OXA-35 is an OXA-10-related $\beta$-lactamase from *Pseudomonas aeruginosa*

Daniel Aubert$^a$, Laurent Poirel$^a$, Adel Ben Ali$^b$, Fred W. Goldstein$^b$ and Patrice Nordmann$^a*$

$^a$Service de Bactériologie-Virologie, Hôpital de Bicêtre, Assistance Publique/Hôpitaux de Paris, Faculté de Médecine Paris-Sud, 94275 Le Kremlin-Bicêtre; $^b$Laboratoire de Bactériologie, Hôpital Saint-Joseph, 75014 Paris, France

*Pseudomonas aeruginosa* clinical isolate PA35 is resistant to amino- and ureido-penicillins, has intermediate susceptibility to cefsulodin, cefepime and aztreonam, and is susceptible to imipenem and ceftazidime. Cloning and sequencing revealed a new $\beta$-lactamase variant, OXA-35, sharing 96% amino acid identity with OXA-10. OXA-35 displays a restricted-substrate hydrolysis profile with improved hydrolysis of amoxicillin and cloxacillin compared with OXA-10. OXA-35 differs from derivatives OXA-19 and OXA-28 by one amino acid substitution and may be a progenitor of these OXA-13-like extended-spectrum $\beta$-lactamases.

**Introduction**

Oxacillinases are Ambler class D $\beta$-lactamases that possess active site serine groups like class A and class C $\beta$-lactamases.1,2 Restricted-spectrum oxacillinases usually confer resistance to amino- and ureido-penicillins, and have good hydrolytic activities against oxacillin, cloxacillin and methicillin.1 Extension of the hydrolysis spectrum of oxacillinases to oxyimino cephalosporins is observed for OXA-2 and OXA-10 extended-spectrum derivatives.3 Most of the oxacillinase genes are plasmid-, transposon- and integron-located.3 Here we report on the characterization of a new restricted-spectrum oxacillinase gene that is integron-located and which may be the progenitor of several extended-spectrum oxacillinase genes.

**Materials and methods**

**Bacterial strains and plasmids**

*Pseudomonas aeruginosa* clinical strain PA35 was isolated from a pulmonary brush of an 80-year-old patient hospitalized for pneumonia at the Hôpital Saint-Joseph (Paris, France) in 1999. Identification of *P. aeruginosa* PA35 was confirmed by API 20 NE test (bioMérieux, Marcy-l’Étoile, France). *P. aeruginosa* PAO38-10 encoding OXA-10 $\beta$-lactamase was used as a reference strain. Rifampicin-resistant derivatives of *P. aeruginosa* PU21 and *Escherichia coli* K12 C600,3 and *E. coli* XL1 blue MRF’ Kan (Stratagene, Amsterdam, The Netherlands), were used as recipients for conjugation and cloning experiments, respectively. Plasmid pPCR Script Cam (SK$^+$) (Stratagene) was used as cloning vector.

**Antimicrobial agents and MIC determinations**

Clavulanic acid-, tazobactam- and imipenem-containing discs were used together with ureidopenicillin-containing discs to detect synergy.4 The MICs of selected $\beta$-lactams were determined for *P. aeruginosa* and recombinant *E. coli* strains, as reported previously.3

**Cloning experiments and analysis of recombinant plasmids**

Genomic DNA of *P. aeruginosa* PA35 and PAO38-10 (*blaOXA-10* positive) was extracted as described previously.3 PCR experiments were performed with genomic DNA from these *P. aeruginosa* strains as templates, using specific class 1 integron primers.3 The PCR-generated products were ligated into the SrfI site of pPCR Script Cam (SK$^+$) vector and electrocompetent *E. coli* XL1 blue cells were transformed with recombinant plasmids and selected as described previously.5

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*Corresponding author. Tel: +33-1-45-21-36-32; Fax: +33-1-45-21-63-40; E-mail: nordmann.patrice@bct.ap-hop-paris.fr

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Plasmid content and DNA–DNA hydridizations

Several methods were used to extract plasmid DNA from P. aeruginosa PA35. Plasmid DNA from recombinant E. coli strains was extracted using Qiagen plasmid DNA Midi kits (Qiagen, Courtaboeuf, France). Plasmid preparations were analysed by electrophoresis as reported previously. Hybridization of a PCR-amplified internal probe for blaOXA-10 to total DNA from P. aeruginosa PA35 after Southern transfer from an agarose gel was performed as described previously.

DNA sequencing and protein analysis

Sequencing of the cloned DNA fragment of recombinant plasmid pOXA-35 was performed on both strands as well on the corresponding PCR-amplified fragment of P. aeruginosa PA35, using an automatic DNA sequencer, and the nucleotide and deduced protein sequences were analysed as reported previously.

β-Lactamase assays and isoelectric focusing analysis

Cultures of E. coli (pOXA-10 or pOXA-35) were incubated overnight at 37°C in 50 mL trypticase soy broth containing amoxicillin (100 mg/L). Extracts containing β-lactamase from cultures of E. coli (pOXA-10 or pOXA-35) were obtained as described previously. Determination of β-lactamase-specific activities and of 50% inhibitory concentrations (IC50) of clavulanate and imipenem and analytical isoelectric focusing (IEF) analysis were performed as described previously.

Nucleotide sequence accession number

The nucleotide sequence data reported in this paper will appear in the GenBank nucleotide database under accession number AF315786.

Results and discussion

Preliminary susceptibility testing, transfer and cloning of the antibiotic resistance genes of P. aeruginosa PA35

Susceptibility testing by disc diffusion showed that P. aeruginosa PA35 is resistant to amino- and ureido-penicillins, has intermediate susceptibility to cefsulodin, cefepime, aztreonam and cefotaxime, and is susceptible to ceftazidime and imipenem. A synergy image was detected between ticarcillin-, cefsulodin- and imipenem. A synergy image was detected between ticarcillin-, cefsulodin- and imipenem. A synergy image was detected between ticarcillin-, cefsulodin- and imipenem. A synergy image was detected between ticarcillin-, cefsulodin- and imipenem. A synergy image was detected between ticarcillin-, cefsulodin- and imipenem. A synergy image was detected between ticarcillin-, cefsulodin- and imipenem. A synergy image was detected between ticarcillin-, cefsulodin- and imipenem. A synergy image was detected between ticarcillin-, cefsulodin- and imipenem. A synergy image was detected between ticarcillin-, cefsulodin- and imipenem. A synergy image was detected between ticarcillin-, cefsulodin- and imipenem. A synergy image was detected between ticarcillin-, cefsulodin- and imipenem.

Repeated attempts to isolate transconjugants and to detect plasmid DNA from P. aeruginosa PA35 failed. DNA–DNA hybridization of total DNA from P. aeruginosa PA35 to a probe of blaOXA-10 gave a positive result, consistent with the chromosomal location of a blaOXA-10 related gene. Using class 1 integron primers and total DNA of P. aeruginosa PA35 as template, a 1.7 kb PCR fragment was obtained and cloned in E. coli XL1 blue. A recombinant plasmid, pOXA-35, was analysed.

Sequence analysis of the blaOXA-35-containing integron

Sequence analysis of pOXA-35 revealed two open reading frames (ORFs). One ORF, 798 bp long, encodes a 266 amino acid preprotein with 96, 97 and 99% amino acid identities with OXA-10, OXA-7 and with OXA-13, -13-1, -19 and -28, respectively. Compared with OXA-10, this new oxacillinase variant, named OXA-35, shows eight amino acid substitutions (Table 1). None of these amino acid changes is located in conserved elements of class D β-lactamases (Table 1).

Comparison of extended-spectrum variants with OXA-10 has shown that several amino acid substitutions may be involved in the extensions to their substrate profiles (Table 1). OXA-35 has none of these amino acid changes. However, OXA-35 does possess several amino acid changes found in the extended-spectrum β-lactamases OXA-13-1, OXA-19 and OXA-28 as compared with OXA-10, which are not involved in the extension of hydrolysis profile (Table 1). OXA-35 differs from OXA-13-1, OXA-19 and OXA-28 by one or two amino acid substitutions. Accordingly, OXA-35 may be a progenitor of this subgroup of OXA-13-related enzymes.

The second ORF of the 1.7 kb DNA fragment, located immediately downstream of a class 1 intI1 gene (integrase gene), encodes an aminoglycoside acetyltransferase, AAC(6’)-Ib, that shares 98% amino acid identity with AAC(6’)-Ib, the gene of which is associated with blaOXA-19 in a class 1 integron. AAC(6’)-Ib enzymes usually confer resistance to gentamicin. In this respect, the aac(6’)-Ib gene variant found in P. aeruginosa PA35 is non-functional, since E. coli XL1 (pOXA-35) is susceptible to gentamicin. Loss of AAC(6’)-Ib activity most likely results from two substitutions at positions 132 and 133 in the AAC(6’)-Ib amino acid sequence. The blaOXA-35 and aac(6’)-Ib genes are carried on gene cassettes. The structures of class 1 integrons carrying blaOXA-13, blaOXA-19, blaOXA-28 and blaOXA-35 genes are very similar, reflecting, perhaps, a common origin of the P. aeruginosa isolates (Paris area, France, 1990, 1991 and 1999).

Antibiotic susceptibility and biochemical analyses

The MICs of various β-lactams for P. aeruginosa PA35 show that it is resistant to amino- and ureido-penicillins, has intermediate susceptibility to cefsulodin, cefepime, aztreonam and cefotaxime, and is susceptible to ceftazidime and imipenem (Table 2). The β-lactamase inhibitors clavulanic acid and tazobactam did not significantly decrease the MICs (Table 2). The MICs of β-lactams indi-
Table 1. Amino acid differences between OXA-10-related enzymes

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The standard numbering scheme for class D enzymes according to DBL is used (amino acid residues 10–272).\(^2\)

\(^a\)The italicized amino acids correspond to conserved regions among class D enzymes potentially involved in the active site.

\(^b\)Mutants obtained in vitro.\(^4,8\)

\(^c\)The bold-underlined amino acids may contribute to the extension of substrate hydrolysis in extended-spectrum variants.
cate that OXA-35, when expressed from *E. coli* XL1 blue (pOXA-35), confers resistance to amoxicillin, ticarcillin, piperacillin and cefsulodin, but not to other restricted- and extended-spectrum cephalosporins (Table 2). Resistance to ticarcillin was lowered slightly by imipenem, which has been shown to protect β-lactams from other OXA-10-related enzymes.4

IEF analysis of β-lactamases in extracts of *P. aeruginosa* PA35 and of *E. coli* XL1 blue (pOXA-35) revealed an enzyme with a pI value of 8. In addition, extracts of *P. aeruginosa* PA35 had a second enzyme with a pI value of 8.5, which most likely is an AmpC-type β-lactamase.

Kinetic parameters of the enzymes in extracts of OXA-35 [*E. coli* (pOXA-35)] and of OXA-10 [*E. coli* (pOXA-10)] show that these β-lactamases have similar restricted-spectrum β-lactam hydrolysis profiles (specific activities being 0.04, 0.06, 0.08 and 0.01 U/mg for cefepime, cefotaxime, cefpirome and cefsulodin, respectively, and no detectable hydrolysis of ceftazidime, in both cases). However, OXA-35 has three- to four-fold increases in specific activities with amoxicillin and cloxacillin as compared with OXA-10 (0.2 versus 0.05 U/mg for amoxicillin, and 0.7 versus 0.2 U/mg for cloxacillin). Inhibition studies showed that OXA-35 is weakly inhibited by clavulanic acid (IC₅₀ 5 µM) and efficiently by imipenem (IC₅₀ 0.03 µM).

Finally, the OXA-35 sequence and biochemical properties indicate that it may be the progenitor of the extended-spectrum β-lactamases OXA-19 and OXA-28.

### Acknowledgements

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OXA-35 from *Pseudomonas aeruginosa*


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