A novel mobilizing tool based on the conjugative transfer system of the IncM plasmid pCTX-M3

Michał Dmowski[a] and Izabela Kern-Zdanowicz[#]

Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Department of Microbial Biochemistry, Warsaw, Poland

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[#]Address correspondence to Izabela Kern-Zdanowicz, iza@ibb.waw.pl

[a]Present address: Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Laboratory of Mutagenesis and DNA Repair, Warsaw, Poland

ABSTRACT

Conjugative plasmids are the main players in horizontal gene transfer in Gram-negative bacteria. DNA transfer tools constructed on the basis of such plasmids enable gene manipulation even in strains of clinical or environmental origin, which are often difficult to work with. The conjugation system of the IncM plasmid pCTX-M3 isolated from a clinical strain of *Citrobacter freundii* has been shown to enable efficient mobilization of $oriT_{pCTX\text{-}M3}$-bearing plasmids into a broad range of hosts comprising *Alpha-*, *Beta-*, and *Gammaproteobacteria*. We constructed a helper plasmid pMOBS mediating such mobilization with an efficiency up to 1000-fold higher than that achieved with native pCTX-M3. We also constructed *E. coli* donor strains with chromosome-integrated conjugative transfer genes: S14 and S15, devoid of one putative regulator (*orf35*), and S25 and S26, devoid of two putative regulators (*orf35* and *orf36*) of the pCTX-M3 *tra* genes. Strains S14 and S15, and S25 and S26 are, respectively, up to 100 and 1000 times more efficient in mobilization than pCTX-M3. Moreover, they also enable plasmid mobilization to the Gram-positive bacteria *Bacillus subtilis* and *Lactococcus lactis*. Additionally, the constructed *E. coli* strains carried no antibiotic resistance genes that are present in pCTX-M3 to facilitate manipulations with antibiotic-resistant recipient strains, such as those of clinical origin. To demonstrate possible application of the constructed tool, an antibacterial conjugation-based system was designed. Strain S26 was used for introduction of a mobilizable plasmid coding for a toxin, resulting in the elimination of over 90% of recipient *E. coli* cells.

34 **IMPORTANCE**

35 The conjugation of donor and recipient bacterial cells resulting in conjugative
36 transfer of mobilizable plasmids is the preferred method enabling the introduction of DNA
37 into strains for which other transfer methods are difficult to establish (e.g., clinical strains).
38 We have constructed *E. coli* strains carrying the conjugation system of the IncM plasmid
39 pCTX-M3 integrated into the chromosome. To increase the mobilization efficiency up to
40 1000-fold, two putative regulators of this system, *orf35* and/or *orf36*, were disabled. The
41 constructed strains broaden the repertoire of tools for the introduction of DNA into the
42 Gram-negative *Alpha-*, *Beta-*, and *Gammaproteobacteria*, as well as into Gram-positive
43 bacteria such as *Bacillus subtilis* and *Lactococcus lactis*. The antibacterial procedure based
44 on conjugation with the use of the *orf35*- and *orf36*-deficient strain lowered the recipient cell
45 number by over 90% owing to the mobilizable plasmid-encoded toxin.
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INTRODUCTION

The transfer of conjugative plasmids is one of the major mechanisms of horizontal gene transfer between bacteria, which plays a key role in bacterial ecology and evolution. Notably, it can also be utilized for biotechnological and laboratory purposes. For a conjugative DNA transfer event, several elements are necessary in the donor cell of a Gram-negative bacterium: i/ a DNA transporter of the type IV secretion system (T4SS) with the pilus responsible for establishing physical contact between the mating cells; ii/ a relaxase complex that nicks DNA, prepares it for transport and enables the start of DNA replication; iii/ *oriT*, a specific DNA sequence where the process of transfer begins, recognized and cut by the relaxase complex to generate single-stranded DNA with the relaxase covalently bound at its 5' end; and iv/ a coupling protein that brings together the DNA - relaxase complex and the T4SS transporter (1). Conjugative plasmids encode all the elements necessary for their conjugative transfer during mating. Such plasmids can also serve as helpers in the mobilization and transfer of mobilizable plasmids bearing compatible *oriT* sequences. Apart from their importance in nature, mobilizable plasmids are commonly used in laboratories because, being transferred as single-stranded DNA, they avoid the host restriction system (2, 3).

Genetic manipulations performed on diverse bacteria require effective methods for introducing DNA into recipient cells. Although many bacteria are not naturally competent, laboratory bacterial strains are easy to manipulate using methods such as chemical transformation and electroporation. However, these methods are often inefficient on clinical or environmental isolates. In this case, conjugative transfer is the most powerful method for introducing DNA into bacterial cells, and one of the most popular systems is based on the broad-host-range IncP-1α plasmid RP4/RK2 (4). Its conjugative transfer system is used to introduce DNA into a broad range of hosts, including virtually any Gram-negative bacteria,

73 certain Gram-positive ones (5), yeast (6, 7), and even mammalian cells (8). However, new
74 and/or alternative systems in the repertoire of laboratory methods are still required to
75 broaden the spectrum of recipients to include "difficult" bacteria such as multiresistant
76 isolates and also clinical or environmental strains already bearing the IncP-1α plasmids.

77 Multiresistant clinical strains pose one of the greatest health risks due to a lack of
78 effective therapies. Therefore, novel antibacterial treatments are urgently needed. One of
79 such alternatives is the bacterial conjugation-based technology (BCBT) that relies on a
80 transfer of killing agents during bacterial conjugation (9). In principle, it works as a Trojan
81 horse: acquisition of a mobilizable plasmid by the recipient should result in its death.
82 Simultaneously, the donor is protected from the deleterious action of the killing agent by an
83 agent-specific mechanism. The toxin - antidote (TA) systems (10) of the plasmid addiction
84 modules can be used in BCBT. The Zeta-Epsilon module, the TA system of the
85 streptococcal pSM19035 plasmid, has been shown to act as a plasmid addiction system not
86 only in various *Firmicutes* species but also in *E. coli* (11). Zeta toxin is a kinase
87 phosphorylating the peptidoglycan precursor UDP-*N*-acetylglucosamine (UNAG), which
88 inhibits cell wall synthesis (12). The Zeta-encoding gene located on a mobilizable plasmid
89 and introduced *via* conjugation into recipient bacteria may be used in BCBT. Noteworthy, in
90 Gram-negative bacteria no homologs of the gene encoding Epsilon, the antidote of the Zeta
91 toxin, have been found (13).

92 The IncM plasmid pCTX-M3 (GenBank Acc. No. AF550415) was isolated in 1996
93 from a clinical *Citrobacter freundii* strain in Poland as a vector of the $bla_{\text{CTX-M-3}}$ gene
94 encoding an extended-spectrum β-lactamase (14, 15). Plasmids of the IncL and IncM groups
95 (formerly constituting a single IncL/M group) are widespread in bacterial populations
96 worldwide and are responsible for the dissemination of different antibiotic resistance genes
97 (16–18), mostly through the conjugative transfer system (19). In addition to $bla_{\text{CTX-M-3}}$,

98 pCTX-M3 bears other genes, conferring resistance to β-lactams (*bla*$_{TEM-1}$), aminoglycosides
99 (*aacC2*, *aadA2*, and *armA*) and trimethoprim-sulfamethoxazole (*dhfrA12* and *sul1*) (15).
100 Recently, the host range of the pCTX-M3 replicon was verified to be much narrower than
101 previously determined (20) and restricted to *Enterobacteriaceae* (21). However, the range of
102 hosts of the conjugative transfer system of this plasmid is much broader than the host range
103 of its replicon and comprises *Alpha-*, *Beta-*, and *Gammaproteobacteria* (21). The closest
104 homologs of the pCTX-M3 conjugation system, besides other IncM and IncL plasmids, are
105 the IncI1 representatives R64 and ColIb-P9 (21), prototypes of the I-type conjugation system
106 (22). pCTX-M3 is able to mobilize plasmids bearing *oriT*$_{ColIb-P9}$ and *vice versa*, ColIb-P9
107 mobilizes plasmids containing *oriT*$_{pCTX-M3}$ (15), in both instances with low efficiency.
108 Elements of the conjugative transfer system of pCTX-M3 are encoded in two distant
109 regions, the *tra* and *trb* operons (15). Surprisingly, the replacement of *orf35*, a gene located
110 in the leading region and unnecessary for the conjugative transfer of pCTX-M3, with the *cat*
111 gene increased the mobilization of an *oriT*$_{pCTX-M3}$-bearing plasmid one-hundred-fold.
112 Moreover, a similar replacement of *orf36* from the *tra* region had no impact on the
113 conjugative transfer of pCTX-M3 but led to a ten-fold increase in the mobilization
114 efficiency (21). The deletion of *orf35* increased the transcript levels of the *nikA*, *nikB*, and
115 *traH* genes, whereas deletion of *orf36* increased the *traH* transcript level. The *tra* genes
116 located downstream of *traH* most likely also are subject to an *orf35*- and *orf36*-dependent
117 regulation (21), but its mode is currently unknown. In view of that greatly increased
118 mobilization efficiency of pCTX-M3 devoid of *orf35* alone or of *orf35* and *orf36* was a
119 promising candidate for the preparation of a novel conjugative donor strain.

120 Here, we present the construction of a helper plasmid, pMOBS, and a set of donor *E.*
121 *coli* strains, S14 and S15, all devoid of *orf35*, and S25 and S26, devoid of *orf35* and *orf36*,
122 as new tools for plasmid mobilization that are up to 1000-fold more efficient than parental

123 pCTX-M3. We show that the constructed strains enable conjugative transfer of mobilizable
124 plasmids into multiresistant clinical strains. Additionally, we found that a mobilizable
125 plasmid encoding the Zeta toxin is highly efficient in BCBT against a laboratory *E. coli*
126 strain, but not equally effective against several clinical *E. coli* strains. We also show that the
127 range of recipients in the conjugative transfer system is broader than previously determined
128 and comprises not only *Alpha-*, *Beta-*, and *Gammaproteobacteria* but also Gram-positive
129 bacteria such as *Bacillus subtilis* and *Lactococcus lactis*.
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RESULTS

We have shown previously (21) that in the presence of the helper plasmid pCTX-M3*orf35::cat* (devoid of a functional *orf35*, the first gene of the pCTX-M3 leading region), pToriT, a broad-host-range plasmid with $oriT_{pCTX\text{-}M3}$, was mobilized into the recipients *Escherichia coli* or *Agrobacterium tumefaciens*, with almost 100-fold higher efficiency than it was in the presence of intact pCTX-M3. Deletion of another gene, *orf36*, resulted in ca. 10-fold increase in the mobilization efficiency to both recipients (21). We have also shown that *orf46* located next to the *trb* region, is dispensable for conjugative transfer and that its deletion does not influence plasmid mobilization. Therefore, we decided to use the *orf46::cat* cassette for antibiotic selection of a pCTX-M3-based efficient helper plasmid devoid of the mobilization limiting *orf35* and *orf36.* The latter, due to its localization in the middle of the *tra* operon, was deleted at a later stage of plasmid construction.

Construction of the helper plasmid pMOBS and the *E. coli* donor strain S14. First, the pLMAB212 plasmid was constructed by multistep sub-cloning of the *tra*, *trb*, and *rep* regions from pCTX-M3*orf46::cat* (Figs. S1A and S1B,); pLMAB212 contains the IncM replicon, lacks *orf35*, and has *orf46* replaced by *cat* to allow for subsequent selection of transformants. The *cat* gene was integrated with two flanking FRT sequences (the Flp recombinase recognition targets) and therefore it could be removed in the presence of Flp recombinase (21, 23). Notably, pLMAB212 also lacks mobile genetic elements (insertion sequences and transposons) and antibiotic resistance genes from pCTX-M3, except for $bla_{TEM\text{-}1}$.

The structure of $oriT_{pCTX\text{-}M3}$ was predicted based on sequence similarity with *oriT* of R64, an IncI1 plasmid (Fig. S1C). This enabled the introduction of four mutations in the predicted nick region (ACATCTTGT → **CG**A**A**CT**A**GT) in the *oriT* sequence of pLMAB212 to construct pMOBS. These changes made *oriT* nonfunctional and introduced a

157 unique *Spe*I restriction site (A↓CTAGT). The ability of pMOBS to self-transfer was thereby
158 eliminated, but the plasmid mobilization efficiency remained unchanged (Fig. 1).

159 To construct a convenient *E. coli* donor strain, a pMOBS fragment comprising the
160 *tra* and *trb* genes, *attP* and *cat*, was integrated into the DH5α chromosome to yield the S14
161 strain (Fig. S2A). The correctness of the integration was verified by PCR (Fig. S2B) using
162 specific primers indicated in Table 3. The S14 chromosome carries the conjugative transfer
163 regions: *tra* - pos. 31300 – 54398, with *oriT* mutated in pos. 31626, 31629, 31631, and
164 31632; and *trb* - pos. 84101 – 89015, according to the pCTX-M3 GenBank sequence (Acc.
165 No. AF550415). Additionally, the S14 strain is resistant to chloramphenicol.

166 **Mobilization efficiency of the pMOBS helper plasmid and the S14 strain.** *E. coli*
167 strains DH5α(pCTX-M3), DH5α(pMOBS), and S14 were used as helpers in the
168 mobilization of pToriT, a vector based on the broad-host-range and low-copy-number
169 replicon $oriV_{pBBR1}$ (24). As shown in Fig. 2, the *E. coli* strain with pMOBS as a helper
170 plasmid mobilized pToriT almost 1000-fold more efficiently, and the S14 helper strain ca.
171 100-fold more efficiently in comparison with the strain with pCTX-M3.

172 **Construction of S14-derived strains.** The S14 strain is devoid of *orf35* regulating
173 the expression of the *tra* genes in pCTX-M3 (21). To delete the second regulatory gene,
174 *orf36*, we first removed the *cat* gene from S14 (Cm^r; *Δorf35*, *orf46::cat*) to obtain the S15
175 strain (Cm^s; *Δorf35*, *Δorf46*). Then, we constructed an *orf36* deletion mutant by replacing
176 this gene with *cat* to obtain the S25 strain (Cm^r; *Δorf35*, *orf36::cat*, *Δorf46*). Finally, *cat* was
177 removed from S25, resulting in the S26 strain (Cm^s; *Δorf35*, *Δorf36*, *Δorf46*) (Fig. S2C).

178 **Mobilization efficiency of helper strains S15, S25, and S26.** We tested the
179 efficiency of pToriT mobilization by the newly constructed helper strains S15, S25, and S26
180 in matings with the JE2571Rif^r *E. coli* recipient and compared with that of the S14 strain
181 (Fig. 3). Additionally, we verified the pToriT mobilization efficiency in interspecies matings

using the constructed helper strains as donors and representatives of *Alpha-*, *Beta-*, and *Gammaproteobacteria* as recipients. The recipients tested were: *A. tumefaciens*, *Cupriavidus necator* (previously *Ralstonia eutropha*) and *Pseudomonas putida* (as a non-enteric *Gammaproteobacterium*). As shown in Figs. 3 and S3A, when S25 or S26 were the donors, the mobilization efficiencies were ca. 50-100× higher than those of the S14 or S15 donors regardless of the recipient.

Additionally, we tested the ability of the S14, S15, S25, and S26 helper strains to mobilize pABB19oriT, a high-copy-number plasmid. Again, strains S25 and S26 were 100× more effective as donors than were S14 and S15 (Fig. S3B).

The S25 helper strain enables plasmid mobilization to Gram-positive bacteria. Because the pCTX-M3-derived system could efficiently mobilize plasmids into a wide range of Gram-negative bacteria, we sought to determine its ability to transfer plasmids into Gram-positive bacteria as well. For that purpose, we used pBSUoriT, which is a shuttle vector that replicates in *E. coli* using $oriV_{pMB1}$ and uses $oriV_{pAM\beta1}$ for replication in Gram-positive bacteria with low GC content. One of the most efficient helper strains, S25 (Cm^r) bearing pBSUoriT, was used as a donor in mating experiments. As recipients, we used a *B. subtilis* subsp. *subtilis* 168-derived laboratory strain YB1015Rif^r, a biofilm-forming *B. subtilis* subsp. *spizizeni* strain PCM2021Rif^r, and rifampicin-resistant *L. lactis*, a derivative of the laboratory plasmid-free strain IL1403. In each mating, interspecies transconjugants were selected (Fig. 4). Interestingly, a large difference in the mobilization efficiencies was observed between the two *B. subtilis* strains. PCM2021Rif^r gave transconjugants with a mobilization efficiency four orders of magnitude higher (over 10^{-2}/donor) than did YB1015Rif^r. Transconjugants of *L. lactis* were obtained at an efficiency of ca. 10^{-6}/donor. To verify the presence of pBSUoriT in the transconjugants, plasmid DNA from several *B.*

206 *subtilis* and *L. lactis* Sp^r Rif^r clones was isolated. Their restriction analysis confirmed the
207 presence of pBSUoriT (data not shown).

208 Additionally, to exclude the possibility that the Sp^r Rif^r clones of *B. subtilis* resulted
209 from transformation due to its natural competence, we tested mobilization of pBSUoriT or
210 pBSU1 (lacking $oriT_{pCTX\text{-}M3}$) into *B. subtilis* YB1015Rif^r. The appearance of the Sp^r Rif^r
211 *B.subtilis* clones was shown to be strictly $oriT_{pCTX\text{-}M3}$ dependent (Fig. S4).

212 **The S26 strain enables bacterial conjugation-based recipient killing.** To test the
213 usefulness of strain S26 in conjugation-based elimination of bacteria, we used pAZAKT, an
214 $oriT_{pCTX\text{-}M3}$-containing plasmid with the *zeta* gene coding for the toxin of the pSM19035
215 toxin-antidote (TA) system (11). The expression of *zeta* was controlled by the arabinose
216 operon P_{BAD} promoter (25). As a donor of pAZAKT, we used strain S26 additionally
217 carrying the pUC-epsi plasmid coding for Epsilon, the Zeta antidote. JE2571Rif^r was used as
218 a recipient. To avoid the killing of donor cells, the level of Zeta must be adequately balanced
219 to permit its inactivation by formation of complexes with Epsilon. Simultaneously, upon
220 conjugative transfer of pAZAKT, P_{BAD} should allow the *zeta* gene expression and
221 production of the toxin in recipient cells. In both the recipient and donor strains, the P_{BAD}
222 regulator AraC is encoded chromosomally. In the absence of arabinose, the chromosomally
223 encoded AraC protein tightly represses the chromosomally-encoded arabinose operon
224 *araBAD* by binding to the P_{BAD} promoter (26). However, in the presence of arabinose, AraC
225 stimulates transcription from P_{BAD}. In fast growing *E. coli* cells the level of AraC is low - ca.
226 20 molecules per cell (27). In S26(pAZAKT, pUC-epsi) cells the P_{BAD} promoter controlling
227 the *zeta* gene was on a plasmid present at 15-30 copies per cell (due to the $oriV_{P15A}$), so
228 AraC could be titrated out.

229 Plasmid pAZAKT was mobilized by S26(pUC-epsi) into JE2571Rif^r. In a control
230 experiment, we used the S26(pAAKT, pUC-epsi) strain bearing the inactive *zeta* gene as a

231 donor. Additionally, the same experiment was repeated with DH5αRif^r as a recipient. For
232 both recipients, the number of pAZAKT transconjugants was ca. three orders of magnitude
233 lower than that of the pAAKT ones, indicating *zeta*-dependent killing of transconjugants
234 (Fig. 5).
235 To determine whether arabinose supplementation was necessary to better observe the results
236 of Zeta toxicity, we compared the efficiencies of pAAKT and pAZAKT mobilization from
237 S26(pUC-epsi) to JE2571Rif^r in the presence or absence of 0.1% arabinose in conjugation
238 medium and/or medium for transconjugants selection. The results indicated that the addition
239 of arabinose to the conjugation medium or to the transconjugants selection medium did not
240 affect the number of transconjugants and hence did not increase the negative effect on
241 survival of the transconjugants (Fig. S5).
242 To check whether the transfer of the pAZAKT and pAAKT plasmids was dependent
243 on the conjugation system encoded by S26, in a control experiment we used DH5α(pUC-
244 epsi) as a donor. As expected, no transconjugants were detected (Fig. S6A), indicating the
245 strict dependence of pAZAKT and pAAKT transfer on the S26 donor strain.
246 **Characteristics of the JE2571Rif^r transconjugants surviving pAZAKT transfer.**
247 In principle, a cell could survive the transfer of *zeta* either if the *zeta* gene became inactive
248 or if the cell became resistant to Zeta action. To distinguish between these possibilities, both
249 the plasmids and the host cells of ten survivor clones were investigated in more detail. All of
250 them were found to be not only Km^r Rif^r but also Ap^r, which suggested that they carried the
251 pUC-epsi plasmid, probably as a cointegrate with pAZAKT.
252 i/ **Analysis of plasmids**. Digestion with *Hin*dIII, *Hin*cII, and *Eco*RI revealed
253 identical restriction patterns of all plasmids isolated from the survivor clones. Restriction
254 analysis of plasmids isolated from the survivors revealed a recombination of pAZAKT and
255 pUC-epsi within the 623-bp fragment identical in both plasmids. Additionally, the presence

of the *epsilon* gene in these plasmids was confirmed by PCR with primers EpsiS and EpsiE (Table 3). Moreover, sequencing of the *zeta* gene revealed no mutations in any of the plasmids. Finally, we showed that these plasmids could be transformed into *E. coli* DH5α, indicating that the presence of the antidote gene in the incoming plasmid prevents the toxic effect of Zeta.

ii/ **Analysis of bacterial hosts.** Six survivor clones were cured of plasmids by culturing in nonselective conditions (at 37°C in LB medium with rifampicin) for 5 days with 10^{-3} dilutions every 24 hours, to get Ap^s Km^s Rif^r cells. Next, the cured clones were transformed with pAZAKT and pUC-epsi, and transformants were selected on kanamycin-containing plates. Then, 100 transformants of each clone were tested for ampicillin resistance. All the transformants were Ap^r and Km^r, indicating that the establishment of pAZAKT required co-transformation with pUC-epsi (11). These results demonstrated that survivors of pAZAKT transfer did not acquire resistance to the Zeta toxin.

To address the problem of recombination between pAZAKT and pUC-epsi, the 623 bp-region common to both plasmids was removed from pUC-epsi to give a shortened version of the *epsilon*-bearing plasmid, pUC-epsiSH. This plasmid was introduced into the S26 strain which was then used to mobilize pAZAKT and pAAKT into the $JE2571Rif^r$ recipient. Transconjugants with pAZAKT were observed at an efficiency ca. 4200-fold lower than those with pAAKT (Fig. S6B). Plasmids from eight survivor clones were analysed. They could be introduced into DH5α by transformation, suggesting that either *zeta* was inactive or *epsilon* was additionally present in the transforming plasmid. Indeed, in four plasmids the *zeta* gene was disrupted: i/ in two cases, with a 4-bp (CTAG) insertion after the 85^{th} codon of *zeta*, ii/ in two cases, with an IS*1* insertion after 94^{th} or 196^{th} codon. The remaining four plasmids were found to confer resistance to ampicillin and had a higher copy number than pAZAKT, suggesting that they were recombinants with pUC-epsiSH. The

281 presence of *epsilon* was detected by PCR with EpsiS and EpsiE primers. Sequence analysis
282 revealed that none of the survivors contained a single plasmid with an active *zeta* gene.

283 **Optimization of recipient killing upon mobilization of the Zeta-encoding**
284 **plasmid.** To optimize the mobilization-mediated Zeta killing of recipient cells conditions of
285 the conjugation procedure were varied. S26(pUC-epsi) was used as a donor of pAZAKT and
286 JE2571Rif^r was the recipient. As a control pAAKT was mobilized. We used the donor and
287 recipient in the stationary phase of growth with the donor-to-recipient ratios of 130:1, 14:1
288 and 0.9:1. The recipient survival rate was calculated by comparing the number of recipients
289 (Rif^r cells) in the conjugation mixture after mating to their initial number. With the
290 pAZAKT donor-to-recipients ratio equal to 0.9, no reduction of the number of JE2571Rif^r
291 cells was observed (Fig. 6A). With an excess of donors over recipients efficient killing of
292 the latter was observed as their number was reduced to 35% and to 10% with the respective
293 ratios of 14:1 and 130:1. As expected no killing was observed when the pAAKT plasmid
294 was mobilized.

295 Since Zeta toxin is an inhibitor of cell wall synthesis, we expected that the killing of
296 recipient cells upon the pAZAKT transfer should be more efficient for actively dividing
297 cells. To check this, we used JE2771Rif^r in the exponential phase of growth (OD_{600}=0.4) as
298 the recipient, with S26(pUC-epsi) serving as a donor of pAZAKT or pAAKT. The donor-to-
299 recipient ratios were 3:1, 32:1, 273:1, and 2727:1, with the number of donors constant. The
300 respective recipient survival rates were 2.1%, 1.1%, 1%, and 0.7% when pAZAKT was
301 mobilized, while with the pAAKT transfer the recipient number actually increased (Fig. 6B).
302 These results confirmed, that indeed the recipient in the exponential phase of growth is more
303 susceptible to Zeta killing.

304 **The S26 helper strain enables plasmid mobilization to multiresistant bacteria of**
305 **clinical origin.** Plasmid mobilization is a convenient method for introducing DNA into

306 environmental or clinical strains for which conventional transformation methods are
307 ineffective. Such strains frequently contain plasmids of the IncP groups (28, 29). Since the
308 most widely used mobilization system utilizes genes coding for the conjugative transfer
309 system of RK2/RP4, it cannot introduce DNA into bacteria bearing plasmids from the IncP-
310 1α group. In contrast, S26, which encodes a highly efficient IncP-compatible mobilizing
311 system from pCTX-M3, was a good candidate for a plasmid donor. To test this we used
312 S26(pToriT) in matings with two clinical *E. coli* strains as recipients, 1355/2004 and
313 1149/2004, both carrying IncP replicons as determined using the plasmid replicon typing
314 method performed according to Carattoli et al. (30). Transconjugants were obtained for the
315 both recipients, albeit with different efficiencies: over 10^{-1}/donor for 1149/2004 and
316 10^{-4}/donor for 1355/2004 (Fig. 7).

317 Importantly, S26 is devoid of any antibiotic-resistance determinants, which is a
318 desired feature of mobilizing systems for introduction of DNA into clinical or environmental
319 strains.

320 **The *zeta* gene transferred from the S26 strain eliminates bacteria of clinical**
321 **origin.** To check whether the mobilization system described above could also be used
322 against clinical isolates, the clinical *E. coli* strains 1355/2004 and 1149/2004 were used as
323 recipients. As for the JE2571Rif^r strain, in both cases the number of pAAKT transconjugants
324 was higher than that of the pAZAKT ones, indicating *zeta*-dependent killing (Fig. 8A). That
325 difference was 1275-fold for 1149/2004 and only 40-fold for 1355/2004. At the same time
326 the overall efficiency of plasmid mobilization (determined for pAAKT) was ca. five orders
327 of magnitude lower for 1355/2004 than for either 1149/2004 or JE2571Rif^r.

328 **The low mobilization efficiency into the 1355/2004 strain is not caused by its**
329 **resident IncP plasmid.** Conjugative plasmids encode mechanisms, such as entry exclusion
330 systems acting in recipients to prevent acquisition of identical plasmid backbones (31). Such

a system could be present on the IncP plasmid residing in the 1355/2004 strain. To verify this hypothesis first, the IncP plasmid conferring tetracycline resistance in 1355/2004 was transferred by conjugation to JE2571Rif^r. Then, three independent transconjugants of JE2571Rif^r with the 1355/2004 plasmid, named R$^+$[*E. coli* 1355/2004], were used as recipients in matings with the pair of donors S26(pAZAKT, pUC-epsi) and S26(pAAKT, pUC-epsi). The efficiencies of mobilization into R$^+$[*E. coli* 1355/2004] were identical to those to JE2571Rif^r (Fig. 8B), indicating that the features determining the low mobilization efficiency of the *oriT*$_{pCTX-M3}$-bearing plasmid into 1355/2004 were not encoded by the IncP plasmid.

DISCUSSION

Based on the I-type conjugation system of the IncM plasmid pCTX-M3 we constructed and characterized a set of bacterial strains for efficient mobilization of *oriT*$_{pCTX-M3}$-bearing plasmids. First, the mobilizing pMOBS plasmid with an inactivated *oriT*$_{pCTX-M3}$ was constructed. This plasmid bears the IncM replicon, therefore it can replicate in *Enterobacteriaceae*. pMOBS is devoid of *orf35* found earlier to be involved in the regulation of *tra*$_{pCTX-M3}$ genes (21). The conjugation system from the pMOBS helper plasmid was also introduced into the *E. coli* chromosome to create the S14 donor strain. An additional deletion of *orf36*, a gene unique to the IncL and IncM plasmids, involved in the regulation of expression of T4SS transporter-encoding genes (21), produced strains S25 (Cm^r) and S26 (Cm^s). These strains were ca. 50-100× more efficient as donors than was S14 in matings with all *Alpha-*, *Beta-*, and *Gammaproteobacteria* recipients tested. Moreover, these pCTX-M3-conjugation system-based strains enabled plasmid mobilization even into the Gram-positive bacteria *B. subtilis* and *L. lactis*, indicating that the range of the recipients of the pCTX-M3 conjugation system is even broader than previously shown.

356 The present system is a good alternative to the S17-1/SM10 mobilization system and
357 its derivatives based on the IncP-1α plasmid RP4/RK2, in which the entire plasmid was
358 integrated into the *E. coli* chromosome using phage Mu (32). S17-1/SM10 allows the
359 introduction of $oriT_{RP4/RK2}$-bearing plasmids into various species (for examples see 30, 31).
360 Its major drawback is that it contains all of the antibiotic resistance genes present in
361 RK2/RP4 and can also promote the transfer of chromosomal genes because of the
362 combination of $oriT_{RP4/RK2}$ functionality and phage Mu mobilization (35–37). The strain was
363 improved after twenty years by *oriT* inactivation, but it still retained the entire integrated
364 plasmid (36, 38). The Mu activation problem was solved by inactivating the phage (37) or
365 by constructing the broad-host-range plasmid pTA-Mob with $oriT_{RP4/RK2}$ inactivated (36).
366 In contrast to the widely used S17-1/SM10 donor strain, the newly constructed strain
367 S14 and its derivatives S15, S25, and S26 contain neither the IS sequences nor antibiotic
368 resistance genes present in the parental plasmid pCTX-M3. The selective marker *cat* was
369 easily deleted using Flp recombinase (39) to obtain the chloramphenicol-sensitive S15 and
370 S26 strains. Importantly, strain S26 enabled efficient plasmid mobilization also into *E. coli*
371 strains of clinical origin which contained IncP-1α replicons (30) and thus could not be
372 recipients in matings with RK2/RP4-based donors. Similarly, IncM plasmid-carrying strains
373 cannot be recipients in matings with donors bearing pMOBS as well as with the S14 strain
374 and its derivatives due to the presence of the entry exclusion system encoded by the *traY* -
375 *excA* genes (40).
376 To illustrate the usefulness of the constructed strains as potential conjugative
377 antibacterial agents we used the S26 strain in BCBT as a vehicle of the Zeta toxin-encoding
378 gene. The number of transconjugants upon mobilization of the active *zeta*-encoding plasmid
379 pAZAKT was over three orders of magnitude lower than that of the control plasmid pAAKT
380 indicating Zeta-dependent killing of transconjugants. Moreover, the transfer of a Zeta-

encoding plasmid eliminated up to 90% of recipient *E. coli* cells in the stationary phase of growth when the number of donors exceeded that of recipients 130-fold. For an exponentially growing population of recipients the efficiency of killing by *zeta* mobilization was much higher, between ca. 98% and 99.3% for the donor excess of 3-fold to 2727-fold. One should note, however, that in a natural setting the recipient population is unlikely to comprise exclusively actively dividing cells. Nevertheless, this limitation results from the toxin used, not from the mobilizing system, thus using another killing agent could overcome this issue.

Preliminary results obtained for one of the clinical strains, 1149/2004, used as a recipient were highly promising regarding potential use indicating a decrease of over three orders of magnitude of the number of transconjugants upon pAZAKT mobilization. However, the number of pAZAKT transconjugants of another *E. coli* clinical strain tested, 1355/2004, was reduced to a lesser extent (only by a factor of 40). The mobilization efficiency to the 1355/2004 recipient was ca. five orders of magnitude lower compared to the laboratory strain and that effect was not related to the presence of a conjugative IncP plasmid in the recipient. Another potential reason for the poor plasmid mobilization into the 1355/2004 strain could stem from its mucoid phenotype. It has been suggested that the capsule constitutes a physical barrier for DNA (41), lowering the rate of horizontal gene transfer. However, recent findings (42) indicate that bacteria with capsule systems are more genetically diverse and have fast-evolving gene repertoires, suggesting intensive genetic exchange. At present, the reason for the lower population-reducing effect observed for the 1355/2004 strain remains unknown.

The analysis of the JE2571Rif^r transconjugants surviving pAZAKT transfer showed that it was not due to the development of Zeta-resistance. The survivors contained either an

405 inactivated *zeta* gene or a recombinant of pAZAKT and the *epsilon*-encoding plasmid from
406 the donor, most probably due to the activity of chromosomally encoded IS*1* (43).

407 In summary, we have constructed a novel tool for plasmid mobilization based on the
408 pCTX-M3 conjugation system: the pMOBS helper plasmid; the *E. coli* helper strains S14,
409 S15, S25, and S26; and mobilizing vectors that can easily be modified to fit specific
410 requirements. The system can mobilize $oriT_{pCTX\text{-}M3}$-containing plasmids into a broad range
411 of hosts, including not only *Alpha-*, *Beta-*, and *Gammaproteobacteria*, but also the Gram-
412 positive bacteria *B. subtilis* and *L. lactis*, and thus can be used in a variety of
413 biotechnological applications.

414

415 **MATERIALS AND METHODS**

416 **Bacterial strains and growth conditions.** The strains used in this work are listed in
417 Table 1. *E. coli* DH5α was used as the host strain for DNA cloning. In mating experiments,
418 DH5α bearing pCTX-M3 or its derivatives (Table 2) was used as a donor. *E. coli* strain
419 JE2571Rif^R or the clinical *E. coli* isolates 1355/04 and 1149/04 were used as recipients. In
420 trans-species matings, *Pseudomonas putida*, *Cupriavidus necator*, *Agrobacterium*
421 *tumefaciens*, *Bacillus subtilis*, and *Lactococcus lactis* were used as recipients. Bacteria were
422 cultured with agitation in LB medium (Biocorp, Warsaw, Poland) or on agar-solidified LB
423 plates (44) at either 37°C (*E. coli*, *P. putida* and *B. subtilis*) or 30°C (*A. tumefaciens* and *C.*
424 *necator*). *L. lactis* was cultured without agitation in GM17 (M17 broth from Oxoid Ltd.,
425 Basingstoke, United Kingdom, with 0.5% glucose) or on agar-solidified GM17 plates. When
426 required, antibiotics were added to the medium at the following final concentrations (μg/ml):
427 ampicillin, 100; chloramphenicol, 20; kanamycin, 50; rifampicin, 100; spectinomycin, 100;
428 tetracycline, 20.

DNA cloning and manipulation. Plasmid DNA was isolated by the alkaline lysis method using A&A Biotechnology Mini or Midi Plasmid kits (Gdańsk, Poland) according to the manufacturer's instructions. For isolation of plasmid DNA from *B. subtilis* or *L. lactis*, 20 µg/ml lysozyme (Serva, Heidelberg, Germany) was added to solution L1, followed by a 30-min incubation at 37°C. Cloning procedures were performed according to standard protocols (44). All enzymes used for cloning were obtained from Thermo Fisher Scientific (Waltham, MA, USA).

Plasmid construction. Plasmids used in this study are listed in Table 2. The pMOBS plasmid was constructed as follows. First, short sequences flanking the *tra* and *trb* regions (flanks) were PCR-amplified from the pCTX-M3 template except for the downstream flank of *trb*, which was amplified from pCTX-M3*orf46::cat* using primers listed in Table 3. Initially, the four amplified flanks were cloned independently into the pUC18 vector to give pUCA0118, pUCA0218, pUCA0318, pUCB0219 and pUCB0318 plasmids (Fig. S1A). Next, both flanks of *tra* were cloned together, the pUCA0218 *Kpn*I-*Sal*I fragment was transferred into *Kpn*I-*Sal*I digested pUCA0318 to give pUCA3218. Similarly, both *trb* flanks were cloned together, the *Sal*I-*Kpn*I fragment from pUCB0318 was introduced into the *Sal*I-*Kpn*I site of pUCB0219 to give pUCB3219. pUCB3219 was cut with *Bsp*1407I to give pUCB3219B for subsequent cloning. pUCA3218 and pUCB3219B contained terminal parts of the *tra* and *trb* regions, respectively. Further cloning was carried out in pLD1 (a derivative of pLDR10 devoid of the chloramphenicol resistance gene, carrying an *attP* sequence): the *Eco*RI-*Bam*HI fragment from pUCB3219B was cloned into pLD1, resulting in pLDB, which then received the *Hin*dIII-*Bam*HI fragment of pUCA3218 to generate pLDAB (Fig. S1A). The high copy-number replicon ($oriV_{pMB1}$) from pLDAB was replaced by the PCR-amplified (primers FrepCNI and RrepANB2, pBS3-1 as a template) low copy-number replicon of pCTX-M3 ($oriV_{pCTX-M3}$) to obtain pLMAB2. Then, the central *Bsp*1407I-

454 *Bsp*1407I fragment of the *trb* region from pSN17 was introduced into pLMAB2 to give
455 pLMAB202, which next received the central *Aat*II-*Nhe*I fragment of the *tra* region from
456 pSS29 to give pLMAB212 (33614 bp), as presented in Fig. S1B.

457 To make pLMAB212 unable to self-propagate, mutations in the nick region were
458 introduced as follows. Regions surrounding the nick region were amplified from
459 pLMAB212 (primer pairs FAatII-RnicSpe and FnicSpe-RPshAI) to introduce a site
460 recognized by the *Spe*I restrictase in the nick region. These fragments were cloned
461 individually in pAL3 (plasmids pAL-AS14 and pAL-SP3) and then combined to give
462 pALAP. Next, the kanamycin resistance gene amplified from pET28a+ (primers FKanSpe2
463 and RKanSpe) was cloned into the *Spe*I site introduced into the nick region of pALAP to
464 produce pALAPK1. Then, the *Aat*II-*Psh*AI fragment from pALAPK1 replaced the
465 appropriate fragment in pLMAB212 to create pMOBSK (transformants were selected on
466 kanamycin-containing LB plates). Finally, the kanamycin resistance gene (the *Spe*I-*Spe*I
467 fragment) was removed from pMOBSK to give pMOBS. The mutated $oriT_{pCTX\text{-}M3}$ sequence
468 in pMOBS is shown in Fig. S1C.

469 **Construction of pAZAKT.** The *zeta* gene was PCR-amplified from pBT233
470 (primers EcoZetaFor and ZetaRevBam) and then the *Eco*RI (blunted)-*Bam*HI fragment was
471 cloned into pET28a+ digested with *Nde*I (blunted)-*Bam*HI to give pET-zeta12. The *Bgl*II-
472 *Sal*I fragment of pET-zeta12, comprising *zeta*, was cloned into *Bgl*II-*Sal*I-digested
473 pACYC184 to produce pACYC-zeta. Then, the 114-bp *Xba*I-*Nhe*I fragment containing
474 P_{BAD}, the arabinose operon promoter from pBAD24, amplified with upTEM and
475 ARA1down (Table 3), was cloned into *Xba*I-digested pACYC-Zeta. The plasmid with
476 proper orientation of P_{BAD} was called pAZA. Next, the *Nae*I-*Bsi*WI fragment comprising
477 $oriT_{pCTX\text{-}M3}$ and the kanamycin resistance gene from pABB20oriT was introduced in *Tat*I-
478 *Pvu*II-digested pAZA to give pAZAKT. All *zeta* bearing plasmids were constructed in

479 DH5α(pUC-epsi), an Epsilon producing strain. The activity of the *zeta* gene in each of the
480 constructed plasmids was verified by a co-transformation assay with pUC-epsi (11).

481 **Construction of pAAKT.** pAZAKT was digested with *Spe*I, blunted and re-ligated,
482 resulting in pAAKT with a frame-shift in the 78th codon of the *zeta* gene. The lack of
483 activity of the *zeta* gene in pAAKT was verified by co-transformation with pUC-epsi (11).

484 **Strain construction.** The S14 strain, with the *tra* and *trb* modules integrated into the
485 chromosomal *attB* site, was constructed by transforming *E. coli* DH5α(pLDR8) (45),
486 carrying the λ phage integrase gene, with the circularized DNA comprising the pMOBS
487 plasmid devoid of the *Eco*31I-*Eco*31I fragment containing $oriV_{pCTX\text{-}M3}$ and $bla_{TEM\text{-}1}$ (Fig.
488 S2A). A strain devoid of pLDR8 was selected by colony purification. The correct
489 chromosomal integration of the *tra* and *trb* regions was verified by multiplex PCR (Fig.
490 S2B) with primers specified in Table 3.

491 Strain S15 was constructed by elimination of the *cat* gene from the chromosome of
492 S14 with the use of the Flp recombinase encoded by pCP20 according to the method
493 described by Datsenko and Wanner (23). Next, S15(pKD46) was transformed with *Dpn*I-
494 treated PCR-amplified *orf36::cat* (generated using primers orf36uP1 and orf36dP2 on pKD3
495 as a template, Table 3) to inactivate *orf36* by replacement with the *cat* gene to give the S25
496 strain. S26 is an S25 derivative with *cat* eliminated with the use of Flp recombinase encoded
497 by pCP20. The correctness of the *cat* elimination or insertion was verified by PCR with
498 primers pCTX96 and orf36sU (Table 3).

499 **PCR conditions.** PCR was performed in a Veriti Thermal Cycler (Applied
500 Biosystems, Foster City, CA, USA) using DreamTaq DNA polymerase with supplied
501 buffers (Thermo Fisher Scientific), dNTP mixture and a template (purified DNA or bacterial
502 cells), with appropriate primer pairs listed in Table 3, according to manufacturer's
503 recommendations. *Pfu* DNA polymerase was used for the generation of DNA fragments that

504 were used in the construction of pMOBS and strain S14 as well as for amplification of the
505 P_{BAD} promoter and of *orf36::cat* for construction of the S25 strain.

506 **Plasmid conjugative transfer.** Generally, matings were performed as described
507 previously (21). *B. subtilis* was grown in LB to stationary phase (approximately 10^8 CFU
508 ml^{-1}), washed twice with LB medium and resuspended in one-fourth of the initial culture
509 volume. The mixture of the donor and recipient was filtered through a sterile Millipore HA
510 0.45 µm filter (Millipore, Billerica, MA, USA). The filter was then incubated on an LB plate
511 at 30°C for 24 h (*B. subtilis*) or 2 h (Gram-negative bacteria). When *B. subtilis* was the
512 recipient, LB plates containing DNase I (100 U/ml) were used. The *E. coli – L. lactis*
513 matings were performed similarly, except that the *L. lactis* recipient was prepared as
514 described by Bogusławska et al. (46) from exponentially grown culture, and after the donor
515 and recipient were filtered, the filter was incubated on a BHI (Oxoid Ltd) plate with DNase I
516 (100 U/ml) at 30°C for 24 h. The conjugation was stopped by vigorous vortexing of the
517 mating mixture for 30 s and then placing it on ice. Serial dilutions of the donor, recipient,
518 and mating mixture were plated on selective LB agar (or GM17 for *L. lactis*) supplemented
519 with appropriate antibiotics. The efficiency of conjugative transfer is expressed as the
520 number of transconjugants per donor cell. As a control, dilutions of the donor and recipient
521 cells were plated on LB (or GM17 for *L. lactis*) supplemented with the antibiotics
522 appropriate for transconjugant selection.

523 **Mobilization-mediated Zeta killing assays**. The mobilization-mediated Zeta killing assay
524 was performed following mating as described previously (21) with modifications involving
525 the use of an excess of donors. The recipients were in either the stationary or the exponential
526 phase of growth. In experiments with recipient cells in the stationary phase, 50 µl of a
527 recipient suspension (4.6×10^5 per ml) was mixed with 950 µl of a donor suspension (initial
528 concentration 7.0×10^9 per ml) diluted 1-, 10- or 100-fold. For recipients in the exponential

529 phase of growth (OD_{600}=0.4) the conjugation mixture was composed of 500 µl of the donor
530 suspension (1.2×10^9 per ml) and 500 µl of 1-, 10-, 100- or 1000-fold diluted recipient
531 suspension (initial concentration 4.0×10^8 per ml). Following conjugation the number of Rif^r
532 cells was compared with the initial number of recipients to establish the recipient survival
533 rate.
534

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TABLES

Table 1. Bacterial strains used in this study.

Species	Strain	Genotype or relevant feature	Source
Escherichia coli	DH5α	*ϕ*80 *lacZ*ΔM15 *deoR endA1 gyrA96 hsdR17 recA1 relA1 supE44 thi-1* Δ(*lacZYAargF*)*U169*	(47)
	DH5αRif^r	DH5α selected on LB with rifampicin	This work
	JE2571Rif^r	JE2571 selected on LB with rifampicin	(21)
	S14	DH5α with the pCTX-M3 *tra-trb* genes integrated, *Δorf35*, *orf46::cat*; Cm^r	This work
	S15	S14 with Δ*orf46*, Cm^s	This work
	S25	S15 with *orf36::cat*; Cm^r	This work
	S26	S25 with Δ*orf36*; Cm^s	This work
	1149/2004	Clinical isolate; Ap^r, Tc^r, Str^r; replicons: IncFIB, IncI1, IncP	Collection of National Institute of Medicines (48)
	1355/2004	Clinical isolate; Ap^r, Tc^r, Str^r; replicon: IncP	Collection of National Institute of Medicines (48)
Bacillus subtilis	YB1015	*amyE metB trpC xin-1 attSPβ recA*	(49)
	YB1015Rif^r	YB1015 selected on LB with rifampicin	This work
	PCM 2021	Biofilm forming strain	Polish Collection of Microorganisms
	PCM 2021Rif^r	PCM 2021 selected on LB with rifampicin	This work
Lactococcus lactis	IL1403		(50)
	IL1403Rif^r	IL1403 selected on GM17 with rifampicin	This work
Agrobacterium tumefaciens	LBA1010	Rif^r	(51)
Pseudomonas putida	KT2442	Rif^r	(52)
Cupriavidus necator (previously *Ralstonia eutropha*)	JMP228	Rif^r, *gfp*, Km^r	(53)

729 **Table 2.** Plasmids used in this work.

Name	Relevant feature or construction description	Source
	pCTX-M3 and its derivative	
pCTX-M3	IncM plasmid, 89468 bp; Ap^r, Pi^r, Azt^r, Caz^r, Cft^r, Km^r, Gen^r, To^r	(15, 54)
pCTX-M3*orf46::cat*	pCTX-M3 with *orf46* replaced with the *cat* gene; Ap^r, Pi^r, Azt^r, Caz^r, Cft^r, Km^r, Gen^r, To^r, Cm^r	(21)
	Cloning vectors	
pABB19	cloning vector; $oriV_{pMB1}$, Ap^r	(55)
pACYC184	cloning vector; $oriV_{P15A}$, Tc^r, Cm^r	(56)
pAL3	cloning vector $oriV_{P15A}$, Tc^r	(21)
pBAD24	vector, P_{BAD} promoter of *araBAD* (arabinose) operon, $oriV_{pMB1}$, Ap^r	(57)
pBBR1 MCS-2	vector; $oriV_{pBBR1}$, $oriT_{RK2}$, Km^r	(24)
pBSU100	*E. coli – Firmicutes* shuttle vector; $oriV_{pMB1}$, $oriV_{pAMβ1}$, *egfp*, Sp^r, Ap^r	(58)
pBSU1	pBSU100 with deleted *egfp* containing fragment *Sac*I-*Sph*I ($oriV_{pMB1}$, $oriV_{pAMβ1}$, Sp^r)	This work
pCP20	Flp recombinase expression plasmid; *repA101*(ts), $oriV_{R101}$, Ap^r, Cm^r	(39)
pET28a+	vector, $oriV_{pMB1}$, Km^r	Novagen
pKD3	template for generation of the *cat* gene containing flanks for gene disruption, *pir*-dependent replicon; $oriV_{R6Kγ}$, Ap^r, Cm^r	(23)
pKD46	λRed recombinase expression plasmid; *repA101*(ts), $oriV_{R101}$, Ap^r	(23)
pLDR8	helper plasmid for integration; *int*(λ) gene, $oriV_{pSC101}$, Km^r	(45)
pLDR10	vector for integration into *attB*; *attP*, $oriV_{P15A}$, Ap^r, Cm^r	(45)
pUC18	cloning vector; $oriV_{pMB1}$, Ap^r	(59)
pUC19	cloning vector; $oriV_{pMB1}$, Ap^r	(59)
	Plasmids carrying $oriT_{pCTX-M3}$	
pALoriT	pOriT *Eco*RI-*Pst*I fragment containing $oriT_{pCTX-M3}$ cloned into *Eco*RI-*Pst*I pAL3 ($oriV_{p15A}$, Tc^R)	This work
pABB19oriT	pOriT BamHI-PstI fragment containing $oriT_{pCTX-M3}$ (31616-31721)* cloned into *Bam*HI-*Pst*I of pABB19 ($oriV_{pMB1}$, Ap^r)	This work
pBBToriT	pALoriT *XbaI-PvuI* fragment containing tetracycline resistance gene and $oriT_{pCTX-M3}$ cloned into *PvuI-XbaI* pBBR1MCS-2 ($oriV_{pBBR1}$, Tet^R)	This work
pBSUoriT	pOriT *Pae*I-*Sac*I fragment containing $oriT_{pCTX-M3}$ cloned into *Pae*I-*Sac*I of pBSU1 ($oriV_{pMB1}$, $oriV_{pAMβ1}$, Sp^r)	This work
pOriT	$oriT_{pCTX-M3}$ (31616-31721)* in pMI3 vector ($oriV_{pMB1}$, Cm^r)	(15)
pToriT	pBBToriT derivative, fragment BsaI-Bst1107I with MOB_{RK2} removed ($oriV_{pBBR1}$, Km^r, Tc^r)	This work
	Plasmids used for *tra* and *trb* assembly and pMOBS construction	
pALAP	pAL-SP3 *Spe*I-*Pst*I fragment cloned into *Spe*I-*Pst*I of pAL-AS14	This work
pALAPK1	pET28a+ fragment (3943-4832), contains kanamycin resistance gene amplified with primers FKanSpe2# and RKanSpe# (*Spe*I) cloned into *Spe*I of pALAP	This work
pAL-AS14	pLMAB212 fragment (92-359) amplified with primers FAatII# and RnicSpe#, cloned into *Sma*I of pAL3	This work
pAL-SP3	pLMAB212 fragment (356-1622) amplified with primers FnicSpe# and RPshAI#, cloned into *Sma*I of pAL3	This work

pBS3-1	pCTX-M3 minireplicon; fragment *Bst*1107I-*Stu*I (54309-57986)*, Ap^r	This work
pHS11	pCTX-M3 derivative *Sex*AI-*Sna*BI (36645-40568)* and *Nru*I-*Sal*I (51663-58653)* fragments	This work
pLD1	pLDR10 derivative, removed fragment *Bsm*I (1713-2120)	This work
pLDAB	pUCA3218 *Hin*dIII-*Bam*HI fragment cloned into *Hin*dIII-*Bam*HI of pLDB	This work
pLDB	pUCB3219B *Eco*RI-*Bam*HI fragment cloned into *Eco*RI-*Bam*HI of pLD1	This work
pLMAB2	pBS3-1 (3624-2152) fragment containing pCTX-M3 replicon, amplified with primers FRepCNI# and RepANB2# (*Not*I), cloned into *Not*I of pLDAB	This work
pLMAB202	pSN17 *Bsp*1407I (11410-14854; 84420-87864)* fragment cloned into *Bsp*1407I of pLMAB2	This work
pLMAB212	*Aat*II-*Nhe*I pSS29 fragment cloned into *Aat*II-*Nhe*I of pLMAB202; *tra* (30634-59557)* and *trb* (84101-89015)* of pCTX-M3,$oriT_{pCTX-M}$, $oriV_{pCTX-M3}$, Ap^r	This work
pMOBS	pMOBSK derivative, removed the *Spe*I-*Spe*I fragment with the kanamycin resistance gene; *tra* (30634-59557)* and *trb* (84101-89015)* of pCTX-M3, $oriV_{pCTX-M3}$, Cm^r, Ap^r	This work
pMOBSK	pALAPK1 *Aat*II-*Psh*AI fragment cloned into *Aat*II-*Psh*AI of pLMAB212	This work
pSN17	pCTX-M3 *orf46::cat* derivative, *Nde*I-*Sph*I (53187-59797)* and *Sph*I –*Nde*I(80753–626)* fragments	This work
pSS29	pCTX-M3 derivative, *Swa*I-*Sal*I (30630–59552)* and *Sal*I-*Swa*I (64145–64426)* fragments	This work
pUCA0118	pCTX-M3 (31285-32022)* fragment amplified with primers FtraHind# and RtraPst # (*Hin*dIII, *Pst*I), cloned into *Hin*dIII-*Pst*I of pUC18	This work
pUCA0218	pCTX-M3 (52154-54408)* fragment amplified with primers FtraSa#l and RtraXba# (*Sal*I, *Xba*I), cloned into *Sal*I-*Xba*I of pUC18	This work
pUCA0318	pUCA0118 derivative with substitutions in the *nic* sequence introduced with primers FnicM# and RnicM#	This work
pUCA3218	*Kpn*I-*Sal*I pUCA0218 fragment cloned into *Kpn*I-*Sal*I of pUCA0318	This work
pUCB0219	pCTX-M3 (87807-89020)* fragment amplified with primers FtrbNco-Sac#-RtrbEco# (*Sac*I, *Eco*RI), cloned into *Sac*I-*Eco*RI of pUC19	This work
pUCB0318	pCTX-M3 *orf46::cat* fragment (83021-85053)* amplified with primers FtrbXba# and RtrbBam#, cloned into *Sma*I of pUC18	This work
pUCB3219	*Sal*I-*Kpn*I pUCB0318 fragment cloned into *Sal*I-*Kpn*I of pUCB0219	This work
pUCB3219B	pUCB3219 derivative, fragment *Bsp*1407I (1543-1729) removed	This work
	Plasmids coding for *zeta* or *epsilon*	
pUC-epsi	pACE1 *Eco*RI-*Hin*cII fragment containing *epsilon* gene cloned into *Eco*RI-*Hin*cII digested pUC18; $oriV_{pMB1}$, Ap^r	This work
pUC-epsiSH	shortened pUCepsi after *Bsp*119I and *Ehe*I digestion, the sticky ends were blunted and re-ligated ; $oriV_{pMB1}$, Ap^r	This work
pBT233	pSM19035 derivative, Acc. No. X64695	(60)
pET-zeta12	pET28a+ with *zeta* cloned in *Nde*I (blunted)-*Bam*HI	This work
pACYC-zeta	pACYC184 with *zeta* gene, $oriV_{P15A}$, Cm^r	This work
pACE1	pACYC184 with *epsilon* gene of pSM19035, $oriV_{P15A}$, Tc^r	(11)
pAZA	pACYC-Zeta with P_{BAD}, $oriV_{P15A}$, Cm^r	This work
pAZAKT	pAZA with $oriT_{pCTX-M3,}$ the *zeta* gene under control of P_{BAD}, $oriV_{P15A}$, Km^r, $oriT_{pCTX-M3}$	This work
pAAKT	pAZAKT *Spe*I-digested, filled-in, re-circularized, inactive *zeta* gene under control of P_{BAD}, $oriV_{P15A}$, Km^r, $oriT_{pCTX-M3}$	This work

Ap – ampicillin, Azt - aztreonam, Cft - cefotaxim, Caz - ceftazidime, Cm – chloramphenicol, Gen - gentamicin, Pi - piperacillin, Sp - spectinomycin, Tc –tetracyclin, To - tobramycin; (ts) - thermosensitive

*pCTX-M3 coordinates (Acc. No. AF550415);

primers listed in Table 3

734 **Table 3**. Primers used in this work.

Primer	Sequence 5'-3'	PCR template
	Primers for *tra trb* assembly	
FtraHind	CATACCCTTTCGAAGCTTTCAGC	pCTX-M3
RtraPst	CTCCTGCTGCAGTTTCTGTGC	pCTX-M3
FnicM	GTACGGGACAATATTGGTTTTTGGAGTACCGC	pCTX-M3
RnicM	CTCCAAAAACCAATATTGTCCCGTACTTAAATACC	pCTX-M3
FtraSal	GCAGGGTCGACTTCTATCTTCGCTAGCGG	pCTX-M3
RtraXba	ACTCTCTCTAGAACTCCGGGTTAC	pCTX-M3
FtrbXba	AGATCTAGAAAACGTTGCTTAACGTGAG	pCTX-M3 *orf46::cat*
RtrbBam	TTCCAGGATCCCCTGGTACGCAGCGCAG	pCTX-M3 *orf46::cat*
FtrbNco-Sac	CGGTTGAGCTCGTCGAGAATGGATTTAGC	pCTX-M3
RtrbEco	AATAGAATTCCTCTGACACCCTCTC	pCTX-M3
FrepCNI	GTGGCGGCCGCGTAAGAAACCATTATTATC	pBS3-1
RrepANB2	TAGGCGGCCGCGGTCTCGCACCCCTGCCGTCTTACG	pBS3-1
	Primers for nick region mutagenesis	
FAatII	TTCTGACGTCACATCAGGCAAGTCG	pLMAB212
RnicSpe	AACCGAACTAGTCCCGTACTTAAATACCTC	pLMAB212
FKanSpe2	GAACTAGTCATGAACAATAAAACTGTCTGC	pET28a+
RKanSpe	AGACTAGTATCCGCTCATGAATTAATTC	pET28a+
FnicSpe	GGACTAGTTCGGTTTTTGGAGTACCGCCGACAC	pLMAB212
RPshAI	GAAGACCGATGTCTGCAAATGTCTTATGC	pLMAB212
	Primers for Kan-*oriT* cloning	
FKanAatII	ATGGACGTCAGCTACTGGGCTATCTGG	pToriTB
oriTminDAatII	TTGGACGTCTGCAGAGATAGCTAACCTCGTTAGG	pToriTB
	Primers for *orf36* replacement with *cat*	
orf36uP1	ATGCAAACAGTGATGCATTCCCGTTCCATTTGTAACGTGTAGGCTG GAGCTGCTTCG	pKD3
orf36dP2	GAACAATGAGGTATACATGAGCGAACATAATGATTATATATGAAT ATCCTCCTTA	pKD3
	Primers for integration verification	
ybhB122	CTGGCAAGCGCCTCGATTAC	
ybhC159	ACCAGGCGCGGTTTGATCAG	
orf35UEc	TCGAATTCGACATTATTGGGAGGGC	
FtrbNco-Sac	CGGTTGAGCTCGTCGAGAATGGATTTAGC	
pCTX96	CCGAGTCAGTTTGATCCATA	
orf36sU	GGATGAGGTATGCAATACGG	
	Primers for cloning of *zeta* gene	
EcoZetaFor	GCC GAA TTC ATG GCA AAT ATA GTC AAT TTT ACT	pBT233
ZetaRevBam	GCC GGA TCC TTA AAT ACC TGG AAG TTT AGG TGT	
	Primers for cloning of P_{BAD} promoter	
upTEM	CACCAGCGTTTCTGGGTGAG	pBAD24
ARA1down	GCTCTAGAGGCGTCACACTTTGCTATGC	
	Primers for *epsilon* detection	
EpsiS*	TGAA**ATG**GCAGTTACGTATG	
EpsiE*	TGCCATA**TTA**AGCCACTTTC	

735 relevant restriction sites are underlined

736 * *epsilon* start and stop codons are bolded

737
738

739 **FIGURES**

740

741 **Figure 1**. Conjugation and pALoriT (*oriT*$_{pCTX-M3,}$ *oriV*$_{p15A}$, Tc^{R}) mobilization efficiencies
742 from *E. coli* donors with pLMAB212 or pMOBS into the JE2571Rif^{r} recipient. Each result is
743 the mean of four experiments. # undetectable transfer. Error bars indicate SD.

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745

746 **Figure 2.** Mobilization efficiency of pToriT from strains: DH5α(pCTX-M3),
747 DH5α(pMOBS), and S14 into *E. coli* JE2571Rif^{r}. Each result is the mean of four
748 experiments. Error bars indicate SD.

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752 **Figure 3**. Mobilization efficiency of pToriT by strains S14, S15, S25, and S26 into *E. coli*
753 JE2571Rif^{r} and by S15 and S26 into different *Proteobacteria* recipients. Each result is the
754 mean of four experiments. Error bars indicate SD.

755

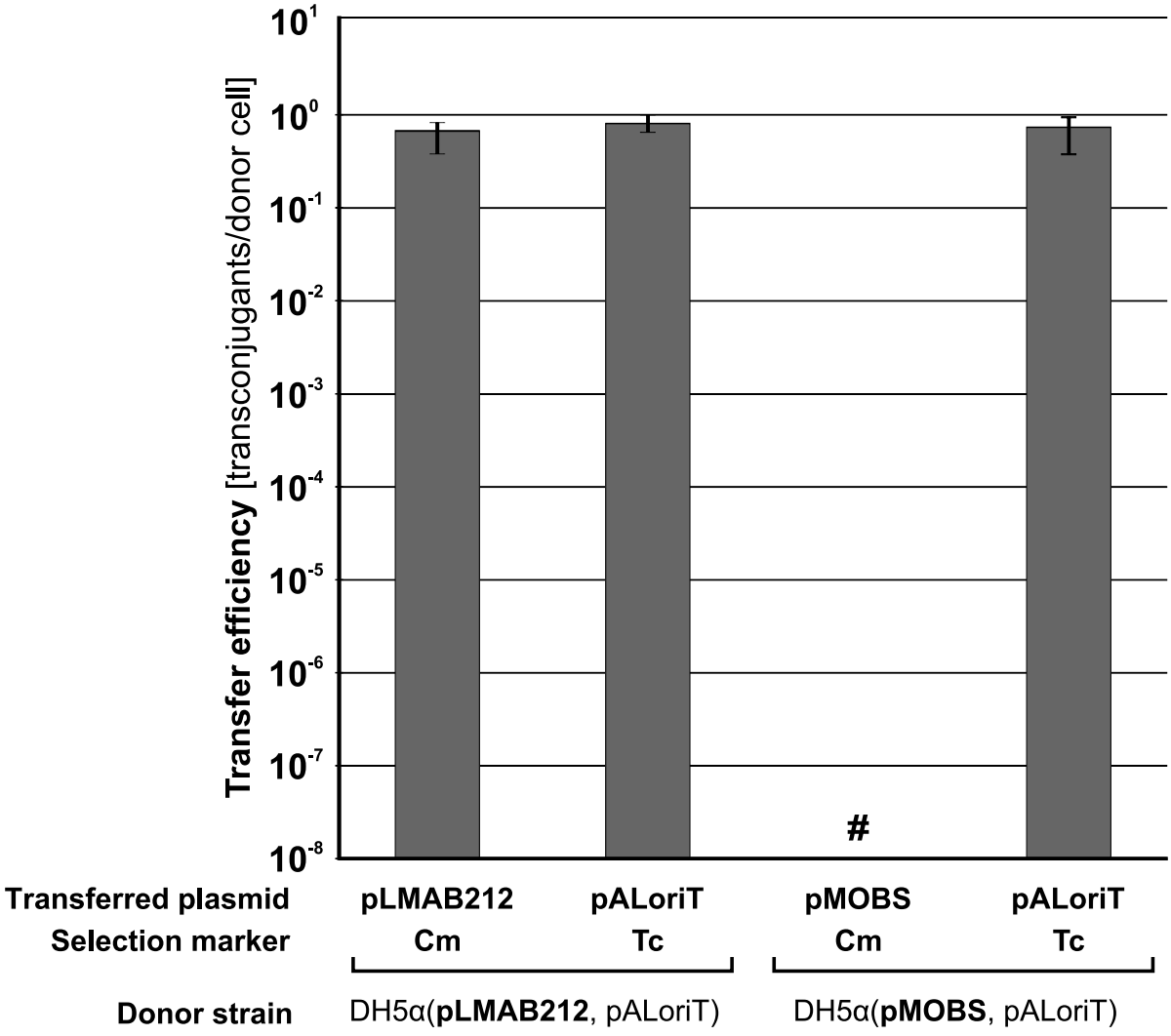
756

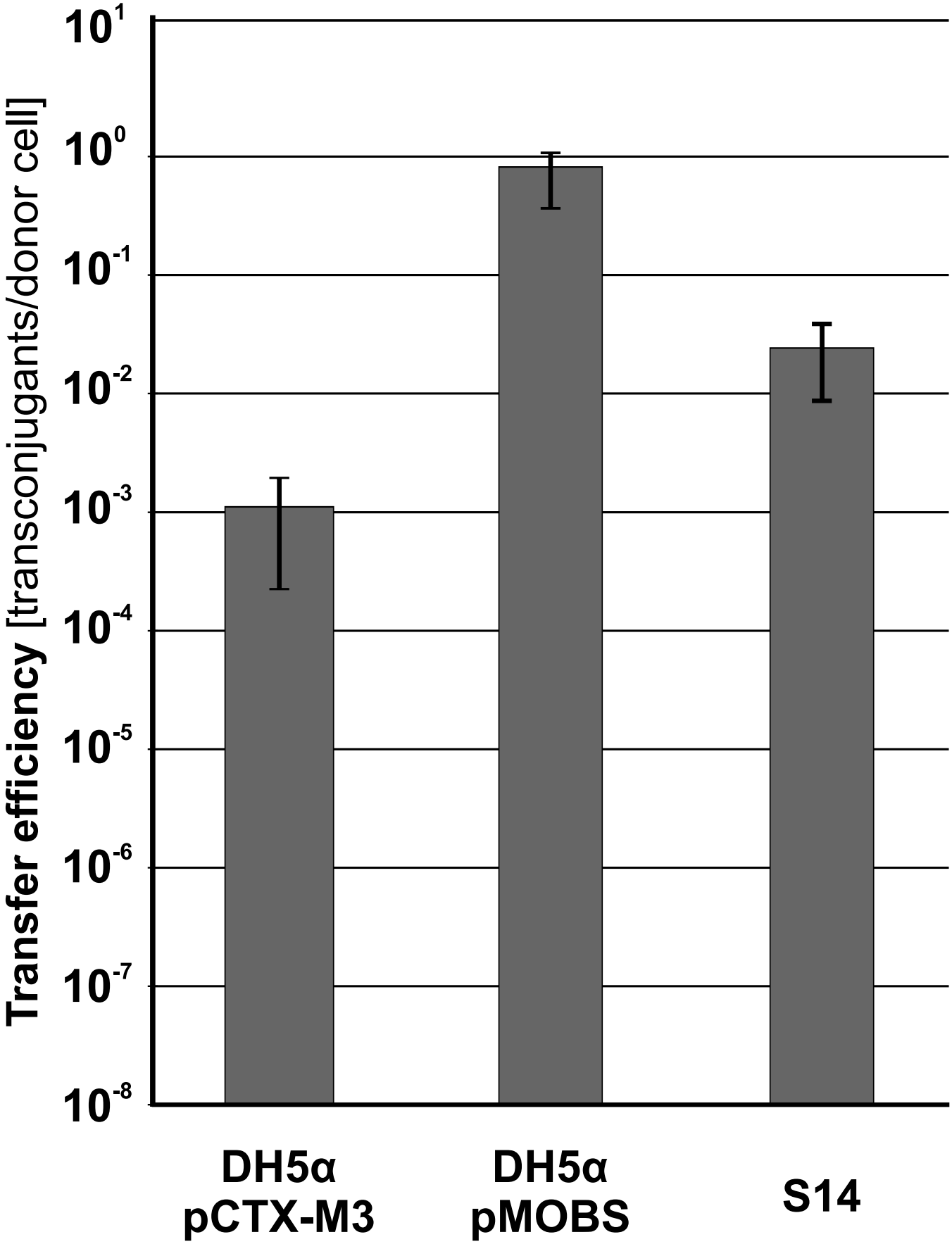
757 **Figure 4.** Mobilization efficiency of pBSUoriT from strain S25 into the Gram-positive
758 recipients *B. subtilis* (A) and *L. lactis* (B). For comparison, efficiency of plasmid
759 mobilization into *E. coli* JE2571Rif^{r} is shown. Each result is the mean of four experiments.
760 Error bars indicate SD.

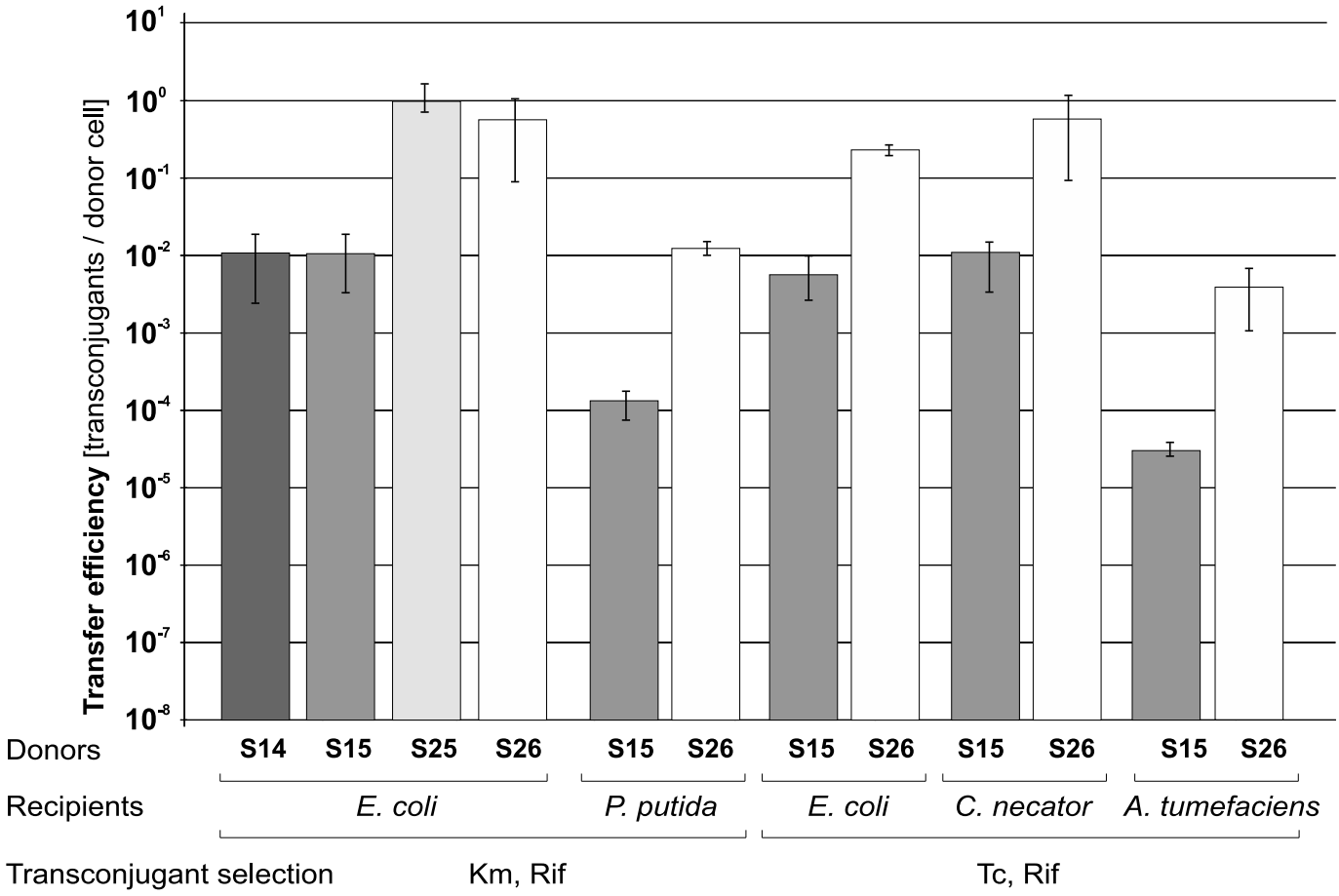
761

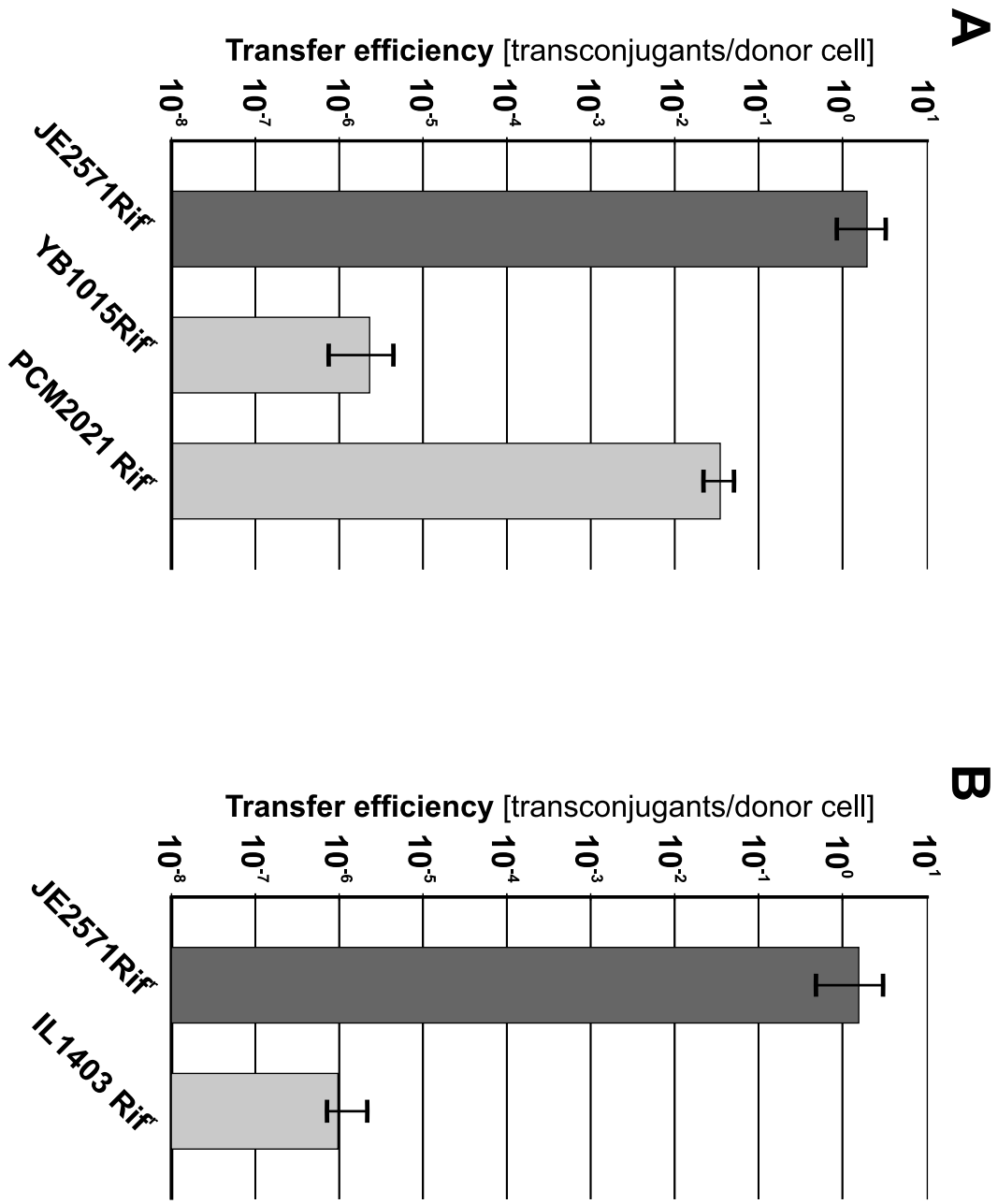
762

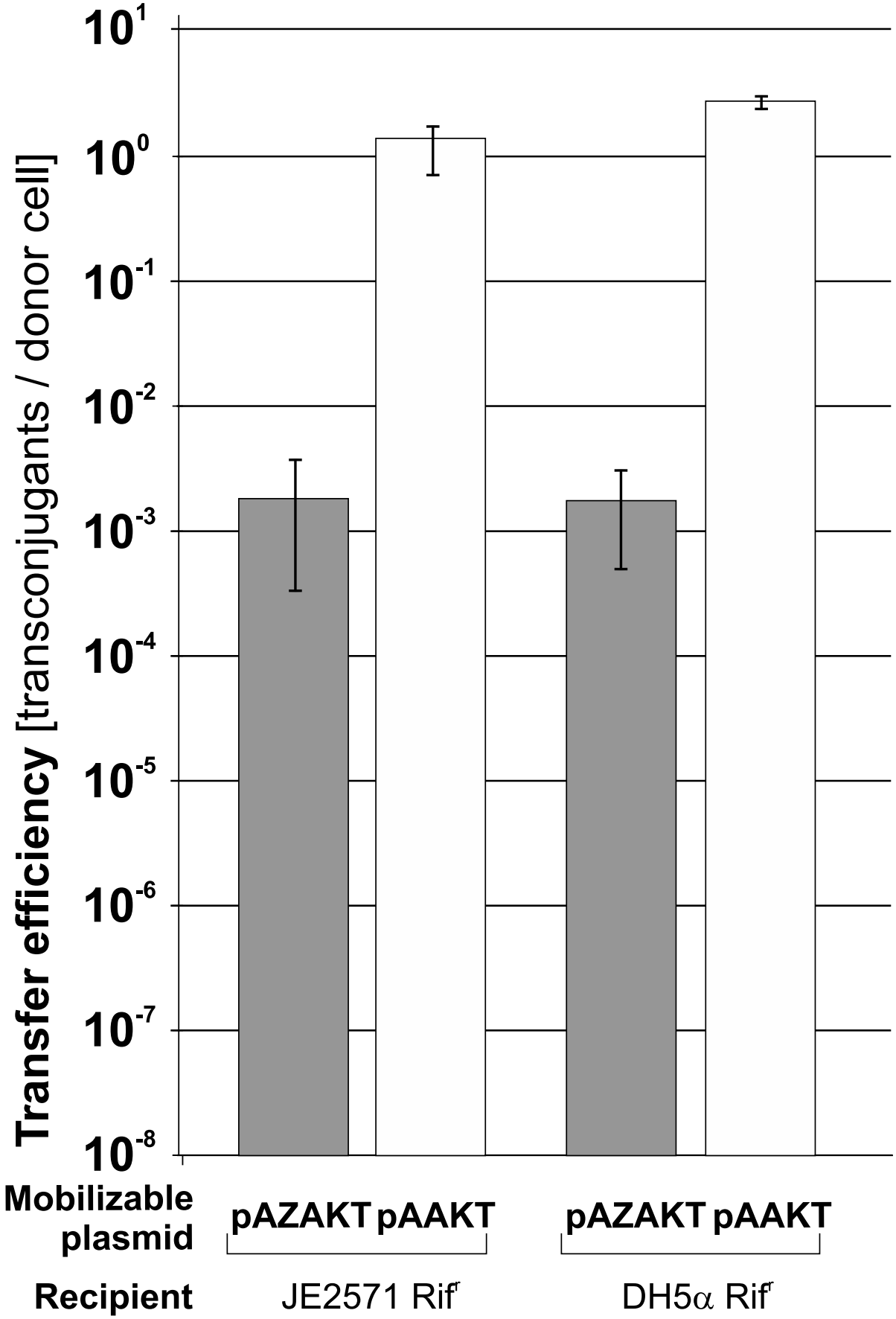
763 **Figure 5.** Mobilization efficiencies of pAZAKT and pAAKT by strain S26(pUC-epsi) into
764 *E. coli* recipients JE2571Rif^r and DH5αRif^r. Each result is the mean of four experiments.
765 Error bars indicate SD.
766
767
768 **Figure 6.** Mobilization-mediated Zeta killing. Mobilization of pAZAKT or pAAKT from
769 the S26(pUC-epsi) donor into *E. coli* JE2571Rif^r as a recipient in stationary growth phase
770 (A) and exponential growth phase (B) with various donor-to-recipient ratios. Each result is
771 the mean of three experiments. Error bars indicate SD.
772
773
774 **Figure 7.** Mobilization efficiency of pToriT from strain S26 into clinical *E. coli* strains
775 1355/2004 and 1149/2004. For comparison, efficiency of plasmid mobilization into *E. coli*
776 JE2571Rif^r is shown. Each result is the mean of four experiments. Error bars indicate SD.
777
778
779 **Figure 8.** Effect of mobilization of pAZAKT and pAAKT from the S26(pUC-epsi) donor
780 into clinical strains 1149/2004 and 1355/2004 (A) and JE2571Rif^r carrying IncP plasmid
781 from strain 1355/2004 - R$^+$[*E. coli* 1355/2004] (B). For comparison, efficiency of plasmid
782 mobilization into *E. coli* JE2571Rif^r is shown. Each result is the mean of at least three
783 experiments. Error bars indicate SD.

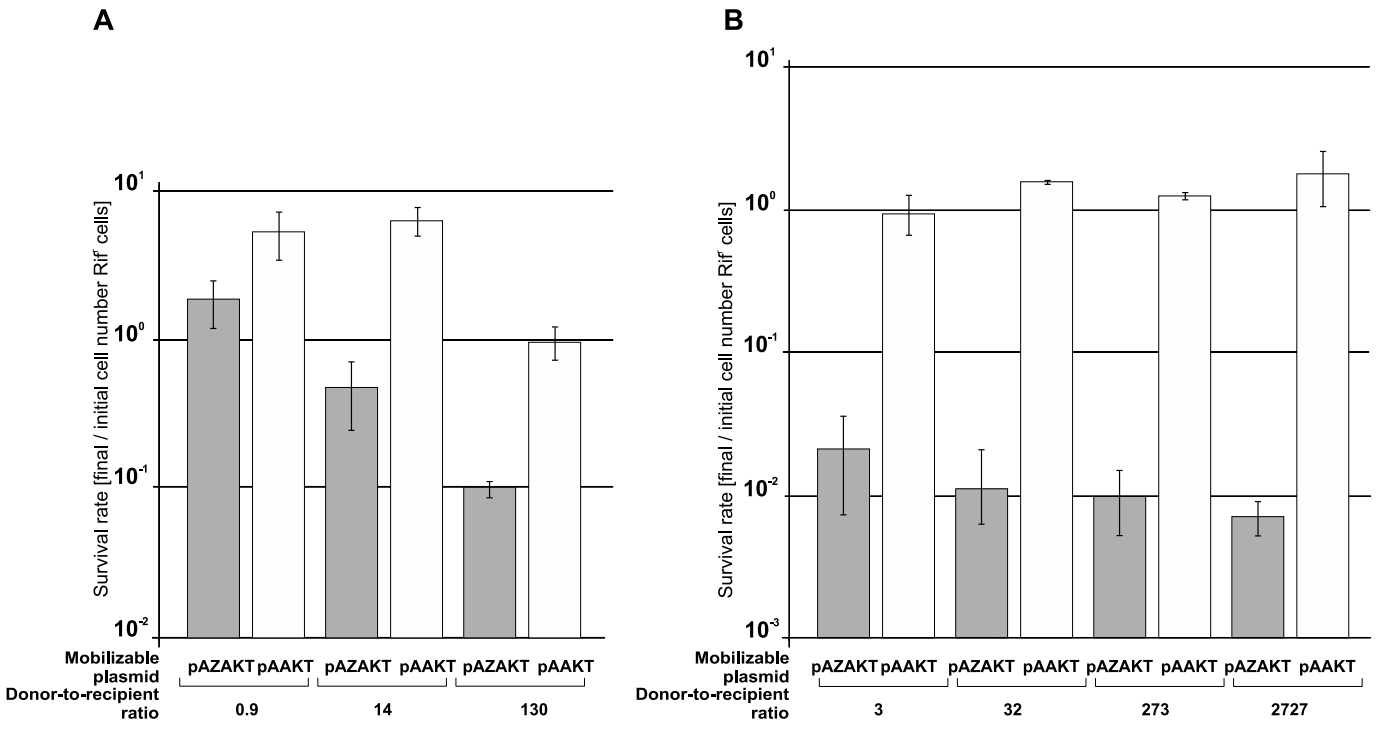


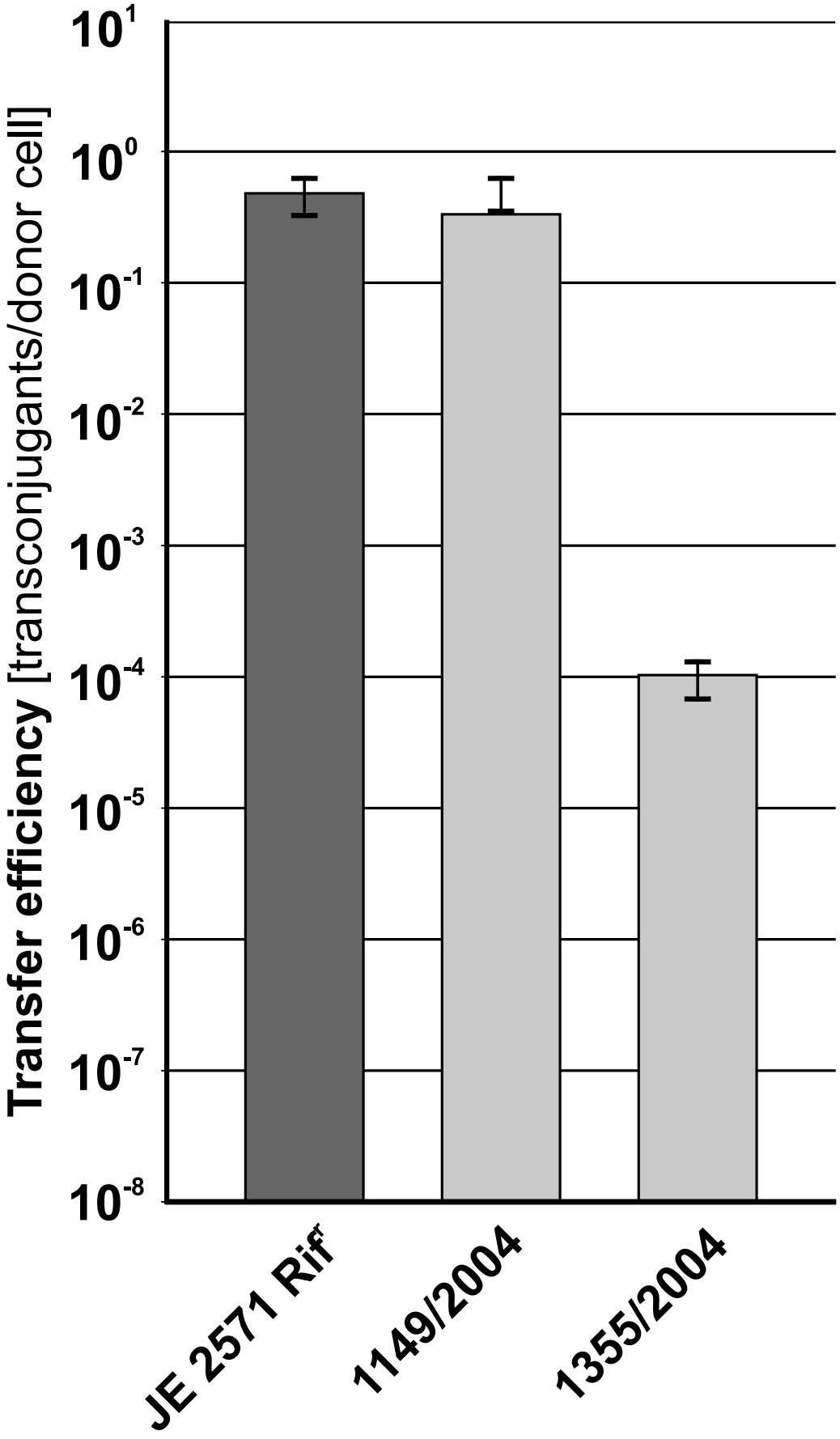












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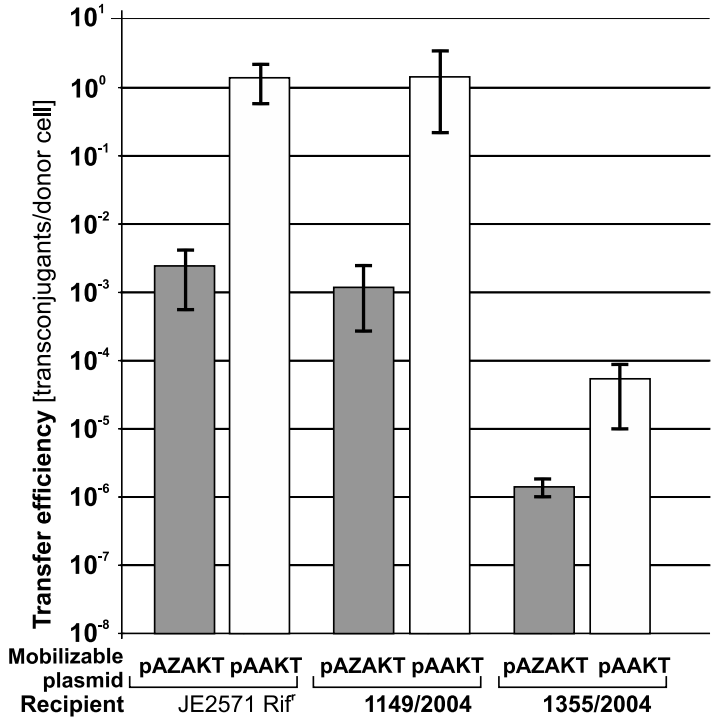

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