S

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Received: August 9, 2016
Revised: October 20, 2016
Accepted: October 28, 2016

First published online
November 4, 2016

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Supplementary data for this paper are available on-line only at http://jmb.or.kr.

pISSN 1017-7825, eISSN 1738-8872

The complexity of the bacterial recombination system is a barrier for the construction of bacterial mutants for the further functional investigation of specific genes. Several protocols have been developed to inactivate genes from the genus *Pseudomonas*. Those protocols are complicated and time-consuming and mostly do not enable easy construction of multiple knock-ins/outs. The current study describes a single and double crossover-recombination system using an optimized vector-free allele-exchange protocol for gene disruption and gene replacement in a single species of the family Pseudomonadaceae. The protocol is based on self-ligation (circularization) for the DNA cassette which has been obtained by overlapping polymerase chain reaction (Fusion-PCR), and carries an antibiotic resistance cassette flanked by homologous internal regions of the target locus. To establish the reproducibility of the approach, three different chromosomal genes (*ncRNA31*, *rpoN*, *rpoS*) were knocked-out from the root-associative bacterium *Pseudomonas stutzeri* A1501. The results showed that the *P. stutzeri* A1501 mutants, which are free of any plasmid backbone, could be obtained via a single or double crossover recombination. In order to optimize this protocol, three key factors that were found to have great effect on the efficiency of the homologous recombination were further investigated. Moreover, the modified protocol does not require further cloning steps, and it enables the construction of multiple gene knock-in/out mutants sequentially. This work provides a simple and rapid mutagenesis strategy for genome editing in *P. stutzeri*, which may also be applicable for other gram-negative bacteria.

Keywords: Vector-free allele-exchange (VFAE), homologous recombination, *Pseudomonas stutzeri*, mutagensis, genome editing

Introduction

Pseudomonas is a gram-negative member of the bacterial family Pseudomonadaceae; it comprises 191 validly described species widely distributed in various environments, including water, soil, and within various living host organisms [1–3]. Some representatives, such as *Pseudomonas aeruginosa*, represent some of the most prevalent causes of opportunistic infections in humans [4]. Others, such as *Pseudomonas fluorescens*, comprise physiologically diverse species that contribute greatly to the turnover of organic matter [5]. One well-studied strain of the genus *Pseudomonas* is *P. stutzeri*, which is a rod-shaped, motile, single polar-flagellated soil bacterium that is increasingly recognized as a member of the nitrogen fixative group [6]. Functional investigation of *P. stutzeri* A1501 opens up new perspectives in the study of nitrogen fixation processes [7–9].

One of the most useful ways of furthering our understanding of the genetics and molecular mechanisms of specific metabolic pathways is the creation of artificial mutations. Several methods have been available to generate such knock-in/out mutations in bacteria; all are based on the homologous recombination principle, including recombineering and CRISPR-Cas9, which are the most recent approaches in that area [10–18]. However, the method most commonly used to generate gene replacement in gram-negative bacteria is two-step homologous recombination [19–21]. The first step involves the integration of a plasmid into the chromosome

by single crossover recombination (diploid), and the second step involves applying stress to splice the plasmid backbone and create a double crossover event (deletion mutant) [22]. Generally, the prerequisite for two-step homologous recombination is a resistance cassette flanked by homologous fragments taken from the target gene and cloned into a non-replicative plasmid vector [22–25]. The non-replicative plasmid, which is called a suicide vector, contains a replicon for the intermediate-host cell but not for the target host cell. Experimentally, it is known that suicide vectors have low-frequency integration into the chromosome, which affects the selection process of identifying gene replacement candidates. Moreover, the mentioned gene replacement methodologies require several subcloning steps [25].

The method described in the present study is a vectorless homologous recombination approach based on electroporation of a recipient A1501 strain with a circularized PCR fragment carrying an antibiotic resistance cassette flanked by homologous fragments taken from the target locus. The present protocol rapidly inactivates selected chromosomal gene(s) and does not require cloning steps, unlike the suicide vector pK18mob-*sacB*, which is one example frequently used with multiple strains of *Pseudomonas* and other bacterial species [26–33]. Moreover, the mutants grow directly on agar plates containing appropriate antibiotics and are confirmed by a simple PCR assay and sequencing.

The current protocol has been evaluated through several trials to inactivate three chromosomal loci in A1501 (*ncRNA31*, *rpoN*, *rpoS*). Three main factors appeared to control the current protocol, including DNA cassette construction, DNA concentration, and homology length. These three factors were studied extensively to identify the optimum conditions. The result comprised vector-free and high-frequency specific gene mutations in A1501 compared with other gene replacement and disruption methods.

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions

The bacterial strains and plasmids used in this study are listed in Table S1. The A1501 strain was grown in Luria-Bertani (LB) medium. When necessary, kanamycin (50 μg/ml) or gentamicin (20 μg/ml) was added to the growth medium. All cultures were incubated at 30°C, 200 rpm. The mutants were left to grow for 48 h.

DNA Manipulations

Three forms of DNA cassettes (as illustrated in Fig. S1) were used in the current study, which included a linear DNA cassette flanked with homologous fragments (Fig. S1A) and two circularized PCR products with single or double flanks (Figs. S1B and S1C).

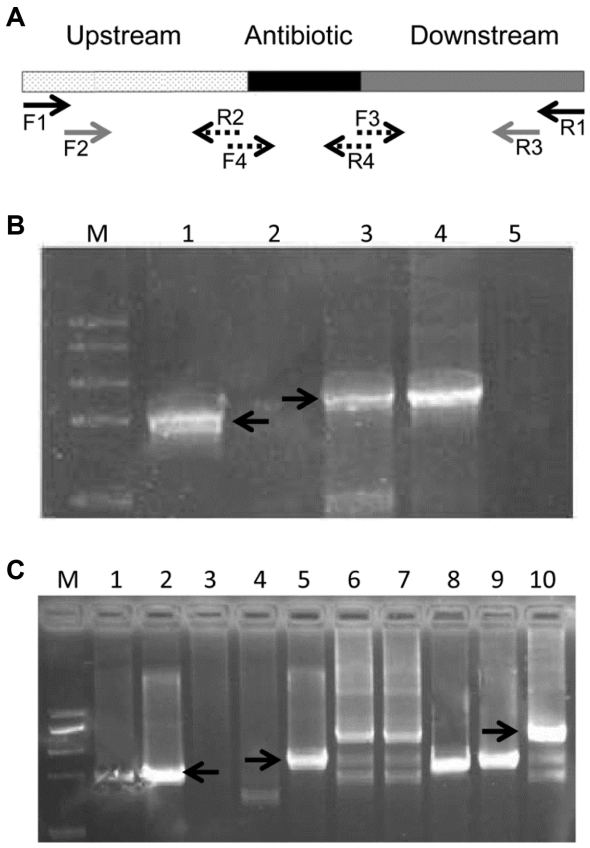

Fig. 1. Primer locations and validation of the *rpoS* and *rpoN* mutants.

(**A**) Location of the primers used in the current study on the target locus; F1 and R1, primer pairs used for sequencing; F2 and R3 are 5′ end phosphorylated primer pairs; R2, F3, F4, and R4 are overlapped primer pairs. (**B**) Screening of the *rpoS* mutant; lane 1, arrow shows the wild-type pattern; lane 2, arrow shows the mutant band pattern. (**C**) *rpoN* band pattern; lane 2, arrow shows the wild type; lane 5 shows the deletion mutant; lane 10 shows the insertion mutant (diploid).

Genomic and plasmid isolation were performed with a Tiangen Bacterial Genomic Purification Kit (Tiangen, China) and a Plasmid Miniprep DNA Purification System (Tiangen), respectively. Standard PCR amplifications were performed with Prime-Star DNA polymerase (Takara, China). Primer locations are shown in Fig. 1A. All cassette constructions and oligonucleotides used in the study are listed in Tables S2–S4. PCR products were purified with a PCR-Purification Kit (Tiangen). The obtained overlapped PCR products were sequenced by the BGI Tech sequencing service (China). The optimization parameters of the current protocol include DNA concentrations and homologous length.

Three genomic locations from A1501 (*ncRNA31*, *rpoN*, *rpoS*) were selected for cassette construction using overlapping PCR with one of either two resistance genes for kanamycin (*nptII*) or gentamicin (*aacC1*). The experimental conditions were optimized first using ncRNA31's locus as a model with the kanamycin

resistance gene *nptII*. Amplified fragments ranging from 30 to 600 bp were taken from ncRNA31's location of the A1501 genome using the primer pairs shown in Tables S3 and S4; they were then overlapped with *nptII* to construct 10 DNA cassettes, as shown in Table S2. The PCR product was adjusted to different concentrations along with ligation and electroporation, as shown in Table S5. Self-ligation was performed using T4 ligase NEB overnight (self-circularization), and the circularized product DNA cassette was electroporated into A1501. Mutants were selected on kanamycin-containing plates.

Electroporation, Grouped Colonies, and Colony PCR

The electrocompetent cells of *P. stutzeri* A1501 were made through a single bacterial colony taken from a fresh LB plate and grown overnight (14–16 h) in 40 ml of LB broth. Cultures were grown at an OD_{600} of 1.9–2.0. Cells were pelleted by centrifugation at 6,000 ×*g* for 8 min and then washed 2–3 times with 300 mM sucrose and finally resuspended in 1 ml of the same solution. A 200 µl aliquot of the cell suspension was mixed with the recombinant DNA (up to 20 µl). The mixture was placed in a pre-chilled sterile electroporation cuvette (2 mm electrode gap; Bio-Rad, USA) and immediately pulsed by a Bio-Rad Gene Pulser (2.5 kV, 200 W, and 25 µF). The mixture was incubated at 30°C for 1 h with 1 ml of LB broth. Cells were spread on LB agar containing the appropriate antibiotics and incubated at 30°C.

Colonies resistant to the antibiotic were grouped into five colonies per tube to carry out the PCR, followed by colony PCR for each colony within the selected group to find the desired mutant (shown in Fig. S2A). Each gene deletion or disruption was confirmed by sequencing using primers S2F20 and S2R20 in addition to S2F24 and S2R24, as shown in Fig. 1A and Table S3.

Double and Triple Mutagenesis

Double knock-in/out was performed using two approaches; first by gathering and the electroporation of two or three circularized DNA cassettes flanked by two homologous fragments taken from the *ncRNA31* and *rpoN* genes. The second approach was through sequential electroporation of the two DNA cassettes, one by one, into the regenerated bacterial strain.

Mutant Stability Assays

Five colonies, which had been confirmed to be deletion mutants of *ncRNA31*, were purified via screening three times on selective plates. The colonies were then cultured in 5 ml of LB broth without kanamycin and then incubated at 30°C. During the following 10 days, 100 µl of each culture was diluted in 5 ml of fresh medium and incubated for 24 h. On days 1, 5, and 10, all cultures were diluted 100-fold and plated on selective and non-selective plates to determine the frequency of cell viability in regard to the percentage of kanamycin-susceptible colonies.

Transcription Analyses

A bacterial culture was grown overnight in LB broth supplemented with kanamycin at 30°C. RNA isolation was performed with the RNAeasy Bacterial Mini Kit (Qiagen, Germany), followed by RT-PCR performed with the First Strand cDNA Transcription Synthesis Kit (PrimeScript RT; Takara). The primers used in the first strand synthesis and the PCR amplification are listed in Table S3. The PCR product was visualized in a 1.0% agarose gel.

Motility Assay

A swimming assay as a phenotypic analysis was performed for the *rpoN* mutants. The preparation of semisolid "swimming" agar plates was carried out as previously described [34].

K-minimal medium was supported with 0.3% Bacto agar (BM; Becton) dissolved in distilled water (DW). The solution was then filled into Petri dishes and dried under laminar flow. The semisolid agar plates were used on the same day that they were prepared. For the motility tests, overnight cultures of the different strains were diluted in fresh LB broth and incubated for additional 2 h at 30°C. Ten microliters of this bacterial suspension was inoculated in the middle of a semisolid agar plate and incubated at 30°C for 72 h.

Salt Stress Sensitivity Assay

The intrinsic resistance of the bacterial isolates against salinity was evaluated by observing growth on LB medium just after exposing several dilutions of the cells (10^{-1}:10^{-4}) to a final concentration of 1.3 M NaCl for 1 h. A control plate was also maintained in LB medium for the same duration. The plates were incubated for 48–72 h at 28 ± 2°C.

Statistical Analysis

T-tests were applied to find the significance between the numbers of colonies that appeared on the selective and non-selective plates of the stability test. The test was performed using GraphPad Prism ver. 5.00 for Windows (GraphPad Software, USA).

Results

DNA Cassette Construction

Three strategies were used to find out the simplest and fastest method to edit the A1501 genome. The first strategy was by linear DNA cassettes containing the kanamycin resistance gene *nptII* flanked by homologous fragments taken from *rpoS*, as shown in Fig. S1A and Table S3. Most *Pseudomonas* species were genome edited using suicide vectors to be integrative with the genome as a non-replicated plasmid, so based on the same principle, the second strategy involved circularizing a kanamycin DNA cassette flanked by just one homologous fragment taken from the *ncRNA31* locus, with different lengths (as shown in Fig. S1B, and Tables S3 and S4). In the third attempt, self-ligation was performed to circularize a DNA cassette containing *nptII* flanked by two homologous fragments with different lengths

and orientations taken from in- and outside *ncRNA31*, as shown in Fig. S1C. The only difference between the suicide vector and the second and third strategy is that the current protocol is done without using a vector backbone. The optimum experimental conditions were later replicated with *rpoN* and *rpoS*. All PCR products were visualized and confirmed using agarose gel electrophoresis and then sequencing.

Optimum Conditions

A linear DNA cassette and a circular cassette with one-side flanking homology failed to achieve recombination. No colonies appeared on the kanamycin plates related to the latter two strategies. The third strategy achieved single and double crossover recombination in specific and non-specific locations within 48–72 h for cassettes with > 200 bp flanking homology. As shown in Fig. 2, the smaller homologous fragments showed no colonies resistant to the applied antibiotic; in contrast, the longer homologous fragments showed direct contact with a number of resistant colonies. An impure DNA cassette (overlapping-PCR product with less purity) achieved a very low frequency of single and double crossovers. Among the five concentrations that were tested for the DNA cassettes, 300–500 ng/μl achieved the highest rate of colonies with correct single and double crossovers (shown in Figs. S3 and S4, and Table S6). Concentrations outside the range of 300–500 ng electroporated circularized DNA resulted in random mutants that were able to grow rapidly on the antibiotic-supplemented plates while showing false-positive clones that carry the incorrect insertion regarding the target loci.

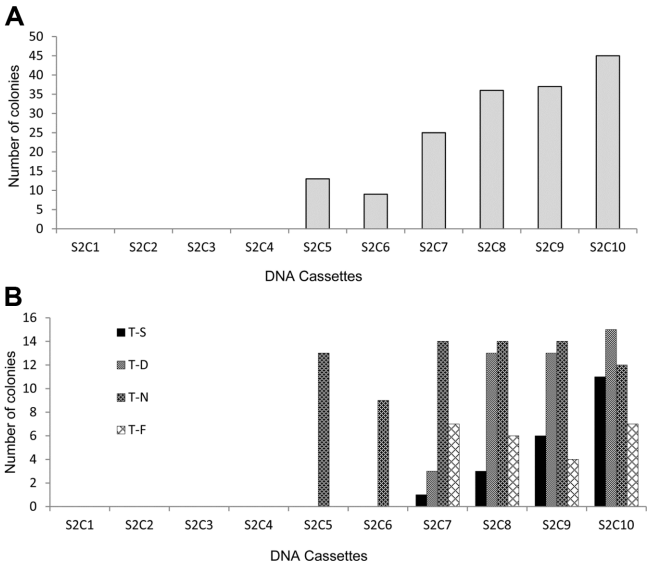

Fig. 2. Impact of different homolog fragment sizes on the number and pattern of the bacterial colonies.
(**A**) Impact of the size of homology taken from the target locus *ncRNA31* (represented in each DNA cassette S2C1-S2C10) on the number of colonies appearing. (**B**) Curve showing the total colony PCR patterns for each DNA cassette, which demonstrates the impact of the size of homology taken from the target locus *ncRNA31* (represented in each DNA cassette S2C1–S2C10) on the colony type. T-S, Single crossover mutant. T-D, Double crossover mutant. T-N, Wild-type pattern. T-F, Failed PCR.

Single, Double, and Triple Mutagenesis

All three gene locations selected (*ncRNA31*, *rpoN*, *rpoS*) were successfully mutated as single mutants using the third-mentioned strategy. Double and triple mutants were obtained via two procedures; first by mixing two or three circularized cassettes with different antibiotics and electroporating into the same competent cells. The frequency of colonies appearing resistant to the two antibiotics was too low and mostly nonspecific. In regard to the triple mutagenesis, no colonies appeared. The second procedure used was by sequentially mutating the cells by two or three individual single mutations, separated by screening and recultivating each of the obtained mutants. These results suggest that sequential mutagenesis is the way to achieve double or multiple mutations.

Mutant Stability Assays

The difference between the numbers of colonies that appeared on the selective and non-selective plates was non-significant, as demonstrated with a *p*-value of 0.8, confirming the fact that the deletion mutants were stable.

Mutant Phenotype and Transcriptional Analyses

Transcription analysis showed no result regarding the expression of the three genes in the deletion mutagenesis. As shown in Fig. 3A, the *rpoN* mutant phenotype data on plates showed a lack of swimming in comparison with the wild type. Defective swimming ability is considered to be a general bacterial phenotype for *rpoN* (Sigma 54) deletion mutants, as this result has already been reported previously with other bacterial species [35–37]. The bacterial *rpoS* gene encodes the general stress response sigma factor [38]. The *rpoS* negative strain showed sensitivity to salt stress in comparison with the wild type (as shown in Fig. 3B). The same stress-response phenotype has been detected with other *Pseudomonas* species in regard to the deletion of *rpoS* [39].

False-Positive Clones and Grouped-Colony PCR

As a matter of fact, allele-exchange methodologies produce

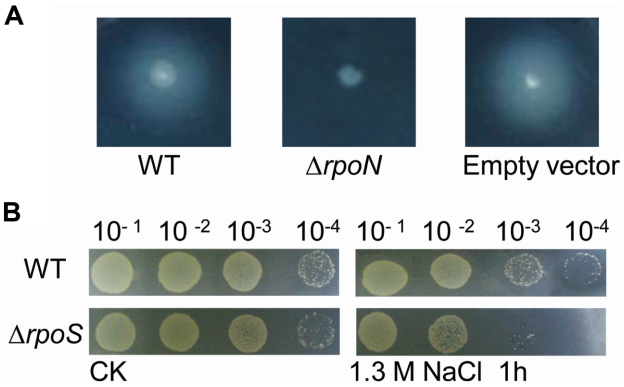

Fig. 3. Phenotypic data of the A1501 mutants.
(**A**) *rpoN* (swimming pattern). (**B**) *rpoS* (sensitivity to salt stress).

numerous false-positive clones that have the antibiotic resistance but the insertion is not in the target locus. Thus, an approach was used to avoid the massive screening among colonies searching for the correct deletion mutant, as shown in Fig. S2A. Each colony that appeared on the plate was diluted in a PCR tube with 50 µl of DW, followed by collecting 1 µl from every five tubes to form a group of mixed colonies in one new PCR tube. Then, 0.5 µl was taken from the final grouped-colonies tube to represent a group of mixed templates in a PCR, resulting in a band pattern that determines the location of the correct deletion mutant, as shown in Fig. S2B.

Discussion

Genome editing via homologous recombination, with special regard to the double crossover, is a rare event in gram-negative bacteria like *Pseudomonas* species. Several protocols were developed to enhance such double crossover events, such as CRISPR-Cas9, suicide vectors, and recombineering. However, most of the previous protocols require a plasmid vector backbone in addition to several cloning steps.

In the current study, a simple vectorless protocol was developed to edit the genome of one of the *Pseudomonas* species (*P. stutzeri* A1501). To find the simplest protocol for homologous recombination, three strategies were tested. However, only one strategy succeeded to achieve knock-in/out in A1501, which was a cassette harboring an antibiotic flanked by two homologous fragments located up- and downstream of the target locus. There was positive relation between the frequency of getting mutants and the length of the flanked homology. Colonies with the right insertion were observed to be dependent mainly on three factors,

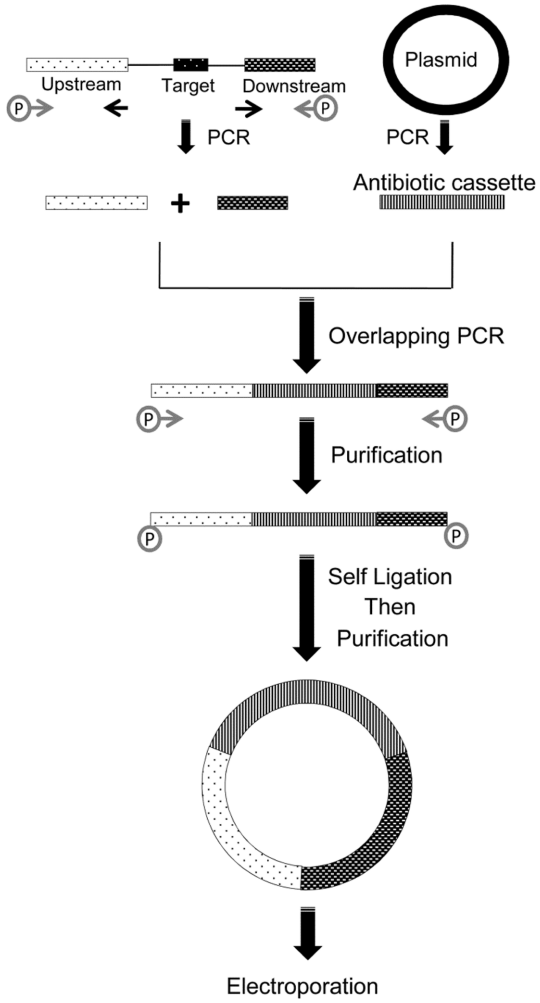

Fig. 4. Schematic representation of the current protocol.

which were cassette purity, DNA concentration, and the length of the homologous fragments. The tested homology lengths began in the range of 30–600 bp; only fragments with >200 bp homology were observed to achieve high frequency of insertion into the genome and yield an antibiotic-resistant strain, although the insertion could be at random locations along the whole genome. Increasing the homology length enhances the possibility of getting single and double crossover mutants (diploid and deletion mutant). The DNA cassette concentration and purity play an important role in the enhancement of the overall process. Moreover, purification of the circular DNA from the ligation mixture decreases the occurrence of the false-positive clones. Finally, grouped-colony PCR could facilitate the screening to find the desired mutant. Overall, the current methodology, as described in Fig. 4, shows the most straightforward strategy to create mutations in gram-

negative bacteria in comparison with other protocols that mostly need a vector backbone or cloning intermediates to achieve genome editing.

Acknowledgments

We would like to thank Dr. Atef Omar for the valuable revision of the manuscript. This work was supported by the National Basic Research Program of China (2015CB755700), the National Science Foundation of China (No. 31470174, 31470205, and 31570081), and Guangdong Innovative and Entrepreneurial Research Team Program (No. 2013S033).

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