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

Running title: Evolutionary rescue of bacteria via conjugation

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Description

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

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

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

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

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

residing next to several resistance genes, suggesting that this plasmid carries a DNA-integrating resistance island. Indeed, mobile genetic islands such as these are common among multi-drug resistant bacteria. Also, sequence assembly of four of the plasmids revealed variable sequence within the original DNA sample, resulting in two versions of the plasmid sequences (named pEC3II_1 and pEC3II_2, pEC14II_1 and pEC14II_2, pEC15I_1 and pEC15I_2 and pEC16I_1 and pEC16I_2). In all cases, this sequence variability was observed in IncI plasmid shufflon area[10]. Overall, the selected plasmids provide a decent coverage of common features of mobile resistance elements.

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

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

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

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

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

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

Experimental procedures

Conjugation of ESBL plasmids, plasmid sequencing and analysis

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

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

Evolutionary rescue experiments

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

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

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

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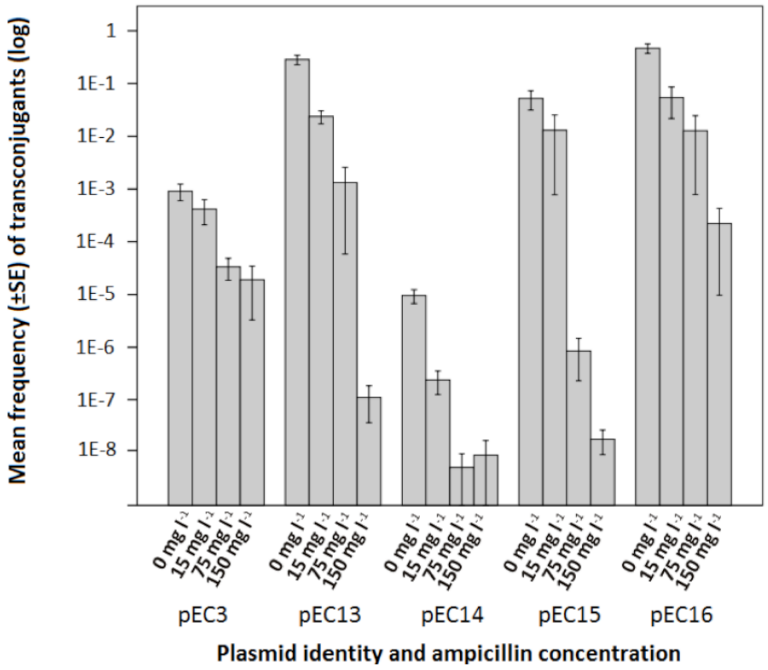
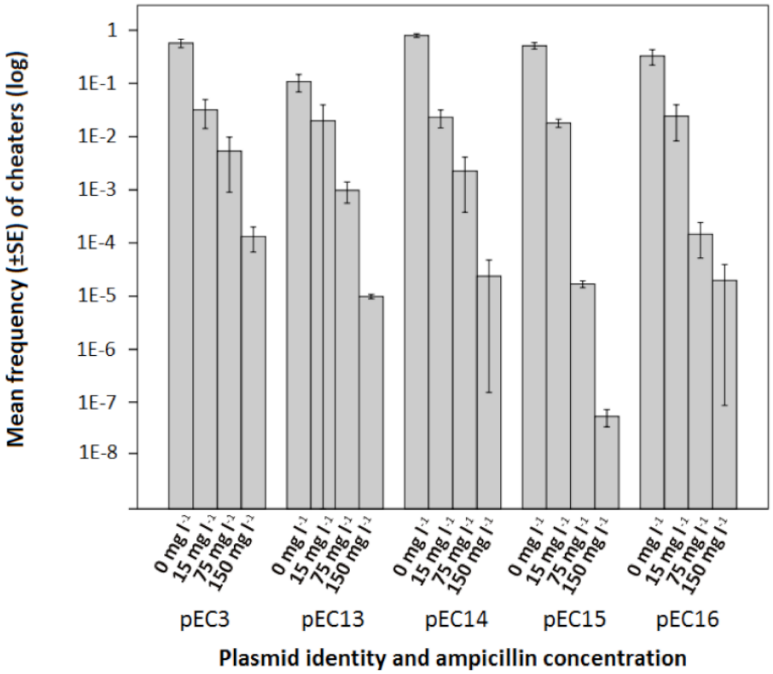
Figure legends

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^{-1}, n=5).

A


Mean frequency (±SE) of transconjugants (log)
1
1E-1
1E-2
1E-3
1E-4
1E-5
1E-6
1E-7
1E-8
0 mg l-1
15 mg l-1
75 mg l-1
150 mg l-1
pEC3
pEC13
pEC14
pEC15
pEC16
Plasmid identity and ampicillin concentration

B


Mean frequency (±SE) of cheaters (log)
1
1E-1
1E-2
1E-3
1E-4
1E-5
1E-6
1E-7
1E-8
0 mg l-1
15 mg l-1
75 mg l-1
150 mg l-1
pEC3
pEC13
pEC14
pEC15
pEC16
Plasmid identity and ampicillin concentration

C

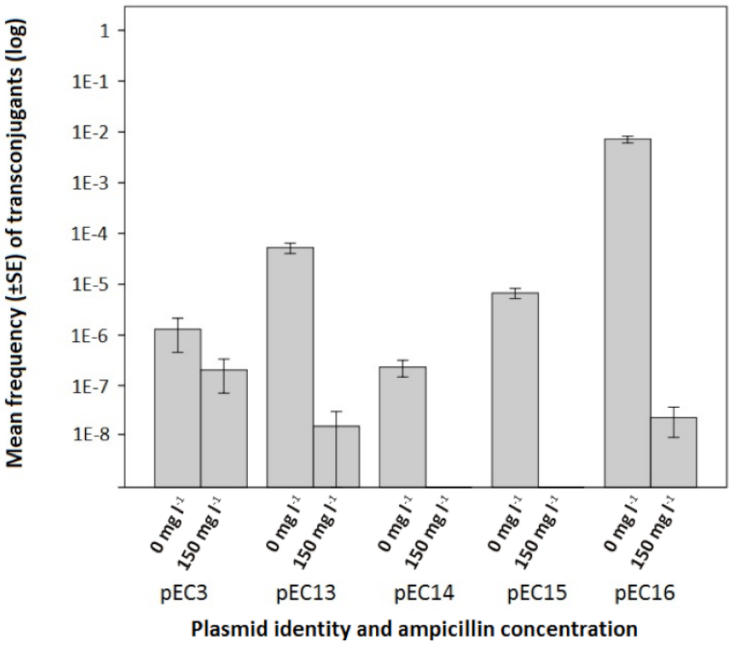

Mean frequency (±SE) of transconjugants (log)
1
1E-1
1E-2
1E-3
1E-4
1E-5
1E-6
1E-7
1E-8
0 mg l-1
150 mg l-1
pEC3
pEC13
pEC14
pEC15
pEC16
Plasmid identity and ampicillin concentration

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

* non-conjugative mobilizable plasmid

Supplementary material

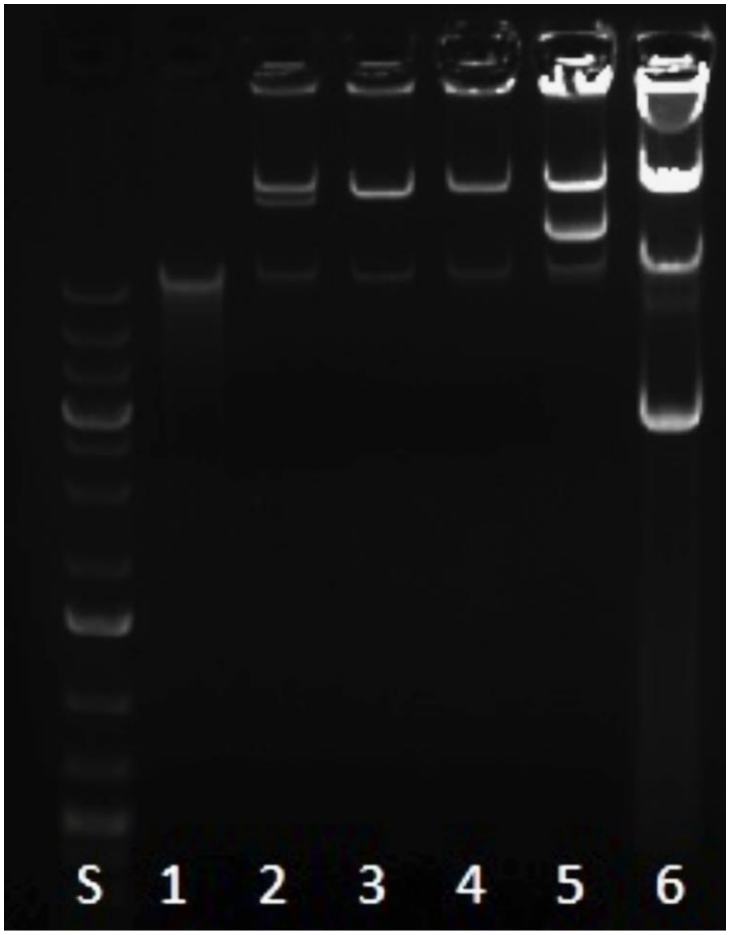


Figure S1. AGE (0.8 %) profiles of selected plasmids. S= GeneRuler 1kb Plus DNA Ladder, 1=HMS174 genome 2=pEC3, 3=pEC13, 4=pEC14, 5=pEC15, 6=pEC16.

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

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

** 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