Tn*3*-derived inverted-repeat miniature elements (TIMEs) that mobilize antibiotic resistance genes

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Running title: TIME-COMPs associated with ARGs

16 **ABSTRACT**

17 Miniature inverted-repeat transposable elements (MITEs) are nonautonomous mobile
18 genetic elements (MGEs) that can be mobilized by transposases provided by the relevant
19 autonomous MGEs. MITEs originating from Tn*3*-family transposons were previously
20 termed Tn*3*-derived inverted-repeat miniature elements (TIMEs). Composite transposon-
21 like structures bounded by two copies of TIME, called TIME-COMPs, were shown to
22 mobilize the intervening sequences. However, their association with antibiotic resistance
23 genes (ARGs) has not yet been systematically studied. This study thus aimed to identify
24 new TIME-COMP-like structures containing ARGs in the genomic sequences of the
25 clinically important bacterial family Enterobacteriaceae in public databases. TIME-
26 COMP-like structures were first searched for in the plasmid database PLSDB, focusing
27 on small plasmids, using a self-against-self blastn approach to identify repeated elements.
28 Then, newly and previously identified MITEs (including TIMEs) were searched for in
29 the NCBI core nucleotide database to identify TIME-COMP-like structures located on
30 other replicons. Bioinformatic analysis identified multiple previously unreported TIME-
31 COMPs containing ARGs, which are bounded by directly or inversely oriented TIMEs,
32 namely, IS*101*, MITESen1, and a novel 244-bp TIME termed TIME244. TIME244
33 contains a putative resolution site related to that of Tn*21*. These TIMEs were

34 predominantly detected in plasmids and very rarely in chromosomes. The ARGs
35 embedded in newly identified TIME-COMPs were *bla*$_{KPC-2}$, *floR*, *qnrS1*, and *tet*(A).
36 Notably, the *bla*$_{KPC-2}$ carbapenemase gene was found in TIME-COMPs bounded by
37 TIME244 and a TIME-COMP bounded by IS*101*. These findings highlight a potential
38 role for TIMEs in the spread of diverse ARGs.
39

40 **IMPACT STATEMENT**

41 Bacterial miniature inverted-repeat transposable elements (MITEs) are a group of short
42 (50 bp–500 bp) nonautonomous transposable elements that are thought to have originated
43 from insertion sequences or transposons. Although MITEs can theoretically mobilize
44 antibiotic resistance genes (ARGs) in the presence of transposases, only a few studies
45 have reported their association with ARGs, probably due to difficulties in identifying
46 MITEs in genomic sequences. This study provides evidence, based on bioinformatic
47 analysis of public Enterobacteriaceae genomes, that a subset of MITEs, called Tn*3*-
48 derived inverted-repeat miniature elements (TIMEs), mobilizes ARGs by forming
49 composite transposon-like structures. A novel 244-bp TIME, designated TIME244, was
50 present in more than 100 Enterobacteriaceae plasmids in the current RefSeq database,
51 suggesting its further transmission in bacterial populations through horizontal gene

transfer. This study reveals that TIMEs were often overlooked when analyzing the genetic contexts of ARGs in previous studies. These findings highlight the importance of TIMEs in bacterial gene acquisition and underscore the need for new tools that can detect TIMEs in bacterial genomes for ARG surveillance.

KEYWORDS

antibiotic resistance, mobile genetic element, miniature inverted-repeat transposable element, Enterobacteriaceae

DATA SUMMARY

Accession numbers of sequence data analyzed in this study are provided within the article or in supplementary data files.

64 **INTRODUCTION**

65 Antibiotic-resistant bacteria, particularly those with multidrug resistance (MDR), are a
66 global health concern. Bacteria can accumulate antibiotic resistance genes (ARGs) with
67 the help of mobile genetic elements (MGEs), which can often lead to MDR. MGEs can
68 be categorized into two types: those mobilizing ARGs from one cell to another cell (i.e.,
69 intercellular mobilization) and those mobilizing ARGs from one replicon to another
70 replicon or to different locations within the same replicon (i.e., intracellular
71 mobilization).[1] Intercellular mobilization mechanisms such as conjugation can directly
72 contribute to horizontal transfer of ARGs and thus the emergence and spread of MDR.
73 Intracellular mobilization can also contribute to the increase of MDR in multiple ways;
74 for example, by mobilizing ARGs on a plasmid to different plasmids, thereby increasing
75 the potential of the ARGs to be transmitted to additional bacterial recipients via
76 conjugation.[2]

77 MGEs mediating intracellular mobilization of ARGs include well-studied elements such
78 as insertion sequences (ISs) and transposons. These are autonomous (i.e., capable of self-
79 transposition), in contrast to nonautonomous elements, which can also mediate
80 intracellular mobilization but need the transposase activity provided by autonomous
81 elements in the same cell.[1] Among the known nonautonomous elements are structures

82 called miniature inverted-repeat transposable elements (MITEs).[3] MITEs are thought to
83 have originated from ISs or transposons. They retain the terminal inverted repeats (IRs)
84 but lack the internal regions, including the transposase gene(s). They are typically 50 bp–
85 500 bp and create direct repeats (DRs) upon insertion.[4]
86 A subset of MITEs originating from Tn*3*-family transposons and carrying Tn*3*-related
87 IRs are called Tn*3*-derived inverted-repeat miniature elements (TIMEs), and they include
88 elements such as IS*101*, integron mobilization unit (IMU), MITESen1, and TIME1–
89 TIME4.[5-8] Composite transposon-like structures bounded by two copies of TIME, known
90 as TIME-COMPs, were shown to mobilize the intervening sequences when the
91 transposase activity of autonomous Tn*3*-family elements was provided in *trans*.[5, 7]
92 However, to date, known TIME-COMPs carrying an ARG(s) are limited to IMU-bounded
93 TIME-COMPs carrying a carbapenemase gene $bla_{\text{GES-5}}$ and an IS*101*-bounded TIME-
94 COMP carrying a quinolone resistance gene *qnrD1*, even in the clinically important
95 bacterial family Enterobacteriaceae (**Figure S1**).[7, 9, 10] MITEs other than TIMEs should
96 also be capable of mobilizing an ARG when the ARG is embedded within a TIME-
97 COMP-like structure; however, to our knowledge, no such non-TIME MITE-bounded
98 structures have been reported in Enterobacteriaceae.

99 Tn*3*-family transposons have been identified in nearly all bacterial phyla and are
100 classified into several subgroups (including Tn*3* and Tn*21*) based on the phylogeny of
101 their transposases.[11] These elements mobilize through a "paste-and-copy" mechanism
102 involving two distinct steps: cointegrate formation and cointegrate resolution. The first
103 step is mediated by the transposase (the *tnpA* gene product), IRs, and the host replication
104 machinery, generating a fusion replicon in which the donor and target replicons are
105 bridged by directly repeated copies of the transposon. In the second step, the cointegrate
106 is resolved into two separate molecules, each carrying a transposon insertion. This
107 resolution is typically mediated by the transposon-encoded resolvase (the *tnpR* gene
108 product) and its cognate target site (*res*), or alternatively through homologous
109 recombination between the duplicated transposon sequences. TIME and TIME-COMP
110 elements are hypothesized to employ a similar "paste-and-copy" mechanism; however,
111 whether these elements contain functional resolution sites remains unclear in most cases,
112 with the exception of IS*101*, which possesses a functional *res* site.[12]
113 Transposition of an IMU-bounded TIME-COMP was previously demonstrated by
114 providing a transposase of IS*Sod9* belonging to the Tn*21* subgroup.[7] IS*101* cointegrate
115 formation and resolution were demonstrated in the presence of Tn*1000* (also called
116 gamma-delta) belonging to the Tn*3* subgroup.[12] Known TIMEs and TIME-COMPs create

117 5-bp (or sometimes 6-bp) DRs upon transposition like Tn*3*-family transposons.[5,7,11] Thus,
118 past transposition of a TIME-COMP can be inferred from the presence of 5-bp or 6-bp
119 DRs flanking the outermost IRs of the TIME-COMP.
120 MITEs (including TIMEs) tend to be overlooked in standard annotation pipelines due to
121 their lack of transposase gene(s). Furthermore, as of January 2026, the number of MITE
122 sequences registered in the ISFinder database remained at 65, with only three originating
123 from Enterobacteriaceae.[13] Thus, the contribution of MITEs to the intracellular mobility
124 of ARGs is likely underestimated. Hence, this study aimed to identify previously
125 overlooked TIME-COMP-like structures carrying ARGs in Enterobacteriaceae, by data-
126 mining.
127

128 **METHODS**

129 **Plasmid sequences**

130 Plasmid sequences (n = 72,556) were downloaded from PLSDB (v. 2024_05_31_v2),
131 which is a curated and non-redundant plasmid database sourced from the NCBI
132 database.[14] Plasmid sequences meeting the following criteria were extracted: (i) at least
133 one ARG was detected by Abricate (v1.0.1, https://github.com/tseemann/abricate) with
134 the ncbi database;[15] (ii) at least one plasmid replicon was detected by Abricate (v1.0.1)

135 with the "--db plasmidfinder" option[16] (this was to exclude sequences that are not likely
136 to be true plasmids and to be conservative; for example, NZ_CP107371 is composed
137 almost entirely of ARGs and ISs/transposons, suggesting a non-plasmid circular element
138 or a misassembled circular contig); (iii) the plasmid host is Enterobacteriaceae; and (iv)
139 the length is 20,000 bp or shorter. Here, we focused on small plasmids because their small
140 size allows manual curation of the results of bioinformatic analysis (described in detail
141 below), and previously reported TIME-COMPs with ARGs in Enterobacteriaceae were
142 located on small plasmids (<10 kbp).[7, 9, 10] Plasmid sequences meeting the above criteria
143 (n = 1,007) were subjected to self-against-self blastn analysis as described below to detect
144 repeated sequences that could be MITEs.

145 **Detection of TIME-COMP-like structures carrying ARGs**

146 The BLAST+ (v2.16.0) program was used for similarity searches. The makeblastdb and
147 blastn functions were used with defaults parameters. Plasmid sequences with self-against-
148 self blastn hits meeting the following criteria were retained: (i) the alignment length is
149 between 50 bp and 500 bp; (ii) the percentage of identical matches is 98% or higher; (iii)
150 a sequence longer than 500 bp is present between the aligned part of the query sequence
151 and that of the subject sequence. These criteria were applied to detect repeat sequences
152 (50 bp–500 bp) surrounding a stretch of sequence (> 500 bp) in a plasmid (see **Figure S2**

153 for the rationale for these criteria). To confirm that the 50-bp to 500-bp repeat sequences
154 are actually MITEs, a self-against-self blastn search was performed for each repeat
155 sequence to identify IRs, which are the signature of MITEs. The blastn search was
156 performed by using a word size of six to increase the sensitivity, and the result was
157 visualized using CLC Main Workbench 24 (Qiagen, Hilden, Germany) (**Figure S3**). The
158 putative MITE sequences were checked for identity to any known elements in the ISfinder
159 database.[13] Only TIME-COMP-like structures flanked by DRs, which are typically
160 created upon insertion of the entire structure, were considered for further analysis. This
161 was to exclude cases where two MITEs were independently inserted and coincidentally
162 formed a TIME-COMP-like structure. The lengths of DRs looked for were determined
163 with reference to those of the related ISs or transposons. Plasmids with TIME-COMP-
164 like structures carrying ARGs were annotated using ISfinder and the blastn web tool
165 (https://blast.ncbi.nlm.nih.gov/Blast.cgi).
166 The MITE(s) found in the above approach, previously described TIMEs associated with
167 ARGs in Enterobacteriaceae (i.e., IMU and IS*101*),[7, 9, 10] and Enterobacteriaceae MITEs
168 listed in the ISfinder database (i.e., MITEKpn1 and MITESen1, excluding MITEEc1,
169 which is an enterobacterial repetitive intergenic consensus [ERIC] sequence and present
170 in multiple copies on the chromosome of *Escherichia coli*),[13, 17, 18] were searched for in the

171 NCBI core nucleotide database (core_nt) using the blastn web tool (last accessed July
172 2025). Replicons with two or more blastn hits with ≥95% identity and ≥95% coverage
173 were manually screened to detect additional TIME-COMP-like structures carrying an
174 ARG in Enterobacteriaceae. This enabled identification of TIME-COMP-like structures
175 in small plasmids not indexed in PLSDB, in longer plasmids, and also in chromosomes.
176 Putative ancestral plasmids without insertion of TIME-COMP-like structures were
177 identified by performing the online blastn analysis against the NCBI core_nt database,
178 using a sequence, which was prepared by joining the sequences flanking the TIME-
179 COMP-like structure minus one copy of the DR, as a query sequence (last accessed
180 January 2026).

181 **Detection of MITEs in RefSeq Enterobacteriaceae genomes**

182 To investigate the prevalence of MITEs that were found to be associated with ARGs in
183 the above analysis, we downloaded RefSeq Enterobacteriaceae genomes with an
184 assembly level of "complete genome" using the NCBI Datasets command-line tools
185 (v18.9.0) in October 2025 (n = 11,572).[19] MITE sequences were searched for in the
186 retrieved genomes using makeblastdb and blastn in BLAST+ (v2.16.0) with default
187 parameters. We used the same criteria as the online blastn search to define the presence
188 of MITE sequences (i.e., ≥95% identity and ≥95% coverage).

189

190 **RESULTS AND DISCUSSION**

191 **Identification of TIME-COMP-like structures carrying ARGs**

192 First, we conducted self-against-self blastn analysis to identify TIME-COMP-like
193 structures in a subset of small Enterobacteriaceae plasmids registered in the curated
194 plasmid database, PLSDB. This relatively small dataset (n = 1,007) made manual curation
195 of the blastn results feasible, which includes confirmation of the IRs in putative MITEs
196 and DRs flanking the entire TIME-COMP-like structures. These procedures revealed
197 TIME-COMP-like structures carrying ARGs in six plasmids (**Table S1**). All these
198 structures are bounded by TIMEs and thus are TIME-COMPs. One of these plasmids is
199 pKFu015_4, which was previously reported to carry the IS*101*-bounded TIME-COMP
200 containing *qnrD1* (**Figure S1**).[10] The remaining five plasmids contain the same TIME-
201 COMP structure with $bla_{\text{KPC-2}}$ and a truncated bla_{TEM} gene, which is bounded by two
202 copies of a 244-bp TIME (hereafter named TIME244). pCHE-A and pCHE-A1, which
203 were previously reported to contain IMU-bounded TIME-COMPs (**Figure S1**), were
204 detected by the self-against-self blastn searches but were filtered out in manual curation,
205 because the TIME-COMP structures in these plasmids are not flanked by DRs.[7,9] We also
206 detected six other plasmids carrying TIME-COMP structures with ARGs but not flanked

207 by DRs, all bounded by MITESen1. These TIME-COMP structures may have the
208 potential to transpose as a unit; however, these cases were filtered out to restrict the
209 dataset to plasmids showing direct evidence of transposition.

210 To identify additional TIME-COMP-like structures in replicons other than small plasmids
211 indexed in PLSDB, we performed online blastn searches using TIME244 and previously
212 reported MITEs, including those classified as TIMEs (IS*101*, IMU, and MITESen1) and
213 one non-TIME MITE (MITEKpn1), as query sequences (see **Table S2** for the sequence
214 of each MITE). This online blastn analysis identified 141 additional replicons carrying
215 TIME-COMPs with ARGs. These included 136 plasmids and one chromosome carrying
216 TIME244-bounded TIME-COMPs (most of which contain *tet*(A)), one plasmid carrying
217 an IS*101*-bounded TIME-COMP, and three plasmids carrying MITESen1-bounded
218 TIME-COMPs (**Table S1**).

219 The genetic contexts of TIME-COMPs identified using the above approaches were
220 thoroughly inspected as described below.

221 **TIME244-bounded TIME-COMPs**

222 As mentioned above, we detected TIME244-bounded TIME-COMPs in a total of 142
223 replicons (i.e., five identified by the self-against-self blastn analysis, and 137 identified

224 by the online blastn analysis). Three main types of TIME244-bounded TIME-COMPs

225 were identified.

226 One of them is a TIME244-bounded TIME-COMP carrying *tet*(A) detected in 133

227 replicons (**Table S1**). This TIME-COMP is bounded by inversely oriented TIME244

228 copies and contains part of Tn*1721*, an 11-kb Tn*3*-family element carrying tetracycline

229 resistance determinant genes originally identified on an *E. coli* plasmid (**Figure 1a**).[20, 21]

230 This TIME-COMP was inserted in three unique locations with DRs of AGCAA (n = 114),

231 TATAA (n = 18), and TACTT (n = 1) (**Figure 1a** and **Table S1**). This indicates that three

232 independent transposition events of this TIME-COMP had occurred in the past. In 114

233 replicons with DRs of AGCAA, two types of the TIME-COMP were identified: one with

234 an inverted internal structure (n = 52) and the other without (n = 62) (see below for

235 detailed discussion). Furthermore, there were several indels within the TIME-COMP

236 structure, two of which involved alterations of ARG contents (**Figure S4**, see **Table S1**

237 for detailed description). Briefly, in a plasmid, a resistance region containing $bla_{\text{TEM-1B}}$ and

238 $bla_{\text{CTX-M-206}}$ is inserted within the left TIME244 copy. In the other plasmid, the Tn*1721*-

239 derived region is partially deleted and replaced by a truncated Tn*3*-like transposon and a

240 truncated Tn*4401*-like transposon containing $bla_{\text{KPC-2}}$ (i.e., a variant of non-Tn*4401*

241 elements [NTE_{KPC}], which contains only a portion of Tn*4401*, the most common bla_{KPC}-

242 containing mobile element belonging to the Tn*3* family[22]). In these cases, the DR
243 sequences flanking the TIME-COMPs are the common ones (AGCAA); thus the indels
244 were likely introduced after the insertion of the original TIME-COMP carrying *tet*(A).
245 The outermost IRs within the altered TIME-COMP structures are intact, implying the
246 feasibility of further transposition if the relevant transposase activity is provided in *trans*
247 (**Figure S4**).
248 Another TIME-COMP carries bla_{KPC-2} and a truncated bla_{TEM} gene, which is bounded by
249 directly oriented TIME244 copies (**Figure 1b**). This genetic context was previously
250 described as a variant of NTE_{KPC}, designated NTE_{KPC}-IId.[23] The authors noted 243-bp
251 repeats at its boundaries, but did not identify them as MITEs or TIMEs. This TIME-
252 COMP was inserted in the same site in six highly related IncQ1 plasmids, with the same
253 DRs (TGTTTA), and in a single Col-like plasmid with DRs of TATAT (**Figure 1b** and
254 **Table S1**). In one of the IncQ1 plasmids (pKPN535a), three copies of TIME244 are
255 present, which could be explained by the fusion of two plasmids.
256 We also detected another TIME-COMP, which contains *qnrS1* and is bounded by
257 inversely oriented TIME244 copies, in two untypeable plasmids (**Figure 1c**).[24, 25] In these
258 plasmids, the TIME-COMP structure is inserted in the same position with DRs of
259 ATCCA; therefore, a total of one unique transposition event was detected. These two

260 plasmids and their putative ancestral plasmid were classified as phages by geNomad
261 (v1.11.2).[26] Thus, these are putative phage-plasmids, which transfer horizontally between
262 cells as viruses and vertically within cellular lineages as plasmids.[27]

263 **An IS*101*-bounded TIME-COMP**

264 IS*101*, a 209-bp element, is among the earliest identified TIMEs and was originally
265 discovered on a recombinant *E. coli* plasmid, pSC101.[6] We identified a previously
266 unreported IS*101*-bounded TIME-COMP in one *Enterobacter kobei* IncX3 plasmid,
267 pEkFL23_IncX3 (**Figure 2**). This TIME-COMP is bounded by inversely oriented copies
268 of IS*101* and carries $bla_{\text{KPC-2}}$ embedded within a truncated Tn*4401b* structure.[28] This
269 genetic context was previously reported as Tn*4401k*, but the presence of IS*101* was not
270 addressed in the previous study.[29] The sequence between the two IS*101* copies in this
271 TIME-COMP corresponds to the partial sequence of plasmids such as pKPC_FCF/3SP,
272 and the whole TIME-COMP structure was flanked by 5-bp DRs (ATTCT). These indicate
273 that IS*101*-mediated transposition of part of a pKPC_FCF/3SP-like plasmid into another
274 plasmid generated pEkFL23_IncX3.

275 **MITESen1-bounded TIME-COMPs**

276 MITESen1 is a 256-bp TIME first identified on an IncX1 plasmid from *Salmonella*
277 *enterica*.[8] We identified two different MITESen1-bounded TIME-COMPs in the same

278 location with the same flanking 5-bp DRs (TAATA) in two IncX1-IncX2 hybrid plasmids
279 (**Figure 3a**).[30, 31] One TIME-COMP contains *qnrS1*, and the other contains *floR*.
280 MITESen1 copies are directly oriented in these TIME-COMPs. Interestingly, there are
281 IncX1-X2 hybrid plasmids carrying a single MITESen1 copy inserted in the same
282 location. We speculate that the two plasmids with TIME-COMPs were generated by
283 integration of a hypothetical circular molecule containing MITESen1 and an ARG into a
284 plasmid already carrying a single copy of MITESen1. The hypothetical circular molecules
285 might be generated by recombination between MITESen1 in a TIME-COMP or by
286 intramolecular replicative transposition of MITESen1 (also see **Figure S5** for another
287 possible pathway for generation of the *floR*-containing circular element). Integration of
288 the circular molecule might occur by homologous recombination between MITESen1
289 copies.
290 A plasmid with the repE(pEh60-7) replicon (pECC-003-1) was found to carry a remnant
291 of a MITESen1-bounded TIME-COMP carrying *qnrD1* (**Figure 3b**).[32] One copy of
292 MITESen1 is interrupted by IS*26*, while the truncated remnant of MITESen1 is present
293 next to another copy of IS*26* that is oriented in the opposite direction. This arrangement
294 is consistent with intramolecular replicative transposition (in *trans*) of an IS*26* element
295 into MITESen1 in the intact TIME-COMP structure.[33] The presence of 8-bp DRs

296 (ATATCAAC or its reverse complement) next to each IS*26* element on the MITESen1-
297 sides supports this, though the intact version of this TIME-COMP structure was not
298 present in the NCBI core_nt database.

299 **Putative resolution sites in TIMEs**

300 All TIMEs forming the TIME-COMP structures identified in the present study (i.e.,
301 TIME244, IS*101*, and MITESen1) carry IRs related to Tn*3*-family transposons (**Figure**
302 **4a**). IRs of TIME244 and the previously reported IMU show more similarities to IRs of
303 Tn*21* than to those of Tn*3*, IS*101*, MITESen1, and *Pseudomonas*-derived TIME2 that is
304 not associated with ARGs.[5] Thus, TIME244 and IMU seem to have originated from the
305 Tn*21* subgroup of the Tn*3* family.[11] To deduce whether TIME244 contains a *res* site, we
306 conducted all-against-all SSEARCH using sequences of TIMEs, Tn*21 res*, Tn*1721 res*,
307 and Tn*3 res*.[34] A significant match (Smith-Waterman score [S-W] >100) was detected for
308 pairs of TIME244-Tn*21 res* (S-W = 148), TIME244-Tn*1721 res* (S-W = 152), Tn*21 res*-
309 Tn*1721 res* (S-W = 188), and IS*101*-Tn*3 res* (S-W = 139). **Figure 4b** shows alignment of
310 the TIME244 internal sequence and the experimentally determined *res* sites of Tn*21* and
311 Tn*1721*.[35] The *res* sites of the Tn*3* family consist of three subsites (site I, site II, and site
312 III) where TnpR dimers bind. Strand exchange takes place at the center of site I (C/O in
313 **Figure 4b**).[11] The TIME244 internal sequence shows similarity to both Tn*21 res* and

314 Tn*1721 res* at all three subsites. Therefore, we speculate that TIME244 contains a
315 functional *res* site of the Tn*21* subgroup. On the other hand, the internal sequences of
316 IMU and MITESen1 did not show similarity to well-characterized *res* sites from either
317 autonomous Tn*3*-family elements or other TIMEs (S-W <70), although this does not
318 exclude the possibility that these TIMEs carry resolution sites that serve as targets of site-
319 specific recombinase(s).
320 In principle, the functionality of *res* affects the stability of the TIME-COMP structures.
321 When *res* sites are directly oriented, the segment between two *res* sites can be deleted in
322 the presence of TnpR. According to studies on autonomous Tn*3*-family elements, when
323 *res* sites are inversely oriented, the segment between the two *res* sites can be inverted by
324 TnpR *in vivo*,[36, 37] likely because knotted substrates carrying inversely oriented *res* sites,
325 which are efficient substrates for TnpR,[38] are produced *in vivo*. Among 142 replicons
326 containing TIME244-bounded TIME-COMPs, only seven replicons contain directly
327 oriented TIME244 copies, while 135 replicons contain inversely oriented TIME244
328 copies (**Table S1**). This orientation bias is consistent with the idea that TIME-COMPs
329 carrying directly repeated TIME244 copies are structurally unstable due to the intrinsic
330 resolution activity in the presence of Tn*21*-related TnpR.

331 The most prevalent TIME244-bounded TIME-COMP was that carrying *tet*(A). This
332 TIME-COMP carries TIME244 copies in inverse orientation. The TIME-COMPs
333 carrying *tet*(A) in the 114 replicons with DRs of AGCAA are classified into those with an
334 inverted internal structure (n = 52) and those without (n = 62) (**Figure 1a** and **4c**). This
335 inversion could be explained by TnpR-mediated site-specific recombination at the *res*
336 sites. Because TIME244 contains a putative *res* site, the transposition of TIME244-
337 bounded TIME-COMPs with inversely oriented TIME244 copies should follow a two-
338 step process: cointegrate formation and cointegrate resolution in the presence of Tn*21*-
339 related transposons (**Figure 4d**). Cointegrate resolution can occur by RecA-dependent
340 homologous recombination or site-specific recombination involving Tn*21*-related TnpR
341 and *res* sites.

342 **Prevalence of TIMEs in RefSeq Enterobacteriaceae genomes**

343 The NCBI core_nt database, which we used in the online blastn analysis, contains a
344 mixture of complete genomes and sequences that are not derived from complete genomes
345 (e.g., plasmid sequences without the host chromosomal sequences). This prevents the
346 accurate estimation of the prevalence of MITEs associated with ARGs (i.e., TIME244,
347 IS*101*, MITESen1, and IMU, all of which are TIMEs) in Enterobacteriaceae genomes.
348 Thus, to circumvent this problem, we downloaded RefSeq complete Enterobacteriaceae

349 genomes (n = 11,572) and detected these TIMEs in the retrieved genomes. The frequency
350 of genomes carrying each TIME was as follows: TIME244 (n = 109, 0.94%), IS*101* (n =
351 49, 0.42%), MITESen1 (n = 264, 2.28%), and IMU (n = 7, 0.06%) (**Figure 5** and **Table**
352 **S3**). The frequency of genomes with a replicon(s) carrying multiple copies of each TIME
353 was as follows: TIME244 (n = 71, 0.61%), IS*101* (n = 4, 0.03%), MITESen1 (n = 54,
354 0.47%), and IMU (n = 2, 0.02%). These TIMEs were mainly detected in clinically
355 important genera, namely *Klebsiella*, *Escherichia*, *Enterobacter*, and *Citrobacter*.
356 However, these taxa are more likely to be sequenced due to their clinical importance, and
357 care should be taken to interpret the results. In total, 408 genomes (3.53%) harbored at
358 least one of these TIMEs, indicating that these elements, while rare, are present at a
359 detectable frequency. Interestingly, these TIMEs were predominantly detected in
360 plasmids (n = 444, 97.16%) and very rarely in chromosomes (n = 13, 2.84%).

361 **Study limitations**

362 This study has limitations. First, the self-against-self blastn approach, which allows *de*
363 *novo* identification of MITEs that form TIME-COMP-like structures, was performed only
364 on small plasmids (<20 kbp). Thus, novel MITEs potentially present in other types of
365 replicons might have been missed. Performing the self-against-self blastn analysis also
366 on large plasmids and chromosomes may enable the identification of more MITE

367 elements; however, this approach is unwieldly because it will return exponentially more
368 blastn hits, which makes manual curation of the results (e.g., confirmation of DRs at the
369 ends) almost impossible. A direction for future work would be to develop software for
370 automated detection of TIME-COMP-like structures. A second limitation of this study is
371 that we analyzed only the genomes of Enterobacteriaceae. Although rare, there seem to
372 be TIME-COMP-like structures containing ARGs in other bacteria as well, such as
373 *Acinetobacter* spp.[39] Extending the approach employed in the present study to other
374 bacteria may uncover additional TIME-COMP-like elements.

375 **Conclusions**

376 The present study identified multiple previously unreported TIME-COMPs containing
377 ARGs. These novel TIME-COMPs are bounded by three types of TIMEs (i.e., TIME244,
378 IS*101*, and MITESen1). The sequences of these three TIMEs and also IMU are divergent
379 and seem to have independently emerged from distinct transposons of the Tn*3* family.
380 Interestingly, the same TIMEs were associated with different ARGs, and the same ARGs
381 were associated with different TIMEs. This study revealed that TIMEs contribute to the
382 intracellular mobilization of ARGs and highlights the importance of taking these elements
383 into account when analyzing the genetic contexts of ARGs.

384

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Author contributions

Conceptualization: R.G. Methodology: R.G. Formal analysis: R.G. and H.Y. Data Curation: R.G. Visualization: R.G. and H.Y. Project administration: R.G. Funding acquisition: R.G. and H.Y. Writing - Original Draft: R.G. and H.Y. Writing - Review & Editing: R.G. and H.Y.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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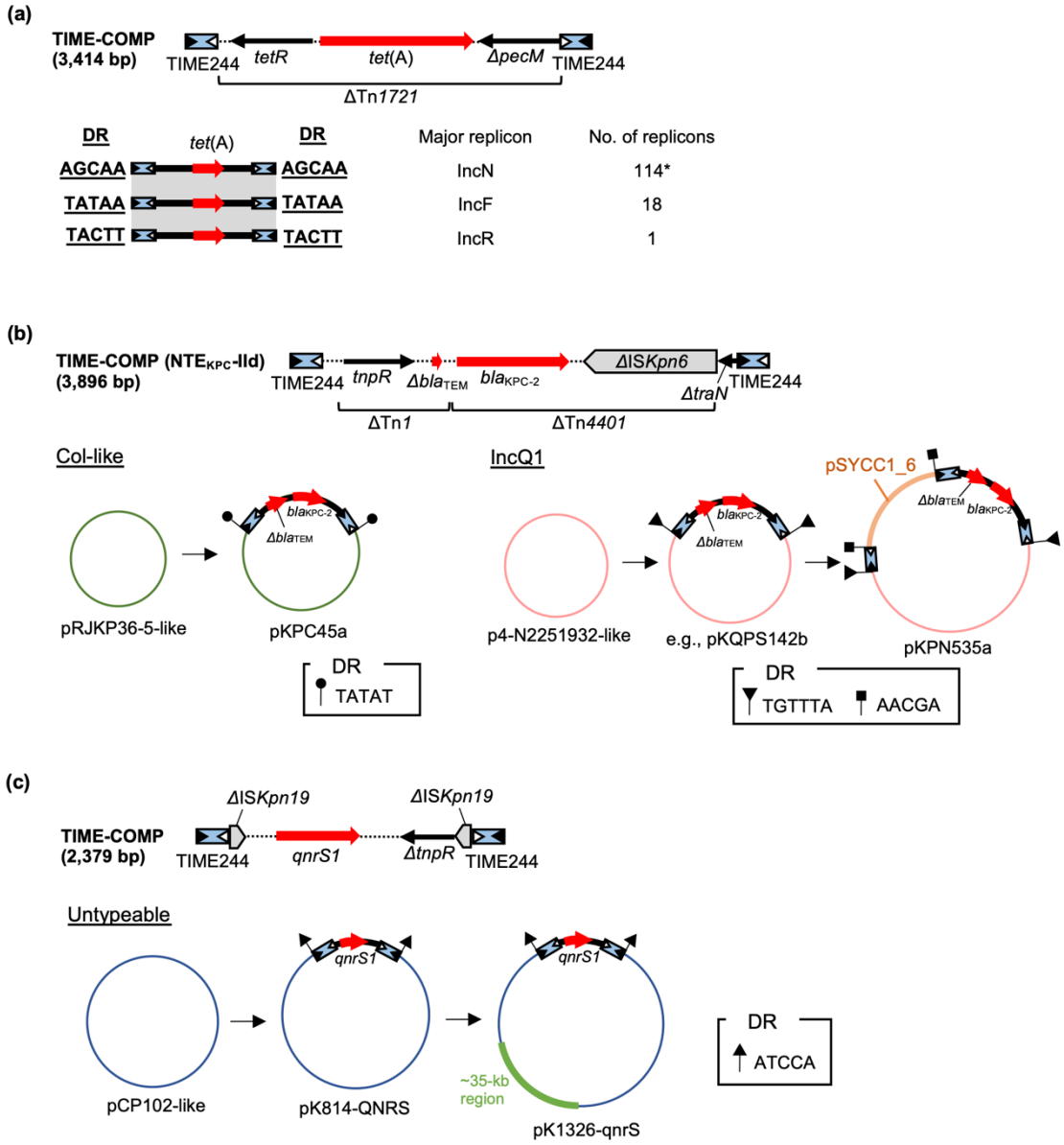
503

504 **Figures**



505

506 **Figure 1**. (a) TIME244-bounded TIME-COMP carrying *tet*(A) and DRs flanking the
507 TIME-COMP. The expanded diagram of the TIME-COMP is shown on the top. Major
508 replicon types and the numbers of replicons are shown on the right side. *There are
509 variants in TIME-COMPs flanked by AGCAA (see **Table S1** for details). (b) TIME244-
510 bounded TIME-COMP carrying bla_{KPC-2} and a truncated bla_{TEM} gene, and plasmids
511 carrying the TIME-COMP. The expanded diagram of the TIME-COMP is shown above
512 the plasmids. For the TIME-COMP structure in each plasmid, only TIMEs and ARGs
513 are depicted for clarity. The TIME-COMP was inserted into a pRJKP36-5-like plasmid
514 and a p4-N2251932-like plasmid, leading to pKPC45a and pKQPS142b (and four other
515 IncQ plasmids showing >99% identity and >99% coverage against pKQPS142b, as in

516 **Table S1**), respectively. In pKPN535a, three copies of TIME244 are present, and a
517 sequence identical to pSYCC1_6 is present between two of the TIME244 copies. This
518 indicates a cointegrate formation by "paste-and-copy" transposition of TIME244,
519 leading to fusion of a pKQPS142b-like plasmid and pSYCC1_6. The online blastn
520 analysis identified eight putative ancestral plasmids (including pRJKP36-5) for pKPC45
521 and seven putative ancestral plasmids (including p4-N2251932) for pKQPS142b,
522 showing >90% identity and >98% coverage against the uninterrupted version of each
523 plasmid. (c) TIME244-bounded TIME-COMP carrying *qnrS1*, and plasmids carrying
524 the TIME-COMP. The TIME-COMP was likely inserted in a pCP102-like plasmid,
525 leading to pK814-QNRS. pK814-QNRS then acquired a ~35-kb region with high
526 similarity to the partial sequence of *Klebsiella* phage KP12 clone KP12_2 (OM835952)
527 (96% identity and 93% coverage), leading to pK1326-qnrS. The online blastn analysis
528 identified three putative ancestral plasmids (including pCP102) for pK814-QNRS,
529 showing >90% identity and >80% coverage against the uninterrupted version of pK814-
530 QNRS. Red arrows indicate ARGs, gray pointed boxes indicate ISs, blue boxes indicate
531 TIMEs, and black arrows indicate other genes. The IRs within each TIME are shown by
532 a black (left, IRL) or white (right, IRR) triangle (see **TableS2** for the basis of the
533 orientations of TIMEs). DRs are shown as differently shaped flags, and their sequences
534 are shown in boxes. The accession numbers of the plasmids are as follows: pRJKP36-5,
535 CP100081; pKPC45a, MH595534; p4-N2251932, CP165866; pKQPS142b, CP023480;
536 pKPN535a, MH595533; pSYCC1_6, CP113183; pCP102, CP104015; pK814-QNRS,
537 CP183858; and pK1326-qnrS, CP183864.
538

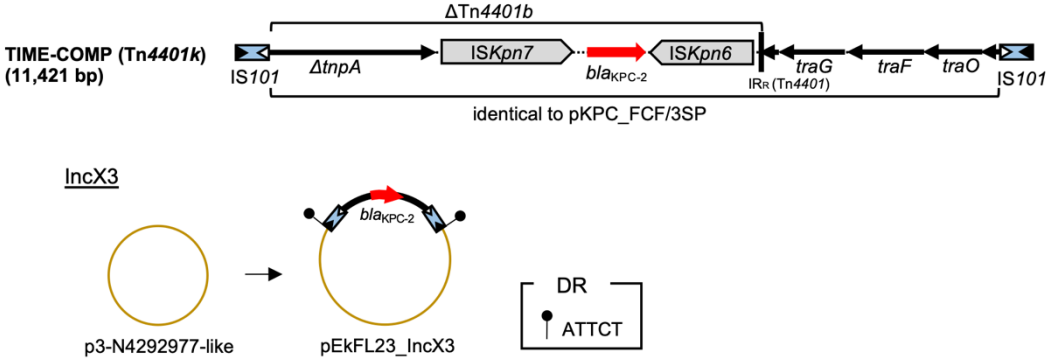


Figure 2. IS*101*-bounded TIME-COMP carrying *bla*$_{KPC-2}$ and a plasmid carrying the TIME-COMP. The TIME-COMP was inserted into a p3-N4292977-like plasmid, leading to pEkFL23_IncX3. The online blastn analysis identified 19 putative ancestral plasmids (including p3-N4292977) for pEkFL23_IncX3, showing >99% identity and >90% coverage against the uninterrupted version of pEkFL23_IncX3. Genes and elements are shown as in **Figure 1**. The accession numbers of the plasmids are as follows: pKPC_FCF/3SP, CP004367; p3-N4292977, CP165819; and pEkFL23_IncX3, OQ434669.

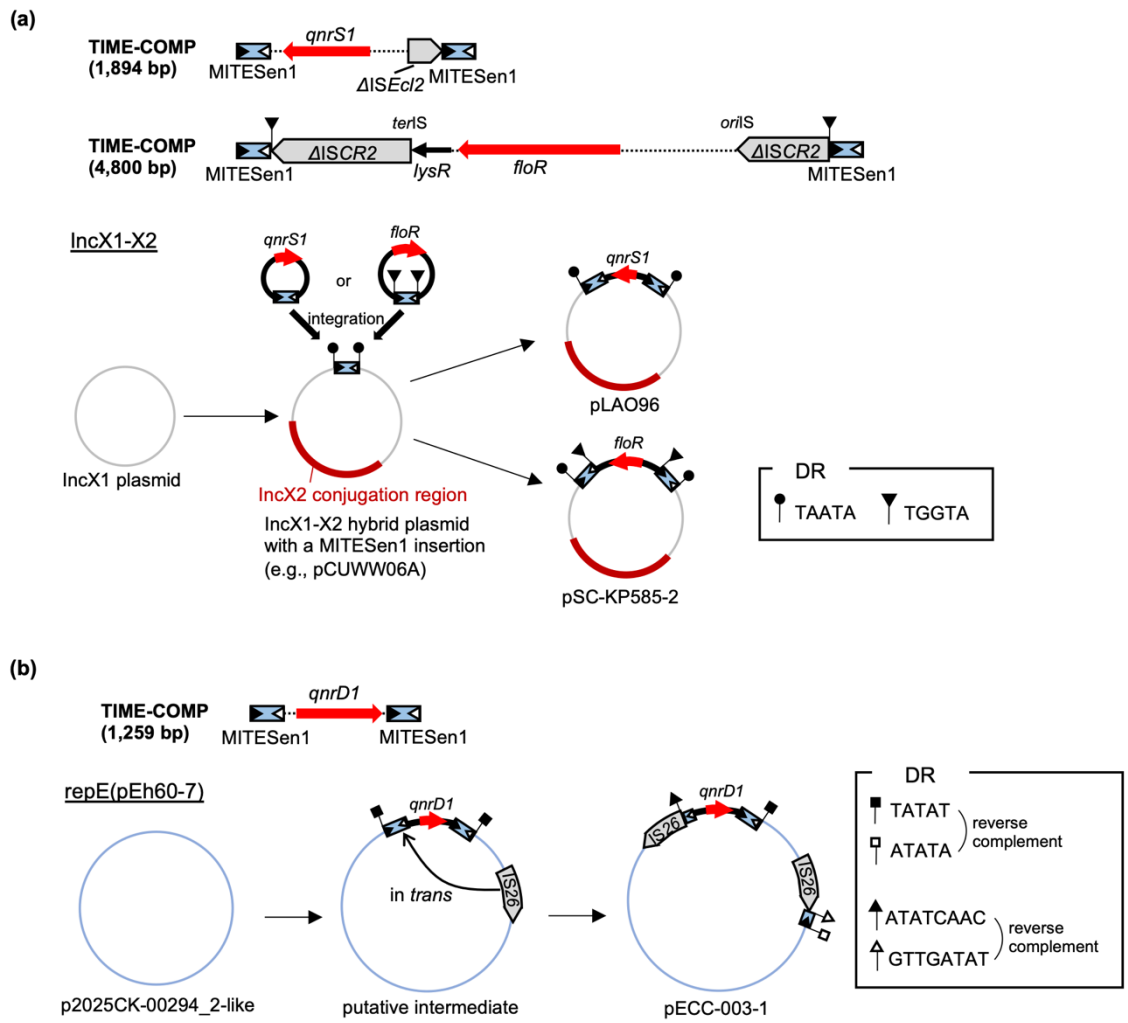


Figure 3. (a) MITESen1-bounded TIME-COMPs carrying *qnrS1* and *floR*, and plasmids carrying these TIME-COMPs. pLAO96 and pSC-KP585-2 might have been generated by integration of a hypothetical circular molecule into a pCUWW06A-like plasmid. pLAO96 (45,300 bp) and pSC-KP585-2 (44,092 bp) share ~35.6 kb of highly similar sequence (>99% identity), excluding the TIME-COMP regions (1,894 bp and 4,800 bp) and regions bounded by IS*26* (7,824 bp and 3,609 bp). (b) MITESen1-bounded TIME-COMP carrying *qnrD1* and a plasmid carrying the interrupted TIME-COMP. A putative intermediate plasmid carrying the intact TIME-COMP is also shown. "in *trans*" indicates intramolecular replicative transposition (in *trans*) of IS*26*. The online blastn analysis identified one putative ancestral plasmid (p2025CK-00294_2) for pECC-003-1, showing >99% identity and 86% coverage against the uninterrupted version of the putative intermediate. Genes and elements are shown as in the previous figures. The accession numbers of the plasmids are as follows: pCUWW06A,

563 CP182140; pLAO96, OP242301; pSC-KP585-2, CP123874; p2025CK-00294_2,
564 CP194151; and pECC-003-1, CP143772.
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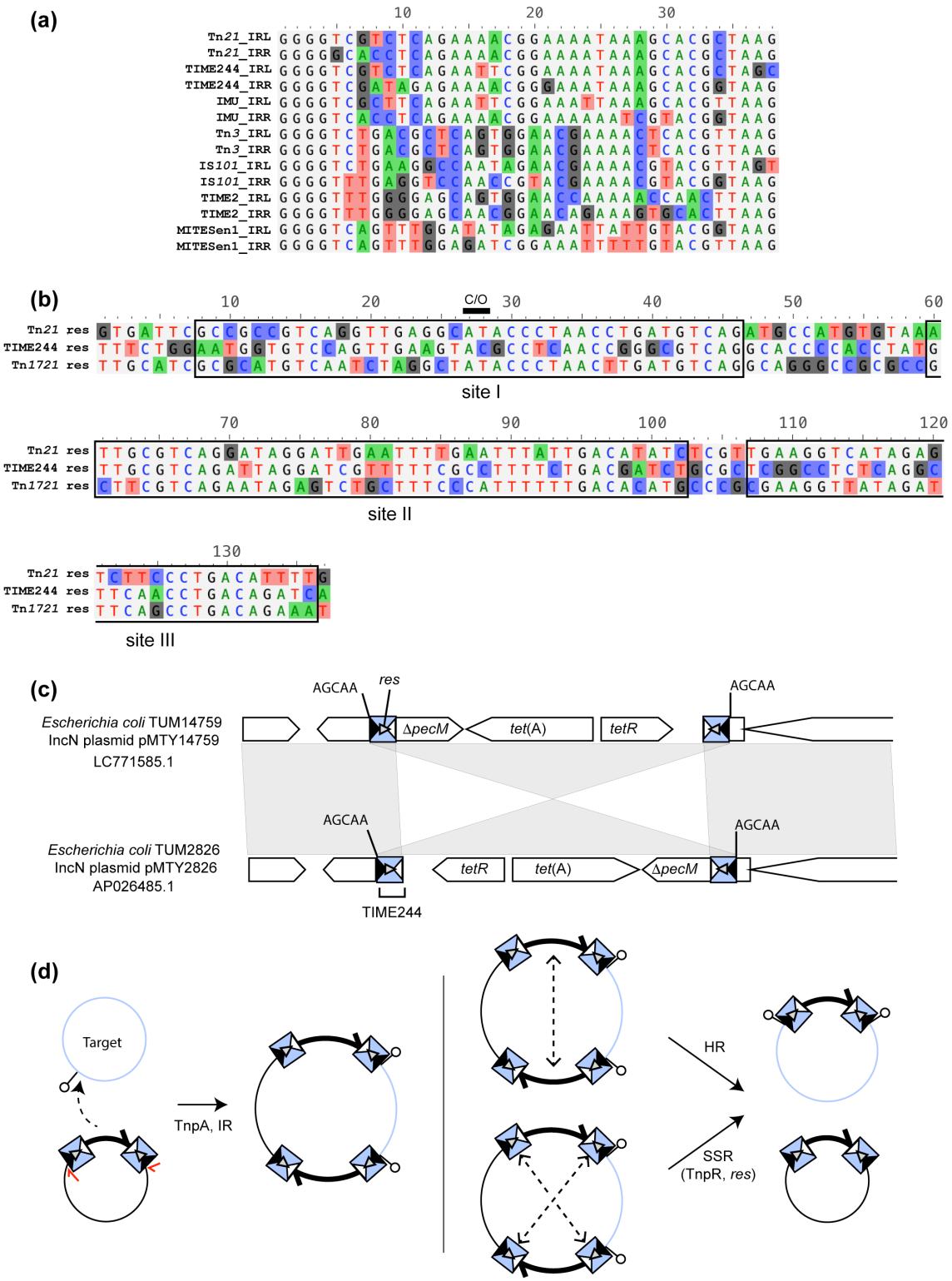

Figure 4. (a) Multiple alignment of IRs of TIMEs, Tn*3*, and Tn*21*. (b) Multiple alignment of *res* sites of Tn*21*, TIME244, and Tn*1721*. Definition of subsites follows a previous study.[35] See **Table S2** for the sequences of TIME244, IMU, IS*101*, and MITESen1. The remaining sequences are derived from the following accession

571 numbers: Tn*21*, AF071413; Tn*3*, HM749966; TIME2, KJ920398; and Tn*1721*, X61367.
572 (c) Two frequent forms of TIME244-bounded TIME-COMPs carrying *tet*(A) inserted in
573 the same location. The genetic contexts in two IncN plasmids (LC771585 and
574 AP026485) are shown as examples.[40] (d) Deduced transposition pathways of TIME-
575 COMPs bounded by inversely oriented TIME244 copies. Cointegrate formation (left) is
576 mediated by TnpA and IRs, followed by replication. Cointegrate resolution (right) is
577 mediated by RecA-dependent homologous recombination (HR) between duplicated
578 TIME-COMPs or by TnpR/*res*-dependent site-specific recombination (SSR). SSR can
579 occur between either pair of the directly oriented TIME244 copies. Nicking sites in the
580 donor molecule are indicated by red arrows.
581

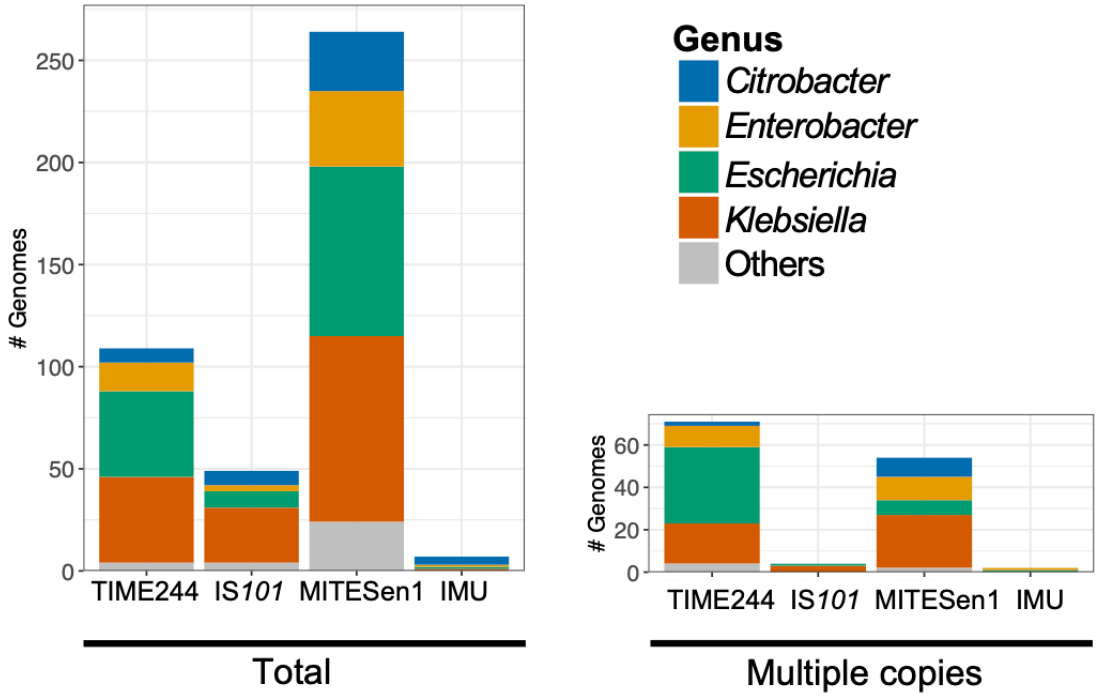

Figure 5. Numbers of RefSeq Enterobacteriaceae genomes carrying TIMEs (TIME244, IS*101*, MITESen1, and IMU) among the downloaded genomes (n = 11,572). "Total" indicates the total number of genomes carrying each TIME. "Multiple copies" indicates the number of genomes with a replicon(s) carrying multiple copies of each TIME. Genera detected in >10 genomes are shown in different colors, while genera detected in ≤ 10 genomes are grouped as "Others" and shown in gray. See also **Table S3** for information on individual genomes.