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Supplementary Information for

Increased copy number couples the evolution of plasmid horizontal transmission and plasmid-encoded antibiotic resistance.

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Dataset S1

Supplementary Information Text

Evidence for contamination during the evolution experiment

The evolution experiment was carried out using 96-well culture plates, which is associated with a higher risk for cross-contamination. We detected evidence of some contamination, based on molecular markers of ancestral / immigrant strains, and on whole-genome sequencing results, both described below:

- The experiment was designed with initial plasmid hosts being "blue" (no red fluorescence, and presence of the active lac operon, leading to blue colonies when plating on XGal+IPTG), early immigrants being red, and late immigrants being "white" (lac deletion leading to white colonies when plating on XGal+IPTG). The 'no immigration' or 0% treatment was founded with only initial plasmid hosts, thus should have contained only blue cells. However, from passage 16, all 6 mut lineages contained some red clones, with 3 of 6 lineages having only red clones. This might be due to cross-contamination between wells, or (most likely in our opinion) a pipetting mistake that introduced once some red immigrants to all 6 wells.

- Plasmid-free controls were run in parallel to the plasmid evolution treatments, on the same 96-well plate. One out of twelve sequenced "plasmid-free" hosts resulted in plasmid reads, mapping to R1 but with the loss of the whole antibiotic resistance region (shown in Figure S5), a definitive evidence for cross-well contamination.

Genomic analysis and variants of interest

- Manual inspection of plasmid mutation data:

Breseq was run in polymorphism mode to account for the presence of high copy number variants, and cases in which mutations were not present on all plasmid copies. In order to exclude sequencing errors, we then excluded mutations that were present in <20% frequency in all samples. Two other types of variants were excluded:

i) The mutation 58157 C>T was present in all samples, including the ancestral plasmids, and represents a divergence from the published sequence.

ii) We observed a cluster of complex mutations positions 87616-87664, within the coding sequence of *traD*, in the ancestor and many (but not all) of the evolved plasmid sequences. They were all insertions or deletions of 9 nucleotides, leading to amino-acid changes but no frameshift or stop codon. A similar 9bp insertion is also found in AY684127 accession of R1 *traD* sequence, suggesting it is not specific to this evolution experiment. Thus, all variants at these positions were excluded from the analysis.

Large deletions were also added manually, after analysis of coverage plots. Four large deletions were detected on evolved R1 plasmids:

Clone	Genome coordinates of deletions
W90b	18390 to 40664
W90e	6200 to 17100
	22124 to 40664
M90b	6200 to 15335
M90c	18289 to 40664

In addition, coverage plots (Figure S6) show changes in coverage that suggest coexistence of plasmids with full and deleted genomes, with changes in coverage corresponding to the location of insertion sequences on R1 genome.

- *Variants of interest:*

Mutations suggesting parallel evolution are described in the main text. In addition, here we present more detailed information on other variants that might have phenotypic effects on R1 transfer rate.

Three mutations were detected in or close to the *traJ* gene. Clones m99a and w90f each had a point mutation respectively 49bp and 30bp before the start of the coding sequence; clone w97a had a point mutation within the coding sequence, leading to a change in protein sequence (K42E).

The clone mut68d carried a point mutation from A to G at position 83395, within the coding sequence of *traG*, leading to an amino-acid change, Q663R in protein TraG. TraG is involved in mating pair stabilisation, but also entry exclusion (1): when a plasmid is present in the recipient cell, it expresses TraS entry exclusion protein at its surface, and TraG -TraS interaction decreases transfer rate by 1000-fold. In F plasmid, the specific recognition of TraS was shown to be due to the region between aa 610 and 673, and plasmid R100 TraG, which differs from F TraG only in this same region, does not cause entry exclusion from F (1). This suggests that mutation Q663R might allow the evolved mut68d variant to avoid entry exclusion from the wildtype R1 plasmid, similarly to what has been observed with a variant of R1 previously (2).

Finally, we sequenced an additional clone, m68e-t12. We first noticed that R1 plasmid from clone m68e isolated after 19 days of evolution has high transfer rate when present in the ancestor *E. coli* host but not when in its coevolved clone (Figure 1D), suggesting that the coevolved host repressed transfer. Thus, we analysed a clone from the same lineage but isolated earlier, hoping that we might obtain the same plasmid variant but different regulation by the host. Instead, m68e-t12 plasmid carries only one single mutation within the *finO* gene, similar to the ones described in the main text for other evolved variants. We used this *finO* variant in phenotypic and fitness assays as a example of derepression of transfer, as midpoint evolved *finO* clones (Figure 4A) all carry other mutations as well.

Evolution of plasmid conjugation rates: detailed analysis and statistical tests

We examined the effect of two factors in the evolution of plasmid conjugation rates: immigration treatment, and host background (wt or mut). As seen below, immigration treatment had the strongest effect on evolved conjugation rates, but the interaction between immigration treatment and host background was significant, mut hosts being associated to the evolution of higher transfer rates particularly for high immigration treatments. Evolution in high immigration treatments might be the most dependent on high mutation rates because plasmid-bearing lineages will be subject to strong bottlenecks limiting genetic diversity.
We also present below the results of models including only immigration treatment, from which the statistical values presented in the main text are taken.

- Conjugation from coevolved donor hosts:

Model: lm($\log_{10}$ transfer rate~strain*treatment)

```
                  Df Sum Sq Mean Sq F value   Pr(>F)
strain             1   0.64   0.643   2.537 0.112831
treatment          5  59.55  11.911  47.013  < 2e-16 ***
strain:treatment   5   5.47   1.094   4.319 0.000955 ***
Residuals        192  48.64   0.253
```

Coefficients:

```
                          Estimate Std. Error t value Pr(>|t|)
(Intercept)               -10.64945    0.14530 -73.292  < 2e-16 ***
Strain mut                 -0.07987    0.20549  -0.389 0.697935
treatment 99%               0.61097    0.18758   3.257 0.001331 **
treatment 97%               0.73612    0.18758   3.924 0.000121 ***
treatment 90%               1.33969    0.18758   7.142 1.85e-11 ***
treatment 68%               0.11721    0.18758   0.625 0.532802
treatment 0%               -0.35686    0.18758  -1.902 0.058616 .
strainmut:treatment 99%     0.60526    0.26528   2.282 0.023612 *
strainmut:treatment 97%     0.47830    0.26528   1.803 0.072960 .
strainmut:treatment 90%    -0.38143    0.26528  -1.438 0.152113
strainmut:treatment 68%     0.22230    0.26528   0.838 0.403088
strainmut:treatment  0%     0.16436    0.26528   0.620 0.536269
```

Model: lm($\log_{10}$ transfer rate~treatment)

```
             Df Sum Sq Mean Sq F value Pr(>F)
treatment     5  59.55  11.911   43.07 <2e-16 ***
Residuals   198  54.76   0.277
```

Coefficients:

```
              Estimate Std. Error t value Pr(>|t|)
(Intercept)    -10.6894     0.1073 -99.581  < 2e-16 ***
Treatment 99%    0.9136     0.1386   6.593 3.84e-10 ***
Treatment 97%    0.9753     0.1386   7.038 3.13e-11 ***
Treatment 90%    1.1490     0.1386   8.291 1.69e-14 ***
Treatment 68%    0.2284     0.1386   1.648   0.1010
Treatment 0%    -0.2747     0.1386  -1.982   0.0489 *
```

- Conjugation of evolved plasmids from unevolved standard host:

Model: lm($\log_{10}$ transfer rate~strain*treatment)

```
                  Df Sum Sq Mean Sq F value   Pr(>F)
strain             1  11.75  11.747  23.964 1.94e-06 ***
treatment          5  56.85  11.371  23.197  < 2e-16 ***
strain:treatment   5  12.66   2.533   5.167  0.00017 ***
Residuals        212 103.92   0.490
```

Coefficients:

```
                          Estimate Std. Error t value Pr(>|t|)
```

```
(Intercept)                -11.42673    0.18077 -63.211  < 2e-16 ***
strainmut                   -0.04456    0.25565  -0.174  0.86179
treatment 99%                0.27577    0.23669   1.165  0.24527
treatment 97%                0.79269    0.24182   3.278  0.00122 **
treatment 90%                1.34303    0.24182   5.554 8.32e-08 ***
treatment 68%                0.47300    0.24477   1.932  0.05464 .
treatment 0%                -0.05751    0.24477  -0.235  0.81448
strainmut:treatment 99%      1.24338    0.33473   3.715  0.00026 ***
strainmut:treatment 97%      0.63664    0.34408   1.850  0.06566 .
strainmut:treatment 90%     -0.01676    0.34408  -0.049  0.96119
strainmut:treatment 68%      0.80598    0.33617   2.398  0.01737 *
strainmut:treatment 0%       0.10797    0.34615   0.312  0.75542
```

Model: lm($\log_{10}$ transfer rate~treatment)

```
             Df Sum Sq Mean Sq F value   Pr(>F)
treatment     5  56.88  11.377   19.33 6.49e-16 ***
Residuals   218 128.30   0.589
```

Coefficients:

```
               Estimate Std. Error t value Pr(>|t|)
(Intercept)    -11.449013   0.140062 -81.742  < 2e-16 ***
Treatment 99%    0.897462   0.183384   4.894 1.92e-06 ***
Treatment 97%    1.103006   0.188477   5.852 1.76e-08 ***
Treatment 90%    1.335483   0.188477   7.086 1.89e-11 ***
Treatment 68%    0.930379   0.183384   5.073 8.35e-07 ***
Treatment 0%    -0.003522   0.189645  -0.019    0.985
```

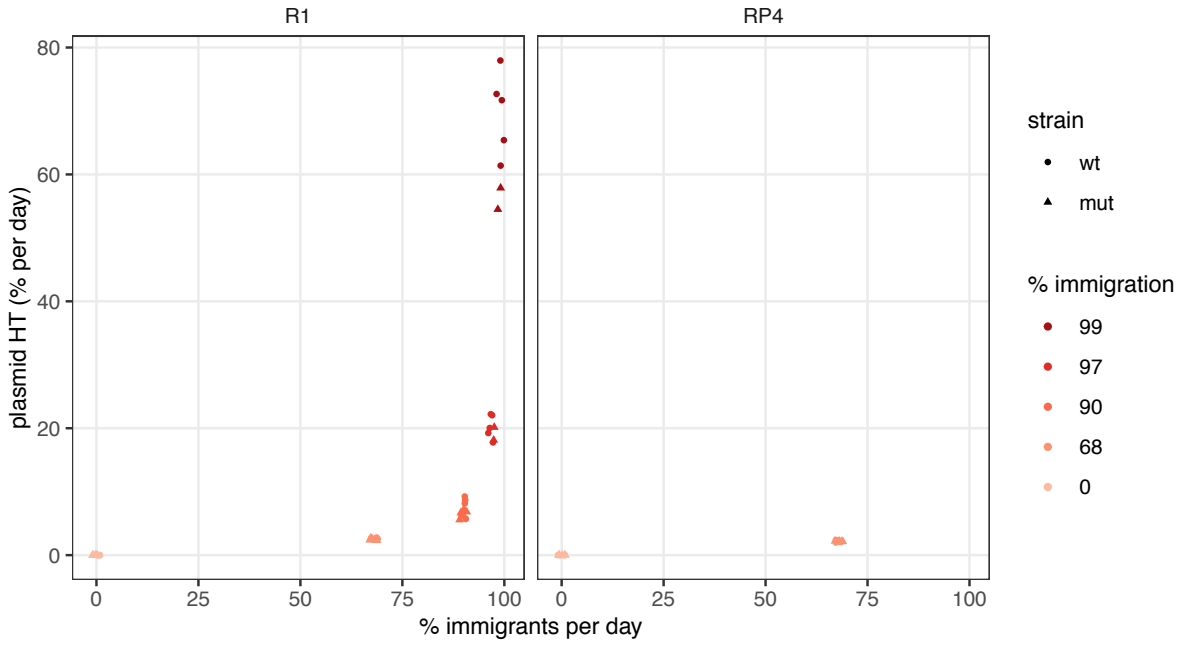

Figure S1: Average horizontal transmission per day. Theoretical plasmid-bearing cell proportions with only vertical transmission, $pred_{19}$, were calculated after 19 days assuming equal fitness for all hosts ($pred_{19} = (1 - \%immigration)^{19}$). Plasmid spread by horizontal transmission (HT) was defined as the average daily increase in plasmid-bearing cell proportion necessary to explain observed plasmid-bearing cell proportions at day 19 ($obs_{19} = (1 - \%immigration + HT)^{19}$). Thus, HT per day is calculated as HT = $\left(1 - \% \frac{immigration}{100}\right) * (1 + \sqrt[19]{\frac{obs_{19}}{pred_{19}}})$.

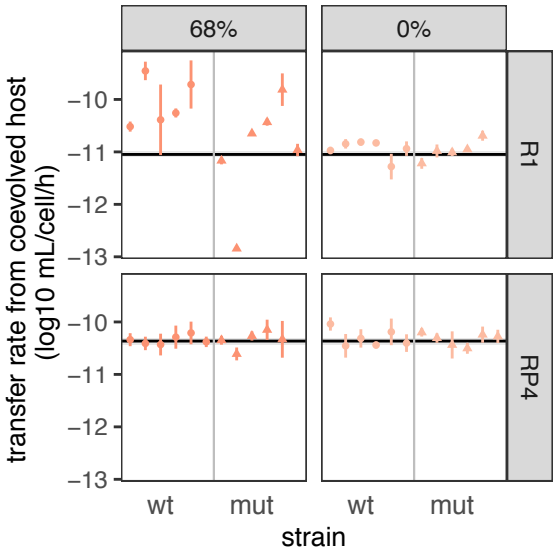

Figure S2: Endpoint (29 days) plasmid conjugation rates. The black line and shaded area are respectively the geometric mean and standard error (SE) of ancestral plasmid transfer rate; each coloured dot and line indicates respectively the geometric mean and SE of evolved clones (N=3).

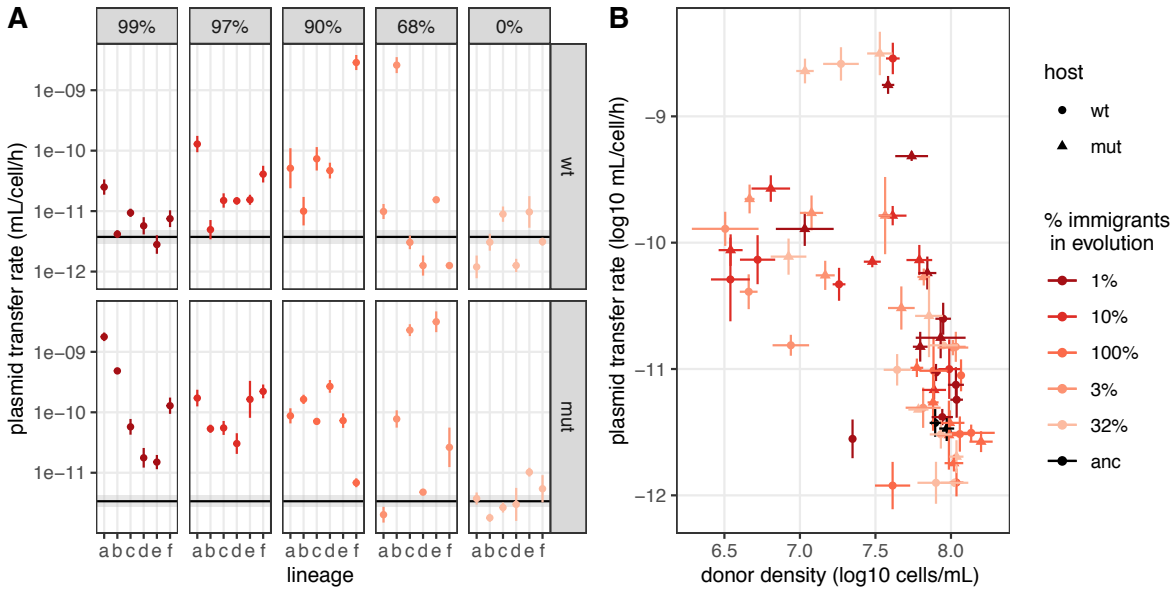

Figure S3: Midpoint R1 plasmid specific conjugation rates (A) and associated trade-off with donor density (B). Plasmid transfer rates were measured from plasmid-bearing ancestral hosts towards the standard recipient strain MG1655 Nal^R. Dots are geometric averages and lines indicate geometric standard errors (N=3). In A, the black line and shaded area are respectively the geometric average and standard error of ancestral plasmid transfer rate. In B, transfer rates measured from ancestral hosts are shown as a function of donor host cell density at the end of the conjugation assay. Statistics are described in Supplementary Text.

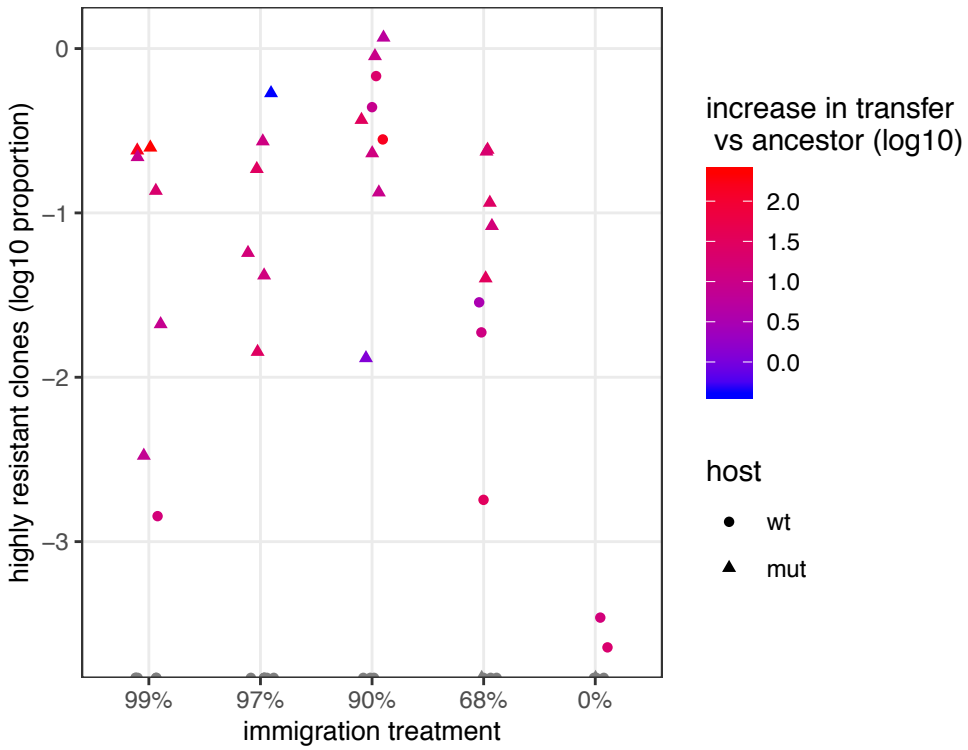

Figure S4: Proportion of highly resistant plasmids after selection for transfer. The proportion of plasmid-bearing cells able to grow in the presence of 0.5g/L Amp is shown as a function of immigration treatment in 9 day evolved populations. For each population, a clone was picked randomly, and its transfer rate was measured (N=2). Dot colour indicates the ration of evolved transfer rate to ancestral R1 transfer rate (log_{10}).

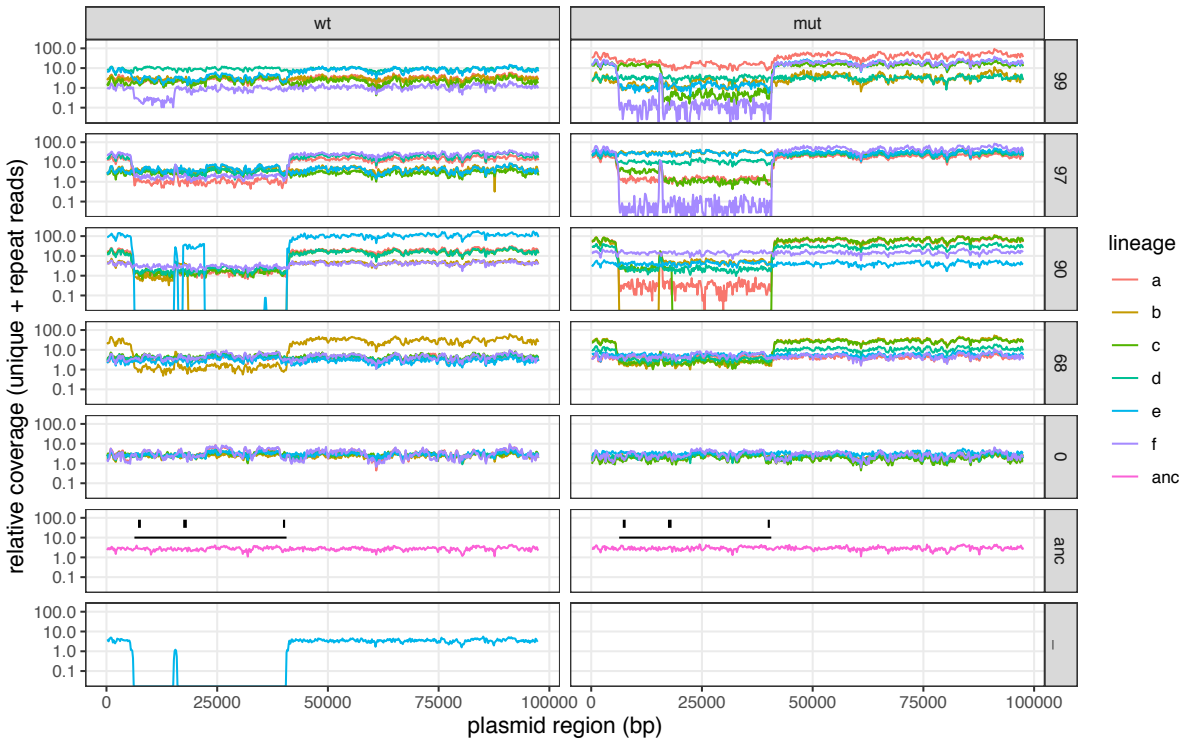

Figure S5: Variation in sequencing coverage across R1 plasmid map. Relative coverage of sequencing reads is shown for all clones across $R1_{wt}$ sequence map. Relative coverage was measured as the sum of coverage of both unique and repeat reads, divided by the overall average coverage of reads mapped to the chromosome. Black rectangles above the ancestor graph indicate the position of genes conferring antibiotic resistance (from left to right: kanamycin, ampicillin and chloramphenicol), and the black line indicates the whole resistance determinant region (bordered by insertion sequences). In the plasmid-free control treatment (bottom), reads mapping to the plasmid were detected for lineage w_e, indicating contamination by a plasmid missing the whole resistance determinant. All other clones retained at least the *bla* gene conferring Amp resistance, as they were obtained by plating on Amp-containing medium.

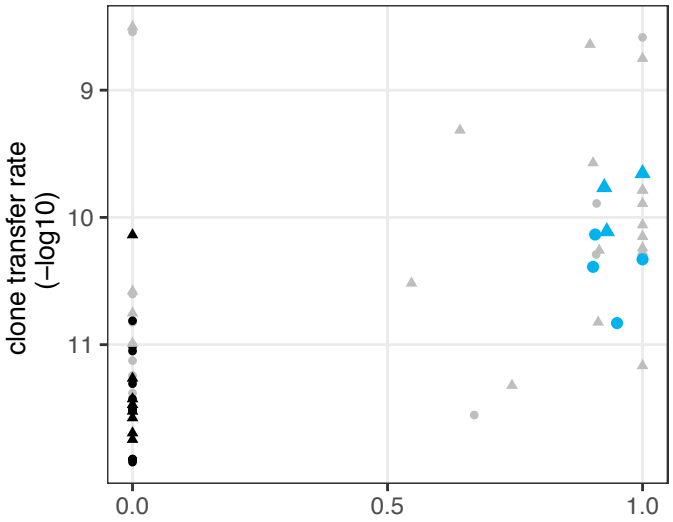

Figure S6: Effect of *copA variants on evolved plasmid transfer rate from the ancestral host.** Evolved rate is shown as a function of *copA** mutation frequency in the evolved clone. Black dots indicate clones for which no mutation was detected on the plasmid; blue dots clones for which no other mutation than *copA** was present on the plasmid.

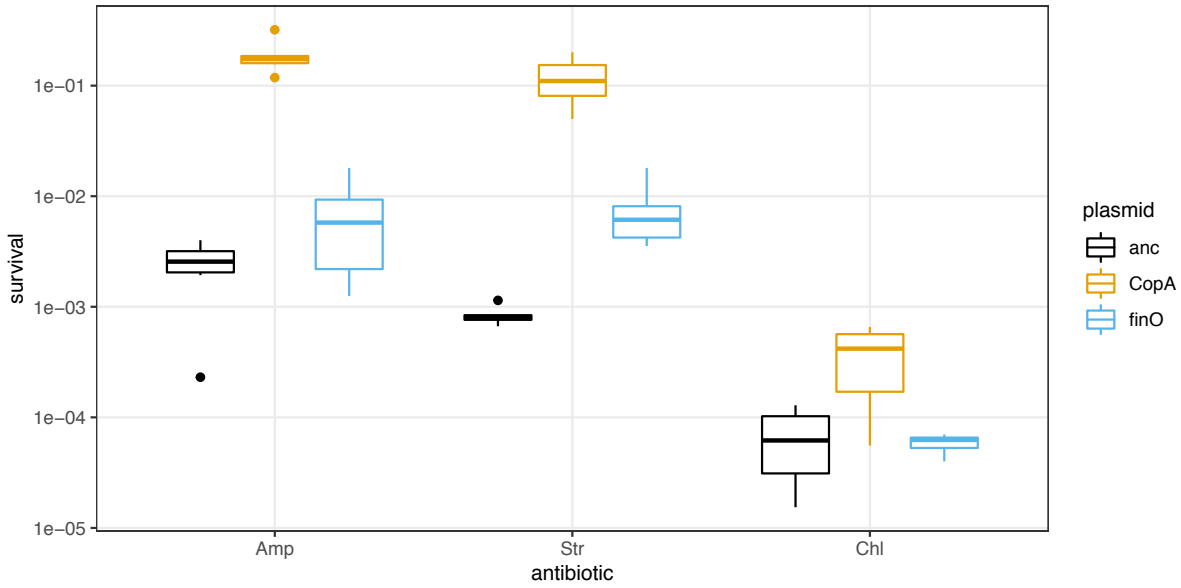

Figure S7: Effect of antibiotics on cells carrying R1 variants. Survival in the presence of high doses of antibiotics to which R1 carries resistance determinants. Antibiotics tested were ampicillin 500 mg/L (Amp), streptomycin 200 mg/L (Str) and chloramphenicol 600 mg/L (Chl); R1 carries resistance determinants to the three last antibiotics. Dots indicate individual populations; the centre value of the boxplots is the median and boxes denote the interquartile range (*N=8*).

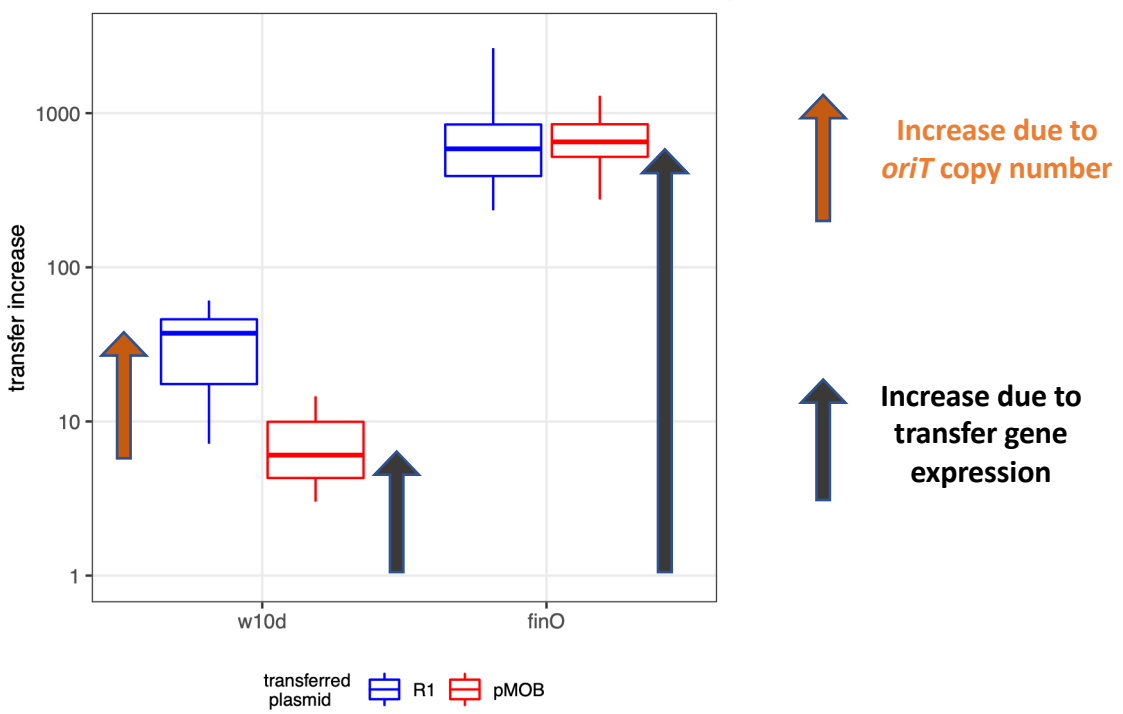

Figure S8: Impact of evolved *copA variants on plasmid mobilisation *in trans*.** R1 transfer was measured together with mobilisation of pMOB carrying R1 *oriT*. Increased transfer operon expression will increase transfer of both plasmids, whereas increased *oriT* copy number will only increase R1 transfer. The increase in transfer compared to $R1_{wt}$ is shown for each R1 variant and associated pMOB plasmid. The centre value of the boxplots shows the median, boxes the first and third quartile, and whiskers represent 1.5 times the interquartile range (*N=8*).

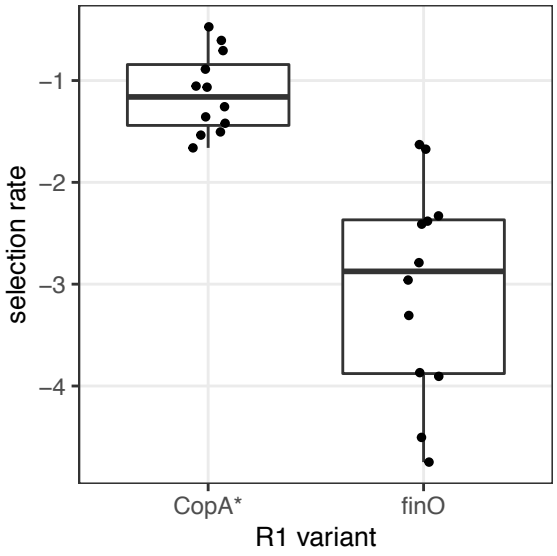

Figure S9: selection rate of CopA* and finO variants in competition against R1wt, in the absence of plasmid-free immigrants. The centre value of the boxplots shows the median, boxes show the first and third quartile, and whiskers represent 1.5 times the interquartile range; individual data points are shown as dots.

Table S1: Detail of characterized R1 clones. Midpoint clones were isolated between passage 16 and passage 19: one Amp resistant clone per lineage was chosen randomly for each lineage. When possible, clones from passage 19 were isolated but in lineages where plasmid-bearing cells were already extinct at passage 19, one clone was isolated from populations from passages 17 or 16. Two additional clones from the m68e lineage were isolated from different timepoints and sequenced; the m68e_t12 clone was found to carry only one mutation in *finO* (see Dataset S1).

clone name	background	plasmid	immigration	lineage	day clone isolation	day last detected	marker
wt_R1	mut	R1	ancestor	ancestor			blue (lac+)
mut_R1	mut	R1	ancestor	ancestor			blue (lac+)
MGred	wt	/	ancestor	ancestor			red (td-Cherry)
MGΔlac	wt	/	ancestor	ancestor			white (Δlac)
m68e_t12	mut	R1	68%	e	12		red
m68e_t29	mut	R1	68%	e	29		white
w99_a	wt	R1	99%	a	19	19	white
w99_b	wt	R1	99%	b	19	19	white
w99_c	wt	R1	99%	c	19	19	white
w99_d	wt	R1	99%	d	19	19	white
w99_e	wt	R1	99%	e	17	17	white
w99_f	wt	R1	99%	f	19	25	white
m99_a	mut	R1	99%	a	19	19	white
m99_b	mut	R1	99%	b	17	17	white
m99_c	mut	R1	99%	c	16	16	red
m99_d	mut	R1	99%	d	17	17	white
m99_e	mut	R1	99%	e	19	19	white
m99_f	mut	R1	99%	f	16	16	red
w97_a	wt	R1	97%	a	19	19	white
w97_b	wt	R1	97%	b	17	17	white
w97_c	wt	R1	97%	c	19	25	white
w97_d	wt	R1	97%	d	19	19	white
w97_e	wt	R1	97%	e	19	19	white
w97_f	wt	R1	97%	f	19	19	white
m97_a	mut	R1	97%	a	19	19	white
m97_b	mut	R1	97%	b	19	19	red
m97_c	mut	R1	97%	c	17	17	white
m97_d	mut	R1	97%	d	16	16	red
m97_e	mut	R1	97%	e	17	17	white

m97_f	mut	R1	97%	f	17	17	white
w90_a	wt	R1	90%	a	19	25	white
w90_b	wt	R1	90%	b	19	29	white
w90_c	wt	R1	90%	c	19	19	red
w90_d	wt	R1	90%	d	19	19	white
w90_e	wt	R1	90%	e	19	25	white
w90_f	wt	R1	90%	f	19	29	red
m90_a	mut	R1	90%	a	17	17	white
m90_b	mut	R1	90%	b	19	19	white
m90_c	mut	R1	90%	c	19	19	white
m90_d	mut	R1	90%	d	19	19	red
m90_e	mut	R1	90%	e	19	25	red
m90_f	mut	R1	90%	f	19	19	red
w68_a	wt	R1	68%	a	19	29	blue
w68_b	wt	R1	68%	b	19	29	red
w68_c	wt	R1	68%	c	19	29	blue
w68_d	wt	R1	68%	d	19	29	red
w68_e	wt	R1	68%	e	19	29	blue
w68_f	wt	R1	68%	f	19	29	red
m68_a	mut	R1	68%	a	19	29	red
m68_b	mut	R1	68%	b	19	29	red
m68_c	mut	R1	68%	c	19	29	red
m68_d	mut	R1	68%	d	19	29	red
m68_e	mut	R1	68%	e	19	29	red
m68_f	mut	R1	68%	f	19	29	red
w0_a	wt	R1	0%	a	19	29	blue
w0_b	wt	R1	0%	b	19	29	blue
w0_c	wt	R1	0%	c	19	29	blue
w0_d	wt	R1	0%	d	19	29	blue
w0_e	wt	R1	0%	e	19	29	blue
w0_f	wt	R1	0%	f	19	29	blue
m0_a	mut	R1	0%	a	19	29	red
m0_b	mut	R1	0%	b	19	29	blue
m0_c	mut	R1	0%	c	19	29	red
m0_d	mut	R1	0%	d	19	29	blue
m0_e	mut	R1	0%	e	19	29	red
m0_f	mut	R1	0%	f	19	29	blue

Dataset S1: Sequence variants detected in evolved clones. A table was generated with breseq option gdtools COMPARE, then manually cleaned. For the plasmid, two types of mutations were discarded. One was a point mutation 58157 C>T, present in the ancestor and all evolved plasmids. The second was a cluster of complex mutations in positions 87616-87664, within *traD* CDS (see details in Supplementary Text). For the chromosome, variants with less than 100% allele frequency were discarded.

SI References

1. Audette GF, Manchak J, Beatty P, Klimke WA, Frost LS. Entry exclusion in F-like plasmids requires intact TraG in the donor that recognizes its cognate TraS in the recipient. Microbiology. 2007;153(2):442–51.
2. Smith J. Superinfection drives virulence evolution in experimental populations of bacteria and plasmids. Evolution. 2011;65(3):831–41.