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Cloning and Expression of the Metallo-Proteinase Inhibitor (S-MPI) Gene From Streptomyces nigrescens

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The coding and regulatory regions of a proteinaceous metallo-proteinase inhibitor (S-MPI) gene have been cloned from the genomic DNA of Streptomyces nigrescens using a deoxyinosine-containing synthetic probe designed from the amino acid sequence of the S-MPI protein. The S-MPI gene was located to the DNA region of about 1.2 kilobase pairs, which was verified to be sufficient for the gene expression in S. lividans.

Streptomyces strains are mycelial Gram-Positive bacteria which reveal a very complex development (1). They produce a number of extracellular proteins besides various important antibiotics.

A metallo-proteinase inhibitor, S-MPI, produced extracellularly by S. nigrescens TK-24, is a small protein consisting of only 102 amino acids, which specifically inhibits some of metallo-proteinases such as thermolysin (2-4). S-MPI is a sole proteinaceous inhibitor specific to metallo-proteinases. It has been demonstrated from the amino acid sequence that the S-MPI protein having a rigid conformation inhibits metallo-proteinase by a specific binding (4). A small protein like S-MPI is, therefore, considered to be quite useful for the study of a specific protein-protein interaction.

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In the present paper we describe the cloning and expression of the S-MPI gene from the producer strain.

EXPERIMENTAL PROCEDURES

Microorganisms and media. S. lividans 66 was used for the host of plasmid(5,6). S-MPI producer strains, S. nigrescens TK-24 was described in previous papers (2,3). Plasmid pIJ702 used for a cloning vector in S. lividans was described in a previous paper(7). Escherichia coli JM109 was used for the host of pUC18 (8) and pTZ18R (Pharmacia Co., Uppsala, Sweden). Single stranded DNA of pTZ18R derivatives were prepared by the superinfection of helper phage M13 K07. E. coli and Streptomyces strains were cultivated in TY (9) and YEME (6) medium, respectively.
Recombinant technology. Preparation of plasmid DNA from S. lividans and E. coli strains were performed as described in previous papers (5,10). Methods of DNA cleavage and joining were carried out using the standard methods (6,9). A synthetic oligonucleotide containing deoxyinosine was kindly provided by Mr. Y. Ohtani (Yamasa Shoyu Co., Chosi, Japan), and used as a probe after end-labeled by [γ-^{32}P]-ATP (3,000 Ci/mmole, Amersham Co., UK) and T4 polynucleotide kinase (Takara Shuzo Co., Kyoto, Japan)(9). Hybridization with the inosine-containing probe was performed as described in a previous paper (11). Autoradiography was done using X-ray film Rx (Fuji Film Co., Japan). Nucleotide sequence was determined by the dideoxy-chain termination method(12).
Assay of S-MPI activity. Inhibitory activity against metallo-proteinase was assayed using the method of Oda et al. (3). Thermolysin (Sigma Co., pronase type X) was used as the standard metallo-proteinase for the assay.

RESULTS AND DISCUSSION

(a) Cloning of the S-MPI gene

The strategy used for the isolation of clones containing the S-MPI gene from the producer strain S. nigrescens was to screen a genomic library using a synthetic probe. A similar strategy was successfully used for the cloning of a proteinaceous alpha-amylase inhibitor, HaimII gene, from S. griseosporeus(11). Since the complete amino acid sequence of the S-MPI protein was known, we designed an oligonucleotide containing deoxyinosine at the degenerated third codon positions (Fig. 1). The oligonucleotide synthesized was complementary to the mRNA deduced from a portion corresponding to the 67th to 75th amino acid residues, which was chosen by taking the codon degeneracy into account.

Procedures adopted for the cloning of the S-MPI gene are illustrated in Fig.2. DNA of S. nigrescens was cleaved partially with Sau3A and the DNA fragments were fractionated on a sucrose density gradient centrifugation. Fractions corresponding to about 10- to 15-kilobase pairs (kbp) were pooled and used for

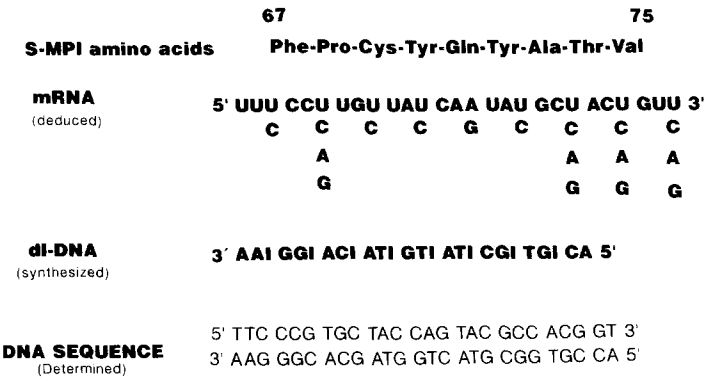

Fig. 1. Design of a synthetic probe used for the cloning of S-MPI gene. the top lane is the amino acid sequence of S-MPI protein used for the probe design. The second lane shows the messnger RNA deduced from the amino acid sequence, and the third lane presents an oligonucleotide containing inosines at the degenerated third codon positions, which was designed to be complemental to the mRNA sequence. The last lane shows the experimentally determined DNA sequence of the cloned DNA.

construction of a genomic library. About 10,000 ampicillin resistant transformants were screened by colony hybridization using the synthetic probe shown in Fig. 1. Four positive clones were confirmed to contain DNA inserts hybridizable with the probe. One of the plasmids isolated those clones, named pMPI3, was used for the source of sub-cloning.

A 1.2-kbp SalI fragment from the pMPI3 was confirmed to hybridize with the probe and sub-cloned in the SalI site of pTZ18R (Fig. 2). Two plasmids, named pMPI31 and pMPI32, which had the 1.2-kbp insert in opposite orientations, were constructed and used for further analysis. The orientation of S-MPI gene in pMPI31 and pMPI32 was determined by hybridization between the single-stranded DNA and the synthetic probe used for the identification of S-MPI clones. Also partial nucleotide sequence around the probe hybridizing region was determined and that corresponding to the probe is shown in the last lane of Fig. 1, which indicates that the cloned DNA has a sequence predicted from the synthetic probe used. The entire nucleotide sequence of the regulatory and coding regions of S-MPI gene will be published elsewhere.

(b) Production of metallo-proteinase inhibitor in S. lividans.

In order to know if the 1.2-kbp S-MPI fragment in pMPI32 is sufficient for the expression in Streptomyces cell, PstI-cleaved pMPI32 was ligated with the S. lividans vector pIJ702 which has

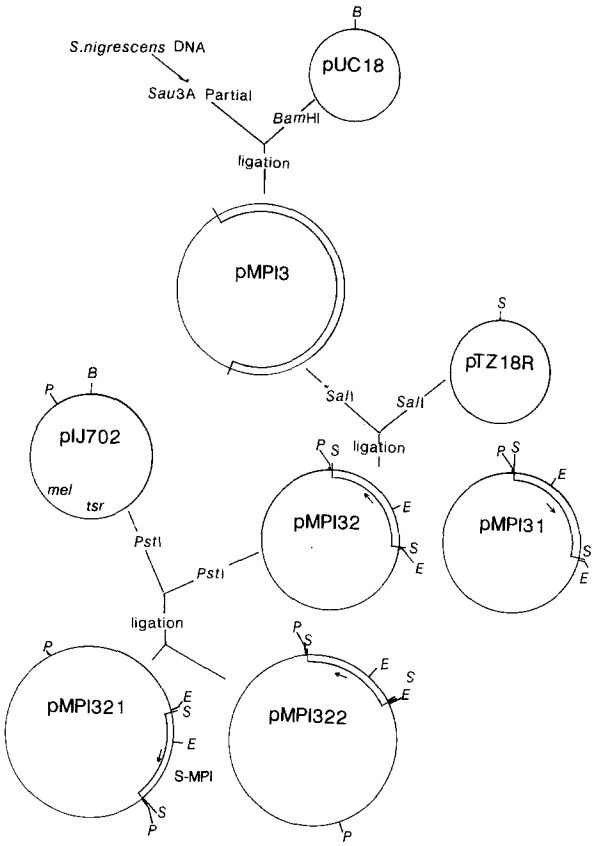

Fig. 2. Procedures for the cloning of S-MPI gene and the construction of subclones. pIJ702 is a cloning vector of S. lividans, which comprises thiostreptone resistance (tsr) and melanin genes (mel). Transformants to E. coli and S. lividans were selected by ampicillin and thiostreptone resistance, respectively. The arrows in plasmids indicate the region corresponding to the S-MPI gene and the orientation of the S-MPI open reading frame determined by the hybridization between the single-stranded DNA and the synthetic probe. Abbreviations: B, BamHI; E, EcoRI; P, PstI; S, SalI.

been cleaved with the same enzyme. Thus two plasmids, named pMPI321 and pMPI322, which can replicate in both E. coli and S. lividans, were constructed (Fig.2). Both pMPI321 and pMPI322 in S. lividans 66 produced extracellularly an inhibitory activity having the same specificity as the original S-MPI. Essentially no proteinase inhibitor activity was detected in the cell extract of S. lividans carrying pMPI321 or pMPI322. Furthermore, S. lividans carrying the vector plasmid pIJ702 did not produce such an inhibitor (data not shown). Thus we have concluded that the 1.2-kbp DNA fragment originated from the producer strain S. nigrescens contains regions essential for the expression in Streptomyces.

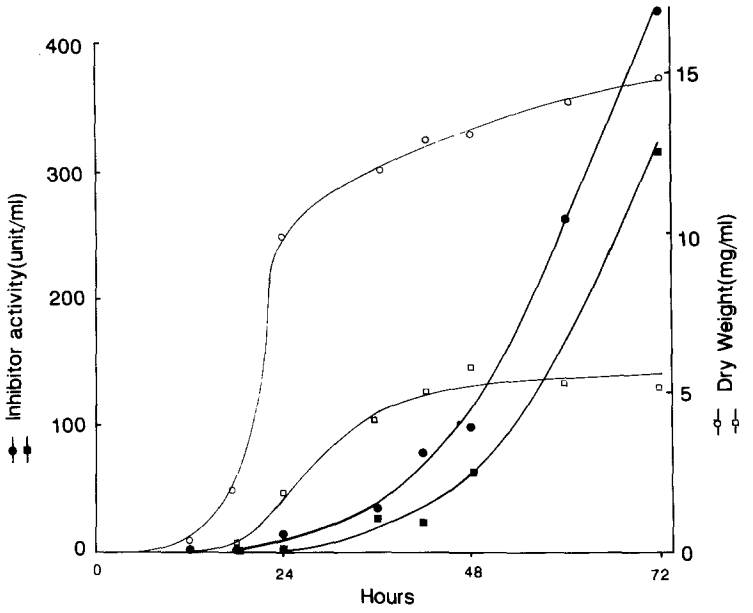

Fig. 3. Kinetics of S-MPI production in S. nigrescens and S. lividans carrying pMPI321. Cells were periodically sampled and thermolysin inhibitory activity in the culture filtrate was assayed (unit / ml). Cell growth was monitored by weighing dry mycelia (mg / ml). (●, ○), S. nigrescens; (■, □), S. lividans carrying pMPI321.

The kinetics of the inhibitor production in S. lividans carrying pMPI321 is shown in Fig. 3. The inhibitory activity was detected exclusively in the culture filtrate. The production started at the mid-logarithmic phase and continued to increase linearly more than 72 hours. The kinetics of S-MPI production in the original strain was essentially the same as that in S. lividans carrying pMPI321. The continuous expression of the S-MPI gene might be attributed to the multi-promoter systems verified in some of Streptomyces genes. The kinetics data of S-MPI production in S. nigrescens and S. lividans carrying pMPI321 indicate that the cloned 1.2-kbp DNA region comprises the regulatory and coding regions of S-MPI gene.

S-MPI is a small protein with a rigid structure, which is amenable for the analysis of three dimensional conformation by the X-ray crystallography or other analytical methods (12,13). The cloned S-MPI gene can be utilized as a source for the in vitro mutagenesis to obtain S-MPI protein with altered amino acid sequence. Productivity of the inhibitor protein in S. lividans is quite important to obtain a large quantity of the protein. S. lividans carrying a plasmid clone with the S-MPI gene produced,

however, not more than the original strain in spite of the gene dosage effect by the high-copy plasmid vector. This might be due to either inefficient expression of the S-MPI gene in S. lividans or to the instability of the S-MPI protein produced in this strain.

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