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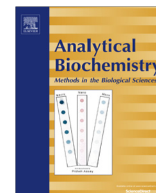

Notes & Tips

Assembling the *Streptococcus thermophilus* clustered regularly interspaced short palindromic repeats (CRISPR) array for multiplex DNA targeting

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ABSTRACT

In addition to the advantages of scalable, affordable, and easy to engineer, the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) technology is superior for multiplex targeting, which is laborious and inconvenient when achieved by cloning multiple gRNA expressing cassettes. Here, we report a simple CRISPR array assembling method which will facilitate multiplex targeting usage. First, the *Streptococcus thermophilus* CRISPR3/Cas locus was cloned. Second, different CRISPR arrays were assembled with different crRNA spacers. Transformation assays using different *Escherichia coli* strains demonstrated efficient plasmid DNA targeting, and we achieved targeting efficiency up to 95% with an assembled CRISPR array with three crRNA spacers.

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Recently, the technology of clustered regularly interspaced short palindromic repeats (CRISPR)[2]/CRISPR-associated proteins (Cas) derived from a bacteria-adaptive immune system has emerged as a powerful and versatile platform for biotechnological, biomedical, and transgenic research. For the type II CRISPR/Cas system, three minimal components, the Cas9 protein, the crRNAs transcribed from the CRISPR locus, and the auxiliary *trans*-activating crRNA (tracrRNA), are sufficient for DNA recognition and targeting [1,2]. The crRNA::tracrRNA duplex, which can further be fused with a loop to generate a functional single guiding RNA (gRNA or sgRNA), directs the Cas9 protein for the recognition and targeting [2,3]. The single RNA guided CRISPR/Cas9 technology is scalable, affordable, and easy to engineer, making it a focus of intense development for genome engineering applications. Thus, a dozen CRISPR/Cas systems derived from different bacterial strains have been developed using similar strategies. Furthermore, compared with the designed zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), CRISPR/Cas technology is superior for multiplex targeting. However, we found that the currently popular multiplex usages mostly involved several different RNA Pol III promoters in a single vector with each promoter driving one gRNA expression [4–6]. An alternative method used a single RNA Pol III promoter (tRNA) for multiple gRNA coexpression. The gRNA coexpression product was designed to be processed internally by HDV ribozymes [7]. Unfortunately, it is very laborious and inconvenient for these methods to clone and assemble the multiple Pol III-gRNA or HDV-gRNA cassettes [4–7]. Then, we asked whether we could simply mimic the bacterial CRISPR array for multiplex targeting, and the answer was yes as previously reported with a synthesized CRISPR array containing three direct repeats (DRs) and two crRNA spacers [8,9]. But when we designed a CRISPR array harboring four DRs and three crRNA spacers, the synthesis was not recommended by Genscript (Nanjing, China) due to the difficulty and high mutagenesis. Hence, there remains a need for approaches to artificially assemble the CRISPR array, which will facilitate the use of multiplex targeting for CRISPR/Cas technology. Here, we report the cloning of the *Streptococcus thermophilus* (*S. thermophilus*) CRISPR3/Cas locus, a detailed design for assembling CRISPR arrays (Fig. 1) and functional assays using different *Escherichia coli* (*E. coli*) strains (Fig. S2).

The native *S. thermophilus* CRISPR3 array consists of short conserved direct repeats (36 bp) interspaced by unique crRNA spacers of the same size (30 bp), which involves a complicated spacer assembly and precrRNA maturing processes. Considering that a guide sequence of 20 nt within a gRNA, which is derived from the crRNA spacer sequence, is long enough for guiding the CRISPR/Cas9 nuclease activity [3,8,10], we hypothesized that the

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[2] *Abbreviations used:* Cas, CRISPR-associated proteins; CRISPR, clustered regularly interspaced short palindromic repeats; DRs, direct repeats.

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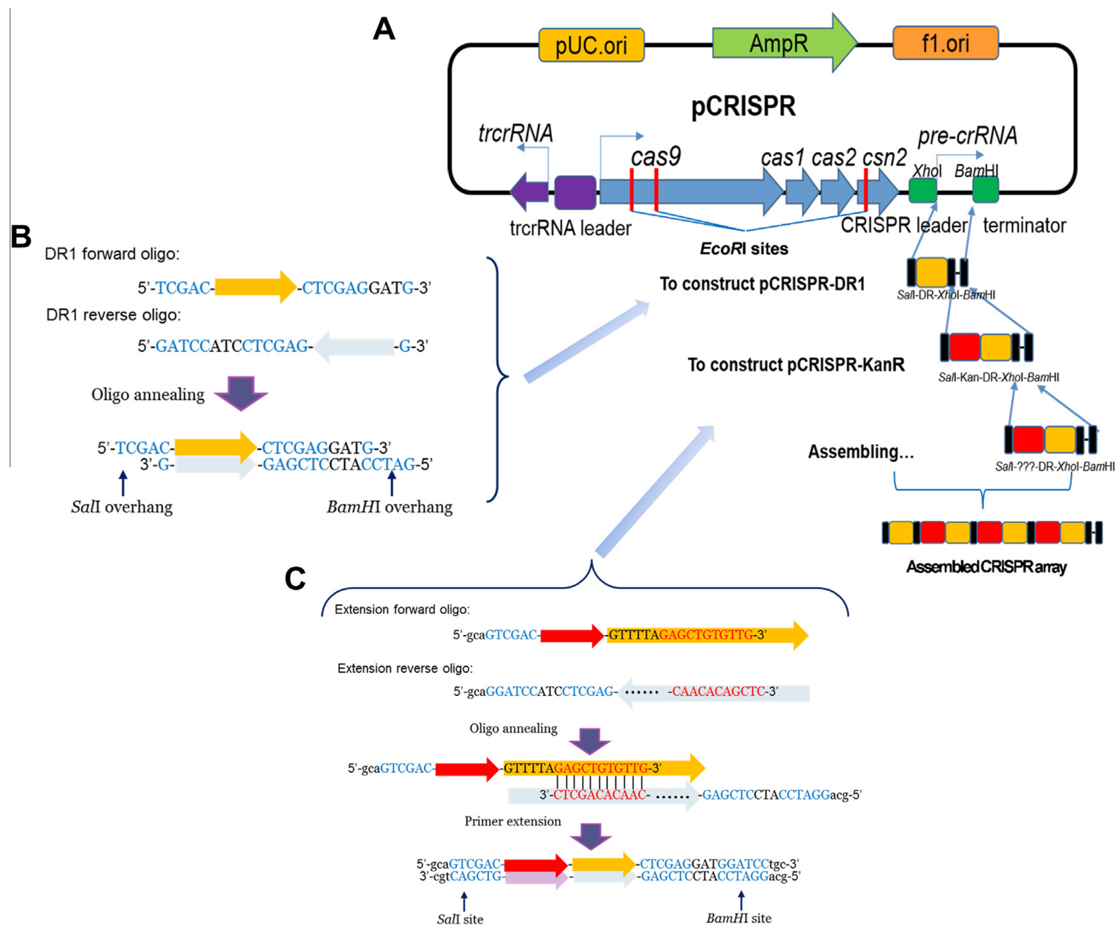

Fig.1. Schematic diagram for assembling the CRISPR array. (a) Cloning the *Streptococcus thermophilus* CRISPR3/Cas locus and assembling the CRISPR array. (b) Generating the first direct repeat (DR, yellow arrow) fragment by annealing oligonucleotides. (c) Generating the assembling fragment containing the interested crRNA spacer sequence (Red arrow), and the adjacent DR (yellow arrow) by primer extension. The sites or overhangs for restriction enzymes are indicated with pale blue font. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

crRNA spacer sequence can be reconstituted with a restriction enzyme site (6 bp) and the interested guide sequence (24 bp). By employing a compatible restriction enzyme strategy, we can simply assemble multiple crRNA spacers into the CRISPR array for multiplex targeting. First, we isolated industrial *S. thermophilus* stains from local yogurt as we did previously [3]. The strains were confirmed by PCR for amplifying the tracrRNA cassette and partial Cas9 fragment (Fig. S1) with primers tracrRNA.F1/R1 and Cas9.F2/R2, respectively (Table S1). Second, colony PCRs were performed with primers tracrRNA.F1/crLeader.R1 and Term.F3/R3 (Table S1) for a ~6.7 kb fragment (consisting of the tracrRNA cassette, the locus of four *Cas* genes, and the CRISPR leader sequence) and the 103 bp CRISPR terminator. The two fragments were cloned into the ampicillin-resistant (AmpR) pBlueScript II SK cloning vector by *Sac*I/*Bam*HI and *Bam*HI/*Kpn*I sites successively, to generate the parental C0:pCRISPR vector (Fig. 1a). Then, we assembled the first DR fragment by directly annealing oligonucleotides (Fig. 1b, Table S2) using a normal procedure [3,11], generating the C1:pCRISPR-DR1 subcloning vector which would serve as the backbone for further assembling and the negative control for the transformation assays. To mimic the Spacer-DR tandem CRISPR array, different Spacer-DR DNA fragments containing the interested crRNA spacer sequence, the adjacent DR, and designed restriction enzyme sites [*Sal*I-Interested guide (24 bp)–DR (36 bp)–*Xho*I–*Bam*HI] were generated by primer extension [12] (Fig. 1c, Table S3) and cloned into the *Xho*I/*Bam*HI sites of the C1:pCRISPR-DR1 subcloning vector or the generated pCRISPR serial plasmids. Through the compatibility between the isocaudarners *Xho*I/*Sal*I, we can assemble multiple Spacer-DR fragments into the CRISPR array consecutively (Fig. 1a). By employing this strategy, we assembled two crRNA spacers (KanR1/KanR2) targeting the kanamycin/neomycin-resistant gene and one crRNA spacer (CMV3) from the CMV promoter (Table S3), resulting in a series of pCRISPR plasmids harboring different CRISPR arrays (Fig. S2a).

To validate these assembled CRISPR arrays for efficient DNA targeting, we selected three plasmids [pAdTrack-CMV (Addgene), pEGFP-C1 (Clontech), and pDsRed1-C1 (Clontech); Fig. S2a] containing the kanamycin/neomycin-resistant gene and the CMV promoter sequence. In the transformation assay, the targeting by CRISPR/Cas9 nucleases driven by pCRISPR plasmids was supposed to destroy the kanamycin-resistant plasmids, and the destroyed kanamycin-resistant plasmids would fail to support colony survival under the pressure of kanamycin on LB/+Amp+Kan agar plates. With C1:pCRISPR-DR1 as the negative control, different ampicillin-resistant pCRISPR plasmids (0.5 μg per transformation) and kanamycin-resistant plasmids (0.25 μg per transformation) were used to cotransform (using electric transformation) different *E. coli* strain cells. Proper volume and equivalent proportion of different transformants were spread on LB agar plates with 37.5 μg/ml kanamycin and 100 μg/ml ampicillin for selecting survival colonies. The survival efficiencies (%) for different pCRISPR plasmids were calculated by comparing with the C1:pCRISPR-DR1 negative control and the clearances were used to estimate the targeting efficiencies. All transformation experiments were repeated at least three times. Transformation assay with *E. coli* DH5α first demonstrated that the assembled CRISPR array functioned successfully

and the targeting efficiency was improved dramatically with the number of crRNA spacers increased. The targeting efficiencies were 53.31–74.74% for arrays with one crRNA spacer, 75.71–89.94% for arrays with two spacers, and around 95% for the array with all there spacers (Fig. S2b). Also, the assembled CRISPR arrays were further tested and confirmed to function in another two *E. coli* strains, JM109 and TOP10 (Fig. S2c and d). These revealed that the residual *Xho*I/*Sal*I hybrid sites in the front of crRNA spacers did not block the transcription, processing, and function of the CRISPR arrays. The more efficient targeting with three spacers, compared with one or two spacers, further indicated that multiplex DNA targeting did exist and can be achieved successfully by assembling the CRISPR array.

On the other hand, DH5α cells were cotransformed with the control C1 or the C6 pCRISPR plasmid (Fig. S2a) and pET28a (+) (Novagen), a plasmid employing a different kanamycin-resistant gene (KanR) and harboring none of the three crRNA target sites (Table S3). A transformation assay generated similar survival colonies between the experiment and the control groups (Fig. S3). This result suggested that the assembled CRISPR arrays possess specificity for DNA targeting as the native array, and could be used to support bacterial cells for specific plasmid interference and phage resistance [1]. Furthermore, we inactivated the Cas9 protein by deleting the fragments among the three *Eco*RI sites (Fig. 1a) in the C1 and C6 pCRISPR plasmids. The resulting D1 and D2 plasmids (Figs. S2a and S4) were used with pAdTrack-CMV to cotransform DH5α cells. The result validated that the CRISPR array without Cas9 was not sufficient for initiating the targeting in *E. coli* (Fig. S5), which was consistent with the report that there are no Cas9 orthologs in *E. coli* strains [13]. In addition, we adapted the system for *Lactobacillus reuteri* (*L. reuteri*) by cloning the assembled CRISPR arrays, the tracrRNA cassette, the kanamycin/neomycin-resistant gene sequence, and CMV3 crRNA target site into the pLEM415 plasmid (BBa_K128007). A transformation assay using the bacterial strain without Cas9 ortholog generated similar results with *E. coli* DH5α (data not shown). Thus, for multiplex targeting usage in bacterial strains without Cas9 orthologs, we need to import the tracrRNA and CRISPR array, as well as Cas9. However, perhaps for bacterial strains with native Cas9 orthologs such as *S. pyogenes* and *S. thermophilus*, we may simply introduce an assembled CRISPR array without Cas9 coupled to achieve efficient plasmid interference, phage resistance, or even multiplex genome engineering.

Actually, the CRISPR array could be assembled precisely by employing type II restriction enzymes, such as *Bsa*I and *Bsm*BI, as we previously reported for assembling TALEs [14]. However, this may need longer primers for the extension reaction (Fig. 1c). Besides, there is no need to use only a 30 bp crRNA spacer, because a 20 nt guide sequence for crRNA or gRNA has been proven to be sufficient for plasmid and genome DNA targeting. Our original purpose for developing the CRISPR assembling technique was to facilitate the multiplex targeting usage in prokaryotes. As previously reported that the use of two crRNAs encoded within a single CRISPR array enables multiplex mutagenesis in mammalian cells without specially introducing RNase III [8], our method could also be adapted to and help with the eukaryotic multiplex targeting. Also, CRISPR/Cas technology can be exploited as sequence-specific antimicrobials against bacteriophage [15], virus [16], and bacteria [17,18]. However, our results indicated that multiplex targeting with increased crRNA spacers (three or even more) may improve the performance for sufficient clearance of intent microbes. Thus, our method provides a potential tool for preparing efficient and powerful sequence-specific antimicrobials.

In conclusion, we developed a simple CRISPR array assembling method based on a compatible restriction enzyme strategy with isocaudarners *Xho*I/*Sal*I, which was proven to function in the transformation assay for multiplex plasmid DNA targeting and was supposed to facilitate the multiplex targeting usage for CRISPR/Cas technology. Compared to previously described approaches involving multiple Pol III-gRNA or HDV-gRNA cassettes [4–7], our technique mimics the bacterial native CRISPR array and allows the more convenient assembly of three or more interested crRNA spacers within a short array for multiplex targeting.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ab.2015.02.028.

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