

**This is a self-archived version of an original article. This version may differ from the original in pagination and typographic details.**

**Author(s):** Mattila, Sari; Ruotsalainen, Pilvi; Ojala, Ville; Tuononen, Timo; Hiltunen, Teppo; Jalasvuori, Matti

**Title:** Conjugative ESBL plasmids differ in their potential to rescue susceptible bacteria via horizontal gene transfer in lethal antibiotic concentrations

**Year:** 2017

**Version:** Accepted version (Final draft)

**Copyright:** © 2017, Japan Antibiotics Research Association

**Rights:** In Copyright

**Rights url:** <http://rightsstatements.org/page/InC/1.0/?language=en>

**Please cite the original version:**

Mattila, S., Ruotsalainen, P., Ojala, V., Tuononen, T., Hiltunen, T., & Jalasvuori, M. (2017). Conjugative ESBL plasmids differ in their potential to rescue susceptible bacteria via horizontal gene transfer in lethal antibiotic concentrations. *Journal of Antibiotics*, 70, 805-808.  
<https://doi.org/10.1038/ja.2017.41>

1 **Conjugative ESBL-plasmids differ in their potential to rescue**  
2 **susceptible bacteria via horizontal gene transfer in lethal antibiotic**  
3 **concentrations**

4  
5 **Running title: Evolutionary rescue of bacteria via conjugation**

6  
7 **Sari Mattila<sup>1</sup>, Pilvi Ruotsalainen<sup>1</sup>, Ville Ojala<sup>1</sup>, Timo Tuononen<sup>1</sup>, Teppo Hiltunen<sup>2</sup> and Matti**  
8 **Jalasvuori<sup>1\*</sup>**

9  
10  
11 <sup>1</sup>University of Jyväskylä, Centre of Excellence in Biological Interactions, Nanoscience Center,  
12 Department of Biological and Environmental Science, Jyväskylä, Finland

13 <sup>2</sup>Department of Food and Environmental Sciences / Microbiology and Biotechnology, P.O. Box 56,  
14 00014 University of Helsinki, Finland

15  
16 \*Correspondence:

17 Matti Jalasvuori

18 Ambiotica building, Survontie 9 C

19 P.O.Box 35, 40014 University of Jyväskylä

20 Jyväskylä, Finland

21 Phone: +358504135092

22 Email: matti.jalasvuori@jyu.fi

23

24 Keywords: ESBL / conjugative plasmids / antibiotic resistance / beta-lactams / evolutionary rescue /  
25 horizontal gene transfer

## 26 **Description**

27 Emergence (and proliferation) of resistant pathogens under strong antibiotic selection is an  
28 evolutionary process where bacteria overcome the otherwise growth inhibiting or lethal  
29 concentration of antimicrobial substances. In this study, we set to investigate a largely unexplored  
30 mechanism, namely evolutionary rescue (i.e. adaptive evolutionary change that restores positive  
31 growth to declining population and prevents extinction) via horizontal gene transfer, by which new  
32 resistant bacteria may emerge both in and out of clinical environments.

33 At sufficiently high concentrations, the likelihood of pre-existing single-step resistant mutants  
34 to exist in the population is very low, hence therapies aim to maintain the drug-levels above this so-  
35 called “mutation selection window” (MSW)<sup>1</sup>. However, when we take a look at the multi-resistant  
36 bacteria in hospital settings, they often carry mobile genetic elements such as conjugative  
37 plasmids<sup>2</sup>. These elements contain genes that encode molecular machineries for mediating the  
38 transfer of the mobile element from one bacterium to another allowing the spread of antibiotic  
39 resistance. Often, it is the mobile elements that carry resistance genes and thus provide pathogens  
40 with their phenotype. Clearly, the evolution of resistance is not just a matter of mutations but also,  
41 and even more so, it is about the lateral movement of selfish genetic replicators among (even  
42 distantly) related bacterial cells<sup>3</sup>. This notion served as an incentive for our study: even if it is  
43 reasonable to maintain antibiotic-level above the MSW in order to avoid the emergence of resistant  
44 mutants, the presence of other - even harmless - bacteria that carry mobile resistance conferring  
45 elements may nevertheless compromise the outcome of antibiotic treatments. In other words, it is  
46 possible that resistance element gets transferred horizontally to the susceptible bacteria during  
47 antibiotic therapy, thus generating novel resistant pathogens.

48 Studies have shown that beta-lactamase producing bacteria allow other susceptible (non  
49 producer) individuals to co-exist with them as “cheaters”<sup>4</sup>. That is, non-resistant bacteria take the  
50 benefit of the “altruistic” nature of the resistance mechanism as beta-lactamases reduce the  
51 concentration of the antibiotic for everyone in their immediate vicinity. Indeed, it was shown that a  
52 conjugative resistance plasmid can get transferred to the “cheaters” even when the “altruistic”  
53 plasmid-harboring bacteria were added only afterwards to the high-antibiotic environment<sup>5</sup>. Due to  
54 the transfer, cheaters became genuinely resistant entities. Extrapolating this result to clinical context  
55 would hint that the effectiveness of antibiotic treatment might depend on the bacterial community to  
56 which the patient is exposed during treatment.

57 We selected sixteen ESBL *E. coli* -strains isolated from patients from the University Hospital of  
58 Turku, Finland, and transferred the resistance plasmids from these strains to a second bacterium (*E.*  
59 *coli* K-12 HMS174) then to third strain (*E. coli* K-12 JM109(pSU18)) and finally back to HMS174.  
60 We isolated total plasmid-DNA from HMS174 strains after the third transfer and selected five  
61 strains with differing DNA-profiles for detailed analysis (Supplementary Figure 1). All the plasmids  
62 were sequenced, resulting in total of ten plasmid sequences originating from five ESBL *E. coli*  
63 strains. The plasmids were named pEC3I, pEC3II, pEC13I, pEC14I, pEC14II, pEC14III, pEC15I,  
64 pEC15II, pEC16I and pEC16II, where ECx is the name of the original host strain<sup>6</sup> and the Roman  
65 numeral is the number of the plasmid. Three of the strains (EC3, EC15, EC16) carried two  
66 mobilizable plasmids whereas EC14 had three plasmids and EC13 had only one. Hereafter, pEC14,  
67 for example, describes all the different plasmids originally derived from the strain EC14.

68 General features of the isolated plasmids are listed in Table 1. All the sequences are available on  
69 GenBank (accession numbers KU932021-KU932034). The incompatibility types of the isolated  
70 plasmids (including IncI, IncF, IncX) resemble those of ESBL-plasmids in bacteria causing  
71 nosocomial infections<sup>7,8</sup>. The beta-lactamase genes similarly provided a good coverage of the most  
72 common types (TEM, CTX-M, SHV<sup>9</sup>). One of the plasmids, pEC14I, contains a Class 1 integron

73 residing next to several resistance genes, suggesting that this plasmid carries a DNA-integrating  
74 resistance island. Indeed, mobile genetic islands such as these are common among multi-drug  
75 resistant bacteria. Also, sequence assembly of four of the plasmids revealed variable sequence  
76 within the original DNA sample, resulting in two versions of the plasmid sequences (named  
77 pEC3II\_1 and pEC3II\_2, pEC14II\_1 and pEC14II\_2, pEC15I\_1 and pEC15I\_2 and pEC16I\_1 and  
78 pEC16I\_2). In all cases, this sequence variability was observed in IncI plasmid shufflon area<sup>10</sup>.  
79 Overall, the selected plasmids provide a decent coverage of common features of mobile resistance  
80 elements.

81 In order to evaluate the evolutionary rescue potential of each of the plasmid combinations, an  
82 ampicillin-susceptible strain HMS174 was used as the recipient for the resistance plasmids. Around  
83  $2 \times 10^6$  HMS174 cells were transferred to a medium containing differing concentrations of  
84 ampicillin (0, 15, 75 and 150 mg/l). Few minutes later, 5  $\mu$ l of overnight grown plasmid-harboring  
85 JM109-cells (their respective average cell densities are listed in Supplementary table 1) was added  
86 to the medium. Notably, bacteria carrying plasmid pEC3 reached about four times higher density  
87 than the rest of the donor cells and were thus diluted accordingly before the rescue experiments.  
88 The co-culture was let to grow for 24 hours in 37 °C. The number of beta-lactam resistant HMS174  
89 cells in these cultures was measured with colony forming assay (Figure 1a).

90 The rate of horizontal transfer of beta-lactam resistance providing elements differed  
91 substantially between the strains. In the absence of antibiotics, pEC13, pEC15 and pEC16 were the  
92 most efficient in getting transferred to the recipient strain. This conjugation frequency reflected  
93 their rescue potential in 15 mg/ml ampicillin concentrations. However, when the antibiotic level  
94 increased, there were substantial differences between plasmids. In particular, pEC15 lost most of its  
95 rescue potential in higher concentrations since the number of resulting transconjugants was almost  
96 four orders of magnitude lower than that of pEC13 and pEC16. On the other hand, pEC3 was less  
97 efficient in transferring its plasmids to the recipient in the absence of antibiotics but it relatively

98 well maintained the rescue potential as the concentration increased. It is also notable that pEC14,  
99 which consists of three different large plasmids along with the plasmid-integrated resistance  
100 cassette, was very poor at disseminating the resistance to susceptible bacteria even in the absence of  
101 antibiotics. In practice, pEC14's potential for evolutionarily rescuing other bacteria in its vicinity  
102 was abolished as the concentration of antibiotic increased.

103 We measured the number of cheaters (i.e. bacteria that are not resistant but which survive due to  
104 the presence of "altruistic" beta-lactamase producers) in an attempt to explain the differences in  
105 rescue potentials (Figure 1b). Indeed, in the sole case of pEC15, the lower levels of cheaters could  
106 explain its rapidly diminishing rescue potential as the antibiotic concentration rises. Interestingly,  
107 however, the potential to support cheaters did not differ much for other plasmid combinations, thus  
108 the prevalence of cheaters is not directly related to their capability to rescue susceptible bacteria.  
109 Also, we tested whether only some of the plasmids (in those strains that harbor multiple plasmids)  
110 are transferred during the rescue event by amplifying each plasmid sequence with specific primers  
111 from the rescued clones. In most cases it appears that even in adverse conditions for the recipient  
112 strain, all plasmids get through the conjugation channel. However, pEC3II plasmid was detected  
113 only in half of the tested transconjugants, indicating that sometimes rescue via horizontal gene  
114 transfer can lead to the loss of plasmids that are not coding for the necessary resistance.  
115 Furthermore, we attempted to evaluate the sensitivity of rescue frequency on resource availability.  
116 As suspected, lower concentration of resources (5% L-broth) significantly reduces the rescue  
117 potential in 150 mg/l ampicillin concentration, but the rescue pattern remained similar (i.e. the best  
118 rescuing strains were the same in both nutrient levels) (Figure 1c).

119 In practical and evolutionary terms, the obtained results could help infer the resistance dynamics  
120 during antibiotic treatments as well as in farming environments and sewage. In particular, ESBL-  
121 carriage (i.e. people diagnosed with ESBL-positive bacteria, but with no acute infections) is  
122 becoming more common among healthy individuals<sup>11</sup>. Often carriage itself is not dangerous, but it

123 may compromise the outcome of future antibiotic therapies. Previous studies have highlighted the  
124 importance of maintaining antibiotic concentration above MSW<sup>12</sup>. However, given that ESBL-  
125 genes often reside in mobilizable elements, the efficiency of treatment may be more dependent on  
126 preventing the pathogen's access to global gene pool rather than preventing novel mutations *per se*.  
127 Measures that block horizontal gene transfer<sup>13</sup> could help prevent evolutionary rescue during  
128 treatment and thus improve the success-rates of treating ESBL-positive patients. Especially in the  
129 case of ESBL-carriage, the mobilizable resistance element(s) can be identified beforehand and thus  
130 the treatment, if meaningful, could be modified accordingly. Using our results as an example, in  
131 case of pEC14, the evolutionary rescue can be prevented simply by increasing the effective  
132 concentration of antibiotics. On the contrary, such approach would be ineffective for most of the  
133 other plasmid combinations, thus calling for alternative ways to prevent conjugation. Speculatively,  
134 these could utilize plasmid-dependent phages, pilus-binding phage-derived proteins or other  
135 components that disrupt plasmid transfer and/or maintenance<sup>14,15,16,17</sup>. Overall, extending the use of  
136 existing antibiotics requires us to acknowledge that resistance among bacteria is often a feature of  
137 the whole microbial community where lateral genetic transfer can play a notable role.

138

139

140

141

## 142 **Experimental procedures**

### 143 **Conjugation of ESBL plasmids, plasmid sequencing and analysis**

144 *Escherichia coli* strains 10UU11258, 57253, 55027, 56895 and 57361 were obtained from Medix  
145 Laboratories and Turku University Hospital and were named EC3, EC13, EC14, EC15 and EC16,  
146 respectively (see 6). Plasmids were isolated HMS174 with QIAGEN Large-construct Kit according  
147 to manufacturer's instructions. Their sequences were determined with PacBio next-generation

148 sequencing technology (DNA sequencing and Genomics laboratory, University of Helsinki,  
149 Finland). Sequence analysis was performed with NCBI's Prokaryotic Genomes Automatic  
150 Annotation Pipeline (PGAAP). Highly similar plasmid sequences were searched using BLASTn  
151 (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Plasmid Inc groups were determined using  
152 PlasmidFinder<sup>18</sup> (<https://cge.cbs.dtu.dk/services/PlasmidFinder/>) and antimicrobial resistance genes  
153 using ResFinder<sup>19</sup> (<https://cge.cbs.dtu.dk/services/ResFinder/>). Mating pair formation (MPF) types  
154 and Mobility (MOB) groups of plasmids were determined using Geneious version 9.0.5 and  
155 BLASTx to compare the amino acid and nucleotide sequences of mobility region proteins and  
156 relaxase, respectively, with reference sequences described before<sup>20</sup>.

157

#### 158 **Evolutionary rescue experiments**

159 Five donor strains (see above) and recipient strain HMS174 were grown to carrying capacity 46 h at  
160 +37 °C, 200 rpm in the presence of appropriate antibiotics. To initiate the experiments, equal  
161 volumes of recipient and donor strain (5 µl) were added in 5 ml of L-broth (containing varying  
162 concentrations of ampicillin, when appropriate) in this order. After 24 h incubation (+37 °C, 200  
163 rpm) number of different cell types were determined by plating various dilutions on appropriate  
164 antibiotic-containing plates. Three replications of each experiment were performed in the presence  
165 of lethal ampicillin concentrations (15, 75 or 150 mg/l). Natural transfer rates of plasmids were  
166 conducted in similar experimental conditions lacking the antibiotic. Additionally, all used  
167 ampicillin concentrations were shown to be lethal in experimental conditions without the presence  
168 of resistance plasmid. In other words, after using the same amounts of HMS174 and JM109(pSU18)  
169 cultures as in original evolutionary rescue experiments, no colonies formed on L-plates.

170 In order to determine which plasmids actually transferred during experiments, the plasmid  
171 contents of at least five transconjugants from each experiment were analyzed with colony PCR  
172 using plasmid-specific primers (Supplementary table 2). The PCR-products were analyzed with



173 agarose gel electrophoresis In case we were unable to assure the presence of a certain plasmid via  
174 colony PCR, the total plasmid content was isolated with Agencourt CosMCPrep (Beckman Coulter)  
175 kit according to manufacturers' instructions before PCR.

176

177

## References

- 178 1. Drlica, K. & Zhao, X. Mutant selection window hypothesis updated. *Clin. Infect. Dis.* **44**, 681-  
179 688 (2007).
- 180 2. Schultz, C. & Geerlings, S. Plasmid-Mediated Resistance in Enterobacteriaceae Changing  
181 Landscape and Implications for Therapy. *Drugs* **72**, 1-16 (2012).
- 182 3. Stokes, H. W. & Gillings, M. R. Gene flow, mobile genetic elements and the recruitment of  
183 antibiotic resistance genes into Gram-negative pathogens. *FEMS Microbiol. Rev.* **35**, 790-819  
184 (2011).
- 185 4. Yurtsev, E. A., Chao, H. X., Datta, M. S., Artemova, T. & Gore, J. Bacterial cheating drives the  
186 population dynamics of cooperative antibiotic resistance plasmids. *Mol. Syst. Biol.* **9**, 683 (2013).
- 187 5. Ojala, V., Mattila, S., Hoikkala, V., Bamford, J. K. H. & Jalasvuori, M. Evolutionary rescue of  
188 bacteria via horizontal gene transfer under a lethal beta-lactam concentration. *Journal of Global*  
189 *Antimicrobial Resistance* **2**, 198-200 (2014).
- 190 6. Mattila, S., Ruotsalainen, P. & Jalasvuori, M. On-Demand Isolation of Bacteriophages Against  
191 Drug-Resistant Bacteria for Personalized Phage Therapy. *Front. Microbiol.* **6**, 1271 (2015).
- 192 7. Bielak, E. *et al.* Investigation of diversity of plasmids carrying the blaTEM-52 gene. *J.*  
193 *Antimicrob. Chemother.* **66**, 2465-2474 (2011).
- 194 8. Kim, J. *et al.* Characterization of IncF plasmids carrying the blaCTX-M-14 gene in clinical  
195 isolates of Escherichia coli from Korea. *J. Antimicrob. Chemother.* **66**, 1263-1268 (2011).
- 196 9. Bush, K. & Jacoby, G. A. Updated functional classification of beta-lactamases. *Antimicrob.*  
197 *Agents Chemother.* **54**, 969-976 (2010).
- 198 10. Brouwer, M. S. *et al.* Complete Genome Sequences of IncII Plasmids Carrying Extended-  
199 Spectrum beta-Lactamase Genes. *Genome Announc* **2**, 10.1128/genomeA.00859-14 (2014).
- 200 11. Oteo, J., Perez-Vazquez, M. & Campos, J. Extended-spectrum [beta]-lactamase producing  
201 Escherichia coli: changing epidemiology and clinical impact. *Curr. Opin. Infect. Dis.* **23**, 320-326  
202 (2010).
- 203 12. Canton, R. & Morosini, M. I. Emergence and spread of antibiotic resistance following exposure  
204 to antibiotics. *FEMS Microbiol. Rev.* **35**, 977-991 (2011).
- 205 13. Williams, J. J. & Hergenrother, P. J. Exposing plasmids as the Achilles' heel of drug-resistant  
206 bacteria. *Curr. Opin. Chem. Biol.* **12**, 389-399 (2008).
- 207 14. Garcillan-Barcia, M. P. *et al.* Conjugative transfer can be inhibited by blocking relaxase activity  
208 within recipient cells with intrabodies. *Mol. Microbiol.* **63**, 404-416 (2007).
- 209 15. Lujan, S. A., Guogas, L. M., Ragonese, H., Matson, S. W. & Redinbo, M. R. Disrupting  
210 antibiotic resistance propagation by inhibiting the conjugative DNA relaxase. *Proc. Natl. Acad. Sci.*  
211 *U. S. A.* **104**, 12282-12287 (2007).
- 212 16. Lin, A. *et al.* Inhibition of bacterial conjugation by phage M13 and its protein g3p: quantitative  
213 analysis and model. *PLoS One* **6**, e19991 (2011).
- 214 17. Ojala, V., Laitalainen, J. & Jalasvuori, M. Fight evolution with evolution: plasmid-dependent  
215 phages with a wide host range prevent the spread of antibiotic resistance. *Evol. Appl.* **6**, 925-932  
216 (2013).
- 217 18. Carattoli, A. *et al.* In silico detection and typing of plasmids using PlasmidFinder and plasmid  
218 multilocus sequence typing. *Antimicrob. Agents Chemother.* **58**, 3895-3903 (2014).

- 219 19. Zankari, E. *et al.* Identification of acquired antimicrobial resistance genes. *J. Antimicrob.*  
220 *Chemother.* **67**, 2640-2644 (2012).
- 221 20. Shintani, M., Sanchez, Z. K. & Kimbara, K. Genomics of microbial plasmids: classification and  
222 identification based on replication and transfer systems and host taxonomy. *Front. Microbiol.* **6**,  
223 242 (2015).

224

225

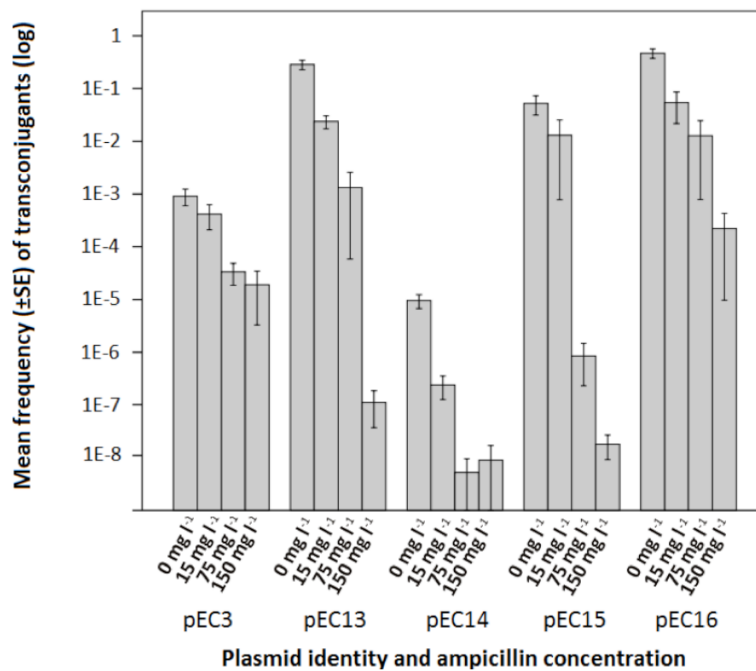
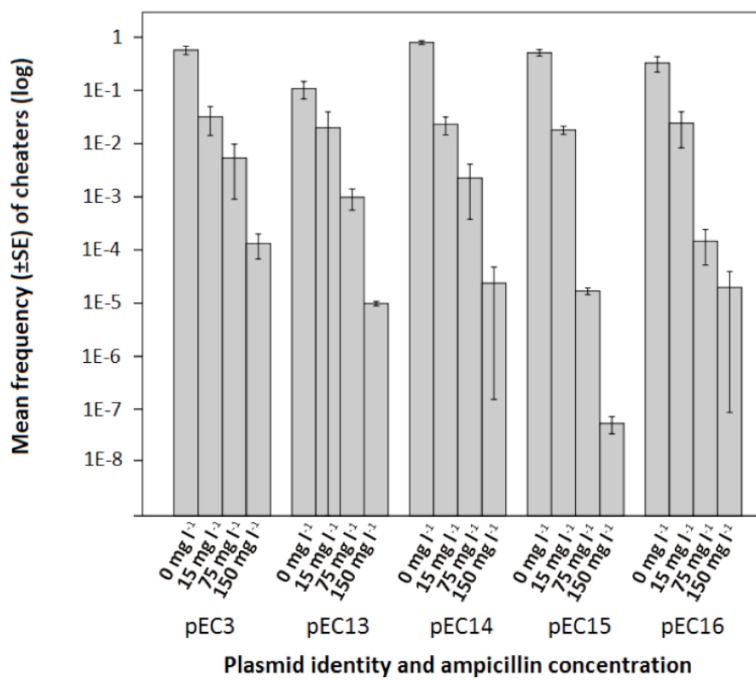
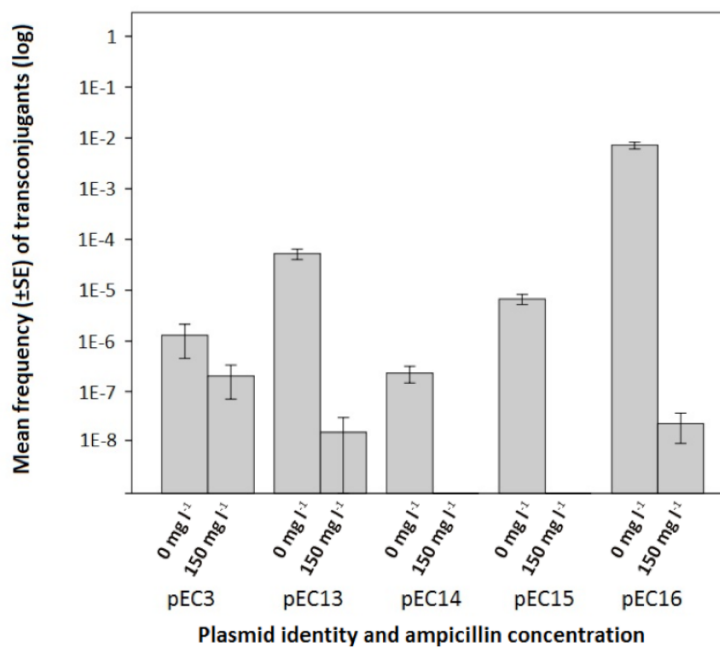
## 226 **Acknowledgements**

227 This work was supported by the Academy of Finland Centre of Excellence Program of the  
228 Academy of Finland (Biological Interaction 2012-2017, 252411) and personal grant to MJ  
229 (297049).

230

## Figure legends

**Figure 1:** (A) Number of transconjugants (HMS174) after 24 h in different ampicillin concentrations at 37 °C (n=3). (B) Number of cheaters (HMS174) after 24 h in different ampicillin concentrations at 37 °C (n=3). In the absence of antibiotics (0 mg ampicillin), the value presents the standard frequency of the recipient bacterial strain. (C) Number of transconjugants (HMS174) after 24 h in 5 % L-broth at 37 °C with and without antibiotic selection (150 mg l<sup>-1</sup>, n=5).

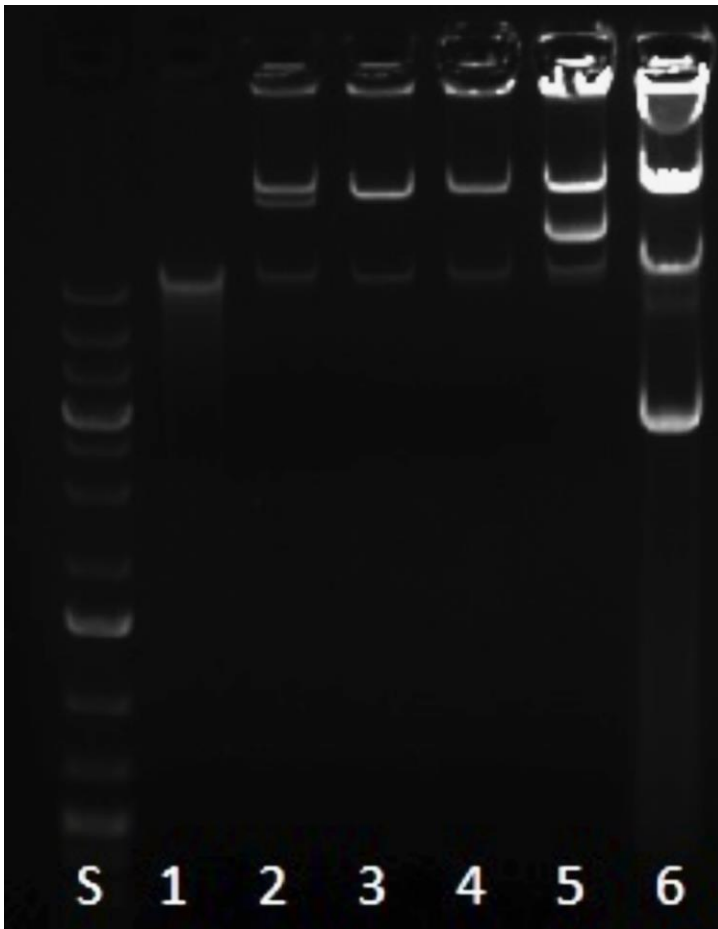
**A****B****C**

**Table 1: Plasmid features.** Alterations to plasmid size due to shufflon area are indicated in parenthesis.

Plasmid	Plasmid size bp	Inc type	MPF type	MOB class	$\beta$ -Lactamase identified	Other resistance genes
pEC3I	91 885	<i>IncB/O/K/Z</i>	MPFI	MOBP	<i>blaTEM-1C</i>	<i>strA, strB, sul2</i>
pEC3II	59 192 (59 192)	<i>IncI2</i>	MPFT	MOBP	-	-
pEC13	71 656	<i>IncFII</i>	MPFF	MOBF	<i>blaCTX-M-14</i>	-
pEC14I	143 590	<i>IncFII, IncQ1, IncP, IncFIB(AP001918)</i>	MPFF	MOBF	<i>blaTEM-1B</i>	<i>strA, strB, aadA1, mph(B) sul1, sul2, tet(A), dfrA1</i>
pEC14II	87 848 (87 666)	<i>IncI1</i>	MPFI	MOBP	-	-
pEC14III	80 057	<i>IncFII</i>	MPFF	MOBF	-	-
pEC15I	87 811 (87 767)	<i>IncI1</i>	MPFI	MOBP	-	-
pEC15II	38 611	<i>IncX1</i>	MPFT	MOBQ	<i>blaTEM-52B</i>	-
pEC16I	94 325 (95 380)	<i>IncI1</i>	MPFF	MOBP	<i>blaSHV-12</i>	-
pEC16II*	7 939	<i>ColRNAI</i>	-	MOBP	-	-

\* non-conjugative mobilizable plasmid

*Supplementary material*



**Figure S1.** AGE (0.8 %) profiles of selected plasmids. S= GeneRuler 1kb Plus DNA Ladder, 1=HMS174 genome 2=pEC3, 3=pEC13, 4=pEC14, 5=pEC15, 6=pEC16.

**Table S1:** Average cell densities at the beginning of the evolutionary rescue experiments.

Cell strain JM109	cfu/ml	cells/ experiment
pEC3	$8.1 \cdot 10^8$	$1.01 \cdot 10^6$ **
pEC13	$2.4 \cdot 10^8$	$1.20 \cdot 10^6$
pEC14	$2.5 \cdot 10^8$	$1.25 \cdot 10^6$
pEC15	$2.1 \cdot 10^8$	$1.05 \cdot 10^6$
pEC16	$2.5 \cdot 10^8$	$1.25 \cdot 10^6$

\*\* 1:4 dilution of saturated culture was used

**Table S2:** Primers used in the study

Target sequence	Forward primer	Reverse primer
pEC3 I	CGGACATATGGACTGGAACAG	GACAGGTGTTTCCCAGCGCAG
pEC3 II	CTGTCGGCATGTCTGTCTCCC	TTCACCAGATCAACTCCCAGC
pEC14 I	GTCCGCAACGGCGATGCGCCG	CTTTTGACGACACCAAGGCCAG
pEC14 II	GCAAAACGATAGTTTCCCCTG	TTTCTTGTCACCTTCCACATC
pEC14 III	AGATGCTCTGTCTGTAATAC	ACCCTTATCCGGAGAGAG
pEC15 I	GACTCTCATTTCCGACGCTC	GAAGAACTGCTGAATGATAC
pEC15 II	CTGCCGAAAATCTGAAAGCTG	CCTATCAGTTTATATTTTCTG
pEC16 I	TCCGTGGGTATGTAATAACTG	GAAATCAACTATACATCTTAG
pEC16 II	GAAGAAGTTCGGGAACTCATC	CCTGGTGCTCCACCCACAGG