Physiological Regulation of Sporulation of *Kitasatosporia setae* in Submerged Culture

Yōko Takahashi, Yuzuru Iwai and Satoshi Ōmura*

Research Center for Biological Function, The Kitasato Institute, 5-9-1, Shirokane, Minato-ku, Tokyo 108, Japan

(Received Sep. 26, 1995 / Accepted Dec. 19, 1995)

Kitasatosporia setae KM-6054^T produces both filamentous mycelia and submerged spores (SS) in the submerged culture with shaking. SS were not produced in a chemically defined medium, MMT, but were produced when the medium was added with yeast extract or casamino acids. In MMT supplemented with casamino acids (MMT-CA), SS began to elongate within 1 hour incubation and grew up to form long filamentous mycelia after 6 hours. Spore chains were produced at the tips of the hyphal branches after 10 hours, and then short spore chains were fragmented to yield SS after 15 hours.

In feeding experiments, the strain produced SS in MMT when casamino acids were added within 4 hours after inoculation, but not when added after 6 hours. In MMT-CA, additional feeding of casamino acids did not influence the initiation time of SS formation.

In a nutritional shift-down experiment, the strain formed SS when casamino acids were removed from MMT-CA after 8 hours of cultivation, but not when removed within 6 hours. The strain starts to form cells committing SS already after about 8 hours under nutrient rich condition. Cells that commit SS formation started to be formed within about 8 hours incubation under nutrient-rich conditions. It seems thus likely that the SS formation of *K. setae* KM-6054^T is not regulated with nutritional starvation, but by a clock mechanism.

Actinomycetes have developed their differentiation mechanisms, as they exhibit diverse morphologies such as mycelia, sporangia, spores or zoospores. It has been reported that submerged spores (SS) are produced in the submerged culture of *Streptomyces griseus*¹⁻⁷⁾, *S. roseosporus*⁸⁾, *S. acrimycin*⁹⁾, *S. albus*⁹⁾ or *S. venezuelae*¹⁰⁾. Ochi^{5,6)} reported that a strain of *S. griseus* IFO 13189 forms SS along with stringent response to nutritional starvation. By contrast, Ensign^{2,4)} reported that the SS production of *S. griseus* NRRL 2682 was not controlled by nutrient limitation, but by a clock mechanism. Huber⁸⁾ also reported that the sporulation of a *S. roseosporus* strain was initiated earlier compared with *S. griseus* and its process was not dependent on nutrient deprivation. All of these examples are the case of *Streptomyces* of which filamentous mycelia (FM) and SS contain LL-diaminopimelic acid (DAP) in their cell wall.

We discovered and established the genus *Kitasatosporia*^{11,12)} with the following specific feature. *Kitasatosporia* forms both FM and SS in submerged culture. The type of DAP in the cell wall was found to be mainly *meso* type in FM and vegetative mycelia, and only LL-type in SS and aerial spores formed in both solid and liquid media¹³⁻¹⁵⁾. It seems thus likely that the differentiation of *Kitasatosporia* is more complicated than that of *Streptomyces* in terms of the relationship between cell morphology and chemical composition of cell wall. Amounts of SS in submerged culture can be estimated by HPLC analysis of LL-DAP¹⁶⁾.

We previously reported that in an organic medium containing yeast extract, SS of a *Kitasatosporia* strain begin to elongate within 1 hour after incubation, and grow up to form a long FM after 6 hours of incubation¹⁷⁾. Spore chains are produced at the tips of hyphal branches after 10 hours and then the short spore

chains are fragmented to yield SS at 15 hours. These data suggested that the SS production of the strain is not related to nutrient starvation. In the present paper we report the relationship between nutrients and SS formation time in *K. setae* KM-6054^T.

In relation to taxonomy of *Kitasatosporia*, Wellington *et al.* proposed that *Kitasatosporia* is a synonym of the *Streptomyces* on the basis of hybridization study using a 16S rRNA/DNA probe for *Streptomyces* spp¹⁸⁾. Consequently, the description of genus *Streptomyces* has been emended as the cell wall peptidoglycan contains major amounts of LL-DAP in spores and hyphae; and possibly major amounts of *meso*-DAP in the vegetative hyphae. However, we think that their proposal should be discussed more in detail, because some strains of *Streptomyces* do not contain DNA sequence hybridizing to the 16SrRNA/DNA probe Wellington *et al.* used. In addition, it has been known that genus *Sporichthya* contains DNA sequence hybridizing to the same probe (DDBJ data base). Moreover, we found out many different sequences in the conserved region among *Streptomyces* and *Kitasatosporia* by sequencing of entire 16S rRNA/DNA¹⁹⁾. On the other hand, *K. setae* was tentatively designated as '*Streptomyces setae* group' by Nakagaito *et al.*²⁰⁾, however this group name is not a valid name in the Bacteriological Code²¹⁾. Therefore, we still use the name of *Kitasatosporia* in this paper.

MATERIALS AND METHODS

Actinomycete strain: *Kitasatosporia setae* KM-6054^T (ATCC 33774, IFO 14216, JCM 3304) was used.

Morphology observation: The morphology of the actinomycete strain was observed with a light microscope (AH-2, Olympus) and a scanning electron microscope (Model S-430, Hitachi).

Medium: Yeast extract - dextrose medium

(YD) and a chemically defined medium MMT with the following composition were used. The former consisted of 1.0% yeast extract (Difco) and 1.0% dextrose (Difco). The latter consisted of glucose 1.0%, (NH₄)₂SO₄ 0.5%, K₂HPO₄ 0.05%, MgSO₄·7H₂O 0.05%, NaCl 0.001%, N-tris(hydroxymethyl)2-aminoethansulfonic acid 25 mM (pH7.0), FeSO₄·7H₂O 0.0001%, MnCl₂·4H₂O 0.0001%, ZnSO₄·7H₂O 0.0001%, CuSO₄·7H₂O 0.0001% and CoCl₂·2H₂O 0.0001%. MMT supplemented with 1% caseamino acids (CA; Difco) designated as MMT-CA was also used.

Cultivation: For the synchronous-submerged cultures, SS were inoculated into media and incubated at 27°C on a reciprocal shaker. Feeding and shift-down cultures were carried out according to the time schedule shown in Fig. 3. In the feeding cultures, 1.0% CA were added as indicated. In the shift down cultures, after CA were removed from MMT-CA by washing with sterilized water, the washed mycelia were transferred into MMT medium and incubation was continued.

Fig. 1. Scanning electronmicrograph of a submerged culture of *K. setae* KM-6054^T in YD medium for 24 hrs. Bar represents 1 μ m.

Fig. 2. Submerged culture of *K. setae* KM-6054^T in MMT. Bars represent 10 μ m.

Estimation of diaminopimelic acid isomer:

The isomer of DAP was analyzed quantitatively by HPLC according to the method of Takahashi *et al.*¹⁶⁾.

RESULTS AND DISCUSSION

Conditions for submerged spore production: Fig. 1 shows photomicrograph of FM and SS of *K. setae* KM-6054^T grown at 27°C for 24 hours in YD medium with shaking. SS appeared after 15 hours of incubation.

In MMT, however, SS were not observed at any incubation time (Fig. 2-a, b and c). Subsequently, effect of nutrient sources on the growth and SS production of *K. setae* KM-6054^T in MMT was examined. Table 1 shows a summarized results monitored with a light microscope at 15, 24, 48 and 72 hours after inoculation. SS were produced by addition of 1.0% yeast extract, 1.0% CA (hydrolyzed milk casein) or 1.0% vitamin free-CA, but not by addition of 1.0~3.0% glucose, 0.2~0.8% (NH₄)₂SO₄, 0.05% K₂HPO₄ or 0.44% CaCl₂. These results suggest that some factors in yeast extract or CA

induced SS formation in *K. setae* KM-6054^T.

Nutritional dependence of submerged spore production: Nutritional dependence of the SS production was examined by feeding and shift-down cultures according to the time

Table 1. Effect of nutrients on submerged spore (SS) formation of *K. setae* KM-6054^T

	Growth	SS
YD	++	++
MMT	+	—
plus 1.0% Dextrose	+	—
1.0% Yeast extract	++	++
1.0% Casamino acids	++	++
1.0% Casamino acids (vitamin free)	++	++
0.001% Fatty acids*	+	—
0.8% (NH ₄) ₂ SO ₄	+	—
0.05% K ₂ HPO ₄	+	—
0.44% CaCl ₂	+	—

* Fatty acids; oleic acid, arachidonic acid, linoleic acid, α -linoleic acid, and stearic acid.

schedule shown in Fig. 3. In the feeding culture, two kinds of examination were carried out. First, MMT medium was used for the initial cultivation to which 1.0% CA were added at 0, 2, 4, 6 and 8 hours after inoculation. Then the cultivation was continued until 15 hours in order to check SS formation as indicated in Fig. 3. SS were observed clearly when CA were added within 4 hours after inoculation of the strain as shown in Fig. 3-(1)~(3) and slightly when added at 6 hours (Fig. 3-(4)). No SS were observed when CA were added at 8 hours (Fig. 3-(5)). This indicates that the SS formation in MMT requires CA addition within 6 hours of incubation.

In the other feeding experiment, MMT-CA was used for the initial cultivation to which 1.0

% CA were additionally fed at 6 or 8 hours (Fig. 3-(6) and (7), respectively), and the cultivation was continued until 15 hours. SS formation was observed in both cultures as in the case of MMT-CA without additional CA feeding (Fig. 3-(1)). Thus, additional feeding of CA into MMT-CA did not result in the change of the initiation time of SS formation. These data suggest that sporulation of this organism is not correlated to nutrient limitation.

The shift-down cultures were carried out as follows. The initial cultivations were started in MMT-CA. After 4, 6 or 8 hours of incubation, cells were washed with sterilized water, transferred into MMT and cultivations were continued until 15 hours as indicated in Fig. 3-(8)~(10). SS did not emerge by the shift-down at 4 or 6 hours, but emerged by the shift down at 8 hours. In the cultures downshifted at 4 and 6 hours, SS were not observed even if the cultivation was continued until 72 hours.

Yeast extract (1%) showed the same effect as that obtained with CA (data not shown).

It seems thus likely that at least 6 to 8 hours of cultivation in the presence of CA or yeast extract is necessary for the organism to form cells that commit SS formation.

Commitment to sporulation: The time course of the SS formation in *K. setae* KM-6054^T in MMT-CA was monitored by morphological observation (Fig.4) and quantitative analysis of LL-DAP which is contained in SS (Fig. 5).

When only SS were inoculated, FM were observed at 6 hours after inoculation and occupied the culture at 8 hours. Premature spore chains shown by arrow emerged at 10 hours and spore chains appeared at 12 hours. At 15 hours, large numbers of the spore chains came off from FM and then these spore chains were dis-jointed to yield SS (Fig.4).

Fig. 5 shows the time course of growth and accumulation of LL-DAP and *meso*-DAP. Since SS and FM of *K. setae* KM-6054^T contain LL-

Fig. 3. Effect of nutritional feeding or shift-down cultures on submerged spore formation of *K. setae* KM-6054^T.

Fig. 4. Morphological change of *K. setae* KM-6054^T in a submerged culture in MMT-CA medium. Bar represents 10 μ m. FM; filamentous mycelia, SS; submerged spores.

DAP and *meso*-DAP, respectively, in their cell walls, the amount of LL-DAP or *meso*-DAP corresponds with the number of SS or FM, respectively, in the strain cultured in MMT-CA. The data (Fig. 5) indicated that *meso*-DAP began to increase after 6 hours of incubation, and LL-DAP began to increase after 8 hours. Thereafter both LL-DAP and *meso*-DAP kept increasing until 15 hours. Rapid increase in LL-DAP apparently correlated with the emergence of SS or spore chains. It seemed therefore likely

that SS of this strain originated from premature spore chains derived from the elongating FM at a certain stage of growth, but not from matured FM. In other words, this strain started to form cells committing SS formation already at about 8 hours, even though SS were not observed by microscopy.

Sporulation of this strain is not clearly caused by nutrient deprivation. The cells grown in the MMT medium supplemented with CA within about 8 hours of cultivation commit to

Fig. 5. Time course of LL-DAP and *meso*-DAP contents in the whole cells of *K. setae* KM-6054^T grown in MMT-CA medium.

sporulate regardless of the nutritional environment. It appears that *K. setae* KM-6054^T has a clock mechanism for spore formation. To study the mechanism of SS production, we are planning to identify the effective components in CA for the SS production.

ACKNOWLEDGMENTS

We thank Yoshiko Seki of The Kitasato Institute, Masashi Shimbo and Satoshi Aoyama of Science Univ. of Tokyo for their assistance, and Dr. Kunitomo Hotta of National Institute of Health and Dr. A. Seino and Dr. H. Takeshima of The Kitasato Institute for kindly suggestions.

REFERENCES

- 1) Carvajal, F.: The Production of spores in submerged cultures by some *Streptomyces*. *Mycologia*. 39: 426-440, 1947.
- 2) Ensign, J. C.: Physiological regulation of sporulation of *Streptomyces griseus*. In "Proceedings of Seventh International Symposium on Biology of Actinomycetes, Biology of Actinomycetes '88. Japan", p.309-315, Scientific Societies Press Tokyo. 1988.
- 3) Kendrick, K. & J. C. Ensign: Sporulation of *Streptomyces griseus* in submerged culture. *J. Bacteriol.* 155: 357-366, 1983.
- 4) Stastna, J.; P. Kvapil, J. Caslavská & J. C. Ensign: Microcycle sporogenesis in some streptomycetes without shift down treatment. *Arch. Microbiol.* 156: 263-265, 1991.
- 5) Ochi, K.: Metabolic initiation of differentiation and secondary metabolism by *Streptomyces griseus*: Significance of the stringent response (ppGpp) and GTP content in relation to A factor. *J. Bacteriol.* 169: 3608-3616, 1987.
- 6) Ochi, K.: Changes in nucleotide pools during sporulation of *Streptomyces griseus* in submerged culture. *J. Gen. Microbiol.* 133: 2787-2795, 1987.
- 7) Wilkin, G. D. & A. Rhodes: Observations on the morphology of *Streptomyces griseus* in submerged culture. *J. Gen. Microbiol.* 12: 259-264, 1955.
- 8) Huber, F. M.; R. L. Pieper & F. P. Mertz: Sporulation of *Streptomyces roseosporus* in submerged culture. *J. Ind. Microbiol.* 2: 235-241, 1987.
- 9) Daza, A.; J. F. Marti, A. Dominguez & J. A. Gil: Sporulation of several species of *Streptomyces* in submerged cultures after nutritional downshift. *J. Gen. Microbiol.* 135: 2483-2491, 1987.
- 10) Glazebrook, M. A.; J. L. Doull, C. Stuttard & L. C. Vining: Sporulation of *Streptomyces venezuelae* in submerged cultures. *J. Gen. Microbiol.* 136: 581-588, 1990.
- 11) Ōmura, S.; Y. Takahashi, Y. Iwai & H. Tanaka: *Kitasatosporia*, a new genus of the order Actinomycetales. *J. Antibiot.* 35: 1013-1019, 1982.
- 12) Ōmura, S.; Y. Takahashi, Y. Iwai & H. Tanaka: Revised nomenclature of *Kitasatosporia setalba*. *Int. J. Syst. Bacteriol.* 35: 221, 1985.
- 13) Ōmura, S.; Y. Iwai, Y. Takahashi, K. Kojima, K. Otaguro & R. Iwa: Type of diaminopimelic acid different in aerial and vegetative mycelia of setamycin-producing actinomycete KM-6054. *J. Antibiot.* 34: 1633-1634, 1981.
- 14) Takahashi, Y.; Y. Iwai & S. Ōmura: Relationship between cell morphology and the types of diaminopimelic acid in *Kitasatosporia setalba*. *J. Gen. Appl. Microbiol.* 29: 459-425, 1983.
- 15) Takahashi, Y.; T. Kuwana, Y. Iwai & S. Ōmura: Some characteristics of aerial and submerged spores of *Kitasatosporia setalba*. *J. Gen. Appl. Microbiol.* 30: 223-229, 1984.
- 16) Takahashi, Y.; Y. Iwai, H. Tomoda, R. Nimura,

- T. Kinoshita & S. Ōmura: Optical resolution of 2, 6,-diaminopimelic acid stereoisomers by high performance liquid chromatography for the chemotaxonomy of actinomycete strains. *J. Gen. appl. Microbiol.* 35: 27-32, 1989.
- 17) Takahashi, Y.; Y. Iwai & S. Ōmura: Mode of submerged spore formation in *Kitasatosporia setae*. *J. Gen. Appl. Microbiol.* 37: 261-266, 1991.
- 18) Wellington, E.M. H.; E. Stackebrandt, D. Sanders, J. Wolstrup & N.O.G. Jorgensen: Taxonomic status of *Kitasatosporia*, and proposed unification with *Streptomyces* on the basis of phenotypic and 16S rRNA analysis and emendation of *Streptomyces* Waksman and Henrici 1943, 339^{AL}. *Int. J. Syst. Bacteriol.* 42: 156-160, 1992.
- 19) Takahashi, Y.; M. Shinose, H. Ishikawa, Y. K. Shin, A. Seino, Y. Iwai & S. Ōmura: Characteristics of the genus *Kitasatosporia* as examined by restriction fragment length polymorphism analysis of 16S rRNA genes. In "Abstract of The 1995 Annual Meeting of SAJ" p.6, The Society for Actinomycetes Japan, 1995.
- 20) Nakagaito, Y.; A. Shimazu, A. Yokota & T. Hasegawa: Proposal of *Streptomyces atroaurantiacus* sp. nov. and *Streptomyces kifunensis* sp. nov. and transferring *Kitasatosporia cystarginea* Kusakabe and Isono to the genus *Streptomyces* as *Streptomyces Cystargineus* comb. nov. *J. Gen. Appl. Microbiol.* 38: 627-633, 1992.
- 21) Lapage, S.P.; P.H.A. Sneath, E.F. Lessel, V.B.D. Skerman, H.P.R. Seeliger & W.A. Clark (Editors): 1975. International Code of Nomenclature of Bacteria, 1990 Revision, American Society for Microbiology, Washington, D.C.