



# Cis-Acting Relaxases Guarantee Independent Mobilization of MOB<sub>Q4</sub> Plasmids

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Plasmids are key vehicles of horizontal gene transfer and contribute greatly to bacterial genome plasticity. In this work, we studied a group of plasmids from enterobacteria that encode phylogenetically related mobilization functions that populate the previously non-described MOB<sub>Q4</sub> relaxase family. These plasmids encode two transfer genes: *mobA* coding for the MOB<sub>Q4</sub> relaxase; and *mobC*, which is non-essential but enhances the plasmid mobilization frequency. The origin of transfer is located between these two divergently transcribed *mob* genes. We found that MPF<sub>I</sub> conjugative plasmids were the most efficient helpers for MOB<sub>Q4</sub> conjugative dissemination among clinically relevant enterobacteria. While highly similar in their mobilization module, two sub-groups with unrelated replicons (Rep\_3 and ColE2) can be distinguished in this plasmid family. These subgroups can stably coexist (are compatible) and transfer independently, despite origin-of-transfer cross-recognition by their relaxases. Specific discrimination among their highly similar *oriT* sequences is guaranteed by the preferential *cis* activity of the MOB<sub>Q4</sub> relaxases. Such a strategy would be biologically relevant in a scenario of co-residence of non-divergent elements to favor self-dissemination.

**Keywords:** mobilizable plasmids, horizontal gene transfer, MOB<sub>Q</sub> relaxase, *cis*-acting relaxase, plasmid coexistence, bacterial conjugation

## INTRODUCTION

Mobilizable plasmids are small genetic elements transmissible by conjugation with the assistance of a helper conjugative plasmid. They encode a relaxase, and usually a relaxase accessory protein (RAP), which are in charge of the conjugative DNA processing at a specific site of the origin of transfer (*oriT*) called *nic*. Mobilizable plasmids lack the transfer genes required for establishing a conjugative bridge (mating pair formation system, MPF) to the recipient cell, as well as the type IV coupling protein (T4CP) that puts in contact relaxosome and MPF and thus depend on conjugative plasmids to be transferred (Garcillán-Barcia and de la Cruz, 2013).

According to their relaxase, transmissible plasmids were phylogenetically classified into MOB families (Francia et al., 2004; Garcillán-Barcia et al., 2009). Currently, nine relaxase MOB classes are defined, and five of them (MOB<sub>P</sub>, MOB<sub>F</sub>, MOB<sub>Q</sub>, MOB<sub>H</sub>, and MOB<sub>C</sub>) are prevalent in transmissible plasmids hosted in  $\gamma$ -Proteobacteria. Plasmids gathered in a relaxase MOB family share similar genomic traits. Relaxase MOB classification has thus shown to be a good predictor of the plasmid backbone (Garcillán-Barcia and de la Cruz, 2013; Fernandez-Lopez et al., 2017). Mobilizable plasmids resident in  $\gamma$ -Proteobacteria form phylogenetically related clusters mainly within two relaxase MOB classes: MOB<sub>P</sub> and MOB<sub>Q</sub> (Garcillán-Barcia et al., 2009). Relevant examples are ColE1-like plasmids, grouped in family MOB<sub>P5</sub>; IncQ1 plasmids, such as RSF1010/R1162, gathered in MOB<sub>Q11</sub>; and IncQ2 plasmids, such as pTC-F14, in family MOB<sub>P14</sub> (Garcillán-Barcia et al., 2009; Garcillán-Barcia and de la Cruz, 2013). An additional clade of small plasmids encoding MOB<sub>Q</sub> relaxases, previously classified as MOB<sub>Qu</sub>, and here redefined as MOB<sub>Q4</sub>, was observed in a phylogenetic reconstruction of this relaxase family (Garcillán-Barcia et al., 2009).

A pair of degenerate primers specific for MOB<sub>Q4</sub> plasmids was implemented in the Degenerate PCR MOB Typing (DPMT) approach developed by Alvarado et al. (2012) to detect and classify transmissible plasmids. This method revealed the abundance of MOB<sub>Q4</sub> plasmids in clinical isolates of enterobacteria (Alvarado et al., 2012; Garcillán-Barcia et al., 2015), previously unnoticed by other plasmid typing methods. Whole-genome sequencing of clinical *E. coli* isolates also uncovered the presence of this kind of plasmids (Brolund et al., 2013; de Toro et al., 2014; Lanza et al., 2014). Prototype plasmids pIGWZ12 and ColE9-J (ColE2-like) cluster within the MOB<sub>Q4</sub> clade. They are stable, theta-replicating, high copy-number, narrow host-range plasmids, whose replication systems have been extensively studied (Yasueda et al., 1989, 1994; Yagura et al., 2006; Zaleski et al., 2006, 2015). Here, we uncovered the diversity of MOB<sub>Q4</sub> plasmids, determined the helper conjugative plasmids responsible for their dissemination, and established their behavior in terms of stability and transfer.

## MATERIALS AND METHODS

### Plasmid Construction

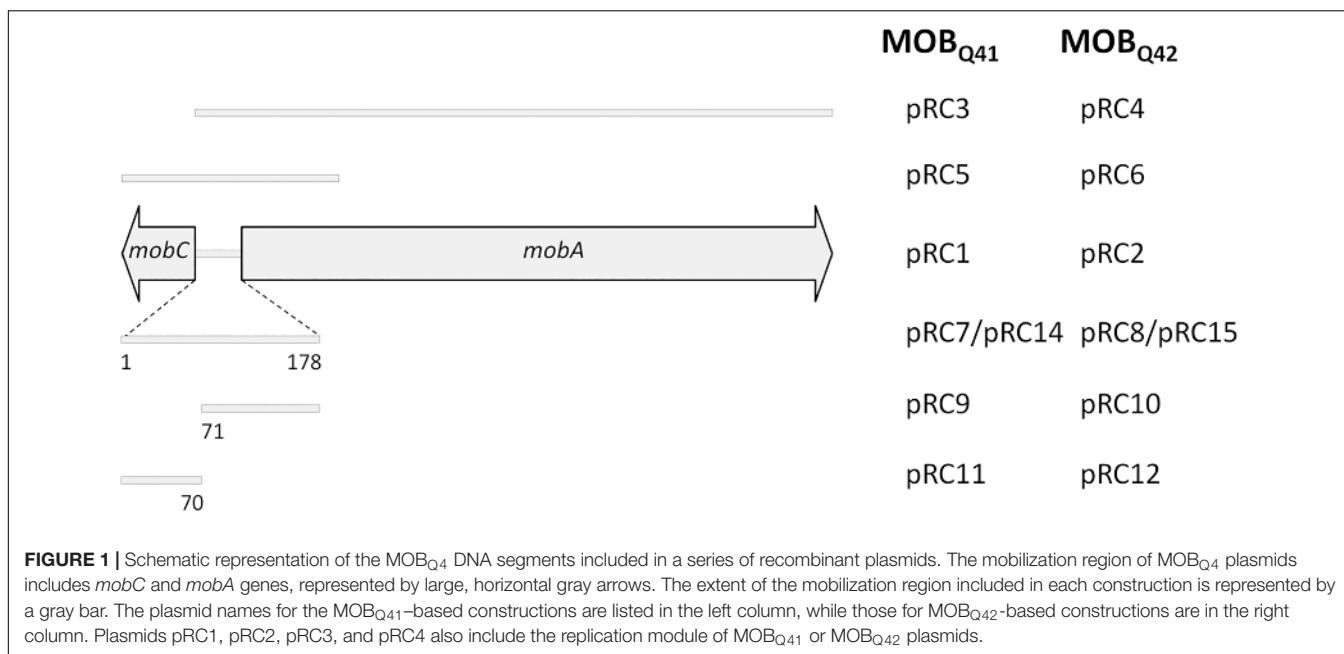
MOB<sub>Q4</sub> plasmid derivatives were constructed by isothermal assembly of linear DNA fragments from PCR reactions, following the Gibson method (Gibson et al., 2009, 2015). The MOB<sub>Q41</sub> backbone (replication and mobilization regions), based on the complete sequence of the pE2022\_4 plasmid [GenBank Acc. No. KT693143 (Lanza et al., 2014)], was linked to a kanamycin-resistance gene [coordinates 272 to 1216 of pSEVA211, GenBank Acc. No. JX560326 (Silva-Rocha et al., 2013)] and a cerulean fluorescent protein gene [coordinates 41 to 1091 of pNS2- $\phi$ VL (Dunlop et al., 2008)], generating plasmid pRC1. The MOB<sub>Q42</sub> backbone (replication and mobilization regions) was obtained by PCR amplification from the *E. coli* isolate HUMV 04/979 (Garcillán-Barcia et al., 2015), which contains

a ColE9-J-like plasmid (coordinates 5102 to 7577, GenBank Acc. No. NC\_011977.1). It was joined to a chloramphenicol resistance gene (coordinates 272–1072 of pSEVA311, GenBank Acc. No. JX560331 (Silva-Rocha et al., 2013)] and mCherry fluorescent protein gene (*cfp*, coordinates 1092–2117 of pNS2- $\phi$ VL (Dunlop et al., 2008)], generating plasmid pRC2. MOB<sub>Q4</sub> plasmids lacking the *mobC* ORF (from start to stop codon) were constructed by self-ligation of a single PCR fragment from either pRC1 or pRC2, producing plasmids pRC3 and pRC4, respectively.

Additional plasmids were constructed to delimit the *oriT* region. A schematic representation of the fragments included in each construction is depicted in **Figure 1**. Such fragments were individually assembled to coordinates 1–1030 and 1360–3001 of vector pSEVA631 (GenBank Acc. No. JX560348). Plasmids pRC5 and pRC6 contained a fragment including the *mobC* gene, the 178bp intergenic region between *mobC* and *mobA* and the first 400 nucleotides of the *mobA* gene from pRC1 and pRC2, respectively. Plasmids pRC7 and pRC8 included only the 178bp intergenic fragment (**Supplementary Figure S1**), located between genes *mobA* and *mobC* of pRC1 and pRC2, respectively. Plasmids pRC14 and pRC15 contain the *oriT* regions of pRC7 and pRC8 but cloned in the inverse orientation. Plasmids pRC11 and pRC9, respectively included portions 1–70 and 71–178 of the intergenic fragment between genes *mobA* and *mobC* of pRC1, while the same portions from pRC2 were included in pRC12 and pRC10, respectively. A pSEVA631 fragment containing coordinates 1–1030 and 1360–3001 was self-ligated, generating the non-mobilizable vector pRC13, which was used as a control in the mating experiments.

### Stability Assays

Plasmids pRC1 and pRC2 were introduced in the *recA*<sup>+</sup> and *recA*<sup>-</sup> isogenic strains UB1636 (F<sup>-</sup> *lys his trp rpsL*) (Achtman et al., 1971) and UB1637 (F<sup>-</sup> *lys his trp rpsL recA56*) (de la Cruz and Grinsted, 1982), either independently to check for their stability or both together to check for their compatibility. Single colonies were inoculated in Lysogeny-Broth (LB) supplemented with kanamycin at 50  $\mu$ g/ml (for pRC1-containing strains) or chloramphenicol at 25  $\mu$ g/ml (for pRC2-containing strains) and grown to saturation at 37°C with agitation (150 rpm). A volume of 9.7  $\mu$ l was transferred from saturated cultures to 10 mL of fresh LB media without antibiotics and grown to saturation in the same conditions. Rounds of transfer and growth were repeated up to 80 generations. The proportion of plasmid-bearing cells in the population was monitored by replica-plating 100 colonies in LB-agar supplemented with the appropriate antibiotics every 10 generations. A larger number of cells was inspected by fluorescence microscopy and, in the case of pRC1-containing cells, also by flow cytometry. Live cells were visualized using a Leica AF6500 microscope at 63x magnification. CFP and mCherry signals were monitored using BP filters (Excitation 434/17 – Emission 479/40 for CFP, Excitation 562/40 – Emission 641/75 for mCherry). Images were obtained using an iXon885 EM CCD Camera (Andor) and up to 1000 cells were analyzed in each case. Fluorescence emission was measured by flow cytometry using a FACS Canto II flow



cytometer (Becton Dickinson) equipped with a 488 nm solid state laser for excitation. The cyan fluorescence of 20,000 events was detected using a 525/20 filter.

## Mating Assays

Conjugative plasmids used in this work are listed in **Supplementary Table S1**. They were tested as helpers of the MOB<sub>Q4</sub> plasmids in surface mating experiments, following the procedure described by del Campo et al. (2012). *E. coli* strain DH5 $\alpha$  (F<sup>-</sup> *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20  $\phi$ 80dlacZ $\Delta$ M15  $\Delta$ (lacZYA-argF)U169, hsdR17(*rK*<sup>-</sup> *mK*<sup>+</sup>),  $\lambda$ <sup>-</sup>) (Grant et al., 1990) containing different plasmid combinations was used as donor and BW25113 (*lacI*<sup>q</sup> *rrnBT14  $\Delta$ lacZWJ16 hsdR514  $\Delta$ araBADAH33  $\Delta$ rhaBADLD78*), BW25993 (*lacI*<sup>q</sup> *hsdR514  $\Delta$ araBADAH33  $\Delta$ rhaBADLD78*) (Datsenko and Wanner, 2000) as recipient. Donor and recipient strains were mixed in a 1:1 ratio, deposited onto an LB-agar surface and incubated for 1 h at 37°C (except when drR27 was used as a helper, in which case matings were carried out at 25°C). Then, the mixture was resuspended in LB and plated in the presence of appropriate antibiotics. Conjugation frequencies were expressed as the number of transconjugants per donor cell.*

## Phylogenetic Analysis

The 300 N-terminal residues of the MobA relaxase of plasmid Cole9-J were used as a query in a BLASTP search (Altschul et al., 1997) (*e*-value: 1x E-3). The homologous sequences were aligned using MUSCLE (Edgar, 2004). TrimAl v1.4 was used to calculate the average identity between sequences in the alignment (Capella-Gutiérrez et al., 2009). ProtTest 3 was used to estimate the best model of protein evolution for our set (Guindon and Gascuel, 2003; Darriba et al., 2011). RAxML version 7.2.7 (Stamatakis, 2006) was used for phylogenetic reconstruction.

Using the JTTGAMMA model 10 maximum likelihood (ML) searches trees were inferred and support values were assigned to each node of the best tree from 1000 bootstrap searches. Relaxase of the pXF5847 plasmid (GenBank Acc. no. YP\_009076807.1) was used as outgroup.

## 3D Structure Prediction

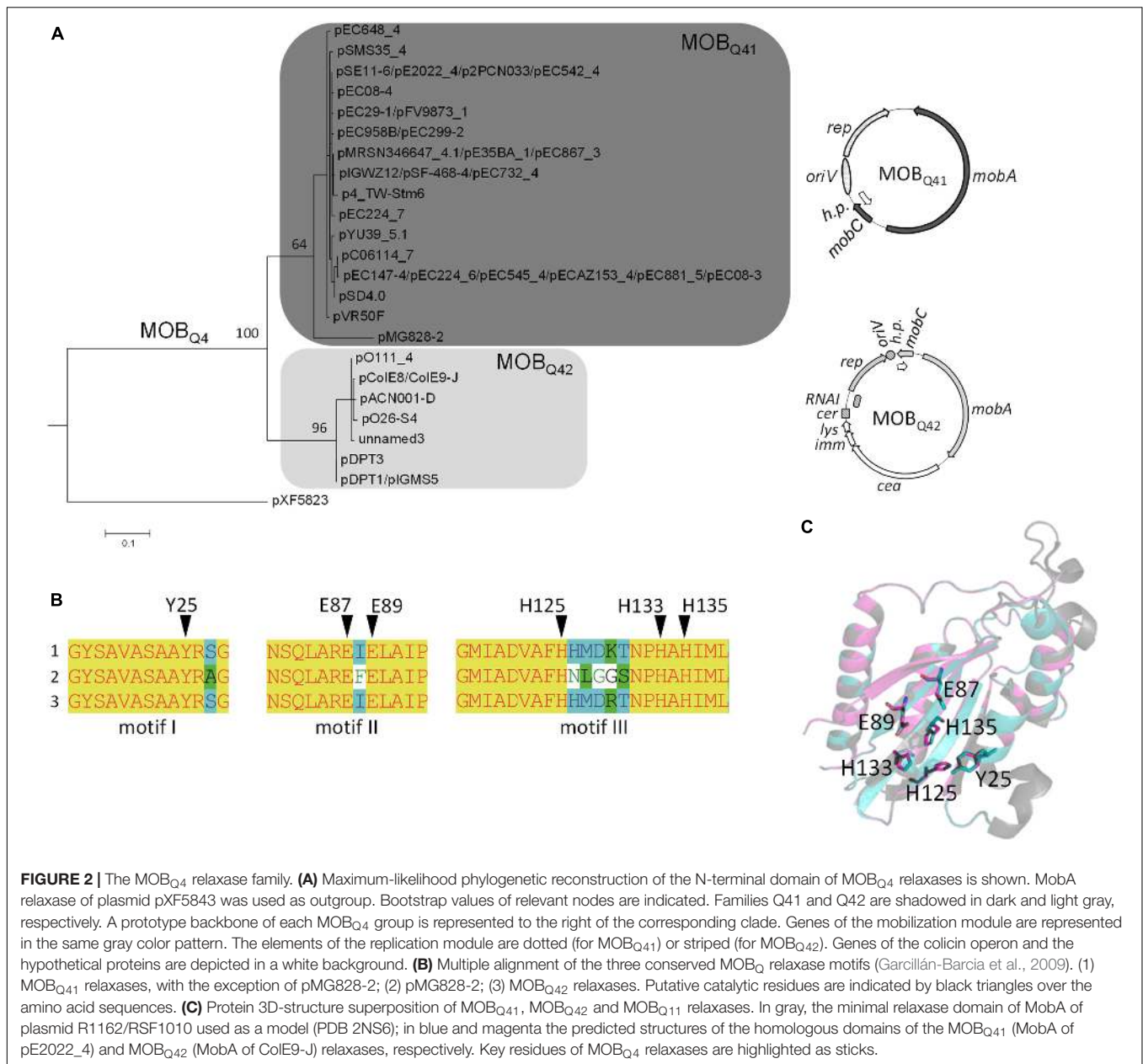
Phyre2 was used to predict the 3D structure of the MobA relaxase domains of plasmids pE2022\_4 and Cole9-J (Kelley et al., 2015), which were visualized using PyMOL (Schrödinger, 2015).

## RESULTS AND DISCUSSION

### Analysis of MOB<sub>Q4</sub> Plasmids

MOB<sub>Q</sub> is a broad relaxase class that encompasses several families, each of which includes related plasmid backbones: MOB<sub>Q1</sub> comprises relaxases of mobilizable broad host-range IncQ1-like plasmids; MOB<sub>Q2</sub>, conjugative relaxases of pTi and many rhizobial plasmids; MOB<sub>Q3</sub>, conjugative broad host-range plasmids resident in gram-positive, such as pIP501 (Garcillán-Barcia et al., 2009). In this previous study, many MOB<sub>Q</sub> plasmids were not ascribed to a specific subclassification due to either low resolution of the clades or lack of information on the plasmid members. Here, we focused on one of these poorly defined clades, now named MOB<sub>Q4</sub>, prompted by the fact that these relaxases have been recurrently detected in enterobacterial clinical isolates (Alvarado et al., 2012; Brolund et al., 2013; de Toro et al., 2014; Lanza et al., 2014; Garcillán-Barcia et al., 2015).

The phylogenetic reconstruction, based on the first N-terminal 300 residues of MOB<sub>Q4</sub> relaxases produced two clusters, MOB<sub>Q41</sub> and MOB<sub>Q42</sub> (**Figure 2A** and **Supplementary Table S2**). This relaxase domain contains the three relaxase motifs (**Figure 2B**)



and share 84% average amino acid identity (97 and 90% for individual MOB<sub>Q41</sub> and MOB<sub>Q42</sub> groups, respectively). The 3D structure prediction of the relaxase domain of MOB<sub>Q41</sub> and MOB<sub>Q42</sub> plasmids rendered MOB<sub>Q4</sub> relaxases NES [plasmid pLW1043, PDB Acc. No. 4HT4 (Edwards et al., 2013)] and MobA [plasmid R1162/RSF1010, PDB Acc. No. 2NS6, (Monzingo et al., 2007)] as best hits (100% confidence). The superimposed structures pointed to MOB<sub>Q4</sub> amino acids Y25 (motif I), E87 and E89 (motif II), and H125, H133 and H135 (motif III) as homologs of the MobA<sub>R1162</sub> catalytic residues Y25, E74 and E76, and H112, H120 and H122, respectively (Figure 2C). Contrary to the high conservation of the N-terminal domain among members of both MOB<sub>Q4</sub> subgroups, the amino acid identity of the C-terminal part of the MOB<sub>Q4</sub> relaxases dropped

to 35%. This C-terminal domain exhibited low homology to SogL primases of IncII plasmids.

Each MOB<sub>Q4</sub> subclade groups highly related backbones (Figure 2A). MOB<sub>Q41</sub> are cryptic, small-size plasmids (Supplementary Table S2). Their backbone contains only four genes encoding a replication initiation protein (Rep), a relaxase (MobA), a putative relaxase accessory protein (MobC) and a hypothetical protein. The genes for the last two are generally not annotated. Besides the above-mentioned replication and mobilization genes, MOB<sub>Q42</sub> plasmids also contain a colicin operon, including colicin, immunity and lysis genes, following the synteny of Group A nuclease colicins (Cascales et al., 2007). Plasmids ColE9-J and pO111\_4 contain a second, partial colicin operon.

The MOB<sub>Q4</sub> subdivision in two relaxase groups matches with the presence of two different replicons (**Supplementary Table S2**) and this family thus encompasses at least two plasmid species as defined by Fernandez-Lopez et al. (2017). MOB<sub>Q41</sub> plasmids encode a replication initiation protein that belongs to the Rep\_3 superfamily [PF01051 in the Pfam classification (Finn et al., 2016)], with no defined group in the PlasmidFinder classification (Carattoli et al., 2014). MOB<sub>Q42</sub> plasmids encode ColE2-like initiators (Pfam PF03090 + PF08708), classified as Col156 by PlasmidFinder. Plasmids pIGWZ12 and ColE9-J exemplify each cluster. They are stable, theta-replicating, high copy number plasmids (15 and 10 copies per chromosome molecule, respectively) (Takechi et al., 1994; Zaleski et al., 2012). The origin of replication of plasmid pIGWZ12 was located upstream the *rep* gene. It contains iterons, an A+T rich region and four DnaA boxes (Zaleski et al., 2006, 2015). The iterons were found to be the incompatibility determinants (Zaleski et al., 2015). ColE2-like plasmids, such as ColE9-J, form a group of closely related elements that share an identical priming mechanism, mediated by the plasmid-encoded Rep protein (Horii and Itoh, 1988; Itoh and Horii, 1989; Yasueda et al., 1989; Hiraga et al., 1994). The origin of replication consists of 32 bp located downstream of the *rep* gene, containing two directly repeated sequences (Kido et al., 1991; Nomura et al., 1991; Yagura and Itoh, 2006; Yagura et al., 2006). In ColE2-like plasmids, the *rep* gene expression is post-transcriptionally controlled by a plasmid-encoded RNA (*RNAI*), which binds the untranslated 5' region of the *rep* mRNA, preventing its translation (Sugiyama and Itoh, 1993; Takechi et al., 1994; Yasueda et al., 1994). MOB<sub>Q42</sub> plasmids contain a *cer*-like site (Hiraga et al., 1994), an indication that they use a host site-specific recombination system for resolving multimers to monomers as ColE1-like plasmids do (Summers and Sherratt, 1984, 1988; Summers, 1998).

All completely sequenced MOB<sub>Q4</sub> plasmids come from hosts of the Enterobacteriaceae family (**Supplementary Table S2**). They were isolated from different backgrounds: *Salmonella enterica* isolated from pork meat (pSD4.0) (Bleicher et al., 2013), pork feces (p4\_TW-Stm6) (Dyall-Smith et al., 2017) and human systemic infection (pYU39\_5.1) (Calva et al., 2015), multidrug-resistant environmental *E. coli* (pSMS35\_4) (Fricke et al., 2008), commensal *E. coli* (pSE11-6) (Oshima et al., 2008), enterohemorrhagic *E. coli* strains of the O26 and O111 serogroups (pO26-S4 and pO111\_4) (Ogura et al., 2009; Fratamico et al., 2011), extended-spectrum beta-lactamase producing *E. coli* clinical isolates (pE2022\_4, pFV9873\_1, pEC147-3 and pEC08-6) (Brolund et al., 2013; Lanza et al., 2014), *E. coli* isolated from human urinary tract (pVR50F) (Beatson et al., 2015) and bloodstream infections (pSF-468-4) (Stephens et al., 2015), as well as porcine extraintestinal pathogenic *E. coli* strain (p2PCN033) (Liu et al., 2015), among others (**Supplementary Table S2**). None of these plasmids contain antibiotic-resistance genes. There is still no clue on the selective advantage provided by the cryptic MOB<sub>Q41</sub> plasmids. In the case of MOB<sub>Q42</sub> plasmids, the fact that all carry colicin operons, *a priori* an advantageous trait for the bacterial host, could explain the abundance of this type of plasmids. For example, the MOB<sub>Q42</sub> plasmid pDPT1 was stably acquired by a Vietnamese *Shigella*

*sonnei* strain in the mid-1990s, and became fixed in the evolving bacterial population (Holt et al., 2013). The colicin E5 produced by pDPT1 was highly bactericidal against non-immune *Shigella* and *E. coli* strains. The acquisition of the pDPT1 colicin plasmid, coinciding with the high increase of dysentery produced by this strain, suggests that pDPT1 conferred a beneficial function to its host (Holt et al., 2013).

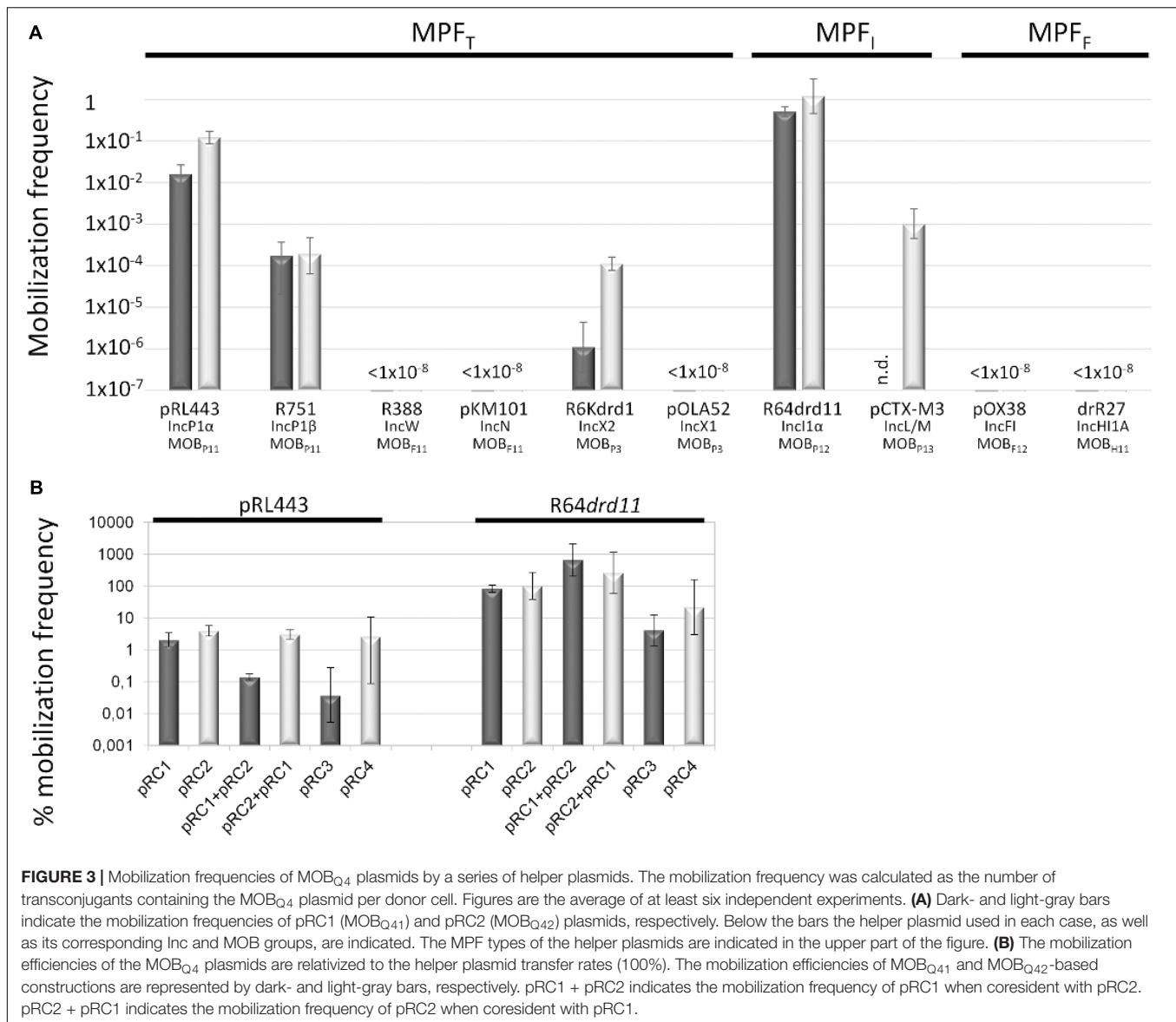
## Stability and Co-residence of MOB<sub>Q4</sub> Plasmids

To study the MOB<sub>Q4</sub> plasmids, two derivatives were constructed, pRC1 and pRC2. They included the replication and mobilization modules of the MOB<sub>Q41</sub> and MOB<sub>Q42</sub> backbones, respectively. Antibiotic-resistance and fluorescent protein genes were also included as reporters. Plasmid stability and compatibility were assayed in *recA*<sup>+</sup> and *recA*<sup>-</sup> *E. coli* strains by propagating the plasmids either alone or in combination during 80 generations. Despite the cargoes loaded in plasmids pRC1 and pRC2, the percentage of plasmid retention in the bacterial population was 100%, suggesting that the MOB<sub>Q4</sub> backbone confers a minimized fitness cost to its enterobacterial host (San Millan and MacLean, 2017). Besides stability in *E. coli*, both MOB<sub>Q4</sub> plasmid species also exhibited full compatibility (100% retention of both after 100 generations), as could be expected due to their different replicons (Novick, 1987), and ruling out other plasmid-encoded traits out of the replication module that could interfere with the stable vertical inheritance of each other.

## Mobilization of MOB<sub>Q4</sub> Plasmids by Different MPF Systems

Since mobilizable plasmids do not encode the mating pair formation system neither the T4CP, their transfer relies on auto-transmissible plasmids. We wondered which conjugative plasmids could be responsible for the dissemination of the MOB<sub>Q4</sub> plasmids. Not all conjugative plasmids are equally efficient at supplying these functions to a specific mobilizable plasmid (Cabezón et al., 1994, 1997). The contacts established between the relaxosome of the mobilizable plasmid and the T4CP-MPF of the helper plasmid are crucial in the transfer process. ColE1-like MOB<sub>P5</sub> plasmids are efficiently mobilized by IncF-MOB<sub>F12</sub> (e.g., F) and IncI1-MOB<sub>P12</sub> (e.g., R64*drd11*) plasmids (Cabezón et al., 1997). IncQ1-MOB<sub>Q1</sub> plasmids, such as RSF1010, are transferred by IncP1-MOB<sub>P11</sub> helper plasmids (e.g., RP4) (Cabezón et al., 1997; Meyer, 2009). pMV158-like plasmids (MOB<sub>V1</sub>) are mobilized by IncP1-MOB<sub>P11</sub> and Inc18-MOB<sub>Q3</sub> (e.g., pIP501) plasmids (Lorenzo-Díaz et al., 2014).

We looked for reports providing indirect evidence on MOB<sub>Q4</sub> plasmid mobilization through conjugation. In a survey for the presence of transmissible plasmids in a multidrug *E. coli* collection, MOB<sub>Q4</sub> transconjugants were obtained from seven out of the eight MOB<sub>Q4</sub> containing clinical isolates (Garcillán-Barcia et al., 2015). In all cases, a MOB<sub>P12</sub>-MPF<sub>I</sub> plasmid, presumptively the helper, was also present in both, donor and transconjugant cells. Similarly, the MOB<sub>Q41</sub> plasmid pSD4.0 and the IncI1 plasmid pSD107 were found in *E. coli*



transconjugants arisen from a mating with *Salmonella enterica* (Bleicher et al., 2013).

Three conjugative MPF types (MPF<sub>T</sub>, MPF<sub>F</sub>, and MPF<sub>I</sub>) are prevalent in Enterobacteriaceae (Smillie et al., 2010; Guglielmini et al., 2014), the taxonomic family where MOB<sub>Q4</sub> plasmids have been found. In this study, a set of conjugative plasmids representative of these MPF families were tested as helpers for the mobilization of MOB<sub>Q4</sub> plasmids (**Supplementary Table S1**). Not all of them were equally efficient (**Figures 3A,B** and **Supplementary Table S3**). R64drd11, the prototype of IncI $\alpha$ -MOB<sub>P12</sub> plasmids, which encodes a MPF<sub>I</sub> conjugative apparatus, was the most efficient helper. Another MPF<sub>I</sub> plasmid, pCTX-M3 (IncL/M-MOB<sub>P13</sub>), was also an efficient helper. Co-residence with MPF<sub>I</sub> plasmids has been reported for the MOB<sub>Q4</sub> plasmids pSE11-6 (Oshima et al., 2008), pSD4.0 (Bleicher et al., 2013), pEC147-4 (Brolund et al., 2013), pO26-S4

(Fratamico et al., 2011), pDPT1 (Holt et al., 2013), and pE2022\_4 (Lanza et al., 2014).

On the other hand, MPF<sub>F</sub>-type plasmids [e.g., IncF-MOB<sub>F12</sub> (F) or IncHI1-MOB<sub>H11</sub> (R27) plasmids], which show high prevalence in enterobacteria, were not appropriate for MOB<sub>Q4</sub> mobilization. MPF<sub>T</sub> plasmids behaved unevenly as MOB<sub>Q4</sub> mobilizers. IncP1-MOB<sub>P11</sub> (RP4 and R751) and IncX2-MOB<sub>P3</sub> (R6Kdrd1) plasmids rendered MOB<sub>Q4</sub> transconjugants, while IncW-MOB<sub>F11</sub> (R388), IncN-MOB<sub>F11</sub> (pKM101) or IncX1-MOB<sub>P3</sub> (pOLA52) did not. Contrary to IncP, IncW and IncN plasmids, most IncF, IncI1, IncH, and IncX plasmids are naturally repressed for conjugation. In this study, we used derepressed variants of IncF (pOX38 and R100-1), IncI $\alpha$  (R64drd11), IncHI1 (drR27), and IncX2 (R6Kdrd1) plasmids, but not a derepressed IncX1. IncX1 and IncX2 plasmids are highly similar in their conjugation genes. Taking into account

that the IncX2 derepressed plasmid R6K*drd1* was not efficient at mobilizing MOB<sub>Q4</sub> plasmids (Figure 3A and Supplementary Table S3), and that the IncX1 plasmid pOLA52 self-transfers at low frequency (around 10<sup>-4</sup> per donor) (Sørensen et al., 2003), the lack of mobilization of the MOB<sub>Q4</sub> plasmids pRC1 and pRC2 by pOLA52 is not surprising. The widely different mobilization efficiencies displayed by the two IncP1-MOB<sub>P11</sub> helpers used is more curious. RP4 and R751 are prototypes of the  $\alpha$  and  $\beta$  divisions of the IncP1 backbones, respectively. Despite the high conservation of their transfer genes, the kanamycin-sensitive RP4 derivative, pRL443, was 100–1000 times more efficient than R751 as a MOB<sub>Q4</sub> helper. Noticeable differences were also observed for these two conjugative plasmids at transferring IncQ2-MOB<sub>P14</sub> mobilizable plasmids pTC-F14 and pTF-FC2 (van Zyl et al., 2003). The common characteristic of the MOB<sub>Q4</sub> mobilizers was their belonging to the MOB<sub>P</sub> relaxase class. This could indicate that the MOB<sub>Q4</sub> relaxosomes interact more efficiently with the T4 encoded by these MOB<sub>P</sub> plasmids.

## Effect of Co-residence in the MOB<sub>Q4</sub> Plasmid Mobilization

Bacterial co-infection with multiple plasmids is common in nature (San Millan et al., 2014). Co-residence of compatible plasmids may lead to intracellular interactions that negatively or positively affect plasmid transfer rates (Gama et al., 2017a,b,c; Getino et al., 2017). Among them, plasmid-encoded fertility inhibition systems that block transmission of unrelated plasmids from the same donor cell have been intensively studied (Maindola et al., 2014; Gama et al., 2018; Getino and de la Cruz, 2018). Besides, competition of two relaxosomes for the same T4CP-MPF can result in the preponderance of one them (Cascales et al., 2005), a fact relevant for any mobilizable plasmid. Cohabitation of two or more mobilizable plasmids that use the same mating apparatus could affect each other's transfer. To test whether the mobilization of the MOB<sub>Q41</sub> plasmid was affected by co-residence with a MOB<sub>Q42</sub> plasmid and vice versa, pRC1 and pRC2 were introduced conjointly with the helper plasmid (either pRL443 or R64*drd11*) in the same cell (Figure 3B). Curiously, presence of pRC1 did not produce a significant variation in pRC2 transfer. In turn, pRC2 produced one-log decrease in pRC1 transfer by pRL443. However, this moderate negative effect was not exhibited when using R64*drd11* as a helper: on the contrary, pRC2 presence resulted in one-log increase in pRC1 transfer. Testing different combinations of MOB<sub>Q41</sub>, MOB<sub>Q42</sub> and helpers would be necessary to deeper assess the impact of residing together in MOB<sub>Q4</sub> horizontal propagation.

## *mobC* Deletion Effect in the Mobilization Efficiency

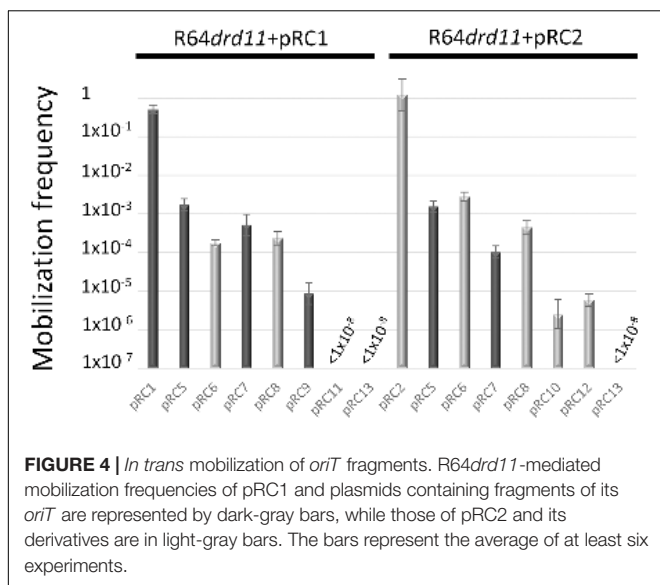
Many conjugative and mobilizable plasmids encode RAPs that recognize and bind their cognate *oriT* sequence probably favoring a single-stranded state around the *nic* site (de la Cruz et al., 2010). Deletion of RAP genes *trwA* of R388 (Moncalián et al., 1997), *nika* of R64 (Furuya et al., 1991), *mobB* and *mobC* of plasmids pTC-F14 and pTF-FC2 (van Zyl et al., 2003),

*traJ* and *traK* of RP4 (Guiney et al., 1989), *mobC* of R1162/RSF1010 (Brasch and Meyer, 1986), and *mbeC* of ColE1 (Varsaki et al., 2009) resulted in drastic decrease of plasmid transfer. All MOB<sub>Q4</sub> plasmids encode a gene, called *mobC*, which is located adjacent to *oriT* and transcribed opposite to the *mobA* relaxase gene (Figure 1). Most of the *mobC* genes are not annotated, so we updated their annotation, as listed in Supplementary Table S2. The MobC proteins of MOB<sub>Q4</sub> plasmids are small (less than 100 amino acids) and showed no homology to other RAPs (by using PSI-Blast). To check whether MobC plays a role in the MOB<sub>Q4</sub> plasmid mobilization, *mobC* deletion mutants were constructed from pRC1 and pRC2, respectively producing pRC3 and pRC4 (Figure 1). A moderate decrease in mobilization was observed in the *mobC*<sup>-</sup> variants: 1.5-log reduction for pRC3 and 0.6-log for pRC4, when using R64*drd11* as a helper (Figure 3B). MobC is thus not absolutely essential for MOB<sub>Q4</sub> plasmid mobilization. This is an interesting difference to other plasmid groups, which should be further investigated. It is conceivable that some MOB<sub>Q4</sub> plasmids can be found, the mobilization of which is independent of RAPs.

## *In trans* Mobilization of *oriT*\_MOB<sub>Q4</sub>-Containing Vectors

The 178 bp intergenic region comprised between the *mobC* and *mobA* genes of MOB<sub>Q4</sub> plasmids was assembled with an *oriT*-lacking fragment of vector pSEVA631. The resulting constructions, pRC7 (for MOB<sub>Q41</sub>) and pRC8 (for MOB<sub>Q42</sub>) (Figure 1), were introduced in donor strains to check for their mobilization. The transfer proteins were supplied *in trans*: the corresponding mobilizable plasmid (pRC1 or pRC2) provided the relaxosomal proteins, while the conjugative plasmid (R64*drd11*) supplied the T4CP and MPF. Plasmids pRC7 and pRC8 were transferred to the recipient population, but 1000-fold less efficiently than their corresponding *mobA*<sup>+</sup>*mobC*<sup>+</sup> partners (pRC1 and pRC2) (Figure 4). This result was confirmed by using plasmids pRC14 and pRC15, instead of pRC7 and pRC8, in the mobilization experiments. Plasmids pRC14 and pRC15 contained the same *oriT* region present in pRC7 and pRC8, but cloned in the inverse orientation. Besides, to avoid losing any *oriT*-related function, larger segments including also the *mobC* gene and the first 431 bp of the *mobA* gene [pRC5 and pRC6 (Figure 1)], were analyzed. Here again relaxase, T4CP and MPF components were provided *in trans*. Plasmids pRC5 and pRC6 behave similarly to pRC7 and pRC8, and were mobilized at least 500-fold less than pRC1 and pRC2 (Figure 4).

MOB<sub>Q4</sub> relaxases showed thus a *cis*-acting preference for their *oriT*s, performing at least 500-fold better on a *cis* than on a *trans oriT* substrate. The *cis*-acting preference is a characteristic exhibited by some DNA-binding proteins, such as the TnpA transposases of Tn10, Tn5 and Tn903 (Morisato et al., 1983; Derbyshire et al., 1990; DeLong and Syvanen, 1991). Relaxases generally lack a *cis* preference for their *oriT*s. There are only a few examples of relaxases that show preference for a *cis*-encoded substrate. The MOB<sub>P</sub> relaxase of transposon Tn1549 was found to be *cis*-acting (Tsvetkova et al., 2010). Notably,



all plasmid-encoded *cis*-acting relaxases have been reported in members of the MOB<sub>Q</sub> class: TraA of plasmid pRetCFN42d (MOB<sub>Q2</sub>) (Pérez-Mendoza et al., 2006) and TraA of plasmid pIP501 (MOB<sub>Q3</sub>) (Arends et al., 2012). Nevertheless, other MOB<sub>Q</sub> relaxases, such as Nes\_pSK41 (Pollet et al., 2016), as well as MobA of plasmids R1162/RSF1010 and pSC101 (Brasch and Meyer, 1986; Derbyshire and Willetts, 1987; Meyer, 2000) worked efficiently *in trans*.

The MOB<sub>Q4</sub> relaxases were also tested for their specificity to act on a non-cognate MOB<sub>Q4</sub> *oriT*. The *oriT*s of MOB<sub>Q41</sub> and MOB<sub>Q42</sub> plasmids differ in 10 nucleotides along their 178bp sequence (Supplementary Figure S1). Mobilization frequencies of *oriT*\_MOB<sub>Q42</sub> plasmids pRC6 or pRC8 by the MOB<sub>Q41</sub> plasmid pRC1 + R64drd11, as well as *oriT*\_MOB<sub>Q41</sub> plasmids pRC5 or pRC7 by the MOB<sub>Q42</sub> plasmid pRC2 + R64drd11, were similar to that obtained for the cognate systems, varying no more than one log (Figure 4).

To further delimit the *oriT* of MOB<sub>Q4</sub> plasmids, the 178bp *oriT* fragments cloned in pRC7 and pRC8 (see Supplementary Figure S1) were subdivided in two portions, one containing *oriT* nucleotides 1–70 (pRC11 and pRC12) and the other containing *oriT* nucleotides 71–178 (pRC9 and pRC10) (Figure 1 and Supplementary Figure S1). Disruption of the 178bp *oriT* region resulted in a drastic loss of conjugation efficiency of the *oriT*-containing plasmid (Figure 4), as previously reported for pIGWZ12 (Zaleski et al., 2015).

The *cis*-acting preference of the MOB<sub>Q4</sub> relaxases shown here is an example of biological orthogonality (de Lorenzo, 2011), that is, a mechanism to avoid interference. It implies that when two MOB<sub>Q4</sub> plasmids are present in the same cell, the contribution of *oriT* cross-recognition by the heterologous MOB<sub>Q4</sub> relaxase to plasmid transfer is not substantial. This feature could be essential to guarantee their efficient transfer, given the fact that both types of MOB<sub>Q4</sub> plasmids use the same repertoire of conjugative helpers and share the same hosts.

## CONCLUSION

MOB<sub>Q41</sub> and MOB<sub>Q42</sub> plasmids are able to coexist and spread in the *E. coli* population without affecting each other largely. They disseminate through bacterial conjugation, aided specially by MPF<sub>I</sub> conjugative plasmids, but neither of the MOB<sub>Q4</sub> plasmids dominates the horizontal transfer process. Co-residence of MOB<sub>Q41</sub> and MOB<sub>Q42</sub> plasmids in the same host neither hindered nor boosted considerably their respective mobilization frequencies. Since both plasmids (MOB<sub>Q41</sub> and MOB<sub>Q42</sub>) have a narrow host-range (they circulate among enterobacteria), their coexistence in natural environments is likely. In such ecological setting, specific discrimination among their highly similar *oriT* sequences would be guaranteed by the preferential *cis* activity of the MOB<sub>Q4</sub> relaxase. Such strategy would be biologically relevant in a scenario of co-residence of non-divergent elements to favor self-dissemination.

## DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/Supplementary Material.

## AUTHOR CONTRIBUTIONS

MG-B and FC conceived the study and designed the experiments. RC-L, AC, and MG-B performed the experiments. RC-L, AC, MG-B, and FC interpreted the data. MG-B and FC wrote the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2019.02557/full#supplementary-material>



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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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