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Regulation of Cephalosporin Biosynthesis

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Abstract The filamentous fungus *Acremonium chrysogenum* is the natural producer of the β-lactam antibiotic cephalosporin C and is as such used worldwide in major biotechnical applications. Albeit its profound industrial importance, there is still a limited understanding about the molecular mechanisms regulating cephalosporin biosynthesis in this fungus. This review focuses on various regulatory levels of cephalosporin biosynthesis. In addition to precursor and antibiotic biosynthesis, molecular genetic characteristics of cephalosporin biosynthesis genes and the knowledge of multiple layers of their regulatory expressional control, as well as the function of activators or repressors on cephalosporin biosynthesis are jointly being surveyed. Furthermore, this review summarizes (i) molecular features, which distinguish strains with different production levels and (ii) examples of molecular engineering approaches to *A. chrysogenum*.

Keywords *Acremonium chrysogenum* · Cephalosporin · Gene regulation · Transcription factors · Genetic engineering

1 Introduction

Cephalosporin C and its semisynthetic derivatives are very potent and widely used β-lactam antibiotics of general and applied interest. However, the knowledge of the molecular regulation of β-lactam biosynthesis in the corresponding host is still limited. In the case of cephalosporin biosynthesis, even the total number of involved biosynthesis genes is not known and has yet to be identified. Cephalosporin is exclusively produced by *Acremonium chrysogenum* (syn. *Cephalosporium acremonium*), but compared to other filamentous fungi, genetic manipulation of this fungus is rather difficult. *Acremonium chrysogenum* belongs to the Deuteromycetes, which lack a sexual cycle and are thus not accessible for any conventional genetic analysis. In addition, this fungus produces only very few conidiospores, which in other biotechnically relevant fungi are the preferred cells for DNA-mediated transformations.

In 1945, *A. chrysogenum* was first isolated from Sardinian coastal seawater by Prof. Brotzu. Brotzu was also the first to describe the antibiotic effect of extracts generated from this fungus and, some years later, the structure of the active compound was determined [1]. Cephalosporin C was shown to be active against Gram-positive as well as Gram-negative bacteria. Today, *A. chrysogenum* is cultured worldwide to yield approximately 2500 tons of cephalosporin derivatives. Semisynthetic derivatives are mainly used as broad-spectrum antibiotics for the treatment of bacterial infections.

In biotechnical applications, intensive strain improvement programs resulted in production strains that yield a significantly higher titer of the antibiotic than wild-type strains. Approximately 40 years of mutation and selection cycles separate today's industrial strains from the genetic potential of the original isolates. For basic as well as for applied research, the comparison of wild-type and production strains is of specific interest when differences of

cephalosporin biosynthesis regulation are being investigated. A deeper knowledge of regulatory changes that occurred during strain improvement of cephalosporin production strains can be highly valuable for the directed improvement of novel, so far not optimized, fungal antibiotic producers by genetic engineering.

Future work will show whether or not further significant improvements of cephalosporin production strains are feasible. One perspective is a combined approach, which uses genetic engineering techniques together with conventional strain improvement procedures.

The following sections focus on molecular and genetic mechanisms of cephalosporin biosynthesis that were elucidated in recent years. This review starts with a summary of precursors of cephalosporin biosynthesis and their competing pathways, followed by an overview of the biosynthesis and the structural genes involved in the production of cephalosporin C. Then an outline of regulatory parameters and mechanisms is given, and the transcriptional control of the biosynthesis genes by transcription factors is detailed in section 6. The last two sections deal with the molecular differences that occurred during classical strain improvement of industrial strains and attempts to use a rational approach via molecular engineering.

2
Precursors and Competing Pathways

The biosynthesis of all occurring β-lactams is primarily based on the three amino acids L-α-aminoadipic acid (L-α-AAA), L-cysteine and L-valine. These amino acids play also an important role in the regulation of the cephalosporin C biosynthesis. L-Cysteine and L-valine are ubiquitous amino acids, whereas the non-proteinogenic amino acid L-α-AAA is synthesized as an intermediate in the L-lysine biosynthesis pathway.

2.1
L-α-Aminoadipic Acid (L-α-AAA) Marks a Biosynthesis Branch Point

In fungi, the non-proteinogenic amino acid L-α-AAA is synthesized by a specific aminoadipate pathway, which leads to the formation of lysine, whereas in β-lactam producing bacteria, a specific pathway for the formation of L-α-AAA has been identified (reviewed in [2, 3]).

The L-lysine biosynthesis pathway in higher fungi, including *A. chrysogenum*, starts with the condensation of α-ketoglutarate and acetyl-CoA to form homocitrate, which is then subjected to isomerization, oxidative decarboxylation and amination to yield L-α-AAA. Subsequently, this precursor amino acid is converted into α-AA-δ-semialdehyde by the action of the α-aminoadipate reductase (α-AAR) to finally form L-lysine [4–6]. Furthermore, L-α-AAA can also be obtained for β-lactam biosynthesis by reversal of the last steps of the L-ly-

sine biosynthesis pathway; however, the influence of this catabolic pathway on cephalosporin production remains to be shown [7].

Since L-α-AAA marks the branch point between cephalosporin and the competing L-lysine biosynthesis pathway, its intracellular availability is an important parameter in the regulation of cephalosporin biosynthesis. Mehta et al. [8] showed that L-lysine concentrations reduce the synthesis of cephalosporin C in *A. chrysogenum* and that this inhibition is derepressed by L-α-AAA. Furthermore, recent studies demonstrated that L-lysine concentrations inhibit α-aminoadipate reductase (α-AAR) activity but do not repress its synthesis [9]. These results and the fact that L-lysine caused inhibition of the homocitrate synthase in *Penicillium chrysogenum* indicated that the L-α-AAA pool available for β-lactam production is reduced by L-lysine through feedback inhibition or through repression of several L-lysine biosynthesis genes and enzymes [10].

The initiation of the ACV tripeptide formation depends not only on the availability of L-α-AAA but also on the affinity of the two enzymes for this intermediate. The α-aminoadipate reductase (α-AAR) encoded by the *lys2* gene of *A. chrysogenum* acts as a key enzyme in the branched pathway for lysine and cephalosporin C biosynthesis, since it competes with ACVS for their common substrate L-α-AAA. α-AAR catalyzes the activation and reduction of L-α-AAA to its α-AA-δ-semialdehyde using NADPH as cofactor [11, 12].

Hijarrubia et al. [9] revealed that a lower α-AAR activity could be detected in high cephalosporin producing strains of *A. chrysogenum*. It was suggested that this lower activity might lead to channeling of L-α-AAA towards the formation of cephalosporin. These results concur with the increased availability of the precursor amino acid L-α-AAA, suggesting that more L-α-AAA is shifted from the primary metabolism (lysine formation) to a higher cephalosporin yield in production strains [13].

Furthermore, the α-AAR activity peaked during the growth phase preceding the onset of cephalosporin production and then drastically decreased. At the end of the growth phase, a metabolic switch appears to occur that correlates with an increased availability of L-α-AAA for its use as precursor of cephalosporin production. This switch also coincides with the beginning of mycelium fragmentation into arthrospores in *A. chrysogenum* [9]. Immunoblotting analysis has shown a strong negative effect of nitrate on α-AAR formation. A possible explanation could be the requirement of large amounts of NADPH by the nitrate reductase [14]. Such activity would constitute a competitive inhibitor for the reduction of L-α-AAA to its semialdehyde. The possible reversal of the nitrate effect by lysine addition [9] can be explained by the well-known fact that lysine represses nitrate uptake as well as the metabolic route from nitrate to ammonium [15, 16]. Thus, the L-α-AAA biosynthesis pathway in *A. chrysogenum* is regulated by several control mechanisms such as the feedback inhibition at the α-AAR or homocitrate synthase. However, there is a decided lack of knowledge concerning the L-lysine pathway and its influence on the cephalosporin C production in *A. chrysogenum*.

2.2 L-Valine as a Metabolic Signal

Another crucial factor for the initiation of the ACV tripeptide formation is the availability of the precursor amino acid L-valine. The biosynthesis pathway of this ubiquitous amino acid is closely connected to the biosynthesis of leucine. Valine biosynthesis comprises four enzymatic steps with pyruvate as precursor metabolite. Two moles of pyruvate are converted to the intermediate α-acetolactate, which is then reduced to α, β-dihydroxyisovalerate and ketoisovalerate to finally form L-valine. In *A. chrysogenum*, high levels of L-valine result in a feedback inhibition of the first reaction step catalyzed by acetohydroxy acid synthase [17]. So far, no further data on the regulation of the L-valine biosynthesis pathway and its competing effect on the cephalosporin C biosynthesis have become available.

2.3 Non-Conventional Biosynthesis of L-Cysteine

Another limiting step for cephalosporin C biosynthesis is the availability of the amino acid L-cysteine, which can generally be formed through four different biosynthesis pathways (reviewed in [18–20]). In the direct sulfhydrylation pathway, reduced sulfur is incorporated into the intermediate *O*-acetyl-L-serine to give L-cysteine, whereas in the transsulfuration pathway, sulfide incorporation is catalyzed by *O*-acetylhomoserine sulfhydrylase. The third possibility is the reverse transsulfuration in which the sulfur of L-methionine is transferred to L-cysteine via four intermediates [21] (see Fig. 1). The incorporated sulfur is known to be the efficient precursor of the sulfur atom contained in cephalosporin C [22]. In addition, L-cysteine is synthesized by the so-called autotrophic pathway, which leads to the assimilation of inorganic sulfur via serine *O*-acetyltransferase and *O*-acetylserine sulfhydrylase [23, 24]. All of these pathways seem to exist in *A. chrysogenum* [19]. However, results of mutant analysis showed that the fungus prefers to generate L-cysteine for optimal cephalosporin C biosynthesis via the reverse transsulfuration pathway, which has been detailed in Fig. 1 [25], and to a certain extent via the autotrophic pathway [26]. The relative contributions of the two pathways to the cephalosporin C biosynthesis are still to be determined.

High levels of methionine, particularly the D-isomer, significantly stimulate the synthesis of β-lactam antibiotics. In methionine-supplemented cultures of *A. chrysogenum*, a two to threefold increase in cephalosporin C titers was determined [27]. Additionally, a transient enlargement of the endogenous pool of methionine has been observed in advance of cephalosporin C formation, and the specific biosynthesis seemed to be proportional to the intracellular D-methionine concentration [28]. The addition of high levels of methionine is necessary to achieve optimum cephalosporin C biosynthesis, possibly due to methionine degradation by the intracellular amino acid oxidases [29, 30].

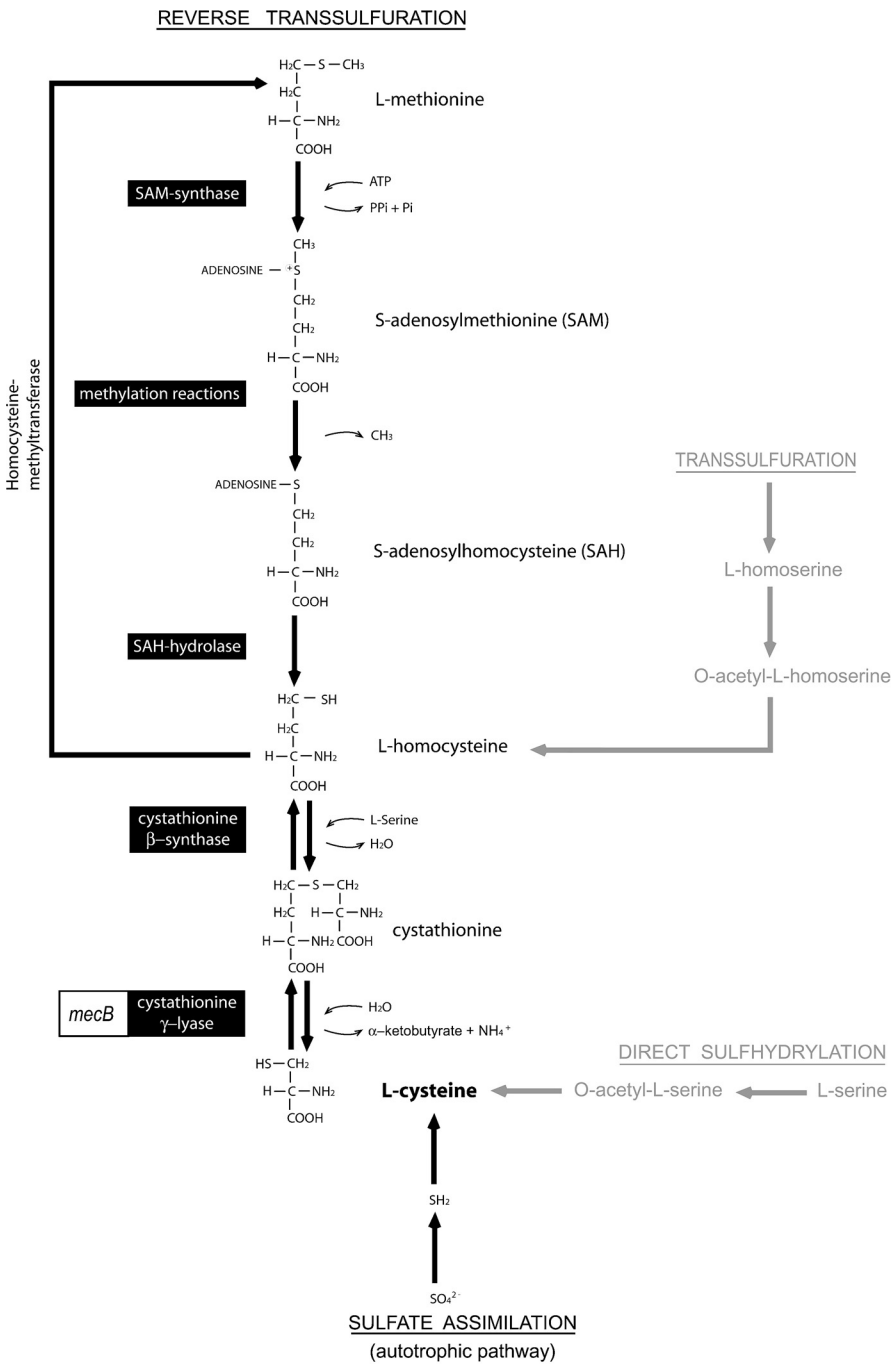

Fig. 1 Biosynthesis of L-cysteine in *A. chrysogenum*. 'Reverse transsulfuration' is the preferred pathway to generate L-cysteine in *A. chrysogenum*. Alternatively, sulfate assimilation is used, while 'transsulfuration' and 'direct sulfhydrylation' seem to exist in *A. chrysogenum*, but are not used for L-cysteine biosynthesis

Early analyses have shown that the enzyme cystathionine-γ-lyase, which catalyzes the conversion of cystathionine to L-cysteine in the reverse transsulfuration is crucial for the methionine induced titer-enhancing effect. This reaction was proposed to induce the transfer of L-cysteine from the primary metabolism to the cephalosporin C biosynthesis pathway [31]. In recent studies, the so-called *mecB* gene encoding cystathionine-γ-lyase was cloned from *A. chrysogenum*. The encoded protein was shown to be functional by complementing the *Aspergillus nidulans* C47 mutant, which is defective in cystathionine-γ-lyase activity. The expression of the *mecB* gene is not regulated by the addition of DL-methionine [32].

Targeted inactivation of the *mecB* gene indicated that the supply of L-cysteine through the reverse transsulfuration pathway is required for high-level cephalosporin C production but not for low-level biosynthesis proving that the essential L-cysteine is obtained from both the autotrophic and the reverse transsulfuration pathways [33]. The supply of methionine results in the complete repression of sulfate assimilation [34]. *mecB*-disruption did not affect the methionine induction of the cephalosporin C biosynthesis genes. Thus, their expression is not mediated by a putative regulatory mechanism exerted by cystathionine-γ-lyase, but the induction may be triggered by methionine itself or by a catabolite derived from methionine [33]. Amplification of the *mecB* gene and the resulting overproduction of the cystathionine-γ-lyase in moderate doses lead to an increased cephalosporin C formation, whereas high cystathionine-γ-lyase activity is likely to produce high intracellular levels of L-cysteine, which are known to be toxic and inhibit β-lactam synthesizing enzymes [35].

Taken together, methionine presumably has a double effect on cephalosporin C biosynthesis in *A. chrysogenum*. On the one hand it seems to be the main supplier of L-cysteine via the reverse transsulfuration pathway and on the other hand it has an induction effect on cephalosporin biosynthesis genes (reviewed in [36, 37]).

3 Biosynthesis of Cephalosporin

Cephalosporins are members of the large group of β-lactam antibiotics, which inhibit the growth of Gram-negative as well as Gram-positive microorganisms at already low concentrations. β-lactam antibiotics are specified by the typical cephem nucleus and are produced by a wide variety of microorganisms, including the filamentous fungus *A. chrysogenum*, Gram-positive streptomycetes and a small number of Gram-negative bacteria (reviewed in [38]). All of them produce β-lactams essentially through the same biosynthesis pathway, which is chemically and kinetically well characterized owing to the considerable industrial potential of these antibiotics [39].

3.1 General β-Lactam Biosynthesis

As shown in Fig. 2, the *first step* of cephalosporin biosynthesis results in the formation of the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) from the amino acid precursors and is catalyzed by a single multifunctional enzyme designated δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase (ACVS).

ACVS are monomers with a molecular mass of about 420 kDa, which function similarly to other peptide synthetases from bacterial or fungal sources. They mediate the non-ribosomal synthesis of peptides via a multiple carrier thiotemplate mechanism [40–42].

ACVS contains three repeated modules with conserved amino acid sequences [43]. Each module consists of functional domains for amino acid recognition, activation and thiolation. During condensation, peptide bond formation occurs from the amino to the carboxy terminus of the peptide. In addition, the last module of the ACVS contains an epimerization module, which is involved in the conversion of the activated intermediates ([41], reviewed [44]).

A detailed analysis showed that ACVS catalyzes the activation of the carboxyl group of the first amino acid in the presence of Mg^{2+} and ATP by the formation of the corresponding aminoacyl adenylate and the release of pyrophosphate [45]. This step is followed by the transfer of the activated carboxyl group to the 4′-phosphopantheteine cofactor to generate the thioester bond between the enzyme and the amino acid. This thioesterified amino acid represents the target for nucleophilic attack by the amino group of the second amino acid, resulting in the formation of the first peptide bond between the L-aminoadipic acid and L-cysteine. The resulting dipeptidyl intermediate remains bonded to the enzyme. After condensation of the dipeptide with the third

Table 1 Designation of genes, which have been isolated and characterized from *Acremonium chrysogenum*

Gene abbreviation	Product
pcb AB (syn. *acvA*)	δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase
pcbC (syn. *ipnA*)	isopenicillin N synthase
cefD1	acyl-CoA-synthetase
cefD2	acyl-CoA-racemase
cefEF	deacetoxycephalosporin C/deacetylcephalosporin C synthetase
cefG	acetyl-CoA: deacetylcephalosporin C acetyltransferase
lys2	α-aminoadipate reductase
mecB	cystathionine-γ-lyase
cpcR1	cephalosporin C regulator 1
cre1	carbon catabolite repressor CRE1
pacC	pH-dependent transcription factor PACC

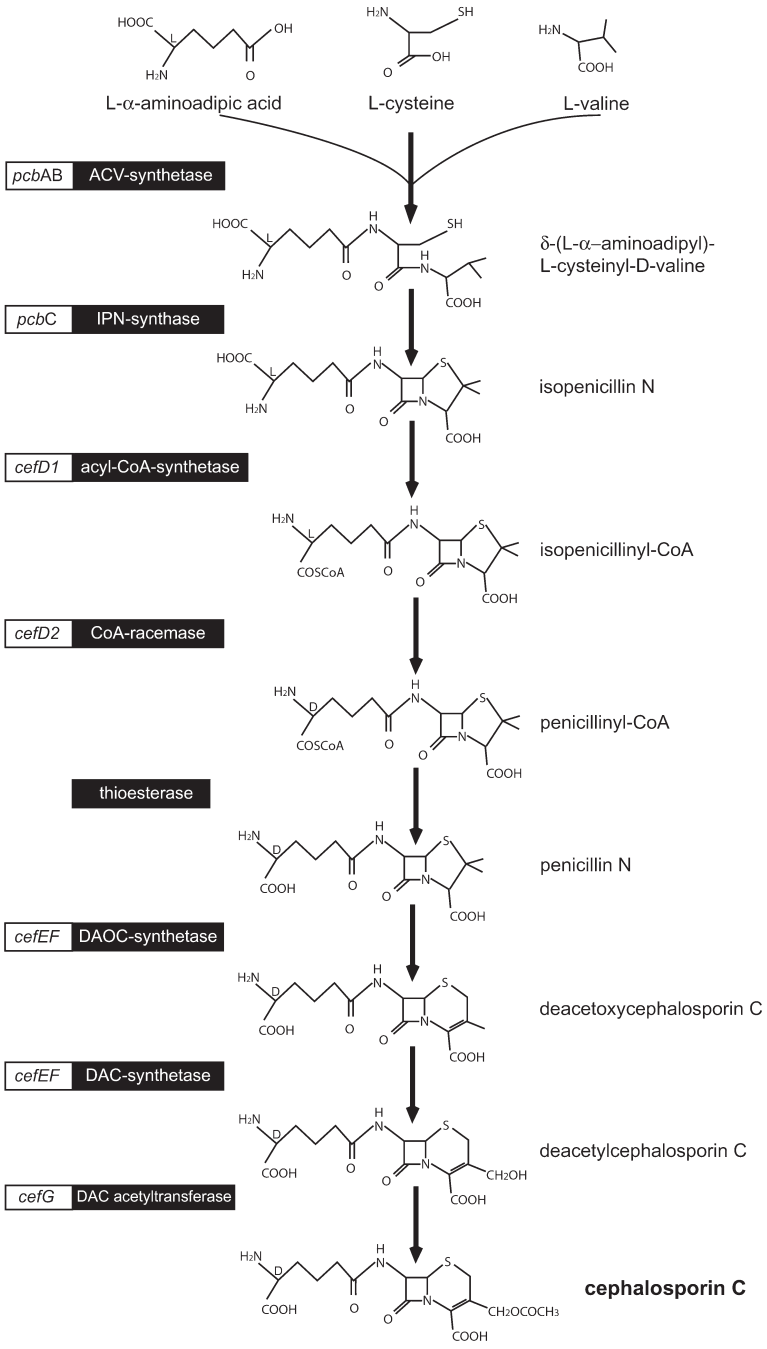

Fig. 2 Cephalosporin C biosynthesis, which exclusively occurs in *A. chrysogenum*. Biosynthesis genes as well as their products were framed. With the exception of the predicted gene encoding a thioesterase, all others have been cloned. For details see main text

amino acid, L-valine is epimerized at the tripeptide stage to its D-enantiomer and is followed by the formation of the final ACV tripeptide. The selective release of the tripeptide with the correct LLD configuration is arranged via the integrated thioesterase domain in the C-terminal region of ACVS [41, 46, 47].

The *second reaction* is a key step in the cephalosporin biosynthesis pathway, which implies the cyclization of the linear ACV tripeptide to form the first bioactive intermediate isopenicillin N (IPN) (see also Fig. 2).

This reaction is mediated by the isopenicillin N synthase (IPNS), a non-heme monoferrous-dependent oxidase of a molecular mass of about 38 kDa, which binds ferrous iron, uses dioxygen as co-substrate and ascorbate as electron donor to form the bicyclic nucleus [48, 49]. In a unique enzymatic reaction, IPNS catalyzes the transfer of four hydrogen atoms from the precursor ACV tripeptide to dioxygen associated with the desaturative ring closure and the formation of two water molecules [38, 49, 50]. X-ray crystallography determined that the four-membered β-lactam ring system is primarily formed in conjunction with a highly oxidized iron-oxo (ferryl) group, which then mediates the closure of the corresponding thiazolidine ring [51, 52].

3.1.1 Cellular Localization and Structure of IPNS

The IPNS enzyme is localized in the cytoplasm as a soluble protein [53]. It exists in two interconvertible forms, one is an oxidized state forming a disulfide linkage and the other exists in a reduced state [54]. IPNS consists of a catalytic center containing a highly conserved H-Xaa-D-(53–57 residues)-Xaa-H motif for iron coordination and of a specific substrate-binding pocket with a common R-X-S motif crucial for its catalytic activity [49, 55–57]. A third amino acid residue tyrosine (189–191) is also involved in binding of the valine carboxylate moiety of the ACV tripeptide, but it is not as crucial as the R-X-S motif [58]. Analysis of the crystal structure has shown that the active site is unusually buried within an eight-stranded "jelly-roll" motif and lined by hydrophobic residues [49]. This structural characteristic of the IPNS proteins and many other keto-acid-dependent oxygenases is probably necessary for the isolation of the reactive complex and of subsequent intermediates from the external environment.

Combined application of Mössbauer electron paramagnetic resonance as well as nuclear magnetic resonance spectroscopy, has determined a mechanism for isopenicillin N formation. This involves direct ligation of ACV to the active iron site of the IPNS via the corresponding cysteinyl thiol, or more precisely, via the sulfur atom of the ACV [59, 60] and the creation of a vacant iron coordination site into which dioxygen may bind. The binding of ACV leads to the initiation of the reaction and the replacement of the amino acid residue Q 330 side chain, which coordinates the metal in absence of a substrate. Subsequently, iron-dioxygen and iron-oxo species remove the essential hydrogens from ACV [49, 55, 61]. Thus, in the generated Fe^{2+}:ACV:IPNS complex, three of

the five coordination sites are occupied with protein ligands. The remaining two sites are filled by a water molecule and the ACV thiolate resulting in a penta-coordinated iron active site (reviewed in [62]). In this reaction step, only the thiol form of the ACV tripeptide serves as a substrate, the spontaneously formed bis-disulfide state shows no binding activity [63]. Due to the broad substrate specificity of IPNS in particular with alterations in the L-AAA as well as with the valine residue of ACV tripeptide, it is possible to generate new penicillins/cephalosporins in vivo or to generally improve the enzyme activity [64].

3.2 Cephalosporin Specific Biosynthesis

The formation of isopenicillin N is the branch point of penicillin and cephalosporin biosynthesis. The reaction step to follow is shown in Fig. 2 and leads to the formation of penicillin N. This step establishes the pathway that is specific for the synthesis of cephalosporins. An epimerization reaction is involved that converts isopenicillin N to penicillin N, which, despite its industrial relevance, had remained uncharacterized for a long time. Konomi et al. [65] has first shown epimerase activity in cell-free extracts of the cephalosporin C producing fungus *A. chrysogenum*, but it was suggested that the epimerizing enzyme was extremely instable preventing purification of the protein [66–68]. Until recently, no further data on the fungal enzyme have been obtained.

Since all known cephalosporin biosynthesis genes of *A. chrysogenum* are clustered in two separate loci, Ullán et al. [69] suggested that the gene encoding the enzyme involved in the conversion of isopenicillin N into penicillin N might be located in one of the cephalosporin gene clusters. A transcriptional analysis of a 9 kb region located downstream of the *pcbC* gene revealed the presence of two open reading frames that were cloned and sequenced on both strands. ORF1 corresponds to the gene designated *cefD1* and encodes a protein with a molecular mass of about 71 kDa, which shows a high degree of similarity to long chain acyl-CoA synthetases, particularly to those from *Homo sapiens* (26.3% identity), *Rattus norvecigus* or *Mus musculus* (25.5% identity). The encoded protein contains all characteristic motifs of the acyl-CoA ligases involved in the activation of the carboxyl moiety of fatty acids or amino acids [70]. The second identified gene designated *cefD2* encodes a protein with a deduced molecular mass of 41.4 kDa, which is similar to α-methyl-acyl-CoA racemases from *H. sapiens* (42.1% identity) or *M. musculus* (39.4% identity) [71–73].

Based on the identified homology of the CEFD1 and CEFD2 proteins with known eukaryotic enzymes, it seems feasible to establish a mechanism for the *A. chrysogenum* two-component epimerization system which is different from epimerizations found in prokaryotes. Such systems have been reported to be involved for example in the inversion of 2-arylpropionic acids (e.g. ibuprofen), which is an important group of non-steroidal anti-inflammatory drugs in hu-

mans [74–76]. Therefore, it was suggested that the epimerization reaction in the cephalosporin biosynthesis pathway begins with the activation of the substrate isopenicillin N to its CoA-thioester by the acyl-CoA-synthetase. The product of the *cefD2* gene, the α-methylacyl-CoA racemase, catalyzes the epimerization of isopenicillinyl-CoA to D-enantiomer penicillinyl-CoA. Finally, the required hydrolysis of the CoA-thioesters seems to occur in a non-stereoselective manner by different thioesterases [75]. The resulting product, penicillin N, is the direct precursor of all cephalosporins and cephamycins and, thus, available as a substrate for further reactions in the biosynthesis pathway.

The next committed step of the cephalosporin pathway leads to the conversion of penicillin N to deacetoxycephalosporin C (DAOC) by expanding the five-membered thiazolidine ring to the six-membered dihydrothiazine ring characteristic for the class of cephalosporins. This reaction is catalyzed by DAOC synthetase, which ensures the required expandase function [77]. In the following reaction of the biosynthesis pathway, DAOC hydroxylase, also designated deacetylcephalosporin C synthetase, catalyzes the incorporation of an oxygen atom from O_2 into the exocyclic methyl moiety at the C-3 atom of DAOC thus forming deacetylcephalosporin C (DAC) (reviewed in [78–81]). The enzymatic expansion of the five-membered thiazolidine ring was first observed in cell-free extracts of the cephalosporin C producer *A. chrysogenum* [77, 82].

The enzyme involved is responsible for the two-step reaction in *A. chrysogenum*, which leads to the conversion of penicillin N to deacetylcephalosporin C, while in streptomycetes like *Streptomyces clavuligerus* [83, 84], the two enzymatic activities could be distinctly separated by anion-exchange chromatography [85]. Analysis of the amino acid sequence of the DAOC/DAC synthetase of *A. chrysogenum* revealed a ten amino acid region containing a cysteine residue at position 100, which is 50% identical to the corresponding region containing the cysteine residue at position 106 of isopenicillin N synthetase. This region is of special interest because the cysteine residue of the IPNS is important for substrate binding and specific activity [86]. Thus, it seems to be possible that the corresponding residue C-100 of the DAOC expandase/hydroxylase may either be directly or indirectly involved in substrate binding [87]. The existent sulfhydryl groups in the enzyme were apparently essential for both ring expansion and hydroxylation [80]. In addition to penicillin N, the DAOC/DAC synthetase exhibits a diverse substrate specificity, which differs in the efficiency of ring expansion [88].

3.2.1 Final Reaction of Cephalosporin Biosynthesis

In the last reaction of the cephalosporin biosynthesis pathway, the transfer of an acetyl moiety from the acetyl coenzyme A to the hydroxyl group on the sulfur-containing ring of deacetylcephalosporin C leads to the formation of the

final product cephalosporin C, which possesses high antibiotic activity [89–92]. This acetylation reaction is catalyzed by the acetyl-coenzyme A (CoA):DAC acetyltransferase, which behaves like a soluble cytosolic enzyme without any known targeting signals or other indications for compartmentalization [93].

The pure enzyme shows a molecular mass of about 50 kDa and the N-terminal end possesses the sequence M-P-S-A-Q-V-A-R-L, which perfectly matches the deduced amino acid sequence starting at the first ATG codon. This enzyme seems to be a monomer, which shows no dissociation into subunits.

The amino acid sequence of the *A. chrysogenum* acetyl-CoA:DAC acetyltransferase reveals significant similarity with sequences of several *O*-acetyltransferases, especially with homoserine-*O*-acetyltransferases of the fungi *Saccharomyces cerevisiae* and *Ascobolus immersus* (55.8% and 48.5% identity) [94, 95]. This is probably due to the structural similarity of the exocyclic CH_2OH moiety in DAC and the homoserine molecules [96]. Even though this similarity results from mutant analysis, it suggests that there are two independent *O*-acetyltransferases for DAC and homoserine in *A. chrysogenum* [19].

The acetylation reaction of DAC to cephalosporin C seems to be very inefficient in most strains of *A. chrysogenum*. The *cefG* gene is expressed very poorly when compared with other genes of the pathway [96, 97] and it is well known that high levels of DAC accumulated in many cephalosporin C producing strains [90]. Consequently, the conversion of DAC to cephalosporin C seems to be the limiting step in the pathway.

4
Structural Genes of Cephalosporin Biosynthesis

As shown in Fig. 3, the genes involved in cephalosporin biosynthesis are organized in at least two clusters in *A. chrysogenum*. The *pcbAB* and *pcbC* genes as well as the newly discovered *cefD1* and *cefD2* genes are linked in the so-called "early" cephalosporin cluster. The "late" cluster contains the *cefEF* and *cefG* genes, which are involved in the last two steps of the biosynthesis pathway (reviewed in [69, 98]). In most strains, the "early" cluster could be mapped to chromosome VI and the "late" cluster to chromosome II [99, 100]. Analysis of high cephalosporin producing strains, such as C10, has shown a different localization of the biosynthesis gene cluster on chromosomes I and VII [101], which indicates that significant chromosome rearrangements have occurred during strain improvement (reviewed in [98]). Both cephalosporin clusters are available as a single copy in the genome of all analyzed *A. chrysogenum* strains.

The cluster formation of biosynthesis genes in many antibiotic producing organisms gives rise to the hypothesis that linkage has occurred during evolution conferring an ecological selective advantage [102]. Furthermore, the or-

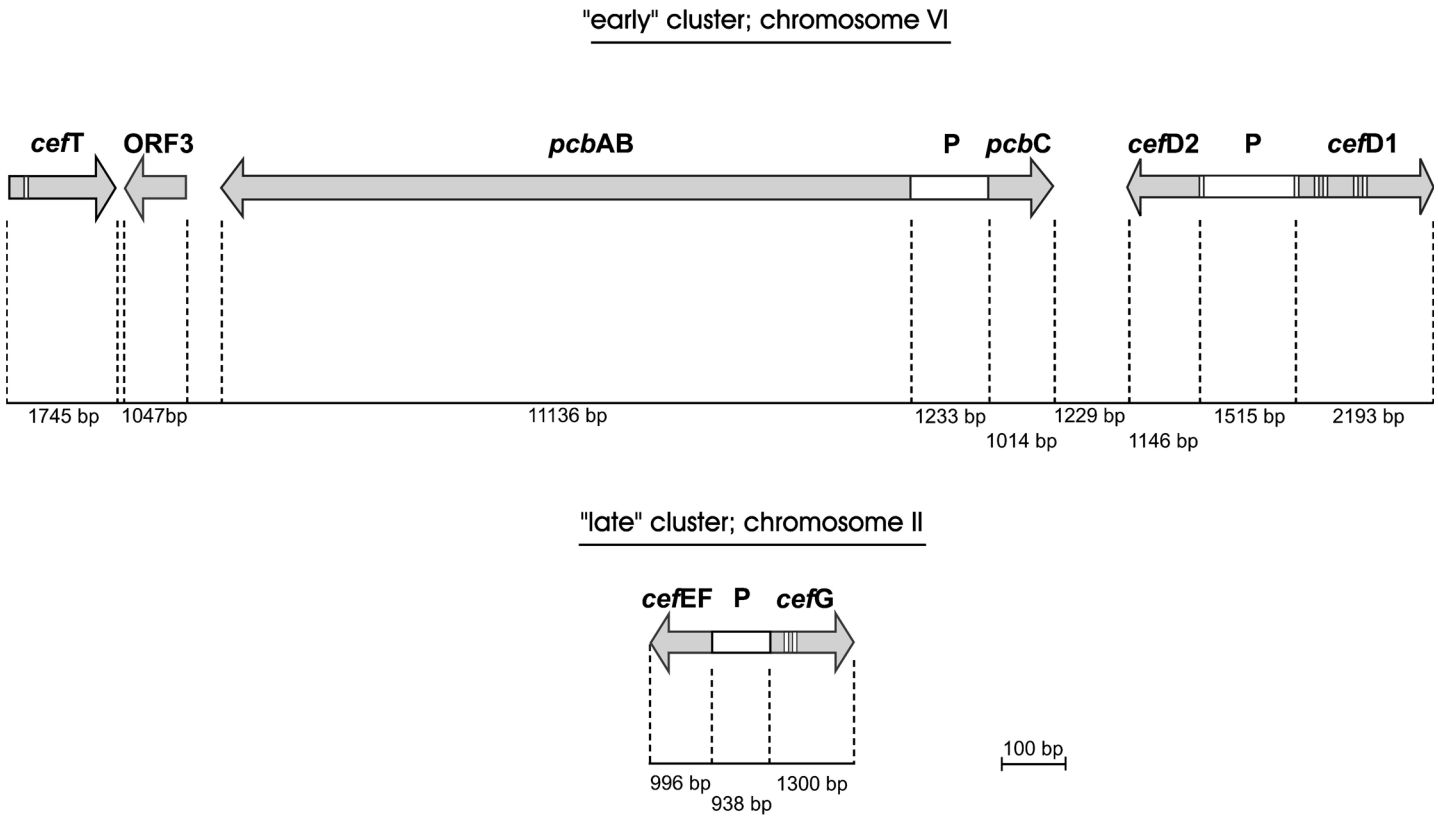

Fig. 3 Gene organization of the 'early' and 'late' cephalosporin biosynthesis genes in *A. chrysogenum*. Intronic sequences are *kept in white* and the *arrows* indicate the direction of transcription

ganization of the biosynthesis genes into large operons controlled by a single promoter could allow a coordinated regulation of the biosynthesis genes [103]. However, in eukaryotic fungi like *A. chrysogenum*, β-lactam biosynthesis genes are transcribed separately and are expressed through different promoters (reviewed in [50]). In this light, gene expression does not seem to be coordinated as a result of genomic linkage and, hence, it seems more likely that cluster formation reflects only a common ancestral origin.

4.1 "Early" Cephalosporin Genes

ACVS, the first acting enzyme of the cephalosporin C pathway in *A. chrysogenum*, is encoded by a single structural gene designated *pcbAB* (syn. *acvA*) with a size of about 11 kb. The *pcbAB* gene was first identified and cloned in *P. chrysogenum* by complementation of mutants blocked in penicillin biosynthesis and by transcriptional mapping of the genome [104]. For the cephalosporin C producer *A. chrysogenum*, a 32 kb DNA fragment was identified in several phages using the *pcbAB* and *pcbC* genes of *P. chrysogenum* as heterologous probes [43]. Complementation studies using the *npe5* strain from *P. chrysogenum* carrying a defective *pcbAB* gene confirmed in vivo that the functional *pcbAB* gene is located on a 15.6 kb fragment. Northern analysis of total RNA using probes internal to the *pcbAB* gene identified a transcript of 11.4 kb. It has been shown that the *pcbAB* open reading frame of 11136 bp, which matched the 11.4 kb transcript initiation and termination regions, was located upstream of the *pcbC* gene.

The ORF does not contain any intron sequences and the translational start codon of the gene is not yet clearly defined, because attempts to obtain the N-terminal amino acid sequence have been unsuccessful so far [105, 106]. In *A. chrysogenum,* the *pcbAB* gene is separated by an intergenic region of 1233 bp from the *pcbC* gene and is divergently orientated with respect to *pcbC* [43].

In industrial strains of *A. chrysogenum*, the expression of the *pcbAB* gene was much weaker compared to that of *pcbC* [107]. Furthermore, differences may even exist in the temporal expression among genes of the same cluster. In *A. chrysogenum*, the *pcbAB* gene seems to be coordinately regulated with the *pcbC* gene, whereas the later genes of the pathway appear to be sequentially induced [108–110]. Disruption of the *pcbAB* gene in *A. chrysogenum* resulted in loss of ACVS activity without affecting the other cephalosporin biosynthesis genes [110].

The corresponding *pcbAB* genes were cloned and sequenced from different prokaryotic and eukaryotic microbial β-lactam producers [2, 104, 111–114]. A comparison of the nucleotide sequences encoding the three repeated domains [115] demonstrated a high similarity among the fungal and bacterial *pcbAB* genes. The fungal domains showed on average 71% nucleotide sequence identity to each other, whereas fungal and bacterial domains revealed about 48% identity. Only little similarity was found between domain-

separating regions. These results imply a close relationship between all *pcbAB* genes [116, 117].

The second structural gene of the "early" cephalosporin gene cluster in *A. chrysogenum* is the *pcbC* (syn. *ipnA*) gene, which encodes the IPNS enzyme. The *pcbC* gene is divergently linked by an intergenic promoter region to the *pcbAB* gene. The *pcbC* gene of *A. chrysogenum* was the first β-lactam biosynthesis gene to be cloned and sequenced [118]. This was achieved by purification of the IPNS enzyme, determination of the N-terminal amino acid sequence and the design of two pools of oligonucleotides, which contain all possible sequences encoded by two short peptides of the IPNS amino terminus. Using this DNA as probe for screening a cosmid library, it was possible to identify a clone possessing an ORF with a size of 1014 bp, which encodes a protein of 338 amino acids. Expression of this ORF in *E. coli* resulted in IPNS activity of the corresponding cell extracts [118].

The *pcbC* gene does not contain any introns and the corresponding transcript size was determined to lie between 1.15 and 1.5 kb. While primer extension established two major [–56 and –77] and at least two minor transcription start sites [–58 and –78], the corresponding values obtained from S1 endonuclease mapping were –51/–73 and –54/–80/–97, respectively [118, 119]. These transcription initiation sites appeared as major and minor pairs on either side of one of the pyrimidine-rich blocks, which characterize the promoter sequence.

After identification of the *pcbC* gene in *A. chrysogenum*, the corresponding structural genes have been cloned and sequenced from several different fungi and bacteria, such as *P. chrysogenum*, *A. nidulans* or *S. clavuligerus* (reviewed in [3, 38]). Alignments using *pcbC* sequences from different prokaryotic and eukaryotic organisms revealed a degree of identity greater than 60%. Most of the sequence identity is scattered throughout the protein, which makes it difficult to identify functionally important domains [57, 120, 121].

The "early" cephalosporin gene cluster was completed with the newly discovered genes *cefD1* and *cefD2*, which encode two enzymes that act in a two-protein system for formation of the cephalosporin C intermediate penicillin N. Identification of the two genes was obtained by transcriptional studies of a 9 kb region located downstream of the *pcbC* gene. Analysis was performed using RNA extracted from mycelia of *A. chrysogenum* strain C10 grown for 48 h, since at this time the expression of the other cephalosporin C biosynthesis genes is known to be high. A 5.8 kb subfragment containing two open reading frames was cloned and sequenced on both strands. ORF1 corresponded to the gene *cefD1* with a size of 2193 nucleotides and was interrupted by the presence of five introns with sizes varying between 28 and 150 bp. The corresponding transcript revealed a size of 2 kb. This gene was also cloned from a previously constructed cDNA library [122] and the sequence confirmed the presence of five introns. The *cefD2* gene consists of 1146 nucleotides and is interrupted by the presence of a single intron with a size of 92 bp. RT-PCR and sequence analysis have shown that the intron had been removed at the splicing sites corresponding to nucleotides 64–157 relative to the

ATG translation initiation codon. The corresponding transcript could be detected in the 48 h cultures with a size of 1.2 kb. The *cefD1* and *cefD2* genes, which are located closely downstream the *pcbC* gene are expressed in opposite orientation from a bi-directional promoter region with a size of 1515 bp, which is characteristic for cephalosporin C biosynthesis genes. Functional analyses of the *cefD1* and *cefD2* genes have been performed by targeted inactivation of both genes using DNA-mediated transformation and resulting in strains lacking iso-penicillin epimerase activity [69].

4.2 "Late" Cephalosporin Genes

The *cefEF* gene of *A. chrysogenum*, which encodes DAOC/DAC synthetase is one of the two genes organized in the "late" cephalosporin cluster. Isolation and characterization of the gene was achieved by the design of oligonucleotide probes based on the amino acid sequence of the purified DAOC/DAC synthetase and a subsequent screen of a cosmid genomic library of *A. chrysogenum*. One ORF with a size of 996 bp coding for a protein of 332 amino acids matched the sequence predicted for the peptide fragments. After expression of this ORF in *E. coli*, cell extracts harbored both expandase and hydroxylase activities [87]. The *cefEF* gene does not possess any intron sequences suggesting a prokaryotic origin.

In antibiotic producing streptomycetes, a clearly different system was detected. There are two different genes designated *cefE* and *cefF* encoding two different enzymes namely DAOC expandase and hydroxylase. Both genes are linked together with the *cefD* gene in a single cluster (reviewed in [38]).

In *A. chrysogenum*, the *cefEF* gene is closely linked to the *cefG* gene, but it is transcribed in the opposite direction. The intergenic region with a size of 938 bp contains the promoters for both genes [96, 123]. The *cefG* gene of *A. chrysogenum* encoding the last enzyme of the cephalosporin C pathway, namely the acetyl-CoA:DAC acetyltransferase, was cloned and sequenced independently by three research groups [96, 97, 124, 125].

Mathison and co-workers [97] achieved the *cefG* gene isolation by sequencing the ambient region of the *cefEF* gene and identification of an open reading frame. An alternative for gene cloning was the screening of an *A. chrysogenum* lambda phage library with a probe specific for the *cefEF* gene. Northern blotting and DNA sequence analysis revealed the existence of the *cefG* gene close to the *cefEF* gene [96]. In both cases, the identity of the *cefG* gene was demonstrated by complementation of *A. chrysogenum* mutants, which are deficient in acetyl-CoA:DAC acetyltransferase activity. In addition, overexpression of the gene in *Aspergillus niger,* which lacks these genes, demonstrated such an activity. Matsuda et al. [124] used another strategy by screening a cDNA library with oligonucleotides based on the N-terminal sequence of the corresponding acetyl-CoA:DAC acetyltransferase enzyme. In this case, the identity of *cefG* has been proven by gene disruption experiments resulting in strains that failed to produce cephalosporin C but accumulated its precursor DAC.

Based on the identification of three different ATG translation-initiation codons in the *cefG* gene, different sizes for the open reading frame and the resulting proteins have been proposed [96, 97, 124]. This was elucidated by Velasco and co-workers [126], who synthesized all estimated proteins in *E. coli* and purified the native *A. chrysogenum* acetyl-CoA:DAC acetyltransferase using immunoaffinity chromatography.

The *cefG* gene has a size of about 1.3 kb and contains two intronic sequences as demonstrated by sequencing of its cDNA [97, 124, 125].

5
Multiple Layers of Control

The complexity of the biosynthesis of cephalosporin C and its precursors implicates different layers of regulation. In fact, evidence is available for regulatory mechanisms that act on the transcript level of biosynthesis genes and on the activity of the enzymes involved. Furthermore, cellular investigations suggest a correlation between cephalosporin C biosynthesis and mycelial morphology and differentiation. In addition, the uptake of precursors, the compartmentalization of biosynthesis and the export of cephalosporin are regulatory processes, which influence the overall production of cephalosporin C (see separate sections for details).

5.1
Transcript Level

Until the early 1990s, almost all of our knowledge of the regulation of cephalosporin biosynthesis was derived from measurements of the product and intermediates. With cloning and sequencing of the cephalosporin biosynthesis genes, a new era started in as much as detailed analysis of the biosynthesis by monitoring transcript levels was facilitated. Smith et al. [119] mapped the transcription start points of the *pcbC* gene and analyzed the transcript level of *pcbC* during a seven day fermentation. The transcript level was not constitutive and found to be highest between day two and day four. Their results suggested a transcriptional regulation of the *pcbC* gene. This assumption was later confirmed and more refined analyses revealed several external parameters that act at the transcriptional level.

Velasco et al. [27] used wild-type and two industrial strains from *A. chrysogenum* to establish the influence of methionine on the transcript levels of the four biosynthesis genes *pcbAB*, *pcbC*, *cefEF* and *cefG*. Previous reports indicated that methionine has a stimulatory effect on the production of cephalosporin C, and a higher enzyme activity in these cultures has been described before [127, 128]. The inhibition of de novo protein synthesis by addition of cycloheximide prevented increased antibiotic production that usually occurs in cultures supplemented with methionine only. It was concluded that methionine does not

simply stimulate already present enzymatic activity, but rather acts on another, earlier level. The assumed transcriptional regulation could be confirmed in the wild-type strain for the *pcbAB* and *pcbC* genes, which exhibited increased transcript levels in the presence of methionine. Velasco et al. [27] also reported that in the two industrial strains C10 and CW19, the transcript level of the *cefEF* gene was increased by methionine supplementation. However, for all strains the highest induction rate could be detected for the *pcbAB* transcript.

In this context, Velasco et al. [27] detected several consensus CANNTG sequences in the intergenic region of the *A. chrysogenum* biosynthesis genes *pcbAB* and *pcbC*, which are recognized by members of the basic region-helix-loop-helix (bHLH) protein family. Some of these known transcription factors are involved in the transcriptional control of the sulfur network in *S. cerevisiae* [129–132]. Thus, the authors suggested that a member of the bHLH proteins might mediate these methionine-inducing effects.

Besides methionine, the influence of other factors such as carbon source and ambient pH on the transcript levels of the biosynthesis genes was investigated. Jekosch and Kück [133] were able to show that the *pcbC* transcript in a wild-type strain of *A. chrysogenum* was completely repressed in the presence of 6.3% glucose. The amount of transcript correlated well with the amount of isopenicillin N synthase in a Western blot analysis indicating the importance of transcriptional regulation in biosynthesis. However, in the semi-producer strain A3/2, the transcription of the *pcbC* gene was not repressed by glucose. The higher transcript levels of all biosynthesis genes in the improved strain allowed the analysis of the *cefEF* gene in addition to the *pcbC* gene. A clear reduction of the *cefEF* transcript and protein level in the presence of 6.3% glucose was observed in strain A3/2.

A pH-dependent transcription of the *pcbC* gene was reported for both industrial and wild-type strains. In the wild-type *A. chrysogenum* strain, highest transcript levels could be detected under neutral and mild alkaline conditions at pH 7 and 8, whereas in the semi-producer A3/2, the pH optimum was at pH 6 [123]. So far, no detailed analysis has yet been published, which might illustrate transcriptional regulation with respect to all parameters, also including different nitrogen sources, known to influence cephalosporin C production [134].

The expression of the *cefG* gene is limiting for cephalosporin C production in all studied strains of *A. chrysogenum* [135]. Only a very weak transcript of about 1.4 kb, which corresponds to the *cefG* gene, could be detected in *A. chrysogenum* cells grown in a defined production medium for 48 h and 96 h [96, 97]. The fact that the acetyl-CoA:DAC acetyltransferase showed high protein levels in cultures at 72 and 96 h, which decreased dramatically thereafter, corresponds with the late conversion of DAC to cephalosporin C during the fermentation process [126, 136]. Thus, *cefG* seems to be expressed at a later stage of fermentation and at a lower transcriptional level than the *cefEF* gene suggesting a different control mechanism for these genes, although they are expressed divergently from the same promoter region [96]. Furthermore, transcriptional analysis revealed that the *cefG* gene appears not to be the target of

glucose-dependent regulation [137], and its expression is not significantly stimulated by the addition of methionine unlike that of other cephalosporin C biosynthesis genes [27].

All these examples indicate that the transcriptional regulation of the biosynthesis genes is an important aspect of the regulation of cephalosporin C production. Undoubtedly, major regulatory effects in the biosynthesis of cephalosporin C result from transcriptional changes. One striking observation is that all industrial producer strains of *A. chrysogenum* have increased transcript levels from all biosynthesis genes. Nevertheless, in addition to transcriptional control, other regulatory levels exist in the biosynthesis of cephalosporin C and the modification of enzyme activity will be described in the following section.

5.2 Enzyme Activity

As described above, glucose has a significant impact on transcription of the *pcbC* and the *cefEF* gene. There is no negative effect of glucose on the activity of purified isopenicillin N synthase or resting cells (e.g. [138]). In contrast, ACV synthetase activity is directly inhibited by glucose in vitro [139]. Actually, the inhibitory effect on ACV synthetase, the first enzyme of the biosynthesis, results from glyceraldehyde 3-phosphate and not from glucose itself [140].

Another enzyme with relevance for cephalosporin biosynthesis that shows inhibited enzyme activity under certain circumstances is α-aminoadipate reductase encoded by the *lys2* gene in *A. chrysogenum*. This enzyme is involved in lysine biosynthesis and competes with ACV synthetase for the precursor α-aminoadipic acid. α-Aminoadipate reductase activity was quantified in the presence of 0 to 10 mmol/L lysine. A wild-type strain from *A. chrysogenum* showed 80% inhibition of α-aminoadipate reductase activity in extracts of 1 mmol/L lysine [9]. The inhibition of this enzyme of the lysine pathway is of relevance for cephalosporin C biosynthesis, because a high intracellular level of α-aminoadipic acid is required for efficient antibiotic production. It has been described that in vitro, the energy requirement for tripeptide formation through ACV synthetase is rather high under unfavorable conditions, which could be caused, e.g. through limited amino acid concentrations. At saturated conditions, the consumption amounts to 3 ATPs per ACV tripeptide, whereas under unfavorable conditions it can be more than 20 ATPs [141].

5.3 Correlation Between Secondary Metabolism and Morphogenesis

The biosynthesis of secondary metabolites in filamentous fungi is often associated with cell differentiation and development. In *Aspergillus nidulans*, there is a link between biosynthesis of secondary metabolites and asexual sporulation. In recent years, the involvement of a common G-protein-mediated growth

pathway has been demonstrated (reviewed in [142]). The α-subunit FadA of the trimeric G-protein binds GTP in its active form and then favors vegetative growth by inhibiting conidiogenesis. Upon inactivation of the G-protein through intrinsic GTPase activity, the inhibitory effect on signaling cascades to sporulation and toxin biosynthesis is released. Interestingly, a dominant activating *fadA* allele stimulates the expression of the *pcbC* gene and penicillin biosynthesis in *A. nidulans* [143].

It cannot be excluded that an FadA homologue also influences β-lactam biosynthesis in *A. chrysogenum*, as such a G-protein-mediated regulation of secondary metabolite production has already been described for different fungi other than *A. nidulans*. Examples are cyclopiazonic acid and aflatoxin biosynthesis in *Aspergillus flavus*, trichothecene production in *Fusarium sporotrichioides* and pigment synthesis in *Cryphonectria parasitica*. The influence of an active G-protein can be both positive and negative depending on the respective biosynthesis (overview see [142]).

In *A. chrysogenum,* sporulation is very weak and little is known about a possible coupling to cephalosporin biosynthesis. Bartoshevich et al. [144] describe three differentiation types for *A. chrysogenum* and their correlation with cephalosporin production. Type 1 is the transition from the vegetative stage into a reproductive one with the formation of conidia. In this reproductive stage, cephalosporin production is lowered. It should be noted that conidia are usually not formed by high titer strains of *A. chrysogenum*. Type 2 is described for the late stages of development and characterized by the formation of arthrospores with thick cell walls and probably retarded metabolism. These arthrospores may be considered as simplified reproductive spores serving the survival of the organism under stress conditions and are accompanied by a lowered production of cephalosporin C. Type 3 differentiation is a multi-stage transformation of the mycelial organization into swollen fragments or yeast-like cells, which are capable of periodical polycyclic development. This alternating mycelial and yeast-like organization is most pronounced under conditions of high cephalosporin production [144].

It has been known for a long time that the phase of hyphal differentiation coincides with the maximum rate of cephalosporin synthesis and that methionine enhances fragmentation and antibiotic production [22, 145, 146]. The stimulatory effect of methionine on the transcription of biosynthesis genes was mentioned earlier, and the pleiotropic action of methionine in *A. chrysogenum* has been reviewed by Martín and Demain [37]. Norleucine, a non-sulfur analogue of methionine, also stimulated cephalosporin C production and mimics the methionine's effect on mycelial morphology [147].

The capability of yeast-like cells to produce high amounts of cephalosporin C might rely on the alternative respiration pathway in *A. chrysogenum*. This cytochrome-independent and cyanide-insensitive respiration seems to be an obligate feature of yeast-like, but not filamentous cells and is important when cytochrome-dependent respiration cannot completely regenerate the reduced coenzyme [148]. It was also reported that the alternative respiration exhibits a

more than two-fold increase when *A. chrysogenum* was grown on sugars or soybean oil. The addition of soybean oil even doubled the specific production of cephalosporin C [149].

However, no strict correlation and interdependency exists between mycelial fragmentation and cephalosporin production rate. Allosamidin, a potent chitinases inhibitor, retarded the fragmentation of hyphae but did not affect cephalosporin C production [150]. An analysis of carbon source, growth rate and antibiotic synthesis revealed that the fragmentation has a causal relationship with growth rate. Low growth rates may weaken the hyphae and agitation could possibly cause breakages [151].

6
Transcription Factors as Activators and Repressors of Cephalosporin Biosynthesis

The isolation and analysis of transcription factors from filamentous fungi began about 15 years ago. First examples came from two model organisms *Aspergillus nidulans* and *Neurospora crassa*. Transcription factors like CREA from *A. nidulans*, which acts as a major glucose repressor [152] and NIT2 from *N. crassa* that regulates structural genes of nitrogen metabolisms are involved in primary metabolism. *A. nidulans* is not only a model organism but also a penicillin producer and many investigations on the regulation of β-lactam biosynthesis were performed in this fungus over the last several years. Due to low titer, penicillin production in *A. nidulans* is not of any biotechnical interest and, therefore, other fungi such as *Penicillium chrysogenum* are used for penicillin production.

The first transcription factor, which was isolated from this fungus is the PACC protein [153]. The *pacC* gene was cloned using the heterologous sequence from *A. nidulans* as hybridization probe.

Transcription factors from *A. chrysogenum* have only recently been isolated and can be divided into two groups: the zinc finger proteins PACC and CRE1 already known from other fungal β-lactam producers, and the RFX transcription factor CPCR1 initially discovered in *Acremonium chrysogenum*. As shown in Fig. 4, a detailed sequence analysis revealed that all promoter sequences of cephalosporin biosynthesis genes contain potential DNA-binding sites for all of these transcription factors.

6.1
PACC – pH-Dependent Transcriptional Control

Many filamentous fungi are capable of surviving and growing in a broad range of ambient pH, which might be as acidic as 2 or as alkaline as 10. Apart from their ability of homeostasis, they adapt the secretion of enzymes and secondary metabolites in response to the respective pH environment. Penicillins and

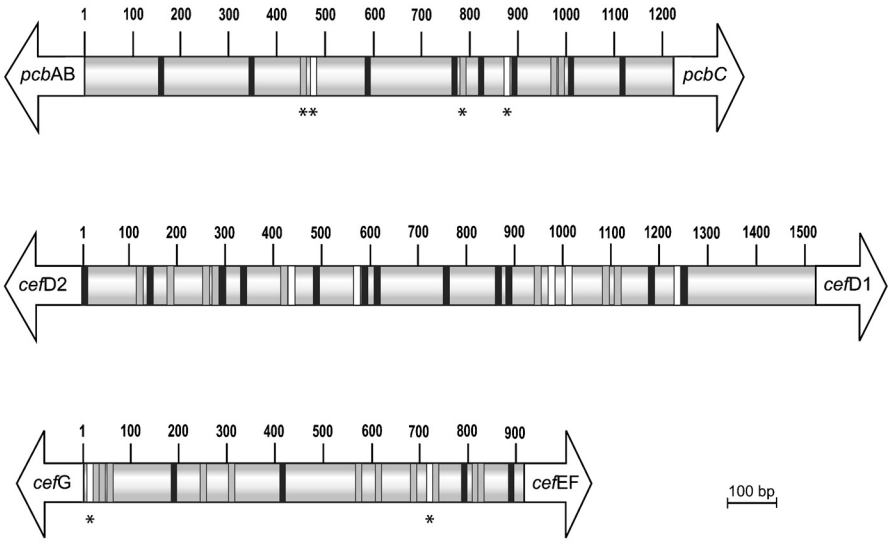

Fig. 4 Transcription factor binding sites in the divergently orientated promoter sequences from the *pcbAB-pcbC*, *cefD1-cefD2*, and *cefEF-cefG* cephalosporin biosynthesis genes. *Bars* indicate recognition sites for the transcription factor CPCR1 (*grey*), CRE1 (*black*), and PACC (*white*). Those binding sites, which have been shown to exist experimentally, are marked by asterisks. The CPCR1 consensus binding site is highly complex. Therefore, it is not certain that all predicted sites show in vitro the expected binding activity

cephalosporins are produced in elevated amounts under alkaline ambient pH. The intensive study of mutants with defects in pH regulation led to the isolation of the *pacC* gene from *A. nidulans* by complementation of a mutant strain [154]. PACC is a zinc finger transcription factor of the C2H2-type with three zinc fingers. The success in *A. nidulans* was followed by the isolation of *pacC* genes from *P. chrysogenum*, *A. niger* and *A. chrysogenum* [123, 153, 155].

The *pacC* gene from *A. chrysogenum* (*pacCAc*) was isolated from a lambda genomic library using the *P. chrysogenum pacC* gene (*pacCPc*) as a heterologous DNA probe encompassing the highly conserved zinc finger region. Sequencing of a 3-kb fragment allowed the identification of a DNA fragment encoding an ORF of 621 amino acids. The ORF is interrupted by three introns of which two are located in the zinc finger region. PacCAc is 20 and 57 amino acids shorter than the corresponding genes from the penicillin producers *A. nidulans* and *P. chrysogenum*, respectively. PACCAc shows approximately 35% sequence identity to other PACC proteins, which are much more alike with about 60% identity. The observed differences are consistent with the taxonomic classification of the three fungi: Acremonium belongs to the Pyrenomycetes, whereas Aspergillus and Penicillium are Plectomycetes. Southern analysis revealed that *pacC* is a single copy gene and is located on identical restriction fragments in wild-type and semi-industrial strains of *A. chrysogenum* [123].

PACC proteins are zinc finger transcription factors with highly conserved DNA recognition positions in the first two of the three zinc fingers [156]. They bind to a consensus binding site 5′GCCAAG3′ with high affinity in vitro. In *A. chrysogenum* the *pcbAB-pcbC* promoter region contains two binding sites for PACC (see Fig. 4). Both are recognized efficiently in vitro by an *E. coli* synthesized protein fragment of PACCAc encompassing the DNA-binding zinc finger region [123]. The promoter region between the *cefEF* and *cefG* genes, which is specific to cephalosporin C biosynthesis and *A. chrysogenum* also contains two PACC binding sites that are recognized in vitro (see Fig. 4). Experimental results from *A. chrysogenum* suggest that the PACCAc protein functions in the regulation of cephalosporin gene expression in a pH dependent manner. In wild-type strains of *A. chrysogenum*, the expression of cephalosporin biosynthesis genes is stronger under alkaline conditions, which probably results from an activated PACC protein [123].

The PACC protein is activated through proteolytic processing, which results in the mature, shorter polypeptide. In Aspergillus, a detailed study of the regulation of gene expression in a pH dependent manner resulted in the identification of the Pal signal transduction pathway (e.g. [157]). Each of the six *pal* gene products is required for the proteolytic processing of the PACC transcription factor in Aspergillus. Only the processed and shorter form of the protein can act as a transcriptional activator [158, 159].

The activated PACC protein is involved in the pH dependent regulation of many genes and probably also of the *pacC* gene itself. The existence of five putative PACC binding sites in the promoter of the *pacC* gene from *A. chrysogenum* suggests a strong autoregulation of the gene. A similar conclusion has been drawn for the *pacC* genes from *P. chrysogenum* and *A. niger* [153, 155].

As already stated, the regulation of gene expression in a pH-dependent manner is not restricted to β-lactam biosynthesis only. Many different genes and pathways in fungi and yeasts are regulated in response to ambient pH, and PACC is a key player in all of these processes. Although PACC is not a specific regulator of β-lactam antibiotics or cephalosporin biosynthesis, it has a general effect on gene expression of biosynthesis genes.

6.2 CRE1 – A Glucose Repressor Protein

Another important parameter for fungal growth and the induction of secondary metabolism is the available carbon source. It has been described for all β-lactam producing fungi that growth is often promoted by glucose, but that higher concentrations of glucose have a negative effect on antibiotic production (reviewed in [160]). This negative effect is suspected to stem from transcriptional and post-transcriptional mechanisms. There are also a number of differences between the three β-lactam producers *P. chrysogenum, A. nidulans* and *A. chrysogenum* regarding the extent of glucose repression for certain biosynthesis genes and enzyme activities.

In *A. chrysogenum*, it was reported that the enzyme activity of the gene products from *pcbAB*, *pcbC* and *cefEF* decreased in the presence of 6.3% glucose and cephalosporin production was reduced [138, 139, 161]. Jekosch and Kück [133] showed that in the wild-type strain, both the *pcbC* and the *cefEF* gene are transcriptional repressed in the presence of glucose. In *A. nidulans* and *Trichoderma reesei*, repression of gene transcription by glucose is regulated by the carbon catabolite repressors CREA (syn. CRE1) [152, 162]. A PCR-based approach using degenerative primers derived from amino acid sequences of published CRE proteins led to the amplification of a partial *cre1* gene from *A. chrysogenum*. The gene fragment was used to screen a lambda genomic library, and a 2.9 kb fragment carrying the complete *cre1* gene could be isolated. An intronless ORF of 1218 bp codes for 406 amino acids. The deduced CRE1 protein sequence showed an overall similarity of 69% to the *T. reesei* CRE1 and 56% to the *A. nidulans* CREA [137]. CRE proteins contain two C2H2-type zinc fingers and recognize a consensus binding motif 5′-SYGGRG-3′ in a context-dependent manner. In addition to the zinc fingers, *A. chrysogenum* CRE1 carries all conserved domains, which were previously described for the *T. reesei* CRE1 [162]. These include in particular the acidic and regulatory regions, which have been shown to be involved in the regulation of the DNA-binding ability by phosphorylation [163]. The *cre1* gene is a single copy gene in wild-type and producer strains of *A. chrysogenum* and comparison of both strains showed no chromosomal rearrangement within the *cre1* gene region [137].

The promoters of the *pcbC* and the *cefEF* gene contain several putative binding sites for CRE1 through which the transcription factor might repress these genes. This idea is supported by results obtained with *A. chrysogenum* transformants that contain ectopically integrated multiple copies of the *cre1* gene [133]. In the wild-type strain, the *pcbC* and the *cefEF* gene are repressed by glucose. This repression does not significantly differ in transformants carrying multiple copies of the *cre1* gene. However, a similar approach using the semi-producer strain A3/2 yielded in changed transcript levels. In strain A3/2, the *pcbC* gene is no longer subject to glucose repression. However, in transformants with multiple *cre1* gene copies, transcript levels of *pcbC* are lower in the presence of glucose indicating a restored glucose repression mechanism due to insertion of multiple copies of the *cre1* gene. The *cefEF* gene is glucose-repressed in strain A3/2 and in the corresponding *cre1*-transformants with the repression being more pronounced in the transformants described above [133]. These experiments indicate that in *A. chrysogenum*, the CRE1 transcription factor acts as a carbon catabolite repressor on the biosynthesis genes of cephalosporin.

Like PACC, CRE1 is also regulating its own gene expression via binding sites in the promoter region of *cre1*. This autoregulation is of special interest in *A. chrysogenum* since in contrast to *A. nidulans* and *T. reesei*, transcript levels of the *cre1* gene increased in the wild-type strain in the presence of glucose. In the wild-type strain ATCC14553, the *cre1* transcript level was increased about sixfold after two days of cultivation in the presence of glucose. In contrast, changes in the transcript levels in the semi-producer strain A3/2 could not be observed,

thus indicating the absence of a glucose regulation of *cre1* transcriptional expression [137].

6.3 CPCR1 – Cephalosporin C Regulator 1

PACC and CRE1 are known to be involved in the transcriptional regulation of β-lactam biosynthesis in various filamentous fungi. These could also be isolated from and investigated in *A. chrysogenum.* A different approach led to the identification of the CPCR1 (Cephalosporin C Regulator 1) transcription factor in *A. chrysogenum*. A 24-bp sequence from the *pcbAB-pcbC* promoter was used in the yeast one-hybrid-system to isolate cDNAs from *A. chrysogenum* encoding DNA-binding proteins that interact with the promoter sequence. The sequence is located –441 to –418 relative to the translational start of the *pcbC* gene and contains a CCAAT-box and an imperfect palindrome. A cDNA was identified in the one-hybrid-screen that encodes a polypeptide of 788 amino acids. Analysis of the genomic DNA extended the ORF of the *cpcR1* gene to 830 amino acids and revealed the position of two short introns. Southern analysis of genomic DNA from wild-type and semi-producer strains with a *cpcR1* gene probe detected only a single hybridizing band of identical size. This indicates that *cpcR1* is a single copy gene in *A. chrysogenum* [164].

CPCR1 is the first member of the RFX-family of transcription factors in filamentous fungi. RFX proteins form a subfamily of the winged-helix proteins that are characterized by a DNA-binding domain of the helix-turn-helix type [165, 166]. Another novel RFX gene was isolated from *P. chrysogenum* through sequence homology with *cpcR1* [164]. PcRFX1 and CPCR1 share about 29% amino acid identity (Fig. 5). A data library search of completely sequenced fungal genomes showed that *cpcR1* homologues are present in *Neurospora crassa*, *Magnaporthe grisea*, as well as in *Aspergillus nidulans*. From the amino acid sequence comparison in Fig. 5 can be concluded that all predicted polypeptides show highest homology with regard to the DNA-binding and dimerization domain. A similar degree of identity exists also to the yeast RFX proteins SAK1 from *Schizosaccharomyces pombe* and CRT1 from *Saccharomyces cerevisiae* [167, 168]. The yeast proteins are involved in DNA repair and meiotic divisions, whereas the human members of the RFX family function in a tissue and lineage specific manner. RFX5 is an interesting example as it is part of a protein complex that regulates the immune response. Therefore, mutation in RFX5 can lead to severe defects in the immune system (e.g. [169, 170]).

CPCR1 is a typical member of the RFX/winged-helix family. Besides the RFX-type DNA-binding domain at amino acid positions 224 to 298, it contains a characteristic C-terminal dimerization domain. The C-terminus is necessary for homodimerization of CPCR1 (Fig. 5). Truncation of the dimerization domain results not only in the inability of CPCR1 to form homodimers but also in a loss of its DNA-binding activity. Thus, CPCR1 only binds DNA in a dimeric state [164]. Interestingly, *cpcR1* homologues have been found in DNA sequence data

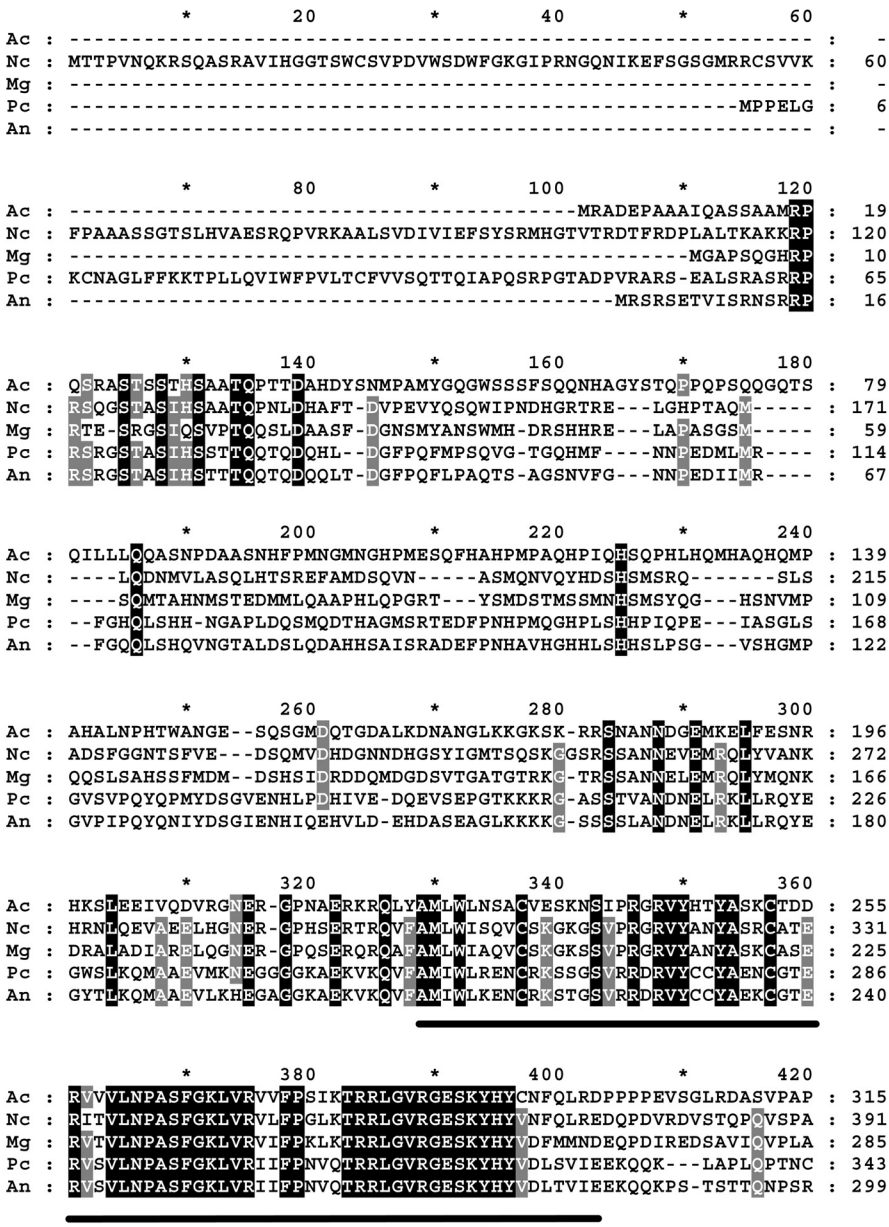

Fig. 5 Alignment of primary amino acid sequences of predicted CPCR1 polypeptides from different fungal species. Using available sequences from data libraries, *cpcR1* homologous genes have been identified in five different fungi. The DNA-binding domain and the dimerization domain are underlined with *black or grey bars*, respectively. Identical residues in all (*black*) or in four (*grey*) sequences are *shaded*. Abbreviations: Ac, *Acremonium chrysogenum*; An, *Aspergillus nidulans*; Mg, *Magnaporthe grisea*; Nc, *Neurospora crassa*; Pc, *Penicillium chrysogenum*. For Fig. 5 see also following pages

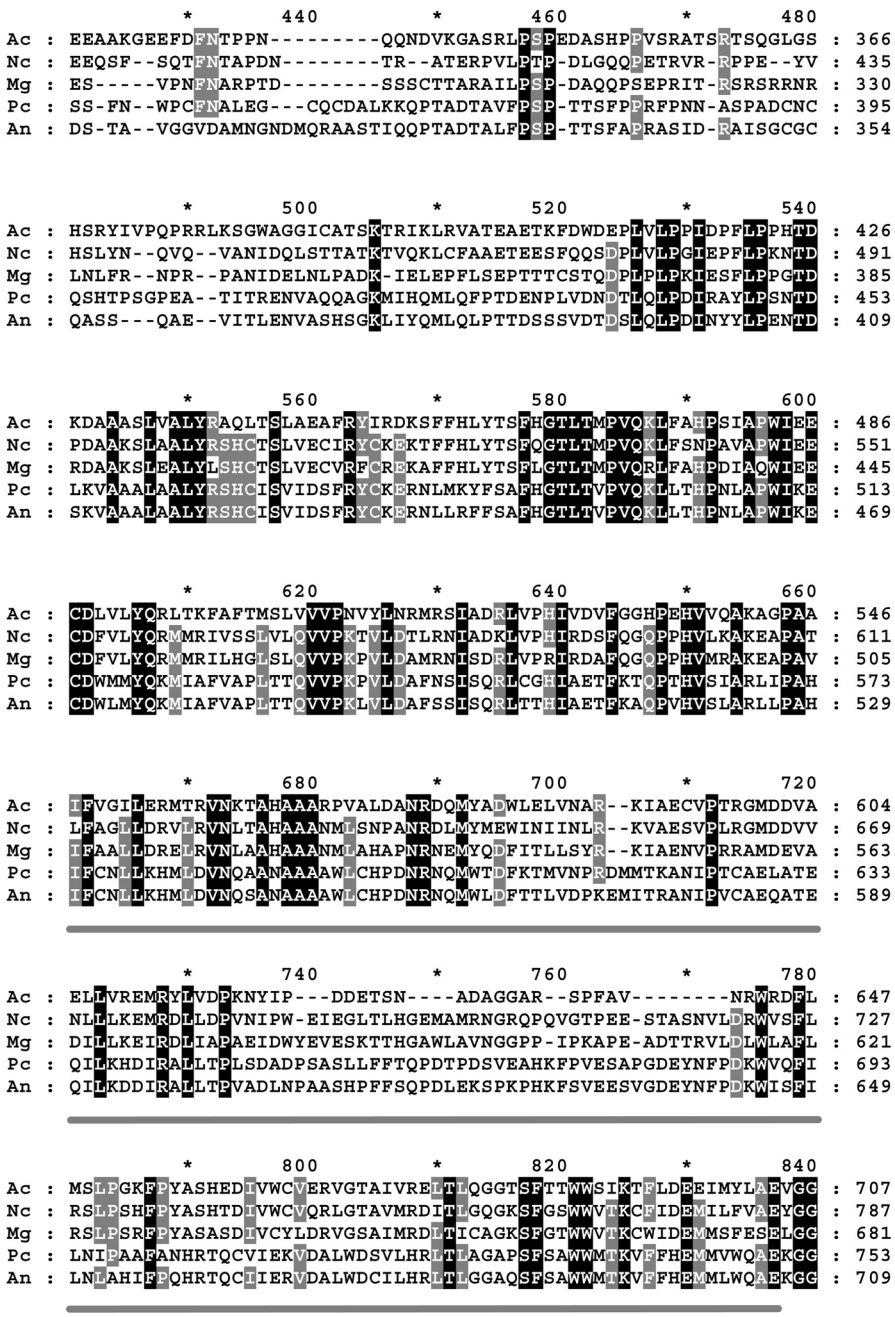

Fig. 5 (continued)

```
                *       860         *       880         *       900
Ac : LMHTKSLRDEPSRQQATTVVTERGQKGATADDDGEVVMGDAGQEEPMAVAASPVHTDRAP : 767
Nc : FLKQK------STHEYTSLVSQQQGASN--EASNEASRYRSGSDDSSASRMSESQPDRAR : 839
Mg : FLRMT------STKSVSAEEAQKSYASDNRETSVQESTLGRASEEHHGLSQSQPQPDRAP : 735
Pc : FMRNT-----PGSLQSATFGPESLGHFQMKQASVPETSPFEASNDSQAKSNPSPPVAHNG : 808
An : FMHYT-----PSTLQKAGLDSDQSGPGGLVLRNSPKVVGNESS-TSNAQDGASLERSRMG : 763

                *       920         *       940         *       960
Ac : FP---PKQGHAAN-MGDAASDAHDDSGIGIRTPET---DFPVDKFGLHEAEAGVTELAYE : 820
Nc : FPM--PAST-------ATQPSVHDDSGIGIRTPDE---DSPIQKFGFQTTTAN-SQDIFA : 886
Mg : FPGCSPANGPPED---ASTSMNPDDSGIGIRTPDE---DFPMDSIKFDFPPEP-TSLATH : 788
Pc : HQSHPPSQHPEFKHPPTEKRLSLQDPGFQGHNNDDSAIDLEDDTMLLAVGKYG-DMMVSD : 867
An : VETN-PSRMDDEK--PTDISENLTS--FHAPNHDDSAIDLDDDSMLMTVGKYG-DMMVSD : 817

                *
Ac : GLDREW--AAPA- : 830
Nc : GTELQDEDHLPDI : 899
Mg : GTDTP---VTPS- : 797
Pc : PADAEGDVVVI-- : 878
An : PADAEGDVIVI-- : 828
```

Fig. 5 (continued)

from different fungal sources (Fig. 5) indicating that RFX proteins fulfill a different regulatory function which is not restricted to β-lactam biosynthesis.

For the functional elucidation of CPCR1, several fungal transformants with varying *cpcR1* gene copy numbers were generated [171]. A knockout strain showed changed *pcbC* transcript levels indicating a direct involvement of CPCR1 in transcriptional regulation of this cephalosporin biosynthesis gene. Reporter gene analysis of *pcbC* promoter derivatives with deleted CPCR1 binding sites revealed a clear dependence of reporter gene activity on functional binding sites. The deletion of two CPCR1 binding sites resulted in the total loss of reporter gene activity after cultivation of seven days. Interestingly, the levels for cephalosporin C were not significantly altered in the *cpcR1* knockout strain. However, the amount of the biosynthesis intermediate penicillin N was drastically reduced. This underlines the assumption that CPCR1 is involved in the regulation of the early biosynthesis genes such as *pcbC*. The observed reduction of penicillin N was completely reverted in a strain with a complemented *cpcR1* gene [171].

To summarize, CPCR1 is a transcription factor, which is involved in the regulation of cephalosporin C biosynthesis. The deletion of the *cpcR1* gene does not prevent the production of cephalosporin C, but has significant effects on the level of antibiotic biosynthesis. CPCR1 probably binds to the promoter of the *pcbC* gene as a dimer with a molecular mass of nearly 200 kDa and it is most likely that one or several other regulatory proteins interact with CPCR1. Thus, the full function of CPCR1 and putative additional transcription factors or mediator proteins has first to be identified before the complete scenario of *pcbC* gene regulation can be pictured. So far, it has not been investigated whether PACC or CRE1 are protein interaction partners of CPCR1 under defined physiological conditions.

6.4
Comparison of Cephalosporin and Penicillin Biosynthesis Regulation

Penicillin and cephalosporin biosynthesis share the *pcbAB* and the *pcbC* genes encoding enzymes for the first catalytic steps resulting in the formation of isopenicillin N. Although differences between the β-lactam producers exist in promoter length and structure, and in the transcriptional response to environmental parameters, it is not unlikely that a basic set of similar transcription factors is involved in the regulation of β-lactam biosynthesis in other fungi as well. Even if a transcription factor and its target gene are conserved between two fungi, it still remains to be clarified if all regulatory details are identical. One good example is the PACC transcription factor. Differences have been observed between *P. chrysogenum* and *A. nidulans* with regard to the transcriptional response of β-lactam biosynthesis genes to the ambient pH [153].

Nevertheless, in *A. chrysogenum* are some additional transcription factor candidates with a role of regulating cephalosporin biosynthesis genes, which have not been described so far. Both candidates are not specific for penicillin biosynthesis but function in a more general way. General transcription factors can act on a broad range of promoters of target genes that are involved in unrelated pathways. One example is the AREA family of fungal transcription factors including NIT2 and NRE that regulate nitrogen control in *A. nidulans, N. crassa* and *P. chrysogenum*. It was shown that the NRE transcription factor binds to promoter sequences from penicillin biosynthesis genes [172].

The second example of a general transcription factor is the HAP-complex from *A. nidulans*. This multi-protein-complex, which has been designated PENR1 [173] is involved in the regulation of many genes, but also binds to the *pcbC* promoter of *A. nidulans*.

Finally, there is the question of how much could be learnt from non-cephalosporin producing fungi. Recently, we have discovered sequences with similarity to β-lactam biosynthesis genes in the genomic sequence of the human pathogen *Aspergillus fumigatus* [174]. This finding invites to speculate whether or not cephalosporin biosynthesis genes could be residual in genomes of anamorphs or teleomorphs of *A. chrysogenum*. Additionally, it remains to be determined whether or not these species have retained regulatory systems for β-lactam biosynthesis genes, similar to those of *A. chrysogenum*.

7
Molecular Differences in Production Strains

All *A. chrysogenum* strains that are currently used for the production of cephalosporin C have been derived from the Brotzu isolate found in 1945. Repeating cycles of mutation and selection methods resulted in strains that produce under ideal fermentation conditions more than 20 g/L [39]. Selection was aimed at achieving highest possible production rates under conditions suitable

for fermentation in a cost efficient way. Bearing this in mind, most changes inevitably occurred at the molecular level that have accumulated in these strains. In general, transcript levels of biosynthesis genes are significantly higher in producer strains, an observation, which has also been made for optimized *P. chrysogenum* strains. However, there are striking differences between penicillin and cephalosporin producer strains. For *A. chrysogenum* producer strains, no report is available stating that the gene copy number of biosynthesis genes has increased as it is the case for the penicillin biosynthesis cluster. This has been amplified by a factor of between 5 to greater than 10 in most production strains of *P. chrysogenum* (e.g. [175]). One explanation for this finding is that cephalosporin biosynthesis genes are not located in a single cluster, but rather are distributed over two different chromosomes. However, several investigations have shown that the transcript level of biosynthesis genes is increased and, thus, changes must have occurred at the level of transcriptional regulation of structural genes. There are two main explanations for these findings: mutations in the promoter region may be responsible for example for a different recruitment of transcriptional activator proteins and/or molecular changes occurred in the regulatory genes and, consequently, in the corresponding proteins.

To date, no changes in the copy number of the identified regulatory genes of cephalosporin C biosynthesis have been observed. All transcription factor genes of *A. chrysogenum* described so far, namely *cpcR1*, *cre1* and *pacC*, seem to be single copy genes in wild-type and producer strains. From hybridization analysis with restricted genomic DNA can be concluded that no major intrachromosomal DNA rearrangements have taken place when producer strains were generated from wild-type strains [123, 137, 164]. However, it is not known whether mutations occurred in the transcription factors, which might for example increase the transcriptional activation capacity or their interaction with other regulatory factors.

Other relatively constant parameters are promoter sequences. It was described that no significant changes could be detected when the *pcbC* promoter DNA sequences of *A. chrysogenum* strains with different antibiotic production rates were compared [176]. A similar result was obtained for the promoter regions of penicillin biosynthesis genes from wild-type and producer strains [175]. Nevertheless, a number of changes have already been described. Relatively soon after the discovery of the biosynthesis genes, the chromosomal localization of the gene clusters was investigated using pulsed-field gel electrophoresis. This technique allows the separation of intact chromosomes with a size of up to 10 Mb. Walz and Kück [177] have reported that different *A. chrysogenum* strains were indistinguishable with respect to restriction fragment patterns, but six out of eight chromosomes differed in size. In addition, the rDNA gene cluster seemed to be relocated from its original site at chromosome II in wild-type strains to chromosome VII in an improved production strain. Another investigation revealed chromosome changes only in the minority of strains from a lineage with improved cephalosporin C production. In one strain, the size of the chromosome was altered on which the *pcbC* gene is

located [101]. Until now, it could not be clarified whether these chromosomal rearrangements have occurred accidentally during industrial strain improvement programs and whether they are at least partially responsible for titer improvement through increased gene expression rates of the translocated biosynthesis genes.

The increasing knowledge of transcription of biosynthesis genes and the regulatory proteins involved in this process has led to the discovery of some interesting differences in production strains. As already mentioned, the transcript levels in production strains are generally increased. Besides different transcript levels, improved strains often show an altered regulation in response to certain parameters. One example is provided by Velasco et al. [27]. They report that in production strain C10, the supplementation of methionine correlates with an increased transcription of the *pcbAB*, *pcbC* and *cefEF* genes. This is in contrast to the Brotzu strain, where only *pcbAB* and *pcbC* transcript levels are higher in the presence of methionine. This suggests that changes might have occurred in the regulation of the *cefEF* gene during strain improvement.

Another example is related to the transcription factor PACC and the pH-dependent transcriptional control. In the semi-producer strain A3/2 of *A. chrysogenum*, transcript analysis revealed a pH optimum for *pcbC* and *cefEF* transcript levels at pH 6 [123]. This is in contrast to the general observation that higher yields of β-lactam antibiotics are obtained at pH 7 to 8. Indeed, in a non-optimized strain, the highest level of the *pcbC* transcript could be detected at pH 7 to 8. The change of the optimum pH to pH 6 is justified for industrial fermentation of *A. chrysogenum*, as this process is usually run at a slightly acid pH to increase the stability of the product in the fermentation broth. Thus, classical strain improvement resulted in an adaptation of the transcriptional regulation towards an optimized antibiotic production under the employed fermentation conditions. Another interesting observation was that not only the transcript level was increased at pH 6, but that the transcription rate showed a real optimum curve and was lower at an alkaline pH. If this altered pH-dependent transcription still depends on PACC and the related PAL signal cascade, the pH sensing for the induction of the cascade must have changed in the production strain.

The last example is derived from investigations of carbon source regulation and the transcription factor CRE1, a glucose repressor protein of the zinc finger type. One difference between the wild-type strain and strains with enhanced antibiotic production is the regulation of the *cre1* gene itself. The transcript level of the gene encoding the repressor protein CRE1 is increased sixfold in the presence of glucose in a wild-type strain [137]. Interestingly, this glucose-dependent transcriptional upregulation of *cre1* does not take place in strain A3/2 with improved production of cephalosporin C. This deregulation of the glucose repressor gene might be related to the increased antibiotic production rate. *cre1* promoter sequences of the two strains were determined and found to be completely identical, indicating that the deregulation of the *cre1* gene does not result from mutations in its own promoter region. The idea of an altered

glucose regulation in improved strains is supported by the finding that in the production strain, the *pcbC* gene is no longer repressed by glucose as it is the case in the wild-type strain [133]. Northern analysis revealed a complete reduction of the *pcbC* transcript level in the wild-type strain in the presence of 6.3% glucose and in the producer strain a reduction to less than 50% for the *cefEF* transcript. As already mentioned, the level of the *pcbC* transcript was not reduced in the producer strain. The involvement of CRE1 in the glucose effect was shown by the transfer of several copies of the repressor gene *cre1* into the producer strain. The resulting transformants have a glucose-dependent regulation of the *pcbC* and the *cefEF* gene. This suggests that transcription factors are important targets, which have been directly or indirectly subjected to alterations during strain improvement.

8
Examples of Molecular Engineering of *A. chrysogenum*

The directed manipulation of genetic material of an organism with the aim to change its biosynthesis capabilities can be regarded as an alternative to classical strain improvement to complement current strain breeding strategies. Compared to classic approaches of titer improvement, molecular engineering of biosynthesis genes requires much more knowledge of the relevant molecular details.

8.1
Genetic Tools for Molecular Engineering

One prerequisite for molecular engineering is an established set of genetic tools. For *A. chrysogenum*, the available tools are still very limited. There are few examples for strong or inducible promoters for the expression of homologous and heterologous genes, selection markers for transformation, plasmids and methods for efficient homologous integration of genes and gene disruptions. When the codon usage of *A. chrysogenum* was investigated in 1999, the DNA sequences of only 19 nuclear genes were accessible in public databases [178]. Today, the number of known genes from *A. chrysogenum* is approximately 30 of which 6 are cephalosporin C biosynthesis genes and 3 encode transcription factors that are involved in the regulation of the biosynthesis genes.

In the 1980s, an efficient integrative transformation system was described for *A. chrysogenum* [179]. Transformation usually results in the ectopic integration of plasmid DNA at one or several genomic loci due to illegitimate recombination. So far, only three different selection markers have been used repeatedly: the bacterial genes for hygromycin B and phleomycin resistance (e.g. [171, 180]), and a mutated version of the *β-tubulin* gene from *A. chrysogenum* providing a homologous transformation system [181]. The substitution of phenylalanine by tyrosine at codon 167 results in a mutated *β-tubulin* gene, which conveys benomyl resistance as a dominant selection marker. The mu-

tated *β-tubulin* gene can be expressed using its own promoter and flanking regions. Transformation of a gel-purified DNA fragment encompassing the *β-tubulin* selection marker, but no bacterial DNA sequences from cloning vectors, is feasible and results in *A. chrysogenum* transformants without integrated heterologous DNA [182]. This aspect of the homologous transformation system is important with respect to governmental restrictions concerning the use of recombinant strains in biotechnical production processes. It is also possible to integrate a DNA fragment or a plasmid without a suitable selection marker in the genomic DNA of *A. chrysogenum* when a co-transformation experiment is conducted together with a vector harboring a dominant selection marker [107, 182].

Further molecular tools that are important for studying the regulation of cephalosporin C biosynthesis genes include reporter genes. Menne et al. [107] fused the intergenic region between the *pcbAB* and the *pcbC* gene with the two reporter genes *lacZ* and *gusA* and compared the specific enzyme activity of *A. chrysogenum* transformants harboring the four different gene fusions. They could show that the specific activity of the β-galactosidase encoded by the *lacZ* gene is higher than the enzyme activity obtained with the *gusA* gene, making the *lacZ* gene more suitable for the analysis of weak promoters in *A. chrysogenum.*

The ability to disrupt a gene is of high importance for molecular engineering. The disrupted gene can be for example a structural gene of the biosynthesis resulting in truncated biosynthesis, or a regulatory gene whose product is involved in the regulation of cephalosporin C biosynthesis. In the latter case, gene disruption can alter the transcription of one or several biosynthesis genes. So far, only a few examples of gene disruption in *A. chrysogenum* are available, because the required homologous recombination is a rare event in this filamentous fungus. The first investigation used the disruption of the *pcbC* gene to determine that 3 kb are the required length of homologous DNA sequences at both sides of the resistance cassette to yield knock-out transformants [183]. Velasco et al. [184] also used several kb of homologous DNA flanking the resistance cassette for their disruption of the *cefEF* gene in *A. chrysogenum*. The disruption of a gene encoding a biosynthesis enzyme results in the accumulation of pathway intermediates, which can often be detected using bioassays, allowing a fast identification of the desired gene disruption transformant [183, 184]. The disruption of a regulatory gene often lacks a phenotype, which in principle could be used to identify the desired knockout transformants. Therefore, a PCR strategy is applied in order to detect the knockout strain. This approach was followed for the transcription factor gene *cpcR1* [171]. For the disruption of the *mecB* gene encoding cystathione-γ-lyase, which is involved in cysteine synthesis, a double-marker technique was used. Transformants with a correct double-crossover were hygromycin-resistant and phleomycin-sensitive, whereas ectopic integration led to transformants with resistance against both antibiotics [33].

The tools available for the investigation and alteration of cephalosporin C biosynthesis can also be utilized to establish the synthesis of other products in

A. chrysogenum. So far, a few heterologous proteins were synthesized in different strains of *A. chrysogenum*, but no secondary metabolites besides cephalosporin derivatives. Examples are alkaline proteases from *Fusarium sp*., human lysozyme and recombinant hirudin [185–187].

8.2 Optimization of Cephalosporin C Biosynthesis

The determination of rate-limiting steps in the biosynthesis is the first goal of a rational approach to improve cephalosporin titer. This can be performed by looking for intermediates that accumulate in fermentation broth, measurements of specific enzyme activity and the comparison of wild-type and high-titer production strains [188].

Several biosynthesis genes have been transformed into *A. chrysogenum* to yield strains with a higher copy number of these genes. The amplification of the *pcbC* gene did not result in significantly increased cephalosporin C production indicating that the cyclase activity is not rate-limiting in β-lactam biosynthesis [100].

In contrast to the results with the *pcbC* gene, an increase in the copy number of the *cefG* gene had a positive effect on cephalosporin C titer. Mathison and co-workers [97] cloned the *cefG* gene encoding the acetyl transferase, which catalyzes the last step in the biosynthesis. By transforming the *cefG* gene in the *A. chrysogenum cefG* mutant M40, they restored the synthesis of cephalosporin C and observed a correlation between *cefG* copy number and cephalosporin C titer. The transformation of a wild-type strain with up to five additional copies of the *cefG* gene increased the cephalosporin C titer from 0.625 mg/mL to 1.9 mg/mL. Mathison et al. [97] concluded that at least in the wild-type strain, the acetyl transferase activity is a rate-limiting step. In another investigation, the *cefG* gene was expressed from the homologous promoter and from four heterologous promoters. This study used for example promoters from the *gpd* gene of *A. nidulans* as constitutive promoters and the *pcbC* promoter from *P. chrysogenum* as the non-constitutive one [135]. In general, a higher steady-state transcript level of the *cefG* gene was observed in all transformants. Transformants of the producer strain C10 showed a doubled acetyl transferase activity when the *cefG* gene was fused to the *pcbC* promoter of *P. chrysogenum*, and a better conversion of deacetylcephalosporin C to cephalosporin C. Again, it was concluded that the expression of the *cefG* gene is limiting for cephalosporin C biosynthesis.

A similar amplification of gene copy number with the goal to increase the corresponding enzymatic activity was tried for a gene, which is involved in precursor synthesis. L-cysteine is a precursor of the ACV tripeptide in cephalosporin C biosynthesis and can be supplied through an autotrophic pathway and through the reverse transsulfuration pathway. In the latter case, it is produced from methionine via cystathionine, which is split into cysteine and α-ketobutyrate enzymatically by cystathionine-γ-lyase. This enzyme is en-

coded by the *mecB* gene and is required for high-level cephalosporin production [33]. Some transformants with multiple copies of the *mecB* gene showed higher cystathionine-γ-lyase activity and one transformant produced higher amounts of cephalosporins [35]. It was concluded that moderately increased levels of cystathionine-γ-lyase stimulate cephalosporin production, but very high levels are deleterious for growth and production.

Titer improvement was also reached in transformants of *A. chrysogenum* with a bacterial hemoglobin gene. The oxygen-binding heme protein from the bacterium *Vitreoscilla* has been synthesized in fungal transformants to improve the oxygen supply during fermentation [189]. It is not known, whether oxygen supply directly affects the three oxidation reactions in cephalosporin biosynthesis or indirectly benefits the production by a more efficient overall metabolism. Several transformants expressed the *heme* gene under the control of the strong constitutive TR1 promoter from *Trichoderma reesei* and produced a higher cephalosporin titer than control strains in batch culture experiments. Ten out of 17 transformants with the *Vitreoscilla heme* gene produced 7–64% higher cephalosporin C levels than the non-transformed control strain C10 [189].

In principle, transcription factors are promising candidates for use in molecular engineering. Overexpression of a transcriptional activator gene or the disruption of a repressor gene are relatively simple scenarios. More sophisticated approaches could be in vitro optimized transcription factors, e.g. with stronger transactivation capacities or different DNA-binding specificities. Due to the limited knowledge of transcription factors from *A. chrysogenum*, the future will show whether this kind of experimentation can considerably contribute to cephalosporin titer improvement. First results obtained from *A. chrysogenum* transformants with altered transcription factor gene copies are listed above.

Besides titer improvement, an objective of gene amplification in genetically engineered strains can be the reduction of an intermediate, which may be an undesirable by-product. The *cefEF* gene encodes a bifunctional expandase/hydroxylase that converts penicillin N to deacetoxycephalosporin C and then to deacetylcephalosporin. Deacetoxycephalosporin C accumulates in the fermentation broth to a concentration of 1–2% of the final cephalosporin C yield and is an undesired contaminant in the extraction process [190]. Genetically engineered strains with increased copy number of the *cefEF* gene resulted in the reduction of desacetoxycephalosporin C to 50% or less of the control. The *cefEF* gene was expressed from its own promoter and Southern analysis indicated the integration of only a single additional gene copy. The reduction of desacetoxycephalosporin content relative to the cephalosporin C production was verified in upscale fermentation of 30,000 L, but the total production of cephalosporin C was not increased significantly [190].

Another reason for the construction of genetically engineered strains is the attempt to produce cephalosporin derivatives that are more suitable for chemical modifications than cephalosporin C itself. Semisynthetic cephalosporins are made from 7-aminodeacetoxycephalosporanic acid (7-ADCA) or 7-

aminocephalosporanic acid (7-ACA), which can be derived enzymatically or chemically from cephalosporin C or penicillin G. The direct conversion of cephalosporin C into 7-ACA in *A. chrysogenum* was performed by transforming two heterologous genes into one recipient strain. The genes encoding D-amino acid oxidase from the fungus *Fusarium solani* and glutaryl acylase from the bacterium *Pseudomonas diminuta* expanded the biosynthesis potential in the engineered strain. However, the amounts of 7-ACA were detectable but not commercially significant [191].

Recently, a different approach was followed where desacetoxycephalosporin (DAOC) from the fermentation broth of *A. chrysogenum* was used as starting material for the production of 7-ADCA. In order to accumulate DAOC in *A. chrysogenum*, a two step approach was used. In the first step, the *cefEF* gene encoding the bifunctional expandase/hydroxylase was disrupted and subsequent transformants of *A. chrysogenum* accumulated penicillin N. In the following step, a gene fusion was integrated into the Δ*cefEF* strain, which consisted of the *pcbC* promoter from *Penicillium chrysogenum* and the *cefE* gene from *S. clavuligerus* [184]. The *cefE* gene from the bacterial cephem-producer encodes the expandase enzyme that catalyzes the ring-expansion step in cephalosporin biosynthesis. Resulting recombinant strains were tested for production of DAOC in bioassays analyzing penicillinase-resistant inhibition of *E. coli* growth. HPLC analysis of the most promising transformant revealed a DAOC production of 75–80% of the total β-lactams produced by the parental production strain that was used for genetic engineering. It is worth mentioning that about 20% of the β-lactams produced by engineered strains accumulate in the fermentation broth as penicillin N indicating that the heterologous expandase activity is a rate limiting step. The accumulation of penicillin N might be reduced by a higher expression rate of the *cefE* gene or a classical strain improvement program starting with the engineered strain [184]. The purified DAOC from the fermentation broth of *A. chrysogenum* was bioconverted into 7-ADCA in two enzymatic steps using D-amino acid oxidase from the basidiomyceteous fungus *Rhodotorula gracilis* and the bacterial glutaryl acylase originating from *Acinetobacter spec.*

9 Outlook

After the development of different molecular tools for *Acremonium chrysogenum*, one of the major interests in this field was to elucidate molecular changes that have occurred in strains during production improvement programs. The simple idea in the beginning that gene copy number, DNA rearrangements or promoter sequence mutations are mainly responsible for different gene expression levels has not yet been confirmed. Instead, one of the more interesting lessons learned from extensive molecular investigations in recent years has been the realization that multiple layers of control exist in

cephalosporin biosynthesis. Therefore, future efforts will focus on deciphering regulatory networks that control cephalosporin biosynthesis. Thus, the development of genomic tools including genome-wide location and expression analysis can be foreseen to allow the simultaneous interrogation of the expression of thousands of genes in a high-throughput fashion. Microarray analysis responds to physiological or genetic changes and will provide indispensable information that ultimately may lead to improved strain development programs. Knowledge of this kind seems to be the necessary prerequisite together with conventional procedures for the efficient generation of novel strains, which are constantly required in competitive production processes.

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