RESEARCH ARTICLE

In Vitro CRISPR/Cas9 System for Efficient Targeted DNA Editing

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ABSTRACT **The clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) system, an RNA-guided nuclease for specific genome editing *in vivo*, has been adopted in a wide variety of organisms. In contrast, the *in vitro* application of the CRISPR/Cas9 system has rarely been reported. We present here a highly efficient *in vitro* CRISPR/Cas9-mediated editing (ICE) system that allows specific refactoring of biosynthetic gene clusters in *Streptomyces* bacteria and other large DNA fragments. Cleavage by Cas9 of circular pUC18 DNA was investigated here as a simple model, revealing that the 3′→5′ exonuclease activity of Cas9 generates errors with 5 to 14 nucleotides (nt) randomly missing at the editing joint. T4 DNA polymerase was then used to repair the Cas9-generated sticky ends, giving substantial improvement in editing accuracy. Plasmid pYH285 and cosmid 10A3, harboring a complete biosynthetic gene cluster for the antibiotics RK-682 and holomycin, respectively, were subjected to the ICE system to delete the *rkD* and *homE* genes in frame. Specific insertion of the ampicillin resistance gene (*bla*) into pYH285 was also successfully performed. These results reveal the ICE system to be a rapid, seamless, and highly efficient way to edit DNA fragments, and a powerful new tool for investigating and engineering biosynthetic gene clusters.**

IMPORTANCE **Recent improvements in cloning strategies for biosynthetic gene clusters promise rapid advances in understanding and exploiting natural products in the environment. For manipulation of such biosynthetic gene clusters to generate valuable bioactive compounds, efficient and specific gene editing of these large DNA fragments is required. In this study, a highly efficient *in vitro* DNA editing system has been established. When combined with end repair using T4 DNA polymerase, Cas9 precisely and seamlessly catalyzes targeted editing, including in-frame deletion or insertion of the gene(s) of interest. This *in vitro* CRISPR editing (ICE) system promises a step forward in our ability to engineer biosynthetic pathways.**

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Microbial natural products have been a focus for drug discovery and development for almost a century and have afforded valuable therapeutics, including antibiotics (1), anticancer agents (2), immunosuppressants (3), and insecticides (4). The discovery and characterization of biosynthetic gene clusters that produce such bioactive metabolites remain critical for the development of new natural product-based drugs. Recent advances in next-generation sequencing of microbial genomes (5) have revealed a wealth of information about potential secondary metabolite pathways (6). However, many clusters appear silent, and the size of these gene clusters (50 to 130 kb) has hindered their manipulation (7). New and efficient methods are required to develop our understanding of these biosynthetic pathways (8).

Traditionally biosynthetic gene cluster analysis has relied upon *in vivo* gene editing via homologous recombination, but more recently heterologous expression of gene clusters has emerged as a valuable alternative (9), and the method of choice for clusters from genetically intractable strains or from metagenomic analysis of noncultured microorganisms (10). Cloning of gene clusters on cosmids is slow and inappropriate for larger clusters, so direct methods such as RecET-mediated homologous recombination in *Escherichia coli* (11), transformation-associated recombination (TAR) in *Saccharomyces cerevisiae* (12), *oriT*-directed cloning (13), and artificial chromosome library construction were established (14, 15). Moreover, site-specific recombination to assemble biosynthetic gene clusters (16–18) and direct Gibson assembly of a gene cluster have been reported (19). In contrast, strategies for *in vitro* manipulation of these giant DNA fragments are extremely underdeveloped. Routine cloning strategies, including PCR amplification and restriction digestion are severely constrained by errors, restriction site limitation, and low efficiency when a gene cluster spans dozens of kilobases (20). Even λ *red* recombination-mediated PCR targeting may cause unintended recombination because of the repetitive sequences typical of such clusters (21). Therefore, a rapid, specific, and seamless DNA editing system is required for functional investigation of biosynthetic gene clusters.

Recently, the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) system, an

adaptive microbial immune system has been identified and been used in numerous model organisms to edit the genome with high efficiency and specificity *in vivo* (22–27). The CRISPR/Cas system from *Streptococcus pyogenes*, which consists of Cas9 protein and two RNAs (CRISPR RNA [crRNA] and *trans*-activating crRNA [tracrRNA]), is most commonly used (28). First, Cas9 assembles the mature single guide RNA (sgRNA), which is composed of crRNA- and tracrRNA-derived sequences connected by an artificial tetraloop, into an effector complex. The complex will be enabled to recognize target DNA guided by a 20-nucleotide (nt) guide sequence on 5′ sgRNA (29). The Cas9-sgRNA complex specifically cleaves double-stranded DNA (dsDNA) 3 bp upstream of the protospacer adjacent motif (PAM) (29, 30), and the PAM has been demonstrated to be an indispensable part for DNA interrogation (31). Although the CRISPR/Cas9 system has repeatedly been shown to edit the chromosome of various organisms with high efficiency and specificity and has been widely used in animal models and non-animal research, the application of the CRISPR/Cas9 system *in vitro* for design and refactoring of a DNA fragment has received much less attention (32–35). In the present study, the CRISPR/Cas9 system was exploited as an *in vitro* programmable DNA endonuclease to edit typical actinomycete biosynthetic gene clusters. Two large constructs, plasmid pYH285 (~20 kb) containing an intact tetronate RK-682 biosynthetic gene cluster (36) and cosmid 10A3 (~40 kb) containing an intact dithiolopyrrolone holomycin biosynthetic gene cluster (37), were successfully engineered by the *in vitro* CRISPR/Cas9-mediated editing (ICE) system. The *rkD* gene was deleted in frame and an ampicillin resistance gene (*bla*) was inserted in pYH285, respectively, and the *homE* gene was deleted in frame in cosmid 10A3. The integrity of the recombinants was checked, and heterologous expression of the recombinant constructs confirmed loss of RK-682 and holomycin production in each targeted mutant. The ICE system should be of wide applicability for the seamless and convenient editing of biosynthetic gene clusters, especially in conjunction with expression in a heterologous host strain.

RESULTS

Investigation of DSB ends of circular dsDNA created by Cas9. In the CRISPR/Cas9 system, Cas9 complexes with sgRNA to form the effector complex that binds to and cleaves dsDNA either *in vivo* or *in vitro*. After cleavage by Cas9, both circular dsDNA and linear dsDNA undergo a double-strand break (DSB) 3 bp upstream of PAM (29). Based on our current understanding of the specific cleavage activity of Cas9, it is reasonable to consider CRISPR/Cas9 system as a programmable DNA endonuclease. The cleavage of linear dsDNA by CRISPR/Cas9 is reported to generate blunt ends, with the end without PAM of the noncomplementary strand being subsequently trimmed by the 3′→5′ exonuclease activity of Cas9 to generate a sticky end (30). We reasoned that "noncomplementary strand trimming" should also happen to a DSB in circular dsDNA and likewise generate one blunt end and one sticky end. To examine this, we investigated "noncomplementary strand trimming" on the general cloning plasmid pUC18 as a model system. Cas9 in complex with sgRNAS3 (Cas9-sgRNAS3) was applied to pUC18, the resulting linearized dsDNA was treated with T4 DNA polymerase, then self-ligated and was introduced into *E. coli* DH10B by transformation (Fig. 1A). Ten colonies were randomly selected and sequenced to check the editing junction. Sequencing showed that 6 to 7 bp nucleotides 5′ of the cleavage site on the noncomplementary strand had disappeared in the majority of those sequenced colonies not treated with T4 DNA polymerase (Fig. 1B). However, half of colonies treated with T4 DNA polymerase contained intact sequence, and the rest showed only 1 bp missing, compared to the intact sequence (Fig. 1F). This result confirmed that "noncomplementary strand trimming" also happens at a DSB in circular dsDNA.

To investigate the influence of sgRNA design on DSB production, protospacers S1, S2, and S4 on pUC18 were selected to transcribe sgRNAS1, sgRNAS2, and sgRNAS4, respectively. As shown in Fig. 1A, when one pair of sgRNAs was complexed with Cas9, cleavages guided by sgRNAS1/sgRNAS4, sgRNAS2/sgRNAS3, or sgRNAS2/sgRNAS4 were generated. Theoretically, three groups of different DSB ends should be generated according to different combinations of sgRNAs. pUC18 cleaved by Cas9-sgRNAS1 and Cas9-sgRNAS4 will generate a backbone fragment with two blunt ends, in contrast, pUC18 cleaved by Cas9-sgRNAS2 and Cas9-sgRNAS3 will produce a fragment with two sticky ends. Cas9-sgRNAS2 and Cas9-sgRNAS4 should generate a fragment with one blunt end and one sticky end. The backbone fragments of interest were recovered and directly self-ligated and introduced into *E. coli* DH10B by transformation. Subsequent plasmid sequencing revealed that the joints from blunt end-blunt end ligation (Cas9-sgRNAS1/Cas9-sgRNAS4) are all the same, as expected (Fig. 1C). However, only one plasmid from sticky end-blunt end ligation (Cas9-sgRNAS2/Cas9-sgRNAS4) harbored an accurate sequence at the joint, the others containing a mutated sequence (Fig. 1D). Furthermore, sticky end-sticky end ligation (Cas9-sgRNAS2/Cas9-sgRNAS3), resulted in random loss of 5 to 14 bp from the joint (Fig. 1E). Clearly, the different sgRNA combinations will greatly affect the nature of DSB ends generated in the fragment. Accordingly, a rational design of sgRNA combination is essential to generate a DNA fragment with blunt ends to ensure accurate DNA editing.

End repair increases the flexibility of target site selection for cleavage by Cas9-sgRNA. Although rational design of a combination of sgRNAs can ensure generation of a fragment with two blunt ends, the requirements of sgRNA transcription, together with limited PAM selectivity, will constrain the routine use of Cas9 *in vitro*. An end repair strategy could overcome the sticky end barrier to accurate cloning raised by noncomplementary strand trimming. To test this, three different combinations of ends, in DNA fragments generated by Cas9 complexed with sgRNAS1/sgRNAS4, sgRNAS2/sgRNAS3, and sgRNAS2/sgRNAS4, respectively, were further treated by T4 DNA polymerase, and the products were self-ligated and introduced into *E. coli* DH10B. Sequencing of the editing joints showed that half of colonies from sticky end-sticky end joints (Cas9-sgRNAS2/Cas9-sgRNAS3) have the expected sequence at the joint after end repair by T4 DNA polymerase (Fig. 1I). For sticky end-blunt end joints generated from Cas9 complexed with sgRNAS2/sgRNAS4, 70% of colonies with the expected joint were obtained after end repair (Fig. 1H). Consistent with our expectation, little influence on the joint sequence in blunt end-blunt end (Cas9-sgRNAS1/Cas9-sgRNAS4) joints (Fig. 1G). Thus, end repairing by T4 DNA polymerase means that guide RNA (gRNA) design is not restricted to generation of two blunt ends. This modification greatly expands the choice of target sequence when Cas9 is used as a programmable endonuclease. This system, which we refer to as ICE, enables specific editing of DNA sequence unconstrained by selection of re-

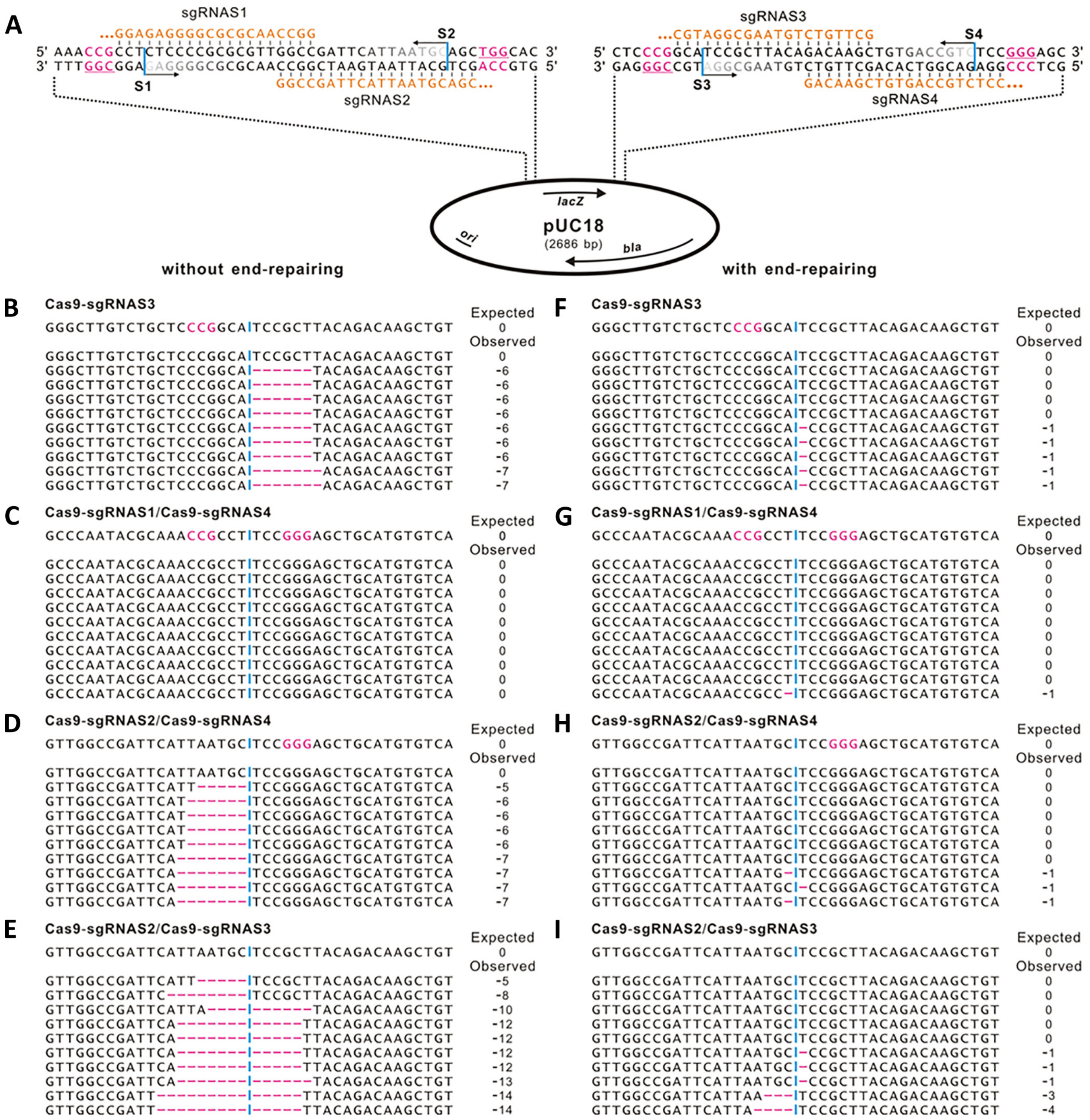

FIG 1 Recombination of a Cas9-created DNA fragment with and without end repair. (A) Schematic representation of four selected protospacers in the pUC18 plasmid. The transcribed sgRNAs matched the specific protospacers are shown in orange. PAM sequences are highlighted in pink, and "NGG" is underlined. The vertical blue lines between nucleotides indicate the Cas9-mediated DSB sites matched with specific protospacers. The 3′→5′ exonuclease trimming is shown with horizontal arrows, and the sequential trimming occurred in the noncomplementary strand is shown in gradient gray. *ori*, origin of replication of plasmid pUC18; *bla*, ampicillin resistance gene; *lacZ*, β-galactosidase gene. (B to E) End-joint sequencing results from cleavage of Cas9-sgRNAS3 (B), Cas9-sgRNAS1/Cas9-sgRNAS4 (C), Cas9-sgRNAS2/Cas9-sgRNAS4 (D), and Cas9-sgRNAS2/Cas9-sgRNAS3 (E) without end repair. For each combination of Cas9-sgRNA complex, the desired sequence is shown at the top, with the PAM sequence highlighted in pink and the joint interface indicated with a vertical blue line. Deletions are shown as a pink dashed line. The net change in length caused by exonuclease trimming is shown to the right of each sequence (−, deletion). (F to I) End-joint sequencing results from cleavage of Cas9-sgRNAS3 (F), Cas9-sgRNAS1/Cas9-sgRNAS4 (G), Cas9-sgRNAS2/Cas9-sgRNAS4 (H), and Cas9-sgRNAS2/Cas9-sgRNAS3 (I) with an additional end-repairing procedure that is parallel to the left column, respectively.

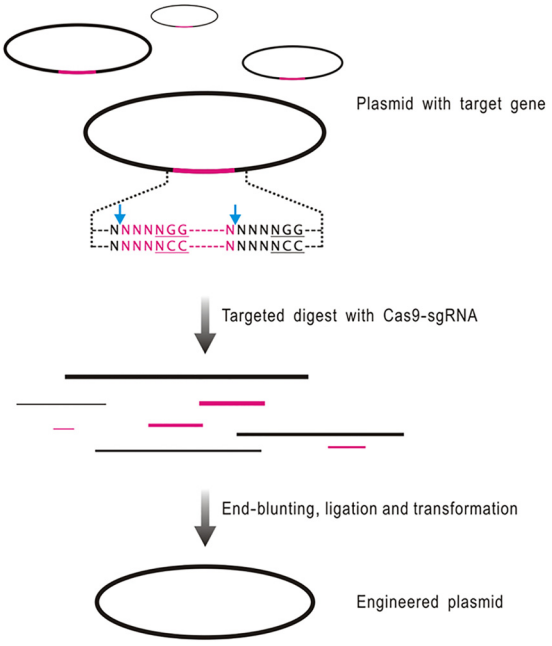

FIG 2 Flowchart of targeted gene deletion of biosynthetic gene cluster using ICE system. The target gene (in pink) flanking by protospacers is cleaved 3 bp upstream of PAM by the Cas9-sgRNA complex. After phenol-chloroform extraction and ethanol precipitation, the linearized DNA fragments mixture is subsequently end blunted, self-ligated, and then introduced into *E. coli*. The desired colonies will be screened out from the pool of recirculation plasmids by PCR and subsequently confirmed by restriction mapping.

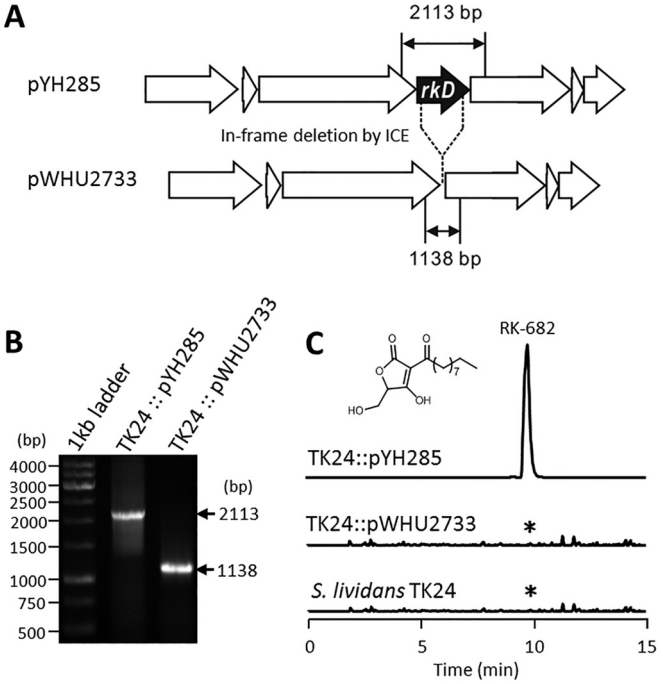

FIG 3 Targeted in-frame deletion of *rkD* in plasmid by ICE system. (A) Schematic representation of in-frame deletion of *rkD*. The numbers above bidirectional arrows indicate the expected size of the PCR fragments before and after deletion. (B) PCR confirmation of heterologous strains harboring plasmids pYH285 and pWHU2733, respectively. (C) LC-ESI-HRMS analysis of RK-682 production in strains TK24::pYH285, TK24::pWHU2733, and blank host *S. lividans* TK24, respectively. The selective ion monitoring was carried out on $[M+H]^-$ (*m*/*z*, 367.2479) ions of RK-682.

striction sites. Moreover, it facilitates seamless editing of even large DNA fragments and is particularly valuable for investigating the function of genes clustered in one operon without polarity effects on downstream genes.

Targeted editing of biosynthetic gene clusters *in vitro*. In general, biosynthetic gene clusters of microbial natural products span many tens of kilobases, and numerous gene clusters, especially those encoding polyketide and nonribosomal peptide biosynthesis, even exceed 100 kb. The ICE system therefore appears a promising method to edit these giant gene clusters efficiently and specifically to construct in-frame deletion recombinants (Fig. 2).

To investigate this, we used plasmid pYH285 (~20 kb) harboring a complete biosynthetic gene cluster from *Streptomyces* sp. 88-682 for the protein phosphatase inhibitor RK-682 as a target for testing (38). In this cluster, the *rkD* gene encodes a FabH-like 3-oxoacyl-ACP synthase III that is essential for RK-682 biosynthesis (36). Protospacers rk1 and rk2 inside *rkD* were selected, and sgRNArk1 and sgRNArk2 were subsequently transcribed for in-frame deletion of *rkD* in pYH285 *in vitro*. pYH285 DNA was cleaved by Cas9 complexed with sgRNArk1 and sgRNArk2, resulting in two DNA fragments (~19 kb and 975 bp). After phenol-chloroform extraction and ethanol precipitation, the mixture of DNA fragments was directly end repaired and self-ligated before introduction into *E. coli* DH10B. Thirty-one colonies with the expected deletion were obtained from 96 transformants by PCR screening. Of these, 10 were further confirmed by digestion (see Fig. S2A in the supplemental material) and one colony designated pWHU2733 (see Table S1 in the supplemental material) was sequenced for examination of the sequence at the joint. The result showed that *rkD* was successfully knocked out in frame from pYH285 as expected (Fig. 3A).

Cosmid library cloning has been the strategy most frequently used in cloning biosynthetic gene clusters for natural products. DNA insert size in these vectors most commonly spans 40 to 50 kb. To further investigate the editing efficiency of ICE on large DNA fragments, *homE* encoding a multidomain nonribosomal peptide synthase in cosmid 10A3 (~40 kb) from a *Streptomyces clavuligerus* library harboring the holomycin biosynthetic gene cluster (35), was selected for in-frame deletion. PCR screening of 96 clones obtained after application of the ICE procedure identified 15 that appeared to have the expected deletion. This was confirmed by restriction digestion (see Fig. S2B in the supplemental material). One of these plasmids was then selected for sequencing of the joint. The result proved the successful in-frame deletion of *homE*, generating pWHU2736 (Fig. 4; see Table S1 in the supplemental material). These data suggest that the editing efficiency is maintained when the DNA insert size was increased to 40 kb, which is highly encouraging for the application of this method to the investigation of microbial biosynthetic gene clusters.

Gene editing can be used not only for precise gene deletion but also for accurate gene insertion or replacement, especially for combinatorial biosynthesis of natural products or for promoter exchange to stimulate production from so-called "silent" or "cryptic" biosynthetic gene clusters. To test the utility of the ICE system in gene insertion, we generated sgRNA285 to target plasmid pYH285. After digestion of Cas9-sgRNA285 and end repair,

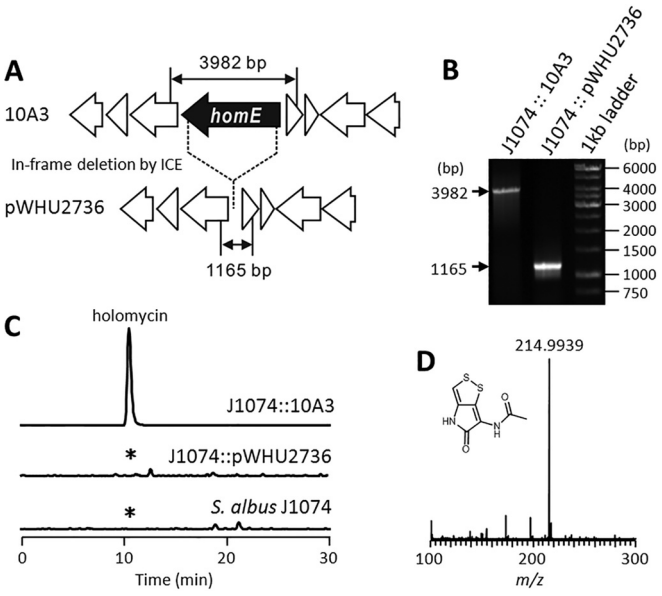

FIG 4 Targeted in-frame deletion of *homE* in a cosmid by the ICE system. (A) Schematic representation of in-frame deletion of *homE*. The numbers above bidirectional arrows indicate the expected size of the PCR fragments before and after deletion. (B) PCR confirmation of heterologous strain harboring cosmid 10A3 and pWHU2736, respectively. (C) LC-ESI-HRMS analysis of holomycin production in strains J1074::10A3, J1074::pWHU2736, and blank host *S. albus* J1074, respectively. The selective ion monitoring was carried out on $[M+H]^+$ (*m/z*, 214.9945) ions of holomycin.

we ligated a PCR fragment amplified from the region containing a promoter and ampicillin resistance gene in pUC18 plasmid and used the resulting ligation mixture to transform *E. coli* DH10B. The resulting colonies were selected on medium containing ampicillin and confirmed by digestion (see Fig. S2C in the supplemental material). Sequencing of DNA isolated from the selected colonies confirmed that the PCR fragment was inserted at the desired position and no mutations were introduced upon Cas9 cleavage (Fig. 5). Taken together, these results suggest that the ICE system not only can be used for efficiently targeted knockout in cosmid DNA but also can be applied for seamless insertion at a specific site.

To determine the production of RK-682 and holomycin in the constructs housing ICE-edited gene clusters, heterologous expression was carried out in *Streptomyces lividans* TK24. For RK-682 production, pWHU2733 and pYH285 (positive control) were each introduced into TK24 by conjugation. Analysis of fermentation extracts of TK24::pYH285 by liquid chromatography-electrospray ionization–high-resolution mass spectrometry (LC-ESI-HRMS) showed the presence of RK-682. However, no RK-682 was found in TK24::pWHU2733 (Fig. 3; see Table S2 in the supplemental material). This result showed that ICE-mediated *rkD* in-frame deletion ablated RK-682 production, as previously reported using time-consuming homologous recombination (36). Cosmid 10A3 allows successful heterologous expression in *Streptomyces albus* J1074 of the antibiotic holomycin (38). ICE-mediated editing of this cosmid gave pWHU2736 with *homE* deleted in frame, and this cosmid was transferred into *S. albus* J1074 by conjugation. LC-ESI-HRMS analysis revealed that

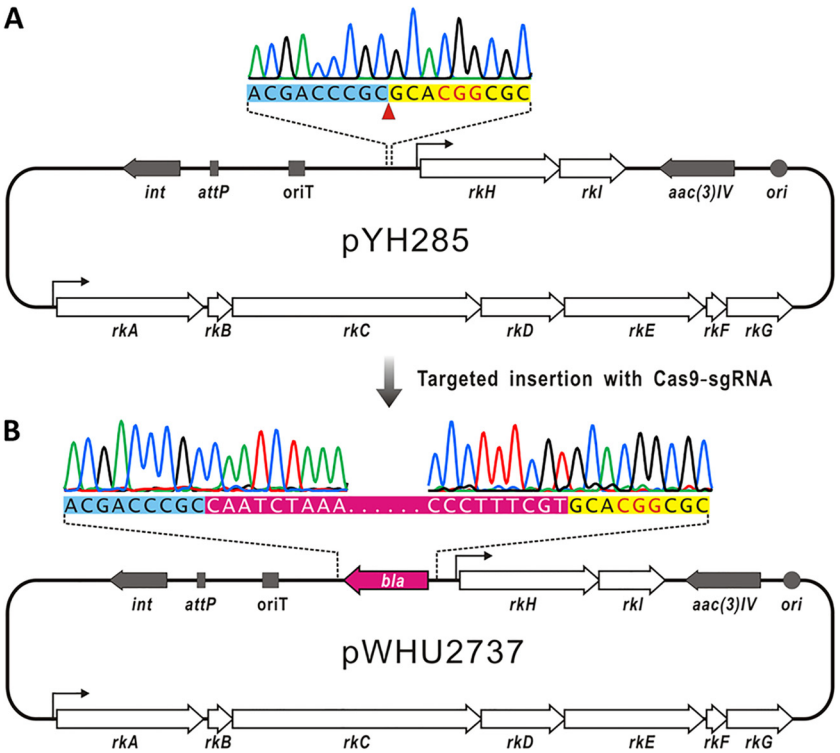

FIG 5 Targeted insertion of *bla* gene in plasmid pYH285 by ICE system. (A) Schematic representation of the target site in pYH285 plasmid. The target region is shown as the top panel, in which the Cas9 cleavage site is indicated by a red triangle and the PAM sequence is highlighted in red. (B) DNA sequencing confirmation of *bla* insertion in a recombinant plasmid.

J1074::10A3 (positive control) produced holomycin with an exact mass value of $[214.99435]^+$. In contrast, J1074::pWHU2736 produced no holomycin (Fig. 4; see Table S2).

DISCUSSION

The CRISPR/Cas9 system facilitates site-specific cleavage of the chromosome from various organisms. The DSBs generated are repaired *in vivo* by nonhomologous end joining (NHEJ), microhomology-mediated end joining (MMEJ), and homology-directed repair (HDR), resulting in insertion, deletion, or replacement mutations (39–41). Recently, the CRISPR/Cas9 system has been developed as a universal genome-editing tool and has been widely adopted for specific editing of various organisms *in vivo*, ranging from bacteria to humans (42). The high efficiency and specificity of Cas9 suggest its use in routine cloning as a programmable endonuclease. In this study, we have demonstrated that nucleotide trimming is specific to the noncomplementary strand of the blunt end without PAM, in circular dsDNA as well as in linear dsDNA, and that this seriously compromises the accuracy of subsequent cloning. By using a simple end repair procedure, the ICE system greatly increases the likelihood of accurate cloning. Furthermore, we have demonstrated that even large biosynthetic gene clusters can be rapidly edited with high efficiency and accuracy by ICE. Compared with the commonly used PCR-targeting system for refactoring DNA, ICE is easier in manipulation, with no scar remaining in the engineered DNA, and avoids unintended recombination. The ICE system has the potential for high-throughput production of a series of engineered DNAs, thus laying the foundation for systematically knocking out every single gene in biosynthetic gene clusters *in vitro*.

Previously, *Streptococcus thermophilus* Cas9 (*St*Cas9) rather than *Streptococcus pyogenes* Cas9 (*Spy*Cas9) had been used as a programmable endonuclease (32). However, *Spy*Cas9 has been widely adopted as an RNA-guided nuclease and is commercially available. Also, the application of *St*Cas9 is severely constrained by the need to adapt CRISPR/*St*Cas9 to recognize PAM consisting of 5 bp (43). The use of T4 DNA polymerase for end repair allows much more flexible design of editing experiments and opens the way to convenient manipulation of large DNA fragments.

Although we did not test inserts of greater than 40 kb, it should be possible to extend the method to clusters larger than this. In the ICE experiments reported here, the DNA of the edited biosynthetic gene cluster was directly used for self-ligation after ethanol precipitation, omitting the tedious agarose recovery step. Modifications to improve cloning efficiency will be important to optimize the extension of the method to larger DNA fragments. It is known that manipulation of a giant DNA fragment turns more difficult when DNA size increases, which may greatly influence the cloning efficiency. Therefore, procedure optimization like desalting after end repair, using supercompetent cells, and applying electrotransformation may further improve cloning efficiency.

Further development of the ICE system could make it even more convenient to use. The guide sequence of sgRNA is transcribed *in vitro* by T7 RNA polymerase, and the 5′ nucleotides have to be chosen to ensure precise initiation at the T7 promoter, which reduces the flexibility of selection of protospacers, especially when the gene of interest is too small to harbor the appropriate protospacers. It may be possible to use self-processing of ribozyme-flanked RNA to expand the scope of target sequence, or T3 or SP6 RNA polymerase may be used instead to transcribe sgRNA *in vitro* (44). Even as it stands, the ICE system described here represents the first practical means of generating a series of in-frame gene deletions and/or insertions rapidly and simultaneously, enabling heterologous expression of numerous gene-null mutants to provide comprehensive analysis of biosynthetic gene clusters.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *E. coli* DH10B was used as the cloning host, *E. coli* Rosetta(DE3) was used for Cas9 expression, and *E. coli* ET12567/pUZ8002 was used for intergeneric conjugation. All *E. coli* strains were cultured in 2×TY medium (1.6% tryptone, 1% yeast extract, 0.5% NaCl) at 37°C.

Streptomyces sp. 88-682 is a wild-type strain producing RK-682. *S. lividans* TK24 was used as surrogate host for RK-682 production, and *S. albus* J1074 was used for holomycin production (see Table S2 in the supplemental material). *S. lividans* and *S. albus* were grown on SFM agar medium (2% mannitol, 2% soybean meal, 2% agar [pH 7.2]) at 28°C for sporulation.

Expression and purification of Cas9. A 4,107-bp DNA encoding *Spy*Cas9 was amplified using primers (28+cas9-S/A) from pMJ806 (29). The resulting product was assembled with EcoRI- and NdeI-linearized pET28a(+) by Gibson assembly to generate pWHU2739 (see Table S1 in the supplemental material) in order to express the N-terminally His-tagged Cas9 protein, which was subsequently introduced into *E. coli* Rosetta(DE3). The recombinant strain was cultured in 2×TY (containing 25 μg/ml kanamycin) at 37°C overnight. Five milliliters of an overnight culture was inoculated into 500 ml of 2×TY containing 25 μg/ml kanamycin and incubated at 37°C until the A_{600} reached 0.6 to 0.8. Cas9 expression was induced by adding isopropyl-β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.2 mM and the culture was further incubated at 18°C overnight. The cells were harvested by centrifugation and the pellet was resuspended in lysis buffer containing 20 mM Tris-Cl, 500 mM NaCl, and 1 mM tris(2-carboxyethyl)phosphine (TCEP) (pH 8.0) and lysed by sonication. The cell lysate was clarified by centrifugation at 12,000 × *g* for 30 min and applied to a column of Ni-nitrilotriacetic acid (NTA) agarose (GE Life Sciences). After washing of the column, Cas9 was eluted using elution buffer (250 mM imidazole, 20 mM Tris-Cl, 250 mM NaCl, 10% glycerol [pH 8.0]). Cas9 was desalted using a PD-10 desalting column (GE Life Sciences) and eluted with storage buffer (500 mM NaCl, 10 mM Tris-Cl, 0.1 mM EDTA, 1 mM dithiothreitol [DTT], 50% glycerol [pH 7.4]). Cas9 protein was ultimately concentrated to ~4 mg/ml by Amicon Ultra centrifugal filters (Millipore) and stored at −25°C.

gRNA transcription *in vitro*. For sgRNA design, the gene sequence to be edited was screened for the presence of a 20-bp guide and PAM sequence ($5'-X_{20}$NGG–$3'$), and nucleotides between two PAMs were specified to be a multiple of 3 when gene in-frame deletion was to be performed. A guide sequence for sgRNA had to meet the rules governing efficient and precise initiation of the T7 promoter, and the target sequence $5'-GX_{19}-3'$ lying upstream of PAM was preferred (see Table S3 and Fig. S1 in the supplemental material). Transcription templates of the sgRNA were amplified by overlap extension PCR with the primers listed in Table S3. *In vitro* transcription of sgRNA was performed using the TranscriptAid T7 high-yield transcription kit (Thermo; Fisher Scientific) according to the manufacturer's instructions. The sgRNA product was pretreated by heating at 95°C for 10 min and slowly cooling down to room temperature before use in the ICE system.

***In vitro* Cas9-mediated editing (ICE) system.** First, a 50-μl reaction mixture containing ~3 μg Cas9 protein, 10 μg sgRNA, and 3 μg DNA was prepared and incubated in buffer 3.1 (New England Biolabs) at 37°C overnight. The reaction was terminated by addition of 0.2 mg/ml RNase (Tiangen Biotech) and continued incubation at 37°C for 15 min. Then the reaction mixture was treated with SDS (to 1%), 1 mg/ml proteinase K, and

10 mM $CaCl_2$ and incubated at 55°C for 30 min. Finally, the Cas9-digested DNA was recovered by ethanol precipitation.

T4 DNA polymerase (New England Biolabs) was used to repair the sticky end generated by 3′→5′ exonuclease activity of Cas9. A mixture of 3 μg Cas9-digested DNA, 100 μM deoxynucleoside triphosphates (dNTPs), 1× bovine serum albumin (BSA), and 0.5 μl T4 DNA polymerase was prepared in 1×T4 DNA ligase reaction buffer (New England Biolabs). The end repair mixture was then incubated at 12°C for 15 min, and reaction was terminated by incubation at 75°C for 20 min. Subsequently, end-repaired DNA was self-ligated or ligated with an additional DNA fragment in the ligation mixture, which contained 0.2×T4 DNA ligase reaction buffer (New England Biolabs), 15% (vol/vol) polyethylene glycol 4000 (PEG 4000), and 1 μl T4 DNA ligase (Thermo, Fisher Scientific), and then the mixture was incubated at 16°C overnight.

Fermentation, isolation, and analysis of RK-682. For RK-682 production, *Streptomyces* sp. strain 88-682, and the *S. lividans* TK24 wild type and mutants were incubated on TWM agar medium (0.5% glucose, 1% sucrose, 0.5% tryptone, 0.25% yeast extract, 0.0036% EDTA, 2% agar [pH 7.1]) at 28°C for 6 days. All cultures were filtered and extracted with 2 volumes of ethyl acetate; extracts were then evaporated to dryness and redissolved in methanol for further analysis. Analysis of RK-682 was performed with a Phenomenex C_{18} column (5 μm, 4.6 by 250 mm) with elution at a flow rate of 1 ml/min over a 15-min gradient as follows: time $T = 0$, 60% B; $T = 6$ min, 95% B; $T = 9$ min, isocratic gradient with 95% B; $T = 12$ min, 60% B; $T = 15$ min, isocratic gradient with 60% B (phase A, ammonium acetate; phase B, methanol). LC-ESI-HRMS analysis was carried out on an LTQ XL Orbitrap (Thermo, Fisher Scientific) coupled with an Accela photodiode array (PDA) detector, Accela PDA autosampler, and Accela pump, using electrospray ionization in the negative-ion mode.

Fermentation, isolation, and analysis of holomycin. For holomycin production, *S. albus* mutants were incubated in TSBY broth (3% tryptone soy broth, 10.3% sucrose, 0.5% yeast extract) at 28°C for 2 days and then transferred to YD broth (0.5% yeast extract, 1% malt extract, 0.4% glucose, 0.2% $MgCl_2$, 0.15% $CaCl_2$ [pH 7.2]) and incubated for 4 days. The fermentation culture was centrifuged at 5,000 × *g* for 10 min, and the supernatant was extracted with two volumes of ethyl acetate. The extract was evaporated to dryness under reduced pressure and redissolved in methanol for further analysis. Analysis of holomycin was performed with a Phenomenex C_{18} column (5 μm, 4.6 by 250 mm) eluted at a flow rate of 1 ml/min over a 30-min gradient as follows: $T = 0$, 10% B; $T = 2$ min, 10% B; $T = 7$ min, 30% B; $T = 27$ min, 100% B; $T = 30$ min, 90% B (phase A, H_2O; phase B, methanol). The LC-ESI-HRMS analysis was carried out on an LTQ XL Orbitrap (Thermo, Fisher Scientific) coupled with an Accela PDA detector, Accela PDA autosampler, and Accela pump, using electrospray ionization in the positive-ion mode.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01714-15/-/DCSupplemental.

Figure S1, PDF file, 0.3 MB.
Figure S2, PDF file, 0.4 MB.
Table S1, PDF file, 0.2 MB.
Table S2, PDF file, 0.2 MB.
Table S3, PDF file, 0.1 MB.

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