

RESEARCH PAPER



## An ancient family of mobile genomic islands introducing cephalosporinase and carbapenemase genes in *Enterobacteriaceae*

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### ABSTRACT

The exchange of mobile genomic islands (MGIs) between microorganisms is often mediated by phages, which may provide benefits to the phage's host. The present study started with the identification of *Enterobacter cloacae*, *Klebsiella pneumoniae* and *Escherichia coli* isolates with exceptional cephalosporin and carbapenem resistance phenotypes from patients in a neonatal ward. To identify possible molecular connections between these isolates and their  $\beta$ -lactam resistance phenotypes, the respective bacterial genome sequences were compared. This unveiled the existence of a family of ancient MGIs that were probably exchanged before the species *E. cloacae*, *K. pneumoniae* and *E. coli* emerged from their common ancestry. A representative MGI from *E. cloacae* was named MIR17-GI, because it harbors the novel  $\beta$ -lactamase gene variant *bla*<sub>MIR17</sub>. Importantly, our observations show that the MIR17-GI-like MGIs harbor genes associated with high-level resistance to cephalosporins. Among them, MIR17-GI stands out because MIR17 also displays carbapenemase activity. As shown by mass spectrometry, the MIR17 carbapenemase is among the most abundantly expressed proteins of the respective *E. cloacae* isolate. Further, we show that MIR17-GI-like islands are associated with integrated P4-like prophages. This implicates phages in the spread of cephalosporin and carbapenem resistance amongst *Enterobacteriaceae*. The discovery of an ancient family of MGIs, mediating the spread of cephalosporinase and carbapenemase genes, is of high clinical relevance, because high-level cephalosporin and carbapenem resistance have serious implications for the treatment of patients with enterobacteriaceal infections.

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## Introduction

The shape of bacterial genomes, as we know them today, is the outcome of many successive evolutionary events that occurred ever since the respective bacterial species branched off from their common ancestors [1]. This is clearly evidenced through comparisons of the genome sequences of individual bacterial species, where a distinction can be made between the core genome and the accessory genome. Here, the accessory genome reflects those elements that were either lost through genomic erosion, or gained through horizontal gene transfer [2–4]. Of note, the accessory genome is only the echo of those events that happened relatively recently on the evolutionary timeline, namely after the particular species emerged. In this context, it is frequently overlooked that also the core genome has


been shaped from elements that were recruited through genetic exchanges, but before speciation occurred. Thus, also the core genome is the offspring of evolution and includes ancient mobile genomic elements (MGIs), which turned out beneficial for the species within its ecological niche to the extent that they are no longer readily lost [5–7]. As a consequence of their co-evolution with the species, such ancient MGIs may have lost particular traits of present-day MGIs, such as a difference in the GC content. Yet, other traits can still be discerned, such as the typical sites of integration and an overrepresentation of certain classes of genes [8]. In the present study we focus attention on an ancient family of MGIs, first discovered within the “core genome” of *Enterobacter cloacae*.

*E. cloacae* is a rod-shaped, non-spore forming, facultative anaerobic, Gram-negative bacterium belonging to the

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family of *Enterobacteriaceae*. The occurrence of *E. cloacae* is widespread, ranging from soil and sewage to the human gastrointestinal tract, where it is a frequent component of the gut microbiota. Importantly, *E. cloacae* can cause opportunistic infections and has recently emerged as a nosocomial pathogen, especially in intensive care units (9). In the clinical setting, *E. cloacae* has been identified as the causative agent of skin and soft tissue infections, respiratory and urinary tract infections, intra-abdominal infections, bacteremia, endocarditis, septic arthritis, and osteomyelitis in immunocompromised patients [9,10]. The antimicrobial therapy of patients with *E. cloacae* infections often faces complications due to the intrinsic drug resistance of this bacterium, and its propensity to acquire multiple resistance genes. Thus, resistance has been reported against ampicillin, amoxicillin, first-generation cephalosporins and cefoxitin owing to constitutive expression of the AmpC  $\beta$ -lactamase. *E. cloacae* also exhibits a high frequency of enzymatic resistance to broad-spectrum cephalosporins [11], which is typically caused by overproduction of AmpC  $\beta$ -lactamases. Such extended spectrum cephalosporinases (e.g. ACT, CMY and MIR) confer resistance to third-generation cephalosporins, and they are not inhibited by common  $\beta$ -lactamase inhibitors [12]. Until now, fourth- and fifth-generation cephalosporins maintain reasonable activity against such strains. While ACT and MIR in *Enterobacter* species are encoded by intrinsic chromosomal genes, CMY genes may be plasmid-borne [13,14]. In general, extended spectrum cephalosporinases of *Enterobacteriaceae* have been associated with mobile genomic elements.

In contrast to cephalosporinases, acquired carbapenemase genes are still fairly uncommon in *E. cloacae* on a global scale [12,15]. However, many extended spectrum cephalosporinases have a weak affinity for carbapenems and can hydrolyze carbapenems with low efficiency. As a consequence, clinical resistance of *E. cloacae* to these last-line  $\beta$ -lactam antibiotics is mostly brought about by outer membrane permeability defects combined with a derepression of constitutive AmpC cephalosporinases [11,12,16].

The present study started with the identification of *E. cloacae*, *K. pneumoniae* and *E. coli* isolates with exceptional  $\beta$ -lactam resistance phenotypes, including one *E. cloacae* isolate with decreased susceptibility for carbapenems, in patients on a neonatal ward of the University Medical Center Groningen (UMCG). To assess possible molecular connections between these isolates and their antibiotic resistance phenotypes, the respective bacterial genomes were compared. This led to the identification of four highly conserved MGIs of ~ 140 kb in the investigated isolates. This observation is of general importance, since it focuses attention on a

family of genomic islands carrying a diversity of cephalosporinase-encoding genes that was already widely transduced before the species *E. cloacae*, *K. pneumoniae* and *E. coli* emerged from their common ancestry.

## Results

### Description of the study isolates and clinical background

In the present study, we investigated four epidemiologically linked Gram-negative bacterial isolates with decreased susceptibility to carbapenems as reflected by minimal inhibitory concentration (MIC) values for meropenem and/or imipenem  $\geq 0.5$  mg/L. Table 1 summarizes the results of the antibiotic susceptibility testing in these four isolates.

The first study isolate (isolate 1) was a carbapenem-resistant *E. cloacae* that was obtained from a rectal swab of a neonate (patient 1), who had been repatriated from Curaçao to the UMCG. Prior to admission in the UMCG, the patient had been treated with meropenem. Isolate 1 showed an atypical growth phenotype resulting in small fatty colonies on Blood Agar (BA) and Mueller Hinton Agar (MHA) plates. Automated resistance analysis with the VITEK 2 system revealed increased MIC values to the carbapenems meropenem (8 mg/L) and imipenem (8 mg/L), which were subsequently confirmed by Etests. Imipenem Etests on MHA with or without 250 mg/L cloxacillin revealed that imipenem resistance was significantly reduced in the presence of cloxacillin, which is an inhibitor of  $\beta$ -lactamases of the AmpC-type [17]. Specifically, the MIC was

**Table 1.** Antibiotic susceptibility of the bacterial study isolates.

Antibiotic	Isolate 1	Isolate 2	Isolate 3	Isolate 4
	Patient 1 <i>E. cloacae</i> ST-232	Patient 1 <i>E. cloacae</i> ST-97	Patient 1 K. <i>pneumoniae</i> ST-20	Patient 2 <i>E. coli</i> ST-131
	MIC (mg/L)	MIC (mg/L)	MIC (mg/L)	MIC (mg/L)
Amoxicillin + clavulanic acid	> 32	> 32	> 32	> 32
Cefuroxime	> 64	32	> 64	> 64
Cefotaxime	> 64	< 1.0	16	> 64
Ceftazidime	> 64	< 1.0	1	> 64
Cefoxitin	> 64	> 64	> 64	32
Cefepime	2	< 1.0	2	16
Meropenem	8	< 0.25 <sup>#</sup>	0.032	4
Imipenem	8	< 0.25 <sup>#</sup>	0.5	0.75
ESBL*	negative	negative	negative	positive

Antibiotic susceptibility of the four bacterial isolates described in this study.

All four isolates carried a MIR17-GI-related MGI. In three of the four isolates, a cephalosporinase gene (*ampC*) was integrated in this island.

<sup>#</sup>Carbapenem-resistant colony variants grew into the carbapenem inhibition zone.

\*ESBL production was tested by E-tests and indicated by a > 8-fold reduction in MIC in the presence of clavulanic acid compared to cefepime, ceftazidim or cefotaxim alone.

reduced to 0.125 mg/L (Figure 1). This showed that the reduced carbapenem sensitivity of isolate 1 is largely due to the production of a carbapenem-degrading enzyme. Of note, confluent plating of isolate 1 on MHA results in a patchy growth phenotype that is suppressed in the presence of cloxacillin (Figure 1). Multi-locus sequence type (MLST) analysis assigns isolate 1 to the sequence type (ST-)232 (*dnaA88*, *fusA25*, *gyrB49*, *leuS72*, *pyrG49*, *rplB12*), where *dnaA88* represents a novel *dnaA* allele. Phenotypic ESBL tests with isolate 1 were negative. Whole-genome sequencing revealed a novel  $\beta$ -lactamase gene variant, which is homologous to the MIR lineage. In fact, this gene is 99.3% identical to the *bla*<sub>MIR-1</sub> gene from *Klebsiella pneumoniae* (ENA accession M37839.2) and it was thus designated *bla*<sub>MIR-17</sub> (NCBI accession CEA29752.1). Specifically, MIR17 is distinct from MIR1 due to five amino acid substitutions.

Upon withdrawal of meropenem treatment for 2 weeks, a carbapenem-sensitive *E. cloacae* isolate (i.e. isolate 2) was obtained from an intravascular catheter tip from patient 1. This isolate 2 was susceptible to third-generation cephalosporins and carbapenems, and the sensitivity to imipenem was not influenced by the presence of cloxacillin (not

shown). Genome sequencing revealed the presence of an *ampC* gene encoding the ACT-5 cephalosporinase. Further, MLST analysis showed that isolate 2 belongs to ST-97.

After 3 months of hospitalization, patient 1 acquired a *K. pneumoniae* (i.e. isolate 3) with a remarkable antibiotic resistance pattern. Specifically, isolate 3 was shown to be resistant to cefuroxime and cefotaxime, but susceptible to ceftazidime. Phenotypic ESBL-testing was negative. For the molecular detection of resistance genes, first a microarray analysis was performed and, subsequently, isolate 3 was subjected to whole genome sequencing followed by screening of the sequence against the Resfinder database. This showed the presence of a  $\beta$ -lactamase gene variant with 99% identity to *bla*<sub>SHV-140</sub> of *K. pneumoniae*. This gene was therefore designated *bla*<sub>SHV-187</sub> (NCBI accession LN515533.1). Specifically, SHV187 is distinct from SHV140 due to two N-terminal amino acid substitutions. MLST showed that isolate 3 belongs to ST-20.

Patient 2 was hospitalized in the same ward of the UMCG as patient 1. During this hospital stay, patient 2 acquired an *E. coli* (isolate 4) resistant to ceftaxime and third-generation cephalosporins. Further, isolate 4

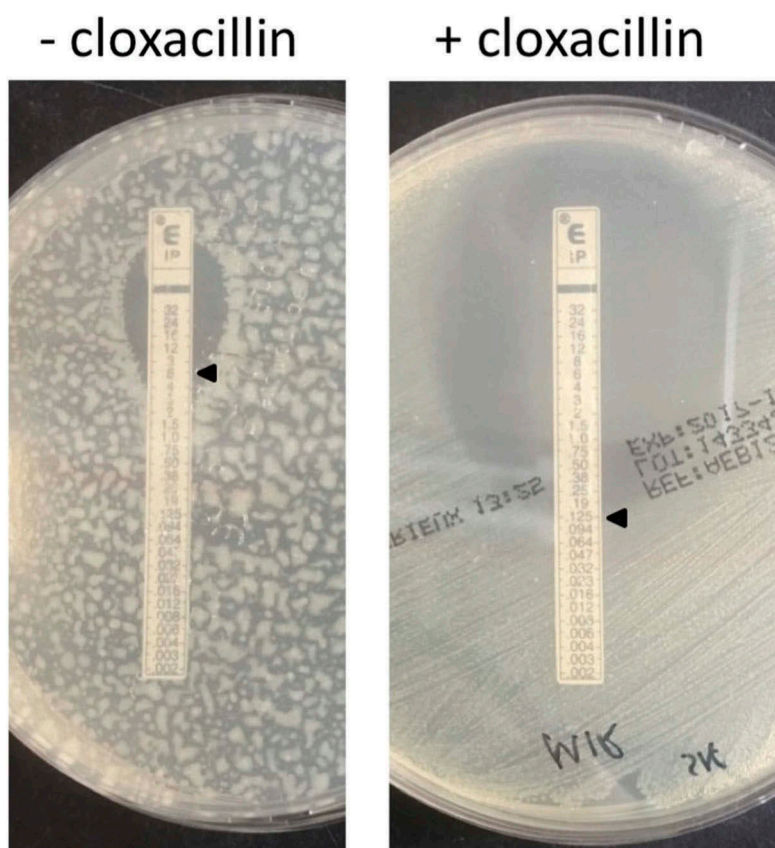


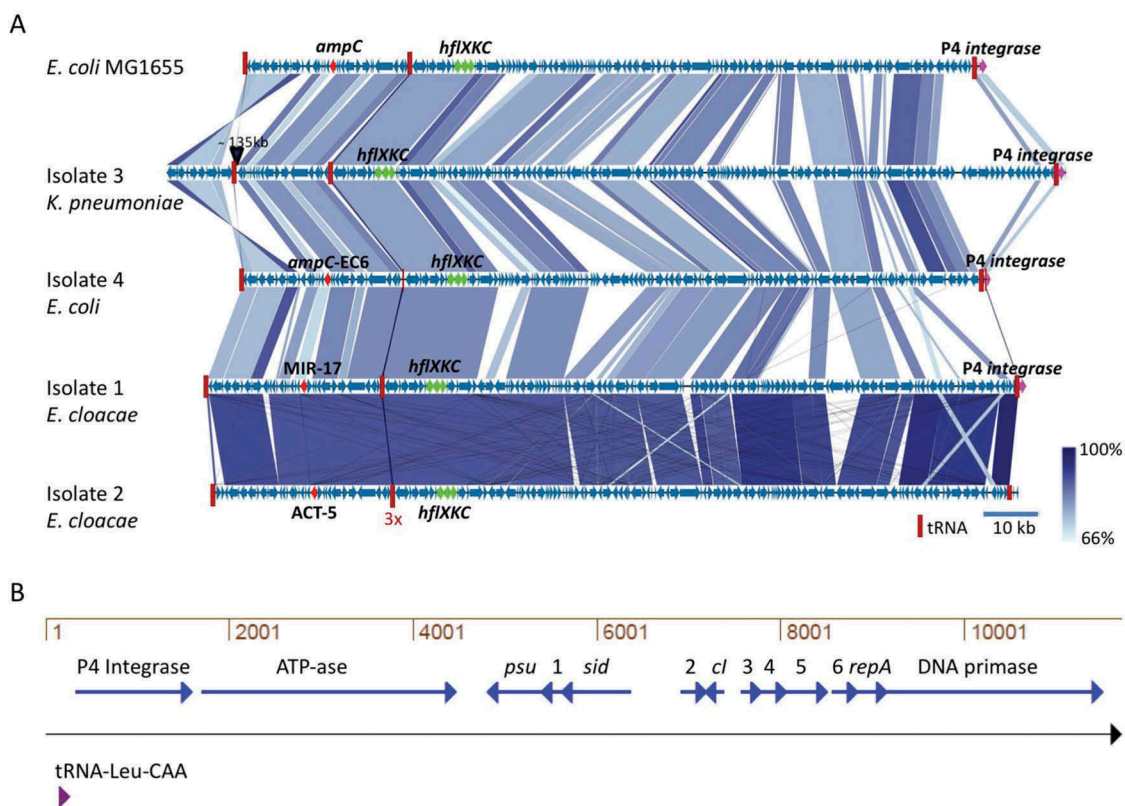
Figure 1. Imipenem Etest for *E. cloacae* study isolate 1 on Muller Hinton agar with or without cloxacillin.

displayed a meropenem MIC value of 4 mg/L. ESBL-tests were positive and, upon whole genome sequencing, Resfinder detected two acquired  $\beta$ -lactamase gene variants designated  $bla_{\text{TEM-1b}}$  and  $bla_{\text{CTX-M-147}}$ . Additionally, an EC-6 AmpC  $\beta$ -lactamase-encoding gene was detected [18]. Isolate 4 was shown to belong to ST-131.

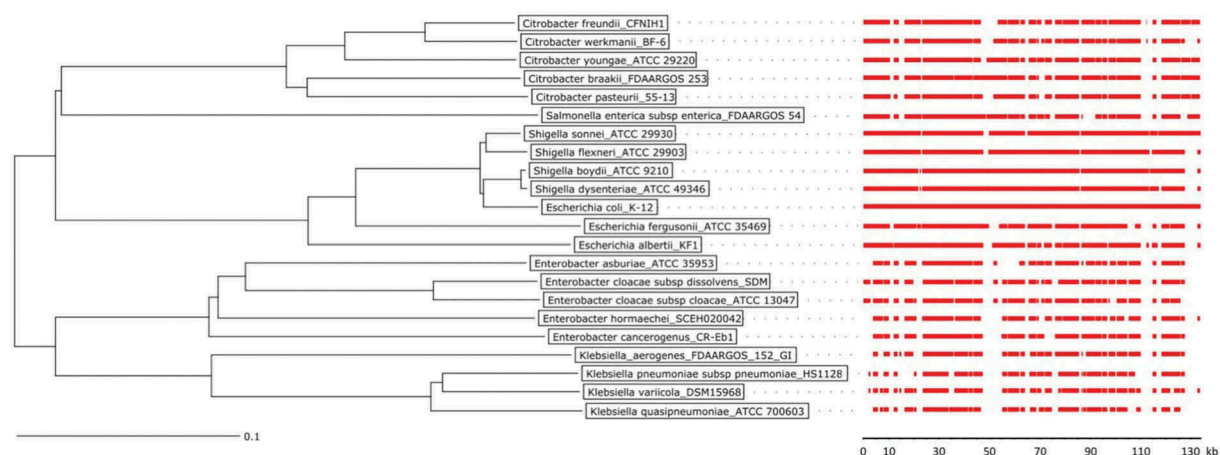
### The genomic neighborhood of $bla_{\text{MIR-17}}$ is conserved in *Enterobacteriaceae*

Since the cephalosporinase genes identified in the four study isolates were distinct, the respective antibiotic resistance phenotypes could not be directly related to recent horizontal gene transfer events between these isolates. However, comparison of the whole genome sequencing data uncovered ~140-kb regions of high similarity in the four isolates, which harbor  $ampC$  genes in the case of isolates 1, 2 and 4 (Figure 2(a)). This prompted us to investigate the spread of this conserved region among

*Enterobacteriaceae*. As shown in Figure 3 and Supplemental Figure S1, the  $ampC$ -containing region, as present in *E. coli*, is highly conserved in the genera *Citrobacter*, *Escherichia*, *Enterobacter*, *Salmonella*, *Shigella* and *Klebsiella*, where the sequences cluster according to the respective species. Of note,  $ampC$  genes are absent from the respective conserved regions in *Salmonella* and *Klebsiella* species, as is the case in our *K. pneumoniae* study isolate 3 (Figure 2(a)). Further, we observed that parts of this conserved region are present in other *Enterobacteriaceae*, such as *Cronobacter*, *Dickya*, *Proteus*, *Providencia*, *Serratia* and *Yersinia* species, where it lacks  $ampC$  and can be positioned at different chromosomal loci (not shown). The conserved region is completely absent from non-enterobacteriaceal Gram-negative bacteria, such as *Acinetobacter*, *Haemophilus*, *Pasteurella*, *Prevotella* and *Pseudomonas* species. Combined with previous studies on the phylogeny of *Enterobacteriaceae* [19–23], these observations are



**Figure 2.** Similarity among MIR17-GI-like MGIs and schematic representation of the P4-like prophage at the 3' end of MIR17-GI. (a) MIR17-GI-like MGIs identified in the four study isolates and *E. coli* K12 MG1655. The alignment of MIR17-GI-like MGIs was performed with Easyfig (<http://mjsull.github.io/Easyfig/>). The positions of relevant genes are indicated. (b) Genetic map of the early operon of the P4-like prophage associated with the 3' end of MIR17-GI, following the tRNA-Leu-CAA gene. The relative positions of the genes for the phage integrase, an ATP-ase of unknown function (ATP-ase), the phage polarity suppression protein ( $psu$ ), the phage capsid and scaffold protein ( $sid$ ), the Ash family secondary immunity repressor ( $cl$ ); the DNA replication protein ( $repA$ ), and the DNA primase are indicated. Numbers correspond to the following gene annotations: 1, late gene regulator; 2, phage DNA binding protein; 3, phage immunity repressor protein; 4, immunity derepression protein; 5, phage protein of unknown function; 6, hypothetical protein for heme transfer during cytochrome c biogenesis.



**Figure 3.** Phylogenetic tree of *Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Salmonella*, and *Shigella* species and conservation of MIR17-GI-like MGIs.

The phylogenetic tree was created with the Ridom SeqSphere+ software v4.1.9 using a neighbor joining algorithm. It is based on a SNP analysis of 39430 targets of the MGI from the reference genome of the *E. coli* K12 strain MG1655. The scale bar under the tree represents the phylogenetic distance (in %). The red bars indicate the conservation of the MIR17-GI using the respective MGI of *E. coli* K12 strain MG1655 as the reference. For the MGI comparisons, an 80% DNA similarity cut-off was used in DNA plotter. The scale bar under the MGI alignment indicates the sequence position of the reference MGI. Bacterial isolates and the respective genome sequence accession codes are presented in Supplemental Table S2.

indicative of an ancient MGI that spread among the enterobacteriaceal ancestry, before genera like *Enterobacter*, *Escherichia*, and *Klebsiella* evolved.

### The *bla*<sub>MIR-17</sub> $\beta$ -lactamase gene of *E. cloacae* isolate 1 is located on an ancient mobile genomic island

Analysis of the conserved genomic neighborhood of the *bla*<sub>MIR-17</sub> gene in *E. cloacae* isolate 1 revealed the presence of several genes that are commonly found on MGIs, as described in detail in the following paragraph. The idea that *bla*<sub>MIR-17</sub> could be part of a MGI was further corroborated by inspection of the genome sequence of *E. cloacae* isolate 1, which revealed that indeed a 140-kb MGI including *bla*<sub>MIR-17</sub> had integrated in the Phe-GAA tRNA gene. Of note, this tRNA gene contains a phage P4-associated attachment site (GAGTCCGGCCTTCGGCACCA) [24] in the 3'-5' direction (Supplemental Figure S2). Since the identified MGI carries the *bla*<sub>MIR-17</sub> gene, we named it MIR17-GI. At the 3' end of the MIR17-GI, downstream of a Leu-CAA tRNA gene, an integrated P4 prophage was detected (Figure 2(b)). A BLAST-x analysis of the replicative helicase gene of this P4 prophage indicated that it is most closely related to a P4 bacteriophage of *Salmonella enterica* (GI:380464247, 87% identity, 100% coverage).

The 5' side of MIR17-GI is schematically represented in Supplemental Figure S2. The *bla*<sub>MIR-17</sub> gene located

on the reverse strand is flanked by an *ampR* regulator gene on the forward strand. The *bla*<sub>MIR-17</sub>-*ampR* genes are positioned within a 32-kb region of MIR17-GI that is flanked by the Phe-GAA tRNA gene with the P4 attachment site at the 5' end, and a triplet of Gly-GCC tRNA genes at the 3' end. Of note, this region contains several additional resistance genes, potentially providing resistance to cations and heavy metals (*cutA1*, *cutA2*) [25], or quaternary ammonium compounds (*sugE*) [26]. Further, it includes the gene for a mechano-sensitive potassium efflux pump (*kefA*) that could be involved in osmoprotection [27], and genes for two outer membrane lipocalins (*blc*, *yjel*) implicated in the transport of small hydrophobic molecules [28]. The first gene of the island encodes a regulator of the TetR transcription regulator family, and this gene is located downstream of the *cutA1* and *cutA2* genes. Regulators of the TetR family repress gene transcription, and transcription is derepressed in response to stress [29].

MIR17-GI also carries several genes that are known to have housekeeping functions. In particular, on the forward strand, the *groES-groEL* genes encode chaperones implicated in protein folding and cell cycle regulation [30]. On the reverse strand of MIR17-GI, there are the *frdABCD* genes potentially involved in anaerobic respiration. Such *frd* genes were previously implicated in transduction by phages [31]. Lastly, MIR17-GI carries genes involved in maintenance of DNA and mobile genetic elements. The *ecnA* and *ecnB* genes located downstream of *bla*<sub>MIR-17</sub> encode the Entericidin A and

B toxin-antitoxin pair previously reported to prevent loss of plasmids [32]. Also located downstream of *bla*<sub>MIR-17</sub>, the *fxsA* gene encodes a polytopic membrane protein that prevents bacteriophages from exclusion [33–35]. Upstream of *bla*<sub>MIR-17</sub>, the DNA repair gene *mutL* and the high frequency of lysogeny operon *hflQXKC* are located (Supplemental Figure S2).

Notably, the above-listed genes represent the common context of chromosomal *ampC* genes in *Citrobacter*, *Escherichia*, *Enterobacter*, and *Shigella* species, which implies that MIR17-GI is an ancient mobile genomic element acquired before speciation. The latter view is supported by the fact that the GC content of the MIR17-GI and its left and right flanking sequences is quite similar among *Enterobacteriaceae*, as exemplified in Figure 4 for the *E. cloacae* study isolate 1 and the *E. coli* K12 reference strain MG1655. This is consistent with the notion that horizontally transferred DNA will adapt to the host genome over time, a process known as genome amelioration [36].

#### Landmark features of MIR17-gi-like MGIs in *Enterobacteriaceae*

Several “landmark” features of the MIR17-GI homologous MGIs can be distinguished. In the first place, the respective tRNA genes are highly conserved in all these islands, starting with the Phe-GAA tRNA gene at the 5' end, followed by the triplet Gly-GCC tRNA genes, and ending with the Leu-CAA tRNA gene at the 3' end. The Phe-GAA tRNA and the Leu-CAA tRNA genes define the borders of the MGIs, which vary in length from 136 to 148 kb in *E. coli*, *E. cloacae* and *K. pneumoniae* (Figure 2(a)). Further, these MGIs share the aforementioned *fxsA*, *groES/EL*, *encB*, *frdABCD* and *hflQXKC* genes, as well as a trehalose operon, a putative sugar

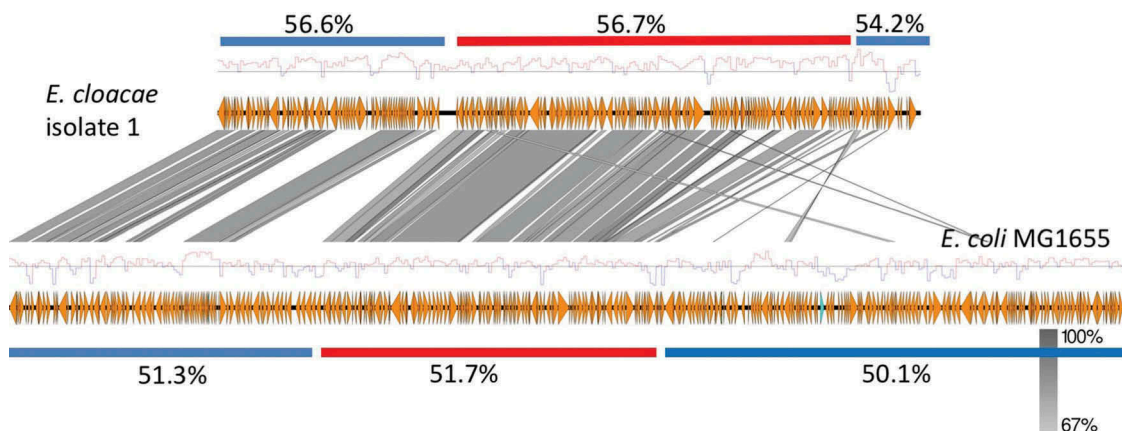
transport gene cluster, a primosomal replication protein N gene cluster, and an L-ascorbate utilization gene cluster. Lastly, a P4-associated integrase gene is located next to the LEU-CAA tRNA gene (Figure 2).

#### Diversity of *ampC* genes in MIR17-GI-like MGIs

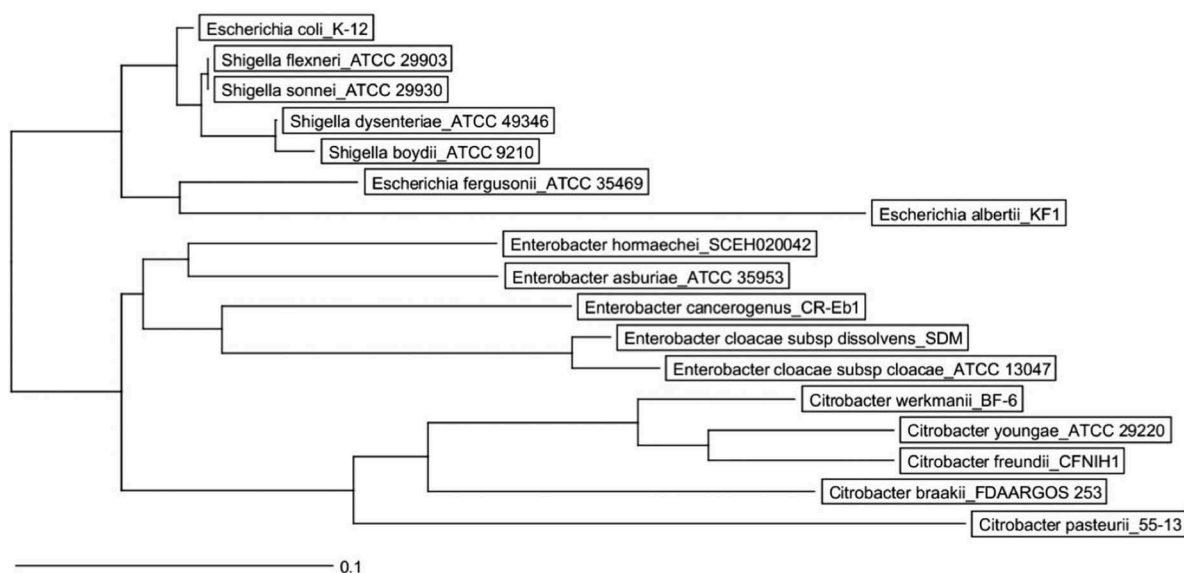
The *ampC* genes of MIR17-GI-like MGIs are integrated at the same location in all *Citrobacter*, *Escherichia*, *Enterobacter*, and *Shigella* genomes, next to *sugE-blc* genes. Nevertheless, the sequences of these *ampC* genes are quite diverse. As shown in the phylogenetic tree in Figure 5, the *ampC* genes of *Citrobacter* and *Enterobacter* species are relatively closely related to each other, forming a distinct cluster from the *ampC* genes encountered in *Escherichia* and *Shigella* species. Within *E. coli* and *Shigella*, the clustering of *ampC* genes corresponds well with the different phylogenetic groups and species, although the *ampC* genes of *E. coli* isolates belonging to phylogenetic group A are divided over four clusters (Supplemental Figure S3). These findings are indicative of separate acquisitions of *ampC* by the MIR17-GI-like MGIs over time.

#### Expression profile of MGI-encoded proteins from isolates 1 and 2

The frequent occurrence of MIR17-GI-like MGIs in *Enterobacteriaceae* raised the question to what extent proteins encoded by such MGIs are expressed. To approximate protein expression from MIR17-GI-like islands, cells of the *E. cloacae* study isolates 1 and 2 were investigated using liquid chromatography and tandem mass spectrometry (LC-MS/MS). A total number of 1300 different *E. cloacae* proteins was identified for both strains, including



**Figure 4.** GC content of the MIR17-GI from *E. cloacae* and the related MGI from *E. coli* and their flanking regions. Red bars indicate the position of the respective MGI and blue bars the different flanking regions. The overall GC% per region is indicated and the red-blue diagrams mark variations in the respective GC profiles.



**Figure 5.** Neighbor joining tree of the *ampC* genes in *Citrobacter*, *Escherichia*, *Enterobacter* and *Shigella* species. The phylogenetic tree of *ampC* genes in *Citrobacter*, *Escherichia*, *Enterobacter* and *Shigella* species was created with the Ridom SeqSphere+ software v4.1.9 using a neighbor joining algorithm. The scale bar represents the % difference among 77 nucleotides of *ampC*, using the *ampC* gene of the *E. coli* K12 strain MG1655 as a reference. Bacterial isolates and the respective genome sequence accession codes are presented in Supplemental Table S2.

857 proteins of isolate 1 and 1116 proteins of isolate 2 (Supplementary Table S1). The MS analysis identified the largest numbers of different proteins in the stationary phase of growth (Figure 6; Supplemental Figure S4). Further, label-free quantification of the proteins identified by MS (i.e. spectral counting) showed similar patterns of representation of the proteins detected at the highest levels among the two investigated isolates (Figure 7(a)). In contrast, there was substantially more variation in the identified proteins from both isolates that were detectable at relatively low levels. Importantly, for isolate 1, eleven of the 45 MIR17-GI-encoded proteins were identified, including the MIR17 carbapenemase that was expressed at the highest levels in the stationary growth phase (Figure 7(b, c)). In fact, judged by spectral counting, MIR17 is one of the 40 most abundant proteins that were identified in isolate 1. Further, the MIR17-GI-encoded GroEL protein was the second most abundant protein identified by MS. Nine proteins encoded by the MIR17-GI-like MGI of isolate 2 were identified. Of note, the ACT-5  $\beta$ -lactamase of isolate 2 remained undetected, which is consistent with the susceptibility of this isolate to third-generation cephalosporins and carbapenems.

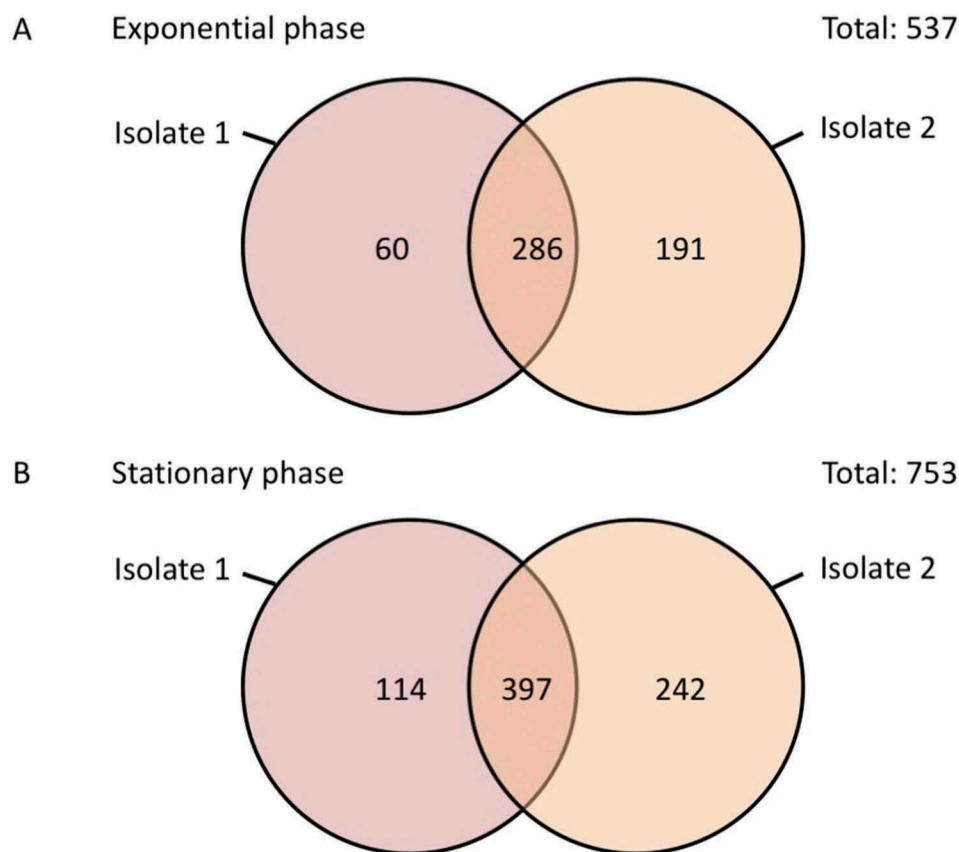
## Discussion

The present study highlights the identification of an ancient MGI-family integrating *ampC*-like cephalosporinase genes

in a wide range of *Enterobacteriaceae*. This MGI was first identified in a carbapenem-resistant clinical isolate of *E. cloacae*, where it was shown to carry the *bla*<sub>MIR-17</sub> gene for an AmpC-like cephalosporinase. Accordingly, the respective MGI was named MIR17-GI. Further inspection of MIR17-GI revealed a high abundance of different resistance genes of which the MIR17 enzyme was found to be highly expressed. Importantly, MIR17 displays carbapenemase activity that can be inhibited with cloxacillin, suggesting that the carbapenem resistance of isolate 1 has to be attributed to this enzyme.

Interestingly, the MIR17-GI of study isolate 1 was found to be associated with a P4 prophage. This suggests that the transmission of MIR17-GI-like MGIs between *Enterobacteriaceae* may have been phage-mediated. Consistent with this idea, all MIR17-GI-like MGIs were found to be integrated into Phe-GAA tRNA genes that include a phage P4-associated attachment site at the 5' end of the MGI. Moreover, they are all flanked by a conserved P4 integrase gene, with the Leu-CAA tRNA gene as attachment site at the 3' end. These features are typical for chromosomal P4-like gene clusters [24].

Resistance genes are frequently exchanged between microorganisms via MGIs and this seems to be the case also for the MIR17-GI-like islands of which most, but not all, were found to carry an *ampC*-like cephalosporinase gene. Such resistance genes that are carried and integrated into the bacterial chromosome by phages are sometimes referred to as “morons”. In general, morons



**Figure 6.** Numbers of consistently or uniquely identified proteins in the *E. cloacae* study isolates 1 and 2 during the exponential and stationary growth phases. The diagrams were created using the Venn diagram web tool of the VIB and the University of Gent in Belgium (<http://bioinformatics.psb.ugent.be/webtools/Venn/>).

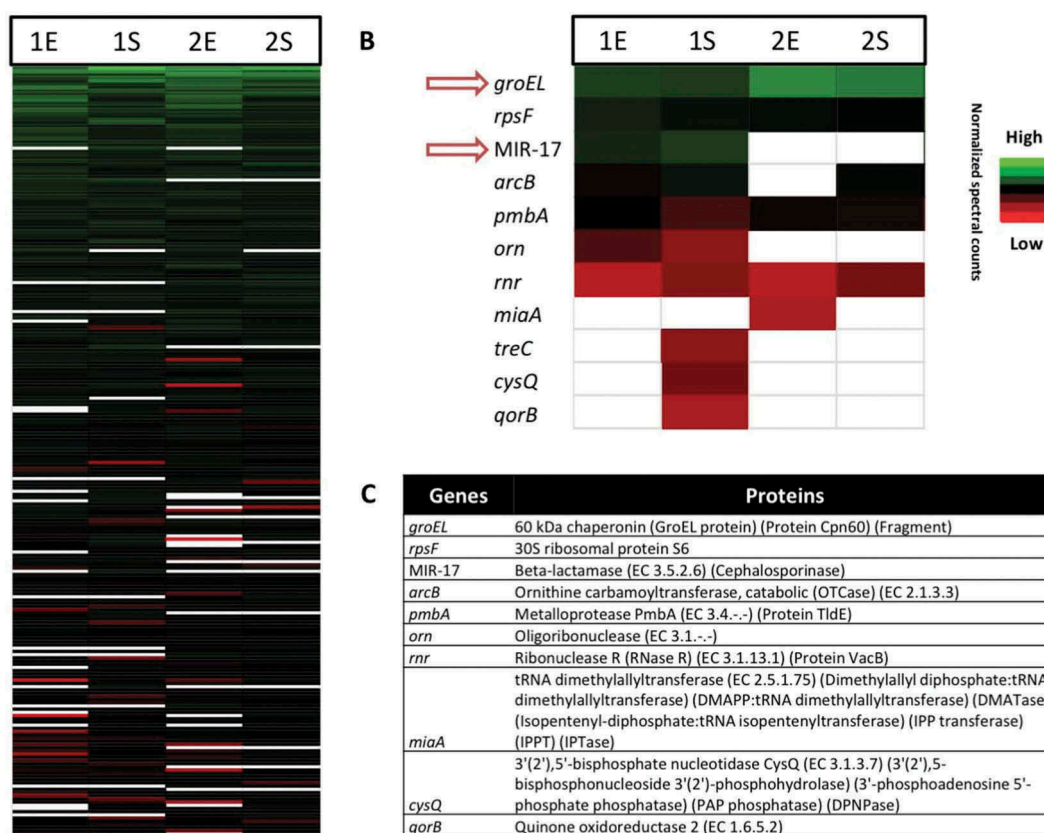
are composed of one or more genes that have no particular function in the phage's lysogenic cycle, but that may provide benefits to the phage's host. This coevolution of prophages and bacteria would be important for species to adapt to various environmental niches [37]. In the case of MIR17, the benefit for the *E. cloacae* host would be the acquisition of resistance to cephalosporins or even carbapenems. In this respect, it is important to note that such antibiotics are produced in nature by fungi and *Streptomyces* that may compete for the same ecological niches as *E. cloacae*. Morons are frequently flanked by tRNAs and this was also found to be the case for *bla*<sub>MIR-17</sub>. The idea that *bla*<sub>MIR-17</sub> is a moron is further supported by the observation that it is localized in the vicinity of the bacterial high frequency lysogeny operon *hflQXKC* [38].

Lastly, we observed a diversity of genes encoding cephalosporinases among the identified MIR17-GI-like mobile genomic islands. The integration of broad spectrum cephalosporinase genes, as found in the *E. cloacae* study isolates is of major clinical importance. Particularly, upon high-level expression, both the ACT- and the MIR-families of  $\beta$ -lactamases are capable of hydrolyzing third-

generation cephalosporins [11]. Moreover, our study shows that the carbapenem resistance of *E. cloacae* study isolate 1 can be attributed to the new allele *bla*<sub>MIR-17</sub> and the high-level production of the encoded MIR17 enzyme. The carbapenem resistance of study isolate 1 may be further enhanced by a change in outer membrane protein expression. In particular, the *ompF* gene of isolate 1, homologous to *ompK35*, contains two point mutations compared to reference *ompF* gene of *E. cloacae* MGH 54 (aebl-supercont1.1.C3), one of which results in a stop codon at position 200. Therefore, an additional factor contributing to the carbapenem resistance of isolate 1 could be the absence of an outer membrane porin, especially *OmpF*. The latter would be consistent with previous studies on the role of porins in the resistance of *Enterobacteriaceae* to antibiotics [29,39,40].

In conclusion, we identified a novel family of MGIs harboring genes associated with the resistance to cephalosporins, phage immunity, and accessory metabolic functions. These MIR17-GI-like islands are present in various *Enterobacteriaceae*, including *K. pneumoniae*, *E. coli*, and *E. cloacae*. Importantly, the MIR17-GI-like islands are associated with integrated P4-like prophages,





**Figure 7.** Proteomic profiles of the investigated *E. cloacae* study isolates 1 and 2 in the exponential and stationary growth phases. (a) Bar diagram depicting the relative amounts of 500/1300 identified *E. cloacae* proteins in the exponential (E) and stationary (S) growth phases based on normalized spectral counts. Relative amounts of identified proteins encoded by the MIR17-GI (b), and their respective functions (c).

which implicate phages in the spread of high-level cephalosporin and carbapenem resistance amongst *Enterobacteriaceae*. In view of the serious consequences of high-level cephalosporin and carbapenem resistance for treatment of patients with enterobacteriaceal infections, the discovery of an ancient mobile genomic island that has facilitated the spread of “cephalosporinase and carbapenemase morons” is of high clinical relevance.

## Materials and methods

### Bacterial isolates

The bacterial isolates used in this study and their antibiotic resistances are summarized in Table 1. Species determination was performed by matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) MS, using a Bruker Microflex (Bruker Corporation, Billerica, USA).

### Antibiotic susceptibility testing

Antibiotic susceptibility was routinely determined with the VITEK 2 system with an ASTn344 card

(bioMérieux, Marcy l’Etoile, France). The VITEK 2 minimum inhibitory concentration results were interpreted according to the advanced expert system following EUCAST guidelines ([www.eucast.org](http://www.eucast.org)). The susceptibility to meropenem or imipenem was subsequently verified on MHA using the AB Biodisk Etest according to manufacturer’s guidelines (AB Biodisk, Mannheim, Germany). The presence of genes for carbapenemase, ESBL and AmpC was verified with the Check-MDR CT103 XL microarray assay (Check-Points, Wageningen, the Netherlands).

### DNA sequence analyses

#### Nanopore sequencing

Bacteria were grown overnight at 37°C on Blood Agar (BA) plates. Then single colonies were picked for overnight culture at 37°C on BA plates again. For DNA extraction the DNeasy UltraClean Microbial Kit (Qiagen) was used with minor modifications. A 10 µl-loopful of bacteria was directly transferred into a tube with microbeads and microbeads solution. The incubation period was prolonged

to 20 min, instead of 5 min. The quality and quantity of isolated DNA was determined using a Qubit® 2.0 fluorometer (ThermoFisher Scientific), an Agilent TapeStation 2200 (Agilent) and a NanoDrop (ThermoFisher Scientific). Libraries were prepared without shearing to maximise sequencing read length. The library was prepared using the 2D ligation sequencing kit (SQK-LSK208). The protocol for 2D ligation sequencing kit was followed as described by the manufacturer. The final library was loaded onto an FLO-MIN106 R9.4 flow cell. The run was performed on a MinION device using the NC\_48Hr\_Sequencing\_Run\_FLO-MIN107\_SQKLSK208 protocol with 976 available pores (464, 314, 161 and 37 pores per group). The run proceeded for the full 48 hours. Base calling was performed after the run, using Albacore v1.2.2 (Nanopore) with the r94\_250bps\_2d.cfg workflow. Lastly, the quality of the data was analysed with Poretools v0.6.0 [41] and the fast5 files were transformed into a fastq file.

### **Illumina sequencing**

Total DNA extraction for whole-genome sequencing was performed directly from colonies of the respective isolates using the Ultraclean Microbial DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, US) according to the manufacturer's protocol. DNA concentrations were determined using a Qubit® 2.0 fluorometer and the dsDNA HS and/or BR assay kit (Life technologies, Carlsbad, CA, US). Subsequently, DNA libraries were prepared using the Nextera XT v3 kit (Illumina, San Diego, CA, US) according to the manufacturer's instructions. Sequence analysis was performed with an Illumina Miseq System generating paired-end reads of 300 bp as described previously [42]. *De novo* assembly of paired-end reads was performed using CLC Genomics Workbench v8.5.3 (QIAGEN, Hilden, Germany) after quality trimming ( $Q_s \geq 20$ ) establishing a word size of 30. This resulted in 159 and 66 contigs ( $\geq 500$  bp) with an average coverage of 72-fold. The acquired antimicrobial resistance genes and multi-locus sequence types (MLST) were identified by uploading the assembled genome sequences onto the Resfinder server v2.1. [43] and the MLST 1.7 server [44], respectively. The raw WGS datasets generated in the current study are available in the European Nucleotide Archive (ENA) repository under Bioproject PRJEB22119 (<http://www.ebi.ac.uk/ena/>).

### **Hybrid assembly**

The hybrid assembly of the Illumina short reads and the MinION long reads of isolate 339389L was performed using SPAdes version: 3.10.1 (<http://bioinf.spbau.ru/en>). The resulting assembly was submitted to NCBI under accession number CP026536. Sequence similarity

analyses between MIR17 and MGIs from *Escherichia coli* were performed using the rapid annotation using subsystem technology (RAST) server 4.0 [45].

### **Phylogenetic analyses**

Phylogenetic trees based on single nucleotide polymorphisms (SNPs) were built using the SNP comparison tool in Ridom SeqSphere v4.1.9 (Münster, Germany) with default settings [46]. Properties of the enterobacteriaceal genomes used for phylogenetic analyses are summarized in Supplemental Table S2. The relatedness of MIR17-GI-like MGIs was analyzed using a local *ad hoc* cgMLST scheme based on 124 genes of the respective MGI from *E. coli* K12 strain MG1655 (Supplemental Table S3). For visualization of the MGI comparisons, BLAST+ 2.6.0 (NCBI, Bethesda, USA) and DNA plotter v 1.11 (Wellcome Sanger Institute, Cambridge, UK) were used with default settings.

### **Identification of orthologous proteins**

To identify orthologous proteins, reciprocal best hits (RBHs) were calculated. Galaxy, a python script [47], was used to perform reciprocal protein BLAST searches (NCBI BLAST+ v. 2.3.0 [48]. Default parameters (minimum percentage identity: 70%; minimum High Scoring Pair (HSP) coverage: 50%) were used and all redundancies were removed. RBHs were calculated by blasting: (i) the two whole genome sequences; (ii) the MGI from isolate 1 and the whole genome of isolate 2; (iii) the MGI from isolate 2 and the whole genome of isolate 1; and (iv) the two MGIs.

### **Proteome analyses**

*E. cloacae* isolates 1 and 2 were cultured in triplicate in brain heart infusion broth (BHI; Oxoid, Basingstoke, UK) at 37°C with vigorous shaking at 250 rpm. For sample preparation, cells were collected in the mid-exponential and stationary growth phases by centrifugation and disrupted by bead-beating with glass beads (~ 0.1 mm diameter) in a Precellys 24 homogenisator (Bertin Technologies, France) as described previously [49,50]. Glass beads and cell debris were removed by centrifugation ( $21,000 \times g$ , 10 min, 4°C). The cell extract protein fraction was prepared and analyzed by LC-MS/MS using an Orbitrap Velos Pro mass spectrometer (ThermoFisher, Waltham, MA USA) as described previously [49]. Briefly, proteins were concentrated with Strataclean beads, subsequently reduced and alkylated, digested with trypsin and then purified through StageTip purification. Desalted peptides were loaded on

an EASY-nLC™ II nano-flow LC system (ThermoFisher) with 10 µl buffer A (0.1% (v/v) acetic acid) and a constant flow rate of 0.5 µL/min. Afterwards, the peptides were separated by reversed phase chromatography with a 155 min non-linear gradient from 1 to 50% buffer B (0.1% (v/v) acetic acid in acetonitrile) with a constant flow rate of 0.3 µl/min and injected online into the mass spectrometer. The 20 most abundant precursor ions were selected for collision-induced dissociation (CID) fragmentation after a survey scan in the Orbitrap with a resolution of 60,000 and activated lockmass correction. MS/MS scans were recorded in the dual pressure linear ion trap after fragmentation was performed for 10 msec with a normalized collision energy of 35.

Data analysis was performed according to Bonn et al. [49,51]. In brief, database searching was done with Sorcerer-SEQUEST 4 (Sage-N Research, Milpitas, USA). After data extraction from raw files, the \*.dta files were searched with Sequest against a target-decoy database with a set of common laboratory contaminants. A non-redundant database for peptide/protein searches was created from the genome sequences of isolate 1 (339389L) and isolate 2 (141024K), the genome sequence of the *E. cloacae* type strain ATCC 13047 as downloaded from Uniprot (<http://www.uniprot.org>; 23<sup>rd</sup> of October 2015), plus five additional genome sequences from unrelated clinical *E. cloacae* isolates. The used database includes protein sequences that differ in at least 1 amino acid, and it contains 30486 proteins in total. Only strict tryptic peptides with up to two missed cleavages were used for the database search. Fixed modifications were not considered. Oxidation of methionine and carbamidomethylation of cysteine were considered as variable modifications. Mass tolerance for precursor ions was set to 10 ppm, and for fragment ions to 0.5 Da. Validation of the MS/MS-based peptide and protein identification was performed with Scaffold v.4.4.1.1 (Proteome Software, Portland, USA). Peptide identifications were only accepted if they exceeded the following specific database search engine thresholds: the SEQUEST identifications required at least deltaCn scores of > 0.1 and XCorr scores of > 2.2, 3.3 and 3.7 for doubly, triply and all higher charged peptides, respectively. Protein identifications (Supplemental Table S1) were accepted if at least 2 identified peptides were detected with above mentioned filter criteria in 2 out of 3 biological replicates. This resulted in a false-positive discovery rate (FDR) below 0.2% on protein level as was verified by a search against a concatenated target-pseudoreversed decoy database. All MS data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD007113 [52].

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## Disclosure statement

No potential conflict of interest was reported by the authors.

## Ethics statement

The bacterial isolates used for the present analyses were collected in the course of routine diagnostics and infection prevention control. Oral consent for the use of such clinical samples for research purposes is routinely obtained upon patient admission to the UMCG, in accordance with the guidelines of the Medical Ethics Committee of the University Medical Center Groningen. All experiments were performed in accordance with the guidelines of the Declaration of Helsinki and the institutional regulations, and all samples were anonymized.

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