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Prolonged Outbreak of Infection Due to TEM-21-Producing Strains of *Pseudomonas aeruginosa* and Enterobacteria in a Nursing Home

Véronique Dubois,[1]* Corinne Arpin,[1] Patrick Noury,[2] Catherine Andre,[1] Laure Coulange,[1] and Claudine Quentin[1]

Laboratoire de Microbiologie, Faculté de Pharmacie, EA 525, Université de Bordeaux 2, Bordeaux, France,[1] *and Laboratoire d'Analyses Médicales, Villenave d'Ornon, France*[2]

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Over a 6-year period, 24 extended-spectrum β-lactamase (ESBL)-producing isolates of *Pseudomonas aeruginosa* were collected from 18 patients living in a nursing home. These isolates had a delayed development of a red pigment and exhibited a similar antibiotype (resistance to all β-lactams except for imipenem and to gentamicin, tobramycin, netilmicin, ciprofloxacin, and rifampin) associated with the production of the TEM-21 β-lactamase and a type II 3′-*N*-aminoglycoside acetyltransferase [AAC(3)-II] enzyme. Surprisingly, serotyping showed that these isolates belonged to four successive serotypes (P2, P16, P1, and PME), although molecular typing by PCR methods and pulsed-field gel electrophoresis yielded identical or similar profiles. Moreover, in all isolates the bla_{TEM-21} gene was part of a chromosomally located Tn*801* transposon truncated by an IS*6100* element inserted within the resolvase gene, and the *aac(3)-II* gene was adjacent to this structure. During the same period, 17 ESBL-producing isolates of enterobacteria were also collected from 10 of these patients. These isolates harbored a similar large plasmid that contained the bla_{TEM-21} and the *aac(3)-II* genes and that conferred additional resistance to sulfonamides and chloramphenicol, as well as to kanamycin, tobramycin, netilmicin, and amikacin, conveyed by an AAC(6′)-I enzyme. The bla_{TEM-21} gene was part of the Tn*801* transposon disrupted by IS*4321*. Thus, a single clone of *P. aeruginosa* that had undergone a progressive genetic drift associated with a change in serotype appeared to be responsible for an outbreak of nosocomial infections in a nursing home. This strain has probably acquired the bla_{TEM-21}-encoding plasmid that was epidemic among the enterobacteria at the institution, followed by chromosomal integration and genomic reorganization.

Pseudomonas aeruginosa is a saprophytic organism widespread in nature, particularly in moist environments (water, soil, plants, and sewage). This bacterial species is endowed with only a weak pathogenic potential in immunocompetent persons (2, 17, 21, 23) but can cause severe and even fatal infections in patients with impaired specific or nonspecific defense systems (40). Thus, *P. aeruginosa* is rarely involved in community-acquired infections, while it is responsible for a wide range of hospital-acquired infections, such as pneumonia, urinary tract infections (UTIs), and bacteremia. Moreover, a series of outbreaks due to this important and frequent nosocomial pathogen has been reported in hospital intensive care, burn wound, and cancer units (6, 13, 42). In contrast, little has been reported on *P. aeruginosa*-induced infections in long-term-care facilities, including nursing homes; and most of the studies that have reported such infections have described sporadic cases rather than outbreaks (16, 22, 44).

Linked to its nosocomial status, *P. aeruginosa* is intrinsically resistant to most antimicrobials. In addition, resistance to the main active agents, i.e., β-lactams and aminoglycosides, by gene acquisition is common. However, in this bacterial species, extended-spectrum β-lactamases (ESBLs) are rare and are generally of the OXA type, which are poorly inhibited by clavulanic acid, and belong to class D of the Ambler classification scheme (1). Clavulanic acid-susceptible ESBLs of the TEM and SHV families of class A, which are widespread among enterobacteria (35), are highly infrequent in *P. aeruginosa*. Nevertheless, some of these enzymes, i.e., TEM-4, TEM-21, TEM-24, TEM-42, SHV-2a, SHV-5, and SHV-12, as well as unaffiliated class A β-lactamases such as PER-1, VEB-1, VEB-2, GES-1, GES-2, and IBC-2, have occasionally been found (48). These observations suggest that ESBL-producing enterobacteria, which are often responsible for intra- and interhospital outbreaks due to the spread of strains, plasmids, or genes, might be the sources of these ESBLs for *P. aeruginosa* (33), which could become a reservoir for these types of enzymes. Aminoglycoside resistance is mainly mediated by antibiotic-modifying enzyme production, principally, the type II 6′-*N*-aminoglycoside acetyltransferase [AAC(6′)-II] (kanamycin, gentamicin, tobramycin, and netilmicin resistance phenotype) in *P. aeruginosa*, while the type II 3′-*N*-aminoglycoside acetyltransferase [AAC(3)-II] enzyme (gentamicin, tobramycin, and netilmicin resistance phenotype), the type I 2″-*O*-nucleotidyltransferase [ANT(2″)-I] enzyme (kanamycin, gentamicin, and tobramycin resistance phenotype), and the type I AAC(6′) enzyme (kanamycin, tobramycin, netilmicin, and amikacin resistance phenotype) are prevalent among members of the family *Enterobacteriaceae* (32).

Over a 6-year period, 24 isolates of *P. aeruginosa* and 17 enterobacteria producing the TEM-21 β-lactamase and the AAC(3)-II enzyme were isolated from 18 patients living in a nursing home. The aim of this study was to investigate this prolonged outbreak and to analyze the genetic backgrounds of the resistance genes in order to understand their route of dissemination between these taxonomically distant bacteria.

* Corresponding author. Mailing address: Laboratoire de Microbiologie, Faculté de Pharmacie, Université de Bordeaux 2, 146 rue Léo Saignat, 33076 Bordeaux Cedex, France. Phone: 33 5 57 57 10 75. Fax: 33 5 56 90 90 72. E-mail: veronique.dubois@bacterio.u-bordeaux2.fr.

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MATERIALS AND METHODS

Bacterial strains and culture conditions. Between November 1996 and December 2002, 24 ESBL-producing isolates of *P. aeruginosa* were collected from 18 patients by a private laboratory (Table 1). All isolates were identified by conventional methods (18) and serotyped by three independent assays by using the antisera provided by Bio-Rad (Marnes la Coquette, France). Seventeen ESBL-producing enterobacteria were also recovered from 10 of these 18 patients (Table 1). Identification to the species level was performed with the API 20E system (bioMérieux, Marcy l'Etoile, France). The wild-type strain *P. aeruginosa* ATCC 27853 was used as the control for MIC determination and pulsed-field gel electrophoresis (PFGE). An azide-resistant (Az^r) mutant of *Escherichia coli* HB101, a spontaneous rifampin- and nalidixic acid-resistant (Rif^r Nal^r) mutant of *E. coli* K-12, and a rifampin-resistant (Rif^r) mutant of *P. aeruginosa* ATCC 27853 were the recipient strains in conjugation experiments; and *E. coli* JM109, *E. coli* DH5α, and *P. aeruginosa* ATCC 27853 were used in transformation assays. All bacterial strains were routinely cultured at 37°C on Mueller-Hinton (MH) agar medium (Diagnostics Pasteur, Marnes la Coquette, France) or were grown in brain heart broth (Bio-Rad) or Trypticase soy broth (Diagnostics Pasteur).

Antibiotic susceptibility testing. The susceptibilities of the 41 isolates to 32 antimicrobial agents were determined by the disk diffusion method in MH agar medium, according to French guidelines (http://www.sfm.asso.fr). The presence of an ESBL was detected by the double-disk synergy test. For the 24 *P. aeruginosa* isolates, the MICs of 10 β-lactams alone or in combination with β-lactamase inhibitors (clavulanic acid, 2 μg/ml; tazobactam, 4 μg/ml) and of four aminoglycosides were determined by a standard agar dilution method in MH medium with an inoculum of 10^4 CFU per spot (http://www.sfm.asso.fr).

β-Lactamase extraction and IEF. β-Lactamases of the 24 isolates of *P. aeruginosa*, the 17 isolates of enterobacteria, and their transconjugants were released by ultrasonic treatment; and their pIs were determined by isoelectric focusing (IEF) on a pH 3.5 to 10 ampholine polyacrylamide gel, as described by Matthew et al. (31). Enzyme activities were detected by the iodine procedure in a gel with benzylpenicillin (75 μl/ml) as the substrate. β-Lactamases of known pIs, TEM-1 (pI 5.4), TEM-21 (pI 6.4), SHV-3 (pI 7.0), and SHV-1 (pI 7.6), were used as pI markers.

Conjugation, transformation experiments, and plasmid content analysis. The transfer of resistance genes to *E. coli* K-12 Rif^r Nal^r, *E. coli* HB101 Az^r, and *P. aeruginosa* ATCC 27853 Rif^r was attempted by a filter mating technique. Transconjugants were selected on MH agar plates containing ceftazidime (2 μg/ml) and rifampin (200 μg/ml) or ceftazidime (2 μg/ml) and sodium azide (300 μg/ml). Plasmid DNA was extracted from the clinical isolates, the transconjugants, and the *E. coli* JM109 strain harboring the recombinant plasmid by using an alkaline lysis method (5) and a plasmid DNA midi kit (QIAGEN, Courtaboeuf, France). Putative plasmid DNA extracts obtained from the *P. aeruginosa* isolates was electroporated into *E. coli* DH5α and *P. aeruginosa* ATCC 27853 with selection on MH agar plates containing ticarcillin (100 μg/ml). Plasmid DNA extracted from the transconjugants of *Enterobacteriaceae* was restricted by the EcoRI enzyme and was analyzed on a 0.8% agarose gel in the presence of ethidium bromide.

PCR, sequencing, and cloning experiments. Total DNA of the *P. aeruginosa* and enterobacterial isolates was extracted as described previously (39). The detection of the resistance genes was carried out under standard PCR conditions (41) with primers specific for the bla_{TEM} and *aac(6′)-Ib* genes or primers designed in the laboratory (Table 2) and 0.5 μg of whole-cell DNA of the ESBL-expressing isolates. A combination of primers designed in the laboratory was also used for the determination of the bla_{TEM-21} genetic environment (Table 2). Amplification of large fragments (>3 kb) was performed with the GeneAmp XL-PCR kit (Applied Biosystems, Courtaboeuf, France), according to the manufacturer's instructions. The amplicons were revealed by electrophoresis on a 1.5% agarose gel and subsequent exposure to UV light in the presence of ethidium bromide. For sequencing purposes, the PCR products, purified through spin columns (Sephacryl S400; Amersham Biosciences, Orsay, France), and recombinant plasmid pEc223H were used as templates in a single-cycle reaction by using the primers designed in the laboratory and the DYEnamic ET dye terminator kit (Amersham Biosciences). The sequences were analyzed with an automatic sequencer (ABI 310; Perkin-Elmer, Courtaboeuf, France) by using the Sequencing Analysis software and were compared to each other and to homologous sequences with the Sequence Navigator software. The nucleotide and the deduced protein sequences were analyzed by using the software available over the Internet at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov).

The whole-cell DNA of the Ec223 isolate was totally restricted by the HindIII enzyme and was ligated into the HindIII site of the pBK-CMV cloning vector. The *E. coli* JM109 strain, which harbored recombinant plasmid pEc223H, was selected on MH agar plates containing 100 μg/ml of ampicillin and 50 μg/ml of kanamycin. The resistance patterns of the transconjugants were assessed by the disk diffusion method.

Hybridization. DNA-DNA hybridization was carried out as described by Sambrook et al. (41) by Southern transfer on an agarose gel containing total DNA from the 24 *P. aeruginosa* isolates and the 17 enterobacterial isolates digested with the NruI restriction enzyme as the templates. The probe consisted of a 956-bp PCR fragment that was generated from the total DNA of the Pa141 isolate and that corresponds to the bla_{TEM-21} gene. Nonradioactive labeling of the probe and signal detection were achieved according to the manufacturer's instructions (Roche, Applied Science, Meylan, France).

Molecular typing. For the 24 isolates of *P. aeruginosa*, a random amplified polymorphic DNA (RAPD) assay with primers AP12h (50) and primers 208 and 272 (28) and enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) with primers ERIC2 and ERIC1R (47) were performed. After a first cycle of denaturation for 10 min at 94°C, the 45 subsequent cycles of amplification consisted of denaturation for 1 min at 94°C, annealing for 1 min at 42°C, and extension for 1 min at 72°C, with a final extension step for 10 min at 72°C. The amplification products were analyzed by electrophoresis of 10-μl samples on 1.5% agarose gels in the presence of ethidium bromide.

PFGE analysis was performed with the 24 isolates of *P. aeruginosa* by using the whole-cell DNA digested with the SpeI restriction enzyme, the Gene Path kits, and the CHEF-DR III variable angle system, according to the manufacturer's recommendations (Bio-Rad, Marnes la Coquette, France).

Nucleotide sequence accession number. The nucleotide sequence of strain Pa141 has been submitted to GenBank and can be found under accession number AF46656.

RESULTS

Patients and strains. From November 1996 to December 2002, 18 patients (patients A to R) living in a nursing home near the city of Bordeaux, France, were infected by ESBL-expressing *P. aeruginosa* isolates that produced a green pigment that turned to red after 24 h. The nursing home was a 70-bed long-term-care facility that accommodates elderly and mostly invalid individuals. The 18 patients, 12 females and 6 males, ranged in age from 54 to 97 years (mean age, 80.5 years) and carried urinary catheters or condom drainage systems. A total of 24 *P. aeruginosa* isolates were collected from 19 urine specimens, 4 bedsores, and 1 sputum sample (Table 1). Seventeen ESBL-producing enterobacteria (five *Proteus mirabilis*, four *Klebsiella pneumoniae*, three *Providencia stuartii*, three *E. coli*, and two *Morganella morganii* isolates) were also isolated from 10 of these patients during the same period. These strains were collected from 15 urine specimens and 2 bedsores (Table 1). Unfortunately, the histories of these patients were unavailable, and no epidemiological investigation could be conducted.

Antibiotic susceptibility testing. By the disk diffusion method, the 24 *P. aeruginosa* isolates were insensitive to all potentially active β-lactams except imipenem and produced an ESBL. In addition, all isolates were resistant to gentamicin, tobramycin, netilmicin, ciprofloxacin, and rifampin. These data were confirmed by MIC determination (Table 3). Five isolates were also resistant to fosfomycin.

The 17 enterobacterial isolates were also ESBL producers and were additionally resistant to kanamycin, gentamicin, tobramycin, netilmicin, amikacin, sulfonamides, and chloram-

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TABLE 1. Characteristics of the patients and the bla_{TEM-21}-producing isolates of *P. aeruginosa* and enterobacteria[a]

Patient/age (yr)/sex	Bacterial species	Strain no.	Specimen	Date of isolation (day/mo/yr)	*P. aeruginosa* serotype	β-Lactamase content		Antibiotype[b]
						pI(s)	Enzyme(s)	
A/78/M	*P. aeruginosa*	Pa192	Urine	27/11/1996	P2	6.4	TEM-21	GTNt, FQ
B/85/M	*P. aeruginosa*	Pa133	Urine	09/09/1997	P2	6.4	TEM-21	GTNt, FQ
	P. aeruginosa	Pa190	Urine	31/10/1997	P2	6.4	TEM-21	GTNt, FQ
	P. stuartii	Ps202	Urine	31/10/1997		6.4	TEM-21	**KGTNt(A)**, **SSS**, TMP, **C**, TE, FQ, FOS
	K. pneumoniae	Kp447	Urine	06/08/1998		5.4, 6.4, 7.6	TEM-1, TEM-21, SHV-1	**KGTNt(A)**, **SSS**, TMP, **C**, FQ
C/68/F	*P. aeruginosa*	Pa141	Urine	19/09/1997	P2	6.4	TEM-21	GTNt, FQ
	P. stuartii	Ps1737	Urine	12/09/2001		6.4	TEM-21	**KGTNt(A)**, **SSS**, TMP, **C**, TE, FQ, FOS
	P. mirabilis	Pm1890	Urine	05/03/2002		5.4, 6.4	TEM-1, TEM-21	**KGTNt(A)**, **SSS**, **C**, TE, FQ
D/74/M	*E. coli*	Ec223	Urine	23/01/1999		6.4	TEM-21	**KGTNt(A)**, **SSS**, **C**
	K. pneumoniae	Kp276	Urine	23/02/1999		6.4, 7.6	TEM-21, SHV-1	**KGTNt(A)**, **SSS**, **C**
	P. aeruginosa	Pa504	Urine	23/04/1999	P2	6.4	TEM-21	GTNt, FQ
E/94/M	*P. aeruginosa*	Pa678	Bedsore	09/10/1999	P2	6.4	TEM-21	GTNt, FQ
F/91/F	*P. mirabilis*	Pm749	Urine	28/01/2000		5.4, 6.4	TEM-1, TEM-21	**KGTNt(A)**, **SSS**, **C**, TE
	K. pneumoniae	Kp750	Urine	28/01/2000		6.4, 7.6	TEM-21, SHV-1	**KGTNt(A)**, **SSS**, **C**, TE
	P. aeruginosa	Pa773	Sputum	02/02/2000	P16	6.4	TEM-21	GTNt, FQ
	P. stuartii	Ps763	Bedsore	10/02/2000		5.4, 6.4	TEM-1, TEM-21	**KGTNt(A)**, **SSS**, TMP, **C**, TE, FQ, FOS
G/88/F	*P. aeruginosa*	Pa815	Bedsore	25/02/2000	P16	6.4	TEM-21	GTNt, FQ
	P. aeruginosa	Pa943	Urine	08/04/2000	P1	6.4	TEM-21	GTNt, FQ
H/72/M	*M. morganii*	Mm109	Urine	15/12/1998		6.4, 7	TEM-21, AmpC	**KGTNt(A)**, **SSS**, TMP, **C**, TE, FQ, FOS
	P. aeruginosa	Pa813	Urine	29/02/2000	P16	6.4	TEM-21	GTNt, FQ
I/80/F	*P. aeruginosa*	Pa942	Urine	13/04/2000	P16	6.4	TEM-21	GTNt, FQ
J/91/F	*P. aeruginosa*	Pa1152	Urine	18/10/2000	P16	6.4	TEM-21	GTNt, FQ
	M. morganii	Mm1344	Urine	02/02/2001		6.4, 7	TEM-21, AmpC	**KGTNt(A)**, **SSS**, TMP, **C**, TE, FQ, FOS
	P. mirabilis	Pm1615	Bedsore	16/05/2001		5.4, 6.4	TEM-1, TEM-21	**KGTNt(A)**, **SSS**, **C**, TE, FQ, FOS
K/89/F	*P. aeruginosa*	Pa1276	Bedsore	02/01/2001	P16	6.4	TEM-21	GTNt, FQ
L/97/F	*P. mirabilis*	Pm1345	Urine	02/02/2001		5.6, 6.4	TEM-2, TEM-21	**KGTNt(A)**, **SSS**, **C**, TE, FQ, FOS
	P. aeruginosa	Pa1486	Urine	08/03/2001	P16	6.4	TEM-21	GTNt, FQ, FOS
	P. aeruginosa	Pa1513	Urine	21/03/2001	P1	6.4	TEM-21	GTNt, FQ, FOS
	P. mirabilis	Pm1587	Urine	11/04/2001		5.4, 6.4	TEM-1, TEM-21	**KGTNt(A)**, **SSS**, **C**, TE, FQ, FOS
M/91/F	*P. aeruginosa*	Pa1727	Urine	30/08/2001	P1	6.4	TEM-21	GTNt, FQ
	E. coli	Ec1728	Urine	30/08/2001		6.4	TEM-21	**KGTNt(A)**, **SSS**, **C**, TE, FQ
	P. aeruginosa	Pa1782	Urine	23/11/2001	P1	6.4	TEM-21	GTNt, FQ, FOS
N/77/F	*P. aeruginosa*	Pa1891	Urine	05/03/2002	P2	6.4	TEM-21	GTNt, FQ
O/72/F	*P. aeruginosa*	Pa1885	Urine	11/03/2002	PME	6.4	TEM-21	GTNt, FQ
	P. aeruginosa	Pa2030	Urine	27/11/2002	PME	6.4	TEM-21	GTNt, FQ
P/79/F	*P. aeruginosa*	Pa1884	Urine	15/03/2002	PME	6.4	TEM-21	GTNt, FQ
Q/54/M	*P. aeruginosa*	Pa1920	Bedsore	15/05/2002	PME	6.4	TEM-21	GTNt, FQ
	E. coli	Ec1927	Urine	12/06/2002		6.4	TEM-21	**KGTNt(A)**, **SSS**, **C**
R/80/F	*K. pneumoniae*	Kp1966	Urine	28/09/2002		5.6, 6.4, 7.6	TEM-2, TEM-21, SHV-1	**KGTNt(A)**, **SSS**, TMP, **C**
	P. aeruginosa	Pa2017	Urine	13/11/2002	PME	6.4	TEM-21	GTNt, FQ, FOS
	P. aeruginosa	Pa2044	Urine	10/12/2002	PME	6.4	TEM-21	GTNt, FQ, FOS

[a] M, male; F, female; FQ, fluoroquinolones; FOS, fosfomycin; SSS, sulfonamides; C, chloramphenicol; TE, tetracycline; TMP, trimethoprim; K, kanamycin; G, gentamicin; T, tobramycin; Nt, netilmicin; (A), amikacin at low levels.

[b] The drug to which resistance is cotransferred by the ESBL is indicated in boldface.

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TABLE 2. Oligonucleotides used as primers for PCR in this study

Primer	Sequence (5′ to 3′)	Position (nt)[a]	Reference
TEM-A2	GTATCCGCTCATGAGACAAT	1285–1304	14
TEM-ext	GTATATATGAGTAAACTTGGTCTG	2234–2211	14
aac(3)-IIa	ATATCGCGATGCATACGCGG	2380–2399	14
aac(3)-IIb	GACGGCCTCTAACCGGAAGG	3256–3237	14
5′AAC6′	CATCATAGGAGGTGATCCAATGACCAACAGCAACGATTCCG		15
3′AAC6′	CCTCGATGGAAGGGTTAGGC		15
C6T7	GCCTGAAGCACTGGGAAAAG	410–429	14
C6T7bis	TTTATTGACGACGGGATCAG	879–898	14
C6T3P	TTTGAACGTCCGCTGGCTGTG		This study
Resinv	ATCCTCCGGCGTTCAGCTTG	970–951	14
TnpA4R	GGACACTCAAACGAAGCCG		This study
IRL4321	ATGGTCACTCCCTCCTTCC		This study

[a] nt, number of the nucleotide, according to the sequence from the strain Pa141 submitted to GenBank under accession number AF46656.

phenicol. Among them, 12 were also resistant to tetracycline, 11 to fluoroquinolones, 8 to fosfomycin, and 7 to trimethoprim.

Transfer of resistance and plasmid content. Conjugation experiments between the ESBL-producing *P. aeruginosa* isolates and *E. coli* HB101 Az^r or *P. aeruginosa* ATCC 27853 Rif^r as the recipients did not yield any ESBL-producing transconjugants ($<10^{-8}$). Despite repeated attempts, plasmid DNA could be detected in only two pseudomonal isolates, and transformation of all plasmid DNA extracts into *E. coli* DH5α or *P. aeruginosa* ATCC 27853 by electroporation remained unsuccessful.

In contrast, mating assays between the ESBL-producing enterobacterial isolates and *E. coli* K-12 Nal^r Rif^r as the recipient always led to ESBL-producing transconjugants, as indicated by the antibiogram. Moreover, the transconjugants displayed the same pattern of coresistance as the donor strains, i.e., kanamycin, gentamicin, tobramycin, netilmicin, amikacin, sulfonamide, and chloramphenicol resistance. Plasmid DNA analysis revealed the presence of a single large plasmid, which gave an identical EcoRI restriction profile in all transconjugants (data not shown).

β-Lactamase characterization. The β-lactamase contents of the clinical isolates and transconjugants was first analyzed by IEF. All the ESBL-producing *P. aeruginosa* isolates presented a band of pI 6.4 that cofocused with that of Pa141, which was previously shown by sequencing to contain the $bla_{\text{TEM-21}}$ gene (14). Moreover, PCR products of the expected size were obtained with primers specific for the TEM family of β-lactamases for all *P. aeruginosa* isolates. All the ESBL-producing transconjugants obtained from the enterobacteria also displayed a single band of pI 6.4 and gave positive TEM-specific PCR amplifications. The clinical donor isolates occasionally presented additional bands consistent with species-specific β-lactamases (chromosomal penicillinase of pI 7.6 in *K. pneumoniae* or cephalosporinase with a pI of ≈7 in *M. morganii*) or acquired TEM-1 or TEM-2 enzymes (pI 5.4 and 5.6, respec-

TABLE 3. Antimicrobial susceptibilities of the 24 resistant strains of *P. aeruginosa* isolated during the outbreak

Antimicrobial agent(s)	Resistant strains of *P. aeruginosa*		MIC (μg/ml) for *P. aeruginosa* ATCC 27853	MIC breakpoint (μg/ml)[c]
	Range of MICs (μg/ml)	Mode MIC (μg/ml)		
Ticarcillin	>512	>512	8	≤16–>64
Ticarcillin + CLA[a]	16–64	32	8	≤16–>64
Piperacillin	32–128	64	4	≤16–>64
Piperacillin + TZB[b]	4–16	8	4	≤16–>64
Imipenem	1–4	2	2	≤4–>8
Aztreonam	8–32	16	4	≤4–>32
Aztreonam + CLA	2–8	4	4	
Ceftazidime	8–32	16	1	≤4–>32
Ceftazidime + CLA	1–4	4	1	
Cefotaxime	128–512	256	16	≤4–>32
Cefotaxime + CLA	16–128	128	16	
Cefsulodin	256–>512	512	2	≤8–>32
Cefoperazone	32–128	32	4	≤4–>32
Cefepime	16–64	32	2	≤4–>32
Cefpirome	32–128	128	4	≤4–>32
Gentamicin	512–512	>512	2	≤4–>8
Tobramycin	64–256	128	1	≤4–>8
Netilmicin	>512	>512	4	≤4–>8
Amikacin	8–32	16	4	≤8–>16

[a] CLA, clavulanic acid, 2 μg/ml.
[b] TZB, tazobactam, 4 μg/ml.
[c] According to the French guidelines (http://www.sfm.asso.fr).

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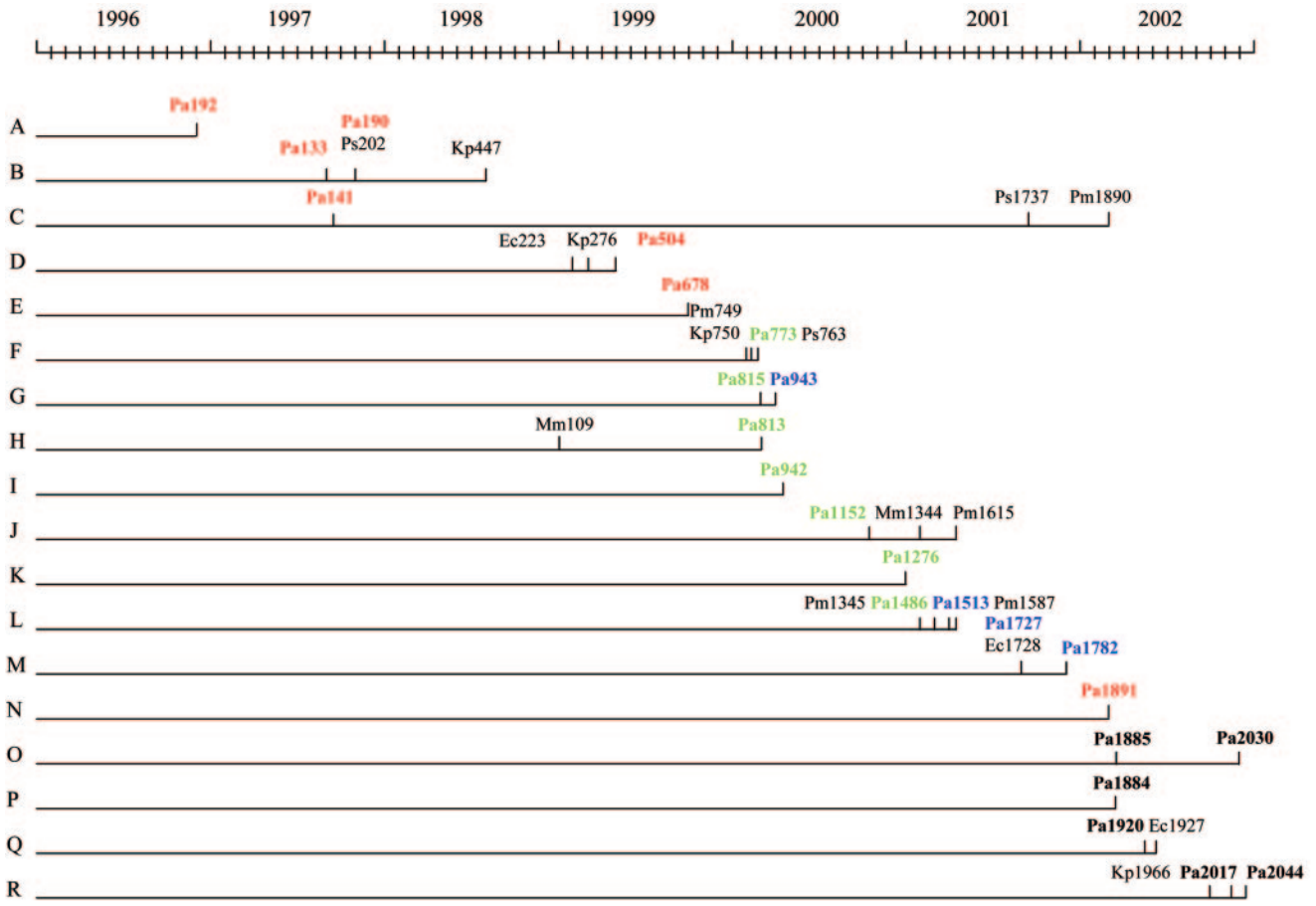

FIG. 1. Temporal relationship among the 18 patients with the TEM-21-producing isolates of *P. aeruginosa* and enterobacteria in the nursing home. The *P. aeruginosa* isolates are in boldface (red for the O:2 serotype; green for the O:16 serotype; blue for the O:1 serotype, and black for the PME serotype).

tively, in five *P. mirabilis* isolates, two *K. pneumoniae* isolates, and one *P. stuartii* isolate.

Serotyping and molecular typing. The isolates of *P. aeruginosa* were serotyped by using polyvalent antisera and then monovalent antisera. They were found to belong to three serotypes (Fig. 1), O:2 (seven isolates), O:16 (seven isolates), and O:1 (four isolates); or they agglutinated with the serotype PME-specific polyvalent antiserum (six isolates) but with none of the corresponding monovalent antisera (O:2, O:5, O:15, and O:16). In two patients (patients G and L), *P. aeruginosa* isolates belonging to different serotypes (O:16 and O:1) were isolated at intervals of 6 and 2 weeks, respectively.

Molecular typing of *P. aeruginosa* isolates was undertaken at first by RAPD and ERIC-PCR methods. The results obtained with primers Ap12h and ERIC1R were insufficiently discriminant (the profiles were composed of two or three bands). The profiles given by primers ERIC2, 208, and 272 allowed better differentiation (8 to 11 bands) and appeared to be very similar (data not shown). The PFGE profiles of SpeI-restricted DNA of all TEM-21-producing *P. aeruginosa* isolates were identical or similar and were clearly different from that of the *P. aeruginosa* ATCC 27853 strain, used as a control (Fig. 2). Compared to the predominant pattern (e.g., Pa773), additional fragments were observed: ca. 500 kb (Pa1513), 400 kb (Pa133, Pa190, Pa1920, and Pa2044), 220 kb in four isolates of serotype PME (Pa1885, Pa1920, Pa2017, and Pa2030), and 140 kb (Pa133, Pa141, Pa190, and Pa192). On the other hand, some fragments were missing: ca. 360 kb (Pa2044), 320 kb (Pa133 and Pa190), and 80 kb (Pa815, Pa942, Pa1152, Pa1276, Pa1486, Pa1885, Pa1920, and Pa2017). Thus, the maximum number of fragment differences was three, which corresponds to the "closely related" category of isolates, according to Tenover et al. (45), and indicates that the isolates were probably part of an outbreak. Moreover, there was no evident relationship between the PFGE profiles and the serotypes.

Characterization of the genetic environment of the $bla_{\text{TEM-21}}$ gene. Based on the genetic environment of $bla_{\text{TEM-21}}$ previously determined for isolate Pa141 (Fig. 3) (14), PCR amplifications with primers C6T7 and aac(3)-IIb were performed for all *P. aeruginosa* isolates and generated a fragment of the expected size, 2,847 bp. The specificity of the amplicon was checked by restriction with the SspI enzyme and led to the predicted profile of two bands of 919 and 1,928 bp. Then, primer C6T3P, located ca. 2,000 bp downstream from the *aac(3)-II* gene, was combined with primer C6T7, generating a large fragment of about 5 kb for all *P. aeruginosa* isolates. These data strongly suggested an identical genetic environment of $bla_{\text{TEM-21}}$ in all pseudomonal isolates. In order to confirm

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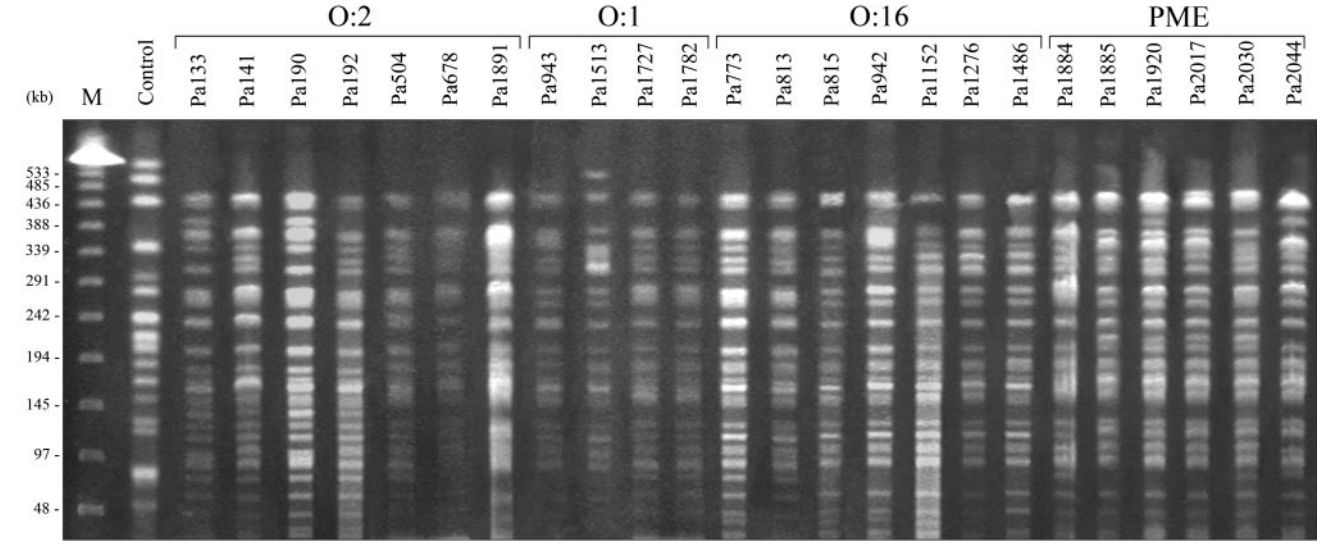

FIG. 2. PFGE profiles of SpeI-digested whole-cell DNA of the 24 *P. aeruginosa* isolates. Lane M, DNA ladder; lane control, *P. aeruginosa* ATCC 27853.

these observations and the presence of a single bla_{TEM-21} gene, Southern blot experiments were carried out with the DNA of *P. aeruginosa* isolates restricted with the NruI enzyme and a bla_{TEM}-specific probe. A single band of the expected size of 2,260 bp was observed in all cases (Fig. 4A). In contrast, Southern blotting carried out under the same experimental conditions and with DNA from the isolates of enterobacteria revealed the presence of at least one band, generally of about 6.5

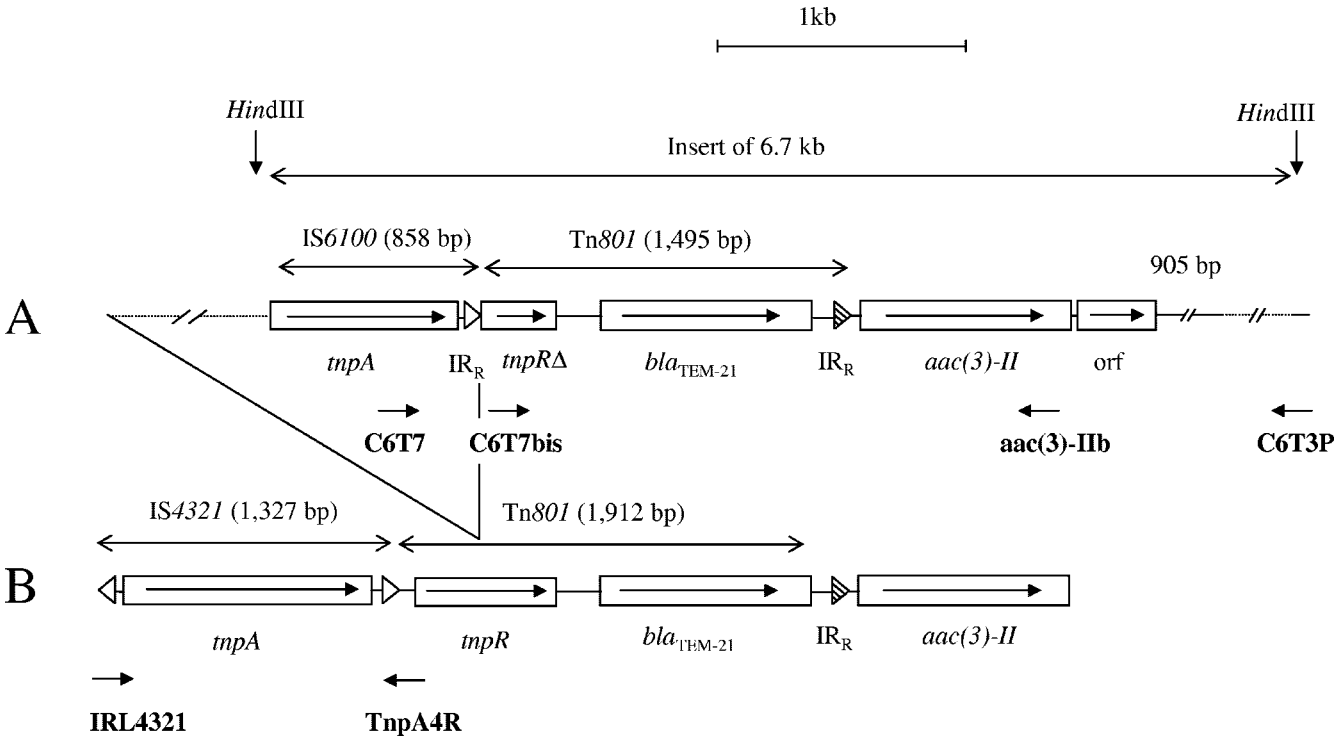

FIG. 3. (A) Schematic representation of the 4,486-bp cloned DNA fragment from *P. aeruginosa* Pa141 (reprinted from reference 14); (B) schematic representation of part of the cloned DNA fragment from *E. coli* Ec223. The solid lines represent the sequenced fragments, with the different genes boxed, and the dotted lines indicate the unanalyzed sequences. The horizontal arrows indicate the translation orientation. The arrowheads represent the terminal inverted repeats, and the double-headed arrows indicate the sizes of the insertion sequence and the transposon found in the fragment. The horizontal arrows in boldface represent the locations and the orientations of the primers. Dashes are used to indicate that the corresponding sequences are not to scale.

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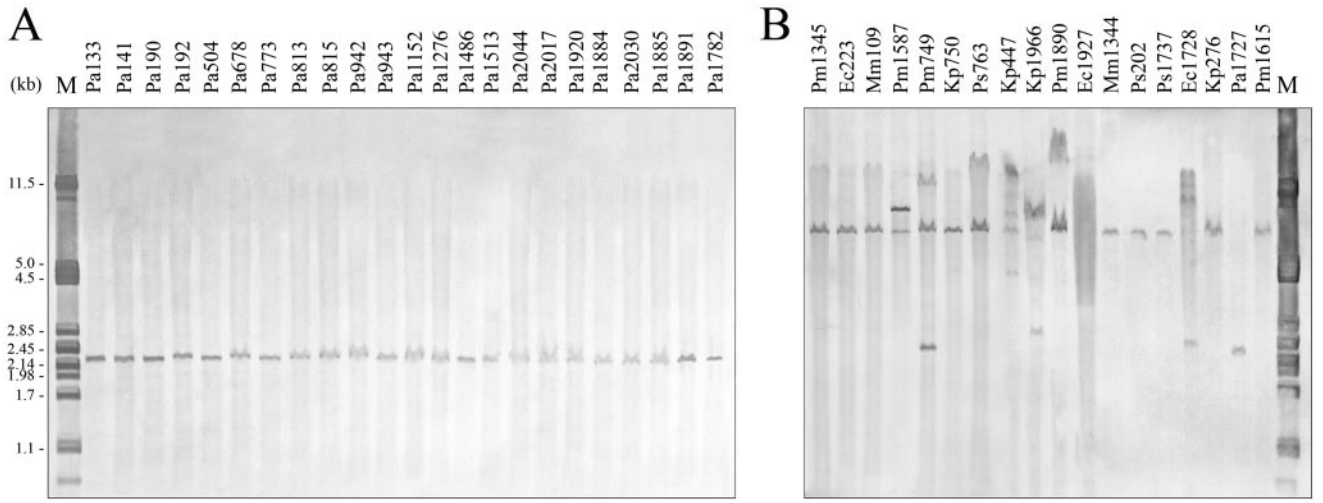

FIG. 4. (A) Southern blot of NruI-digested whole-cell DNA of 23 isolates of *P. aeruginosa* and hybridization with a bla_{TEM}-specific probe. Lane M, size marker consisting of λ phage DNA restricted by PstI. (B) Southern blot of NruI-digested whole-cell DNA of 14 isolates of enterobacteria and 1 isolate of *P. aeruginosa* and hybridization with a bla_{TEM}-specific probe. Lane M, size marker.

kb (Fig. 4B), suggesting a common genetic environment different from that observed in the *P. aeruginosa* isolates. Moreover, PCR amplifications with primers C6T7 and aac(3)-IIb did not give any band with the enterobacteria, suggesting the absence of the IS*6100* element upstream from the bla_{TEM-21} gene. Then, primer C6T7bis was combined with primer aac(3)-IIb, which yielded a fragment of 2,378 bp for all enterobacteria, indicating an identical genetic background of bla_{TEM-21}. Further digestion with the SspI restriction enzyme confirmed the specificity of the amplicon. Thus, bla_{TEM-21} was present within transposon Tn*801* and was colocated with the *aac(3)-II* gene. Then, the whole-cell DNA of the Ec223 strain was totally restricted by HindIII and ligated into the HindIII site of the pBK-CMV cloning vector. Sequencing experiments with the recombinant plasmid showed the presence of IS*4321* (37) upstream from the Tn*801* resolvase gene (Fig. 3). Primer IRL4321, within the left inverted repeat sequence of IS*4321*, in association with primer TnpA4R, upstream from the Tn*801* resolvase gene, generated an expected fragment of 1,443 bp for all enterobacteria. These primers also allowed an amplicon of the same size to be obtained for all isolates of *P. aeruginosa* except Pa133 and Pa190, the two isolates from patient B, suggesting the presence of IS*4321* upstream from IS*6100*. Thus, a sequence encompassing IS*6100* has probably inserted within the resolvase gene of Tn*801* in *P. aeruginosa*. However, this inserted fragment seemed to be very large, since GenAmp XL-PCR did not allow a specific amplification to be obtained.

Since the TEM-21-producing enterobacteria and their transconjugants were resistant to amikacin, the presence of the *aac(6′)-I* gene in these isolates was confirmed by PCR amplification, but it was absent from *P. aeruginosa*.

DISCUSSION

Between November 1996 and December 2002, infections associated with ESBL-producing isolates of *P. aeruginosa* were observed in a nursing home. Most of them were collected from urine specimens. Although *E. coli* is the most common organism responsible for UTIs, other enterobacteria, such as *P. mirabilis*, *P. stuartii*, or *K. pneumoniae*, and *P. aeruginosa*, occur frequently in older patients, especially those in long-term-care facilities, as in this study (16, 34, 43, 44). UTIs are the most common bacterial infections in the older population (34). Moreover, among individuals who use urinary devices, as was the case among all patients in this study, colonization of these devices and subsequent infection by transmission between patients on the hands of caregivers or through contaminated equipment tend to occur (7, 34). The urinary tract is thus the most prevalent site of nosocomial infections in long-term-care facilities (16, 43). Although the term "nosocomial infections" has traditionally meant infections acquired in a general hospital, it should be broadened to include infections acquired in nursing homes (43). UTIs are also one of the most frequent indications for the prescription of systemic antimicrobials in the older population. This contributes to the excessive use of antibiotics and promotes the emergence of antimicrobial resistance (7). Consistently, older residents of long-term-care facilities have a higher prevalence of resistant organisms than older patients who are not institutionalized (44, 51).

The *P. aeruginosa* isolates involved exhibited an unusual red pigment whose development was delayed and a similar antibiotype. Moreover, the presence of a TEM-21 enzyme was demonstrated by IEF, PCR experiments, and sequencing of representative isolate Pa141 (14). These data were suggestive of an outbreak of resistant strains due to a clone that persisted over at least a 6-year period in the environment or in the patients of this nursing home. Surprisingly, serotyping revealed that these isolates belonged to four chronologically successive serotypes, O:2, O:16, O:1, and PME. The bla_{TEM-21} gene was found to be chromosomally located, as demonstrated by the absence of transfer by conjugation, transformation of a putative plasmid extract, and Southern blot experiments with unrestricted DNA (data not shown). Thus, the plasmid profile could not be used as an epidemiological marker. Consequently,

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whole-DNA typing was undertaken by RAPD and ERIC-PCR experiments. RAPD analysis is considered a robust, simple, and highly reproducible method and has successfully been used to study the epidemiology of *P. aeruginosa* in patients with cystic fibrosis (8) and during a nosocomial outbreak (24). Primers ERIC2, 208, and 272 gave the most informative results and indicated a high similarity among the isolates, despite their different serotypes. Thus, the "gold standard" typing method, PFGE, was performed and confirmed that all isolates were closely related, suggesting a progressive genetic drift from the parental isolate. Indeed, members of the species *P. aeruginosa* are known to have variable bacterial characteristics and to undergo changes in serotype and O-antigen structure during bacteriophage infection (27) or antipseudomonal drug use (25).

Moreover, because the genetic environment of antibiotic resistance genes may represent an epidemiological marker for closely related strains (4), the sequences surrounding the $bla_{\text{TEM-21}}$ gene in *P. aeruginosa* isolates were analyzed by PCR amplification and Southern blot experiments. All pseudomonal isolates appeared to possess an identical genetic environment for the $bla_{\text{TEM-21}}$ gene; i.e., the $bla_{\text{TEM-21}}$ gene was part of a Tn*801* transposon truncated by an IS*6100* element inserted within the resolvase gene, and the *aac(3)-II* gene was adjacent to this structure (14). In order to elucidate the origin of the $bla_{\text{TEM-21}}$ gene in the *P. aeruginosa* isolates, the ESBL-producing enterobacteria carried by 10 of the 18 patients were examined. These isolates, which belonged to five different species, were found by IEF and PCR experiments to produce the TEM-21 β-lactamase. This enzyme was encoded by the same large conjugative plasmid, according to analysis of the cotransferred drug resistance and restriction profiles. This plasmid carried the $bla_{\text{TEM-21}}$ and the *aac(3)-II* genes and conferred additional resistance to amikacin, conveyed by an AAC(6′)-I enzyme. Indeed, an outbreak of nosocomial infections due to the spread of TEM-21-producing enterobacteria and a TEM-21-encoding plasmid among different species of the family *Enterobacteriaceae* has previously been demonstrated for other patients in this nursing home during a 6-month survey in 1999 (3). Outbreaks of ESBL-producing enterobacteria due to the spread of resistant plasmids have been described in hospitals (9, 10, 20, 26, 36) as well as in long-term-care facilities (49), but the persistence of the ESBL-producing enterobacteria in the nursing home described here during at least a 6-year period reflected an endemic situation that has, until now, been reported only in hospitals (19, 29). Among the ESBLs elaborated by enterobacteria, TEM-21 is less frequent than TEM-24 and TEM-3 in France (11, 12, 19), but it has been already detected in the Bordeaux area (3, 46) and was unknown in *P. aeruginosa* until our description (14). Thus, the hypothesis that the $bla_{\text{TEM-21}}$ gene found in *P. aeruginosa* isolates originated from enterobacteria by gene transfer, as previously evidenced for $bla_{\text{TEM-24}}$ (30) and $bla_{\text{SHV-2a}}$ (33) and suspected for $bla_{\text{SHV-12}}$ and $bla_{\text{SHV-15}}$ (9, 38), appeared to be likely. However, cloning and sequencing experiments for a representative isolate, Ec223, followed by PCR amplifications of all transconjugants of the 16 remaining strains, indicated an organization different from that in the *P. aeruginosa* isolates. Indeed, the $bla_{\text{TEM-21}}$ gene was part of Tn*801* and was adjacent to the *aac(3)-II* gene, but the transposon was disrupted by IS*4321*, inserted between the resolvase and the transposase genes.

Thus, the $bla_{\text{TEM-21}}$ gene was carried on a conjugative plasmid in the enterobacterial isolates, while it was chromosomally located in the *P. aeruginosa* isolates. Differences in origins of replication may lead to the elimination of plasmids from *Enterobacteriaceae* in *P. aeruginosa*. Therefore, the persistence of the resistance genes requires their chromosomal integration. Since the $bla_{\text{TEM-21}}$ gene was located within a truncated Tn*801* and the *aac(3)-II* gene was situated outside of the transposon in both types of organisms, both genes could not have been transferred by a Tn*801*-mediated transposition. Thus, chromosomal integration of the plasmid itself might have happened after intergeneric transfer, as was suggested for *P. aeruginosa* isolates containing the $bla_{\text{SHV-12}}$ gene (9) and the $bla_{\text{SHV-5}}$ gene (38). However, if such chromosomal integration occurred in TEM-21-producing *P. aeruginosa* isolates, it was incomplete since the *aac(6′)-I* gene present on the plasmid from *Enterobacteriaceae* was lacking. Alternatively, a large mobile element encompassing the resistance genes might have ensured this genetic transfer. In this regard, one can notice that IS*4321* is a member of the IS*1111* family and is known to target the terminal inverted repeats of the Tn*21* family of transposons (37). Moreover, the genetic environment of the $bla_{\text{TEM-21}}$ gene was different and was characterized by the presence of IS*6100* in *P. aeruginosa* isolates, replaced by an IS*4321* at a slightly different position in the enterobacteria. The insertion of IS*4321* in Tn*801* of most *P. aeruginosa* isolates indicated that a very large fragment that includes IS*6100* might have been secondarily integrated in the chromosome within Tn*801*. Only isolates Pa133 and Pa190, from patient B, lacked this fragment; and both isolates were indistinguishable by PFGE, but their PFGE patterns were slightly different from the outbreak isolate PFGE pattern, suggesting a specific genetic rearrangement.

In conclusion, we report here on an endemic situation in a nursing home due to the dissemination of ESBL-producing strains and an ESBL-encoding plasmid, which shows that such a situation is not restricted to hospitals and clinics. A *P. aeruginosa* strain once probably acquired the ESBL-encoding plasmid that was epidemic among enterobacteria in this institution, and subsequent chromosomal integration followed by genetic reorganization allowed the persistence of the ESBL-encoding gene. Then, this strain spread among patients of the nursing home, undergoing genetic drift under antibiotic treatment or other pressures which became associated with modifications of the serotype. After a change in the directory staff of the nursing home, the number of prescriptions of microbiological analyses decreased considerably, and no ESBL-producing *P. aeruginosa* isolates have been isolated from the residents since 2003; but ESBL-producing enterobacteria are still being recovered.

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