Lagging strand DNA replication origins are required for conjugal transfer of the promiscuous plasmid pMV158

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Short title: Transfer of pMV158
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 inter-species mobilization. Whereas either sso supported transfer between strains of *Streptococcus pneumoniae*, only those plasmids 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.

Key words: lagging-strand origins; plasmid mobilization; plasmid transfer; pMV158; pneumococci; enterococci
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 (G+) bacteria, two pioneer findings led to the discovery of the rolling circle mechanism of replication (RCR-plasmids; reviewed in (20, 21). Firstly, the strand-specific single-stranded DNA (ssDNA) molecules which act as replication intermediates were identified (41), and secondly 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 initiator, but also a Mob protein with relaxase activity involved in mobilization mediated by auxiliary plasmids. Such is the case of the promiscuous plasmid pMV158 which can be mobilized between various bacterial species by the pMV158-encoded MobM protein and by helper conjugative plasmids belonging to the Inc18 plasmid family, like pAMβ1 (15) or even by IncP plasmids, like RP4 (11). The relaxing activity of MobM on supercoiled DNA of pMV158 and the site of cleavage were first demonstrated in vitro (5, 17) and later the same activity of the MobA protein of the Staphylococcus aureus RCR-plasmid pC221 was demonstrated (3, 39).

Initiation of transfer, like initiation of RCR, involves cleavage of the phosphodiester bond of a specific di-nucleotide 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 lagging-strand 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 Pol 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_{rpo}, 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). Presence of ssDNA promoters have also been shown in plasmids pMV158 (27) and ColI-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 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, ssoA and ssoU (23). Two conserved regions were found in the ssoA of pLS1 plasmid (a non-mobilizable pMV158-derivative lacking ssoU): a short region termed recombination site B, RS_B, supposedly involved in plasmid cointegration (16, 38), and a 6-nt consensus sequence (5'-TAGCGT-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 long pRNA in the loop of the hairpin (27). The predicted intrastrand pairings in the pMV158-ssoA showed the presence of a 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'-TAcgcT-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 the pMV158-ssoU (24).

In the present work we have addressed the question of whether and, eventually, which of the two pMV158-ssos plays a role in conjugal transfer. With this objective, we constructed pMV158-derivatives defective in one or both sso and tested their role on intra- and inter-species 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 G+ bacteria for the pMV158 promiscuous plasmid, and demonstrate that efficient transfer and replication in enterococci depend upon a functional ssoU. We suggest that those
ssos lacking functionality for vegetative replication in a specific host should not be efficient in conjugative transfer and viceversa, 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 (Pronadisa, Spain) media and growth conditions for S. pneumoniae and E. faecalis have been described (28, 33). Competent S. pneumoniae 708 cells bearing plasmid pAMβ1 were transformed with DNA from pMV158wt and its derivatives as reported (10). These strains were used as donors in filter-mating experiments. To construct S. pneumoniae MP3008, the novobiocin-resistant (Nov R) 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 (Nov R) and E. faecalis OG1RF (resistant to rifampicin, Rif R) were employed 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 (9). Selection for plasmids pAMβ1 and pMV158 was 1 μg/ml erythromycin (Em) and 1 μg/ml tetracycline (Tc) in S. pneumoniae and 1 μg/ml Em and 4 μg/ml Tc in E. faecalis. Purified pMV158 plasmid DNA was prepared by two consecutive CsCl/ethidium bromide gradients as described (7). Plasmid pMV158ΔBD, a derivative of pMV158 lacking the ssoU was constructed
by deletion of a 205-bp *Bsa*-*Dra* I DNA fragment (coordinates 3223 to 3428 of pMV158; see Fig. 1). To generate pMV158-derivatives in the *ssoA*, the small 1132-bp *Eco* RI fragment from pMV158 was cloned into plasmid pLS1G3G7 carrying nucleotide changes in the RS₈ and CS-6 conserved sequences of the *ssoA* (27); see Fig. 1). The resulting plasmid, pMV158G3G7, was functionally defective in the *ssoA*. Similarly, the small *Eco* RI restriction fragment from pMV158ΔBD (927-bp) was cloned into pLS1G3G7 thus generating pMV158G3G7ΔBD, with defective functionality of both *sso*. All constructions were rescued by transformation of competent pneumococcal cells and the mutations were confirmed by sequencing with specific primers. U1 (GGGATCAACTTTGGGAGAGA) and U2 (GCGTCTCAAAAAACACGTCCA) were employed to confirm the *ssoU* deletion, A1 (TCACAACGCTCACCTCCA) was used to confirm the G3G7 mutations of *ssoA* and U1 and M1 (AAAGCACCCCTCACATGC) were used to confirm the orientation of the small *Eco* RI fragment (see Figure 1A).

Filter mating experiments. Mobilization assays of pMV158 and its derivatives from *S. pneumoniae* donor cells harbouring pAMβ1 as auxiliary plasmid were performed as described (37) with minor modifications. Donor and recipient cultures were grown without aeration at 37 °C to 5 x 10⁸ cells/ml. Cells were centrifuged and resuspended in pre-warmed AGCH medium supplemented with 10 mM MgCl₂, 2mg/ml bovine serum albumin and 100 units of DNase I. Donor/recipient mixtures (1/5 ratios) were filtered onto sterile 25-mm nitrocellulose filters (0.22 µm). Then filters were placed cell-side down over another filter previously placed on a plate with conjugation medium (AGCH with 10 mM MgCl₂, 2mg/ml bovine serum albumin and 2% agar). After 4 h incubation at 37 °C, 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 0.22 µm nitrocellulose filter (Millipore), filtered and incubated for 4 hours at 37 ºC. With this device, 8 transfer experiments were done for each condition, thus obtaining a high degree of reproducibility and enough repetitions in the same experiment. Transconjugants were selected on AGCH medium with Tc (1 µg/ml), Nov (10 µg/ml), 0.3% maltose, and 1.5% agar for *S. pneumoniae* MP3008 or on ESTY plates with Tc (4 µg/ml), 0.3% glucose, and 1.5% agar for *E. faecalis* OG1RF. After serial dilutions, the number of colony-forming units (cfu) of recipient strains was calculated in the same media but without selection for Tc-resistance. Conjugative mobilization efficiencies were calculated as the number of transconjugant cells per recipient.

**Determination of plasmid copy number and intracellular ssDNA accumulation.**

Total DNA preparations from cultures harbouring plasmids (28) were loaded on 0.7% agarose gels in Tris/borate buffer containing 0.5 µg/ml ethidium bromide. After electrophoresis, plasmid copy number was determined by fluorescence densitometry with the Gel-Doc system and QuantityOne software (BioRad). The DNAs in the same gels were denatured and transferred to positively-charged nylon membranes (Roche), instead of the nitrocellulose ones used earlier (9, 41) since the nylon membranes offered superior performance. Membranes were hybridized with a specific ³²P-labelled PCR-DNA probe (coordinates 609 to 924 of pMV158), amplified with P1 (GCACGGTTATGCTACT) and P2 (CAGCTCCCAGTCGCTT) primers. The total amount of ssDNA and dsDNA were quantified with Phosphorimager equipment and using ImageQuant software (Molecular Dynamics).
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 ssos in such a way that, during conjugation, the ssoA, generated in the transferred ssDNA, enters the recipient cell first, whereas the ssoU is the last plasmid region transferred (Fig. 1A, see Fig. 5). Computer-assisted and structural analyses showed that both ssos could generate complex secondary structures on the ssDNA intermediates by intrastrand pairing. In these stem-loop structures the consensus sequences RS_B (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 the ssoA, a single hairpin would be generated, whereas up to five hairpins may be formed in the region encompassing the ssoU (Fig. 1A). Homologies between the two lagging strand origins were observed especially around the RS_B conserved sequences, homologies that were partially maintained when the four types of sso reported so 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 ssoU could be involved in the determination of the broad host range of ssoU-containing RCR-plasmids thus contributing to the horizontal spread 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 the \textit{ssoA}, a 6-nucleotide change was introduced in the unpaired RS\textsubscript{B} sequence (mutation G7), as well as another 9-nucleotide change 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 the \textit{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 RS\textsubscript{B}) and affected termination of the pRNA (changes at CS-6) (27). The resulting mutant plasmid, pMV158G3G7 (Fig. 1B and Table 1) has a non-functional \textit{ssoA}. In the case of the pMV158-\textit{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 pMV158\textDelta BD, lacks the two most important sequences (RS\textsubscript{B} and CS-6) within the \textit{ssoU}. Finally, a derivative affected in both origins was also generated (plasmid pMV158G3G7\textDelta BD). The wild type (wt) pMV158 and its three derivatives were rescued in competent \textit{S. pneumoniae} cells, and the mutations confirmed by sequencing of the affected regions.

**Defects in \textit{sso} functionality lead to reduced mobilization frequency.** To analyze the usage of the \textit{ssos} during conjugative mobilization, each of the plasmids (pMV158wt and its derivatives) were next transferred to competent \textit{S. pneumoniae} 708 cells carrying pAMβ1 (the plasmid providing the auxiliary functions for conjugation), and intra- and interspecific conjugal transfer assays were performed using \textit{S. pneumoniae} MP3008 (Nov\textsuperscript{R}) or \textit{E. faecalis} OG1RF as recipients. To have several transfer experiments with high reproducibility, we made use of a recently developed multiwell-plate set-up coupled with a filter device so that up to 8 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 intraspecies transfers were assayed between *S. pneumoniae* strains, it was evident that plasmids lacking both *sso* 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 the *ssoA* or the *ssoU* (Fig. 2). These findings indicate that in the pneumococcal host, both pMV158-*sso* 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 latter bacteria being aerophilic. 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 those observed for transfer between pneumococci or even higher, that is in the order of $10^{-4}$ transconjugants per ml of recipient cells (Fig. 2). 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 non-functional *ssoA* (pMV158G3G7; Fig. 2). Other mutations in the *ssoA* were also tested (i.e. mutations in the CS-6 or in the RS₃) 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 (near 60-fold).
when the plasmid tested lacked both origins (pMV158G3G7ΔBD; Fig. 2). We conclude that, in *E. faecalis*, there is a strong preference for employment of the *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 (not shown). Further, the ss/dsDNA ratio, which indicates efficient replication, was the same for the two bacterial hosts tested here (see 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 harbouring pMV158wt or its derivatives were selected and grown for 30 generations (the minimum period of time for a colony to become a full-grown liquid culture; ref. 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 Figs. 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’ in Figs. 3 and 4). In the case of *S. pneumoniae*, transconjugants harbouring plasmids with either *sso* intact accumulated ssDNA intermediates in amounts similar to pMV158wt, which were very
low and only detectable after long exposures (Fig. 3A, R). These findings
demonstrate that both origins were equally functional in *S. pneumoniae*, in agreement
with the conjugation frequencies observed (Fig. 2), allowing us to conclude that both
lagging–strand origins supported post-conjugative conversion of ssDNA to dsDNA in
*S. pneumoniae* with similar levels of efficiency. However, cells harbouring
pMV158G3G7ΔBD exhibited a 3-4 fold reduction in copy number (from 30 to 8-10
copies per genomic equivalent, see below) and the ss/ds DNA ratios increased from
nearly undetectable for pMV158wt to large amounts (Fig. 3B, R). A very different
picture was observed for *E. faecalis* transconjugants (Fig. 4). In this case, it was
apparent that cells harbouring pMV158ΔBD (defective in the *ssoU*) accumulated
substantially increased levels of ssDNA (Fig. 4A, R). This amount was similar to that
found for plasmids with both origins inactivated (pMV158G3G7ΔBD; Fig. 4B, R),
indicating that the *ssoU* was the only highly efficient functional origin in the
enterococcal host.

Comparative quantifications of plasmid copy numbers and ss/ds DNA ratios
were calculated from determination of the plasmid copy numbers and of the
radioactivity counted in the different plasmid bands. A parameter, termed
“accumulation coefficient” (AC), was introduced to calculate the relationship between
ss/ds DNA ratios of the different *sso*-mutants with respect to the ss/ds DNA ratio of
pMV158wt (Table 2). In the pneumococcal transconjugants, a fourfold reduction in
plasmid copy number was found only for pMV158G3G7ΔBD concomitant with a
strong (40-fold) increase in the AC ratio. No significant variations in copy numbers
were observed for plasmids defective in either *sso*, although a slight increase in the
AC ratios were detected (Table 2). In the case of the *E. faecalis* transconjugants, the
eight-fold reduction in plasmid copy numbers was greater than the values determined for pneumococci, although number of copies of pMV158wt was lower in enterococci (around 17 copies per genome equivalent) than in pneumococci (around 30 copies). Furthermore, 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 the ssoA might still be partially functional (AC ratio of 2 for pMV158G3G7), but not so for ssoU (AC ratio ~ 14 for plasmid pMV158ΔBD). The null-mutant for both sso (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*, the ssoU is the main origin employed by the plasmid, but there is a partial functionality of the 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 re-circularization of the T-DNA by the strand-transfer activity of the MobM relaxase, efficient conversion of ss- 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 ssos 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 “post-conjugative” conversion of ssDNA to dsDNA in S. pneumoniae, but not in E. faecalis. Here, we have conscientiously used the term “post-conjugative” 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 the ssoU in plasmids with an intact ssoA (pMV158ΔBD) resulted in a 60-fold reduction in the interspecies transfer frequency. The ssoA origins seem to function efficiently only in the plasmid natural host, and it was hypothesized that specific host factors may be required for the ssoA-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 the pMV158-ssoA was not functional in S. aureus cells, it supported lagging strand synthesis on staphylococcal cell-free extracts, pointing to a problem of efficiency rather than to 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 ssoA-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, independently 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 previous to cell division, since 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 comparable number of transfer events to 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 non-functional sso) transferred very poorly and accumulated ssDNA (30- to 40-fold) in both *E. faecalis* and *S. pneumoniae*. Thus, it appears 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. As 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 so 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 is supported by: i) a derivative of
plasmid pVA380-1 (isolated from *S. ferus* 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 a *ssoU* identical to that of pMV158, a highly homologous *mob* cassette
and a kanamycin-resistance gene) to *S. pneumoniae* which have failed (our
unpublished observations). Thus, we have invoked fitness/adaptation of the bacterial-
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
like pMV158, reconstitution of a dsDNA plasmid after transfer takes place by
synthesis of a pRNA starting at one of the plasmid *sso* by a mechanism equivalent to
the vegetative plasmid lagging strand replication (Fig. 5). Therefore, we could predict
that plasmids with non-functional *sso* for vegetative replication should not be
efficient in conjugative transfer and *viceversa*. The results obtained for the plasmid
lacking both *sso* (pMV158G3G7ΔBD) corroborated this assumption, since vegetative
replication and conjugative transfer were negatively affected, independently of the
host tested. If pMV158 colonizes a new pneumococcal host by 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
electro-transformation (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 simultaneous to) lagging-strand (post-conjugative) replication from the
*sso*. If this kind of replication were independent of the *sso* efficiency, we should
expect the same conjugation frequencies for plasmids having or not defects in the
sso. However, no increased numbers of transconjugants were obtained in any case
by prolonged incubation times, which mirror a direct effect of ssoU functionality on
plasmid transfer. In addition, we have analyzed the transformation efficiency by
electroporation (where plasmid DNA enters as 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 conjugative
transfer.

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**LEGENDS TO THE FIGURES**

**Fig. 1** Features of pMV158 and its two lagging strand origins. **A.** Schematic map of the plasmid transfer module indicating relevant restriction sites and the relative positions (shadowed) of the two lagging-strand origins of replication (ssoA and ssoU). Plasmid-encoded MobM protein (arrow below the map) and the position of the oriT are depicted. Direction of DNA transfer is indicated. The *Eco*RI fragment deleted to construct the pLS1 derivative (28) and the positions of primers used are also shown. Representation of the secondary structures of ssoA (left) and of ssoU (right), indicates the positions of the CS-6 and the RS_B regions (boxed). The location of the G3 and G7 mutations in the ssoA and of the restriction sites used to generate deletions in the ssoU are shown. Start point and direction for the RNA primer (pRNA) synthesis, downstream to CS-6 sequence, is indicated by a wavy arrow. **B.** Relevant sequence features of the two pMV158 sso. The RS_B and CS-6 sequences are shown in boxes. Restriction sites of *Bsal* and *Dral* used to generate ssoU-ΔBD mutant are also indicated as well as the nucleotides changed (boldface) to construct the ssoA-G3G7 mutant (sequence indicated beneath).

**Fig. 2** Transfer frequencies of pMV158 and derivatives from *S. pneumoniae* harbouring pAMβ1 as donor cells to *S. pneumoniae* and to *E. faecalis* as recipient cells. Donor and recipient cells were incubated together on nitrocellulose filters (0.22 µm) at a ratio 1:5, for 4 hours at 37°C. The transfer frequencies (plotted in logarithmic scale) were calculated as the number of transconjugants colony-forming units per ml of recipient cells. The bar above each column indicates the standard error of 8 independent experiments in a 96-well filter plate. The reduction in transfer
frequencies of plasmids with mutations in the ssoU relative to frequencies of pMV158wt from S. pneumoniae to E. faecalis were statistically significant (P < 0.001).

Number of colonies of S. pneumoniae MP3008 and E. faecalis OG1RF recipients were 5.5x10^8 and 3.2x10^8 CFU per filter, respectively.

**Fig. 3** Accumulation of intracellular ssDNA intermediates in S. pneumoniae MP3008 carrying pMV158 wild type (wt) or derivatives with mutations in the ssoA (G3G7) or in the ssoU (ΔBD) (panel A), and mutations in both origins (panel B). Total DNA was prepared from plasmid-containing pneumococcal cultures and the different forms were separated by electrophoresis on 0.7% agarose gels in 1xTBE buffer with 0.5 µg/ml ethidium bromide (L, left). After denaturation, the DNA was transferred to nylon membranes and hybridized with a ³²P-labelled probe (R, right). The various DNA forms are: chr, chromosomal; oc, open circles; ccc, circular covalently closed supercoiled, and ssDNA intermediates.

**Fig. 4** Intracellular ssDNA accumulated in E. faecalis OG1RF transconjugants harbouring wild type or mutations in the ssoA (G3G7) or in the ssoU (panel A) and mutations in both origins (panel B). Total DNA prepared from enterococcal cells was treated as in Fig. 3.

**Fig. 5** Proposed model for reconstitution of pMV158 dsDNA after transfer. After nicking by the MobM relaxase, the cleaved strand is piloted by MobM into the recipient cell. The ssoA lagging-strand enters first and as ssDNA undergoes intramolecular pairing and then acts as a conversion signal for the host-recipient RNAP to synthesize the pRNA which initiates lagging-strand replication. If the
recipient RNAP does not recognize efficiently the ssoA (crossed), then only when the
entering ssoU is properly folded, may the synthesis of the pRNA take place.
Table 1. Bacterial strains and plasmids used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotypea</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. pneumoniae</em> 708</td>
<td>end-1, exo-1, trt-1, hex-4, malM594</td>
<td>(28)</td>
<td></td>
</tr>
<tr>
<td><em>S. pneumoniae</em> MP517</td>
<td>end-1, exo-1, trt-1, hex-4, malM594; NovR</td>
<td>Lab collection</td>
<td></td>
</tr>
<tr>
<td><em>S. pneumoniae</em> MP3008</td>
<td>end-1, exo-1, trt-1, hex-4; NovR</td>
<td>This work</td>
<td></td>
</tr>
<tr>
<td><em>E. faecalis</em> OG1RF</td>
<td>Gel+, Spr+ (gelatinase and serine protease production positive)</td>
<td>A gift of D. Le Blanc</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Size (bp)</th>
<th>Featuresa</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAMβ1</td>
<td>26500</td>
<td>Conjugative Inc18 plasmid identified in <em>E. faecalis</em> DS5; EmR</td>
<td>A gift of L. Janniere</td>
</tr>
<tr>
<td>pMV158</td>
<td>5540</td>
<td>Isolated from a clinical isolate of <em>S. agalactiae</em> MV158; TcR</td>
<td>(2)</td>
</tr>
<tr>
<td>pLS1G3G7</td>
<td>4408</td>
<td>ssoA mutant; non-mobilizable pMV158 derivative (ΔmobM, ΔssoU); TcR</td>
<td>(27)</td>
</tr>
<tr>
<td>pMV158G3G7</td>
<td>5540</td>
<td>ssoA mutant; changes in RS_B (6-nt) and CS-6 (9-nt) sequences; TcR</td>
<td>This work</td>
</tr>
<tr>
<td>pMV158ΔBD</td>
<td>5335</td>
<td>ssoU mutant; deletion of 205-bp <em>Bsal-Dral</em> fragment (RS_B-, CS-6-); TcR</td>
<td>This work</td>
</tr>
<tr>
<td>pMV158G3G7ΔBD</td>
<td>5335</td>
<td>Double ssoA-ssoU mutant; contains ΔBD deletion and the G3G7 mutation; TcR</td>
<td>This work</td>
</tr>
</tbody>
</table>

EmR, NovR, TcR: resistance to erythromycin, novobiocin and tetracycline, respectively.
Table 2. Copy number, molecular ratios of ss/ds DNA and accumulation coefficient (AC).

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>S. pneumoniae MP3008</th>
<th>E. faecalis OG1RF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Copy number&lt;sup&gt;a&lt;/sup&gt;</td>
<td>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>pMV158ΔBD</td>
<td>32±4</td>
<td>0.009±0.0007</td>
</tr>
<tr>
<td>pMV158G3G7ΔBD</td>
<td>8±1</td>
<td>0.31±0.05</td>
</tr>
</tbody>
</table>

<sup>a</sup> Measured as ds plasmid DNA per genomic equivalent.

<sup>b</sup> Relationship between mutants and wild-type ss/ds DNA molecular ratios.