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Review

Determination of the rate-limiting step(s) in the biosynthetic pathways leading to penicillin and cephalosporin

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SUMMARY

This paper is a review of strategies that have been used, or that could be used, to determine the rate-limiting step(s) in the biosynthetic pathways leading to penicillin or cephalosporin. Information is summarized from published material that involves studies with low-producing strains of *Penicillium chrysogenum* and *Cephalosporium acremonium*. We also summarize information derived from some high-producing production strains. Identification of the rate-limiting step(s) was of great interest to us as the first step in a rational program to further improve antibiotic titers of these highly developed strains. A number of approaches that could be used to elucidate the rate-limiting step(s) are described herein.

INTRODUCTION

The β-lactam biosynthetic pathways in *Penicillium chrysogenum* and *Cephalosporium acremonium* leading to penicillin V and cephalosporin C are well known (see Figure 1). The nomenclature of the genes encoding these enzymes follows published recommendations [5]. As part of the fermentation process improvement program, we are most interested in the yield of antibiotics produced by these organisms.

The yield of β-lactam antibiotic produced by a particular strain is probably limited by one or more rate-limiting enzymes and/or regulatory proteins. If the 'bottlenecks' in the biosynthetic pathways can be identified then it may be possible to clone the genes encoding for these rate-limiting proteins. Construction of recombinant strains with multiple copies of these genes would theoretically lead to amplification of enzymes important in antibiotic biosynthesis. This can result in improved strains with higher levels of production of penicillin V or cephalosporin C. Alternatively, identification of rate-limiting step(s) may allow us to develop screening strategies for higher producing strains using classical mutagenic methods.

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This paper is a review of ways that have been used or that may be used to determine the rate-limiting step(s) in the biosynthetic pathways leading to penicillin V or cephalosporin C. A number of possible approaches are described.

LOOK FOR INTERMEDIATES THAT ACCUMULATE IN FERMENTATION BROTHS

The rationale for this method is that an intermediate in the β-lactam biosynthetic pathway will be excreted into the fermentation broth if the enzyme for which it is a substrate is limiting. A fundamental problem with this method is that, because of the potential for negative feedback ('end-product inhibition'), this accumulation may not be observed. Another problem is that the intermediate may not accumulate because it is unstable in broth.

An example of the use of this approach was in trying to decide whether the level of isopenicillin N synthetase (IPNS) is limiting in certain of our *P. chrysogenum* or *C. acremonium* production strains. A search was made by HPLC for δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) or, after oxidation with performic acid, ACV sulphonic acid, in fermentation broths of both organisms. In neither case was any tripeptide detected. It is concluded, therefore, that in neither production strain is IPNS the rate-limiting step. However, if the ACV tripeptide had

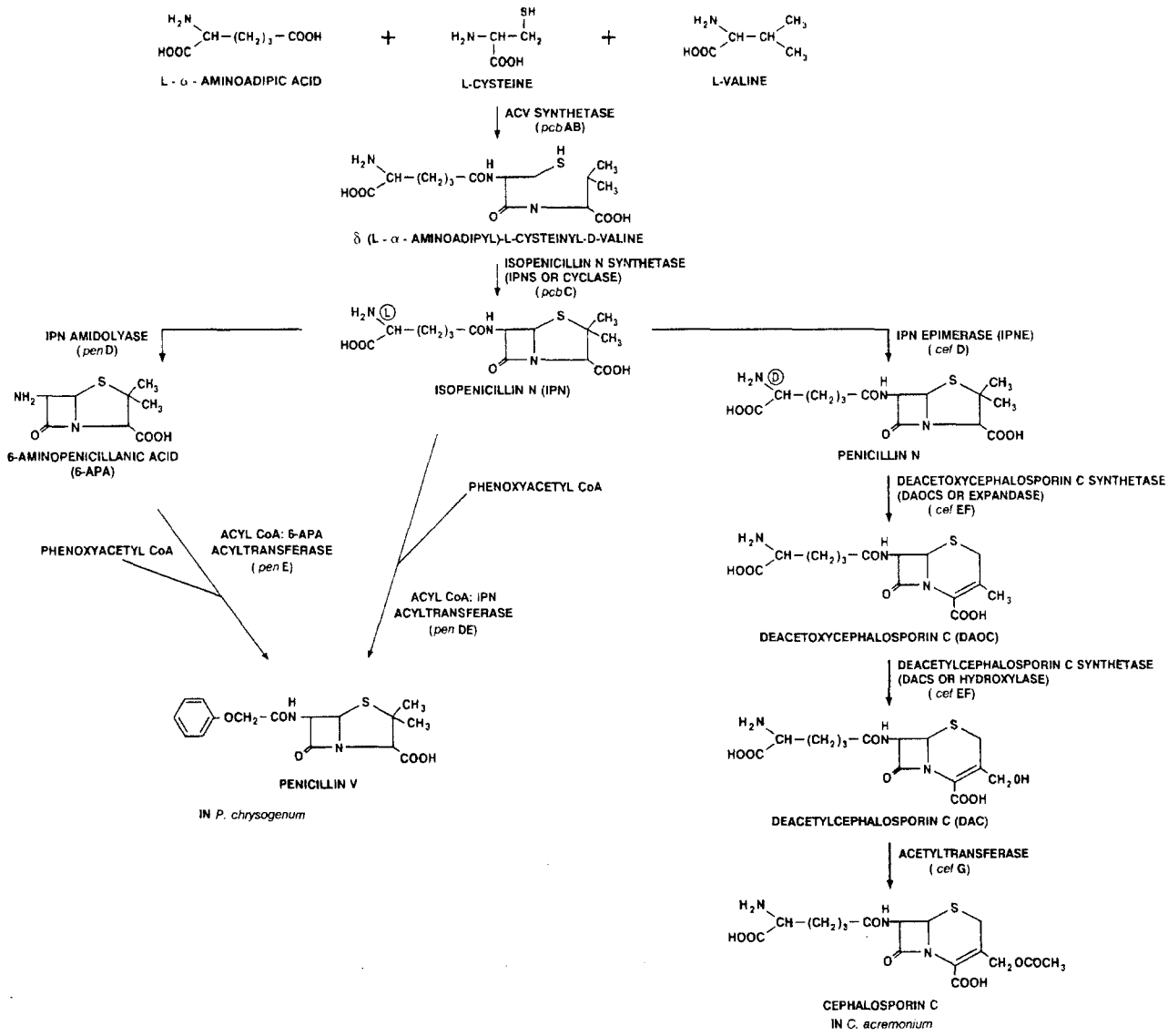


Fig. 1. Biosynthetic pathways for penicillin V and cephalosporin C.

been broken down to its amino acids or further derivatized, it would not have been detected. Further experiments to test this primary observation by other approaches are described later.

Most of the intermediates of secondary metabolism are structurally related (most are δ-linked derivatives of α-aminoadipic acid) and might be detected simultaneously with one general analytical system. The method described earlier for detection of compounds involved in the biosynthesis using *o*-phthaldialdehyde/*N*-acetyl-L-cysteine [19] was used in this manner. In our work with the cephalosporin fermentation, the only intermediates clearly identified in broth were penicillin N and deacetylcephalosporin C and traces of deacetoxycephalosporin C. The last two probably arose, respectively, from breakdown of cephalosporin C and from disruption of cells during the fermentation. The accumulation of penicillin N in broth indicates that the deacetoxycephalosporin C synthetase (DAOCS) enzyme might be rate limiting. Skatrud et al. [15] introduced extra copies of the DAOCS gene and showed an improvement in cephalosporin titer with a concomitant increase of expandase enzyme activity and reduction in penicillin N level, confirming that this was the rate-limiting enzyme. In the penicillin fermentation, traces of α-aminoadipic acid were found in broths. This probably arose from hydrolysis of the lactam of α-aminoadipic acid known to be present in broth [2]. No other intermediates were identified.

It is more difficult to design an analytical system that could be used to detect accumulating precursor compounds from primary metabolism. It might be possible to feed the fermentation culture with ^{14}C-labeled glucose and search for intermediates that accumulate using a general analytical procedure for sugars, amino acids, etc.

MEASURE THE SPECIFIC ACTIVITY OF ENZYMES IN THE PATHWAY

The specific activity of most β-lactam biosynthetic enzymes can be measured in vitro. This measurement of one enzyme can be compared with the specific activities of other enzymes in the pathway or with the rate of formation of the final product. A rate-limiting step might then be identified.

An example of this method is provided by measurement of the IPNS enzyme in a strain of *C. acremonium* [7]. The rate of production of isopenicillin N by the IPNS extracted from a given weight of mycelium was at least an order of magnitude higher than the rate of production of penicillin N and cephalosporin C together by the same weight of mycelium at the time of harvesting. There was, therefore, much more IPNS activity than necessary for the synthesis of penicillin N and cephalosporin C, suggesting that IPNS was not limiting in this strain. This conclusion requires that in vitro measurements reflect in vivo activities. This will not be true if there is compartmentalization of the different enzymes in vivo (as there is in *P. chrysogenum* [6], for example), or if the enzyme being measured is unstable or subject to enzymatic breakdown (by protease in the lysate, for example), or if the assay conditions are not optimized.

In another example, it was found in this laboratory that cells from a cephalosporin fermentation produced cephalosporin C linearly from 40 h up to 200 h, although the cephalosporin C titers leveled off at 160–170 h because of chemical instability of the antibiotic [20]. The specific activity of the IPNS enzyme (in IU/g dry cell weight) for cells taken from the fermentor reached a maximum at about 90 h, then fell to about one-fifth of the value at 200 h [21]. As the cells were still fully active at 200 h, there was, therefore, in the period from 40–200 h, up to 5-times more IPNS than needed for good productivity of the cells. This showed that the IPNS enzyme was probably not rate-limiting in this production strain.

USE OF SPECIFIC ACTIVATORS OR INHIBITORS OF ENZYMES TO IDENTIFY RATE-LIMITING STEP(S) IN VIVO

This method involves the use of specific activators or inhibitors of enzymes involved in the synthesis of β-lactam antibiotics. With the current *P. chrysogenum* production strains, we found one activator, dichloroacetate, which led to improvements in penicillin titers in shake flasks.

Dichloroacetate activates the pyruvate dehydrogenase complex through inhibition of the kinase subunit which phosphorylates and inactivates the complex [17]. Activation of the complex promotes the conversion of pyruvate into acetylcoenzyme A; this would be expected to have a profound effect on the metabolism of the organism. When added to a penicillin shake flask fermentation, it led to an increase in penicillin V production of ~20%. This represents, therefore, a positive correlation between penicillin titer and the activity in vivo of one enzyme on the pathway to penicillin; i.e., it suggests that raising the level of the pyruvate dehydrogenase enzyme complex might lead to increased titers.

The use of specific inhibitors or activators of individual enzymes to obtain a correlation between the level of a particular enzyme and the final yield of antibiotic is an approach with some advantages. First, the method may provide information about in vivo levels of the enzymes critical for β-lactam biosynthesis. Second, this type of experiment is relatively easy to perform. A possible disadvantage of the method is that the inhibitor or activator may not be specific for one enzyme or may have other effects on the organism tested.

GUESS THE RATE-LIMITING STEP AND RAISE THE LEVEL OF THAT ENZYME BY GENETIC ENGINEERING TECHNIQUES

Cloned β-lactam biosynthetic genes can be transformed into *C. acremonium* and *P. chrysogenum* to provide additional copies of these genes. If the cloned gene corresponds to a rate-limiting step, increased production of cephalosporin C and penicillin may result. This is a direct way to test the hypothesis that a particular enzyme is rate limiting.

Skatrud et al. [14] transformed both wild-type and high-producing strains of *C. acremonium* with a plasmid containing the *Cephalosporium* IPNS gene. Most of the transformants showed unchanged cephalosporin C titers even though cell extracts had 3–4-fold more IPNS enzyme activity. In this laboratory, extra copies of the *Penicillium* or *Cephalosporium* IPNS genes were introduced into a production strain of *P. chrysogenum*. There was an improvement in penicillin titer, but it was small and not proportional to the copy number of the IPNS gene within the host genome [4]. It appears likely, therefore, that in neither *C. acremonium* nor *P. chrysogenum* is the formation of isopenicillin N by the IPNS enzyme the rate-limiting step.

A high-producing strain of *C. acremonium* was trans-

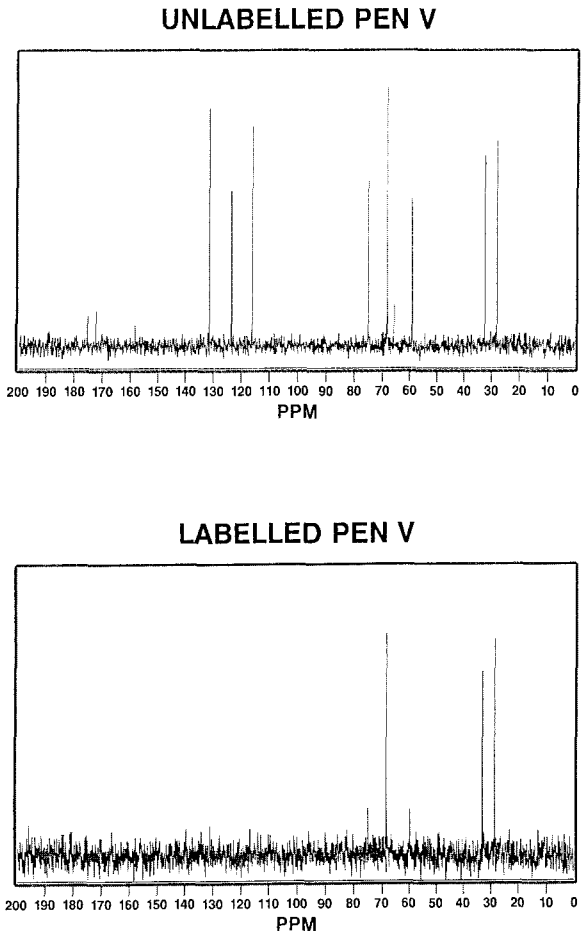


Fig. 2. ^{13}C-NMR spectrum of unlabelled penicillin V and penicillin V produced in a washed cell system containing [1-^{13}C]glucose.

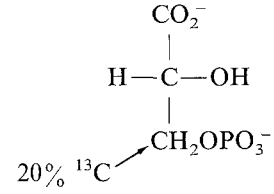
formed with a plasmid containing the DAOCS gene (Skatrud et al. [15]). After screening a small number of transformants, one was found which produced approx. 40% more cephalosporin C than the parental strain in shake flask fermentations. Extracts of this transformant contained nearly 2-fold more DAOCS activity than corresponding extracts of the parent. The recombinant strain also accumulated much less penicillin N. This data shows that the DAOCS enzyme is the rate-limiting step in cephalosporin C biosynthesis in this strain.

Two quotes are relevant. The first is from Skatrud et al. [14]: "Introduction of other biosynthetic genes should provide – by process of elimination – knowledge of what step is rate-limiting...". Unfortunately, this "process of elimination" requires an enormous amount of work. Furthermore, as Pirt [8] states: "... current methods in genetics and recombinant DNA technology cannot be applied rationally unless the 'bottlenecks' in the (biosynthetic) pathway have been identified".

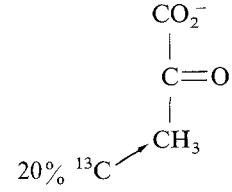
MEASURE THE OVERALL KINETICS OF THE ORGANISM

The first four approaches focus on the kinetics of a single step, although the first – looking for accumulation of one of the intermediates of secondary metabolism – might be used to look at several steps at once. We were interested in ways of gaining information about several steps at once.

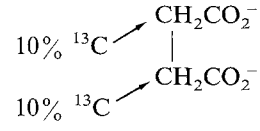
One possible way was examined in a current penicillin production strain using in vivo labeling techniques. Washed cells from a penicillin fermentation were incubated with [1-^{13}C]glucose, ammonium sulphate, phosphate, and phenoxyacetate in buffer. The resulting ^{13}C-labeled penicillin V was isolated by preparative HPLC and its ^{13}C-spectrum recorded and compared with that for unlabeled penicillin V (Fig. 2). The extent of enrichment with ^{13}C of the nucleus could be calculated from the spectrum (Fig. 3). The metabolic pathways used for conversion of glucose through to penicillin were deduced from this labeling pattern. About 60% of the C-1 label of glucose is lost as CO_2 when [1-^{13}C]glucose flows through the pentose phosphate pathway. The remaining label passes through the Embden-Meyerhof pathway to give eventually, after equilibration of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate, 3-phosphoglycerate labeled approximately as shown:



This is converted to pyruvate:



Passage through the TCA cycle leads to randomization of label in succinate:



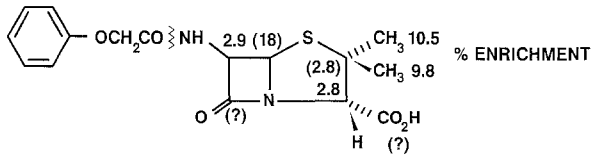


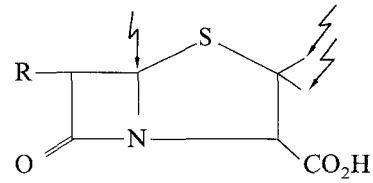
Fig. 3. Distribution of ^{13}C label in isolated penicillin V.

This is converted to oxalacetate, then back to phosphoenolpyruvate, pyruvate, and 3-phosphoglycerate, all with scrambled label. With a simplistic kinetic model, it was possible to arrive at pyruvate and 3-phosphoglycerate with the labeling patterns shown in Fig. 4. These would be converted by standard pathways to cysteine and valine and then penicillin with the labeling as indicated. This model pattern is close to the labeling pattern observed.

This analysis throws light on many different enzymes and indicates flux through different metabolic pathways. The method is powerful because the labeling pattern of the penicillin isolated contains a lot of information. For example, the extent of labeling of pyruvate (deduced from the penicillin spectra) imposes very stringent constraints on the possible pathways from [1-^{13}C]glucose through to pyruvate. This single experiment does not prove the pathway but imposes strong limits on what it must be.

To improve the quality of the information obtained, experiments could be performed with other labeled glucose molecules such as [2-^{13}C]glucose and [6-^{13}C]glucose. It would also be useful to have measurements on some intermediates; for example, a direct measurement of label in pyruvate would confirm the above deductions. Finally, it should be possible to perturb the cells using specific activators or inhibitors of metabolism and observe the effect on penicillin V labeling; this would throw more light on the pathways.

An indication of the effect of cell age on the pathways was obtained in the following way. Labeled penicillin V was isolated after incubation with cells taken from the fermentation at 72, 120, and 168 h. In the passage to older cells, the percentage of label going directly into the three sites indicated



increased, and there was less scrambling of label (at succinate). In older cells, therefore, there is a lower flux of carbon through the TCA cycle.

Measurement of flux through the biosynthetic pathways, determined in this way, might be extended to the point where a rate-limiting step could be identified.

LOOK FOR GENE AMPLIFICATION

It is possible that gene amplification has been responsible for improved titers in commercial strains. In this laboratory [22], a search was made for amplified genes by the technique of Roninson [12] using a molecular hybridization method to specifically detect amplified DNA sequences. Banding patterns for DNA isolated from a wild-type *Penicillium* strain were compared with those for DNA isolated from more recent production strains. No bands were identified which became more intense on passing to higher-producing strains. Gene amplification did not ap-

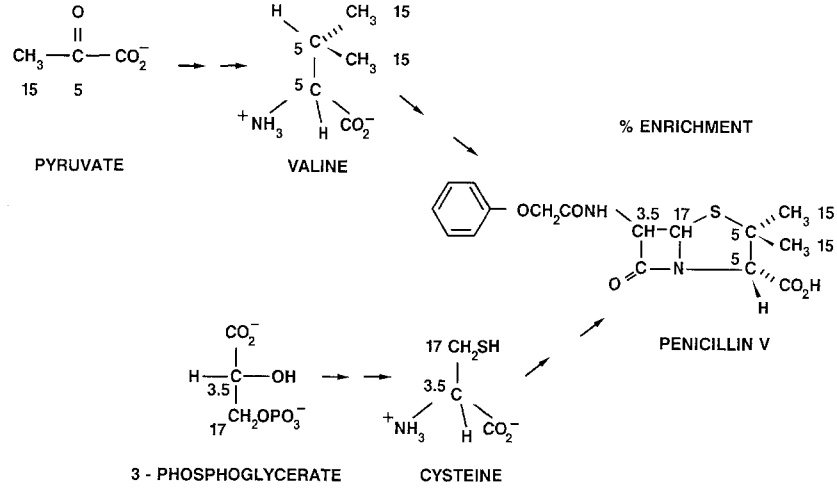


Fig. 4. Labeling of penicillin V from pyruvate and 3-phosphoglycerate.

pear, therefore, to be responsible for the improved antibiotic titers observed.

Cloned β-lactam biosynthetic genes can be used as probes to detect the copy number of each gene in a production strain. Southern hybridization revealed that wild-type and high-producing strains of *P. chrysogenum* have the same intensity of hybridization signal, i.e., the same copy number of the gene, when using the *Penicillium* IPNS gene as a probe (Chiang, unpublished data; Soliday et al. [18]). However, Smith et al. [16], and Barredo et al. [1] recently demonstrated that the IPNS and acyltransferase genes were amplified between 8–16-fold in two high penicillin-producing strains.

It might be possible to make a correlation between gene dosage, enzyme activity, and antibiotic production per cell. This might then indicate which enzyme or enzymes are rate limiting. Alternatively, it might also indicate that some regulatory factor (approach 7) is responsible for the expression of certain amplified genes leading to the final yield of antibiotic.

SEARCH FOR REGULATORY GENES

It has been shown that a high penicillin-producing strain of *P. chrysogenum* produces higher levels of IPNS enzyme than a low-producing control strain [9]. Moreover, it has been reported that high glucose levels repress the synthesis of several penicillin synthesizing enzymes, including ACV synthetase and IPNS [11]. Also it has been shown that a high cephalosporin C-producing strain of *C. acremonium* produces higher levels of three enzymes of the cephalosporin C biosynthetic pathway compared to a low-producing strain [10,13]. These observations indicate that some of the genes coding for penicillin and cephalosporin C biosynthetic enzymes in *P. chrysogenum* and *C. acremonium* are coordinately regulated and, therefore, may be expressed under the regulation of one or more common effectors [10]. It will be important to identify the existence of these regulatory genes.

It has been reported that in *Streptomyces* certain mutations have pleiotropic effects on both antibiotic synthesis and morphological differentiation. Most of these mutants are morphologically abnormal. The restoration of morphology of these mutants by shotgun cloning experiments can serve to identify these regulatory genes. It has been demonstrated that with the presence of extra copies of these kinds of genes there is a stimulation of antibiotic production [3]. This approach of generating similar types of pleiotropic mutants can probably be used with the β-lactam-producing filamentous fungi.

The genes directly involved in β-lactam production might be found in the following manner. Radioactive labeled mRNA isolated from fermentation cultures of β-lactam-producing and nonproducing strains at either logarithmic phase (trophophase) or production phase (idiophase) could be hybridized with a genomic DNA bank of *P. chrysogenum* and *C. acremonium*. After subtraction of clones that hybridize to the mRNA isolated from trophophase, the clones that hybridize solely with mRNA from the idiophase of a producer culture will presumably contain genes related to β-lactam biosynthesis and microbiological differentiation. With the information accumulated from a variety of pleiotropic nonproducing mutants, this plus/minus hybridization approach will narrow down the genes to those directly involved with β-lactam production. Presumably, this can be used further in gene disruption experiments to distinguish which regulatory genes are important in determining the yield of the β-lactam-producing organism.

CONCLUSION

This paper has described several approaches that can be used to elucidate the rate-limiting step(s) in biosynthetic pathways leading to penicillin and cephalosporin. The strategies require the application of a range of different techniques to attempt to solve what is a difficult problem. At the present time, classical methods of mutation and screening still continue to be a tried, tested, and reliable way to make improvements in β-lactam antibiotic production strains. It is anticipated that the newer and more rational techniques will begin to make a significant impact in the not-too-distant future.

REFERENCES

1 Barredo, J.L., B. Diez, E. Alvarez and J.F. Martin. 1989. Large amplification of a 35-kb DNA fragment carrying two penicillin biosynthetic genes in high penicillin producing strains of *Penicillium chrysogenum*. Curr. Genet. 16: 453–459.

2 Brundidge, S.P., F.C.A. Gaeta, D.J. Hook, C. Sapino, R.P. Elander and R.B. Morin. 1980. Association of 6-oxo-piperidine-2-carboxylic acid with penicillin V production in *Penicillium chrysogenum* fermentations. J. Antibiot. 33: 1348–1351.

3 Chater, K.F. 1990. The improving prospects for yield increase by genetic engineering in antibiotic-producing streptomycetes. Bio/Technol. 8: 115–121.

4 Chiang, S-J.D., L.T. Chang, Y.S. Chen, H.H. Hou and R.P. Elander. 1991. Strain improvement in *Penicillium chrysogenum*: From classical genetics to genetic engineering. In: 50 Years of Penicillin Application – History and Trends (Kleinkauf, H. and H. von Dohren, eds.), in press.

5 Ingolia, T.D. and S.W. Queener. 1989. Beta-lactam biosynthetic genes. Med. Res. Rev. 9: 245–264.

6 Muller, W.H., T.P. van der Krift, A.J.J. Krouwer, H.A.B. Wosten, L.H.M. van der Voort, E.B. Smaal and A.J. Verkleij.

1991. Localization of the pathway of the penicillin biosynthesis in *Penicillium chrysogenum*. EMBO J 10: 489–495.

7 Pang, C.-P., B. Chakravarti, R.M. Adlington, H.-H. Ting, R.L. White, G.S. Jayatilake, J.E. Baldwin and E.P. Abraham. 1984. Purification of isopenicillin N synthetase. Biochem. J. 222: 789–795.

8 Pirt, S.J. 1987. Microbial physiology in the penicillin fermentation. Trends Biotechnol. 5: 69–72.

9 Ramos, F.R., M.J. Lopez-Nieto and J.F. Martin. 1985. Isopenicillin N synthetase of *Penicillium chrysogenum*, an enzyme that converts δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine to isopenicillin N. Antimicrob. Ag. Chemother. 27: 380–387.

10 Ramos, F.R., M.J. Lopez-Nieto and J.F. Martin. 1986. Coordinate increase of isopenicillin N synthetase, isopenicillin N epimerase and deacetoxycephalosporin C synthetase in a high cephalosporin-producing mutant of *Acremonium chrysogenum* and simultaneous loss of the three enzymes in a non-producing mutant. FEMS Microbiol. Lett. 35: 123–127.

11 Revilla, G., F.R. Ramos, M.J. Lopez-Nieto, E. Alvarez and J.F. Martin. 1986. Glucose represses formation of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine and isopenicillin N synthase but not penicillin acyltransferase in *Penicillium chrysogenum*. J. Bacteriol. 168: 947–952.

12 Roninson, I.B. 1983. Detection and mapping of homologous, repeated and amplified DNA sequences by DNA renaturation in agarose gels. Nucleic Acids Res. 11: 5413–5431.

13 Shen, Y.-Q., S. Wolfe and A.L. Demain. 1986. Levels of isopenicillin N synthetase and deacetoxycephalosporin C synthetase in *Cephalosporium acremonium* producing high and low levels of cephalosporin C. Bio/Technol. 4: 61–64.

14 Skatrud, P.L., S.W. Queener, D.L. Fisher and J.L. Chapman. 1987. Strain improvement studies in *Penicillium chrysogenum* using the cloned *P. chrysogenum* isopenicillin N synthetase gene and the amdS gene of *Aspergillus nidulans*. SIM Annual Meeting Abstr. P-46.

15 Skatrud, P.L., A.J. Tietz, T.D. Ingolia, C.A. Cantwell, D.L. Fisher, J.L. Chapman and S.W. Queener. 1989. Use of recombinant DNA to improve production of cephalosporin C by *Cephalosporium acremonium*. Bio/Technol. 7: 477–485.

16 Smith, D.J., J.H. Bull, J. Edwards and G. Turner. 1989. Amplification of the isopenicillin N synthetase gene in a strain of *Penicillium chrysogenum* producing high levels of penicillin. Mol. Gen. Genet. 216: 492–497.

17 Snell, K. and D.A. Duff. 1984. Branched-chain amino acid metabolism and alanine formation in rat diaphragm muscle in vitro. Biochem. J. 223: 831–835.

18 Soliday, C.L., M.S. Crawford and J.A. Rambosek. 1990. Strains of *Penicillium chrysogenum* producing high levels of penicillin show no amplification of genes in the biosynthetic pathway. SIM Annual Meeting Abstr. P-43.

19 Usher, J.J., M.A. Lewis and D.W. Hughes. 1985. Determination by high-performance liquid chromatography of some compounds involved in the biosynthesis of penicillin and cephalosporin. Anal. Biochem. 149: 105–110.

20 Usher, J.J., M.A. Lewis, D.W. Hughes and B.J. Compton. 1988. Development of the cephalosporin C fermentation taking into account the instability of cephalosporin C. Biotechnol. Lett. 10: 543–548.

21 Usher, J.J., M.A. Lewis and D.W. Hughes. 1990. Measurement of β-lactam biosynthetic enzymes in *Cephalosporium acremonium*. Anal. Biochem., submitted for publication.

22 Whitkop, C. and J. Tabor. 1986. Correlation of gene amplication and formation of secondary metabolites. Abstracts in Fungal Genetics Conference 1986.