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Molecular Cloning of the Gene for α-Acylamino-β-lactam Acylhydrolase from *Acetobacter turbidans* by Immunochemical Detection Method

Nam, Doo Hyun[1*] and Dewey D.Y. Ryu[2]

[1]*Genetic Engineering Center, KAIST, Seoul 136-791, Korea*
[2]*University of California, Davis, CA 95616, U.S.A.*

면역화학적 방법에 의한 *Acetobacter turbidans*의 α-Acylamino-β-lactam Acylhydrolase의 유전자 클론화

남두현[1*]·유두영[2]

[1]한국과학기술원 유전공학센터 [2]캘리포니아대학교 데이비스분교

Molecular cloning of gene for α-acylamino-β-lactam acylhydrolase (ALAH) III from *Acetobacter turbidans* has been attempted by immunochemical detection method, in which polyclonal antibody from mouse Balb/c against this enzyme was employed as a probe. As a cloning vector, λ gtll was chosen for this purpose. Two positive clones has been selected from genomic libraries of *A. turbidans*, which had somewhat different binding affinities on anti-ALAH III serum and anti-β-galactosidase. By restriction analysis, both clones has been turned out to lose one of *Eco*RI sites. From these results, it concluded that deletion of DNA between *lacZ* gene and inserted DNA has occurred during replication of these clones in host cells.

The enzymes capable of catalyzing the hydrolysis and synthesis of β-lactam antibiotics including penicillins and cephalosporins have been strongly investigated for a long time and are still one of the industrially important enzymes. There are four different enzymes in this category based on their substrate specificities, but they have been revealed to have some common biochemical properties in reaction modes from their kinetic behaviors. These enzymes catalyze three different reactions simultaneously including the hydrolysis of side chains of β-lactam antibiotics, the hydrolysis of chemically activated compounds of side chains in ester or amide forms, and the semisynthesis of β-lactam antibiotics from their nuclei and their activated side chains. Based upon these findings of ours and other investigators, and from the expansion of its applicabilities to new other β-lactam antibiotics, we suggested a new name for this category of enzyme as α-acylamino-β-lactam acylhydrolase (ALAH) I, II, III, and IV (15). According to the former researchers, ALAH I was called as penicillin V (phenoxymethylpenicillin) acylase, ALAH II as penicillin G (benzylpenicillin) acylase, ALAH III as ampicillin acylase, and ALAH IV as glutarylcephalosporin acylase.

In the same pace with the development of recombinant DNA technology, the genes for this important enzymes have been cloned from several different microorganisms including *Bacillus sphaericus* producing ALAH I enzyme (17), *Bacillus megaterium* (13, 14), *Escherichia coli* (12, 14), *Proteus rettgeri* (5), and *Kluyvera citrophila* (1, 6) producing ALAH II, and *Pseudomonas* sp. (11) producing ALAH IV. Throughout these works, several interesting findings have been reported. One is that the two different subunits of *E. coli* ALAH II enzyme is processed from a common precursor protein through the cleavage by endopeptidases (1, 2, 3, 16). Another interesting report is that two non-

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*Corresponding author

identical subunits of *P. rettgeri* ALAH II enzyme play different roles in enzyme reactions, that is, one for enzyme activity and the other for substrate specificity (4).

In this paper, we will report to clone the gene for ALAH III enzyme produced by *Acetobacter turbidans* ATCC 9325, the studies on which have been mainly focussed on the enzymatic synthesis of semi-synthetic cephalosporins (18, 19).

Materials and Methods

Materials

7-Amino-3-deacetoxycephalosporanic acid (7-ADCA) was kindly supplied by Lilly Laboratories, IN, and D-α-phenylglycine methyl ester (PGM) hydrochloric acid was purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Cephalexin, D-α-phenylglycine, isopropyl-β-D-thiogalactoside (IPTG), 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal), protease type XIV (pronase), lysozyme, and ribonuclease type II was purchased from Sigma Chemical Co., MO. Restriction enzymes and T4 polynucleotide kinase from Bethesda Research Laboratories, MD, calf intestinal alkaline phosphatase from Boeringer Mannheim Biochemicals, IN, and T4 DNA ligase, ***Eco*RI methylase, *Eco*RI linker,** and Klenow fragment of DNA polymerase from New England Biolabs, MA were used in recombinant DNA works. As a cloning vehicle, *EcoRI*-cut and bacterial alkaline phosphatase (BAP)-treated λ gtll was commercially available from Promega Biotech., WI. The reagents for the immunological detection was also purchased as a kit from Promega Biotech. The complete Freund's adjuvant from Gibco Laboratories, NY was used in immunization of mouse.

Preparation of chromosomal DNA from *Acetobacter turbidans*

From 1 *l* of cultivated media (18), the cells of *A. turbidans* ATCC 9325 were harvested by centrifugation, and washed with 10 *mM* Tris, 1 *mM* EDTA buffer (TE). The cell wall was disrupted by lysozyme in 25% sucrose, 20 *mM* Tris, and 5 *mM* EDTA solution at 37 °C for 2 hours, and debris were removed by centrifugation at 20,000 rpm for 1 hour. The proteins in supernatant were hydrolyzed with pronase at 37 °C for 2 hours, and extracted with phenol/chloroform (1:1) mixture. The nucleic acids in an aquous phase were precipitated by ethanol, and dissolved in TE buffer. The treatment of RNase at 37 °C for 2 hours afforded the removal of RNAs, and the remaining DNAs were recovered by ethanol precipitation after extraction with phenol/chloroform, and then dissolved in TE buffer after drying.

Construction of genomic library in λgtll

The obtained chromosomal DNA of *A. turbidans* was fractionated into fragments having a size between 5 to 10 kilobases by two different methods. The first methods was sucrose gradient (10-40%) ultracentrifugation technique after partial digestion of chromosomal DNA with *EcoRI*. The other shearing method was also employed, which accompanied shearing DNA through a small bore needle, blocking *Eco*RI sites by *Eco*RI methylase, filling both ends up with Klenow fragment, adding *Eco*RI linker followed by digestion with *Eco*RI, and finally purifying the fragments on agarose gel (20). The chromosomal DNA fragments having *Eco*RI ends obtained from above were ligated with *Eco*RI-cut and BAP-treated λgtll by T4 DNA ligase. After packaging the ligated λgtll DNA in packaging system (Promega Biotech.), these phage particles were infected into *E. coli* Y1090 (r^- m^-). The phage particles in plaques on Luria-Bertani (LB) media were harvested by extraction with SM buffer (0.1 *M* sodium chloride 2 *g/l* magnesium sulfate, 50 *mM* tris), and used as a genomic library of *A. turbidans*.

Immunochemical detection of positive clones

In the selection of positive clones from genomic library in λ gtll, the polyclonal antibody (antiserum) against ALAH III enzyme of *A. turbidans* was used as a probe. This polyclonal antibody was obtained by immunization of mouse BALB/c BYJ, supplied from Jackson Laboratory, ME, with intraperitoneal injection after mixing to make emulsion of 0.25 m*l* of purified enzyme solution (18) with equal volume of complete Freund's adjuvant solution. Two more boosting was made by using the same enzyme solution without Freund's adjuvant 2 weeks and 3 weeks later from the first injection. After confirming the production of antibody, all the blood was collected and the serum fraction was separated by centrifugation after clotting. Using the obtained serum as polyclonal antibody, the proteins

liberated in each plaques of λgtll library were immunologically tested, after induction of gene expression of λgtll phages with 10 *mM* IPTG-soaked nitrocellulose filter paper. The proteins bound on the filter was saturated with 1% bovine serum albumin in 10 *mM* tris, 150 *mM* sodium chloride, and 0.05% Tween 20 (TTBS, pH 8.0), and was treated with polyclonal antibody which could be bound with anti-mouse IgG conjugated with alkaline phosphatase. The colorization of positive plaques was underwent by reacting with nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) as substrates. The plaques showing dark blue color were selected as positive clones.

Confirmation of positive clones

The preparation and purification of phage DNAs in recombinant λgtll was followed by the protocol of Maniatis *et al.* (9). The produced fusion proteins in *E. coli* lysates were confirmed by the electroimmunoblotting technique after running sodium dodecyl sulfate polyacrylamide gel electrophoresis (7), following the production of recombinant proteins from *E. coli* Y1089 lysogenized with the λgtll clones (8).

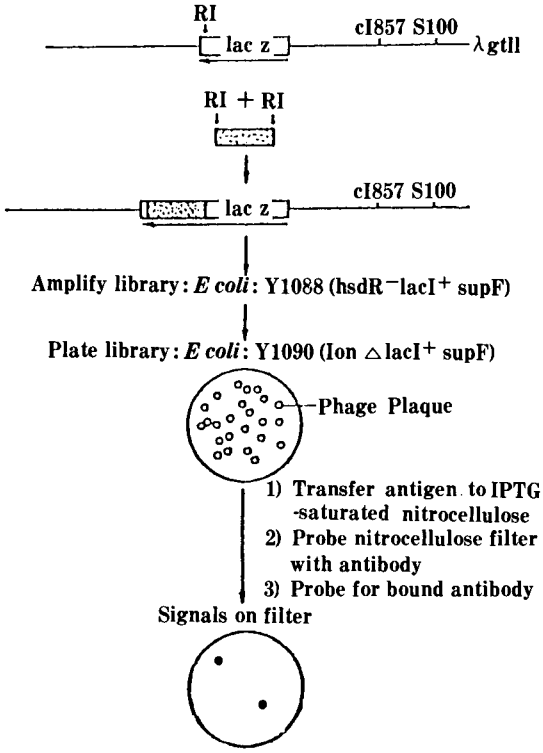

Fig. 1. The representative diagram for molecular cloning of foreign genes in an expression vector, λgtll.

Results and Discussion

Molecular cloning of the gene for ALAH III enzyme in λgtll

In molecular cloning of the gene for ALAH III enzyme from *A. turbidans,* λgtll phage DNA was chosen as a cloning vector in order to overcome an expression problem when foreign DNA was inserted in bacterial plasmids. The λgtll was originally designed as an expression vector capable of producing a polypeptide specified by the DNA insert fragment (8, 20). Thus, phage DNA libraries cloned in λgtll could be screened with polyclonal antibody probe (antiserum) to isolate DNA sequences encoding the antigens (target proteins) against which the antibodies are directed, as illustrated in Fig. 1.

In this study, the commercial λgtll vector DNA, which was already digested with *Eco*RI and treated with BAP enzyme, was ligated with the genomic libraries of *A. turbidans* having *Eco*RI-end and sized between 4 to 10 kilobases, and infected into *E. coli* Y1090 after packaging in λ envelope proteins. The obtained titer of ligated phages was 2.2×10^4 plaque forming unit (pfu)/ml of λgtll which was nearly 50% of total plaques when genomic libraries were made by partial digestion with *Eco*RI, whereas it was 4.2×10^4 pfu/ml of λgtll DNA which was 70% of total plaques when made by shearing chromosomal DNA and ligated with *Eco*RI linker, based on the calculation of white plaques among total in the presence of X-gal by inactivation of gene for β-galactosidase.

By immunoblotting method after transferring proteins produced by induction with IPTG in λgtll lysates to nitrocellulose filter, two positive clones which could bind with polyclonal antibody for ALAH III of *A. turbidans* were screened out through the test of all the phages as seen in Fig. 2. These two positive clones, however, showed slightly different immunochemical responses, one of which gave a darker color with the larger plaque (λgtll dn1) compared with the other one (λgtll dn2).

Analysis and confirmation of positive clones

In order to confirm the size of proteins produced by inserts in λgtll clones, the selected phages

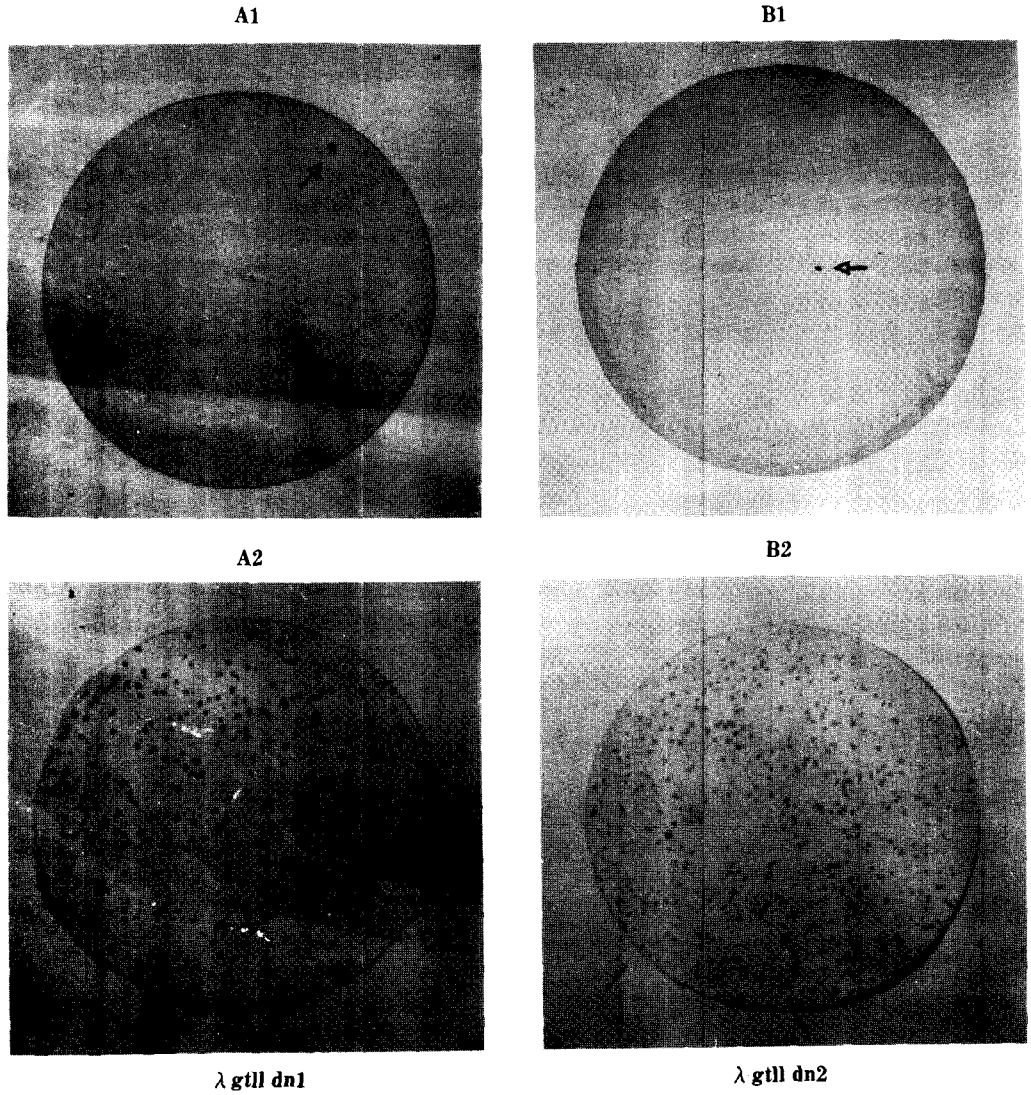

Fig. 2. Selection of positive clones by immunochemical detection method using antiserum (polyclonal antibody) against ALAH III enzyme of *A. turbidans* as a probe.
A1, B1; positive clones obtained at the first screening. A2, B2; the purified plaques of A1 and B1 positive clones.

were infected to *E. coli* Y1089 (*hflA*) to lysogenize, and the produced proteins were separated as crude cell lysates by freezing and thawing technique. By immunoelectroblotting method after running sodium dodecyl sulfate polyacrylamide gel (Fig. 3), it was found that the product from first clone was 105 kilodaltons which bound with anti-ALAH III serum very well but bound with anti-β-galactosidase weakly (λ gtll dn1), and that the other product from second clone had a molecular weight of 180 kilodaltons which showed opposite properties in binding with antibodies (λ gtll dn2).

The restriction mapping of these two phage DNA extracted from each clones was performed by using *Eco*RI, *Bam*HI, *Hind*III, *Xba*I, and *Pvu*I endonucleases (Fig. 4). It was so strange that both of two phage recombinants lost one of two *Eco*RI sites located at both ends of inserts. However, both of two recombinants have somewhat identical sequences at the downstream of cloned inserts, as drawn in Fig. 5 based on the results of restriction digestion.

It has been reported previously that ALAH III enzyme of *A. turbidans* is composed of two pairs of two nonidentical subunits as large as 70 kilodaltons (18). This means that the sizes of DNA encoding each subunits are around 2 kilobases, and that total gene size for this enzyme should be larger than 4 kilobases at least. Another consideration can be taken that the maximal insertional size of DNA in λ gtll cannot exceed 7.5 kilobases for proper packaging and infection of host cells (8).

From the above facts, it could be imagined that inserts larger than 7.5 kilobases were incorporated into λ gtll DNA, and that some parts of *lac*Z gene and inserted DNA were deleted during replication in host cells. This is reason why λ gtll dn1 could synthesize the protein product smaller than β-galactosidase fused originally (114 kilodaltons) but larger than ALAH III subunits (70 kilodaltons) as shown in Fig. 3. It could be also explained by this phenomenon that both clones lost *Eco*RI site between β-galactosidase gene and inserted DNA portion.

The restriction analysis showed that λ gtll dn1 is 3.5 kilobases larger than λ gtll whereas λ gtll dn2 is 5.3 kilobases larger. The real inserted sizes in both recombinants might be bigger than these values, if some parts of gene had been deleted.

Using the inserted DNAs in these clones as probes, molecular cloning of full gene for ALAH III

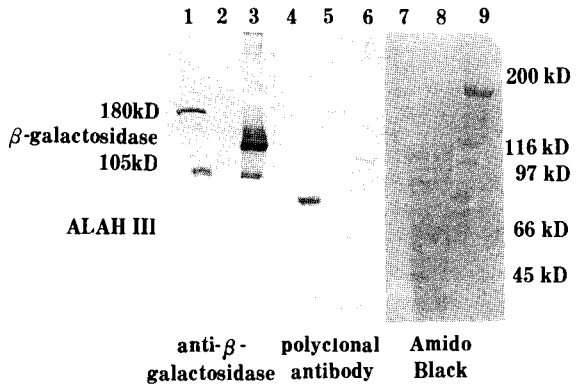

Fig. 3. Immunochemical identification of proteins produced from two recombinant phages by using Western blotting technique after running 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
Lane 2, 6, 8; total cell proteins of *E. coli* Y1089 (λ gtll dn1) Lane 1, 5, 7; total cell proteins of *E. coli* Y1089 (λ gtll dn2) Lane 3, 9; high molecular weight protein marker. Lane 4; ALAH III enzyme from *A. turbidans*.

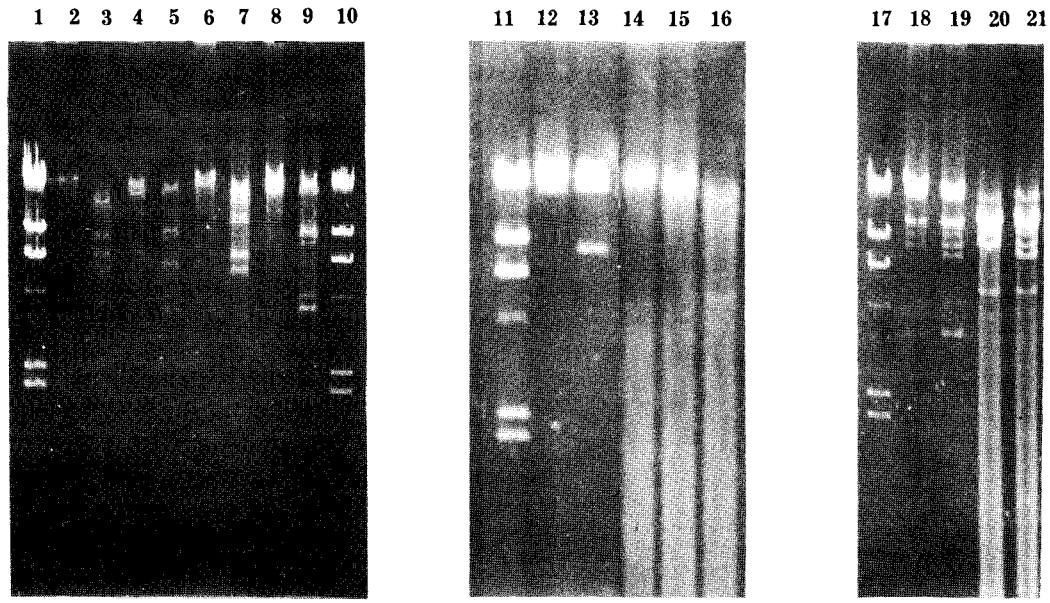

Fig. 4. Restriction pattern of two positive clones.
Lane 1, 10, 11, 17; λ DNA/*Hind*III. Lane 2; λgtll dn1 DNA. Lane 3; λgtll dn1/*Bam*HI. Lane 4; λgtll dn1/*Eco*RI. Lane 5; λgtll dn1/*Hind*III. Lane 6, 14; λgtll dn2 DNA. Lane 7; λgtll dn2/*Bam*HI. Lane 8; λgtll dn2/*Eco*RI. Lane 9; gtll dn2/*Hind*III. Lane 12; λgtll dn1/*Xba*I. Lane 13; λgtll dn1/*Xba*I and *Eco*RI. Lane 15; λgtll dn2/*Xba*I. Lane 16; λgtll dn2/*Xba*I and *Eco*RI. Lane 18; λgtll dn1/*Pvu*I. Lane 19; λgtll dn1/*Pvu*I and *Eco*RI. Lane 20; λgtll dn2/*Pvu*I. Lane 21; λgtll dn2/*Pvu*I and *Eco*RI.

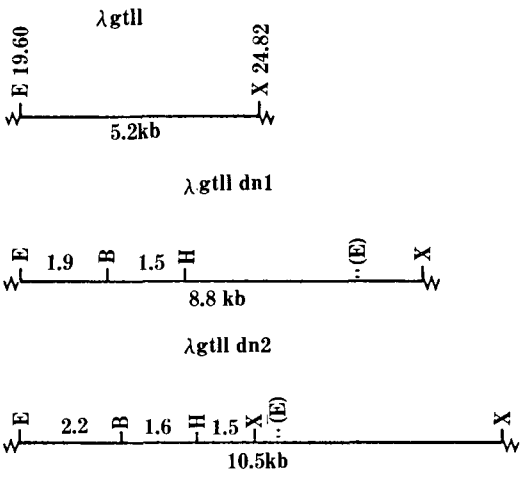

Fig. 5. Restriction maps between *Eco*RI and *Xba*I sites in two positive clones.
E; *Eco*RI. B; *Bam*HI. H; *Hind*III. X; *Xba*I. (E); *Eco*RI site considered to be deleted.

enzyme is now under study.

요 약

반합성 베타 락탐 항생물질의 가수분해 및 합성을 촉매하는 효소인 α-acylamino-β-lactam acylhydrolase(ALAH)의 유전자를 *Acetobacter turbidans* 로부터 클론화하기 위한 연구를 수행하였다. 우선 순수 분리정제된 효소에 대한 항혈청(폴리클론 항체)을 제조한 다음 이를 probe로 하여 면역화학적 방법으로 유전자의 선별을 시도하였다. 이러한 용도로 개발된 운반체인 λgtll에다 *A. turbidans*의 유전자 단편들을 삽입하여 genomic library를 제조한 후 이 library에서 유전자를 선별한 결과 두개의 positive clone을 얻을 수 있었다. 그러나, 이 두 clone들은 면역화학적으로 서로 다른 반응을 나타내었는데, 그 중 하나는 효소의 항혈청과는 잘 결합하나 융합되어진 베타 갈락토시다아제에 대한 항체와는 잘 결합하지 못하였고(λgtll dnl), 또다른 clone은 이와 반대의 양상을 보여주었다(λgtll dn2). 더구나 이들 clone을 여러 제한효소들로 분석해본 결과, 유전자가 삽입된 부분인 *Eco*RI 부위중 하나가 없어진 것을 알 수 있었다. 따라서 *A. turbidans*의 효소에 대한 유전자가 λgtll에 클론화되었으나 이 유전자와 베타 갈락토시다아제의 유전자(*lac*Z)간에 염기배열상 동위성이 있은 부위가 존재하여 재조합된 λgtll 파지의 복제과정에서 삭제되어진 것으로 간주되어진다.

References

1. Barbero, J.L., J.M. Buesa, G.G. de Buitrago, E. Mendez, A. PerezAranda, and J.L. Garcia: *Gene* **49,** 69 (1986).
2. Boeck, A., R. Wirth, G. Schmid, G. Schimacher, G. Lang, and P. Buckel: *FEMS Microbiol. Letters* **20,** 141 (1983).
3. Bruns, W., J. Hoppe, H. Tsai, H.J. Bruening, F. Maywald, J. Collins, and H. Mayer: *J. Mol. Appl. Genet.* **3,** 36 (1985).
4. Daumy, G.O., D. Danley, and A.S. McColl: *J. Bacteriol.* **163,** 1279 (1985).
5. Daumy, G.O., J.A. Williams, A.S. McColl, T.J. Zuzel, and D. Danley: *J. Bacteriol.* **168,** 431 (1986).
6. Garcia, J.L. and J.M. Buesa: *J. Biotechnol.* **3,** 187 (1986).
7. Gooderham, K.: Methods in Molecular Biology, Vol. 1. Proteins (Walker J.M., ed.) Humana Press, 165 (1984).
8. Hyunh, T.V., R.A. Young, and R.W. Davis: DNA Cloning (Glover, D.M., ed.) IRL Press, Vol. 1, 49 (1985).
9. Maniatis, T., E.F. Fritsch, and J. Sambrook: Molecular Cloning. A Laboratory Manual, Cold Spring Harbor, 75 (1982).
10. Marquez, G., J.M. Buesa, J.L. Garcia, and J.L. Barbero: *Appl. Microbiol. Biotechnol.* **28,** 144 (1988).
11. Matsuda, A. and K. Komatsu: *J. Bacteriol.* **163,** 1222 (1985).
12. Mayer, H., J. Collins, and F. Wagner: *Enzyme Eng.* **5,** 61 (1980).
13. McCullough, J.E.: *Bio/Technol.* **1,** 879 (1983).
14. Meevootisson, V. and J.R. Saunders: *Appl. Microbiol. Biotechnol.* **25,** 372 (1987).
15. Nam, D.H., C. Kim, and D.D.Y. Ryu: *Biotechnol. Bioeng.* **27,** 953 (1985).
16. Oliver, G., F. Valle, F. Rosetti, M. Gomez-Pedrozo, P. Santamaria, G. Gosset, and F. Bolivar: *Gene* **40,** 9 (1985).
17. Olsson, A., T. Hagstroem, B. Nilssom, M. Uhlen, and S. Gatenbeck: *Appl. Environ. Microbiol.* **49,** 1084 (1985).
18. Ryu, Y.W. and D.D.Y. Ryu: *Enzyme Microbiol. Technol.* **9,** 339 (1987).
19. Takahashi, T., Y. Yamazaki, and K. Kato: *Biochem. J.* **137,** 497 (1974).
20. Young, R.A. and R.W. Davis: Genetic Engineering (Setlow, J. and A. Hollander, ed.) Plenum Press, Vol. 7, 29 (1985).

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