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Initiation signals for the conversion of single stranded to double stranded DNA forms in the streptococcal plasmid pLS1

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ABSTRACT

We have characterized a region in the streptococcal plasmid pLS1 located between nucleotides 4103 and 4218 which is a signal involved in the conversion of single stranded intermediates of replication to double stranded plasmid forms. This region has a large axis of dyad symmetry resulting in the formation of a secondary structure as revealed by the location of endonuclease S1-cleavage sites in supercoiled covalently closed circular pLS1 DNA. Deletions affecting this region caused a fivefold reduction in plasmid copy number, plasmid instability and the accumulation of single-stranded DNA intermediates. The conversion signal of pLS1 has homologues in other staphylococcal plasmids, sharing a consensus sequence located in the loop of the signal. Computer assisted analysis showed that the signal detected in pLS1 has a high degree of homology with the complementary strand origin of the Escherichia coli single stranded bacteriophages ØX174 and M13.

INTRODUCTION

pLS1 is a fully sequenced 4408 bp broad-host-range multicopy plasmid, derived from the streptococcal plasmid pMV158 (1) by deletion of a 1.1-kb EcoRI fragment (2). Plasmid pLS1 specifies constitutive resistance to tetracycline, and has been shown to replicate in a variety of hosts, Gram-positive and Gram-negative (2). Studies performed in cell-free extracts of E. coli strains devoid of plasmid have shown that replication of pLS1 required the host RNA polymerase and de novo protein synthesis (3). In addition, two plasmid-encoded polypeptides, RepA (5.1 KDa) and RepB (24.2 KDa), are required for plasmid replication (del Solar et al., manuscript in preparation). The origin of pLS1 has been located within a 443-bp HinfI restriction fragment and replication proceeds unidirectionally in the direction of transcription of the plasmid mRNAs (Puyet et al., sub-

mitted). Copy number control of pLS1 seems to be regulated by a small countertranscribed RNA located between the coding regions for RepA and RepB, although high copy number mutants affecting only RepA have also been isolated (del Solar et al., to be published).

Small plasmids isolated from Gram-positive hosts show various degrees of homology either in the antibiotic-resistance markers or in the replication systems (4). In the case of the streptococcal plasmid pLS1, the gene encoding tetracycline resistance in highly homologous to the tet gene of the staphylococcal plasmid pT181 (5) and almost identical to the Bacillus plasmid pTHT15 (6); however no homology upstream of the tet promoter has been found between these three plasmids (2). In addition, the Staphylococcus aureus plasmids pT181, pC221 and pS194 have considerable mutual homology (7), and key elements of the control system of pT181 and pC221 are closely conserved between the two plasmids (4). Thus, it seems that all small Gram-positive plasmids have conserved a general functional structure. Nevertheless, pLS1 seems to be different in some aspects to the other plasmids, in the sense that it is also able to replicate in Gram-negative hosts, both "in vivo" (2) and "in vitro" (3), which indicates that it should share some characteristics with the replicative machinery of the Gram-negative replicons.

Another common feature of many of these small Gram-positive plasmids is their replication mechanism: initiation of plasmid replication seems to involve the recognition of a specific site, at which the replication protein produces a single-stranded nick (8, 9; Ballester et al., submitted). Then, by a mechanism of rolling circle replication, the nicked strand is displaced while the other strand is used a template for DNA synthesis. When strand separation goes to completion, single--stranded circular plasmid forms are generated (10). The second step of this mode of replication should involve the conversion of the single-stranded intermediates into double-stranded plasmid forms by a mechanism still unknown.

In this work we present evidence that a specific plasmid sequence is needed in the conversion of single- to double-

stranded pLS1 plasmid DNA. This sequence has a strong potential secondary structure and is located within a non-coding region, left of the origin of replication (plus origin). Deletion of this signal region reduces plasmid copy number, porduces plasmid instability and leads to the accumulation of single-stranded DNA intermediates. The signal present in pLS1 seems to be recognized not only by Streptococcus pneumoniae and B.subtilis but also by E. coli, although the efficiency of conversion was higher in the pneumococcal host. Computer assisted analysis showed about 50% homology between this conversion signal of pLS1 and the complementary strand origins of bacteriophages M13 (11) and ØX174 (12). In addition, large regions of potential secondary structure in the staphylococcal plasmids pC221, pC194 and pE194 were found to share homology with the conversion signal of pLS1.

MATERIALS AND METHODS

Bacterial strains and plasmids

S. pneumoniae 708 (end-1 exo-2 trt-1 hex-4 malM594) was used throughout this work; it was grown in a casein-hydrolysate based medium (13). B. subtilis MB11 (lys-3 metB10 hisH2) was grown in L broth or, for the development of competence for transformation, in a minimal medium (14). Competence development and transformation procedures for these two Gram-positive hosts have been reported elsewhere (14). E. coli C600 (thr-1 thi-1 leu-6 lacY1 tonA21 supE44) was grown in L broth and transformed as described (2). Plasmids used are described in the Results section. Selections for the antibiotic resistance markers were: tetracycline at 1, 15 and 10 µg/ml; chloramphenicol at 5, 7.5 and 10 µg/ml, for S. pneumoniae, B. subtilis and E. coli, respectively.

Plasmid DNA preparations

For rapid screening of plasmids, total cell extracts containing chromosomal and plasmid DNA were prepared from 1.5-ml cultures at 5 to 10 x 10^8 colony forming units (c.f.u.) per ml, as previously reported (2). Alkaline lysates were prepared from E. coli and B. subtilis by the procedure of Birboim and Doly (15) and from S. pneumoniae by a modification of that method (16). Purification of plasmid DNA was performed as described

(3). For plasmid copy number determinations, cells were grown in minimal media as described below.

For the analysis of single-stranded plasmid DNA, 1.5-ml cultures were grown to 4 x 10^8 c.f.u./ml and washed with 0.15 M NaCl. Cells of E. coli and B. subtilis were suspended in 25 µl of a mixture containing 25% sucrose, 0.1 M NaCl, 50 mM Tris. HCl (pH 8.0), 5 µg pancreatic RNase, and 40 µg lysozyme; they were incubated at 37º C until a change in viscosity occurred (usually between 7 and 12 min) and then clear lysis was obtained by addition of 1 µl of 10% SDS. Cells of S. pneumoniae were suspended in 100 µl of solution containing 25% sucrose, 0.15 M sodium citrate, 0.1% sodium deoxycholate, 0.01% SDS, and 20 µg pancreatic RNase; they were incubated at room temperature for 10 min. For the three bacterial species, the lysed cell extracts were subjected to vortex treatment for 3 min and twice frozen and thawed. For nuclease S1 treatment, lysates (without previous removal of SDS from the samples) were adjusted to 30 mM sodium acetate (pH 4.6), 0.5 mM $ZnCl_2$ and 250 mM NaCl and incubated for 20 min at 37º C in the presence of 1000 units/ml of endonuclease S1. The lysates were then adjusted to 1% SDS, vortexed for 2 min, frozen and thawed, and treated with Proteinase K, 300 µg/ml. After incubation at room temperature for 10 min, the tracking dye was added and the samples were immediately subjected to electrophoresis as described below.

Detection and analysis of single-stranded plasmid DNA

Samples (50 µl) of cell extracts were loaded in 0.7% agarose gels in Tris/borate buffer containing 0.5 µg/ml ethidium bromide. Electrophoresis was conducted at room temperature at the constant voltage of 2 V/cm for 18 h. The gel was photographed and the position of the supercoiled covalently closed circular (ccc) plasmid DNA, recorded. DNA was transferred to nitrocellulose filters by diffusion, as described (10).

Radioactive probes were prepared either by nick-translation of purified plasmid DNA or by the method of Feinberg and Vogelstein involving the use of oligonucleotides (17) in the presence of (α-^{32}P)dCTP. Filters were hybridized and exposed to X-ray films as described (10).

Positional specificity of endonuclease S1 nicks

S1 nuclease reactions were performed in buffer containing 30 mM sodium acetate, pH 4.6, 50 mM NaCl, 1mM $ZnSO_4$. Typical reaction conditions were performed in 20 µl volumes containing purified plasmid DNA (1 µg), 30 units nuclease S1 and buffer. Incubations were at 10º C for 24 h or at 37º C for 7 h, followed by heating at 68º C for 10 min. Plasmid DNA was then dialyzed against 10 mM Tris, 1 mM EDTA, pH 8.0 in Millipore filters (type VS) (18) and subjected to restriction with EcoRI or with Bg1I.

Copy number determination

Cells were grown in synthetic media (2) containing 4 µM (^{3}H)thymidine, 15 Ci/mmol. Total DNA extracts were prepared as indicated above and the amount of radioactivity incorporated into plasmid or chromosomal DNAs was determined as described (2).

Stability test

Cultures of S. pneumoniae harbouring plasmids were grown in liquid medium with selective pressure for 30 generations until 4 x 10^8 c.f.u./ml. Then the culture was successively diluted (1:1024) into antibiotic-free medium and grown to the same cell density; the number of generations in each passage was thus kept at the constant value of 10. The number of colonies in medium with or without selective pressure was determined and the frequency of plasmid loss was calculated according to Gerdes et al. (19).

Computer work

Searching for secondary structures in plasmid and phage DNAs, repeats and other features in the complete DNA sequences analyzed in this work were performed by using DNASTAR computer programs (DNASTAR, Inc., UK).

RESULTS

Location of cleavage sites by endonuclease S1

To locate secondary structures in plasmid pLS1, we made use of the finding that endonuclease S1 is able to introduce selective cleavages into single-stranded regions of hairpin

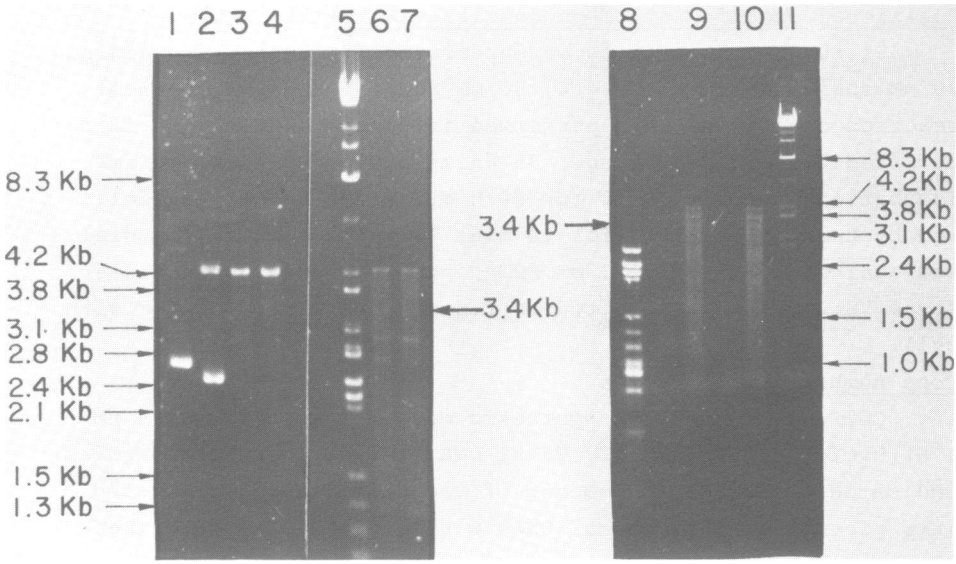


Figure 1. Agarose gel electrophoresis showing supercoiled pLS1 (lane 1) and pLS5 (lane 2) DNAs and the cleavage of supercoiled pLS1 DNA by S1 nuclease at 10º C (lane 3) or at 37º C (lane 4). Open circular forms are also observed, specially in lane 2. The 10º C S1-linearized pLS1 DNA was digested with EcoRI (lane 6) or with BglI (lanes 9 and 10; 0.15 and 0.2 µg of DNA were loaded, respectively); the S1-cleaved pLS1 DNA at 37º C was digested with EcoRI (lane 7). Lanes 5, 8 and 11: molecular weight standards. Heavy arrows indicate the position of the 3.4 kb bands generated between the S1-cleavage sites and the EcoRI or the BglI restriction sites. Linear plasmid forms observed in lanes 6, 7, 9 and 10 resulted from restricted relaxed molecules and they are visible because twice or thrice more DNA was loaded in these lanes than in lanes 1 to 4.

structures present only in supercoiled ccc-DNA molecules (20). These cleavage sites can be mapped by restriction analysis of the S1-linearized molecules. As shown in Fig. 1, treatment of supercoiled pLS1 DNA with nuclease S1 resulted in the conversion of these forms into linear molecules, whereas the minor relaxed forms were insensitive to the enzyme (lanes 3 and 4). When the S1-linearized pLS1 DNA was cleaved with EcoRI (lanes 6 and 7) (which cuts this plasmid once at coordinate 3170), discrete fragments of 3.4, 3.0 and 2.7 kb were observed. In

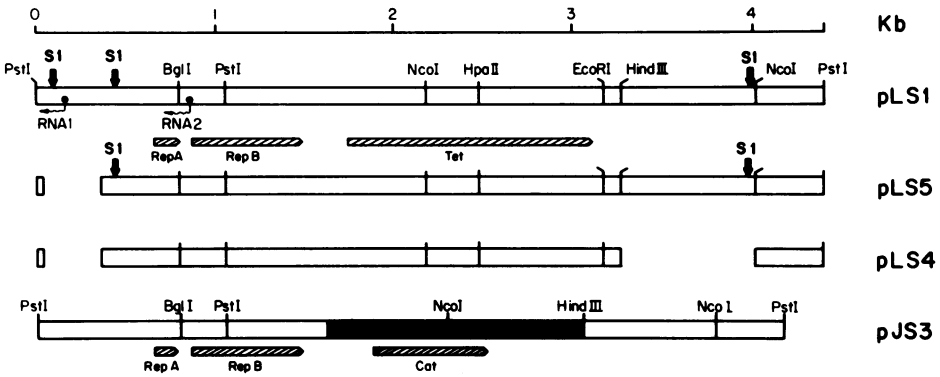


Figure 2. Plasmids used in this work: maps of plasmids pLS1, pLS5, pLS4 and pJS3. Only relevant restriction sites are indicated. Missing regions in pLS5 and pLS4 indicate deletions. Heavy arrows (S1) show endonuclease S1 cleavage sites for pLS1 and pLS5. They are not indicated in pLS4 nor pJS3 since they have not been identified in this work. Wavy arrows indicate putative countertranscribed RNAs.
Polypeptides synthesized by pLS1 DNA are indicated as shadowed boxes with pointed ends corresponding to carboxyl termini. Filled part in pJS3 corresponds to the pC194 segment.

addition, linear forms were also detectable due to cleavage by the restriction enzyme of S1-insensitive relaxed plasmid molecules. The above three fragments were mainly observed when the S1 treatment was performed at 10º C (lane 6). The 3.4 kb fragment corresponds to a specific S1 cleavage sites that could be mapped either about coordinate 2170 or 4170 in the pLS1 map (Fig. 2). Computer analysis showed that no significant secondary structure could be formed at coordinate 2170, but a large hairpin structure could be formed around coordinate 4170 (see below). When S1-linearized pLS1 DNA was cleaved with BglI (which cuts once at coordinate 800), bands of about 4.1, 3.7 and 3.4 kb were detectable (lanes 9 and 10). If we add the 3.4 kb band to the 0.8 kb (position of BglI), we may unequivocally locate the above specific S1 site around coordinate 4200,which corresponds to the hairpin structure detected by computer analysis. In addition, we have mapped the two other S1-sensitive sites in pLS1 (Fig. 2) which are located near the origin of re-

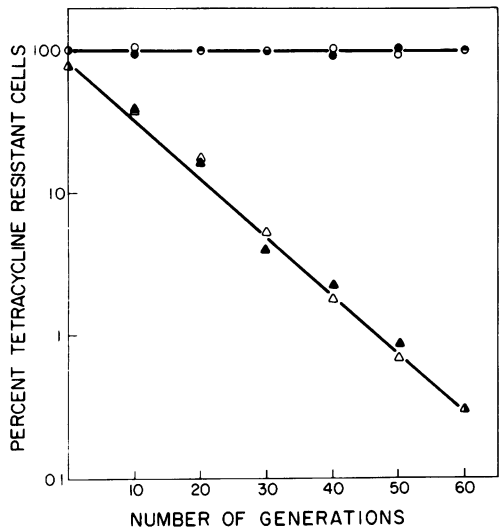


Figure 3. Stability of plasmids pLS1 (○), pLS5 (●), pLS4 (△) and pLS4-t (▲) in exponentially growing cells of S. pneumoniae. Plasmid pLS4-t contains a bacteriophage Ø29 bidirectional transcription terminator cloned into the EcoRI site of pLS4.

plication and that will be presented elsewhere (Puyet et al., submitted).

From plasmid pLS1, several derivatives have been isolated; maps of some of them are depicted in Fig. 2. Plasmid pLS5 was isolated in B. subtilis and contains a deletion of 332 bp extending from nucleotide 34 to 365 in pLS1 that resulted in a doubling in its copy number (2). Plasmid pLS4 was constructed from pLS5 by digestion with HindIII (coordinate 3278) followed by partial digestion with NcoI (coordinates 2181 and 4220) and end-filled with the Klenow fragment of the DNA polymerase I. Plasmid pJS3 contains a 1432-bp fragment from plasmid pC194 specifying resistance to chloramphenicol. It also contains the pLS1 replicon and was isolated as a spontaneous deletion from a hybrid plasmid (termed pJS37) between pLS1 and pC194 (21). Plasmids pLS5 and pLS4 lack a putative small RNA reading leftward (2), located within a region of harpin structures that are sensitive to endonuclease S1 (Puyet et al., submitted). Furthermore, the deletion in pLS4 resulted in the removal of

TABLE 1. Efficiencies of plasmid transfers

Plasmid	DNA concentration (µg)	Transformants/ml in the bacterial host		
		S.pneumoniae	E.coli	B. subtilis[a]
pLS1	0.1	16,000	2,000	100
	1.0	40,000	10,000	860
PLS5	0.1	21,000	3,000	3,800
	1.0	53,000	12,000	30,500
pLS4	0.1	4,000	0	500
	1.0	9,000	0	4,500

(a): Plasmid DNAs were purified from S. pneumoniae: the heterospecific origin of the plasmid DNA results in a decrease in the efficiency of plasmid transfer in B. subtilis (14).

the S1-cleavage site located around coordinate 4170 and of an open reading frame (orfD; 2) with no identified promoter and which is not synthesized in pLS1 nor in pJS3 (results not shown).

Differential characteristics of pLS4

When pLS4 was constructed in S. pneumoniae, it was apparent that a drastic reduction in its copy number had occurred. Copy number measurements, demonstrated that pLS4 has 5 copies per cell genome, as compared to the 50 or 25 copies determined for pLS5 or pLS1, respectively. Since the stability of a plasmid may be related to its copy number, it was of interest to test the stability of pLS4 as compared to its parental plasmids pLS1 and pLS5. As depicted in Fig. 3, pLS4 showed a high instability in S. pneumoniae, with a frequency of loss (19) of 0.09 per cell per generation, whereas no detectable loss of pLS1 or pLS5 was observed during the 60 generations of the assay. In addition, it should be noted that, in the presence of selective pressure (Fig. 3, generation 0), already 10% of the cells were plasmid-free. The instability of pLS4 was not due to transcriptional interference of the tet gene with plasmid replication, since a bidirectional transcription terminator from bacteriophage Ø29 (cloned in plasmid pEB116, a gift of J.C. Alonso) inserted into the unique EcoRI site of pLS4, did not stabilize the

plasmid (Fig. 3). Furthermore, the low copy number and instability of pLS4 cannot be attributed to alterations in the origin of replication (plus origin), since the origin lies outside the deletions. Therefore, the differential characteristics of pLS4 must reflect alterations in its replication. It was thus of interest to know whether pLS4 was able to replicate in E. coli and if so, the frequencies of its establishment in this host and in pneumococcus. To this end, competent cultures of S. pneumoniae, E. coli or B. subtilis were treated with pLS4 DNA at subsaturating (0.1 µg/ml) or saturating (1.0 µg/ml) concentrations. As controls, the same amounts of pLS1 or pLS5 DNAs were used. Results are shown in Table 1. As can be seen, pLS4 failed to transform E.coli and showed a reduced transforming ability (4 to 5 times) in S. pneumoniae as compared to pLS1 or pLS5, independent of the concentration of DNA used. No significant differences in the efficiency of establishment were found for pLS1 or pLS5. When the same experiments were performed using B. subtilis as a host, plasmids containing the deletion of pLS5 transformed with higher efficiency than the parental pLS1, although no accumulation of multimeric forms (which are the only plasmid forms with transforming activity in this bacterium) was detectable (results not shown). At present we do not have an explanation for this finding.

Nucleotide sequence features within the deletion in pLS4.

Computer assisted analysis of the region included in the deletion HindIII-NcoI of pLS4 showed that the most relevant characteristic is a large hyphenated inverted repeat from coordinates 4103 to 4218. This sequence forms a hairpin structure the loop of which could be recognized by endonuclease S1 as a site of preferential cleavage (Figs. 1 and 2). However our results do not allow us to precisely locate the sequence where the break is made. Large palindromes have been found in other Gram-positive plasmids (4), but no function has been ascribed so far. By computer analysis of secondary structures, we have found that at least three staphylococcal plasmids, viz. pC221 (4), pC194 (22) and pE194 (23) have also the potential to form stem loop structures resembling that of pLS1 (Fig. 4). All plasmids share the common sequence: 5'-TAGCGT-3' located on the

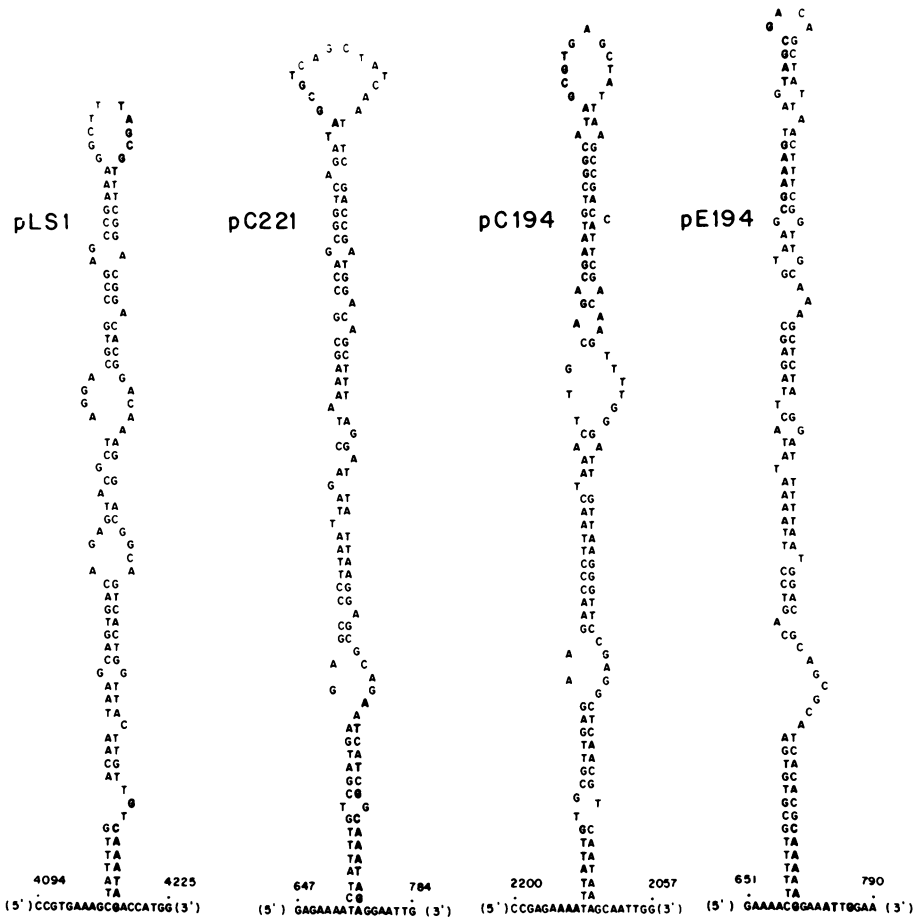
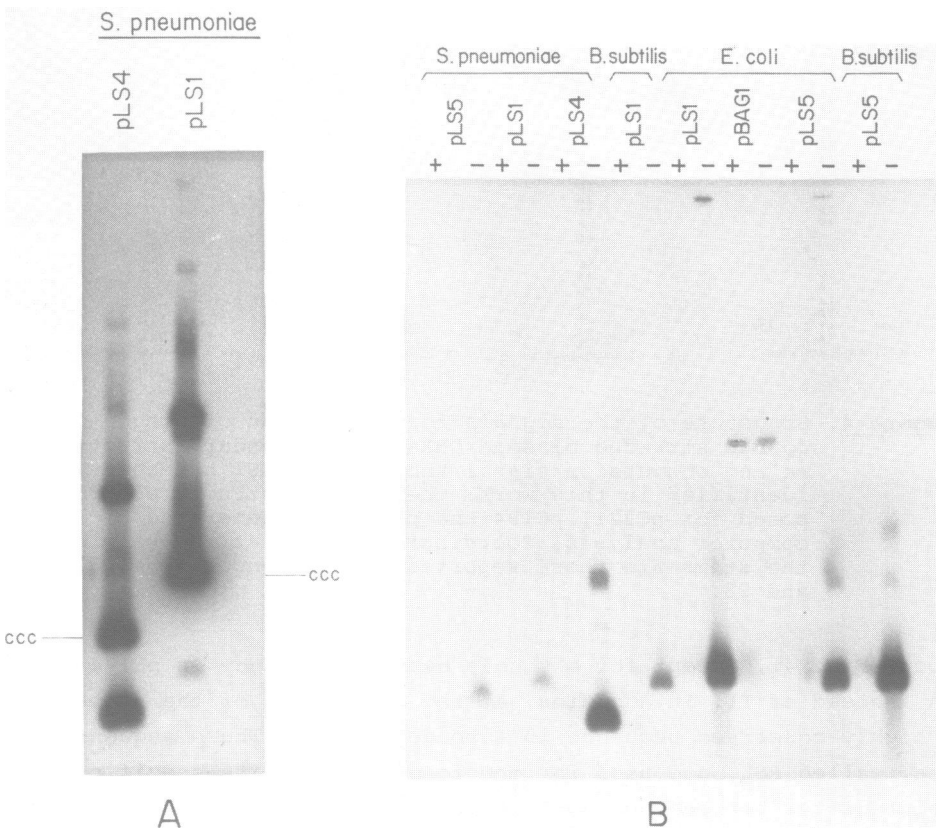


Figure 4. Structure of the signals for conversion of single to double stranded plasmid DNAs. The secondary structure and conversion signal showed for pLS1 has been identified in this work; the hairpin structures proposed for pC221, pC194 and pE194 are generated by computer analysis. Coordinates showed at the base of the stems are those reported in references 2, 4, 22 and 23.

loop (Fig. 4), except for a single base discrepancy in pE194 (A instead of T). In addition, at the bottom end of the stems, a highly conserved sequence is also detectable. These sequences, called RS_B, are used for the formation of site-specific cointegrates between non-homologous plasmids (24).

Accumulation of single-stranded intermediates in pLS4.

Single-stranded circular plasmid DNA can be detected in total cell extracts as molecules that have a higher electrophoretic mobility in agarose gels than double-stranded supercoiled ccc-monomeric forms. In addition, single-stranded forms bind to nitrocellulose filters without previous denaturation and are fully sensitive to endonuclease S1 (10). It has been postulated that single-stranded DNA circles are intermediates in the replication of staphylococcal plasmids (9) and that these intermediates are generated by replication through a rolling circle mechanism (8). If these assumptions are correct, it should be expected that a signal must exist in plasmid DNA which is involved in the conversion of single-stranded forms to double-stranded molecules, similarly to the complementary



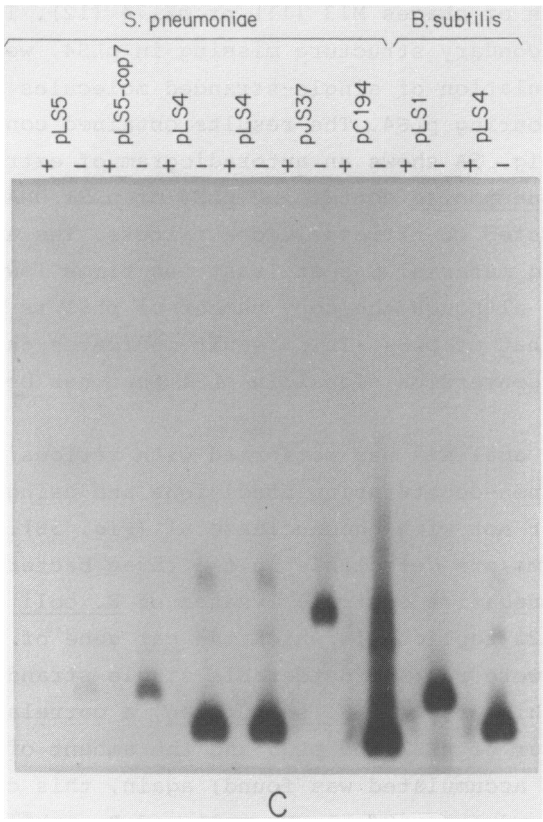


Figure 5. Single stranded plasmid DNA generated in S. pneumoniae, E. coli and B. subtilis. Lysates prepared from strains harbouring the indicated plasmids were treated (+)or not (-) with endonuclease S1, electrophoresed and the DNA transferred to nitrocellulose.The filters were then hybridized to the appropriate probes prepared from purified plasmid DNA. In A, the DNA was denatured prior to transfer and was not treated with nuclease S1; ccc indicates the position of supercoiled double-stranded plasmid forms. In B and C, the DNA was not denatured prior to transfer and consequently only single-stranded DNA is detectable. Bacterial strains and plasmids used are indicated in the upper part of the figures. In some cases the autoradiograms were overexposed to reveal the weak bands of single-stranded plasmid forms generated by pLS1 and pLS5 in S. pneumoniae.

strand origins of phages M13 (11) or ØX174 (12). If this signal is the secondary structure missing in pLS4, we should expect an accumulation of single-stranded molecules in lysates of cells harbouring pLS4. The results obtained confirmed this hypothesis. Fig. 5A shows an autoradiogram of extracts prepared from S. pneumoniae containing pLS4 of pLS1 DNAs denatured prior to transfer to nitrocellulose filters. The amount of fast migrating material was at least ten times lower in pLS1 than in pLS4, although the copy number of pLS1 is five times higher than that of pLS4. This result indicates that there is an efficient conversion signal in pLS1 that has been removed in pLS4.

The same analysis was performed with various plasmids and hosts, under non-denaturating conditions and using cell lysates treated or not with endonuclease S1 (Fig. 5B). Single--stranded forms are detectable in the three bacterial systems tested. As a negative control, lysates of E. coli carrying pBAG1 (a pBR322 replicon in which the cat gene of pJS3 has been cloned) were run; no detectable single-stranded DNA was observed in this case. In S. pneumoniae, a correlation between the copy number of pLS1 and pLS5 and the amount of single--stranded DNA accumulated was found; again, this correlation was not observed for pLS4. In E. coli and B. subtilis, the amount of single-stranded DNA generated by pLS1 and pLS5 was higher than in pneumococci, even though the copy number of these two plasmids in E. coli and in B. subtilis is six times lower than in S. pneumoniae (2). This indicates that the conversion signal is more efficiently recognized in S. pneumoniae. When the copy number mutation cop7 (which increases the copy number up to 160; del Solar et al., to be published) was introduced in pLS5, once again correlation was found between number of copies and amount of single-stranded DNA in S.pneumoniae (Fig. 5C). Taking all these results together, we may conclude that the copy number mutations in pLS5 and cop7 affect the plus origin of replication and that the conversion signal is absent in pLS4 but not in pLS5. When S. pneumoniae carrying plasmids that use the pC194 replicon (either pJS37 or a high copy number mutant of pC194; Ballester et al., submitted)

were assayed for the generation of single-stranded DNA, accumulation of endonuclease S1-sensitive fast migrating material was observed (Fig. 5C). This finding indicates that the conversion signal in pC194 is poorly recognized by S. pneumoniae.

In the case of B. subtilis, the absence of the conversion signal in pLS4 only resulted in a slight increase in the amount of single-stranded DNA as compared to the already high accumulation of these forms generated by pLS1 (Fig. 5C). This was in clear contrast to the case of S. pneumoniae, which indicates either that the conversion signal in pLS1 is not recognized by B. subtilis or that it is recognized as poorly as the alternative signal (see Discussion).

DISCUSSION

The results presented here demonstrate that the streptococcal plasmid pLS1 has a specific sequence acting as a signal for the conversion of plasmid single-stranded DNA to the double-stranded form. This sequence probably forms a large secondary structure similar to other hairpin structures present in non-coding regions of the staphylococcal plasmids pC221, pC194 and pE194 (Fig. 4). In the loop of such structures a consensus sequence: 5'-TAGCGT(A)-3' has been observed. Deletion of the conversion signal of pLS1 leads to plasmid instability, reduction in the copy number and accumulation of single-stranded intermediates. However, even with this deletion, the defective pLS4 plasmid can be maintained with selective pressure in S. pneumoniae. This implies that the deleted plasmid should have an alternative conversion signal(s). We may postulate that to be efficiently recognized, such a putative signal must be placed in the "correct" strand, that is the strand in which the direction of replication of pLS1 DNA proceeds from 5' to 3' (Puyet et al., submitted). Furthermore, it can be assumed that the putative signal should have the potential to form a secondary structure with a loop containing the above consensus sequence. Computer search for this consensus in pLS1 DNA sequence showed the existence of five of them in addition to the one located at 4160 (Fig. 4). Two were located in the complementary strand: consequently they could not function as a part

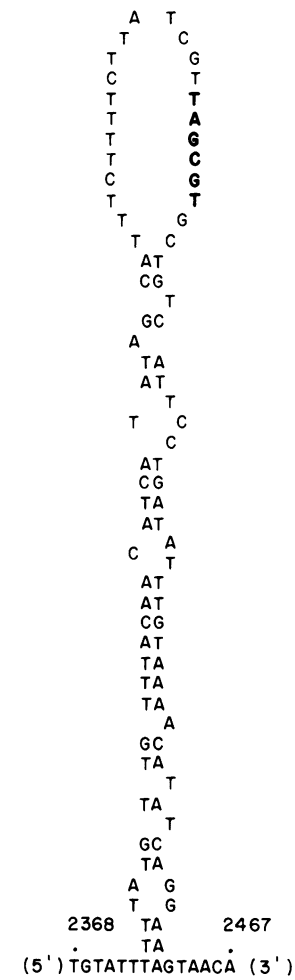


Figure 6. Proposed secondary structure acting as alternative conversion signal in pLS4. Boldface letters: consensus sequence. Coordinates as in reference 2.

of a conversion signal. Another two were in the appropriate strand (coordinates 149 and 1810) but no secondary structure could be found. The fifth was placed at coordinate 2419 on the loop of a potential secondary structure (Fig.6). We propose that this region could act as an alternative conversion signal. The same analysis performed in pC194 showed that only one consensus sequence located at coordinate 2134 (22) existed. This sequence

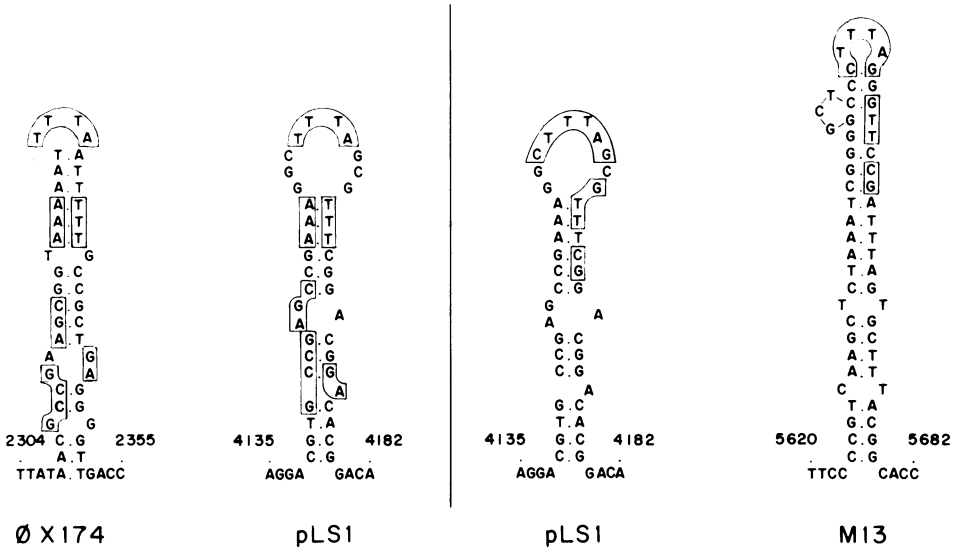


Figure 7. Comparison of the conversion signal of pLS1 with the complementary strand origins of bacteriophages ØX174 (12) and M13 (11). Stretches of homology are boxed.

is located in the strand that carries the sense message for Rep H (25) and is placed on the loop of a large potential secondary structure (Fig. 4). Plasmid pJS3 (Fig. 2) contains the genuine conversion signal of pLS1 (Fig. 4), lacks the proposed alternative signal (Fig. 6) and has the pC194 signal in the "incorrect" strand. Various attempts to remove the genuine conversion signal in pJS3 failed to give plasmids able to transform S. pneumoniae thus favouring the hypothesis of the proposed alternative conversion signal.

A peculiar characteristic of pLS1 is that it is able to be established in E. coli (2) demonstrating that its plus origin is functional in this host (3). In addition, pLS1 replicates in E. coli by the same proposed rolling circle mechanism (8) as in S. pneumoniae (Fig. 5). However, a different requirement could exist in the Gram-negative host since the alternative signal in pLS4 was not sufficient for the establishment of this plasmid in E. coli (Table 1). Comparison of homologies between the conversion signal of pLS1 and the complementary strand origins of single-stranded DNA coliphages, showed that

pLS1	(4149)	CGAAAGGCTT**TAGCGT**TTCGGACGGA
ØX174	(2320)	GT***AAT****ATT***GCCG*T**
M13	(5643)	G*GCTCC******G***C**ATTTAG
pE194	(698)	GCG**A*TAG*****ACAGCT*TTA*
pC221	(695)	A*CGGTCAGA******CAGCT*TCA*
pC194	(2148)	AATGTC*GCA******GAGCT*TTA*
pLS1	alternative (2409)	TTCTTAT*G*******GCT*TCATTC

Figure 8. Nucleotide sequence comparison of DNA regions containing the conversion signals of phages and plasmids. The comparison was limited to 26 bp and searched for maximal homology without allowing any gap. Nucleotide numbers are those reported in references 2, 11, 12, 23, 4 and 22. The consensus sequence in pLS1 is underlined and in boldface. A star indicates that an identical nucleotide is present in the sequence.

stretches of homologous bases existed (Fig. 7). In the case of ØX174 (12), although it lacked the proposed consensus sequence, regions of homology located both in the stem and on the loop are detectable. Comparison with M13 (11) showed that a sequence similar to the consensus: 5'TAGGGT-3' is partially located on the loop. In addition, the six bases placed on the loop of M13 constitute a stretch of perfect homology with the loop of pLS1 (Fig. 7). Calculation of homologies between the conversion signal of pLS1 and those shown in this work (Fig. 8), demonstrated that within a region of 26 bases, 50% and 46% homology was found for ØX174 and M13, respectively. The degree of homology was lower for the staphylococcal plasmids pE194 (38%), pC221 (35%) and pC194 (35%). In the case of pC194, the poor recognition of its conversion signal by S. pneumoniae as compared to the efficient recognition for pLS1 (Fig. 5C), may reflect differences in the ability of the host machinery to identify a specific plasmid structure. This could be also the situation for the proposed alternative signal of pLS1 that showed a homology with the genuine conversion signal similar to that of pC194 (35%). We are tempted to assume that the high homology between the conversion signal of pLS1 and the complementary strand origins of ØX174 and M13 is a requisite for the streptococcal plasmid to replicate in E. coli. If our assumption is correct, it would explain the failure of pLS1 to be established in polA mutants of E. coli (2) since DNA polyme-

rase I is required for the replication of single-stranded DNA coliphages (12). In this respect it is interesting to point out that the pLS1 replicon carrying the S. pneumoniae polA gene was successfully established in E. coli polA mutants (2).

Note: While this manuscript was being reviewed, a paper describing a palindromic sequence involved in replication of plasmids in S. aureus has been published (A.D. Gruss, H.F. Ross and R.P. Novick, Proc. Natl. Acad. Sci. USA (1987) 84, 2165-2169).

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