Lagging-strand origins of the promiscuous plasmid pMV158: physical and functional characterization

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The streptococcal plasmid pMV158 replicates by a rolling circle mechanism, which involves the generation of single-stranded plasmid DNA intermediates. This plasmid has the unique feature of having two lagging-strand origins of replication. One of these origins, termed *ssoU*, is functional in *Streptococcus pneumoniae* and in *Bacillus subtilis* in an orientation-dependent manner. The other origin, *ssoA*, is only functional in the former host. RNA polymerase seems to be involved in the initiation of the conversion of single- to double-stranded plasmid DNA from both *ssoA* and *ssoU*. Mutational and deletion analyses have allowed us to define *ssoA* as being within a highly structured, non-coding 199 bp region. Within this region, two elements which are conserved in several rolling-circle replicating plasmids are located, the recombination site *RS_g*, and a 6 base consensus sequence. Both elements may play a role in the conversion of single- to double-stranded plasmid DNA.

Keywords: Lagging-strand origins, RNA polymerase, rolling-circle replication, single-stranded DNA

INTRODUCTION

Circular, double-stranded (ds) bacterial plasmids replicate by mechanisms involving the generation of theta-shaped, D-looped, or sigma-shaped DNA intermediates. The last mechanism is used by many small multicopy plasmids, and is termed asymmetric rolling-circle (RC) replication because of the uncoupling of leading- and lagging-strand syntheses (reviewed by Novick, 1989; Gruss & Ehrlich, 1989; del Solar *et al.*, 1993a). Initiation of RC-replication is mediated by the plasmid-encoded Rep protein which introduces a strand- and site-specific nick on supercoiled plasmid DNA at the plasmid double-strand origin (*dso*). Rep activity leaves a 3'-OH end which is extended by host proteins while the parental strand is being displaced, and replication proceeds around the whole plasmid, until the entire *dso* is synthesized. As a consequence of the termination reactions, the parental (+) strand is released as a single-stranded (ss) DNA intermediate (te Riele *et al.*, 1986), which is the hallmark of RC-replicating plasmids (Gruss & Ehrlich, 1989). The last stage of RC-replication involves the conversion of ss- to ds-plasmid DNA by the synthesis of the lagging strand (Gruss *et al.*, 1987; del Solar *et al.*, 1987). This synthesis initiates in a plasmid

region distant from the *dso*, the single-strand origin, *sso* (Gruss *et al.*, 1987; del Solar *et al.*, 1987, 1993b). RC-replicating plasmids are constructed like cassettes of genetic information (Projan & Novick, 1988), one of the modules being *sso* (del Solar *et al.*, 1993a). Based on sequence homologies, various types of *sso* have been described (Novick, 1989), including *ssoA*, which is present in plasmids pT181, pLS1 and pC194 (Novick, 1989), and *ssoU*, which is characteristic of plasmid pUB110 (Boe *et al.*, 1989; Birch & Khan, 1992). A more complex situation is found in the streptococcal plasmid pMV158 (from which plasmid pLS1 is derived; Lacks *et al.*, 1986; Priebe & Lacks, 1989), because it bears both types of *sso* (van der Lelie *et al.*, 1989). This feature may provide the plasmid with greater flexibility for adapting itself to the different hosts in which it replicates, but poses the question of whether there is preferential lagging-strand origin utilization within a given host.

In general, the *sso* regions are located within highly structured non-coding regions (Novick, 1989), perhaps with the exception of plasmid pR (Gigliani *et al.*, 1993). The host machinery recognizes the *sso* in an orientation-dependent manner (Birch & Khan, 1992; del Solar *et al.*, 1993b). These findings suggest that unpaired sequences, within the secondary structures that constitute the *sso*, are important for its recognition by host proteins involved in

Abbreviation: RC, rolling circle.

ss → dsDNA conversion. However, it is not clear to what extent these secondary structures contribute to efficient *sso* function. The mechanisms involved in ss → dsDNA conversion are not well understood, although some features of the process have been defined. First, it seems that only host proteins are involved in the lagging-strand synthesis, most likely RNA polymerase, as shown *in vivo* for the *ssoU* of pUB110 (Boe *et al.*, 1989), and *in vitro* for the *ssoA* of pT181 (Birch & Khan, 1992). However, in the case of the lactococcal plasmid pWVO1 (an *ssoA*-containing plasmid) this conversion has been proposed to be mediated by the host DNA primase (Leenhouts *et al.*, 1991). Second, the amount of ssDNA molecules accumulated during RC-replication is plasmid- and host-dependent, indicating that the efficiency of this conversion depends upon the effectiveness with which the host machinery recognizes a given plasmid *sso* (Gruss *et al.*, 1987; del Solar *et al.*, 1987, 1993b; van der Lelie *et al.*, 1989). However, it is not clear how or where the RNA primer synthesis initiates, and with which specific plasmid sequences it would pair to initiate the lagging-strand synthesis. To answer some of the above questions, we have started a systematic analysis of the regions of the streptococcal plasmid pMV158 involved in ss → dsDNA conversion. We show here that two plasmid elements belonging to *ssoA* (namely, the recombination site RS_B and a consensus 6 base sequence) contribute to the ss → dsDNA conversion in *Streptococcus pneumoniae*.

METHODS

Bacterial strains and plasmids. Strains employed for copy number determinations and stability assays were *S. pneumoniae* 708 (*end-1 exo-2 trt-1 hex4 malM594*) and *B. subtilis* MB11 (*lys-3 metB-10 hisH-2*). Media and growth conditions were as described by Lacks *et al.* (1986). The plasmids employed, all harbouring the pMV158 replicon and conferring resistance to tetracycline, are listed in Table 1. Selective pressure for the antibiotic was kept at 1 µg ml⁻¹ for *S. pneumoniae* and at 10 µg ml⁻¹ for *B. subtilis*. In addition, plasmid pALTER and the *Escherichia coli* strains from a kit for site-directed mutagenesis (Promega) were used. Copy number determinations were performed as described by del Solar *et al.* (1987), with the aid of Computer Densitometer-ImageQuant equipment and software (Molecular Dynamics).

DNA preparation and manipulation. Purified plasmid DNAs were prepared from *S. pneumoniae* by two consecutive CsCl/ethidium bromide gradients as described by del Solar *et al.* (1987). Total DNA preparations (crude extracts) were made according to published procedures (Lacks *et al.*, 1986). BAL31 deleted derivatives were constructed from plasmid pLS1 linearized at the single *Afl*II site, treated as described by Puyet *et al.* (1988), and rescued by transformation of the pneumococcal host. The extent of the deletions was analysed by fine restriction mapping and by determination of the nucleotide sequence of the deletion junctions. Enzymes to manipulate the plasmid DNA were purchased from New England Biolabs or from Boehringer Mannheim, and used as specified by the vendors. All constructions were checked by determining the nucleotide sequence of the desired changes using the T7 Sequencing kit (Pharmacia).

Determination of the intracellular amounts of ssDNA. Analyses of ssDNA in cultures harbouring the plasmids of interest were made by electrophoresis on 1% (w/v) agarose gels of total

DNA samples, followed by transfer to nitrocellulose filters with or without prior denaturation (te Riele *et al.*, 1986). Filters were hybridized using ³²P-labelled pLS1 DNA as a probe. When accumulation of ssDNA was measured in cultures treated with rifampicin and/or erythromycin, pneumococcal cultures harbouring the desired plasmids were grown to middle exponential phase (about 2 × 10⁸ c.f.u. per ml culture). Then rifampicin (to 100 µg ml⁻¹), erythromycin (to 100 µg ml⁻¹) or both antibiotics were added. Culture samples were removed at various times and total DNA was analysed as described above. Direct quantification of the amount of ss- and dsDNA transferred to the filters, and calculation of the molecular ratios of ss/dsDNAs, were done with the aid of PhosphorImager-ImageQuant equipment and software (Molecular Dynamics).

Site-directed mutagenesis. The 'Altered Sites' kit (Promega), designed for *in vitro* mutagenesis was used. To perform the mutagenesis, the 1243 bp *Eco*RI-*Pst*I fragment of pLS1 (coordinates 3170 and 5, respectively; see Fig. 1a) was cloned into pALTER digested with the same enzymes. The consensus sequence: 5'-TAGCGT-3' of the *ssoA* was changed into the sequence 5'-TATCGA-3' by the use of the oligonucleotide 5'-CGAGCCGAAAGGCTTATCGATTTTCGGACGGACACGGA-3' (underlined letters indicate the mutation introduced within the wild-type sequence). This generates a new *Cla*I site (5'-ATCGAT-3'), which facilitated the screening of mutated plasmids. The desired mutant plasmid was isolated in *E. coli*, and the mutation was identified by digestion with *Cla*I. Then the cloned pLS1 DNA was inserted back into pLS1 by exchanging the *Eco*RI-*Pst*I fragment. The resulting plasmid (termed pLS1-CM) was rescued by transformation of *S. pneumoniae*, and the mutation was characterized by determination of its nucleotide sequence.

Database searching and structure prediction. Computer searches for homologies within the sequence of pMV158 were performed with the programs ALIGN and GAP (Dnastar, UK) with a window of 15 and a minimal homology of 55%. Secondary structure predictions were done with the SQUIGLES program (UWGCG, Wisconsin, USA).

RESULTS AND DISCUSSION

Deletion mapping of *ssoA*

The two *sso* regions of pMV158 are located on the plasmid coding strand. They are separated by the *mob* gene cassette, involved in plasmid conjugative mobilization (Fig. 1a). The *ssoU* has been reported to be the preferred lagging-strand origin in *B. subtilis* (van der Lelie *et al.*, 1989), but its efficiency in the ss → dsDNA conversion had not been analysed in the pneumococcal host. On the other hand, *ssoA* is efficiently recognized by *S. pneumoniae*, but not by *B. subtilis* (del Solar *et al.*, 1987). We had experimentally defined the *ssoA* region as being between the *Hind*III and *Nco*I sites (pMV158 coordinates 4407–5349; Fig. 1a), the fragment removed during construction of plasmid pLS4 (Table 1). Deletion of this 942 bp region in pLS1, which already lacks *ssoU*, led to plasmids exhibiting an increased accumulation of ssDNA in *S. pneumoniae*, but not in *B. subtilis* (del Solar *et al.*, 1987, 1993b). These plasmids, which lack the two *sso* regions, have a lower copy number, and a high segregational instability in both hosts, with a rate of loss of about 0.1 per cell per generation (del Solar *et al.*, 1993b). We predicted that the *ssoA* should be included in a region with a high potential to generate

Fig. 1. Relevant features of pMV158. (a) Physical and genetic map of pMV158 and its deleted derivatives. The positions of the *dso* and the two *sso* are shadowed. Plasmid-encoded proteins, with direction of synthesis (arrowheads), are indicated. Wavy arrows indicate the position of the two antisense RNAs encoded by pMV158. Only relevant restriction sites are shown. (b) Nucleotide sequence around the *ssoA*. Coordinates of pMV158 are those by Priebe & Lacks (1989). The recognition sites for *AflII* and *NcoI* are underlined. Locations of the recombination site B (RS_B ; double underlined), and of the 6 bp consensus (bold) are shown. The borders of the $\Delta 13$ and $\Delta 14$ deletions and the left border of the $\Delta 24$ deletion are indicated. (c) Computer prediction of the secondary structure that could be generated from the sequence shown in (b). Features relevant for this work are indicated.

secondary structures, within which a nuclease-S1-sensitive site has been mapped (del Solar *et al.*, 1987; Fig. 1c). This region is bordered by the restriction sites *AflII* and *NcoI* (coordinates 5150 and 5349 of pMV158, respectively; Fig. 1b). Within this putative *ssoA*-containing region, two conserved sequences have been proposed as being important elements of the lagging-strand origin of replication (Fig. 1c; Gruss *et al.*, 1987; del Solar *et al.*, 1987). The first of these is the recombination site, termed RS_B (Novick *et al.*, 1984), which is involved in plasmid

recombination and generation of co-integrates among homologous plasmids. This region shares homologies with the pSC101 *par* locus, which is involved in stable maintenance and which includes a DNA-gyrase-binding site (Wahle & Kornberg, 1988). The second of these, a 6 base consensus sequence (5'-TAGCGT-3'), is located on the terminal loop of the putative hairpin within the *ssoA* of pLS1, pC221 and pC194 (del Solar *et al.*, 1987; Fig. 1c). This consensus sequence is also present in the *ssoA* of several other RC-plasmids and coliphages, and has

Table 1. Features of the plasmids used in this work

Plasmid	Size (bp)	Mutation	Relevant features	Reference/ source
pMV158	5536	None	Wild-type	Burdett (1980)
pLS1	4408	Δ EcoRI-B fragment of pMV158	Δ ssuU	Lacks <i>et al.</i> (1986)
pLS1 Δ HA	3665	Δ HindIII-AffII of pLS1	Δ ssuU	This work
pLS1 Δ NA	4209	Δ AffII-NcoI small fragment of pLS1	Δ ssuA, Δ ssuU	This work
pLS1-CM	4408	5bp change at the <i>ssuA</i> consensus in pLS1	Δ ssuU, partially affected in <i>ssuA</i>	This work
pLS1 Δ 13	4258	BAL31 deletion of pLS1 linearized at AffII	Δ ssuU, partial deletion of <i>ssuA</i>	This work
pLS1 Δ 14	4193	BAL31 deletion of pLS1 linearized at AffII	Δ ssuU, partial deletion of <i>ssuA</i>	This work
pLS5	4076	<i>in vivo</i> spontaneous deletion of pLS1	Δ ssuU, Δ rnaI (Δ 5)	del Solar <i>et al.</i> (1987)
pLS4	3138	Δ 7: HindIII-NcoI small fragment of pLS5	Δ ssuA, Δ 5 (Δ rnaI)	del Solar <i>et al.</i> (1987)
pLS4-EC	4266	Δ 7, EcoRI-B fragment of pMV158 cloned in pLS4 (correct orientation)	Δ ssuA, Δ 5 (Δ rnaI)	This work
pLS4-EI	4266	Δ 7, EcoRI-B fragment of pMV158 cloned in pLS4 (incorrect orientation)	Δ ssuA, Δ 5 (Δ rnaI) non-functional <i>ssuU</i>	This work
pLS1 Δ 24	3838	BAL31 deletion of pLS1 linearized at BanI	Δ ssuU, Δ rnaI	A gift from P. Acebo

Fig. 2. Location of regions important for a functional *ssuA*. In addition to pMV158, plasmids derived from pLS1 (Δ ssuU) were employed. The intracellular ssDNA accumulated by the indicated plasmid derivatives was detected by Southern blot hybridization of total DNA isolated from plasmid-containing cultures and electrophoresed in 1% agarose. DNA was transferred after denaturation, so that ds- and ssDNA are visible. The positions of ssDNA in *S. pneumoniae* (a) and in *B. subtilis* (b) are bracketed. The leftmost lanes in part (a) are overexposed to visualize the ssDNA.

been used as a criterion for the location of lagging-strand origins (Zaman *et al.*, 1993). Part of the RS_B as well as the consensus sequence are located within unpaired regions within the *ssuA* (Novick, 1989).

To define more precisely the boundaries of the *ssuA*, AffII-linearized pLS1 DNA was treated with BAL31 nuclease. Deleted pLS1-derivatives were rescued by transformation of *S. pneumoniae*, and several of them were

Table 2. Copy number and molecular ratios of ss/dsDNA for plasmids bearing the pMV158 replicon

Plasmid	<i>S. pneumoniae</i>		<i>B. subtilis</i>	
	Copy number*	ss/ds DNA ratio	Copy number	ss/ds DNA ratio
pMV158	36 ± 6	0.005 ± 0.0006	7 ± 3	< 0.001
pLS1	22 ± 2	0.0065 ± 0.0003	4 ± 1	0.67 ± 0.09
pLS1ΔHA	19 ± 4	0.0052 ± 0.0016	4.5 ± 1	1.14 ± 0.10
pLS1Δ13	12 ± 4	0.10 ± 0.02	4 ± 1	0.87 ± 0.02
pLS1Δ14	10 ± 3	0.20 ± 0.04	4.5 ± 1	0.75 ± 0.07
pLS1ΔNA	15 ± 3	0.31 ± 0.01	ND	ND
pLS4	11 ± 3	0.34 ± 0.10	3 ± 1	1.02 ± 0.05
pLS4-EC	20 ± 4	0.007 ± 0.001	5 ± 1	< 0.001
pLS4-EI	12 ± 4	0.10 ± 0.04	5 ± 1	0.84 ± 0.05
pLS1-CM	21 ± 2	0.017 ± 0.004	ND	ND
pLS1Δ24	21 ± 2	0.008 ± 0.002	ND	ND
pLS5	46 ± 3	0.01 ± 0.003	9 ± 1	0.42 ± 0.02

ND, Not done.

* Measured as ds plasmid DNA.

characterized. Two plasmids were selected, both of them lacking the RS_B but preserving the consensus sequence (pLS1Δ13 and pLS1Δ14; Fig. 1). Plasmids lacking the entire *AflI*–*NcoI* fragment (pLS1ΔNA), or the *HindIII*–*AflI* region (pLS1ΔHA) were also constructed (Table 1). As controls, pMV158 (wild-type), pLS1 (Δ*ssoU*), pLS1Δ24 (Δ*ssoU*) and pLS4 (Δ*ssoU*, Δ*ssoA*) were used (Fig. 1a). All these plasmids were transferred to *S. pneumoniae* and to *B. subtilis*, and the ssDNA accumulated was detected (Fig. 2), and quantified (Table 2). In the pneumococcal host (Fig. 2a), no influence of the *HindIII*–*AflI* region in accumulation of ssDNA was observed, since the plasmid pLS1ΔHA accumulated as little ssDNA as pLS1 or pMV158. In addition, sequences located at 3' of the *NcoI* site (on the coding strand, Fig. 1b) do not play any role in the ss → dsDNA conversion, since the amount of ssDNA detected for pLS1Δ24 was similar to that of pLS1 (Fig. 2a). However, a 15-fold increase in the amount of ssDNA was detected in plasmid pLS1Δ13, with respect to pLS1, and a further twofold increase was found in pLS1Δ14. Deletion of the whole *AflI*–*NcoI* region (pLS1ΔNA) further increased the amount of ssDNA accumulated. These results demonstrate that the *ssoA* of plasmid pMV158 is located within the coordinates 5150 and 5349, which experimentally narrows down this lagging-strand origin from 942 bp (del Solar *et al.*, 1993b) to a 199 bp region (Fig. 1b). Regions similar in size have been defined for the *ssoA* of plasmid pT181 (Birch & Khan, 1992), and for the *ssoU* of pUB110 (Boe *et al.*, 1989). In addition to this, we may also conclude that either the DNA sequence or its structure within the RS_B plays an important role in the ss → dsDNA conversion. However, the RS_B is not essential since there is some *ssoA*-dependent conversion in its absence. This indicates that some other elements, in addition to the RS_B , must be involved in the *ssoA*-

Fig. 3. Influence of the consensus sequence (5'-TAGCGT-3') on *ssoA* functionality. (a) Putative secondary structure generated on the loop of the *ssoA* in the wild-type pLS1 (left) and in the mutant pLS1-CM (right). Note that the mutation generates a consensus-like sequence 5'-TATCGa-3'. (b) Accumulation of ssDNA in pneumococcal cultures harbouring the indicated plasmids. Samples were treated as in Fig. 2.

dependent conversion because pLS1ΔNA accumulated more ssDNA than pLS1Δ14. In the case of *B. subtilis*, lack of functionality of the *ssoA* as a lagging-strand origin is demonstrated because none of the pLS1-deleted derivatives exhibited changes in the already high amount of ssDNA accumulated (Fig. 2b, Table 2).

The consensus sequence of *ssoA* plays a role within pLS1 lagging-strand origin

We had earlier postulated that the consensus 5'-TAGCGT-3' sequence, located on the loop of the secondary structure mapped in the *ssoA* region of pLS1, could play a role as part of this conversion signal (del Solar *et al.*, 1987). To determine whether the consensus is involved in the ss → dsDNA conversion, we changed five bases of this sequence without significantly altering intrastrand pairing within the secondary structure (Fig. 3a). Due to the presence of a T just before the consensus, the mutation generated a weak consensus-like sequence 5'-TATCGa-3' (Fig. 3a). Curiously, this sequence has only one mismatch (the central T) as compared with the consensus found at the *ssoA* of plasmid pE194 (del Solar *et al.*, 1987). Some of the features of the mutant plasmid, termed pLS1-CM, were analysed only in *S. pneumoniae* because of the lack of functionality of *ssoA* in *B. subtilis*. The phenotype of pLS1-CM was indistinguishable from its parental pLS1 with respect to copy number (Table 2). To evaluate statistically accumulation of ssDNA in cells harbouring pLS1-CM, eight independent DNA preparations were analysed. The results (Table 2, Fig. 3b) showed that a significant 2.6-fold increase in the amount of ssDNA was observed within cells harbouring pLS1-

Fig. 4. The *ssuU* functionally replaces the *ssuA* in *S. pneumoniae*. Cultures of *S. pneumoniae* or *B. subtilis*, harbouring the indicated plasmids, were treated as described in the legend to Fig. 2.

CM, as compared with pLS1 or pMV158. These results allowed us to conclude that the consensus sequence by itself may play a role within the conversion signal. However, we cannot rule out that the putative configuration of the loop of the *ssuA*, which would be larger in the mutant than in the wild-type (Fig. 3a), could influence the ss → dsDNA conversion.

ssuU is efficiently recognized by *S. pneumoniae*

Pneumococcal cells harbouring pMV158 accumulate as small an amount of ssDNA as pLS1 (Fig. 2a), which could be due solely to the presence of the functional *ssuA*. To test whether *ssuA* could be replaced by the *ssuU* in *S. pneumoniae*, the 1128 bp *EcoRI* fragment B of pMV158, which is removed in pLS1 and its derivatives (Fig. 1a), was cloned into the single *EcoRI* site of plasmid pLS4 in the two possible orientations (Table 1). If the *ssuU* acts as an efficient lagging-strand origin in pneumococci, low amounts of ssDNA should be detected only when the *EcoRI*-B fragment is cloned in the correct (pLS4-EC), but not in the opposite (pLS4-EI), orientation. This was indeed the case, and pLS4-EC accumulated little ssDNA, whereas pLS4-EI behaved similarly to its parental pLS4 (Fig. 4a). In addition, the decrease in copy number (measured as dsDNA) observed for pLS4 was restored to the levels of pLS1 only in the case of pLS4-EC (Table 2). Similar results on ssDNA accumulation were found for *B. subtilis* (Fig. 4b), although the number of copies of the plasmids did not vary (Table 2). This finding supports previous observations indicating that the *ssuU* is the functional lagging-strand origin in *B. subtilis* (van der

Lelie *et al.*, 1989). We can thus conclude that the pMV158-*ssuU* acts as an efficient conversion signal in both hosts. It remains to be shown whether the two *ssu* present in pMV158 play different roles in *S. pneumoniae*. Due to its location within the plasmid (Fig. 1a), we can speculate that the *ssuU* would be the lagging-strand origin preferentially used after conjugative plasmid transfer, since deletion of a region which encompasses the *ssuU* resulted in loss of the mobilization function (Priebe & Lacks, 1989). In this sense, it is curious that the two *ssu* regions are placed just before and just after the *mob* gene. Alternatively, RC-replication from the *dso* would lead to the exposure of *ssuU* as an ssDNA region before the replisome reaches the *ssuA*. If the host machinery follows a 'first-exposed, first-used' pattern for ss → dsDNA conversion, then *ssuU* should be the preferential lagging-strand origin employed in pMV158.

Pneumococcal RNA polymerase is involved in the ss → dsDNA conversion

RC-plasmids pUB110 and pT181 require the host RNA polymerase for ss → dsDNA conversion (Boe *et al.*, 1989; Birch *et al.*, 1992). To determine whether pMV158 uses the *S. pneumoniae* RNA polymerase for lagging-strand synthesis, exponentially growing cells harbouring pLS1 (Δ *ssuU*), pLS4-EC (Δ *ssuA*) or pLS4 (Δ *ssuU* Δ *ssuA*) were subjected to inhibition of RNA and/or protein synthesis, by treatment with rifampicin, erythromycin or both antibiotics simultaneously. Rifampicin treatment would prevent RNA polymerase activity, whereas inhibition of protein synthesis would hinder synthesis of the initiation of replication RepB protein, thus preventing (at least partially) further rounds of replication from the plasmid *dso*. The amount of plasmid DNA was quantified and the molecular ratio of ss/dsDNA was plotted as a function of the time of treatment (Fig. 5). A 10-fold increase in this ratio was observed in the cultures in which RNA or RNA and protein synthesis were inhibited, but only if they harboured plasmids having one or another *ssu* (Fig. 5a, b). A net increase in the amount of ssDNA accumulated upon inhibition of RNA polymerase could be observed in either pLS1 and pLS4-EC, but not in pLS4 (Fig. 6). No such accumulation was found for the *ssu*-containing plasmids in the absence of antibiotics (Fig. 6). Consequently, the accumulation of ssDNA after inhibition of RNA synthesis (but not of protein synthesis) suggests that an active RNA polymerase is needed to avoid accumulation of ssDNA within the cells. This in turn indicates that the pneumococcal RNA polymerase is the enzyme involved in priming DNA synthesis from both lagging-strand origins. These results are supported by the observation that no significant changes were observed for the *ssu*-deleted plasmid pLS4 (Fig. 5c). We conclude that the same host enzyme is involved in synthesis of a primer in two plasmid regions located far apart, and apparently performing the same function. The *ssuA* and the *ssuU* regions exhibit high potential to generate secondary structures (data not shown) and, since both *ssu* regions are recognized by the host RNA polymerase, we expected to find some sequence conservation among them. However, computer searches

Fig. 5. Influence of rifampicin on ss $\rightarrow$ dsDNA conversion. Pneumococcal cultures harbouring pLS1 [*ΔssoU*, panel (a)], pLS4-EC [*ΔssoA*, panel (b)] or pLS4 [*ΔssoA ΔssoU*, panel (c)] were treated with rifampicin (Δ), erythromycin (○) or both antibiotics (●). At the indicated times, samples were removed, and the molecular ratios of ss/dsDNA accumulated were quantified.

Fig. 6. Intracellular accumulation of ssDNA. Pneumococcal cultures harbouring the indicated plasmids were treated (+) or not treated (-) with rifampicin or erythromycin as in Fig. 5. Samples were removed at various times, and total DNA was analysed as in Fig. 2. The position of ssDNA is indicated.

showed neither significant homologies between the *ssoU* and the *ssoA*, nor any putative promoter-like sequence (data not shown). Plasmids having a low G + C content, like pMV158, do have several sequences identical to the promoter -10 consensus region, but their significance is questionable. Consequently, which DNA signals are recognized by the RNA polymerase is unknown.

An interesting finding is that, in spite of the high amount of ssDNA accumulated in cells harbouring plasmids devoid of *sso*, those plasmids could be maintained under selective pressure. This indicates that at least an alternative pathway for the conversion of ss $\rightarrow$ dsDNA should exist. How then could plasmids with defective lagging-strand origins replicate? Based upon indirect evidence, we

proposed that an alternative *ss* could exist within the *tet* gene of pMV158 (del Solar *et al.*, 1987). If this alternative *ss* is functional, a host function, other than RNA polymerase, and insensitive to inhibition of protein synthesis, should be involved in this weak mechanism of ss $\rightarrow$ dsDNA conversion. Based on our results, we believe that both sequence-specific elements (RS_B and 6-mer consensus) and secondary structures could be the best combination for a region to function as an *ss*. The results obtained so far with pMV158 allow us to draw the following sequence of events in the ss $\rightarrow$ dsDNA conversion process. Lagging-strand synthesis would initiate from an ssDNA plasmid template which should be covered by the host single-stranded DNA binding protein, except in those regions having intrastrand pairing. Secondary structures, in conjunction with paired and unpaired sequence-specific sites, could act as signals which would be recognized by the host RNA polymerase. Upon synthesis of an RNA primer, DNA polymerase III would proceed to synthesize DNA until reaching an *ss* which would now be a DNA–RNA hybrid. The last stage would be the removal of the RNA and termination of DNA synthesis, which is performed by the host DNA polymerase I (Díaz *et al.*, 1994).

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