Molecular and Biochemical Analysis of AST-1, a Class A β-Lactamase from Nocardia asteroides Sensu Stricto

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The Nocardia genus includes several species that are opportunistic pathogens in immunocompromised patients (3, 13). Species of Nocardia asteroides sensu stricto are the predominant human pathogens and are involved in pulmonary and brain abscesses (13). Since nocardiosis requires a long treatment (6 to 12 months or longer) and may cause a high mortality rate, the choice of the optimal antibiotic treatment is crucial (7).

β-Lactams have been used as first-line treatment with little concern for the β-lactam susceptibility of Nocardia sp. isolates (13). Knowledge of the mechanisms of β-lactam resistance profiles of Nocardia isolates may be critical for assessing the potential clinical efficacy of β-lactams. A study of the antimicrobial susceptibility patterns of 78 clinical isolates belonging to the N. asteroides complex found that 95% of the isolates exhibit one of the four major antibiotic resistance patterns (24). Type I (20% of the isolates) is susceptible to ampicillin and carbenicillin but intermediate in susceptibility to imipenem; type III (18%) is susceptible to ampicillin and erythromycin; type V (17%) is resistant to broad-spectrum cephalosporins; and type VI, the most prevalent group (35%), is resistant to ampicillin but susceptible to extended-spectrum cephalosporins and imipenem. Type II and type IV are extremely rare and not well characterized. Wallace et al. show that drug resistance patterns of type III and type V correlate with taxonomic groups and have been reclassified as Nocardia nova and Nocardia farcinica, respectively (21, 22). Isolates belonging to types I, IV, and VI are grouped into the same subspecies, named N. asteroides sensu stricto.

Although some nocardial β-lactamases have been characterized biochemically in N. asteroides (9, 17), Nocardia brasiliensis (19, 23), and N. farcinica (11, 20), the accurate role of β-lactamase in the β-lactam resistance pattern has scarcely been explored. Sequences of β-lactamase genes are available only for N. farcinica and the nonhuman pathogen Nocardia lactamica (5, 11).

We report on the molecular and biochemical characterization of a class A β-lactamase named AST-1 from a clinical isolate belonging to the most prevalent group of N. asteroides sensu stricto species. Hydrolytic activity of β-lactamase AST-1 was compared to that of β-lactamase FAR-1, the most closely related enzyme.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** The N. asteroides sensu stricto isolate JPL was from a pulmonary abscess of a 50-year-old human immunodeficiency virus-infected man. It was identified by molecular methods based on the restriction analysis of PCR fragments corresponding to the heat shock protein gene, as described previously (12, 18). The recipient strain Escherichia coli JM109 for cloning experiments and phagemid cloning vector pBK-CMV have been reported previously (11).

**Antimicrobial agents and MIC determinations.** Antibiotic powders and their sources have been described previously (11). Antibiotic disks were used for routine antibiograms (Sanofi-Diagnostics Pasteur, Marnes-La-Coquette, France). MICs were determined by an agar dilution technique on Mueller-Hinton agar (Sanofi-Diagnósticos Pasteur) with an inoculum of 10^6 CFU per spot as reported previously (11). All plates were incubated at 35°C for 18 h for E. coli and for 72 h for N. asteroides according to NCCLS guidelines (15). MICs of β-lactams were determined alone or in combination with a fixed concentration of clavulanic acid (2 μg/ml), sulbactam (8 μg/ml), and tazobactam (4 μg/ml).

**Cloning experiments and genetic analysis.** Genomic DNA from N. asteroides sensu stricto JPL was extracted as previously described (13). Partially digested Sau3AI fragments of genomic DNA of N. asteroides JPL were ligated into BamHI-restricted phagemid pBK-CMV (Stratagene, La Jolla, Calif.). Ligation was performed at a 1:2 vector/insert ratio at a final concentration of 200 ng of DNA in a ligation mixture containing 1 U of T4 DNA ligase at 4°C for 18 h. Recombinant plasmids were transformed by electroporation (Gene Pulser II; Bio-Rad, Ivry-sur-Seine, France) into electrocompetent E. coli JM109 cells. Antibiotic-resistant colonies were selected onto Trypticase soy (TS) agar plates containing amoxicillin (50 μg/ml) and kanamycin (30 μg/ml) that were analyzed as described previously (13). Plasmid DNAs of recombinant strains were obtained using Qiagen columns (Qiagen, Courtaboeuf, France). Plasmid mapping was performed after double restriction analysis. DNA of one recombinant plasmid with the shortest insert was sequenced on both strands by using an ABI 377 sequencer (Applied Biosystems, Foster City, Calif.). The nucleotide and the deduced protein sequences were analyzed with software available over the

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β-Lactamase preparation. Cultures of E. coli JM109 harboring recombinant plasmid pAST-1 were grown overnight at 37°C in 4 liters of TS broth with amoxicillin (50 μg/ml). The bacteria were harvested for 10 min at 6,000 × g, and the bacterial pellet was resuspended in 30 ml of 20 mM bis-Tris (pH 5.5) [bis(2-hydroxyethyl)iminio][tri(hydroxymethyl)methane] at 4°C. The bacterial cells were disrupted by sonication (two times for 20 s at 20 Hz) (Vibra Cell 75022 Phospholyser; Bioblock, Illkirch, France) and were centrifuged (30 min, 10,000 × g, 4°C). The supernatant containing the enzyme extract was purified by ion-exchange chromatography with AGMP-1 exchanger (Bio-Rad). The exchanger in the chloride form was treated with 0.1 M ammonia in water and was then washed extensively with water. After adsorption of the extracts, elution was performed with 0.1 M NaCl. The active fractions were pooled, dialyzed extensively, and lyophilized.

Kinetic measurements. Kinetic measurements were performed with the semi-purified β-lactamase preparation extracted from E. coli JM109(pAST-1). The kinetic constants were determined by the online computerized microacidimetric method at pH 7.0 and 37°C as described previously (10). Vₘₐₓ and Vₘₐₓ/Kₘ were expressed relative to that of benzylpenicillin (Vₘₐₓ = 100). The 50% inhibitory concentrations (IC₅₀) were determined for clavulanate, sulbactam, and tazobactam as the concentration that reduced the hydrolysis rate of 100 μM benzylpenicillin by 50% under conditions in which the enzyme was preincubated with various concentrations of inhibitor for 5 min at 30°C before addition of the benzylpenicillin (10). The specific activity of the semi-purified enzyme from E. coli JM109 harboring pAST-1 (AST-1) was obtained as described previously (16). One unit of the enzyme was defined as the activity which hydrolyzed 100 μmol of cephalothin per min per mg of protein. The total protein content was determined with bovine serum albumin as the standard (Bio-Rad DC protein assay kit).

IEF and determination of relative molecular mass. Cultures of N. asteroides JPL were grown in TS broth at 35°C for 72 h in an aerobic atmosphere. β-Lactamase extracts from these cultures were obtained as described previously (11) and were submitted with the β-lactamase preparation from cultures of E. coli JM109 harboring recombinant plasmid pAST-1 to isoelectric focusing (IEF) analysis on an ampholine polyacrylamide gel, as described previously (11). The relative molecular mass of the β-lactamase from E. coli JM109(pAST-1) culture was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis, as reported previously (16).

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper have been assigned to the GenBank nucleotide database under accession no. AF 279904.

RESULTS AND DISCUSSION

Identification and susceptibility testing of N. asteroides sensu stricto isolate. The N. asteroides isolate JPL, was assigned to the type VI group of Steingrube et al. (18), which includes most of the N. asteroides sensu stricto isolates. MICs of β-lactams that showed this isolate was resistant to aminopenicillins, narrow-spectrum cephalosporins, ceftazidime, and aztreonam (Table 1). Addition of clavulanate partially decreased the MICs of aminopenicillins, while tazobactam and sulbactam did not have any significant effect (Table 1). These results were consistent with those obtained for other N. asteroides isolates (9), for N. farrinica (11, 20), and for Mycobacterium fortuitum (2, 6). Disk susceptibility testing showed that the N. asteroides isolate JPL was also susceptible to aminoglycosides (except kanamycin), tetracycline, and sulfonamides and resistant to fluoroquinolones, macrolides, fosfomycin, and chloramphenicol.

Characterization of the bla_AST-1 gene and its expression in E. coli. Two recombinant plasmids were obtained harboring the same 1.7-kb insert as a result of cloning experiments. One of them, pAST-1, was further characterized, and its insert was sequenced. It contained a 933-bp open reading frame (ORF). bla_AST-1, encoding a 310-amino-acid protein named ASTM-1 (Fig. 1). The G+C content of blast-1 was 71.3%, which lies within the G+C ratios for other chromosomally encoded Nocardia sp. genes as recorded in the EMBL and GenBank sequence databases (64 to 72%). Moreover, 18 bp upstream of this ORF, part of another ORF was identified, the deduced protein of which shared 42% identity with a 561-amino-acid protein of unknown function from Streptomyces coelicolor (GenBank accession no. T35845). Additionally, 244 bp from bla_AST-1, another ORF was identified, the protein of which shared 59% amino acid identity (within 89 amino acids) with a probable phosphorylating protein, UreD, from Mycobacterium leprae (GenBank accession no. S72992). These results are consistent with the Actinomycetales origin of blast-1. Since no ATG initiation codon was found for blast-1, a putative GTG

![Table 1. MICs of β-lactams for N. asteroides sensu stricto JPL, E. coli JM109 harboring recombinant plasmid pAST-1, and reference strain E. coli JM109](http://www.ncbi.nlm.nih.gov)
was retained as its initiation codon (data not shown), as in several *Streptomyces* and *Mycobacterium* sp. genes (11). Within the deduced protein, structural elements characteristic of serine and Ambler class A $\beta$-lactamases were identified (1, 8) (Fig. 1). The comparison of the AST-1 sequence with those of other class A $\beta$-lactamases showed that it was distantly related to class A $\beta$-lactamases, including those of *Streptomyces* and *Mycobacterium* spp. (35 to 50% of amino acid identity). It was related mostly to FAR-1 $\beta$-lactamase from *N. farcinica* VIC, sharing 65% amino acid identity (11).

MICs of $\beta$-lactams for *E. coli* JM109(pAST-1) showed mostly resistance to penicillins that was partially antagonized by addition of clavulanate (Table 1). These MICs mirrored those obtained for *N. asteroides* isolate JPL and for *E. coli* JM109(pFAR-1) expressing $\beta$-lactamase FAR-1, except that in this latter case, a slight increase of the MIC of aztreonam was observed (11).

**Biochemical properties of the $\beta$-lactamase AST-1.** IEF analysis showed that cultures of *N. asteroides* isolate JPL and of *E. coli* JM109(pAST-1) produced an identical $\beta$-lactamase with a pI of 4.8 (data not shown). This pI was similar to those observed for $\beta$-lactamases of other *N. asteroides* isolates (4.2 to 4.6) (9) and was different from the pI of 5.8 of an *N. asteroides* isolate, as reported recently (17). Thus, *N. asteroides* isolates may possess different $\beta$-lactamases of acidic pI values. However, valid comparison of $\beta$-lactamase content based on pI val-
TABLE 2. Compared kinetic parameters for AST-1 from *N. asteroides* sensu stricto and FAR-1 from *N. farcinica*

| Substrate     | Parameter for: | AST-1 | FAR-1 |
|---------------|----------------|-------|-------|
|               | $V_{\text{max}}$ ($K_{\text{m}}$ (µM)) | $V_{\text{max}}/K_{\text{m}}$ | $V_{\text{max}}$ ($K_{\text{m}}$ (µM)) | $V_{\text{max}}/K_{\text{m}}$ |
| Benzylopenicillin | 100 | 30 ± 2 | 100 | 30 ± 2 |
| Aminoacidin    | 53 ± 4 | 50 ± 4 | 32 | 115 ± 12 | 50 ± 3 |
| Ticaricillin   | 8 ± 0.7 | 7 ± 0.5 | 33 | 30 ± 1 | 31 ± 1 |
| Piperacillin   | 90 ± 6 | 330 ± 27 | 8 | 250 ± 26 | 45 ± 2 |
| Cephalothin    | 40 ± 4 | 20 ± 1 | 60 | 85 ± 6 | 104 ± 9 |
| Cephaloridine  | 57 ± 3 | >500 | <3.5 | 80 ± 5 | >500 |
| Cefopazone     | 12 ± 0.7 | >500 | <0.7 | NS$^b$ | NS |
| Cefotaxime     | <1 | >500 | <6 × 10$^{-2}$ | <1 | >500 | <6 × 10$^{-2}$ |
| Aztreonam      | <1 | >500 | <6 × 10$^{-2}$ | 3 ± 0.2 | >500 |

$^{a} V_{\text{max}}$ and $V_{\text{max}}/K_{\text{m}}$ relative to that of benzylopenicillin, which was set at 100. Data are the means and standard deviations from three independent experiments.

$^{b}$ NS, not studied.

umes is difficult, since in the previous studies (9, 17), *N. asteroides* sensu stricto isolates were not differentiated from other *N. asteroides* spp. by molecular techniques.

The relative molecular mass of the β-lactamase AST-1 expressed in *E. coli* JM109(pAST-1) was estimated to be 31 kDa (data not shown), close to the value of 32 kDa for the β-lactamase FAR-1 (11).

The β-lactamase AST-1 was very poorly expressed from *E. coli* JM109(pAST-1) and *N. asteroides* JPL cultures (data not shown). The specific activity of the semipurified extract of *N. asteroides*-lactamases extracted from *N. asteroides* sensu stricto isolates were not differentiated from other *N. asteroides* spp. by molecular techniques.

**Conclusion.** β-Lactamase AST-1 is the second class A β-lactamase characterized in a *Nocardia* sp. clinical isolate. As already mentioned for β-lactamase FAR-1 from *N. farcinica*, AST-1 expression cannot explain the entire β-lactam resistance profile of the *N. asteroides* sensu stricto isolate, especially concerning its resistance to aztreonam and ceftazidime. Additionally, other undetected β-lactamases and/or penicillin-binding affinities may account for this naturally occurring β-lactam resistance profile. Since AST-1 and FAR-1 β-lactamases shared significant amino acid identity and similar biochemical properties, they may derive from a common ancestor.

ACKNOWLEDGMENTS

L.P. and F.L. contributed equally to this work. This work was funded by a grant from the Ministère de l’Education Nationale et de la Recherche (UPRES JE 2227), Université Paris XI, Faculté de Médecine Paris-Sud, France.

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