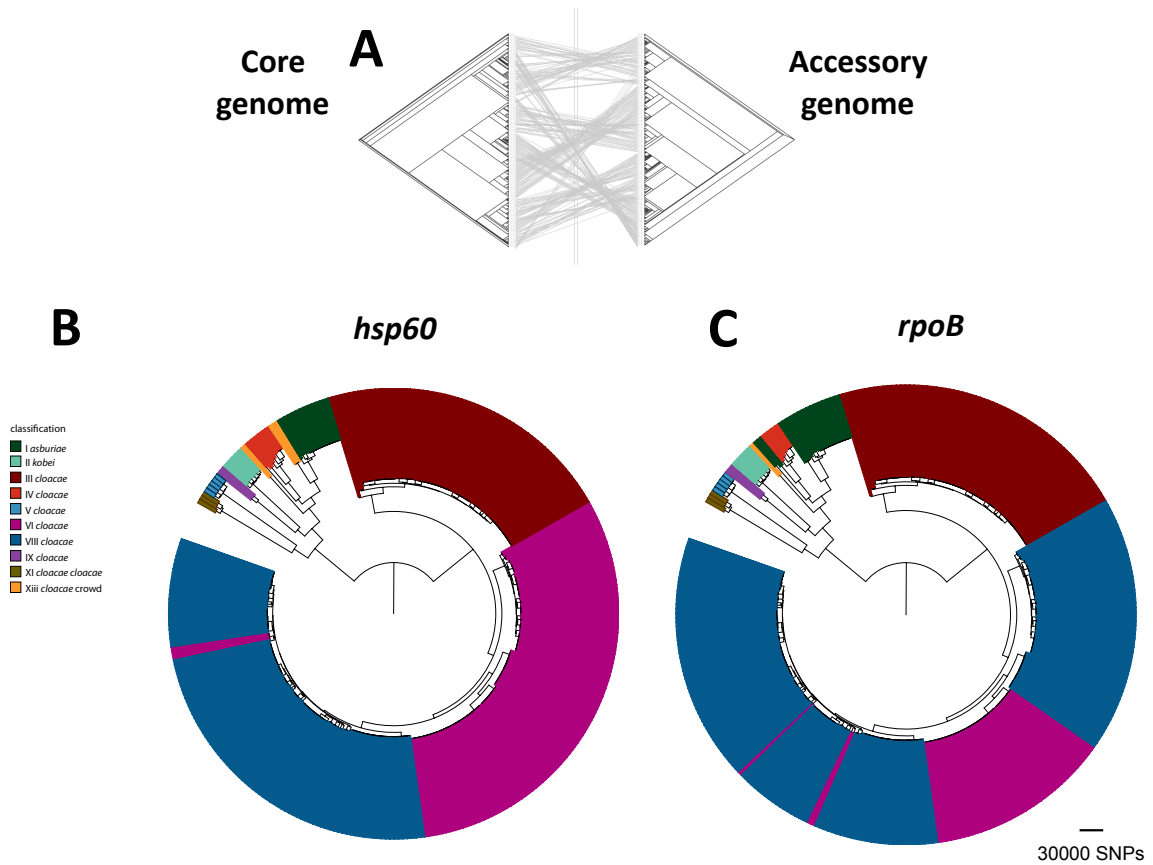**Supplemental Material**

The dissemination of multidrug resistant *Enterobacter cloacae* throughout the UK and Ireland

Danesh Moradigaravand, Sandra Reuter, Veronique Martin, Sharon J. Peacock and Julian Parkhill

Typing and comparative phylogeny

Supplementary Figure 1 A) Comparison between the core genome tree and the binary tree constructed from the absence-presence pattern of accessory elements. B) and C) The phylogenetic tree constructed from the core genome alignment and the species annotation from the variants analysis of *hsp60* and *rpoB* genes.

**Supplementary Notes 2**   **MLST comparison**

To allow comparisons with previous analyses of *E. cloacae*, we determined the MLST composition of the population [1] and found that around half of isolates belonged to eleven major STs (Supplementary Figure 2 A). Of these major STs, ST78, ST108 and ST114 were previously identified as the most widespread STs in a global collection [2]. This confirms that the UK is linked to the proposed global circulation of a limited number of clones [2] and suggests that other clones may have the potential to spread globally. The resolution of MLST was sufficient to discriminate between clones that are greater than 1000 SNPs apart (Supplementary Figure 2 B), and thus our results also show that MLST has satisfactory discriminatory power to identify major clones on the tree (Supplementary Figure 2 C).
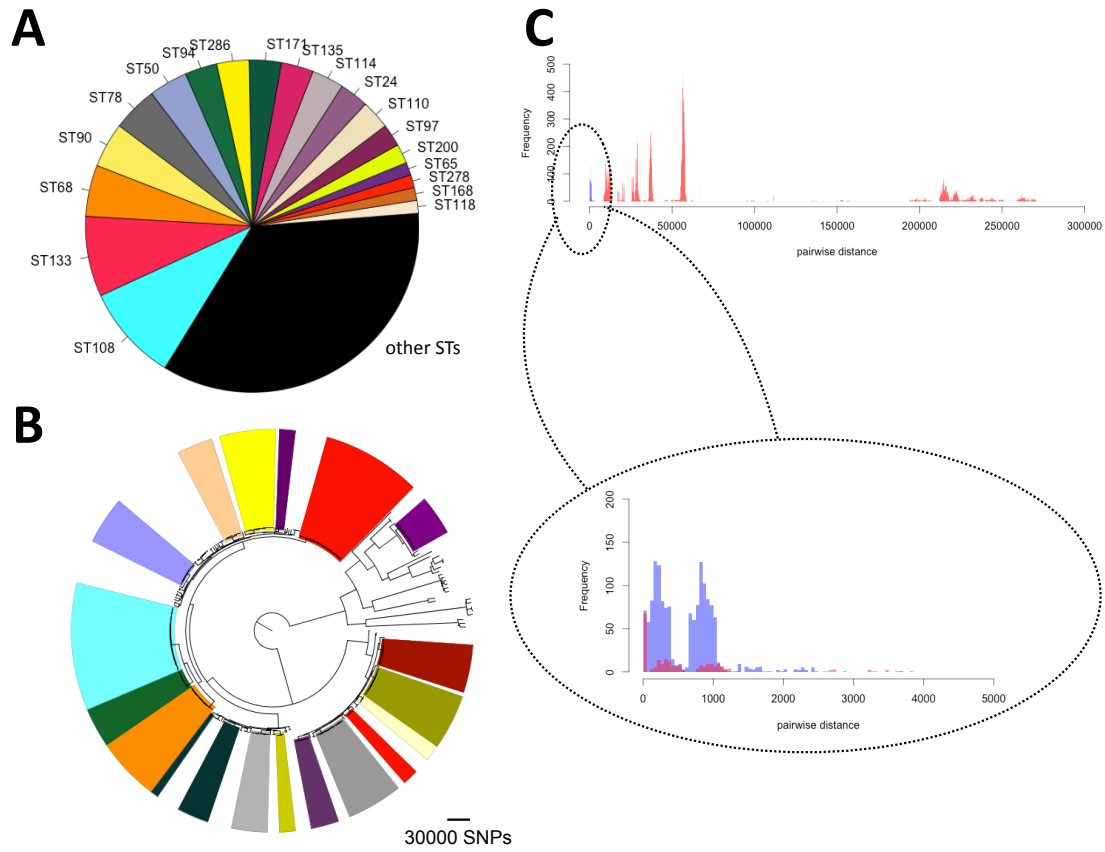
Supplementary Figure 2 A) MLST composition of the dataset. The results are sorted according the frequency of STs. Singleton STs are clustered as the other STs. B) Distribution of STs across the phylogenetic tree. Each colour corresponds to one ST shown in A). C) Pairwise SNP distribution for the core genomes for the pairs with the same (blue) and different STs (red).

# Supplementary Notes 3 Substitution rate estimation and linkage analysis
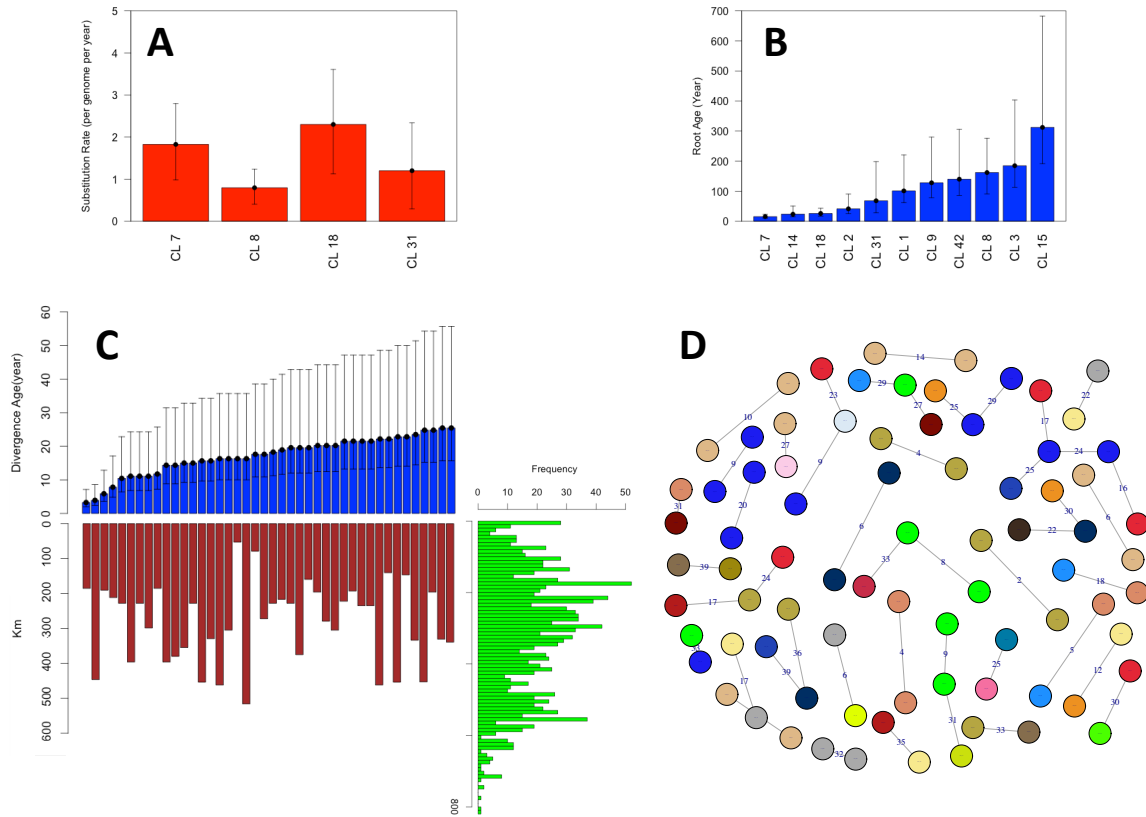
Supplementary Figure 3 A) The substitution rate estimated for four clusters with detectable temporal signals on the phylogenetic tree. The error bars show 95% confidence intervals. B) The calculated age of the Most Recent Common Ancestor (MRCA) for ten clusters detected on the phylogenetic tree. The error bars show 95% confidence intervals. For the clusters without a detectable temporal signal we used the mean substation rate of clusters 7, 8, 18 and 31 to estimate the MRCA, and the means of 95% confidence intervals for clusters 7, 8, 18, 31 to estimate the upper and lower confidence intervals. C) Isolates with close inter-hospital relationships in the last ~20 years. The blue plot shows the estimated time of the recent ancestor for each isolate pair of less than 40 SNPs distance. The results are sorted according to the time of divergence between samples. The error bars show 95% confidence intervals. To compute the upper and lower values of the confidence interval for the error bars, we divided the pairwise distances by the mean upper and lower values of the error bars of the substitution rates for the clusters in A). The brown plot shows the geographical distance between these pairs

and the green histogram shows the distribution of pairwise geographical distances for the whole collection. D) The connectivity network for samples within the collection at a SNP cut-off of 40. Each node corresponds to one sample. Each colour corresponds to one hospital. The edges show the connection between isolates that were <40 SNPs apart. The numbers show pair-wise SNP distances.
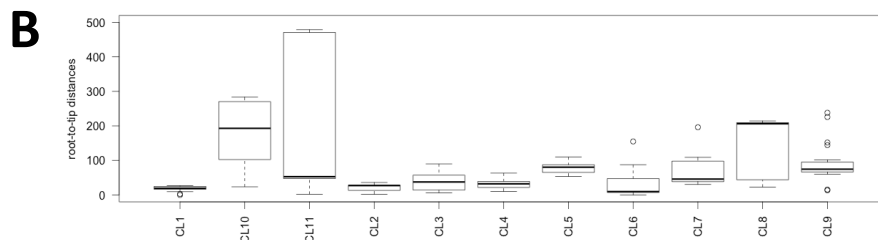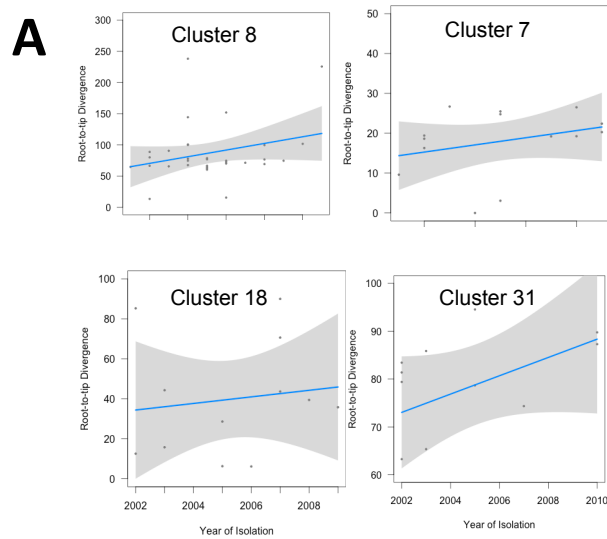

Supplementary Figure 4 A) Root-to-tip distances for the groups with temporal signal. The grey area around the fitted line is 95% confidence interval. B) Distribution of root-to-tip distances for the identified clades. The boxes give the interquartile range, the whiskers indicate the boundary of 1.5 times the interquartile range, and the points beyond that are outliers.
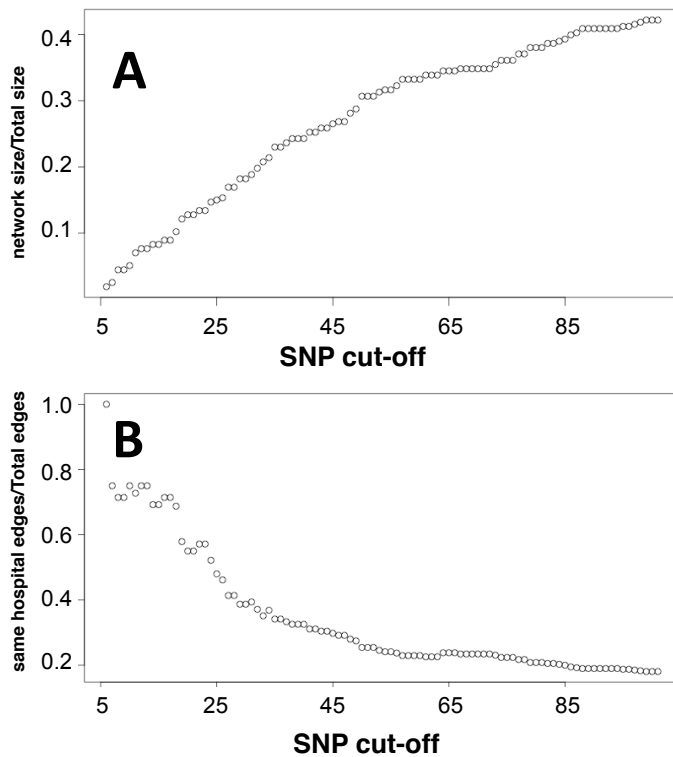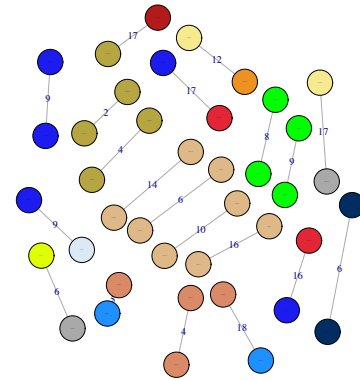
Supplementary Figure 5 A) Plot of the number of isolates in the connectivity network divided by total population size for different values of the SNP cut-off. B) Plot of the number of edges that connect two isolates of the same isolation origin (same hospital) divided by the total number of edges for different values of the SNP cut-off. 3% of total pairwise divergences occurred between isolates of the same hospital origin in the total dataset. C) Connectivity network constructed for the 20 SNP cut-off. The edges and numbers show links and SNP distances, respectively. Each colour corresponds to one hospital. Each node represents one isolate.

Supplementary Notes 4   **Plasmid analysis**

Plasmids can be difficult to identify directly from draft genomes assembled from short reads, as used here. We therefore searched the collection for the presence of known plasmid replicons using a database of known plasmid replicons and discovered several plasmids that appear to have been acquired from other gram-negative species by the isolates in our collection (Supplementary Figure 6). Amongst the major plasmids, HI2A Col156 (from *Escherichia coli*), R (from *Klebsiella pneumoniae*), FIAH1_H1 (from *Salmonella enterica*) and colRNA (from *Klebsiella pneumoniae*) were present in the majority of our samples (Supplementary Figure 3). Some of the plasmids (such as the conjugative R plasmid) are known to harbor virulence and resistance elements and are capable of transferring between gram-negative *Enterobacteriaceae* [3].
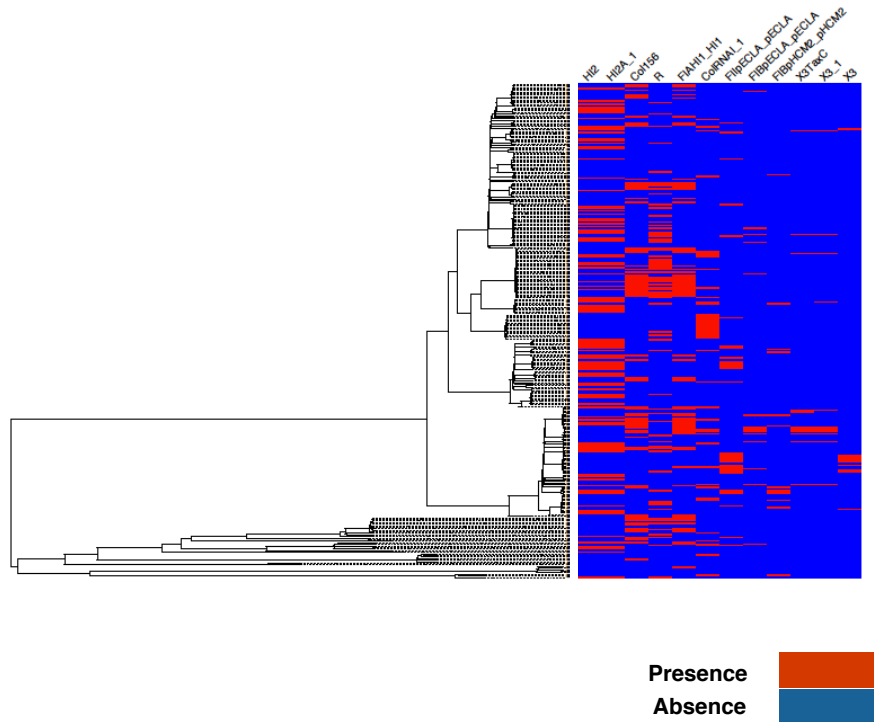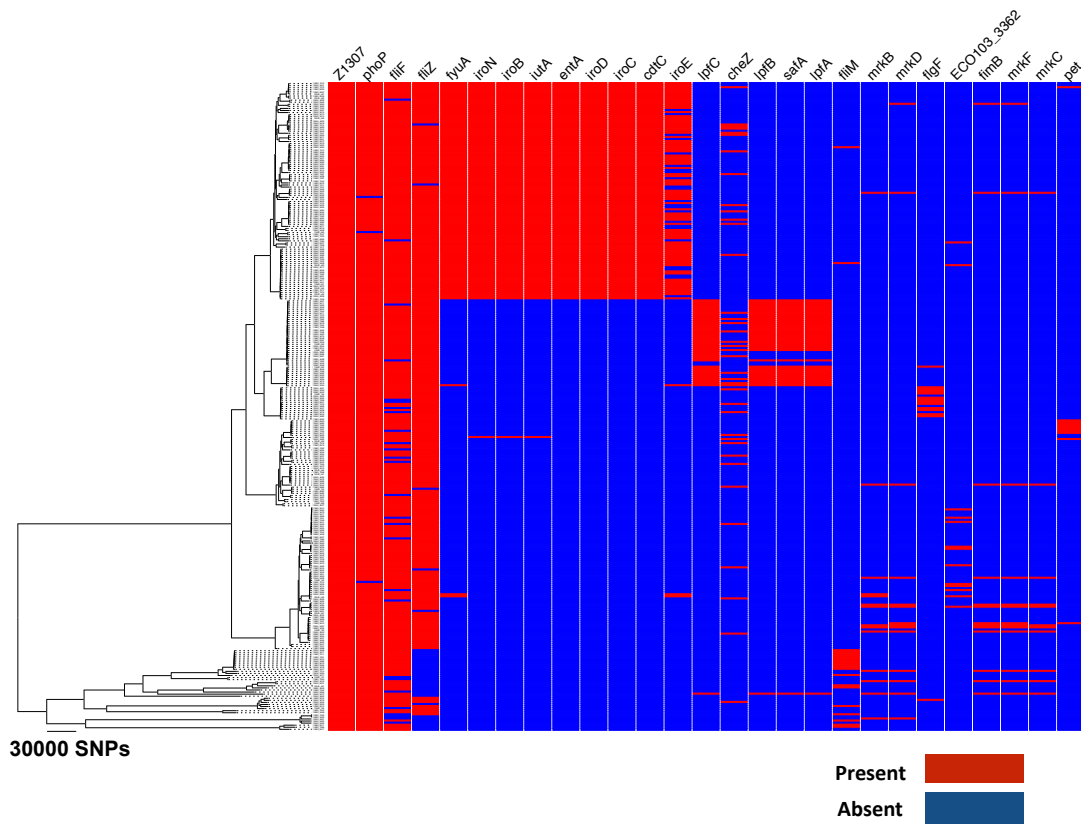
Supplementary Figure 6 Distribution of plasmid replicons from the PlasmidFinder database across the phylogenetic tree constructed for the core genome. Only plasmids present in more than 0.05% of the population are shown here.

Supplementary Notes 5   Virulence factors analysis

Knowledge about virulence factors in *E. cloacae* is scarce [4, 5]. Although recent studies have identified some factors, the exact involvement of these in the progression of disease is still unknown [4]. We therefore screened a database of known virulence factors and were able to identify several putative virulence factors in the population, including the outer membrane protein A (OmpA), the transcriptional regulator PhoP and flagella biosynthesis proteins (Fli) (Supplementary Figure 7). These factors can be involved in host-pathogen interactions and intra-macrophage survival in other pathogens, although they may also have other functions in environmental organisms. Other proteins such as siderophore production (Iro), the toxin subunit CdtC and fimbrial chaperones (Lpf) had only been acquired in some clades. The cytotoxin has been described previously as a virulence factor [6] and fimbrial proteins are believed to contribute to cell adhesion and biofilm formation, both of which are important in the process of disease development [7]. It seems likely, therefore, that pathogenicity of *E. cloacae* may vary both within and between clades.
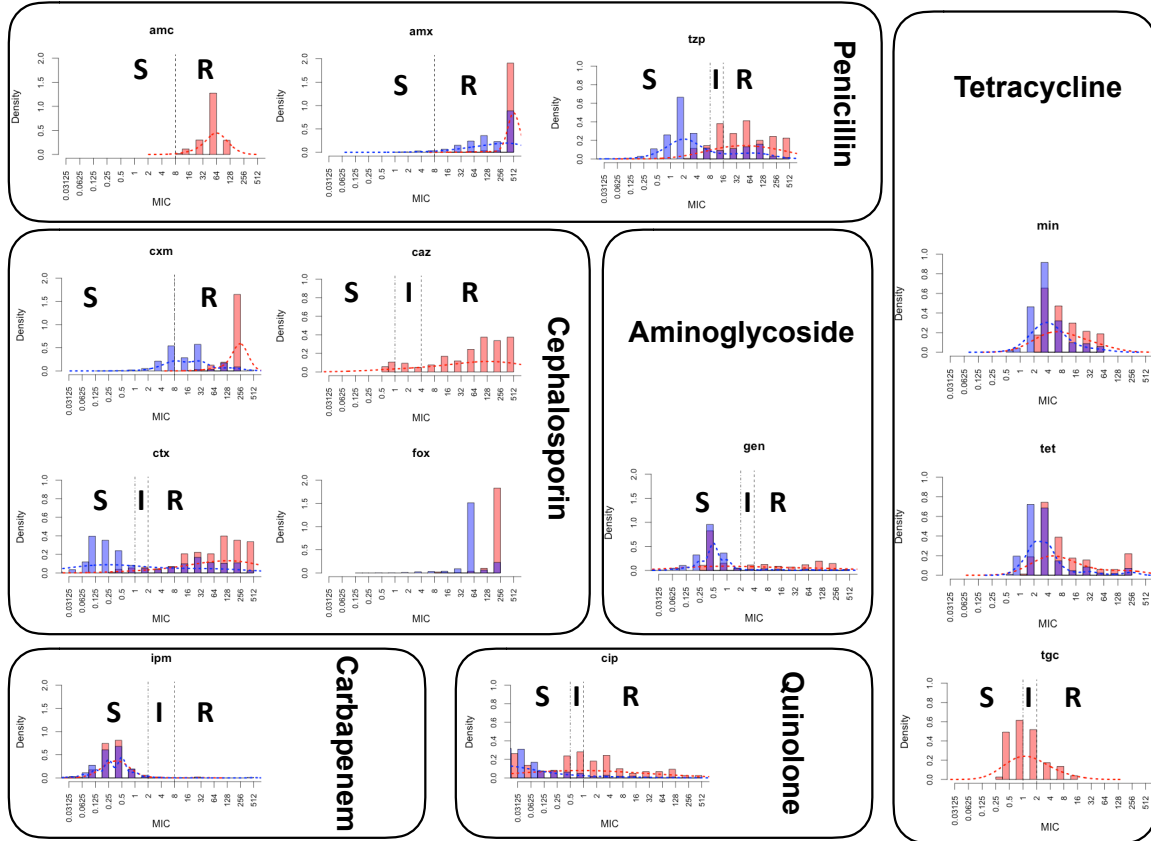
Supplementary Figure 7 Distribution of virulence factors identified by srst2 across the phylogenetic tree constructed for the core genome. Only virulence factors present in more than 0.05% of the population are shown here.

**Supplementary Notes 6**   **Antimicrobial resistance analysis**

Enterobacter cloacae is known to be intrinsically resistant to a wide range of beta-lactams including cephalosporins and penicillins. This is due either to the presence of extended-spectrum beta-lactamases or to the over-production of AmpC, which can be caused by changes in the regulation of the chromosomal *ampC* gene or the acquisition of additional *ampC* genes [4, 8]. Compared to virulence factors, the antimicrobial resistance determinants of *E. cloacae* have been more closely scrutinized. *E. cloacae* is intrinsically resistant to amoxicillin, amoxicillin–clavulanate, first-generation cephalosporins and cefoxitin [4]. By contrast, *E. cloacae* is widely susceptible to fluoroquinolones, aminoglycosides, piperacillin–tazobactam and carbapenems, but for some of these antibiotics increased resistance levels have been reported [9, 10].

The MIC values for thirteen antimicrobials from four different groups were available for the isolates in our collection. With the exception of cefoxitin, amoxicillin, cefuroxime and amoxicillin-clavulanate, there was considerable variation within the MICs (Supplementary Figure 8 and 9). As expected, variations in MIC tended to be correlated for antimicrobials with similar mechanisms of action (e.g. ceftazidime and pipercilin-tazobactam and amoxicillin and cefuroxime, and for the antimicrobials that are derived from each other (e.g. tigecyline and minocycline) (Supplementary Figure 9 A). We determined the phenotypes (resistance status) for the antibiotics with known clinical breakpoints. The findings showed that the collection was extensively resistant to different classes of beta-lactams, including ceftazidime (S:25, I:23, R:268), amoxicillin (S:0, I:0, R: 316), amoxicillin-clavulanate (S:3, I:0, R:313), cefotaxime (S:12, I:8, R:240), cefuroxime (S:0, I:0, R:316) and piperacillin-tazobactam (S:41, I:61, R:214). (Cefotaxime was included in the testing panel of antimicrobials from 2003, but not earlier). However, the collection exhibited a higher susceptibility to impenem (S:310, I:3, R:3), ciprofloxacin (S:136, I:45, R:135), gentamicin (S: 179. I:18, R:119) and tigecycline (S: 173, I:78, R:54) (Supplementary Figure 9 B and 10 A and 10 B). These data are largely in agreement with previous reports for *E. cloacae,* according to which non-beta lactam antimicrobials such as ciprofloxacin and gentamicin along with carbapenems still offer an effective treatment for *E. cloacae* infections [4, 5, 11].

Supplementary Figure 8 Distribution of MIC values for different antimicrobials for the BSAC collection (in red) and, where it was available, for the EUCAST collection (in blue) (see Methods). The dotted lines illustrate clinical breakpoints for the antimicrobials with known breakpoints. The dotted curves are the fitted density distributions. The letters S, I and R stand for susceptible, intermediate and resistant, respectively. The abbreviations of antimicrobials are: amoxicillin (amx), cefuroxime (cxm), amoxicillin-clavulanate (amc), cefotaxime (ctx), cefoxitin (fox), imipenem (imp), piperacillin-tazobactam (tzp), ciprofloxacin (cip), ceftazidime (caz), gentamicin (gen), tigecycline (tgc), minocycline (min) and tetracycline (tet).
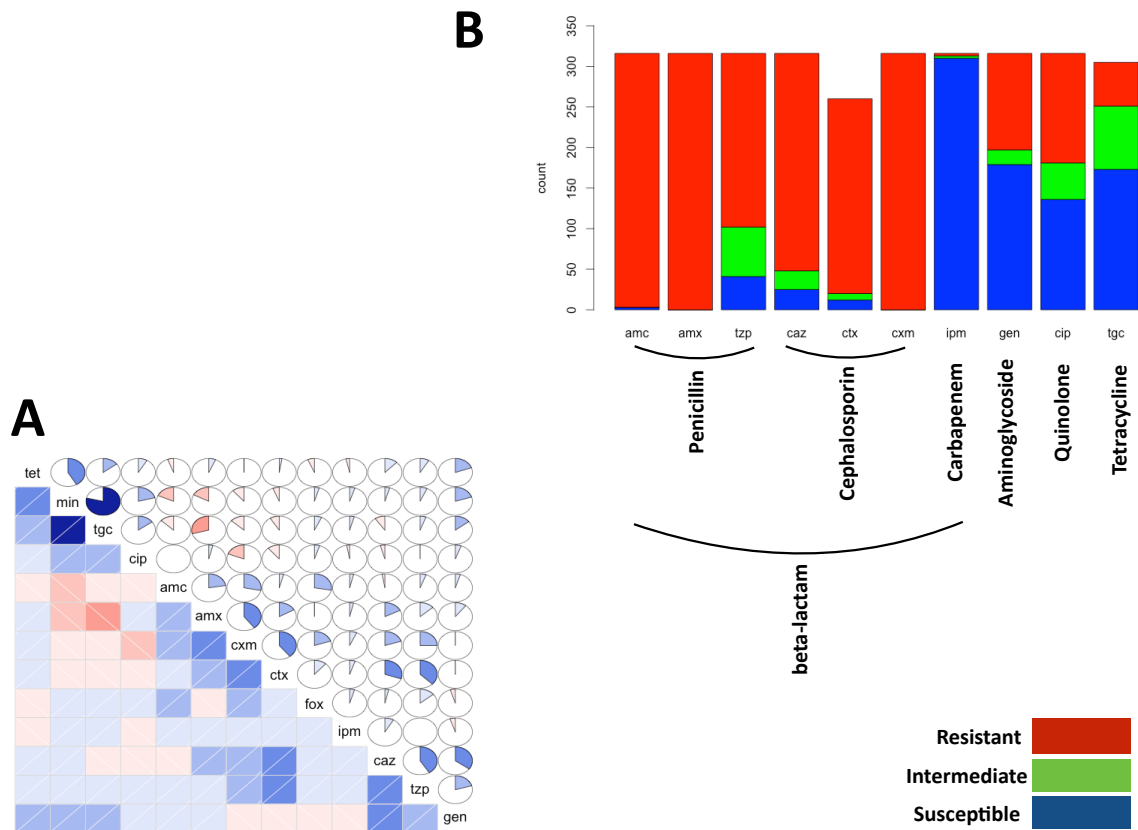
Supplementary Figure 9 A) Correlogram to display correlations between pairwise MIC values for the antimicrobials. The blue and red colours show positive and negative correlations. The colour density illustrates the strength of correlation. B) The frequency of resistant, intermediate, susceptible phenotypes within the population for the antimicrobials studied here. Abbreviations are the same as in Supplementary Figure 8.
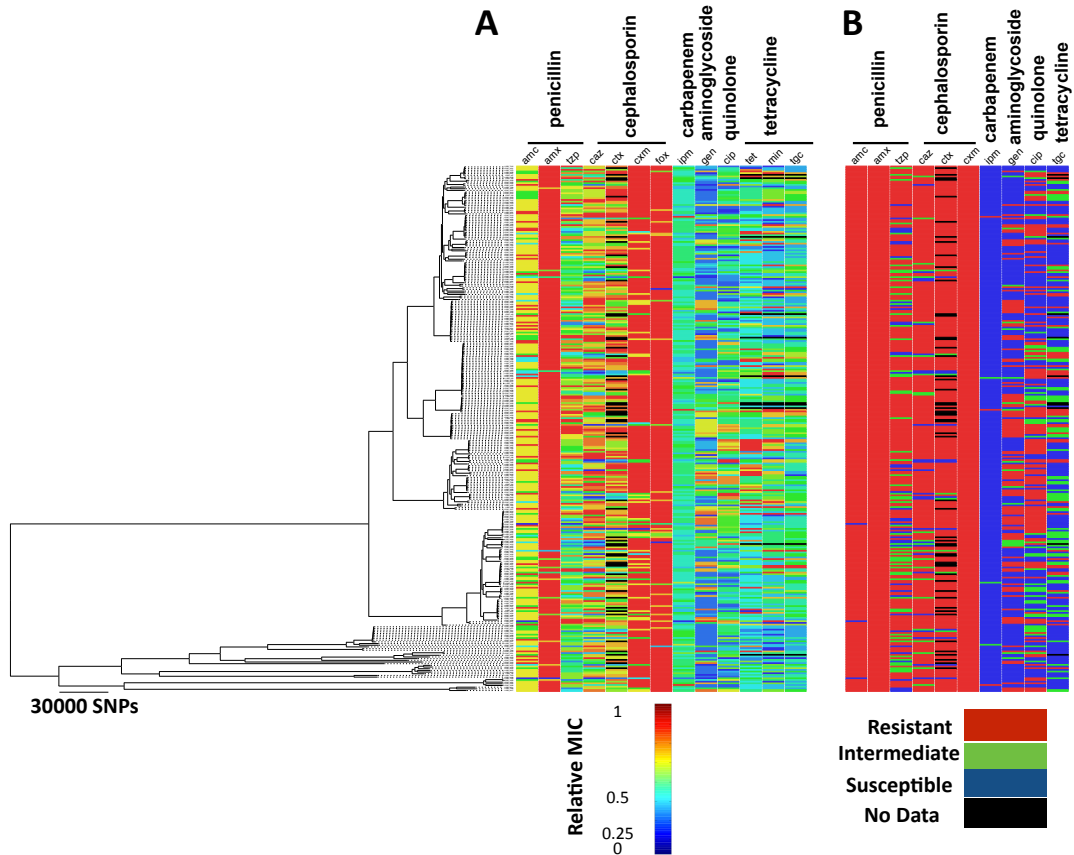
Supplementary Figure 10 A) MIC distribution for antimicrobials across the phylogenetic tree. B) Phenotypic distribution of susceptible, intermediate and resistant categories for the antimicrobials with known clinical cutoffs.

STs and antimicrobials

We compared the MICs for the major STs in the dataset to identify any STs that were associated with increased resistance to specific antimicrobials (Supplementary Figure 11). Compared with other STs, ST68 is relatively more resistant to piperacillin-tazobactam, and ST114 and ST171 are relatively more resistant to ciprofloxacin and tetracycline/minocycline, respectively (Supplementary Figure 8). Even though ST78 and ST114 were previously recognized as ESBL producers, the MIC values of these isolates against the beta-lactams were not significantly different from other isolates [12]. The lack of strict correlation between MICs and STs suggests that *E. cloacae*, like other major nosocomial *Enterobacteriaceae*, has first disseminated and subsequently acquired antimicrobial resistance determinants [12].
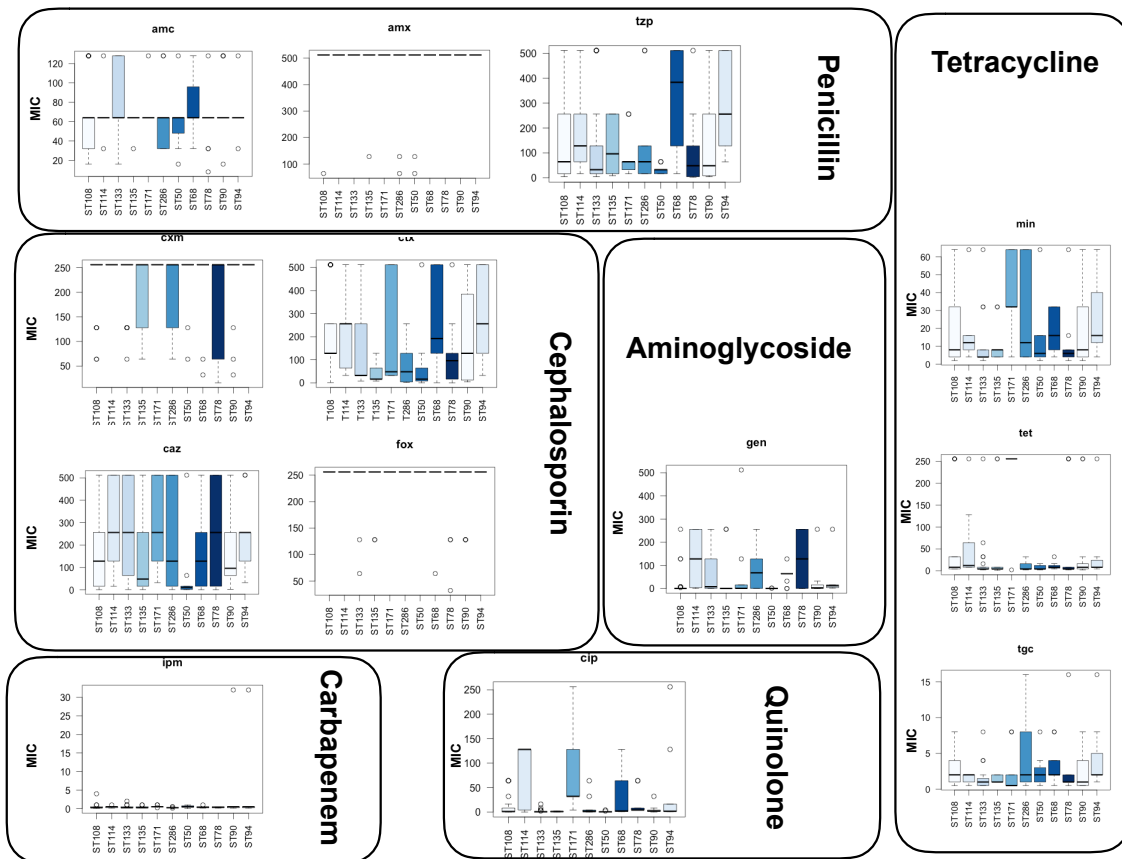
Supplementary Figure 11 MIC distributions for the major STs in our collection. The abbreviations are the same as in Supplementary Figure 8. The boxes give the interquartile range, the whiskers indicate the boundary of 1.5 times the interquartile range, and the points beyond that are outliers.
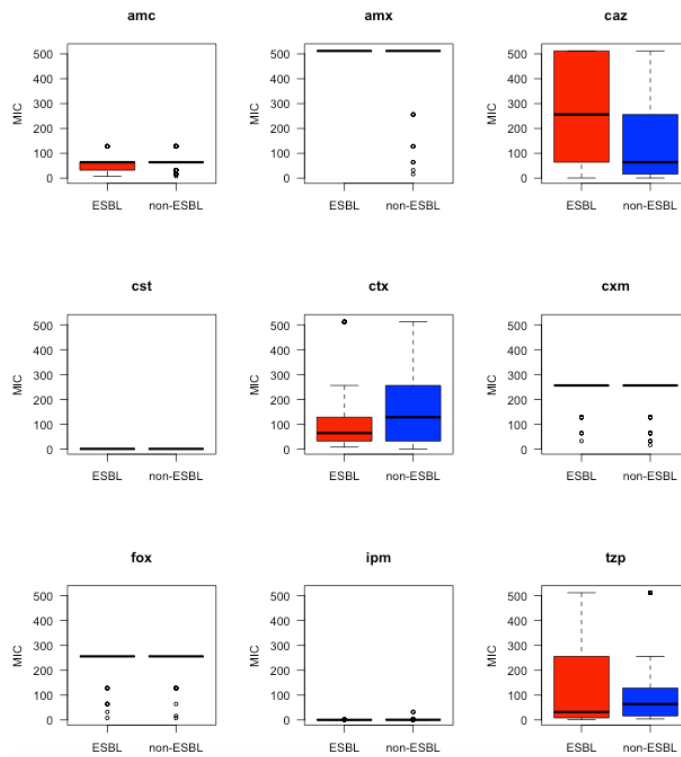
Antimicrobial resistance determinants and ESBL analysis

Enterobacter cloacae is known to be intrinsically resistant to a wide range of beta-lactams including cephalosporins and penicillins. This is due either to the presence of extended-spectrum beta-lactamases or to the over-production of AmpC [4, 8]. Our collection was composed of 85 ESBL-containing isolates and 230 non-ESBL isolates and 281 non-CTX-M and 25 CTX-M producing isolates (Supplementary Figure 12A). Comparing the MICs of these two sub-groups did not show a significant difference in resistance between the two sub-classes for most beta-lactams except ceftazidime (Supplementary Figure 13). This suggests that resistance is predominantly caused by hyper-production of AmpC rather than ESBLs, even when these are present (see also [4]).

Furthermore we found that the isolates in our collection were enriched in beta-lactamases, which were present at varying frequencies, implying that the multiple acquisitions of beta-lactamases have contributed to the resistance (Supplementary Figure 12B). In addition to *ampC* and its homologues, which were found at multiple copies in the core and accessory genome and confer resistance to most beta-lactams except carbapenem drugs, we also found some other known *amp* genes such as those encoding the signal transducers AmpG (in 315 isolates) and AmpE (in 311 isolates), the penicillin binding protein AmpH (in 313 isolates). Copies of genes encoding the beta-lactamase regulator AmpR and AmpD were also present in the collection and have been previously described in *E. cloacae* and *Escherichia coli* [13-15]. Some well-known beta-lactamases like oxacillinase (oxa), bla-OKP and bla-LEN have been gained by isolates throughout the tree but others such as bla-CMG and bla-MIR were restricted to some clades only. Altogether, our results support the idea that the concurrent action of multiple beta-lactamases contributes to the high level of resistance to cephalosporins in our collection.

Supplementary Figure 12 A) Distribution of ESBL and ESBL-CTX-M harboring isolates across the phylogenetic tree. Our collection was composed of 85 ESBL-containing isolates and 230 non-ESBL isolates and 281 non-CTX-M and 25 CTX-M producing isolates. B) Distribution of beta-lactamases, detected by srst2 in the Resfinder database, across the phylogenetic tree.

Supplementary Figure 13 Distribution of MIC values for different antimicrobials for ESBL and non-ESBL sub-populations. The abbreviations are defined in Supplementary Figure 8. The boxes give the interquartile range, the whiskers indicate the boundary of 1.5 times the interquartile range, and the points beyond that are outliers.

There are only a few reports of carbapenemase-producing *E. cloacae*, which make this antimicrobial an effective treatment choice for this species although recently carbapenem resistance has been increasingly seen in some regions [16, 17]. Our samples were extensively susceptible to imipenem, which is consistent with the lack of known carbapenemases such as serine carbapenemase KPC, NDM, GIM, VIM and the oxa-48 genes [9, 18-20]. In the few isolates that were relatively more resistant, the putative beta-lactamase precursor *blm* and the metalloenzyme carbapenemase beta-lactamases *nmc* genes, described in [21], were present.

Our isolates exhibited an intermediate level of resistance to gentamicin. The acquisition of three groups of aminoglycoside modifying enzymes i.e. acetyltransferases (AAC), phosphotransferases (APH) and adenylyltransferases (AAD) mainly account for resistance to aminoglycosides [4]. Screening against the antimicrobial resistance gene database indicates that multiple copies of acetyltransferases (*aac),* phosphotransferases (*str*) and adenylyltransferases (*aad*) genes have been acquired by multiple isolates across the phylogenetic tree. The presence of *aac* and *str* genes is strongly associated with MIC values for gentamicin (p-value < 10^{-5}) (Supplementary Figure 14). Amongst the variants of *aac* genes, *aac(3)* and *aac(6)* genes are frequently reported in members of the *Enterobacteriaceae* family [22]. Moreover, the regression analysis reveals some other putative genes strongly associated with gentamicin resistance. This includes several copies of acetyltransferase and phosphotransferase enzymes (Supplementary Figure 15).

Our isolates appear to have acquired multiple copies of tetracycline resistance genes independently (Supplementary Figure 14) and the presence of these genes is strongly associated with MIC values for tetracycline (p-value < 10^{-5}). The genes include tetracycline efflux proteins Tet(A), Tet(B), Tet(C) and Tet(D) and the monooxygenase Tet(X). The regression analysis on accessory genes also reveals some other putative resistance genes like the tetracycline repressor protein class D in 14 isolates and a tetracycline repressor originating from transposon *Tn*10. These genes were acquired independently in different isolates across the tree and strongly associated with the elevated MICs for minocycline and tetracycline in some isolates (Supplementary Figure 15). We conclude that multiple mechanisms appear to cause the MIC levels for tetracyclines to increase in *E. cloacae*.

Ciprofloxacin appears to be one the few remaining effective antimicrobials to treat *E. cloacae* infections, despite recent observations that quinolone resistance has been globally on the rise in *E. cloacae* isolates [23-25]. Although the enzymatic resistance to ciprofloxacin is attributed to the presence of AAC (6') and gyrase binding protein Qnr [24], in our collection there was no strong correlation between ciprofloxacin MICs and the presence of these genes (Supplementary Figure 14). Similarly although the majority of isolates seem to harbor efflux pumps *oqx* gene, which is known to cause quinolone resistance, there is no association between the presence of the genes and the MIC level for ciprofloxacin (Supplementary Figure 14). These findings seem to exclude the implication of known resistance enzymes in conferring resistance to ciprofloxacin.

Besides enzymatic resistance, mutations in gyrase A and B or subunits of DNA topoisomerase IV genes are widely described as ciprofloxacin resistance mechanisms in *Enterobacteriaceae* [26]. In line with this, we identified three non-synonymous point mutations in position 87 i.e. D87V (in 4 isolates), D87A (in 3 isolates) and D87G (in 7 isolates) in the well-known quinolone resistance-determining regions (QRDR) region of *gyrA* [27] that were strongly associated with MIC values for ciprofloxacin and occurred in parallel across the tree (Supplementary Figure 16 A). Furthermore we identified two putative non-synonymous mutations in position 78 (G78A in 1 isolate) and position 80 (S80I in 43 isolates) of DNA topoisomerase IV subunit A (*parC* with Locus_tag: ECL_04341) that exhibit strong association with resistance and may be studied further in future studies (Supplementary Figure 16 A). In addition, the number of non-synonymous mutations in the DNA topoisomerase IV subunit A was strongly correlated with MIC values, which indicates that a combination of non-synonymous changes rather than a single mutation in this gene may be responsible for the elevated MICs (Supplementary Figure 16 B).
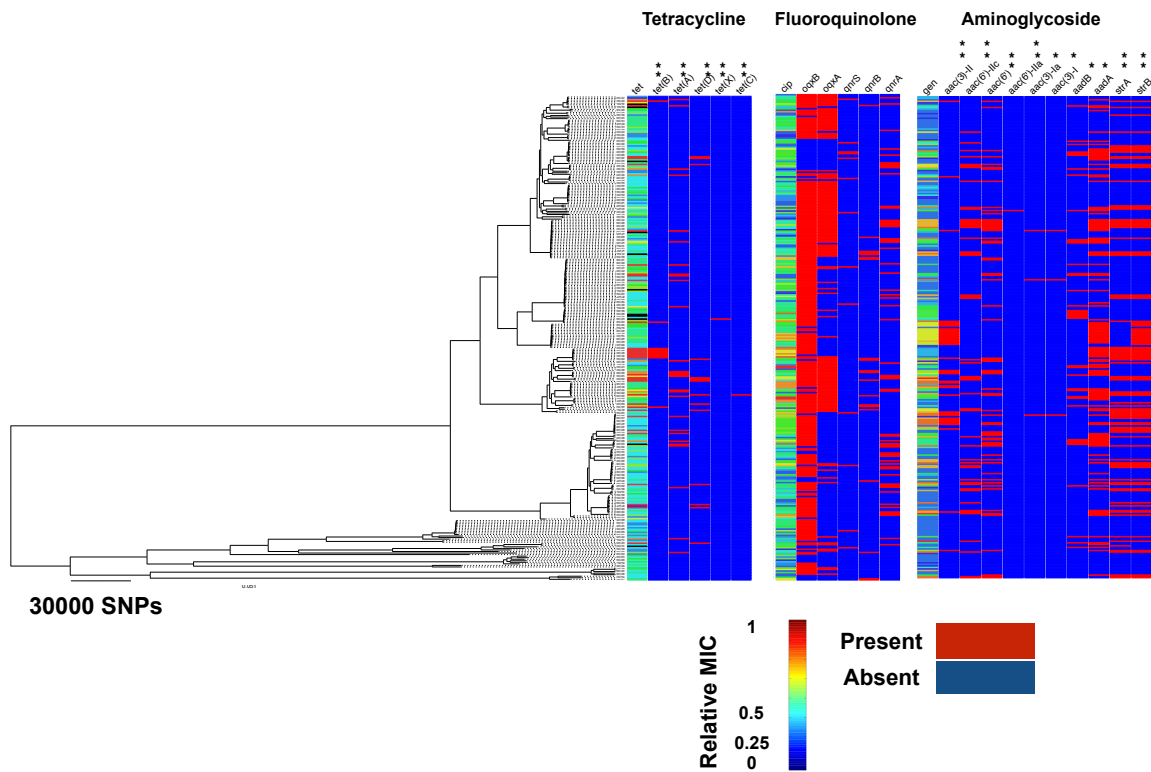
Supplementary Figure 14 Distribution of antimicrobial resistance genes found in the ResFinder database across the phylogenetic tree for three classes of antimicrobials: Tetracycline, Fluoroquinolone and Aminoglycoside, as well as ordered in the figures. The first columns in each block show the relative MIC values for the antimicrobials. The * and ** signs show the significance level (p-value) of association between the presence of the gene and MIC value of $<10^{-2}$ and $<10^{-5}$, respectively.
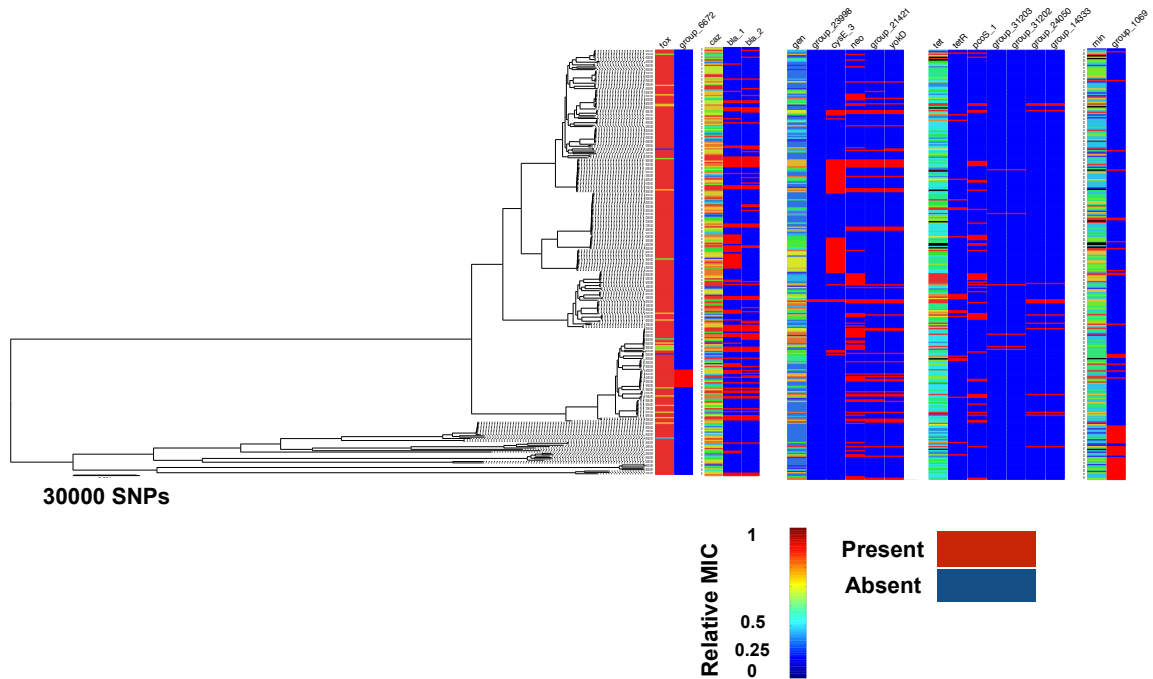
Supplementary Figure 15 Distribution of some putative antimicrobial resistance genes found the in the accessory genome that strongly correlate with MICs. The antimicrobial abbreviations are the same as in Supplementary Figure 8. The *group_6672*, *group_23998*, *cysE_3*, *neo*, *group_21421*, *yok* genes are major facilitator superfamily protein, acetyltransferases and phosphotransferase. The *pcoS_1*, *group_31203*, *group_31202*, *group_24050*, *group_14333* and *group_1069* genes are modifying enzymes, major facilitator superfamily and tetracycline repressor proteins.
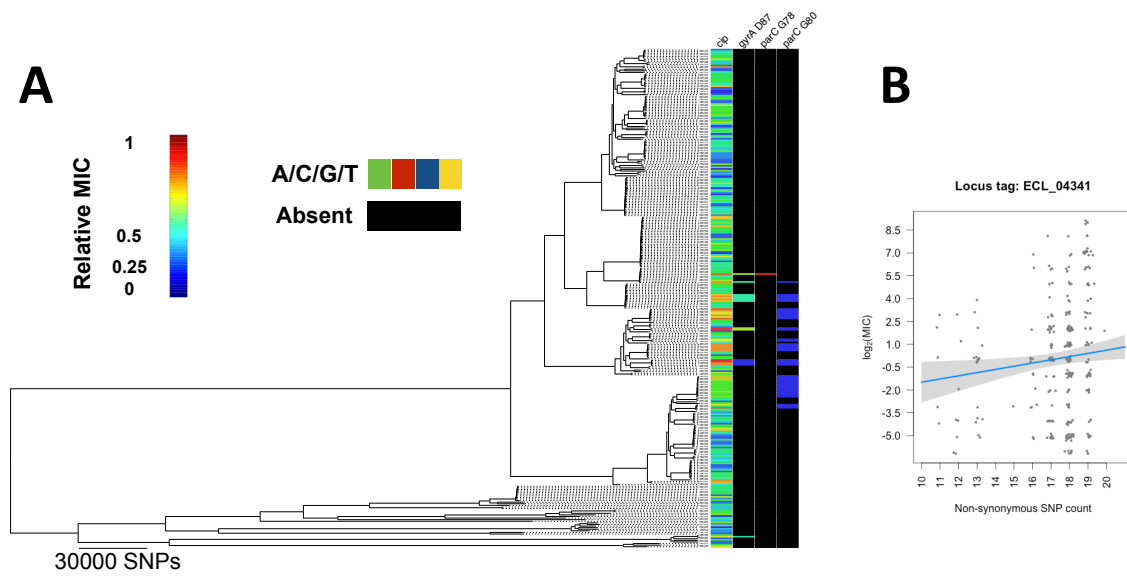
Supplementary Figure 16 A) Distribution of putative resistance SNPs in position 87 of *gyrA* and positions 78 and 80 of the subunit A of DNA topoisomerase IV (*parC*) that are strongly associated with the MIC values for ciprofloxacin (first column). B) Plot of non-synonymous SNP density versus the $\log_2$(MIC) in the subunit of DNA topoisomerase IV subunit A (p-value $< 10^{-5}$). The grey area shows 95% confidence interval. To better illustrate data point density, we have introduced error values taken from a normal distribution with the mean value of 0 and standard deviation of 0.1 along the x and y axes for each data point.
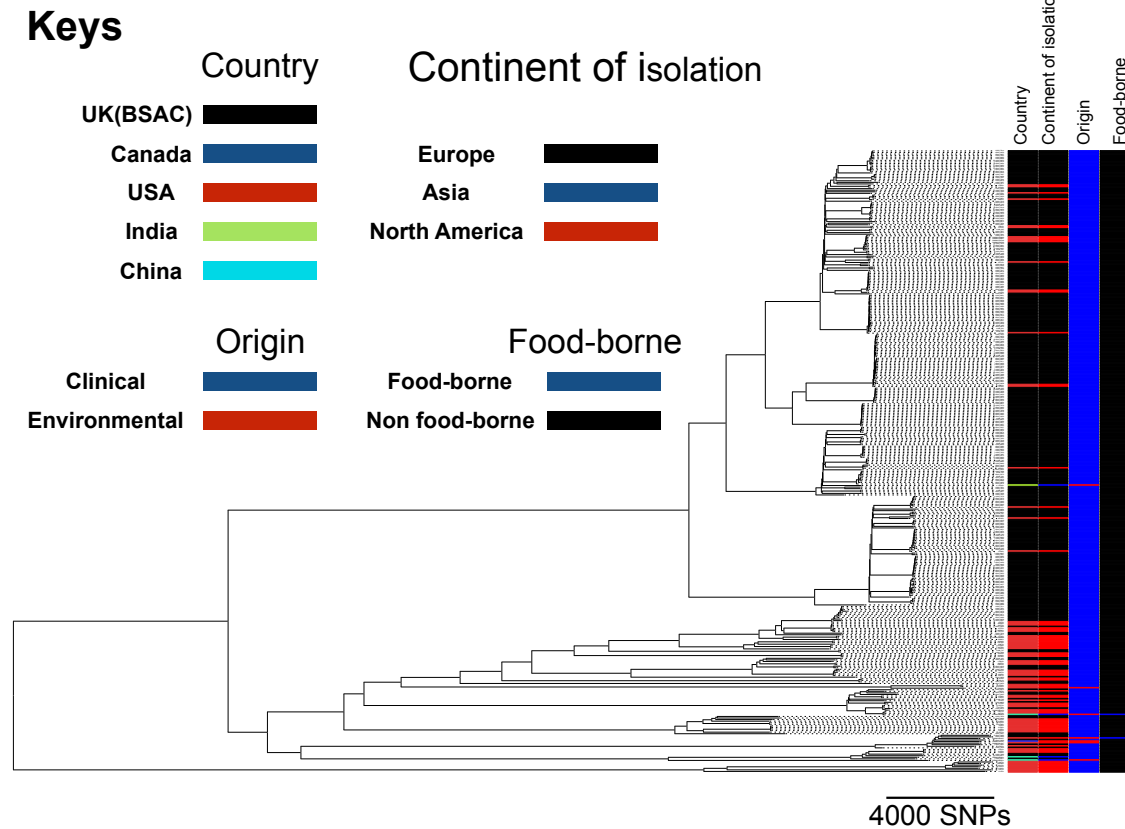
Supplementary Figure 17 Phylogenetic tree of the whole genomes of the isolates studied here (BSAC) in the context of previously published genomes, which are mapped to the reference genome detailed in Methods. The accession numbers and publication IDs for the previously published isolates are provided in Supplemental Table S5. The majority of the isolates that were isolated from the USA and found within the rare sub-species of *E. cloacae* were recovered only from the one hospital in the USA [28] and therefore do not represent the diversity of *E. cloacae* across the USA.
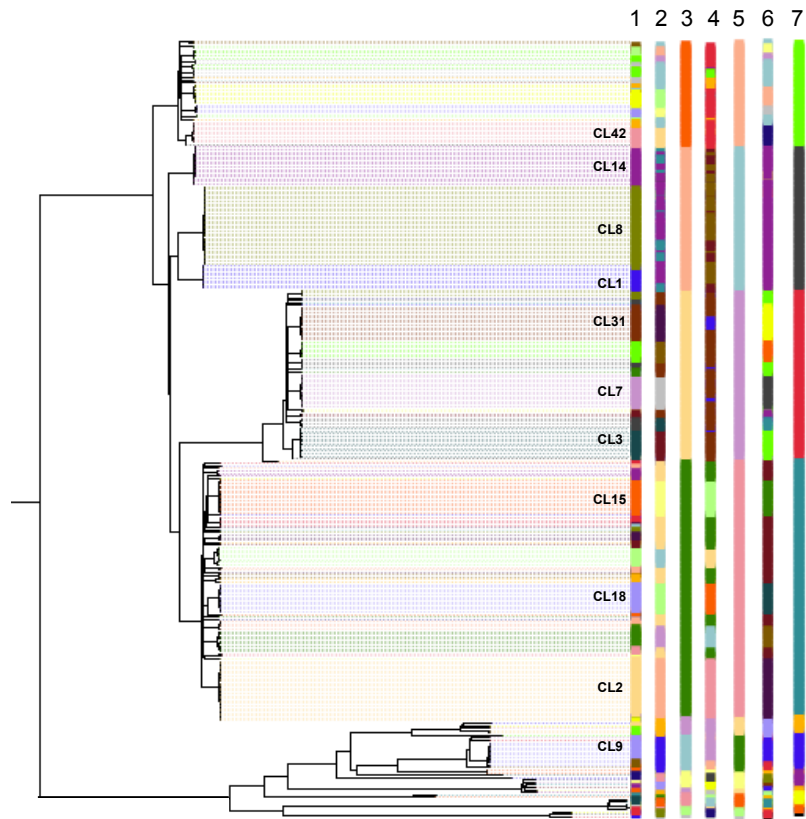
Supplementary Figure 18 Comparison between the clusters we identified in the collection and

those detected by HierBAPS across the phylogenetic tree. Columns are defined as follows:

Column 1: identified clusters

Column 2: 2nd iteration of BAPS with k=50

Column 3: 1st iteration of BAPS with k=50

Column 4: 2nd iteration of BAPS with k=30

Column 5: 1st iteration of BAPS with k=30

Column 6: 2nd iteration of BAPS with k=10

Column 7: 1st iteration of BAPS with k=10

Supplementary Tables:

Supplementary Table 1.csv: Table of accession numbers of isolates studied here and the results of antimicrobial susceptibility testing. Columns are defined as follows:

A) SangerID: Run identification number, as produced by pipelines at the Wellcome Trust Sanger Institute.

B) SampleID: Secondary sample accession at the European Nucleotide Archive (ENA).

C) ContigAcc.: Accession numbers for annotated contigs.

D) HospitalOrCentre: Hospital code.

E) Isolation year: Year of isolation of isolates.

F) CambridgeID: Hospital accession ID for the isolates used for sequencing.

G) ENA run accession ID: Accession ID for sequencing run at the ENA.

H) ESBL: ESBL status of the isolates.

I) ESBL_CTXM: Status of isolates with respect to the possession of bla-CTXM genes.

J-V) MIC values for the antimicrobials. The abbreviations of antimicrobials are: amoxicillin (amx), cefuroxime (cxm), amoxicillin-clavulanate (amc), cefotaxime (ctx), cefoxitin (fox), imipenem (imp), piperacillin-tazobactam (tzp), ciprofloxacin (cip), ceftazidime (caz), gentamicin (gen), tigecycline (tgc), minocycline (min) and tetracycline (tet).

W) Contig.Total.Length: Total lengths of contigs for each isolates.

X) No.Contigs: Number of contigs for each isolate.

Y) Avg.Contig.Length: Average length of contigs for each isolate.

Z) Largest.Contig: Lengths of largest contig for each isolates.

Supplementary Table 2.csv: Results of the regression model based on the presence of genes in the accessory genome. Columns are defined as follows:

A) Gene: name of accessory genes.

B) Annotation: function/product of genes.

C) Count: Number of isolates with each accessory gene.

D) Association value: association expressed as -$\log_{10}$(p-value) of the regression model.

E) Antimicrobial: Antimicrobial names. Abbreviations are the same as in SupplementalTable S1.csv.

Supplementary Table 3.csv: Results of the regression model based on presence of individual SNPs. Columns are defined as follows:

A) Position_in_reference_genome: position of the SNP in the reference *E. cloacae* ATCC 13047 genome.

B) CDS/rRNA/tRNA/Intergenic: Genomic status of the region in which the SNP occurs.

C) Strand: DNA strand of the coding region where the SNP occurs. The values -1 and +1 refer to reverse and forward strands, respectively.

D) CDS_name: Name of the coding region.

E) Product: Protein product of the coding region.

F) Synonymous/Non-synonymous: impact of SNPs at the protein level.

G) Ref_base: Base in the positions of SNPs in the reference genome.

H) SNP_base: Altered base at SNP sites.

I) Count: Number of SNPs.

J) Association value: association expressed as -$\log_{10}$(p-value) of the regression model.

K) Antimicrobial: Antimicrobial names. Abbreviations are the same as in SupplementalTable S1.csv.

Supplementary Table 4.csv: Results of the regression model based on the number of non-synonymous SNPs. Columns are defined as follows:

A) Gene: Locus tag id in the reference *E. cloacae* ATCC 13047 genome.

B) Association value: association expressed as $-\log_{10}$(p-value) of the regression model.

C) Antimicrobial: Antimicrobial names. Abbreviations are the same as in SupplementalTable S1.csv.

D) Product: Product of genes with strong association scores.

Supplementary Table 5.csv: Table of isolates used to contextualize MDR *E. cloacae* isolates. Columns are defined as follows:

A) Sample ID: ID of isolate.

B) Country: Country of isolation.

C) Continent: Continent of isolation.

D) Origin: clinical versus non-clinical/environmental isolates.

E) Pubmed ID: ID of publications where isolates are reported.

F) Comment: Status of isolates with respect to food-borne status.

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