Establishment of a System Suitable for Analysis of Balanced and Unbalanced Growth of *Streptomyces hygroscopicus*

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(Received 18 October 1983; revised 26 April 1984)

A small-scale system was developed in which balanced growth of *Streptomyces hygroscopicus* occurred. Although the balanced growth, verified by corresponding increase of ATP, DNA, RNA, protein and mycelial length, was restricted to a relatively short period of the life cycle, it lasted for at least two doublings. The conditions for balanced growth could be altered by various treatments to induce imbalance. This system could be applied to study regulation in the mycelium under well-defined conditions.

INTRODUCTION

Cultures of unicellular bacteria grow exponentially under unrestricted conditions in a balanced manner, i.e. each component of the cells increases by the same factor (Maaløe & Kjeldgaard, 1966). Analysis of balanced growth and of cellular responses after perturbations (shift up or shift down) have proved to be very useful for physiological studies. The aim of the present investigation was to study the regulation of balanced growth in *Streptomyces hygroscopicus* mycelium, particularly the metabolism and mode of action of nucleoside polyphosphates.

Streptomycetes have a complex life cycle which includes spore germination, development of individual hyphae, formation of substrate mycelium by hyphal elongation and branching, formation of aerial mycelium, and finally sporogenesis (Hopwood *et al.*, 1973; Kalakoutskii & Agre, 1976). The kinetics of germination and growth of *S. hygroscopicus* have been studied under surface (Schuhmann & Bergter, 1976) and submerged (Riesenberg & Bergter, 1979) conditions. After germination, the specific growth rate, determined by rates of apical growth and branching, was constant for several doublings of mycelial length and biomass, although the specific growth rate later declined. Other actinomycetes (Kretschmer, 1978) and fungi (Righelato, 1979) behaved similarly. Growth declined due to mycelial differentiation characterized by different metabolic activities in various mycelial regions such as the apex, branching region and the distal non-branching parts. Experimental results regarding the physiology and growth of streptomycetes are difficult to interpret since they are derived from the whole mycelium and cannot be ascribed to specific mycelial regions. Furthermore, comparisons with unicellular bacteria require caution since such bacteria multiply by complete cell division and do not, therefore, differentiate during ageing.

We have assumed that streptomycete cultures exhibit a restricted period of balanced mycelial growth after germination. In this paper we describe the conditions under which mycelia of *S. hygroscopicus* grow for at least two doublings in a balanced manner. In this well-defined system, mycelial responses after disturbances of balanced growth were reproducible. Our system proved

Abbreviations: α MG, methyl α -D-glucopyranoside; SHX, DL-serine hydroxamate.

to be useful for investigation of carbon source shift down and amino acid starvation in *S. hygroscopicus* (Riesenberger *et al.*, 1984).

METHODS

Strain. *Streptomyces hygroscopicus* IMET JA 6599 (wild-type) from the culture collection of the Central Institute of Microbiology and Experimental Therapy (Jena) was obtained from Dr H. Prauser.

Media. Shift down medium (Tris/GSD medium) contained the following components per litre: 4.7 g NaCl, 1.1 g Na₂SO₄, 41 mg MgCl₂·6H₂O, 5.4 mg FeCl₃, 4 mg MnCl₂, 20 mg CaCl₂, 20.4 mg KH₂PO₄, 118 mg NH₄Cl, 0.4 g glucose and 4.8 g Tris (Serva, FRG). Shift down medium (MOPS/GSD medium) buffered with 8.3 g MOPS l⁻¹ (Sigma) had the same composition as the Tris/GSD medium but without Tris. For determination of substrate yield coefficients and for optimization of growth, the concentrations were varied as indicated in the text. All media were adjusted to pH 7.0 and sterilized by filtration.

Growth conditions. Rapid spore germination and subsequent growth without clumping for at least five doublings was achieved by incubating *S. hygroscopicus* at 28 °C in a glass tube (13 mm internal diameter) containing 5 ml Tris/GSD medium or MOPS/GSD medium. For aeration and mixing, air was bubbled through a hypodermic stainless steel needle (150 × 120, MLW Injecta, Klingenthal, GDR). A spore suspension pretreated for 10 min at 50 °C served as inoculum to give a final spore titre of 3×10^7 spores ml⁻¹. Growth could be followed without sampling simply by placing the glass tube in the TR accessory of a Spekol spectrophotometer (VEB, Carl Zeiss Jena, GDR) and then measuring the intensity of the light (470 nm) scattered by the culture at an angle of 45° (*I*₄₅).

Preparation of spores. Freeze-dried spores were spread on agar slants (AL53) which had the following composition per litre: 3 g sucrose, 15 g dextrin, 1 g urea, 5 g NaCl, 0.5 g KH₂PO₄, 1 g yeast extract, 5 g peptone, 10 mg FeSO₄, adjusted to pH 6.9 prior to sterilization at 120 °C for 35 min. After 10 to 14 d at 28 °C, sporulated aerial mycelia served as the inoculum for new agar slant cultures. Fourteen days later these cultures were used for the preparation of spore suspensions. Spores were collected by rolling glass beads over the aerial mycelia. After adding Tris/GSD medium or MOPS/GSD medium to the spore-coated beads and shaking to remove the spores from the beads, the suspension was filtered (Por-40-filter, VEB Schott & Gen., Jena, GDR) to separate spores from hyphal fragments.

Determination of dry weight. Mycelia from four 5 ml cultures were filtered through membrane filters (Sartorius SM1306, 0.45 µm). The filters containing the mycelia were dried in a desiccator and weighed.

Morphological analysis. Microphotography and determination of hyphal length has been described previously (Riesenberger & Bergter, 1979).

Radioactive labelling. To determine accumulation of DNA, RNA, protein and ATP in growing mycelia, L-[¹⁴C]leucine (194 MBq mmol⁻¹ ml⁻¹), [5-³H]uridine (3.6 GBq mmol⁻¹ ml⁻¹), [³H]thymidine-6 (45 GBq mmol⁻¹ ml⁻¹) (all from the Isotope Institute Prague, CSSR) and KH₂³²PO₄ (185 MBq ml⁻¹, carrier-free) (Isocommerz, Berlin, GDR) were added to separate cultures growing in Tris/GSD medium. Sampling and further processing of the samples was as described by Riesenberger & Kari (1981).

Statistical analysis. Regression analyses were calculated and tested for significance according to Sachs (1972). Tests of normality were done according to Davies (1971). A significance level of 5% was applied.

RESULTS AND DISCUSSION

Optimization of culture conditions for balanced growth

Culture device. Preliminary experiments were carried out with spore inocula in Tris/GSD medium and MOPS/GSD medium in different culture vessels.

No satisfactory results were obtained in reciprocally shaken cylindrical glass vials with different volumes. Some vials had a side arm cuvette for direct measurement of the optical density of the culture. In all cases mycelial clumping occurred independently of shaking frequency. I. Zimmermann (personal communication) found that the presence of a bottom layer of agar, or addition of liquid agar to the medium resulted in a very loose filamentous growth of *Streptomyces levoris*. For *S. hygroscopicus* neither a layer of agar at the bottom of the glass vials nor addition of liquid agar at different final concentrations prevented mycelial clumping. Clumping was greatly reduced but not prevented by incubating the mycelium in conical flasks of different volumes, which were rotated in a New Brunswick gyrotory water bath shaker at speed mark 3. Clumping of the mycelium could be prevented for at least five doublings of biomass when spores were germinated and the growing hyphae were incubated in simple glass tubes. Growth was measured by light scattering, as described in Methods.

Influence of the concentrations of medium buffers on mycelial growth. Microscopic observations

revealed a positive correlation between the concentration of Tris and the degree of mycelial clumping. Growth of *S. hygroscopicus* lowered the pH of the medium. A concentration of Tris of 4.8 g l^{-1} was the lowest which still allowed growth without noticeable clumping and which buffered the medium efficiently for at least four doublings of biomass after germination. No mycelial clumping was observed in media containing the buffer MOPS at a final concentration of 8.3 g l^{-1} .

Influence of calcium ions and heat treatment of spore suspension on germination and growth. Hirsch & Ensign (1976*a*) found a heat activation of *Streptomyces viridochromogenes* spores and a positive effect of calcium ions on germination. The separate influences of calcium ions ($20 \text{ mg CaCl}_2 \text{ l}^{-1}$, according to Hirsch & Ensign, 1976*a*) and of heat treatment of the spore suspension (10 min at 50°C) on germination of *S. hygroscopicus* spores and on subsequent growth in Tris/GSD medium and MOPS/GSD medium were determined. Both heat treatment and calcium ions shortened the duration of germination and increased the growth rate of the germinated mycelia. A combination of the two treatments gave the optimum result, i.e. the shortest germination time and the highest specific growth rate (data not shown). Therefore, subsequent experiments were performed in calcium-supplemented media and the spore suspension was always heat treated.

Germination and growth of S. hygroscopicus

Fig. 1 shows germination of a heat-treated spore suspension in Tris/GSD medium. A similar germination pattern was obtained for MOPS/GSD medium. The time of appearance of the first germings depended on the age of the agar slant as well as on inoculum size; in the experiment shown in Fig. 1 it was 8 h (variation: 7 to 12 h). Spores did not germinate synchronously as the period of germ tube formation lasted 3 h. Germination was not associated with a temporary decrease of intensity of scattered light, as was found for absorption of light (Attwell & Cross, 1973; Hirsch & Ensign, 1976*b*; Hardisson *et al.*, 1978; Riesenberger & Bergter, 1979).

To show that measurement of intensity of scattered light (I_{45°) corresponded to actual mycelial growth, the mean hyphal length (L) of 30 to 60 individual mycelia was determined at different times of cultivation. Statistical comparison of the slopes of regression lines of I_{45° for $11 < t < 15 \text{ h}$ and of L for $10 < t < 15 \text{ h}$ revealed no difference at a significance level of 5%. The strong positive correlation between I_{45° and L was also shown by the high correlation coefficient ($R = 0.9996$) from regression analysis. Therefore, measurement of I_{45° could be used as a measure of mycelial growth under our experimental conditions. However, this was valid only in the phase of exponential increase of I_{45° and until L was $70 \mu\text{m}$.

The growth of *S. hygroscopicus* and incorporation of $\text{KH}_2^{32}\text{PO}_4$, $[^3\text{H}]$ thymidine, $[^3\text{H}]$ uridine and $[^3\text{H}]$ leucine into ATP, DNA, RNA and protein, respectively, are shown in Fig. 2. After about two doublings of I_{45° , radioactivity was incorporated into ATP, DNA, RNA and protein at the same rate. This was verified by statistical analysis at the 5% significance level of the slopes of regression lines for ATP, DNA, RNA, protein and intensity of scattered light, in the cultivation period between 13.5 and 17 h. In this restricted period we considered the growth to be balanced in the sense of Maaløe & Kjeldgaard (1966).

The culture conditions under which *S. hygroscopicus* grew in a balanced manner had to be strictly maintained, otherwise mycelial clumping prevented balanced growth. This was especially important for the cultivation device, although we cannot explain why mycelial clumping occurred in reciprocally shaken cylindrical glass vials, but could be avoided by using glass tubes with aeration.

Disturbance of balanced growth

The response to perturbations in growth conditions by bacteria growing in a balanced manner has been used to investigate regulatory processes. Growth can be perturbed by exhaustion of a substrate in the culture medium. Therefore, we determined the yield coefficients of *S. hygroscopicus* for carbon and nitrogen sources, and investigated the action of certain substrate analogues, e.g. methyl α -D-glucopyranoside (α -methylglucoside, α MG) and DL-serine hydroxamate (SHX) on growth.

Fig. 1

Fig. 2

Fig. 1. Germination of *S. hygroscopicus* spores. ▲, Germinated spores (%); ●, intensity of scattered light; ■, mean length of mycelia, with confidence intervals based on significance level of 5%. Initial values: mean hyphal length, 2.3 μ m; intensity of scattered light, 9 units.

Fig. 2. Growth of *S. hygroscopicus*. Four glass tubes containing Tris/GSD medium were inoculated with samples of a common prepared spore suspension and subsequently treated as described under Methods. Growth of each culture was measured by intensity of scattered light (○). pH was measured (▲). At $t = 9$ h, $\text{KH}_2^{32}\text{PO}_4$, [^3H]uridine, [^{14}C]leucine and [^3H]thymidine were added separately to the glass tubes and incorporation was followed into ATP (●), RNA (□), protein (■) and DNA (△). For specific activities of labelling see Methods. Values are given in a semilogarithmic scale and are displaced for clarity. Initial values were: ATP, 139 pmol ml^{-1} ; RNA, 40 c.p.m.; protein, 124 c.p.m.; DNA, 97 c.p.m.; intensity of scattered light, 8 units. The curves during balanced growth represent the calculated regression lines.

Determination of yield coefficients of glucose, ammonium and phosphate. Yield coefficients (Y) were calculated from the amount of substrate used $\Delta S (\text{g l}^{-1})$ to give an increase in cell mass, $\Delta X (\text{g l}^{-1})$, according to $Y = \Delta X \Delta S^{-1}$. These yield coefficients, $Y_{\text{glucose}} = 0.45$ and $Y_{\text{NH}_4} = 5.8$, for *S. hygroscopicus* were in very good agreement with those of *Escherichia coli* (Pirt, 1975). To enable an efficient labelling of cellular components with ^{32}P , we also determined the yield coefficient of phosphorus, $Y_p \approx 60$, but this value was not reproducible.

Treatment with SHX. After addition of SHX at a concentration of 2.5 mg ml^{-1} to an exponentially growing culture of *S. hygroscopicus*, further growth was prevented.

Treatment with αMG . In *E. coli* αMG was taken up from the medium by the phosphotransferase system to form αMG 6-phosphate. It was not metabolized further (Kessler & Rickenberg, 1963). The decreased uptake of glucose after addition of αMG created the carbon source shift down. αMG was also active in *Klebsiella pneumoniae* (Riesenberg & Kari, 1981). In *S. hygroscopicus*, αMG resulted in reduction of specific growth rate (Fig. 3). A negative correlation was found between the concentration of αMG and the specific growth rate, μ , until 2 h after the shift:

$$\mu = (\log_2 I_{45^\circ, t_{i+1}} - \log_2 I_{45^\circ, t_i}) (t_{i+1} - t_i)^{-1} \ln 2$$

(see upper insert of Fig. 3). Therefore, we concluded that αMG competed with glucose. The post-shift specific growth rates of cultures treated with αMG at low concentrations ($< 3.2\%$)

Fig. 3. Growth of *S. hygrosopicus* before and after treatment with α MG at different concentrations. Δ , Control (i.e. no addition of α MG); $\blacktriangle$, 0.8%; $\diamond$, 1.6%; $\blacklozenge$, 2.4%; $\circ$, 3.2%; $\bullet$, 4%; $\square$, 6%; $\blacksquare$, 8%. The inset shows the dependence of the specific growth rate, μ , on the concentration of α MG. μ was calculated on the basis of regression lines in the time interval $0 < t < 2$ h.

were lower than the pre-shift specific growth rate and remained constant over two doublings. In cultures treated with α MG at high concentrations ($> 3.2\%$), the culture grew, after a lag period, with a post-shift specific growth rate comparable with μ of the control culture. Probably, α MG at high concentrations triggered the synthesis of an enzyme splitting glucose from α MG. Such an enzyme was found in bacilli by Harris & Miller (1976).

The system developed, suitable for analysis of balanced and unbalanced growth of *S. hygrosopicus*, has been successfully applied for studying the effect of α MG as well as the effect of SHX on nucleoside polyphosphate pools, RNA and protein accumulation in this mycelium forming bacterium (Riesenberg *et al.*, 1984).

The technical help of Mrs Jutta Günther is gratefully acknowledged.

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