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Emergence of DHA-1-Producing *Klebsiella* spp. in the Parisian Region: Genetic Organization of the *ampC* and *ampR* Genes Originating from *Morganella morganii*

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Eleven *Klebsiella pneumoniae* clinical isolates and one *Klebsiella oxytoca* clinical isolate showing various pulsed-field gel electrophoresis types and producing an inducible DHA-1 class C β-lactamase were isolated in the Parisian region between 1998 and 2003. The aim of this study was to compare the genetic organization of the bla_{DHA-1} genes in this collection of clinical isolates. In four isolates, the *Morganella morganii*-derived genomic region containing bla_{DHA-1} was inserted in an entire complex *sul1*-type integron, including a region common to In6-In7 (CR1), as previously described in a bla_{DHA-1}-producing *Salmonella enterica* serovar Enteritidis KF92 isolate from Saudi Arabia in 1992. Different gene cassette arrays were characterized in each of these integrons. In two of them, an additional 10-kb fragment was inserted between the CR1 and the *M. morganii*-derived region and was similar to the *sap* (ABC transporter family) and *psp* (phage shock protein) operons originated from *Salmonella enterica* serovar Typhimurium. The length of the *M. morganii* region was variable, suggesting that several independent recombination events have occurred and that open reading frame *orf513* encodes a recombinase involved in the mobilization of the resistance genes. The genetic organization of bla_{DHA-1} was identical in the eight other isolates. This structure is likely derived from a complex integron following the insertion of IS*26*, leading to the deletion of the first part of integron. The horizontal transfer of one plasmid carrying that truncated integron was shown for seven of these isolates.

Since the 1980s, several acquired plasmid-encoded Ambler class C β-lactamases have been characterized from *Klebsiella pneumoniae* and other members of the family *Enterobacteriaceae* lacking inducible chromosomal AmpC enzymes (35). These are derivatives of the chromosomally encoded, clavulanate-resistant AmpC cephalosporinases of *Enterobacter* spp. (ACT-1 and MIR-1), *Citrobacter freundii* (CMY type), *Morganella morganii* (DHA type), *Hafnia alvei* (ACC-1), and other gram-negative bacilli (6, 10, 15, 16, 27, 31). The most prevalent of the transferable class C β-lactamases and the most widely geographically distributed are the CMY-type enzymes (35), while DHA-type enzymes have been isolated less often. The first was characterized in a *Salmonella enterica* serovar Enteritidis strain isolated in Dhahran, Saudi Arabia, in 1992 (15). At the same time, a DHA-2-producing strain of *K. pneumoniae* was isolated in France (14). At the end of the 1990s, epidemiology studies in the United States detected DHA-1 in two *K. pneumoniae* isolates from California and Florida (3, 24). In Taiwan, between 1999 and 2001, 10 sporadic infections with *K. pneumoniae* isolates producing DHA-1 β-lactamase were reported (49). At the same time, 36 DHA-1-producing *K. pneumoniae* isolates were collected among 99 cephamycin- and extended-spectrum cephalosporin-resistant *K. pneumoniae* isolates (50). Also, between 1998 and 2002 in Seoul, South Korea, 14 isolates of DHA-1-producing *K. pneumoniae* were reported in cases of bacteremia (30). More recently, bla_{DHA-1} has been found in *Salmonella enterica* serovar Montevideo in Korea (19) and in *Salmonella enterica* serotype Senftenberg in the United Kingdom (20). So, strains producing DHA-type enzymes have been found on each continent, but particularly in the Far East.

Knowledge of the genetic organization of acquired AmpC should allow us to understand the molecular mechanisms of *ampC* gene transfer. Some *ampC* genes are located within or near mobilizing elements, such as transposons or integrons (23, 46). Integrons have been implicated in the horizontal transfer of class C β-lactamases, but not as gene cassettes, as has been seen for the Ambler class A, B, and D β-lactamases (17). Indeed, several *ampC* genes, such as bla_{DHA-1}, bla_{FOX-4}, bla_{CMY-1}, bla_{CMY-8}, and bla_{MOX-1}, have been described in a particular class 1 integron, originally identified in In6 and In7, which contains two partial copies of the 3′ conserved segment (3′-CS1 and 3′-CS2) surrounding a common region (CR) and an antibiotic resistance gene (32, 35, 42, 44). Other antibiotic resistance genes have now been reported adjacent to the CR of similar integrons, such as *qnr* in In37 (47), $bla_{CTX-M-9}$ in In60 (38), *dfrA10* in In34 (32), *dfrA10* in a variant *Salmonella* genomic island (SGI1) from *Salmonella enterica* serovar Agona (8), bla_{CMY-9} in pCMXR1 from *Escherichia coli* (13), and *catA2* in pAr-32 from *Aeromonas salmonicida* (41). Partridge and Hall showed that CR1 containing open reading frame (ORF) *orf513*, CR2 (containing *orfA*), and CR3 (containing *orf2*) form a family of genetic elements involved in the mobi-

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lization of resistance genes in gram-negative bacteria (32). The incorporation of a resistance gene at the 3′ end of the CR is probably catalyzed by *orf513*, but this has yet to be established experimentally.

This study reports on the characterization of 12 *Klebsiella* sp. strains with an inducible DHA-type enzyme. These were isolated between 1998 and 2003 in the Paris, France, region. The aim of this work was to describe the epidemiological features of these strains and to explore the genetic organization of $bla_{\text{DHA-1}}$.

MATERIALS AND METHODS

Bacterial strains. Twelve clinical isolates from 12 patients in eight hospitals in the Parisian region between 1998 and 2003 were studied. Eleven *K. pneumoniae* isolates and one *Klebsiella oxytoca* isolate were identified by using the API system (bioMerieux, Marcy l'Etoile, France). The characteristics of the strains are summarized in Table 1.

Previously described DHA-1-type β-lactamase-producing strain *Salmonella* serovar Enteritidis KF92 (5) and *M. morganii* SLM01 (4) were used as controls.

Escherichia coli K-12 strain J53-2 (Rif^r), *E. coli* K-12 strain C600 (Nal^r), and *E. coli* strain DH10B (Invitrogen SARL, Cergy-Pontoise, France) were used as recipient strains for conjugation and electroporation experiments.

Plasmid vector pBKCMV (Stratagene, La Jolla, CA) was used for cloning.

Antibiotic susceptibility. The MICs for several β-lactams were determined by the standard agar dilution method on Mueller-Hinton agar (Bio-Rad, Marnes la Coquette, France). The resistance phenotype was determined by the disk diffusion method on Mueller-Hinton agar. Disk antagonism tests with imipenem or cefoxitin as inducing agents were used to detect the inducibilities of the β-lactamases (22). The antagonism was detected by the blunting of the oxyimino β-lactam zone of inhibition surrounding the imipenem or cefoxitin disk.

Class A extended-spectrum β-lactamases (ESBLs) were detected by the standard double-disk synergy test with amoxicillin-clavulanic acid and cefotaxime, ceftazidime, or cefepime (9).

Transfer of resistance. Mating experiments were done by mixing equal volumes of each test strain and either *E. coli* K-12 C600 (Nal^r) or *E. coli* K-12 J53 (Rif^r). β-Lactam-resistant *E. coli* transconjugants were then selected on Drigalski agar (Bio-Rad) containing either nalidixic acid (50 μg/ml) or rifampin (250 μg/ml) and either cefoxitin (10 μg/ml) or ceftazidime (2 μg/ml).

Plasmid DNA was isolated by alkaline lysis (QIAGEN Plasmid Purification, Courtaboeuf, France) and was used to transform *E. coli* DH10B cells by electroporation, according to the manufacturer's instructions (Bio-Rad). Transformants were incubated for 2 h at 37°C and plated on Drigalski agar supplemented with cefoxitin (10 μg/ml).

Plasmid DNA fingerprinting. Plasmid DNA was purified from transconjugants or transformant cells by using a Plasmid Midi kit (QIAGEN), according to the manufacturer's recommendations. For fingerprinting analysis, plasmid DNA was digested with the EcoRI restriction enzyme (New England Biolabs Inc., Saint Quentin en Yvelines, France) and subjected to electrophoresis in a 1% agarose gel at 80 V for 4 h.

PFGE analysis. Genomic DNA, prepared as described by Decré et al. (11) and digested with XbaI (New England Biolabs), was subjected to pulsed-field gel electrophoresis (PFGE) in a CHEF DRIII device (Bio-Rad). DNA fragments were separated in a 1% (wt/vol) agarose gel in 0.5× Tris-borate-EDTA buffer at 200 V for 20 h with pulse times ranging from 5 to 30 s.

PCR and DNA sequencing: characterization of *bla* genes. Total DNA was extracted by using a QIAamp DNA Mini Kit (QIAGEN). Primers OT3 (5′-ATGAGTATTCAACATTTCCG-3′) and OT4 (5′-CCAATGCTTAATCAGTGAGG-3′) were used to amplify putative bla_{TEM} genes, and primers OS5 (5′-TTATCTCCCTGTTAGCCACC-3′) and OS6 (5′-GATTTGCTGATTTCGCTCGG-3′) were used to amplify bla_{SHV} genes. The bla_{TEM} and bla_{SHV} genes were characterized by sequencing and were then analyzed by using tables available on the website www.lahey.org/Studies. The entire region containing the bla_{DHA} gene and its regulator gene, *ampR*, was amplified by using three pairs of primers generating overlapping PCR fragments on a 2,243-bp region: DHA upper1 and DHA lower1, DHA upper2 and DHA lower2, and DHA upper3 and DHA lower3 (Table 2, PCRs M, N, and O, respectively). The PCR conditions were as follows: 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C. All enzymes were purchased from Sigma (St. Quentin Fallavier, France).

TABLE 1. Origins and epidemiological features of the clinical strains investigated[a]

Strain	Collection date (yr)	Source	Origin	DHA-1 plasmid transfer	TEM type	SHV type	PFGE type	DHA-1 plasmid fingerprint	*orf513*-specific PCR result
Salmonella serovar Enteritidis KF92	1992	Stool	Dhahran Saudi Arabia	Tc *E. coli* HB101(pSAL-1)	–	–	ND	ND	+
K. pneumoniae Kp760	1998	Blood	Rothschild Hospital, Paris	Tc *E. coli* C600(pKp760)	–	–	I	ND	+
K. pneumoniae TN58647	1999	Urine	Tenon Hospital, Paris	Tc *E. coli* J53(pTN58647)	TEM-1	SHV-2a	A	1a	–
K. pneumoniae RDDHA	1999	Urine	R. Debré Hospital, Paris	Tc *E. coli* C600(pRDDHA)	–	SHV-2	G	ND	+
K. pneumoniae RBDHA	2002	Urine	R. Ballanger Hospital, Parisian region	Tc *E. coli* J53(pRBDHA)	–	–	H	2	+
K. pneumoniae JDDHA	2002	Urine	Joffre-Dupuytren Hospital, Parisian region	Tc *E. coli* J53(pJDDHA)	TEM-1	SHV-2a	A	1a	–
K. oxytoca MPDHA	2002	Urine	Massy-Palaiseau Hospital, Parisian region	No	TEM-1	–	ND	NT	+
K. pneumoniae TN60013	2002	Urine	Tenon Hospital, Paris	Tc *E. coli* C600(pTN60013)	TEM-1	SHV-2a	A	1c	–
K. pneumoniae STA1DHA	2003	Mesentery	St. Antoine Hospital, Paris	Ep *E. coli* DH10B(pSTA1DHA)	–	–	B	1b	–
K. pneumoniae TN26033	2003	Catheter	Tenon Hospital, Paris	No	–	–	D	NT	–
K. pneumoniae STA2DHA	2003	Urine	St. Antoine Hospital, Paris	Ep *E. coli* DH10B(pSTA2DHA)	–	–	C	1a	–
K. pneumoniae IGR1DHA	2003	Urine	Institut Gustave Roussy, Parisian region	Tc *E. coli* J53(pIGR1DHA)	–	–	E	1a	–
K. pneumoniae IGR2DHA	2003	Stool	Institut Gustave Roussy, Parisian region	Tc *E. coli* J53(pIGR2DHA)	–	–	F	1a	–

[a] Abbreviations: Tc, transconjugant; Ep, transformant; –, negative; NT, no transfer; ND, not determined.

TABLE 2. Sequences of oligonucleotides used for PCR mapping

PCR[a]	Primer	Target	Primer sequence
H	orf341 5′ rot 3′	Between CR1 and p(Kp760-2_{ind})	5′-TGCATGGAAAAGCGTGGGAT-3′ 5′-AACATCACCCGCTCGCTGG-3′
I	sapB 5′ pspF 3′	Between *sap* operon and *psp* operon	5′-GCCAGCTAAAGACCATCTCG-3′ 5′-CCAGCGATTACCCACTGAAT-3′
J	pspF 5′ pspA 3′	Inside *psp* operon	5′-CAGATCCAGCGGTAGGGTTA-3′ 5′-CAACATCAATGCCTGCTGAC-3′
K	Faz 5′ morg 3′	Between *psp* operon and *M. morganii* region	5′-AAGAAACAGTGTGCCGCCAT-3′ 5′-GGTAACGCTGATCGGTAAAC-3′
L	pspD 5′ ampC 3′	Between *psp* operon and *M. morganii* region	5′-CCGGCAATCAGGCTAAATAA-3′ 5′-TCCGGAAAAACAGGTGGCGA-3′
M	DHA-1 upper DHA-1 lower	*M. morganii* region	5′-CTCATCCTCCATAAAACAGC-3′ 5′-TTATCTCACACCTTTATTACT-3′
N	DHA-2 upper DHA-2 lower	*M. morganii* region	5′-AGATACATTGCCATTTCCAG-3′ 5′-ACTTGCCGCCGTTACTCACA-3′
O	DHA-3 upper DHA-3 lower	*M. morganii* region	5′-TGTGCCATCAGCGGTTTATT-3′ 5′-TGGAAGGTGAGTGAGTTTTA-3′
P	ampR 5′ orf5 3′	Second copy of 3′-CS	5′-CGTGAGCGGGTGAAAGTCG-3′ 5′-AAGTGTCGACGTGGGTGAAT-3′
Q	sul5′ IS6100 3′	Downstream from second copy of 3′-CS	5′-ATCAGATGCACCGTGTTTCA-3′ 5′-GGTGATCGCTGCACCATAG-3′
R	orf5 5′ tniB 3′	Downstream from second copy of 3′-CS	5′-CCGCATATCTGCACAAGCTC-3′ 5′-AGTTGGAAAATCGCTTCGAG-3′
S	orf5 5′ IS26 3′	Downstream from second copy of 3′-CS	5′-CCGCATATCTGCACAAGCTC-3′ 5′-CCCAGGGGATCACCATAATA-3′
T	amont KpB pspF 3′	Upstream from p(TN60013-2_{ind})	5′-CTTATCATCCCCTTTTGCTG-3′ 5′-CCAGCGATTACCCACTGAAT-3′
U	morg 5′ morg 3′	*M. morganii* chromosome	5′-GCGGGCTCATAAAGATTTCA-3′ 5′-TGGCGTTAGGACTGGAAAAC-3′
i	GSP1i *GSP2 i[b]	Downstream from CR1	5′-TACCGCCAAATCGAACCTTATTAGAGC-3′ 5′-TTTAGGCTGGACCGGCAGTTAAAAT-3′
ii	GSP1ii *GSP2 ii[b]	Upstream from *ampC*	5′-ATAACCGTGCTTCCTGGGTGCATAAAAC-3′ 5′-AAAAACAGGTGGCGATTGTGATTCTGG-3′

[a] i and ii, PCR with the Universal Genome Walker kit.
[b] Primer for nested PCR.

Cloning of *ampC* and *ampR* genes and DNA sequencing. Plasmids pKp760 and pTN60013 were isolated from the *E. coli* C600 transconjugant by alkaline lysis (QIAGEN Plasmid Purification).

The plasmids were totally digested with EcoRI, and the fragments were ligated into the EcoRI site of pBKCMV. Each recombinant plasmid was introduced into *E. coli* DH10B. Transformants were selected on Mueller-Hinton agar supplemented with cefoxitin (50 μg/ml) and kanamycin (25 μg/ml).

The insert was sequenced as described by Sanger et al. (39), using an ABI 373 DNA sequencer (PE Applied Biosystems, Foster City, CA).

The BLAST and the FASTA programs were used to search databases for similar nucleotide and amino acid sequences. The ClustalX program was used to align multiple protein sequences (1, 2, 34).

PCR mapping and DNA sequencing: genetic organization of *bla*$_{\mathbf{DHA-1}}$. Previously described PCR primers for integron pSAL-1 were used to map the unknown region surrounding the *ampC* and the *ampR* genes in DHA-1-producing strains. PCRs A, B, F, D, and G were performed (46). The PCR conditions were as described above for all fragments except those that could exceed 3 kb, most notably, for PCR B, which included the gene cassettes. In this case the extension time was increased to 5 min and the DNA polymerase was adapted for long-time PCR: DyNAzyme EXT (Finnzymes, Espoo, Finland).

In strains RBDHA and MPDHA, the unknown sequences downstream from *ampC* or downstream from CR1 were amplified by using the Universal Genome Walker kit (Clontech Laboratories, Palo Alto, CA) (40). Genomic DNA was digested with EcoRV, DraI, PvuII, StuI, NruI, and AviII; and the DNA fragments were ligated with a Genome Walker adapter. Then, the following steps were performed according to the manufacturer's recommendations by using *ampC*-specific or CR1-specific primers (Table 2).

New PCR primers were designed in this study to map the *bla*$_{DHA-1}$ region by using as templates the DNA sequences of the clones obtained from pKp760 or pTN60013, the results obtained for RBDHA and MPDHA with the Universal Genome Walker kit, or, finally, the DNA sequences of various In6- and In7 type-integrons. The primers are listed in Table 2.

TABLE 3. MICs of β-lactams and inhibitors

Strain	MIC (μg/ml)[a]											
	AMX	AMC	TIC	TCC	PIP	TZP	FOX	CTX	CAZ	ATM	FEP	IPM
KF92	>512	>512	>256	NT	>512	NT	256	256	512	4	0.25	1
HB101(pSAL-1)	>512	>512	128	NT	128	NT	128	16	64	1	0.12	1
Kp760	>256	>256	>256	>256	256	8	>256	8	32	8	0.06	1
C600(pKp760)	>256	>256	128	64	128	2	128	4	32	2	0.06	1
TN58647	>256	16	256	256	256	64	256	4	64	4	0.5	2
J53(pTN58647)	>256	64	4	4	2	1	64	0.125	4	0.25	0.06	2
RDDHA	>256	64	256	256	256	32	256	64	256	16	0.5	2
C600(pRDDHA)	>256	32	256	8	8	2	32	2	2	1	0.25	1
RBDHA	>256	32	256	256	32	16	256	4	32	8	0.25	4
J53(pRBDHA)	>256	128	256	32	4	4	64	0.125	2	0.125	0.06	2
JDDHA	>256	32	256	256	256	64	256	4	32	4	0.5	2
J53(pJDDHA)	>256	64	16	8	8	2	128	0.25	4	0.5	0.06	2
MPDHA	>256	32	256	256	16	1	64	0.5	1	0.5	0.06	1
TN60013	>256	32	256	256	256	64	128	8	64	2	1	1
C600(pTN60013)	>256	64	8	8	4	1	128	0.25	4	0.25	0.06	1
STA1DHA	>256	64	256	256	64	8	128	2	128	4	0.06	2
DH10B(pSTA1DHA)	>256	128	16	8	4	2	128	0.25	4	0.25	0.06	2
TN26033	>256	32	256	128	64	32	32	2	128	4	0.125	1
STA2DHA	>256	64	256	256	64	8	256	4	32	1	0.06	1
DH10B(pSTA2DHA)	>256	128	256	256	32	64	64	16	256	0.5	0.125	1
IGR1DHA	>256	64	64	16	8	4	128	2	32	4	0.06	2
J53(pIGR1DHA)	>256	32	4	8	4	1	128	0.5	2	0.5	0.06	1
IGR2DHA	>256	64	256	256	64	8	128	4	16	4	0.06	2
J53(pIGR2DHA)	>256	32	4	4	2	1	64	0.25	2	0.5	0.06	1
E. coli J53	1	1	0.25	0.25	0.125	0.125	1	<0.06	0.06	0.06	<0.06	0.25
E. coli C600	2	2	0.5	0.5	0.125	0.125	1	0.125	0.25	0.125	0.125	0.25

[a] AMX, amoxicillin; AMC, amoxicillin-clavulanic acid (2 μg/ml); TIC, ticarcillin; TCC, ticarcillin-clavulanic acid (2 μg/ml); PIP, piperacillin; TZP, piperacillin-tazobactam (4 μg/ml); FOX, cefoxitin; CTX, cefotaxime; CAZ, ceftazidime; ATM, aztreonam; FEP, cefepime; IPM, imipenem; NT, not tested.

The resulting PCR products were sequenced and then analyzed with the BLAST, FASTA, and ClustalX programs. AMIGene software was used to characterize the most likely coding sequences in a large bacterial genome sequence (7).

Several PCR products obtained from strains TN58647, JDDHA, STA1DHA, TN26033, STA2DHA, IGR1DHA, and IGR2DHA were not sequenced but only compared by their restriction fingerprints. Two aliquots of the products of PCRs J, K, L, P, and S were totally digested, one with TacI (New England Biolabs) and the other with HaeIII (New England Biolabs). The digestion products were separated on 2.5% agarose gels and compared to that obtained from strain TN60013 under the same conditions.

Nucleotide sequence accession numbers. The nucleotide sequences in strains Kp760, RDDHA, RBDHA, MPDHA, and TN60013 have been submitted to GenBank and have been assigned accession numbers AJ971341, AJ971342, AJ971343, AJ971344, and AJ971345, respectively.

RESULTS

Antibiotic susceptibility. According to the criteria of the French Society for Microbiology (www.sfm.asso.fr/), the 12 isolates were highly resistant to amoxicillin, ticarcillin, and cefoxitin. Clavulanic acid did not significantly restore susceptibility to penicillins. The isolates were also resistant or showed decreased susceptibility to ceftazidime and cefotaxime, whereas they remained susceptible to cefepime and imipenem (Table 3). Antagonism between imipenem or cefoxitin and cefotaxime or ceftazidime was apparent in disk diffusion assay. This phenotype suggested the presence of an inducible AmpC-type β-lactamase. In four *K. pneumoniae* isolates (isolates TN58647, RDDHA, JDDHA, and TN60013), synergy between cefepime and amoxicillin-clavulanic acid was also observed, suggesting the presence of a class A extended-spectrum β-lactamase.

Transfer of resistance. *E. coli* J53-2 or *E. coli* C600 transconjugants were obtained by mating out with rifampin or nalidixic acid and cefoxitin selection (Table 1). Electroporation of plasmids extracted from *K. pneumoniae* STA1DHA and STA2DHA into *E. coli* DH10B successfully transferred cefoxitin resistance. The transconjugants and transformants exhibited an antagonism between imipenem and either cefotaxime or aztreonam. No transfer of resistance was obtained from *K. oxytoca* MPDHA or *K. pneumoniae* TN26033 either by mating

out or by electroporation. Moreover, the synergy between cefepime and amoxicillin-clavulanic acid observed in four donor strains was conserved in the transconjugant for one strain only, *K. pneumoniae* RDDHA, suggesting the transfer of both class A and class C β-lactamase-encoding genes.

Transconjugants showing typical synergy between clavulanic acid and broad-spectrum β-lactams were obtained by mating out strains TN58647, JDDHA, and TN60013 in *E. coli* C600 by using ceftazidime for selection.

Epidemiological analysis. According to the interpretation criteria of Tenover et al. (43), of the 11 *K. pneumoniae* isolates analyzed in this study, 9 different PFGE profiles were obtained (data not shown). Three isolates, isolates TN58647, JDDHA, and TN60013, had identical patterns, indicating their clonal origin (Table 1).

Plasmid DNA fingerprinting carried out with transconjugants or transformants identified similar plasmids in seven isolates (Fig. 1; Table 1).

Characterization of β-lactamases: PCR and DNA sequencing of *bla* genes. PCR experiments were carried out with the clinical isolates to detect bla_{DHA} and bla_{TEM} genes.

PCRs were positive for the three overlapping DHA fragments with all isolates. The analysis of the 2.2-kb DNA sequence yielded two ORFs in opposite orientations: *ampC* and *ampR*. This region was 98% identical to the *M. morganii* nucleotide sequence. For each isolate, the deduced amino acid sequence of the AmpC-like enzyme was 100% identical to that of DHA-1.

By PCR and sequence analysis, 4 of the 12 isolates were also shown to be TEM-1 positive (Table 1).

Likewise, the extended-spectrum β-lactamase characterized in the transconjugants obtained from strains TN58647, JDDHA, and TN60013 by selection with ceftazidime was SHV-2a and that of the transconjugant *E. coli* C600(pRDDHA) was SHV-2 (Table 1).

Characterization of the genetic contexts of bla_{DHA-1}. As bla_{DHA-1} from KF92 is inserted in a *sul1*-type integron that includes *orf513*, it was hypothesized that bla_{DHA-1} in the other isolates would exist in the same genetic configuration. Therefore, we used PCR to look for the presence of *orf513* in all isolates (PCR D) (46). PCR D was negative for eight isolates and positive for the remaining four isolates (Table 4). Therefore, we explored the genetic organizations of the bla_{DHA-1} genes in several ways: (i) isolates that were *orf513* positive were considered to include a backbone of an In6- and In7-like *sul1*-type integron, and they were explored by PCR mapping and cloning experiments. (ii) We considered that isolates that were *orf513* negative belonged to the same DHA-1 plasmid fingerprint and that, in these cases, bla_{DHA-1} would have the same genetic organization. TN60013, an *orf513*-negative strain, was then chosen for the cloning and sequencing of the *ampC* and *ampR* regions. Subsequently, the other isolates were tested by PCR mapping.

Isolates carrying *orf513*: characterization of the In6- and In7-like integrons. We used PCRs A, B, and F to successfully amplify fragments from the four *orf513*-positive isolates (isolates Kp760, RDDHA, RBDHA, and MPDHA; Table 4). These fragments overlapped a region typical of a complex *sul1*-type integron, from the *int* gene to CR1, with variable gene cassette arrays (Fig. 2) (46).

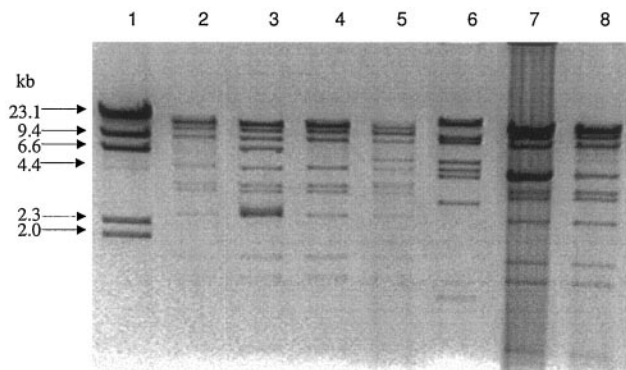

FIG. 1. Plasmid DNA fingerprinting after restriction with EcoRI. Lane 1, molecular size marker (Bio-Rad); lane 2, transconjugant *E. coli* J53(pJDDHA); lane 3, transformant *E. coli* DH10B(pSTA1DHA); lane 4, transconjugant *E. coli* J53(pTN58647); lane 5, transconjugant *E. coli* C600(pTN60013); lane 6, transconjugant *E. coli* J53(pRBDHA); lane 7, transconjugant *E. coli* J53(pIGR1DHA); lane 8, transconjugant *E. coli* J53(pIGR2DHA).

(i) In the integron from Kp760 we detected two gene cassettes, *aadB* and *aadA2*.

(ii) In the integron from RDDHA we detected three gene cassettes, *dhfr*, *arr2*, and *cmlA7*, and a fused gene cassette *oxa10-aadA1*. An open reading frame, *orf437*, of unknown function was found downstream of *dhfr* in an opposite orientation; this ORF encodes a 437-amino-acid protein that is 79% identical to that of *orfII* (GenBank accession number AAK54203). The *cmlA7* and *oxa10* genes are interrupted by two putative insertion sequences, IS_X and IS_Y, respectively (1,202 bp and 1,039 bp, respectively), that are homologous to ISXCD1 (68% protein identity) found in *Xanthomonas campestris* pv. dieffenbachiae (GenBank accession number AF263433) and to ISXo2 (45% protein identity) found in *Xanthomonas oryzae* pv. oryzae (GenBank accession number AAF61102). The transposase in both putative insertion sequences (ISs) was surrounded by two inverted repeats (IRs), with a direct repeat site found on the target flanking the IS: GGGC for *cmlA* and TGACCTTA for *oxa10*.

(iii) In the integron from strain RBDHA we detected four gene cassettes: *aac(6′)-Ib*, bla_{OXA-30}, *catB3*, and *arr-3*.

(iv) In the integron from strain MPDHA we detected two gene cassettes, *aac(6′)-IIc* and *ereA2*. *ereA2* is interrupted at position 188 by a 3,924-bp region surrounded by a 4-bp direct repeat site, TTAA. This 3,924-bp region included an insertion sequence, IS*1247*, previously described in *Xanthobacter autotrophicus* (45), which was characterized with imperfect IRs on either side. IS*1247* was followed by two ORFs whose protein products were 79% and 72% identical to aminoglycoside acetyltransferase *aac3-Vb* from *S. marcescens* (GenBank accession number Q01515) and a putative rifampin ADP-ribosyltransferase from *Rhodopseudomonas palustris* (accession number NP_948241), respectively.

We attempted PCR G with the four *orf513*-positive isolates to cover the region between CR1 and bla_{DHA-1}. However, PCR G was positive only for strain RDDHA. Thus, bla_{DHA-1} from RDDHA was mobilized from a 3,085-bp region of the *M. morganii* chromosome and inserted downstream from CR1 in

TABLE 4. Results for PCR mapping: length of PCR products

PCR[a]	PCR product length (kb)						
	pSAL-1	pKp760	pRDDHA	pRBDHA	MPDHA	pTN60013	SLM01
A	0.9	0.9	0.9	0.9	0.9	Negative	ND
B	1.9	2.5	9.8	4.1	7.1	ND	ND
F	1.9	1.9	1.9	1.9	1.9	ND	ND
D	0.6	0.6	0.6	0.6	0.6	Negative	ND
G	1.2	Negative	1.6	Negative	Negative	ND	ND
H	ND[b]	1.4	ND	Negative	Negative	ND	ND
I	ND	ND	ND	7.0	7.0	Negative	ND
J	ND	ND	ND	1.4	1.4	1.4	ND
K	ND	ND	ND	2.8	2.8	2.8	ND
L	ND	ND	ND	1.2	1.2	1.2	ND
M	0.6	0.6	0.6	0.6	0.6	0.6	0.6
N	0.9	0.9	0.9	0.9	0.9	0.9	0.9
O	1.1	1.1	1.1	1.1	1.1	1.1	1.1
P	2.6	2.6	2.6	Negative	2.6	2.6	ND
Q	ND	ND	ND	4.6	ND	ND	ND
R	Negative	0.6	0.6	Negative	Negative	Negative	ND
S	1.0	ND	ND	ND	1.0	1.0	ND
T	ND	ND	ND	ND	ND	2.1	ND
U	ND	0.8	ND	ND	ND	ND	0.8
i	ND	ND	ND	1.8[c]	1.8[c]	ND	ND
ii	ND	ND	ND	2.0[d]	2.0[d]	ND	ND

[a] i and ii, the library was obtained by digestion of genomic DNA.
[b] ND, not done.
[c] PvuII restriction genomic library.
[d] AviII restriction genomic library.

an In6- and In7-like *sul1*-type integron with a particularly long (9-kb) gene cassette region (Fig. 2).

By cloning the region encompassing *ampC* and *ampR* in strain Kp760, a recombinant plasmid (pKp760-2_{ind}) with an 8.1-kb insert that conferred inducible resistance to ceftazidime was selected. The insert sequence was determined (Fig. 2). A 5.9-kb region at the 5′ end of the insert that contained *ampC* and *ampR* was 98% identical to the *M. morganii* chromosome. The 3′ end of the insert includes a 3′-CS of an integron. We designed a reverse primer that binds to the 5′ region of the insert of pKp760-2_{ind} (PCR H) to generate an overlap between the CR1 and recombinant plasmid pKp760-2_{ind}. We used the overlapping fragments from PCRs A, B, F, and H and pKp760-2_{ind} to map the 13.5-kb region surrounding *bla*$_{DHA-1}$ in pKp760 (Fig. 2). The *ampC* and *ampR* genes were found to be inserted in a 6,133-bp region identified as the *M. morganii* chromosome. However, the first 561 bp was unknown in nucleotide data banks. We designed primers to generate an overlap between this unknown region and the available *M. morganii* sequence (GenBank accession number AF055067) (PCR U). PCR U was successful for both pKp760 and *M. morganii* SLM01. The nucleotide sequences of these PCR products were 99% identical. Therefore, we suggest that the entire 6,133-bp region comprising *ampC* and *ampR* came from the *M. morganii* chromosome. This 6,133-bp region is followed by a 3′-CS with the same deletion of *quacE*Δ*1* at the 5′ end (180 bp) observed in pSAL-1. A partial *tniB* gene was found downstream of *orf5* at the 3′ end of the insert in a reverse orientation and contained the EcoRI site (Fig. 2).

We then explored *bla*$_{DHA-1}$ in strains RBDHA and MPDHA. Although we assumed that the genetic organization was similar to that in Kp760, PCR H failed for both RBDHA and MPDHA. Therefore, we explored the unknown regions of RBDHA and MPDHA downstream from CR1 and downstream from *ampC* with the Universal Genome Walker kit. With both strains, a 1.8-kb PCR product was obtained downstream from CR1 (Table 4, PCR i). We characterized two ORFs whose predicted protein sequences were about 90% identical to the *sapB* and *sapA* products of the *sap* operon (ABC transporter family) from *Salmonella enterica* serovar Typhimurium LT2 (GenBank accession number AE008775). We analyzed a 2-kb PCR product obtained downstream from *ampC* in RBDHA and MPDHA (Table 4, PCR ii). The sequence of the 3′ end was identical to that of the *M. morganii* chromosome. The 5′ end provided several ORFs of the *psp* operon: *pspF*, *pspA*, *pspB*, and *pspC*. Both the *sap* and the *psp* operons are usually found in the genomes of members of the family *Enterobacteriaceae* and can be adjacent to each other, as in *Salmonella enterica* serovar Typhimurium LT2, section 79 of 220 of the complete genome, in which *sapA* is directly followed by *pspF*. Consequently, we attempted PCR I to link the region downstream from CR1 (s*apB*) and the region upstream from *M. morganii* (*pspF*). The products of PCRs I, J, and K were obtained from strains RBDHA and MPDHA, and the sequences were identical in both strains. The region between the end of CR1 (TATACCC site) and the beginning of the *M. morganii* region was found to be 10,317 bp long (Fig. 2). It includes a part of the *sap* operon at the 5′ end (3,049 bp), followed by an unknown region (4,216 bp) and then part of the *psp* operon at the 3′ end (3,052 bp).

The structure of the 3′-CS2 region present in pSAL-1 and in the *orf513*-containing integrons from strains RDDHA, RBDHA, and MPDHA was investigated by a PCR mapping approach

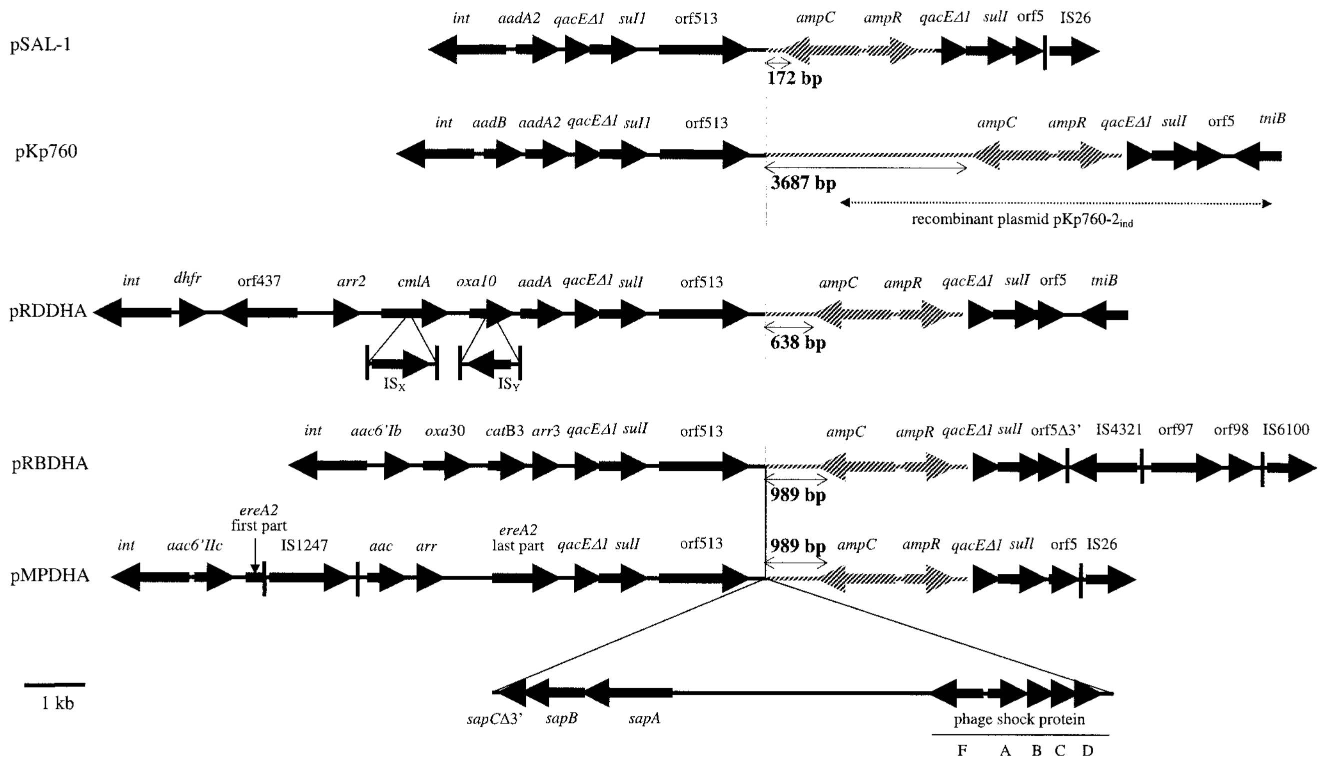

FIG. 2. Comparison of regions surrounding *orf513* and $bla_{\text{DHA-1}}$. Arrows with slashes, *M. morganii* region; vertical bars, IR sequences.

based on known sequence data (8, 32, 47). As expected, we identified the second copy of a 3′-CS (3′-CS2). However, variable structures were found downstream of *orf5* (Fig. 2). In strains KF92 and MPDHA, the IS*26* insertion sequence followed the 3′-CS2, as in strain TN60013, whereas a partial *tniB* was found downstream of 3′-CS2 in RDDHA, as in Kp760. In RBDHA, the last 314 bp of the 3′-CS2 is deleted by the insertion of an IS similar to IS*4321* (33). This is followed first by two ORFs similar to *orf97* (GenBank accession number AAR05756.1) and *orf98* (GenBank accession number AAR05757.1), as described in *S. enterica* serovar Typhimurium plasmid pU302L (GenBank accession number AY333434), and then by IS*6100*.

Isolates not carrying *orf513*. By cloning the region encompassing *ampC* and *ampR* in strain TN60013, we selected a recombinant plasmid (pTN60013-2_{ind}) with a 12-kb insert that conferred inducible resistance to oxyimino-cephalosporins, and we partially determined (10.2 kb) the sequence of the insert (Fig. 3). As IS*26* was found at the 3′ end of the recombinant plasmid, we looked for and detected IS*26* upstream from pTN60013-2_{ind} using PCR T (Table 4). The PCR T prod-

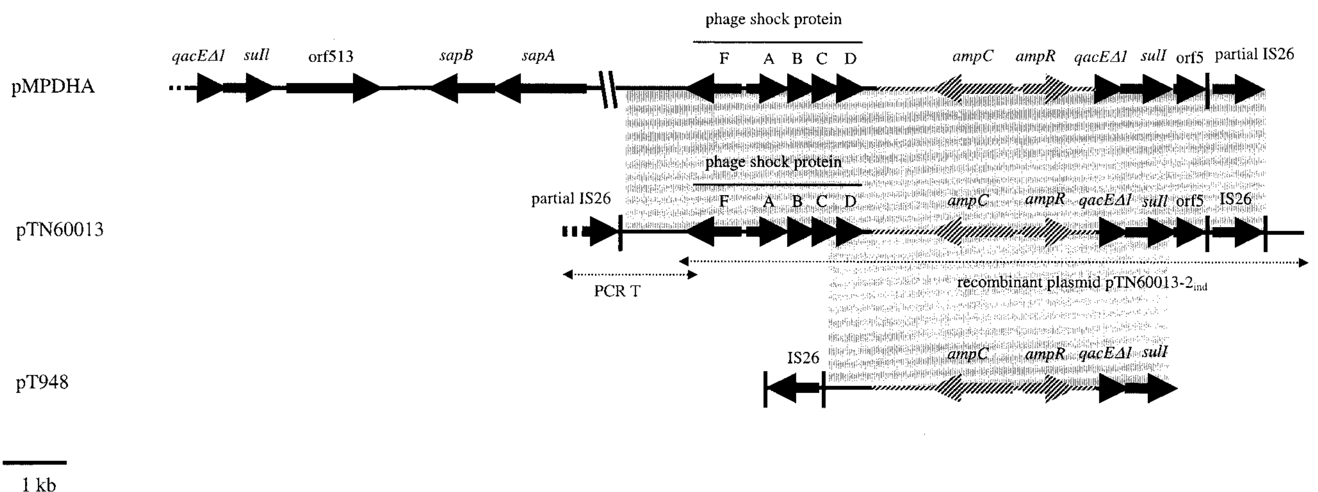

FIG. 3. Genetic organization of pTN60013 in comparison with those of pMPDHA and pT948. Arrows with slashes, *M. morganii* region; shaded region, regions of 100% identity; vertical bars, IR sequences.

uct, which overlapped the recombinant plasmid (pTN60013-2_{ind}), was sequenced; and this allowed us to map a 12.2-kb region surrounding bla_{DHA-1} in pTN60013.

The *ampC* and *ampR* genes are inserted in a 3,437-bp region that originated from the *M. morganii* chromosome. Downstream of *ampR* there is a 1,737-bp 3′-CS, including partial *qacE*Δ*1*, *sul1*, and *orf5* sequences, with the same length of deletion (180 bp) detected for 3′-CS2 in pSAL-1. This partial 3′-CS is followed by an insertion sequence, IS*26*, that includes a *tnpA* gene encoding a transposase oriented in the same way as *orf5*. Upstream from the *M. morganii* region, a 2,691-bp segment with five ORFs was characterized by using AMIGene software (7). Each protein product was 100% identical to the products of the *psp* operon in strains RBDHA and MPDHA: PspF, PspA, PspB, PspC, and PspD. Neither 3′-CS1 nor 5′-CS was found on plasmid pTN60013.

By using the results obtained with TN60013, several PCRs (PCRs J, K, L, P, and S) (Table 4) were then used to characterize the genetic context of bla_{DHA-1} in the other seven *orf513*-negative isolates. The PCR products and the restriction profiles obtained with these PCR products were the same for each isolate (data not shown), suggesting that the genetic environment of bla_{DHA-1} was at least similar, if not identical, in the eight *orf513*-negative isolates.

DISCUSSION

We have studied 12 DHA-1-producing clinical strains isolated between 1998 and 2003 from 12 patients in eight hospitals of the Parisian area. To date, all DHA-type class C β-lactamases described seem to be inducible. However, the expression in the strains isolated in the United Kingdom remains unknown (20). A previous study of *Salmonella* serovar Enteritidis KF92, which produces DHA-1, showed that *ampR* was mobilized together with *ampC* from the *M. morganii* chromosome and that the plasmid-encoded AmpR was functional (5). Most plasmid-mediated *ampC* genes are expressed constitutively, as they lack *ampR* mobilization (35). Few other inducible acquired non-DHA class C β-lactamases have been described: ACT-1 in a *K. pneumoniae* isolate from New York City in 1996 (36), CFE-1 in an *E. coli* isolate from Japan in 1997 (28), and CMY-13 in an *E. coli* isolate from Greece in 2001 (23). In these strains, *ampC* and *ampR* had the same configuration and had a high degree of identity with the corresponding chromosomally encoded genes of *Enterobacter asburiae* for ACT-1 (37) and *C. freundii* for CFE-1 and CMY-13. However, plasmid-mediated bla_{AmpC} originating from *C. freundii* is not often found with *ampR*. AmpC enzymes, for example, MIR-1 and CMY-2, therefore have a high level of constitutive expression (6, 18). By contrast, bla_{AmpC} originating from *M. morganii* is nearly always mobilized together with *ampR*. Why *ampC* alone or *ampC* and *ampR* are mobilized from a chromosome onto a plasmid has yet to be determined.

Many strains with plasmid-encoded AmpC enzymes also produce TEM-1, TEM-2, or even an ESBL such as SHV-5 (3, 18, 25). To date, DHA-producing *K. pneumoniae* strains isolated in Taiwan or Seoul, South Korea, have been shown to have acquired bla_{TEM-1} and, sometimes, an additional ESBL, either a CTX-M-type or SHV-type enzyme (30, 49, 50). Our study of 12 isolates of *K. pneumoniae* and *K. oxytoca* producing DHA-type β-lactamases showed that 4 isolates produced an ESBL of the SHV type. Epidemiological results and the β-lactam resistance profiles showed that three identical isolates produced SHV-2a, which was encoded by a plasmid different from that encoding DHA-1, while with another isolate, the DHA-1- and SHV-2-encoding determinants were cotransferred.

Among the three identical isolates, both TN58647 and TN60013 were isolated in the same medical care unit in Tenon Hospital in Paris in 1999 and 2002, respectively. Strain JDDHA was isolated in 2002 in Joffre-Dupuytren Hospital in the Parisian region. Although many patients admitted to Joffre-Dupuytren Hospital are transferred from either Tenon Hospital or St. Antoine Hospital, no unambiguous epidemiologic link could be established between these patients. The similarity of the plasmids of four other *K. pneumoniae* strains led us to conclude that these plasmids spread by horizontal transfer. The emergence of DHA-1 in the Parisian region is primarily due to the dissemination of one plasmid.

Previous outbreaks with AmpC-type β-lactamases (ACC-1 or CMY-type enzymes) have been described in Paris, with strains being either imported from a region of endemicity or isolated after a secondary horizontal spread inside the hospital (12, 27, 29). However, in our study, a common geographical origin could not be established for the DHA-1-producing strains, as most of the patients were French and had not traveled abroad immediately prior to infection. The higher rate of isolation in recent years suggests that DHA-1 β-lactamases are becoming more prevalent in the Parisian region, which may be a cause of concern for public health. Considering the number of hospitals affected, we believe that our study shows one of the most significant instances of the emergence of plasmid-mediated class C β-lactamases described in France.

Although DHA-1-producing strains have been present in the Far East for several years, there have been few data about the genetic organization of bla_{DHA-1}. We compared epidemiological data with the genetic organization for our 12 isolates. Seven *orf513*-negative isolates with the same plasmid fingerprint also had the same genetic organization: the 3,437-bp *M. morganii*-derived segment bounded by two directly repeated IS*26* elements within a 12.2-kb region (Fig. 3). The plasmid fingerprint of an eighth isolate, isolate TN26033, could not be obtained, as cefoxitin resistance was not transferable either by electroporation or by conjugation. However, we classified this isolate with the other seven strains because it had the same genetic organization.

Four strains differed from the eight *orf513*-negative isolates in their plasmid fingerprints and by the presence of an integron-type backbone in the environment of bla_{DHA-1}. These four strains all have the same complex *sulI*-type integron, similar to In6, In7, and pSAL-1, with a CR1 that includes *orf513*, which encodes a putative recombinase (42, 44, 46). We propose that these integrons be named InKp760, InRDDHA, InRBDHA, and InMPDHA (Fig. 2). As with similar integrons, the variable region (*M. morganii*) is inserted at the same right-hand boundary of CR1, which is presumed to be a site recognized by *orf513*. The deletion of the 5′ part of 3′-CS2 is the same for each bla_{DHA-1}-containing integron (180 bp), whereas this deletion is variable in other In6- and In7-type integrons (8, 38, 42, 44). However, each of the five DHA-1-carrying inte-

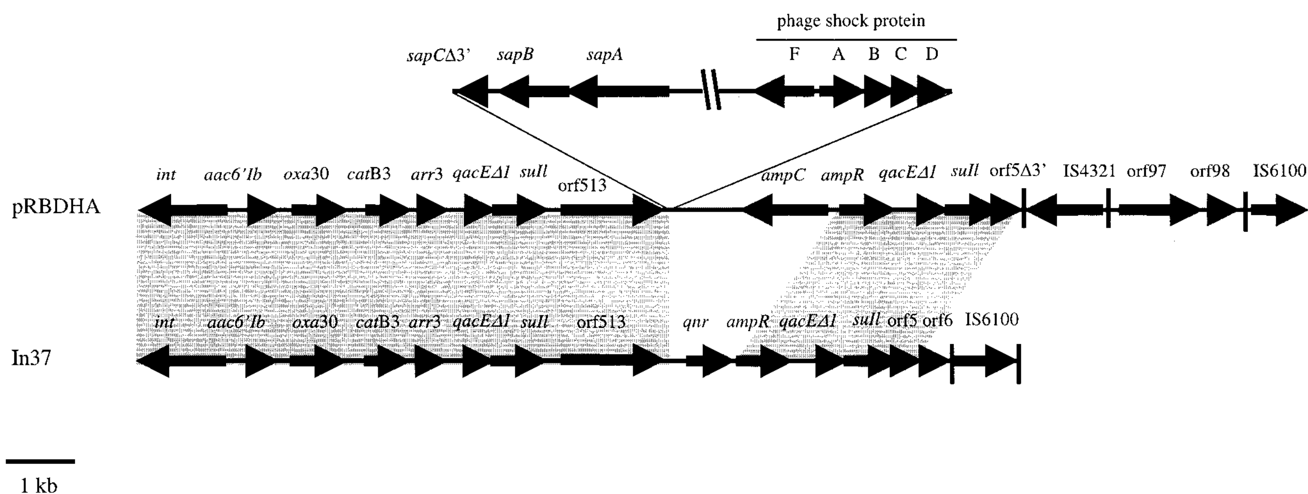

FIG. 4. Comparison of pRBDHA and In37. Shaded region, regions of 100% identity; vertical bars, IR sequences.

grons differs in several ways: (i) the gene cassette region varies in number and identification (Fig. 2). The integron of plasmid pSAL-1 carries the usual gene cassette *aadA2*, and the integron InKp760 carries the usual gene cassettes *aadB* and *aadA2* (Fig. 2).

The integron InRDDHA is unusual by the presence of ORFs that are not well characterized (Fig. 2). The first gene cassette, *dhfr*, is followed by *orf347*, which codes for a protein close to *orfII* and which has been described several times, always in a reverse orientation, in integrons comprising the $bla_{\text{VIM-2}}$ gene, in *Pseudomonas aeruginosa* YMC 95/1/704 (GenBank accession number AY029772), or in *Acinetobacter* genomospecies 3 YMC 99/11/160 (GenBank accession number AF369871). Following *orf347*, the last four genes cassette, *arr2*, *cmlA7*, *oxa10*, and *aadA*, are in the same array as part of In53, described in *E. coli* MG-1, which encodes an extended-spectrum β-lactamase, $bla_{\text{VEB-1}}$ (26). A notable difference with In53 is the presence of a putative IS-type structure inserted inside *cmlA7* and *oxa10*. Their presence most likely leads to the inactivation of both genes. To our knowledge, this is the first time that a putative IS-type structure has been found to be inserted inside a gene cassette. Their presence is surprising, as a gene cassette can simply be excised if its expression needs to be stopped. The *cmlA7* gene confers nonenzymatic chloramphenicol resistance and contains a promoter-like sequence (26). The fused gene cassette *oxa10-aadA1*, previously described by Naas et al. (26), likely provides its own promoter.

Integron InRBDHA carries four gene cassettes, identical to that found in In37 from an *E. coli* strain isolated in Shanghai, China, between March 2000 and March 2001 (Fig. 4): *aac6′Ib*, $bla_{\text{OXA-30}}$, *catB3*, and *arr3* (47).

The region of gene cassettes in integron InMPDHA is unusual, with the insertion of IS*1247* inside a well-characterized gene cassette, *ereA2*, which leads to the duplication of a 4-bp target site. We suggest that IS*1247* mediated the insertion of a large region of unknown origin comprising two new antibiotic resistance genes: a putative aminoglycoside acetyltransferase and a putative rifampin ADP-ribosyltransferase. Both genes will be explored in a future study.

(ii) In InRBDHA and InMPDHA, the integron-type backbone is unusual, with the insertion, between the CR1 and the *M. morganii* region, of an additional 10.3-kb large region that includes the *psp* and *sap* operons (Fig. 2).

(iii) The length of the *M. morganii* region is variable, with the length of the segment between CR1 and *ampC* varying—172 bp (strain KF92), 638 bp (strain RDDHA), 989 bp (strains RBDHA and MPDHA), and 3,687 bp (strain Kp760)—while the segment downstream from *ampR* is always 321 bp long (Fig. 2). The sequence at the left boundary of the *M. morganii* region is different for each strain. Thus, its insertion downstream from CR1 seems to be randomly determined and not target site specific.

(iv) The extension of the 3′-CS2 beyond *sulI* was only partially sequenced. However, we established differences between the five integrons (Fig. 2). Unfortunately, PCR mapping did not allow us to characterize the boundaries of the integrons with IRi and IRt.

The variability of such a complex *sulI*-type integron suggests that this structure evolves. Each different integron was found in a single isolate, which suggests an overall high rate of recombination events. On the contrary, the genetic organization of the $bla_{\text{DHA-1}}$ context in the *orf513*-negative isolates was conserved, with a mobile genetic element apparently capable of spreading. A trace of a *sul1*-type integron, a 3′-CS2, was present in the genetic organization of pTN60013 (Fig. 3). However, a complete integron was not found, with there being no 3′-CS1 or *int* gene present in KpTN60013. The presence of two directly repeated IS*26* copies suggests a composite transposon. This could have been involved in the integration of the 12.2-kb region that includes $bla_{\text{DHA-1}}$. However, the sequences beyond the IS*26* elements could not be determined; and it is unknown whether an 8-bp target site is duplicated, although no marked target selectivity has been described for IS*26* (21). This genetic organization is very close to that of a Chinese isolate of *K. pneumoniae* carrying $bla_{\text{DHA-1}}$ on the plasmid pT948 (GenBank accession number AY705809) (48). The segment comprising part of a *psp* operon, followed by the *M. morganii* region and then part of a 3′-CS2, is 100% identical in both plasmids pT948 and pTN60013 (Fig. 3). How-

ever, the orientation of IS*26* and the sequences adjacent to it differ in these two plasmids. Both of these plasmids are likely to have evolved from a common structure, with the IS*26* elements being inserted independently. Plasmids pMPDHA, pTN60013, and pT948 were analyzed; and the genetic organizations of $bla_{\text{DHA-1}}$ in the three plasmids were compared (Fig. 3). We suggest that pTN60013, as well as pT948, has evolved from a complex integron, such as that carried by pMPDHA, by the insertion of IS*26* upstream from the *psp* operon, for pTN60013, or inside the *psp* operon, for pT948. This then leads to the deletion of the first part of a complex integron from *int* to CR1.

Study of the spread of $bla_{\text{DHA-1}}$ in the Parisian region allowed us to describe the genetic organization of this acquired class C β-lactamase. The involvement of In6- and In7-type integrons, especially *orf513* including CR1, is clearly demonstrated in the horizontal transfer of antibiotic resistance genes. The function and mobility of *orf513*, which is assumed to interact with the right-hand boundary of CR1, remain to be explored. This study allowed the description of new IS or IS-like structures in the variable gene cassette region from *sulI*-type integrons.

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