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16 **A self-transmissible plasmid from a hyperthermophile that**
17 **facilitates genetic modification of diverse Archaea**

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30

31 **ABSTRACT**

32 **Conjugative plasmids are self-transmissible mobile genetic elements which transfer**
33 **DNA between host cells via Type IV Secretion Systems (T4SS). While T4SS-mediated**
34 **conjugation has been well-studied in bacteria, information is sparse in Archaea and**
35 **known representatives exist only in the Sulfolobales order of Crenarchaeota. Here we**
36 **present the first self-transmissible plasmid identified in a Euryarchaeon, *Thermococcus***
37 ***sp.* 33-3. The 103 kbp plasmid, pT33-3, is seen in CRISPR-spacers throughout the**
38 **Thermococcales order. We demonstrate that pT33-3 is a bona fide conjugative plasmid**
39 **that requires cell-to-cell contact, and is dependent upon canonical, plasmid-encoded**
40 **T4SS-like genes. Under laboratory conditions pT33-3 transfers to various**
41 **Thermococcales, and transconjugants propagate at 100°C. Using pT33-3, we developed**
42 **a genetic toolkit which allows modification of phylogenetically diverse Archaeal**
43 **genomes. We demonstrate pT33-3-mediated plasmid mobilization and subsequent**
44 **targeted genome modification in previously untransformable Thermococcales species,**
45 **and extend this process to interphylum transfer to a Crenarchaeon.**

46

47 **MAIN**

48 Conjugative plasmids (CPs) are self-transmissible mobile genetic elements which encode
49 Type IV Secretion Systems (T4SS) to facilitate their transfer between host cells. The
50 paradigmatic plasmid “F” of *E. coli* has been well-studied for over 70 years¹, and other CPs
51 have been identified throughout both gram-positive and gram-negative bacteria^{2,3}. Such
52 plasmids are important epidemiological markers, often carrying virulence factors and drug
53 resistance genes, contributing to pathogenesis^{4,5}. Even among non-pathogenic organisms, CPs
54 are important contributors not only to host genome evolution⁶, but also to the population
55 dynamics of the mobilome^{7,8}. Despite their prevalence in diverse bacteria, CPs have only
56 been observed in one family of Archaea, the Sulfolobales, where it is estimated that 3% of
57 strains harbour such plasmids⁹. These archaeal CPs encode genes homologous to T4SS of
58 bacterial CPs and phylogenetic analysis suggests they arose via inter-domain transfer from a
59 bacterial ancestor (possibly multiple times)¹⁰. While no CPs have been observed outside of
60 the Sulfolobales family of Crenarchaeota, bioinformatic analyses have identified homologues
61 of bacterial conjugation machinery (e.g. *virB4* and *virD4*) in other archaeal genome and
62 plasmid sequences¹⁰, suggesting they may be more widespread than previously appreciated.
63 Indeed, in sequencing the genomes of isolated Thermococcales strains¹¹, we identified an
64 extrachromosomal element with both *virB4* and *virD4* homologues reminiscent of a bacterial

65 CP. Here, we show that this plasmid, named pT33-3 after the strain in which it was identified,
66 *Thermococcus sp. 33.3*, is a true CP encoding genes effecting its own transfer. This plasmid
67 represents the first archaeal CP outside of Sulfolobales, here in the phylum Euryarchaeota. As
68 DNA uptake is a major limiting factor in establishing genetic techniques in Archaea, we use
69 the conjugative ability of pT33-3 to develop pT33-3-mediated transformation and genome
70 modification techniques for a variety of hyperthermophilic archaea, including those of highly
71 divergent phyla.

72

73 RESULTS

74 Identification and characterization of pT33-3

75 We sequenced the complete genome of the archaeal strain *Thermococcus sp. 33-3*, a
76 hyperthermophilic euryarchaeon isolated from the East Pacific Ridge (depth 2,330m)¹¹ and
77 assembled a complete closed circular genome of 2,013,327 bp (ENA accession
78 GCA_946300405). The 16S rRNA of *T. sp. 33-3* is identical to that of the published species
79 *T. nautili*¹² and *T. sp. 26-2*¹³, suggesting they are strains of the same species; however, other
80 common phylogenetic markers are non-identical (*rpoB* at 97% nucleotide identity, *pheS* at
81 92%), and whole genome alignments reveal significant large-scale rearrangements between
82 these isolates (Extended Data Figure 1), as observed in other Thermococcales species⁶.
83 Phylogenetic reconstruction using RpoB places *T. sp. 33-3* in a clade with *T. nautili*,
84 *T. henrieti*¹⁴ and the as yet unnamed *T. sp. 21S9*¹⁵ (Figure 1B). In addition to the circular
85 chromosome, we identified an extra-chromosomal circular plasmid of 103,230 bp (hereafter
86 referred to as pT33-3) (Figure 1A), representing the largest known plasmid in the
87 Thermococcales order. Illumina read depth for the plasmid was approximately equal to that
88 of the chromosome, suggesting pT33-3 exists at single copy number (1.47±0.33). Marker
89 frequency analysis did not reveal the presence of an active replication origin (unsurprising in
90 Thermococcales¹⁶), though indicators of replication initiation and termination regions (GC
91 skew and keto excess¹⁷) give clear inflection points at 3.6 kbp and 19.0 kbp (Extended Data
92 Figure 2).

93

94 While annotation of archaeal mobile genetic elements (MGEs) remains difficult, we were
95 able to ascribe potential functions to 36/166 genes by a combination of BLAST sequence
96 searching and HHblits homology searching (Supplementary Table). This revealed the
97 presence of many genes which are cogent for a large replicon e.g. toxin-antitoxin pairs
98 (RelBE- and MazEF-family) promoting plasmid stabilisation and infectivity¹⁸, cell

99 division/septum binding proteins (FtsK- and MinD-like) implicated in plasmid
100 segregation/partitioning¹⁹, and an MCM-family helicase likely involved in plasmid
101 replication²⁰. No integrase homologues were identified, and no evidence of integration in the
102 host chromosome was found in long-read sequencing, suggesting pT33-3 is exclusively extra-
103 chromosomal in nature. Analyses also reveal that pT33-3 encodes proteins homologous to
104 VirB4/TrbE and VirD4/TraG (genes *p0019* and *p0132*, respectively) required for plasmid
105 transfer through Type IV Secretion Systems (T4SS) in bacteria²¹. Combined with the
106 detection of many other predicted integral membrane proteins (e.g. *p0012*, *p0071*, *p0101*,
107 *p0150*), we sought to investigate whether pT33-3 is capable of conjugative transfer. If
108 present, this would represent the first self-transmissible plasmid in Archaea outside of the
109 Sulfolobales.

110

111 **Determination of pT33-3 transmissibility**

112 If pT33-3 is mobile between Thermococcales species/strains (or has been in the past), we
113 predict a record of these encounters to be found in the CRISPR arrays of such cells²². Indeed,
114 of the 70 available Thermococcales genome assemblies, 12 strains encode a total of 45
115 CRISPR spacers potentially targeting pT33-3 at >85% identity (Figure 1A). Additionally, the
116 45 spacers target different sites on pT33-3 (Figure 1B), indicating multiple spacer acquisition
117 events and thus multiple infections by pT33-3, rather than a single ancestral infection²³.

118

119 The potential T4SS-like machinery of pT33-3, combined with its apparent historical
120 promiscuity prompted us to investigate its ability to transfer under laboratory conditions.
121 Although genetic tools have been developed for a few select Thermococcales species²⁴,
122 *T. sp. 33-3* is not naturally competent for DNA uptake, so we instead developed the plasmid
123 in a *T. kodakarensis* host. However, the presence of a potential anti-pT33-3 CRISPR spacer
124 in *T. kodakarensis* (CRISPR1 spacer 12 with 87% match to *p0132*) required generation of a
125 strain devoid of CRISPR-Cas activity. To achieve this, we disrupted the entire 20 kb cas gene
126 locus and the upstream CRISPR array ($\Delta TK0445-TK0464, \Delta CR1$) in *T. kodakarensis*²⁵. The
127 resulting strain indeed lacked CRISPR-Cas activity against incoming plasmid DNA
128 (Extended Data Figure 3) and was thus used as a host for pT33-3 in subsequent experiments.

129

130 Using a combination of triparental mating and homologous recombination (see materials and
131 methods), we successfully generated pT33-3 variants encoding an HMG-CoA over-
132 expression cassette (conferring resistance to mevinolin²⁶). Illumina sequencing confirmed the

133 entire, circular pT33-3 plasmid was present in the *T. kodakarensis* recipient, and sequencing
134 read depth confirmed the approximately single copy number per chromosome (0.86 ± 0.25)
135 observed in the native *T. sp. 33-3* host. In addition, the marker-encoding pT33-3 readily
136 transferred to the naturally competent *P. furiosus* COM1, confirming inter-genus host
137 compatibility of this plasmid. To test transfer of pT33-3, auxotrophic strains of the
138 *T. kodakarensis* host were generated which allowed selection against the donor strain, and
139 selection of recipients containing pT33-3 by selection for prototrophy in combination with
140 mevinolin resistance (Figure 2A). Using this methodology, efficient transfer of pT33-3 was
141 achieved across the Thermococcales order, not only between divergent *Thermococcus*
142 species, but also from *Thermococcus* to *Pyrococcus* and *Palaeococcus* species (Figure 1B).
143 Of the tested species, only *T. kodakarensis* and *P. furiosus* are naturally competent for DNA
144 uptake, thus this transfer of pT33-3 to the 10 other non-competent species represents the first
145 genetic transformation of these isolates. Average pT33-3 transfer efficiencies were
146 approximately 10^{-3} - 10^{-4} per donor cell (with recipients provided in ~ 10 -fold excess) (Figure
147 2). Despite the low efficiency of transfer, when (and only when) appropriate donors and
148 recipients were combined, transfer was always detected at least two orders of magnitude
149 above the detection limit (10^{-7} per donor in most cases). In fact, of the 14 species tested as
150 transfer recipients, only two proved inaccessible – *T. peptonophilus* which is naturally
151 resistant to mevinolin and thus not experimentally suitable; and *T. sp. 26-2* which encodes 11
152 CRISPR spacers perfectly matching pT33-3, and 17 at 85% identity (Figure 1).

153

154 The ability of pT33-3 to transfer from a non-native host (*T. kodakarensis*) to various non-
155 competent recipients, and the inability of shuttle vectors to transfer under identical conditions
156 (Figure 2C) suggests that this mobility is plasmid-encoded, rather than host-encoded (as in
157 other Euryarchaeota where plasmid transfer results from cell fusion²⁷). Following
158 identification of virB/D homologues (*p0019* and *p0132*), above, we hypothesised that the
159 transfer of pT33-3 occurs via T4SS-like machinery, relying on this virB/D combination²¹. We
160 successfully deleted either *p0019* (virB4 homologue) or *p0132* (virD4 homologue) from
161 pT33-3 (Extended Data figure 4), indicating that they are not required for plasmid replication
162 or maintenance in hosts. Removal of either completely abolished the ability of pT33-3 to
163 transfer to *T. nautili* or *T. gammatolerans* recipients (Figure 2D). In contrast, the knockout
164 plasmids readily transferred between *T. kodakarensis* strains (likely due to natural
165 competency²⁸ – Extended Data Figure 5) indicating their ability to replicate in naive hosts is

166 unperturbed. The requirement of both VirB4 and VirD4 homologues for transmission
167 confirms a conjugative transfer mechanism for pT33-3 using a T4SS-like machinery.

168

169 True T4SS-mediated conjugation requires cell-to-cell contact with the formation of a pore or
170 protein complex bridging cells (Figure 2A). To test whether pT33-3 transfer occurs through
171 conjugation or through an alternative plasmid-encoded mechanism (such as virions or
172 plasmidions^{29,30}), we used a membrane filter to separate donor and recipient cells. Here,
173 transfer of pT33-3 from *T. kodakarensis* to *T. nautili* and *T. gammatolerans* was completely
174 blocked by the membrane filter (Figure 2). In contrast, plasmid transfer was still observed
175 between strains of the naturally competent *T. kodakarensis*, reaffirming competence-
176 mediated transformation. This confirms that cell-to-cell contact is required for the plasmid-
177 mediated transfer of pT33-3, identical to that of bacterial conjugative plasmids utilising
178 T4SS.

179

180 **Mobilization of shuttle vectors by pT33-3**

181 A further defining feature of conjugative elements is the use of an *oriT* (origin of transfer)
182 sequence as the initiation point for transfer³¹. In bacterial CPs, *oriTs* are often encoded
183 adjacent to the transfer genes³². We identified two candidate *oriT* sequences (hereafter named
184 *oriT1* and *oriT2*, Figure 1) of ~2 kbp due to their proximity to potential transfer genes, being
185 relatively long intergenic spaces, encoding significant potential for stem-loop formation, and
186 being at inflection points of GC-skew (points 3.6 kb and 19.0 kb (Extended Data Figure 2)).
187 We cloned each region into a small pTN1-based replicative vector encoding the mevinolin
188 resistance marker (*poriT1/2*) (Extended Data Figure 6). We then tested the ability of
189 *T. kodakarensis* donors with and without pT33-3 to mobilise these plasmids to other
190 Thermococcales. Strikingly, *oriT2* facilitated transfer to *T. nautili* and *T. gammatolerans* at a
191 higher frequency than pT33-3 itself (Figure 3, Extended Data Figure 6). The elevated transfer
192 frequency may be due to the smaller size of mobilised plasmids, and the higher copy number
193 relative to pT33-3. This transfer was reliant upon the presence of pT33-3, suggesting
194 mobilization of shuttle vectors occurs through the pT33-3 encoded conjugation apparatus. We
195 were able to recover intact plasmid DNA from cultures of *T. litoralis*, *T. gammatolerans*, *T.*
196 *pacificus*, and *T. sp. 5-4* (Figure 3). These species are not amenable to genetic transformation
197 with purified DNA, demonstrating the utility of this conjugation/mobilization-based
198 approach. We further narrowed this *oriT*-encoding region to a 300 bp sequence which
199 facilitated mobilization (Extended Data Figure 6). Deletion of this 300bp *oriT* region from

200 pT33-3 decreased transfer efficiency, but did not completely abolish transfer, suggesting
201 some level of *oriT* redundancy.

202

203 **Evolutionary origin of pT33-3**

204 With the knowledge that pT33-3 encodes true conjugation apparatus including a *virB4*
205 homologue, we wished to investigate the evolutionary source of this conjugation machinery.
206 We reconstructed phylogenies for *virB4* sequences similar to that described previously³³.
207 *VirB4* is the only ubiquitous protein in all 8 mating-pair formation (MPF) classes of T4SS,
208 and thus can be used to generate phylogenetic trees of T4SS. In such phylogenies, all
209 archaeal *VirB4* sequences (almost all encoded chromosomally on integrated conjugative
210 elements) cluster in the MPF_{FATA} class, having been transferred from bacteria³³. In our tree,
211 p0019 of pT33-3 grouped with other archaeal *virB4* sequences; however, p0019 clustered
212 within Crenarchaeotal sequences (Extended Data Figure 7), rather than *virB4* homologues of
213 integrated mobile elements in Euryarchaeota. This phylogenetic position suggests transfer of
214 an ancestral pT33-3-like element from Crenarchaeota (or an ancestral lineage) to *T. sp.33-3*
215 (or an ancestor thereof). These analyses reveal a complex evolutionary history of pT33-3 -
216 phylogenies suggest T4SS emerged in ancestral diderm bacteria and were transferred later to
217 ancestral monoderm bacteria¹⁰, then by our analyses were transferred to an ancestor of
218 Crenarchaeota and Thaumarchaeota, with pT33-3 emerging from a further transfer from
219 Crenarchaeota to Euryarchaeota. This suggests that conjugative plasmids such as pT33-3 are
220 involved horizontal gene transfer between distant taxonomic groups of Archaea.

221

222 **pT33-3 for genomic manipulation of diverse Archaea**

223 We next tested the ability of pT33-3 to transfer non-replicative DNAs with the potential to
224 integrate into recipient genomes. We designed plasmids with a mevinolin resistance cassette
225 flanked by homology arms for allelic exchange with the recipient genome, cloned between
226 tandem *oriT2* sequences. As such, transfer will initiate at the first *oriT2* sequence, passing the
227 allelic exchange cassette to the recipient cell, and terminate at the second *oriT2*³⁴ (Extended
228 Data Figure 8). The resulting fragment does not encode an origin of replication, and thus will
229 only confer mevinolin resistance to hosts upon integration into the host genome (and
230 simultaneous allelic replacement). Using this approach, we replaced the *pyrF* gene from both
231 *T. litoralis* and *P. abyssi* with a mevinolin-resistance marker. Allelic exchange was confirmed
232 in the resulting colonies by PCR, and these cultures displayed resistance to both mevinolin
233 and 5-fluoroorotic acid (*pyrF* confers sensitivity to 5-FOA) (Figure 4). Hence, pT33-3

234 mediated mobilization can also be harnessed to carry out genetic modification in non-
235 competent strains.

236

237 Additionally, we were able to extend this plasmid delivery/genetic manipulation approach
238 beyond Euryarchaeota to the Crenarchaeal species *Staphylothermus marinus*³⁵. Using
239 *T. kodakarensis* as a mobilization donor, we replaced the putative adenosine
240 phosphoribosyltransferase (*apt*) gene (*hpt1/Smar_0750/ABN69851.1*) with a mevinolin-
241 resistance marker from *Sulfolobus islandicus*³⁶. Transformants were selected on a
242 combination of 6-methylpurine and mevinolin, and colonies screened by PCR. In this case,
243 spontaneous resistance was more frequent than true allelic exchange, but knockouts were
244 detectable by PCR (Figure 4, Extended Data Figure 9). To our knowledge, this is the first
245 example of inter-phylum conjugation in Archaea, and the first example of genetic
246 modification of *Staphylothermus marinus*.

247

248 **Conjugation at high temperatures**

249 Finally, we were intrigued by the ability of pT33-3 to transfer to *Pyrococcus abyssi*
250 (maximum growth temperature 102°C³⁷) in our experiments, as well as the presence of pT33-
251 3 CRISPR spacers in various *Pyrococcus* species, suggesting an impressive ability to transfer
252 at very high temperature. We thus sought to test the maximum and minimum temperatures at
253 which we can detect pT33-3 transfer between species. Transconjugants of both *P. abyssi* and
254 *T. litoralis* were isolated following co-culturing at 100°C but not at 105°C; however, this is
255 outside the growth temperature range of the *T. kodakarensis* donor, thus host survival may be
256 the limiting factor rather than thermostability of the pT33-3 transfer machinery. It is difficult
257 to say whether conjugation occurs during the 100°C incubation period, or at some
258 intermediate temperature while cultures warm to 100°C. Regardless, isolation of
259 transconjugants at 100°C sets new upper bounds on the function of known conjugative
260 plasmids. At lower temperatures, transfer was successful at 55°C, but not detected at 50°C.
261 Again, this is outside the growth temperatures of both donor and recipient cells. Such high
262 thermostability makes pT33-3 an ideal candidate for studying the structure of archaeal
263 conjugative apparatus.

264

265 **DISCUSSION**

266 We demonstrate that pT33-3 is the first identified self-transmissible plasmid in Archaea
267 outside of the Sulfolobales, here in the phylum Euryarchaeota, family Thermococcales.

268 Transfer of pT33-3 requires cell-to-cell contact and is dependent upon plasmid-encoded
269 homologues of bacterial conjugation machinery, *virB4* and *virD4*, found in Type IV Secretion
270 Systems. Taken together, these results suggest pT33-3 is a true conjugative plasmid
271 transferring between archaeal cells via a plasmid-encoded T4SS-like apparatus.

272

273 The abundance of CPs in Sulfolobales, and their absence outside of this family has led to the
274 hypothesis that archaeal CPs are restricted to Sulfolobales³⁸, likely resulting from an
275 exceedingly rare bacteria-to-archaea transfer event involving an ancestor of modern
276 Sulfolobales¹⁰. The discovery of pT33-3 shows CPs do exist outside of the Sulfolobales, and
277 in highly divergent archaeal phyla (Sulfolobales being Crenarchaea; Thermococcales being
278 Euryarchaea). Phylogenetic reconstruction of VirB4 evolution suggests that pT33-3 was
279 transferred from an ancestor of Sulfolobales (Extended Data Figure 7), perhaps suggesting
280 pT33-3 reflects a later diversification of archaeal CPs following an original establishment in
281 Sulfolobales. Such interdomain transfers are likely rare - the cell membranes and
282 proteinaceous cell walls of archaea differ greatly from those of bacteria³⁹ thus it is difficult to
283 imagine bacteria-to-archaea CP transfer through a classical T4SS. These differences in the
284 cell envelopes of bacteria and archaea also indicate archaeal CP transfer apparatus must
285 necessarily differ from bacterial T4SS, despite sharing some homologous proteins.
286 Determining the nature of this archaeal transfer system will be important as our
287 understanding of gene flow in Archaea advances.

288

289 Recent studies of the genetics and cell biology of Archaea have revealed important insights
290 into not only this enigmatic domain of life, but into of bacterial and eukaryotic biology. The
291 Thermococcales have proven to be invaluable model organisms for the study of Archaea, as
292 well as the study of hyperthermophiles. There are now 70 Thermococcales genome
293 assemblies available, with most representing culturable isolates. Despite the abundance of
294 data and relative ease of cultivation, genetic tractability of this family has been limited to the
295 few species which are naturally competent for DNA uptake (*T. kodakarensis* KOD1⁴⁰, *P.*
296 *furiosus* COM1⁴¹, and to a lesser extent *T. onnurineus* NA1⁴² and *P. yayanosii* A1⁴³). Our
297 development of a conjugation-based DNA transfer system for Thermococcales breaks this
298 transformation barrier, giving the potential to modify *any* Thermococcales isolate, without
299 reliance upon natural-transformation. Furthermore, the apparent promiscuity of the pT33-3
300 transfer machinery makes this technique extendable to other archaeal families, such as
301 Desulfurococcales, shown here (Figure 4D). This work opens the door for genetic dissection

302 of previously unexplored archaeal species, and the development of a broad-range archaeal
303 genetic toolkit.

304

305

306

307

308 **Methods**

309 **Culturing**

310 All Thermococcales species, except *T. kodakarensis* and *P. furiosus*, were cultured
311 anaerobically in Thermococcales Rich Medium (TRM)⁴⁴ with sulfur at 85°C. *T. kodakarensis*
312 was cultured anaerobically in ASW medium²⁸ at 85°C. *P. furiosus* was cultured in defined
313 medium described previously⁴¹. *S. marinus* was cultured anaerobically in 0.65x TRM with
314 sulfur at 85°C. Where appropriate, agmatine was added at a final concentration of 1 mM;
315 mevinolin at 10 µM; 6-methylpurine at 100 µM; 5-FOA at 1.5% w/v. Solid media was
316 prepared by adding Gelrite to a final concentration of 1% w/v, polysulfide solution at 0.2%
317 v/v²⁶, and starch azure added to a final concentration of 0.15% w/v to aid in colony
318 visualisation (not required for *P. furiosus*)⁴⁵. All manipulations of Thermococcales were
319 carried out under anaerobic conditions.

320

321

322 **DNA sequencing and analyses**

323 Total DNA was isolated from cultures by phenol-chloroform extraction as described
324 previously¹¹. Sequencing of the *Thermococcus sp.* 33-3 strain (ENA accession
325 GCA_946300405) was carried out at Genoscope (Centre National de Séquençage, France),
326 using Illumina MiSeq and Oxford Nanopore MinION. Reads were assembled with NaS
327 (<https://github.com/institut-de-genomique/NaS>) and corrected with Canu⁴⁶.
328 Mapping of Illumina reads to assembled genomes (as an estimate of copy number) was
329 performed and visualised using Geneious (BioMatters). 16S, RpoB and PheS sequences were
330 each aligned with MAFFT⁴⁷. Whole genomes were aligned with Gepard v2.1⁴⁸.
331 Functional annotations of pT33-3 were predicted using each ORF as a query sequence for
332 BLASTp⁴⁹ against the nr database (cutoff E=0.01); HHblits (HHSuite v3)⁵⁰ against the
333 UniRef30_2020_06 database (n=2 iterations, cutoff E=0.01).
334 To analyse CRISPR spacer matches, CRISPR arrays were predicted in all Thermococcales
335 genomes available through NCBI (November 2021) using CRT⁵¹ in Geneious. Spacers were
336 extracted and used as query sequences for BLASTn search against pT33-3 (cutoff parameters
337 of 85% identity over the entire spacer length).

338

339 **Strain generation**

340 To generate a *T. kodakarensis* strain devoid of CRISPR activity, we deleted the region of the
341 genome comprising *TK0445-TK0464* and adjacent the CRISPR1 array²⁵ from strain TS559
342 using techniques described previously⁵². Activity was tested by cloning spacers from each of
343 the 3 *T. kodakarensis* CRISPR arrays²⁵ in to pLC70⁵³, and used to transform CRISPR-
344 knockout strains (and parental strains) of *T. kodakarensis* as described previously⁴⁰.
345 To generate a *T. kodakarensis* strain harboring pT33-3, we generated a pTP2-derived
346 plasmid⁵⁴ encoding TK0664 (conferring 6-methylpurine sensitivity), and a recombination
347 cassette encoding 1kb homology arms identical to pT33-3 flanking a *pdaD* agmatine marker
348 gene. A triparental mating was carried out by mixing cell pellets from 50mL of early
349 stationary phase *T. sp. 33-3*, *T. kodakarensis* (*trp*-) harboring the recombination substrate
350 plasmid, and the *T. kodakarensis* (*trp*+) CRISPR-null strain. The mixed suspension was
351 spotted on solid TRM media (supplemented with agmatine) and incubated anaerobically (N₂
352 atmosphere) at 85°C overnight. The following day, plates were chilled to 4°C before
353 suspending the plated cell mass in 200 µL 0.8x ASW, and spreading on solid ASW-CH
354 media containing 6-methylpurine (media devoid of tryptophan to select for recipient cells
355 over recombination donors; containing 6-methylpurine to select against the full
356 recombination substrate plasmid and against *T. sp. 33-3*; devoid of agmatine to select for the
357 presence of the agmatine marker cassette). Colonies were screened by PCR for the presence
358 of pT33-3 with the agmatine marker cassette, grown in liquid culture, and total DNA
359 sequenced as described above.

360 Once established in *T. kodakarensis*, the marker cassette of pT33-3 was swapped by allelic
361 exchange. Deletion of *virB/D* homologues, and *oriT* was carried out as described previously
362 for chromosomal genes⁵².

363

364 **Mating assays**

365 25 mL cultures of donor and recipient cells were grown to early stationary phase in
366 appropriate media (see above). Cultures were chilled on ice before pelleting cell mass at
367 3,500xg for 20 minutes at 4°C. Media was decanted and pellets resuspended in 100 µL 0.8x
368 ASW. Appropriate cell suspensions were mixed and spotted on the centre of a solid TRM
369 plate containing agmatine. Where donors and recipients were separated by a membrane filter,
370 donor suspension was first spotted directly on the media then overlaid with a 0.2 µm
371 nitrocellulose filter, atop which the recipient suspension was spotted. Plates were incubated at
372 85°C (except when testing temperature limits) for 16 hours to allow mating, before chilling to
373 4°C. Cell mass was scraped from the plate and suspended in 200 µL 0.8x ASW. Serial

374 dilutions were then plated on media selective for donors, recipients, or transconjugants, and
375 incubated at 85°C to allow colony formation. Colonies were enumerated, screened by PCR
376 and amplicon sequencing by Sanger sequencing (Eurofins Genomics), and/or transferred to
377 liquid media where required.

378

379 **Mobilization assays**

380 Candidate *oriT* sequences were cloned into a pLC70-derived plasmid encoding only a
381 mevinolin resistance cassette by Gibson Assembly (sequences and plasmid maps of all
382 plasmids available in supplementary data on Dryad). These plasmids were used to transform
383 CRISPR-null *T. kodakarensis* harboring a trp-selectable pT33-3 plasmid. The resulting
384 strains were used as donors in mating assays (as above), with transconjugants selected by
385 resistance to mevinolin (mobilised plasmid marker) and agmatine prototrophy (donors are
386 agmatine auxotrophs).

387 When mobilised DNA was designed to integrate into recipient genomes, a transfer cassette
388 was cloned as above. The transfer cassette consists of a mevinolin-resistance marker
389 surrounded by 1kb homology arms for pop-in/pop-out allelic exchange with the recipient
390 chromosome, all surrounded by tandem *oriT* sequences. Modified recipients were selected by
391 resistance to mevinolin, screened by PCR, and confirmed by resistance to 5-FOA or 6-
392 methylpurine resulting from gene knockouts. Growth curves generated using MicrobeMeter
393 HighT (Humane Technologies).

394

395 **Phylogeny**

396 For RpoB phylogenetic reconstruction, sequences aligned with MAFFT⁴⁷, alignment gaps
397 removed with BMGE⁵⁵, evolutionary model selected with ModelFinder⁵⁶, and phylogenetic
398 trees generated with IQ-TREE v1.6.12⁵⁷. Trees were visualised with iTol⁵⁸.

399 For VirB4 phylogenetic reconstruction, sequences of VirB4 proteins from MPF_{FATA}, and
400 MPF_{FA} used previously^{10,33} were retrieved from Genbank. Additional archaeal sequences
401 were retrieved by BLASTp search. Sequences of the MPF_F class were used as an outgroup.
402 Phylogenetic trees were generated as for RpoB as above. The maximum likelihood tree was
403 constructed using IQ-TREE with the MFP option for model selection. Branch supports were
404 either computed through SH-like aLRT⁵⁹ (10,000 replicates) and UFBoot⁶⁰ (10,000
405 replicates).

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410 **Data Availability**

411 The genome sequence for *Thermococcus sp.* 33-3 has been deposited with ENA number
412 GCA_946300405. Sequences of all plasmids, genomes, and primers used in this study are
413 available on Dryad with doi 10.5061/dryad.2jm63xst9.

414

415

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422

423 **Author Contributions**

424 RJC and EM performed the experiments, VB and GM performed the sequencing, RJC, JO,
425 and VDC performed bioinformatic analyses. MT, PF, JO, and VDC supervised aspects of the
426 project and provided essential expert analysis. RJC wrote the manuscript, with input from
427 MT, PF, JO, and VDC.

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430 **Competing interests**

431 The authors declare no competing interests.

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433

434 **References**

435

436 1. Lederberg, J., Cavalli, L. L. & Lederberg, E. M. Sex Compatibility in Escherichia Coli.

437 *Genetics* **37**, 720–730 (1952).

438 2. Grohmann Elisabeth, Muth Günther & Espinosa Manuel. Conjugative Plasmid Transfer

439 in Gram-Positive Bacteria. *Microbiol. Mol. Biol. Rev.* **67**, 277–301 (2003).

440 3. De La Cruz, F., Frost, L. S., Meyer, R. J. & Zechner, E. L. Conjugative DNA metabolism

441 in Gram-negative bacteria. *FEMS Microbiol. Rev.* **34**, 18–40 (2010).

442 4. Ramirez, M. S., Traglia, G. M., Lin, D. L., Tran, T. & Tolmasky, M. E. Plasmid-

443 Mediated Antibiotic Resistance and Virulence in Gram-negatives: the Klebsiella

444 pneumoniae Paradigm. *Microbiol Spectr* **2**, 1–15 (2014).

445 5. Montero, I., Herrero-Fresno, A., Rodicio, R. & Rodicio, M. R. Efficient mobilization of a

446 resistance derivative of pSLT, the virulence plasmid specific of Salmonella enterica

447 serovar Typhimurium, by an IncII plasmid. *Plasmid* **70**, 104–109 (2013).

448 6. Cossu, M. *et al.* Flipping chromosomes in deep-sea archaea. *PLoS Genet.* **13**, e1006847

449 (2017).

450 7. Tamminen, M., Virta, M., Fani, R. & Fondi, M. Large-scale analysis of plasmid

451 relationships through gene-sharing networks. *Mol. Biol. Evol.* **29**, 1225–1240 (2012).

452 8. Basta, T., Smyth, J., Forterre, P., Prangishvili, D. & Peng, X. Novel archaeal plasmid

453 pAH1 and its interactions with the lipothrixvirus AFV1. *Mol. Microbiol.* **71**, 23–34

454 (2009).

455 9. Prangishvili, D. *et al.* Conjugation in Archaea: Frequent Occurrence of Conjugative

456 Plasmids in Sulfolobus. *Plasmid* **40**, 190–202 (1998).

457 10. Guglielmini, J., de la Cruz, F. & Rocha, E. P. C. Evolution of conjugation and type IV

458 secretion systems. *Mol. Biol. Evol.* **30**, 315–331 (2013).

459 11. Lepage, E. *et al.* Molecular diversity of new Thermococcales isolates from a single area

- 460 of hydrothermal deep-sea vents as revealed by randomly amplified polymorphic DNA
461 fingerprinting and 16S rRNA gene sequence analysis. *Appl. Environ. Microbiol.* **70**,
462 1277–1286 (2004).
- 463 12. Gorlas, A. *et al.* Thermococcus nautili sp. nov., a hyperthermophilic archaeon isolated
464 from a hydrothermal deep-sea vent. *Int. J. Syst. Evol. Microbiol.* **64**, 1802–1810 (2014).
- 465 13. Soler, N. *et al.* Two novel families of plasmids from hyperthermophilic archaea encoding
466 new families of replication proteins. *Nucleic Acids Res.* **38**, 5088–5104 (2010).
- 467 14. Alain, K. *et al.* Thermococcus henrietii sp. nov., a novel extreme thermophilic and
468 piezophilic sulfur-reducing archaeon isolated from a deep-sea hydrothermal chimney. *Int.*
469 *J. Syst. Evol. Microbiol.* **71**, (2021).
- 470 15. Strang, L. C. Genomic Insights and Ecological Adaptations of Deep-Subsurface and Near
471 Subsurface Thermococcus Isolates. (Western Washington University, 2020).
- 472 16. Gehring, A. M. *et al.* Genome Replication in Thermococcus kodakarensis Independent of
473 Cdc6 and an Origin of Replication. *Front. Microbiol.* **8**, 2084 (2017).
- 474 17. Freeman, J. M., Plasterer, T. N., Smith, T. F. & Mohr, S. C. Patterns of Genome
475 Organization in Bacteria. *Science* (1998) doi:10.1126/science.279.5358.1827a.
- 476 18. Díaz-Orejas, R., Espinosa, M. & Yeo, C. C. The Importance of the Expendable: Toxin-
477 Antitoxin Genes in Plasmids and Chromosomes. *Front. Microbiol.* **8**, 1479 (2017).
- 478 19. Guynet, C. & de la Cruz, F. Plasmid segregation without partition. *Mob. Genet. Elements*
479 **1**, 236–241 (2011).
- 480 20. Krupovic, M., Gribaldo, S., Bamford, D. H. & Forterre, P. The evolutionary history of
481 archaeal MCM helicases: a case study of vertical evolution combined with hitchhiking of
482 mobile genetic elements. *Mol. Biol. Evol.* **27**, 2716–2732 (2010).
- 483 21. Grohmann, E., Christie, P. J., Waksman, G. & Backert, S. Type IV secretion in Gram-
484 negative and Gram-positive bacteria. *Mol. Microbiol.* **107**, 455–471 (2018).

- 485 22. Terns, M. P. & Terns, R. M. CRISPR-based adaptive immune systems. *Curr. Opin.*
486 *Microbiol.* **14**, 321–327 (2011).
- 487 23. Pinilla-Redondo, R. *et al.* Type IV CRISPR-Cas systems are highly diverse and involved
488 in competition between plasmids. *Nucleic Acids Res.* (2019) doi:10.1093/nar/gkz1197.
- 489 24. Atomi, H., Imanaka, T. & Fukui, T. Overview of the genetic tools in the Archaea. *Front.*
490 *Microbiol.* **3**, 337 (2012).
- 491 25. Elmore, J. R. *et al.* Programmable plasmid interference by the CRISPR-Cas system in
492 *Thermococcus kodakarensis*. *RNA Biol.* **10**, 828–840 (2013).
- 493 26. Matsumi, R., Manabe, K., Fukui, T., Atomi, H. & Imanaka, T. Disruption of a sugar
494 transporter gene cluster in a hyperthermophilic archaeon using a host-marker system
495 based on antibiotic resistance. *J. Bacteriol.* **189**, 2683–2691 (2007).
- 496 27. Naor, A. & Gophna, U. Cell fusion and hybrids in Archaea: prospects for genome
497 shuffling and accelerated strain development for biotechnology. *Bioengineered* **4**, 126–
498 129 (2013).
- 499 28. Sato, T., Fukui, T., Atomi, H. & Imanaka, T. Targeted gene disruption by homologous
500 recombination in the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1. *J.*
501 *Bacteriol.* **185**, 210–220 (2003).
- 502 29. Forterre, P., Da Cunha, V. & Catchpole, R. Plasmid vesicles mimicking virions. *Nat*
503 *Microbiol* **2**, 1340–1341 (2017).
- 504 30. Erdmann, S., Tschitschko, B., Zhong, L., Raftery, M. J. & Cavicchioli, R. A plasmid
505 from an Antarctic haloarchaeon uses specialized membrane vesicles to disseminate and
506 infect plasmid-free cells. *Nat Microbiol* **2**, 1446–1455 (2017).
- 507 31. Willetts, N. & Wilkins, B. Processing of plasmid DNA during bacterial conjugation.
508 *Microbiol. Rev.* **48**, 24–41 (1984).
- 509 32. Zatyka, M. & Thomas, C. M. Control of genes for conjugative transfer of plasmids and

- 510 other mobile elements. *FEMS Microbiol. Rev.* **21**, 291–319 (1998).
- 511 33. Guglielmini, J. *et al.* Key components of the eight classes of type IV secretion systems
512 involved in bacterial conjugation or protein secretion. *Nucleic Acids Res.* **42**, 5715–5727
513 (2014).
- 514 34. Draper, O., César, C. E., Machón, C., de la Cruz, F. & Llosa, M. Site-specific
515 recombinase and integrase activities of a conjugative relaxase in recipient cells. *Proc.*
516 *Natl. Acad. Sci. U. S. A.* **102**, 16385–16390 (2005).
- 517 35. Fiala, G., Stetter, K. O., Jannasch, H. W., Langworthy, T. A. & Madon, J.
518 *Staphylothermus marinus* sp. nov. Represents a Novel Genus of Extremely Thermophilic
519 Submarine Heterotrophic Archaeobacteria Growing up to 98 °C. *Syst. Appl. Microbiol.* **8**,
520 106–113 (1986).
- 521 36. Zhang, C., Cooper, T. E., Krause, D. J. & Whitaker, R. J. Augmenting the genetic
522 toolbox for *Sulfolobus islandicus* with a stringent positive selectable marker for agmatine
523 prototrophy. *Appl. Environ. Microbiol.* **79**, 5539–5549 (2013).
- 524 37. Erauso, G. *et al.* *Pyrococcus abyssi* sp. nov., a new hyperthermophilic archaeon isolated
525 from a deep-sea hydrothermal vent. *Arch. Microbiol.* **160**, 338–349 (1993).
- 526 38. Wagner, A. *et al.* Mechanisms of gene flow in archaea. *Nat. Rev. Microbiol.* **15**, 492–501
527 (2017).
- 528 39. Albers, S.-V. & Meyer, B. H. The archaeal cell envelope. *Nat. Rev. Microbiol.* **9**, 414–
529 426 (2011).
- 530 40. Sato, T., Fukui, T., Atomi, H. & Imanaka, T. Improved and versatile transformation
531 system allowing multiple genetic manipulations of the hyperthermophilic archaeon
532 *Thermococcus kodakaraensis*. *Appl. Environ. Microbiol.* **71**, 3889–3899 (2005).
- 533 41. Lipscomb, G. L. *et al.* Natural competence in the hyperthermophilic archaeon
534 *Pyrococcus furiosus* facilitates genetic manipulation: construction of markerless deletions

535 of genes encoding the two cytoplasmic hydrogenases. *Appl. Environ. Microbiol.* **77**,
536 2232–2238 (2011).

537 42. Kim, M.-S. *et al.* CO-dependent H₂ production by genetically engineered *Thermococcus*
538 *onnurineus* NA1. *Appl. Environ. Microbiol.* **79**, 2048–2053 (2013).

539 43. Li, X. *et al.* Genetic tools for the piezophilic hyperthermophilic archaeon *Pyrococcus*
540 *yayanosii*. *Extremophiles* **19**, 59–67 (2015).

541 44. Zeng, X. *et al.* *Pyrococcus* CH1, an obligate piezophilic hyperthermophile: extending the
542 upper pressure-temperature limits for life. *ISME J.* **3**, 873–876 (2009).

543 45. Akpan, I., Bankole, M. O. & Adesemowo, A. M. A rapid plate culture method for
544 screening of amylase producing micro-organisms. *Biotechnol. Tech.* **13**, 411–413 (1999).

545 46. Koren, S. *et al.* Canu: scalable and accurate long-read assembly via adaptive k-mer
546 weighting and repeat separation. *Genome Res.* **27**, 722–736 (2017).

547 47. Katoh, K. & Standley, D. M. MAFFT multiple sequence alignment software version 7:
548 improvements in performance and usability. *Mol. Biol. Evol.* **30**, 772–780 (2013).

549 48. Krumsiek, J., Arnold, R. & Rattei, T. Gepard: a rapid and sensitive tool for creating
550 dotplots on genome scale. *Bioinformatics* **23**, 1026–1028 (2007).

551 49. Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. Basic local
552 alignment search tool. *J. Mol. Biol.* **215**, 403–410 (1990).

553 50. Steinegger, M. *et al.* HH-suite3 for fast remote homology detection and deep protein
554 annotation. *bioRxiv* 560029 (2019) doi:10.1101/560029.

555 51. Bland, C. *et al.* CRISPR recognition tool (CRT): a tool for automatic detection of
556 clustered regularly interspaced palindromic repeats. *BMC Bioinformatics* **8**, 209 (2007).

557 52. Santangelo, T. J., Cubonová, L. & Reeve, J. N. *Thermococcus kodakarensis* genetics:
558 TK1827-encoded beta-glycosidase, new positive-selection protocol, and targeted and
559 repetitive deletion technology. *Appl. Environ. Microbiol.* **76**, 1044–1052 (2010).

- 560 53. Santangelo, T. J., Cubonová, L. & Reeve, J. N. Shuttle vector expression in
561 *Thermococcus kodakaraensis*: contributions of cis elements to protein synthesis in a
562 hyperthermophilic archaeon. *Appl. Environ. Microbiol.* **74**, 3099–3104 (2008).
- 563 54. Catchpole, R., Gorlas, A., Oberto, J. & Forterre, P. A series of new *E. coli*-*Thermococcus*
564 shuttle vectors compatible with previously existing vectors. *Extremophiles* **22**, 591–598
565 (2018).
- 566 55. Criscuolo, A. & Gribaldo, S. BMGE (Block Mapping and Gathering with Entropy): a
567 new software for selection of phylogenetic informative regions from multiple sequence
568 alignments. *BMC Evol. Biol.* **10**, 210 (2010).
- 569 56. Kalyaanamoorthy, S., Minh, B. Q. & Wong, T. K. F. ModelFinder: fast model selection
570 for accurate phylogenetic estimates. *Nature* (2017).
- 571 57. Nguyen, L.-T., Schmidt, H. A., von Haeseler, A. & Minh, B. Q. IQ-TREE: A Fast and
572 Effective Stochastic Algorithm for Estimating Maximum-Likelihood Phylogenies. *Mol.*
573 *Biol. Evol.* **32**, 268–274 (2014).
- 574 58. Letunic, I. & Bork, P. Interactive tree of life (iTOL) v3: an online tool for the display and
575 annotation of phylogenetic and other trees. *Nucleic Acids Res.* **44**, W242-5 (2016).
- 576 59. Guindon, S. *et al.* New Algorithms and Methods to Estimate Maximum-Likelihood
577 Phylogenies: Assessing the Performance of PhyML 3.0. *Syst. Biol.* **59**, 307–321 (2010).
- 578 60. Hoang, D. T., Chernomor, O., von Haeseler, A., Minh, B. Q. & Vinh, L. S. UFBoot2:
579 Improving the Ultrafast Bootstrap Approximation. *Mol. Biol. Evol.* **35**, 518–522 (2018).
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581 **FIGURE LEGENDS**

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Figure 1

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Overview of pT33-3 and evidence for CRISPR spacer targeting. A) Plasmid map of pT33-3, with predicted ORFs indicated by grey block arrows. *virB4* and *virD4* homologues highlighted in blue; potential origins of transfer highlighted in orange. CRISPR spacers of Thermococcales are indicated by black arrowheads on the interior.

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B) Phylogenetic tree of Thermococcales species used/described in this work. Phylogeny based on RpoB with Methanococcales as an outgroup. Number of CRISPR spacers targeting pT33-3 and ability to receive pT33-3 under laboratory conditions is indicated (ND indicates not determined).

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Figure 2

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Interspecies transfer of pT33-3. A) In bacterial CPs, the plasmid-encoded T4SS forms a pore bridging the cytoplasm of donor and recipient cells through which the conjugative plasmid is actively transferred. Phenotypes selected in this work are indicated. B) pT33-3 readily transfers from *T. kodakarensis* donor to species not naturally competent for DNA uptake. Individual points indicate conjugation efficiency in a single biological replicate. C) pT33-3 transfers from *T. kodakarensis* to *T. nautili*; non-conjugative shuttle vector (pLC70) does not, indicating transfer ability is plasmid-encoded (not host-encoded). D) Transfer of pT33-3 to non-competent species requires homologues of known T4SS proteins, p0019 (VirB4 homologue), and p0132 (VirD4 homologue). Transfer also requires cell-to-cell contact, being blocked by a 0.2 µm filter.

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Figure 3

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pT33-3 mediated mobilization of shuttle vectors. A) T4SSs encoded by conjugative plasmids mobilize other DNAs encoding their cognate origin of transfer (*oriT*). Phenotypes selected in this work are indicated. B) A shuttle vector encoding pT33-3 *oriT* (*poriT2*) is mobilized to non-competent donor species in the presence of pT33-3. C) Plasmid DNA isolated from transconjugants has identical *HindIII* digestion patterns to the that of the donor strain (*T. kodakarensis*). Representative data from a single experiment shown, but identical results were obtained in 4 replicate experiments.

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Figure 4

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pT33-3 mediated genome modification. A) pT33-3 *oriT* allows mobilisation of non-replicative DNA to recipient organisms. Here, homology arms allow allelic exchange with the chromosome, facilitating genetic modification of recipients. Phenotypes selected in this work are indicated. B) Deletion of *pyrF* from *T. litoralis* via pT33-3 mediated mobilisation. Top: diagnostic PCR for deletion shows loss of WT gene and replacement with a pop-in/pop-out MevR cassette; Lower: Resulting strains form colonies on 5-FOA, as expected for a Δ *pyrF* genotype. C) As per B, but with a *P. abyssi* recipient. Growth curves show resistance to 5-FOA. D) *Staphylothermus marinus*, a Crenarchaeon, receives DNA designed to replace the *apt* gene with a MevR cassette. Top: Diagnostic PCR of knockout. Lower: Phylogenetic tree based on 16S rDNA, indicating phylogenetic distance between *T. kodakarensis* donor (Thermococci, labelled red), and *S. marinus* recipient (Desulfurococcales, labelled blue). Representative data from a single experiment shown for B-D, but identical knockouts obtained in 3 biological replicates.

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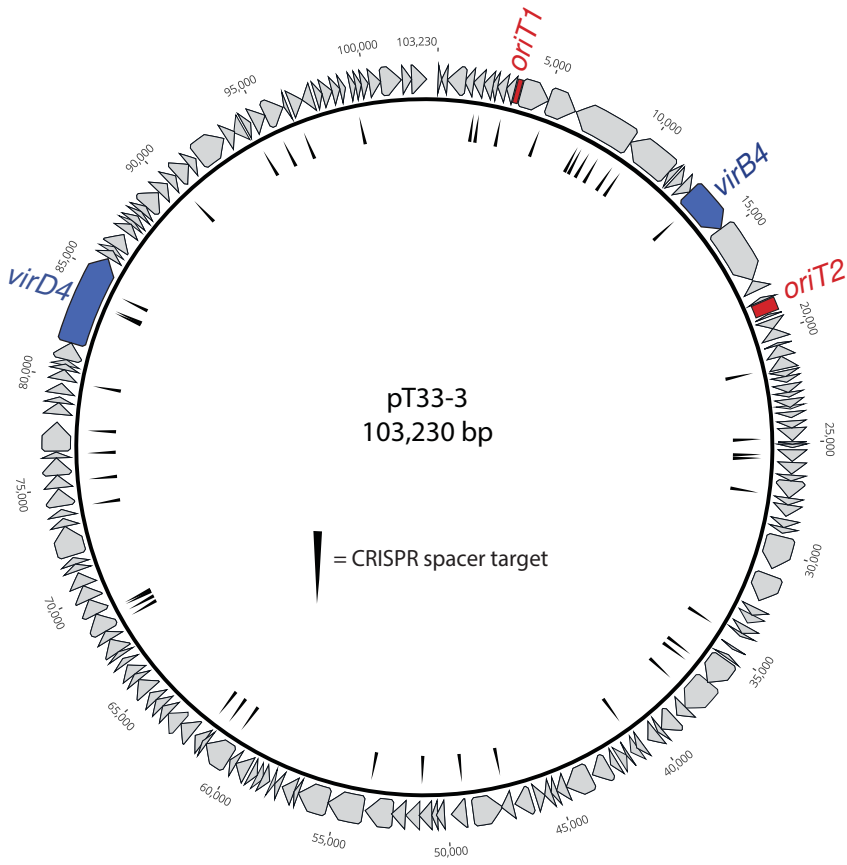
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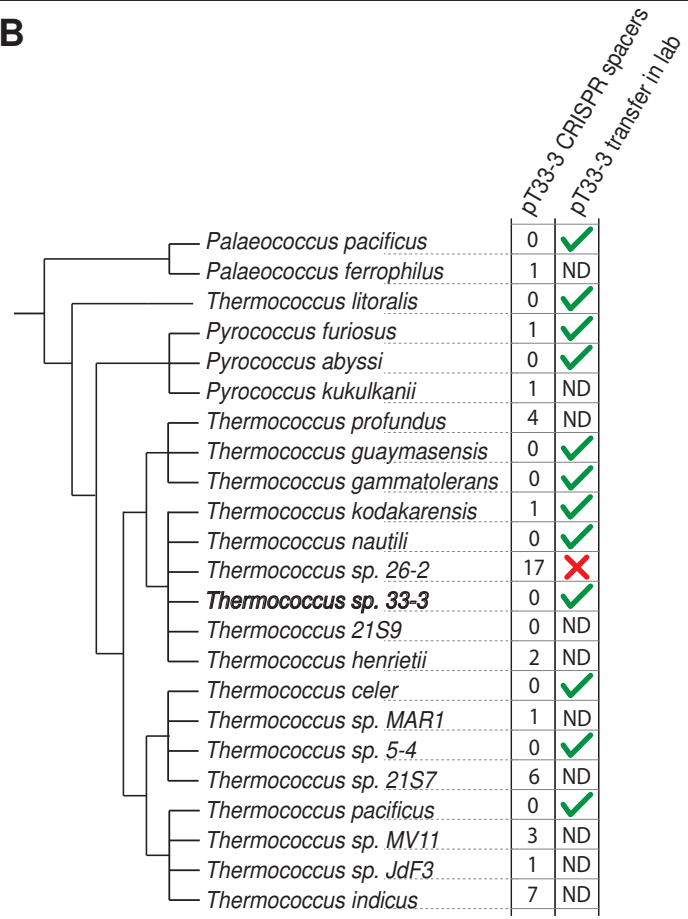
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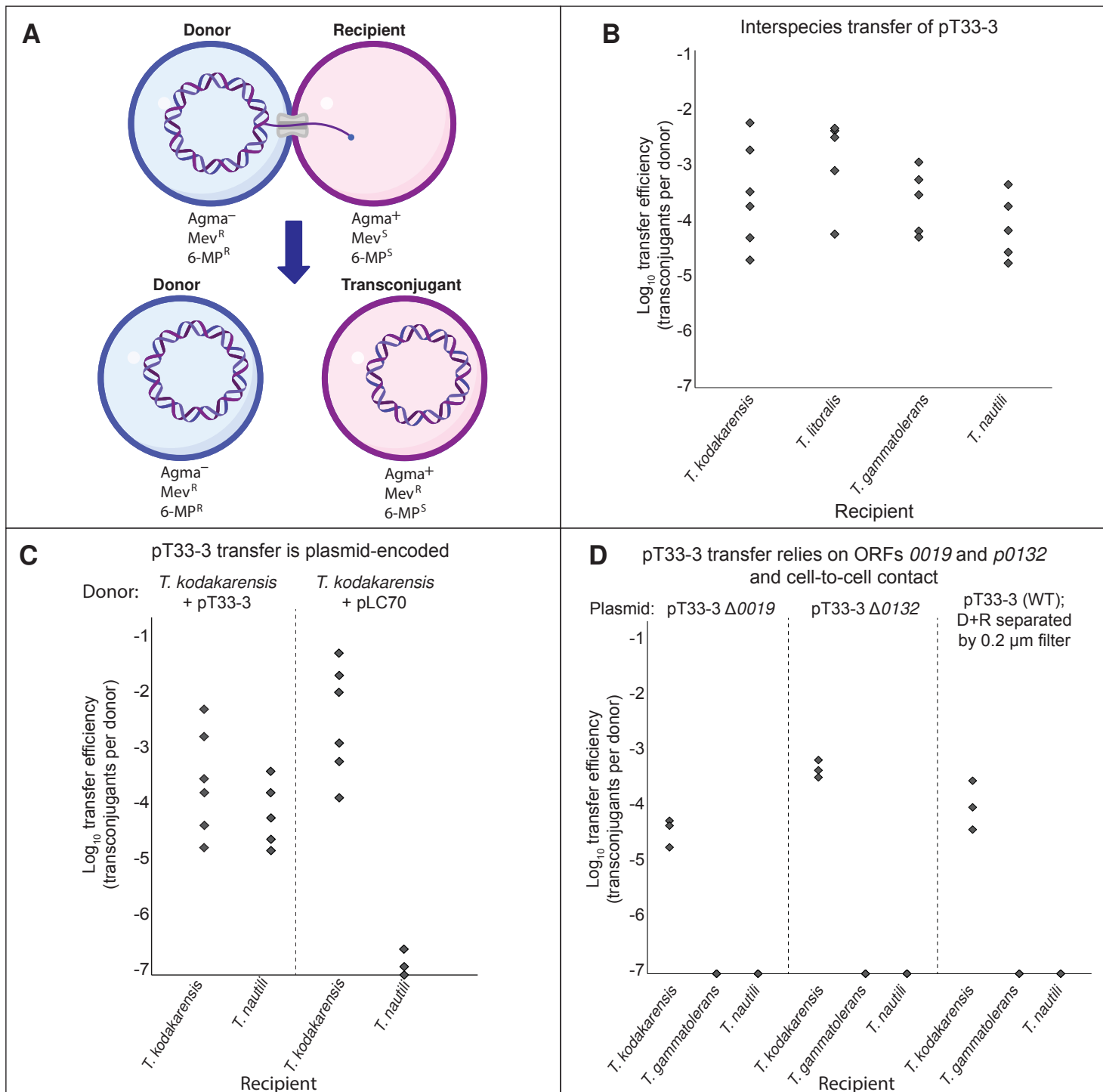
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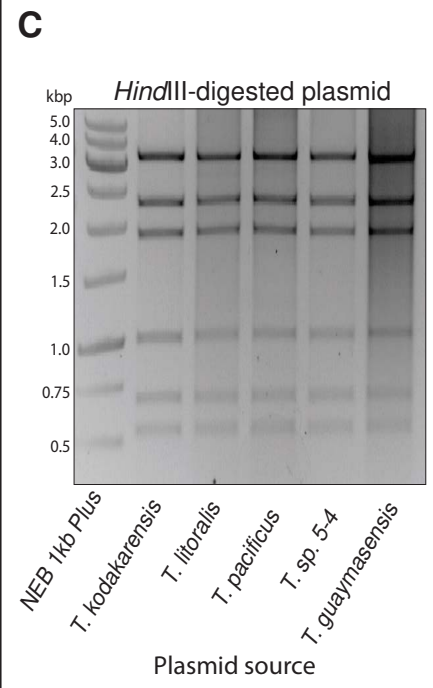
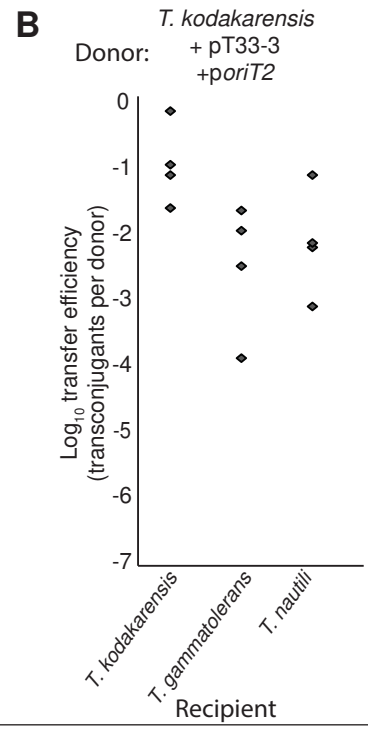
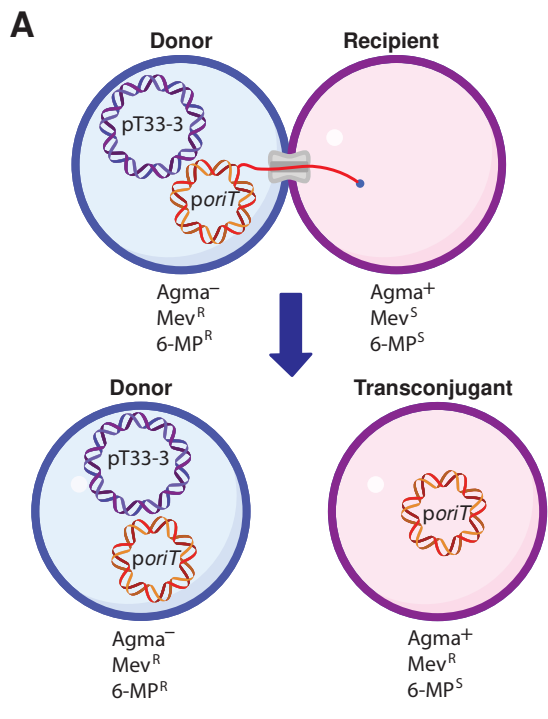
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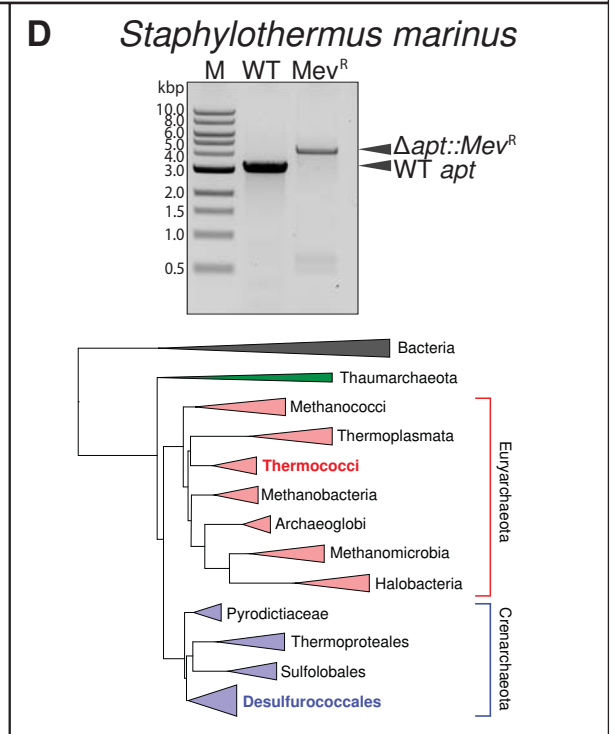
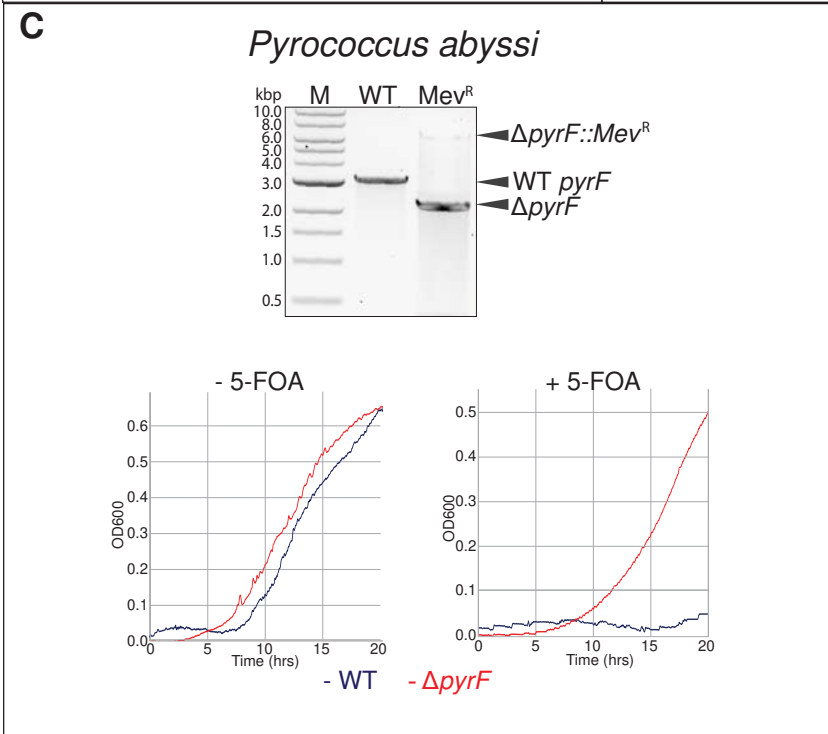
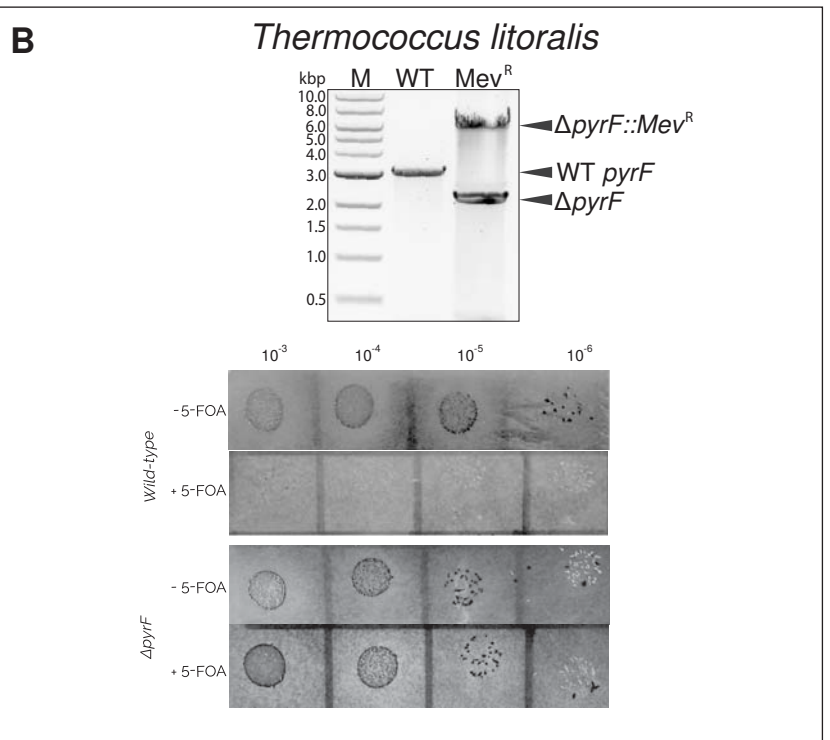
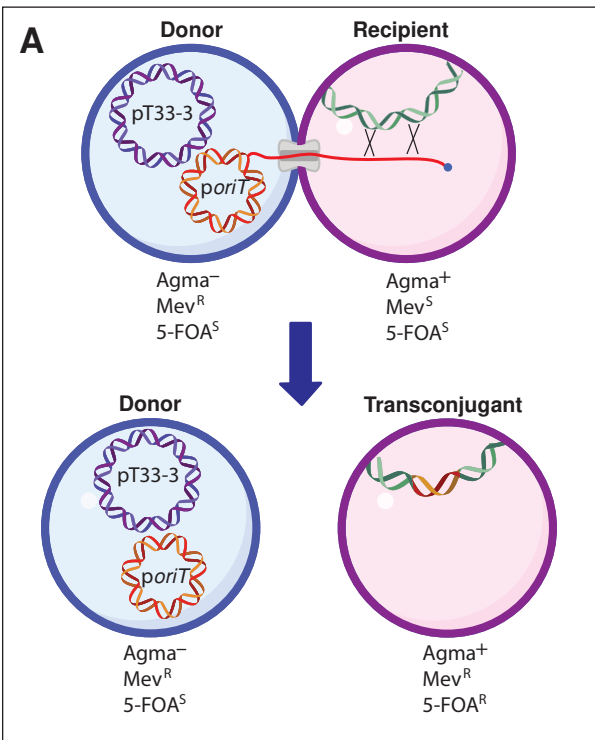


Figure #	Figure title One sentence only	Filename This should be the name the file is saved as when it is uploaded to our system. Please include the file extension. i.e.: <i>Smith_ED_Fig1.jpg</i>	Figure Legend If you are citing a reference for the first time in these legends, please include all new references in the main text Methods References section, and carry on the numbering from the main References section of the paper. If your paper does not have a Methods section, include all new references at the end of the main Reference list.
Extended Data Fig. 1	Similarity of <i>T. sp.</i> 33-3 to other species.	Ex_data_fig_1.eps	Genome alignment between <i>Thermococcus. sp.</i> 33-3 and closely related strains or species, <i>T. nautili</i> (A); <i>T. henrieti</i> EXT12c (B); and <i>T. sp.</i> 26-2 (C). Dots (which form into lines) indicate sequence identity between corresponding regions of the chromosome for the two indicated species. Discontinuities indicate indels; direction changes e.g. change from pointing north-east to south-east, indicate large-scale inversions.
Extended Data Fig. 2	Bioinformatic identification of pT33-3 origin of replication and transfer	Ex_data_fig_2.eps	Plot of cumulative GC skew $[(G-C)/(G+C)]$ and keto excess $[(G+T)-(C+A)]$ for pT33-3. Sharp inflection points are often indicative of origins of replication, origins of transfer, or replication termination sites. These sites are indicated by vertical dashed lines.
Extended Data Fig. 3	Interference assays with CRISPR WT and null strains of <i>T. kodakarensis</i>	Ex_data_fig_3.eps	Diamonds indicate data from a single biological replicate (for WT with target plasmid, overlapping points obscure data, n=3). Wild-type (TS559) <i>T. kodakarensis</i> is unable to be transformed by plasmids encoding a sequence complementary to spacers in its CRISPR array (target plasmid). In contrast, non-target plasmids transform at $\sim 100\text{cfu}/\mu\text{g}$ DNA. Deletion of genes encoding all CRISPR-associated (Cas) proteins abolishes this targeting activity, restoring transformation with target-encoding plasmids.
Extended Data Fig. 4	Deletion of genes homologous to bacterial T4SS	Ex_data_fig_4.eps	PCR of knockouts for predicted transfer genes, <i>p0019</i> and <i>p0132</i> . PCR was carried out using primers binding to pT33-3 outside the homology arms used in pop-in/pop-out recombination
Extended Data Fig. 5	Transfer rates of genetic markers between <i>T. kodakarensis</i> strains does not require conjugation	Ex_data_fig_5.eps	A) <i>T. kodakarensis</i> incubated with purified plasmid DNA (presented as transformants per fg DNA for comparable scale) - <i>T. kodakarensis</i> is naturally competent for DNA uptake. B) Transfer of a non-conjugative plasmid from a <i>T. kodakarensis</i> donor to a plasmid-free recipient – plasmids transfer between <i>T. kodakarensis</i> strains occurs

			<p>by simple co-culturing. C) Transfer of a chromosomal prototrophic marker from a <i>T. kodakarensis</i> donor to an auxotrophic recipient - chromosomal markers transfer between <i>T. kodakarensis</i> strains. D) Transfer of a chromosomal prototrophic marker from a <i>T. gammatolerans</i> donor to an auxotrophic <i>T. kodakarensis</i> recipient - chromosomal markers are unable to transfer between <i>T. gammatolerans</i> and <i>T. kodakarensis</i>, suggesting allelic exchange is mediated by homologous recombination. E) Transfer of a chromosomal prototrophic marker from a <i>T. kodakarensis</i> donor to an auxotrophic recipient where the marker is encoded at a locus encoding a second prototrophic marker – transfer chromosomal markers requires a suitable receptive genomic site (non-essential). Individual biological replicates (-, n=3) are presented with average and standard deviation indicated by error bars.</p> <p>G-I) In contrast to <i>T. kodakarensis</i>, <i>T. nautili</i> is unable to receive a shuttle vector by co-culturing, whereas pT33-3 readily transfers (see main text and Figure 2C).</p>
Extended Data Fig. 6	Identification of <i>oriT</i> -encoding region of pT33-3.	Ex_data_fig_6.eps	<p>1.5kb regions surrounding GC-skew/keto excess minima/maxima were cloned into a shuttle vector and transfer observed in the presence of pT33-3 from <i>T. kodakarensis</i> donors. A) <i>oriT1</i> candidate region. While plasmids encoding this region readily transferred between <i>T. kodakarensis</i> strains, minimal transfer was observed to <i>T. nautili</i> and <i>T. gammatolerans</i> recipients. B) <i>oriT2</i> candidate region. Plasmids encoding this region readily transferred from <i>T. kodakarensis</i> to <i>T. kodakarensis</i>, <i>T. nautili</i>, and <i>T. gammatolerans</i>, indicating this region encodes the <i>oriT</i> of pT33.3. C) <i>oriT2</i> region was split into four overlapping fragments. Plasmids encoding both <i>oriT2.1</i> and <i>oriT2.2</i> regions transferred to the non-competent recipients, whereas <i>oriT2.3</i> and <i>oriT2.4</i> did not, indicating the <i>oriT</i> is encoded by the overlap region between <i>oriT2.1</i> and <i>oriT2.2</i>. D) The 300bp overlap between <i>oriT2.1</i> and <i>oriT2.2</i> (named <i>oriT300</i>) also confers mobilization ability to plasmids, indicating that this region encodes the <i>oriT</i> of pT33.3.</p>

Extended Data Fig. 7	Phylogeny of VirB4	Ex_data_fig_7.eps	A) Figure adapted from Figure 6 of Guglielmini et al. 2014 (doi: 10.1093/nar/gku194) where it was proposed that all archaeal VirB4 sequences arise from a transfer from bacteria within the MPF _{FATA} group. B) Re-creation of MPF _{FATA} and MPF _{FA} phylogeny including VirB4 sequences from integrated elements in other archaeal genomes, and the VirB4 homologue from pT33-3. Phylogeny rooted with MPF _F outgroup. pT33-3 VirB4 groups within a clade of Crenarchaeal sequences, suggesting pT33-3 arose from an inter-phylum transfer. Supported branches, computed by aLRT >80 or UFBoot >95, are indicated by dots at nodes (the full tree is provided as an extended data file). The scale bar indicates the average number of substitutions per site.
Extended Data Fig. 8	Strategy employed for allelic exchange in non-competent recipient cells	Ex_data_fig_8.eps	A) Schematic diagram of mobilizable plasmid (pMob) encoding pT33-3 <i>oriT</i> sequences and homologous recombination + selectable marker cassette for allelic exchange. B) Transfer of pMob initiating at <i>oriT1</i> and terminating at <i>oriT2</i> results in transfer of a non-replicative DNA encoding recombination/marker cassette. This can be a substrate for homologous recombination and allelic exchange with the recipient chromosome. C) Transfer of pMob initiating at <i>oriT2</i> and terminating at <i>oriT1</i> results in transfer of a replicative DNA without any selectable marker. Transformant selection on drug-containing media renders these cells non-viable.
Extended Data Fig. 9	PCR screen of <i>S. marinus</i> colonies following conjugation mediated mobilisation of a recombination substrate	Ex_data_fig_9.eps	Eight colonies were screened using primers binding to the <i>S. marinus</i> chromosome, outside the homology arms used in allelic exchange. Clean allelic exchange ($\Delta apt::SimR$) was observed in 2/8 colonies. Data from a representative replicate is shown, but similar data obtained on 3 occasions.

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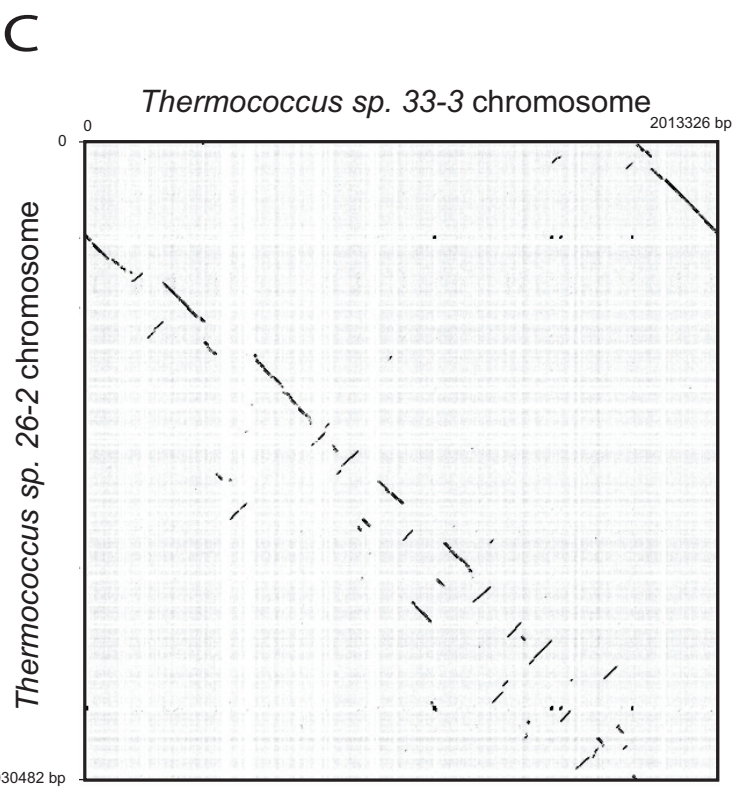
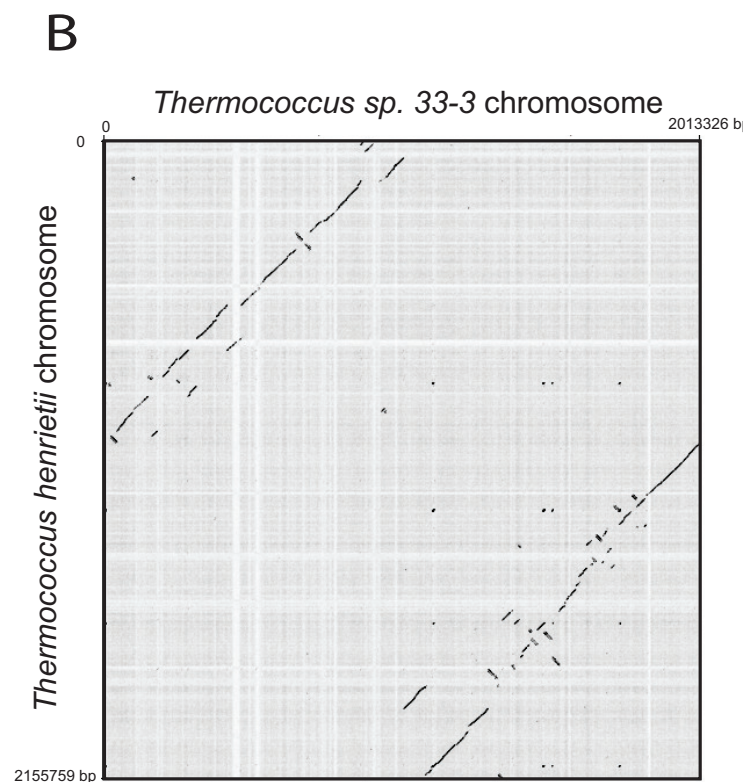
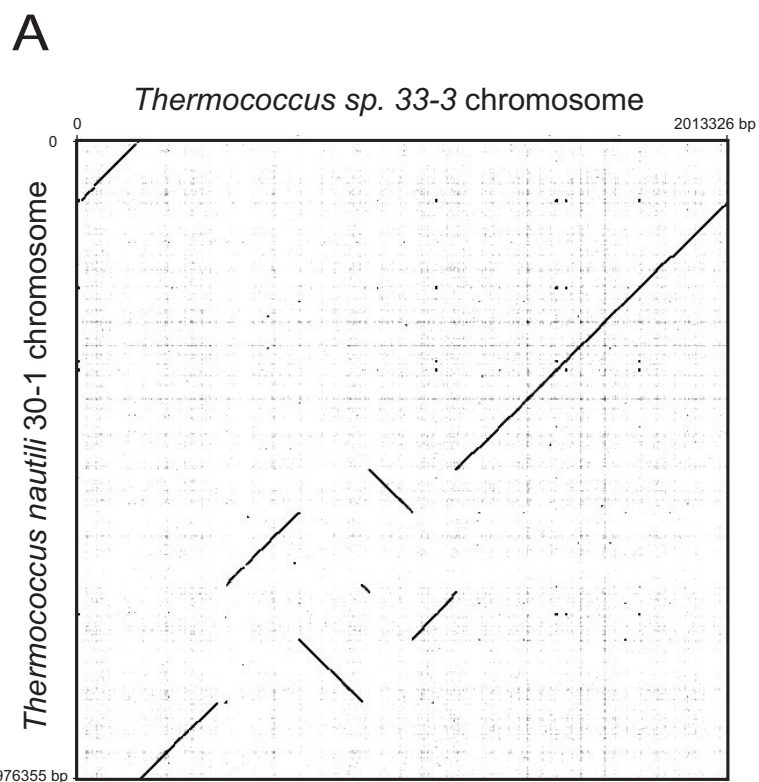
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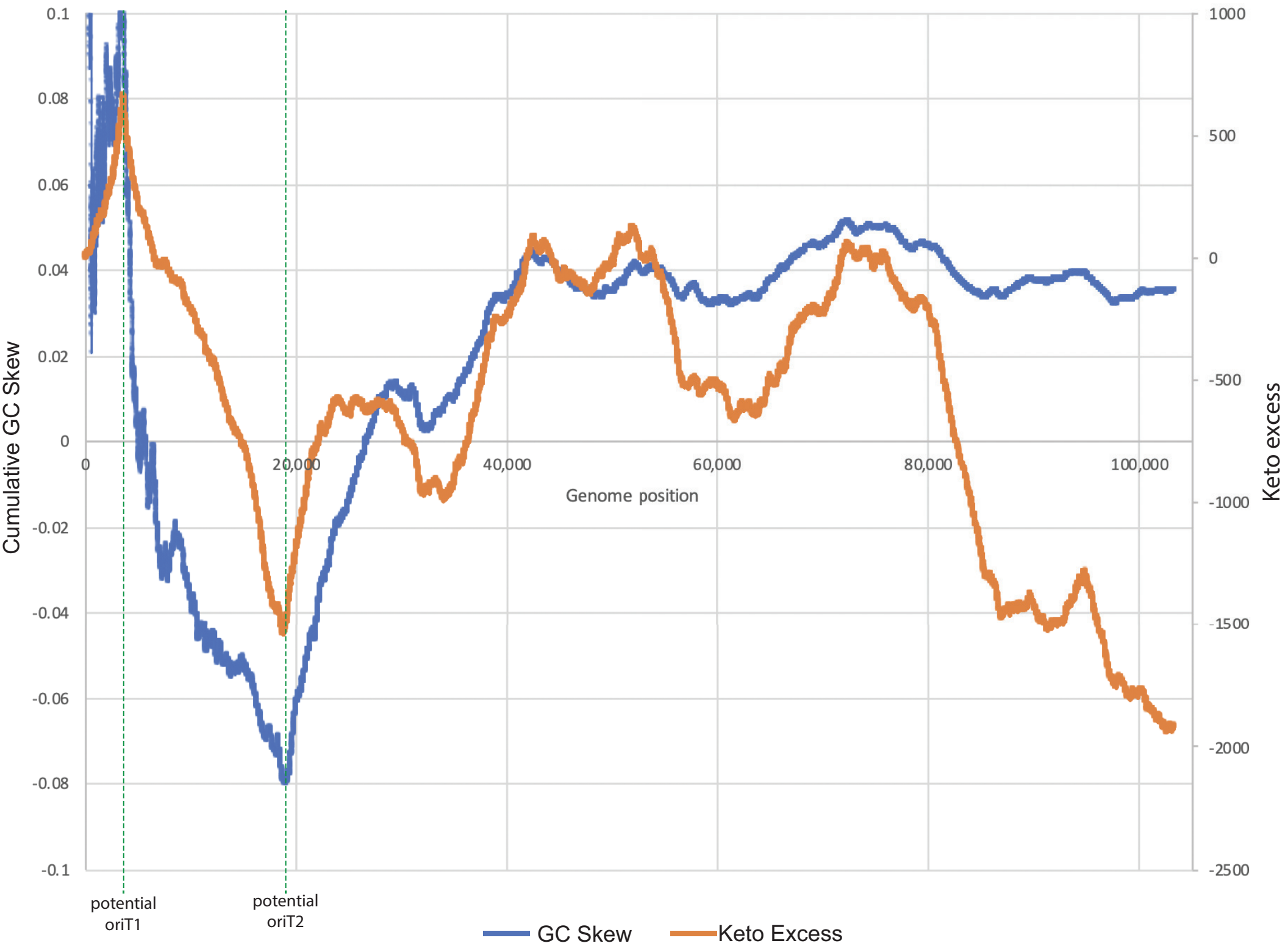
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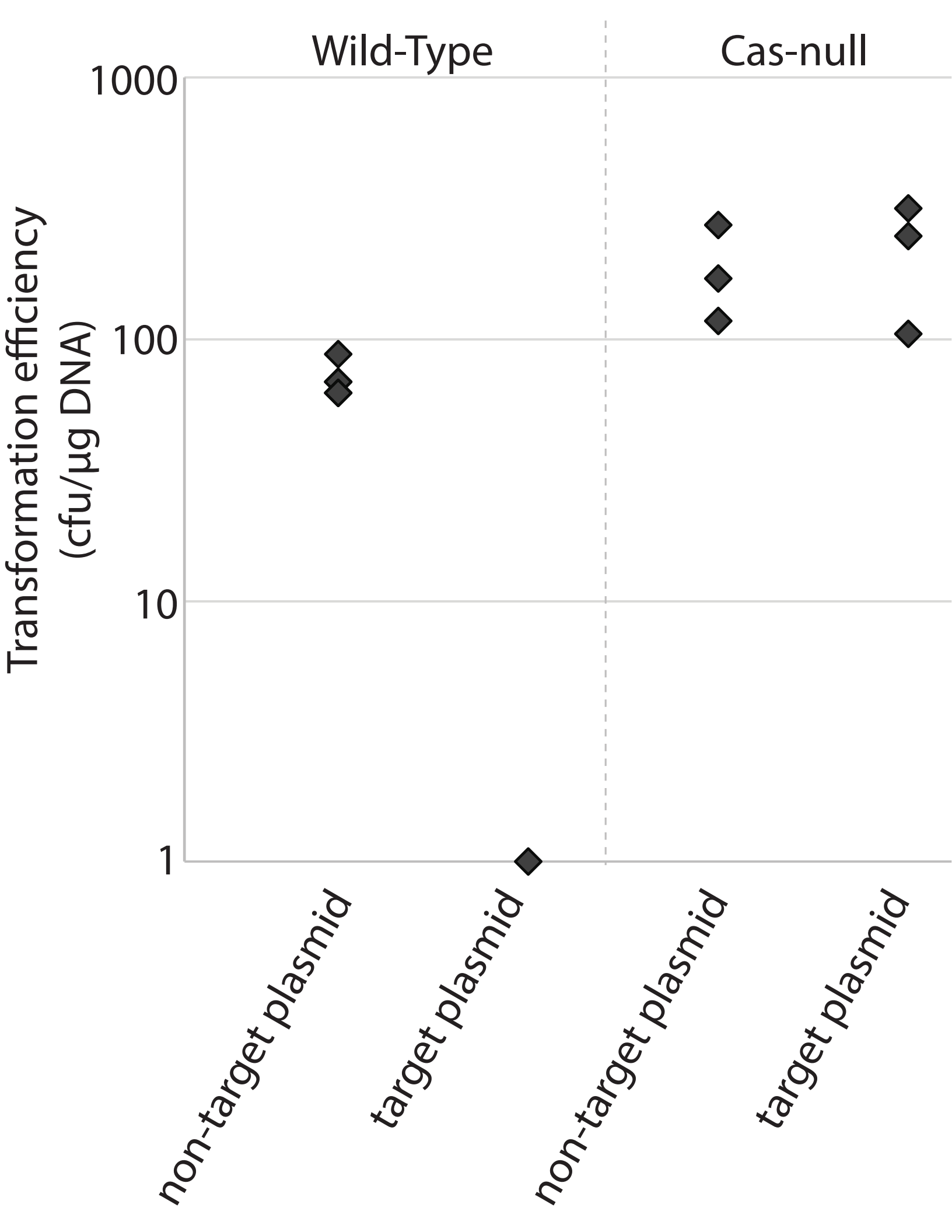
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Supplementary Table		Supplementary Table 1.xlsx	Bioinformatic prediction of pT33-3 ORF function. A combination of BLAST and HHblits was used to predict functions for each ORF in pT33-3. BLAST was performed against the non-redundant protein database. Hits with E-value<0.01 are highlighted in yellow. HHblits was performed against the Uniprot database; however, the majority of hits returned 'hypothetical protein' or 'uncharacterised protein'. Thus, hits were further limited to those with functional annotations.

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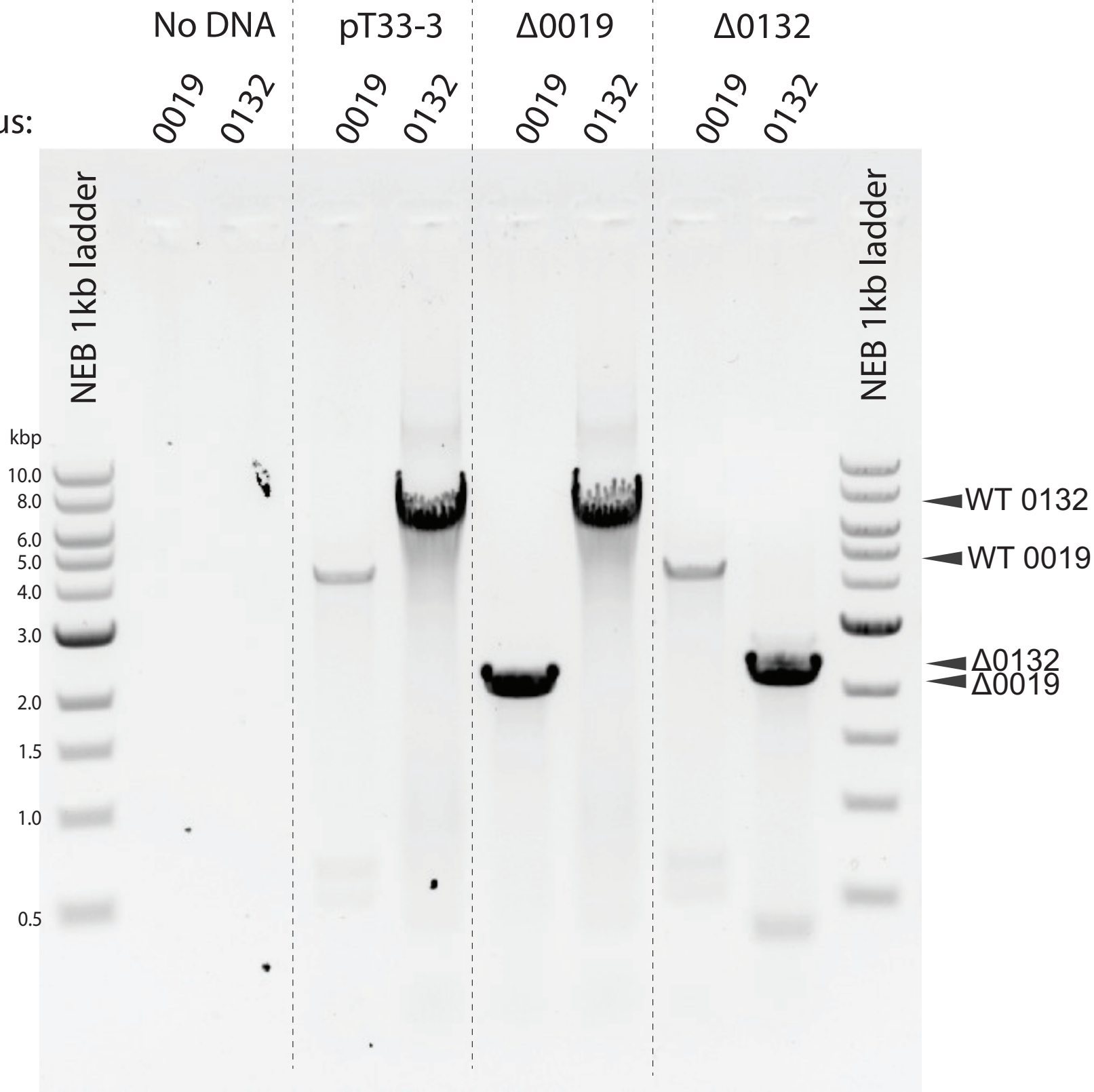
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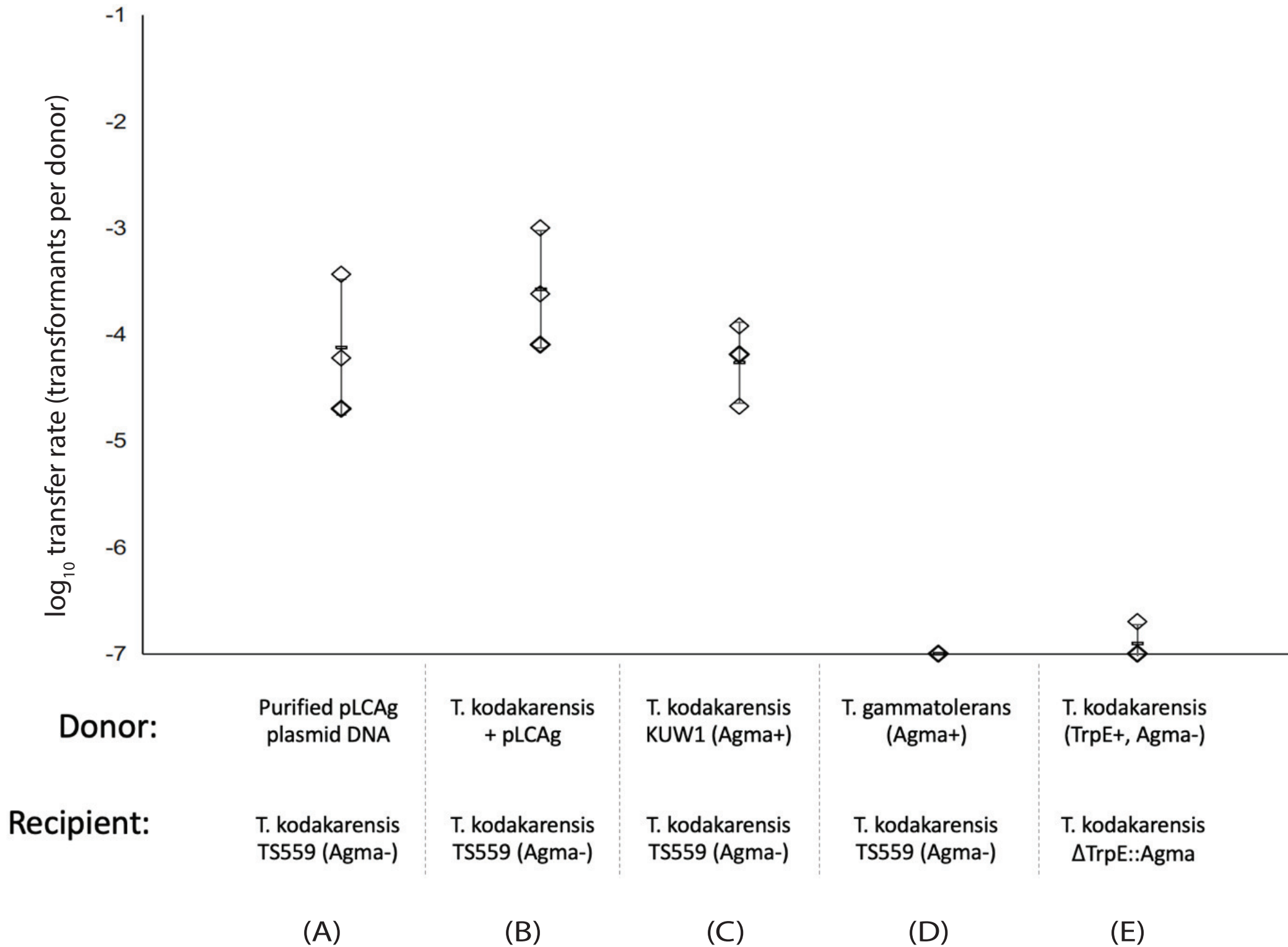


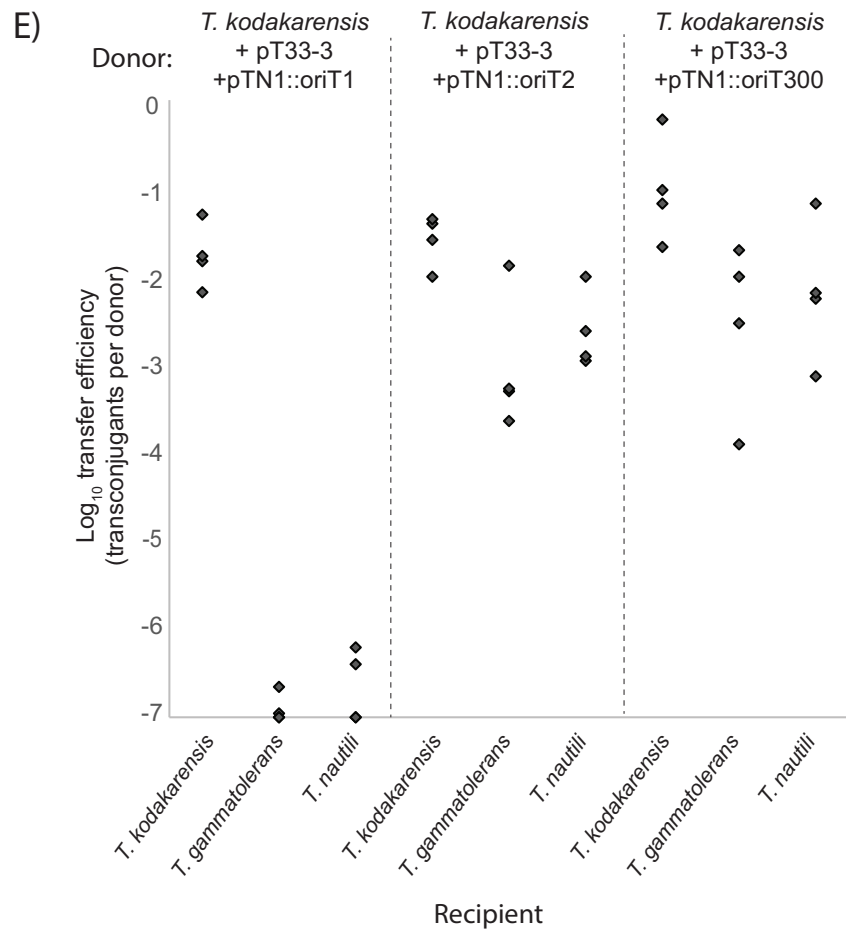
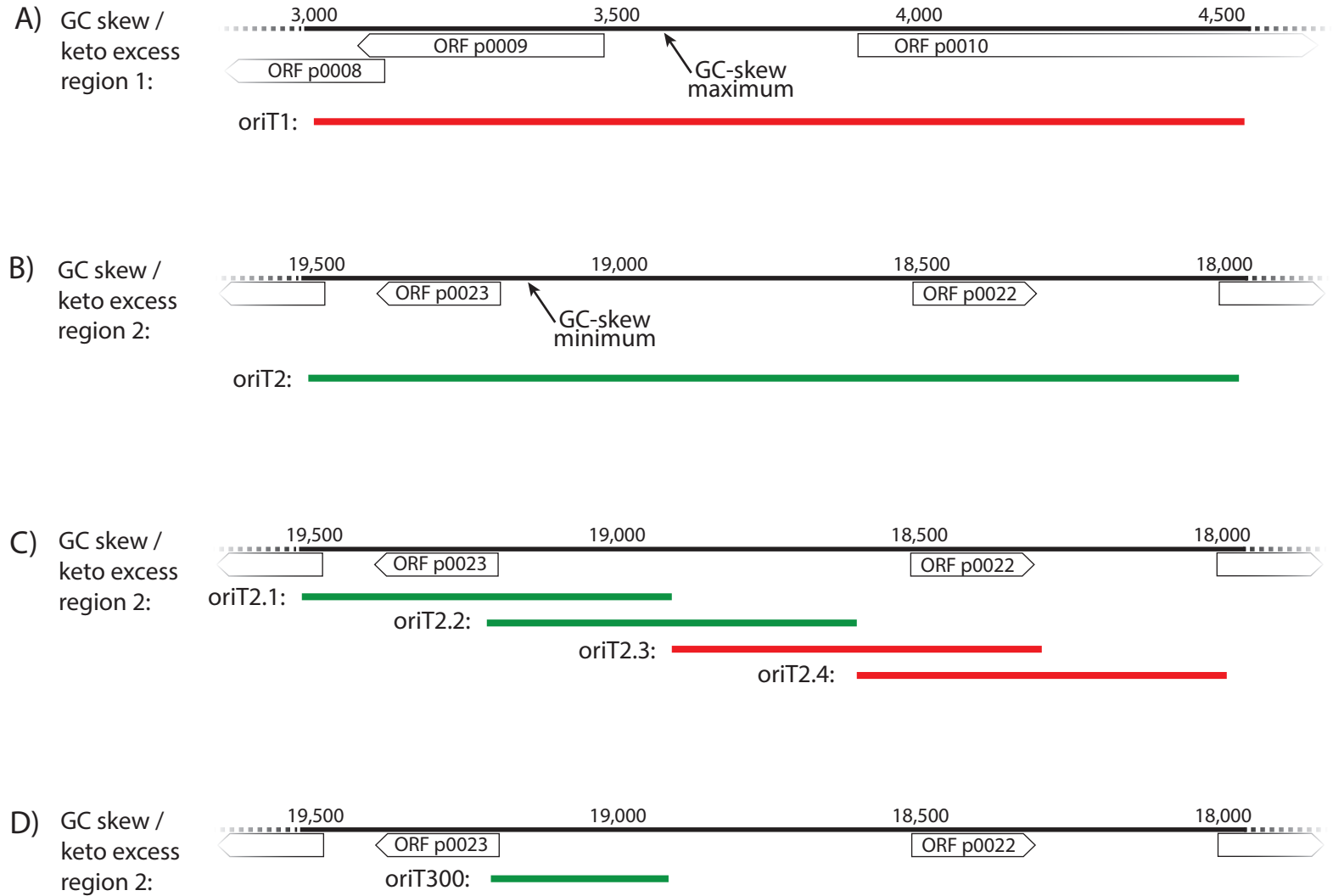


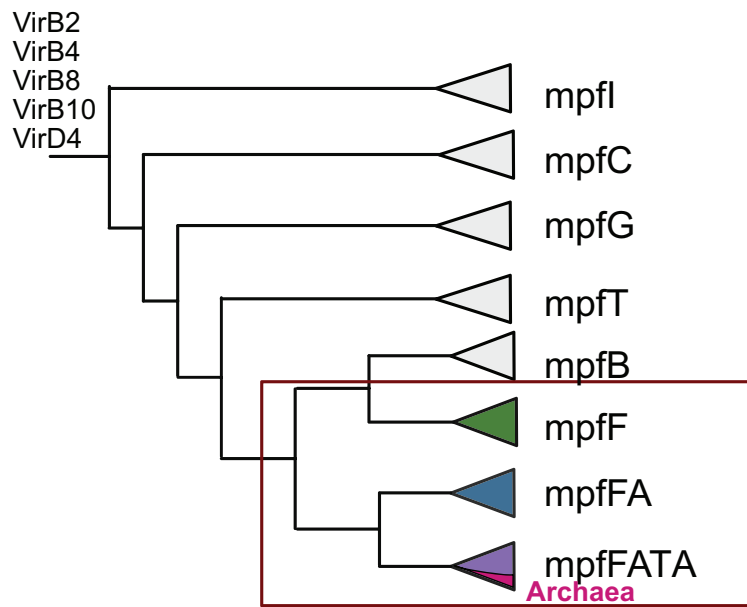
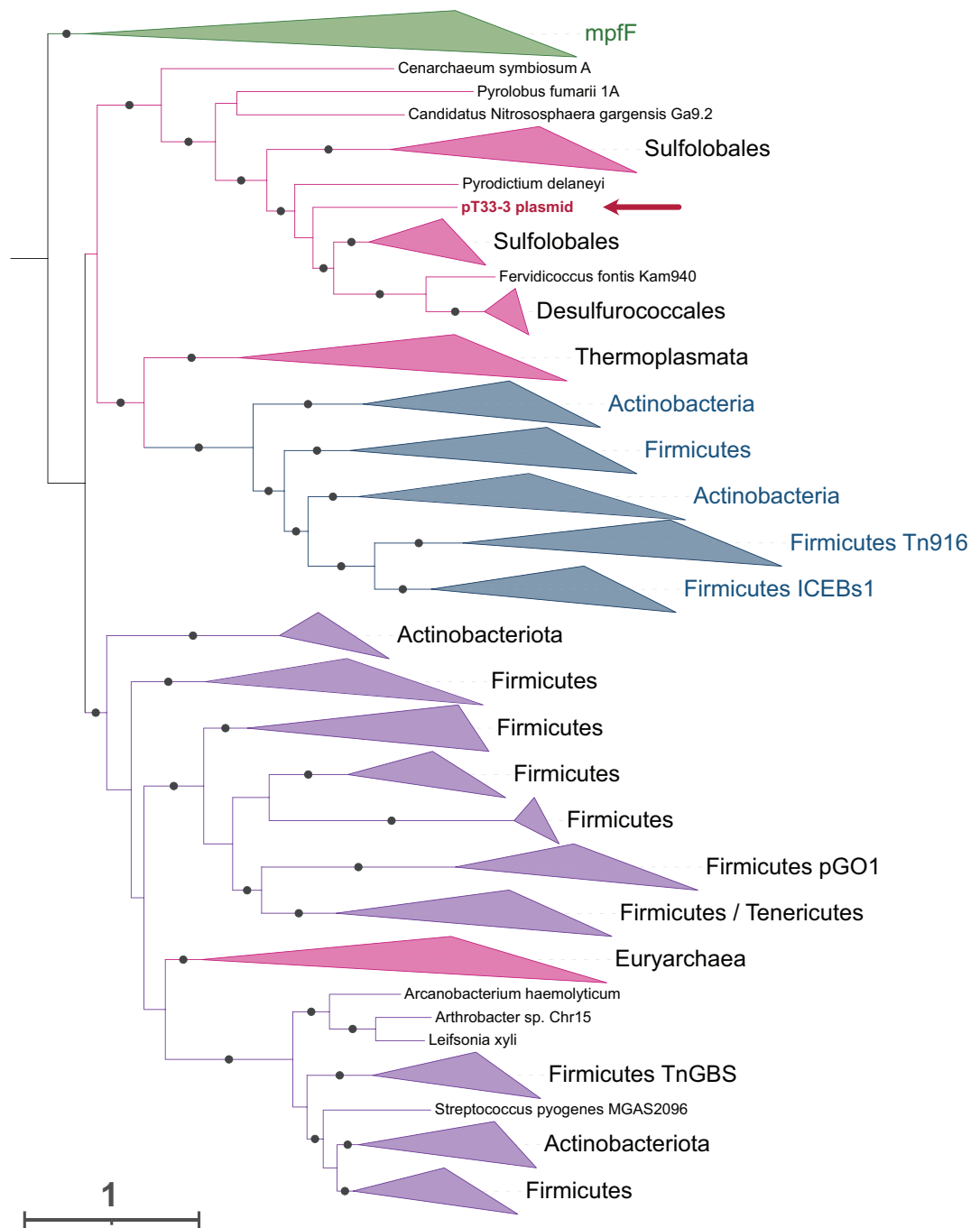


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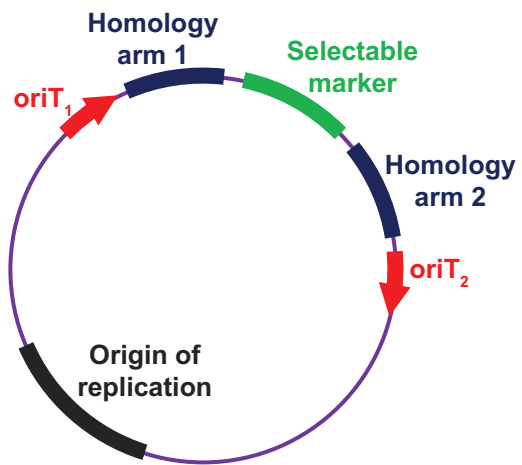




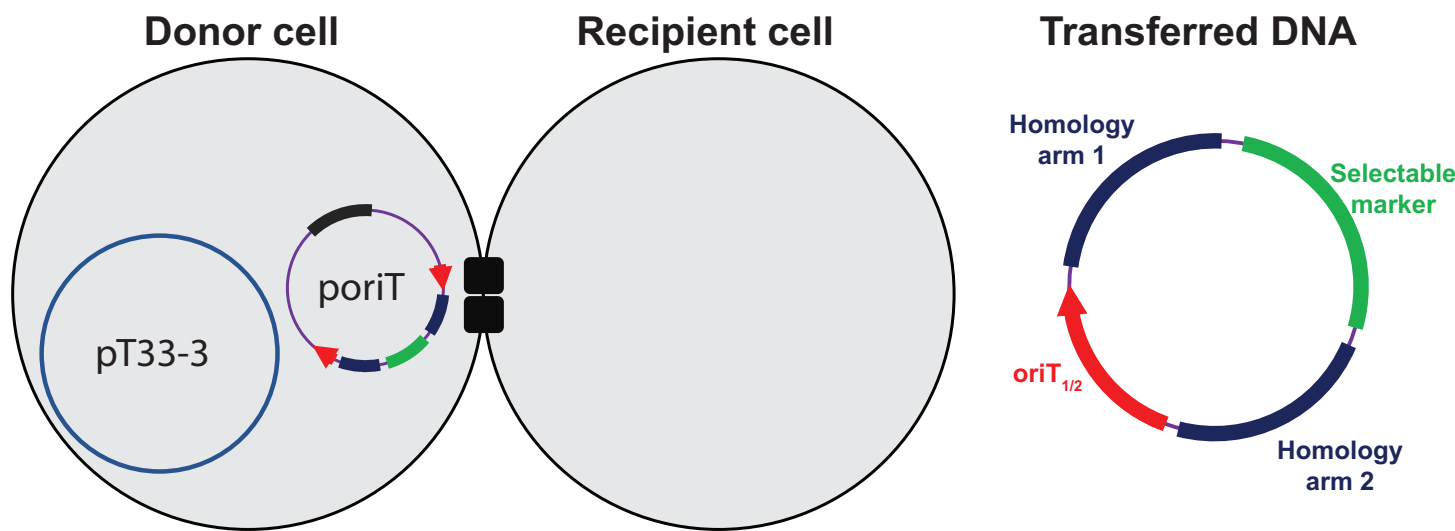


A**B**

A)



B)



C)

