Identification of essential genes for linear replication of an SCP1 composite plasmid

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Abstract

The 365,023 bp linear plasmid Streptomyces coelicolor plasmid 1 (SCP1) of S. coelicolor A3(2) belongs to the model systems of actinomycetes linear plasmids. Although the plasmid was detected more than two decades ago, little functional information about replication and maintenance of the linear topology exists. Here, for the first time, is the description of a region in SCP1 that is essential for linear replication of the plasmid. The SCP1 linear replication (Slr) region contains three putative genes. Intriguingly, one of the genes, SlrC, does show homology to PPA1294 of Propionibacterium acne that possesses a conserved DNA_pol_B_2 domain, which is found in organellar and viral DNA polymerases as, for example, in the Bacillus phi29 polymerase.

Keywords

Streptomyces coelicolor; SCP1; linear plasmid; replication.

Introduction

Members of different Actinomycetales genera possess large linear chromosomes (Redenbach et al., 2000a) and, additionally, several species were reported to contain linear extrachromosomal DNA elements independent of the host chromosome topology (Netolitzky et al., 1995; Picardeau & Vincent, 1997; Polo et al., 1998; Saeki et al., 1999; Redenbach et al., 2000b; Huang et al., 2003; Ma et al., 2003; Zhang et al., 2006).

Replication of actinomycetes linear elements (chromosome as well as plasmid) is supposed to start from a centrally located replication origin, moving toward ends that consist of terminal inverted repeats (Calcutt & Schmidt, 1992; Zakrzewska-Czerwinska & Schrempf, 1992; Chang & Cohen, 1994; Musialowski et al., 1994), leading to 3′ overhangs. The 5′ recessed ends, resembling the situation at eukaryotic chromosome telomers, are patched requiring specific proteins as well as the terminal 200 bp. This relies mainly on results obtained from analysis of the Streptomyces rochei plasmid pSLA2 that has been developed as a model in terms of actinomycetes linear DNA replication. Based on pSLA2 analysis, genes necessary for linear DNA replication and maintenance in streptomycetes were discovered as terminal protein genes (tpg’s) (Bao & Cohen, 2001), genes for terminal associated proteins (tap’s) (Bao & Cohen, 2003) and loci required for linear replication (rlrA/rlrO) (Qin et al., 2003). In addition, a DNA polymerase I (PolA) and a topoisomerase I were recently copurified with the TAP protein, and it was demonstrated that they possess reverse transcriptase activity (Bao & Cohen, 2004).

Although most of the linear Streptomyces replicons that were investigated showed a conserved terminal sequence pattern, the giant linear plasmid Streptomyces coelicolor plasmid 1 (SCP1) and several recently identified plasmids of soil isolated streptomycetes species are different (Kinashi et al., 1991; Huang et al., 1998; Zhang et al., 2006). This is confirmed by the lack of any recognizable tpg and tap homologs in these plasmids. The differences between the SCP1 end sequence and other Streptomyces termini suggest that SCP1 is using a unique set of proteins in the terminal complex that are not chromosomally encoded. Although SCP1 is completely sequenced (Bentley et al., 2004), no genetic function involved in SCP1 end replication has been identified so far. Investigations into initiation of SCP1 replication (Redenbach et al., 1999), as well as analysis of SCP1 terminal replication, have been started. Here, for the first time is a report of an SCP1 region including three putative genes that are necessary for stable maintenance of an artificial linear composite plasmid containing SCP1 ends.
Materials and methods

Bacterial strains, plasmids, general methods

Streptomyces strains used for this study were *S. coelicolor* M138, M145, 2612 (Kieser et al., 2000) and *Streptomyces lividans* TK64 (Hopwood et al., 1983). *Streptomyces* liquid cultures were grown in YEME media. SM was used as solid media. Protoplast transformations were carried out according to the method used by Kieser et al. (2000). *Escherichia coli* cultures were grown using standard procedures (Sambrook et al., 1989). *Escherichia coli* DH5α (Life Technologies) was used for cloning all plasmid constructs except cosmids which were amplified in *E. coli* Sure™ (Greener, 1990). DNA for *Streptomyces* protoplast transformation was isolated from *E. coli* ET12567 (MacNeil et al., 1992). Transformation in *E. coli* was achieved using electroporation (BioRad, E. coli Pulse™). Vectors for pST plasmids were pUC18 (Yanisch-Perron et al., 1985) and pBluescript® II KS (Alting-Mees & Short, 1989). pOJ436 (Bierman et al., 1992) was used for establishing the integrative SCP1 cosm library. The fragment containing the pSLA2-S replication origin was obtained from pQC48 (Qin & Cohen, 1998) that was kindly provided by Stan Cohen. The tsr gene was derived from p1702 (Katz et al., 1983). Plasmid DNA was isolated by alkaline lysis (Birnboim & Doly, 1979). Transfection of *E. coli* cells for the generation of the pOJ436 cosm library was carried out according to the manufacturer’s instructions with the Gigapack® III Kit (Stratagene).

Reagents and enzymes

T4 DNA Ligase, Klenow-Polymerase and restriction enzymes used were either from MBI Fermentas or New England Biolabs. PCR reactions were carried out according to the manufacturer’s instructions using *Taq*-DNA-Polymerase (Q BIO, LifeTechnologies) with standard protocols. Blunt ending of 3’ or 5’ overhangs was carried out according to the method used by Sambrook et al. (1989). DNA was gel purified with the GeneClean II kit from Dianova. Nonradioactive labeling and filter hybridizations were carried out with the dig-11dUTP labeling kit following the protocol of the supplier (Roche Molecular Biochemicals).

Pulsed field gel electrophoresis (PFGE)

PFGE DNA was isolated in accordance with the method used in Redenbach et al. (1996). One percent PFGE gels in 0.5 × TBE buffer were used in all experiments. PFGE DNA was eluted using low melting point agarose. A CHEF-DR® II chamber (BioRad) was used for all experiments with a temperature setting of 14 °C and a reorientation angle of 120°. Separation of *S. lividans* DNA was carried out applying 100 μM thiourea in buffer and gel (Kieser et al., 1992).

DNA sequencing

The sequencing of DNA fragments was performed by use of the dideoxy chain termination method using the sequense fluorescent-labeled primer cycle sequencing kit (Amersham/Life Science). A LI-COR sequencer (Model 4000) was used for sequencing. Standard sequencing primers were used.

Results

Construction of an artificial SCP1 mini plasmid pST108/109

For identification and analysis of genes involved in SCP1 terminal replication, an SCP1 minimal replicon similar to pQC48 (Qin & Cohen, 1998) was constructed. The terminal 1.3 kb BamHI fragment of SCP1, which contains the outer protein bound end and DNA until the first BamHI restriction site, was enriched from *S. coelicolor* M138 total DNA using the glass milk method (Stoll & Cullum, 2000). The terminally bound protein was digested with Proteinkase K before gel electrophoresis. Then the DNA fragment was gel eluted and subsequently cloned in BamHI and HindIII (generating blunt ends) digested pBluescriptII KS, which in turn generated pST100 and pUC18, which generated pST101 (Fig. 1). Inserts of recombinant DNA molecules were sequenced and two constructs with the expected fragment were used for further analysis. pST100 was digested with BamHI and KpnI to release the cloned SCP1 terminus that was gel eluted. pST101 was linearized with a BamHI and KpnI digest, gel-eluted and ligated with the BamHI–KpnI SCP1 terminal fragment plus an 800 bp spacer molecule with BamHI ends. The spacer molecule was initially derived from BamHI digested chromosomal *S. coelicolor* M145 DNA and chosen due to its size. The resulting plasmid pST102 possesses two identical SCP1 termini in inverted orientation, which are separated by the 800 bp spacer fragment.

PstI and XhoI linker with rare cutting sites SmiI and AseI (Asel restriction site not shown in Fig. 1) were inserted into the PstI and XhoI site of pST102 that are flanking the terminal inverted repeats. The resulting plasmid pST104 was digested with BamHI releasing the 800 bp spacer fragment. The SCP1 termini containing core replicon of pST104 was gel eluted and BamHI ends were blunted with Klenow polymerase. For selection of the artificial plasmid construct in *Streptomyces*, the DraII fragment of pKV130 containing the thioesteron resistance gene was filled with Klenow polymerase and ligated with modified linear pST104 plus a Pmel/MunI linker leading to pST105. The MunI site was used to introduce another linker, including an Ascl and a BssHI site. The resulting plasmid pST107 was cleaved with BssHI and ligated with the 6 kb MluI fragment of pQC48, which contained the pSLA2 replication origin. Clones with...
Fig. 1. Construction of the composite replicons pST108/109. See the text for details.
two different orientations of the pSLA2 fragment were identified and named pST108 and pST109.

**Linear replication of the composite SCP1 mini plasmid depends on SCP1 specific functions**

The two constructed plasmids pST108 and pST109 were used in independent experiments and transformed into different *S. coelicolor* variants (M138, 2612, M145) and *S. lividans* TK64 to analyze if the artificial plasmid is stably maintained and if it might replicate in a linear form. Circular as well as SmiI digested linearized pST108/109 DNA was used to transform *S. coelicolor* M138 (SCP1<sup>+</sup>), *S. coelicolor* 2612 (carrying the SCP1 plasmid in integrated form, the NF state), *S. coelicolor* M145 (SCP1<sup>-</sup>) and *S. lividans* TK64 (SCP1<sup>-</sup>) protoplasts. Thioestrepton resistant transformants were obtained for all *Streptomyces* strains transformed with ccc DNA of pST108/109. In contrast, transformation with SmiI linearized pST108/109 DNA only revealed thioestrepton resistant clones with *S. coelicolor* M138.

PFGE DNA of selected thioestrepton resistant strains was isolated and undigested DNA was analyzed by PFGE (Fig. 2). Linear pST108/109 DNA could, without further hybridization, be detected in *S. coelicolor* M138 which was transformed with circular pST108/109 (lane 5), and in *S. coelicolor* M138 which was transformed with linearized plasmid DNA (lane 6). In both cases a single 9.7 kb band was migrating with the same size as the control (lane 7).

Hybridization was carried out using the *tsr* gene as a probe and revealed strong bands corresponding to the linear pST108/109 in *S. coelicolor* M138 transformed with circular and linear DNA (lanes 5 and 6). In addition, the already obtained weak band seen in the ethidium bromide stained gel with *S. coelicolor* 2612 DNA hybridized with the probe, indicating that 2612 contains linear pST108/109 DNA (lane 4), but less than pST108/109 transformed M138. ccc DNA of pST108/109 could be clearly seen by hybridization in M145 (lane 3), in 2612 (lane 4) and faintly in M138 (lane 5); all transformed with circular mini plasmid DNA. PFGE DNA of thioestrepton resistant M138 strains obtained after transformation with linearized plasmid DNA only showed hybridization with the 9.7 kb band already detected after ethidium bromide staining. No circular form of pST108/109 was detected by this hybridization. *Streptomyces lividans* TK64 thioestrepton resistant strains obtained after transformation with pST108/109 did show a similar picture as M145, harbored no linear pST108/109 but the circular form of the plasmid (data not shown).

![Fig. 2. Linear replication of the composite vector depends on the presence of SCP1.](image-url)

(a) Undigested PFGE DNA of *Streptomyces coelicolor* transformants (M145, M138, 2612) with pST109, separated by PFGE (60 s, 200 V, 20 h). Lanes 1 and 9 contain λ-HindIII as size standard. Lane 2, *S. coelicolor* AS14 with linear plasmids SCP1 and SLP2; lane 3, M145 transformed with circular pST109; lane 4, 2612 transformed with circular pST109; lane 5, M138 transformed with circular pST109; lane 6, M138 transformed with linear pST109; lane 7, SmiI linearized pST109 DNA (pST109лин); lane 8, circular pST109 DNA (pST109ccc). Inconsistent migration between the marker DNA (λ-HindIII) and the *Streptomyces* plasmid SLP2 is due to the GC content of the plasmid as described by Gravius *et al.* (1994). (b) Hybridization of the filter with the dig labeled *tsr* gene as a probe.
Hybridizations of the BamHI restricted total DNA of all transformants with labeled pST108/109 as a probe confirmed the PFGE results (Fig. 3). Figure 3 shows representative samples of the obtained results for pST109 transformed S. coelicolor variants and S. lividans TK64 transformed with pST108.

Strains without copies of SCP1, S. coelicolor M145 and S. lividans TK64 only showed signals at 10.8 and 1.6 kb (M145/pST109, lane 3) and at 8.0 and 4.4 kb (TK64/pST108, lane 7), respectively. These signals fit with the circular topology of the constructs as compared with the BamHI digested controls of circular pST109 (lane 8) and circular pST108 DNA (lane 10). The strains which contained SCP1 (M138 and 2612) transformed with circular pST109 DNA (2612, lane 4 and M138, lane 5) showed hybridization signals at 10.8, 6.8, 1.6 and 1.3 kb. Hybridization bands at 10.8 and 1.6 kb are obtained when the circular topology of pST109 is present, as described above. Linearization of the plasmid in vivo is indicated by hybridization bands at 6.8, 1.6 and 1.3 kb, which are revealed with a SmI1/BamHI digest in the pST109 control (lane 9). 2612 and M138 therefore do contain, after introducing the circular pST109, the linear derivative of pST109 as well as the circular pST109. The additional 2.7 kb fragment (pUC) appearing in controls (lanes 9 and 11) corresponds to the vector pUC18, which is cut out of pST109 with the BamHI–SmI1 restriction digest, and is not detectable in the BamHI restricted transformant DNA. M138 transformants obtained by transformation with linearized pST109 (lane 6) showed exclusive signals of 6.8, 1.6 and 1.3 kb indicating a linear topology of the plasmid, confirming the already obtained PFGE data.

These results indicated that:

1. The circularized composite mini SCP1 plasmids (pST108/109) replicated stably in all S. coelicolor variants as well as in S. lividans TK64.
3. Transformation with linearized pST108/109 DNA only leads to replication of linear pST108/109 in S. coelicolor strains with free SCP1, as seen in M138.

Screening for SCP1 genes necessary for linear replication

A cosmid library with SCP1 DNA in the integrative vector pOJ436 was constructed to identify SCP1 genes involved in the demonstrated maintenance of the linear composite plasmid pST108/109 in M138 and 2612. pOJ436 contains the apramycin resistance gene (aac(3)IV), the integrase gene (int) and the attachment site of the actinophage φC31. Thus, cosmid clones integrate into the chromosomal DNA of S. coelicolor and S. lividans strains by restriction digests and hybridization. (a) BamHI restricted genomic DNA of S. coelicolor strains and S. lividans TK64 transformed with pST108/109 DNA. Size standards are λ-HindIII DNA (lanes 1 and 13) and Fermentas 1 kb-Gene Ruler DNA Ladder Mix (lanes 2 and 12). Restricted genomic DNA of strains transformed with circular pST109 DNA: S. coelicolor M145 (lane 3), S. coelicolor 2612 (lane 4), S. coelicolor M138 (lane 5). Restricted genomic DNA of S. coelicolor M138 transformed with linear pST109 (lane 6). Restricted genomic DNA of S. lividans TK64 transformed with circular pST108 DNA (lane 7). BamHI restricted pST109 DNA (lane 8); BamHI–SmI1 restricted pST109 DNA (lane 9); BamHI restricted pST108 DNA (lane 10); BamHI–SmI1 restricted pST108 DNA (lane 11). (b) Hybridization of the filter with dig labeled pST109 as a probe.
attachment site \textit{attB}^{C31} in \textit{S. lividans} TK64 and \textit{S. coelicolor}. The SCP1 library was established with partially \textit{Sau}3A digested total DNA of \textit{S. coelicolor} M138, which was then ligated into \textit{Bam}HI restricted pOJ436. About 1500 recombinant pOJ436 clones were screened with gel-eluted, labeled SCP1 DNA, and 154 SCP1 crosshybridizing cosmid clones were detected.

The ends of 29 recombinant pOJ436 plasmids were sequenced and the data obtained were aligned with the available complete SCP1 sequence (Table 1). A minimal alignment of twelve overlapping cosmid clones spanning SCP1, except the terminal 15 kb, was established (Fig. 4). DNA of the aligned SCP1 cosmids was used to transform \textit{S. lividans} TK64 protoplasts, and transformants were selected for apramycin resistance. Successful integration of cosmid DNA of the aligned SCP1 cosmids was used to transform \textit{SCP1}, except the terminal 15 kb, was established (Fig. 4). Alignment of twelve overlapping cosmid clones spanning available complete SCP1 sequence (Table 1). A minimal sequenced and the data obtained were aligned with the SCP1 DNA, and 154 SCP1 crosshybridizing cosmid clones were detected.

Identification of SCP1 genes required for linear replication

Cosmid pOCP66 contains 33.7 kb of SCP1 DNA and possesses 39 ORFs. To identify putative ORFs required for linear replication, inserts of partially pOCP66 overlapping cosmids were analyzed for supporting linear replication of pST108/109. Cosmids pOCP3, 45, 77, 109 and 151 provided linear plasmid replication when integrated in the \textit{S. lividans} chromosome. Analysis of these cosmid inserts indicated that the required SCP1 region is located between SCP1 basepair positions 116.442 and 120.700 (Table 1). This region contains three complete ORFs SCP1.125, SCP1.126, SCP1.127 and three quarters of the ORF SCP1.128 (Fig. 6). No significant homology was obtained in BLASTP searches for SCP1.126 and SCP1.127. ORF SCP1.125 revealed significant homology with three hypothetical proteins from members of two separate actinomycetes genera: KradDRAFT_3662 (42% homology), KradDRAFT_3143 (44% homology) of \textit{Kineococcus radiotolerans} SRS30216 and PPA1294 (40% homology) of \textit{Propionibacterium acnes} KPA171202.

Generation of an autonomous composite plasmid replicating in \textit{S. lividans} TK64

PCR products of different ORF combinations were generated for cloning into the PmeI site of pST109 to reveal which of the identified ORFs were necessary for linear replication. PCR products containing SCP1.125, SCP1.125–SCP1.126 and SCP1.125–SCP1.127 were cloned into pST109. DNA of recombinant molecules in both orientations were subsequently used to transform \textit{S. lividans} TK64 protoplasts. PFGE DNA of thioestrepton resistant clones was isolated and analyzed for the presence of possible linear and circular pST109 derivatives. Only the complete region, including the ORFs SCP1.125–SCP1.127 in both orientations, restored linear replication of pST109 in \textit{S. lividans} TK64, indicating that at least SCP1.127 is necessary for the complementation.

Discussion

Although SCP1 was the first \textit{Streptomyces} plasmid predicted by Hopwood and colleagues (Vivian, 1971), its basic mode of replication and the genes involved are still unknown. To obtain insights into the essential components of stable SCP1

Table 1. Details of integrative cosmids covering SCP1

<table>
<thead>
<tr>
<th>Cosmid clone</th>
<th>T3inv</th>
<th>T7</th>
<th>Insert size (kb)</th>
</tr>
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<tbody>
<tr>
<td>8</td>
<td>275.363</td>
<td>317.500</td>
<td>38.574</td>
</tr>
<tr>
<td>11</td>
<td>207.356</td>
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<td>150.469</td>
<td>34.0</td>
</tr>
</tbody>
</table>

End sequences of individual cosmids indicated as T3inv and T7 are assigned to the sequence of Bentley et al. (2004). Cosmids in bold support linear replication of the composite replicons pST108/109. Insert sizes are in kb.
replication, screening of SCP1 was started for functions involved in replication initiation (Redenbach et al., 1999) and accessory genetic functions. In contrast to other investigated Streptomyces linear replicons, SCP1 termini do not possess palindromic sequence motives at the end. The sequence GGGGGGCGG with a run of 6 G residues represents the terminal 9 nucleotides of the ends (Kinashi et al., 1991). This sequence is followed by seven perfect
palindromic regions of which six contain four central nucleotides. Only one palindrome is centered by three nucleotides, as seen in all other "Streptomyces" termini, highlighting the differences of the SCP1 end. In contrast, the sequence potentially provides a similar secondary structure, which in principle enables the foldback patching mechanism.

To reveal if specific functions are necessary to drive linear replication of SCP1, we designed a composite "Streptomyces" replicon, which consisted of the two 1.3 kb inverted SCP1 termini, the "tsr" gene and the "S. rochei" pSLA2 plasmid origin. We could prove that the recombinant circular plasmid replicated stably in "S. coelicolor" M145 and in "S. lividans" TK64. Both strains do not possess any SCP1. In contrast, transformation of the circular composite replicon into "Streptomyces" strains harboring SCP1 (2612 and M138) did lead to the formation of linear replicating SCP1 composite plasmids. This provides, for the first time, functional evidence that SCP1 requires specific components for linear DNA replication. All the chromosomally located genes in "S. coelicolor" as well as in "S. lividans", affiliated with end replication, were not sufficient.

To track down the putative SCP1 genes required, an SCP1 insertion library was generated in "S. lividans" TK64 with SCP1 cosmids based on pOJ436. By using recombinant "S. lividans" TK 64 mutants with specific SCP1 segments integrated into the chromosome, it was possible to assign a single region (SCP1 linear replication; "slr"), including the recently annotated ORFs SCP1.125–SCP1.127, with the ability to complement linear replication of the established composite replicon. In addition, it was possible to demonstrate that at least "slrA" (SCP1.127) is necessary for linear replication. Although "slrA" does not show any valuable homology with database entries, "slrC" (SCP1.125) showed significant homology in BLASTP searches with hypothetical proteins of two different actinomycete species: KradDRAFT_3143 (44% homology) and KradDRAFT_3143 (42% homology), KradDRAFT_3143 (44% homology) of "K. radiotolerans" SRS30216 and PPA1294 (40% homology) of "P. acne" KPA171202.

Based on our experiments, the function of "slrA"-"slrC" is not clear. The genes might code, for example, for the terminal protein, terminal associated protein or other essential components of the SCP1 terminal complex. Intriguingly, the PPA1294 homolog of "P. acne" possesses a conserved DNA_pol_B_2 domain that is found in organellar and viral DNA polymerases, as for example, in the Bacillus φ29 polymerase. φ29 is replicated, in contrast to pSLA2, from the φ29 polymerase by strand displacement replication using a terminal protein as a primer in a sliding back process (Blanco & Salas, 1996; Kamtekar et al., 2006). "SlrC" has neither any conserved pol_B_2 domain nor any of the typical motives described with DNA polymerases of type B.

Further functional analysis is required to study the role of "slrA", as well as the adjacent genes, to gain more detailed insights into terminal SCP1 replication.

Acknowledgements

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References


