ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 1999, p. 2081–2083 Vol. 43, No. 8
0066-4804/99/$04.00+0

Molecular Characterization of the SHV-11 β-Lactamase of *Shigella dysenteriae*

JASIMUDDIN AHAMED AND MANIKUNTALA KUNDU*

Department of Chemistry, Bose Institute, Calcutta 700 009, India

Received 16 November 1998/Returned for modification 3 March 1999/Accepted 6 June 1999

A β-lactamase with an M_r of 29,000 and a pI of 7.6 was partially purified from a clinical isolate of *Shigella dysenteriae*. The *bla* gene encoded the SHV-11 enzyme carrying the substitution Leu→Gln at position 35 and was linked to a strong promoter. This variant, unlike the prototype SHV-1 enzyme, hydrolyzed oxacillin, cloxacillin, and oxyiminocephalosporins such as cefotaxime.

Shigella species are a major cause of diarrheal diseases and mortality in developing countries (4), and the frequency of strains multiply resistant to ampicillin, trimethoprim-sulfamethoxazole, and streptomycin is causing growing concern. β-Lactamases are primarily responsible for β-lactam resistance in gram-negative bacteria. The presence of TEM-, OXA-1, and OXA-3 β-lactamases in *S. flexneri* and *S. sonnei* has been previously reported (21). However, detailed characterization of these enzymes in *S. dysenteriae* is lacking. We report a clinical strain of *S. dysenteriae* PB-10 (obtained from the National Institute of Cholera and Enteric Diseases [NICED], Calcutta, India) harboring the SHV-11 β-lactamase capable of hydrolyzing oxacillin, cloxcillin, and oxyimnocephalosporins.

Bacteria were routinely grown in tryptic soy broth on a rotary shaker at 37°C. The β-lactamase was partially purified from sonic extracts of cells by size-exclusion chromatography on Sephadex G-75, followed by successive chromatography on a Q-Sepharose anion exchanger at pH 7 (at which time β-lactamase activity remained in the flowthrough) and then at pH 10 (where β-lactamase activity eluted at a salt concentration of 0.1 M NaCl in 20 mM ethanolamine containing 5% glycerol and 5% ethylene glycol). During purification of the enzyme, β-lactamase activity was routinely determined at 30°C by monitoring the hydrolysis of nitrocefin spectrophotometrically at 482 nm (17). One unit of β-lactamase activity is the amount of enzyme hydrolyzing 1 μmol of nitrocefin per min.

Hydrolysis of β-lactams was monitored at 30°C in 100 mM sodium phosphate buffer, pH 7.2, at wavelengths that gave a maximum in the difference spectrum of the hydrolyzed antibiotic against the unhydrolyzed antibiotic. The relative V_{max} values of the substrates were obtained after absorbance data were fit to the integrated Michaelis-Menten equation (7). The rate of hydrolysis of benzylpenicillin was set at 100.

Conjugative transfer of *S. dysenteriae* PB-10 antibiotic resistance plasmid into *Escherichia coli* HB-101 was performed as described previously by Philippon et al. (18). Transconjugants were selected by plating on medium containing 25 μg of ampicillin/ml and 10 μg of rifampin/ml.

For cloning of the β-lactamase gene, plasmid DNA from the transconjugant was partially digested with *Bam*HI and ligated to the *Bam*HI-digested vector pK19 (20). Transformants were selected on Luria-Bertani agar supplemented with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), 50 μg of ampicillin, and 50 μg of kanamycin per ml. Plasmid DNA containing an ≈9-kb insert was subsequently subcloned in several steps by using successively the enzymes *Sst*I, *Sa*II, and *Ava*I and selecting each time for Amp Kan^r recombinants. The plasmid obtained in the final step, pMK105, contained a 1.8-kb insert. A library of nested deletions of the clone pMK105 was finally generated in the vector pK19 to sequence the entire cloned DNA, by using the double-stranded nested deletion kit from Amersham Pharmacia Biotech. DNA sequencing was done by using the Thermosequenase cycle sequencing kit (Amersham Pharmacia Biotech). Both strands of DNA were sequenced from two clones. The nucleotide sequence and the deduced protein sequence were analyzed by using the Genetics Computer Group software package. Similarity searches were performed by using the Gap BLAST algorithm (1).

The β-lactam resistance of strain PB10 could be transferred to a rifampin-resistant strain of *E. coli*, HB101, by transconjugation. MICs were determined on Mueller-Hinton agar plates containing serial dilutions of antibiotics by inoculating with 10^4 CFU per spot and reading after 18 h of growth at 37°C. The clinical isolate PB-10 was resistant toward all the penicillins and some of the cephalosporins tested (Table 1). Cefoxitin and imipenem appeared to be the most effective antibiotics. The MICs of selected antibiotics were determined in combination with that of clavulanic acid (MIC, 32 μg/ml) at a concentration of 4 μg/ml. The decreased MICs indicated the involvement of a transferable β-lactamase resistant to strain PB-10. The cefotaxime and ceftazidime MICs are high compared to data reported earlier for *Shigella* species (6, 21). For cefotaxime, this could be due, at least in part, to the presence of the β-lactamase reported here, since a decrease in the MIC was observed in the presence of clavulanic acid for strain PB-10. The higher ceftazidime MIC could not be correlated with β-lactamase activity, particularly since there was no decrease in the MIC in the presence of clavulanate.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the cell lysate and the partially purified β-lactamase of strain PB-10, followed by renaturation and overlay of gels with nitrocefin (12), showed the presence of a single band of β-lactamase activity, with an approximate M_r of 29,000 ± 2,000 (mean ± standard deviation). Isoelectric focusing of the reference SHV-1 enzyme as well (3) as crude and partially purified β-lactamase of PB-10, followed by overlay with nitrocefin, showed in each case a single band with β-lactamase activity at an isoelectric point of 7.6 ± 0.2, which corresponded to that reported for SHV enzymes (2, 5).

The hydrolysis of cephaloridine and of almost all other cephalosporins tested was much slower than that of benzylpenicil-

* Corresponding author. Mailing address: Department of Chemistry, Bose Institute, 93/1 Acharya Prafulla Chandra Rd., Calcutta 700 009, India. Phone: 91 33 350 6619. Fax: 91 33 3506790. E-mail: mani@boseinst.ernet.in.

TABLE 1. In vitro susceptibilities of *S. dysenteriae* and transconjugant to β-lactam antibiotics

Antibiotic(s)	MIC (μg/ml) for:			
	E. coli HB-101	*S. dysenteriae* C152[a]	*S. dysenteriae* PB-10	Transconjugant
Ampicillin	8	8	>1000	>1,000
Ampicillin + CA[c]	8	8	40	52
Ticarcillin	8	ND[b]	>1000	>1,000
Ticarcillin + CA	8	ND	100	100
Cefotaxime	0.25	0.25	16	16
Cefotaxime + CA	0.25	0.25	4	4
Ceftazidime	0.16	1	16	16
Ceftazidime + CA	0.16	1	16	16
Cefuroxime	ND	ND	250	ND
Piperacillin	ND	8	500	ND
Carbenicillin	ND	ND	1,000	ND
Cefoxitin	ND	4	4	ND
Imipenem	ND	2	2	ND

[a] *S. dysenteriae* C152 was a susceptible clinical isolate obtained from NICED, Calcutta, India.
[b] ND, not determined.
[c] CA, clavulanate.

lin, nitrocefin being the only exception (Table 2). The oxyiminocephalosporins cefotaxime, cefuroxime, and ceftizoxime were hydrolyzed at measurable rates. No detectable hydrolytic activity was observed for ceftazidime, cefoxitin, cefsulodin, aztreonam, or imipenem. Also, these antibiotics failed to inhibit the enzyme. Unlike other SHV enzymes, the PB-10 enzyme hydrolyzed oxacillin and cloxacillin at appreciable rates. Comparison of relative rates of hydrolysis of β-lactams by a reference SHV-1 enzyme and the β-lactamase harbored by pMK105 demonstrate that, unlike the reference SHV-1 enzyme, the β-lactamase produced by pMK105 hydrolyzed oxacillin. Cefotaxime was hydrolyzed by this enzyme, whereas its hydrolysis could not be measured with the reference SHV-1 enzyme. Sensitivity to clavulanic acid is characteristic of TEM and SHV enzymes. The 50% inhibitory concentrations (IC_{50}) of clavulanic acid and sulbactam were determined by incubating the enzyme with either inhibitor for 10 min prior to the addition of benzylpenicillin (1 mM). The IC_{50} was determined graphically. For clavulanic acid, the values were 2 and 40 nM, while for sulbactam, the values were 17,000 and 30,000 nM for the reference strain SHV-1 and the *S. dysenteriae* enzyme, respectively. The *S. dysenteriae* enzyme was therefore less susceptible to clavulanic acid and sulbactam than the SHV-1 enzyme.

TABLE 2. Hydrolysis parameters for SHV-1 and SHV-11 β-lactamases for selected β-lactams[c]

Antibiotic	Wavelength	$\Delta\varepsilon(M^{-1}cm^{-1})$	SHV-1 J53R1010 (reference)[a]	SHV-11 (*S. dysenteriae*)
Benzylpenicillin	232	800	100	100
Ampicillin	235	670	150	300
Oxacillin	260	400	<0.1	96
Cloxacillin	260	140	<0.1	45
Carbenicillin	235	830	20	45
Nitrocefin	482	15,000	ND[c]	150
Cephaloridine	255	9,000	50	35
Cefazolin	273	6,600	10	10
Cefuroxime	273	7,500	<0.1	6
Cefotaxime	264	6,700	<0.1	8
Cefacetrile	260	7,400	5	4
Cefoperazone	260	5,700	ND	4
Cefaclor	260	7,200	8	15
Ceftizoxime	255	5,000	<0.1	18
Ceftazidime	260	8,600	<0.1	<0.1
Cefsulodin	265	5,800	<0.1	<0.1
Cefoxitin	265	7,380	<0.1	<0.1
Imipenem	297	9,000	<0.1	<0.1

[a] SHV-1 J53R1010 was a gift from Antone A. Medeiros.
[b] ND, not determined.
[c] Results are the means of three determinations (standard deviation, ≤10%). Rates of hydrolysis (V_{max}) of β-lactams are expressed relative to the rate of hydrolysis of benzylpenicillin, which was set at 100.

The nucleotide sequence (EMBL nucleotide sequence database, accession no. Y18299) of the plasmid pMK105 revealed a 286-amino-acid open reading frame. The nucleotide sequence corresponding to positions 1 through 286 of the deduced amino acid sequence was identical to that reported for the *Klebsiella pneumoniae* SHV-1a (or SHV-11) (GenBank accession no. X98101), except for a silent CAC-to-CAT mutation encoding histidine 108. Mutations of the plasmid-determined TEM, SHV, and OXA β-lactamases that enhance their affinity for expanded-spectrum cephalosporins usually occur between positions 104 and 240. These mutations have resulted in roomier active-site cavities that permit expanded-spectrum β-lactams with bulky side chains to enter and bind to the active site serine-70 (10, 13). With site-directed mutants it has been reported that the leu→gln substitution at position 35 on the protruding NH_2 terminus (16) increases resistance to ceftazidime but reduces the MICs of all other cephalosporins tested compared to SHV-2. In our case, the situation was not comparable, since the SHV-11 enzyme has glycine at position 238 rather than serine, as in SHV-2. (8) A survey of SHV β-lactamases in Switzerland previously led to the identification of the variant enzyme SHV-11 (15) harboring the Leu→Gln substitution at position 35. In this report, *K. pneumoniae* KPZU-12, harboring the SHV-11 β-lactamase, showed β-lactam susceptibilities virtually identical to *K. pneumoniae* KPAA-I harboring the SHV-1 β-lactamase. We demonstrate that the SHV-11 enzyme from *S. dysenteriae* hydrolyzes oxyiminocephalosporins. The hydrolysis of oxacillin and cloxacillin was intriguing.

There was little similarity of the nucleotide sequence upstream of the −10 with that of the prototype SHV-1 *bla* gene (14). Changes in the −35 and −10 regions are a powerful influence on promoter strength (11). In *E. coli*, the closer the sequence is to the consensus, the stronger the promoter (−35 consensus sequence, TTGACA; −10 consensus sequence, TATAAT) (9). The promoter comprising 5′TTGCAA′3′ (−35 box) and 5′TATTCT3′ (−10 box) identified in the pMK105 *bla* gene has been reported to increase β-lactam resistance when coupled to the SHV-2 gene. (19). The association of the *bla* gene with a strong promoter likely influenced susceptibilities to β-lactams in the present study as well.

The continued emergence of β-lactam-resistant *S. dysenteriae* is of particular concern to developing countries. Surprisingly, reports of noteworthy efforts to characterize the β-lactamases most likely to be associated with this resistance phenomenon are sparse in the literature, particularly in terms of nucleotide sequence information. Our report describes perhaps the first serious attempt both to characterize the structural *bla* gene of a β-lactamase from a clinical isolate of *S. dysenteriae* and to biochemically characterize the elaborated enzyme. Although the leu-35→gln substitution had been reported while this work was in progress (15), its effect on substrate profile had not been investigated. Rather, conclusions had been drawn merely from an evaluation of MIC data, which may be influenced by an interplay of factors, including the permeability of the outer membrane of the hosts, the amount

of β-lactamase produced, the affinities of the target penicillin-binding proteins for the tested β-lactam, and the involvement of efflux pumps. We demonstrate, using the SHV-1 J35R1010 as a reference enzyme, that the SHV-11 enzyme hydrolyzes oxacillin, cloxacillin, and oxyiminocephalosporins such as cefotaxime and is less sensitive to clavulanic acid. The results from our biochemical characterization of this enzyme suggest that an amino acid substitution in the protruding amino terminus of the β-lactamase likely alters the overall conformation of the molecule and, consequently, its substrate profile. The structural basis for this needs to be investigated further.

This work was supported in part by grants from the Department of Science and Technology and the Council of Scientific and Industrial Research, government of India.

REFERENCES

1. **Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
2. **Ambler, R. P., F. W. Coulson, J.-M. Frere, J.-M. Ghuysen, B. Joris, M. Forsman, R. C. Levesque, G. Tiraby, and S. G. Waley.** 1991. A standard numbering scheme for the class A β-lactamases. Biochem. J. **276:**269–270.
3. **Barthelemy, M., J. Peduzzi, and R. Labia.** 1988. Complete amino acid sequence of p453-plasmid-mediated PIT-2 β-lactamase (SHV-1) Biochem. J. **251:**73–79.
4. **Bennish, M. L., and M. A. Salam.** 1992. Rethinking options of the treatment of shigellosis. J. Antimicrob. Chemother. **30:**243–247.
5. **Bush, K., G. A. Jacoby, and A. A. Medeiros.** 1995. A functional classification scheme for β-lactamase and its correlation with molecular structure. Antimicrob. Agents Chemother. **39:**1211–1233.
6. **Cavallo, J. D., J. Bericon, J. M. Bandet, T. Samson, M. France, and M. Meyran.** 1993. Antibiotic sensitivity of 140 strains of *Shigella* isolated in Djibouti. Bull. Soc. Pathol. Exot. **86:**35–40.
7. **De Meester, F., B. Joris, G. Reckinger, C. Bellefroid-Bourguignon, J. M. Frere, and S. G. Waley.** 1987. Automated analysis of enzyme inactivation phenomena. Biochem. Pharmacol. **36:**2393–2403.
8. **Garbarg-Chenon, A., V. Godard, R. Labia, and J.-C. Nicolas.** 1990. Nucleotide sequence of SHV-2 β-lactamase gene. Antimicrob. Agents Chemother. **34:**1444–1446.
9. **Hawley, D., and W. R. McClure.** 1983. Compilation and analysis of *Escherichia coli* promoter DNA sequences. Nucleic Acids Res. **11:**2237–2255.
10. **Herzberg, O., and J. Moult.** 1987. Bacterial resistance to β-lactam antibiotics: crystal structure of β-lactamase from *Staphylococcus aureus* PC1 at 2.5 A resolution. Science **236:**694–701.
11. **Kobayashi, M., K. Nagata, and A. Ishihama.** 1990. Promoter selectivity of *Escherichia coli* RNA polymerase: effect of base substitutions in the promoter-35 region on promoter strength. Nucleic Acids Res. **18:**7367–7372.
12. **Matthew, M., A. M. Harris, M. J. Marshall, and G. W. Ross.** 1975. The use of analytical isoelectric focusing for detection and identification of β-lactamase. J. Gen. Microbiol. **88:**169–178.
13. **Medeiros, A. A.,** 1997. Evolution and dissemination of β-lactamases accelerated by generations of β-lactam antibiotics. Clin. Infect. Dis. **24:**S19–S45.
14. **Mercier, J., and R. C. Levesque.** 1990. Cloning of SHV-2, OHIO-1, and OXA-6 β-lactamases and cloning and sequencing of SHV-1 β-lactamase. Antimicrob. Agents Chemother. **34:**1577–1583.
15. **Nüesch-Inderbinen, M. T., F. H. Kayser, and H. Hächler.** 1997. Survey and molecular genetics of SHV β-lactamases in *Enterobacteriaceae* in Switzerland: two novel enzymes, SHV-11 and SHV-12. Antimicrob. Agents Chemother. **41:**943–949.
16. **Nüesch-Inderbinen, M. T., H. Hächler, and F. H. Kayser.** 1995. New system based on site-directed mutagenesis for highly accurate comparison of resistance levels conferred by SHV β-lactamases. Antimicrob. Agents Chemother. **39:**1726–1730.
17. **O'Callaghan, C. H., A. Morris, S. M. Kirby, and A. H. Shingler.** 1982. Novel method for the detection of β-lactamases by using a chromogenic cephalosporin substrate. Antimicrob. Agents Chemother. **1:**283–288.
18. **Phillipon, A. M., G. C. Paul, and G. A. Jacoby.** 1983. Properties of PSE-2 β-lactamase and genetic basis for its production in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **24:**362–369.
19. **Podbielski, A., J. Schonling, B. Melzer, K. Warnatz, and H.-G. Leusch.** 1991. Molecular characterization of a new plasmid-encoded SHV-type β-lactamase (SHV-2 variant) conferring high-level cefotaxime resistance upon *Klebsiella pneumoniae*. J. Gen. Microbiol. **137:**569–578.
20. **Pridmore, R. D.** 1987. New and versatile cloning vectors with kanamycin-resistance marker. Gene **56:**309–312.
21. **Verbist, L., and J. Verhaegen,** 1980. GR-20263: a new aminothiazolyl cephalosporin with high activity against *Pseudomonas* and *Enterobacteriaceae*. Antimicrob. Agents Chemother. **17:**807–812.