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Plasmidome Analysis of a Hospital Effluent Biofilm:

Status of Antibiotic Resistance

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Abbreviations

Antibiotic Resistance Genes (ARGs)

Antibiotic Resistant Bacteria (ARB)

Next-Generation Sequencing (NGS)

Insertion Sequences (IS)

Incompatibility (Inc)

26	Extended-Spectrum Beta-Lactamase (ESBL)
27	Coding Sequence (CDS)
28	Cluster of Orthologous Groups (COG)
29	Kyoto Encyclopedia of Genes and Genomes (KEGG)
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ABSTRACT

Plasmids are widely involved in the dissemination of characteristics within bacterial communities. Their genomic content can be assessed by high-throughput sequencing of the whole plasmid fraction of an environment, the plasmidome. In this study, we analyzed the plasmidome of a biofilm formed in the effluents of the teaching hospital of Clermont-Ferrand (France). Our analysis discovered more than 350 new complete plasmids, with a length ranging from 1,219 to 40,193 bp. Forty-two plasmid incompatibility (Inc) groups were found among all the plasmid contigs. Ten large plasmids, described here in detail, were reconstructed from plasmid contigs, seven of which carried antibiotic resistance genes. Four plasmids potentially confer resistance to numerous families of antibiotics, including carbapenems, aminoglycosides, colistin, and chloramphenicol. Most of these plasmids were affiliated to *Proteobacteria*, a phylum of Gram-negative bacteria. This study therefore illustrates the composition of an environmental mixed biofilm in terms of plasmids and antibiotic resistance genes.

Keywords: hospital effluents, plasmidome, biofilm, antibiotic resistances

1. Introduction

Antibiotic resistance in nosocomial bacteria is a major health concern. The large amounts of antibiotics used in hospitals can promote the selection of antibiotic resistance bacteria (ARB) and genes (ARGs) in patients and hospital wastewater. Indeed, hospital effluents are loaded with human-associated microorganisms, as they collect the feces and urine of hospitalized patients. However, ARB occur naturally and ubiquitously in the environment (Buelow et al., 2018), regardless of antibiotic pressure. Certain environmental microorganisms, such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Aeromonas* sp., and *Stenotrophomonas maltophilia*, are intrinsically multidrug resistant, since they naturally produce genes conferring multiple antibiotic resistances. These bacteria have emerged as opportunistic pathogens in the hospital environment in particular (Oliveira & Reygaert, 2021). Outbreaks of *P. aeruginosa* (Jefferies et al., 2012), *S. maltophilia* (Guyot et al., 2013), and *A. baumannii* (Warde et al., 2019) occur frequently in hospital wards. Resistances conferred by chromosomic genes are not easily transmissible to other microorganisms and do not constitute a real potential for dissemination (Yoon et al., 2020), even though transposons could also “jump” to and from bacterial chromosomes (CDC, 2013; Griffith et al., 2000). Both enteric (e.g. *E. coli*) and environmental bacteria (e.g. *Pseudomonas* sp., *Acinetobacter* sp.) can acquire antibiotic resistances through acquisition of ARGs (Botelho et al., 2019). Plasmids are mobile genetic elements that can carry various genes useful for bacterial survival, including ARGs. They are highly diverse in their genetic content, size (1–1000 kb), and copy number (1–500) (Kothari et al., 2019). Bacteria can exchange plasmids with each other, even across the species or phylum barrier, by horizontal gene transfer. This plasmid transfer facilitates the transmission of ARGs, making plasmid detection and reconstruction of the highest importance for understanding the spread of resistance genes and for improving risk assessment. The prevalence of ARGs or ARB

101 in hospital effluents has previously been monitored by culture on selective medium or by
102 detection by PCR with primers specific for diverse antibiotic resistance genes (Harris et al.,
103 2014; Huerta et al., 2013; Liang et al., 2020; Rodriguez-Mozaz et al., 2014; Szekeres et al.,
104 2017; Varela et al., 2016). However, these methods are limited and do not give exhaustive
105 indications of the plasmid content.

106 A study specifically exploring the whole plasmid community (including cultivable and non-
107 cultivable bacteria) in an environment (plasmid metagenome) by next-generation sequencing
108 (NGS) was first performed by Zhang et al. (Zhang et al., 2011). Total DNA was isolated from
109 activated sludge with a plasmid-specific DNA purification kit to enrich extrachromosomal
110 elements, and the remaining sheared genomic DNA from the samples was removed by an ATP-
111 dependent plasmid-safe DNase. More recently, several approaches have been proposed that
112 specifically enrich plasmid DNA from community samples (Jørgensen et al., 2015). Plasmid
113 prediction programs have also been developed to automatically predict plasmids from short-
114 read sequencing data. Some of these determine whether a preassembled contiguous fragment
115 comes from a plasmid and others aim to reconstruct entire plasmid sequences from sequencing
116 reads or from the assembly graph (Arredondo-Alonso et al., 2017).

117 To date, most plasmid analyses of effluents have been performed with planktonic cells
118 (Buelow et al., 2018; Manoharan et al., 2021; Perry et al., 2021). The plasmid content of biofilm
119 microbial communities formed in hospital effluents has been poorly investigated and yet
120 biofilms are a refuge for bacteria, contribute to the dissemination of virulence and resistance
121 factors, and represent an environmental and public health risk (Calhau et al., 2015).

122 Therefore, in this study we adopted a metagenomic approach with plasmid enrichment
123 combined with high-throughput sequencing and bioinformatics tools for assessing the whole
124 plasmid fraction of a hospital effluent biofilm. We focused on the plasmid content and its ARG
125 content.

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2. Material and Methods

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2.1 Biofilm Formation and DNA Extraction

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The effluent wastewater of the Gabriel Montpied teaching hospital (Clermont-Ferrand, France) is collected in a single pipe before being mixed with the urban domestic water without any pre-treatment. A three-month-old biofilm was allowed to form on glass slides attached to a rectangular granite slab placed on the bottom of this pipe (Ory et al., 2016). This biofilm is an environmental, polymicrobial, and mature biofilm. The glass slides were collected in a Falcon tube filled with 50 mL of saline to rinse the biofilm and remove planktonic cells. The glass slides were then scratched with a razor blade and sonicated for 5 min in a total volume of 50 ml saline medium with 0.1% (w/v) Na-pyrophosphate to disrupt the biofilm, and its plasmid DNA was extracted as described previously (Birnboim & Doly, 1979; Hilpert et al., 2020). Briefly, bacteria were centrifuged twice for 10 min at 8,000 rpm. The cells were then resuspended in 200 µl ice-cold resuspension buffer [(50 mM glucose/10 mM EDTA/10 mM Tris-Cl, pH 8.0) + 5 µl RNase A/T + fresh lysozyme 4 mg ml⁻¹, proteinase K 20 µg ml⁻¹] with a mini-potter and incubated for one hour at 37°C. The bacterial cells were lysed with 400 µl of freshly made lysis solution (0.2 M NaOH/1% (w/v) SDS) and the tubes were mixed by gentle inversion. The tubes were incubated for 5 min at room temperature and then neutralized by the addition of 300 µl of 7.5 M ammonium acetate followed quickly by 300 µl of chloroform, mixed by gentle inversion of the tube. The tubes were then chilled on ice for 10 min. After centrifugation at 4°C at 12,000 rpm for 10 min, the samples were cleaned up by phenol/chloroform/isoamyl alcohol (25:24:1) extraction, and the DNA was precipitated with isopropanol (0.7 vol.). After two washes with ethanol 70%, the DNA was resuspended in 100 µl of TE buffer, transferred to 200 µl of

precipitation solution in a fresh tube (30% polyethylene glycol 8000/1.5 M NaCl), mixed by inversion and chilled on ice overnight to clean the DNA before centrifugation and resuspension in 5 mM Tris buffer.

The DNA was sequenced by GATC Biotech using an Illumina platform (HiSeq 2500, 2 x 150 bp, paired end). The reads were deposited with the NCBI accession number ERX3666225 (Hilpert et al., 2020).

2.2 Plasmidome Analysis

The cleaned reads were assembled with Megahit v1.0.5 (D. Li et al., 2015), then processed with the bioinformatic workflow (Hilpert et al., 2020) composed of several scripts (<https://github.com/meb-team/PlasSuite>): plasmid contigs were predicted by PlasPredict and annotated by PlasAnnot, the assembly taxonomy was provided by PlasTaxo, and the resistance gene abundance matrix was provided by PlasAbund. PlasPredict also listed all contigs corresponding to complete plasmids available in the NCBI database (Figure 1). The search for plasmid contigs was combined with alignment against plasmid databases and detection of circular contigs. In parallel, training with PlasFlow (<https://github.com/smaegol/PlasFlow>) was performed. Finally, chromosomal contaminants were excluded by alignment against microbial databases.

The main software options are described with the software in brackets: Megahit (default), Prodigal (-c -m -p meta), hmmer (-E 1e-5), minimap2 (-x asm5), Blast (E-value 1e-6), PlasFlow (--thres 0.7 with default models), prokka (--metagenome --hmms --rawproduct --force "RESFAM profiles" in the plasmid database). Complete circular plasmids were detected by looking for exact identical k-mer at the two ends of the contigs.

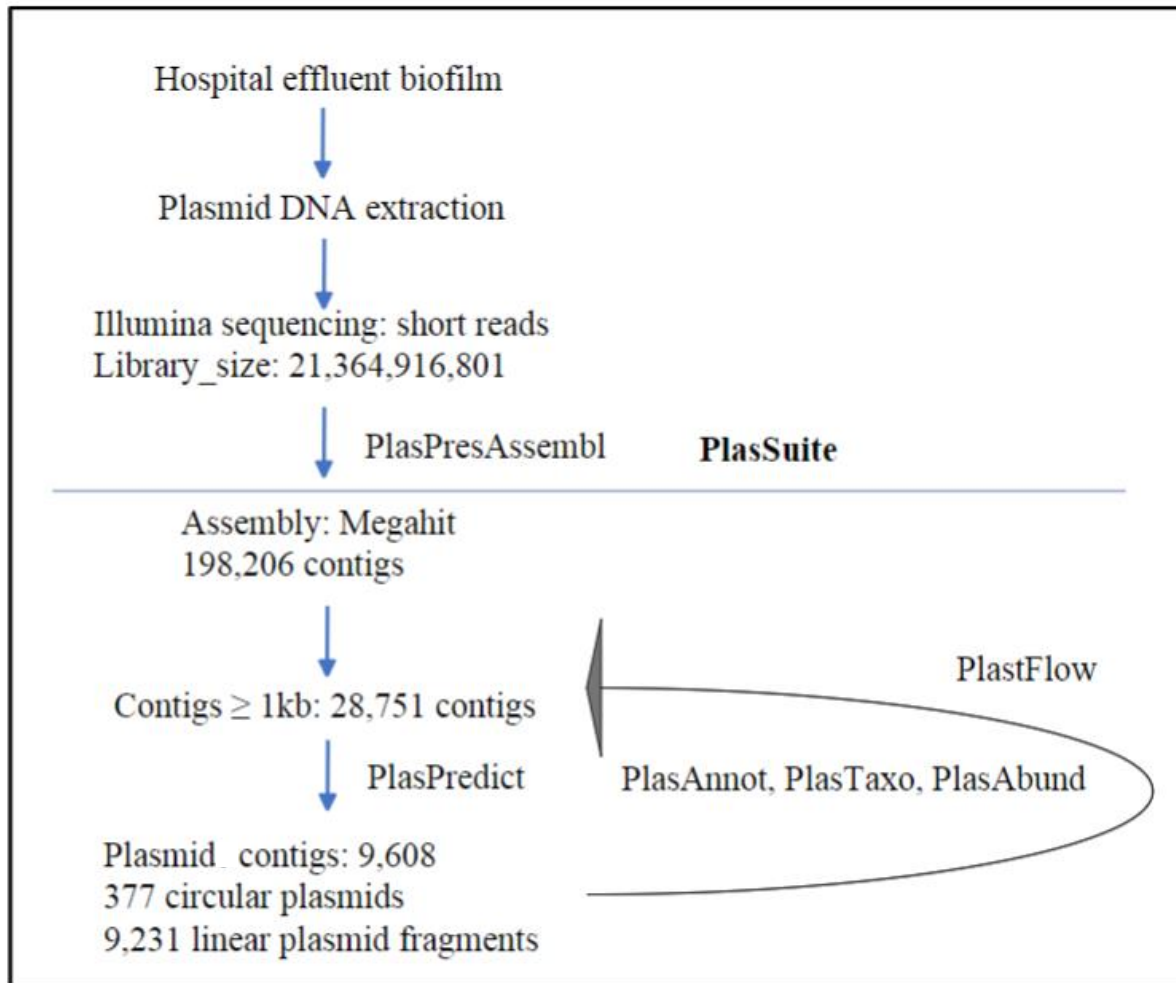


Figure 1: Summary of the First Steps of the Analysis

Two different strategies were applied to reconstruct other potential complete plasmids from contigs (Figure 2). The largest contigs (> 40,000 bp) were blasted against the NCBI plasmid database with the GenBank BLAST tool to find homologies with previously described plasmids, as they obtained a limited number of hits. All the linear plasmid contigs matching the reference sequence were then ordered against the suitable reference plasmid by Mauve v snapshot_2015-02-13 (Darling et al., 2010). When successful, this method enables the detection of insertions or deletions compared to the reference sequence. The final polishing was performed with Pilon v1.22 (Walker et al., 2014).

Smaller linear plasmid contigs were blasted against the NCBI plasmid database with blastn 2.2.31+. (E-value = 0.001, length of plasmid contigs > 1,000 pb). A list of candidate plasmids was thus obtained (plasmid sequences with the highest number of hits). Raw data were mapped to these reference sequences using bwa v0.7.17-r1188 (Frank et al., 2016; H. Li & Durbin, 2009) and the final polishing of the assembly was performed with Pilon v1.22 (Walker et al., 2014).

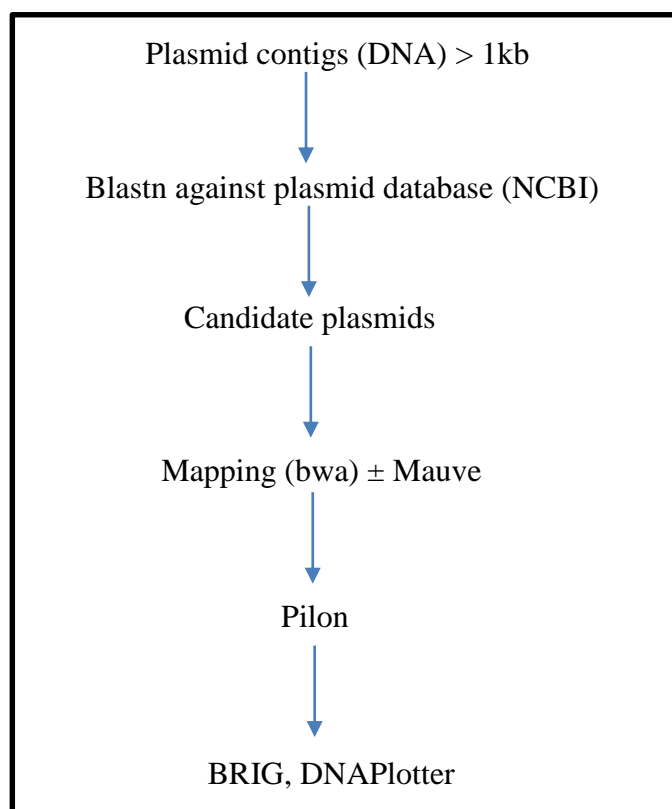


Figure 2: Summary of the Strategy Used to Reconstruct Large Plasmids

BRIG v0.95 was used to compare plasmids (reference against reconstructed sequence) (Alikhan et al., 2011). An overview of the plasmids was produced with DNAPlotter v1.11 (Carver et al., 2009). Proteins were blasted with blastp 2.9.0+ (E-value = 0.001) against the EggNOG 4.0 database to determine their cluster of orthologous groups (COG) functional categories (Powell et al., 2014). Proteins were also aligned against the Uniprot Reference

Clusters (Uniref90) database (www.uniprot.org), to determine their Kyoto Encyclopedia of Genes and Genomes (KEGG) (www.genome.jp/kegg/) pathways, and the Refseq (non-redundant) nr database (NCBI) (O’Leary et al., 2016).

3. Results

3.1 Plasmidome

3.1.1 Description

More than 350 circular and complete plasmids with an average size of 3,606 bp and 9,231 linear contigs (fragments of circular plasmid sequences, linear and complete plasmid sequences, or fragments of linear plasmid sequences) were obtained with an assembly length of 23,729,318 bp. The largest contig was 98,842 bp long. Only a few large plasmid contigs could be identified (e.g. four contigs $\geq 50,000$ bp and 29 contigs $\geq 25,000$ bp), due to the presence of numerous mobile elements with insertion sequences (IS), usually flanked by inverted repeats (608 transposases, transposons, and IS were found on the contigs). Genes of *tra* operons involved in plasmid transfer and their homologs *virB*, *virD*, and *virC* encoding the type IV secretion system proteins were found on numerous contigs, of which 62 and 88 harbored *vir* and *tra* genes, respectively. The type IV mechanism involves three key molecular systems: the relaxosome, the type 4 coupling protein, and the type IV secretion system (De la Cruz, 2020). Most of these plasmids might not be able to promote their own transfer but could be mobilizable, since 38 and 18 genes encoding the mobilization proteins MbeC and MobA, respectively, were found.

The taxonomy results are presented in Tables S1 and S2 (Supplementary Data). This is a predicted taxonomy based on reference databases, which could be biased by multiple events

such as horizontal transfers between bacteria. The Gram-negative *Proteobacteria* phylum was predominant, which is consistent with the presence of feces in hospital effluents. Environmental bacterial such as *Aeromonas* sp. and *Pseudomonas* sp. were those most frequently assigned to chromosomal contigs. These bacteria are known for their natural and multiple chromosomal resistance to antibiotics (Figueira et al., 2011). Concerning plasmid taxonomy, *Enterobacteriaceae* and *Aeromonas* sp. accounted for, respectively, 45% and 19% of the affiliated contigs.

The abundance of resistance mechanisms is presented in Table S3 (Supplementary Data). Impermeability by efflux pumps was the most common mechanism of antibiotic resistance-associated genes, followed by enzymatic destruction.

3.1.2 Functional Annotation by EggNOG and KEGG

The alignment of putative plasmid proteins against the EggNOG database enabled determination of the COG functional categories, which are abbreviated to one letter (Tatusov et al., 2000). The alignment of putative plasmid proteins against the Uniref90 database enabled determination of KEGG. Some of these potential proteins clearly belong to the plasmid machinery. The results are summarized in Tables S4 and S5 in Supplementary Data.

3.1.3 Origins of Replication (Table S6, Supplementary Data)

We identified 42 replicons of plasmids (one replicon per contig) belonging to major Inc groups of *Enterobacteriaceae* species among the 9,608 plasmid contigs (0.43%). Of the 22 contigs that had Inc group sequences specific to small plasmids (< 20 kb) (Carattoli et al., 2014), 17 (40%) belonged to the small-size Col plasmid group and five to the IncQ-like plasmids

(12%). The most frequent Inc group among large plasmids was IncF, which was found on 10 contigs (24%). This is not surprising, since IncF plasmids play a major role in the dissemination of antibiotic resistance genes in the *Enterobacteriaceae* family (Villa et al., 2010). Seventeen other contigs, including four circular contigs, were annotated with replication proteins or replication initiation proteins.

3.2 Antibiotic Resistance Genes

3.2.1 Beta-Lactamase-Encoding Genes

Of the 22 plasmid contigs annotated with beta-lactamase proteins encoding genes, four belonged to class A, two to class B, eight to class C, four to class D, two to subclass B3, and two were GES (class A), according to the Ambler classification (Ambler, 1980). These contigs were short, measuring from 1,001 bp to 9,716 bp (mean of 2,959 bp), and therefore the genetic environment was poor. Only five of these genes encoded proteins whose functions as plasmid beta-lactamases have been demonstrated experimentally. Other genes were either previously annotated as chromosomal or encoding for proteins whose functions were putative or whose genetic environment was not large enough to predict the location (chromosome or plasmid).

A contig of 1,827 bp carried both a *bla*_{BEL-1} gene (identity 100%, coverage 100%) encoding the class A extended-spectrum beta-lactamase (ESBL) BEL-1 and an *aac*(6')-II gene (identity 99.78%, coverage 100%) encoding an aminoglycoside N(6')-acetyltransferase type 1. These two genes have been previously observed in numerous *P. aeruginosa* isolates (Bouheraoua et al., 2018) on a class 1 integron. The genetic organization of the 1,827 bp contig was the same. Glupczynski *et al.* showed that BEL-1 was the most frequently found ESBL, present in 80% of

P. aeruginosa clinical ESBL-positive isolates, and they highlighted its potential for local dissemination in hospitals via patient and personnel transfer (Glupczynski et al., 2010).

A contig of 3,702 bp carried a *bla*_{CTX-M-15} gene (identity 100%, coverage 100%). When the contig was blasted on NCBI, 100 results were obtained with 100% coverage and identity. Indeed, this plasmid gene was previously shown to be epidemic (Cantón et al., 2008).

A contig of 1,246 bp carried a *bla*_{GES-7} gene (identity 100%, coverage 100%). The ESBL GES-7 was initially detected on a 60-kb plasmid in an environmental *Aeromonas* sp. isolated from water samples of the river Seine (Paris) in 2009 by Girlich *et al.* (Girlich et al., 2011). These *Aeromonas* sp. strains were therefore considered to be a potential aquatic reservoir of antibiotic resistance genes.

A contig of 1,017 bp carried *bla*_{NPS} (identity 99.87, coverage 100%). NPS-1 is a plasmid-mediated-lactamase that was reported in 1986 in two isolates of *P. aeruginosa* from a hospital in the United Kingdom (Pai & Jacoby, 2001), but so far has never been described in environmental strains.

3.2.2 Aminoglycoside Resistance Genes

Ten plasmid contigs (1,204 to 4,189 bp) were annotated with genes encoding aminoglycoside modifying enzymes, three aminoglycoside N-acetyltransferases (*aac*(3)-II_d, *aac*(3)-I_a, *aac*(6')-II), four aminoglycoside nucleotidyltransferases (*aadA13*, *aadA5*, *aadD*, *ant*(2'')-I_a), and three aminoglycoside phosphotransferases (*aph*(3')-I_a, *aph*(3')-VI_a, *aph*(3')-XV). Ferro et al. suggested that this mechanism of resistance is associated with improved fitness in the environment (Ferro et al., 2019), which would explain the prevalence of these genes in the biofilm sample of this study.

3.2.3 Quinolone Resistance Genes

A contig of 1,595 bp carried a *qnrS2* gene, and a contig of 1,284 bp carried a *qnrVC4* gene.

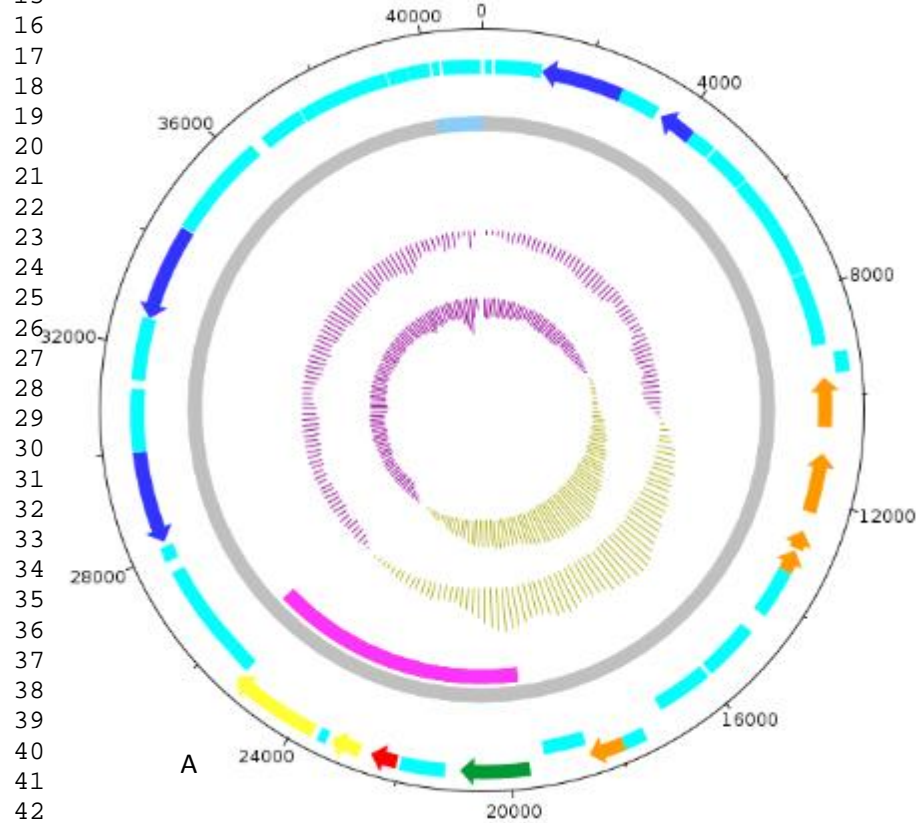
These plasmid genes encoded proteins of the pentapeptide repeat family, which protect topoisomerase IV and DNA gyrase from quinolone inhibition (Rodríguez-Martínez et al., 2011). Their detection was not unexpected, since the aquatic environment has been shown to be a reservoir of *qnr* genes (Ory et al., 2016; Yan et al., 2017).

3.3 Circular Plasmids

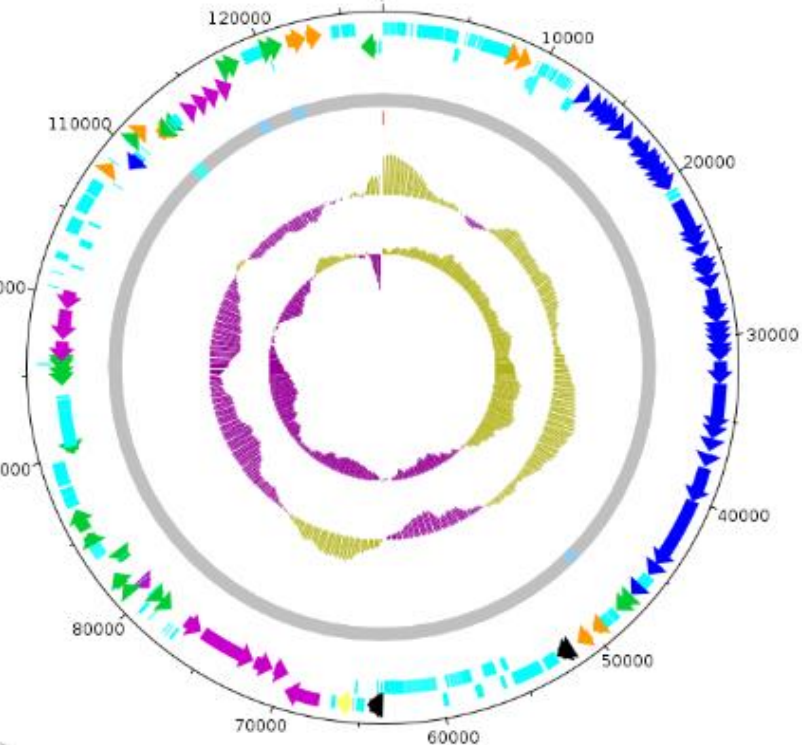
3.3.1 Small Plasmids

Only three of these circular plasmids among the 377 detected by PlasSuite were longer than 20 kb but shorter than 50 kb, meaning they were small plasmids (Arredondo-Alonso et al., 2017). Annotation of the coding sequence (CDS) showed that most of these corresponded to hypothetical proteins. The rest of the CDS mainly encoded toxin antitoxin proteins, mobilization proteins, IS-family transposases, and replication or transposon proteins. Thirteen plasmids from PlasSuite were already present in the NCBI database and therefore known, e.g. a first circular contig of 2,075 bp and a second one of 4,171 bp matched with plasmid pEco70745_4 and plasmid pE35BA_1 of *E. coli*, respectively (Table S7, Supplementary Data). Two plasmids carried genes encoding aminoglycoside-modifying enzymes, an aminoglycoside N(6')-acetyltransferase type 1, already mentioned above, and an aminoglycoside acetyltransferase (AAC3-I). The latter plasmid is also the largest circular plasmid (40,193 pb) (Figure 3A). It was also composed of genes encoding Flavodoxin, an IS1380 family transposase ISEcp1, and a methyl-accepting chemotaxis sensor/transducer. This plasmid shares

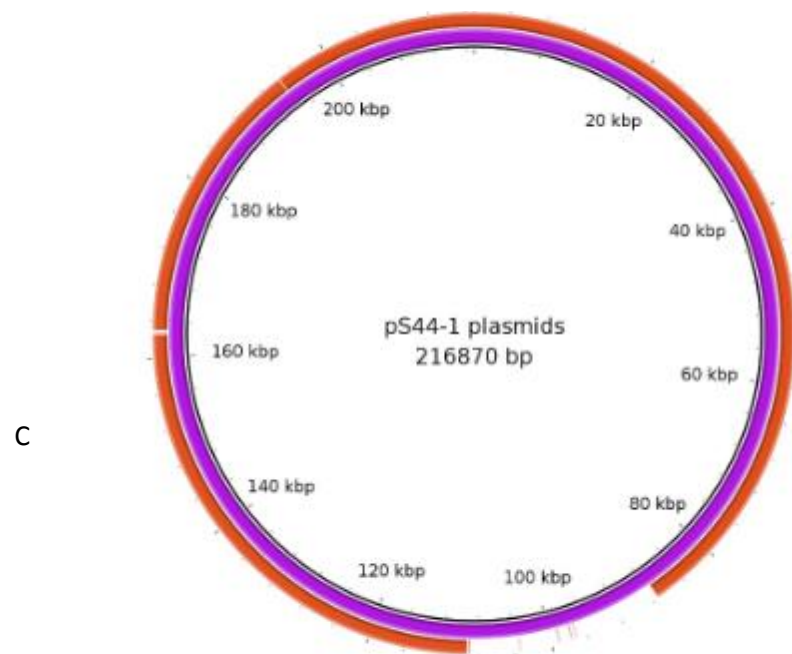
336 homologues with a plasmid previously isolated in the *Acetoanaerobium noterae* strain ATCC
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2 337 35199 (accession number GCA_900168025.1) (coverage: 68%, identity from 95.80 to 99.30%),
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4 338 which is an acetate-producing anaerobic bacterium belonging to the Firmicutes phylum.
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pS44-1
100% identity
70% identity
50% identity
biofilm_pS44-1
100% identity
70% identity
50% identity



B



C

B

D

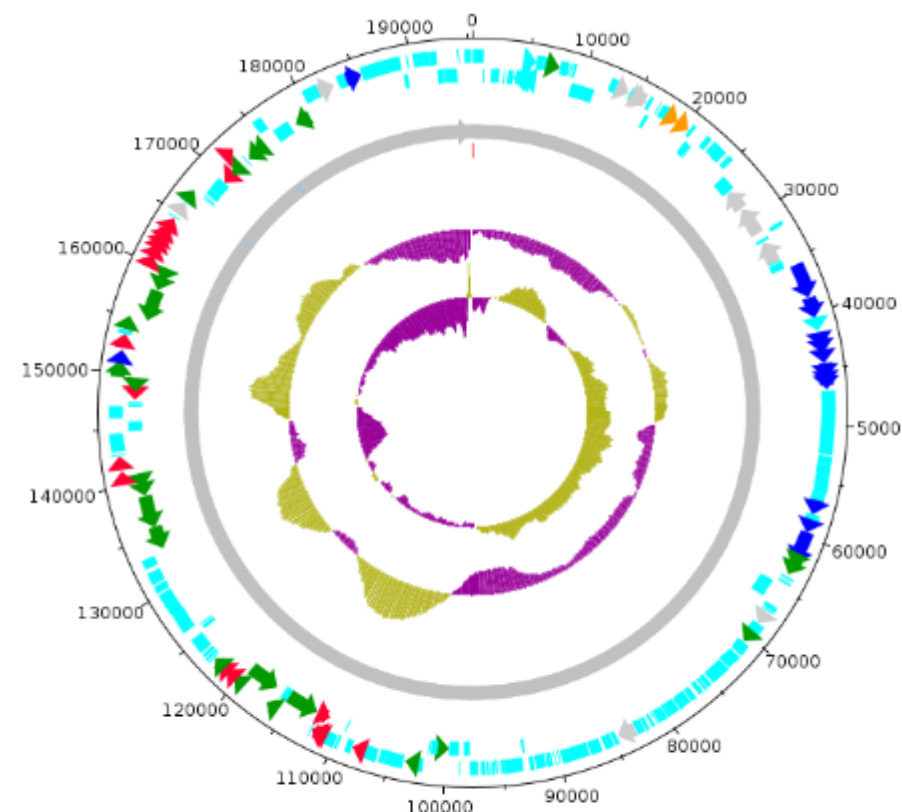


Figure 3: (A) Overview of the 40 kb plasmid k141_113735 made with DNAPlotter. The first ring shows the entire plasmid. On the second ring, transfer genes are indicated in blue; mobile genes in green; genes encoding plasmid proteins in orange; genes involved in resistance in red; genes with other annotations in yellow; and genes encoding unknown proteins in cyan. Genes not affiliated to *A. noterae* are outlined in pink. %GC plot and GC skew are the two inner circles. Blue on the grey circle indicates the repeat regions. (B): Overview of the pColV-C1-type plasmid made with DNAPlotter. The first ring shows the entire plasmid. On the second ring, transfer genes are indicated in blue; mobile genes in green; plasmid proteins in orange; and genes involved in virulence or resistance in pink. Toxin-antitoxin genes are indicated in black and aromatic genes in yellow. %GC plot and GC skew are the two inner circles. Blue on the grey circle indicates the repeat regions. (C): Comparison of the pS44-1 plasmids in the study using BRIG. The reference plasmid is pS44-1 (accession number NZ_CP022176.1). (D): Overview of the pS44-1-type plasmid made with DNAPlotter. Transfer genes are indicated in blue, resistance genes in red, genes encoding unknown proteins in cyan, mobile genes in green, and genes encoding plasmid proteins in orange. Genes with other annotations are indicated in grey.

3.3.2 Large Plasmids with Homologies with Public Databases

Ten plasmids were recovered from linear plasmid contigs. Details and those not described here are presented in Supplementary Data (Table S7 and supplementary doc).

3.3.2.1 Virulence Plasmids

3.3.2.1.1 pColV-C1-type Plasmid (Figure 3B)

The reference plasmid was extracted from an *E. coli* strain isolated from influent municipal wastewater in Japan. pColV-C1 plasmids have been associated with virulence in this species (T. J. Johnson et al., 2006), and these strains of *E. coli* are pathogenic in birds. This IncFIB plasmid harbors the *tra* operon that enables its transfer by conjugation; it was devoid of antibiotic resistance genes.

The pColV-C1-type plasmid of 127,389 bp found in this study had 185 CDS including 67 CDS encoding for hypothetical proteins, nine encoding for the plasmid machinery, and 22 for mobile elements. It was 2,998 bp longer than the pColV-C1 plasmid with several short insertions, including two genes encoding proteins involved in conjugative transfer. Three virulence genes were detected: *iroN* (identity 100%) encoding for the Enterobactin siderophore receptor protein (accession number AF449498), *iss* involved in increased serum survival (accession number DQ381420), and *mchF* encoding an ABC transporter protein MchF (accession number CP002089). The IncF transfer system contained 36 genes corresponding to the conjugal type IV system of Gram-negative bacteria, and the Salmochelin siderophore encoded by *iroN*, *iroE*, *iroD*, *iroC*, and *iroB*. The virulence region was found to contain one gene for ColV export (*cvaA*) and one gene for ColV synthesis (*cvaC*) (Hayashi et al., 2019). It also contained the *sit* operon (*sitABCD*), a manganese ABC transporter, and two Phd-Doc toxin antitoxin (programmed cell death) systems. One gene encoded a 2-keto-3-deoxy-D-arabino-heptulosonate-7-phosphate synthase I alpha, which has a role in the synthesis of aromatic compounds.

3.3.2.1.2 Unnamed Plasmid

A plasmid of 44,381 bp was also found. This IncP plasmid carried 50 CDS including 24 genes potentially encoding conjugative pili (Tra/Trb), components of the type IV secretion system, five genes encoding plasmid machinery, and 16 genes encoding hypothetical proteins.

It was initially isolated from a strain of *Xanthomonas hyacinthi*, a plant pathogenic bacterium that causes yellow disease in *Hyacinthus* (Cohen et al., 2020) , and was part of a collection in the Netherlands in 1958.

3.3.2.2 Resistance Plasmids

3.3.2.2.1 pNDM-KN plasmid

The pNDM-KN-type plasmid found in this study had a length of 162,747 bp. The original plasmid was initially isolated from a *K. pneumoniae* strain, itself isolated from urine in 2009 in Nairobi (Kenya). It carried several antibiotic resistance genes encoding the carbapenemase NDM-1 (New Delhi metallo- β -lactamase), the cephalosporinase CMY-6, the methylase RmtC conferring high-level resistance to all aminoglycosides, the chloramphenicol resistance protein CmlA7, the dihydropteroate synthase Sul1, the adenylyltransferase AadA1, the erythromycin esterase EreC, and the rifampicin ADP-ribosylation transferase ARR-2. All these genes were supported by a class I integron. Downstream of the *bla*_{NDM-1} gene, a *ble*_{MBL} gene involved in bleomycin resistance was found. These *bla*_{NDM-1} and *ble*_{MBL} genes are often found together (A. P. Johnson & Woodford, 2013). The IncA/C plasmids are involved in nosocomial outbreaks and have widely diffused among clinical and environmental bacteria (Poirel et al., 2011).

3.3.2.2.2 PU25001 plasmid

The plasmid found in this study had a length of 211,814 bp. The conjugative IncF plasmid PU25001 was extracted from a multidrug resistant strain of *K. pneumoniae*, strain U25 isolated from urine in a tertiary care hospital in Chennai (India). It had two plasmids and was shown to form a biofilm (Rafiq et al., 2016). Plasmid PU25001 had only one resistance gene

(*catA1*), which encoded resistance to chloramphenicol. The IncF plasmid was conjugative with numerous *tra* and *trb* genes and had genes encoding proteins involved in copper resistance and ABC transporters. The second plasmid of strain U25 harbored 20 resistance genes, seven encoding aminoglycoside resistance, one encoding aminoglycoside and fluoroquinolone resistance, two encoding sulphonamide resistance, seven encoding beta-lactam resistance, one encoding phenicol resistance, and two encoding macrolide resistance.

3.3.2.2.3 pS44-1 Plasmid (Figure 3C and 3D)

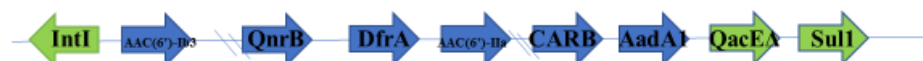
The pS44-1 plasmid was isolated from a strain of *Aeromonas salmonicida* (S44) found in an Atlantic salmon in 2014 in Yantai (China). The recomposed plasmid found in our study has only 195,364 bp (coverage 89%) and has a deletion of 21,867 bp (87,358 to 109,225 bp) compared to pS44-1, including genes encoding mobile elements (transposons), transcriptional regulators, CRISPR-associated proteins, and efflux RND transporters compared to the reference plasmid. It carries 232 CDS, of which 147 (63%) were annotated as hypothetical proteins. Our plasmid is conjugative with 14 genes encoding IncF plasmid conjugative transfer proteins Tra. Of the 14 antibiotic resistance genes found, six potentially encoded aminoglycoside modifying enzymes, one encoded beta-lactamase (*bla_{OXA-1}*), two were involved in resistance to phenicol — including one encoding a chloramphenicol acetyltransferase (*catB4*), two encoded a dihydropteroate synthase (*sulI*) involved in resistance to sulfonamide, *qnrVC6* involved in resistance to quinolones, one encoded a rifampicin ADP ribosylation transferase (*arr-2*), and, finally, one encoded a macrolide 2'-phosphotransferase (*mphA*). Genes encoding proteins involved in resistance to chromium and mercury were also found. We recorded genes encoding mobile elements (transposase, integrase, recombinase) and proteins involved in plasmid stabilization. The other annotated proteins were involved in DNA or protein metabolism and DNA replication.

3.3.2.2.4 pAER-f909 Plasmid

This plasmid was isolated in 2014 in the USA from a strain of *Aeromonas* sp. ASNIH4 found in wastewater. The recomposed plasmid found in our study was 170,935 bp long. Our plasmid had mobile genes but no conjugative function. Antibiotic (aminoglycoside 2"-nucleotidyltransferase and chloramphenicol O-acetyltransferase) and mercury resistance genes were found in a particular class 1 integron of *sul1* type, which contains a copy of the conserved segment *qacDEI**sul1* (Figure 4A).



(A) pAER-f909 plasmid



(B) pMCR5 plasmid

Figure 4: Genetic Context of Antibiotic Resistance Genes in pAER-f909-Type Plasmid (A) and pMCR5_045096-Type Plasmid (B).

(IntI1: integrase; ANT(2''): aminoglycoside adenylyltransferase; CmlA: chloramphenicol efflux MFS transporter; CatB type B chloramphenicol O-acetyltransferase; QacEA: quaternary ammonium compound-resistance protein; Sul1: sulfonamide-resistant dihydropteroate synthase; Mer: mercury resistant system; AAC: aminoglycoside 6'-N-acetyltransferase; QnrB:

quinolone resistance pentapeptide repeat protein; DfrA: trimethoprim-resistant dihydrofolate reductase; CARB: beta-lactamase; AadA1: aminoglycoside nucleotidyltransferase).

3.3.2.2.5 pMCR5_045096 Plasmid

Plasmid pMCR_045096 was found in 2015 in Chengdu (China) in a strain of *Aeromonas hydrophila subsp. hydrophila* (strain WCHAH045096) isolated from sewage. The recomposed plasmid found in our study was 241,363 bp long. Our plasmid had only two *tra* genes but carried numerous resistance genes: *bla*_{CARB-12} (beta-lactamase), *aac(3)-IId*, *aac(6')-IIa*, *aac(6')-Ib3*, *aadA1*, *aph(3'')-Ib*, *aph(3')-Ia*, *aph(6)-Id* (aminoglycoside resistance), *mcr-5.1* (colistin resistance), *qnrVC6* (quinolone resistance), *sul1* (sulphonamide resistance), *tet(E)* (tetracycline resistance), *dfrA1* (trimethoprim resistance), mercury resistance proteins (MerT, MerP), and chromate resistance protein (ChrA). Some of these genes were in a class 1 integron of *sul1* type, as described for the pAER-f909-type plasmid (Figure 4B). Four genes involved in restriction modification systems were also detected (DNA metabolism).

4. Discussion

More than 350 plasmids were assembled from this study's hospital effluent biofilm sequences, most of which were small in size. Only ten large plasmids were reconstructed by comparison with the databases. Most of the plasmids were well conserved compared to the originally described plasmids (whatever their geographical origin, whether from Japan, Kenya, Netherlands, China, Israel, or India), which is evidence of their ability to disseminate. Alignment of the plasmidome reads or contigs to completely sequenced plasmids was also used by Shi *et al.* to identify previously described plasmids in a specific environment (Shi *et al.*,

2018). Using other methods such as Nanopore and PacBio technologies would have resulted in longer reads and made it possible to efficiently construct large plasmids. However, the quantity and quality of DNA required for such technologies and the cost of sequencing are still an obstacle to their use. Progress in sequencing technology of long fragments will certainly help to reconstruct large plasmids in the future. This study on a hospital effluent biofilm had the advantage of combining several technologies to extract the maximum information from data obtained by NGS and resulted in the discovery of potentially new plasmids.

Proteobacteria was the main phylum attributable to the biofilm plasmidome, and Gram-negative bacteria (Genus *Aeromonas* sp., *Pseudomonas* sp., and those belonging to the *Enterobacteriaceae* family) were the most frequently found among the plasmid contigs. In their study of the plasmidome of cultivable bacteria from a biological wastewater treatment reactor supplemented with oxytetracycline, Shi *et al.* reported that the dominant phyla were *Proteobacteria* (37.21%), *Planctomycetes* (16.99%), *Bacteroidetes* (9.89%), and *Chloroflexi* (6.79%) (Shi *et al.*, 2018). *Salmonella* sp., *Acinetobacter* sp., *Escherichia-Shigella* sp., *Pseudomonas* sp., *Staphylococcus* sp., and *Vibrio* sp. were also found in the rumen plasmidome with a metagenomic approach (Ekwanzala *et al.*, 2020). *Aeromonas* sp. and *Pseudomonas* sp. are considered autochthonous to aquatic environments and *Enterobacteriaceae* bacteria are members of the gut microbiota; it is therefore not surprising to find large amounts of these bacteria in effluents. They are both highly resistant to environmental conditions, unlike certain other phyla of the gut microbiota. Another possible explanation for their abundance is that they host more plasmids than other bacteria. Finally, all these bacteria have the capacity to form a biofilm (Avila-Novoa *et al.*, 2019; Balestrino *et al.*, 2005; Magalhães *et al.*, 2016; Ory *et al.*, 2019): the functional analysis of the biofilm plasmidome showed the presence of several protein-encoding genes involved in biofilm formation, and this specific bacterial lifestyle is known to facilitate DNA exchanges. The relationship between bacterial conjugation and

biofilm formation (Ghigo, 2001) is probably an important ecological consequence of the use of antibiotics in clinical medicine. Bacteria use the type IV secretion system to inject proteins, DNA, or nucleoproteins involved in many bacterial processes, some of which play a role in virulence and biofilm formation (Ogunyemi et al., 2019). Several studies have shown the role of histidine kinase proteins (Ali-Ahmad et al., 2017; Cheng et al., 2019) and quorum sensing processes (Balestrino et al., 2005) in biofilm formation and the link between chemotaxis and biofilm formation (Huang et al., 2019), which demonstrates the adaptive response of bacteria to their environment.

Large amounts of antibiotics and multiresistant bacteria are released every day into hospital effluents in patients' urine and feces (Chagas et al., 2011). ARGs, such as *bla*_{TEM} (resistance to β -lactams), *qnrS* (reduced susceptibility to fluoroquinolones), *ermB* (resistance to macrolides), *sulI* (resistance to sulfonamides), and *tet* (resistance to tetracyclines) have been detected in very high concentrations in hospital effluent (Rodriguez-Mozaz et al., 2014). These ARGs are also predominantly found within biofilm plasmidome. As described elsewhere (Caltagirone et al., 2017; Chagas et al., 2011; Suzuki et al., 2020), ARGs involved in high levels of resistance (inducing resistance to all or almost all molecules of an antibiotic family) have also been observed in our study. Antibiotic resistance to third generation cephalosporins (ESBLs, cephalosporinases) and carbapenems (carbapenemases) and to methylase RmtC, conferring high-level resistance to all aminoglycosides, and *mcr-5*, conferring colistin resistance, were found — all of these representing resistance to antibiotics of last chance or last resort. The presence of these ARGs in hospital wastewater is of concern and could have a detrimental impact on public health, principally by dissemination of both the plasmids harboring the genes and the microorganisms (Chagas et al., 2011).

Nucleotide Accession Numbers

530

531 The plasmidome contigs and the large plasmids were deposited in the ENA (European
532 Nucleotide Archive; <https://www.ebi.ac.uk/ena/browser/home>) with the accession numbers
533 ERS4825915, ERS4825916, ERS4825917, ERS4825918, ERS4825919, ERS4825920,
534 ERS4825921, ERS4825922, ERS4825923, ERS4825924, and ERS4825925. Circular and
535 linear (> 5 kb) plasmid contigs were deposited in Figshare
536 (<https://doi.org/10.6084/m9.figshare.13656620.v1>).

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542 Declaration of Competing Interest

543 None.

545 Appendix A. Supplementary Data

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Supplementary Table S1: Contigs with Consistent Taxonomy

Taxon	Number of contigs
Actinobacteria	3
Bacteroidetes	4
Firmicutes	4
Proteobacteria	2,556
unclassified	718

Supplementary Table S2: Taxonomies Assigned to Chromosomal and Plasmid Contigs (only the 10 most abundant species are represented)

Chromosomal contigs (total: 2,843)	Number of contigs
<i>Pseudomonas</i>	697
<i>Enterobacteriaceae</i>	751
<i>Aeromonas</i>	745
<i>Proteobacteria</i>	116

Plasmid contigs (total: 9,608)	Number of contigs
<i>Enterobacteriaceae</i>	208
<i>Gammaproteobacteria</i>	62
<i>Enterobacterales</i>	37
<i>Aeromonas</i>	88
<i>Acinetobacter</i>	35
<i>Desulfovibrio vulgaris</i>	24

Supplementary Table S3: Matrix with Counts Group by "Antibiotics Category" Defined by Resfam

Mechanism of resistance	Number of reads normalized to the total reads x 10 ⁷ (Library size: 21,364,916,801)	Numbers of plasmid contigs annotated with a mechanism of resistance
Efflux pump	220	201
ABC transporter	123	28
Aminoglycoside modifying enzyme	101	10
Beta-lactamase	95	22
Glycopeptide resistance	70	36
RND antibiotic efflux	66	16
Gene modulating resistance	21	10
Chloramphenicol resistance	13	7
Quinolone resistance	7	8
rRNA methyltransferase	5	5
MFS transporter	5	5
Aminoglycoside resistance	2	1
Tetracycline MFS efflux	2	2
Tetracycline inactivation	1	0

Supplementary Table S4: Most Frequent Functional Annotations of the Plasmid Proteins by EggNOG (15,624 proteins were annotated among the 33,180 found)

Functional annotation	COG functional categories	Number of proteins
Transposase	[u'L']	484
Histidine kinase	[u'T']	185
Transcriptional regulator	[u'K']	183
Resolvase	[u'L']	169
Sel1 domain protein repeat-containing protein	[u'M']	153
Integrase	[u'L']	103
ABC transporter	[u'P']	100
Relaxase mobilization nuclease	[u'S']	84
Membrane	[u'S']	64
TraG TraD family protein	[u'U']	58
Conjugal transfer protein	[u'U']	54
Plasmid recombination enzyme	[u'S']	52
Type I restriction-modification system	[u'V']	51
MobA MobL family protein	[u'L']	48
Hemolysin-type calcium-binding	[u'Q']	43
Cytosine-specific methyltransferase	[u'L']	42

INFORMATION STORAGE AND PROCESSING

[K] Transcription

[L] Replication, recombination, and repair

CELLULAR PROCESSES AND SIGNALING

[V] Defense mechanisms

[T] Signal transduction mechanisms

[M] Cell wall/membrane/envelope biogenesis

[U] Intracellular trafficking, secretion, and vesicular transport

METABOLISM

[P] Inorganic ion transport and metabolism

[Q] Secondary metabolites biosynthesis, transport, and catabolism

POORLY CHARACTERIZED

[S] Function unknown

Supplementary Table S5: Details on the Functional Annotation by EggNOG and KEGG

COG functional categories	Number of proteins	Percentage of the annotated proteins	Involvement in plasmid machinery
[S]	6,066	38%	Relaxase /mobilization nuclease and plasmid recombination enzyme
[L]	2,286	15%	Transposase, resolvase, and integrase
[K], [M], [U], [P]	782	5%	[U]: conjugative transfer (TraG, TraD family proteins, conjugal transfer proteins)
[T]	185	1%	Histidine kinase (signal transduction mechanisms)

KEGG pathway	Percentage of the annotated proteins
Metabolism	58%
Cellular processes (quorum sensing, bacterial chemotaxis, and biofilm formation)	11%
Human diseases (beta-lactam resistance)	7%

Supplementary Table S6: Rapid identification of plasmids in *Enterobacteriaceae* species by plasmidFinder v2.1 (Carattoli et al., 2014) (each result corresponds to a unique contigs)

Plasmid	Identity	Query / Template length	Accession number
Col(IMGS31)	100.0	198 / 198	NC011406
Col(MGD2)	100.0	103 / 136	NC003789
Col(MGD2)	100.0	103 / 136	NC003789
Col(pHAD28)	95.83	96 / 131	KU674895
Col(pHAD28)	96.67	120 / 131	KU674895
Col(pHAD28)	95.54	112 / 131	KU674895
Col(pWES)	100.0	178 / 178	DQ268764
Col440I	96.36	110 / 114	CP023920
Col440I	95.19	104 / 114	CP023920
Col440I	95.61	114 / 114	CP023920
Col440I	96.49	114 / 114	CP023920
Col440I	95.61	114 / 114	CP023920
Col440I	95.61	114 / 114	CP023920
Col440I	96.49	114 / 114	CP023920
Col440II	97.15	281 / 282	CP023921
Col440II	96.34	273 / 282	CP023921
ColKP3	96.97	198 / 280	JN205800
IncA	100.0	417 / 417	FJ705807
IncC	100.0	417 / 417	JN157804
IncFIA(HI1)	98.45	387 / 388	AF250878
IncFIB(AP001918)	98.53	682 / 682	AP001918
IncFIB(K)	100.0	560 / 560	JN233704
IncFIB(pQil)	100.0	740 / 740	JN233705
IncFII	96.56	262 / 261	AY458016
IncFII	100.0	261 / 261	AY458016
IncFII(K)	97.97	148 / 148	CP000648
IncFII(Yp)	99.57	230 / 230	CP000670
IncFII(pCRY)	96.96	593 / 593	NC005814
IncFII(pCoo)	95.06	263 / 262	CR942285
IncM1	100.0	739 / 739	U27345
IncN2	99.79	477 / 477	JF785549
IncN3	100.0	477 / 477	EF219134
IncP1	99.14	583 / 582	U67194
IncQ2	100.0	319 / 450	FJ696404
IncQ2	98.9	272 / 450	FJ696404
IncQ2	98.56	277 / 450	FJ696404
IncQ2	95.78	450 / 450	FJ696404
IncQ2	100.0	272 / 450	FJ696404
IncR	100.0	251 / 251	DQ449578
IncU	97.17	565 / 565	DQ401103
IncX5	99.83	576 / 576	MF062700
pKPC-CAV1321	100.0	596 / 596	CP011611

Supplementary Table S7: Circular Contigs Found in the NCBI Database and Assemblies of Contigs Created by Homologies with Plasmids from the NCBI Database

Contigs	Reference plasmid	Accession number	Size (bp)	Organism	Features	Country	Contig name	Contig length (bp)	Identity* Coverage** Coverage/ Pilon***
Circular contigs	pEco70745_4	NZ_CP023262	2,061	<i>E. coli</i>	/	Sweden	k141_64611	2,075	99.95% 100%
	pE35BA_1	KT693145	4,130	<i>E. coli</i>	/	Spain	k141_46178	4,171	98.70% 100%
	pBI143	U30316.1	2,747	<i>B. fragilis</i>	/	USA	k141_33698	2,824	97.89% 100%
	pRGRH1852	LN854346.1	5,604	/	/	Denmark	k141_67643	5,741	100% 100%
	pCd13_cfrC	MH229772.1	6,961	<i>C. difficile</i>	antibiotic resistance plasmid	Greece	k141_24103	6,194	99.83% 85%
	p35_E	NZ_CP050072.1	5,406	<i>K. aerogenes</i>	/	Switzerland	k141_106360	4,941	99.96% 98%
	pWP5-S18-ESBL-05_10	NZ_AP022152.1	4,048	<i>K. quasipneumoniae</i>	/	Japan	k141_119889	4,189	99.83% 99%
	pRHBSTW-00184_5	NZ_CP055557.1	6,352	<i>K. pneumoniae</i>	/	United Kingdom	k141_160628	6,494	99.91% 100%
	pRHBSTW-00486_3	NZ_CP056468.1	4,183	<i>C. freundii</i>	/	United Kingdom	k141_205210	4,324	99.97% 100%
	pRHBSTW-00570_5	NZ_CP056403.1	2,925	<i>Citrobacter sp.</i>		United Kingdom	k141_212688	3,066	99.97% 100%
	pKAM330_9	NZ_AP023407.1	2,624	<i>A. hydrophila</i>	/	Japan	k141_217174	2,743	98.94% 100%
	unnamed2	NZ_CP060082.1	1,240	<i>E. coli</i>		USA	k141_219832	1,381	100% 99%
	pG	NZ_CP062145.1	1,443	<i>K. quasipneumoniae</i>	/	China	k141_224557	1,584	99.55% 100%
Linear contigs >40,000bp	pColV-C1	LC484363.1	124,394	<i>E. coli</i>	virulence plasmid	Japan	k141_34238	60,607	99.82% 99% 1184

	pNDM-KN	JN157804	162,747	<i>K. pneumoniae</i>	antibiotic resistance plasmid	Kenya	k141_58583	98,842	99.88% 100% 1274
	Unnamed plasmid	CP043477.1	44,381	<i>X. hyacinthi</i>	virulence plasmid	Netherlands	k141_120197	41,000	99.89% 100% 162
	pMC64A	CP047963.1	283,486	<i>A. media</i>	antibiotic resistance plasmid	China	k141_118895 k141_108335	46,532 61,112	99.89% 99% 298
	pCTEL-2	CP045203.1	235,057	<i>Citrobacter telavivensis</i>	heavy metal resistance plasmid	Israel	k141_173082	49,799	99.93% 99% 298
Linear contigs < 40,000bp	pQnrS2_045523	NZ_CP032896.1	9,639	<i>E. kobei</i>	mixed plasmid	China	k141_144924	1,595	92.25% 100% 9681
	PU25001 tig00000080_ pilon	KT203286 CP026752.1	211,813	<i>K. pneumoniae</i>	resistance plasmid	India	k141_103650	38,269	99.86% 100% 869
	pS44-1	NZ_CP022176.1	216,870	<i>A. salmonicida</i>	resistance plasmid	China	k141_135849	20,332	99.28% 89% 760
	pAER-f909	NZ_CP026221.1	171,236	<i>Aeromonas sp.</i>	resistance plasmid	USA	k141_57307	30,609	99.85% 99% 626
	pMCR5_045096	NZ_CP028567.1	241,090	<i>A. hydrophila</i>	resistance plasmid	China	k141_86147	23,023	99.75% 99% 934

* Percent identity describes how similar the sequence plasmid found in the study is to the reference plasmid sequence

** Percent coverage is the percentage of the sequence plasmid found in the study that is included in the reference plasmid sequence

*** A mean total coverage with Pilon represents the mean number of reads that align to the reference sequence

Details of Five Circular Plasmids Found in Plasmid Databases

Contig k141_64611 shared 99.95% identity and 100% coverage with the plasmid pEco70745_4 of *E. coli* strain CCUG 70745. This strain was isolated in Gothenburg (Sweden) and was sequenced as part of a study on clinical carbapenem-resistant pathogens of different species. The plasmid pEco70745_4 had three CDS, two of them encoding replication proteins.

Contig k141_46178 shared 98.70% identity and 100% coverage with the plasmid pE35BA_1 of *E. coli* strain E35BA. This strain was isolated from urine in 2008 in Spain and belongs to the clonal group of extra intestinal pathogenic *E. coli*. In addition to this plasmid, the strain harbored two IncF plasmids. The pE35BA_1-type plasmid had four CDS, two of which encoded a replication initiation protein and a mobilization protein, MobA.

Contig k141_33698 shared 97.89% identity and 100% coverage with the circular plasmid pBI143 of *Bacteroides fragilis* strain IB143. This plasmid, described by Smith et al. in 1996, harbors genes encoding a MobA and a RepA protein (Smith & Parker, 1996).

Contig k141_67643 shared 100% coverage and 100% identity with the plasmid pRGRH1852. This plasmid was isolated from a rat gut bacterial strain. It carried genes encoding replication (TrfA) and mobilization proteins (e.g. MobC).

Contig k141_24103 shared 99.83% identity and 85% coverage with the circular plasmid pCd13_cfrC of *Clostridioides difficile* strain Cd-13Lar, isolated from human stools in Greece. k141_24103 carried genes encoding an aminoglycoside 6'-N-acetyltransferase, a toxin-antitoxin system (relB/DinJ family), and a plasmid recombination enzyme.

Detail of the Plasmids Reconstructed with Linear Contigs Association and Not Described in the Text

Contigs > 40,000 bp

pMC64A Plasmid

This plasmid was isolated from a clinical strain of *Aeromonas media* (MC64A) in 2017 in Shandong (China). The plasmid found in our study, which was 280,925 bp long, carried 324 CDS, including 179 genes encoding hypothetical proteins, 53 genes encoding transposases and IS-like elements of the transposase family, and 27 genes encoding mobile element proteins. Several antibiotic resistance genes encoding beta-lactamases (the

carbapenemase KPC-2 and the TEM-1A and TEM-1B enzymes), an aminoglycoside-modifying enzyme (AAC(3)-IId), and a Mph(A) protein involved in macrolide resistance were found. Twelve of the other genes were involved in DNA metabolism, including six genes encoding the CRISPR Cmr cluster.

pCTEL-2 Plasmid

We detected a plasmid of 232,893 bp in length that shared 99.93% identity with a previously identified pCTEL-2 plasmid described in a clinical strain of *Citrobacter telavivensis* 6105, itself isolated from the rectum of a man in 2010 in Tel Aviv (Israel). It had a deletion of 2,126 pb, encompassing IS family transposase and AAA family ATPase. This Inc FIB plasmid harbors the conjugative *tra* operon. Apart from classical plasmid genes (encoding transposases, mobile elements, toxin-antitoxin systems, DNA repair systems, recombinases, etc.), the plasmid was devoid of antibiotic resistance genes, but carried genes involved in resistance to mercury and cobalt/zinc/cadmium. It also had the particularity of having eight genes involved in glycerol metabolism (glycerol dehydrogenase).

Contigs < 40,000 bp

pQnrS2_045523 Plasmid

We detected a pQnrS2_045523-type plasmid of 9,633 bp. pQnrS2_045523 was isolated from a clinical strain of *Enterobacter kobei* in 2017 in Chengdu (China). In addition to the *qnrS2* gene involved in quinolone resistance, the plasmid found in this study had two genes encoding replication proteins and a gene encoding a methyl-accepting chemotaxis sensor/transducer.