Kinetic analysis of morphological differentiation and protease production in *Streptomyces albidoflavus SMF301*

Sung Gyun Kang and Kye Joon Lee

Author for correspondence: Kye Joon Lee. Tel: +82 2 880 6705. Fax: +82 2 882 9285/888 4911. e-mail: lkjl2345@plaza.snu.ac.kr

Department of Microbiology and Research Centre for Molecular Microbiology, Seoul National University, Seoul 151-742, Korea

The effects of specific growth rate and specific nutrient uptake rate on morphological differentiation of *Streptomyces albidoflavus SMF301* were determined in chemostat cultures. Production of three types of proteases: chymotrypsin-like protease (CTP), trypsin-like protease (TLP) and metalloprotease (MTP) were analysed in relation to mycelium growth and spore formation. Production of CTP was closely linked to mycelium growth, whereas spore formation, TLP synthesis and MTP synthesis were inversely related to growth. Evaluation of various kinetic parameters [specific production rates of spores ($q_{spo}$), TLP ($q_{TLP}$), MTP ($q_{MTP}$) and CTP ($q_{CTP}$)] showed that mycelium growth rate and CTP production were optimal at 0.1 h$^{-1}$, but submerged spore formation, TLP production and MTP production were optimal at 0.025 h$^{-1}$. Changes in specific nutrient uptake rates [glucose ($q_{glu}$), ammonium ion ($q_{am}$) and phosphate ($q_{pho}$)] affected sporulation and protease production; limitation of carbon, nitrogen and phosphate stimulated spore, TLP and MTP production.

**Keywords**: *Streptomyces albidoflavus* SMF301, submerged spore, kinetics of sporulation, extracellular proteases, differentiation

INTRODUCTION

Physiological differentiation, such as secondary metabolite production and morphological differentiation, in *Streptomyces* spp. may be induced by limitation of essential nutrients (Chater, 1984; Champness, 1988; Champness et al., 1989). The effects of essential nutrients on differentiation have been reported: limitation of nitrogen and phosphate can initiate sporulation (Ensign, 1988; Glazebrook et al., 1990), but the effect of glucose addition differs in various species of *Streptomyces* (Babycock & Kendrick, 1988; Chater et al., 1988; Daza et al., 1989).

Morphological differentiation of *Streptomyces coelicolor* is accompanied by the production of degradation enzymes (Chater, 1989; Granotzzi et al., 1990). Various extracellular proteases participate in the assimilation of external proteinaceous nitrogen sources (Shapiro, 1989) and a recent study of the regulation of protease activities during growth and mycelium development of *Streptomyces exfoliatus* SMF13 showed that the production of trypsin-like protease (TLP) and leupeptin-inactivating protein are closely related to morphological differentiation (Kim & Lee, 1995, 1996).

The effects of nutrient limitation on morphological and physiological differentiation have been determined mostly in agar cultures where the concentration of nutrients and products change significantly with time. Under these conditions, the roles of specific nutrients or products cannot be distinguished. Although only a minority of *Streptomyces* form spores in submerged liquid cultures (Kendrick & Ensign, 1983; Daza et al., 1989; Koepsel & Ensign, 1984; Glazebrook et al., 1990), such cultures provide advantages over agar cultures for determining the specific role of environmental changes on differentiation.

We have investigated a strain of *Streptomyces albidoflavus* SMF301 that produces extracellular proteases and abundant spores in both submerged and agar cultures (Rho et al., 1992; Shin & Lee, 1986). The cellular composition of submerged spores differs from those of mycelium and aerial spores (Lee & Rho, 1993). Kinetic analysis showed that the specific submerged spore formation rate ($q_{spo}$) is inversely related to the specific mycelium growth rate ($\mu$). In the batch cultures, limi-
S. G. KANG and K. J. LEE

Fig. 1. Effect of \( \mu \) on (a) mycelium growth, \( Y_{\text{siglu}} \) and mycelium productivity; (b) spore formation, \( q_{\text{spo}} \) and spore productivity (s.f.u., spore-forming units); and (c) changes in CTP, TLP and MTP in \( S. \) albido-flavus SMF301. ○, Carbon-limited culture; ●, nitrogen-limited culture.

In this study we analysed quantitatively the effects of specific growth rate and specific nutrient uptake rate on mycelium growth, spore formation and extracellular protease production in chemostat cultures of \( S. \) albido-flavus SMF301.

METHODS

Organism and media. \( S. \) albido-flavus SMF301 has been described by Rho et al. (1992). Stock agar culture medium consisted of (w/v): 1\% glucose, 0.2\% peptone, 0.3\% yeast extract, 0.1\% beef extract and 1.8\% agar. The carbon-limited medium for the chemostat consisted of (w/v): 0.5\% glucose, 0.2\% NH\(_4\)Cl, 0.01\% KH\(_2\)PO\(_4\), 0.03\% MgSO\(_4\) . 7H\(_2\)O, 0.001\% FeSO\(_4\) . 7H\(_2\)O, 0.001\% CuSO\(_4\) . 5H\(_2\)O, 0.001\% CaCl\(_2\) . 2H\(_2\)O and 0.0003\% MnCl\(_2\) . 4H\(_2\)O. In the nitrogen-limited cultures, 1\% glucose and 0.05\% NH\(_4\)Cl were used as carbon and nitrogen sources, respectively. In the phosphate-limited cultures, 1\% glucose, 0.2\% NH\(_4\)Cl and 0.01\% KH\(_2\)PO\(_4\) were used as carbon, nitrogen and phosphate sources, respectively. The initial pH of the media was adjusted to 7.0 before steam sterilization. Phosphate and other salts were separately sterilized by membrane filtration (0.2 \( \mu \)m, Millipore).

Strain maintenance and culture conditions. Strains were maintained by transfer to slopes of stock culture medium each month, and were stored at 4 °C. For agar cultures, a spore suspension harvested from the stock culture was spread evenly and incubated at 28 °C. For submerged batch cultures, one loopful of mycelium and spores was used to inoculate 30 ml of the seed culture medium and incubated at 28 °C for 2 d. The seed culture was used to inoculate 1-3 l of the chemostat culture medium contained in a jar fermenter (Model KF-5L, Korea Fermentor Co.). Cultures were maintained at 28 °C and the pH was controlled at 7-0 with agitation at 200 r.p.m. and aeration at 1 v v\(^{-1}\) min\(^{-1}\).

Observation of mycelium morphology and growth. Morphological characteristics were observed with a phase-contrast microscope (Nikon Laphot). To measure growth, mycelium was harvested by centrifugation (10000 g, 10 min) and washed twice with physiological saline solution and once with distilled water. The washed mycelium was collected by vacuum
Analysis of chemical changes in the culture medium.

The concentration of glucose was determined with dinitrosalicylic acid reagent (Miller, 1959). The concentration of ammonium ion was measured with a specific ion analyser (Model EA940, Orion Research). The phosphate concentration was determined by the method of Pierpoint (1957).

Measurement of submerged spore formation. The culture broth (5 ml) was sonicated for 5 min at 100 W using a sonic dismembrator (Model 300, Fisher). The sonicated suspension (0.5 ml) was mixed gently with 0.1 M HCl (4.5 ml) and after 5 min the mixture was diluted in physiological saline and spread on stock agar medium. Colonies were counted after 4 d incubation to estimate the number of spores in the inoculum (Lee & Rho, 1993).

Assay of proteases. The culture broth was centrifuged at (10000 g, 10 min) and enzyme activities were measured in the supernatant. Total protease activity was estimated by measuring tyrosine liberated from Hammarsten casein (Merck) during 15 min incubation at 37 °C and pH 7.5 (0.1 M Tris/HCl buffer). One unit of casein hydrolytic activity (caseinase) was defined as the amount of enzyme needed to produce 1 µg tyrosine equivalent min⁻¹ from the casein (Narahashi, 1970). Other hydrolytic activities were estimated by measuring the amount of p-nitroanilines liberated from the following synthetic substrates: N-benzylytyrosine p-nitroanilide (BTPNA; a specific substrate for CTP), N-benzyloxycarbonyl p-nitroanilide (BAPNA; a specific substrate for TLP) and leucyl p-nitroanilide (LPNA; a specific substrate for MTP).

Solutions containing 200 µM aminoacyl p-nitroanilides were incubated at 35 °C and pH 7.5 (0.1 M Tris/HCl buffer). Activity was calculated from the linear part of the curve using ε₉₆₀ = 9620 mol⁻¹ cm⁻¹. One unit of hydrolytic activity was defined as the amount of enzyme needed to produce 1 µmol p-nitroaniline min⁻¹ (Sarath et al., 1989).

Gel electrophoresis. Protease activity was visualized by incorporating 0.1% gelatin into a SDS-PAGE gel (12%). After electrophoresis at 4 °C, the gel was incubated at 35 °C for 3 h in 0.1 M potassium phosphate buffer (pH 7.5) and then stained with Coomassie brilliant blue.

Fermentation kinetic parameters. Data from chemostat cultures were analysed for specific growth rate (µ), specific ammonium ion uptake rate (qₐₙₐ), specific glucose uptake rate (qₐ₈ₖ), specific phosphate uptake rate (q₉₉ₙ₉), specific rate of protease production (q₉₉ₙ₉₉), CTP activity and TLP activity.

Kinetic analysis of differentiation in *Streptomyces albidoflavus*

**RESULTS**

**Kinetic analyses of growth, spore formation and protease production**

In chemostats limited by glucose or ammonium ion, the steady-state values for biomass concentration increased with µ (Fig. 1a); on the other hand, the number of submerged spores in the chemostats was inversely related to D (Fig. 1b). Kinetic parameters calculated from the steady-state values showed that Yₓₐₙ₉₉ was optimal at ca 0·1 h⁻¹, while qₛₐₒ was optimal at ca 0·025 h⁻¹ (Fig. 1b). q₉₉₉₉ and qₐ₉ₐ₉₉ increased with increasing µ (Fig. 1), indicating that those nutrients were essential for mycelium growth but not for spore formation.

Patterns of extracellular protease production were

---

**Fig. 2.** Relationship between (a) CTP or (b) TLP activity and changes in µ (○, ○) and qₛₐₒ (△, △). ○, △, Carbon-limited culture; △, △, nitrogen-limited culture.
S. G. KANG and K. J. LEE

Effects of nutrient uptake rate on growth, spore formation and protease production at a fixed growth rate

Although spore formation and TLP production were enhanced at the low growth rates of both carbon- and nitrogen-limited cultures, it was not clear whether the nutrients played a role in the differentiation of mycelium and protease production. To distinguish the effect of the growth rate from the effects of the nutrient limitation, chemostat cultures were carried out at a fixed \( \mu \) of 0.06 h\(^{-1}\) where the concentration of glucose, ammonium ion or phosphate was varied by changing the feed concentration.

In the chemostat where the glucose feed rate was varied, the changes in growth and \( q_{\text{CTP}} \) were directly related to \( q_{\text{glu}} \). In contrast, \( q_{\text{spo}} \), \( q_{\text{MTP}} \) and \( q_{\text{TLP}} \) were inversely related to \( q_{\text{glu}} \) (Fig. 3a). Similar patterns were found in chemostats where the ammonium ion or phosphate feed rate was varied (Fig. 3b and c).

Fig. 3. (a) Effect of \( q_{\text{glu}} \) on mycelium growth (○), \( q_{\text{spo}} \) (●), \( q_{\text{TLP}} \) (■), \( q_{\text{MTP}} \) (△) and \( q_{\text{CTP}} \) (□). (b) Effect of \( q_{\text{amn}} \) on mycelium growth (○), \( q_{\text{spo}} \) (●), \( q_{\text{TLP}} \) (■), \( q_{\text{MTP}} \) (△) and \( q_{\text{CTP}} \) (□). (c) Effect of \( q_{\text{pho}} \) on mycelium growth (○), \( q_{\text{spo}} \) (●), \( q_{\text{TLP}} \) (■), \( q_{\text{MTP}} \) (△) and \( q_{\text{CTP}} \) (□). \( D \) was fixed at 0.06 h\(^{-1}\) and pH was controlled at 7 with 1 M HCl and 1 M NaOH.

Effect of nutrient balance on growth, spore formation and protease production at a fixed growth rate

In chemostat cultures at a fixed \( \mu \) of 0.06 h\(^{-1}\) in chemically defined media with different C/N ratios, \( q_{\text{spo}} \) was affected by the change in the C/N ratio, and the changes in \( q_{\text{TLP}} \) and \( q_{\text{MTP}} \) were correlated with the change in \( q_{\text{spo}} \) (Fig. 4). The analysis of results with varied C/N ratios also shows that the production of spores, TLP and MTP was very high at both low and high C/N ratios. The results from changing nutrient feed rates and C/N ratios indicate that limitation of any one of the essential nutrients (carbon and nitrogen) brings about differentiation. On the other hand, a change in \( q_{\text{CTP}} \) was associated with mycelium growth change rather than spore formation.

DISCUSSION

As reported previously (Kang et al., 1995a), morphological differentiation of \( S. \ text{albidoflavus} \ SMF301 \) is accompanied by sequential production of CTP, TLP and MTP. Each protease was thought to have a specific role in growth and differentiation, CTP production being linked to mycelium formation, whereas TLP and MTP were associated with spore formation. In chemostats, the kinetic analyses of changes in protease and spore formation can be carried out with variation in only one parameter such as growth rate or supply of an essential nutrient.

The specific rate of CTP production and the mycelium
Kinetic analysis of differentiation in *Streptomyces albidosflavus*

![Graph](image)

**Fig. 4.** Effect of C/N ratio in the nutrient input on the steady-state values in chemostat cultures (D, 0.06 h⁻¹). ○, mycelium growth; ●, spore; ■, q<sub>TLP</sub>; ▲, q<sub>MTP</sub>; □, q<sub>CTP</sub>

growth rate responded similarly to changes in growth rate and nutrient feed rates. In addition, the activity of CTP in various chemically defined media was much lower than that in rich media containing sodium caseinate as sole nitrogen source (Kang et al., 1995a; Shin & Lee, 1986). This suggests that CTP could be a major protease supporting mycelium growth in complex media by hydrolysis of proteinaceous compounds.

On the other hand, previous reports (Kang et al., 1995a, b) demonstrated that the activities of TLP and MTP were not affected by medium composition. The kinetic analyses also showed that the specific production rates of TLP and MTP were associated with the changes of in the spore production rate observed under various conditions. These results suggest that TLP and MTP are closely correlated with spore formation.

Limitation of essential nutrients is reported to have a variety of effects on differentiation in *Streptomyces*. Sporulation of several species was not inhibited by high concentrations of glucose (Daza et al., 1989) and the repression of sporulation by glucose has been attributed to acid accumulation and pH (Babcock & Kendrick, 1988; Rho & Lee, 1994). However, in our experiment (Fig. 3), where only the carbon feed rate was changed, kinetic analyses show that the rate of spore formation was clearly inhibited by high concentrations of glucose and stimulated by low concentrations. Since the pH of all chemostats was maintained at 7, the inhibition was not caused by acid accumulation or pH variation. These results are consistent with the observation that cellular differentiation in surface cultures was inhibited by high concentrations of glucose (Chater et al., 1988).

Submerged spore formation is initiated by depletion of ammonium ions or phosphate in *Streptomyces griseus* (Kendrick & Ensign, 1983; Rho & Lee, 1994) and is suppressed by excess nitrogen source (Glazebrook et al., 1990; Rho & Lee, 1994). The kinetic data reported here show that limitation of carbon, nitrogen or phosphate brings about differentiation. It is also noteworthy that nutrient imbalances in the C/N ratio (high C/N, nitrogen limitation; low C/N, carbon limitation) were detrimental to mycelium proliferation but beneficial to spore formation, TLP and MTP synthesis (Fig. 4).

Thus, it was concluded that morphological differentiation in *S. albidosflavus* is triggered by limitation of any one of the essential nutrients (glucose, nitrogen or phosphate). In addition, nutrient limitation immediately affected the production of proteases that play important physiological roles in mycelium growth and spore formation: CTP participated in mycelium growth, and MTP and TLP were associated with the formation of spores.

**ACKNOWLEDGEMENTS**

This work was supported by a research grant from the Research Centre for Molecular Microbiology (RCMM) sponsored by the Korea Science and Engineering Foundation (KOSEF). The authors are very grateful to Professor K. F. Chater for his thoughtful discussion.

**REFERENCES**


2713


Received 31 December 1996; revised 30 April 1997; accepted 7 May 1997.