ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 2011, p. 118–123 Vol. 55, No. 1
0066-4804/11/$12.00 doi:10.1128/AAC.01062-10
Copyright © 2011, American Society for Microbiology. All Rights Reserved.

Metallo-β-Lactamase Production by *Pseudomonas otitidis*: a Species-Related Trait▽

Maria Cristina Thaller,[1] Luisa Borgianni,[2] Gustavo Di Lallo,[1] Yunsop Chong,[3] Kyungwon Lee,[3] Joseph Dajcs,[4] David Stroman,[4] and Gian Maria Rossolini[2]*

Dipartimento di Biologia, Università di Roma "Tor Vergata," I-00133 Rome, Italy[1]; Dipartimento di Biologia Molecolare, Sezione di Microbiologia, Università di Siena, I-53100 Siena, Italy[2]; Department of Laboratory Medicine, Yonsei University College of Medicine, Seoul, South Korea[3]; and Alcon Research Ltd., Fort Worth, Texas[4]

Received 2 August 2010/Returned for modification 17 September 2010/Accepted 29 October 2010

Susceptibility to several β-lactams and β-lactamase production was investigated in a collection of 20 strains of *Pseudomonas otitidis*, a new *Pseudomonas* species that has been recently recognized in association with otic infections in humans. All strains appeared to be susceptible to piperacillin, cefotaxime, ceftazidime, and aztreonam, while resistance or decreased susceptibility to carbapenems was occasionally observed. All strains were found to express metallo-β-lactamase (MBL) activity and to carry a new subclass B3 MBL gene, named bla_{POM}, that appeared to be highly conserved in this species. *P. otitidis*, therefore, is the first example of a pathogenic *Pseudomonas* species endowed with a resident MBL. The POM-1 protein from *P. otitidis* type strain MCC10330 exhibits the closest similarity (60 to 64%) to the L1 MBL of *Stenotrophomonas maltophilia*. Expression in *Escherichia coli* and *Pseudomonas aeruginosa* revealed that, similar to L1 and other subclass B3 MBLs, POM-1 confers decreased susceptibility or resistance to carbapenems, penicillins, and cephalosporins but not to aztreonam. Expression of the POM MBL in *P. otitidis* is apparently constitutive and, in most strains, does not confer a carbapenem-resistant phenotype. However, a strong inoculum size effect was observed for carbapenem MICs, and carbapenem-resistant mutants could be readily selected upon exposure to imipenem, suggesting that carbapenem-based regimens should be considered with caution for *P. otitidis* infections.

Pseudomonas otitidis is a new *Pseudomonas* species that has recently been recognized in association with otic infections in humans, including acute otitis externa, acute otitis media, and chronic suppurative otitis media (2). Genotypically and phenotypically, *P. otitidis* is closely related to *Pseudomonas aeruginosa* (2), and this similarity likely accounts for the belated identification of this new pathogenic species within the *Pseudomonas* genus.

The susceptibility of *P. otitidis* was previously investigated with several antimicrobial agents, including aminoglycosides, fluoroquinolones, macrolides, β-lactams, tetracycline, chloramphenicol, and polymyxin B, and overall, the behavior of this species appeared to be similar to that of *P. aeruginosa* (2). However, of β-lactams, only piperacillin was tested, while no information is available on the susceptibility of this species to antipseudomonal cephalosporins, aztreonam, and carbapenems.

In this work, we investigated the susceptibility of *P. otitidis* to several β-lactams and the production of β-lactamase activity by this species. Results revealed that *P. otitidis* strains constitutively produce a novel subclass B3 metallo-β-lactamase (MBL), that was named POM (after P. otitidis metallo-β-lactamase), which is active on carbapenems and other β-lactams.

MATERIALS AND METHODS

Bacterial strains. The *P. otitidis* strains investigated in this work included 19 strains from the collection previously described by Clark et al. (2) and a clinical isolate from South Korea (isolate YMC-Po/06) cultured from the purulent discharge of a patient suffering from chronic suppurative otitis media (Table 1). Identification of the latter isolate was carried out by 16S rRNA gene sequencing (7) and biochemical profiling (2). *Escherichia coli* strain MC1061 and *Pseudomonas aeruginosa* strain PAO1 were used as hosts for recombinant plasmids.

Antimicrobial susceptibility testing. MICs were determined by Etest (bioMérieux SA, Marcy l'Etoile, France) according to the manufacturer's instructions or by an agar dilution technique in Mueller-Hinton (MH) agar (3). The latter method was used to evaluate the inoculum size effect, using inocula of 10^5 and 10^7 CFU per spot, respectively. Results were recorded after incubation at 37°C for 18 to 20 h. *P. aeruginosa* ATCC 27853 was used for quality control of susceptibility testing.

***In vitro* selection of carbapenem-resistant mutants.** Approximately 10^9 CFU of *P. otitidis* strain MCC10330^T were plated on MH agar containing a gradient concentration of imipenem (from 0 to 5 μg/ml) and incubated overnight at 37°C. Colonies grown in the presence of the antibiotic were subcultured in antibiotic-free MH agar and subjected to reidentification by 16S rRNA gene sequencing and biochemical profiling (2, 7).

β-Lactamase assays. Production of carbapenemase activity in crude extracts was assayed spectrophotometrically as described previously (9) in buffer containing 50 mM HEPES plus 50 μM $ZnSO_4$ at pH 7.5 and 30°C, with 150 μM imipenem as the substrate. Inhibition by EDTA was tested by measuring carbapenemase activity in the presence of 5 mM EDTA after preincubation of the sample for 20 min at 30°C with the same EDTA concentration. Isoelectric focusing (IEF) analysis was performed as described previously (5). β-Lactamase induction experiments were carried out with exponentially growing cells in MH broth, using imipenem to induce β-lactamase. β-Lactamase activity was determined in crude extracts, using imipenem and nitrocefin as substrates as described previously (9).

DNA analysis and manipulation techniques. Basic recombinant DNA methodology was performed essentially as described by Sambrook et al. (10). Genomic DNA was extracted from *P. otitidis* strains as described previously (9). The genomic library from *P. otitidis* MCC10330^T was constructed in the *E. coli* pACYC184 plasmid vector as described previously (9). The shuttle vector pPME6001 (1) was used as the subcloning vector. The presence of the bla_{POM-1}-like genes in *P. otitidis* isolates was investigated by PCR. Amplification reactions were carried out in a 50-μl volume, with the Pomseq/F and Pomseq/R forward and reverse primers, respectively (Table 2) (25 pM each), 125 μM (each)

* Corresponding author. Mailing address: Dipartimento di Biologia Molecolare, Sezione di Microbiologia, Università di Siena, I-53100 Siena, Italy. Phone: 39 0577 233455. Fax: 39 0577 233334. E-mail: rossolini@unisi.it.
▽ Published ahead of print on 8 November 2010.

TABLE 1. *P. otitidis* strains investigated in this work, MBL activity measured in crude extracts, and MICs of various β-lactams

Source of strain or MIC parameter[a]	Strain	MBL production[b]	MIC (μg/ml) of β-lactam[c]:					
			IMP	MEM	PIP	AZT	CTX	CAZ
CU	MCC04446	46.0	0.75	3	1.5	6	6	6
CU	MCC04511	28.7	>32	>32	4	6	4	2
CU	MCC04517	37.8	3	6	3	4	2	6
CSOM	MCC09159	44.7	2	−12	0.75	2	1.5	4
AOE	MCC10330[T]	42.3	3	3	4	4	2	6
AOE	MCC10429	58.1	2	2	6	4	6	8
AOE	MCC10744	66.7	1	2	4	6	4	3
AOE	MCC11140	42.4	1.5	1	4	6	3	6
AOE	MCC11061	28.5	1	2	4	8	3	4
AOE	MCC11338	41.1	1	3	1.5	2	2	4
AOE	MCC11683	30.3	1	1.5	0.75	6	4	4
AOE	MCC12065	38.8	0.5	4	1.5	6	2	4
AOE	MCC12178	36.2	1.5	2	4	8	3	3
AOM	MCC40150	17.9	1	2	1.5	3	1.5	3
AOM	MCC40159	16.6	0.75	6	4	8	4	3
AOM	MCC51196	15.1	0.75	0.25	1	6	3	3
AOMT	MCC61379	97.2	2	16	3	6	4	4
AOMT	MCC61485	16.1	2	2	2	6	2	3
AOMT	MCC61433	31.5	2	24	3	4	4	4
CSOM	YMC-Po/06	81.7	>32	>32	2	4	3	4
MIC range			0.50–>32	0.25–>32	0.75–6	2–8	1.5–6	2–8
MIC_{50}			1.5	3	3	6	3	4
MIC_{90}			3	24	4	8	4	6

[a] CU, corneal ulcer; CSOM, chronic suppurative otitis media; AOE, acute otitis externa; AOM, acute otitis media; AOMT, otitis media with otorrhoea drainage through tympanostomy tube.
[b] MBL production is shown in nanomoles of imipenem hydrolyzed per minute per milligram of protein. Data are the mean values of measurements for three replicate samples (the standard deviation was always <10%); in all cases, the activity was inhibited by at least 80% in the presence of EDTA. Carbapenemase activity measured with the negative control (*P. aeruginosa* ATCC 27853) yielded a value of <10 nmol of imipenem hydrolyzed/min/mg of protein.
[c] The MICs were determined by Etest. IMP, imipenem; MEM, meropenem; PIP, piperacillin; AZT, aztreonam; CTX, cefotaxime; CAZ, ceftazidime.

deoxynucleoside triphosphates (dNTPs), and 50 ng of template DNA. GoTaq (Promega, Madison, WI) was used according to the manufacturer's instructions. The following cycling conditions were used: 5 min at 96°C, 30 cycles (1 cycle consists of 45 s at 95°C, 30 s at 56°C, and 30 s at 72°C), and 7 min at 72°C. *P. otitidis* MCC10330[T] and *P. aeruginosa* ATCC 27853 were used as the positive control and negative control, respectively. Amplification reactions with primers PomOrf/F and POM948/R (Table 2) were also performed with some strains to determine the sequences of bla_{POM} genes. The following cycling conditions were used: 5 min at 96°C, 30 cycles (1 cycle consists of 45 s at 95°C, 30 s at 51°C, and 45 s at 72°C), and 7 min at 72°C. Sequencing was carried out on both strands at an external sequencing facility (Macrogen, Seoul, South Korea) using custom primers.

Database search and sequence analysis. Database search was performed using BLAST software (version 2.2.23) available at the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi). DNA and protein sequence alignments were performed with ClustalW software (version 2) available at the EBI web server (http://www.ebi.ac.uk/tools/clustalw2), that was also used to create phylogenetic trees. Signal peptide cleavage site was predicted using SignalP (version 3.0). Putative promoter sequences were detected using Bprom software (Softberry, Inc., Mount Kisco, NY).

Nucleotide sequence accession numbers. The nucleotide sequences reported in this paper have been submitted to the GenBank/EMBL sequence database and assigned accession no. EU315252 (for the bla_{POM-1} gene from *P. otitidis* MCC10330[T]) and GU002288 to GU002298 for partial bla_{POM} gene sequences from other *P. otitidis* strains.

TABLE 2. Oligonucleotide primers used in this work

Primer	Sequence (5′→3′)	Location[a]
Pom-seq/F (primer 1)	CTGCACAGCCACGCCCAC	+298/+315
Pom-seq/R (primer 2)	GTCATGCCGCCCAGCTCC	+509/+492
POTSEQ/F (primer 3)	ACGTCGCTGATGCTCAG	−265/−249
Pom948/R (primer 4)	CCTGCGTCATCAGAGACCTC	−879/−870
C5ft/F (primer 5)	TGTCCTTGATGTGGTTGTGG	−1091/−1071
CT1Prom/R (primer 6)	GGGGGGTCAGGGTACGCA	+20/+2

[a] +1 corresponds to the first base of the GTG start codon of the bla_{POM-1} ORF in the GenBank entry (GenBank accession no. EU315252). The locations of primers are also indicated in Fig. 2, using the corresponding primer numbers.

RESULTS AND DISCUSSION

β-Lactam susceptibility and β-lactamase production of *P. otitidis* strains. The susceptibility of 20 *P. otitidis* strains to various β-lactams, including piperacillin, cephalosporins, carbapenems, and aztreonam, was determined by Etest. MICs of piperacillin, aztreonam, cefotaxime, and ceftazidime were quite homogeneous, with a 4- to 8-fold variability at maximum between different strains. Higher variability was observed with carbapenems, with a few strains showing high-level carbapenem MICs (Table 1). Using the CLSI breakpoints for *P. aeruginosa* (4), all *P. otitidis* strains were susceptible to piperacillin, cefotaxime, ceftazidime, and aztreonam, while two strains (10%) were resistant to imipenem and 7 strains (35%) were intermediate or resistant to meropenem. Using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints for *P. aeruginosa* (http://www.eucast.org), all *P. otitidis* strains were susceptible to piperacillin and ceftazidime and intermediate to aztreonam, while two strains (10%) were resistant to imipenem and 11 strains (55%) were

intermediate or resistant to meropenem. Imipenem MICs were usually lower than those of meropenem, with a few exceptions (Table 1). The two imipenem-resistant strains exhibited high-level MIC values (>32 μg/ml) for both imipenem and meropenem (Table 1).

Analytical IEF, carried out with five strains, including the type strain (MCC10330^T), two additional carbapenem-susceptible strains (MCC12065 and MCC61485) and the two high-level carbapenem-resistant strains (MCC04511 and YMC-Po/06) revealed in all cases the presence of a single β-lactamase band with a pI of around 6 to 6.2 (data not shown).

Analysis of crude extracts from exponential-phase cultures revealed the presence of carbapenemase activity in all *P. otitidis* strains (Table 1). The specific activity was variable within an approximately 5-fold range, and in all cases, the activity was inhibited (≥80%) by EDTA. No clear relationship was observed between the amount of carbapenemase activity and carbapenem susceptibility (Table 1).

Following exposure to subinhibitory concentrations of imipenem (0.25× MIC), the MBL activity measured at 2 h after induction was not significantly different from that observed under basal conditions either with MCC10330^T or with YMC-Po/06 (data not shown), showing that production of the MBL activity was independent of β-lactam exposure. Activity against nitrocefin was also unaffected by exposure to imipenem (data not shown).

According to these data, the behavior of *P. otitidis* to β-lactams and the β-lactamase profile of *P. otitidis* appeared to be different from those of *P. aeruginosa*. Unlike *P. aeruginosa*, *P. otitidis* constitutively produces an MBL while it apparently lacks additional inducible β-lactamase genes.

Cloning and characterization of the MBL-encoding gene from *P. otitidis* MCC10330^T. A clone producing MBL activity was isolated from a genomic library of the *P. otitidis* type strain, constructed in the *E. coli* plasmid vector pACYC184 and transformed in the *E. coli* strain MC1061, after replica plating of the library onto LB medium containing imipenem (1 μg/ml).

The MBL-producing clone, named CT-1, carried a DNA insert of about 6 kb. Sequencing of the insert revealed the presence of an 859-bp open reading frame (ORF) encoding a protein similar to MBLs of subclass B3 (Fig. 1) that was named POM-1 (after P. otitidis metallo-β-lactamase). The ORF starts with a GTG codon and is preceded by a recognizable ribosomal binding site and by a putative promoter region.

The POM-1 enzyme exhibits the closest similarity (60 to 64% amino acid identity) to the several known variants of the L1 enzyme of *S. maltophilia* and a lower similarity to other enzymes of subclass B3 (Fig. 1), suggesting a closer ancestry with the L1 enzyme. BLAST search also showed that POM-1 was almost identical to a protein fragment (amino acids [aa] 23 to 308) deduced from the translation of the genome of *Culex quinquefasciatus* (the southern house mosquito), suggesting the occurrence of bacterial contamination in that genome project (GenBank no. AAWU00000000).

Genetic context of the bla_{POM-1} gene. Sequencing of the regions flanking the cloned bla_{POM-1} gene revealed the following: (i) upstream of bla_{POM-1}, the presence of the 5′ moiety of an ORF encoding a putative sensor-histidine kinase homologous to the *P. aeruginosa* PA_2882 protein; (ii) downstream of bla_{POM-1}, an operon for phosphonate utilization and the 5′ end of a *selD* homologue, which are highly conserved among different *Pseudomonas* species (Fig. 2). Altogether, the region downstream of bla_{POM-1} exhibited colinearity with the genomes of *Pseudomonas syringae*, *Pseudomonas entomophila*, and *Pseudomonas putida* at least until the *selD* gene, and with the genomes of *P. aeruginosa*, *Pseudomonas mendocina*, *Pseudomonas fluorescens*, and *Pseudomonas stutzeri* until the phosphonate operon (Fig. 2). On the other hand, the region upstream of bla_{POM-1} did not show any significant homology to other *Pseudomonas* genomes except for *P. aeruginosa* in which, however, the gene for the PA_2882 homologue is not linked with the phosphonate operon (Fig. 2).

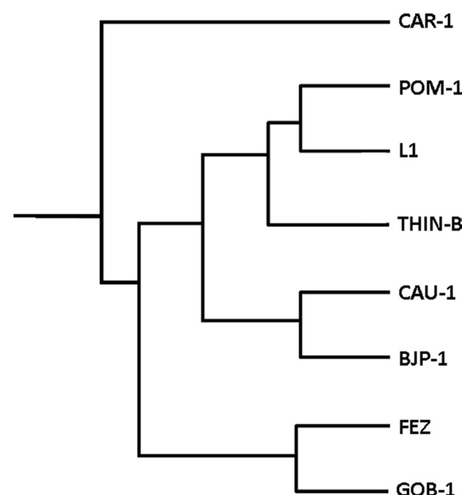

FIG. 1. Tree showing the similarity of the POM-1 protein (GenBank/EMBL accession no. EU315252) with other known subclass B3 MBLs. The tree has been constructed using the Clustal2 program (http://www.ebi.ac.uk/tools/clustalw2). Other MBLs (Swiss-Prot accession nos.) are FEZ-1 from *Fluoribacter gormanii* (Q9K578), GOB-1 from *Elizabethkingia meningoseptica* (Q9RB00), CAR-1 from *Erwinia carotovora* (ECA2849), L1 from *S. maltophilia* (Q9RBQ3), THIN-B from *Janthinobacterium lividum* (Q9AEF9), CAU-1 from *Caulobacter crescentus* (Q8KKG1), and BJP-1 from *Bradyrhizobium japonicum* (Q89GW5).

Finding of bla_{POM-1} within a similar genetic context in the absence of homologues in any other *Pseudomonas* genome suggests that the gene was acquired by horizontal transfer, followed by recombination into the chromosome downstream of the conserved phosphonate operon, after divergence of *P. otitidis* from the other species.

Prevalence and conservation of bla_{POM}-like MBL genes in *P. otitidis* strains. Since MBL production was a constant feature of *P. otitidis*, we investigated the presence of bla_{POM}-related sequences in the genomic DNAs of the 20 *P. otitidis* strains. By PCR amplification of an internal fragment, using the Pomseq/F and Pomseq/R primers (Table 2 and Fig. 2), an amplicon of the expected size (212 bp) was obtained from all tested strains (data not shown), suggesting that bla_{POM-1}-like genes are actually resident in this species.

To investigate the variability of bla_{POM-1}-like genes carried by different strains, the coding region was amplified using primers POTSEQ/F and Pom984/R (Table 2 and Fig. 2) from the genomic DNAs of 10 randomly selected *P. otitidis* strains from the United States and from the South Korean strain

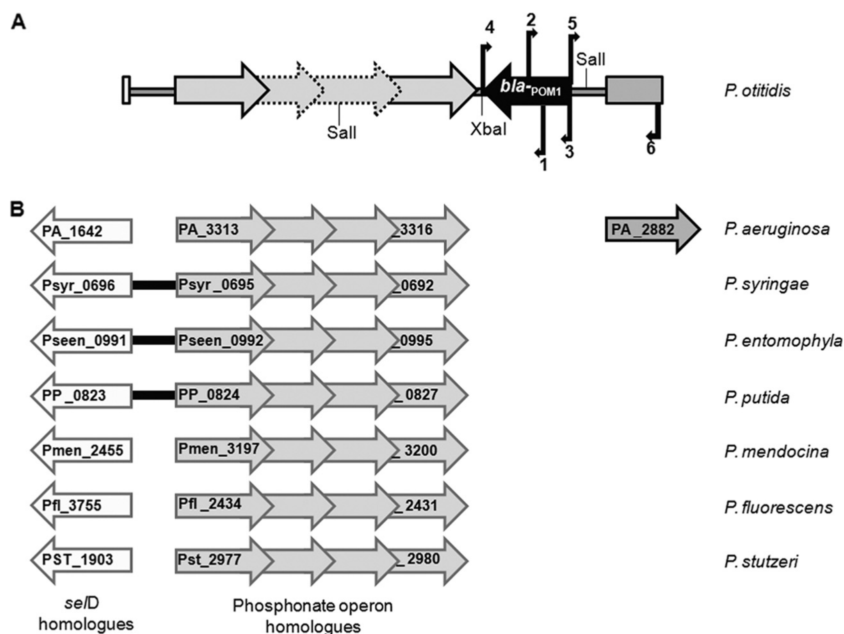

FIG. 2. (A) Genetic context of bla_{POM-1} and (B) homologies of flanking regions with the genomes of other *Pseudomonas* species. The location of primers used for PCR amplification (Table 2) is shown by numbered arrows: 1, Pom-seq/F; 2, Pom-seq/R; 3, POTSEQ/F; 4, POM948/R; 5, CT1ft/F; 6, CT1Prom/R. The NCBI accession numbers for the *Pseudomonas* genome sequences are as follows: NC_002516.2 for *P. aeruginosa* PAO1, NC_007005.1 for *P. syringae* pv. syringae B728a, NC_008027.1 for *P. entomophila* L48, NC_002947.3 for *P. putida* KT22440, NC_009439.1 for *P. mendocina ymp*, NC_007492.2 for *P. fluorescens* Pf-01, and NC_009434.1 for *P. stutzeri* A1501. Colinearity of genes/operons is indicated by thick black lines; where no such lines are drawn, the homologues are present but located apart from each other on the chromosome.

YMC-Po/06. Amplicons of the expected size (1,143 bp) were obtained from all tested strains. Amplicon sequencing allowed comparison of most of the coding sequences (except for a 45-bp region at the 3′ end) and revealed an overall high degree of sequence conservation, with a nucleotide homology ranging from 97.0 to 99.1% and an amino acid homology ranging from 97.0 to 100% for the aligned region (Fig. 3). All key residues known to be involved in metal binding in subclass B3 enzymes (His/Gln116, His118, His196, Asp120, His121, and His263), in the BBL numbering scheme (6) were conserved (Fig. 3). In one case (strain MCC51196), a three-amino-acid deletion in the putative leader peptide was observed (Fig. 3). Interestingly, this strain was the one with the lowest meropenem MIC, although carbapenemase activity was detectable in the crude extract (Table 1). This could reflect some impairment in the secretion process of this enzyme variant.

Functional characterization of POM-1. A 2,213-bp XbaI fragment, including the bla_{POM-1} gene and flanking regions, was subcloned from pCT-1 into the shuttle plasmid pPME6001 to obtain the recombinant plasmid pPom6/11. This plasmid was used to transform *E. coli* MC1061 and *P. aeruginosa* PAO1 to investigate the impact of POM-1 production on β-lactam resistance. MBL production was detected in both transformants, although the specific activity was much higher in *P. aeruginosa* PAO1(pPom6/11) (Table 3). Expression of POM-1 in *E. coli* and *P. aeruginosa* affected the susceptibility of the bacterial hosts to penicillins, cephalosporins, and carbapenems, while aztreonam MICs were not affected, revealing a broad substrate specificity. The impact on MIC values was most evident with ampicillin in *E. coli* and with carbapenems in *P. aeruginosa* (Table 3). The different impact observed with *E. coli* and *P. aeruginosa* could reflect the different expression level and/or different outer membrane permeability and/or a different contribution of efflux systems in the two bacterial hosts. Investigation of these issues and biochemical characterization of the POM-1 enzyme will be the subject of future studies.

Effect of inoculum size on carbapenem susceptibility of *P. otitidis* and selection of carbapenem-resistant mutants. Carbapenem susceptibility of the *P. otitidis* type strain was remarkably affected by the inoculum size. The imipenem and meropenem MICs increased by 16-fold (from 2 to 32 μg/ml) and 64-fold (from 2 to 128 μg/ml), respectively, when the inoculum size was increased from 10^5 to 10^7 CFU. This behavior was consistent with the production of carbapenemase activity and was not observed with *P. aeruginosa* PAO-1 whose carbapenem MICs increased by only 1- to 4-fold by a similar increase of inoculum size.

By plating a large inoculum (10^9 CFU) of *P. otitidis* MCC10330^T on a plate containing a gradient concentration (0 to 5 μg/ml) of imipenem, colonies growing in the presence of the highest antibiotic concentrations were reproducibly obtained. Analysis of three of these colonies, selected at random from three independent experiments, revealed carbapenem MICs of >32 μg/ml, while the susceptibility to other β-lactams (including piperacillin, ceftazidime, cefotaxime, and aztreonam) was unchanged. The high-level carbapenem-resistant phenotype was stable upon subculturing in the absence of selective pressure and was not related to modification of the carbapenemase activity or mutations in the bla_{POM-1} gene sequence (data not shown). Analogous to other species (e.g., *P. aeruginosa*), a mutation leading to an outer membrane perme-

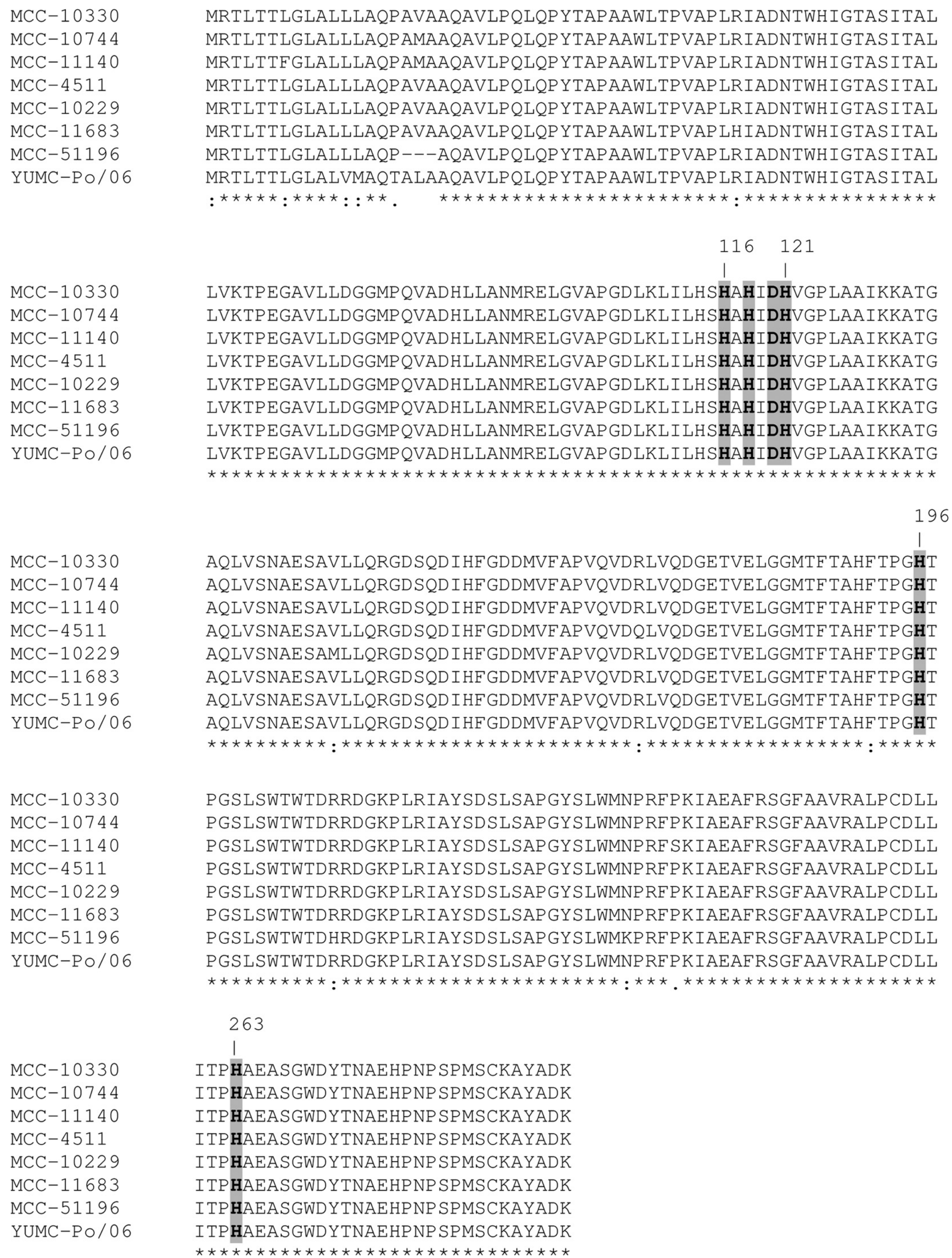

FIG. 3. Sequence alignment of the POM-1-like proteins from different *P. otitidis* strains. The conserved residues known to be involved in metal binding are indicated by gray shading. Identical residues (*), strongly similar residues (:), and weakly similar residues (.) are indicated below the sequences. Gaps introduced to optimize alignment are indicated by dashes.

ability defect and/or to upregulation of an efflux system could be the most likely cause of the resistance observed in this case as for MCC04511 and YMC-Po/06 isolates. Investigation of this issue will be the subject of future studies.

Concluding remarks. Investigation of the β-lactamase profile of *P. otitidis* carried out in this work revealed a unique pattern which consists of constitutive production of a subclass B3 MBL (named POM-1), while other β-lactamases (e.g., AmpC-type β-lactamases) that are resident in *P. aeruginosa*, *P. putida*, and *P. fluorescens* (NCBI completed microbial genomes accessed on 28 July 2010) are apparently lacking. As such, *P. otitidis* is the first example of a pathogenic *Pseudomonas* species endowed with a resident MBL.

The fact that several *P. otitidis* strains were not categorized

TABLE 3. MICs and carbapenemase activity conferred by bla_{POM-1} gene carriage in *E. coli* MC1061 and in *P. aeruginosa* PAO1

Strain	Relevant characteristics	MIC (μg/ml) of antimicrobial agent[a]:										Carbapen. act.[b]
		AMP	PIP	LOT	FOX	CTX	CAZ	IMP	MEM	ERT	AZT	
MC1061(pPME6001)	*E. coli* host with empty vector	4	2	4	2	0.047	0.38	0.25	0.047	0.012	0.25	<10
MC1061(pPom6/11)	*E. coli* host carrying the cloned bla_{POM-1} gene	>256	16	32	4	0.125	1	1	0.75	0.25	0.25	16 ± 2
PAO1(pPME6001)	*P. aeruginosa* host with empty vector	ND	3	ND	ND	ND	0.75	1	0.38	ND	1.5	<10
PAO1(pPom6/11)	*P. aeruginosa* host carrying the cloned bla_{POM-1} gene	ND	12	ND	ND	ND	2	>32	32	ND	1.5	230 ± 18

[a] AMP, ampicillin; PIP, piperacillin; LOT, cephalothin; FOX, cefoxitin; CTX, cefotaxime; CAZ, ceftazidime; IMP, imipenem; MEM, meropenem; ERT, ertapenem; AZT, aztreonam; ND, not determined.

[b] Carbapen. act., carbapenemase activity. Carbapenemase activity is shown in nanomoles of imipenem hydrolyzed per minute per milligram of protein. A value of <10 indicates that no significant activity was detected in the extract.

as carbapenem resistant in conventional susceptibility testing despite the carbapenemase activity of the POM-1 enzyme is not entirely surprising. In fact, a similar phenomenon is well documented with carbapenemase-producing members of the family *Enterobacteriaceae* (8) and could reflect a relatively high outer membrane permeability to carbapenems in wild-type *P. otitidis* strains. However, the strong inoculum size effect observed with these drugs and the ease with which resistant mutants could be selected upon carbapenem exposure suggest that the use of carbapenem-based regimens should be considered with caution for treating *P. otitidis* infections.

ACKNOWLEDGMENTS

The skillful technical contribution of Gianluca Lentini to some experimental work is acknowledged.

This work was partially supported by grant 223031 from the European FP7 TROCAR project.

REFERENCES

1. **Blumer, C., S. Heeb, G. Pessi, and D. Haas.** 1999. Global GacA-steered control of cyanide and exoprotease production in *Pseudomonas fluorescens* involves specific ribosome binding sites. Proc. Natl. Acad. Sci. U. S. A. **96:**14073–14078.
2. **Clark, L. L., J. J. Dajc, C. H. McLean, J. G. Bartell, and D. W. Stroman.** 2006. *Pseudomonas otitidis* sp. nov., isolated from patients with otic infections. Int. J. Syst. Evol. Microbiol. **56:**709–714.
3. **Clinical and Laboratory Standards Institute.** 2009. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. M7-A8. Clinical and Laboratory Standards Institute, Wayne, PA.
4. **Clinical and Laboratory Standards Institute.** 2010. Performance standards for antimicrobial susceptibility testing. Supplement M100-S20. Clinical and Laboratory Standards Institute, Wayne, PA.
5. **Docquier, J. D., F. Pantanella, F. Giuliani, M. C. Thaller, G. Amicosante, M. Galleni, J. M. Frere, K. Bush, and G. M. Rossolini.** 2002. CAU-1, a subclass B3 metallo-β-lactamase of low substrate affinity encoded by an ortholog present in the *Caulobacter crescentus* chromosome. Antimicrob. Agents Chemother. **46:**1823–1830.
6. **Garau, G., I. García-Sáez, C. Bebrone, C. Anne, P. Mercuri, M. Galleni, J. M. Frère, and O. Dideberg.** 2004. Update of the standard numbering scheme for class B β-lactamases. Antimicrob. Agents Chemother. **48:**2347–2349.
7. **Lane, D. J.** 1991. 16S/23S rRNA sequencing, p. 115–175. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. John Wiley & Sons, Inc., New York, NY.
8. **Miriagou, V., G. Cornaglia, M. Edelstein, I. Galani, C. G. Giske, M. Gniadkowski, E. Malamou-Lada, L. Martinez-Martinez, F. Navarro, P. Nordmann, L. Peixe, S. Pournaras, G. M. Rossolini, A. Tsakris, A. Vatopoulos, and R. Canton.** 2010. Acquired carbapenemases in Gram-negative bacterial pathogens: detection and surveillance issues. Clin. Microbiol. Infect. **16:**112–122.
9. **Rossolini, G. M., M. A. Condemi, F. Pantanella, J. D. Docquier, G. Amicosante, and M. C. Thaller.** 2001. Metallo-β-lactamase producers in environmental microbiota: new molecular class B enzyme in *Janthinobacterium lividum*. Antimicrob. Agents Chemother. **45:**837–844.
10. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, New York, NY.