

# Characterization of Epidemic IncI1-I $\gamma$ Plasmids Harboring Ambler Class A and C Genes in *Escherichia coli* and *Salmonella enterica* from Animals and Humans

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The aim of the study was to identify the plasmid-encoded factors contributing to the emergence and spread of epidemic IncI1-I $\gamma$  plasmids obtained from *Escherichia coli* and *Salmonella enterica* isolates from animal and human reservoirs. For this, 251 IncI1-I $\gamma$  plasmids carrying various extended-spectrum  $\beta$ -lactamase (ESBL) or AmpC  $\beta$ -lactamase genes were compared using plasmid multilocus sequence typing (pMLST). Thirty-two of these plasmids belonging to different pMLST types were sequenced using Roche 454 and Illumina platforms. Epidemic IncI1-I $\gamma$  plasmids could be assigned to various dominant clades, whereas rarely detected plasmids clustered together as a distinct clade. Similar phylogenetic trees were obtained using only the plasmid backbone sequences, showing that the differences observed between the plasmids belonging to distinct clades resulted mainly from differences between their backbone sequences. Plasmids belonging to the various clades differed particularly in the presence/absence of genes encoding partitioning and addiction systems, which contribute to stable inheritance during cell division and plasmid maintenance. Despite this, plasmids belonging to the various phylogenetic clades also showed marked resistance gene associations, indicating the circulation of successful plasmid-gene combinations. The variation in *traY* and *excA* genes found in IncI1-I $\gamma$  plasmids is conserved within pMLST sequence types and plays a role in incompatibility, although functional study is needed to elucidate the role of these genes in plasmid epidemiology.

*Escherichia coli* strains producing extended-spectrum  $\beta$ -lactamases (ESBLs) have emerged globally in humans as well as in animals during the last decade (1–3). Conjugative plasmids, integrons, and IS elements play a dominant role in the dissemination of the resistance genes in *Enterobacteriaceae* (3). Many plasmid families have been associated with the carriage of genes encoding specific ESBLs or plasmid-borne AmpC type  $\beta$ -lactamases (pAmpC) and were considered to be epidemic resistance plasmids due to their role in the dissemination of these genes (4). Among them, IncI1-I $\gamma$  plasmids have been predominantly associated with ESBL/pAmpC-producing isolates from animals (5–8). Genetically related IncI1-I $\gamma$  plasmids carrying *bla*<sub>CTX-M-1</sub> genes were identified in *E. coli* isolates from poultry sources and in those sustaining infections in humans in The Netherlands, suggesting that transfer of plasmids occurred among strains from food animals and humans (9).

Predominant plasmid-gene combinations have been described in *Enterobacteriaceae* from both animals and humans and help to determine the epidemiology of ESBL genes. In isolates of animal origin, the *bla*<sub>CTX-M-15</sub>, *bla*<sub>TEM-52</sub>, and *bla*<sub>SHV-12</sub> genes predominate, each on IncI1-I $\gamma$  plasmids, while *bla*<sub>CMY-2</sub> genes can be associated with IncI1-I $\gamma$  or IncK plasmids. In contrast, *bla*<sub>CTX-M-15</sub> predominates in human isolates and is typically located on IncFII plasmids and to a much lesser extent on IncI1-I $\gamma$  plasmids (7, 8, 10, 11). IncI1-I $\gamma$ -associated *bla*<sub>CTX-M-15</sub> genes are also present in isolates from livestock animals but at a very low prevalence (12). The predominant gene-plasmid combinations spread epidemically in bacterial communities and animal or human enterobacte-

rial populations (7). The successful dissemination of epidemic IncI1-I $\gamma$  plasmids will most probably be related to plasmid-encoded factors, possibly in association with factors encoded by the host strain.

Plasmid multilocus sequence typing (pMLST) using five conserved genes can discriminate between IncI1-I $\gamma$  plasmid subtypes (<http://pubmlst.org/plasmid/>) (13), and the IncI1-I $\gamma$  plasmids deposited in the pMLST database actually belong to 158 different sequence types (STs) and 10 clonal complexes (CCs), indicating their high level of variability. The most frequent IncI1-I $\gamma$  ST identified is ST7 (<http://pubmlst.org/plasmid/>). Recent data showed that dominant STs can be identified among the IncI1-I $\gamma$  plasmids

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and are associated with specific ESBL/pAmpC genes (4, 14). IncI1-I $\gamma$  plasmids belonging to ST7 were most frequently associated with *bla*<sub>CTX-M-15</sub>, whereas IncI1-I $\gamma$  plasmids belonging to ST12 and ST31 were associated with *bla*<sub>CMY-2</sub> and *bla*<sub>CTX-M-15</sub> genes, respectively (<http://pubmlst.org/plasmid/>). Complete nucleotide sequences of a number of IncI1-I $\gamma$  plasmids are available in the GenBank database. All IncI1-I $\gamma$  plasmids characterized contained a set of highly conserved core genes involved in essential functions, such as replication, maintenance, stability, and transfer as well as some unique accessory elements (15).

To understand better the plasmid-encoded factors contributing to the emergence and spread of epidemic IncI1-I $\gamma$  plasmids, we performed pMLST and comparative sequence analysis of epidemic and rarely detected IncI1-I $\gamma$  plasmids obtained from *E. coli* and *Salmonella enterica* from animal and human reservoirs.

## MATERIALS AND METHODS

**Bacterial isolates.** *S. enterica* and *E. coli* isolates containing IncI1-I $\gamma$  plasmids carrying ESBL/pAmpC genes were selected from existing strain collections in The Netherlands, United Kingdom, and Germany from isolates obtained from 2005 to 2009. These collections were obtained in National Resistance Surveillance programs or dedicated studies for ESBL/pAmpC producers (16) and integrons (17) in which the plasmids were characterized by PCR-based replicon typing (PBRT) (18, 19). This resulted in a collection of 251 *E. coli* and *Salmonella* isolates with ESBL, pAmpC, or class 1 integrons on IncI1-I $\gamma$  plasmids (see Table S1 in the supplemental material).

**$\beta$ -Lactamase identification.** Whole-cell DNA of the isolates was used for screening for ESBL and/or pAmpC genes by miniaturized microarray (Alere, Jena, Germany). The identity of ESBL/pAmpC genes was confirmed by PCR and sequencing as described previously (20).

**Plasmid isolation and characterization.** IncI1-I $\gamma$  plasmids were extracted using a crude lysis (miniprep) method (21) or a Qiafilter plasmid midikit (Qiagen). Extracted plasmid DNA was used for electrotransformation of *E. coli* DH10B cells (Invitrogen), and transformants were selected on Luria-Bertani agar with 1 mg/liter cefotaxime (Sigma).

All 251 ESBL/pAmpC-encoding IncI1-I $\gamma$  plasmids were subtyped by pMLST analysis as described previously (13) using DNA extracted from transformants with the DNeasy blood and tissue kit (Qiagen). pMLST amplicons obtained using pubmlst-derived primers (<http://pubmlst.org/plasmid/primers/incI1.shtml>) were purified and sequenced using an ABI 3730 DNA sequencer (Applied Biosystems, Foster City, CA). Plasmids were assigned to sequence types (STs), and minimum-spanning trees were generated from the allelic profiles using Bionumerics 6.5. A PCR result negative on one of the selected loci was designated an incomplete sequence type. The sizes of the plasmids were estimated using pulsed-field gel electrophoresis (PFGE) of S1 nuclease digests of total DNA (22).

**Plasmids selected for sequence analysis.** Based on the variations in pMLST types, 32 transformants were selected for complete plasmid sequence analysis (see Table S1 in the supplemental material). These transformants harbored IncI1-I $\gamma$  plasmids that were all obtained from individual *E. coli* strains, and 28 of the 32 plasmids belonged to the most prevalent pMLST types identified. For each ST (if possible), representative plasmids were chosen that (i) differed in their sizes and (ii) were obtained from *E. coli* isolates that originated from several animal or human sources and differed in their years of isolation. For comparative reasons, four IncI1-I $\gamma$  plasmids belonging to unique or incomplete STs were also selected for sequence analysis.

**Plasmid sequencing.** Deep sequencing of the plasmid genomes was performed using Roche 454 XL shotgun sequencing technology or using 150-bp paired-end sequencing libraries (Nextera TAG-mentation sequencing kits [Epicentre]) on an Illumina MiSeq sequencer (23). High-quality filtered reads were subsequently assembled *de novo* using the Newbler algorithm (v2.5.3) for 454 reads and the AbySS algorithm (abyss

version 1.3.3) for Illumina-derived reads. The sequence coverage of the *de novo* assemblies was on average over 100, with a minimum (for some 454-sequenced plasmids) of 60 sequence reads of coverage per assembled base. High-coverage scaffolds of the genomes were reconstructed by scaffolding the contigs against the closely related IncI1 reference R64 (15). Putative open reading frames (ORFs) were identified by GeneMarkHMMp version 2.6p (24). BLASTP analyses of the putative ORFs against the NCBI nonredundant proteins (NR) database, Pfam (25), and Interpro scan (26) were used to assess their putative functions by identification of structural features and motifs.

**Sequence clustering and phylogenetics.** Plasmid sequences were hierarchically clustered and displayed as a phenogram using the BioNJ algorithm (27), where the underlying distance matrix was calculated from the pairwise nonoverlapping maximal unique matches (MUMs) using Nucmer version 3.07 (28). Relative pairwise distances were obtained by dividing the pairwise MUMs' sum by the average genome size of the two paired genomes (MUMi genomic distance) (29). BioNJ trees were generated from the MUMi distance matrix using SplitsTree4 (30).

**Nucleotide sequence accession numbers.** The NCBI GenBank accession numbers for the plasmid samples have been submitted under NCBI-BioProject PRJNA263774 and BioSample accession no. SAMN03168474 to SAMN03168504. The accession numbers of the reference plasmids used in this study for comparison are as follows: R64, AP005147; ColIb-P9, AB021078; pEC-Bactec, GU371927; pND11-107, HQ114281; pEK204, EU935740; and R621a, AP011954.

## RESULTS AND DISCUSSION

**pMLST.** Two hundred fifty-one IncI1-I $\gamma$  plasmids carrying ESBL/pAmpC-encoding genes obtained from *S. enterica* and *E. coli* isolates from different countries were used in this study. The plasmids were assigned to 28 different pMLST types (Fig. 1 and 2; see Table S1 in the supplemental material). In addition, six incomplete pMLST profiles were observed. The 153 IncI1-I $\gamma$  plasmids containing *bla*<sub>CTX-M-1</sub> were assigned to 14 different STs, but the majority belonged to either ST7 (70%) or ST3 (27%) (Fig. 1 and 2). IncI1-I $\gamma$  plasmids harboring *bla*<sub>CTX-M-1</sub> were obtained from *S. enterica* as well as from *E. coli* isolates, which originated mainly from humans and poultry (9) (see Table S1). IncI1-I $\gamma$  plasmids containing the *bla*<sub>CTX-M-15</sub> gene were mainly obtained from *E. coli* isolates from humans or cattle, and a high percentage (60%) of these plasmids belonged to ST31 (14) (see Table S1). Most (92%) IncI1-I $\gamma$  plasmids containing *bla*<sub>TEM-52</sub> were assigned to ST36, ST10, or ST21, all of which belong to CC5. IncI1-I $\gamma$  plasmids containing the *bla*<sub>TEM-52</sub> gene were obtained from *S. enterica* and *E. coli* isolates and were mainly found in human and poultry reservoirs. IncI1-I $\gamma$  plasmids containing the *bla*<sub>CMY-2</sub> gene were mainly (81%) assigned to ST12 (<http://pubmlst.org/plasmid/>) (31) (Fig. 2). These data confirm the presence of predominant ST-gene combinations among IncI1-I $\gamma$  plasmids as previously described (4, 14), suggesting circulation of a number of prevalent IncI1-I $\gamma$  plasmids among bacterial species of animal and human reservoirs. However, some pMLST types were associated with a number of different ESBL/AmpC genes: e.g., ST3 plasmids carrying the *bla*<sub>CTX-M-1</sub>, *bla*<sub>TEM-20</sub>, *bla*<sub>TEM-1</sub>, *bla*<sub>SHV-12</sub>, and *bla*<sub>CTX-M-3</sub> genes. Previously, ST3 IncI1-I $\gamma$  plasmids have been found to be associated with the *bla*<sub>CTX-M-1</sub> gene among *E. coli* strains of avian and human origins in The Netherlands (9), as well as among *E. coli* strains isolated from several animal species in other European countries (13, 32, 33).

**Characteristics of sequenced plasmids.** Twenty-eight plasmids belonging to the most prevalent STs and four plasmids belonging to rarely detected STs were selected for complete plasmid

ESBL/AmpC	numbers	STs (numbers)													
CTX-M-1	153	ST7 (87)	ST3 (33)	IC (12)*	ST63 (5)	ST26 (4)	ST58 (3)	ST35 (2)	ST10 (1)	ST30 (1)	ST36 (1)	ST59 (1)	ST61 (1)	ST62 (1)	ST64 (1)
TEM-52	46	ST36 (34)	ST10 (10)	IC (2)	ST21 (1)	ST27 (2)	ST60 (1)								
CTX-M-15	21	ST31 (13)	ST37 (3)	ST16 (3)	ST61 (1)	IC (1)									
CMY-2	11	ST12 (8)	ST65 (1)	ST2 (1)	IC (1)										
Int11**	6	ST26 (2)	ST3 (1)	ST29 (1)	ST24 (1)	ST50 (1)									
TEM-1	4	ST3 (3)													
TEM-20	4	ST3 (4)													
CTX-M-3	3	ST57 (2)		ST3 (1)											
SHV-12	3	ST3 (1)		ST25 (1)		ST95 (1)									

\* IC = incomplete ST, indicating that 1 out of the 5 primer sets did not result in an amplicon of the expected size

\*\* IncI1 plasmids not containing an ESBL/AmpC gene but carrying a class I integron were selected for comparison

belonging to CC3

belonging to CC2

belonging to CC5

belonging to CC7

belonging to CC12

belonging to CC26

belonging to CC31

belonging to CC58

belonging to CC61

FIG 1 Distribution of plasmids to pMLST STs and CCs.

sequence analysis (Table 1). All plasmids shared the typical IncI1-Iγ-associated genetic modules, including the replication region, maintenance and stability regions, and the transfer regions. Moreover, the plasmids were supplemented with one to three unique accessory elements, containing the antibiotic resistance genes. Major parts of the plasmid backbone sequences were highly conserved. Based on a phylogenetic analysis of the whole plasmid sequences, including the segments containing the antibiotic resistance genes, the plasmids were classified into a number of distinct clades (Fig. 3A). Similar phylogenetic trees were obtained using the plasmid backbone sequences exclusively (Fig. 3B), which shows that the differences observed between the plasmids belonging to distinct clades were mainly caused by differences in their backbone sequences. Despite this, plasmids belonging to the various phylogenetic clades showed predominant plasmid-gene combinations, indicating the circulation of successful plasmid-gene combinations. Moreover, similar plasmid-gene combinations were obtained from different animal sources as well as from humans, which suggests transmission between animal and human isolates, as was previously assumed (9). Rarely detected plasmids clustered together as a distinct clade.

**Differences between the sequenced plasmids.** The plasmids belonging to distinct clades mainly differed in the presence/absence of (i) genes encoding colicins Collb, TraD, PilJ, FinQ, and CcgAII, (ii) genes encoding the proteins involved in partitioning and in surface exclusion, (iii) the number of genes encoding addiction factors, and (iv) the composition of the shufflon (Fig. 4 and Table 1).

ST3 *bla*<sub>CTX-M-1</sub>, ST7 *bla*<sub>CTX-M-1</sub>, and ST12 *bla*<sub>CMY-2</sub> plasmids as well as the uncommonly isolated plasmid STs contained *cib* and *imm* genes similar to the colicin-encoding and immunity-confering genes of plasmid Collb-P9 (34). All other plasmids of the present study lacked the *cib* and *imm* genes. Expression of colicins can benefit the bacterial host by causing lethality to related enteric bacteria that would otherwise compete for scarce nutrients under conditions of stress (35).

For the correct partitioning of the plasmids among daughter cells, most of the plasmids studied here contained the *parAB* genes as in the IncI1 reference plasmid R64 (15). Two of the plasmids representing ST37 *bla*<sub>CTX-M-15</sub> and ST35 *bla*<sub>CTX-M-1</sub> contained the *parAB* genes as in the IncIγ plasmid R621a (36), whereas the plas-

mids belonging to the uncommon STs contained *parAB* genes highly homologous to those of pND11\_107 (14). The R621a and pND11-107 *parAB* genes differed significantly both from each other and from those of R64 (15). The ST31 *bla*<sub>CTX-M-15</sub> plasmids lacked the R64/R621a/pND11-107 type of *parAB* genes. Instead, these plasmids contained the *soj-yfhA* genes previously suggested to act as a partitioning system in the plasmid pEC-BacTec (37).

Entry exclusion systems inhibit the transfer of closely related conjugative plasmids to prevent plasmid redundancy through recognition of the donor target protein TraY by the recipient protein ExcA or ExcA and ExcB during conjugation (38, 39). Genetic variation between these genes in R64 and R621a allows for cointroduction of these plasmids into a single host cell, resulting in the phenotypic classification of these plasmids into IncI1 and IncIγ groups, respectively (38). Current classification of the plasmids in this study was done based on the PBRT scheme, which detects the presence of RNA interference (RNAi) present in both IncI1 and IncIγ plasmids and cannot discriminate between these (1, 18). Sequence comparison of the plasmids studied here showed that the ST7 *bla*<sub>CTX-M-1</sub>, ST31 *bla*<sub>CTX-M-15</sub>, ST37 *bla*<sub>CTX-M-15</sub>, ST25 *bla*<sub>SHV-12</sub>, and ST26 plasmids belonged to the R621a exclusion group, whereas the ST35 *bla*<sub>CTX-M-1</sub> plasmid contained the exclusion system found in R64 (Table 1). The ExcAB proteins encoded by ST12 *bla*<sub>CMY-2</sub> plasmids (40) were exactly like those in R64, while the TraY protein shared 98% identity to the TraY protein of R64. The effect of these amino acid changes in the TraY proteins on the specificity of the entry exclusion is unknown. The ExcAB and TraY proteins encoded by the plasmids belonging to the ST3 *bla*<sub>CTX-M-1</sub>, incomplete ST (IC) *bla*<sub>CTX-M-1</sub>, ST3 *bla*<sub>SHV-12</sub>, ST36 *bla*<sub>TEM-52</sub>, and ST24 IntI1 types were identical to ExcAB and TraY proteins of plasmid pH2291-112 (40). The ExcAB proteins encoded by the ST50 *bla*<sub>TEM-1</sub> plasmid were identical to the ExcAB proteins in R64, whereas the TraY protein shared 98% identity to the TraY protein of *E. coli* ECH489. Based on these results, it is expected that some of the plasmids studied here will phenotypically belong to IncI1 and some to IncIγ, but until functional studies have been carried out, we will adhere to the genotypic classification and refer to these plasmids as IncI1-Iγ.

Addition systems, such as the toxin-antitoxin modules encoded by plasmids will contribute to the maintenance of the plasmids in their hosts by killing daughter cells that do not inherit the



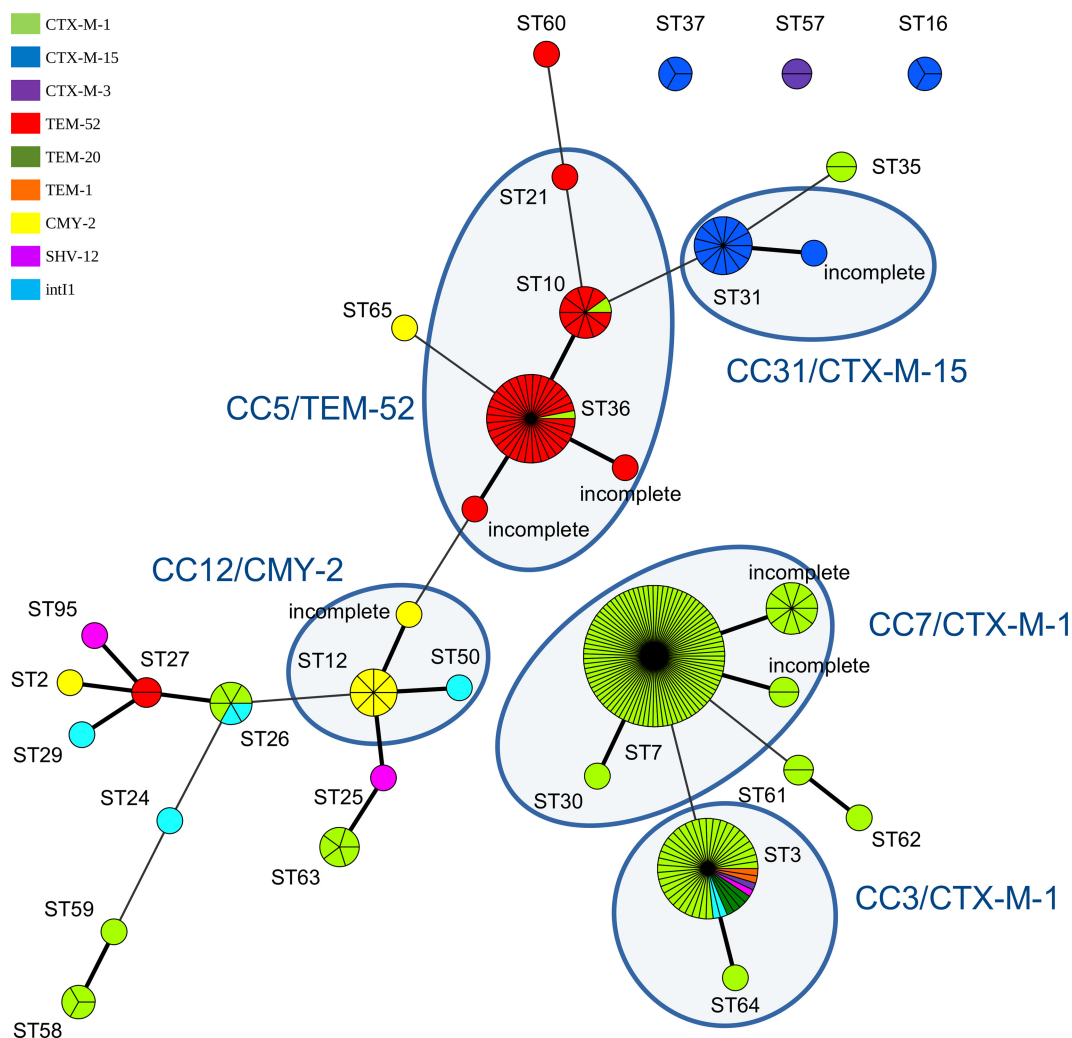


FIG 2 pMLST analysis of 251 selected IncI1-I $\gamma$  plasmids as visualized as a minimum-spanning tree. Plasmids with identical sequence types (STs) were assigned to one circle. Single-locus variants are indicated by a thick line and double-locus variants by a thin line. Sequence types belonging to one clonal complex were grouped by a blue circle. The colors represent the various antibiotic resistance genes found to be associated with the sequence types of the plasmids.

plasmids during cell division (41, 42). One to three different addition systems were observed in the plasmids included in this study. As expected, all plasmids contained the *pndAC* system characteristic for IncI1-like plasmids (42). Moreover, except for the *bla*<sub>TEM-52</sub>-containing plasmids, including the ST31 *bla*<sub>CTX-M-15</sub>, ST37 *bla*<sub>CTX-M-15</sub>, and ST35 *bla*<sub>CTX-M-1</sub> plasmids, all plasmids also contained the *relBE* system (43). The *bla*<sub>TEM-52</sub>-containing isolates had, except for the *pndAC* system, the *vapBC* system previously described as being a toxin-antitoxin system for maintenance of R64 (44). ST12 *bla*<sub>CMY-2</sub> IncI1-I $\gamma$  plasmids contained three addition systems: the *pndAC* and *relBE* systems, as well as the *ccdAB* system. The *ccdAB* system has previously been characterized in IncF replicons mainly (42).

**Conjugation systems.** All IncI1-I $\gamma$  plasmids are characterized by the presence of genes for two types of conjugal pili: a thick pilus required for both liquid and surface matings and a thin pilus required exclusively for liquid matings (45). All plasmids included in this study contained the genes essential for conjugation. Conjugation under liquid conditions was confirmed “*in vitro*” for a

selected number of the plasmids that lacked the *traD* gene (data not shown).

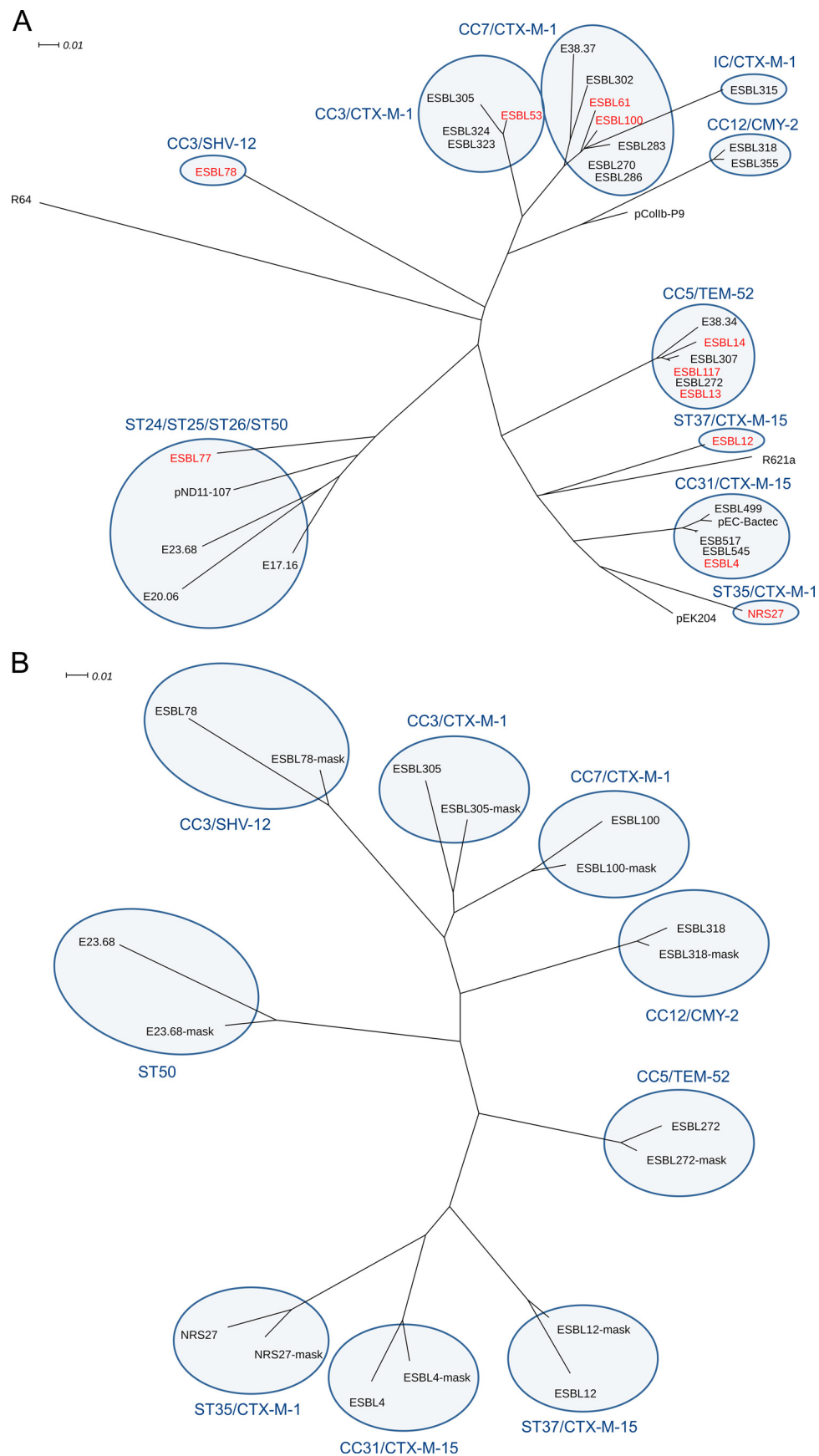
The shufflon, originally described in plasmid R64, consists of a number of invertible DNA segments, which are separated and flanked by recombination sites (46). The *rci* gene, located next to the shufflon, mediates site-specific recombination between any of the inverted repeat regions (46). The number of invertible repeats present in the plasmids included in this study varied considerably (Table 1), indicating that the plasmids varied in their ability to conjugate to various host recipients.

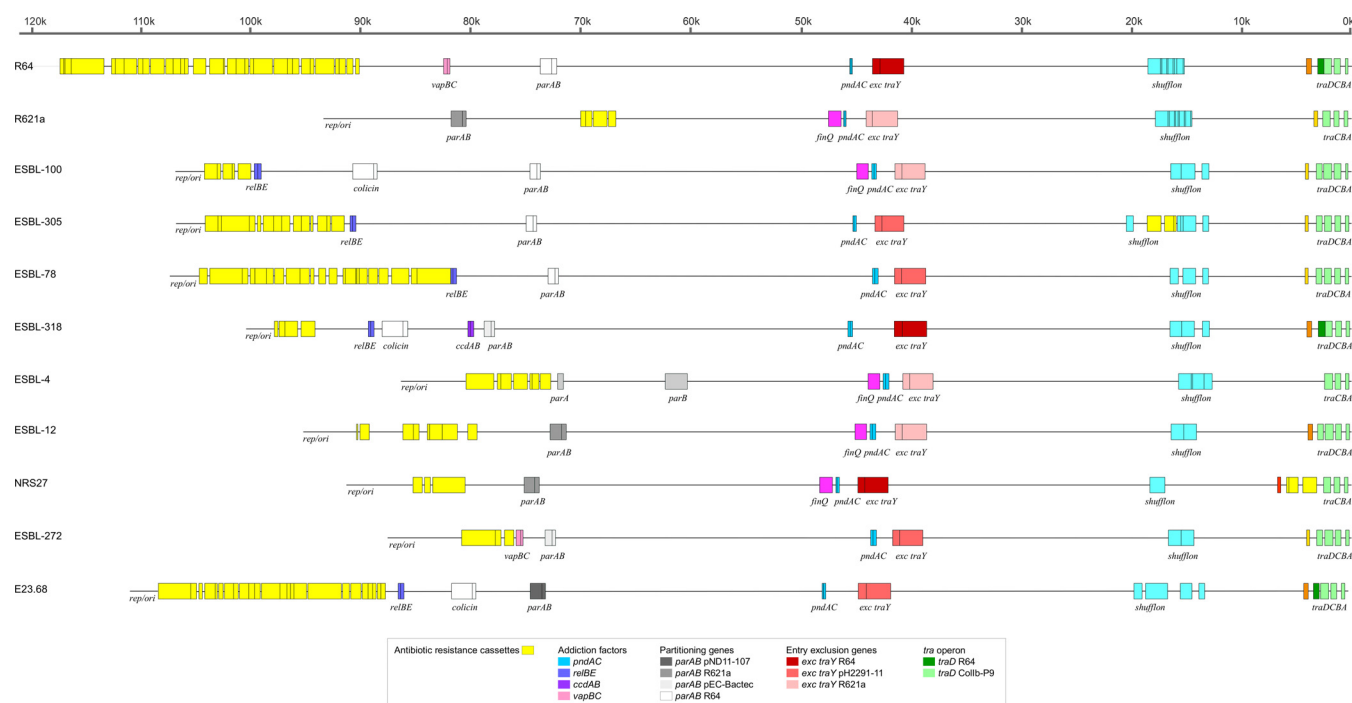
**Accessory modules and resistance genes.** Accessory modules representative for the various plasmid clades are shown in Table S2 in the supplemental material. All plasmids contained one or two accessory modules encoding in total one to six antimicrobial resistance genes. Although most of the modules were inserted near the replication functions of the plasmids, the exact locations of the accessory modules in the IncI1-I $\gamma$  plasmid backbones differed between the various plasmid clades. Some plasmids harbored accessory modules at other locations in the plasmid backbone as well.

TABLE 1 Characteristics of sequenced IncI1 plasmids

Plasmid	Origin	ESBL	Yr of isolation	Country of origin <sup>a</sup>	Estimated size (kb)	pMLST result		Presence of colicin	Plasmid partitioning gene <i>parB</i> <sup>b</sup>	Plasmid(s) with entry exclusion gene:		Gene(s) for addition factors <sup>c</sup>	Shaftion	Presence of gene:		Accession no.
						ST	CC			<i>excB</i> <sup>d</sup>	<i>traI</i> <sup>e</sup>			<i>puI</i> <sup>f</sup>	<i>traD</i> <sup>g</sup>	
E38-27	Poultry	CTX-M-1	2006	NL	88	ST7	CC7	–	R64 <sup>h</sup>	R621a	R621a	<i>puIAC</i> and <i>relBE</i>	BB'C'CA'	ColIb-P9 <sup>h</sup>	ColIb-P9	3168474
ESBL-100	Human	CTX-M-1	2009	NL	100	ST7	CC7	+	R64	R621a	R621a	<i>puIAC</i> and <i>relBE</i>	BB'C'CA'	ColIb-P9	ColIb-P9	3168475
ESBL-286	Poultry	CTX-M-1	2008	NL	110	ST7	CC7	+	R64	R621a	R621a	<i>puIAC</i> and <i>relBE</i>	BB'C'CA'	ColIb-P9	ColIb-P9	3168476
ESBL-270	Poultry	CTX-M-1	2007	NL	105	ST7	CC7	+	R64	R621a	R621a	<i>puIAC</i> and <i>relBE</i>	BB'C'CA'	ColIb-P9	ColIb-P9	3168477
ESBL-283	Pigs	CTX-M-1	2008	NL	105	ST7	CC7	+	R64	R621a	R621a	<i>puIAC</i> and <i>relBE</i>	BB'C'CA'	ColIb-P9	ColIb-P9	CO008736
ESBL-61	Human	CTX-M-1	2009	NL	105	ST7	CC7	+	R64	R621a	R621a	<i>puIAC</i> and <i>relBE</i>	BB'C'CA'	ColIb-P9	ColIb-P9	3168479
ESBL-302	Poultry	CTX-M-1	2008	NL	95	IC <sup>d</sup>	CC7	ND <sup>e</sup>	R64	R621a	R621a	<i>puIAC</i> and <i>relBE</i>	BB'C'CA'	ColIb-P9	ColIb-P9	3168480
ESBL-315	Human	CTX-M-1	2008	NL	90	IC	CC3	+	R64	R621a	R621a	<i>puIAC</i> and <i>relBE</i>	BB'C'CA'	ColIb-P9	ColIb-P9	CF008738
ESBL-53	Human	CTX-M-1	2009	NL	95	ST3	CC3	–	R64	pH2291-11	pH2291-11	<i>puIAC</i> and <i>relBE</i>	CC'-cas-BB	ColIb-P9	ColIb-P9	3168482
ESBL-305	Poultry	CTX-M-1	2008	NL	105	ST3	CC3	–	R64	pH2291-11	pH2291-11	<i>puIAC</i> and <i>relBE</i>	CC'-cas-BB	ColIb-P9	ColIb-P9	3168483
ESBL-323	Pigs	CTX-M-1	2008	NL	105	ST3	CC3	+	R64	pH2291-11	pH2291-11	<i>puIAC</i> and <i>relBE</i>	CC'-cas-BB	ColIb-P9	ColIb-P9	3168484
ESBL-324	Pigs	CTX-M-1	2008	NL	105	ST64	CC3	+	R64	pH2291-11	pH2291-11	<i>puIAC</i> and <i>relBE</i>	BB'C'CA'	ColIb-P9	ColIb-P9	3168485
ESBL-78	Human	SHV-12	2009	NL	105	ST3	CC3	–	R64	pH2291-11	pH2291-11	<i>puIAC</i> and <i>relBE</i>	BB'C'CA'	ColIb-P9	ColIb-P9	3168486
ESBL-318	Poultry	CMY-2	2008	NL	95	ST12	CC12	+	R64	R64	R64 (98%)	<i>puIAC</i> , <i>ccdB</i> , and <i>relBE</i>	BB' A	ColIb-P9	ColIb-P9	3168487
ESBL-355	Poultry	CMY-2	2007	NL	100	ST12	CC12	+	R64	R64	R64 (98%)	<i>puIAC</i> , <i>ccdB</i> , and <i>relBE</i>	A	R64	R64	3168488
ESBL-4	Human	CTX-M-15	2009	NL	90	ST31	CC31	–	pEC-Bactec <sup>i</sup>	R621a	R621a	<i>puIAC</i>	BB'D'C'CA'	None	None	3168489
ESBL-499	Cattle	CTX-M-15	2009	UK	ND	ST31	CC31	–	pEC-Bactec	R621a	R621a	<i>puIAC</i>	BB'C'CD'A'	None	None	3168490
ESBL-517	Cattle	CTX-M-15	2009	UK	ND	ST31	CC31	–	pEC-Bactec	R621a	R621a	<i>puIAC</i>	BB'C'CD'A'	None	None	3168491
ESBL-545	Cattle	CTX-M-15	2009	UK	ND	ST31	CC31	–	pEC-Bactec	R621a	R621a	<i>puIAC</i>	BB'C'CD'A'	None	None	3168492
ESBL-12	Human	CTX-M-15	2009	NL	100	ST37	CC31	–	R621a	R621a	R621a	<i>puIAC</i>	BB'C'CD'A'	R64	ColIb-P9	CF008735
NRS27	Human	CTX-M-1	2009	NL	90	ST35	CC5	–	R621a	R64	R64	<i>puIAC</i>	ND	pH1519-88 <sup>m</sup>	None	3168494
ESBL-272	Poultry	TEM-52	2007	NL	90	ST36	CC5	–	R64	pH2291-11	pH2291-11	<i>puIAC</i> and <i>vopBC</i>	BB'C'CA'	ColIb-P9	ColIb-P9	3168495
ESBL-14	Human	TEM-52	2009	NL	90	ST36	CC5	–	R64	pH2291-11	pH2291-11	<i>puIAC</i> and <i>vopBC</i>	BB'C'CA'	ColIb-P9	ColIb-P9	3168496
ESBL-307	Poultry	TEM-52	2008	NL	90	ST36	CC5	–	R64	pH2291-11	pH2291-11	<i>puIAC</i> and <i>vopBC</i>	BB'C'CA'	ColIb-P9	ColIb-P9	3168497
ESBL-117	Human	TEM-52	2009	NL	90	ST36	CC5	–	R64	pH2291-11	pH2291-11	<i>puIAC</i> and <i>vopBC</i>	BB'C'CA'	ColIb-P9	ColIb-P9	CF008734
ESBL-13	Human	TEM-52	2009	NL	95	ST36	CC5	–	R64	pH2291-11	pH2291-11	<i>puIAC</i> and <i>vopBC</i>	BB'C'CA'	ColIb-P9	ColIb-P9	3168499
E38-34	Chicken	TEM-52	2009	NL	97	ST36	CC5	–	R64	pH2291-11	pH2291-11	<i>puIAC</i> and <i>vopBC</i>	BB'C'CA'	ColIb-P9	ColIb-P9	3168500
E17-16	Poultry	Int1 <sup>j</sup>	2004	NL	100	ST24	CC12	+	pND11-107 <sup>k</sup>	pH2291-11	pH2291-11	<i>puIAC</i> and <i>relBE</i>	BB'C'CA'	R64	R64	CF008733
E23-68	Poultry	Int1	2004	NL	110	ST50	CC12	+	pND11-107	R64	Ech489	<i>puIAC</i> and <i>relBE</i>	D'BB'C'CA'	R64	R64	3168502
E20-06	Poultry	Int1	2004	NL	110	ST26	CC26	+	pND11-107	R621a	R621a	<i>puIAC</i> and <i>relBE</i>	BB'C'CA'	R64	R64	3168503
ESBL-77	Human	SHV-12	2009	NL	105	ST25	CC26	+	pND11-107	R621a	R621a	<i>puIAC</i> and <i>relBE</i>	BB'C'CA'	R64	R64	3168504

<sup>a</sup> NL, The Netherlands; UK, United Kingdom.<sup>b</sup> The plasmid genome with the highest level of identity is indicated.<sup>c</sup> Identified addition factors are indicated.<sup>d</sup> IC, incomplete sequence type.<sup>e</sup> ND, not detected/not determined.<sup>f</sup> IncI1 plasmids not containing an ESBL/AmpC gene but carrying a class I integron were selected for comparison.<sup>g</sup> Accession no. AP005147.<sup>h</sup> Accession no. AB021078.<sup>i</sup> Accession no. AP011954.<sup>j</sup> Accession no. GU371927.<sup>k</sup> Accession no. HQ114281.<sup>l</sup> Accession no. K1484630.<sup>m</sup> Accession no. K1484629.





**FIG 4** Genetic organization of IncI1-I $\gamma$  plasmids (aligned at the origin of replication) belonging to various phylogenetic clades. Backbone sequences found to be identical among the various plasmids are indicated by a straight line. Conserved genes are indicated by colors and refer to colors representative for the reference plasmids R64, R621a, pEC-Bactec, ColIb-P9, pND11-107, and pH1519, as indicated below.

Resistance genes of plasmids harboring a *bla*<sub>CTX-M-1</sub> or *bla*<sub>CTX-M-15</sub> gene were all linked to an *ISEcp1* element. In the ST7 *bla*<sub>CTX-M-1</sub> and the IC *bla*<sub>CTX-M-1</sub> plasmids, the *ISEcp1-bla*<sub>CTX-M-1</sub> element was inserted near the replication function, whereas in the ST3 *bla*<sub>CTX-M-1</sub> plasmids, the *ISEcp1-bla*<sub>CTX-M-1</sub> element was inserted into the *shufflon*. The ST7 *bla*<sub>CTX-M-1</sub> and IC *bla*<sub>CTX-M-1</sub> plasmids harbored a second accessory module associated with a class 1 integron, including *dfrA1*, *aadA1*, *qacEΔ*, and *sul1* genes.

The plasmid obtained from NRS27 (ST35 *bla*<sub>CTX-M-1</sub>) was highly homologous to pEK204 (44). Instead of the *bla*<sub>CTX-M-3</sub> and the *bla*<sub>TEM-1</sub> genes present in pEK204, the ST35 *bla*<sub>CTX-M-1</sub> plasmid contained a *bla*<sub>CTX-M-1</sub> gene and a *bla*<sub>TEM-33</sub> gene. In pEK204, the *ISEcp1-bla*<sub>CTX-M-3</sub> element and the *bla*<sub>TEM-1</sub> gene were both linked to the Tn3 elements and were located close to the replication functions (44). In contrast, in the ST35 *bla*<sub>CTX-M-1</sub> plasmid, the *ISEcp1-bla*<sub>CTX-M-1</sub> element was located in the transfer region between the genes encoding Pilf and TraC.

The ST31 *bla*<sub>CTX-M-15</sub> plasmids were highly homologous to pEC-Bactec, originally obtained from an *E. coli* isolate from a horse (37). As in pEC-Bactec, the ST31 *bla*<sub>CTX-M-15</sub> plasmids carried an *ISEcp1-bla*<sub>CTX-M-15</sub> element linked to Tn3 elements as well as to a *bla*<sub>TEM-1</sub> gene. The ST37 *bla*<sub>CTX-M-15</sub> plasmid showed a high level of identity to R621a (36), which was originally isolated from *Salmonella enterica* serovar Typhimurium and classified as belonging to incompatibility group I $\gamma$ . The ST37 *bla*<sub>CTX-M-15</sub> plas-

mid lacked the Tn10-like and IS2 elements present in R621a and had an accessory element containing the *bla*<sub>CTX-M-15</sub> and *bla*<sub>TEM-1</sub> resistance genes instead. CC5 *bla*<sub>TEM-52</sub> plasmids formed a distinct clade and did not show a high level of similarity to any IncI1-I $\gamma$  plasmids sequenced previously. The resistance-associated module contained the *bla*<sub>TEM-52</sub> gene linked to Tn3 elements. ST12 *bla*<sub>CMY-2</sub> plasmids carried the *bla*<sub>CMY-2</sub> gene associated with an *ISEcp1* element and the *blc* and *sugE* genes, as previously described for pCVM29188-101 (47). Highly similar resistance elements were previously identified in *Salmonella* and other *Enterobacteriaceae* (48), supporting the concept of a common resistance gene pool available to various bacterial communities (47, 49). The accessory module of the ST3 *bla*<sub>SHV-12</sub> plasmid was very similar to the accessory module of the IncI1-I $\gamma$  plasmid pND11-107 (14) but contained an additional *bla*<sub>SHV-12</sub> gene associated with a Tn1722 element (50). The rarely detected IncI1-I $\gamma$  plasmids not associated with ESBL/pAmpC genes established a distinct clade with the plasmids pSD107 (51), TY474p2 (CP002489) (52), and pND11-107 (14). The resistance regions of these plasmids differed from those of the plasmids in other clades with respect to the sizes and compositions of the incorporated resistance genes. In addition to the antimicrobial resistance genes, some of the rarely detected IncI1-I $\gamma$  plasmids also contained genes putatively providing protection against mercury.

**Concluding remarks.** All of the epidemic IncI1-I $\gamma$  plasmids

**FIG 3** Relationship of sequenced IncI1-I $\gamma$  plasmids. (A) Thirty-two IncI1-I $\gamma$  plasmid sequences were hierarchically clustered and are displayed as a phenogram using the BioNJ algorithm, in which the underlying distance matrix was obtained from the pairwise comparison of nonoverlapping maximal unique matches. Clades (tree groups as defined by MLST data) are indicated by colored clouds. Reference plasmids R64, R621a, pEK204, pEC-Bactec, pND11-107, and pColIb-P9 were included for comparison. (B) Randomly selected IncI1-I $\gamma$  plasmids from individual clades were hierarchically clustered with their plasmid backbones (lacking the antibiotic resistance cassettes), indicated as the ESBL number followed by “-mask.”



studied encoded ESBLs or pAmpC enzymes and differed mainly by the presence/absence of genes contributing to partitioning systems and addiction systems, which contribute to stable inheritance during cell division and plasmid maintenance. The sequence variation identified in *traY* and *excA* genes is expected to represent the divide between the IncI1 and IncI $\gamma$  incompatibility groups, and functional analysis is under way to be able to explain what roles these genes have played in the evolution of the IncI1-I $\gamma$  plasmids. In addition to plasmid-encoded factors, undefined host strain-encoded factors are also likely to contribute to the successful dissemination of these epidemic IncI1-I $\gamma$  plasmid lineages.

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