Conjugation of bacterial plasmids is, together with transposition, the most important source of horizontal gene transfer among bacteria of the same or of different species (42). Conjugation implies the unidirectional transfer of one plasmid DNA strand from a donor to a recipient cell. This is initiated by the activity of a plasmid-encoded protein generically termed relaxase, in a process that resembles replication by the rolling-circle mechanism (13, 29). In the case of numerous, small plasmids (<10 kb) isolated primarily from gram-positive bacteria, two pioneer findings led to the discovery of the rolling-circle mechanism of replication (RCR) plasmids (reviewed in references 20 and 21). First, the strand-specific single-stranded DNA (ssDNA) molecules which act as replication intermediates were identified (41) and, second, the relaxing activity on the supercoiled DNA via the recognition of a specific sequence (the double-strand origin) of the Rep initiator proteins were described (22). Most RCR plasmids are not self-transmissible; instead, they encode not only the Rep topoisomerase-like relaxase that had an intact ssoU could be efficiently mobilized from *S. pneumoniae* to *Enterococcus faecalis*. Thus, it appears that ssoU is a critical factor for pMV158 promiscuity and that the presence of a functional *sso* plays an essential role in plasmid transfer.

The promiscuous streptococcal plasmid pMV158 is mobilizable by auxiliary plasmids and replicates by the rolling-circle mechanism in a variety of bacterial hosts. The plasmid has two lagging-strand origins, *ssoa* and *ssou*, involved in the conversion of single-stranded DNA intermediates into double-stranded plasmid DNA during vegetative replication. Transfer of the plasmid also would involve conversion of single-stranded DNA molecules into double-stranded plasmid forms in the recipient cells by conjugative replication. To test whether lagging-strand origins played a role in horizontal transfer, pMV158 derivatives defective in one or in both *sso* were constructed and tested for their ability to colonize new hosts by means of intra- and interspecies mobilization. Whereas either *sso* supported transfer between strains of *Streptococcus pneumoniae*, only plasmids that had an intact *ssou* could be efficiently mobilized from *Staphylococcus aureus*.

Initiation of transfer, like initiation of RCR, involves cleavage of the phosphodiester bond of a specific dinucleotide on one of the plasmid strands. Cleavage is mediated either by the plasmid-encoded Mob protein at the origin of transfer (oriT) during conjugation or by the plasmid-encoded Rep protein at the *dso* during replication. In both processes, this initial stage is followed by displacement of the cleaved strand in a unidirectional manner (8, 21, 29, 36). Thus, RCR and conjugal transfer are equivalent processes in the sense that they generate strand-specific ssDNA plasmid intermediates that correspond only to the cleaved strand (9, 16, 41). The ssDNA intermediates are generated in the plasmid host by the activity of the Rep initiator protein (replication) or generated and transferred to the recipient cell (T-DNA) and closed by the Mob relaxase (conjugation), where they are converted into double-stranded plasmid DNA (dsDNA) molecules by laggingstrand synthesis. Replication of the lagging strand is initiated at the single-strand origins (*sso*) by the host RNA polymerase (RNAP), upon recognition of a specific site on ssDNA and synthesis of a short RNA primer (pRNA). The pRNA is used by DNA polymerase I for limited extension synthesis, followed by replication of the lagging strand by DNA polymerase III (27). Features of the *sso* include the potential to generate stem-loop structures on ssDNA (9, 16, 41) that can conform a ssDNA promoter, which is inactive in the dsDNA configuration. This kind of promoter was described in the coliphage N4 (18), as recognized by the virion RNAP (4, 14). A different kind of ssDNA promoter, F*pro*, was reported for the *Escherichia coli* plasmid F and was demonstrated to be used for gene expression and appeared to play a role during plasmid conjugation (34). The presence of ssDNA promoters has also been demonstrated in plasmids pMV158 (27) and Coll-P9 (1, 35). The organization of this kind of promoters showed that they are placed on the DNA strand that is partially complementary to the template strand.

The first *sso* was described in the staphylococcal RCR plasmid...
mid pT181, in which a deletion located out of the replicon led to instability, reduction in copy number, and accumulation of ssDNA intermediates (16). Plasmid pMV158 exhibits two sso’s, ssoA and ssoU (23). Two conserved regions were found in the ssoA of pLS1 plasmid (a nonmobilizable pMV158 derivative lacking ssoU): a short region termed recombination site B, RS_B, supposedly involved in plasmid cointegration (16, 38), and a 6-nucleotide (nt) consensus sequence (5’-TAGGGT-3’, termed CS-6). Determination of the roles of these two conserved sites showed that, whereas RS_B was the primary site of RNAP binding (located at the stem of the hairpin), CS-6 was the termination site for the synthesis of a 20-nt RNA in the loop of the hairpin (27). The predicted intradonor pairings in the pMV158-ssoA showed the presence of an ssDNA promoter in the vicinity of the RS_B, which would have a consensus −35 region (5’-TTGACA-3’) but a weak −10 region (5’-TAgcgT-3’). With this situation, RNA synthesis should start and proceed in the direction toward the binding site of RNAP, being thus opposite to RNA synthesis from classic promoters (27) (see Fig. 1A). Sites homologous to RS_B and CS-6 were later observed in pMV158-ssoU (24).

In the present study we have addressed the question of whether and, eventually, which of the two pMV158-ssoA plays a role in conjugal transfer. With this objective, we constructed pMV158-derivatives defective in one or both sso’s and tested their role on intra- and interspecies mobilization. Whereas either sso supported transfer between strains of Streptococcus pneumoniae with the same efficiency as the parental pMV158, only the ssoU could do so when conjugal transfer was assayed between S. pneumoniae and Enterococcus faecalis. Our findings show that the functionality of ssoU is a critical factor in the colonization of a broad range of gram-positive bacteria for the pMV158 promiscuous plasmid and demonstrate that efficient transfer and replication in enterococci depend upon a functional ssoU. We suggest that sso’s lacking functionality for vegetative replication in a specific host should not be efficient in conjugal transfer and vice versa, since both events are mechanistically identical. As far as we know, this is the first report that shows the effect of sso functionality on horizontal gene transfer by plasmid conjugation, as well as the efficiency of the ssoA and ssoU in E. faecalis.

### MATERIALS AND METHODS

**Bacterial strains, plasmids, and DNA manipulations.** Bacteria and plasmids used are listed in Table 1. AGCH and ESTY (Promadisa, Spain) media and the growth conditions for S. pneumoniae and E. faecalis have been described (28, 33). Competent S. pneumoniae 708 cells bearing plasmid pAM1 were transformed with DNA from pMV158wt and its derivatives as reported elsewhere (10). These strains were used as donors in filter-mating experiments. To construct S. pneumoniae MP3008, the novobiocin-resistant (Novr) strain MP517, which is unable to grow in maltose as the only carbon source, was transformed with the PstI DNA fragment from plasmid pLS70 that contains part of the pneumococcal wild-type mal operon (40). S. pneumoniae MP3008 (Novr) and E. faecalis OG1RF (resistant to rifampin) were used as recipients in intra- and interspecies transfer assays, respectively. Cultures of transconjugants were used to determine plasmid copy numbers and to detect intracellular ssDNA intermediates as described previously (9). Selection for plasmids pAM1 and pMV158 was performed using 1 μg of erythromycin/ml and 1 μg of tetracycline/ml in S. pneumoniae and 1 μg of erythromycin/ml and 4 μg of tetracycline/ml in E. faecalis. Purified pMV158 plasmid DNA was prepared by two consecutive CsCl-ethidium bromide gradients as described previously (7). Plasmid pMV158ΔBD, a derivative of pMV158 lacking the ssoU was constructed by deletion of a 205-bp Bsal-DraI DNA fragment (coordinates 3223 to 3428 of pMV158; see Fig. 1). To generate pMV158ΔBD, the small EcoRI fragment from pMV158 was cloned into plasmid pLS1G3G7 carrying nucleotide changes in the RsB and CS-6 conserved sequences of the ssoU (27) (see Fig. 1). The resulting plasmid, pMV158ΔG3G7, was functionally defective in the ssoU. Similarly, the small EcoRI restriction fragment from pMV158ΔBD (927 bp) was cloned into pLS1G3G7, thus generating pMV158G3G7ΔBD, with defective functionality of both sso genes. All constructions were rescued by transformation of competent pneumococcal cells and the mutations were confirmed by sequencing with specific primers. U1 (GGGATCAACTTGGG GAGAGA) and U2 (CGGTCTCAAA A CGGTTCA) were used to confirm the ssoU deletion, A1 (TCACAAAGCT CACCTCA) was used to confirm the G3G7 mutations of ssoU, and U1 and M1 (AAAGCAACCTCTCACATG) were used to confirm the orientation of the small EcoRI fragment (see Fig. 1A).

**Filter-mating experiments.** Mobilization assays of pMV158 and its derivatives from S. pneumoniae donor cells harboring pAM1 as auxiliary plasmid were performed as described previously (37) with minor modifications. Donor and recipient cultures were grown without aeration at 37°C to 5 × 10⁶ cells/ml. Cells were centrifuged and resuspended in prewarmed AGCH medium supplemented with 10 mM MgCl₂, 2 mg of bovine serum albumin/ml, and 100 μg of DNaše 1. Donor-recipient mixtures (1:5 ratios) were filtered onto sterile 25-nm nitrocellulose filters (0.22-μm pore size). The filters were then placed cell-side-down over another filter previously placed on a plate with conjugal transfer medium (AGCH with 10 mM MgCl₂, 2 mg of bovine serum albumin/ml, and 2% agar). After 4 h of incubation at 37°C, the cells were recovered by washing the filters in 1 ml of AGCH medium. A recent method to perform multiple simultaneous conjugations was also applied (33), where several donor cell densities were mixed with a fixed recipient cell density and placed onto a multiwell plate equipped with a 0.22-μm-pore-size nitrocellulose filter.
(Millipore), filtered, and incubated for 4 h at 37°C. With this device, eight transfer experiments were performed for each condition, resulting in a high degree of reproducibility and enough repetitions of the same experiment. Transconjugants were selected on AGCH medium with tetracycline (1 g/ml), novobiocin (10 g/ml), 0.3% maltose, and 1.5% agar for *S. pneumoniae* MP3008 or on ESTY plates with tetracycline (4 g/ml), 0.3% glucose, and 1.5% agar for *E. faecalis* OG1RF. After serial dilutions, the number of CFU of recipient strains was calculated in the same media, but without selection for tetracycline resistance. Conjugative mobilization efficiencies were calculated as the number of transconjugant cells per recipient.

**RESULTS**

**Construction of pMV158 derivatives deficient in sso functionality.** Plasmid pMV158 contains a gene cassette devoted to its mobilization, which is composed of the origin of transfer, oriT, and the gene encoding the MobM relaxase (Fig. 1A). This cassette is encompassed by the two *sso* genes in such a way that, during conjugation, *sso*A, generated in the transferred ssDNA, enters the recipient cell first, whereas *sso*U is the last plasmid region transferred (Fig. 1A; see also Fig. 5). Computer-assisted and structural analyses showed that both *sso*s could generate complex secondary structures on the ssDNA intermediates by intrastrand pairing. In these stem-loop structures the consensuses sequences RSB (the RNAP binding sites) is partially paired and the CS-6 would be unpaired and placed on the loop of the hairpin (24, 27); schematized in Fig. 1A. In the case of *sso*A, a single hairpin would be generated, whereas up to five hairpins may be formed in the region encompassing *sso*U (Fig. 1A). Homologies between the two lagging-strand origins were observed especially around the RSB conserved sequences, homologies that were partially maintained when the four types of *sso* reported thus far were aligned (24). A strong interaction between the host RNAP and the lagging-strand origin(s) could be an important factor in determining the host range of RCR plasmids (24). This, in conjunction with the genetic structure surrounding the *mobM* cassette and the promiscuity of pMV158, led us to hypothesize that the *sso*U could be involved in the determination of the broad host range of *sso*U-containing RCR plasmids.
between the different hosts they colonize (6). To address this hypothesis, we constructed pMV158 derivatives carrying modifications in their sso, either individually or together, that affected their functionality. To modify ssoA, a 6-nt change was introduced in the unpaired R5 nucleotides (mutation G7), as well as another 9-nt change introduced into the CS-6 sequence (mutation G3), as shown in Fig. 1B. These changes did not alter the high potential for secondary structure formation within ssoA, and only local changes in the general organization of this region were predicted by computer analysis (not shown). However, the mutations hindered binding of RNAP (changes at the R5) and affected termination of the pRNA (changes at CS-6) (27). The resulting mutant plasmid, pMV158G3G7 (Fig. 1B and Table 1), has a nonfunctional ssoA. In the case of the pMV158-ssoU, a fine characterization of the nucleotides important for its functionality has not yet been made. Thus, we generated a 205-bp deletion that affected hairpins I, II, and III (Fig. 1A). The resulting plasmid, termed pMV158ΔBD, lacks the two most important sequences (RS and CS-6) within the ssoU. Finally, a derivative affected in both origins was also generated (plasmid pMV158G3G7ΔBD). The wild-type pMV158 and its three derivatives were rescued in competent S. pneumoniae cells, and the mutations were confirmed by sequencing of the affected regions.

Defects in sso functionality lead to reduced mobilization frequency. To analyze the usage of the sso’s during conjugative mobilization, each of the plasmids (pMV158wt and its derivatives) were next transferred to competent S. pneumoniae 708 cells carrying pAMBl (the plasmid providing the auxiliary functions for conjugation), and intra- and inter-specific conjugal transfer assays were performed using S. pneumoniae MP3008 (Nov+) or E. faecalis OG1RF as recipients. To have several transfer experiments with high reproducibility, we made use of a recently developed multiwell plate setup coupled with a filter device so that up to eight transfers per plasmid and per donor-recipient cells were done simultaneously (33). The results obtained have a high confidence index and a low experimental standard deviation (usually below 10%). When interspecies transfers were assayed between S. pneumoniae strains, it was evident that plasmids lacking both sso’s exhibited a near-150-fold reduction in the conjugation frequencies (Fig. 2). No significant reduction in transfer frequencies were found for plasmids impaired in the functionality of either ssoA or ssoU (Fig. 2). These findings indicate that in the pneumococcal host, both pMV158-ssoU were functionally replaceable, and that a 205-bp deletion encompassing most of the ssoU did not affect the functionality of the pMV158 transfer module.

In interspecies transfer experiments from pneumococci to E. faecalis, we took advantage of this bacteria being aerobic. Since pneumococcus is unable to grow on the surface of agar plates (being a microaerophilic bacteria), there was no need to apply selection for the recipients other than growing them on the surface of the plates, a strategy that has proven to be useful in plasmid transfer from S. pneumoniae to aerobic bacteria (12, 33). In the case of pMV158wt, the frequencies of transfer were similar to or even greater than, i.e., ca. 10−4 transconjugants of recipient cells per ml (Fig. 2), those observed for transfer between pneumococci. This is around the maximum value ever attained for pMV158 transfers (11, 37, 43). The values obtained for pMV158wt were nearly identical to those observed when the plasmid carried a nonfunctional ssoA (pMV158G3G7; Fig. 2). Other mutations in the ssoA were also tested (i.e., mutations in the CS-6 or in the RS5), and the results obtained did not differ from the ones obtained for pMV158G3G7 (not shown). However, there was a strong 30-fold reduction in the transfer frequency when the plasmid had a defective ssoU (pMV158ΔBD) with a further decrease (nearly 60-fold) when the plasmid tested lacked both origins (pMV158G3G7ΔBD; Fig. 2). We conclude that, in E. faecalis, there is a strong preference for the use of ssoU as the plasmid lagging-strand replication signal. Although the copy number of pMV158wt was lower in E. faecalis than in S. pneumoniae, the plasmid was segregationally stable in the former host, since 100% of the cells retained the plasmid after 100 generations in the absence of selective pressure (data not shown). Further, the ssDNA/dsDNA ratio, which indicates efficient replication, was the same for the two bacterial hosts tested here (Table 2).

Intracellular accumulation of ssDNA depends on the plasmid sso-host interactions. The reduction in the conjugation efficiency when ssoU-deficient plasmids were mobilized to E. faecalis indicates that, in contrast to mobilization to S. pneumoniae, the ssoU origin may have a critical role. If this were the case, we would expect ssoU-deficient plasmids in E. faecalis to generate large amounts of ssDNA intermediates. To test this hypothesis, colonies of transconjugants harboring pMV158wt or its derivatives were selected and grown for 30 generations (the minimum period for a colony to become a full-grown liquid culture [9]). Total DNA was prepared, and the different DNA forms were separated by agarose gel electrophoresis in
the presence of ethidium bromide. Gels were recorded (gels marked as “L” in Fig. 3 and 4), and the DNA was transferred to filters and analyzed by Southern hybridization. The plasmid DNA forms bound to the membranes showed that ssDNA intermediates had a higher electrophoretic mobility than supercoiled circular covalently closed (ccc) monomeric forms (“R” gels in Fig. 3 and 4). In the case of \textit{S. pneumoniae}, transconjugants harboring plasmids with either ssA intact accumulated ssDNA intermediates in amounts similar to pMV158wt, which were very low and only detectable after long exposures (Fig. 3A, R gels). These findings demonstrate that both origins were equally functional in \textit{S. pneumoniae}, in agreement with the conjugation frequencies observed (Fig. 2), allowing us to conclude that both lagging–strand origins supported postconjugative conversion of ssDNA to dsDNA in \textit{S. pneumoniae} with similar levels of efficiency. However, cells harboring pMV158G3G7BD exhibited a 3 to 4-fold reduction in copy number (from 30 to 8 to 10 copies per genomic equivalent, see below) and the ss/ds DNA ratios increased from nearly undetectable for pMV158wt to large amounts (Fig. 3B, R gels). A very different picture was observed for \textit{E. faecalis} transconjugants (Fig. 4). In this case, it was apparent that cells harboring pMV158ABD (defective in ssU) accumulated substantially increased levels of ssDNA (Fig. 4A, R gels). This amount was similar to that found for plasmids with both origins inactivated (pMV158G3G7BD; Fig. 4B, R gels), indicating that the ssU was the only highly efficient functional origin in the enterococcal host. Comparative quantifications of plasmid copy numbers and ssDNA/dsDNA ratios were calculated from determination of the plasmid copy numbers and of the radioactivity counted in the different plasmid bands. A parameter, termed the accumulation coefficient (AC), was introduced to calculate the relationship between the ssDNA/dsDNA ratios of the different ss mutants with respect to the ssDNA/dsDNA ratio of pMV158wt (Table 2). In the pneumococcal transconjugants, a fourfold reduction in plasmid copy number was found only for pMV158G3G7BD concomitant with a strong (40-fold) increase in the AC ratio. No significant variations in copy numbers were observed for plasmids defective in either ssA, although a slight increase in the AC ratios was detected (Table 2). In the case of the \textit{E. faecalis} transconjugants, the eightfold reduction in plasmid copy numbers was greater than the values determined for pneumococci, although the number of copies of pMV158wt was lower in enterococci (around 17 copies per genome equivalent) than in pneumococci (around 30 copies). Furthermore, the AC is the relationship between mutant and wild-type ssDNA/dsDNA molecular ratios.

### Table 2. Copy number, molecular ssDNA/dsDNA ratios, and ACα

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>\textit{S. pneumoniae} MP3008</th>
<th>\textit{E. faecalis} OG1RF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Copy no.</td>
<td>ssDNA/dsDNA ratio</td>
</tr>
<tr>
<td>pMV158</td>
<td>31 ± 5</td>
<td>0.007 ± 0.0003</td>
</tr>
<tr>
<td>pMV158G3G7</td>
<td>33 ± 6</td>
<td>0.012 ± 0.0006</td>
</tr>
<tr>
<td>pMV158ABD</td>
<td>32 ± 4</td>
<td>0.009 ± 0.0007</td>
</tr>
<tr>
<td>pMV158G3G7BD</td>
<td>8 ± 1</td>
<td>0.31 ± 0.05</td>
</tr>
</tbody>
</table>

αThe copy number was measured as the plasmid dsDNA per genomic equivalent. Copy numbers and ratios are expressed as means ± the standard deviation. The AC is the relationship between mutant and wild-type ssDNA/dsDNA molecular ratios.

![FIG. 3. Accumulation of intracellular ssDNA intermediates in \textit{S. pneumoniae} MP3008 carrying pMV158 wild-type (wt) or derivatives with mutations in ssA (G3G7) or in ssU (ΔBD) (A) and mutations in both origins (B). Total DNA was prepared from plasmid-containing pneumococcal cultures and the different forms were separated by electrophoresis on 0.7% agarose gels in 1× Tris-borate-EDTA buffer with 0.5 μg of ethidium bromide/ml (L, left). After denaturation, the DNA was transferred to nylon membranes and hybridized with a 32P-labeled probe (R, right). The various DNA forms are indicated: chromosomal (chr), open circle (oc), circular covalently closed supercoiled (ccc), and ssDNA intermediates.](http://jb.asm.org/ on September 22, 2017 by guest)
a severe drop in copy numbers (from 17 to 4) was measured for plasmid pMV158ΔBD, with a further twofold reduction when neither plasmid origin was functional (pMV158G3G7ΔBD; Table 2). Decreases in the AC ratios show that sso4 might still be partially functional (AC ratio of 2 for pMV158G3G7) but not so for ssoU (AC ratio of ~14 for plasmid pMV158ΔBD). The null mutant for both sso’s (pMV158G3G7ΔBD) exhibited a further twofold reduction in the AC ratio. These results are consistent with the observed drop in the conjugation frequencies from S. pneumoniae to enterococcal cells (Fig. 2). From these results we may draw the following conclusions: (i) in S. pneumoniae, both ssoA and ssoU are equally functional and can replace each other, and (ii) in E. faecalis, ssoU is the main origin used by the plasmid, but there is a partial functionality of ssoA, as shown by further reductions in copy numbers and AC coefficients when the plasmid bears mutations in both origins.

DISCUSSION

Our understanding of a successful conjugative transfer of pMV158 not only contemplates that the transferring ssDNA molecule (the T-DNA) physically penetrates the recipient cell but also implies an efficient establishment in the new host. In this sense, after recircularization of the T-DNA by the strand transfer activity of the MobM relaxase, efficient conversion of ssDNA to dsDNA would be critical for the process to reconstitute the pMV158 plasmid that would undergo vegetative replication, repopulation of plasmid molecules, and antibiotic resistance expression in the recipient cells. Thus, the sso’s would be the first elements participating into the transfer process within the recipients. To analyze the sso functionality on replicative transfer and/or on vegetative replication, we have taken into account three parameters: (i) plasmid transfer frequencies, (ii) copy numbers, and (iii) ssDNA accumulation of the wild-type and derivatives of pMV158. From the results obtained we can conclude that both pMV158-ssos could support “postconjugative” conversion of ssDNA to dsDNA in S. pneumoniae but not in E. faecalis. Here, we have conscientiously used the term “postconjugative” to include both events, conjugative and vegetative lagging-strand replication, because they are mechanistically identical. In the case of transfer between pneumococci, either origin was functional, whereas deletion of ssoU in plasmids with an intact ssoA (pMV158ΔBD) resulted in a 60-fold reduction in the interspecies transfer frequency. The sso4 origins seem to function efficiently only in the plasmid natural host, and it was hypothesized that specific host factors may be required for the sso4 functionality present in their native hosts and the absence of such factors would be responsible for their poor functionality in heterologous hosts (9). However, while pMV158-ssoa was not functional in S. aureus cells, it supported lagging-strand synthesis on staphylococcal cell extracts, pointing to a problem of efficiency rather than to a lack of specificity of the initiation process (25, 26). Thus, an efficient RNAP-ssoa interaction could be a determinant of the host specific functionality of the sso4-type origins. In the case of ssoU, it would appear that its ability to interact efficiently with RNAPs from various hosts could provide these plasmids with an expanded host range. The mobilization cassette (oriT and gene mobM) of the pMV158 derivatives used here was intact. If transfer is independent of plasmid copies in the donor, then the number of mobilization events from S. pneumoniae donors to the recipient cells should be similar, independent of the activity of the sso. The transferred molecules (as ssDNA) of a plasmid lacking a functional sso would not support conversion to dsDNA or would do so very poorly, thus compromising its establishment in the new host because of its low copy number prior to cell division, since the distribution of RCR plasmid copies is based on random events (8). Alternatively, it could be that plasmid copy number in the donors may affect the number of transfer events. If this were the case, plasmids replicating with a similar efficiency in S. pneumoniae, such as pMV158ΔBD and pMV158wt (Table 2), should maintain a number of transfer events comparable to those of the recipients. However, the frequency of transfer of pMV158ΔBD to E. faecalis was significantly lower than those of pMV158wt and pMV158G3G7 (Fig. 2), demonstrating that the absence of the ssoU signal is essential for a successful conjugation. Plasmid pMV158G3G7ΔBD (with nonfunctional sso) transferred very poorly and accumulated ssDNA (30- to
that, whereas pneumococcal intraspecies transfer was equally efficient, provided that the plasmid bears a functional sso, it was not the case for interspecies transfer from pneumococci to enterococci, where a strong dependence of an intact ssoU is essential for pMV158 propagation. Since the ssoU functions efficiently in both hosts, this origin seems to be an important determinant for the promiscuity of pMV158. However, other factors may contribute to the extraordinary host range of this plasmid (it has been established in more than 20 different hosts thus far). Stable inheritance of a plasmid after colonizing a new host does not necessarily need a functional lagging-strand origin (19), so that establishment of a plasmid bearing a functional ssoU does not seem to be enough for productive replication in S. pneumoniae. This view is supported by the following: (i) a derivative of plasmid pVA380-1 (isolated from Streptococcus fereus and carrying a kanamycin gene [30, 31]) could be easily established in pneumococci, where it stably replicates with a high copy number (15); and (ii) attempts to transfer the staphylococcal RCR plasmid pUB110 (which bears an ssoU identical to that of pMV158, a highly homologous mob cassette and a kanamycin resistance gene) to S. pneumoniae, have failed (our unpublished observations). Thus, we have invoked the fitness and/or adaptation of the bacterium-plasmid pair as one of the main reasons for the plasmid broad host range (6).

The current model for conjugation predicts that, at least in small RCR plasmids such as pMV158, reconstitution of a dsDNA plasmid after transfer takes place by synthesis of a RNA starting at one of the plasmid sso’s by a mechanism equivalent to the vegetative plasmid lagging-strand replication (Fig. 5). Therefore, we could predict that plasmids with non-functional sso’s for vegetative replication should not be efficient in conjugal transfer and vice versa. The results obtained for the plasmid lacking both sso’s (pMV158G3G7BD) corroborated this assumption, since vegetative replication and conjugal transfer were negatively affected, independently of the host tested. If pMV158 colonizes a new pneumococcal host by the transformation of competent cells, its DNA should be taken up as ssDNA segments by the degradative process of DNA transport (32). Thus, intracellular reconstitution of an intact plasmid molecule by DNA synthesis and/or recombination is followed by vegetative leading-strand replication from the dso by the RCR mechanism. Entry of plasmid DNA by electrotransformation (in a dsDNA conformation), as in E. faecalis, would also result in vegetative leading-strand replication. However, colonization of a new host by conjugation would imply Mob-mediated closing of a full-length ssDNA intermediate followed by (or simultaneously with) lagging-strand (postconjugative) replication from the sso. If this kind of replication were independent of the sso efficiency, we should expect the same conjugation frequencies for plasmids with or without defects in the sso. However, no increased numbers of transconjugants were obtained in any case by prolonged incubation times, which mirrors a direct effect of ssoU functionality on plasmid transfer. In addition, we have analyzed the transformation efficiency by electroporation (where plasmid DNA enters as a double strand) of E. faecalis OG1RF with pMV158 and sso mutants. The results showed no differences in the number transformed colonies recovered with pMV158ΔBD and pMV158, although the colony size of the former was smaller and the plasmid copy number was lower, as expected. Thus, it could be envisaged that the role of the sso on plasmid establishment may be more relevant in the first stages of plasmid colonization of a new host via conjugal transfer.

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