ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 2008, p. 3898–3904 Vol. 52, No. 11
0066-4804/08/$08.00+0 doi:10.1128/AAC.00403-08

Genetic Structure Associated with *bla*$_{OXA-18}$, Encoding a Clavulanic Acid-Inhibited Extended-Spectrum Oxacillinase[▽]

Thierry Naas,[1]* Fatemeh Namdari,[1] Pierre Bogaerts,[2] Te-Din Huang,[3] Youri Glupczynski,[2,3] and Patrice Nordmann[1]

Service de Bactériologie-Virologie, INSERM U914: Emerging Resistance to Antibiotics, Hôpital de Bicêtre, Assistance Publique-Hôpitaux de Paris, Faculté de Médecine, Université Paris-Sud, Paris, France,[1] and Laboratoire de Bactériologie, Cliniques Universitaires Mont-Godinne,[2] and Laboratoire de Bactériologie, Clinique Universitaire Saint-Luc,[3] Université Catholique de Louvain, Louvain, Belgium

Received 25 March 2008/Returned for modification 28 May 2008/Accepted 18 July 2008

The genetic environment of the *bla*$_{OXA-18}$ gene encoding a peculiar clavulanic acid-inhibitable Ambler class D extended-spectrum β-lactamase was determined from the prototype OXA-18-producing *Pseudomonas aeruginosa* MUS clinical isolate. An 8.2-kb genomic DNA fragment containing *bla*$_{OXA-18}$ was cloned from *P. aeruginosa* MUS. Although most oxacillinases are located in integrons, *bla*$_{OXA-18}$ lacked gene cassette-specific features. It was bracketed by two duplicated sequences containing IS*CR19*, a novel insertion sequence of the IS*CR* family of mobile elements; Δ*intI1*, a truncated integrase gene; and a truncated Δ*aac6′-Ib* gene cassette. It is likely that IS*CR19* was at the origin of the *bla*$_{OXA-18}$ gene mobilization by a rolling-circle transposition event followed by homologous recombination. Furthermore, analysis of the cloned genomic DNA fragment revealed the presence of the integron-containing *bla*$_{OXA-20}$ gene. Concomitantly, three *P. aeruginosa* clinical isolates, displaying a synergy image as determined by double-disk diffusion tests on cloxacillin-containing plates, were isolated from three patients hospitalized in different wards over a 9-month period at the Saint-Luc University hospital (Brussels, Belgium). These isolates were positive by PCR for *bla*$_{OXA-18}$ and *bla*$_{OXA-20}$ genes, genetically related to *P. aeruginosa* MUS as determined by pulsed-field gel electrophoresis, and carried the same *bla*$_{OXA-18}$/*bla*$_{OXA-20}$-associated genetic structures. This report characterized the genetic elements likely at the origin of *bla*$_{OXA-18}$ gene mobilization in *P. aeruginosa* and suggests the spread of oxacillin-type extended-spectrum β-lactamases in *P. aeruginosa* at the Saint-Luc University hospital of Brussels, Belgium.

Pseudomonas aeruginosa is a predominantly nosocomial pathogen that is increasingly resistant to antibiotics (18). Whereas *P. aeruginosa* is naturally susceptible to most expanded-spectrum cephalosporins, resistance to these molecules may result from overexpression of the naturally occurring cephalosporinase and from acquired Ambler class A, B, and D extended-spectrum β-lactamases (ESBLs) (2, 18).

Clavulanic acid-inhibitable Ambler class A ESBLs have been identified in *P. aeruginosa* and include TEM-, SHV-, CTX-M-, PER-, GES-, and VEB-type and BEL-1 β-lactamases (1, 18, 21, 24, 27). Although the definition of ESBLs is often restricted to class A β-lactamases, several oxacillinases with extended-spectrum activity may be also included, and these have been termed OXA-ESBLs (19, 21, 24). These enzymes predominantly occur in *P. aeruginosa* and mostly derive from OXA-10 (OXA-11, -14, -16, and -17) or OXA-13, which is a 10-amino-acid derivative of OXA-10 (OXA-19 and -28) or to a lesser extent from OXA-2 (OXA-15 and -32) (19, 21, 24). Others OXA-ESBLs are unrelated to any broad-spectrum OXA enzymes, e.g., OXA-18 and OXA-45 (21, 25, 34). Although most OXA-type enzymes are resistant to β-lactamase inhibitors, OXA-18 and OXA-45 are well inhibited by clavulanic acid (19, 25, 34).

* Corresponding author. Mailing address: Service de Bactériologie-Virologie, Hôpital de Bicêtre, 78 Rue du Général Leclerc, 94275 Le Kremlin-Bicêtre Cedex, France. Phone: 33 1 45 21 29 86. Fax: 33 1 45 21 63 40. E-mail: thierry.naas@bct.aphp.fr.

▽ Published ahead of print on 28 July 2008.

The *bla*$_{OXA-18}$ gene was initially reported in France from the *P. aeruginosa* MUS isolate, along with another oxacillinase gene, *bla*$_{OXA-20}$ (22, 25). The *bla*$_{OXA-18}$ gene has recently been detected in a clinical epidemic clone of *P. aeruginosa* in Tunisia (15). Unlike the prototype OXA-18-producing *P. aeruginosa* MUS strain, the Tunisian isolates were *bla*$_{OXA-20}$ negative but positive for either TEM-1 or mostly for SHV-1 β-lactamases. In both studies, the *bla*$_{OXA-18}$ gene was chromosomally encoded (15, 25), and the Tunisian isolates were clonally related but different from the prototype OXA-18-producing *P. aeruginosa* MUS strain, as revealed by pulsed-field gel electrophoresis (PFGE) analysis (15).

IS*CR* are peculiar mobile elements, since they lack the terminal inverted repeats (IRs) typical of most insertion sequence (IS) elements (35). Instead, their termini are distinctive and have different functions (35). Their transposition mechanism may differ from that of most transposable elements in that it involves a rolling-circle (RC) transposition mechanism (6, 33, 35). The single protein encoded by each element is the cognate transposase, which is responsible for initiating replication at one end of the element (called *ori*IS) and is also believed to be involved in terminating replication at the other end of the element (called *ter*IS) (35). IS*CR* have been found to be associated with several resistance genes of different families of antibiotics that are disseminating (35).

In the present study, the genetic environment of the *bla*$_{OXA-18}$ gene has been characterized from the prototype strain *P. aeruginosa* MUS clinical isolate and compared to that

TABLE 1. Primers used in this study

Primer	No.[a]	Sequence (5′–3′)	Source or reference
OXA-18B	1	TTGGCATCGGAAAGCGAACC	25
OXA-18F	2	ATTTCAACGGTTTGCGACG	25
5′CSINV	3	GCTCCATAACATCAAACATC	This study
T3-666	4	GGGCGCAGATGGTGATGTCG	This study
T3.4	5	TCAGCTCGATGAAGGTTTCCA	This study
T7.2	6	GGAAACCTTCATCGAGCTGAT	This study
MD32.9	7	TCGGTCTCCACGCATCG	This study
T3-811	8	CCCCGATGGCGTCAACTGTG	This study
5′CS	9	GGCATCCAAGCAGCAAG	29
AAC6′Ib-B	10	CGTTTGGATCTTGGTGACCT	This study
STR-A-B	11	ATTGATCAACCGATAGGCTG	This study
OXA-20B	12	AGAATAGCACGCGCAATTGC	22
OXA-20F	13	CTGTTGTACTTGTCTCTCTTGG	22
Oxa18-GPS1	14	TTCCGGCTTGTAATCCCAG	This study
Oxa18-GPS2	15	GTCGATAACAAGCGTGCAGG	This study
Oxa18-GPS3	16	CATGGACAGGCTCCGTTGCAT	This study
Oxa18-GPS4	17	CACTCGACGATATGAGATCG	This study

[a] See numbering in Fig. 1.

of OXA-18 expressing *P. aeruginosa* strains isolated from patients hospitalized at Saint-Luc University hospital in Brussels, Belgium. Unlike most oxacillinases, bla_{OXA-18} was not in a form of gene cassette. The bla_{OXA-18} gene was surrounded by insertion sequences IS*CR19* of the IS*CR* family, likely at the origin of its acquisition. Furthermore, the present study demonstrates the ongoing emergence of this type of resistance determinant now in clinical *P. aeruginosa* isolates.

MATERIALS AND METHODS

Bacterial strains, plasmids, electroporation, and culture conditions. The clinical *P. aeruginosa* isolate MUS produces OXA-18 and OXA-20 β-lactamases (22, 25). *P. aeruginosa* 1-63, *P. aeruginosa* 1-52, and *P. aeruginosa* 1-22 strains were isolated in 2006 and in 2007 from in-patients hospitalized at Saint-Luc University Hospital, Brussels, Belgium (Table 1), and were identified by conventional microbiological methods (API-20NE; bioMérieux, Marcy l'Etoile, France).

Electrocompetent *Escherichia coli* DH10B (Invitrogen, Eragny, France) and *P. aeruginosa* KG2505, which does not express the naturally and chromosome-encoded AmpC β-lactamase and is deficient for the multidrug efflux pump MexAB-OprM (23) were used as a recipient in electroporation experiments. *E. coli* J53Az^R strain, which is resistant to sodium azide, and ciprofloxacin-resistant *P. aeruginosa* PU21 (12) were used for conjugation experiments. *E. coli* 50192 was used as a source of high size plasmid marker (12, 25). The plasmid vector pBKCMV carrying a kanamycin resistance marker was used for cloning experiments (25). Bacterial cells were grown in Trypticase soy (TS) broth and on TS agar plates (Bio-Rad, Marnes-La-Coquette, France).

Antimicrobial agents and susceptibility testing. Routine antibiograms were determined by the disk diffusion method on Mueller-Hinton (MH) agar (Bio-Rad). The antimicrobial agents and their sources have been described elsewhere (25). MICs of β-lactams were determined and interpreted as described previously (4). The double-disk synergy test was performed with expanded-spectrum cephalosporins and ticarcillin-clavulanic acid disks on MH cloxacillin (250 μg/ml)-containing agar plates (13, 20).

Plasmid content, mating out, and electroporation experiments. Direct transfer of resistance into azide-resistant *E. coli* J53 and ciprofloxacin-resistant *P. aeruginosa* PU21 was attempted as previously reported (20). Plasmids were introduced by electroporation into *E. coli* DH10B (25) and *P. aeruginosa* KG2505 (23, 31) using a Gene Pulser II (Bio-Rad). The mating cultures were plated onto TS agar plates containing ticarcillin (100 μg/ml) and sodium azide (100 μg/ml) or ciprofloxacin (15 μg/ml), and the electroporants were plated onto on TS agar plates containing ticarcillin (100 μg/ml).

Nucleic acid extractions. Recombinant plasmids were extracted by using Qiagen plasmid Mini-Midi kits (Qiagen, Courtaboeuf, France), whereas natural plasmids were extracted according to the Kieser technique (16). Plasmid extracts were subsequently analyzed by electrophoresis on a 0.7% agarose gel. Total DNA from *P. aeruginosa* isolates was extracted as described previously (25).

Cloning experiments and analysis of recombinant plasmids. Unless specified, standard molecular techniques were used (30). Whole-cell DNAs were extracted as described previously (25). The ligation products of HindIII-digested total DNA of *P. aeruginosa* MUS into HindIII-restricted pBKCMV were electroporated into *E. coli* DH10B, and selection was performed on TS agar plates containing amoxicillin (100 μg/ml) and kanamycin (30 μg/ml).

PCR screening and genetic environment of bla_{OXA-18}. *Taq* DNA polymerase was from Roche Diagnostics (Meylan, France). Standard PCR amplification experiments (20, 25) were attempted. Primers specific for genes coding for the β-lactamases OXA-10, OXA-20, OXA-18, TEM, SHV, PER, VEB, GES, and BEL have been detailed previously (20, 21, 22, 25, 28, 27). The PCR products were purified by using QIAquick columns (Qiagen).

PCR experiments were performed on an ABI 2720 thermocycler (Applied Biosystems, Les Ulis, France) using laboratory-designed primers (Table 1) as previously described (25). PCR products were then analyzed on an agarose gel and sequenced.

Mapping the bla_{OXA-18} transcription start site. Reverse transcription and RACE (rapid amplification of cDNA ends) were performed with a 5′RACE system (version 2.0; Invitrogen). Then, 5 μg of total RNAs extracted from *P. aeruginosa* MUS (Qiagen RNeasy Maxi kit) was used to determine the bla_{OXA-18} initiation site of transcription.

After a reverse transcription step with gene-specific primer OXA-GSP1 and reverse transcriptase, the cDNA was tailed with cytosines by using the terminal deoxynucleotidyltransferase and was subsequently amplified with another gene-specific primer, OXA-GSP2 combined with an oligo-dG adapter primer provided with the kit. This PCR product was used as a template for a nested PCR assay with a second adapter-primer (provided with the kit) and OXA-GSP3 primer located at the very beginning of bla_{OXA-18} or OXA-GSP4 primer located at the very beginning of *aac6′-Ib* gene (Table 1). The amplicon obtained was directly sequenced. The transcription initiation site was determined as the first nucleotide following the sequence of the adapter primer.

DNA sequencing and protein analysis. Both strands of the PCR products and of the cloned DNA fragment of recombinant plasmid pJOA-1 were sequenced by using laboratory-designed primers with an automated sequencer (ABI Prism 3100; Applied Biosystems). The nucleotide and the deduced protein sequences were analyzed by using software available at the National Center of Biotechnology Information website (http://www.ncbi.nlm.nih.gov).

Genotyping and hybridization. PFGE was performed using SpeI (Amersham Biosciences) as previously described (10). SpeI macrorestriction patterns were interpreted according to the recommendations of Tenover et al. (32).

DNA-DNA hybridizations were performed as described by Sambrook et al. (30), with a Southern transfer of a PFGE agarose gel that contained total DNA of *P. aeruginosa* isolates. The probe consisted of a 600-bp PCR-generated fragment from recombinant plasmid pJOA-1 and was internal to the bla_{OXA-18} gene. Labeling of the probe and signal detection were carried out by use an ECL nonradioactive labeling and detection kit (Amersham Biosciences) according to the manufacturer's instructions.

IEF analysis. β-Lactamase extracts were prepared as described previously (10, 20) and subjected to analytical isoelectric focusing (IEF) on a pH 3.5 to 9.5 ampholine polyacrylamide gel (Amersham Biosciences), as described elsewhere (25).

Nucleotide sequence accession number. The nucleotide sequences reported in the present study have been assigned to the EMBL/GenBank nucleotide database under the accession no. EU503121. The nucleotide sequences of the IS*CR* insertion sequences reported in the present study have been submitted to M. Toleman and assigned the number IS*CR19*.

RESULTS

Cloning of the bla_{OXA-18} gene from *P. aeruginosa* MUS. HindIII-restricted genomic-DNA of *P. aeruginosa* MUS was cloned into pBKCMV vector. Several *E. coli* transformants were obtained for the cloning experiment and selected on medium supplemented with kanamycin and amoxicillin. Two phenotypes were observed: an AmpC phenotype that was not further studied (data not shown) and an ESBL phenotype that corresponded to *E. coli* containing a recombinant plasmid, pJOA-1, with an 8.2-kb HindIII insert (Fig. 1) that was further analyzed.

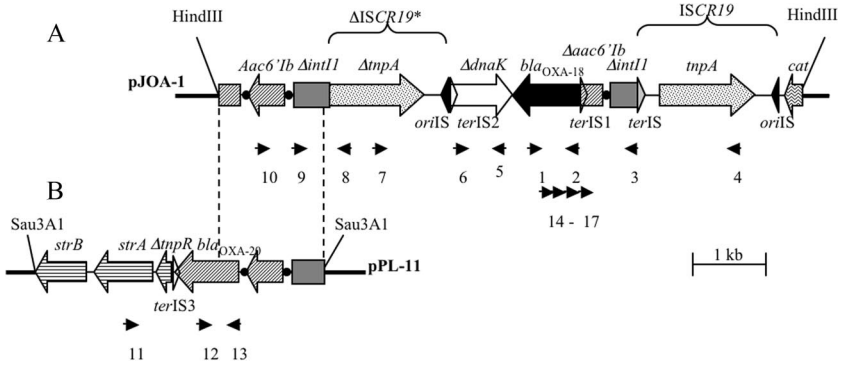

FIG. 1. Schematic representations of the genetic environment of the bla_{OXA-18} gene (A) and the bla_{OXA-20} gene (B) (22) in *P. aeruginosa* MUS. The coding regions are shown as boxes, with an arrow indicating the orientation of transcription. Black circles indicate the integron- and gene cassette-specific recombination sites *attI* and *attC*, respectively. Restriction sites that were used for cloning are indicated. Primers used for PCR-mapping experiments are indicated by small horizontal arrows (Table 1). Vertical dashed lines indicate the identity between the plasmids pJOA-1 and pPL11. Filled and empty triangles represent IS*CR*-specific sequences (*ori*ISs are black and *ter*ISs have the color of the ORF they are located within).

Characterization of the genetic environment of the bla_{OXA-18} gene in *P. aeruginosa* MUS. The nucleotide sequence of the ~8.2-kb insert of plasmid pJOA-1 was determined and revealed several open reading frames (ORFs) (Fig. 1). The immediate genetic environment of bla_{OXA-18} gene was identical to that previously described (25), being upstream of a 3′-truncated *aac6′-Ib* (Δ*aac6′-Ib*) gene cassette and downstream a 5′-truncated gene that codes for a putative chaperone protein DnaK that shares 79% sequence identity with a gene from *Rhizobium etli* CFN42 (80% amino acid identity) (9, 11, 25).

Further upstream of the Δ*aac6′-Ib* gene cassette a class 1 integron-specific recombination site, *attI*, preceded by the 5′ end of an integrase gene, *intI1*, of class 1 integrons was identified (Δ*intI1*) (8, 9). The Δ*intI1*gene was interrupted by a novel insertion sequence of the IS*CR* family, termed IS*CR19*.

Downstream of Δ*dnaK*, a truncated copy of IS*CR19* was found that lacked 300 bp, including its *ter*IS and the 40 first amino acids of the transposase gene. This truncated copy shared 92% nucleotide identity with IS*CR19* and was thus named ΔIS*CR19**. At the site of truncation, ΔIS*CR19** was fused to another truncated copy of the *intI1* gene (Δ*int*I*1*) in opposite orientation (Fig. 1). Farther downstream, another copy of the *aac6′-Ib* gene cassette followed by the 5′ end of bla_{OXA-20} gene cassette was identified. The sequence downstream of bla_{OXA-20} was matched by PCR to be identical to that characterized on plasmid pPL11 (Fig. 1B) (22).

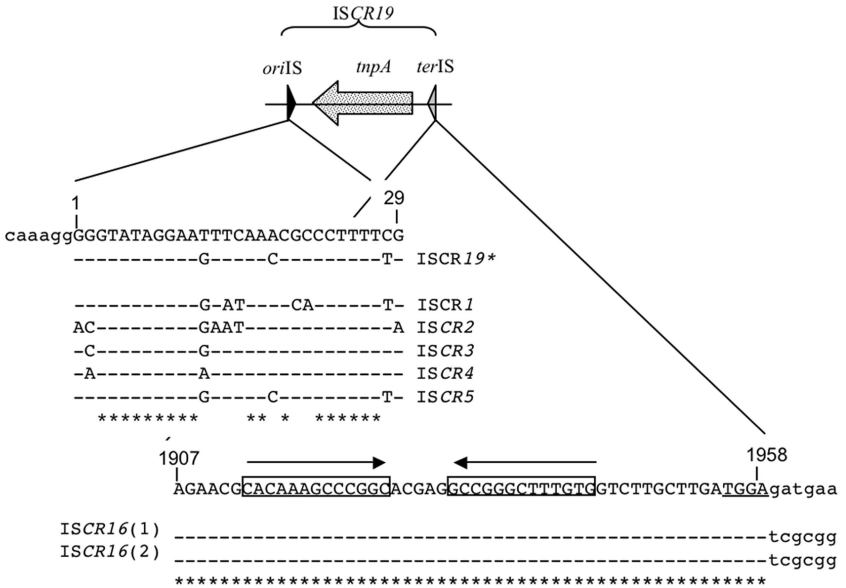

FIG. 2. Alignment of the *ori*IS and *ter*IS of IS*CR19* with that of IS*CR* elements. *ori*IS and *ter*IS are the initiation and termination sites of IS*CR19* transposition, respectively, and *tnpA* represents the transposase gene. Identical bases compared to IS*CR19* are indicated by dashes, and conserved bases found in all of the sequences are indicated by asterisks. (Top panel) Alignment of the first 29 bp of the various IS*CR* elements with those of IS*CR19*, showing *ori*IS. (Bottom panel) Alignment of *ter*IS of IS*CR19* with the sequences found at the equivalent termini of IS*CR16.* IRs are underlined. Accession numbers are given in the text.

Sequence analysis of IS*CR19* and IS*CR19.** IS*CR19* is 1,958 bp long and is delimited by two sequences: *ori*IS and *ter*IS (Fig. 2). IS*CR19* belongs to the IS*CR3*/IS*CR5* group (88% nucleotide identity with IS*CR5*) and is structurally related to the IS*CR16* elements found in the avian pathogenic *E. coli* plasmid pAPEC-01-R (14) (GenBank no. DQ517526) and in the *Salmonella enterica* plasmid sequence (36) (GenBank no. CP000604) (91% nucleotide identity). The *ori*IS sequence is located 245 bp downstream of the stop codon of the transposase gene. This sequence is conserved and matches those of well-characterized IS*CR*s (35) (Fig. 2).

The *terIS* sequence is often difficult to determine precisely for IS*CR*s, since the flanking sequences are often identical or deleted (35). In the case of IS*CR19*, similar elements, such as IS*CR16* (sharing 90% nucleotide identity), have been characterized on naturally occurring plasmids (14, 36). Alignment of the region encompassing the beginning of the transposase gene up to the flanking sequences revealed sequence identity until a TGGA motif (Fig. 2), thus suggesting the likely end of the IS*CR19*/IS*CR16* elements. Furthermore, as suggested for *ter*IS sequences of other IS*CR* elements (35), the IS*CR19*/IS*CR16 ter*IS sequences contain a 13-bp IR region. To date, IS*CR19* is associated only with the bla_{OXA-18} gene in a manner similar to that of IS*CR5*, which is exclusively associated with OXA-45, another OXA-ESBL (35).

IS*CR19** is 1,663 bp long and is delimited by an *ori*IS that is very close to that of IS*CR19* (3 changes out of 29). The *ori*IS sequence is located 259 bp downstream of the stop codon of the transposase gene. This sequence is conserved and matches well with those of well-characterized IS*CR*s (35) (Fig. 2).

Mapping of the bla_{OXA-18} transcription start site. Computer-assisted promoter analysis had suggested that the promoter upstream of the bla_{OXA-18} gene was 84 bp from the translational start site (25). However, using 5′RACE PCR experiments, we could not confirm this site; instead, the site of initiation of transcription of the bla_{OXA-18} gene was mapped in *P. aeruginosa* MUS to be 690 bp upstream of the translational start codon, corresponding to the class 1 *Pc* (formerly known as *Pant*) promoter located in the integron (7, 8; data not shown).

Identification of the bla_{OXA-18} gene in Belgian *P. aeruginosa* isolates. ESBL-producing ceftazidime-resistant isolates of *P. aeruginosa* were isolated from respiratory tract specimens of three patients over a 9-month period in 2006 and 2007 at the Saint-Luc University Hospital in Brussels, Belgium. In the three patients, the *P. aeruginosa* isolates were recovered at least 48 h after admission, suggesting that the organisms were hospital acquired. The patients had been hospitalized in different wards at different time periods, and no common source or diagnostic invasive procedures could be found. The three isolates were resistant to ticarcillin, aztreonam, and ceftazidime, but they were susceptible to ticarcillin-clavulanate, piperacillin, and piperacillin-tazobactam and intermediate to meropenem and cefepime according to Clinical and Laboratory Standards Institute breakpoints. A synergy image could be observed for the three isolates between ceftazidime, cefepime, and ticarcillin-clavulanic acid on MH agar only with disks placed 2 cm apart and on cloxacillin-containing plates. PCR amplification identified bla_{OXA-18} and bla_{OXA-20} in these isolates. Sequencing of the PCR fragments revealed 100% sequence identity with the previously described bla_{OXA-18} and bla_{OXA-20} genes (22, 25). These results were confirmed by IEF analysis. β-Lactamase extracts of cultures of *P. aeruginosa* 1-52, 1-63, 1-22, and MUS that were subjected to analytical IEF expressed three β-lactamases with pI values of 5.5, 6.0, and 8.6, respectively, a finding consistent with those of β-lactamases OXA-18, OXA-20, and AmpC, respectively, from *P. aeruginosa* (23).

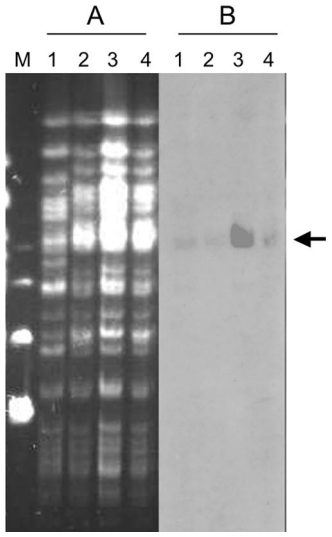

FIG. 3. Molecular comparison of bla_{OXA-18}-producing *P. aeruginosa* isolates. (A and B) PFGE with SpeI-restricted DNA (A) and bla_{OXA-18} hybridization of the SpeI PFGE gel (B). Lane 1, *P. aeruginosa* MUS; lane 2, *P. aeruginosa* 1-63; lane 3, *P. aeruginosa* 1-22; lane 4, *P. aeruginosa* 1-52. Molecular weight markers (lane M) correspond to the lambda ladder (Bio-Rad). The arrow indicates the 200-kb band that hybridizes with the OXA-18 specific probe.

Plasmid content and transfer of resistance. No plasmid DNA was detected in *P. aeruginosa* 1-52, 1-63, and 1-22 isolates despite repeated analyses. Transfer by electroporation of the ticarcillin resistance marker from *P. aeruginosa* 1-52, 1-63, 1-22, and MUS isolates to *E. coli* J53 or *P. aeruginosa* PU21 failed, suggesting a chromosomal location for the β-lactamase genes. These results are in agreement with those found previously for *P. aeruginosa* MUS (25).

Strain typing and bla_{OXA-18}-bla_{OXA-20} genetic environment. PFGE analysis using the SpeI restriction enzyme revealed only slight differences between the three Belgian *P. aeruginosa* isolates, thus suggesting their epidemiological relationship. These strains differed, however, more extensively from *P. aeruginosa* MUS (more than seven-band differences [Fig. 3A]). The SpeI-restricted DNA separated on the PFGE gel was transferred onto a nylon membrane and hybridized with an internal bla_{OXA-18}-specific probe. A hybridization signal of high molecular weight (ca. 200 kb) (Fig. 3B) was detected for all *P. aeruginosa* isolates, indicating that the genomic DNA fragment carrying the bla_{OXA-18} gene is likely the same in all isolates.

Using the genetic environment determined for the bla_{OXA-18} gene in *P. aeruginosa* MUS, primers were designed to amplify the genetic environment in the Belgian strains (Fig. 1). Similar-sized PCR products were obtained for all of the *P. aeruginosa* isolates (data not shown), and subsequent sequencing of these fragments revealed identical genetic environments.

DISCUSSION

The most common mechanisms of resistance to oxyimino-cephalosporins in *P. aeruginosa* correspond to overexpression of the AmpC chromosomal enzyme (18). The prevalence of ESBLs in *P. aeruginosa* is variable depending on the type of ESBL and the geographic origin (10, 17, 21). The prevalence of OXA-ESBLs is difficult to estimate, most of these enzymes being reported in single *P. aeruginosa* clinical isolates from Turkey, France, and Korea (21). OXA-18 had been identified previously from a single *P. aeruginosa* MUS clinical isolate in France in 1996 (25) and 10 years later in Tunisia in 2006, during an outbreak involving a *P. aeruginosa* clone that was unrelated to *P. aeruginosa* MUS (15) In the present study, OXA-18-producing isolates from Belgium were also detected and compared to the prototype *P. aeruginosa* MUS strain.

The three OXA-18-producing *P. aeruginosa* isolates were gathered over a 9-month period from patients located in three different wards of the same hospital. The patients had not been in contact with one another during hospitalization and had no apparent source exposure or invasive procedure in common. The three *P. aeruginosa* strains were deemed to be colonizing rather than pathogenic organisms (data not shown). The prevalence of this OXA-18-producing *P. aeruginosa* strain in the hospital is not known, but it might be present on an endemic basis, probably due to underdetection, especially when chromosomal cephalosporinase is overexpressed.

The failure to identify a plasmid suggested that the gene encoding OXA-18 is chromosomally mediated, which is in agreement with previous studies (15, 22, 25). The Belgian *P. aeruginosa* isolates were closely related to each other as determined by PFGE analysis, suggesting a strong epidemiological link between these isolates. The three Belgian strains were different from *P. aeruginosa* MUS. However, detailed analysis of the PFGE patterns revealed some genetic relatedness between *P. aeruginosa* MUS and the Belgian isolates, especially the bla_{OXA-18}-containing fragment that is conserved in all of the strains. Moreover, sequencing analysis confirmed that the immediate genetic environments of bla_{OXA-18} and bla_{OXA-20} were identical in French and Belgian strains. The recently identified OXA-18-producing *P. aeruginosa* isolates from Tunisia were bla_{OXA-20} negative (15) (but positive for either SHV-1 or, less frequently, TEM-1) and genetically different from *P. aeruginosa* MUS, thus suggesting that at least two OXA-18-producing *P. aeruginosa* clones are currently identified worldwide.

The genetic environment of bla_{OXA-18} was different from that of most oxacillinase genes, since this gene was not located in an integron, in contrast to the bla_{OXA-10}, bla_{OXA-2}, and bla_{OXA-1} genes of *P. aeruginosa* (19, 21), and it was not composite transposon-borne, such as the bla_{OXA-48} gene of *K. pneumoniae* (3) and the bla_{OXA-23} and bla_{OXA-58} genes of *A. baumannii* (5, 26). Rather, bla_{OXA-18} was associated with IS*CR* elements. IS*CR* elements have been found associated with many resistance genes, including β-lactam resistance genes of different Ambler classes (2). The only other example of an oxacillinase gene associated with IS*CR* elements is bla_{OXA-45} gene, which is associated with the IS*CR5* element. Interestingly, OXA-45 is another OXA-ESBL and IS*CR19*, like IS*CR5*, belongs to a subset of IS*CR3*-type elements (35). bla_{OXA-18} was surrounded by two copies of IS*CR19*-like elements that share 92% sequence identity. It is likely that these elements derive from a common ancestor and have diverged (35). Insertions of IS*CR* have been reported to provide downstream inserted genes with a promoter sequence (35). Here, the element seems not to fulfill this function, but IS*CR19*, by mobilizing the bla_{OXA-18} gene into the *aac6′-Ib* gene, allowed its expression by the 5′ conserved sequence (CS)-located promoter *Pc* (formerly known as *Pant*) that is equally well recognized in *P. aeruginosa*, *A. baumannii*, and *Enterobacteriaceae* (3, 7, 8).

IS*CR19* may have likely been at the origin of bla_{OXA-18} gene

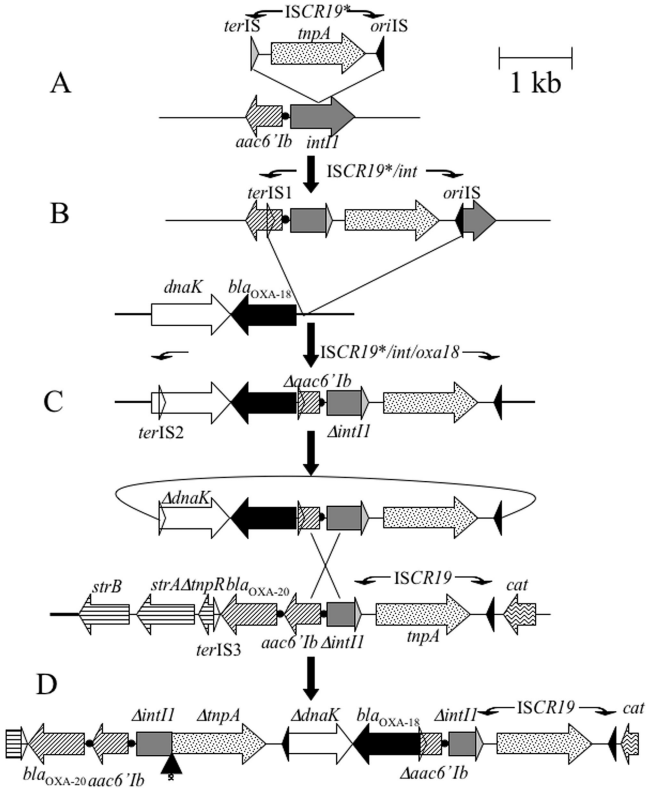

FIG. 4. Proposed model of IS*CR19*-mediated mobilization of bla_{OXA-18} and genesis of a bla_{OXA-18}-containing *intI1* complex class 1 integron. The construction of bla_{OXA-18}-containing complex class 1 integrons has been inspired from the model proposed by Toleman et al. Several steps are necessary. (A) Insertion of IS*CR19** into a class 1 integrase gene. Aberrant RC replication of the IS*CR19** element (inserted into the *int1* gene) generates a transposition intermediate starting at *ori*IS, ending at another *ter*IS, and then its cognate *ter*IS at *ter*IS1, located inside the *aac6′-Ib* gene. (B) This intermediate then transposes adjacent to the bla_{OXA-18} gene in another location. (C) A second aberrant RC replication event produces circular intermediates that now include the bla_{OXA-18} gene. (D) These circular intermediates may then be rescued by recombination events between *aac6′-Ib* or integrase genes on another a class 1 integron already including a copy of IS*CR19*, generating the bla_{OXA-18}-containing complex integron. Boxes represent the ORFs of the genes, with arrows indicating the direction of their transcription. The transposase gene of the IS*CR19* elements are dotted, the integrase gene is shaded in gray, and the bla_{OXA-18} gene is black. The integrase-specific recombination sites are indicated as black dots. *ori*IS is represented as an black triangle, *ter*IS is represented as a gray triangle, and *ter*IS1, -2, and -3 represent secondary *ter*IS (open triangles). The vertical arrow in panel D indicates the deletion event that occurred fusing the 5′CS sequence with that of *tnpA* of IS*CR19**.

mobilization. At some time in the past, the IS*CR19** transposed into the 5′CS (integrase gene, *intI1*) of a class 1 integron (Fig. 4A). From this point on, IS*CR19** is able to mobilize part of the integrase gene and any antibiotic resistance gene cassettes therein by an IS*91*-like RC mechanism possibly recognizing various putative termination sequence (*ter*IS-1, *ter*IS-2 or *ter*IS-3 in Fig. 1 and 4), similar or not to its original and cognate *ter*IS. It is also possible that IS*CR19** does not possess an intrinsic termination site and, accordingly, terminates transposition randomly, thereby mobilizing varied lengths of 5′-located (upstream) DNA. As suggested for IS*91*-like elements (6) (Fig. 4C), free circular intermediates could be generated, carrying IS*CR19* and sequences adjacent to it and distal to *ori*IS, including at least the truncated 5′CS and the linked resistance gene. These circular entities can then in turn insert by transposition or be rescued by homologous recombination into either the 5′CS of conventional class 1 integrons or that of an integron-IS*CR19*-like variant, which seemed to be the case here. The arrangement that would arise from the recombination event (Fig. 4C and D) is that there would be a direct duplication of the IS*CR* element following the duplication of the Δ5′CS-Δ*aac6′-Ib*.

While IS*CR1* or IS*CR3* and IS*CR4* elements mobilize adjacent DNA sequences that are subsequently rescued by homologous recombination via flanking 3′CS sequences or via flanking *groEL* sequences, respectively (35), IS*CR19* mobilizes adjacent DNA sequences by homologous recombination via flanking Δ5′CS-Δ*aac6′-Ib* sequences. IS*CR19** appeared to have lost its original *ter*IS sequence (Fig. 4D), possibly by a deletion event linking *intI1* to the 5′ end of the transposase gene. A similar deletion appeared with IS*CR4*, which has lost its original *ter*IS sequence, possibly by a deletion event linking *groEL* to the 5′ end of IS*CR4* (35). In our case, however, due to interruption of the transposase gene, it is likely that IS*CR19** can only function when a transposase is provided in *trans*. It is, however, not possible to clearly position this deletion event in our transposition model; the deletion could have occurred early and then the transposase would have been trans-complemented by the intact IS*CR19*-copy, or it could have occurred at a very late stage.

The present study highlights the spread of OXA-18-producing *P. aeruginosa* isolates in a Belgium hospital and suggests that their prevalence might be underestimated due to their underdetection. Furthermore, the present study identified a novel IS*CR* element, IS*CR19* associated with the bla_{OXA-18} resistance gene and likely at the origin of its genetic mobilization. Clinically, the most interesting aspect of IS*CR* elements is that they are increasingly being reported with powerful resistance determinants, such as metallo-β-lactamases and now OXA-ESBLs.

ACKNOWLEDGMENTS

We thank C. Bauraing for assistance in preliminary PCR experiments. We are grateful to T. Walsh and M. Toleman for helpful discussions.

This study was funded by a grant from the Ministère de l'Education Nationale et de la Recherche (UPRES-EA3539); the Université Paris XI, Paris, France; and mostly by the European Community (6th PCRD, LSHM-CT-2005-018-705).

REFERENCES

1. **Al Naiemi, N., B. Duim, and A. Bart.** 2006. A CTX-M extended-spectrum beta-lactamase in *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia*. J. Med. Microbiol. **55:**1607–1608.
2. **Ambler, R. P., A. F. Coulson, J.-M. Frere, J. M. Ghuysen, B. Joris, M. Forsman, R. C. Levesque, G. Tiraby, and S. G. Waley.** 1991. A standard numbering scheme for the class A beta-lactamases. Biochem. J. **276:**269–270.
3. **Aubert, D., T. Naas, C. Héritier, L. Poirel, and P. Nordmann.** 2006. Functional characterization of IS*1999*, an IS*4* family element involved in mobilization and expression of beta-lactam resistance genes. J. Bacteriol. **188:** 6506–6514.
4. **Clinical and Laboratory Standards Institute.** 2005. Performance standards for antimicrobial susceptibility testing; 15th informational supplement. M100-S15. Clinical and Laboratory Standards Institute, Wayne, PA.
5. **Corvec, S., L. Poirel, T. Naas, H. Drugeon, and P. Nordmann.** 2007. Genetics and expression of the carbapenem-hydrolyzing oxacillinase gene bla_{OXA-23} in *Acinetobacter baumannii*. Antimicrob. Agents Chemother. **51:**1530–1533.
6. **Del Pilar Garcillán-Barcia, M., I. Bernales, M. V. Mendiola, and F. de la Cruz.** 2001. Single-stranded DNA intermediates in IS*91* rolling-circle transposition. Mol. Microbiol. **39:**494–501.
7. **Depardieu, F., I. Podglajen, R. Leclercq, E. Collatz, and P. Courvalin.** 2007. Modes and modulations of antibiotic resistance gene expression. Clin. Microbiol. Rev. **20:**79–114.
8. **Fluit, A. C., and F. J. Schmitz.** 2004. Resistance integrons and super-integrons. Clin. Microbiol. Infect. **10:**272–288.
9. **Galimand, M., T. Lambert, G. Gerbaud, and P. Courvalin.** 1993. Characterization of the *aac(6′)-Ib* gene encoding an aminoglycoside 6′-*N*-acetyltransferase in *Pseudomonas aeruginosa* BM2656. Antimicrob. Agents Chemother. **37:**1456–1462.
10. **Girlich, D., T. Naas, A. Leelaporn, L. Poirel, M. Fennewald, and P. Nordmann.** 2002. Nosocomial spread of the integron-located *veb-1*-like cassette encoding an extended-spectrum β-lactamase in *Pseudomonas aeruginosa* in Thailand. Clin. Infect. Dis. **34:**603–611.
11. **Gonzalez, V., R. I. Santamaria, P. Bustos, I. Hernandez-Gonzalez, A. Medrano-Soto, G. Moreno-Hagelsieb, S. C. Janga, M. A. Ramirez, V. Jimenez-Jacinto, J. Collado-Vides, and G. Davila.** 2006. The partitioned *Rhizobium etli* genome: genetic and metabolic redundancy in seven interacting replicons. Proc. Natl. Acad. Sci. USA **103:**3834–3839.
12. **Hall, L. M., D. Livermore, D. Gur, M. Akova, and H. E. Akalin.** 1993. OXA-11, an extended-spectrum variant of OXA-10 (PSE-2) β-lactamase from *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **37:**1644–1649.
13. **Jarlier, V., M. H. Nicolas, G. Fournier, and A. Philippon.** 1988. Extended broad-spectrum β-lactamases conferring transferable resistance to newer β-lactam agents in *Enterobacteriaceae*: hospital prevalence and susceptibility patterns. Rev. Infect. Dis. **10:**867–878.
14. **Johnson, T. J., Y. M. Wannemeuhler, J. A. Scaccianoce, S. J. Johnson, and L. K. Nolan.** 2006. Complete DNA sequence, comparative genomics, and prevalence of an IncHI2 plasmid occurring among extraintestinal pathogenic *Escherichia coli* isolates. Antimicrob. Agents Chemother. **50:**3929–3933.
15. **Kalai Blagui, S., W. Achour, M. S. Abbassi, M. Bejaoui, A. Abdeladhim, and A. Ben Hassen.** 2007. Nosocomial outbreak of OXA-18-producing *Pseudomonas aeruginosa* in Tunisia. Clin. Microbiol. Infect. **13:**794–800.
16. **Kieser, T.** 1984. Factors affecting the isolation of cccDNA from *Streptomyces lividans* and *Escherichia coli*. Plasmid **12:**19–36.
17. **Kolayli, F., G. Gacar, A. Karadenizli, A. Sanic, H. Vahaboglu, and the Study Group.** 2005. PER-1 is still widespread in Turkish hospitals among *Pseudomonas aeruginosa* and *Acinetobacter* spp. FEMS Microbiol. Lett. **249:**241–245.
18. **Livermore, D. M., and N. Woodford.** 2006. The beta-lactamase threat in *Enterobacteriaceae*, *Pseudomonas*, and *Acinetobacter*. Trends Microbiol. **14:** 413–420.
19. **Naas, T., and P. Nordmann.** 1999. OXA-type beta-lactamases. Curr. Pharmaceut. Des. **5:**865–879.
20. **Naas, T., D. Aubert, T. Lambert, and P. Nordmann.** 2006. Complex genetic structures with repeated elements, a sul-type class 1 integron, and the bla_{VEB} extended-spectrum β-lactamase gene. Antimicrob. Agents Chemother. **50:** 1745–5172.
21. **Naas, T., L. Poirel, and P. Nordmann.** 2008. Minor extended-spectrum β-lactamases. Clin. Microbiol. Infect. **14**(Suppl. 1)**:**42–52.
22. **Naas, T., W Sougakoff, A Casetta, and P. Nordmann.** 1998. Molecular characterization of OXA-20, a novel class D β-lactamase, and its integron from *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **42:**2074–2083.
23. **Okamoto, K., N. Gotoh, and T. Nishino.** 2001. *Pseudomonas aeruginosa* reveals high intrinsic resistance to penem antibiotics: penem resistance mechanisms and their interplay. Antimicrob. Agents Chemother. **45:**1964–1971.
24. **Paterson, D. L., and R. A. Bonomo.** 2005. Extended-spectrum β-lactamases: a clinical update. Clin. Microbiol. Rev. **18:**657–686.
25. **Philippon, L. N., T. Naas, A.-T. Bouthors, V. Barakett, and P. Nordmann.** 1997. OXA-18, a class D clavulanic acid-inhibited extended-spectrum β-lac-

tamase from *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **41:** 2188–2195.

26. **Poirel, L., and P. Nordmann.** 2006. Genetic structures at the origin of acquisition and expression of the carbapenem-hydrolyzing oxacillinase gene bla_{OXA-58} in *Acinetobacter baumannii*. Antimicrob. Agents Chemother. **50:** 1442–1448.
27. **Poirel, L., L. Brinas, A. Verlinde, L. Ide, and P. Nordmann.** 2005. BEL-1, a novel clavulanic acid-inhibited extended-spectrum β-lactamase, and the class 1 integron In120 in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **49:**3743–3748.
28. **Poirel, L., P. Gerome, C. De Champs, J. Stephanazzi, T. Naas, and P. Nordmann.** 2002. Integron-located OXA-32 gene cassette encoding an extended-spectrum variant of OXA-2 β-lactamase from *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **46:**566–569.
29. **Recchia, G. D., H. W. Stokes, and R. M. Hall.** 1994. Characterization of specific and secondary recombination sites recognized by the integron DNA integrase. Nucleic Acids Res. **22:**2071–2078.
30. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
31. **Smith, A. W., and B. H. Iglewski.** 1989. Transformation of *Pseudomonas aeruginosa* by electroporation. Nucleic Acids Res. **17:**10509.
32. **Tenover, F. C., R. Arbeit, R. V. Goering, P. A. Mickelsen, B. E. Murray, D. H. Persing, and B. Swaminathan.** 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J. Clin. Microbiol. **33:**2233–2239.
33. **Tavakoli, N., A. Comanducci, H. M. Dodd, M. C. Lett., B. Albiger, and P. Bennett.** 2000. IS*1294*, a DNA element that transposes by RC transposition. Plasmid **44:**66–84.
34. **Toleman, M. A., K. Rolston, R. N. Jones, and T. R. Walsh.** 2003. Molecular and biochemical characterization of OXA-45, an extended-spectrum class 2d′ β-lactamase in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **47:**2859–2863.
35. **Toleman, M. A., P. M. Bennett, and T. R. Walsh.** 2006. IS*CR* elements: novel gene-capturing systems of the 21st century? Microbiol. Mol. Biol. Rev. **70:** 296–316.
36. **Welch, T. J., W. F. Fricke, P. F. McDermott, D. G. White, M. L. Rosso, D. A. Rasko, M. K. Mammel, M. Eppinger, M. J. Rosovitz, D. Wagner, L. Rahalison, J. E. Leclerc, J. M. Hinshaw, L. E. Lindler, T. A. Cebula, E. Carniel, and J. Ravel.** 2007. Multiple antimicrobial resistance in plague: an emerging public health risk. PLoS ONE **2:**E309.