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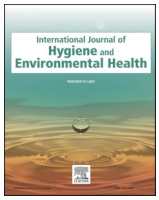

Seawater from Bergen harbor is a reservoir of conjugative multidrug-resistance plasmids carrying genes for virulence

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ABSTRACT

Aquatic environments play important roles in the dissemination of clinically-relevant antibiotic resistance genes (ARGs) and pathogens. Limited knowledge exists about the prevalence of clinically-relevant acquired resistance genes in the marine environment, especially in Norway. The aim of the current study was to investigate the presence of and characterize self-transmissible resistance plasmids from Bergen harbor seawater, with exogenous-plasmid capture, using a green fluorescent protein (GFP)-tagged *Escherichia coli* strain as a recipient. We obtained transconjugants resistant against ampicillin and cefotaxime from four of the 13 samples processed. Nine transconjugants, selected on the basis of antibiotic sensitivity patterns, were sequenced, using Illumina MiSeq and Oxford Nanopore MinION platforms. Ten different plasmids (ranging from 35 kb to 136 kb) belonging to incompatibility groups IncFII/IncFIB/Col156, IncFII, IncI1 and IncB/O/K/Z were detected among these transconjugants. Plasmid p1A1 (IncFII/IncFIB/Col156, 135.7 kb) carried resistance genes bla_{TEM-1}, *dfrA17, sul1*, *sul2*, *tet*(A), *mph(A), aadA5, aph(3″)-Ib* and *aph(6)-Id*, conferring resistance against six different classes of antibiotics. Plasmid p1A4 carried $bla_{CTX-M-55}$, *lnu(F)*, *aadA17* and *aac(3)-IId*. Cephalosporinase bla_{CMY-2} was detected on plasmids captured from an area impacted by wastewater from a local marine aquarium. Along with ARGs, some plasmids also carried virulence factors, such as enterotoxins, adhesion factors and siderophores. Our study demonstrates the presence of clinically-important multidrug-resistance conjugative plasmids in seawater from Bergen harbor, which have the potential to be transferred to human microbiota. The results highlight the need for surveillance of antibiotic resistance in the environment, as suggested by the World Health Organization, especially in low prevalence settings like Norway.

1. Introduction

Antimicrobial resistance (AMR) is an increasing public health threat, in which bacterial pathogens are becoming resistant to nearly all the antibiotic treatment options available (WHO 2021). The most critical group of pathogens, for which the World Health Organization (WHO) has indicated research and development of new diagnostic protocols and new antibiotics are urgently needed, includes *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and members of the family *Enterobacteriaceae* (WHO 2017).

The transmission and emergence of AMR is caused by certain high-risk clones within the broad microbial population and the movement of antibiotic resistance genes (ARGs), due to horizontal transfer (Peirano and Pitout 2019). Horizontal gene transfer occurs through the uptake of free DNA (transformation), the incorporation of genetic elements via phages (transduction), or the exchange of plasmids between bacteria (conjugation) (Aminov 2011). Plasmids can be divided into conjugative (self-transmissible) and non-conjugative plasmids. An intermediate category of plasmids, mobilizable plasmids, are plasmids that are non-conjugative, themselves, but can be mobilized by the presence of conjugative plasmids (Smillie et al., 2010). Conjugative plasmids play an important role in the transmission of ARGs across species (Carattoli

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2013). The ability of plasmids to capture genes through mobile genetic elements (MGEs), such as transposons or insertion sequences, and to replicate in a wide range of bacterial hosts, makes them effective vectors for the spread of AMR (Che et al., 2021). Plasmids are classified based on incompatibility (Inc), that is, the inability of the plasmids that carry similar replication and partition systems to stably propagate in the same host. Twenty-eight plasmid Inc groups have been reported in *Enterobacteriaceae* (Shintani et al., 2015). Among these, IncF, I, A/C, B/O, K, H, N and X groups are commonly associated with AMR (Rozwandowicz et al., 2018).

Environmental niches, including aquatic environments, have been acknowledged as a source of and a dissemination route for clinically important ARGs and pathogens (Bengtsson-Palme et al., 2018; Wellington et al., 2013). Clinically relevant ARGs and pathogens, as well as antimicrobial substances, can be introduced into the environment via different routes, such as wastewater (Marathe et al., 2017), waste from animal production (Zhu et al., 2013), or runoff from the land (Heuer et al., 2011). We have shown the presence of *Escherichia coli*, harboring CTX-M-type extended-spectrum β-lactamases (ESBLs) on MGEs, isolated from blue mussels (*Mytilus edulis*) collected along the Norwegian coast (Grevskott et al., 2020). In another study, we have shown that treated wastewater effluent discharge represents a potential source of *E. coli* carrying clinically important mobile ARGs, such as ESBL of the CTX-M family and carbapenemases, such as OXA-244 and VIM-1, into the receiving marine environment (Grevskott et al., 2021; Marathe et al., 2022). Bergen harbor is known to periodically be polluted with urban stormwater runoff and overflow from wastewater networks (Svanevik and Lunestad 2015). Although known, there is a lack of understanding of the risks associated with the spread of clinically relevant mobile ARGs in the marine environment. The aim of this study was to capture and characterize resistance plasmids capable of propagation in *Enterobacteriaceae* from seawater from Bergen harbor, Norway, using exogenous plasmid capture with a green fluorescent protein (GFP)-tagged *E. coli* strain. Our study demonstrates that the seawater in Bergen harbor contains multidrug-resistance virulence conjugative plasmids that can easily replicate in human-associated bacteria, thus, highlighting the risk of transmission of AMR from the marine environment back to humans.

2. Material and methods

2.1. Collection of seawater samples

Seawater samples were collected from 13 sites along the Bergen harbor, Norway, in April 2019 (Supplementary Fig. S1). From each site, 500 ml of seawater was collected in a sterile bottle and transported to the laboratory immediately after collection and kept at 2–8 °C prior to further processing. From each seawater sample, 50 ml was transferred to a sterile tube containing 50 ml 2X Mueller-Hinton (MH) broth (Oxoid, UK), with a final ampicillin (AMP) (Sigma-Aldrich, Germany) concentration of 100 μg/ml; the samples were incubated at room temperature for 24 h. In addition, 100 ml of seawater was vacuum-filtered through S-Pak filters (pore size 0.22 μm) (Millipore, USA), after which the filters were placed on the surface of m-FC agar (BD, USA) and incubated at 44 °C for 24 h before counting colonies, to estimate the number of fecal coliform bacteria, according to the Norwegian Standard NS 4792:1990.

2.2. Preparation of the bacterial donor and the recipient for conjugation

The green fluorescent protein (GFP)-marked recipient strain, *E. coli* CV601 (Heuer et al., 2002), resistant to kanamycin (KAN) and rifampicin (RIF), was pre-grown at 30 °C, shaking overnight in MH broth (Oxoid, UK) (Sigma-Aldrich, Germany). The seawater community enriched in 2X MH broth served as a donor community. Both the donor and recipient cells were centrifuged at 10,000×*g* and suspended in sterile saline to get a suspension of optical density of 0.6, using a Den-1 Densitometer (Grant Instruments, UK).

2.3. Conjugation assay

Equal aliquots (1 ml) of the prepared donors and recipients were mixed and this conjugation mixture was vacuum-filtered onto S-Pak filters (pore size 0.22 μm) (Millipore, USA), after which the filters were placed on the surface of MH agar (Oxoid, UK). The plates were incubated at 30 °C for 4 h. Conjugation was disrupted by vortexing the filters in tubes containing 10 ml of sterile phosphate buffered saline (PBS) and sterile glass beads for 1 min, in order to facilitate cell detachment from the filters. The samples were serially diluted ten-fold with PBS before plating 100 μl aliquots on CHROMagar™ MH Orientation agar (CHROMagar™, France) with KAN (50 μg/ml), RIF (50 μg/ml) and either AMP (100 μg/ml) or cefotaxime (FOT) (2 μg/ml), for selection of transconjugants. In addition, MH plates with 50 μg/ml KAN and 50 μg/ml RIF were used to estimate the number of recipients. The plates were incubated overnight at 37 °C. Transconjugants were distinguished, based on KAN and RIF resistance markers, as well as the GFP production checked under the UV light (Heuer et al., 2002). In addition, the transconjugants were identified by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) at the Institute of Marine Research (IMR) (Bruker Daltonics, Germany). Subsequently, the transconjugants were stored at −80 °C in MH broth (Oxoid, UK) with 20% glycerol until further use. A schematic diagram of the experimental set up of the study is presented in Fig. 1.

2.4. Antibiotic susceptibility testing

Obtained transconjugants were grown overnight on MH agar with 100 μg/ml AMP at 37 °C. The resistance profiles for nine transconjugants against 14 antibiotics was determined, using a broth microdilution assay with Sensititre® EUVSEC plates (Thermo Scientific, USA), following the manufacturer's protocol. Each isolate was tested for AMP, azithromycin (AZI), chloramphenicol (CHL), ciprofloxacin (CIP), colistin (COL), FOT, gentamicin (GEN), meropenem (MERO), nalidixic acid (NAL), sulfamethoxazole (SMX), ceftazidime (TAZ), tetracycline (TET), tigecycline (TGC), and trimethoprim (TMP). The plates were incubated at 37 °C for 20–22 h. The isolates were defined as, 'susceptible' or 'resistant', according to the EUCAST clinical breakpoints tables v.11.0 (EUCAST 2021). When defining the 'cut-off' for resistance to AZI, NAL, SMX and TET, the minimum inhibitory concentration (MIC) breakpoints were used, as described in Grevskott et al. (2021). The recipient strain was included as negative control.

2.5. DNA extraction and sequencing

Nine transconjugants were selected for plasmid sequencing. Selected transconjugants were grown overnight on MH agar with 100 μg/ml AMP or 2 μg/ml FOT (Sigma-Aldrich, Germany) at 37 °C. Plasmid DNA was extracted from the isolates, using the Plasmid Mini kit (Qiagen, Germany), following the manufacturer's protocol. Sequencing was carried out, using a combination of Illumina and Nanopore sequencing, to obtain complete plasmid sequences. For Oxford Nanopore sequencing, plasmid DNA samples were concentrated, using a SpeedVac instrument (Thermo Fisher Scientific, USA). Additionally, for one sample, high-molecular weight genomic DNA was obtained, following the protocol described by Salvà-Serra et al. (2018). Integrity of the DNA was verified, using a Genomic ScreenTape kit, with a 2200 TapeStation system (Agilent Technologies, USA). The extracted DNA was quantified, using a NanoDrop™ 2000 Spectrophotometer and a Qubit™ 2.0 Fluorometer with the dsDNA BR (Broad-Range) kit (Thermo Fisher Scientific, USA). For Illumina sequencing, libraries were prepared, using Nextera DNA Flex Library Prep kit (Illumina, USA), following the manufacturer's instructions. Sequencing was performed, using the Illumina MiSeq platform (Illumina, USA), with 2 × 300 bp chemistry, at the Norwegian Sequencing Centre (Ullevål University Hospital, Oslo, Norway). For Oxford Nanopore sequencing, libraries were prepared, using the Rapid

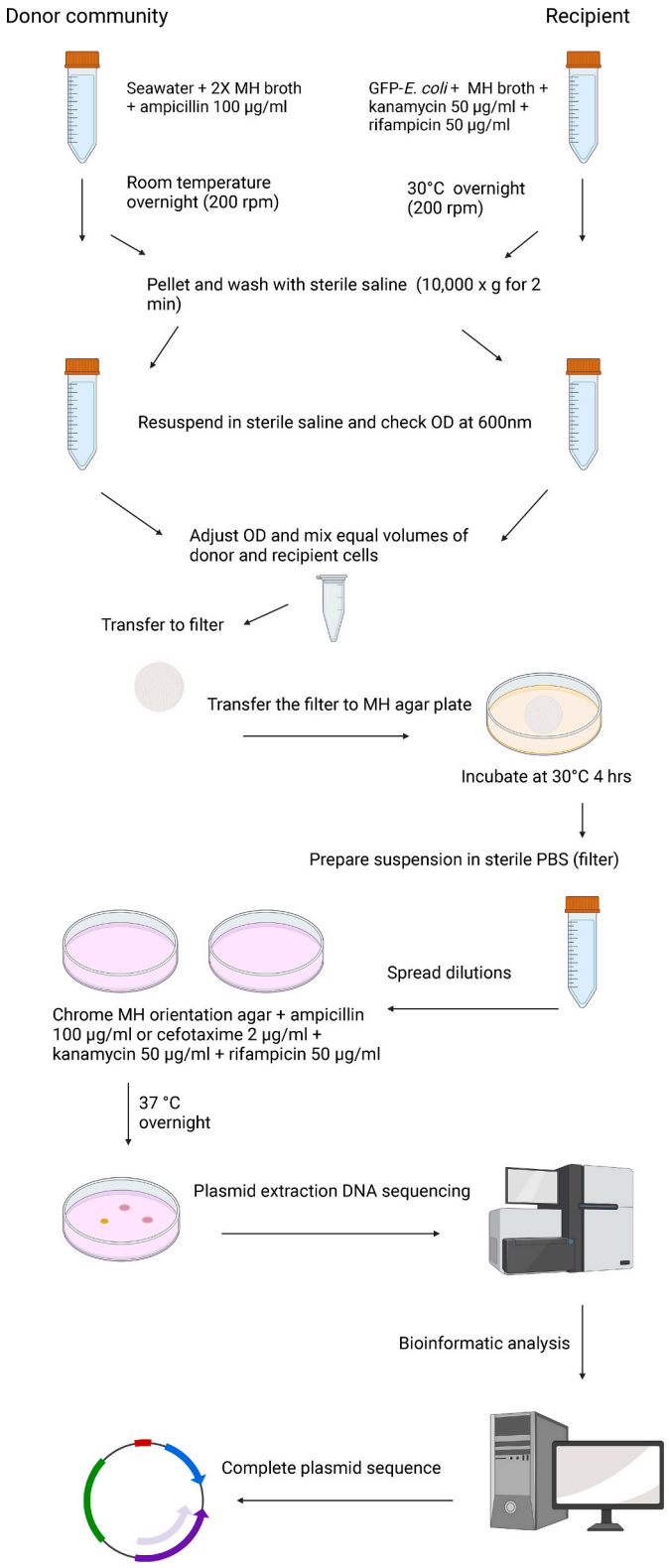

Fig. 1. Schematic diagram of the method used for plasmid capture experiments. The figure was created with BioRender.com.

Barcoding kit (SQK-RBK004). Sequencing was performed, using a MinION sequencer (Oxford Nanopore Technologies, UK) and a FLO-MIN106 v.R9.4, with the 72 h sequencing script of the software MinKNOWN v.3.6.5 and default parameters.

2.6. Plasmid assembly

For Illumina sequencing, the raw reads generated were quality trimmed, using Trimmomatic v.0.36 (Bolger et al., 2014) and assembled, using SPAdes v.3.13.0 (Bankevich et al., 2012). The quality of the Illumina reads was evaluated, using FastQC v.0.11.3 (Andrews 2010). For Nanopore sequencing, the raw reads generated were base-called, using Guppy v.3.4.5+. The quality of the reads was evaluated, using NanoPlot v.1.26.3 (De Coster et al., 2018). Subsequently, hybrid *de novo* assemblies of Illumina and Nanopore reads were performed, using Unicycler v.0.4.7 (Wick et al., 2017). The Oxford Nanopore reads were also assembled *de novo* and the assemblies polished, using the Long Read Support plug in of CLC Genomics Workbench v.20 (QIAGEN Aarhus A/S, Denmark). Subsequently, the Nanopore-solo assemblies were mapped with Illumina reads BWA v.0.7.17.4 (Li and Durbin 2009) and polished, using Pilon v.1.2 (Walker et al., 2014). The quality and accuracy of the assemblies was assessed, using QUAST v.5.0.2 (Gurevich et al., 2013) and the Illumina-only assemblies as reference, the on-line server DFAST v.1.2.4 (Tanizawa et al., 2017) and by mapping the Illumina reads and performing a variant detection using CLC Genomics Workbench v.20, with a minimum frequency of 70%.

Plasmids were annotated, using the Prokaryotic Genome Annotation Pipeline (PGAP) v.4.13 at the National Center for Biotechnology Information (NCBI) (Tatusova et al., 2016). Overview of the plasmids was obtained, using CGView (Grant and Stothard, 2008). Plasmid replicons were typed, using PlasmidFinder 2.0 (Camacho et al., 2009; Carattoli et al., 2014). The presence of ARGs was examined, using ResFinder 4.1 (Bortolaia et al., 2020; Camacho et al., 2009; Zankari et al., 2017) and the Comprehensive Antibiotic Resistance Database (CARD) (Alcock et al., 2020). Virulence genes were analyzed, using the VFanalyzer at the Virulence Factor Database (VFDB) (Liu et al., 2019). Biocide/metal resistance genes (BMRGs) and conjugal transfer genes were detected by searching through the GenBank files of the annotated plasmids.

3. Results and discussion

Total colony-forming units (CFUs) for fecal coliforms varied between 4 and 215 (mean 58) CFU/100 mL (Supplementary Table S1). The concentrations of fecal coliforms were higher in the inner parts of the harbor (sampling site 1–7), compared to the outer parts (sampling site 8–13), suggesting pollution of seawater in the harbor with sewage, as well as a lower degree of water circulation and less dilution of the water masses after pollution.

Using the plasmid capture method previously described (Jutkina et al., 2018), we obtained a total of nine different transconjugants resistant against AMP and FOT from four of the 13 samples processed. The conjugation frequency was between 1×10^{-6} and 4×10^{-6} transconjugants per recipient cell. The MICs for nine transconjugants are presented in Table 1. Ten unique plasmids, ranging from 34,855 bp to 135,739 bp, were detected in nine transconjugants (Table 2). Plasmids p1A1 and p1A3 are identical except for 3 additional bases in p1A3, similarly plasmid p9C1 and p9C8 are identical except for 1 additional base in p9C8. The plasmids belonged to four broad range plasmid incompatibility groups IncFII/IncFIB/Col156, IncFII, IncI1 and IncB/O/K/Z, and carried multiple ARGs and virulence factors. There was no correlation between fecal coliform counts and detection of conjugative plasmids in our study.

3.1. Plasmids carrying both virulence genes and ARGs

The plasmid p1A1 belonging to IncFII/IncFIB/Col156 (GenBank accession number: CP075626) has a size of 135,736 bp and carries several ARGs, two BMRGs and two virulence genes (Fig. 2A). All ARGs are located between positions 42,098 bp and 61,709 bp, flanked by a truncated *IntI1* integrase and a truncated IS*1* transposase. This multidrug-resistance determining (MDR) region harbors $bla_{\text{TEM-1}}$,

Table 1
Minimum inhibitory concentrations (MICs) against 14 antibiotics determined for nine transconjugants and *E. coli* strain CV601.

Isolate	Species	AMP	AZI	FOT	TAZ	MERO	NAL	CIP	TMP	SMX	TET	TGC	GEN	CHL	COL
1A1	*E. coli*	**>64**	**32**	<0.25	<0.5	<0.03	8	0.03	**>32**	**>1024**	**64**	<0.25	1	<8	<1
1A3	*E. coli*	**>64**	**32**	<0.25	<0.5	<0.03	8	0.03	**>32**	**>1024**	**64**	<0.25	<0.5	<8	<1
1A4	*E. coli*	**>64**	4	**>4**	**>8**	<0.03	8	0.03	<0.25	<8	<2	<0.25	**>32**	<8	<1
2A2	*E. coli*	**>64**	8	<0.25	<0.5	<0.03	8	0.03	<0.25	<8	<2	<0.25	<0.5	<8	<1
8A2	*E. coli*	**>64**	4	<0.25	<0.5	<0.03	8	0.03	<0.25	**>1024**	**64**	<0.25	2	**>128**	<1
8A3	*E. coli*	**>64**	4	<0.25	<0.5	<0.03	8	0.03	<0.25	**>1024**	<2	<0.25	<0.5	**128**	<1
9A3	*E. coli*	**>64**	4	<0.25	<0.5	<0.03	8	0.03	<0.25	**>1024**	<2	<0.25	2	<8	<1
9C1	*E. coli*	**>64**	4	**>4**	**>8**	<0.03	8	0.03	<0.25	<8	<2	<0.25	<0.5	<8	<1
9C8	*E. coli*	**>64**	4	**>4**	**>8**	<0.03	8	0.03	<0.25	<8	<2	<0.25	1	<8	<1
GFP	*E. coli*	4	4	<0.25	<0.25	<0.03	8	0.03	<0.25	<8	<2	<0.25	<1	<8	<1

GFP - green fluorescent protein expressing *E. coli* strain CV601-GFP (control). ampicillin (AMP), azithromycin (AZI), chloramphenicol (CHL), ciprofloxacin (CIP), colistin (COL), cefotaxime (FOT), gentamicin (GEN), meropenem (MERO), nalidixic acid (NAL), sulfamethoxazole (SMX), ceftazidime (TAZ), tetracycline (TET), tigecycline (TGC), and trimethoprim (TMP). Note: MICs above clinical breakpoints for Enterobacterales have been marked in bold (https://www.eucast.org/clinical_breakpoints/).

Table 2
Summary of the plasmids captured from seawater from Bergen harbor, Norway.

Sample site	Plasmid (GenBank accession nr.)	Size (bp)	G + C (%)	CDSs (total)	Plasmid type	Group	ARGs	Virulence genes
1	p1A1 (CP075626)[a]	135,736	51.7	165	conjugative	IncFII/IncFIB/Col156	bla_{TEM-1}, *dfrA17*, *sul1*, *sul2*, *tet*(A), *mph(A)*, *mrx*, *aadA5*, *aph(3″)-Ib*, *aph(6)-Id*	*senB*, *artA*
1	p1A3 (CP075625)[a]	135,739	51.7	165	conjugative	IncFII/IncFIB/Col156	bla_{TEM-1}, *dfrA17*, *sul1*, *sul2*, *tet*(A), *mph(A)*, *mrx*, *aadA5*, *aph(3″)-Ib*, *aph(6)-Id*	*senB*, *artA*
1	p1A3_2 (CP075624)	80,609	53.3	91	conjugative	IncB/O/K/Z	*fosA*	
1	p1A4 (CP075623)	105,608	50.2	120	conjugative	IncI1-I (α)	$bla_{CTX-M-55}$, *lnu(F)*, *aadA17*, *aac(3)-IId*	*cia*
3	p2A2 (CP075622)	76,834	51.4	96	conjugative	IncFII	bla_{TEM-1}	*artA*
11	p8A2 (CP075621)	115,456	50.4	130	conjugative	IncI1-I (α)	bla_{TEM-1}, *sul2*, *tet*(A), *floR*, *aph(3″)-Ib*, *aph(6)-Id*	
11	p8A3 (CP075620)	96,196	53.3	107	conjugative	IncB/O/K/Z	bla_{TEM-1}, *sul2*, *floR*, *aph(3″)-Ib*, *aph(6)-Id*	
13	p9A3 (CP075619)	89,841	53.2	102	conjugative	IncB/O/K/Z	bla_{TEM-1}, *sul2*, *aph(3″)-Ib*, *aph(6)-Id*	
13	p9A3_2 (CP075618)	34,855	48.9	49	mobilizable	pSL483	–	
13	p9C1 (CP075617)[b]	99,872	52.3	117	conjugative	IncB/O/K/Z	bla_{CMY-2}	
13	p9C1_2 (CP075616)	37,503	40.7	50	mobilizable	pSL483	–	
13	p9C8 (CP075615)[b]	99,873	52.3	117	conjugative	IncB/O/K/Z	bla_{CMY-2}	

ARGs: Antibiotic resistance genes, bp: base pair, G + C: GC content, CDSs: Coding sequences, [a, b] plasmids are identical.

dfrA17, sul1, sul2, tet(A), *mph(A), mrx, aadA5, aph(3″)-Ib* and *aph(6)-Id* genes, conferring resistance to penicillins, trimethoprim, sulfonamides, tetracyclines, macrolides and aminoglycosides. Although, aminoglycoside resistance genes were present on the plasmid, no resistance against gentamicin was observed. This could be attributed to the narrow spectrum of these genes, which confer resistance mainly against streptomycin and kanamycin, but not gentamicin (Ramirez and Tolmasky 2010). The plasmid also carries virulence genes like *senB* coding for enterotoxin TieB, which is an important virulence factor for uropathogenic *E. coli* (Meza-Segura et al., 2020), as well as a gene for ArtA toxin, an ADP-ribosyltransferase homologue of the pertussis toxin (Ptx) that has been identified in *Salmonella enterica* serovar Typhimurium and endometrial pathogenic *E. coli* (Lopes et al., 2020). The plasmid also carries genes conferring resistance to chromate (*chrA*) and quaternary ammonium compounds (*qacE*Δ1). Plasmid p1A1 has a DNA fragment (positions 0–60,365 bp) that shows high sequence similarity (>99.9% nucleotide identity) to a segment of approximately 60 kb of plasmid pAVS0096-a (~101 kb) from *E. coli* (CP076345) reported in Switzerland. This plasmid also has a DNA fragment (positions 64, 753–125,227 bp) that shares high identity (99.9%) to a segment of ~60 kb from plasmid p6-Pyelo_1 (120.6 kb) from *E. coli* (CP048871) reported in Belgium. The plasmid p1A1 has two competent replication initiation systems *repB* and *repA* (Fig. 2A), indicating that this plasmid is a composite plasmid (Villa et al., 2010). Plasmid p1A3 (CP075625) is identical (100%) to plasmid p1A1 (Supplementary Fig. S2), but has three extra nucleotides compared to p1A1.

Plasmids p1A1 and p1A3 encode two type II toxin-antitoxin systems (TAs), viz., PemI/K and CcdA/B. Both *ccdAB* and *pemIK* are found to be the most common TAs occurring in plasmids found in *E. coli* and *Klebsiella pnemoniae* (Wu et al., 2020). Moreover, PemI/K TAs was found to be important in adaptation to the presence of an antimicrobial biocide and is believed to play a role in persistence and biofilm formation (Bleriot et al., 2020). Type II TAs have both toxin and antitoxin as proteins; these kinds of TAs are the most common in bacteria (Zhang et al., 2020). Toxin-antitoxin systems are involved in plasmid maintenance and are commonly found on bacterial plasmids (Alonso 2021). TAs typically carry two gene loci, one encoding a stable toxin that induces cell death or growth inhibition and a second antitoxin that neutralizes the toxin. If the daughter cell does not receive a plasmid during cell division, the active toxin kills the daughter cells favoring selection of plasmid-carrying cells, thus, leading to maintenance of the plasmid (Song and Wood 2018). The presence of TAs on plasmids p1A1 and p1A3, thus, signifies their potential for persistence in the environmental microbiota.

The plasmid p1A4, belonging to IncI1 group (CP075623), has a size of 105,608 bp and carries several ARGs, as well as a virulence gene (Fig. 2B). This plasmid encodes type II TA RelE/ParE, which is important for plasmid stability, and stress response, which increases the tolerance of beta-lactams, aminoglycosides and quinolones in *E. coli* (Kamruzzaman and Iredell 2019). Genes conferring resistance to lincosamides (*lnu (F)*) and aminoglycosides (*aadA17* and *aac(3)-IId*) are located between positions 11,855 bp and 19,506 bp, flanked by a complete IS*26* transposase on either end. A $bla_{CTX-M-55}$ gene, conferring resistance to the third-generation cephalosporins (3GCs), is located between positions 6234 bp and 11,830 bp, flanked by a truncated Tn3 transposase and a complete IS*Ec9* transposase. This plasmid also carries colicin 1A (*cia*), which kills non-host *E. coli* strains (Cascales et al., 2007). The plasmid p1A4 has two DNA fragments (54,755 bp and 9030 bp) that show high sequence similarities (99.9% nucleotide identity) to segments of a plasmid pCE-R2-11-0435_92 (~92 kb) from *S. enterica* serovar

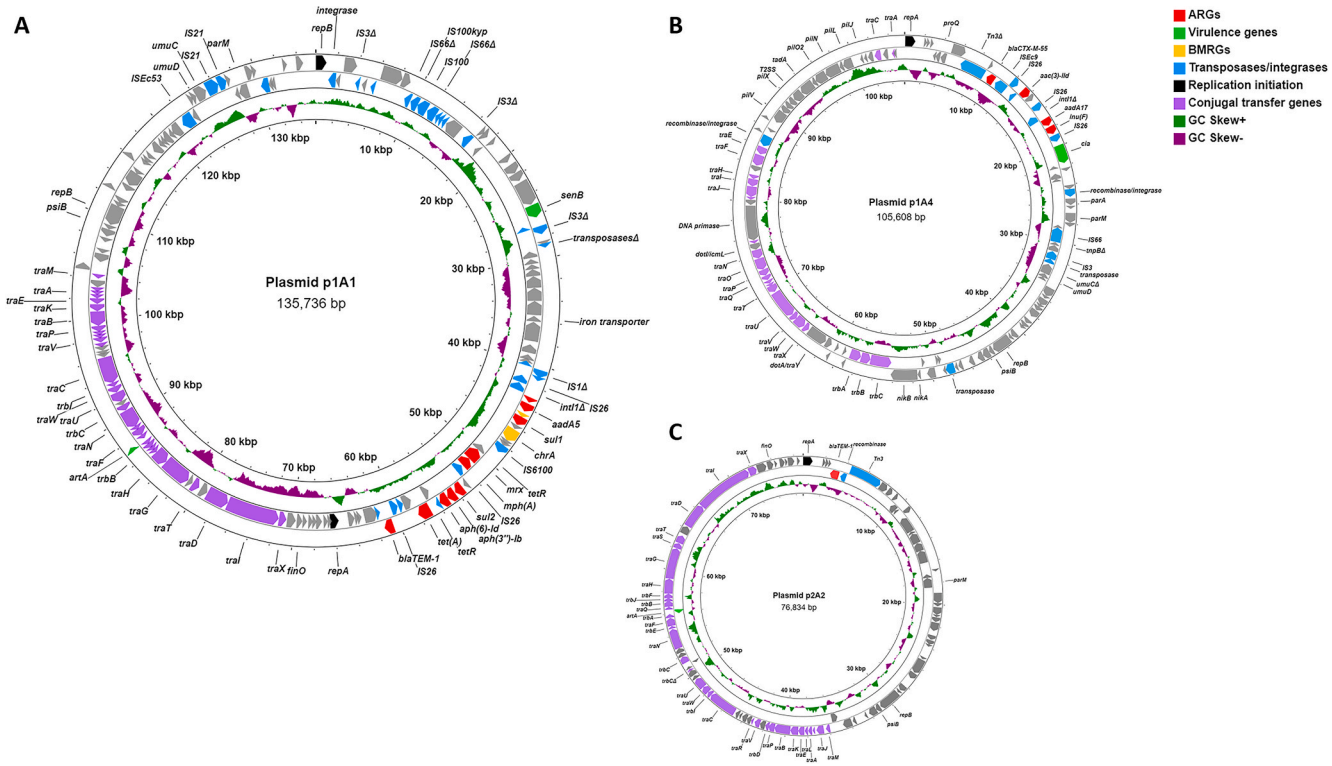

Fig. 2. Map of (A) plasmid p1A1 (GenBank accession number: CP075626), (B) plasmid p1A4 (CP075623), and (C) plasmid p2A2 (CP075622). Arrows indicate the sizes of the ORFs and their orientations. Antibiotic resistance genes (ARGs) are highlighted in red, virulence genes in green, biocide/metal resistance genes (BMRGs) in orange, transposases/integrases in blue, replication initiation in black, conjugal transfer genes in purple and other genes in gray. Δ represents truncated genes. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article)

Heidelberg (CP016520) reported from Canada. The region of the plasmid carrying *aadA17, aac(3)-IId, lnu(F)* and the flanking IS*26* transposases, detected on the plasmid p1A4, share high similarities (>99.9%) to segments of plasmids carried by *E. coli* strains reported from China (CP019261, CP059044, CP059285 and CP058950). The region of the plasmid carrying $bla_{\text{CTX-M-55}}$ and the flanking transposases is identical (100%) to segments of plasmids carried by different members of the *Enterobacteriaceae* family, including *E. coli* (AP023198), *K. pneumoniae* (CP024461), *S. enterica* serovar Agona (CP048776) and *Enterobacter hormaechei* (CP079936). In Norway, the prevalence of CTX-M-55 in the clinics (NORM/NORM-VET 2020), as well as in the local population (Grevskott et al., 2021), is low, to date. However, the detection of $bla_{\text{CTX-M-55}}$ on a conjugative plasmid captured from seawater highlights the potential for spread of this gene in the environment and, subsequently, to the clinics.

Plasmid p1A4 carries a bacteriocin (colicin 1A) and colicin immunity protein. Colicins are secreted antimicrobial proteins (bacteriocins) that are produced by *E. coli* and some other members of the family *Enterobacteriaceae* that kill susceptible related bacterial species (Šmajs and Weinstock 2001). Colicin production in *E. coli* is an important trait, with respect to competition and survival in the complex ecosystem (Samuels et al., 2020). Colicins and their immunity proteins are found on many plasmids. These represent a toxin-antitoxin couple that kills plasmid-free cells (Jeziorowski and Gordon 2007). A major difference between the colicins and other TAs is that the colicins are secreted from cells producing them and destroy plasmid-free cells in contact with the toxin. These systems also helps maintenance of the plasmids carrying them within the microbial community (Lauffenburger 1987). The presence of colicin and colicin immunity protein suggests that p1A4 originated in a member of *Enterobacteriaceae* and has a potential for maintenance and propagation in the environmental microbiota.

The plasmid p2A2, belonging to IncFII group (CP075622), is 76,834 bp and carries a $bla_{\text{TEM-1}}$ gene conferring resistance to penicillins (Fig. 2C). This plasmid also encodes an ArtA toxin, as well as a type II TA RelE/ParE. The $bla_{\text{TEM-1}}$ gene is located between positions 2645 bp and 7412 bp, flanked by a complete Tn3 transposase. This plasmid shares high similarity (99.61% nucleotide identity) to plasmid pH1519-76 (~76 kb), reported in *E. coli* (KJ484631) from Ireland. The region of the plasmid carrying $bla_{\text{TEM-1}}$ and the flanking transposase is identical (100%) to segments of plasmids carried by *E. coli* (CP039843), *S. enterica* serovar Schwarzengrund (CP048298), *A. baumannii* (MK531543), and *S. enterica* serovar Wien (MK797990).

3.2. Plasmids carrying ARGs

The plasmid p8A2 belonging to IncI1 group (CP075621) has a size of 115,456 bp and carries several ARGs (Fig. 3A). All ARGs are located between positions 28,776 bp – 42,970 bp, flanked by a complete IS*26* transposase on either end. This MDR region harbors $bla_{\text{TEM-1}}$, *tet*(A), *floR, sul2*, *aph(6)-Id* and *aph(3″)-Ib*, conferring resistance to penicillins, tetracyclines, chloramphenicol, sulfonamides and aminoglycosides. The plasmid p8A2 has three DNA fragments (54,085 bp, 17,147 bp and 8173 bp, respectively) that show high sequence similarities (>99.9% nucleotide identity) to segments of a plasmid pSNE2-1928 (~97 kb) from *S. enterica* serovar Newport (CP025239) reported from the USA. The MDR region and the flanking IS*26* transposases, detected on the plasmid p8A2, are identical (100%) to segments of plasmids carried by different members of the *Enterobacteriaceae* family, including *S. enterica* serovar Newport (CP025233) and *E. coli* (CP024140). This suggests a possibility that the MDR region on plasmid p8A2 may have been transferred between plasmids by transposition (Rubio-Cosials et al., 2018).

The plasmid p9C1 belonging to IncB/O/K/Z group (CP075617) is 99,872 bp (Fig. 3B). This plasmid carries genes conferring resistance to second-generation cephalosporins ($bla_{\text{CMY-2}}$) and quaternary ammonium compounds (*sugE*), which is located between 27,206 bp and 30,329 bp flanked by a complete IS*Ec12* transposase. The plasmid p9C1 has five

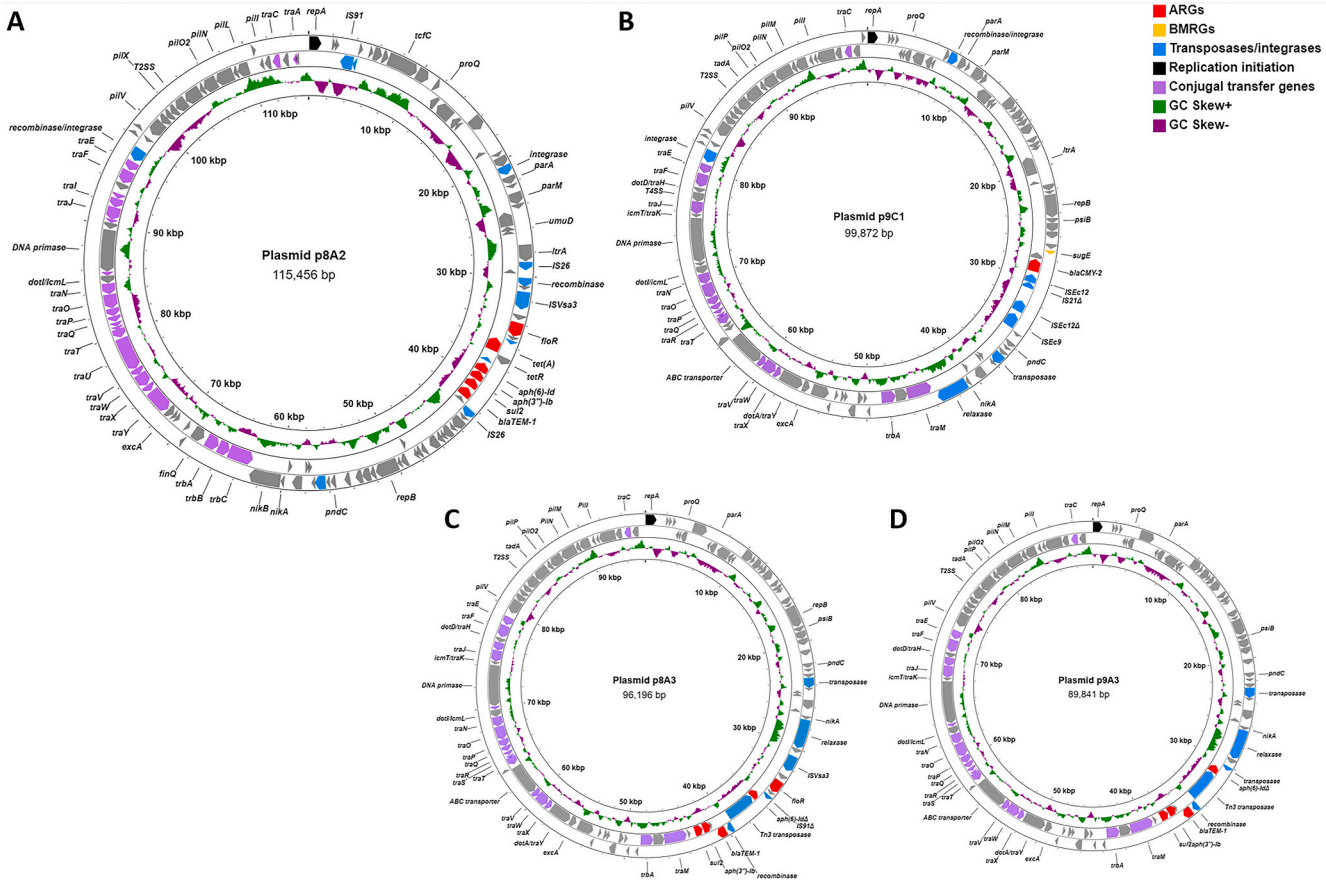

Fig. 3. Map of (A) plasmid p8A2 (GenBank accession number: CP075621), (B) plasmid p9C1 (CP075617), (C) plasmid p8A3 (CP075620), and (D) plasmid p9A3 (CP075619). Arrows indicate the sizes of the ORFs and their orientations. Antibiotic resistance genes (ARGs) are highlighted in red, biocide/metal resistance genes (BMRGs) in orange, transposases/integrases in blue, replication initiation in black, conjugal transfer genes in purple and other genes in gray. Δ represents truncated genes. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

DNA fragments (31,200 bp, 18,617 bp, 16,165 bp, 8757 bp and 6096 bp) that show high sequence similarities (>99.9% nucleotide identity) to segments of a plasmid pRHBSTW-00321_4 (~87.9 kb) from *E. coli* (CP056607) reported from the UK. The region of the plasmid carrying bla_{CMY-2}, *sugE*, and the flanking transposase are identical (100%) to segments of plasmids carried by *S. enterica* serovar Typhimurium (CP025339) and *E. coli* (KC788405). Dissemination of bla_{CMY-2} has been linked to specific plasmids that are frequently detected in *E. coli* strains from animal origin (Hong et al., 2020; Mulvey et al., 2009; Pietsch et al., 2018). Plasmid p9C1 was isolated from a site impacted by the effluent from the local aquarium. This suggests a probable animal origin of this plasmid. The conjugative plasmid p9C8 (CP075615) is identical to plasmid p9C1 (Supplementary Fig. S3).

The plasmid p8A3, belonging to IncB/O/K/Z group (CP075620), is 96,196 bp and carries several ARGs (Fig. 3C). All ARGs are located between positions 33,392 bp and 43,032 bp, flanked by a complete IS*Vsa3* transposase. The MDR region harbors bla_{TEM-1}, *sul2, floR, aph(3″)-Ib* and *aph(6)-Id* genes, conferring resistance to penicillins, sulfonamides, amphenicols and aminoglycosides. This plasmid has two DNA fragments (59,887 bp and 24,075 bp) that share high similarities (99.9% nucleotide identity) to segments of a plasmid unnamed2 (~148 kb) reported in *K. pneumoniae* (CP058363) from Poland.

The plasmid p9A3, belonging to IncB/O/K/Z group (CP075619), is 89,841 bp and carries several ARGs (Fig. 3D). All ARGs are located between positions 30,047 bp and 38,778 bp. This MDR region harbors genes conferring resistance to penicillins (bla_{TEM-1}), sulfonamides (*sul2*), and aminoglycosides (*aph(3″)-Ib* and *aph(6)-Id*). Plasmid p9A3 shares high sequence similarity (99.9% nucleotide identity) to plasmid p86 (~86 kb) reported in *E. coli* (CP023387) from the UK. The MDR region shares high sequence similarity (99.9%) to segments of plasmids carried by *E. coli* (CP041565) and *Shigella sonnei* (LR213460) reported from the USA and Australia, respectively. Plasmids p8A2, p9C1, p8A3 and p9A3 all encode type II TAs RelE/ParE.

3.3. Co-mobilized plasmids

We captured three mobilizable plasmids in strains carrying conjugative antibiotic resistance plasmids (Fig. 4). Plasmid p1A3_2, belonging to IncB/O/K/Z (GenBank accession number: CP075624), is 80,609 bp and encodes type II TA RelE/ParE. This plasmid also carries a *fosA* gene, conferring resistance to fosfomycin (Fig. 4A). The *fosA* gene is located between positions 36,407 bp and 38,332 bp, flanked by a IS*Vsa5* transposase. This plasmid shares high sequence similarity (98.2% nucleotide identity) to plasmid pSCU-116-1 (~84 kb), reported in *E. coli* (CP051720) from the USA. The region of the plasmid carrying *fosA* and the flanking transposase is identical (100%) to a segment of a plasmid pHNPK9-FOS (~54 kb) reported in *E. coli* (MT074415) from China. This fragment is also identical (100%) to a segment (1322 bp) from the chromosome of *K. pneumoniae* (CP020061) reported from the USA, suggesting this fragment to be mobile.

The plasmids p9C1_2 (GenBank accession number: CP075616) and p9A3_2 (GenBank accession number: CP075618) are 37,503 bp and 34,855 bp, respectively (Fig. 4B and C, Table 2). The plasmids carry putative virulence genes belonging to the *virB* complex of type IV secretion systems, such as *virB2-B4* and *virB9-B11*, capable of transporting toxic effectors into target bacteria resulting in cell death (Sgro

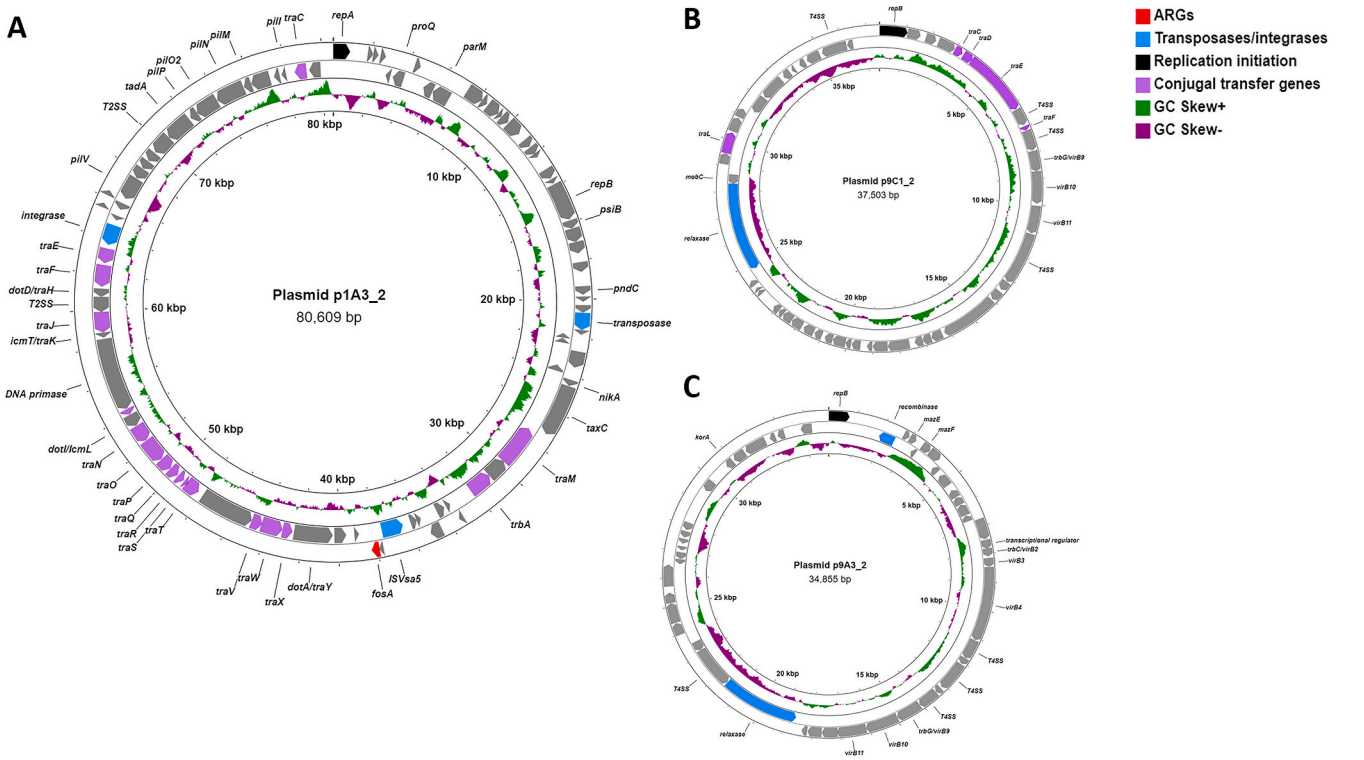

Fig. 4. Map of (A) plasmid p1A3_2 (GenBank accession number: CP075624), (B) plasmid p9C1_2 (GenBank accession number: CP075616), and plasmid p9A3_2 (GenBank accession number: CP075618). Arrows indicate the size of the ORFs and their orientation. Antibiotic resistance genes (ARGs) are highlighted in red, transposases/integrases in blue, replication initiation in black, conjugal transfer genes in purple and other genes in gray. Δ represents truncated genes. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

et al., 2019). The plasmid p9C1_2 shares high similarity (99.50% nucleotide identity) to plasmid pSL483 (~38 kb), reported in *S. enterica* serovar Agona (CP001137) from the USA. The plasmid p9A3_2 shares high sequence similarity (99.77% nucleotide identity) to plasmid pR16.0676_34k (~34 kb) reported in *S. enterica* serovar Anatum (CP029801) from Taiwan. Although the plasmids p9C1_2 and p9A3_2 are not self-transmissible by conjugation, these plasmid can mobilize in the presence of conjugative plasmids (Carattoli 2009, 2013).

We captured several plasmids, ranging in size from 35 to 136 kb. These plasmids represent both, conjugative and mobilizable plasmids, carrying antibiotic resistance and/or virulence genes. The captured plasmids belonged to several incompatibility groups, including IncFII, IncB/O/K/Z and IncI1 (Table 2). IncFII and IncI1 groups carrying ARGs and virulence factors are considered to be epidemic plasmids and are detected predominantly in *Enterobacteriaceae* from different sources and geographical origins (Carattoli 2009; Martínez and Baquero 2002). The IncF group is the most described plasmid type within the *Enterobacteriaceae* family and usually encodes several replicons, often carrying the FII replicon, together with FIA or FIB (Villa et al., 2010). Members of this Inc group are frequently associated with the spread of clinically important ARGs, particularly CTX-M-type ESBLs (Coque et al., 2008; Woodford et al., 2009). The plasmids detected in this study are, thus, of clinical relevance.

Beta-lactams such as penicillins and cephalosporins are the first choice drugs used for treatment in Norway (NORM/NORM-VET 2021). Third-generation cephalosporins (3GCs) are critically important antibiotics for treating Gram-negative pathogens (Bush 2018; Rossolini et al., 2021). Although overall resistance against 3GCs currently is low in Norway, a gradual increase in cephalosporin resistance and ESBL production in *Enterobacteriaceae* from blood cultures has been observed in clinics in Norway over the last decade (NORM/NORM-VET 2021). Our study demonstrates the presence of CTX-M-type ESBLs and cephalosporinase $bla_{\text{CMY-2}}$ on conjugative plasmids, capable of propagating in *E. coli,* in seawater contaminated with sewage. Moreover, the plasmids carried TAs, suggesting their potential for persistence in the environmental microbiota. One of the limitations of the study is that we do not have quantitative data on the plasmid abundance in the seawater. Nevertheless, the presence of MDR plasmids, that can be transferred to the members of family *Enterobacteriaceae,* in seawater represents a risk of transfer of such plasmids to the human associated bacteria and human pathogens. A previous study has shown that exposure to ESBL-carrying *E. coli* through swimming is likely, if the waters are contaminated by wastewater discharge (Schijven et al., 2015). Such resistant bacteria from seawater can also be transferred back to humans via exposure to contaminated seafood (Grevskott et al., 2017; Håkonsholm et al., 2022). Our study, thus, demonstrates that polluted seawater represents a potential dissemination route for clinically relevant ARGs and MGEs.

4. Conclusion

This is the first study characterizing conjugative plasmids carrying ARGs and virulence genes captured from seawater from Norway. Our study reveals the presence of several multidrug-resistance conjugative plasmids belonging to incompatibility groups that are prevalent among human pathogens and are usually associated with AMR. We show the presence of plasmids in seawater, carrying both ARGs and virulence genes, that are capable of propagating in human pathogens. Furthermore, we show the presence of clinically important cephalosporinases, such as $bla_{\text{CMY-2}}$ and $bla_{\text{CTX-M-55}}$, on plasmids with potential for persistence in the environmental microbiota. Our study, thus, demonstrates that seawater contaminated with sewage effluent represents a possible dissemination route for clinically important ARGs and MGEs. Our study also highlights the importance and need for surveillance of antibiotic resistance in the environment, especially in low prevalence settings like Norway.

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Data availability statement

The complete plasmid sequences are deposited in GenBank under the following accession numbers: CP075615, CP075616, CP075617, CP075618, CP075619, CP075620, CP075621, CP075622, CP075623, CP075624, CP075625 and CP075626.

Declaration of competing interest

No competing interests exist.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2022.114108.

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