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High-yield actinorhodin production in fed-batch culture by a *Streptomyces lividans* strain overexpressing the pathway-specific activator gene *act*II-ORF4

P Bruheim[1], H Sletta[2], MJ Bibb[3], J White[3] and DW Levine[1]

[1]*Department of Biotechnology, Norwegian University of Science and Technology, Trondheim, Norway;* [2]*Sintef Applied Chemistry, Group of Industrial Biotechnology, Trondheim, Norway;* [3]*Department of Molecular Microbiology, John Innes Centre, Norwich NR4 7414, UK*

***Streptomyces lividans* 1326 usually does not produce the red/blue colored polyketide actinorhodin in liquid culture even though it carries the entire actinorhodin biosynthesis gene cluster. The bacterium can be forced to produce this secondary metabolite by introducing *act*II-ORF4, the actinorhodin pathway-specific activator gene from *Streptomyces coelicolor*, on a multicopy plasmid. The production of actinorhodin by such a strain has been optimized by medium and process manipulations in fed-batch cultures. With high-yield cultivation conditions, 5 g actinorhodin/l are produced during 7 days of cultivation; or approximately 0.1 g actinorhodin/g dry weight (DW)/day in the production phase. The yield in this phase is 0.15 Cmol actinorhodin/Cmol glucose, which is in the range of 25% to 40% of the maximum theoretical yield. This high-level production mineral medium is phosphate limited. In contrast, nitrogen limitation resulted in low-level production of actinorhodin and high production of α-ketoglutaric acid. Ammonium as nitrogen source was superior to nitrate supporting an almost three times higher actinorhodin yield as well as a two times higher specific production rate. The wild-type strain lacking the multicopy plasmid did not produce actinorhodin when cultivated under any of these conditions. This work examines the actinorhodin-producing potential of the strain, as well as the necessity to improve the culture conditions to fully utilize this potential. The overexpression of biosynthetic pathway-specific activator genes seems to be a rational first step in the design of secondary metabolite overproducing strains prior to alteration of primary metabolic pathways for redirection of metabolic fluxes.**
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Introduction

Actinorhodin is a red/blue (pH-dependent) secondary metabolite belonging to the aromatic polyketides. Acetyl CoA is the only carbon precursor for its synthesis. Study of the biosynthesis of actinorhodin especially in *Streptomyces coelicolor* from the genetic point of view, for many years [5,8]. There have also been some reports about actinorhodin production in the closely related strain *Streptomyces lividans*. The wild type of this bacterium does not usually produce actinorhodin when grown in liquid culture — even though it carries the entire cluster for actinorhodin production, it usually remains unexpressed [23].

Secondary metabolism in streptomycetes is regulated through a complex cascade of regulatory factors. Both pleiotropic and pathway-specific activator proteins have been identified and characterized [5,8]. It has been shown at the transcriptional level in *S. coelicolor* that introducing extra copies of the positively acting pathway-specific regulatory genes *redD* and *act*II-ORF4 forces the cells to turn on, respectively, the undecylprodigiosin and actinorhodin biosynthesis earlier than normal [13,25]. The results indicated that for rapidly growing cultures, the synthesis of actinorhodin is limited by the availability of the activator protein. Overexpression of the ActII-ORF4 activator protein may therefore be a rational first step in the development of an actinorhodin-overproducing *S. lividans* strain.

Nutrient limitation or reduced growth opportunities often trigger the onset of secondary metabolism, the extent of which is influenced by carbon catabolic, nitrogen metabolite and phosphate regulation [20]. Some physiological studies of actinorhodin production in liquid cultures of *S. coelicolor* have been reported. Production levels in these studies were in the range of 10–200 mg actinorhodin/l/4–5 days, reflecting low volumetric productivities [3,16,21]. Nutritional status and cell morphology have also been addressed in these studies. Ates *et al* [3] studied carbon catabolite repression of actinorhodin production in *S. coelicolor* and found increased production for fed-batch culture when the glucose concentration was kept below a critical level. Doull and Vining [10] avoided glucose catabolite repression by using glutamate and starch as carbon sources, and they found that actinorhodin production in *S. coelicolor* was relatively insensitive to the carbon source concentration. However, they observed that nitrogen depletion, phosphate depletion or a decline in growth rate triggered actinorhodin production. The morphological status of both the inoculum and the resulting culture and its effect on secondary metabolite production have also been analysed [4,11,12,15].

There are few reports about strain development by metabolic engineering in *Streptomyces* spp. even though these bacteria are

Correspondence: Dr P Bruheim, Department of Biotechnology, Norwegian University of Science and Technology, Sem Selands vei 6/8, N-7491 Trondheim, Norway
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very important commercially. The aim of this study was to investigate the nutritional demands for actinorhodin synthesis in an *S. lividans* strain overexpressing the pathway-specific activator gene *act*II-ORF4. Preliminary studies had suggested that this strain had the ability to produce large amounts of actinorhodin. Such a high yielding system is desirable for physiological studies including flux analysis. Such analysis will indicate which enzymatic steps should be altered to increase actinorhodin yield even further. It was therefore of importance to establish medium and process conditions that would produce high yielding actinorhodin cultures.

Materials and methods

Microorganisms

These studies were carried out with *S. lividans* 1326 and various plasmid-containing derivatives. Strain RpdS 102 is *S. lividans* 1326 containing *act*II-ORF4 cloned on a multicopy plasmid (pIJ68), RpdS 105 is *S. lividans* containing the vector alone (pIJ486), while the wild-type *S. lividans* was designated RpdS 101. pIJ68 contains the *act*II-ORFIV promotor [22]. Unless otherwise stated, the plasmids were maintained by selecting for thiostrepton (10 μg ml^{-1}) (pIJ68 and pIJ486 both carry the thiostrepton resistance gene, *tsr*).

Media and growth conditions

A two-state inoculum protocol was used as provided by Dr Paul Ives (DSM, Delft, Netherlands). Medium for the first stage contained (g/l of distilled water): 15.0 glucose, 15.0 glycerol, 15.0 soya peptone, 3.0 NaCl, 1.0 $CaCO_3$, and the second stage (g/l): 33.0 glucose, 15.0 yeast extract. Mycelial cells in 15% glycerol were stored in 1-ml cryotubes at -80°C and used to inoculate the first stage of the inoculum. Cultures were stirred (400 rpm) for 48 and 24 h, respectively, in baffled 500-ml Erlenmeyer flasks with 150 ml medium; 12-ml cultures were transferred from stage one to two. A 5% inoculum was used in the fermenters. The mineral medium (actinorhodin mineral medium, AMM) was prepared as follows (g/l of distilled water): 9.4 NH_4Cl (alt 14.9 $NaNO_3$), 1.78 $Na_2HPO_4{\cdot}2H_2O$, 0.2 $MgCl_2{\cdot}6H_2O$, 0.7 KCl, 0.85 Na_2SO_4, 2.0 NaCl 1 ml 0.05 M $CaCl_2$. The medium was supplemented with 2 ml trace mineral solution containing (g/l of distilled water): 13.5 $FeCl_3{\cdot}6H_2O$, 1.5 $CuCl_2{\cdot}2H_2O$, 9 $ZnCl_2$, 3.6 $MnCl_2{\cdot}4H_2O$, 0.6 $Na_2MoO_4{\cdot}2H_2O$, 0.4 $CoCl_2{\cdot}6H_2O$ and 0.3 H_3BO_4. Glucose was used as carbon source with a starting concentration of 45 g/l. The glucose concentration in the fermenters was kept above 10 g/l by continuous addition of a concentrated solution of glucose (450 g/l as required). This was adjusted individually for each fermentor depending upon respiratory activity. Fermentations were run at 28°C for 6–7 days in 3-l Applicon fermenters with an operating volume of 1 l. The stirrer speed was set to 900 rpm and the airflow rate at 0.5 vvm. Dissolved oxygen was never below 40% of saturation. Silicon-based antifoam was added manually as required. Fermenters were maintained at pH 7.0 by addition of 2 M HCl or 2 M NaOH.

Analysis

Percentage CO_2 in the off gas was continuously monitored on a Rosemount (Rosemount GmbH & Co, Hanau, Germany) Binos 100 CO_2 analyzer and subsequently logged together with data for pH, flow, and gram glucose solution added in the time period between sample withdrawals. Oxygen in the off gas was measured on a Rosemount Oxynos 200 O_2 analyzer discontinuously at every sample point. Dry weight (DW) was measured from 10-ml samples of cultures centrifuged twice, and washed with distilled water before being dried at 110°C for 24 h. Actinorhodin content was measured both in the supernatant and in the pellet. Depending upon the concentration of actinorhodin in the cultures, 10-ml samples were withdrawn from the fermenters and washed one to four times. All supernatants from the subsequent washings were collected and the actinorhodin concentration was determined with the 1 M KOH assay measuring the absorbance at 640 nm [6]. The concentration of actinorhodin in milligrams per milliliter was calculated using 25,320 as extinction coefficient and taking into consideration the dilution from the washing and the KOH solution in the cuvettes [6]. The intracellular concentration of actinorhodin was measured by subsequent extraction of actinorhodin from the pellets into 1 M KOH until there was no blue color in the last KOH supernatant.

Glucose and organic acids were analyzed on a Shimadzu (Shimadzu, Kyoto, Japan) LC 9A HPLC with an Aminex (Aminex, Biorad, Hercules, CA) -HPX-87H column at 45°C using 5 mM H_2SO_4 (0.6 ml/min) as eluant. A Shimadzu RID 6A RI detector and a Shimadzu SPD 6A UV detector were mounted in series for the detection of sample peaks. Glucose and α-ketoglutaric acid concentrations were also determined by enzymatic assays as a control, (glucose oxidase enzymatic kit from Biomerieux (Biomerieux, Marcy l'Etoile, France), and glutamate dehydrogenase enzymatic kit from Boehringer (Boehringer Mannheim, Mannheim, Germany). In addition, the reference and the samples from the cultures with high organic acid production were assayed for pyruvate and acetate by enzyme analysis (Boehringer Mannheim) even though these compounds were never found on the HPLC analysis. The enzyme assays also demonstrated that pyruvate and acetate were not produced under the conditions tested by any of the three strains evaluated in this study.

Spectrophotometric assays were used to determine the extracellular concentration of ammonium [26] and phosphate [14].

Results

AMM composition was optimized through successive series of fermentations focusing on the production of actinorhodin by an *S. lividans* 1326 strain (RpdS 102) carrying the pathway-specific activator gene *act*II-ORF4 on a multicopy plasmid (pIJ68). The high-yield medium formulation was included as a control (called reference — ref) in subsequent fermentation experiments (AMM — 10 mM phosphate-limited version). This was necessary since there was a slight variation in production levels under reference conditions. Variation in the reference condition within one series of experiments was assessed using three identical fermenters. The variation in the fermentation data was less than 5%. Variation in samples (e.g., dry weight, etc.) taken at the same time was below 1%. Data in the tables are taken from the phase with highest production rates — the production phase with constant DW. The consumption rate of glucose (r_{glucose} (mmol C/g DW h)) and the production rates of actinorhodin and of α-ketoglutaric acid were calculated from the difference in concentration between two representative sample points. Data are corrected for the loss of water in the off gas and the dilution due to the addition of glucose

and base in the case of acid production. The production rate of CO_2 is an average based on the logged data in the time period between the two sample points. R_Q (respiration coefficient, mole CO_2/ mole O_2) is calculated from CO_2 and O_2 measurements at the two sample points. The R_Q was stable throughout the production phase. The yield of actinorhodin on glucose was not constant throughout the production phase; r_{CO_2} and $r_{glucose}$ decreased while r_{act} was more or less constant. The yield of actinorhodin is therefore highest at the end of each individual fermentation and it is this yield that is presented in the tables.

The effect of the inoculum morphology and quantity on growth and actinorhodin production was investigated. This is reported first since it is important to separate these effects from the effects of the nutritional status of the mediums.

Inoculum morphology

Different morphologies of the RpdS inoculum were obtained that ranged from fluffy pellets 200 μm in diameter to dispersed hyphal fragments. These were obtained by varying the inoculum growth condition: incubation in a shaking incubator gave a pelleted inoculum, while agitation with a triangular magnet yielded a dispersed inoculum. The morphological state of the inoculum significantly influenced the growth profile in the fermenters as shown in Figures 1 and 2 for a dispersed and pelleted inoculum of RpdS 102, respectively. The optimum medium formulation for actinorhodin was used in this experiment. Most of the cell mass increase for the dispersed inoculum occurred in one phase (0–40 h). The CO_2 evolution rate curve showed the same profile, although a deceleration phase was observed at the end of the growth phase (22–40 h). There was a slight increase in DW after cessation in the CO_2 evolution rate (CER). Henceforth, this growth profile will be referred to as the one-phase growth situation. Another growth profile was obtained by using an inoculum with a pelleted morphology (Figure 2). The cell mass increase can roughly be separated into two phases, the first with the lowest rate ending at 60–70 h. The CER profile gave a somewhat different picture since three phases were observed. The cell mass increased in the intermediate phase but the CER was constant. (A control experiment with samples taken more frequently confirmed this picture.) Analysis of the fermentation broth showed that there was no nutrient limitation that caused this transition phase (glucose, phosphate, and nitrogen were present in excess). This growth profile is termed the two-phase growth situation.

Over 5 g actinorhodin/l was produced during 7 days of cultivation. In the production phase 15–17% of the glucose was converted to actinorhodin. The inoculum morphology had little effect on the overall performance of the culture with regard to specific production rates and yields of actinorhodin in the production phase (Table 1). This was a desirable result since the inoculum morphology varied among the different fermentation series. To minimize variation in inoculum among the different fermentation series, fermenters were inoculated with cells showing the most dispersed morphology from parallel inoculum cultures. The variation among the different fermentation series made it necessary to include a reference condition for quantitative comparison among the various series. Reducing the dispersed inoculum concentration from 5.0% to 2.5% (data not shown) resulted in two-phase growth, implying that the growth profile also depends on inoculum quantity (i.e., the number of growth kernels). It was therefore decided to use 5% dispersed inoculum in the medium optimization experiments.

Nutrient limitation and choice of nitrogen source

Nitrogen limitation had a much more significant physiological impact than phosphate limitation for RpdS 102. This can be seen

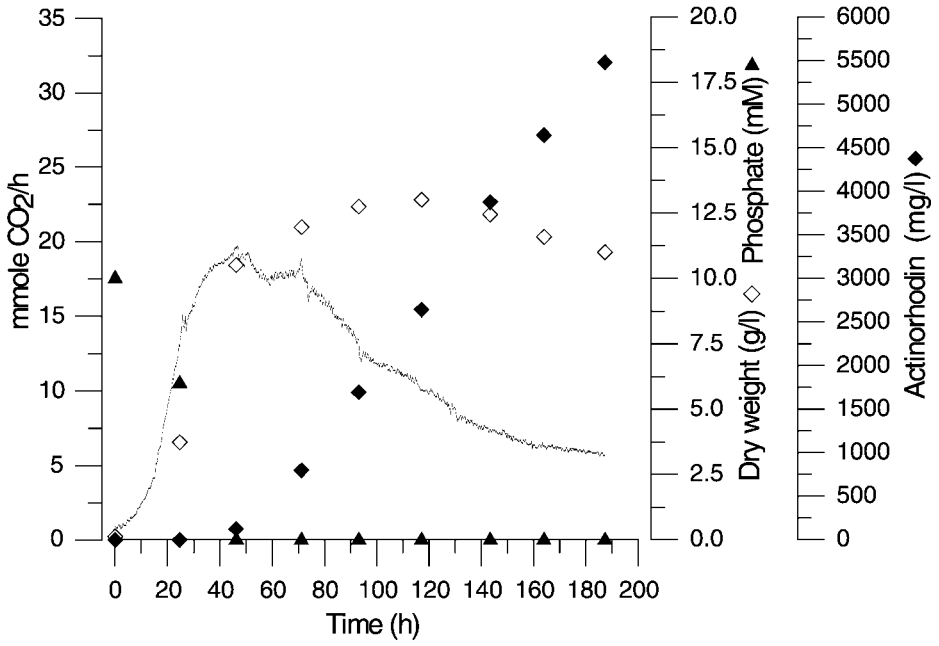

Figure 1 Time course for growth and actinorhodin production in fed-batch fermentation in phosphate-limited AMM inoculated with a dispersed RpdS 102 inoculum. ◇ DW (g/l), ◆ actinorhodin (mg/l), ▲ phosphate (mM), — CO_2 production (mmol C/h).

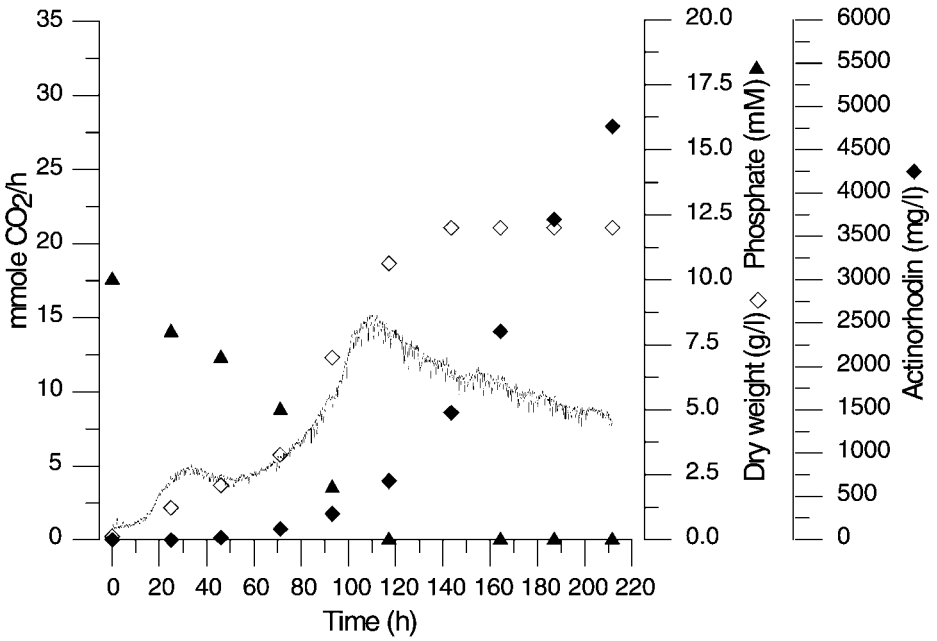

Figure 2 Time course for growth and actinorhodin production in fed-batch fermentation in phosphate-limited AMM inoculated with pelleted RpdS 102. ◇ DW (g/l), ◆ actinorhodin (mg/l), ▲ phosphate (mM), — CO_2 production (mmol C/h).

on the CO_2 curve in Figure 3, which shows a nitrogen-limited culture with ammonium as nitrogen source (Figure 1) is the phosphate-limited control). When the culture reached nitrogen limitation, the CER dropped instantly by almost 50%, while phosphate limitation resulted in a gradual decline in CER. It was determined in a preliminary experiment that the ammonium concentration was below detection limits at the time of the CO_2 drop (this cannot be seen directly from Figure 3 since the sample frequency was too low in this experiment). Conceivably, phosphate limitation may allow some increase in cell mass, while the nitrogen limitation presumably stops protein synthesis. Fermentation data comparing phosphate and nitrogen limitations are given in Table 2. The phosphate-limited culture had a much higher specific production rate of actinorhodin than the nitrogen-limited one, independent of the nitrogen source (ammonium and nitrate were tested). In the nitrogen-limited situation, much of the carbon was instead converted to α-ketoglutaric acid. The production of this acid started after the culture reached growth limitation. The α-ketoglutaric acid production was much higher on a Cmole basis than the production of actinorhodin, and even much higher than actinorhodin production under phosphate limitation. The specific substrate consumption was significantly higher under nitrogen limitation especially with nitrate as nitrogen source.

The choice of nitrogen source was also critical for the production efficiency of actinorhodin by RpdS 102 in phosphate-limited conditions. The specific actinorhodin production doubled when nitrate was replaced with ammonium, and the yield increased three times (Table 2). The specific substrate consumption was much higher than with ammonium even in stationary phase, after growth had ceased. The growth profile was also different with nitrate (Figure 4). Growth was divided in two phases with different CO_2 evolution rates, and cell mass also increased with different rates in these two periods. This might be explained by initial growth on inoculum carry-over nitrogen sources. Then when this source is depleted induction of nitrate-reducing enzymes takes place.

The optimal phosphate concentration was determined from a phosphate gradient experiment (data not shown). The specific actinorhodin production rate was twice as high with 10 mM initial phosphate concentration (reference) as with 2.5 and 20 mM. The growth profile for the 20 mM culture showed an interesting feature since it had two-phase growth (the growth curve is not shown but

Table 1 Fermentation data for RpdS 102 with two different inocula grown on AMM — 10 mM phosphate-limited version

Reactor conditions	DW	Data from production phase[a]				Y_{SA}	Carbon recovery
		r_s	r_c	r_a	r_k		
Dispersed inoculum	11.5	1.11	0.86	0.17	0.00	0.15	0.93
Pellet inoculum	12.0	1.13	0.86	0.19	0.00	0.17	0.93

Data from the production phase are average values between two sampling times in a production phase with constant production rates. DW (g/l), r_s (mmol $C_{glucose}$/g DW h), r_c (mmol C_{CO_2}/g DW h), r_a (mmol C_{act}/g DW h), r_k (mmol $C_{\alpha\text{-ketoglutaric acid}}$/g DW h), R_Q (mol CO_2/mol O_2), Y_{SA} (mol C_{act}/mol $C_{glucose}$).
[a]Not included slight increase in dry mass.

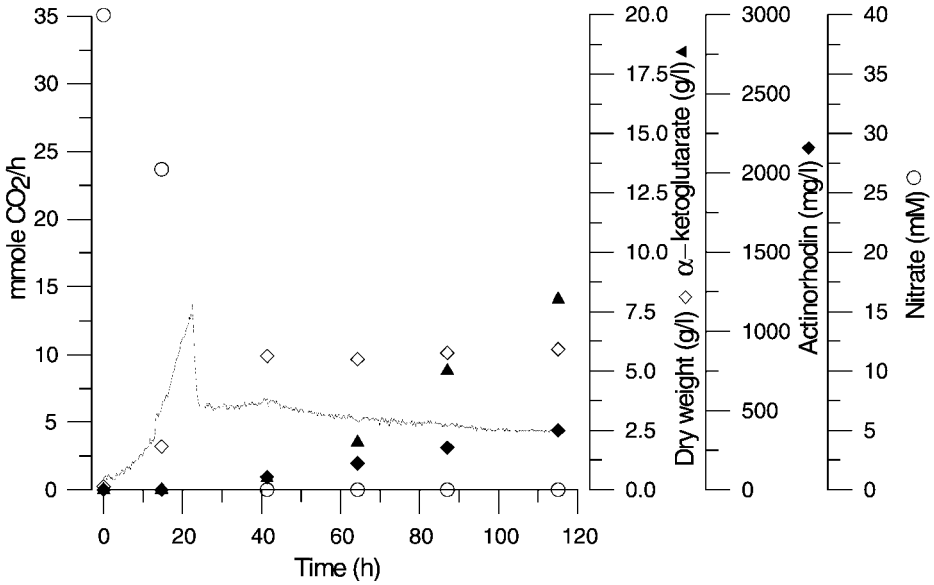

Figure 3 Time course for growth and actinorhodin production in fed-batch fermentation in nitrogen-limited AMM inoculated with dispersed RpdS 102. ◇ DW (g/l), ◆ actinorhodin (mg/l), ▲ α-ketoglutaric acid (g/l), ○ ammonium, — CO_2 production (mmol C/h).

the CO_2 profile was similar to that in Figure 5), while the reference with 10 mM phosphate had only one-phase growth (Figure 1). The same was observed in another experiment where the whole medium concentration was varied from 0.5AMM to 1.5AMM (data not shown). The reference had the highest yield and specific production rate, but the 1.5AMM culture had two-phase growth (like Figure 5). The nutritional status of the medium is therefore important for the growth profile.

Glucose limitation was tested by controlling the feeding rate of the glucose solution based on holding the CO_2 evolution rate at a level 60–70% of the reference (Table 2). This was done after phosphate limitation was reached. The DW decreased 20%, but the yield for actinorhodin remained the same as for the reference.

Effects of varying the concentration of other medium constituents

The effect on actinorhodin production of varying concentrations of other medium components. The most pronounced effects were found for variations in magnesium and calcium concentration. These effects were nonetheless minor compared to the choice of nutrient limitation and choice of nitrogen source. All of these compounds were also tested as the limiting compound for growth and gave a negative effect on actinorhodin production relative to phosphate limitation (data not shown).

When the magnesium concentration was increased 10 times, the specific actinorhodin production rate stayed the same, but the specific glucose consumption rate increased 40% (Table 3). A very low level production of α-ketoglutaric acid was observed in the high Mg culture. Thus, increased glucose consumption results in a lower yield of actinorhodin from glucose and an increase in specific CO_2 production.

A significant increase in DW was observed when the calcium concentration was increased 10 times (Table 3). The specific glucose consumption rate increased, implying a lower actinorhodin yield since the specific actinorhodin rate did not increase. The high

Table 2 Fermentation data for RpdS 102 — nutrient limitations and choice of nitrogen source

Reactor conditions	DW	Data from production phase				Y_{SA}	Carbon recovery
		r_s	r_c	r_a	r_k		
Phosphate-limited ammonium (ref)	11.3	1.24	0.89	0.18	0.00	0.14	0.86
Phosphate-limited nitrate	14.0	1.74	1.39	0.09	0.02	0.05	0.86[a]
Ammonium-limited	5.8	1.76	0.78	0.03	0.79	0.02	0.91
Glucose-limited, P-limited[b]	9.1	0.77	0.65	0.11	0.00	0.14	0.98
Nitrate-limited 40 mM N	5.0	2.44	1.56	0.09	0.47	0.04	0.87
Ref — P-limited ammonium	9.9	1.49	1.12	0.22	0.00	0.15	0.90

Data from the production phase are average values between two sampling times in a production phase with constant production rates. DW (g/l), r_s (mmol $C_{glucose}$/g DW h), r_c (mmol C_{CO_2}/g DW h), r_a (mmol C_{act}/g DW h), r_k (mmol $C_{\alpha\text{-ketoglutaric acid}}$/g DW h), R_Q (mol CO_2/mol O_2), Y_{SA} (mol C_{act}/mol $C_{glucose}$).
[a]Not included slight increase in dry mass.
[b]Glucose limitation in production phase (see text).

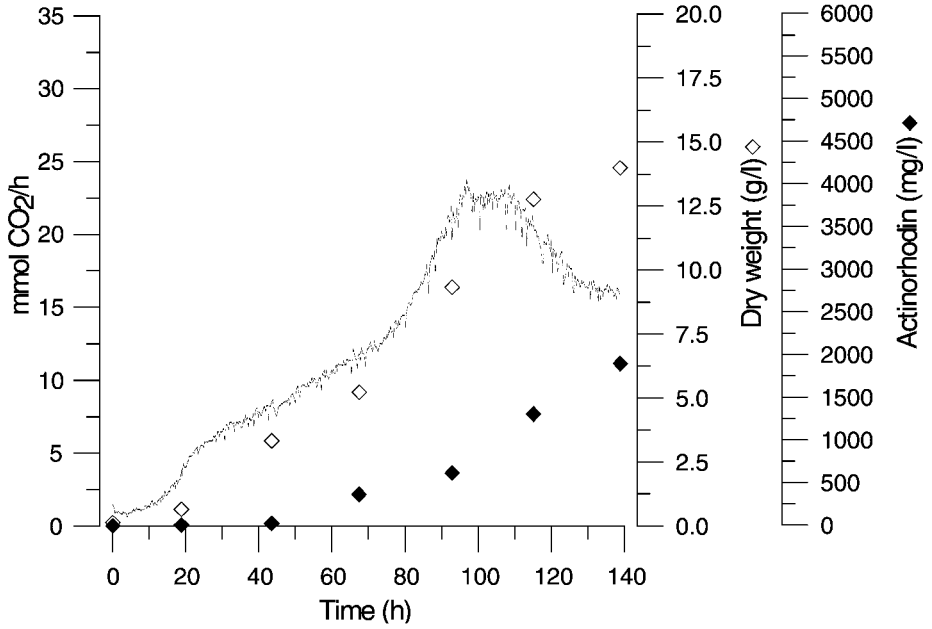

Figure 4 Time course for growth and actinorhodin production in fed-batch fermentation with nitrate as nitrogen source inoculated with dispersed RpdS 102. ◇ DW (g/l), ◆ actinorhodin (m/l), — CO_2 production (mmol C/h).

calcium culture had a two-phase growth profile (Figure 5). Variation in the amount of trace minerals (0.2–5 ml) or the concentration of sodium chloride (0.5–6 g/l) did not significantly influence either growth or production of actinorhodin. A minor negative effect was observed by increasing the potassium concentration five times: this reflected an increased specific respiration rate, while specific actinorhodin production was the same as the reference.

Comparison with the wild-type strain and vector-only control

The wild-type strain (RpdS 101) and a strain containing the vector alone (RpdS 105) were tested as controls (data not shown). Neither RpdS 101 nor RpdS 105 produced actinorhodin when grown under reference conditions. Trace amounts of the red pigment undecylprodigiosin were produced by both strains. Large amounts of α-ketoglutaric acid were produced by both

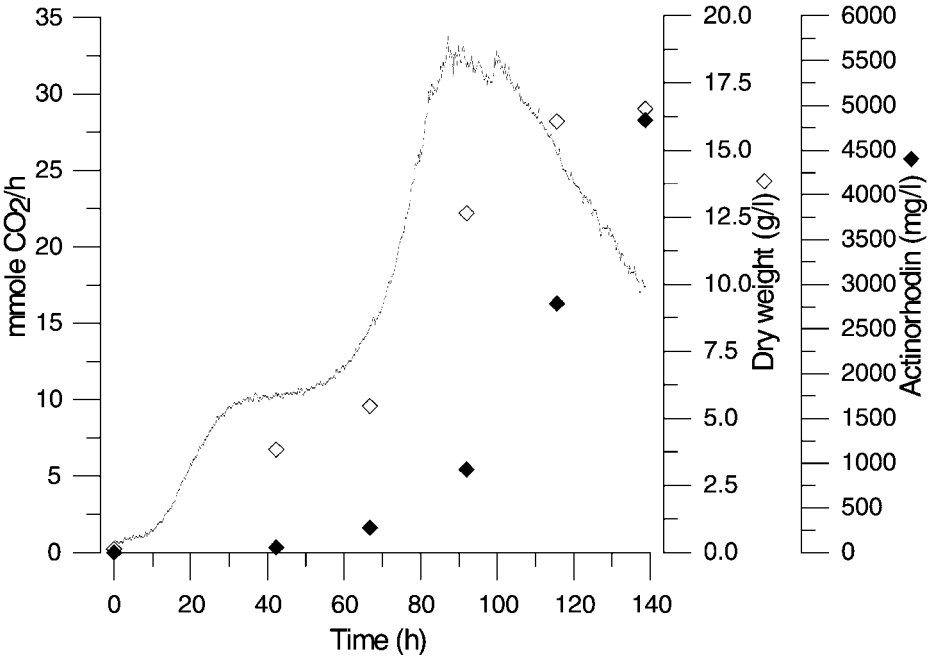

Figure 5 Time course for growth and actinorhodin production in fed-batch fermentation with high concentration of calcium inoculated with dispersed RpdS 102. ◇ DW (g/l), ◆ actinorhodin (mg/l), — CO_2 production (mmol C/h).

Table 3 Fermentation data for RpdS 102 — variation of the concentration of magnesium and calcium in phosphate-limited AMM

Reactor conditions	DW	Data from production phase[a]				Y_{SA}	Carbon recovery
		r_s	r_c	r_a	r_k		
High Mg 10AMM	10.7	1.74	1.32	0.19	0.02	0.11	0.88
Ref — P-limited ammonium	11.3	1.24	0.89	0.18	0.00	0.14	0.86
High Ca 10AMM	16.5	1.80	1.58	0.14	0.00	0.09	0.96
Ref — P-limited ammonium	9.9	1.31	1.06	0.14	0.00	0.13	0.92

Data from the production phase are average values between two sampling times in a production phase with constant production rates. DW (g/l), r_s (mmol $C_{glucose}$/g DW h), r_c (mmol C_{CO_2}/g DW h), r_a (mmol C_{act}/g DW h), r_k (mmol $C_{\alpha\text{-ketoglutaric acid}}$/g DW h), R_Q (mol CO_2/mol O_2), Y_{SA}(mol C_{act}/mol $C_{glucose}$).
[a]Not included slight increase in dry mass.

strains when subjected to nitrogen-limiting conditions. This shows that overexpression of the activator gene turns in the actinorhodin production in *S. lividans*, and that the production of α-ketoglutaric acid is not only a property of the actinorhodin-overproducing strain.

Plasmid stability

Thiostrepton (10 μg/ml) was added for plasmid maintenance both at the inoculum and fermenter stages. The same growth and production data were obtained when thiostrepton was not added to the fermentor. The onset of actinorhodin production was delayed by 20 h when thiostrepton was excluded from the second stage of the inoculum and from the fermenter. However, the same growth profile and production data in the production phase were obtained. Samples taken at the end of the fermentation were plated on SFM (soya flavor-mannitol) agar plates and the colonies were inspected for their ability to produce color (*S. lividans* 1326 produced no blue color on the SFM medium). Only very rarely were there colonies of RpdS 102 that did not produce actinorhodin, and this was also true for cultures grown in the presence and absence of thiostrepton. This indicated that the phenotype of 102 was stable even without selection.

Discussion

The introduction of multiple copies of the pathway-specific activator gene *act*II-ORF4 turns on the synthesis of actinorhodin in *S. lividans* 1326. We report medium and process conditions for high-level production of actinorhodin in such a strain. The focus has been on improving the yield based on carbon source and specific production rate and not the volumetric productivity and the end point concentration. The final cell mass concentration was not very high (11 g/l), implying that the end point concentration of actinorhodin could be higher than shown here (5 g/l), possibly by several fold with extended fermentation time and increased cell mass. More importantly, 15% of the glucose carbon was converted to actinorhodin in the production phase, which is 25% to 40% of the theoretical maximum depending on how this is calculated [7]. This is a significant figure for a mineral medium, and also in comparison with processes developed using industrial media. Actinorhodin production has been studied extensively especially in *S. coelicolor* [3,16,21] but the end point concentration has been almost exclusively between 1% and 5% of the values obtained here with RpdS 102. The yields were not presented in those studies, but the specific productivity was almost certainly significantly lower than that presented in this report. When the medium presented by Hobbs *et al* [15] for actinorhodin production in *S. coelicolor* was used for RpdS 102, a cell mass of 6 g/l and an end point concentration of only 50 mg actinorhodin/l were obtained after 5 days of fermentation (data not shown). The results presented here emphasize both the actinorhodin-producing potential of the strain as well as the necessity to optimize cultivation conditions. The choice of growth-limiting nutrient and nitrogen sources is highly critical, while the concentrations of other ions in the medium have to be varied to obtain high-yield conditions.

There are several reports concerning the morphology of *Streptomyces* in liquid culture [4,9,12,18]. Most of these studies have been on the shake flask level. In contrast, this report contributes results from carefully controlled experiments in fermenters, where continuous recording of the CO_2 production has given new information on the growth patterns of *Streptomyces* in liquid culture. The morphological status of the inoculum influenced the growth profile, while the actinorhodin yield and production rates were at the same level. The growth profile of RpdS 102 is a function of inoculum quality (morphology) and quantity (the number of growth kernels) and the growth-supporting potential of the medium defined as cell mass yield and not the specific growth rate the medium is supporting. An inoculum of a certain quantity and morphology can grow only up to a certain DW before a transition process has to take place so that the rest, if any, of the medium growth potential can be utilized. The cell mass concentration increased during this period, possibly by accumulation of storage compounds such as glycogen.

Phosphate limitation is much more favorable than nitrogen limitation for actinorhodin production in RpdS 102 (Table 2). This is contrary to the findings of Doull and Vining [10], who studied actinorhodin production in *S. coelicolor*, and reported that nitrogen limitation stimulated actinorhodin synthesis most. A comparison between the experiments is difficult to draw since this work was done with *S. lividans*. The introduction of multicopy plasmids may also change the effects of nutritional factors. There was no production of organic by-products when RpdS 102 cells were grown under reference conditions (AMM — 10 mM phosphate limitations). The carbon is therefore converted only to two products in the production phase for

RpdS 102: actinorhodin and CO_2. Carbon recovery was usually in the range of 85–95% indicating that most of the carbon can be accounted for.

It is well known that *Streptomyces* spp. produce organic acids. Pyruvic acid and α-ketoglutaric acid are the most usual [2,15,19,24]. Both Madden *et al* [19] and Ahmed *et al* [2] reported that both the composition and quantity of the acid excretion depended upon nitrogen source, but it was not reported if the cultures were nitrogen or phosphate limited. The production of α-ketoglutaric acid was high for nitrogen limited RpdS 102 (almost 45% of the carbon was converted to α-ketoglutaric acid (Table 2), which is 55% of the maximum theoretical yield). α-Ketoglutaric acid is a central metabolite in the quest for nitrogen. Therefore, the production of α-ketoglutaric acid by nitrogen-limited cultures may reflect the response of the cells to nitrogen starvation. The production of α-ketoglutaric acid may also be a response to a "metabolic imbalance" since carbon sources other than glucose yield significant production of α-ketoglutaric acid (and pyruvic acid) by RpdS 102 even under phosphate limitation [7]. This cannot be explained by the quest for nitrogen, but may be a consequence of a metabolic imbalance — excretion of α-ketoglutaric acid may allow the bacterium to remain in "metabolic balance." RpdS 101 and 105 also produced high amounts of α-ketoglutaric acid under nitrogen-limiting conditions. There seems to be no correlation between decreased production of actinorhodin and increased production of organic acids under nitrogen limitation. Remarkably, more acetyl CoA was used for α-ketoglutaric acid production under conditions of nitrogen limitation than was used for actinorhodin production under conditions of phosphate limitations (acetyl CoA is the only carbon precursor for actinorhodin).

When the production of by-products is high, limiting the glucose supply, forcing the cells to convert relatively more of the substrate to the desired product, might increase the yield. There was no production of by-products in the reference fermentation for RpdS 102. The aim of running glucose-limited cultures in this case would then be to try to influence the relative carbon fluxes between the two products, CO_2 and actinorhodin — maybe there are futile cycles running making the CO_2 evolution rate higher. The only precursor for actinorhodin is acetyl CoA, which can be used for the synthesis of actinorhodin or oxidized in the TCA cycle to CO_2. Table 2 shows that the yield on a substrate basis (Y_{SA}) is the same in the glucose limited culture as in the reference culture with 10 g glucose/l in excess. The flux-split ratio of acetyl CoA for actinorhodin synthesis versus the TCA seems therefore not to be influenced by availability of carbon source. A theoretical analysis of the biosynthesis of actinorhodin on various carbon sources is extensively treated elsewhere [7].

The specific actinorhodin production rate for the high magnesium culture was the same as the reference (Table 3), but a significantly increased specific glucose consumption rate resulted in a lower actinorhodin yield. The flux-split ratio at the acetyl CoA node is therefore influenced by the magnesium concentration with all extra consumed glucose being oxidized to CO_2 in the TCA cycle implying a relatively higher carbon flux to the latter. Increased activity of the oxidative part of the pentose phosphate pathway might also explain these results, but this seems unlikely since in stationary phase there would be no demand for pentose phosphates as biomass constituents. In any event, a high concentration of magnesium is not desirable in actinorhodin fermentations. The level of calcium is also important. A 10-fold increase resulted in a completely different growth profile and 70% higher DW (Figure 5). This suggested calcium limitation in addition to the phosphate limitation. However, an additional experiment with limiting calcium concentrations showed that the concentration of calcium in the reference medium should support over 30 g DW/l, assuming direct proportionality to DW obtained in the calcium-limited study (data not shown). Abbas and Edwards [1] also reported increased biomass yield and reduced actinorhodin production at high calcium concentrations. This is in agreement with the results presented here. Calcium gradients exist in fungal hyphae, with the concentration being highest at the apical tip, and very important for regulation and coordination of cellular processes accompanying hyphal extension [17]. The same may be true for streptomycetes, explaining the marked effect on DW and growth profile by changes in the calcium concentration of the medium.

In summary, cultivation conditions for high-level production of actinorhodin in *S. lividans* have been developed. A basis has been established for further physiological studies of secondary metabolism in a strain where the central metabolic pathways have not been changed, but where a large part of the carbon flux is converted to a desired product.

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