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The Impact of Horizontal Gene Transfer on the Evolution of New Functions in *Salmonella enterica*

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Various aspects of evolution have been the focus of many experimental studies. In this project an attempt was made to develop an experimental system for studying how horizontal gene transfer affects the evolution of new functions.

For this, we constructed a library of strains of *Salmonella enterica* containing the *dhfr* gene, different variants of the *tem*-gene, including the wild type *tem-1*, the mutants E102K, R162S and G236S, and genes encoding fluorescent proteins. We analyzed the susceptibility of the strains to the β-lactam antibiotics Ceftazidime, Cefotaxime and Ceftriaxone to find out the antibiotic and the type of *tem*-gene that result in the highest minimal inhibitory concentration compared to the wild type *tem-1* gene. Thereafter a competition experiment was performed at different antibiotic concentrations in order to find an appropriate antibiotic concentration to use in a later evolution experiment, in which a modified P22 prophage, called GTA22, would be used to ensure a high frequency of HGT.

In the competition experiment a decrease of the wild type was observed during the first cycle at concentrations just below MIC_{wt}, after which the wild type was enriched at higher rates than the mutant during the next two cycles. This result was probably due to an induction of P22 by Ceftazidime, which was also supported by another study, in which induction of the SOS response by β-lactams through inactivation of PBP3 was observed. The SOS response, in turn, can induce P22. A transduction experiment was performed to detect possible induction of P22,however, very low levels of transduction were detected. Another explanation of the results from the competition experiment could be that the wild type had a fitness advantage compared with the mutant at low concentrations of the antibiotic ceftazidime during the second and third cycles. Further competition experiments should be done to find right conditions for an evolution experiment.

Keywords: P22, DIRex, lambda-red recombineering, ceftazidime, transduction, transformation

Populärvetenskaplig sammanfattning

Evolution av nya gener är en central frågeställning inom evolutionsbiologi och inte minst evolutionen av antibiotikaresistensgener som har visat sig vara bland de snabbast evolverande DNA-sekvenserna. En drivkraft bakom evolution är mutationer, men vilka mutationer som förs vidare till kommande generationer beror bl.a. på vilken typ av population de uppstår inom.

Genetiskt material kan överföras mellan olika organismer via horisontell genöverföring (HGT) och denna process skiljer sig från vertikal genöverföring som innebär överföring av genetiskt material från en förälder till deras avkomma. Horisontell genöverföring kan ske via flera olika mekanismer, varav de vanligaste är transformering, transduktion och konjugering. Denna typ av genöverföring bidrar bland annat till den ökande spridningen av gener som orsakar antibiotikaresistens.

I kontrollerade evolutionsexperiment där man har studerat evolution av resistensgener har förekomsten av mutationer i β-laktamaser visat sig vara låg. Det motsatta har observerats i naturen, nämligen att mutationer i β-laktamaser är ofta förekommande. En potentiell orsak till den här skillnaden kan vara avsaknaden av HGT i evolutionsexperiment. Mot bakgrund av detta var syftet med det här projektet att utveckla ett experimentellt system för att studera hur HGT kan påverka evolution av nya funktioner.

Experimenten gjordes genom att först konstruera olika varianter av *Salmonella enterica* som sedan skulle tävla med varandra i ett kompetitionsexperiment. Stammarna som användes innehöll bl.a. gener som kodar för olika varianter av β-laktamaser, inklusive en vild typ β-laktamas och flera muterade β-laktamaser. Med hjälp av transformation integrerades olika fluorescensgener i stammarna för att kunna urskilja och mäta antalet av dessa i nästkommande experiment. I kompetitionsexperimentet studerades konkurrensfördelen av varje tem-variant över resten av populationen efter exponering för olika koncentrationer av antibiotikumet ceftazidim. I alla biologiska replikat observerades att vildtypen hade en fördel över mutanten vid en viss koncentration varefter den dog bort. Eventuell inducering av fager undersöktes i andra experiment, för att kunna förklara observationerna från kompetitionsexperimentet. Dock kunde experimenten inte påvisa förekomst av höga nivåer av inducerade fager.

Med hjälp av resultaten av experimenten kunde inte rätt förhållanden hittas för ett evolutionsexperiment för att studera hur HGT påverkar evolutionen av nya funktioner. Ett nytt kompetitionsexperiment kan göras med stammarna som konstruerades under den här studien. Tätare koncentrationsintervall kan användas i kompetitionsexperimentet för att hitta koncentrationer där mutanten har en konkurrensfördel över vildtypen.

Abbreviations

ant	antirepressor gene
cam	chloramphenicol
cat	chloramphenicol acetyl transferase
dhfr.	dihydrofolate reductase gene
GTA	gene transfer agent
HGT	horizontal gene transfer
TZ	ceftazidime
tmp	trimethoprim

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1. Introduction

Horizontal gene transfer (HGT), also known as lateral gene transfer (LGT) plays a fundamentally important role in the evolution of new genes and functions in prokaryotes[1,2]. HGT is the transmission of genetic material between organisms other than from parent to offspring and it can take place by three main mechanisms (Figure 1), namely, transformation, transduction and conjugation[3]. The importance of each mechanism for genetic transfer depends on the environment and the genetic material involved[4].

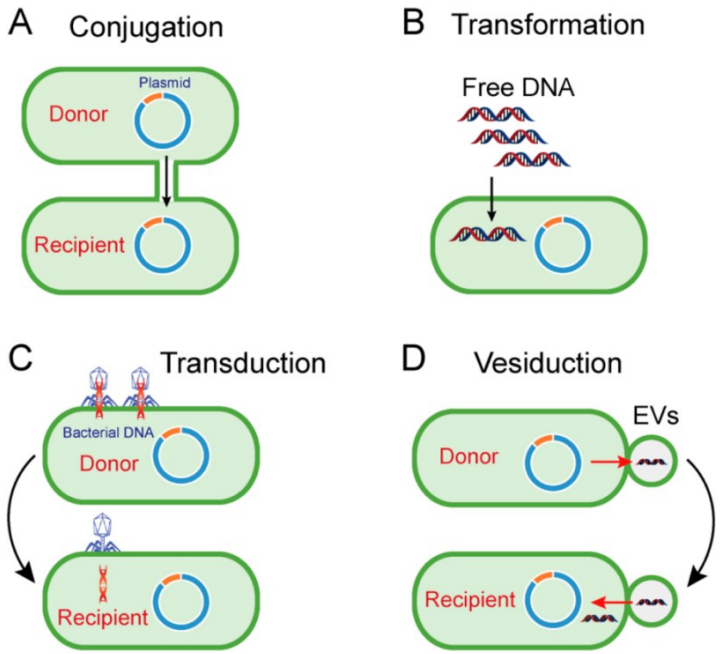

Figure 1. Mechanisms of HGT. A) Conjugation: genetic material is transferred from a donor to a recipient cell through a direct contact B) Transformation: the prokaryotic cell takes up free DNA from the environment. C) Transduction: DNA is transferred from one cell to another with the help of bacteriophages. D) Vesiduction: DNA is encapsulated into vesicles and transferred to another cell [2, 3, 5, 6].

1.1. Transformation and conjugation

Transformation is a process by which a bacterium takes up free DNA from the environment and the origin of the free DNA can be, for instance, lysed cells or bacterial biofilms[2]. This mode of HGT does not require contact between the source of DNA and the recipient cell, unlike the process of conjugation, in which the transfer of genetic material from a donor to a recipient cell is facilitated by a conjugation pilus and the DNA is transferred through a type IV secretion system (T4SS)[3,19].

1.2. Transduction

Transduction is the process of transferring DNA from one cell to another by a bacteriophage, whose capsid contains DNA[2]. There are three types of transductions – generalized, specialized, and lateral[20]. In generalized transduction, random fragments of host DNA are packed in the capsids before cell lysis with the help of terminases that recognize pac site homologs in host DNA and initiate packaging[21]. In specialized transduction, the prophage excises imprecisely from the chromosome together with some bacterial genes that are adjacent to the integrated prophage[21]. Lateral transduction that was recently discovered, begins with *in situ* prophage replication. Some of the integrated prophages can excise and enter the lytic cycle, while others become substrates for *in situ* DNA packaging in which terminases recognize pac sites on the integrated prophages, excise a DNA fragment consisting of both phage DNA and chromosomal DNA and fill it in capsids, and after that they continue to fill more capsids with bacterial chromosome. These DNA fragments are then transferred to other cells and can be integrated into the recipient genome by homologous recombination[20].

1.3. Vesiduction

Another mode of gene transfer, recently termed vesiduction, involves incorporating host DNA in extracellular vesicles and transferring it to a recipient cell. However, its contribution to evolution is still unknown[5].

1.4. Evolution

The driving force of evolution is genetic variation and it arises due to mutations[7]. In clonal populations, multiple beneficial mutations can occur and compete with each other, a phenomenon called clonal interference. This can lead to the disappearance or fixation of beneficial mutations and the fate of these mutations is highly determined by their rate of occurrence[8]. In contrast, sexual populations recombine competing beneficial mutations and result in a new lineage, where the most beneficial combination of mutations is fixed[9]. It is believed that HGT acts in a similar manner, so the most beneficial combination of mutations can come to dominate within the population[9].

1.5. β-lactamases in evolution experiments

TEM-1 β-lactamase is one of the most well-known determinants of antibiotic resistance[10]. Unlike in nature, in evolution experiments it has been observed that mutations in β-lactamases

are very rare[10]. This may be due to clonal interference, because mutations with a small rate of occurrence are outcompeted by mutations with a high rate of occurrence. Moreover, controlled evolution experiments lack HGT, which may also be a reason for the underestimated rate of evolution of antibiotic resistance genes in this kind of experiment (Joakim Näsvall, personal communication).

1.6. P22 prophage

One approach to ensure the lateral transfer between different bacterial strains through generalized transduction in an experimental study is using an artificial gene transfer agent (GTA), for example, a P22 prophage[11]. An unmodified phage P22 can insert its DNA into a susceptible host cell by binding to the O-antigen of the lipopolysaccharide (LPS) of the bacterial cell. After being injected, the DNA is circularized because its two ends are identical to each other, while the O-antigen is modified in order to prevent other P22 phages from injecting their DNA into the infected cell. The injected DNA can either go into the lytic cycle or the lysogenic cycle. If it "decides" to go into the lytic cycle, then the phage DNA is replicated and encapsulated in new phage particles. This is followed by cell lysis and release of the new phage particles[12]. If the lysogenic cycle is chosen, the injected DNA becomes incorporated into the host cell´s chromosome (a prophage is formed) and when the host cell divides the prophage DNA is inherited by the daughter cells[12]. The entrance into the lytic cycle can be induced by the SOS response. Under stressful conditions, the lytic cycle is induced and then the prophage DNA is excised from the chromosome[12].

In this study, a modified P22 prophage was used. The P22 prophage was engineered in the *Salmonella enterica* genome into a gene transfer agent (GTA, J. Näsvall, unpublished) by deleting the integrase/excisionase genes, which are responsible for the excision of the phage genome from the host chromosome after induction of the lytic cycle. This mutation makes the phage unable to package a complete copy of its genome in a single virion, making it avirulent. A mutation (HT105/1) in the DNA packaging machinery increases the frequency of mispackaging of host DNA in new virions and increases the rate of generalized transduction[17], and deletion of the O-antigen modification locus (*gtrABC*) makes it possible touse bacteria carrying the prophage as recipients in transductions. Finally, the P22 antirepressor (*ant*) was introduced under the control of the chromosomal *araB* promoter, providing a means to induce the prophage to generate high efficiency transducing lysates.

2. Aim

The aim of this project was to develop an experimental system for studying how HGT affects the evolution of a new function. For this, I used *S. enterica* strains with the *tem-1* gene, which encodes a β-lactamase that normally acts on penicillins and 1st generation cephalosporins and strains with the *tem*(R162S), *tem*(E102K) and *tem*(G236S) genes that *tem*-1 can evolve to and act on extended spectrum β-lactams. Moreover, the possibility of P22 induction by the β-lactam antibiotic ceftazidime was investigated.

3. Materials and Methods

3.1. Bacterial strain and growth conditions

All strains used in these experiments were derivatives of *Salmonella enterica* subsp. *enterica* serovar Typhimurium strain LT2 (Table 1, Table S2). Solid (in plates) and liquid (in tubes) media were used for growing bacteria. Lysogeny broth (LB; 10 g/L NaCl, 10 g/L Tryptone and 5 g/L yeast extract) was used as rich liquid media[18]. M9 was used as minimal media and it was supplemented with 0.2% (w/v) glycerol. LB agar (LA) plates were made with Bacto agar (15 g/L) and LB or M9. Salt-free LB supplemented with 0.2% (w/v) glucose was used to grow cultures prior to electroporation. Sucrose selection agar plates (salt-free LB and 30g/L sucrose) were used for counter-selection against *sacB* carrying strains. SOC (20 g/L Tryptone, 5 g/L Yeast extract, 0.5 g/L NaCl, 2.4 g/L MgSO4, 0.186 g/L KCl and 0.2 % [w/v] glucose) was used for cell recovery after electroporation[18].

Tetracycline (tet), 7.5 mg/L; chloramphenicol (cam), 12.5 mg/L; trimethoprim (tmp), 10 mg/L; L-histidine (0.1 mM) or L-tryptophan (0.1 mM) were added when appropriate. Strains with pSIM5-Tet[16] plasmid were used for λ Red recombineering. The bacteriophage used for generalized transduction was P22 *HT 105/1 int*[17]. The cassettes *Acatsac1* and *Acatsac3*[18] were used for DIRex[18].

3.2. λ Red recombineering

Linear transformation with a temperature inducible λ Red system was initiated by growing strains O/N at 30°C in salt-free LB + glucose + tet, after which the cultures

were diluted 1:100 in the same medium that was prewarmed to 30°C. The cultures were then grown at 30°C for one hour. The flasks were moved to a 42°C shaking water bath with a shaking speed of 185 rpm for 15 minutes to induce expression of the temperature controlled λ Red system genes (Figure 2). After that they were moved to an ice-water bath for 10 minutes to cool down. The cells were made competent for electroporation by first spinning them down at 4500 rpm for 7 minutes at 4°C in precooled 50 ml tubes. Then the liquid was poured off and the cells were washed with 1 ml of ice-cold 10% glycerol and spun again at 4500 rpm, 4°C, this time for 3 minutes. This was followed by aspiration of the liquid and resuspension of the cells in 200 µl of ice-cold 10% glycerol. For doing the electroporation 20 µl of cells were mixed with DNA in a precooled electroporation cuvette on ice. The DNA was purified, concentrated, and de-salted with SureClean prior to the electroporation. The amount of DNA to be used for electroporation was determined by measuring its concentration with Qubit fluorometer and then calculating the amount with the formula: DNA length (kb) x 10^{-4} ≈ amount DNA (ng) for 0.15 pmol. The cells were electroporated in a Gene Pulser Xcell (Bio Rad) under the following conditions: 2.5kV, 25µF, 200Ω. Immediately after the electroporation, the cells were resuspended in 200 µl 42°C warm SOC and transferred to a 10 ml tube in a 42°C water bath for about 15 minutes to let the cells recover. The cells were then plated on 37°C prewarmed selective plates to allow cells containing the desired gene cassette to grow and then, they were incubated O/N at 37°C.

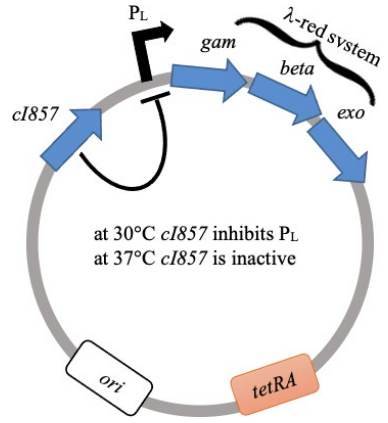

Figure 2. An illustration of the pSIM5-Tet plasmid. It contains the λ Red system (with its components gam, beta and exo). The genes of the λ Red system encode Exo, Beta and Gam proteins. Gam inhibits the RecBCD enzyme to protect the dsDNA fragment that has entered the cell from degradation and allow recombination to occur. Exo binds to the linear DNA and degrades it from each end in a 5´ 3 → ´direction, creating a dsDNA with 3´overhangs. The Beta protein binds to these overhangs and anneals the ssDNA to a complementary ssDNA in the cell, and in this way a DNA sequence is integrated into a cell's genome[15]. There is a promoter on the left side of the λ Red system, which is inhibited by the repressor called cI857. At 30°C the repressor is active, and it inhibits the promoter of the λ Red system. At higher temperatures the repressor becomes inactive, allowing transcription from the promoter. The plasmid also contains the *tetRA* genes conferring resistance to tetracycline and enables the positive selection of cells containing pSIM5-Tet in the presence of tetracycline[16].

3.3. Direct and Inverted Repeat stimulated excision (DIRex)

DIRex is a method used to generate deletions, mutations, small insertions and replacements in DNA sequences by using λ Red recombineering to generate a semi-stable DIRex intermediate, followed by isolation of the final mutant (Figure 3)[18].

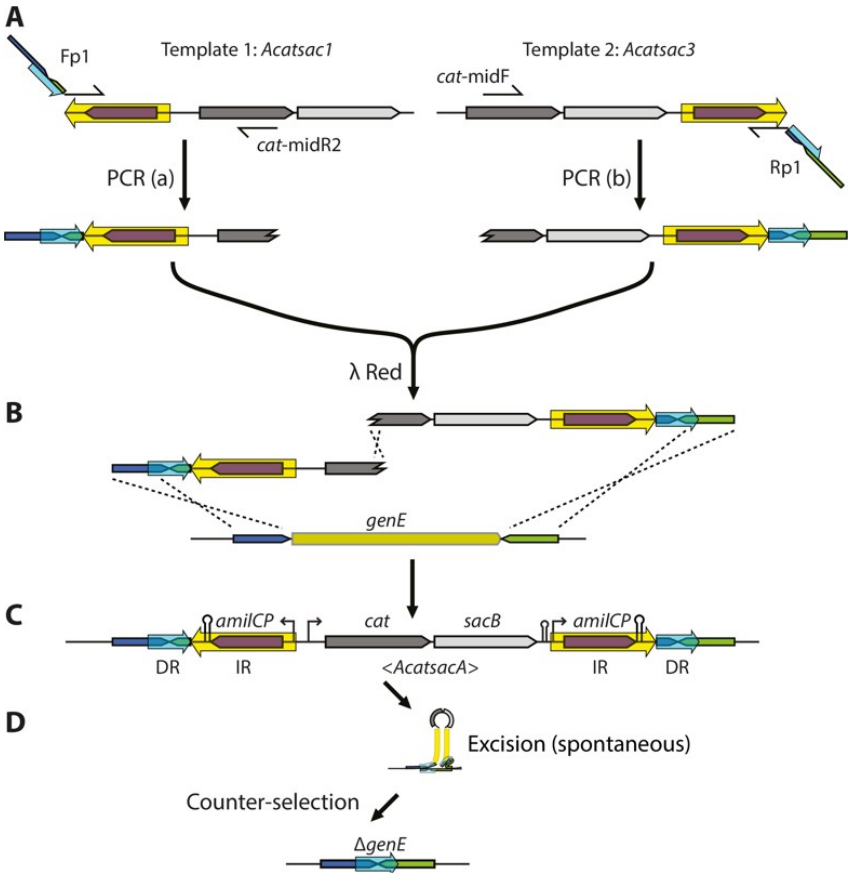

Figure 3. *DIRex. "The method is illustrated with an example for generating a precise deletion of a hypothetical gene. (A) Two overlapping "half-cassettes" are generated in separate PCR reactions (which can be run in parallel in the same PCR cycle) using one locus specific long primer "Fp1" or "Rp1" in combination with the cassette specific primers "cat-midR2" or "cat-midF", respectively. Each PCR fragment contain one copy of the IR (yellow arrow) and DR (light blue arrrow), as well as one of the recombinogenic 5'-homology extensions. The templates (Acatsac1 and Acatsac3) differ in the location and orientation of the IR sequence, which contains the gene encoding the blue chromoprotein AmilCP. (B) The two "half-cassettes" are mixed in equimolar amounts and electroporated into λ Red induced cells. For formation of a functional cat gene recombination has to occur between the recombinogenic ends and the chromosome, as well as in the sequence overlap between the two "half-cassettes". (C) The structure of the semi-stable DIRex intermediate. (D) The structure of the final deletion after spontaneous excision of the DIRex intermediate".* The design of the primers used for generating a point mutation differs from those used for generating a deletion. One of the locus specific primers used to generate a point mutation has a homology tail containing the mutation and the other primer is designed with a homology tail that ends just next to the mutation, which results in no deletion in the DNA sequence and a point mutation positioned just next to one of the DRs. The figure and the part of the legend that is in italics is from Näsvall J (2017)[18], which is published under a Creative Commons Attribution License (CC BY 4.0).

3.4. Construction of strains

Construction of:

- ***dhfr-tem1-xfp***
- ***dhfr-tem*(E102K)-*xfp***
- ***dhfr-tem*(R162S)-*xfp***
- ***dhfr-tem*(E102K, R162S)-*xfp***

- ***dhfr-tem*(E102K, G236S)- *xfp***

DNA sequences containing the *dhfr* and *tem* genes were amplified with the primers IS*200*#6f-P2V2 and PCP25r-P1 (Table S1). For all PCR reactions Phusion DNA polymerase was used. The PCR program is shown in Table S3. 24 nucleotide bases of the 3' extension of IS*200*#6f-P2V2 are homologous to a sequence beyond the *dhfr* gene and 40 bases of the 5' extension are homologous to a sequence outside an IS*200*-element on the chromosome of *S. enterica*. The primer PCP25r-P1 carries a 3' extension (20 bases) homologous to a sequence just beyond the *tem*-gene and a 5' extension (40 bases) complementary to the promoter PCP25 that is located in front of the fluorescent protein genes. The DNA was purified, concentrated, and de-salted with SureClean. λ Red recombineering was carried out to transfer the amplified DNA into strains containing genes encoding the fluorescent proteins mtagBFP2, CometGFP, sYFP2, and mScarlet (herafter collectively called *xfp* genes). After the electroporation and recovery of the cells in SOC they were plated on 37°C prewarmed trimethoprim plates and incubated over night at 37°C to allow transformants containing the *dhfr* gene to grow.

Construction of:

- ***dhfr-*Δ*tem-xfp***
- ***dhft-tem*(E102K G236S R162S)-*xfp***

A deletion in *tem-1* and a triple mutant were generated with DIRex[18]. For the construction of *dhfr-*Δ*tem-xfp* the cassette *Acatsac1* was amplified with the primers catmidR2 and DEL-tem_rCP, and *Acatsac3* with catmidF and DEL-tem_fCP (Table S1). The primers DEL-tem_rCP and DEL-tem_fCP contain 40 nt homology extensions followed by 15 nt from the other side of the sequence to delete and a 20 nt 3' primer. The PCR program used for these amplifications is shown in Table S6.

To construct the triple mutant the primers catmidR2 and Tem-R164rCP were used to amplify *Acatsac1* and catmidF and Tem-R164fCP to amplify *Acatsac3* (Table S1). Tem-R164rCP has a 5' extension identical to 40 nt on the right side of the codon containing the mutation and a 3' extension complementary to the 20 nt in the end of amilCP that is included in the IRs. The primer Tem-R164fCP is complementary to a 25 nt sequence on the left side of the codon 162 containing the mutation, 25 nt on the right side of the same codon and 20 nt homology to the end of amilCP. The PCR program used for these amplifications is shown in Table S6.

Then, the recipient cells containing *dhfr-tem1* (for constructing the *tem-1* deletion) or *dhfr-tem*(E102K G236S; for construction of the triple mutant) genes were grown, λ Red system genes were induced and cells were made competent for electroporation using the same approach that is described in section 3.2. The two DNA fragments were transformed into the recipient cells by electroporation, after which they were plated on cam plates and incubated O/N at 37°C. Transformants were purified by single cell streaks on new cam plates to separate the transformants from any surviving recipient cells and these plates were also incubated at 37°C. Colonies were picked and streaked out on sucrose-trimethoprim selection plates to allow spontaneous excision of the DIRex intermediate, leaving a single copy of the DR behind. An overnight incubation of the plates at 37°C resulted in white colonies, which indicated that an excision of the intermediate containing the gene encoding blue AmilCP had occurred.

The sequence of the white colonies was confirmed by PCR (Table S7) with primers P1 and tmp_midR and sequencing with P1 (Table S1). After confirming the sequences, DNA fragments of the new strains containing *dhfr*-Δ*tem* or *dhfr-tem*(E102K G236S R162S) were amplified (Table S3) with the primers IS*200*#6f-P2V2 and PCP25r-P1 (Table S1). λ Red recombineering was used to transfer *dhfr*-Δ*tem* or *dhfr-tem*(E102K G236S R162S) to strains containing genes coding for mtagBFP2, CometGFP, sYFP2, and mScarlet. The cells were plated on trimethoprim plates and incubated O/N at 37°C.

3.5. DNA sequencing

- ***dhfr-tem1-xfp***
- ***dhfr-tem*(E102K)-*xfp***
- ***dhfr-tem*(R162S)-*xfp***
- ***dhfr-tem*(E102K, R162S)-*xfp***
- ***dhfr-tem*(E102K, G236S)-*xfp***

The DNA cassettes *dhfr-tem*(R162S)-*yfp*, *dhfr-tem*(R162S)-*rfp* and *dhfr-tem*(E102K, G236S)-*rfp* were amplified with the primers tmp_midR and CP25-chk_rev (Table S1) with the PCR program shown in Table S5. The *rfp* gene in each strain was coding for mScarlet. The rest of the DNA cassettes were amplified with the primers IS*200*#6-chk_fwd and CP25-chk_rev (Table S1) and the PCR program shown in Table S4. Then the DNA was purified, concentrated, and de-salted using SureClean (Bioline). 30 µl of water was added to the sample

and then it was divided into two Mix2Seq tubes. 4 µl P1 primer was added to the tubes, after which they were sent for sequencing.

- ***dhfr-Δtem-xfp***
- ***dhfr-tem*(E102K G236S R162S)-*xfp***

The sequences of *dhfr-*Δ*tem* and *dhfr-tem*(E102K G236S R162S) were confirmed by PCR (Table S7) with the primers P1 and tmp_midR and sequencing with P1 (Table S1). The cassettes *dhfr-*Δ*tem-xfp* and *dhfr-tem*(E102K G236S R162S)-*xfp* were amplified with PCR (Table S5) with the primers tmp_midR and CP25-chk-rev (Table S1) and then they were confirmed with sequencing with the primer P1 (Table S1).

3.6. Antibiotic susceptibility testing

The antibiotics used to test the susceptibility of the strains were cefotaxime (CT), ceftazidime (TZ) and ceftriaxone (TX). Bacterial strains (Table 1) were plated on trimethoprim plates and incubated O/N at 37°C. Colonies were transferred in triplicate to 1 ml LB and incubated O/N at 37°C. 50µl of every culture was mixed with 950µl of PBS (200xdilution) and cells were spread on LA-plates using sterile cotton swabs that were dipped only once in the diluted cell suspension. An Etest strip was applied to the inoculated plates, after which they were incubated for 18h and the MIC value was read from the scale where the ellipse intersects the strip.

Table 1. Bacterial strains used in the antibiotic susceptibility testing

Strain	**Gene cassette[a]**	**Made by**
DA69458	*dhfr-tem1*	Joakim Näsvall
DA69569	*dhfr-tem*(G236S)	Joakim Näsvall
DA69956	*dhfr-tem*(E102K G236S)	Joakim Näsvall
DA69958	*dhfr-tem*(R162S)	Joakim Näsvall
DA69960	*dhfr-tem*(E102K)	Joakim Näsvall
DA69962	*dhfr-tem*(G236S R162S)	Joakim Näsvall
DA69994	*dhfr-tem*(E102K R162S)	Joakim Näsvall
DA74893	*dhfr-Δtem*	Nevin Muhamer
DA74894	*dhfr-tem*(E102K R162S G236S)	Nevin Muhamer

[a] *All strains in this experiment had the dhfr-tem cassette inserted between the genes res and stm0360 on the S. enterica chromosome, and no fluorescent protein genes.*

3.7. Competition experiment

The strains used in this experiment were those containing *tem-1* and *tem*(R162S) with all the fluorescent protein encoding genes (Figure 4). After growing the cultures, they were mixed and diluted in different TZ concentrations (Table 2). Flow cytometer (MACSQuant VYB; Miltenyi Biotec), which was fitted with 405 nm, 561 nm and 488 nm lasers was used to analyze the cultures. The V1 channel (450/50 filter) was used to measure BFP, the V2 channel (525/50 filter) was used to measure GFP, the B1 channel (525/50 filter) was used for YFP and the Y2 channel (615/20) for RFP.

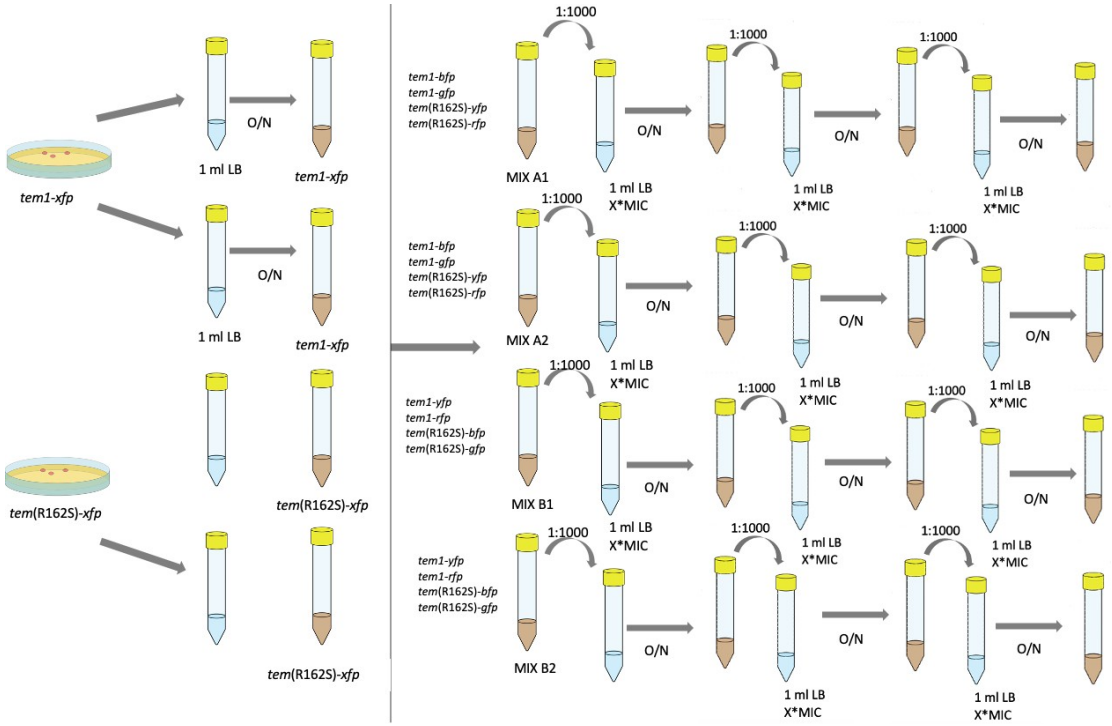

Figure 4. Illustration of the competition experiment. Strains containing the following tem-variants and fluorescent protein encoding genes were used: *tem1-bfp*, *tem1-gfp*, *tem1-yfp*, *tem1-rfp*, *tem*(R162S)-*bfp*, *tem*(R162S)-*gfp, tem*(R162S)-*yfp*, *tem* (R162S)-*rfp*. On day 1 strains were plated on LA plates and incubated O/N at 37°C. On day 2 a colony from each plate was picked and transferred to two 10 ml tubes containing 1 ml LB and incubated O/N with shaking at 37°C. On day 3 the cultures containing *tem1-bfp* and *tem1-gfp* were mixed with *tem*(R162S)-*yfp* and *tem*(R162S)-*rfp* in two 10 ml tubes (MIX A1 and MIX A2) and *tem1-yfp*, *tem1-rfp* were mixed with *tem*(R162S)-*bfp* and *tem*(R162S)-*gfp* in other two 10 ml tubes (MIX B1 and MIX B2). All mixes were diluted 1:1000 in 1 ml LB with different TZ concentrations and incubated at 37°C O/N with shaking. The TZ concentrations used were 0xMICwt, 1/16xMICwt, 1/8xMICwt, 1/4xMICwt, 1/2xMICwt, 1xMICwt, 1.25xMICwt, 1.5xMICwt, 1.75xMICwt, 2xMICwt, 2.25xMICwt, 2.5xMICwt. On day 4 the cultures were diluted again in 1 ml LB with the same antibiotic concentrations as the day before and were incubated O/N at 37°C with shaking. The O/N cultures were analyzed with flow cytometry. On day 5 they were diluted once again in the same antibiotic concentrations and analyzed with flow cytometry. On day 6 cultures from day 5 were analyzed with flow cytometry.

Table 2. [TZ] calculated based on the MIC-value 0.19 µg/ml from the Etests and used in the competition experiment.

MIC$_{wt}$	[TZ] g/ml
1/16	0.01
1/8	0.02
1/4	0.05
1/2	0.10

1.00	0.19
1.25	0.24
1.50	0.29
1.75	0.33
2.00	0.38
2.25	0.43
2.50	0.48

Table 3. [TZ] calculated based on the MIC-value of 0.2 µg/ml for the wild type tem-1.

MIC_{mutant}	[TZ] g/ml
1/8	0.03
1/4	0.05
1/2	0.10
1	0.20

Table 4. [TZ] calculated based on the MIC-value of 0.5 µg/ml for the mutant tem(R162S).

MIC_{wt}	[TZ] g/ml
1/8	0.06
1/4	0.13
1/2	0.25
1	0.5

3.8. Detection of transduction induced by Sub-MIC[TZ] in overnight bacterial cultures

The strains used in this experiment were the wild type *tem1-xfp* and the mutant *tem*(R162S)-*xfp*. O/N cultures were grown without antibiotics in 1 ml LB and incubated at 37°C, after which they were diluted 1:1000 in different TZ concentrations: 0xMIC, 1/8xMIC, 1/4xMIC, 1/2xMIC and 1xMIC and incubated again O/N at 37°C. MIC of 0.20 µg/ml was used for TEM-1 β-lactamase, and MIC of 0.5 µg/ml for the TEM(R162S) β-lactamase (Table 3-4). 100 µl chloroform was added to all the tubes in order to kill all cells but leave any phage particles intact. A culture of a strain missing the *tem*, *hisA* and *trpF* genes was grown for several hours and then 100 µl of it was mixed with 100 µl of the supernatants of the chloroform-killed cultures. The samples were transferred to histidine, tryptophan and trimethoprim plates and incubated O/N at 37°C. The number of colonies that appeared on respective plate was counted.

3.9. Detection of transduction induced by Sub-MIC [TZ] in growing bacterial cultures and identifying the direction of transduction

1) Construction of strains for the experiment

Two strains that were ceftazidime and trimethoprim sensitive and lack *hisA* and *trpF* (TZs, Tmps, His-, Trp-) were used for the construction of two new strains. A strain that lacks a *hisA* gene is not able to grow in the absence of histidine, while a strain that lacks a *trpF* gene cannot grow in a medium lacking tryptophan. In order to make the new strains markers containing a fluorescent protein gene (dTomato or sYFP) and a chloramphenicol resistance gene (*cat*) were transduced into both of the strains using phage P22, but before that P22 transducing lysates were prepared in the following way:

Cultures of the two donor strains (Δ*galK*::*cat-sYFP2* or Δ*galK*::*cat-dTomato*) were grown in 1 ml LB for 7 hours. 200 µl of the cultures were mixed with 1 ml of a P22 stock (diluted to approximately $1x10_6$ pfu/ml) in a 10 ml tube and incubated over night with shaking at 37°C for lysis to occur. The debris is spun down at 16100 x g for 1 minute and the supernatants were transferred to 1.5 ml eppendorf tubes and 50 µl of $CHCl_3$ was added, after which $CHCl_3$ was spun down at 16100 x g for 1 minute.

For making the P22 transduction, an overnight culture of the recipient strain missing the *tem*, *hisA* and *trpF* genes (TEM-, His-, Trp-) was grown in 1 ml LB. Then, a serial dilution of the P22 lysate was made (100x, 1000x and 10000x) in LB. 100 µl of each diluted lysate was mixed with 100 µl of the recipient culture in a 10 ml tube and incubated at 37°C for 30 minutes. The cells were plated on LA + cam plates and incubated over night at 37°C. Colonies from every plate were picked and streaked out on new cam plates and incubated again O/N at 37°C. These two new strains TZs, Tmps, His-, Trp-, Δ*galk::cat-sYFP2* and TZs, Tmps, His-, Trp-, Δ*galK*::*cat-dTomato* were used in the experiment.

2) Experiment: detection of transduction

Two O/N cultures of the newly constructed strains and two O/N cultures of ΔIS*200#6::dhfr-tem(R162S)-sYFP2* and ΔIS*200#6::dhfr-tem(R162S)-mScarlet* that were TZ_R, Tmp_R, His+, Trp+ were grown in triplicates at 37°C. The fluorescent marker in the TZs strains was inserted in galK, while the TZ_R strains had its fluorescent marker in IS*200*#6. Thereafter the cultures were mixed (1:1), as the Δ*galK*::*cat-dTomato* was mixed with Δ*IS200#6::dhfr-tem(R162S)-*

sYFP2 and Δ*galk::cat-sYFP2* was mixed with Δ*IS200#6::dhfr-tem(R162S)-mScarlet*. Each of the mixes was diluted (1:1000) in LB supplemented with different concentrations of TZ: 0xMIC, 1/8xMIC, ¼xMIC, ½xMIC and 1xMIC (Table 4) and incubated over night at 37°C. The overnight cultures were first diluted in the same TZ concentrations and incubated over night at 37°C and then different volumes of it (50 µl, 150 µl and 300 µl) were plated on LA + cam + tmp plates, which were also incubated at 37°C. The number of colonies that appeared on the plates was counted. The dilution and plating on agar plates was repeated the day after, but this time just one volume of 100 µl was plated. The number of colonies after the second dilution was also counted.

3) Experiment: identifying the direction of transduction

Several colonies from every plate from 2) were patched on new LA + cam + tmp plates and incubated O/N at 37°C. After that the plates were patched on different types of minimal medium: M9 + glucose, M9 + glucose + histidine, M9 + glucose + tryptophan, M9 + glucose + histidine + tryptophan. In order to see which fluorescent marker the cells contained the plates were analyzed on a VisiBlue table after incubating them over night at 37°C.

4. Results

4.1. Construction of strains

To investigate the impact of HGT on the evolution of *tem*-genes a library of *S. enterica* strains was made by PCR amplification of a gene cassette, transformation with λ Red recombineering and/or transduction. The gene cassettes (Table 1) that were PCR-amplified to use in transformations contained two important genes, the dihydrofolate reductase (*dhfr*) gene coding for the trimethoprim resistant enzyme dihydrofolate reductase and a variant of a *tem*-gene providing resistance to penicillins and cephalosporins. The amplified gene cassette was transferred to and integrated next to a gene encoding a fluorescent protein on the chromosome of a bacterial strain. Four strains containing different fluorescent protein-encoding genes: *bfp, gfp, yfp and rfp* (which I term collectively *xfp*) were used and along with the fluorescent protein-encoding gene, the bacterial strains also contained a pSIM5-Tet plasmid (temperature-controlled λ Red system; Figure 2), P22 [Δ(*gtr-'thrW*) Δ(*thrW-kil*) HT 105/1 sieA44]) and Δ *araBAD::ant*P22. The *tem*-genes that were used for construction of new strains were *tem-1* (wild type), *tem*(E102K), *tem*(R162S), *tem*(E102K R162S) and *tem*(E102K G236S).

4.2. Antibiotic susceptibility testing

The aim of the antibiotic susceptibility testing was to find which mutant and which antibiotic was suitable to use in later experiments. In order to determine the susceptibility of the strains expressing the different β-lactamases to the three different β-lactam antibiotics ceftazidime (TZ), ceftriaxone (TX) and cefotaxime (CT) I used Etests. Of the tested single mutants (*tem* (G236S), *tem*(R162S), *tem*(E102K)), the *tem*(R162S) variant was the only one to show a decreased susceptibility to any of the tested drugs compared to strains with *tem-1* or a strain without any β-lactamase (Table 5). In a comparison of the strains containing a single mutation of *tem*(E102K), *tem*(R162S) or *tem*(G236S) it was observed that the MIC value of *tem* (R162S) was more than twice as high as the MIC value of *tem-1* (0.5 vs 0.19), while the MIC value of *tem*(E102K) and *tem*(G236S) was almost the same as the one of *tem-1* (0.25 and 0.125 vs 0.19). Therefore, *tem*(R162S) and TZ were chosen for further experiments. The antibiotic susceptibility testing was performed in triplicate to increase the reliability of the results. The small variability of the MIC values obtained by the E-tests are acceptable in this case since we were only interested in differences in MIC-values that are big enough in order to be useful in an evolution experiment.

4.3. Competition experiment

The aim of this experiment was to find a concentration at which both the wild type and the mutant are alive, and at which the mutant has a higher fitness than the wild type. For this, the number of cells per milliliter was measured in mixes containing different combinations of *tem*-genes (*tem*-1 and *tem*(R162S)) and fluorescent encoding genes (*bfp, gfp, yfp and rfp).* There were two biological replicates (MIX A1 and A2; MIX B1 and B2), each having a technical replicate. MIX A1 and A2 contained *tem1-bfp*, *tem1-gfp*, *tem*(R162S)-*yfp*, *tem* (R162S)-*rfp*. MIX B1 and B2 contained *tem1-yfp*, *tem1-rfp*, *tem*(R162S)-*bfp*, *tem*(R162S)-*gfp* . TZ in different concentrations (0xMIC_{wt} – 2.5xMIC_{wt}) was present in the mixtures, which were diluted in the same antibiotic concentrations three days in a row and after every dilution the mixtures were incubated O/N. The change in size of the populations in every mix after the O/N incubation was calculated for each of the days by relating every culture to one reference culture (Table S8-S10). The reference used in MIX A1 and A2 was the strain containing *tem1-bfp* and in MIX B1 and B2: *tem1-yfp*. The reason for choosing these strains as references is that they showed a lowest fitness cost (BFP has even lower fitness cost than

YFP) compared to the rest of the fluorescent markers in another experiment (J. Näsvall, personal communication).

Table 5. MIC values of the antibiotics cefotaxime (CT), ceftazidime (TZ) and ceftriaxone (TX) to derivatives of S. enterica. Three cultures of each strain were tested, hence the three MIC values of every strain. Among the single mutants tem(R162S) was the only one to show a decreased susceptibility to an antibiotic compared to tem-1.

Strain	Genotype	MIC (CT) (µg/ml)	MIC (TZ) (µg/ml)	MIC (TX) (µg/ml)
DA74893	no TEM	0.047	0.190	0.032
		0.064	0.190	0.032
		0.047	0.190	0.023
DA69458	*tem*-1	0.047	0.125	0.023
		0.032	0.190	0.023
		0.064	0.125	0.023
DA69569	*tem*(G236S)	0.094	0.125	0.023
		0.064	0.125	0.032
		0.064	0.125	0.016
DA69958	*tem*(R162S)	0.047	0.380	0.023
		0.047	0.500	0.016
		0.047	0.380	0.023
DA69960	*tem*(E102K)	0.047	0.190	0.023
		0.047	0.250	0.023
		0.047	0.190	0.023
DA69956	*tem*(E102K G236S)	0.190	0.750	0.125
		0.125	0.250	0.094
		0.125	0.380	0.125
DA69962	*tem*(G236S R162S)	0.047	0.190	0.032
		0.047	0.125	0.032
		0.047	0.190	0.032
DA69994	*tem*(E102K R162S)	0.064	1.500	0.032
		0.064	3.000	0.032
		0.047	3.000	0.032
DA74894	*tem*(E102K R162S G236S)	0.064	0.750	0.047
		0.064	1.500	0.047
		0.094	1.500	0.047

The competition between the populations at different TZ concentrations during 30 generations of growth was analyzed with flow cytometry (Figure 5-8, S1-S4). As expected, in the absence of an antibiotic the fitness differences between the strains are negligible. However, at 1/2xMIC_{wt} the wild type died, which was not expected, since this concentration is lower than the earlier determined MIC. It was expected that an increasing antibiotic concentration would result in higher fitness for the mutant relative to the wild type and that this scenario would be observed in all generations. However, at ¼MIC it was observed that the fitness of the wild

type became higher than that of the mutant (Figure 5-8) from generations 20 to 30, even though it apparently had lower fitness during the first 10 generations, which could be seen when comparing it to the populations at [TZ]=0, at which the fitness differences were negligible. A hypothesis was made that this result was due to an induction of P22 that was present in the cells by TZ. The possibility of P22 induction was investigated in the next experiments.

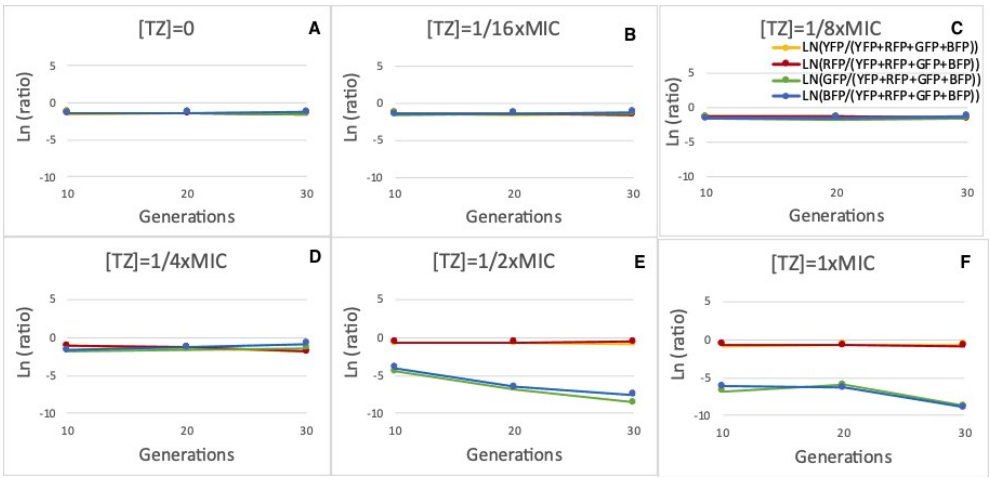

Figure 5. MIX A1, technical replicate 2. Ratios of the different fluorescent markers in the different cultures from generation 10 to 30 at the TZ concentrations 0xMIC (A), 1/16xMIC (B), 1/8xMIC (C), 1/4xMIC (D), 1/2xMIC (E) and 1xMIC (F). YFP/(YFP+RFP+GFP+BFP) and RFP/(YFP+RFP+GFP+BFP) represent the mutant *tem*(R162S) while GFP/(YFP+RFP+GFP+BFP) and BFP/(YFP+RFP+GFP+BFP) represent the wild type *tem-1*.

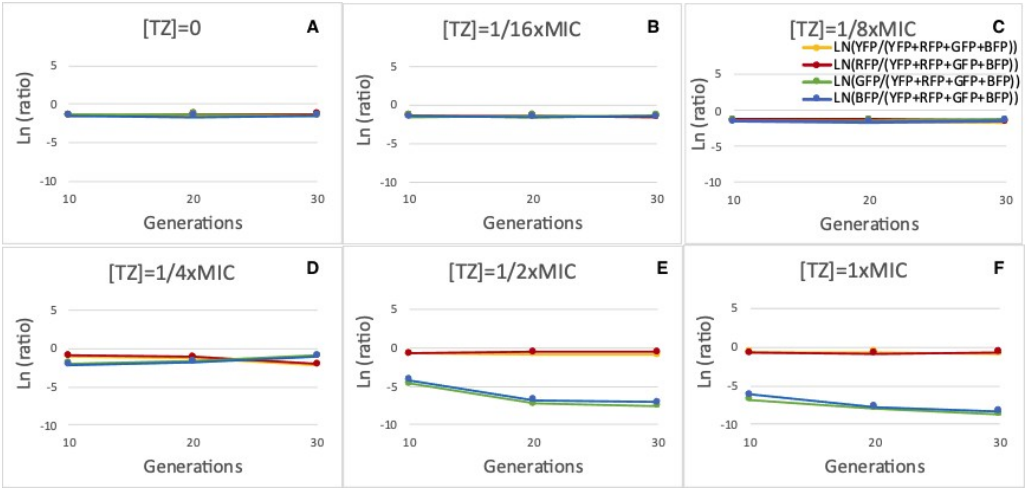

Figure 6. MIX A2, technical replicate 1. Ratios of the different fluorescent markers in the different cultures from generation 10 to 30 at the TZ concentrations 0xMIC (A), 1/16xMIC (B), 1/8xMIC (C), 1/4xMIC (D), 1/2xMIC (E) and 1xMIC (F). YFP/(YFP+RFP+GFP+BFP) and RFP/(YFP+RFP+GFP+BFP) represent the mutant *tem*(R162S) while GFP/(YFP+RFP+GFP+BFP) and BFP/(YFP+RFP+GFP+BFP) represent the wild type *tem-1*.

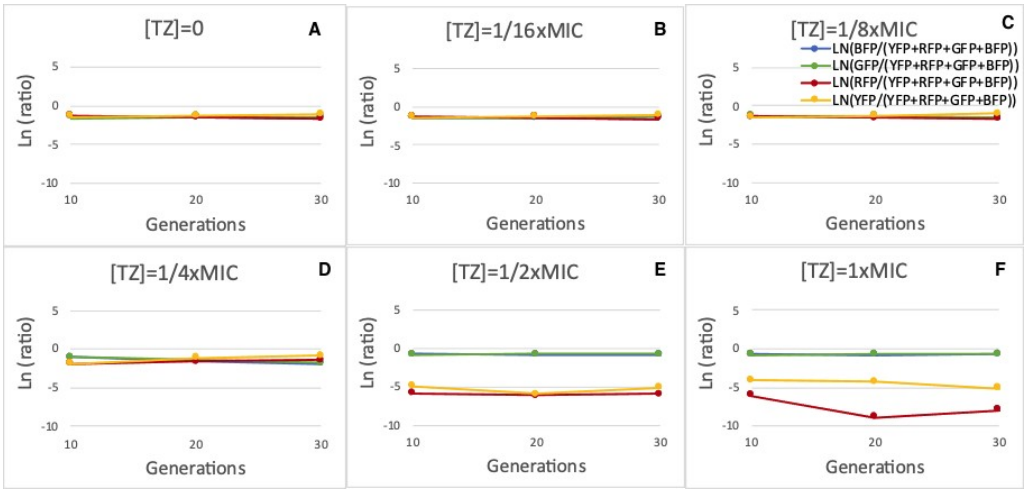

Figure 7. MIX B1, technical replicate 1. Ratios of the different fluorescent markers in the different cultures from generation 10 to 30 at the TZ concentrations 0xMIC (A), 1/16xMIC (B), 1/8xMIC (C), 1/4xMIC (D), 1/2xMIC (E) and 1xMIC (F). BFP/(YFP+RFP+GFP+BFP) and GFP/(YFP+RFP+GFP+BFP) represent the mutant *tem*(R162S), while YFP/(YFP+RFP+GFP+BFP) and RFP/(YFP+RFP+GFP+BFP) represent the wild type *tem-1*.

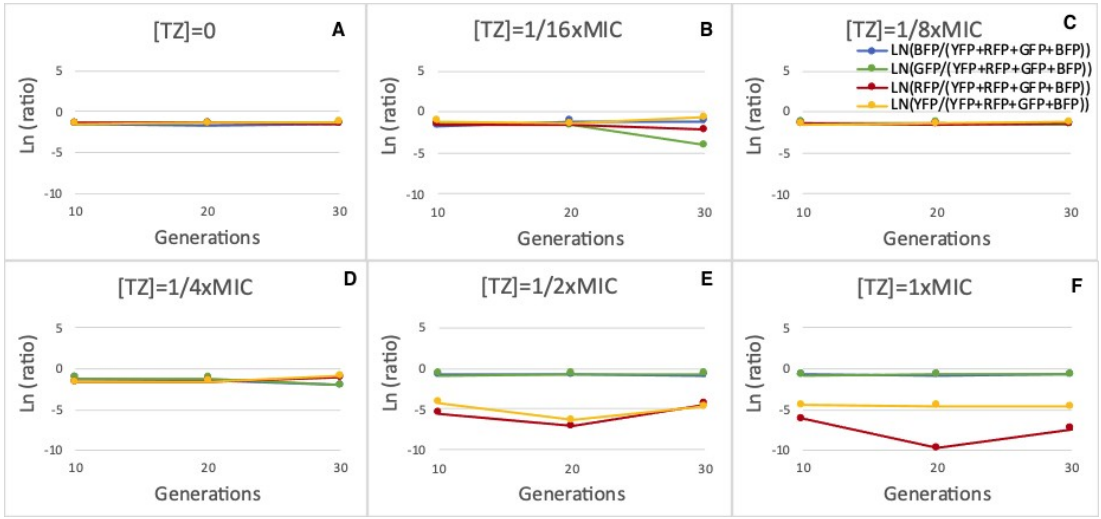

Figure 8. MIX B2, technical replicate 1. Ratios of the different fluorescent markers in the different cultures from generation 10 to 30 at the TZ concentrations 0xMIC (A), 1/16xMIC (B), 1/8xMIC (C), 1/4xMIC (D), 1/2xMIC (E) and 1xMIC (F). BFP/(YFP+RFP+GFP+BFP) and GFP/(YFP+RFP+GFP+BFP) represent the mutant *tem*(R162S), while YFP/(YFP+RFP+GFP+BFP) and RFP/(YFP+RFP+GFP+BFP) represent the wild type *tem-1*.

4.4. Detection of transduction induced by Sub-MIC[TZ] in overnight bacterial cultures

In this experiment the induction of GTA22 by Sub-MIC concentrations of TZ in O/N cultures was tested. This was done due to the observations from the competition experiment that showed that the wild type *tem*-1 outcompeted the resistant mutant *tem*(R162S) during the second and third growth cycle at 1/4xMIC. The strains used as donors in this experiment were *tem1-xfp* and *tem*(R162S)-*xfp*. The cultures were grown at the same TZ concentrations 0xMIC, 1/16xMIC, 1/8xMIC, 1/4xMIC, 1/2xMIC and 1xMIC (Table 3-4). The cells were

killed with chloroform and the supernatants (containing any transducing phage particles that may have been released during the growth of the cultures) were mixed with recipient cells that were auxotrophic for histidine and tryptophan (due to the Δ*hisA* and Δ*trpF* mutations) and TmpS (missing the *dhfr*-gene). If there were phages released from the *dhfr-tem1-xfp* or *dhfr-tem*(R162S)-*xfp* that could transduce their DNA into the recipient cells, it would be possible to select *dhfr-tem-xfp* transductants on LA+tmp plates, and *hisA*+ or *trpF*+ transductants on minimal media lacking histidine or tryptophan, respectively.

No colonies appeared on the LA + tmp plates (Table 6), which indicated that the *dhfr-tem-xfp*-cassettes were not transduced to the recipient strain in this experiment. There were few colonies on the minimal medium plates (Table 6), which means these markers were transduced at very low frequency. One explanation for these results could be that the phages were induced in growing cultures where all cells were susceptible to P22 transduction. Perhaps the new phage particles would quickly inject their DNA into a cell, and there would be very few free phages left in the overnight cultures, hence almost no transductants on the plates.

Table 6. Number of colonies formed on +tmp, -his and -trp plates after mixing tem1-xfp and tem(R162S)-xfp with a strain with no tem-gene, nor histidine or tryptophan gene in the TZ concentrations 0xMIC, 1/16xMIC, 1/8xMIC, 1/4xMIC, 1/2xMIC and 1xMIC.

			n colonies				
Strain	**Genotype**	**Plate**	**0xMIC**	**1/8xMIC**	**1/4xMIC**	**1/2xMIC**	**1xMIC**
DA73894	*tem1-bfp*	+tmp[a]	0	0	0	0	0
		-his[b]	1	1	5	3	0
		-trp[c]	6	0	1	0	0
DA73903	*tem1-gfp*	+tmp	0	0	0	0	0
		-his	2	1	4	0	0
		-trp	0	0	1	0	0
DA73910	*tem1-yfp*	+tmp	0	0	0	0	0
		-his	1	0	0	3	0
		-trp	1	0	0	0	0
DA73918	*tem1-rfp*	+tmp	0	0	0	0	0
		-his	1	9	3	4	0
		-trp	0	0	0	0	0
DA73898	*tem*(R162S)-*bfp*	+tmp	0	0	0	0	0
		-his	1	0	0	1	1
		-trp	0	0	0	0	0
DA73906	*tem*(R162S)-*gfp*	+tmp	1	0	0	0	0
		-his	0	0	2	0	1
		-trp	0	0	0	0	0
DA73914	*tem*(R162S)-*yfp*	+tmp	0	0	0	0	0
		-his	0	2	3	3	2
		-trp	0	0	0	0	0
DA73923	*tem*(R162S)-*rfp*	+tmp	0	0	0	0	0
		-his	1	1	0	0	3
		-trp	0	0	0	0	0

[a] *+tmp plates contained trimethoprim (10 mg/l)*
[b] *-his plates contained M9 + glucose + tryptophan*
[c] *-trp plates contained M9 + glucose + histidine*

4.5. Detection of transduction induced by Sub-MIC[TZ] in growing bacterial cultures and identifying the direction of transduction

This experiment was also performed to detect transduction due to induction of the GTA. Unlike the previous experiment that was carried out to detect transduction in supernatants from chloroform-killed overnight cultures, this experiment had the aim to detect transduction in growing cultures. To do this I made two strains that were trimethoprim and ceftazidime sensitive (Tmps, TZs), one of which contained an *rfp* marker (DA75229) and the other contained a *yfp* marker (DA75230). Each of the fluorescent protein genes were linked to a chloramphenicol resistance gene (Camr). Furthermore, two trimethoprim and ceftazidime resistant strains (Tmpr, TZr) were used, and one of them contained *rfp* (DA73923) and the other one contained *yfp* (DA73914). None of these strains were chloramphenicol resistant (Cams).

A culture of Δ*galK*::*cat-rfp* was mixed with a culture of ΔIS*200#6*::*dhfr-tem*(R162S)-*yfp*, and Δ*galK*::*cat-yfp* was mixed with ΔIS*200#6*::*dhfr-tem*(R162S)-*rfp* to see if chloramphenicol resistance could be transferred from the TZs strains to the TZr strains or if trimethoprim resistance could be transferred from TZr to TZs. Thereafter, the cells were diluted and grown in different TZ concentrations: 0xMIC, 1/8xMIC, 1/4xMIC, 1/2xMIC and 1xMIC. 1xMIC is the MIC-value that was determined with E-tests and the rest of the concentrations were calculated based on it. Then the cells were plated on a selective medium and incubated to allow transductants to grow. On the following day it was observed that the number of transductants on the plates was very low (Table 7, day 1), which means that the phage was not induced by TZ in these cultures. A second dilution and growth were carried out in the same procedure and resulted in an increase of the transductants on some of the plates and decrease on others (Table 7, day 2), which could not clearly demonstrate if GTA22 was induced by TZ.

To be able to identify which strains acted as donors and which ones acted as recipients the phenotype of the transductants was characterized. This was done by streaking out the transductants on new LA + cam + tmp plates, after which they were incubated and then patched on different types of minimal media agar plates: M9+glucose, M9+glucose+histidine, M9+glucose+tryptophane and M9+glucose+histidine+tryptophane. M9 + glucose plates were used to select for transductants in which TZs is a donor and TZr is a recipient. M9 + glucose + histidine plates were selective for transductants lacking a *trpF* gene, while M9 + glucose +

tryptophan plates were selective for transductants lacking a *hisA* gene. M9 + glucose + histidine + tryptophan plates were not selective, thus all Cam^R, Tmp^R transductants could grow on them. All plates were analysed on a VisiBlue table (Figure 9-10).

Since the most of the transductants from cam + tmp plates grew well on M9 + glucose (and M9 + glucose + histidine and M9 + glucose + tryptophan) plates it was concluded that they were Cam^R, Tmp^R, His+, Trp+, which means that TZ^S acted as a donor and TZ^R as a recipient. Transductants formed by both strain combinations contained both *yfp* and *rfp* fluorescence markers. The red fluorescent marker in one of the strains is located in *galK* and it is expressed by a weaker promoter than the fluorescent marker of the other strain, in which it is located in IS*200*#6. The weaker promoter is the reason for the weak red fluorescence of some of the colonies observed on the VisiBlue table (Figure 9-10). There were a few colonies on the plates that also grew poorly, perhaps due to a mutation that made the chloramphenicol sensitive cells chloramphenicol resistant without having received the *cat*-gene through transduction.

Table 7. Number of transductants on LA + cam + tmp plates at the TZ concentrations 0xMIC, 1/8xMIC, 1/4xMIC, 1/2xMIC and 1xMIC. Three replicates of each cell mixture (DA75229 & DA73914; DA75230 & DA73923) were made and the number of transductants per ml was calculated for each of the days. The number of colonies per ml that increased from day 1 to day 2 is highlighted in yellow.

Strains	*xfp* in *galK*	*xfp* in IS*200#6*	[TZ]	Replicate	Transd./ml (day 1)	Transd./ml (day 2)
DA75229+DA73914	*rfp*	*yfp*	0xMIC	1	42	310
DA75229+DA73914	*rfp*	*yfp*	1/8xMIC	1	54	400
DA75229+DA73914	*rfp*	*yfp*	1/4xMIC	1	96	0
DA75229+DA73914	*rfp*	*yfp*	1/2xMIC	1	16	0
DA75229+DA73914	*rfp*	*yfp*	1xMIC	1	0	0
DA75229+DA73914	*rfp*	*yfp*	0xMIC	2	108	170
DA75229+DA73914	*rfp*	*yfp*	1/8xMIC	2	72	710
DA75229+DA73914	*rfp*	*yfp*	1/4xMIC	2	68	1120
DA75229+DA73914	*rfp*	*yfp*	1/2xMIC	2	20	0
DA75229+DA73914	*rfp*	*yfp*	1xMIC	2	0	0
DA75229+DA73914	*rfp*	*yfp*	0xMIC	3	36	100
DA75229+DA73914	*rfp*	*yfp*	1/8xMIC	3	38	540
DA75229+DA73914	*rfp*	*yfp*	1/4xMIC	3	52	0
DA75229+DA73914	*rfp*	*yfp*	1/2xMIC	3	32	0
DA75229+DA73914	*rfp*	*yfp*	1xMIC	3	0	0
DA75230+DA75923	*yfp*	*rfp*	0xMIC	1	20	30
DA75230+DA75923	*yfp*	*rfp*	1/8xMIC	1	20	0
DA75230+DA75923	*yfp*	*rfp*	1/4xMIC	1	8	0
DA75230+DA75923	*yfp*	*rfp*	1/2xMIC	1	2	0
DA75230+DA75923	*yfp*	*rfp*	1xMIC	1	0	0

DA75230+DA75923	*yfp*	*rfp*	0xMIC	2	24	60
DA75230+DA75923	*yfp*	*rfp*	1/8xMIC	2	0	10
DA75230+DA75923	*yfp*	*rfp*	1/4xMIC	2	16	0
DA75230+DA75923	*yfp*	*rfp*	1/2xMIC	2	0	0
DA75230+DA75923	*yfp*	*rfp*	1xMIC	2	0	0
DA75230+DA75923	*yfp*	*rfp*	0xMIC	3	38	80
DA75230+DA75923	*yfp*	*rfp*	1/8xMIC	3	10	40
DA75230+DA75923	*yfp*	*rfp*	1/4xMIC	3	4	0
DA75230+DA75923	*yfp*	*rfp*	1/2xMIC	3	0	0
DA75230+DA75923	*yfp*	*rfp*	1xMIC	3	0	0

5. Discussion

In this project different derivatives of *S. enterica* were constructed in order to use them in a competition experiment, the results of which can be used to develop an experimental system for studying the effects of HGT on the evolution of new functions. Furthermore, the possibility of phage induction by the antibiotic ceftazidime that causes transduction within growing cultures was investigated to see if this could be a source of error affecting the results from the competition experiment.

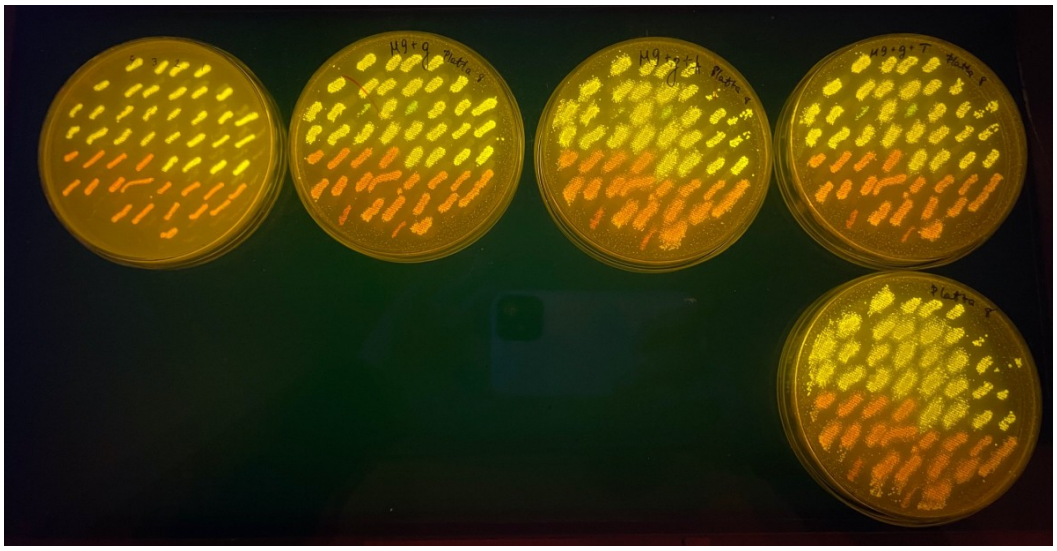

Figure 9. Plates patched with different types of transductants. The first plate to the left is a cam + tmp plate and the rest are different types of M9 plates (from left to right: M9 + glucose, M9 + glucose + histidine, M9 + glucose + tryptophan, at the bottom: M9 + glucose + histidine + tryptophan). Yellow colonies contain *yfp* expressed by the strong CP25-promoter in IS *200#6::dhfr-tem-xfp* and *rfp* expressed by the weak j23101-promoter in *galK*::*cat-xfp*. Red colonies, on the other side, contained *rfp* expressed by the CP25-promoter and *yfp* by the j23101-promoter. Perhaps yellow-green colonies express only *yfp* from the CP25-promoter, while dark red colonies express only *rfp* from the same promoter.

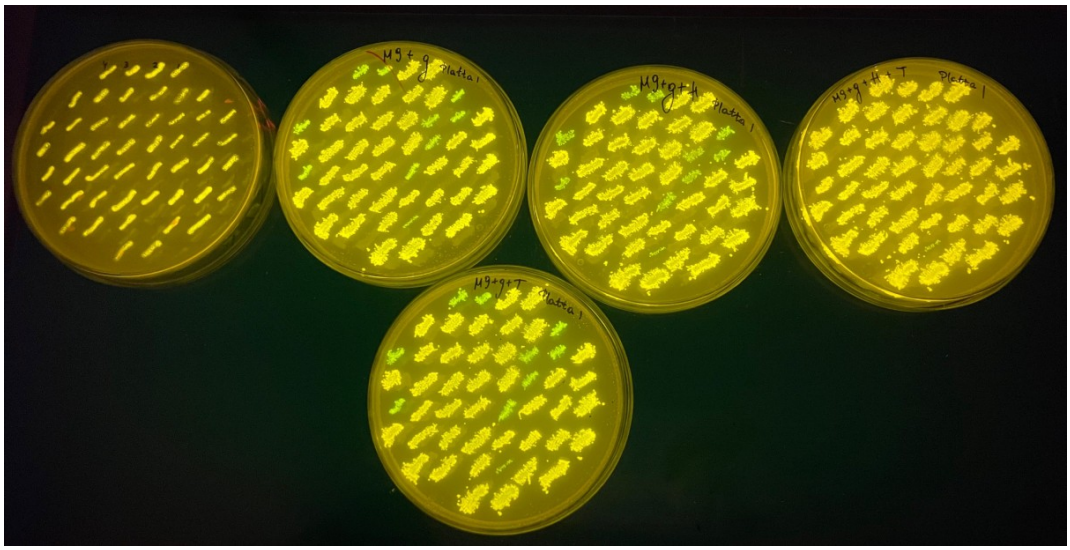

Figure 10. Plates patched with different types of transductants. The first plate to the left is a cam + tmp plate and the rest are different types of M9 plates (from left to right: M9 + glucose, M9 + glucose + histidine, M9 + glucose + tryptophan, at the bottom: M9 + glucose + histidine + tryptophan. Yellow colonies contain *yfp* from the strong CP25-promoter and *rfp* from the weak j23101-promoter. Perhaps yellow-green colonies express only *yfp* from the CP25-promoter.

The constructed strains contained different variants of *tem*-genes, one strain contained the wild type *tem*-1, another contained a deletion in *tem*-1 (Δ*tem*), and others contained one, two or three mutations in their *tem*-gene, called E102K, R162S and G236S. Δ*tem* was almost entirely identical with the rest of the strains, with the only difference being that a large piece of the *tem*-gene was missing and this strain was made in order to be used as a control in competition and evolution experiments. These three mutations are often found in clinical isolates and they contribute to resistance to extended spectrum β-lactam antibiotics[10], which is the reason for involving these particular mutants in this study. The *dhfr*-gene, encoding trimethoprim resistance, and the *cat*-gene, encoding chloramphenicol resistance were used as selection markers. Genes coding for the fluorescent proteins mtagBFP2, CometGFP, sYFP2, mScarlet and dTomato were involved to be able to track different cells in the experiments.

The three antibiotics ceftazidime, ceftriaxone and cefotaxime that were used for the antibiotic susceptibility testing are broad spectrum third generation cephalosporins. The reason for testing these antibiotics was that they were expected to contribute to a higher fitness of the mutants *tem*(E102K), *tem*(R162S) and *tem*(G236S), since they had a lower susceptibility to the antibiotic than the wild type. E102K showed almost no difference compared to the wild type in the antibiotic susceptibility testing, which was also seen in other studies, according to which E102K does not drastically alter the resistance spectrum, unlike G236S and R162S[10]. These studies also observed that E102K has a higher influence on the antibiotic susceptibility

when present in the background of other mutations, such as G236S and R162S and this was also seen in this study where the MIC value of *tem*(E102K, R162S) was 3 µg/ml and the MIC value of the single mutant *tem*(E102K) was 0.19 µg/ml. R162 alone has been shown to give increased resistance to both cefotaxime and ceftazidime[10], however in this study R162S resulted in no change in susceptibility to cefotaxime compared to *tem*-1. The influence of R162 to ceftazidime susceptibility, though, was consistent with previous studies[10], since its MIC value was more than twice as high as the MIC value of *tem*-1 (0.5 vs 0.19). Hence the decision to use *tem*(R162S) and ceftazidime in the competition experiment.

In the competition experiment we wanted to find a concentration at which both the wild type and the mutant are alive, and at which the mutant has a higher fitness than the wild type. This would enable us to find the right conditions for an evolution experiment where we could test if HGT increases the probability of mutations in *tem-1* to remain in the population despite the occurrence of mutations in other genes than *tem-1* that also confer an antibiotic resistance. However, a right concentration could not be found since the mutant did not show to have an advantage at concentrations at which the wild type was alive.

The wild type was already dead at concentration 1/2xMIC_{wt}, which lead to the assumption that perhaps the MIC-value determined on solid medium differs from the MIC-value in liquid medium that was used in the competition experiment. In other studies, it has also been observed that liquid medium gave commonly lower MICs than agar medium[22]. Perhaps a right antibiotic concentration for HGT was not found due to the wrong MIC-value and the wide concentration intervals used in the competition experiment. The correct concentration should be between 1/4xMIC and 1/2xMIC (0.05 and 0.10 g/ml) because none of the strains had an advantage beneath 1/4xMIC and the wild type died at 1/2xMIC.

Besides the premature cell death in the competition experiment, it was also observed that at 1/4xMIC the fitness of the wild type became higher than that of the mutant from generations 20 to 30. A hypothesis was made that this was due to induced transduction of phages by the antibiotic. Perhaps the phages transferred *tem*-1 to the cells containing *tem*(R162S) and resulted in a lower fitness of the mutant. This assumption was supported by another study, in which induction of the SOS response by β-lactams in *E. coli* was observed, through inactivation of the penicillin-binding protein 3 (PBP3) and increased expression of the *dpiBA* operon[23]. β-lactam antibiotics have been found to induce SOS response in *S. aureus* as well[24].

The SOS response, in turn, induces P22, which can promote the dissemination of antibiotic resistance genes[21, 24].

The experiments carried out to detect induced transduction by TZ showed that Sub-MIC [TZ] did not induce GTA[22]. It was observed that the number of transductants in media lacking TZ and in media with low TZ concentrations increased from day 1 to day 2 (Table 7), but not in the media containing higher TZ concentrations. Therefore, the hypothesis about the TZ induced transfer of the *tem*-1 gene to a strain containing *tem*(R162S) was disproved. Apart from this, the increase of transductants was more noticeable on the plates with strains having *rfp* in *galK* than those containing *yfp* in *galK*, which indicated that perhaps the increased number of transductants was affected by the fluorescence markers, but not by the antibiotics. Perhaps another experiment can be designed to detect transductants, since the experiment carried out with growing cultures was not capable of detecting all types of transductants, for example His- Trp+ and His+ Trp-. Nevertheless, it was interesting to know if the direction of transduction in those few transductants was from the antibiotic sensitive cells to the antibiotic resistant cells and the results from the last experiment confirmed this assumption.

Understanding how HGT affects the evolution of new functions, or the driving force of evolution in general, can help us to combat the constantly increasing problem of antibiotic resistance. We cannot stop the evolution, but perhaps we can slow it down and we can also be one step ahead in terms of treatment recommendations or development of new more efficient drugs.

6. Conclusion

Another attempt can be made to find optimal conditions for studying how HGT can affect the evolution of new functions. First, the MIC-value of TZ needs to be determined in liquid media to confirm that the MIC-value determined with E-tests in this study is higher than the actual one. Then based on this MIC-value new and smaller antibiotic concentration interval needs to be determined for the competition experiment. Besides the concentrations, the competition experiment can be repeated under the same conditions as in this study. If the wild type still has a fitness advantage over the mutant, then another reason for this than antibiotic-induced GTA should be investigated.

7. References

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Supplementary information

Table S1. Primers used for construction of strains and sequencing

Primer name	Primer sequence
IS200#6f-P2V2	CTCCTCATATTTGCGGCAGCGTAGTCTGCCGCTTTTTTG**CATATGAATATCCTCCTTAGTTCC**[a]
PCP25r-P1	CACTACATGTCAAGAATAAACTGCCAAAGCTCTAGAAGC**GTGTAGGCTGGAGCTGCTTC**[a]
IS200#6-chk_fwd	CGGATTACTTTGTGGTGTAG
CP25-chk_rev	CCTCCTAAGGTCTCGAAAAGTTA
tmp_midR	GGAAGAAGGCGTCACCCTCG
P1	GTGTAGGCTGGAGCTGCTTC
cat_midR2	CCGACATGGAAGCCATCAC
cat_midF	CGACGATTTCCGGCAGTTTC
Tem-R164rCP	GCTCGTCGTTTGGTA*TGGCTTCATTCAGCTCCGGTTCCCA***TTAGGCGACCACAGGTTTGC**[a]
Tem-R164S-fCP	GGGGGATCATGTAACTCGCCTTGATtcg*TGGGAACCGGAGCTGAATGAAGCCA***TTAGGCGACCACAGGTTTGC**[a]
DEL-tem_fCP	TATTGAAAAAGGAAGAGTATGAGTA*TTCAACATTTCCGTGAGATAGGTGCCTCAC***TTAGGCGACCACAGGTTTGC**[a]
DEL-tem_rCP	TATTTTTATTACCAATGCTTAATCA*GTGAGGCACCTATCTCACGGAAATGTTGAA***TTAGGCGACCACAGGTTTGC**[a]

[a] Long primers for lambda Red. The 3'-end that acts as primer for the first cycles of PCR is indicated in bold text. In primers for DIRex, the part of the primer that generates the direct repeats is indicated in italics, and the point mutation in Tem-R164SrCP is indicated in lower case letters.

Table S2. Strains constructed in this study

Strain	Genotype	Source
DA73894	*Δ*(IS*200#6*)*::*[*dhfr-bla*$_{TEM1}$*-CP25*-mTagBFP2] *Δ*(*flhE-flhD*) *ΔaraBAD::ant*(P22) / P22 *[Δ(gtr-'thrW) Δ(thrW-kil)* HT 105/1 sieA44]	This study
DA73895	*Δ*(IS*200#6*)*::*[*dhfr-bla*$_{TEM1}$*-CP25*-mTagBFP2] *Δ*(*flhE-flhD*) *ΔaraBAD::ant*(P22) / P22 *[Δ(gtr-'thrW) Δ(thrW-kil)* HT 105/1 sieA44]	This study
DA73896	*Δ*(IS*200#6*)*::*[*dhfr-bla*$_{TEM(E102K\ G236S)}$*-CP25*-mTagBFP2] *Δ*(*flhE-flhD*) *ΔaraBAD::ant*(P22) / P22 *[Δ(gtr-'thrW) Δ(thrW-kil)* HT 105/1 sieA44]	This study
DA73897	*Δ*(IS*200#6*)*::*[*dhfr-bla*$_{TEM(E102K\ G236S)}$*-CP25*-mTagBFP2] *Δ*(*flhE-flhD*) *ΔaraBAD::ant*(P22) / P22 *[Δ(gtr-'thrW) Δ(thrW-kil)* HT 105/1 sieA44]	This study
DA73898	*Δ*(IS*200#6*)*::*[*dhfr-bla*$_{TEM(R162S)}$*-CP25*-mTagBFP2] *Δ*(*flhE-flhD*) *ΔaraBAD::ant*(P22) / P22 *[Δ(gtr-'thrW) Δ(thrW-kil)* HT 105/1 sieA44]	This study
DA73899	*Δ*(IS*2000#6*)*::*[*dhfr-bla*$_{TEM(R162S)}$*-CP25*-mTagBFP2] *Δ*(*flhE-flhD*) *ΔaraBAD::ant*(P22) / P22 *[Δ(gtr-'thrW) Δ(thrW-kil)* HT 105/1 sieA44]	This study
DA73900	*Δ*(IS*200#6*)*::*[*dhfr-bla*$_{TEM(E102K\ R162S)}$*-CP25*-mTagBFP2] *Δ*(*flhE-flhD*) *ΔaraBAD::ant*(P22) / P22 *[Δ(gtr-'thrW) Δ(thrW-kil)* HT 105/1 sieA44]	This study
DA73901	*Δ*(IS*200#6*)*::*[*dhfr-bla*$_{TEM(E102K\ R162S)}$*-CP25*-mTagBFP2] *Δ*(*flhE-flhD*) *ΔaraBAD::ant*(P22) / P22 *[Δ(gtr-'thrW) Δ(thrW-kil)* HT 105/1 sieA44]	This study
DA73902	*Δ*(IS*200#6*)*::*[*dhfr-bla*$_{TEM1}$*-CP25*-cometGFP] *Δ*(*flhE-flhD*) *ΔaraBAD::ant*(P22) / P22 *[Δ(gtr-'thrW) Δ(thrW-kil)* HT 105/1 sieA44]	This study
DA73903	*Δ*(IS*200#6*)*::*[*dhfr-bla*$_{TEM1}$*-CP25*-cometGFP] *Δ*(*flhE-flhD*) *ΔaraBAD::ant*(P22) / P22 *[Δ(gtr-'thrW) Δ(thrW-kil)* HT 105/1 sieA44]	This study
DA73904	*Δ*(IS*200#6*)*::*[*dhfr-bla*$_{TEM(E102K\ G236S)}$*-CP25*-cometGFP] *Δ*(*flhE-flhD*) *ΔaraBAD::ant*(P22) / P22 *[Δ(gtr-'thrW) Δ(thrW-kil)* HT 105/1 sieA44]	This study
DA73905	*Δ*(IS*200#6*)*::*[*dhfr-bla*$_{TEM(E102K\ G236S)}$*-CP25*-cometGFP] *Δ*(*flhE-flhD*) *ΔaraBAD::ant*(P22) / P22 *[Δ(gtr-'thrW) Δ(thrW-kil)* HT 105/1 sieA44]	This study
DA73906	*Δ*(IS*200#6*)*::*[*dhfr-bla*$_{TEM(R162S)}$*-CP25*-cometGFP] *Δ*(*flhE-flhD*) *ΔaraBAD::ant*(P22) / P22 *[Δ(gtr-'thrW) Δ(thrW-kil)* HT 105/1 sieA44]	This study
DA73907	*Δ*(IS*200#6*)*::*[*dhfr-bla*$_{TEM(R162S)}$*-CP25*-cometGFP] *Δ*(*flhE-flhD*) *ΔaraBAD::ant*(P22) / P22 *[Δ(gtr-'thrW) Δ(thrW-kil)* HT 105/1 sieA44]	This study
DA73908	*Δ*(IS*200#6*)*::*[*dhfr-bla*$_{TEM(E102K\ R162S)}$*-CP25*-cometGFP] *Δ*(*flhE-flhD*) *ΔaraBAD::ant*(P22) / P22 *[Δ(gtr-'thrW) Δ(thrW-kil)* HT 105/1 sieA44]	This study
DA73909	*Δ*(IS*200#6*)*::*[*dhfr-bla*$_{TEM(E102K\ R162S)}$*-CP25*-cometGFP] *Δ*(*flhE-flhD*) *ΔaraBAD::ant*(P22) / P22 *[Δ(gtr-'thrW) Δ(thrW-kil)* HT 105/1 sieA44]	This study
DA73910	*Δ*(IS*200#6*)*::*[*dhfr-bla*$_{TEM1}$*-CP25*-SYFP2] *Δ*(*flhE-flhD*) *ΔaraBAD::ant*(P22) / P22 *[Δ(gtr-'thrW) Δ(thrW-kil)* HT 105/1 sieA44]	This study
DA73911	*Δ*(IS*200#6*)*::*[*dhfr-bla*$_{TEM1}$*-CP25*-SYFP2] *Δ*(*flhE-flhD*) *ΔaraBAD::ant*(P22) / P22 *[Δ(gtr-'thrW) Δ(thrW-kil)* HT 105/1 sieA44]	This study
DA73912	*Δ*(IS*200#6*)*::*[*dhfr-bla*$_{TEM(E102K\ G236S)}$*-CP25*-SYFP2] *Δ*(*flhE-flhD*) *ΔaraBAD::ant*(P22) / P22 *[Δ(gtr-'thrW) Δ(thrW-kil)* HT 105/1 sieA44]	This study
DA73913	*Δ*(IS*200#6*)*::*[*dhfr-bla*$_{TEM(E102K\ G236S)}$*-CP25*-SYFP2] *Δ*(*flhE-flhD*) *ΔaraBAD::ant*(P22) / P22 *[Δ(gtr-'thrW) Δ(thrW-kil)* HT 105/1 sieA44]	This study
DA73914	*Δ*(IS*200#6*)*::*[*dhfr-bla*$_{TEM(R162S)}$*-CP25*-SYFP2] *Δ*(*flhE-flhD*) *ΔaraBAD::ant*(P22) / P22 *[Δ(gtr-'thrW) Δ(thrW-kil)* HT 105/1 sieA44]	This study
DA73915	*Δ*(IS*200#6*)*::*[*dhfr-bla*$_{TEM(R162S)}$*-CP25*-SYFP2] *Δ*(*flhE-flhD*) *ΔaraBAD::ant*(P22) / P22 *[Δ(gtr-'thrW) Δ(thrW-kil)* HT 105/1 sieA44]	This study
DA73916	*Δ*(IS*200#6*)*::*[*dhfr-bla*$_{TEM(E102K\ R162S)}$*-CP25*-SYFP2] *Δ*(*flhE-flhD*) *ΔaraBAD::ant*(P22) / P22 *[Δ(gtr-'thrW) Δ(thrW-kil)* HT 105/1 sieA44]	This study
DA73917	*Δ*(IS*200#6*)*::*[*dhfr-bla*$_{TEM(E102K\ R162S)}$*-CP25*-SYFP2] *Δ*(*flhE-flhD*) *ΔaraBAD::ant*(P22) / P22 *[Δ(gtr-'thrW) Δ(thrW-kil)* HT 105/1 sieA44]	This study
DA73918	*Δ*(IS*200#6*)*::*[*dhfr-bla*$_{TEM1}$*-CP25*-mScarlet] *Δ*(*flhE-flhD*) *ΔaraBAD::ant*(P22) / P22 *[Δ(gtr-'thrW) Δ(thrW-kil)* HT 105/1 sieA44]	This study
DA73919	*Δ*(IS*200#6*)*::*[*dhfr-bla*$_{TEM1}$*-CP25*-mScarlet] *Δ*(*flhE-flhD*) *ΔaraBAD::ant*(P22) / P22 *[Δ(gtr-'thrW) Δ(thrW-kil)* HT 105/1 sieA44]	This study
DA73920	*Δ*(IS*200#6*)*::*[*dhfr-bla*$_{TEM(E102K\ G236S)}$*-CP25*-mScarlet] *Δ*(*flhE-flhD*) *ΔaraBAD::ant*(P22) / P22 *[Δ(gtr-'thrW) Δ(thrW-kil)* HT 105/1 sieA44]	This study
DA73921	*Δ*(IS*200#6*)*::*[*dhfr-bla*$_{TEM(E102K\ G236S)}$*-CP25*-mScarlet] *Δ*(*flhE-flhD*) *ΔaraBAD::ant*(P22) / P22 *[Δ(gtr-'thrW) Δ(thrW-kil)* HT 105/1 sieA44]	This study
DA73922	*Δ*(IS*200#6*)*::*[*dhfr-bla*$_{TEM(R162S)}$*-CP25*-mScarlet] *Δ*(*flhE-flhD*) *ΔaraBAD::ant*(P22) / P22 *[Δ(gtr-'thrW) Δ(thrW-kil)* HT 105/1 sieA44]	This study
DA73923	*Δ*(IS*200#6*)*::*[*dhfr-bla*$_{TEM(R162S)}$*-CP25*-mScarlet] *Δ*(*flhE-flhD*) *ΔaraBAD::ant*(P22) / P22 *[Δ(gtr-'thrW) Δ(thrW-kil)* HT 105/1 sieA44]	This study
DA73924	*Δ*(IS*200#6*)*::*[*dhfr-bla*$_{TEM(E102K\ R162S)}$*-CP25*-mScarlet] *Δ*(*flhE-flhD*) *ΔaraBAD::ant*(P22) / P22 *[Δ(gtr-'thrW) Δ(thrW-kil)* HT 105/1 sieA44]	This study
DA73925	*Δ*(IS*200#6*)*::*[*dhfr-bla*$_{TEM(E102K\ R162S)}$*-CP25*-mScarlet] *Δ*(*flhE-flhD*) *ΔaraBAD::ant*(P22) / P22 *[Δ(gtr-'thrW) Δ(thrW-kil)* HT 105/1 sieA44]	This study
DA73975	*Δ*(IS*200#6*)*::*[*dhfr-bla*$_{TEM(E102K)}$*-CP25*-mTagBFP2] *Δ*(*flhE-flhD*) *ΔaraBAD::ant*(P22) / P22 *[Δ(gtr-'thrW) Δ(thrW-kil)* HT 105/1 sieA44]	This study
DA73976	*Δ*(IS*200#6*)*::*[*dhfr-bla*$_{TEM(E102K)}$*-CP25*-cometGFP] *Δ*(*flhE-flhD*) *ΔaraBAD::ant*(P22) / P22 *[Δ(gtr-'thrW) Δ(thrW-kil)* HT 105/1 sieA44]	This study
DA73977	*Δ*(IS*200#6*)*::*[*dhfr-bla*$_{TEM(E102K)}$*-CP25*-SYFP2] *Δ*(*flhE-flhD*) *ΔaraBAD::ant*(P22) / P22 *[Δ(gtr-'thrW) Δ(thrW-kil)* HT 105/1 sieA44]	This study
DA73978	*Δ*(IS*200#6*)*::*[*dhfr-bla*$_{TEM(E102K)}$*-CP25*-mScarlet] *Δ*(*flhE-flhD*) *ΔaraBAD::ant*(P22) / P22 *[Δ(gtr-'thrW) Δ(thrW-kil)* HT 105/1 sieA44]	This study
DA73979	*Δ*(IS*200#6*)*::*[*dhfr-bla*$_{TEM1}$*-CP25*-mTagBFP2] *Δ*(*flhE-flhD*) *ΔaraBAD::ant*(P22) / P22 *[Δ(gtr-'thrW) Δ(thrW-kil)* HT 105/1 sieA44]/ pSIM5-Tet	This study
DA73980	*Δ*(IS*200#6*)*::*[*dhfr-bla*$_{TEM(E102K\ G236S)}$*-CP25*-mTagBFP2] *Δ*(*flhE-flhD*) *ΔaraBAD::ant*(P22) / P22 *[Δ(gtr-'thrW) Δ(thrW-kil)* HT 105/1 sieA44]/ pSIM5-Tet	This study
DA73981	*Δ*(IS*200#6*)*::*[*dhfr-bla*$_{TEM1}$*-CP25*-cometGFP] *Δ*(*flhE-flhD*) *ΔaraBAD::ant*(P22) / P22 *[Δ(gtr-'thrW) Δ(thrW-kil)* HT 105/1 sieA44]/ pSIM5-Tet	This study

Strain	Genotype	Source
DA73982	*Δ*(IS*200#6*)*::*[*dhfr-bla*$_{TEM(E102K\ G236S)}$*-CP25*-cometGFP]*Δ*(*flhE-flhD*) *ΔaraBAD::ant*(P22) / P22 *[Δ(gtr-'thrW) Δ(thrW-kil)* HT 105/1 sieA44]/ pSIM5-Tet	This study
DA73983	*Δ*(IS*200#6*)*::*[*dhfr-bla*$_{TEM1}$*-CP25*-SYFP2] *Δ*(*flhE-flhD*) *ΔaraBAD::ant*(P22) / P22 *[Δ(gtr-'thrW) Δ(thrW-kil)* HT 105/1 sieA44]/ pSIM5-Tet	This study
DA73984	*Δ*(IS*200#6*)*::*[*dhfr-bla*$_{TEM(E102K\ G236S)}$*-CP25*-SYFP2] *Δ*(*flhE-flhD*) *ΔaraBAD::ant*(P22) / P22 *[Δ(gtr-'thrW) Δ(thrW-kil)* HT 105/1 sieA44]/ pSIM5-Tet	This study
DA73985	*Δ*(IS*200#6*)*::*[*dhfr-bla*$_{TEM(E102K\ G236S)}$*-CP25*-mScarlet] *Δ*(*flhE-flhD*) *ΔaraBAD::ant*(P22) / P22 *[Δ(gtr-'thrW) Δ(thrW-kil)* HT 105/1 sieA44]/ pSIM5-Tet	This study
DA74823	*Δ*(IS*200#6*)*::*[*dhfr-bla*$_{TEM1}$*-CP25*-mScarlet] *Δ*(*flhE-flhD*) *ΔaraBAD::ant*(P22) / P22 *[Δ(gtr-'thrW) Δ(thrW-kil)* HT 105/1 sieA44] / pSIM5-Tet	This study
DA74893	"headful-2"::[*Δbla*$_{TEM}$*-dhfr*] *ΔaraBAD::ant*(P22) *treB*(+) *malT*(+) *malQ*(+) *malP*(+) / P22 [*Δ(gtr-'thrW) Δ(thrW-kil)* HT 105/1 sieA44]	This study
DA74894	"headful-2"::[*bla*$_{TEM(E102K\ R162S\ G236S)}$*-dhfr*] *ΔaraBAD::ant*(P22) *treB*(+) *malT*(+) *malQ*(+) *malP*(+) / P22 [*Δ(gtr-'thrW) Δ(thrW-kil)* HT 105/1 sieA44]	This study
DA75229	*galK::cat-J23101*-dTomato *ΔaraBAD::ant*(P22) *ΔhisA ΔtrpF ΔParaE::J23106* / P22 [*Δ(gtr-'thrW) Δ(thrW-kil)* HT 105/1 sieA44]	This study
DA75230	*galK::cat-J23101*-SYFP2 *ΔaraBAD::ant*(P22) *ΔhisA ΔtrpF ΔParaE::J23106* / P22 [*Δ(gtr-'thrW) Δ(thrW-kil)* HT 105/1 sieA44]	This study

Table S3. PCR program used for amplification of tem and dhfr gene with the primers IS200#6f-P2V2 and PCP25r-P1

First step	Temperature	Time	Number of cycles
Initial denaturation	98°C	2 min	1
Denaturation	98°C	20 sec	
Annealing	72°C	5 sec	5
Annealing (slow)	57°C	20 sec	
Extension (slow)	72°C	2 min	
Second step			
Denaturation	98°C	20 sec	25
Extension	72°C	2 min	
Third step			
Extension	72°C	10 min	1

Table S4. PCR program used for amplification of tem and dhfr gene with the primers IS200#6-chk_fwd and CP25-chk_rev

First step	Temperature	Time	Number of cycles
Initial denaturation	98°C	2 min	1
Denaturation	98°C	20 sec	
Annealing	61°C	20 sec	30
Extension	72°C	2 min	
Second step			
Extension	72°C	5 min	1

Table S5. PCR program used for amplification of tem and dhfr gene with the primers tmp_midR and CP25-chk_rev

First step	Temperature	Time	Number of cycles
Initial denaturation	98°C	2 min	1
Denaturation	98°C	20 sec	
Annealing	62°C	20 sec	30
Extension	72°C	2 min	
Second step			
Extension	72°C	5 min	1

Table S6. PCR program used for amplification of DIRex cassettes: Acatsac1 and Acatsac3 with the primers catmidR2, catmidF, Tem-R164rCP, Tem-R164fCP, DEL-tem_rCP and DEL-tem_fCP

First step	Temperature	Time	Number of cycles
Initial denaturation	98°C	2 min	1
Denaturation	98°C	20 sec	
Annealing	72°C	5 sec	5
Annealing (slow)	57°C	20 sec	
Extension (slow)	72°C	3 min	
Second step			
Denaturation	98°C	20 sec	
Annealing	65°C	20 sec	25
Extension	72°C	3 min	
Third step			
Extension	72°C	10 min	1

Table S7. PCR program used for amplification of tem deletion and tem(E102K G236S R162S) with the primers P1 and tmp_midR

First step	Temperature	Time	Number of cycles
Initial denaturation	98°C	2 min	1
Denaturation	98°C	20 sec	
Annealing	66°C	20 sec	30
Extension	72°C	1.5 min	
Second step			
Extension	72°C	5 min	1

Table S8. Data from day 4 of the competition experiment. Two biological replicates (MIX A1 and A2; MIX B1 and B2) and two technical replicates of each of the mixes were analyzed with MACSQuant VYB. The ratio of every culture and the reference culture (BFP in MIX A1 and A2; YFP in MIX B1 and B2) was calculated for the TZ concentrations 0xMIC, 1/16xMIC, 1/8xMIC, 1/4xMIC, 1/2xMIC and 1xMIC.

				Concentrations					
MIX	**Biological replicate**	**Technical replicate**	**Ratio**	**0xMIC**	**1/16xMIC**	**1/8xMIC**	**1/4xMIC**	**1/2xMIC**	**1xMIC**
A1	1	1	YFP/(YFP+BFP+GFP+RFP)	0.250	0.250	0.259	0.334	0.267	0.500
A1	1	1	RFP/(YFP+BFP+GFP+RFP)	0.260	0.261	0.281	0.358	0.272	0.496
A1	1	1	GFP/(YFP+BFP+GFP+RFP)	0.247	0.256	0.232	0.150	0.203	0.001
A1	1	1	BFP/(YFP+BFP+GFP+RFP)	0.243	0.233	0.228	0.158	0.259	0.002
A1	1	2	YFP/(YFP+BFP+GFP+RFP)	0.251	0.249	0.258	0.310	0.469	0.494
A1	1	2	RFP/(YFP+BFP+GFP+RFP)	0.265	0.261	0.273	0.337	0.501	0.502
A1	1	2	GFP/(YFP+BFP+GFP+RFP)	0.245	0.251	0.238	0.168	0.011	0.001
A1	1	2	BFP/(YFP+BFP+GFP+RFP)	0.239	0.239	0.231	0.184	0.019	0.002
A2	2	1	YFP/(YFP+BFP+GFP+RFP)	0.251	0.248	0.249	0.352	0.472	0.521
A2	2	1	RFP/(YFP+BFP+GFP+RFP)	0.261	0.261	0.270	0.384	0.502	0.475
A2	2	1	GFP/(YFP+BFP+GFP+RFP)	0.255	0.259	0.250	0.141	0.010	0.001
A2	2	1	BFP/(YFP+BFP+GFP+RFP)	0.233	0.232	0.230	0.123	0.015	0.002
A2	2	2	YFP/(YFP+BFP+GFP+RFP)	0.250	0.247	0.253	0.413	0.467	0.519
A2	2	2	RFP/(YFP+BFP+GFP+RFP)	0.262	0.266	0.271	0.448	0.506	0.479
A2	2	2	GFP/(YFP+BFP+GFP+RFP)	0.258	0.254	0.250	0.072	0.011	0.001
A2	2	2	BFP/(YFP+BFP+GFP+RFP)	0.230	0.232	0.226	0.067	0.017	0.002
B1	1	1	BFP/(BFP+GFP+RFP+YFP)	0.242	0.239	0.255	0.347	0.483	0.489
B1	1	1	GFP/(BFP+GFP+RFP+YFP)	0.247	0.251	0.268	0.357	0.507	0.492
B1	1	1	RFP/(BFP+GFP+RFP+YFP)	0.263	0.266	0.255	0.147	0.003	0.002
B1	1	1	YFP/(BFP+GFP+RFP+YFP)	0.247	0.244	0.223	0.149	0.008	0.017
B1	1	2	BFP/(BFP+GFP+RFP+YFP)	0.238	0.238	0.257	0.293	0.483	0.482
B1	1	2	GFP/(BFP+GFP+RFP+YFP)	0.252	0.252	0.268	0.306	0.502	0.503
B1	1	2	RFP/(BFP+GFP+RFP+YFP)	0.255	0.255	0.240	0.175	0.004	0.002
B1	1	2	YFP/(BFP+GFP+RFP+YFP)	0.255	0.254	0.235	0.227	0.011	0.012
B2	2	1	BFP/(BFP+GFP+RFP+YFP)	0.240	0.183	0.261	0.307	0.482	0.492
B2	2	1	GFP/(BFP+GFP+RFP+YFP)	0.246	0.264	0.272	0.309	0.500	0.495
B2	2	1	RFP/(BFP+GFP+RFP+YFP)	0.264	0.219	0.246	0.191	0.004	0.002
B2	2	1	YFP/(BFP+GFP+RFP+YFP)	0.250	0.333	0.221	0.193	0.015	0.012
B2	2	2	BFP/(BFP+GFP+RFP+YFP)	0.258	0.252	0.260	0.408	0.501	0.507
B2	2	2	GFP/(BFP+GFP+RFP+YFP)	0.236	0.246	0.230	0.358	0.476	0.479
B2	2	2	RFP/(BFP+GFP+RFP+YFP)	0.239	0.240	0.287	0.110	0.006	0.002
B2	2	2	YFP/(BFP+GFP+RFP+YFP)	0.267	0.262	0.223	0.124	0.017	0.012

Table S9. Data from day 5 of the competition experiment. Two biological replicates (MIX A1 and A2; MIX B1 and B2) and two technical replicates of each of the mixes were analyzed with MACSQuant VYB. The ratio of every culture and the reference culture (BFP in MIX A1 and A2; YFP in MIX B1 and B2) was calculated for the TZ concentrations 0xMIC, 1/16xMIC, 1/8xMIC, 1/4xMIC, 1/2xMIC and 1xMIC.

				Concentrations					
MIX	**Biological replicate**	**Technical replicate**	**Ratio**	**0xMIC**	**1/16xMIC**	**1/8xMIC**	**1/4xMIC**	**1/2xMIC**	**1xMIC**
A1	1	1	YFP/(YFP+BFP+GFP+RFP)	0.004	0.239	0.252	0.282	0.438	0.487
A1	1	1	RFP/(YFP+BFP+GFP+RFP)	0.293	0.253	0.262	0.293	0.562	0.510
A1	1	1	GFP/(YFP+BFP+GFP+RFP)	0.333	0.253	0.237	0.182	0.000	0.001
A1	1	1	BFP/(YFP+BFP+GFP+RFP)	0.371	0.255	0.248	0.243	0.000	0.001
A1	1	2	YFP/(YFP+BFP+GFP+RFP)	0.259	0.243	0.259	0.252	0.491	0.503
A1	1	2	RFP/(YFP+BFP+GFP+RFP)	0.232	0.250	0.263	0.264	0.507	0.492
A1	1	2	GFP/(YFP+BFP+GFP+RFP)	0.251	0.256	0.233	0.202	0.001	0.002
A1	1	2	BFP/(YFP+BFP+GFP+RFP)	0.258	0.250	0.245	0.281	0.002	0.002
A2	2	1	YFP/(YFP+BFP+GFP+RFP)	0.238	0.240	0.251	0.307	0.445	0.551
A2	2	1	RFP/(YFP+BFP+GFP+RFP)	0.270	0.261	0.275	0.344	0.553	0.448
A2	2	1	GFP/(YFP+BFP+GFP+RFP)	0.265	0.271	0.259	0.182	0.001	0.000
A2	2	1	BFP/(YFP+BFP+GFP+RFP)	0.227	0.228	0.215	0.167	0.001	0.000
A2	2	2	YFP/(YFP+BFP+GFP+RFP)	0.247	0.237	0.246	0.324	0.441	0.553
A2	2	2	RFP/(YFP+BFP+GFP+RFP)	0.265	0.266	0.289	0.374	0.555	0.446
A2	2	2	GFP/(YFP+BFP+GFP+RFP)	0.270	0.273	0.254	0.167	0.002	0.000
A2	2	2	BFP/(YFP+BFP+GFP+RFP)	0.219	0.225	0.212	0.135	0.003	0.000
B1	1	1	BFP/(BFP+GFP+RFP+YFP)	0.232	0.229	0.238	0.221	0.473	0.472
B1	1	1	GFP/(BFP+GFP+RFP+YFP)	0.257	0.251	0.272	0.246	0.522	0.514
B1	1	1	RFP/(BFP+GFP+RFP+YFP)	0.232	0.254	0.218	0.199	0.002	0.000
B1	1	1	YFP/(BFP+GFP+RFP+YFP)	0.279	0.266	0.272	0.335	0.003	0.013
B1	1	2	BFP/(BFP+GFP+RFP+YFP)	0.225	0.226	0.257	0.283	0.495	0.471
B1	1	2	GFP/(BFP+GFP+RFP+YFP)	0.259	0.253	0.291	0.317	0.503	0.522
B1	1	2	RFP/(BFP+GFP+RFP+YFP)	0.258	0.240	0.189	0.173	0.001	0.000
B1	1	2	YFP/(BFP+GFP+RFP+YFP)	0.258	0.282	0.264	0.227	0.001	0.008
B2	2	1	BFP/(BFP+GFP+RFP+YFP)	0.232	0.325	0.246	0.268	0.467	0.474
B2	2	1	GFP/(BFP+GFP+RFP+YFP)	0.257	0.201	0.271	0.298	0.530	0.516
B2	2	1	RFP/(BFP+GFP+RFP+YFP)	0.257	0.231	0.239	0.213	0.001	0.000
B2	2	1	YFP/(BFP+GFP+RFP+YFP)	0.254	0.243	0.244	0.221	0.002	0.011
B2	2	2	BFP/(BFP+GFP+RFP+YFP)	0.250	0.238	0.221	0.239	0.494	0.496
B2	2	2	GFP/(BFP+GFP+RFP+YFP)	0.250	0.260	0.213	0.218	0.503	0.484
B2	2	2	RFP/(BFP+GFP+RFP+YFP)	0.240	0.234	0.320	0.239	0.001	0.000
B2	2	2	YFP/(BFP+GFP+RFP+YFP)	0.260	0.269	0.247	0.304	0.002	0.019

Table S10. Data from day 6 of the competition experiment. Two biological replicates (MIX A1 and A2; MIX B1 and B2) and two technical replicates of each of the mixes were analyzed with MACSQuant VYB. The ratio of every culture and the reference culture (BFP in MIX A1 and A2; YFP in MIX B1 and B2) was calculated for the TZ concentrations 0xMIC, 1/16xMIC, 1/8xMIC, 1/4xMIC, 1/2xMIC and 1xMIC.

				Concentrations					
MIX	**Biological replicate**	**Technical replicate**	**Ratio**	**0xMIC**	**1/16xMIC**	**1/8xMIC**	**1/4xMIC**	**1/2xMIC**	**1xMIC**
A1	1	1	YFP/(YFP+BFP+GFP+RFP)	0.269	0.252	0.240	0.187	0.415	0.530
A1	1	1	RFP/(YFP+BFP+GFP+RFP)	0.203	0.205	0.153	0.188	0.584	0.469
A1	1	1	GFP/(YFP+BFP+GFP+RFP)	0.243	0.233	0.269	0.248	0.000	0.000
A1	1	1	BFP/(YFP+BFP+GFP+RFP)	0.285	0.310	0.339	0.378	0.000	0.000
A1	1	2	YFP/(YFP+BFP+GFP+RFP)	0.267	0.254	0.242	0.169	0.417	0.521
A1	1	2	RFP/(YFP+BFP+GFP+RFP)	0.212	0.200	0.216	0.161	0.583	0.478
A1	1	2	GFP/(YFP+BFP+GFP+RFP)	0.239	0.252	0.254	0.250	0.000	0.000
A1	1	2	BFP/(YFP+BFP+GFP+RFP)	0.282	0.294	0.289	0.420	0.001	0.000
A2	2	1	YFP/(YFP+BFP+GFP+RFP)	0.244	0.258	0.226	0.117	0.404	0.495
A2	2	1	RFP/(YFP+BFP+GFP+RFP)	0.272	0.240	0.239	0.127	0.594	0.505
A2	2	1	GFP/(YFP+BFP+GFP+RFP)	0.252	0.264	0.282	0.388	0.001	0.000
A2	2	1	BFP/(YFP+BFP+GFP+RFP)	0.231	0.238	0.253	0.369	0.001	0.000
A2	2	2	YFP/(YFP+BFP+GFP+RFP)	0.256	0.242	0.223	0.157	0.380	0.515
A2	2	2	RFP/(YFP+BFP+GFP+RFP)	0.263	0.264	0.261	0.185	0.619	0.484
A2	2	2	GFP/(YFP+BFP+GFP+RFP)	0.262	0.267	0.289	0.339	0.000	0.000
A2	2	2	BFP/(YFP+BFP+GFP+RFP)	0.219	0.227	0.226	0.319	0.000	0.000
B1	1	1	BFP/(BFP+GFP+RFP+YFP)	0.231	0.200	0.203	0.146	0.472	0.483
B1	1	1	GFP/(BFP+GFP+RFP+YFP)	0.258	0.262	0.259	0.168	0.520	0.510
B1	1	1	RFP/(BFP+GFP+RFP+YFP)	0.191	0.211	0.182	0.248	0.003	0.000
B1	1	1	YFP/(BFP+GFP+RFP+YFP)	0.320	0.327	0.356	0.438	0.006	0.006
B1	1	2	BFP/(BFP+GFP+RFP+YFP)	0.208	0.210	0.200	0.442	0.495	0.252
B1	1	2	GFP/(BFP+GFP+RFP+YFP)	0.247	0.236	0.230	0.266	0.501	0.188
B1	1	2	RFP/(BFP+GFP+RFP+YFP)	0.200	0.199	0.205	0.100	0.001	0.231
B1	1	2	YFP/(BFP+GFP+RFP+YFP)	0.345	0.354	0.365	0.192	0.003	0.329
B2	2	1	BFP/(BFP+GFP+RFP+YFP)	0.234	0.328	0.218	0.127	0.453	0.485
B2	2	1	GFP/(BFP+GFP+RFP+YFP)	0.263	0.017	0.234	0.130	0.525	0.505
B2	2	1	RFP/(BFP+GFP+RFP+YFP)	0.220	0.120	0.242	0.343	0.012	0.001
B2	2	1	YFP/(BFP+GFP+RFP+YFP)	0.283	0.535	0.305	0.400	0.009	0.010
B2	2	2	BFP/(BFP+GFP+RFP+YFP)	0.250	0.230	0.201	0.098	0.497	0.499
B2	2	2	GFP/(BFP+GFP+RFP+YFP)	0.260	0.253	0.200	0.089	0.502	0.495
B2	2	2	RFP/(BFP+GFP+RFP+YFP)	0.206	0.220	0.286	0.353	0.000	0.000
B2	2	2	YFP/(BFP+GFP+RFP+YFP)	0.284	0.296	0.314	0.460	0.001	0.006

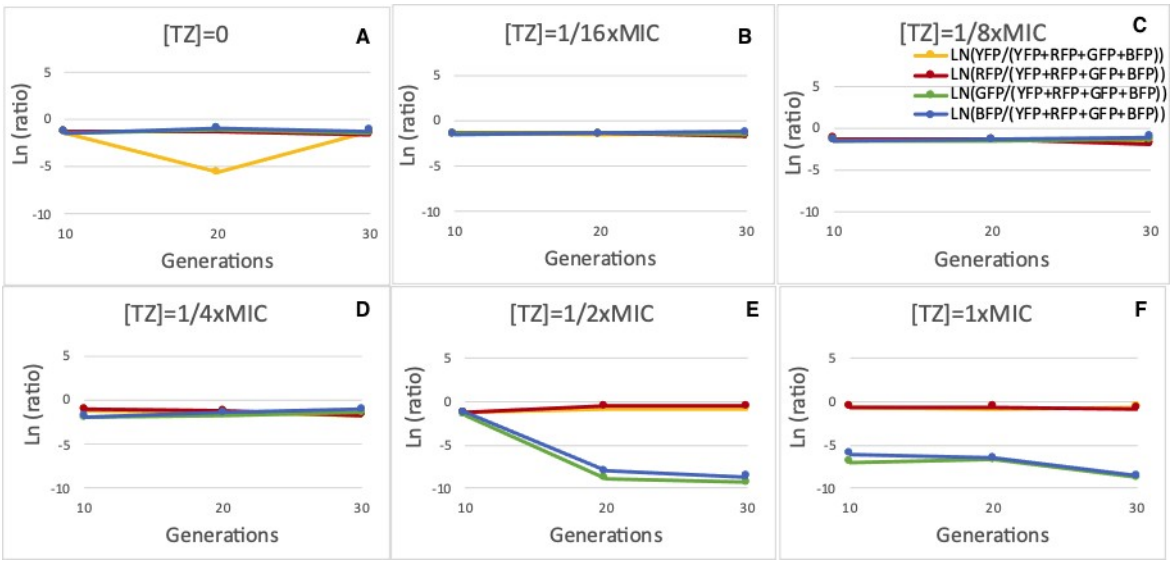

Figure S1. MIX A1, technical replicate 1. Ratios of the different fluorescent markers in the different cultures from generation 10 to 30 at the TZ concentrations 0xMIC (A), 1/16xMIC (B), 1/8xMIC (C), 1/4xMIC (D), 1/2xMIC (E) and 1xMIC (F). YFP/(YFP+RFP+GFP+BFP) and RFP/(YFP+RFP+GFP+BFP) represent the mutant *tem*(R162S) while GFP/(YFP+RFP+GFP+BFP) and BFP/(YFP+RFP+GFP+BFP) represent the wild type *tem-1*.

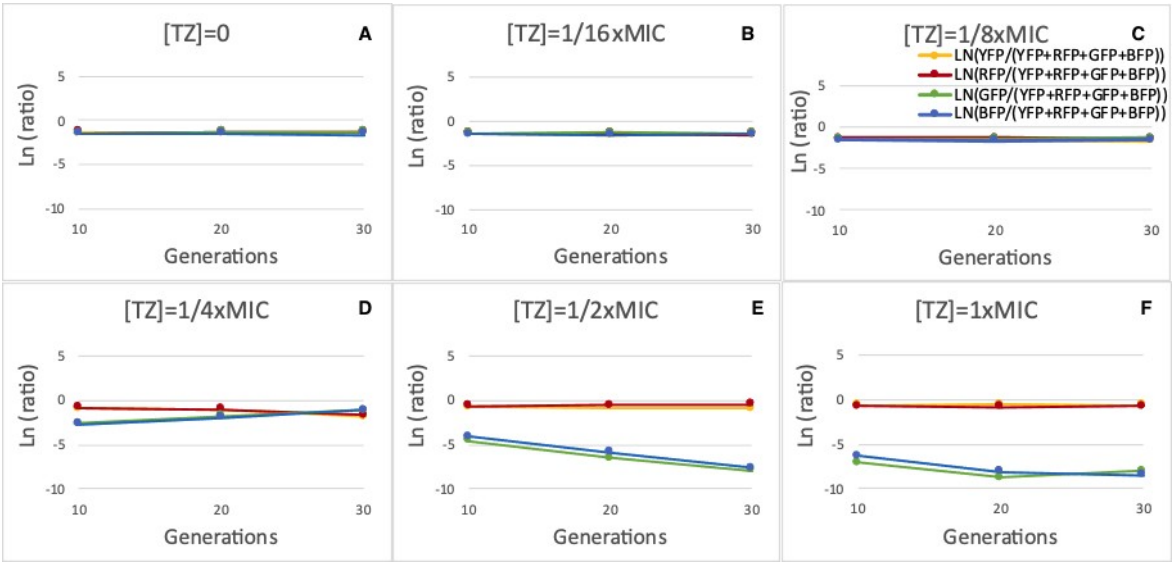

Figure S2. MIX A2, technical replicate 2. Ratios of the different fluorescent markers in the different cultures from generation 10 to 30 at the TZ concentrations 0xMIC (A), 1/16xMIC (B), 1/8xMIC (C), 1/4xMIC (D), 1/2xMIC (E) and 1xMIC (F). YFP/(YFP+RFP+GFP+BFP) and RFP/(YFP+RFP+GFP+BFP) represent the mutant *tem*(R162S) while GFP/(YFP+RFP+GFP+BFP) and BFP/(YFP+RFP+GFP+BFP) represent the wild type *tem-1*.

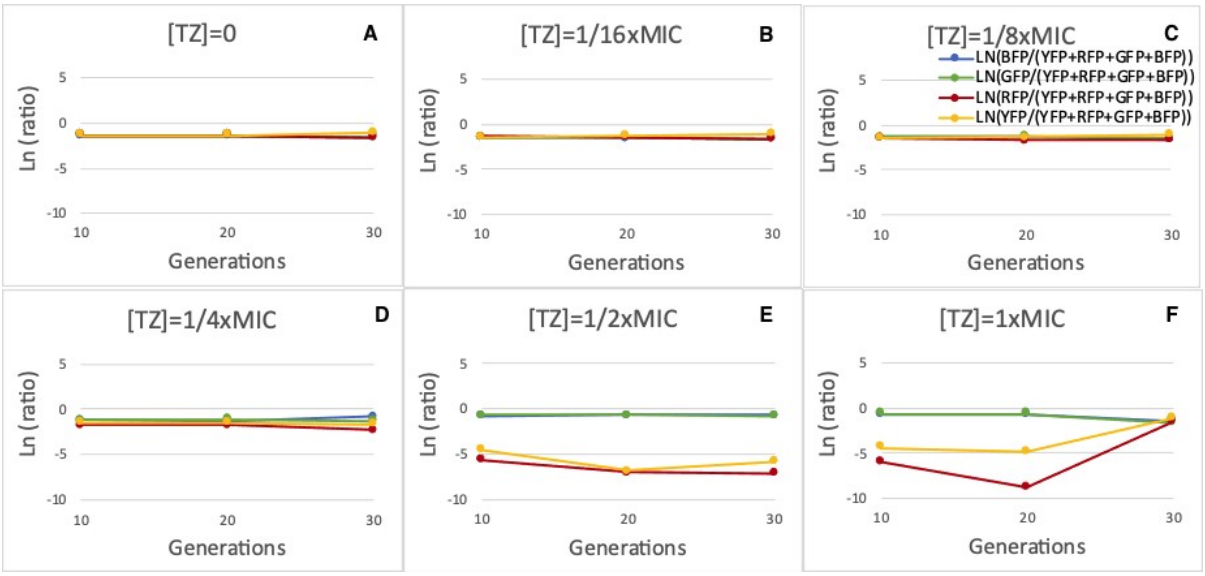

Figure S3. MIX B1, technical replicate 2. Ratios of the different fluorescent markers in the different cultures from generation 10 to 30 at the TZ concentrations 0xMIC (A), 1/16xMIC (B), 1/8xMIC (C), 1/4xMIC (D), 1/2xMIC (E) and 1xMIC (F). BFP/(YFP+RFP+GFP+BFP) and GFP/(YFP+RFP+GFP+BFP) represent the mutant *tem*(R162S), while YFP/(YFP+RFP+GFP+BFP) and RFP/(YFP+RFP+GFP+BFP) represent the wild type *tem-1*.

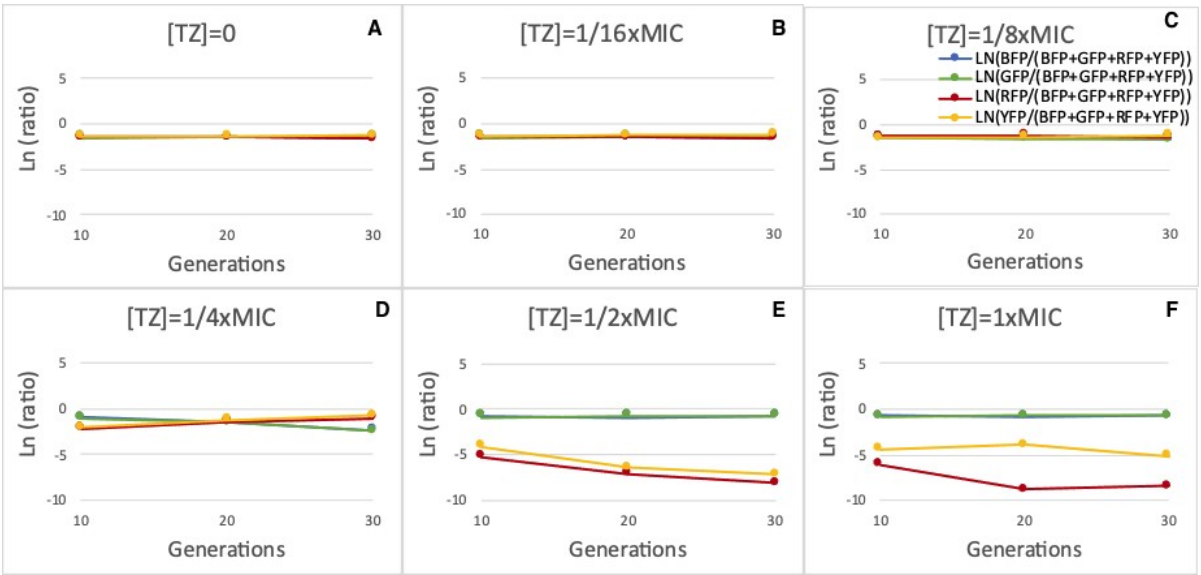

Figure S4. MIX B2, technical replicate 2. Ratios of the different fluorescent markers in the different cultures from generation 10 to 30 at the TZ concentrations 0xMIC (A), 1/16xMIC (B), 1/8xMIC (C), 1/4xMIC (D), 1/2xMIC (E) and 1xMIC (F). BFP/(YFP+RFP+GFP+BFP) and GFP/(YFP+RFP+GFP+BFP) represent the mutant *tem*(R162S), while YFP/(YFP+RFP+GFP+BFP) and RFP/(YFP+RFP+GFP+BFP) represent the wild type *tem-1*.