Subtractive Hybridization Yields a Silver Resistance Determinant Unique to Nosocomial Pathogens in the *Enterobacter cloacae* Complex

Anita N. Kremer^{a,c} and Harald Hoffmann^{b,c}

Department of Medicine 5, Hematology and Oncology, University of Erlangen, Erlangen, Germany^a; IML red GmbH, synlab Bayern, Asklepios Fachkliniken, Gauting, Germany^b; and Department of Bacteriology, Max von Pettenkofer Institute of Microbiology, Munich, Germany^c

The heterogeneity and the increasing clinical importance of the *Enterobacter cloacae* complex have often been discussed. However, little is known about molecular factors causing pathogenicity within this nomenclature. Here, we analyzed the genetic differences between an avirulent plant isolate and a pathogenic strain causing an outbreak with septicemia in three patients. We identified an IncHI-2 plasmid as a major difference between these two strains. Besides resistance to several antibiotics, this plasmid encoded a silver resistance determinant. We further showed that this *sil* determinant was present not only in the analyzed outbreak strain but also in the vast majority of clinical isolates of the *E. cloacae* complex, predominantly in (sub)species that frequently cause nosocomial infections. The identified *sil* determinant was highly conserved within the *E. cloacae* complex and mediated resistance to up to 600 μ M silver nitrate. As silver is often used as a disinfectant and treatment for burn wounds, we present here an important fitness factor within the clinically most prevalent subspecies of the *E. cloacae* complex. This provides a possible explanation for their unequal involvement in nosocomial and especially burn wound infections.

The term “*Enterobacter cloacae*” is often used for a complex consisting of nine species and subspecies and at least three additional genovars, which are all genetically and phenotypically closely related to each other. Members of this complex have been isolated from plants (38), insects (11), and open bodies of water (19), reflecting the wide distribution of this intestinal commensal in the environment. However, the increasing number of epidemiological studies highlighting severe cases of bacteremia caused by the *E. cloacae* complex reflects the particular importance of these microorganisms as clinical pathogens (4, 21, 36). Especially at (neonatal) intensive care (2, 10, 12, 24, 25, 29, 34, 44, 45) and burn units (31, 32), (sub)species of the *E. cloacae* complex often emerge as the cause of severe outbreaks, with several patients being infected by the same clone.

Despite this clinical importance, few pathogenicity factors have so far been characterized within the *E. cloacae* complex, and most of them have been found by screening for known virulence-associated properties. Keller et al. (26) demonstrated broad distribution of serum resistance, aerobactin production, and hemagglutinin production, whereas Simi et al. (42) found a low-molecular-weight enterotoxin hemolysin in seven of 50 *E. cloacae* strains analyzed. Sevcik et al. (39) described a paralytic toxin called saxitoxin found in two species of the *E. cloacae* complex, and the level of the outer membrane protein OmpX has been directly linked to invasiveness of *E. cloacae* in rabbit ileal tissue (9). Unfortunately, these studies did not account for the diversity of the *E. cloacae* complex. This might be of importance, as some genovars were repeatedly overrepresented in clinical isolates (15, 22, 30), while others were significantly underrepresented. Furthermore, our recent data show that some genotypes are significantly associated with certain infection sites (27).

Paauw et al. (36) studied the pathogenicity of the *E. cloacae* complex by performing comparative genomic hybridization to identify genes specific for an *Enterobacter hormaechei* outbreak strain. They thereby found the large IncHI-2 plasmid pQC, which harbored many resistance determinants, probably leading to a greater likelihood of survival in the hospital environment.

In the present study, we sought to identify virulence-associated

genes within the *E. cloacae* complex by direct whole-genome comparison of an outbreak strain and an avirulent plant isolate using a subtractive hybridization assay (1). With this approach, we also found a large IncHI-2 plasmid as a major difference between the two strains. Besides several antibiotic resistance determinants, this plasmid harbored a silver resistance determinant (*sil*). We could demonstrate that the *sil* determinant was phenotypically functional and was present significantly more often in genotypes that frequently cause nosocomial infections. This resistance to silver ions represents a potent fitness factor and could therefore partly explain clonal outbreaks as well as the general predominance of members of the *E. cloacae* complex with certain genotypes as nosocomial pathogens.

MATERIALS AND METHODS

Bacterial strain collection. The strains used in the subtraction studies belonged to the species *Enterobacter asburiae*. The presumed virulent strain EN-373 (= M40, tester) had infected three infants nursed at a neonatal intensive care unit (12) and was kindly provided by Victoria Fernandez-Baca, Palma de Mallorca, Spain. This outbreak strain (tester) was compared with strains 501R3 and E6 (driver), both plant-beneficial bacteria that have been recovered independently from cucumber (38) and were considered avirulent. These strains were kindly provided by Daniel P. Roberts, Beltsville, MD.

All clinical isolates were recovered from specimens sent from the University Hospital Großhadern, Munich, Germany, to its diagnostic bacteriology department (Max von Pettenkofer Institute). One hundred sixty-four isolates were enrolled, including all blood culture isolates ($n = 67$)

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Address correspondence to Anita N. Kremer, anita.kremer@uk-erlangen.de.

The two authors contributed equally to this work.

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identified as *E. cloacae* complex between 1997 and 2002. Furthermore, study isolates included 97 isolates randomly taken from our *E. cloacae* strain bank collected from 2000 to 2002. This included isolates recovered from respiratory tract ($n = 39$), gastrointestinal tract ($n = 13$), genitourinary tract ($n = 16$), wound swabs ($n = 12$), central venous lines and Foley catheters ($n = 9$), and abdominal ($n = 6$) and pleural cavities ($n = 2$). All isolates were identified as members of the *E. cloacae* complex by API20E (bioMérieux, Marcy l'Étoile, France) and were assigned to their genovars by partial *hsp60* sequence analysis as described previously (22). To our knowledge, none of the patients was treated with silver compounds for respective infections. However, silver-coated devices such as central venous lines and Foley catheters were widely used in the hospital. In all analyses, *Escherichia coli* DH5 α served as negative control and *E. coli* DH5 α pMG101 (kindly provided by Simon Silver, Chicago, IL) as positive control. All bacteria were grown in Luria-Bertani (LB) medium and cryopreserved at -80°C with 20% glycerol.

Preparation of chromosomal DNA. For preparations of chromosomal DNA, strains were incubated overnight in 5 ml LB broth, washed and incubated in Tris-HCl buffer (pH 8.5), and lysed with 0.5% SDS and 100 $\mu\text{g}/\text{ml}$ proteinase K for 1 h at 37°C . After the addition of NaCl to a concentration of 0.7 M, cell debris was removed using cetyltrimethylammonium bromide (CTAB) and chloroform-isoamyl alcohol extraction. DNA was recovered by precipitation in 60% isopropanol and dissolved in 500 μl of Tris Cl buffer (pH 8.5). Small and medium-sized plasmids (up to 20 kb) of the clinical isolate EN-373 were prepared using a Nucleobond PC100 kit (Macherey & Nagel, Düren, Germany). Chromosomal DNA and plasmid DNA solutions of clinical strain EN-373 were mixed in a proportion of 3:1 (vol/vol). DNA (10 μg) of each of the clinical and the environmental strains was cleaved with RsaI (Fermentas GmbH, St. Leon-Roth, Germany).

Subtractive hybridization. For DNA subtraction, a bacterial PCR-Select DNA subtraction kit (Clontech, La Jolla, CA) and GeneAmp PCR system 9700 (Applied Biosystems, Darmstadt, Germany) were used, following the manufacturer's instructions. After subtractive hybridization, DNA fragments unique to the clinical isolate EN-373 were PCR amplified, cloned into pCR2-1-TOPO vector, and transformed into *E. coli* TOP-10 One Shot chemically competent cells (Invitrogen BV, Groningen, The Netherlands). After beta-galactosidase read-out of the transformants, white colonies were separated twice on LB agar containing 100 $\mu\text{g}/\text{ml}$ ampicillin. After quick heat lyses of single colonies in 100 μl H_2O , the cloned inserts were PCR amplified with the vector-specific primers M13F and M13R. The primers bind 114 bp upstream and 95 bp downstream, respectively, from the insertion site of the TOPO cloning vector. Hence, PCR products obtained corresponded to the DNA fragment unique to the outbreak strain plus 209 bp of the cloning vector. PCR products were visualized in 2% agarose gel after ethidium bromide staining. After purification using a Nucleospin PCR cleaning kit (Macherey-Nagel, Düren, Germany), PCR products were sequenced with the dye terminator method (BigDye reaction solution, ABI Prism 277 sequencer).

PCR amplifications and sequencing. Amplification reactions were performed with an Applied Biosystems GeneAmp PCR system 2700 and Taq Gold DNA polymerase (Perkin-Elmer, Branchburg, NJ) under standard conditions (500 pmol of each primer, 200 μM nucleotide mix, 10 mM Tris-HCl [pH 8.3], 5 mM KCl, 1.5 mM MgCl_2). Primers are listed in Table 1. Reactions were carried out in a total volume of 50 μl and started after a preheating time of 10 min at 95°C . Samples were subjected to 30 cycles of 30 s denaturation at 95°C , 30 s annealing (annealing temperatures are listed in Table 1), and 2 min of extension at 72°C . Cycles were followed by a final elongation step of 15 min at 72°C . PCR products were visualized in 2% agarose gels after electrophoresis and ethidium bromide staining. DNA was cleaned using a Nucleospin extraction kit (Macherey-Nagel, Düren, Germany) for further processing, following the manufacturer's guidelines.

PCR-amplified and cleaned DNA was sequenced using the dye terminator method. To prepare cycle sequencing reaction mixtures, 0.2 μg of

purified DNA was added to a 20- μl reaction solution containing 4 μl of BigDye dye terminator sequencing mix and 0.7 μl of a 5-pmol/ μl primer solution. Cycle sequencing was performed in an Applied Biosystems GeneAmp 2700 PCR system. Cycle parameters were 25 cycles with an initial 96°C denaturation step of 10 s, followed by an annealing step at 55°C of 10 s and an extension step of 4 min at 60°C . Sequences were determined by electrophoresis with the ABI Prism 377 DNA sequencer.

Phenotypic tests. Inhibition of bacterial growth was assessed by measuring the OD₆₀₀ after incubation (16 h, $36 \pm 1^{\circ}\text{C}$, room air) of five representative isolates in liquid LB medium supplemented with different concentrations of AgNO_3 (dissolved to a stock solution of 40 mM in H_2O ; Sigma-Aldrich). Three PCR-negative isolates (114, 501R3, and E6), two PCR-positive isolates (244 and 373), and *E. coli* strains DH5 α and DH5 α (pMG101) (negative and positive controls, respectively) were included. To measure growth inhibition in the absence of NaCl ions, LB agar plates were prepared without addition of NaCl but supplemented with different concentrations of AgNO_3 medium as previously described (17). After incubation (16 h, $36 \pm 1^{\circ}\text{C}$, room air), growth of strains was assessed and documented by photography.

RESULTS

Subtractive hybridization of EN-373 and 501R3. One hundred seventy-eight white colonies were obtained by subtraction of strains 373 and 501R3. Fifty of them were randomly selected for further analysis. Twenty-one of the colonies analyzed (42%) yielded PCR products of 210 bp, meaning that they did not contain a DNA fragment. The PCR products of the other 29 clones were longer than 210 bp.

BLAST search for homologies yielded the results listed in Table 2. Four sequences were not usable. Ten clones contained novel sequences with no homology to any gene previously identified. Fifteen inserts gave homologies to known sequences. Their GenBank accession numbers are listed in Table 2. Six sequences (A-051, D-007, C-005, C-007, C-006, and A-000) showed homology to housekeeping genes of various members of the *Enterobacteriaceae*, one (A-053) to a two-component regulatory system, one (A-088) to a hypothetical protein, and one (B-031) to a putative kinase, both of unknown functions. Two clones (A-007 and B-007) showed homology to *nirAB*, *nreAB*-like genes, and *ncrABC*, genes of the nickel/cobalt resistance determinants present in *Klebsiella oxytoca*, *Legionella pneumophila*, and the *Hafnia alvei* plasmid NRS148.

Four (A-114, A-073, B-036, and A-104) of the five clones with the best BLAST scores contained DNA fragments with high-level identity to genes on plasmid R478, found in *Serratia marcescens*. pR478 is a large group H incompatibility (IncHI) conjugative plasmid with a length of 274,762 bp which harbors determinants of resistance to several heavy metals (*ter* operon for tellurium resistance, *mer* operon for mercuric resistance, *sil* operon for silver resistance, *cop* operon for copper resistance, and *ars* operon for arsenic resistance) and antibiotics (*tetA* gene for tetracycline resistance and *aphA* gene for kanamycin resistance). While three clones (A-114, A-073, and B-036) showed homology to plasmidic genes with unknown functions, one clone (A-104) showed homology to the *silS* gene. *silS* is one of seven genes of a silver resistance determinant characterized by Gupta et al. (16). It encodes a membrane sensor functioning as part of a regulatory system of the resistance operon. The *silPABCRSE* silver resistance determinant has been identified on a variety of group H incompatibility plasmids, i.e., MG101, MIP233, WR23, and MIP235 from *Salmonella enterica* serovars and R476b and R478 from *Serratia marcescens*. Two accession numbers for sequences with homologies are listed

TABLE 1 Primers and annealing temperatures used for PCR amplifications

PCR ^a	Primer	Primer sequence ^b	Size of PCR product (bp) and temp (°C)	Position on plasmid (accession no)
<i>silE-silS</i>	sil 1105	GTT CGT CAT GGT TTC ATG AGC ^c	1,915; 58.0	SATY pMG101, <i>silE</i> 1105–1125 (AF067954)
	sil 3019	CTG ACC TTT TTT ATC AGC CTG ^c		SATY pMG101, <i>silS</i> 3019–2999 (AF067954)
<i>silS</i>	sil 3019	CTG ACC TTT TTT ATC AGC CTG ^c	741; 58.0	SATY pMG101, <i>silS</i> 3019–2999 (AF067954)
	sil 2279	GCG GGT AAA RAC ATC CTC AAT ^c		SATY pMG101, <i>silS</i> 2279–2299 (AF067954)
<i>silS-silR</i>	sil 2999	CAG GCT GAT AAA AAA GGT CAG ^c	574; 58.0	SATY pMG101, <i>silS</i> 2999–3019 (AF067954)
	sil 3572	AAC GGC TGG GAT ATC RTS CG ^c		SATY pMG101, <i>silR</i> 3572–3553 (AF067954)
<i>silR</i>	sil 3244	CCC TGA TGG CGA AKG AAG AA ^c	329; 58.0	SATY pMG101, <i>silR</i> 3244–3263 (AF067954)
	sil 3572	AAC GGC TGG GAT ATC RTS CG ^c		SATY pMG101, <i>silR</i> 3572–3553 (AF067954)
<i>silS-silC</i>	sil 2999	CAG GCT GAT AAA AAA GGT CAG ^c	1,472; 58.0	SATY pMG101, <i>silS</i> 2999–3019 (AF067954)
	sil 4470	CCA GTT GCT GRY TGA AAT AGC ^c		SATY pMG101, <i>silC</i> 4470–4449 (AF067954)
<i>silC</i>	sil 4090	CGG GCT GGC GHA MCT TTT TTG ^c	381; 58.0	SATY pMG101, <i>silC</i> 4090–4111 (AF067954)
	sil 4470	CCA GTT GCT GRY TGA AAT AGC ^c		SATY pMG101, <i>silC</i> 4470–4449 (AF067954)
<i>silC-silB</i>	sil 4090	CGG GCT GGC GHA MCT TTT TTG ^c	1,942; 58.0	SATY pMG101, <i>silC</i> 4090–4111 (AF067954)
	sil 6031	TCC ATA TCC ATA AAV GGC GAT ^c		SATY pMG101, <i>silB</i> 6031–6011 (AF067954)
<i>silB-silA</i>	sil 6549	ATT CAG GGD ATG GAC CCG GT ^c	732; 58.0	SATY pMG101, <i>silB</i> 6549–6568 (AF067954)
	sil 7280	CCG GGA TAG CTG GTT TTR ATA ^c		SATY; pMG101, <i>silA</i> 7280–7260 (AF067954)
<i>silA-silP</i>	sil 9936	TGG CGT GGT SAT GCT GAT GTA ^c	1,228; 58.0	SATY pMG101, <i>silA</i> 9936–9956 (AF067954)
	sil 11163	TGT TCA CTG GCA TCT TCG GTA ^c		SATY pMG101, <i>silP</i> 11163–11143 (AF067954)
<i>silP</i>	sil 11882	GGC GAT AAG CTC CGC ATC AGA ^c	524; 58.0	SATY pMG101, <i>silP</i> 11882–11902 (AF067954)
	sil 12405	TCC ACT TTT TCA AGA CGC TCA ^c		SATY pMG101, <i>silP</i> 12405–12385 (AF067954)
RepHIA	RepHIA-F RepHIA-R	ACC TTT CTT AAC YTC TTG ATA ^d TGT GGT ATC AGT TCW ACA AAC AT ^d	450; 51.1	SEMA pR478, 813–793 (BX664015) SEMA pR478, 364–386 (BX664015)
M13	M13-F M13-R	GTA AAA CGA CGG CCA G CAG GAA ACA GCT ATG AC	Depending on insert; 50.0	pCR2.1-TOPO, 391–406 pCR2.1-TOPO, 205–221

^a The structure of the complete *sil* operon is depicted in Fig. 2.

^b A = adenine, C = cytosine, G = guanosine, T = thymine, I = inosine; D = G, T or A; H = A, C or T; K = G or T; M = A or C; R = A or G; S = G or C; V = G, C or A; W = A or T; Y = C or T.

^c Primers were designed based on DNA sequences of accession numbers AF067954 (pMG101) and BX664015 (pR478).

^d Primers were designed based on DNA sequences of accession no. AF250878 (pR27) and BX664015 (pR478).

in Table 2, i.e., AF067954 for pMG101 and BX664015 for pR478. Both are members of IncHI subgroup 2 (IncHI-2). The DNA sections with homology to IncHI plasmids were the most important genomic differences found between clinical outbreak strain EN-373 and plant isolate 501R3.

Subtractive hybridization of EN-373 and E6. A second subtractive hybridization experiment was performed with another plant isolate as a driver (E6), in order to confirm the results of the first experiment. After subtractive hybridization, about 220 white clones were isolated, of which 40 were randomly selected for further analysis. Eight of them did not contain an insert. Four clones yielded nonsense sequences with large clusters of repeats. The remaining 28 sequences were compared to sequences in the GenBank database. BLAST search results of this experiment are listed in Table 2. Fifteen clones yielded unknown sequences. Thirteen sequences had homology to transferable DNA, i.e., transposons (E-002 and E-009), IS elements (E-011) or plasmid DNA. Again, five (E-001, E-007, E-022, E-010, and E-037) of the clones with the best BLAST scores

showed homologies to the IncHI-2 plasmid R478 of *S. marcescens*. Three clones (E-023, E-029, and E-033) had homology to IncHI-1 plasmids of *Salmonella enterica* serovar Typhi. Two further clones were homologous to plasmids of *Escherichia coli* (E-013) and *Enterobacter aerogenes* (E-034), respectively.

RepHIA PCR. In both subtractive hybridizations, the main differences between the two strains were homologies to IncHI plasmids. Beside multiple resistance genes, few functional genes have been described as being located on IncHI-1 as well as on IncHI-2 plasmids, which carry, for example, silver resistance genes of the *sil* type found in clone A-104. Homologues of the autoreplicative region RepHIA were found on both IncHI-1 and IncHI-2 plasmids (37, 40). In order to verify whether EN-373 truly harbored an IncHI plasmid and the plant isolates did not, a PCR amplifying 450 bp of RepHIA was performed. The clinical outbreak strain EN-373 was PCR positive, while plant isolates 501R3 and E6 were negative, suggesting that EN-373 harbored an IncHI plasmid of subgroups IncHI-1 or IncHI-2 while the plant isolates did not (Fig. 1B). This experiment confirmed the specificity of the

TABLE 2 BLAST GenBank search with sequences of DNA fragments found in 28 clones after subtractive hybridization of clinical outbreak strain EN-373 and plant isolates 501R3 and E6

Database information about homologous genes				Database information about homologous genes			
Clone		Segment (bp) with homology ^b	% C+G	Species, serovar, and plasmid ^c			
Subtractive hybridization	Name ^a			Accession no.	Homologous gene(s)	Homologous region ^e	Protein function
EN-373 and 501R3	A-114	1–288	56	BX664015	Hypothetical	71129–70840	Putative ATP/GTP-binding protein integrated in tellurium resistance operon
	A-073	1–275	56	BX664015	Hypothetical	71123–70848	Putative ATP/GTP-binding protein integrated in tellurium resistance operon
	B-036	21–214	56	BX664015	Hypothetical	71169–70976	Putative ATP/GTP-binding protein integrated in tellurium resistance operon
	B-007	87–321	48	AY492000	<i>nirA</i> and <i>-B</i> and <i>mreA</i> and <i>-B</i> homologues	1318–1553	Nickel/cobalt resistance determinant; CCUG15788 integral membrane protein
	A-104	19–447	55	AY030282 AF067954	<i>siS</i>	2624–2196	<i>siI</i> operon (plasmid silver resistance genes)
	A-007	87–267	49	BX664015 AY492000	<i>nirAB</i> , <i>mreAB</i> , and <i>ncrABC</i> homologues	127176–126824 1318–1499	Nickel/cobalt resistance determinant CCUG15788 integral membrane protein
	A-051	245–415	52	AY030282 AF322866		1690–1875	
	D-007	32–170	63	AE016840	<i>ibpB</i> and <i>ibpA</i>	12266–12437	Small heat shock protein
	C-005	54–212	58	AE016762	<i>hcr</i>	255337–255475	NADH oxidoreductase
	B-031	26–188	57	AE005674	<i>yefW</i>	261785–261943	Putative enzyme D-alanyl-D-alanine carboxypeptidase
	A-000	23–165	55	AE008886	<i>trkH</i>	1166030–1166192	Putative kinase
	A-053	174–244	59	AE008803	<i>yfjE</i>	6464–6322	Trk family potassium transport protein
	C-007	13–48	48	AE008706	<i>yafB</i>	4172–4242	Putative DegT/Dmr1/EryC1/StrS family regulatory system
	A-088	139–225	61	BA000007	Hypothetical	15741–15706	2,5-Diketo-D-glucuronate reductase B
	C-006	1–24	60	KPN293850	EVGA	3370219–3370306	Ecs3386, unknown function
	E-013	1–624	48	AF550679	<i>orf18</i>	56–79	Putative positive transcription regulator EVGA
	E-001 and E-007	1–571	47	SMU60283	<i>tragI</i>	54236–54860	Unknown
	E-022	19–125, 491–510	42	AF250878	PR478 intergenic region between RepH11A and <i>tra2</i>	386–958	Putative restriction methylase
	E-010	1–386	42	SMU62007	Like <i>traE</i> of F plasmid	47386–47468	None; RepH11A, putative Rep protein Tra2; putative pilin precursor; similar to TrhA from SATY plasmid HCM1
	E-009	22–204	42	AF250878.1		2288–2738	Plasmid transfer protein
EN-373 and E6	E-011	153–457	42	STYFIMCLUS	Putative <i>tnpB</i>	4201–4586	Putative transposase B
	E-034	1–275	44	AE000134	<i>tnsA-2</i>	36704–36522	IS element 30
	E-037	8–188	44	EAU67194	IS4321R	1356–1052	Putative transposase binding site
	E-002	1–169	31	SMU62007	<i>htrIV</i>	4764–5038	Putative transfer-associated protein
	E-023	531–612	42	AF550679	<i>tnpA</i>	14134–13954	Transposase
				AF342826		7640–7472	
				AF250878	R0009, putative gene	68019–68100	Hypothetical cell surface glycoprotein (S-layer protein)

Clones	Accession number	Sequence section of insert of respective clone with homology to the genes with the indicated accession numbers	STYPPHCM1	R0135, putative gene	SATY CT18 pHCM1 SATY pR27	69452–69699 121746–121500	80 79	103 86	Cell envelope; putative outer membrane constituents
E-029	83–330 1–247	48	STYPPHCM1 AF250878	R0135, putative gene	SATY CT18 pHCM1 SATY pR27	69452–69699 121746–121500	80 79	103 86	Cell envelope; putative outer membrane constituents
E-033	1–247 51–139, 216–268, 330–450	50	STYPPHCM1 AF250878	R0156	SATY CT18 pHCM1 SATY pR27	187835–188081 142955–143354	79 83	86 68	Unknown
			STYPPHCM1	Putative gene	SATY pHCM1	137009–137129	83	68	

^a Clones A-117, A-008, A-018, A-056, A-087, B-008, B-018, C-001, C-004, C-011, E-004, E-005, E-008, E-014, E-015, E-017, E-019, E-020, E-021, E-024, E-025, E-030, E-031, E-032, and E-035 are novel sequences.
^b Sequence section of insert of respective clone with homology to the genes with the indicated accession numbers.
^c ENAE, *Enterobacter aerogenes*; ENCL, *Enterobacter cloacae*; ESCO, *Escherichia coli*; HAAL, *Hafnia alvei*; LEPN, *Legionella pneumophila*; KLOX, *Klebsiella oxytoca*; KLPN, *Klebsiella pneumoniae*; SAEN, *Salmonella enterica* serotype Enteritidis; SATY, *Salmonella enterica* serotype Typhimurium; SHFL, *Shigella flexneri*.
^d Percent nucleotide identity.
^e Sequence section of accession number with homology to insert of respective clone.
^f BLASTN 2.2.5 score (bits).

second subtractive hybridization in which clone E-022 showed 99% nucleotide identity to the pR478 intergenic region of RepHI1A and *tra2*. *tra2* is one of two clusters encoding transfer genes in IncH plasmids. This transfer region varies widely between the subgroups IncHI-1 and IncHI-2, and clone E-022 has shown homology only to *tra2* of IncHI-2 plasmid R478. This suggests that the plasmid of our clinical outbreak strain EN-373 belongs to IncHI subgroup 2 rather than to subgroup 1. This also fits with reports of other authors, who found the majority of *sil* genes on IncHI-2 plasmids rather than on IncHI-1 plasmids (18).

***sil* PCRs.** To confirm existence of the *sil* operon in the tester strain, PCRs of four of the *sil* genes (*silS*, *silR*, *silC*, and *silP*) were performed. PCR results are displayed in Fig. 1A. 501R3 was negative and EN-373 positive in all *sil* PCRs, suggesting that the clinical outbreak strain did harbor the complete silver resistance determinant while the plant isolate did not. *hsp60* PCR, amplifying 341 bp of the gene encoding the 60-kDa heat shock protein, was performed as a positive-control PCR as previously described (22).

To verify whether the *sil* operon has the same configuration as on *Salmonella* plasmid pMG101, PCRs overlapping from one gene to the other were performed (*silE* to *silS*, *silS* to *silR*, *silS* to *silC*, *silC* to *silB*, and *silA* to *silP*). All amplification products were comparable to those of pMG101 (Fig. 2).

Sequencing of *sil* genes. Partial *sil* gene sequencing revealed that *sil* genes in the *E. cloacae* complex were highly conserved (Table 3). ORF105 was the least conserved gene (mean nucleotide identity, 88%). Partial sequences of *silS*, *silR*, *silC*, *silB*, *silA*, and *silP* revealed nucleotide identities of 99%, 98%, 99%, 97%, 94%, and 95%, respectively, confirming their higher degree of conservation between species. Deduced amino acid sequences were all more than 90% identical to that of pMG101 (Table 3).

Distribution of the *sil* operon within the *E. cloacae* complex. One hundred sixty-four clinical isolates of all genotypes of the *E. cloacae* complex were screened for *silS*, *silR*, *silC*, and *silP*. Of these isolates, 63% were positive in all *sil* PCRs, suggesting that about two-thirds of clinical isolates of the *E. cloacae* complex harbor the complete silver resistance determinant. The genovars most prevalent in the population structure of the *E. cloacae* complex, i.e., clusters III, VI (*Enterobacter hormaechei* subsp. *oharae*) and VIII (*Enterobacter hormaechei* subsp. *hormaechei*) (22), were also most frequently positive in the *sil* PCRs (80% were positive in all four PCRs performed). Of clusters IX and xiii, which were rarely recovered from clinical specimens, no isolate was positive in all PCRs, although most isolates of cluster xiii seemed to harbor at least one or two *sil* genes. Similarly, most isolates of cluster V (*Enterobacter ludwigii*) were positive in the *silS* PCR, and half were positive in the *silC* or *silR* PCRs, but only two were positive in all PCRs performed, suggesting that isolates of this cluster could harbor either partial *sil* determinants or *sil* genes too divergent to be amplified by the PCRs employed. Cluster V represents about 6% of strains of the recently presented population structure (22) and about 11% of isolates in the present study. Clusters I, II, and IV represented 5 to 10% of our clinical isolates (27) and had an intermediate rate of *sil* PCR positivity (60 to 63%). It is noteworthy that isolates from these clusters yielded 100% positive results in the *silC* PCR. To summarize, genovars III, VI, and VIII had the complete silver resistance determinant significantly more often and were more frequently recovered from clinical specimens than genovars V, IX, and xiii (chi-square test, $P < 0.0001$). Data are shown in Table 4.

Phenotypic tests. Plasmids harboring the *sil* determinant were

FIG 1 Silver resistance genes and IncHI plasmids in pathogenic isolate EN-373. (A) PCRs of four genes of the *sil* resistance determinant in strains EN-373, a clinical outbreak strain, and 501R3, a plant isolate. Plasmid MG101 was used as a positive control; *E. coli* strain DH5 α was the negative control (NK). The *hsp60* PCR was used as control PCR. (B) PCR of *repA*, a marker gene for IncHI-1 and IncHI-2 plasmids. 501R3 and E6, two plant isolates, were negative in the *repA* PCR, suggesting that they did not carry IncHI-1 or -2 plasmids. EN-373 was PCR positive, confirming the presence of an IncHI-1 or -2 plasmid, presumably carrying the silver resistance genes.

reported to transfer resistance to up to 600 μ M AgNO₃ (17). In order to verify a potential correlation between the presence of silver resistance genes and phenotypic behavior, the extent of silver resistance was determined for five representative isolates of the *E. cloacae* complex. Inhibition of bacterial growth in the presence of increasing amounts of AgNO₃ was assessed by measuring the optical density at 600 nm (OD₆₀₀) after overnight incubation with different concentrations of AgNO₃ (Fig. 3A). PCR-negative isolates 114, 501R3, and E6 and the negative control DH5 α did not grow in AgNO₃ concentrations of more than 100 μ M. In contrast, PCR-positive isolates 244 and 373 and the positive control DH5 α (pMG101) sustained concentrations of up to 800 μ M. This finding was confirmed on solid Luria-Bertani (LB) medium without added NaCl (Fig. 3B).

DISCUSSION

In this study, we assessed the molecular background of the imbalanced distribution of genotypes within the *E. cloacae* complex as nosocomial pathogens by direct whole-genome comparison of an outbreak strain and an avirulent strain. Using subtractive hybridization, we found an IncHI plasmid harboring a silver resistance determinant as a major difference between pathogenic and non-pathogenic isolates. Serving as a hygienic fitness factor, the presence of silver resistance determinants could improve survival in hospital environments and might be an explanation for the rising numbers of nosocomial infections caused by the *E. cloacae* complex. Moreover, the distinct distribution of the *sil* genes among different (sub)species of the *E. cloacae* complex could partially explain their unequal prevalence as nosocomial pathogens (22, 27, 28).

Silver ions are frequently used as antiseptics in medicine and health (41). The best known uses of silver preparations are Crds

prophylaxis and treatment of burn wounds, e.g., in topical creams containing 1% silver sulfadiazine (14). Ag-coated bandages are used to cover burns and traumatic injuries. Silver-impregnated polymers are commonly used in medical devices such as catheters to prevent the formation of bacterial biofilms (7, 43). Besides these and other medical applications, silver compounds are employed in a variety of nonmedical products. For example, silver ions are used in disinfectants (5), water purifiers, and sterilizers in hospitals and hotels, e.g., to control *Legionella* growth in water pipe systems (41). Hence, a resistance mechanism against silver ions would serve as a potent hygienic fitness factor for bacteria, facilitating their survival in hospital environments and creating a potential infection risk for patients. This has directed our attention to the *silS* gene, a silver resistance regulator gene, which was found to be unique to the clinical outbreak strain by subtractive hybridization. We showed that the complete silver resistance determinant was present and functional in the majority of isolates of the *E. cloacae* complex. The distribution of the *sil* operon among the different genotypes of the *E. cloacae* complex was significantly shifted in favor of genotypes frequently isolated in hospital environments (Table 4). The prevalence of 63% found in our cohort was surprisingly high; however, the study set consisted of clinical isolates collected over several years without any specific selection criteria and without knowledge of their susceptibility or resistance to silver. Woods et al. (46) screened 172 bacterial strains isolated from human and equine wounds for the presence of the *sil* determinant. They found only six positive strains; however, all of them were *E. cloacae*, representing 60% of all investigated *E. cloacae* strains. In our study cohort, we observed that a number of isolates were positive for some but not all *sil* genes. This might have been partly due to amplifications being missed by the PCR assays de-

FIG 2 The *sil* operon in *Enterobacter cloacae*. (A) Graphic illustration of the *sil* operon with marked PCR amplification sites. (B) PCRs of the *sil* operon; Lanes a, positive control DH5α pMG101; lanes b, tester strain EN-373. Lane pairs: 1, *silE* to *silS*; 2, *silS*; 3, *silS* to *silR*; 4, *silR*; 5, *silS* to *silC*; 6, *silC*; 7, *silC* to *silB*; 8, *silB* to *silA*; 9, *silA* to *silP*; 10, *silP*. M, 100-bp DNA marker.

signed from reference sequences. However, it is interesting to speculate whether these genes also might have additional functions independent of the context of the *sil* operon. This would be of particular interest for *silC*, which is consistently present in all isolates of genotypes I, II, and IV.

In the 1960s, the advent of silver compounds as therapeutics in medicine revolutionized the care of surgical and especially burn patients. Before topical silver nitrate was available, burn wound sepsis accounted for about two-thirds of mortality in burn patients. Since the late 1960s, when silver compounds were routinely applied to burn wounds (e.g., silver sulfadiazine in Flamazine), mortality rates rapidly reduced to about 1/4 (35). A decade later, a series of studies reported silver resistance in *Enterobacteriaceae* and nonfermenters (3, 6, 20, 33). Since that time, an increasing number of outbreaks due to silver-resistant strains of *Enterobac-*

teriaceae have been reported. In 1983, Markowitz et al. (31) reported a strong correlation between silver resistance and the presence of a typical “epidemic” plasmid pattern in *E. cloacae* outbreak strains causing infections in burn patients. These silver-resistant *E. cloacae* strains contained a 55-MDa plasmid which was not found in strains susceptible to silver sulfadiazine.

Irrespective of the medical impact of silver resistance in bacteria, the molecular genetics of silver resistance were not deciphered until the late nineties (16). Gupta et al. described a silver resistance determinant composed of nine open reading frames (ORFs), of which seven are named and two are still called ORFs. The unique feature of this determinant is the composition of two parallel membrane Ag(I) efflux pumps (*silCBA* and *silP*) and a periplasmic metal-binding protein (*silE*). Furthermore, Gupta et al. (18) described that *sil* genes were highly conserved in different IncHI plasmids of *Salmonella* serovars (MIP233, MIP235, and WR23) and *Serratia marcescens* (R476b and R478). Also, in the present study, partial *sil* sequences proved to be highly conserved compared to the prototype of the *sil* determinant in plasmid MG101 (16). Nucleotide identities ranged from 94% for *silA* to 99% for *silS* and *silC* (Table 3).

These results, together with the other plasmid-derived sequences found in the subtractive hybridization and the verification with the RepHIA PCR, clearly favor a plasmidic origin of the silver resistance in our *E. cloacae* strains. An extrachromosomal origin was also suggested by Davis et al. (8), who isolated two highly silver-resistant *E. cloacae* strains from teeth. This is also in line with the recent findings by Paauw et al. (36), who identified a large plasmid, pQC, in an *E. hormaechei* strain that caused a hos-

TABLE 3 Identity of *sil* nucleotide and amino acid sequences of *Enterobacter* species with pMG101

Type of identity ^a	% Identity with pMG101 ^b						
	<i>SilS</i>	<i>SilR</i>	<i>SilC</i>	<i>SilB</i>	<i>SilA</i>	ORF105	<i>SilP</i>
Nucleotide	99	98	99	97	94	88	95
Amino acid							
Identical	100	97	99	94	97	96	97
Positive	100	98	100	96	98	98	98

^a “Positive” amino acids are those that are different but have the same characteristics.

^b PCR products resulting in partial sequences of 818 bp for *silS*, 525 bp for *silR*, 381 bp for *silC*, 243 bp for *silB*, 277 bp for *silA*, 317 bp for ORF105, and 720 bp for *silP*.

TABLE 4 *sil* PCRs of clinical isolates from different genetic clusters

Genetic cluster ^a	<i>n</i>	No. (%) positive in PCR				
		SilC	SilP	SilR	SilS	All ^b
I	9	9 (100)	6 (67)	6 (67)	9 (100)	6 (67)
II	11	11 (100)	9 (82)	11 (100)	10 (91)	8 (73)
III	33	27 (82)	27 (82)	27 (82)	27 (82)	27 (82)
IV	15	15 (100)	9 (60)	14 (93)	15 (100)	9 (60)
V	18	9 (50)	11 (61)	5 (28)	17 (94)	2 (11)
VI + VII	19	17 (89)	16 (84)	17 (89)	16 (84)	16 (84)
VIII	40	32 (80)	32 (80)	32 (80)	32 (80)	32 (80)
IX	6	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
XI + XII ^c	7	6 (86)	5 (71)	6 (86)	4 (57)	4 (57)
xiii	6	5 (83)	3 (50)	3 (50)	3 (50)	0 (0)
Total	164	131 (80)	118 (71)	121 (74)	133 (81)	104 (63)

^a Genetic clusters according to Hoffmann and Roggenkamp (22).
^b Distribution of isolates positive for all PCRs in genetic clusters; chi-square test, *P* < 0.0001.
^c Includes *E. cloacae* type strain ATCC 13047.

pital-wide outbreak in the Netherlands. This plasmid also belonged to the IncHI-2 group and showed the highest similarity to plasmid R478. Besides resistance to several antibiotics, it was reported to carry genes for resistance to heavy metals, including the

sil operon. The presence and absence of the putative IncHI-2 plasmid are then the major genomic differences between clinical outbreak strain EN-373 and two independent plant isolates. However, as the large plasmid was isolated in the chromosomal DNA fraction, it might have been overrepresented in the tester DNA and—depending on the number of plasmidic copies—may have obscured other genomic differences between the pathogenic and nonpathogenic strains.

In conclusion, by subtractive hybridization we identified a silver resistance determinant selectively present in a pathogenic *Enterobacter* strain. Furthermore, this *sil* operon was found in 63% of all isolates of the *E. cloacae* complex and was predominantly present in genotypes with high clinical prevalence. This distribution could partially explain the unequal prevalence rates of the (sub) species of the *E. cloacae* complex in the hospital setting and might also provide some explanation for the high infection rates in burn patients; however, the relatively high frequency of infections caused by *Enterobacter* in newborns is still not sufficiently elucidated by our findings. The association of such a plasmid with certain genotypes, however, also raises the question of why it is not more extensively distributed among other genotypes. This again suggests further differences between the pathogenic and nonpathogenic variants. Furthermore, the question of whether silver resistance is only a fitness factor promoting survival in hospital environment or whether the *sil* determinant itself confers pathogenicity-associated functions needs to be investigated. The amino acid sequence of the product of *silC*, for instance, shows 70% identity with that of *ibeB*, a gene which has been described as being required in *E. coli* for the penetration of brain microvascular endothelial cells (23). This speculation is supported by a recent report demonstrating that isolates of the *E. cloacae* complex are able to adhere to and penetrate epithelial cells (28). Other studies have shown that mutations in the copper transport ATPase reduce the virulence in *Listeria monocytogenes* (13). In line with those results, it is intriguing to speculate that silver resistance might not only promote survival but also increase bacterial pathogenicity. In this context, therapeutic usage of silver compounds would not only be ineffective but could even be harmful, as they would select for more pathogenic strains. Whether there are indeed such associations is currently unknown, and further studies are needed to focus on this issue.

FIG 3 Phenotypic silver resistance. (A) Killing of bacteria by AgNO₃. OD₆₀₀ was measured after incubation (16 h, 36 ± 1°C, room air) of the indicated strains in LB medium supplemented with increasing amounts of AgNO₃. *E. coli* strains DH5α and DH5α(pMG101) served as negative (AgNO₃ MIC, 50 μM) and positive (AgNO₃ MIC, 600 μM) controls, respectively. Two *sil* PCR-positive strains (244 and 373) as well as three *sil* PCR-negative strains (114, 501R3, and E6) were included. Symbols in the key are labeled with the strain name, genetic cluster, and PCR results. Clinical outbreak strain 373 of genetic cluster I (22) was positive in all *sil* PCRs and showed an AgNO₃ MIC of 600 μM. Both strains 501R3 and E6 yielded negative PCRs with all *sil* genes and had AgNO₃ MICs equal to or below 50 μM. (B) Growth of strains on LB agar containing rising concentration of AgNO₃. The positions of the strains on the plates are depicted on the left.

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