Antimicrobial Agents and Chemotherapy, Apr. 2010, p. 1619–1622 Vol. 54, No. 4
0066-4804/10/$12.00 doi:10.1128/AAC.01585-09
Copyright © 2010, American Society for Microbiology. All Rights Reserved.

PER-6, an Extended-Spectrum β-Lactamase from *Aeromonas allosaccharophila*▽

Delphine Girlich, Laurent Poirel, and Patrice Nordmann*

Service de Bactériologie-Virologie, INSERM U914 Emerging Resistance to Antibiotics, Hôpital de Bicêtre, Assistance Publique/Hôpitaux de Paris, Faculté de Médecine et Université Paris-Sud, Le Kremlin-Bicêtre, France

Received 9 November 2009/Returned for modification 8 January 2010/Accepted 28 January 2010

An *Aeromonas allosaccharophila* environmental isolate recovered from the Seine River (Paris, France) produced a novel extended-spectrum β-lactamase, PER-6, that shared 92% amino acid identity with the closest ß-lactamase, PER-2. The kinetic properties of PER-6 showed a slightly increased affinity for carbapenems. The bla_{PER-6} gene was chromosomally located and bracketed by non-transposon-related structures.

Aeromonas species are ubiquitous in aquatic environments. Formerly considered strict fish pathogens, *Aeromonas* spp. are reported increasingly as emerging human pathogens for both immunocompetent and immunocompromised patients as a source of gastroenteritis, bacteremia, meningitis, and skin and soft tissue infections (10).

Extended-spectrum β-lactamases (ESBLs) have rarely been reported to occur in *Aeromonas* species, with TEM-24 and CTX-M-1 identified in *Aeromonas hydrophila* (6, 24) and PER-1 in *Aeromonas media* (18). Although β-lactamases of the PER type are not the most common ESBLs (15) identified, they have been reported increasingly, with PER-1 being detected mainly in Europe, especially in Turkey (20) and in Italy (17). It has been also identified in Korea in *Providencia* spp. (12) and more recently in Algeria in *Proteus vulgaris* and *Providencia stuartii* (8). β-Lactamase PER-2, which is distantly related to PER-1 (86% amino acid identity), has been reported to occur in *Enterobacteriaceae* in South America, mostly in Argentina (2, 23). The PER-3, -4, and -5 β-lactamases, which are closely related to PER-1 (99% amino acid identity), have been identified in *Aeromonas punctata*, *P. vulgaris*, and *Acinetobacter baumannii*, according to data available in GenBank (accession no. AY740681, EU748544, and EU687473, respectively).

Aeromonas spp. are Gram-negative, mostly environmental species frequently containing plasmids and integrons with multiple genes for antibiotic resistance (9). *Aeromonas* spp. have been shown to be the source of plasmid-mediated resistance to quinolones of the Qnr type associated with novel genetic elements (3, 19).

The bla_{PER-1} gene has been identified mostly as part of a Tn*1213* composite transposon (20). The bla_{PER-2} gene has been identified on a large self-conjugative plasmid downstream of the IS*Pa12 tnpA* gene in *Citrobacter freundii* (23).

The present study was initiated by isolation of an *Aeromonas allosaccharophila* isolate from a water sample of the Seine River in Paris in January 2009, as a result of screening for multidrug-resistant isolates. The sampling procedure consisted of filtrating 100 ml of water through nitrocellulose membranes (0.45 μm; Millipore, Molsheim, France), resuspending the filters in 1 ml of sterile water, and plating 100-μl aliquots on ceftazidime (2 μg/ml)-containing MacConkey agar plates. Isolate AL-1 was identified by the API 32GN system (bioMérieux, Marcy-l'Etoile, France) and by sequencing of the 16S rRNA genes. MICs were determined by Etest (AB Biodisk, Solna, Sweden) and by the agar dilution method with Mueller-Hinton agar plates for imipenem and imipenem clavulanate and were interpreted according to the CLSI guidelines (4). *A. allosaccharophila* AL-1 was resistant or reduced in susceptibility to amoxicillin, ticarcillin, ceftazidime, and cefotaxime (Table 1). A synergy image between aztreonam or ceftazidime and clavulanic acid-containing disks suggested expression of an ESBL gene. Standard PCR amplification experiments performed with primers specific for the genes encoding β-lactamases TEM, SHV, PER-1, VEB, and GES (7) failed. Shotgun cloning using EcoRI-restricted genomic DNA and EcoRI-restricted pBKCMV plasmid (Invitrogen, Life Technologies, Cergy-Pontoise, France) was performed as previously described (16). Selection on amoxicillin (100 μg/ml)- and kanamycin (30 μg/ml)-agar plates yielded a recombinant *Escherichia coli* DH10B(pEco-1) clone expressing an ESBL phenotype. The cloned insert of 4,716 bp was sequenced by using universal primers T3 and T7 and the primers listed in Table 2. Recombinant *E. coli*(pEco-1) expressed a novel β-lactamase, PER-6 (www.lahey.org/Studies/). PER-6 shares 92% amino acid identity with PER-2, with the bla_{PER-6} gene sharing 79% nucleotide identity with the bla_{PER-1} gene, explaining the lack of PCR detection with the use of the bla_{PER-1}-specific primers. The G+C content of the bla_{PER-6} gene (45.5%) was much lower than that of the *A. allosaccharophila* genes (56.9%) (5), suggesting that the bla_{PER-6} gene in *A. allosaccharophila* Al-1 originated from another bacterial species. Transfer of the β-lactam resistance marker by conjugation or by transformation as described previously (21) from *A. allosaccharophila* AL-1 to an *E. coli* reference strain failed. Plasmid extraction performed as described previously (11) identified four plasmids of ca. 5, 12, 22, and 160 kb. None of these plasmids gave a hybridization signal with a bla_{PER-6}-specific probe after Southern transfer (Hybond N^+; GE Healthcare), suggesting a

* Corresponding author. Mailing address: Service de Bactériologie-Virologie, Hôpital de Bicêtre, 78 Rue du Général Leclerc, 94275 Le Kremlin-Bicêtre Cedex, France. Phone: 33-1-45-21-36-32. Fax: 33-1-45-21-63-40. E-mail: nordmann.patrice@bct.aphp.fr.
▽ Published ahead of print on 9 February 2010.

TABLE 1. MICs of β-lactams for *A. allosaccharophila* AL-1 and *E. coli* DH10B harboring recombinant plasmids pPER-1, pPER-2, and pPER-6 and the *E. coli* DH10B reference strain

β-Lactam(s)	MIC (μg/ml)				
	A. allosaccharophila AL-1(PER-6)	*E. coli* DH10B harboring:			*E. coli* DH10B
		pPER-6[a]	pPER-1	pPER-2	
Amoxicillin	>256	>256	>256	>256	4
Amoxicillin-CLA[b]	12	4	4	6	4
Ticarcillin	>256	>256	>256	>256	4
Ticarcillin-CLA	256	12	16	16	4
Piperacillin	4	256	32	256	1
Cephalothin	16	>256	>256	>256	2
Cefoxitin	0.5	2	2	2	1
Ceftazidime	128	>256	>256	>256	0.5
Cefotaxime	2	64	48	64	0.12
Cefepime	0.5	32	16	32	0.06
Cefpirome	0.5	8	16	16	0.06
Moxalactam	<0.06	2	1	0.5	0.12
Aztreonam	1	>256	>256	>256	0.25
Imipenem	1	0.25	0.25	0.25	0.25
Imipenem-CLA	0.5	0.25	0.25	0.25	0.25
Meropenem	0.06	0.0	0.03	0.03	0.06
Ertapenem	0.5	<0.03	<0.03	<0.03	<0.03

[a] *E. coli* DH10B(pPER-6) expressed β-lactamase PER-6 from *A. allosaccharophila* AL-1.
[b] CLA, clavulanic acid at a fixed concentration of 4 μg/ml.

chromosomal location for the bla_{PER-6} gene (data not shown). This location was confirmed by restricting total DNA with the I-CeuI enzyme (New England Biolabs, Saint-Quentin-en-Yvelines, France), followed by pulsed-field gel electrophoresis and hybridization with the bla_{PER-6} and rRNA probes, as described previously (13) (data not shown).

Analysis of the surrounding sequences did not identify composite transposon Tn*1213* (20, 23). Instead, part of an open reading frame of 550 bp was identified 80 bp upstream of the bla_{PER-6} gene, encoding a putative protein that shared weak identity (<28% amino acid identity) with a DNA translocase domain-containing protein (FtsK-like) (Fig. 1). Downstream of the bla_{PER-6} gene, the 5′ extremity (only 180 bp) of a gene encoding a glutathione *S*-transferase was identified, exhibiting 86% nucleotide identity with a gene identified downstream of the bla_{PER-1} and bla_{PER-2} genes (23). The location of part of an identical *gst* gene downstream of all those bla_{PER}-like genes indicates a common origin for these β-lactamase genes. That *gst* gene was truncated by a genetic structure containing a gene encoding a PecM-like protein, together with the *tetA* and *tetR* genes, usually part of the Tn*1721* transposon (22) (Fig. 1).

In order to compare the catalytic properties of PER-1, PER-2, and PER-6 and their contributions to β-lactam resistance, the corresponding genes were cloned and expressed in an isogenic *E. coli* background under the control of the same promoter (*E. coli* DH10B; Invitrogen, Cergy-Pontoise, France). Cloning experiments were performed with the pCR-BluntII-TOPO vector (Invitrogen) by following the manufacturer's instructions, using external primer PERextS and specific primers PER-1extAS, PER-2extAS, and PER-6extAS (Table 2), encompassing the entire group of bla_{PER} genes. It gave rise to recombinant strains *E. coli* DH10B(pPER-1), *E. coli* DH10B(pPER-2), and *E. coli* DH10B(pPER-6), expressing β-lactamases PER-1, PER-2, and PER-6, respectively. The MIC values of β-lactams showed no significant differences between those three different *E. coli* recombinant strains (Table 1).

In order to characterize more precisely whether PER-6

TABLE 2. Nucleotide sequences of primers used for amplification and sequence analysis

Primer	Sequence (5′ → 3′)
T3.1	TTAAGTTCATGGGTCGTCTCTG
T3.2	ATTCCGCCGAATCAGCAAGAAC
T7.1	ATCAGAAATGAGCGCCAGTC
T7.2	ATTGCCGATATCACTGATGG
PERextS	AAGGACARTCSKATGAATGTC[a]
PER-1extAS	TAGTGTACAACCAGAGTCAGC
PER-2extAS	TTGCTCAATCCGGACTCACTGC
PER-6extAS	TCGTTTAATCCGGACTTACTGCGG

[a] R represents A or G, S represents C or G, and K represents G or T.

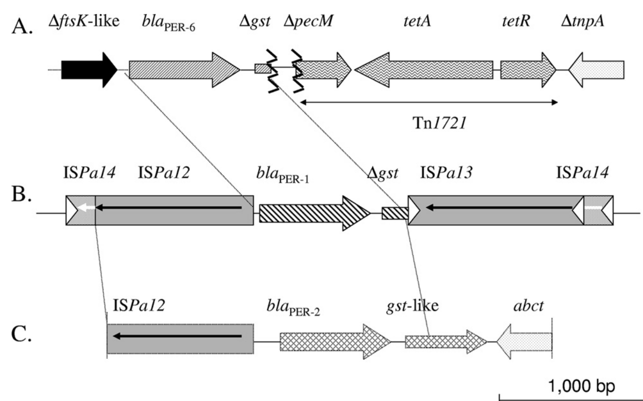

FIG. 1. Schematic map of the bla_{PER-6}-containing structure identified in *A. allosaccharophila* (this study) (A), the bla_{PER-1}-containing structure identified in *Pseudomonas aeruginosa* (20) (B), and the bla_{PER-2}-containing structure identified in *C. freundii* (23) (C).

TABLE 3. Steady-state kinetic parameters of the β-lactamase PER-6 and comparison of parameter values obtained for β-lactamase PER-2 (23)[a]

β-Lactam	PER-6			PER-2
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
Benzylpenicillin	5	200	25	120
Ampicillin	1	20	50	330
Ticarcillin	0.4	9	50	ND
Piperacillin	0.1	4	25	200
Cephalothin	8	55	145	670
Ceftazidime	12	1,000	10	430
Cefotaxime	40	900	45	760
Cefepime	10	2,000	5	20
Cefpirome	12	1,500	8	NA
Cefoxitin	ND	ND	ND	<10
Aztreonam	3	40	75	120
Moxalactam	ND	ND	ND	NA
Imipenem	0.006	1.5	4	<10
Meropenem	0.004	10	0.4	NA
Ertapenem	0.002	7	0.3	NA

[a] Data are means of results from three independent experiments. Standard deviations were within 10% of the means. ND, not determinable or no detectable hydrolysis ($<0.01\ s^{-1}$); NA, not available.

might possess specific catalytic properties, a kinetic study was initiated. *E. coli* DH10B(pPER-6) produced a β-lactamase with a pI value of 6.4 according to isoelectric focusing analysis performed as described previously (14). This pI value was different from those of PER-1 (16) and PER-2 (23) (both with a pI value of 5.4). PER-6 was purified (>90% as estimated by SDS-PAGE analysis; data not shown) from the *E. coli* DH10B(pPER-6) crude extract by using a two-step chromatography process (a cation exchange at pH 6.8 using an S-Sepharose column, followed by an anion exchange at pH 8 using a Q-Sepharose column). This protocol allowed 5 mg of purified PER-6 β-lactamase to be obtained (specific activity of 36,800 nmol/min · mg of protein with the use of 100 μM cephalothin as a substrate). β-Lactamase PER-6 had a broad-spectrum hydrolysis profile, including penicillins, broad-spectrum cephalosporins, and, surprisingly, carbapenems but excluding cephamycins (Table 3). The activity of PER-6 was less susceptible to inhibitions by clavulanic acid or tazobactam than that of PER-2. The fifty percent inhibitory concentrations (IC_{50}) for clavulanic acid were 0.3 and 0.07 μM for PER-6 and PER-2 (23), respectively, and those for tazobactam were 1 and 0.1 μM for PER-6 and PER-2 (23), respectively. ß-Lactamase PER-6 was weakly inhibited by sulbactam, with an IC_{50} of 4 μM. In comparison to PER-2, β-lactamase PER-6 showed overall low catalytic efficiencies (k_{cat}/K_m) for most β-lactams (Table 3). This was due to a significant alteration of the K_m values for all substrates, particularly for cephalosporins, which were from 20-fold (cefotaxime) to 120-fold (cefepime) higher than those observed for PER-2 (Table 3) (23). A significant hydrolytic activity against carbapenems was detected with PER-6. The k_{cat} values for PER-6 for imipenem, meropenem, and ertapenem were low, but the K_m values were also very low, showing a good affinity of PER-6 for those molecules. To better assess whether PER-6 may possess specific properties in comparison to other PER derivatives, specific activities for PER-1, PER-2, and PER-6 were determined with imipenem as a substrate, using culture extracts of isogenic *E. coli* recipient strains. No significant difference could be observed between those different extracts (data not shown), and no difference in the MICs of carbapenems could be observed (Table 1). However, among the 20 amino acid changes identified between PER-6 and PER-2, two were located in the Ω loop: alanine 161 was replaced by an aspartic acid residue, and glutamine 178 was replaced by a lysine residue in PER-6. These changes may be involved in the peculiar catalytic properties of PER-6.

Conclusion. This study emphasizes the spread of PER-type ESBLs in *Aeromonas* species. A novel PER-type β-lactamase was identified, the corresponding gene being associated with a novel genetic environment. However, the fact that identical structures were found immediately downstream of all bla_{PER} genes indicates a likely common origin.

Finally, the recent identification of PER-1 in *A. media* from a Swiss alpine lake, together with the present identification of PER-6 in *A. allosaccharophila* from the Seine river, emphasizes the spread of these PER-type determinants in the environment, at least on the European continent, with reservoirs likely being waterborne bacterial species.

Nucleotide sequence accession number. The nucleotide and protein sequences of the PER-6 ß-lactamase have been registered in GenBank under accession no. GQ396303.

This work was funded mostly by a grant from the INSERM (U914) and by grants from the European Community (TROCAR, HEALTH-F3-2008-223031) and from the Ministère de l'Education Nationale et de la Recherche (UPRES-EA3539), Université Paris XI, France.

REFERENCES

1. Reference deleted.
2. **Bauernfeind, A., I. Stemplinger, R. Jungwirth, P. Mangold, S. Amann, E. Akalin, O. An, C. Bal, and J. M. Casellas.** 1996. Characterization of ß-lactamase gene bla_{PER-2}, which encodes an extended-spectrum class A ß-lactamase. Antimicrob. Agents Chemother. **40:**616–620.
3. **Cattoir, V., L. Poirel, C. Aubert, C. J. Soussy, and P. Nordmann.** 2008. Unexpected occurrence of plasmid-mediated quinolone resistance determinants in environmental *Aeromonas* spp. Emerg. Infect. Dis. **14:**231–237.
4. **Clinical and Laboratory Standards Institute.** 2008. Performance standards for antimicrobial susceptibility testing; 18th informational supplement M100-S18. Clinical and Laboratory Standards Institute, Wayne, PA.
5. **Esteve, C., M. C. Gutiérrez, and A. Ventosa.** 1995. DNA relatedness among *Aeromonas allosaccharophila* strains and DNA hybridization groups of the genus *Aeromonas*. Int. J. Syst. Bacteriol. **45:**390–391.
6. **Fosse, T., C. Giraud-Morin, I. Madinier, F. Mantoux, J. P. Lacour, and J. P. Ortonne.** 2004. *Aeromonas hydrophila* with plasmid-borne class A extended-spectrum ß-lactamase TEM-24 and three chromosomal class B, C, and D ß-lactamases, isolated from a patient with necrotizing fasciitis. Antimicrob. Agents Chemother. **48:**2342–2343.
7. **Girlich, D., L. Poirel, A. Leelaporn, A. Karim, C. Tribuddharat, M. Fennewald, and P. Nordmann.** 2001. Molecular epidemiology of the integron-located VEB-1 extended-spectrum ß-lactamase in nosocomial enterobacterial isolates in Bangkok, Thailand. J. Clin. Microbiol. **39:**175–182.
8. **Iabadene, H., C. Dallenne, Y. Messai, D. Geneste, R. Bakour, and G. Arlet.** 2009. Emergence of extended-spectrum ß-lactamase PER-1 in *Proteus vulgaris* and *Providencia stuartii* isolates from Algiers, Algeria. Antimicrob. Agents Chemother. **53:**4043–4044.
9. **Jacobs, L., and H. Y. Chenia.** 2007. Characterization of integrons and tetracycline resistance determinants in *Aeromonas* spp. isolated from South African aquaculture systems. Int. J. Food Microbiol. **114:**295–306.
10. **Jones, B. L., and M. H. Wilcox.** 1995. *Aeromonas* infections and their treatment. J. Antimicrob. Chemother. **35:**453–461.
11. **Kieser, T.** 1984. Factors affecting the isolation of CCC DNA from *Streptomyces lividans* and *Escherichia coli*. Plasmid **12:**19–36.
12. **Lee, H. W., H. Y. Kang, K. S. Shin, and J. Kim.** 2007. Multidrug-resistant *Providencia* isolates carrying bla_{PER-1}, bla_{VIM-2}, and *armA*. J. Microbiol. **45:**272–274.
13. **Liu, S. L., A. Hessel, and K. E. Sanderson.** 1993. Genomic mapping with I-Ceu I, an intron-encoded endonuclease specific for genes for ribosomal RNA, in *Salmonella* spp., *Escherichia coli*, and other bacteria. Proc. Natl. Acad. Sci. U. S. A. **90:**6874–6878.

14. **Mammeri, H., L. Poirel, and P. Nordmann.** 2003. In vivo selection of a chromosomally encoded β-lactamase variant conferring ceftazidime resistance in *Klebsiella oxytoca*. Antimicrob. Agents Chemother. **47:**3739–3742.
15. **Naas, T., L. Poirel, and P. Nordmann.** 2008. Minor extended-spectrum ß-lactamases. Clin. Microbiol. Infect. **14**(Suppl. 1)**:**42–52.
16. **Nordmann, P., E. Ronco, T. Naas, C. Duport, T. Michel-Briand, and R. Labia.** 1993. Characterization of a novel extended-spectrum ß-lactamase from *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **37:**962–969.
17. **Perilli, M., F. De Santis, C. Mugnaioli, G. M. Rossolini, F. Luzzaro, S. Stefani, M. L. Mezzatesta, A. Toniolo, and G. Amicosante.** 2007. Spread of *Enterobacteriaceae* carrying the PER-1 extended-spectrum ß-lactamase gene as a chromosomal insert: a report from Italy. J. Antimicrob. Chemother. **59:**323–324.
18. **Picão, R. C., L. Poirel, A. Demarta, O. Petrini, A. R. Corvaglia, and P. Nordmann.** 2008. Expanded-spectrum ß-lactamase PER-1 in an environmental *Aeromonas media* isolate from Switzerland. Antimicrob. Agents Chemother. **52:**3461–3462.
19. **Picão, R. C., L. Poirel, A. Demarta, C. S. Silva, A. R. Corvaglia, O. Petrini, and P. Nordmann.** 2008. Plasmid-mediated quinolone resistance in *Aeromonas allosaccharophila* recovered from a Swiss lake. J. Antimicrob. Chemother. **62:**948–950.
20. **Poirel, L., L. Cabanne, H. Vahaboglu, and P. Nordmann.** 2005. Genetic environment and expression of the extended-spectrum ß-lactamase $bla_{\mathrm{PER-1}}$ gene in gram-negative bacteria. Antimicrob. Agents Chemother. **49:**1708–1713.
21. **Poirel, L., M. Guibert, S. Bellais, T. Naas, and P. Nordmann.** 1999. Integron- and carbenicillinase-mediated reduced susceptibility to amoxicillin-clavulanic acid in isolates of multidrug-resistant *Salmonella enterica* serotype Typhimurium DT104 from French patients. Antimicrob. Agents Chemother. **43:**1098–1104.
22. **Post, V., and R. M. Hall.** 2009. AbaR5, a large multiple-antibiotic resistance region found in *Acinetobacter baumannii*. Antimicrob. Agents Chemother. **53:**2667–2671.
23. **Power, P., J. Di Conza, M. M. Rodríguez, B. Ghiglione, J. A. Ayala, J. M. Casellas, M. Radice, and G. Gutkind.** 2007. Biochemical characterization of PER-2 and genetic environment of $bla_{\mathrm{PER-2}}$. Antimicrob. Agents Chemother. **51:**2359–2365.
24. **Radice, M., P. Power, J. Di Conza, and G. Gutkind.** 2002. Early dissemination of CTX-M-derived enzymes in South America. Antimicrob. Agents Chemother. **46:**602–604.