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MINIREVIEW

Molecular Biology of Penicillin and Cephalosporin Biosynthesis

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INTRODUCTION

In 1984, Sir Edward P. Abraham and I initiated a collaboration to clone the gene responsible for forming the bicyclic ring structure of penicillins. D. Perry, E. P. Abraham, and J. E. Baldwin (Oxford University) supplied a homogeneous preparation of isopenicillin N (IPN) synthetase [synonym, δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) cyclase] from *Cephalosporium acremonium* (*Acremonium chrysogenum*) to T. D. Ingolia and colleagues (Eli Lilly & Co.), who determined its N-terminal amino acid sequence, cloned the corresponding gene, *pcbC*, by reverse genetics, and expressed the *pcbC* open reading frame (ORF) in *Escherichia coli* (35). From that time forward, the understanding of the genes responsible for the biosynthesis of penicillin and cephalosporin antibiotics has rapidly increased. Recent progress is highlighted in this short overview. For brevity, citations are representative, rather than exhaustive, and emphasize first examples.

pcbC, THE GENE ENCODING ACV CYCLASE

In the biosynthesis of penicillins and cephalosporins (Fig. 1) (1, 4, 33), a tripeptide ACV, is oxidatively cyclized to IPN (2). IPN synthetase (synonym, ACV cyclase) (30) catalyzes this reaction, and the *pcbC* gene encodes the enzyme (35). The designation *pcb* stands for penicillin and cephalosporin biosynthesis and denotes genes encoding enzymes catalyzing steps between amino acid precursors (L-α-aminoadipic acid, L-cysteine, L-valine) and IPN. IPN may be viewed as a "branchpoint" intermediate which is converted, by separate pathways, to either cephalosporins in fungi and bacteria or acyltransfer penicillins in fungi.

In the 3 years following its initial cloning, the *pcbC* gene was cloned from *Penicillium chrysogenum* (14), *Aspergillus nidulans* (34, 45), *Streptomyces clavuligerus* (25), and *Streptomyces lipmanii* (45). The ORFs of several *pcbC* genes have been expressed in *E. coli* (14, 35, 45).

Exploration of the substrate specificity of IPN synthetase by Baldwin and colleagues (5–7, 9) and Gesellchen and colleagues (P. D. Gesellchen, R. B. Rothenberger, J. J. Osborne, D. Preston, G. Huffman, R. D. G. Cooper, A. Hunt, J. L. Chapman, and S. W. Queener, Abstr. 10th Am. Peptide Symp., 1987) was encouraged by the availability of large quantities of ACV cyclase obtainable from *E. coli*, although many conversions were achieved with ACV cyclase from nonrecombinant sources by other investigators (46). The variety of products formed by the action of ACV cyclase on chemically synthesized ACV analogs has strengthened arguments for a free radical mechanism in the cyclization step (4). ACV cyclase produced via the *C. acremonium pcbC* gene in *E. coli* appears to have the same substrate specificity as the ACV cyclase from the fungus (10).

Samson and colleagues changed the two cysteine codons of the *pcbC* gene from *C. acremonium* to serine codons, separately or together, and characterized the corresponding enzymes to establish the relative importance but nonessential nature of these residues in the formation of the first antibiotically active intermediate in cephalosporin biosynthesis (36).

cefEF AND *cefE*, GENES ENCODING PENICILLIN N EXPANDASE; AND *cefF* AND *cefEF*, GENES ENCODING DAOC 3′-HYDROXYLASE

In the biosynthesis of cephalosporins (Fig. 1), penicillin N is oxidatively ring expanded to deacetoxycephalosporin C (DAOC) (22) and DAOC is hydroxylated at its 3′-methyl carbon to deacetylcephalosporin C (13).

In *C. acremonium*, purification and amino acid sequence determination of a bifunctional penicillin N expandase/DAOC 3′-hydroxylase (16), reverse genetics, and expression of the cloned *cefEF* gene in *E. coli* (37) established unequivocally, as suggested earlier (38), that in this fungus, one protein is responsible for the ring expansion of penicillin N to DAOC and the 3′ hydroxylation of DAOC to deacetylcephalosporin C. In *S. clavuligerus*, penicillin N expandase (synonyms, DOAC synthetase and DAOC synthase) and DAOC 3′-hydroxylase (synonym, deacetylcephalosporin C synthetase) are separable (19). A *cefE* gene which encoded penicillin N expandase was cloned from *S. clavuligerus* and expressed in *E. coli* (23). Recombinant expandase from *E. coli* was purified to homogeneity (16a).

The designation *cef* denotes a gene encoding an enzyme catalyzing a step or steps committed to the biosynthesis of cephalosporins, i.e., steps between IPN and the final cephalosporin end product (18).

There is only 11% similarity between the *pcbC* gene and the *cefEF* gene of *C. acremonium* (18), and the enzymes differ fundamentally in their mechanisms. Ring expansion of penicillin N and 3′-hydroxylation of DAOC require concomitant conversion of 2-oxoglutarate to succinate, but cyclization of ACV to IPN does not require the presence of 2-oxoglutarate as a cosubstrate (16, 19, 24). The enzymes catalyzing each of these reactions require Fe^{2+} and are stimulated by dithiothreitol. Expandase from *C. acremonium* exhibits stringent substrate specificity (8, 16); for example, it does not utilize IPN or penicillin V as a substrate.

There is 57% identity at the amino acid level (sequence deduced from the DNA sequence; Fig. 2) and there is 65% identity at the nucleotide level between the *cefEF* gene of *C. acremonium* and the *cefE* gene of *S. clavuligerus*. The *cefEF*

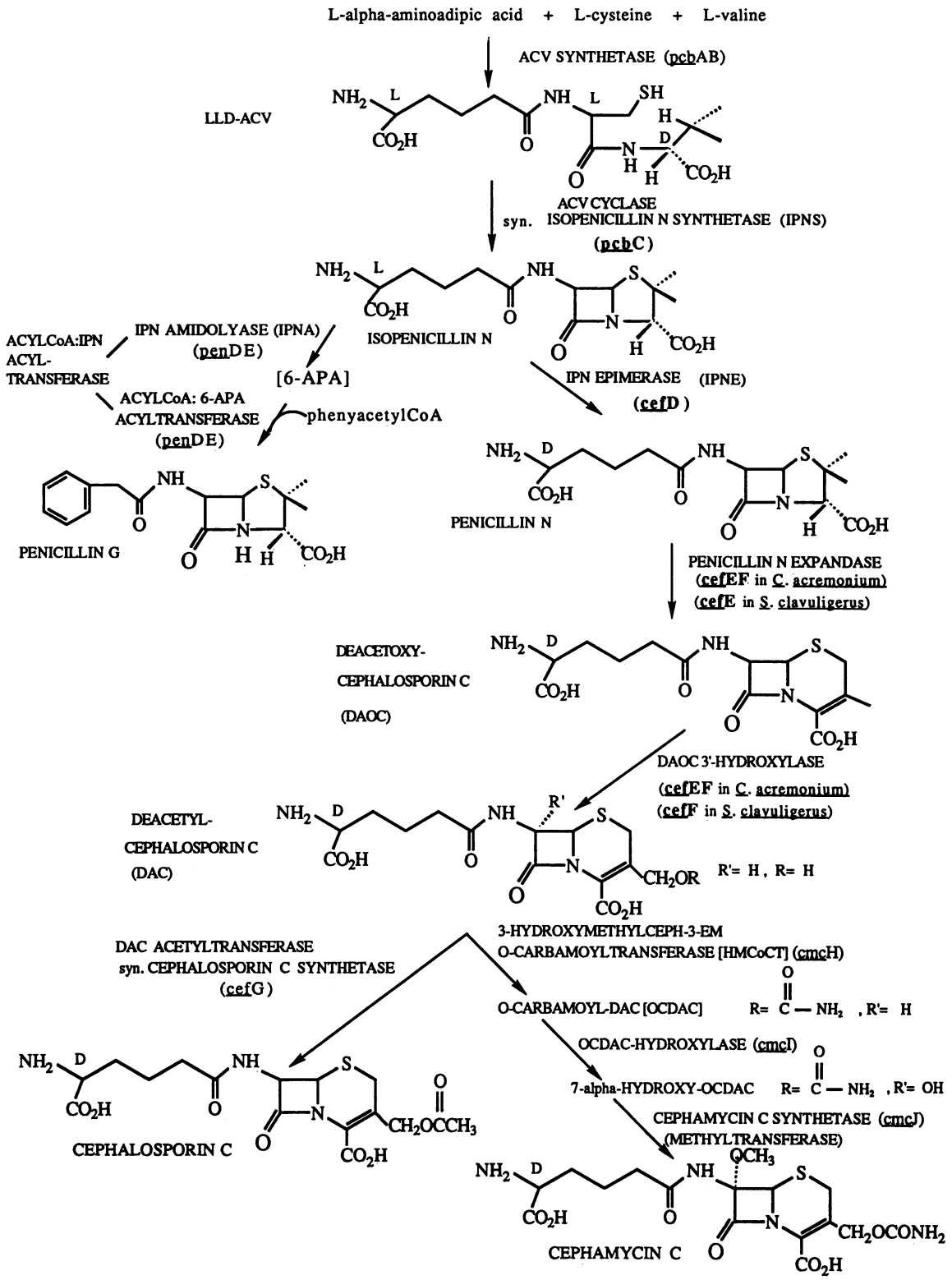

FIG. 1. Biosynthetic pathways for the sulfur-containing β-lactam antibiotics penicillin G, cephalosporin C, and cephamycin C. When gene designations are shown in boldface type, the gene has been cloned and expressed in *E. coli*; gene designations indicated in lightface type are provisional and predict the nature of genes based on the current understanding of a corresponding gene product. The designation *penDE* for acyl-CoA:IPN acyltransferase is in lightface type to indicate the concept of a gene encoding a polypeptide with an IPN amidolyase domain and an acyl-CoA:6-APA acyltransferase domain; an acyltransferase gene has been cloned (44), but the domains in the gene product have not been proven. *cmc* stands for cephamycin and denotes genes encoding enzymes catalyzing the conversion of DAC to a cephamycin antibiotic (cephalosporin with a 7-α-OCH_3 moiety). DAC may be viewed as a branchpoint intermediate which is converted, by separate pathways, to cephalosporin C in *C. acremonium* and to cephamycin C in *S. clavuligerus*.

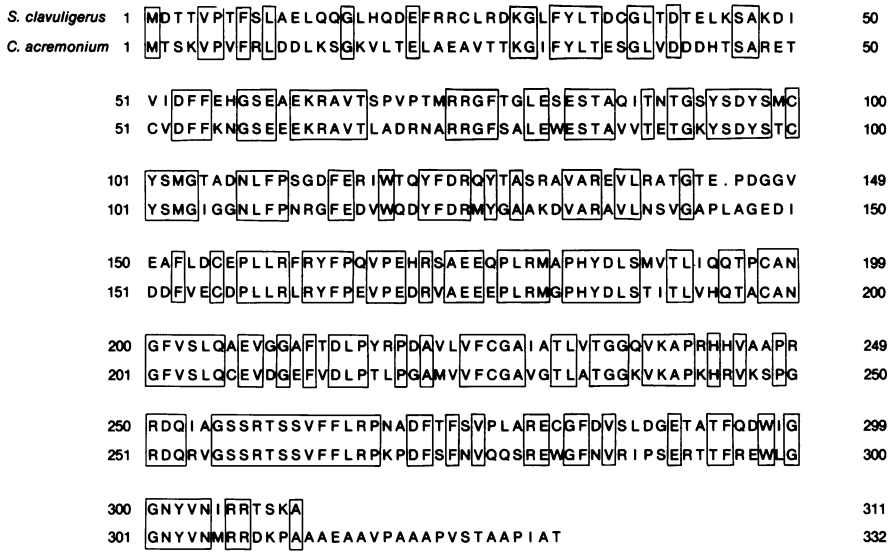

FIG. 2. Comparison of the amino acid sequence of the *S. clavuligerus* expandase to the amino acid sequence of the *C. acremonium* expandase/hydroxylase (28). The amino acid sequence of the *S. clavuligerus* expandase is that predicted by the nucleotide sequence of the *cefE* gene (23); the amino acid sequence of the *C. acremonium* expandase/hydroxylase is that predicted by the nucleotide sequence of the *cefEF* gene (37).

gene codes for 19 carboxy-terminal amino acids which are not encoded by the shorter *cefE* gene (18, 28).

A practical use has been identified for the cloned *cefEF* gene of *C. acremonium*. A highly developed production strain of *C. acremonium* was shown to produce significant amounts of penicillin N as an unwanted by-product in the production of cephalosporin C. By use of a transformation system (32, 40) based on a hybrid dominant maker constructed (40) from a *C. acremonium* promoter and a bacterial ORF (21), an extra copy of the *cefEF* gene was heterologously integrated into this *C. acremonium* stain (41). A transformant, LU4-79-6, exhibited twice as much penicillin N expandase activity as did the recipient and converted virtually all penicillin N to cephalosporin C, with a resulting increase in cephalosporin C production (41).

Recently, DAOC 3′-hydroxylase was purified to homogeneity from *S. clavuligerus* (W. K. Yeh, personal communication), and amino acid sequence information obtained from this protein was used in cloning the *cefF* gene, which encodes the hydroxylase (J. R. Miller, personal communication).

A direct comparison of very active enzyme preparations has recently shown that *C. acremonium* expandase/hydroxylase, *S. clavuligerus* penicillin N expandase, and *S. clavuligerus* DAOC 3′-hydroxylase all catalyze ring expansion of penicillin N and 3′-hydroxylation of DAOC. Both reactions are efficiently catalyzed by the bifunctional fungal enzyme, but only one reaction, ring expansion or hydroxylation, is efficiently catalyzed by the bacterial enzymes (W. K. Yeh, personal communication).

cefD, THE GENE ENCODING IPN EPIMERASE

In the biosynthesis of cephalosporins (Fig. 1), IPN must be epimerized to penicillin N; penicillin N but not IPN is a substrate for ring expansion enzymes. IPN epimerase catalyzes this reaction (27), and the *cefD* gene encodes this enzyme (18). The designation *cef* rather than *pcb* is used because penicillin N is not found in fungi that produce acyltransfer penicillins; i.e., the gene appears to be associated with the biosynthesis of cephalosporin end products and not penicillin end products.

Usui and Yu (42) purified and characterized IPN epimerase. They determined the N-terminal sequence of the enzyme. Using this information, Kovacevic and colleagues cloned and expressed in *E. coli* the ORF encoding this enzyme (S. Kovacevic, M. B. Tobin, and J. R. Miller, submitted for publication).

A GENE ENCODING ACYL-CoA:IPN ACYLTRANSFERASE

In the biosynthesis of acyltransfer penicillins (e.g., penicillin G, penicillin V) (Fig. 1), the L-α-aminoadipyl side chain of IPN is exchanged for the acyl side chain of coenzyme A (CoA) derivatives of a variety of monosubstituted acetic acids, most notably, phenylacetic acid (for penicillin G biosynthesis) and phenoxyacetic acid (for penicillin V biosynthesis). The enzyme catalyzing this reaction is acyl-CoA:IPN acyltransferase (3, 33) and is encoded by a gene recently cloned by Veenstra and colleagues (44). The designation *pen* has been suggested for this gene, since it is involved in the biosynthesis of acyltransfer penicillins but is not present in the biosynthetic pathways for cephalosporins (18).

The literature on acyl-CoA:IPN acyltransferase can be confusing because the enzyme utilizes a variety of acyl substrates and has been assayed in many ways, often in crude form in extracts in which other acylases may have been present. The acyltransferase partially purified by Preuss and Johnson 20 years ago (31) using a side chain exhange assay is probably the same enzyme recently purified to homogeneity (3) using an acyl-CoA:6-aminopenicillanic acid (6-APA) acyltransferase assay.

Alvarez and co-workers isolated a 29,000-dalton acyl-CoA:6-APA acyltransferase from *P. chrysogenum* (catalyzes the condensation of 6-APA with phenylacetyl-CoA to form penicillin G and CoA (Fig. 1) (3). Using reverse genetics, Veenstra and colleagues (44) cloned a gene encoding a 39,000-dalton protein, which probably comprises both

the 29,000-dalton protein and a 10,000-dalton protein. The gene complemented a mutation that prevents the conversion of IPN to penicillin G in a *P. chrysogenum* strain unable to make penicillin G.

P. Whiteman, E. P. Abraham, and colleagues (Oxford University) purified the 39,000-dalton protein to homogeneity (personal communication). The Oxford group demonstrated copurification of enzyme activities that catalyzed the following reactions: IPN + H_2O → L-α-aminoadipic acid + 6-APA (IPN amidolyase); IPN + phenylacetyl-CoA → penicillin G + CoA (acyl-CoA:IPN acyltransferase); and 6-APA + phenylacetyl-CoA → penicillin G + CoA (acyl-CoA:6-APA acyltransferase). They supplied the amino acid sequence to M. B. Tobin, J. R. Miller, and colleagues (Eli Lilly & Co.), who used the sequence to design a probe for the corresponding gene (personal communication). The gene encoding the 39,000-dalton protein was cloned from a cDNA library and was expressed in *E. coli*. Both the 6-APA → penicillin G and IPN → penicillin G activities were detected.

It is likely that 6-APA remains bound to acyl-CoA:IPN acyltransferase when the enzyme is saturated with appropriate acyl-CoA substrates but is released in their absence. Significant amounts of 6-APA are produced when exogenous side chain precursors are not fed to *P. chrysogenum*.

In their purification of the 39,000-dalton protein, P. Whiteman, E. P. Abraham, and colleagues observed the following relative specific activities: IPN amidolyase reaction < acyl-CoA:IPN acyltransferase reaction ≪ acyl-CoA:6-APA acyltransferase reaction (E. P. Abraham, personal communication).

The amidolyase activity, which must function to catalyze the rupture of the C-N bond in the amido moiety of IPN, may act coordinately with the activity that forms the C-N bond in the amido moiety of the end-product penicillin, e.g., penicillin G or V. The molar yield of 6-APA in fermentations not fed with an appropriate monosubstituted acetic acid precursor is low relative to that of penicillin G or V produced in fermentations fed phenylacetic or phenoxyacetic acid, respectively. The high relative specific activity for the acyl-CoA:6-APA acyltransferase reaction suggests that for acyl-CoA:IPN acyltransferase, C-N bond formation would not limit the rate of conversion of IPN to penicillin G.

ACV SYNTHETASE

The first step in the biosynthesis of penicillins and cephalosporins (Fig. 1) involves the formation of the tripeptide ACV from L-α-aminoadipic acid, L-cysteine, and L-valine (17). ACV synthetase catalyzes this reaction, which requires the hydrolysis of ATP (11); hence, ACV synthetase appropriately denotes a ligase-type mechanism. (Several commonly used names of penicillin and cephalosporin biosynthetic enzymes include synthetase or synthase in a trivial sense, i.e., unrelated to mechanistic class and simply to denote a synthetic reaction.) A *pcb* designation would be appropriate for a gene encoding ACV synthetase, since it functions in the biosynthesis of cephalosporins and acyltransfer penicillins.

ACV synthetase has been observed in *C. acremonium* (11, 17), *S. clavuligerus* (20), *A. nidulans* (43), and *P. chrysogenum* (17). Catalysis of the subreactions A + C → AC and AC + V → ACV has been observed with extracts of *C. acremonium*, but the overall reaction A + C + V → ACV appears to be more efficient (11). Epimerization of L-valine occurs concomitantly with its incorporation into ACV; D-valine is not a substrate for ACV synthetase. The ACV synthetase from *A. nidulans* has been purified and appears to be a single enzyme with a molecular weight of ca. 220,000 (43). With methods for purifying ACV synthetase at hand, the use of reverse genetics to clone the ACV synthetase gene is possible. Progress in cloning has been made by several groups.

ORGANIZATION OF *pcb*, *pen*, and *cef* GENES IN FUNGI AND *STREPTOMYCES* SPP.

In an industrial strain of *C. acremonium*, eight chromosomes have been separated by pulsed-field gel electrophoresis and the *pcbC* and *cefEF* genes have been located to chromosomes VI and II, respectively (39). This separation of genes is in contrast to the situation in *Streptomyces* spp., in which the *pcbC* gene and the *cefE* gene are clustered, with only ca. 25 kilobases of DNA separating them (23). The *pcbC* gene and the gene for acyl-CoA:IPN acyltransferase in *P. chrysogenum* are clustered (44). The *pcbAB*, *pcbC*, and *penDE* genes are clustered within a 20-kilobase region of genomic DNA in *A. nidulans* (27a).

EVOLUTION OF THE GENES FOR CEPHALOSPORIN AND PENICILLIN BIOSYNTHESIS

Analysis and comparison of the DNA sequences and deduced amino acid sequences of five *pcbC* genes from *Streptomyces* spp. and fungi caused T. Ingolia to conclude that cephalosporin biosynthetic genes probably evolved in bacteria, were transferred to a primordial fungus, and diverged separately in fungi and bacteria (14, 18, 45). The origin in bacteria was suggested by the lack of introns in these genes and the lack of introns in genes encoding proteins with penicillin N expandase activity. That evolution occurred first in bacteria was also suggested by the fact that cephalosporin pathways are more elaborate in *Streptomyces* spp. For example, 7-α-hydroxylations and 3′-carbamylations of cephalosporins occur in these bacteria but have not been observed in fungi. On the basis of a mutation rate of 10^{-9} base changes per year per nucleotide (26), the differences in DNA sequence place the divergence of bacterial and fungal *pcbC* genes 370 million years in the past. However, a variety of studies have indicated that bacteria and fungi diverged 2 billion years ago. Thus, clustered genes encoding the main portion of a cephalosporin pathway are suggested to have been transferred from an ancestral bacterium to an early fungus.

Both *cefE*, encoding penicillin N expandase, and *cefF*, encoding DAOC 3′-hydroxylase, have been demonstrated in bacteria. Modification of either a *cefE* or *cefF* gene may have given rise to fully bifunctional forms like the *cefEF* gene of *C. acremonium*.

The acyl-CoA:IPN acyltransferase gene in *P. chrysogenum* contains introns (44; M. B. Tobin and J. R. Miller, unpublished results) and thus is likely to have evolved in a eucaryote. Penicillin N expandase and DAOC 3′-hydroxylase have not been observed in fungi that produce acyltransfer penicillins. Loss of the *cefD*, *cefE*, and *cefF* genes (or the *cefD* and *cefEF* genes) from an early cephalosporin-producing fungus and the subsequent evolution of a eucaryotic acylase-encoding gene to an acyl-CoA:IPN acyltransferase gene may have occurred to form the present-day fungi that produce acyltransfer penicillins.

CONSTRUCTION OF NOVEL BIOSYNTHETIC PATHWAYS

Penicillin N expandase and DAOC 3′-hydroxylase are not produced in fungi that produce acyl-CoA:IPN acyltransfer-

ase. Acyltransfer penicillins are easier to process and are produced in a higher yield from *P. chrysogenum* than are natural cephalosporins with the D-α-aminoadipyl side chain from *C. acremonium* and bacteria. Therefore, clinical oral cephalosporins are prepared industrially by isolating an acyltransfer penicillin (e.g., penicillin G) and ring expanding this natural product by synthetic chemistry. For many years, the utility of combining ring expansion and acyl-CoA:IPN acyltransfer in one biosynthetic pathway to produce acyltransfer cephalosporins has been recognized. Investigators fused protoplasts of *P. chrysogenum* and *C. acremonium*, hoping to obtain desirable recombinants, without success.

Recently, transformants of *P. chrysogenum* that exhibit penicillin N expandase activity were prepared. A hybrid gene was constructed by splicing the promoter from the *P. chrysogenum pcbC* gene to the ORF encoding *S. clavuligerus* expandase (C. A. Cantwell, R. J. Beckmann, J. E. Dotzlaf, D. L. Fisher, P. L. Skatrud, W.-K. Yeh, and S. W. Queener, Curr. Genet., in press). A dominant marker-based transformation system developed for *P. chrysogenum* independently by different groups (12; P. L. Skatrud, S. W. Queener, D. L. Fisher, and J. L. Chapman, SIM News **37**:77, 1987) was used by Cantwell and colleagues (Cantwell et al., in press) to insert the hybrid expandase gene into *P. chrysogenum*; some of the transformants produced the same amount of penicillin as the recipient.

Genetic engineering of the *cefE* gene to forms which encode modified versions of expandase is of interest. A form, $cefE^V$, capable of encoding an expandase that could utilize penicillin V rather than penicillin N as a substrate, could make possible the construction of *P. chrysogenum* transformants that produce acyltransfer cephalosporins.

SUMMARY AND PROGNOSIS

In a span of 4 years, investigators have cloned and expressed in *E. coli* genes encoding enzymes for all but one of the steps required for the biosynthesis of the penam and cephem bicyclic ring structures that characterize penicillins and cephalosporins, respectively. The utility of gene dosage with the cloned *cefEF* gene has been demonstrated. Expression of a bacterial expandase ORF in *P. chrysogenum* is an important step towards constructing a novel biosynthetic pathway for acyltransfer cephalosporins. This expression of a recombinant gene in an industrially important fungus and the many novel β-lactams produced in vitro from analogs of ACV by ACV cyclase, taken together, suggest that in the future some novel β-lactams may be made by feeding appropriate simple precursor analogs to fungi or bacteria. Cloning and modification of a gene for ACV synthetase to broaden its substrate specificity would enhance the likelihood of developing such fermentations. It would be more efficient to feed amino acid analogs than to feed synthetic tripeptide analogs.

Much remains to be done. However, there is a new force to drive further efforts. There is a growing realization of how narrow the focus has been with respect to compounds bearing the now famous "enchanted" acetidinone (β-lactam) ring, and there is a growing literature on nonantibacterial effects of β-lactam compounds (29).

The extreme utility of many β-lactams in fighting bacterial infections derives in part from their highly specific covalent interaction with bacterial cell wall biosynthetic enzymes and in part from their low toxicity for mammals. The interaction is particularly effective and occurs at a low concentration because the β-lactam ring does react covalently with nucleophilic active site residues in the cell wall enzymes.

Modifications of the chemical moieties attached to the acetidinone ring can target other medically important enzymes with nucleophilic active sites (15). If low toxicity can be maintained, entirely new uses for β-lactam compounds will be developed for clinical practice. If new uses can be identified, the potential utility of manipulating the biosynthesis of penicillins, cephalosporins, and other β-lactams by molecular biology will be increased significantly.

LITERATURE CITED

1. **Abraham, E. P.** 1986. Biosynthesis of penicillins and cephalosporins, p. 103–116. *In* S. F. Queener, J. A. Weber, and S. W. Queener (ed.), Beta-lactam antibiotics for clinical use. Marcel Dekker, Inc., New York.
2. **Abraham, E. P., J. A. Huddlestone, G. S. Jayatilake, J. O'Sullivan, and R. L. White.** 1981. Conversion of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine to isopenicillin N in cell-free extracts of *Cephalosporium acremonium*, p. 125–134. *In* G. I. Gregory (ed.), Recent advances in the chemistry of β-lactam antibiotics. The Royal Society of Chemistry, London.
3. **Alvarez, E., J. M. Cantoral, J. L. Barredo, and J. F. Martín.** 1987. Purification to homogeneity and characterization of acyl coenzyme A:6-aminopenicillanic acid acyltransferase of *Penicillium chrysogenum*. Antimicrob. Agents Chemother. **31**:1675–1682.
4. **Baldwin, J. E., and E. P. Abraham.** 1988. The biosynthesis of penicillins and cephalosporins. Nat. Prod. Rep. **1988**:129–145.
5. **Baldwin, J. E., R. M. Adlington, A. Basak, S. L. Flitsch, A. K. Forrest, and H.-H. Ting.** 1986. Penicillin biosynthesis: structure-reactivity profile of unsaturated substrates for isopenicillin N synthetase. J. Chem. Soc. Chem. Commun. **3**:273–275.
6. **Baldwin, J. E., R. M. Adlington, A. Basak, S. L. Flitsch, S. Petursson, N. J. Turner, and H.-H. Ting.** 1986. Enzymatic synthesis of a new type of penicillin. J. Chem. Soc. Chem. Commun. **12**:975–976.
7. **Baldwin, J. E., R. M. Adlington, A. Basak, and H.-H. Ting.** 1986. Penicillin biosynthesis: structure-reactivity profile of allenic substrates for isopenicillin N synthetase. J. Chem. Soc. Chem. Commun. **16**:1280–1281.
8. **Baldwin, J. E., R. M. Adlington, M. J. Crabbe, G. Knight, T. Nomoto, C. J. Schofield, and H.-H. Ting.** 1987. Enzymatic ring expansion of penicillins to cephalosporins: side chain specificity. Tetrahedron **43**:3009–3014.
9. **Baldwin, J. E., R. M. Adlington, S. L. Flitsch, H.-H. Ting, and N. J. Turner.** 1986. Penicillin biosynthesis: the origin of hydroxy groups in beta-lactams derived from unsaturated substrates. J. Chem. Soc. Chem. Commun. **17**:1305–1308.
10. **Baldwin, J. E., S. J. Killin, A. J. Pratt, J. D. Sutherland, N. J. Turner, M. J. C. Crabbe, E. P. Abraham, and A. C. Willis.** 1987. Purification and characterization of cloned isopenicillin N synthetase. J. Antibiot. **40**:652–659.
11. **Banko, G., A. L. Demain, and S. Wolfe.** 1987. δ-(L-α-Aminoadipyl)-L-cysteinyl-D-valine synthetase (ACV synthetase): a multifunctional enzyme with broad substrate specificity for synthesis of penicillin and cephalosporin precursors. J. Am. Chem. Soc. **109**:2858–2860.
12. **Beri, R. K., and G. Turner.** 1987. Transformation of *Penicillium chrysogenum* using the *Aspergillus nidulans* amdS gene as a dominant selective marker. Curr. Genet. **11**:639–641.
13. **Brewer, S. J., J. E. Farthing, and M. K. Turner.** 1977. The oxygenation of the 3-methyl group of 7-beta-(5-D-aminoadipamido)-3-methylceph-3-em-4-caboxylic acid, desacetoxycephalosporin C, by extracts of *Acremonium chrysogenum*. Biochem. Soc. Trans. **5**:1024–1026.
14. **Carr, L. G., P. L. Skatrud, M. E. Sheetz II, S. W. Queener, and T. D. Ingolia.** 1986. Cloning and expression of the isopenicillin N synthetase gene from *Penicillium chrysogenum*. Gene **48**: 257–266.
15. **Doherty, J. B., B. M. Ashe, L. W. Argenbright, P. L. Barker, R. J. Bonney, G. O. Chandler, M. E. Dahlgren, C. P. Dorn, Jr., P. E. Finke, R. A. Firestone, D. Fletcher, W. K. Hagmann, R.**

Mumford, L. O'Grady, A. L. Maycock, J. M. Pisano, S. K. Shah, K. R. Thompson, and M. Zimmerman. 1986. Cephalosporin antibiotics can be modified to inhibit human leukocyte elastase. Nature (London) **322**:192–194.

16. **Dotzlaf, J. E., and W.-K. Yeh.** 1987. Copurification and characterization of deacetoxycephalosporin C synthetase/hydroxylase from *Cephalosporium acremonium*. J. Bacteriol. **169**:1611–1618.

16a. **Dotzlaf, J. E., and W. K. Yeh.** 1989. Purification and properties of deacetoxycephalosporin C synthase from recombinant *Escherichia coli* and its comparison with the native enzyme purified from *Streptomyces clavuligerus*. J. Biol. Chem. **264**:10219–10227.

17. **Fawcett, P. A., J. J. Usher, J. A. Huddleston, R. C. Bleaney, J. J. Nisbet, and E. P. Abraham.** 1976. Synthesis of δ-(α-aminoadipyl)cysteinylvaline and its role in penicillin biosynthesis. Biochem. J. **157**:651–660.

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

19. **Jensen, S. E., D. W. S. Westlake, and S. Wolfe.** 1985. Deacetoxycephalosporin C synthetase and deacetoxycephalosporin C hydroxylase are two separate enzymes in *Streptomyces clavuligerus*. J. Antibiot. **38**:263–265.

20. **Jensen, S. E., D. W. S. Westlake, and S. Wolfe.** 1988. Synthesis of the penicillin precursor, δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV), by an immobilized enzyme preparation. FEMS Microbiol. Lett. **49**:213–218.

21. **Kaster, K. R., S. G. Burgett, and T. D. Ingolia.** 1984. Hygromycin B resistance as a dominant marker in yeast. Curr. Genet. **8**:353–358.

22. **Kohsaka, M., and A. L. Demain.** 1976. Conversion of penicillin N to cephalosporins by cell-free extracts of *Cephalosporium acremonium*. Biochem. Biophys. Res. Commun. **70**:465–473.

23. **Kovacevic, S., B. J. Weigel, M. B. Tobin, T. D. Ingolia, and J. R. Miller.** 1989. Cloning, characterization, and expression in *Escherichia coli* of the *Streptomyces clavuligerus* gene encoding deacetoxycephalosporin C synthetase. J. Bacteriol. **171**:754–760.

24. **Kupka, J., Y.-Q. Shen, S. Wolfe, and A. L. Demain.** 1983. Studies on the ring-cyclization and ring-expansion enzymes of β-lactam biosynthesis in *Cephalosporium acremonium*. Can. J. Microbiol. **29**:488–496.

25. **Leskiw, B. K., Y. Aharonowitz, M. Mevarech, S. Wolfe, L. C. Vining, D. W. S. Westlake, and S. E. Jensen.** 1988. Cloning and nucleotide sequence determination of the isopenicillin N synthetase gene from *Streptomyces clavuligerus*. Gene **57**:187–196.

26. **Li, W.-H., C.-C. Luo, and C.-I. Wu.** 1985. Evolution of DNA sequences, p. 1–94. *In* R. J. MacIntyre (ed.), Molecular evolutionary genetics. Plenum Publishing Corp., New York.

27. **Lubbe, C., S. Wolfe, and A. L. Demain.** 1986. Isopenicillin N epimerase activity in a high cephalosporin-producing strain of *Cephalosporium acremonium*. Appl. Microbiol. Biotechnol. **23**:367–368.

27a. **MacCabe, A. P., M. B. R. Riach, S. E. Unkles, and J. R. Kinghorn.** 1990. The *Aspergillus nidulans npe*A locus consists of three contiguous genes required for penicillin biosynthesis. EMBO J. **9**:279–297.

28. **Miller, J. R., and T. D. Ingolia.** 1989. Cloning and characterization of beta-lactam biosynthetic genes. Mol. Microbiol. **3**:689–695.

29. **Neftel, K. A., and U. Hubscher.** 1987. Effects of β-lactam antibiotics on proliferating eucaryotic cells. Antimicrob. Agents Chemother. **31**:1657–1661.

30. **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.

31. **Preuss, D. L., and M. J. Johnson.** 1967. Penicillin acyltransferase in *Penicillium chrysogenum*. J. Bacteriol. **94**:1502–1508.

32. **Queener, S. W., T. D. Ingolia, P. L. Skatrud, J. L. Chapman, and K. R. Kaster.** 1985. A system for genetic transformation of *Cephalosporium acremonium*, p. 468–472. *In* L. Leive (ed.), Microbiology—1985. American Society for Microbiology, Washington, D.C.

33. **Queener, S. W., and N. Neuss.** 1982. Biosynthesis of β-lactam antibiotics, p. 1–81. *In* R. B. Morin and M. Gorman (ed.), Chemistry and biology of β-lactam antibiotics, vol. 3. Academic Press, Inc., New York.

34. **Ramon, D., L. Carramolino, C. Patino, F. Sanchez, and M. A. Penalva.** 1987. Cloning and characterization of the isopenicillin N synthetase gene mediating the formation of the β-lactam ring in *Aspergillus nidulans*. Gene **57**:171–181.

35. **Samson, S. M., R. Belagaje, D. T. Blankenship, J. L. Chapman, D. Perry, P. L. Skatrud, R. M. VanFrank, E. P. Abraham, J. E. Baldwin, S. W. Queener, and T. D. Ingolia.** 1985. Isolation, sequence determination and expression in *E. coli* of the isopenicillin N synthetase gene from *Cephalosporium acremonium*. Nature (London) **318**:191–194.

36. **Samson, S. M., J. L. Chapman, R. Belagaje, S. W. Queener, and T. D. Ingolia.** 1987. Analysis of the role of cysteine residues in isopenicillin N synthetase activity by site-directed mutagenesis. Proc. Natl. Acad. Sci. USA **84**:5705–5709.

37. **Samson, S. M., J. E. Dotzlaf, M. L. Slisz, G. W. Becker, R. M. Van Frank, L. E. Veal, W. K. Yeh, J. R. Miller, S. W. Queener, and T. D. Ingolia.** 1987. Cloning and expression of the fungal expandase/hydroxylase gene involved in cephalosporin C biosynthesis. Bio/Technology **5**:1207–1214.

38. **Scheidegger, A., M. T. Küenzi, and J. Nüesch.** 1984. Partial purification and catalytic properties of a bi-functional enzyme in the biosynthetic pathway of beta-lactams in *Cephalosporium acremonium*. J. Antibiot. **37**:522–531.

39. **Skatrud, P. L., and S. W. Queener.** 1989. An electrophoretic molecular karyotype for an industrial strain of *Cephalosporium acremonium*. Gene **78**:331–338.

40. **Skatrud, P. L., S. W. Queener, L. G. Carr, and D. L. Fisher.** 1987. Efficient integrative transformation of *Cephalosporium acremonium*. Curr. Genet. **12**:337–348.

41. **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/Technology **7**:477–485.

42. **Usui, S., and C.-A. Yu.** 1989. Purification and properties of isopenicillin N epimerase from *Streptomyces clavuligerus*. Biochim. Biophys. Acta **999**:78–85.

43. **van Liempt, H., H. von Dohren, and H. Kleinkauf.** 1989. δ-(L-α-Aminoadipyl)-L-cysteinyl-D-valine synthetase from *Aspergillus nidulans*. J. Biol. Chem. **264**:3680–3684.

44. **Veenstra, A. E., P. van Solingen, H. Huininga-Muurling, B. P. Koekman, M. A. M. Groenen, E. B. Smaal, A. Kattevilder, E. Alvarez, J. L. Barredo, and J. F. Martín.** 1989. Cloning of penicillin biosynthetic genes, p. 262–269. *In* C. L. Hershberger, S. W. Queener, and G. Hegeman (ed.), Genetics and molecular biology of industrial microorganisms. American Society for Microbiology, Washington, D.C.

45. **Weigel, B. J., S. G. Burgett, V. J. Chen, P. L. Skatrud, C. A. Frolik, S. W. Queener, and T. D. Ingolia.** 1988. Cloning and expression in *Escherichia coli* of isopenicillin N synthetase genes from *Streptomyces lipmanii* and *Aspergillus nidulans*. J. Bacteriol. **170**:3817–3826.

46. **Wolfe, S. W., A. L. Demain, S. E. Jensen, and D. W. S. Westlake.** 1984. Enzymatic approach to syntheses of unnatural beta-lactams. Science **226**:1386–1392.