# CLONING OF MACROMOMYCIN APOPROTEIN GENE FROM *STREPTOMYCES MACROMOMYCETICUS* BY USE OF 50-MER DEOXYNUCLEOTIDE PROBES

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A mixed probe consisting of two synthetic deoxynucleotides (52 and 54 mers referred to as 50-mer) with arbitrarily chosen C or G for the third letters was prepared based on the amino acid sequences No. 31~48 and No. 72~90 of macromomycin (MCM) apoprotein and successfully used to clone the MCM apoprotein gene. Digestion with *Sph* I of total DNA of MCM-producing *Streptomyces macromomyceticus* M480-M1 yielded a 2.6-kb fragment that hybridized strongly to the probes. The hybridized probe was stable to washing with 3×SSC at 75°C. Radioactivity derived from the hybridized probe was comparable to that expected theoretically from hybridization between the probe and the true target sequence. The 2.6-kb fragment was cloned into *Escherichia coli* RR1 with pBR322 and subsequently subcloned into *Streptomyces lividans* TK21 with pIJ702. Nucleotide sequence analysis of the cloned fragment verified the exsistence of the sequence corresponding to the amino acid sequence of MCM apoprotein and about 90% homologies with the probes. Thus, the use of relatively long deoxynucleotide probes with arbitrarily chosen C or G for the third letters will be advantageous in cloning *Streptomyces* protein genes where more than 90% of the third letters have been known to be C or G. In addition, theoretical diagnosis of hybridization should be a great help to distinguish true positives from false ones.

Actinomycetes have been best remarked by their productivity of wide varieties of biologically active secondary metabolites such as antibiotics and enzyme inhibitors. Biosynthesis of these metabolites results from a concerted action of a series of biosynthetic enzymes involving relatively complicated regulations. Genes involved in antibiotic biosynthesis have been cloned and characterized for their structures, arrangements and regulation of expression[1~13].

We have studied the enzymes involved in the biosynthesis of leupeptin[14] (an enzyme inhibitor), 2-deoxystreptamine[15] and valanimycin[16] and now are interested in mechanisms controlling expression of various *Streptomyces* enzyme genes.

Macromomycin (MCM) is a complex protein with antitumor activity produced by a strain of *Streptomyces macromomyceticus*[17]. MCM consists of a chromophore capable of cleaving DNA strands and an apoprotein comprizing 112 amino acids and the latter is believed to stabilize the former[18,19]. Since the amino acid sequence of MCM apoprotein was determined[20], it seemed possible to clone the MCM apoprotein gene and thereby to study its structure and mechanism controlling its expression.

In order to clone protein genes, mixed probes consisting of all possible combination of chemically synthesized deoxyoligonucleotides corresponding to appropriate amino acid sequences of the target proteins have been widely used. In the case of *Streptomyces* genes, however, it seems not necessary to use such mixed probes because more than 90% of the third letters of their codons are C or G[21]. In fact, an $\alpha$-amylase gene[22] and a tylosin biosynthetic gene[23], whose codons were postulated to comprize C or G at their third letters, were cloned by use of mixed deoxynucleotide probes consisting of two 14 mers and all possible 44 mers, respectively. In such experiments, however, one can often encounter false positive bands or clones. Although numbers of reports have been published in this field, little has been described about how to distinguish true positives from false ones. In order to clone the MCM apoprotein gene, we used a mixed probe consisting of two relatively long deoxynucleotides (52~56 mers) in which the third letters were arbitrarily C or G. We report here that a high thermostability of hybridization between the probe and the true target sequence and a logical diagnosis of hybridization led to the successful cloning of the MCM apoprotein gene.

## Materials and Methods

### Strains and Plasmids

MCM-producing *S. macromomyceticus* M480-M1 was obtained from the Institute of Microbial Chemistry. *Escherichia coli* RR1 and *Streptomyces lividans* TK21, and plasmids pBR322 and pIJ702 were used as hosts and vectors, respectively, for gene manipulation.

### Preparation of DNA

Total DNA from *S. macromomyceticus* M480-M1 was prepared as reported by HOTTA *et al.*[24]. Plasmid DNAs (pBR322 and pIJ702) were extracted from *E. coli* RR1 and *S. lividans* TK21 by the method of BIRNBOIM and DOLY[25], and cccDNAs were prepared by the CsCl-EtBr density gradient centrifugation. DNA fragments to be cloned were electroeluted from electrophoresed agarose gels by the method of GIRVITZ *et al.*[26].

### Deoxynucleotide Probes

As shown in Fig. 1, four different deoxynucleotides (52~56 mers) corresponding to No. 31~48 (probes I and II) and No. 72~90 (probes III and IV) amino acid residues of MCM apoprotein were synthesized by the phosphite coupling method[27] using a DNA synthesizer (Applied Biosystems Model 380A), electrophoresed on a 7-M urea - 8% polyacrylamide gel and eluted from the gel as described by MAXAM and GILBERT[28]. The probes I and III are complementary with the probes II and IV, respectively. These deoxynucleotides were labeled with [$\gamma$-$^{32}$P]dCTP and T4 polynucleotide kinase

Fig. 1. Base sequence of probes for macromomycin apoprotein.
R: Purine (A/G), Y: pyrimidine (T/C), L: A/C/T, * A/G/C/T.

| | 31 | | | | 35 | | | | | 40 | | | | | 45 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Amino acid | -Tyr | —His | —Val | —Gly | —Gln | —Cys | —Ala | —Val | —Val | —Glu | —Pro | —Gly | —Val | —Ile | —Gly | —Cys | —Asp | —Ala- | |
| DNA | TAY | CAY | GT* | GG* | CAR | TGY | GC* | GT* | GT* | GAR | CC* | GG* | GT* | ATL | GG* | TGY | GAY | GC* | |
| Probe I | TAC | CAC | GTC | GGC | CAG | TGC | GCC | GTC | GTC | GAG | CCC | GGC | GTC | ATC | GGC | TGC | GAC | GC | |
| Probe II | | TG | GTG | CAG | CCG | GTC | ACG | CGG | CAG | CAG | CTC | GGG | CCG | CAG | TAG | CCG | ACG | CTG | CG |

| | 72 | | | 75 | | | | 80 | | | | | 85 | | | | | 90 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Amino acid | -Phe | —Gln | —Ala | —Val | —Val | —Gly | —Ala | —Asp | —Gly | —Thr | —Pro | —Trp | —Gly | —Thr | —Val | —Asn | —Cys | —Lys | —Val- |
| DNA | TTY | CAR | GC* | GT* | GT* | GG* | GC* | GAY | GG* | AC* | CC* | TGG | GG* | AC* | GT* | AAY | TGY | AAR | GT* |
| Probe III | TTC | CAG | GCC | GTC | GTC | GGC | GCC | GAC | GGC | ACC | CCC | TGG | GGC | ACC | GTC | AAC | TGC | AAG | GT |
| Probe IV | | G | GTC | CGG | CAG | CAG | CCG | CGG | CTG | CCG | TGG | GGG | ACC | CCG | TGG | CAG | TTG | ACG | TTC | CA |

(Takara Shuzo Co., Ltd., Japan) to a specific activity of $1.4\sim2.0\times10^8$ cpm/$\mu$g as described[29].

Southern Blot Hybridization

Total genomic DNA from *S. macromomyceticus* M480-M1 or plasmid DNA was completely digested with appropriate restriction endonucleases (Takara Shuzo Co., Ltd., or Toyobo Co., Ltd., Japan), electrophoresed on a 0.8% agarose gel in 1×TAE buffer containing 0.5 $\mu$g/ml of EtBr and then transferred onto a nitrocellulose filter (Schleicher and Schuell BA85, pore size 0.45 $\mu$m) as described by SOUTHERN[30]. The filter was prehybridized for 5 hours at 42~55°C in a solution (5 ml) consisting of 6×SET, 10×DENHARDT's solution, 0.1% SDS and 100 $\mu$g/ml of sonicated and heat-denatured calf thymus DNA in a plastic bag. For hybridization, the solution was replaced with a fresh one containing the heat-treated probes at a concentration of 6 ng/ml ($4.2\sim6.0\times10^6$ cpm in total) and incubated at 42~55°C for 40~45 hours. After hybridization, the filter was washed with 4×SSC (100~200 ml) followed by the same volume of 3×SSC, each at 40~60°C for 30~60 minutes and air-dried. Autoradiography was performed by exposing an X-ray film (Fuji X-ray film RX) to the air-dried filter at −80°C on an intensifying screen (Cronex Lighting Plus, Du Pont).

Cloning of MCM Apoprotein Gene

After the total DNA (8 $\mu$g) of strain M480-M1 was digested with *Sph* I and subsequently electrophoresed on a 0.8% agarose gel, 2.0~4.4 kb fragments were electroeluted from the gel, phenol-extracted and ethanol-precipitated. The fragments (about 400 ng) were ligated with T4 DNA ligase (Toyobo Co., Ltd.) to pBR322 which had been cut with *Sph* I and dephosphorylated with calf intestine alkaline phosphatase (CIAP; Takara Shuzo Co., Ltd.) in advance. *E. coli* RR1 was transformed with the ligated mixture and transformants were allowed to grow on LB agar plates containing 40 $\mu$g/ml of ampicillin at 37°C overnight. Colonies were replica plated onto nitrocellulose filters which were then transferred on LB agar plates containing 150 $\mu$g/ml of chloramphenicol. After incubation at 37°C overnight, the filters were subjected to denaturation with 0.5 N NaOH - 1.5 M NaCl and neutralization with 0.5 M Tris-HCl (pH 8.0) - 1.5 M NaCl. The filters were then air-dried, baked at 80°C for 2 hours and subjected to hybridization with the 50-mer probes as described above. Prehybridization and hybridization were performed at 55°C for 18 and 44 hours, respectively. The filters were washed at 55°C with 4×SSC followed by 3×SSC. Positive colonies were transferred onto a LB agar plate containing 12.5 $\mu$g/ml of tetracycline to check the insertional inactivation of the tetracycline resistance gene in pBR322. Plasmid DNAs were extracted from the positive clones and examined for the 2.6-kb *Sph* I insert hybridizable to the probes by Southern blot hybridization. For subcloning, the 2.6-kb fragment from the recombinant plasmid (pMCM1) was ligated to pIJ702 which had been cut with *Sph* I and dephosphorylated with CIAP. *S. lividans* TK21 was transformed as described by CHATER *et al.*[31].

Thermal Dissociation of Hybridized Probe

After autoradiography of the hybrids between the probes and genomic DNA fragments, a band with clear hybridization signal was cut out of the nitrocellulose filter and placed into a plastic scintillation vial (Beckman, U.S.A.) containing 1 ml of 3×SSC prewarmed at 50°C. After incubation for 10 minutes, the filter was taken out of the vial, blot-dried and measured for radioactivity with a scintillation counter (Beckman LS 1801). The radioactivity released into the buffer was also measured. Then, the filter was put back into the plastic vial containing fresh 3×SSC kept at an elevated temperature by 5°C and incubated for 10 minutes. This procedure was repeated until the filters were free of radioactive counts.

Nucleotide Sequence Analysis

The 1.2-kb *Sac* I region of the cloned 2.6-kb *Sph* I fragment that contains the sequence hybridizing to the probes was subcloned into pUC118 and sequenced by an improved dideoxy chain termination method[32] by use of 7-deaza-dGTP and a sequencing kit (Takara 6015). Deletion fragments[33] were also constructed using a deletion kit (Takara 6030). Single stranded DNA templates for sequence determination were prepared using the pUC118-M13K07 system[34] (Takara 3318). The 7-M urea - 8% polyacrylamide gel electrophoresis was performed using Fuji Gensor gel sheet (Fuji Photo Film

Co., Ltd., Japan). DNA sequence analysis was performed using DNASIS programs (Hitachi SK, Japan).

## Results

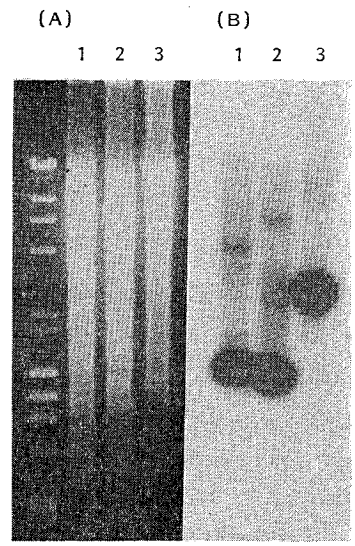
### Genomic DNA Fragments Hybridizing to the Probes

Prior to cloning the MCM apoprotein gene from *S. macromomyceticus* M480-M1, we examined Southern blot hybridization between the fragments of total genomic DNA digested with appropriate restriction endonucleases and the 50-mer deoxynucleotide probes. As shown in Fig. 2, single digestion with *Bam*H I, *Sac* I and *Sph* I provided 1.2~1.3 kb, 1.3~1.4 kb and 2.5~3.0 kb fragments, respectively, hybridizing strongly to a mixture of probes II and IV. Furthermore, dobule and triple digestions with these enzymes were also attempted. All of the digestions even in combination with *Eco*R I and *Sal* I provided one clear positive band as summarized in Table 1. The bands were the same in size with every probe and the smallest band of 1.2~1.3 kb was the one observed with single digestion with *Sac* I.

A thermal dissociation experiment with the hybrid between the probe and the *Sph* I fragment was conducted to know thermal stability of the hybridization (Fig. 3). The radioactivity released from the band was about 2,300 cpm. The hybrid started dissociating at 80°C and completely dissociated at 85°C.

Based on the following logic, the result of this experiment further led us to believe that the hybridized fragment was the right one. Eight $\mu$g of total DNA used for genomic hybridization was expected to contain about 1.2 fmol of MCM apoprotein gene on the assumption that the genome size of *Streptomyces* was $1\times10^4$ kb, the size of the MCM apoprotein gene was 1 kb (MW=$6.6\times10^5$ daltons) and a single copy of the gene existed per genome. If the probe hybridize with the gene at a mol/mol ratio, the radioactivity of the hybridized probe (1.2 fmol) was expected to be 2,750 cpm by calculation based on the MW of the 50-mer (16,500 daltons) and its specific activity ($1.4\times10^8$ cpm/$\mu$g). The actual cpm value obtained (2,300 cpm) was comparable to the value expected. Thus, it was strongly suggested that the sequence of the probe was highly complementary to that of the MCM apoprotein gene and that the two sequences hybridized with each other at the molar ratio of 1:1. The spots observed on the lanes of *Bam*H I and *Sac* I (Fig. 2) provided similar results to that obtained with *Sph* I fragment (data not shown).

Fig. 2. Genomic DNA hybridization with a mixed probe.




1: *Bam*H I digestion, 2: *Sac* I digestion, 3: *Sph* I digestion. (A) Agarose gel electerophoresis. A mixture of *Hind* III-digested $\lambda$ and *Hae* III-digested $\phi$X 174 DNAs were used as size markers. (B) Southern blot hybridization with a mixed probe consisting of probes II and IV.

Hybridization was performed at 42°C and the filter was washed at 60°C. X-Ray film was exposed for 20 hours.

Table 1. Genomic DNA fragments hybridized to the probes.

| Digestion | Hybridized fragments (kb) | | | |
|---|---|---|---|---|
| | Probe II | Probe III | Probe IV | Probe II & IV |
| *Eco*R I | >20 | >20 | >20 | |
| *Sal* I | ~3.2 | ~3.2 | ~3.2 | |
| *Sal* I/*Eco*R I | ~3.2 | | | |
| *Sph* I | ~2.6 | ~2.6 | | ~2.6 |
| *Sph* I/*Eco*R I | ~2.6 | ~2.6 | | |
| *Bam*H I | ~1.4 | ~1.4 | ~1.4 | ~1.4 |
| *Sac* I | | ~1.3 | | ~1.3 |
| *Sac* I/*Eco*R I | | ~1.3 | | |
| *Sac* I/*Eco*R I/*Sph* I | | ~1.3 | | |

Fig. 3. Dissociation of hybridized probe.

● cpm remained on the filter, ○ cpm released into the buffer.

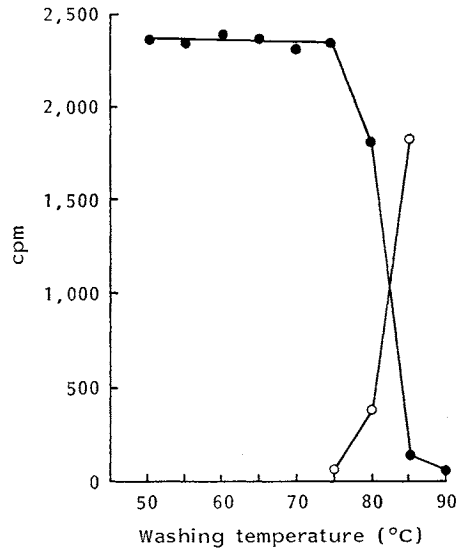
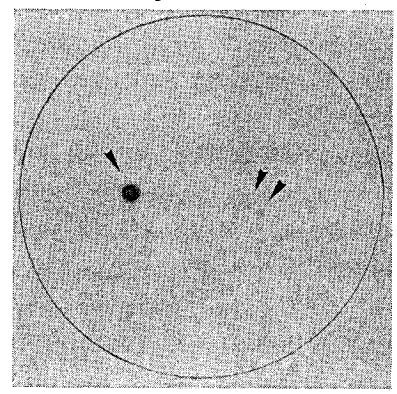


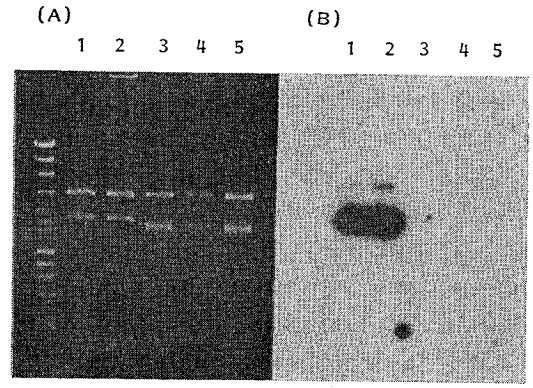

Fig. 4. Colony hybridization of transformed *Escherichia coli* with a mixed probe.

Arrows indicate positive clones.



After hybridization with a mixture of probes II and IV. X-Ray film was exposed for 18 hours.

Fig. 5. Southern hybridization of plasmid pMCM1 with a mixed probe.

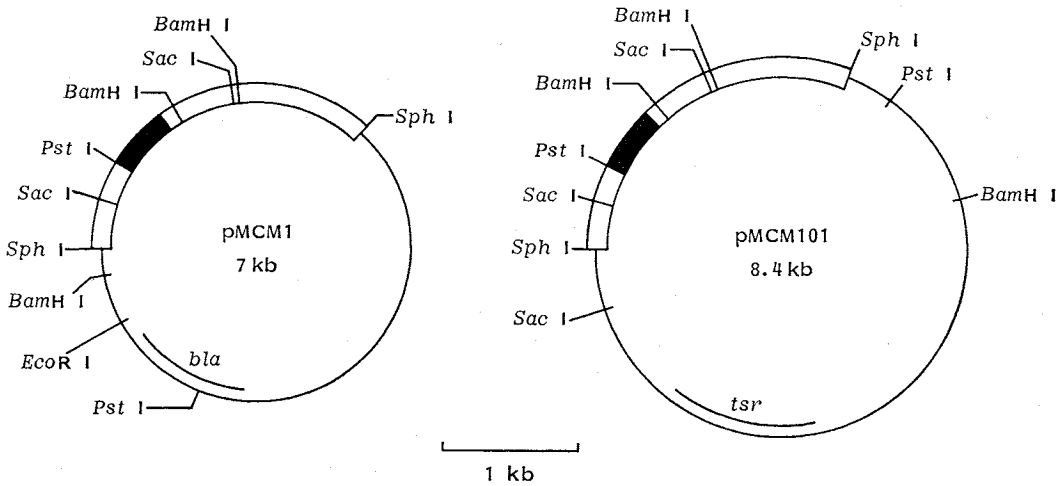



Plasmid DNAs extracted from positive colonies upon colony hybridization were digested with *Sph* I, electrophoresed (A) and hybridized with a mixture of probes II and IV (B). X-Ray film was exposed for 18 hours.

## Cloning of the 2.6-kb *Sph* I Fragment

Since the MCM apoprotein consists of 112 amino acid residues, its gene will span about 0.5 kb including its control region and leader sequences. Although the *Sac* I and *Bam*H I fragments, 1.3 kb and 1.4 kb, respectively, were larger than the expected gene size, it seemed risky to believe that they cover the entire MCM apoprotein gene. So we chose a longer one, the 2.5~3.0-kb *Sph* I fragment, that should cover the entire *Sac* I region. About 400 ng of the *Sph* I fragments extracted from a 2.0~4.4-kb

Fig. 6. Restriction map of the cloned 2.6-kb *Sph* I fragment.




Open boxes and shaded regions in plasmids refer to the 2.6-kb *Sph* I fragment and the coding sequence region of MCM apoprotein gene, respectively.

Fig. 7. Homology between probes and true target sequence.

Asterisks indicate mismaches.

```
Probe II   TGGTGCAGCCGGTCACGCGGCAGCAGCTCGGGCCGCAGTAGCCGACGCTGCG
                 *        * *     *      *           *
           ~ACCACGTGGGCCAGTGTGCGGTCGTGGAGCCGGGCGTCATCGGCTGTGACGC~

Probe IV   GGTCCGGCAGCAGCCGCGGCTGCCGTGGGGGACCCCGTGGCAGTTGACGTTCCA
                 *        *     * *       *
           ~CCAGGCCGTGGTCGGCGCCAACGGCACGCCGTGGGGCACGGTCAACTGCAAGGT~
```

area of an electrophoresed agarose gel was ligated to the *Sph* I site of pBR322. *E. coli* RR1 was transformed with the ligated mixture. Transformants were checked for ampicillin resistance and tetracycline susceptibility and screened for the DNA sequence hybridizing to the probe by colony hybridization. Consequently, a couple of tens of apparently positive colonies with varied intensities of hybridization signal appeared among about 2,500 transformants tested (Fig. 4). Plasmids were extracted from these clones and examined for insertion of the target sequence by Southern hybridization using the probes II and IV (Fig. 5). The clones with weak intensities were found to be false positives, while those with strong hybridization signals were found to contain the 2.6-kb *Sph* I segment hybridizing to the probes as shown in Fig. 5. A restriction map of the insert in a recombinant plasmid, pMCM1, is shown in Fig. 6. The 2.6-kb *Sph* I fragment from pMCM1 was then subcloned into *S. lividans* TK21 with pIJ702 after ligation with the *Sph* I site of pIJ702. The recombinant plasmid was designated as pMCM101 (Fig. 6).

## Homology between the 50-mer Probes and the Cloned Sequence

Nucleotide sequence analysis verified the nucleotide sequence corresponding to the MCM apoprotein in the 1.2-kb *Sac* I region of the cloned 2.6-kb *Sph* I fragment (Fig. 6), indicating that our clone was the right one. Although the entire sequence was not elaborated here, the sequences cor-

responding to the amino acids No. 31~48 and No. 72~90 derived from the cloned fragment were shown in comparison with the 50-mer probes II and IV (Fig. 7). In total, only 5 base mismatches were found only in the third letters between the probes and their corresponding sequences and the homology was about 90% with both probes. It should be noted that more than 90% of the third letters were C or G; 15 out of 17 in the region of No. 31~48 and 18 out of 18 in the region of No. 72~90.

## Discussion

In *Streptomyces* genes, it has been known that more than 90% of the third letters of codons are C or G[21]. On the basis of this fact, the synthesis and use of a few species of 52~56 base long deoxynucleotide probes with arbitrarily chosen C or G as the third letters led to the successful cloning of the MCM apoprotein gene. High homology (90%) between the probes and the gene sequence and relatively high thermal stability (75°C) of their hybridization should suggest that single use of any one of the four probes would be good enough for the cloning. Generally, mixed probes consisting of all possible combination of deoxyoligonucleotides complementary to an appropriate amino acid sequence of a given target protein have been widely used for this type of experiment. Before this study, we also attempted the use of mixed 14-mer deoxyoligonucleotide probes containing all possible sequences for cloning of the MCM apoprotein gene under a relaxed hybridization condition. However, we observed several bands upon genomic hybridization and a shot gun cloning resulted in only false positive fragments although true-positive clones should have been obtained (data not shown). By contrast, the use of the 50-mer deoxynucleotide probes visualized only one fragment that hybridized strongly under relatively high-stringent condition of hybridization. The hybrid was stable to washing at 75°C with 3×SSC in spite of 10% mismatch between the probes and the cloned nucleotide sequence. In the hybridization using deoxyoligonucleotide probe, such stringent conditions can not be employed because of much lower thermo-stability. In addition, it has been known that one base mismatch (less than 10%) between the deoxyoligonucleotide probe and the target sequence loweres the thermo-stability of the hybrid by more than 10°C[29]. Thus, the use of a limited species of relatively long deoxynucleotide probes, which is getting popular recently[35], will be more advantageous for the detection and cloning of *Streptomyces* protein genes. A similar strategy with long probes was also attempted for cloning biosynthetic genes for cephalosporin[36] and tylosin[23]. In any case, however, it seems likely that false positives are detected and cloned. Usually, false positives can be distinguished after a laborious work of nucleotide sequence analysis. In this regard, the logical analysis for true positive bands should be a very important procedure for this type of study as we described in this paper.

Possible production of the MCM apoprotein by *S. lividans* TK21 harboring pMCM101 was also tested by determining the aminopeptidase activity of the protein[37] in the fermentation broths. However, since strain TK21 without pMCM101 also produced an aminopeptidase with similar substrate specificity, the production level of the MCM apoprotein could not be estimated. The molecular size and stability of these enzymes were somewhat different (data not shown).

Uniqueness of the MCM apoprotein gene, if any, is another interest. In *S. macromomyceticus* M480-M1, there was a fragment hybridized weakly to the probes upon genomic DNA hybridization in addition to the one containing the MCM apoprotein gene. This probably resulted in cloning a false gene in the shot gun cloning experiment. On the other hand, it is of special interest to know the gene structures for MCM-related antibiotics such as actinoxanthin and neocarzinostatin, share some homology with MCM apoprotein in amino acid sequence[20]. The neocarzinostatin gene was already cloned[38]. The entire sequence of the MCM apoprotein gene is being analyzed and will be reported in our next paper.

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