Reports

A strategy for seamless cloning of large DNA fragments from Streptomyces

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We report a novel method for the seamless cloning of large DNA fragments (SCLF) of up to 44 kb or larger from \textit{Streptomyces} chromosomal DNA. SCLF is based on homologous recombination in \textit{Streptomyces} and is easy to perform. The strategy of SCLF is to flank the target sequence in the chromosomal DNA with two identical restriction sites by the insertion of plasmids containing that site at either end of the fragment, which is then isolated by plasmid rescue through the self-ligation of restriction digested genomic DNA. The method involves three steps: (i) placing a certain restriction site (CRS) at the 3’-end of the target sequence by insertion through homologous recombination of a plasmid containing the CRS; (ii) inserting through homologous recombination at the 5’-end of the target sequence a linearized self-suicide vector with the identical CRS; (iii) digesting the genomic DNA with the certain restriction enzyme followed by self-ligation in order to plasmid rescue the target fragment. SCLF can be applied to other Actinomycetales, and further optimizations may reduce the amount of time required to perform this technique.

Large DNA fragment cloning is an important technique in molecular biology. Traditional methods such as PCR, genome library construction, and chemical synthesis have their respective limitations. PCR is widely used for DNA cloning, but it is difficult to amplify DNA sequences longer than 10 kb. Another popular method for large fragment cloning is via the construction of a genome library using cosmids (1–2), bacterial artificial chromosomes (BAC) (3–4), or other vectors, but extra sequences flanking the target sequence are undesirable yet unavoidable in the isolated clones. Chemical synthesis is a powerful approach but can be very expensive. Moreover, it is also a challenge to synthesize complex sequences with special secondary structures resulting from high G+C content, repeats, etc. To overcome these obstacles, some improved methods for long DNA sequence synthesis have been proposed, such as thermodynamically balanced inside-out PCR-based gene synthesis (6), the method developed by Smith et al. (6), PTDS (PCR-based two-step DNA synthesis) (7), SLiCE (seamless ligation cloning extract) (8), etc. Yet most of these techniques (5–7) can have difficulty obtaining exact sequences larger than 6 kb, and the utility of SLiCE is restricted because it cannot facilitate DNA cloning with long flanking heterologous sequences (8).

Recently, several methods for large DNA fragment cloning (9–11) have been reported. Zhang et al. (9) devised a strategy based on redET recombination to clone foreign DNA from DNA pools. The target fragments obtained from the BAC clones could be up to 28 kb, but this approach does not allow direct cloning of DNA regions larger than a certain size, e.g., 10 kb, from genomic DNA. Techniques based on in vivo recombination in yeast (10–11) allow the cloning of fragments up to 80 kb, but the target large fragments are assembled from a number of relatively small fragments with overlaps. However, when used for cloning PKS (polyketide synthase) genes, the assembly efficiency can be low, and most of the constructs undergo severe gene deletions. This failure is due to the repeating PKS domains, which share very similar sequences (10). Similarly, large target fragments with repeats are difficult to clone via such methods.

Seamless cloning of DNA fragments plays an important role in many applications, such as synthetic biology (12,13), gene fusion (14), etc. Conventional seamless cloning techniques involve type II restriction enzyme digestion and DNA ligation. Such techniques rely on restriction sites and cannot be widely applied. To date, several effective methods

METHOD SUMMARY

Seamless cloning of large DNA fragments (SCLF) is a method for cloning DNA fragments up to 44 kb or larger from \textit{Streptomyces}. SCLF consists of steps that are well-established and can be easily carried out. Our new approach would greatly facilitate large DNA fragment manipulation in Actinomycetales.
have been developed. These methods are either based on in vitro recombination (8,15) or on the introduction of special restriction sites (16–17) and are applicable in most circumstances.

Here we introduce a novel strategy, seamless cloning of large DNA fragments (SCLF), which is based on in vivo recombination in Streptomyces. Via SCLF, we can clone not only long target DNA fragments from Streptomyces chromosome precisely but also generate seamless junctions between the target sequence and its flanking sequences if necessary. To illustrate the application of SCLF, we present two examples: one is the cloning of a 44-kb DNA fragment directly from the Streptomyces avermitilis chromosome to fill in the gaps of a genome library we constructed previously (2), and the other is the construction of a plasmid for the seamless replacement a large gene.

Material and methods

Bacterial strains and culture conditions

Escherichia coli DH5α was used for cloning, and E. coli ET12567 (pIJ9320) (18) was used for conjugation. Both E. coli strains were cultured in LB medium (18). S. avermitilis G8–17 (19) and Streptomyces hygroscopicus HS023 (CGMCC No. 7677) were cultured as described previously (19). Antibiotics were added at the concentrations specified in the protocol (18).

Plasmids used for conjugation

Three suicide conjugative vectors were used in this study: pUAMT14 was constructed previously (19), and pMSST4 and pSNT3 (Figure 1) were constructed as follows: Plasmid pMSST4 was constructed by inserting the spectinomycin resistance cassette into the SspI-DraI site of pMD19-Simple (TaKaRa Biotechnology, Dalian, China). The resistance cassette was obtained from pUA778 (18) by restriction digestion with SacI and BstBI. This 1281-bp fragment containing aadA (spectinomycin resistance gene) and oriT (origin of transfer) was blunted before ligation. Plasmid pSNT3 was generated by replacing the EcoRI-HindIII fragment of supercos-1 (Stratagene, La Jolla, CA) with an oriT fragment that was PCR amplified from pUA773. The primers used in this reaction were orf (5'-ccggtttggtctacctcaaccggcgtcagc-3') and AVI3R (5'-aaccaagcttcgaatctgccgaagcagc-3'), the EcoRI restriction site is underlined) and oriR (5'-acaagcttcagaagttccgcccagc-3'), the HindIII restriction site is underlined).

Molecular biology techniques

Isolation of Streptomyces genomic DNA was performed using standard protocols (20). PCR amplification with PrimeSTAR HS DNA Polymerase (TaKaRa Biotechnology) was carried out according to the manufacturer’s manual. Plasmids were isolated from E. coli by alkaline lysis (21). DNA fragments were phosphorylated and blunted using the Blunting Kination Ligation Kit (TaKaRa Biotechnology) when needed. The Inoue (22) or electroporation (18) methods were used for the preparation and transformation of E. coli competent cells. Streptomyces strains were transformed by conjugation (18).

Strategy for SCLF

Three steps are involved in cloning a fragment from Streptomyces using SCLF (Figure 2A): (i) introduction of a certain restriction site (CRS) at the 3’-end of the target fragment by insertion of a linearized suicide plasmid containing the CRS; (ii) introduction of the same CRS at the 5’-end of the target fragment by insertion of another linearized suicide plasmid containing the CRS; and (iii) digestion of the genomic DNA from Step (ii) with the certain restriction enzyme followed by self-ligation in order to plasmid rescue the target fragment.

The strategy for the gene replacement plasmid construction needs extra insertions of the homologous arms of the replaced gene at both ends of the target DNA fragment in Steps (i) and (ii) (Figure 2B).

Construction of plasmid pSNT-MA1

The aim of this example is to clone a 43,899-bp DNA fragment from the chromosomal DNA of S. avermitilis G8–17. The sequence of the fragment is the same as that from 1165664 to 1205664 of the S. avermitilis MA-4680 genome (GenBank: BA000030.3).

In Step (i), the 2497-bp fragment homologous to the target fragment’s 3’ end was amplified with primers AVI3F (5’-ACCTGAGCACTCCGCTCAGC-3’) and AVI3R (5’-GATCTAGGTTCTCGAAGTGG-3’). The HindIII site used for conjugation is the same as that from 1165664 to 1205664 of the S. avermitilis MA-4680 genome (GenBank: BA000030.3).

In Step (ii), the 2446-bp segment of the target fragment’s 3’ end was amplified with oligonucleotides AVI3F (5’-GATCTAGGTTCTCGAAGTGG-3’) and AVI3R (5’-GATCTAGGTTCTCGAAGTGG-3’); HindIII) and AVI5R (5’-GATCTAGGTTCTCGAAGTGG-3’; HindIII). The PCR product was digested by HindIII and inserted into the same site of pMSST4 to give rise to plasmid pMSST-S3, which was subsequently introduced into G8–17 by conjugation. Since this suicide plasmid cannot self-replicate in Streptomyces unless integrated into the chromosome of the host, colonies growing under selection of spectinomycin meant that the plasmid had integrated into the chromosome by single-crossover homologous recombination.

In Step (iii), the 2446-bp segment of the target fragment’s 5’ end was amplified with oligonucleotides AVI5F (5’-AGGCTGGCAGAAGTGG-3’) and AVI5R (5’-AGGCTGGCAGAAGTGG-3’); HindIII) and AVI5R (5’-AGGCTGGCAGAAGTGG-3’; HindIII). The PCR product was digested by HindIII and inserted into the same site of pSNT3, and the resulting plasmid, pSNT-S5, was transformed into one of the transformants obtained in Step (i).

In Step (iii), the HindIII-fragment containing the linearized pSNT3 and the target fragment was plasmid rescued by HindIII digestion of the chromosomal DNA.

Figure 1. Maps of vectors pMSST4 and pSNT3. oriT: conjugative transfer origin; aadA: spectinomycin resistance gene; bla: carbenicillin resistance gene; neo: kanamycin resistance gene.
DNA of a transformant obtained in Step (i) followed by self-ligation of the HindIII-fragments, transformation of the self-ligated fragments into E. coli, and selection for kanamycin-resistant colonies to obtain the final plasmid, pSNT-AVS1 (see details in the section “Efficiency of construction for plasmids containing fragments of different sizes” below).

Construction of seamless gene replacement plasmid pUAmT-AMA1

In this example, the purpose was to construct a plasmid for replacing the aveA1 gene of S. avermitilis seamlessly with the milA1 gene from S. hygroscopicus.

Step (i) is to seamlessly place a 3266-bp downstream fragment of aveA1 (AD) at the 5’ end of milA1. The 3212-bp fragment homologous to the milA1 fragment was PCR amplified with primers MA13F (5′-GCCTGCCACTCCGCCCGTATC-3′) and MA15R (5′-TTGCCCAAAGCCCAGAAC-3′) from S. hygroscopicus HS023. AD was amplified from S. avermitilis G8–17 using oligonucleotides AA1DF (5′-ACCGGACGCCTGCCACTCCGCCCGTATC-3′) and AA1DR (5′-attaatatGCCCTGTGTCGCCGCTC-3′). These two fragments were successively inserted into the conjugative vector pMSST4: M3 was phosphorylated and cloned into the EcoRI site before ligation, and the SwaI site was set as the CRS. pMHJ03 was then transformed into S. hygroscopicus H5023. The 2899-bp segment of milA1 5′-end (M5) was generated by PCR from S. hygroscopicus with primers MA15F29 (5′-TTGCCCAAAGCCCAGAACGAGTTCG-3′) and MA15R (5′-attaatatCCACGGGCTTGTCCACGTGC-3′, Swal). These two fragments were subcloned into pUAmT14 in turn: AU was inserted as an EcoRI-SphI fragment, and M5 was then inserted into the 3′-end of AU using the blunt-end SphI site of AU. The resulting plasmid, pMHJ08, was transformed into one of the transformants obtained in Step (i). In this step, the seamlessness of junction between AU and M5 was achieved by blunting of the SphI-digested-end before ligation.

In Step (iii), the arranged DNA fragments were digested from the chromosomal DNA by SwaI, self-ligated, and transformed into E. coli. Apramycin-resistant colonies were selected to obtain the gene replacement plasmid pUAmT-AMA1 (see details in the section “Efficiency of construction for plasmids containing fragments of different sizes” below).

Efficiency of construction for plasmids containing fragments of different sizes

In order to calculate the efficiency of construction, we standardized the amounts used. The electroporation and inoue methods were used for transformation. The transformation efficiencies of the two methods were ~3 × 10^6 and ~2 × 10^7 per µg of pUC19 DNA (TaKaRa Biotechnology), respectively. Approx-
imately 6 μg of genomic DNA was digested by the corresponding restriction endonuclease. After alcohol precipitation, 20 μl 2 mM Tris-HCl (pH 8.0) was used to dissolve the recovered fragment. The recovered fragment was self-ligated with T4 DNA ligase (TaKaRa Biotechnology, Japan) and recovered by alcohol precipitation. The ligation product was divided into two equal portions and transformed into DH5α competent cells by the electroporation and Inoue methods. The cultures of the transformed cells were concentrated by centrifugation and spread on an LB plate containing the appropriate antibiotic. All of the experiments starting from the restriction digestion were repeated three times. The number of transformants growing on each plate was counted manually.

Results and discussion

To obtain the desired target DNA fragment (43899 bp) directly from the chromosomal DNA of *S. avermitilis* G8–17, plasmid pSNT-AVS1 was constructed via SCLF (Figure 2A). The self-suicide conjugative vectors pSNT-5S and PMSST-S3 were inserted at the 5’ and 3’ ends, respectively, of the target DNA region in *S. avermitilis* G8–17, which then flanked the target DNA fragment with a *Hind*III site from each plasmid such that the vector backbone of the inserted pSNT-5S is contained in the same *Hind*III fragment as the target DNA sequence. The chromosomal DNA of this transformed cell line was digested by *Hind*III and, after self-ligation, was transformed into *E. coli* DN5α by the Inoue method. Twelve kanamycin-resistant colonies were randomly selected from the transformation plates for plasmid extraction. The plasmids were digested with *Pst*I to screen for the correct plasmids containing the target DNA region, and nine of the plasmids were confirmed to be correct (Figure 3).

Plasmid pUAmT-AMA1 was constructed for seamless replacement of the *aveA1* gene in *S. avermitilis* G8–17 with the *milA1* gene from *S. hygroscopicus* HS023 (13). The aim of the procedure (Figure 2B) was to place the *milA1* gene exactly between the upstream and downstream fragments of *aveA1* (AU and AD, respectively) that were to be used for homologous recombination in the gene replacement. This was done in Steps (i) and (ii) by inserting the self-suicide conjugative vectors pMHJ08 (containing AU and a *Swa*I site as the CRS) and pMHJ03 (containing AD and a *Swa*I site as the CRS) at the 5’ and 3’ ends, respectively, of the *milA1* gene. This resulted in a *Swa*I fragment containing the vector backbone of pMHJ08 and the two arms for homologous recombination in *S. avermitilis* pMHJ08 and downstream fragments of *aveA1* (AU and AD, respectively) that were to be ligated in Step (iii), 12 randomly selected apramycin-resistant colonies were cultured for plasmid extraction. These plasmids were then analyzed by restriction digestion using Sacl, and all 12 plasmids were confirmed to be correct (Figure 4).

The numbers of transformants are listed in Table 1. Although the transformants growing on the Inoue transformation plate were sparse when the target fragment was up to 44 kb, the high rate of correct clones (75%) made them sufficient for routine cloning. The numbers of transformants were decreased when the target fragment size increased. It seems that the maximum size of the target fragment is ~44 kb if the Inoue transformation method is used in the last step of SCLF. In order to obtain even larger DNA fragments, transformation methods of higher efficiency (e.g., electroporation, in which the efficiency can be as high as 10¹⁰ per μg of pUC19 DNA if carefully prepared) can be adopted.

Generally, digestion of chromosomal DNA by frequently cutting restriction endonucleases generates numerous

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Size of entire plasmid (bp)</th>
<th>Size of cloned fragment (bp)</th>
<th>Origin of fragments</th>
<th>Amount of colonies</th>
<th>Correct rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUAmT-AMA1</td>
<td>22518</td>
<td>13032</td>
<td><em>S. hygroscopicus</em></td>
<td>560 (142 ± 23)</td>
<td>100</td>
</tr>
<tr>
<td>pSNT-AV1</td>
<td>48611</td>
<td>43899</td>
<td><em>S. avermitilis</em></td>
<td>98 ± 8 (12 ± 4)</td>
<td>75.0</td>
</tr>
</tbody>
</table>

a Approximately 3 μg of self-ligated digested genomic DNA was used for each transformation.

b The numbers represent transformants grown in electroporation plates, while the numbers in parentheses represent transformants grown in Inoue plates.

c Twelve transformants of each Inoue plate were randomly selected for restriction analysis.

(A) Locations of the *Pst*I sites in the target fragment. (B) Expected restriction fragment gel patterns for *Pst*I digestion of pSNT-AVS1. (C) *Pst*I restriction analysis of the 12 randomly selected recombinants. The digestion products were fractionated on a 1% agarose gel. M: DNA ladder (Fermentas Inc., Burlington, Canada).
small fragments. Since these small fragments are present in the self-ligation reaction of the plasmid rescue step, they can be easily recruited into the rescued plasmid. Hence, restriction endonucleases whose recognition sequences are rare in the chromosomal DNA should be preferentially considered as candidates for CRSs. In dealing with Streptomyces, the rarely cutting restriction endonuclease SwaI is usually suitable. Since the genomes of most Streptomyces species have a G+C content >60%, the SwaI recognition sequence ATTTAAC seldom appears in such genomes. For instance, there are only 1 and 2 SwaI sites in the chromosomal DNA of *S. avermitilis* and *S. hygroscopicus*, respectively. However, during the process of constructing plasmid pSNT-AV1, numerous small fragments from 18 to 100 bp were generated when the *Streptomyces* chromosomes were digested by HindIII, but no inserted small fragments were detected in the final plasmids. A possible reason for this might be that the efficiency of self-ligation of a large DNA fragment is much higher than that of ligation with other fragments. Therefore, restriction sites that do not exist in the final plasmid can be used as CRSs. Nevertheless, in some exceedingly rare instances, if the target sequence includes almost all of the known restriction sites, then there is no available CRS, and SCLF cannot be used to obtain that target sequence.

Perhaps the main advantage of SCLF is that large DNA fragments up to 44 kb can be precisely captured from chromosomes. Current methods in the same field (10–11) are unable to clone large target fragments with repeat sequences, whereas SCLF is able to. In fact, the size of the target fragment could be larger, since conventional plasmids have the ability to harbor DNA fragments ranging from 40 to 100 kb (23), and there is no difference in the procedures for cloning fragments of different sizes. Although SCLF is time-consuming and is not superior to traditional methods for constructing gene replacement plasmids, it is still a robust technique when the desired plasmid is for a large gene replacement and seamless junctions are simultaneously needed. Also, SCLF can partially substitute for the use of a genome library. Construction of a genome library involves several laborious steps, such as partial digestion of genomic

![Figure 4. Restriction digestion analysis of pUAmT-AMA1.](image)
DNA and screening of the target library, and the target DNA fragment is usually not contained within a single clone and is accompanied by undesired flanking sequences, which need to be removed. In contrast, SCLF does not suffer these problems.

In addition, target fragments obtained by SCLF will be of high fidelity. For those methods based on PCR amplification, mutations can be easily introduced if the target fragment is too long. SCLF uses several steps involving PCR to obtain homologous arms. In general, the relatively small sizes of these PCR products, for example, less than 700 bp in *S. avermitilis* (24), are sufficient for homologous recombination. As most high fidelity polymerases such as *Pfu* (Beyotime, Nanjing, China) and PrimerStar (TaKaRa Biotechnology) can generate high-quality PCR products, mutations would seldom happen and are easily excluded by sequencing. In the first example, 7 fragments with sizes from 574 to 643 bp were PCR amplified from the cloned fragment of pSNT-AV1. The sequencing results all agreed with those published in GenBank (data not shown).

Finally, SCLF does not require special materials and equipment. The vector can be derived from almost any plasmid that can replicate in *E. coli*. All related molecular techniques have been well-established and can be easily performed in a typical laboratory.

Although the examples presented here were all performed with *Streptomyces*, SCLF definitely could be applied to other Actinomycetales, given the genetic manipulation system that has been established. In fact, most Actinomycetales can be transformed by conjugation, even those that have been considered difficult to transform, such as *Saccharopolyspora spinosa* (25) or *S. hygroscopicus* (26). Furthermore, the current basic protocol could be simplified by adopting other alternative techniques. For example, the seamless cloning procedure will be also feasible if the SLiCE method (8) is used or the *Eam1401* (16) or type IIB restriction enzyme (17) recognition sites are introduced, and the *EtNa* method (27) might make genomic DNA extraction easier.

In conclusion, SCLF is an innovative technique for large DNA fragment cloning from Actinomycetales that will supplement the few other available methods in the field.

**Author contributions**

J.H. contributed to the conception of the study, acquisition of data, analysis, and interpretation of data, and drafting of the article. Z.Y., M.H.L., and N.L. performed experiments. J.Z. contributed to the analysis and interpretation of data and drafting of the article. Y.G.Z. contributed the conception of the study and general supervision.

**Competing interests**

The authors declare no competing interests.

**References**


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