Correlation between carbon flux through the pentose phosphate pathway and production of the antibiotic methylenomycin in *Streptomyces coelicolor* A3(2)

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Radiorespirometry was employed to study carbon metabolism during the growth of *Streptomyces coelicolor* A3(2) in a minimal medium which permitted the production of methylenomycin as the sole detectable secondary metabolite. A switch in the pattern of carbon metabolism from the Embden–Myerhof–Parnas pathway to the pentose phosphate pathway occurred during the period of slower growth in batch culture which immediately preceded entry into the stationary phase. This coincided with the period of methylenomycin production. It is proposed that the biosynthesis of methylenomycin is supported by the generation of NADPH during the latter part of growth.

Keywords: secondary metabolism, pentose phosphate pathway, methylenomycin, *Streptomyces coelicolor* A3(2), radiorespirometry

INTRODUCTION

Streptomycetes are filamentous soil bacteria whose life cycle involves the formation of a prostrate mycelium which, following the cessation of vegetative growth, produces aerial hyphae and spores. This period of morphological differentiation is usually accompanied by a change in metabolism toward the synthesis of secondary products which include biologically active molecules, such as antibiotics and herbicides. Although the synthesis of antibiotics and other secondary metabolites is not linked to growth (by definition; Demain, 1992), it may occur in either the growth or the stationary phase of batch culture (Hobbs *et al.*, 1990). The particular growth phase with which secondary product formation is associated depends, to a large extent, upon the nutritional environment in which the producing organism is grown.

Streptomyces coelicolor A3(2) has been adopted as a model system in which it has been possible to identify clusters of genes responsible for the synthesis of, and resistance to, antibiotics (Narva & Feitelson, 1990; Malpartida *et al.*, 1990; Malpartida & Hopwood, 1984, 1986). A number of genes which regulate both physiological differentiation (antibiotic production) and morphological differentiation

(sporulation) have also been defined (Hopwood, 1988). In spite of these achievements, our understanding of the precise physiological changes which trigger secondary product formation, as well as the mechanisms by which metabolism is redirected towards this end, are still rudimentary. Although this species is not the most amenable of subjects for physiological research, systems have been developed which facilitate such experiments (Hobbs *et al.*, 1989) and permit the integration of the molecular genetical and physiological approaches to the study of secondary metabolism (Hobbs *et al.*, 1992).

Methylenomycin is representative of a class of antibiotics possessing cyclopentanone nuclei, of which several have been isolated (McGahren *et al.*, 1969; Umino *et al.*, 1973; Noble *et al.*, 1978). It is derived, principally, from acetyl-CoA and pyruvate (Hornemann & Hopwood, 1981). In *S. coelicolor* A3(2) grown on a minimal medium containing alanine as the sole nitrogen source, glucose as the principal carbon source and high levels of phosphate, methylenomycin is the only secondary product formed and it accumulates during the period of slow growth which marks the transition between the exponential and stationary phases in batch culture (Hobbs *et al.*, 1992). Both pyruvate and 2-oxoglutarate are produced during the acidogenic exponential growth phase of these cultures. The period of methylenomycin production precedes glucose exhaustion, but coincides with the apparent re-absorption of pyruvate from the medium.

Abbreviations: EMP, Embden–Meyerhof–Parnas; PP, pentose phosphate; TCA, tricarboxylic acid.

Fig. 1. Rationale of experiments. The ratios investigated in this work were obtained by considering the degree to which the C1, C3,4 and C6 carbon atoms of glucose were released from the respective metabolic pathways in the form of CO_2 . According to the simplified model presented here, C1 CO_2 is released both from the oxidative branch of the PP pathway and from the oxidative processes of the tricarboxylic acid (TCA) cycle. However, as C1 and C6 CO_2 are released to the same extent from the TCA cycle (due to the equilibration reaction at the level of triose phosphate isomerase in the EMP pathway), the amount of CO_2 released from the oxidative branch of the PP pathway is obtained as the value C1-C6 CO_2 . Flux through the TCA cycle is represented by the value C6 CO_2 , while flux through the EMP pathway (CO_2 released by oxidation via pyruvate dehydrogenase) is represented by C3,4 CO_2 .

In *S. coelicolor*, little is known about carbon metabolism and how this might alter during the period of antibiotic production. In *Streptomyces aureofaciens*, DiMarco (1956; quoted in Cochrane, 1961) has suggested that the balance of the Embden-Myerhof-Parnas (EMP) and pentose phosphate (PP) pathways is affected by the phosphate concentration in the medium. He found that the initial phase of growth was dominated by the EMP pathway, while a second phase (during which the medium phosphate concentration was depleted) was typified by an active PP pathway. Chlortetracycline was produced by *S. aureofaciens* during this second phase of growth. Penzikova & Levitov (1966) have also suggested that the rate of antibiotic biosynthesis in *Streptomyces (Actinomyces) fradiae* was dependent, to a large extent, upon the nature of flux through the pathways by which carbohydrates are degraded, and that the rate of antibiotic synthesis is regulated by carbon metabolism. Primary metabolism and antibiotic production have been correlated in a number of other streptomycetes (Hostalek, 1969; Vorisek *et al.*, 1969; Thiericke & Zeeck, 1988; Dekleva & Strohl, 1988a, b; Canovas & Kornberg, 1965). Particularly relevant is the discovery by Ikeda *et al.* (1988) that the activity of 6-phosphogluconate dehydrogenase of the PP pathway was correlated with avermectin production in *Streptomyces avermitilis*. These investigators emphasized the importance of this enzyme in providing NADPH for avermectin biosynthesis.

In this study we report on the balance between the EMP and PP pathways of carbon catabolism in *S. coelicolor*

A3(2) during batch and continuous culture, using an adaptation of the radiorespirometric methodology of Wang *et al.* (1958). The rationale governing this approach is explained in Fig. 1.

METHODS

Bacterial strain. *S. coelicolor* A3(2) strain 1147 was used throughout the project.

Culture conditions. All experiments were carried out using Hobbs' minimal medium (HMM; Hobbs *et al.*, 1989), adapted for the exclusive synthesis of methylenomycin by the substitution of alanine for nitrate as the sole source of nitrogen (Hobbs *et al.*, 1992). Methods for inoculum preparation and fermentation conditions in small shake flasks and 2 l bioreactors were as described by Hobbs *et al.* (1989, 1992).

Sampling procedures. Samples (1 ml) were withdrawn from shake flasks or fermenters and the OD_{550} was determined. Further aliquots (1 ml) were centrifuged in a microcentrifuge, after which the supernatant and the cell pellet were separated and both stored at -20°C .

Radiorespirometric analysis. The metabolism of exogenously supplied glucose was monitored using the following radiorespirometric technique. Duplicate aliquots (1 ml) were withdrawn from the same 250 ml conical flask as used for biomass and supernatant characterization, and placed in a 15 ml capacity glass vial (Aldrich) which contained the radioactive metabolite (C1- and C6-labelled glucose, Amersham; C3,4-labelled glucose, Dupont NEN Research). The final vial concentration of C3,4-labelled glucose was 0.0019 mM and its specific activity was $10.3 \text{ mCi mmol}^{-1}$ ($381 \text{ MBq mmol}^{-1}$). The respective values for C1- and C6-labelled glucose were 0.0036 mM and $55.8 \text{ mCi mmol}^{-1}$ ($2064 \text{ MBq mmol}^{-1}$). The vial was sealed with a rubber stopper (Fisons Scientific Apparatus) through which was suspended a Konte centre well (Burkard Scientific) containing a filter paper plug. Vials were incubated at 30°C for 40 min in a shaking water bath (Infors). Incubation was terminated by injecting 150 μl of 1 M HCl through the rubber cap into the culture. The CO_2 released was trapped by injecting 300 μl of a 1:1 mixture of β -phenylethylamine/methanol into the Konte well. Trapping was conducted for 1 h. The incubation and trapping time, as well as the amounts of trapping agent and HCl required, were established in separate experiments (Obanye, 1994). After the CO_2 had been trapped, the Konte centre well and filter paper were transferred to 4 ml scintillation cocktail (Optiphase 'Hi-Safe' 3; Wallac) in a plastic scintillation vial (Sterilin). This was subsequently analysed using an LKB Wallac 1216 Rackbeta II liquid scintillation counter. The window settings were 50–165 and the counts were corrected to d.p.m. using the external standard channels ratio method (Herberg, 1963). CCl_4 was used as the quenching agent. The accuracy of counting lay within $\pm 5\%$.

Metabolic assays. Concentrations of organic acids, glucose and methylenomycin in the culture supernatants were measured as described previously (Hobbs *et al.*, 1992).

RESULTS

Variation of radiorespirometric ratios during batch growth

In batch culture, during the exponential phase of growth, there was a fall in the C1-C6/C3,4 ratio (i.e. [flux through the PP pathway]/[flux through the EMP pathway]) to a

Fig. 2. Radiorespirometric analysis of C-metabolism in batch culture. Biomass (■) and methylenomycin (○) concentrations and the radiorespirometric ratios: PP/EMP (△) and EMP/TCA (▲). Values shown are the mean of two measurements; the bars indicate the range of values.

Table 1. Minimum and maximum values of PP/EMP and EMP/TCA

Ratio*	Expt ...	1	2	3	4	5	6	7	Mean (SD)
A = Min. [PP/EMP]		0.26	0.19	0.16	0.14	0.35	0.33	0.20	0.23 (0.08)
B = Max. [PP/EMP]		0.59	0.93	1.64	0.74	1.02	1.74	1.13	1.11 (0.43)
C = Min. [EMP/TCA]		3.07	2.73	2.12	2.97	2.81	2.86	2.91	2.78 (0.31)
D = Max. [EMP/TCA]		3.91	3.43	4.42	3.92	3.93	3.64	5.62	4.12 (0.73)
B/A		2.27	4.98	10.12	5.29	2.91	5.27	5.65	5.21 (2.53)
D/C		1.27	1.26	2.08	1.32	1.37	1.27	1.93	1.50 (0.35)

* A is the minimum value that the PP/EMP ratio attains during exponential growth. B is the maximum value that the PP/EMP ratio attains just prior to, or during, the production of methylenomycin. C is the minimum value of the EMP/TCA ratio attained prior to the production of methylenomycin. D is the maximum value of the EMP/TCA ratio attained at or around the onset of methylenomycin production.

minimum value at the mid-exponential phase (Fig. 2). The value of this ratio at its *minimum* varied from 0.14 to 0.35 (Table 1). After this point, this ratio rose again to achieve a maximum at or around the point at which growth slowed and methylenomycin production was initiated. During this slow growth phase, the PP/EMP ratio remained elevated. The value of the maximum during the latter stages of growth varied from between about 0.6 and 1.74 in six replicate experiments.

From these results, it is apparent that the carbon flux through the PP cycle was less than that passing through the EMP pathway during the exponential growth phase. When the specific growth rate had slowed, and methylenomycin was being produced, relative flux through the PP pathway increased and, in some experiments, exceeded that through the EMP pathway. However, the variation observed between replicate experiments suggests that the data provide only a qualitative picture of the balance of carbon flux between the two pathways.

With regard to the specific rates of production of C1-C6 CO₂ (PP flux; mmol per OD₅₅₀ unit min⁻¹) and of C6 CO₂ (TCA flux; mmol per OD₅₅₀ unit min⁻¹), the increases in the PP/EMP ratio during the production of methyleno-

mycin appeared to be associated predominantly with a real increase in the PP flux rather than a fall in the TCA flux (Table 2). The other important metabolic parameter was the EMP/TCA ratio, which was also seen to fall during the exponential phase, reaching a minimum value at or around the time that the PP/EMP ratio attained its maximum (Fig. 2). The value of the EMP/TCA ratio, at its minimum, varied between 2.12 and 2.97 in six replicate experiments (Table 1). Thereafter, the ratio rose again during the production phase, attaining a maximum of between 3.64 and 5.62 (Table 1). Thus not only was there a real increase in the specific rate of C1-C6 CO₂ production (mmol per OD₅₅₀ unit min⁻¹), representing a real increase in PP flux, but there was also an increase in the specific rate of C3,4 CO₂ production (flux through pyruvate dehydrogenase; mmol per OD₅₅₀ unit min⁻¹; Fig. 2). This latter observation is not shown in Table 2, which simply compares the two time points immediately before the onset of methylenomycin production with those immediately after this point.

The highest values of the PP/EMP ratio were found during the periods of slowest growth; however experiments using continuous-flow cultures (data not shown)

Table 2. Mean rate of CO₂ release over the two time points subsequent to the onset of methylenomycin production relative to the two time points prior to methylenomycin production, from six independent experiments

Expt	Change in mean rate of CO ₂ release (%)*		
	C1-C6 CO ₂	C6 CO ₂	C3,4 CO ₂
1	152 (4.7 × 10 ⁻³)	229 (2.1 × 10 ⁻³)	203 (6.3 × 10 ⁻³)
2	115 (2.7 × 10 ⁻³)	94 (9.7 × 10 ⁻⁴)	96 (3.3 × 10 ⁻³)
3	224 (1.2 × 10 ⁻³)	101 (2.8 × 10 ⁻³)	99 (9.1 × 10 ⁻³)
4	375 (4.7 × 10 ⁻³)	79 (1.0 × 10 ⁻³)	123 (5.5 × 10 ⁻³)
5	266 (3.6 × 10 ⁻³)	125 (1.2 × 10 ⁻³)	67 (4.0 × 10 ⁻³)
6	133 (4.0 × 10 ⁻³)	125 (2.5 × 10 ⁻³)	142 (9.2 × 10 ⁻³)
Mean	210 (3.5 × 10 ⁻³)	126 (1.8 × 10 ⁻³)	122 (6.2 × 10 ⁻³)
(n = 6)			
SD	99 (1.3 × 10 ⁻³)	49 (8.1 × 10 ⁻⁴)	47 (2.5 × 10 ⁻³)
(n - 1)			

* The quantitative value of the mean rate of CO₂ released (mmol per OD₅₅₀ unit min⁻¹) in each case is shown in parentheses.

established that specific growth rate itself was not responsible for the variations in both ratios.

DISCUSSION

The use of radiorespirometry demonstrated that significant changes in carbon catabolism occurred around the time at which methylenomycin was produced. In this work, this was observed as the result of ordinary batch growth processes. A similar switch in glucose metabolism was seen as a consequence of artificially lowering the medium pH in a chemostat (Obanye, 1994). The most important change observed was a significant increase in the flux through the oxidative limb of the pentose phosphate pathway.

All phases of growth were associated with the presence of organic acids in the culture medium (Hobbs *et al.*, 1992). Organic acid production by micro-organisms is a common phenomenon. 2-Oxoglutarate and pyruvate are the keto-acids most commonly reported to be produced by streptomycetes (Ahmed *et al.*, 1984). In our own experiments, it was during the slow transition phase that the medium concentration of 2-oxoglutarate stabilized while that of pyruvate declined. The latter event suggested active reassimilation of pyruvate and, possibly, the utilization of this organic acid for incorporation into methylenomycin (Hornemann & Hopwood, 1981). Pyruvate is also a gluconeogenic substrate. Such changes in acidogenesis intimate either metabolic or transport changes occurring during the change in the growth phase. Although transport studies were not carried out, it is likely that the excretion of organic acids was dependent on a combination of transport and metabolic factors.

Our radiorespirometric results suggest that there may be a relationship between the regulation of methylenomycin production and the carbon catabolic changes that occur

throughout the latter part of the growth cycle of *S. coelicolor* A3(2). This relationship is demonstrated by Table 2, in which the mean values of the specific rates of CO₂ released from the PP pathway, the TCA cycle, and the EMP pathway immediately after the onset of methylenomycin production are compared with those recorded just prior to the production of methylenomycin, for six independent experiments. The production phase is accompanied by an increased rate of glucose catabolism in all cases, the greatest effect being seen for CO₂ released via the PP pathway. We suggest that the relationship between the regulation of methylenomycin production and the carbon catabolic changes observed at or around the start of the the production phase involves the utilization of NADPH, generated by the flux through the PP pathway.

Many antibiotics, notably those derived from polyketides, are synthesized reductively and the operation of pathways that generate NADPH is often essential to secondary metabolite production (Ikeda *et al.*, 1988). In all our experiments, the bulk of methylenomycin production occurred after a real elevation in the rate of flux through the PP pathway had taken place. The other important parameter was the EMP/TCA ratio (i.e. the C3,4/C6 ratio). It may be significant that this ratio was seen to rise at approximately the same time that methylenomycin was first detected in the medium, at a point where the increased generation of NADPH was probably crucial, and during a period where the concentration of pyruvate was falling. The tendency for the decrease in the pyruvate concentration to coincide with an increase in the EMP/TCA ratio supports the idea that there was an increase in flux through pyruvate dehydrogenase, perhaps due to the reduction of the NADH/NAD⁺ ratio. Such a reduction might have arisen from the combination of a fall in the extracellular pH and utilization of NADH in a transhydrogenase reaction involved in the generation of NADPH either via the operation of nicotinamide nucleotide transhydrogenase (Yamaguchi & Hatefi, 1991; Yamaguchi *et al.*, 1988; Ahmad *et al.*, 1992) or indirectly via coupled enzyme systems.

It is not known definitively whether a supply of NADPH is necessary for the production of methylenomycin. An increase in the EMP/TCA ratio would be consistent with a flow of carbon into acetyl-CoA in that the flux from the EMP pathway did not seem to be transmitted to the TCA cycle to a proportionate extent. Acetyl-CoA is an important precursor for the synthesis of methylenomycin (Hornemann & Hopwood, 1981) and polyketides alike. In the latter case, it is known that NADPH plays a crucial part in this process (Herbert, 1989).

ACKNOWLEDGEMENTS

This work was supported by a SERC studentship to A.I.C.O. and by grants from SERC and the SERC/DTI/Glaxo Wellcome/SmithKline Beecham Link initiative to S.G.O. and C. P. Smith. We would also like to thank SmithKline Beecham and Glaxo Wellcome for additional financial support to A.I.C.O. We are grateful to Dr Phil Butler for his critical appraisal of the manuscript.

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Received 13 July 1995; revised 11 September 1995; accepted 19 September 1995.