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HAL Id: hal-01658988
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Submitted on 8 Dec 2017

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Replication of the linear chromosomal DNA from the centrally located oriC of Streptomyces ambofaciens revealed by PFGE gene dosage analysis

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SUMMARY

From a cosmid clone of Streptomyces ambofaciens containing the dnaA and gyrAB genes, a 2.7-kb self-replicating DNA fragment containing the chromosome replication origin oriC was isolated. This cosmid was previously mapped physically to a region near the middle of the 8-Mb linear chromosomal DNA. A pulsed-field gel electrophoresis time-course analysis revealed that sequences flanking oriC were overrepresented relative to the rest of the chromosomal DNA during rapid growth, indicating that this origin is active. In addition, the terminal regions of the chromosomal DNA showed a slight overrepresentation at the onset of stationary phase.

Key-words: Streptomyces ambofaciens, oriC; Linear chromosome, Replication, PFGE.

INTRODUCTION

A physical map of the linear chromosome of three strains of Streptomyces ambofaciens has recently been constructed (Leblond et al., 1996). The DNA sequences of the chromosome ends are inversely repeated over 210 kb in strain DSM40697, an unusually long distance. The 5' ends of the chromosome are covalently associated with proteins. This kind of structure has been called an “invertron” by Sakaguchi (1990) and seems to be typical of Streptomyces genomes (Lezhava et al., 1995; Lin et al., 1993; Pandza et al., 1997; Redenbach et al., 1996). Two different replication mechanisms have been demonstrated for invertrons. (1) Replication of Φ29 of Bacillus subtilis, by a bacteriophage-encoded DNA polymerase, is primed at both ends by the terminal
proteins; it proceeds over the whole length of the phage DNA, resulting in semi-conservative replication without formation of Okazaki fragments (reviewed in Salas, 1991). (2) The linear plasmid pSLA2 of *Streptomyces rochei* has a functional centrally located, bidirectional replication origin; most of pSLA2 is replicated bidirectionally from the central origin. Protein-primed DNA synthesis contributes only about 280 nucleotides at the end of the lagging strands (Chang and Cohen, 1994).

The chromosome replication origins (*oriC*) of *Streptomyces lividans* 66 and *Streptomyces coelicolor* A3(2) were mapped close to the middle of the chromosome associated with *dnaA* and *gyrB* genes (Musialowski et al., 1994). In the latter species, gene dosage experiments indicated that *oriC* is active during vegetative growth (Calkcutt and Schmidt, 1992; Musialowski et al., 1994; Zakrzewska-Czerwinska and Schrempf, 1992). Here we show that *S. ambofaciens* also has an *oriC* locus near the middle of the chromosome and that replication proceeds from this locus by a bidirectional mechanism.

**MATERIALS AND METHODS**

**Bacterial strains**

*S. ambofaciens* DSM40697 (Hütter, 1967), *S. ambofaciens* ATCC 15154 (Pinnert-Sindico et al., 1955), and *S. lividans* TK21 (Hopwood et al., 1985) were the *Streptomyces* strains used in this work. *Escherichia coli* HB101 (Boyer and Roulland-Dussoix, 1969; Boehringer Mannheim, Germany) and *E. coli* GM33 (Yanish-Perron et al., 1985; Stratagene, USA) were used as hosts for the cloning experiments.

**Media, culture conditions and transformation**

*E. coli* cells were grown in Luria-Bertani medium, according to Sambrook et al. (1989) and transformed by electroporation (Biorad, Hercules, Calif., USA). *Streptomyces* were grown at 30°C in yeast extract malt extract (YEME) medium and transformed according to Hopwood et al. (1985).

**DNA purification, Southern analysis**

Plasmid extractions were performed according to Sambrook et al. (1989) for *E. coli*. For *Streptomyces*, plasmid extractions and subsequent purifications by caesium chloride-ethidium bromide gradients were carried out according to Hopwood et al. (1985) and Sambrook et al. (1989), respectively. Genomic DNA extractions and restriction analyses were carried out as previously described (Demuyter et al., 1988; Leblond et al., 1996). Restriction enzymes and molecular biology reagents were purchased from New England Biolabs (USA) and Boehringer Mannheim (Mannheim, Germany).

For Southern transfers, "Hybond-N" membranes (Amersham, UK) and either the "Vacugene" system (LKB, Sweden) or the Smith and Summers method (1980) were used. For colony transfers, "Qiabrane" membranes (Qiagen, Germany) were used according to the instructions of the supplier. DNA (purified or in agarose) was labelled with digoxigenin (Dig)-labelled dUTP, and specific hybrids were detected using the "DIG DNA" labelling and detection kit (Boehringer Mannheim). Alternatively, probes were labelled with $^{32}$P according to Demuyter et al., (1988) using the "Multiprime" labelling system (Amersham). Hybridizations were performed at 68°C corresponding to a stringency consistent with the detection of 100% homologues.

**Gene bank preparation**

An *S. ambofaciens* cosmid gene library was constructed using the "Packagene" kit (Promega, USA). DNA was partially *PstI*-digested, ligated into the *PstI* site of pHC79 (Hohn and Collins, 1980; Boehringer Mannheim) and introduced into *E. coli* HB101.

**Cloning of *oriC* from *S. ambofaciens***

The non-replicating 1.1-kb *BclI* fragment of plasmid pJ702 (Hopwood et al., 1985) containing the thiostrepton resistance gene (*tsr*) was purified from an agarose gel using the GeneClean$^{TM}$ procedure (Bio 101, La Jolla, CA, USA) and ligated to *BclI*-digested...
DNA of pNSA2001, a cosmid harbouring the S. ambofaciens oriC region. Plasmid pNSA2001 was isolated from E. coli GM33 [Dam-] to allow cleavage with BclI restriction. The ligation mixture was introduced by transformation into S. lividans TK21 protoplasts, and thiostrepton-resistant colonies were selected (50 μg/ml; Hopwood et al., 1985).

PFGE and gene dosage analysis

Pulsed-field gel electrophoresis (PFGE) experiments were carried out according to Leblond et al. (1993). Electrophoresis was performed in a contour-clamped homogeneous electric fields system (CHEF, Biorad; Chu et al., 1986). Gene dosage experiments were carried out at different growth times. After growth of S. ambofaciens DSM40697 in YEME medium (Hopwood et al., 1985) growth curves were calculated, followed by measuring of the DNA concentrations as described by Burton (1956). For each AseI fragment, the intensity was measured from a densitometric reading using the “GelDoc 1000” system and the Molecular Analyst™ software (Bio-rad), and was expressed relative to its molecular weight.

RESULTS

Cloning of the gyrAB-dnaA region of the S. ambofaciens DSM40697

A gene library was constructed from partially PstI-digested S. ambofaciens DSM40697 ligated to pHC79 and propagated into E. coli HB101. Among 2,000 recombinant clones, 11 hybridized to the gyrB probe pLST182 (Thiara and Cundliffe, 1993). Two cosmids, pNSA2001 and pNSA2002, overlapping only by a 1.7-kb PstI fragment, were selected for further analysis (fig. 1). The 1.7-kb PstI fragment hybridizing to pLST182 was sequenced partially from both ends (389 and 264 nucleotides, Genbank accession numbers AF004299 and AF004298, respectively). The amino acid sequences deduced from the right and left ends resemble, respectively, GyrA and GyrB proteins of S. coelicolor A3(2) (Calcutt, 1994), closely related to S. ambofaciens. Both genes read in the same direction. Hybridization with the 1.1-kb pFF911-derived S. lividans dnaA (Musialowski et al., 1994) probe located this gene to a 4.4-kb BamHI fragment upstream of the gyrAB sequences in pNSA2001 (fig. 1).

Isolation of oriC of S. ambofaciens on a 2.7-kb self-replicating DNA fragment

Since oriC of S. ambofaciens was expected to be close to dnaA, within pNSA2001, we undertook the subcloning of pNSA2001 recombinant cosmid (inserted DNA circa 35 kb) with BclI in order to isolate the DNA fragment conferring autonomous replication. Plasmid pNSA2001 BclI fragments were ligated to the 1.1-kb BclI tsr (thiostrepton resistance) fragment (see “Materials and methods”). The ligation mixture was introduced into S. lividans TK21. Forty thiostrepton-resistant clones were isolated, and for two of them the extrachromosomal content was studied by caesium-chloride gradient analysis. Both of them contained pNSA2004, a 3.8-kb circular molecule consisting of a 2.7-kb BclI fragment from pNSA2001 and the 1.1-kb BclI tsr fragment. The 2.7-kb fragment is wholly contained within the 4.4 kb-BamHI fragment, as shown by hybridization of pNSA2004 DNA onto BamHI-digested DNA of pNSA2001 (fig. 1). In addition, the former fragment hybridized to the dnaA probe pFF911 (Musialowski et al., 1994) and to pUOR2, which contains oriC of S. lividans (Zakrzewska-Czerwinska and Schrempf, 1992). Furthermore, pJ702 tsr DNA (1.1-kb BclI fragment) was used as a probe onto Southern blots of total DNA of one of the S. lividans TK21 tsr-resistant transformants digested with restriction enzymes cutting once in the tsr sequence but not in the 2.7-kb insert. For two such enzymes (EcoRV and Clal), only a DNA fragment whose size corresponded to the linearized extrachromosomal form of the plasmid (3.8 kb) was revealed by the probe. No other fragment that could have corresponded to integrated forms of the plasmid was observed (data not shown). This result ruled out apparent autonomous replication by integration-excision process.

Time-course gene dosage in S. ambofaciens

The chromosomal localization of oriC was determined by probing of S. ambofaciens DNA
Cosmids pNSA2001 and pNSA2002 are partially represented. The 2.7-kb \( \text{Bcll} \) fragment (flanked by the arrows) containing \( \text{oriC} \) and \( \text{dnaA} \) is totally included in the 4.4-kb \( \text{BamHI} \) fragment, but was not precisely mapped.

Similarly, ratios H/J and I/J were 1.6 at 20 h. In contrast, the ratios C/D, C/E, H/J and I/J decreased to 1.0, 1.1, 1.0 and 0.9 at 148 h, respectively (fig. 3). The overrepresentation of fragments \( \text{AseI-C} \), -H and -I is always observed when DNA is prepared during the exponential growth phase. Our early PFGE analysis of the \( S. \ ambofaciens \) genome concluded that doublets were present (Leblond et al., 1990), while mapping experiments showed that they were single bands. Thus, these data support the hypothesis of reinitiations of the chromosomal replication at this point during rapid growth.

Bidirectional processing of \( \text{oriC} \) was demonstrated taking advantage of the restriction polymorphism between the two strains DSM40697 and ATCC 15154 (Leblond et al., 1996). In strain ATCC 15154, fragment \( \text{AseI-N}'' \) replaces fragment B of strain DSM40697 (fig. 2). The intensity ratios \( \text{AseI-N}''/\text{L}'' \) and \( \text{M}''/\text{L}'' \) were 1.5 and 2.0 at 20 h of growth, respectively. The unbalanced position of \( \text{oriC} \) relative to \( \text{N}'' \) and \( \text{M}'' \) (\( \text{N}'' \) and \( \text{M}'' \) are distant from \( \text{oriC} \) by at least 750 and 280 kb, respectively; fig. 2) could explain the slightly fainter overrepresentation of \( \text{N}'' \) relative to that of \( \text{M}'' \). Fragments \( \text{AseI-J}'' \) (280 kb) and K'' (260 kb) comigrated in this experiment and were left out.
Overrepresentation of the terminal fragments at the onset of the stationary phase

In *S. ambofaciens*, the densitometric analysis revealed an overrepresentation of the fragments corresponding to the extremities of the chromosomal DNA between 36 and 60 h. At 60 h, the ratios D/C and D/E were 1.4 and 1.2, respectively (fig. 3). Similarly, the ratio G/F was 1.2 at 60 h. This phenomenon is reproducibly observed, although the values vary with the experiment. This indicated a noticeable overrepresentation of fragments D and G, corresponding to the ends of the chromosomal DNA (fig. 2).

DISCUSSION

A locus exhibiting an oriC-like organization similar to oriC from *S. lividans* 66 and *S. coelicolor* A3(2) was isolated from *S. ambofaciens* DSM40697 (Calcott and Schmidt, 1992; Zakrzewka-Czerwinska and Schrempf, 1992). The localization of the gyrAB genes close to oriC observed in *Streptomyces* was also reported in several other bacteria such as *B. subtilis*, *Staphylococcus aureus*, *Borrelia burgdorferi* and *Mycoplasma pneumoniae*, but it was different in *E. coli* (Reece and Maxwell, 1991).

The localization of oriC was previously reported in *S. coelicolor* (Kieser et al., 1992) and *S. lividans* (Leblond et al., 1993) to map approximately to the middle of the linear 8Mb chromosomal DNA as in *S. ambofaciens* (Berger et al., 1996) but not in *S. rimosus* (34 to 44% from one end, Pandza et al., 1997).

Several lines of evidence showed that oriC functions bidirectionally in *S. ambofaciens* during the exponential phase. First, a 2.7-kb fragment
Fig. 3. PFGE gene dosage experiments during growth.

Growth curve (A) and DNA concentration measurements (B) of *S. ambofaciens* DSM40697 are described in “Materials and methods”. Vertical lines in A indicate times (h) when PFGE analysis was carried out. PFGE running conditions were 0.85% agarose gel, 200 V for 24 h with ramped pulse times from 40 to 160 s. Nomenclature of fragments, A-J, is according to Leblond *et al.* (1996). Densitometric readings were carried out using “GelDoc 1000” and Molecular Analyst™ (Biorad).
showed autonomous replication in *S. lividans* TK21 and hybridized to both *dnaA* and *oriC* (from *S. lividans*). Secondly, PFGE time-course experiments showed that when mycelia were harvested from rapidly growing cultures, the regions flanking the *oriC* locus were overrepresented. This data strongly suggested that reinitiation of replication takes place at this locus during exponential growth. A similar strategy based on gene dosage experiments led Musialowski *et al.* (1994) to the same conclusion for the centrally located *oriC* locus of the *S. coelicolor* chromosome. Our approach constitutes a rapid and simple way to initiate the study of the replication mechanisms and could be applied in other organisms.

In addition, the cloning of the *rrn* genes was recently achieved in *S. ambofaciens* DSM40697 (Berger *et al.*, 1996). These experiments enabled the precise positioning of the *rrn* loci relative to the physical map and the determination of their transcriptional orientation. For five of six of them it was found to be divergent from *oriC* (fig. 2). Considering that highly expressed genes are usually transcribed in the direction of the migration of the replication forks (Brewer, 1988), altogether, these results show that the linear chromosome of *S. ambofaciens* would be essentially replicated by a bidirectional replication mechanism from *oriC* towards the extremities.

Furthermore, at the onset of the stationary phase, a slight overrepresentation of the terminal fragments (flanking the ends of the chromosome) was noticed. Overrepresentation could be interpreted as reinitiation of replication in the terminal regions. DNA replication might be initiated either from the chromosomal ends and require the terminal proteins as primers, or from subterminal loci by a bidirectional mechanism. Secondly, overrepresentation of a DNA fragment might reveal its relative resistance to DNA degradation. DNA degradation could happen *in vivo* during the transition phase or during DNA preparation. These data could support the existence of a "racket frame" structure (Sakaguchi, 1990) keeping the terminal regions including terminal inverted repeats associated. This structure might be related to the high structural instability occurring in the terminal regions of the linear chromosomal DNA in *S. ambofaciens* (Fischer *et al.*, 1997).

Acknowledgements

We are grateful to T. Kieser for his help with the preparation of the manuscript. Many thanks to E. Cundliffe, F. Flett and H. Schrempf for giving us some of the plasmids used in this work.

**Réplication de l'ADN chromosomique linéaire de *Streptomyces ambofaciens* à partir de l'origine de réplication *oriC* localisée au milieu du chromosome: mise en évidence par dosage de gènes en PFGE.**

L'origine de réplication principale du chromosome de *Streptomyces ambofaciens* a été isolée sur un fragment d'ADN de 2,7 kb *BclI* à partir d'un cosmide recombinant contenant les gènes *dnaA* et *gyrAB*. Ce cosmide a été localisé dans une région située approximativement au milieu de l'ADN chromosomique linéaire de 8 Mb. L'analyse de l'intensité relative des fragments de restriction séparés par électrophorèse en champs pulsés en fonction du temps de croissance montre que les séquences proches d'*oriC* sont sur-représentées en phase exponentielle de croissance. Ce résultat indique que cette origine de réplication est active. Par ailleurs, à l'entrée de la phase stationnaire de croissance, les régions terminales de l'ADN chromosomique présentent une légère sur-représentation par rapport au reste du chromosome.

**Mots-clés : Streptomyces ambofaciens, oriC ; ADN linéaire, Réplication, PFGE.**

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