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High plasmidome diversity of extended-spectrum beta-lactam-resistant *Escherichia coli* isolates collected during one year in one community hospital

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ABSTRACT

Plasmid-encoded antibiotic resistance encompasses many classes of currently used antibiotics. In globally distributed *Escherichia coli* lineages plasmids, which spread via horizontal gene transfer, are responsible for the dissemination of genes encoding extended-spectrum β-lactamases (ESBL). In this study, we combined 2nd and 3rd generation sequencing techniques to reconstruct the plasmidome of overall 97 clinical ESBL-*E. coli* isolates. Our results highlight the enormous plasmid diversity in respect to size, replicon-type and genetic content. Furthermore, we emphasize the diverse plasmid distribution patterns among the clinical isolates and the high intra- and extracellular mobility potential of resistance conferring genes. While the majority of resistance conferring genes were located on large plasmids of known replicon type, small cryptic plasmids seem to be underestimated resistance gene vectors. Our results contribute to a better understanding of the dissemination of resistance-conferring genes through horizontal gene transfer as well as clonal spread.

1. Introduction

Infections caused by multidrug-resistant bacteria pose an increasing challenge for public health systems. The rise in 3rd generation cephalosporin resistance in *E. coli* has led to its classification as a critical pathogen [59]. In Germany, the fraction of cefotaxime resistant *E. coli* increased from 6.4% in 2008 to 11.3% in 2019 ([42]; Robert Koch-Institut, 2021). Many antibiotic resistance conferring genes (ARG) such as those encoding for extended-spectrum β-lactamases (ESBL) are located on plasmids [43]. Plasmids replicate autonomously, spread via horizontal gene transfer (HGT) and exhibit a wide range of different copy numbers and sizes [10]. In addition to antimicrobial resistance [46], they confer important traits to their host, such as virulence, and can give rise to new outbreak situations [32].

The spread of resistance conferring plasmids plays an important role in the global increase of antimicrobial resistance (AMR) [14]. Large epidemic plasmids, such as those encoding the $bla_{CTX\text{-}M\text{-}15}$ gene in the globally dominant high risk *E. coli* sequence type (ST) 131 [12,13,32], contribute to the dissemination of ARG via HGT. In addition, the frequently observed fluoroquinolone resistance in the ST131 subclades C3/H30Rx and C1/H30R [41,44] limits treatment options in infected patients.

Sequencing of complete plasmids is important to understand the molecular mechanisms of resistance and its dissemination [8,30]. Multiple approaches have been undertaken to reconstruct plasmids from short sequencing reads or draft assemblies. As an example the tool plasmidSPADES was benchmarked in various studies [3,45,58]. However, the tool proved not to be suitable for low copy number plasmids [58], such as IncF ARG-encoding plasmids that are frequently found in ST131 isolates [46]. Long reads of 3rd generation sequencing

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technology can help to span repetitive regions of plasmids and aid the scaffolding process during assembly. Furthermore, long-read sequencing seems to be particular important for resolving the nested structure of mobile genetic elements (MGEs) on resistance conferring cassettes [54]. If combined with less error prone short reads, as produced by Illumina technology (hybrid assembly), de novo assemblies at a low error rates can be obtained [30].

Here we used a whole genome sequencing (WGS) approach to capture the plasmid variability found in 97 ESBL positive clinical *E. coli* (ESBL-Ec) isolated in a German community hospital within one year. By applying 2nd and 3rd generation sequencing techniques, we fully reconstructed 372 plasmids. In addition, we identified 77 contigs as putative ARG-encoding plasmids. While the overall variation of ARG harboring plasmids was limited, the plasmidome of the individual clinical *E. coli* isolates was remarkably diverse. Furthermore, the mobility potential of the almost 500 plasmid-encoded ARGs was high and their acquisition explained phenotypic resistance to 3rd generation cephalosporins in the great majority of clinical isolates.

2. Results

2.1. Data generation and exploratory data analysis

We combined Illumina short-read sequencing of genomic DNA and

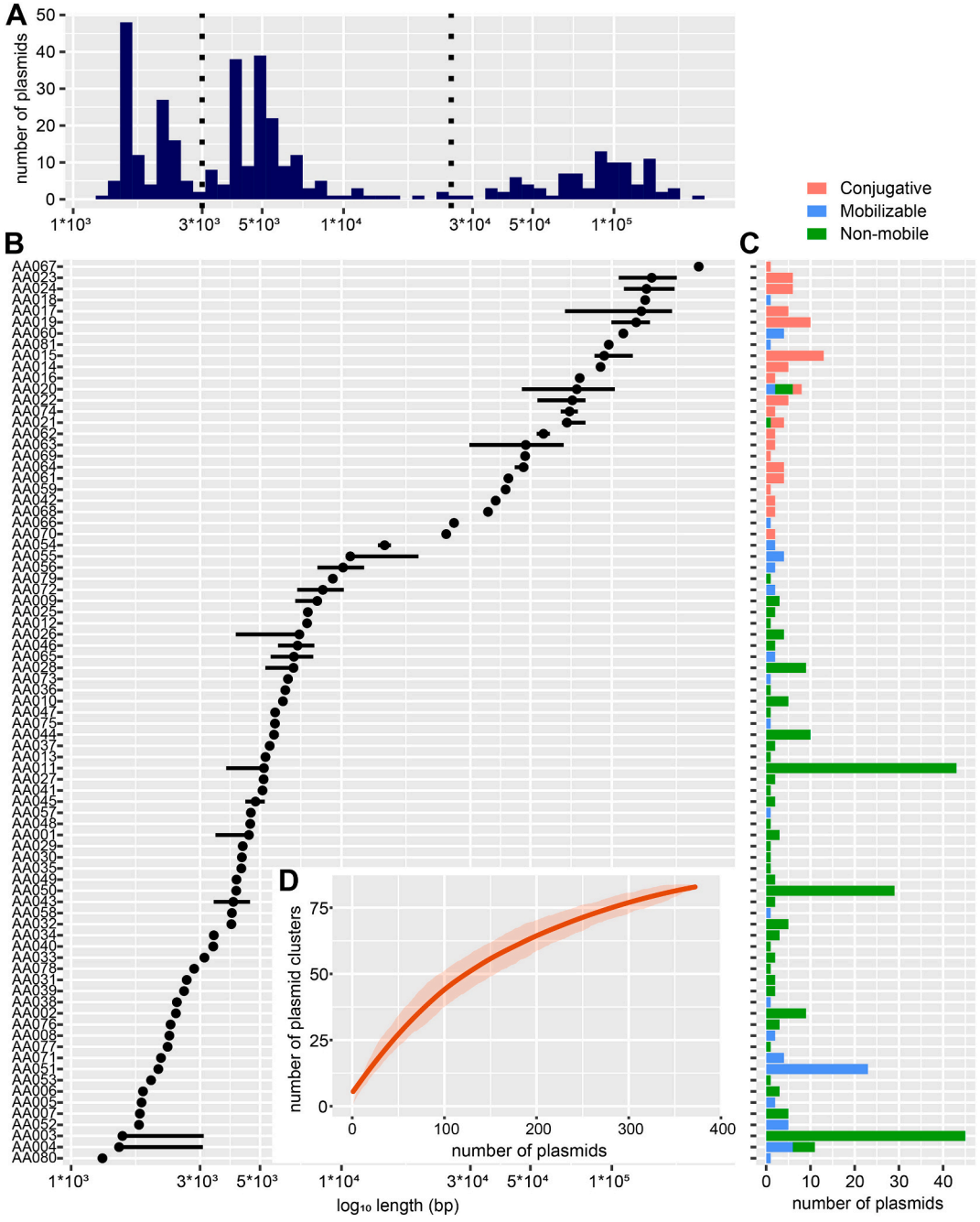

Fig. 1. Genomic variation landscape of 372 complete sequences of plasmids from 97 clinical ESBL-Ec isolates. (A) Plasmid length distribution. Dashed lines indicate size group boundaries. (B) Length range of plasmids in 83 clusters (cluster ID is shown on the left) as defined by mob-suite based on a mash-distance cut-off of 0.06 Points show median length of the cluster. Lines show length range of plasmids. (C) Number of plasmids per cluster, bars are colored according to their mobility. (D) Rarefaction plot of 372 complete plasmids and plasmid cluster accumulation curve of 83 clusters.

Oxford Nanopore Technologies (ONT) long-read sequencing of plasmid enriched DNA in order to reconstruct and characterize the plasmidome of 97 clinical ESBL-Ec isolates. Those were recovered within a one-year sampling period from overall 89 patients in a large German community hospital. The globally dominant sequence type (ST) 131 was also most dominant in our selection (59 isolates, Supplementary Table S1).

Overall, 372 complete (circularized) plasmids were reconstructed. For the majority of the plasmids (301), at least a 15× short read coverage and a 5× long read coverage for >95% of the respective plasmid length was achieved. In order to ensure high quality, we based our initial analysis of the plasmid variation landscape on the dataset of the circularized plasmid assemblies.

However, to comprehensively cover all ARG carrying plasmids, we additionally compared the content of all contigs (including those that were not part of the circularized, primary plasmid collection) against the CARD database [33]. We identified 77 additional contigs representing putative ARG-bearing plasmids by filtering based on size (< 250 kb) and mob-recon analysis [45]. We also identified seven $bla_{\text{CTX-M}}$ carrying contigs in the short-read only assemblies that had been lost during Unicycler hybrid assembly, probably due to low coverage support. They were not considered further in the analysis of the plasmid landscape.

2.2. Genomic variation landscape of closed circular plasmids

The sizes of the 372 circularized plasmids (P1-P372) ranged from 1.3 kb to 209.7 kb. They grouped into 119 small (< 3 kb), 161 medium (3 to 25 kb) and 92 large plasmids (> 25 kb) (Fig. 1 A). A very similar size distribution of clinical ESBL-Ec plasmids (255 plasmids in 61 isolates) has been described previously [21].

They separated into 83 clusters based on a mash-distance cut-off of 0.06 [45] (Fig. 1 B). Of those 83 clusters, 27 contained only one plasmid (Fig. 1 C). Replicon types were found for 76% of the plasmids based on an expanded set of the PlasmidFinder replicons, also including known replication genes without incompatibility information [15,45]. In total, we identified 54 different replicon types. Of note, single replicon types were not necessarily restricted to one plasmid cluster. For example, the 33 plasmids of replicon type 2350 were assigned to 14 different clusters and their sizes ranged from 2.7 kb to 7.9 kb (Supplementary Table S2). Furthermore, we found 24 IncF replicons in various combinations with other replicons on the same plasmids. Overall, up to five replicon types were detected in a single complete plasmid. Forty-seven plasmids that were not replicon typeable were grouped into 21 clusters. Twenty-one of those exceeded 0.06 mash distance to a known neighbor from the mob-typer database, indicating that these types of plasmids might not have been described previously.

Of the 83 clusters, the largest (AA003) consisted of 45 small (1.5 kb), mobilizable plasmids of replicon type Col(MG828). The second largest cluster (AA011) comprised 43 plasmids (3.8–5.3 kb), the majority of which belonged to Col156 or replicon type 2131. No complete conjugative plasmid smaller than 24.4 kb was found (Fig. 1 C) confirming the observation that plasmids encoding a conjugation machinery have a minimum size of approx. 25 kb [21,56]. However, we also found non-conjugative plasmids among the larger ones.

Overall, clusters with larger plasmids were significantly less conserved than clusters of smaller plasmids (mash distance within clusters, Tukeys HSD, p-value = 0.0008).

In order to estimate the sampling effort and to evaluate plasmid diversity of our strain collection, we performed a rarefaction analysis on the 372 closed plasmids. The plasmid cluster accumulation served as an assessment of plasmid richness. Although the estimator curve did not reach an asymptotic state, it indicated a stabilization of the number of expected new clusters (Fig. 1 D).

2.3. Assignment of putative ARG-bearing plasmids

To visualize similarities between the closed circularized plasmids, we generated a network based on the pairwise mash distances. In general, the plasmids clustered according to their size, and large and small plasmids were clearly separated (Fig. 2 A). Sixty-eight (18%) of the 372 closed plasmids harbored ARGs (Fig. 2 B). They were mainly found in a large group containing mainly large and medium size plasmids. All 77 putative ARG-bearing plasmids fell into the main cluster of large and medium-sized closed resistance plasmids (Fig. 2 C). Rarefaction analysis based on the 449 closed and putative plasmids, falling into overall 98 clusters, confirmed that the putative ARG bearing plasmids added only a limited amount of diversity to the primary plasmidome landscape of the 97 clinical isolates (Fig. 2 D).

Of note, we found two clusters of closed resistance plasmids clearly distinct from the main group (Fig. 2 C). One cluster contained the two smallest ARG-bearing plasmids found in this study (P114, P115 in cluster AA040). They were identical to the quinolone resistance conferring plasmid KU674895.1, which was isolated from a *Salmonella enterica* contaminated food source [24]. The other cluster contained two plasmids (P348 and P349 in AA060) harboring an intact *Salmonella* SSU5 prophage (each >100 kb). The presence of these phage-like plasmids in ESBL-Ec has been described previously [17,60].

2.4. Replicon-types and ARG-content of plasmids

Among the overall 145 ARG harboring closed and putative plasmids we found IncFIA to be the dominant replicon type (n = 53, in 21 different multi-replicon combinations). Twelve plasmids belonged to IncFIB (in six different multi-replicon combinations) and ten plasmids were typed to be IncI-gamma/K1. Three plasmids were IncFII, two IncQ1 and four IncX1. Some replicon types were only detected once (IncK2/Z, IncX1/IncX3, IncX4, IncY). We also detected ARGs on 50 plasmids that were of unknown replicon type. These plasmids fell into 15 different clusters, indicating a high variability among them. Six plasmids were harboring not-yet described replicon types without incompatibility scheme information (Supplementary Table S2).

These plasmids contained overall 491 distinct ARGs of 38 families, conferring resistance to different classes of antibiotics including aminoglycosides, tetracycline, fluoroquinolones and cephalosporins (Supplementary Table S3). The median number of ARGs per plasmid was three. 40% of the plasmids carried only one ARG (Fig. 3). One clinical isolate harbored a plasmid (P306) containing an array of 13 identical copies of TEM-135 (a single long read spanning 10 of the 13 elements confirmed the multi-copy gene structure, see Supplementary Fig. S1). Furthermore, another clinical isolate harbored overall 12 ARGs located on a 126.5 kb conjugative plasmid with 5 replicons (IncFIA, IncFIB, IncFII, IncFII, P355 and replicon type cluster 2131).

Larger plasmids carried ARGs more often than smaller plasmids (Wilcoxon rank sum test with continuity correction, $p < 2.2e^{-16}$). While 55 of the closed large plasmids harbored ARGs, only 2 small plasmids and 11 medium-sized plasmids contained ARGs. Most of the small and medium sized plasmids were non-typeable (n = 10) or belonged to the replicon type cluster 2335. Small cryptic plasmids (SCPs) have been described before to harbor genes that confer resistance against aminoglycosides, colistin, tetracycline, sulphonamides and trimethoprim, although the ARGs were not clearly associated with a specific SCP family [9]. In our dataset we also found genes conferring resistance to the previously described classes on small and medium plasmids with the exception of colistin.

2.5. Plasmid profiles of clinical ESBL-Ec isolates

The mean number of plasmids per isolate was four, and up to 13 plasmids were found in a single strain (Fig. 4). Up to ten SCPs were reconstructed from a single isolate (ec_00074). The 97 ESBL-Ec isolates showed overall 96 different plasmid profiles, indicating a high diversity – although we might have missed some plasmids. However, we also found two isolates of different STs that showed a similar plasmid profile

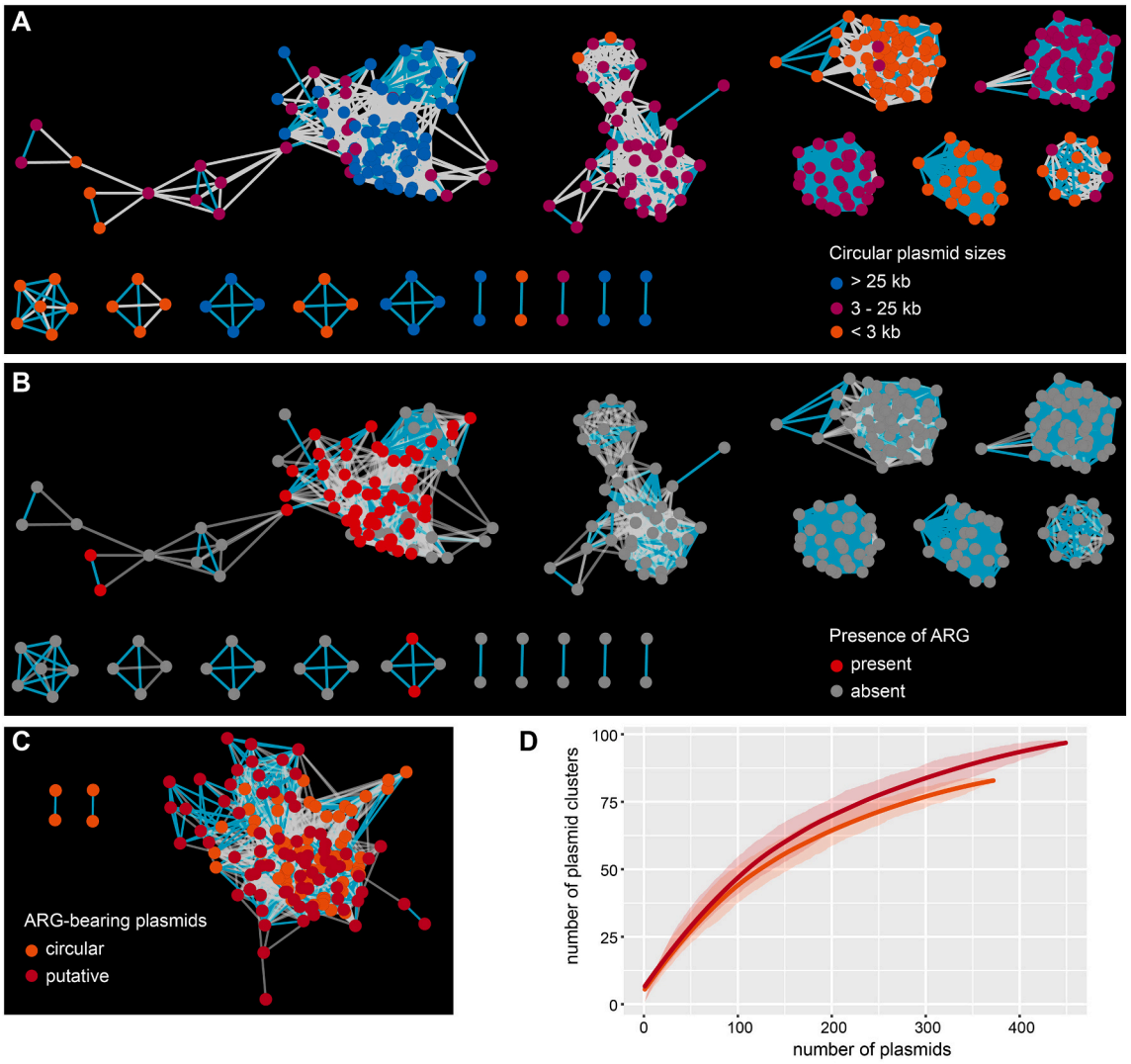

Fig. 2. Network based on pairwise mash distances between plasmids.Edges with >0.12 mash distance had been removed prior to network construction, edges with <0.06 mash distance are highlighted in green. Closed plasmids (372) are colored according to (A) their size or (B) the presence of an ARG. (C) All 77 putative ARG-bearing plasmids fell into the main group of large and medium-sized circular resistance plasmids. (D) Rarefaction plot of 372 complete plasmids (orange) and all 449 plasmids (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

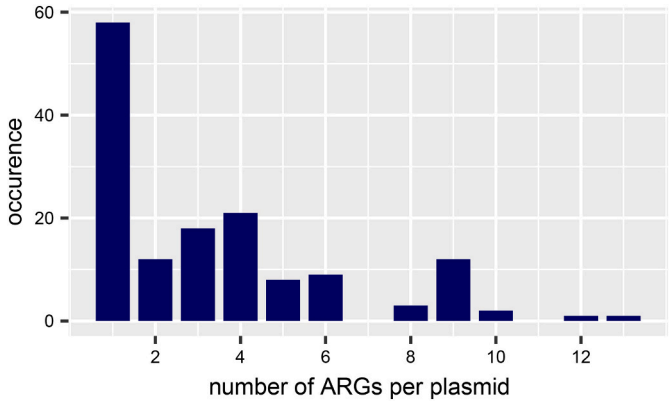

Fig. 3. Distribution of the number of ARGs one complete and putative resistance plasmids. Only the 145 ARG harboring plasmids are shown.

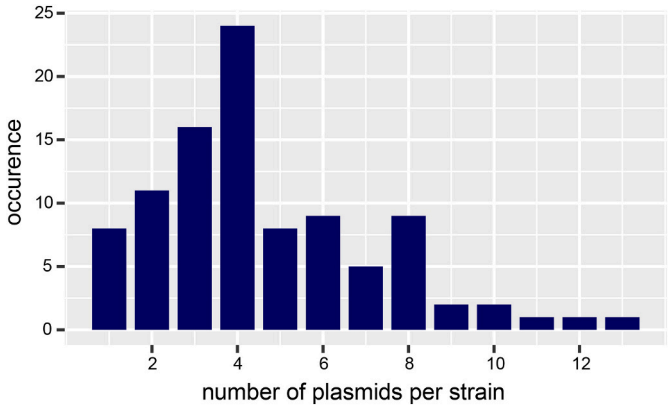

Fig. 4. Distribution of the number of closed and putative resistance plasmids per strain.

(ec_00024, ec_00180). Each isolate harbored a large (123 kb and 143 kb, respectively) conjugative plasmid of cluster AA026, containing IncFIA, IncFIA, IncFIB, IncFIC replicons.

More than half of the isolates belonged to ST131 ($n = 59$). Among those isolates, we found 47 plasmids (in 23 clusters), which were not observed outside ST131. Vice versa, in the non-ST131 isolates, 54 plasmids (in 29 different clusters) were not found in ST131 isolates. Since both, the ST131 and the non-ST131 specific plasmids fell into diverse small clusters, it seems that, in general, there is no specific association of sequence types and distinct plasmid clusters. Plasmid incompatibility occurs when multiple plasmids within one cell have the same replicon and/or partitioning system. Nevertheless, we found more than one plasmid of a single cluster in two clinical isolates. In the first clinical isolate (ec_00102) two plasmids of the same cluster were identified, which were typed as replicon type Col(MG828). Plasmid incompatibility in ColE-1-like plasmids can be circumvented by the introduction of SNPs in replication controlling genes [22]. We found 72 SNPs across the sister plasmids, which included the coding sequences of replication genes. In the second clinical isolate (ec_00020) we found two plasmid pairs, each of which was assigned to the same cluster (AA011, AA051). The first plasmid pair belonged to replicon type cluster 1778 with the two different replication initiation genes *repE_3* and *repE_4* for P148 (4072 bp) and P155 (4082 bp), respectively. The second plasmid pair belonged to two different replicon types (replicon type cluster 2150 and 3131). These plasmids were similar to replicon type Col156 plasmids.

2.6. Mobility and co-occurrence of ARGs in clinical isolates

More than half of the ARGs were located on conjugative plasmids (259 of 491 genes). We further identified associations between ARGs and transposons (Tn) or insertion sequence elements (ISE) on the plasmid. Additionally, we evaluated whether a conjugative plasmid was present in an isolate, so that other plasmids became co-mobilizable (Fig. 5 A). 373 ARGs were located within or in close proximity to a Tn or ISE site. Only 43 ARGs (9%) were not associated with a mobile genetic element (MGE) of any type (Fig. 5 B, Supplementary Fig. S2). Of these ARGs, 33 were found on putative plasmids (Supplementary Table S3).

Certain combinations of ARGs were repeatedly found. The joint presence of *catB3, AAC(6`)-lb-cr* and *OXA-1* was detected in five isolates on conjugative plasmids (in cluster AA017, AA019), but also on four non-mobile putative plasmids without a Tn in close proximity (in cluster AA093) and on a non-mobile plasmid but with Tn or ISE association (AA055). We detected $bla_{\text{CTX-M-15}}$ and *AAC(6`)-lb-cr* on four IncF plasmids with multiple replicons. Furthermore, we repeatedly found co-occurrence of *mphA, sul1, aadA5, dfrA17* as well as $bla_{\text{CTX-M-27}}$*, tet(A), sul2, APH(3″)-Ib, APH(6)-Id*. Both cassettes were found co-occurring in the same isolate (Supplementary Fig. S2). Interestingly, we discovered that two genes, which confer resistance to macrolides (*mphA* and *ermB*), were present together on two different plasmids (P307 and P311) in a single clinical isolate (ec_00166). Two highly similar plasmids of replicon type IncX1 (AA067, P296 + P297) contained *mphA and* $bla_{\text{TEM-158}}$*, qnrS1,* respectively.

Further combinations of ARGs were found only once. These included the presence of $bla_{\text{SHV-134}}$, which so far has only been described in *Klebsiella pneumoniae* [31], together with *tet(A)* on a 106.5 kb conjugative plasmid of replicon type IncI-gamma/K1. Moreover, one clinical isolate harbored a gene encoding the narrow-spectrum β-lactamase *LAP-2*. This β-lactamase has been described in an *E. coli* isolate from Vietnam for the first time and has since then has been found in isolates from Canada [1]. To our knowledge, it has not been described in Europe before. In this study, the *LAP-2* gene was found in an isolate that also harbored *qnrS1,* $bla_{\text{CTX-M-27}}$*, sul2* as well as *APH(6)-Id* and *APH(3″)-Ib* on a 80 kb large plasmid of IncFIA, IncFIC replicon type. Of note, *LAP-2* and *qnrS1* have previously been described to occur together [29].

The two strains ec_00140 and ec_00141 belonged to different STs (ST131, ST636). Both strains harbored two very distinctive ARG incorporating plasmids. Each had a conjugative plasmid with *mphA, QnrS1,* $bla_{\text{TEM-158}}$ (cluster AA067) and a phage like plasmid with $bla_{\text{CTX-M-15}}$ (cluster AA060). This could indicate a HGT mechanism transferring both plasmids together.

2.7. Prevalence and distribution of $bla_{CTX\text{-}M}$ variants

β-lactam resistance in clinical *E. coli* isolates is largely conferred by $bla_{\text{CTX-M}}$ genes on plasmids [40]. In the present study, we detected

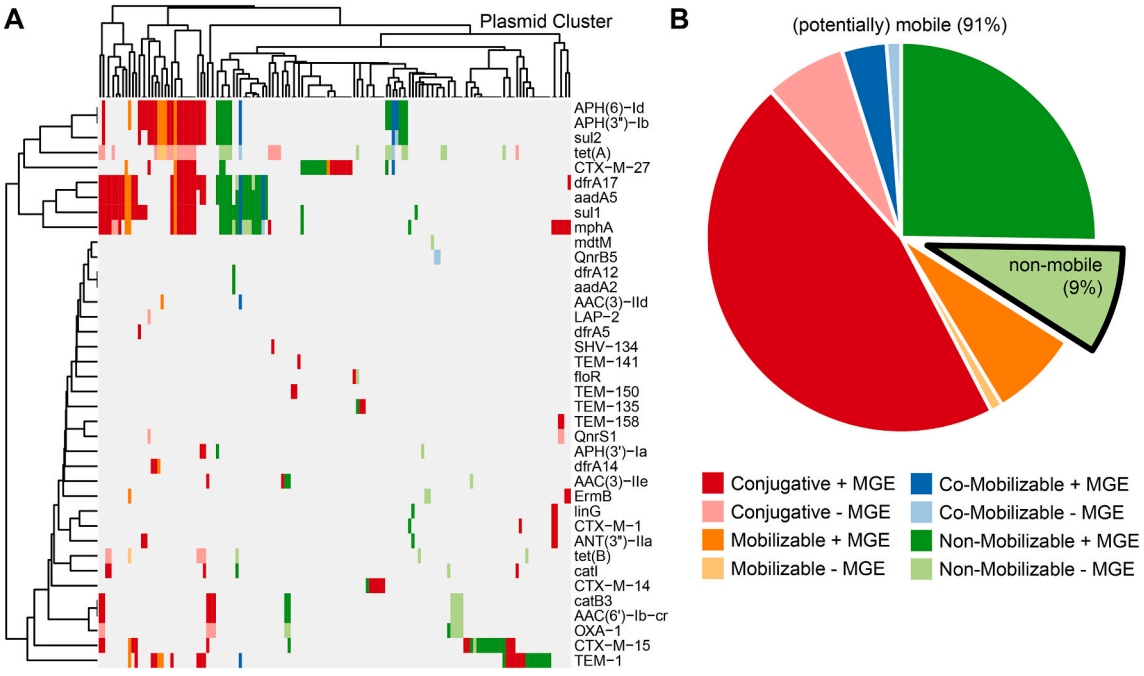

Fig. 5. Mobility potential of ARGs on plasmids. (A) Co-localization and mobility of ARGs within plasmid clusters. A detailed version can be found in Supplementary Fig. S2. (B) Distribution of different levels of mobility of ARGs on plasmids. See Material and Methods section for a definition of the levels of mobility.

bla$_{CTX-M}$ genes on plasmids in 61 out of 97 strains. *bla*$_{CTX-M-15}$ ($n = 23$, associated with ST131 clade C2) and *bla*$_{CTX-M-27}$ ($n = 29$, associated with ST131 clade C1) were most frequently found. Six and four plasmids harbored *bla*$_{CTX-M-14}$ and *bla*$_{CTX-M-1}$, respectively. Different *bla*$_{CTX-M}$ alleles were never detected together on the same plasmid or in the same strain (Supplementary Table S3). However, one strain (ec_000204) harbored two *bla*$_{CTX-M-15}$ genes on putative plasmids (PutP41 + PutP21). All *bla*$_{CTX-M}$ genes which we found on putative plasmids were Tn or ISE associated, with only one exception (PutP4, 1.4 kb), for which the genomic surrounding remains unclear due to incomplete assembly.

Eleven plasmids harbored *bla*$_{CTX-M-15}$ as the only ARG. Of those, eight were of unknown replicon type (AA069), two of replicon type 488 (AA060) and one IncX1. Furthermore, *bla*$_{CTX-M-15}$ was present in conjunction with other ARGs on two IncI−gamma/K1 plasmids and on seven IncFIA plasmids. Overall, we found four plasmids with *bla*$_{CTX-M-15}$ or *bla*$_{CTX-M-27}$ genes that were < 25 kb and not typeable. They clustered with the IncX plasmids and despite their smaller size, they might have originated from them. The smallest plasmid on which we detected *bla*$_{CTX-M-15}$ was 3.9 kb (P138), non-mobile and with no association of a transposon nor other ARGs.

Within the C1 clade [7], *bla*$_{CTX-M-27}$ was found on plasmids in 20 of 24 strains (Fig. 6). The dissemination of *bla*$_{CTX-M-27}$ among strains of the ST131 subclade C1 might indicate a clonal origin [26]. Alignment of closed plasmids with *bla*$_{CTX-M-27}$ in the ST131 isolates showed that the smaller plasmids were deprived of locally collinear blocks (LCB), which held replication genes that were found in larger plasmids (Supplementary Fig. S3). Thus, the smaller plasmids might have lost the redundant replication genes from a common ancestor plasmid. 18 *bla*$_{CTX-M-27}$ carrying plasmids were of multireplicon type IncFIA, three with multireplicon IncFIB and additional eight plasmids with no replicon type were identified. Outside ST131, we found *bla*$_{CTX-M-27}$ on multi-replicon IncFII or non-typeable plasmids in ST1193. The different incompatibility group may indicate different evolution in ST1193 as compared to ST131 after acquisition via HGT or acquisition from a different source.

2.8. Genetic basis of the ESBL phenotype in the clinical isolates

145 ARG-bearing plasmids were isolated from overall 88 of the 97 (91%) clinical ESBL-Ec isolates. The phylogenetic relationships among all strains and information on the presence/absence of all plasmid encoded ARGs in the individual isolates can be found in Supplementary Fig. S4. Plasmid encoded genes that confer resistance to cephalosporins (*bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$) were found in 73 strains (75%). Of those, 61 isolates harbored plasmid encoded *bla*$_{CTX-M}$ genes, which confer the ESBL phenotype.

In order to unravel the genetic basis for the ESBL phenotype also in those clinical *E. coli* isolates for which no plasmid-associated resistance-conferring gene could be identified, we searched for additional ARGs on chromosomal contigs. Among the 30 identified chromosomal *bla*$_{CTX-M}$ genes, *bla*$_{CTX-M-15}$ was found most often (in 25 isolates), *bla*$_{CTX-M-14}$ and *bla*$_{CTX-M-27}$ was found twice each and *bla*$_{CTX-M-55}$ once. Five strains had two chromosomal copies of *bla*$_{CTX-M-15}$ and one strain had two chromosomal copies of *bla*$_{CTX-M-27}$. Three ESBL-Ec isolates (ec_00080, ec_00132, ec_00209) had a chromosomal and a plasmid located *bla*$_{CTX-M-15}$ gene. Most *bla*$_{CTX-M}$ genes on chromosomal contigs were in close proximity to an ISE.

In conclusion, we found 97 *bla*$_{CTX-M}$ genes (on plasmids and/or chromosomal) in 87 out of the 97 ESBL-Ec isolates, based on the hybrid assemblies (Supplementary Table S4). We then went back to the draft assemblies based on short sequencing reads only and searched again for ARGs in the remaining 10 ESBL-Ec isolates. We detected *bla*$_{CTX-M}$ genes in additional seven isolates. For one of the remaining three *E. coli* isolates (ec_00021) with no detected *bla*$_{CTX-M}$ gene, the resistance phenotype could not be verified. This leaves us with two of the 97 ESBL-Ec isolates with undetected genomic resistance determinants (ec_00012, ec_00161).

3. Discussion

3.1. Highly diverse plasmidome of clinical isolates

In this study, we used a combination of 2nd and 3rd generation sequencing technologies to describe the plasmidome of overall 97

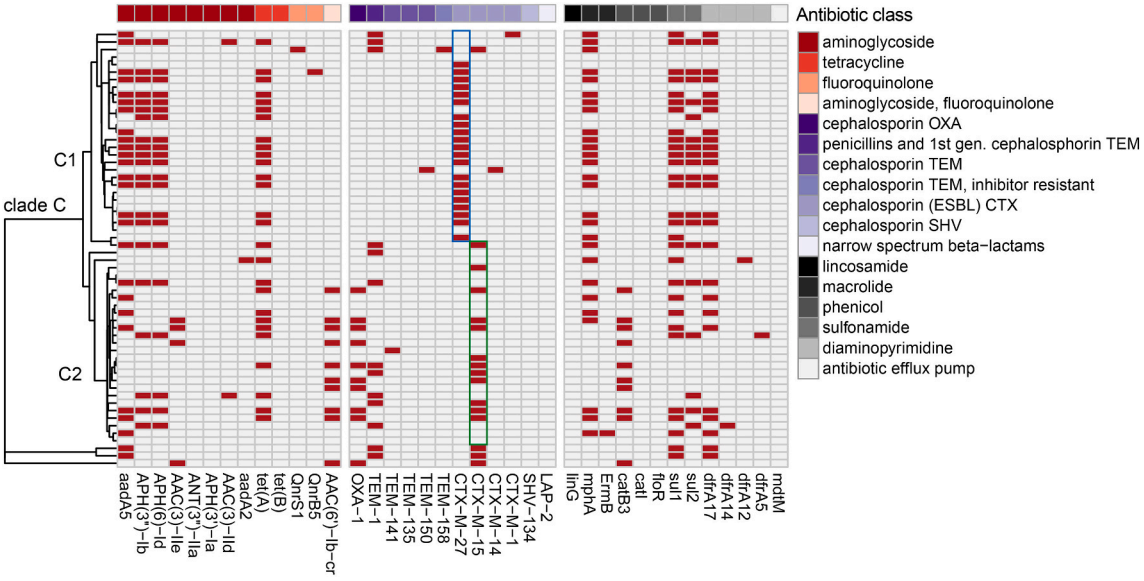

Fig. 6. Presence of ARG families on plasmids in phylogenetically related isolates of ESBL-Ec ST131. The various classes of antibiotics against which the genes (listed at the bottom) confer resistance to are represented in colour codes at the top. Red and grey boxes in the heatmap indicate presence and absence of the ARG, respectively. The distribution of *CTX-M-27* and *CTX-M-15* alleles in Clade C1/C2 isolates is highlighted. Empty columns indicate the absence of this gene among ST131 isolates, but presence in non-ST131 isolates. Supplementary Fig. S4 shows ARG presence in isolates across all STs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

clinical ESBL positive *E. coli* isolates. Our results on 372 fully and 77 incompletely reconstructed plasmids highlight the enormous plasmid diversity not only in respect to their size, replicon-types and genetic content, but also in respect to their distribution pattern among the various clinical isolates. Only 76% of the plasmids were typable using the PlasmidFinder scheme, reflecting what has been described before for plasmids of *Enterobacteriaceae* [15,36]. The plasmid richness and diversity of ESBL-Ec isolates reflects a species that it is open to plasmid acquisition and loss [28]. However, associations between certain ARG bearing plasmids and lineages are extremely prevalent in the clinical context and might be the result of positive selection for plasmid-encoded traits and/or the acquisition of compensatory mutations to reduce the fitness costs [48]. The flattening of the plasmid-cluster accumulation-curve observed in this study might reflect these lineage-plasmid associations, which limit overall plasmid diversity.

In general, we found that larger plasmids were more diverse. Particularly, IncF plasmids harbor a multitude of nested MGEs, which makes them prone to insertions, deletions and recombination (Coque, 2008, Caralotti, 2013, [39]). This mosaicism, which is also reflected in the occurrence of various multireplicon combinations [46], challenges biomarker typing [15,36]. It also contributes to the large number of plasmid clusters described in this study, although some of the plasmids in different clusters seem to have emerged from a common ancestor.

3.2. Large and small plasmids as ARG vectors

We found ARGs in different combinations mainly on IncF plasmids, as has been described before [13,46]. Those large and medium-sized IncF plasmids harbored almost all of the overall 491 ARG identified in this study. Nevertheless, small cryptic plasmids were remarkable abundant and significantly contributed to the high plasmid profile diversity seen among our clinical isolates. The majority of the 97 isolates harbored at least one SCP, one clinical isolate harbored 10 SCPs. Up to six SCPs in ESBL-Ec isolates have been previously described [10]. This diversity corresponds to the observed frequent flux of SCPs between different ESBL strains in the microbiota of patients [11,28]. Without bringing an obvious benefit to the host, the presence of SCPs is still of largely unknown biological consequences. SCPs have been suggested to be possible “moldable vectors” for the transfer of ARGs [4,61]. We found genes conferring resistance against various classes of antibiotics on SCPs, however, at a much lower rate than on large plasmids. Two SCPs in two clinical isolates of different ST-types harbored quinolone resistance conferring (*qnrB*) genes. Those were identical to pHAD28 (KU674895.1) from a *S. enterica* isolated from German poultry abattoirs in the years 2007–2010 [24]. This indicates a stable circulation of *qnrB* alleles on SCPs in *Enterobacteriaceae* and a connection to food production. *QnrB* alleles are also found on large plasmids within ARG cassettes, but their widespread dissemination also on small ColE1-like plasmids [37] indicate that SCPs might be an underestimated yet frequent ARG vector.

Overall, 60% of the plasmids analysed in this study carried more than one ARG (Supplementary Table S3). This finding supports the genetic capitalism principle suggesting that AMR genotypes are often gained but not lost, thus leading to the accumulation of ARG [25]. We found 66% of all ARGs to be Tn or ISE associated, which highlights their multi-level mobility. Since our cut-off for a Tn or ISE association was very strict and possible MGE association of the ARG on non-fully assembled plasmids might have been missed, the potential mobility of ARG genes might even exceed 91%. Our findings imply a high potential for HGT of ARGs via plasmids or other MGEs and emphasize the mosaicism of many ARG-bearing plasmids. One question left to be answered is the role of multiple copies of an AMR gene like the 13 copies of TM-135 on one plasmid of isolate ec_00067. While this high number of genes probably is a burden to the cell, it did not result in an exceptional AMR profile (Supplementary Table S1).

3.3. Genomic context of $bla_{CTX\text{-}M}$ genes

Although no ST131-specific plasmid cluster association was found in this study, we clearly observed an Inc-type/$bla_{\text{CTX-M}}$ association within the ST131 C1/C2 subclades as previously described (Kondratyeva et al., 2020). While the ST131 C2/H30Rx clade is internationally predominant and associated with the $bla_{\text{CTX-M-15}}$ allele, there are reports in Europe of an emerging subgroup (C1/H30R) with other $bla_{\text{CTX-M}}$ alleles, such as $bla_{CTX\text{-}M\text{-}14}$ and $bla_{\text{CTX-M-27}}$ [7,26,34]. The reasons for the association of certain clonal lineages with certain epidemic resistance plasmids are uncertain [32]. It seems that the $bla_{\text{CTX-M-27}}$ allele was incorporated independently into different plasmids in various strains of the C1 subclade, as the genomic surrounding of the gene is different to isolates from other regions and species [6]. All ST131 $bla_{\text{CTX-M-27}}$ alleles identified in this study were associated with a Tn or ISE on either a multireplicon IncFIA or a non-typeable plasmid, while in ST1193 isolates $bla_{\text{CTX-M-27}}$ was localized on IncFIB plasmids. The various ST131 C1 subclade isolates also differed only by a maximum of 109 SNPs, suggesting their clonal spreading. Furthermore, the alignment of the closed $bla_{\text{CTX-M-27}}$ carrying plasmids revealed that they consisted of the same building blocks. This indicates that the $bla_{\text{CTX-M-27}}$ harboring plasmids might have a common ancestor, arguing for a clonal distribution of ST131 C1 isolates in Germany and other European countries, rather than HGT events to disseminate $bla_{\text{CTX-M-27}}$ [6,26].

We found chromosomal localization of all $bla_{\text{CTX-M}}$ genes in only 33% ($n = 29$) of the 97 ESBL positive strains. Chromosomal integration of $bla_{\text{CTX-M}}$ genes via ISE has been described before, however at low frequency [16,18]. It seems that the frequency of chromosomal integration is approximately 10 times lower for $bla_{\text{CTX-M-27}}$ compared to $bla_{\text{CTX-M-15}}$ [27]. In the present study, one single strain harbored $bla_{\text{CTX-M-27}}$ chromosomally. The isolate harbored even two copies probably due to recurrent transposition and chromosomal integration [27]. Chromosomal integration of $bla_{\text{CTX-M-15}}$ genes was observed at a higher rate. However, as it was not restricted to a specific phylogenetic group, it does not seem to play a major role in strengthening the ST131/$bla_{\text{CTX-M}}$ gene association.

Of the total of 97 *E. coli* isolates in this study (92 of which were confirmed to have an ESBL phenotype), we were unable to identify ESBL-encoding genes in only two cases. Five isolates were without a phenotypic profile from the clinic, of those all exhibited an ESBL positive genotype. However, this detection rate required detailed re-analysis of also the draft short read based assembly, generated with Spades (see Material and Methods), as not all $bla_{\text{CTX-M}}$ alleles were detected in the hybrid assemblies. Obviously, the different filtering parameters, which were applied in the hybrid assembly approach, led to a loss of contigs with $bla_{\text{CTX-M}}$ genes. This loss of genomic information occurred at different stages during the assembly process, indicating that the single steps and filtering parameters of the hybrid assembly process need to be optimized, so that robust retrieval of complete genome sequence data becomes possible.

4. Material and methods

4.1. Clinical isolates collection

ESBL-Ec isolates were kindly provided by a large municipal hospital in Lower Saxony. The isolates were collected from April 2019–April 2020. In total 234 isolates were collected and sequenced with Illumina technology. Short read sequencing was performed prior to ONT long read sequencing. Based on the short read assemblies the ST of the isolates was determined in silico. Of this strain collection, 97 isolates were chosen that represent the ST variation and yielded a sufficient amount of plasmid DNA. Eight patient duplicates are included, i.e. strains originating from the same patient but not necessarily from the same sampling site. Phenotypic resistance testing and interpretation was done in the hospital, based on the EUCAST guidelines implemented in the VITEK2

system v8.02 (parameter set 2018 and 2019). For six strains we did not obtain an ESBL phenotypic resistance profile, but these were however included in the ESBL strain collection and therefore included in the present study.

4.2. Illumina sequencing

Several colonies from an LB agar plate after overnight incubation were resuspended in 50 µL PBS and frozen until further processing. DNA extraction was performed using the DNeasy Blood & Tissue Kit with standard protocol for bacterial pellets on the Qiacube automated system (Qiagen). The library preparation was based on a modified version of the Illumina Nextera XT protocol with unique dual indices [5,57]. Different from the protocol the input DNA concentration was increased to 0.8 ng/µL to avoid overtagmentation. The library pool was size selected on the BluePippin™ (Sage Science) instrument with parameters set to 500–1500 bp. Prior to size selection and afterwards the fragment length profile was resolved again on the Agilent 2100 Bioanalyzer Pico Chip (Agilent Genomics) to confirm the exclusion of small fragments. Paired end sequencing in 150 bp mode was conducted either on a NextSeq 500 or on NovaSeq 6000 instrument.

4.3. Plasmid preparation

Plasmid enriched DNA was extracted with the QIAprep Spin Miniprep Kit (Qiagen) with the following modifications from the manufacturer's protocol. The bacterial pellet from overnight culture in LB Medium was frozen at −20 °C for at least 15 min. After the extraction and washing steps the DNA was eluted by application of 50 µL of prewarmed nuclease free water (50 °C), incubation at room temperature for 2 min and centrifugation at 13.000 rpm for 1 min. Isolates that did not yield the required amount of DNA for long read sequencing after repeated plasmid preparations were excluded from the study.

4.4. Library preparation and long read sequencing

Plasmid enriched DNA extracts of the majority of the strains was prepared for sequencing with the Rapid Barcoding Sequencing Kit (SQK-RBK004) according to manufacturer's instructions. For three strains (ec_00012, ec_00019, ec_00029) the Ligation Sequencing Kit in addition with Native Barcoding Kit library preparation (SQK-LSK109, EXP-NBD104/114), was applied. In these cases, a shearing step with Covaris g-tubes (Covaris) was included to linearize circular structures.

However, as part of another study high molecular weight DNA of few clinical isolates (ec_00003 and ec_00022) was prepared with the Ligation Sequencing Kit in addition with Native Barcoding Kit library preparation. For that, the DNA was extracted as previously described [47] but not sheared during library preparation.

After flow cell priming and library loading were done with the Flow cell Priming Kit (EXP-FLP002), sequencing was performed on a MIN106D R9 Flow Cell (ONT).

4.5. ONT base calling and genome assembly

ONT base calling was performed with Guppy v3.1.5 in high accuracy mode. Short (150 bp, obtained on either Illumina NextSeq or NovaSeq) were combined with long reads generated with ONT MinION. Unicycler v0.4.8 [62] was used with default settings to construct high quality hybrid assemblies. Completeness of the assembly was confirmed by determining the coverage by the longest ONT read (median: 99%, Supplementary Table S3).

4.6. Phylogenetic analysis and ST determination

To relate the presence of plasmids in clinical *E. coli* strains to the phylogenetic background we used a reference based core genome approach. As reference the chromosome of ec_00010 (ST131) was used. The alignment of short reads with variant calling was generated in *snippy* v4.6.0 [52]. The snippy-core functions was used to generate a core genome alignment of all strains within the ST. An initial alignment calculated by *snippy-core* was analysed regarding false positive positions. If false positive variants were called in core positions between the reference genome and corresponding short reads, these positions were corrected using the consensus sequence provided by the tool. The observed variant positions were first checked for support in the variant calling file. The mapping procedure was then repeated. The full alignment produced by *snippy-core* was then processed in *gubbins* v2.4.1 to exclude regions of recombination [19]. SNP positions that were masked by *gubbins* were subsequently excluded by *snp-sites* v2.5.1 [38]. The pairwise SNP distance was determined with *snps-dists* v0.7.0 [49] based on the final alignment.

In silico multilocus sequence typing (MLST) was perfomed with pubMLST v2.19.0.

For the determination of subclades of ST131 we used the marker region described by Birgy et al. [7]. The fragment of the closed chromosome of ec_00010 was used as blast database against which the short read assemblies of all strains were blasted with coverage and identify set to 100% [7].

4.7. Assembly of illumina reads

The paired and unpaired reads of each isolate were used to produce the assemblies with Spades v3.13.0. Draft assemblies were analysed using *QUAST* v5.0.2 (Gurevich et al. 2013). Only assemblies with a minimum N50 value of 100 kbs were considered for subsequent analysis steps to avoid fragmentation.

4.8. Determination of plasmid contigs

Presumed circular plasmid contigs were extracted from the WG assembly, if they were circular and their length below 1000 kb. The size criterion is based on Orlek et al. who found the largest plasmid in Enterobacteriaceae to be 750 kb [36]. Plasmids that are linear are not accounted for in this analysis. According to a recent publication [23] the mob-typer software could type more plasmids than the classical PlasmidFinder database, for this reason the typing was performed using mob-typer.

Mob-recon and mob-typer v3.0 software (database download 20.06.2020) was used to find typical plasmid genes, perform a mobility typing and find a close relative in the database that is part of the software. If these typical genes not found and mob-recon designated the contig as chromosomal, the contig was excluded from the plasmid dataset. However, we found the smallest plasmid (P1) to match an entry in NCBI (accession: CP058111) despite being not designated as plasmid by mob_typer, we included the contig. The entry in NCBI was submitted after the database download (07.08.2020) and not included in the mob_typer database yet. The tool allows for expanded incompatibility typing by also including replication proteins have not been assigned to a known incompatibility group, designated replicon types are marked with numbers [45]. A plasmid was classified 'conjugative'if at least a relaxase and a mate-pair formation marker was located on the contig. Plasmids are 'mobilizable', if they either have a relaxase or an *oriT* but are missing the mate-pair formation marker and 'non-mobilizable' if they are missing a relaxase and an *oriT*. The plasmids were annotated using PROKKA v1.14.6 [51].

4.9. Detection of AMR genes

Plasmid-encoded AMR conferring genes (ARGs) were identified in silico by using ABRicate v0.9.9 [50] searching the integrated Comprehensive Antibiotic Resistance Database (CARD, [33]), downloaded on 20.02.2020, with a coverage filter set to 50%.

4.10. ISFinder/Phaster/PlasmidFinder analysis

For the detection of transposable elements on plasmids the dataset was blasted against the ISFinder database ([55], database accessed: 16.09.2020). The following settings were used: gap open penalties for existence 5 and for extension 2; word size 11; e-value 1e-50.

To elucidate single structures further about replicon type and phage content the web servers PlasmidFinder ([15], database access: 13.07.2020) and PHASTER [2] were used.

4.11. Analysis and visualization

To test if clusters of plasmids shared more identities according to the sizes within the clusters Analysis of Variance (AOV) was performed in R (R Core Team (2020). The ARG content was linked to the ISFinder output and filtered for transposons, which include ARGs or are located within 300 bp next to the ARG.

Rarefaction analysis was done by randomly subsampling the rarefied numbers of strains (1–97) and counting the respective plasmid clusters. Wilcoxon rank sum test with continuity correction was used to test plasmid size and ARG association.

Alignment of plasmids were created in progressiveMAUVE v20150226 [20] using locally colinear block (LCB) size of 1 kb.

4.12. Cytoscape network visualization

For the analysis of between cluster similarity and with cluster variation we determined the mash distances between each plasmid pair using mash v2.2.2 [35]. To visualize the distances between plasmids we used Cytoscape v3.8.1 [53] in prefused force directed layout default settings.

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Data availability

Long- and short-read sequencing raw data has been uploaded to the NCBI Sequence Read Archive under accession PRJNA769856 (http://www.ncbi.nlm.nih.gov/bioproject/769856).

CRediT authorship contribution statement

Lisa Neffe: Methodology, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Lisa Abendroth:** Investigation, Visualization. **Willfried Bautsch:** Resources. **Susanne Häussler:** Funding acquisition, Supervision, Writing – original draft, Writing – review & editing. **Jürgen Tomasch:** Methodology, Investigation, Visualization, Supervision, Writing – original draft, Writing – review & editing.

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Appendix A. Supplementary data

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