Improved Production of Phenomycin by a Genetically Engineered *Escherichia coli*

YUICHI NAGANO, NAOTO TAKEUCHI, RYO MURAMATSU, YOICHI OHBA,
SHIN-ICHIRO ABE*, HIDEYA HAYASHI and YOSHIO INOUYE

Pharmaceuticals & Biotechnology Laboratory, Japan Energy Corporation,
Niizo-minami, Toda-shi, Saitama, Japan
†Department of Environmental Hygiene, Tohoh University School of Pharmaceutical Sciences,
Miyama, Funabashi-shi, Chiba, Japan

(Received for publication December 12, 1994)

We established an improved production of an antitumor polypeptide antibiotic, phenomycin (PHM), by using a genetically engineered *Escherichia coli*. Phenomycin consists of 89 natural amino acids without intramolecular disulfide bridge. PHM gene was synthesized as a fusion gene in which PHM at the C-terminus and the residues 1~20 of Hirudin variant 1 (HV1) at the N-terminus connected by the factor Xa recognition sequence (Ile-Glu-Gly-Arg). *E. coli* JM 109 transformed with a plasmid containing the synthesized gene expressed a fusion protein and the trypsinization of the fusion protein purified by ultrafiltration and ion-exchange chromatography gave efficiently recombinant PHM at a final yield of 50 mg/liter of culture. This PHM yield was six times higher than that obtained by a natural PHM producing strain of *Streptoverticillium baldacci*. Recombinant PHM was not distinguishable from natural PHM in all aspects observed.

Phenomycin (PHM), an antitumor antibiotic, was isolated from a culture of *Streptoverticillium baldacci* Ma564-C1[1]. The inhibition of protein synthesis in tumor cells[1] and the inhibition of initiation of globin synthesis in a rabbit cell-free system[2] are the biological features of PHM. It also had a significant inhibitory effect on the growth of Ehlrich carcinoma, sarcoma 180, and adenocarcinoma 755 in mice[3]. In contrast, there was no inhibition against Gram-positive and Gram-negative bacterial growth up to 100 μg/ml[1].

We have shown previously that PHM consists of 89 amino acid residues (MW 9,524) and has no disulfide bridges[4]. The amino acid sequence was identical to that deduced from the nucleotide sequence of the PHM gene isolated from the strain Ma564-C1[5]. However, since production level of PHM by the strain Ma564-C1 remains at a low level, we tried to improve PHM production by a genetically engineering; *Escherichia coli* transformed with a plasmid containing the synthetic PHM gene. Expectedly, a markedly improved PHM production was achieved and the biological activity of recombinant PHM was comparable to that of natural PHM *in vivo*.

Materials and Methods

Organisms and Plasmids

Escherichia coli JM 109 and a plasmid, pUC18, were purchased from Pharmacia (Uppsala, Sweden). Plasmid pMTSHV1, which had been constructed as a Hirudin variant 1 (HV1) expression vector[6] was provided by S. MISAWA, Japan Energy Corporation. Meth A cells was kindly donated by Dr. C. TASHIRO, Japanese Foundation for Cancer Research, Tokyo, Japan. *Streptoverticillium baldacci* Ma564-C1 was obtained from Institute of Microbial Chemistry.

Cultivation of *S. baldacci* Ma564-C1 and Purification of Natural PHM

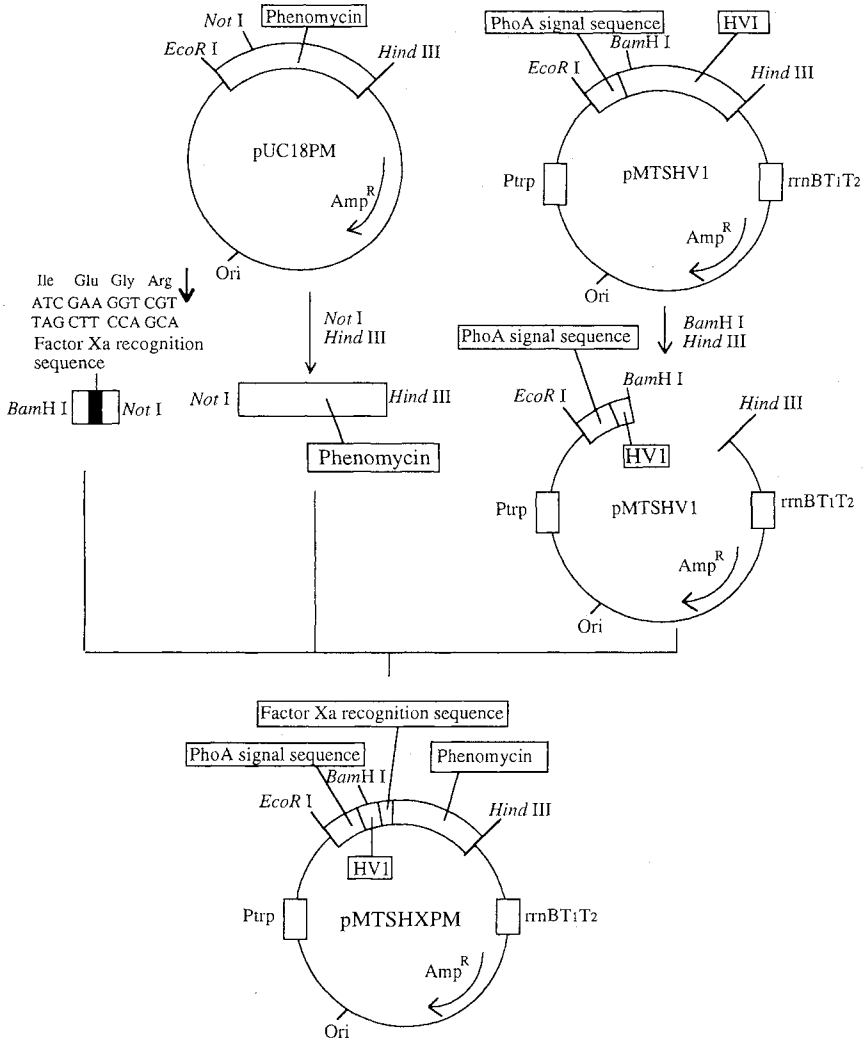
A medium consisting of peptone 1%, meat extract 1% and NaCl 0.5% (pH 7.0) was used for the cultivation of *S. baldacci* Ma564-C1. After 48 hour-shake culture at 27°C, 200 ml of the cultured broth was transferred into 2 liters of the fresh medium and cultured for 72 hours at 27°C with shaking.

Culture supernatant was collected by centrifugation and applied to an S Sepharose Fast Flow (Pharmacia) column (1.6 × 5 cm) previously equilibrated with 10 mM sodium phosphate buffer (pH 7.0). After washing the column, PHM was eluted with 10 mM sodium phosphate buffer (pH 7.0) containing 1 M NaCl and each fraction thus obtained was analyzed by SDS-PAGE. The fractions containing the same molecular weight as PHM, 9,500 daltons, were pooled. This partially purified PHM was subjected to reverse-phase HPLC using a Vydac (The Separation Group Inc., California, U.S.A.) C_{18} column (2.2 × 25 cm). PHM was eluted with a linear gradient of acetonitrile concentrations from 29 to 30% in 0.1% TFA over 15 minutes. The PHM-containing fractions were pooled, evaporated, dialyzed against distilled water and then lyophilized.

Plasmid Construction

The scheme of construction of expression vector pMTSHXPM is outlined in Fig. 1. In order to construct

Fig. 1. Construction of the expression vector.



The linker, joining HV1 region and PHM region contains the factor Xa recognition sequence. The fusion protein can be cleaved by factor Xa or trypsin. The vertical arrow immediately after the arginine residue indicates the factor Xa and trypsin cleavage site.

a PHM gene, 12 oligonucleotides of average length of 50 bases were designed on the basis of the amino acid sequence of PHM and synthesized using a DNA synthesizer model 380B (Applied Biosystems, California, U.S.A.). Codons found most frequently in strongly expressed genes of *E. coli*[7] were used together with unique restriction sites. Oligonucleotides were provided to endow the final PHM gene with cohesive ends compatible with the *Eco* RI and *Hind* III sites at the 5′ and 3′ ends, respectively. All oligonucleotides were phosphorylated by T4 polynucleotide kinase (Toyobo, Osaka, Japan) except for the two oligo-nucleotides overhanging on 5′ ends of both strands of the full-length gene. The kinated complementary oligonucleotides were annealed and ligated to pUC18 by T4 DNA ligase (Takara Shuzo, Kyoto, Japan) in order to yield pUC18PM. The synthetic DNA gene from pUC18PM and a DNA fragment encoding the tetrapeptide recognition sequence of factor Xa were inserted into the fragment generated by *Bam* HI/*Hind* III digestion of pMTSHV1.

Cultivation of *E. coli* and Purification of the Fusion Protein

One hundred ml of a seed culture which was incubated at 30°C for 16 hours in 2×TY medium consisting of Bactotryptone 1.6%, yeast extract 1% and NaCl 0.5% (pH 7.0) was inoculated into 2.5 liters of 2×TY medium supplemented with 2% glycerol in a 5-liter jar fermenter and cultivated at 37°C for 20 hours. The expression level of the fusion protein was monitored by SDS-PAGE. Cells were harvested by centrifugation, suspended in sterile water (1 mg cells/ml) and sonicated for 3 minutes using a Sonifier 250 (Branson, Connecticut, U.S.A.). A crude fusion protein from the soluble fraction was obtained by ultracentrifugation (Beckman, California, U.S.A.) at 20,000×*g* at 4°C for 30 minutes, followed by ultrafiltration using a Prep/Scale-TFF Cartridge (MW cut off

10,000, Millipore). The crude fusion protein was applied to a CM-Toyopearl 650M (Tosoh, Tokyo, Japan) column (2.2 × 20 cm) previously equilibrated with 10 mM sodium phosphate buffer (pH 6.5). The column was washed with the same buffer at a flow rate of 40 ml/minute and eluted with 10 mM sodium phosphate buffer (pH 6.5) containing 0.3 M NaCl at a flow rate of 4 ml/minute. Each fraction was analyzed by SDS-PAGE and the fractions showing a single band at about 14,000 daltons were pooled and concentrated by ultrafiltration using a YM-3 membrane (MW cut off 3,000, Amicon).

Cleavage of the Fusion Protein and Purification of Recombinant PHM

The purified fusion protein (1 mg/ml) was digested with bovine pancreas L-1-tosylamide-2-phenylethyl chloromethyl ketone-treated Type XIII trypsin (Sigma, Missouri, U.S.A.) at 37°C for 6 hours at an enzyme substrate ratio of 1 : 100 (w/w). After 4-amidinophenylmethanesulfonyl fluoride (Wako Pure Chemical Industry, Osaka, Japan) had been added to a final concentration of 1 mM, the reaction mixture was left on ice for 1 hour.

The sample was subjected to reverse-phase HPLC using a Vydac C_{18} column, and the PHM was eluted with a linear gradient of acetonitrile concentrations from 28 to 30% in 0.1% TFA over 30 minutes. The PHM-containing fractions were pooled, evaporated, dialyzed against distilled water and then lyophilized.

Amino Acid Analysis

Recombinant PHM (10 μg) was hydrolyzed with 6 N HCl containing 1% phenol in a Millipore Picotag system at 110°C for 24 hours. Each hydrolysate was dried and analyzed on a Beckman model 7300 amino acid analyzer.

In Vivo Antitumor Activity

The male BALB/c mice weighing 21 ~ 25 g (Japan Clea, Tokyo, Japan) were implanted intraperitoneally with 2×10^6 Meth A cells on day 0, and randomly divided into three groups; control (n = 13), natural PHM (n = 6), and recombinant PHM (n = 6). These mice were treated once daily for 7 consecutive days by intraperitoneal administration of the respective test sample or saline (control) starting at 24 hours after the implantation. Survival periods were observed, and the antitumor activity was evaluated in terms of the increase in life span.

Results

Production of Natural PHM by *S. baldacci* Ma564-C1

From 400 ml of the culture supernatant of the strain Ma564-C1, 3.36 mg of purified PHM was obtained as a lyophilized powder. A final yield of 8.4 mg/liter was consistent with the reported value, 8 mg/liter[1].

Expression and Purification of the Fusion Protein

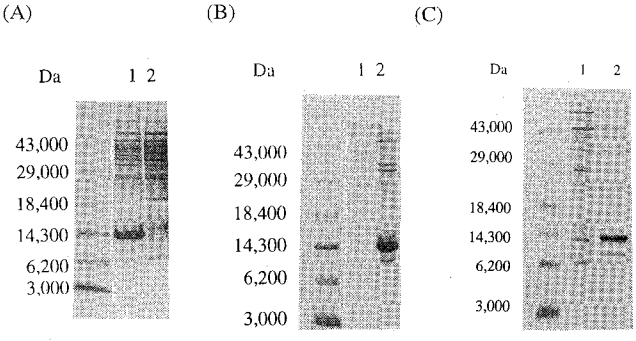
The fusion protein accumulated in *E. coli* cells carrying pMTSHXPM accounted for about 35% of the total cellular protein on the basis of SDS-PAGE result of whole cell lysate. A predominant band with an apparent molecular weight of about 14,000 dalton was observed in the lysate of the transformed cells, but not in that of the non-transformed ones (Fig. 2A; lanes 1, 2). The apparent molecular weight corresponded to that calculated from the amino acid sequence. This overexpressed protein was not found in the periplasmic fraction (Fig. 2B), but in the soluble fraction (Fig. 2C).

The 14,000 dalton protein was purified by ultrafiltration and ion-exchange chromatography, and approximately 90 mg purified protein per liter of culture was obtained. The sequence of the five amino-terminal amino acids of this purified protein, determined with an automatic protein sequencer (Applied Biosystems), was shared by that of the *E. coli* alkaline phosphatase signal sequence.

Cleavage of the Fusion Protein and Purification of Recombinant PHM

Recombinant PHM was released from the fusion protein by cleavage with factor Xa in a very low yield (Fig. 3A). Trypsin, unlike factor Xa, released recom-

Fig. 2. Localization of the fusion protein in *E. coli* by SDS-PAGE.

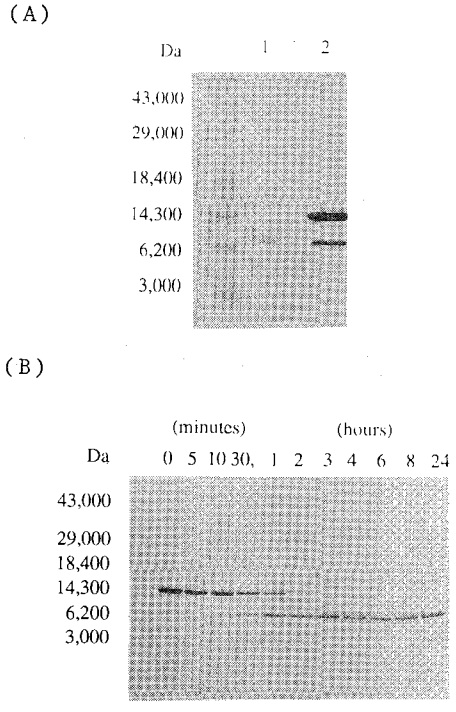


(A) Total proteins from transformed cells (lane 1) and non-transformed cells (lane 2).

(B) Periplasmic fraction (lane 1) and non-periplasmic fraction from transformed cells (lane 2). Cells were harvested from 1.5 ml of transformed cell culture by centrifugation, suspended in 1.5 ml of 25% sucrose and 10 mM EDTA solution, and stood at room temperature for 10 minutes. After centrifugation, the pellet was suspended in 1.5 ml of cold sterile water and left on ice for 30 minutes. The suspension was then centrifuged to obtain supernatant (periplasmic fraction) and pellet (non-periplasmic fraction).

(C) Insoluble fraction (lane 1) and soluble fraction (lane 2) from transformed cell extract prepared by sonication as described in "Materials and Methods".

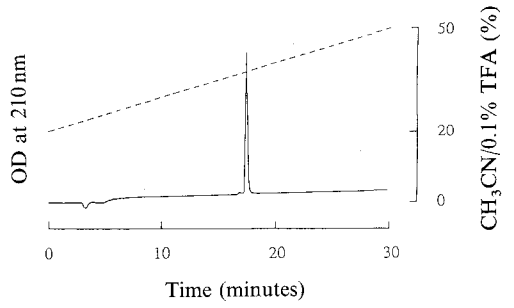
Fig. 3. SDS-PAGE of the purified fusion protein and the cleavage product.



(A) Natural PHM (lane 1) and recombinant PHM (lane 2). The purified fusion protein was denatured by dialysis against 20 mM Tris-HCl (pH 7.6) containing 6 M guanidine hydrochloride, and then dialyzed against 20 mM Tris-HCl (pH 7.6) containing 200 mM NaCl to remove the denaturant. Three μg of this fusion protein was cleaved with factor Xa at 37°C for 72 hours at an enzyme substrate ratio of 1 : 30 (w/w).

(B) Time-course of the cleavage of fusion protein by trypsin. One hundred μl of the purified fusion protein (0.4 μg/μl) was incubated with bovine pancreas L-1-tosylamide-2-phenylethyl chloromethyl ketone-treated Type XIII trypsin at an enzyme substrate ratio of 1:100 (w/w). At each time point, 5 μl of the reaction mixture was taken out, mixed with 5 μl of 0.2 M Tris-HCl (pH 8.0), 1% SDS, 10% glycerol and 10% 2-mercaptoethanol and analyzed by SDS-PAGE.

Fig. 4. Purity of the purified recombinant PHM.



Purified recombinant PHM was applied onto a Tosoh TSK Gel ODS 120T column (0.46 × 15 cm) and was eluted with a linear gradient of acetonitrile concentrations from 20 to 50% in 0.1% TFA over 30 minutes at a flow rate of 1 ml/minute.

Table 1. Amino acid composition of the recombinant PHM.

Amino acid	Number of residues
Asx	9.8 (10)
Glx	3.3 (3)
Ser	7.3 (8)
Thr	6.6 (7)
Arg	4.0 (4)
Gly	4.3 (4)
Ala	16.5 (17)
Cys	0.0 (0)
Pro	3.9 (3)
Val	2.9 (3)
Met	1.9 (2)
Ile	3.8 (4)
Leu	4.1 (4)
Phe	1.0 (1)
Trp	ND (1)
Lys	9.9 (10)
His	3.9 (4)
Tyr	3.0 (3)

Numbers in parentheses are taken from the reported amino acid sequence of natural PHM.
ND: Not detected.

binant PHM efficiently, with cleavage being completed within 4 hours. The fusion protein was mainly cleaved at a single, specific site by trypsin, even though the protein contained 5 arginine and 12 lysine residues (Fig. 3B).

The reaction mixture of trypsin cleavage was directly subjected to reverse-phase HPLC. Recombinant PHM and natural PHM were eluted at the identical retention time in the same HPLC system. The recovery yield of recombinant PHM from the fusion protein was approximately 55% by weight, that is 80% yield on the molar basis.

The purity of recovered recombinant PHM was more than 95% when assessed by analytical HPLC (Fig. 4). Furthermore the amino acid composition of recombinant PHM was in good agreement with that calculated on the basis of the amino acid sequence of PHM (Table 1). Taken all together, the data indicated that the trypsin-cleavage site was confined to the carboxyl side of arginine residue of the factor Xa recognition sequence.

Contamination by trypsin was checked with a fluorogenic peptide substrate[8], and no activity of trypsin was found in recombinant PHM preparation.

The trypsinization of the fusion protein purified by ultrafiltration and ion-exchange chromatography gave efficiently recombinant PHM at a final yield of 50 mg/liter of culture, which was six times higher than that obtained by natural PHM producing microorganism.

In Vivo Antitumor Activity

Both natural and recombinant PHM prolonged the survival period of tumor-bearing mice. The results are summarized in Table 2. The antitumor activities of recombinant and natural PHM were equivalent at a dose of 250 μg/kg/day.

Table 2. *In vivo* antitumor activity.

	Dose (μg/kg/day)	n	Survival days[a]	T/C (%)
Control		13	12.8 ± 1.5	100
Natural PHM	250	6	16.7 ± 4.5 [b]	130
Recombinant PHM	250	6	18.7 ± 3.6 [b]	146

[a] Mean ± SD.
[b] Significantly different from the control value (STUDENT's t-test), $P<0.01$.

Discussion

To our knowledge, there has been no literature reporting an enhanced production of a polypeptide antibiotic by a genetically engineered *E. coli*. The productivity of this new production system is six times higher than that from the natural microorganism. This high level production of biologically active recombinant PHM without any folding procedures can be attributed to the nature of PHM that consists of only L-amino acid residues and has no disulfide bridges. In this expression system, a native protein is released from its fusion protein by treatment with trypsin or factor Xa. Our first attempt to cleave the fusion protein with factor Xa[9,10] resulted in a poor recovery. WINGENDER *et al.* reported that a cro-β-galactosidase-hPTH fusion protein containing the factor Xa recognition site was not cleaved by factor Xa, suggesting that the cleavage site in this protein was blocked from the enzyme[11]. However, after the fusion protein was denatured with guanidine hydrochloride to render the factor Xa site accessible to the enzyme, the efficiency of factor Xa cleavage remained unimproved (Fig. 3A). Therefore, it seems likely that the Gly-Ser-Arg domain is blocked from trypsin in this protein owing to its secondary or higher dimensional structures.

Due to the establishment of a high-level PHM-expression system, it became possible to obtain a large amount of PHM for the further biological studies. This expression system is also suitable for site-directed mutagenesis to study the effect of individual amino acid residues on the function of PHM and offers a convenient method for the production of other polypeptide antibiotics resembling to PHM.

References

1) NAKAMURA, S.; T. YAJIMA, M. HAMADA, T. NISHIMURA, M. ISHIZUKA, T. TAKEUCHI, N. TANAKA & H. UMEZAWA: A new antitumor antibiotic, phenomycin. J. Antibiotics, Ser. A 20: 210~216, 1967
2) YAMAKI, H.; T. NISHIMURA, K. KUBOTA, T. KINOSHITA & N. TANAKA: Selective inhibition of initiation of globin synthesis by phenomycin. Biochem. Biophys. Res. Commun. 59: 482~488, 1974
3) NISHIMURA, T.: Activity of phenomycin against transplantable animal tumors. J. Antibiotics 21: 106~109, 1968
4) MURAMATSU, R.; S. ABE, H. HAYASHI, K. YAMAGUCHI, K. JINDA, K. SAKANO, Y. INOUYE & S. NAKAMURA: Complete amino acid sequence of phenomycin, an antitumor polypeptide antibiotic. J. Antibiotics 44: 1222~1227, 1991
5) SAKATA, N.; T. OKA, S. IKENO, M. HORI, K. YAMAGUCHI, Y. INOUYE & S. NAKAMURA: Nucleotide sequence of the phenomycin gene from *Streptoverticillium baldacci* Ma564-C1. J. Antibiotics 47: 370~371, 1994
6) MISAWA, S.; H. FURUYA, H. MATSUDA, S. ABE & H. HAYASHI: High level expression and secretion of biologically active leech hirudin variant 1 (HV1) by *Escherichia coli*. Proceeding of Asia-Pacific Biochemical Engineering Conference, 1992
7) IKEMURA, T.: Correlation between the abundance of *Escherichia coli* transfer RNAs and the occurrence of the respective codons in its protein genes. J. Mol. Biol. 151: 389~409, 1981
8) KAWABATA, S.; T. MIURA, T. MORITA, H. KATO, K. FUJIKAWA, S. IWANAGA, K. TAKADA, T. KIMURA & S. SAKAKIBARA: Highly sensitive peptide-4-methyl-coumaryl-7-amide substrates for blood-clotting protease and trypsin. Eur. J. Biochem. 172: 17~25, 1988
9) NAGAI, K. & H. C. THØGERSEN: Generation of β-globin by sequence-specific proteolysis of a hybrid protein produced in *Escherichia coli*. Nature 309: 810~812, 1984
10) NAGAI, K. & H. C. THØGERSEN: Synthesis and sequence-specific proteolysis of hybrid proteins produced in *Escherichia coli*. Methods Enzymol. 153: 461~481, 1987
11) WINGENDER, E.; B. GISELA, B. HELMUT, F. RONALD & M. HUBERT: Expression of human parathyroid hormone in *Escherichia coli*. J. Biol. Chem. 264: 4367~4373, 1989