JOURNAL OF CLINICAL MICROBIOLOGY, June 2004, p. 2523–2529 Vol. 42, No. 6
0095-1137/04/$08.00+0 DOI: 10.1128/JCM.42.6.2523–2529.2004

Multifocal Detection of Multidrug-Resistant *Pseudomonas aeruginosa* Producing the PER-1 Extended-Spectrum β-Lactamase in Northern Italy

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Received 7 October 2003/Returned for modification 20 November 2003/Accepted 19 February 2004

Forty-four nonreplicate clinical isolates of *Pseudomonas aeruginosa* that were resistant to extended-spectrum cephalosporins (ceftazidime and cefepime) and aztreonam, that putatively produced an acquired extended-spectrum β-lactamase (ESBL), according to the results of a double-disk synergy test, and that had been involved in nosocomial outbreaks were obtained from six different hospitals in northern Italy and screened for the presence of bla_{PER} ESBL determinants. Twenty isolates, associated with nine independent outbreaks that occurred in five hospitals in the Milan area and its surroundings during 1995-2000, were found to carry an acquired bla_{PER-1} gene. PER-1 producers representative of the nine outbreaks exhibited a multidrug resistance (MDR) phenotype, including resistance to extended-spectrum cephalosporins, aztreonam, meropenem, aminoglycosides, and in most cases, imipenem and ciprofloxacin. An analysis of macrorestriction profiles of their genomic DNAs by pulsed-field gel electrophoresis revealed an overall clonal diversity of the PER-1 producers, although interhospital clonal spread was also observed. The bla_{PER-1} gene was not transferable and appeared to be chromosomally located. An analysis of the EcoRI and EcoRV restriction fragment length polymorphisms of the bla_{PER-1} locus revealed identical patterns for all isolates, and the characterization of a 1.9-kb region containing bla_{PER-1} revealed a conserved structure in representatives of the various clonal lineages. The present findings indicate that MDR *P. aeruginosa* clones producing the PER-1 ESBL are endemic to this area of northern Italy, where they have been circulating since the mid-1990s and have been associated with several nosocomial outbreaks.

Pseudomonas aeruginosa is a leading cause of nosocomial infections, including pneumonia, urinary tract infections, and bacteremia. Infections can be particularly severe in cases of an impaired specific or nonspecific defense, such as that in neutropenic or cancer patients (21).

P. aeruginosa exhibits intrinsic resistance to several antimicrobial agents. The antipseudomonal β-lactams (such as ticarcillin, piperacillin, ceftazidime, cefepime, aztreonam, and the carbapenems) represent a major weapon against *Pseudomonas* infections, either for monotherapy or for combination therapy, for which β-lactams almost invariably represent one of the components (5, 21). Therefore, acquired resistance to these agents constitutes a major challenge for anti-*Pseudomonas* chemotherapy, especially when it is associated with resistance to other classes of drugs, such as aminoglycosides and fluoroquinolones (11).

Several mechanisms can contribute to acquired β-lactam resistance in *P. aeruginosa*, including β-lactamase production, the upregulation of efflux systems, and a decreased outer membrane permeability (11). Concerning β-lactamase production, mutational derepression of the chromosomally mediated AmpC enzyme remains one of the leading mechanisms of broad-spectrum β-lactam resistance (10). However, acquired extended-spectrum β-lactamases (ESBLs) encoded by mobile genetic elements, such as serine-β-lactamases of molecular classes A and D and metallo-β-lactamases of molecular class B, are important emerging resistance mechanisms in *P. aeruginosa* (11, 15, 29).

Among the acquired enzymes, PER-1 is a class A ESBL of notable clinical importance, due to its high level of activity toward oxyimino-cephalosporins and to the broad diffusion achieved by it in some epidemiological settings (16, 17, 20, 28), for which a poorer outcome of infections caused by PER-1 producers has been reported (27). In Italy, PER-1 was previously reported from two hospitals. At the Varese University hospital, sporadic isolates of PER-1-producing *P. aeruginosa* and *Alcaligenes faecalis* were detected (20; F. Luzzaro, G. M. Rossolini, M. Perilli, L. Pagani, R. Belloni, L. Lauretti, G. Amicosante, and A. Toniolo, Abstr. 99th Gen. Meet. Am. Soc. Microbiol., abstr. A-73, 1999), and a PER-1-producing *P. aeruginosa* clone recently caused a major outbreak in the general intensive care unit (ICU) (12). At the Pavia University hospital, PER-1-producing isolates of *Proteus mirabilis* that are resistant to extended-spectrum cephalosporins have recently been detected in various wards (19).

In this study, we report the detection of multidrug-resistant *P. aeruginosa* isolates producing the PER-1 ESBL that have been involved in nosocomial outbreaks in various hospitals in northern Italy since 1995. A molecular characterization of

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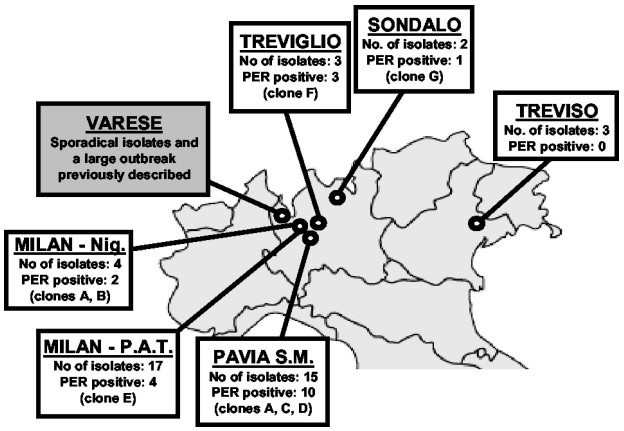

FIG. 1. Map of northern Italy showing the locations of the six hospitals from which the *P. aeruginosa* isolates investigated in this study were isolated. P.A.T., Pio Albergo Trivulzio Hospital; Nig., Niguarda Hospital; S.M., San Matteo Hospital. For each hospital, the total number of isolates and the number of PER-1-positive isolates are reported. The location of the hospital in Varese, from which PER-1-positive *P. aeruginosa* isolates were previously detected (12), is also shown (shaded in gray).

these isolates was performed to investigate their relationships and the spreading patterns of the bla_{PER-1} determinant.

MATERIALS AND METHODS

Clinical isolates. The *P. aeruginosa* strains analyzed in this study were nonreplicate (one per patient) clinical isolates from six different hospitals in Northern Italy (Fig. 1) that were isolated during the period 1995-2000. All isolates were resistant to oxyimino-cephalosporins (ceftazidime and cefepime) and aztreonam and putatively produced a secondary ESBL, according to the results of a double-disk synergy test performed as described below. All isolates had been involved in nosocomial outbreaks, defined on the basis of epidemiological data (three or more nonreplicate nosocomial isolates that showed identical or similar resistance profiles and were spatially and temporally related according to clinical records). In some cases, a single representative per outbreak was obtained, while in other cases multiple isolates from the same outbreak were present. The identification of the isolates was always double-checked by using the GNI card of the Vitek System (BioMérieux, Rome, Italy).

Susceptibility testing. In vitro susceptibility was determined by a broth macrodilution procedure using cation-supplemented Mueller-Hinton (MH) broth (Difco Laboratories, Detroit, Mich.), as recommended by the National Committee for Clinical Laboratory Standards (NCCLS) (13). The results of susceptibility testing were interpreted according to the criteria of the NCCLS (14). Antimicrobial agents were purchased from Sigma Chemical Co. (St. Louis, Mo.) unless otherwise specified. Cefepime and aztreonam were purchased from Bristol-Myers Squibb (Wallingford, Conn.), imipenem was from Merck (Rome, Italy), meropenem was from Astra-Zeneca (Milan, Italy), piperacillin and piperacillin-tazobactam were from Wyeth (Catania, Italy), and ciprofloxacin was from Bayer (Milan, Italy). *P. aeruginosa* ATCC 27853 was used for quality control purposes in susceptibility testing. Susceptibility to tellurite was tested as described previously (7), using brain heart infusion agar (Oxoid Unipath, Milan, Italy) containing 0.5 mM K_2TeO_3 (Sigma Chemical Co.). *P. aeruginosa* K3913/SI, a tellurite-resistant strain from our collection, and *P. aeruginosa* PAO1 were used as a positive and negative control, respectively.

β-Lactamase assays. The double-disk synergy test for the detection of ESBL activity was performed essentially as described previously (12) by screening for synergism between clavulanate (represented by a disk of amoxicillin-clavulanate) and ceftazidime, cefepime, or aztreonam. The disks were placed at a distance of 20 mm (center to center). A potentiation of the inhibitory zones of any of the expanded-spectrum β-lactams by clavulanate was considered suggestive of ESBL production. *P. aeruginosa* ATCC 27853 and *P. aeruginosa* 87SM (a clinical isolate from our collection that overproduces the AmpC enzyme) were used as negative controls. *P. aeruginosa* VA-463/98, which produces the PER-1 enzyme (12), was used as a positive control.

Analytical isoelectric focusing (IEF) of crude bacterial lysates for the detection of β-lactamases was carried out in polyacrylamide gels containing ampholines (pH range, 3.5 to 10), as described previously (18). Crude extracts were prepared by sonication from early-stationary-phase cultures grown aerobically at 37°C in antibiotic-free brain heart infusion broth (Biokar Diagnostics, Milan, Italy). β-Lactamase bands were visualized with the chromogenic substrate nitrocefin (Oxoid Unipath) as described previously (18). The activities of the β-lactamase bands separated by IEF against cefotaxime, ceftazidime, cefepime, and aztreonam were assayed by a substrate overlay procedure as described previously (18).

Molecular analysis. Colony blot hybridization was performed as described previously (20) with bacteria grown directly on sterile nitrocellulose filters layered onto MH agar plates (Schleicher & Schuell, Dassel, Germany). *P. aeruginosa* VA-463/98 and *P. aeruginosa* ATCC 27853 were included as positive and negative hybridization controls, respectively. Southern blot hybridization was carried out on dried gels, as described previously (26). Genomic DNA was extracted from *P. aeruginosa* as described previously (8). Plasmid DNA extraction was done by the alkaline lysis method (23), by the method of Kado and Liu (9), and by the method of Hansen and Olsen (6). The probe used for hybridization experiments was a PCR-generated amplicon comprising the entire bla_{PER-1} open reading frame (20) labeled with ^{32}P by the random priming technique by use of a commercial kit (Rediprime II DNA labeling system; Amersham, Milan, Italy). PCR amplification of the bla_{PER} alleles was carried out with primers BLA-PER/F (5′-GGGACARTCSKATGAATGTCA) and BLA-PER/R (5′-GG YSGCTTAGATAGTGCTGAT), as described previously (20). The sequenced region included the coding sequence for the mature PER-1 enzyme and the last 10 amino acids of the signal peptide. The region containing the bla_{PER-1} gene of isolate Ps101PAT was cloned from a genomic library constructed in the plasmid vector pBC-SK (Stratagene Inc., La Jolla, Calif.) by screening the library with the same bla_{PER-1} probe used for the hybridization assays (described above). The characterization of the region containing the bla_{PER-1} gene in other PER-1-positive clones was performed by a PCR mapping and sequencing approach. Primers PER-EXT1/F (5′-CC GGG GAA ATG CAG GCT GA) and PER-EXT1/R (5′-GAA TTC GCT CAT GCT GAA GAT TA), designed for the terminal regions of the cloned fragment, were used for the amplification reaction, and custom primers were used for amplicon sequencing. PCRs were always carried out with the Expand high-fidelity PCR system (Roche Molecular Biochemicals, Mannheim, Germany) and 10 ng of a genomic DNA template under the reaction conditions recommended by the manufacturer. Amplicon sequences were always determined for both strands, as described previously (20).

Analysis of macrorestriction patterns of genomic DNAs by PFGE. Genomic DNAs for pulsed-field gel electrophoresis (PFGE) analysis were extracted from stationary-phase cultures grown in Luria broth (Sigma) by using a pathogen group reagent kit 3 (Genepath system; Bio-Rad, Richmond, Calif.). Digestion of the genomic DNAs was carried out with the enzyme SpeI, used as recommended by the enzyme manufacturer (Bio-Rad). PFGE was performed with 1% agarose gels in 0.5× Tris-borate-EDTA buffer (23) at 14°C and 6 V/cm, using a Gene Path apparatus (Bio-Rad). After electrophoresis, the restriction fragments were stained with ethidium bromide and visualized under UV light. Comparisons of the PFGE patterns were interpreted according to the criteria of Tenover et al. (24).

Gene transfer experiments. Conjugation experiments were performed on a solid medium (MH agar), with *P. aeruginosa* 10145/3 (an *rpoB his* derivative of the reference strain ATCC 10145^T) used as a recipient. The initial donor/recipient ratio was 0.1. Mating plates were incubated at 37°C for 8 h. Transconjugants were selected on MH agar containing ceftazidime (50 μg/ml) plus rifampin (300 μg/ml). The detection sensitivity of the assay was $\geq 5 \times 10^{-8}$ transconjugants/recipient.

Nucleotide sequence accession number. The nucleotide sequence reported in this paper has been submitted to the EMBL and GenBank databases and assigned accession number AJ621265.

RESULTS

Detection of PER-1-producing *P. aeruginosa* isolates from different hospitals in northern Italy. A total of 44 nonreplicate clinical isolates of *P. aeruginosa* were investigated for the presence of acquired bla_{PER} ESBL determinants. The isolates were collected from six different hospitals in northern Italy (Fig. 1) during the period 1995-2000 and fulfilled the following criteria: (i) resistance to oxyimino-cephalosporins (ceftazidime and cefepime) and aztreonam; (ii) putative ESBL production, as

indicated by a positive double-disk synergy test between clavulanate and at least one of the compounds mentioned above; and (iii) involvement in a nosocomial outbreak.

A colony blot hybridization assay using a bla_{PER-1}-specific probe yielded positive results for a total of 20 isolates from five different hospitals (Fig. 1). PCR analysis with primers designed for the amplification of known bla_{PER} genes (20) yielded an amplification product of the expected size (966 bp) from each of the 20 hybridization-positive isolates (data not shown). Direct sequencing of the amplification products revealed, in all cases, the presence of a bla_{PER} allele encoding a mature enzyme that was identical to PER-1 (16). The putative ESBL producers that did not carry bla_{PER-1}-related sequences were not investigated further in this study.

A review of the epidemiological data for the 20 PER-1-positive isolates revealed that, overall, they had been involved in nine independent outbreaks in five different hospitals. A single representative of each outbreak (the earliest isolate available) was selected for further investigation (Table 1).

The susceptibilities to various antimicrobial agents of the nine PER-1-positive isolates that are representative of the nine outbreaks are reported in Table 1. In addition to a uniform resistance to ceftazidime, cefepime, and aztreonam, all isolates were also resistant to meropenem, gentamicin, tobramycin, and netilmicin, and most of them were also resistant to ciprofloxacin and amikacin. More than half of the isolates retained susceptibility to piperacillin and piperacillin-tazobactam. In the double-disk test for ESBL detection, all of the PER-1 producers exhibited synergy between clavulanate and each extended-spectrum β-lactam (ceftazidime, cefepime, and aztreonam). None of the nine isolates grew in the presence of 0.5 mM tellurite.

Analytical IEF of crude extracts of the nine isolates, coupled with a bioassay to unravel the activities of the separated enzymes against oxyimino-cephalosporins and aztreonam, revealed in each isolate a β-lactamase with a pI of 5.3 that was active on cefotaxime, ceftazidime, cefepime, and aztreonam, consistent with the production of the PER-1 ESBL. An enzyme with an alkaline pI (range, 8.0 to 8.4) which did not show ESBL activity in the bioassay was also detectable in each isolate and most likely corresponded to the chromosomally encoded AmpC protein. Finally, an additional enzyme with a pI of 6.9 that exhibited ESBL activity was present in a single isolate (Table 1). The nature of this enzyme was not investigated further in this work.

Clonal relationships of PER-1-producing isolates. The clonal relationships of the nine PER-1-producing isolates that were representative of the nine outbreaks were investigated by comparing the PFGE profiles of genomic DNAs after digestion with SpeI.

The PFGE profiles exhibited a notable degree of diversity. Only a couple of isolates (Pa34SM and Pa6NI) showed identical profiles (PFGE pattern A), and another couple (Pa105TR and Pa144TR) showed profiles that differed by only three bands (PFGE patterns F_0 and F_1). All other isolates exhibited profiles that differed from each other by more than four bands (Fig. 2), so the nine PER-1 producers appeared to be distributed in seven different clonal lineages (Table 1). It should be noted, however, that members of some clonal lineages differed

TABLE 1. Sources, antimicrobial susceptibilities, IEF profiles, and PFGE profiles of the nine epidemiologically unrelated PER-1-producing *P. aeruginosa* clinical isolates investigated in this study

Hospital	Isolate	Date	Ward[a]	Specimen[b] type	MIC of antibiotic (μg/ml)[c]									pIs by IEF[d]	PFGE pattern
					CZ	FP	AT	PI	PT	IP	ME	CP	GM	TO	NE	AK		
San Matteo, Pavia	Pa34SM	November 1997	HAEM	Cutaneous swab	>32	>32	>32	32	32	>16	>16	>4	>16	>16	>32	>64	5.3, 8.0	A
	Pa50SM	March 1998	PAED-SU	Urine	>32	>32	>32	>128	>128	>16	>16	>4	>16	>16	>32	32	5.3, 8.3	C
	Pa66SM	April 1998	ICU	Cutaneous swab	>32	>32	>32	>128	>128	<4	>16	>4	>16	>16	>32	>64	5.3, 8.0	D
Treviglio	Pa105TR	September 1995	ICU	BAW	>32	>32	>32	64	16	≤4	>16	>4	>16	>16	>32	32	5.3, 8.0	F_0
	Pa144TR	September 1998	ICU	BAW	>32	>32	>32	>128	16	>16	>16	>4	>16	>16	>32	>64	5.3, 8.0	F_1
Pio Albergo Trivulzio, Milan	Ps101PAT	August 1999	LCTF	Urine	>32	>32	>32	>128	>128	>16	>16	>4	>16	>16	>32	>64	5.3, 6.9, 8.2	E
Niguarda, Milan	Pa6NI	February 1999	BMT	Pharyngeal swab	>32	>32	>32	32	16	>16	>16	>4	>16	>16	>32	>64	5.3, 8.0	A
	Pa40NI00	February 2000	HAEM	Pharyngeal swab	>32	>32	>32	16	≤8	≤4	>16	≤1	>16	>16	>32	>64	5.3, 8.4	B
Sondalo	Pa130SO	December 1999	NSU	Sputum	>32	>32	>32	32	16	>16	>16	>4	>16	>16	>32	>64	5.3, 8.0	G

[a] HAEM, hematology; PAED-SU, pediatric surgery; LCTF, long-term care facility; BMT, bone marrow transplantation unit; NSU, neurosurgery.
[b] BAW, bronchoalveolar washing.
[c] CZ, ceftazidime; FP, cefepime; AT, aztreonam; PI, piperacillin; PT, piperacillin-tazobactam (tazobactam at a fixed concentration of 4 μg/ml); IP, imipenem; ME, meropenem; CP, ciprofloxacin; GM, gentamicin; TO, tobramycin; NE, netilmicin; AK, amikacin.
[d] pIs of β-lactamase activities in crude cell extracts, as detected by analytical IEF. In the bioassay, the pI 5.3 enzyme always exhibited activity against cefotaxime, ceftazidime, cefepime, and aztreonam; the pI 6.9 enzyme of isolate Ps101PAT exhibited activity against ceftazidime, cefepime, and aztreonam; and the pI 8.0 to 8.4 enzymes did not exhibit ESBL activity.

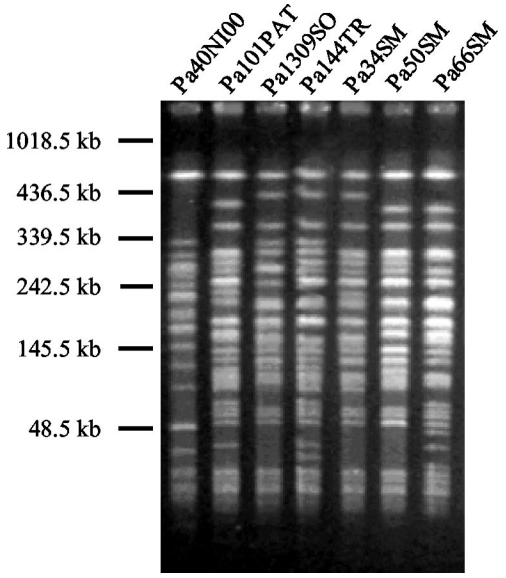

FIG. 2. PFGE profiles of genomic DNAs of PER-1-positive *P. aeruginosa* isolates after digestion with SpeI. The profile of isolate Pa6NI, which was identical to that of Pa34SM, and that of isolate Pa105TR, which was different from that of Pa144TR by three bands, are not shown. DNA size standards are reported on the left.

from each other by fewer than seven bands (Fig. 2), suggesting a potential (although more distant) relatedness.

Different outbreaks were usually associated with different clonal lineages. However, one clone (PFGE profile F) was detected in the same hospital (Treviglio) over an extended period of time, causing two apparently unrelated outbreaks, while another one (PFGE profile A) was detected in two different hospitals (San Matteo Hospital, Pavia, Italy, in 1997 and Ni-

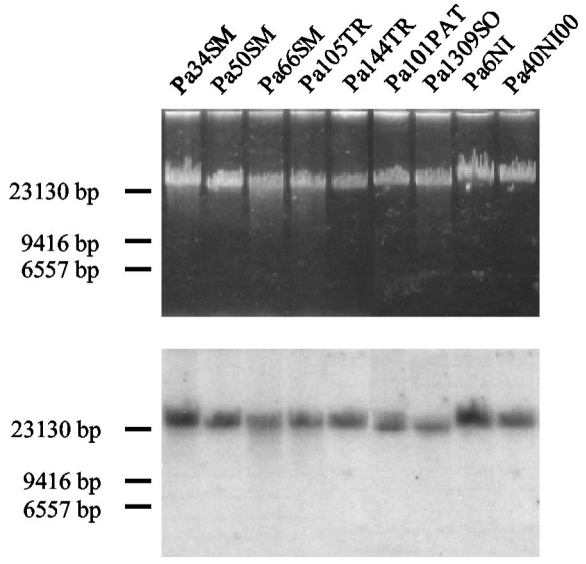

FIG. 3. Agarose gel electrophoresis of undigested genomic DNAs of the nine epidemiologically unrelated PER-1-positive *P. aeruginosa* isolates investigated in this study (top) and results of Southern blot analysis of the same samples with a bla_{PER-1} probe (bottom). DNA size standards are reported on the left.

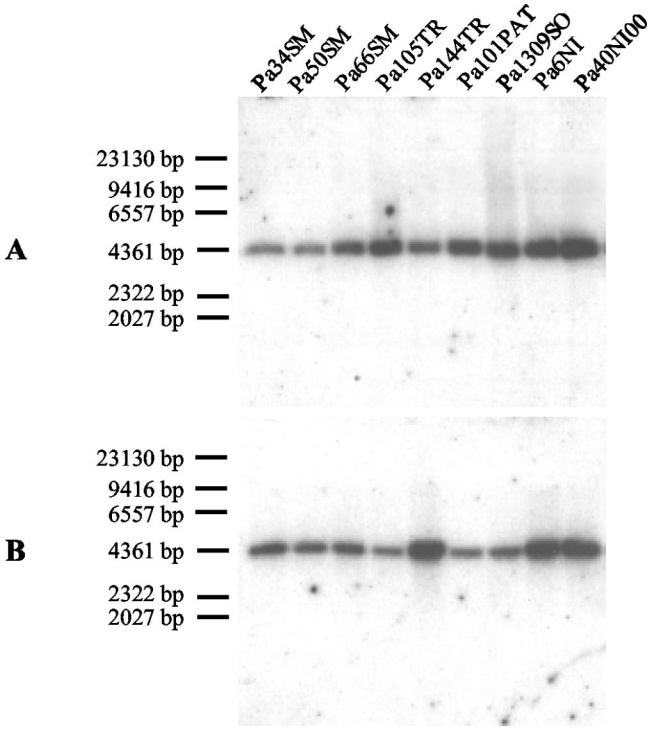

FIG. 4. Results of Southern blot analysis of genomic DNAs of the nine epidemiologically unrelated PER-1-positive *P. aeruginosa* isolates investigated in this study with a bla_{PER-1} probe after digestion with EcoRI (A) or EcoRV (B). DNA size standards are reported on the left.

guarda Hospital, Milan, Italy, in 1999 [Fig. 1; Table 1]). In some hospitals, multiple clones were present (Fig. 1; Table 1).

None of the PER-1 producers that were representative of the nine outbreaks was related to the isolate responsible for a nosocomial outbreak that previously occurred in the general ICU of the Varese Hospital (12), according to PFGE analysis (data not shown).

Transferability, genetic support, and environment of bla_{PER-1} gene. The transferability in conjugation experiments of the bla_{PER-1} determinant to a *P. aeruginosa* recipient was tested for each of the nine PER-1-positive isolates that were representative of the nine outbreaks. Conjugational transfer of the ESBL determinant was not detected in any case.

Plasmid DNAs were not detectable by agarose gel electrophoresis of either genomic or plasmid DNA preparations from any of the nine PER-1-positive isolates, even when plasmid DNAs were extracted by the method described by Hansen and Olsen (6), which is suitable for detecting large Inc-P2 plasmids, some of the most common plasmids in *P. aeruginosa* (Fig. 3, top panel; also data not shown). The absence of similar plasmids was also supported by the susceptibility to tellurite exhibited by all isolates (described above). For all of the isolates, Southern blot hybridization with a bla_{PER-1} probe yielded a hybridization signal corresponding to the band of chromosomal DNA (Fig. 3, bottom panel), suggesting that the bla_{PER-1} determinant was inserted in the chromosome.

The restriction fragment length polymorphism of the bla_{PER-1} locus was investigated by Southern blotting using the bla_{PER-1} probe after the digestion of genomic DNAs with EcoRI or

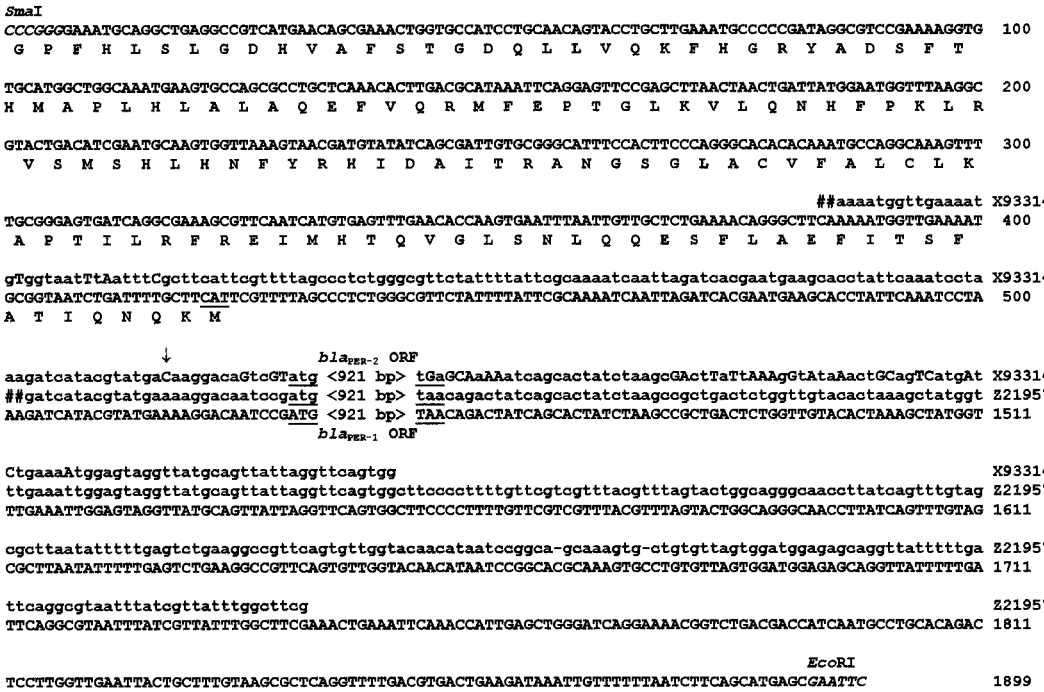

FIG. 5. Structure of SmaI-EcoRI genomic fragment containing the bla_{PER-1} gene from *P. aeruginosa* Ps101PAT and comparison with the region containing bla_{PER-1} from *P. aeruginosa* RNL-1 (16) (EMBL/GenBank database entry Z21957) and the region containing bla_{PER-2} in plasmid pMVP-5 from *S. enterica* serovar Typhymurium JMC (1) (EMBL/GenBank database entry X93314). The sequence from Ps101PAT is shown in capital letters. The start and stop codons of the bla_{PER} genes are underlined. The start codon of the open reading frame, encoding a transposase-like protein whose translation is shown below the nucleotide sequence, is also underlined. The other sequences are shown in lowercase letters, except when they are different from that of Ps101PAT; in the region upstream of the bla_{PER} genes, "##" indicates that the sequence completely diverges beyond that point. A vertical arrow indicates the point of insertion of an extra 115-bp sequence in the region upstream of bla_{PER-2}.

EcoRV (neither enzyme cuts inside bla_{PER-1} [16]). With either enzyme, an apparently identical hybridization profile was observed for all isolates, consisting of a single band of approximately 4.2 kb after digestion with EcoRI and a single band of approximately 6.4 kb after digestion with EcoRV (Fig. 4).

A 1.9-kb SmaI-EcoRI genomic DNA fragment containing the bla_{PER-1} gene was cloned from isolate Ps101PAT (representative of clonal lineage E) into the plasmid vector pBC-SK. Sequencing of the cloned fragment revealed that the bla_{PER-1} gene from Ps101PAT was identical to that from *P. aeruginosa* RNL-1 (16). The region downstream of bla_{PER-1} was virtually identical to that downstream of bla_{PER-1} in RNL-1 and was remarkably conserved compared to that downstream of bla_{PER-2} in plasmid pMVP-5 from *Salmonella enterica* serovar Typhimurium JMC (1) (Fig. 5). The region upstream of bla_{PER-1} was identical to that upstream of bla_{PER-1} in RNL-1 for the first 27 bp but abruptly diverged beyond that point, while it exhibited a high degree of similarity for a longer tract with the sequence upstream of bla_{PER-2} (except for the presence of a 115-bp insertion in the latter) (Fig. 5). An open reading frame (truncated by the SmaI cloning site) is present in the region upstream of bla_{PER-1} (Fig. 5), and it encodes a putative protein that exhibits 31% similarity with the transposase of an insertion sequence from a marine psychrotrophic bacterium (GenBank/EMBL database entry CAC84124) and less similarity with other (putative) transposases.

The structure of the region containing bla_{PER-1} was investigated, by a PCR mapping and sequencing approach, in representative isolates of the other clonal lineages. The results of these experiments revealed a structure identical to that of Ps101PAT for the bla_{PER-1}-containing regions of isolates Ps34SM (clonal lineage A), Pa40NI00 (clonal lineage B), Pa50SM (clonal lineage C), Pa66SM (clonal lineage D), and Pa105TR (clonal lineage F) (data not shown).

DISCUSSION

The antimicrobial susceptibility of *P. aeruginosa* has been reported to be on the decrease in several settings (4). In Europe, a significant decline in susceptibility rates to β-lactams, quinolones, and aminoglycosides was recently observed for this species (4), and nosocomial outbreaks of multidrug-resistant *P. aeruginosa*, usually caused by clonal spread, have been described for various European hospitals (for examples, see references 2, 12, 22, and 25). Although surveillance studies that establish baseline resistance patterns by geographic areas are numerous, the mechanisms underlying acquired antimicrobial resistance in *P. aeruginosa* isolates have been investigated in only a limited number of cases. A better knowledge of those mechanisms could be important for understanding the spreading patterns of resistant strains and of resistance determinants and for devising suitable control strategies.

For this study, we investigated the presence of PER-like ESBL determinants in isolates of *P. aeruginosa* that exhibited a resistance phenotype that was suggestive of ESBL production and that had been associated with nosocomial outbreaks in

various hospitals in northern Italy during 1995-2000. The results showed that *P. aeruginosa* isolates producing the PER-1 enzyme have been present in hospitals in the Milan area and the surrounding region, where they have been associated with multifocal nosocomial outbreaks, since at least the mid-1990s. The fact that similar isolates were also found at Varese University Hospital (12; Luzzaro et al., Abstr. 99th Gen. Meet. Am. Soc. Microbiol.; J. D. Docquier, F. Luzzaro, G. Amicosante, A. Toniolo, and G. M. Rossolini, Letter, Emerg. Infect. Dis. **7:**910-911, 2001), located in the same region, further supports the notion that PER-1-producing *P. aeruginosa* isolates have been endemic to this area for several years. In contrast, PER-1 producers were not detected at the Treviso hospital, which is located in a different region of northeastern Italy, suggesting that the resistance determinant may be absent or less widespread in that area. All of the PER-1 producers were not only resistant to extended-spectrum cephalosporins and monobactams, but also exhibited a multidrug-resistant phenotype that included most other antipseudomonal agents, leaving few therapeutic choices. Piperacillin-tazobactam was the most active among the tested drugs, although one-third of the strains were also resistant to this agent.

PER-1 producers associated with epidemiologically independent outbreaks belonged to multiple clonal lineages, with different lineages usually being present in different hospitals. However, the presence of the same clone in different hospitals was also observed (PFGE type A, detected in two different hospitals, in Milan and Pavia), likely reflecting interhospital spread of the resistant clone caused by the transfer of patients or by subsequent admissions of patients to different hospitals in that area. The polyclonal nature of the PER-1-producing strains was similar to that observed in Turkey, where a remarkable endemicity of PER-1 producers has been reported for several hospitals (28). The relatively broad distribution and polyclonal nature of PER-1-positive *P. aeruginosa* isolates detected in this area of northern Italy, along with the presence of the bla_{PER-1} determinant in strains of other species from the same area (19, 20), indicate that the bla_{PER-1} determinant is also endemic to this region of northern Italy, where it may represent an important emerging resistance determinant in various gram-negative species.

In *P. aeruginosa*, the bla_{PER-1} gene has been found either in plasmids (3) or on the chromosome (28, 29). In the isolates investigated in this study, the bla_{PER-1} determinants were apparently located on the chromosome, as suggested by the results of gene transfer, plasmid extraction, and hybridization experiments. An analysis of the bla_{PER-1} locus by Southern blotting and by PCR mapping and sequencing revealed a conserved structure in isolates from different clonal lineages, suggesting an overall conservation of the genetic element that delivered the resistance determinant to these strains. Interestingly, for our isolates the size of the EcoRI fragment recognized by the bla_{PER-1} probe was apparently the same as that found with most isolates from Turkey (28). However, the region upstream of bla_{PER-1} was different from that in *P. aeruginosa* RNL-1 (16), suggesting that a certain variability can also exist in the structure of the cognate genetic elements.

The fact that *P. aeruginosa* isolates producing the PER-1 enzyme tended to exhibit a multidrug-resistant phenotype that usually included aminoglycosides (12, 28; this study) could be related to a linkage between bla_{PER-1} and aminoglycoside resistance determinants within the same genetic element. We are currently investigating the structure of the element(s) carrying bla_{PER-1}. A preliminary characterization of bla_{PER-1}-flanking regions from these isolates revealed the presence of a gene for an original transposase that could pertain to the genetic element carrying bla_{PER-1}.

Finally, the results of this study also indicated that additional ESBLs are likely present in *P. aeruginosa* isolates that are resistant to extended-spectrum cephalosporins and aztreonam. Investigations of these enzymes are currently under way.

ACKNOWLEDGMENTS

This work was supported in part by grant no. 0035/2002 from Ricerca Corrente IRCCS Policlinico San Matteo, Pavia, Italy, and by grant no. 20011068755_003 from MIUR (PRIN 2001).

We thank F. Luzzaro, E. Viganò, A. Berlusconi, G. Ortisi, and P. Troupioti for providing some of the investigated isolates.

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