Cloning of the Enomycin Structural Gene from *Streptomyces mauvecolor* and Production of Recombinant Enomycin in *Escherichia coli*

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A genomic DNA library from the enomycin (ENM) producer, *Streptomyces mauvecolor*, was screened for the ENM structural gene (*enm*) by use of a segment of the phenomycin gene (*phm*) as the probe, and a plasmid, pEN1, was constructed. By primer-walking along the insert, a 573 bp DNA sequence that contain an ORF corresponding to preENM was determined. The deduced amino acid composition of ENM was close to that previously reported (MIZUNO, S.; K. NITTA & H. UMEZAWA: Mode of action of enomycin, an antitumor antibiotic of high molecular weight. I. Inhibition of protein synthesis. J. Biochem. 61: 373~381, 1967). The producer cells expressed *enm* during an ENM-productive fermentation. An *enm*-expression plasmid, pENE 1, was constructed, with which *E. coli* AD202 was transformed. The transformant produced a fusion protein consisting of glutathione-S-transferase (GST) and ENM. The genetically engineered ENM (rENM) inhibited the growth of Hela cells *in vitro*. Comparison of the base sequence spanning *enm* with that spanning *phm* showed that the structural genes were conserved more extensively than were the flanking regions, though the genes were unlikely to be essential to the lives of the producers.

Enomycin (ENM) and phenomycin (PHM) are polypeptide antitumor antibiotics isolated by H. UMEZAWA's group^{1,2)}. Based on the amino acid sequence of PHM³⁾, we previously cloned the PHM structural gene (*phm*) from the producer strain, *Streptovercillium baldaccii* and found that PHM was produced as prePHM that had a leader peptide of 45 amino acid residues ahead of the N-terminus⁴⁾. Structural similarity between PHM and ENM was expected because of the similar amino acid compositions⁵⁾, though the amino acid sequence of ENM remained unknown.

In the present study, we cloned the ENM structural gene (*enm*) from *Streptomyces mauvecolor* A390-P4 (ENM producer) using a partial sequence of *phm* as the probe, constructed an expression system of *enm* in *E. coli*, and confirmed the biological activity of the genetically engineered ENM.

Materials and Methods

Materials

E. coli AD202^{6,7)}, a protease-negative strain, was kindly provided by T. SAITO, Chiba University. Vector pGEX-KT⁸⁾ was kindly provided by JACK E. DIXON, University of Michigan Medical School. Glutathione-Sepharose 4B and thrombin were purchased from Pharmacia (Uppsala, Sweden). Restriction enzymes were purchased from TOYOBO (Tokyo, Japan) and

TAKARA (Tokyo, Japan) and sequencing kits were from TAKARA, United States Biochemicals (Cleveland, U.S.A.) and Pharmacia. *Streptomyces mauvecolor* A390-P4, producer of ENM, was obtained from the Institute of Microbial Chemistry.

Shotgun Cloning and Sequencing of *enm*

Total DNA from the ENM producer was digested with *Bam*HI, etc., at 10 units enzyme/ μ g DNA, electrophoresed in a 0.7% agarose gel, Southern transferred⁹⁾, and hybridized with a ³²P-labeled DNA probe corresponding to the amino acid sequence from Arg¹⁴ to Ala⁷² of PHM. *Bam*HI digests of 4.8 Kb were extracted from the gel, ligated to pUC118 and the products were used to transform *E. coli* DH5 α . Colonies positive to the same probe were selected. From an isolate, a plasmid was extracted, purified and identified as pEN1 (Fig. 2). Primer walking along pEN1 was initiated with a 24-mer primer that corresponded to a partial sequence of *phm*.

Northern Blot Analysis of *enm*-mRNA

The *Bam*HI insert in pEN1 was amplified by PCR, and the product was ³²P-labeled by the random primer method (10⁹ cpm/ μ g)¹⁰⁾ and used as the probe. Total RNA was extracted from ENM producer cells (2g wet weight) that had been withdrawn from a productive fermentation on day 4 or 6. The RNA samples, 20 μ g each, were electrophoresed in a 1.2% formaldehyde gel, Northern blotted, and hybridized with the above (prehybridization and hybridization at 42°C, final washing in 0.2 $\times$ SSC at 45°C for 30 minutes) prior to

autoradiography.

Expression of *enm* in *E. coli*

pEN1 was double-digested with *Sma*I and *Sph*I, the products were electrophoresed, and 1.15 Kb fragments including *enm* were extracted from the gel. Using the fragment as a template, *enm* (not including the segment for the leader peptide codons) was amplified by PCR with Bam 5' primer (5'-CGGTGGGATCCAACCC-GAAGACG-3') and Eco 3' primer (5'-CGAGGC-GAATTCGGCCTCACCAG-3'), in repeating 30 cycles of heating at 100°C for 40 seconds and at 63°C for 90 seconds, and the reaction was finished by heating at 63°C for 5 minutes. The products were double-digested with *Bam*HI and *Eco*RI and electrophoresed in a 2% agarose gel. The 297 bp fragment, extracted from the gel, was ligated to vector pGEX-KT and submitted to transformation of *E. coli* DH5 α . Among ampicillin resistant colonies, a transformant that included pENE1 was cloned. The insert and the short flanking regions were sequenced to confirm that *enm* was ligated behind the GST gene in the correct reading frame. *E. coli* AD202 was transformed with pENE1 and a transformant was selected, cloned and named AD202/pENE1. The clone was cultured with shaking at 37°C in 100 ml $\times$ 10 flasks of L-broth containing ampicillin at 50 μ g/ml, until the turbidity (660 nm) reached 0.8. IPTG was then added to 0.1 mM final concentration and incubation with shaking was continued for 2.5 hours. Cells were harvested by centrifugation at 4°C (all lysis and fractionation procedures were also carried out at 4°C, except when otherwise described), washed with PBS, suspended in 25 ml of PBS, and disrupted in a Branson Sonifier Model 350 (Duty Cycle at 25%, Output Control at 1, Pulsed). The homogenate was centrifuged at 6,600 $\times$ g for 10

minutes. To the resulting supernatant of about 25 ml, was added 0.4 ml of 50% (v/v) slurry of Glutathione Sepharose 4B, prepared as recommended by the manufacturer (Pharmacia). The mixture was shaken for 40 minutes at a room temperature and centrifuged. The pelleted resin that adsorbed the fusion protein was washed five times with 2.5 ml of PBS, incubated for 16 hours at a room temperature with 200- μ l of PBS containing 10 units of thrombin to release ENM, and centrifuged. The supernatant was combined with the washings of the resin (2 $\times$ 200 μ l of PBS) to obtain approximately 600 μ l of an ENM solution. The purification steps were monitored by SDS-polyacrylamide gel electrophoresis (See Legend to Fig. 5). Protein concentrations were determined by the Bradford method¹¹.

Results and Discussion

Cloning of Enomycin Gene

Total DNA from the ENM producer was digested with various restriction enzymes, electrophoresed and Southern hybridized with the DNA probe that corresponded to a partial amino acid sequence of PHM, i.e., Arg¹⁴ to Ala⁷². The shortest hybridization target observed was an *Bam*HI fragment of about 4.8 Kb, as shown in Fig. 1. Therefore, DNA was extracted from this region of the gel and ligated to pUC118. With the products, *E. coli* DH5 α was transformed. Out of nine candidate colonies, two were confirmed to include the desired plasmid, pEN1 (Fig. 2), that included the 4.8 Kb insert. The DNA sequence (573 bp) that included *enm* was determined, as shown in Fig. 3. By analogy with the PHM precursor protein⁴, the leader peptide of ENM, from Met⁻⁴⁶ to Ala⁻¹, was deduced, which showed several characteristics of leader peptides of streptomycetes¹². ENM was thought to be produced as a precursor protein of 135 amino acid residues (preENM) that would be split into

Fig. 1. Genomic southern blot analysis of *enm*.
(A) Ethidium bromide staining, (B) autoradiogram.

Total DNA from the enomycin producer, *Streptomyces mauveicolor* was digested with restriction enzymes and probed with *phm*. The arrow indicates the 4.8 Kb *Bam*HI band. Enzymes used were 1. *Bam*HI, 2. *Eco*RI, 3. *Pst*I, 4. *Sac*I, and 5. *Sph*I.

Fig. 2. Restriction map of pEN 1.

Fig. 3. Nucleotide sequence of *enm*.

1	GCC	AGC	GGC	ACT	CGT	COC	TGT	TTG	CCC	GCA	TGC	GGC	36
37	AGG	CTT	CCC	GGT	GCA	AGG	TTC	AGC	ACG	CTT	TTC	ATC	72
73	CAG	TAA	<u>GCG</u>	<u>AGG</u>	AAT	CGT	ATG	CAG	TTC	ATC	GTC	CGT	108
-46							M	Q	F	I	V	R	-41
109	ACG	GGT	ATG	GCC	GCC	GCG	TTG	GCG	GTC	GGC	GCG	CTT	144
-40	T	G	M	A	A	A	L	A	V	G	A	L	-29
145	GCC	GCG	GTC	GTC	GCG	COC	GCC	TOC	GCC	TOC	GCC	GTC	180
-28	A	A	V	V	A	P	A	S	A	S	A	V	-17
181	GTC	GCC	GAC	GGT	GCG	CAC	GCG	GTG	AGC	GGC	GCC	CAC	216
-16	V	A	D	G	A	H	A	V	S	G	A	H	-5
217	CCG	GTG	GCG	GCC	AAC	CCG	AAG	ACG	ATC	AAG	CCT	GCC	252
-4	P	V	A	A	/ N	P	K	T	I	K	P	A	8
253	GCG	TAC	AAC	CAG	GCG	CGG	AGC	ATC	CTG	GCC	AAT	GCC	288
9	A	Y	N	Q	A	R	S	I	L	A	N	A	20
289	GGG	AGT	CAG	ACG	GCG	GCC	AAG	TGC	CAT	CCC	GTG	CAC	324
21	G	S	Q	T	A	A	K	S	H	P	V	H	32
325	GGA	AAG	GAC	GAC	GTG	CCC	GTC	AGC	TAC	GGC	ACG	AGC	360
33	G	K	D	D	V	P	V	S	Y	G	T	S	44
361	CTG	CTC	GCG	GCC	GCG	CGC	GAC	GAG	TTC	CCG	CAC	ACG	396
45	L	L	A	A	A	R	D	E	F	R	H	T	56
397	GAC	CGG	AAC	CTG	CCG	GCG	AAG	CAG	AAG	AAG	TCC	GAC	432
57	D	R	N	L	P	A	K	Q	K	K	S	D	68
433	ATG	TGC	ATC	CCG	CAC	TAC	AAC	GCC	ATT	CAC	AGC	GCG	468
69	M	S	I	P	H	Y	N	A	I	H	S	A	80
469	GCG	AAG	ACG	ATG	GCC	ATC	GAC	CGC	TGG	TGA	GCC	CGT	504
81	A	K	T	M	G	I	D	R	W	*			89
505	AGG	CGC	CTC	GGG	CCA	AGG	<u>GCC</u>	<u>GCC</u>	<u>GCT</u>	CGC	<u>GGC</u>	<u>CGG</u>	540
541	CCG	ACG	CGG	GCT	CAG	CGC	GCG	GCG	GCG	CTG	CTC		573

The slash indicates the splitting site between the leader peptide and ENM. The putative ribosome binding site is double-underlined. The potential transcription terminator is underlined. The nucleotide sequence data of *enm* will appear in the DDBJ, EMBL and GeneBank Nucleotide Sequence Databases with the accession number D85170.

the leader peptide and ENM. Throughout the structural gene for preENM, the total GC content was 70%, while that for the 3rd codon position was 93%, consistent with data for other structural genes of *Streptomyces*. The deduced amino acid composition of ENM agreed with the composition reported⁵⁾ except for the presence of a previously-undetected Phe residue (encoded by TTC, codon 54). A possible ribosome-binding sequence (GAGG) was found 6 residues upstream of the initiator ATG, while there were two inverted repeat sequences commence at residues 24 and 54 downstream of the terminator TGA, respectively, possibly acting as transcription terminators. Northern blot analysis showed that this gene was transcribed into an about 630 nucleotides long RNA on the 4th day of ENM-producing fermentation. The RNA seemed long enough to code preENM that was 135 amino acids long.

Production of Recombinant Enomycin

The DNA fragment (297 bp) that included the ENM-encoding sequence but not the leader peptide codons was amplified by PCR and the product was ligated to an expression vector pGEX-KT to give pENE1 (Fig. 4), with which *E. coli* AD202 was transformed and the

Fig. 4. Production of glutathione-S-transferase (GST)-enomycin (ENM) fusion protein.

Fig. 5. SDS-polyacrylamide gel analysis of purified rENM.

A 10 μ l sample was mixed with 3 μ l of a buffer containing 62.5 mM Tris-HCl pH 7.0, 10% glycerol, 2% SDS, 720 mM β -mercaptoethanol, 0.00125% Bromophenol Blue, heated on a boiling water bath for 5 minutes, and electrophoresed in a 14% SDS-polyacrylamide minigel (TEFCO) at 20 mA for 80 minutes. The gel was immersed successively in 100 ml of a staining solution (0.2% Coomassie Brilliant Blue R250 in 20% (V/V) acetic acid/methanol) at 65°C for 10 minutes and in 150 ml of a destaining solution (50% methanol, 37.5% acetic acid) overnight. Lane 1: Protein size markers. Lane 2: Total proteins from uninduced cells. Lane 3: Total proteins from induced cells. Lane 4: Purified rENM after thrombin cleavage. Lane 5: Protein size markers.

clone was named *E. coli* AD202/pENE1. The clone was grown in L-broth, induced using IPTG, and the fusion protein GST-ENM was isolated from cell lysates by adsorption to glutathione-Sepharose 4B. ENM was released from the affinity resin on digestion with

Fig. 6. Comparison between amino acid sequences for enomycin and phenomycin.

ENM	Met	Gln	Phe	Ile	Val	Arg	Thr	Gly	Met
PHM	---	Lys	Leu	---	---	---	---	---	Leu
Ala	Ala	Ala	Leu	Ala	Val	Gly	Ala	Leu	Ala
---	---	---	Val	Val	Leu	---	---	Ala	---
Val	Val	Ala	Asp	Gly	Ala	His	Ala	Val	Ser
---	Thr	Asp	---	---	Pro	Arg	Pro	---	Ala
Thr	Ile	Lys	Pro	Ala	Ala	Tyr	Asn	Gln	Ala
---	---	Ala	---	---	---	---	---	---	Arg
Thr	Ala	Ala	Lys	Ser	His	Pro	Val	His	Gly
---	---	---	---	---	Ile	---	---	---	---
Ser	Leu	Leu	Ala	Ala	Ala	Arg	Asp	Glu	Phe
---	---	---	---	---	---	---	---	---	---
Gln	Lys	Lys	Ser	Asp	Met	Ser	Ile	Pro	His
Asp	---	---	---	---	---	---	---	Ala	---
Met	Gly	Ile	Asp	Arg	Trp	*	---	---	---
---	---	---	---	---	---	---	---	---	---

Dashed amino acid residues are identical between the two proteins.

thrombin. The isolation procedure was monitored by electrophoretic analysis of proteins, as shown in Fig. 5. From a 1,000 ml culture of the clone, 3 mg of purified ENM was obtained. The productivity could possibly be improved if the codons of *enm* were replaced with those of *E. coli* type. The genetically engineered ENM, having an extra Gly-Ser dipeptide on the *N*-terminal, inhibited *in vitro* the growth of Hela cells by 50% at 2.2 µg/ml.

Why ENM and PHM are Produced

As shown in Fig. 6, the amino acid sequences of ENM and PHM shared 84% homology in their mature protein regions. The conserved amino acid residues should include important ones for the biological activity of the proteins, *i.e.*, inhibition of eukaryotic protein synthesis. The leader peptide regions showed 59% homology, apparently lower than that for the mature proteins. We suggest that the producer strains have been under evolutionary pressure to produce and secrete proteins with such biological activity. Comparison between the DNA sequences supports this idea more clearly. Similarity between the segments "outside the structure gene", between "leader peptide codons" and between "mature protein codons" were 44% (74/165 bases), 71% (98/138 bases) and 86% (232/270 bases), respectively. In the natural environment for the producer strains, *i.e.*, in the soil, what eukaryote is the natural enemy to be killed with ENM or PHM?

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