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Cloning, Sequencing, and Site-Directed Mutagenesis of β-Lactamase Gene from *Streptomyces fradiae* Y59

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The β-lactamase gene from *Streptomyces fradiae* Y59 was cloned and sequenced. To determine which amino acid residues are critical in binding activity to blue dextran, chimera β-lactamases were constructed and their binding abilities were determined. The results suggested that blue dextran binding may depend more on overall conformation of about two-thirds of the β-lactamase molecule from the N terminus than on the primary structure.

Many *Streptomyces* species produce β-lactamase constitutively regardless of their resistance to β-lactam antibiotics (9, 14). The amino acid sequences of these β-lactamases show clearly that like other β-lactamases from gram-positive bacteria (11), they are class A enzymes. However, some *Streptomyces* species also produce β-lactamases which can hydrolyze cloxacillin at a rate of about 10% of that of benzylpenicillin, and the rate of hydrolysis of benzylpenicillin is not retarded in the presence of methicillin. Thus, β-lactamases from *Streptomyces* species can be further classified into two groups, group I and group II, on the basis of their amino acid sequences. Moreover, these groups coincide well with classifications on the basis of their properties in binding blue dextran (11, 20). Group I β-lactamases, which do not bind blue dextran, are similar to β-lactamases from *Bacillus licheniformis* and *Bacillus cereus*, whereas group II β-lactamases, which bind blue dextran, are similar to β-lactamases encoded by genes on the chromosomes of gram-negative bacteria such as *Yersinia enterocolitica* (21) and *Citrobacter diversus* (17). This property of binding to blue dextran is similar to that among class D β-lactamases. Interestingly, however, while blue dextran-binding proteins are in general known to bind dinucleotides such as $NADP^+$ (23), spectroscopic analysis suggested that the β-lactamase isolated from fermentation broth did not contain NAD^+ or $NADP^+$. In addition, the presence of these nucleotides did not affect the enzymatic activity of the β-lactamase used. Thus, the physiological significance of the property of dinucleotide binding remains to be clarified. It is therefore interesting to know the evolutionary relationship between group II β-lactamases and proteins which bind $NADP^+$, such as dehydrogenases, and between group II β-lactamases and group I β-lactamases.

Previously, we cloned and sequenced the genes of group II β-lactamases from *S. cellulosae* (12, 25) and *S. lavendulae* (20). Here, we describe the cloning, sequencing, and results of site-directed mutagenesis of amino acid residues important in binding to blue dextran.

S. lividans 1326 (8) was the kind gift of D. A. Hopwood (John Innes Institute, Norwich, United Kingdom). *Streptomyces* plasmid DNA was prepared by alkaline lysis (16) and spermine treatment (5). *Escherichia coli* plasmid DNA was isolated by the method of Holmes and Quigley (3). DNA fragments were purified by electroelution (22). Site-directed mutagenesis was carried out by the method of Kunkel et al. (7) with a Mutan-K kit (Takara Shuzo Co., Kyoto, Japan). Oligonucleotides were synthesized with a 381-A DNA synthesizer from Applied Biosystems Co. The nucleotide sequence was determined by the method of Sanger et al. (19) by using Sequenase 2 (U.S. Biochemical Corp., Cleveland, Ohio) and 7-deaza-dGTP. Thiostrepton was the kind gift of S. J. Lucania, Squibb and Sons, Inc. (New Brunswick, N.J.). β-Lactamase activity was determined by modified iodometric and microiodometric methods as described previously (9, 14).

DNA fragments from *S. fradiae* Y59 were hybridized with a 1.9-kb *Sac*I-*Xba*I fragment from *S. cellulosae* KCCS0127 (25). By using this property, the hybridized fragment was cloned. The nucleotide sequence of the essential 2.2-kb fragment was then determined with the Sequenase 2 kit. Comparison of the nucleotide sequence with a β-lactamase from *S. fradiae* (2) showed that only one nucleotide in the open reading frame region was different; namely, the guanine at nucleotide 497 was replaced by an adenine in the *S. fradiae* Y59 β-lactamase gene. Comparison of the amino acid sequence with those of other β-lactamases indicated that it was a class A, group II β-lactamase (11, 20). This classification is supported by the finding that mutants A (S-82→A), E (E-182→N), and W (P-183→Q) showed no enzymatic activity.

To date, two types of conserved amino acid sequences have been implicated in dinucleotide binding: a glycine-rich sequence, like GXGXXG (18), and an RHXXXXR sequence (6), in which X is any amino acid. However, it was difficult to find such a sequence in the β-lactamase from *S. fradiae* Y59. Thus, we wanted to know which amino acid residues or what part of the molecule is implicated in the binding of blue dextran and/or dinucleotides. To investigate this problem, we first constructed chimeric β-lactamases between blue dextran-binding (*S. fradiae*) and non-blue dextran-binding (*S. cacaoi*) β-lactamases and measured their blue dextran binding activities. Figure 1 compares the amino acid sequence of *S. fradiae* β-lactamase with that of *S. cacaoi* β-lactamase (15, 24). To construct chimera β-lactamase genes, each gene was divided into three parts (Fig. 2A). In junction I, each gene was digested with *Eco*47III and the fragments from *S. fradiae* were mixed with those from *S. cacaoi* (Fig. 2B). Ligation of these mixtures gave two chimera genes. In junction II, in contrast, a sequence of 10 bases (GAGGGTCACC) from *S. fradiae* (underlined in Fig. 2B) was changed to TCGACCCCGG by using the synthetic oligonucleotide, and the product was then digested with *Sma*I.

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S. fradiae          MDRTTARPNRRAVLATGVGAALAATAAAAGPAHAAPGRGARVEGRLRALERTHDARLGAFAYDTGTGRTVAYRADERFPIASMFKTIAVAAVLRDLDRDGEVLARRVHY  109
                                                *. .. .. .* . .*.*...  *  .****.. **. *..* ** .*..... ******. .* **..... *.* .. *** . **
S. cacaoi     MRIRPTRRLLLGAVAPLALVPLVACGQAASGSESGGQPGLGGCGTSAHGSADAHEKEFRALEKKFDAHPGVYAIDTRDGQEITHRADERFAYGSTFKALQAGAILAQVLRDGREV-RRGAE  119

S. fradiae    TADYVKRSGYSPVTGLP------ENVANGMTVAELCEATLTRSDNTAANLLLHDLGGPTAVTRFCRSVGDHVTRLDRWEPELNSAEPGRVTDTTSPRAIGRTYGRLILGD----LLAAHD  219
              ...  *  *.. . **      ..**.**.. ***.*... *********...***. . **  . .***.*..**.*.**.** **  **..***.. .  . ..*    **..*
S. cacaoi     ADGMDKVVHYGQDAILPNSPVTEKHVADGMSLRELCDAVVAYSDNTAANLLFDQLGGRRGSTRVLKQLGDHTTSMDRYEQELGSAVPGDPRDTSTPRAFAEDLRAFAVEDGEKAALAPND  239

S. fradiae    RERLTRWMLDNRTSDERFRKGLPADWLLADKTGGGDYGTNNDAGVAWPPGRPPVVLAVQTTRFTPDAEADNVLVAEAARLLAEAMTD  306
              **.*. ** ..**.*. .* *.* ** ..**.*  .***.** .*..***.*.*..* .   *.***... *****. ..*...
S. cacaoi     REQLNDWMSGSRTGDALIRAGVPKDWKVEDKSGQVKYGTRNDIAVVRPPGRAPIVVSVMSHGDTQDAEPHDELVAEAGLVVADGLK  325
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FIG. 1. Comparison of amino acid sequences of β-lactamases from *S. fradiae* Y59 and *S. cacaoi* KCCS0352. Each number refers to the position of the amino acid residue in the corresponding enzyme. Asterisks and dots, identical and homologous amino acid residues between the two enzymes, respectively. Hyphens, no corresponding amino acid.

The gene from *S. cacaoi* was also digested with *Sma*I, and the products were mixed with the modified digested products of *S. fradiae*. As was the case for junction I, ligation of these mixtures yielded two chimera genes. Four types of chimera plasmid were constructed (f1, f2, f3, and f4 [Fig. 2A]). These plasmids were introduced into *S. lividans* 1326, and transformants were cultured in modified E medium (9) that contained $CaCl_2$ instead of $CaCO_3$. The β-lactamase enzymes were isolated as crude powders by the addition of ammonium sulfate, dialyzed against distilled water, and lyophilized. A Sephadex G-75 column (1 by 30 cm) in 0.1 M phosphate buffer (pH 7) was used to determine whether β-lactamase enzyme was bound to the column when a β-lactamase sample was applied to the column together with blue dextran 2000, as described previously (13, 25). When the crude enzyme from *S. fradiae* Y59 was loaded onto the column and eluted with the same buffer, the peak of enzyme activity appeared in the position expected from its molecular weight (Fig. 2A). In contrast, when the same

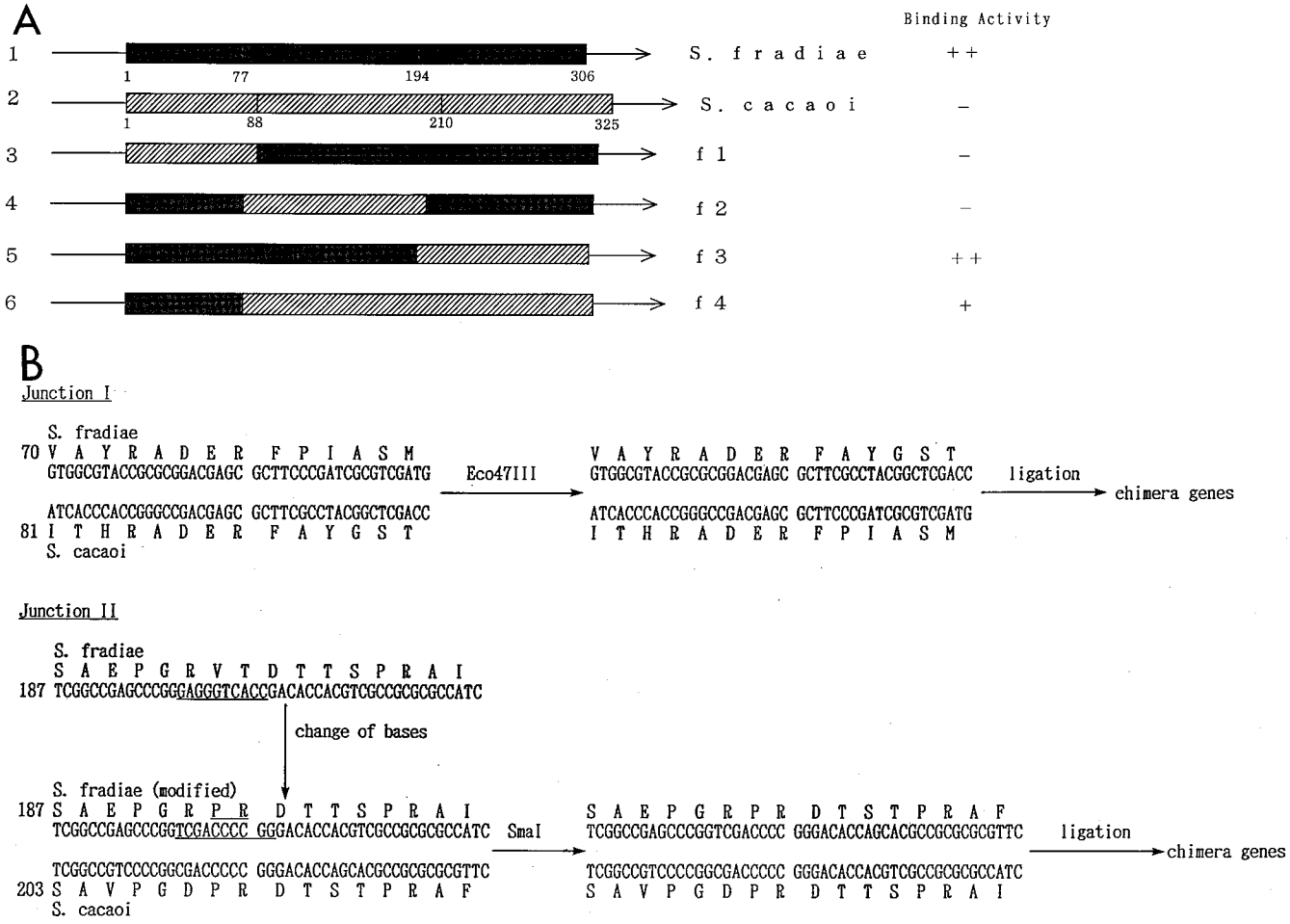

FIG. 2. Structures of chimera genes (A) and the nucleotide and corresponding amino acid sequences in two junction regions and the construction scheme of chimera genes between β-lactamases from *S. fradiae* and *S. cacaoi* (B). (A) Dark box, sequence from *S. fradiae*; hatched box, sequence from *S. cacaoi*. Numbers below boxes refer to the positions of corresponding amino acid residues. Binding activity to blue dextran is indicated at the right. ++, strong binding; +, moderate binding; −, no binding. (B) Numbers refer to the positions of the amino acid residues from the N terminus.

TABLE 1. Distribution of β-lactamase activity in fractions from the blue dextran gel

Strain	% Total activity in elution buffer with NaCl concn (M) of:												Relative β-lactamase activity
	Pass-through[a]	0	0.2	0.4	0.6	0.8	1.0	1.2	1.4	1.6	1.8	2.0	
S. fradiae Y59	—[b]	—	2.6	7.8	11.7	17.2	17.2	18.2	13.3	9.7			100
S. cacaoi KCCS0352	81.3	18.5	—	—	—	—	—	—	—	—			320
f1	68.4	25.3	4.5	1.7	—	—	—	—	—	—			9
f2	87.3	12.7	—	—	—	—	—	—	—	—			0.1
f3	3.0	—	4.8	7.9	14.8	15.5	18.4	15.9	11.9	7.9			0.1
f4	41.9	14.2	20.1	11.1	6.6	6.1	—	—	—	—			0.04
J (G-65→R and T-66→D)	—	—	4.4	9.5	13.3	15.7	16.7	15.4	13.4	9.2			240
M (R-51→K and T-52→K)	—	—	1.9	6.6	14.4	19.7	21.8	19.5	10.6	4.2			150
N (R-56→H and L-57→P)	2.0	—	3.5	7.7	12.7	16.7	20.3	15.2	13.4	8.5			0.2
P (D-95→Q)	2.0	—	—	3.6	8.3	15.0	19.7	19.2	16.6	13.9			100
Q (E-50→Q)	—	—	—	—	3.8	7.8	12.5	15.5	18.7	15.5	15.5	10.8	20
R (P-79→A and I-80→Y)	—	—	—	—	—	—	100	—	—	—			0.01
S (R-94→L and D-97→L)	—	—	—	5.1	15.4	24.0	24.0	18.0	9.9	3.4			4
T (E-101→R)	—	1.5	—	3.3	7.6	14.2	19.4	19.6	17.3	15.1			90
U (D-157→Q)	7.9	—	2.6	2.6	2.6	23.7	19.7	14.5	14.5	11.8			2

[a] Passed through the fraction.
[b] —, no detectable activity.

preparation was applied to the column together with blue dextran, the peak of enzyme activity appeared in the void volume (Fig. 2A). With the crude enzyme from *S. cacaoi*, the peak of enzyme activity did not shift whether or not blue dextran was present. When the sample from mutant f3 was applied to the column together with blue dextran, the peak of enzyme activity was shifted to the void volume (Fig. 2A). In contrast, when the sample from mutant f1 was applied with blue dextran, most of the enzyme activity appeared in the same position as it did without blue dextran (Fig. 2A). The β-lactamases of point mutants B (M-83→T), G (V-193→T and T-194→R), and O (A-88→Q) behaved similarly to that of *S. fradiae* itself (data not shown). These results clearly show that one-third of the molecule from the C terminus of the β-lactamase of *S. fradiae*, that is, amino acids 194 to 306, is not implicated in the binding of blue dextran but exerts a negative effect on binding (compare the results for f2 and f4 in Fig. 2A).

To investigate binding ability more quantitatively, TSK AF-blue Toyopearl 650ML gel was used. This gel is TSK Toyopearl HW-65 gel covalently bound to Cibacron blue F3GA (Tosoh Co., Tokyo, Japan). When the crude enzyme of *S. fradiae* was applied to a column composed of 1 ml of the gel and was eluted with 0.1 M phosphate buffer (pH 7.0) containing increasing (stepwise) amounts of NaCl (0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, and 1.6 M) and fractions of 5 ml each were collected at each step, enzyme activity appeared as shown in Table 1. Thus, most of the β-lactamase activities of mutants f1 and f2 passed through the column, while those of mutant f3 behaved similarly to the *S. fradiae* enzyme. On the other hand, while about one-half of the β-lactamase activity of mutant f4 passed through the column, the rest interacted weakly with blue dextran, indicating that two-thirds of the molecule from the N terminus of the β-lactamase of *S. fradiae* has an important role in binding to the blue dextran gel and that the exchange of one-third of the molecule from the N terminus of the β-lactamase of *S. fradiae* with that of *S. cacaoi* provided the *S. cacaoi* enzyme with weak binding ability to the blue dextran gel.

To study which amino acid residues play a critical role in this binding activity, several mutant genes of β-lactamases were constructed by site-directed mutagenesis, mainly in consideration of two points. First, the common amino acid residues in the three group II β-lactamases were changed to the corresponding amino acid residues in the β-lactamase of *S. cacaoi*. Second, the region of mutation was restricted to amino acid residues 40 to 194. Among these, the behavior of β-lactamases of mutants B (M-83→T), G (V-193→T and T-194→R), and O (A-88→Q) with the Sephadex G-75 column was similar to that of *S. fradiae* itself, as described above. As expected, mutant E (E-182→N) showed no β-lactamase activity, indicating that glutamic acid at residue 182 is involved in this β-lactamase activity (4). The β-lactamases from mutants J, M, N, P, R, S, and T showed behavior toward the blue dextran gel very similar to that of *S. fradiae* β-lactamase (Table 1). Similarly, the peak of enzyme activity did not shift with the β-lactamases of mutants X (H-53→V), Y (S-170→Q and V-171→L), Z (T-86→I and I-87→L), δA (δ115K and δ116R), and δB (δ125L and δ126P) (data not shown). Interestingly enough, however, the β-lactamase of mutant Q was bound more strongly and that of mutant U was bound slightly less strongly to the blue dextran gel column than was *S. fradiae* β-lactamase. We thus concluded that blue dextran and/or dinucleotide binding may depend more on the overall three-dimensional structure of dye binding and dinucleotide binding sites than on the primary structure of this domain. Branlant and Branlant (1) reported a similar situation in noting that the NAD^+ binding of *E. coli* D-glyceraldehyde-3-phosphate dehydrogenase depended more on the overall conformation of the coenzyme binding site than on the primary structure of this domain.

Apart from blue dextran binding, these exchanges of parts of the molecule gave β-lactamases less than 1% of the specific enzyme activity, except for mutant f1, whose specific activity was about 10% of that of the original molecule (Table 1). In addition, the replacement of amino acid residues by site-directed mutagenesis produced β-lactamases with various specific enzyme activities. While mutants J and M showed greater specific activities, other mutants had lower activities despite

the exchange of amino acid residues with the corresponding residues of *S. cacaoi* β-lactamase (Table 1). Thus, mutant R produced almost no enzyme activity (Table 1), suggesting that the replacement of amino acid residues adjacent to the essential SXFK region with those of *S. cacaoi* affected the three-dimensional structure drastically, even though these β-lactamases share the essential SXFK sequence. Therefore, it is suggested that although the physiological role of β-lactamases in *Streptomyces* species is unclear at present (10), the differences in various properties between group I and group II β-lactamases from *Streptomyces* species are much greater than expected from the differences in these amino acid sequences.

Nucleotide sequence accession number. The nucleotide sequence reported here has been deposited in the DDBJ and GenBank databases under accession number D13898.

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