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Mobilizable Rolling-Circle Replicating Plasmids from Gram-Positive Bacteria: A Low-Cost Conjugative Transfer

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Chapter summary

Conjugation is a key mechanism for horizontal gene transfer in bacteria. Some plasmids are not self-transmissible but can be mobilized by functions encoded *in trans* provided by other auxiliary conjugative elements. Although the transfer efficiency of mobilizable plasmids is usually lower than that of conjugative elements, mobilizable plasmidsare more frequently found in nature. In this sense, replication and mobilization can be considered as important mechanisms influencing plasmid promiscuity. Here we review the present available information on two families of small mobilizable plasmids from Gram-positive bacteria that replicate via the rolling-circle mechanism. One of these families, represented by the streptococcal plasmid pMV158, is an interesting model since it contains a specific mobilization module (MOB_V) that is widely distributed among mobilizable plasmids. We discuss a mechanism in which the promiscuity of the pMV158 replicon is based on the presence of two origins of lagging strand synthesis. The current strategies to assess plasmid transfer efficiency as well as to inhibit conjugative plasmid transfer are presented. Some applications of these plasmids as biotechnological tools are also reviewed.

1. Introduction

Bacteria are everywhere simply because they can colonize and adapt to different ecological niches in a very short-term period. One important molecular mechanism underlying the abilities of bacteria to colonize new niches is the acquisition of novel traits by conjugative DNA transfer. Under these circumstances, the so-called ‘variable genome’ (as opposed to the ‘core genome’), which encodes an array of accessory functions (such as antibiotic-resistance, specific degradation pathways, symbiosis, and virulence, to name a few), is freely exchanged among bacteria (1). These newly acquired DNA pieces are represented by intra- or extra-chromosomal elements, which may or may not have self-replication and/or auto-transferable capacities. However, all of them participate in the fitness of the bacteria to colonize and to adapt to new niches; thus they contribute to create new evolutionary patterns

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(2). Mobile genetic elements (MGEs) constitute a reservoir of DNA that is shared among bacterial species (3) and being so, they contribute to the virulence and to the colonization of different niches by their bacterial hosts. Among MGEs, bacterial plasmids play a key role in horizontal gene transfer (HGT) and thus are important in the co-evolution and fitness of the bacterial/plasmid pair. Bacterial conjugation (described in depth elsewhere in this book) involves the unidirectional transfer of plasmid DNA from a donor to a recipient cell through physical contact (4). In the donor cell, the pre-requisite for transfer is the assembly of the plasmid-encoded relaxase and other plasmid- or host-encoded proteins on a specific *cis*-acting DNA plasmid region, the origin of transfer (*oriT*). This protein-DNA complex, the relaxosome, initiates the DNA transfer to the recipient cell by the relaxase-mediated cleavage of a phosphodiester bond at a specific dinucleotide (the nick site) within the *oriT* (5). It has been proposed that the relaxosome is already preformed on supercoiled DNA even before the transfer signals reach the donor-recipient cell pair (6). However, this hypothesis poses a yet unsolved question when the plasmid replicates by the rolling circle (RC) mechanism: in these RC-replicating (RCR) plasmids, initiation of replication and initiation of conjugative transfer are exerted by two different plasmid-encoded initiation proteins: Rep in the case of replication, and Mob for transfer (7). Each of these proteins recognizes a different origin on the plasmid DNA (Rep recognizes the origin of double-stranded replication, *dso*, whereas Mob recognizes the origin of transfer *oriT*); both proteins require that their DNA substrate is super coiled (7–9). If the relaxosome was already preformed, the plasmid DNA would be relaxed and the Rep-initiator could not recognize its cognate *dso*. As a result, the plasmid could not replicate. On the other hand, if the Rep-initiator nicks the plasmid DNA to initiate the replication, the relaxosome could not be formed and thus transfer will be hindered.

The initiation of transfer involves a Mob relaxase-mediated DNA cleavage at the *oriT*, the result of it being one covalent amino acyl-DNA adduct which would be actively pumped into the recipient cell by the plasmid-encoded coupling protein (CP) and the transferosome, a Type IV Secretion System (T4SS; (10–13). Thus, like almost all the nucleotidyl transferase cation-dependent enzymes, relaxases leave a free 3'-OH end while the protein remains stably bound to the 5'-phosphate product. Once in the recipient, the relaxase–DNA intermediate would restore the original circular plasmid molecule after termination of transfer by a reversion of the strand transfer reaction. Such reactions resemble the RCR termination mechanism elegantly solved in the Novick's lab (reviewed in (14), although no indication of one modified relaxase with a short oligonucleotide covalently bound to it (as the by-product of the termination reaction) has been shown for conjugation (see (8, 14) for a detailed explanation of the termination mechanism). The last stage of transfer, the conversion of the single-stranded (ss) DNA intermediates into double-stranded (ds) plasmid molecules would take place in the recipient cell by conjugative lagging strand replication through transcription of a small primer RNA (15–18).

Since the *oriT* is the only *cis*-acting element required for DNA transfer (6), every *oriT*-containing plasmid should be transferred by conjugation if the protein resources (relaxase, CP, and T4SS) are available *in trans* from any compatible self-transferable element, a phenomenon defined as *mobilization*[1]. This interaction is therefore an important factor that

can determine the frequency with which a given mobilizable element and its associated genes are involved in HGT (19). In general, self-transferable elements are transferred at higher frequencies than their mobilizable partner elements (20–21). A wide variety of mobilizable elements harbouring the *oriT* and the relaxase-and CP-codifying genes have been found (reviewed in (20–21). Further, many small plasmids contain a single gene cassette (*oriT* and relaxase-encoding gene) that allows them to be mobilized with the aid of the machinery provided by helper (auxiliary) plasmids. This is the case of many small RCR-plasmids from Gram-positive (G+) bacteria that can be mobilized by their Mob relaxase when they co-reside with an auxiliary self-transferable element (22–24). Whether the relationship between mobilizable and conjugative elements is considered as parasitic or altruistic is arguable, it seems reasonable to propose mobilization as a strategy to travel around the microbial world at low cost.

2. Nature and Diversity of Mobilizable Elements

Many MGEs share the ability to be transferred by conjugation between bacteria when they co-reside with a compatible auto-transferable element in the donor cell. Furthermore, due to their modular structure and their dynamic genetic nature, any MGE can be considered as a platform where new events of bi-directional mobilization/integration (in and out of the MGE) of other gene cassettes can occur, making it difficult to determine its original genomic location. Since the discovery of the first mobile element in 1953 (25), the diversity of the entire mobilome that one could expect in nature has been found to be very rich (21, 26). Several aspects can be considered to study the diversity of mobilizable elements, leaving aside the bacteriophages. Depending on the location of the mobile elements, they can be classified into extra- (plasmids), or intra-chromosomal elements. In the former case, they constitute the so-called plasmidome (27), whereas within the MGEs having an intra-chromosomal location two categories can be distinguished: i) Integrative and Mobilizable Elements (IMEs; (28), and ii) Mobilizable Conjugative Transposons (MCTn), which usually show non-specific integration/excision (29). The adaptive functions provided by the MGE are important because a large number of MGEs encode one or several genes involved in resistance to antibiotics, heavy metals or metabolic pathways (21, 30).

Concerning their replication autonomy, plasmids keep self-replication machinery whereas IMEs and MCTn retain the ability to integrate and excise from the host chromosome and, consequently, they do not necessarily have to be self-replicative. In the case of the extra-chromosomal MGEs, they can be generally classified considering their replication strategies, namely RC, theta, and strand displacement, although some different replication strategies can be found in linear plasmids (8, 31). Analysis of the genetic structure of the plasmid transfer-cassette shows that some plasmids contain only the *oriT* (plasmid pCI411), the *oriT* and genes encoding relaxosome components (plasmids pMV158 and pC221), or the *oriT* and the genes encoding the relaxase and the CP (plasmid CloDF13) (32–34). An important aspect to consider in plasmids is their host range, because replication, stability and transfer

[1] *mobilization* is used here to define the transfer of conjugative elements that do not self-transfer, and therefore they need the aid of the machinery provided by an auxiliary plasmid. It might be confused with *mobile*, a more general and global term to define every element that either changes from the location within a genome or moves between genomes.

mechanisms contribute to the promiscuity of a plasmid as shown for pMV158 (7) and for IncQ plasmids (35).

3. The Mobilizable RCR-Elements: Small Plasmids Could Do It

The finding that genetic elements could be transferred by conjugation between G+ bacteria took a long while after the discovery of conjugation in *Escherichia coli* (36). In fact, the first reports on conjugation in G+ bacteria were over-cautious about naming 'conjugation' to the transfer of genetic elements encoding antibiotic-resistance determinants (the today Integrative and Conjugative Elements, ICEs) so that they were referred to as 'DNase-resistant transfer in filter mating assays' (37). Once accepted that the observed genetic transfer was due to genuine conjugation, it was soon apparent that small plasmids, needing helper plasmids of the Inc18 family (such as pIP501 or pAMβ1), could be also transferred by conjugation between *Streptococcus pneumoniae* strains (38). It was unclear which the mechanism was, since the generally believed process for transfer of these small plasmids was their co-integration with the helper, a process termed conduction ('piggyback'). Three relevant findings at the time were: i) all RCR-plasmids studied generated large amounts of ssDNA intermediates, which were shown to be highly recombinogenic; ii) some RCR-plasmids like the staphylococcal plasmids pE194 and pT181 and, later on, pUB110 and pMV158 shared conserved sequences termed recombination site A (RS_A) (22, 39); and iii) plasmid-encoded proteins, termed Pre, could be responsible for the plasmid co-integration. These findings led to the proposal that the interaction of the RS_A site and the Pre protein could not only play a role in plasmid maintenance but also in the distribution of small antibiotic resistance plasmids among G+ bacteria (22, 39). These Pre proteins were claimed to contain positively charged amino acids, which were considered as probably involved in the binding of the Pre protein to the RS_A site, as it is still found in some databases (http://www.uniprot.org/uniprot/P13015; http://www.ebi.ac.uk/interpro/entry/IPR001668). In spite of this, it was also clear that, at least, the 'Pre' protein of the streptococcal plasmid pMV158 was needed for its transfer (24), so that the term Pre/Mob to name these proteins is still retained. However, demonstration that: i) the pMV158-encoded MobM protein was able to cleave supercoiled plasmid DNA, and ii) MobM behaved like a *bona fide* relaxase, led to the conclusion that inter-plasmidic recombination was a by-product of the primary mobilization function (34, 40).

The only Mob proteins of RCR-plasmids that have been characterized in some detail so far are the pMV158-MobM (34, 41–42), and the mobilization proteins from plasmid pC221 (43–45). However, there are nearly 30 Mob proteins that show a high degree of similarity to MobM, including Mob proteins from well-characterized staphylococcal plasmids like pUB110, and pE194 (Table 1). Curiously enough, the staphylococcal plasmid pC194, which is closely related to pUB110, does not appear to carry a clear mobilization cassette (see below). Cross-recognition of heterologous *oriT*s by Mob proteins could play a role in the plasmid cassettes shuffling and spreading between bacterial species and, in fact, we have recently demonstrated the *in vitro* cross-recognition of *oriT*s of three RCR-plasmids by the pMV158-encoded MobM relaxase (41).

Historically, mobilizable plasmids have been studied in greater detail than the intra-chromosomal MGEs. In 2004, Francia and colleagues presented the first comprehensive classification of plasmids based on the *oriT*-relaxase module and the homology of the relaxases identified in small mobilizable plasmids (26). Later on, this classification was updated and extended by analysing the available sequences of conjugative plasmids on the GenBank database; six MOB families were defined: MOB_F, MOB_H, MOB_Q, MOB_C, MOB_P and MOB_V (20–21). Some remarkable conclusions from these studies were: i) 39% of the prokaryotic plasmids could be conjugative (although it has been experimentally demonstrated only for part of them), from which 61.5% of them may be mobilizable; ii) the majority of mobilizable plasmids are small in size (mean peak of 5 kb) but some could also be larger than 1 Mb; iii) small mobilizable plasmids have a high copy number, making them more promiscuous than the conjugative elements; iv) relaxase MOB typing can be useful to classify conjugative plasmids (46), and v) MOB families are distributed differentially among bacterial phyla: for instance, MOB_V is overrepresented in Firmicutes but rare in Proteobacteria and totally absent in Actinobacteria. Whereas MOB_F and MOB_H are almost absent, MOB_V is the most frequent module in mobilizable plasmids, followed by MOB_C, MOB_P and MOB_Q. The following sections will summarize the representative systems belonging to mobilizable RCR-plasmid families.

4. The MOB_V Family

After years of research on mobilization of pMV158 and on its MobM-relaxase (24, 34), MobM is today the prototype of the MOB_V family of relaxases (clade MOB_{V1}; (20). Plasmid pMV158 (5540 bp) was originally isolated from *Streptococcus agalactiae*, strain MV158; historically, it was one of the first useful cloning tools since it specifies resistance to tetracycline (*tetL*-type determinant) which is selectable in many G+ and Gram-negative (G−) bacteria (47–50). The promiscuity of plasmid pMV158 is very high, and so far it has been established in more than 20 bacterial species. In fact, it is naturally mobilizable between G+ bacteria by functions supplied by auxiliary plasmids such as pIP501 (51), or even between G+ and G− bacteria helped by plasmids RP4 or R388 (40). In addition, its replication by the RC mode (52), and the presence of four origins, namely *dso* for leading strand replication, *ssoA* and *ssoU* for lagging strand synthesis, and $oriT_{pMV158}$ for mobilization, has made it an ideal molecule to study macromolecular interactions between the RepB initiator of replication and the MobM initiator of transfer with their cognate origins, *dso* and $oriT_{pMV158}$, respectively (reviewed by (7–8, 53).

During the writing of this review, we performed an in-depth search on the entire database of sequenced plasmids and bacterial genomes, and found that the family of plasmids sharing the organization of the pMV158 replicon (54) has increased up to 76 members. The search was based on homologies with the RepB-initiator of replication and with its cognate dso_{pMV158}. Out of the 76 replicons, 29 harbour a MOB cassette that includes a putative relaxase gene or an experimentally verified relaxase (Table 1). There are a few plasmids, like pFX2, pCI411, and pXY3, which contain just an *oriT* but lack any recognizable relaxase gene. Another interesting exception was the case of plasmid pA1 from *Lactobacillus plant arum*, which appears to harbour a relaxase pseudogene that could codify a truncated Mob protein of 103 residues. However, no promoter could be identified and synthesis of the

truncated protein was not detected by *in vitro* assays (55). Plasmid pLFE1 also seems to encode a putative truncated Mob protein (83 amino acids), which would belong to the MOB_V family and harbours an *oriT* sequence similar to that of pMV158. Whether these pseudogenes exist as such or they are due to sequence errors remains to be determined. In the rest of the 29 plasmids, the organization of the mobilization region is similar: the *oriT* is placed upstream of the Mob-encoding gene. Moreover, such plasmids harbour one or two lagging-strand origins, *sso*s; in the latter case, the two *sso* are flanking the MOB cassette (Figs. 1A and 3). Out of the 29 RCR-plasmids that harbour a MOB cassette, pE194 and pMV158 are the only ones for which the nick site at the *oriT* has been determined *in vivo* (56). Both nick sites coincided with the one previously mapped for pMV158 *in vitro*, namely 5'-TAGTGTG↓TTA-3', being '↓' the dinucleotide cleaved by the Mob proteins (Fig. 1B; (34)). Putative nick sites for other replicons of the MOB_V family, such as the *S. thermophilus* plasmid pSMQ172 (57) or the *L. plantarum* plasmid pPB1 (58) have been proposed on the basis of the pMV158 nick site. However, whereas the proposed nick site of pSMQ172 (AGTGNG↓TT) coincides with that of pMV158 (AGTGTG↓TT), the nick site of pPB1 (AGTGG↓GTT) was proposed to be located just one nucleotide upstream in the plasmid sequence. We believe that these nick sites should be experimentally determined or revised.

5. The MOB_P Family

The closely related plasmids pC221 and pC223 (4.6 kb) were originally isolated from *Staphylococcus aureus* and confer chloramphenicol resistance (59–60). They belong to the MOB_P family (clade MOB_{P7}; (20) and can be mobilized by a co-resident self-transmissible plasmid such as pGO1 (61). The transfer module of pC221/pC223 contains the *oriT* and three overlapping genes required for the plasmid DNA processing and mobilization. MobC and MobAreading frames overlap 16 nucleotides whereas MobA and MobBoverlap 266 nucleotides (43, 45). These genes encode the MobA relaxase and the MobB and MobC accessory proteins (Fig. 1A). Nicking of supercoiled plasmid DNA by MobA was shown to be dependent on the presence of MobC and divalent metal ions (Mg^{2+}) both *in vitro* and *in vivo* (43, 45). Whereas the MobC proteins of pC221 and pC223 are interchangeable, the MobA proteins display specificity towards their cognate DNAs (43). The *oriT*s of pC221 and pC223 span a 77-bp sequence (Fig. 1B) that includes, in addition to the nick site, the MobA binding site (*sra*) which only differs in 4 bp between both plasmids (5'-**A**C**GAC**GC-3' and 5'-**C**C**AGT**GC-3' in pC221 and pC223, respectively; differences in boldface letters). Remarkably, the substrate specificity of MobA could be effectively exchanged by swapping such a small region between the two plasmid *oriT*s (44).Little is known about the function of the MobB accessory protein, but more information is available for MobC. Protein MobC recognizes three different sites that share a consensus sequence: two sites within the $oriT_{pC221}$ (*mcb1* and *mcb2*, at 106 and 15 bp upstream of the nick site, respectively) and another site downstream of the $oriT_{pC221}$ and within the *mobC* gene (*mcb3*). All the MobC binding sites are required for an efficient plasmid mobilization but only the one closest to the nick site is necessary for DNA nicking, which supports the idea that MobC works at more than one level, such as: i) assisting MobA in its nicking activity, probably by altering the substrate structure (i.e. nick site melting), and ii) positioning MobA

on its recognition sequence, and mediating in a high order protein-complex structure to yield a mobilizable substrate (44).

6. Other Mobilizable RCR-Plasmids

Detailed experimental information on the features of other RCR-plasmids that can be mobilized is scarce, except for early reports on: i) transfer of staphylococcal plasmids (62–63); ii) those referring to the transfer of pUB110 from *Bacillus* (64), and iii) the transfer of the streptococcal plasmid pVA380-1 (65); reviewed by (9). Joint transfer of the staphylococcal plasmids pC194 and pUB110 by Tn*916* from *B. subtilis* to *B. thuringiensis* has been reported (66). However, it appeared that pC194 was either not stable or non-replicative in the recipient host, which raise the question whether pC194 was conducted by the transposable element. Recently, the mobilization of pC194 (and two other plasmids lacking mobilization cassettes) by ICE*Bs1* was also reported, achieving higher transfer frequencies than with Tn*916*. These findings led the authors to propose that the initiator of replication of the plasmid could also act as a conjugative relaxase (67). Whether conduction was operating in these transfers remains to be evaluated in depth.

7. Plasmid pMV158

As stated above, the streptococcal plasmid pMV158 is the representative of the RepB-family of replicons and also of the MOB_V family of relaxases. Due to its promiscuity (7) and to the numerous genetic tools based on it and developed to be used in low G+C Firmicutes (see below), we will describe here some of the main features of the pMV158 MOB-cassette (Fig. 1A).

The $oriT_{pMV158}$ is located within a 41-bp region upstream of the *mobM* gene. The DNA sequence spanning this region exhibits a high A+T content and includes three inverted repeats (IR1, IR2, and IR3) that partially overlap (68). Such organization would permit generation of three mutually exclusive hairpin-loop structures in which the position of the dinucleotide cleaved by MobM (G/T at coordinates 3595–3596 in the pMV158 sequence; accession number NC_010096) would be different. There is a 6-bp direct repeat (5'-ACTTTA-3'), which is placed in the left arm of IR1/IR3 and in the right arm of IR2 (Fig. 1B). These features confer the $oriT_{pMV158}$ a particularly complex configuration since most plasmids studied harbour only one or two IR sequences at their *oriT*s (69). How would MobM recognize the different IRs? This may depend on the contextual DNA topology, since, for example, extrusion of the hairpin generated by IR3, would exclude extrusion of the hairpin generated by IR2 (68). In addition, the affinity of MobM for binding to supercoiled DNA or ssDNA is much higher than for binding to linear dsDNA. Further, MobM binds to the ssDNA-encompassing IR1/3 with higher affinity (K_d ~ 60 nM) than to ssDNA-encompassing IR2 (K_d>320 nM). In fact we have shown that on ssDNA substrates the minimal $oriT_{pMV158}$ sequence for an efficient MobM-binding spans 26 nucleotides that are located just upstream of the nick site (68). This minimal origin includes IR1 and eight adjacent nucleotides (Fig. 1B). Thus, differences in MobM binding could be due to the relative position of the nick site, which would be located eight nucleotides downstream of

the IR1 hairpin, within the loop of the IR2 hairpin structure or just at the 3'-end of the stem of the IR3 hairpin (68).

The MobM protein (494 residues) is a dimer of identical subunits with two main domains: the N-terminal moiety harbours the DNA binding and cleaving activities (the relaxase domain), whereas the C-terminal moiety is involved in dimerization (probably through a leucine zipper motif) and, most likely, in the interaction with other proteins of the conjugative machinery (CP and association to membrane components) through coiled-coil regions (33, 41). The MobM N-terminal region has been studied in detail, and three conserved motifs have been defined: i) motif I (HxxR) of yet unknown function; ii) motif II (NYEL), which contains the proposed catalytic tyrosine, and iii) motif III (HxDExxPHuH), also known as the 3H motif of the HUH proteins, involved in the coordination of a divalent metal (70). The catalytic residue of MobM was predicted to be Tyr44 (20, 33). However, in the case of the Mob relaxase of plasmid pBBR1 (a broad-host-range theta replicating plasmid from *Bordetella bronchiseptica*), which belongs to the MOB_V family (clade MOB_{V2}), it was shown that none of its Tyr residues was involved in transfer (71). This finding suggested that perhaps the MOB_V relaxases might present a new mechanism of conjugative DNA transfer mediated by a non-Tyr catalytic residue or by a water-mediated nucleophilic attack. This is a subject that awaits the resolution of the structure of any member of the MOB_V family of relaxases.

Interestingly, MobM was able to relax super coiled DNA of other RCR-plasmids from the MOB_V family (41). However, the efficiency of cleavage depended upon the homologies of the *oriT*s: the efficiency of MobM-mediated cleavage was similar for pMV158 and pUB110 (50% of nicking), whereas it was lower for plasmid pDL287 and pE194 (40% and 30% of nicking, respectively; (41)). Previous experiments performed with the pMV158-RepB initiator of replication protein showed that the degree of super helicity of the DNA substrate was essential for successful cleavage (72). This held true also for the RepB activity on other RCR-plasmids with origins of replication similar to the pMV158-*dso* (72). We postulate that, in the case of MobM, the activity of the protein would also depend on the degree of super helicity of the DNA substrate. Biochemical studies have been performed with the full length MobM protein and with two truncated derivatives that conserved the first 199 (MobMN199) or the first 243 (MobMN243) residues (41, 73). The results showed that the specificity of MobM for the DNA substrate also depends on the protein length. The three proteins relaxed super coiled DNA with the same efficiency. However, compared to MobMN199, the 44 additional residues present in MobMN243, which are predicted to form an extra α-helical region, resulted to be essential for efficient ssDNA cleavage (73). Biophysical studies showed that the thermal stability of MobMN199 increased in the presence of Mn^{2+} and/or DNA and that the protein retained its enzymatic activity after thermal denaturation and renaturation (68). These findings are consistent with a model in which the relaxase–DNA complex needs the protein to be unfolded to enter the recipient cell through the T4SS (74–75).

We have shown that MobM is able to negatively regulate the transcription of its own gene (76). This does not seem to be the case of several other plasmids, which require an accessory protein for transcriptional regulation of the relaxase synthesis (77). We have found that the

two main protein players interacting at the *oriT*$_{pMV158}$ region are the host RNA polymerase (RNAP) and the plasmid-encoded MobM. However, depending on the host in which the plasmid replicates, transcription of the *mobM* gene is controlled by different promoters (Fig. 2). Two promoters placed nearly in tandem, and termed P_{mob1} and P_{mob2}, are used by the host RNAP: the latter is preferentially used in *E. coli* (76), whereas the former is used in *L. lactis* (51). Whereas promoter P_{mob1} was mapped to reside within *oriT*$_{pMV158}$, promoter P_{mob2} was found to be adjacent to it; and yet both promoters were subjected to self-regulation by MobM in *E. coli* (76). Given the relative positions of RNAP and MobM proteins in the region encompassing the two promoters (Fig. 2), we could speculate that control of the *mobM* gene expression would be exerted by the relaxase hindering the accessibility of the RNAP to the promoters which, in turn, could also depend on the efficiency of the host RNAP to recognize one or the other promoter. Which of these two promoters is used in *S. pneumoniae* is a subject under investigation. Control of gene expression exerted by only a protein seems to be inefficient when a fast response to intracellular changes is required. The control role is better performed by a short-lived antisense RNA than by a relatively long-lived protein. In the case of RCR-plasmids, which are apparently devoid of any partition system, control of the synthesis of the essential Rep initiator protein is exerted at a post-transcriptional level by one or two antisense RNAs (78). However, there are instances, like replication of pMV158 or of pIP501, in which synthesis of the initiator is controlled by the joint contribution of an antisense RNA and a transcriptional regulator protein (79–80). We have no indication of the existence of an antisense RNA to control expression of the *mobM* gene, and we do not know yet the half-lives of the MobM-DNA and of the RNAP-P_{mob1} or -P_{mob2} complexes. We also lack information on the competition of the two proteins for binding to the *oriT*$_{pMV158}$ promoter region; thus it is presently difficult to evaluate the efficiency of MobM as a transcriptional self-regulator. Several streptococcal plasmids such as pER13 (81), pSMQ172 (57), pVA380-1 (82), and pRW35 (83) maintain a similar structure at their *oriT* regions, with two putative tandem promoters for expression of the relaxase gene. Thus, we propose that an auto-regulation mechanism, similar to that of pMV158, would also be involved in the synthesis of their respective relaxases.

8. Factors contributing to the promiscuity of RCR-plasmids

The RCR-plasmids are constituted by a small number of gene modules and, in general, show an unusual promiscuity (84). Their capacity to colonize new hosts depends on their ability to: i) express their Rep initiator protein and trigger over-replication until they surpass their average copy number (around 20–30 plasmid copies per bacterial genome equivalent at the steady state); ii) control efficiently their replication once they are established in the new host and the average number of copies is reached; iii) have an efficient mechanism to convert ssDNA intermediates into dsDNA molecules, and iv) use the host machinery for their own replication and/or transfer. Thus, the philosophy of ‘travel light, not constituting a genetic load, and making a clever use of the host resources’ applies to RCR-plasmids (84). Their replication needs the Rep initiator which is provided by the plasmid, while the rest of the machinery is provided by the host: RNAP, DNA polymerases (I and III), ssDNA-binding protein, DNA gyrase, and a DNA helicase, most likely PcrA (85–87). These host proteins

are synthesized in sufficient quantities so that the plasmid would not be a heavy genetic load to its host (88). Conversion of ssDNA intermediates into dsDNA forms is initiated by the host RNAP and would require the same proteins used for leading strand synthesis (15, 89).

It was soon demonstrated that small RCR-plasmids were able to replicate in several G+ bacterial hosts (49, 90), and also in G− bacteria like *E. coli* (50, 91–92). Some of the RCR-plasmids, like pC194, could even replicate in yeasts (93). However, some of these plasmids exhibited either structural (deletions) or segregational (loss of plasmid in the absence of selective pressure) instability. This was, most likely, associated to their replication functions (94–98), and especially to the functionality of the plasmid *sso* (99–100). The lagging strand origins of the RCR-plasmids are located at specific non-coding regions that can fold into imperfect hairpin structures on the ssDNA plasmid intermediates (Fig. 3). They operate in an orientation-dependent manner, indicative of the need of unpaired sequences within the hairpins that would be recognition sites of the host-encoded machinery (99, 101–102). Sequence homology analyses led to the description of various types of *sso* (63): *ssoA* (in pT181 and pC194), *ssoU* (in pUB110), *ssoT* (in pTA1060 and pBAA1), and *ssoW* (in pWV01 and closely related replicons; (97)). The streptococcal plasmid pMV158 harbours both *ssoA* and *ssoU* ((22, 99). Deletions affecting the *ssoA* functionality lead to plasmid copy number decrease, segregational instability, and accumulation of large amounts of intracellular ssDNA plasmid intermediates (22, 99, 102). Analysis of the DNA sequence and structure of the *ssoA* of several plasmids showed the presence of conserved unpaired regions (63) and homologies between *ssoA* and *ssoU* sequences (16, 103). One of the conserved regions encompasses part of the so-called plasmid recombination site B (Pre_RS_B), which is highly conserved in nearly all the reported *ssoAs*. Later on, it was demonstrated that the RS_B site was the binding site of the host RNAP for a class of promoters located on ssDNA (15, 18). There is another unpaired region, which was termed CS-6 (consensus sequence 6: 5'-TAGCGt/a-3'), which is a transcriptional terminator for the synthesis of a small primer RNA by the host RNAP ((15); see Fig. 3). Lagging strand synthesis is continued by DNA polymerase I, followed by DNA polymerase III and DNA gyrase to complete the plasmid replication (8, 89). An indication of a good adaptation of RCR-plasmids to their hosts is provided by the ratio of ss/ds DNA, which should be substantially low because accumulation of intracellular ssDNA can be harmful for the host bacteria (16). The MOB cassette of pMV158 ($oriT_{pMV158}$ and *mobM* gene) is flanked by the *ssoU* and *ssoA* origins (Fig. 3). Transfer of pMV158 between *S. pneumoniae* and *Enterococcus faecalis*, allowed us to study which pMV158-*sso* is used in homologous and heterologous hosts (Table 2). Determination of the frequencies of transfer, plasmid copy number in the two hosts, and the efficiency of replication measured as the ratio of ss/ds DNA showed that: i) either *sso* supported transfer between strains of *S. pneumoniae* with the same efficiency as the parental pMV158; ii) only the *ssoU* efficiently supported transfer when it was tested between *S. pneumoniae* and *E. faecalis*, and iii) a functional *ssoU* is a critical plasmid region in the colonization of hosts by pMV158 (16).

9. Inhibitors of Conjugative Transfer

Antibiotic resistance genes and virulence genes are commonly associated with MGEs that can be transferred among bacteria sharing the same niche. Many human activities related to

the hospital use and to the industrial waste of antibiotics constitute a major driving force in the selection of MGEs carrying multiple antibiotic resistance genes (104). Thus, strategies for inhibiting conjugation may be crucial to preserve the effectiveness of antibiotics and to avoid the spread of resistance traits. It must be considered that many mobilizable plasmids not only are promiscuous but also harbour one or more resistance genes. Considering the molecular mechanism of conjugative transfer, several components of the relaxosome/CP/T4SS machinery could be looked at as potential targets for the development of inhibitory drugs (conjugation inhibitors, or COINS; (105)). For instance, it has been shown that unsaturated fatty acids are effective inhibitors of plasmid R388 conjugation (105). The underlying mechanism involved in this inhibition is presently unknown, but it might be related to either direct effects on the transfer machinery or to modifications of plasmatic membrane fluidity. Remarkably, whereas R388 plasmid conjugation was inhibited by unsaturated fatty acids, R388-mediated mobilization of plasmid CloDF13 was not affected, suggesting a possible effect of unsaturated fatty acids on the assembly of the R388 relaxosome. A second example of possible COINS has been provided: conjugation of plasmid F was shown to be inhibited by filamentous bacteriophages (like M13), a family of ssDNA phages that attach to the tip of the conjugative pilus (106). The proposed model contemplates that the phage coat protein gp3 occludes or reduces the assembly of the T4SS components. Therefore, these ssDNA phage coat proteins could be considered as a natural source of proteins that can compete with the conjugative T4SS, thus acting like *bona fides* COINS. Using again plasmid F, a third example of COINS was provided by showing that conjugative DNA relaxase was inhibited by bisphosphonates (107). However, this approach was directed against plasmids, like F, that encode multi-tyrosine relaxases and that are represented by the MOB_F family (20). This may not be the general feature for all plasmid-encoded relaxases, so that bisphosphonates may be considered as limited-value COINS. A fourth approach was designed to inhibit the activity of the TrwC_R388 relaxase, and it was done by blocking its relaxation activity once the relaxase-DNA complex was within the recipient cell. This was achieved by employment of single-chain antibodies (108). All the above approaches may not be of wide usefulness, so that it would be interesting to search for COINS that target general transfer processes such as relaxosome assembly in the donor or lagging strand synthesis in the recipient bacteria.

10. Applications of the pMV158 Replicon

Due to their small size, it was soon envisaged that many of the RCR-plasmids could be used to construct useful cloning and expression vectors for G+ bacteria, especially because they were scarce or non-existent compared to those developed for G− bacteria (48). It was also apparent that generation of ssDNA intermediates, as a consequence of the RCR mechanism, could result in plasmid structural instability (94, 98, 109–111). Thus, deletions of cloned DNA happened with a certain frequency especially when the recombinant plasmids were transferred among hosts of different species (112). Nevertheless, cloning of DNA fragments up to 10 kb in size was shown to be feasible and the recombinant plasmids were structurally stable in the homologous host (48). Furthermore, employment of mobilizable RCR-plasmids to assess the HGT as well as their use as possible tools to test novel COINS has been important in the development of novel strategies to deal with the spread of antibiotic-

resistance traits. To date, many different genetic tools based on RCR-plasmids have been constructed. However, small sized plasmids replicating by the theta mechanism are frequently the preferred vectors for employment in the dairy industry (113). We review below the applications of some of the plasmid vectors (mobilizable or not) that have been recently constructed using the pMV158 replicon.

10.1.Construction of Green Fluorescent Bacteria using the pMV158 Replicon

Plasmid pMV158 has been employed to construct useful genetic tools for G+ bacteria in both versions, mobilizable or non-mobilizable vectors. In the latter case, a derivative of pMV158 lacking the *mobM* gene (plasmid pLS1) was used to construct vectors that harbour a variant of the *gfp* gene, which encodes a green fluorescent protein (GFP) that carries the F64L and S65T mutations (114). The F64L mutation increases GFP solubility, while the S65T mutation increases GFP fluorescence and causes a red shift in the excitation spectrum. In addition, this variant of the *gfp* gene contains translation initiation signals that are optimal for its expression in prokaryotes (114). In the pLS1GFP vector, the *gfp* gene is under the control of a pneumococcal promoter (P_M) that is inducible by maltose. The MalR transcriptional repressor controls the activity of the P_M promoter in *S. pneumoniae* (115). A second version, termed pLS1RGFP, has the *malR* gene placed *in cis* to provide an increased gene dosage of the repressor (116). Pneumococcal cells harbouring either of these plasmids would be unable to synthesize GFP by growing them in a sucrose-containing medium (repression conditions). However, when the culture medium is supplemented with maltose the MalR repressor will be inactivated and the strong P_M promoter will direct synthesis of GFP (see Fig. 4).

The mobilizable pMV158GFP vector is a pMV158 derivative that also carries the *gfp* gene fused to the maltose-inducible promoter P_M (117). Thus, in pneumococcal cells carrying pMV158GFP and the chromosomal *malR* gene, expression of *gfp* depends on the carbon source added to the culture medium (repression or activation in the presence of sucrose or maltose, respectively). Nevertheless, in other G+ bacteria (like *E. faecalis*), the *gfp* gene is expressed constitutively (Fig. 4). Plasmid pMV158GFP has been mobilized not only between strains of *S. pneumoniae* but also from *S. pneumoniae* to *L. lactis* or *E. faecalis* (117), and to *S. aureus* (118). Plasmid pMV158GFP was shown to be a powerful tool for the construction of green fluorescent bacteria, which have been used for many different aims, such as (to name a few): i) investigation of the phagocytosis and bactericidal activity of granulocytes against live *S. pneumoniae* (119); ii) analysis of the intracellular localization of pneumococcal cells within murine microglial cells (120); iii) detection of *L. lactis* during cheese production (121); iv) visualization of the binding of *E. durans* to human Caco-2 cells (122); v) quantitative analysis of the intra- and inter-species conjugal transfer of pMV158 (123), and vi) quantification of adhesion of green-fluorescent-protein producing *S. aureus* (118). More interestingly, pMV158GFP has been used in the development of *Caenorhabditis elegans* as an infection model for *E. faecalis*. *E. faecalis* harbouring plasmid pMV158GFP allowed for visualization of the infection in gut of the transparent animal (Fig. 5) and can be used to quantify colonization under different conditions (D.A.G., not shown). Because the P_M promoter drives constitutive expression in *E. faecalis* so well, it was used in the construction of an *E. faecalis* chromosomal integration vector to drive *gfp* expression in

stable integrants. The expression of *gfp* in such a strain (SD234) was very even and stable (124).

10.2. Construction of a Promoter-Probe Vector Based on the pMV158 Replicon

Promoter-probe vectors have been shown to be very useful for studies on regulation of gene expression. Although many algorithms have been developed for the prediction of promoters in bacterial genomes (125–126), definitive identification of promoter sequences requires the use of several experimental approaches, both *in vivo* and *in vitro* (127). Among these approaches, the use of promoter-probe plasmid vectors facilitates the identification of sequence elements that are essential for promoter activity. Moreover, such vectors make possible to investigate promoter activity in a variety of genetic backgrounds and under diverse environmental stimuli. Compared to *E. coli*, promoter-probe vectors are still scarce in many G+ bacteria. Ruiz-Cruz *et al.* (128) designed the pAST plasmid vector (5456 bp) (Fig. 6) that derives from a non-mobilizable pMV158 replicon (plasmid pLS1). Vector pAST contains a highly efficient transcriptional terminator signal (the tandem terminators *T1* and *T2* of the *E. colirrnB* operon) just upstream of a multiple cloning site (MCS). The MCS is followed by a promoter-less *gfp* reporter gene (114). The pAST vector was shown to be suitable to assess the activity of homologous and heterologous promoters in *S. pneumoniae* and *E. faecalis*. It is very likely that it can be used in numerous G+ bacteria because pMV158 replicates autonomously in streptococci, enterococci, staphylococci, bacilli and lactococci. In addition, pAST is a valuable tool for the design of novel expression vectors (see below).

10.3. Expression Vectors Based on the pMV158 Replicon

Vector pLS1ROM (6805 bp) is a non-mobilizable pMV158 derivative that was designed for regulated gene expression in *S. pneumoniae* (129). It carries the *malR* gene under the control of a constitutive promoter and the MalR-repressed promoter P_M, which is followed by a MCS. The P_M promoter is activated when bacteria are grown in maltose-containing media. Under such induction conditions, pLS1ROM was shown to be structurally and segregationally stable. The functionality of this system was tested using the *gfp* reporter gene.

Plasmids pAST-*Pung* and pAST-*PfcsK* are a new category of plasmid pAST-derivatives (128) that can be also used as expression vectors in *S. pneumoniae*, and that contain useful pneumococcal promoters. Plasmid pAST-*Pung* (5615 bp) was constructed by cloning the promoter region (*Pung*) of the pneumococcal *ung* gene (uracil-DNA glycosylase) into the *Bam*HI site of the pAST promoter-probe vector (Fig. 6). Promoter *Pung* was shown to increase 10-fold the expression of the *gfp* gene in pneumococcal cells (fluorescence assays). Plasmid pAST-*Pung* has a MCS between the *Pung* promoter and the *gfp* gene. Thus, promoter-less genes inserted into the MCS can be expressed in pneumococcus. For the construction of plasmid pAST-*PfcsK* (5573 bp), the *PfcsK* promoter of the fucose operon (130) was inserted into the *Xba*I site of the pAST vector (Fig. 6). Expression of *gfp* from the *PfcsK* promoter increased about 5-fold (as measured by fluorescence assays) when bacteria were grown in the presence of fucose (128). Since the putative fucose regulator gene *fcsR* is widely conserved in *S. pneumoniae*strains (131), it can be expected that plasmid pAST-

PfcsK will be valuable as inducible-expression vector in pneumococcus, and the promoter-less gene of interest can be inserted into the MCS positioned between the *PfcsK* promoter and the *gfp* gene.

11. High-throughput Assessment of the Mobility of RCR-Plasmids

Conjugation implies physical contact between the pair of donor/recipient cells, which can be established by flexible retractile pili (exemplified by F), by means of rigid pili (R388 and RP4) or, like in some G+ bacteria, by surface proteins of the sortase-type (132) or peptidoglycan degrading enzymes (133). Thus, depending on the specific conjugative system involved, cell mating can take place in liquid media or on solid/semisolid surfaces. Several high-throughput formats have been developed in order to study plasmid transfer in liquid (134), semisolid (105), or solid surfaces (123) using fluorescent as well as bioluminescent detection technologies. Such approaches permit testing plasmid transfer in single multi-well plates under the same or different conditions, reducing experimental variations and hands-on time. For instance, mobilization of plasmid pMV158GFP between different G+ bacteria has been analyzed by using microtiter plates coupled to a filtration device with sterile 0.22 μm filters (Fig. 7) to quantify: i) intra-species plasmid transfer, where the R61 pneumococcal donor strain harbouring pIP501 was mated with another pneumococcal recipient strain (carrying different selectable markers), and ii) inter-species plasmid transfer, using *S. pneumoniae* as donors and *E. faecalis* as recipients (123). Detection of transconjugants was performed by measurement of the fluorescence provided by pMV158GFP (*gfp* expression) and by plating suitable dilutions on selectable-marker containing plates followed by colony-forming-units counting. The frequency of transfer was high enough to be quantified: as on average, 1 out of 10,000 recipient cells received pMV158GFP. A further advantage of the employment of this format is the use of microaerophilic bacteria (like *S. pneumoniae*) as donors and aerobic bacteria (i.e. *E. faecalis*) as recipients. This improves counter-selection of donors when testing the number of transconjugants and makes the assays much easier. Therefore, this large-scale filter mating assay could be employed to analyse the parameters involved in interspecies RCR-plasmid transfers as well as to test lead compounds that could act as COINS.

12. Final considerations

Within the present ‘ome’ era, we are confronted with the still scarce knowledge and attention that it is given to the mobilome as a whole because the spread of the antibiotic resistance is due to the presence of mobile elements among bacteria. The discovery that many G+ bacteria encode relatively simple T4SSs (4, 13, 135), which are accompanied by surface proteins involved in the DNA traffic during conjugation or genetic competence for transformation (136), raised the concern on the massive transfer of resistance genes to many pathogens. Since these ‘minimal’ T4SSs are generally associated to MGEs (such as Tn*916*, ICEs, pathogenicity islands, and plasmids), the risks of explosive outbreaks of G+-mediated bacterial infectious diseases are evident. Due to their small size and their promiscuity, we cannot but be astonished that only two RCR-plasmids (pC221 and pMV158) have merited attention as to study their transfer properties. It is no a matter to be taken slightly the assumption that *all* mobilizable RCR-plasmids will be transferred by the *same* mechanism

or will have only one single type of relaxase or CP, so that 'once known one, we know them all' is a poor scientific philosophy.

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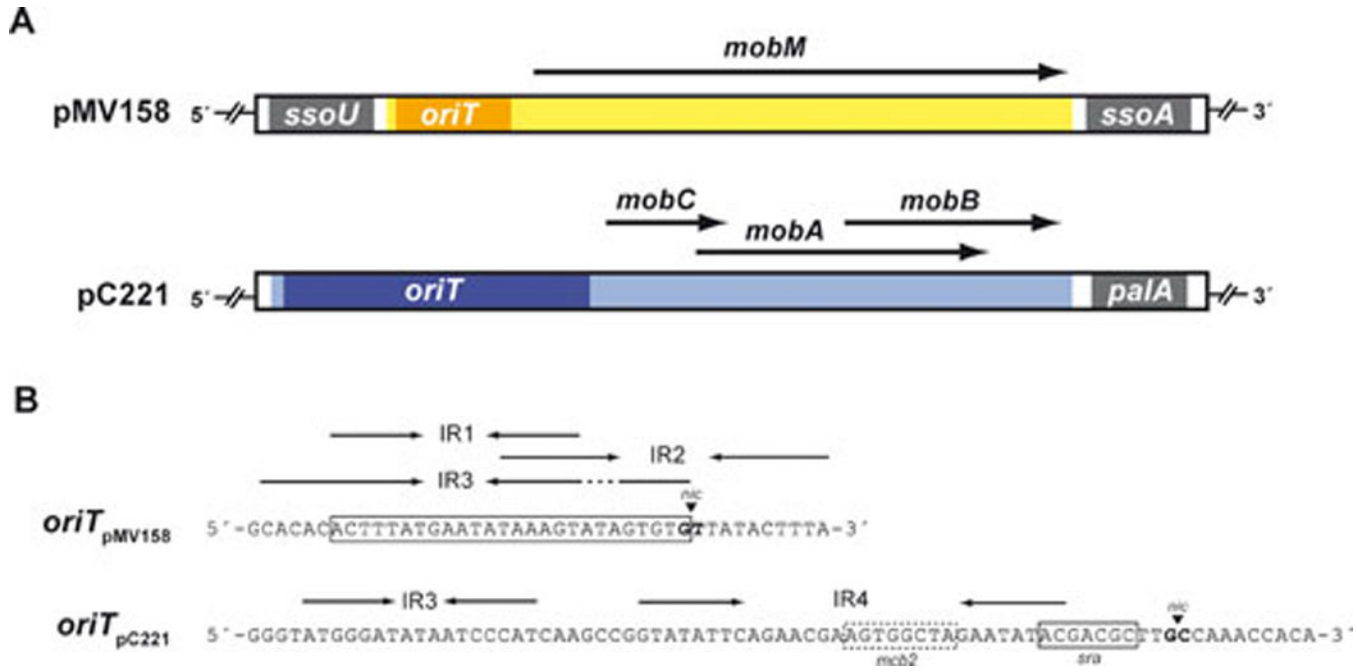


FIG. 1.

(A) The MOB modules of plasmids pMV158 and pC221. The MOB cassette of pMV158, which contains a single gene encoding the MobM relaxase, is flanked by two *sso*s of the types U and A, respectively (see Fig. 3). In the case of pC221, there is a single *ssoA* (also termed *palA*). Its MOB cassette contains three genes encoding the MobC, MobA, and MobB proteins. (B) Relevant features of the *oriT*s of plasmids pMV158 and pC221. The nucleotide sequences of the $oriT_{pMV158}$ (coordinates 3564–3605) and $oriT_{pC221}$ (coordinates 3084–3160) are shown. The inverted repeats (IRs, arrows) and the nick site (*nic*, arrowhead) are indicated. Demonstrated minimal binding site for MobM (lined box), and MobA recognition site (*sra*) and one of the MobC binding sites (*mcb2*) are also specified.

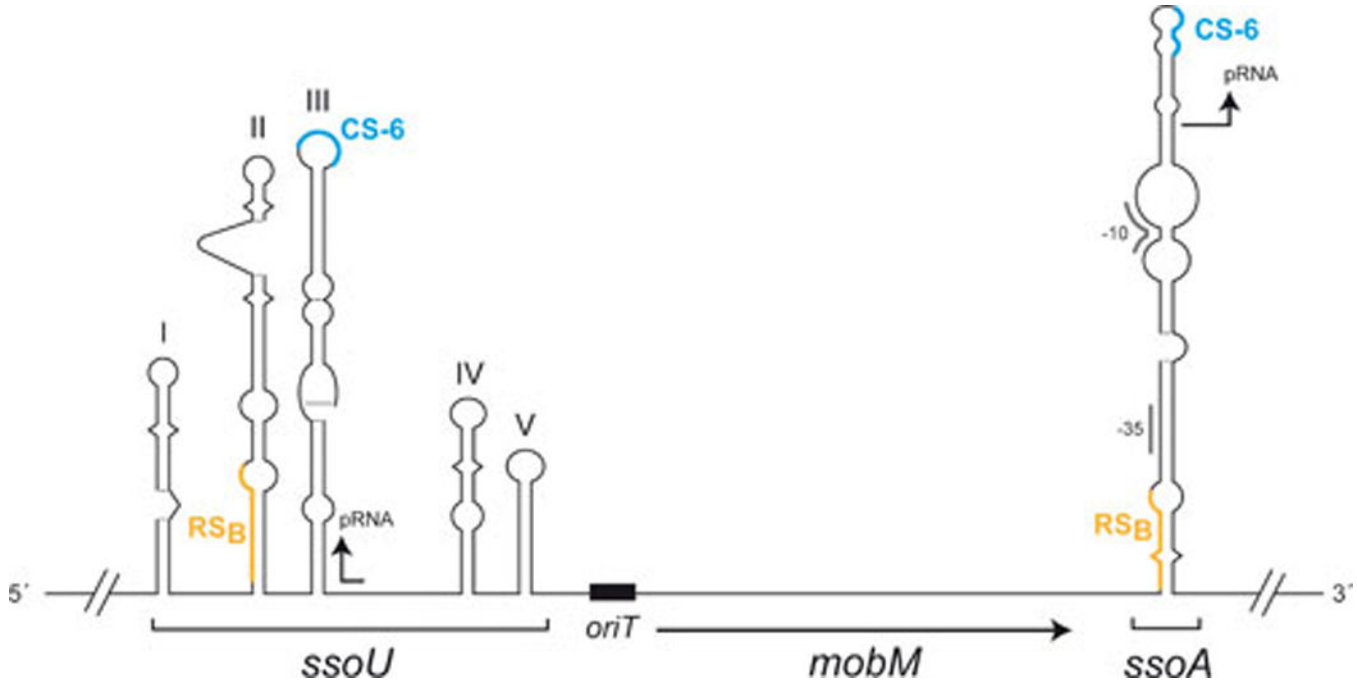


FIG. 2.

Autoregulation of *mobM* gene expression. The region of pMV158 that includes the *Pmob1* and *Pmob2* promoters of the *mobM* gene is shown. In *E. coli*, *mobM* transcription is mostly initiated from promoter *Pmob2* (in blue) (76). Promoter *Pmob1* (in black), which is located within the *oriT* sequence, is used in *L. lactis* ((51). The main sequence elements of both promoters and the nick site (*nic*) are indicated. Transcription start sites are also indicated with arrows. The regions recognized by MobM and *E. coli* RNA polymerase (RNAP) were determined by DNase I foot printing assays using linear double-stranded DNAs (42, 76). MobM is able to repress *in vivo* the transcription initiated from both promoters, P_{mob1} and P_{mob2} (76).

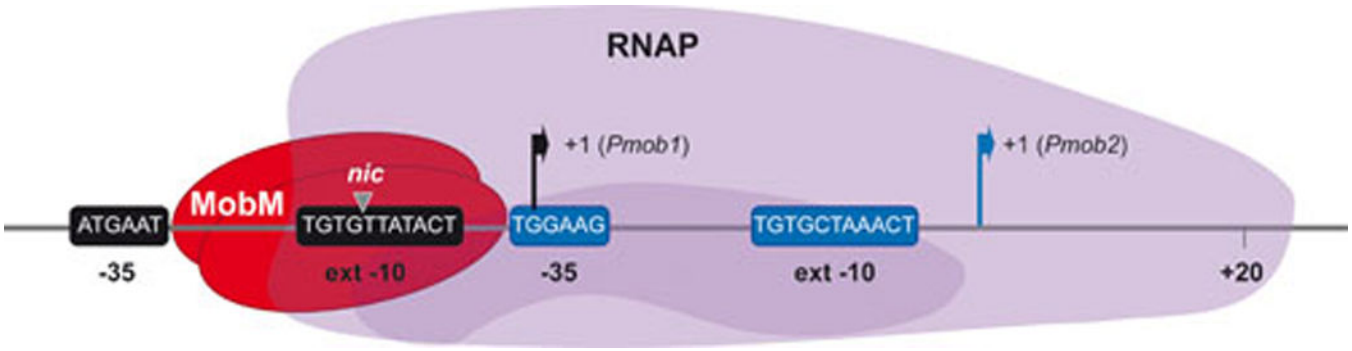
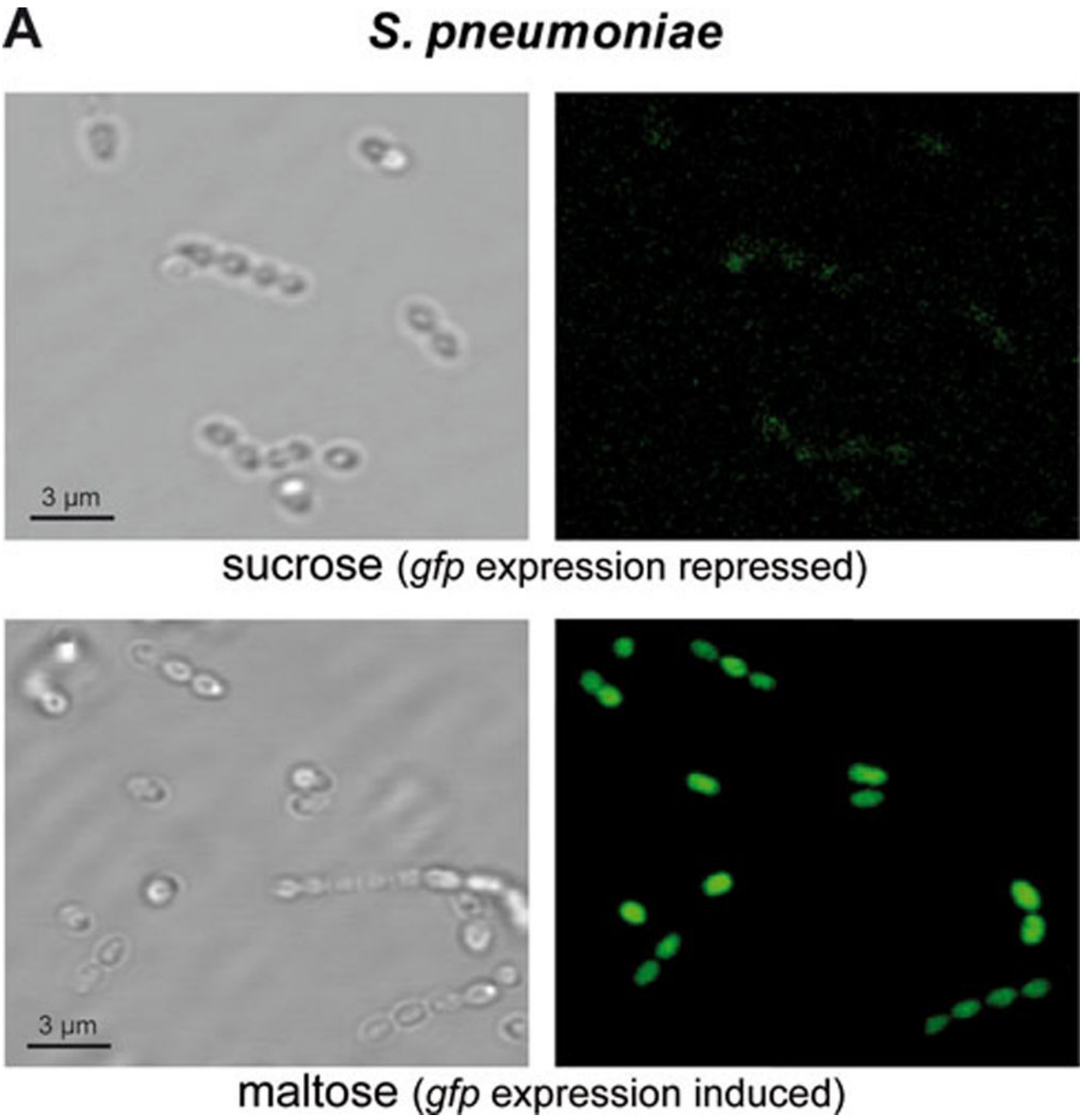


FIG. 3.

Genetic organization of the MOB cassette in pMV158. Plasmid-*oriT* and the *mobM* gene are flanked by two lagging-strand origins of replication (*ssoA* and *ssoU*). Both*sso*s have the possibility of generating long hairpin-loop structures exhibited as 'ssDNA promoters' (15, 18) where the RNAP-binding site (RS_B) is recognized by the RNAP to synthesize a short RNA primer (pRNA). A consensus sequence (CS-6) located in the loop of the hairpin acts as the termination point for the pRNA synthesis. The pRNA is then used by DNA polymerase I for limited extension synthesis, followed by replication of the lagging strand by DNA Pol III.



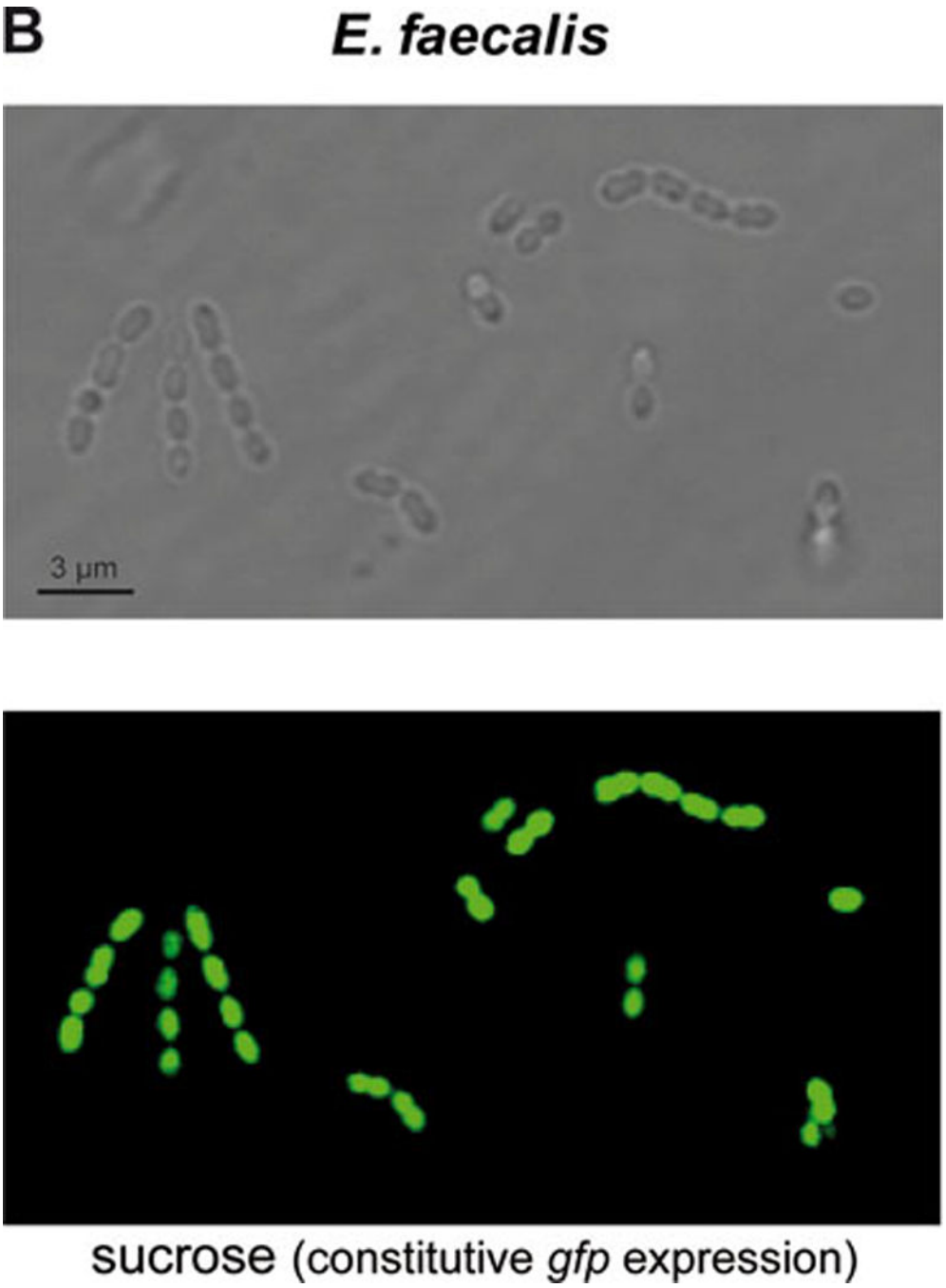


FIG. 4.

Expression of gene *gfp* in cells harboring plasmid pMV158GFP. Phase-contrast and fluorescence microscopy of: A) *S. pneumoniae* cells expressing *gfp* after growth in sucrose (maltose promoter P_M repressed in pneumococci; top panel) or in maltose (P_M induced; bottom panel), and B) *E. faecalis* cells expressing constitutively *gfp* after growth in sucrose (right panel). See text for details.

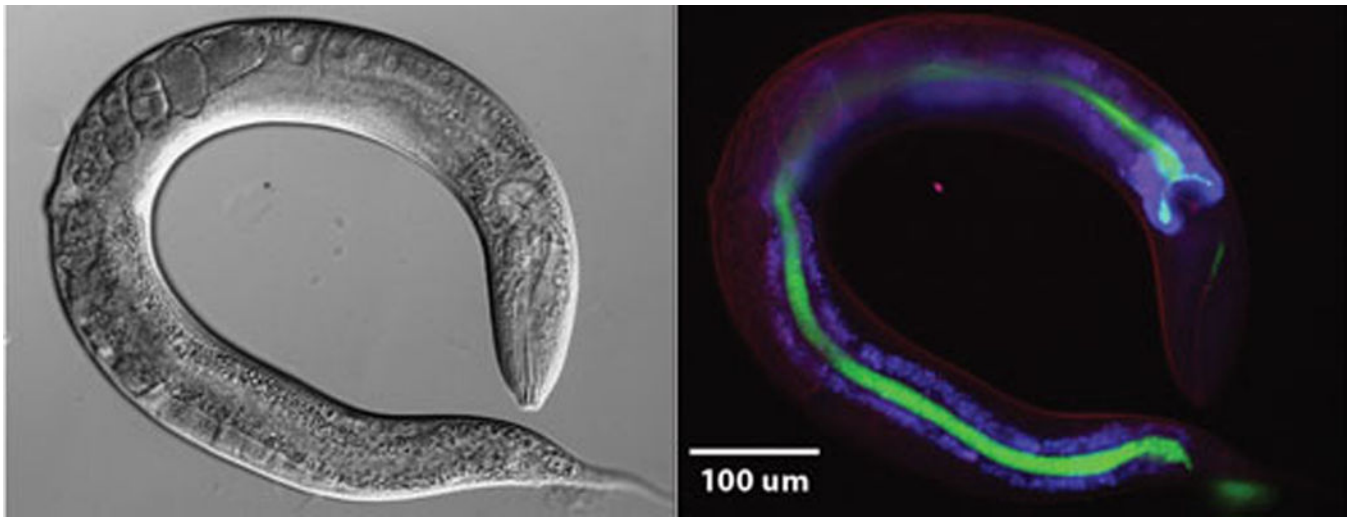


FIG. 5.

Enterococcus faecalis OG1RF expressing Green Fluorescent Protein (GFP) colonizes the intestine of *C. elegans*. L4 larvae were exposed for 3 hours to *E. faecalis* OG1RF carrying plasmid pMV158GFP. Subsequently, worms were washed with M9 medium and anesthetized with 0.25mM levamisol before imaging. DIC and fluorescence microscopy were used to visualize the worms. GFP expressing bacteria and auto-fluorescence generated by lipofuscin granules in the body of the worm were observed using FITC and DAPI filters respectively.

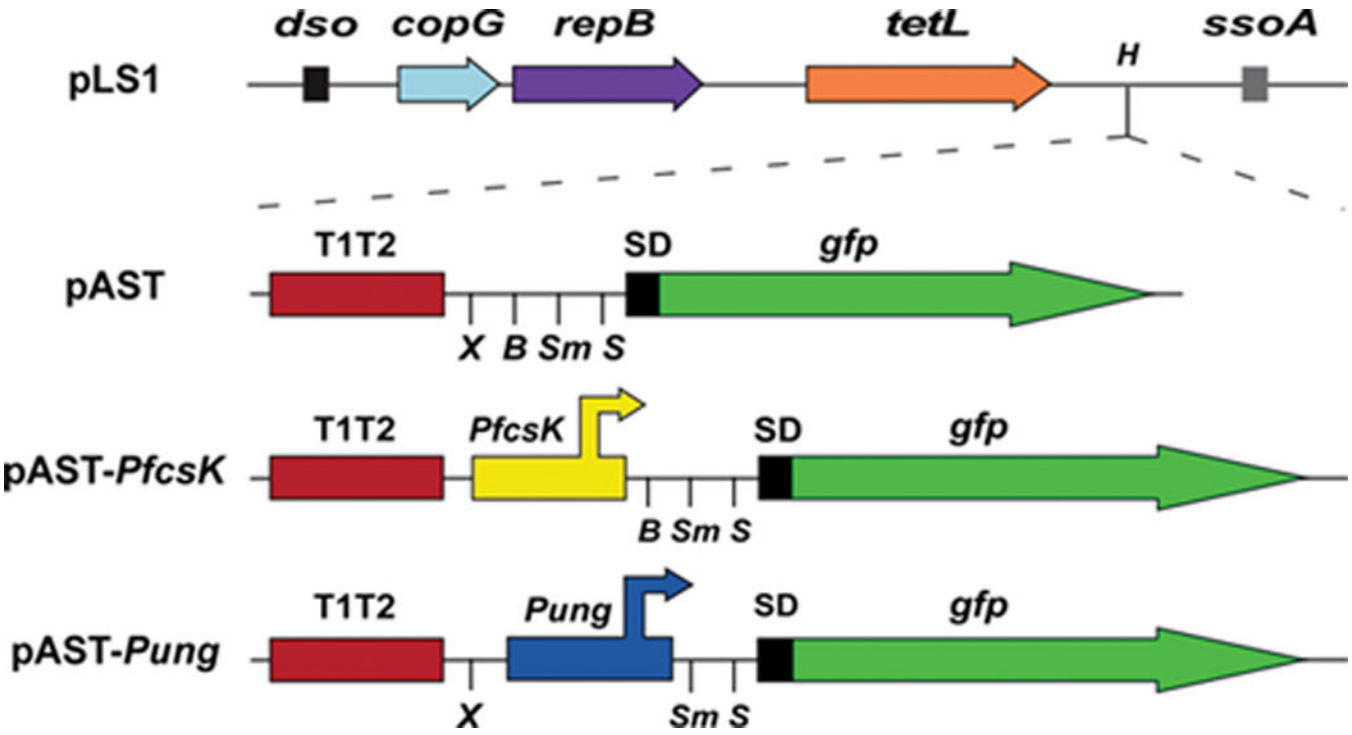


FIG. 6.

Genetic maps of plasmid pLS1 (a non-mobilizable pMV158 replicon) and its derivatives pAST, pAST-*PfcsK* and pAST-*Pung* (128). Only relevant features are indicated. *copG* and *repB* genes are involved in plasmid DNA replication. The location of the replication origins *dso* (double-strand origin) and *ssoA* (single-strand origin) is indicated. The *tetL* gene confers resistance to tetracycline. T1T2: tandem terminators *T1* and *T2* of the *E. colirrnB* rRNA operon. SD: translation initiation signals optimized for the expression of the *gfp* gene in prokaryotes (114). The position of the *PfcsK* and *Pung* promoters is indicated. *H*: *Hind*III, *X*: *Xba*I, B: *Bam*HI, *Sm*: *Sma*I, *S*: *Sac*I.

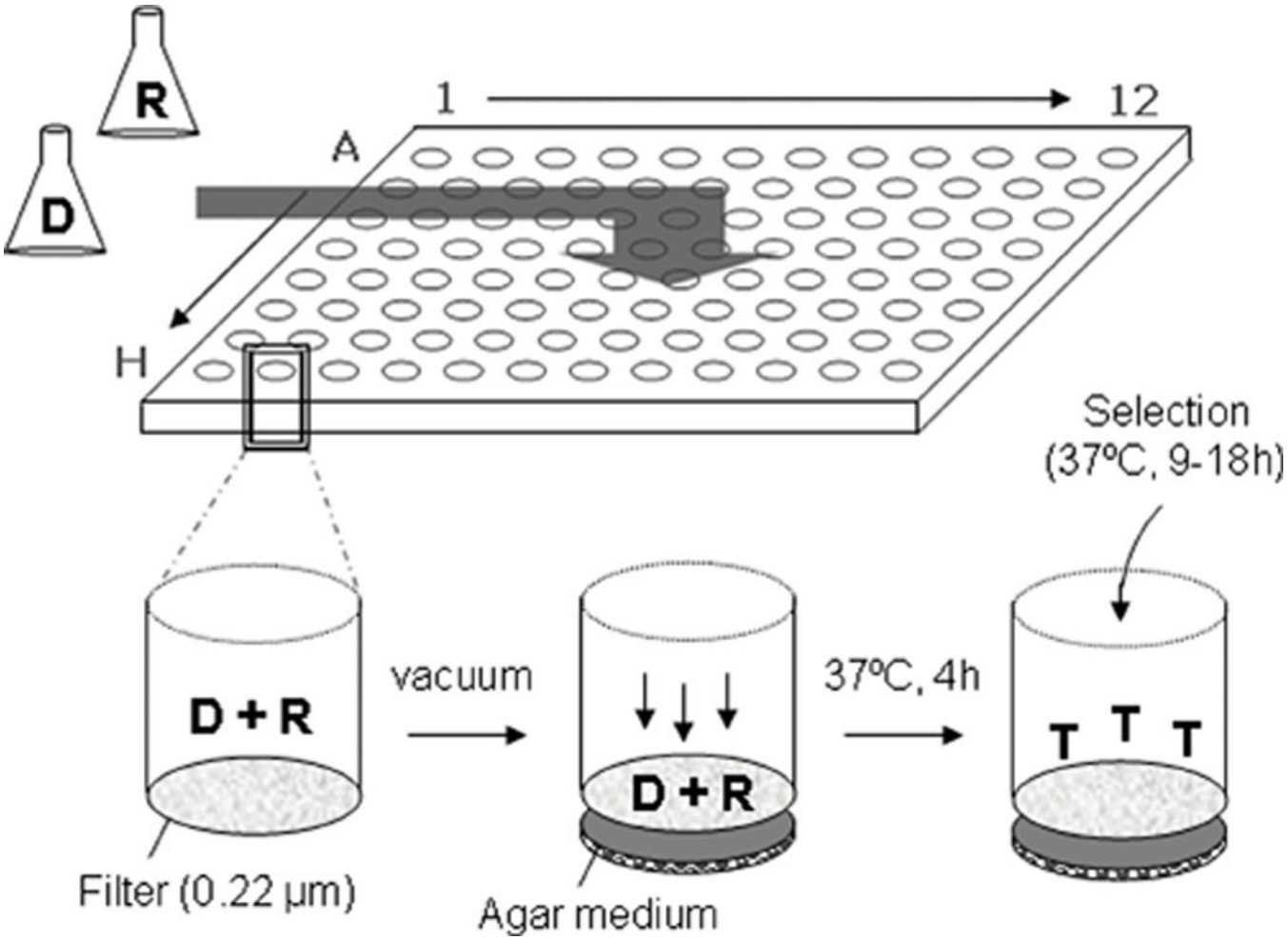


FIG. 7. Strategy for high-throughput plasmid transfer detection

Plasmid-containing donor (D) and recipient (R) cells are mixed (it can be done at different ratios), filtered in a 96-filter-well plate (0.22 µm) and placed on a layer of solid conjugation medium (containing 5 µg/ml DNase I). After 4 h at 37°C, selection for transconjugants (T) is applied by adding antibiotic-containing medium and application of gentle vacuum. Thus, for each well the transfer frequencies can be assessed by a number of methods, such as plating on selective medium, fluorescent confocal microscopy (*gfp*-expressing plasmids), LacZ measurements, or quantitative PCR (Modified from (123)).

Table 1

RCR-plasmids from G+ bacteria belonging to the MOB_V family[a]

Plasmid[b]	Accession number	Size (bp)[c]	Bacterial source	Mob (aa)[c]	Reference
pMV158	NC_010096	5540	*Streptococcus agalactiae*	494	(47)
pGB2001	NC_015973	4967	*Streptococcus agalactiae*	500	(137)
pGB2002	NC_015971	6825	*Streptococcus agalactiae*	500	(137)
p5580	NC_019370	4950	*Streptococcus dysgalactiae*	500	(138)
pSBO2	AB021465	3582	*Streptococcus equinus*	527	(139)
pSMA198	NC_016750	12728	*Streptococcus macedonicus*	410	(140)
pRW5	NC_010423	4968	*Streptococcus pyogenes*	500	(83)
pGA2000	NC_019252	4967	*Streptococcus pyogenes*	500	(137)
pSSU1	NC_002140	4975	*Streptococcus suis*	499	(141)
pSMQ172	NC_004958	4230	*Streptococcus thermophilus*	499	(57)
pER13	NC_002776	4139	*Streptococcus thermophilus*	499	(81)
pE194	NC_005908	3728	*Staphylococcus aureus*	403	(142)
pCPS49	NC_019142	5292	*Staphylococcus aureus*	409	(143)
pLA106	NC_004985	2862	*Lactobacillus acidophilus*	411	(144)
pCD034-2	NC_016034	2707	*Lactobacillus buchneri*	363	(145)
pRCEID2.9	NC_017466	2952	*Lactobacillus casei*	436	(146)
pWCZ	NC_019669	3078	*Lactobacillus paracasei*	436	Unpublished
pTXW	NC_013952	3178	*Lactobacillus paracasei*	432	(147)
pMRI 5.2	NC_019900	5206	*Lactobacillus plantarum*	408	(148)
pLFE1	NC_012628	4031	*Lactobacillus plantarum*	83	(149)
pLB4	M33531	3547	*Lactobacillus plantarum*	361	(150)
pA1	NC_010098	2820	*Lactobacillus plantarum*	103	(55)
pPB1	NC_006399	2899	*Lactobacillus plantarum*	355	(58)
pLS55	NC_010375	5031	*Lactobacillus sakei*	439	(151)
pYSI8	NC_010936	4973	*Lactobacillus sakei*	403	(152)
pGL2	NC_016981	4572	*Lactococcus garvieae*	504	(153)
pBM02	NC_004930	3854	*Lactococcus lactis*	304	(154)
pMBLR00	NC_019353	3370	*Leuconostoc mesenteroides*	361	(155)

Plasmid[b]	Accession number	Size (bp)[c]	Bacterial source	Mob (aa)[c]	Reference
pMA67	NC_010875	5030	*Paenibacillus larvae*	435	(156)

[a] Data were collected by BLASTP, using as query the pMV158-initiator of replication RepB protein. We extracted 76 plasmids, and out of these, the 29 plasmids showed in this Table 1 harbour an open reading frame that could encode a relaxase.

[b] In plasmid pSBO2 the ORF-Mob is annotated as a protein of 144 residues (corresponding to coordinates 3149–3582). Extending the analyzed DNA sequence from coordinates 1 to 1150, a larger ORF-Mob (527 residues) could be identified. Plasmids pGA2000, pGB2001, pGB2002 and p5580 share the same replicon and mobilization cassettes (137–138).

[c] Plasmids and Mob protein sizes are indicated in number of base pairs (bp) and amino acids (aa), respectively.

Table 2

Indicators of pMV158 promiscuity: relative efficiencies of conjugative transfer from donor *S. pneumoniae* to recipients *S. pneumoniae* and *E. faecalis*.

pMV158	*Streptococcus pneumoniae*			*Enterococcus faecalis*		
	Transfer[a]	Copy number[b]	ss/dsDNA[c]	Transfer[a]	Copy number[b]	ss/dsDNA[c]
WT	1	30	1	10	20	1
Δ*ssoA*	1	30	1	10	20	1
Δ*ssoU*	1	30	1	0.01	5	15
Δ*ssoA/ssoU*	0.01	10	50	0.01	3	30

[a] Relative transfer in comparison to the pMV158 wild-type (WT) plasmid.

[b] Number of plasmid molecules per chromosomal equivalent.

[c] Ratio of ssDNA dsDNA, indicative of efficiency of conversion.