Within-patient evolution to piperacillin/tazobactam resistance in a clinical isolate of *Escherichia coli* due to IS*26*-mediated amplification of bla_{TEM-1B}.

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Abstract

A novel phenotype of *Escherichia coli* and *Klebsiella pneumoniae* resistant to piperacillin/tazobactam (TZP), but susceptible to carbapenems and 3rd generation cephalosporins has recently emerged. The resistance mechanism of this phenotype has been identified as hyperproduction of the β-lactamase bla_{TEM}, however the mechanism of hyperproduction in isolates lacking promoter region mutations is not well understood. We sought to understand this mechanism by focussing on a pair of isolates obtained from an individual patient across two infection episodes and displaying within-patient evolution to TZP resistance. Following confirmation that the two isolates were clonal, we found that the TZP-resistant isolate hyperproduced a β-lactamase but lacked mutations within β-lactamase promoter regions. Hybrid assembly of long and short sequencing reads of the two isolates revealed both harboured a novel IS*26*-flanked composite transposon containing several antibiotic resistance genes, including bla_{TEM-1B}, which was designated Tn*6762*. These resistance genes are also found to be present on a translocatable unit which had excised from Tn*6762* in the TZP-resistant isolate. By replicating the evolutionary event leading to TZP resistance we were able to observe excision of the translocatable unit from Tn*6762* following exposure to TZP and capture the TU in a plasmid containing a copy of IS*26*. Subsequent amplification of the TU, and by extension bla_{TEM-1B}, leads to β-lactamase hyperproduction and TZP resistance. Despite a significant increase in gene copy number (P value = <0.0001), we found that the TZP-resistant isolate was as fit as the susceptible ancestor. This mechanism of gene amplification, and the subsequent hyperproduction, of bla_{TEM-1B} is an important consideration when using genomic data to predict resistance/susceptibility to TZP.

Introduction

β-lactam/β-lactamase inhibitor combinations were developed to overcome the activity of class A (e.g. TEM-1) and class C β-lactamases (BLs) (e.g. AmpC) [1, 2], which inactivate β-lactam antibiotics by hydrolysing the β-lactam ring. New β-lactamase inhibitors, particularly metallo-β-lactamase inhibitors, are still urgently required and their discovery and development is the topic of intense investigation [3]. β-lactam/β-lactamase inhibitor combinations currently in clinical use include amoxicillin/clavulanic acid, ampicillin/sulbactam and piperacillin/tazobactam (TZP), with ceftolazone/tazobactam and ceftazidime/avibactam recently introduced into clinical use. TZP has broad spectrum antibacterial activity and is routinely used for intra-abdominal infections and febrile neutropenia [4, 5]. TZP usage has increased year on year in the UK, from just under 2.1% of all antibiotics prescribed in 2008-2009 to 3.6% in 2012-2013 [6]. During the 2-year period between April 2012 and March 2014 10.2% of bacteraemia causing *Escherichia coli* isolates in England tested for TZP susceptibility were resistant [7]. Resistance to TZP has been previously linked to AmpC hyperproduction, and the co-production of multiple β-lactamases, which also confer resistance to 3rd generation cephalosporins [8]. Additionally, tazobactam is a poor inhibitor of metallo-β-lactamase enzymes, which cause resistance to TZP, alongside 3rd generation cephalosporins and carbapenems [9-11].

A novel phenotype in *Klebsiella pneumoniae* and *E. coli* clinical isolates has emerged which has been classified as TZP-non-susceptible but susceptible to 3rd generation cephalosporins and carbapenems [12-14], indicating a different resistance mechanism. While still relatively rare, one study in the United States found that the frequency of this phenotype was between 1.9% and 5.6% of *E. coli* and *K. pneumoniae* isolated from the bloodstream between 2011 and 2015, and specifically 4.1% of all *E. coli* over the study period [14]. The same study reported risk factors associated with the TZP-non-susceptible but 3rd generation cephalosporin and carbapenem susceptible phenotype, including exposure to β-lactam/β-lactamase inhibitors and cephalosporins within the previous 30 days [14]. Resistance to TZP, but 3rd generation cephalosporin and carbapenem susceptible, has been linked to

hyperproduction of the β-lactamase *bla*$_{TEM,}$ which can hydrolyse piperacillin but not 3rd generation cephalosporins. Hyperproduction is hypothesised to overcome the inhibitory activity of tazobactam, allowing the hydrolysis of piperacillin [15]. Mechanisms leading to hyperproduction include mutations in the promoter region of *bla*$_{TEM}$, changing it from a weak promoter (*P3*) to a stronger promoter (*P4* or *P5*) [16] or a single point mutation further upstream resulting in the overlapping, stronger promoter *Pa*/*Pb* superseding the weaker *P3* promoter [17], and increased production of *bla*$_{TEM}$. Another such mechanism proposed to cause TZP-resistance but 3rd generation cephalosporin and carbapenem susceptibility is increase in copy number of *bla*$_{TEM}$ present in the chromosome [15, 18]. Gene amplification has been linked to the cause of temporary antibiotic resistance seen in a sub-population of bacteria and is known as heteroresistance. Heteroresistance is often lost after multiple generations in the absence of antibiotic selective pressure, due to the fitness cost imposed by the production of extra proteins as a result of amplification [19, 20]. While the mechanism of amplification of the *bla*$_{TEM}$ is not well known, recent studies have found that the amplified *bla*$_{TEM}$ has been co-located on a segment of DNA containing other antibiotic resistance genes, such as *aadA* and *sulI*, termed a genomic resistance module [15]. Amplification of *bla*$_{TEM}$ leading to TZP resistance via β-lactamase hyperproduction has also been suggested to be mediated by the presence of the insertion sequence, IS*26* [18]. IS*26* is often linked with the movement of antibiotic resistance genes; for example a translocatable unit (TU) containing IS*26* has been shown to be able to excise from the transposon Tn*4352*B, which itself was located on a plasmid, between two IS*26*, leaving one in the plasmid [21, 22]. Following excision, the single IS*26* and antibiotic resistance gene(s) found between the two insertion sequences forms a circular TU, which then can insert into a plasmid via a conservative Tnp*26*-dependent but RecA-independent mechanism, Tnp26 replicative transposition or RecA-dependent homologous recombination, preferentially adjacent to another IS*26* insertion sequence [21-23].

Here, using a clonal pair of *E. coli* isolated from a single patient from two separate infection episodes which displayed within-patient evolution to TZP resistance, we sought to determine the mechanism

of gene amplification resulting in hyperproduction of bla_{TEM-1B}. We found that a TU was excised from a novel composite transposon flanked by IS*26* present in the chromosome, leading to gene amplification which did not carry any fitness cost. We were also able to replicate the evolutionary event leading to the excision in the TZP-susceptible isolate and captured the excised TU in a plasmid containing a copy of IS*26*.

Materials and methods

Bacterial isolates, media and antibiotics

Clinical isolates of *E. coli* isolated from blood cultures between 2010 and 2017 at the Royal Liverpool University Hospital (Liverpool, UK) which were found to be carbapenem and cephalosporin susceptible but TZP resistant using the disc diffusion method of antimicrobial susceptibility testing (AST) were initially identified from isolate records. Isolate records were then searched for a corresponding carbapenem, cephalosporin and TZP susceptible isolate, isolated in the same or a previous infection episode from the same patient. Using these criteria, we identified five paired clinical isolates of *E. coli*. All isolates had been stored at the time of blood culture isolation in glycerol broth at -80°C.

All isolates were grown on LB (Lennox) agar at 37°C for 18 hours followed by growth in LB (Lennox) broth, (LB, Sigma, UK), iso-sensitest (ISO, Oxoid, UK) or M9 (50% (v/v) M9 minimal salts (2x) (Gibco, ThermoFisher Scientific, USA), 0.4% D-glucose, 4mM magnesium sulphate (both Sigma, UK) and 0.05 mM calcium chloride (Millipore, USA)) at 37°C for 18 hours at 200 rpm.

Piperacillin, tazobactam (both Cayman Chemical, USA), gentamicin (GEN), and amoxicillin trihydrate:potassium clavulanate (4:1, AMC) were solubilised in molecular grade water (all Sigma, UK), while chloramphenicol (CHL) and tetracycline (TET) (both Sigma, UK) were solubilised in ethanol (VWR, USA) and ciprofloxacin (CIP) was solubilised in 0.1N hydrochloric acid solution (both Sigma, UK). All stock solutions of antibiotics were filter sterilised through a 0.22µm polyethersulfone filter

125 unit (Millipore, USA). In all assays, unless stated, tazobactam was used at a consistent concentration
126 of 4 µg/ml and the piperacillin concentration was altered.

127 *Restriction Enzyme Digestion*

128 Restriction fragment length polymorphism (RFLP) analysis of 1 µg of 16S rRNA PCR amplicon from
129 the 10 putative clonal isolates were digested with AlwNI, PpuHI and MslI (all New England Biolabs,
130 USA) and 1 µg of long fragment genomic DNA extracts of 153964, 152025, 190693 and 169757 were
131 digested with SpeI and MslI (both New England Biolabs, USA) for 1 hour at 37°C. Both RFLP digest
132 reactions were incubated for 5 minutes at 80°C and immediately run on a 1% agarose gel. Enzyme
133 digest of 500 ng of plasmid DNA extracted from 190693 and 169757 was performed with PpuMI and
134 XhoI (both New England Biolabs, USA) and immediately run on a 1% agarose gel following incubation
135 for 1 hour at 37°C.

136 *Antimicrobial susceptibility testing*

137 Initial AST for cefpodoxime (CPD), cefoxitin (FOX), TZP, meropenem (MEM), CIP, cefotetan (CTT),
138 amikacin (AMK), ertapenem (ETP), AMC, chloramphenicol (CHL) and ampicillin (AMP) was performed
139 in clinic using the disk diffusion method, according to the CLSI guidelines for Antimicrobial
140 Susceptibility Testing [24].

141 AST for TZP, GEN, CIP, CHL, AMC and TET were performed using the broth microdilution method for
142 minimum inhibitory concentrations, performed in cation adjusted Mueller Hinton Broth (CA-MHB),
143 following CLSI Guidelines. Efflux pump inhibition was performed using phenylalanine-arginine β-
144 naphthylamide (PAβN) as a supplement in CA-MHB at a final concentration of 50µM.

145 *Nitrocefin assay*

146 β-lactam hydrolysis was evaluated using a colorimetric nitrocefin assay. Cell lysates were obtained
147 from triplicate cultures of 190693 and 169757 in LB, adjusted to an OD_{600} of 0.1. Cultures (10ml)
148 were centrifuged at 14,000 g for 5 minutes, the supernatant discarded, and the pellet resuspended

in 5ml phosphate buffered saline (PBS). The cultures were sonicated for three intervals of ten seconds, on ice, using a Soniprep 150 plus (MSE centrifuges, UK). The lysed cultures were centrifuged at 14,000 g for 5 minutes, and the supernatant taken as the culture lysate.

A total of 90µl of this lysate was then added to 10µl of 0.5mg/ml nitrocefin solution (Sigma, UK) in a 96 well microplate, in triplicate. The absorbance of the plate was read at 450nm every 20 seconds for 25 minutes, using a SPECTROstar OMEGA spectrophotometer (BMG lab systems).

Whole genome sequencing and bioinformatics

Illumina MiSeq 2 x 250 bp short-read sequencing of long fragment DNA extractions from isolates 190693 and 169757, as well as adapter trimming of the sequencing reads, were provided by MicrobesNG (MicrobesNG, UK).

The same long fragment DNA extracts were processed using the SQK-LSK109 ligation and SQK-RBK103 barcoding kit and sequenced on an R9.4.1 flow cell with an Oxford Nanopore Technologies (ONT) MinION. Sequencing reads were basecalled during the sequencing run using MinKNOW, de-multiplexing and adapter trimming of the basecalled reads were performed using Porechop (v0.2.4) and finally sequencing reads were filtered for a quality score of 10 via Filtlong (v0.2.0).

Both Illumina short-read and MinION long-read sequences were assembled using Unicycler (v0.4.7 [25]), with the quality of the assembly assessed using QUAST (v5.0.2 [26]), annotated using Prokka (v1.14.0 [27]) and visualised using Bandage (v0.8.1 [28]).

Sequence type and serotype of both 190693 and 169757 were determined using Multi-Locus Sequence Typing (MLST, v2.0.4 [29]) and SerotypeFinder (v2.0.1 [30]), respectively. The relatedness of the two genomes were compared using MUMmer (v3.23 [31]) and the average nucleotide identity (ANI) was calculated using OrthoANI (v0.93.1 [32]). Presence of acquired antimicrobial resistance genes within the two genomes were assessed using ResFinder with minimum threshold of 90% and a minimum length of 60% (v3.2 [33]) and segments of the two genomes were characterised using

SnapGene® software (from GSL Biotech; available at snapgene.com). Finally, plasmid replicons were identified using PlasmidFinder (v2.0.1) [34].

Competent cell preparation

The TZP-susceptible isolate was made competent according to Chung *et al.* [35].

Quantitative PCR

Changes in gene copy number of bla_{TEM-1B}, bla_{OXA-1}, *aac*(3)-*lla, aac*(6')-*lb-cr*, *tet*(D) were calculated via qPCR, using the ΔΔCT method for relative quantitation of these genes against the single copy *uidA* housekeeping gene.

Each qPCR reaction contained 6.25µl QuantiTect® SYBR Green PCR buffer (Qiagen, UK), 0.4 µM forward and reverse primers (Table S1), 1ng of extracted DNA, and molecular grade water to a final volume of 12.5µl. Reactions were processed using a Rotor-Gene Q (Qiagen, Germany), using the following protocol; an initial denaturation step of 95°C for 5 minutes, followed by 40 cycles of; DNA denaturation at 95°C for 10 seconds, primer annealing at 58°C for 30 seconds, and primer extension at 72°C for 10 seconds with fluorescence monitored in the FAM channel. HRM analysis was carried out over a temperature range of 75°C to 90°C, increased in 0.1°C increments, in order to confirm specific amplification. Fluorescence thresholds were set manually for calling Ct values, at 5% of the difference between baseline and maximum fluorescence.

The mean qPCR Ct value for the *uidA* gene from each strain was taken using four replicate qPCR reactions, and the ΔΔCT method was utilised to determine fold change using quadruplicate qPCR reactions for each AMR gene.

In vitro evolution of susceptible isolate

The clinical isolate 190693 (TZP-susceptible isolate) and 190693 transformed with pHSG396:IS*26* were subcultured into 10 ml LB and 10 ml LB plus 35 µg/ml chloramphenicol, respectively, and incubated at 37°C for 18 hours at 200 rpm. Following incubation 10 µl of 190693 was subcultured

197 into 10 ml LB and 10 ml LB plus 8/4 µg/ml TZP and 10 µl of 190693 with pHSG396:IS*26* was
198 subcultured into 10 ml LB plus 35 µg/ml chloramphenicol and 10 ml LB plus 35 µg/ml
199 chloramphenicol and 8/4 µg/ml TZP and incubated at 37°C for 24 hours at 200 rpm. Genomic DNA
200 from each of the four cultures were extracted for qPCR following the protocol described above,
201 however triplicate biological replicates were used instead of quadruplicate qPCR reactions

202 *Translocatable unit capture*

203 The TZP-susceptible isolate 190693 transformed with pHSG396:IS*26* was grown in the presence of
204 TZP and chloramphenicol as previously stated. Following selection, the culture was serially diluted
205 1/10 in PBS down to 10^{-7} dilution and 50 µl of each dilution was plated out on to LB agar
206 supplemented with 35 µg/ml chloramphenicol and 16/4 µg/ml TZP. Five single colonies were
207 selected and subcultured into 10 ml LB plus 35 µg/ml chloramphenicol and 16/4 µg/ml TZP for 18
208 hours at 37°C and 200 rpm and the plasmid extracted following the protocol above. The purified
209 plasmids were transformed into NEB® 5-alpha competent *E. coli* following the protocol in the
210 supplementary material and plated out on to LB agar supplemented with 35 µg/ml chloramphenicol
211 and 16/4 µg/ml TZP and incubated at 37°C for 18 hours. A single colony from each transformation
212 was subcultured into 10 ml LB supplemented with 35 µg/ml chloramphenicol and 16/4 µg/ml TZP
213 and incubated at 37°C, 200 rpm for 18 hours and the plasmid extracted following the protocol in the
214 supplementary material. The initial pHSG396:IS*26* plasmid extract and pHSG396:IS*26* plasmid
215 selected in TZP and extracted from NEB® 5-alpha *E. coli* were digested with XhoI (New England
216 Biolabs, US) and EcoRI for 1 hour at 37°C, followed by a 20 minute incubation at 65°C and run on a
217 1% agarose gel.

218 *Competitive fitness*

219 The relative fitness of 169757 (TZP-resistant) and 190693 (TZP-susceptible) grown in the presence of
220 8/4 µg/ml TZP, compared to 190693 and 190693 grown in the absence of TZP, were assessed
221 comparatively in LB, ISO and M9. Each culture was diluted to an OD_{600} of 0.1 in the respective media,

222 then further diluted 1/1000 in the same media and 150 µl of each diluted culture added to a flat
223 bottom, 96 well microtitre plate in duplicate as well as 150 µl of the media as a negative control. The
224 96 well plate was incubated at 37°C and the OD_{600} of each well was measured with 100 flashes every
225 10 minutes over 24 hours, with orbital shaking at 200 rpm between readings, using a Clariostar Plus
226 microplate reader (BMG Labtech, Germany). The relative fitness compared to either 190693 or
227 190693 grown in the absence of TZP between absorbance values 0.2 and 0.08 and a minimum R
228 value of 0.9799 was estimated using BAT version 2.1 [36].

229 *Statistical analysis*

230 Statistical analysis of comparison for qPCR of the antibiotic resistance genes on the RM of 190693
231 and 169757 was performed using the 2way ANOVA test. Statistical analysis of comparison for qPCR
232 of the antibiotic resistance genes on the RM of 190693 grown in the presence or absence of TZP,
233 with and without pHSG396:IS*26* was performed using the Ordinary One-Way ANOVA with
234 Uncorrected Fisher LSD test. Statistical analysis of relative fitness of 169757 and 190693 grown in
235 the presence of TZP was performed using Ordinary One-Way ANOVA with Uncorrected Fisher LSD
236 test. All statistical tests were performed using GraphPad Prism version 8.2.1.

237

238 **Results**

239 *Identification of clonal isolates*

240 Initially, we identified five isolates in the collection of TZP-resistant, 3rd generation cephalosporin
241 and carbapenem susceptible *E. coli* from blood cultures at the Royal Liverpool University Hospital
242 which had a corresponding TZP-susceptible isolate from the same or previous infection episode, and
243 therefore could have evolved to become TZP-resistant within a patient. RFLPs of the 16S rRNA
244 amplicons from the five pairs of isolates indicated that three pairs of TZP-susceptible/TZP-resistant
245 clinical isolates had identical digestion patterns (Fig. S1A). Two of these three pairs of isolates had an
246 identical resistance profile generated during routine disk-based susceptibility testing, aside from TZP

(Table S2). RFLPs of genomic DNA identified one pair of isolates with identical banding patterns indicating clonality; 190693 (TZP-susceptible) and 169757 (TZP-resistant) which were isolated from different infection episodes from the same patient approximately 3 months apart (Fig. S1B). During the first infection episode, the TZP-susceptible *E. coli* was isolated and the patient was initially treated with a five day course of TZP, followed by a seven day course of TZP with teicoplanin and then a third seven day course of TZP although a second blood culture was found to be negative. A second infection episode occurred approximately 6-7 weeks after the final course of TZP was completed, and again the patient was treated initially with TZP until the TZP-resistant *E. coli* was isolated, when the treatment was changed to meropenem. Putative clonality of these two isolates was confirmed with whole genome sequencing; both isolates were identified as serotype H30 O86, sequence type 8 and had an ANI of 100%, with 36 single nucleotide polymorphisms difference between the two isolates.

Confirmation of TZP susceptibility/resistance and mechanism of resistance

We determined the MIC of the pair of isolates and verified that TZP-susceptible isolate was susceptible to TZP (2-4/4 μg/ml) and TZP-resistant isolate was resistant to TZP (64/4 μg/ml) according to EUCAST clinical breakpoints [37] (Table 1). Using the efflux pump inhibitor PAβN as a supplement in the MIC assay, we were able to rule out overexpression of efflux pumps as a possible mechanism of resistance as there was less than a 4-fold reduction [15] in MIC of both the TZP-susceptible (2/4 μg/ml) and TZP-resistant isolates (32/4 μg/ml, Table 1). Whole genome sequencing revealed no differences in the predicted resistance genes present in the genome between the TZP-susceptible and TZP-resistant isolate (Table 2) and no mutations in the promoter region of any of the BLs present within the genome (bla_{TEM-1B}, bla_{OXA-1} or *ampC*) of the TZP-resistant isolate. We confirmed that the TZP-resistant isolate hyperproduced a BL due to the significant increase in nitrocefin hydrolysis compared to the TZP-sensitive isolate (P value = <0.0001, Fig. 1A).

Hybrid assembly and comparison of the genomes of the TZP-susceptible and TZP-resistant isolates

Using a hybrid assembly of ONT long and Illumina short sequencing reads, we were able to complete the genome of the TZP-susceptible isolate, which was found to be 5151952 bp in length, with a GC content of 50.64% and did not contain any plasmids (Fig. S4A). In contrast, we unable to complete the genome of the TZP-resistant isolate as a 530bp segment remained unresolved, and a complete, low copy number (2.59x) 106637 bp plasmid containing an IncFII replicon (Fig. S4B) was detected. A complete, smaller (10899 bp) circular DNA molecule was also found to be present in the TZP-resistant isolate, at a copy number of 8.51x, however this small circular DNA molecule did not contain a plasmid replicon (Fig. S4B). The large plasmid did not to contain any predicted antimicrobial or metal resistance genes, but did contain three bacteriocins, both colicin B and M (with cognate immunity proteins) and linocin. Comparison of the predicted resistance genes present on the chromosome of the TZP-susceptible and TZP-resistant isolates highlighted that $bla_{\text{TEM-1B}}$, $bla_{\text{OXA-1}}$, *aac*(3)-*lla, aac*(6')-*lb-cr*, *tet*(D) and *cat*B3 were missing from the assembled chromosome of the TZP-resistant isolate (Table 2). Characterisation of the small circular DNA molecule found that it contained these missing resistance genes, as well as several putative transposable elements including three copies of IS*26* (Table 2), and aligned exactly to the chromosome of the TZP-susceptible isolate and was no longer present in the chromosome of the TZP-resistant isolate. The predicted *cat*B3 resistant gene was truncated to 69.8% length and therefore unlikely to be functional, which was confirmed as both the TZP-susceptible and TZP-resistant isolates were sensitive to chloramphenicol according to EUCAST guidelines (Table 1). Further analysis of the TZP-susceptible genome uncovered that the circular DNA molecule from the TZP-resistant isolate aligned with 100% identity to a novel integrated composite transposon flanked by two copies of IS*26* in the same orientation, which we subsequently registered as Tn*6762* via the transposon registry [38], however the circular DNA molecule only contained one IS*26*. The antibiotic resistance gene $bla_{\text{TEM-1B}}$, $bla_{\text{OXA-1}}$, *aac*(3)-*lla, aac*(6')-*lb-cr* and *tet*(D) were identified to be present on Tn*6762* (Fig. 2A). This suggested that a translocatable unit (TU) [21-23], containing one IS*26* and the antibiotic resistance genes, was excised from the chromosomally located Tn*6762* while the other IS*26* stayed in the

298 chromosome (Fig. 2A). Interestingly, we also found that the tetracycline resistance regulator, *tetR*,
299 on the TU had been disrupted 52 bp from the end of the *tetR* due to the excision which overlaps the
300 start of the copy of IS*26* remaining in the chromosome. Following excision, a copy of IS*26* is present
301 at the start of the TU which then connects to the end of the TU, containing the disrupted *tetR*,
302 forming a circular DNA molecule. As the two copies of IS26 are identical and in the same orientation,
303 and therefore containing the same 52 bp bases missing from *tetR*, *tetR* was reformed when the TU
304 circularised completing the 657 bp gene.

305 *Confirmation of the presence of Tn6762 and the translocatable unit*

306 Firstly, we confirmed the amplification of the resistance genes found on the TU in the TZP-resistant
307 isolate and Tn*6762* in the chromosome of the TZP-susceptible isolate. Comparing the fold change in
308 copy number of each resistant gene to the housekeeping gene *uidA*, we found that each resistance
309 gene on the TU increased in copy number in the TZP-resistant isolate compared to the TZP-
310 susceptible isolate (Fig. 1B, P value = <0.0001). The increase in copy number of the resistance genes
311 found on the TU in the TZP-resistant isolate also corresponded to an increase in MIC of all
312 antimicrobials that the genes confer resistance to (Table 1), further confirming the amplification of
313 the entire TU. By PCR of the left and right junctions of the chromosomally located Tn*6762*, with one
314 primer specific for Tn*6762* and one for chromosome either before or after IS*26* (Fig. 2B), we were
315 able to confirm that Tn*6762* was present in the chromosome of both the TZP-susceptible isolate and
316 TZP-resistant isolate by yielding the expected 1640 bp and 2402 bp products, respectively (Fig. S2).
317 Using primers that would only yield a 1942 bp product if the circular TU was present (Fig. 2C), we
318 were able to detect the presence of the TU in the TZP-resistant isolate and absence in the TZP-
319 susceptible isolate (Fig. S2). This suggests that Tn*6762* has been excised and existed as a circular TU,
320 as well as in the chromosome of the TZP-resistant isolate.

321 *Replication of excision of the translocatable unit*

Completed assembly of the large plasmid present in the TZP-resistant isolate showed that the plasmid contained a single copy of IS*26*. We sought to determine whether the presence of another mobile IS*26* and/or the use of TZP induced the excision of the TU by replicating the evolutionary event that led to the TZP-sensitive isolate becoming resistant to TZP *in vitro*. We found that there was a significant increase in copy number of all the resistance genes present on Tn*6762*, relative to the housekeeping gene *uidA*, following exposure to 2 x MIC of TZP (8/4 μg/ml) while there was no evidence of amplification in the same isolate grown in the absence of TZP (P value = 0.0012). There was also an increase in copy number when the isolate containing the pHSG396:IS*26* plasmid was exposed to TZP but, again, no increase in copy number when TZP was absent (P value = <0.0001), therefore TZP can either select for the maintenance of the excised TU or induce the excision event, leading to an increase in copy number of the genes present on Tn*6762*. In contrast, there was no significant difference in gene copy number between the TZP-susceptible with and without the pHSG396:IS*26* plasmid grown in the absence of TZP (P value = 0.5064), underlining that the presence of an extra chromosomal IS*26* does not induce excision of the TU from Tn*6762*.

Capture of the translocatable unit

We sought to capture the excised TU, and therefore observe the excision and insertion events, using a pHSG396 plasmid containing IS*26* transformed into the TZP-sensitive isolate in the same orientation relative to the origin of replication as found on Tn*6762* and selected for TZP-resistant derivatives by growing the isolate in the presence of TZP. We detected the insertion of a >10kb fragment into the pHSG396:IS*26* after TZP selection following digestion with XhoI and EcoRI (Fig. S3A). Insertion of the TU from the TZP-susceptible chromosome was confirmed through PCR amplification across the two newly formed junctions on the pHSG396:IS*26*, with one primer specific for pHSG396 and one for either *aac*(6')-*Ib-cr* (left) or *tet*(D) (right) on the TU for each junction yielding the expected 1458 bp (left) and 1385 bp (right) products (Fig. 2D), consistent with the insertion of the TU adjacent to IS*26* in the pHSG396 and reforming Tn*6762* in pHSG396 (Fig. S3B).

Fitness effect of extensive amplification

Hyperproduction of a protein could result in a fitness cost to the cell due to the increased metabolic activity. Yet, we found that the amplification of the TU and carriage of the large plasmid in the TZP-resistant isolate did not result in a significant change in fitness compared to the TZP-susceptible isolate in LB (P value = 0.9968), ISO (P value = 0.2836) and M9 (P value = 0.2204, Fig. 4). We also assessed the relative fitness of the TZP-susceptible isolate following exposure to TZP (resulting in the increase in copy number of the resistance genes present on the RM (Fig. 3)) compared to the TZP-susceptible grown in the absence of TZP (which did not result in an increase in copy number (Fig. 3)). Again, we found no significant change in fitness in LB (P value = 0.8047), ISO (P value = 0.1242) and M9 (P value = 0.2803, Fig. 4).

Discussion

Tazobactam is able to inhibit the activity of class A β-lactamases [39], and therefore the presence of bla_{TEM-1} within the genome of an *E. coli* isolate should not result in resistance to TZP. However, two studies have linked amplification of bla_{TEM}, and therefore hyperproduction of the BL, with this phenotype [15, 18], with one linking amplification and the presence of IS*26* [18]. However, the exact mechanism of amplification/hyperproduction has remained elusive. Due to the emergence of a novel TZP-resistant but 3rd generation cephalosporins and carbapenems susceptible *E. coli* and *K. pneumoniae* phenotype [12-14], as well as the increasing reliance on TZP as an empirical treatment [6] and the recent interest in the use of TZP as a carbapenem sparing treatment for ESBL infections [40, 41], it is of growing importance to understand the mechanism of resistance. In this study we had the unique opportunity to compare a pair of clonal isolates which have evolved within a patient to become TZP-resistant but remain cephalosporin/carbapenem sensitive, allowing us to build on recent studies and identify the mechanism of IS*26*-mediated amplification of bla_{TEM-1B} which leads to TZP-resistance.

We found multiple antibiotic resistance genes, including *bla*$_{TEM-1B}$, co-located on a novel IS*26* composite transposon Tn*6762* on both the TZP-resistant and TZP-susceptible isolate. This is the first time that the resistance genes *tet*D and *bla*$_{TEM-1B}$ have found to be present on the same transposable element and a result of insertion of several transposons into the same location, demonstrated by the presence of several insertion sequences and transposable elements on Tn*6762*. Amplification of *bla*$_{TEM-1B}$ is achieved when Tn*6762* is excised from the chromosome forming a TU, evidenced by the hybrid assembly of the TZP-resistant isolate, increase in copy number of the antibiotic resistance genes present on Tn*6762* and the capture of the TU in pHSG396:IS*26*. Precise excision and formation of a TU containing the antibiotic resistance gene *aphA1a* from a Tn*4352*B transposon present in a plasmid has been demonstrated before as a mechanism of movement of antibiotic resistance gene [21, 22, 42], this is the first time it has been shown to directly result in gene amplification resulting in resistance. We found no evidence from the whole genome sequencing of the TZP-resistant isolate of insertion of the TU anywhere else in the chromosome, except for a gap in sequencing where Tn*6762* was originally situated in the TZP-susceptible isolate adjacent to an IS*26*. Through PCR of the left and right junctions of the gap in sequencing, we confirmed that Tn*6762* was still present at this location in the chromosome of the TZP-resistant isolate and therefore existed both as Tn*6762* and TU, which the hybrid assembly was unable to resolve. We also found no evidence of a tandem repeat of the TU in the chromosome of the TZP-resistant isolate and only evidence of the TU through hybrid assembly of the genome and confirmation by PCR. Using the chloramphenicol-resistant pUC vector, pHSG396, containing a copy of IS*26* from the TZP-susceptible chromosome, we were able to replicate the evolutionary event and capture a single copy of the excised TU adjacent to the IS*26* copy in the plasmid, providing evidence of the insertion event, but again there was no evidence of tandem repeats of the TU in the pHSG396:IS*26* plasmid. Therefore, the TU preferentially re-inserts into the chromosome adjacent to the chromosomally located IS*26* via a conservative Tnp26-dependent but RecA-independent mechanism, Tnp26 replicative transposition or RecA-dependent homologous recombination [22]. While this mechanism has previously been demonstrated in terms of movement

of antibiotic resistance genes [21, 22], this is the first time this mechanism has been shown to cause gene amplification resulting in antibiotic resistance in a clinical isolate, be able to replicate the evolutionary event resulting in amplification *in vitro* and show that use of TZP either selects for or induces this phenotype. This mechanism is of concern as IS*26* has been associated with the transfer of bla_{NDM-1} in a recent nosocomial outbreak in Germany [42], and other carbapenemases [43] and therefore represents a risk of clinical resistance to any carbapenemase inhibitor currently in development [44] which needs to be investigated further. The method of capture of the TU used in this study can be used to investigate whether the same mechanism will result in amplification to other β-lactam/β-lactamase inhibitors, as well as to further confirm the role of TZP in the induction of excision of the TU, and therefore gene amplification.

The absence of insertion of the TU into the plasmid in the TZP-resistant isolate was notable. After determining the direction of IS*26* in relation to the origin of replication in both the chromosome of the TZP-susceptible isolate and the plasmid, IS*26* was in the reverse direction in the plasmid but in the forward direction on both the TU and the chromosome. The orientation of IS*26* may have bearing on whether insertion of the TU can occur, as previous studies on the insertion of a TU via a conservative mechanism found that both IS*26* were in direct orientation [21-23], however this needs further investigation to confirm whether the direction of the IS*26* is critical for insertion of the TU.

Hansen *et al.* 2019 associated amplification of bla_{TEM-1} in *E. coli* clinical isolate with a significant fitness cost [18];Fitness of this isolate was compared to other unrelated clinical isolates of *E. coli* which hyperproduced Bla_{TEM-1} due to promoter mutations, rather than the same isolate with and without amplification. This approach can lead to over- or under-estimation of fitness cost, as genetic background of the isolate can have an impact on the overall fitness affect, as can the environment fitness is assessed in [45, 46]. Adler *et al.* 2014, however, identified a fitness cost associated with IS*26*-mediated amplification of an antibiotic resistance cassette from a plasmid in all lineages tested [20]. In this study, we were able to compare the fitness of the paired clinical isolate and *in vitro* evolved isolates with and without amplification of the chromosomally located composite transposon

and found that there was no significant difference in fitness cost, despite amplification of a >10kb region with multiple functionally transcribed genes and, in terms of the TZP-resistant clinical isolate, has acquired a large plasmid. If this lack of fitness cost is translated into a physiological environment, it may result in the TZP-resistant phenotype persisting. While there was no observed fitness effect of amplification on this single isolate, the effect of amplification on bacterial fitness needs to be extensively investigated as it may not be a global phenomenon.

Conclusions

Resistance to the β-lactam/β-lactamase inhibitor TZP can be the result of hyperproduction of the β-lactamase bla_{TEM-1B} due to the IS*26*-associated excision and circularisation of a translocatable unit containing bla_{TEM-1B} from the chromosome either selected by or in response to exposure to TZP. In this clinical isolate and an *in vitro* evolved isolate, we found that there was no effect on fitness due to the amplification and subsequent carriage of high numbers of the TU. This mechanism of amplification, and the subsequent hyperproduction, of bla_{TEM-1B} is an important consideration if treatment failure involving TZP occurs, as well as other β-lactam/β-lactamase inhibitor combinations, and when using genomic data to predict resistance/susceptibility to β-lactam/β-lactamase inhibitor combinations.

Author contributions

ATMH and TE conceptualised the study. JM, PR, CMP, CC, JvA, and AH collated isolate metadata, clinical antimicrobial susceptibility data and patient treatment data. ATMH, AJF, ERA, APR and TE contributed to the experimental design and data analysis. ATMH, AJF and TE contributed to carrying out the experiments. ATMH and TE wrote the first draft of the manuscript, which was then edited and approved all authors by all authors.

Data availability

Unicycler hybrid assemblies of the two clonal isolates of *E. coli* were submitted to GenBank under the BioProject PRJNA607545. Accession number CP048934 corresponds to TZP-susceptible isolate (190693) and accession number JAAKGF000000000 corresponds to TZP-resistant isolate (169757).

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579 **Tables and figures**

580 **Table 1:** Minimum inhibitory concentrations of gentamicin (GEN), tetracycline (TET),
581 chloramphenicol (CHL), ciprofloxacin (CIP), amoxicillin/clavulanic acid (AMC) and
582 piperacillin/tazobactam (TZP) (with and without PAβN) towards the TZP-susceptible and TZP-
583 resistant isolates

	GEN	TET	CHL	CIP	AMC	TZP	TZP + PAβN
TZP-	128	256	4	64	32	2 - 4/4	2/4

susceptible							
TZP-resistant	1024 - >1024	512	4	128	64 - 128	64/4	32/4

584

585

586 **Table 2:** Predicted antimicrobial resistance genes by ResFinder found on the genome of the TZP-
587 susceptible and TZP-resistant isolates and their position in genome. *The resistance gene catB3 was
588 predicted by ResFinder to be present with 69.8% length, but both isolates were phenotypically
589 chloramphenicol susceptible

Antimicrobial Resistance Gene	TZP-susceptible isolate	TZP-resistant isolate
***bla*$_{OXA-1}$**	Chromosome	Translocatable unit
***bla*$_{TEM-1B}$**	Chromosome	Translocatable unit
aac*(3)-*IIa	Chromosome	Translocatable unit
aac*(6')-*Ib-cr	Chromosome	Translocatable unit
***aadA*1**	Chromosome	Chromosome
aph*(3")-*Ib	Chromosome	Chromosome
aph*(6)-*Id	Chromosome	Chromosome
***tet*(D)**	Chromosome	Translocatable unit
***dfrA*1**	Chromosome	Chromosome
***sul*2**	Chromosome	Chromosome
***mdf*(A)**	Chromosome	Chromosome

catB3*	Chromosome	Translocatable unit

590

591 **Figure 1: A**) Increase in nitrocefin hydrolysis by the TZP-resistant isolate compared to the TZP-

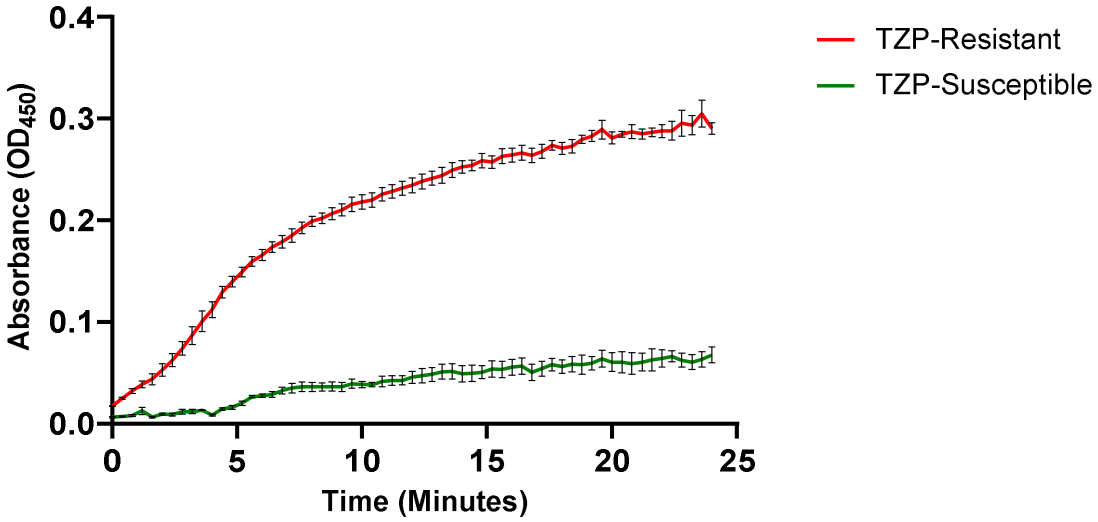
592 susceptible, isolate as measured at $OD_{450,}$ due to hyperproduction of a BLS in the TZP-resistant

593 isolate. **B**) Comparison of the fold change in copy number of the antimicrobial resistance genes

594 present on the composite transposon of the TZP-susceptible isolate/translocatable unit of the TZP-

595 susceptible isolate as assessed by qPCR compared to the housekeeping gene *uidA*

596 **A**



597
598

599 **B**

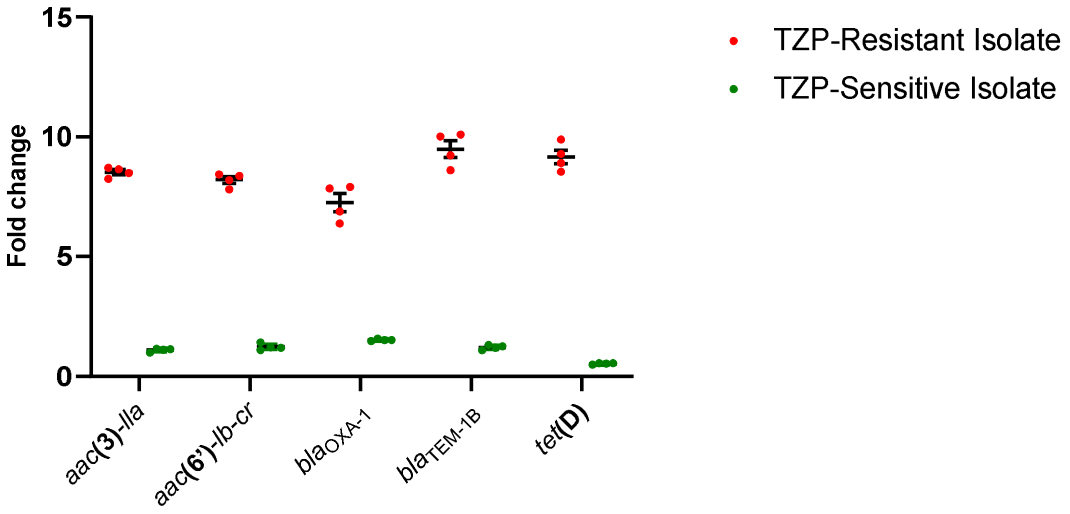
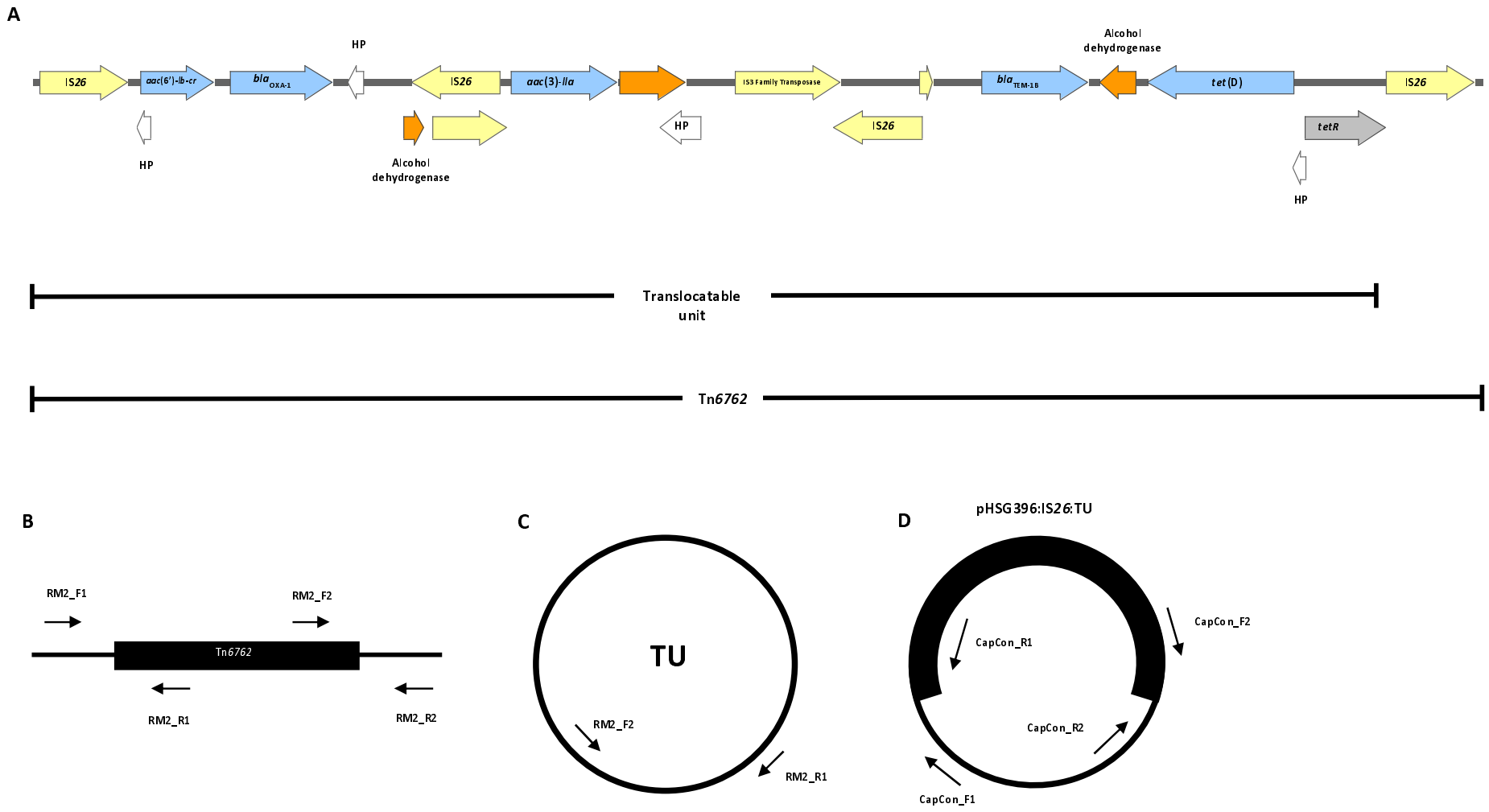


Figure 2: Schematic showing **A**) the characterisation of Tn*6762* (HP = hypothetical protein) and the position of the primer pairs to detect **B**) the junctions of the Tn*6762* in the chromosome, **C**) the presence of the TU and **D**) the junctions of the insertion of the TU into pHSG396:IS*26*. **E**) Schematic of the proposed mechanism of leading to hyperproduction of bla_{TEM-1B}; **1**) a composite transposon, Tn*6762,* is present on the chromosome flanked by two copies of IS*26*. Due to selective pressure from TZP, **2**) Tn*6762* is excised from the chromosome, **3**) which then forms a translocatable unit (TU) which **4**) re-inserts and excises from the chromosome adjacent to the chromosomally located IS*26*, increasing the copy number of bla_{TEM-1B}



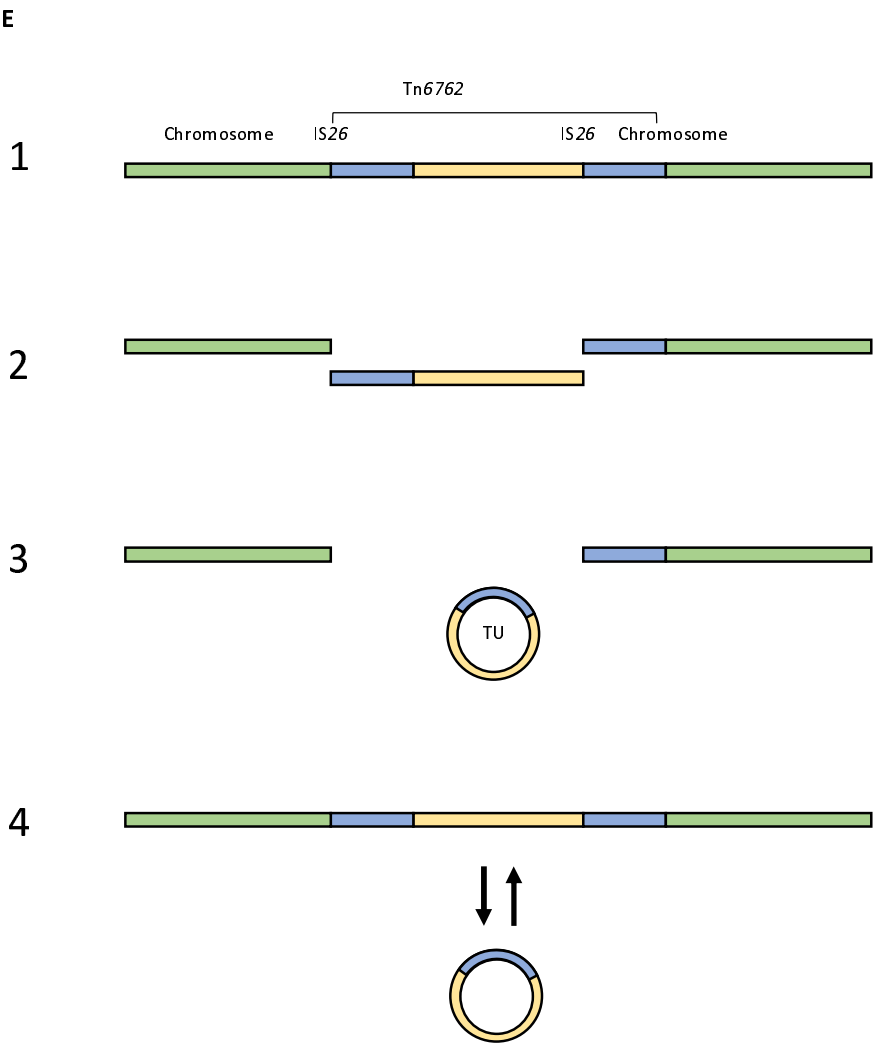


Figure 3: Fold change in copy number of all the antimicrobial resistance genes found on the composite transposon compared to the housekeeping gene *uidA* following growth of the TZP-susceptible isolate in the absence of antibiotics, TZP-susceptible isolate in the presence of 8/4 µg/ml TZP, TZP-susceptible isolate transformed with pHSG396 plasmid containing IS*26* in the presence of 8/4 µg/ml TZP and 35 µg/ml chloramphenicol and TZP-susceptible isolate transformed with pHSG396 plasmid containing IS*26* in the presence of 35 µg/ml chloramphenicol only

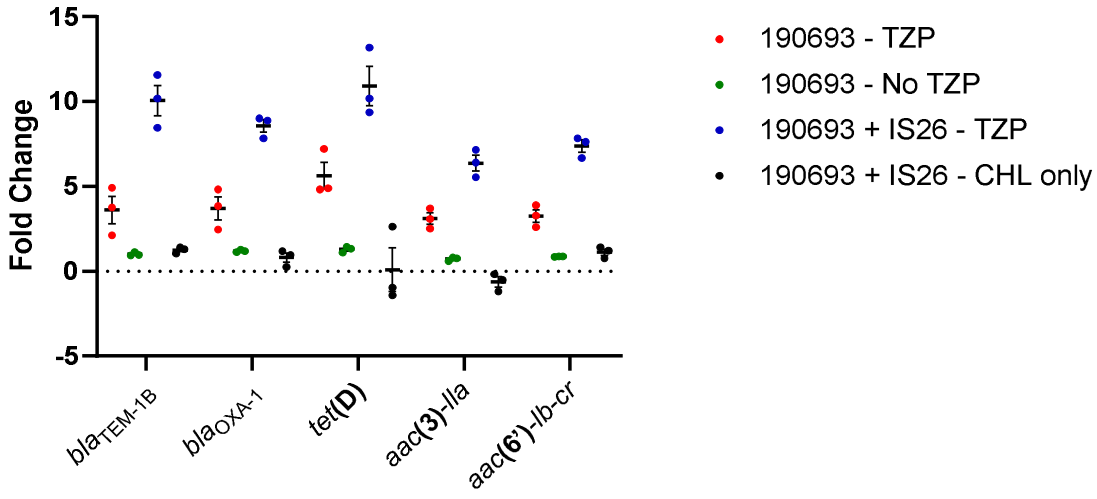


Figure 4: Relative fitness of the TZP-resistant isolate compared to the TZP-susceptible isolate and the TZP-susceptible isolate grown in the presence of TZP compared to the TZP-susceptible isolate grown in the absence of TZP, assessed comparatively in LB, ISO and M9