Determination of specific DNA strand discontinuities with nucleotide resolution in exponentially growing bacteria harboring rolling circle-replicating plasmids

Elisabeth Grohmann a, Ellen L. Zechner b, Manuel Espinosa a,*

a Centro de Investigaciones Biológicas, CSIC, Velázquez 144, E-28006 Madrid, Spain
b Institut für Mikrobiologie, Karl-Franzens-Universität Graz, Universitätsplatz 2, A-8010 Graz Austria

Received 14 April 1997; revised 9 May 1997; accepted 12 May 1997

Abstract

Plasmid replication by the rolling circle mechanism and conjugative transfer of plasmids require the generation of a specific strand discontinuity in the DNA. In both processes cleavage at the so-called nic site is catalyzed by plasmid-encoded proteins. The strand discontinuities at the conjugative origins of transfer of plasmid pE194 and pMV158 were determined in Bacillus subtilis and Streptococcus pneumoniae, respectively, with a recently developed runoff DNA synthesis assay. The positions of intracellular cleavage within the respective transfer origins were shown to coincide with the site predicted for pE194 and with the nic site determined in vitro for pMV158. For pMV158, the influence of a mutation in the S. pneumoniae polA gene on the efficiency of replication was investigated. In addition, the nic site within the double-stranded origin of the rolling circle-replicating plasmid pMV158 in S. pneumoniae as well as that of pFX2 in Escherichia coli was mapped with nucleotide resolution.

Keywords: Rolling circle replication; Strand-specific cleavage; DNA polymerase I; Runoff DNA synthesis assay

1. Introduction

Site- and strand-specific breakage of double-stranded (ds) DNA is a prerequisite for several interrelated DNA processing reactions, including conjugal transfer, rolling circle (RC)-type replication, and transfer of T-DNA of the Ti plasmid of Agrobacterium [1-4]. In addition, site-specific recombination processes such as bacteriophage λ integration, transposition and formation of plasmid multimers also involve DNA strand transfer reactions [5]. In the case of plasmids replicating by the RC mechanism (generically termed RCR plasmids), the protein initiating replication, Rep, acts at the double strand origin, dsO, to catalyze a strand- and site-specific cleavage reaction. Leading strand replication begins at the free 3’ hydroxyl generated by Rep at the nic site. Some RCR plasmids also encode a Mob protein that functions similarly to generate a strand- and site-specific nick in the plasmid origin of trans-
fer, oriT, in preparation for conjugative mobilization of the plasmid [3]. The actual mechanisms of conjugative mobilization remain obscure [5]. Current models, developed for self-transmissible plasmids, would predict that transfer of the single-stranded DNA takes place with a 5' to 3' directionality, and proceeds by a replicative RC mechanism [2,3]. Synthesis of a DNA strand complementary to transferred F- and I-like plasmid DNA in the recipient bacterium was shown to be dependent on the Escherichia coli dnaE gene [6]. This finding implies that E. coli DNA polymerase III (Pol III) is essential for complementary strand DNA synthesis following plasmid transfer. In the same study, an involvement of DNA polymerase I (Pol I) of E. coli in conjugative DNA synthesis was not suggested as polA mutations in donor or recipient strains had no effect on the conjugative DNA synthesis measured. An involvement of the pneumococcal Pol I (Spn Pol I) in RC replication has only been suggested in the establishment and replication of plasmid pLS1 [7], a derivative of the streptococcal plasmid pMV158 lacking the mobilization functions [8].

We are interested in understanding the mechanisms of initiation and termination of plasmid-mediated DNA processing reactions involved in asymmetric RC replication, especially in plasmids of the pMV158 family [4]. One approach to study these mechanisms is to monitor the cleavage reactions occurring in vivo at the replication and transfer origins. To that end, we recently developed an assay system that allowed us to map the precise position and monitor the efficiency of DNA cleavage reactions occurring in vivo on native replicons [9]. In addition, although part of the dso region of plasmids of the pMV158 family shows a high overall similarity, their Rep proteins are not interchangeable in vivo. This feature is thought to be due to differences at their putative C-terminal domains, which are probably involved in DNA recognition of directly repeated sequences (iterons) located within their respective dso [4]. Indications that the iteron-binding domain of the Rep proteins of this plasmid family is located at their C-moiety are some relevant similarities extending towards the C-terminal domains of Rep proteins en-

---

Table 1

<table>
<thead>
<tr>
<th>Oligonucleotides used in this work</th>
<th>Oligo</th>
<th>Size (bp)</th>
<th>Plasmid</th>
<th>Nucleotide position</th>
</tr>
</thead>
<tbody>
<tr>
<td>pFX2ro</td>
<td>19</td>
<td>pFX2</td>
<td></td>
<td>1227-1209*</td>
</tr>
<tr>
<td>pFX2op</td>
<td>19</td>
<td>pFX2</td>
<td></td>
<td>959-977*</td>
</tr>
<tr>
<td>pCR25w</td>
<td>25</td>
<td>pFX2</td>
<td></td>
<td>1558-1573*+9 b 5'-extension</td>
</tr>
<tr>
<td>PCR27lw</td>
<td>27</td>
<td>pFX2</td>
<td></td>
<td>2364-2347*+9 b 5'-extension</td>
</tr>
<tr>
<td>pEl194oriT</td>
<td>20</td>
<td>pEl194</td>
<td></td>
<td>3221-3202b</td>
</tr>
<tr>
<td>pEl194oriTop</td>
<td>19</td>
<td>pEl194</td>
<td></td>
<td>2810-2828b</td>
</tr>
<tr>
<td>pLS1ro</td>
<td>17</td>
<td>pMV158</td>
<td></td>
<td>659-643*</td>
</tr>
<tr>
<td>pLS1op</td>
<td>19</td>
<td>pMV158</td>
<td></td>
<td>1-19*</td>
</tr>
<tr>
<td>pMV158oriT</td>
<td>18</td>
<td>pMV158</td>
<td></td>
<td>3732-3715*</td>
</tr>
<tr>
<td>pMV158oriTop</td>
<td>22</td>
<td>pMV158</td>
<td></td>
<td>3430-3451*</td>
</tr>
</tbody>
</table>

*Coordinates from [10].

b Coordinates from [19].

Coordinates from [13].

---

Fig. 1. The nic site within oriT of pMV158 in S. pneumoniae coloindes with the in vitro mapped site. A: Reaction mixtures containing 25 ng primer pMV158oriT and S. pneumoniae polA^-/pMV158 (lane 1), S. pneumoniae polA^-/pMV158 (lane 2), or S. pneumoniae polA^-/pMV158-III (lane 3) were subjected to the runoff nucleotide sequencing assay. B: Synthesis in vitro of nic-terminated reaction products was observed when B. subtilis pEl194cop6 was analyzed (lane 1), but not when the same strain harbored pEl194cop6CAT, which lacks oriT and mob (lane 2). For A and B the nucleotide size markers obtained with oligo pMV158oriT on pMV158 DNA and oligo pEl194oriT on pEl194cop6 DNA were loaded on the same gels to determine the precise oriT cleavage site. The vertical nucleotide sequence depicted at the left of each gel indicates the sequence of the cleaved DNA strand, which is complementary to the markers. The arrows indicate the sites of cleavage, nic, in oriT of each plasmid. Note that samples were run in the same gel, but the exposure times were different. C: The origins of transfer of pEl194 and pMV158. The coordinates in parentheses represent the first nucleotide of the oriT sequence. Conserved nucleotides are indicated by vertical lines. The cleavage site is shown by an arrow.
2. Materials and methods

2.1. Bacterial strains and plasmids

*E. coli* JM109 (F', traD36, lacI, Δ(lacZ)M15, proA*B'14le14' (MerA'), Δ(lac-proAB), thi, gyrA96 (Nal'), endA1, hsdR17 (rK-mK'), relA1, supE44, recA1) was used for maintenance of plasmid pFX2 [10] (a gift of Dr. L.E. Pierce) and pGEX-2T-repX. The latter construction contains a 819 bp *BamHI*-EcoRI DNA fragment with the repX coding region, but missing the *dso*, inserted into pGEX-2T (Pharmacia Biotech), digested with the above-mentioned enzymes. This fragment was generated by PCR with the aid of two oligonucleotides, PCR25rw (annealing from nucleotide position 1558–1573 to the pFX2 sequence) and PCR27lw (hybridizing from 2364–2347 to pFX2) which have 5' ends compatible with the recognition sequences for the above-mentioned enzymes (Table 1). *S. pneumoniae* 708 (trtl, hex4, end1, exo2, malM594) and *S. pneumoniae* MP-551 (*polA*2(1052bp::cat), malM*, end-1, noz-19, exo-3) [8] were used to propagate plasmids pMV158, pMV158-HIII and pCGA12 [11]. pCGA12, containing the entire *dso* of pMV158 inserted into the RCR plasmid pC194 [12], was used as a control in some experiments. Another control plasmid, pMV158-HIII, was constructed analogously to [13] by *HindIII* linearization of pMV158 at the unique site within the coding region of *mobM*, followed by filling-in with the Klenow fragment of the DNA Pol I. The *mobM* gene is inactivated as a result. *B. subtilis* 1E7 (trpC2 thr5; Bacillus Genetic Stock Center) harbored pE194cop6, a copy-up mutant of plasmid pE194 [4]. *B. subtilis* MB11 (metB10, lys3, hisB2; our lab collection) harbored pE194cop6CAT. This plasmid was constructed by deletion of the 1443 bp *TagI* fragment of pE194cop6 (containing the putative *oriT* and *mob* gene) and, after filling in with the Klenow fragment of Pol I, insertion of a 1861 bp *DpnII* fragment of pJS3, carrying the *cat* gene of this plasmid [14].

2.2. DNA techniques and transformation

Routine techniques used for plasmid isolation, construction and transformation of *E. coli* strains were as described [15]. Plasmid DNA from *E. coli*...
was purified with the Qiagen Plasmid Midi kit, and plasmid DNA from *S. pneumoniae* was isolated by preparation of cleared lysates followed by two consecutive CsCl/ethidium bromide equilibrium gradients [16]. Cultures of *S. pneumoniae* were grown and transformed as previously described [8,16,17].

2.3. **PCR amplification and runoff DNA synthesis assay**

Bacterial strains harboring plasmids were cultured overnight to stationary phase and then diluted between 1:20 and 1:50 in fresh medium. Then they were grown at 37°C until a final optical density, OD_{600}, of 0.3–0.5 was reached. The runoff DNA synthesis assay [9] is based on thermal lysis of bacterial cells in a thermocycler, followed by in situ extension of DNA with an appropriate oligonucleotide and a thermostable DNA polymerase. For these assays, an aliquot of each culture was taken at the time of harvest and diluted serially in ice-cold medium. The dilutions were spread on solid medium with the appropriate selection to quantify the number of viable cells as colony forming units (cfu). Aliquots of culture medium were taken as follows: 4 × 10^5 cfu for *B. subtilis* pE194cop6 and pE194cop6CAT; 1–4 × 10^6 cfu for *S. pneumoniae* pMV158 and pMV158-HIII; 1–4 × 10^6 cfu for *E. coli* pFX2 and pGEX2-T-repX; and 4–8 × 10^6 cfu of *S. pneumoniae* pCGA12. Cultured bacteria were collected and added to the reaction mixtures, as described [9]. Reaction mixtures were as follows: *E. coli* pFX2 or pGEX2-T-repX, contained 50 ng oligonucleotide pFX2ra; those with *S. pneumoniae* pMV158 and pCGA12 (in *dso* mapping) received 10–25 ng of oligo pLSI1ro; assays with *S. pneumoniae* pMV158 and pMV158-HIII (in *oriT* mapping) 25–100 ng of oligo pMV158oriT; and those with *B. subtilis* pE194cop6 and pE194cop6CAT contained 200 ng of oligo pE194oriT.

The cycle programs were set as follows: (i) 95°C × 90 s; 60°C × 1 min; 72°C × 1 min (35 cycles) for *E. coli* pFX2 cells or pGEX2-T-repX; (ii) 95°C × 90 s; 60°C × 40 s; 72°C × 40 s (35 cycles) for the reactions with *S. pneumoniae* pMV158 or pCGA12 (*dso* mapping); (iii) 96°C × 30 s; 54°C × 30 s; 72°C × 90 s (31 cycles), followed by a cycle with a long extension step (72°C × 5 min) for cells of *S. pneumoniae* pMV158 or pMV158-HIII (oriT mapping); (iv) the program 96°C × 90 s; 57°C × 30 s; 72°C × 40 s (35 cycles) proved to be the most appropriate for *B. subtilis* pE194cop6 and pE194cop6CAT. These conditions were chosen empirically by first optimizing the yield and specificity of DNA amplification from plasmid-carrying bacteria with the nicking oligonucleotide plus a second primer (see Table 1).

2.4. **Denaturing polyacrylamide gel electrophoresis and preparation of polynucleotide size markers**

Following in vitro DNA synthesis, the reaction products were treated as described [9], and analyzed on 6% polyacrylamide gels containing 8 M urea. Gels were visualized by autoradiography at −70°C, for 1–10 days, with Kodak X-Omat film and intensifying screens. All the autoradiographs shown in Figs. 1 and 2 are composed of two parts of the same 6% polyacrylamide gel. In these, the time of exposure varied between 12 h (the part containing the polynucleotide size markers) and 5–10 days (for the runoff DNA synthesis samples). To generate nucleotide size markers, the same oligonucleotide primer used in the nicking assay was used to form primed DNA templates containing the respective *dso* or *oriT*. Reactions were carried out as specified by the manufacturers of the T7 sequencing kit (Pharmacia Biotech) in the presence of [α-32P]dCTP (3000 Ci/mmol, Amersham).

3. **Results**

3.1. **Transfer of pMV158 to a poloA**− mutant of *S. pneumoniae* and of pFX2 to *E. coli* JM109

The *S. pneumoniae* *polA*− mutant strain MP-551 has 1052 bp of the *cat* gene from plasmid pJS3 [14] inserted into its chromosome. This insertion is located within the *polA* gene such that the DNA polymerase activity of Spn Pol I is disrupted but the 5′-3′ exonuclease activity is intact [8]. Competent cultures of this *polA* strain were transformed [16,17] with 1 μg of purified DNA from plasmid pMV158 and transformants were selected in agar medium containing 1 μg ml⁻¹ tetracycline. Purified DNA from plasmid pFX2 was used to transform *E. coli* JM109.
Transformants were selected with chloramphenicol (30 µg ml⁻¹). It is noteworthy that this is the third plasmid of the pMV158 family that is able to replicate in the Gram-negative host, E. coli [4].

3.2. Determination of the nic sites within the transfer origins of pMV158 and pE194 in Gram-positive hosts

The nic site at the oriT of pMV158 has been determined in vitro by the use of purified, plasmid-encoded MobM protein [18]. To determine the feasibility of using the run-off DNA synthesis assay to confirm a variety of known or predicted nic sites in Gram-positive hosts harboring RCR plasmids, we tested the procedure with cultures of B. subtilis 1-E7/pE194cop6, B. subtilis MB11/pE194cop6CAT, and S. pneumoniae strains 708 and MP-551 carrying the pMV158 or pMV158-HIII plasmids. Cells harboring plasmids were harvested from liquid culture by centrifugation, and an aliquot of culture was spread on selective solid medium to determine the viable cell count. Cells were suspended directly in a reaction mixture for the in vitro DNA synthesis. For each strain tested, the assay was first optimized with regard to the number of bacteria, the concentration of oligonucleotide primer, and the temperature and duration of each incubation (not shown). For this purpose a second oligonucleotide (Table 1) was included in the reaction such that a typical PCR amplification of the plasmid DNA would result. The yield and specificity of the amplification was examined afterwards using DNA agarose gels. Cell concentrations of 4×10⁶ cfu for B. subtilis/pE194cop6 and pE194cop6CAT, and 1–4×10⁶ cfu for S. pneumoniae/pMV158 and pMV158-HIII, were chosen for use in the nicking assay.

Electrophoretic analysis of the run-off DNA synthesis products (Fig. 1A) showed that the site-specific single-strand breakage (⁎) at the oriT of the staphylococcal plasmid pMV158 was located between G-3591 and T-3592 within the sequence 5'-AGTGTG'TTATAC-3' of the pMV158 oriT (Fig. 1A,C). This result coincided exactly with the site mapped in vitro by isolation of MobM-treated pMV158 DNA, followed by terminal transferase labeling in the presence of [α-³²P]dATP [18]. When cells carrying a mob⁻ plasmid, pMV158-HIII, were analyzed, DNA molecules nicked in oriT were not detected, presumably due to the lack of a functional MobM protein. The specific in vitro DNA reaction products observed appeared to migrate as two bands which differed in length by one nucleotide. We assume that formation of two specific products is due to the terminal transferase activity of the thermostable DNA polymerase and not to the presence of two independent cleavage sites in the pMV158 plasmid DNA, as similar observations were made with purified MobM [18].

In this experiment, the position of the nic site cleaved in vivo was detected with the S. pneumoniae polA⁻ mutant/pMV158 (Fig. 1A, lane 2). In comparison, assays with the isogenic polA⁺ strain under the same conditions resulted in almost undetectable levels of nicked molecules (Fig. 1A, lane 1). Since more nicked molecules of pMV158 were detected in the polA mutant strain than in the wild-type strain, this would suggest that Spn Pol I affects: (i) the copy number of pMV158; (ii) the equilibrium of nicking and closing catalyzed in vivo at the pMV158 oriT, or (iii) both (i) and (ii). The first assumption could be proved, as S. pneumoniae polA⁻ cells indeed harbored pMV158 at an approximately twofold elevated copy number. As this phenomenon was already considered in the amount of cells employed in the run-off DNA synthesis assay, the detected amount of nicked pMV158 molecules in polA⁻ cells should really reflect an effect of Spn Pol I on the equilibrium of nicking and closing at oriT. Therefore, we would be in favor of suggestion (iii), indicating an influence of the pneumococcal Pol I on plasmid copy number as well as on the nicking and closing processes taking place at the oriT.

The nic site of the Staphylococcus aureus plasmid pE194 was predicted according to sequence homology with the oriT sequences of other RCR plasmids from Gram-positive bacteria [18]. When we performed this assay using B. subtilis cells harboring a copy-up mutant of pE194, pE194cop6, the strand discontinuity (⁎) was mapped between G-3092 and T-3093 (sequence coordinates according to [19]) within the sequence 5'-AGTGTG'TTAGAC-3' of the pE194 oriT (Fig. 1B,C). This position coincided exactly with the predicted site of cleavage. The negative control, pE194cop6CAT, which lacks the pE194-mob coding region as well as oriT, did not
produce a specific extension product (Fig. 1B, lane 2).

3.3. Identification of the site of initiation of leading strand synthesis: plasmid pMV158 in S. pneumoniae, and pFX2 in E. coli

We next sought to detect the strand-specific nicks introduced in vivo by Rep proteins of different plasmids at the initiation step of RCR. E. coli JM109/pFX2 was employed to determine the nic site at the dos of the lactococcal plasmid pFX2. The position cleaved in vivo was expected to map between the G (coordinate 1129) and the A (coordinate 1130) within the consensus sequence of the plus origin of replication of the pMV158 family: 5'-TACTACG*A-3' ([4] and Fig. 2C). The specific reaction product generated by extension of oligonucleotide pFX2r annealed to the nicked replication intermediates downstream of the nic site demonstrated that this was indeed the case (Fig. 2A). The nic site within the dos of pMV158, which was defined in vitro [20], was also mapped in vivo in S. pneumoniae. As the nic site within the dos of the Δmob plasmid pLS1 was determined recently [9], we aimed to verify the strand discontinuity in the parental plasmid pMV158 as well as to compare replication efficiencies in S. pneumoniae polA− and polA+ strains. For this experiment, S. pneumoniae 708, and the isogenic polA− strain, MP-551, both harboring pMV158, and MP-551/pCGA12 cells were subjected to the runoff DNA synthesis assay. Shown in Fig. 2B are the reaction products analyzed on a 6% denaturing polyacrylamide gel. The site- and strand-specific breakage at the pMV158 dos was mapped to the identical site as that determined in vitro, namely between nucleotides G-448 and A-449 (Fig. 2C). No significant difference in the amount of nicked replication intermediates between the polA− and the polA+ host systems could be detected. Therefore, a contribution of Spn Pol I to the initiation of replication starting at the 3′ site of the nick as suggested [8] remains to be further analyzed.

4. Discussion

The runoff DNA synthesis assay has proved to be a very useful tool for mapping DNA strand discontinuities without prior isolation of the relaxation complexes, but with the DNA substrates in their natural Gram-negative or Gram-positive hosts. As shown here (Fig. 1B, Fig. 2A), the assay was suited to determine the position of the nick introduced by Rep proteins (RepX of pFX2) or by Mob proteins (Mob of pE194), which were not demonstrated before. In addition, the assay proved to be a useful tool to confirm whether site-specific strand scissions mapped in vitro reflect the position of the strand breakage catalyzed in vivo. Consequently, this procedure can be used to identify unambiguously the replication and transfer origins of the RCR plasmids which have been sequenced so far (presently more than 50) without the necessity of first purifying their Rep and/or Mob proteins. Furthermore, extension of the method to yet unidentified plasmids should also be feasible, provided some sequence data are available. This can be a powerful tool to examine RCR plasmids in their natural environment, a study which has not been approached so far.

We were additionally interested in investigating the contribution of the Spn DNA Pol I to the replicative processes taking place after the introduction of the strand- and site-specific breakage at the dos of RCR plasmids, as well as at their oriTs. In contrast to a previous report [8], where an increased persistence of the break at the dos in S. pneumoniae polA− mutants harboring pLS1 was observed, we did not detect a significant difference in the amount of nicked replication intermediates in the polA− host (Fig. 2B). We cannot rule out, however, that differences in the approaches used and/or the existence of host factors other than Spn Pol I can account for these differences. In addition, the involvement of S. pneumoniae Pol I in the initiation and/or completion of synthesis of the lagging strand [8] would agree with our results as it would not result in an increase of nicked leading strand replication intermediates. Although to date no evidence exists demonstrating an involvement of DNA Pol I in replacement strand synthesis in the donor cell nor in synthesis of a complementary strand in the recipient [2,6] as part of the plasmid transfer process, the increased abundance of plasmids nicked at oriT observed here with the S. pneumoniae polA− mutant harboring pMV158 (Fig. 1A) makes it tempting to speculate on a possible role.
of Pol I in the elongation step of the complementary strand synthesis of RC plasmid DNA in the donor and/or the recipient cell. The experimental data showing a requirement for the larger Pol III enzyme in conjugal DNA synthesis stem from Gram-negative host systems supporting conjugal transfer of plasmids replicating via the theta type [6]. Therefore, in future studies we plan to investigate the putative role of Pol I in establishment and replication of small promiscuous RCR plasmids in different Gram-positive and -negative hosts.

Acknowledgments

Thanks are due to M.T. Alda for help in the transfer of plasmids to the various hosts, and to Dr. L.E. Pierce for his gift of plasmid pFX2. This joint research has been done within the framework of the European Science Foundation Network on Molecular Biology and Ecology of Plasmid-mediated Gene Spread. Research at the CIB was financed by CI-CYT, Grants BIO94-1029 and BIO95-0794. Research at the KFUniGraz was financed by P9141-MED and P11844-MED. The Integrated Action Program (numbers 13-B and 30/96) between Spain and Austria supported this cooperation. E.G. is the recipient of an ‘Erwin Schroedinger postdoctoral grant’ of the Austrian Funds Supporting Science (‘FWF’).

References
