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Nutritional Regulation of Morphological and Physiological Differentiation on Surface Culture of *Streptomyces exfoliatus* SMF13

KIM, IN SEOP AND KYE JOON LEE*

Department of Microbiology, College of Natural Science, and Research Center for Molecular Microbiology, Seoul National University, Seoul 151-742, Korea

Nutritional factors regulating the morphological differentiation and physiological differentiation of *Streptomyces exfoliatus* SMF13 on surface cultures were evaluated. *S. exfoliatus* SMF13 produced leupeptin and chymotrypsin-like protease (CTP) at the stage of substrate mycelium growth, and leupeptin-inactivating enzyme (LIE) and trypsin-like protease (TLP) at the stage of aerial mycelium growth. The activity of leupeptin and CTP was high in the region of active growing substrate mycelium, whereas the activity of LIE and TLP was high in the region of aerial mycelium or spores. The differentiations were induced in glucose-limited conditions or by the addition of glucose anti-metabolite (methyl α-glucopyranoside), but repressed by high concentrations of glucose or casamino acids. Morphological differentiation (formation of aerial mycelia and spores) was closely related with physiological differentiation (formation of brown-pigment, LIE and TLP). The local distribution of leupeptin, CTP, LIE, and TLP in a developing colony showed that colony development correlated with the production and functions of the compounds: CTP is essential for providing a nitrogen source for mycelium growth: leupeptin regulates TLP activity: LIE inactivates leupeptin: TLP hydrolyzes non-growing mycelium.

Members of the genus *Streptomyces* are gram positive bacteria with an unusual morphological complexity. On solid medium a spore germinates, grows vegetatively as a substrate mycelium and then develops into an aerial mycelium, which segments into chains of spores (2, 4).

There have been some reports on the nutritional regulation of morphological differentiation in *Streptomyces* sp. (3, 4, 9). The formation of aerial mycelium is thought to be a reaction to unsuitable conditions in the natural environment of the organism. One of the triggering stimuli of the differentiation is nutrient-limitation. Autoradiographic studies have shown that substrate mycelium was a nutrient support for aerial mycelium growth (14, 15). In the later stage of morphological differentiation, many extracellular enzymes as well as secondary metabolites such as antibiotics and pigments were produced. There have been increasing reports regarding the possible relationship between secondary metabolism and extracellular protease production (5-8, 12, 13). However, the regulatory influences governing morphological and physiological differentiation is still very poorly understood.

S. exfoliatus SMF13 sequencially produced leupeptin, chymotrypsin-like protease (CTP), leupeptin-inactivating enzyme (LIE), and trypsin-like protease (TLP). Production of leupeptin was closely associated with mycelial growth, but it was inactivated by LIE when mycelium growth turned to stationary phase in a submerged culture, or aerial mycelium started to form on the surface culture. TLP functioned as an essential enzyme involved in the metabolism of mycelial proteins in the decline phase of submerged cultures or during aerial mycelium formation on surface cultures (12, 13).

To help understand the roles of leupeptin, CTP, LIE, and TLP in mycelium development of *S. exfoliatus* SMF13, it is desirable to determine how their synthesis is regulated. In this study, the effect of nutritional factors on morphological differentiation and production of leupeptin, CTP, LIE, and TLP will be discussed.

MATERIALS AND METHODS

Microorganism and Media

The microorganism used was *Streptomyces ex-*

*Corresponding Author
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foliatus SMF13 (10, 11). Stock culture medium and main culture medium (Bennett medium) consisted of (w/v): 1% glucose, 0.1% yeast extract, 0.1% beef extract, 0.2% casamino acids and 1.8% agar. Increasing concentrations of glucose or casamino acids were added to the main culture medium in order to study the effect of glucose or casamino acids on the differentiation of *S. exfoliatus* SMF13. An increasing concentration of methyl α-D glucopyranoside was added to the main culture medium supplemented with 2% glucose in order to study the derepressible effect of methyl α-D glucopyranoside on the glucose repression.

Strain Maintenance and Culture Conditions

The strain was transferred to slopes of stock culture medium each month, and stored at 4°C. For surface cultures, spores from stock culture plates were inoculated to agar plates of the culture medium by sterile toothpick transfer, and incubated at 28°C.

CTP, TLP, Leupeptin, and LIE Assay

Agar plugs containing colonies were cut-off from the plates, homogenized in 10 ml of Tris-HCl buffer (0.1 M, pH 7.5) and centrifuged (10,000 g for 10 min). The activities of CTP, TLP, and LIE and the concentration of leupeptin in the supernatant were measured.

The activity of CTP and TLP was estimated by measuring the amount of *p*-nitroanilides liberated from the N-benzoyl tyrosine *p*-nitroanilide and N-benzoyl arginine *p*-nitroanilide, respectively. Enzyme reactions were carried out with 200 μmol of substrates at 35°C and pH 7.5 (Tris-HCl buffer, 0.1 M). Activity was calculated from the linear part of the curve, using E_{405}=9620 mol^{-1} cm^{-1}. One unit of CTP and TLP activity was defined as the amount of enzyme needed for the production of 1 μmol of product (*p*-nitroanilide) per min (18).

Leupeptin concentration was determined as follows: Inhibition activity of leupeptin was measured using 80 μg of papain as the target protease. The amount of leupeptin was calculated from the standard inhibition curve using leupeptin purchased from Sigma Co. (12).

The activity of LIE was determined as follows: 1.0 ml of the supernatant was preincubated with 50 μg of leupeptin at 4°C and pH 7.5 (Tris-HCl buffer, 0.1 M) for 10 min in order to compensate for the possible interaction between leupeptin and TLP existing in the supernatant. The preincubated reaction mixture was incubated at 35°C for 10 min, then heated for 5 min at 80°C for complete inactivation of any protease and LIE in the reaction mixture. The remaining activity of leupeptin was assayed (A). In parallel, the preincubated reaction mixture was heated at 80°C for 5 min for complete inactivation of LIE, then incubated at 35°C for 10 min. The remaining activity of leupeptin was assayed (B). The difference between A and B was defined as the leupeptin-inactivating activity. 1 unit of LIE was defined as the amount of enzyme needed for the inactivation of 10 μg of leupeptin per min (12).

Scanning Electron Microscopy

Colonies developed on agar medium were fixed using the following procedures: 8% phosphate-buffered glutaraldehyde solution (pH 7.4) was poured into holes punched around colonies (16). Plates were left for 24 hours at 4°C; colonies were cut out to the minimal size from the agar medium and then dried in a sealed Revco box under P_2O_5 at 4°C. Dried colonies were gold-coated with Polaron SC502 Sputter Coater (Fisons, U.K.) at 15 mA for 1 min in vacuum conditions. The morphology of colonies were observed with a Streoscan 260 scan electron microscope (Cambridge Ltd., U.K.).

RESULTS

Glucose Repression of Aerial Mycelium Formation

Streptomyces exfoliatus SMF13 formed aerial mycelium and sporulated well when grown on surface culture using Bennett agar medium. The effect of glucose on aerial mycelium formation and sporulation on Bennett agar medium was tested. The results after 8 days growth are shown in Fig. 1A. The surface of the culture growing on Bennett agar medium was tough and leathery, which was due to the presence of spores. Scanning electron micrograph showed that surface of the colony on Bennett agar plates consisted primarily of the long rectiflexible spore chains and the surface of spore was smooth (Fig. 2A). Glucose did repress the formation of aerial mycelium and spores as well as brown-pigment production. The surface of the colony grown on Bennett media with 2% glucose added was bald (Fig. 1A). Scanning electron micrograph showed that the surface consisted of substrate mycelial growth with no spores (Fig. 2B).

The effect of glucose on the production of leupeptin, CTP, LIE, and TLP is presented in Table 1. The production of leupeptin was increased with increasing concentrations of added glucose. The production of CTP was somewhat stimulated with the addition of 1% glucose, but partially repressed above 2% glucose addition. However, the production of LIE and TLP was decreased with the increasing concentration of glucose and completely repressed above 2% glucose addition.

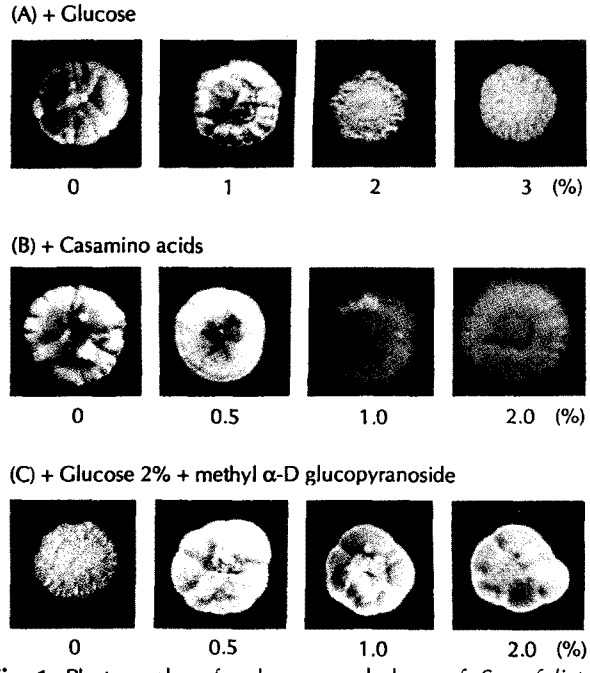

Fig. 1. Photographs of colony morphology of *S. exfoliatus* SMF13 grown on Bennett medium added with increasing concentration of glucose.
(A) Bennett medium added with increasing concentration of casamino acids, (B) Bennett medium added with 2% glucose and increasing concentration of methyl α-D glucopyranoside, (C) after 8-days culture.

Casamino Acids Repression of Aerial Mycelium Formation

The effect of casamino acids on aerial mycelium formation and sporulation on Bennett agar medium were tested. The results after 8 days culture are shown in Fig. 1B. The addition of 2% casamino acids did repress formation of aerial mycelium and spores as well as brown-pigment production. The surface of growth on Bennett media with 2% casamino acids was bald. Scanning electron micrograph showed that the surface consisted of substrate mycelial growth with no spores (Fig. 2C).

The effect of casamino acids on the production of leupeptin, CTP, LIE, and TLP are presented in Table 2. The production of leupeptin was increased with the increasing concentration of added casamino acids. The production of CTP was partially repressed by the addition of casamino acids. However, the production of LIE and TLP was decreased by increasing the concentration of added casamino acids and completely repressed by the addition of 2% casamino acids.

Derepression of Aerial Mycelium Formation by Glucose Anti-Metabolite, Methyl α-D Glucopyranoside

The glucose repression of aerial mycelium formation and sporulation was relieved by the addition of methyl α-D glucopyranoside (Fig. 1C). Also the

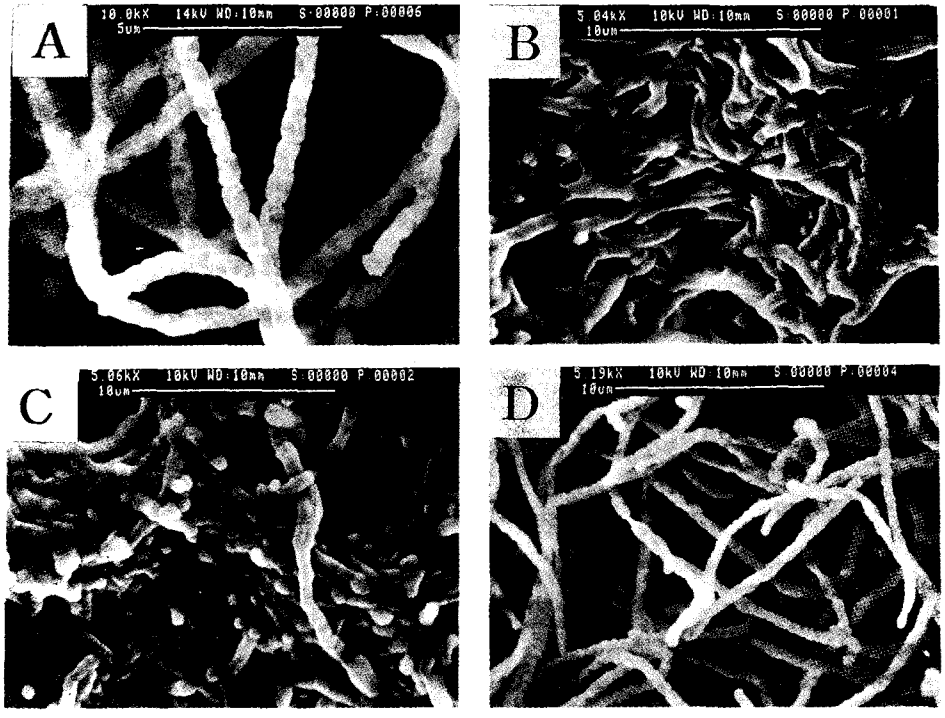

Fig. 2. Scanning electron micrographs of *S. exfoliatus* SMF13 grown on Bennett medium.
(A) Bennett medium added with 2% glucose, (B) Bennett medium added with 2% casamino acids, (C) Bennett medium added with 2% glucose and 0.5% methyl α-D glucopyranoside, (D) after 8-days culture.

Table 1. Effect of glucose on morphological and physiological differentiation of *S. exfoliatus* SMF13 on surface culture[a].

Glucose (%)	Aerial mycelium (%)	Sporulation (%)	CTP	LIE	TLP	Leupeptin (μg colony^{-1})
			(mU colony^{-1})			
0.0	100	100	13.78	19.33	13.80	7.2
1.0	100	80	24.09	14.65	3.36	54.1
2.0	0	0	6.40	0.13	ND[b]	258.0
3.0	0	0	5.09	0.10	ND	265.5

[a]Basal medium was Bennett agar medium. 50 agar plugs containing colony were removed from the plates after 7-days culture. Concentrations of leupeptin, CTP, LIE, and TLP were measured as discribed in Materials and Methods.
[b]ND: not-detected.

Table 2. Effect of casamino acids on morphological and physiological differentiation of *S. exfoliatus* SMF13 on surface culture[a].

Casamino (%)	Aerial mycelium (%)	Sporulation (%)	CTP	LIE	TLP	Leupeptin (μg colony^{-1})
			(mU colony^{-1})			
0.0	100	100	13.78	19.33	13.80	7.0
0.5	100	18	7.63	2.32	1.28	283.6
1.0	80	0	6.68	1.63	1.19	329.2
2.0	0	0	6.79	0.50	0.45	581.8

[a]Basal medium was Bennett agar medium. 50 agar plugs containing colony were removed from the plates after 7-days culture. Concentrations of leupeptin, CTP, LIE, and TLP were measured as discribed in Materials and Methods.

Table 3. Effect of glucose analogue, methyl α-D glucopyranoside (MG) on derepression of morphological and physiological differentiation of *S. exfoliatus* SMF13 on surface culture[a].

MG (%)	Aerial mycelium (%)	Sporulation (%)	CTP	LIE	TLP	Leupeptin (μg colony^{-1})
			(mU colony^{-1})			
0.0	0	0	6.40	0.13	ND[b]	258.0
0.5	100	100	18.37	2.59	2.16	74.8
1.0	100	100	20.38	4.06	2.52	60.5
2.0	100	100	19.61	4.55	2.70	51.0

[a]Basal medium was Bennett agar medium added with 2% glucose. 50 agar plugs containing colony were removed from plates after 7-days culture. Concentrations of leupeptin, CTP, LIE and TLP were measured as discribed in Materials and Methods.
[b]ND: not-detected.

production of brown-pigment was derepressed. The scanning electron micrograph of 8 days culture on Bennett media with 2% glucose and 0.5% methyl α-D glucopyranoside added was smiliar to that of 8 days culture on Bennett media (Fig. 2D). The effect of methyl α-D glucopyranoside on the production of leupeptin, CTP, LIE, and TLP is presented in Table 3. The production of LIE and TLP was clearly derepressed by the addition of methyl α-D glucopyranoside. The production of CTP was also derepressed. However, the

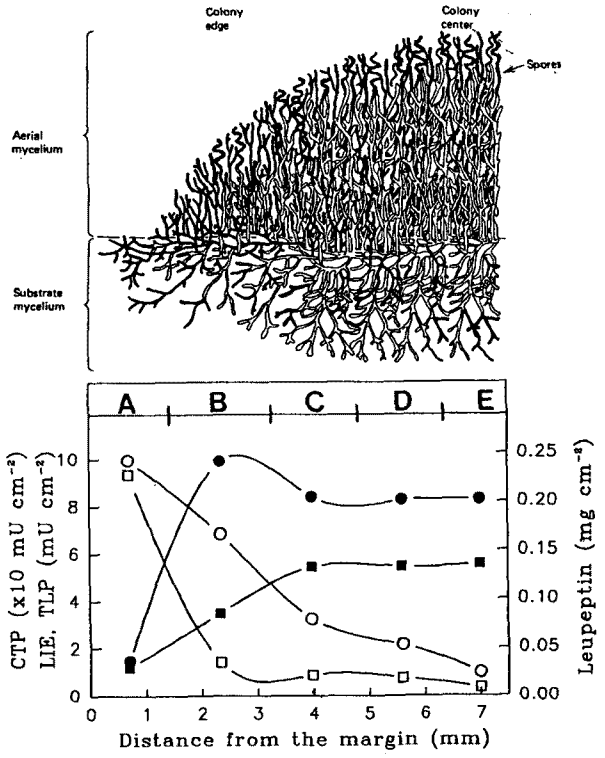

Fig. 3. Local distribution of leupeptin (□), LIE (■), CTP (○), and TLP (●) in a developing colony of *S. exfoliatus* SMF13.
Agar slices were cut-off by the developmental stages such as substrate mycelium (A), aerial mycelium (B), and sporulation (C, D, E). The activities of leupeptin, LIE, CTP, and TLP were extracted from the cutted agar slices collected within 4 cm^2 rectangle. The activities were measured and expressed as activity per surface area. Cross-section of an Streptomycete colony was cited from Wildermuth (20). The cross-section showed the substrate mycelium, aerial mycelium, chains of spores, and lysis of hyphae in the center of the colony. Shaded hyphae are living; white hyphae are lysed.

production of leupeptin was decreased by the addition of methyl α-D glucopyranoside.

Local Distribution of Leupeptin, CTP, LIE, and TLP in a Developing Colony

The filamentous growth habit of *Streptomyces* permits three different types of develpomental stages within one colony. In *S. exfoliatus* SMF13, vegetative mycelium developed from the swollen germinated spore to form a branched mycelium which resulted in the formation of circular colonies. Aerial mycelium initials developed directly from the substrate mycelium at approximately 28 h after inoculation. Substrate mycelium continued to extend at the colony margins during aerial mycelium formation which started from the central part of the colony. At 48 h after inoculation, aerial mycelium started to form spores and three different types of developmental stages were observable in a single colony. Local distribution

of leupeptin, CTP, LIE, and TLP within one colony in relation to developmental stages was measured (Fig. 3). The concentration of leupeptin was very high only in the region of substrate mycelium and it was very low in the region of aerial mycelium and spore. Also the activiy of CTP was higher in the region of substrate mycelium than in the region of aerial mycelium and spore. However the activity of LIE and TLP was higher in the region of aerial mycelium and spore than in the region of substrate mycelium.

DISCUSSION

The morphological differentiation of *S. exfoliatus* SMF13 was completely repressed by high concentrations of glucose and casamino acids. Glucose repression of morphological differentiation in *Streptomyces* seemed to be dependant on species. *S. alboniger, S. scabies* and *S. coelicolor* formed aerial mycelia when grown on a complex medium (Hickey-Tresner agar), but the addition of 2% glucose to the medium resulted in repression of aerial mycelium formation (17). The cause of this inhibition was assumed to be due to accumulation of undissociated organic acids. However, the addition of 2% glucose to Hickey-Tresner and GMS media had no effect on formation of aerial mycelia or spores by *S. viridochromogenes* or *S. griseus* (3).

Streptomyces sporulate well in media containing inorganic nitrogen and sporulate poorly or not at all in media containing a high concentration of complex organic nitrogen (4, 9). The addition of casein hydrolysate to the medium stimulated growth while completely repressing the formation of aerial mycelium and spores by *S. viridochromogenes*. However, the repression mechanism was not elucidated (3).

The repression of morphological differentiation by glucose or casamino acids in *S. exfoliatus* SMF13 was correlated with the repression of physiological differentiation (production of brown-pigment, LIE and TLP). Previous report has shown that a *bld* mutant of *S. exfoliatus* SMF13 could not produce LIE and TLP, and that the mycelium autolysis rate in submerged cultures was very reduced in the *bld* mutant (13). These results suggested that morphological differentiation and derepression of LIE and TLP are coordinately regulated or that the activity of LIE or TLP is essential for differentiation in *S. exfoliatus* SMF13.

Glucose repression of morphological differentiation was derepressed by the addition of glucose anti-metabolite, methyl α-D glucopyranoside. As a rule, methylated sugar interferes with glucose utilization, creating glucose-limited growth conditions (19). Glucose-limitation also stimulated the production of LIE and TLP (12). Therefore, it was concluded that glucose-limitation was a triggering factor for induction of morphological differentiation.

The growth of *Streptomyces* on solid media progresses as a sequential formation of substrate mycelium and aerial mycelium (20). The aerial mycelium appears to grow at least partially by the utilization of degraded substrate mycelium (14, 15), because the aerial mycelium has little access to other direct mechanisms of nourishment for its growth and development (1, 2). We suggested that TLP produced in the growth-limited mycelia plays an important role in the degradation of substrate mycelium protein and that leupeptin protects growing mycelium from the hydrolytic invasion of TLP (12, 13). Therefore, the leupeptin accumulated in the culture must be inactivated for the exhibition of TLP activity. The inactivation of leupeptin is made by LIE. Consequently, TLP is unlocked and starts to hydrolyze non-growing mycelium. The local distribution of leupeptin, CTP, LIE, and TLP in a colony showed that colony development correlated with the production and functions of the compounds. The activity of CTP, essential for primary growth, was higher in the active growing substrate mycelium. The concentration of leupeptin was very high only in the region of substrate mycelium. The low concentration of leupeptin in the region of aerial mycelium or spore clearly was due to LIE activty which was induced only where carbon and energy source were exhausted. The activity of TLP was high in the region of aerial mycelium or spore where substrate mycelium had to be degraded for aerial mycelium growth.

The current work is a report on the identification of the roles and regulation of leupeptin, CTP, LIE and TLP in terms of mycelium morphological changes. The regulatory actions of the compounds given here will provoke increased discussion of the morphological differentiation of *Streptomyces* spp.

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