ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 2003, p. 2938–2945 Vol. 47, No. 9
0066-4804/03/$08.00+0 DOI: 10.1128/AAC.47.9.2938–2945.2003

Insertion Sequence IS*Ecp1B* Is Involved in Expression and Mobilization of a *bla*$_{\text{CTX-M}}$ β-Lactamase Gene

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Received 14 April 2003/Accepted 17 May 2003

The genetic structures (ca. 10-kb DNA fragment) surrounding the plasmid-borne extended-spectrum β-lactamase *bla*$_{\text{CTX-M-19}}$ gene in a *Klebsiella pneumoniae* clinical isolate were determined. This β-lactamase gene was part of a 4,797-bp transposon inserted inside *orf1* of Tn*1721*. Inside this transposon, *bla*$_{\text{CTX-M-19}}$ was bracketed upstream and downstream by insertion sequences IS*E cp1B* and IS*903D*, respectively, and further downstream by a truncated gene encoding an outer membrane protein for iron transport. The single-copy IS*Ecp1B* element was probably involved alone in the mobilization process that led to a 5-bp duplication at the target site of the transposed fragment. This mobilization event probably involved one inverted repeat of IS*E cp1B* and a second sequence farther away, resembling its second inverted repeat. Additionally, IS*Ecp1B* provided −35 and −10 promoter sequences, contributing to the high-level expression of the *bla*$_{\text{CTX-M-19}}$ gene. Southern blot analysis failed to identify a reservoir of IS*Ecp1*-like sequences among a series of gram-negative and gram-positive bacterial species usually found in the skin and intestinal human floras. The ability of IS*Ecp1*-like elements to mobilize and to promote the expression of β-lactamase genes may explain, in part, the current spread of CTX-M-type enzymes worldwide.

The clavulanic acid-inhibited Ambler class A extended-spectrum β-lactamases (ESBLs) of the CTX-M type are increasingly reported worldwide in members of the *Enterobacteriaceae* (2, 5, 9, 10, 16–18, 25, 39, 42). They may be classified according to their amino acid identity in four groups: CTX-M-1 (CTX-M-1, CTX-M-3, CTX-M-15, . . .), CTX-M-2 (CTX-M-2, CTX-M-4, . . .), CTX-M-8, and CTX-M-9 (CTX-M-9, CTX-M-14/-18, CTX-M-16, CTX-M-17, CTX-M-19, . . .) (42). It is known now that the progenitors of several plasmid-located CTX-M-type genes may be enterobacterial species such as *Kluyvera cryocrescens* (14), *Kluyvera georgiana* (36), and *Kluyvera ascorbata* (24), which are rarely a source of human infections and belong to the human intestinal flora. A recent spread of these plasmid-located ESBL genes may threaten further the clinical efficacy of expanded-spectrum cephalosporins and raises the question of which genetic background(s) may have contributed to their selection in recent years.

Although rare ESBL genes are associated with class 1 integrons (that enhance their expression [37, 38]), integron location of CTX-M genes has not been reported. Insertion sequences (IS) are also an important source of genetic plasticity in prokaryotes. In the case of β-lactamase genes, association with IS elements and transposons has been found mostly in *Enterobacteriaceae* and *Pseudomonas aeruginosa.* Tn*1*/Tn*3* and Tn*21* derivatives are associated with genes encoding narrow-spectrum β-lactamases such as TEM-1/TEM-2 and oxacillinases, respectively (4, 28) whereas IS*26* and IS*1*-like elements are associated with genes encoding the ESBLs SHV-2a, TEM-3, and TEM-6 (19, 33).

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We have published part of the sequence of an IS element (IS*Ecp1*-like element) that was associated with *bla*$_{\text{CTX-M-15}}$ identified in several enterobacterial isolates from India (27). Subsequently, several reports identified IS*Ecp1*-like sequences upstream of other *bla*$_{\text{CTX-M}}$-type genes (5, 9, 10). These results raise the question of the role of IS*Ecp1*-like sequences in the spread and expression of *bla*$_{\text{CTX-M}}$ genes.

Recently, we have reported another plasmid-borne CTX-M-type gene, *bla*$_{\text{CTX-M-19}}$, from a *Klebsiella pneumoniae* isolate from Vietnam (39). CTX-M-19 is a point mutant derivative of CTX-M-14/-18 that is able to hydrolyze ceftazidime to a significant degree (39). In the present study, we have investigated the genetic environment of the *bla*$_{\text{CTX-M-19}}$ gene and described the surrounding sequences that included two IS elements, IS*Ecp1B* and IS*903D.* One of these IS elements, IS*Ecp1B*, may have mobilized the β-lactamase gene through a transposition mechanism and may drive the expression of the β-lactamase at a high level. We failed to identify the reservoir of IS*Ecp1*-like elements among many gram-positive and gram-negative species screened.

MATERIALS AND METHODS

Bacterial strains, plasmids, and susceptibility testing. *K. pneumoniae* clinical isolate ILT-3, which produces CTX-M-19 β-lactamase, has been reported previously (39). *Escherichia coli* reference strains and phagemids used in this study are referenced in Table 1. Antibiotic-containing disks (Bio-Rad, Marnes-la-Coquette, France) on Mueller-Hinton agar plates were used for routine antibiograms (www.sfm.asso.fr). A series of bacterial species were screened for the presence of IS*Ecp1*-like sequences; they were representative (type strains in each case) of several bacterial species usually isolated from the skin and intestinal human flora. These include, among the *Enterobacteriaceae*, *Citrobacter freundii* (two strains), *Citrobacter koseri* (two strains), *Citrobacter braakii*, *Citrobacter werkmanii*, *Citrobacter murliniae*, *Citrobacter gillenii*, *Edwarsiella hoshinae*, *Erwinia carotovora*, *Erwinia amylovora*, *Erwinia chrysanthemi*, *Erwinia quecina*, *E. coli* (six strains), *Enterobacter cloacae*, *Enterobacter aerogenes*, *Enterobacter gergoviae*, *Klebsiella pneumoniae*, *Kluyvera ascorbata* (four strains), *Kluyvera cryocrescens* (two strains), *Pantoea* spp., *Proteus mirabilis*, *Proteus vulgaris*, *Proteus pen-*

TABLE 1. Reference strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype	Source or reference
Strains		
E. coli JM109	*endA1 gyrA96 hsdR17* Δ(*lac proA*) *relA recA1 supE44 thi* F (*lacI*^{q}ZΔM15 *proAB*$^+$ *traD36*)	Gibco BRL-Life Technologies
E. coli DH10B	*araD139* Δ(*ara leu*)7697 *deoR endA1 galK1 galU nupG recA1-rpsL* F′ *mrcA* Δ(*mrr-hsdRMS-mrcBC)*Φ80d*lacZ*ΔM15 Δ*lacX74*	Stratagene
Plasmids		
pPCRScript Amp SK(+)	Ampicillin resistant	Stratagene
pACYC184	Chloramphenicol resistant, low copy number	11
pBK-CMV	Kanamycin resistant, high copy number	Stratagene
pILT-3	Natural ca. 50-kb plasmid of *K. pneumoniae* ILT-3 producing CTX-M-19 β-lactamase	39
pA-1	Recombinant pPCR-Script Amp with a 6.5-kb PCR fragment from whole-cell DNA of *K. pneumoniae* ILT-3 containing IS*Ecp1B* and a truncated $bla_{CTX-M-19}$ gene	This study
pB-1	Recombinant pPCR-Script Cam with a 1.1-kb PCR fragment from whole-cell DNA of *K. pneumoniae* ILT-3 containing IS*Ecp1B* promoter sequences and the entire $bla_{CTX-M-19}$ gene	This study
pB-2	Recombinant pPCR-Script Cam with a 1-kb PCR fragment from whole-cell DNA of *K. pneumoniae* ILT-3 lacking IS*Ecp1B*-promoting sequences and containing the entire $bla_{CTX-M-19}$ gene	This study

neri, *Providencia stuartii*, *Rahnella aquatilis* (two strains), *Salmonella enterica* (four strains), *Serratia plymuthica*, *Serratia rubidaea*, *Serratia odorifera*, and *Shigella* spp. (nine strains); among the gram-negative rods, *Acinetobacter baumannii*, *Acinetobacter johnsonii*, *Aeromonas hydrophila*, *Alcaligenes xylosoxydans*, *Alcaligenes denitrificans*, *Bordetella bronchiseptica*, *Brevium diminuta*, *Comamonas acidovorans*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Pseudomonas putida*, and *Stenotrophomonas maltophilia*; among the gram-positive rods and anaerobes, *Bifidobacterium* spp., *Clostridium difficile* (two strains), *Clostridium perfringens* (two strains), and *Propionibacterium* spp.; among the gram-positive cocci and aerobes, *Enterococcus faecium* (three strains), *Enterococcus faecalis* (two strains), *Staphylococcus aureus* (two strains), coagulase-negative *Staphylococcus* spp. (three strains), *Streptococcus equinus*, *Streptococcus pneumoniae* (two strains), and viridans streptococci (two strains); and among anaerobic gram-positive cocci, *Peptostreptococcus* spp., and *Campylobacter* spp. (five strains).

Cloning experiments, recombinant plasmid analysis, and DNA sequencing. Whole-cell DNAs of *K. pneumoniae* ILT-3 and of bacterial strains tested in screening for IS*Ecp1*-like presence were extracted as described previously (38). Partially digested *Sau*3AI fragments of whole-cell DNA of *K. pneumoniae* ILT-3 were ligated into *Bam*HI-restricted phagemid pBK-CMV (Stratagene, Amsterdam, The Netherlands) (38). Ligation was performed at 4°C for 18 h at a 1:3 vector/insert ratio, at a final concentration of 1 μg of DNA in a ligation mixture containing 1 U of T4 DNA ligase. Recombinant plasmids were transformed by electroporation (Gene Pulser II; Bio-Rad, Ivry-sur-Seine, France) into electrocompetent *E. coli* DH10B cells.

Then, cloning experiments were performed with PCR-generated fragments, using a series of primers (Table 2) at the *Srf*I site of pPCRScript Amp SK(+) or at the *Sma*I site of the high-copy-number plasmid pBK-CMV or the *Eco*RV site of the low-copy-number vector pACYC184 (Tables 1 and 2). Long-range PCRs were performed under specific conditions (extension step, 72°C for 6 min; 6 U of *Taq* polymerase). Cloning of the INT2F/CTX-MB PCR fragment into pPCRScript Amp to identify integron features gave recombinant plasmid pA-1 (Fig. 1). Cloning of the PROM+/PRECTX-M3B and PROM−/PRECTX-M3B fragments containing the entire $bla_{CTX-M-19}$ gene with or without the IS*Ecp1B*-mediated promoter sequences into pACYC184 gave recombinant plasmids pB-1 and pB-2, respectively (Fig. 1). Antibiotic-resistant colonies were selected onto Trypticase soy agar plates containing 50 μg of amoxicillin per ml, except for *E. coli* harboring recombinant pA-1, which was subjected to a classical blue-white selection (β-galactosidase test). Recombinant plasmids were purified using Qiagen (Courtaboeuf, France) columns. Inserts of recombinant plasmids were sequenced on both strands by using an ABI 377 sequencer (Applied Biosystems, Foster City, Calif.). The nucleotide and the deduced protein sequences were analyzed with software available over the internet at the National Center of Biotechnology Information website (http://www.ncbi.nlm.nih.gov), and at the Institut Pasteur website (http://www.bioweb.pasteur.fr/seqanal/interfaces/clustalw-simple.html).

Hybridization experiments. A 527-bp PCR-obtained internal fragment of the IS*Ecp1B* transposase gene was generated using primers ISEcp1A and ISEcp1B, and a 472-bp PCR-obtained internal fragment of the IS*903D* transposase was generated with primers IS903A and IS903B (Fig. 1; Table 2) and with *Bam*HI- and *Pvu*I-restricted whole-cell DNA of *K. pneumoniae* ILT-3 and *E. coli* recom-

TABLE 2. Sequences of primers used in this study

Primer	Sequence (5′→3′)	Location	No. as shown in Fig. 1
ISEcp1A	GCAGGTCTTTTTCTGCTCC	IS*Ecp1B* transposase	1
ISEcp1B	TTTCCGCAGCACCGTTTGC	IS*Ecp1B* transposase, reverse primer	2
PRECTX-M-3B	CCGTTTCCGCTATTACAAAC	3′ end of bla_{CTX-M}, reverse primer	3
PROM+	TGCTCTGTGGATAACTTGC	Right part of IS*Ecp1B* including −35 and −10 promoter sequences	4
PROM−	GCAGTCTAAATTCTTCGTG	Right part of IS*Ecp1B* lacking −35 and −10 promoter sequences	5
CTX-M-REV	CCGCGAACATCATCCGTTGC	5′ end of $bla_{CTX-M-19}$, reverse primer for primer extension experiments	6
IS903-A	CATATGAAATCATCTGCGC	IS*903D* transposase	7
IS903-B	CCGTAGCGGGTTGTGTTTTC	IS*903D* transposase, reverse primer	8
INT2F	TCTCGGGTAACATCAAGGCCC	3′ end of the *int11* integrase gene	9
3′-CS	AAGCAGACTTGACCTGA	5′ end of the *qacEΔ1* gene, reverse primer	10
CTX-MA	ACCGCGATATCGTTGGT	$bla_{CTX-M-19}$ gene	11
CTX-MB	CGCTTTGCGATGTGCAG	$bla_{CTX-M-19}$ gene, reverse primer	12
ORF1B	ATACTCTTGCTCATATGGGG	5′ end of ORF1 of Tn*1721,* reverse primer	13

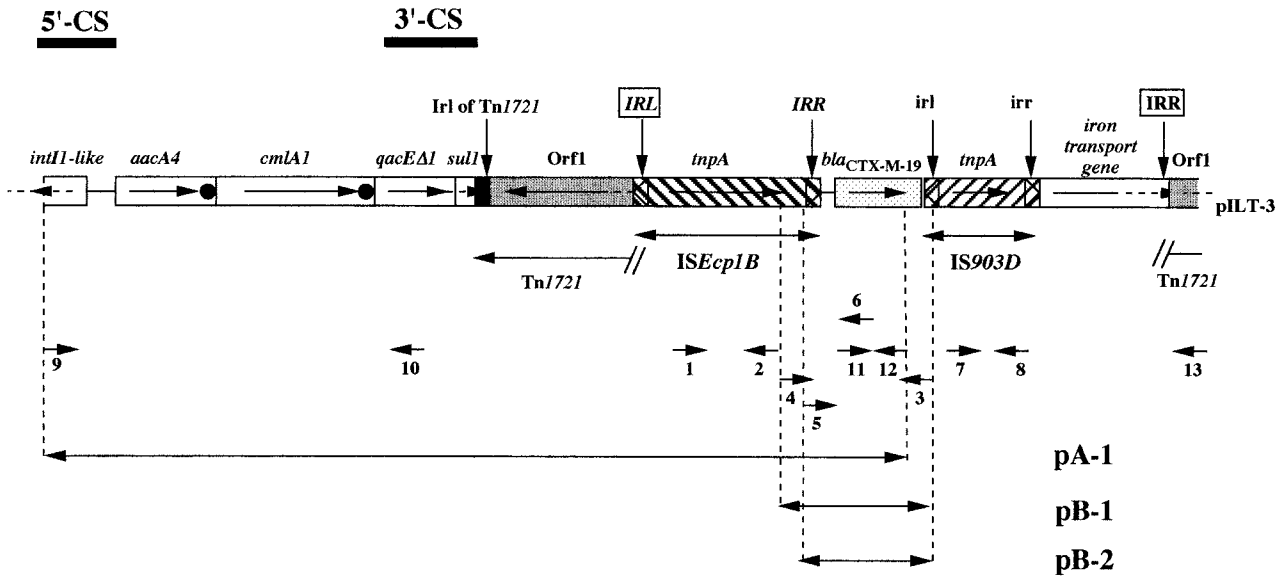


FIG. 1. Schematic map of a 9,590-bp DNA fragment of the natural plasmid pILT-3 from *K. pneumoniae* ILT-3 containing IS*Ecp1B*, IS*903D*, and the $bla_{CTX\text{-}M\text{-}19}$ gene. The 5′ and 3′ conserved sequences (5′-CS and 3′-CS) of class 1 integrons are indicated in the upper left part of the figure. ORFs and genes are shown as boxes with an arrow indicating the transcription orientation. Black dots are for 59-be sequences. IRL and IRR motifs are indicated by vertical arrows. The IRL and IRR of the IS*Ecp1B*-mobilized DNA fragment are boxed, those of the IS*Ecp1B* element are italicized, and those of IS*903D* are in lowercase; the inverted repeat left of Tn*1721* (Irl) is also shown. The cloned sequences of recombinant plasmids are indicated by arrows at both ends, with the corresponding plasmid names indicated on the right.

binant clones as templates. These PCR fragments were end labeled using the ECL nonradioactive labeling kit (Amersham Pharmacia Biotech). Hybridizations with these PCR products used as probes were performed as previously described (37). Similarly, Southern experiments were performed with *Apa*I- and *Hin*dIII-restricted DNA of strains of a series of bacterial species with the internal probe of IS*Ecp1B*.

β-Lactamase activity. Specific β-lactamase activities were determined with cultures of *E. coli* DH10B harboring recombinant plasmids pB-1 and pB-2 derived from pACYC184 (Fig. 1), which does not possess strong promoter sequences near its *Eco*RV cloning site. Overnight cultures were performed in 10 ml of Trypicase soy broth containing 50 μg of amoxicillin per ml and 30 μg of chloramphenicol per ml. Bacterial cultures were harvested by centrifugation for 15 min at 5,000 × *g*, and the bacterial pellets were resuspended in 500 μl of 50 mM phosphate buffer (pH 7) at 4°C, disrupted by sonication (1 min at 4 W), and centrifuged (30 min at 10,000 × *g* and 4°C), and the supernatants were analyzed. Hydrolytic activities were measured spectrophotometrically with 100 μM cephalothin as the substrate, as described previously (39). The protein content was measured by the Bio-Rad DC protein assay.

RNA extraction and primer extension analysis. Total RNAs of *E. coli* DH10B (pA-1) and *E. coli* DH10B(pB-1) were extracted using the Qiagen RNeasy maxi-kit. Samples were treated with 1 U of DNase (Amersham Pharmacia Biotech) at 37°C for 30 min. Primer extension experiments were performed with the primer extension system avian myeloblastosis virus reverse transcriptase kit (Promega, Charbonnières, France) as specified by the manufacturer. The cDNA was generated from the CTX-M-REV primer (Table 2), which had been previously 5′ end labeled with [γ-^{32}P]ATP and polynucleotide kinase.

Nucleotide sequence accession number. The nucleotide sequence data for the entire IS*Ecp1B*-mobilized DNA segment have been submitted to the GenBank nucleotide sequence database under accession number AF458080.

RESULTS

Identification and characterization of the genetic structures surrounding the β-lactamase gene. Cloning of *Sau*3AI partially digested fragments of whole-cell DNA of *K. pneumoniae* ILT-3 into kanamycin-resistant pBK-CMV followed by selection on ampicillin-containing plates was performed. Despite several attempts, *E. coli* DH10B clones harboring recombinant plasmids were not obtained but ampicillin-resistant and kanamycin-susceptible *E. coli* DH10B clones were isolated. These clones displayed a β-lactam-resistant phenotype consistent with the expression of the $bla_{CTX\text{-}M\text{-}19}$ gene. In these cases, the $bla_{CTX\text{-}M\text{-}19}$ gene could have been integrated into the chromosome of these recombinant strains.

Since several ESBL genes may be transposon or integron borne (23, 37, 38, 40, 41), the surrounding sequences of the $bla_{CTX\text{-}M\text{-}19}$ gene were explored further to identify a class 1 integron that may contain this gene (22). This has been done using PCR amplification experiments and the natural plasmid pILT-3 carrying the $bla_{CTX\text{-}M\text{-}19}$ gene as the template (39).

Plasmid DNA of *E. coli* DH10B(pILT-3) was used as a template in long-range PCR experiments using primers INT2F and 3′-CS (located in the 5 and 3′ conserved sequences [5′-CS and 3′-CS] of class 1 integrons, respectively) in combination with primers CTX-MA and CTX-MB (located at the 5′ and 3′ ends of the $bla_{CTX\text{-}M\text{-}19}$ gene, respectively) (Fig. 1; Table 2). A positive result was not obtained with primers CTX-MA and 3′-CS whereas a ca. 6.5-kb PCR product was obtained using primers INT2F and CTX-MB. This fragment, which contained a truncated $bla_{CTX\text{-}M\text{-}19}$ β-lactamase gene, was cloned into ampicillin-resistant plasmid pPCRScript Amp SK(+), giving rise to pA-1 (Fig. 1). In addition to ampicillin resistance, plasmid pA-1 conferred resistance to chloramphenicol, kanamycin, gentamicin, netilmicin, and tobramycin. Sequencing of the insert of this recombinant plasmid identified several open reading frames (ORFs) (Fig. 1 and 2).

Part of an *intI1*-like integrase gene was found, which contained typical P_c promoter features consisting of −35 (TTG ACA) and −10 (TAAACT) sequences able to promote the expression of gene cassettes, whereas the secondary P_2 pro-

moter was in its inactive form (12, 27) (Fig. 1 and 2). An *aacA4* gene cassette encoding an aminoglycoside 6′-*N*-acetyltransferase (26) and conferring resistance to gentamicin and kanamycin was found immediately upstream of the integrase gene (Fig. 1 and 2). A second gene cassette that carried a *cmlA1* variant gene conferring resistance to chloramphenicol was identified downstream of the *aacA4* cassette (Fig. 1 and 2) (41). The deduced 419-amino-acid sequence contained a protein that differed from CMLA1 by five amino acid substitutions. This gene was preceded by its own putative promoter sequences, P_a (−35 [TTGCAG] and −10 [TACGAT]) (Fig. 2), that may drive the expression of the chloramphenicol resistance determinant (41) independently of the promoter sequences located in the integrase sequence. The 59-be that was associated with this chloramphenicol resistance gene differed from that of the *cmlA1* gene cassette by only a 7-bp substitution (41), suggesting a common origin.

Downstream of these gene cassettes, typical features of a 3′-CS of class 1 integrons were found, with a *qacEΔ1* gene fused with a *sul1*-type gene (22) (Fig. 1 and 2). The *sul1*-type gene was interrupted by insertion of the inverted repeat left (IRL) of Tn*1721* (1), thus explaining the susceptibility to sulfonamides of *E. coli* DH10B harboring natural plasmid pILT-3. The putative site of insertion of Tn*1721* consisted of an AT-rich 5-bp target site (TTAGA), as reported previously (1) (Fig. 2, bp 3501 to 3505). On the right side of the IRL of Tn*1721*, the 3′ end of the so-called ORF1 of Tn*1721* encoding a putative methyl-accepting chemotaxis protein was identified (Fig. 1 and 2). This coding sequence was truncated at its 5′ end by insertion of an IS*Ecp1*-like element designated IS*Ecp1B* (Fig. 1 and 2).

IS*Ecp1B* (1,655 bp long) differed from IS*Ecp1* (GenBank accession no. AJ242809) by three nucleotide substitutions and was weakly related to other IS elements, with the amino acid sequence of its transposase having only 25% identity to that of transposases of the IS*1380* family elements (29). IS*Ecp1B* contained two imperfect 18-bp inverted-repeat sequences (four mismatches) surrounding a *tnpA* gene that encoded a putative transposase with one amino acid change (Q179R) compared to the IS*Ecp1* transposase. The same "DDE" motif found in most phosphotransferases (29) was present in the transposase sequence of IS*Ecp1B.* The $bla_{CTX\text{-}M\text{-}19}$ gene was located on the right side of IS*Ecp1B* (Fig. 1 and 2), with its ATG start codon being located 42 bp downstream of the inverted repeat right (IRR) of IS*Ecp1B* (Fig. 2).

To identify further DNA sequences surrounding the $bla_{CTX\text{-}M\text{-}19}$ gene, several long-range PCR experiments were performed using a series of primers (Fig. 1 and Table 2). The CTX-MA primer annealing to the 5′ internal part of the $bla_{CTX\text{-}M\text{-}19}$ gene was used in combination with primers annealing to (i) the IS*Ecp1B*-IRR or IS*Ecp1B*-IRL sequences in order to search for a putative insertion of a second IS*Ecp1B* element downstream of $bla_{CTX\text{-}M\text{-}19}$, (ii) the 5′ end of ORF1 of Tn*1721,* or (iii) the 3′ end of class 1 integrons (Table 2). Only PCR experiments with primer located in the 5′ end of ORF1 and primer CTX-MA gave positive results, suggesting that the 3′ end of a class 1 integron or a second IS*Ecp1B* insertion sequence (able to constitute a composite transposon) was not present in the immediate vicinity of $bla_{CTX\text{-}M\text{-}19}$. Sequencing of this ca. 3-kb PCR product revealed that an IS*903*-like insertion sequence was located downstream of the $bla_{CTX\text{-}M\text{-}19}$ gene.

The 1,056-bp IS element, designated IS*903D,* was bracketed by two imperfect 19-bp inverted-repeat sequences (one mismatch) and contained a transposase-coding sequence. The sequence of this transposase differed by 7 of the 467 residues of IS*903* (20) and by 3 residues from that of IS*903B* (V105I, N156K, A189S) (31). No significant identity was found between the DNA sequences of the IRs of IS*Ecp1B* and IS*903D.* Furthermore, target site duplications on each side of IS*903D* were not identified (15). On the right side of IS*903D,* a DNA sequence was found that likely encodes the carboxy-terminal 365 of 700 amino acids of a protein with 63% amino acid identity to an outer membrane lipoprotein involved in iron transport and identified in *E. coli* K-12 (8).

Inside this lipoprotein gene, a second 18-bp sequence with consistent nucleotide identity to the IRR of IS*Ecp1B* was identified at the place where truncation has occurred and was followed by a TAACA sequence identical to that found on the left side of IS*Ecp1B*-IRL. This 5-bp duplication was probably the signature of an insertion generated by transposition (Fig. 2). Thus, the 4,797-bp fragment, bracketed on one side by the IRL of IS*Ecp1B* and on the other side by this 18-bp sequence used as the IRR, probably constituted a transposon.

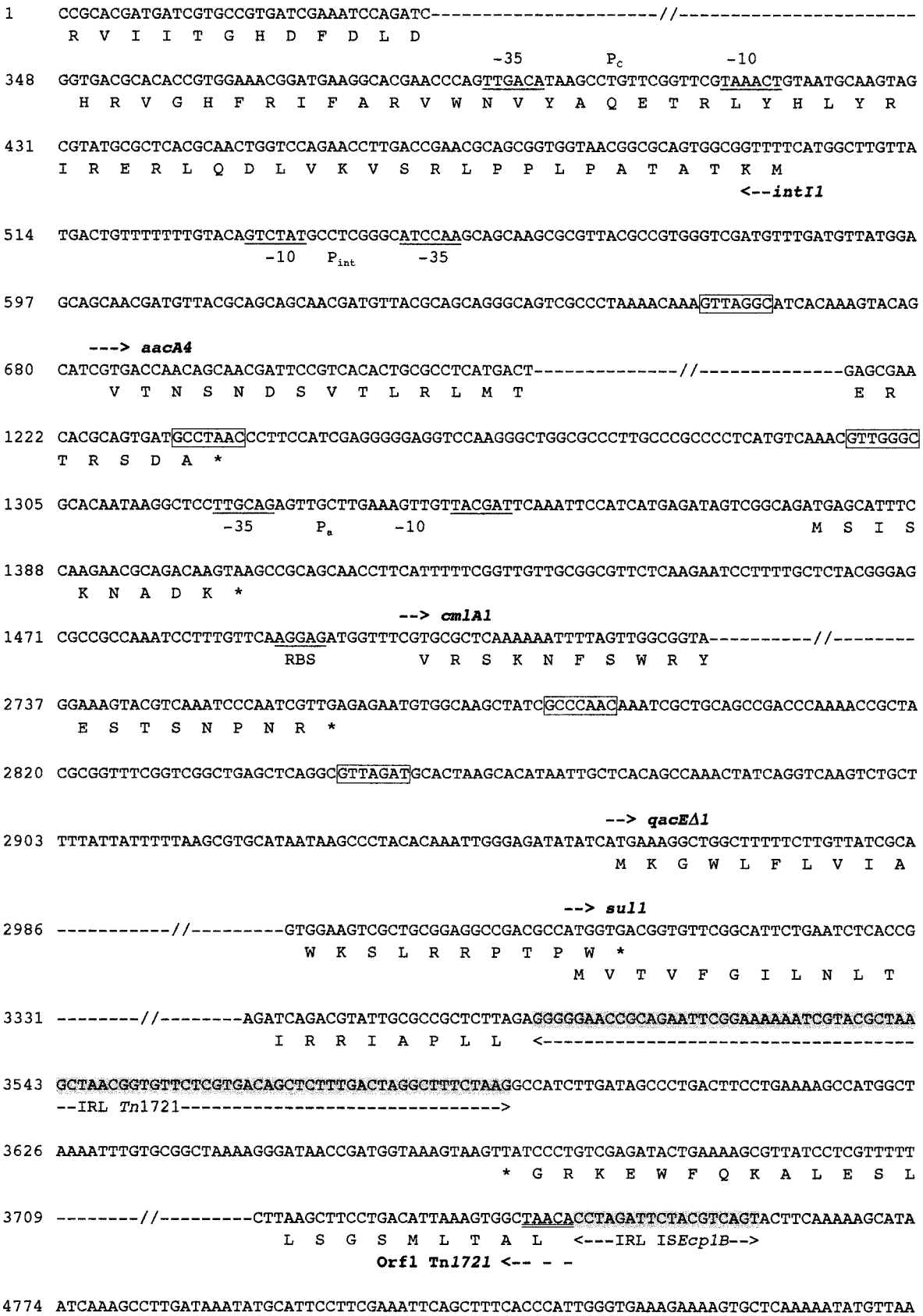
Finally, another part of ORF1 of Tn*1721* was identified on the right side of the second IRR of IS*Ecp1B* (Fig. 2). No deletion had occurred in the ORF1 sequence resulting from the insertion of the DNA fragment that contained IS*Ecp1B,* $bla_{CTX\text{-}M\text{-}19}$, IS*903D,* and the truncated gene encoding the outer membrane lipoprotein (Fig. 1 and 2).

IS*Ecp1B* as an enhancer of $bla_{CTX\text{-}M\text{-}19}$ gene expression. No putative promoter sequences were found in the 42-bp sequence that separated the IRR of IS*Ecp1B* from the ATG site of the $bla_{CTX\text{-}M\text{-}19}$ gene. This suggested a role of IS*Ecp1B* in β-lactamase expression. Indeed, as we have reported previously (27), upstream of another $bla_{CTX\text{-}M}$ gene ($bla_{CTX\text{-}M\text{-}15}$), −35 (TTGAAA) and −10 (TACAAT) regions corresponding to a putative promoter were identified in IS*Ecp1B* near its IRR (Fig. 2). To determine the role of IS*Ecp1B* in $bla_{CTX\text{-}M\text{-}19}$ expression, β-lactamase activities were determined with cell cultures of *E. coli* DH10B harboring several recombinant plasmids, and primer extension experiments were performed.

Two recombinant plasmids containing the $bla_{CTX\text{-}M\text{-}19}$ gene were constructed with or without the putative promoter sequences located next to the IRR of IS*Ecp1B;* these were recombinant plasmids pB-1 and pB-2, respectively (Fig. 1). Specific β-lactamase activities with cultures of *E. coli* DH10B harboring pB-1 and pB-2 were 35 ± 4 and 2 ± 0.8 U per mg of protein, respectively, corresponding to a ca. 17-fold difference. Thus, the role of IS*Ecp1B* sequences in $bla_{CTX\text{-}M\text{-}19}$ expression was demonstrated.

Primer extension experiments were performed with RNAs extracted from cultures of *E. coli* DH10B(pA-1) and *E. coli* DH10B(pB-1) by using extension primer CTX-M-REV (Table 2). The +1 signal was identified 110 bp upstream of the ATG start codon of the $bla_{CTX\text{-}M\text{-}19}$ gene, and the deduced −35 and −10 promoter sequences were located at the end of IS*Ecp1B,* as expected (Fig. 2).

Copy number of IS*Ecp1B*. To determine the copy number of IS*Ecp1B* in *K. pneumoniae* ILT-3 and *E. coli* DH10B clones resulting from preliminary *Sau*3AI cloning experiments, whole-cell DNAs of these strains were digested with *Bam*HI



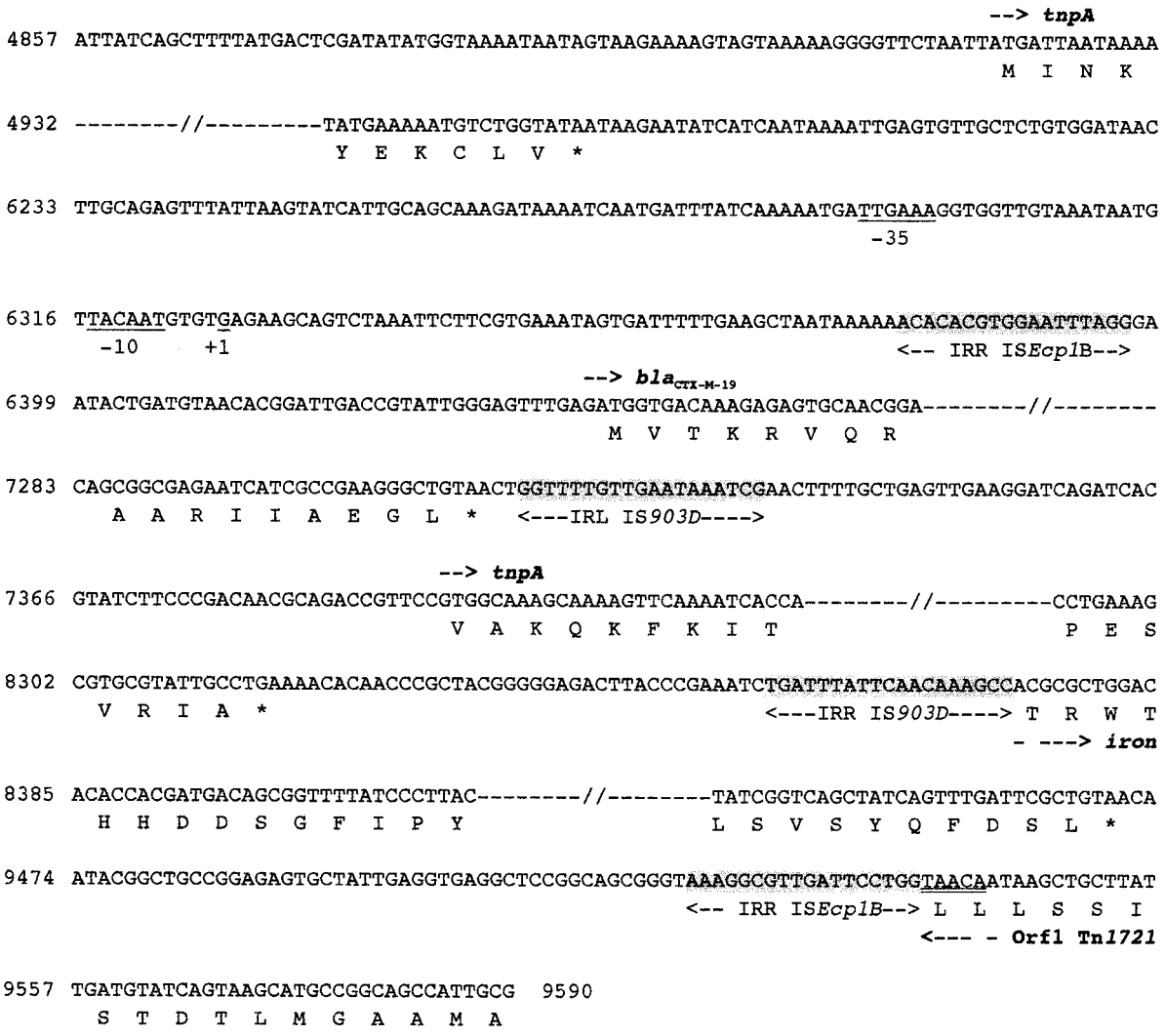


FIG. 2. Nucleotide sequence of a 9,590-bp DNA fragment of the natural pILT-3 plasmid of *K. pneumoniae* ILT-3 containing IS*Ecp1B,* β-lactamase CTX-M-19, and IS*903D* coding sequences. The deduced amino acid sequence is indicated in single-letter code below the nucleotide sequence. The start codons of the ORFs are indicated by horizontal arrows, and stop codons are indicated by asterisks. The −35 and −10 promoter sequences of the P_c, P_{int}, and P_a promoters are underlined, as well as the +1 position of the transcriptional start of the $bla_{CTX\text{-}M\text{-}19}$ site. The 5-bp duplicated target sites of the putative insertion site for the DNA fragment resulting from an IS*Ecp1B*-mediated transposition process are doubly underlined. RBS indicates the putative ribosome binding site for the *cmlA1*-like gene, and the 9-amino-acid leader peptide sequence of the variant of *cmlA1* cassette is indicated. Core and inverse core sites located at each cassette boundary are boxed. *TnpA*, transposase gene; *iron,* gene encoding a putative outer membrane lipoprotein for iron uptake; and Orf1, the gene encoding the putative methyl-accepting chemotaxis protein of Tn*1721.*

and *Pvu*I, which did not cut inside the IS*Ecp1B* and IS*903D* DNA sequences. These restricted fragments were then hybridized with internal probes for IS*Ecp1B* transposase and IS*903D* transposase genes. A single copy of IS*Ecp1B* and IS*903D* was found in all strains (data not shown). Since $bla_{CTX\text{-}M\text{-}19}$ was plasmid borne and was associated with IS*Ecp1B* in *K. pneumoniae* ILT-3 (37), this result indicated that the IS*Ecp1B*-transposed fragment was not chromosome located in *K. pneumoniae* ILT-3. Positive hybridization results obtained with *E. coli* DH10B clones resulting from preliminary *Sau3*AI cloning experiments indicated a likely chromosomal integration of at least IS*Ecp1B* and $bla_{CTX\text{-}M\text{-}19}$ in those cases.

Screening for progenitor of IS*Ecp1*. Since the $bla_{CTX\text{-}M}$ genes have spread worldwide, we hypothesized that the reservoir of IS*Ecp1*-like elements could have been either the progenitor of $bla_{CTX\text{-}M}$ genes (*Kluyvera* spp.) or bacterial species that may have been in close contact with those enterobacterial species. Thus, selected bacterial species that are common components of human intestinal and skin floras were studied.

*Apa*I- and *Hin*dIII-restricted whole-cell DNA of strains of these bacterial species were subjected to a Southern blot analysis. Hybridizations with an IS*Ecp1B*-specific probe did not give positive signals (data not shown), except for a single *Salmonella enterica* serotype Blockley strain. This result was confirmed by PCR analysis with primers specific for the transposase gene of IS*Ecp1B* (data not shown). Sequence analysis revealed perfect DNA identity to IS*Ecp1B.* Analysis of the antibiotic susceptibility of that *Salmonella* isolate showed that it remained susceptible to

β-lactams, and PCR experiments failed to detect any bla_{CTX-M} genes. Other *S. enterica* strains belonging to the same serotype were IS*Ecp1B* negative (data not shown).

DISCUSSION

This work showed that the IS*Ecp1B* element seems to act as a key factor in the dissemination of CTX-M-type β-lactamase genes. IS*Ecp1B* also acts as a strong positive factor for $bla_{CTX-M-19}$ gene expression. The −35 and −10 promoter sequences for $bla_{CTX-M-19}$ expression are located at the end of IS*Ecp1B* near its IRR, as described for the −35 and −10 promoter sequences driving the expression of the carbapenem-hydrolyzing *cfiA* gene of *Bacteroides fragilis,* which are located in the IS*1186* element (35). Other IS elements may enhance the expression of β-lactamase genes, but, in most of these cases, the −35 promoter sequences are located in the IS inverted repeats whereas the −10 promoter sequences are part of the downstream β-lactamase genes (19, 30, 33).

The mechanism of mobilization generated by IS*Ecp1B* seems to correspond to a normal transposition mechanism and not to a one-ended transposition mechanism as suggested previously (P. D. Stapleton, Program Abstr. 39th Intersci. Conf. Antimicrob. Agents Chemother, abstr. 1457, 1999). Indeed, one-ended transposition requires only a single copy of an IS element but does not use specific inverted repeat sequences (32). Transposition usually requires IR sequences located at both ends of a transposon that are recognized by a transposon-encoded transposase (13). It is likely that the IS*Ecp1B* transposase may recognize structurally related IR sequences and use them as ends in regular transposition (21). As observed for IS*1247,* which is a also a member of the IS*1380* family, transposition of the insertion element together with adjacent sequences is possible without the requirement for a second IS element (43).

While this work was in progress, Cao et al. detailed the genetic structure of the 7-kb natural pIP843 plasmid of a *K. pneumoniae* isolate encoding β-lactamase CTX-M-17, which differed from CTX-M-19 by two amino acid substitutions (9). This *K. pneumoniae* isolate was from Vietnam, whereas *K. pneumoniae* strain ILT-3, analyzed in the present study, was isolated in Paris (France) but from a Vietnamese patient. A very similar structure was found bracketing the $bla_{CTX-M-17}$ gene, with an upstream IS*Ecp1*-like element (five amino acid substitutions in the transposase sequence compared to that of IS*Ecp1B*) and a downstream IS*903C* element (two amino acid substitutions in that transposase compared to that of IS*903D*). Additionally, a truncated gene encoding an outer membrane lipoprotein was identified in both cases, and these genes differed by a few nucleotide substitutions. In fact, a similar transposon was present compared to that in *K. pneumoniae* ILT-3, containing an IS*Ecp1*-like sequence, $bla_{CTX-M-17}$, IS*903C,* and a truncated outer membrane lipoprotein gene. These transposons have 99% nucleotide identity, differing by 19 of 4,797 bp. In addition, a careful analysis of the published sequence led us to identify a 6-bp duplication consisting of the motif AT AATA on each side of the transposon carrying the $bla_{CTX-M-17}$ gene. However, in the case of the $bla_{CTX-M-17}$ gene, the fragment was not bracketed by DNA structure related to Tn*1721* and was inserted in a smaller plasmid of 7 kb.

Additionally, Chanawong et al. (10) reported clinical *E. coli* isolates from Southern China (near Vietnam) that harbored a structurally related bla_{CTX-M} gene, $bla_{CTX-M-14}$ (a point mutant analogue of $bla_{CTX-M-19}$), which is bracketed by the ends of an IS*Ecp1*-like element and of an IS*903*-like element. This latter result suggests dissemination of similar genetic structures carrying structurally related bla_{CTX-M} genes in that part of the world.

Cao et al. have shown that the −35 and −10 promoter sequences at the end of the IS*Ecp1*-like element near its IRR were located 110 bp upstream of the ATG site of the $bla_{CTX-M-17}$ gene and drove the expression of the β-lactamase exactly as for the expression of $bla_{CTX-M-19}$ (9). In other cases, the ATG sites of $bla_{CTX-M-3}$ and of its point mutant derivative $bla_{CTX-M-15}$ from Polish isolates were located 128 and 48 bp downstream of the −10 promoter sequences of the IS*Ecp1*-like sequences, respectively (5, 25). This result indicates the variety of genetic events that have enabled associations between IS*Ecp1*-like sequences and several bla_{CTX-M} genes in clinical strains of different geographic origins.

We hypothesized that bla_{CTX-M} genes and IS*Ecp1*-like sequences must have been in close contact somewhere to enable the formation these hybrid genetic structures. The bla_{CTX-M} genes may possess low-strength promoter sequences in *Kluyvera* spp. that do not allow a high level of their expression. In recipient species such as *E. coli, K. pneumoniae*, and *S. enterica* serovar Typhimurium, IS*Ecp1*-like sequences may provide a higher level of expression of the plasmid-located bla_{CTX-M} genes. Although it has been shown that several enterobacterial species of the *Kluyvera* genus are a natural reservoir of bla_{CTX-M}-like genes (24, 36), we did not identify the reservoir of IS*Ecp1*-like sequences. Future studies should look for bacterial species in animal floras, since bla_{CTX-M} genes have been identified in animal isolates, especially in *Salmonella* spp. that are a source of anthropozoonotic infections.

Additionally, two recent reports describe genetic structures surrounding a $bla_{CTX-M-2}$ gene, described as a complex *sul1*-type integron, similar to In*6* and In*7* (2, 16). These structures possess an ORF for a putative transposase that is not related to an IS*Ecp1*-like transposase and that may enhance $bla_{CTX-M-2}$ mobilization. Thus, genetic plasticity linked to bla_{CTX-M} genes may be more complex than expected.

Finally, IS*Ecp1*-like sequences may be more general mobilizing and expression elements for β-lactamase genes since analysis of GenBank databases also identified them upstream of plasmid-located cephalosporinase genes (6, 7, 34).

ACKNOWLEDGMENTS

We thank Daniel Aubert for help with the primer extension experiments.

This work was financed by a grant from the Ministère de l'Education Nationale et de la Recherche (grant UPRES-EA3539), Université Paris XI, Paris, France.

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