Three regions in the DNA of plasmid pLS1 show sequence-directed static bending

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ABSTRACT

Three regions showing abnormal electrophoretic mobility, which is an indication of the existence of bends in DNA, have been observed in the DNA of plasmid pLS1. These loci have been characterized by assays designed to detect sequence-directed bending in DNA (temperature-dependence migration and two dimensional electrophoresis). The first region (locus B-1) was located within a fragment that contains a proposed inhibitor countertranscribed RNA (RNAII). The second locus (B-2) contains the plasmid plus origin of replication and the third region (locus B-3) was located in the vicinity of a putative antisense RNA (RNAI) of unknown function. The centres of the first two bent DNA regions were located by circular permutation assays at nucleotides 882 (locus B-1) and 634 (locus B-2). The bend centre of locus B-1 was found to be upstream of the promoter for the putative antisense RNAII. The centre of curvature in locus B-2 was located in the vicinity of the putative promoter of the replication proteins RepA and RepB and of a sequence that has three 11-bp direct repeats. The DNA sequence at this region showed the existence of A.T tracts, with an internal repeat of 10 to 11 base pairs, for five helix turns. A complex curvature in the DNA of pLS1 at locus B-2 that may have a regulatory role in plasmid replication is postulated.

INTRODUCTION

Plasmid pLS1 is a 4408-bp long multicopy streptococcal replicon that has the unusual property of exhibiting a very broad host range. It is able to replicate in a variety of hosts, Gram-positive and Gram-negative (1). Replication of pLS1 has been shown to follow a mechanism of asymmetric rolling circle (2,3), similar to other small multicopy staphylococcal plasmids (4,5). Replication functions of pLS1 include at least two polypeptides codified by the same mRNA: a 5.1 kDa trans-acting repressor, RepA (del Solar et al., manuscript in preparation) and the replication initiator protein, RepB, that introduces a strand- and site-

specific nick to initiate the synthesis of the leading strand (de la Campa et al., to be published). A sequence involved in incompatibility and copy number control, and with the potential to specify a small antisense RNA (RNAII), has been located between the coding regions for RepA and RepB (del Solar et al., unpublished).

At the structural level, we have reported previously that the minus origin of pLS1 is partially homologous with those of single stranded coliphages (2). No homologies with other replicons at the plus origin of the plasmid have been detected so far (3). In contrast to Gram-positive staphylococcal replicons such as pT181 (6) or pC194 (7), pLS1 has three 11-bp directly repeated sequences included in, or situated near the plus origin of replication (1,3). Secondary structures, located in the regions containing the plasmid plus and minus origins of replication have been mapped "in vitro" (2,3).

During the course of this structural analysis, we noticed that the fragments PstIB and HinfID of plasmid pLS1 DNA exhibited anomalous migration in polyacrylamide gels. These fragments include the plasmid plus origin of replication (3) and their electrophoretic behaviour suggested that a bend in the DNA could exist. Sequence-directed static bending, due to the presence of alternate A.T tracts phased with the helix turn, has been shown to exist in kinetoplast DNA (8,9,10). Static DNA bending at the origins of replication of plasmid R6K (11) and pT181 (12), as well as at the origins of replication of phage lambda (13) and simian virus 40 (14) is well documented. Protein-induced bends in DNA also seem to be a common feature in regions of DNA subjected to regulation of gene expression (15) or involved in the initiation of replication (12,16,17).

In this work, we report the existence of three regions with static bending in the DNA of plasmid pLS1. The first region which exhibits bending has been located upstream of the putative promoter for the proposed RNAII (1). The second bend is the strongest, and has been located near the three 11-bp direct repeats and the putative promoter for RepA and RepB. The third locus showing curvature is included in a fragment that contains

the minus origin of replication and a putative antisense RNA (RNAI) of unknown function (3).

MATERIALS AND METHODS

Bacterial strains and plasmids

S. pneumoniae 708 (end-1 exo-2 trt-1 hex-4 malM594) and *E. coli* JM109 (recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 Δ (lac-proAB) (F' traD36 proAB lacI^q lacZ Δ M15)) were used throughout this study. Plasmids used were pLS1, pLS5 (1), pLS4 (2) and the constructions described in this work. Plasmid pLS5 derives from pLS1 and has a deletion extending from coordinates 34 to 365 (1), whereas plasmid pLS4 derives from pLS5 and has a further deletion extending from coordinates 3278 to 4220 (2).

Preparation and manipulations of DNA

Plasmid DNA was isolated from *S. pneumoniae* and purified by two consecutive CsCl gradients as previously described (18). Restriction endonucleases, DNA ligase and DNA polymerase I (Klenow fragment) were obtained commercially and used as specified by their suppliers.

Constructions of circular permutations

Circular permutations of the desired pLS1 DNA fragments were constructed by separately cloning these fragments at the HincII and SmaI sites of pUC18. The cloned fragments were: ApaLI-TaqI (coordinates 607 to 1130) from pLS1, to construct plasmid pLSM1S and pLSM1H; and XmnI-StyI (coordinates 4366 to 758) from pLS4, to construct plasmid pLSM2S and pLSM2H. Duplications to construct pLSM1B and pLSM2B were performed as described (16). After the filling-in of the cohesive ends, and taking into consideration the 11-bp distance between the sites BamHI and HincII in the multicloning site of pUC18, each of the repeats that were duplicated were of 540 bp (pLSM1B) and 484 bp (pLSM2B). Attempts to construct the circular permutation designed to characterize the centre of the third region of bending were unsuccessful (see Results).

Gel electrophoresis of DNA fragments

One-dimension gel electrophoresis was performed in 5% polyacrylamide gel slabs (0.7mm thick) in 40mM Tris (pH 8.0), 5mM

Na acetate, 1mM EDTA with buffer circulation. Electrophoresis was usually conducted at the temperature of 23°C (unless otherwise stated) at a constant voltage of 8 V/cm until the tracking dye reached the bottom of the gel. Two-dimensional electrophoresis was carried out under similar conditions, but the first dimension was conducted at 60°C and the second dimension at 6°C, as described (19).

Computer work

Cylindrical projection of DNA sequences and bending analysis were performed by the use of the DNASTAR computer programs (DNASTAR, Inc., UK). The A-A wedge angle was estimated to be 8.6°, as calculated by Trifonov (20).

RESULTS AND DISCUSSION

One of the most relevant features of a curvature in a linear duplex DNA fragment is its abnormal slow migration when it is electrophoresed in polyacrylamide gels (8). When pLS1 DNA was digested with enzymes that give several fragments and their electrophoretic pattern at 23°C was analyzed in polyacrylamide gels, it was apparent that at least one of the resulting fragments migrated more slowly than expected. Figure 1A shows the eight restriction bands generated when pLS1 DNA was digested with HphI (lane 1, see map in Fig. 1C). In this instance, fragment HphIF (443 bp) migrated more slowly than fragment HphIE (469 bp). The anomalous migration of fragment F was detected because a Bal-31 deletion derivative at the unique BglI site of pLS1 (that had lost part of the fragment HphIF), resulted to have this fragment with a reduced electrophoretic mobility, whereas fragment E was unchanged (lane 2 and Fig.1C). The abnormal mobility of fragment HphIF was also confirmed afterwards (Fig. 2; see below). Digestion of pLS1 DNA with HinFI gives seven bands (lane 4 and Fig 1C), and it was clear that fragment HinFIID (443 bp) exhibited a mobility corresponding to a fragment of 600 bp (Fig.1B). These were the indications that the DNA region of pLS1 located between the coordinates 242 (left end of fragment HinFIID; Fig 1C) and 1102 (right end of fragment HphIF; Fig. 1C) contained at least one bend. This region in pLS1 includes the plus origin of replication, the three 11-bp direct repeats, the coding sequences for RepA and

Figure 1. One- and two-dimensional polyacrylamide gel electrophoresis of pLS1 DNA restriction fragments and their positioning in the map of the plasmid. **A:** HphI digestions of pLS1 DNA (lane 1) and of a Bal-31 deletion at fragment F (lane 2). **B:** HinfI digestion of pLS1 DNA. Lanes 3 and 5: phage T7 DNA digested with HpaII, as molecular weight standard, with the fragment sizes indicated to the right. Letters and numbers at the left of panels A and B indicate the actual size of the fragments as deduced from the nucleotide sequence of pLS1 (1). **C:** linear map of pLS1 showing the pertinent restriction sites, the putative mRNAs (wavy lines) and their products. **D:** two dimensional electrophoresis of pLS1 DNA digested with HinfI and SspI; numbers indicate the actual size of the fragments. Arrows point to the fragments migrating off the diagonal.

part of that for RepB (Fig. 1C). During the course of these experiments, it was observed that fragment PstIB (Fig. 1C) exhibited a high rate of self-ligation (results not shown), which is another test used for the identification of regions of curvature in DNA (16).

It has been shown that bent DNA segments can be selectively

Figure 2. **A:** The effect of temperature on the apparent length of pLS1 DNA restriction fragments generated by digestion with HinfI (lanes 1, 4 and 7) or with HphI (lanes 2, 5 and 8). Lanes 3, 6 and 9: pBR322 DNA digested with HinfI as an unbent control. **B:** Plot of the relative gel mobility of the restriction fragments as a function of the temperature; numbers in brackets indicate their actual size.

detected by two-dimensional acrylamide gel electrophoresis (19). The first dimension is conducted at a high temperature to eliminate the anomalous gel migration of the DNA fragment, whereas the second dimension is performed at a low temperature to increase it (21). This type of assay was performed with pLS1 DNA digested with HinfI and SspI, to split HinfIA (1295 bp) into two fragments

of 870 bp and 425 bp, thus avoiding interference with fragment HinfIB (1162 bp). As can be seen in Fig. 1D, two of the DNA spots were located above the diagonal line, behaviour expected for bent DNA (19). These two bands corresponded to the HinfIB (1162 bp) and, more clearly, to the HinfID (443 bp) fragments. These results support the existence of a static bend, located between coordinates 242 and 685 of pLS1. They also indicate the existence of another bent region in the HinfIB fragment.

To quantitatively analyze the migration of the fragments generated by digestion of pLS1 DNA with either HphI or HinfI, three gels were run at the temperatures of 6°C, 23°C and 50°C. The pictures of the gels are shown in Figure 2A and the plots of the apparent length of the fragments as a function of the temperature, in Figure 2B. Calibrations of these gels were performed from semilogarithmic plots of the relative gel mobility of the fragments and by the use of a least-squares fit. As a control, a sample of HinfI-cleaved pBR322 DNA was run in all gels (lanes 3, 6 and 9), because this DNA is employed as an unbent control (13,19). As can be observed, for the HinfI digestions (lanes 1, 4 and 7), the 443-bp fragment D showed an apparent mobility of about 600 bp, 530 bp and 450 bp at 6°C, 23°C and 50°C, respectively. The rest of the HinfI fragments showed no anomaly in their electrophoretic mobility, except for a slight reduction in the apparent size of fragment B (from an apparent mobility of 1250 bp at 6°C to 1160 bp at 50°C). From the HinfI restriction pattern of pLS1, we calculated that the ratios of apparent lengths at 6°C to the actual size of the fragments was nearly 1.0, except for fragment HinfID that has a ratio of 1.35 and HinfIB that has a ratio of 1.07. From the HphI digestions (lanes 2, 5 and 8), it was observed that fragments B and F exhibited a temperature-dependent anomalous behaviour. Fragment HphIB (which covers fragment HinfID almost completely), showed the apparent mobilities of 950 bp (6°C), 860 bp (23°C) and 720 bp (50°C). Fragment F exhibited apparent lengths of 520 bp, 510 bp and 450 bp at those three temperatures, respectively. The calculated ratios of their apparent lengths at 6°C to their actual sizes were 1.35 and 1.17 for HphI fragments B and F, respectively.

The results presented so far, demonstrate that a strong bend

Figure 3. Mapping of the bending centres of loci B-1 (A) and B-2 (B) of pLS1 DNA by circular permutation assays. The upper open bars show the restriction map of the duplicated DNA fragments with the appropriate coordinates of pLS1; sites marked with * belong to the multicloning site of pUC18. The fragments generated by digestion with the indicated restriction enzymes are depicted below the map. The relative gel mobilities of the circularly permuted pLS1 DNA fragments are plotted against the position of the restriction cleavage sites in one unit of the tandem dimer. Arrow points to the centre of the bending. The positions of the bent loci in the map of pLS1 are indicated below the plottings. P: promoters, 3 D.R.: 11-bp direct repeats; $\Delta 5$: deletion in pLS5.

is located within the 443-bp HinfID fragment. Two other regions exhibiting weaker bendings are located in fragment HphIF, contiguous to the HinfID fragment and in fragment HinfIB (see Fig. 1C). We have termed these three bent loci as B-2, B-1 and B-3, respectively.

To locate the centre of the bends in the above mentioned plasmid regions, circular permutation tests (9) were designed. Two plasmids were constructed: pLSM1B that contains a tandem duplication of the locus B-1 (ApaLI-TaqI, coordinates 607 to 1130 from pLS1), and pLSM2B that contains a tandem duplication of the locus B-2 (XmnI-StyI, coordinates 4366 to 758 from pLS4). Construction of a tandem duplication of the bent region B-3 was not feasible (see below). Each of the repeated fragments in plasmids pLSM1B and pLSM2B was eluted, digested with the appropriate restriction enzymes and the resulting fragments were run in 5% polyacrylamide gels. The electrophoretic mobilities of

Figure 4. Mapping of the bending centre of locus B-3 by two dimensional polyacrylamide (8%) gel electrophoresis (panel C) of partial restriction fragments (panel A) generated from the cloned *AflII*-*BanI* pLS1 DNA fragment. Panel B: diagram of the gel. Numbers indicate the actual size of the fragments and arrows indicate the position of the bent 350-bp fragment.

the circularly permuted fragments were plotted against the position of the restriction sites in the pLS1-DNA curved loci. The results are depicted in Figure 3, and they allowed us to estimate the bend centre of loci B-1 (Fig. 3A) and B-2 (Fig. 3B) at coordinates 882 (± 8 bp) and 634 (± 5 bp), respectively. The bending site of locus B-1 is located about 12 bp upstream of the putative promoter for the antisense RNAII, whereas the bend centre of locus B-2 is calculated to be about 5 nucleotides downstream of the putative promoter for RepA and RepB. However, the mobility curve for the locus B-2 is neither symmetrical nor sinusoidal as expected if there was only one major bending locus in this fragment (9). This could be an indication that more than one bending locus exists in this region (see below). A very weak, but

reproducible, bending could also be detectable near the PstI site (Fig. 3A). This locus was not further analyzed.

We could only clone the bending locus B-3 (AflII-BanI, coordinates 4022 to 214 from pLS1) in one copy; the resultant recombinant plasmid showed a dramatic reduction in its copy number as compared to the pUC18 vector used. Analysis of the DNA sequence of pLS1 at the cloned B-3 region showed 60% homology, in a stretch of 32 nucleotides, between the origin region of pUC18 and the putative antisense RNAI of pLS1 (1). It could be conceivable that the cloned RNAI would interfere with the replication of pUC18, causing the observed reduction in its copy number and making it impossible to construct a tandem duplication of this region. In this sense, it is worth noting that hybrids between pBR322 and pLS1-based replicons have been obtained only when pLS5 (that lacks RNAI) has been employed (Espinosa et al., unpublished). Due to these difficulties, a different approach was followed: the cloned AflII-BanI pLS1 fragment was eluted and partially digested with StyI and AvaI. The resulting fragments were subjected to a two-dimensional electrophoretic analysis (Fig. 4). As can be seen, out of the five fragments generated, only the partial AflII-AvaI (350 bp; Figs. 4B and C) fragment migrated off the diagonal. This indicates that the curved B-3 locus could have its centre around the StyI site, because cuts with this enzyme destroy the bending of this region, which is located in the vicinity of the plasmid minus origin of replication (Fig. 4A).

Inspection of the DNA sequence of pLS1 at the bend loci B-1 and B-3 showed no stretches of A and T periodically phased with the helix turn, one of the causes for sequence-directed static bending (10). At present we do not have any clear role for the curvature of pLS1 DNA at these regions. However, the region B-1 is located upstream of the putative promoter for the proposed countertranscribed RNAII, implicated in plasmid copy number control (del Solar et al., unpublished). It has been postulated that transcription at the promoters for inhibitor plasmid RNAs should be much higher than that of the target (22). Bending at regions upstream of prokaryotic promoters with high transcription rates seems to be a common characteristic, at least for E. coli promoters (15).

Figure 5. Scheme from a computer-generated cylindrical projection of pLS1 DNA from coordinates 530 to 630. Stretches of A's and T's are boxed. At the right, the position of the three 11-bp direct repeats is indicated.

The centre of curvature of locus B-2 in pLS1 DNA was located about coordinate 634, in the vicinity of a region that includes the three 11-bp direct repeats and the putative promoter for the

Figure 6. Computer-generated structure of pLS1 DNA from coordinates 430 to 700. Plot is shown in the YZ plane to show the three predicted bent regions. The small square at the bottom right is the 5' end of the fragment.

repA and repB genes. Cylindrical projection of the DNA helix between coordinates 530 to 630 (Fig. 5) showed that runs of four to five A or T residues are found almost exactly at every helix turn, throughout five consecutive turns. This phasing of A.T residues could be responsible for the strong bending detected at locus B-2, since this is a structure similar to the well characterized bending of kinetoplast DNA (8,9,10). However, such a DNA configuration would place the locus B-2 bending centre at around coordinate 580 instead of the experimentally determined coordinate 634. Downstream of this region, and starting at coordinate 634, the sequence $T(A)_4(T)_4G \dots 13bp \dots G(A)_7G$ is observable. This could indicate the presence of a second curvature at locus B-2, since it has been shown that oligodeoxynucleotides with various numbers of A_6 (or T_6) tracts are bent (23). In addition, upstream of this region, and starting at coordinate 472, the sequence $C(A)_4C(A)_{10}C$ is found, suggesting a third bend in this locus. The presence of various bent regions in locus B-2 could explain the asymmetry of the mobility curve of this locus (Fig. 3B). Although the explanation of the physical basis of helix deformation is not fully known (23,24), computer-assisted models facilitate a structural analysis of bend loci in a DNA fragment (25). When such an analysis was performed with the previously defined parameters (20,26) for the DNA of pLS1 between coordinates 430 to 700, three bends within locus B-2 were observable (Fig. 6). The predicted centres of these bends are located around

coordinates 470, 580 and 630. Similar predictions were obtained when a model that emphasizes the values of the roll angles (27) was used (not shown). A more detailed analysis of the circular permutation assay (9; Fig. 3B) was not possible in this region, since no known restriction sites exist from coordinates 510 to 610 in pLS1 DNA. This apparent bent locus B-2 of pLS1 includes the plus origin of replication, the three 11-bp direct repeats (coordinates 534 to 566) and the putative promoter for RepA and RepB (coordinates 597 to 629). The curvature of the DNA at this region could then have a functional role in the control of plasmid replication. We could speculate that the first bend of locus B-2 would be implicated in the recognition of the plasmid plus origin of replication by the replication initiator protein RepB. The second, at the vicinity of the repA-repB promoter might be the target of the repressor RepA. In this respect, we have observed a very strong bend induced by RepA at the fragment HinfID that protects the ApaLI site from restriction by this enzyme (del Solar et al., manuscript in preparation). Since RepA seems to be a trans-acting repressor of its own synthesis and of the synthesis of RepB, it is likely that static DNA bending at the target of RepA would facilitate the recognition and interaction between the repressor and its target. Control of plasmid pLS1 replication would then be exerted at three or more levels: i) by the antisense RNAII, ii) through transcriptional repression of the initiator protein RepB by RepA, and iii) by structural conformations at the plus origin of replication: secondary structures (2,3) and/or curvatures that would facilitate the target recognition by RepB. However, whether the three 11-bp direct repeats are actually a part of the plasmid origin of replication implicated in its recognition by RepB is a question under current investigation.

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