ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 1997, p. 2757–2759 Vol. 41, No. 12
0066-4804/97/$04.00+0

Cloning and Nucleotide Sequence Analysis of a Gene Encoding an OXA-Derived β-Lactamase in *Acinetobacter baumannii*

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Received 13 February 1997/Returned for modification 15 May 1997/Accepted 17 September 1997

A clinical strain of *Acinetobacter baumannii* (strain Ab41) that was resistant to all β-lactam antibiotics tested except ceftazidime, ceftriaxone, ceftizoxime, and imipenem produced three β-lactamases: a presumptive chromosomal cephalosporinase, a TEM-1-like β-lactamase (pI 5.4), and a novel OXA-derived β-lactamase named OXA-21 (pI 7.0). The gene encoding OXA-21 was located in an integron. The nucleotide sequence showed three mutations compared with the sequence of OXA-3, with two being silent; the nonsilent mutation generated a substitution of Ile-217 to Met.

Acinetobacter baumannii is recognized as an important opportunistic pathogen which mainly causes pneumonia, bacteremia, and meningitis in immunocompromised patients (2, 13, 14). Currently, it is resistant to a wide variety of antibiotics, and this complicates the treatment of serious infections (11, 12, 15, 17). The low level of susceptibility of this microorganism to β-lactam antibiotics is linked to either an intrinsic or an acquired resistance. Sato and Nakae (10) showed that the outer membrane permeability of *Acinetobacter* to β-lactam antibiotics was 1 to 3% of that observed in *Escherichia coli*, suggesting that one of the causes for the high level of antibiotic resistance of *Acinetobacter calcoaceticus* is attributable to the presence of a small number of small porins. However, the most common mechanism of resistance to β-lactam antibiotics is due to the inactivation of these antibacterial agents by β-lactamases encoded either by the chromosome or by plasmids (3, 17). The plasmid-encoded β-lactamases TEM-1 and CARB-5 are the β-lactamases most frequently found in *Acinetobacter* (3, 17). Chromosomally encoded enzymes in *Acinetobacter* have also been extensively studied (3). In a previous study (17), β-lactamase detection was performed with 54 epidemiologically unrelated clinical isolates of *A. baumannii*, yielding a TEM-type β-lactamase in 16% of the clinical isolates analyzed and an unknown β-lactamase with a pI of 7.0 in 11% of the clinical isolates analyzed. The main purpose of the present work was to clone and sequence the gene encoding this unknown β-lactamase.

A strain of *A. baumannii* (strain Ab41) that was isolated during an outbreak in an intensive care unit in our hospital (16) and that has been epidemiologically and biochemically analyzed (16, 19) was studied. The susceptibility testing of this strain was performed by an agar dilution method in accordance with the guidelines established by the National Committee for Clinical Laboratory Standards (8). Approximately 10^4 CFU of the isolate was inoculated onto freshly prepared medium containing serial dilutions of the following antimicrobial agents: ampicillin (Antibioticos, S.A., León, Spain); amoxicillin, amoxicillin plus clavulanic acid, ticarcillin, and ticarcillin plus clavulanic acid (Beecham Laboratories, Brentford, United Kingdom); ceftazidime (Glaxo Wellcome, Greenford, United Kingdom); cefotaxime (Hoechst, Frankfurt, Germany); ceftriaxone (Roche, Basel, Switzerland); piperacillin (Lederle Laboratories, Pearl River, N.Y.); imipenem (Merck Research Laboratories, Rahway, N.J.); ampicillin plus sulbactam (Pfizer, Inc., New York, N.Y.); ceftizoxime (Smith Kline & French, Philadelphia, Pa.); and aztreonam (Squibb, Princeton, N.J.). Ampicillin plus clavulanic acid and ampicillin plus sulbactam were tested at ratios of 2:1, whereas ticarcillin plus clavulanic acid was tested at 2 μg of clavulanic acid per ml.

A. baumannii Ab41 was resistant to all β-lactam antibiotics except ceftazidime, ceftriaxone, ceftizoxime, and imipenem. The MICs were >256 μg/ml for ampicillin, amoxicillin and ticarcillin; 256 μg/ml for piperacillin and ticarcillin plus clavulanic acid; 64 μg/ml for aztreonam; 16 μg/ml for cefotaxime; 16/8 μg/ml for amoxicillin plus clavulanic acid; 8 μg/ml for ceftazidime, ceftizoxime, ceftriaxone, and ampicillin plus sulbactam; 0.5 μg/ml for imipenem; and 4 μg/ml for sulbactam.

β-Lactamases were analyzed by isoelectric focusing as described by Matthew et al. (7). The pIs were determined by comparison with those of enzymes with known pIs. The extract of the strain contained three β-lactamases: one gave a band with a pI above 8.0, likely corresponding to a chromosomal cephalosporinase, the other focused at pI 5.4 (TEM-1 type), and the third focused at pI 7.0. By using PCR, the presence of an integron was detected in this strain. PCR was carried out with a 50-μl volume containing 25 μl of a suspension of the strain which was prepared as described previously (18) and 25 μl of a reaction mixture containing 20 mM Tris-HCl (pH 8.8), 100 mM potassium chloride, 3.0 mM magnesium chloride, 0.1% (wt/vol) gelatin, 400 μM deoxynucleoside triphosphates, 1 μM primers, and 2.5 U of *Taq* polymerase (GIBCO-BRL). The primers 5′-AAGCAGACTTGACCTGA3′ (upper primer) and 5′GGCATCCAAGCAGCAAG3′ (lower primer) were used (6). The reaction mixture was overlaid with sterile mineral oil and was submitted to the following program of amplification: 1 min at 94°C, 1 min at 55°C, and 5 min at 72°C, with a final extension of 16 min at 72°C. The amplified DNA product was resolved by electrophoresis in a 1% (wt/vol) agarose gel containing ethidium bromide. This strain yielded a PCR product of approximately 1.5 kb, and this product was extracted from the agarose gel by using the Gene-Clean kit (Bio 101, Inc., La Jolla, Calif.) and was cloned by using the TA cloning kit (Invitrogen BV, Leek, The Netherlands). Sequencing was done with the *Taq* DyeDeoxiTerminator Cycle Sequencing Kit, and the sequence was analyzed in an automatic

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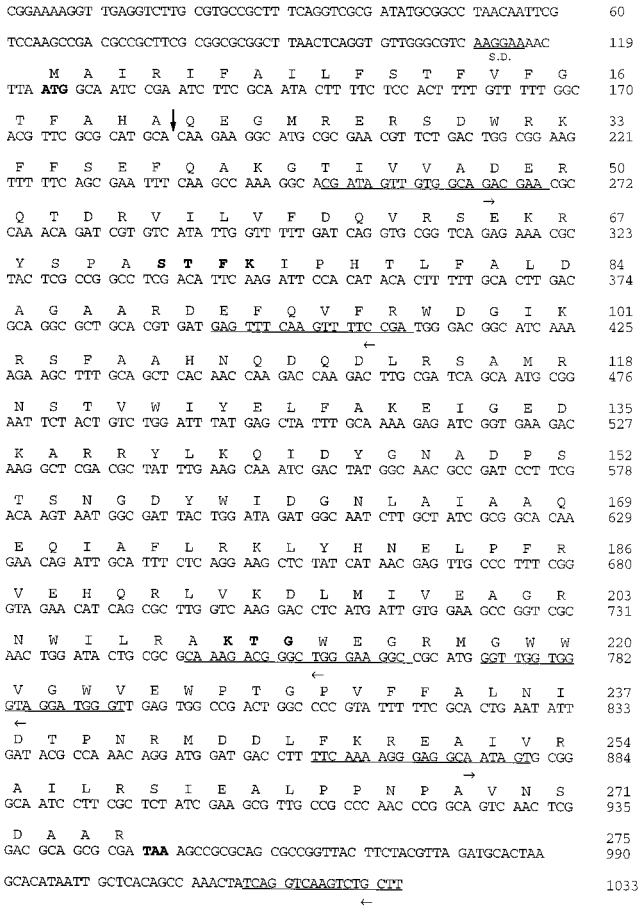

FIG. 1. Nucleotide and amino acid sequences of the OXA-21 gene and the OXA-21 protein from *A. baumannii* Ab41. The locations of the primers used to sequence the gene are underlined, and the direction of the sequence is indicated with an arrow. S.D., a putative Shine-Dalgarno ribosomal recognition site. The boldface ATG and TAA represent the initiation and termination codons, respectively. The β-lactamase-active site S-T-F-K and the conserved triad K-T-G are presented in boldface. A proposed cleavage site generating a possible signal sequence is indicated with a vertical arrow.

DNA sequencer (373A; Applied Biosystems). The entire sequence of the gene was determined twice in order to prove the accuracy. The DNA sequence of this integron in both directions was determined by using both PCR primers. A 665-bp fragment was sequenced, with the lower primer showing a high level of homology with the *aadB* gene, whereas the upper primer yielded a 505-bp fragment, which was sequenced and which showed homology with a type OXA β-lactamase. The complete nucleotide sequence obtained by the sequencing strategy described above is presented in Fig. 1. An ATG codon initiated an 825-bp open reading frame, which ended with a TAA codon. The initiation codon was preceded by a Shine-Dalgarno ribosome-binding sequence, AAGGAA. The nucleotide sequence differed from that of OXA-3 β-lactamase by three point mutations, which represented 99.5% similarity. However, two of these three mutations were silent. The gene was flanked at the 3′ end by a short recombination element. At the 5′ end another antibiotic resistance gene, *aadB*, was found, and this gene was also flanked by another recombination element, indicating that the two genes are inserted independently in the integron. Therefore, this *oxa* gene is integrated as a cassette in an integron, similar to other oxacillinases. The deduced amino acid sequence is presented in Fig. 1. The enzyme was 275 amino acids long and began with about 21 hydrophobic residues suitable for a signal peptide. The Ser-Thr-Phe-Lys (positions 72 to 74) and Lys-Thr-Gly (positions 210 to 212) conserved regions corresponding to the enzyme active site and the conserved triad, similar to that found in class A β-lactamases, were observed. The amino acid sequence homology between this β-lactamase and the OXA-3 β-lactamase was 99.6%. In comparison with the OXA-3 sequence (9), the nonsilent mutation generated a substitution of Ile-217 to Met, which is located four amino acids from the conserved triad (Table 1).

TABLE 1. Nucleotide and amino acid changes between OXA-3 and OXA-21

Nucleotide position	Nucleotide change (OXA-3→OXA-21)	Amino acid change (OXA-3→OXA-21)
18	T→C	No change
651	T→G	I→M
696	C→T	No change

The oxacillinases are often plasmid-mediated enzymes which belong to the molecular class D β-lactamases (1) and are included in group 2d of the recent functional classification by Bush et al. (4). They share a number of unusual characteristics and show hydrolytic activity for isoxazolyl penicillins such as oxacillin and cloxacillin and other penicillins such as methicillin. Eighteen oxacillinases have been characterized so far. Phylogenetic data have revealed five groups of class D β-lactamases, with large evolutionary distances between each group (9). Group II includes OXA-2, OXA-3, and OXA-15. The OXA-3 β-lactamase shows properties similar to those of OXA-2 and shares a significant degree of immunological cross-reactivity with OXA-2 (5). Comparison of the amino acid sequence of the β-lactamase investigated in this study with those of OXA-2 and OXA-15 shows similarities of 91 and 90.5%, respectively. Met-217 was found in both OXA-2 and OXA-15 but was not found in OXA-3. The high degree of similarity among this group of class D β-lactamases indicates a common ancestor.

In summary, the present study has described a new OXA-derived β-lactamase in *A. baumannii*. It is the first of its class described in this microorganism. We suggest the designation OXA-21 for the enzyme.

Nucleotide sequence accession number. The nucleotide sequence of the OXA-21 gene has been given EMBL database accession no. Y10693.

This work was supported in part by grants PM93/1229 and FIS 95/0875 from Spain.

We thank Servicios Científico Técnicos from the University of Barcelona for helping us with the DNA sequencing.

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