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

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ABSTRACT 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 plasmids are more frequently found in nature. In this sense, replication and mobilization can be considered important mechanisms influencing plasmid promiscuity. Here we review the currently 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 (MOBV) 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.

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 extrachromosomal 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 (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 and thus are important in the coevolution 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 prerequisite 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 oriT); both proteins require that their DNA substrate is supercoiled (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 nicked the plasmid DNA to initiate the replication, the relaxosome could not be formed, and thus transfer would be hindered.

The initiation of transfer involves a Mob relaxase-mediated DNA cleavage at the oriT; the result of the cleavage is one covalent amino acyl-DNA adduct that 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 restores 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 Novick’s lab (reviewed in reference 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 references 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. 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 horizontal gene transfer (19). In general, self-transferable elements are transferred at higher frequencies than their mobilizable partner elements (20–21). A wide variety of mobilizable elements harboring the oriT and the relaxase- and CP-coding genes have been found (reviewed in references 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 bacteria that can be mobilized by their Mob relaxase when they coreside with an auxiliary self-transferable element (22–24). Whether the relationship between mobilizable and conjugative elements is considered parasitic or altruistic is arguable; it seems reasonable to propose mobilization as a strategy to travel around the microbial world at low cost.

**NATURE AND DIVERSITY OF MOBILIZABLE ELEMENTS**

Many MGEs share the ability to be transferred by conjugation between bacteria when they coreside 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 a platform where new events of bidirectional 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 (23), 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 intrachromosomal elements. In the former case, they constitute the so-called plasmidome (27), whereas within the MGEs that have an intrachromosomal location two categories can be distinguished: (i) integrative and mobilizable elements (28) and (ii) mobilizable conjugative transposons, which usually show nonspecific 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 integrative and mobilizable elements and mobilizable conjugative transposons retain the ability to integrate and excise from the host chromosome and, consequently, they do not necessarily have to be self-replicative. The extrachromosomal MGEs 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 pCl411), 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 mechanisms contribute to the promiscuity of a plasmid as shown for pMV158 (7) and for IncQ plasmids (35).

THE MOBILIZABLE RCR-ELEMENTS: SMALL PLASMIDS COULD DO IT

The finding that genetic elements could be transferred by conjugation between Gram-positive bacteria took a long while after the discovery of conjugation in Escherichia coli (36). In fact, the first reports on conjugation in Gram-positive bacteria were overcautious about naming “conjugation” to the transfer of genetic elements encoding antibiotic-resistance determinants (the current integrative and conjugative elements [ICES]), so they were referred to as “DNase-resistant transfer in filter mating assays” (37). Once it was 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 pLP501 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 cointegration with the helper, a process termed conduction (“piggyback”). Three relevant findings at the time were that (i) all RCR-plasmids studied generated large amounts of ssDNA intermediates, which were shown to be highly recombinogenic; (ii) some RCR-plasmids such as the staphylococcal plasmids pE194 and pT181 and, later on, pUB110 and pMV158 shared conserved sequences termed recombination site A (RSA) (22, 39); and (iii) plasmid-encoded proteins, termed Pre, could be responsible for the plasmid cointegration. These findings led to the proposal that the interaction of the RSA site and the Pre protein could play a role not only in plasmid maintenance but also in the distribution of small antibiotic-resistance plasmids among Gram-positive bacteria (22, 39). These Pre proteins were claimed to contain positively charged amino acids, which were considered to be probably involved in the binding of the Pre protein to the RSA site, as it is still found in some databases (http://www.uniprot.org/uniprot/P13015; http://www.ebi.ac.uk/interpro/entry/3PR001668). 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 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 that (ii) MobM behaved like a bona fide relaxase led to the conclusion that interplasmidic 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 such as 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 oriTs 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 oriTs of three RCR-plasmids by the pMV158-encoded MobM relaxase (41).

Historically, mobilizable plasmids have been studied in greater detail than the intrachromosomal 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 analyzing the available sequences of conjugative plasmids on the GenBank database; six MOB families were defined: MOBF, MOBH, MOBQ, MOBC, MOBP, and MOBV (20–21). Some remarkable conclusions from these studies were that (i) 39% of the prokaryotic plasmids could be conjugative (although it has been experimentally demonstrated only for some of them), 61.5% of which may be mobilizable; (ii) the majority of mobilizable plasmids are small in size (mean peak of 5 kb), but some could 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, MOBV is overrepresented in Firmicutes but rare in Proteobacteria and totally absent in Actinobacteria. Whereas MOBF and MOBH are almost absent, MOBV is the most frequent module in mobilizable plasmids, followed by MOBC, MOBP, and

**TABLE 1** RCR-plasmids from Gram-positive bacteria belonging to the MOBV family

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Accession Number</th>
<th>Size (bp)</th>
<th>Bacterial Source</th>
<th>Mob (aa)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMV158</td>
<td>NC_010096</td>
<td>5,540</td>
<td><em>Streptococcus agalactiae</em></td>
<td>494</td>
<td>47</td>
</tr>
<tr>
<td>pGB2001</td>
<td>NC_015973</td>
<td>4,967</td>
<td><em>S. agalactiae</em></td>
<td>500</td>
<td>137</td>
</tr>
<tr>
<td>pGB2002</td>
<td>NC_015971</td>
<td>6,825</td>
<td><em>S. agalactiae</em></td>
<td>500</td>
<td>137</td>
</tr>
<tr>
<td>pSS80</td>
<td>NC_019370</td>
<td>4,950</td>
<td><em>Streptococcus dysgalactiae</em></td>
<td>500</td>
<td>138</td>
</tr>
<tr>
<td>pSBO2</td>
<td>AB021465</td>
<td>3,582</td>
<td><em>Streptococcus equinus</em></td>
<td>527</td>
<td>139</td>
</tr>
<tr>
<td>pSM1A98</td>
<td>NC_016750</td>
<td>12,728</td>
<td><em>Streptococcus macedonicus</em></td>
<td>410</td>
<td>140</td>
</tr>
<tr>
<td>pRW5</td>
<td>NC_010423</td>
<td>4,968</td>
<td><em>Streptococcus pyogenes</em></td>
<td>500</td>
<td>83</td>
</tr>
<tr>
<td>pGA2000</td>
<td>NC_019252</td>
<td>4,967</td>
<td><em>S. pyogenes</em></td>
<td>500</td>
<td>137</td>
</tr>
<tr>
<td>pSSU1</td>
<td>NC_002140</td>
<td>4,975</td>
<td><em>Streptococcus suis</em></td>
<td>499</td>
<td>141</td>
</tr>
<tr>
<td>pSM0172</td>
<td>NC_004958</td>
<td>4,230</td>
<td><em>Streptococcus thermophilus</em></td>
<td>499</td>
<td>57</td>
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<tr>
<td>pER13</td>
<td>NC_002776</td>
<td>4,139</td>
<td><em>S. thermophilus</em></td>
<td>499</td>
<td>81</td>
</tr>
<tr>
<td>pE194</td>
<td>NC_005908</td>
<td>3,728</td>
<td><em>Staphylococcus aureus</em></td>
<td>403</td>
<td>142</td>
</tr>
<tr>
<td>pCPS49</td>
<td>NC_019142</td>
<td>5,292</td>
<td><em>S. aureus</em></td>
<td>409</td>
<td>143</td>
</tr>
<tr>
<td>pLA106</td>
<td>NC_004985</td>
<td>2,862</td>
<td><em>Lactobacillus acidophilus</em></td>
<td>411</td>
<td>144</td>
</tr>
<tr>
<td>pCD034-2</td>
<td>NC_016034</td>
<td>2,707</td>
<td><em>Lactobacillus buchneri</em></td>
<td>363</td>
<td>145</td>
</tr>
<tr>
<td>pRCieiD2.9</td>
<td>NC_017466</td>
<td>2,952</td>
<td><em>Lactobacillus casei</em></td>
<td>436</td>
<td>146</td>
</tr>
<tr>
<td>pWCZ</td>
<td>NC_019669</td>
<td>3,078</td>
<td><em>Lactobacillus paracasei</em></td>
<td>436</td>
<td>Unpublished</td>
</tr>
<tr>
<td>pTXW</td>
<td>NC_013952</td>
<td>3,178</td>
<td><em>L. paracasei</em></td>
<td>432</td>
<td>147</td>
</tr>
<tr>
<td>pMRI 5.2</td>
<td>NC_019900</td>
<td>5,206</td>
<td><em>Lactobacillus plantarum</em></td>
<td>408</td>
<td>148</td>
</tr>
<tr>
<td>pLFE1</td>
<td>NC_012628</td>
<td>4,031</td>
<td><em>L. plantarum</em></td>
<td>83</td>
<td>149</td>
</tr>
<tr>
<td>pLB4</td>
<td>M33531</td>
<td>3,547</td>
<td><em>L. plantarum</em></td>
<td>361</td>
<td>150</td>
</tr>
<tr>
<td>pA1</td>
<td>NC_010998</td>
<td>2,820</td>
<td><em>L. plantarum</em></td>
<td>103</td>
<td>55</td>
</tr>
<tr>
<td>pPB1</td>
<td>NC_006399</td>
<td>2,899</td>
<td><em>L. plantarum</em></td>
<td>355</td>
<td>58</td>
</tr>
<tr>
<td>pLS55</td>
<td>NC_010375</td>
<td>5,031</td>
<td><em>Lactobacillus sakei</em></td>
<td>439</td>
<td>151</td>
</tr>
<tr>
<td>pYS16</td>
<td>NC_010936</td>
<td>4,973</td>
<td><em>L. sakei</em></td>
<td>403</td>
<td>152</td>
</tr>
<tr>
<td>pGL2</td>
<td>NC_016981</td>
<td>4,572</td>
<td><em>Lactococcus garvieae</em></td>
<td>504</td>
<td>153</td>
</tr>
<tr>
<td>pBM02</td>
<td>NC_004930</td>
<td>3,854</td>
<td><em>Lactococcus lactis</em></td>
<td>304</td>
<td>154</td>
</tr>
<tr>
<td>pMBL000</td>
<td>NC_019353</td>
<td>3,370</td>
<td><em>Leuconostoc mesenteroides</em></td>
<td>361</td>
<td>155</td>
</tr>
<tr>
<td>pMA67</td>
<td>NC_010875</td>
<td>5,030</td>
<td><em>Paenibacillus larvae</em></td>
<td>435</td>
<td>156</td>
</tr>
</tbody>
</table>

| 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 harbor an open reading frame (ORF) that could encode a relaxase. |
| b | In plasmid pSBO2 the ORF-Mob is annotated as a protein of 144 residues (corresponding to coordinates 3149 to 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 pSS80 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. |
MOB\(\text{V}\). The following sections summarize the representative systems belonging to mobilizable RCR-plasmid families.

THE MOB\(\text{V}\) FAMILY

After years of research on mobilization of pMV158 and on its MobM-relaxase (24, 34), MobM is today the prototype of the MOB\(\text{V}\) family of relaxases (clade MOBV\(\text{V}\)) (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 Gram-positive and Gram-negative 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 Gram-positive bacteria by functions supplied by auxiliary plasmids such as pIP501 (51) or even between Gram-positive and Gram-negative 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 in references 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 (34) has increased 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 harbor a MOB cassette that includes a putative relaxase gene or an experimentally verified relaxase (*Table 1*). There are a few plasmids, such as pFX2, pCH411, and pXY3, that contain just an *oriT* but lack any recognizable relaxase gene. Another interesting exception was the case of plasmid pA1 from *Lactobacillus plantarum*, which appears to harbor a MOB cassette (*Table 1*). Nicking of supercoiled plasmid DNA by MobA was shown to be dependent on the presence of MobC and confers chloramphenicol resistance (59–60). They belong to the MOB\(\text{P}\) family (clade MOBP) (20) and can be mobilized by a coexpressed self-transmissible plasmid such as pG01 (61). The transfer module of pC221/pC223 contains the *oriT* and three overlapping genes required for the plasmid DNA processing and mobilization. The MobC and MobA reading frames overlap 16 nucleotides, whereas MobA and MobB overlap 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 toward their cognate DNAs (43). The *oriT* 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′-ACGACGC-3′ and 5′-CCAGTGCG-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 oriTs (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).

**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 in reference 9). Joint transfer of the staphylococcal plasmids pC194 and pUB110 by Tn916 from *B. subtilis* to *B. thuringiensis* has been reported (66). However, it appeared that pC194 was either not stable or nonreplicative in the recipient host, which raises the question of 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 Tn916. 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.
**PLASMID pMV158**

As stated above, the streptococcal plasmid pMV158 is the representative of the RepB-family of replicons and also of the MOBV family of relaxases. Due to its promiscuity (7) and to the numerous genetic tools based on it and developed for use 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<sub>pMV158</sub> 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 the 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<sub>pMV158</sub> a particularly complex configuration since most plasmids studied harbor only one or two IR sequences at their oriT<sub>s</sub> (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<sub>d</sub> ~ 60 nM) than to ssDNA-encompassing IR2 (K<sub>d</sub> > 320 nM). In fact, we have shown that on ssDNA substrates the minimal oriT<sub>pMV158</sub> 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 harbors 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 conjugal 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

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**FIGURE 2** 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 ssoA have the possibility of generating long hairpin-loop structures exhibited as “ssDNA promoters” (15, 18) where the RNAP-binding site (RSB) 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. doi:10.1128/microbiolspec.PLAS-0008-2013.f2
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 MOBV family (clade MOBV2), it was shown that none of its Tyr residues was involved in transfer (71). This finding suggested that perhaps the MOBV 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 MOBV family of relaxases.

Interestingly, MobM was able to relax supercoiled DNA of other RCR-plasmids from the MOBV family (41). However, the efficiency of cleavage depended upon the homologies of the oriTs: 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 superhelicity 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-RepB initiator of replication protein (73). However, compared to MobMN199, the 44 additional residues present in MobMN243, which are predicted to form an extra α-helical region, were found to be essential for efficient ssDNA cleavage (73). Biological studies showed that the thermal stability of MobMN199 increased in the presence of Mn²⁺ 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. 3). Two promoters placed nearly in tandem, termed Pmob1 and Pmob2, are used by the host RNAP: the latter is preferentially used in E. coli (76), whereas the former is used in Lactococcus lactis (51).

**FIGURE 3** 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 (blue) (76). Promoter Pmob1 (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 footprinting assays using linear double-stranded DNAs (42, 76). MobM is able to repress in vivo the transcription initiated from both promoters, Pmob1 and Pmob2 (76). doi:10.1128/microbiolspec.PLAS-0008-2013.f3
Whereas promoter $P_{mob1}$ was mapped to reside within oriT$_{pMV158}$, promoter $P_{mob2}$ was found to be adjacent to it; 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. 3), 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 posttranscriptional level by one or two antisense RNAs (78). However, there are instances, such as replication of pMV158 or of pLP501, 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 yet know 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.

**FACTORS CONTRIBUTING TO THE PROMISCUITY OF RCR-PLASMIDS**

The RCR-plasmids are constituted of a small number of gene modules and, in general, show unusual promiscuity (84). Their capacity to colonize new hosts depends on their ability to (i) express their Rep initiator protein and trigger overreplication until they surpass their average copy number (around 20 to 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 constitute a genetic load, and making a clever use of the host resources” applies to RCR-plasmids (84). Their replication needs the Rep initiator that 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 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 Gram-positive bacterial hosts (49, 90), and also in Gram-negative bacteria such as *E. coli* (50, 91–92). Some of the RCR-plasmids, such as 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 with their replication functions (94–98), especially the functionality of the plasmid *sso* (99–100). The lagging strand origins of the RCR-plasmids are located at specific noncoding regions that can fold into imperfect hairpin structures on the ssDNA plasmid intermediates (Fig. 2). 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 harbors 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$_{RSB}$), 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′-TAGC Gt/a-3′), which is a transcriptional terminator for the synthesis of a small primer RNA by the host RNAP (15; see Fig. 2). 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 very 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. 2). 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).

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 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 harbor 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 T4SS relaxosome. A second example of possible COINS has been provided: conjugation of plasmid F was shown to be inhibited by filamentous bacteriophages (such as 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 a natural source of proteins that can compete with the conjugative T4SS, thus acting like bona fide COINS. Using plasmid F again, 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, such as F, that encode multityrosine relaxases and that are represented by the MOB family (20). This may not be the general feature for all plasmid-encoded relaxases, so bisphosphonates may be considered 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

**TABLE 2** Indicators of pMV158 promiscuity: relative efficiencies of conjugative transfer from donor S. pneumoniae to recipients S. pneumoniae and E. faecalis

<table>
<thead>
<tr>
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<th>S. pneumoniae</th>
<th>E. faecalis</th>
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</thead>
<tbody>
<tr>
<td>pMV158</td>
<td>Transfer^a</td>
<td>Copy Number^b</td>
</tr>
<tr>
<td>Wild type</td>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td>ΔssoA</td>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td>ΔssoU</td>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td>ΔssoA/ssoU</td>
<td>0.01</td>
<td>10</td>
</tr>
</tbody>
</table>

^a Relative transfer in comparison to the pMV158 wild-type plasmid.
^b Number of plasmid molecules per chromosomal equivalent.
^c Ratio of ssDNA/dsDNA, indicative of efficiency of conversion.
single-chain antibodies (108). All the above approaches may not be of wide usefulness, so 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.

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 Gram-positive bacteria, especially because they were scarce or nonexistent compared to those developed for Gram-negative 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 horizontal gene transfer 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.

Construction of Green Fluorescent Bacteria by Using the pMV158 Replicon

The plasmid pMV158 has been employed to construct useful genetic tools for Gram-positive bacteria in both versions, mobilizable and nonmobilizable vectors. In the latter case, a derivative of pMV158 lacking the mobM gene (plasmid pLS1) was used to construct vectors that harbor 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 (PM) that is inducible by maltose. The MalR transcriptional repressor controls the activity of the PM 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 harboring 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 PM 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 PM (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 Gram-positive bacteria (such as E. faecalis), the gfp gene is expressed constitutively (Fig. 4). The 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 Enterococcus durans to human Caco-2 cells (122), (v) quantitative analysis of the intra- and interspecies conjugal transfer of pMV158 (123), and (vi) quantification of adhesion of GFP 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 harboring the 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 PM 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).
Construction of a Promoter-Probe Vector Based on the pMV158 Replicon

Promoter-probe vectors have been shown to be very useful for studies of the 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 it 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 Gram-positive bacteria. Ruiz-Cruz et al. (128) designed the pAST plasmid vector (5456 bp) (Fig. 6) that derives from a nonmobilizable pMV158 replicon (plasmid pLS1). The vector pAST contains a highly efficient transcriptional terminator.

FIGURE 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 constitutive *gfp* after growth in sucrose. See text for details. doi:10.1128/microbiolspec.PLAS-0008-2013.f4

FIGURE 4  continues on next page
signal (the tandem terminators \(T1\) and \(T2\) of the \(E. coli\) \(rrnB\) 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 Gram-positive 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).

**Expression Vectors Based on the pMV158 Replicon**

The vector pLS1ROM (6805 bp) is a nonmobilizable 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 an 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.

The 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. The plasmid pAST-\(Pung\) (5615 bp) was constructed by cloning the promoter region (\(Pung\)) of the pneumococcal \(ung\) gene (uracil-DNA glycosylase) into the \(BamHI\) site of the pAST promoter-probe vector \((\text{Fig. 6})\). The promoter \(Pung\) was shown to increase 10-fold the expression of

**FIGURE 4 (continued)**
the *gfp* gene in pneumococcal cells (fluorescence assays). The plasmid pAST-*Pung* has an 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 the 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

**FIGURE 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 0.25 mM levamisol before imaging. DIC and fluorescence microscopy were used to visualize the worms. GFP expressing bacteria and autofluorescence generated by lipofuscin granules in the body of the worm were observed using FITC and DAPI filters, respectively. doi:10.1128/microbiolspec.PLAS-0008-2013.f5

**FIGURE 6** Genetic maps of plasmid pLS1 (a nonmobilizable 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. coli* rrnB rRNA operon; SD, translation initiation signals optimized for the expression of the *gfp* gene in prokaryotes (114). The positions of the *PfcsK* and *Pung* promoters are indicated. *H*, *HindIII*; X, *XbaI*; B, *BamHI*; S, *SacI*. doi:10.1128/microbiolspec.PLAS-0008-2013.f6
fucose (128). Since the putative fucose regulator gene fcsR is widely conserved in S. pneumoniae strains (131), it can be expected that the plasmid pAST-PfcsK will be valuable as an 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.

**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 Gram-positive 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 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 multiwell plates under the same or different conditions, reducing experimental variations and hands-on time. For instance, mobilization of the plasmid pMV158GFP between different Gram-positive bacteria has been analyzed by using microtiter plates coupled to a filtration device with sterile 0.22-μm filters (Fig. 7) to quantify (i) intraspecies plasmid transfer, where the R61 pneumococcal donor strain harboring pLP501 was mated with another pneumococcal recipient strain (carrying different selectable markers), and (ii) interspecies 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-unit counting. The frequency of transfer was high enough to be quantified: 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 (such as S. pneumoniae) as donors and aerobic bacteria (i.e., E. faecalis) as recipients. This improves counterselection 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 analyze the parameters involved in interspecies RCR-plasmid transfers as well as to test lead compounds that could act as COINS.

**FIGURE 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 frequency 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 Lorenzo-Díaz F, Espinosa M, 2009. Large-scale filter mating assay for intra- and inter-specific conjugal transfer of the promiscuous plasmid pMV158 in Gram-positive bacteria. *Plasmid* 61:65–70 [123]. Copyright 2009, with permission from Elsevier.) doi:10.1128/microbiolspec.PLAS-0008-2013.17

**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 Gram-positive 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 about the massive transfer of resistance genes to many pathogens. Since these “minimal” T4SSs are generally associated with MGEs (such as Tn916, ICEs, pathogenicity islands, and plasmids), the risks of explosive outbreaks of Gram-positive-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 study of their transfer properties. One should not take lightly the assumption that all mobilizable RCR-plasmids will be
transferred by the same mechanism or will have only one type of relaxase or CP, so that “once known one, we know them all” is a poor scientific philosophy.

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