CLONING FROM *STREPTOMYCES CELLULOSAE* OF THE GENE ENCODING β-LACTAMASE, A BLUE-DEXTRAN BINDING PROTEIN

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A β-lactamase gene was cloned from *Streptomyces cellulosae* as a 2.3-kb DNA fragment using *Streptomyces lividans* 1326 and PIJ385 as a host-vector system. During the course of cloning, a part of the chromosomal DNA fragment cloned together with a part of the vector plasmid were deleted, indicating instability of this contiguous DNA region. The enzyme from the clone showed similar properties with respect to binding of blue dextran and isoelectric point to the enzyme from *S. cellulosae*. The cloned gene hybridized not only to DNA of *S. cellulosae*, the source of DNA, but also to DNAs of several *Streptomyces* species, irrespective of their formation of β-lactamase. These results suggest that this gene may have homology to genes other than the one for β-lactamase.

β-Lactamases are produced by a large number of different prokaryotic organisms with differing chemical, physical and enzymatic properties[1]. The designation β-lactamase is based on only one common feature: they catalyze the hydrolysis of the β-lactam ring of penicillins and cephalosporins to yield the antibacterially inactive products, penicilloic acids and cephalosporoic acids, respectively. As a consequence of this reaction pathogenic bacteria become resistant to the β-lactam antibiotics. Further, these enzymes are produced by non-pathogenic bacteria including *Streptomyces*[2,3] and the cyanobacteria[4]. The role of these enzymes in non-pathogenic bacteria is not well understood.

One group of β-lactamases present in *Streptomyces* has the unique property of binding blue dextran[5]. This type of β-lactamase is very interesting from an evolutionary point of view, because such proteins in general have a dinucleotide fold and bind ATP, NAD^+ or $NADP^+$[6]. The β-lactamase from *Streptomyces cellulosae* binds blue dextran and $NADP^+$ and was purified by means of Blue Sepharose[7]. Similar β-lactamases are found in clinical isolates[8]. This paper describes the cloning of the gene encoding the β-lactamase of *S. cellulosae* and some of its properties.

Materials and Methods

Bacterial Strains and Plasmids

S. cellulosae KCCS127 and *Streptomyces lividans* 1326[9] were kind gifts from A. SEINO and D. A. HOPWOOD, respectively. pIJ385[10] was a gift from M. J. BIBB. pMCP180 is the plasmid which contains the β-lactamase gene cloned in this paper from *S. cellulosae*. pMCP181 was constructed by inserting the *Sac* I-*Cla* I fragment of pMCP180 into pHSG396 digested with *Sac* I and *Cla* I. pHSG396 was purchased from Takara Shuzo Co., Ltd. The β-lactamase gene from *Streptomyces lavendulae* ATCC 8664 was a generous gift from B. JAURIN.

Genetic Manipulation

Preparation of chromosomal and plasmid DNA from *Streptomyces* and transformation of *Streptomyces* protoplasts were performed as described by HOPWOOD *et al.*[10] Treatment with restriction endonucleases

or calf intestine alkaline phosphatase, ligation experiments, ^{32}P-labelling by nick translation and Southern hybridization were carried out as described by MANIATIS *et al.*[11] Restriction endonucleases and calf intestine alkaline phosphatase were purchased from Takara Shuzo Co., Ltd. Radioactive compounds were obtained from Amersham Co.

β-Lactamase Activity

Nitrocefin (Oxoid Co.) was used for the selection of β-lactamase producing clones. The β-lactamase activity in culture supernatants was determined using benzylpenicillin as a substrate by the method of OGAWARA[2]. E medium described previously[2] was used for liquid culture. One unit of enzyme activity is defined as the amount of the enzyme which catalyzes the hydrolysis of 1 μmol of benzylpenicillin per minute at 30°C.

Results and Discussion

Cloning of the Gene

Chromosomal DNA from *S. cellulosae* was completely digested with *Sac* I or *Pst* I and the unfractionated fragments were used in shotgun cloning experiments using *S. lividans* 1326 and pIJ385 as a host-vector system. The same restriction enzymes, *Sac* I or *Pst* I, were used for the digestion of pIJ385. In the case of *Pst* I, one β-lactamase positive clone was obtained among 1,518 thiostrepton-resistant but neomycin-sensitive recombinants; by comparison, with *Sac* I, three positive clones were isolated among 1,725 recombinants. However, only one clone from the *Sac* I experiment showed β-lactamase activity in a liquid culture, whereas the clone from the *Pst* I experiment showed no activity under the same conditions. A plasmid named pMCP180 was isolated from the positive clone which was obtained by the *Sac* I digestion. When this plasmid was transformed into *S. lividans* 1326, the liquid culture of the transformant again revealed β-lactamase activity. The endonuclease restriction map of pMCP180 is shown in Fig. 1.

Fig. 1. Endonuclease restriction map of pMCP180.

The dark box indicates the inserted DNA from *Streptomyces cellulosae* KCCS127.

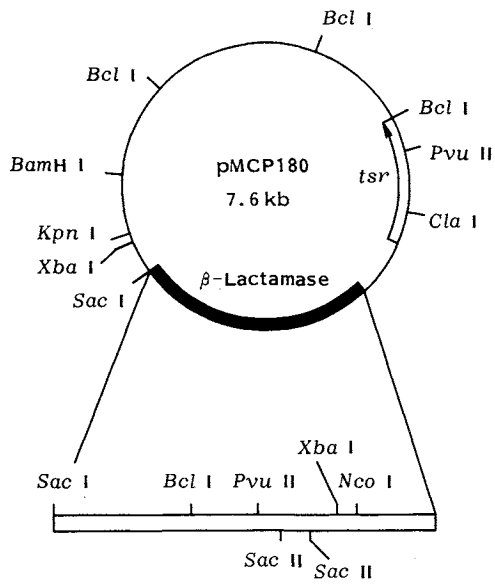
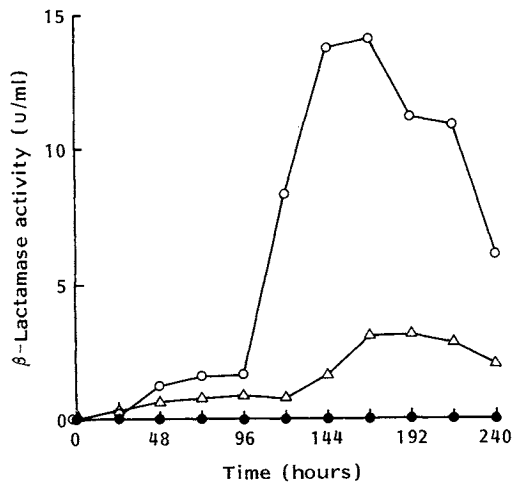


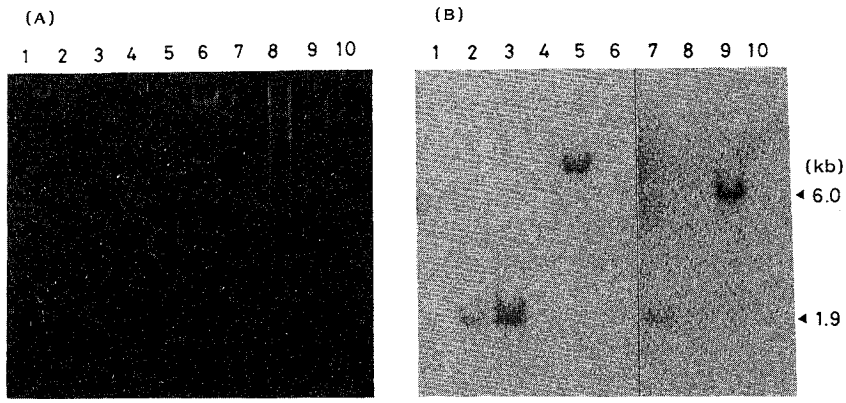
Fig. 2. Time course of β-lactamase production.

○ *Streptomyces lividans* 1326 containing pMCP180 (clone CMA180), △ *S. cellulosae* KCCS127 and ● *S. lividans* 1326 containing pIJ385.



Each strain was cultured in 100 ml of E medium[2] in 500 ml flask and β-lactamase activity in the supernatant of 1 ml aliquot was determined at the indicated time by the method of OGAWARA[2].

Fig. 3. Southern hybridization of various plasmids and chromosomal DNAs.



Probe used was the 1.9-kb *Sac* I-*Xba* I fragment (see Fig. 1). (A) Ethidium bromide stain. (B) autoradiography. 1: pIJ385 digested with *Sac* I and *Xba* I. 2: pMCP180 digested with *Sac* I and *Xba* I. 3: pMCP181 digested with *Sac* I and *Xba* I. 4: pIJ385 digested with *Sac* I. 5: pMCP180 digested with *Sac* I. 6: DNA digested with *Hind* III. 7: chromosomal DNA of *Streptomyces cellulosae* digested with *Sac* I and *Xba* I. 8: chromosomal DNA of *S. lividans* digested with *Sac* I and *Xba* I. 9: chromosomal DNA of *S. cellulosae* digested with *Sac* I. 10: chromosomal DNA of *S. lividans* digested with *Sac* I.

It is clear that the right part of the insert in the figure has no *Sac* I site, although the *Sac* I enzyme was used for the cloning. As described later, the size of the corresponding *Sac* I fragment in the chromosome was 6.0 kb, indicating that the *Sac* I fragment derived from the chromosome gave rise to a deletion of 3.7 kb during the course of cloning. This type of unstable character of the DNA region may explain why only one β-lactamase positive clone was isolated from the four positive clones obtained initially.

S. lividans 1326 containing pMCP180 (clone CMA180) produced β-lactamase activity at 14.3 U/ml in E medium, which was about 6-fold higher than that obtained with *S. cellulosae* KCCS127. The time course of β-lactamase synthesis by CMA180 was similar to that of *S. cellulosae* (Fig. 2). On the other hand no β-lactamase activity was detected in *S. lividans* 1326 or *S. lividans* 1326 transformed with pIJ385.

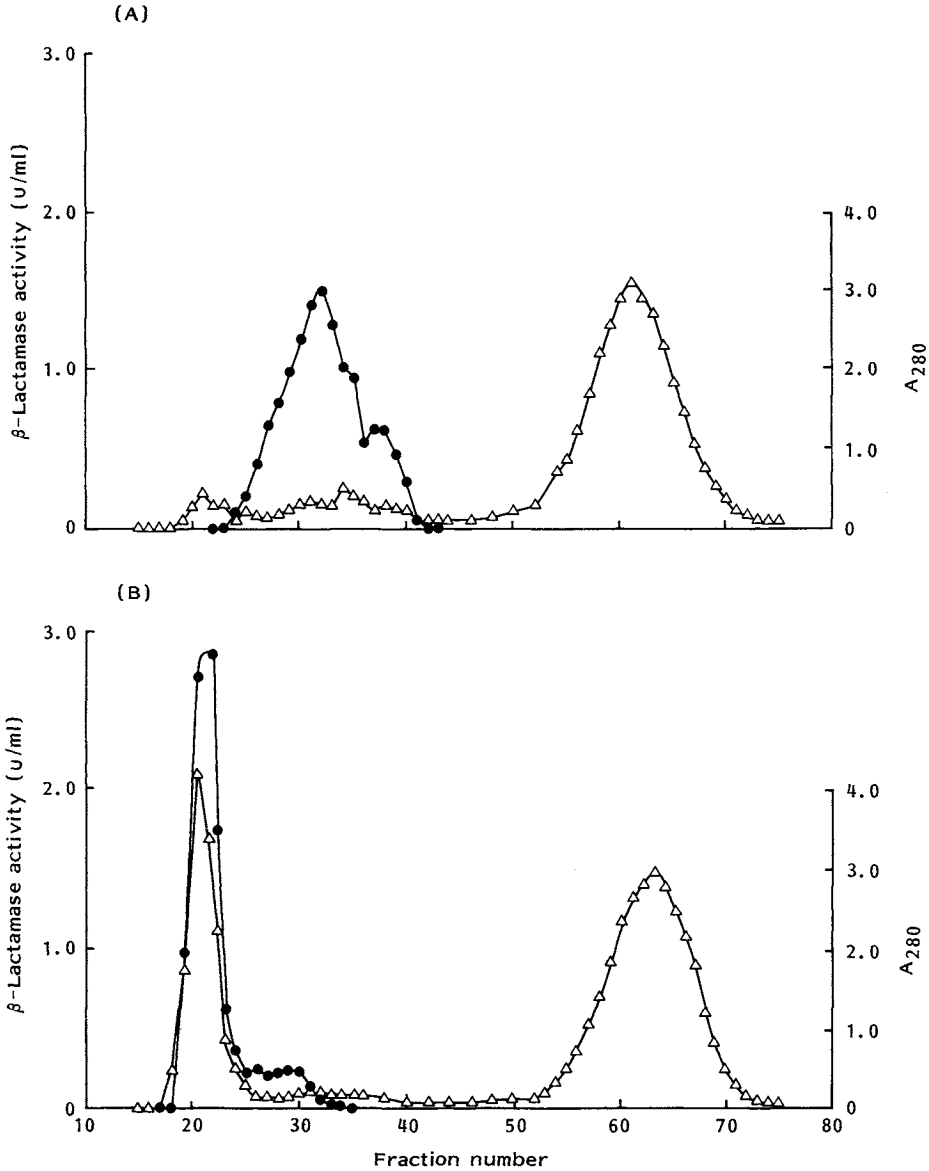
To confirm that the 2.3-kb DNA fragment was derived from *S. cellulosae* DNA, Southern hybridization experiments were performed using the 1.9-kb *Sac* I-*Xba* I fragment as a probe. The results in Fig. 3 revealed that the 1.9-kb probe hybridized to a 1.9-kb fragment from pMCP181, pMCP180 or chromosomal DNA of *S. cellulosae* digested with *Sac* I and *Xba* I. It also hybridized to a 6.0-kb sequence from the chromosomal DNA of *S. cellulosae* (Fig. 3, lane 9) and to a 7.6-kb sequence of pMCP180 when they were digested with *Sac* I (Fig. 3, lane 5). These results suggest that the 2.3-kb fragment in pMCP180 was surely derived from *S. cellulosae* DNA and that during the course of cloning a 3.7-kb fragment was lost to give pMCP180. In addition, a 0.3-kb region of pIJ385 was also deleted.

The Elution Profile of the Enzyme

When the $(NH_4)_2SO_4$ fractionated enzyme from extracts of clone CMA180 was applied to a column of Sephadex G-75, the peak of the enzyme activity eluted with 0.1 M phosphate buffer, pH 7.0, appeared in similar fractions as the corresponding enzyme from *S. cellulosae* (Fig. 4A). By comparison, when the same preparation was applied to a column of Sephadex G-75 together with blue dextran, the peak of the enzyme activity appeared in the void volume (Fig. 4B). The property of binding to blue dextran and its exclusion from the gel matrix is characteristic of the β-lactamase from *S. cellulosae*[7]. The isoelectric point

Fig. 4. Elution pattern of an ammonium sulfate precipitated enzyme of clone CMA180 from a Sephadex G-75 column.

● β-Lactamase activity, △ A_{280}.



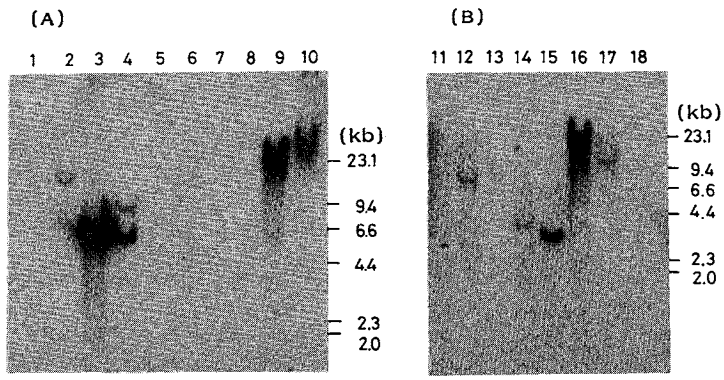
(A) an appropriate amount of the enzyme together with DNP-alanine was eluted from a 0.9 × 55 cm column of Sephadex G-75 equilibrated with 0.1 M phosphate buffer of pH 7.0. (B) An appropriate amount of the enzyme together with blue dextran and DNP-alanine was eluted under the same condition as above. Each fraction contains 0.5 ml.

in alkaline region of the β-lactamase from clone CMA180 was also similar to that of *S. cellulosae* (pI = about 9.5).

Properties of the Gene

As described above, when the 1.9-kb *Sac* I-*Xba* I DNA fragment was used as a probe, it hybridized

Fig. 5. Southern hybridization of various species of DNAs.



The probe used was *Sac* I-*Xba* I fragment (see Fig. 1). The hybridization was carried out under the same condition as in Fig. 3. (A) *Sac* I digestion. (B) *Bam*H I digestion. 1, 11: *Streptomyces phaeochromogenes* KCCS070; 2, 12: *S. fradiae* Y59; 3: *S. cellulosae* KCCS127; 4, 14: *S. lavendulae* KCCS055; 5: *S. lavendulae* KCCS057; 6, 15: *S. lavendulae* KCCS263; 7: *S. lavendulae* KCCS985; 8, 13: *S. cacaoi* KCCS352; 9, 16: *S. coelicolor* KCCS006; 10, 17: *S. diastaticus* KCCS128; and 18: *S. lividans* 1326.

to a DNA region from *S. cellulosae* KCCS127 but not from *S. lividans* 1326. However, it also hybridized to DNAs from *Streptomyces fradiae* Y59, *S. lavendulae* KCCS055, *S. lavendulae* KCCS263, *Streptomyces coelicolor* KCCS006 and *Streptomyces diastaticus* KCCS128 but not to the DNAs from *Streptomyces phaeochromogenes* KCCS070, *S. lavendulae* KCCS057, *S. lavendulae* KCCS985 and *Streptomyces cacaoi* KCCS352 (Fig. 5). *S. phaeochromogenes* KCCS070, *S. fradiae* Y59, *S. cacaoi* KCCS352, *S. coelicolor* KCCS006 and *S. diastaticus* KCCS128 produce β-lactamase constitutively[5], while *S. lavendulae* KCCS055, *S. lavendulae* KCCS057, *S. lavendulae* KCCS263 and *S. lavendulae* KCCS985 do not produce any β-lactamase. In addition, β-lactamases from *S. phaeochromogenes* KCCS070 and *S. fradiae* Y59 also possess a high affinity for blue dextran similar to that observed with the *S. cellulosae* KCCS127 enzyme. The other β-lactamases do not exhibit this property. In contrast, neither the β-lactamase gene from *S. lavendulae* ATCC 8664[12] nor that from *S. cacaoi* KCCS352[13] hybridized to any other ofthe DNA fragments from the strains listed above with the exception of its own DNA under the same hybridization conditions (data not shown). Moreover, the three β-lactamase genes did not cross-hybridize to each other. These results suggest strongly that the hybridization by the 1.9-kb *Sac* I-*Xba* I DNA fragment took place without the corresponding presence of β-lactamase activity. Moreover, even when the probe bound to DNAs of the β-lactamase producing strains, it was not directly related to the property of β-lactamase binding to blue dextran. It is anticipated that the evolutionary relationship of these β-lactamases to the other proteins and their physiological role in the organisms will be clarified by further study.

Acknowledgments

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References

1) HAMILTON-MILLER, J. M. T. & J. T. SMITH (*Ed.*): Beta-Lactamases. Academic Press, 1979

2) OGAWARA, H.: Production and property of beta-lactamases in *Streptomyces*. Antimicrob. Agents Chemother. 8: 402~408, 1975
3) OGAWARA, H.: Antibiotic resistance in the pathogenic and the producing bacteria, with special reference to beta-lactam antibiotics. Microbiol. Rev. 45: 591~619, 1981
4) KUSHNER, D. J. & C. BRENIL: Penicillinase (beta-lactamase) formation by blue-green algae. Arch. Microbiol. 112: 219~223, 1977
5) OGAWARA, H.; S. HORIKAWA, S. SHIMADA-MIYOSHI & K. YASUZAWA: Production and property of beta-lactamases in *Streptomyces*: Comparison of the strains isolated newly and thirty years ago. Antimicrob. Agents Chemother. 13: 865~870, 1978
6) THOMPSON, S. T.; K. H. CASS & E. STELLWAGEN: Blue-dextran Sepharose: An affinity column for the dinucleotide fold in protein. Proc. Natl. Acad. Sci. U.S.A. 72: 669~672, 1975
7) OGAWARA, H. & S. HORIKAWA: Purification of β-lactamase from *Streptomyces cellulosae* by affinity chromatography on Blue Sepharose. J. Antibiotics 32: 1328~1335, 1979
8) BUSH, K.: Characterization of β-lactamases. Antimicrob. Agents Chemother. 33: 259~263, 1989
9) LOMOVSKAYA, N. D.; N. M. NKRTUMIAN, N. L. GOSTIMSKAYA & V. N. DANILENKO: Characterization of temperate actinophage ϕc31 isolated from *Streptomyces coelicolor* A3(2). J. Virol. 9: 258~262, 1972
10) HOPWOOD, D. A.; M. J. BIBB, K. F. CHATER, T. KIESER, C. J. BRUTON, H. M. KIESER, D. J. LYDIATE, C. P. SMITH, J. M. WARD & H. SCHREMPF: Genetic Manipulation of *Streptomyces*. A Laboratory Manual. *Ed.*, D. A. HOPWOOD *et al.*, The John Innes Foundation, 1985
11) MANIATIS, T.; E. F. FRITSCH & J. SAMBROOK (*Ed.*): Molecular Cloning. Cold Spring Harbor Lab., 1982
12) FORSMAN, M.; B. HAGGSTROM, L. LINDGREN & B. JAURIN: Molecular analysis of β-lactamases from species of *Streptomyces*: comparison of amino acid sequences with those of other β-lactamases. J. Gen. Microbiol. 136: 589~598, 1990
13) LENZINI, V. M.; S. NOJIMA, J. DUSART, H. OGAWARA, P. DOHOTTAY, J. M. FRERE & J. M. GHUYSEN: Cloning and amplified expression in *Streptomyces lividans* of the gene encoding the extracellular β-lactamase from *Streptomyces cacaoi*. J. Gen. Microbiol. 133: 2915~2920, 1987