Proc. KSAM Annual Meeting
June 20-22, 2001, Chonan, Korea

Functional Proteins Regulating Physiological and Morphological Differentiation in *Streptomyces* spp

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Streptomycetes are Gram-positive bacteria with an unusual morphological complexity, forming a substrate mycelium on solid medium and an aerial mycelium which differentiates into arthrospores. The bacteria are important of their unique metabolism to produce secondary metabolites such as antibiotics and pigments (physiological differentiation). Both of the morphological and physiological differentiations are induced by shift-down of some of essential nutrients such as carbon, nitrogen, and phosphate. A quantitative analysis of the differentiations in *Streptomyces* spp. and evaluation some of the important proteins are reviewed in conjunction with the relationships between the morphological differentiation and physiological differentiation.

Roles of proteases and protease inhibitor on the morphological differentiation and the quantitative analysis of the state

In order to characterize of spores formed in submerged culture a strain producing abundant spores in submerged culture was selected from soil and the strain (SMF301) was classified as *Streptomyces albidoflavus* by numerical analysis of taxonomic characters. Spores formed in submerged cultures (submerged spores) and formed on solid media (aerial spores) were compared. The resistance of aerial spores to lysozyme digestion, mild acid treatment, heating and desiccation was higher than that of submerged spores, but the submerged spores were more resistant to sonication.

Quantitative analysis of sporulation kinetics in submerged cultures was done using a chemically defined medium. Kinetic parameters calculated from batch and chemostat cultures showed that specific submerged spores formation rate (q_{spo}) was inversely related to the specific mycelium growth rate (u), being optimum for sporulation at 0.05 h^{-1} The turnover rate of biomass at maximum growth yield was 0.029 h^{-1} and 5.6×10^6 spores was formed from 1 g of mycelium.

The state of the morphological differentiations of *S. albidoflavus* SMF301 was analyzed with the combination of pyrolysis mass spectrometry (PyMS) and artificial neural networks (ANNs). Cells of *S. albidoflavus* taken from a batch culture at various intervals were subjected to PyMS. It was evident that the PyMS spectra varied with growth phase and sporulation and too complex to identify the distinct state of growth and sporulation of *S. albidoflavus* SMF301.

In order to predict the differentiation states, the normalized PyMS data were analyzed by using multivariate statistics techniques including PCA and PC-CVA. And ANNs were trained on PyMS data using two different algorithms: a back propagation and a radial basis function classifier. The momentum was set to 0.7 to avoid local minima and the number of hidden nodes was set to 5. The learning curve showed a typical learning pattern, and the final error was 0.0049 at minimum validation error. The estimated values of input vectors of training, validation, and test sets were well fitted to the differentiation states.

As results, the morphological differentiation was clearly distinguished and more interestingly the dynamic changes from mycelium growth state to sporulation state could be quantitatively demonstrated by applying the multivariate statistics and ANNs.

Streptomyces exfoliatus SMF13 producing leupeptin was also isolated from Korean soil. And it was found that the strain produced in due order of leupeptin, a metallo-protease, and a trypsin-like protease (TLP) in a batch culture. The production of leupeptin was very closely related to the mycelium growth and the activity decreased with simultaneous production of a metallo-protease. The metallo protease (34,7 kDa) were found to inactivate leupeptin by hydrolysis of peptide bond of leupeptin at the P1 position. Hence the protease was named as leupeptin-inactivating enzyme (LIE). It was found that TLP (31.8 kDa) produced at the later stage of the batch culture could hydrolyzed the mycelial protein of the producing strain. Even more it was evident that the hydrolytic activity was inhibited competitively by leupeptin.

As result, it was thought that TLP was essential for the formation of aerial mycelium in relation with the endogeneous metabolism of resting form of substrate mycelium, whereas the growing mycelium could be protected from the TLP by accumulation of leupeptin on the surface of mycelium. This is the first report to demonstrate the role of CTP, leupeptin, LIE, and TLP specifically in relation with mycelium differentiation of *Streptomyces* spp.

[Publications: 1~9]

Regulation of antibiotic production by intra-cellular signaling and quantitative analysis of the physiological differentiation

Antibiotic production in *Streptomyces* spp. is generally confined to occur at the stationary phase cultures, or on the condition where growth rate is very low. And it usually coincides with the onset of morphological differentiation such as, aerial mycelium and spore formation in agar-based solid cultures. With an intensive investigation on the factors regulating the unique differentiations, the role of ppGpp have been proposed. In this context, ppGpp in antibiotic production was analysed, as a model, in *Streptomyces coelicolor* A3(2), the most genetically characterized and tractable streptomycete.

Studies of batch cultures, some of which were subjected to amino acid starvation, indicated a correlation between ppGpp synthesis and transcription of pathway-specific regulatory genes for the both of actinorhodin (Act) and undecylprodigiosin (Red), the two pigmented antibiotics made by the strain. Even more recently, the ppGpp synthetase gene, *relA*, of *S. coelicolor* was cloned and characterized, and used to construct a *relA* null-mutant.

The effects of growth rate and nutrient feed rate on the production of actinorhodin (Act) and undecylprodigiosin (Red) were determined in *Streptomyces coelicolor* A3(2) and in a congenic *relA* null-mutant known to be deficient in ppGpp synthesis and antibiotic production under conditions of nitrogen limitation. Production of Act and Red in the *relA* mutant was lower than that of the parental strain, particularly under conditions of glucose- and ammonium-limitation, indicating an important and general role for ppGpp in determining the onset of the antibiotic biosynthesis under conditions of nutrient limitation.

Analysis of clavulanic acid production in *Streptomyces clavuligerus* was also considered. The steady state values of glycerol, ammonium ion, biomass, and clavulanic acid production in the chemostats limited by glycerol were measured. The steady-state values of biomass in the chemostats increased with the specific growth rate; on the other hand, clavulanic production was inversely related to growth rate.

The specific uptake rate of glucose and ammonium ion (q_{glu} and q_{amm}) increased with the specific growth rate, indicating that those nutrients were more essential for mycelium growth but not for clavulanic acid production.

In order to distinguish the effect of the growth rate from the limitation of the nutrients, chemostat cultures were carried out at fixed specific growth rate (0.04 h^{-1}) where the concentration of glycerol or ammonium ion were varied by changing the feeding concentration. The steady-state values of biomass increased with the nitrogen feed rate, while clavulanic acid production was inversely related with nitrogen feed rate, and was optimum at 0.9 mM h^{-1}.

[Publications: 9~11]

Fact and roles of β-lactamase-inhibitory proteins

A strain producing β-lactamase-inhibitory proteins was isolated from the Korean soil and the strain was identified to be *S. exfoliatus* SMF19 by using by numerical taxonomy using Taxon program. *S. exfoliatus* SMF19 produced two potent β-lactamase inhibitory proteins, BLIP-I (17.5 kD) and BLIP-II (28 kDa). The *Ki* value of BLIP-I and BLIP-II against TEM-1 -lactamase was determined to be 0.085 nM and 0.46nM, respectively.

Gene (*bliA*) for BLIP-I consists of 558 bp and encodes a mature protein of 157 amino acid residues preceded by a 29 amino acid signal sequence (GenBank: AF201389). The gene (*bliB*) for BLIP-II consists of 116bp amd encode 332 amino acids preceded by a 40 amino acid signal sequence (GeneBank: U97057). While BLIP-I is homologous to BLIP (38% sequence identity), BLIP-II shows no discernible sequence identity with BLIP of *S. clavuligerus*. An expression system was developed to produce large amount of functional BLIP-I using translational fusion with His-tag. BLIP-II did not show any similarity to that of BLIP-I and BLIP, but shares significant sequence identity with the RCC1 (regulator of chromosome condensation) family of proteins (24% identity with *Schizosacchromyces pombe* PIM112), 23% identity with *Drosophila melanogaster* RCC1 and 21% with human RCC1.

Based on the recently determined seven-bladed-propeller fold of human RCC1, the structure of BLIP-II was predicted to be entirely distinct from that of BLIP. The structure of the BLIP-II in complex with the TEM-1 -lactamase has been determined to 2.3° resolution. BLIP-II is a seven-bladed -propeller with a unique blade motif consisting of only three antiparallel -strands. The overall fold is highly similar to the core structure of the human regulator of chromosome condensation (RCC1). Although BLIP-II does not share the same fold with BLIP, a comparison of the two complexes reveals a number of parallels and provides further insights into key components of the TEM-1BLIP and TEM-1BLIP-II interfaces. In addition to providing insights into the design of novel peptide-based -lactamase inhibitors, kinetic, mutagenesis and crystallographic studies of BLIP and its complex with the TEM-1 -lactamase have provided a model system for studying protein-protein interactions.

BLIPs showed *in vitro* inhibitory activity against various -lactamases, while the biological roles of BLIP and BLIP-II are not still well understood. One hypothesis is that it functions as a regulator of cell wall synthesis by interacting with penicillin-binding proteins (PBPs). Studies exploring this possibility and aimed at elucidating the regulatory mechanism are now in progress. The insertional disruption of the each gene (*bliA* and *bliB*) and double disruption both the gene (*bliA/bliB*) were carried out. And the phenotypic characteristics of the disruptants of *S. exfoliatus* SMF19 were apparent in significant changes in the morphological differentiation. Hence the direct or indirect role of the BLIPs on the mor-

phological differentiation would be the mostly interesting in the next works.

[Publications: 12~15]

References

1. Rho, Y. T. and K. J. Lee. (1993). Characteristics of spores formed on solid cultures and in submerged cultures of *Streptomyces albidoflavus* SMF301. *J. Gen. Microbiol.* **139**, 3131-3137.
2. Rho, Y. T. and K. J. Lee. (1994). Kinetic studies on spore formation of *Streptomyces albidoflavus* SMF301 in submerged culture. *Microbiology.* **140**, 2061-2065.
3. Kang, S. G., I. S. Kim, Y. T. Rho and K. J. Lee. (1995). Production dynamics of extracellular proteases accompanying morphological differentiation of *Streptomcyes albidoflavus* SMF301. *Microbiology.* **141**, 3095-3103.
4. Kim, I. S. and K. J. Lee. (1995). Physiological roles of leupeptin and extracellular proteases in mycelium development of *Streptomyces exfoliatus* SMF13. *Microbiology* **141**(4), 1017-1025.
5. Kim, I. S. and K. J. Lee. (1995). Kinetic study on the production and degradation of leupeptin in *Streptomyces exfoliatus* SMF13. *J. Biotechnol.,* **42**, 35-44.
6. Kim, I. S. and K. J. Lee. (1996). Trypsin like protease in *Streptomyces exfoliatus* SMF13, as a potential agent for mycelium differentiation. *Microbiology* **142**, 1797-1806. [103]. Kim, I. S., Kim, Y. B. and K. J. Lee. (1997). Characterization of leupeptine inactivating enzyme from *Streptomyces exfoliatus* SMF13 which produces leupeptin. *Biochem. J.* **331**, 539-545.
7. Kang, S. G. and K. J. Lee. (1997). Kinetic analysis of morphological differentiation and protease production in *Streptomyces albidoflavus* SMF301. *Microbiology* **143**, 2709-2714.
8. Kang, S. G., R. G. W. Kenyon, A. C. Ward and K. J. Lee. (1998). Analysis of the differentiation state in *Streptomyces albidoflavus* SMF301 by the combination of pyrolysis mass spectrometry and neural network. *J. Biotech.* **62,** 1-10.
9. Lee, K. J. (1998). Dynamics of morphological and physiological differentiation in actinomycetes group and quantitative analysis of the differentiation. *J. Microbiol. Biotech.* **8**, 1-7.
10. Kang, S. G., W. Jin, M. Bibb and K. J. Lee. (1998). Actinorhodin and undeecylprodigiosin production in wild-type and relA- mutant strains of *Streptomyces coelicolor* A3(2). *FEMS Letters,* **168**, 221-226.
11. Jin W. J. Y. Kim, H. K Kim and K. J. Lee. (2001). Identification of the genes for the synthesis of ppGpp in *Streptomyces clavuligerus* and the roles of the gene products on the biosynthesis of clavulanic acid. (Manuscrip in preparation).
12. Kim, M. K. and K. J. Lee. (1994). Characteristics of β-lactamase inhibiting protein from *Streptomyces exfoliatus* SMF19. *Appl. Env. Microbiol.* **60** (3), 1029-1032.
13. Park, H. U. and K. J. Lee. (1998). Cloning and heterologus expression of the gene for BLIP-II, a β-lactamase inhibitory protein from *Streptomyces exfoliatus* SMF19. *Microbiology.* **144**, 2161-2167.
14. Kang, S. G., H. U. Park, H. S. Lee and K. J. Lee. (2000). A new β-lactamase inhibitory protein (BLIP-I) from *Streptomyces exfoliatus* SMF19 and the role on the morphological differentiation. *J. Biol. Chem.* **275**, 16851-16856.
15. Lim D., H.U. Park, L.De Castro, S. G. Kang, S. Jensen, K. J. Lee, and N. Strynadka (2001). Crystal structure and kinetic analysis of β-lactamase inhibitor protein-II, a novel seven-bladed -propeller, in complex with TEM-1 -lactamase. *Nature Structual Bioloogy* (In press).