Journal of Infection 87 (2023) 220–229

Contents lists available at ScienceDirect

Journal of Infection

journal homepage: www.elsevier.com/locate/jinf

Role of bla_{TEM} and OmpC in the piperacillin-tazobactam resistance evolution by *E. coli* in patients with complicated intra-abdominal infection

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ARTICLE INFO

Article history:
Accepted 9 July 2023
Available online 11 July 2023

Keywords:
Escherichia coli
ß-lactamase
OmpC
Piperacillin-tazobactam
Resistance
Complicated intra-abdominal infection

SUMMARY

Piperacillin-tazobactam resistance (P/T-R) is increasingly reported among *Escherichia coli* isolates. Although *in vitro* experiments have suggested that bla_{TEM} gene plays a key role in the P/T-R acquisition, no clinical *in vivo* study has yet confirmed the role of bla_{TEM} or other genes. Therefore, we aimed to identify the mechanisms underlying P/T-R by following up patients with *E. coli* complicated intra-abdominal infections (cIAI) who experienced P/T treatment failure. Four pairs of strains, clonally related from four patients, were isolated both before and after treatment with P/T dosed at 4 g/0.5 g intravenously. The P/T MIC was tested using broth microdilution, and β-lactamase activity was determined in these isolates. Whole-genome sequencing (WGS) was performed to decipher the role of bla_{TEM} and other genes associated with P/T-R. Changes in the outer membrane protein (OMP) profile were analyzed using SDS-PAGE, and bla_{TEM} and *ompC* transcription levels were measured by RT-qPCR. In addition, *in vitro* competition fitness was performed between each pairs of strains (P/T-susceptible *vs.* P/T-resistant). We found a higher copy number of bla_{TEM} gene in P/T-R isolates, generated by three different genetic events: (1) IS*26*-mediated duplication of the bla_{TEM} gene, (2) generation of a small multicopy plasmid (ColE-like) carrying bla_{TEM}, and (3) adaptive evolution *via* reduction of plasmid size, leading to a higher plasmid copy number. Moreover, two P/T-R strains showed reduced expression of OmpC. This study describes the mechanisms involved in the acquisition of P/T-R by *E. coli* in patients with cIAI. The understanding of P/T-R evolution is crucial for effectively treating infected patients and preventing the spread of resistant microorganisms.

Introduction

The ß-lactamases are the main cause of ß-lactam resistance in Gram-negative bacteria[1], whose mechanisms of action are based on the hydrolysis of the ß-lactam ring. Combinations of ß-lactams/ß-lactamase inhibitors (BL/BLI) were introduced to address ß-lactamase activity.[1,2] Some of the most commonly used combinations in

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https://doi.org/10.1016/j.jinf.2023.07.005

clinical practice include ampicillin/sulbactam (A/S), amoxicillin/clavulanic acid (A/C), and piperacillin/tazobactam (P/T), as well as the recently introduced ceftazidime/avibactam (CZA) and ceftolozane/tazobactam (C/T).

P/T is a broad-spectrum BL/BLI commonly used for the empiric treatment of severe infections, including complicated intra-abdominal infection (cIAI) and febrile neutropenia.[1] The use of P/T has increased in recent years, as well as the number of P/T-resistant *Escherichia coli* isolates, limiting its therapeutic use.[3–6] bla_{TEM} enzymes have been described to lead the P/T resistance acquisition in *E. coli*, either through an overproduction of bla_{TEM-1} or an evolution of inhibitor-resistant TEM variants (IRT), or an evolution of bla_{TEM} variants with increased hydrolytic capabilities.[3,4,6–9] Other ß-lactamases such as bla_{OXA-1} or bla_{ampC}, as well as loss of porins are also frequent causes of P/T resistance.[10–14]

In *E. coli*, BL/BLI resistance is a gradual and unidirectional process that extends first from A/S, then A/C and finally P/T. We have previously described a new concept on antimicrobial resistance, called extended-spectrum BL/BLI resistance (ESRI), which is defined as the acquisition of high-level BL/BLI resistance (resistance to A/S, A/C and P/T) from a low- (resistance to A/S with susceptibility to A/C and P/T) or moderate-level (resistance to A/S and A/C and susceptibility to P/T) resistance phenotype, in which the presence of bla_{TEM} plays an important role.[7,15] Exposure to sub-inhibitory concentrations of P/T has been shown to trigger ESRI isolates *in vitro* by two different mechanisms: the first based on duplication of bla_{TEM} through the action of Insertion Sequence 26 (IS*26*); and the other based on transposition of the bla_{TEM} gene into a multicopy plasmid (such as ColE1-like plasmids).[7] Two other studies have demonstrated that the bla_{TEM-1} gene, located on a 10 kb tandem repeated genomic resistance module, was associated with P/T resistance when the *E. coli* 907355 strain was grown *in vitro* in the presence of subinhibitory concentrations of P/T,[4] and the IS26-associated amplification of bla_{TEM1b} gene was found to be associated with P/T resistance in the *E. coli* EC78 strain.[5] It is important to note that both studies only analyzed individual *E. coli* strains that were resistant to P/T.

Here, we have identified four pairs of *E. coli* isolates, recovered from four different patients over two different times, prior and after P/T-treatment, which showed the acquisition of P/T resistance after its administration. Our aim was to determine, for the first time, whether treatment with P/T in patients with cIAI could select for resistance to P/T in *E. coli*, using genomic sequencing with long-read and short-read techniques, along with molecular and microbiological assays. Additionally, we sought to characterize the bacterial resistance mechanisms that contribute to the development of this BL/BLI resistance. Understanding how bacteria acquire resistance to antibiotics is crucial for effective treatment of infected patients and for preventing the spread of resistant microorganisms.

Materials and methods

Study design, patients and ethics

This is a prospective observational cohort study from February 1st, 2020 to December 31th, 2021. Adult patients (≥18 years) with cIAI and bloodstream infection (BSI) caused by *E. coli* at the Virgen del Rocío University Hospital (Seville, Spain) were included in the study. The role of *E. coli* isolated from intra-abdominal and blood samples, as colonization or infection, was evaluated according to previously defined criteria.[16] The study was approved by the Ethics Committee of Virgen Macarena and Virgen del Rocío University Hospitals (approval number C.I. 0088-N-20). Written informed consent was signed by all patients before the inclusion in the study.

Bacterial isolates

Four isolates pairs (AEC-11/AEC-51, AEC-15/AEC-24, AEC-99/AEC-106 and PT3/PT4) from four patients were isolated in the pre- and post-therapy with P/T dosed 4 g/0.5 g intravenously every 8 h in extended infusion. *E. coli* isolates were identified by MALDI-TOF (Bruker Daltonik, GmbH, Germany). In addition, *E. coli* MG1655 and its *ompC* mutant (Δ*ompC*) strains were used.[17] Finally, *E. coli* ATCC 25922 strain was used as a standard control in all the experiments.

Resistance profile

The A/C and P/T antimicrobial susceptibility profiles of the four isolates pairs were initially tested by broth microdilution using the MicroScan WalkAway NMDR-1 panels. P/T MICs for these isolates and for the MG1655 and Δ*ompC* strains were subsequently confirmed by broth microdilution reference method and interpreted according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines.[18] Additionally, the P/T MIC was determined using an approved gradient diffusion assay (E-test) (Liofilchem, Italy) following the protocol published recently by Rafael Canton research group.[19]

Antimicrobial selection pressure

The diluted bacterial inoculum (10^5 CFU/mL) of the different isolates and strains were incubated with sub-inhibitory concentrations of P/T (Merck, Spain) corresponding to dilutions 1-fold below the MICs at 37 ºC for 24 h. Positive bacterial growth was re-adjusted to 10^5 cfu/mL for P/T MIC determinations and for further incubation with a 2-fold increased concentration of P/T concentration of 256/4 mg/L or a P/T concentration that did not allow bacterial growth.

Removal of antimicrobial selection pressure

In order to evaluate the stability of the resistance, daily passages of the P/T-resistant isolates (AEC-51, AEC-24, AEC-106 and PT4) on sheep blood agar plates (ThermoFisher, Spain) were made for 15 days and the P/T MICs were determined on days 5, 10 and 15 for each isolate.

Whole-genome sequencing and bioinformatic analysis

Whole-genome sequencing (WGS) was performed with two different techniques: short-read Illumina sequencing and long-read Oxford Nanopore technology to obtain a consensus sequence. Total DNA was extracted from colonies using (i) the Invitrogen PureLink™ Genomic DNA Mini Kit following the manufacturer's instructions (ThemoFisher, France) and (ii) the Magcore® extractor system H16 with a Magcore Genomic DNA Large Volume Whole Blood Kit (RBC Bioscience Corp., Taiwan). A DNA library was prepared either (i) using a NEB Ultra II FS DNA library prep kit for Illumina (NEB, France) and then run on the MiSeq sequencer (Illumina, France) to generate paired-end 150 pb reads and (ii) by using a R9.4 flow cell and the 1D native barcoding genomic DNA kit (SQK-LSK109) on a MinION sequencer (Oxford Nanopore Technologies, UK). *De novo* assembly of Illumina reads was performed using CLC Genomics Workbench 21.0 (QIAGEN, France). Basecalling was performed in a GPU cluster with four Tesla v100 using guppy 5.0.16 in high accuracy mode to obtain the Nanopore reads. Then and in order to perform a genomic analysis, nanopore long reads were assembled using flye 2.9. The acquired antimicrobial resistance genes were identified using ResFinder v.4.1 (Center for Genomic Epidemiology, CGE, https://cge.food.dtu.dk/services/ResFinder/). The genome was annotated using the Rapid Annotations using Subsystems Technology (RAST) server tool.[7] The ISfinder database (https://isfinder.biotoul.fr/) was used to

identify insertion sequence (IS) elements. The plasmid content was analyzed using PlasmidFinder (https://cge.cbs.dtu.dk/services/PlasmidFinder/) and manually searched by homology. Additionally, MLST tool was used to determine the clonality of the different pairs of isolates from each patient.[7] The bla_{TEM} gene copy number was estimated using the coverage ratio of the bla_{TEM-1b} gene (or its genetic context) and the mean of three chromosomally encoded genes (replication initiator protein *dnaA*, the ß-subunit of RNA polymerase *rpoB* and the malate dehydrogenase *mdh*). To obtain the consensus sequence, reads were filtered with fastp and assembled with flye (version 2.9). Later, the assembly was corrected with medaka (version 1.7.2). Finally, the consensus sequence produced by medaka was corrected by pilon (version 1.20.1).

BLAST analysis and sequence comparison using BRIG

Consensus sequences of the plasmid harboring bla_{TEM} gene were blasted by pairs (P/T-susceptible *vs* P/T-resistant) using the BLAST Ring Image Generator (BRIG) software (version 0.95-dist). The blastn algorithm was used to generate a circular BLAST between the different plasmids.

Quantification of ß-lactamase activity

The ß-lactamase activity was determined using the ß-lactamase Activity Kit (BioVision Inc, Milpitas, CA, USA).[7] Briefly, the bacterial culture pellets were weighed and resuspended in ß-lactamase assay buffer. Samples were sonicated and centrifuged. Five microliters of each sample were incubated with nitrocefin, which is a chromogenic cephalosporin substrate frequently used to find beta-lactamase enzymes. The optical density (OD) at 490 nm was immediately measured in kinetic mode by the CLARIOstar reader (BMG LABTECH, France).

RNA extraction and RT-qPCR

Bacterial RNA was purified using the RNeasy Mini Kit (Qiagen, Netherlands) from overnight cultures. Contaminating DNA was removed from the RNA samples and the reverse transcription step was carried out using the QuantiTect Reverse Transcription kit (Qiagen, Spain), according to the manufacturer's instructions. The primer3 v.0.4.0 software (http://bioinfo.ut.ee/primer3-0.4.0/) was used to design the primers. We used specific primers to amplify the bla_{TEM} and *ompC* genes and the housekeeping *rrsG* gene[7,20] (Table S1). The quantitative real-time PCR assay was carried out with SYBR Premix Ex Taq (Takara, Japan) in a MxPro 3005p system (Stratagene, CA, USA). Gene expression was relatively quantified using the Comparative CT (ΔΔCt) Method (Applied Biosystems Guide, USA).

Growth rate measurements

Growth rate was measured as the maximum increase in OD over time during exponential growth. OD measurements were performed in 96-well plates containing 200 µL Luria Bertani (LB) or Mueller Hinton (MH) (Merck, Spain) broth/well by the Clariostart plate reader. OD at 600 nm was measured with 20 min intervals for 12 h and incubated at 37ºC with shaking for 5 s every measure point. The doubling time (DT) was determined by the exponential curve fitting function to generate an equation in the form $y = Ae^{Bx}$, where A and B are numbers and x is the time between doubling of y ($A_{600\ nm}$). The equation simplifies to DT = ln2/B. Colonies were pre-cultured during 2–3 h with shaking at 37ºC to reach the exponential phase before the inoculation of the final plate.

In vitro competition experiments

The *in vitro* competition index (CI) was determined from growth cultures, consisting of AEC-11 or AEC-51, and a mixed inoculum of equivalent CFUs from AEC-11 and AEC-51 as previously described.[20] Briefly, bacteria with a titer of 5.5 log CFU/mL were grown in LB broth. At 0, 4, 8 and 24 h, 10 µL aliquots were taken and AEC-11 and AEC-51 CFUs were determined by plating serial log dilutions on LB agar (Sigma, Spain) or LB agar plus P/T (64/4 µg/mL). The AEC-51 in competition with AEC-11 was determined after plating the mixed inoculum on LB agar plus P/T to select only the AEC-51 colonies. The AEC-11 in competition with AEC-51 was determined after plating the mixed inoculum on LB agar. The obtained log CFU/mL was subtracted from the log CFU/mL of AEC-51 in competition. The CI was defined as the number of AEC-51 CFUs recovered per number of AEC-11 CFUs recovered at 4, 8 and 24 h, divided by the number of AEC-51 CFUs inoculated per number of AEC-11 CFUs inoculated at 4, 8 and 24 h, respectively. The same protocol was used with the rest of the paired isolates (AEC-15 *vs* AEC-24, AEC-99 *vs* AEC-106 and PT3 *vs* PT4).

Analysis of outer membrane proteins by SDS-PAGE

Bacterial cells were grown in LB to the logarithmic phase and lysed by sonication. Outer membrane proteins (OMPs) were extracted with sodium lauroylsarcosinate (Merck, Spain) and recovered by ultracentrifugation as described previously.[21] The OMP profiles were determined by SDS-PAGE using 10% SDS gels and 6 µg of OMP, followed by Simply Blue SafeStain gel (ThermoFisher, Spain). For peptide mass fingerprinting, a Simply Blue SafeStain band representing an unidentified protein with molecular weight 40 kDa was excised from the SDS-PAGE gel and sent for MALDI-TOF-TOF (MS-MS/MS) analysis. Data obtained from peptide MS fingerprinting were matched against the National Centre for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov) using the Mascot program.

Statistical analysis

Group data are presented as means ± standard errors of the means (SEM). The student *t*-test was used to determine differences between means using the GraphPad Prism 9. A p-value < 0.05 was considered significant.

Results and discussion

Demographics and clinical data analysis

Demographics and clinical features of the four patients included in this study are summarized in Fig. 1 and Table 1.

Patient #1 was an 63-year-old male who had been diagnosed with a neuroendocrine tumor of the pancreas and was undergoing surgery. He was carrying an abdominal drainage catheter due to an infection of the surgical site. He was admitted due to an increase in flow through the drainage, and a diagnosis of intra-abdominal abscess was made. Samples were taken and multisusceptible *E. coli* (AEC-11) was isolated. Treatment with P/T dosed 4 g/0.5 g every 8 h iv. in extended infusion was started. After 12 days, treatment was simplified to A/C, and he was discharged from the hospital. Treatment with A/C was continued at home. However, 73 days later, he was readmitted for removal of the drainage catheter and sampling, where P/T-resistant *E. coli* (AEC-51) was isolated.

Patient #2 was an 66-year-old woman diagnosed with colorectal adenocarcinoma who underwent emergency surgery due to intestinal obstruction. On the 8th day after admission, she was diagnosed with a postsurgical intra-abdominal abscess which was drained, and samples were taken. Multisusceptible *E. coli* (AEC-15)

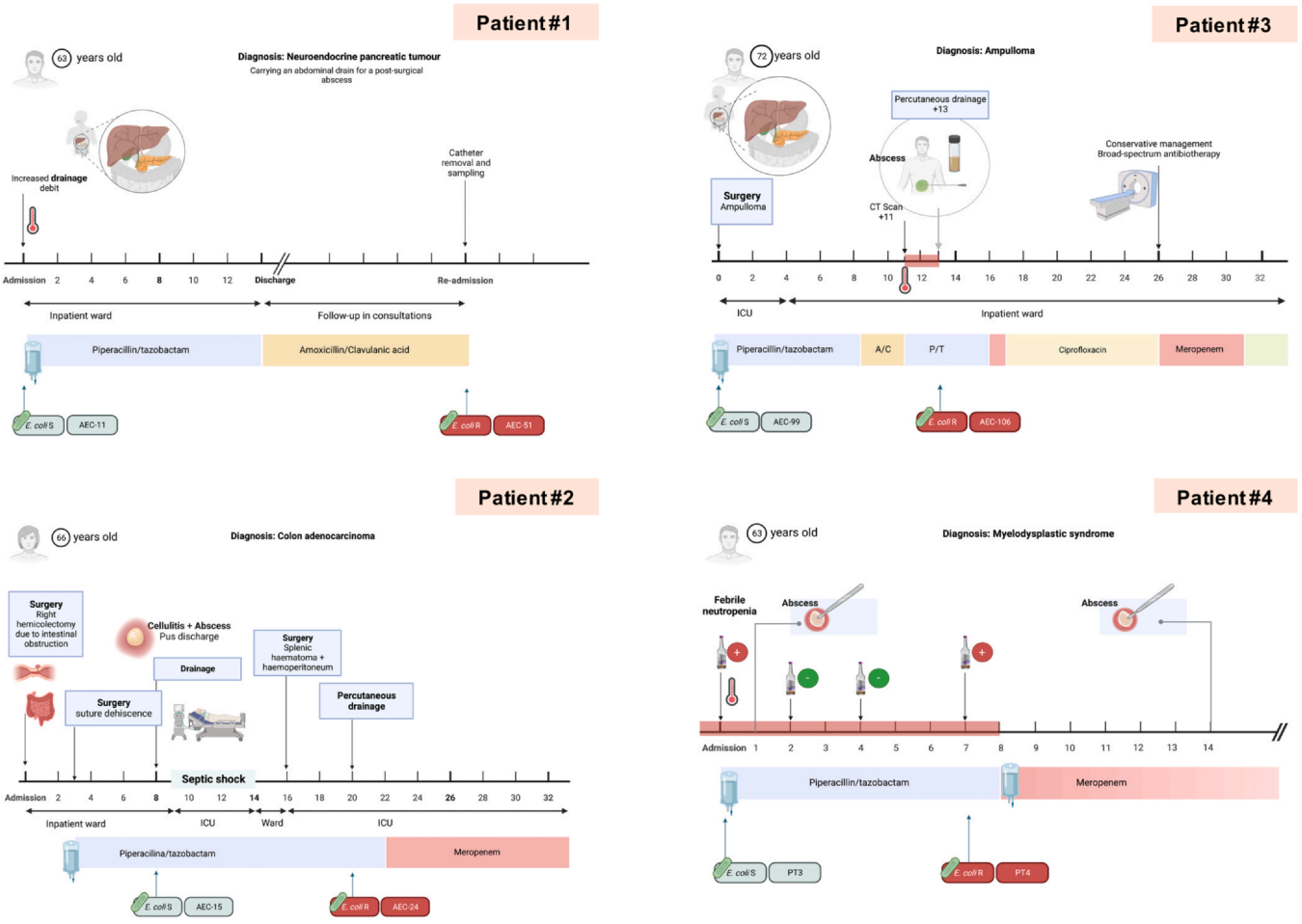

Fig. 1. Cartoon of the four patients hospitalization, treatments and samples obtention.

was isolated, and she started treatment with P/T on the 3rd day since admission, dosed 4 g/0.5 g every 8 h iv. in extended infusion for 19 days. Seventeen days after the start of treatment, a new sample was taken from the abscess, where P/T-resistant *E. coli* (AEC-24) was isolated.

Patient #3 was a 72-year-old man diagnosed with ampulloma who was admitted with symptoms suggestive of cholangitis. On the 13th day of admission, a post-surgical intra-abdominal abscess was drained, and *E. coli* P/T-susceptible (AEC-99) was recovered. He began treatment with P/T dosed 4 g/0.5 g every 8 h iv. in extended infusion on the 1st day of admission, continuing for 14 days. Ten days after the start of treatment, a new sample was taken due to fever onset, and *E. coli* P/T-resistant (AEC-106) was recovered.

Patient #4 was an 63-year-old man diagnosed with myelodysplastic syndrome with excess blasts. He was admitted for febrile neutropenia with an abdomino-pelvic abscess focus. Blood cultures were taken on admission, and multisusceptible *E. coli* (PT3) was isolated. Treatment was started with P/T, dosed 4 g/0.5 g every 8 h iv. in extended infusion, which was maintained for 8 days. On the 7th day, a new blood culture was taken, and P/T-resistant *E. coli* (PT4) was isolated.

MLST analysis

The MLST analysis performed with the assembled Whole Genome sequences revealed the same ST type for each pairs of isolates from the same patient, being ST162, ST48, ST73, and ST88 recovered from the patient #1, #2, #3, and #4, respectively (Table 1).

Analysis of P/T resistance in E. coli isolates

For each patient, a pair of *E. coli* isolates with the same ST were recovered from each single patient (AEC-11 and AEC-51 from patient #1; AEC-15 and AEC-24 from patient #2; AEC-99 and AEC-106 from patient #3, PT3 and PT4 from patient #4) from two separate infection episodes, suggesting within-patient evolution to P/T resistance. All these isolates were carbapenem and 3rd to 4th generation cephalosporins susceptible and none produced an extended-spectrum ß-lactamase (Table S2). The broth microdilution assay demonstrated that the MIC for P/T of the second isolate was 32-fold higher than that of the first isolate in all four cases, prior to intravenous therapy with P/T (Fig. 2A). Almost similar or even higher fold changes were observed with the E-test assay (Table S3).

Since *E. coli* harboring bla_{TEM} acquired resistance to P/T after incrementally increasing the selection pressure (*i.e.* concentration) of P/T,[5,7] we hypothesized that P/T-susceptible isolates (AEC-11, AEC-15, AEC-99 and PT3) became resistant to P/T after exposure to P/T. In the *in vitro* selection pressure, the new P/T MIC of these isolates increased 32-fold with respect to the initial MIC; these isolates thus became resistant to P/T (Table S4).

Of note, the P/T resistance observed for AEC-51, AEC-24, AEC-106 and PT4 remained stable when the antibiotic pressure was removed from the medium. Indeed, after growth in medium free of P/T for 15 days, none had returned to their initial phenotype, except for one isolate (PT4) that decreased its P/T MIC from 256 to 32 mg/L (which is still considered as resistant by EUCAST) (Fig. 2B). This latter is in line with our previous *in vitro* study, in which different *E. coli* isolates

Table 1
Demographics, clinical, epidemiological and microbiological data of the patients and the *Escherichia coli* isolates.

Patient	Sex	Age	Source	Dose regimen	Treatment duration (days)	Isolates	MLST	Resistance phenotype	Resistance genotype
#1	Male	63	Pancreatic abscess	P/T dosed 4 g/0.5 g IV (extended-infusion 4 h)[a]	12	AEC-11	162	AMP, TIC, PIP, CIP, LEV, TMP-SXT	***bla*$_{TEM-1}$**, *tet(B), sul2, dfrA17, aadA5, aph(6)-ld, aph(3")-lb*
						AEC-51	162	AMP, AMC, TIC, TCC, PIP, P/T, CIP, LEV, TMP-SXT	***bla*$_{TEM-1}$**, *tet(B), sul2, dfrA17, aadA5, aph(6)-ld, aph(3")-lb*
#2	Female	66	Post-surgical intra-abdominal abscess	P/T dosed 4 g/0.5 g IV (extended-infusion 4 h)[a]	18	AEC-15	48	AMP, TIC, PIP	***bla*$_{TEM-1}$**, *tet(A)*
						AEC-24	48	AMP, AMC, TIC, TCC, PIP, P/T	***bla*$_{TEM-1}$**, *tet(A)*
#3	Male	72	Post-surgical intra-abdominal abscess	P/T dosed 4 g/0.5 g IV (extended-infusion 4 h)[a]	14	AEC-99	73	AMP, AMC, TIC, PIP, TMP-SXT	***bla*$_{TEM-1}$**, *tet(B), sul2, dfrA8, aph(6)-ld, aph (3")-lb*
						AEC-106	73	AMP, AMC, TIC, TCC, PIP, P/T, TMP-SXT	***bla*$_{TEM-1}$**, *sul2, dfrA8, aph(6)-ld, aph(3")-lb*
#4	Male	63	Blood (Perianal abscess)	P/T dosed 4 g/0.5 g IV (extended-infusion 4 h)[a]	8	PT3	88	AMP, AMC, TIC, PIP, TMP-SXT	***bla*$_{TEM-1}$**, *tet(A), sul2, dfrA5, aph(6)-ld, aph (3")-lb*
						PT4	88	AMP, AMC, TIC, TCC, PIP, P/T, TMP-SXT	***bla*$_{TEM-1}$**, *tet(A), sul2, dfrA5, aph(6)-ld, aph (3")-lb*

AMP, ampicillin; AMC, amoxicillin/clavulanic acid; TIC, ticarcillin; TCC, ticarcillin/clavulanic acid; PIP, piperacillin; P/T, piperacillin/tazobactam; CIP, ciprofloxacin; LEV, levofloxacin; TMP-SXT, trimethoprim/sulfamethoxazole. [a]extended infusion every 8 h.
The sources are the same for the first and second isolates of each patient.

acquiring P/T resistance by selective pressure kept their resistance to P/T in medium free of P/T for 15 days.[7]

Genomic analysis of the region surrounding the bla$_{TEM-1}$ gene in E. coli

To better understand and describe the underlying mechanisms responsible for the significative increase in P/T MICs, the four pairs of isolates were sequenced by Oxford Nanopore technologies (long read) and corrected using Illumina short reads, allowing complete assembly of the plasmids and of the genomes. Analysis of the different pairs of isolates revealed the following results:

(i) in the AEC-11 isolate the mean chromosomal coverage (MCC) was 12.06x, and the *bla*$_{TEM-1}$ coverage was 9.07x, representing a single copy per cell. Meanwhile, in AEC-51 isolate, the MCC was 12.21x, and the *bla*$_{TEM-1}$ coverage was 233.75x representing 25 copies per cell. Analysis of the coverage of the region surrounding the *bla*$_{TEM-1}$ gene in AEC-51 isolate revealed five copies of IS*26* bracketing the *bla*$_{TEM-1}$ gene, generated through a series of ~ 15 kb DNA fragment translocation events by IS*26*. The repeated fragment is composed by resistance genes (*bla*$_{TEM-1}$, *tet (B), dfrA*, and *ant(3′)-la*), and MGEs (IS*4*, IS*26*, and *IntI*) (Fig. 3A). The latter finding aligns with a study by Hubbard et al., in which they identified, in a pair of clonal *E. coli* isolates obtained from a single patient across two distinct infection episodes, an increase in *bla*$_{TEM-1b}$ gene amplification when a translocable unit containing IS*26* was reintroduced into the chromosome, resulting in the formation of a tandem array of translocable units.[6] Indeed, resistance to antibiotics by increase of resistance gene copy numbers has already been described among *Enterobacterales in vitro* and *in vivo*.[7,22]

(ii) in the AEC-15 isolate the MCC was 51.44x, and the *bla*$_{TEM-1}$ gene coverage was 4.63x, representing a single copy per cell. Meanwhile, in AEC-24 isolate, the MCC was 48.14x, and the *bla*$_{TEM-1}$ coverage was 108.67x representing 25 copies per cell. Similarly, in the AEC-99 isolate the MCC was 5.39x, and the *bla*$_{TEM-1}$ coverage was 2.51x, representing a single copy per cell. Meanwhile, in AEC-106 isolate, the MCC was 2.56x, and the *bla*$_{TEM-1}$ coverage was 45.17x representing 35 copies per cell. Genetic context analysis revealed that plasmids of AEC-24 and AEC-106 isolates underwent similar modifications with slight differences. While a single copy of *bla*$_{TEM-1}$ gene and no single nucleotide polymorphisms (SNPs) were identified in the two pairs of *E. coli*, a consistent deletion of approximately 60 kb and 30 kb was observed in the pAEC-24 and the pAEC-106 plasmids respectively (Fig. 3C and 3D). In pAEC-24, the consistently deleted region encoded SOS system inhibitor genes, plasmid partition system, methyltransferase genes, and the main conjugation machinery. Interestingly, deletions was surrounded by IS*26*, suggesting that intramolecular transposition and recombination lead by the insertion sequence allowed for the observed loop-out dynamics (Fig. 3C).

On the other hand, pAEC-106 lost regions encoding transporter system, iron related genes, and two resistance determinants. In this case, deletions was surrounded by IS*1*, IS*4*, and IS*26*, which could be the responsible of the reorganization of the plasmid backbone (Fig. 4D). ISs and mainly IS*26* is often found on bacterial plasmid associated with antibiotic resistance genes, and believed to be involved in the plasmid reorganization through intramolecular replicative transposition events.[23–26] Hence, we showed that the reported adaptative evolution mechanism (deletions of costly plasmid genes) in pAEC-24 and pAEC-106 is also used as a resistance mechanism by increasing plasmid copy number in order to overcome antibiotics, even when this change entails an increased fitness cost.

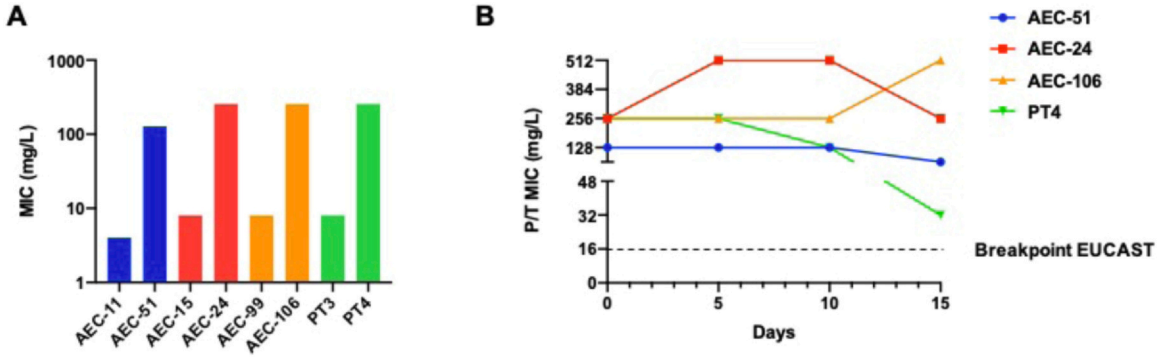

Fig. 2. P/T resistance and stability in *Escherichia coli* isolates. (A) MIC of P/T in the four pairs isolates of *E. coli*. (B) Stability of P/T MIC during 15 days in the absence of P/T.

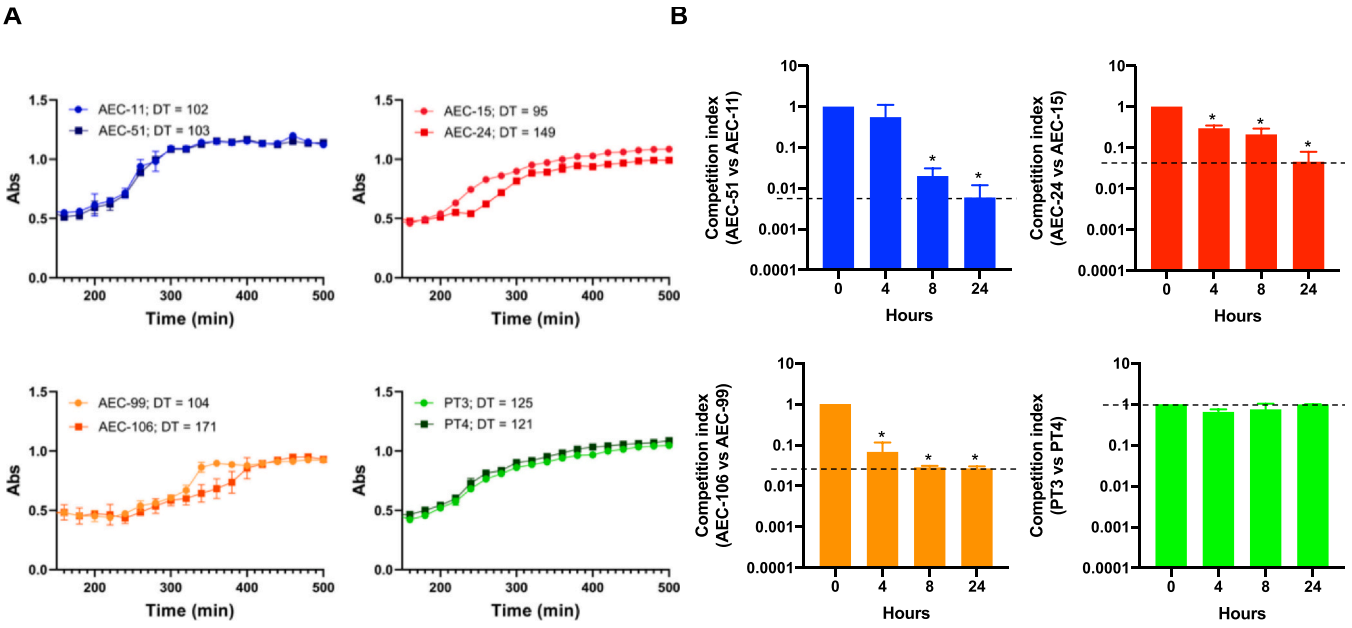

Fig. 3. BLAST atlas of the bla_{TEM-1} gene harboring plasmids. (A) pAEC-11 plasmid (IncFIB) compared to the pAEC-51 plasmid (IncFIB). (B) pPT3 plasmid (IncFIB) compared to the pPT4 (IncFIB) and pSmPT4 plasmids. (C) pAEC-15 plasmid (IncFII) compared to the pAEC-24 plasmid (IncFII). (D) pAEC-99 plasmid (IncFII/FIA/FIB) compared to the pAEC-106 plasmid (IncFII/FIA/FIB). GC content is represented on the distance scale (in kbp) on the inner map. Resistance genes (red), Mobile Genetic Elements [MGEs] (blue), replication genes (orange), conjugative transfer genes (green) and other genes (black) are also indicated in the external ring. The maps were drawn using BLAST Ring Image Generator (BRIG) (http://sourceforge.net/projects/brig/).

(i) in the PT3 isolate the MCC was 26.24x, and the bla_{TEM-1} coverage was 21.66x, representing a single copy per cell. In PT4 isolate, the MCC was 28.75x, and the bla_{TEM-1} coverage was 517.89x representing 18 copies per cell. Genetic context analysis of PT4 isolate reveals that two copies of bla_{TEM-1} gene localized in the pPT4-IncFIB plasmid (150 kb), which showed 100% identity with the pPT3-IncFIB plasmid (150 kb), and in the pSmPT4 plasmid (8 kb), which was generated through the action of the IS26 and harbor resistance genes such as bla_{TEM-1}, *dfrA*, *aph(6′)-Ic* and *aph (3′)-I*, and two origin of replication (*repA* and *repC*) (Fig. 3B). The reduced size of the pSmPT4 plasmid is commonly associated to a increased copy number, leading to a major expression and production of bla_{TEM-1} with the consequent elevated MIC of P/T. Similar adaptative evolution in conditions selecting for plasmid carriage was observed in *E. coli* after an gradual pressure by P/T *in vitro*.[7] The latter study described the transposition event of a transposon (Tn*2*) from a plasmid *IncI1* to a *ColE1-like* plasmid, in contradiction to our study, where the pSmPT4 was the product of the circularization of a plasmidic fragment induced by the action of the IS*26*.[7]

Impact of the P/T resistance on bacterial fitness

Previous studies on plasmid evolution have described that the deletions of large DNA fragments increased the plasmid stability in the host through reducing the fitness cost of the original plasmid.[27,28] In order to test the effect on the fitness of the P/T-susceptible and -resistant bacteria, growth rates were determined. Surprisingly, growth rate assays revealed increased doubling times (DT) for the resistant isolates AEC-24 (DT = 149 min) and AEC-106 (DT = 171 min) in comparison with their paired isolates AEC-15 (DT = 95.5 min) and AEC-99 (DT = 104.5 min), respectively (Fig. 4A). This increase in doubling time is probably related to their reduced fitness. For the two other pairs of isolates (AEC-11/AEC-51 and PT3/PT4), the growth rates were identical, suggesting no fitness cost in those types of resistance mechanisms (Fig. 4A).

To confirm this observation, *in vitro* competition experiments between the four pairs of isolates have been performed. The competition index between AEC-51 and AEC-11, and PT4 and PT3 did not show significant decrease of AEC-51 and PT4 growth from 1 to 0.55 and 0.65 at 4 h of culture, respectively. Meanwhile, the competition

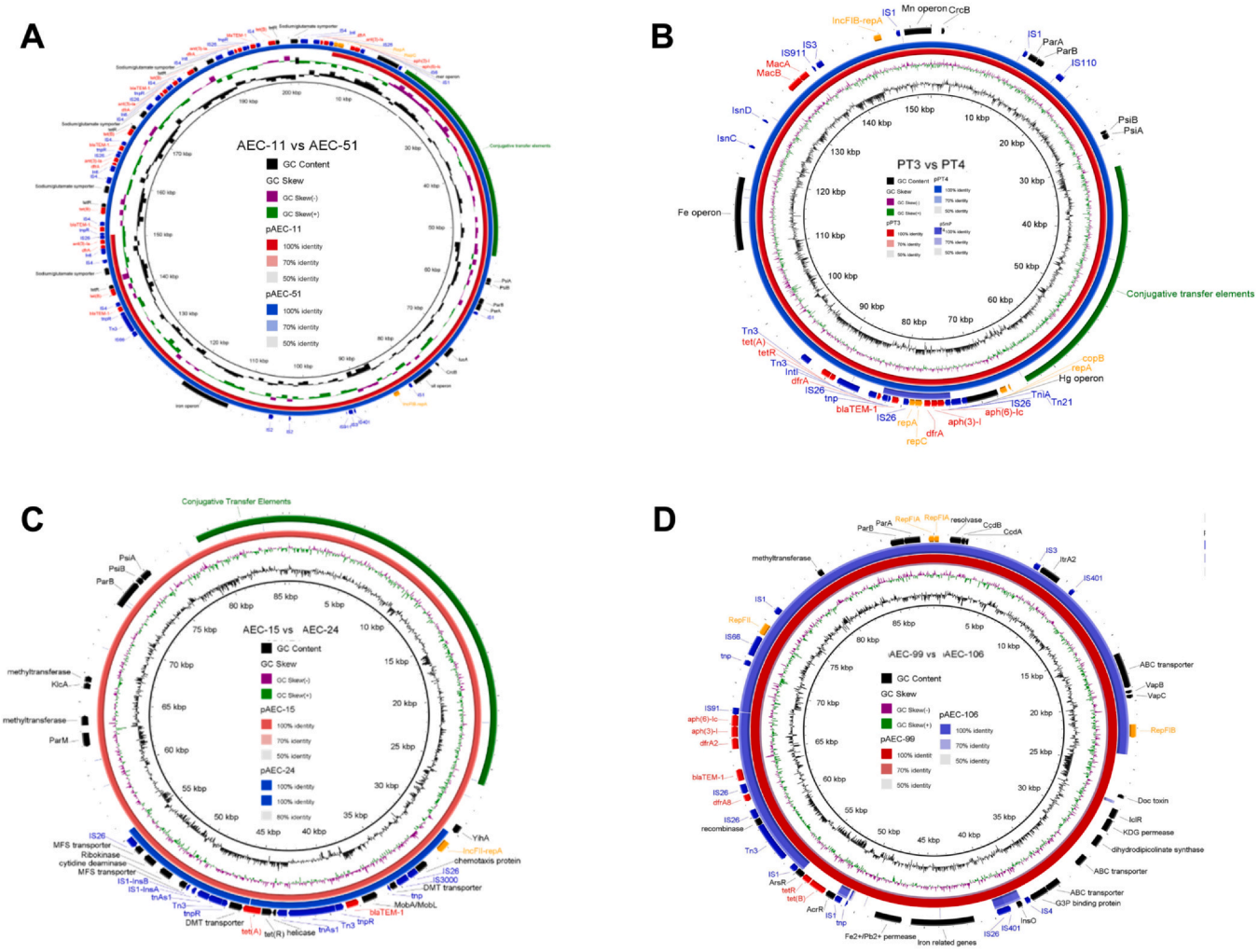

Fig. 4. Impact of the P/T resistance on bacterial fitness. (A) Growth of the four pairs isolates of *E. coli*, and (B) Competition index between each pairs of isolates during 24 h. * $P < 0.05$ for 4, 8 and 24 h *vs* 0 h.

index between AEC-24 and AEC-15, and AEC-105 and AEC-99 showed a significant decrease of AEC-24 and AEC-105 growth from 1 to 0.29 and 0.007, respectively, at 4 h of culture. Finally, at 24 h of culture all the resistant isolates have reduced their competition index significantly, from 1 to 0.006–0.099 (Fig. 4B). These data indicate that, despite the IS*26*-based duplication and generation of smaller plasmids (ColE-like) in P/T-R isolates, their fitness cost remained unaffected. Hubbard et al. reported similar findings, where no significant difference in fitness cost was observed in *E. coli* harboring IS*26*-based duplications associated with P/T resistance.[6] However, Adler et al. identified a fitness cost associated with IS*26*-mediated amplification of other antibiotic resistance genes.[29] While the absence of fitness cost could result in the persistence of the P/T-R phenotype even in the absence of P/T pressure (Fig. 2B), the two isolates (AEC24 and AEC106) exhibited a reduction in plasmid size and fitness cost, resulting in a higher plasmid copy number. However, despite these changes, they still maintained the P/T-R phenotype. Further investigations are needed to understand why AEC24 and AEC106 exhibit persistence of P/T resistance.

bla_{TEM-1} is involved in the acquired resistant to P/T

Since isolates of *E. coli* carrying bla_{TEM} enzymes could acquire P/T resistance by expressing higher β-lactamase activity,[7] we suggest that the increase in P/T MIC values of the study isolates would be due to higher β-lactamase activities after hyperproduction of bla_{TEM} enzymes. Indeed, Fig. 5A shows that AEC-51, AEC-24, AEC-106 and PT4 isolates resistant to P/T showed higher β-lactamase activity than their paired susceptible *E. coli* isolates. Noteworthy, in the absence of selective pressure, these isolates maintained higher β-lactamase activity, which may explain the long-term stability of P/T resistance in the absence of this antibiotic observed in this and in another study.[7] The hyperproduction of bla_{TEM-1} in the isolates of this study has been previously reported and is widely associated with P/T resistance.[4–7,15,30] However, other β-lactamases such as the inhibitor variant of bla_{TEM-1}, bla_{OXA-1}, and $bla_{CTX-M-15}$ hyperproduction have also been linked to P/T resistance.[7,15,30] None of these β-lactamases were found in any of the studied isolates, suggesting that bla_{TEM-1} may play a significant role in inducing resistance to P/T.

In addition, we analysed the transcription of bla_{TEM} in these isolates, and found that the PT-R isolates contained higher levels of transcripts of bla_{TEM} (Fig. 5B). Of note, other ß-lactamases genes were not detected in these isolates.

In order to verify whether differential transcription of bla_{TEM-1} gene was due to different promoter sequences, upstream regions were analyzed. In all cases, the promoter upstream the bla_{TEM-1} gene was *Pa/Pb,* which has been shown to be a strong promoter responsible of bla_{TEM-1} hyperproduction, leading to resistance to

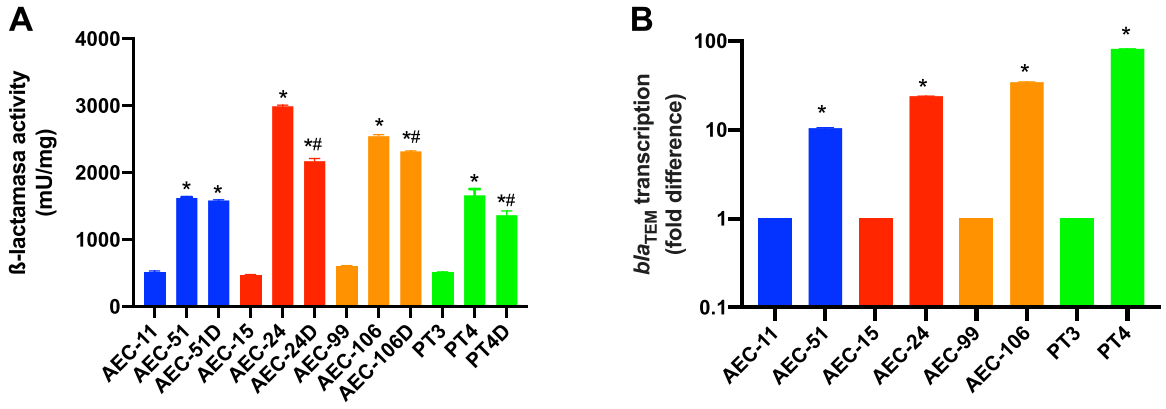

Fig. 5. bla_{TEM} is involved in the acquired resistance to piperacillin/tazobactam. (A) ß-lactamase activity of the four pairs of *E. coli* isolates. (B) bla_{TEM} gene transcription in the four pairs of *E. coli* isolates. * $P < 0.05$ for AEC-51 and AEC-51D *vs* AEC-11, AEC-24 and AEC-24D *vs* AEC-15, AEC-106 and AEC-106D *vs* AEC-99, PT4 and PT4D *vs* PT3. # $P < 0.05$ for AEC-24 *vs* AEC-24D, AEC-106 *vs* AEC-106D, PT4 *vs* PT4D. Deselected (D) indicates an isolate without selective P/T pressure.

ß-lactamase inhibitors such as clavulanate and sulbactam[31] and also tazobactam.[6,8] No mutation was evidenced in the *Pa/Pb* region of any P/T resistant isolates as compared to the P/T susceptible ones.

Role of OmpC in the P/T resistance

Additional comparative analysis of the WGS revealed mutations in *ompC* and *ompR* genes, a regulator of OmpC expression,[32] in the P/T-resistant AEC-51 and AEC-24 isolates as compared with P/T-susceptible AEC-11 and AEC-15, respectively. The mutation in *ompC* gene consist in an chromosomal-insertion of adenosine duplication at position 51 of the gene (c.51 dupA) generating an frame shift change, and the mutation of *ompR* gene consist in single nucleotide variation (c 0.136 C > T) in the position 136 of the *ompC* gene leading to an amino acid substitution (Arg46Cys). Transcription analysis of *ompC* gene revealed that *ompC* gene was less transcribed in P/T-resistant AEC-51 and AEC-24 isolates (Fig. 6A). Furthermore, SDS-PAGE analysis of OMPs showed a reduction of the expression of a protein identified as OmpC by MALDI-TOF-TOF (MS-MS/MS) (Fig. 6B). Notably, after exposure of MG1655 wt and Δ*ompC* strains with 2-fold increased P/T concentration, the MIC of Δ*ompC* strain was increased progressively from 8 to 32-folds as compared with the MG1655 wt strain (Fig. 6C).

OmpC constitute the main OMP in *E. coli* that is necessary for drug transport across cellular membranes.[33] A reduction in the OmpC expression has been associated with the ß-lactam reduced susceptibility/resistance.[34–36] The importance of the OmpC in the P/T resistance is less clear.[37] Additional studies are need to decipher how P/T regulates the expression of OmpC.

Long read sequencing has shown to be crucial in the elucidation of the antimicrobial resistance mechanisms, mainly those involving

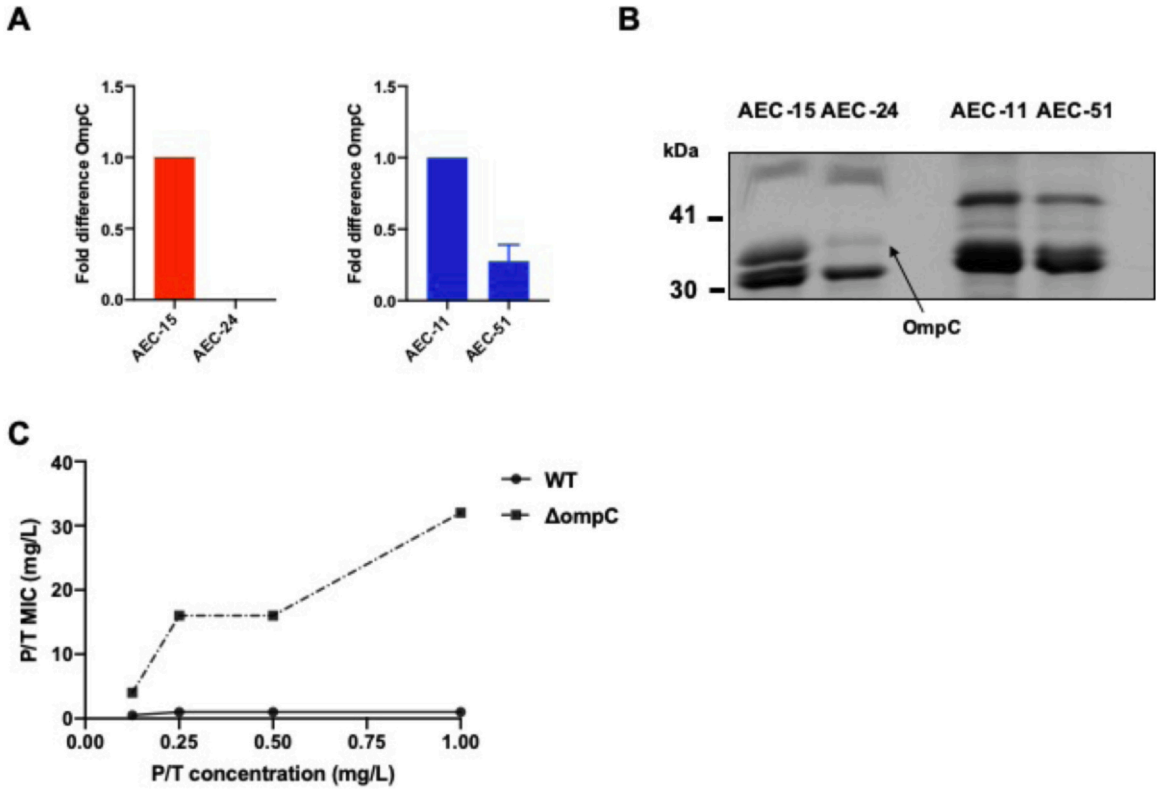

Fig. 6. Role of OmpC in the P/T resistance. (A) *ompC* gene transcription (B) OmpC expression in the two pairs of *Escherichia coli* isolates, and (C) MIC of P/T in MG1655 and its *ompC* mutant strains.

numerous IS elements and repeat sequences. The acquisition of P/T resistance induced by P/T treatment is not attributed to a clonal outbreak of high-risk clones but rather to multiple acquisitions of resistance mechanisms within the circulating population of clinical strains. In this study, we have found three ways in the acquisition of resistance to P/T: (i) IS*26*-based duplication, as previously shown.[6,7] We found modifications of a pre-existing plasmid by transposition, rather than acquisition of foreign DNA; (ii) generation of a smaller plasmid (ColE-like) harboring the $bla_{\text{TEM-1}}$ gene as previously reported in *in vitro* studies,[7] and; (iii) adaptative evolution through the reduction of the plasmid size and fitness cost, leading to a higher plasmid copy number. To the best our knowledge, this is the first study describing IS-mediated deletions of important traits of the plasmid as a driving force of bacterial resistance by increasing the plasmid copy number. This kind of genetic event (recombination, transposition or conjugation) occurs frequently, but the genetic events leading to antibiotic resistance, such as, higher production levels of bla_{TEM}, seem to be responsible in clinical failures of P/T treatments.

Conclusions

We demonstrated that treatment of *E. coli* cIAI with P/T can result in the development of resistance to this antibiotic. Three different mechanisms of resistance to P/T were found, in all of which the $bla_{\text{TEM-1}}$ gene played a central role and OmpC expression was reduced. The novelty of this study lies in the identification of a new evolutionary mechanism based on adaptive evolution through the reduction of plasmid size and fitness cost, leading to an increased plasmid copy number and the emergence of P/T-R *E. coli* isolates. This work represents a significant advancement in our understanding of the mechanisms underlying the acquisition of P/T resistance by *E. coli* in patients with cIAI. This enhanced understanding of P/T resistance evolution is crucial for effectively treating infected patients and preventing the spread of resistant microorganisms.

Funding

This work was funded by the Instituto de Salud Carlos III, Subdirección General de Redes y Centros de Investigación Cooperativa, Ministerio de Economía, Industria y Competitividad (grant PI19/01009), by Plan Nacional de I+D+i 2013–2016, and by the Instituto de Salud Carlos III, Subdirección General de Redes y Centros de Investigación Cooperativa, Ministerio de Economía, Industria y Competitividad, Spanish Network for Research in Infectious Diseases (grant RD16/0016/0009), cofinanced by the European Development Regional Fund “A way to make Europe”/”Investing in your future”. A.R.V, R.A-M, J.A.L. and J.M.C. were supported (grant CB21/13/00006) by CIBERINFEC - Consorcio Centro de Investigación Biomédica en Red, Instituto de Salud Carlos III, Ministerio de Ciencia e Innovación and Unión Europea – NextGenerationEU.

L.G.B. was partly financed by a grant from the Spanish Society of Infectious Diseases and Clinical Microbiology (SEIMC) and is supported by the Subprograma Rio Hortega, Instituto Carlos III, Subdirección General de Redes y Centros de Investigación Cooperativa, Ministerio de Ciencia, Innovación y Universidades, Spain (CM22/00196). J.M.O.R. is supported by the Subprograma Sara Borrell, Instituto de Salud Carlos III, Subdirección General de Redes y Centros de Investigación Cooperativa, Ministerio de Ciencia, Innovación y Universidades, Spain (CD21/00098). A.R.V. is supported by the Subprograma Juan Rodés, Instituto de Salud Carlos III, Subdirección General de Redes y Centros de Investigación Cooperativa, Ministerio de Ciencia, Innovación y Universidades, Spain (JR20/00023), and Y.S. has received a Miguel Servet Tipo II contract from the Instituto de Salud Carlos III, Subdirección General de Redes y Centros de Investigación Cooperativa, Ministerio de Economía y Competitividad, Spain (grant CPII20/00018). Funding for open access publishing: Universidad Pablo de Olavide

Data availability

Whole-genome sequences were deposited in GenBank under the BioProject accession number PRJNA949846.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We like to thank Andrea Vila Domínguez for technical help and Sanna Koskiniemi for the gift of the MG1655 and its *ompC* mutant strains.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jinf.2023.07.005.

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