REVIEW ARTICLE

Strategies for manipulation of genes in microbes for production of β-lactam antibiotics*

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The deployment of recombinant DNA techniques to β-lactam antibiotic producing fungi and various *Streptomyces* spp. have made it possible to understand in considerable details, the biosynthetic pathways, the mechanism of synthesis, and the genes involved in the process. As a result, it is becoming possible to manipulate the individual gene or cluster of genes and thereby the pathways in order to develop microbes which can produce β-lactams at rates higher than the traditional strains. By the judicious incorporation of genes such as *cef* E of *Streptomyces clavuligerus* into *Penicillium chrysogenum* it has been possible to produce the cephalosporin intermediate, 7-amino-3-deacetoxycephalosporanic acid (7-ADCA) in the fermenters. The incorporation of *VHb* gene in *Acremonium chrysogenum* increased the productivity of the strain for cephalosporin C. The understanding of the substrate specificity of *pcb C* genes has enabled the synthesis of complex β-lactams from a variety of synthetic tripeptides. The future years are expected to witness the mixing, shuttling, and reshuffling of bacterial and fungal genes among themselves and the maximization of expression of the target gene(s) by modifying the regulatory and the control mechanisms of the biosynthetic pathways in order to synthesize the target β-lactam molecules at economic costs by engineered microbes.

THE β-lactam antibiotics have enjoyed a long history of use as very effective therapeutic agents against a wide range of bacterial infections. The usage of fermentation-based, chemically unmodified β-lactams started with combatting gram-positive bacterial infections like *Streptococcus pneumoniae*, *S. aureus*, and *S. pyogenes*; the chemically modified β-lactams are currently being effectively used to cure infections caused by a large number of rod-shaped pathogenic gram-negative bacterial like *Escherichia coli, Enterobacter, Proteus, Serratia, Klebsiella*, and *Pseudomonas* spp. Presently the β-lactams account for more than 60% of all the antibiotics prescribed world-wide, and are mainly represented by several penicillins and the structurally related cephalosporins, all of which have sulphur-containing 5- or 6-membered rings fused to four-membered β-lactam ring. There are several other nonsulphur containing β-lactams such as clavulanic acid, thienamycin, nocardium A, etc. which have much lower antibacterial activities and which are used in very limited quantities, exception being clavulanic acid which is being used as a β-lactamase inhibitor in combination with ampicillin or amoxycillin. Penicillins and cephalosporins are produced by a number of microorganisms: for example by the fungal species of *Penicillium chrysogenum, Cephalosporium acremonium, Aspergillus nidulans*, and several *Streptomyces* spp. including *S. clavuligerus, S. lipmanii, S. cattleya*, and *Nocardia lactamdurans*. The extensive use of β-lactams is attributed to their therapeutic effectiveness, less toxicity, and abundant availability at cheaper prices. The price reduction has become possible due to tremendous technological developments in the fermentation process, in the genetic improvements of the producer microbial strains, and in the downstream processing and isolation systems. The starting materials for the production of β-lactams are the fermentation-based potassium penicillin G or V (K-Pen G or K-Pen V), which in turn are utilized for the production of 6-aminopenicillanic acid (6-APA) or 7-amino-3-deacetoxycephalosporanic acid (7-ADCA) or The 6-amino-3-chlorocephalo-sporanic acid (7-ACCA). The 6-APA is further converted into a large number of finished β-lactam semisynthetic penicillins, of which ampicillin, amoxycillin and cloxacillin are the most significant ones in terms of usage and may account for more than 90% of the consumption of 6-APA; 7-ADCA is used primarily for the synthesis of cephalexin and partly for cefradine and cefdroxil, and minor quantities (less than 5%) go in for other semisynthetics; 7-ACCA is consumed mainly for the synthesis of cefaclor. The current combined global consumption of K-Pen G and K-Pen V is of the order of 40,000 ton (64000 MMU approximately)[1] with K-Pen G representing nearly 76% of the total. There are two other fermentation-based β-lactam products, namely, cephalosporin C and cephamycin C, which in turn are converted into the intermediates of 7-amino-cephalosporanic acid (7-ACA) and 7-amino-7-alpha-methoxy-3-carbamoyloxy methyl-3-cephem-4-carboxylic acid (7-α-methoxy ACA) respectively, and thereafter they are converted into finished bulk drugs. The 7-ACA is used mainly for the production of cephalothin sodium,

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and 7-α-methoxy ACA for cefoxitin sodium. The combined use of 7-ACA-derived products is of the order of 2400 ton, and requires nearly 5000 ton of the fermentation-based cephalosporin C (ref. 1). The consumption of 7-α-methoxy ACA is considerably lower, about 200 ton. The fermentation-based cephamycin C is consequently required to the extent of 400 ton only[1]. Totally, nearly 60 semisynthetic β-lactams are used globally for therapy, but the finished and formulated drugs mentioned above would account for more than 90% of the global consumption, and consequently the primary attention of the industry is on these products. As the producers of β-lactams depend upon the primary fermentation products, the availability and pricing of these basic products are the most important factors which would shape the future directions of this important industry. It is these basic products which will eventually decide the pricing of the commonly used β-lactam formulations dispensed as tablets, capsules, drypowders, injectables, etc. in therapy. This paper focuses on the recent advances in genetics and molecular biology of the fermentation-based basic β-lactam antibiotic products and their biosynthetic pathways; the basic understandings and the capacity to manipulate the regulatory genes are expected to enable the development of more productive and useful microbial strains for use in the industry in the coming years.

Organization of genes of penicillin, cephalosporin and cephamycin biosynthesis

The first step in the biosynthesis of all the three above-mentioned β-lactam antibiotics involves a complete set of biochemical reactions including the activation and the condensation of three precursor amino acids namely, L-α-aminoadipic acid, L-cysteine, and L-valine to form the tripeptide δ-(L-aminoadipyl)-L-cysteinyl-D-valine (LLD-ACV) as shown in Figure 1. While the L-valine provides for the D-penicillamine fragment in all penicillins, the L-α-aminoadipic acid gets transformed into a D-α-aminoadipyl moiety in penicillin N, and thereafter in all cephalosporins (Figure 1). The LLD-ACV has been found in *P. chrysogenum* mycelium[2–4]. This tripeptide was also shown to be present in *C. acremonium* which produces penicillin N and cephalosporins[5]. From the cell-free extract of *C. acremonium* it was possible to synthesize LLD-ACV using δ-(L-α-amino adipyl)-L-cysteine, and L-valine[6,7]. But the use of either α-aminoadipic acid and L-cysteinyl-L-valine or L-cysteinyl-D-valine as also D-valine and δ-(L-α-aminoadipyl)-L-cysteine could not synthesize the LLD-ACV. It was further demonstrated that α-aminoadipic acid was essential for the initial stages of biosynthesis of penicillins[8] in *P. chrysogenum*, and the mutant strain of *P. chrysogenum* not synthezing α-aminoadipic acid started synthezing penicillins as soon as α-aminoadipic acid was added into the culture medium. The L-lysine is used as the precursor by the microbes for synthesizing their own α-aminoadipic acid, with the help of the enzyme lysine amino transferase which in turn is coded by late stage gene in the biosynthetic pathways, designated as *lat* gene[9,10]. Supplementation of α-aminoadipic acid to mutants not encoding lysine amino transferase could reverse the inhibition of the production of penicillin[11]. In cephalosporin-producing streptomycetes, it was always found that penicillin N was produced as an intermediate and its concentration could be modulated in *C. acremonium* by changing the conditions of production. Furthermore, the mass spectrometric analysis of penicillin N and cephalosporin C products in *C. acremonium* for the appropriately labelled radioactive valine showed that the common precursor LLD-ACV tripeptide was transformed into isopenicillin N (IPN), which is then diverted into penicillin N route for its eventual transformation into the cephalosporins in *Streptomyces* species[12]. In *P. chrysogenum*, the LLD-ACV after transformation into IPN got diverted into penicillins without following the pathways of cephalosporins (Figure 1). In this organism, the LLD-ACV was later cyclized to form isopenicillin N (IPN), as in various *Streptomyces* spp. This is the first intermediate in the penicillin pathway which shows some antibiotic activity. After epimerization to penicillin N, the five-membered thiazolidine ring of this intermediate is expanded to six-membered dihydrothiazine ring of deacetylcephalosporin C in *C. acremonium, S. clavuligerus,* and *N. lactamdurans.* From this point of the pathway, the biosynthetic pathway diverges, leading to the formation of penicillin G in *P. chrysogenum*, cephalosporin C in *C. acremonium*, and cephalosporin C as well as cephamycin C in microbial strains *S. clavuligerus* and *N. lactamdurans*, respectively.

LLD-ACV synthetase (pcb AB)

Invariably in all these organisms, the first condensation reaction of the three amino acids to LLD-ACV tripeptide is catalysed and carried out by the multifunctional enzyme LLD-ACV synthetase encoded by the *pcb AB* gene[13]. Although LLD-ACV synthetase is the first important enzyme of the complex β-lactam biosynthetic pathway, the gene *pcb AB* was the last gene to be cloned. The *pcb AB* gene was initially cloned by the complementation of blocked mutants deficient in LLD-ACV synthetase in *P. chryosegenum*[14], where this gene showed an open reading frame (ORF) of 11376 nucleotides that encodes a large protein with a deduced molecular weight (M_r) of 425,971. This gene was subsequently used as a probe to clone *pcb AB* gene of *C. acremonium, A. nidulans* and of *N. lactamdurans.* The nucleotide sequence of *pcb AB* of *C. acremonium* showed 62–69% homology to *pcb AB* gene of

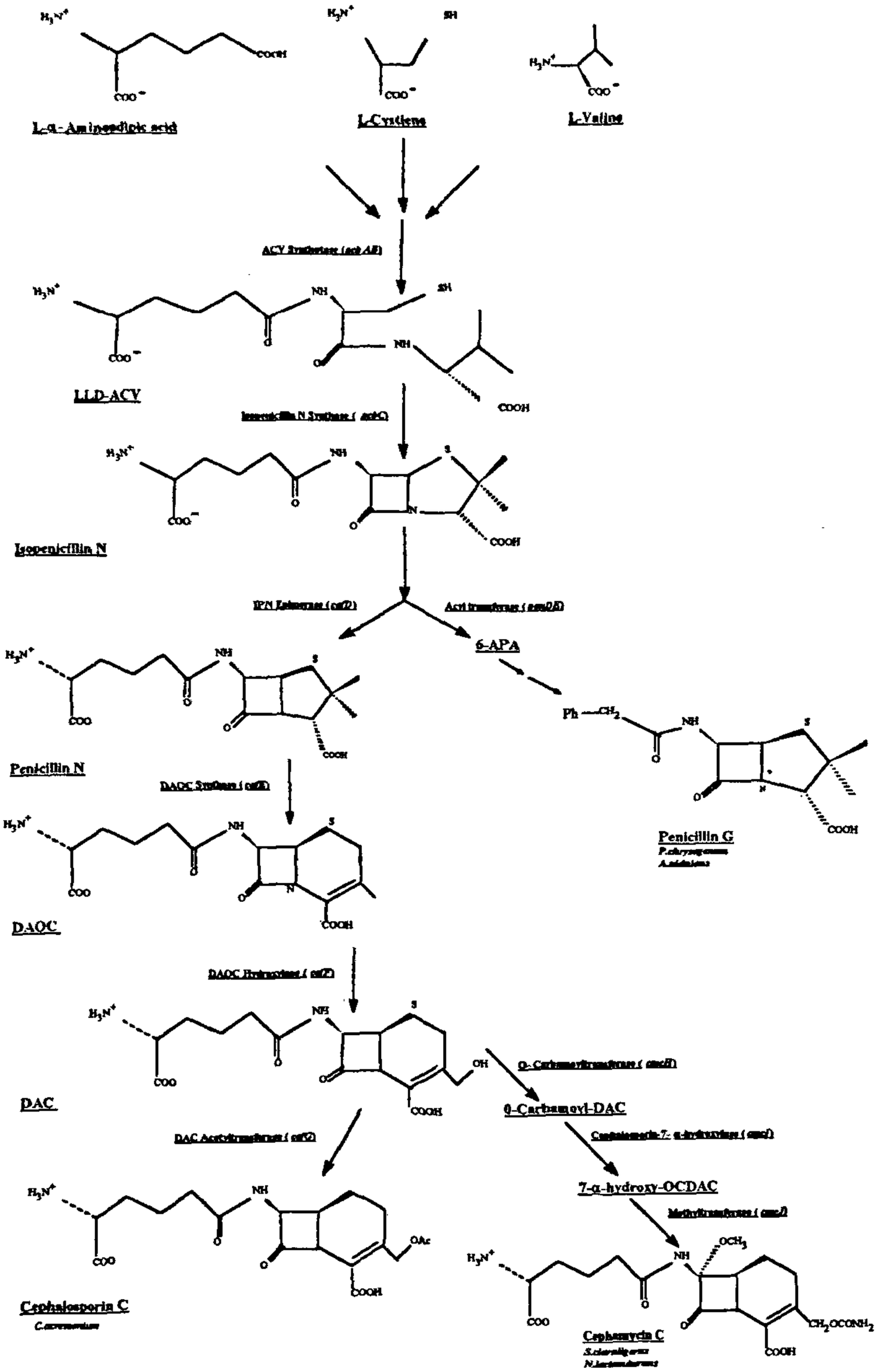

Figure 1. *The common pathway for biosynthesis of penicillins, cephalosporins and cephamycins in bacteria and fungi.* LLD-ACV: D-(L-α-aminoadipyl)-L-cysteinyl-D-valine; 6-APA: 6-aminopenicillanic acid; DAC: deacetylcephalosporin C, DAOC: deacetoxycephalosporin C; IPNS: isopenicillin N; OCDAC: *O*-carbomoyl-deacetoxycephalosporin C. The genes are indicated in parentheses.
*The *pcb* prefix is currently carried to denote the penicillin and cephalosporin biosynthetic genes based on the earlier studies in *P. chrysogenum*, and *cef* to denote genes responsible for the biosynthesis of cephalosporin, initially studied in *C. acremomium*. Depending upon the stages where the gene products code for enzymes that are responsible for the stage product transformation, the genes are designated as *pcb* AB, *pcb* C, etc. and cephalosporins biosynthetic genes as *cef* D, *cef* E, *cef* F, etc.

P. chrysogenum. The *pcb AB* genes studied up to the present time show the presence of three domains[15]. These domains not only align with each other but also with similar domains present in the gramicidin synthetase I of *Bacillus subtilis*[16,17]. However unlike multisubunit organization of *Bacillus* peptide synthetase, all enzyme functions involved in LLD-ACV synthase activity namely activation, epimerization, and polymerization

are contained within different domains of one large polypeptide[9], which is a multifunctional polypeptide. In addition, its smaller size of 420 kDa makes it an attractive system for genetic manipulations (Figure 2).

Isopenicillin N synthase (pcb C)

The next enzyme of the biosynthetic pathway is isopenicillin N synthase (IPNS), which catalyses the cyclization of the tripeptide LLD-ACV to form isopenicillin N, the first β-lactam-structure-containing-intermediate in the pathway. The IPNS captures and uses one molecule of oxygen and converts the LLD-ACV tripeptide into IPN (Figure 3)[18,19]. The IPNS is a nonheme ferrous-dependent enzyme and can transfer one or both atoms of oxygen into the substrates. Suitable incorporation of other genes at the right position in the chromosome of the microbe which would facilitate the capture, transfer and use of molecular oxygen at this stage or other stages requiring oxygen (like VHb gene), could increase and ease the capacity of the microbes in the biochemical transformation and may prove useful. IPNS is coded by a gene *pcb C*. This gene was first cloned from *C. acremonium* and was also expressed in *E. coli*[20]. It was established that the *pcb C* genes are closely linked on the same chromosome with *pcb AB* located upstream of *pcb C* (ref. 21). The *pcb AB* and *pcb C* genes in different β-lactam-producing fungi, are expressed from divergent promotors[14,22] and in actinomycetes producing cephamycin C such as *N. lactamdurans* and *S. clavuligerus*, the two genes are arranged head to tail and are expressed in the same orientation. Further, in these microbes *pcb AB* as well as *pcb C* genes are linked to *lat* (which also code lysine

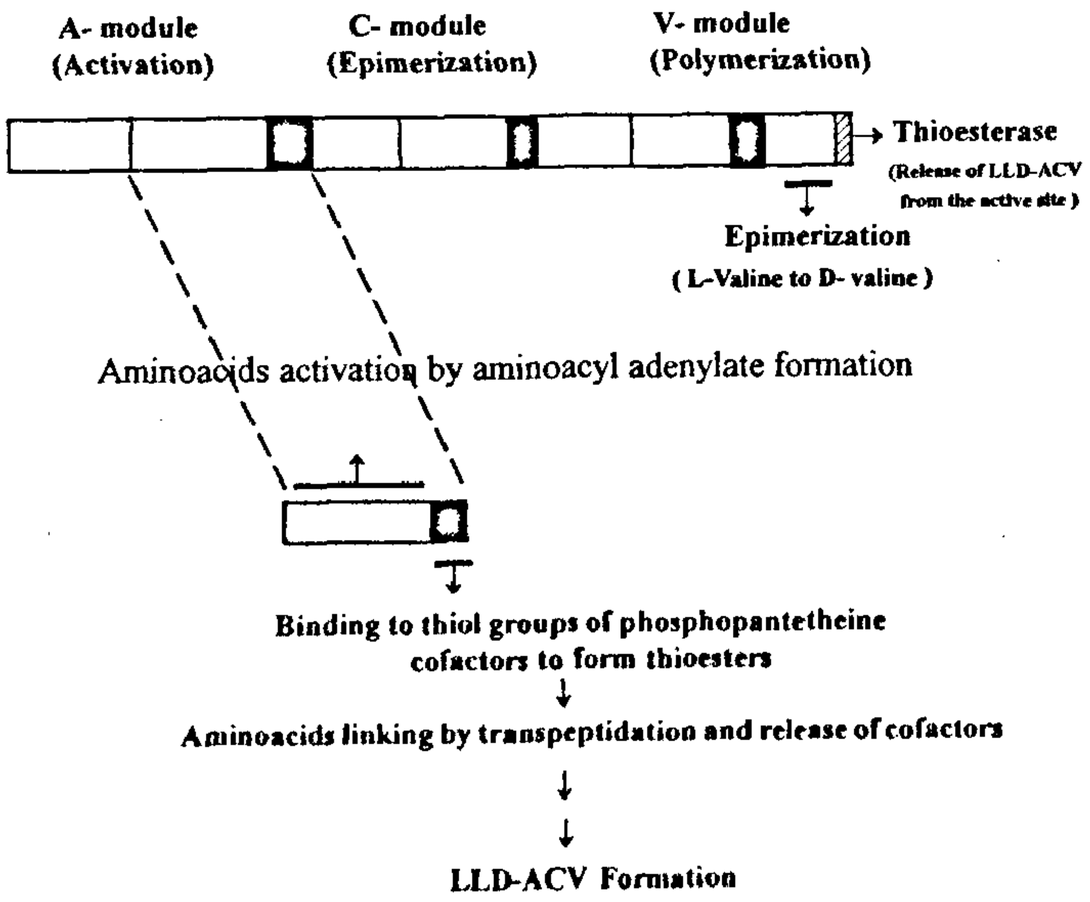

Figure 2. The modular organization of the ACV synthase polypeptide. A: α-aminoadipicate domain; C; cystein domain; V: valine domain.

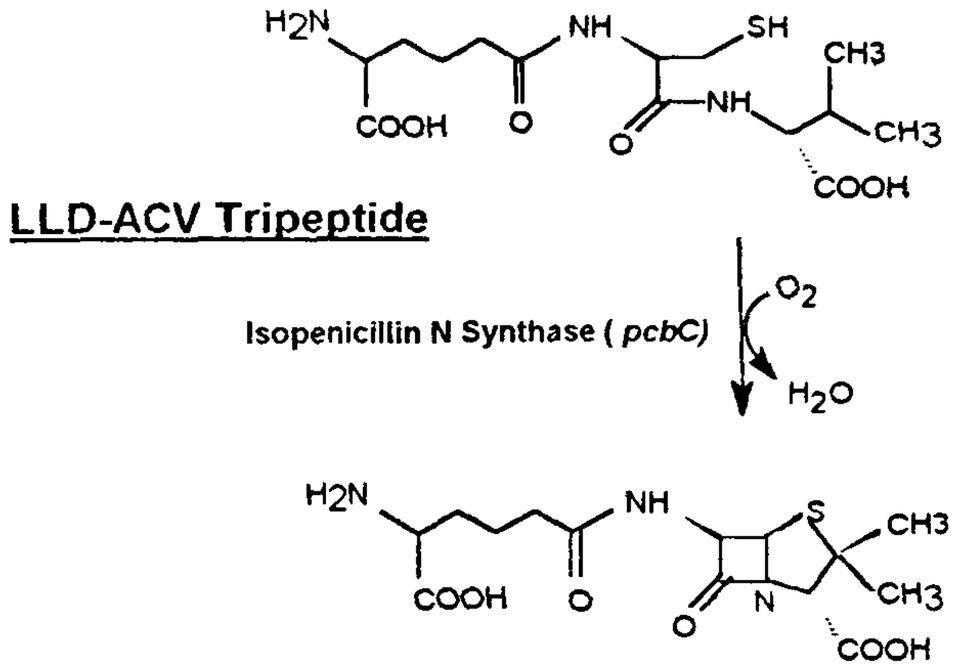

Figure 3. Biosynthetic enzyme (*pcb* C gene) for the conversion of LLD-ACV tripeptide into IPN.

aminotransferase) genes which participates in the antibiotic biosynthetic pathway during earlier stages[9,23] (not shown in Figure 1). As with *pcb AB*, the *pcb C* sequences from different sources show extensive nucleotide similarly at the levels of both the nucleotides and deduced amino acid sequence level[22,24,25]. Because of the central importance of *pcb C* gene in the biosynthesis of penicillin, cephalosporin and cephamycin, this gene has been the major target for genetic manipulations (Figure 3) the world over. Isopenicillin N is considered an important junction point intermediate in the penicillin/cepholosporin pathway as mentioned earlier. All the reported IPNS enzymes have been found to have molecular weight of 30–40 kDa, and the sequences are highly conserved[26]. Methods for maximizing the copy number of *pcb C* gene into organisms are expected to increase the productivity of the microbial strains, if they are otherwise competent to process the produced IPN faster or if the earlier steps are not rate limiting.

The conversion of isopencillin N to various penicillins, and intermediates of the cephalosporins requires several enzymatic steps in two branched pathways (Figure 1). Many genes encoding these steps have already been cloned from bacteria as well as from fungi. For instance, during penicillin G synthesis in *P. crysogenum* the *pen DE* gene encodes the acyltransferase that catalyses the final step in penicillin synthesis[27]. The *pen DE* gene of *P. chrysogenum* and *A. nidulans* is similar, but is absent in cephalosporin-producing prokaryotes. The mechanisms of conversion and properties of the enzymes encoded by *pen DE* have already been described in detail elsewhere[28]. The branching of isopenicillin N into the cephalosporin pathway occurs only in cephalosporin-producing fungi and prokaryotes. The *cef D* gene, which encodes isopenicillin N epimerase, could not be cloned from *C. acremonium* and has

been cloned from *Streptomyces clavuligerus*[29]. It was identified[9,30] that the *lat* genes which apart from encoding enzymes of the late biosynthetic steps also encode lysine amino transferase which forms α-aminoadipic acid (a precursor of β-lactam antibiotics) from lysine. Further studies on the *cef D* and the *cef E* genes[31,32] of *Nocardia lactamdurans* revealed that these two genes are located 0.63 kb upstream from *lat* gene, and further, the *cef* E gene was found to contain an ORF of 1197 nucleotides encoding a protein of 398 amino acids with M_r of 43622. The deduced amino acid sequence exhibited 62.2% identity to the *cef D* gene product of *S. clavuligerus*.

Thiazoline ring expansion

The branching step in the cephalosporin biosynthesis is the expansion of five-membered thiazolidine ring of penicillin N into six-membered dehydrothiazine ring, which is characteristic of all cephalosporins (Figure 1). The ring-expanding reaction is catalysed by deacetoxycepholosporin C synthase, encoded by *cef E*. The *cef E* gene in *N. lactamdurans* was found to contain an ORF of 946 nucleotides encoding a protein of 314 amino acids with a M_r of 34532 which is similar to the deacetoxycephalosporin C synthase of *Streptomyces clavuligerus*[32]. Expression of both the genes, *cef D* and *cef E*, in *S. lividans* resulted in deacetoxycephalosporin C synthase and isopencilin N epimerase activities 10–12 times higher than those in *N. lactamdurans*. The *cef D* and *cef E* genes of *N. lactamdurans* are closely linked; however, the overall organization of the cephalosporin gene cluster differed among these organisms. In *C. acremonium*, the *cef* EF encodes the bifunctional expandase/hydroxylase which expands the ring penicillin N to deacetoxycephalosporin C (DAOC) and hydroxylates DAOC to form deacetylcephalosporin C. The *cef G* gene encodes DAC acetyltransferase which catalyses the final step in the synthesis, leading to the formation of cephalosporin C.

Although the *pcb AB* and the *cef EF* genes in *P. chrysogenum* and *C. acremonium* are single ORFs, each encoding single polypeptides possessing two or more distinct enzymatic activities, the *cef E* and *cef F* genes in *S. clavuligerus* and *N. lactamdurans* encode two separate enzymes with DAOC synthetase (expandase DAOCS) and DAC synthetase (hydroxylase DAOCH) activities respectively[31,32]. This resulted in early hypothesis that DAOCS and DAOCH were also separate enzymes in *C. acremonium*. However, the difficulties of separating DAOCS from DAOCH activity, and the observation of the constant ratio of these activities from one purification step to the next, led to the identification of only a single enzyme having dual activity in *C. acremonium*. Comparison of the *cef E* and *cef F* genes from *S. clavuligerus* to the *cef EF* gene from *C. acremonium* shows the two genes to be homologous except that the *cef E* gene lacks the nucleotides corresponding to 19 amino acids at the carboxy terminal end of the DAOCS/DAOCH enzyme[33].

Manipulation of *cef EF* genes

The discovery, successful cloning and characterization of genes involved in β-lactam biosynthesis made it possible to enter into more practical phases of engineering β-lactam antibiotic biosynthetic pathways. The first successful attempt at the engineering of a β-lactam antibiotic biosynthetic pathway of filamentous fungi was the improvement of cephalosporin C production by *C. acremonium*[34]. Analysis of this industrial strain which produces cephalosporin C revealed that it accumulates penicillin N in substantial quantities in the broth. Secretion of penicillin N also suggested that the next enzymatic step mediated by DAOCS encoded by the *cef EF* in *C. acremonium* in the biosynthetic pathway limited the rate of formation of cephalosporin C. A plasmid vector carrying a copy of the *cef EF* gene from *C. acremonium* was transformed into this strain of *C. acremonium*. About 25% of the transformants recovered had exhibited an enhanced level of cephalosporin C production (50% increase in the laboratory scale fermentations). One transformant (LU4-79-6) revealed the presence of one extra copy of the *cef EF* gene. In this case an additional copy of the *cef EF* gene had integrated heterologously on chromosome III, while the endogenous copy was in chromosome II (ref. 35). Cell extracts of recombinant strain LU4-79-6 possessed approximately twice the amount of DAOCS activity compared to the untransformed recipient strain. No penicillin N accumulated in the fermentation broth. This removed the rate limiting step in the biosynthesis of cephalosporin C in *C. acremonium*. In large scale fermentation, the productivity increased by about 15%, by using this recombinant strain; this is a significant improvement on the commercially used industrial strain which was already a highly developed one for cephalosporin C production. Thus in *C. acremonium*, the strain improvement via gene dosage was effective because whereas the original industrial strain possessed only a single copy of *cef EF* gene, the recombinant strain contained two copies of *cef EF* gene. This example clearly shows how recombinant DNA technology can help to overcome the nature of rate-limiting conversion steps and so further improve a highly developed industrial strain (in which the classical strain improvement programme has already become less productive). The relief of the bottleneck with respect to penicillin N still leaves other possible improvements to be made. The next step in the cephalosporin biosynthesis is the ex-

pansion of five-membered thiozolidine ring of penicilline N into six-membered dihydrothiazine. The ring expending reaction is catalysed by deacetoxycephalosporin C synthase encoded by *cef E*. There may be some limitations of carbon flow at the last step of the pathway which involves DAC acetyltransferase. To this end, it was observed[36] that the *cef G* gene, responsible for DAC acetyltransferase, is closely linked to the *cef G* gene in *C. acremonium* and it was suggested that cloning of *cef G* gene and its further manipulations may further increase the production of cephalosporin C. But similar studies performed in *P. chrysogenum* using *pcb C* gene were unsuccessful. On further studies, this was attributed to the presence of several copies of this gene already present in the organism. Thus, as several copies of this gene were already present, this step was no more rate limiting; the rate limitation in product formation shifted most likely to the depletion of the precursor amino acids (α-aminoadipic acid, cysteine and valine) formed during primary metabolism[37]. Attention would be consequently drawn in future on how to speed up this step; one of the strategies would be to increase the copy number of *pcb AB* gene and to maximize its expression.

Manipulations of *cef G* gene

Some years back, the *cef G* gene, encoding DAC acetyltransferase, was cloned from a wide-type strain of *C. acremonium*; the gene was located just upstream of the *cef EF* gene[38]. When *cef G* was reintroduced into a DAC acetyltranferase mutant of *C. acremonium*, a gene dosage effect was observed; the transformants showed a direct correlation between the number of copies of *cef G* integrated into the mutant genome and the quantity of cephalosporin C produced. This effect was also observed when the *cef G* gene was reinduced into the wild-type strain, strongly suggesting thereby that DAC acetyltransferease was rate-limiting in this strain. Moreover, it was noted that the *cef EF*-encoding DNA fragment[39], which enhanced cephaloporin C production in *C. acremonium*, also contained the closely linked *cef G* gene. Consequently, it can be questioned whether it was the *cef EF* gene or *cef G* that caused the improved production in that study. If the production strain used[25] was rate-limiting for DAC acetyltransferase rather than DAOCS/DACS, then the penicillin N that accumulated in that strain might have resulted from the feed-back inhibition of DAOCS/DACS by DAC. The introduction of a fragment containing the *cef EF* and *cef G* genes, therefore would have resolved the rate-limiting reaction as well as feed-back loop. However as it is also well known that industrial production strains from different lineages have very different biosynthetic capabilities, it is quite possible that DAOCS/DAOCH was the actual rate-limiting step in the strain used[39].

The above description provides an introduction that a closer understanding of the organization and manipulation of atleast the three genes, namely, the *pcb AB*, *pcb C*, and *pen DE*, in the target organism are required to have success in the developmental work for the generation of more efficient strains for the production of penicillins, while for improving the productivities of cephalosporins, the different stage-regulating genes such as *cef D*, *cef E*, *cef F*, and *cef G* besides *pcb AB* and *pcb C* genes and their interrelationships should be clearly understood. The developments up to the present time are summarized in Figure 4.

Production of novel β-lactams through manipulation of *pcb C* and *cef E* genes

The understanding of the genetics of biosynthesis of β-lactam antibiotics has not only enabled the scientists to overcome the bottlenecks posed by limited enzymatic activity but it has also made possible to produce novel β-lactam antibiotics. In the past, several novel β-lactams have been produced through expensive process of chemical modifications of penicillin, but only few of them (around 20) were found to be of clinical significance[40]. These studies also revealed that there is a need to create more molecules with β-lactam core structure to be used subsequently in the screening programme to create still more unique β-lactams. This became possible due to the cloning and expression of the *pcb C* gene in *E. coli*[20]. As mentioned earlier, the *pcb C* gene encodes isopenicillin N-synthase (IPNS), and this enzyme has broad substrate range. In one study in which around 150 unnatural substrates were tested for their conversion in β-lactams through *pcb C*-encoded IPNS, nearly 80% were converted to β-lactams[40] and 25% of them possessed antibacterial activity (Figure 5). These results indicated that *pcb C* gene can be modified to generate IPNS with broader substrate activity. This may further help in generating many novel β-lactam antibiotics. The understanding of the substrate-specific-binding of IPNS through its amino acid cysteine at positions 206 and 255 (ref. 41) is a step toward this direction. Not only the manipulations of *pcb C* gene can suitably lead to the production of several novel β-lactam antibiotics but other important genes of the pathway can also be suitably modified to increase their range of activity. In addition, there is a need to find out suitable enzymatic or biotransformation method to bypass the chemical synthesis, e.g. 7-ADCA. For instance, the process of chemical conversion of penicillin G to produce cephalosporin intermediates like 7-ADCA as illustrated in Figure 6 *a* is highly expensive and the overall efficiency of conversion is low. An alternative route proposed for its production involves enzymatic modification (Figure 6 *b*). This would require modifying the monofunctional

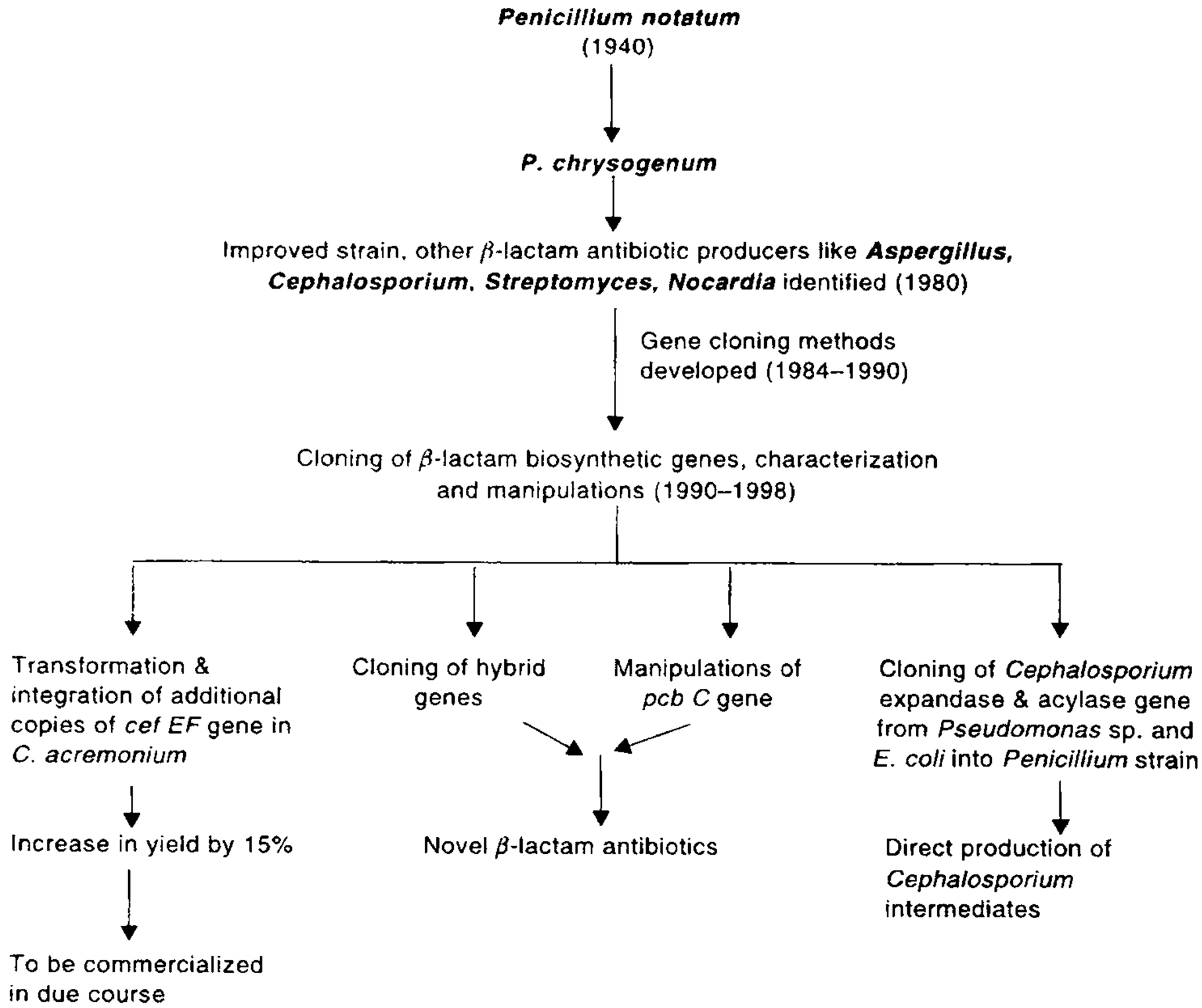

Figure 4. Current status of genetic manipulations of β-lactam antibiotic producers.

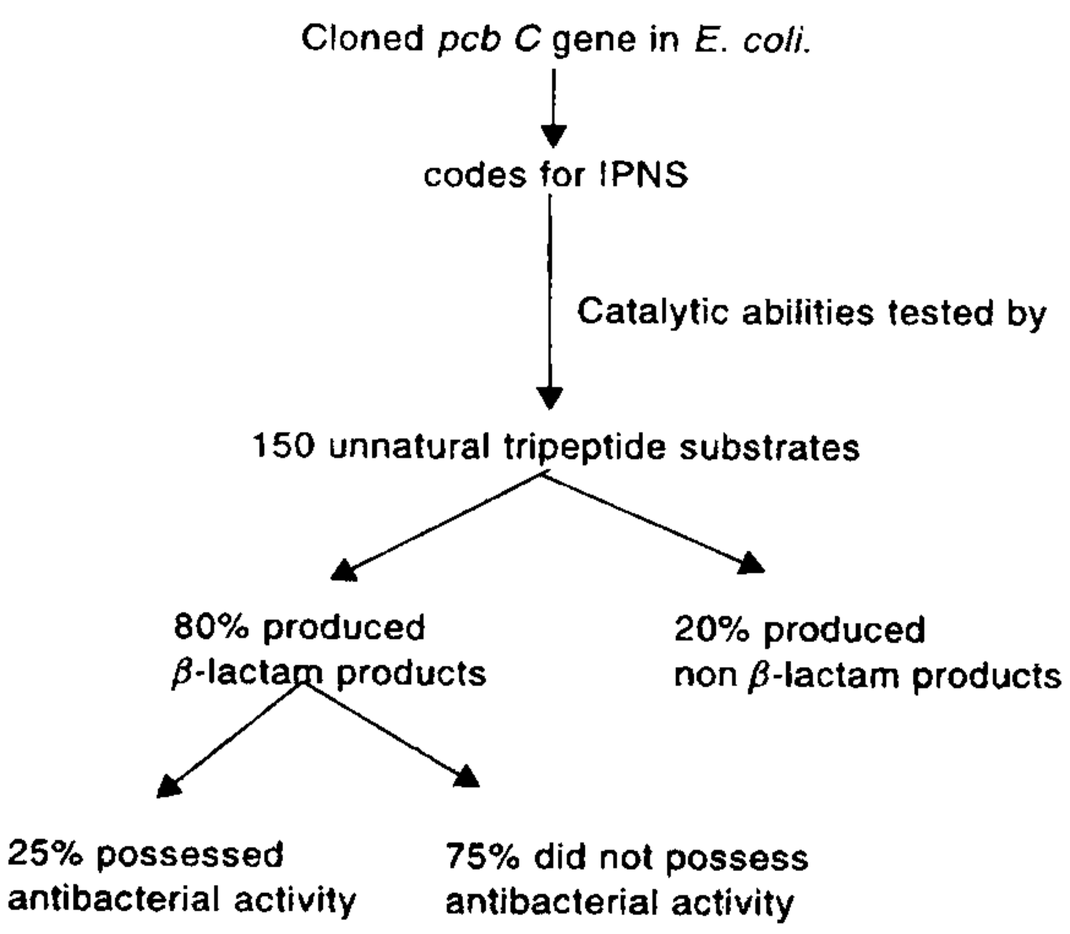

Figure 5. *In vitro* enzymatic formation of novel β-lactams.

cef E gene of *S. clavuligerus* to produce an expandase which will accept penicillin V as well as penicillin N as the substrates. The *cef E* gene is required to be cloned and expressed in *P. chrysogenum*, using an appropriate promoter of *cef E*. On adopting this strategy[42] and by the addition of the 3-regulatory sequence from the *P. chrysogenum pcb C* gene to the hybrid *cef E* gene, it enhanced their expression to useful level and it also increased the substrate range of the expandase thus produced; both the substrates like penicillin V (Figure 6 *b*) and penicillin N (Figure 6 *c*) could be used in such strategies. The deacetoxycephalosporin V or the side-chain-containing D-α-aminoadipyl group thus produced, could then be enzymatically converted to 7-ADCA. This process when industrially applied would be responsible for avoiding the use of significant quantities of polluting organic chemicals and solvents presently used in the chemical conversion steps. There are likewise several genetic approaches which could be followed to generate novel β-lactam antibiotics. For instance, the vectors containing hybrid bacterial fungal *cef D* and *cef E* genes of *S. clavuligerus* and *P. chrysogenum* were used in *P. chrysogenum* for the production of DAOC (refs 42, 43). The hybrid *cef D* gene was controlled by the regulatory

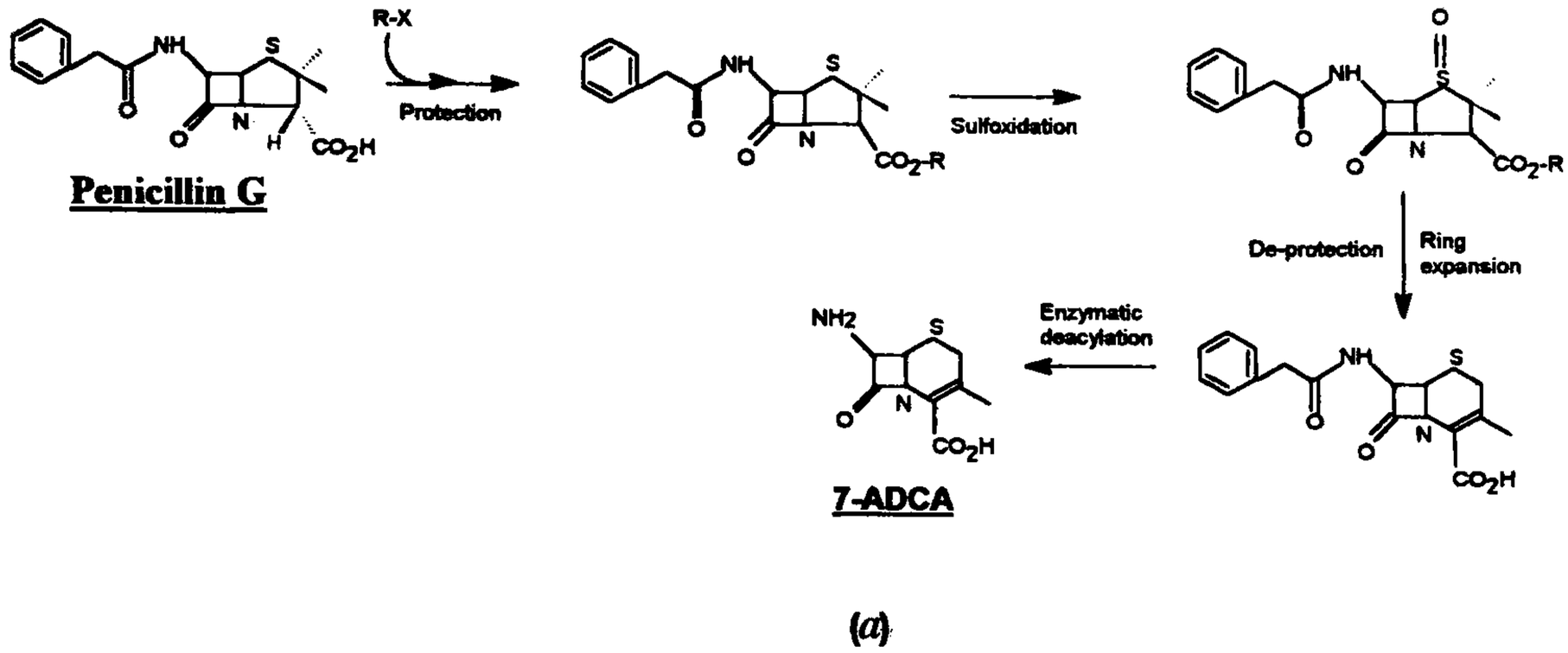

Figure 6. ***a***, Currently used route of chemical synthesis of 7-ADCA; ***b***, Schematic representation of a proposed route of 7-ADCA production based on combining biosynthetic and enzymatic reactions. ***c***, This shows the side chain found when the reaction catalysed by natural expandase of *S. clavuligerus* utilizes penicillin N as a substrate.

sequences of the *P. chrysogenum pcb C* gene, while the *cef E* gene was expressed using those of *pen DE*. Expression of these genes in *P. chrysogenum* to produce isopenicillin N epimerase enables conversion of isopenicillin N to penicillin N, which could then be ring-expanded by DAOC synthase (DAOCS). The *P. chrysogenum* transformants carrying these hybrid genes produced detectable concentrations of DAOC, thus indicating the successful construction of a novel β-lactam biosynthetic pathway in *P. chrysogenum*. Optimizing DAOC production in such transformants would require the removal of the acetyltransferase activity (encoded by *pen DE*). However, gene deletion may not be easy in those strains which carry multiple copies of the penicillin biosynthetic pathway genes.

Expression of cephalosporin acylase of *E. coli* and *Pseudomonas* strains in *C. acremonium*

As mentioned earlier, the 7-aminocephalosporanic acid (7-ACA) is used for the production of the starting material for a larger number of clinically important cepholosporin antibiotics[40]. Traditionally, the 7-ACA is produced by a complex chemical route from the basic fermentation product cephalosporin-C. Several cephalosporin acylases, the enzymes which hydrolyse cephalosporins to give 7-ACA, have been discovered[45]. Cephalosporin acylase activity was first found in penicillin G acylase of *E. coli* which deacylated cephalosporins possessing aromatic or heterocyclic carboxylic acid linked to the 7-amino group of the 7-ACA as amides. Later, cephalosporin acylases were discovered which attacked glutaryl 7-ACA and other cephalosporins possessing an aliphatic decarboxylic acid at the 7th position substituent[46,47]. Glytaryl 7-ACA stands important from the view point of industrial production of 7-ACA, because glytaryl 7-ACA can be easily obtained by oxidation of cephalosporin C and, accordingly, 7-ACA can be produced from cephalosporin C by two step conversion.

Several organisms have been investigated and their acylases identified. For instance molecular cloning and nucleotide sequencing of genes encoding these three types of cephalosporin acylases from *E. coli*. ATCC 11105 penicillin G (ref. 48), GK 16 glutaryl 7-ACA acylase[49] and SE83 cephalosporin C acylase[50,51] have already been done. All of these acylases were found to be composed of two nonidentical subunits which are processed from a common precursor polypeptide. There is also a fairly good homology among the deduced amino acid sequences of these three acylases. New glutaryl 7-ACA and cephalospoin C acylases from *E. coli* and *Pseudomonas* strains were obtained subsequently[52,53] after an intensive survey of such acylases was carried out to select the best performer that could be used industrially. The process for the enzymatic conversion or for the direct production of 7-ACA by fermentation, using appropriately modified recombinant strains of *P. chrysogenum*, have been made to extend the existing biosynthetic pathway constructs in *C. acremonium*. For instance, a group[54] devised an *in vitro* chemoenzymatic process of 7-ACA production and another group[55] has since created *in vivo* system which produces detectable amounts of 7-ACA in fermentation broths of *C. acremonium*. Two bacterial genes were modified by the addition of expression signals from the cloned *C. acremonium* alkaline protease gene[55]. The bacterial genes modified were responsible for the production of D-amino acid oxidase (DAO) from *Fusarium solani* C, and cephalosporin acylase from *Pseudomonas diminuta*[50]. Expression of these bacterial fungal genes generated a new β-lactam biosynthetic pathway in *C. acremonium* in which cephalosporin C was converted by DAO to 7-β-cephalosporanic acid. In addition, cephalosporin C was hydrolysed (with different efficiencies) by cephalospoin C acylase to form 7-ACA within *C. acremonium*. Although only low concentration of 7-ACA were detected, its production by fermentation has been shown to be feasible which in itself is an important milestone achieved in this area.

As mentioned earlier the chemical process of removal of the natural D-aminoadipyl side chain from cephalosporins is inefficient and attempts are on to find out superior enzymes to remove the side chain directly or by using a two-step enzymatic process[53]. Development of an analogous route to 7-ADCA is also hindered by the lack of strains producing acceptable quantities of deacetoxycephalosporin C; although adipyl side chain can be enzymatically removed, this invariably leads to the formation of other cephalosporin intermediates[54,56]. In addition, the production of adipyl 6-APA under different experimental conditions from recombinant strains as well as from ordinary bacterial strains faced difficulties[40,53,57,58]. It was proposed by a group[43] that expandase might be modified through protein engineering to expand penicillin G to phenyl acetyl-7-ADCA from which the aromatic side chain could be removed with a penicillin amidase. However this could not be achieved[40,59]. Subsequently it was demonstrated[43] that DAOC could be produced by *penicillium*-transformants carrying the *S. clavuligerous* expandase and epimerase gene. The information generated from the experiments of one group[42,43] formed the basis of the experimental strategy of another group[60] to develop an efficient bioprocess for the production of cephalosporin intermediates; this group fed adipic acid directly to penicillin-producing strains of *P. chrysogenum* expressing *S. clavuligerous* expandase/hydroxylase activity which led to the production of cephalosporin with an adipyl side chain (Figure 7). They also thus demonstrated a novel and efficient bioprocess for the production of cephalosporin intermediates 7-ACA or 7-ADCA through the recombinant *P. chrysogenum* strains in which *S. clavuligerous* expandase gene or *C. acremonium* expandase hydroxylase gene with and without acetyl transferase gene were expressed. The exogenous factor which resulted in the final efficient production of cephalosporin intermediates was through the addition of adipic acid to the medium as the side chain precursor, proving that adipyl 6-APA is a substrate for either enzyme *in vivo*. Interestingly strains expressing expandase hydroxylase produced both adipyl 7-ACA and adipyl 7-ADCA. In addition, strains expressing expandase hydroxylase and acetyltransferase produced adipyl-7-ADCA. The adipyl side chain of these cephalosporins could then be easily removed with a *Pseudomonas*-derived amidase to yield the cephalospoin intermediate 7-ADCA. Panlabs (USA) engineered[60] the *Pencillium* strains developed by the

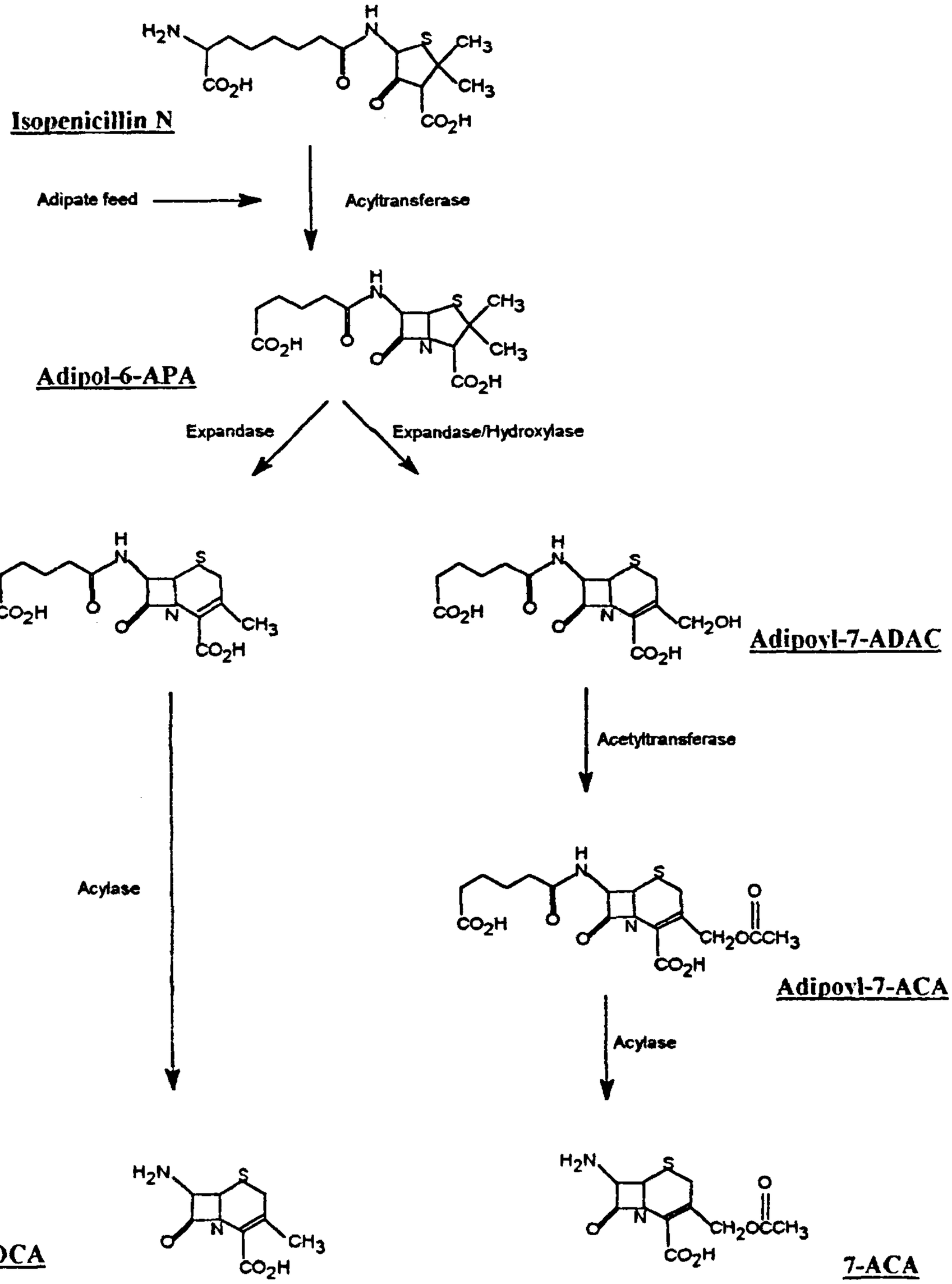

Figure 7. Schematic representation of the production of cephalosporin intermediates via adipoylcephalosporins in recombinant strains of *P. chrysogenum*.

earlier group[42,43] for this process. This recombinant strain, when optimally developed, is expected to replace worldover the currently popular but expensive chemical routes for the manufacture of 7-ADCA with a simpler, less effective and novel bioprocess.

Concluding remarks

The β-lactam antibiotics are still the most frequently prescribed antibacterial agents, despite the availability of an ever growing number of alternative antimicrobials. From the time Sir Alexander Fleming made his first publication in 1929 on penicillin[61] to the present time, there has been phenomenal developments, and the American Chemical Abstracts have described over 17,000 molecules belonging to β-lactam family, which signifies the enormous interest the world community has taken in these molecules. The overwhelming clinical success of a sizable number of them of this family has stimulated not only the industries producing them but

also the academic laboratories, to focus intense efforts for improving the performance of the microbes which produce these, by using recombinant DNA technologies. As a result, the biosynthetic pathways and the genes involved have been extensively studied. This has now set the stage for further genetic manipulations of fungi and bacteria which produce them. Amplification of the stage-specific genes in chosen target hosts of *Streptomyces* spp. or fungi, which are more tolerent to increased concentrations of commonly used substrates including metal and ammonium ions (tolerance to ammonium ion is particularly important as use of corn steep liquor or soybean meal, etc. as substrate produces substantial quantities of ammonia) would be the areas of immediate relevance for further developments. More attention would be put to engineering (mutations), amplification and maximization of expression of *pcb AB* genes of different lineages for all classes of β-lactams. The application of molecular genetics to strain improvement in the β-lactam-producing species has just begun, as the basic understanding is getting revealed. Up to the present time, it has allowed us to manipulate only a few of the parameters controlling secondary metabolism and has resulted in very modest gains in increasing the productivity of cephalosporin C. Further, uses of molecular techniques are also likely to revolve around identifying the transcriptional and regulatory mechanisms that limit or restrict the expression of native or foreign genes in the producing industrial strains. In several stages of the biosynthetic pathways, oxygen has been found to play a significant role. Such pathways include[62] the cyclization of the LLD-ACV tripeptide, the ring expansion from the five-membered thiazolidine ring of penicillin N into the six-membered dehydrothiazine ring which is characteristic of all cephalosporins, the hydroxylation of deacetoxycephalosporin C, etc. Intracellular expression of vitreoscilla hemoglobin or *VHb* was found to increase the productivity of a polyketide actinorhodin by *Streptomyces coelicolor*[63] and cephalospoin C by *Acremonium chrysogenum*[64]. Therefore, successful expression of *VHb* gene in *Penicillium chrysogenum* is expected to increase the productivity of penicillins significantly, and it is also expected that this strategy would be useful in improving the individual microbial strains of cephalosporins and cephamycins too. Processes are relatively simpler to adopt random integration of the *VHb* genes into the microbial chromosomes; however the knowledge of the site specific integration of the gene into the chromosome alongwith the choice of appropriate promoters for its optimum expression seems to be the key to success to obtain maximum gain out of the *VHb* gene expression. It is likely that at several stages of the microbial gene expression requiring the utilization of molecular oxygen, the *VHb* gene could be integrated in parallel to augment the maximization of the stage-specific biochemical reaction rate. It would also be worthwhile to spot the nucleotide sequence which would be negatively regulating the specific gene expression; modification or deletion of such sequences would also be the other strategies for enhancing the speed of the rate-controlling steps in the overall antibiotic biosynthetic train. Furthermore, a growing knowledge of the biochemical and biophysical properties of the biosynthetic enzymes guided by X-ray-crystallographic analysis of active-site structure will ultimately permit their manipulation at the molecular levels; this may in turn allow the synthesis of a wider range of bioactive precursors and metabolites. By adopting the right strategies of cloning or disruption of specific stages, the target organisms could be modified genetically to suppress the production of undesirable byproducts; it should also be possible to alter the cell wall of the organisms to enable the permeation of only the desired product. Further, it should also be possible to engineer a product which would pass through the cell wall of the organism, and which would have affinity for a separation matrix. Such strategies would be explored by the techniques of genetic engineering. The elucidation of the knowledge of the genes responsible for the biosynthesis at various stages is already being utilized for the development of new and effective β-lactans[20,40,65]. The future years are expected to witness the mixing, shuttling and reshuffling of bacterial and fungal genes of the β-lactam-producing strains, and maximizing the expression of the target gene(s) of the industrially used microbes as well as of the cheaper biosynthesis of a wide range of analogs by adopting a combination of strategies like, improving the promoter stretches; inserting newer genes; increasing the gene dosage; making newer but effective chimeric genes; modifying the physiology of the microbial cells; and altering the regulatory mechanisms of the pathways by the recombinant gene technology.

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