RESEARCH LETTER

Recombinational cloning of the antibiotic biosynthetic gene clusters in linear plasmid SCP1 of Streptomyces coelicolor A3(2)

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Abstract
The model organism Streptomyces coelicolor A3(2) harbors a 356-kb linear plasmid, SCP1. We report here development of a recombinational cloning method for deleting large segment from one telomere of SCP1 followed by replacing with the telomere of pSLA2 and sequentially inserting with the overlapping cosmids in vivo. The procedure depends on homologous recombination coupled with cleavage at telomere termini by telomere terminal protein. Using this procedure, we cloned the 81-kb avermectin and the 76-kb spinosad biosynthetic gene clusters into SCP1. Heterologous expression of avermectin production in S. coelicolor was detected. These results demonstrate the utility of SCP1 for cloning large DNA segments such as antibiotic biosynthetic gene clusters.

Introduction
Streptomyces species are Gram-positive, high G+C, mycelial bacteria that are a major source of natural products, producing about half of all known microbial antibiotics (Bérdy, 2005). Unlike most other bacterial chromosomes, the 6.6- to 11-Mb chromosomes of Streptomyces are linear (Lin et al., 1993), and Streptomyces species often harbor linear as well as circular plasmids (Kinashi et al., 1987; Hopwood & Kieser, 1993). Linear plasmids range in size from 12 (Keen et al., 1988) to 580 kb (Kinashi & Shimaji, 1987). Their ‘telomeres’ contain inverted repeat sequences from 44 bp (Chen et al., 1993) to 180 kb long (Pandza et al., 1998), and the 5′ telomeric ends are linked covalently to telomere terminal proteins (Tpg: Bao & Cohen, 2001; Yang et al., 2002). Most Streptomyces linear plasmids and linear chromosomes have conserved telomeric sequences (e.g. pSLA2, SLP2, S. coelicolor, and S. lividans; Huang et al., 1998) and telomere replication genes (e.g. tap and tpg; Bao & Cohen, 2001, 2003; Yang et al., 2002), while some contain novel telomereres (e.g. SCP1, pRL1, pRL2, and S. griseus; Kinashi et al., 1991; Goshi et al., 2002; Zhang et al., 2006) and telomere replication proteins/genes (Huang et al., 2007; Stoll et al., 2007; Suzuki et al., 2008).

Unlike the terminal protein-capped linear replicons of adenoviruses and Bacillus bacteriophage ɸ29 (Salas, 1991), replication of Streptomyces linear plasmids starts at a central locus (Shiffman & Cohen, 1992) and proceeds bidirectionally toward the telomeres (Chang & Cohen, 1994). This leaves short single-stranded overhangs at the 3′ telomeric ends of linear plasmids as a replication intermediate (Chang & Cohen, 1994), to be converted to a fully double-stranded form by DNA synthesis primed by the telomere terminal proteins (Bao & Cohen, 2001; Yang et al., 2006). Although replication and inheritance of Streptomyces linear plasmids have been studied for a long time, only a few vectors derived from linear plasmids have been developed for cloning DNA segments (e.g. < 20 kb; Qin et al., 2003).

Streptomyces coelicolor A3(2) is the genetically most studied Streptomyces species from the in vivo through in vitro to in silico years (Hopwood, 1999). Streptomyces coelicolor A3(2) harbors a 356 023-bp linear plasmid, SCP1 (Bentley et al., 2004); a 31 317-bp circular plasmid, SCP2 (Haug et al., 2003); and a 17-kb chromosome-
integrating plasmid, SLP1 (Bibb et al., 1981; Omer & Cohen, 1984). SCP1 was genetically identified 40 years ago (e.g. Hopwood et al., 1969), but was physically detected by pulsed-field gel electrophoresis much later (Kinashi et al., 1987). This large linear plasmid contains long inverted repeats of 75 122 bp (Kinashi & Shimaji-Murayama, 1991; Bentley et al., 2004), in the formation of which the insertion element IS466 may be involved (Kinashi et al., 1991). SCP1 carries the methylenomycin biosynthetic gene cluster (Chater & Bruton, 1985). Almost identical methylenomycin biosynthetic gene clusters are also present on a circular plasmid pSV1 of Streptomyces violaceoruber (Aguilar & Hopwood, 1982; Chater & Bruton, 1985; Kinashi et al., 1987; Yamasaki & Kinashi, 2004). SCP1 can integrate into chromosome of Streptomyces coelicolor (Hopwood & Wright, 1976), and a variety of co-integrates and hybrids have been found between SCP1 and the host chromosome, which can increase chromosome transfer; for example, strain 2612 can undergo exchange with the chromosome to produce a 1843-kb linear plasmid and a 7.2-Mb linear chromosome (Hopwood & Wright, 1976; Yamasaki & Kinashi, 2004). It suggests that single crossover recombination between a linear plasmid and the chromosome to generate molecules with heterologous telomeric ends and very large genomic segments may be cloned in SCP1 in vivo. Recombination between the linear plasmid pZG101 and the linear chromosome of Streptomyces rimosus to exchange of their ends is also observed (Pandza et al., 1998). It appears that linear plasmids have played key roles in the architecture, accessory gene content, and rapid evolution of Streptomyces chromosomes (Chater & Kinashi, 2007).

To exploit the potential of SCP1 for cloning large genomic segments, such as Streptomyces antibiotic biosynthetic gene clusters (usually ranging in size from 20 to 140 kb; e.g. Deng & Bai, 2006), we designed here a recombinational procedure. Using this new method, we cloned the 81-kb avermectin (Ikeda et al., 2003) and the 76-kb spinosad biosynthetic gene clusters (Waldron et al., 2001) in SCP1 in S. coelicolor A3(2).

Materials and methods

Bacterial strains, plasmids, and general methods

Strains and plasmids used in this work are listed in Table 1. The primers used for vector construction, cloning the X fragment, and PCR-targeting in this article are listed in Supporting Information, Table S1. The vector constructions and the restriction sites were described in Fig. S1. Plasmid isolation, transformation of Escherichia coli DH5α, and PCR amplification followed Sambrook et al. (1989). Streptomyces culture, antibiotic selection, conjugation from E. coli into Streptomyces, plasmid isolation, and pulsed-field gel electrophoresis followed Kieser et al. (2000). Cosmid libraries of Streptomyces avermitilis NRRL 8165 and Saccharopolyspora spinosa were constructed in pHAQ34 (Xia et al., 2009) using the Giga-pack® III XL Gold Packaging Extract Kit (Stratagene). The ordered cosmid libraries were obtained by PCR-sequencing the ends of inserts of c. 1000 cosmids and compared with the complete nucleotide sequence of the S. avermitilis chromosome. Gene disruption and replacement in Streptomyces followed the Red/ET-mediated PCR-targeting method (Gust et al., 2003). The complete nucleotide sequence of the 338-kb plasmid pZR695-p was obtained on the Genome Sequencer FLX 454 System (Roche) at the Chinese Human Genome Center in Shanghai. The nucleotide sequence of pZR695-p was submitted to GenBank under accession number KC907349.

Linear plasmid embedding in gel for enzyme digestion and Southern hybridization

Strain ZR695 containing linear plasmid pZR695-p was inoculated into tryptone soya broth (TSB, Oxoid) liquid medium, and mycelium was embedded in agarose plugs for pulsed-field gel electrophoresis. After electrophoresis of pZR695-p in low-melting-point agarose, gel slices containing the appropriate band were cut for digestion with restriction enzymes (EcoRI, PstI, and XhoI) overnight. The digested DNA was separated by electrophoresis in a 0.7% agarose gel at 2 V cm⁻¹ for 8 h and blotted onto a nylon membrane (Amersham). Southern hybridization was performed with mixed probes of cosmids 6-09, ave-2, and ave-3 labeled with ‘digoxigenin (DIG) high prime’ according to product manual of ‘Detection Starter Kit’ (Roche).

Fermentation and detection of avermectin production

Spore of strain ZR695 from fresh YMS (0.4% yeast extract, 1% malt extract, 0.4% soluble starch, 2% agar, pH 7.0) plates was inoculated into 30 ml preculture medium (2.5% corn starch, 0.8% soybean cake flour, 1.0% peanut cake flour, 0.5% yeast powder, 0.3% corn steep liquor, 0.003% cobalt chloride, pH 7.2) in a 250-ml flask and incubated at 28 °C, 250 r.p.m. on orbital shaker for 40 h. About 2 ml preculture was inoculated into 30 ml production medium [12% corn starch (liquefied at 85 °C with 0.02% alpha-amylase), 3.0% soybean cake flour, 1.0% yeast powder, 0.1% zeolite powder, 0.002% cobalt chloride, 0.0002% MnSO₄, 0.0001% Na₂MoO₄, 0.00025% ZnSO₄, pH 7.2] and incubated under the same condition.
for 8 days. Culture broth was collected by centrifugation at 13 000 g for 10 min. The pellet was extracted with equal volume ethanol for 24 h. The ethanol phase was examined by Agilent 1200 high-performance liquid chromatography (HPLC) machine (Agilent) to determine the production of avermectin. The avermectins produced

<table>
<thead>
<tr>
<th>Strain and plasmid</th>
<th>Genotype or description</th>
<th>Source or reference</th>
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</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
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<tr>
<td>Streptomyces coelicolor A3(2)</td>
<td>Harbors linear plasmid SCP1</td>
<td>Kieser et al. (2000)</td>
</tr>
<tr>
<td>Streptomyces avermitilis MMR630</td>
<td>An avermectin producer</td>
<td>Xia et al. (2009)</td>
</tr>
<tr>
<td>ZR495-m</td>
<td>S. coelicolor harbors SCP1-derived pZR495-m and tsr</td>
<td>This work</td>
</tr>
<tr>
<td>ZR458-p</td>
<td>S. coelicolor harbors SCP1-derived pZR458-p and aac(3)IV</td>
<td>This work</td>
</tr>
<tr>
<td>ZR692-p</td>
<td>S. coelicolor harbors SCP1-derived pZR692-p and aadA</td>
<td>This work</td>
</tr>
<tr>
<td>ZR695-p</td>
<td>S. coelicolor harbors SCP1-derived pZR695-p and aac(3)IV</td>
<td>This work</td>
</tr>
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<td>Saccharopolyspora spinosa</td>
<td>A spinosad producer</td>
<td>Waldron et al. (2001)</td>
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<tr>
<td>ZR577-m</td>
<td>S. coelicolor harbors SCP1-derived pZR577-m and tsr</td>
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</tr>
<tr>
<td>ZR588-p</td>
<td>S. coelicolor harbors SCP1-derived pZR588-p and aac(3)IV</td>
<td>This work</td>
</tr>
<tr>
<td>ZR595-p</td>
<td>S. coelicolor harbors SCP1-derived pZR595-p and aadA</td>
<td>This work</td>
</tr>
<tr>
<td>ZR648-p</td>
<td>S. coelicolor harbors SCP1-derived pZR648-p and aac(3)IV</td>
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<tr>
<td>ZR669-p</td>
<td>S. coelicolor harbors SCP1-derived pZR669-p and aadA</td>
<td>This work</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
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<td>pBluescript II SK</td>
<td>amp colEI-ori lacZ</td>
<td>Stratagene, Inc.</td>
</tr>
<tr>
<td>pQC156</td>
<td>A 2.6-kb BclI fragment of melC/tsr cloned in pSP72 (BglII)</td>
<td>Qin et al. (2003)</td>
</tr>
<tr>
<td>pZR131</td>
<td>Two 381-bp pSLA2 telomeres tsr melC amp colEI</td>
<td>Xia et al. (2009)</td>
</tr>
<tr>
<td>pHAQ34</td>
<td>amp tsr melC cos colE1</td>
<td>Zhang et al. (2008)</td>
</tr>
<tr>
<td>SuperCos1</td>
<td>neo cos colE1</td>
<td>Kieser et al. (2000)</td>
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<tr>
<td>Cosmid 6-09</td>
<td>SuperCos1 contains a 42-kb insert of S. avermitilis</td>
<td>This work</td>
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<tr>
<td>Cosmid ave-2</td>
<td>pHAQ34 contains a 46-kb insert of S. avermitilis</td>
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<tr>
<td>Cosmid ave-3</td>
<td>pHAQ34 contains a 47-kb insert of S. avermitilis</td>
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<td>pZR495</td>
<td>pZR131 contains a 1-kb oriT/a 1.7-kb fragment of SCP1/a 2.3-kb fragment of S. avermitilis (PCR)</td>
<td>This work</td>
</tr>
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<td>pZR445</td>
<td>pBluescript II SK contains an aac(3)IV-oriT-SLP2 telomere cassette (PCR)</td>
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<td>pZR568</td>
<td>pBluescript II SK contains an aadA-pSLA2 telomere cassette (PCR)</td>
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</tr>
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<td>pZR546</td>
<td>pBluescript II SK contains an aac(3)IV-pSLA2 telomere cassette (PCR)</td>
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<td>pZR458</td>
<td>Cosmid 6-09 contains an aac(3)IV-oriT-SLP2 telomere cassette of pZR445 (PCR-targeting)</td>
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<td>pZR692</td>
<td>Cosmid ave-2 contains an aadA-pSLA2 telomere cassette of pZR568 (PCR-targeting)</td>
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</tr>
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<td>pZR695</td>
<td>Cosmid ave-3 contains an aac(3)IV-pSLA2 telomere cassette of pZR546 (PCR-targeting)</td>
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<tr>
<td>Cosmid spn-1</td>
<td>pHAQ34 contains a c. 40-kb insert of S. spinosa</td>
<td>This work</td>
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<td>Cosmid spn-2</td>
<td>pHAQ34 contains a c. 40-kb insert of S. spinosa</td>
<td>This work</td>
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<tr>
<td>Cosmid spn-3</td>
<td>pHAQ34 contains a c. 40-kb insert of S. spinosa</td>
<td>This work</td>
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<tr>
<td>Cosmid spn-4</td>
<td>pHAQ34 contains a c. 40-kb insert of S. spinosa</td>
<td>This work</td>
</tr>
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<td>pZR557</td>
<td>pZR131 contains a 1-kb oriT/a 1.7-kb fragment of SCP1/a 1.7-kb fragment of S. spinosa (PCR)</td>
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<td>pZR547</td>
<td>pBluescript II SK contains an aac(3)IV-SLP2 telomere cassette (PCR)</td>
<td>This work</td>
</tr>
<tr>
<td>pZR569</td>
<td>pBluescript II SK contains an aadA-SLP2 telomere cassette (PCR)</td>
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<td>pZR588</td>
<td>Cosmid spn-1 contains an aac(3)IV-SLP2 telomere cassette of pZR547 (PCR-targeting)</td>
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<td>pZR595</td>
<td>Cosmid spn-2 contains an aadA-SLP2 telomere cassette of pZR569 (PCR-targeting)</td>
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<td>pZR648</td>
<td>Cosmid spn-3 contains an aac(3)IV-pSLA2 telomere cassette of pZR546 (PCR-targeting)</td>
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<tr>
<td>pZR669</td>
<td>Cosmid spn-4 contains an aadA-pSLA2 telomere cassette of pZR568 (PCR-targeting)</td>
<td>This work</td>
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</table>
by the strains were confirmed by HPLC-MS analysis. HPLC is developed on Venusil ABS C18 (Agela Technologies) column at flow rate 0.2 mL min⁻¹ under 30 °C. The wavelength detector was set as 245 nm. The mobile phase methanol/water = 85 : 15. MASS machine was Agilent 6520 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF).

**Results**

**A recombinational deletion and insertion procedure**

This strategy was based on (1) large antibiotic biosynthetic gene clusters were often cloned and identified in a set of overlapping cosmids, with various sizes of homologous sequences at the ends of the inserts (e.g. Deng & Bai, 2006); (2) introduction of a circular plasmid containing telomeres into *S. lividans* (and *S. coelicolor*) resulted in the formation of linear replicons (i.e. precise deletion of an *E. coli* portion bracketing the two telomeres of plasmids) in some clones and circular molecules in others (Qin & Cohen, 1998; Zhang et al., 2009). These linear and circular molecules could be distinguished by inserted selection makers within the bracketed telomeres and at the *E. coli* vector portion. The 356-kb SCP1 linear molecule contains terminal inverted repeats (TIRs) of about 75 kb, and its internal replication locus lies near the center of the molecule (Redenbach et al., 1999; Bentley et al., 2004). In the first, one TIR of SCP1 was completely deleted and recombinationally replaced by a linear plasmid pSLA2 telomere. As illustrated in Fig. 1, pZR495 containing a 1.7-kb SCP1 segment (X, 250 073–251 785 bp) was introduced by conjugation from *E. coli* ET12567 (pUZ8002) into *S. coelicolor* A3(2). Among 12 thiostrepton-resistant colonies, we obtained one clone containing a c. 257-kb linear plasmid (detected by pulsed-field gel electrophoresis, designated pZR495-m), while other clones contained DNA band similar to SCP1. PCR amplification and DNA sequencing proved deletion of a c. 104-kb SCP1 segment and no *E. coli* portion on pZR495-m.

To perform subsequent cloning of antibiotic biosynthetic gene clusters on pZR495-m, pZR495 also contained a 2.3-kb sequence (1 130 183–1 132 510 bp, ‘A’) from one end of the 80-kb avermectin biosynthetic gene cluster for homologous recombination of cosmids (e.g. 6-09) containing a homologous sequence. To delete the *E. coli* portion of cosmid 6-09, an aac(3)IV–oriT–SLP2 telomere cassette from pZR445 was inserted near the other end of the gene cluster to obtain pZR458. In the first step (Fig. 2), pZR458 was introduced by conjugation from *E. coli* into *S. coelicolor* containing pZR495-m (designated ZR495-m). Among 15 apramycin-resistant colonies, three clones also showed both thiostrepton- and kanamycin-sensitive phenotypes and harbored a c. 292-kb linear plasmid (pZR458-p), suggesting Tpg cleavage at the SLP2 telomere terminus, and others still contained the *tsr* and *neo* genes. Thus, in one recombinational deletion step, a c. 37-kb sequence of the avermectin biosynthetic gene cluster from cosmid 6-09 was cloned via homologous recombination in linear plasmid pZR495-m in *S. coelicolor*.

Because there were no *tsr* and *neo* genes on pZR458-p (determined by PCR), as illustrated in Fig. 2, the next recombinational insertion step was available for insertion of another overlapping cosmid containing a homologous sequence of pZR458-p. Thus, the recombinational insertion step could be performed more than once in principle.

**Fig. 1.** Schematic of the recombinational step for deletion of a SCP1 segment. pZR495 containing a 1.7-kb SCP1 segment (X, indicated by a striped box) and two pSLA2 telomeres (filled arrowheads) was introduced by conjugation from *Escherichia coli* into *Streptomyces coelicolor* A3(2) containing SCP1. After thiostrepton selection, the co-integration of SCP1 and pZR495 was obtained. Deletions of a 104-kb SCP1 segment and the *E. coli* portion of pZR495 (i.e. pZR495-m) among individual clones were determined by pulsed-field gel electrophoresis and PCR. *tsr*, thiostrepton-resistant gene.
Cloning and heterologous expression of the 81-kb avermectin biosynthetic gene cluster

To clone the 81-kb avermectin biosynthetic gene cluster (Ikeda et al., 2003) in SCP1, as required by the recombinational deletion and insertion method, we constructed a cosmid library of S. avermitilis using pHAQ34 containing two cos sites and the tsr selection marker (Xia et al., 2009). By sequencing the ends of the inserts and comparing with the published avermectin gene sequences (Ikeda et al., 2003), we identified two overlapping cosmids (ave-2 and ave-3) plus cosmid 6-09 covering the entire 81-kb avermectin gene sequences (Fig. 3). After insertion of an aadA–pSLA2 telomere cassette from pZRS68 in cosmid ave-2 by PCR-targeting to obtain pZRS692 and an aac(3)IV–pSLA2 telomere cassette from pZRS46 in ave-3 to obtain pZRS695, we continued the sequential insertion steps in pZRS48 (Fig. 2). Similarly, after introduction of pZRS692 into strain ZR458-p and of pZRS695 into ZR692-p by conjugation, respectively, pZRS692-p and pZRS695-p were obtained by antibiotic selections and PCR confirmation.

These SCP1-derived linear plasmids containing various sizes of the avermectin genes in the recombinational deletion and insertion experiments were electrophoresed in a pulsed-field gel. As shown in Fig. 4, bands representing plasmids called large deletion of SCP1 (pZRS495-m) and then insertions (i.e. pZRS48-p, pZRS692-p, and pZRS695-p) were observed. To determine whether the final construct (pZRS695-p) contained the intact 81-kb avermectin biosynthetic gene cluster, we employed Southern hybridization with labeled probes of the three cosmids (6-09, ave-2, and ave-3). As shown in Fig. S2, the expected sizes of pZRS695-p bands digested with EcoRI (40, 20.7, 13.8, 5.4, 4.1, and 3.6 kb), PstI (19.5, 13.9, 11, 5.6, 5.4, 5.1, 5, 4.7, 4, and 3.1 kb), and XhoI (15.8, 15, 12.4, 10.2, 8.6, 7.4, 6.3, and 3.6 kb) were detected, indicating no visible deletions of the 83 744-bp sequence (1 213 927–1 130 183 bp) of the S. avermitilis genome in pZRS695-p. The 338-kb plasmid pZRS695-p was completely sequenced, and no mutation was found in the cloned avermectin biosynthetic genes.

Strains ZR695 and A3(2) were cultured in R5 or YEME liquid media, but no avermectin production was detected by HPLC. After optimization of culture media and fermentation, including using the modified preculture and production media and enriching extracts of culture, three peaks were detected in strains ZR695-p by HPLC under 245 nm wavelength. These peaks were further con-
firmed to be avermectin A2a, B1a, and A1a, respectively, by mass spectrum analysis (Fig. 5).

Cloning of the 76-kb spinosad biosynthetic gene cluster

Another example of assembling a large genomic segment using the recombinational method was provided by cloning the 76-kb spinosad biosynthetic gene cluster. Unlike the avermectin producer, *S. avermitilis*, there is no published complete genome sequence for the spinosad producer, *S. spinosa*, and only about 76 569 bp of the entire spinosad biosynthetic gene cluster is publicly available (GenBank number AY007564: Waldron et al., 2001).

A similar recombinational deletion and insertion strategy was employed to clone the spinosad biosynthetic gene cluster (Figs S3 and S4). As a prerequisite of the procedure, we constructed a cosmid library of *S. spinosa* in pHAQ34 and identified cosmids containing the spinosad genes by Southern hybridization. After sequencing the ends of the inserts and comparing with the complete nucleotide sequence of the spinosad genes, a set of overlapping cosmids was obtained (Fig. S5). Four kinds of cassettes [aac(3)IV–SLP2 telomere, aadA–SLP2 telomere, aac(3)IV–pSLA2 telomere, and aadA–pSLA2 telomere] from pZR547, pZR569, pZR546, and pZR568 were inserted by the Red/ET-mediated ‘PCR-targeting’ in cosmids (spn-1–4) to obtain pZR588, pZR595, pZR648, and pZR669, respectively (Fig. S4). As in the cloning and confirmation of the avermectin biosynthetic gene cluster (Figs 4 and S2), bands representing one large deletion and then four insertions on SCP1 were observed in a pulsed-field gel (Fig. S6). The expected sizes of pZR669-p bands digested with XhoI (32, 11, 5.6, 2.7, and 1.9 kb) and EcoRV (35, 16.9, 9.4, 5, and 1.8 kb) and then probed with cosmids (spn 1–4) were confirmed (Fig. S7).

Discussion

*Escherichia coli* F plasmid-based bacterial artificial chromosomes (BACs) and bacteriophage Pl-derived artificial chromosomes (PAC) are powerful genetic tools for cloning large genomic segments (up to 300 kb; Shizuya et al., 1992; Ioannou et al., 1994). By combined PAC with a *Streptomyces* φC31 site-specific integration system, Sosio et al. (2000) constructed an *E. coli–Streptomyces* artificial chromosome (ESAC). Using iterative rounds of homologous recombination in *E. coli*, a 90-kb genomic segment...
was assembled into an ESAC (Sosio et al., 2001). However, no *Streptomyces* autonomous-replicating circular or linear plasmid-derived BAC has been developed. We report here a recombinational deletion and insertion procedure in the 356-kb linear plasmid SCP1 of *S. coelicolor* A3(2), for first deleting large segment from one telomere of SCP1 and then sequentially adding the overlapping cosmids in vivo. Cloning the antibiotic biosynthetic gene cluster in an integrating BAC leads single copy of the vector to integrate into host chromosome; SCP1 is in single copy with respect to the chromosome and therefore similar to a chromosomal integration event.

Heterologous expression of *Streptomyces* antibiotic gene clusters is summarized in the review by Baltz (2010). The reference cited several expression systems that ranged from low to high expression levels. The complete A54145 (structurally related to daptomycin) biosynthesis gene cluster in an integrating BAC heterologously expresses in *S. ambofaciens* and *S. roseosporus* strains in yields of > 100 mg L⁻¹ (Alexander et al., 2010). Daptomycin production is initially much lower in *S. lividans* than in *S. roseosporus*; however, its production in *S. lividans* was improved by deletion of genes encoding the production of actinorhodin and by medium optimization to control the chemical form of the calcium-dependent antibiotic (Penn et al., 2006). In our experiment, using YEME medium for antibiotic fermentation to detect possible productivity of avermectin in strain ZR695-p or spinosad in strain ZR699-p failed. However, after using the modified preculture and production media and enriching extracts of culture, we detected three peaks of avermectin compounds.

During the course of cloning more antibiotic biosynthetic gene clusters (e.g. oligomycin of *S. avermitilis*) by the recombinational method, we found that the first deletion step was accomplished easily, but two or more insertion steps were often needed to screen many colonies and sometimes failed to yield desired clones. The homologous

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**Fig. 5.** Detection and confirmation of avermectin production. The extracts of culture broth of strains ZR695 and A3(2) were detected by HPLC, and the peaks were collected for mass spectra. The molecular ion[M+K] of avermectin A2a, B1a, and A1a are shown.
sequences of multiple pks genes (encoding polyketide synthases) in some antibiotic biosynthetic gene clusters could recombine to result in deletions or rearrangements in the insertion steps. Selection of overlapping cosmids with long identical sequences would reduce undesirable homologous recombination events in other regions.

While screening desirable clones, we found that 'cleavage' by the telomere terminal proteins of S. coelicolor at telomere termini of linear pSLA2 was more efficient than that of the S. lividans chromosome (i.e. SLP2 telomere: Lin et al., 1993). Qin & Cohen (1998) found that introduction of a circular plasmid containing two pSLA2 telomeres into S. lividans ZX7 by transformation also results in the formation of linear plasmids in some clones and circular molecules in others, but only linear plasmids arose in S. lividans 1326, suggesting that the context of strain 1326 is a better host than that of strain ZX7 [and also strain A3(2)] for telomere 'cleavage'. In the recombinational cloning procedure, insertion of an antibiotic marker–telomere cassette in cosmids is tedious, and 'cleavage' at one telomere terminus is less frequent than at two telomere termini (as in the deletion step). Thus, using a cosmid vector containing two pSLA2 telomeres may save time for screening.

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Authors’ contribution

R.Z. and H.X. contributed equally to this work.

References


**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Schematic maps of plasmids and their restriction sites.

**Fig. S2.** Determination of the cloned avermectin biosynthetic gene cluster in linear plasmid pZR695-p by Southern hybridization.

**Fig. S3.** Schematic of the recombinational step for deletion of a SCP1 segment.

**Fig. S4.** Schematic of the recombinational insertion steps for adding large DNA segments from overlapping cosmids.

**Fig. S5.** Locations of a PCR segment (‘A’) and four overlapping cosmid inserts (spn-1–4) in the spinosad biosynthetic gene cluster.

**Fig. S6.** Detection of SCP1 and its derived linear plasmids in the recombinational experiments.

**Fig. S7.** Determination of the cloned spinosad biosynthetic gene cluster in linear plasmid pZR669-p by digestion with EcoRV and XhoI and Southern hybridization with mixed probes (1-kb ladder, four cosmids spn-1–4).

**Table S1.** The primers used for cloning the avermectin biosynthetic gene cluster into SCP1.