1 **Title:** “Comparative analysis of MOB_{Q4} plasmids demonstrates that MOB_Q is a
2 *cis*-acting-enriched relaxase protein family”

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10 **ABSTRACT**

11 A group of small mobilizable plasmids is increasingly being reported in
 12 epidemiology surveys of enterobacteria. Some of them encode colicins, while
 13 others are cryptic. All of them encode a relaxase belonging to a previously non-
 14 described group of the MOB_Q class, MOB_{Q4}. While highly similar in their
 15 mobilization module, two families with unrelated replicons can be
 16 distinguished, MOB_{Q41} and MOB_{Q42}. Members of both groups were compatible
 17 between them and stably maintained in *E. coli*. MOB_{Q4} plasmids were mobilized
 18 by conjugation. They contain two transfer genes, *mobA* coding for the MOB_{Q4}
 19 relaxase and *mobC*, which was non-essential but enhanced the plasmid
 20 mobilization frequency. The origin of transfer was located between these two
 21 divergently transcribed *mob* genes. MPF_I conjugative plasmids were the most
 22 efficient helpers for MOB_{Q4} conjugative transmission. No interference in
 23 mobilization was observed when both MOB_{Q41} and MOB_{Q42} were present in the
 24 same donor cell. Remarkably, MOB_{Q4} relaxases exhibited a *cis*-acting
 25 preference for their *oriTs*, a feature already observed in other MOB_Q plasmids.
 26 These findings indicate that MOB_{Q4} plasmids can efficiently spread among
 27 enterobacteria aided by coresident IncI1, IncK and IncL/M plasmids, while
 28 ensuring their self-dissemination over highly-related elements.

29 **IMPORTANCE**

30 Plasmids are key vehicles of horizontal gene transfer and contribute greatly to
 31 bacterial genome plasticity. A group of plasmids, called mobilizable, is able to
 32 disseminate aided by helper conjugative plasmids. Here, we studied a group of
 33 phylogenetically-related mobilizable plasmids, MOB_{Q4}, commonly found in clini-
 34 cally-relevant enterobacteria, uncovering the helper plasmids responsible for

their dissemination. We found that the two plasmid species encompassed in the MOB_{Q4} group can coexist and transfer orthogonally, 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_{Q4} relaxases. Such 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_Q relaxase, *cis*-acting relaxase

45 INTRODUCTION

46 Mobilizable plasmids are small genomes transmissible by conjugation. They
 47 encode a relaxase, and usually a relaxase accessory protein (RAP), which are in
 48 charge of the conjugative DNA processing at a specific site of the origin of
 49 transfer (*oriT*) called *nic*. Mobilizable plasmids lack the transfer genes required
 50 for establishing a conjugative bridge (mating pair formation system, MPF) to
 51 the recipient cell, as well as the type IV coupling protein (T4CP) that puts in
 52 contact relaxosome and MPF and thus depend on conjugative plasmids to be
 53 transferred (1).

54 According to their relaxase, transmissible plasmids were phylogenetically
 55 classified into MOB families (2) (3). Currently, nine relaxase MOB classes are
 56 defined, and five of them (MOB_P, MOB_F, MOB_Q, MOB_H and MOB_C) are prevalent in
 57 transmissible plasmids hosted in γ-Proteobacteria. Plasmids gathered in a
 58 relaxase MOB family share similar genomic traits. Relaxase MOB classification
 59 has thus shown to be a good predictor of the plasmid backbone (1, 4).
 60 Mobilizable plasmids resident in γ-Proteobacteria form phylogenetically-related
 61 clusters mainly within two relaxase MOB classes: MOB_P and MOB_Q (3). Relevant
 62 examples are ColE1-like plasmids, grouped in family MOB_{P5}; IncQ1 plasmids,
 63 such as RSF1010/R1162, gathered in MOB_{Q11}; and IncQ2 plasmids, such as pTC-
 64 F14, in family MOB_{P14} (1, 3). An additional clade of small plasmids encoding
 65 MOB_Q relaxases, previously classified as MOB_{Qu}, and here redefined as MOB_{Q4},
 66 was observed in a phylogenetic reconstruction of this relaxase family (3).

67 A pair of degenerate primers specific for MOB_{Q4} plasmids was implemented in
 68 the Degenerate PCR MOB Typing (DPMT) approach developed by (5) to detect

69 and classify transmissible plasmids. This method revealed the abundance of
 70 MOB_{Q4} plasmids in clinical isolates of enterobacteria (5, 6), previously unnoticed
 71 by other plasmid typing methods. Whole-genome sequencing of clinical *E. coli*
 72 isolates also uncovered the presence of this kind of plasmids (7-9). Prototype
 73 plasmids pIGWZ12 and ColE9-J (ColE2-like) cluster within the MOB_{Q4} clade. They
 74 are stable, theta-replicating, high copy-number, narrow host-range plasmids,
 75 whose replication systems have been extensively studied (10-14). Here, we
 76 uncovered the diversity of MOB_{Q4} plasmids, determined the helper conjugative
 77 plasmids responsible for their dissemination, and established their orthogonal
 78 behavior in terms of stability and transfer.

79 MATERIALS AND METHODS

80 **Plasmid construction.** MOB_{Q4} vectors were constructed by isothermal
 81 assembly of linear DNA fragments from PCR reactions, following the Gibson
 82 method (15, 16). The MOB_{Q41} backbone, based on the complete sequence of
 83 the pE2022_4 plasmid (GenBank Acc. No. KT693143 (8)), was linked to a
 84 kanamycin-resistance gene (coordinates 272 to 1216 of pSEVA211, GenBank
 85 Acc. No. JX560326 (17)) and a cerulean fluorescent protein gene (coordinates
 86 41 to 1091 of pNS2-φVL (18)), producing plasmid pRC1 (see Supplementary
 87 Information S1). The MOB_{Q42} backbone was obtained by PCR amplification from
 88 the *E. coli* isolate HUMV 04/979 (6), which contains a ColE9-J-like plasmid
 89 (coordinates 1814 to 6114 of the sequence in Supplementary Information S2).
 90 It was joined to a chloramphenicol resistance gene (coordinates 272-1072 of
 91 pSEVA311, GenBank Acc. No. JX560331 (17)) and mCherry fluorescent protein
 92 gene (*cfp*, coordinates 1092-2117 of pNS2-φVL (18)), rendering plasmid pRC2
 93 (see Supplementary Information S2). MOB_{Q4} vectors lacking the *mobC* ORF
 94 (from start to stop codon) were constructed by self-ligation of a single PCR
 95 fragment from either pRC1 or pRC2, generating plasmids pRC3 and pRC4,
 96 respectively.

97 Additional plasmids were constructed to delimit the *oriT* region. A schematic
 98 representation of the fragments included in each construction is depicted in
 99 Figure 1. Such fragments were individually assembled to coordinates 1-1030
 100 and 1360-3001 of vector pSEVA631 (GenBank Acc. No. JX560348). Plasmids
 101 pRC5 and pRC6 contained a fragment including the *mobC* gene, the 178bp
 102 intergenic region between *mobC* and *mobA* and the first 400 nucleotides of the
 103 *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, rendering the non-mobilizable vector pRC13, which was used as a control in the mating experiments.

Stability assays. Plasmids pRC1 and pRC2 were independently introduced in the *recA*⁺ and *recA*⁻ isogenic strains UB1636 (19) and UB1637 (20) to check for their stability. Single colonies were inoculated in Lysogeny-Broth (LB) supplemented with kanamycin at 50 µg/ml (for pRC1-containing strains) or chloramphenicol at 25 µg/ml (for pRC2-containing strains) and grown to saturation at 37°C with agitation (150 rpm). A volume of 9.7 µl 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 MOB_{Q4} plasmids in surface mating experiments, following the procedure described by (21). Briefly, donor and recipient strains were mixed in a 1:1 ratio, deposited onto an LB-agar surface and incubated for 1h 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 (22) (e-value: 1xE-3). The homologous sequences were aligned using MUSCLE (23). TrimAl v1.4 was used to calculate the average identity between sequences in the alignment (24). ProtTest 3 was used to estimate the best model of protein evolution for our set (25, 26). RAxML version 7.2.7 (27) was used for phylogenetic reconstruction. Using the JTTGAMMA model ten 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.

153 **3D structure prediction.** Phyre2 was used to predict the 3D structure of the
154 MobA relaxase domains of plasmids pE2022_4 and ColE9-J (28), which were
155 visualized using PyMOL (29).

RESULTS

Analysis of MOB_{Q4} plasmids. The phylogenetic tree of MOB_{Q4} relaxases shows that they constitute a well-supported clade, which is in turn divided in two groups, MOB_{Q41} and MOB_{Q42} (Figure 2). The reconstruction was based on the first N-terminal 300 residues of MOB_{Q4} relaxases, which contain the three relaxase motifs (Figure 3A) and share 84% average amino acid identity (97 and 90% for individual MOB_{Q41} and MOB_{Q42} groups, respectively). Each MOB_{Q4} subclade groups highly related backbones (Figure 2). MOB_{Q41} 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 (Figure 2). The genes for the last two are generally not annotated. Besides the replication and mobilization genes, MOB_{Q42} plasmids also contain a colicin operon, including colicin, immunity and lysis genes, following the synteny of Group A nuclease colicins (30) (Figure 2). Plasmids ColE9-J and pO111_4 contain a second, partial colicin operon. The MOB_{Q4} subdivision in two relaxase groups matches with the presence of two different replicons (Supplementary Table S2). MOB_{Q41} plasmids encode a replication initiation protein that belongs to the Rep₃ superfamily (pfam PF01051), while MOB_{Q42} plasmids encode ColE2-like initiators (pfam PF03090 + PF08708). Thus, at least two plasmid species are included in MOB_{Q4}, according to the criteria established by (31).

The 3D structure prediction of the relaxase domain of MOB_{Q41} and MOB_{Q42} plasmids pE2022_4 and ColE9-J rendered MOB_Q relaxases NES (plasmid pLW1043, PDB Acc. No. 4HT4, (32)) and MobA (plasmid R1162 / RSF1010, PDB Acc. No. 2NS6, (33)) as best hits (100% confidence). The superimposed

structures pointed to MOB_{Q4} amino acids Y25 (motif I), E87 and E89 (motif II), and H125, H133 and H135 (motif III) as homologs of the MobA_R1162 catalytic residues Y25, E74 and E76, and H112, H120 and H122, respectively (Figure 3B). Contrary to the high conservation of the N-terminal domain among members of both MOB_{Q4} subgroups, the amino acid identity of the C-terminal part of the MOB_{Q4} relaxases dropped to 35%. This C-terminal domain exhibited low homology to SogL primases of IncI1 plasmids.

Stability and co-residence of MOB_{Q4} plasmids. To study the MOB_{Q4} plasmids, two synthetic plasmids were constructed, pRC1 and pRC2. They included the replication and mobilization modules of MOB_{Q41} and MOB_{Q42} backbones, respectively. Antibiotic-resistance (AbR) and fluorescent protein genes were also included as reporters for the stability and mating experiments. Plasmid stability was assayed in *recA*⁺ and *recA*⁻ *E. coli* strains, containing either one or both plasmids (to test for stability and incompatibility, respectively). In all cases, the percentage of plasmid retention in the bacterial population after 80 generations was 100%. Thus, besides stability in *E. coli*, both MOB_{Q4} plasmid species exhibited full compatibility.

Mobilization of MOB_{Q4} plasmids by different MPF systems. Conjugative plasmids encoding three different MPF systems, MPF_T, MPF_I and MPF_F, the most abundant in γ -Proteobacteria, were used as helpers to mobilize the MOB_{Q4} plasmids pRC1 and pRC2 (Table 1 and Figure 4). Their mobilization by MPF_F plasmids (pOX38, R100-1 and drR27) was not detected. Three of the MPF_T-type helper plasmids (R388, pKM101, and pOLA52) did not mobilized pRC1 neither pRC2, while pRL443, R751, and R6K*drd1* did mobilize them. pRL443 and R751 are closely related plasmids, respectively belonging to the α and β subdivisions

206 of the IncP1 group. Nevertheless, their efficiency at mobilizing MOB_{Q4} plasmids
 207 differed widely, being that of IncP1 α plasmid approximately 1000-fold higher.
 208 Both MPF_I plasmids (R64*drd11* and pCTX-M3) mobilized MOB_{Q4} plasmids
 209 efficiently. The highest mobilization frequencies obtained for both MOB_{Q4}
 210 plasmids, pRC1 and pRC2, were achieved by using R64*drd11* as a helper.
 211 Besides, the mobilization efficiency of the MOB_{Q4} plasmids in relation to the
 212 transfer of the helper conjugative plasmid was also the highest for R64*drd11*
 213 (Figure 5). So, R64*drd11* was the most efficient helper plasmid.

214 To test whether the mobilization of the MOB_{Q41} plasmid was affected by co-
 215 residence with a MOB_{Q42} plasmid and vice versa, pRC1 and pRC2 were
 216 introduced conjointly with the helper plasmid (either pRL443 or R64*drd11*) in
 217 the same host. The values obtained for pRC1 and pRC2 mobilization when
 218 residing together in the same host did not differ significantly from those
 219 obtained when only one of them was present in the donor cells (Figure 5).

220 ***mobC* deletion effect in the mobilization efficiency.** To check whether
 221 MobC plays a role in the MOB_{Q4} plasmid mobilization, *mobC* deletion mutants
 222 were constructed from pRC1 and pRC2, respectively producing pRC3 and pRC4
 223 (Figure 1). A moderate decrease in mobilization was observed in the *mobC*⁻
 224 variants (two-log reduction for pRC3 and one-log for pRC4, when using
 225 R64*drd11* as a helper (Figure 5)).

226 ***In trans* mobilization of *oriT*-MOB_{Q4}-containing vectors.** The 178 bp
 227 intergenic region comprised between the *mobC* and *mobA* genes of MOB_{Q4}
 228 plasmids was assembled with an *oriT*-lacking fragment of vector pSEVA631.
 229 The resulting constructions, pRC7 (for MOB_{Q41}) and pRC8 (for MOB_{Q42}) (Figure 1),
 230 were introduced in donor strains to check for their mobilization. The transfer

231 proteins were supplied *in trans*: the corresponding mobilizable plasmid (pRC1
 232 or pRC2) provided the relaxosomal proteins, while the conjugative plasmid
 233 (R64*drd11*) supplied the T4CP and MPF. Plasmids pRC7 and pRC8 were
 234 transferred to the recipient population, but 1000-fold less efficiently than their
 235 corresponding *mobA⁺mobC⁺* partners (pRC1 and pRC2) (Figure 6). This result
 236 was confirmed by using plasmids pRC14 and pRC15, instead of pRC7 and pRC8,
 237 in the mobilization experiments. Plasmids pRC14 and pRC15 contained the
 238 same *oriT* region present in pRC7 and pRC8, but cloned in the inverse
 239 orientation. Besides, to avoid losing any *oriT*-related function, larger segments
 240 including also the *mobC* gene and the first 431bp of the *mobA* gene (pRC5 and
 241 pRC6 (Figure 1)), were analyzed. Here again relaxase, T4CP and MPF
 242 components were provided *in trans*. Plasmids pRC5 and pRC6 behave similarly
 243 to pRC7 and pRC8, and were mobilized at least 500-fold less than pRC1 and
 244 pRC2 (Figure 6).

245 To further delimit the *oriT* of MOB_{Q4} plasmids, the 178bp *oriT* fragments cloned
 246 in pRC7 and pRC8 (see Supplementary Figure S1) were subdivided in two
 247 portions, one containing *oriT* nucleotides 1-70 (pRC11 and pRC12) and the
 248 other containing *oriT* nucleotides 71-178 (pRC9 and pRC10) (Figure 1 and
 249 Supplementary Figure S1). Disruption of the 178bp *oriT* region resulted in a
 250 drastic loss of conjugation efficiency (Figure 6).

251 The MOB_{Q4} relaxases were also tested for their specificity to act on a non-
 252 cognate MOB_{Q4} *oriT*. Mobilization frequencies of *oriT*_MOB_{Q42} plasmids pRC6 or
 253 pRC8 by the MOB_{Q41} plasmid pRC1 + R64*drd11*, as well as *oriT*_MOB_{Q41} plasmids
 254 pRC5 or pRC7 by the MOB_{Q42} plasmid pRC2 + R64*drd11*, were similar to that
 255 obtained for the cognate systems (Figure 6).

DISCUSSION

MOB_Q is a broad relaxase class that encompasses several families, each of which includes related plasmid backbones: MOB_{Q1} comprises relaxases of mobilizable broad host-range IncQ1-like plasmids; MOB_{Q2}, conjugative relaxases of pTi and many rhizobial plasmids; MOB_{Q3}, conjugative broad host-range plasmids resident in gram-positive, such as pIP501 (3). In the referred study, many MOB_Q 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_{Q4}, prompted by the fact that these relaxases have been recurrently detected in enterobacterial clinical isolates (5-9).

MOB_{Q4} relaxases formed two clusters that correlated with the presence of two different replicons, thus containing at least two plasmid species as defined by (31) (Figure 2 and Supplementary Table S2). 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 (34, 35)). 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 (10, 11). The iterons were found to be the incompatibility determinants (10). The corresponding replication initiation protein belongs to the Rep_3 superfamily (PF01051) (11). 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 (13, 36-38). The origin of replication consists of 32 bp located downstream of the *rep* gene, containing two directly repeated sequences (12, 39-41). 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 (14, 35, 42). MOB_{Q42} plasmids contain a *cer*-like site (38), an indication that they profit from a host site-specific recombination system for resolving multimers to monomers as ColE1-like plasmids do (43-45).

Completely sequenced MOB_{Q4} plasmids all originate in Enterobacteriaceae family hosts (Supplementary Table S2). They were isolated from different backgrounds: *Salmonella enterica* isolated from pork meat (pSD4.0) (46), pork feces (p4_TW-Stm6) (47) and human systemic infection (pYU39_5.1) (48), multidrug-resistant environmental *E. coli* (pSMS35_4) (49), commensal *E. coli* (pSE11-6) (50), enterohemorrhagic *E. coli* strains of the O26 and O111 serogroups (pO26-S4 and pO111_4) (51, 52), extended-spectrum beta-lactamase producing *E. coli* clinical isolates (pE2022_4, pFV9873_1, pEC147-3 and pEC08-6) (7, 8), *E. coli* isolated from human urinary tract (pVR50F) (53) and bloodstream infections (pSF-468-4) (54), as well as porcine extraintestinal pathogenic *E. coli* strain (p2PCN033) (55), among others. None of these plasmids contain AbR genes. There is still no clue on the selective advantage provided by the cryptic MOB_{Q41} plasmids. In the case of MOB_{Q42} 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_{Q42} plasmid pDPT1 was stably acquired by a Vietnamese *Shigella sonnei* strain in the mid-1990s, and became fixed in the evolving bacterial population (56). The colicin E5 produced by pDPT1 was highly bactericidal against nonimmune *Shigella* and *E. coli* strains. The acquisition of the pDPT1 colicin plasmid, coinciding with the high increase of dysentery

306 produced by this strain, suggests that pDPT1 conferred a beneficial function to
307 its host (56).

308 To get insight into the MOB_{Q4} family, mobilizable synthetic plasmids based on
309 two prototype backbones (pE2022_4 from MOB_{Q41} and ColE9-J from MOB_{Q42})
310 were constructed (pRC1 and pRC2). They included the natural replication and
311 mobilization modules, and AbR and fluorescent protein genes as markers.
312 Firstly, they were assayed for their maintenance in the bacterial population.
313 Stability experiments indicated that MOB_{Q4} backbones were highly stable in *E.*
314 *coli* (100% retention in 80 generations) despite the cargoes loaded in plasmids
315 pRC1 and pRC2. Furthermore, both plasmids were 100% stable when
316 replicating in the same host cell, which indicates that they are compatible and
317 do not interfere with the stable vertical inheritance of each other.

318 Since mobilizable plasmids do not encode the mating pair formation system
319 neither the T4CP, their transfer relies on auto-transmissible plasmids. We
320 wondered which conjugative plasmids could be responsible for the
321 dissemination of MOB_{Q4} plasmids. Not all conjugative plasmids are equally
322 efficient at supplying these functions to a specific mobilizable plasmid (57, 58).
323 The contacts established between the relaxosome of the mobilizable plasmid
324 and the T4CP-MPF of the helper plasmid are crucial in the transfer process.
325 ColE1-like MOB_{P5} plasmids are efficiently mobilized by IncF-MOB_{F12} (e.g. F) and
326 IncI1-MOB_{P12} (e.g. R64drd11) plasmids (58). IncQ1-MOB_{Q1} plasmids, such as
327 RSF1010, are transferred by IncP1-MOB_{P11} helper plasmids (e.g. RP4) (58, 59).
328 pMV158-like plasmids (MOB_{V1}) are mobilized by IncP1-MOB_{P11} and Inc18-MOB_{Q3}
329 (e.g. pIP501) plasmids (60).

330 We looked for reports providing indirect evidence on MOB_{Q4} plasmid
331 mobilization through conjugation. In a survey for the presence of transmissible
332 plasmids in a multidrug *E. coli* collection, MOB_{Q4} transconjugants were obtained
333 from seven out of the eight MOB_{Q4} containing clinical isolates (6). In all cases, a
334 MOB_{P12}-MPF_I plasmid, presumptively the helper, was also present in both, donor
335 and transconjugant cells. Similarly, the MOB_{Q41} plasmid pSD4.0 and the IncI1
336 plasmid pSD107 were found in *E. coli* transconjugants arisen from a mating
337 with *Salmonella enterica* (46).

338 Three conjugative MPF types (MPF_T, MPF_F, and MPF_I) are prevalent in
339 Enterobacteriaceae (61, 62), the taxonomic class where MOB_{Q4} plasmids have
340 been found. In this study, a set of conjugative plasmids representative of these
341 MPF families were tested as helpers for the mobilization of MOB_{Q4} plasmids
342 (Figures 4 and 5). Not all MPF types were equally efficient. R64*drd11*, the
343 prototype of IncI1α-MOB_{P12} plasmids, which encodes a MPF_I conjugative
344 apparatus, was the most efficient helper. Plasmid pCTX-M3 (IncL/M-MOB_{P13}),
345 was also an efficient helper. Co-residence of MOB_{Q4} with MPF_I plasmids has
346 been observed for MOB_{Q4} plasmids pSE11-6 (50), pSD4.0 (46), pEC147-4 (7),
347 pO26-S4 (Fratamico *et al.*, 2011), pDPT1 (56) and pE2022_4 (8).

348 On the other hand, MPF_F-type plasmids (e.g. IncF-MOB_{F12} (F) or IncHI1-MOB_{H11}
349 (R27) plasmids), which show high prevalence in enterobacteria, were not
350 appropriate for MOB_{Q4} mobilization. MPF_T plasmids behaved unevenly as MOB_{Q4}
351 mobilizers. IncP1-MOB_{P11} (RP4 and R751) and IncX2-MOB_{P3} (R6K*drd1*) plasmids
352 rendered MOB_{Q4} transconjugants, while IncW-MOB_{F11} (R388), IncN-MOB_{F11}
353 (pKM101) or IncX1-MOB_{P3} (pOLA52) did not. Contrary to IncP, IncW and IncN
354 plasmids, most IncF, IncI1, IncH and IncX plasmids are naturally repressed for

355 conjugation. In this study, we used derepressed variants of IncF (pOX38 and
 356 R100-1), IncI1 α (R64*drd11*), IncHI1 (drR27) and IncX2 (R6K*drd1*) plasmids, but
 357 not a derepressed IncX1. IncX1 and IncX2 plasmids are highly similar in their
 358 conjugation genes. Taking into account that the IncX2 derepressed plasmid
 359 R6K*drd1* was not efficient at mobilizing MOB_{Q4} plasmids (Table 1, Figures 4 and
 360 5), and that the IncX1 plasmid pOLA52 self-transfers at low frequency (around
 361 10⁻⁴ per donor)(63), the lack of mobilization of the MOB_{Q4} plasmids pRC1 and
 362 pRC2 by pOLA52 is not surprising. The widely different mobilization efficiencies
 363 displayed by the two IncP1 helpers used is more curious. RP4 and R751 are
 364 prototypes of the α and β divisions of IncP1 backbones, respectively. Despite
 365 the high conservation of their transfer genes, the kanamycin-sensitive RP4
 366 derivative, pRL443, was 100-1000 times more efficient than R751 as a MOB_{Q4}
 367 helper. Noticeable differences were observed for these two conjugative
 368 plasmids at transferring IncQ2-MOB_{P14} mobilizable plasmids pTC-F14 and pTF-
 369 FC2 (64).

370 Many conjugative and mobilizable plasmids encode RAPs that recognize and
 371 bind their cognate *oriT* sequence probably favoring a single-stranded state
 372 around the *nic* site (65). Deletion of RAP genes *trwA* of R388 (66), *nikA* of R64
 373 (67), *mobB* and *mobC* of plasmids pTC-F14 and pTF-FC2 (64), *traJ* and *traK* of
 374 RP4 (68), *mobC* of R1162/RSF1010 (69), and *mbeC* of ColE1 (70) resulted in
 375 drastic decrease of plasmid transfer. All MOB_{Q4} plasmids encode a gene, called
 376 *mobC*, which is located adjacent to *oriT* and transcribed opposite to the *mobA*
 377 relaxase gene (Figure 1). Most of the *mobC* genes are not annotated, so we
 378 updated their annotation, as listed in Supplementary Table 2. The MobC
 379 proteins of MOB_{Q4} plasmids are small (less than 100 amino acids) and showed

380 no homology to other RAPs (by using PSI-Blast). Here, we demonstrated that
 381 they are not absolutely essential for MOB_{Q4} plasmid mobilization. Nevertheless,
 382 *mobC* deletion resulted in 10 to 100-fold reduction in MOB_{Q4} plasmid
 383 mobilization frequencies (Figure 5). This is an interesting difference to other
 384 plasmid groups, which should be further investigated. It is conceivable that
 385 some MOB_{Q4} plasmids can be found, the mobilization of which is independent of
 386 RAPs.

387 We delimited the *oriT* of MOB_{Q4} plasmids to the 178 bp intergenic region
 388 between genes *mobC* and *mobA* (Supplementary Figure S1). *oriT*-containing
 389 plasmids (pRC5 and pRC7 for MOB_{Q41}, or pR6 and pRC8 for MOB_{Q42}) were
 390 mobilized by the relaxase proteins provided by the corresponding mobilizable
 391 plasmid (either pRC1 or pRC2) and the T4CP+MPF functions of the helper
 392 plasmid (R64*drd11*) (Figure 6). Disruption of such 178 bp segment resulted in a
 393 drastic loss of conjugation efficiency of the *oriT*-containing plasmid, as
 394 previously reported for pIGWZ12 (10). Furthermore, MOB_{Q41} relaxases were able
 395 to recognize and process the heterologous *oriT*_MOB_{Q42} with the same
 396 efficiency as the cognate *oriT*_MOB_{Q41}. The same situation accounted for MOB_{Q42}
 397 relaxases (Figure 6). These two *oriTs* showed 10 differences in their 178bp
 398 sequence.

399 Remarkably, MOB_{Q4} relaxases showed a *cis*-acting preference for their *oriTs*.
 400 They acted at least 500-fold better on a *cis* than on a *trans oriT* substrate
 401 (Figure 6). The *cis*-acting preference is a characteristic exhibited by some DNA-
 402 binding proteins, such as the TnpA transposases of Tn10, Tn5 and Tn903 (71-
 403 73). Relaxases generally lack a *cis* preference for their *oriTs*. There are only a
 404 few examples of relaxases that show preference for a *cis*-encoded substrate.

Notably, all plasmid-encoded *cis*-acting relaxases have been reported in members of the MOB_Q class: TraA of plasmid pRetCFN42d (MOB_{Q2}) (74) and TraA of plasmid pIP501 (MOB_{Q3}) (75). The MOB_P relaxase of transposon Tn1549 was also found to be *cis*-acting (76). Nevertheless, other MOB_Q relaxases, such as Nes_pSK41 (77), as well as MobA of plasmids R1162 / RSF1010 and pSC101 (69, 78, 79) worked efficiently in *trans*. The *cis*-acting preference of the MOB_{Q4} relaxases implies that when two MOB_{Q4} plasmids are present in the same cell, the contribution of *oriT* cross-recognition by the heterologous MOB_{Q4} 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_{Q4} plasmids use the same repertoire of conjugative helpers.

CONCLUSIONS

MOB_{Q41} and MOB_{Q42} plasmids are orthogonal in terms of replication and mobilization. That is, they are able to coexist stably in the *E. coli* population without negatively interfering between each other. They disseminate through bacterial conjugation, aided specially by MPF_I conjugative plasmids, but neither of the MOB_{Q4} plasmids dominates the horizontal transfer process. Co-residence of MOB_{Q41} and MOB_{Q42} plasmids in the same host neither hindered nor boosted their respective mobilization frequencies. So, MOB_{Q41} relaxosome interaction with the transfer machinery of the helper plasmid was not affected by the presence of a MOB_{Q42} plasmid and vice versa. Since both plasmids (MOB_{Q41} and MOB_{Q42}) 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*

429 activity of the MOB_{Q4} relaxase. Such strategy would be biologically relevant in a
430 scenario of co-residence of non-divergent elements to favor self-dissemination.

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FIGURE LEGENDS

Figure 1. Schematic representation of the MOB_{Q4} DNA segments

included in a series of recombinant plasmids. The mobilization region of MOB_{Q4} plasmids includes *mobC* and *mobA* genes, represented by large, horizontal gray arrows. The extent of the mobilization region included in each construction is represented by a gray bar. The plasmid names for the MOB_{Q41}-based constructions are listed in the left column, while those for MOB_{Q42}-based constructions are in the right column. Plasmids pRC1 and pRC2 also include the replication module of MOB_{Q41} and MOB_{Q42} plasmids, respectively.

Figure 2. Phylogenetic tree of the MOB_{Q4} relaxase subclass.

A maximum-likelihood phylogenetic reconstruction of the N-terminal domain of MOB_{Q4} relaxases is shown. MobA relaxase of plasmid pXF5843 were used as outgroup. Bootstrap values of relevant nodes are indicated. Families Q41 and Q42 are shadowed in dark and light gray, respectively. A prototype backbone of each MOB_{Q4} subfamily is represented to the right of the corresponding clade. The common genes of the mobilization module are represented in the same gray color pattern. The elements of the replication module are dotted (for MOB_{Q41}) or striped (for MOB_{Q42}). Genes of the colicin operon are depicted in a white background.

Figure 3. The MOB_{Q4} relaxase domain.

A) Multiple alignment of the three conserved MOB_Q relaxase motifs (3). (1) MOB_{Q41} relaxases, with the exception of pMG828-2; (2) pMG828-2; (3) MOB_{Q42} relaxases. Putative catalytic residues are indicated by black triangles over the amino acid sequences. B) Protein 3D-structure superposition of MOB_{Q41}, MOB_{Q42} and MOB_{Q11} relaxases. Structure

prediction of MOB_{Q4} relaxases was carried out with Phyre2 (28). In gray, the minimal relaxase domain of MobA of plasmid R1162 / RSF1010 used as a model (PDB 2NS6); in blue and magenta the predicted structures of the homologous domains of the MOB_{Q41} (MobA of pE2022_4) and MOB_{Q42} (MobA of ColE9-J) relaxases, respectively. Key residues of MOB_{Q4} relaxases are highlighted as sticks.

Figure 4. Mobilization frequencies of MOB_{Q4} plasmids by a series of helper plasmids. The mobilization frequency was calculated as the number of transconjugants containing the MOB_{Q4} plasmid per donor cell. Figures are the average of at least six independent experiments. Dark- and light-gray bars indicate the mobilization frequencies of pRC1 (MOB_{Q41}) and pRC2 (MOB_{Q42}) plasmids, respectively. Below the bars the helper plasmid used in each case, as well as its corresponding Inc and MOB groups, are indicated. The MPF types of the helper plasmids are indicated in the upper part of the figure.

Figure 5: Mobilization frequencies of different plasmid combinations. The mobilization efficiencies of the MOB_{Q4} plasmids are relativized to the helper plasmid transfer rates (black bars, 100%). pRC1 and pRC2 efficiencies are represented by dark- and light-gray bars, respectively. pRC1 + pRC2 (dotted bar in a dark-gray background) indicates the mobilization frequency of pRC1 when coresident with pRC2. pRC2 + pRC1 (dotted bar in a light gray background) indicates the mobilization frequency of pRC2 when coresident with pRC1. The mobilization efficiencies of the *mobC*⁻ derivatives pRC3 and pRC4 are represented by striped bars in dark and light gray backgrounds, respectively. The bars represent the average of at least six experiments.

485 **Figure 6: In *trans* mobilization of *oriT* fragments.** R64*drd11*-mediated
 486 mobilization frequencies of pRC1 and plasmids containing fragments of its *oriT*
 487 are represented by dark-gray bars, while those of pRC2 and its derivatives are
 488 in light-gray bars. The bars represent the average of at least six experiments.

489 **Table 1. Mobilization frequencies of MOB_{Q4} plasmids by different MPF type helpers.**

490

Mobilizable plasmid	MPF type of the helper plasmid										
	MPF _T						MPF _I		MPF _F		
	<u>RP4</u>	<u>R751</u>	<u>R388</u>	<u>pKM101</u>	<u>R6Kdrd1</u>	<u>pOLA52</u>	<u>R64drd11</u>	<u>pCTX-M3</u>	<u>pOX38</u>	<u>R100-1</u>	<u>R27dr</u>
pRC1 (MOB _{Q41})	1.6x10 ⁻² (1x10 ⁻² – 2.7x10 ⁻²)	1.7x10 ⁻⁴ (2.1x10 ⁻⁵ – 7.6x10 ⁻⁵)	<1x10 ⁻⁷	<1x10 ⁻⁷	1.1x10 ⁻⁶ (2.7x10 ⁻⁷ – 4.3 x10 ⁻⁶)	<1x10 ⁻⁷	5.1x10 ⁻¹ (3.9x10 ⁻¹ – 6.7x10 ⁻¹)	n.d.	<1x10 ⁻⁷	n.d.	<1x10 ⁻⁷
pRC2 (MOB _{Q42})	1.2x10 ⁻¹ (8.4x10 ⁻² – 1.8x10 ⁻¹)	7.8x10 ⁻⁵ (6.3x10 ⁻⁵ – 9.7x10 ⁻⁵)	<1x10 ⁻⁷	<1x10 ⁻⁷	1.1x10 ⁻⁴ (7.8x10 ⁻⁵ – 1.6 x10 ⁻⁴)	<1x10 ⁻⁷	1.2 (4.6x10 ⁻¹ – 3.2)	1.0x10 ⁻³ (4.5x10 ⁻⁴ – 2.4x10 ⁻³)	<1x10 ⁻⁷	<1x10 ⁻⁷	<1x10 ⁻⁷

491 n.d.: Not determined due to the lack of an appropriate AbR marker.

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Fig. 1

Fig. 2

Fig. 3

B

Fig. 4

Fig. 5

Fig. 6