# Replacement of Serine 237 in Class A β-Lactamase of *Proteus vulgaris* Modifies Its Unique Substrate Specificity[†,‡]

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ABSTRACT: The chromosomal β-lactamase gene of *Proteus vulgaris* K1 was cloned and sequenced. The gene comprises 813 nucleotides and codes for the mature enzyme of 29 655 Da, comprising 271 amino acids. The K1 β-lactamase showed 30–70% similarity, in the overall amino acid sequence, to class A β-lactamases of Gram-negative bacteria. However, the K1 β-lactamase differs from most class A enzymes in having a unique substrate specificity as a cephalosporinase, its spectrum extending to even oxyiminocephalosporins. To clarify the relationship between its unique substrate specificity and specific amino acid residues, alignment of the amino acid sequence of the K1 β-lactamase with those of class A β-lactamases was performed, and Ala104 and Ser237 were found to be candidates. Ala104 and Ser237 were replaced with glutamic acid and alanine, respectively, which are commonly found in other class A β-lactamases. The substitution at position 104 had no effect on the enzyme activity or the substrate specificity. The amino acid replacement at position 237, however, reduced the $k_{cat}/K_m$ value for an oxyiminocephalosporin (cefuroxime) to 17% of that in the case of the wild-type enzyme, whereas the mutant enzyme showed a higher $k_{cat}/K_m$ value for benzylpenicillin, 3 times, than that of the wild-type enzyme. These results indicated that Ser237 is one of the residues responsible for the unique substrate specificity of the *P. vulgaris* β-lactamase.

Serine β-lactamases (EC 3.5.2.6) are bacterial enzymes that inactivate β-lactam antibiotics and have an active-site serine that participates in the formation of an acyl-enzyme intermediate with a β-lactam (Fisher et al., 1980; Knott-Hunziker et al., 1982). Serine β-lactamases are further classified into three classes, A, C, and D, according to the homology of the primary amino acid sequence (Ambler et al., 1991). According to the traditional grouping of β-lactamases based on substrate specificity, class A and D β-lactamases are penicillinases and the class C enzymes are so-called cephalosporinases. In Gram-negative bacteria, most penicillinases are produced as constitutive enzymes with mediation by genes on plasmids, while all known cehalosporinases are essentially inducible enzymes with mediation by chromosomal genes. Thus, there are sigificant differences in the substrate profile and mode of production between usual class A and class C β-lactamases of Gram-negative bacteria.

*Proteus vulgaris* is well-known to produce an inducible cephalosporinase with a unique substrate specificity, being able to hydrolyze cefuroxime, an oxyiminocephalosporin stable to the usual cephalosporinases. Okuguchi et al. (1986) cloned the chromosomal cephalosporinase gene from *P. vulgaris* 5E78-1 cells and found that the enzyme belongs to class A on the basis of the deduced amino acid sequence. This preliminary result was against the general concept of class A β-lactamases, as mentioned above, and so it seemed interesting to determine the amino acid residues contributing to the unique substrate specificity. We cloned and sequenced the chromosomal cephalosporinase gene from *P. vulgaris* K1, a clinical isolate highly resistant to cephalosporins as well as penicillins. The deduced amino acid sequence of the K1 enzyme supported the observation by Okuguchi et al. (1986), and we tried to assign candidates for the amino acids contributing to the unique substrate specificity through sequence alignment of the *P. vulgaris* enzyme and nine known β-lactamases, including six class A and three class C β-lactamases. The alanine at position ABL-104 and the serine at position ABL-237 in the K1 enzyme were selected as candidates, and the possibility was investigated by means of site-directed mutagenesis.

Here we report the nucleotide sequence of the K1 β-lactamase gene, the deduced amino acid sequence, and the results of the new approach for understanding the unique substrate specificity.

## MATERIALS AND METHODS

*Materials.* *P. vulgaris* K1 is a clinical isolate producing a chromosomal cephalosporinase with a broad substrate profile. *Escherichia coli* TG1 (Carter et al., 1985), a derivative of K12, was employed for DNA technology. *E. coli* AS226-51 (Tsukamoto et al., 1990), an *ampD* mutant of C600, which also has a deletion mutation in *ampC*, was used for measuring the β-lactam susceptibility of cells bearing the cloned β-lactamase gene, and as host cells for enzyme preparation in order to avoid contamination by the *ampC* β-lactamase of *E. coli*. *E. coli* CJ236 (Kunkel, 1985) was used for preparation of single-strand DNA, and *E. coli* strains BMH71-18 (Kunkel, 1985) and MV1184 (Vieira & Messing, 1987) were used as recipients for transfection in site-directed mutagenesis.

Plasmid pPVCF1 is a derivative of pHSG398 into which the cloned β-lactamase gene from the K1 strain was inserted parallel with the *lacZ* direction. pHSG398 carrying the mutant enzyme genes is termed pPVCF-A104E and pPVCF-S237A, respectively. The mutant genes were named using a one-letter amino acid code, i.e., A104E means the mutant gene in which Ala104 was changed to Glu. M13mp18 (Yanisch-Perron et al., 1985) was used as the vector for site-directed mutagenesis and DNA sequencing, and the vector carrying the wild-type β-lactamase gene antiparallel with the *lacZ* direction is termed M13m18-PVCR1.

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Nutrient broth (Eiken Chemical Co., Tokyo, Japan) was used as the culture medium for chromosomal DNA preparation. For transformation and transfection, 2× yeast extract/tryptone (2× YT) broth and YT agar (Miller, 1972) were employed, respectively. For β-lactamase preparation, bacteria were grown in heart infusion broth (Eiken Chemical Co., Tokyo, Japan). Heart infusion agar (Eiken Chemical Co., Tokyo, Japan) was used for measuring bacterial susceptibility to β-lactams.

Enzymes and enzyme kits for DNA technology were purchased from Stratagene, La Jolla, CA; Takara Shuzo Co., Kyoto, Japan; Toyobo Co., Osaka, Japan; and Wako Junyaku Co., Tokyo, Japan. [α-$^{32}$P]dCTP was purchased from Amersham Inc., Buckinghamshire, U.K. The antibiotics used in this study were kindly provided by the following pharmaceutical companies: benzylpenicillin and ampicillin, Meiji Seika Kaisha Ltd., Tokyo, Japan; cephalothin and cephaloridine, Shionogi & Co., Ltd., Osaka, Japan; cefuroxime and ceftazidime, Nippon Glaxo Ltd., Tokyo, Japan; aztreonam, Eisai Co., Tokyo, Japan; clavulanic acid, SmithKline Beecham Seiyaku, Tokyo, Japan; sulbactam, Pfizer Seiyaku, Tokyo, Japan; and chloramphenicol, Yamanouchi Pharmaceutical Co., Tokyo, Japan.

*Cloning of the β-Lactamase Gene and DNA Sequencing.* A 1.26-kb DNA fragment, containing the β-lactamase gene, was cloned from the chromosomal DNA fraction of *P. vulgaris* K1 by the polymerase chain reaction (PCR) method (Saiki et al., 1985; Lawyer et al., 1989). PCR primers were designed with reference to the nucleotide sequence of the *P. vulgaris* 5E78-1 β-lactamase gene (Okuguchi et al., 1986), and the primers were as follows: primer 1, 5′-TTGCTG AAGCTTCAAATGCACGCAG-3′; and primer 2, 5′-CGG AAGCTTTTTTATTACAGTCTAA-3′. The fifth nucleotide from the 5′ end of primer 2 was changed from G to A in order to make a *Hin*dIII site, the cleavage sites in both primers being underlined. PCR was performed using a MiniCycler (MJ Research Inc.) in a 100-μL reaction mixture comprising 3.26 μg of template DNA and 2.0 nmol each deoxynucleotide and PCR buffer. The reaction was initiated with 5 units of *Taq* polymerase and with 20 cycles of 80 s at 93 °C, 80 s from 52 to 48 °C, downing 0.2 °C at a time, and 180 s at 72 °C, and then 15 cycles of 80 s at 93 °C, 80 s at 48 °C, and 180 s at 72 °C. The amplified DNA fragments were digested with *Hin*dIII and then ligated into the *Hin*dIII site of pHSG398. The recombinant plasmid DNAs were transformed into *E. coli* AS226-51, and the positive transformants were selected as to cephalothin resistance and β-lactamase activity.

The 1.26-kb DNA fragment was inserted antiparallel with the *lacZ* direction into the *Hin*dIII site of the vector, M13mp18, and then sequenced by the dideoxy chain-termination method (Sanger et al., 1977) using a Bca BEST dideoxy sequencing kit (Takara Shuzo Co.).

*Site-Directed Mutagenesis.* Two 25-mer oligonucleotides were synthesized using a Cyclone Plus DNA/RNA synthesizer (Milligen Biosearch Co.); see Table 3. In addition to mismatches for the amino acid replacement, the primer for mutation at ABL104 contained silent DNA mismatches in order to eliminate a restriction site, and the primer for mutation at ABL237 contained silent mismatches to create a new restriction site. Mutagenesis was performed by the method of Kunkel (1985) using M13mp18-PVCR1 as a template. The mutant genes were entirely sequenced by the chain-termination method using a specific oligonucleotide primer to confirm the desired exchange in the nucleotide sequence, and then the mutant gene was inserted into the *Hin*dIII site of pHSG398.

*β-Lactamase Purification and Assay. E. coli* AS226-51 cells carrying the wild-type or a mutant β-lactamase gene were grown overnight in heart infusion broth containing a sublethal concentration of chloramphenicol (30 μg/mL) at 37 °C. The preculture was then diluted with a 40-fold volume of fresh medium, followed by growth at the same temperature under aeration until the midlogarithmic phase. Crude β-lactamase was prepared by disruption of the cells with a French press in 50 mM sodium phosphate buffer, pH 7.0, followed by centrifugation for 1 h at 40000*g* and 4 °C after removal of cell debris. The crude enzyme was further purified to homogeneity by ion-exchange chromatography on a SP-Sephadex C-50 column in 10 mM sodium malonate buffer, pH 5.5, with a 0–0.6 M linear NaCl gradient, followed by gel filtration on a Sephadex G-75 column in 100 mM sodium phosphate buffer, pH 6.0; its purity was confirmed by SDS–PAGE. β-Lactamase activity was assayed by the microiodometric method (Novick, 1962), with slight modifications, and a UV spectrophotometric method (Yamaguchi et al., 1983). One unit of enzyme was defined as the amount of enzyme which hydrolyzed 1 μmol of substrate in 1 min at pH 7.0 and 30 °C. The kinetic parameters, $K_m$ and $K_i$, were determined by procedures reported previously (Yamaguchi et al., 1983).

*Isoelectric Focusing.* Isoelectric focusing was carried out with an Atto Model SJ-1071 apparatus (Atto Co., Tokyo, Japan) and a gel plate containing 5% Ampholine (pH 3.5–9.5). The enzyme protein on the gel plate was detected by staining with Coomassie brilliant blue.

*Antibiotic Susceptibility Testing.* Bacterial susceptibility to β-lactams was measured by the serial agar dilution method (Sawai et al., 1981), the susceptibility being expressed as the minimum inhibitory concentration (micrograms per milliliter) of a drug.

*Nucleotide Sequence Accession Number.* The nucleotide sequence data reported in this paper will appear in the GSDB, DDBJ, EMBL, and NCBL nucleotide sequence databases under accession number D29982.

## RESULTS

*Properties of P. vulgaris K1 β-Lactamase. P. vulgaris* K1 exhibits high ability to produce a cephalosporinase, and its specific enzyme activity per milligram of bacterial protein increases significantly in the presence of sublethal concentrations of β-lactams such as benzylpenicillin and cefoxitin, being 135 times that in the absence of the inducer. In order to identify the inducible enzyme as the species-specific β-lactamase of *P. vulgaris*, the enzymic properties of the purified K1 β-lactamase was examined and compared with those of other β-lactamases. The relative $V_{max}$ values and $K_m$ and $K_i$ values of the β-lactamases for cephalothin, benzylpenicillin, and cefuroxime are summarized in Table 1. Cephalothin and benzylpenicillin are well-known as favorable substrates for cephalosporinases (CSases) and penicillinases (PCases), respectively. The kinetic behavior of the K1 β-lactamase on interaction with cephalothin and benzylpenicillin is consistent with that of other two cephalosporinases belonging to class C. However, the β-lactamases, except for the K1 enzyme, hardly hydrolyze cefuroxime, a cephalosporin with an oxime group in the side chain at the 7-position of the cephalosporin nucleus. These characteristics of the K1 β-lactamase are in agreement with those of the *P. vulgaris* β-lactamase (Sawai et al., 1982). The isoelectric point of the K1 β-lactamase was determined to be 7.40.

Table 1: Comparison of the Kinetic Constants of *P. vulgaris* β-Lactamase with Those of Other β-Lactamases[a]

| | | BP | | | CET | | | CXM | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| β-lactamase | type | $V_{max}$ (%) | $K_m$ (μM) | $K_i$ (μM) | $V_{max}$ (%) | $K_m$ (μM) | $K_i$ (μM) | $V_{max}$ (%) | $K_m$ (μM) | $K_i$ (μM) | reference |
| TEM-1 | PCase | 100 | 36 | | 17 | 677 | | <2 | | >100 | Delaire et al., 1992; this paper |
| *P. mirabilis* | PCase | 100 | 11 | | <2 | | 100 | <2 | | >100 | Takahashi et al., 1983; this paper |
| *P. vulgaris* | CSase | 14 | | 4 | 100 | 22 | | 276 | 159 | | this paper |
| *C. freundii* | CSase | 9 | 5 | | 100 | 19 | | 0.03 | 0.03 | | this paper |
| *E. cloacae* | CSase | 7 | | 0.6 | 100 | 9 | | 0.03 | | 0.02 | Galleni et al., 1988a; Galleni & Frére, 1988 |

[a] The relative $V_{max}$ is expressed as a percentage of benzylpenicillin hydrolysis in the case of penicillinase and of cephalothin hydrolysis in the case of cephalosporinase. The abbreviations are as follows: BP, benzylpenicillin; CET, cephalothin; CXM, cefuroxime; PCase, penicillinase; CSase, cephalosporinase.

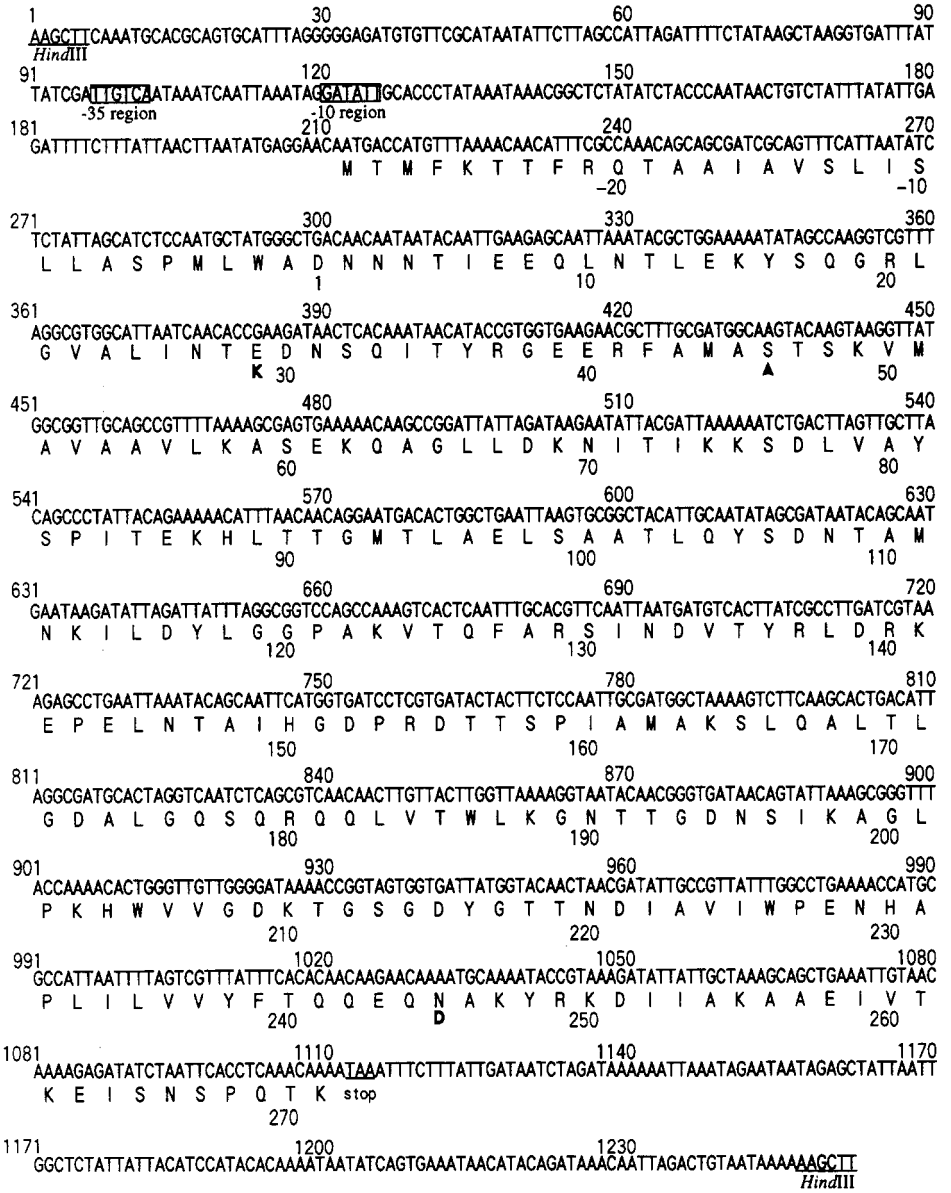


FIGURE 1: DNA sequence of the *P. vulgaris* K-1 β-lactamase gene and flanking regions and the predicted amino acid sequence for the enzyme. The nucleotides are numbered from the *Hind*III site. The −35 and −10 regions are boxed. The position of the N-terminal amino acid of the mature enzyme is designated as position 1 of the amino acid sequence. The amino acid sequence from −29 to −1 is assumed to be the signal peptide. The active-site serine at position 46 is indicated by an arrowhead. The amino acid sequence of the mature enzyme differs from that of the *P. vulgaris* 5E78-1 β-lactamase (Okuguchi et al., 1986) at positions 29 and 245, and the corresponding amino acids are shown in boldface type under the sequence.

*DNA Sequence Determination.* The *Hind*III fragment, with a molecular size of 1.25 kb, on pHSG398 was transferred into *E. coli* AS226-51. The crude cell extract of the transformant showed higher β-lactamase activity per bacterial protein than that of the *P. vulgaris* K1, i.e., about 35 times that found in K1 cells fully induced by an inducer (benzylpenicillin). This high specific activity in the *E. coli* cells may be attributed to the gene dose effect of the multicopy plasmid.

The 1252-bp *Hind*III fragment was completely sequenced, as shown in Figure 1. The sequenced region was found to contain two possible open reading frames starting from nucleotide positions 212 and 218, respectively. Two possible

Shine–Dalgarno sequences, GAGG and AGGA, were located at nucleotide positions 204–207 and 205–208, respectively. The putative −35 and −10 regions are indicated in Figure 1. The TAA terminal codon was found to be located at nucleotide positions 1112–1114. The N-terminal amino acid sequence of the mature enzyme was determined using an Applied Biosystems gas-phase sequencer (Applied Biosystems, Foster City, CA) to be $NH_2$-DNNNTIEEQLNT, coinciding with nucleotide positions 299–334. The mature enzyme is composed of 271 amino acids and has a molecular mass of 29 655 Da.

There are several conserved amino acids or amino acid sequences commonly found in serine β-lactamases and penicillin-binding proteins (Joris et al., 1988). A Ser-X-X-Lys tetrad characteristic of the active-site region was found at amino acid positions 46–49, Ser-Thr-Ser-Lys, Ser46 being the active-site serine. A KTG/KSG triad and a SDN sequence are common in serine β-lactamases and were found at positions 210–212 and 106–108, respectively. A glutamic acid (ABL166) essential for the deacylation reaction (Adachi et al., 1991) is presumed to be Glu142. When the deduced amino acid sequence is compared with that of the *P. vulgaris* 5E78-1 β-lactamase, they differ from one another only in two amino acid residues, i.e., Glu29 and Asn245. In the 5E78-1 β-lactamase, these residues are Lys29 and Asp245, respectively. The K1 β-lactamase showed 30–70% similarity in the amino acid sequence to other class A β-lactamases examined, with especially high similarity (70%) to the β-lactamase of *Klebsiella oxytoca* (Arakawa et al., 1989). On the other hand, low similarity of less than 1% was found between the K1 β-lactamase and class C β-lactamases. These results confirmed the earlier conclusion of Okuguchi et al. (1986) that the chromosomal β-lactamase of *P. vulgaris* is a class A β-lactamase.

*Substitution of Ala104 with Glutamine and Ser237 with Alanine.* The substrate spectra of plasmid-mediated class A β-lactamases such as TEM and SHV-type β-lactamases are knwon to extend to oxyimino β-lactams on substitution of a few amino acids at specific positions (Philippon et al., 1989; Jacoby & Medeiros, 1991). The purpose of this study was to determine the amino acid residues in the *P. vulgaris* β-lactamase contributing to the hydrolysis of oxyimino β-lactams. We performed alignment of the sequence of the K1 β-lactamase with six class A and three class C β-lactamases and found significant differences in the residues at positions Ala104 and Ser237 (Table 2). The class A β-lactamases produced by Gram-negative bacteria, except the *P. vulgaris* enzyme, have an acidic amino acid or threonine at position 104 and alanine at position 237. It should be noted that position 237 is located just after the KTG/KSG triad. The conserved triad constitutes the active-site structure and the lysine is known to be a functional amino acid in the enzyme reaction (Lamotte-Brasseur et al., 1991; Moews et al., 1990; Strynadka et al., 1992).

On the basis of the assumption that Ala104 and Ser237 contribute to the unique substrate specificity of the *P. vulgaris* β-lactamase, Ala104 and Ser237 were replaced with TEM-type residues, i.e., glutamic acid and alanine, respectively. The oligonucleotides used for the preparation of the mutant genes are shown in Table 3. Plasmids carrying the mutant genes, A104E and S237A, were introduced into *E. coli* A226-51 cells. The tranformants were examined as to their β-lactamase activity and susceptibility to two pencillins and three cephalosporins, including an oxyiminocephalosporin, cefuroxime (Table 4). The extracts of the cells carrying the A104E and S237A mutant genes showed 105% and 58% of the activity of the wild-type enzyme with cephalothin as the substrate, respectively. These specific activities per bacterial protein may be closely related to the molecular activities of the enzymes, for the gene expression in *E. coli* cells was not affected by site-directed mutagenesis (Tsukamoto et al., 1990). These results indicated that Ala104 and Ser237 are not the functional amino acids essential for the enzymic catalysis. The cells carrying the A104E mutant gene showed about the same levels of resistance to the β-lactams as the cells producing the wild-type enzyme. On the other hand, the drug-resistance level in the case of cefuroxime was significantly reduced on the substitution at position 237 and this result could not be explained only by the decrease in the specific enzyme activity.

Table 2: Amino Acid Sequence Around Residues ABL-104 and ABL-237 in β-Lactamases[a]

| β-Lactamase | Class | Amino acid sequence: ABL-104 residue | Amino acid sequence: ABL-237 residue |
|---|---|---|---|
| TEM-1 | A | NDL...VEYSPVT | ADKSGAGE.RG |
| SHV-1 | A | QDL...VDYSPVS | ADKTGAGE.RG |
| PSE-4 | A | ADL...VTYSPVI | ADRSGAGG.FG |
| *K.oxytoca* E23004 | A | SDL...VVWSPIT | GDKTGAGD.YG |
| *P.vulgaris* K-1 | A | SDL...VAYSPIT | GDKTGSGD.YG |
| *P.vulgaris* 5E78-1 | A | SDL...VAYSPIT | GDKTGSGD.YG |
| *S.aureus* PC-1 | A | DDI...VAYSPIL | ADKSGQAITYA |
| *E.coli* K12 | C | PELTAKQWNGI.. | VHKTGAT..GG |
| *C.freundii* GN346 | C | PELTGKQWRGI.. | VHKTGST..GG |
| *E.cloacae* P99 | C | PQLTGKQWQGI.. | VHKTGST..GG |

[a] The alignment of the sequences of the *P. vulgaris* β-lactamases with six class A and three class C enzymes was performed with the aid of a computer program (PILE UP software in the software package of Genetics Computer Group Inc.). The amino acids at ABL-104 and 237 are underlined. The references for the amino acid sequences are as follows: TEM-1 (Sutcliffe, 1978), SHV-1 (Barthelemy et al., 1988), PSE-4 (Boissinot & Levesque, 1990), *K. oxytoca* E23004 (Arakawa et al., 1989), *P. vulgaris* K-1 (this paper), *P. vulgaris* 5E78-1 (Okuguchi et al., 1986), *S. aureus* PC-1 (Chan, 1986), *E. coli* K12 (Jaurin & Grundström, 1981), *C. freundii* GN346 (Tsukamoto et al., 1990), and *E. cloacae* P99 (Galleni et al., 1988b).

*Purification and Kinetic Properties of the A104E and S237A Mutant β-Lactamases.* Prior to the enzyme purification, the relative $V_{max}$ and $K_m$ values of the crude enzyme preparations as to cephalothin, cephaloridine, cefuroxime, benzylpenicillin, and ampicillin were measured. There were no significant differences in these kinetic properties between the wild-type β-lactamase and the A104E mutant β-lactamase, while the S237A mutant enzyme showed a significantly decreased relative $V_{max}$ for cefuroxime and increased ones for benzylpenicillin and ampicillin.

To evaluate the effect of the substitution at position 237 on kinetic properties, both the wild-type β-lactamase and the S237A mutant β-lactamase were purified to homogeneity. The isoelectric point of the S237A β-lactamase was estimated to be 7.40, i.e., identical with that of the wild-type enzyme. However, the optimum pH of the S237A mutant with cephalothin as the substrate was 6.21, which was somewhat lower than that of the wild-type enzyme, 7.40. The catalytic activity and affinity for substrates or inhibitors were examined as to seven β-lactams, i.e., two traditional cephalosporins (cephalothin and cephaloridine), two traditional penicillins (benzylpenicillin and ampicillin), and three oxyimino β-lactams (cefuroxime, ceftadizime, and aztreonam), and two β-lactamase inhibitors (clavulanic acid and sulbactam). The

Table 3: Mutagenic Primers Used for Site-Directed Mutagenesis and the Amino Acids Changed in the Mutant Enzymes[a]

| Plasmid | Primer sequence | Substitution | | |
|---|---|---|---|---|
| | | ABL No. | Codon | Amino acid |
| pHSG398 | * ** | | | |
| -A104E | 5'-TCTGATTTAGTTGAATACAGCCCTA-3' | 104 | GCT → GAA | Ala → Glu |
| | *DdeI* | | | |
| pHSG398 | **** | | | |
| -S237A | 5'-AAAACCGGCGCCGGTGATTATGGTA-3' | 237 | AGT → GCC | Ser → Ala |
| | *NarI* | | | |

[a] Asterisks indicate the mismatches. Underlining indicates the restriction site eliminated (in the case of A104E) or created (in the case of S237A).

Table 4: β-Lactamase Activity of *E. coli* AS226-51 Strains Carrying the Wild and Mutant Genes and the Levels of Resistance to β-Lactams[a]

| strain | β-lactamase activity (units/mg of protein) | MIC (μg/mL) | | | | |
|---|---|---|---|---|---|---|
| | | BP | APC | CET | CER | CXM |
| AS226-51/pPVCF-1(wild) | 49.7 | >1600 | >1600 | 1600 | 200 | 1600 |
| AS226-51/pPVCF-A104E | 52.3 | 1600 | 1600 | 1600 | 200 | 800 |
| AS226-51/pPVCF-S237A | 28.7 | >1600 | >1600 | 400 | 200 | 100 |
| AS226-51 | $<10^{-5}$ | 6.3 | 6.3 | >1.6 | >1.6 | >1.6 |

[a] β-Lactamase activity was measured using cell extracts of *E. coli* AS226-51 cells harboring the wild-type or a mutant gene. The enzyme assay was performed by the microiodometric method with 200 μM cephalothin. The β-lactams used were as follows: BP, benzylpenicillin; APC, ampicillin; CET, cephalothin; CER, cephaloridine; CXM, cefuroxime.

Table 5: Kinetic Parameters for β-Lactams of the Wild-Type and S237A Mutant Enzymes[a]

| β-lactam | wild type | | | | S237A | | | |
|---|---|---|---|---|---|---|---|---|
| | $k_{cas}$ ($s^{-1}$) | $K_m$ (μM) | $K_i$ (μM) | $K_{cat}/K_m$ ($s^{-1}$ $\mu M^{-1}$) | $k_{cat}$ ($s^{-1}$) | $K_m$ (μM) | $K_i$ (μM) | $k_{cat}/K_m$ ($s^{-1}$ $\mu M^{-1}$) |
| CET | 262 | 21.5 | | 12.2 | 106 | 19.4 | | 5.46 |
| CER | 231 | 119 | | 1.94 | 310 | 122 | | 2.54 |
| CXM | 570 | 177 | | 3.22 | 151 | 278 | | 0.543 |
| CAZ | | | | 0.00143 | | | | 0.000498 |
| AZT | 1.96 | 8.97 | | 0.219 | 3.52 | 14.6 | | 0.241 |
| BP | 21.3 | 5.33 | | 4.00 | 64.7 | 4.99 | | 13.0 |
| APC | 31.4 | 12.0 | | 2.62 | 200 | 47.4 | | 4.22 |
| CVA | | | 1.80 | | | | 2.37 | |
| SBT | | | 3.87 | | | | 12.8 | |

[a] The $k_{cat}$, $K_m$, and $K_i$ values were determined by a UV spectrophotometric method. In the case of the $K_i$ values, cephalothin was used as the reporter substrate. The β-lactams used were as follows: CET, cephalothin; CER, cephaloridine; CXM, cefuroxime; CAZ, ceftazidime; AZT, aztreonam; BP, benzylpenicillin; APC, ampicillin; CVZ, clavulanic acid; SBT, sulbactam.

results are summarized in Table 5 together with the structures of the β-lactams (Figure 2).

The $k_{cat}/K_m$ value of the S237A mutant enzyme for cephalothin was about half that of the wild-type enzyme. On the other hand, the $k_{cat}/K_m$ values of the mutant enzyme for oxyiminocephalosporins such as ceftazidime and cefuroxime were 3–6-fold lower than those of the wild-type enzyme. The $k_{cat}/K_m$ values for ampicillin and benzylpenicillin were 1.6–3-fold higher than those of the wild-type enzyme. Thus, the substitution of Ser237 in the K1 β-lactamase to a TEM-1-type residue evidently decreased its characteristic action as a cephalosporinase with a broad substrate spectrum. The alteration in the $k_{cat}/K_m$ value is mainly due to the alteration in the $k_{cat}$ value. The K1 β-lactamase showed high susceptibility to the two inhibitors, clavulanic acid and sulbactam, like usual class A β-lactamases, and the substitution at position 237 tended to increase the $K_i$ value, suggesting some alteration in the active-site configuration.

## DISCUSSION

So-called cephalosporinases, a group of β-lactamases preferring cephalosporins as substrates, are found in a large number of Gram-negative bacteria as species-specific β-lactamases (Sawai et al., 1982). These cephalosporinases belong to class C, according to Ambler's classification based on the amino acid sequence (Ambler et al., 1991). The species-specific β-lactamase of *P. vulgaris* has been known to be a cephalosporinase with a broad substrate spectrum since 1968 (Sawai et al., 1968) and is assumed to be a class C β-lactamase from the similarity of its substrate profile to those of known class C β-lactamases. Contrary to this expectation, this study confirmed that the *P. vulgaris* K1 β-lactamase is a class A enzyme showing 30–70% similarity to the known class A enzymes and less than 1% similarity to the known class C enzymes. This is an example of a difference between the classification based on the substrate specificity and that based on the amino acid sequence.

Glu104 is one of the amino acids forming the active-site hollow in the TEM-1 β-lactamase (Sougakoff et al., 1988). The replacement of Glu104 in the TEM-1 enzyme by lysine resulted in a marked increase in the activity toward oxyiminocephalosporins (Sougakoff et al., 1988; Sowek et al., 1991). Sowek et al. (1991) assumed that such a phenomenon is due to the hydrogen-bonding interaction between the N and O

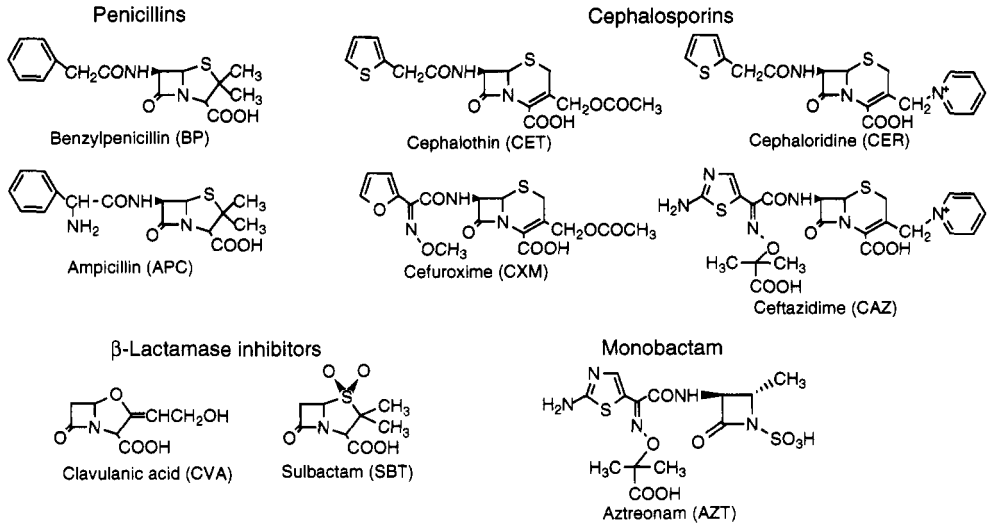


FIGURE 2: Structures of the β-lactams used in this study. Two penicillins, four cephalosporins, including two oxyiminocephalosporins, a monobactam with an oxime group in the side chain, and two β-lactamase inhibitors were used.

atoms of an oxime group and the hydrogens on the N atom of the lysine. As observed in our study, Ala104 in the K1 β-lactamase does not contribute to the hydrolytic activity toward oxyiminocephalosporins, which is consistent with the observations by other workers.

Healey et al. (1989) reported that the substitution of Ala237 in the TEM-1 β-lactamase to threonine or asparagine resulted in an increase in hydrolytic activity toward cephalosporins such as cephalothin and cephalosporin C. In the case of K1 β-lactamase, the $k_{cat}/K_m$ value of the S237A mutant enzyme for cefuroxime was 18% that of the wild-type enzyme, while the mutant enzyme showed 52–340% the $k_{cat}/K_m$ value of the wild-type enzyme for usual cephalosporins and penicillins. These results indicate that Ser237 in the K1 β-lactamase mainly contributes to its ability to hydrolyze oxyimino β-lactams. On the other hand, the S237A mutant still retains higher activity for cephalothin and cephaloridine, favorable substrates for cephalosporinases, than that for benzylpenicillin, the most common substrate for penicillinases. It can therefore be presumed that there are some amino acid residues other than Ser237 which contribute to the cephalosporinase activity of the *P. vulgaris* β-lactamases.

Most class A β-lactamases produced by Gram-negative bacteria have alanine at position 237, and Ala237 is located on the β3 strand of the TEM-1 β-lactamase, being located across the active-site space from Ala104 (Sougakoff et al., 1988). From a three-dimensional model of a TEM-1 β-lactamase complex with benzylpenicillin (Strynadka et al., 1992), the backborn amide NH of Ala237 was assumed to participate in the formation of the oxyanion hole together with the active-site Ser70, and a hydrogen bond between the carbonyl oxygen of Ala237 and the amide NH of the side chain of benzylpenicillin was suggested. The class A β-lactamase of *Staphylococcus aureus* PC1 has glutamine at position 237, and the carbonyl oxygen of Gln237 undergoes an electrostatic interaction with the amino group of the side chain of ampicillin (Herzberg & Moult, 1987). The substitution of Ser237 in the K1 β-lactamase by alanine resulted in an about 4-fold increase in the $K_m$ value for ampicillin, even though the $K_m$ values for other substrates were little affected by the substitution, only changing in the range of 0.8–1.6-fold. These results may suggest the interaction of Ser237 with the amino group of ampicillin.

In previous papers, we reported that the substitution of Glu219 in the loop structure of the class C β-lactamase of *Citrobacter freundii* by tryptophan, lysine, or cysteine broadens its substrate spectrum to oxyiminocephalosporins, and this alteration was confirmed to be due to a significant increase in the deacylating reaction for the acyl-enzyme intermediate (Tsukamoto et al., 1992). The order of increasing cefroxime hydrolysis was approximately proportional to the molecular volume of the amino acid substituted and independent of the ionic character of the amino acid. We assumed that this phenomenon may be due to alteration in the configuration of the enzyme around position 219, which residues at the base of the active-site hollow. On the basis of these observations, it can be presumed that Ser237 in the *P. vulgaris* K1 β-lactamase plays a role in maintaining the active-site hollow in an adequate configuration for the hydrolysis of oxyimino β-lactams.

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