Characterization of the Upstream Region of the β -Lactamase Gene of *Streptomyces fradiae* Y590

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The effect of the upstream region of the β -lactamase structural gene was studied on the production of β -lactamase from *Streptomyces fradiae* Y59. In contrast to the case in *S. cacaoi*, β -lactamase activity was weakly enhanced by the presence of the upstream 5-kb DNA fragment but was not induced by 7-ACA. The sequence analysis of this region revealed a couple of putative regulatory proteins, although their actual functions were not clear. It was therefore suggested that at least one of the ORFs might be involved in the weak enhancement of β -lactamase gene expression of *S. fradiae* Y59.

β -lactamases (E.C.3.5.2.6) have been found in a large number of bacteria and divided into four or six classes on the basis of their amino acid sequences¹⁻³. Some are inducible, whereas the others are constitutive. The regulatory mechanism of induction of β -lactamase production was studied in *Bacillus licheniformis* as well as in gram-negative enteric bacteria⁴⁻⁶. Although both β -lactamases from *Citrobacter freundii* and *Bacillus* are inducible, their mechanisms of induction are completely different. In *Citrobacter freundii*, a trans-acting protein, AmpR, is implicated in the induction together with a couple of other regulatory proteins⁶, whereas PenI (repressor) and PenJ (antirepressor) are involved in *Bacillus*⁴. In addition, the arrangement of the genes involved in the induction is also different, even within gram-negative bacteria as well as within gram-positive bacteria.

Most *Streptomyces* strains produce β -lactamases⁷. In *S. cacaoi*, two kinds of β -lactamases are produced. In one case, the expression was induced by the presence of β -lactam antibiotics, 6-aminopenicillanic acid (6-APA) and 7-aminoccephalosporanic acid (7-ACA)⁸. We have previously cloned and sequenced the gene encoding another β -lactamase of *S. cacaoi*⁹. It turned out that the upstream sequence of the β -lactamase gene had an enhancing effect on the β -lactamase pro-

duction in *Streptomyces lividans*. The nucleotide sequence of this region contained two regulatory genes, *blaA* and *blaB*^{10, 11}. The polypeptide BlaA encoded by *blaA* had a high homology with AmpR, and was found to be a DNA-binding protein with a helix-turn-helix motif. Recently, we cloned and sequenced another gene from *Streptomyces fradiae* Y59¹². In contrast to the case of *S. cacaoi*, this β -lactamase is not inducible. In this paper, we characterize the upstream region of this β -lactamase gene in terms of regulatory proteins and effect on β -lactamase activity.

S. fradiae Y59 used for the DNA donor was described previously¹². *S. lividans* 1326¹³ was a kind gift of D. A. Hopwood (John Innes Institute, Norwich, United Kingdom). *Streptomyces* plasmid DNA was prepared by alkaline lysis¹⁴ and spermine treatment¹⁵. *Escherichia coli* plasmid DNA was isolated by the method of Holmes and Quigley¹⁶. DNA fragments were purified by electroelution¹⁷. The nucleotide sequence was determined as described previously^{10, 18}. β -Lactamase activity was determined by modified iodometric and microiodometric methods as described previously^{7, 19}.

To determine the effect of the upstream DNA sequences on the β -lactamase activity, two plasmids (Fig. 1) were constructed: pMCP229 with the

Fig. 1. Plasmids, pMCP229 and pMCP261. pMCP261 contains about 700-bp nucleotide segment upstream of the β -lactamase structural gene. The dark area is from *S. fradiae* Y59 and the shaded area is from pIJ487. The solid line indicates the nucleotide from pUC118.

upstream region and pMCP261 without the upstream region. pMCP229 was built by inserting 7.7-kb *Bam*HI fragment containing β -lactamase structural gene from *S. fradiae* into *Bam*HI site of pIJ486. The β -lactamase structural gene is located about 5-kb downstream from the *Bam*HI site. pMCP261 was constructed as follows. pUC118 containing the 7.7-kb fragment was digested with *Mlu*I and treated with Klenow fragment to form the blunt ends. Then, the 8-bp *Eco*RI linker (Takara Shuzo Co.) was inserted into the blunt ends, digested with *Eco*RI, and then the plasmid was self-ligated to give plasmid A. Subsequently, plasmid A was digested with *Xba*I and *Hind*III and the resulting longer fragment containing β -lactamase gene was ligated with pIJ487 fragment digested with *Xba*I and *Hind*III to give pMCP261. The fragment in pMCP261 contained a about 730-bp DNA segment upstream of the β -lactamase structural gene. These two plasmids were introduced into *S. lividans* 1326 and β -lactamase activity was determined in the presence or the absence of 7-ACA (Fig. 2). *S. lividans* /pMCP229 showed only four times higher β -lactamase activity than *S. lividans* /pMCP261. Furthermore, 7-ACA did not affect β -lactamase activity irrespective of the presence of the upstream region. This is in contrast to the case of the β -lactamase of *S. cacaio*¹⁰⁾, where the presence of about 2-kb upstream region enhanced the

β -lactamase activity about 50 times and the β -lactamase activity was distinctively induced by 7-ACA¹¹⁾. For the enhancement and the induction of β -lactamase activity, at least two genes were involved in *S. cacaio*^{10, 11)}. Thus, it is interesting to know the organization of genes upstream of the structural gene of β -lactamase from *S. fradiae* Y59.

Fig. 3 shows the nucleotide and the deduced amino acid sequences of the upstream region. "The Frame analysis" developed by Bibb et al.²⁰⁾ revealed four possible open reading frames (ORFs) in this region. ORF1 (411 amino acids) consisted of nucleotide numbers 4403 to 3168, ORF2 (244 amino acids) of nucleotide numbers 2360 to 1626 and ORF4 (139 amino acids) of nucleotide numbers 2337 to 2756. ORF3 starts at nucleotide number 1310 but the end of the ORF could not be detected in the sequence. The β -lactamase structural gene is from nucleotide numbers 4917 to 5837 (sequence data not shown). To clarify whether proteins encoded by these ORFs have any functional similarity to other proteins, the amino acid sequences were screened for homologous proteins by the current version of GenBank and Swiss Plot databases, using fasta program. ORF1 was found to exhibit high score of homology with sialidases from *Actinomyces viscosus* (Genbank accession number L06898) and *Caenorhabditis elegans* (Genbank accession number U23181) and colla-

Fig. 2. Time course of β -lactamase activity of *S. lividans* containing pMCP229 or pMCP261 in the presence or the absence of 7-ACA. + means the presence and - indicates the absence of 7-ACA.

gens from *C. elegans* (GenBank accession numbers Z79694-2 and Z70756-5) but only weak homology with subunit of RNA polymerase II transcription factor TFIID (GenBank accession number Y11354), DNA-binding protein A from human (GenBank accession number L29071) and *Mycobacterium tuberculosis* hypothetical protein Rv1157c (GenBank accession number Z95584). It is interesting to note that ORF1 carries two blocks (Blocks number BL01063A and BL01063B) corresponding to sigma-70 factor ECF subfamily proteins, that is, amino acids 94 to 128 and amino acids 153 to 199. On the other hand, ORF4 showed very high scores of homology with an unknown protein from *Actinosynnema pretiosum* subsp. *auranticum* (GenBank accession number U33059-2), which was involved in biosynthesis of rifamycin, and with a putative regulatory protein from *Streptomyces coelicolor* A3(2) (GenBank accession number AL031031-2). That is, ORF4 gave 35.79 % identity in 95 amino acid overlap with U33059-2 protein and 37.11 % identity in 97 amino acid overlap with AL031031-2 protein. In addition, ORF4 has a block (amino acids 82 to 123) corresponding to β -lactamase class-A proteins. BlaB protein from *S. cacaoi* also possesses β -lac-

tamase-like sequence. Although it is not clear whether these ORFs function actually as regulatory proteins at the present time and no definitive induction was observed with this β -lactamase, it is possible that at least one of these ORFs may be implicated in 4-times enhancement of β -lactamase expression. To confirm this possibility, it is necessary to disrupt these genes and check the β -lactamase activity. On the other hand, ORF2 and ORF3 were found to show no significant homology with other proteins by blast and fasta analyses.

Another point to note will be that the regulatory mechanisms diverge very differently in bacterial β -lactamases including *Streptomyces*, although the properties of β -lactamases themselves are not so much different²¹.

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[illegible]

Fig. 3. The nucleotide sequence of the 4.4-kb DNA fragment upstream of the structural gene of β -lactamase from *S. fradiae* Y59 and the deduced amino acid sequences. GTCGAC indicated in bold shows the *Sal*I site which overlaps with the sequence reported previously¹²; C at nucleotide 4379 is corresponding to nucleotide 1 in the previous report (GenBank accession number D13898). The *Mlu*I site into which *Eco*RI linker was inserted is underlined. The nucleotide sequence reported here has been deposited in the DDBJ and GenBank databases under accession number AB027763.

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3960 TACACCTGGTGGATCAGGGGGGGCCGCCCTCGTAGAGGCGCGTCCAACTCCACCCGACCCCTGGCGAGGGGGGCAAGGGGGCCCGGCCACCGCGGGCGCGCCGTTCGGGCGCG
 Y V Q H I L A P A A R E Y L A D F E V R G R G V A P V P A A G G P A A T A G
 4080 GCGACGGGGCGCGCTCGCCCGCGGCTCGGGCGGCTCGGACCGGTGGCGGCTCACCGCCGACCCGGGGGGGGGACCCGCTGGGCAAGGGGCACTTCGGCGGTGGTCTCC
 A S P A A D G G P E A A D P G T A T P A E G S G P A A S G T P L P V E A T T E
 4200 GCGTCACCGGCTCGGCAACCGGCGCGGCTCGCGGCGGCGGTGCTCTCGGGGGCGGCTGGCGGTGGTGGGGCCCGTGCCTGGGGGGGGGGGACCGGTTCGGCG
 A D V P D A G A P A D A S R P A T D E P P R T A T T P G T G P E P A G S A D A
 4320 GCGGTTTCGGGCGGCGGCGGACGATGCGGTCCACCGAGCGCGCTCAACGGGGCGCGGCTTCGGGGGGCGGGGGGACCGGATCGGGGTCCACCGAGCGGTCAACGGGGCGCG
 A P E A P A P A V S A P G G L A D V P A P A V S A P G G L A D V P A P A V S A P
 4440 CCACCGGGCGGTCAACGGGGCGGGGGGACGATGCGGGTCCACCGGGCGGTCCACCGAGCGGGCTCCACCGAGCGGGGCTCTCGGGGGGGGGCGCGCGGGGGC
 G G P A D V P A P A V S A P G G P A D V P A D A G P E M (ORF1)

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