Decision phase regulation of streptomycin production in *Streptomyces griseus*

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The streptomycin (Sm) producer, *Streptomyces griseus* N2-3-11, shows medium-independent biphasic kinetics of the vegetative or exponential growth phase (EGP), reflecting an innate clock-like behaviour of growth and differentiation. The *S. griseus* growth and development cycle has the following characteristics: (1) after the developmental cycle commences, it cannot be influenced by environmental conditions; (2) the first EGP (decision phase) and its duration seem to be genetically determined, and it is also exhibited in pleiotropic mutants deficient in differentiation and antibiotic production; (3) during this early phase of growth, the decision to produce Sm is established and the fixation of later production and differentiation can only be influenced by effector molecules, e.g. A-factor, during this period; (4) after the onset of the second EGP, the commitment to Sm production cannot be reversed by dilution into fresh medium, nor by effector molecules; (5) the length of time of this effector-insensitive growth phase (second EGP or execution phase) can be extended by dilution into fresh medium; (6) the differentiation cycle of *S. griseus* is completed on entering stationary phase. The cells of *S. griseus* then return to a decision-making stage and recover sensitivity to effector molecules. Evidence that this type of phasing is valid for the growth and developmental cycles of all streptomycetes is discussed.

Keywords: *Streptomyces griseus*, streptomycin, biosynthesis, regulation, A-factor

INTRODUCTION

Due to their great importance in the production of a large variety of enzymes and low-molecular-mass secondary metabolites, members of the genus *Streptomyces* are the best-studied group of the Gram-positive mycelium-forming *Actinomycetales*. Knowledge of the biosynthetic pathways of secondary metabolites has improved at the biochemical and genetic levels during recent years (for reviews see Hopwood & Sherman, 1990; Piepersberg, 1995). It is both of biological interest and of great importance for the production of secondary metabolites by fermentation to understand the molecular events involved in (1) regulating these complex biosynthetic pathways, (2) inducing secondary metabolism and differentiation and (3) linking primary and secondary pathways.

Streptomycetes undergo a complex life-cycle on solid media, including spore germination, vegetative growth of

substrate mycelium, formation of aerial mycelia and sporulation. In liquid medium the growth of *Streptomyces* is similar to that of non-filamentous bacteria in showing the three classical growth phases: lag, exponential and stationary. This picture of the growth of *Streptomyces* in liquid medium was first challenged by Thompson and co-workers (Holt *et al.*, 1992). *Streptomyces hygroscopicus*, which produces the herbicide bialaphos, exhibited a biphasic exponential growth phase (EGP) with a reproducible deceleration of growth between 14 and 16 h following inoculation. Interestingly, this pause in growth was associated with a peak level of ppGpp and the induction of expression of the bialaphos biosynthetic genes. Maximum bialaphos synthesis was only attained in stationary phase about 8 h later.

The physiology, enzymology, genetics and regulation of the synthesis of streptomycin (Sm) by *Streptomyces griseus* has been intensively studied (reviewed by Piepersberg, 1995). The synthesis of Sm is regulated by a complicated network including pleiotropic regulators (Horinouchi & Beppu, 1992) and the regulator protein, StrR, encoded by a gene of the biosynthetic gene cluster for Sm (Retzlaff *et*

Abbreviations: 2-D, two-dimensional; EGP, exponential growth phase; SGP, stationary growth phase; t_d , doubling time.

al., 1993; Retzlaff & Distler, 1995). In addition to these regulators, a hormone-like autoregulator, A-factor, controls the production of Sm in *S. griseus* at the level of gene expression via a still-unknown mechanism (Horinouchi & Beppu, 1994). The induction of Sm synthesis had commonly been described as a response to nutrient limitation in the stationary growth phase (SGP), probably mediated by ppGpp (Martin, 1989; Demain, 1989; Ochi, 1987a, b). However, Ensign (1988) showed that the sporulation of *S. griseus* NRRL 2682 in liquid medium is not caused by nutrient deprivation, but is dependent on a developmental cycle based on a clock-like mechanism.

In this paper, we demonstrate that the physiological decision of *S. griseus* cells to produce or not to produce Sm, is made very early in the growth phase. This 'decision phase' of Sm synthesis is part of a periodic developmental cycle which occurs independently of changes in the environment. After the decision-making point has been passed, there follows a factor-independent phase, called the execution phase, which can be influenced in its extension by physiological parameters. Cells are returned to a decision-making state in the early stationary phase.

METHODS

Strains and culture media. The strains used in this study are given in Table 1. The standard minimal medium (MM) contained 50 mM glucose, 60 mM, L-asparagine, 15 mM $(\text{NH}_4)_2\text{SO}_4$, 20 mM Tris/HCl, 17 mM NaCl, 3 mM K_2SO_4 , 2.5 mM KH_2PO_4 , 0.8 mM MgSO_4 , 0.7 mM CaCl_2 and 10 ml trace elements solution. The pH of the medium was adjusted to 7.2. The 100-fold-concentrated stock solution of trace elements contained 5 mM each of FeSO_4 , ZnCl_2 , MnCl_2 , CoCl_2 and $\text{NH}_4\text{Mo}_7\text{O}_{24}$. L-Asparagine (100 ml 0.6 M, pH 7.2) and potassium phosphate (10 ml 0.25 M, pH 7.2) were autoclaved separately and added aseptically to the other ingredients dissolved in 880 ml distilled water. The trace element solution was sterilized by filtration. Tryptic Soy Broth (TSB; 30 g l⁻¹) was used as a complete medium.

All *Streptomyces* strains were maintained on soy-flour mannitol agar (SMA) (Distler *et al.*, 1985). *S. griseus* and mutant strains were routinely grown in 1 l Erlenmeyer flasks containing 50 ml medium at 29 °C. The seed cultures (10 ml MM) were inoculated with *Streptomyces* spores from SMA plates. Seed culture (500 µl) grown for 4 d was used as an inoculum for the main cultures resulting in a final OD₅₆₀ of 0.1–0.15 (Kontron Uvikon 710 spectrophotometer). For the measurement of growth, nutrient concentrations, and intracellular nucleotide pools, *S. griseus* was fermented in 1.5 l standard MM using a 2 l fermenter model F-1000 (New Brunswick Scientific). The cultures were stirred at 300 r.p.m. at a temperature controlled to 29 °C. Air was supplied to the fermenter at a rate of 1.5 l min⁻¹. The pH and oxygen were continuously monitored throughout the experiment, but were not adjusted.

Measurement of growth. The growth was routinely controlled by measuring the OD₅₆₀. Samples with OD₅₆₀ values higher than 0.5 or 1.0 were diluted 1:4 or 1:10 with MM prior to measurement. The cell dry weight was determined by harvesting the mycelium by filtration through a nitrocellulose filter (0.2 µm pore size; Schleicher & Schuell). Before determination of the weight, the mycelium was washed with 10 mM NaCl and the filters were dried at 65 °C until the weight remained constant. Total cell protein was determined by the method described by

Virolle & Bibb (1988), with the following modifications. The mycelium of 1 ml culture was collected by centrifugation at 14000 r.p.m. The pellet was washed twice in distilled water and finally resuspended in 500 µl H₂O. The cells were disintegrated by sonication and the protein concentration was determined by the method of Bradford (1976), using BSA as a standard.

Medium transfer. For culture-shift experiments, *S. griseus* mycelium was harvested at the indicated times by centrifugation at 2000 r.p.m. for 5 min at 25 °C. The mycelia were subsequently resuspended in 2 ml MM at 29 °C. These cells were used to inoculate 50 ml fresh MM to give a final OD₅₆₀ of 0.1. Prior to inoculation, the fresh medium was placed in a 1 l Erlenmeyer flask on a rotary shaker at 29 °C for 1 h. Further incubation of the shifted cultures was as described above.

Measurement of nutrient concentration. *S. griseus* was grown in 1.5 l MM at 29 °C. At given times, 2 ml samples were taken and the supernatants were obtained by centrifugation at 10000 g for 15 min. The supernatants were heated at 90 °C for 10 min to inactivate any extracellular enzyme activity and stored at –70 °C. The concentrations of D-glucose and L-asparagine were measured enzymically using D-glucose and L-asparagine assay kits (Boehringer), respectively. Ammonium concentrations were determined by a glutamate-dehydrogenase-based assay kit (Sigma). The concentration of phosphate was assayed colorimetrically using a commercially available assay (Merck). The values of pH and saturation of oxygen were determined continuously using electrodes Ingold 405/S7 (Roth) and YSI model 25 (Kipp & Zonen), respectively.

The concentration of Sm was measured by a bioassay using a Sm-sensitive and a Sm-resistant strain of *Bacillus subtilis* DSM 347 as described by Distler *et al.* (1985). A-factor was extracted and identified as described earlier (Horinouchi & Beppu, 1992). The concentration of A-factor was assayed on SMA essentially as described by Horinouchi & Beppu (1992). The *S. griseus* mutant strain M881 was used as an indicator for A-factor. Chemically synthesized A-factor (Funakoshi) was used as a standard.

Extraction and assay of nucleotides. The extraction and quantification of the intracellular pools of NTPs and of ppGpp were performed by an HPLC method as described elsewhere (Ochi, 1987a). Routinely, mycelium from 100–200 ml cultures was harvested by filtration on a nitrocellulose filter (pore size 0.2 µm; Schleicher & Schuell) and extracted. Finally the preparations were dissolved in 100 µl H₂O and the NTPs and ppGpp were identified and quantified by co-chromatography with authentic standard solutions. A sample of ppGpp was kindly provided by A. Böck Institut für Genetic und Mikrobiologie, Universität München, Germany. The detection ranges for the NTPs and ppGpp were > 2 pmol. Extracellular cAMP was assayed by reversed-phase HPLC using a C₁₈-Partisil column (4.6 × 250 mm; Beckman). After centrifugation at 14000 r.p.m., 100 µl samples of culture supernatant were directly applied onto the column and subsequently eluted with a 90 ml gradient (curve no. 4 of the System Gold; Beckman) from 0 to 40 % (v/v) solvent B (100 % acetonitrile) with a flow rate of 1.5 ml min⁻¹. Solvent A comprised 30 mM KH_2PO_4 , 5 mM tetrabutylammoniumhydrogen-sulfate and 2 % (v/v) acetonitrile, pH 6.0. The elution of cAMP was assayed by the determination of A₂₆₀. cAMP was identified and quantified by co-chromatography with a standard solution of 10–100 pmol cAMP. For the determination of the intracellular level of cAMP, mycelium of a 200 ml culture was extracted as described above, dissolved in 100 µl H₂O, and immediately analysed by HPLC.

Preparation of pulse-labelled protein samples. For pulse-chase labelling of proteins, *S. griseus* N2-3-11 was grown in MM.

At indicated times 150 μCi [^{35}S]TRAN-Mix (specific activity $> 37 \text{ TBq mmol}^{-1}$; ICN Biochemicals) was added to 300 μl culture and incubated for an additional 20 min. Subsequently, 10 μl of a solution containing 10 mg ml^{-1} each of L-methionine and L-cysteine was added and the *S. griseus* was incubated for a further 10 min. The cells were collected by centrifugation, washed twice in crack-buffer (10 mM Tris/HCl, 5 mM EDTA, 1 mM PMSF and 5 mM 2-mercaptoethanol, pH 7.4) and resuspended in 100 μl ice-cold crack-buffer. The mycelium was disintegrated by sonication (power 50%, cycle 70%, pulse 15 s) using a sonifier model UW 60 (Bandelin). After sonication, 10 μl of a solution containing 0.5% (w/v) SDS and 10% (v/v) 2-mercaptoethanol was added immediately and the sample was frozen in liquid nitrogen. To inactivate proteases, samples were incubated at 85 $^{\circ}\text{C}$ for 15 min. The samples were supplemented with 6 μl 0.5 M MgCl_2 prior to treatment with DNase I and RNaseA at concentrations of 100 and 40 $\mu\text{g ml}^{-1}$, respectively. The nuclease treatment was performed at 5 $^{\circ}\text{C}$ for 30 min. The samples were frozen in liquid nitrogen and lyophilized overnight, then resuspended in 100 μl 2-D sample buffer [9 M urea, 4% (v/v) Nonidet P-40, 1.8% ampholytes pH 4–8, 0.2% ampholytes pH 3–10 (Promochem BDH) and 5 mM DTT] and centrifuged (10 min, 10000 g) prior to loading onto the IEF gel.

Two-dimensional (2-D) gel electrophoresis. 2-D gel electrophoresis of total cellular proteins was essentially as described by Garrels (1979). The gels were run with the ISO-DALT System (Hoefer Scientific). In the first dimension the proteins were separated in IEF gels (280 $\times$ 1.5 mm). The IEF gel mixture had the following composition: 4.5% (w/v) polyacrylamide, 8 M urea, 2% Nonidet P-40, 1.8% ampholytes pH 4–8 and 0.2% ampholytes pH 3–10. Each gel was loaded with approximately 2×10^6 c.p.m. trichloroacetic-acid-precipitable material and the focusing of the proteins was achieved at 36 kV h^{-1} overnight. After electrophoresis, the IEF gels were equilibrated with 10 ml ISO-equilibration buffer (125 mM Tris/HCl, pH 6.8, 10% (v/v) glycerol, 2% SDS, 5 mM 2-mercaptoethanol and a trace of bromphenol blue) for 15 min. Polyacrylamide gels (12.5%) (250 $\times$ 220 $\times$ 1.5 mm; Laemmli, 1970) containing 0.1% SDS were used for the second dimension. Separation of proteins was achieved at a constant voltage of 100 V for 22 h at 10 $^{\circ}\text{C}$. The gels were incubated overnight in a solution of 10% (v/v) acetic acid and 20% (v/v) methanol. The gels were dried in a vacuum on a Whatman 3MM paper and the radioactively labelled proteins were visualized by autoradiography. The protein spots in the autoradiograms were quantified densitometrically using the BioImage system (Millipore) and Visage 110 (release 4.4) software.

RESULTS

Growth of *S. griseus* N2-3-11 in a defined MM

The growth of the Sm-overproducing strain *S. griseus* N2-3-11 in batch fermentation was determined by measuring the optical density, cell dry weight and total protein (Fig. 1). *S. griseus* N2-3-11 reproducibly showed a temporal phasing of growth and Sm production in the standard liquid MM employed in this study. The phasing was characterized by an initial lag phase, two EGPs separated by a short transient lag phase of reduced growth, and a stationary phase (Figs 1, 2a). Using mycelium from a stationary culture as inoculum, *S. griseus* entered the first EGP after a short lag of 0.5–1 h. The first EGP [duration 10 h; doubling time (t_d) = 4 h] was followed by a diminution of growth ('transient lag phase'; $t_d > 8$ h) for

Fig. 1. Measurement of the growth of *S. griseus* N2-3-11 in standard defined medium. Growth was determined by measuring the optical density (■), total cell protein (○) and cell dry weight (◇).

at least 30–45 min. Growth resumed and the culture entered the second EGP (duration 14 h; $t_d = 3$ h), showing a significantly higher rate of growth when compared to the first EGP. After 24 h, the culture reached stationary phase. *S. griseus* N2-3-11 showed the same growth behaviour, with the exception of a prolonged initial lag phase of 16 h, when spores were used as inoculum for the cultures (data not shown). The biphasic EGP and the positioning of the transient lag phase after 10 h growth were also found when the amount of biomass used as inoculum was changed by a factor of 0.5–4 (data not shown).

Since *S. griseus* was being grown in the presence of both glucose and L-asparagine, this chronological course of growth could easily be explained by the well-known diauxic growth of a bacterial population on carbon sources used sequentially. Therefore, the most important parameters such as biomass, pH, and the concentrations of glucose, L-asparagine, ammonium, phosphate, dissolved oxygen, Sm and A-factor were measured during the course of a typical batch fermentation (Fig. 2a–c). Glucose and L-asparagine were consumed simultaneously throughout the growth of *S. griseus*. Under the conditions used, glucose and phosphate seemed to be the only nutrients to become exhausted in stationary phase (Fig. 2c). The concentration of dissolved oxygen did not fall below 85% at any point during the whole course of fermentation (Fig. 2b). The concentrations of ammonium and L-asparagine remained in excess throughout the growth of *S. griseus* (Fig. 2c). The concentration of ammonium even increased after 24 h growth and reached a final concentration of 58 mM. Entering the stationary phase, the pH in the medium dropped to about 6.0 and started to increase after 30 h, reaching values around 7.8 by the end of fermentation (stopped at 36 h). The transient lag phase of *S. griseus* does not seem to be caused by diauxic growth, as during the period from 8 to 12 h there was no limitation of any nutrients in the culture.

Fig. 2. Growth of *S. griseus* N2-3-11 in standard defined medium. (a) Production of biomass (■); Sm (●); A-factor in a 1.5 l batch culture of *S. griseus* N2-3-11 (◇). (b) Concentration of oxygen (—) and pH value (---) throughout the fermentation. (c) Concentrations of glucose (□), L-asparagine (▼), ammonium (○) and phosphate (●) in the medium. (d) Intracellular concentrations of ATP (■), GTP (▷), CTP (▴), UTP (◄), and ppGpp (◆). (e) Intracellular (△) and extracellular (▲) cAMP.

Although Sm was already detectable at a low concentration of about 0.5 mg l^{-1} after 14 h growth, its concentration increased dramatically only after 24 h, when *S. griseus* entered stationary phase (Fig. 2a). The maximum Sm concentration of 115 mg l^{-1} was produced 36 h after inoculation under the standard conditions employed. No A-factor was detectable in the *S. griseus* cultures within the first 6 h growth. After the transient lag phase the concentrations of A-factor increased in parallel with growth reaching a maximum amount of approximately 30 μg l^{-1} in early stationary phase. The concentration of A-factor decreased after 36 h growth and became undetectable in the culture supernatant or mycelium at the end of the fermentation (Fig. 2a).

Previously, Thompson and co-workers (Holt *et al.*, 1992) reported that a similar pause during vegetative growth of *S. hygroscopicus* in complete medium was associated with dramatic changes in the intracellular levels of ppGpp and NTPs. Although *S. griseus* N2-3-11 was able to accumulate

Table 1. *S. griseus* strains used in this work

Strain	Phenotype			Reference/source
	Sm*	A-factor†	Spo‡	
N2-3-11	+	+	+	Distler <i>et al.</i> (1985)
40236	+	+	+	DSM§
M881	—	—	—	Distler <i>et al.</i> (1987)
W67	—	+	—	Distler <i>et al.</i> (1990)
M66	—	+	+	Distler <i>et al.</i> (1985)

* Sm production was measured on SMA.

† A-factor was extracted from cultures as described in Methods. The presence of A-factor was determined by the induction of production of Sm in strain M881.

‡ Sporulation was analysed after growth on SMA for 3 d.

§ Deutsche Sammlung für Mikroorganismen, Braunschweig, Germany.

up to $50 \text{ pmol (mg cell dry wt)}^{-1}$ ppGpp in nutrient shift down experiments (data not shown), the amount of ppGpp was always below the level of detection during growth of *S. griseus* N2-3-11 in MM, even when as much as $500 \text{ mg dry cell weight}$ cells was extracted (Fig. 2d). In TSB complete medium, *S. griseus* produced ppGpp at a basal level of $3 \text{ pmol (mg cell dry wt)}^{-1}$ during exponential growth and a maximum concentration of $10 \text{ pmol (mg cell dry wt)}^{-1}$ after entering stationary phase, but no increased level of ppGpp was found during the transient lag phase (data not shown). The concentrations of GTP, UTP and CTP showed no significant alteration during the growth of *S. griseus* (Fig. 2d). On the other hand, the concentration of ATP had a basal level of $240\text{--}290 \text{ pmol (mg cell dry wt)}^{-1}$ in the first and second EGP. A peak level of $480 \text{ pmol (mg cell dry wt)}^{-1}$ ATP was detected in the transient lag phase. With the end of the second EGP, the concentration of ATP increased to $800 \text{ pmol (mg cell dry wt)}^{-1}$ by the end of the fermentation (Fig. 2d). As in the case of ppGpp, the intracellular level of cAMP was hardly detectable throughout the course of the batch fermentation; however, a peak concentration of $5 \text{ pmol (mg cell dry wt)}^{-1}$ cAMP was measured after 8 h growth, just before *S. griseus* entered the transient lag phase (Fig. 2e). Surprisingly, cAMP was found in concentrations as high as 100 nM in the fermentation broth of *S. griseus*. Extracellular cAMP was detected in the medium supernatant for the first time when *S. griseus* passed the transient lag phase (Fig. 2e). The concentration of extracellular cAMP reached a maximum from 24 to 34 h growth and disappeared again during the late stationary phase (Fig. 2e).

Effects of variations in content of individual nutrients on the growth of *S. griseus*

To analyse possible influences of the initial concentrations of the major nutrients in the medium on the growth curve, *S. griseus* N2-3-11 was cultivated in differently

Table 2. Growth and Sm production of *S. griseus* N2-3-11 in different media

Medium	Characteristics of growth phase				Sm production	
	1st EGP t_d (h)*	Transient start at (h)	2nd EGP t_d (h)†	Stationary start at (h)	Initial detection (h)	Yield after 36 h (mg l ⁻¹)
MM	4.2 ± 0.5	10	3.0 ± 0.3	24	14	115 ± 10
+ 2 × glucose	4.3 ± 0.2	10	3.1 ± 0.2	24	14	85 ± 10
+ 2 × asparagine	4.2 ± 0.3	10	3.5 ± 0.2	25	18	95 ± 5
+ 2 × ammonia	4.1 ± 0.2	10	3.1 ± 0.2	25	18	90 ± 5
+ 2 × phosphate	4.3 ± 0.2	10	3.4 ± 0.2	25	23	50 ± 5
+ 2 × trace elements	4.2 ± 0.5	10	3.0 ± 0.1	24	14	115 ± 5
+ 5 × ammonia	4.0 ± 0.1	10	3.3 ± 0.2	25	22	65 ± 5
+ 10 × ammonia	3.9 ± 0.1	10	3.6 ± 0.2	26	35	15 ± 5
2 × MM	4.3 ± 0.4	10	3.7 ± 1.0	26	23	20 ± 5
TSB	2.3 ± 0.1	5	1.25 ± 0.1	12	22	40 ± 5

* Determined for the period 2–9 h, or 1–4 h for TSB.

† Determined for the period 11–24 h, or 6–10 h for TSB.

Table 3. Growth characteristics and Sm production in cultures of various *S. griseus* wild-type and mutant strains grown in defined MM

Strain	Characteristics of growth phase				Sm production	
	1st EGP t_d (h)*	Transient start at (h)	2nd EGP t_d (h)†	Stationary start at (h)	Initial detection (h)	Yield after 48 h (mg l ⁻¹)
N2-3-11	4.2 ± 0.5	10	3.0 ± 0.3	24	14	115 ± 10
DSM 40236	4.2 ± 0.4	10	2.9 ± 0.1	24	36	30 ± 20
M881	4.7 ± 0.5	10	3.1 ± 0.5	24	—	0
M881 + A-factor‡	4.9 ± 0.5	10	3.0 ± 0.5	24	13	100 ± 10
M66	4.2 ± 0.5	10	2.9 ± 0.3	24	—	0
W67	3.9 ± 0.5	3.5	2.4 ± 0.1	22	—	0

* Determined for the period 2–9 h, or 0–3 h for W67.

† Determined for the period 11–20 h, or 5–18 h for W67.

‡ Grown in the presence of 2 mg A-factor l⁻¹.

composed MM and TSB complete medium (Table 2). There was no significant alteration in growth and Sm production of *S. griseus* when the concentration of all or of single components of the MM were increased by a factor of 2–10 (Table 2, not all data shown). The growth of *S. griseus* always showed two EGPs separated by the transient lag phase 10 h post-inoculation. The rates of growth within the first and second EGPs were not significantly changed by the various alterations of the synthetic medium. In contrast, the time-course of Sm production and its maximal yield varied considerably under the same conditions (Table 2).

In TSB, *S. griseus* also showed a biphasic EGP, separated by a transient lag phase after 5 h growth. The growth rates within the two EGPs were almost doubled to 0.3 and 0.55, respectively, as compared to those found in synthetic medium (Table 2). After 12 h growth, *S. griseus* entered

the stationary phase and Sm was detected for the first time after 22 h. Comparing the characteristics of growth of *S. griseus* in the minimal and complete media, it was conspicuous that *S. griseus* cells entered the transient lag phase on both media after growing exponentially for approximately 2.5 generations. Therefore, the general course of growth of *S. griseus*, but not the production of Sm, seems to be independent of the medium. This probably reflects a programmed developmental cycle, as already postulated by Ensign (1988).

Growth of other *S. griseus* strains and mutants deficient in differentiation

The growth of *S. griseus* N2-3-11 used as the principal strain in our investigations was compared to that of the Waksman strain (DSM 40236) and mutants of strain N2-

Fig. 3. Growth of the *S. griseus* N2-3-11 mutant strains W67 (●) and M881 (▲) in standard MM determined by measuring the optical density.

3-11 (Table 3, Fig. 3). These mutants were M66, unable to produce Sm, and the pleiotropic mutants M881 (A-factor-deficient; Distler *et al.*, 1990, 1992) and W67 (A-factor-insensitive; Distler *et al.*, 1990), both lacking secondary metabolism and differentiation. The *S. griseus* wild-type strain DSM 40236 and strains M66 and M881, grown with or without A-factor, showed the same biphasic EGP interrupted by the transient growth phase after 10 h growth. The t_d of these *S. griseus* strains within the first and the second EGPs were roughly the same as in strain N2-3-11 (Table 3). The time-course and yield of Sm by the mutant M881 grown in the presence of A-factor was very similar to that of the overproducing strain *S. griseus* N2-3-11. Sm was detectable in the culture supernatant for the first time after 13 h growth and a maximal amount of 100 mg Sm l⁻¹ was found after 36 h growth. In contrast, in the cultures of *S. griseus* DSM 40236 Sm was reproducibly detectable only after 36 h growth. A maximal amount of 30 mg Sm l⁻¹ was produced after 72 h. No Sm production was detected in the cultures of *S. griseus* mutants M66 and M881, grown without A-factor. Therefore, the overall time-course and phasing growth of *S. griseus* is independent of its ability to produce either A-factor or Sm.

In contrast to the growth characteristics of all the other *S. griseus* wild-type and mutant strains analysed so far, that of strain W67 deviated significantly (Table 3, Fig. 3). Although the growth rate of W67 within the first EGP was comparable to the other *S. griseus* strains, the onset of the transient lag phase reproducibly occurred after 3.5 h, equivalent to one generation of exponential growth. Also, within the second EGP, strain W67 showed a higher growth rate as compared to all other *S. griseus* strains.

Fig. 4. Induction or repression of Sm synthesis and dependence of the time of addition of effector molecules. The yield of Sm produced after 48 h by M881 and *S. griseus* N2-3-11 supplemented at the indicated time with effector molecules: M881 with 2 mg A-factor l⁻¹ (▲); *S. griseus* N2-3-11 with 50 mM L-valine (○) or 10 μM staurosporine (●).

Biological relevance of the first EGP for the production of Sm

The biosynthesis of Sm in various strains of *S. griseus* is repressible by either L-valine (Ensign, 1988) or protein kinase inhibitors like staurosporin (Hong *et al.*, 1993). In the *S. griseus* mutant strain M881, deficient in A-factor synthesis, Sm production can be restored to wild-type levels by addition of exogenous A-factor (Khokhlov, 1982). A-factor showed the strongest effect when added to a culture of M881 at the time of inoculation, suggesting the existence of a physiological state soon after start of growth (or during the preceding stationary phase) in which the action of A-factor is required, the A-factor-sensitive stage. To analyse this assumption in detail, parallel cultures of M881 were supplemented at different times with 2 μg A-factor ml⁻¹ and the yield of Sm produced after 48 h growth was determined (Fig. 4). The effect of A-factor on the induction of Sm synthesis was strictly dependent on the age of the M881 culture. A-factor (2 μg ml⁻¹) added at the beginning of fermentation induced the production of 110 mg Sm l⁻¹. The final amount of Sm produced dropped, reaching non-detectable levels, when A-factor was added upon entering the transient lag phase. In cultures of M881, there was no induction of Sm production when A-factor was added up to concentrations as high as 20 μg ml⁻¹ 10 h after inoculation or later (Fig. 4, not all data shown). Also, the repression of Sm synthesis in the *S. griseus* wild-type by L-valine and staurosporin was found to be dependent on the age of the culture. Within the first 10 h growth, addition of either valine or staurosporin repressed the production of Sm without affecting the time-course of growth or the amount of biomass produced (Fig. 4, and data not shown). However, there was no repression of Sm production when these inhibitors were added to a culture of *S. griseus* which had already passed the transient lag phase after 10 h growth (Fig. 4).

Growth-phase-dependent expression of Sm biosynthetic genes

The growth-phase-dependent expression of three Sm biosynthetic genes, *stsC* encoding the L-glutamine:scyllo-inosose aminotransferase, and the related putative aminotransferase genes *stsA* and *strS*, each located in a different transcription unit of the *S. griseus str/sts* gene cluster (Retzlaff *et al.*, 1993; Piepersberg, 1995) were analysed using two-dimensional gel electrophoresis of [³⁵S]methionine pulse-labelled proteins of *S. griseus* N2-3-11 (Fig. 5a). The StrS, StsA, and StsC proteins were identified by co-migration with these proteins individually overexpressed from the T7 promoter in *E. coli* recombinant strains (J. Ahlert, personal communication). 2-D electrophoregrams from several independent experiments were analysed and the relative rates of expression were calculated (Fig. 5a). No expression of proteins StrS, StsA and StsC was found during the first EGP (Fig. 5a, b). The expression of the proteins StsC and StrS is characterized by an increasing level of synthesis during the second EGP reaching a maximal expression rate in the SGP (Fig. 5a). The expression of StrS was already detectable 2 h after the transient lag phase. It reached a peak of synthesis 22 h post-inoculation and no expression of StrS was found in stationary phase after 24 h (Fig. 5a, c, d). The protein StsC was found to be expressed for the first time after 14 h growth and the rate of expression reached a maximum after 24 h (Fig. 5a, d). In contrast, the protein StsA was detectably synthesized only during early stationary phase between 20 and 24 h growth (Fig. 5a, d). These results showed that at least two of the Sm biosynthetic genes were expressed immediately after the *S. griseus* culture had passed the transient lag phase and entered the second EGP, during which the factor-induced physiological switch is executed. This explains the synthesis of Sm already within the second EGP. Thus the decision-making process in *S. griseus*, to produce Sm, became irreversible within the first 10 h growth (first EGP), with the susceptibility for signals inducing secondary metabolism and cell differentiation being highest at the time immediately after inoculation. Hence, we define the first EGP as a factor-sensitive 'decision phase' and the second EGP as an 'execution phase' which cannot be further influenced by external switch-setting factors. The transient lag phase is called the 'decision lag'.

Decision phase model of the Sm production and developmental cycle of *S. griseus*

To further understand the physiological characteristics of the two EGPs, several shift experiments were carried out.

Fig. 5. Growth, Sm production and kinetics of the expression of selected Sm biosynthetic genes of *S. griseus* N2-3-11 in standard MM. (a) Bar graphs show the relative expression rates of the pulse-labelled StrS, StsA, and StsC proteins, which were resolved in 2-D gel autoradiograms and densitometrically quantified using Visage software. The production of biomass (■) and Sm (○) are indicated. (b–d) Autoradiography of 2-D gels of *S. griseus* proteins pulse-labelled after 8 h (b), 18 h (c) and 24 h (d). The positions of the proteins StrS (△) StsA (□) and StsC (○) are shown.

Fig. 6. Time-course of growth of *S. griseus* cells shifted from a primary culture (a) into fresh MM after 8 h (c), 12 h (e) and 24 h (g) growth. Growth was determined by measuring the optical density. (b, d, f, h) Determination of the time period in which the Sm production of *S. griseus* was sensitive to repression by L-valine. Sm production after 48 h in the primary culture (b) and the 8 h (d), 12 h (f) and 24 h (h)-shift cultures supplemented at the indicated times with 50 mM L-valine are shown.

S. griseus mycelium grown for different times in standard synthetic medium was shifted into fresh medium. The time-course of growth of the shift cultures was then analysed by (1) measuring the optical density of the cultures and (2) by determining the time period in which the production of Sm was sensitive to repression by L-valine (Fig. 6). When *S. griseus* cells grown for 8 h were shifted into fresh MM, the culture entered a decision lag phase after an additional 2 h, corresponding to the overall time between inoculation and the onset of the transient growth phase in the primary culture (Fig. 6c). Also, L-valine only repressed the synthesis of Sm when added to the 8-h-shift culture within the first 2 h growth. Therefore, the first EGP or decision phase in the 8-h-shift culture was reduced to a period of 2 h (Fig. 6d). When *S. griseus* cells which had entered the second EGP were transferred after 12, 14 or 16 h into fresh medium (Fig. 6e, and data not shown), these cultures continued to grow exponentially but without a decision lag, entering stationary phase after 24 h. Consistent with these results, there was no repression of Sm synthesis in cultures shifted after

12–16 h when L-valine was added at any time thereafter (Fig. 6f, and data not shown). The regular time-course of growth in the shifted cultures was regained when *S. griseus* cells were transferred from the stationary phase of the primary culture into fresh medium (Fig. 6g). Thus the 24-h-shift culture exhibited all normal growth phases, and L-valine added within the first 10 h growth was again able to repress the production of Sm (Fig. 6h).

DISCUSSION

The initial studies of the life-cycle of a *Streptomyces* strain in batch culture presented here were initiated to obtain a more fundamental and precise knowledge of the various physiological stages of the growth of *Streptomyces*. This knowledge is a prerequisite for further analyses of the processes governing secondary metabolism and cell differentiation. *S. griseus* was chosen as a model organism since it has the advantages of both being an intensively studied streptomycete and reproducibly showing all the features of a normal life-cycle in a defined medium,

including sporulation and antibiotic production (Shapiro, 1989; Distler *et al.*, 1990, 1992; Ensign, 1988). We have shown that the vegetative or exponential growth of various Sm-producing and A-factor-dependent strains of *S. griseus* follow medium-independent biphasic kinetics. This behaviour is caused by passing through a phase named 'decision lag' during which the cells undergo a fundamental change regarding their sensitivity to signals inducing or repressing differentiation and secondary metabolism. Under normal conditions, a strain-specific signalling cascade induces the decision-making process in the early vegetative growth phase ('decision phase'; Fig. 1). As the culture enters the 'decision lag', the decision-making process is completed and seems to become unchangeable within the second EGP which might be regarded as an 'execution phase' (Fig. 6). Later, during the interval between 18 and 24 h growth of a standard batch culture, the cells return again to a state in which they are sensitive to external signal molecules priming them for a following round of decision-making events.

In the case of several strains of *S. griseus*, comprising isolates producing Sm, leucaemomycin, nonactin and daunorubicin, A-factor is a basic and essential effector of the switch-inducing machinery (Eritt *et al.*, 1984; Horinouchi & Beppu, 1994). To induce Sm synthesis, A-factor needs to be present either during the stationary phase or directly upon onset of growth in the early vegetative growth phase of *S. griseus*. This is suggested by the rapid loss of A-factor-sensitivity of an A-factor-negative mutant (strain M881) directly after the onset of growth, with the complete abolition of any effects of A-factor addition when the decision lag is reached (Fig. 4). In contrast to this observation, the highest productivity for A-factor in the culture is at the beginning of stationary phase (Figs 2 and 4). Therefore it is tempting to speculate that (1) the much higher level of A-factor in the second EGP is necessary for the complete induction of sporulation or (2) the extracellular A-factor is responsible for inducing the mechanism(s) for the switch back to the signalling-sensitive state, possibly via the activation of membrane-bound GTPases (Penyige *et al.*, 1992).

Although A-factor is currently the only known signal molecule switching the cultures towards cell differentiation and secondary metabolism, other still-unknown factors or physiological effects could also be prerequisites for a successful decision-making process in *S. griseus* or other *Streptomyces* species. For example, this view is supported by the availability of other types of pleiotropic mutants, such as the *S. griseus* mutant strain W67, in which differentiation and secondary metabolism are suppressed by events which probably do not influence the A-factor-dependent cascade (Distler *et al.*, 1990; Babcock & Kendrick, 1988). The *S. griseus* mutant strain W67 seems to be impaired in the programmed cell-cycle which possibly brings about its A-factor-insensitive pleiotropic deficiency. The properties of the differentiation-cycle mutant W67 support the concept that the course of growth of *S. griseus* described here is based on a genetically determined programme. Internal components of the signalling cascade could be G-proteins (Penyige *et al.*,

1992), as well as protein kinases/phosphatases and inhibitors thereof (Hong *et al.*, 1993). The participation of protein kinases in the switch-setting process is supported by the growth-phase-dependent repression of Sm synthesis in *S. griseus* by staurosporin (Fig. 4).

ppGpp has also been postulated to be involved in the induction of secondary metabolism in Sm-producing *S. griseus* (Ochi, 1987b). However, the data presented in this report do not support a direct correlation between the accumulation of ppGpp and the decision-switch phenomenon. Indeed, there was no accumulation of ppGpp during the growth of *S. griseus* N2-3-11 in the synthetic medium used. This in turn suggests that regulatory processes based on the stringent response phenomenon are not a prerequisite for the induction of the secondary metabolism in *S. griseus*. Similar results were previously reported for other streptomycetes (Bascarán *et al.*, 1991). Another generally distributed intracellular signal molecule in bacteria, cAMP, which was discussed as being involved in the regulation of aminoglycoside production in *Streptomyces* species (Ragan & Vining, 1978), is accumulated intracellularly in *S. griseus* just before the culture enters the decision phase lag (Fig. 2). Whether the intracellular peak level of cAMP is the stimulus for the diminution of growth during the decision lag or simply another phenomenon accompanying the decision-making process will have to be investigated in the future. There is also no experimental evidence available that the cAMP found in the culture supernatant at the beginning of the SGP is correlated with events switching the culture back towards a signal-sensitive state (see above). The biochemical mechanism(s) of the L-valine-dependent physiological effect(s) leading to the repression of secondary metabolism (Fig. 4) and of sporulation in *S. griseus* N2-3-11 is still unclear. The L-valine-dependent effect in *S. griseus* strain 2682 is explained by the observed repression of A-factor synthesis (Ensign, 1988). However, A-factor biosynthesis is not inhibited by L-valine in *S. griseus* N2-3-11, nor does A-factor relieve the valine effect when added in concentrations up to 10 mg l⁻¹ (data not shown). Further investigations into the processes involved must be conducted with these different *S. griseus* strains to explain the conflicting results.

These results suggest that a decision-making process occurs during the first EGP of the *S. griseus* fermentation, in which secondary metabolism and cell differentiation are switched on. This phenomenon is probably a generally distributed property of streptomycetes and other actinomycetes. This concept is supported by the description of a biphasic exponential growth behaviour of *S. hygroscopicus* and *S. coelicolor* in batch fermentations (Holt *et al.*, 1992; Blanco *et al.*, 1994). Therefore, the traditional picture of the growth of *Streptomyces* having a uniform vegetative growth phase has to be changed to a more complex one. There is no longer only a vegetative growth phase in which biomass is accumulated, followed by a SGP 'responsible' for the production of secondary metabolites and sporulation. Instead, we postulate the presence of an intrinsic physiological cycle only in the course of which the regulatory switch-settings for metabolic and cell

differentiation can be made. During the first EGP, the decision to switch to the genetic programme(s) for later differentiation and secondary metabolite production can be induced by general or strain-specific effector molecules. The cells are then committed to secondary metabolism. If this decision lag has been passed without switch-inducing conditions and the second EGP has been entered, these decision-making processes are transiently blocked until resuming a sensitive, decision-prone state after entering the SGP. The nature of the physiological block in the post-decision phase, and the mechanism(s) of returning to the state in which cells become sensitive to A-factor or L-valine, are the most interesting phenomena for an understanding on the molecular level of this type of control of a differentiation cycle in streptomycetes.

ACKNOWLEDGEMENTS

We thank August Böck for the sample of ppGpp and Annette Mehling for the critical reading of the manuscript. This work has been supported in part by grants from the Deutsche Forschungsgemeinschaft and the Bundesministerium für Forschung und Technologie.

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Received 30 October 1995; revised 31 January 1996; accepted 4 March 1996.