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Molecular and Biochemical Characterization of OXA-45, an Extended-Spectrum Class 2d′ β-Lactamase in *Pseudomonas aeruginosa*

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As part of the CANCER Antimicrobial Surveillance Program in North America, a clinical strain of *Pseudomonas aeruginosa*, strain 07-406, isolated in Texas was found to be resistant to all antimicrobials except polymyxin B. Genetic analysis of this isolate identified two unique extended-spectrum β-lactamase genes. One, *bla*$_{VIM-7}$, encoded a metallo-β-lactamase (unpublished data), and the other, *bla*$_{OXA-45}$, described here, encoded a class D extended-spectrum β-lactamase. *bla*$_{OXA-45}$ was isolated on a *Sau*3A1 genomic fragment of 1.8 kb and encodes a protein of 264 amino acids with the highest identities to OXA-18 (65.9%), OXA-9 (42.8%), OXA-22 (40.2%), OXA-12 (38.6%), and OXA-29 (35.2%) but weak identities with other class D β-lactamases. *bla*$_{OXA-45}$ was found to be harbored on a 24-kb plasmid in a region that displays high identities with a section of the 43-kb genomic island of *Salmonella enterica* serovar Typhimurium DT104. Biochemically OXA-45 is most similar to OXA-18 in its substrate profile and inhibition by clavulanic acid and is a member of the 2d′ class of β-lactamases.

β-Lactamases inactivate penicillins, cephalosporins, and to a lesser extent carbapenems by hydrolyzing the amide bond of the β-lactam ring. They can be categorized into four classes (A through D) based on their sequence similarities and substrate profiles (2, 6). Class B enzymes are metalloenzymes and generally have a broad spectrum of hydrolytic activity against all β-lactam classes (14), whereas enzymes of classes A, C, and D are serine hydrolases and have a more restricted spectrum of activity. Extended spectrum in class A enzymes is usually due to small sequence changes in TEM or SHV plasmid-encoded β-lactamases, and this class now also includes the prevalent CTX-M-type enzymes (5, 21). Class A enzymes are usually inhibited by clavulanic acid; however, inhibitor-resistant TEM- and SHV-type enzymes are now well documented, although they lack an extended-spectrum profile (5, 6). Conversely, class D β-lactamases are almost entirely resistant to inhibition by clavulanic acid or are poorly inhibited (6), exceptions being the restricted-spectrum OXA-12 and ImiS (23, 26) and the extended-spectrum OXA-18 (22) enzymes. Class D β-lactamases generally have a preference for penam substrates, including oxacillin and related compounds; they exhibit “burst kinetics” with initial hydrolysis rates declining more rapidly than can be explained by substrate depletion (13) and are inhibited by chloride ions (typical 50% inhibitory concentration [IC_{50}], ~75 mM) (16). The crystal structures of OXA-10 (19), OXA-13 (20), and OXA-1 (24) have been determined and indicate that the catalytic mechanism of class D enzymes is different from catalytic mechanisms of the other serine hydrolases (15). Mechanistic studies support this hypothesis and demonstrate the involvement of a carbamylated lysine in the catalytic function of class D enzymes (11). The crystal structures of OXA-10 and OXA-13 also demonstrate that these enzymes form dimers in solution with elevated kinetic activity relative to the monomer, which may explain the complex biphasic kinetics these enzymes show with some substrates (19). The class D β-lactamase sequences vary extensively, with identities ranging from 16 to 99% between individual enzymes. Distinct phylogenetic subgroups can be identified where the degree of structural relatedness between members of each group is higher than that between groups (16). The majority of the oxacillinases are chromosomally encoded (3); however, the majority of the clinically relevant class D β-lactamases are acquired enzymes, whose genes are resident on plasmids in gram-negative pathogens such as pseudomonads, acinetobacters, and members of the *Enterobacteriaceae* (10, 16) and contained within integrons or transposons. A number of extended-spectrum oxacillinases have been identified; several that are similar to TEM and SHV class A enzymes are variants of their restricted-spectrum counterparts, e.g., mutants of OXA-10 (7, 9, 12) and OXA-2 (8). Others have distinct sequences, e.g., OXA-18 (20) (a cefotaxime- and ceftazidime-hydrolyzing enzyme) or the carbapenem-hydrolyzing oxacillinases (1). In the course of cloning a metallo-β-lactamase from *Pseudomonas aeruginosa* strain 07-406, we discovered a new oxacillinase, encoded by *bla*$_{OXA-45}$, with activity against aztreonam and oxacillin which extended the profile of the resistant strain to include all antibiotics except polymyxin B.

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MATERIALS AND METHODS

Bacterial strains and plasmids. *P. aeruginosa* strain 07-406 was isolated from a 58-year-old woman suffering from cancer (Texas) as part of the CANCER

TABLE 1. MICs of β-lactams for *P. aeruginosa* 07-406 and three *E. coli* strains

β-Lactam	MIC (μg/ml)[a]			
	P. aeruginosa 07–406	*E. coli* SNO3	*E. coli* SNO3 (pMAT40611)	*E. coli* JM109 (bla_{OXA-18})[b]
Ceftazidime	>256	0.06	128	64
Ceftazidime + clavulanate	>256	ND	0.12	0.5
Cefotaxime	>256	0.125	4	2
Cefotaxime + clavulanate	ND	ND	0.03	0.06
Cefepime	>256	0.06	4	1
Cefepime + clavulanate	ND	ND	0.03	0.03
Cefoxitin	>256	2	4	8
Ceftriaxone	>256	0.125	8	ND
Imipenem	>256	0.06	0.12	0.06
Meropenem	>256	0.06	0.03	ND
Piperacillin	>256	0.5	32	16
Ampicillin	>256	4	128	ND
Amoxicillin	>256	4	128	32
Oxacillin	>256	4	64	ND
Aztreonam	>256	0.06	64	64

[a] ND, not determined.
[b] Data are for reference 21.

Antimicrobial Surveillance Program in North America and was resistant to all antimicrobials except polymyxin B (Table 1). *Escherichia coli* strain DH5α [*supE44 ΔlacU169* (φ80*lacZΔM15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*] was used as the host strain for genomic library construction and together with *P. aeruginosa* PAO1 was utilized as recipients of the 07-406 plasmid. *E. coli* SNO3 was used as the recipient for assessing MICs and hydrolytic profiles (18). The library was generated in the cloning vector pK18 as previously described (25).

Antimicrobials and reagents. β-Lactam antibiotics used in this study were ceftazidime and clavulanic acid (GlaxoSmithKline, Worthing, United Kingdom); kanamycin, penicillin, ampicillin, oxacillin, cefotaxime, and aztreonam (Sigma Chemical Co., St. Louis, Mo.); piperacillin (Lederle, Carolinas, Puerto Rico); cefoxitin and imipenem (Merck Sharpe & Dohme, West Point, Pa.); meropenem (AstraZeneca Pharmaceuticals, Macclesfield, United Kingdom); and nitrocefin (Becton Dickinson, Cockeysville, Md.). Reagents used for DNA manipulation were obtained from Gibco-BRL (Life Technology Ltd., Paisley, United Kingdom). Other general reagents were purchased from Sigma Chemical Co. or BDH (Poole, United Kingdom).

MICs. MICs of β-lactam antibiotics were determined by agar dilution according to the National Committee for Clinical Laboratory Standards method (17).

Recombinant DNA methodology. DNA procedures were performed as previously described (22). Genomic DNA was isolated from *P. aeruginosa* strain 07-406 by the cetyltriammonium bromide method. Plasmids were purified by the alkaline lysis method with a Qiagen miniprep kit. For construction of the genomic library, *Sau*3A1 genomic fragments were purified after gel electrophoresis with a Qiagen gel purification kit. Five micrograms of purified size-fractionated genomic fragments (>1 kb) were ligated to 1 μg of pK18 that had previously been linearized and dephosphorylated by using *Bam*HI and calf intestinal alkaline phosphatase, respectively. The ligation mixture was dialyzed and used to transform *E. coli* DH5α by electroporation using a Bio-Rad gene pulser (2.5 kV, 25 μF, 200 Ω). Plating of the library onto X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 30 μg/ml) and kanamycin (25 μg/ml) plates yielded more than 500,000 recombinants/500 ng of recombinant DNA. The library was amplified by purifying the plasmids from the recombinants. This amplified library was used to transform DH5α, and cloned β-lactamase genes were selected on media containing 10 μg of ceftazidime per ml and 25 μg of kanamycin per ml.

Transfer of resistance. The pseudomonas plasmid was extracted with a Qiagen miniprep kit and transformed by electroporation into *E. coli* DH5α as well as another *P. aeruginosa* strain, PAO1, containing no plasmids.

DNA sequencing and sequence analysis. Several hundred clones were isolated from the *P. aeruginosa* 07-406 library with plasmid inserts ranging from 1.8 to 9 kb. One clone, pMAT40611, containing a 1.8-kb insert was sequenced on both strands by the dideoxy-chain termination method with a Perkin Elmer Biosystems 377 DNA sequencer. Sequence analysis was performed with the Lasergene DNASTAR software package. Nucleotide searches were undertaken by using BLAST (http://www.ebi.ac.uk/blast2) against the EMBL prokaryotic database (http://www.ebi.ac.uk/embl/index.html). Protein alignments and protein phylogenetic analysis were done with Clustal W (http://www.ebi.ac.uk/clustalW) and the PAM 250 matrix (http://www.blc.arizona.edu). The putative cleavage site of the signal sequence was identified by computer analysis using the Center for Biological Sequence Analysis website (http://www.cbs.dtu.dk/services/SignalP/#submission).

Oligonucleotide primers for sequencing and PCR. The primers used to sequence the cloned insert of pMAT40611 are listed in Table 2. Primers used to back-probe the *P. aeruginosa* 07-406 host were P673-693F and P1221-1201R (bla_{OXA-45} internal primers), giving an expected PCR product of 548 bp, and P673-693F and P1750-1730R, which amplify a 1,057-bp product including most of the pMAT40611 insert.

PCR conditions. For amplification using OXA primers, PCR was performed with AB-gene Expand high-fidelity master mix containing a mixture of *Pfu* and nonproofreading *Taq* polymerases and deoxynucleoside triphosphates. Primers were used at 10 pM concentrations, and 1 μl of bacterial culture at an optical density of 1 at 600 nm was used as the template. Cycling parameters were 95°C for 5 min followed by 30 cycles of 95°C for 1 min, annealing at 45°C for 1 min, and extension 68°C for 1 min and ending with a 5-min incubation at 68°C. PCR products were visualized by electrophoresis on 0.8% (wt/vol) agarose gels in Tris-boric acid–EDTA buffer (pH 7.0) and stained with 1% ethidium bromide as previously described (25).

Isoelectric focusing. Isoelectric focusing (IEF) was carried out as previously described (25). β-Lactamases from *P. aeruginosa* 07-406 and recombinant clones expressed in *E. coli* SNO3 were visualized by staining the IEF gels with 100 μM nitrocefin. Confirmation of the metallo-β-lactamase pI value was preformed by preincubating the crude cell extract with either 20 mM EDTA or 5 μM BRL42715 and repeating the IEF.

Determination of β-lactamase activity. Cellular extracts of *E. coli* SNO3 carrying recombinant β-lactamase genes had hydrolytic activity determined for penicillin, ampicillin, piperacillin, oxacillin, cefoxitin, ceftazidime, cefotaxime, aztreonam, imipenem, meropenem, and nitrocefin. The penicillins were used at a concentration of 500 μM, cephalosporins were used at 250 μM, and carbap-

TABLE 2. Primers used to sequence pMAT40611 and to back-probe the *P. aeruginosa* 07-406 chromosome to verify origin of clones

Primer	Sequence (5′-3′)
M13F	GTAAAACGACGGCCAGTG
pK18R	GCAAGGCGATTAAGTTGG
P673–693F	ATGGCGATATCTCCGGTGAG
P626–606R	GCGAATACCAGACGATCGAC
P1175–1195F	GGTTCATGCGTGGGTTTTCG
P1221–1201R	GCGATGTCGAAAACCCACGC

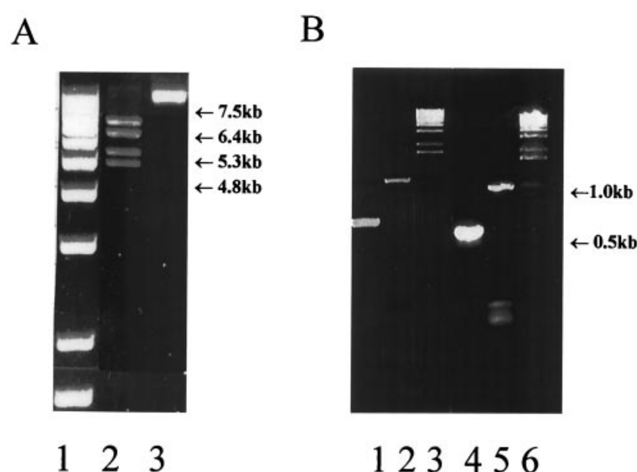

FIG. 1. (A) A 0.8% agarose gel showing pMAT40611 DNA digested with *Eco*RI and *Bam*HI. Lane 1, 1 kb plus DNA marker; lanes 2 and 3, pMAT40611 from strain 07-406 digested with *Eco*RI and *Bam*HI, respectively. (B) Back-probing of *P. aeruginosa* 07-406 with bla_{OXA-45}-specific primers and detection of bla_{OXA-45} on pMAT40611. Lanes 1 and 2, PCR products produced from *P. aeruginosa* 07-406 genomic DNA using bla_{OXA-45} primers p673-693F/p1221-1201R and p673-693F/P1750-1730R, respectively; lanes 3 and 6, 1-kb Plus DNA marker; lanes 4 and 5, PCR products produced by using bla_{OXA-45}-specific primers p673-693F/p1221-1201R and p673-693F/P1750-1730R and pMAT40611 isolated from *E. coli* DH5α as the template.

enems were used at 300 μM. Assays were carried out by measuring the breakdown of substrate at a specific wavelength for that β-lactam, except for nitrocefin and oxacillin, where the accumulation of product was measured at wavelengths of 482 and 235 nm, respectively. Specific activity was measured as nanomoles of substrate hydrolyzed per minute per milligram of protein and reported as a percentage of activity against penicillin (reported as 100). The inhibitory activities of EDTA, NaCl, clavulanic acid, and imipenem were determined by using nitrocefin as a reporter substrate (150 μM). The IC_{50} for each inhibitor was calculated by using serial dilutions of inhibitor until 50% of enzyme activity was observed.

Nucleotide sequence accession number. The sequence of the pMAT40611 insert has been deposited in the EMBL database under accession number AJ519683.

RESULTS

Antibiotic susceptibility. β-Lactam MICs for *P. aeruginosa* strain 07-406, *E. coli* SNO3, and *E. coli* SNO3 carrying pMAT40611 were compared to those for *E. coli* carrying bla_{OXA-18} (Table 1). *E. coli* SNO3 carrying pMAT40611 was completely inhibited by clavulanic acid at 4 μg/ml.

Plasmid DNA and transfer of resistance. A plasmid of approximately 24 kb was readily isolated from *P. aeruginosa* 07-406 and transferred to both *E. coli* DH5α and *P. aeruginosa* PAO1 by electroporation conferring high-level resistance to all antimicrobials tested. PCR using primers designed against the sequenced bla_{OXA-45} gene confirmed its presence in the original strain and also the 24-kb plasmid isolated from *E. coli* DH5α(pMAT40611) (Fig. 1).

Preliminary plasmid characterization. The plasmid was further characterized by restriction mapping. The plasmid was purified from 1.5 ml of *P. aeruginosa* 07-406 overnight culture, yielding approximately 5 μg of DNA. *Eco*RI was found to cut the plasmid four times, leaving fragments of 4.8, 5.3, 6.4, and 7.5 kb and giving an approximate size for the plasmid of 24 kb (Fig. 1).

Cloning and sequence analysis of the extended-spectrum enzyme encoded by bla_{OXA-45}. Initial screening of the *P. aeruginosa* 07-406 gene bank on agar plates containing ceftazidime (10 μg/ml) yielded a large number of resistant colonies. Twelve colonies not exhibiting reduced activity against ceftazidime in the presence of EDTA were selected. Restriction analysis of the plasmids indicated that they contained inserts of between 1.8 and 9 kb. The ceftazidime MIC was greatest for one clone containing pMAT40611 (>256 μg/ml), and an insert of 1.8 kb was chosen for further study.

Sequence analysis of the pMAT40611 insert revealed the presence of a 792-bp open reading frame coding for a protein of 264 amino acids that displays a wide range of homologies with a number of previously cloned oxacillinases. The amino terminus of the predicted protein exhibits characteristic features of a bacterial signal peptide that target protein secretion into the periplasmic space. The cleavage site was predicted to be between amino acid positions 23 and 24, AGA-QM, which would produce a mature peptide with a predicted molecular mass of 26,324 Da and a pI of 9.43. The STFK tetrad (positions 55 to 58), which is known to be the active site of class A, C, and D β-lactamases, was found within the protein. In addition, other structural elements characteristic of class D β-lactamases were detected, including YGN (positions 130 to 132), QXXFX (positions 161 to 164), and KTG (positions 200 to 202) (Fig. 2). A putative start codon (ATG) was found at nucleotide bp 281, which was preceded 6 bp upstream by a suitable ribosome-binding site (GGAG). A stem-loop structure consistent with a rho-independent terminator element was found at bp 63 to 120, 161 nucleotides upstream of the start codon. This indicates that the oxacillinase promoter is likely positioned between bp 120 and 150, although relevant promoter sequences were not immediately obvious. The overall GC content of this gene and the downstream sequence was 62.7 and 63%, respectively, which is consistent with the expected GC content of *P. aeruginosa* (60.1 to 69.5%) genes (except for pilin genes).

Analysis of the genetic locus of bla_{OXA-45}. Sequence analysis of the DNA sequences surrounding bla_{OXA-45} revealed that the sequence upstream had 100% identity with a segment of DNA at the 3′ end of an ABC transporter operon from mycobacterium, including its putative terminator. The sequences downstream of bla_{OXA-45} displayed 88.2% identity over a 740-nucleotide overlap with a region of DNA from the 43-kb genomic island associated with the multidrug resistance region of *Salmonella enterica* serovar Typhimurium DT104. In particular, the overlap extended from the last 150 nucleotides of the remnants of *qacEΔ1* through 488 bp of a putative transposase-like gene. No homologies either upstream or downstream of bla_{OXA-45} were found to conserved core or inverse core recombination sites or 59b elements characteristic of gene cassettes.

Back-probing of *P. aeruginosa* 07-406 DNA. The deduced sequence of pMAT40611 was used to design the primers P673-693F and P1750-1730R, which amplify a 1,057-bp product including most of the pMAT40611 insert. These primers were used to amplify the correct-size PCR product from *P. aeruginosa* 07-406, thus confirming its origin (Fig. 1).

Homology with other β-lactamases. The putative product of bla_{OXA-45} showed the highest identity to the oxacillinases

FIG. 2. Multiple alignment of the OXA β-lactamases displaying the most identity with OXA-45 together with representative members of each phylogenetic subgroup. Amino acid residues found in the majority of sequences are highlighted with asterisks.

OXA-18 (65.9%), OXA-9 (42.8%), OXA-22 (40.2%), OXA-12 (38.6%), and OXA-29 (35.2%), as determined by using the Clustal W (http://www.ebi.ac.uk/clustalW) search program. The BLAST analysis involving DNA comparisons gave similar results, i.e., 70.3% identity with bla_{OXA-18}. Homology to the other oxacillinases varied between 16 and 29%. The enzyme is thus a new class D β-lactamase and therefore named OXA-45. An alignment and a phylogenetic tree were constructed to relate OXA-45 to the most closely related class D β-lactamases and other oxacillinases taken as representatives of each phylogenetic subgroup (Fig. 2 and 3).

Biochemical characterization of OXA-45. The relative hydrolytic activities of *E. coli* SNO3(pMAT40611) (with penicillin held at a value of 100) for penicillin, ampicillin, piperacillin, oxacillin, cefoxitin, ceftazidime, cefotaxime, aztreonam, imipenem, meropenem, and nitrocefin were 100, 98, 55, 34, <1, 1,150, 192, 110, <0.1, <0.1, and 755, respectively. These values are similar to those reported by Philippon et al. for OXA-18 (22). The inhibitory activities of EDTA, NaCl, clavulanic acid, and imipenem were determined by using nitrocefin as a reporter substrate. The IC_{50}s for NaCl, clavulanic acid, and imipenem were 260 mM, 0.04 μM, and 0.6 μM, respectively. EDTA had no inhibitory effect.

E. coli SNO3(pMAT40611) gave a single isoelectrophoretic band after staining with nitrocefin at 8.8, which is in reasonable agreement with the theoretical pI of 9.4 predicted from the primary amino acid sequence. This band was resistant to the effects of EDTA but not to the serine β-lactamase inhibitor BRL42715.

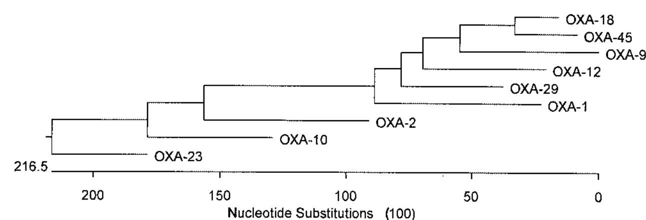

FIG. 3. Phylogenetic tree of class D β-lactamases displaying the greatest homology to OXA-45, together with representative members of each phylogenetic subgroup. The length of each pair of branches represents the distance between sequence pairs, while the units at the bottom of the tree indicate the number of nucleotide substitution events (http://www.ebi.ac.uk/clustalW).

DISCUSSION

This work started with the identification of a clinical isolate of *Pseudomonas aeruginosa* from a 58-year-old woman suffering from cancer that was resistant to all antimicrobials tested except polymyxin B. The isolate was shown to harbor a metallo-β-lactamase gene as detected by use of the metallo-β-lactamase Etest strip (AB Biodisk, Solna, Sweden) and cloning and sequence analysis of the resistance determinant (unpublished data). In the course of cloning the metallo-β-lactamase gene, bla_{OXA-45} was also cloned. Analysis of the bla_{OXA-45} gene and flanking DNA determined that the GC content was consistent with genetic information originating from a *Pseudomonas* sp. The deduced amino acid sequence of this gene displayed the greatest identity (65.9%) with the extended-spectrum enzyme OXA-18 and showed various degrees of identity (16 to 42.8%) with other members of class D β-lactamases. It also contained an active-site serine motif and several other motifs consistent with its placement among the Ambler class D β-lactamases and has therefore been designated OXA-45.

Recombinant bla_{OXA-45} harbored by *E. coli* DH5α confers high-level resistance to ceftazidime, aztreonam, and to a lesser extent cefotaxime. It was also inhibited by imipenem and clavulanic acid and was most similar functionally and genetically to its nearest extent relative, OXA-18. OXA-45 and OXA-18 are therefore the only two members of the novel subgroup 2d′, related to other group 2d β-lactamases but with extended-spectrum hydrolytic properties and full inhibition by clavulanic acid, previously proposed by Philippon et al. (22).

bla_{OXA-45} was harbored along with a metallo-β-lactamase gene (data not shown) on a multicopy 24-kb plasmid that could be transferred to *E. coli* and *P. aeruginosa* by electroporation, implying that the plasmid had a broad host range. The majority of plasmids harbored by pseudomonas have been large—often >100 kb—and contain the genetic machinery for their own mobility into different strains and/or species. The small size of this plasmid suggests that it is not autotransmissible, but it is large enough to contain *mob* genes, enabling transfer to other strains or species, along with an autotransmissible plasmid (27).

The genetic locus of bla_{OXA-45} was partially analyzed, and the downstream sequence showed the highest identity to the remnants of *qacE*Δ*1* and the transposase gene (88.2%) from the 43-kb genomic island of *S. enterica* serovar Typhimurium DT104 coding for multiantimicrobial resistance (4). This area in the genomic island was close to a class I integron; however, bla_{OXA-45} does not appear to have any sequences displaying homology to core or inverse core sites and 59-bp elements characteristic of genes harbored on gene cassettes. Interestingly, bla_{SPM-1}, a novel metallo-β-lactamase gene recently isolated from a *P. aeruginosa* isolate from Brazil (25), was also flanked by a very similar segment of DNA. Neither of these genes was flanked by core and inverse core sequences characteristic of class I integron systems, and it may be that this segment of DNA is a "hot spot" for the integration and dispersion of antibiotic resistance genes. The combination of bla_{OXA-45} and the metallo-β-lactamase gene on a small broad-host-range multicopy plasmid gives *P. aeruginosa* 07-406 resistance against all β-lactam antibiotics. The plasmid therefore constitutes a very attractive acquisition for other bacteria trying to survive against the onslaughts of modern anti-infective therapy.

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