Piperacillin/tazobactam resistant, cephalosporin susceptible *Escherichia coli* bloodstream infections driven by multiple resistance mechanisms across diverse sequence types

Thomas Edwards[1,*], Eva Heinz[2,3,4], Jon van Aartsen[5], Alex Howard[5], Paul Roberts[5,6], Caroline Corless[5], Alice J. Fraser[1], Christopher T. Williams[1], Issra Bulgasim[1], Luis E. Cuevas[3], Christopher M. Parry [3,7,8,9], Adam P. Roberts[1], Emily R. Adams[1], Jenifer Mason[5] and, Alasdair T. M. Hubbard[1,*]

[1]Department of Tropical Disease Biology, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool, L3 5QA

[2]Department of Vector Biology, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool, L3 5QA

[3]Department of Clinical Sciences, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool, L3 5QA

[4]Wellcome Sanger Institute, Wellcome Genome Campus, Hinxton, CB10 1SA

[5]Liverpool University Hospital Foundation Trust, Prescot street, Liverpool, L7 8XP

[6]Faculty of Science and Engineering, University of Wolverhampton, Wulfruna Street, Wolverhampton WV1 1LY

[7]Alder Hey Children's NHS Foundation Trust, Eaton Road, Liverpool, L12 2AP

[8]Department of Clinical Infection, Microbiology and Immunology, University of Liverpool, L69 7BE

[9]School of Tropical Medicine and Global Health, University of Nagasaki, Japan

[*]Corresponding authors:

Dr. Thomas Edwards; email: thomas.edwards@lstmed.ac.uk, telephone: 0151 705 3308

Dr. Alasdair Hubbard; email: alasdair.hubbard@lstmed.ac.uk, telephone: 0151 705 3163

Abstract

Resistance to piperacillin/tazobactam in *Escherichia coli* is usually mediated by mechanisms providing resistance to 3rd generation cephalosporins, such as extended-spectrum β-lactamases and carbapenemases. Recent reports have identified *E. coli* strains with resistance to piperacillin/tazobactam but susceptibility to 3rd generation cephalosporins, achieved through hyperproduction of penicillinases, but the genetic diversity of this phenotype and the diversity of resistance mechanisms are unknown. We analysed the genomes of 63 clinical isolates of *E. coli* with this phenotype, isolated between 2014-2017 at a single tertiary hospital in Liverpool, UK. The phenotype was displayed in a broad range of sequence types which, after comparison with a UK-wide collection, reflected the overall diversity of *E. coli* clinical isolates. Resistance mechanisms were also diverse, and included predicted hyperproduction of penicillinases, either via strong promoters or gene amplification, carriage of inhibitor resistant β-lactamases, and an S133G $bla_{\text{CTX-M-15}}$ mutation detected for the first time in clinical isolates.

Introduction

Escherichia coli is the most common cause of bacterial blood stream infections globally [1], accounting for 27% of all bacteraemic episodes, with a case fatality rate of 12% [2], and in the UK caused 78·8 blood stream infections per 100 000 people in 2014 [3]. Antimicrobial resistance (AMR) in *E. coli* is increasingly prevalent as highlighted by major public health agencies [4-6]. Extended spectrum β-lactamase (ESBL) production, mediating resistance to 3rd generation cephalosporins (3GCs) and other β-lactam antibiotics [7], is of particular concern and was recorded in approximately 11% of *E. coli* isolated from blood stream infections isolated in 2018 in the UK [8].

One strategy to provide therapeutic options for antimicrobial resistant infections has been the combined use of β-lactamase inhibitors with β-lactam antibiotics to block the activity of β-lactamase enzymes, rendering the bacteria *de facto* susceptible [9]. The inhibitor tazobactam, which inhibits class A β-lactamases and thus includes most ESBL enzymes, is commonly utilised in combination with the penicillin class antibiotic piperacillin [10]. Tazobactam is described as a "suicide inhibitor" as it irreversibly binds to the β-lactam ring of antibiotics permanently inactivating the enzyme via secondary reactions in the enzyme active site [11]. Piperacillin/tazobactam (TZP) has broad spectrum activity against Gram-negative and -positive bacteria [12], is well tolerated [13], available for paediatric use, and is utilised in the UK as a first line empirical agent for a range of serious infections including pneumonia, intra-abdominal infection and sepsis [14]. Its broad coverage and effectiveness make it an important agent for reducing the usage of carbapenem drugs, which are globally important last line antibiotics. Limiting carbapenems is essential for preventing the spread of carbapenem resistance, which is a critical element of antimicrobial stewardship [15], as treatment options for carbapenem resistant bacteria are often limited to poorly tolerated drugs such as colistin or tigecycline [16]. Whilst TZP does provide coverage against ESBLs, the MERINO trial demonstrated TZP to be inferior to meropenem in treating patients with ESBL *E.*

coli and *K. pneumoniae* blood stream infections [17], and carbapenems are now recommended for this patient group [18].

In 2018, resistance to TZP occurred in 9.1% of invasive *E. coli* isolates in the UK [19]. This can be caused by the production of carbapenemase enzymes [20], multiple β-lactamases [21] or ESBLs in combination with increased efflux or porin loss [22], which also provide resistance to 3GCs. Recently, a phenotype of resistance to TZP with susceptibility to 3GCs has emerged in *E. coli* and *Klebsiella pneumoniae*, indicating an alternative resistance mechanism. The major cause of this phenotype has been determined to be hyperproduction of class A or D β-lactamases such as TEM-1 [23,24], SHV-1 [25] and OXA-1 [26]. Increased production of β-lactamase overcomes the inhibitive effect of tazobactam, ostensibly through saturation of the inhibitor, allowing the excess enzyme to hydrolyse and degrade the antibiotic, piperacillin [25]. β-lactamase hyperproduction can occur via increased gene expression modulated by a stronger promoter [27], or an increase in gene copy number mediated by insertion sequences [28,29] or plasmids [24] .

Routine blood culture surveillance identified the occurrence of this phenotype in *E. coli* at the Royal Liverpool University Hospital (RLUH), Liverpool, UK, between 2014 and 2017, and we sought to identify the diversity of *E. coli* strains and distribution of known mechanisms of TZP. Our analysis demonstrates that the introduction and subsequent increase of this phenotype in the hospital is not the spread of a single clone or resistance mechanism, but in fact arose multiple times into phylogenetically distant lineages with diverse resistance mechanisms. This demonstrates TZP resistance has the potential to occur in a wide variety of *E. coli* lineages and must be closely monitored to prevent unnecessary over-use of carbapenems on 3GC-susceptible infections.

Methods

Study setting

The RLUH is the primary hospital in Liverpool, UK, providing secondary and tertiary care, with a catchment area of >2 million people in Merseyside, Cheshire, North Wales, and the Isle of Man. In 2019 the hospital recorded around 95,000 daily inpatients; and over 587,000 outpatient appointments. On-site blood culture facilities are used for adult patients with presumptive bacteraemia. All cultured isolates undergo antimicrobial susceptibility testing and are stored at -80ºC for future studies

Ethics statement

The study utilised bacterial isolates collected by the RLUH for standard diagnostic purposes. All isolates were anonymised and de-linked from patient data. As no human samples or patient data were utilised in the study, ethical approval was not required. This was confirmed using the online NHS REC review tool http://www.hra-decisiontools.org.uk/ethics/.

Surveillance data & Isolate collection

Blood stream bacterial pathogens at RLUH were first isolated using BacTAlert 3D blood culture system (bioMérieux, France) and identified to a species level using MALDI-TOF (Bruker, US). Antimicrobial susceptibility testing (AST) was carried out using disk diffusion-based testing according to British Society of Antimicrobial Chemotherapy guidelines [30] between 2014 and August 7th 2017, after which these were replaced by the European Committee for Antimicrobial Susceptibility Testing (EUCAST) guidelines [31]. In 2014 ceftazidime was used as the indicator 3GC, which was changed to cefpodoxime between 2015 and 2017. Isolate details and AST results were recorded within the Laboratory Information System (Telepath, CSC, US). All isolates were retained in glycerol stocks at -80°C in the RLUH Biobank. Data for the study was extracted into a central database, and included susceptibility data for ampicillin, cefpodoxime/ceftazidime, TZP, meropenem, ertapenem, cefoxitin, ciprofloxacin, gentamycin, amikacin, amoxicillin/clavulanic acid, tigecycline, and chloramphenicol. This dataset was used to identify the proportion of *E. coli* isolates per year with TZP resistance, with and without associated 3GC resistance. In

cases were multiple isolates were obtained from a single infectious episode, only the first isolate was included for further investigation and sequencing, to avoid duplication. Isolates that were TZP resistant/3GC susceptible were identified, retrieved from the Biobank and resurrected from glycerol stocks using Luria-Bertani agar (Oxoid, UK) and incubated at 37°C for 18 hours.

Minimum inhibitory concentrations

Minimum inhibitory concentrations (MIC) for the isolates were obtained using the E-TEST method (Biomerieux, France) [32] according to EUCAST guidelines [33], to determine the MICs for TZP and for the 3GC ceftriaxone (CTX).

DNA extraction and sequencing

Genomic DNA was extracted using the PureGene Yeast/Bacteria Kit (Qiagen, Germany), following the manufacturer's instructions for Gram-negative bacteria. Genome sequencing of 65 isolates was performed by MicrobesNG (http://www.microbesng.uk), using 2 x 250 bp short-read sequencing on the Illumina MiSeq (Illumina, US) (Table S1).

Genome analysis, sequence typing and AMR gene prediction

All genomes were *de novo* assembled and annotated using SPAdes version 3.7 [34], and Prokka 1.11 [35], respectively, by MicrobesNG, in addition to providing the trimmed and quality filtered sequencing reads. The presence and copy number of AMR genes was determined using ARIBA [36], with the strst2-argonnot database [37]. *In silico* multi locus sequence typing (MLST), and plasmid replicon typing were also carried out using ARIBA and the MLSTFinder [38] and PlasmidFinder [39] databases, respectively. β-lactamase promoters were identified by constructing databases with promoter sequences for $bla_{\text{TEM-1}}$ [27] and $bla_{\text{SHV-1}}$ [40] and screening using ARIBA . Copy number was estimated by dividing the sequencing coverage of β-lactamase genes by the coverage of the chromosomal single copy gene *ampH*.

Phylogenetic analysis of study isolates

A pan genome analysis of all sequences was generated using roary [41], and the core gene alignment was used as input for snp-sites [42] to extract ACGT-only SNPs (-c option). A maximum likelihood tree was produced using iqtree [43], with the general time reversible (GTR) model and gamma correction using ASC ascertainment bias correction (ASC) for SNPs-only alignments (-m GTR+G+ASC) and 1000 bootstrap replicates (-bb 1000). Phylogenetic trees were annotated using the Interactive Tree of Life [44] (https://itol.embl.de/). Core genome trees for ST131 and ST73 were generated by mapping the reads against the reference chromosomes of *E. coli* strains EC958 (HG941718.1) and CFT073 (AE014075.1), respectively, using snippy (https://github.com/tseemann/snippy). Recombination blocks were removed with gubbins [45], and extraction of SNPs-only of the recombination-free alignment, and tree calculation, were performed as described above, using snp-sites and iqtree.

To investigate the positioning of the study isolates in the UK *E. coli* population, the sequences from a large UK-wide comparative analysis were included (PRJEB4681, [46]). These sequences included 1094 isolates submitted to the UK wide Bacteraemia Resistance Surveillance Programme (www.bsacsurv.org) between 2001–2011 by 11 hospitals across England, and 415 isolates provided by the Cambridge University Hospitals NHS Foundation Trust (CUH), Cambridge.

A core gene alignment and phylogenetic tree were constructed as described above. Isolates from the UK-wide collection with the same phenotype of TZP resistance/3GC susceptibility (defined as susceptible to both ceftazidime and cefotaxime) were identified from the phenotypic AMR data provided [46], and highlighted alongside study isolates.

Data availability

Raw read data and assemblies were submitted under BioProject ID PRJNA644114. Detailed per-strain information on accession numbers, resistance profiles, resistance gene predictions and sequence types (STs) are given in Table S1.

Results

Isolate collection and antimicrobial susceptibility testing

The RLUH recorded 1472 BSI *E. coli* isolates between 2014 and 2017 and antimicrobial susceptibility testing showed 172 of these isolates (11.8%) were resistant to TZP (Fig.S.1). The proportion of E. coli resistant to TZP declined between 2014 (21%) and 2017 (9%, Fig. 1C). Of the 1258 TZP susceptible isolates, the majority (1129, 89.7%) were also susceptible to 3GC, while 129 (10.3%) were 3GC non-susceptible. In contrast, 86/172 (50%) TZP-resistant isolates were non-susceptible and 86/172 (50%) were susceptible to 3GC (Fig.1.A).

Resistance to carbapenems was only seen in the TZP resistant/3GC resistant isolates, with 3.9% resistant to meropenem and 2.7% to ertapenem, and the TZP resistant resistant/3GC susceptible isolates had increased resistance to amoxicillin/clavulanic acid in comparison with TZP resistant/3GC resistant isolates (96.4% vs 81.1%) (Fig.1.B).

Of the 86 isolates with TZP resistance and 3GC susceptibility, 14 isolates derived from repeated sampling of long-term patients were excluded from further study, resulting in 72 isolates derived from unique patients, which we subsequently reduced to 66 after excluding those with TZP MICs under the EUCAST breakpoint for susceptibility. A single isolate was determined to be a contaminant (*Staphylococcus aureus*) based on colony morphology and confirmed as such by 16S PCR. After whole genome sequencing, two of the 65 isolates were removed from the study as they contained more than one *E. coli* genome, either due to mixed infections, or possible contamination (assembly sizes were 9602556bp and 9552068bp, associated with 1448 and 1180 contigs respectively), leaving 63 isolates for further analysis.

The minimum inhibitory concentration of TZP ranged between 12 and 258 mg/L. Fifty eight isolates had an MIC over the EUCAST breakpoint for resistance (16 mg/L), whilst 5 were determined to be intermediately resistant (MIC 12mg/L). The CTX MICs ranged between 0.016 and 0.25 mg/L, all below the break point for resistance (2mg/L), confirming the TZP resistant/3GC susceptible phenotype.

Population structure of the TZP resistant/3GC susceptible population within the nationwide context

The TZP resistant/3GC susceptible phenotype occurred in a diverse number of sequence types. The 63 study isolates represented 16 STs; the most representative were ST131 (n=22), ST73 (n=11), 12 (n=5), and ST69 (n=3). When placing them into the phylogenetic context of a large UK-wide collection of bloodstream isolates collected from 2001 to 2011 from 12 hospitals, it is apparent that our isolates reflect the overall population structure with the exception of ST127, a uropathogenic and highly virulent lineage (Fig.2A, B). This indicates that the TZP-resistant/3GC-susceptible phenotype is not driven by a clonal outbreak, but rather by multiple acquisitions of resistance mechanisms in the circulating population of hospital strains. The AMR gene profile of the RLUH isolates varied between STs (Fig.2C), with ST131 carrying more identifiable resistance genes than the other major STs as previously reported [47].

Varied putative genetic determinants of the TZP resistant/3GC susceptible phenotype

The study isolates harboured a variety of β-lactamase genes, including TEM-type (n=49; $bla_{\text{TEM-1}}$ [46], $bla_{\text{TEM-33}}$ [2], $bla_{\text{TEM-148}}$ [1]), $bla_{\text{SHV-1}}$ (n=9), $bla_{\text{CTX-M-15}}$ (n=4) and $bla_{\text{OXA-1}}$ (n=3) (Fig.3A.). No carbapenemase genes were predicted to be present within the genomes of all study isolates, although four ST131 isolates harboured the ESBL $bla_{\text{CTX-M-15}}$ gene, normally associated with 3GC resistance. However, these all carried an S133G mutation, that was recently reported to result in a non-ESBL phenotype [48]. The copy numbers of β-lactamase genes, as estimated by sequencing coverage, varied between 1 and 186 copies for $bla_{\text{TEM-1}}$ and between 5 and 51 for $bla_{\text{SHV-1}}$. All $bla_{\text{CTX-M-15}}$ and $bla_{\text{OXA-1}}$ were present at between 1 and 6

copies. Of the isolates with *bla*$_{TEM-1}$, 29 had the weak *P3* promoter, four had the strong promoter *P4* and 16 contained the strong, overlapping promoter *Pa/Pb*. All *bla*$_{SHV-1}$ genes were associated with the strong PS promoter. Additionally, all isolates carried the chromosomal *bla*$_{AmpC1}$ [49] which is constitutively expressed at a low level, and 57/63 of the isolates carried *bla*$_{AmpC2.}$ A graphical depiction of the resistance mechanisms is shown in Fig.3B. To identify predicted resistance to TZP due to an increase in β-lactamase copy number, we visualised the distribution of gene copy numbers (Fig.S2A) and found them to cluster above or below 10 copies. In general, the copy numbers in isolates with weak promoters clustered >10, whilst those with strong promoters clustered <10 (FigS2B), therefore >10 copies was chosen as the cut off level for classifying isolates as likely resistant due to gene amplification.

The AMR genotypes correlated well with the phenotypic data obtained by disk testing, with most isolates susceptible to ciprofloxacin and gentamycin. Replicons usually associated with large resistance plasmids such as IncFIA and IncFIB, IncFIA and IncFIIA were detected in only 22.5% of the study isolates (Fig.S3.), reflecting the low proportion of isolates with multiple resistance genes and therefore the unusual resistance profile characteristic of the TZP-resistant/3GC-susceptible phenotype (Fig.5.).

The presence of β-lactamase genes correlated with resistance to ampicillin and also TZP, whilst resistance to ciprofloxacin (18/63 isolates, 29%) was accounted for by *gyrA* mutations D78N (10/18, 56%) and S83L (12/18, 67%), and *parC* S80I mutation (10/18, 55%); only a single isolate was found to have plasmid-derived fluoroquinolone resistance determinants (*qepA*), in accordance to the low proportion encoding F-, R-, S- or N-type resistance plasmids. Aminoglycoside resistance was explained by the *O*-adenylyltransferases *aadA* (6/6, 100%), in combination with the genes *aac(3)-IIa* or *aadB* (3/6, 50%).

Multiple independent acquisitions of different resistance mechanisms

To get a higher-resolution insight into the within-ST diversity of the isolates, we calculated core genome trees of the main STs by mapping the reads against selected reference genomes and extracting the conserved, non-recombinant SNPs (see methods for details). This shows that the acquisition of the phenotype was not a single event even in these closely related organisms, but occurred on several occasions for both of these main sequence types, with no (ST73; Fig. S4) or very few (ST131; Fig. S5) isolates closely related which may indicate within-hospital transmission.

Impact of putative resistance mechanism on TZP MIC

Putative mechanisms of TZP resistance were found in 57 (90%) of the 63 strains (Fig.4A), including increased (>10 fold) copies of β-lactamase genes (21/57, 37%), strong β-lactamase promoters with (10/57, 18%) and without (18/57, 32%) copy number increases, inhibitor resistant bla_{TEM} and $bla_{CTX\text{-}M}$ variants (6/57, 11%), and carriage of $bla_{OXA\text{-}1}$ (2/57, 4%).

The TZP MIC of the strains varied according to the resistance mechanism(s) present (p=0.0153, Kruskal-Wallis test) (Fig.4B). Generally, increases in the β-lactamase copy number led to an increase in MIC (Fig.4C), most notably for strains carrying $bla_{TEM\text{-}1}$ with a weak promoter. The highest MICs were encountered in strains with increased copy number of β-lactamase, paired with strong promoters, and also those with the modified CTX-M-15 enzyme.

Six isolates with $bla_{TEM\text{-}1}$ had a weak promoter and copy number below 10; and thus, as yet undetermined resistance mechanisms. The MIC of the strains with undetermined mechanisms was significantly lower than the MICs recorded for the strains with detectable resistance mechanisms (p=0.0031, Mann Whitney test).

Discussion

This phylogenetic analysis of TZP resistant/3GC susceptible isolates demonstrates that the phenotype derives from repeated, independent acquisition events throughout the *E. coli* population. The

comparison of the isolates with a large UK wide collection [46] show that this is not unique to our study site, but broadly reflective of the genotype corresponding to this phenotype in a large collection of hospital isolates from multiple sites across the UK. As the TZP resistant/3GC susceptible phenotype reflects the overall UK population structure of *E. coli* bacteraemia isolates, this is potentially the result of repeated or sustained antimicrobial pressure, rather than fixation in a certain lineage and their subsequent spread. The phenotype was encountered in the typically drug resistant ST131 [50], and the often highly virulent but drug susceptible ST73 [51], reflecting the overall dominance of these STs, and was not associated with an overall elevated level of resistance to other classes of antibiotic. The majority of TZP resistant/3GC susceptible strains in the study relied on hyperproduction of class A β-lactamase enzymes, particularly those encoded by $bla_{\text{TEM-1}}$ and $bla_{\text{SHV-1}}$, which can hydrolyse piperacillin but not 3GCs, and are inhibited by tazobactam. Hyperproduction can occur via gene amplification, in which tandem repeats of AMR genes are generated, for example via the IS*26* mediated amplification of pseudo-compound transposons [28,52], or the transfer of β-lactamase genes to high copy AMR plasmids [24]. A number of the isolates were lacking a detectable increase in gene copy number, but had a route to hyperproduction via strong promoters of $bla_{\text{TEM-1}}$ [27] or $bla_{\text{SHV-1}}$ [40].

We also detected $bla_{\text{TEM-33}}$, encoding an inhibitor resistant variant of TEM-1b [53], and $bla_{\text{OXA-1}}$, either as the only β-lactamase or in combination with $bla_{\text{CTX-M-15}}$ or $bla_{\text{TEM-1}}$. OXA-1 is poorly inhibited by tazobactam [54], and in a recent UK study was the major contributor to TZP resistance amongst ESBL *E. coli* [55]. However, the carriage of $bla_{\text{OXA-1}}$ does not always confer resistance to TZP, which appears to depend on the genetic background of the strain; the risk ratio of $bla_{\text{OXA-1}}$ being associated with TZP resistance in ESBL *E. coli* is higher in ST131 strains (12.1) compared with ESBL *E. coli* as a whole (6.49) [55]. The single isolate with only a OXA-1 β-lactamase gene was ST88, and this combination of sequence type and β-lactamase has been identified in an outbreak of amoxicillin/clavulanic acid resistant *E. coli* in Spain [56].

All detected *bla*$_{CTX-M-15}$ genes encoded the S133G mutation, which has been in shown to enhance TZP MIC ten-fold whilst reducing the 3GC MIC by the same margin in a strain harbouring an error prone PCR derived *bla*$_{CTX-M-15}$ genes [48]. To our knowledge this is the first report of this *bla*$_{CTX-M-15}$ variant in clinical isolates, and these genes were associated with 6% of TZP resistance/3GC susceptibility in our setting. All *bla*$_{CTX-M-15}$ genes were found in ST131 isolates, which exhibited some of the highest MICs to TZP in our study. The mutation of *bla*$_{CTX-M}$ genes in order to better hydrolyse mecillinam has been reported during urinary tract infection treatment [57], but not for TZP or other β-lactam/inhibitor combinations. The circulation of *bla*$_{CTX-M}$ variants that do not confer the ESBL phenotype, but provide resistance to TZP, has implications for molecular testing for ESBL organisms [58], which would misclassify these isolates as 3GC resistant, and as a consequence might lead to unnecessary use of carbapenems.

Strategies to increase the effectiveness of TZP include increasing dosage, which in one study increased the coverage of TZP from 83.2% to 93% of bacterial blood stream pathogens [59]. Increasing the concentration of tazobactam alongside a fixed dose of piperacillin has also rescued TZP effectiveness against TEM-1 hyperproducers in a neutropenic mouse model [25], and could be a viable strategy to protect the future effectiveness of this drug and improve treatment outcomes. The TZP resistant/3GC susceptible isolates had increased resistance to amoxicillin/clavulanic acid, another penicillin drug combined with a suicide inhibitor [11]. This is in agreement with reports that hyperproduction of β-lactamase accounts for a large proportion of resistance to this alternative β-lactam/inhibitor combination [60], and reports that lower levels of hyperproduction of TEM-1 in *E. coli* are required for resistance to amoxicillin/clavulanic acid compared to TZP [24].

The rapid diagnosis of TZP resistant/3GC susceptible *E. coli* would enable de-escalation from TZP to a 3GC [61], both reducing the likelihood of treatment failure, and preventing overuse of carbapenems, which is key for antimicrobial stewardship in the UK [62]. The isolates were mostly susceptible to ciprofloxacin, gentamicin and amikacin, and de-escalation to these antibiotics would avoid unnecessary use of

carbapenems. Recent work on methicillin resistant *Staphylococcus aureus* has described frequent collateral sensitivity to narrow spectrum penicillin/inhibitor combinations, highlighting that targeted de-escalation rather than escalation can be possible when treating organisms highly resistant to first line drugs [63].

Whilst molecular diagnostics can be used to rapidly detect AMR genes [58,64], the diverse causes of TZP resistance, including copy number increases and promoter mutations of β-lactamases that are inhibited by tazobactam would make this difficult. Rapid colorimetric tests are available for carbapenemase detection [65] and a similar test for TZP resistance/3GC susceptibility would potentially enable rapid identification of this phenotype and faster clinical management. A greater understanding of the evolution of TZP resistance could be useful for predicting resistance to the new generation of β-lactam/β-lactamase inhibitor combinations, including 5th generation cephalosporins such as ceftolozane-tazobactam, and carbapenems such as meropenem-vaborbactam and imipenem-relebactam [66].

Our study has some limitations, including that we only sequenced TZP resistant/3GC susceptible isolates at our site, and utilised a large and UK wide collection of isolates for comparison. These comparison isolates were collected between 2001 and 2012, and so are not contemporary with the study isolates collected between 2014 and 2017 but serve as the best available large-scale comparator of the same demographics. The isolates from our site and in the UK wide collection were similarly diverse and reflected the overall population structure, which has been shown to be stable over time [46].

This work highlights the diversity of *E. coli* strains capable of developing the TZP resistant/3GC susceptible phenotype, and the range of possible mechanisms involved, including β-lactamase hyperproduction via gene amplification and promoter mutations, inhibitor resistant TEM-1 and CTX-M-15 variants and OXA-1 production. The investigation of β-lactamase copy number and promoter type is essential when inferring TZP resistance from genomic data.

Funding

This work was supported by an LSTM Directors Catalyst Award to TE. EH acknowledges support from a Wellcome SEED Award (217303/Z/19/Z). APR would like to acknowledge funding from the AMR Cross-Council Initiative through a grant from the Medical Research Council, a Council of UK Research and Innovation (Grant Number; MR/S004793/1), and the National Institute for Health Research (Grant number; NIHR200632).

Author contributions

TE, EH, JM, CMP and ATMH conceptualised the study. JvA, AH PR, CC, CMP, JM, and AH collated isolate metadata, and clinical antimicrobial susceptibility testing data. TE, EH, ERA, APR, LEC and ATMH contributed to the experimental design and data analysis. Bioinformatic analysis was carried out by TE and EH. TE, CTW, AJF, IB and ATMH carried out microbiological experiments. TE, EH and ATMH wrote the first draft of the manuscript. All authors reviewed and edited the final manuscript.

Acknowledgments

We acknowledge the technical staff in the diagnostic microbiology laboratories in the Royal Liverpool University Hospital and expert informatics support from the Pathogen Informatics team at the Wellcome Sanger Institute.

References

1 Laupland, K. B. Incidence of bloodstream infection: a review of population-based studies. *Clin Microbiol. Infect.* **19**, 492-500. (2013).
2 Bonten, M. *et al.* Epidemiology of Escherichia coli Bacteremia: A Systematic Literature Review. *Clin. Infect. Dis.* ciaa210. (2020).
3 Public Health England. Annual epidemiological commentary: mandatory MRSA, MSSA and E coli bacteraemia and C difficile infection data. (2014).
4 Worl Health Organization. Global priority list of antibiotic-resistant bacteria to guide research, discovery, and developmentof new antibiotics. (2017).
5 Tacconelli, E. *et al.* Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. *Lancet. Infect. Dis.* **18**, 318-327, (2018).

6 World Health Organisation. Landscape of diagnostics against antibacterial resistance, gaps and priorities. (2019).
7 Temkin, E. *et al.* Estimating the number of infections caused by antibiotic-resistant Escherichia coli and Klebsiella pneumoniae in 2014: a modelling study. *Lancet Glob. Health* **6**, e969-e979, (2018).
8 European Centres of Disease Control. Surveillance of antimicrobial resistance in Europe 2018 (2018).
9 Tooke, C. L. *et al.* β-Lactamases and β-Lactamase Inhibitors in the 21st Century. *J. Mol. Biol.* **431**, (2019).
10 Gin, A. *et al.* Piperacillin-tazobactam: a beta-lactam/beta-lactamase inhibitor combination. *Expert Rev. Anti. Infec. Ther.* **5**, 365-383 (2007).
11 Drawz, S. M. & Bonomo, R. A. Three decades of beta-lactamase inhibitors. *Clin. Microbiol. Rev.* **23**, 160-201 (2010).
12 Johnson, D. M., Biedenbach, D. J. & Jones, R. N. Potency and antimicrobial spectrum update for piperacillin/tazobactam (2000): emphasis on its activity against resistant organism populations and generally untested species causing community-acquired respiratory tract infections. *Diagn. Microbiol. Infect Dis.* **43**, 49-60 (2002).
13 Kim, M.-K., Xuan, D., Quintiliani, R., Nightingale, C. H. & Nicolau, D. P. Pharmacokinetic and pharmacodynamic profile of high dose extended interval piperacillin–tazobactam. *J. Antimicrob. Chemother.* **48**, 259-267, (2001).
14 Barton, G. J., Morecroft, C. W. & Henney, N. C. A survey of antibiotic administration practices involving patients with sepsis in UK critical care units. *Int. J. Clin. Pharm.* **42**, 65-71, (2020).
15 Wilson, A. P. R. Sparing carbapenem usage. *J. Antimicrob. Chemother.* **72**, 2410-2417 (2017).
16 Morrill, H. J., Pogue, J. M., Kaye, K. S. & LaPlante, K. L. Treatment Options for Carbapenem-Resistant Enterobacteriaceae Infections. *Open Forum Infect. Dis.* **2**, ofv050, (2015).
17 Harris, P. N. A. *et al.* Effect of Piperacillin-Tazobactam vs Meropenem on 30-Day Mortality for Patients With E coli or Klebsiella pneumoniae Bloodstream Infection and Ceftriaxone Resistance: A Randomized Clinical Trial. *Jama* **320**, 984-994, (2018).
18 Gutiérrez-Gutiérrez, B. & Rodríguez-Baño, J. Current options for the treatment of infections due to extended-spectrum beta-lactamase-producing Enterobacteriaceae in different groups of patients. *Clin. Microbiol. Infect.* **25**, 932-942, doi: (2019).
19 England, P. H. Laboratory surveillance of Escherichia coli bacteraemia in England, Wales and Northern Ireland. *Health Protection Report* **13** (2018).
20 van Duin, D. & Bonomo, R. A. Ceftazidime/Avibactam and Ceftolozane/Tazobactam: Second-generation β-Lactam/β-Lactamase Inhibitor Combinations. *Clin. Infect. Dis.* **63**, 234-241 (2016).
21 Lee, J., Oh, C. E., Choi, E. H. & Lee, H. J. The impact of the increased use of piperacillin/tazobactam on the selection of antibiotic resistance among invasive Escherichia coli and Klebsiella pneumoniae isolates. *Int. J. Infect. Dis.* **17**, e638-643 (2013).
22 Suzuki, Y. *et al.* Contribution of beta-lactamase and efflux pump overproduction to tazobactam-piperacillin resistance in clinical isolates of Escherichia coli. *Int. J. Antimicrob. Agents*. 105919, (2020).
23 Schechter, L. M. *et al.* Extensive Gene Amplification as a Mechanism for Piperacillin-Tazobactam Resistance in Escherichia coli. *mBio* **9**, doi:10.1128/mBio.00583-18 (2018).
24 Rodríguez-Villodres, Á. *et al.* Extended-spectrum resistance to β-lactams/β-lactamase inhibitors (ESRI) evolved from low-level resistant Escherichia coli. *J. .Antimicrob. Chemother.***75**, 77-85 (2019).

25 Abdelraouf, K. *et al.* Piperacillin-Tazobactam-Resistant/Third-Generation Cephalosporin-Susceptible Escherichia coli and Klebsiella pneumoniae Isolates: Resistance Mechanisms and In vitro-In vivo Discordance. *Int. J. Antimicrob. Agents* **55**, 105885 (2020).
26 Shropshire, W. C. *et al.* β-lactamase Amplification and Porin Loss Drive Progressive β-lactam Resistance in Recurrent ESBL Enterobacteriaceae Bacteremia. *bioRxiv*, 616961, (2019).
27 Zhou, K., Tao, Y., Han, L., Ni, Y. & Sun, J. Piperacillin-Tazobactam (TZP) Resistance in Escherichia coli Due to Hyperproduction of TEM-1 β-Lactamase Mediated by the Promoter Pa/Pb. *Front. Microbiol.* **10**, 833-833. (2019).
28 Hubbard, A. T. M. *et al.* Within-patient evolution to piperacillin/tazobactam resistance in a clinical isolate of Escherichia coli due to IS26>-mediated amplification of bla<sub>TEM-1B. *Nat. Commun.* In Press. doi:10.1101/2020.02.28.969360 (2020).
29 Hansen, K. H. *et al.* Resistance to piperacillin/tazobactam in Escherichia coli resulting from extensive IS 26-associated gene amplification of bla TEM-1. *J. Antimicrob. Chemother.* **74**, 3179-3183 (2019).
30 Andrews, J. M. & Howe, R. A. BSAC standardized disc susceptibility testing method (version 10). *J. Antimicrob. Chemother* **66**, 2726-2757 (2011).
31 European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. (2013).
32 García-Fernández, S. *et al.* Multicenter Evaluation of the New Etest Gradient Diffusion Method for Piperacillin-Tazobactam Susceptibility Testing of Enterobacterales, Pseudomonas aeruginosa, andAcinetobacter baumannii Complex. *J. Clin. Microbiol.* **58**, e01042-01019, (2020).
33 uropean Committee on Antimicrobial Susceptibility Testing. Clinical Breakpoints - bacteria (v10.0). *https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_10.0_Breakpoint_Tables.pdf* (2020).
34 Bankevich, A. *et al.* SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol.* **19**, 455-477 (2012).
35 Seemann, T. Prokka: rapid prokaryotic genome annotation. *Bioinformatics* **30**, 2068-2069 (2014).
36 Hunt, M. *et al.* ARIBA: rapid antimicrobial resistance genotyping directly from sequencing reads. *Microb. Genom.* **3**, e000131-e000131 (2017).
37 Inouye, M. *et al.* SRST2: Rapid genomic surveillance for public health and hospital microbiology labs. *Genom. Med.* **6**, 90, (2014).
38 Larsen, M. V. *et al.* Multilocus sequence typing of total-genome-sequenced bacteria. *J. Clin Microbiol* **50**, 1355-1361 (2012).
39 Carattoli, A. *et al.* In silico detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. *Antimicrob. Agents Chemother.* **58**, 3895-3903 (2014).
40 Zhai, Y. *et al.* Relative Strengths and Regulation of Different Promoter-Associated Sequences for Various blaSHV Genes and Their Relationships to β-Lactam Resistance. *J. Molec. Microbiol Biotechnol.* **27**, 91-101 (2017).
41 Page, A. J. *et al.* Roary: rapid large-scale prokaryote pan genome analysis. *Bioinformatics* **31**, 3691-3693 (2015).
42 Page, A. J. *et al.* SNP-sites: rapid efficient extraction of SNPs from multi-FASTA alignments. *Microb. Genom.* **2.** (2016).
43 Nguyen, L. T., Schmidt, H. A., von Haeseler, A. & Minh, B. Q. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Molec. Biol. Evol.* **32**, 268-274 (2015).

44 Letunic, I. & Bork, P. Interactive tree of life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other trees. *Nucleic Acids Res.* **44**, W242-245 (2016).
45 Croucher, N. J. *et al.* Rapid phylogenetic analysis of large samples of recombinant bacterial whole genome sequences using Gubbins. *Nucleic Acids Res.* **43**, e15 (2015).
46 Kallonen, T. *et al.* Systematic longitudinal survey of invasive Escherichia coli in England demonstrates a stable population structure only transiently disturbed by the emergence of ST131. *Genom. Res.* **27**, 1437-1449 (2017).
47 McNally, A. *et al.* Diversification of Colonization Factors in a Multidrug-Resistant Escherichia coli Lineage Evolving under Negative Frequency-Dependent Selection. *mBio* **10**, e00644-00619 (2019).
48 Rosenkilde, C. E. H. *et al.* Collateral sensitivity constrains resistance evolution of the CTX-M-15 β-lactamase. *Nat. Commun.* **10**, 618 (2019).
49 Peter-Getzlaff, S. *et al.* Detection of AmpC beta-lactamase in Escherichia coli: comparison of three phenotypic confirmation assays and genetic analysis. *J. Clin. Microbiol.* **49**, 2924-2932, (2011).
50 Whitmer, G. R., Moorthy, G. & Arshad, M. The pandemic Escherichia coli sequence type 131 strain is acquired even in the absence of antibiotic exposure. *PLOS Pathog* **15**, e1008162 (2019).
51 Cunha, M. P. V. *et al.* Pandemic extra-intestinal pathogenic Escherichia coli (ExPEC) clonal group O6-B2-ST73 as a cause of avian colibacillosis in Brazil. *PLOS ONE* **12**, e0178970, (2017).
52 Harmer, C. J., Pong, C. H. & Hall, R. M. Structures bounded by directly-oriented members of the IS26 family are pseudo-compound transposons. *Plasmid* **111**, 102530 (2020).
53 Sirot, D., Chanal, C., Bonnet, R., De Champs, C. & Bret, L. Inhibitor-resistant TEM-33 beta-lactamase in a Shigella sonnei isolate. *Antimicrob. Agents Chemother.* **45** (2001).
54 Che, T., Bethel, C. R., Pusztai-Carey, M., Bonomo, R. A. & Carey, P. R. The different inhibition mechanisms of OXA-1 and OXA-24 beta-lactamases are determined by the stability of active site carboxylated lysine. *J. Biol. Chem.* **289**, 6152-6164, doi:10.1074/jbc.M113.533562 (2014).
55 Livermore, D. M. *et al.* OXA-1 β-lactamase and non-susceptibility to penicillin/β-lactamase inhibitor combinations among ESBL-producing Escherichia coli. *J. Antimicrob. Chemother.***74**, 326-333 (2018).
56 Ortega, A. *et al.* Spanish Multicenter Study of the Epidemiology and Mechanisms of Amoxicillin-Clavulanate Resistance in Escherichia coli. *Antimicrobial Agents and Chemotherapy* **56**, 3576-3581 (2012).
57 Nielsen, K. L. *et al.* Mutational change of CTX-M-15 to CTX-M-127 resulting in mecillinam resistant Escherichia coli during pivmecillinam treatment of a patient. *MicrobiologyOpen* **8**, e941 (2019).
58 Edwards, T. *et al.* A highly multiplexed melt-curve assay for detecting the most prevalent carbapenemase, ESBL and AmpC genes. *Diagn. Microbiol. and Infect. Dis.* 115076 (2020).
59 Rothe, K. *et al.* Antimicrobial resistance of bacteraemia in the emergency department of a German university hospital (2013–2018): potential carbapenem-sparing empiric treatment options in light of the new EUCAST recommendations. *BMC Infect. Dis.* **19**, 1091 (2019).
60 Davies, T. J. *et al.* Reconciling the potentially irreconcilable? Genotypic and phenotypic amoxicillin-clavulanate resistance in Escherichia coli. *Antimicrob. Agents Chemother*, AAC.02026-02019 (2020).
61 Carlisle, L., Justo, J. A. & Al-Hasan, M. N. Bloodstream Infection due to Piperacillin/Tazobactam Non-Susceptible, Cephalosporin-Susceptible Escherichia coli: A Missed Opportunity for De-Escalation of Therapy. *Antibiotics (Basel, Switzerland)* **7** (2018).
62 Robson, S. E. *et al.* Optimizing carbapenem use through a national quality improvement programme. *J. Antimicrob. Chemother.* **73**, 2223-2230 (2018).

63 Harrison, E. M. *et al.* Genomic identification of cryptic susceptibility to penicillins and β-lactamase inhibitors in methicillin-resistant Staphylococcus aureus. *Nat. Microbiol.* **4**, 1680-1691 (2019).

64 Williams, C. T., Edwards, T., Adams, E. R., Feasey, N. A. & Musicha, P. ChloS-HRM, a novel assay to identify chloramphenicol-susceptible Escherichia coli and Klebsiella pneumoniae in Malawi. *J. Antimicob. Chemother.* (2019).

65 Coppi, M. *et al.* Multicenter evaluation of the RAPIDEC® CARBA NP test for rapid screening of carbapenemase-producing Enterobacteriaceae and Gram-negative nonfermenters from clinical specimens. *Diagn. Microbiol. Infect. Dis* **88**, 207-213 (2017).

66 Palwe, S., Khobragade, K. & Kharat, A. S. Preserving the Dwindling β-lactams-Based Empiric Therapy Options for Gram-Negative Infections in Challenging Resistance Scenario: Lessons Learned and Way Forward. *Microb. Drug Resist.* (2019).

Figures

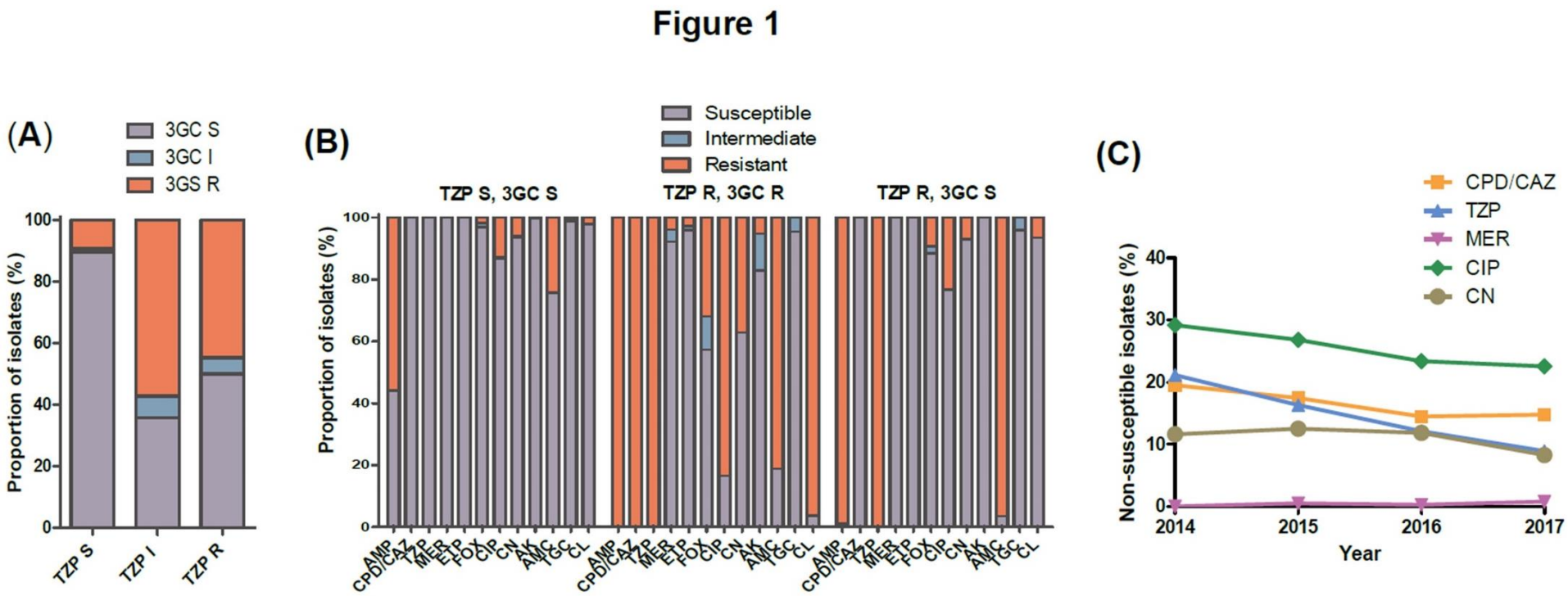


Fig.1.(A) Proportion of TZP susceptible (TZP S), intermediate (TZP I) and resistant (TZP R) isolates that are 3rd generation cephalosporin susceptible (3GC S), intermediate (3GC I) and resistant (3GC R). **(B)** Antimicrobial susceptibilities of *E. coli* isolates from the RLUH, grouped by their susceptibility to piperacillin/tazobactam (TZP) and 3rd generation cephalosporins (3GC). Susceptibility data is shown for isolates that are TZP susceptible and 3GC susceptible (TZP S, 3GC R). TZP resistant and 3GC resistant (TZP R, 3GC R), and TZP resistant and 3GC susceptible (TZP R,

3GC S), for the antibiotics ampicillin (AMP), cefpodoxime/ceftazidime (CPD/CAZ), piperacillin/tazobactam (TZP), meropenem (MER), ertapenem (ETP), cefoxitin (FOX), ciprofloxacin (CIP), gentamycin (CN), amikacin (AK), amoxicillin/clavulanic acid (AMC), tigecycline (TGC), and chloramphenicol (CL). **(C)** Trends in non-susceptibility to CPD/CAZ, TZP, MER, CIP and CN between 2014 and 2017 at RLUH.

Figure 2

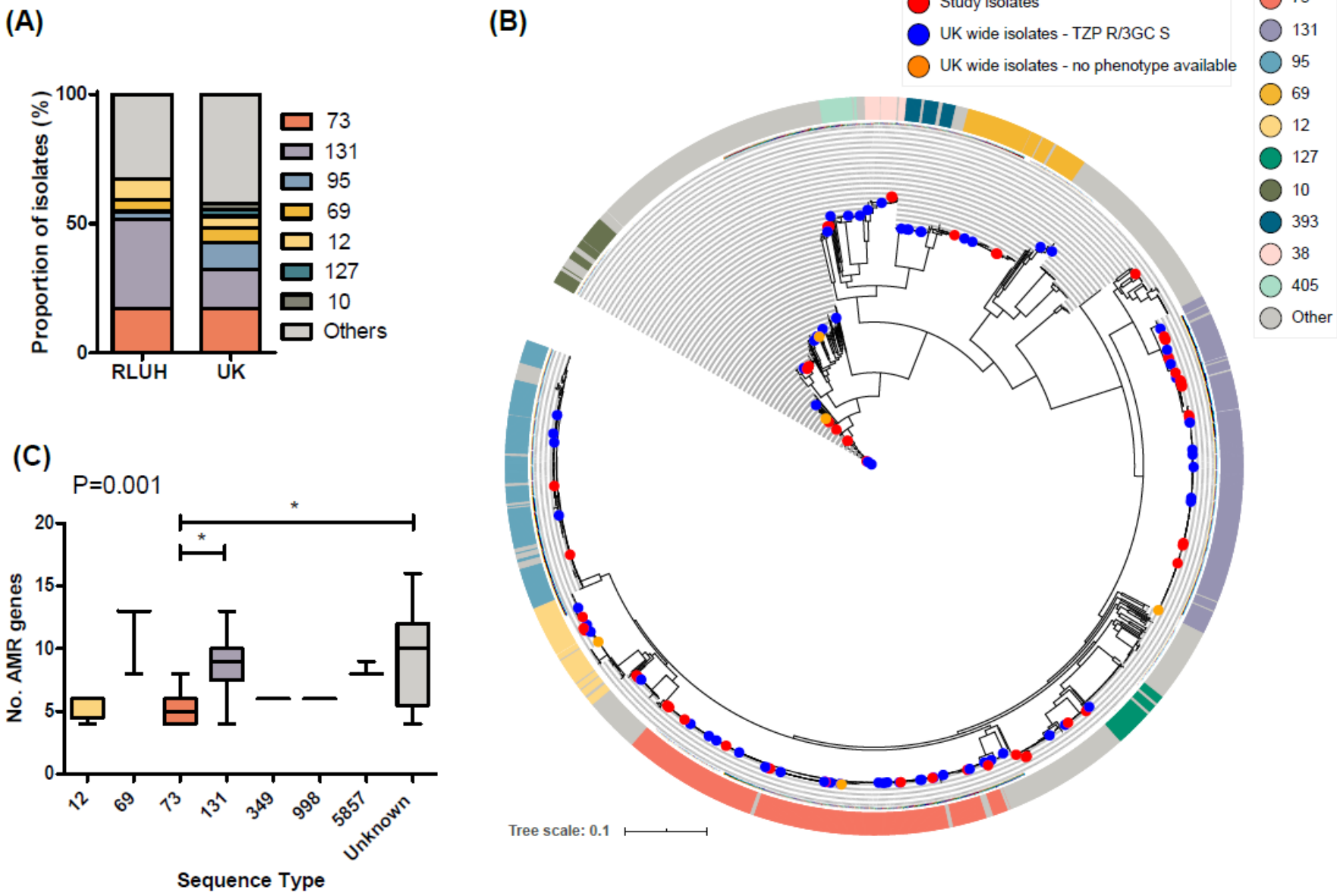
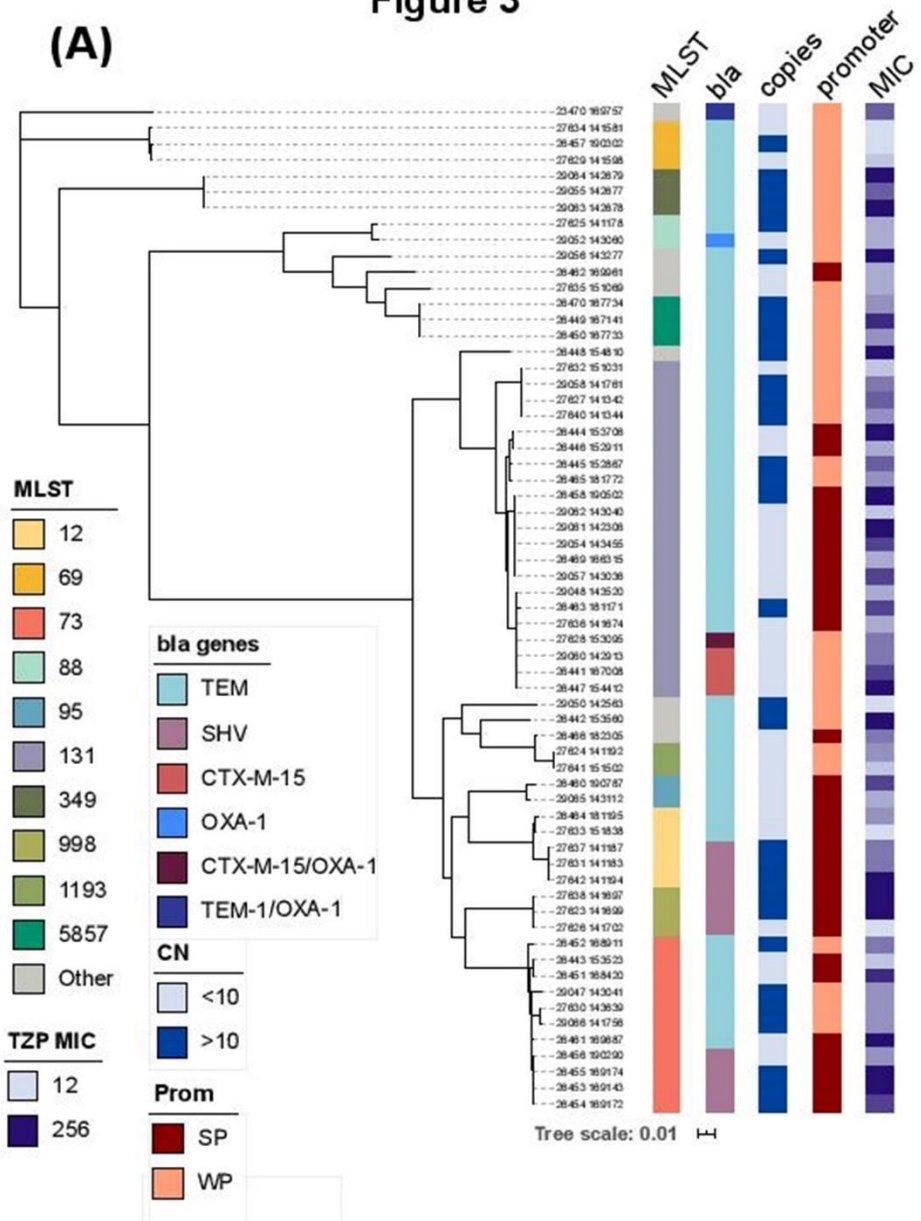

(A)
Proportion of isolates (%)
100
50
0
RLUH
UK
73
131
95
69
12
127
10
Others
(B)
Isolates
Study isolates
UK wide isolates - TZP R/3GC S
UK wide isolates - no phenotype available
MLST
73
131
95
69
12
127
10
393
38
405
Other
Tree scale: 0.1
(C)
P=0.001
*
*
No. AMR genes
20
15
10
5
0
12
69
73
131
349
998
5857
Unknown
Sequence Type

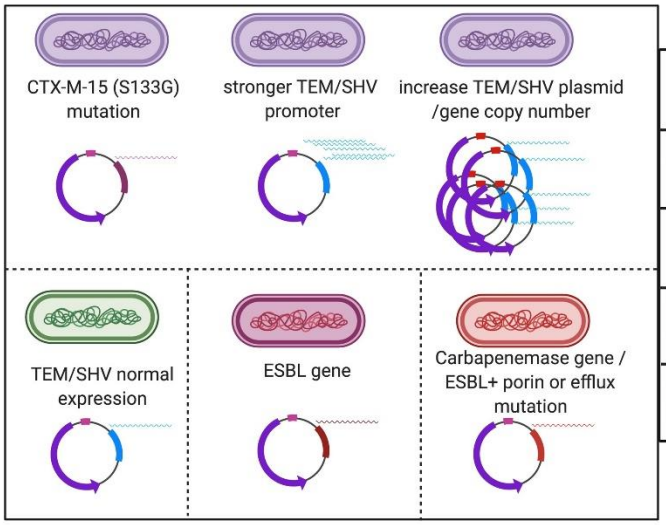
Fig.2.(A) Bar chart showing the proportion of isolates belonging to common sequence types in the RLUH study isolates in comparison with those in the collection of 1509 isolates taken from a UK wide study [46]. **(B)** Circular Maximum Likelihood core genome phylogenetic tree of the 68 study isolates in combination with 1509 UK wide study isolates. The ring indicates the ten most commonly encounteredSTs. Dots at the terminus of branches indicate study isolates, UK wide isolates with the TZP resistant/3GC susceptible phenotype (TZP-R/3GC -S) or isolates from the UK wide collection missing sufficient phenotypic data to assign an accurate AMR phenotype. **(C)** The number of AMR genes in isolates from the major sequence types encountered in the study. Whiskers show minimum and maximum values. Significance determined by Kruskal-Wallis test, * indicates a p value of <0.05.

Figure 3

(A)



(B)



	TZP	3rd gen Ceph
	Resistant	Sensitive
	Sensitive	Sensitive
	Sensitive	Resistant
	Resistant	Resistant

Fig.3.(A) Maximum likelihood phylogeny of the study isolates from RLUH. The colour strips, from left to right, show the MLST classification (MLST), β-lactamase gene carriage (bla), copy number of bla genes (CN), presence of strong or weak promoter (Pr) and the MIC of TZP. **(B)** proposed mechanisms of TZP resistance

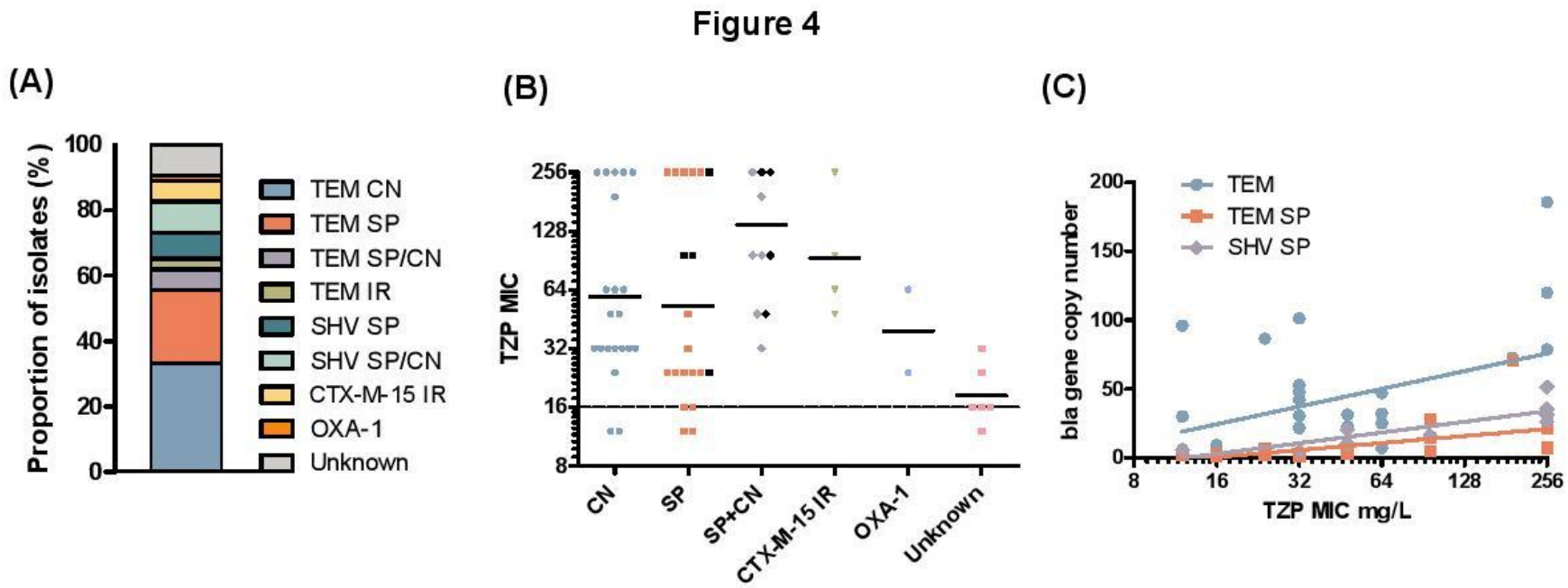


Fig.4.(A) Proportions of TZP resistance mechanisms in study isolates, including copy number >10 (CN), strong promoters (SP), and inhibitor resistant enzymes (IR). **(B)** Minimum inhibitory concentration of TZP of strains with various resistance mechanisms. Black points indicate bla_{SHV}, coloured indicate bla_{TEM} Dotted line indicates EUCAST breakpoint between resistant and intermediate. Lines on plots indicate geometric mean. **(C)** Effect of β-lactamase (bla) gene copy number on TZP MIC, for isolates with bla_{TEM} coupled with a weak promoter (TEM), bla_{TEM} coupled with a strong promoter (TEM SP), and bla_{SHV} with a strong promoter (SHV SP).

Supplementary Figures

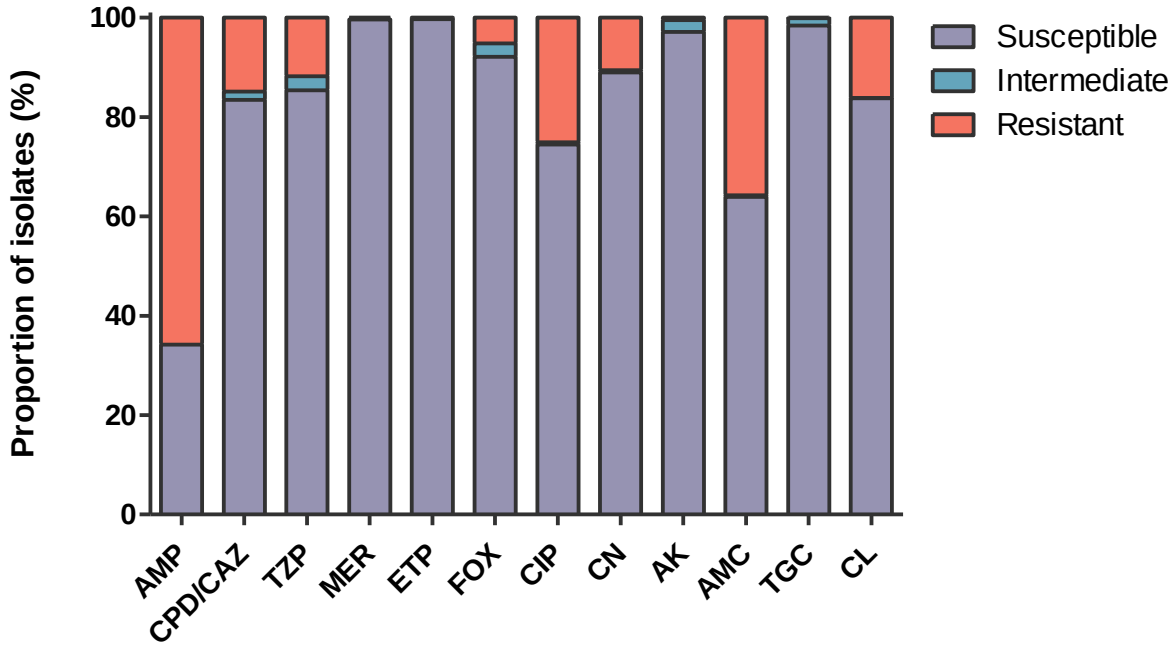


Fig.S1. Proportion of the total *E. coli* isolated from blood stream infections between 2014 and 2017 at RLUH that were susceptible, intermediate or resistant to ampicillin (AMP), cefpodoxime/ceftazidime (CPD/CAZ), piperacillin/tazobactam (TZP), meropenem (MER), ertapenem (ETP), cefoxitin (FOX), ciprofloxacin (CIP), gentamycin (CN), amikacin (AK), amoxicillin/clavulanic acid (AMC), tigecycline (TGC), and chloramphenicol (CL).

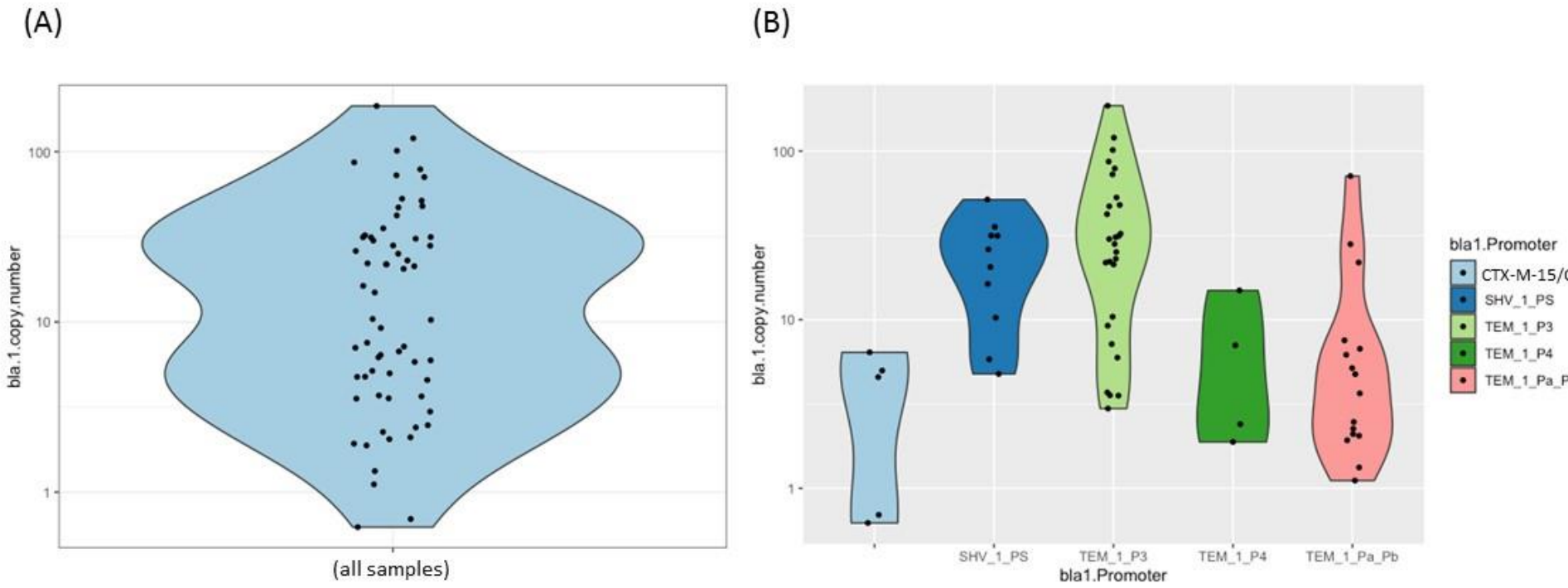


Fig.S2. (A) The distribution of β-lactamase gene copy number across all isolates. (B) Distribution of β-lactamase gene copy number by promoter type.

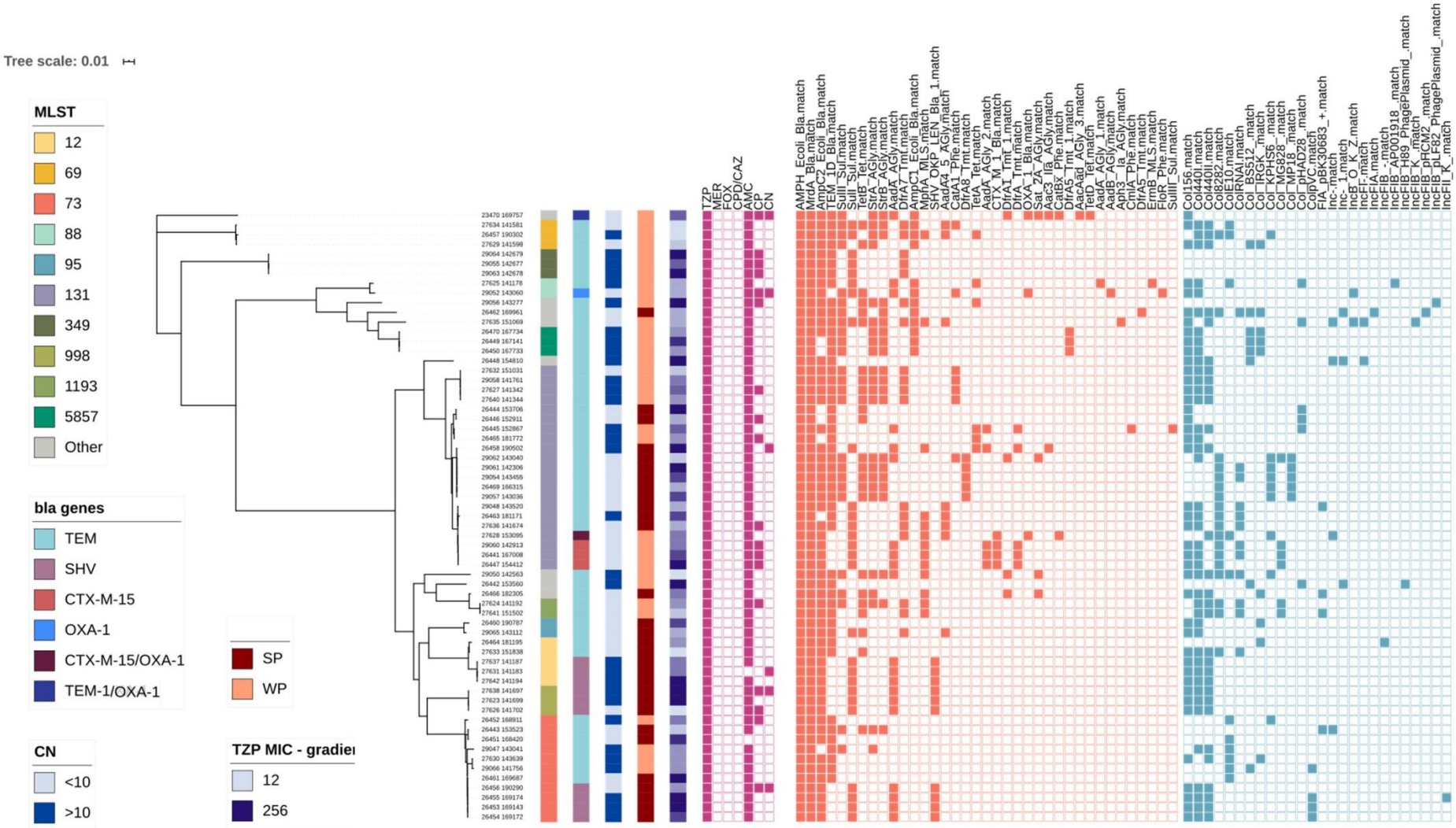

Tree scale: 0.01
MLST
12
69
73
88
95
131
349
998
1193
5857
Other
bla genes
TEM
SHV
CTX-M-15
OXA-1
CTX-M-15/OXA-1
TEM-1/OXA-1
CN
<10
>10
SP
WP
TZP MIC - gradie
12
256
TZP
MER
FOX
CPD/CAZ
AMC
CP
CN

Fig.S3. Maximum likelihood phylogeny of the study isolates from RLUH. The colour strips, from left to right, show the year of isolation, MLST classification, β-lactamase gene carriage, promoter strength, β-lactamase gene copy number, TZP minimum inhibitory concentration, AMR phenotype as determined by disk diffusion testing during routine hospital microbiology, distribution of predicted AMR genes and plasmid replicons.

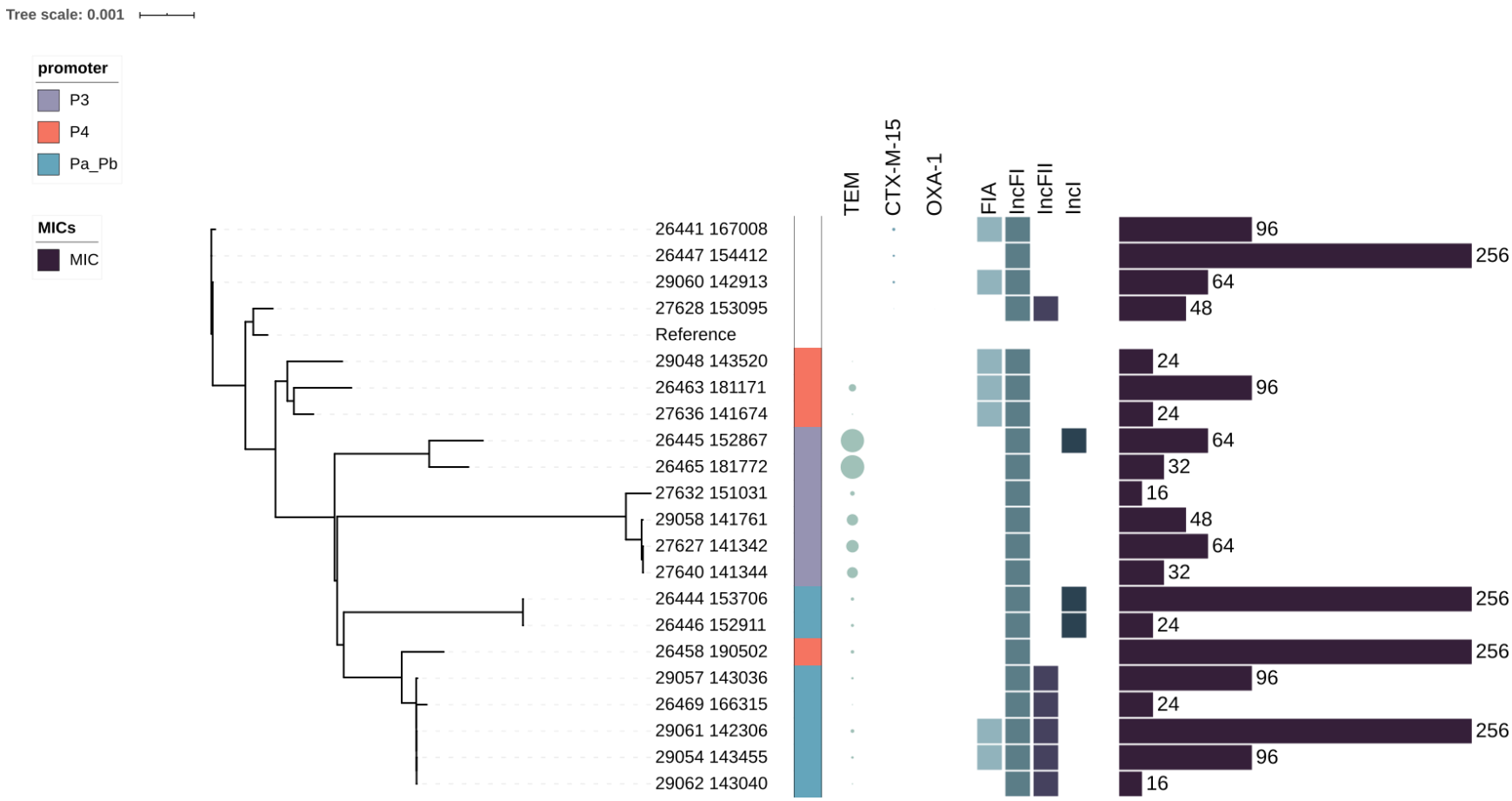


Fig.S4. High resolution core genome – based phylogeny of TZP resistant/3GC susceptible ST131 isolates. Indicated are promoter types, β-lactamase copy numbers (size of circle represents relative copy number), plasmid replicons, and TZP MIC.

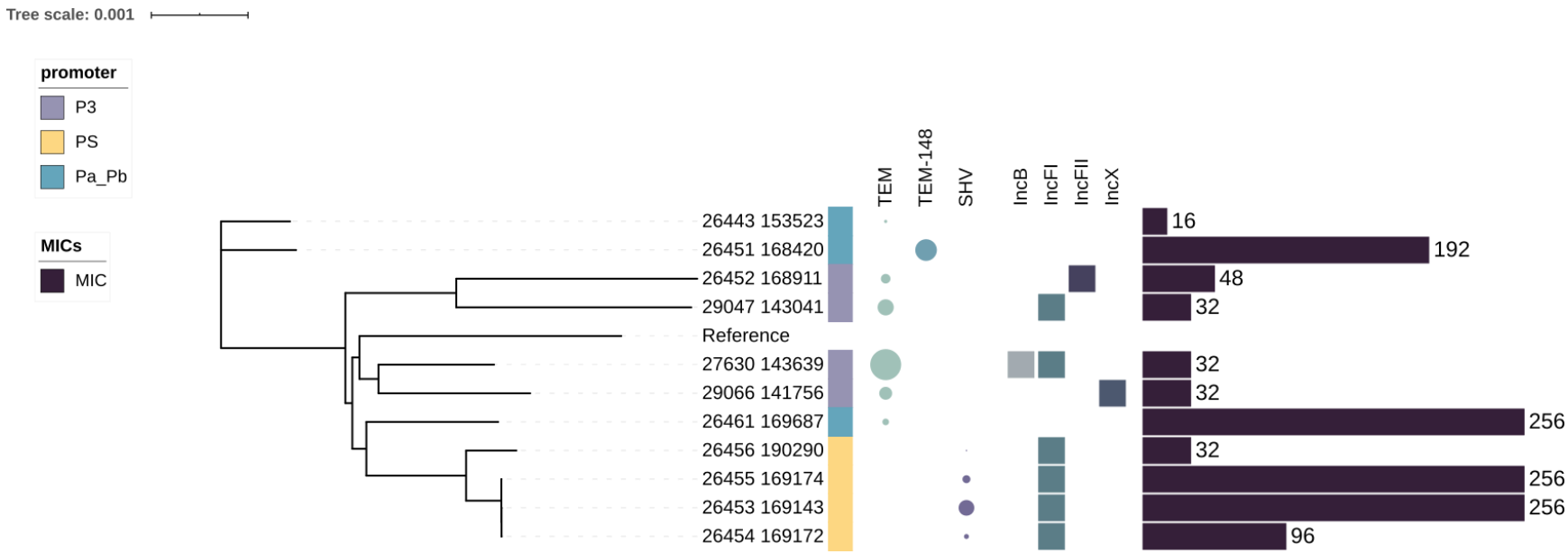


Fig.S5. High resolution core genome – based phylogeny of TZP resistant/3GC susceptible ST73 isolates. Indicated are promoter types, β-lactamase copy numbers (size of circle represents relative copy number), plasmid replicons, and TZP MIC.