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A survey of a functional amino acid of class C β-lactamase corresponding to Glu[166] of class A β-lactamases

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The class C β-lactamase of *Citrobacter freundii* GN346 is a typical cephalosporinase comprising 361 amino acids. The aspartic acid at position 217 and glutamic acid at position 219 in this β-lactamase were, respectively, previously shown not to be the counterpart of Glu[166] (ABL166) in class A β-lactamases, even though sequence alignment of class A and C enzymes strongly suggested this possibility [(1990) FEBS Lett. 264, 211–214; (1990) J. Bacteriol. 172, 4348–4351]. We tried again to assign candidates for the counterpart of Glu[166] through sequence alignment based on other criteria, the glutamic acids at positions 195 and 205 in the class C β-lactamase being selected. To investigate this possibility, these two glutamic acids were changed to glutamine, lysine or alanine, respectively. All the mutant enzymes showed more than 50% of the activity of the wild-type enzyme, indicating that the possibility was ruled out. These results strongly suggested the possibility that the class C β-lactamase lacks a functional acidic residue corresponding to Glu[166] in class A enzymes.

β-Lactamase; *Citrobacter freundii*; Cephalosporinase; Active site; Glu[166]; Site-directed mutagenesis

1. INTRODUCTION

Penicillin interactive enzymes in bacterial cells are a general term for β-lactamase and penicillin-binding proteins (PBPs). Most of these enzymes, which have a serine residue as the catalytic site, are believed to originate from a common ancestral protein [1]. β-Lactamases play a large part in bacterial resistance to β-lactam antibiotics [2], and the enzymic reaction of serine β-lactamases proceeds through an acyl-enzyme mechanism [3]. Serine β-lactamases are classified based on the homology of their amino acid sequences into three distinct classes, A, C and D [4]. Class A enzymes comprise penicillinases, and are produced by both Gram-positive and -negative bacteria. On the other hand, class C enzymes are so-called cephalosporinases, which are mainly produced by Gram-negative bacteria. Class D enzymes are unique penicillinases with the ability to hydrolyse oxacillin, a semisynthetic penicillin stable to usual β-lactamases [5].

When the primary amino acid sequences are compared, the similarity of the sequences between two enzymes of different classes is less than 1% [6]. Sequence alignment, however, revealed at least seven conserved regions in the three classes of enzymes and PBPs [1]. Several amino acids in the conserved regions were confirmed to be functional residues by means of site-directed mutagenesis. Ser[70], Lys[73], Glu[166] and Lys[234] in class A β-lactamases are the most well known functional residues [7]. On the basis of the crystal structure of the class A β-lactamase of *Staphylococcus aureus* PC1, Herzberg and Moult [8] suggested a salt-bridge interaction between the conserved Lys[73] and Glu[166] in this class A enzyme, and the significant role of Glu[166] in the catalytic reaction was confirmed by Madgwick and Waley [9]. Glu[166] in the class A enzyme of RTEM-1 was reported to be essential for the deacylation process [10]. A counterpart of Glu[166] was also found in PBP 2 of *Escherichia coli* by means of site-directed mutagenesis, i.e. aspartic acid at position 447 [11].

The class C β-lactamase of *Citrobacter freundii* GN346 is a typical cephalosporinase comprising 361 amino acids [12]. Ser[64] and two lysines (Lys[67] and Lys[315]) in this class C enzyme were confirmed to be the counterparts of Ser[70], Lys[73] and Lys[234] in the class A enzymes, respectively [12–14]. However, a counterpart of Glu[166] has not yet been identified in a class C enzyme. On the basis of the comparative method proposed by Joris et al. [1], Asp[217] in the class C β-lactamases was assumed to be a candidate for the counterpart of Glu[166], but this assumption and another possibility of Glu[219] being the counterpart were ruled out by site-directed mutagenesis [15,16]. These observations result in the interesting speculation that class C β-lactamases have no acidic amino acid corresponding to Glu[166]. This

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study was carried out to survey the acidic amino acid by means of sequence alignment based on new criteria.

2. MATERIALS AND METHODS

2.1. E. coli *strains and plasmids*

E. coli TG1 [17], a derivative of K12, was employed for DNA technology. *E. coli* AS226-51 [12], an *ampD* mutant of C600, which also has a deletion mutation in *ampC*, was used for measuring the antibiotic susceptibility of cells bearing the β-lactamase gene, and as host cells for enzyme preparation in order to avoid contamination by the ampC β-lactamase of *E. coli*. Plasmid pCFC-1 is a derivative of pHSG398 into which a 1.8 kb *Bam*HI–*Pst*I fragment carrying the wild-type β-lactamase gene from *C. freundii* GN346 has been inserted [12]. pHSG398 carrying the mutant cephalosporinase gene is called pCFC-E195Q. The mutant genes were named using a one-letter amino acid code, i.e. E195Q means a mutant gene in which Glu^{195} was changed to glutamine. M13mp18 [18] was used as the vector for mutagenesis.

2.2. *Media, chemicals and enzymes*

For the transformation and transfection experiments, 2 × yeast-extract/tryptone (2 × YT) broth [19] and yeast-extract/tryptone (YT) agar [19] were employed. Heart infusion agar (Eiken Chemical Co., Tokyo, Japan) was used for measuring the bacterial susceptibility to antibiotics. For enzyme preparation, bacteria were grown in 2 × YT broth. The β-lactam antibiotics used in this study were kindly provided by the following pharmaceutical companies: cephalothin, Shionogi Pharmaceutical Co., Osaka, Japan; and cefuroxime, Nihon Glaxo Co., Tokyo, Japan. [α-^{32}P]dCTP and an in vitro mutagenesis kit were purchased from Amersham Inc., Buckinghamshire, UK. An M13 sequencing kit and enzymes for DNA technology were purchased from: Boehringer-Mannheim GmbH, Mannheim, Germany; Takara Shuzo Co., Kyoto, Japan; and Nippon Gene Co., Toyama, Japan.

2.3. *Oligonucleotides and site-directed mutagenesis*

Four oligonucleotides, 21- to 23-mers, were synthesized using a Cyclone Plus DNA/RNA Synthesizer (Milligen, Biosearch Co.). The mutagenic oligonucleotides are listed in Table I. Site-directed mutagenesis was performed by the method of Eckstein [20]. The mutant genes were sequenced to confirm the desired exchange in the nucleotide sequence by the chain-termination method [21] using a specific oligonucleotide primer.

2.4. β-*Lactamase purification and* β-*lactamase assay*

E. coli AS226-51 cells carrying the wild or a mutant β-lactamase gene were grown overnight in 2 × YT medium containing sublethal concentrations of chloramphenicol (30 μg/ml) and cephalothin (50 μg/ml) at 37°C. The addition of these antibiotics for the preculture was desirable because the vector plasmid was relatively unstable in the host cells. The preculture was then diluted with a 40-fold volume of fresh medium, followed by growth at the same temperature under aeration until the middle of the logarithmic phase. Crude β-lactamase was prepared by sonic disruption of the bacterial cells, followed by centrifugation for 20 min at 14,000 × *g* and 4°C, and the supernatant was used for the enzyme assay. β-Lactamase activity was assayed with 200 μM substrate by means of the microiodometric method [22] with slight modifications. One unit of enzyme was defined as the amount of enzyme which hydrolyzed 1 μmol of substrate per minute at pH 7.0 and 30°C.

2.5. *Antibiotic susceptibility testing*

The bacterial susceptibility to antibiotics was measured by the serial agar dilution method, according to the procedure described previously [2]. The susceptibility was expressed as the minimum inhibitory concentration of a drug (μg/ml).

3. RESULTS

3.1. *Possible candidates for the counterpart of* Glu^{166}

We reexamined the alignment of the sequence of the *C. freundii* GN346 β-lactamase with those of 16 class A β-lactamases and 5 class C β-lactamases with the aid of a computer program (PILE UP and LINE UP software in the software package of Genetics Computer Group Inc.). The information, derived through crystallographic studies, on the secondary and tertiary structures of the class A enzyme of *S. aureus* PC1 [23] and the class C enzyme of *C. freundii* OS60 [24] were utilized for superpositioning of these two enzymes. The amino acid sequence of the *C. freundii* GN346 enzyme differs from that of the OS60 enzyme in only nine of 361 amino acids [12]. The β-lactamases subjected to the alignment, other than the enzymes of PC-1 and OS60, were as follows: TEM-1, SHV-1, SHV-2, OHIO-1, PSE-4, LEN-1, *Klebsiella pneumoniae* E23004, *Bacillus cereus* 5/B type i, *B. cereus* 569/H9 type i, *B. cereus* 569/H type iii, *B. licheniformis* 749/C, *Rhodobacter capsulatus* SP108, *Streptomyces cacaoi* DSM40057, *S. albus* G, *S. aureofaciens*, *Enterobacter cloacae* P99, *E. cloacae* MNH1, *E. coli* K12 and *Serratia marcescens* SR50. The multiple sequence alignment was carried out stepwise; the alignment between enzymes of the same class was mainly made on the basis of the optimum-matching of the amino acid sequences, and that between enzymes of different classes was carried out based on the correspondence of their secondary and tertiary structures in

Table I

Sequences of the oligonucleotides used for site-directed mutagenesis and the codons changed in the mutant plasmids

Plasmid	Oligonucleotide primer	Substitution	
		Amino acid	Codon
pCFC-E195Q	5′-AGTTTTTTTGTTCGGCTTTGCGG-3′	Glu → Gln	GAA → CAA
pCFC-E195K	5′-AGTTTTTTTGTTCTGCTTTGCGG-3′	Glu → Lys	GAA → AAA
pCFC-E195A	5′-AGTTTTTTTGGGCGCTTTGCGC-3′	Glu → Ala	GAA → GCC
pCFC-E205Q	5′-AGGCTTCCCTTGGCGATAGC-3′	Glu → Gln	GAA → CAA
pCFC-E205K	5′-AGGCTTCCCTTTGCGATAGC-3′	Glu → Lys	GAA → AAA
pCFC-E205A	5′-AGGCTTCCCGGCGCGATAGCC-3′	Glu → Ala	GAA → GCC

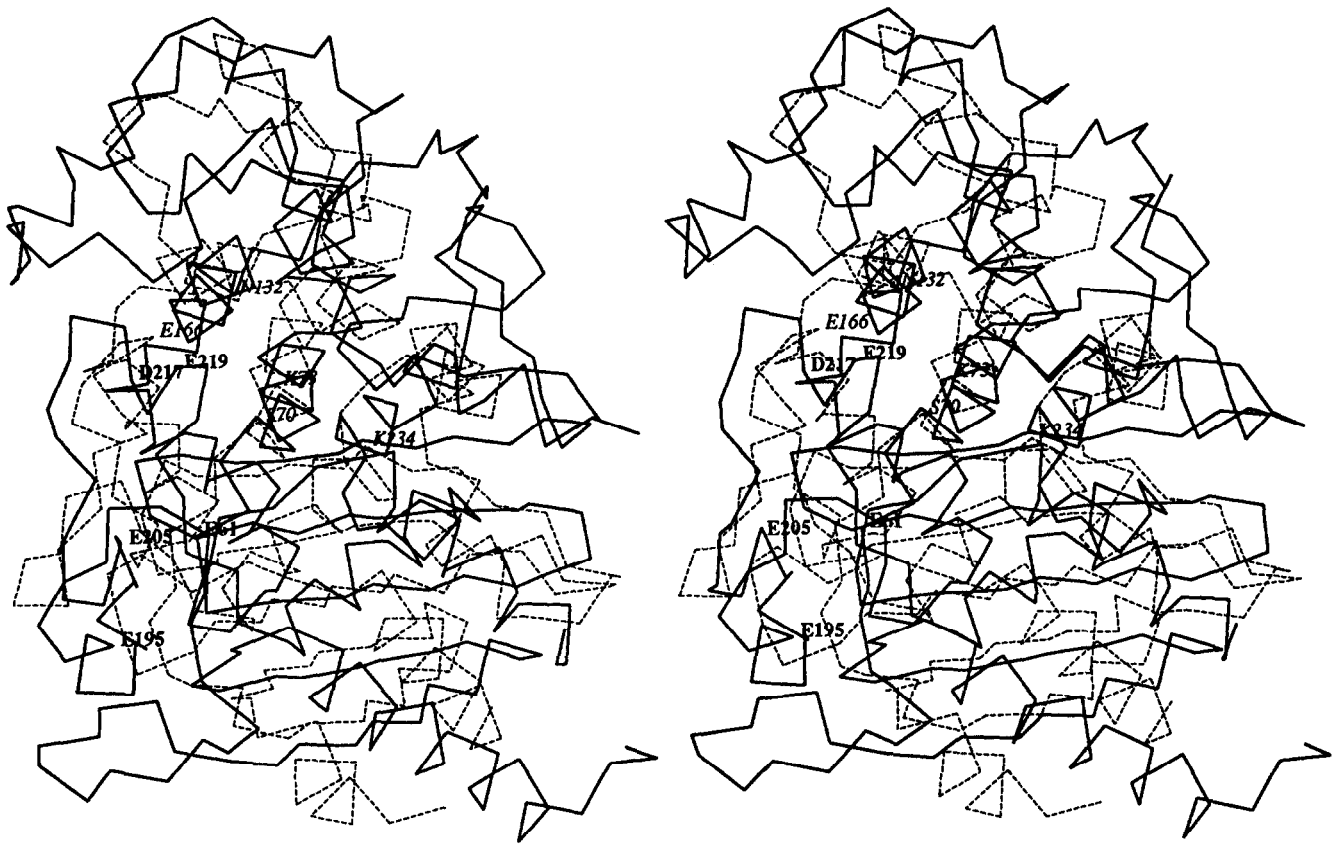


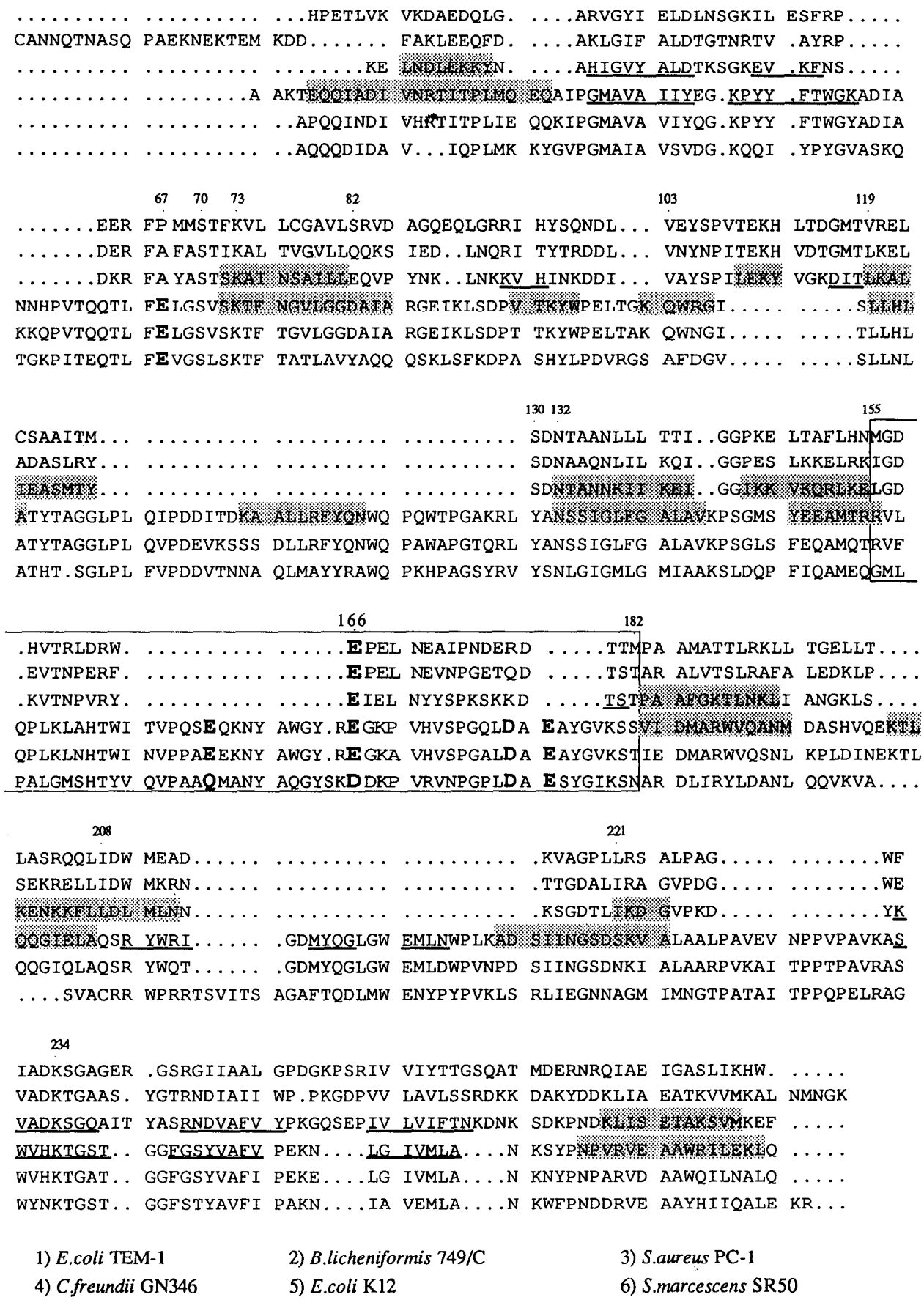
Fig. 1. α-Carbon backbone comparison of the *S. aureus* PC1 class A β-lactamase (dotted line) and the *C. freundii* OS60 class C β-lactamase (solid line). Ser[70], Lys[73], Asn[132] and Lys[234] of the class A enzyme were superimposed upon Ser[64], Lys[67], Asn[152] and Lys[315] of the class C enzyme at the Cα position, respectively. The positions of the respective amino acids are denoted by italic (class A) or bold characters (class C).

addition to their sequence homology. Fig. 1 shows the three-dimensional structures of the PC-1 and OS60 enzymes, in which the α-carbons of the functional amino acids of the PC-1 enzyme, such as Ser[70], Lys[73], Asn[132] and Lys[234], were superimposed on those of the counterparts in OS60, i.e. Ser[64], Lys[67], Asn[152] and Lys[315], by means of root-mean square fitting.

The obtained alignment between the *C. freundii* enzyme and five representative β-lactamases is shown in Fig. 2. The amino acids were numbered according to the ABL numbering proposed by Ambler et al. [25]. This sequence alignment suggested that Glu[166] (ABL166) in class A β-lactamases corresponds to Glu[205] in the *C. freundii* enzyme. Fig. 3 shows the amino acids surrounding the active-site serine in the PC-1 and OS60 enzymes, the presence of three acidic residues, Glu[61], Asp[217] and Glu[219], within 10 Å distance from Ser[64] of the class C enzyme being indicated. Asp[217] is the closest to the serine. The loop containing Asp[217] in the class C enzyme extends in the opposite direction compared with the corresponding loop (omega loop) in the *S. aureus* PC1 enzyme. However, the omega loop was suggested by Herzberg to exhibit high flexibility [23], and it can be assumed that the Asp[217] loop is in the same situation as that of the corresponding loop of a class A enzyme in a physiological environment. This assumption makes it possible for Glu[205] or Glu[195] in the class C enzyme to be the real counterpart of Glu[166]. It should be emphasized that this sequence alignment indicates an appropriate position for a conserved proline (ABL174) found in the two enzymes.

3.2. *Substitution of Glu[195] and Glu[205] with glutamine, lysine or alanine, and properties of the mutant enzymes*

On the assumptions described above, site-directed mutagenesis was performed as to the glutamic acids at positions 195 and 205 in the class C enzyme from *C. freundii* GN346. Mutant genes prepared by the Eckstein method were constructed on pSHG398. Plasmids carrying the mutant genes, E195Q, E195K, E195A, E205Q, E205K and E205A, were introduced into *E. coli* AS226-51 cells. These bacterial cells were examined as to their susceptibility to cephalothin and their β-lactamase activity. All the cells carrying the mutant genes showed the same level of resistance to cephalothin as that of the cells with the wild-type enzyme. The β-lactamase activity per bacterial protein was compared with cells having



1) *E.coli* TEM-1
2) *B.licheniformis* 749/C
3) *S.aureus* PC-1
4) *C.freundii* GN346
5) *E.coli* K12
6) *S.marcescens* SR50

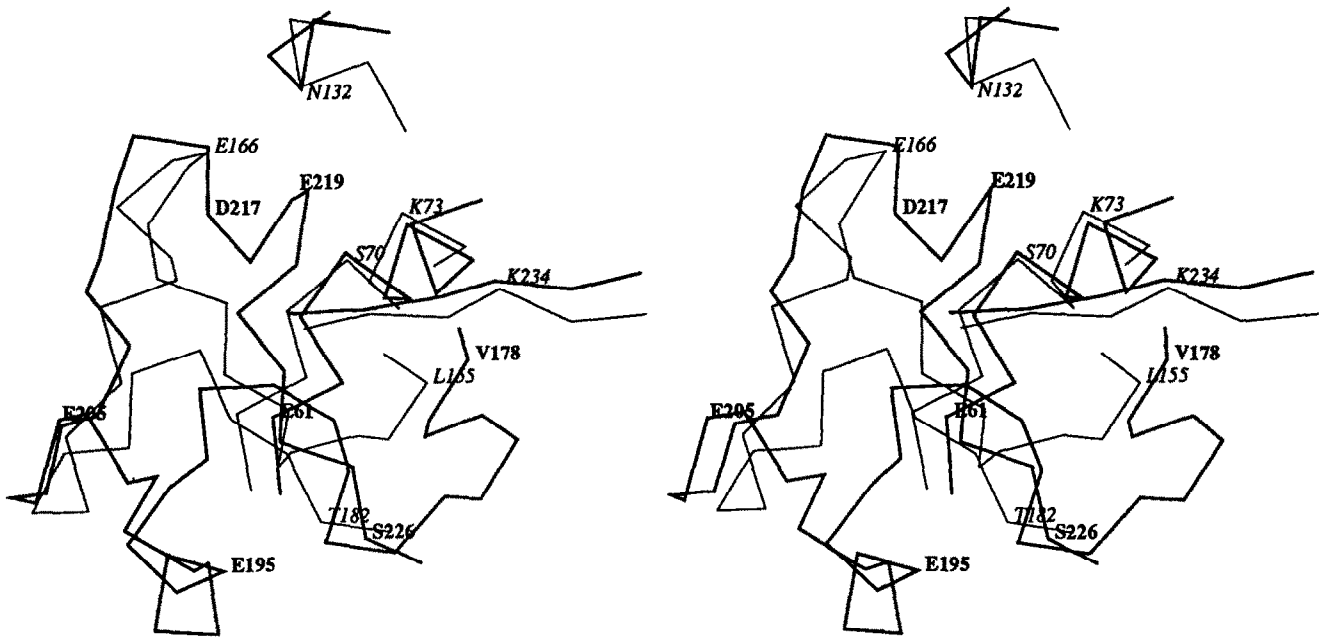


Fig. 3. Comparison of polypeptide segments near the active-site serine between the *S. aureus* PC1 and *C. freundii* OS60 β-lactamases. The α-carbon backbones are superimposed as described in Fig. 1, and denoted by a thin line (class A) and a thick line (class C).

the wild-type gene using a favourable substrate, cephalothin. As summarized in Table II, all the mutant enzymes retained more than 50% of the cephalothin hydrolysis activity of the wild- type enzyme, strongly suggesting that the glutamic acids at positions 195 and 205 in the class C β-lactamase are of no functional importance in the enzyme reaction.

In previous studies [15,16], we found the interesting phenomenon that the substitution of Asp^{217} or Glu^{219} for other amino acids, such as lysine and tryptophan, broadens the substrate specificity to oxyimino β-lactams such as cefuroxime, which are essentially unfavorable substrates for the wild-type enzyme. Such a modification of the substrate profile was not observed for the mutant enzymes (data not shown).

4. DISCUSSION

In the classical grouping of β-lactamases based on substrate profiles, class C β-lactamases belong to the cephalosporinase type family. In addition to the difference in the substrate profile, most class C enzymes exhibit lower molecular activity than class A enzymes [31]. The production of class C β-lactamases is mediated by chromosomal genes in Gram-negative bacteria, and they are basic proteins with larger molecular masses and higher isoelectric points than class A enzymes [31]. Class C enzymes also differ from class A enzymes in their reactivity to β-lactamase inhibitors. Bush et al. [32] reported that aztreonam is a strong progressive inhibitor of class C enzymes but not of class A enzymes. On the other hand, class C enzymes show lower susceptibility to clavulanic acid, which is a strong progressive inhibitor of class A enzymes. Such a marked contrast in enzymological properties is particularly interesting in connection with the difference in the active-site structure between the two classes of enzymes.

Ser^{70}, Lys^{73}, Glu^{166} and Lys^{234} are the most important functional residues in class A β-lactamases, and their significant role in the catalytic mechanism is known [7]. The active-site serine in the *C. freundii* GN346 enzyme was directly identified as Ser^{64} using dansyl-penicillin as a fluorescent probe [13]. Lys^{67} and Lys^{315} of the GN346 enzyme were also confirmed to be the counterparts of Lys^{73} and Lys^{234}, respectively, by means of site-directed mutagenesis. When these two basic amino acids in the GN346 enzyme were substituted with glutamic acid, the enzyme completely lost the catalytic activity [12,14].

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Fig. 2. Amino acid sequence alignment of the *C. freundii* β-lactamase with other five β-lactamases: (1) RTEM1 [26], (2) *B. licheniformis* 749/C [27], (3) *S. aureus* PC1 [28], (4) *C. freundii* GN346 [12], (5) *E. coli* K12 [29], (6) *S. marcescens* SR50 [30]. The residues are numbered according to the standard numbering scheme for class A β-lactamases [25]. The shadowed and underlined regions in the sequences of the PC1 and GN346 enzymes indicate α-helices and β-strands, respectively, the indications being made on the basis of the results of crystallographic studies [23,24]. Bold characters denote Glu^{61} (ABL67) and possible counterparts to Glu^{166} in the class C enzyme. The region examined in this study is boxed.

Table II

β-Lactamase activity of *E. coli* AS226-51 cells harbouring the wild-type or mutant enzyme genes

β-Lactamase gene	β-Lactamase activity (U/mg bacterial protein)
Wild-type	1.6
E195Q	0.8
E195K	0.9
E195A	0.8
E205Q	1.0
E205K	1.0
E205A	1.0

β-Lactamase activity in sonically disrupted cells was measured by the microiodometric method, with cephalothin as the substrate.

Glu^{166} in class A enzymes was assumed to act as the general base in the acylation or deacylation reaction [8,33]. Madgwick and Waley [9] confirmed this assumption, and the substitution of Glu^{166} in β-lactamase I with glutamine markedly reduced its catalytic activity. Recent studies on the *B. licheniformis* 749C enzyme and the RTEM-1 enzyme revealed a significant role of Glu^{166}, i.e. contributed to the deacylation step in the enzyme reaction [10,35]. In this study, we confirmed the lack of this functional acidic residue on the Asp^{217} loop. Although a localization of this functional residue on other regions could not be ruled out, this survey strongly suggested the possibility that the class C β-lactamases lack a functional acidic residue corresponding to Glu^{166}.

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