Identification of the origin and direction of replication of the broad-host-range plasmid pLS1

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ABSTRACT

The replication origin of the fully sequenced broad-host-range streptococcal plasmid pLS1 has been determined by the use of an in vitro replication system prepared from Escherichia coli, a host in which the plasmid can be established. Replicative intermediates were isolated from reaction mixtures that contained dideoxythymidine triphosphate, thus limiting the average extent of in vitro synthesis. Analysis of HinfI-cleaved intermediates demonstrated that the origin of replication is included within a 443-bp fragment. Replication proceeds unidirectionally in the same direction as transcription of plasmid mRNAs. Isolation of deletion derivatives allowed us to define the replication origin of pLS1 within a region of 284 bp. Replication of pLS1 occurs through single-stranded intermediates by a rolling circle mechanism. Cleavage of supercoiled plasmid DNAs with endonuclease S1 followed by restriction mapping, allowed the positioning of three major specific S1 sites in regions of high potential to form secondary structures. One of these inverted repeats is located in the region where the origin of replication of pLS1 has been defined.

INTRODUCTION

Amongst small multicopy plasmids isolated from Gram-positive bacteria, only some streptococcal replicons have been shown to transform Gram-negative hosts (1,2). Since this unusual host range must be a consequence of special features in their replicative machinery, it is of general interest to study the mechanisms of replication of these plasmids. One of them, pLS1, has been used as a vector for cloning pneumococcal genes (3,4) as well as for the construction of additional useful cloning vectors (5,6,7). Plasmid pLS1 was constructed from the Streptococcus agalactiae plasmid pMV158 (8) by removal of a 1.1-kb EcoRI fragment (3). It specifies constitutive resistance to tetracycline, has a molecular length of 4408 bp (2) and is

able to replicate in S. pneumoniae (3), Bacillus subtilis (5),
and Escherichia coli (2).

An in vitro system prepared from E. coli cells (9) has been
optimized for plasmid pLS1 replication (10). Extracts from
plasmid-free cells were able to support replication of exogenous
plasmid. The inhibition of the host DNA gyrase totally blocked
DNA synthesis in extracts prepared from both plasmid-containing
and plasmid-free cells. These characteristics are different to
those reported for the in vitro system developed for the
staphylococcal plasmid pT181 (11). pLS1 replication requires the
host RNA polymerase (at least for the expression of plasmid-
coded proteins) and de novo protein synthesis. Replication
functions of pLS1 include at least two plasmid-encoded
polypeptides, RepA (5.1 kDa) and RepB (24.2 kDa) and two
putative small countertranscribed RNAs involved in copy number
control and incompatibility (2; del Solar et al., to be
published). Although the tet gene of pLS1 is highly homologous
to the tet gene of plasmid pT181 (12), and almost identical to
that of the Bacillus plasmid pTHT15 (13), no similarity in the
replication functions has been observed between these three
plasmids (2). However, it seems that the replication of all
small Gram-positive multicopy plasmids analyzed so far follows a
general pattern. Firstly, the initiation protein(s) recognizes a
specific site located at the (+) strand origin, introducing a
strand and site-specific nick. The 3'-OH end is likely to be
used as a primer for the synthesis of the leading strand by a
rolling circle mechanism (14,15). The products of this step are
single-stranded circular plasmid molecules (16) which, in the
following steps, are converted to duplex DNA molecules. For the
lagging strand synthesis, the host machinery recognizes plasmid
specific initiation signals that, in the case of pLS1, share
about 50% homology with the complementary strand origin of phage
øX174 and with the hairpin B of the minus origin of M13 (17).

In our present work, we have located the replication origin
of pLS1 (plus strand origin) within a region of 284 bp.
Replication of pLS1 is unidirectional, proceeding in the
direction of plasmid mRNAs synthesis. Single-stranded
intermediates, generated from the plasmid coding strand, are

detectable in the three bacterial species analyzed viz. S. pneumoniae, B. subtilis and E. coli. One potential hairpin structure has been located in the region where the origin of replication of pLS1 has been defined.

MATERIALS AND METHODS

Bacterial strains and plasmids.

E. coli C600 (thr-1 thi-1 leu-6 lacY1 fhuA21 supE44), B. subtilis MB11 (lys-3 metB10 hisH2) and S. pneumoniae 708 (end-1 exo-2 trt-1 hex-4 malM594) were used throughout this study. Plasmids used were: pLS1 and pLS5 (2); pLS4 (17), the high copy number mutant pLS1 cop7 (17), and the deleted derivatives constructed in this work.

Plasmid DNA preparations and DNA manipulations.

Preparation of plasmid DNA has been previously described (10,17). Restriction endonucleases were obtained comertially and used as specified by their suppliers. For Bal-31 deletion experiments, 4 µg of plasmid pLS1 DNA were linearized with BanI and treated with 1 U of Bal-31 (New England Biolabs) as specified by the supplier to allow digestion of about 500 to 1000 bp (incubation times of 4, 9 and 14 min). The extent of digestion was checked by agarose gel electrophoresis, the DNA ends were filled in with the Klenow fragment of the DNA polymerase I, ligated and used to transform competent pneumococcal cultures. Nuclease S1 reactions and detection of single-stranded plasmid DNA have been published elsewhere (17).

Mapping of the pLS1 origin by ddTTP inhibition.

Optimal conditions for the preparation of the E. coli cell-free extracts, DNA synthesis and characterization of the reaction products have been described (10). Standard reaction mixtures (25 µl) containing pLS1 DNA (1 µg) and 0.8 µM-[^{3}H]-dTTP (specific activity 50 Ci/mmol) were incubated in the presence of various amounts of 2',3'-ddTTP for 120 min at 37°C. Plasmid DNA was purified, digested with HinfI and run in 1.2% agarose gels. Gels were then stained, photographed, fluorographed and exposed to Kodak XO-Mat films.

Specific strand hybridization.

To identify the plasmid strand that is rendered single

stranded, radioactive probes were prepared by end repair of
restriction fragments. pLS1 DNA (10 µg) was cleaved with HinfI
(recognition sequence 5'-G/ANTC-3') and the seven resulting
restriction fragments (see Fig. 2) were treated with 5 U of the
Klenow fragment of the DNA polymerase I in the presence of
$[\alpha-^{32}P]$-dATP and $[\alpha-^{32}P]$-dTTP (40 µCi each). After incubation at
$25^{\circ}C$ for 15 min, further incorporation of labelled nucleotides
was stopped by an excess of cold dNTPs. Then, samples were
treated with phenol-chloroform, precipitated and the labelled
restriction fragments were separated on a preparative 5%
polyacrylamide gel and identified by exposure to X-ray films.
The HinfI F fragment (213 bp) having the sequence 5'-G/AATC-3' in
both ends, was isolated from the gel by crashing and elution
(18). The recovered labelled fragment was denatured by heating
at $92^{\circ}C$ for 5 min in 30% dimethyl sulfoxide containing 0.05%
each of xylene cyanol and bromophenol blue. The DNA was chilled
and subjected to electrophoresis on a 2 mm-thick 8%
polyacrylamide sequencing gel (18). The two strands were
separated, purified as above and used as probes for
hybridization as described (17). Part of each strand of the
denatured DNA fragment was employed to its identification by
specific strand hybridization. To this end, pLS1 DNA (5 µg) was
digested with HinfI and mixed separately with each of the end-
labelled complementary strands of the pLS1 HinfI F DNA fragment.
The DNA mixture was denatured (in 80% formamide, 0.4 M NaCl,
$85^{\circ}C$, 15 min) and allowed to hybridize overnight at $37^{\circ}C$ (in 50%
formamide and 0.9 M NaCl). After precipitation, the annealed DNA
was cleaved with BglI which cuts once the HinfI F fragment
asymmetrically. Samples were run in 12% polyacrylamide gel and
exposed to X-ray films.

DNA sequence determination and analysis.

DNA restriction fragments were either treated with
intestinal phosphatase and labelled at their 5'ends with
$[\gamma-^{32}P]$-ATP and phage T4 polynucleotide kinase or by the
aforementioned fill in procedure. Nucleotide sequences were
determined by the chemical method of Maxam and Gilbert (18).
Searching for secondary structures, repeats and other features

in the DNA sequence of pLS1 (2) was performed by using DNASTAR
computer programs (DNASTAR, Inc.,UK).

RESULTS
In vitro location of the replication origin of pLS1.

Functions essential for replication of plasmid pLS1 have
been located within a 1054-bp PstI fragment by in vivo isolation
of deletion derivatives and by in vitro constructions (2,17). To
characterize the replication origin in greater detail, we used a
recently developed in vitro system prepared from plasmid-free E.
coli cells that supports replication of exogenously added
plasmid pLS1 DNA (10). Incorporation of [^{3}H]-dTMP into pLS1 DNA
added to E. coli cell-free extracts was analyzed by agarose gel
electrophoresis of the reaction products. The use of this in
vitro system allowed the detection of newly synthesized
supercoiled monomeric pLS1 DNA that has completed at least one
round of replication (Fig. 1A). Since supercoiled monomer DNA is
the only molecule used as substrate, initiation of replication
in this in vitro system may be considered as almost synchronous.
Consequently, a family of molecules that have initiated, but not
completed a round of replication can be isolated from reaction
mixtures containing different concentrations of the chain
terminator ddTTP. Subsequent restriction cleavage of these
molecules and determination of the radioactivity incorporated
into the restriction fragments, would reveal their replication
order and the location of the fragment in which replication
initiates. To this end, increasing amounts of ddTTP, in addition
to the four deoxynucleotides and plasmid pLS1 DNA, were added to
extracts. Incorporations of the radioactive precursor [^{3}H]-dTTP
into plasmid DNA were 5%, 11%, 16%, 26%, 51% and 65% of the
maximal synthesis obtained in the absence of ddTTP. After the
incubation period, pLS1 DNA was extracted from the reaction
mixtures, digested with HinfI and subjected to electrophoresis
in agarose gels. The photograph of the ethidium bromide stained
gel and the autoradiogram of the same gel are depicted in Figure
1, B and C, respectively. No differences can be seen in the
electrophoretic mobilities of the fragments at any ddTTP
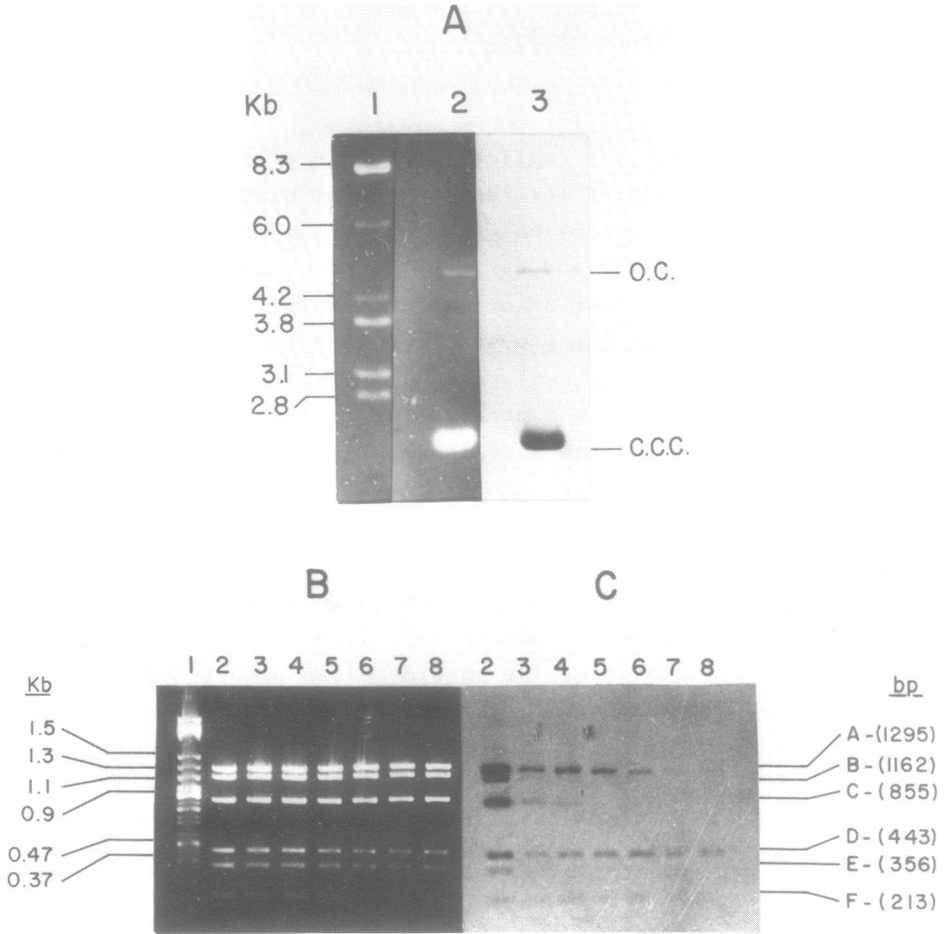concentration, indicating the absence of branched intermediates.

Figure 1. Agarose gel analysis of pLS1 DNA synthesized _in vitro_ in cell-free extracts of <u>E. coli</u>. **A** Reaction products of pLS1 DNA extracted from reaction mixtures: ethidium bromide stained gel (lane 2), fluorograph of the same gel (lane 3), and phage T7 DNA cleaved with <u>Mbo</u>I as molecular weight standard (lane 1). On the right, size of the fragments is indicated. O.C., open circular, and C.C.C., closed circular plasmid forms. **B** <u>Hinf</u>I cleaved intermediates of pLS1 DNA replicated _in vitro_ in the presence of various amounts of ddTTP. In lane 1, phage T7 DNA cleaved with <u>Hpa</u>II was used as molecular weight standard, with sizes indicated on the left. **C** Fluorograph of the same gel. For B and C, the amounts of ddTTP and percentages of maximal synthesis were: 0 μM, 100% (lane 2); 7.5 μM, 65% (lane 3); 10 μM, 51% (lane 4); 25 μM, 26% (lane 5); 60 μM, 16% (lane 6); 150 μM, 11% (lane 7) and 330 μM, 5% (lane 8). On the right, the sizes of the pLS1 DNA <u>Hinf</u>I fragments are indicated. Note that fragment G (84 bp) has run out of the gel.
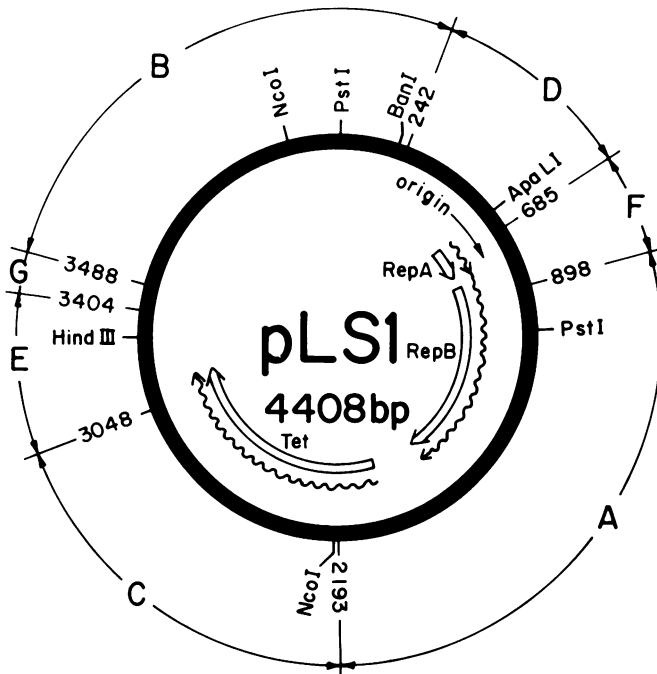
Figure 2. Map of the (+) strand of pLS1. Inside are depicted the location of the origin and the direction of replication, the putative mRNAs for RepA and RepB and for the tetracycline resistance marker and their products. Outside, some relevant restriction sites and the HinfI fragments are shown; numbers indicate the coordinates of the cleavage sites by HinfI.

The HinfI D fragment (443 bp) is the only one labelled at the highest ddTTP concentration (lane 8), indicating that replication starts within this fragment. The sequential order of labelling is: F (213 bp), A (1295 bp), C (855 bp), E (356 bp) and B (1162 bp). This sequence of labelling demonstrates that the order of replication is DFACE(G)B, thus allowing to determine the direction of replication of pLS1 in vitro. In the absence of ddTTP (lane 2), the intensity of fragments A and B is almost equal, indicating that replication of pLS1 in the cell-free system has gone to completion in most of the molecules. These in vitro results demonstrate that the plasmid is replicated unidirectionally in the same direction as the plasmid mRNAs are synthesized (2), that is in clockwise direction according to the circular map of pLS1 (Fig. 2).

Deletion analysis to characterize the plus strand origin.

Plasmid pLS5 carries a well characterized deletion
(coordinates 34-365 in pLS1) extending 123 bp into the HinfI D
fragment (2,17). This deletion resulted in a two-fold increased
plasmid copy number. To construct deletion derivatives of pLS1
that entered further into this fragment, Bal-31 deletions were
prepared from pLS1. To this end, pLS1 DNA was linearized with
BanI, an enzyme that cuts once at coordinate 215 and deletions
of 500 to 1000 bp were made. Sixty derivatives were analyzed by
restriction mapping. All large deletions analyzed were
asymmetric. Derivatives affecting the putative RNAI (2) but not
the (-) strand origin (coordinates 4103 to 4218), had duplicated
their copy number; plasmids missing this (-) origin showed a
dramatic reduction in their copy number (17). Amongst plasmids
of the first category, the smallest one (named pLS1Δ24) was
selected to determine its nucleotide sequence at the deletion
endpoints. The deletion of pLS1Δ 24 extended 384 bp to the left
(to nucleotide 4239) and only 187 bp to the right (to nucleotide
402) of the unique BanI site. This plasmid replicated normally
in vivo, thus allowing us to define the (+) strand origin of
pLS1 in the 284-bp region between nucleotide 402 and the right
end of the HinfI D fragment at nucleotide 685.

**Single-stranded intermediates generated during in vivo pLS1
replication.**

Single-stranded plasmid DNA is detectable in total cell
extracts by three criteria: i) it has a higher electrophoretic
mobility in agarose gels than double-stranded supercoiled ccc-
monomeric forms; ii) it binds to nitrocellulose filters without
previous denaturation and iii) it is fully sensitive to nuclease
S1 (16). Analysis of single-stranded DNA generated in cells of
S. pneumoniae, B. subtilis and E. coli harbouring pLS1 showed
the presence of fast-migrating, nuclease S1-sensitive plasmid
DNA (Fig. 3). The amount of single-stranded DNA is much lower in
S. pneumoniae than in the other two bacteria, whilst the number
of copies of pLS1 in pneumococcus is 25 and in B. subtilis and
in E. coli is only 5. This demonstrates that conversion of
single- to double-stranded plasmid DNA is much more efficient in
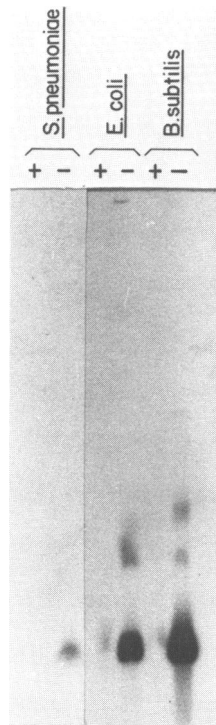S. pneumoniae than in B. subtilis or E. coli.

Figure 3. Single-stranded pLS1 DNA synthesized by S. pneumoniae, E. coli and B. subtilis. Lysates were treated (+) or not (−) with nuclease S1 prior to electrophoresis and transfer (without denaturation) to nitrocellulose. Filters were hybridized with ^{32}P-labelled pLS1 DNA and exposed to X-ray films.

Plasmid pLS1 has a specific conversion signal (coordinates 4103 to 4218) that acts as a (−) strand origin (17). This sequence has been located to the left of the HinfI D fragment and its removal leads to accumulation of single-stranded monomeric plasmid forms in S. pneumoniae (17). If these single-stranded DNA molecules are replicative intermediates generated by a rolling circle mechanism, one should expect that only one plasmid strand would act as a (+) strand, i.e. the displaced strand that would generate the single-stranded molecules, similar to the single-stranded coliphages (19). To prove this assumption, lysates of S. pneumoniae harbouring plasmids pLS1 cop7 (containing the conversion signal; 120 copies
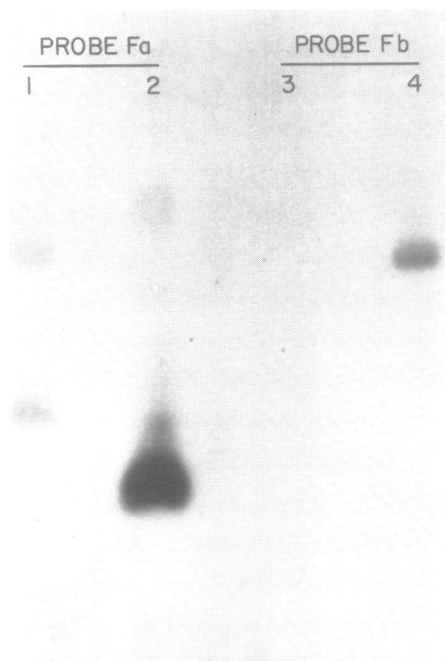
Figure 4. Autoradiogram of single-stranded DNA generated in S. pneumoniae harbouring pLS1 cop7 (lanes 1 and 4) or pLS4 (lanes 2 and 3). Lysates were transferred to nitrocellulose without denaturation, and the filters were then hybridized separately to purified HinfI F single-stranded probes labelled by end-repair. In lanes 1 and 2, samples were hybridized with probe F_a and in lanes 3 and 4 with probe F_b. For identification of the probes see Figure 5.

per cell genome) or pLS4 (deleted in the conversion signal; 5 copies per cell genome) were prepared and analyzed. DNA tethered to nitrocellulose filters (without prior denaturation) was hybridized separately with each one of the strands of the HinfI F fragment. Single-stranded plasmid intermediates hybridize only with one of the probes that we have arbitrarily termed F_a, (Fig. 4, lanes 1 and 2). No significant hybridization was observed between the single-stranded plasmid molecules and the complementary probe F_b (lanes 3 and 4) even though a large amount of single-stranded DNA was generated by pLS4 (compare lanes 2 and 3). These results demonstrated that only one plasmid strand is rendered single-stranded. Slow migrating material,

more visible in lanes 1 and 4, corresponds to hybridization of
the probes with some supercoiled ccc plasmid DNA.

To identify which strand corresponds to probes F_a and F_b,
we have developed a hybridization technique based on
reconstruction of double-stranded DNA fragments from a mixture
of the above single-stranded labelled probes and denatured pLS1
restriction fragments (Fig. 5A). Probes F_a and F_b were
separately mixed with unlabelled denatured HinfI-cleaved pLS1
DNA, hybridized and digested with BglI. The resulting fragments
were separated in polyacrylamide gels. Then, the labelled
fragment of each sample was visualized by autoradiography (Fig.
5B). Fragment HinfI F (213 bp; lane 1), digested with BglI
yielded two fragments of 117 and 96 bp (lane 3). From probe F_a,
only the 117-bp fragment was detectable (lane 2), whereas the
fragment of 96 bp was the one observed for the hybridization
experiment with probe F_b (lane 4). All radioactive single-
stranded DNA probes hybridized with the cold DNA, since the only
detectable single-stranded labelled fragment was the control
sample F_b (lane 5).

From Figures 4 and 5, we conclude that the pLS1 strand
which is rendered single-stranded corresponds to the coding
strand of the plasmid (2). The results support the rolling
circle type of replication of pLS1 and allow us to determine its
direction of replication in vivo: since the displaced strand is
the one that carries the sense message for RepA and RepB, DNA
synthesis can only proceed in the direction of mRNA synthesis.
We also conclude that the direction of replication of pLS1 is
the same in vivo as in vitro.

Secondary structures in the origin of replication of pLS1.

Prokaryotic genomes exhibit negative supercoiling which
produces torsional stress in DNA (20). These tensions probably
affect replication because of helix unwinding and/or unpairing
at sites involved in the initiation of DNA replication (21). It
has been shown that single-strand-specific endonucleases
selectively recognize DNA unpairing at inverted repeat sequences
which generate stem-loop structures (21,22). These hairpin
structures only occur in ccc-supercoiled DNA to release the
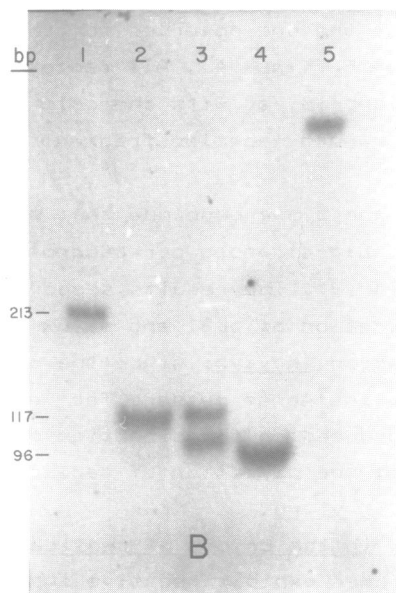torsional stress; their loops can be a substrate for single-
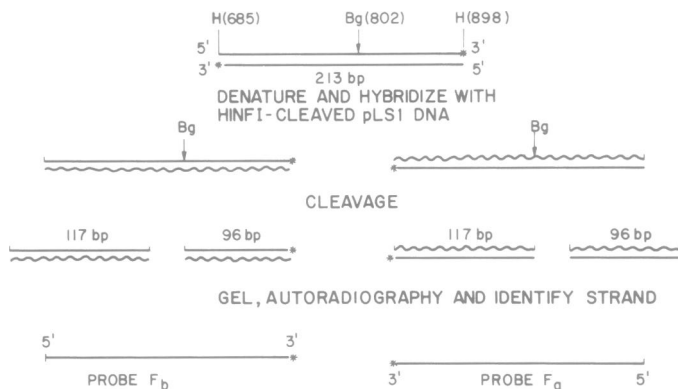
Figure 5. Identification of the strands of fragment HinfI F by specific hybridization. A Diagram of the experimental approach. Purified single strands of fragment HinfI F labelled at the 3'ends (*) were hybridized with total unlabelled denatured pLS1 DNA cleaved with HinfI (wavy lines), digested with BglI (Bg), electrophoresed and autoradiographed. B Autoradiogram showing the results of the above hybridization. Native HinfI F fragment uncleaved (lane 1) or cleaved with BglI (lane 3). Molecules hybridized with the labelled strand F_a (lane 2) or with the labelled strand F_b (lane 4) and cleaved with BglI. Lane 5: control single stranded probe F_b.

strand-specific endonucleolytic cleavage, converting circular
plasmids into linear molecules. Since origin sequences usually
contain secondary structures that can be recognized by single-
strand-specific endonucleases, we wanted to know whether the
first-replicating Hinf1 D fragment of pLS1 contained any
experimentally demonstrable hairpin structures. Two main
palindromes were found in the pLS1 DNA sequence between
nucleotides 80 to 470, one of which was located within the Hinf1
D fragment. In addition, a large inverted repeat located between
nucleotides 4103 and 4218, corresponding to the (-) strand
origin of the plasmid has been identified (17). Supercoiled
ccc-pLS1 DNA was cleaved with nuclease S1 (Fig. 6). The S1
cleavage sites were localized in the resulting linear molecules
(Fig. 6A, lanes 4 and 5) by digesting them with EcoRI or with
EcoRI and HpaII, enzymes that cut once within pLS1 (coordinates
3170 and 2484, respectively). For this analysis, in addition to
pLS1, we chose pLS5 because this derivative lacks one of the
potential hairpin structures. As shown in Figure 6A, incubation
of plasmid DNAs with nuclease S1 at 37°C, followed by digestion
with EcoRI, revealed two major complementary bands of 3.0 kb
(S1-EcoRI) and 1.4 kb (EcoRI-S1) in pLS1 (lane 6). These two
bands were not seen in pLS5 (lane 7). Double digestion of
pLS1-S1 DNA with EcoRI and HpaII (lane 8), showed that the 3.0
kb S1-EcoRI fragment yielded two bands of 2.3 kb (S1-HpaII) and
0.7 kb (HpaII-EcoRI), whereas the EcoRI-S1 1.4 kb-band remained
unchanged. The large EcoRI-HpaII fragment (3.7 kb) generated
from S1-uncleaved molecules was also visible. In the case of
pLS5-S1 DNA (lane 9), the two EcoRI-HpaII bands were mainly
visible. However, several minor bands, not detectable in pLS1,
were observed for pLS5 (compare lane 6 with 7 and lane 8 with
9). From the results of Figure 6A, we deduce that the major pLS1
S1-site at 37°C maps at about coordinate 170, within a region
included in the deletion of pLS5. A control experiment performed
with pBR322 DNA (Fig. 6A, lanes 10 to 12), cleaved with S1 (lane
11) and further with EcoRI (lane 12), allowed to confirm
previous results locating the S1 sites in this plasmid at 3.2
and 3.05 kb from the unique EcoRI site (22).

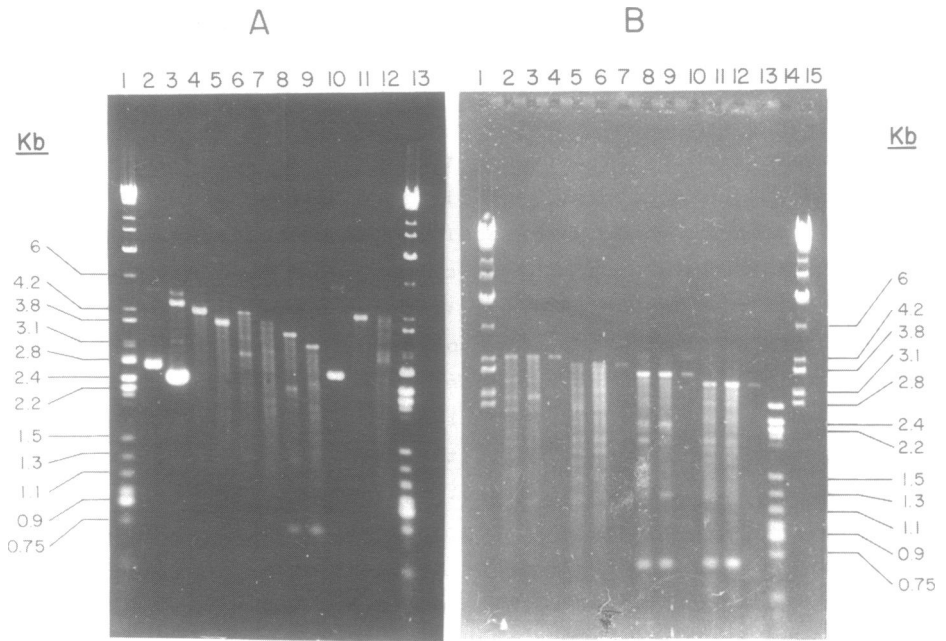   It has been reported that selectivity of S1 cleavage is

Figure 6. Nuclease S1 positional mapping of secondary
structures in supercoiled plasmid DNA by agarose gel
electrophoresis. A Supercoiled pLS1 (lane 2), pLS5 (lane 3)
and pBR322 (lane 10) DNAs and their cleavage by nuclease S1 at
37°C (lanes 4, 5 and 11, respectively). The S1-linearized pLS1
DNA was digested with EcoRI (lane 6) or with EcoRI and HpaII
(lane 8); the same treatment was used for pLS5 DNA (lanes 7 and
9, respectively), whereas the control pBR322 DNA was cleaved
only with EcoRI (lane 12). Lanes 1 and 13: molecular weight
standards (a mixture of T7 DNA digested with MboI and HpaII),
with the relevant sizes indicated on the left. B Effect of the
temperature on the selectivity of nuclease S1 cleavage of pLS1
and pLS5 DNAs. S1-linearized pLS1 DNA at 10°C was digested with
EcoRI (lane 2) or with EcoRI and HpaII (lane 8); the same
analysis was performed for S1-linearized pLS1 DNA at 37°C
(lanes 3 and 9, respectively) or at 42°C (lanes 4 and 10,
respectively). A similar analysis was performed for S1-
linearized pLS5 DNA at 10°C (lanes 5 and 11), 37°C (lanes 6 and
12) or 42°C (lanes 7 and 13); lanes 5 to 7: pLS5-S1 DNA
digested with EcoRI; lanes 11 to 13: pLS5-S1 DNA digested with
EcoRI and HpaII. Lanes 1 and 15: phage T7 DNA digested with
MboI; lane 14: the same DNA digested with HpaII, used as
molecular weight standards, with the relevant sizes indicated
on the right.

mantained at temperatures between 7°C and 45°C (22). To find out

if any temperature-dependent conformational change could be

detected in the streptococcal plasmids, we repeated the previous

kind of analysis at different temperatures (Fig. 6B). At 42°C, most of the plasmid DNA was degraded, probably due to unspecific S1 cleavage; at 37°C, the results were identical to the previous assay. However, a notable difference was observed for pLS1 when the S1 treatment was performed at 10°C instead of 37°C (lanes 2 and 3). At 10°C, pLS1-S1 DNA cleaved with EcoRI (lane 2) exhibited three major bands (in addition to their less visible complementaries and the 4.4 kb linears) of 2.7, 3.0 (the only band visible at 37°C), and 3.4 kb. Digestion with EcoRI and HpaII (lanes 8 and 9), showed the following bands: EcoRI-HpaII (3.7 and 0.7 kb); S1-HpaII (2.0, 2.3, and a faint band of 2.7 kb) and EcoRI-S1 (1.4 and 1.7 kb). The S1-HpaII band of 2.0 kb was better visible at 10°C than at 37°C both in pLS1 (lanes 8 and 9) and in pLS5 (lanes 11 and 12). This S1 site corresponded to a major site in the plasmid and mapped at about coordinate 450, that is, inside the HinfI D fragment. A third S1 site is located at 2.7 kb from HpaII. This site was easier to detect when pLS1-S1 was cleaved only with EcoRI (lane 2, band of 3.4 kb). It corresponds to the complementary strand origin of pLS1 and its more detailed characterization has been published elsewhere (17). No significant variations in the pattern of bands depending upon the temperature of incubation with endonuclease S1 was observed when the same experiment was performed for pLS5 (lanes 5, 6, 11 and 12).

DISCUSSION

Plasmid pLS1 replicates in S. pneumoniae, B. subtilis and E. coli through a rolling circle mechanism, similar to the coliphages M13 and øX174 (19,23) and to the staphylococcal plasmids pT181 (30) and pC194 (15). This conclusion is based on the following findings: i) No fragments with altered electrophoretic mobility, indicative of forked intermediates, have been observed in partially replicated plasmid molecules (Fig. 1), similar to pT181 (24) and opposed to RSF1030 (25); ii) We have detected single-stranded plasmid forms which are intermediates of this type of replication (Figs. 3 and 4); iii) There is a signal in pLS1, to the left of the (+) strand origin of replication, involved in the conversion of single to double

stranded plamid DNA and its removal leads to accumulation of single-stranded intermediates (Fig. 4; 17); iv) Only the coding strand is rendered single-stranded (Figs. 4 and 5).

Replication of pLS1 is unidirectional and proceeds clockwise (Fig. 2). The plasmid single (+) strand origin is included within a 284-bp region. However, cloning of this region in pC194 followed by inactivation of the pC194 origin and trans-complementation with pLS1 did not result in replication driven by the streptococcal replicon (not shown), indicating that other plasmid components are needed in cis. Nevertheless, in vivo replicating plasmids derived from pLS1, lacking RepB, the putative RNAII and RepA have been isolated by trans-complementation with the parental plasmid (not shown).

A S1-sensitive site, mapping at around nucleotide 450 is detectable at $10^{\circ}C$ (Fig. 6B), coinciding with a region that contains two inverted repeats with potential to form hairpin structures. We propose that this region contains the (+) strand origin of replication of pLS1 with the structure presented in Figure 7. At the physiological temperature of $37^{\circ}C$, hairpin III is the major S1 site, although hairpin I has the lowest free energy. Since hairpin III and the putative RNAI are missing in pLS5 and in pLS1Δ24, their increase in copy number could be due to the lack of any of these two structures. A restriction fragment containing the putative RNAI, cloned in another replicon neither reduced the copy number of pLS5 or pLS1 nor exhibited incompatibility (not shown). At present the biological role of the putative RNAI if any, remains to be elucidated. Although sensitivity to nuclease S1 of supercoiled pLS1 DNA does not prove that hairpins are the target for the replication protein(s), superhelicity is an absolute requirement for in vitro replication of pLS1: open circular plasmid forms are not substrates for in vitro DNA synthesis and inhibition of DNA gyrase by novobiocin (resulting in relaxation of plasmid DNA) totally blocked plasmid replication in extracts prepared from plasmid-free (10) or from plasmid-containing cells (unpublished results). Thus, it would appear that secondary structures may be involved in replication of pLS1. In connection with this, it is interesting to point out that phage f1 gpII protein binds to DNA
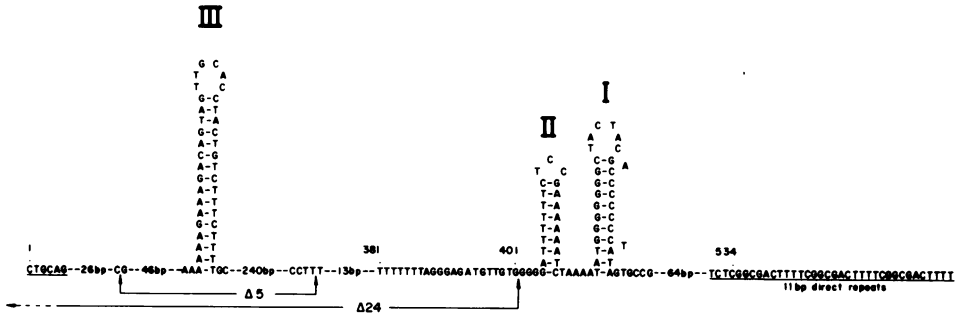
Figure 7. Proposed secondary structures at the origin of
replication of pLS1. Deletion in pLS5 and the right end of
deletion in pLS1Δ24 are indicated. Hairpin II may not
necessarily be formed.

independently of superhelicity whereas the nicking reaction by

gpII occurs only in superhelical DNA (26).

In addition to the secondary structures located beyond

nucleotide 404 in pLS1, three adjacent 11-bp direct repeats are

found 73 bp downstream (Fig. 7). Direct repeats are usually

found in Gram-negative replicons as F, R1, RK2, RSF1010 and R6K.

They seem to function as binding sites for the replication

proteins in the activation of the origins (27,28,29). Similar

structural elements have not been found in other small Gram-

positive plasmids so far. We believe that the three direct

repeats in pLS1 could play a role in the recognition of the

plasmid origin by replication protein(s). Nevertheless, cloning

of these repeats in other replicons did not result in

incompatibility towards pLS1 (not shown). No significant

similarities between the direct repeats of pLS1 and those of the

aforementioned plasmids have been observed.

Unlike in pT181 (24), the origin sequence of pLS1 is not

contained within regions encoding the initiator protein(s).

However, like pT181 (30) the origin region (coordinates 401-567)

of pLS1 is relatively G+C rich (about 50%), in comparison to

that of the whole plasmid (37%). Plasmid pLS1 exhibits a

striking broad host range which indicates that this replicon may

share homologies with both Gram-negative and Gram-positive

replicons. Similarities in the complementary strand origin of

pLS1 and M13 or øX174 (about 50%) have been reported (17). A
search for homologies between (+) strand origins of pLS1 and
various single-stranded coliphages or staphylococcal plasmids
did not reveal any significant similarities. However, at the
structural level it is interesting to note that hairpins I and
II in pLS1 resemble the structure proposed for the plus strand
origin of phage f1 (26) and that the consensus sequence in
origins and promoters of phage DNAs (19):

<div align="center">

5'-CACTAT-3'

3'-GTGATA-5'

</div>

is present at the right end of hairpin I. DnaA boxes (31) or
homology with oriC in pLS1 were not found.

A unique feature of pLS1 amongst small Gram-positive
plasmids is the presence of a polypeptide of 5.1 kDa, RepA, with
strong affinity for DNA (data not shown). RepA is translated
from a polycistronic mRNA together with the replication protein
RepB and seems to be involved in the control of replication of
pLS1 (del Solar et al., manuscript in preparation). Whether this
small protein modulates the recognition of RepB for its target
or the synthesis of RepB is a question that remains to be solved.

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