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AMMONIUM REGULATION IN *Saccharopolyspora erythraea.* PART II: REGULATORY EFFECTS UNDER DIFFERENT NUTRITIONAL CONDITIONS.

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Summary

Non-limiting nutritional conditions revealed a growth-associated erythromycin production with ammonium sulfate while the relation was growth-dissociated for ammonium nitrate. Feeding experiments suggested that the residual nitrate level might be the key regulatory element. A nitrate-induced glutamine synthetase pathway was postulated according to the nature and initial concentration of the ammonium salt.

Introduction

A previous paper (see Part I) reported different metabolic behavior for an erythromycin industrial producer based on the ammonium salt fed. Trilli *et al.*, 1987, and McDermott *et al.*, 1993, observed that the nature and the concentration of the medium can reverse the growth/production relationship in the case of erythromycin. Such a relationship (Luedeking and Piret, 1959) was determined for two ammonium salts under non-limiting nutritional conditions with our mutant strain. Shapiro and Vining, 1984, demonstrated that a mixture of ammonium and nitrate affects chloramphenicol production differently. Since ammonium nitrate provoked a distinctive metabolic behavior with our strain, we also intended to study more precisely some physiological parameters under different feeding conditions of this salt.

Ammonium ions are assimilated via the glutamate dehydrogenase to form glutamate (high concentration) or through glutamine synthetase to form glutamine (low concentration) (Shapiro, 1989). Glutamate, as a sole nitrogen source, does not contribute to erythromycin formation in *Saccharopolyspora erythraea* but glutamine is believed to be a precursor in erythromycin biosynthesis (Flores and Sanchez, 1989). Nitrate was shown in some antibiotic-producing *Streptomyces* to derepress or induce glutamine synthetase activity (Shapiro, 1989). Ammonium assimilatory enzymes were investigated (Flores and Sanchez, 1989) in erythromycin biosynthesis. High ammonium concentrations repressed glutamine synthetase and lowered the antibiotic production. Glutamine lowered the glutamine synthetase level but maintained a

high erythromycin yield. We also intended to study more precisely the physiological parameters under different feeding conditions. Based on these observations and our results, a speculative regulation scheme is proposed.

Materials and methods

Strain: *Saccharopolyspora erythraea* P-1060, a mutant strain selected for its erythromycin hyperproductivity, was kindly provided by Ciba-Geigy AG, Basel, Switzerland. The strain was kept lyophilized.

Media: A medium was developed for erythromycin production (Potvin and Péringer, 1993). The medium was sterilized 20 min at 121°C. Inorganic nitrogen sources ($(NH_4)_2SO_4$ and NH_4NO_3) were added as mentioned in each experiment.

Inoculation and culture conditions: All the experiments presented in this paper were performed according to the procedure described in Potvin and Péringer, 1993. Feeding experiments were conducted using concentrated nutrient solutions fed by a peristaltic pump (Lambda, Naters, Switzerland).

Analysis: The biomass and erythromycin concentrations were determined as in Potvin and Péringer, 1993. Ammonium and glucose concentrations were measured by two enzymatic UV methods (Boehringer Mannheim, Mannheim, Germany) and phosphorus by the colorimetric method of Fiske and Subbarow, 1925.

Data: Results are presented as in Potvin and Péringer, 1993, and are very reproducible. The Luedeking and Piret, 1959, relationship was used to establish how antibiotic production was correlated with growth: $Q_{ery} = \alpha\,(\mu) + \beta$, where Q_{ery} is the specific erythromycin production rate, μ is the growth rate, and α and β are coefficients related to growth rate and biomass concentration respectively.

Results

Non-limiting nutrient supply - A set of fermentations was performed to determine whether maximum growth rate could be improved or extended with non-limiting nutrient supply. Under these conditions, erythromycin appearence should be more retarded and its titer reduced for a longer period. The runs were conducted in a repeated fed-batch mode. A concentrated substrate solution was fed continuously and samples, equal to the expansion volume, were taken periodically according to the feeding rate. This quasi steady-state process was also meant to determine a more accurate growth/production relationship.

The antibiotic onset is not retarded more then what we previously observed in the batch trials (Part I) even with large residual substrate concentrations (Figure 1). Erythromycin formation appeared when the growth phase became linear. Figure 1 also shows that non-limiting substrate feeding increases the final biomass concentration to an elevated value: 3 fold for $(NH_4)_2SO_4$ and 1.5 fold for NH_4NO_3, as compared to batches with high levels of the ammonium salts (not shown). Erythromycin final titers are less in both cases, leading to a circa 4 fold reduction in production yield at the time feeding was stopped (16 mg ery/g bio for NH_4NO_3 and 10 mg ery/g bio for $(NH_4)_2SO_4$) compared to high concentrated batch trials (not shown). It should be noted that the nutrient concentrations in solution remained high at all time under the above experimental conditions. Here again the formation of micropellets was observed when the growth rate has reached its maximum value after the short exponential growth phase. The maximum growth rate value (μ_{max} = 0.1 h^{-1}) was therefore not maintained or increased. Figure 2 shows a clear

correlation between biomass growth and antibiotic production during the production phase. Erythromycin production is growth-associated (β=0) with ammonium sulfate but the secondary metabolism activity is reduced, showing an α value of 13.0 (half the batch value from previous work). Ammonium nitrate provides a non-growth associated relationship (α=0). The 0.75 β value is also half reduced as compared to batch experiments.

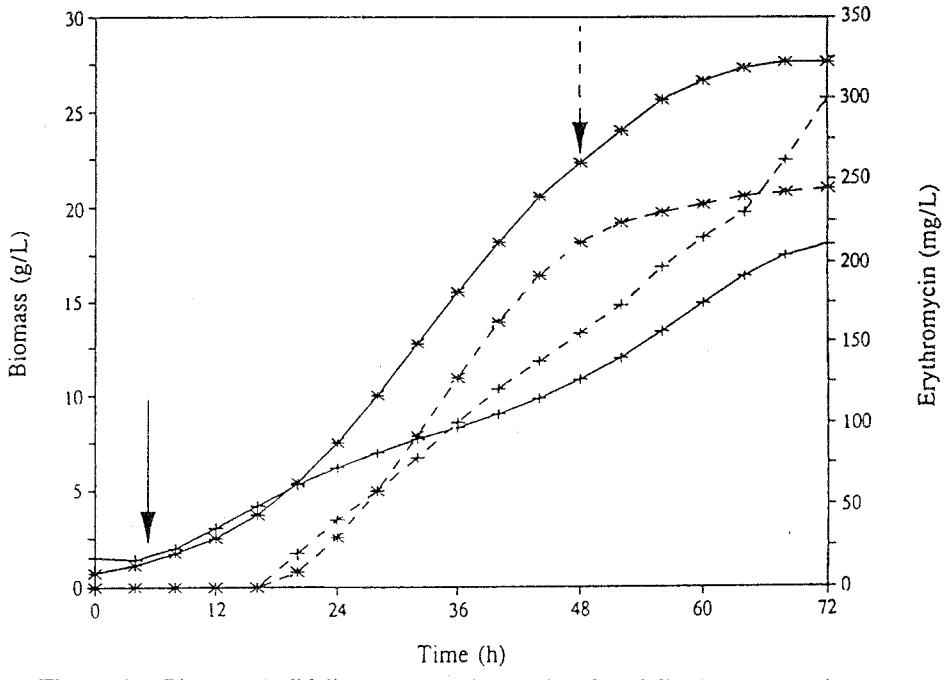

Figure 1: Biomass (*solid lines*) and erythromycin (*dotted lines*) concentrations as a function of time under non-limiting nutrient conditions. [NH_4NO_3 medium (+), $(NH_4)_2SO_4$ medium (*)]. (The solid arrow indicates feed start and the dotted one, feed stop).

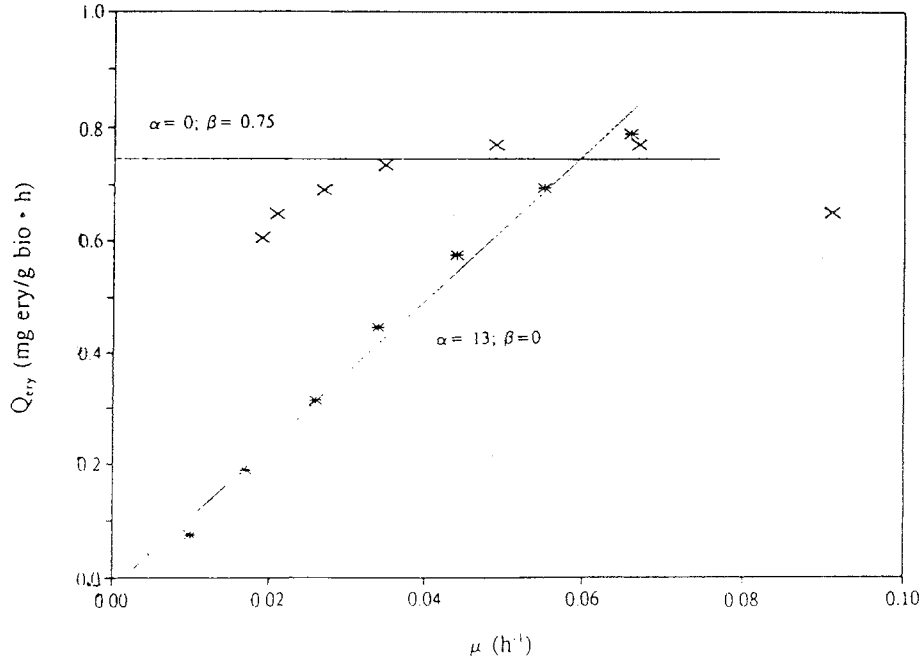

Figure 2: Growth/production relationship under non-limiting nutrient conditions. [NH_4NO_3 medium (x), $(NH_4)_2SO_4$ medium (*)]. (Q_{ery}: specific production; μ: growth).

Ammonium nitrate feeding conditions - The next experiments were conducted to evaluate the importance of the residual ammonium nitrate concentration as well as its feeding regime on both trophophase and idiophase. The residual ammonium level was kept to zero throughout the fermentation for both feeding rates. The final ammonium content added was equivalent to batch runs for comparison purposes.

The high feeding regime produces more biomass than in batch (12.7 g/L versus 6.0 g/L) for the same inorganic nitrogen amount supplemented (0.8 g NH_4^+/L) (Table 1). The maximum growth rate is also improved. A smaller feeding rate does not affect the growth metabolism with respect to final biomass concentration and μ_{max}). On the other hand, the erythromycin final concentration is raised from 335 mg/L to 430 mg/L when 0.2 g NH_4^+/L (ammonium nitrate) is fed at a low rate (versus batch). It doubles the erythromycin maximum specific production rate (Q_{ery} max) under low feeding conditions (Table 1). The antibiotic onset is not delayed more from the growth phase, and the gradual erythromycin increase explains the 30 h maximum Q_{ery} value with low feeding conditions. At 0.8 g NH_4^+/L, the batch trial shows an extended and greater Q_{ery} maximum value versus the high feeding rate.

Table 1: Influence of ammonium nitrate feeding mode and feeding rate on growth and erythromycin production metabolisms of *Saccharopolyspora erythraea* P-1060.

	0.2 g NH_4^+/L		0.8 g NH_4^+/L	
	Batch	Fed/25[a]	Batch	Fed/185[a]
Biomass (g/L)	6.5	6.0	6.0	12.7
Eryhtromycin (mg/L)	335	430	430	430
μ_{max} (h^{-1})	0.07 (12 h)	0.06 (12 h)	0.10 (12 h)	0.15 12 h)
Q_{ery} max (mg ery/g bio · h)	1.8 (18 h)	3.5 (30 h)	3.0 (36 h)	1.6 (18 h)
$Y_{ery/bio}$ (mg ery/g bio)	51.5	71.7	71.7	33.8
$Y_{PO4\text{-}3/bio}$ (g PO_4^{-3}/g bio)	0.08	0.18	0.20	0.09

[a]: feeding regimes are in mg NH_4^+/L · h

From Table 1, a high feeding rate of ammonium nitrate twicely reduces $Y_{ery/bio}$ from 71.7 to 33.8 mg ery/g bio (compare to batch). The low feeding regime increases the production yield to a value of 71.7 mg ery/g bio, identical to the high concentrated batch. Both high erythromycin production yield cases showed a phosphorus consumption yield twice better (0.18-0.20 versus 0.08-0.09 g PO_4^{-3}/g bio).

Discussion

Attempts to increase the maximum growth rate and to extend the exponential growth phase were not successful with our particular strain under non-limiting conditions. The production metabolism is however suppressed to some extend with high concentrated nutritional conditions. Early mycelial pellets formation may have created growth limitations and initiated secondary metabolism as reported by Bader, 1986. It was also suggested (Bushell, 1989) that secondary metabolism is initiated following a nutrient limitation. The pellets could have created diffusional limitations of a nutrient, causing some cells to shift to an early production. The Luedeking and Piret, 1959, relationship clearly indicated different metabolic behaviors between ammonium sulfate and nitrate for our mutant strain during the production phase. The later, showing growth-dissociated production, favored erythromycin production. Such an effect was never reported on either erythromycin or any other macrolide antibiotic production.

Our results (Part I and II) combined with other observations from the literature led us to propose an hypothetical regulatory mechanism based on the biosyntetic precursors formed. High content of ammonium (sulfate salt) was transformed to glutamate via the "low affinity" route (Shapiro, 1989). Erythromycin production was maintained at its basic level and the greater amount of glutamate derived from the ammonium assimilatory pathway enhanced mycelial growth. However, with a high starting content of ammonium nitrate, the erythromycin production metabolism was enhanced but not the biomass concentration. Based on these observations, we hypothesized that the glutamate produced from ammonium can be partly transformed to glutamine, via a nitrate-induced glutamine synthetase (Shapiro, 1989) when ammonium nitrate is fed in larger amount. Glutamine could then be used as a precursor of erythromycin (Flores and Sanchez, 1989), leaving less glutamate available to the primary metabolism. This assumption is suppported by the higher and extended phosphorus uptake rate observed (Part I and Table 1 of this paper) which would provide more ATP needed as a cofactor of the induced glutamine synthetase pathway. The growth/production relationship also became growth-dissociated with ammonium nitrate. It seems that a sufficient residual amount of nitrate is necessary to induce glutamine synthetase; otherwise, nitrate could then become a complementary ammonium source. Table 1 strongly suggest that residual nitrate is the key element in our hypothesis. More details on that proposed mechanism are presented in Potvin, 1992.

The slight increase in erythromycin titer could not account for the lack of biomass produced with ammonium nitrate. One explanation could be that the glutamine (transformed-glutamate) is no longer available for biomass accretion but its high intracellular concentration (or any eryhtromycin precursor) inhibits at the same time one of the biosynthetic enzyme involved in the production of erythromycin. An intracellular inhibition mechanism was already proposed by McDermott *et al.*, 1993, and by Sanchez *et al.*, 1984, in erythromycin biosynthesis. Measurements of the specific activity of the key ammonium assimilatory enzymes will be undertaken under the conditions described above in an attempt to confirme the proposed mechanism (nitrate induction). Experiments combining the two inorganic nitrogen ions

(Shapiro and Vining, 1984) should emphasize the key role of nitrate in regulating erythromycin biosynthesis.

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