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Molecular epidemiology of OXA-1054, a novel carbapenem-hydrolysing Class D β -lactamase, in Enterobacteriaceae isolated from wastewaters



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Class D carbapenemase-producing Enterobacteriaceae have spread globally in recent decades. The increasing detection of novel variants, often differing by amino acid substitutions or deletions in the β 5- β 6 loop of OXA-48 or OXA-372 lineages, indicated ongoing dissemination. In this work we describe the molecular epidemiology of a novel OXA-372-like carbapenemase gene, *bla*_{OXA-1054}, that was identified in *Citrobacter*, *Enterobacter* and *Raoultella* isolates collected from hospital discharges, river water and raw wastewater in Seville, Spain. Cloning of the *bla*_{OXA-1054} gene in *Escherichia coli* confirmed its contribution to carbapenem resistance within the Enterobacterales background. The genetic context of *bla*_{OXA-1054} was highly variable, but a conserved backbone, characterized by the IS21 family transposition genes, *istA* and *istB*, was consistently present upstream. Frequent co-localization with an *ampC* gene and heavy metal resistance operons (arsenic and mercury) suggests potential for co-selection under multiple selective pressures. Our study shows that *bla*_{OXA-1054} is embedded within a mobile genetic module with strong potential for horizontal transfer, which underscores the importance of monitoring emerging OXA variants in both clinical and environmental settings.

The increasing emergence of Carbapenem-Resistant Enterobacteriaceae (CRE), leading to severe infections in clinical settings, represents a major challenge given the limited availability of effective treatments^{1,2}. Carbapenem-Hydrolysing class D β -lactamases (CHDLs) are widespread and frequently detected in Gram-negative bacteria worldwide^{3–5}. The description of OXA-48 Class D β -lactamases (DBL) in Turkey in 2001⁶ was the first time that a CHDL was found in Enterobacterales, and then multiple OXA-48-like variants have been reported. These variants, differing by only a few amino acid substitutions or deletions, have become the predominant carbapenemases among Enterobacterales in Europe in both clinical and community settings^{7,8}. Three OXA-48-like clusters have been proposed, each with a different mechanism of capture and mobilization from the chromosomes of aquatic *Shewanella* spp⁹. Differences in the kinetic properties of OXA-48-like β -lactamases have been observed, being only some of

them true carbapenemases¹⁰. In contrast, other CHDL lineages are phylogenetically distinct from the OXA-48-like group and have been mobilized independently. OXA-372 represents a separate class D β -lactamase protein lineage, which was detected subsequently and shares 43% similarity with OXA-48¹¹, as well as other OXA-372-like (OXA-641 and OXA-1014), according to BLDB¹². Despite this distinct protein nature, characterization of OXA-372 demonstrated a resistance profile consistent with class D carbapenemases, conferring high-level resistance to penicillins, reduced susceptibility to imipenem was also observed, whereas MICs for extended-spectrum cephalosporins and aztreonam remained low¹¹.

Wastewater environments are increasingly recognized as important reservoirs and hotspots for the emergence and dissemination of carbapenemase resistance, including OXA-48-producing bacteria^{11,13,14}. Wastewater treatment plants (WWTPs) receive discharges from hospitals,

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communities, and agricultural sources, creating conditions that favor the selection and horizontal transfer of resistant genes¹⁴. In addition, several studies have also demonstrated the presence of carbapenemase-producing bacteria in hospital wastewater^{15,16}. Both hospital and WWTP effluents harbor significantly higher levels of diversity and abundance of carbapenemase-producing bacteria compared to other environments. In this study, we analyze and describe the molecular epidemiology of OXA-1054, a novel OXA-372 variant that was isolated from different Enterobacteriaceae species recovered from river water, urban wastewater, and hospital discharge samples in Seville, Spain. The genetic location of this variant and structural characterization enabled us to analyze its transmission pathways.

Results

The proportions of CHDL producers accounted for 52.3%, 30%, and 17.5%, of carbapenemase-producing isolates from urban wastewater, river water and hospital discharge samples, respectively. Analysis of all *bla*_{OXA-372-like} genes revealed a new protein sequence, assigned as OXA-1054 by GenBank. This novel OXA β -lactamase showed a high level of identity (93.8%, 96%, and 91%) with OXA-372, OXA-641 and OXA-1014. A comparison of protein sequences showed that OXA-1054 was distantly related to the OXA-48 carbapenemase, with a maximum identity of 43.1%. OXA-1054 and OXA-372 differed by sixteen amino-acid substitutions: M7K, L11F, C16Y, I34L, S36T, T37A, D41Q, R64S, A67T, S89A, A92T, R94K, D96N, H100Y, I102F and V117F. The comparison of OXA-1054, OXA-372, OXA-641, OXA-1014, and OXA-48 showed that four conserved motifs were maintained: STFK (amino acids 70-73; nucleotides 207-219), QXXXL (amino acids 169-173; nucleotides 505-519), KTG (amino acids 208-210; nucleotides 622-630), and PXXG (amino acids 217-220; nucleotides 649-660) (Fig. S2). Amino acid and nucleotides positions are numbered according to the OXA-48 reference sequence.

The 27 OXA-1054 producers were predominantly found in raw wastewater samples from three WWTPs ($n = 23$; 85.2%), while the hospital discharge and river water samples yielded two isolates each. Of the 23 raw wastewater isolates, 19 (82.6%) came from WWTP-A, while three (13.0%) and one (4.3%) were obtained from WWTP-D and WWTP-C, respectively. Multiple Enterobacteriales species were identified: 11 *Citrobacter freundii*; 5 *Enterobacter soli*; 4 *Citrobacter portucalensis*; 2 *Raoultella ornithinolytica*; 2 *Citrobacter braakii*; 1 *Citrobacter farmeri*; 1 *Enterobacter kobei*; and 1 *Raoultella planticola*. A wide variety of clones were found among the different Enterobacteriales. The most prevalent bacterial species, *C. freundii*, was found to have eight distinct sequence types (STs): ST112, ST150, ST167, ST273, ST511, ST701, ST770 and ST898. The two *C. braakii* strains belonged to different ST (ST516 and ST769), and 3 different STs were found among the four *C. portucalensis* isolates (ST51, ST175, and ST961) (Fig. 1A). All *E. soli* isolates belonged to ST1811 and were recovered from the same WWTP in two different quarters of the same year (Fig. 1B). The two *R. ornithinolytica* strains, which were identified in different years (2018 and 2022) and sources (river water and WWTP-D influent), differed by 94 different SNPs (Fig. 1C). The original isolates, STs, year of isolation, location of *bla*_{OXA-1054} gene, source and MIC values are summarized in Table S4.

A wide variety of other *bla* genes were found in addition to the *bla*_{OXA-1054} gene. The *bla*_{TEM-1} gene was found in 10 isolates, as well as *bla*_{KPC-2} ($n = 6$), *bla*_{OXA-9} ($n = 5$), *bla*_{BEL-1} ($n = 4$), *bla*_{CMY-2} ($n = 4$), *bla*_{FOX-3} ($n = 3$), *bla*_{CMY-75} ($n = 2$), *bla*_{OXA-1} ($n = 1$), *bla*_{OXA-2} ($n = 2$), *bla*_{OXA-10} ($n = 1$), *bla*_{SHV-2a} ($n = 1$), and *bla*_{GES-7} ($n = 1$) genes (Fig. S3). The isolates also carried genes for resistance to other antibiotic families such as aminoglycosides (74.1%), plasmid quinolone resistance (PMQR) (48.1%), and sulfonamides (*sul*) (29.6%) (Fig. S3). Heavy-metal resistance operon genes were also identified. Six (33.3%) isolates (337, 17-12, 17-13, 25-15, 25-16, and 33-23) carried the complete arsenic operon. Two (11.1%) isolates (371 and 29-14) harbored the complete mercury operon.

The number of *rep* genes among the 27 strains ranged from three to 11, with an average of 5.9 genes per isolate. Of the 18 isolates that underwent long-read sequencing, the *bla*_{OXA-1054} gene was predominantly found on plasmids (94.4%), with only one strain carrying it chromosomally. The

*bla*_{OXA-1054} gene was found on plasmids belonging to different incompatibility groups (*IncF*, *IncL/M*, *IncN*, *IncR*, *IncU*, and *IncX*), as well as on multireplicon plasmids in 5 out of the 18 long-read sequenced strains (28%) (Fig. 2). One strain (25-16 *C. braakii*) was found to have a double copy of the *bla*_{OXA-1054} gene. One *IncF*/OXA-1054 plasmid also carried *bla*_{SHV-2a}. Apart from the *bla*_{OXA-1054}-carrying plasmids, other incompatibility group plasmids were detected in the isolates that had undergone long-read sequencing: three of these carried carbapenemase and ESBL genes (39-22 carrying *IncQ2*/GES-7; 29-14 carrying *IncP-6*/KPC-2; and 17-15 carrying *IncP-6*/KPC-2) (Fig. 2). It was predicted that 38.9% ($n = 7/18$) of the plasmids carrying the *bla*_{OXA-1054} gene would be predominantly non-mobilizable, 33.3% would be mobilizable ($n = 6/18$), and 27.8% ($n = 5/18$) conjugative. Two of the predicted mobilizable plasmids co-occurred with conjugative plasmids not carrying the *bla*_{OXA-1054} gene. A total of seven plasmids (38.9%) could spread by conjugation. Despite these genetic contents, conjugation assays using *E. coli* J53Azi^R did not yield transconjugants for the 17 long-read sequenced strains under the experimental conditions tested.

Sixteen out of the 18 (88.9%) isolates that underwent long-read sequencing contained phage-like proteins on the *bla*_{OXA-1054}-carrying plasmids, but none of them appeared to represent complete prophages (simultaneous capsid, head and tail) (Fig. S4). Interestingly, six of the 17 *bla*_{OXA-1054}-carrying plasmids (37.5%) harbored tail and integrase, or tail and capsid of bacteriophage RCS47. Analysis of plasmids carrying the *bla*_{OXA-1054} gene revealed the presence of multiple and varied toxin-antitoxin (TA) systems. A total of 98 TA genes were identified on *bla*_{OXA-1054}-carrying plasmids. On average, 6.1 TA modules (ranging from one to 18) were detected per plasmid. The most frequently identified toxin-antitoxin modules, representing 38.8% of all TA modules found, belonged to the *parDE*-like family, the *Arc*-like DNA binding domain, the helix-turn-helix (HTH), and the *ParB*-like nuclease domain. No TA genes were detected on only one plasmid (39-22/*IncX5*) (Fig. S5).

Genetic environment analysis of *bla*_{OXA-1054} gene

The close genetic environments of *bla*_{OXA-1054}, whether chromosomal or plasmid, were highly similar, despite the diversity of plasmid location. The comparative analysis revealed that the genetic environment of *bla*_{OXA-1054}, although dynamic, displays some conserved structural features. A platform containing IS91-IS91-IS91-IS21-IS21-*bla*_{OXA-1054} was observed in 16/18 (88.9%) of the long-read sequenced genomes. The *istB* gene, located upstream of *bla*_{OXA-1054}, was present in all long-read sequences, as was the *istA* gene in 17/18 (94%) of them. Both genes belong to IS21 (Fig. 3). In 11/18 (61.1%) genomes, a Class C β -lactamase gene (accession number PX474618) was found in the region downstream of the *bla*_{OXA-1054} gene (355 bp). This *ampC* sequence was identical to a Class C β -lactamase gene previously detected in a *Sphingobium* sp. strain (accession number CP119128.1) recovered from an activated sludge sample in China in 2021. Five out of the 18 long-read sequences (27.8%) contained a distinct accessory module comprising genes encoding a DNA-methyltransferase, a DEAD/DEAH-box helicase, and an *Smc* ATPase. Beyond these conserved elements, the surrounding regions exhibited substantial variability, with multiple *Tn3*-like transposases and other insertion sequences frequently being inserted into, inverting, or disrupting neighboring genes. Heavy-metal resistance operons for arsenic operon genes (33-23 *C. freundii* and 17-13 *C. farmeri* *bla*_{OXA-1054}-carrying plasmids), and mercury operon genes (371 *C. braakii* *bla*_{OXA-1054}-carrying plasmid) were found in more distant regions, all embedded in transposon-like structures (Fig. 3).

Cloning experiments

Antimicrobial susceptibility testing revealed that the original isolates and their *E. coli* TOP10 transformants displayed distinct resistance profiles (Table S5). OXA-1054 conferred increased minimum inhibitory concentrations (MIC) values than OXA-48 for ceftazidime, imipenem and ceftolozane/tazobactam, while isolates producing these enzymes exhibited very high MICs for piperacillin/tazobactam. The presence of the native promoter increased resistance in both OXA-48 and OXA-1054 constructs.

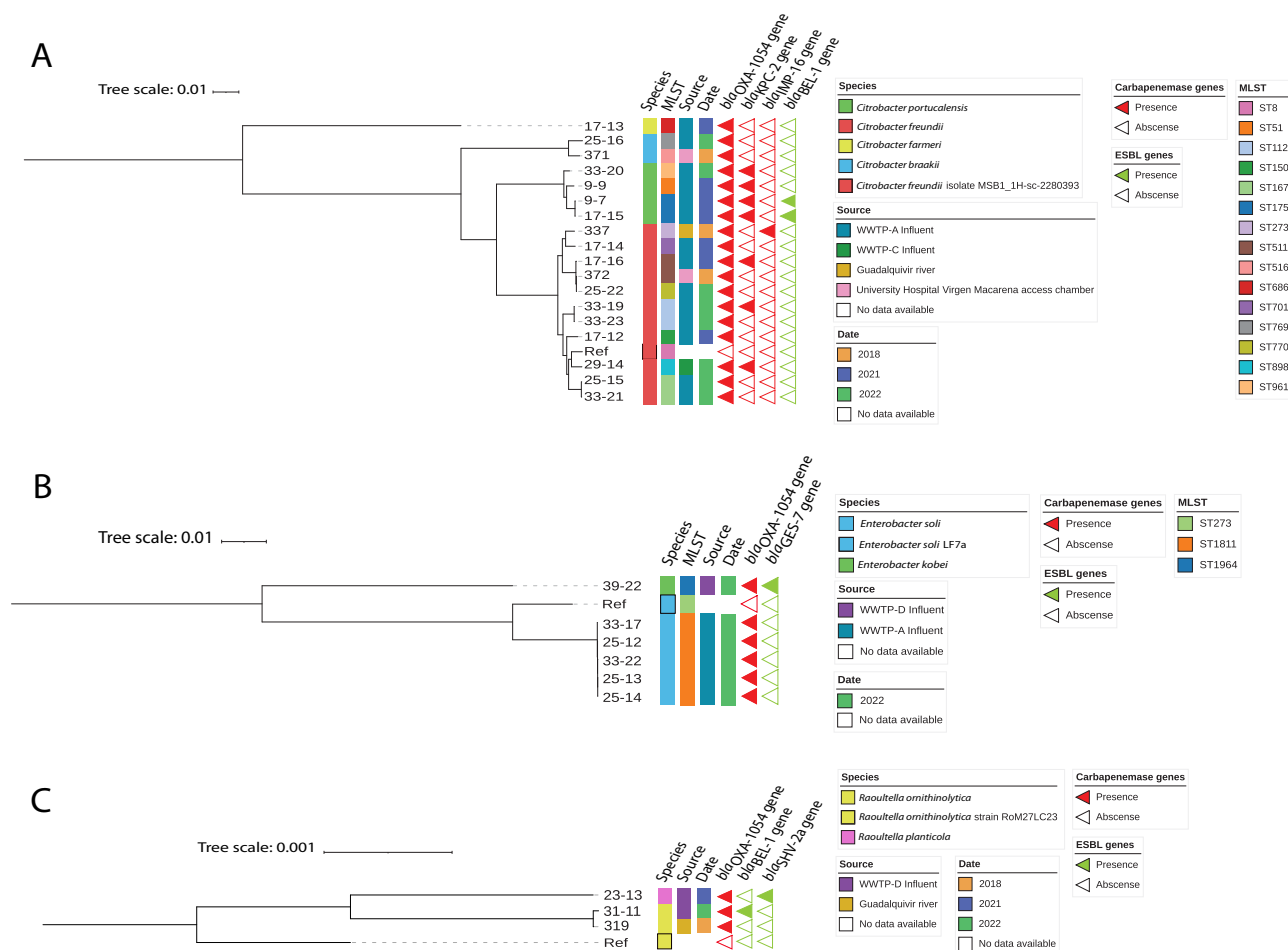


Fig. 1 | Phylogenetic trees showing single-nucleotide polymorphism (SNP) differences in *Citrobacter* (A), *Enterobacter* (B) and *Raoultella* (C) isolates. SNIPPY-core was used to calculate distances.

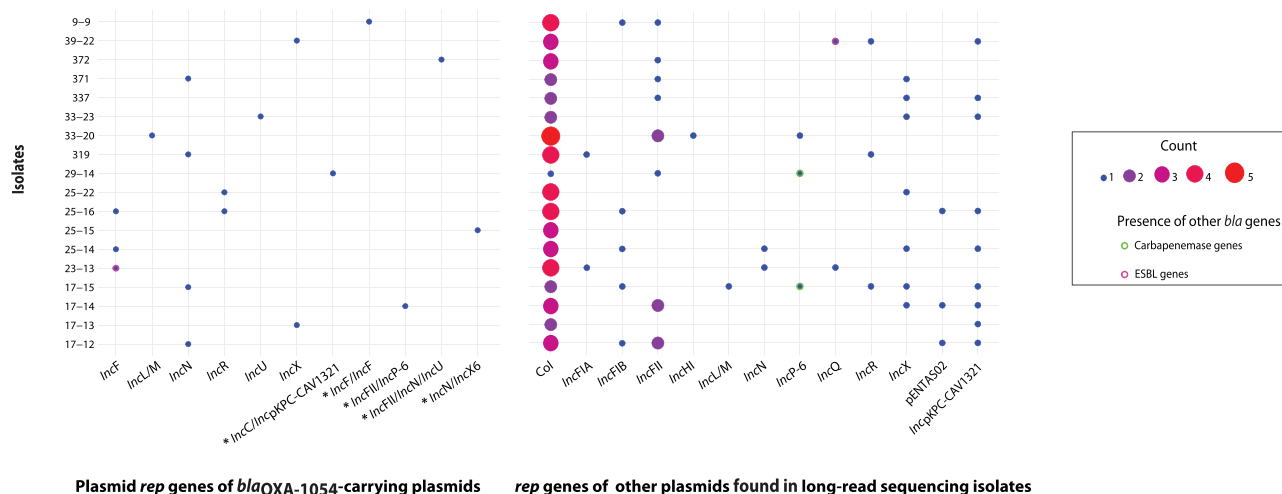


Fig. 2 | Plasmid rep genes identified among 18 long-read sequencing OXA-1054-producing isolates. Different rep genes were identified on the same plasmid.

Expression of *bla*_{OXA-1054} under its native promoter led to a 1.3 - to 63-fold increase in MICs for all the tested β -lactams (except piperacillin/tazobactam) compared with the promoterless construct. Furthermore, the OXA-1054 construct with its native promoter exhibited MICs that were 2- to 15-fold higher than those of the OXA-48 construct carrying its native promoter (Table S5).

Discussion

This study describes the presence of OXA-1054, a novel OXA-372 variant, in multiple Enterobacteriaceae species recovered from different locations within Seville's wastewater system and from the river downstream. To our knowledge, this is the first epidemiological analysis of OXA-1054-producing Enterobacteriaceae, which highlights the potential role of

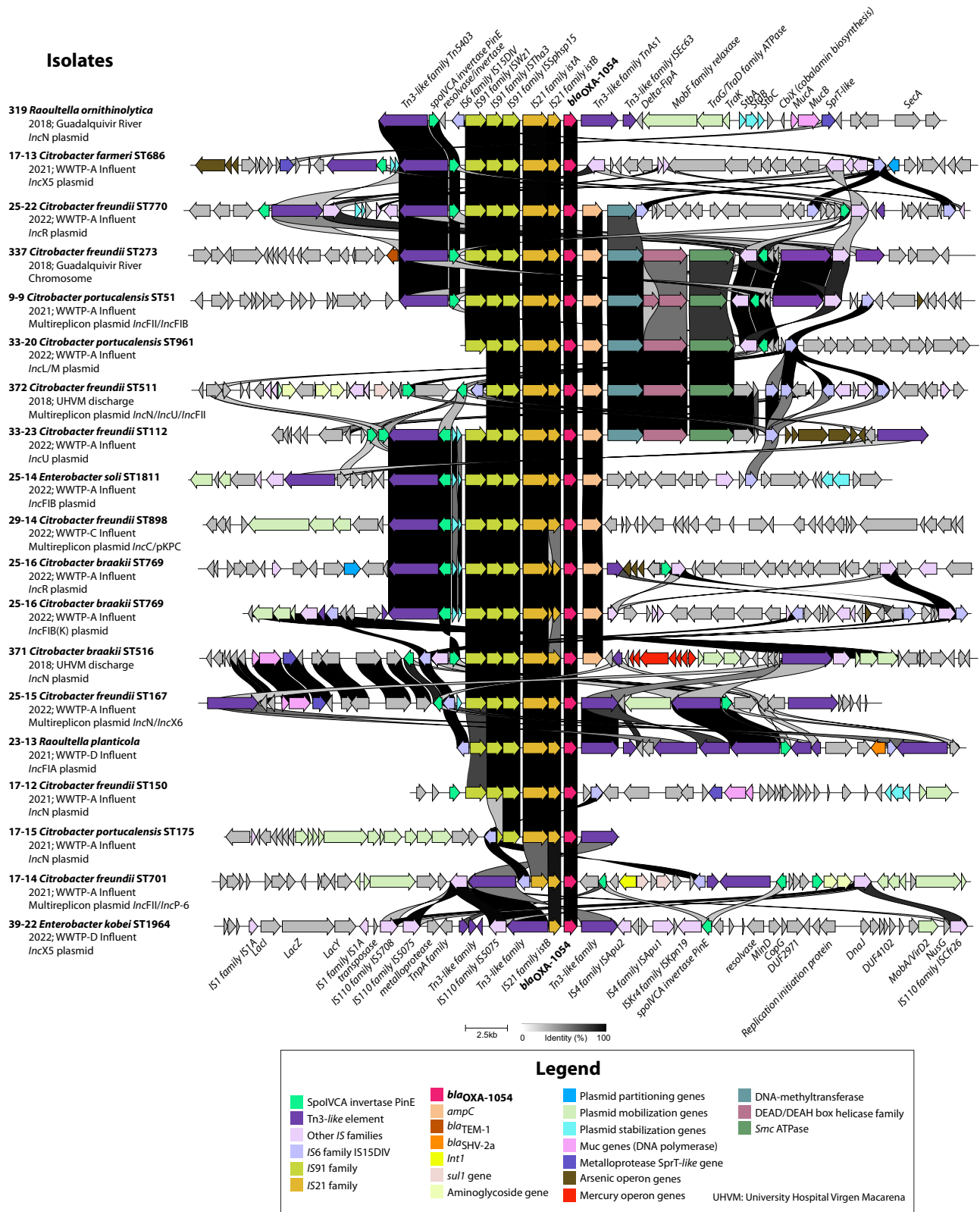


Fig. 3 | The genetic context of the *bla*_{OXA-1054} gene, showing approximately 20 kb upstream and downstream of *bla*_{OXA-1054}. Nucleotide sequence identity is represented using grayscale gradients, ranging from 50% (light gray) to 100% (black). The *bla*_{OXA-1054} gene is shown at the center of each alignment. Arrows represent predicted coding sequences, with the direction of the arrow indicating the

transcriptional direction. Colors denote different functional categories, including insertion sequences, resistance determinants, heavy metal operons, and accessory genes, as indicated in the legend. The names of the isolates, species, MLST, year of isolation, source of isolation, and location of the *bla*_{OXA-1054} gene are shown on the left.

environmental niches in generating, evolving and/or spreading clinically relevant resistance mechanisms.

To date only two variants of OXA-372 have been previously detected: OXA-641 (GenBank: MH211331.1, unpublished, source not reported), shown in a *Morganella morganii* isolate in Germany, and OXA-1016 (GenBank: MZ449319.1, unpublished), detected in a human-associated *Ectopseudomonas oleovorans* in France. These variants share 96% and 91% of amino acid identity with OXA-372 and OXA-1054, respectively. However, no data on their resistance phenotype or further functional characterization are currently available. The resistance phenotype of OXA-1054 revealed in this study was evaluated using heterologous expression in *E. coli* TOP10. The pMiniT2.0 vector confers ampicillin resistance, baseline β -lactamase activity in the host may slightly contribute to observed MIC values. Nonetheless, the relative differences in MICs between OXA-1054 and OXA-48 constructs, including the impact of the native promoter, reflect the intrinsic properties of the expressed enzymes.

The acquisition of *bla*_{OXA-48-like} genes has consistently been linked to plasmid carriage, which is consistent with reports for *bla*_{OXA-163} and *bla*_{OXA-181}¹⁷. Similarly, *bla*_{OXA-372} has previously been identified on a multireplicon plasmid (*IncA/C* and *IncN*) in a *C. freundii* isolate found in wastewater, embedded within a defective transposon, designated *Tn6255* involving *IS26* elements and recombination-derived transposon remnants¹¹. The *bla*_{OXA-1054} gene was chromosomally located in one isolate in this study, it was primarily carried by a variety of plasmids belonging to different incompatibility groups, including two different copies on two different incompatibility group plasmids (*IncFIB(K)* and *IncR*) on a single isolate. The diversity of plasmids observed among OXA-1054-producing isolates, the conserved genetic close environment, as well as the conjugation results suggest that the *bla* gene dissemination is not probably driven by the spread of plasmids, but rather by a conserved mobile genetic module in aquatic ecosystems. Furthermore, the presence of multiple TA systems on plasmids carrying *bla*_{OXA-1054} gene also suggests that this mobile genetic module is highly to persist over time under stressful conditions, even in the absence of antibiotic pressure¹⁸. Finally, the fact that these plasmids are mainly non-autonomous mobilizable, lacking mate-pair formation (MPF) marker, *oriT*, and relaxase (MOB), as well as complete functional prophages, reinforces the idea that dissemination is unlikely to occur through classical conjugative transfer. This is consistent with our conjugation assays, in which none of the 17 isolates yielded a transconjugant, and with previous findings showing that the original *bla*_{OXA-372}-harboring plasmid was not transferable under experimental conditions¹¹. Altogether, these findings suggest that the spread of this resistance determinant is more likely driven by the successful propagation of a conserved genetic platform rather than by efficient plasmid-mediated conjugation. A third part of the analyzed *bla*_{OXA-1054}-carrying plasmids harbored elements of bacteriophage RCS47, a P1-like prophage that has been associated with the *bla*_{SHV-2} gene acquisition. Nevertheless, this prophage has been assessed as a defective phage that is no longer able to lysogenize *E. coli* hosts¹⁹.

The recurrent co-location of *istA* and *istB* genes alongside the *bla*_{OXA-1054} gene suggests that these insertion sequences may have captured the latter and enhanced its expression through strong promoters⁴. Comparative genomic analysis revealed that the *bla*_{OXA-1054} is consistently associated with the *IS21* family transposition genes *istA* and *istB*, forming an *IS21*-associated transposition module that is inserted into different plasmid scaffolds, and occasionally into the chromosome. This module was identified with variable flanking regions indicative of independent insertion events. In the *IS21* family of mobile elements, transposition requires the cooperative activity of *IstA* (the main transposase) and *IstB* (an AAA+ ATPase that modulates transposase activity and facilitates the assembly of the transposition complex). This coordinated process, which is controlled by ATP-dependent assembly and protein-protein interactions, prevents uncontrolled transposition while maintaining module stability^{20,21}. The presence of accessory modules, such as DNA methyltransferases, DEAD/DEAH-box helicases and *Smc* ATPases, suggests that these regions may be involved in

maintaining and stabilizing resistance determinants. DEAD-box helicases are widely recognized for their roles in RNA metabolism and stress adaptation²², while *Smc* ATPases are essential for chromosome segregation and plasmid stability²³. Together, these features probably enable horizontal gene transfer and the stability and persistence of *bla*_{OXA-1054} in different bacterial hosts and genetic contexts. This would increase its potential for dissemination.

The detection of *bla*_{OXA-1054}-carrying Enterobacteriaceae in different environmental sources suggests a potential transmission pathway from hospital wastewater discharges to the natural environment. In contrast to OXA-48-like, which is widely established in clinical settings, current evidence suggests that members of the OXA-372 lineage are primarily associated with environmental reservoirs, but sporadic human sources have been seen. OXA-372 itself has been detected in wastewater and urban drainage systems^{11,24}. OXA-1016 variant has been reported in a human-associated *E. oleovorans* isolate; however, no additional clinical, epidemiological, or functional data are currently available. Therefore, the ecological distribution and potential clinical relevance of this lineage remain largely unexplored. Although this *bla*_{OXA-1054} gene has not previously been detected either in clinical isolates at the University Hospital Virgen Macarena, or in other Andalusian (data until 2024) or Spanish hospitals (data until 2022)²⁵, the hospital discharge was identified as the first positive sampling point within the city's wastewater system. The highest number of isolates in the wastewater system was found in the WWTP influents. Different drivers could be at play in WWTP influents, as well as in recombination events and competition^{26,27}. Although wastewater treatment reduced the load of OXA-1054-producing bacteria to undetectable levels using culture methods¹⁴, OXA-1054 producers continued to be detected in river water downstream of one of the WWTPs in different sampling seasons.

The association of the *bla*_{OXA-1054} gene with co-resistance modules, including antibiotic resistance genes and heavy metal resistance operons, suggests that environmental pollutants may exert a selective effect in favor of carbapenemase maintenance. These co-selection mechanisms are increasingly recognized as important indirect drivers of the spread of antimicrobial resistance beyond clinical settings^{28,29}. Other features serve to reinforce the association with an environmental origin. Firstly, ancestral OXA-372 has so far only been identified in a wastewater isolate, where it was first described¹¹. More recently, in 2025, *bla*_{OXA-372} gene was detected in the drainage network of Sabadell city (Spain), but only through metagenomic analysis, not in cultured bacteria²⁴. Secondly, the co-occurrence of *bla*_{OXA-1054} with an *ampC* gene found only in environmental bacterial species such as *Sphingobium* sp. suggests a common source²⁸. Higher MIC values observed in OXA-1054-producing isolates compared with OXA-48-producing isolates suggest that human waste could be a source of novel β -lactamase variants as well as an incubator for the evolution of enzymes with enhanced antibiotic resistance.

This study has some limitations that should be considered when interpreting the findings. Firstly, not all *bla*_{OXA-1054}-containing genomes have been fully characterized using long-read sequencing, and the specific genetic location of the *bla*_{OXA-1054} gene remains unknown. Nevertheless, genomes of each species and sequence type were selected to ensure that all groups were represented in the analysis. Secondly, the geographical scope of the analyzed isolates was limited, and broader sampling is required to accurately determine the global distribution and prevalence of OXA-1054. To date, no environmental studies have detected this enzyme outside Andalusia. Furthermore, it is not known whether OXA-1054 producers are present in other hospital wastewater discharges inside the city or whether they persist further down the river beyond the urban area of Seville. To better understand the potential implications of this enzyme for human infections, future prevalence studies should also evaluate its activity against the new β -lactamase inhibitors.

We have identified for the first time different Enterobacteriaceae species carrying OXA-1054. This novel OXA-372 variant exhibits higher MIC values for cephalosporins, carbapenems and the novel combination of ceftolozane/tazobactam, compared with OXA-48. The presence of this gene

in human-derived discharges alongside heavy metal resistance genes indicates ongoing environmental selection and dissemination, despite its absence in isolates from human clinical and surveillance samples. Furthermore, the conserved mobile genetic environment harboring the *bla* gene has been detected in multiple different plasmids, many non-conjugative, which suggests that plasmid or clonal spread is unlikely.

Methods

Sample collection, bacterial manipulation and sequencing

This study focused on the wastewater system in the urban area of the city of Seville and used three different sampling sources (Fig. S1). Firstly, water samples were collected 200 meters downstream of WWTP-D effluent in the Guadalquivir river (which flows through the city of Seville from north to south) as part of the multicentre MODERN project focused on ESBL screening³⁰. The samples were filtered and processed according to previously described protocols³⁰. Secondly, samples of discharge water from the University Hospital Virgen Macarena were taken monthly as part of a monitoring programme in southern Spain, and handled as previously described³¹. Thirdly, composite water samples were collected once a quarter during the year from the raw and treated effluent of the WWTPs in Seville (Spain). All samples were transported to the laboratory at 4 °C and processed immediately upon arrival. Serial dilutions were prepared and plated onto chromogenic media (ChromID® CARBA, OXA-48 agar, bioMérieux, Marcy-L'Etoile, France). In all cases, 50 µL of each sample was inoculated onto the plates using an Eddy Jet spiral plater (IUL Instruments, Barcelona, Spain)³⁴. The plates were then incubated for 18 ± 2 h at 37 °C under aerobic conditions. Quantitative culture methodology and species identification were performed, as previously described¹⁴.

All colored colonies identified on the previously mentioned chromogenic media were screened for carbapenemase resistance, using disk diffusion method to test for susceptibility to ertapenem, imipenem and meropenem (10 µg), according to the 2025 guideline of the European Committee on Antimicrobial Susceptibility Testing (EUCAST). If the inhibition zone of carbapenems was smaller than the screening cut-off values³², carbapenemase activity was assessed using β-CARBA® test (Bio-Rad, Marnes-la-Coquette, France), which detects carbapenem hydrolysis through a pH-based colorimetric change (color change from the initial yellow to reddish was interpreted as positive). When carbapenemase activity was detected, the NG-Test CARBA 5 immunochromatographic assay (NG Biotech, Guipry, France) and the KPC, MBL and OXA-48 Confirm Kit (Rosco Diagnostica A/S, Taastrup, Denmark) were performed for carbapenemase group detection. Nevertheless, all β-CARBA positive strains were sequenced, even when a NG-Test CARBA 5 result was found negative, because not all carbapenemases are detected by the immunochromatographic assay.

To further characterize the OXA-48-like genes, all potential OXA-48-like producers were sequenced. Libraries were prepared using the Nextera DNA Sample Preparation kit (Illumina San Diego, CA, USA) and sequenced on an Illumina MiSeq platform with 300 bp paired-end reads. De novo assembly was performed using CLC Workbench 9.5.2 software (Qiagen). Resfinder v.4.1.1 (<https://cge.food.dtu.dk/services/ResFinder/>) and the CARD database (<https://card.mcmaster.ca/analyze/rgi>) were used to identify acquired resistance determinants. For this study, the genomes of 27 environmental strains belonging to *Citrobacter*, *Enterobacter*, and *Raoultella* spp., in which *bla*_{OXA-372} was detected with a homology threshold of 90–95%, were selected. An environmental OXA-48-producing *Klebsiella pneumoniae* strain obtained from raw wastewater was also included as control for further cloning analyses.

Plasmid analysis

For each Multilocus Sequence Typing (MLST) profile, one representative isolate was selected for long-read sequencing. The DNA was extracted with the DNeasy Blood and Tissue Kit, and quantified using a Qubit 2.0 Fluorometer. Libraries were prepared at a minimum concentration of 20 ng/µL using the Rapid Barcoding Kit 96 v14 (SQK-RBK114.96; Oxford Nanopore Technologies, Oxford, UK), following the manufacturer's

recommendations. The libraries were loaded onto a FLO-MIN106 R10.4.1 MinION flow cell (Oxford Nanopore Technologies, Oxford, UK) and sequenced for 24 hours. The Illumina fastq files were trimmed and quality-checked using Trimmomatic v.0.39; MinION fastq files were filtered with Porechop v.0.2.4 and quality-checked with Filtlong v.0.2.1. De novo assemblies were created using Autocycler v.0.1.2. Draft assemblies were polished with two rounds of Medaka v.2.0.1 using the Nanopore reads, and a final round of Polypolish v.0.6.0 using the Illumina reads. Following polishing, the assemblies were ready for further analysis. Clinker software v.0.0.31 was used to model the genetic environment of the *bla*_{OXA-1054} gene³³. Quality control (QC) of the hybrid assemblies was analyzed with QUAST v.5.2.0³⁴. BWA-MEM v.0.7.17-r1188³⁵ was used to align the reads of the assembled genome, and SAMtools v.1.11³⁶ to assess coverage across the genome (Table S1). Each circularized plasmid was annotated using Bakta v.1.9.4³⁷ with the default settings, and mobility was predicted using MOB-Typer v.3.1.9 in the MOB-Suite³⁸. Toxin-antitoxin systems were characterized using the TASER analysis pipeline provided by the TASmania TAS database (<https://shiny.bioinformatics.unibe.ch/apps/taser/>)³⁹. The mobile genetic elements surrounding the *bla*_{OXA-1054} gene were identified and annotated using ISFinder (www-is.biotoul.fr)⁴⁰. Phage-related proteins were sought in *bla*_{OXA-1054}-carrying plasmids using the web server PHASTER (<https://phaster.ca/>)⁴¹.

Phylogenetic analysis

SNIPPY-core v.4.6.0 from SNIPPY was used for the comparative analysis of *Citrobacter*, *Raoultella* and *Enterobacter* isolates. The reference genomes used to construct the phylogenetic trees were selected from the NCBI database (Table S2). iTOL software (version 7) was used to visualize the phylogenetic trees and show relationships among the isolates.

Cloning and expression experiments of *bla*_{OXA-1054} gene

The genes encoding OXA-1054 and OXA-48 were amplified by PCR amplification using pre-designed primers (Table S3). The PCR products were then purified using Qiaquick gel extraction kit (Qiagen) and cloned into the pMiniT2.0 vector (New England Biolabs). *Escherichia coli* TOP10 (Invitrogen) was used as recipient strain in electroporation experiments to express the *bla*_{OXA-1054} and *bla*_{OXA-48} genes. The clones were grown overnight at 37 °C and selected on Mueller-Hinton Agar (MHA) plates supplemented with 100 µg/mL ampicillin. The cloned DNA fragments in the pMiniT2.0 vector were confirmed by sequencing.

Conjugation experiments

All identified *bla*_{OXA-1054}-carrying plasmids that underwent long-read sequencing were used for conjugation experiments as donor strains, and *E. coli* J53Azi^R was used as recipient strain. Donor and recipient strains were mixed at a ratio of 1:1 on 0.45 µm nitrocellulose filters and placed on agar plates supplemented with sodium azide (100 µg/mL) and agar plates with sodium azide (Azi) (100 µg/mL) and ceftazidime (CAZ) (1 µg/mL) and incubated at 37 °C for 6 h. After incubation, bacteria were recovered from the filters, serially diluted up to 10⁻⁶ and plated onto Azi-CAZ supplemented agar plates to select for potential transconjugants. Parallel Azi supplemented agar plates were used for receivers. Colonies were counted manually and transconjugants were picked and grown onto Azi-CAZ supplemented agar plates at 37 °C for 18 ± 2 h. Transconjugants were checked with PCR assays for *bla*_{OXA-1054} gene using the previous primers mentioned (Table. S3).

Antimicrobial susceptibility testing

The minimum inhibitory concentrations (MICs) of cefotaxime, ceftazidime, cefepime, ceftolozane/tazobactam, ertapenem, meropenem, and imipenem for the original environmental isolates were determined in triplicate, using the reference broth microdilution method, according to EUCAST clinical breakpoints (https://www.eucast.org/clinical_breakpoints). For ceftolozane/tazobactam susceptibility testing, the concentration of tazobactam was fixed at 4 mg/L. The MICs for the transformants were determined using the gradient strip E-test method (Liofilchem,

Spain). The results were interpreted according to the 2025 EUCAST clinical breakpoints.

Data availability

The *bla*_{OXA-1054} nucleotide sequence data reported in this work has been deposited in the GenBank nucleotide sequence database under the accession number: OM322820. Raw Illumina reads of isolates are available on the NCBI website under the BioProject numbers PRJNA1026089 and PRJNA1329985. Raw Nanopore reads of the isolates are available on the NCBI website under BioProject number: PRJNA1329997.

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Author contributions

M.R.P. and L.L.C. developed the methodology. L.M.O., L.G.P., J.A.S. and L.L.C. curated the raw data. L.M.O. and M.R.P. managed software applications and formal analysis. L.M.O., M.R.P., and A.B.R. performed tables and figures visualization. L.M.O. and L.L.C. wrote the draft. M.R.P., A.P., and L.L.C. supervised experimental data and analysis. A.P. and L.L.C. obtained funding. L.L.C. administered the projects. All authors reviewed the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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