Regulation of Secondary Metabolism and Morphogenesis in *Streptomyces*

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1. The A-factor-receptor protein of *Streptomyces griseus* as a repressor-type regulator

A-factor (2-isocaprolyl-3R-hydroxy-methyl- γ -butyrolactone) is a low-molecular-weight autoregulating factor originally found in the culture broth of *Streptomyces griseus* by Khokhlov *et al.* It is akin to hormones in eucaryotes because it switches on several phenotypes, such as streptomycin production, streptomycin resistance, and sporulation, at an extremely low concentration, as low as 10^{-9} M. The data from our laboratory and Piepersberg's group have suggested that A-factor switches on transcription of the gene cluster for streptomycin production. The functional similarity of A-factor to eucaryotic hormones has led us to assume that A-factor exerts its regulatory function with the aid of some receptor protein.

The optically active form of tritium-labeled A-factor was chemically synthesized. By using the radioactive A-factor, a binding protein specific to A-factor was detected in the cytoplasmic fraction of this organism. The binding protein had an apparent molecular weight of approximately 26,000, as determined by gel filtration chromatography. Scatchard plot analysis suggested that A-factor bound the protein in the molar ratio of 1:1 with a binding constant, K_d , of 0.7 nM. The number of the binding protein molecules was roughly estimated to be 37 per genome. The "inducing material" virginiae butanolide C (VB-C), which has a structure very similar to that of A-factor and is essential for virginiamycin production in *Streptomyces virginiae*, did not inhibit binding of A-factor by the protein. In addition, no protein capable of specifically binding ^3H -labeled VB-C was found in *S. griseus*. Together with the observation that VB-C had almost no biological activity on the restoration of streptomycin production or sporulation in an A-factor-deficient mutant of *S. griseus*, these results indicated that

the binding protein had a strict ligand specificity. Examination for an A-factor-binding protein in *Streptomyces coelicolor* A3(2) and *Streptomyces lividans*, both of which have been most extensively genetically studied, showed the absence of any specifically binding protein.

In order to obtain definitive evidence for functional involvement of these binding proteins, we conducted experiments to isolate and characterize mutants deficient in the A-factor-binding protein. Our success in isolating the mutants is due to an idea implied by the characteristics of a mutant strain, *S. griseus* 2247, that requires no A-factor for its streptomycin production or sporulation. *S. griseus* 2247 was found to have a defect in the A-factor-binding protein. This observation implied that the A-factor-binding protein in the absence of A-factor repressed the expression of both phenotypes in the wild-type strain. Screening among mutagenized *S. griseus* colonies for strains producing streptomycin and sporulation in the absence of A-factor yielded three mutants that were also deficient in the A-factor-binding protein. The reversal of the defect in the A-factor-binding protein of these mutants led to simultaneous loss of streptomycin production and sporulation. All these data suggested that the A-factor-binding protein played a role in repressing both streptomycin production and sporulation and that the binding of A-factor to the protein released its repression. The mutants deficient in the A-factor-binding protein began to produce streptomycin and sporulate at an earlier stage of growth than the wild-type strain. The amount of streptomycin produced by these mutants were approximately 10 times as large as that by the parental strain. These findings are consistent with the idea that the intracellular concentration of A-factor determines the timing of derepression of the gene(s) whose expression is repressed by the A-factor-binding

protein. These findings provide an important and rational insight for breeding antibiotic-producing strains.

II. Identification of A-factor-dependent promoters in the streptomycin biosynthetic cluster

The streptomycin (Sm) resistance gene encoding streptomycin-6-phosphotransferase is located in the streptomycin biosynthetic cluster. By using an extremely heat-stable malate dehydrogenase gene of *Thermus flavus* as a reporter, we examined A-factor-dependence of the promoters in part of the cloned cluster that contains at least three transcriptional units. Piepersberg's group reported that the three promoters are all inactive in the absence of A-factor. We found that A-factor enhanced transcription of only the promoter for *strR*, a putative regulatory gene, by 2-fold. The 2-fold increase was also observed when the amounts of mRNA transcribed from the *strR* gene were compared by S1 mapping. This suggests that A-factor stimulates transcription of *strR* and then the StrR protein, in turn, induces transcription of other promoters.

Together with the observation that the A-factor-binding protein is acting as a repressor-type regulator, we may assume the following model as our working hypothesis. The A-factor-binding protein binds the promoter region of *strR* at an early stage of growth and represses the expression. When the intracellular concentration of A-factor reaches a certain level at a specific growth phase, the binding protein binds A-factor, as a consequence of which the protein is released. The expression of *strR* is then open for transcription, leading to switch-on of the other Sm production genes.

III. A global regulatory gene, *afsR* for secondary metabolite formation from *Streptomyces coelicolor* A3(2)

The *afsR* gene of *Streptomyces coelicolor* A3(2), which was originally assumed to be the *afsB* gene, is required in this organism for the biosynthesis of A-factor and the pigmented antibiotics actinorhodin and undecylprodigiosin. We have found that the AfsR protein contains two regions which resemble conserved domains of known DNA-binding proteins and that it stimulates transcription of the actinorhodin biosynthetic pathway. We have also found that a region (*afsC*) upstream of *afsR* enhances the stimulatory function of *afsR*. Nucleotide sequencing and subsequent experiments revealed that *afsC* and *afsR* comprised actually a single open reading frame. Furthermore, the amino acid sequence derived from the *afsC* region contains two consensus sequences for ATP-binding. These ATP-binding consensus sequences were found to be important for exerting the regulatory function of AfsR because amino acid exchanges of these regions, generated by site-directed mutagenesis, resulted in complete loss of its regulatory function. Incubation of [γ - 32 P]ATP and the cell extract prepared from *S. griseus* containing the *afsR* gene on a high-copy number plasmid yielded a 32 P-labeled protein that had the same molecular size as expected from the nucleotide sequence and was cross-reactive with the anti-AfsR antibody. No 32 P-labeling occurred when [α - 32 P]ATP instead of [γ - 32 P]ATP was used. These results indicated the presence of a regulatory system of secondary metabolism whose function is modulated by phosphorylation of the regulatory protein.