Identification of a Structural Gene Encoding a Metallothionein-like Domain that Includes a Putative Regulator Protein for *Streptomyces* Protease Gene Expression

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An open reading frame (termed ORF-PR) encoding a metallothionein-like domain-including protein was found upstream of a previously identified *Streptomyces* chymotrypsin-type protease gene (*sam-P20*). Promoter and terminator activities of ORF-PR were detected using the promoterless *Streptomyces* tyrosinase gene as a reporter gene and expression of ORF-PR was supposed to occur before that of *sam-P20* gene. Frameshift mutation analysis showed that the ORF-PR product might act as a repressive regulator of the *sam-P20* gene.

Key words: *Streptomyces*; metallothionein-like domain; sequence comparison; DNA-binding motif; protease gene

Metallothionein has been found in a wide variety of organisms ranging from bacteria to vertebrates and its biosynthesis may be involved in detoxification of heavy metals and oxidative damage, metal storage, development, and differentiation.11 We have been establishing the concept that a potent involvement of molecular interaction between proteases and protease inhibitors in physiological or morphological regulation of their producing cells of *Streptomyces*.2,3 Extracellular chymotrypsin-type proteases with sequences similar to each other, termed SAM-P20 and SAM-P26, were isolated, as the endogenous non-producing mutant strain of *S. alboargaeus S-3253*.4,5 Interestingly, *sam-P20* and *sam-P26* genes were found to be tandemly located close to each other and transcribed by their own individual promotors.6 During the course of analyzing the regulation of *sam-p20* gene expression, we identified, upstream of *sam-P20* gene, an open reading frame encoding a possible regulator protein (termed SAM-PR) containing a cysteine-rich metallothionein-like domain and a potential DNA-binding motif.

Here, we present the deduced structural features of the open reading frame (termed ORF-PR) encoding SAM-PR in comparison with the other metallothionein homologs and discuss a possible function of ORF-PR for *sam-P20* gene expression.

Survey of the new ORF was done based on the consensuss rule in *Streptomyces* genome that the coding region is extremely high in G+C content, particularly, at the third letter of codons.7 ORF-PR was found to be approximately 330 bp from the initiation codon of the *sam-P20* gene as depicted in Fig. 1. The nucleotide sequence data reported in this article will appear in the DDBJ, EMBL, and GenBank nucleotide sequence data bases with the accession number AB012144. ORF-PR includes 930 nucleotides corresponding to 310 amino acids with a deduced molecular mass of 31954 and a predicted isoelectric point of 5.2. ORF-PR potentially encodes, in its C-terminal portion consisting of approximately 60 amino acids, a domain of similar sequence to metallothioneins, which comprise a group that is a superfamily of small cysteine-rich proteins with high and selective affinity for heavy metals such as cadmium, copper, and zinc.12 Although the arrangement of cysteine residues is overall conserved among aligned members, one large insertion consisting of 11 amino acids and one large deletion consisting of 12 amino acids were found respectively at the N-terminal region and C-terminus of the predicted metallothionein domain in the ORF-PR product, as shown in Fig. 2. It is noteworthy that, in ORF-PR product, two negatively charged amino acids are present abundantly in the second and third metal-binding motifs in contrast with the other mammalian metallothionein members, which have positively charged amino acids at the corresponding sites. Prokaryotic metallothionein were reported in *Synecochoccus sp.*8,9 and *Pseudomonas putida*.9,10 Also, a plausible helix-turn-helix DNA binding motif, LAG-CAVWLGLACTCQLCCGT, with similarity to that of human DNA repair protein ERRCC-1,10 was detected adjacent to the metallothionein-like domain with some overlapping. Previously, we constructed the promoter- and terminator-probe vectors, termed pMP and pMT, respectively.5 These vectors were designed to verify the presence of promoter and terminator by allowing visual monitoring the tyrosinase-mediated generation of a brown diffusible pigment, melamin. Using these probe vectors, the test DNA fragments were inserted into the multi-cloning sites upstream from the tyrosinase reporter gene. The resultant plasmid vectors were introduced into *S. coelicolor* and the recombinant clones were test-
ed for melanin pigmentation on the TSB plate as described previously. Promoter activities were detected in the upstream regions between Bgl II-Bam HI sites for ORF-PR and between Sal I-Xho I sites for sam-P20 gene, and terminator activities were also detected in the downstream regions of ORF-PR (between Pst I-Sal I sites) and sam-P20 gene (see Figs 1 and 3, data not shown). It was of interest to observe that the promoter of ORF-PR has stronger activity and functions earlier than that of sam-P20 gene. Although a variety of diverse promoter sequences were identified in many Streptomyces genes, no distinct sequence similar to those already defined could be found in the cloned DNA fragment with promoter activity. Inverted repeat sequences capable of forming potential stable hairpin structures were observed downstream from the ORF-PR and sam-P20 gene, respectively. The free energy values (JG) for hairpin terminator-like structures was calculated to be \(-19.1\) kcal/mol and \(-42.8\) kcal/mol, respectively. These values were in good agreement with the fact that terminator activity of ORF-PR was weaker than that of sam-P20 gene using the terminator-probe vector, pMT (data not shown).

To investigate whether ORF-PR is involved in the sam-P20 gene expression, we constructed a new vector, pMPW, in which a Bgl II-Xho I DNA fragment containing the complete transcriptional unit for ORF-PR and promoter region of the sam-P20 gene was inserted into the pMP vector using Bam HI and Sal I sites, as shown in Fig. 3. Then, a frameshift mutation was introduced into ORF-PR by digestion with Mlu I and modification with T4 DNA polymerase to generate another vector, pMPM. The two vectors thus constructed were individually introduced into S. coelicolor and analysis of melanin pigmentation was done on the TSB plate for each transformant. Fig. 3 shows representative results. The frameshift mutation of ORF-PR (pMPM) drastically increased melanin pigmentation, indicating that the ORF-PR product might negatively regulate the sam-P20 gene expression. However, it is also true that substrate hydrolysis of SAM-P20 was inhibited effectively by endogenous SSI in vitro. Therefore, it is of interest to clarify the regulation of SAM-P20 protease activity by either a transcriptional repression mechanism or interaction with SSI in the extracellular environment.

To our knowledge, this domain-containing protein might be the first such discovery in Streptomyces and the finding obtained here would provide some insight into consideration of physiological significance of this sort of protein with no relation to the well-known roles of detoxification of heavy metals and metal storage. Also, from the viewpoint of practical applications, this protein derived from Streptomyces, a major member of soil bacteria, would be a powerful catalyst capable of bioremediating metallic pollutants in soil.

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Fig. 2. Alignment of Amino Acid Sequence Deduced from ORF-PR with Those of Several Members of Metallothionein Superfamily. CXC, XXCXC, CCXC, and CXXC metal-binding motifs [1] are shaded and Cys residues are written in bold. The numbers on the top of this alignment correspond to the amino acid numbers deduced from ORF-PR.

production of melanin

Fig. 3. Frameshift Mutation Analysis for the Effects of ORF-PR on sam-P20 Gene Expression. sam-P20 gene expression in an S. coelicolor transformant strain carrying each vector was semi-quantitatively monitored as melanin pigmentation on the test plate using the tyrosinase reporter gene on the promoter-probe vector, pMP [6]. A representative example is presented. D and MT are abbreviations of putative DNA-binding motif and metallothionein-like domain, respectively. Asterisk denotes the termination of translation caused by the frameshift mutation. Amino acid sequence of metallothionein-like domain truncated form of ORF-PR product is presented in a box.

References
4) Taguchi, S., Odaka, A., Watanabe, Y., and Momose, H.,


