Chromosomal Integration of the Extended-Spectrum β-Lactamase Gene \( \text{bla}_{\text{CTX-M-15}} \) in Salmonella enterica Serotype Concord Isolates from Internationally Adopted Children\(^7\)

Laëtitia Fabre,1† Aurélie Delauné,1† Emmanuelle Espié,2 Karin Nygard,3 Maria Pardos,1 Lucette Polomack,1 Françoise Guesnier,1 Marc Galimand,4 Jörgen Lassen,3 and François-Xavier Weill1*  

Institut Pasteur, Centre National de Référence des Salmonella, Laboratoire des Bactéries Pathogènes Entériques, Paris, France; Institut de Veille Sanitaire, Saint-Maurice, France; Norwegian Institute of Public Health, Division of Infectious Disease Control, Oslo, Norway; and Institut Pasteur, Unité des Agents Antibactériens, Paris, France

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We report the emergence of Salmonella enterica isolates of serotype Concord (and its monophasic variant 6,7,1,114v:-) producing the extended-spectrum β-lactamases (ESBLs) SHV-12 and CTX-M-15 in France and Norway between 2001 and 2006 (43 in France and 26 in Norway). The majority of these isolates were from adopted children from Ethiopia, most of whom were healthy carriers. Several symptomatic secondary cases were found in the adoptive families and health care facilities in France. Serotype Concord isolates collected before 2003 produced SHV-12 encoded on a 340-kb conjugative plasmid of replicon IncI1. Isolates collected after 2003 produced CTX-M-15. We detected two conjugative plasmids carrying \( \text{bla}_{\text{CTX-M-15}} \). One plasmid, approximately 300 kb in size, was positive for the IncHI2 replicon and the plasmid-mediated quinolone resistance gene \( \text{qnrA}1 \). The other plasmid, from one of the earliest CTX-M-15-producing isolates collected, was a fusion plasmid with IncY and IncA/C2 replicons and was 200 kb in size. However, we showed, using Southern hybridization of I-CeuI-digested chromosomal DNA and S1 nuclease analysis of plasmid DNA, that most isolates had a \( \text{bla}_{\text{CTX-M-15}} \) gene located on chromosomal DNA. Analysis of the flanking regions of the chromosomally located \( \text{bla}_{\text{CTX-M-15}} \) gene by cloning revealed an \( \text{ISEcp1} \) truncated by an intact IS26 upstream from the \( \text{bla}_{\text{CTX-M-15}} \) gene and a truncated orf477 gene downstream from \( \text{bla}_{\text{CTX-M-15}} \). We found regions beyond the IS26 and the orf477 genes that were derived from IncA/C2 plasmids, suggesting the chromosomal integration of part of the \( \text{bla}_{\text{CTX-M-15}} \)-carrying IncY and IncA/C2 fusion plasmid from early CTX-M-15-producing isolates.

Extended-spectrum cephalosporins (ESC) are the drugs of choice in children requiring effective chemotherapy for non-typhoidal salmonellosis. Indeed, fluoroquinolones are contraindicated for use in children, and Salmonella isolates resistant to classical first-line antibiotics, such as aminopenicillins and cotrimoxazole, have emerged over recent decades. The emergence of Salmonella isolates resistant to ESC is a new public health concern (1). Resistance to these drugs is mainly mediated by the bacterial production of β-lactamases that degrade ESC. Two main classes of plasmid β-lactamases that inactivate ESC have been identified in Salmonella: the Ambler class A extended-spectrum β-lactamases (ESBLs), the most prevalent class in the genus, and the Ambler class C cephamycines. Most ESBLs belong to three families, TEM, SHV, and CTX-M (2). Over the last decade, CTX-Ms have been described in many species (including several Salmonella serotypes) and plasmids from nosocomial and community settings and have become the most prevalent family of ESBLs globally (7). There are currently at least 40 enzymes (a dozen of which have been identified from Salmonella isolates) divided into five major phylogenetic groups (1, 5). CTX-Ms (at least four groups) appear to be derived from Kluyvera chromosomal β-lactamases from the environment, in particular, Kluyvera ascorbata and Kluyvera georgiana (23, 30, 31, 32). Various insertion sequences, such as \( \text{ISEcp1} \), or putative transposases, such as CR1 (for common region 1; formerly orf513), are involved in the mobilization of \( \text{bla}_{\text{CTX-M}} \) genes.

The number of records of ESC-resistant human isolates of S. enterica serotype Concord has been increasing in France since 2004 and in Norway since 2001. Concord is a very rare serotype with the antigenic formula 6,7,1,114v:1,2. It was identified for the first time in 1944 from four cultures, three of which were isolated from fatal infections in chicks in the United States and one from the stools of a patient affected in a small outbreak of food poisoning in England (14). In the early 1980s, Concord was isolated in chicken-breeding farms in Saudi Arabia (2). Two sporadic ESBL-producing isolates of serotype Concord were reported in recent studies. One, containing \( \text{bla}_{\text{SHV-12}} \), was isolated in Holland in 2001 (21). The other, a CTX-M-15-producing isolate, was isolated in Ireland in 2005 in a patient originally from Ethiopia (28).

We studied the French and a selection of the Norwegian S. enterica serotype Concord isolates (i) to determine the genomic diversity of these isolates by standardized pulsed-field gel electrophoresis (PFGE), (ii) to determine the genetic basis...
for antibiotic resistance, and (iii) to briefly describe the epidemiology of infections with the organisms.

### MATERIALS AND METHODS

**Salmonella isolates.** A total of 146,160 Salmonella clinical isolates were registered at the French National Reference Centre for Salmonella (FNRC-Salm), Institut Pasteur, Paris, France, between January 1996 and December 2006. The FNRC-Salm network is based on approximately 1,400 voluntary hospital or private clinical laboratories (approximately 30% of all French clinical laboratories) and covers a population of approximately 23.7 million people (39.4% of the French population based on the 1999 census). During this 10-year period, 54 serotype Concord isolates (0.04%) isolated from 54 different cases were identified (Table 1). One isolate was not recovered in subculture, and consequently, 53 serotype Concord isolates were included in this study. We also studied seven isolates with the antigenic formula 6,7:1,v:- (probable monophasic variants of serotype Concord) received at the FNRC-Salm between 2004 and 2006.

In Norway, 16,849 cases of Salmonella infections were reported to the Norwegian Surveillance System for Communicable Diseases at the Norwegian Institute of Public Health, Oslo, Norway, between January 1996 and December 2006. About 80% of the case patients had acquired the infection abroad. All clinical Salmonella isolates were submitted to the Norwegian National Reference Laboratory for Bacterial Enteropathogens at the Norwegian Institute of Public Health for verification and characterization. During this period, 27 serotype Concord isolates (0.16%), corresponding to 27 cases, were identified. Eight isolates, selected on the basis of their diversity...
year of isolation and resistance phenotype), were sent to the FRNRC-Salm and fully characterized (Table 1).

The S. enterica serotype Concord reference strain 156K (isolated from a chick in 1944 in California), obtained from the World Health Organization Collaborative Centre for Reference and Research on Salmonella, Institut Pasteur, was used for molecular typing methods. S. enterica serotype Braenderup H9812 was used as a molecular size marker for PFGE.

EPIDEMIOLOGICAL INVESTIGATIONS. Demographical, clinical, and epidemiological data were collected for each case identified between 2004 and 2006 in France by contacting physicians by mail. Eight associations certified by the French Government for international adoption from Ethiopia were contacted by e-mail. In Norway, it is recommended, but not required, that all adopted infants be screened for intestinal parasites and Salmonella infection. However, the extent to which this recommendation is followed is unclear. Clinicians and microbiological laboratories report all laboratory-confirmed Salmonella infections to the Norwegian Surveillance System for Communicable Diseases. Demographical, clinical, and epidemiological information is collected on the clinical notification forms. The Norwegian association responsible for adoption from Ethiopia was informed.

Antimicrobial susceptibility. Antibiotic susceptibility was determined by the disk diffusion method with 32 antimicrobial drugs (Bio-Rad, Marnes la Coquette, France), as previously described (35). The MICs of ceftriaxone (CRO) and ceftazidime (CAZ) were determined by Etest (AB Biodisk, Solna, Sweden). The ESBL phenotype was detected by using the ESBL detection Etest strips (AB Biodisk) and the double-disk synergy method (25). Isolates were categorized as susceptible, intermediate, or resistant according to Antibiotic Committee of the French Society for Microbiology cutoff values (http://www.sfm.asso.fr/nouveautes/). The cutoff values used for CRO and CAZ are slightly different from those determined by the Clinical and Laboratory Standards Institute (CLSI) susceptibility strains were thus defined by MICs of ≤4 μg/ml (CLSI ≤8 μg/ml) and resistant strains by MIC of >32 μg/ml (CLSI >64 μg/ml for CRO and >32 μg/ml for CAZ).

PCR amplification of antimicrobial resistance genes and sequence analysis. Total DNA was extracted using the InstaGene matrix kit (Bio-Rad) according to the manufacturer’s recommendations. The resistance genes bla

CTXM,  

bla

SHV,  

bla

TEM,  

ble

CTX-M,  

ble

SHV,  

ble

TEM, and group I and class 1 integron cassette were amplified by PCR as described previously (15, 35). Sequencing was performed at Genome Express (Meylan, France) or at the Plateforme de Génotypage des Pathogènes et Santé Publique, P8 (Institut Pasteur). The nucleotide sequences and the deduced protein sequences were analyzed with EditSeq and Megalign software (DNASTAR, Madison, WI). The BLASTN program of NCBI was used for database searches (http://www.ncbi.nlm.nih.gov/BLAST/).

PFGE typing. The genetic diversity of 68 clinical Salmonella isolates of serotype Concord or the monophasic variant 6,7,15:- (60 from France and 8 from Norway) and serotype Concord reference strain 156K was assessed by PFGE of genomic DNA digested with XbaI (Roche, Mannheim, Germany), as described previously (34). The running conditions and the molecular size marker were as described in the standardized PulseNet protocol (24). BioNumerics 4.0 (Applied Maths, Sint-Martens-Latem, Belgium) was used for image normalization and construction of similarity matrices. Bands were manually assigned. Clustering was carried out by the unweighted pair-group method with arithmetic averages based on the Dice similarity index, using a 1% optimization parameter and 1% band position tolerance. Each profile that differed by one or more extra bands of >100 kb in size was assigned a type (e.g., type X10). Profiles that differed only by the position of a high-molecular-weight band (400- to 600-kb region) were assigned to subtypes (e.g., subtypes X10a, X10b, and X10c).

Resistance transfer determination. We carried out a resistance transfer experiment using a subset of 12 (8 French and 4 Norwegian) ESBL-producing isolates on liquid and solid media, using either Escherichia coli K-12 BM12 resistant to sodium azide or E. coli C1a resistant to nalidixic acid (NAL) as the recipient strain (35). Transconjugants were selected on Drigalski agar (Bio-Rad) supplemented with cefotaxime (CTX) (4 μg/ml) and sodium azide (500 μg/ml) or NAL (64 μg/ml). Three E. coli transconjugants were arbitrarily selected for each experiment.

Plasmid DNA from four S. enterica serotype Concord isolates that was unsuccessfuly transferred by conjugation was used to transform electocompetent E. coli DH10B by standard electroporation techniques with a MicroPulser electroporation apparatus (Bio-Rad). Transformsants were selected on Mueller-Hinton agar containing CTX (4 μg/ml).

Plasmid analysis. Plasmids were characterized for a subset of 19 Salmonella isolates (and four E. coli transconjugants) according to the year of isolation, antimicrobial resistance phenotype, and PFGE profile. Two plasmid-profiling methods were used: alkaline lysis and S1 nuclelease analysis. Plasmid DNA extracted by alkaline lysis (33) was analyzed by electrophoresis in 0.8% agarose gels. The molecular sizes of plasmids were estimated by comparison with plasmids of known sizes: pPF173 (125.8 kb), RP4 (56 kb), and P1p16 (210 kb), mixed with supercoiled DNA ladder (Invitrogen, Groningen, The Netherlands).

We used S1 nuclelease treatment and PFGE to accurately determine the molecular sizes of large bacterial plasmids. Proteinase K-treated gel plugs prepared for PFGE analysis were cut into 1-mm slices and digested with 1 U of S1 nuclease (Roche) as described previously (3). DNA fragments separated by PFGE were transferred onto a nylon membrane (Hybond N+; Amersham) and hybridized with bla

CTXM, bla

SHV, Inca/C, and Inca probes (8, 15). Hybridization, labeling, and detection were performed according to the manufacturers’ recommendations, using either an enhanced-chemiluminescence nonradioactive kit (GE Healthcare, United Kingdom) or a radioactive kit (MegaprimeTM DNA-labeling system; GE Healthcare).

PCR-based replica-typing analysis was performed as described by Carattoli et al. (8). The 18 primer pairs targeting FIA, FIB, FIC, H11, H12, H1-Fy, LM, N, P, T, A/C, K, B/O, X, Y and FII replicons were used in separate PCRs.

Chromosomal localization of the bla

CTXM gene by PFGE-I-CEuI. To determine the chromosomal localization of the bla

CTXM gene, plugs of 11 isolates were prepared as described above and digested with the I-CEuI endonuclease (New England Biolabs). The digested fragments were separated using the CHEF DRIII system as described previously (27). The sizes of I-CEuI restriction fragments were determined using known I-CEuI fragment sizes of chromosomal DNA of E. coli DH122 (27) and XbaI-restricted Typhimurium LT2 (27) and XbaI-restricted fragments from the chromosomal DNA of S. enterica serotype Braenderup H9812. The I-CEuI restriction fragments were subjected to Southern hybridization with PCR-generated probes for bla

CTXM and bla

SHV and 16S rRNA gene probes (15, 20).

Analysis of the genetic environment of the bla

CTXM gene by cloning. We used cloning techniques to determine the DNA sequences flanking the bla

CTXM gene in isolate 65-0004 (a representative Concord isolate with a chromosomal bla

CTXM gene, as determined by PFGE I-CEuI and S1 nuclease experiments). DNA prepared using the Promega Wizard kit was partially digested with Sau3AI, purified with Qiaquick PCR purification kits (Qiagen, Courtaboeuf, France), and ligated into dephosphorylated BamHI-restricted phagemid pBK-CMV (Roche, Meylan, France). Recombinant plasmids were introduced into E. coli DH10B by electroporation (Gene Pulser II; Bio-Rad). Antibiotic-resistant colonies were selected on Luria-Bertani agar containing kanamycin (KAN) (30 μg/ml) and CRO (4 μg/ml). Recombinant plasmid DNA was recovered using Qiaprep spin miniprep columns (Qiagen).

The following primers were used for PCR mapping: CP604A, 5'-GGGTTAT TTACCGAGATGGCAGC-3' (nucleotides [nt] 15721 to 15723); pSN254 of S. enterica serotype Newport SL254; GenBank accession number CP000604, 15'-GAATTTCTCGGGTGACTTCCT-3' (nt 12446 to 12466; accession number CP000604); and CP6041200, 5'-TGTGGAATCTCGGTGGTATG-3' (nt 12000 to 12020; accession number CP000604).

Nucleotide sequence accession number. The nucleotide sequence of the class 1 integron cassette qacH-aadA1 was assigned GenBank accession number EU200458.

RESULTS AND DISCUSSION

EPIDEMIOLOGICAL BACKGROUND. Serotype Concord was rare in Norway before 2000 and in France before 2004 (Fig. 1A and B).

In France, of the 14 serotype Concord isolates collected before 2004, 13 were tested (1 was not viable), and all were pan susceptible. In two cases, the subjects had traveled to (Madagascar or Ethiopia) before becoming ill. No epidemiological information was available for the remaining 12 cases. Among isolates collected between 2004 and 2006, 36/40 were multidrug resistant (MDR) with resistance to ESC (Table 1). The seven monophasic isolates were also MDR with resistance to ESC. Most of the 43 ESC-resistant Salmonella (Concord and monophasic) isolates were recovered from children aged <1 year (34/43) or between 1 and 4 years (6/43). Three cases

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were adults (36, 36, and 93 years old). Epidemiological data were available for 35 of the 43 cases. Of the 35 cases, 28 were children recently adopted from Ethiopia. The seven remaining cases were probable or possible secondary cases in the household or in a health care setting. Clinical data were available for 22 adopted children and six French autochthonous cases. For the adopted children, clinical symptoms were absent in 12, whereas bronchopulmonary infections were seen in 8, pyelonephritis (due to E. coli) in 1, and gastroenteritis in 1. For the French autochthonous cases, five had gastroenteritis and one died.

In Norway, the single serotype Concord isolate collected between 1996 and 2000 (acquired after travel to Eritrea) was susceptible to all antibiotics tested. Between 2000 and 2006, 27 Concord isolates were registered. All but one isolate were from children adopted from Ethiopia. Twenty-three of the 27 Concord isolates were MDR with resistance to ESC (not shown and Table 1). The first ESBL-positive isolate was registered in 2000 in an adopted child from Ethiopia.

We evaluated the prevalence of carriers using data obtained from the systematic initial screening of children arriving in France (clinical examination, stool bacteriology and parasitology, and viral serologies). To this end, a French-certified association contacted all 187 families that adopted children from Ethiopia through the association between January 2005 and August 2006. One hundred and four families participated, and Table 1). The first ESBL-positive isolate was registered in 2000 in an adopted child from Ethiopia.

infections. The strain may have then been disseminated due to the promiscuity between infants and perhaps through the hands of the staff, thus explaining the long-lasting circulation of the strain. Overcrowding and understaffing are risk factors commonly observed in neonatal units in developing countries, for example, in Tunisia in 1988 (serotype Wien producing SHV-2) (19) and in 2002 (serotype Livingstone producing CTX-M-27) (6). However, the source of this strain in Ethiopia has yet to be investigated. As it was very difficult to prevent transmission in Ethiopia, we focused on preventing dissemination of the strain in France by informing all certified associations about the risk of transmission of resistant Salmonella and the adoptive families about the importance of basic hygiene practice. In 2007, the number of ESBL-producing Salmonella isolates in adopted children from Ethiopia was decreasing in France, with only 13 isolates, and no secondary cases, detected at the FRNC-Salm. In Norway, the adoption agency changed transit home in 2005, and in 2006, no new cases were reported.

Antimicrobial resistance phenotypes and genes and molecular typing. The antimicrobial resistance phenotypes of the 60 French isolates are described in Table 1. Thirty-six MDR serotype Concord and seven MDR monophasic isolates resistant to ESC contained the bla_{CTX-M-15} ESBL gene. The MICs of CRO, CAZ, and cefepime (FEP) were >256 μg/ml. Of the 43 ESBL-producing isolates, 38 were positive for the penicillinase bla_{TEM-1} gene and none were positive for the penicillinase bla_{OXA-1} group gene. All but three MDR strains harbored a class 1 integron containing a novel qacH-aadA1 gene cassette. One isolate harbored an additional class 1 integron containing a dfrA7 gene cassette. Three CTX-M-15-producing isolates with decreased susceptibility to ciprofloxacin (0.25 to 0.5 μg/ml) were found to contain the plasmid-mediated quinolone resistance gene qnrA1. One of these isolates, 05-3728, was studied previously (11).

The resistance profiles of the eight selected Norwegian isolates are described in Table 1. All of the isolates produced an ESBL, with the exception of one, which was positive for the penicillinase bla_{TEM-1} gene. We identified bla_{SHV-12} in five ESBL isolates collected before 2004 and bla_{CTX-M-15} in the remaining two ESBL isolates collected from 2004 to 2005. The MICs of CRO, CAZ, and FEP were >256 μg/ml for the CTX-M-15-producing isolates. The SHV-12-producing isolates had CAZ MICs of >256 μg/ml, CRO MICs of 64 μg/ml, and FEP MICs of 8 to 16 μg/ml. Several different class 1 integron gene cassettes were identified (Table 1).

The clonal relatedness of S. enterica serotype Concord and monophasic 6,7,14-,: isolates was assessed by standard PFGE analysis of XbaI-digested chromosomal DNA (Fig. 2 and Table 1). Twenty different PFGE profiles were obtained from 68 clinical isolates and the reference strain. All ESBL-producing isolates clustered together (Dice correlation coefficient, 72%). SHV-12-producing isolates displayed the same profile, X10 (divided into three subtypes), distinct from the eight obtained from CTX-M-15-producing isolates. Among the CTX-M-15-producing isolates, X12 was predominant (n = 20). This profile was close to other CTX-M-15 isolate profiles, X11 and X13 to X16. However, two profiles, X17 and X18, differed more significantly. Profile X17 was observed in the three isolates containing the qnrA1 gene, and profile X18 was observed in four isolates displaying additional resistance to KAN. The two other
clusters contained 15 pansusceptible isolates. The profiles obtained from the seven monophasic isolates were X11c (n = 1), X12 (n = 2), X15 (n = 3), and X16 (n = 1), suggesting that they were derived from the Concord serotype.

Two types of ESBL were identified in the Concord strains from Ethiopia: the \( \text{bla}_{\text{SHV-12}} \) gene was observed between 2000 and 2003 and the \( \text{bla}_{\text{CTX-M-15}} \) gene between 2004 and 2006. In France, between 2001 and 2003, there was an outbreak in infants adopted from Mali caused by \( S. \text{enterica} \) serotypes Bablsberg and Enteritidis producing SHV-12 (35). The shift in the ESBL distribution (i.e., CTX-Ms, in particular CTX-M-15 replacing SHV-12) is consistent with the epidemiology of the ESBLs in the genus \( S. \text{Salmonella} \) reported in African countries, such as Senegal (17, 36), Tanzania (4), and South Africa (18, 26).

**Transferability and location of the ESBL genes.** Conjugation experiments were carried out on liquid and solid media with a subset of 12 ESBL-producing isolates selected based on the year and country of isolation, the antibiotic resistance phenotype, and the PFGE type. ESC transfer was successful only for the two Norwegian SHV-12-producing isolates tested and two French CTX-M-15-producing isolates (Table 2).

The \( \text{bla}_{\text{SHV-12}} \) genes were carried by similar plasmids approximately 340 kb in size and belonging to the IncI1 group, as determined by PCR-based replicon typing, whereas the plasmids carrying the \( \text{bla}_{\text{CTX-M-15}} \) gene were of different types (Table 2). One belonged to IncHI2, while the other was positive for both IncY and IncA/C\(_2\) replicons. A positive hybridization of the S1 nuclease-treated plasmid 200-kb band by both IncY and IncA/C\(_2\) probes confirmed the double-replicon structure (data not shown).

A large plasmid of approximately 300 kb that did not hybridize to the \( \text{bla}_{\text{CTX-M}} \) Probe, but which did hybridize to a \( \text{bla}_{\text{TEM}} \) probe, was present in all CTX-M-15-producing \( S. \text{Salmonella} \) isolates, with the exception of isolate 04-347 and the two monophasic isolates tested. This plasmid was also absent in the

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**FIG. 2.** Representative Xbal-PFGE profiles of \( S. \text{enterica} \) serotype Concord isolates studied. A dendrogram was generated with Bionumerics software. The PFGE profile, number of isolates, and resistance type are indicated.
single susceptible isolate tested (data not shown). Although the Norwegian isolate 07-699 did not undergo transfer of ESC resistance to \textit{E. coli}, it contained at least two plasmids, one of approximately 250 kb that hybridized to the \textit{bla} \textit{CTX-M-15} probe and one of approximately 140 kb that hybridized to an IncA/C probe (data not shown).

Two French isolates resistant to KAN, 06-8636 and 06-8404, which did not undergo transfer of ESC resistance, were found to contain a large plasmid of approximately 320 kb that did hybridize to the \textit{bla} \textit{CTX-M-15} probe (data not shown). PCR typing targeting IncA/C and IncY replicons was performed in all other \textit{Salmonella} isolates. An IncA/C replicon was found only in isolate 07-699, whereas the IncY replicon was found in all isolates except 06-8404 and 07-699, and the susceptible isolates tested. S1 nuclease and Southern blotting showed that the IncY plasmid in \textit{Salmonella} isolates (except that of 04-347) was approximately 100 kb in size (data not shown). In a previous study, two different replicons were associated with \textit{bla} \textit{CTX-M-15}-positive plasmids from \textit{Salmonella} and \textit{E. coli} isolates collected between 2001 and 2003 from the United Kingdom, Honduras, and Pakistan (22). Nine of 22 plasmids were positive for the IncI1 replicon, and 12 of 22 plasmids were positive for IncFII (in some strains associated with IncFIA and/or IncFIB replicons in fusion plasmids).

The fact that the ESC resistance transfer experiments were unsuccessful for the majority of the isolates, with no hybridization of their plasmid DNAs to a \textit{bla} \textit{CTX-M-15} gene probe, suggested a chromosomal location of the \textit{bla} \textit{CTX-M-15} gene in these isolates. This hypothesis was confirmed by I-CeuI PFGE, which clearly showed that the \textit{bla} \textit{CTX-M-15} gene was located in a chromosomal fragment of approximately 900 kb (Fig. 3). This 900-kb fragment hybridizing to the 16S rRNA gene probe was absent in isolates 05-5343 and 04-347 (carrying \textit{bla} \textit{CTX-M-15} on a plasmid), 06-8636 (an isolate additionally resistant to KAN), and 07-699 (a Norwegian isolate) and in susceptible isolates (Fig. 3 and data not shown). These isolates that did not have the 900-kb fragment displayed increased intensity of the band at 700 kb, suggesting that two DNA fragments comigrated at this position, as observed for 779-kb and 738-kb fragments of \textit{S. enterica} serotype Typhimurium strain LT2 (27). Because the endonuclease I-CeuI cleaves only \textit{rrn} operons and because the number (\(n = 7\)) and locations of the \textit{rrn} genes are highly conserved in \textit{Salmonella} (27), this ~900-kb chromosomal fragment was interpreted to derive from one of the two

### Table 2. Cefotaxime resistance transfer experiments

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Yr/country of isolation</th>
<th>Antimicrobial resistance pattern(^a)</th>
<th>ESBL gene</th>
<th>PFGE type</th>
<th>Transfer of resistance to ESC by:</th>
<th>Transferred resistance traits(^a)</th>
<th>Plasmids carrying ESBL genes in transconjugants (size [kb], replicon type)</th>
</tr>
</thead>
<tbody>
<tr>
<td>04-347</td>
<td>2004/France</td>
<td>AMX CRO CAZ STR SPT GEN SXT CHL TET</td>
<td>\textit{bla} \textit{CTX-M-15}</td>
<td>X11a</td>
<td>Yes</td>
<td>AMX CRO CAZ STR SXT CHL TET</td>
<td>200, IncY/IncA/C(_2)</td>
</tr>
<tr>
<td>04-7498</td>
<td>2004/France</td>
<td>AMX CRO CAZ STR SPT GEN SXT CHL TET</td>
<td>\textit{bla} \textit{CTX-M-15}</td>
<td>X12</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>04-8041</td>
<td>2004/France</td>
<td>AMX CRO CAZ STR SPT GEN SXT CHL TET</td>
<td>\textit{bla} \textit{CTX-M-15}</td>
<td>X12</td>
<td>No</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>05-0004</td>
<td>2005/France</td>
<td>AMX CRO CAZ STR SPT GEN SUL TET</td>
<td>\textit{bla} \textit{CTX-M-15}</td>
<td>X11b</td>
<td>No</td>
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<td></td>
</tr>
<tr>
<td>05-2657</td>
<td>2005/France</td>
<td>AMX CRO CAZ STR SPT GEN SUL CHL TET</td>
<td>\textit{bla} \textit{CTX-M-15}</td>
<td>X11b</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>05-5343</td>
<td>2005/France</td>
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<td>\textit{bla} \textit{CTX-M-15}</td>
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<td>Yes</td>
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<tr>
<td>06-8636</td>
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<td>\textit{bla} \textit{CTX-M-15}</td>
<td>X18</td>
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<td>No</td>
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<tr>
<td>06-8404</td>
<td>2006/France</td>
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<td>X18</td>
<td>No</td>
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<td>07-690</td>
<td>2001/Norway</td>
<td>AMX CRO CAZ SXT CHL</td>
<td>\textit{bla} \textit{SHV-12}</td>
<td>X10c</td>
<td>Yes</td>
<td>AMX CRO CAZ</td>
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<td>07-693</td>
<td>2002/Norway</td>
<td>AMX CRO CAZ SXT CHL</td>
<td>\textit{bla} \textit{SHV-12}</td>
<td>X10c</td>
<td>Yes</td>
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<td>340, IncI1</td>
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<td>07-698</td>
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<td>X11c</td>
<td>No</td>
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<td>2005/Norway</td>
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<td>No</td>
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\(^a\) AMX, amoxicillin; STR, streptomycin; SPT, spectinomycin; GEN, gentamicin; CHL, chloramphenicol; SUL, sulfonamides; SXT, sulfamethoxazole-trimethoprim; TET, tetracycline.
DNA chromosomal fragments contained in the \(-700\)-kb band by insertion of the \(bla_{\text{CTX-M-15}}\) gene and additional DNA. The \(bla_{\text{TEM}}\) gene was not detected on chromosomal fragments (data not shown). Chromosomal location of ESBL genes in enterobacteria is extremely rare, with only two reports showing chromosomal integration of \(bla_{\text{CTX-M-15}}\) in \(E. coli\) (12, 16).

**Genetic context of the \(bla_{\text{CTX-M-15}}\) gene.** To study the flanking DNA sequences of the chromosomal \(bla_{\text{CTX-M-15}}\) gene, we cloned the gene from isolate 05-0004. This representative isolate contained the \(bla_{\text{CTX-M-15}}\) gene on the 900-kb chromosomal I-CeuI fragment (data not shown). We obtained 31 \(E. coli\) DH10B recombinant clones. Seven of these were tested, and the longest recombinant plasmid, p05-0004-C1 (9 kb), was retained for further analysis. The schematic representation of recombinant plasmid p05-0004-C1 is shown in Fig. 4B. Sequence analysis identified the insertion sequence \(\text{ISEcp}1\) 48 bp upstream from, and in the same orientation as, the \(bla_{\text{CTX-M-15}}\) gene (identical to accession number AY604722). \(\text{ISEcp}1\) was truncated at nucleotide position 1102 by an intact IS26, both in the same orientation. \(orf477\) (identical to accession number AY604722) was located downstream from the \(bla_{\text{CTX-M-15}}\) gene in the opposite orientation. This open reading frame was truncated at amino acid position 70, corresponding to the end of an 18-bp sequence homologous to the inverted repeat right (IRR) of \(\text{ISEcp}1\). We identified 1.9 kb and 4.3 kb of DNA sequence upstream from IS26 and downstream from \(orf477\), respectively, which shared 100% identity with sequences in three MDR IncA/C plasmids (GenBank accession numbers CP000602, CP000603, and CP000604). However, these sequences were located at two distant regions in the plasmids (Fig. 4B). One of these plasmids, pSN254 (accession number CP000604) (37), is the large MDR IncA/C\(_2\) plasmid carrying the cephamycinase \(bla_{\text{CMY-2}}\) gene that has been increasingly detected in \(S. enterica\) serotype Newport in the United States (9, 10, 13).

To verify the flanking structures of the region IS26-\(\text{ISEcp}1\)-\(bla_{\text{CTX-M-15}}\)-\(orf477\) on other isolates, we designed primers CP604Av and CP604Am to target pSN254 regions upstream of IS26 and downstream of \(orf477\), respectively (Fig. 4B). PCRs with CP604Av and CTX-M-R and with CP604Am and CTX-M-F were performed on a selection of 15 isolates (comprising 10 tested for resistance transfer and chromosomal or plasmid localization of \(bla_{\text{CTX-M-15}}\)). PCR products with the expected sizes of 2.2 kb and 1.2 kb were observed in all the isolates with a chromosomally located \(bla_{\text{CTX-M-15}}\) gene, whereas no amplification was observed for isolates 05-5343 (IncHI2 plasmid-located \(bla_{\text{CTX-M-15}}\) gene), 06-8636 (a representative 2006 French isolate resistant to KAN), and Norwegian isolate 07-699. However, an amplicon of 1.2 kb using CTX-M-F and CP604Am was obtained in the early French isolate 04-347 (IncA/C\(_2\)-IncY plasmid-located \(bla_{\text{CTX-M-15}}\) gene), 06-8636 (a representative 2006 French isolate resistant to KAN), and Norwegian isolate 07-699. However, an amplicon of 1.2 kb using CTX-M-F and CP604Am was obtained in the early French isolate 04-347 (IncA/C\(_2\)-IncY plasmid-located \(bla_{\text{CTX-M-15}}\) gene), 06-8636 (a representative 2006 French isolate resistant to KAN), and Norwegian isolate 07-699. However, an amplicon of 1.2 kb using CTX-M-F and CP604Am was obtained in the early French isolate 04-347 (IncA/C\(_2\)-IncY plasmid-located \(bla_{\text{CTX-M-15}}\) gene), and used it for PCR with CTX-M-R on it and its \(E. coli\) transconjugant. We obtained an amplicon of 2.8 kb. Sequencing analysis revealed that the complete \(\text{ISEcp}1\) was present and that the \(\text{ISEcp}1\)-\(bla_{\text{CTX-M-15}}\)-\(orf477\) region was flanked by 5-bp direct repeats (GACTA) and inserted within IncA/C\(_2\) plasmid pSN254 at nucleotide position 12411 (Fig. 4A). PCR with primers CP604-12000 and CP604Am was performed on isolates 06-8636 and 07-699. An amplicon of 450 bp was observed only in isolate 07-699, demonstrating that the \(bla_{\text{CTX-M-15}}\) gene was not inserted within the 12000-12200 region of pSN254. We did not further characterize the genetic environment of the \(bla_{\text{CTX-M-15}}\) genes located on nonconjugative plasmids in these rare isolates.

We found that DNA sequences from the pSN254 IncA/C\(_2\) plasmid were in the vicinity of the chromosomal \(bla_{\text{CTX-M-15}}\). One of our earliest Concord isolates (04-347), isolated in 2004, contained a \(bla_{\text{CTX-M-15}}\)-carrying fusion plasmid with replicon...
elements of types IncY (resident plasmid of MDR Concord) and IncA/C-2. The \( \text{bla}_{\text{CTX-M-15}} \) gene was inserted into this IncA/C-2 plasmid at position 12411 by ISEcp1. ISEcp1 was complete (1,656 nt), and 5-bp direct repeats flanked it upstream and downstream from an IRR-like sequence. In isolate 07-699, the \( \text{bla}_{\text{CTX-M-15}} \) gene was also found in an IncA/C-2 plasmid, but in a different position from that identified in isolate 04-347. ESC resistance in isolates 07-699 and 06-8636 did not appear to be transferable, despite the presence of large plasmids; this may have been due to the absence in these isolates of a helper IncY plasmid, which provides functions necessary for mobilization or allows the generation of an autotransferable fusion plasmid (as observed for isolate 04-347). Failure of the transfer of ESC resistance by electroporation might have resulted in a structure in which the two distant described elements of types IncY and IncA/C-2 were brought in proximity. We can also hypothesize that the whole, or part of, the IncA/C-2-IncY fusion plasmid contained in isolate 04-347 was integrated into the chromosome of other Concord-derived isolates collected later; this probably occurred through IS26-mediated cointegration. Naas et al. (29) demonstrated Tn1- or IS26-mediated integration of a plasmid-borne inhibitor-resistant TEM, \( \text{bla}_{\text{TEM-21}} \), from Proteus mirabilis into E. coli chromosomal DNA. In our study, sequencing the ends of the inserted DNA by genome walking will be necessary in order to elucidate the precise mechanism of integration (homologous recombination, IS- or transposon-mediated transposition, or cointegration).

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1816 FABRE ET AL.


