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In Vitro Recognition of the Replication Origin of pLS1 and of Plasmids of the pLS1 Family by the RepB Initiator Protein

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Rolling-circle replication of plasmid pLS1 is initiated by the plasmid-encoded RepB protein, which has nicking-closing (site-specific DNA strand transferase) enzymatic activity. The leading-strand origin of pLS1 contains two regions, (i) the RepB-binding site, constituted by three directly repeated sequences (iterons or the *bind* region), and (ii) the sequence where RepB introduces the nick to initiate replication (the *nic* region). A series of plasmids, belonging to the pLS1 family, show features similar to those of pLS1 and have DNA sequences homologous to the pLS1 *nic* region. In addition, they all share homologies at the level of their Rep proteins. However, the *bind* regions of these plasmids are, in general, not conserved. We tested the substrate specificity of purified RepB of pLS1. The RepB protein has a temperature-dependent nicking-closing action on supercoiled pLS1, as well as on recombinant plasmid DNAs harboring the pLS1 *nic* region. The DNA strand transferase activity of pLS1-encoded RepB was also assayed on two plasmids of the pLS1 family, namely, pE194 and pFX2. DNAs from both plasmids were relaxed by RepB, provided they had a proper degree of supercoiling; i.e., it was necessary to modulate the supercoiling of pE194 DNA to achieve RepB-mediated DNA relaxation. Single-stranded oligonucleotides containing the *nic* regions of various plasmids belonging to the pLS1 family, including those of pE194 and pFX2, were substrates for RepB. In vitro, the RepB protein does not need to bind to the iterons for its nicking-closing activity.

Many multicopy plasmids isolated from gram-positive and -negative bacteria replicate by an asymmetric rolling-circle (RC) mechanism (reviewed in references 10, 13, and 23). Characterization of various staphylococcal replicons has led to the proposal that plasmids replicating by the RC mode are constructed as interchangeable genetic modules (26). Only one of these modules is essential for plasmid replication, the leading-strand initiation and control region (10). On the basis of homologies in the DNA sequence and in the genetic organization of the leading-strand initiation and control region, four families have been defined, and their prototypes are pT181, pSN2, pC194/pUB110, and pMV158 (10, 13, 23). The latter plasmid has been studied in some detail for its derivative pLS1, which lacks the *mob* gene involved in conjugative mobilization (19, 25). The leading-strand initiation and control region includes the plasmid double-strand origin (*dso*) and the gene encoding the initiator of replication (Rep) protein. The *dso* can be physically and functionally divided into two regions, (i) *bind*, where the Rep protein binds, and (ii) *nic*, which includes the Rep-mediated nick site used to initiate replication (11). The two regions can be contiguous (as in pT181) or separated by dozens of bases (as in pLS1). The *nic* region contains one or two stem-loop structures which have been mapped by determining the sensitivity of plasmids pLS1 and pT181 to nuclease S1 in vitro and in vivo (21, 27). The DNA sequences recognized by the Rep proteins to introduce the nick are generally located on the terminal loop of one of these hairpins (IR-II of pT181 or hairpin I of pLS1), which can be generated on supercoiled DNA (21, 27).

Initiation of RC replication is mediated by the plasmid-encoded Rep protein, which shows site-specific DNA strand transferase activity on the plasmid *dso*. Rep proteins initiate replication by exerting a nucleophilic attack on the phosphodiester bond of a specific dinucleotide within the *nic* region. In pT181 and its cognate pC221, the phosphodiester bond 5′-ApT-3′ is cleaved by a conserved Tyr residue of the corresponding Rep protein, which remains covalently bound to the generated 5′-phosphate end (31). For pC194/pUB110, a similar situation has been proposed on the basis of genetic and mutational data (13, 22). In plasmid pLS1, the RepB-mediated nucleophilic attack is exerted between nucleotides G-448 and A-449 of the plasmid coding strand (6). However, in contrast to the above plasmids, the protein does not appear to remain covalently bound to the 5′-phosphoryl group of A-449 when single-stranded oligonucleotides are employed as the RepB substrate (20). Biochemical analysis has shown that IR-II (the *nic* region) of pC221 is sufficient for nicking-closing activity of its Rep protein. Such an in vitro reaction is not specific for the pC221 *nic* region, since pT181 DNA is also nicked by the pC221 RepD protein (31) and pC221 DNA is relaxed by RepC of pT181 (23, 36). The cross-reactivity of the two Rep proteins in vitro occurs because the *nic* regions of the plasmids are identical, whereas differences conferring *dso* specificity in vivo are located in the *bind* region (33, 34, 36). This "relaxed" specificity on heterologous substrates does not seem to be limited to the Rep proteins of RC-replicating plasmids, since similar behavior has been also shown for other DNA strand transferases like the VirD2 protein of *Agrobacterium tumefaciens* Ti plasmids (24). In RC replicons, conservation at the *nic* regions and differences in the *bind* regions seem to be common features of the different plasmid families (23).

To analyze the substrate requirements of the RepB initiator protein, we used supercoiled DNA from pLS1 and from recombinant plasmids harboring part or all of the *dso* of pLS1. In addition, to determine whether RepB is able to relax supercoiled DNAs of related plasmids, we employed two plasmids of the pLS1 family, namely, pE194 (14) and pFX2 (35). Whereas RepB readily cleaved supercoiled pFX2 DNA, it failed to do so

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TABLE 1. Plasmids used in this work

Plasmid	Size (bp)	Resistance marker[a]	Mutation or feature	Construction	Reference(s)
pLS1	4,408	TC	*mob*$^-$	Δ*Eco*RI-B of pMV158	19
pFX2	3,536	CM	Replication functions of pFX1 and *cat* gene of pC194	Δ*Cla*I-B of pFX1	35
pE194	3,728	EM	Natural isolate		14, 32
pC194*cop*	2,907	CM	Unidentified copy no. mutation		1
pCGA7	3,088	CM	*nic* region of pLS1	*Alu*I (position 320)-*Alu*I (position 505) fragment of pLS1 cloned in both orientations at *Hin*dIII site of pC194*cop*	11
pCGA8	3,088	CM	*nic* region of pLS1		
pCGA11	3,139	CM	Entire *dso* of pLS1	*Nco*I-*Apa*LI small fragment of pLS1Δ24 cloned in both orientations at *Hin*dIII site of pC194*cop*	11
pCGA12	3,139	CM	Entire *dso* of pLS1		
pCGA30	3,144	CM	*bind* region of pLS1	*Alu*I (position 505)-*Apa*LI (position 607) and *Hga*I (position 659)-*Bgl*I (position 804) fragments of pLS1 cloned at *Hin*dIII site of pC194*cop*	11

[a] TC, tetracycline; CM, chloramphenicol; EM, erythromycin.

on pE194 unless the degree of DNA supercoiling of pE194 was increased. By employment of single-stranded oligonucleotides as the substrate, we found that RepB efficiently and specifically recognizes the *nic* regions of most plasmids of the pLS1 family (including pE194), independently of whether the oligonucleotide can generate secondary structures. We propose that initiation of RC replication requires exposure of the plasmid *nic* region in a single-stranded configuration and that this step could be a key feature of *dso* recognition by Rep.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Streptococcus pneumoniae* 708 (*end-1 exo-2 trt-1 hex-4 malM594*) was employed for preparation of all plasmid DNAs except pE194, which was purified from *Bacillus subtilis* MB11 (*lys-3 metB10 hisH2*). Selective pressures for the plasmids were tetracycline (1 μg/ml) for pLS1, erythromycin (2 μg/ml) for pE194, and chloramphenicol (3 μg/ml) for the pC194-based recombinants and pFX2. To purify the RepB protein, the *Escherichia coli* BL21(DE3)/pET5 system (a gift of B. Studier) was employed. The other plasmids used are listed in Table 1.

DNAs and RepB protein purification. Supercoiled plasmid DNAs were isolated from bacterial cultures by preparation of cleared lysates followed by purification through two consecutive CsCl-ethidium bromide (EtBr) equilibrium gradients as previously described (8). The RepB protein was purified from *E. coli* BL21(DE3) harboring plasmid pLS19 (7) essentially as already described (6), except that the DEAE-Sephacel step was omitted. The protein was kept frozen at −70°C and maintained its activity for more than 1 year. The concentration of the protein preparation used here was 45 ng/μl, as calculated from its molar extinction coefficient and by determination of its amino acid composition.

Purification and labeling of oligonucleotides. Oligonucleotides were purified essentially as previously described (28). In some cases, the oligonucleotides were not deprotected from tritylation after synthesis. In these cases, purification was performed with Oligonucleotide Purification Cartridges (Applied Biosystems) by following the instructions of the vendor. Labeling of the oligodeoxyribonucleotides at their 5′ ends was performed with [γ-^{32}P]ATP (5,000 Ci/mmol; Amersham) and bacteriophage T4 polynucleotide kinase (Amersham). Procedures for labeling followed published protocols (28).

Nicking-closing action on supercoiled DNA. Unless otherwise stated, mixtures of RepB (45 ng) and plasmid DNA (700 ng) were incubated in a total volume of 30 μl of 20 mM Tris · HCl (pH 8.0)–1 mM EDTA–100 mM KCl–5 mM dithiothreitol–20 mM $MnCl_2$ for 30 min at the temperatures indicated in Results. Samples were then treated with proteinase K (125 μg/ml) and incubated at 20°C for 10 min. Reactions were stopped by addition of loading buffer, and the reaction products were run in 1% agarose gels in 90 mM Tris-borate–2 mM EDTA buffer containing EtBr (0.5 μg/ml). The amounts of DNA relaxed were determined by laser scanning of various negatives on a Densitometer 400 (Molecular Dynamics). Since open circular (FII), relaxed covalently closed (FI′), and supercoiled (FI) plasmid forms give different specific fluorescence intensities, the yield of RepB nicking-closing products was calculated from the decrease in the fluorescence signal of the FI form relative to that of the untreated DNA.

For competition experiments, pLS1 and pMV158 DNAs were employed. One of the DNAs (either pLS1 or pMV158) was kept at a fixed amount (500 ng), whereas the amounts of the other (competitor) DNA used were 125, 250, 500, and 1,000 ng, i.e., ratios of 0.25, 0.5, 1, and 2, respectively. Because of the different sizes of pLS1 (4,408 bp) and pMV158 (5,536 bp), the actual numbers of the competing molecules were slightly different. In these experiments, the DNAs were mixed, allowed to equilibrate in the above-described buffer, and treated with RepB (45 ng) at 60°C for 2 min. Samples were transferred to ice, and the reactions were stopped by addition of 1/10 volume of loading buffer containing proteinase K (0.27% xylene cyanol, 0.27% bromophenol blue, 10 mM EDTA, 65% glycerol, 1.2 mg of proteinase K per ml). Samples were electrophoresed as described above. Quantification of the fluorescence of stained gels was done directly with a Gel-Doc 1000 apparatus (Bio-Rad Laboratories).

Preparation of plasmid topoisomers. Supercoiled plasmid DNAs (1.5 μg) were relaxed with 10 U of wheat germ topoisomerase I (Promega) for 90 min at 37°C in 50 mM Tris · HCl (pH 8.0)–50 mM NaCl–1 mM EDTA–1 mM dithiothreitol–20% glycerol (final volume, 60 μl) in the presence of various concentrations (1 to 16 mM for pLS1 and 4 to 64 μM for pE194) of EtBr, essentially as already described (29). After 90 min of incubation at 37°C, samples were phenol extracted (twice) and ethanol precipitated. The DNAs were dissolved in distilled water (30 μl), samples (15 μl, 750 ng of DNA) were treated with RepB (55 ng) for 30 min at 60°C, and reactions were stopped as described above. Several electrophoresis conditions were tested for separation of topoisomers. Finally, the untreated and RepB-treated DNAs were electrophoresed on 1% agarose gels containing 0.016 (for pLS1) or 0.024 (for pE194) μg of EtBr per ml at 2.5 V/cm for 20 h. After electrophoresis, the gels were stained with EtBr (0.5 μg/ml) and photographed under UV light at 254 nm.

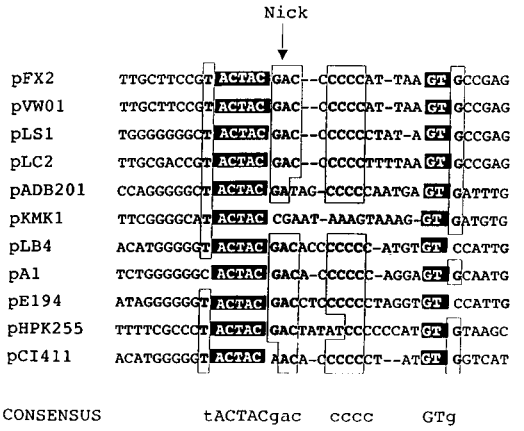
RepB-mediated cleavage of single-stranded oligonucleotides. Labeled oligodeoxyribonucleotides were incubated with RepB (at a molar ratio of 1 to 7.5, respectively) in 20 mM Tris · HCl (pH 8.0)–1 mM EDTA–100 mM KCl–5 mM dithiothreitol–20 mM $MnCl_2$ for 30 min at 37, 45, or 60°C. Reactions were stopped by addition of EDTA (200 mM) or proteinase K (300 μg/ml). The reaction products were separated on 20% polyacrylamide sequencing gels containing 8 M urea. Radioactive bands were detected by autoradiography on Kodak X-OMat film, and the counts in the bands of the gels were directly quantified with a PhosphorImager (Molecular Dynamics).

Computer work. Radioactivity and fluorescence were quantified with ImageQuant (Molecular Dynamics) and Molecular Analyst (Bio-Rad) software, respectively. The PILEUP program from the GCG Program Package (University of Wisconsin) was employed to align the nucleotide sequences of plasmids of the pLS1 family. Other analyses were done with DNASTAR computer software (Dnastar, London, England).

RESULTS AND DISCUSSION

Features of the *dso* of plasmids of the pLS1 family. The pLS1 family of plasmids includes 10 replicons (9, 10), to which recently described plasmid pCI411 (5) should be added. The Rep nick site has been unequivocally determined for pLS1 (6, 20) and genetically shown for pE194 (30). With these two exceptions, little is known about the leading-strand initiation and control region of the other members of the family, except for the information derived from sequence homologies. To determine the degree to which they are related, their *dso* DNA sequences were aligned. The results showed that they all share strong homology in a 9-bp sequence (5′-TACTACGAC-3′)

A



B

Plasmid	Nick site	Distance	Iteron	Number
pFX2	TACTACG/AC	83	TCGCCAACGTTT	3
pWV01	TACTACG/AC	83	TCGCCAACGTTT	3
pLS1	TACTACG/AC	84	TCGGCGACTTT	3
pLC2	TACTACG/AC	35	ACAAACCC	2
pADB201	TACTACG/AT	55	TGTTTTGCTAGCATTTGTTAA	3
pKMK1	TACTACCG/AA	91	GTTTT	3
pLB4	TACTACG/AC	14	GTCCATT	2
pA1	CACTACG/AC	13	GTGCAAT	3
pE194	TACTACG/AC	15	GTCCATT	3
pHPK255	TACTACG/AC	19	AAGCTAC	2
pCI411	TACTACA/AA	13	TGGTCATT	3

C

Plasmid	G+C(%)
pFX2	33.79
pFX2 (1080,1160)	45.68
pWV01	33.47
pWV01 (320,400)	48.15
pLS1	37.23
pLS1 (410,490)	43.21
pLC2	33.66
pLC2 (810,890)	56.79
pADB201	29.41
pADB201 (30,110)	49.38
pKMK1	29.01
pKMK1 (1740,1820)	41.98
pLB4	37.95
pLB4 (1980,2060)	45.68
pA1	34.93
pA1 (760,840)	45.68
pE194	31.71
pE194 (850,930)	49.38
pHPK255	36.77
pHPK255 (240,320)	41.97
pCI411	35.14
pCI411 (1180,1260)	44.44

FIG. 1. *dso* regions of plasmids of the pLS1 family. (A) Highest levels of homology found by PILEUP alignment of *dso* regions. Some gaps (dashes) were allowed for maximal homology. Bases conserved are shown at the bottom; those in capitals are conserved among all of the plasmids, and those in lowercase letters are those conserved in most of them. The Rep-introduced nick (determined for pLS1) is indicated. The following coordinates were aligned: pFX2, 1082 and 1176; pWV01, 322 and 418; pLS1, 400 and 495; pLC2, 807 and 902; pADB201, 31 and 127; pKMK1, 1737 and 1831; pLB4, 1972 and 2068; pA1, 742 and 837; pE194, 843 and 936; pHPK255, 231 and 324; pCI411, 1170 and 1265. Only the regions exhibiting maximal homology are depicted. Data for these plasmids were compiled from references 5 and 10. (B) Predicted Rep nick sites and their iterons. The nick site (G/A or A/A), the iteron numbers and their distances (in base pairs) from the nick site are indicated. (C) G+C contents of plasmids of the pLS1 family. In each case, the overall and the *dso* percentage of G+C content are indicated.

which includes the pLS1 nick site (Fig. 1A). Downstream of it, there are few short stretches of conserved nucleotides and the homologies do not extend further upstream or downstream. Since the purified RepB protein attacks the phosphodiester bond between nucleotides G and A of this 9-bp sequence of pLS1 DNA (coordinates 448 and 449; reference 6), we predict that this should be the nicking site of all of the plasmids in the family (Fig. 1B).

A unique feature of plasmids belonging to the pLS1 family is that they all contain two or three direct repeats (iterons; Fig. 1B) which vary in sequence, in size (5 to 21 nucleotides long), and in distance from the Rep nick site (13 to 91 bp). In pLS1, the iterons are the binding site of the RepB protein in vitro (6) and their deletion leads to failure of pLS1 to replicate in *S. pneumoniae* (data not shown). Another feature of plasmids of the pLS1-family, which is shared with plasmids of the pT181 family (23), is their low overall G+C content, which contrasts with the relatively high G+C content within the *dso* region (Fig. 1C). The heterogeneity of the G+C distribution may be related to the generation of important structural elements that can influence the efficiency of initiation of replication. Three major secondary structures have been mapped on supercoiled pLS1 DNA by determining sensitivity to nuclease S1 (27). One major stem-loop structure (hairpin I) and another putative minor one (hairpin II) are located within the *dso* (see Fig. 3). The other two mapped major secondary structures correspond to (i) hairpin III, located in the region corresponding to the putative terminator of the transcript synthesized from promoter P_1 (RNA I), and (ii) hairpin IV, within the lagging-strand origin of replication (*ssoA*). The latter two structures are dispensable for replication from the *dso* of plasmid pLS1 (11, 18).

Relaxation of supercoiled DNA by RepB protein. Purified RepB protein was able to relax supercoiled pMV158 DNA (FI forms), and the protein exhibited maximum activity at 60°C (20). Two types of molecules are the reaction products of RepB-dependent activity, open circles (FII forms) and relaxed closed circles (FI′ forms). FII molecules should result from a cleavage event, whereas FI′ molecules should be due to the strand transferase (closing) activity of RepB. In pLS1, FI forms were relaxed at 37°C but completion of the reaction could not be achieved (6). To evaluate the influence of temperature on the RepB-mediated nicking-closing action on pLS1, supercoiled pLS1 DNA was incubated with RepB at several temperatures ranging between 37 and 60°C. Quantification of the results (Fig. 2A) showed a progressive temperature-dependent conversion of FI forms into FII and FI′. FI forms almost disappeared after incubation with RepB for 30 min at 60°C. However, at the more physiological temperature of 37°C, only about 50% of the DNA of pLS1 was relaxed by RepB (at 45 ng of the protein and after 30 min of incubation). Little RepB activity was observed at 32°C, and no effect was observed below

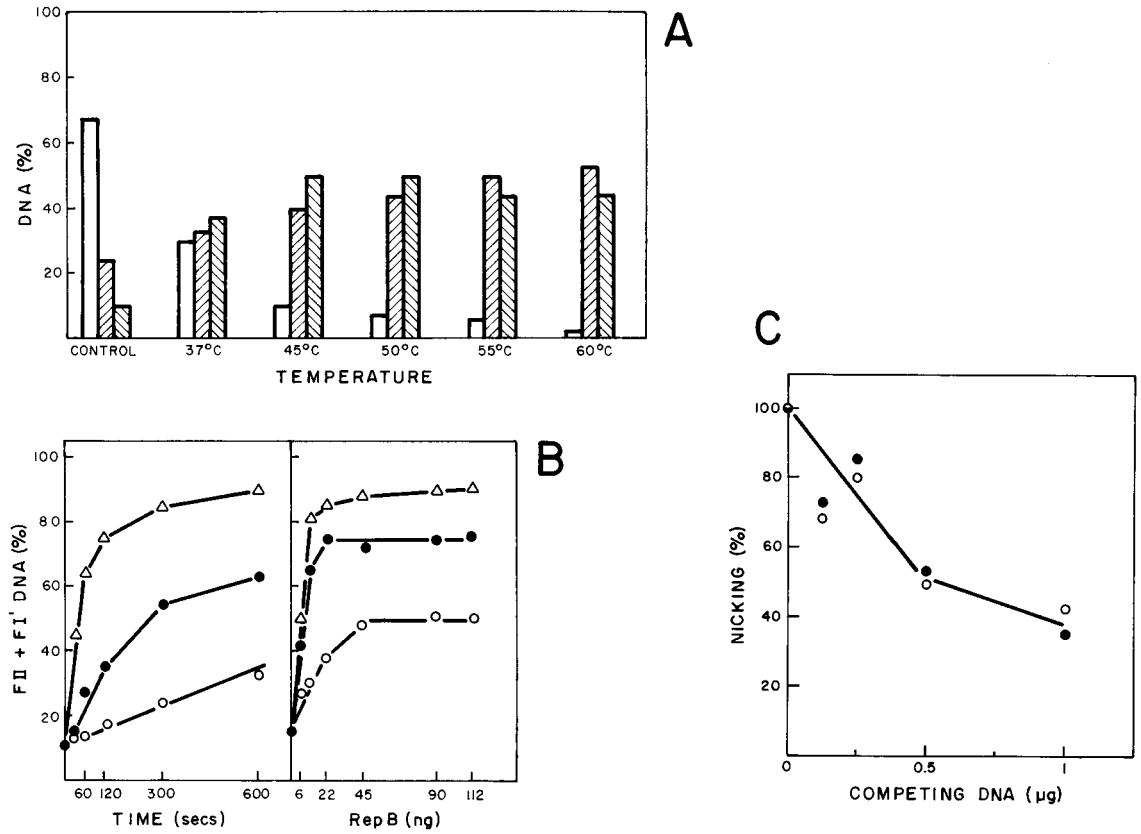


FIG. 2. RepB-mediated nicking-closing action on pLS1 supercoiled DNA. (A) Influence of temperature on the nicking-closing action. DNA (700 ng) from pLS1 was incubated with or without (control) the purified RepB protein (45 ng) for 30 min at the indicated temperatures. The reaction products were analyzed by agarose gel electrophoresis and quantified. The major plasmid forms are supercoiled DNA (FI; □), open circles (FII; ▨), and relaxed closed circles (FI′; ▧). (B) Time course and concentration dependence of the nicking-closing action of RepB on supercoiled pLS1 DNA. Reactions were performed at 37°C (○), 45°C (●), or 60°C (△). (C) Competition experiments. pLS1 or pMV158 DNA (500 ng) was mixed with increasing amounts of pMV158 or pLS1 DNA, respectively. Samples were treated with RepB protein (45 ng) for 2 min at 60°C. The reaction products were quantified by direct measurement of their fluorescence. Results are expressed as the percentage of pLS1 (○) or of pMV158 (●) DNA relaxed by RepB as a function of the amount of competing DNA added. The value obtained in the absence of competing DNA was taken as 100%.

30°C or at 65°C (data not shown). These results are comparable to those obtained with parental plasmid pMV158 (20).

Concentration dependence curves indicate that the reactions were almost saturated at protein levels above 40 ng (Fig. 2B). The final amounts of FII and FI′ generated by RepB depended upon the temperature of the reactions, but in general, the slope of the curves changed when the amount of RepB surpassed 40 ng, without a significant further increase in the amount of the products generated at higher amounts of the protein. Time course experiments showed that the conversion of FI to FII and FI′ was very fast at 60°C, the products reaching almost 80% after 2 min of incubation (Fig. 2B). The process slowed down as the temperature decreased. Comparison of these results with those previously obtained with pMV158 (20) suggested that RepB could act with different efficiency on both plasmid DNAs, since the time required for maximal relaxation of pLS1 DNA at 60°C (about 3 min) was higher than that required for pMV158 DNA relaxation (about 1.5 min; reference 20) under the same conditions. This finding could indicate some differences in the ability of RepB to relax those plasmids. To address this possibility, competition experiments were performed in which the amount of one plasmid DNA (either pLS1 or pMV158) was kept constant while the amount of the other plasmid DNA, used as a competitor, was increased. Quantification of the results (Fig. 2C) showed no preference of RepB cleavage for pMV158 over pLS1 (or vice versa). We interpret the difference between the rates of relaxation of pLS1 and pMV158 as due to the different protein preparations used in the previous study (20) and in the present experiments.

The above results indicate that RepB is able (i) to nick and close supercoiled pLS1 DNA in vitro and (ii) to relax a certain percentage of the supercoiled DNA present in the reaction in a temperature-dependent manner. Since hairpin generation depends upon temperature (4), one possibility would be that only a given fraction of the plasmid DNA substrate may have hairpin I exposed and that this fraction increased as the temperature of the reactions was raised. Thus, at 37°C, only those molecules exhibiting the nick site in a single-stranded configuration would be a substrate for RepB and the rest of the molecules would be insensitive to the protein. Increasing the incubation temperature resulted in an augmented number of specific RepB-relaxed DNA molecules. This supports our previous findings on the need for supercoiled DNA as a substrate for RepB (8). Hairpin generation prior to RepB cleavage could explain the failure of RepB to nick linear pLS1 DNA (data not shown), unlike RepC of pT181 (16). The differences in specificity between the RepB and RepC proteins could be due to the fact that the *bind* and *nic* regions of pLS1 are far apart (which is not the case for pT181), to the existence of a strong curvature between the two regions of pLS1 (our unpublished

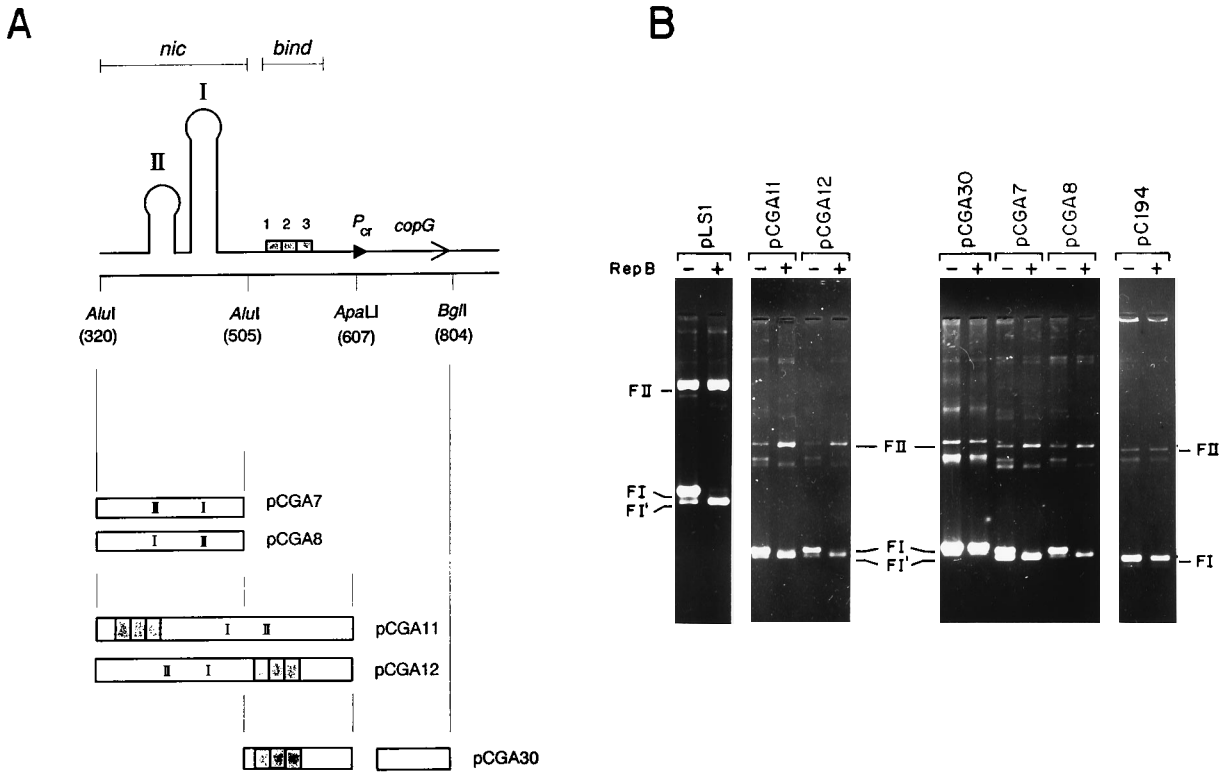


FIG. 3. In vitro structural requirements of RepB protein activity on supercoiled DNA. (A) Physical and functional map of pLS1 fragments cloned into the single pC194 *Hin*dIII site. The depicted segment shows a functional map of the region indicating the positions of hairpins I and II, the iterons (1 to 3, shaded), and promoter p_{CR} (▶) for transcription of genes *copG*, *repB*, and *copG* (arrow). The restriction sites (coordinates are in parentheses) of pLS1 used for cloning and the relevant elements contained within them are depicted below, along with the names of the recombinant plasmids and the orientations of hairpins I and II. (B) Agarose gels containing the DNA-RepB reaction products at 60°C. Conditions were the same as for Fig. 2. The plasmid DNAs, untreated (−) or treated (+) with RepB protein, are indicated above the lanes. Note that the amount of pCGA12 and pCGA8 DNAs loaded was lower than that of the other DNAs.

observations), or to different requirements of the two Rep proteins.

The pLS1 *nic* region is sufficient for RepB topoisomerase activity in vitro. To define more precisely the RepB-*dso* interactions on supercoiled DNA, we made use of recombinant plasmids that harbor pLS1 fragments containing only the *nic* region, the *bind* region, or the entire *dso* (Fig. 3A). These regions were cloned into the single *Hin*dIII site of pC194 (11). The resulting plasmids were pCGA7, pCGA8 (*nic* region in both orientations), pCGA30 (*bind* region), pCGA11, and pCGA12 (the entire *dso* in both orientations). In *S. pneumoniae*, only pCGA11 and pCGA12 exhibited some incompatibility towards pLS1 due to competition for the utilization of RepB, which led us to conclude that an intact *dso* is needed for the in vivo function of the initiator protein (11). To test the in vitro requirements of RepB, supercoiled DNAs from these plasmids were incubated with purified protein at 60°C. As positive and negative controls, DNAs from pLS1 and from the vector pC194, respectively, were used. The results (Fig. 3B) showed that only those plasmids that harbor the *nic* region, independently of its orientation, were sensitive to RepB. Neither vector pC194 nor iteron-containing plasmid pCGA30 was a substrate for the protein, demonstrating the specificity of RepB for the pLS1 *nic* region. Similar results were observed when the experiments were performed at lower temperatures (data not shown). Interestingly, plasmids pCGA7 and pCGA8 were relaxed by RepB even though they contain only the *nic* region and lack the iterons. We may conclude that (i) in vitro, relaxation of supercoiled DNA by RepB does not require previous binding of the initiator protein to its binding site; (ii) although RepB activity is strand dependent (20), it can be orientation independent on supercoiled DNA; and (iii) since the iterons seem to be essential for pLS1 replication in vivo, protein-DNA interactions different from those occurring for in vitro nicking-closing reactions have to take place during initiation of plasmid replication.

The above experiments allowed us to define in vitro the minimal pLS1 region sensitive to RepB as included within a 181-bp region (coordinates 320 to 505, the fragment cloned in pCGA7 and pCGA8). However, this region was not sufficient to show incompatibility towards pLS1, and only a 247-bp fragment harboring the complete *dso* did so (11). This indicates that, in vivo, binding of RepB to the iterons may be a requisite for the generation of hairpin I, RepC promotes hairpin extrusion at the plasmid *dso* (21), and subsequent nicking and initiation of replication for pT181. Alternatively, since the intracellular amount of RepB should be very small, it could be that molecules bound to its target have more advantages for recognition of its nicking site than unbound RepB molecules would have.

RepB recognizes supercoiled DNAs of pLS1-related plasmids. In addition to pLS1, only two other members of the same family are available to us: staphylococcal plasmid pE194 (14) and lactococcal replicon pFX2 (35). We have been unable to establish pE194 in *S. pneumoniae*, whereas pFX2 was readily transferred to this host (our unpublished observations). Although the three plasmids have homologies in the *nic* region (Fig. 1A), only the iterons of pFX2 and pLS1 have sequences

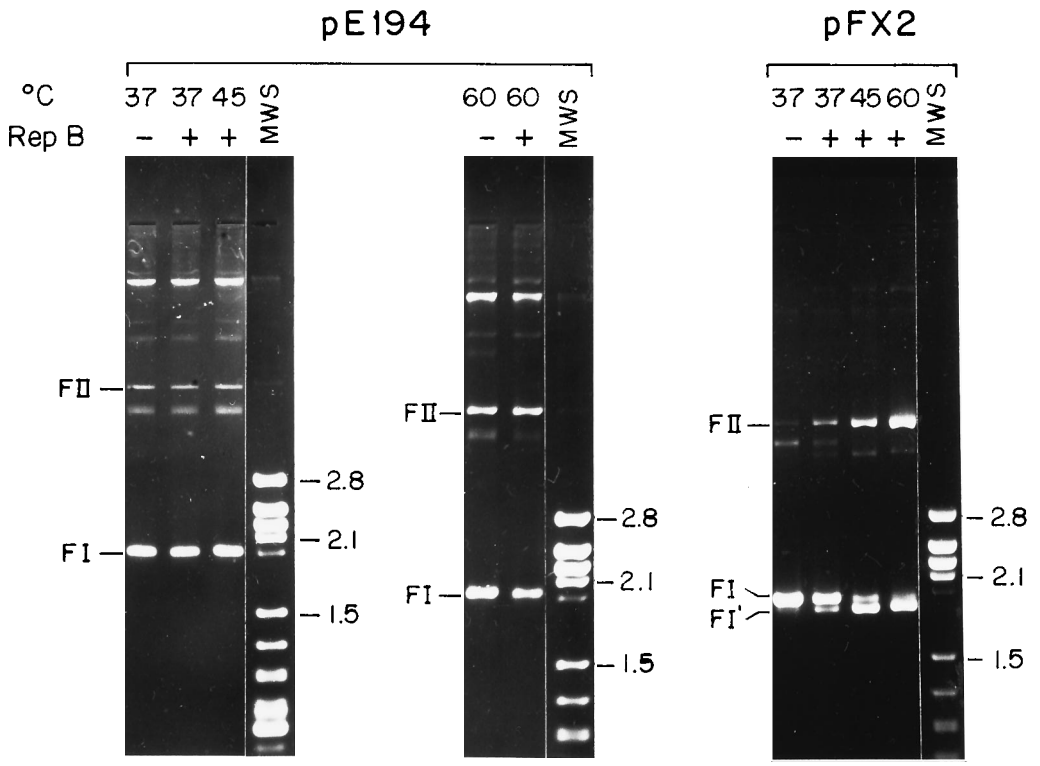


FIG. 4. Action of RepB on pLS1-related plasmid pFX2 and pE194 supercoiled DNAs. Supercoiled plasmid pE194 and pFX2 DNAs (700 ng per reaction) were incubated with (+) or without (−) purified RepB protein (45 ng) for 30 min at the indicated temperatures. MWS, molecular weight standards. Molecular sizes in kilobases are on the right.

in common (Fig. 1B). Computer-assisted searches indicated that the N-terminal moieties of the three Rep proteins are homologous (data not shown). The homologies extend along the whole amino acid sequences only in the Rep proteins of pLS1 and pFX2, whereas RepF (from pE194) has lower levels of homology with the Rep proteins of pLS1 and pFX2.

The above findings, in conjunction with the homologies among Rep proteins of the plasmids of the pLS1 family (10), suggested to us that the N-terminal moieties of the Rep proteins would be involved in the nicking specificity domain, whereas the iteron recognition domain would be located in the C-terminal moiety (10). In the plasmids of the pT181 family, the binding domain of the Rep proteins has been defined within a 6-amino-acid stretch located near the C end (12, 34). However, the *nic* region of plasmids of the pT181 family can be cross-recognized on supercoiled DNA by Rep proteins of the same family (23). To determine whether RepB recognizes the heterologous *nic* regions, supercoiled DNAs from plasmids pFX2 and pE194 were incubated with RepB protein at the above-mentioned temperatures. The results (Fig. 4) showed that pFX2 was relaxed in a way similar to that of pLS1, but pE194 DNA was insensitive to RepB at any temperature tested. Larger amounts of the protein or prolonged incubation times proved to be ineffective (data not shown). These results were somewhat unexpected, because the predicted nick site of pFX2 is located at an internal loop within a predicted plasmid hairpin (35), whereas that of pE194 should be placed on the terminal loop of one of the putative major secondary structures of the plasmid (32), as in pLS1. The inability of RepB to relax pE194 DNA could be due to lack of exposure of the nick site as single-stranded DNA. This, in turn, could be the consequence of either inappropriate superhelical density of the substrate DNA or differences in the abilities of the Rep proteins of pE194 and pLS1 to promote hairpin extrusion at the *dso*. Although there is no evidence of such a Rep-mediated hairpin extrusion in these plasmids, cruciform formation of plasmids of the pT181 family has been shown to be caused by the Rep protein (21). Preliminary experiments (data not shown) indicated that the degree of supercoiling of purified pE194 DNA was lower than that of pLS1 or pFX2 DNA. This could be because the former DNA was isolated from *B. subtilis* whereas the latter were prepared from *S. pneumoniae*. To test whether supercoiling of plasmid DNA influences the access of RepB to the *dso*, the degree of supercoiling of pLS1 and pE194 DNAs was varied in vitro by relaxation of plasmid DNA with topoisomerase I in the presence of increasing concentrations of EtBr (29). After relaxation and removal of the dye, the DNA molecules with different degrees of supercoiling were treated with RepB and electrophoresed in gels containing a low concentration of EtBr. The results (Fig. 5) demonstrated that pE194 could be made sensitive to RepB when its DNA was relaxed by topoisomerase I in the presence of relatively high concentrations of EtBr (over 8 μM), whereas dye concentrations below 4 μM had no effect (data not shown). With pLS1, a lower EtBr concentration (2 μM) was enough to obtain full relaxation by RepB. We can conclude that the degree of plasmid supercoiling modulates RepB activity in vitro, probably through hairpin formation.

RepB-mediated nicking of single-stranded DNAs harboring the *nic* region of pLS1-related plasmids. RepB requires a single-stranded DNA substrate to nick its target (20); the protein is unable to use linear or relaxed double-stranded DNA as a substrate. To define more precisely the RepB-mediated activity, three 23-mer single-stranded oligonucleotides containing

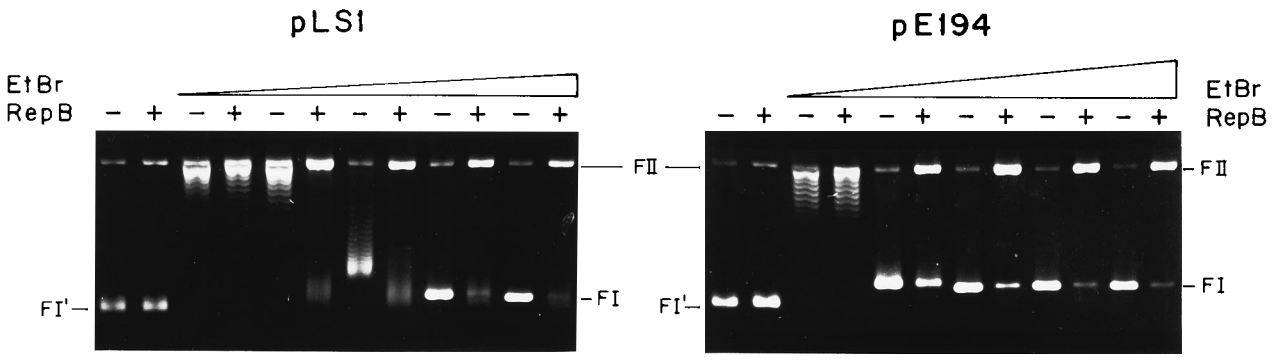


FIG. 5. Changes in the supercoiling of plasmid DNA results in modulation of RepB cleavage of plasmid molecules. pLS1 or pE194 DNA was relaxed with topoisomerase I in the presence of increasing concentrations of EtBr (0, 1, 2, 4, 8, and 16 μM for pLS1 and 0, 4, 8, 16, 32, and 64 μM for pE194). After removal of the enzyme and the dye, samples were treated (+) or not treated (−) with RepB protein and the reaction products were separated by electrophoresis in 1% agarose gels containing EtBr (0.016 μg/ml for pLS1 and 0.024 μg/ml for pE194). The positions of the most supercoiled DNA form obtained under these conditions (FI), of the relaxed covalently closed form (FI′), and of the open circular form (FII) are indicated.

the *nic* region of pLS1, pFX2, and pE194 (oligonucleotides pL, pF, and pE, respectively) were synthesized, labeled at the 5′ ends, and incubated with RepB. The three oligonucleotides have the RepB nick site (GpA) located 15 nucleotides downstream of their 5′ ends (see Table 2). Thus, if RepB were able to cleave these substrates, a 15-mer labeled product should be visible. In addition, whereas oligonucleotides pL and pE have the potential to generate secondary structures by intrastrand pairing, this is not the case for oligonucleotide pF. As a negative control, a 23-mer oligonucleotide complementary to the *nic* region of pLS1 (oligonucleotide comp) was employed. As expected for a protein having sequence-specific activity, oligonucleotide comp was not cleaved by the RepB protein. However, the oligonucleotides containing the *nic* region of the three plasmids were similarly cleaved by RepB, yielding 15-mer labeled products (Fig. 6). Several labeled bands were visible in oligonucleotides pL and comp, and some of them were sensitive to RepB in oligonucleotide pL. We assume that these bands are due to the seven G residues located at the 5′ end of the 23-mer which complicate oligonucleotide synthesis. From the above results, we may draw the following conclusions: (i) intrastrand pairings (i.e., secondary structures) are not essential for RepB activity if the substrate DNAs are in a single-stranded configuration, (ii) the role of the secondary structures of the *nic* region would be to expose this region in a single-stranded configuration on supercoiled DNAs, and (iii) base changes in the *nic* region of pE194 do not affect RepB recognition.

To extend these analyses to other members of the pLS1 family of plasmids, we synthesized single-stranded oligonucleotides harboring the *nic* regions of plasmids pADB201 (3), pHPK255 (15), and pLB4 (2). These DNAs, in addition to the pL, pF, and pE oligonucleotides used as described above, were 5′ end labeled and used as substrates for RepB nicking. The resulting 15-mer products were quantified, and the ability of RepB to nick the different oligonucleotides was expressed as the percentage, at each temperature, of the 15-mer product obtained with oligonucleotide pL (Table 2). All DNAs were cleaved by RepB, with the exception of the one containing the nick site of plasmid pADB201. This result was expected be-

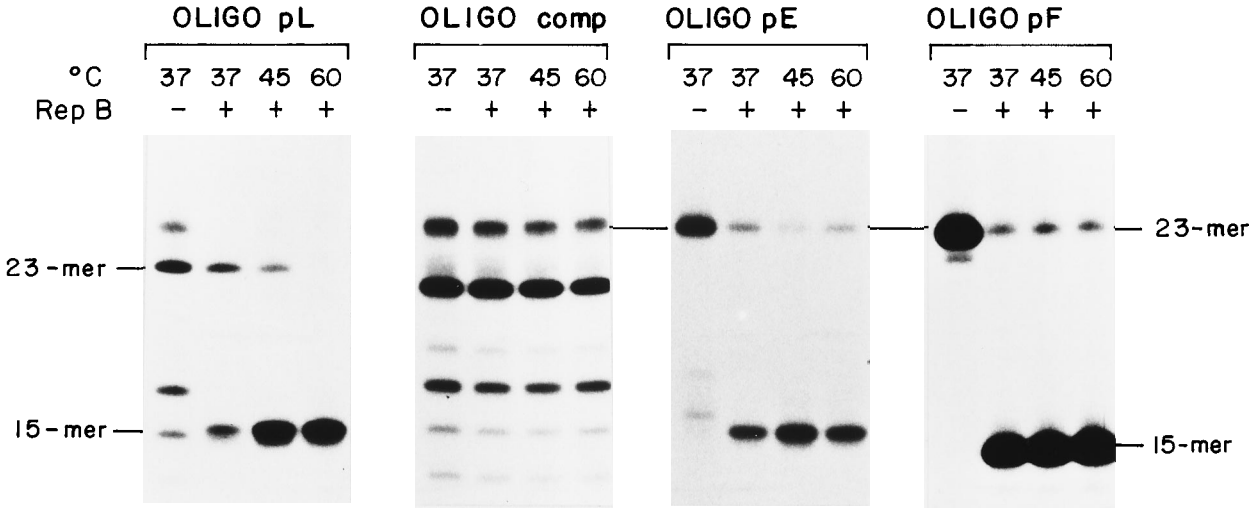


FIG. 6. Specific nicking by RepB of single-stranded oligonucleotides (OLIGOs) containing the *nic* region of plasmid pLS1, pFX2, or pE194 (see Table 2). As a control, a single-stranded oligonucleotide (comp [5′-GGGGGGGTCGTAGTAGCCCCCCC-3′]) containing the sequence complementary to that of oligonucleotide pL, was employed. The reaction mixtures contained 1.26 pmol of the oligonucleotide (2.5 pmol for the oligonucleotide pF) and 9.3 pmol of RepB (18.6 pmol for oligonucleotide pF). Incubations were done at the indicated temperatures, and the reaction products were separated on 20% polyacrylamide–8 M urea sequencing gels and visualized by autoradiography. The positions of the input DNAs (23-mer) and of the reaction products (15-mer) are indicated.

TABLE 2. Specificity of recognition of pLS1-related origins by RepB protein

Oligo (Plasmid)	Sequence (5′ - 3′)	Nicking at[a] 37°C	45°C	60°C
pLS1	GGGGGGGCTACTACG/ACCCCCCCC	100	100	100
pFX2	TGCTTCCGTACTACG/ACCCCCCCA	139	106	106
pE194	TAGGGGGGTACTACG/ACCTCCCCC	111	105	91
pADB201	CAGGGGGGCTACTACG/ATAGCCCCC	<10	<10	<10
pHPK255	TTTCGCCCTACTACG/ACTATATC	132	99	97
pLB4	CATGGGGGTACTACG/ACACCCCCC	50	64	81
	Consensus: TACTACG/AC			

[a] Results are expressed as percentages of the pLS1 values, which where calculated as the ratios of nicked/input DNAs. These values were 0.682 (37°C), 0.902 (45°C), and 0.95 (60°C). Base changes, compared with the sequence of pLS1, are boxed.

cause we have shown that the C just downstream of the GpA dinucleotide is essential for RepB nicking (20). It would be interesting to know whether the Rep protein of pADB201 is able to recognize the *nic* regions of the other pLS1-related plasmids. In addition, the oligonucleotide containing the nick site of plasmid pLB4 was nicked with lower efficiency than that of pHPK255 (Table 2). This suggests that the nucleotide located at the third position after the nick site can be changed to a T (pHPK255), but not to an A (pLB4), without a decrease in RepB activity. We can conclude that (i) the nick site of plasmids of the pLS1 family is located at the place predicted by us (10; Fig. 1A), (ii) in pADB201, the nick site remains to be defined unequivocally, and (iii) RepB recognizes the *nic* regions of plasmids related to pLS1 when they are in a single-stranded configuration, except for that of *Mycoplasma* plasmid pADB201.

The supercoiled FI form is the usual conformation of plasmids within bacteria, and mechanisms to initiate replication are generally designed to open the strands at the origins of replication (17). In RC-replicating plasmids, initiation is achieved in a simple way, since only the plasmid-encoded Rep protein is required to convert the DNA substrate (FI forms) into the initiation product, the nicked FII forms. Upon nicking of plasmid DNA by Rep, a host-encoded helicase would open the strands of the FII molecules and the Rep-generated 3′-OH end would be extended by DNA polymerase III (23). An important requirement for initiation of RC replication is the generation of secondary structures within the plasmid *dso* to expose the Rep nick site in a single-stranded configuration. Hairpin generation at the *nic* region could then be envisaged as a *cis*-acting process that modulates, in a passive way, the initiation of replication. We can speculate that plasmids replicating by the RC mode seem to have developed a strategy to ensure that the hairpins in the *nic* region are formed, since most, if not all, plasmid *dso* regions are located between divergently transcribed promoters, as noted earlier (23). Thus, the waves of transcription would produce enough torsional stress on the *dso* DNA region to facilitate generation of the nick-containing hairpin. In plasmid pLS1, these divergent promoters are (i) p_{CR}, from which the *cop-rep* operon is transcribed (9) and which is located downstream of the *dso*, and (ii) p_L, from which RNA I is transcribed and which is located upstream of the *dso* (our unpublished observations). It remains to be shown whether in vivo transcription from these two divergent promoters plays a role in recognition of the *dso* by RepB.

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