Manipulation of the physiology of clavulanic acid production in *Streptomyces clavuligerus*

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This paper reports a novel use of cluster analysis for the identification of intermediary metabolites that are produced at rates closely correlated with those of antibiotic biosynthesis. This information was used to devise culture feeds resulting in enhanced production of clavulanic acid, an antibiotic of current worldwide commercial interest. The feeding strategies apparently alleviated a rate-limiting supply of the C₃ precursor of clavulanic acid. C₃ limitation may be a consequence of unusual nitrogen and carbon metabolism in *Streptomyces clavuligerus*. This approach has potential as a generic method for influencing biosynthetic pathway fluxes using feeds without knowledge of the biosynthetic pathway.

Keywords: secondary metabolism, clavulanic acid, nutrient feeds, cluster analysis

INTRODUCTION

Bioreactor feeding strategies have been used as a means of (I) controlling growth rate, (II) prolonging stationary phase and (III) supplying feeds of potentially deleterious precursors to the culture at subinhibitory concentrations. Strategy III is well established in antibiotic production processes (Queener & Swartz, 1979) and we have applied strategy II to antibiotic and monoclonal antibody production in, respectively, *Streptomyces* (Bushell, 1988) and hybridoma (Bushell *et al.*, 1994) cell cultures. The control of growth rate (strategy I) has been used to avoid oxygen limitation during yeast production (Reed & Peppler, 1973).

Clavulanic acid is a potent inhibitor of β -lactamases of both Gram-positive and Gram-negative pathogenic bacteria (Reading & Cole, 1977). It exerts a marked synergistic effect with β -lactamase-susceptible penicillins against many penicillin-resistant infections (Brown, 1986). Augmentin, a commercial combination of amoxycillin and clavulanic acid, is currently prescribed in 117 countries, when antibiotic-resistant infections are suspected. Clavulanic acid will shortly attain generic status and is, therefore, of widespread interest.

Streptomyces clavuligerus was isolated from a South American soil sample. It produces at least 21 secondary metabolites (Hopwood, 1981), including several β -

lactam antibiotics. The sugar utilization pattern is unusual in that glucose is not readily assimilated.

Radiolabelled feeding experiments have indicated that arginine and ornithine are efficiently incorporated into clavulanic acid (Townsend & Ho, 1985a; Romero *et al.*, 1986). Despite evidence (Romero *et al.*, 1986) that arginase (converting arginine to ornithine) is active in cultures of *S. clavuligerus*, ornithine is not a direct precursor. A study in which radiolabelled amino acids were fed to arginine auxotrophs demonstrated that ornithine has to be converted to arginine (via a sequence analogous to the mammalian urea cycle) before it can be incorporated into clavulanic acid (Valentine *et al.*, 1993). The remainder of the clavulanic acid molecule is derived from a C₃ precursor. Stirling & Elson (1979) obtained evidence for incorporation of glycerol and a later report suggested that D-glycerate could act as an intermediate between glycerol and clavulanic acid (Townsend & Ho, 1985b). A mechanism accounting for clavulanic acid and valclavam (a related structure produced by *Streptomyces antibioticus*) biosynthesis from arginine and glycerol has been presented (Baldwin *et al.*, 1994). However, more recent evidence (Pitlik & Townsend, 1997) has shown that hydrogen at the C-2 position of glycerate is lost in the biosynthesis of clavulanic acid, suggesting that the C₃ precursor could be pyruvate.

We have examined the necessity for growth-rate down-regulation for the induction of secondary metabolism (Bushell *et al.*, 1997; Clark *et al.*, 1995; Wilson & Bushell, 1995). These studies led to the development of cyclic fed batch cultures for enhanced production of

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antibiotics. This report includes the evaluation of two-stage chemostat cultures, an alternative strategy for achieving growth-rate down-regulation.

METHODS

Strains and culture media. *Streptomyces clavuligerus* NRRL 3585 (ATCC 27064) was used throughout. The chemically defined media contained (maximum concentrations, g l⁻¹ in reverse-osmosis-purified water): glycerol 60, NH₄Cl 7.0, KH₂PO₄ 3.0, K₂HPO₄ 7.0, MgSO₄·7H₂O 0.25, FeSO₄·7H₂O 0.025, CuCl₂ 0.00053, CoCl₂ 0.00055, CaCl₂·2H₂O 0.0138, ZnCl₂ 0.0104, MnCl₂ 0.0062, Na₂MoO₄ 0.0003; reduced to (g l⁻¹) glycerol 15 (C-limited), NH₄Cl 1.5 (N-limited) and KH₂PO₄ 0.1, without K₂HPO₄ (P-limited, also contained MOPS at 21 g l⁻¹) prepared as described previously (Bushell *et al.*, 1997).

Cultures were routinely maintained on SV2 agar (Ives, 1995) and grown in 25 ml cultures in 250 ml magnetically stirred Erlenmeyer flasks at 30 °C. An SV2 broth culture was inoculated with colonies from a plate. The bioreactor inoculum regime was as described previously (Lynch & Bushell, 1995).

Bioreactor culture. The Braun Biolab bioreactors used had working volumes of 1.5 l. Agitation was provided by disc turbine impellers rotating at 750 r.p.m., and sterile air was supplied through a sparger. The temperature was controlled at 30 °C. Cultures were controlled as described previously (Lynch & Bushell, 1995).

Assays. Biomass (Lynch & Bushell, 1995), phosphate and ammonia (McDermott *et al.*, 1993) concentrations were determined as described previously. HPLC was used to assay clavulanic acid (Foulstone & Reading, 1982) and glycerol (Ives, 1995). Experiments showed that clavulanic acid decomposition under the experimental conditions used was insignificant. Other antibiotics produced by this strain were not investigated.

Cell amino acid content was determined using the HPLC-based method of Jones *et al.* (1981), with the modification of Joseph & Marsden (1986). Cells were disrupted following suspension in 0.05 M glycine buffer, pH 9.5 (250 ml 0.2 M glycine, 96 ml 0.2 M NaOH and 0.1 M KCl to 1 l). Single 1 ml volumes were sonicated in 30 s pulses for 2 min at 4 °C at intensity 3 using an Ultrasonic Processor XL sonicator (Heat Systems) fitted with a micro tip. Cell-free extracts were prepared by centrifugation at 12000 g for 15 min at 4 °C and used immediately or frozen at -20 °C. Derivatization was achieved by adding 200 µl OPT-thiol reagent (Sigma) to 50 µl samples. After centrifugation at 10000 g for 10 min, samples (25 µl) were injected onto a 5 µm Hypersil ODS column (HPLC Technology) of length 150 mm and internal diameter 4.6 mm and eluted with two mobile phases. The initial gradient was 0–10% of mobile phase A (800 ml 0.05 M NaH₂PO₄ at pH 5.5 and 200 ml methanol) in mobile phase B (200 ml of the same phosphate buffer solution and 800 ml methanol) applied over 10 min, followed by 10–85% in 30 min, 85–0% in 5 min and finally 10 min equilibration with A at 1 ml min⁻¹. The eluate was monitored at 340 nm. Using this relatively simple single-wavelength detection system we were unable to resolve peaks for glycine, proline or glutamate with sufficient certainty to include concentrations of these amino acids in our calculations. Asparagine concentrations were too small for accurate quantification and were assumed to be insignificant during the subsequent calculations.

Two-stage chemostat culture. The dilution rate was 0.05 h⁻¹ in both vessels; all of the culture flowed from stage 1 to stage 2 without further additions. The rationale behind this experimental design was that the residual concentration of NH₄Cl in the culture flowing from stage 1 would act as the nitrogen source for the culture in stage 2. In two-stage chemostat culture, the growth rate in stage 2 is not equal to the dilution rate, and is determined by comparing the increase in biomass concentration in stage 2 with that in stage 1. The value of the growth rate was calculated using the expression:

$$\mu_2 = D_2 - D_{1,2} \cdot (x_1/x_2) \quad (1)$$

(Pirt, 1975) where: μ_2 is the specific growth rate in stage 2, D_2 is the dilution rate in stage 2, $D_{1,2}$ is the partial dilution rate in stage 2, accounting for the material transferred from stage 1 (in this case, equal to D_2), x_1 is the steady-state biomass concentration in stage 1 and x_2 is the steady-state biomass concentration in stage 2. Estimation of the ammonia concentrations in each stage allowed the calculation of values for substrate affinity and inhibition constants using the equation

$$\mu = \mu_{\max} \left[\frac{s}{K_s + s} \right] \left[\frac{K_i}{K_i + I} \right] \quad (2)$$

where μ is the specific growth rate, $\mu_{\max}$ is the maximum specific growth rate, K_s is the substrate affinity constant, K_i is the inhibition constant, s is the steady-state substrate concentration in the culture vessel and I is the inhibitor concentration (in this case, equal to the substrate concentration).

Substituting the values for s from the two stages results in two simultaneous equations allowing solutions for values of K_i and K_s .

Cluster analysis. Amino acid production rates ($q_{\text{amino acid}}^{\text{biomass}}$) were calculated from the intracellular pool sizes using [amino acid] $\times D$ [$\mu\text{g (mg biomass)}^{-1} \text{ h}^{-1}$]. A data matrix containing the resultant values and clavulanic acid production rates, obtained over the dilution rate range 0.03–0.09 h⁻¹, was standardized and subjected to cluster analysis using average within-groups linkage with the Unistar statistical analysis package. Results were displayed in the form of a dendrogram.

Reproducibility and replication of experiments. All experimental data were obtained from single cultures. Experiments were carried out in triplicate to ensure that the trends and relationships observed in the culture parameters measured were reproducible. Individual assays were replicated fourfold except for the amino acid assays, which were carried out in duplicate. Experiments were rejected where a chi-squared test indicated significant differences between replicates. Where error bars (which represent standard error) are not shown, they were too small to be visible on the figures presented.

RESULTS AND DISCUSSION

Effect of growth-rate-limiting nutrient in batch culture

Bioreactor batch culture using glycerol (C-), NH₄Cl (N-) and KH₂PO₄ (P-) limited medium formulations resulted in growth-dissociated clavulanic acid production under N and P limitation but no production under C limitation (Fig. 1). Exclusive exhaustion of the appropriate nutrients confirmed the identity of the respective growth-limiting substrates (Fig. 1). Specific antibiotic production was threefold higher under P limitation [0.03 g clavulanic acid (g biomass)⁻¹] than

Fig. 1. Biomass concentration (■), clavulanic acid concentration (●) and growth rate-limiting substrate concentration (▲) in (a) C-limited (b) N-limited and (c) P-limited bioreactor batch culture.

obtained under N limitation [0.01 g clavulanic acid (g biomass)⁻¹].

Effect of ammonium chloride concentration

An optimum value for biomass yield at an NH₄Cl concentration of 8 g l⁻¹ was observed in the P-limited medium over the range 4–20 g NH₄Cl l⁻¹. Varying the KH₂PO₄ concentration confirmed that phosphate was the growth-limiting substrate throughout this experiment. It was, therefore, assumed that ammonia was inhibitory to growth.

Reports of arginase activity in *S. clavuligerus* (Romero *et al.*, 1986; Baldwin *et al.*, 1994; Bascaran *et al.*, 1989) are strongly suggestive of a full urea cycle (Mendz & Hazell, 1997). A urea cycle in prokaryotes is unusual and is required for removal of ammonia from cells in which toxic intracellular concentrations are likely to occur, such as those of species that carry out rapid amino acid catabolism (Mendz & Hazell, 1996). Sensitivity to ammonia concentration and presence of a urea cycle may, therefore, be linked. The urea cycle provides a very effective pathway for arginine biosynthesis so it is,

perhaps, not surprising that a pathway for synthesizing an antibiotic using arginine as a precursor has evolved in this species.

Effect of growth-rate down-regulation

To study further the effect of ammonia, and provide a non-inhibitory culture system for complete ammonia utilization, a two-stage NH₄Cl-limited chemostat culture was operated. With a feed NH₄Cl concentration of 3.8 g l⁻¹, 2.88 g l⁻¹ was utilized in stage 1 and a further 0.79 g l⁻¹ in stage 2 at a dilution rate of 0.05 h⁻¹ (Table 1).

Assuming non-competitive substrate inhibition, it was possible to calculate values for substrate affinity constant (K_s) of 1.71 g l⁻¹ and inhibition constant (K_i) of 0.54 g l⁻¹. Thus, at NH₄Cl values approaching these values, significant effects on, respectively, stimulation and inhibition of growth would be expected, consistent with our finding of an optimum ammonia concentration in batch culture of 8 g l⁻¹. Substituting an NH₄Cl concentration of 8 g l⁻¹ into equation (2) indicates that, even at this optimum concentration, the growth rate is only 0.052 times μ_{max} .

The clavulanic acid concentration, and, therefore, production rate, increased by 161% from the first to the second stage. The production rate, relative to ammonia consumption, however, increased by 488% (Table 1). Had the relative production rate remained constant between stage 1 and stage 2, the concentration of clavulanic acid in stage 2 would have increased by 1.93 mg l⁻¹ to 5.03 mg l⁻¹ (Table 1). The observed increase to 8.1 mg l⁻¹ suggests that the change in conditions between stage 1 ($\mu = 0.05$ h⁻¹) and stage 2 ($\mu = 0.022$ h⁻¹) stimulated the rate of antibiotic production. The specific ammonia uptake rate is decreased in stage 2. A possible explanation for the increased production is, therefore, that the clavulanic acid biosynthetic pathway is subject to ammonia repression and that this is relieved by the lower uptake rate in stage 2. Inhibition of clavulanic acid biosynthesis by ammonia has been reported by Romero *et al.* (1984). This non-stoichiometric increase in antibiotic production is, however, also consistent with our findings in other fermentation systems in which growth rate is down-regulated, such as the cyclic fed batch culture system (Bushell *et al.*, 1997; Lynch & Bushell, 1995).

The two-stage system was successful in utilizing the residual ammonia resulting from substrate limitation, and provided a disproportionate increase in the rate of clavulanic acid production arising as a consequence of down-regulating growth rate from 0.05 h⁻¹ to 0.022 h⁻¹ and/or decreasing the ammonia uptake rate between the first and second stages.

Relationship between antibiotic production and amino acid pool size

As the highest clavulanic acid production was obtained in P-limited batch culture, P-limited chemostat culture was used to investigate the relationship between in-

Table 1. Data from two-stage N-limited chemostat culture

The theoretical increase in clavulanic acid concentration in stage 2 is $1.93 \text{ mg l}^{-1} \{[\text{mg (g NH}_4^+)^{-1} \text{ h}^{-1} \text{ in stage 1} \times \text{NH}_4^+ \text{ used in stage 2}]/(\text{growth rate in stage 2})\}$; the theoretical clavulanic acid concentration in stage 2 is, therefore, 5.03 mg l^{-1} (theoretical concentration in stage 2 plus observed concentration in stage 1).

	Stage 1	Stage 2	Notes
Dilution rate (h^{-1})	0.050	0.050	Observation
Specific growth rate (h^{-1})	0.050	0.022	From equation (1)
Residual NH_4Cl concn (g l^{-1})	0.92	0.13	Observation
NH_4Cl used (g l^{-1})	2.88	0.79	Inflow minus residual
Clavulanic acid concn mg l^{-1}	3.1	8.1	Observation
% increase		161	$100 \times (\text{stage 2} - \text{stage 1})/\text{stage 1}$
Clavulanic acid production rate $\text{mg l}^{-1} \text{ h}^{-1}$	0.155	0.405	Concn $\times$ dilution rate
$\text{mg (g NH}_4^+)^{-1} \text{ h}^{-1}$	0.054	0.316	$(\text{Concn} \times \text{dilution rate})/\text{NH}_4^+ \text{ used}$
% increase		485	$100 \times (\text{stage 2} - \text{stage 1})/\text{stage 1}$

Fig. 2. Single-linkage dendrogram obtained by analysing standardized intracellular amino acid and clavulanic acid production rates over a range of dilution rates between 0.03 h^{-1} and 0.09 h^{-1} (see Methods).

tracellular amino acid production rate and clavulanic acid production.

The intracellular amino acid content was quantified over the growth rate range $0.03\text{--}0.09 \text{ h}^{-1}$ and the correlation between values obtained and clavulanic acid production was quantified using cluster analysis (Fig. 2). To test the physiological validity of this analysis, the effect of supplementing the medium with arginine, isoleucine, leucine, serine or valine (to give a final concentration of 10 mM) was examined in chemostat cultures growing at a dilution rate of 0.03 h^{-1} . Valine, serine, leucine and isoleucine feeds resulted in increased clavulanic acid production compared to the control (Table 2).

Hypotheses to account for the effect of amino acid feeds on antibiotic production

If it is assumed that the supply of C_3 precursor is rate limiting for clavulanic acid production whereas the supply of C_5 precursor is not, it can be postulated that

Table 2. Effect of amino acid feeds on the specific productivity of clavulanic acid in a P-limited chemostat culture ($D = 0.03 \text{ h}^{-1}$)

Amino acid in feed (final concn 10 mM)	Clavulanic acid titre	
	$[\text{mg (mg biomass)}^{-1}]$	% of control
Control (none)	0.022	
Arginine	0.022	100
Isoleucine	0.039	177
Leucine	0.039	177
Serine	0.039	177
Valine	0.032	145

Fig. 3. Postulated biosynthetic scheme for clavulanic acid production, illustrating major carbon flux (many intermediate steps omitted). Heavy arrows indicate the fluxes competing with clavulanic acid production that were influenced by the amino acid feeds.

feeding leucine or isoleucine will decrease the anaplerotic carbon flux of pyruvate to the synthesis of these amino acids, thus allowing more C_3 precursor for the synthesis of clavulanic acid (Fig. 3). Increased leucine and isoleucine pool sizes were observed in the fed cultures (Fig. 4d, e) and the synthesis of both amino acids is repressed in many microbial species under these circumstances (Umbarger, 1978). The increase in the ornithine pool size in both cases is consistent with the assumption that the pool size of arginine (its immediate precursor) is not rate limiting and the decrease in arginine pool size is consistent with an increased rate of clavulanic acid production (Fig. 4d, e).

The valine and serine feeds, which resulted in an increase in intracellular valine and serine concentration, respectively (Fig. 4b, c), would also have allowed an increase in the supply of C_3 precursor for clavulanic acid synthesis (Fig. 3). Presumably the valine feed was not as effective as the leucine feed in enhancing antibiotic production because leucine requires carbon skeleton from both α -oxo- β -methylbutyrate (derived from pyruvate) and acetyl-CoA (also derived from pyruvate) and, therefore, accounts for a greater drain on C_3 precursor. It should be noted that the synthesis of the pyruvate family (leucine, isoleucine and valine) can be repressed by any of that group (Umbarger, 1978).

Further evidence that C_3 carbon supply is rate-limiting rather than C_5 was obtained with the arginine feed, which resulted in an increase in the pool size of arginine (Fig. 4a), the immediate precursor of clavulanic acid (Valentine *et al.*, 1993), without increasing clavulanic acid production. Limitation of clavulanic acid synthesis due to C_3 supply is also consistent with the effect of growth-rate-limiting nutrient on antibiotic production (Fig. 1). Under P limitation, which supports the highest clavulanic acid production, gluconeogenesis and glycolysis would be restricted by the supply of phosphate, restricting the flux of glycerol into these pathways and

allowing the flux of C_3 precursor into clavulanic acid to increase. Under N limitation, these restrictions would not apply, so clavulanic acid production would have to compete with gluconeogenesis and glycolysis. Under C limitation, the resultant restriction in glycerol assimilation would limit the global supply of C_3 , presumably accounting for the abolition of clavulanic acid production in a biosynthesis where C_3 supply is rate limiting.

Feeds of arginine and valine resulted in an increase in glutamate pool size. This may be a reflection of the decreased anaplerotic flux, required for their syntheses, which presumably has the effect of decreasing C_3 flux into pyruvate, discussed above. All feeds also resulted in an increase in intracellular tryptophan and tyrosine concentrations, indicating the activity of the shikimate pathway in this organism (Fig. 3). As the shikimate pathway requires both C_3 and C_6 precursors (the latter having to be produced by gluconeogenesis since *S. clavuligerus* has impaired glucose-utilizing ability) this would provide considerable competition for C_3 precursor.

We have not quantified the production of other antibiotics. It is possible that the feeding experiments could have influenced the production of these compounds, particularly where amino acids in the feed are acting as biosynthetic precursors.

Concluding remarks

Clavulanic acid was produced as a secondary metabolite following exponential phase in batch culture and in response to growth-rate down-regulation in two-stage chemostat culture. This latter technique allowed efficient usage of ammonia, despite the fact that it appears to be a growth-inhibitory substrate.

Cluster analysis may be used to identify those intermediary metabolites that are produced at rates most

Fig. 4. Intracellular amino acid pool size, shown as $\mu\text{mol (g biomass)}^{-1}$ above or below the control value (no extra feed; 0 on y axis), of a chemostat culture at a dilution rate of 0.03 h^{-1} . Supplemented feed solutions contained 10 mM (final concentration) of: (a) arginine, (b) valine, (c) serine, (d) leucine, (e) isoleucine.

closely correlated with antibiotic biosynthesis. This information has been used to construct culture feeds resulting in enhanced antibiotic production. This approach may have potential as a generic method for formulating feeds that are capable of enhancing product formation by influencing biosynthetic pathway fluxes. An advantage of this approach is that no knowledge of the biosynthetic pathway would be required to construct the feed.

The effect of various feeds on intracellular amino acid pool sizes indicated that manipulation of physiology to enhance clavulanic acid production requires alleviation of competition from other pathways for a C_3 precursor, the availability of which is critical due to the organism's impaired ability to assimilate glucose. The unusual presence of a urea cycle in a prokaryote would provide an abundant supply of arginine, the C_5 precursor to clavulanic acid.

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