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Regulation of Penicillin Biosynthesis in Filamentous Fungi

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1 Introduction 47
1.1 Fungi as Producers of β-Lactam Antibiotics 47
1.2 Antibiotics as Secondary Metabolites 47
1.3 General Aspects Concerning β-Lactam Biosyntheses 48

2 Biosynthesis of Penicillins and Cephalosporins: An Outline 49

3 Molecular Genetics of Penicillin and Cephalosporin Biosynthesis in Fungi 52
3.1 Genetic Nomenclature 52
3.2 Clustering of Biosynthesis Genes 52
3.3 Structural Genes and Proteins 53
3.3.1 Genes Common to Penicillin and Cephalosporin-Producing Fungi 53
3.3.2 Gene Specific for Penicillin Biosynthesis: *aatA* (*penDE*) Encoding Acyl Coenzyme A:Isopenicillin N Acyltransferase 58
3.4 Compartmentation of Gene Products and Transport of Penicillins 60
3.5 Molecular Regulation of β-Lactam Biosynthesis Genes 60
3.5.1 General Aspects of the Elucidation of the Regulation of Secondary Metabolism Genes 60
3.5.2 Promoter Structures 61
3.5.3 Carbon Source Regulation 62
3.5.4 pH Regulation Mediated by the Transcriptional Activator PACC 63
3.5.5 Nitrogen Regulation 67
3.5.6 Amino Acids as Mediators of Regulation 68
3.5.7 Influence of Oxygen 69
3.5.8 The CCAAT-Box Binding Protein Complex AnCF 69
3.5.9 The *A. nidulans* bHLH Protein AnBH1 71
3.5.10 Velvet A (*veA*) 72
3.5.11 The Cephalosporin C Regulator CPCR1 Identified in *A. chrysogenum* Is very Likely also Present in Both *A. nidulans* and *P. chrysogenum* 73
3.5.12 Recessive *Trans*-Acting Mutations Affecting the Expression of Penicillin Biosynthesis Genes 73
3.5.13 G-Protein-Mediated Signal Transduction 74
3.5.14 Post-Transcriptional Regulation 74
3.6 Regulation of Penicillin Biosynthesis in Fungal Production Strains 75
3.7 Evolution of β-Lactam Biosynthesis Genes in Fungi 76

4 Applied Implications 77
4.1 Increase of Expression of Penicillin Biosynthesis Genes 78
4.2 Genetic Engineering of β-Lactam Biosynthesis Pathways 79

5 Future Prospects 79

References 79

Abstract The β-lactam antibiotic penicillin is one of the mainly used antibiotics for the therapy of infectious diseases. It is produced as end product by some filamentous fungi only, most notably by *Aspergillus* (*Emericella*) *nidulans* and *Penicillium chrysogenum*. The penicillin biosynthesis is catalysed by three enzymes which are encoded by the following three genes: *acvA* (*pcbAB*), *ipnA* (*pcbC*) and *aatA* (*penDE*). The genes are organised into a gene cluster. Although the production of secondary metabolites as penicillin is not essential for the direct survival of the producing organisms, several studies indicated that the penicillin biosynthesis genes are controlled by a complex regulatory network, e.g. by the ambient pH, carbon source, amino acids, nitrogen etc. A comparison with the regulatory mechanisms (regulatory proteins and DNA elements) involved in the regulation of genes of primary metabolism in lower eukaryotes is thus of great interest. This has already led to the elucidation of new regulatory mechanisms. Positively acting regulators have been identified such as the pH dependent transcriptional regulator PACC, the CCAAT-binding complex AnCF and seem also to be represented by recessive *trans*-acting mutations of *A. nidulans* (*prgA1*, *prgB1*, *npeE1*) and *P. chrysogenum* (carried by mutants Npe2 and Npe3). In addition, repressors like AnBH1 and VeA are involved in the regulation. Furthermore, such investigations have contributed to the elucidation of signals leading to the production of penicillin and can be expected to have a major impact on rational strain improvement programs.

Keywords Penicillin biosynthesis · Regulation of penicillin biosynthesis · *Aspergillus nidulans* · *Penicillium chrysogenum*

List of Abbreviations

6-APA	6-Aminopenicillanic acid
A	Adenine
Å	Ångstrom
AA	Amino acids
AAA	Aminoadipic acid
ACV	δ-(L-α-Aminoadipyl)-L-cysteine-D-valine
AF	Aflatoxin
AMP	Adenosine monophosphate
Arg	Arginine
Asp	Aspartic acid
ATP	Adenosine triphosphate
β-GAL	β-Galactosidase
β-GLU	β-Glucuronidase
bp	Base-pairs
bHLH	Basic-region helix-loop-helix
C	Carbon
C	Cytosine

Cys	Cysteine
Δ	Deletion
DAC	Deacetylcephalosporin C
DAOC	Deacetoxycephalosporin C
DNA	Deoxyribose nucleic acid
EMSA	Electrophoretic mobility shift assay
G	Guanine
Gln	Glutamine
Gly	Glycine
h	Hours
His	Histidine
IPN	Isopenicillin N
kbp	Kilo base-pairs
kDa	Kilo Dalton
mg	Milligram
mL	Millilitre
mmol	Millimolar
mRNA	Messenger ribonucleic acid
nt	Nucleotide
ORF	Open reading frame
RT-PCR	Reverse transcription polymerase chain reaction
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
ST	Sterigmatocystin
T	Thymine
Thr	Threonine
Val	Valine

1 Introduction

1.1 Fungi as Producers of β-Lactam Antibiotics

A literature survey covering more than 23,000 microbial products possessing some biological activity, i.e. antifungal, antibacterial, antiviral, cytotoxic and immunosuppressive, shows that the producing strains are mainly from the fungal kingdom (ca. 42%), followed by strains belonging to the genus *Streptomyces* (32.1%) [1]. Hence, fungi are one of the most important sources of bioactive compounds.

1.2 Antibiotics as Secondary Metabolites

The metabolism of fungi can be divided into two parts, the primary metabolism which provides the cells with energy and chemical precursors which are essential for growth and reproduction of the organisms, and the secondary metabolism which seems to possess no obvious function in cell growth [2]. Com-

pounds with antibiotic activity mainly belong to the group of secondary metabolites. Fungi produce numerous secondary metabolites which show antibiotic activity against various microorganisms, antiviral or antitumour and/or fungicidal activity. Some of the secondary metabolites, however, are too toxic for therapeutic applications and are therefore classified as mycotoxins some of which show mutagenic or even carcinogenic potential [3].

1.3 General Aspects Concerning β-Lactam Biosyntheses

The discovery of antibiotics is perhaps the most important discovery in the history of therapeutic medicine. It may conceivably have saved more lives than any other medical therapy [4]. The modern antibiotic therapy started with the discovery of a β-lactam antibiotic in 1929, when Alexander Fleming published his observation about the inhibition of growth of *Staphylococcus aureus* on an agar plate contaminated with *Penicillium notatum* [5]. This discovery led to the development of the β-lactam penicillin, the first clinically used antibiotic. During the late 1940s the fungus *Cephalosporium acremonium* (renamed to *Acremonium chrysogenum*) was isolated from the sea at Cagliari (Italy) by Guiseppi Brotzu [6]. The discovery of cephalosporin C generated a whole new group of clinically significant β-lactams. The success of β-lactams in the treatment of infectious disease is due to their high specificity and their low toxicity. Despite a growing number of antibiotics and the incidence of penicillin-resistant isolates, β-lactams are still by far the most frequently used antibiotic [7, 8].

It is only in the past 20 years that the biosynthesis pathways leading to penicillins and cephalosporins have been elucidated. This is in part due to the fact that industrial production of penicillin and cephalosporin was achieved with *Penicillium chrysogenum* and *Acremonium chrysogenum* (syn. *Cephalosporium acremonium*), respectively. These fungi, however, belong to the deuteromycetes which are in general difficult to analyse genetically. Currently, the greatest progress in elucidation of the molecular regulation of biosyntheses of β-lactams in fungi has been made in the penicillin-producer *Aspergillus (Emericella) nidulans*, since this fungus is an ascomycete with a sexual cycle. Hence, classical genetic techniques can be applied to *A. nidulans* [9] and as the result, a detailed genetic map is available [10]. The genome sequence of *A. nidulans* is publicly available (www.broad.mit.edu/annotation/fungi/aspergillus/index.html). Together with molecular techniques, this facilitated a thorough analysis of the genetic regulation of metabolic pathways, including that of penicillin biosynthesis [11–14].

According to their chemical structures β-lactams can be classified into five groups (Fig. 1). All of these compounds have in common the four-membered β-lactam ring. Apart from the monolactams, which have a single ring only, β-lactams consist of a bicyclic ring system. The ability to synthesise β-lactams is wide-spread in nature. It was found in some fungi, but also in some Gram-pos-

itive and Gram-negative bacteria (Fig. 1). However, whereas for the production of the hydrophilic cephalosporins organisms belonging to all three groups were described, the hydrophobic penicillins are only produced as end-product by filamentous fungi (Fig. 1). For the remaining groups of β-lactams listed in Fig. 1, so far only bacterial producers have been reported. The number of prokaryotic and eukaryotic microorganisms able to synthesize β-lactam antibiotics is continuously increasing [13, 15].

The biosynthesis of β-lactam compounds and their molecular genetics was subject to several recent reviews [13–19]. In particular, the molecular biology of β-lactam biosynthesis in fungi has seen a tremendous increase in knowledge within the last few years. The regulation of the penicillin biosynthesis will be considered mainly in the remainder of this chapter. For regulatory aspects concerning the cephalosporin biosynthesis, the article of Schmitt et al. in this volume is recommended.

2
Biosynthesis of Penicillins and Cephalosporins: An Outline

Penicillins and cephalosporins belong chemically to the group of β-lactam antibiotics. The biosynthesis of both penicillins and cephalosporins have the first two steps in common [13] (Fig. 2). All naturally occurring penicillins and cephalosporins produced by eukaryotic or prokaryotic microorganisms are synthesised from the same three amino acid, L-α-aminoadipic acid (L-α-AAA), L-cysteine and L-valine (Fig. 2). In fungi, the non-proteinogenic amino acid L-α-AAA is derived from the fungus specific aminoadipate pathway which leads to formation of lysine. It can also be provided by catabolic degradation of lysine although the contribution of this pathway to penicillin biosynthesis has not been clarified yet. In bacteria, a specific pathway for formation of L-α-AAA for β-lactam biosynthesis has been found [13].

In the first reaction of the cephalosporin and penicillin biosynthesis pathway, the amino acid precursors are condensed to the tripeptide δ-(L-α-aminoadipyl)-L-cysteine-D-valine (ACV). This reaction is catalysed by a single enzyme, δ-(L-α-aminoadipyl)-L-cysteine-D-valine synthetase (ACVS) (see below). ACVS is encoded by a single structural gene designated *acvA* (*pcbAB*) (Fig. 2). In the second step, oxidative ring closure of the linear tripeptide leads to formation of a bicyclic ring, i.e., the four-membered β-lactam ring fused to the five-membered thiazolidine ring which is characteristic of all penicillins. The resulting compound isopenicillin N (IPN) possesses weak antibiotic activity and is thus the first bioactive intermediate of both penicillin and cephalosporin pathways. This reaction is catalysed by isopenicillin N synthase (IPNS) encoded by the *ipnA* (*pcbC*) gene (see below). IPN is the branch point of penicillin and cephalosporin biosyntheses (Fig. 2).

In the third and final step of penicillin biosynthesis, the hydrophilic L-α-AAA side chain of IPN is exchanged for a hydrophobic acyl group catalysed by

Classes of naturally occurring β-lactams	Antibiotics	Producing microorganisms (Examples)		
		Fungi	Bacteria	
			Gram$^+$	Gram$^-$
Penam	Penicillins	*Penicillium notatum* *P. chrysogenum* *Aspergillus nidulans*		
Ceph-3-em	Cephalosporins Cephamycins Cephabacins Chitinovorins	*Acremonium chrysogenum* *(syn. Cephalosporium acremonium) Paecilomyces persinicus*	*Streptomyces clavuligerus* *Nocardia lactamdurans*	*Flavobacterium* sp. *Lysobacter lactamgenus*
Clavam	Clavulanic acid		*Streptomyces clavuligerus*	
Carbapenem	Thienamycins Olivanic acid Epithienamycins		*Streptomyces clavuligerus* *S. olivaceus*	*Erwinia carotovora* *Serratia*
Monolactam	Nocardicines		*Nocardia uniformis* subsp. *tsuyamanensis*	
	Monobactams			*Agrobacterium radiobacter* *Pseudomonas acidophila*

Fig. 1 Naturally occurring classes of β-lactam antibiotics essentially according to O'Sullivan and Sykes [180] and as shown in Aharonowitz et al. [51] and Brakhage [13]

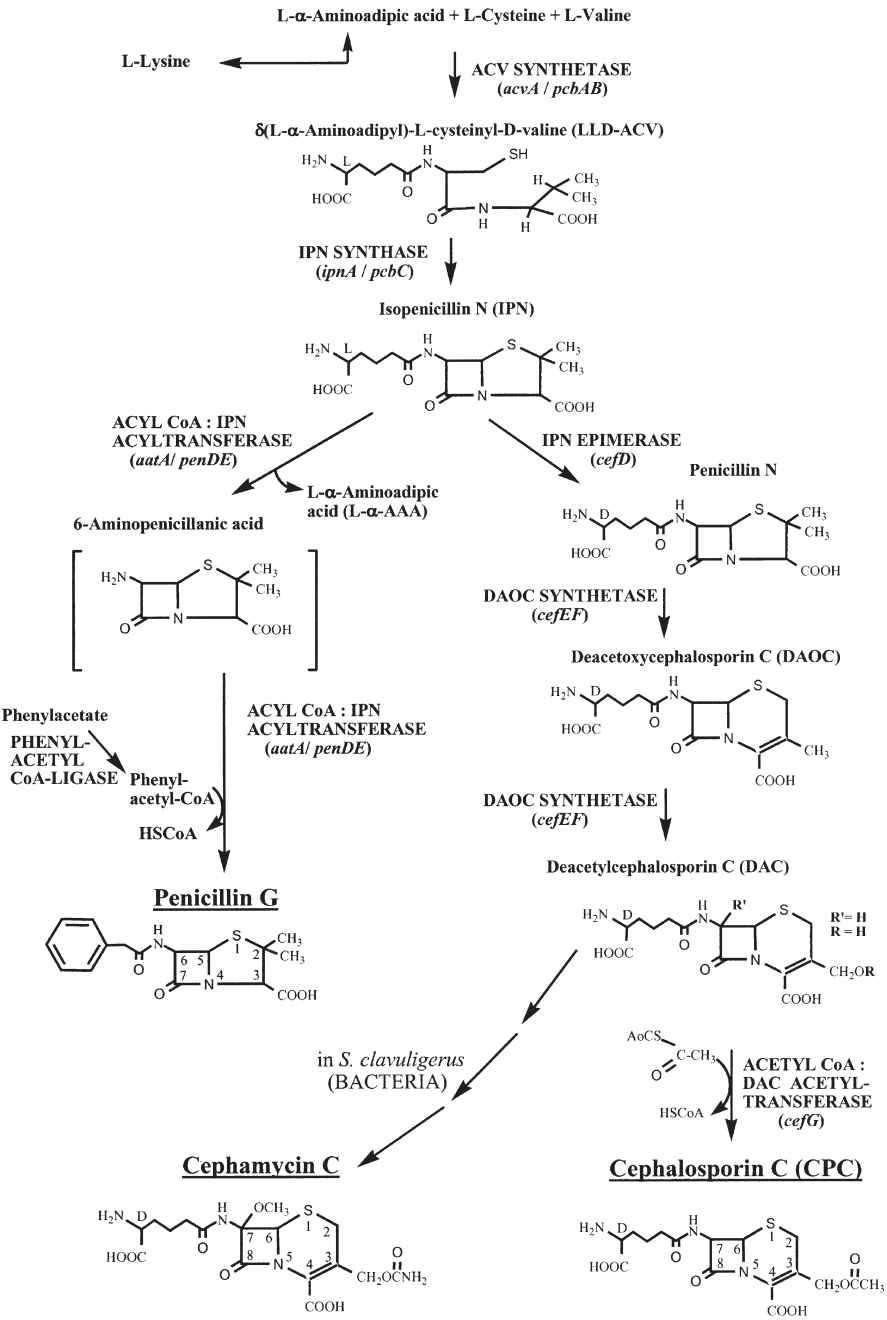

Fig. 2 Biosyntheses of penicillin, cephalosporin C and cephamycin C. Gene and organism names are printed in *italics*, names of enzymes in *capital letters*. L-α-AAA is an intermediate of the L-lysine biosynthetic pathway but can also be provided by catabolic degradation of L-lysine. The penicillin biosynthesis occurs in fungi only, whereas cephalosporins are synthesised in both fungi, e.g. cephalosporin C by *A. chrysogenum*, and bacteria, e.g. cephamycin C by *S. clavuligerus*. Abbreviations: ACV, δ-(L-α-aminoadipyl)-L-cysteine-D-valine; DAC, deacetylcephalosporin C; DAOC, deacetoxycephalosporin C; IPN, isopenicillin N; L-α-AAA, L-α-aminoadipic acid

acyl coenzyme A:isopenicillin N acyltransferase (IAT). The corresponding gene was designated *aatA* (*penDE*). In natural habitats penicillins such as penicillin F and K, which contain D3-hexenoic acid and octenoic acid as side chains, respectively, are synthesised. By supplying the cultivation medium with phenylacetic or phenoxyacetic acid, the synthesis can be directed mainly towards penicillin G and V, respectively [17] (Fig. 2). The side chain precursors have to be activated before they become substrates for the IAT. It is generally believed that the activated forms of the side chains consist of their CoA-thioesters, but the mechanism behind this activation is still not fully elucidated [20] (see below). The formation of hydrophobic penicillins has been reported in fungi only, notably *P. chrysogenum* and *A. nidulans*, whereas the hydrophilic cephalosporins are produced by both fungi and bacteria, e.g. *A. chrysogenum* and *Streptomyces clavuligerus*, respectively (Fig. 2).

3
Molecular Genetics of Penicillin and Cephalosporin Biosynthesis in Fungi

3.1
Genetic Nomenclature

Before their identification, the putative genes encoding ACVS were designated *pcbA* (penicillin cephalosporin biosynthesis) and *pcbB*, because it was believed that two enzymes were involved in the formation of an AC dipeptide and the final ACV tripeptide, respectively (Fig. 2) [21, 22]. Cloning and sequencing of the corresponding gene revealed, however, that a single polypeptide encoded by a single gene is responsible for the formation of the ACV tripeptide. Publications reporting the DNA sequence of the *P. chrysogenum*, *A. nidulans* and *A. chrysogenum* genes named the gene *acvA*, which reflected the involvement of one genetic locus in the synthesis of ACVS [23–26] or, *pcbAB* derived from the combination of *pcbA* and *pcbB* [27, 28]. The gene encoding IAT was named *penDE* or *aat*. In this review the gene is designated *aatA*, reflecting both the correct genetic nomenclature and that one genetic locus encodes the enzyme. The IPNS gene was named *ipnA* [14]. The alternative names are shown in parentheses at the beginning of the relevant sections.

3.2
Clustering of Biosynthesis Genes

So far as we know, in bacteria and fungi all structural genes of β-lactam biosyntheses are clustered (Fig. 3). The penicillin biosynthesis genes form a singe cluster, whereas in *A. chrysogenum*, two clusters containing the cephalosporin biosynthesis genes were identified. By contrast in cephamycin C producing bacteria the cephamycin biosynthesis genes are organised into a single cluster (Fig. 3) [23, 25, 28–31]. The linkage of antibiotic-biosynthesis genes is a well-

known phenomenon in many antibiotic-producing organisms. It has been speculated that linkage has occurred during evolution owing to an ecological selective advantage [32]. Seno and Baltz [33] have suggested that coordinated regulation of antibiotic-biosynthesis genes could be achieved by organising the genes into large operons controlled by a single promoter. For example, genes of the actinorhodin biosynthesis pathway in *Streptomyces coelicolor* are clustered and expressed in several polycistronic messages [34]. In eukaryotic fungi, however, β-lactam biosynthesis genes are transcribed separately, and are expressed from different promoters [14]. Hence, in fungi, there is no obvious need for clustering and it thus seems more likely that linkage reflects a common ancestral origin (see below). However, there is no evidence that the *aatA* gene has a close relative in modern prokaryotes, even though it is part of the cluster. This fact supports the hypothesis that linkage might also confer an ecological advantage to the eukaryotic fungi in their natural habitat, although the reason for this is not yet understood.

3.3 Structural Genes and Proteins

3.3.1 Genes Common to Penicillin and Cephalosporin-Producing Fungi

3.3.1.1 acvA (pcbAB) Encoding δ(L-α-Aminoadipyl)-L-Cysteinyl-D-Valine Synthetase (ACVS)

The first reaction which has been shown for the biosyntheses of penicillin and cephalosporin/cephamycin is the formation of the δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) tripeptide. All of the reactions required for synthesis of the tripeptide are catalysed by a single enzyme, δ-(L-α-aminoadipyl)-L-cysteine-D-valine synthetase (ACVS) which is encoded by the *acvA* (*pcbAB*) gene (Fig. 2). Thus, the ACV tripeptide is formed via a non-ribosomal enzyme thiotemplate mechanism from its amino acid precursors. This is similar in many aspects to the synthesis of other microbial peptides [35–37] (see chapter von Döhren et al.).

The first isolation of an ACVS protein was achieved by van Liempt et al. [38] who partially purified ACVS of *A. nidulans* 118-fold. Since then, ACVS enzymes have been purified from different organisms, including *P. chrysogenum*, *S. clavuligerus*, *A. chrysogenum* and *N. lactamdurans* [35, 36, 39]. Although not entirely clarified, it is believed that ACVS multienzymes are monomers. They exhibit different catalytic activities such as the specific recognition of the three amino acid precursors and their activation, peptide bond formation, isomerisation of the L-valine moiety to the D-form etc. As in ribosomal peptide biosynthesis, the carboxyl function of the amino acid is activated by the formation of a mixed anhydride with the α-phosphate of ATP, resulting in the release of pyrophosphate [38]. After activation of an amino acid, the formed aminoacyl

adenylate is cleaved by the action of an enzyme thiol, resulting in formation of a thioester bond between the enzyme (at an appropriate location on the enzyme) and the amino acid, and in the release of AMP. These thioesterified amino acids are high-energy intermediates which are the targets for a nucleophilic attack by the amino group of a second amino acid, resulting in the formation of a peptide bond. As in the ribosome, the nascent peptide grows from the amino-terminus to the carboxy-terminus and the intermediate peptides remain bound (as thioesters) to the enzyme. Substrate specificity is less strict than in protein synthesis, since a variety of tripeptide analogs are known [13, 35]. L-Valine is apparently epimerized to the D-form at the tripeptide stage since no D-valine intermediate has been detected (Fig. 2) [36, 40].

Each ACVS is encoded by a single structural gene (designated *acvA* or *pcbAB*) with a size of more than 11 kb (Table 1). The translational start codons of the *acvA* genes of all fungi are putative because attempts to obtain the N-terminal amino acid sequence proved to be unsuccessful [24, 41]. The genes were cloned and sequenced from *P. chrysogenum, A. nidulans*, *A. chrysogenum* and bacterial cephamycin producers such as *N. lactamdurans*, *S. clavuligerus* and *Lysobacter lactamgenus* [13, 15, 19, 26, 27, 42]. Even in fungi, the ORF is not interrupted by introns. Fungal *acvA* genes are divergently oriented to the *ipnA* genes (Fig. 3). The genes are separated by about 1 kbp. Sizes of the intergenic regions between both genes vary slightly among the different fungi (Table 1).

The order of the biosynthesis of the AAA-Cys-Val tripeptide is believed to reflect the linear organisation of the ACVS in AAA-, Cys- and Val-activating domains [36]. Sequencing of the ACVS structural genes (Table 1) revealed that in the three repeated regions of about 600 amino acids of each ACVS some similarity to 4′-phosphopantetheine attachment sites described for polyketide synthases (i.e. DSL) is evident [24]. This seems to reflect the attachment of multiple cofactors to ACVS. Because a single phosphopantetheine arm is sufficient for activity of fatty acid synthases, the finding of several phosphopantetheine attachment sites suggest a modified mechanism for the thiotemplate pathway to polypeptides (multiple cofactor model) [24, 43–45]. Although the relevance of all three pantetheine attachment sites of ACVSs has not been proved experimentally yet, it is currently believed that peptide assembly is accomplished by transfer of acyl intermediates between adjacent cofactors [44, 45].

Recently, a putative 4′-phosphopantetheinyl transferase which is essential for penicillin biosynthesis in *A. nidulans* was characterised. It is encoded by the *npgA*/*cfwA* gene. Mutations in this gene led to defects in growth and pigmentation. Furthermore, the mutant did not produce penicillin [46, 47].

In the carboxyl-terminal region of ACVS enzymes, sequence similarities to the thioesterase active site region, GXSXG, have been found which would be required to release the generated tripeptide from the enzyme [24].

ACV synthetases are of special interest since they represent a route for peptide bond formation independent of the ribosome and allow the incorporation

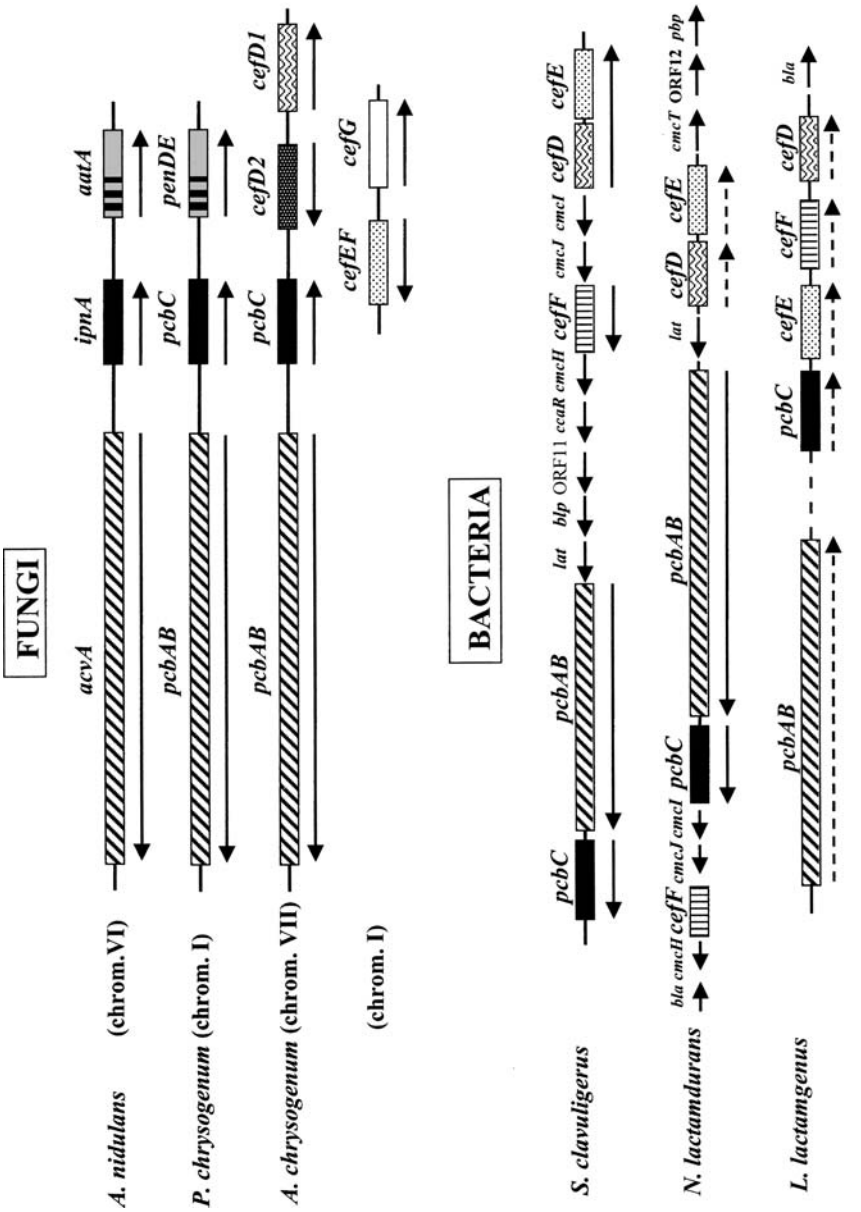

Fig. 3 β-Lactam biosynthesis gene cluster in fungi and bacteria. The *A. chrysogenum* genes *cefD1* and *cefD2* are located next to the *pcbAB* and *pcbC* gene [181]. Bacterial genes with fungal homologs are boxed. The transcriptional orientation and the transcript units (Bacteria), as far as it has been determined, are indicated by *arrows below the boxes*. *Arrows between boxes* (Bacteria) and *arrows with broken lines below boxes* mark the orientation of genes. ORF specifies an open reading frame whose function is unknown. Abbreviations not mentioned in the text: *cmcT*, transmembrane protein; *pbp*, penicillin-binding protein; *bla*, β-lactamase; *blp*, showing similarity to the extracellular β-lactamase inhibitory protein BLIP; ORF, open reading frame [14]

Table 1 *acvA (pcbAB)*. δ(L-α-aminoadipyl]-L-cysteinyl-D-valine synthetase

	DNA (bp)	No of aa	Mr	Transcript-size (kb)	Transcript-start[a]	In-trons	Do-mains	Ref.
A. nidulans	11,310	3770	422,486	> 9.5	Major: −230 Minor: −317 −195 −188	–	3	[23, 24]
P. chrysogenum								
OLI13	11,328[a]	3776[a]	423,996[a]		n.d.	–	3	[25, 26]
AS-P-78	11,376	3792	425,971	11.5	n.d.	–	33	[27]

[a] Correction of published sequence. Translation start moved upstream by 90 bps (Brakhage and Turner, 1995).

of many non-proteinogenic amino acids [48]. Furthermore, since different parts of peptide synthetases are specific for certain amino acids, this can be used to engineer genetically new peptide synthetases producing new compounds, possibly with new pharmacological activities [49] (see chapter von Döhren et al.).

3.3.1.2 ipnA (pcbC) Encoding Isopenicillin N Synthase (IPNS)

The second step of the penicillin/cephalosporin biosynthesis, i.e., the cyclisation of the linear ACV tripeptide to the bicyclic isopenicillin N (IPN), is catalysed by isopenicillin N synthase (IPNS), a nonheme Fe(II)-dependent oxidase (Fig. 2) (Table 2). The enzyme formally catalyses the removal of four hydrogen equivalents of the ACV tripeptide in a desaturative ring closure with concomitant reduction of dioxygen to water [15, 50, 51]. The IPNS reaction requires ferrous iron, molecular oxygen as cosubstrate and ascorbate as electron donor to form the β-lactam and thiazolidine ring of IPN [22].

IPNS was purified to homogeneity from *A. chrysogenum* [52–54] and has subsequently been obtained from *P. chrysogenum*, *A. nidulans*, several actinomycetes such as *S. clavuligerus*, *S. lipmanii*, *N. lactamdurans*, and the Gram-negative bacterium *Flavobacterium* sp. [15]. Only the free thiol form of ACV serves as a substrate, the bis-disulfide dimer, which is spontaneously formed, being inactive [55]. In *P. chrysogenum*, a broad-range disulfide reductase belonging to the thioredoxin family of oxidoreductases was found which efficiently reduced bis-ACV to the thiol monomer. When coupled to IPNS in vitro, it converted bis-ACV to IPN and was therefore suggested to play a role in penicillin biosynthesis [56]. The crystal structure of the *A. nidulans* IPNS was solved at a resolution of 2.5 Å and 1.3 Å complexed with manganese [50], and with

Fe^{2+} and substrate [57], respectively. The active-structure shows the manganese ion attached to four protein ligands (His 214, Asp 216, His 270, Gln 330) and bears two water molecules occupying coordination sites directed into a hydrophobic cavity within the protein [50]. The Fe(II):ACV:IPNS structure has one protein molecule with ferrous ion and ACV bound at the active site. The side chain of Gln 330, which coordinates the metal in the absence of substrate, is replaced by the ACV thiolate [57]. In the substrate complex, three of the five coordination sites are filled with protein ligands: His214, His270 and Asp216 [58]. The remaining two sites are occupied by a water molecule (at position 398) and the ACV thiolate. Such a structural characteristic (an iron-binding site within an unreactive hydrophobic substrate binding cavity) is probably a requirement for this class of enzyme, as it results in the isolation of the reactive complex and subsequent intermediates from the external environment. Thus, the reaction can be channelled along a single path, avoiding the many side reactions potentially open to the highly reactive species resulting from the reduction of dioxygen at the metal [50]. Data on the mechanism of the IPNS reaction suggests that initial formation of the β-lactam ring is followed by closure of the thiazolidine ring [59]. The current model of the catalytic mechanism can be found in Roach et al. [50, 57].

IPNS shows broad substrate specificity in particular with alterations in the L-α-AAA moiety and the valine residue of ACV. This finding has an ingenious use in creating novel penicillins from ACV analogs although cyclisation of unnatural tripeptides occurs at lower efficiency [60, 61].

The genes encoding IPNS enzymes are designated *ipnA* (*pcbC*) (Table 2). *ipnA* (*pcbC*) genes have been isolated from different fungi and bacteria such as *A. chrysogenum*, *A. nidulans*, *P. chrysogenum*, *S. clavuligerus*, *S. griseus*, *S. lipmanii*, *Flavobacterium sp.*, *N. lactamdurans* etc. [15, 62, 63]. The properties of the fungal genes and their corresponding deduced amino acid sequences are summarized in Table 2. In contrast to bacteria, in fungi *ipnA* and *acvA* are bidirectionally oriented (Fig. 3). Fungal IPNS genes identified until now do not possess introns (Table 2).

Table 2 *ipnA (pcbC)*. Isopenicillin N synthase

	DNA (bp)	No of aa	Mr	Transcript-size (kb)	Transcript start[a]	Introns	Ref.
A. nidulans	993	331	37,480	~1.7	Major: –106	–	[88, 170, 182, 183]
P. chrysogenum	993	331	38,012	1.1	Major: –11	–	[86, 161, 162]

[a] Values for transcript starts were determined by primer extension or by S1 mapping.

3.3.2
Gene Specific for Penicillin Biosynthesis: aatA (penDE) Encoding Acyl Coenzyme A:Isopenicillin N Acyltransferase

The third and final reaction of penicillin biosynthesis, which does not occur in cephalosporin biosynthesis and has been found in fungi only, is catalysed by acyl coenzyme A:isopenicillin N acyltransferase (IAT). The hydrophilic L-α-AAA side chain is exchanged for a hydrophobic acyl group, e.g. phenylacetyl in penicillin G (Fig. 2). IAT shows a broad substrate specificity [19, 64, 65]. By addition of appropriate precursor molecules, the fermentation can be directed towards a specific penicillin, e.g. for production of penicillin G, phenylacetic acid is added, for production of penicillin V, phenoxy acetic acid (Fig. 2). Once the precursor has been taken-up, it must be activated to its CoA thioester. A possible candidate to carry out this reaction is the acetyl-CoA synthetase (ACS) which was purified from *P. chrysogenum* and its structural gene *acuA* (=*facA* of *A. nidulans*) cloned. It was shown that the ACS enzymes of both *P. chrysogenum* and *A. nidulans* have the capability to catalyse in vitro the activation (to their CoA thioesters) of some of the side chain precursors required for the production of several penicillins by these fungi [65]. Putatively different ACS-like enzymes have been described as well [66, 67]. In addition, a specific phenylacetic acid-activating ACS-like enzyme designated phenylacetic acid-CoA ligase was isolated and its encoding gene cloned [20, International patent WO97/02349]. Phenylacetic acid activation by ACS appears to be poor [68], and disruption of the *acuA* gene does not affect penicillin biosynthesis [69, International patent WO92/07079]. Furthermore, overproduction of phenylacetic acid-CoA ligase, however, does not seem to result in a higher penicillin production, either. It is interesting to note, that overproduction of the *pcl* gene from *Pseudomonas putida* U encoding a phenylacetic acid-CoA ligase which most probably resides in the fungal cytosol, increased penicillin production two-fold indicating a possible role for a cytosolic enzyme [20, 70].

An efficient penicillin production is apparently hampered by the degradation of the precursors such as phenylacetic acid. A gene of *A. nidulans* was cloned designated *phacA* which encodes a cytochrome P450 monooxygenase. The enzyme catalyses the 2-hydroxylation of phenylacetate. It is involved in the degradation of phenylacetate to fumarate and acetoacetate. *phacA* disruption increased penicillin production three- to fivefold, indicating that catabolism competes with antibiotic biosynthesis for phenylacetate [71]. The corresponding gene of *P. chrysogenum* (*pahA*) was also cloned. In contrast to *A. nidulans*, *P. chrysogenum* is unable to use phenylacetic acid as sole carbon source. This block in phenylacetic acid catabolism could be originated by inactivation or strong reduction of PAHA activity. However, interestingly, PAHA activity displayed an inverse correlation with the penicillin productivity of the *P. chrysogenum* strains studied. Comparison of *pahA* genes of several strains revealed that an L181F mutation was responsible for the reduced function of PAHA in

present industrial strains compared with the wild-type NRRL1951. The mutation was tracked down to strain Wisconsin 49–133 [72].

A two step enzymatic process for conversion of IPN to penicillin G by IAT has been proposed [73]. In the first step, IPN is deacylated to 6-aminopenicillanic acid (6-APA), which in the second step is acylated to penicillin G through addition of a phenylacetyl group from its CoA derivative (Fig. 2). Thus, two enzymatic functions are required, an isopenicillin-N amidohydrolase and acyl-CoA:6-aminopenicillanic acid acyltransferase activity. The cloning and sequencing of the *aatA* (*penDE*) gene encoding IAT revealed that the *P. chrysogenum* enzyme has the required activities [64].

The properties of the *P. chrysogenum* and *A. nidulans aatA* (*penDE*) genes are summarised in Table 3. In contrast to the other penicillin biosynthesis genes (*acvA* and *ipnA*) the *aatA* genes contain three introns in both organisms at similar positions [74–76].

No DNA sequence homologous to the *aatA* gene of *P. chrysogenum* was found in the genome of three different strains of *A. chrysogenum* and actinomycetes [19]. This finding is consistent with the notion that 6-aminopenicillanic acid: acyltransferase activity which is also carried out by IAT is lacking in *A. chrysogenum* and other cephalosporin producers [77]. Therefore, these organisms do not produce penicillin G or any other penicillins with a hydrophobic side chain.

The active form of the IAT enzyme results from processing of the 40 kDa monomeric precursor to a heterodimer containing subunits of 11 and 29 kDa [19, 65, 78]. Both subunits are required for activity [79]. In *P. chrysogenum*, it was shown that the processing event that generated the two subunits from the 40 kDa precursor polypeptide occurred between Gly102/Cys103 [80]. Additional investigations suggest that the formation of recombinant IAT involves cooperative folding events between the subunits and IAT hydrolysis is an autocatalytic event [79]. Site-directed mutagenesis of the *aatA* gene and production of the mutant enzyme in *E. coli* revealed that Cys103 is required for IAT proenzyme cleavage. Whether this requirement reflects a direct participation of Cys103 in cleavage or as part of a cleavage recognition site has not been clarified yet. However, it cannot be entirely excluded yet that Cys103 is involved in IAT enzyme activity because all of these experiments were based on the de-

Table 3 *aatA* (*penDE*). Acyl coenzyme A: isopenicillin N acyltransferase

	DNA (bp)	No of aa	Mr	Transcript-size (kb)	Transcript-start	Introns	Ref.
A. nidulans	1237	357	39,240	1.2	–61 (–60) –52, –82	3	[75, 76, 85, 184, 185]
P. chrysogenum	1274	357	39,943	1.15	n.d.	3	[74, 76, 186]

tection of enzyme specific activity [81]. The encoded amino acid sequence in the cleavage site is identical in *P. chrysogenum* and *A. nidulans* (Arg-Asp-Gly...Cys-Thr-Thr) [80–82].

3.4
Compartmentation of Gene Products and Transport of Penicillins

The penicillin biosynthesis pathway occurs in different compartments of the cell, as reviewed by Driessen and colleagues (see chapter Evers et al.).

3.5
Molecular Regulation of β-Lactam Biosynthesis Genes

3.5.1
General Aspects of the Elucidation of the Regulation of Secondary Metabolism Genes

One of the problems in elucidating the transcriptional control of β-lactam biosynthesis genes is that the physiological meaning of the production of β-lactams for the producing fungi is not entirely understood. It is generally accepted that penicillin and cephalosporin act as antibiotics in the soil against competing bacteria but an experimental proof of this assumption is difficult to obtain. Hence, as long as the physiological meaning of β-lactams for the producing fungi is not fully understood it is not possible to predict the regulatory circuits involved in the regulation of β-lactam biosyntheses. Therefore, an alternative strategy is based on the identification of regulatory proteins and to elucidate to which regulatory circuits these proteins belong. By this means, it should be feasible to unravel the physiology behind the production of β-lactams in fungi.

As for most genes, transcriptional control is a major determinant of the appearance of their products. In case of the β-lactam biosynthesis genes in fungi, there are some studies directly measuring steady state levels of mRNA and, in addition, studies using reporter gene fusions. In the latter case, the promoter regions of the β-lactam biosynthesis genes including codons encoding some of their N-terminal amino acids were fused in frame with the *Escherichia coli* reporter genes *lacZ* or *uidA* encoding β-galactosidase (β-GAL) and β-glucuronidase (β-GLU), respectively. The most sophisticated system offers *A. nidulans*. This fungus has no significant endogenous β-GLU activity and, in addition, there are mutants available which have no endogenous β-GAL activity. Furthermore, this fungus has, in contrast to the deuteromycetes *P. chrysogenum* and *A. chrysogenum* which are employed for industrial production of penicillin and cephalosporin, respectively, a well defined sexual cycle facilitating genetic analyses [14].

Although by using gene fusions it cannot be entirely excluded that also post-transcriptional regulation contributes to the overall measurement of the re-

porter enzymes, in this review the data is formally used as indicative for transcriptional regulation.

Although penicillin is a secondary metabolite, in *A. nidulans* and *P. chrysogenum* with lactose as the carbon source its production occurs right from the beginning of the fermentation run. A strict separation of trophophase (growth phase) and idiophase (metabolite production phase), which has been observed in many antibiotic-producing bacterial cultures, was absent [83–85]. This is consistent with the notion that in fermentation medium, *A. nidulans acvA* and *ipnA* gene fusions were expressed for up to 68 h and 46 h, respectively [83]. In contrast, *aatA* expression was only detected for about 24 h [85]. However, in *P. chrysogenum ipnA* steady-state mRNA levels increased with the age of the culture, indicating preferential transcription of the gene at late growth times [86]. This is consistent with the observation that expression of an *ipnA-uidA* gene fusion was only detectable after 24 h in fermentation medium with lactose as the carbon source. In contrast, an *acvA-uidA* gene fusion seemed to be expressed from the beginning of a fermentation run [87]. Hence, there might even be differences in the temporal expression among genes of the same cluster as well as among genes of clusters in different fungi.

3.5.2 Promoter Structures

Studies to analyse promoters of the penicillin biosynthesis genes have been reported of both fungi, i.e., *A. nidulans* and *P. chrysogenum*. Deletion analyses revealed that the intergenic region between *acvA* and *ipnA* of *A. nidulans* contains several regions containing *cis*-acting DNA elements. Furthermore, the promoters of both genes are, at least in part, physically overlapping and share common *cis*-acting elements [88, 89]. In *P. chrysogenum*, a deletion analysis of the *acvA* (*pcbAB*) promoter region showed that at least three regions are important for regulation under the conditions tested. Together with biochemical assays, such as EMSAs and uracil interference assays a TTAGTAA motif was identified. Point mutations and deletions of the entire TTAGTAA sequence which is a target site, e.g. for BAS2 (PHO2) in *Saccharomyces cerevisiae*, supported the involvement of this sequence in the binding of a transcriptional activator whose biochemical nature is unknown yet. Furthermore, it was shown that this sequence is required for high level expression of the *acvA* gene [90] (see below).

The promoter strengths of β-lactam biosynthesis genes are rather different. On the basis of reporter gene fusions, it became evident that in all three fungi, i.e. *P. chrysogenum*, *A. nidulans* and *A. chrysogenum* expression of *acvA* was much weaker compared to that of *ipnA* [83, 85, 87, 91]. The low expression of *acvA* is, at least in wild-type strains of *A. nidulans*, rate-limiting for penicillin production because overexpression of *acvA* led to drastically increased production of penicillin [92], while similar overexpression of *ipnA* and *aatA* did not [75]. In *A. nidulans*, it was also shown that *aatA* had lower expression than *ipnA* [83, 85].

3.5.3 Carbon Source Regulation

Industrial production of penicillin was usually carried out by using lactose as the carbon source (C-source), which gave the highest penicillin titre. The use of excess glucose leads to a drastic reduction of the penicillin titre [83, 93, 94]. This problem is partially overcome by feeding subrepressing doses of glucose and by the use of lactose as C-source [95]. Since in general the fungus grows better with glucose than with lactose [83], the production of penicillin appears to be favoured by sub-optimal growth conditions. C-source regulation seems to act at several points of the penicillin biosynthesis: (i) in *P. chrysogenum* flux of L-α-AAA to δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) [96]; (ii) both, transcriptional and post-transcriptional regulation of penicillin biosynthesis genes [13]. The expression of both the *acvA* (*pcbAB*) and *ipnA (pcbC)* gene of *P. chrysogenum* strain Q176, both measured by the use of the *uidA* reporter gene, was repressed by glucose [87]. In *P. chrysogenum*, a deletion analysis of the *acvA* gene promoter together with EMSAs using protein crude extract led to the suggestion of a putative region which could be responsible for glucose repression [90]. Renno et al. [84] claimed, however, that steady state mRNA levels of all three *P. chrysogenum* penicillin biosynthesis genes were highest during rapid growth when considerable levels of glucose were present. This shows that measurement of carbon regulation depends, at least in part, on the experimental approach used.

In *A. nidulans*, results obtained with reporter gene fusions showed that the expression of the *ipnA* gene was repressed when glucose or sucrose was used instead of lactose as the C-source during fermentation [83, 93]. This was further supported by the finding that the IPNS specific activity was drastically reduced in glucose-grown mycelia [83]. The repression of *ipnA* expression by repressing C-sources occurs, at least in part, at the transcriptional level because the steady state level of *ipnA* mRNA decreased when mycelia were cultivated with repressing C-sources, such as sucrose [93]. Unexpectedly, in *A. nidulans* the expression of both *acvA* and *aatA* reporter gene fusions was only slightly, if at all, repressed by glucose in fermentation medium [83, 85]. However, the specific activity of the *aatA* gene product, IAT, was reduced in mycelia grown with glucose instead of lactose [83, 85]. This suggests that the glucose regulation of IAT takes place, at least in part, post-transcriptionally (see below). In contrast to the penicillin production strain AS-P-78 of *P. chrysogenum* investigated by Revilla et al. [97], the IAT specific activity of both *A. nidulans* and the *P. chrysogenum* wild-type strain NRRL1951 was clearly reduced in glucose-grown cultures [83].

To study the molecular basis of C-source regulation, several mutants of *A. nidulans* carrying previously characterized loci affecting glucose repression of several genes of the primary metabolism (*creA^d-1*, *creB304*, *creC302*) [98, 99] were analysed. In these mutants, penicillin production was still reduced by glucose [83, 100]. However, in extreme loss-of-function mutations in *creA* slightly

derepressed *ipnA* steady state transcript levels were observed [93]. This was consistent with a deletion analysis of the *ipnA* promoter, demonstrating that a *cis*-acting DNA region crucial to sucrose repression maps between –1334 and –966 relative to the transcriptional start site of the gene [88]. A single CREA binding site was detected in this region, which was protected in DNase I footprint analysis using a GST::CREA protein which contained amino acids 35–240 of CREA [101]. However, the analysis of the expression of an *ipnA-lacZ* gene fusion revealed that the identified putative CREA binding site is not functional in vivo [101], making it unlikely that CREA plays a role in C-source repression of penicillin biosynthesis. This also agrees with the finding that acetate and glycerol, which are repressing and derepressing C-sources, respectively, of some primary metabolism genes in the *creA*-mediated circuit of carbon catabolite repression, behaved opposite to what would be expected from *creA* control. The use of acetate led to increased steady state levels of *ipnA* transcript and penicillin titres and glycerol led to the opposite effects, i.e. decreased *ipnA* transcript levels and penicillin titres [101]. Additional experiments further excluded the possibility of a direct involvement of *creB* and *creC* mutations on C-source repression of *ipnA* transcription [83, 100, 102]. Thus, in *A. nidulans* the mechanism(s) of regulation of penicillin biosynthesis by repressing C-sources remains to be elucidated. (iii) activation of side chain precursors; glucose was also found to cause inactivation of *P. chrysogenum* acetyl-CoA synthetase which has the capability to catalyse the activation (to their CoA thioesters) of some of the side chain precursors required for the production of several penicillins in vitro [68] (see above). Previously, it was reported that the uptake of side chain precursors of phenylacetic acid was regulated by glucose [103]. Recently, it was shown, however, that phenylacetic acid passes the plasma membrane via passive diffusion of the protonated species [104], thus excluding that the uptake could be regulated by the available C-source.

3.5.4
pH Regulation Mediated by the Transcriptional Activator PACC

Penicillin production is subject to regulation by ambient pH [101, 105]. Wild-type strains of *A. nidulans* can grow in media over the pH range of 2.5–10.5 [106]. There was markedly more penicillin in the culture broth when the pH value of the medium was kept constant at 8.1 than at 6.5 or 5.1 [105]. The analysis of the molecular basis of this phenomenon showed that the transcriptional regulator PACC is the key player of the pH regulation (Fig. 4). The DNA sequence of the *pacC* gene consists of 2172 bp interrupted by 2 introns of 85 and 53 bp, respectively. The 678 residue-derived protein (Mr 72,939) revealed that PACC contains three putative Cys_2His_2 zinc fingers. At alkaline ambient pH, PACC activates transcription of alkaline-expressed genes, e.g., of the alkaline phosphatase and protease genes *palD* and *prtA*, respectively, and also of the penicillin biosynthesis genes *ipnA* [107] and very likely of *acvA* [108]. The intergenic region between *acvA* and *ipnA* was found to contain 4 in vitro

PACC binding sites designated ipnA1, ipnA2, ipnA3 and ipnA4AB, recognized by a GST::PACC(amino acids 31–195) fusion protein (Fig. 4). The fusion protein was demonstrated to bind to the core consensus GCCARG [107]. A mutation analysis of each of these sites using *ipnA-lacZ* gene fusions revealed that in vivo the binding site ipnA3 was most important for PACC dependent *ipnA* expression, whereas sites ipnA2 and ipnA4AB were less important, although site ipnA2 was bound with highest affinity by PACC in vitro. Binding site ipnA1 apparently was not required for PACC dependent *ipnA* expression [109]. As observed for expression of an *ipnA-lacZ* gene fusion [101], expression of an *acvA-uidA* gene fusion was increased in a PacC5 mutant strain [108]. *pacC5* is an allele which is active irrespective of the ambient pH [107]. Furthermore, addition of amino acids histidine and valine to the culture medium led to acidification of ambient pH and to reduced *acvA-uidA* expression. This effect was not observed in a deletion strain (Δ183–312) carrying a deletion spanning PACC binding site ipnA3 (at nt 265–270) nor in the PacC5 mutant strain with a constitutively active PACC protein. Taken together, these data suggest that PACC also regulates *acvA* expression of *A. nidulans* predominantly from binding site ipnA3 [108].

At alkaline ambient pH, PACC prevents transcription of acid-expressed genes [107, 110, 111]. PACC must be specifically proteolysed to yield the functional (for both positive and negative roles) version containing the N-terminal 40% of the protein (Fig. 4). The processed form is functional as both activator and repressor. PACC proteolysis occurs in response to a signal provided by the six regulatory *pal* gene products in alkaline environments [107, 110–112]. In wild-type strains, the *pal* pathway is thought to introduce a modification of PACC at alkaline pH, disrupting intramolecular interactions to allow activating proteolysis [112] which leads to the removal of a negative-acting C-terminal domain. The mechanism how PACC can avoid its proteolytic activation in the absence of signal transduction has been studied [113]. The activation of PACC requires two sequential proteolytic steps. First, the 'closed' translation product is converted to an accessible, committed intermediate by proteolytic elimination of the C-terminus (Fig. 4). This ambient pH-regulated cleavage is required for the final, pH-independent processing reaction and is mediated by a distinct signalling protease (possibly PalB) [114]. Interestingly, ambient pH signalling also regulates nuclear localisation of PACC [115].

The *P. chrysogenum pacC* gene of strain NRRL1951 is encoded by 1979 bp interrupted by a single intron of 56 bp. The predicted 641-residue protein (Mr 68,681) exhibits most of the features described for the *A. nidulans* PACC protein, including three zinc fingers of the Cys_2His_2 class. A fusion protein of glutathione thiotransferase with amino acids 46–154 of *P. chrysogenum* PACC (GST::PACC (46–154)) overexpressed in *E. coli* and purified, bound in vitro to the intergenic region between *P. chrysogenum acvA* and *ipnA*. By computer analysis seven PACC binding consensus sites (5′-GCCARG-3′) were found in the intergenic region. This is consistent with the finding that steady state *ipnA* mRNA levels were increased at alkaline pH [86]. The upstream region of the *P.*

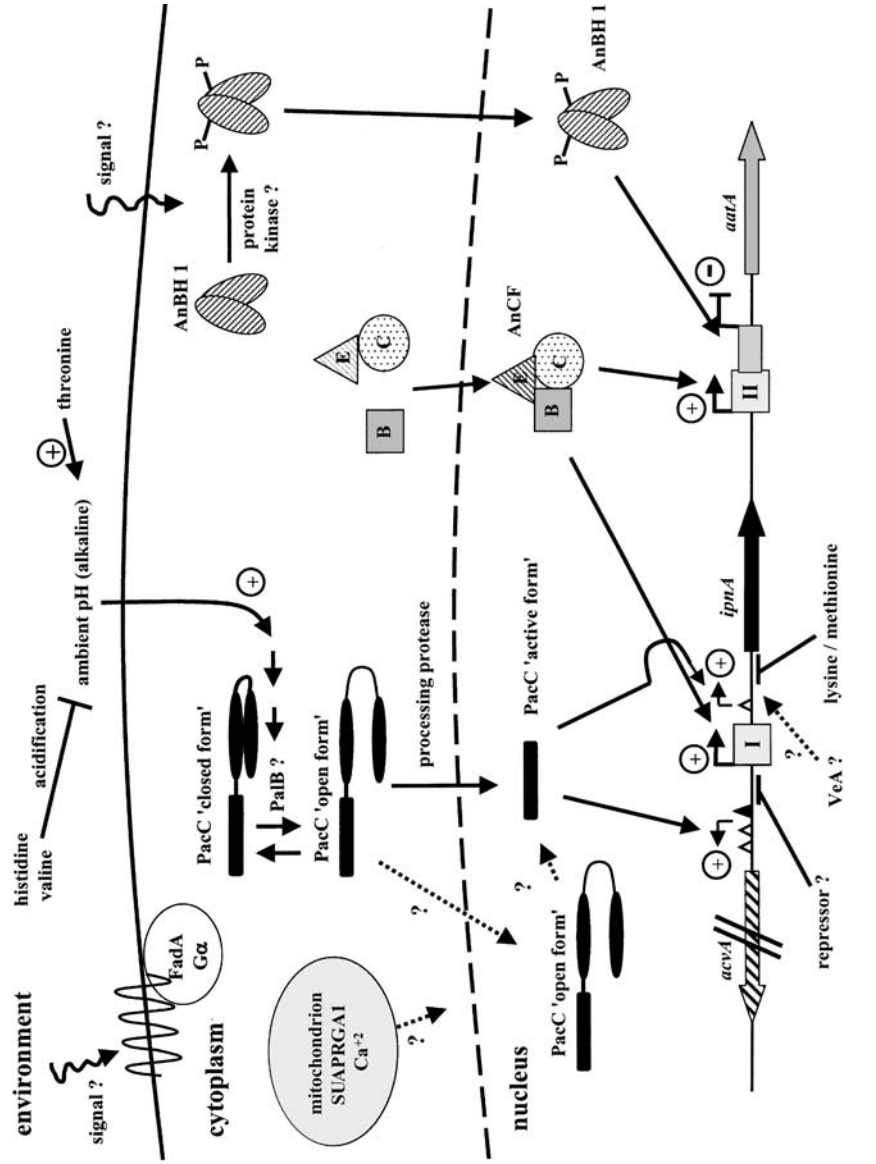

Fig. 4 Regulatory genes involved in the regulation of the penicillin biosynthesis genes of *A. nidulans*. The effects of the indicated amino acids are mediated by the ambient pH most likely via *pal* genes and the central regulatory protein PACC. The four PACC binding sites bound in vitro by PACC are marked by triangles in the intergenic region between *acvA* and *ipnA*. Site 3, which seems to be of major importance for both *ipnA-lacZ* [109] and *acvA-uidA* expression [108], is marked by a *filled triangle*. The model of nuclear import of PACC is adapted from Mingot et al. [71]. The two identified AnCF binding sites are marked with *stippled boxes* and with *Roman numbers* (I and II). The AnBH1 binding site is indicated by a *dark grey box*

chrysogenum aatA (*penDE*) gene contains eight binding sites for PACC, whereas that of *A. nidulans* has just one such sequence, suggesting that these genes might be regulated by PACC as well [86].

In addition, Chu et al. [116] and Feng et al. [117] reported independently of each other that partially purified crude extracts of *P. chrysogenum* bound in vitro to the sequences TGCCAAG and GCCAAGCC, respectively. These binding sites identified almost certainly correspond to PACC binding sites [86]. Furthermore, the *A. chrysogenum* PACC homologue was found to activate transcription of the *ipnA* gene (see chapter Schmitt et al.). *PacC* genes are not confined to β-lactam producing fungi, as the cloning of the *A. niger pacC* gene showed [118]. Hence, PACC represents a wide-domain regulator which is involved in the regulation of expression of β-lactam biosynthesis genes.

Because the use of glucose or sucrose as the C-source leads to acidification of the medium [83, 93, 101] it was conceivable that the glucose/sucrose effect was due to pH regulation [101]. This was further supported by the observation that external alkaline pH could bypass sucrose repression of steady state *ipnA* transcript levels and penicillin titres. Additional experiments confirmed that alkaline pH is the factor derepressing penicillin production in 3% sucrose broth [101]. Furthermore, the analysis of PacC mutants revealed that mutations in *pacC* bypassed C-source regulation of *ipnA* transcript levels, i.e. *pacC* mutations caused derepression of steady state levels of the *ipnA* mRNA in sucrose broth despite external acidic pH resulting from sucrose utilisation [101]. However, neither acidic external pH nor mutations *palA1*, *palB7* and *palF5* mimicking the effects of growth at acidic pH, prevented C-source derepression [101]. Furthermore, the PACC binding sites determined in vitro by the use of a fusion polypeptide containing the PACC DNA-binding domain are not located in the *cis*-acting region which was shown to mediate C-source repression of *ipnA-lacZ* expression [101, 107]. Taken together, these data support the model of independent regulatory mechanisms, one mediating C-source regulation and another mediating pH regulation through the *pacC*-encoded transcriptional regulator [101, 107]. Since alkaline pH values per se seem to derepress *ipnA* transcription, Espeso et al. [101] proposed that alkalinity represents a physiological signal which triggers penicillin biosynthesis. The authors concluded that carbon limitation, either by using less favourable C-sources or by reducing the concentration of favourable C-sources, results in external alkalinisation, whereas sufficient availability of a favourable C-source causes external acidification. Thus, carbon and pH regulation normally act in concert, although through different mechanisms [101].

In contrast to the situation in *A. nidulans*, alkaline ambient pH did not seem to override the negative effect of repressing C-source on *ipnA* transcription in *P. chrysogenum* because full *ipnA* expression was dependent on C-source derepression irrespective of the ambient pH value [86].

The reason for the pH mediated regulation of penicillin biosynthesis is unclear. It might be connected to the observation that β-lactams exhibit increased

toxicity on at least some bacterial species at alkaline pH. Furthermore, bacterial competition with fungi may be more intense at alkaline pH [110].

3.5.5 Nitrogen Regulation

The effect of the availability of nitrogen source on the penicillin biosynthesis has been discussed for a long time. Sanchez et al. [119] reported the inhibition of the penicillin biosynthesis in *P. chrysogenum* by high levels of ammonium. In *A. chrysogenum*, it was found that ammonium concentrations ($(NH_4)_2SO_4$) higher than 100 mmol/L strongly interfered with cephalosporin C production [120]. It was demonstrated that in *P. chrysogenum*, ammonium directly influenced the expression of penicillin biosynthesis genes. By using gene fusions of both penicillin biosynthesis genes *ipnA* and *acvA* with the *E. coli* reporter gene *uidA*, it was shown that the expression of both genes was repressed by addition of 40 mmol/L $(NH_4)Cl$ to lactose-grown mycelia [87].

In *A. nidulans* and *N. crassa*, global nitrogen repression/derepression is mediated by the major positive control genes *areA* and *nit-2*, respectively [121, 122]. The homologous gene of *P. chrysogenum*, *nre*, was shown to complement Nit-2 mutants of *N. crassa* [123]. Each of these three genes encodes regulatory factors with a single Cys-X_2-Cys-X17-Cys-X2-Cys-type zinc finger that in combination with an immediate downstream basic region constitutes a DNA-binding domain. The overall amino acid sequences of these three regulatory proteins show only 30% identity, but they have 98% identity in their DNA-binding domains. These transcription factors recognise the consensus sequence GATA and can be grouped together into a GATA protein family [124]. The optimal binding sites for NIT-2 were found to consist of at least two GATA elements, which can face in the same or opposite directions, with a spacing which can vary from 3 to 30 bp [125].

A protein consisting of 181 amino-acid residues of (the 835-residue) *P. chrysogenum* NRE [126], containing its zinc-finger domain, fused to the N-terminus of *E. coli* β-GAL bound with high affinity to a DNA fragment derived from the intergenic region between *acvA* and *ipnA* of *P. chrysogenum*. Although there are six GATA sequences found in the intergenic region, missing contact experiments using the β-GAL-NRE fusion protein revealed that NRE strongly interacts with a site that contains two of these GATA sequences [126]. In this binding site, the two GATA core sequences are arranged in a head-to-head fashion and separated by 27 bp. Therefore, it appears very likely that nitrogen metabolite regulation of penicillin biosynthesis genes is mediated through NRE, although in vivo studies are clearly needed to relate NRE binding to potential regulatory functions [126]. This suggests that the availability of favoured nitrogen sources and thus good growth conditions, leads to reduced penicillin synthesis by the fungus.

In *A. nidulans*, however, no evidence for nitrogen dependent regulation of the penicillin biosynthesis has been reported so far. This is also consistent with

the observation that the intergenic region between *acvA* and *ipnA* of *A. nidulans* only contains a single GATA motif, whereas six GATA sequences are found in the corresponding *P. chrysogenum* region [126]. Interestingly, the *P. chrysogenum* promoter was shown to respond to nitrogen control when transformed in *A. nidulans* indicating that the nitrogen repressing system of *A. nidulans* acted on the heterologous promoter [127]. Furthermore, it is worth to note that in the intergenic region of the corresponding *A. chrysogenum* genes [91], there are even 15 GATA motifs present. It is thus conceivable that these genes are also regulated by a GATA factor.

3.5.6 Amino Acids as Mediators of Regulation

Because penicillin and cephalosporin are synthesised from the amino acid precursors L-α-AAA, L-cysteine and L-valine, it was conceivable that amino acids play a role in the regulation of their biosyntheses. This was supported by the observation that in both *P. chrysogenum* and *A. nidulans* the addition of L-lysine to fermentation medium led to reduced penicillin titres [83, 128]. Since L-α-AAA is a branch point between L-lysine and penicillin/cephalosporin biosynthesis pathways, L-lysine inhibition of penicillin biosynthesis was suggested to operate at one or more steps of the L-lysine pathway. This was based on the notion that L-lysine feedback inhibited several enzymes of the lysine biosynthesis pathway which might result in a reduced L-α-AAA pool available for penicillin production [13]. However, amino acids also directly affect the expression of β-lactam biosynthesis genes. In *A. chrysogenum*, it was reported that the addition of D,L-methionine to the medium led to a three- to fourfold increase in production of cephalosporin C. The increased production was paralleled by increased steady state levels of mRNAs of cephalosporin biosynthesis genes *acvA*, *ipnA*, *cefEF* and, to a slight extent *cefG* [129].

In *A. nidulans*, differential effects due to various amino acids in the medium on the expression of penicillin biosynthesis genes *acvA* and *ipnA*, and penicillin production were measured. L-Amino acids with a major negative effect on the expression of *acvA-uidA* and *ipnA-lacZ* gene fusions, i.e. histidine, valine, lysine and methionine (only at concentrations greater than 10 mmol/L), led to decreased penicillin titres and a decreased ambient pH during cultivation of the fungus. An analysis of deletion clones lacking binding sites of the pH dependent transcriptional factor PACC (see above) in the intergenic region between *acvA-uidA* and *ipnA-lacZ* gene fusions and in a PacC5 mutant strain suggested that the negative effects of L-histidine and L-valine on *acvA-uidA* expression were due to reduced activation by PACC under acidic ambient conditions caused by these amino acids (Fig. 4). The repressing effect caused by L-lysine and L-methionine on *acvA*, however, was even enhanced in one of the deletion clones and the *pacC5* mutant strain, suggesting that these amino acids act independently of PACC by so far unknown mechanisms on the gene expression [108].

A specific effect of the cross-pathway control on the penicillin biosynthesis was excluded. However, a secondary effect was found. It was shown that amino acid limitation led to significantly increased transcription of *lysA* but not of *lysF*. The *lysF*-encoded homoaconitase acts upstream of the α-aminoadipate branch point, whereas the *lysA* gene product, saccharopine dehydrogenase, catalyses the ultimate step of the lysine-specific branch. Starvation-dependent changes in transcription levels of *lysA* were dependent on the presence of the central transcriptional activator of the cross-pathway control (CPCA) which is the homologue of the *Saccharomyces cerevisiae* GCN4. Overproduction of CPCA decreased expression of *ipnA* and *acvA* reporter gene fusions and even more drastically reduced penicillin production. This data suggests that, upon amino acid starvation, the cross-pathway control overrules secondary metabolite biosynthesis and favours the metabolic flux towards amino acids instead of penicillin in *A. nidulans* [130].

3.5.7
Influence of Oxygen

The availability of oxygen is important for penicillin production. Good aeration of mycelia with oxygen is a prerequisite for high β-lactam titres [95, 131]. Since several enzymes require oxygen for their activity, like IPNS and DAOC synthetase/DAC hydroxylase, it is conceivable that this is the reason for oxygen requirement. The importance of oxygen is also supported by the possibility of increasing cephalosporin production genetically by introducing a bacterial oxygen binding protein in *A. chrysogenum* [132]. However, there is a contradictory report which shows that reduction of oxygen led to increased *acvA* and *ipnA* expression of *P. chrysogenum*, possibly as part of a stress response [84].

3.5.8
The CCAAT-Box Binding Protein Complex AnCF

Based on results with a moving window analysis of the *acvA-ipnA* intergenic region of *A. nidulans* and together with band shift and methyl interference assays, a CCAAT-containing DNA motif (box I) located 409 bp upstream of the ATG initiation codon of the *acvA* gene was identified which is bound by a protein complex designated AnCF (*syn.* PENR1), for *Aspergillus nidulans* CCAAT binding factor [89, 133] (Fig. 4). This CCAAT box I is of major importance for the regulation of both genes, since a 4 bp-deletion within this site (ΔCCA-G) led to an eightfold increase of *acvA* expression and simultaneously, to a reduction of *ipnA* expression to about 30% [89]. Furthermore, the *A. nidulans aatA* promoter region also contains a functional CCAAT element (box II), located about 250 bp upstream of the transcriptional start sites of *aatA*. It was specifically bound by the same AnCF regulatory protein complex. Substitution of the CCAAT core sequence by GATCC led to a fourfold reduction of ex-

pression of an *aatA-lacZ* gene fusion [134] indicating that the identified binding site was functional in vivo and positively influenced *aatA* expression (Fig. 4).

The first CCAAT-box binding factor characterised in detail, was the *S. cerevisiae* HAP complex, which consists of at least four subunits: HAP2, HAP3 and HAP5 form a heterotrimeric complex that is essential for DNA binding, HAP4 is an acidic protein which acts as the transcriptional activation domain [135, 136]. AnCF of *A. nidulans* was found to consist at least of three subunits designated HapB, HapC and HapE, all of which are necessary and sufficient for binding of AnCF to the DNA [137, 138]. Deletion mutants of AnCF subunit-encoding genes in *A. nidulans*, i.e. *hapB*, *hapC* and *hapE*, show an identical phenotype of slow growth and poor conidiation. Band shift experiments and deletion analysis showed that AnCF is also involved in the regulation of the acetamidase gene *amdS*, which is required for the use of acetamide as the nitrogen and C-source [133, 139]. Consequently, *hap* mutant strains hardly grew on acetamide as the sole nitrogen and C-source indicating that AnCF plays a role in regulating *amdS* expression [137, 138]. In addition, the intergenic region of the bidirectionally transcribed genes *lamA* and *lamB* (needed for utilisation of lactams) as well as the promoter region of *gatA* (ɣ-amino butyric acid transaminase) contain CCAAT boxes which were bound by DNA binding factors [140]. An additional CCAAT binding factor (AnCP) of *A. nidulans* had been proposed [141, 142] which bound in vitro to the Taka-amylase gene promoter of *A. oryzae*. It turned out that this complex is in fact AnCF [143, 144] although it is unclear whether AnCF always consists of the same subunits, apart from those essential for DNA binding.

For AnCF, its binding consensus motif was determined by band shift assays as RRCCAATC/ARCR [13]. Deletion or mutagenesis of the AnCF binding sites in the promoters of the penicillin biosynthesis genes had opposite effects; the expression of *acvA* was increased eightfold, while the expression of *ipnA* [89] and *aatA* [134] was reduced. Consistent with data obtained by deletion of the AnCF binding site, in a Δ*hapC* background, expression of both an *ipnA-lacZ* gene fusion and an *aatA-lacZ* gene fusion was reduced to 10% compared to their expression in the wild type. Hence, AnCF is a positively acting factor of *ipnA* and *aatA* expression.

Penicillin titres were reduced in a Δ*hapC* background as well, but only by about 30%. In addition, expression of an *acvA-uidA* gene fusion was hardly affected by the Δ*hapC* mutation [145]. The minor effect of lack of AnCF on penicillin production is consistent with the view that *acvA* expression is rate-limiting in *A. nidulans* wild-type strains [92]. Consistently, decreased expression of *ipnA* and *aatA* even by a factor of five as observed in the Δ*hapC* strain, only results in a reduction of penicillin production of about 30% when the expression of the *acvA* gene is only marginally affected.

The observation that *acvA* expression was less affected in a Δ*hapC* strain was unexpected because it was shown that specific deletion of four nucleotides of the AnCF binding site between *acvA* and *ipnA* (box I) resulted in a strong in-

crease of *acvA* expression [89]. Thus it appears likely that, in addition to AnCF, a repressor protein binds closely to or overlaps the AnCF binding site which would explain that the AnCF binding site exhibits a repressing effect on *acvA* expression in the wild type. Consistent with this view, lack of AnCF binding in the Δ*hapC* mutant did not prevent binding of this putative repressor protein and hence, *acvA-uidA* expression was not increased. Deletion of the AnCF binding site, however, prevented binding of both AnCF and the repressor causing the phenotype of increased *acvA* expression. However, the existence of such a putative repressor remains to be shown experimentally.

Recently, negative regulation could be assigned to AnCF as well. The lysine biosynthesis gene *lysF* of *A. nidulans* is negatively regulated by AnCF [146]. Moreover, it was shown that AnCF is negatively autoregulated by repression of the *hapB* gene [147].

The physiological function of HAP-like regulatory factors in lower eukaryotes remains obscure. In yeast, the HAP complex activates the expression of genes whose products are required for respiration. Hence, HAP mutants are not able to grow on non-fermentable carbon sources [135, 136]. Because *A. nidulans*, however, is an aerobic growing fungus, *S. cerevisiae* may not be a good model for the role of HAP-like complexes in aerobic growing eukaryotes. Furthermore, lack of a functional HAP complex (Δ*hap* strains) is not lethal for *A. nidulans* [137, 138]. In addition, in *A. nidulans* AnCF regulates secondary metabolism genes (penicillin biosynthesis genes). Hence, it will be interesting to elucidate whether this particular function of a HAP-like complex requires so far for HAP-complexes unknown accessory proteins, or, alternatively, which mechanism is involved allowing a HAP-like complex to regulate certain sets of genes (see next section).

It seems very likely that AnCF is conserved among the industrially important β-lactam producing fungi. This assumption is supported by the observation that DNA fragments spanning the corresponding intergenic regions between *acvA* and *ipnA* of *P. chrysogenum* and *A. chrysogenum*, and the promoter region of the *P. chrysogenum aatA* gene were able to dilute the complexes of the corresponding *A. nidulans* probes and AnCF protein [89, 134]. Computer analysis showed that DNA elements with a high degree of sequence identity to the *A. nidulans* AnCF site reside within the intergenic regions of both *P. chrysogenum* and *A. chrysogenum* and the *aatA* promoter region of *P. chrysogenum*. These sites could be potential targets of homologous AnCF complexes in *P. chrysogenum* and *A. chrysogenum*.

3.5.9 The *A. nidulans* bHLH Protein AnBH1

AnCF was shown to bind to a single CCAAT box (box II) present in the promoter of the *A. nidulans aatA* gene. Attempts at purifying components of AnCF by DNA affinity chromatography using a DNA fragment encoding the region of the CCAAT box II of *aatA* led to the identification of several protein bands

in an SDS-PAGE [145]. Some of these did not correspond to the known components of AnCF, i.e. HapB, HapC and HapE [138, 145]. This finding suggested that there might be additional proteins binding to the *aatA* promoter adjacent or overlapping to the CCAAT box. Using affinity chromatography and standard protein purification, a novel transcription factor designated AnBH1 was isolated. The corresponding *anbH1* gene was cloned and found to be located on chromosome IV. The deduced AnBH1 protein belongs to the family of basic-region helix-loop-helix (bHLH) transcription factors. AnBH1 binds in vitro as a homodimer to a not previously described asymmetric E-box within the *aatA* promoter which overlaps with the AnCF binding site. Since deletion of *anbH1* appeared to be lethal the *anbH1* gene was replaced by a regulatable *alcA*p-*anbH1* gene fusion. The analysis of *aatA*p-*lacZ* expression in such a strain indicated that AnBH1 acts as a repressor of *aatA* gene expression and therefore counteracts the positive action of AnCF [148] (Fig. 4).

3.5.10 Velvet A *(veA)*

The velvet gene *veA* gene was previously shown to mediate a developmental light response [149]. In *A. nidulans* strains containing a wild-type allele of the velvet gene (*veA*$^+$), light reduces and delays cleistothecial formation and the fungus develops asexually, whereas in the dark, fungal development is directed toward the sexual stage, forming cleistothecia. Under conditions inducing the sexual development, the *veA* deletion (Δ*veA*) strain is unable to develop sexual structures [150], indicating that *veA* is required for cleistothecium and ascospore formation. Kato et al. [151] demonstrated that *veA* regulates the expression of genes implicated in the synthesis of the mycotoxin sterigmatocystin and penicillin. In a Δ*veA* strain *ipnA* transcripts were abundant. However, surprisingly the *veA* deletion mutant produced less penicillin than the wild type. This contradiction might be explained by the finding that transcript of *acvA* analysed by RT-PCR was only detected in the *veA*$^+$ strain, in both light and dark cultures [151]. Hence the authors concluded VeA repressed the transcription of the *ipnA* gene, and was necessary for the expression of the *acvA* gene. However, by contrast, previously it was shown that *acvA* transcript was present in *veA* mutant strains [24]. Consistently, *acvA* expression when measured via a gene fusion was clearly detectable in *veA* mutant strains because most of the laboratory strains of *A. nidulans* contain a *veA* mutation [83].

3.5.11
The Cephalosporin C Regulator CPCR1 Identified in *A. chrysogenum* Is very Likely also Present in Both *A. nidulans* and *P. chrysogenum*

CPCR1 was identified by Schmitt and Kück [152] as binding to a region in the promoter of the cephalosporin biosynthesis gene (*ipnA*) *pcbC*, located 418 nucleotides upstream of the translational start codon (see chapter Schmitt et al.). By using degenerate oligonucleotides and PCR a putative homologous gene was isolated from *P. chrysogenum* (PcRFX1) which consists of 855 amino acids. PcRFX1 and CPCR1 share an overall similarity of 29% identical amino acids. However, the similarity in the DNA binding domain to CPCR1 is 60% of identical amino acid residues. Also, a putative homologue was identified in the genome of *A. nidulans* (see chapter Schmitt et al.).

3.5.12
Recessive *Trans*-Acting Mutations Affecting the Expression of Penicillin Biosynthesis Genes

In *A. nidulans*, a mutagenesis approach led to the identification of mutants carrying recessive mutations, designated *prg* (for penicillin regulation) [153] and *npeE1* (impaired in penicillin biosynthesis) [154]. Segregation analysis led to the identification of two different complementation groups designated *prgA1* and *prgB1*. For *npeE1*, genetic analysis showed that the gene is located on linkage group IV [154]. To date, it has not been clarified whether *npeE1* differs from *prgA1* and *prgB1*. The mutants exhibited both reduced *ipnA-lacZ* expression and reduced penicillin titres compared with the wild-type strain. For mutants PrgA1 and PrgB1, it was demonstrated that they also differed in *acvA-uidA* expression levels from the wild type and that these mutants contained reduced intracellular amounts of IPNS [153]. The results obtained by genetic and biochemical analyses indicated that the mutants most likely carry mutations in positively acting regulatory genes (Fig. 4).

A gene was isolated designated *suAprgA1* that complemented the *prgA1* phenotype to the wild type, i.e. the expression of both gene fusions and the penicillin production nearly reached wild-type levels. Analysis of *suAprgA1* in the *prgA1* mutant strain did not reveal any mutation in the *suAprgA1* gene or unusual transcription of the gene. This suggested that the gene is a suppressor of the *prgA1* mutation. The *suAprgA1* gene has a size of 1245 bp. Its five exons encode a deduced protein of 303 amino acids. The putative SUAPRGA1 protein showed similarity to both the human p32 protein and the Mam33p of *S. cerevisiae*. The *suAprgA1* gene is located on chromosome VI. Deletion of the *suAprgA1* gene led to a reduction of *ipnA-lacZ* expression to about 50% and to a slight reduction of the *acvA-uidA* expression. The Δ*suAprgA1* strain produced about 60% of the amount of penicillin compared with the wild-type strain [155]. SUAPRGA1 was localised in the mitochondria. It appears to bind Ca^{2+} ions (Gehrke A, Van den Brulle J, Bielen H, Read N, Brakhage AA, unpublished

results). It is likely that SUAPRGA1 is involved in the generation of a physiological signal which is required for the full expression of the penicillin biosynthesis genes and thus the penicillin production [155]. This assumption is also supported by the observation that overexpression of the *suAprgA1* gene in *A. nidulans* using the *alcA* promoter of *A. nidulans* did not result in an increase of penicillin production or expression of penicillin biosynthesis genes beyond the levels observed in wild-type strains (Van den Brulle et al. unpublished data). Using nitrosoguanidine, Cantoral et al. [156] isolated nine mutants of the *P. chrysogenum* Wis54-1255 strain impaired in penicillin production. Biochemical and genetic analyses suggested that two of these mutants (Npe2 and Npe3) carry mutations in regulatory genes affecting the expression of the entire penicillin biosynthesis gene cluster.

3.5.13
G Protein Mediated Signal Transduction

Until now, only little information is available on the signal transduction cascades involved in the β-lactam biosynthesis. Previous work revealed that synthesis of the carcinogenic mycotoxins sterigmatocystin (ST) and aflatoxin (AT) in *Aspergillus* species is negatively controlled by FADA, the α-subunit of a heterotrimeric G-protein. FADA negatively regulated both asexual reproduction (conidiation) and AF/ST synthesis in these aspergilli [157]. In an *A. nidulans* strain containing a constitutively activated FADA (*fadA* G42R), both conidiation and sterigmatocystin production are repressed. Furthermore, the dominant activating *fadA* allele, fadAG42R, also led to an increased steady state mRNA level of the *ipnA* gene and concomitantly increased penicillin titres. Taken together, FADA appears to be a member of a signal transduction cascade activating the penicillin biosynthesis and, interestingly, has opposite roles in regulating the biosynthesis of penicillin and the mycotoxin ST in *A. nidulans* [158].

3.5.14
Post-Transcriptional Regulation

Discrepancies observed between expression of structural genes and enzyme specific activities of the corresponding proteins suggested that besides transcriptional regulation, post-transcriptional regulation of penicillin biosynthesis genes occurs [13]. It was proposed that the glucose effect on IAT specific activity was post-transcriptionally mediated [85]. Furthermore, some discrepancies between *ipnA* expression and detectable IPNS specific activity were observed by comparing a wild-type strain of *A. nidulans* with a strain carrying a disrupted *acvA* gene [159, 160].

3.6 Regulation of Penicillin Biosynthesis in Fungal Production Strains

Apart from the academic interest in elucidating the molecular regulation of biosynthesis of secondary metabolites in lower eukaryotes, there is a strong interest from an industrial point of view because β-lactam compounds are still among the most sold antibiotics in the world's antibiotic market (see chapter Barber et al.). Hence, it is desirable to analyse high producing production strains which are highly mutated and have been derived from several different strain development programmes. This will help to elucidate both the molecular basis of deregulation and thus high production and also any remaining bottlenecks. Nowadays, industrial penicillin and cephalosporin production is mainly carried out with *P. chrysogenum* and *A. chrysogenum*, respectively. Most of these strains have been produced by mutagenesis followed by screening or selection. In 1972, the initial Panlabs Inc. *P. chrysogenum* strain made 20,000 units of penicillin per mL in seven days (an activity equivalent to 12 mg pure penicillin G, Na salt per mL [95]). In 1990, the improved strain made 70,000 units per mL in seven days. Penicillin titres in industry in 1993 were as high as 100,000 units per mL [19, 30].

Two important genetic features of *P. chrysogenum* production strains have been identified: (i) amplification of structural genes and (ii) their massively increased steady state mRNA levels.

Between 8 and 16 copies are present in the high producer strain *P. chrysogenum* BW1890 [161]. The *P. chrysogenum* high titre producing strains P-2 and AS-P-78 (old production strain) carry approximately nine and six copies, respectively, of penicillin biosynthesis genes [162]. Fierro et al. [163] showed that in the high titre *P. chrysogenum* strains E1 and AS-P-78 the amplifications are organised in tandem repeats. A conserved TTTACA hexanucleotide sequence may be involved in their generation. This TTTACA sequence borders the 106.5-kb long penicillin biosynthesis gene cluster in the wild-type strain NRRL 1951 and also the *P. notatum* strain ATCC 9478 (Fleming's isolate). In *P. chrysogenum* mutants independently isolated, it was shown that in all three mutants deletion of the penicillin biosynthesis gene cluster had occurred at a specific site within the conserved hexanucleotide sequence [164]. It was suggested that this site may represent a hot-spot for site specific recombination after mutation with nitrosoguanidine, the process possibly being part of a fungal SOS system similar to that found in *E. coli* [163, 164]. In other members of a strain improvement series, the length of the amplicon was found to be 57.5 kb. Furthermore, cDNA screening has failed to identify any further transcribed elements within the coamplified region apart from those derived from the structural penicillin biosynthesis genes [165]. Taken together, these data indicated the presence of recombinogenic regions flanking the penicillin biosynthesis gene cluster [163, 165].

Sequence analysis has shown that no mutations have been generated within the promoter regions of the penicillin biosynthesis structural genes [165]. In addition, data obtained from several production strains indicated that penicillin

titres were not proportionally increased with copy number. Northern blot analysis established that the *ipnA* mRNA steady state level of strain BW 1890 was 32- to 64-fold that of NRRL1951, an increase too great to be due to the amplification alone [161]. These findings suggest that the increased penicillin production in amplified strains may be due to altered regulation of the biosynthesis pathway through changes in *trans*-acting regulatory factors [165]. Therefore, it will be of considerable interest to compare regulatory genes already found in both *A. nidulans* and *P. chrysogenum* between *P. chrysogenum* wild-type and production strains. Several studies revealed interesting differences [166].

In contrast to the gene amplification of structural genes reported in *P. chrysogenum* production strains, in the cephalosporin C production strain *A. chrysogenum* LU4-79-6, the β-lactam biosynthesis genes seem to be present in single copy (see chapter Schmitt et al.).

There are certainly numerous other mutations involved which lead to a high-producing phenotype. These also include factors such as stability of biosynthesis enzymes and deregulation of enzymes involved in amino acid biosynthesis pathways, and hence the amount of precursor amino acids produced [13]. Furthermore, the transport of intermediates of the penicillin biosynthesis between organelles, and the number of organelles can be predicted to be important for penicillin production strains.

3.7
Evolution of β-Lactam Biosynthesis Genes in Fungi

β-Lactam biosynthesis genes were found in both some bacterial species and some fungi. Based on several observations, a horizontal transfer of β-lactam biosynthesis genes from bacteria to fungi during evolution was proposed by several authors [51, 167–170]. The arguments in favour of a horizontal gene transfer are as follows. (i) *ipnA* genes of fungi and bacteria show high sequence similarities. More than 60% of the nucleotide bases and 50% of the deduced amino acids are identical. (ii) Bacterial as well as fungal β-lactam genes are organized in clusters. In bacteria, the β-lactam biosynthesis genes are organized into a single cluster, as are the penicillin biosynthesis genes in fungi. The cephalosporin biosynthesis genes in *A. chrysogenum* are organized into two clusters located on different chromosomes (Fig. 3). This finding led to the assumption that the β-lactam biosynthesis genes were transferred as a single cluster from an ancestral prokaryote to a common ancestor of the β-lactam synthesising fungi. In the eukaryotic ancestor, the biosynthesis genes were split onto two chromosomes. One part encodes the early genes of β-lactam biosynthesis, the other the late genes. Later in the lineage an ancestor of *A. nidulans* and *P. chrysogenum* diverged from *A. chrysogenum* and has presumably lost the second cluster with the genes for the late stage of cephalosporin biosynthesis [7] (Fig. 3). (iii) The GC content in the third position of codons encoding the *ipnA* gene of *A. nidulans* and *P. chrysogenum* is unusually high and could indicate an evolutionary origin from streptomycetes which show GC contents of

greater than 70% [51]. (iv) Fungal *acvA* and *ipnA* genes do not contain introns indicating a bacterial origin of the genes [13].

Based on the DNA sequences of *ipnA* genes from Gram-positive streptomycetes and fungi and a rate of nucleotide substitution of 10^{-9} nucleotide changes per site per year [171], Weigel et al. [170] proposed that the transfer occurred 370 million years ago. The cloning and sequencing of an *ipnA* gene from a Gram-negative bacterium, *Flavobacterium sp*., however, led to an extension/modification of the hypothesis of horizontal gene transfer. The *ipnA* gene of *Flavobacterium sp.* shares 69% sequence identity with the streptomycetes gene and 64–65% with the fungal genes (*A. chrysogenum*, *P. chrysogenum*) [172]. A recent reevaluation of the divergence times of organisms using a protein clock suggested that Gram-positive and Gram-negative bacteria split about 2 billion years ago, prokaryotes and a eukaryotic ancestor split about 3.2–3.8 billion years ago [173]. If the gene transfer had occurred only 370 million years ago from streptomycetes to fungi as proposed by Weigel et al. [170], it could be expected that the fungal and streptomycetes genes show a greater homology than the Gram-positive (streptomycetes) and Gram-negative genes (*Flavobacterium sp.*). As outlined above, this is not the case [172]. Hence, Aharonowitz et al. [51] suggested that multiple gene transfer events might have occurred from bacteria to fungi. It is difficult to imagine, however, why these multiple gene transfers then happened at about the same time what would be expected from the degree of similarity between the proteins of the various organisms. In addition, Smith et al. [174] argued against a horizontal transfer. The authors criticised that the hypothesis of a horizontal gene transfer, e.g. of the *ipnA* gene, was made with a very limited data set and was based solely on assumptions about rates of change. They rooted the tree with two distantly related β-lactam biosynthesis enzymes. They compared the similarity of both IPNS of *A. nidulans*, *P. chrysogenum*, *A. chrysogenum*, *S. clavuligerus, S. anulatus* and *Flavobacterium sp*., and DAOC synthase/synthetase of *S. clavuligerus* and *A. chrysogenum*. Based on these similarities, a tree arose with conventional evolutionary descent. The authors argued that the simplest interpretation is that the genes for the two enzymes are the result of a duplication that occurred before the prokaryote/eukaryote divergence. However, if the genes appeared very early in the evolution why have most of the eukaryotes and fungi lost the gene cluster? This question cannot be seriously answered at the moment. Thus, the evolutionary origin of β-lactam biosynthesis remains speculative.

4 Applied Implications

The increasing knowledge of the molecular genetics of β-lactam biosynthesis has opened up new possibilities to rationally improve β-lactam production strains and to engineer new biosynthesis pathways. This leads to the question, however, whether an improvement of productivity is still possible. Cephalo-

sporin C production with *A. chrysogenum* is well below the productivity reached with *P. chrysogenum* for penicillin. Therefore, there is much effort needed to increase cephalosporin C production. For penicillin, several theoretical models have been established based on the available experimental data [175]. By using detailed stoichiometric models, the theoretical yield was calculated to be 0.47–0.50 moles penicillin per mole glucose [176, 177]. Until today, the maximum theoretical yields calculated are eight to ten times higher than the overall yields observed in fed-batch cultures, and there is therefore a considerable potential for further improvement of the process. However, it seems unlikely that the maximum theoretical yields can be reached in a real process since the penicillin biosynthesis is indirectly coupled to other cellular reactions. Therefore, taking this into account it was estimated that it should still be possible to improve the current yields by a factor of four to five [175].

Several molecular strategies have been followed to improve or alter β-lactam production. (i) Introducing additional copies or overexpression using strong promoters of β-lactam biosynthesis genes. (ii) Metabolic engineering of β-lactam biosynthesis pathways by expression of heterologous genes, e.g. production of cephalosporin precursors in *P. chrysogenum*. (iii) Use of the increasing knowledge of peptide synthetase genes such as those for ACVS enzymes to produce novel compounds by genetic engineering [178]. (iv) Manipulation of regulatory genes which has not been reported yet because results on the identification of regulatory genes are just being accumulated.

4.1 Increase of Expression of Penicillin Biosynthesis Genes

In an *A. nidulans* wild-type strain *acvA* expression is rate-limiting for penicillin production. The *acvA* gene promoter was replaced by the strong inducible ethanol dehydrogenase promoter (*alcA*p). The expression level of *alcA*p was determined using a strain in which the reporter gene, *lacZ*, is under the control of *alcA*p, and was found to be up to 100 times greater than that from the *acvA* promoter when induced in fermentation conditions with the artificial inducer cyclopentanone. Penicillin yields were found to be increased by as much as 30-fold when the *acvA* gene was overexpressed by induction of the *alcA* promoter. Glucose, which strongly represses transcription from *alcA*p, also repressed penicillin biosynthesis in the overproducing strain [92]. Overexpression of both the *ipnA* and *aatA* gene of *A. nidulans* using the *alcA* promoter resulted in tenfold higher levels of *ipnA* or *aatA* transcripts than those resulting from transcription of the corresponding endogenous genes. This increase caused a 40-fold rise in IPNS activity or an 8-fold rise in IAT activity. Despite this rise in enzyme levels, forced expression of the *ipnA* gene resulted only in a modest increase in levels of exported penicillin (increase by about 25%), whereas forced expression of the *aatA* gene even reduced penicillin production (decrease by about 10–30%), showing that neither of these enzymes is rate-limiting for penicillin biosynthesis [75].

Consistent with data obtained with *A. nidulans*, only transformants of the low-producing, single gene copy strain Wis54-1255 containing extra copies of the whole biosynthesis gene cluster produced more penicillin, whereas transformants carrying only extra copies of individual genes did not [179].

4.2 Genetic Engineering of β-Lactam Biosynthesis Pathways

Processes based on genetic engineering to produce novel cephalosporin derivatives biosynthetically have been introduced (see chapters of Schmitt et al. and Evers et al.).

5 Future Prospects

Although considerable progress has been made in the understanding of the molecular regulation of penicillin/cephalosporin biosynthesis in fungi, our picture is far from being complete. Research on the regulation of biosyntheses of β-lactam antibiotics is heading towards the elucidation of (i) further regulatory circuits involved, (ii) inducing/repressing signals, (iii) signal transduction pathways (missing links between regulatory circuits and regulatory genes), (iv) additional regulators (transcriptional factors, co-activators/co-repressors) and (v) the mode of action of these regulatory proteins. Understanding these aspects will also help to explain the possible physiological and ecological functions of β-lactams for the producing fungi, the evolution of the pathways and also the recruitment of *trans*-acting factors to regulate the biosynthesis genes. The application of this knowledge will contribute not only to a further increase of β-lactam production and to the production of novel related compounds, but also to the identification of new β-lactam producing organisms by genetic means.

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