PCR Conditions for a GC-Rich Gene

Nobuo Sakata*, Souichi Ikeno and
Makoto Hori

Showa College of Pharmaceutical Sciences,
Machida-shi, Tokyo 194, Japan

(Received Oct. 20/

Accepted Oct. 31)

Streptomyces genomes have high GC contents, i.e. $71 \pm 3\%$. We previously sequenced the cloned macromycin apoprotein (mcma) gene of *Streptomyces macromyceticus* and found that the GC content throughout the structural gene was 72%¹⁾. Recently, aphE gene of *Streptomyces griseus* was cloned²⁾ by the polymerase chain reaction (PCR). To our knowledge, this was the first case of PCR cloning of a *Streptomyces* gene but no details of PCR conditions were given. In the present paper, we describe the PCR conditions suitable for amplifying and cloning a GC-rich gene. We attempted to apply PCR as a step to clone the structural gene of antibiotic C-1027 apoprotein from *Streptomyces globisporus* C-1027. The amino acid se-

quence of the apoprotein³⁾ is shown in Fig. 1. We designed 5' and 3' primers based on the amino acid sequences around Y³¹-Q³⁵ and D⁸⁵-A⁸⁹, respectively (Fig. 1). Each primer has a GG clamp followed by a restriction site at the 5' end⁴⁾. The 3rd letters of their codons were arbitrarily C or G considering the *Streptomyces* codon usage. Upon hybridization with C-1027 DNA, dissociation temperature (Td) was estimated to be 74°C for both primers by summing up

10	20	30
APAFS	VSPAS	GLSDG
40	50	60
YYIAQ	CAPVG	GQDAC
70	80	90
ASFSF	VVRKS	YTGST
100	110	
CNLGA	GNSGL	DLGHV
		ALTFG
	Y ³¹	Q ³⁵
5'primer:	5'GGGCATGCTACTACATCGCCAG3'	
	SphI	
	D ⁸⁵	A ⁸⁹
3'primer:	3'CTGACGCGGTGGCGCTTAAGGG5'	
	EcoRI	

Fig. 1. Amino acid sequence of C-1027 apoprotein and PCR primers.

Fig. 2. PCR products from *S. globisporus* C-1027 DNA.

The reaction mixture (100 μ l) contained 20mM Tris-HCl (pH 8.0), 1.5mM MgCl₂, 25mM KCl, 0.05% Tween20, 50 μ M each of dNTP's, 20pmoles of each primer and 1ng of ca. 5Kb *SacI* genomic DNA fragments. The mixture was boiled for 1 min. and added with 2.5 units of AmpliTaq[®] DNA polymerase (Perkin Elmer Cetus, N801-0060). Then the two step or three step PCR was repeated 35 cycles. At the end of the 17th cycle, 2.5 units of the enzyme were supplemented. Ten μ l of each sample was subjected to agarose gel electrophoresis using a 2% agarose gel plate containing 0.5 μ g/ml ethidium bromide, and 40mM Tris-acetate-1mM EDTA buffer. *HaeIII* digests of ϕ X174 DNA served as size markers. A: Three step PCR (96°C, 30 sec./67°C, 30 sec./72°C, 40 sec.). B: Two step PCR (96°C, 30sec./76°C, 40 sec.). The arrow indicates the amplified and sequenced DNA.

*Sph*I Y³¹Y I A Q C A P V G G Q D A C N P A
 5'GCATGCTACTACATCGCCAGTGCCTCCGGTCGGTGGCCAGGACGCGTGAACCCGGCG
 3'CGTACGATGATGTAGCGGTACACGAGGCCAGCCACCGTCTCTGCGCACGTTGGGCCGC

 T A T S F T T D A S G A A S F S F V V R
 ACCGCGACGTCCTTCACCACGGACGCTCCGGAGCGGCGTCGTTACGCTTCGTGTCGC
 TGGCGCTGCAGGAAGTGGTGCCTGCGCAGGCCTCGCCGAGCAAGTCGAAGCAGCACGCG

 K S Y T G S T P E G T P V G S V D C A T
 AAGTCGTACACGGGTCCACGCCCAGGCACGCCGTCGGCAGCGTCGACTGCGCCACC
 TTCAGCATGTGCCCAGGTGCGGGCTTCGTGCGGCCAGCCGTCGAGCTGACGCGGTG

 A⁸⁹*Eco*RI
 CGGAATTC 3'
 CGCTTAAG 5'

Fig. 3. Nucleotide sequence of the amplified fragment by PCR. Amino acid symbols were placed on the first letter of the corresponding codons.

4°C for a G-C pair and 2°C for an A-T pair. A mixture of *Sac*I genomic fragments of about 5 Kb was used as template for PCR. The mixture was expected to include the apoprotein gene by Southern blot analysis by use of a long synthetic probe as in our previous study⁵). The concentration of the template DNA was estimated by Saran Wrap method⁶) and 1ng, which may include 10⁸ target sites, was added. Fig. 2 shows DNA amplification obtained under two different PCR conditions. The conditions for the three thermal steps were 96°C for 30 seconds (DNA denaturation), 67°C for 30 seconds (primer annealing) and 72°C for 40 seconds (primer extension), while those for the two thermal steps were 96°C for 30 seconds (DNA denaturation) and 76°C for 40 seconds (primer annealing and extension). The conventional three thermal steps of PCR resulted in heterogeneous amplification including the expected 194bp fragment (Fig. 2A), while the two thermal step PCR using more stringent conditions for annealing gave rise to a single specific amplified fragment of 194bp (Fig. 2B). The less specific amplification by the three thermal steps (Fig. 2A) was thought to be the result of nonspecific primer-annealing due to many short stretches of G's and C's throughout the genome and hence, we raised the primer

annealing temperature. In addition, the two thermal steps seemed advantageous to minimize heat inactivation of Taq polymerase. Amplification was drastically reduced when the annealing-extension temperature was raised by a few more degrees over 76°C. This may be due to poor primer annealing. Reducing the target sites to 10⁵ (=1pg template DNA) caused poor amplification. The PCR apparatus we used (Bio-Oven, Biotherm Co., Virginia, Cat. 10-202) was an air incubator type requiring over 6 minutes per the two-step cycle (much longer than those of block types). The longer cycle period might also be favorable for denaturing the GC-rich DNA. The PCR product of the two thermal steps (Fig. 2B) was extracted, cloned into pUC118/119, and sequenced. The nucleotide sequence perfectly matched the amino acid sequence of the corresponding region (Fig. 3).

ACKNOWLEDGMENT

We would like to thank Dr. T. Otani, Biological Research Lab., Taiho Pharmaceutical Co., Ltd., Japan, for communicating the amino acid sequence of C1027-AG prior to publication. We also thank Dr. M. Hamada, Institute of Microbial Chemistry for supplying *Streptomyces globisporus*.

REFERENCES

- 1) Sakata, N.; T. Kanbe, M. Tanabe, H. Hayashi, M. Hori, K. Hotta & M. Hamada: Nucleotide sequence of the macromomycin apoprotein gene and its expression in *Streptomyces macromomyceticus*. J. Antibiot. 42: 1704-1712, 1989.
- 2) Trower, M.K. & K.G., Clark: PCR cloning of a streptomycin phosphotransferase (aphE) gene from *Streptomyces griseus* ATCC 12475. Nucl. Acids Res. 18: 4615, 1990
- 3) Otani, T.; T. Yasuhara, Y. Minami, T. Shimazu, R. Zhang & M.-Y. Xie: Purification and primary structure of C-1027-AG, a selective antagonist of antitumor anti-biotic C-1027, from *Streptomyces globisporus*. Agric. Biol. Chem. in press.
- 4) Stephen, J. S.: Cloning with PCR. In "PCR protocols" (ed. M. A. Innis, D. H. Gelfand, J. J. Sninsky, T. J. White), pp. 84-91, Academic Press, California, 1990.
- 5) Hori, M.: N. Sakata, Y. Niino, O. Makabe, M. Hamada, S. Mizuno & K. Hotta: Cloning of macromomycin apoprotein gene from *Streptomyces macromomyceticus* by use of 50-mer deoxynucleotide probes. J. Antibiot. 41: 1462-1470, 1988.
- 6) Sambrook, J.; E.F. Fritsch & T. Maniatis (Ed.): Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory Press, 1989.