Horizontal transmission of a multidrug-resistant IncN plasmid isolated from urban wastewater

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ABSTRACT

Wastewater treatment plants (WWTPs) are considered reservoirs of antibiotic resistance genes (ARGs). Given that plasmid-mediated horizontal gene transfer plays a critical role in disseminating ARGs in the environment, it is important to inspect the transfer potential of transmissible plasmids to have a better understanding of whether these mobile ARGs can be hosted by opportunistic pathogens and should be included in One Health considerations. In this study, we used a fluorescent-reporter-gene based exogenous isolation approach to capture extended-spectrum beta-lactamases encoding mobile determinants from sewer microbiome samples that enter an urban water system (UWS) in Denmark. After screening and sequencing, we isolated a ~73 Kbp IncN plasmid (pDK_DARWIN) that harboured and expressed multiple ARGs. Using a dual fluorescent reporter gene system, we showed that this plasmid can transfer into resident urban water communities. We demonstrated the transfer of pDK_DARWIN to microbiome members of both the sewer (in the upstream UWS compartment) and wastewater treatment (in the downstream UWS compartment) microbiomes. Sequence similarity search across curated plasmid repositories revealed that pDK_DARWIN derives from an IncN backbone harboured by environmental and nosocomial Enterobacterial isolates. Furthermore, we searched for pDK_DARWIN sequence matches in UWS metagenomes from three countries, revealing that this plasmid can be detected in all of them, with a higher relative abundance in hospital sewers compared to residential sewers. Overall, this study demonstrates that this IncN plasmid is prevalent across Europe and an efficient vector capable of disseminating multiple ARGs in the urban water systems.

1. Introduction

Plasmids are extrachromosomal mobile genetic elements (MGEs) capable of self-replication (San Millan, 2018). Bacteria can gain and lose plasmids for the sake of survival under selection pressure and reducing fitness costs in the nonselective environment, respectively (San Millan and MacLean, 2017). In some cases, plasmids persist due to inherency or adaptation to environmental changes, which are attributed to the accompanying accessory genes to help the hosts gain competitive advantages over the others (Carroll and Wong, 2018). These non-core accessory genes promote phenotypic plasticity and significantly increase the diversity of the pan-genome of a given species and can constitute up to 90% of it (McInerney et al., 2017). Moreover, the accessory genes can include various resistance genes (e.g., towards antibiotics, biocides and heavy metals), virulence factors, and even the recent emerging CRISPR-Cas systems. Plasmids also can possess other traits, such as stability and mobility systems, which improve plasmid transfer to new hosts, respectively (Carroll and Wong, 2018). Natural plasmid transfer as an important mechanism of horizontal gene transfer (HGT), normally requires a dedicated cadre of gene systems: the mobility (MOB) genes including oriT, a relaxase and a type IV coupling protein (T4CP), and the mating pair formation genes (the type IV secretion system, T4SS) (Smillie et al., 2010). Plasmid HGT makes it...
possible for plasmid-borne genes to be shared in both closely and
distantly related bacterial species or even in dissimilar ecological niches
(Bolotin and Hershberg, 2017). And these events have led to bacterial
antibiotic resistance becoming prevalent and ubiquitous in diverse
environmental settings (Dolejska and Papagiannitsis, 2018), posing
potential risks to human health.

Plasmids can be classified into various incompatibility (Inc) groups,
based on replication typing. This classification is broad, including 28 Inc
groups documented for Enterobacteriaceae alone (Shintani et al., 2015).
Plasmids that belong to the same Inc group cannot coexist stably in the
same bacterial cell because they share, and thus compete for, compo-
nents of the replication and partitioning machinery. Plasmids of the
same Inc group generally share backbones with similar organizations and
functions. IncF, IncI, IncA/C, IncH plasmids are the most predomi-
nant Inc groups among the human, animal and environment samples
(Rozwandowicz et al., 2018). Together with IncL and IncC, these
plasmid groups are believed to carry the greatest diversity of antibiotic
resistance genes (ARGs) (Rozwandowicz et al., 2018).

Bacterial resistance to beta-lactams can be caused by efflux pumps,
the modification and/or reduced production of outer membrane porins,
alterations of the beta-lactams targeted molecular penicillin-binding
proteins, and enzymatic inactivation (i.e., beta-lactamases) (Descquier
and Mangani, 2018). Among these, the production of beta-lactamases
represents the most relevant mechanism of beta-lactam resistance in
Gram-negative pathogens, thus arguably constituting the biggest chal-
lenge to the use of beta-lactams (Bush, 2018; Bush and Bradford, 2019).

Considering that beta-lactamases are often carried by plasmids and
frequently co-occur with other types of ARGs (Cantón et al., 2012; Cui
et al., 2020), understanding their dissemination routes and potential
hosts is crucial to the call of the “One Health” strategy by the World
Health Organization. Long et al. demonstrated the co-transfer of mer-1,
mer-3.5, blaTEM, and mblI mediated by a dual-replicon IncHI2
(ST3)/IncN plasmid pMCRI 025943 in Escherichia coli strains (Long
et al., 2019). And Kajama et al. suggested IncN plasmid pKPlI-6 favoured
dissemination ofblaIMP-6, and blaCTX-M-2 in some Enterobacteriaceae
members (Klebsiella pneumoniae, Klebsiella oxytoca and Escherichia coli)
(Kajama et al., 2015). Moreover, a ca. 50 Kbp IncN plasmid backbone
with blablM-6 has been widely shared among Enterobacteriales from 22
hospital isolates (Yamagishi et al., 2020). These facts imply some Inc
groups of plasmids can be readily harbour and spread beta-lactamases
genomes in the nosocomial environment.

Wastewater treatment plants (WWTPs) are delineated to be
permissive facilitators in the dissemination of clinically relevant
blaIMP-6 genes into other aquatic ecosystems, thus raising public health concerns
(Coerzte and Beuzidenuhout, 2019). Indeed, WWTPs have been recog-
nised as act as reservoirs of various ARGs and hotspots of HGT
(Che et al., 2019; Rodriguez et al., 2021; Stalder et al., 2019; Suzuki et al.,
2019). Even after wastewater treatment, the effluents of WWTP still
retain diverse and abundant ARGs (Hendriksen et al., 2019; Quintela-Baluja et al.,
2019). One wastewater metagenomic study showed that the detection frequency of extended-spectrum beta-lacta-
mase (ESBL) genes (blaCTX-M) and carbapenemases genes (blaNDM, bla-
VIM, blablaGES and blablaOXA-48) increased during the wastewater treatment
(Makowska et al., 2020). ESBL genes (blaTEM, blaSHV-12, blablaCTX-M-1
and blablaCTX-M-15) carrying and carbapenem-resistance isolates from the family of
Enterobacteriaceae could be discovered in the WWTP effluent (Smyth
et al., 2020). Furthermore, wastewater irrigation practices have been
proven to introduce the ESBL genes (blaCARB-3, blablaOXA-48, blablaOXA-5
and blablaOXA-23) to the agriculture field soils (Bourgrom et al., 2020). Wastewater
flow normally brings together broad-host-range plasmids (e.g., IncN,
IncP, IncQ and IncW) (Moura et al., 2010). Akiyama et al. reported that
IncA/C, IncN, IncP and IncW plasmids could be detected in the WWTP
effluent and receiving streams (Akiyama et al., 2010).

Taken together, WWTPs effectively comprise genetic exchange
compartments, where beta-lactamase genes are often linked to efficient
MGE vehicles, such as plasmids. Therefore, it is crucial to investigate the
potential genetic drivers of beta-lactamase spread and their transfer host
range in the wastewater environment. We aimed to uncover the beta-
lactamase mobile genetic determinants in wastewaters and these ele-
ments’ transfer and retransfer potential in horizontal transmission. In
the present study, we applied an exogenous plasmid isolation method to
capture ESBL plasmids in the residential and hospital sewers from an
urban water system in Odense, Denmark. We used a mCherry red-
fluorescent Escherichia coli CSH26 recipient strain in metaparental
mating assays with sewer microbiomes and screened for transconjugants
exhibiting ampicillin resistance. We identified that the transconjugants
showed multiple-drug-resistance (MDR) in addition to ampicillin resis-
tance. After sequencing the captured plasmids both by Illumina MiSeq
and Oxford Nanopore followed by hybrid assembly, we verified the
MDR-responsible plasmid belonged to the IncN group and encoded
ARGs against aminoglycosides, beta-lactams, fluoroquinolones and
sulfonamides. Consequently, it is critical to investigate the potential host
range of such MDR IncN plasmids. In this study, we performed a
permissiveness test to explore its potential transfer to indigenous sewer/
wastewater bacteria and applied metagenome and PLSDB similarity
search for this plasmid to reveal its favoured habitat (treatment
compartment) and putative ancestors (epidemic sources and lineages).
This study contributes to the understanding of IncN plasmid’s evolution
and its relevant transmissible antibiotic resistance.

2. Materials and methods

The overall methodology applied in this study is illustrated in Fig. 1.

2.1. Sampling and pre-treatment

Hospital and residential sewer samples from a Danish urban water system
(Odense, Denmark) were taken using an ISCO automatic sampler in
24-hour time proportional sampling (50 mL per 5 min) in May 2017.
Three replicates from two hospital sewer pipelines and one residential
sewer pipeline were taken. All samples (n = 9) were immediately cooled
with ice before transport. For each sample, 100 mL was centrifuged at
10,000 g for 8 min at 4 °C (Eppendorf, Hamburg, Germany) in the lab-
atory. Supernatants were then removed, while the pellets were
resuspended in 20% of glycerol (Sigma-Aldrich, St. Louis, MO, USA) to
reach a final volume of 10 mL. After homogenizing for 30 min, samples
were kept at ~80 °C for storage.

Before any experiments, 2 mL of each thawed sample was vortexed
with sterile metal beads for 3 min, then transferred to a 2 mL tube
(Eppendorf) and spun down shortly to settle large debris. The super-
natant was transferred to a new Eppendorf tube and filtered using sterile
10 μm filters (Frisenette Aps, Knebel, Denmark). Afterwards, the sample
buffer was changed to 0.9% of NaCl (Sigma-Aldrich).

2.2. Exogenous plasmid isolation

A modified exogenous plasmid isolation protocol was applied in the
present study using fluorescence-activated cell sorting (FACS). The
hospital and residential sewer samples were used as the donors in the
exogenous plasmid isolation experiment. A chromosomally lacIq-Pipp-
mCherry-Kan2 tagged Escherichia coli CSH26 strain (intrinsically resis-
tant to rifampicin and nalidixic acid) was used as the recipient. The
recipient strain was thus resistant to antibiotics kanamycin (Kan),
rifampicin (Rif) and nalidixic acid (Nal), while sensitive to ampicillin
(Amp).

The live cell numbers of the donors and recipients were detected to
calculate the live cells ratios in the recipient and donor cultures,
respectively. Briefly, cell cultures were diluted in 1xPBS (Sigma-Aldrich)
to obtain ~ 3000 events/sec when loaded on the FACS Aria III instru-
ment (BD, Franklin Lakes, NJ, USA) at the flow rate of 1, each sample
was recorded for 60 s and accordingly, cell numbers were counted. The
number of live bacterial cells in the recipient overnight cultures was
detected in the red fluorescence channel. The live donor cell numbers were determined by using a FilmTrace™ LIVE/DEAD™ Biofilm Viability kit (Invitrogen, Waltham, MA, USA) for the pretreated hospital and residential sewage samples (controls were applied). Conjugation assay was performed by adding live donor and live recipient cells at a ratio of 1:1 and a density of ca. 30,000 bacteria/mm² onto an MCE 0.22 µm membrane (Fisher Scientific, Pittsburgh, PA, USA) (Olesen et al., 2022). This step ensured an equal number of 3.0 × 10⁷ donor and recipient cells on the membrane. The membrane was on a synthetic sewage agar plate (Table S1), which was supplemented with 50 µg/mL of cycloheximide to inhibit fungal growth. All plates were incubated at 25 °C for 48 h. Negative controls were performed for all samples by adding only donor or only recipient onto the membranes at a density of 6.0 × 10⁷ bacteria per membrane. After incubation, the membrane was transferred to a 15 mL Falcon tube (Corning, New York, NY, USA) that contains 2 mL of 1xPBS (Sigma-Aldrich). The tubes were then vortexed for elution of cells for 5 min. Cell eluent was stored at 4 °C for 24 h to increase the fluorescent intensity (Pinilla-Redondo et al., 2018). Afterwards, the recipient and transconjugant cells were sorted out based on their red fluorescence, while donor cells were excluded. Sorted cells were serial diluted 10⁻¹ to 10⁻⁷ times. 100 µL of the diluted cells were plated on selective plates: recipients and transconjugants were selected on Rif/Nal Luria Broth (LB) agar plates, and transconjugants were solely selected on Amp/Rif/Nal LB agar plates. Three replicates and one control were

Fig. 1. Workflow of this study. (1) E. coli CSH26::mCherry was the Escherichia coli CSH26::lacIq-Plpp-mCherry-KanR strain. (2) During the screening (primarily using ampicillin) of the transconjugants obtained from the mCherry channel of FACS, several ampicillin-resistant transconjugants were discovered as shown on the Sierra blue plate. (3) ‘Transconjugant(s) with multiple antibiotic resistance’ means transconjugants showed multiple antibiotic resistance in the AST (antibiotic susceptibility testing). One transconjugant (DK_DARWIN) appeared to be one candidate. (4) CLSM: confocal laser scanning microscopy. (5) E. coli MG1655::mCherry//pDK_DARWIN::pA1O4O3-gfpmut3 was the Escherichia coli MG1655::lacIq-Plpp-mcherry-KanR//pDK_DARWIN::pA1O4O3-gfpmut3 strain.
applied. All plates were incubated at 37°C for 24 h. Afterwards, CFUs were counted, and the transfer ratios were calculated by the equation shown below.

\[
\text{Transfer ratio} = \frac{\text{Transconjugant CFUs}}{\text{Recipient CFUs}} \times 100
\]

Transconjugant colonies were then re-plated and finally harvested from the Amp/Rif/Nal LB agar plates for three times. Stocks of the verified transconjugants were prepared using 15% of glycerol (Sigma-Aldrich) and stored at ~80°C.

2.3. Antibiotic susceptibility testing

Antibiotic susceptibility testing (AST) was performed according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) broth microdilution method for aminoglycosides (kanamycin, gentamicin, streptomycin), beta-lactams (cephalexin, penicillin, ampicillin, cefotaxime, carbenicillin), chloramphenicol, colistin, fluoroquinolones (ciprofloxacin, nalidixic acid, olaquindox), macrolides (erythromycin, tylosin), rifampicin, sulfamethoxazole, tetracycline, thiothreon, trimethoprim, vancomycin (EUCAST, 2020). Meanwhile, the EUCAST disk diffusion method was applied for the rapid test of beta-lactams including ampicillin, cefotaxime, imipenem and meropenem (EUCAST, 2020).

2.4. Plasmid sequencing and data analysis

Fresh transconjugants grown on Amp/Rif/Nal LB agar plates were cultured in Amp/Rif/Nal LB broth overnight. 2 mL of the overnight culture was used for plasmid DNA isolation. A Plasmid Mini AX kit (A&A Biotechnology, Poland) was employed. Thereafter, a digestion with Plasmid-Safe DNase (Lucigen, Madison, WI, USA) was performed to purify the extracted plasmid DNA. After DNA quantity and quality check, sequencing was carried out on a MinION device (Oxford Nanopore Technologies, Oxford, United Kingdom) and a MiSeq machine (illumina, San Diego, CA, USA) with Rapid Barcoding Kit SQK-RBB004 (Oxford Nanopore Technologies) and MiSeq Reagent Kit v3 (Illumina), respectively.

Raw plasmid reads signals from the MinION sequencer were primarily sorted by barcode using deepBinner 2.0.2 (Wick et al., 2018) and base-called using Albacore 2.2.1 (https://nanoporetech.com/), and then were assembled using Unicycler (miniasm + Racon polishing) (Wick et al., 2017). Errors were corrected by Illumina MiSeq short reads polish. Plasmid circular contigs were visually inspected and extracted from the de novo De Bruijn assembly graphs using Bandage 0.81 software (Wick et al., 2015). Circular sequences were primarily submitted for annotation on the RAST server with RASTtk scheme v2.0 (Overbeek et al., 2014). Afterwards, more features of the open reading frames were added by searching the sequences against databases of PlasmidFinder 2.1 (Carattoli et al., 2014; Clausen et al., 2018), RGI 5.1.1/CARD 3.1.0 (McArthur et al., 2013), ResFinder 4.0 (Bortolaia et al., 2020), VirulenceFinder 2.0 (Malberg Tetzchner et al., 2020), and ortFinder 1.0 (Li et al., 2018) to identify plasmid replicon, antibiotic resistance, virulence factors and mobility regions on each plasmid. The unannotated open reading frames (typically known as hypothetical protein CDS after RAST annotations) were translated into protein sequences and blasted against Pfam-A v33.1 databases were applied when no results from blastp were found. HHpred tools were labelled beginning with “Putative”. All detections were run in tools’ built-in default parameters.

Plasmid maps were created and visualized by Geneious Prime 2023.2.1 (Biomatters, Auckland, New Zealand). Similar plasmids were searched by mash dist strategy (tool version: 2.1.1) with default setting (max. p-value=0.1, max. distance=0.1) against the PLSDb database (database version: v.2021_06_23_v2) (Galata et al., 2019; Schmartz et al., 2022). One clinical and one environmental relevant plasmid were randomly picked from those similar plasmids and selected as candidates for plasmid sequence comparison analysis. The comparison analysis was done by Mauve Alignment (progressiveMauve algorithm, match seed weight = 15 minimum LCB score = 30,000, gapped aligner: MUSCLE 3.6) and Geneious Alignment (code matrix=65% similarity [5.0/–4.0]) in Geneious Prime 2023.2.1 (Biomatters).

2.5. Tagging the plasmid with a fluorescent marker

pDK_DARWIN captured from the exogenous isolated was selected for GFP tagging in the permissiveness test. The tagging for pDK_DARWIN was performed by complementing the plasmid sequence with the fragment pA1O4O3-gfpmut3 using the helper plasmid pSPIN-GFP following a previous protocol (Vo et al., 2021). In brief, pSPIN::pA1O4O3-gfpmut3 was initially constructed using the USER® Cloning Kit (New England Biolabs Inc., Ipswich, MA, USA), and the fragment gfpmut3 and plasmid pSPIN (plasmid #160730 from Addgene, Watertown, MA, USA) were assembled according to the manufacturer’s protocol. 32-nt spacer was then cloned into the constructed vector pSPIN::pA1O4O3-gfpmut3 at the Bsal restriction site following the manufacturer’s protocol (Bio-HF, New England Biolabs Inc.; T4 DNA ligase, Thermo Scientific, Waltham, MA, USA). The ligated product was transformed into NEB® 5α Competent Escherichia coli following the manufacturer’s instructions (New England Biolabs Inc.). Selection for Escherichia coli DH5α/pSPIN::pA1O4O3-gfpmut3-space was done overnight at 30°C on LB agar medium supplemented with Kan (50 mg/L). Colonies were then harvested and screened by colony PCR using the pFB50 and pFB51 primers. Details for the strains and primers were documented in Table S2 and Table S3, respectively.

100–150 ng of the plasmid pDK_DARWIN was transformed by electroporation (1.8 KV for ~5 ms) into the competent Escherichia coli DH5α/pSPIN::pA1O4O3-gfpmut3-space. Transformed cells were cultured for 30 h at 30°C in LB agar medium containing cefotaxime (2 mg/L) and Kan (50 mg/L). Plasmid pSPIN::pA1O4O3-gfpmut3-space was then removed and verified by colony PCR with primers pFB50 and pFB51. Afterwards, the inserted fragment on pDK_DARWIN::pA1O4O3-gfpmut3 was amplified using primers Sanger_seq_Primer-F and Sanger_seq_Primer-R (insertion site was indicated on the plasmid map, Fig. 2A). The specific PCR products were purified and confirmed by Sanger sequencing (Eurofins Genomics, Ebersberg, Germany). Candidates with correct insertion of the pA1O4O3-gfpmut3 fragment were transferred to Electrocomp™ GeneHogs Escherichia coli (Invitrogen) with electroporation (1.8 KV for ca. 5 ms) and stored at ~80°C. Subsequently, pDK_DARWIN::pA1O4O3-gfpmut3 was transformed to a plasmid-free Escherichia coli MG1655::lacIq-PpPl-mCherry-Kan® (Klumper et al., 2015) by a conjugation assay. Potential transformants were characterized and sorted out by FACS (BD) at the mCherry channel and plated on Amp LB agar medium for 24 h at 37°C. Colonies on the plate were checked for fluorescence by a confocal laser scanning microscope Zeiss LSM 800 (Carl Zeiss AG, Oberkochen, Germany). Transformants with correct fluorescence phenotype were replated and checked on the confocal laser scanning microscopy (Fig. 5). Accordingly, the Escherichia coli MG1655::lacIq-PpPl-mCherry-Kan®/pDK_DARWIN::pA1O4O3-gfpmut3 strain was constructed.

2.6. Permissiveness test of the captured IncN plasmid

The Escherichia coli MG1655::lacIq-PpPl-mCherry-Kan®/pDK_DARWIN::pA1O4O3-gfpmut3 strain was applied as the donor strain in the permissiveness test. Hospital and residential sewer samples, mixed sewer samples, and wastewater samples were taken from the same urban water treatment process (BTP) of a WWTP were used as recipient communities. All these sewer and wastewater samples were taken from the same urban water
system as aforementioned in the "Exogenous plasmid isolation". The related sampling campaign was conducted in 2018 and has been documented for microbial and beta-lactam ARG profiles together with two other countries’ comparable urban water systems in a previous study (Li et al., 2021).

The live donor cells and WWTP recipient community were mixed at 1:1 cell ratio and placed onto an MCE 0.22 µm sterilized membrane (Fisher Scientific) in a LB medium agar plate and an OECD medium agar plate (Table S1) as the same procedures in the conjugation assay of "Exogenous plasmid isolation". Three replicates and quality controls were applied. After 24 h of incubation at 30 °C and 48 h of GFP maturation at 4 °C, transfer events were detected by a BD FACS machine and transfer ratio was documented as the ratio of transconjugants (only green) to the original WWTP recipient (nonfluorescent) cell number. Sorting was performed in the Single Cell Precision mode targeting to sort ca. 10,500 cells as suggested by Olesen et al. (Olesen et al., 2022) with parameters set as shown in Table S4. A counting step for 10 to 20 min was performed to record events in different gates and calculate the transfer ratio for each sample. All data were stored and analyzed in the BD FACSDiva software version 8.0.3. The detailed permissiveness test method refers to a previous paper (Olesen et al., 2022).

2.7. Abundance of the IncN plasmid pDK_DARWIN in the pan-European urban water systems

Previously, we have performed two sampling campaigns (summer and winter, 2018) at different locations in three urban water systems from three European cities of similar size (Odense, Denmark; Santiago de Compostela, Spain; Durham, United Kingdom) (Li et al., 2021). Samples from the hospital and residential sewers, the end of sewer (i.e., mixed sewer, WWTP inlet) and BTP in the WWTP (n = 78 samples; 3 replicates per location in two seasons) were sequenced using Illumina NovaSeq device (Illumina, San Diego, CA, USA). The detailed metagenomic methodology was documented in Text S1. A detection threshold of >50% of pDK_DARWIN sequence coverage by mapped reads was set to determine if the plasmid is detected in the sample and calculate relative abundance. Relative abundance was calculated by dividing the number of reads mapping to pDK_DARWIN in each sample by the total number of reads in that sample.
3. Results

3.1. Isolation of antibiotic-resistance plasmids from wastewater

Through the exogenous plasmid isolation, we successfully captured several highly transmissible plasmids [transfer ratio (transconjugant CFUs/recipient CFUs): 3.34% ± 1.15%] encoding ampicillin resistance from the sewer communities. These isolated plasmids were constructed by Clasen and thoroughly described in her study (Clasen, 2018). Then we did antibiotic susceptibility testing, and we chose to focus on a functional conjugative gene and multiple ARGs (Fig. 2A). Specifically, these ARGs target antibiotic classes of aminoglycosides (gentamicin, streptomycin), beta-lactams (ampicillin, cefotaxime, carbenicillin), fluoroquinolone (ciprofloxacin), sulfonamides and its combinations (sulfamethoxazole and trimethoprim) as indicated in Table 1.

The BMDT and DDT testing approaches were applied according to EUCAST recommendations (version 10.0) (EUCAST, 2020). The combined results of the two testing methods were shown in the table. Contradictory results were found using the two testing methods for measuring the susceptibility of MEM and CIP for the transconjugant and recipient, respectively. Therefore, both results (MEM and CIP) were not considered acquired resistance.

Next, by plasmid sequencing, we identified the captured plasmid. pDK_DARWIN is a 73,132 bp IncN plasmid carrying a complete set of functional conjugative genes and multiple ARGs (Fig. 2A). Specifically, these ARGs target antibiotic classes of aminoglycosides (aac(3)-Ia, aph (3′)–lb, aph(6)-Id), beta-lactams (bla<sub>CTX-M-15</sub>, bla<sub>OXA-1</sub>, bla<sub>TEM-1B</sub>), chloramphenicol (catB3), fluoroquinolones (aac(6′)-lb-cr, qnrB1), sulfonamides (sul2), trimethoprim (dfrA14) and tunicamycin (mtrB). Meanwhile, we observed antirestriction protein ArdA, Class 1 integron integrase IntI1, diverse transposases and integrases, and miscellaneous putative functional genes on this plasmid.

3.2. Plasmid similarity search and analysis

Sequence similarity searches for pDK_DARWIN were performed against the curated plasmid sequence repositories (PLSDB) (Galata et al., 2019; Schmartz et al., 2022). This resulted in 1169 hits to previously isolated plasmids (derived from the associated BioSample) from diverse sites around the world, particularly in East Asia, Europe and North America. Notably, these plasmids are predominantly documented in the last five years (Figs. 2B and 2C) and harbour by members of Enterobacteriaceae (ca. 97%, 1130 counts out of the 1169 hits), particularly from the Escherichia and Klebsiella genera (ca. 79%), Fig. 2D. The median length of pDK_DARWIN’s similar plasmids is 89 Kbp (Fig. 2E), and the median GC content is 52% (Fig. 2F). Detailed results of the similar plasmids were recorded in Table S5.

A plasmid comparison analysis was performed to detect whether this plasmid shared some common regions with other plasmids and possible evolutionary relations. One clinically and one environmentally relevant plasmid were selected from the PLSDB similarity search result. The results showed that pDK_DARWIN shared the genetic regions encoding conjugation module (relaxase, T4CP, T4SS), ARGs (dfrA14, bla<sub>CTX-M-15</sub>) and MGEs (IntI1, IS1380), stability (StdA, StdB and StdC) and anti-restriction proteins with other IncN plasmids (Fig. 3A). Alignment of these three plasmids showed 67.1% pairwise identity and 58.8% identical sites (Fig. 3B). The main distinct area was the accessory genes region (harbouring a high content of ARGs and MGEs) where the three plasmids showed the lowest identity.

In Fig. 3A, each contiguously coloured region is a locally collinear block (LCB), a region without rearrangement of homologous backbone sequence (progressiveMauve, match seed weight: 15, minimum LCB score: 30,000). LCBs below a sequence’s centre line are in the reverse complement orientation relative to the reference sequence. Lines between plasmid sequences trace orthologous LCBs between plasmids. Whole regions within an LCB signify the presence of lineage-specific sequence at that site. Specifically, the large light blue block stands for the region of the T4SS gene cluster; the large light green block stands for the region of relaxase, T4CP, and several stability and anti-restriction proteins; the red block stands for the region of ARGs towards tetracycline resistance which pDK_DARWIN didn’t carry. In Fig. 3B, in the identity bar, the green-coloured region stands for 100% mean pairwise identity over all pairs in the column, the khaki-coloured region stands for at least 30% and under 100% identity, and the red-coloured region stands for below 30% identity.

3.3. Retransfer of pDK_DARWIN to the wastewater communities

Given that pDK_DARWIN was derived from the hospital sewer, we tested its retransfer potential to the wastewater environment by performing permissiveness tests in different communities from the hospital, residential and mixed sewers, and in the BTP of the receiving WWTP. We observed transfer events of pDK_DARWIN, displayed as green events in the GFP channel (Fig. S2). Transfer ratios were not significantly different (Tukey’s and Wilcoxon’s tests) among the different recipient communities using LB medium, yet significantly different (p < 0.05) between hospital sewer and residential sewer, hospital sewer and mixed sewer, hospital sewer and BTP, residential sewer and mixed sewer, as well as residential sewer and BTP using OECD medium (Turkey’s test) as shown in Fig. 4. In general, we showed that pDK_DARWIN can have high transfer efficiencies (mean transfer ratio: 0.50% ± 0.28% on LB medium and 0.11% ± 0.08% on OECD medium) into the sewer and wastewater samples communities.

Table 1

<table>
<thead>
<tr>
<th>Recipient (Plasmid-free)</th>
<th>KAN</th>
<th>GEM</th>
<th>STR</th>
<th>LEX</th>
<th>PEN</th>
<th>AMP</th>
<th>CTX</th>
<th>CAR</th>
<th>IMP</th>
<th>MEM</th>
<th>CIP</th>
<th>OLA</th>
<th>VAN</th>
<th>COL</th>
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<th>THY</th>
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<th>Transconjugant (Plasmid-harboured)</th>
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<td>BMDT: AST using broth microdilution assay according to EUCAST recommendations (version 10.0)</td>
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<td>DDT: AST using disk diffusion testing method according to EUCAST recommendations (version 10.0)</td>
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* KAN = kanamycin, GEM = gentamicin, STR = streptomycin, LEX = cephalaxin, PEN = penicillin, AMP = ampicillin, CTX = cefotaxime, CAR = carbenicillin, IMP = imipenem, MEM = meropenem, CIP = ciprofloxacin, NAL = nalidixic acid, OLA = olaquindox, VAN = vancomycin, COL = colistin, ERY = erythromycin, TYL = tylosin, THI = thiostrepton, CHL = chloramphenicol, RIF = rifampicin, SMX = sulfamethoxazole, TMP = trimethoprim, TET = tetracycline. BMDT = broth microdilution testing method, DDT = disk diffusion testing method. Resis. = resistant, Sens. = sensitive. Recipient = Escherichia coli CSH26::lacIq-Pipp-mCherry-KanR, Transconjugant = DK_DARWIN (i.e., Escherichia coli CSH26::lacIq-Pipp-mCherry-KanR//pDK_DARWIN).
3.4. Prevalence and abundance of pDK_DARWIN in the sewers and wastewaters

The abundance of pDK_DARWIN was measured using short-reads mapping from shotgun metagenomic datasets from 4 locations in 3 European countries (manuscript under preparation). In all three countries (Denmark, Spain, and the United Kingdom), pDK_DARWIN mapped with the highest relative abundance in hospital sewers, indicating this IncN plasmid was more prevalent in hospital sewers than in any other compartments across all countries (Fig. 5). Significant differences in abundance were observed in Spain and the United Kingdom between hospital and residential sewers samples, and between hospital and end of sewer (i.e., mixed sewer) in Spain. In Denmark, the plasmid was not detected in the BTP of the WWTP, while it was detected in one sample out of 6 both in Spanish and British samples.

4. Discussion

4.1. IncN plasmids encode MDR

IncN plasmids can be multilocus sequence typed by the repA, traJ and korA loci (García-Fernández et al., 2011). They are frequently typed in clinical samples and analysed due to harbouring and disseminating sulphonamide, quinolone, aminoglycoside, tetracycline and streptomycin resistance genes (Rozwandowicz et al., 2018). IncN plasmids are among the most frequently detected ESBL gene carriers (IncFII, IncA/C, IncL/M, IncN and IncI1) in Enterobacteriaceae (Branger et al., 2018; Carattoli, 2011), which are responsible for the spread of blaCTX-M variants (Cantón et al., 2012; Dolejska et al., 2013; Kayama et al., 2015), blaKPC, blaVIM (Carattoli et al., 2010; Chen et al., 2014), and blaNDM-1 (An et al., 2016; Poirel et al., 2011). From a phylogenetic perspective, IncN plasmids are broad-host-range and conjugative plasmids (Rozwandowicz et al., 2018), and they have been frequently detected in various human and food-animal samples (An et al., 2016; Cottell et al., 2013; Dolejska et al., 2013; Kim et al., 2013; Yamagishi et al., 2020; Zurfluh et al., 2014). These indications are alarming since beta-lactam antibiotics are currently the most widely used antibiotics in the hospital settings around the world (Bush and Bradford, 2019), the IncN plasmids are prone to facilitate the dissemination of beta-lactam resistance from nosocomial-relevant systems to adjacent or remote environmental settings via possible pathways such as the urban wastewater network (Coertze and Bezuidenhout, 2019; Pruden, 2014).

Here, we show that the IncN plasmid pDK_DARWIN captured in the hospital sewer promoted MDR when transferred to a previously sensitive host. The plasmid sequence evidenced genes known to confer the observed resistance phenotypes except catB3 (Fig. 2A, Table 1). Plasmids evolve over time and offer the host beneficial traits to be stably maintained and represent a HGT toolbox for exchanging ARGs among bacteria of different origins and sources. Two main antibiotic resistance genetic regions were discovered on pDK_DARWIN: one contained [aac (6’)-Ib-cr, blaCTX-M, aac(3’)-Ila and tmr], the other with [aph(6)-Ld, aph (3’)-Lb and sul2]. A sequence query view for the top 100 blastn results of

![Fig. 3. Plasmid sequence alignment of pDK_DARWIN with its similar plasmids. (A) Mauve alignment of pDK_DARWIN and its similar plasmids. (B) Geneious Alignment of pDK_DARWIN and its similar plasmids. ‘AP019402’ stands for the 51 Kbp IncN plasmid isolated from a patient with accession no. AP019402, and ‘CP056317’ stands for the 56 Kbp IncN plasmid isolated from freshwater downstream of a WWTP with accession no. CP056317.](#)
ARGs for horizontal transmission as shown in this study. However, indicates the IncN backbones do not necessarily or often carry the set of main ARG region we described above for once (Table S6 could hit both left and right regions, but exclusively cover the middle putative functional genes. Interestingly, we found one single sequence ARGs (identified that one ESBL plasmid with an IncN replicon carried multiplelates showed conjugation ability and MDR (Li et al., 2019). Further, they waters of a Chinese WWTP and found that 70.0% (35/50) of these iso groups of highly infective plasmids.

and gene rearrangement, we assumed pDK_DARWIN is among those considering the constantly evolving accessory region (middle hit region). This region mainly contains relaxase, T4CP, multiple stability and tella diverse and larger number of hosts, incorporating T4SS gene clusters and groups of plasmid maintenance and stability pDK_DARWIN indicated that these two resistance genetic blocks might be horizontally acquired from plasmids associated with the genera Citrobacter, Enterobacter, Escherichia, Klebsiella and Salmonella (Fig. S3, Table S6). Meanwhile, we observed the left hit region including Citrobacter, Enterobacter, Escherichia, Klebsiella, Klyvera, Morganella, Pantoaea, Salmonella and Yersinia sourced plasmid sequences overlapped the T4SS gene clusters and groups of plasmid maintenance and stability genes with pDK_DARWIN. The right hit region exhibited an even more diverse and larger number of hosts, incorporating Leclercia and Raoultella. This region mainly contains relaxase, T4CP, multiple stability and putative functional genes. Interestingly, we found one single sequence could hit both left and right regions, but exclusively cover the middle main ARG region we described above for once (Table S6, Fig. S4). This indicates the IncN backbones do not necessarily or often carry the set of ARGs for horizontal transmission as shown in this study. However, considering the constantly evolving accessory region (middle hit region) and gene rearrangement, we assumed pDK_DARWIN is among those groups of highly infective plasmids.

Li et al. isolated ESBLs producing Escherichia coli strains in wastewaters of a Chinese WWTP and found that 70.0% (35/50) of these isolates showed conjugation ability and MDR (Li et al., 2019). Further, they identified that one ESBL plasmid with an IncN replicon carried multiple ARGs (blaCTX.M-15, blatem, qnrS1) (Li et al., 2019). Genes rmb and blatem encoded on IncN plasmids could be commonly captured from pig farms, farmworkers, and the adjacent soil environment (Moodley and Guardabassi, 2009; Yao et al., 2011). Various ESBL genes and qnrS could be found on IncN plasmids harboured by Escherichia coli isolates from waterbirds (Literak et al., 2010). All these investigations imply that IncN-facilitated MDR has occurred and spread in diverse environments. Eikmeyer et al. reported that four IncN plasmids harbouring a common blatem gene emerged in the effluent of a German WWTP (Eikmeyer et al., 2012). Here we displayed that the MDR IncN plasmid pDK_DARWIN possessed a high transfer ratio in the exogenous isolation and preserved an efficient transmission ability transferring back to the sewer and wastewater communities in this study, illustrating that IncN plasmids are potent vehicles for ARG dissemination in sewage water microbial communities. Moreover, pDK_DARWIN shared backbones with both surface water and clinical settings sourcing plasmids, which demonstrates it’s been actively involved in horizontal transmission, constantly evolving its accessory genetic context to be stably maintained in different microbial communities in different environment, arguably bridging the barrier of the ecological niches for HGT (Martínez et al., 2015; Rodríguez-Beltrán et al., 2021; Thomas and Nielsen, 2005).

4.2. Enterobacteriaceae are potential hosts of the sewer derived IncN plasmid

IncN plasmids belong to the MOBf family, size ranging from 30 to 70 Kbp, and are a group of low-copy-number, broad-host-range plasmids (Garcillan-Bartia et al., 2011), which are usually colocalized with IncF plasmids (Smolka et al., 2011). IncN plasmids can be found in most Enterobacteriaceae (Carattoli, 2009). IncN plasmids carrying ARG have already spread throughout Europe and often isolated from Escherichia coli of animal sources (Rozwandowicz et al., 2018). Previously, we have reported that the Danish sewer samples (within the sampling campaign) were dominated by Clostridiales, Bacteroidales, and Enterobacteriales, while the profile shifted to become mainly β-proteobacteriales, Chitino phagales, and Micrococcales in the BTP of the subsequent WWTP (Li et al., 2021). Accordingly, we attempted to investigate the host range (potential recipient) of this exogenously isolated urban sewer derived IncN plasmid pDK_DARWIN in the dynamic wastewater environment. We evidenced conjugation transfer by using a genetically modified
pDK_DARWIN reporter plasmid and FACS (Fig. 4 and S2C–2E). However, due to low sorting speed, we failed to harvest sufficient cells for DNA extraction and subsequent sequencing. Olesen et al. suggested ca. 3000 events/sec was ideal for FACS sorting, whereas we were far away from that number (Olesen et al., 2022). Further concentration of the mating matrix and modification for the permissiveness test protocol can be applied in order to reach a satisfying sorting speed and investigate recipients’ taxonomy by means of 16S-rRNA gene sequencing for example. Despite not identifying putative hosts of IncN pDK_DARWIN in our samples, we documented the transfer ratios of different sampling sites and transfer conditions. Transfer ratios were generally higher using LB medium than OECD medium (Fig. 4). Nonetheless, the OECD medium would probably offer a less biased window to explore those non-fast-growing and/or rare microorganisms since it has alleviated bias. Meanwhile, a lower transfer ratio was detected in hospital sewer compared to other treatment compartments. This was possibly because the hospital sewer communities had already carried other IncN plasmids, resulting in the occurrence of incompatibility with pDK_DARWIN.

Based on the PLSDB similarity search for this IncN plasmid, we retrieved 1169 plasmids from the database. These similar plasmids are dominantly hosted by Enterobacteriaceae (97%), but also can be hosted by the family of Aeromonadaceae, Erwiniaeaceae, Morganellaceae, Pseudomonadaceae, Shewanellaceae, Vibrionaceae and Yersiniaceae. Carattoli et al. showed the MDR IncN plasmid pKOX105 played a crucial role in the propagation of diverse carbapenemase genes in Klebsiella spp (Carattoli et al., 2010). Another MDR IncN plasmid pKP96 that encoded qnrA1, aac(6’)-ib-cr and bldCXM.24 could be determined from clinical Klebsiella pneumoniae isolates (Shen et al., 2008). Sequence alignment of pDK_DARWIN with clinically- and environmentally-relevant plasmids indicated the backbones of this IncN plasmid as well as the possibility of pDK_DARWIN acquiring various cargo genes from remote sources (Fig. 3B).

4.3. Transfer potentials of the IncN plasmid in the urban water systems

Carattoli, A. earlier suggested that IncN plasmids were preferably habited in the nosocomial and animal husbandry surroundings (Carattoli, 2009), whilst IncN plasmids can also emerge in the environmental settings, such as soil (Ansari et al., 2008; Malik et al., 2008), piggy manure (Binh et al., 2008), surface water (Baron et al., 2020) and wastewater (Eikmeyer et al., 2012).

In the present study, we monitored the occurrence and abundance of pDK_DARWIN in a metagenome dataset containing sewer and wastewater samples from different treatment stages of three urban water systems located in three different countries (manuscript in preparation). We found that pDK_DARWIN was detected of a higher relative abundance in hospital sewer samples than in the other treatment stages in Denmark (Fig. 5). Importantly, we showed that pDK_DARWIN can persist up to the mixed sewer (wastewater inlet), but also inside the BTPs of Spanish and British WWTPs. The higher relative abundance of reads mapping to pDK_DARWIN sequence from the British BTP sample can be explained by the aerobic process used in that plant that favours the growth of Enterobacteriaceae (identified as probable hosts of this plasmid), whereas WWTPs from Denmark and Spain use an anaerobic treatment process less suitable for the persistence of these bacteria. Considering the relatively low detection limits of bulk shotgun sequencing, confident detection of pDK_DARWIN sequence, based on reads mapping coverage, in these samples means that this plasmid is present in non-negligible amounts in these samples. Accordingly, we detected a high transfer ratio of pDK_DARWIN to the wastewater communities in the BTP in the permissiveness test (Fig. 4). This implies the transfer potential of pDK_DARWIN was still vigorous in vitro although
the fitness cost in the indigenous circumstances was overwhelming. Meanwhile, our recent plasmidome study (using the same sampling campaign as the metagenome study) revealed the presence of other mobilisable MDR-carrying plasmids in the same wastewater environment, which suggests that plasmid-borne ARGs were widespread with different plasmid replications in such environment (Yu et al., 2024). As such, investigating more transconjugants in the exogenous plasmid isolation is necessary to decipher the responsible plasmid types causing the escalating and imminent antibiotic resistance issue in the urban water systems.

It might also be interesting to search those similar plasmids of pDK-DARWIN in the metagenome dataset as compensatory evolution can occur, and those plasmids with pDK-DARWIN backbones and lowered fitness costs may replicate and maintain themselves in new hosts expanding their host range in the wastewaters. However, the plasmid fitness impact cannot be inferred from results obtained with other host-plasmid combinations, even if these are closely related (Humphrey et al., 2012). Further studies on the evolutionary potential of this IncN plasmid for the purpose of deciphering its adaptive trajectories can be important considering its harboured MDR and high transmission capability in HGT.

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CRediT authorship contribution statement

Madsen Jonas Stenlukke: Conceptualization, Data curation, Methodology, Supervision, Validation, Writing – review & editing. Pinilla-Redondo Rafael: Conceptualization, Formal analysis, Methodology, Writing – review & editing. Sørensen Soren Johannes: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing. Wang Qinqin: Conceptualization, Methodology, Validation, Writing – review & editing. Nesme Joseph: Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Supervision, Validation, Visualization, Writing – review & editing. Yu Zhufeng: Validation, Visualization, Writing – original draft, Writing – review & editing, Conceptualization, Data curation, Formal analysis, Investigation, Methodology. Olesen Asmus Kalckar: Conceptualization, Data curation, Formal analysis, Methodology, Resources, Visualization, Writing – review & editing. Ananbeh Hanadi: Formal analysis, Writing – review & editing. Claesen Kamille Anna Dam: Conceptualization, Formal analysis, Methodology, Resources, Writing – review & editing. Smets Barth: Conceptualization, Funding acquisition, Project administration, Writing – review & editing. Dechesne Arnaud: Conceptualization, Methodology, Project administration, Resources, Writing – review & editing. Yang Nan: Formal analysis, Methodology, Writing – review & editing. Gong Zhuang: Formal analysis, Methodology, Writing – review & editing.

Declaration of Competing Interest

Hereby, the authors declare no other competing financial interests for the manuscript.

Data Availability

Data will be made available on request.

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Transparency declarations

None to declare.

Strain accessibility

Strains of Escherichia coli CSH26::lacIq-Plpp-mCherry-KanR strain (SP1554), Escherichia coli CSH26::lacIq-Plpp-mCherry-KanR/pDK-DARWIN strain (SP1555) and Escherichia coli MG1655::lacIq-Plpp-mcherry-KanR/pDK-DARWIN::pA10403-gfpmut3 (SP1557) are available upon request.

Supplementary data

Supporting information for Tables S1 to S4 and Figs. S1 to S4 are available in Appendix A. Table S5 and Table S6 are in Appendix B and Appendix C, respectively.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2024.115971.

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EcoToxicon and Environmental Safety 271 (2024) 115971

Extended-Spectrum β-Lactamase and carbapenemase genes are substantially and sequentially reduced during conveyance and treatment of urban wastewater. Environ. Sci. Technol. 55 (9), 5905-5913.


