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 PHM[3], we previously cloned the PHM structural gene (*phm*) from the producer strain, *Streptoverticillium baldaccii* and found that PHM was produced as prePHM that had a leader peptide of 45 amino acid residues ahead of the *N*-terminus[4]. Structural similarity between PHM and ENM was expected because of the similar amino acid compositions[5], 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-KT[8] 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/μg DNA, electrophoresed in a 0.7% agarose gel, Southern transferred[9], and hybridized with a ^{32}P-labeled DNA probe corresponding to the amino acid sequence from Arg^{14} to Ala^{72} 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* DH5α. 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 ^{32}P-labeled by the random primer method (10^9 cpm/μg)[10] and used as the probe. Total RNA was extracted from ENM producer cells (2 g wet weight) that had been withdrawn from a productive fermentation on day 4 or 6. The RNA samples, 20 μg 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 × SSC at 45°C for 30 minutes) prior to

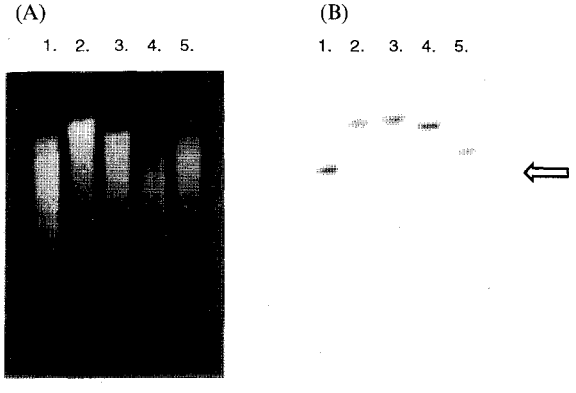
autoradiography.

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* DH5α. 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 × 10 flasks of L-broth containing ampicillin at 50 μg/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 × *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 recomended 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-μl of PBS containing 10 units of thrombin to release ENM, and centrifuged. The supernatant was combined with the washings of the resin (2 × 200 μl of PBS) to obtain approximately 600 μl 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 method[11].

Fig. 1. Genomic southern blot analysis of *enm*.

(A) Ethidium bromide staining, (B) autoradiogram.



Total DNA from the enomycin producer, *Streptomyces mauvecolor* 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.

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.*, Arg^{14} to Ala^{72}. 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* DH5α 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 protein[4], the leader peptide of ENM, from Met^{-46} to Ala^{-1}, was deduced, which showed several characteristics of leader peptides of streptomyces[12]. ENM was thought to be produced as a precursor protein of 135 amino acid residues (preENM) that would be split into

Fig. 2. Restriction map of pEN 1.

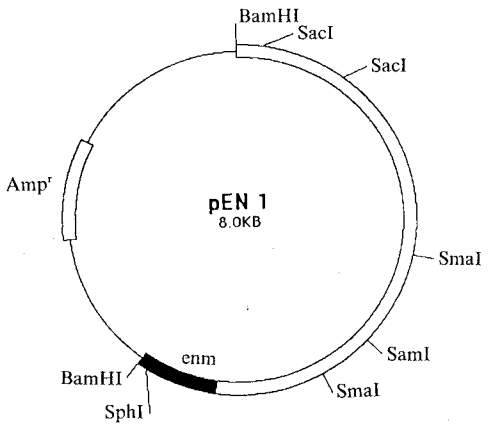


Fig. 3. Nucleotide sequence of *enm*.

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  1  GCC AGC GGC ACT CGT CCC TGT TTG CCC GCA TGC GGC   36
 37  AGG CTT CCC GGT GCA AGG TTC AGC ACG CTT TTC ATC   72
 73  CAG TAA GCG AGG 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 CCC GCC TCC GCC TCC 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 TCG 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 CGG 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 TCG 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 GGC ATC GAC CGC TGG TGA GGC CGT  504
 81   A   K   T   M   G   I   D   R   W   *          89
505  AGG CGC CTC GGG CCA AGG GCC GCC GGT CGC GGC CGG  540
541  CGG ACG CGG GCT CAG CGC GCG CGC 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.

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

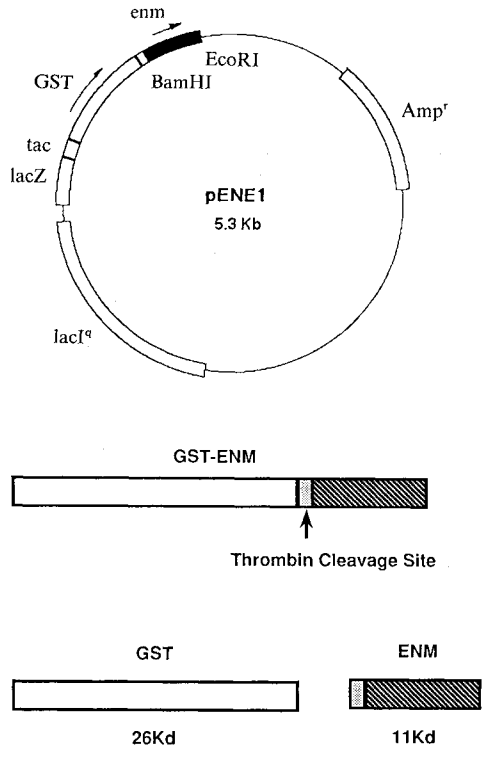
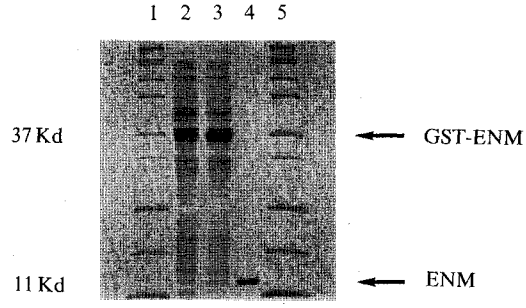


Fig. 5. SDS-polyacrylamide gel analysis of purified rENM.



A 10 μl sample was mixed with 3 μl 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.

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 reported[5] 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 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.

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                                        ENM  Met Gln Phe Ile Val Arg Thr Gly Met
                                        PHM  --- Lys Leu --- --- --- --- --- Leu

Ala Ala Ala Leu Ala Val Gly Ala Leu Ala Ala Val Val Ala Pro Ala Ser Ala Ser Ala
--- --- --- Val Val Leu --- --- Ala --- --- Ala --- Val --- --- --- --- Ala Val

Val Val Ala Asp Gly Ala His Ala Val Ser Gly Ala His Pro Val Ala Ala/Asn Pro Lys
--- Thr Asp ---     Pro Arg Pro --- Ala --- --- --- Ala --- Val Pro/--- --- ---

Thr Ile Lys Pro Ala Ala Tyr Asn Gln Ala Arg Ser Ile Leu Ala Asn Ala Gly Ser Gln
--- --- --- Ala --- --- --- --- --- --- --- --- Thr --- --- --- --- --- --- Arg

Thr Ala Ala Lys Ser His Pro Val His Gly Lys Asp Asp Val Pro Val Ser Tyr Gly Thr
--- --- --- --- --- --- --- Ile --- --- --- Thr --- --- --- --- --- --- ---

Ser Leu Leu Ala Ala Ala Arg Asp Glu Phe Arg His Thr Asp Arg Asn Leu Pro Ala Lys
--- --- --- --- --- --- --- --- --- --- --- Gln Ala --- Lys Lys --- --- --- ---

Gln Lys Lys Ser Asp Met Ser Ile Pro His Tyr Asn Ala Ile His Ser Ala Ala Lys Thr
Asp --- --- --- --- --- --- --- Ala --- --- --- --- Val --- --- --- --- --- ---

Met Gly Ile Asp Arg Trp  *
--- --- --- --- Thr ---  *
```

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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