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Physiological Importance of Trypsin-like Protease during Morphological Differentiation of Streptomycetes

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The relationship between morphological differentiation and production of trypsin-like protease (TLP) in streptomycetes was studied. All the *Streptomyces* spp. in this study produced TLP just before the onset of aerial mycelium formation. Addition of TLP inhibitor, TLCK, to the top surface of colonies inhibited aerial mycelium formation as well as TLP activity. Addition of 2% glucose to the Bennett agar medium repressed both the aerial mycelium formation and TLP production in *S. aburaviensis*, *S. coelicolor* A3(2), *S. exfoliatus*, *S. microflavus*, *S. roseus*, *S. lavendulae*, and *S. rochei*. However the addition of glucose did not affect *S. limosus*, *S. felleus*, *S. griseus*, *S. phaechromogenes*, and *S. rimosus*. The glucose repression on aerial mycelium formation and production of TLP was relieved by the addition of glucose anti-metabolite (methyl α-glucopyranoside). Therfore, it was concluded that TLP production is coordinately regulated with morphological differentiation and TLP activity is essential for morphological differentiation in streptomycetes. The proposed role of TLP is that TLP participates in the degradation of substrate mycelium protein for providing nutrient for aerial mycelial growth.

Key words: streptomycetes, trypsin-like protease, morphological differentiation, TLCK, glucose repression, substrate mycelium, aerial mycelium, scanning elecron microscopy

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 (4, 7, 13, 26). The formation of aerial mycelium is thought to be a reaction to unsuitable conditions in natural environment of the organism.

One of the triggering stimuli on the differentiation is nutrient-limitation. Autoradiographic studies have shown that substrate mycelium was a nutrient support for aerial mycelium growth (15, 16). However the mechanism which substrate mycelium was reused for aerial mycelium growth was not elucidated. During morphological differentiation, many extracellular enzymes as well as secondary metabolites such as antibiotics and pigments were produced. There have been reports on the temporal relationship between secondary metabolism and production of extracellular protease (1, 8, 9, 12). However, the role of extracellular protease on the morphological differentiation remains very poorly understood.

Trypsin-like protease (TLP) is a common extracellular protease in streptomycetes and the characteristics have been reported in many specieses of *Streptomyces* (3, 5, 10, 11, 18, 19, 20, 22, 24, 27). Recently the possible role of extracellular TLP and its specific inhibitor, leupeptin, on morphological differentiation was elucidated in *S. exfoliatus* SMF13 (14). TLP accumulated at the end of the main growth period in conditions of glucose exhaustion, coinciding with extensive mycelial lysis. The activity of TLP and lysis of the mycelium were both inhibited by the leupeptin, suggesting that TLP play a part in lysis.

To understand the roles of TLP in streptomycetes, it is desirable to determine whether TLP production is universal and TLP activity is essential during differentiation of streptomycetes. In the present study, effect of TLP specific inhibitor and glucose on the morphological differentiation of 12 species of streptomycetes which are taxonomically different from each other will be discussed in relation with TLP production and TLP activity.

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Materials and Methods

Microorganism and media

The microorganisms used were *S. exfoliatus* SMF13 (KCTC 8624P), *S. coelicolor* A3(2) (IFO 12854), *S. lavendulae* (IFO 12789), *S. microflavus* (IFO 13062), *S. rochei* (IFO 12908), *S. roseus* (IFO 12818), and *S. aburaviensis* (IFO 12830), *S. felleus* (IFO 12766), *S. rimosus* (IFO 12907), *S. griseus* (KCTC 1080), *S. phaechromogenes* (IFO 12898), and *S. limosus* (ISP 5131). 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. Glucose (2%) was added to the main culture medium in order to study the effect of glucose on the differentiation of *Streptomyces* spp. Methyl α-D glucopyranoside (0.5%) 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℃. For surface cultures, about 10^3 spores were inoculated evenly on agar plates of the main culture medium or spores from stock culture plates were inoculated to agar plates of the culture medium by sterile toothpick transfer, and incubated at 28℃.

TLP assay

Agar plugs containing colony 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 activity of TLP in the supernatant were measured (14).

The activity of TLP was estimated by measuring the amount of p-nitroanilides liberated from the N-benzoyl arginine p-nitroanilide. Enzyme reactions were carried out with 200 μmol of substrates at 35℃ and pH 7.5 (Tris-HCl buffer, 0.1 M). Activity was calculated from the linear part of the curve, using $E_{405}=9{,}620$ mol $^{-1}$ cm $^{-1}$. One unit of TLP activity was defined as the amount of enzyme needed for the production of 1 μmol of product (p-nitroanilide) per min (23).

Scanning electron microscopy

Colonies developed on agar medium were fixed in the following procedures: 8% phosphate-buffered glutaraldehyde solution (pH 7.4) was poured into holes punched around colonies (17). Plates were left for 24 hours at 4℃; colonies were cut out to the minimal size from the agar medium and then dried in sealed Revco box under P_2O_5 at 4℃. Dried colonies were gold-coated with Polaron SC502 Sputter Coater (Fisons, U.K.) at 15 mA for 1 min in the vacuum conditions. The morphology of colonies were observed with Streoscan 260 scan electron microscope (Cambridge Ltd., U.K.).

Results

Relationship between morphological differentiation and production of TLP

S. aburaviensis, S. coelicolor A3(2), *S. exfoliatus, S. limosus, S. microflavus,* and *S. roseus* formed aerial mycelium and sporulated well when grown on surface culture using Bennett agar medium. The production of TLP started just before the onset of aerial mycelium formation and rapidly increased during aerial mycelium growth; the activities were at maxima just before the onset of sporulation and decreased gradually during sporulation (Fig. 1A-F). However *S. felleus, S. griseus, S. lavendulae, S. phaechromogenes, S. rimosus,* and *S. rochei* formed aerial mycelium well but poorly sporulated. The production of TLP started just before the onset of aerial mycelium formation and increased steadily (Fig. 1G-L). The amounts of TLP production were varied in *Streptomyces* spp.

Effect of addition of TLP inhibitor on aerial mycelium formation

The effect of tosyl lysyl chloromethyl ketone (TLCK), a specific inhibitor of TLP, on the morphological differentiation was measured (Table 1). When TLCK (0.1 mg per colony) was added to the top surface of a colony just before the onset of aerial mycelium formation, a significant inhibition of aerial mycelium formation was observed. *S. aburaviensis, S. exfoliatus, S. limosus, S. microflavus, S. roseus, S. griseus, S. lavendulae, S. phaechromogenes,* and *S. rochei* could not form aerial mycelium through the experiment of 6 day cultures. TLP activity was not detected at the extracellular agar medium. *S. coelicolor* A3(2), *S. felleus,* and *S. rimosus* restored aerial mycelium formation after retardation of about 3 days. During the inhibition period of aerial mycelium formation, TLP activity was not detected at the extracellular agar medium. However TLP acivity was detected after initiation of aerial mycelium formation.

The colony surfaces of *S. roseus* grown on Bennett agar medium with or without TLCK were compared. Scanning electron micrographs of *S. roseus* after 6 days culture on Bennett medium without TLCK showed that the surface of the colony consisted primarily of long rectiflexible spore chains (Fig. 2A). On the other hand, the surface of the colony grown with the addition of TLCK consisted of substrate mycelia without aerial mycelia and aerial spores (Fig. 2B).

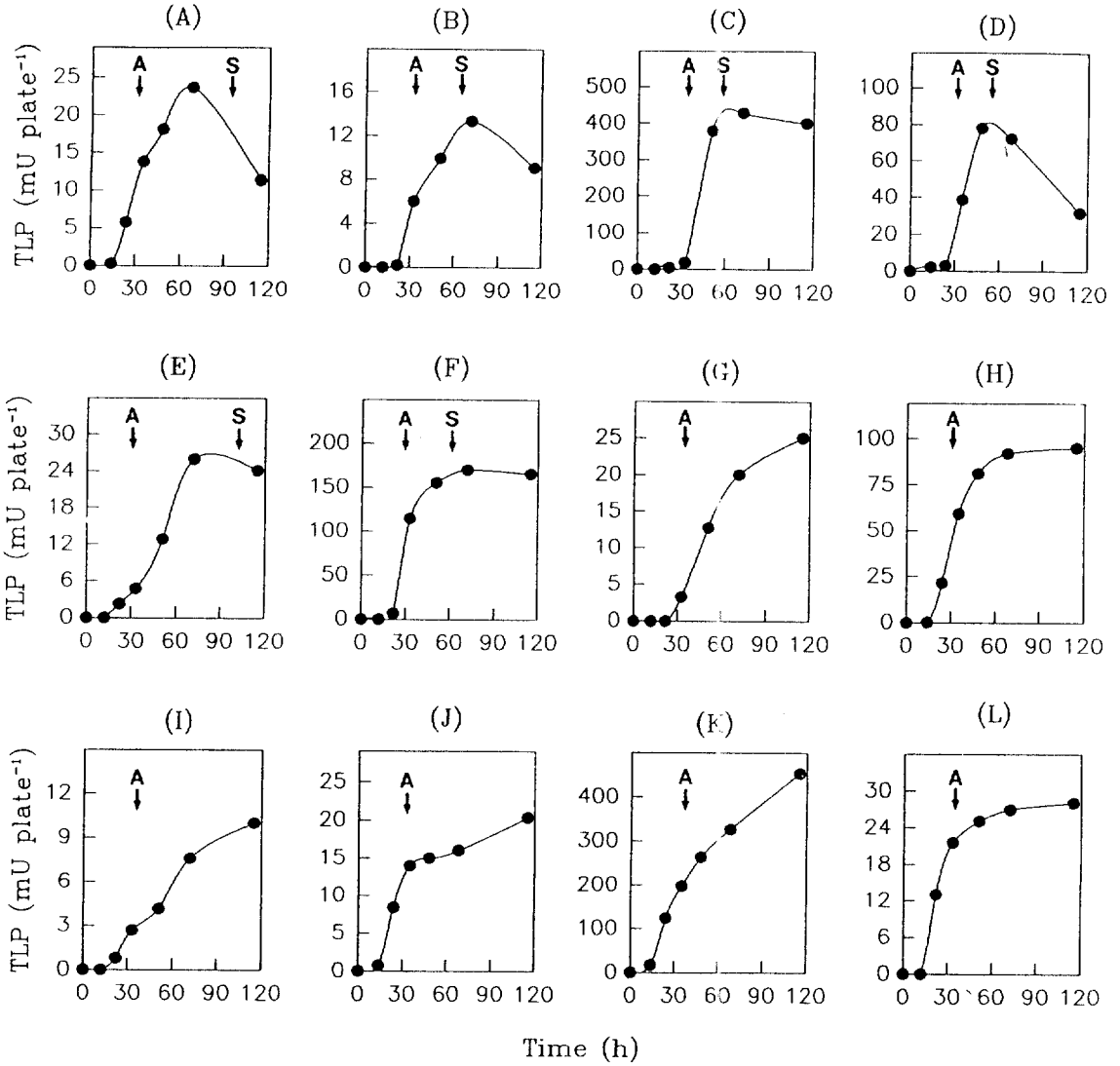

Fig. 1. Relationship between morphological differentiation of mycelium and production of TLP in surface cultures of various specieses of *Streptomyces*. Cultures were grown in Bennett agar medium. Arrows indicate the times initiating aerial mycelium formation (A) or sporulation (S). (A) *S. aburaviensis*, (B) *S. coelicolor* A3(2), (C) *S. exfoliatus*, (D) *S. limosus*, (E) *S. microflavus*, (F) *S. roseus*, (G) *S. felleus*, (H) *S. griseus*, (I) *S. lavendulae*, (J) *S. phaechromogenes*, (K) *S. rimosus*, and (L) *S. rochei*.

Glucose regualtion of mophological differentiation and production of TLP

The effect of glucose on aerial mycelium formation and sporulation on Bennett agar medium was tested (Table 2). Addition of 2% glucose to the Bennett medium repressed the aerial mycelium formation of *S. aburaviensis*, *S. coelicolor* A3(2), *S. exfoliatus*, *S. microflavus*, *S. roseus*, *S. lavendulae*, and *S. rochei*. However, *S. limosus*, *S. felleus*, *S. griseus*, *S. phaechromogenes*, and *S. rimosus* could form aerial mycelium. The production of TLP was repressed only in the species of which morphological differentiation was repressed by glucose (Table 3).

The glucose repression of aerial mycelium formation was relieved by the addition of methyl α-D glucopyranoside (Table 2). Also the production of TLP was derepressed by the addition of methyl α-D glucopyranoside (Table 3).

Fig. 3. shows the scanning electron micrographs of *S. lavendulae* after 8 days culture on Bennett media, Bennett media added with 2% glucose, and Bennett media added with 2% glucose and 0.5% methyl α-D glucopyranoside. Spore chains produced on Bennett agar medium was rectiflexible or spirales (Fig. 3A). However the surface of *S. lavendulae* after 8 days culture on Bennett media with 2% glucose consisted of substrate mycelial growth with no spores (Fig. 3B). The surface of *S. lavendulae* after 8 days culture on Bennett media with 2% glucose and 0.5% methyl α-D glucopyranoside was similar with that of 8 days culture on Bennett media (Fig. 3C).

Table 1. Effect of TLP inhibitor on aerial mycelium formation and extracellular activity of TLP in *Streptomyces* spp.

Streptomyces spp.	Aerial mycelium formation after				TLP activity (mU colony $^{-1}$) after			
	3 days		6 days		3 days		6 days	
	Without TLCK	With TLCK	Without TLCK	With TLCK	Without TLCK	With TLCK	Without TLCK	With TLCK
S. aburaviensis	+	−	+	−	0.41	ND	0.43	ND
S. coelicolor A3(2)	+	±	+	+	1.44	0.41	1.34	0.94
S. exfoliatus	+	−	+	−	12.36	ND	13.27	0.12
S. limosus	+	−	+	−	16.78	ND	15.79	0.89
S. microflavus	+	−	+	−	1.63	ND	1.55	0.09
S. roseus	+	−	+	−	2.28	ND	2.40	0.12
S. felleus	+	±	+	+	2.91	0.87	3.05	2.81
S. griseus	+	−	+	−	3.84	ND	3.90	0.30
S. lavendulae	+	−	+	−	0.18	ND	0.22	0.02
S. phaechromogenes	+	−	+	−	0.42	ND	0.45	ND
S. rimosus	+	+	+	+	3.62	1.01	4.14	3.78
S. rochei	+	−	+	−	2.73	ND	2.86	0.08

Spores from stock culture plates were inoculated to Bennett agar medium in 24 well microplate by sterile toothpick transfer. A specific TLP inhibitor, tosyl lysyl chloromethyl ketone (TLCK), was added to the top surfaces of colonies in the concentration of 100 μg per colony just before the onset of aerial mycelium formation (at 24 h). After 3 days and 6 days, the formation of aerial mycelium and extracellular TLP activity were measured, respectively.

Symbols: +, Abundant aerial mycelium; −, No aerial mycelium; ±, Initiation of aerial mycelium formation.

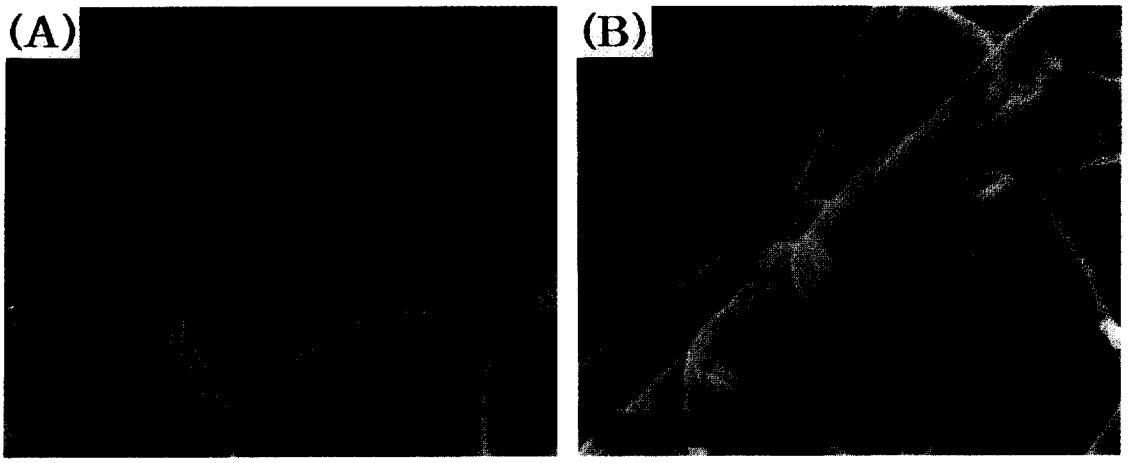

Fig. 2. Scanning electron micrographs of *S. roseus* grown on Bennett medium (A) and Bennett medium added with TLCK (B) after 6 days culture.

Discussion

We have suggested that TLP produced in the growth-limited mycelia of *S. exfoliatus* SMF13 plays an important role in the degradation of substrate mycelium protein for providing nutrient for aerial mycelial growth (14). TLP purified from *S. exfoliatus* SMF13 could degrade mycelium protein very effectively (unpublished result). All the *Streptomyces* species in this study produced TLP just before the onset of aerial mycelium formation. Also the addition of TLP inhibitor inhibited aerial mycelium formation as well as TLP activity. These results indicated that TLP activity is essential for aerial mycelium formation in streptomycetes.

Only three *Streptomyces* species, *S. felleus*, *S. coelicolor*, and *S. rimosus*, restored aerial mycelium formation after retardation of 3 days by TLCK addition. The restoration of aerial mycelium formation was correlated with the restoration of TLP activity. The reason why only three species could restore aerial mycelium formation was not clear. A possible explanation is that TLCK may be inactivated by pH change induced during mycelium growth, because TLCK is unstable at alkaline pH (2) or by certain enzyme produced by the three species.

Glucose repression of morphological differentiation in *Streptomyces* spp. seemed to be dependant on species.

Table 2. Effect of glucose (Glc) and methyl α-D glucopyranoside (MG) on the aerial mycelium formation of *Streptomyces* spp.

Streptomyces spp.	Aerial mycelium formation		
	Bennett	Bennett + Glc	Bennett + Glc + MG
S. aburaviensis	+	−	+
S. coelicolor A3(2)	+	−	+
S. exfoliatus	+	−	+
S. microflavus	+	−	+
S. roseus	+	−	+
S. lavendulae	+	−	−
S. rochei	+	−	−
S. limosus	+	+	+
S. felleus	+	+	+
S. griseus	+	+	+
S. phaechromogenes	+	+	+
S. rimosus	+	+	+

Spores from stock culture plates were inoculated to agar plates containing Bennett medium, Bennett medium + 2% glucose, or Bennett medium + 2% glucose + 0.5% MG by sterile toothpick transfer. After 8 days culture, the formation of aerial mycelium was measured.

Symbols: +, Abudant aerial mycelium; −, No aerial mycelium.

Table 3. Effect of glucose (Glc) and methyl α-D glucopyranoside (MG) on the production of TLP of *Streptomyces* spp.

Streptomyces spp.	TLP (mU colony^{-1})		
	Bennett	Bennett + Glc	Bennett + Glc + MG
S. aburaviensis	0.40	ND	0.39
S. coelicolor A3(2)	1.24	0.23	1.76
S. exfoliatus	13.80	ND	2.16
S. microflavus	1.51	0.17	0.81
S. roseus	2.39	0.24	2.73
S. lavendulae	0.20	0.03	0.14
S. rochei	2.65	0.14	2.40
S. limosus	15.63	14.91	15.20
S. felleus	3.10	3.01	3.05
S. griseus	3.81	3.51	3.79
S. phaechromogenes	0.43	0.42	0.44
S. rimosus	4.21	4.35	4.05

Spores from stock culture plates were inoculated to agar plates containing Bennett medium, Bennett medium + 2% glucose, or Bennett medium + 2% glucose + 0.5% MG by sterile toothpick transfer. After 8 days culture, the production of TLP was measured as described in Materials and Methods.

ND: Not detected.

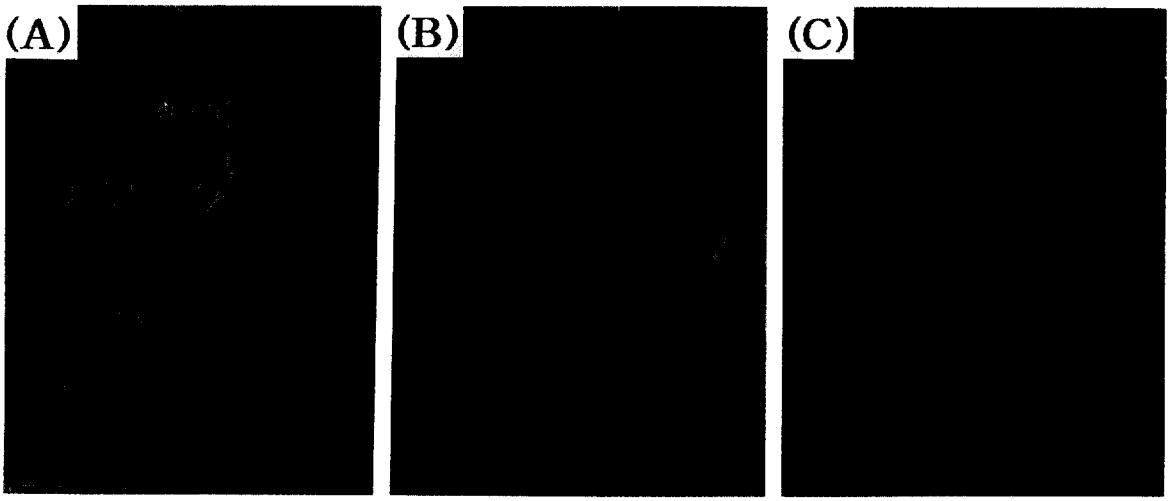

Fig. 3. Scanning electron micrographs of *S. lavendulae* grown on Bennett medium (A), Bennett medium added with 2% glucose (B), Bennett medium added with 2% glucose and 0.5% methyl α-D glucopyranoside (C) after 8 days culture.

Both morphological differentiation and production of TLP were repressed by the addition of 2% glucose to Bennett medium in *S. aburaviensis*, *S. coelicolor* A3(2), *S. exfoliatus*, *S. microflavus*, *S. roseus*, *S. lavendulae*, and *S. rochei*. However *S. limosus*, *S. felleus*, *S. griseus*, *S. phaechromogenes*, and *S. rimosus* could form aerial mycelium and produce TLP; especially *S. limosus* sporulated well. Previous studies have shown that *S. alboniger*, *S. scabies* and *S. coelicolor* formed aerial mycelia when grown on a complex medium (Hickey-Tresner agar), but addition of 2% glucose to the medium resulted in repression of aerial mycelium formation (21). The cause of this inhibition was suggested to be due to accumulation of undissociated organic acids. However, 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* (6).

Glucose repression of morphological differentiation and TLP production 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 (25). Therfore, it was concluded that TLP production is coordinately regulated with morphological differentiation and TLP ac-

tivity is essential for morphological differentiation in streptomycetes.

The filamentous growth habit of streptomycetes permits three different types of developmental stages, substrate mycelium, aerial mycelium, and spore, within one colony. Usually aerial mycelium formation and sporulation start from the central part of the colony, although substrate mycelium continues to extend at the colony margins. Nutrient limitation is most likely to be encountered where biomass is relatively high, and in the most densely-populated parts of colonies. This nutrient limitation may trigger the derepression of TLP as well as the onset of machinary for morphological differentiation, resulting that much of the substrate mycelia in the differentiating central region undergoes lysis; clearly TLP plays a part in the lysis. Finally the substrate mycelial lysate is cannibalised by the aerial mycelium.

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