Biotechnology Letters **22:** 1803–1809, 2000.

Growth limiting substrate affects antibiotic production and associated metabolic fluxes in *Streptomyces clavuligerus*

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Received 13 July 2000; Revisions requested 16 August 2000; Revisions received 11 September 2000; Accepted 14 September 2000

Key words: chemostat culture, clavulanic acid, fermentation technology, metabolic flux analysis

Abstract

Clavulanic acid biosynthesis by *Streptomyces clavuligerus* was dependent on the identity of the growth rate limiting nutrient in chemostat bioreactor culture ($D = 0.05\ h^{-1}$). In phosphate-limited media, a specific production rate of 3.65 $mg_{clav}\ g_{biomass}\ h^{-1}$ was observed while N-limited media supported 0.32 $mg_{clav}\ g_{biomass}\ h^{-1}$. No production was observed in C-limited media. Metabolic flux analysis suggested that changing the nutrient limitation affected the availability of the C_5 precursor. Flux through anaplerotic metabolism was consistent with this, reflecting the lower rate of utilisation of 2-oxo-glutarate from the tricarboxylic acid (TCA) cycle for glutamate and, ultimately, C_5 precursor production, when antibiotic was not produced. We propose that C-limitation restricts the capacity for anaplerotic metabolism, minimising the potential for extensive TCA-cycle derived biosynthesis (the first stage in production of the C_5 precursor). N-Limitation would restrict the availability of nitrogen for amino acid biosynthesis (the next stage). Under P-limitation neither of these restrictions would apply.

Introduction

Streptomyces clavuligerus produces the clinically-important antibiotic clavulanic acid, an effective inhibitor of β-lactamases (Reading & Cole 1977). Antibiotics, in common with many other bioactive microbial metabolites are classified as secondary metabolites, which are produced after the culture growth rate has decayed during batch culture (e.g., Demain *et al*. 1983). This coincides with the exhaustion of the growth rate limiting substrate (gls) in most culture systems, which led to proposals that the gls is acting as a repressor of antibiotic production during the early stages of the culture. However, our studies have suggested that the relationship between growth rate decay and antibiotic production was dependent on whether glucose or nitrate was the gls (McDermott *et al*. 1993). This work and subsequent investigations (e.g., Clark *et al.* 1995, Lynch & Bushell 1995) led us to conclude that growth rate decay, rather than relief from substrate-induced repression, is responsible for induction of antibiotic production, following the down-regulation of intracellular protein synthesis rate (Wilson & Bushell 1995). The dependence of production dynamics on the identity of gls may be a function of affinity between the organism and the substrates studied (Bushell *et al*. 1997).

We have identified those intermediary metabolites whose production rates are closely linked to antibiotic biosynthesis rate (Ives & Bushell 1997) and used this information to devise culture feeds that resulted in enhanced production of clavulanic acid, probably by promoting an increase in the supply of the C_3 precursor of clavulanic acid. Ornithine is converted to arginine (via a sequence analogous to the mammalian urea cycle) prior to incorporation into clavulanic acid (Valentine *et al.* 1993). The remainder of the clavulanic acid molecule is derived from the C_3 precursor (Stirling & Elson 1979, Townsend & Ho 1985).

In non-industrial strains, the concentrations of antibiotics are low (e.g., Bushell & Fryday 1983), and hence the carbon fluxes through precursor pools

Table 1. Nutrient input and bioproduct output in chemostat cultures of *S. clavuligerus* under different nutrient limitations.

Growth limiting substrate	Biomass production rate ($g\ l^{-1}\ h^{-1}$)	Specific glycerol consumption rate ($g\ g^{-1}\ h^{-1}$)	Specific clavulanic acid production rate ($mg\ g^{-1}\ h^{-1}$)	Specific polysaccharide production rate ($g\ g^{-1}\ h^{-1}$)	Specific CO_2 production rate ($g\ g^{-1}\ h^{-1}$)	Specific O_2 consumption rate ($g\ g^{-1}\ h^{-1}$)
C	0.147	0.27	0	0.003	0.26	0.19
P	0.066	1.25	3.65	0.60	0.69	0.49
N	0.198	0.45	0.32	0.22	0.18	0.13

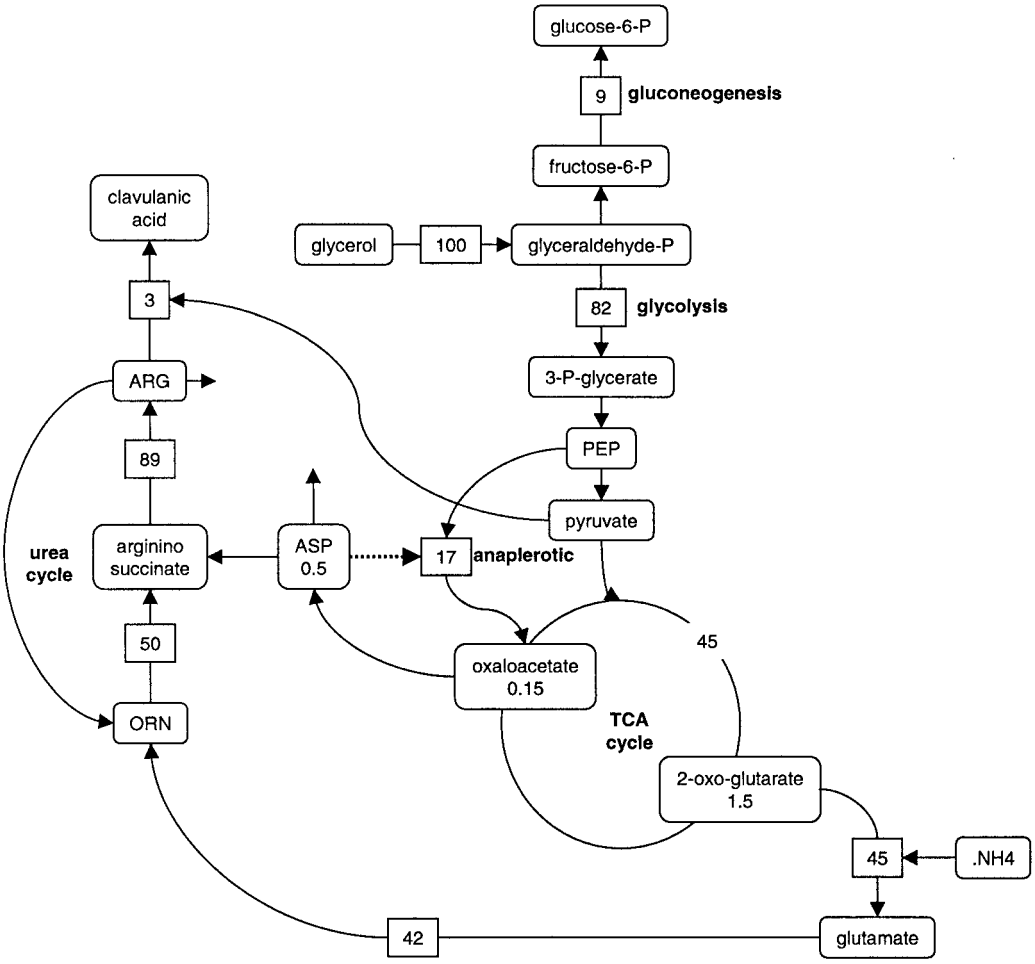

Fig. 1a.

Fig. 1. Bioreaction scheme for clavulanic acid synthesis, in P-limited (a), N-limited (b), and C-limited (c) chemostat cultures of *S. clavuligerus* showing only those reactions discussed in the text. Metabolite fluxes (values in square boxes) are C-mol $l^{-1}\ h^{-1}$. Metabolite pool sizes (values under appropriate metabolites) are $\mu g\ mg^{-1}_{biomass}$. Flux values are normalised to 100 C-$mol_{glycerol\ consumed}\ l^{-1}\ h^{-1}$. The intracellular fluxes were obtained from the solution of the bioreaction network equations, using the observed extracellular metabolite production and consumption rates presented in Table 1.

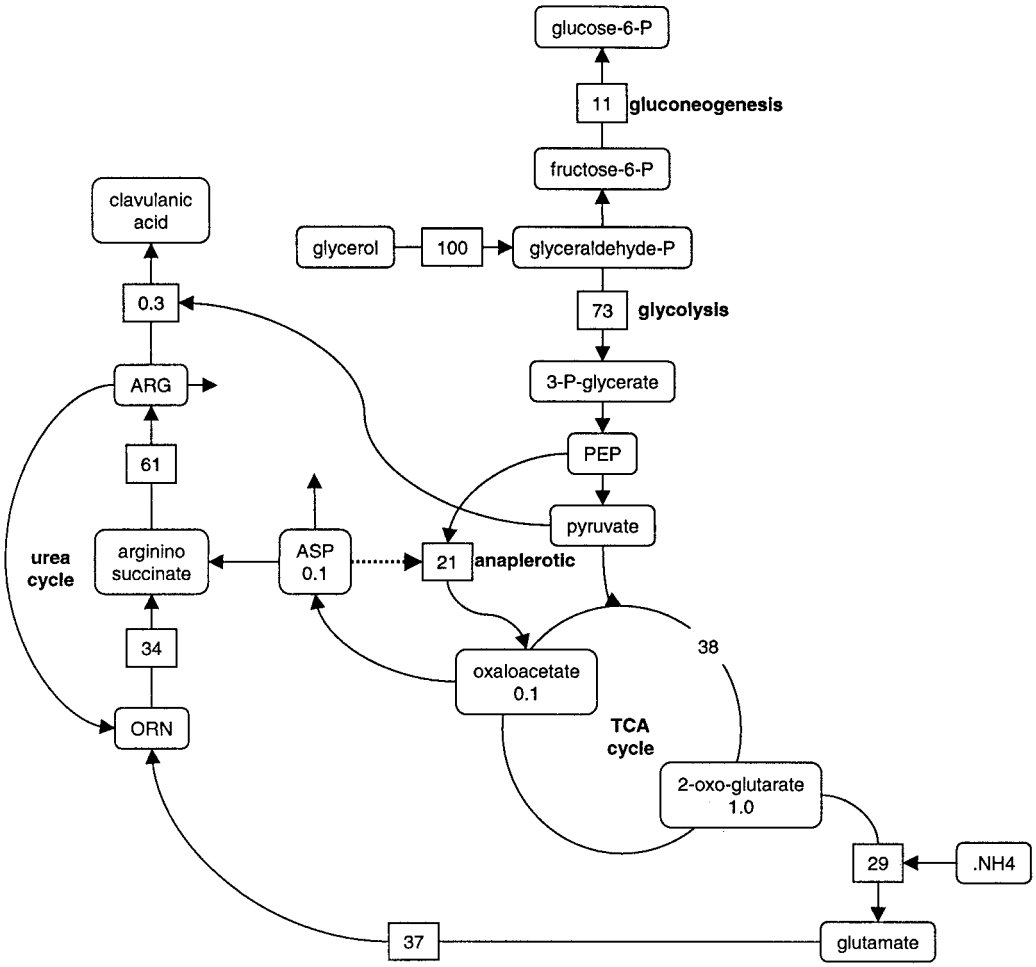

Fig. 1b.

are proportionately lower than those observed in primary metabolite production. However, we propose that MFA of antibiotic-producing cultures can be employed to identify intermediary metabolites, whose production rates are critical to optimising bioproduct formation. This assertion is supported by recent work (van Gulik *et al*. 2000) that established that penicillin production requires significant changes in fluxes through the primary metabolic pathways.

Materials and methods

Strains and culture media

A single spore isolate of *Streptomyces clavuligerus* NRRL 3585, obtained from SmithKline Beecham, was used throughout this work. The P-limited culture medium for *S. clavuligerus* contained: (g l^{-1}, in reverse osmosis purified water) glycerol 60, NH_4Cl 7.0, KH_2PO_4 0.1, MOPS, 21. This was supplemented with trace elements, by addition (10 ml l^{-1} of medium) of a solution containing (g l^{-1}): $MgSO_4 \cdot 7H_2O$ 25, $FeSO_4 \cdot 7H_2O$ 2.5, $CuCl_2$ 0.053, $CoCl_2$ 0.055, $CaCl_2 \cdot 2H_2O$ 1.38, $ZnCl_2$ 1.04, $MnCl_2$ 0.62, Na_2MoO_4 0.03. The pH of this stock solution was adjusted to 7.0. The medium was sterilized by filtration through a sterile cartridge. The pH was adjusted to 7.0 with 5 M KOH before filtration. The C-limited medium was prepared in the same way as the P-limited medium except that it contained 15, 3 g KH_2PO_4 l^{-1}, 7 g K_2HPO_4 l^{-1}. The N-limited medium was prepared in the same way as the P-limited medium except that it contained 45 g glycerol l^{-}, 3 g KH_2PO_4 l^{-1}, 7 g K_2HPO_4 l^{-1} and 1.5 g NH_4Cl l^{-1}.

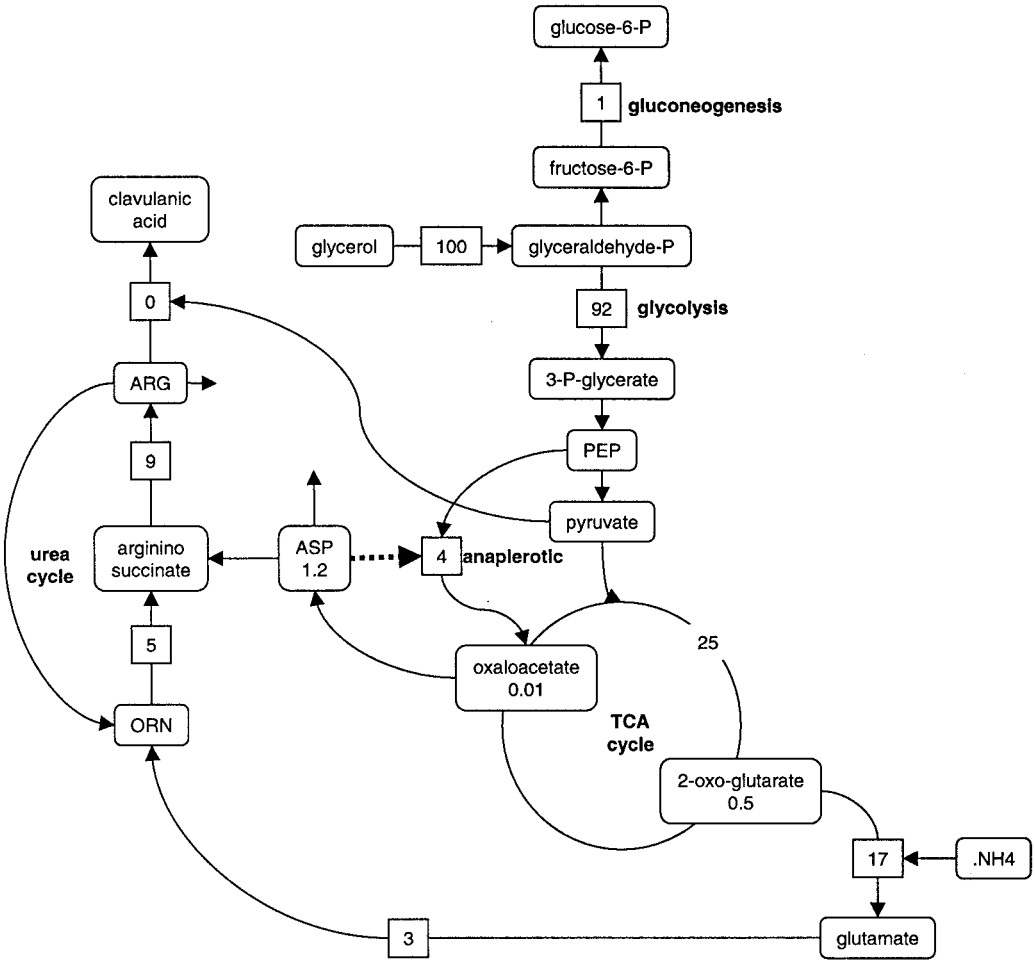

Fig. 1c.

S. clavuligerus cultures were obtained from spores in 250-ml baffled Erlenmeyer flasks containing 25 ml defined medium, and incubated at 30 °C for 48 h on a rotary shaker at 250 rev min^{-1}. Flasks containing 25 ml of minimal medium were inoculated with 2.5 ml of the culture, and incubated for 24 h at the same conditions. Chemostat cultures were inoculated with this culture at a rate of 5% (v/v).

Bioreactor culture

Chemostat cultures were set up in a 2.5 l bioreactor, with a working volume of 1 l. Cultures were aerated at 2 vvm, and stirred at 750 rev min^{-1}. The pH was controlled at 6.8 ± 0.2 by the automatic addition of 0.5 M KOH or 0.5 M HCl. Temperature was controlled at 30 °C. Exhaust gas was analysed by using infrared (CO_2) and paramagnetic (O_2) analysers.

Biomass determination

Dry weights were determined directly in triplicate using 10 ml of membrane filtered culture which was microwave dried. Filtrates were collected and frozen for further assays (clavulanic acid, glycerol, ammonium, and phosphate concentrations).

Clavulanic acid

Antibiotic concentrations were evaluated by HPLC, following the procedure described by Foulstone & Reading (1982).

Analytical determinations

Glycerol was determined enzymatically using a Boehringer kit. Ammonium and phosphate concentrations in the supernatants and in the fresh medium

were determined by reflectometry (RQflex, Merck). Oxaloacetate and 2-oxo-glutarate were determined using spectrophotometric assays (Wahlefeld 1974, Bergmeyer & Bent 1974). Aspartate was determined by hplc as described previously (Ives & Bushell 1997).

The stoichiometric bioreaction network

A model, consisting of a metabolic network was constructed and solved using the FluxMap package (Biotecnol Software, Portugal). A copy of the full matrix used may be obtained by contacting the authors. The network includes the main metabolic pathways (glycolysis, tricarboxylic acid cycle, and pentose phosphate pathway) known to be present in most species of *Streptomyces* (Salas *et al.* 1984, Dekleva & Strohl 1988, Alves 1997), and the reactions for the synthesis of all precursors involved in biomass production. Biomass composition was assumed to be (C-mol per C-mol biomass): protein, 0.523; carbohydrates, 0.13; lipids, 0.134; DNA, 0.029; RNA, 0.195 (Shahab *et al.* 1996, Olukoshi & Packter 1994, Gesheva *et al.* 1997). The equations included the requirements for cofactors such as ATP and NAD(P)H. We have assumed the presence of nicotinamide nucleotide transhydrogenase, which catalyzes the reversibly conversion of NADH into NADPH. Special features of our model were:

(i) the specific pathways for clavulanic acid biosynthesis (see Ives & Bushell 1997) were included;
(ii) only one anaplerotic reaction was considered, namely the carboxylation of phosphoenol pyruvate (Alves 1997);
(iii) the reactions for amino acid biosynthesis included, were considered to be those present in most microorganisms by Moat & Foster (1995);
(iv) the reactions corresponding to the urea cycle were included – several papers have reported that *S. clavuligerus* possesses enzyme activities related to the urea cycle, such as arginase activity (Bascarán *et al.* 1983) and ornithine carbamoyltransferase (de la Fuente *et al.* 1996);
(v) the oxidative step of the pentose phosphate pathway was assumed to be inactive with glycerol as the carbon source (e.g., Holms 1996).

The stoichiometry of clavulanic acid synthesis was deduced using the information reported by Jensen & Paradkar (1999); Elson *et al.* (1993) and Hodgson *et al.* (1995):

$$3\ \text{pyruvate} + 6\ \text{arginine} + 2\ \text{NADPH} + \text{FAD} + 15\ \alpha\text{ketoglutarate} + 3\ O_2 \rightarrow 8\ \text{clavulanic acid} + 2\ \text{NADP} + \text{FADH} + 12\ \text{succinate} + 3\ CO_2 + \text{urea} + NH_4^+.$$

Results and discussion

Antibiotic production

Clavulanic acid titres and production rates were dependent on the identity of the growth rate limiting nutrient in chemostat bioreactor culture. The highest specific production rate was obtained in P-limited medium (3.65 $mg_{clav}\ g_{biomass}\ h^{-1}$), while a significantly lower rate (0.32 $mg_{clav}\ g_{biomass}\ h^{-1}$) was observed in the N-limited medium (Table 1). No production was observed in C-limited medium.

No excretion of organic acids was detected, but extracellular polysaccharides were found in the P- and N-limited cultures. In all the experiments, carbon balances were within the accepted experimental limits ($100 \pm 5\%$).

Metabolic fluxes associated with antibiotic production

Metabolic flux analysis indicated that changing the nutrient limitation exerted its primary effect by affecting the availability of the C_5 precursor. Flux into arginine from arginino-succinate was greatest under P-limitation, lower under N-limitation but decreased by an order of magnitude under C-limitation (Figure 1). Flux through anaplerotic metabolism was significantly lower under C-limitation, restricting the capacity for utilisation of 2-oxo-glutarate from the TCA cycle for glutamate and, ultimately, arginine and C_5 precursor production. The lower antibiotic production under N-limitation compared to P-limitation could reflect the limited capacity of the culture for amination of precursors to the C_5 component of clavulanic acid. This is consistent with the decreased flux into glutamate from 2-oxo-glutarate when comparing P- to N-limitation and the similar decease of flux into ornithine and urea cycle activities. We propose that the reduced fluxes in these reactions under C-limitation reflect the reduced availability of 2-oxo-glutarate due to the significantly lower anaplerotic metabolism.

The decreased oxaloacetate pool size under C-limitation may be a result of the decrease of anaplerotic flux. Aspartate inhibits the activity of

phosphoenol pyruvate carboxylase in some bacteria, consistent with the significantly higher pool size of aspartate observed in this study under carbon limitation. Previous work (Ives & Bushell 1997) has shown that, under P-limitation, antibiotic productivity is limited by flux through C_3 precursor-yielding pathways and that there is potential for increasing productivity under these circumstances by feeding metabolites of these pathways. Taken together, these observations suggest that, under P-limitation, C_5 precursor flux is saturated for clavulanic acid production, limiting under N-limitation and insufficient under C-limitation.

S. clavuligerus is unusual in that it has an impaired capacity for glucose assimilation, necessitating the use of glycerol as carbon source. Under N- and P- limitation (glycerol excess), gluconeogenesis (indicated by flux from fructose 6 phosphate to glucose 6 phosphate) is similar, suggesting that it is saturated. Under C-limitation, however, it is almost an order of magnitude lower, consistent with severe competition for glycerol between glycolysis and gluconeogenisis. Although flux through glycolysis is higher under C-limitation, TCA cycle flux is decreased, compared to the other conditions, which, combined with the significantly lower anaplerotic flux, described above, results in an insufficient supply of C_5 precursor for antibiotic production.

Conclusions

Under C-limitation there appears to be a restricted capacity for anaplerotic metabolism, minimising the potential for extensive TCA-cycle derived biosynthesis. N-limitation would restrict the availability of nitrogen for amino acid biosynthesis, thus restricting the supply of the C_5 precursor further down stream. Under P-limitation neither of these restrictions would apply.

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