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Identification of resistance and virulence factors in an epidemic *Enterobacter hormaechei* outbreak strain

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Bacterial strains differ in their ability to cause hospital outbreaks. Using comparative genomic hybridization, *Enterobacter cloacae* complex isolates were studied to identify genetic markers specific for *Enterobacter cloacae* complex outbreak strains. No outbreak-specific genes were found that were common in all investigated outbreak strains. Therefore, the aim of our study was to identify specific genetic markers for an *Enterobacter hormaechei* outbreak strain (EHOS) that caused a nationwide outbreak in The Netherlands. Most EHOS isolates carried a large conjugative plasmid (pQC) containing genes encoding heavy-metal resistance, mobile elements, pili-associated proteins and exported proteins as well as multiple-resistance genes. Furthermore, the chromosomally encoded high-pathogenicity island (HPI) was highly associated with the EHOS strain. In addition, other DNA fragments were identified that were associated with virulence: three DNA fragments known to be located on a virulence plasmid (pLVPK), as well as phage- and plasmid-related sequences. Also, four DNA fragments encoding putative pili with the most homology to pili of *Salmonella enterica* were associated with the EHOS. Finally, four DNA fragments encoding putative outer-membrane proteins were negatively associated with the EHOS. In conclusion, resistance and putative virulence genes were identified in the EHOS that may have contributed to increased epidemicity. The high number of genes detected in the EHOS that were related to transferable elements reflects the genomic plasticity of the *E. cloacae* complex and may explain the emergence of the EHOS in the hospital environment.

INTRODUCTION

In the pre-antibiotic era, isolates of the *Enterobacter cloacae* complex were not detected in surveys of nosocomial bacteria; they were first described as nosocomial pathogens in the 1970s (Sanders & Sanders, 1997). In 2001, *Enterobacter* spp. caused 7 % of nosocomial infections in intensive care units in the USA (Jones, 2003; Streit *et al.*, 2004). Therefore, the *E. cloacae* complex can be considered as an emerging pathogen, of which *Enterobacter hormaechei* is the most commonly isolated nosocomial pathogen (Delmas *et al.*, 2006; Paauw *et al.*, 2008). A nationwide outbreak occurred with an *E. hormaechei* outbreak strain (EHOS) in The Netherlands (Leverstein-van Hall *et al.*, 2006). This strain disseminated throughout hospitals despite adequate implementation of internationally accepted infection-prevention guidelines and caused invasive infections in more than 100 patients (Paauw *et al.*, 2007). Previous studies showed that most EHOS isolates carried the conjugative plasmid pQC, with several complex integrons containing *aadB*, $bla_{\text{CTX-M-9}}$ and *qnrA1* genes that encoded resistance to aminoglycosides and third-generation cephalosporins, and reduced susceptibility to fluoroquinolones, respectively (Paauw *et al.*, 2006).

However, it was also shown that other *Enterobacteriaceae* including other *E. cloacae* complex isolates with the pQC did not become highly epidemic like the EHOS (Paauw *et al.*, 2007). Furthermore, of aminoglycoside-susceptible *E. cloacae* complex strains, a susceptible variant of the EHOS without pQC had the highest dissemination rate in our hospital (Paauw *et al.*, 2007). The extent of the spread with the pQC-negative EHOS is unknown because the policy in the University Medical Centre Utrecht (UMCU) is to screen for aminoglycoside-resistant isolates; therefore additional cases could be missed. Features other than the

Abbreviations: CGH, comparative genome hybridization; EHOS, *Enterobacter hormaechei* outbreak strain; EPP, estimated probability of presence; HPI, high-pathogenicity island(s); MGA, mixed genome array; MLSA, multi-locus sequence analysis.

Three supplementary tables are available with the online version of this paper.

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resistance phenotype likely played an additional role in the epidemicity of the EHOS.

Comparative genomic hybridization (CGH) is a powerful genetic tool for the study of bacteria (Dobrindt *et al.*, 2003; Fitzgerald *et al.*, 2001, 2003; Fukiya *et al.*, 2004; Israel *et al.*, 2001; Leavis *et al.*, 2007; Lindsay *et al.*, 2006; Paauw *et al.*, 2008; Porwollik *et al.*, 2002; Salama *et al.*, 2000). Most CGH arrays have been designed from annotated whole-genome sequences. Unfortunately, because *E. cloacae* is not a model organism, no whole-genome sequences of *E. cloacae* complex isolates are available. To cover the pan-genome of the genetically diverse *E. cloacae* complex, a mixed genome array (MGA) was constructed from a shotgun library obtained from eight isolates. Previously, this MGA was used for CGH together with multi-locus sequence analysis (MLSA) to determine genetic relationships between clinical *E. cloacae* complex isolates (Paauw *et al.*, 2008). The *E. cloacae* complex was shown to be evolutionarily divided into two clades that are genetically distinct from each other. Based on genetic diversity, the first clade was subdivided into two clusters, of which the second cluster contained the EHOS. The second group, which is genetically more heterogenous, was divided into five clusters (Fig. 1) (Paauw *et al.*, 2008). However, CGH can also be used to identify genes specific for or associated with subpopulations of the tested population. This last property was used in the present study.

The aim of our study was to identify genes specific to the EHOS. These genes may possibly be used as markers to identify EHOS isolates faster, which could lead to faster implementation of control measures preventing further dissemination. In addition, genes that are associated with the EHOS were identified to determine if specific genes contributed to the increased epidemic behaviour of this strain.

METHODS

Bacterial isolates. All 158 isolates that were used in our previous study (Paauw *et al.*, 2008) were also included in this study (Table 1). One hundred and twenty of these originated from the UMCU (Paauw *et al.*, 2008). These isolates were a subset of a large collection of isolates typed with PFGE that represented the *E. cloacae* complex population in the UMCU from 2001 to 2005. Outbreak isolates were identified by >85 % similarity in PFGE patterns. Of the UMCU isolates, 28 belonged to the EHOS (outbreak I). All EHOS isolates were considered to be epidemiologically linked. The strain is endemic in the UMCU and probably also in the other Dutch hospitals (Leverstein-van Hall *et al.*, 2006; Paauw *et al.*, 2006, 2007). Furthermore, six isolates represented two small outbreaks: three isolates of outbreak IV (this aminoglycoside-resistant *E. cloacae* IV caused a small outbreak colonizing six patients in August 2008) and three isolates of outbreak II (an aminoglycoside-susceptible *E. hormaechei*, possibly two cross-transmission events in a 3 month period). The remaining 86 UMCU isolates were not epidemiologically linked.

In addition, 21 aminoglycoside-resistant *E. cloacae* complex isolates from 11 other Dutch hospitals were included (Paauw *et al.*, 2007). These 21 isolates included eight EHOS (outbreak I) isolates, three isolates with a unique genotype, and also 10 isolates involved in four outbreaks in four different hospitals: outbreak III (*E. hormaechei*; a sample of two isolates that represented an outbreak with at least 30 patients), outbreak V (*E. hormaechei*; a sample of three isolates that represented an outbreak with at least six patients), outbreak VI (*E. cloacae* IV; a sample of three isolates that represented an outbreak with at least 20 patients) and outbreak VII (*E. cloacae* IV; a sample of two isolates that represented an outbreak with at least five patients). Four of the seven outbreak strains (I, IV, V, VII) were PCR-positive for *qnrA1* or $bla_{\text{CTX-M-9}}$, indicating the presence of a pQC-like plasmid in these strains.

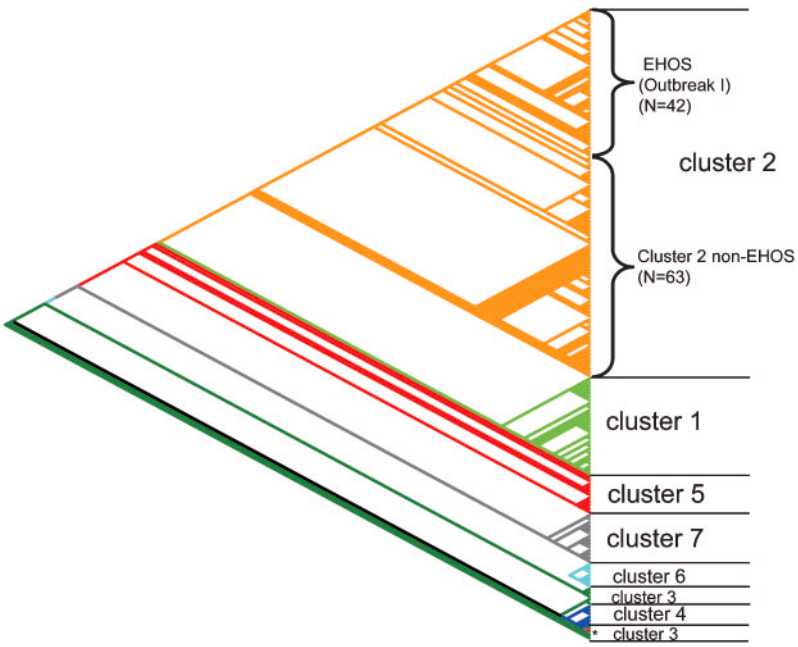

Fig. 1. Tree constructed on the basis of the CGH results of the parsimony method. The slanted cladogram depicts genetic relationships between isolates; each colour represents a different MLSA cluster. *Solitary isolate in MLSA.

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Table 1. Isolates used in the study

Abbreviations: AUS, Austria; ESP, Spain; FRA, France; GBR, Great Britain; ITA, Italy; NL, The Netherlands; POL, Poland: POR, Portugal; SWI, Switzerland; Type (strain), *E. cloacae* ATCC 13047; Type (strain) +, *E. cloacae* ATCC 13047 with pQC.

Isolate no.	No. rep.*	Country	Hospital	Cluster†	Species‡	Same patient	PFGE type	Outbreak no.	*qnr*§	$bla_{CXT-M-9}$‖
88		NL	1	1	*E. hormaechei*		4		0	0
8		NL	1	1	*E. hormaechei*		5		1	1
116		ESP	6	1	*E. hormaechei*		14		0	0
19	3	NL	1	1	*E. hormaechei*		15		0	0
61		NL	1	1	*E. hormaechei*		15		0	0
142		AUS	16	1	*E. hormaechei*		17		0	0
84		NL	1	1	*E. hormaechei*		20		0	0
117		POR	7	1	*E. hormaechei*		26		0	0
145		FRA	19	1	*E. hormaechei*		28		0	0
52		NL	1	1	*E. hormaechei*		29		0	0
54		NL	1	1	*E. hormaechei*		30		0	0
4		NL	1	1	*E. hormaechei*		31		0	0
99	2	NL	1	1	*E. hormaechei*		31		0	0
102		NL	1	1	*E. hormaechei*		32		1	0
23		NL	1	1	*E. hormaechei*	a	33		1	1
47		NL	1	1	*E. hormaechei*		34		0	0
29		NL	1	1	*E. hormaechei*		37		1	1
30		NL	1	1	*E. hormaechei*		38		0	0
122		NL	1	1	*E. hormaechei*		38		0	0
123		NL	1	1	*E. hormaechei*		38		0	0
94		NL	1	1	*E. hormaechei*		39		0	0
56		NL	1	1	*E. hormaechei*		47		0	0
143		GBR	17	1	*E. hormaechei*		50		0	0
22		NL	1	1	*E. hormaechei*		–		0	0
130		NL	1	1	*E. hormaechei*		–		0	0
137		NL	12	2	*E. hormaechei*		1		1	0
152		NL	24	2	*E. hormaechei*		1		0	0
5		NL	1	2	*E. hormaechei*		1	I	1	1
7		NL	1	2	*E. hormaechei*		1	I	0	0
16		NL	1	2	*E. hormaechei*		1	I	0	1
27		NL	1	2	*E. hormaechei*		1	I	0	0
31	4	NL	1	2	*E. hormaechei*		1	I	1	1
37		NL	1	2	*E. hormaechei*	b	1	I	1	1
38		NL	1	2	*E. hormaechei*	b	1	I	0	0
44		NL	1	2	*E. hormaechei*	c	1	I	1	1
45		NL	1	2	*E. hormaechei*	c	1	I	0	0
55		NL	1	2	*E. hormaechei*		1	I	1	1
58		NL	1	2	*E. hormaechei*		1	I	1	1
67		NL	1	2	*E. hormaechei*		1	I	0	1
69		NL	1	2	*E. hormaechei*	e	1	I	1	1
70		NL	1	2	*E. hormaechei*	e	1	I	0	0
74		NL	1	2	*E. hormaechei*		1	I	1	1
77		NL	1	2	*E. hormaechei*	f	1	I	1	1
78	3	NL	1	2	*E. hormaechei*	f	1	I	0	0
79	2	NL	1	2	*E. hormaechei*		1	I	1	1
86		NL	1	2	*E. hormaechei*	g	1	I	1	1
87		NL	1	2	*E. hormaechei*	g	1	I	0	0
89		NL	1	2	*E. hormaechei*		1	I	1	1
92		NL	1	2	*E. hormaechei*		1	I	0	1
95		NL	1	2	*E. hormaechei*		1	I	1	1
96		NL	1	2	*E. hormaechei*		1	I	1	1
98		NL	1	2	*E. hormaechei*		1	I	1	1
100		NL	1	2	*E. hormaechei*		1	I	1	1

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Table 1. cont.

Isolate no.	No. rep.*	Country	Hospital	Cluster†	Species‡	Same patient	PFGE type	Outbreak no.	*qnr*§	$bla_{CXT-M-9}$‖
106		NL	1	2	*E. hormaechei*		1	I	1	1
108		NL	2	2	*E. hormaechei*		1	I	1	1
109		NL	3	2	*E. hormaechei*		1	I	1	1
115		NL	5	2	*E. hormaechei*		1	I	1	1
131		NL	9	2	*E. hormaechei*		1	I	1	1
136		NL	12	2	*E. hormaechei*		1	I	1	1
138		NL	13	2	*E. hormaechei*		1	I	1	1
147		NL	21	2	*E. hormaechei*		1	I	1	1
154		NL	8	2	*E. hormaechei*		1	I	1	1
34		NL	1	2	*E. hormaechei*		2		0	0
72		NL	1	2	*E. hormaechei*		2		1	1
63		NL	1	2	*E. hormaechei*		3		0	0
53		NL	1	2	*E. hormaechei*		3	I	0	0
20		NL	1	2	*E. hormaechei*		5		0	0
148		ESP	22	2	*E. hormaechei*		5		0	0
17	2	NL	1	2	*E. hormaechei*		7		0	0
1		NL	1	2	*E. hormaechei*		8		0	0
14		NL	1	2	*E. hormaechei*		8	II	0	0
41	2	NL	1	2	*E. hormaechei*		8	II	0	0
66		NL	1	2	*E. hormaechei*		8	II	0	0
18		NL	1	2	*E. hormaechei*		9		0	0
33	2	NL	1	2	*E. hormaechei*		9		0	0
50		NL	1	2	*E. hormaechei*		10		0	0
93	2	NL	1	2	*E. hormaechei*		10		0	0
13	2	NL	1	2	*E. hormaechei*		11		0	1
35		NL	1	2	*E. hormaechei*		11		0	0
139		NL	13	2	*E. hormaechei*		11		0	0
155		NL	8	2	*E. hormaechei*		11	III	0	0
156		NL	8	2	*E. hormaechei*		11	III	0	0
124		NL	1	2	*E. hormaechei*		12		0	0
46		NL	1	2	*E. hormaechei*		13		0	0
97		NL	1	2	*E. hormaechei*		13		0	0
112		NL	4	2	*E. hormaechei*		13	V	1	1
113		NL	4	2	*E. hormaechei*		13	V	0	1
114		NL	4	2	*E. hormaechei*		13	V	1	1
126	2	NL	1	2	*E. hormaechei*	h	13		0	0
127		NL	1	2	*E. hormaechei*	h	13		0	0
9		NL	1	2	*E. hormaechei*		14		0	0
48	2	NL	1	2	*E. hormaechei*	d	14		0	0
49		NL	1	2	*E. hormaechei*	d	14		1	0
3		NL	1	2	*E. hormaechei*		16		0	0
90		NL	1	2	*E. hormaechei*		17		0	0
144		ESP	18	2	*E. hormaechei*		17		0	0
2		NL	1	2	*E. hormaechei*		20		0	0
141		FRA	15	2	*E. hormaechei*		21		0	0
71		NL	1	2	*E. hormaechei*		25		0	0
146		ITA	20	2	*E. hormaechei*		27		0	0
73		NL	1	2	*E. hormaechei*		31		0	0
11		NL	1	2	*E. hormaechei*		32		0	0
21		NL	1	2	*E. hormaechei*		36		0	0
59	2	NL	1	2	*E. hormaechei*		36		0	0
24		NL	1	2	*E. hormaechei*	a	37		1	0
57		NL	1	2	*E. hormaechei*		41		0	0
65		NL	1	2	*E. hormaechei*		42		0	0
25		NL	1	2	*E. hormaechei*		43		0	0
75		NL	1	2	*E. hormaechei*		44		0	0

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Table 1. cont.

Isolate no.	No. rep.*	Country	Hospital	Cluster†	Species‡	Same patient	PFGE type	Outbreak no.	*qnr*§	$bla_{CXT-M-9}$‖
36		NL	1	2	*E. hormaechei*		45		0	0
64		NL	1	2	*E. hormaechei*		46		0	0
10		NL	1	2	*E. hormaechei*		49		0	0
60		NL	1	2	*E. hormaechei*		50		0	0
153		POL	25	2	*E. hormaechei*		56		0	0
107		NL	1	2	*E. hormaechei*		57		0	0
103		NL	1	2	*E. hormaechei*		58		0	0
85		NL	1	3	*E. cloacae*		18		0	0
32		NL	1	3	*E. cloacae*		23		0	0
39		NL	1	3	*E. hormaechei*		24		0	0
80		NL	1	3	*E. cloacae*		59		0	0
157		Type	–	3	*E. cloacae*	I	60		0	0
158		Type +	–	3	*E. cloacae*	I	–		1	1
76		NL	1	4	*E. ludwigii*		19		0	0
15	2	NL	1	4	*E. ludwigii*		24		0	0
43		NL	1	4	*E. ludwigii*		46		0	0
28		NL	1	4	*E. ludwigii*		54		0	0
125	2	NL	1	4	*E. ludwigii*		55		0	0
128		NL	1	4	*E. ludwigii*		–		0	0
62		NL	1	5	*E. asburiae*		6		0	0
149		NL	–	5	*E. asburiae*¶		12		0	0
150		NL	–	5	*E. asburiae*¶		12		0	0
151		NL	–	5	*E. asburiae*¶		12		0	0
135		ITA	11	5	*E. asburiae*		16		0	0
140	2	SWI	14	5	*E. asburiae*		20		0	0
6	2	NL	1	5	*E. asburiae*		21		0	1
120		NL	1	5	*E. asburiae*		40		0	0
119		NL	1	5	*E. asburiae*		51		0	0
83		NL	1	6	*E. cloacae* IV		8		0	0
105		NL	1	6	*E. cloacae* IV		12		0	0
81		NL	1	6	*E. cloacae* IV		14		0	0
91		NL	1	6	*E. cloacae* IV		16		1	1
26		NL	1	6	*E. cloacae* IV		22		1	1
82		NL	1	6	*E. cloacae* IV		35		0	0
129		NL	1	6	*E. cloacae* IV		–		0	0
40		NL	1	7	*E. cloacae* IV		12		1	1
101		NL	1	7	*E. cloacae* IV		12		1	1
42		NL	1	7	*E. cloacae* IV		14		0	0
132		NL	10	7	*E. cloacae* IV		27	VI	0	0
133		NL	10	7	*E. cloacae* IV		27	VI	0	0
134		NL	10	7	*E. cloacae* IV		27	VI	0	0
118		NL	1	7	*E. cloacae* IV		36		0	0
121		NL	1	7	*E. cloacae* IV		48		0	0
110		NL	4	7	*E. cloacae* IV		52	VII	1	1
111		NL	4	7	*E. cloacae* IV		52	VII	1	1
12		NL	1	7	*E. cloacae* IV		53	IV	1	1
51		NL	1	7	*E. cloacae* IV		53	IV	1	1
104	2	NL	1	7	*E. cloacae* IV		53	IV	1	1
68		NL	1	–	*E. asburiae*		36		0	0

*Number of replicates tested.
†Cluster according to MLSA and comparative genome hybridization results.
‡Nomenclature is based on *rpoB* genotyping.
§*qnrA* detection by PCR: *qnrA* gene present (1) or absent (0).
‖$bla_{CTX-M-9}$ detection by PCR: $bla_{CTX-M-9}$ gene present (1) or absent (0).
¶From industrial site.

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Also included were 12 unique isolates from 12 different European hospitals (Fluit *et al.*, 2001), three *Enterobacter asburiae* isolates from a contaminated industrial site, and *E. cloacae* ATCC 1307 with and without pQC (Paauw *et al.*, 2008).

Identification of the isolates was based on *rpoB* genotyping; 501 bp fragments of *rpoB* were sequenced bidirectionally (accession numbers EU643264–EU643420). The sequences were compared with a database of *rpoB* sequences representing the major phylogenetic clades in the family *Enterobacteriaceae*; this database is managed by the Pasteur Institute in Paris, France (Salerno *et al.*, 2007). Characteristics of all isolates used in the study are summarized in Table 1.

Microarrays. A shotgun library, previously described, created from eight isolates (seven different strains) was used to construct the microarray (Paauw *et al.*, 2008). Briefly, the library was constructed from eight isolates and contained five isolates from the UMCU, two of which represented the EHOS (outbreak I, PFGE genotype I) with the low-copy-number plasmid pQC (~300 kb), three isolates with different genetic backgrounds based on PFGE (including one with pQC), and three isolates that originated from different European countries (Table 2) (Fluit *et al.*, 2001; Paauw *et al.*, 2006, 2007, 2008).

No high-copy-number plasmid DNA was present in the DNA of the eight isolates used for the shotgun library. Isolates were mixed to create a shotgun library as described by Paauw *et al.* (2008); for each isolate, equal amounts of genomic DNA were mixed and 10 μg of the pooled DNA was sonicated (Branson 250/450 Sonifier, 6 mm microtip, output intensity 1). Fragments of approximately 1.2 kb were extracted from agarose gels (Qiaquick columns, Qiagen) and end-repaired (DNA Terminator End Repair kit, Lucigen). End-repaired fragments were ligated into the pSMART-HC-Kan vector (Clone-SMART, Lucigen). The recombinant plasmids obtained were then transformed into *Escherichia coli* (ElectroMAX DH10B Cells, Invitrogen) and plated on tryptone yeast plates with 30 μg kanamycin ml^{-1}. A total of 3072 recombinant clones were arrayed into 96-well plates. Cloned DNA fragments were amplified by PCR using SMART primers (Lucigen) with 5′-C6 amino linkers to facilitate cross-linking to the aldehyde-coated glass slides. PCR products were ethanol-purified and resuspended in 3× SSC (1× SSC: 0.15 M NaCl, 0.015 M sodium citrate, pH 7). All PCR products were printed on CSS silylated slides (European Biotech Network) by an ESI three-axis DB-3 robot (ChipWriter Pro, Bio-Rad) at a controlled humidity of 55 %. Slides were printed in four batches, after which they were blocked and denatured (15 s dip in boiling water) following the manufacturer's instructions.

Labelling, hybridization and data acquisition. Labelling, hybridization and data acquisition were performed as previously described (Leavis *et al.*, 2007; Paauw *et al.*, 2008). A Bioprime system (Invitrogen) was used to label 0.5 μg DNA. For normalization, equal amounts of the library strains were mixed to form a reference pool and labelled with Cy3-dUTP. Tester isolates were labelled with Cy5-dUTP. To test reproducibility, 15 isolates were tested in duplicate, two in triplicate and one in quadruplicate (Table 1). For all hybridizations, Cy5 and Cy3 DNA fragments were combined with 100 μg yeast tRNA that was dried with a Speedvac (30 min at high temperature), resuspended in 40 μl Easyhyb buffer (Roche) and denatured for 2 min at 100 °C. Printed slides were washed and pre-hybridized in 0.45 μm-filtered pre-hybridization buffer (1 % BSA, 5 × SSC, and 0.1 % SDS) at 42 °C for 45 min with rotation, washed twice with purified water (MilliQ, Millipore), dried with N_2, and prewarmed at 42 °C. The denatured hybridization mix was then pipetted onto the printed side of the slide, covered with a hybrislip (coverslip), placed in hybridization chambers (Corning Life Sciences), and incubated overnight at 42 °C in a water bath. Slides were then thoroughly and sequentially washed in (a) 1× SSC, 0.2 % SDS for 10 s at 37 °C, (b) 0.5× SSC for 10 s at 37 °C, and (c) twice in 0.2× SSC for 10 min at room temperature. Slides were dried with N_2 and scanned using a Scanarray Express 680013 Microarray Analysis System (Perkin Elmer Life Analytical Sciences). Images were obtained and quantified with ImaGene 4.2 software (Biodiscovery).

Processing of MGA data. Inferior spots (empty or less than twice the Cy3 background) were excluded from normalization and data analysis (Leavis *et al.*, 2007; Paauw *et al.*, 2008). Each slide was independently normalized to correct for individual differences. Cy5 (signal minus background)/Cy3 (signal minus background) ratios were calculated. Ratios were normalized by correcting for overall signal intensities in both channels. DNA fragments were selected for further analyses when 95 % of the slides had a hybridization result. Next, the data were log_2 transformed. The estimated probability of presence (EPP) for each insert was determined using GACK transformations (Kim *et al.*, 2002; http://falkow.stanford.edu/whatwedo/software/software.html). For hierarchical clustering, a graded output was selected. In our case, the graded output generated a range of values from −0.5 to 0.5 in increments of 0.05, with −0.5 corresponding to 5 % or less EPP and 0.5 corresponding to 95 % or more EPP.

Table 2. Characteristics of isolates used for construction of DNA library and the calculated gene coverage of each isolate

Abbreviations: AUS, Austria; ITA, Italy; NL, The Netherlands; POL, Poland.

Isolate no.	Country	Hospital	Year	Source	Cluster*	*rpoB* genotyping	PFGE type	Gene coverage (% ± SD†)	Plasmid pQC
31	NL	1	2002	Wound/sst‡	2	*E. hormaechei*	1	58.5 ± 2.2	Positive
55	NL	1	2003	Rectal swab	2	*E. hormaechei*	1	58.5 ± 2.2	Positive
48	NL	1	2002	Urine tract	2	*E. hormaechei*	14	56	Negative
19	NL	1	2003	Pneumonia	1	*E. hormaechei*	15	60 ± 4	Negative
142	AUS	16	1997	Wound/sst	1	*E. hormaechei*	17	58	Negative
146	ITA	20	1998	Blood	2	*E. hormaechei*	27	60	Negative
104	NL	1	2003	Rectal swab	7	*E. cloacae* IV	53	56 ± 3	Positive
153	POL	25	1998	Pneumonia	2	*E. hormaechei*	56	62	Negative

*Cluster according to MLSA results.
†If replicates were tested the standard deviation is shown.
‡Wound or skin or soft tissue infection.

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The *E. cloacae* complex core genome was defined as the set of genomic DNA fragments present in all isolates with a likelihood of >95 % (GACK data >−0.5) (Leavis *et al.*, 2007; Paauw *et al.*, 2008).

For statistical analysis, the data were transformed to binary output, where 0 corresponded to 5 % or less EPP, 1 to 95 % or more EPP, and the values between 5 % and 95 % were discarded.

Determination of R-plasmid pQC-related DNA fragments. Because the approximately 300 kb plasmid pQC appears to play an important role in the outbreaks, it was determined which DNA fragments were derived from pQC as previously described (Paauw *et al.*, 2008). Subsequently, the pQC-derived DNA fragments were sequenced from both sides. Sequencing was performed with SMART primers (SR1 and SL1) directly on purified plasmids using the high-throughput sequencing service of BaseClear. Subsequently, the retrieved sequences were compared with sequences in GenBank.

Analysis of CGH data. For parsimony analysis, the data were transformed into binary output, where 0 corresponded to <50 % EPP, 1 to >50 % EPP, and blanks were represented with a question mark. For this analysis, core- and pQC-related DNA fragments were omitted because these fragments do not contribute to differentiation of isolates of the *E. cloacae* complex. Data were also analysed using Pars from the data package PHYLIP, as previously described (Felsenstein, 2005; Paauw *et al.*, 2008) (http://evolution.genetics.washington.edu/phylip.html).

First, to determine if there was an outbreak-specific DNA fragment, we investigated if there were DNA fragments present in all included outbreak strains (outbreaks I to VII) that were absent in all non-outbreak isolates. Subsequently, we determined if EHOS-specific DNA fragments could be identified. EHOS-specific DNA fragments were defined as present (EPP ⩾95 %) in ⩾95 % of the tested EHOS isolates and absent (EPP ⩾95 %) in ⩾95 % of all other isolates.

As mentioned previously, the *E. cloacae* complex isolates were genotypically compared based on MLSA and CGH. These results indicated that the clinical isolates belonging to the *E. cloacae* complex in the UMCU belonged to seven genetic distinct clusters. The EHOS (outbreak I) belongs to cluster 2. To determine the DNA fragments that were associated with the EHOS (outbreak I), we compared the hybridization data of 36 EHOS isolates (42 slides including replicates) with isolates of a similar genetic background, which are the 55 other isolates (63 slides with replicates) that belonged to cluster 2 (Fig. 1) (Paauw *et al.*, 2008). Associated DNA fragments were identified using paired Student's *t*-test ($P<0.001$) followed by false discovery rate (FDR) correction (Benjamini *et al.*, 2001; Leavis *et al.*, 2007). The sequences of the top 100 most strongly associated DNA fragments were sequenced from both sides. Because not enough isolates were included from the other outbreak strains (outbreak strains II to VII), the analysis was limited to the EHOS.

RESULTS

Validation of the array

Previously, the MGA that contained 3072 random DNA fragments was validated (Paauw *et al.*, 2008). In total, 2614 (85 %) spots passed quality control (for flag-filtered, normalized $\log_2$-transformed data, see Supplementary Table S1, available with the online version of this paper). The mean size of the DNA fragments spotted on the slides was 1202 bp. Of the 2614 spots included, 1358 were considered to represent the core genome. The redundancy of the MGA was less than 5.3 %; the pan-genomic coverage of the *E. cloacae* complex was estimated to be 55–60 % (Akopyants *et al.*, 2001; Paauw *et al.*, 2008). The calculated minimal nucleotide coverage of the core genome is 66 % based on an estimated genome size of 4.5 Mb. Validation of two DNA fragments by PCR showed that 98 % and 97 %, respectively, of the results correlated with the results of the CGH array (Paauw *et al.*, 2008).

All isolates tested in duplicate (15), triplicate (two) or quadruplicate (one) clustered correctly (Paauw *et al.*, 2008). One hundred and six DNA fragments were likely to be derived from pQC. Therefore, 1150 DNA fragments were available for investigation of strain-specific virulence genes.

Potential resistance and virulence genes in pQC

The results of BLAST searches for sequence data of the 106 putative pQC-derived DNA fragments are shown in Supplementary Table S2 and summarized in Table 3. Of these fragments, 15 were not previously found to be associated with plasmids. Two DNA fragments have poor hits in the NCBI database. The remaining 13 DNA fragments have hits only with sequences derived from chromosomal sequences. Because we have included 2614 DNA fragments and the *P*-value was <0.01, we expect 26 false-positive DNA fragments. Therefore, we cannot exclude that these are false-positives. In addition to the previously identified resistance genes ($bla_{\text{CTX-M-9}}$, *qnrA1*, *aadB*, *aadA2*, *sulI* and *sat*) in pQC, a *tetA* gene was found, which is involved in resistance to tetracycline (Ramos *et al.*, 2005). Furthermore, genes encoding heavy-metal resistance were identified: *terA*, *terX*, *terY1*, *terY2* and *terZ* are involved in resistance to tellurium; *merP* and *merR* are involved in resistance to mercury; and *arsH* codes for resistance to arsenic. In addition, *trhA*, *trhC*, *trhL*, *trhI*, *trhE* and *trhV* genes involved in plasmid transfer were identified (Gilmour *et al.*, 2004). Furthermore, seven genes encoding putative exported proteins were detected, but all have unknown functions.

Sequence data of genes involved in plasmid replication showed that pQC belongs to incompatibility group IncHI2

Table 3. Summary of content of 106 pQC-derived DNA fragments

No. of DNA fragments	Contains gene encoding or located on
15	Plasmid replication/conjugation
7	Putative pili proteins/conjugation
7	Exported proteins
26	Enzyme proteins
31	Hypothetical proteins
8	Membrane associated proteins
3	Phages
21	Small mobile elements

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and is related to plasmid R478 of *Serratia marcescens*, pK29 of *Klebsiella pneumoniae* and pAPEC-O1-R from a pathogenic avian *Escherichia coli* (Chen *et al.*, 2007; Chouikha *et al.*, 2006; Gilmour *et al.*, 2004; Johnson *et al.*, 2006). However, the highest similarity of genes (64 %) was found with plasmid R478.

Potential virulence genes in the EHOS (outbreak I)

One hundred and twenty-nine DNA fragments were associated with the EHOS: 96 positively and 33 negatively. Of these, only four DNA fragments had a specificity [percentage of the associated DNA fragment truly not present in the negative isolates (the non-EHOS)] and a sensitivity [percentage of the associated DNA fragment truly present in the positive isolates (the EHOS)] of $\geqslant$95 %. This indicates that these four DNA fragments are specific for the EHOS and can potentially be used to identify the EHOS. The sequences of the top 100 most strongly associated DNA fragments were determined and compared with sequences in the NCBI database (Supplementary Table S3; results summarized in Table 4). The three most associated DNA fragments contained DNA sequences with a low similarity to known sequences and their function is therefore highly speculative. The fourth most associated DNA fragment encoded a putative phage protein. Thirty-two of the 100 DNA fragments were directly linked to transferable elements. Seven DNA fragments (size from 967 to 1511 bp) without any sequence overlap contained sequences present in the high pathogenicity island (HPI), which is a 35–76 kb large genomic island and a known virulence factor in *Enterobacteriaceae* (Bearden *et al.*, 1997; Carniel *et al.*, 1992; de Almeida *et al.*, 1993; Lin *et al.*, 2008; Schubert *et al.*, 2002). Five of these seven DNA fragments belonged to the 25 DNA fragments most highly associated with the EHOS. Three DNA fragments previously described to be located on a virulence plasmid (pLVPK) were also associated with the EHOS.

Table 4. Summary of the comparative results from 100 DNA fragments most associated with the EHOS (all $P<0.001$)

Function (putative)	No. of DNA fragments (negatively associated)
Genes related to transfer or mobile elements	25 (5)
Genes located on the HPI	7
Genes located on virulence plasmid (pLVPK)	3
Putative outer-membrane proteins	4 (4)
Efflux-pump-related genes	2
Proteins involved in regulation	4 (1)
Proteins with a putative metabolic function	25 (9)
Putative, uncharacterized or hypothetical proteins	24 (4)
Fimbriae-associated proteins	4
Resistance genes	2 (1)

Also associated with the EHOS were four DNA fragments encoding putative pili (also called fimbriae) that had most homology with pili of the chaperone/usher pathway producing type 1 fimbriae of *Salmonella enterica*. Fourteen positively associated DNA fragments contained phage-related sequences, whereas four phage-related sequences were negatively associated. Other fragments showing negative association with EHOS contained genes encoding putative outer-membrane proteins, metabolism-associated proteins, a regulation-associated protein and a plasmid-associated protein.

DISCUSSION

The results of this study show that the *E. hormaechei* strain EHOS (outbreak I), causing a nationwide outbreak in The Netherlands, harbours plasmid pQC, which contains resistance genes and putatively exported proteins. Furthermore, chromosomally encoded genes possibly increasing virulence were also associated with the EHOS. It was previously shown that plasmid pQC harbours three large integrons, all containing resistance genes (Paauw *et al.*, 2006). In this study, pQC was identified as an IncHI2 plasmid with a high genetic similarity to plasmid R478. R478, isolated for the first time in 1969 in the USA, is a 274 kb, conjugative low-copy-number plasmid with a highly stable backbone (Gilmour *et al.*, 2004). IncHI2 plasmids are often R-plasmids with $bla_{\text{CTX-M}}$ resistance genes and have been detected in isolates isolated from humans as well as from poultry (García *et al.*, 2007; García Fernández *et al.*, 2007). The previously described $bla_{\text{CTX-M-9}}$ genes have been found in a complex integron (In60) that is located on IncHI2 plasmids (Novais *et al.*, 2006; Paauw *et al.*, 2006).

In addition to antimicrobial resistance genes, *ter* and *mer* genes encoding resistance to heavy metals were identified. This is in accordance with previous studies that showed that IncHI2 plasmids harbour a DNA segment named the 'principal plasticity zone' that encodes resistance determinants such as *ter*, *cat*, *aphA*, *mer*, *sil*, *cop* and Tn*7*. It also contains a large number of IS*26* elements (Gilmour *et al.*, 2004). This further illustrates that in the last 30 years IncHI2 plasmids have evolved by recombination events into multi-antibiotic resistance plasmids that enable their hosts to cope with new antibiotics. pQC provides outbreak strains with a greater likelihood of survival in the hospital environment. This refutes the hypothesis of the early 1980s that expressed the hope that 'new agents' such as cefotaxime and cefoperazone eventually may be valuable for single-drug therapy of infections due to *E. cloacae* (John *et al.*, 1982).

Seven genes coding for putative exported proteins that have been found on other IncHI2 plasmids were also found on pQC. The functions of these exported proteins are unknown, but we speculate that at least some may be virulence factors because proteins exported from bacteria

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are often toxins or proteins that interfere with the immune system (Beuscher *et al.*, 1995; Emody *et al.*, 2003; Jongerius *et al.*, 2007; Rooijakkers *et al.*, 2005). Several genes involved in transfer of the plasmid were identified, supporting previous *in vivo* and *in vitro* observations of horizontal pQC transfer (Paauw *et al.*, 2006). Further proof of transmissibility in the present study was derived from the presence of pQC in four of the seven outbreak strains. The presence of pQC in multiple outbreak strains suggests that pQC likely provides these strains with a strong selective advantage.

In addition, a diverse array of potential virulence genes was identified in the chromosomal DNA of the EHOS when compared to other *E. hormaechei* isolates with a similar genetic background (cluster 2). Seven fragments belonging to HPI that encode an iron uptake and regulation system were highly associated with the EHOS. The siderophore produced by this system, yersiniabactin, is a highly effective iron scavenger. The HPI was first described in *Yersinia* spp., but has also been described in other *Enterobacteriaceae* (Bach *et al.*, 2000; Koczura & Kaznowski, 2003; Mokracka *et al.*, 2004; Petermann *et al.*, 2008; Schubert *et al.*, 1998, 2000, 2002). *Yersinia* spp., *Escherichia coli*, and possibly *K. pneumoniae* isolates containing the HPI are more virulent than isolates lacking this island (Bearden *et al.*, 1997; Carniel *et al.*, 1992; de Almeida *et al.*, 1993; Lin *et al.*, 2008; Schubert *et al.*, 2002). The HPI was recently shown to be transferable by conjugation with HPI-ICE*Kp1* (Lin *et al.*, 2008). This appears to be the first time that the HPI has been described in association with an outbreak of nosocomial pathogenic *Enterobacteriaceae*. Indisputable evidence that HPI increased the virulence of the EHOS is lacking. However, in all *Enterobacteriaceae* tested, virulence was significantly increased by the HPI (Bearden *et al.*, 1997; Carniel *et al.*, 1992; de Almeida *et al.*, 1993; Lin *et al.*, 2008; Schubert *et al.*, 2002). Other genes associated with the EHOS were those previously described on the pLVPK virulence plasmid of *K. pneumoniae* CG43 (Chen *et al.*, 2004). Also interesting were four DNA fragments encoding putative pili with the most homology to pili of the chaperone/usher pathway producing putative type 1 fimbriae from *S. enterica*. Pili are helical filaments that mediate attachment to specific surfaces, e.g. host tissue (Fernandéz & Berenguer, 2000; Sauer *et al.*, 2000). In addition, the EHOS has presumably also lost or altered some membrane proteins, possibly gaining a selective advantage via reduced immune recognition.

In conclusion, resistance and virulence genes were identified in the EHOS, which are likely involved in its epidemic behaviour. Most of the identified genes were located on mobile elements, indicating that the propensity for recombination and genetic exchange in the *E. cloacae* complex is likely an important feature for the development of outbreak strains.

The EHOS thus represents the model of genetic capitalism. In this model, bacteria that already have a selective advantage in a certain setting are able to adapt even further by accumulation of mobile elements (Baquero, 2004; Leavis *et al.*, 2006). This may increase the epidemic behaviour (epidemicity) even further. Because of this cumulative effect it is likely that outbreak strains have an antibiotic-resistant phenotype.

The four specific markers for the EHOS, which is still endemic in the UMCU, will facilitate faster detection, leading to faster implementation of control measures, thus preventing the spread of the EHOS. However, because most specific DNA fragments are located on putative mobile elements, there is a possibility that specificity will be reduced over time.

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