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A β-Lactamase from *Serratia marcescens* Hydrolyzing the 2-Carboxypenam T-5575

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A β-lactamase was purified from *Serratia marcescens* GN16694; it hydrolyzed T-5575 and oxime-type cephalosporins, i.e., cefuroxime and ceftazidime. Its isoelectric point and molecular weight were 8.6 and 42,000, respectively. This enzyme was not inhibited by EDTA and clavulanic acid. This enzyme is an unusual β-lactamase and has been classified as a group 1 cephalosporinase.

T-5575 is a novel 2-carboxypenam antibiotic with potent and broad-spectrum antibacterial activity, and it is stable for a wide range of β-lactamases (Fig. 1) (14). We isolated five *Serratia marcescens* strains resistant to T-5575. All of these strains produced an enzyme which hydrolyzed T-5575. In this study, the enzymatic and biochemical properties of the enzyme produced by *S. marcescens* GN16694 are reported.

The six strains of *S. marcescens* used in this study were isolated in 1985 in Japan. *S. marcescens* GN16666 is a β-lactam-sensitive strain and was used as a control. The five T-5575-resistant strains were isolated from urine samples of patients at different hospitals. T-5575 was supplied by Toyama Chemical Co., Ltd. Ritipenem (FCE 22101; RIPM) was supplied by Farmitalia Carlo Erba Research and Development (15). The following antimicrobial agents were used: cephaloridine (CER), cefuroxime (CXM), ceftazidime (CAZ), cefotaxime, cefmenoxime, ceftriaxone, aztreonam (ATM), benzylpenicillin (PCG), imipenem (IPM), cefazolin, ceftizoxime, and clavulanic acid (CVA). Antibacterial susceptibilities were measured by an agar dilution method with sensitivity disk agar (Nissui Pharmaceutical) (12). β-Lactamase activities were assayed spectrophotometrically with a UV-265 spectrophotometer (11), and the K_m and the maximum rate of hydrolysis (relative V_{max}) were determined with a Lineweaver-Burk plot. The isoelectric point (pI) and the molecular weight were determined as previously described (13).

The preparation and purification of this enzyme was based on the methodology described previously (8, 9, 13). In brief, a culture of *S. marcescens* GN16694 grown in sensitivity test broth (Nissui Pharmaceutical) was harvested and disrupted; it was used as the crude enzyme. For purification, streptomycin sulfate was added to the crude enzyme at a final concentration of 4.0% (wt/vol) for the removal of nucleic acid. After dialysis against 5 mM phosphate buffer (pH 7.0) and centrifugation, the supernatant was treated with ammonium sulfate from 45 to 80%. The fraction with enzyme activity was dialyzed and applied to a carboxymethyl Sephadex C-50 column which was equilibrated with 5 mM phosphate buffer (pH 7.0). Unadsorbed protein was washed out with the same buffer, and then the adsorbed enzyme was eluted with a linear gradient of NaCl from 0.1 to 1.5 M. Fractions with activity were dialyzed against the same buffer and concentrated. The concentrated solution was loaded on a Sephadex G-200 column and eluted with the same buffer. Concentrated fractions with activity were used as the purified enzyme. For inhibitor studies, the enzyme solution was preincubated in 5 mM phosphate buffer (pH 7.0) for 10 min at 30°C with EDTA (final concentration, 100 μM) or with the β-lactamase inhibitor CVA in 5 mM phosphate buffer (pH 7.0) for 2 min at 30°C, and the remaining activity was assayed spectrophotometrically with CXM (100 μM) as the substrate. The 50% inhibitory dose (ID_{50}) of each β-lactamase inhibitor was defined as the concentration giving 50% inhibition of β-lactamase activity.

The molecular weight of the β-lactamase from *S. marcescens* GN16694 was 42,000, and the pI was approximately 8.6. This enzyme was designated SRT-1.

Table 1 presents the stabilities of T-5575 to various β-lactamases. T-5575 was highly stable to hydrolysis by a representative of each group of β-lactamases, except for SRT-1.

The MICs of β-lactam antibiotics for six strains are listed in Table 2. The five strains resistant to T-5575 showed high levels of resistance to cephalosporins. The activities of β-lactamases from six strains were tested on crude enzyme preparations. Each β-lactamase produced by a strain resistant to T-5575 showed hydrolyzing activities for CAZ and T-5575 which were comparable to that for CER but not to that for IPM. The extracted sample from *S. marcescens* GN16666 did not hydrolyze antibiotics. The hydrolyzing activities for β-lactam antibiotics among the other five strains were similar; they showed high levels of resistance to cephalosporins, suggesting that these strains produced the same enzyme (SRT-1). The kinetic parameters (K_m, relative V_{max}, and K_i) of this enzyme for various β-lactam antibiotics were determined. As shown in Table 3, the SRT-1 enzyme showed lower hydrolyzing activity against PCG than against cephalosporins and T-5575. The SRT-1 enzyme also showed significant activities against CXM and CAZ. It should be noted that the SRT-1 enzyme showed activity against T-5575 which was stable to a wide range of β-lactamases (14). The SRT-1 enzyme was inhibited by low concentrations of ATM, IPM, and RIPM, as indicated by their

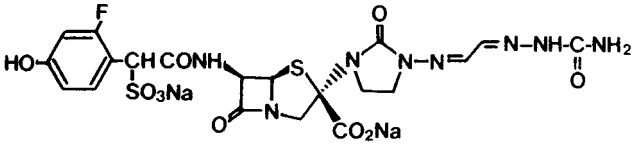

FIG. 1. Chemical structure of the novel 2-carboxypenam T-5575.

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TABLE 1. Stabilities of T-5575 to various β-lactamases

Enzyme source	β-lactamase class[a]	Relative rate of hydrolysis (%)[b]			
		T-5575	IPM	CAZ	ATM
Morganella morganii GN5407	1	<0.01	<0.01	0.3	<0.01
Escherichia coli ML4901/Rms212 (type I)	2b	0.14	<0.01	<0.01	<0.01
E. coli ML4901/Rms149 (type IV)	2c	0.9	0.02	<0.01	<0.01
E. coli ML4901/Rms213 (type II)	2d	3.7	0.7	<0.01	<0.01
Proteus vulgaris GN7919	2e	<0.01	<0.01	<0.01	<0.01
Stenotrophomonas maltophilia GN12873	3	<0.01	1,290	30	<0.01
S. marcescens GN16694	1	80	<0.01	150	<0.01

[a] On the basis of the functional classification of Bush (1–3).
[b] Expressed as a percentage of PCG (100 μM) hydrolysis for each enzyme from an *E. coli* source and as a percentage of CER (100 μM) hydrolysis for each enzyme from another source.

TABLE 3. Kinetics of cephalosporin and penicillin hydrolysis by the SRT-1 enzyme from *S. marcescens* GN16694

Substrate	K_m (μM)	K_i (μM)[a]	Relative V_{max} (%)[b]
CER	680		100
Cefazolin	680		350
CXM	370		320
Ceftriaxone	190		460
Ceftizoxime	540		600
Cefotaxime	430		420
Cefmenoxime	160		190
CAZ	170		25
RIPM		1.5	
IPM		5.2	
ATM		1.7	
T-5575	20		16
PCG	9		8

[a] With CER as a substrate.
[b] Expressed as a percentage of CER hydrolysis.

low K_i values. The activity of the SRT-1 enzyme was not inhibited by EDTA, indicating that this enzyme is not a metalloenzyme. The ID_{50} of CVA for the SRT-1 enzyme was 230 μM, indicating low inhibitory activity.

S. marcescens GN16694 produced an unusual β-lactamase. The purified SRT-1 enzyme showed a unique substrate profile, hydrolyzing T-5575 and cephalosporins, including oxyimiocephalosporins (i.e., CXM, cefotaxime, and CAZ), but not carbapenems, penems, and monobactams. Although this enzyme from each resistant strain did not hydrolyze IPM, RIPM, or ATM, the MICs of these agents for the five resistant strains were high. The data suggest that these increased MICs may have been due to a factor other than the enzyme, such as a mutation of the outer membrane or an alteration in affinities to penicillin-binding proteins.

It has been reported previously that β-lactamases produced by *Burkholderia cepacia* (5), *Proteus vulgaris* (6), *Flavobacterium meningosepticum* (4), and *Branhamella catarrhalis* (16) hydrolyzed cephalosporins, including oxime-type cephalosporins, but did not hydrolyze carbapenems, penems, and monobactams. We have also reported that β-lactamases produced by *Stenotrophomonas maltophilia* (9), *Pseudomonas aeruginosa* (7, 13), *Bacteroides fragilis* (17), and *Flavobacterium odoratum* (10) hydrolyze penicillins, cephalosporins (including oxyiminocephalosporins), cephamycins, carbapenems, and penems. It was most characteristic that the SRT-1 enzyme hydrolyzed the novel 2-carboxypenam T-5575 but not carbapenems. The β-lactamases produced by *Stenotrophomonas maltophilia* GN12873 and *P. aeruginosa* GN17023/pMS350 (data not shown) hydrolyzed carbapenems and oxime-type cephalosporins but not T-5575. The TEM enzymes are well inhibited by CVA, with low ID_{50}s (<1 μM) (2). In contrast, the SRT-1 enzyme was little inhibited by CVA, with an ID_{50} of 230 μM. These TEM enzymes are markedly different from the SRT-1 enzyme in inhibition by CVA. We concluded that the SRT-1 enzyme from *S. marcescens* GN16694 should be classified as a group 1 cephalosporinase in the functional classification scheme (1–3). Detailed genetic studies of this enzyme are in progress.

TABLE 2. Patterns of susceptibility to β-lactam antibiotics of T-5575-resistant *S. marcescens* strains

Antibiotic	MIC (μg/ml) for:					
	GN16666	GN16676	GN16678	GN16679	GN16681	GN16694
T-5575	0.006	100	100	100	100	100
CXM	3.13	100	100	100	100	100
Cefmenoxime	0.1	100	100	100	100	100
Ceftriaxone	0.05	100	100	100	100	100
CAZ	0.2	100	100	100	100	100
IPM	0.39	6.25	12.5	12.5	12.5	12.5
ATM	0.2	100	100	100	100	100
RIPM	0.39	100	100	100	100	100

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