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Phytochemistry 66 (2005) 1200–1210
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Review

Evolution of β-lactam biosynthesis genes and recruitment of *trans*-acting factors

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Received 23 November 2004; received in revised form 3 February 2005

Abstract

Penicillins and cephalosporins belong chemically to the group of β-lactam antibiotics. The formation of hydrophobic penicillins has been reported in fungi only, notably *Penicillium chrysogenum* and *Emericella nidulans*, whereas the hydrophilic cephalosporins are produced by both fungi, e.g., *Acremonium chrysogenum* (cephalosporin C), and bacteria. The producing bacteria include Gram-negatives and Gram-positives, e.g. *Lysobacter lactamdurans* (cephabacins) and *Streptomyces clavuligerus* (cephamycin C), respectively. For a long time the evolutionary origin of β-lactam biosynthesis genes in fungi has been discussed. As often, there are arguments for both hypotheses, i.e., horizontal gene transfer from bacteria to fungi versus vertical descent. There were strong arguments in favour of horizontal gene transfer, e.g., fungal genes were clustered or some genes lack introns. The recent identification and characterisation of *cis*-/*trans*-elements involved in the regulation of the β-lactam biosynthesis genes has provided new arguments in favour of horizontal gene transfer. In contrast to the bacterium *S. clavuligerus*, all regulators of fungal β-lactam biosynthesis genes represent wide-domain regulators which were recruited to also regulate the β-lactam biosynthesis genes. Moreover, the fungal regulatory genes are not part of the gene cluster. If bacterial regulators were co-transferred with the gene cluster from bacteria to fungi, most likely they would have been non-functional in eukaryotes and lost during evolution. Alternatively, it is conceivable that only a part of the β-lactam biosynthesis gene cluster was transferred to some fungi, e.g., the *acvA* and *ipnA* gene without a regulatory gene.

Keywords: *Streptomyces clavuligerus*; *Aspergillus nidulans*; *Penicillium chrysogenum*; *Acremonium chrysogenum*; Horizontal gene transfer; β-Lactam antibiotics; Penicillin; Cephalosporin; Regulators

Contents

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doi:10.1016/j.phytochem.2005.02.030

1. Introduction

β-Lactam antibiotics are produced by both filamentous fungi and bacteria. Bacterial producers include Gram-negative and Gram-positive microorganisms (reviewed in Brakhage, 1998). For a long time the evolutionary origin of β-lactam biosynthesis genes has been discussed. As often, there are arguments for both hypotheses, i.e., horizontal gene transfer versus vertical descent. However, the recent identification and characterisation of *cis*-/*trans*-elements involved in the regulation of the β-lactam biosynthesis genes has provided new arguments in favour of horizontal gene transfer.

2. β-Lactam biosynthesis in bacteria and fungi

2.1. Historical perspective

The modern antibiotic therapy started with the discovery of a β-lactam antibiotic in 1929, when Alexander Fleming published his observation about the inhibition of growth of *Staphylococcus aureus* on an agar plate contaminated with *Penicillium notatum* (Fleming, 1929). Three years later, it was shown that the growth inhibition was due to penicillin (Fig. 1) (Clutterbuck et al., 1932). The first clinical trials with penicillin were undertaken in 1941 (reviewed in Abraham, 1990). During the late 1940s the fungus *Cephalosporium acremonium* (now renamed *Acremonium chrysogenum*) was isolated from the sea at Cagliari (Italy) by Brotzu (1948). This fungus was first found to produce penicillin N and later, another antibiotic was discovered in the culture broth which was established to consist of different derivatives of a β-lactam compound designated cephalosporin (Fig. 1) (reviewed in Abraham, 1990). The discovery of cephalosporin C generated a whole new group of clinically significant β-lactams. Interestingly, it was discovered that both some Gram-negative and some Gram-positive bacteria also produce β-lactam antibiotics. The Gram-positive bacterium *Streptomyces clavuligerus* which has been studied best produces both clavulanic acid and cephamycin C, a derivative of cephalosporin (Fig. 1). The Gram-negative bacterium *Lysobacter lactamgenus* was shown to synthesise cephabacins (reviewed in Liras, 1999).

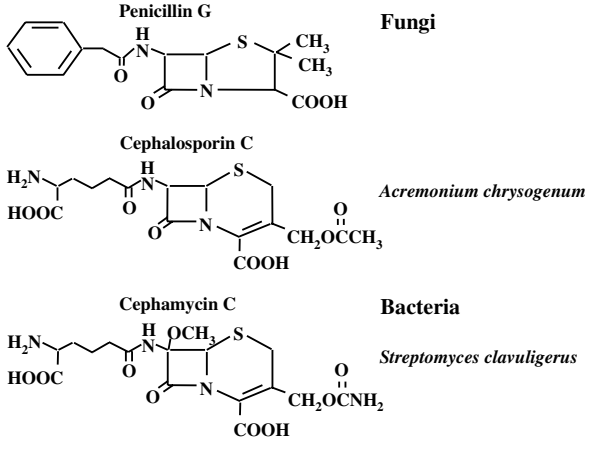

Fig. 1. Structures of some β-lactam antibiotics of fungal and bacterial origin (reviewed in Brakhage, 1998).

Industrial production of penicillin and cephalosporin was achieved with *Penicillium chrysogenum* and *Acremonium chrysogenum*, respectively. These fungi, however, belong to the deuteromycetes which are in general difficult to analyse genetically. Currently, the greatest progress in elucidation of the molecular regulation of biosyntheses of β-lactams in fungi has been made in the penicillin-producer *Aspergillus (Emericella) nidulans*, since this fungus is an ascomycete with a sexual cycle. Hence, classical genetic techniques can be applied to *A. nidulans* (Pontecorvo et al., 1953) and as a result, a detailed genetic map is available (Clutterbuck, 1993). Together with molecular techniques, this facilitated a thorough analysis of the genetic regulation of metabolic pathways, including that of penicillin biosynthesis (reviewed in Arst and Scazzocchio, 1985; Brakhage and Turner, 1995; Brakhage, 1998; Macdonald and Holt, 1976). In addition, the sequence of the whole genome of *A. nidulans* is available at the *Aspergillus nidulans* Database (http://www.broad.mit.edu/annotation/fungi/aspergillus/index.html).

2.2. Biosynthesis of penicillins and cephalosporins

Penicillins and cephalosporins belong chemically to the group of β-lactam antibiotics. The biosynthesis of both penicillins and cephalosporins have the first two steps in common (reviewed in Brakhage, 1998)

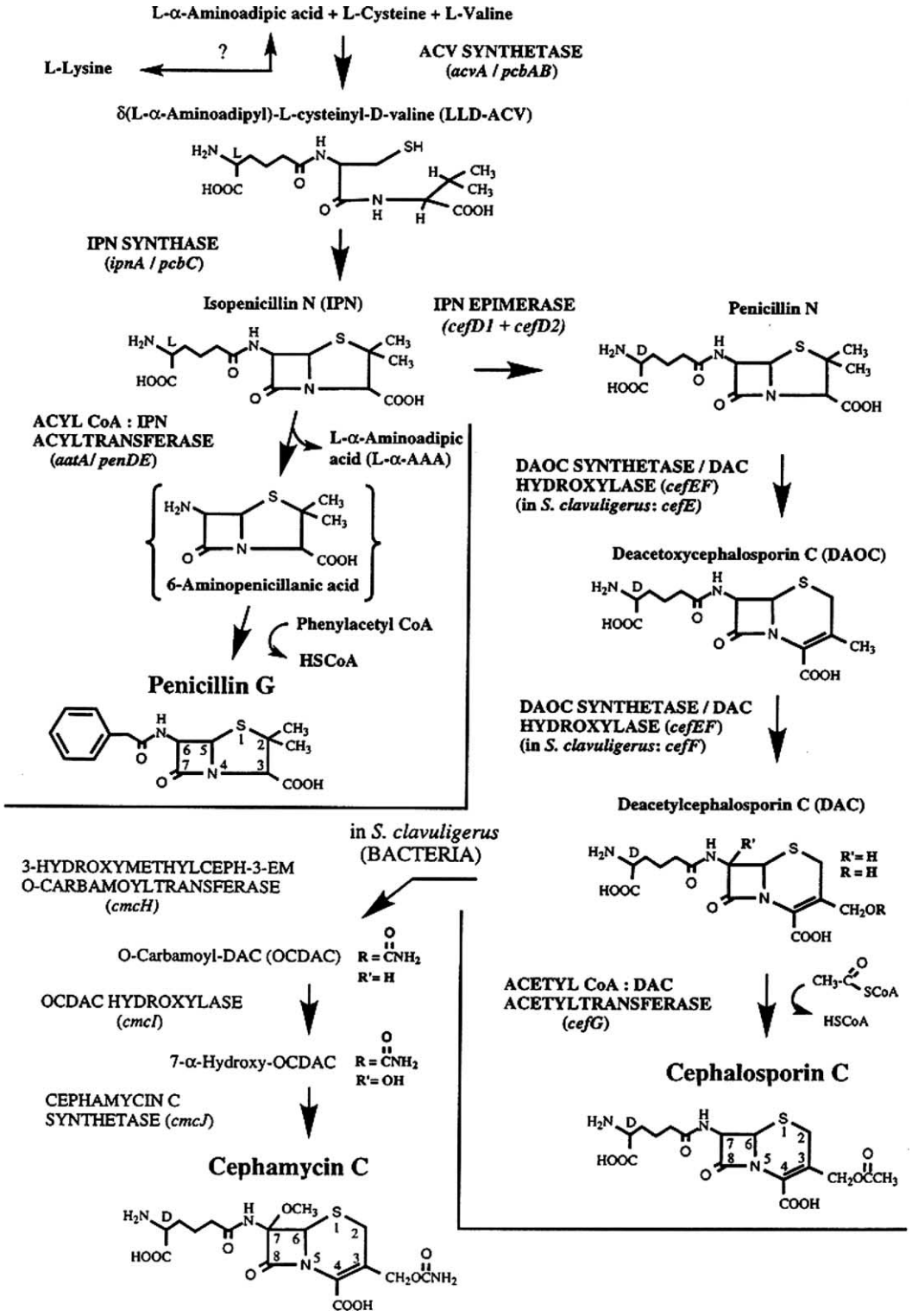

Fig. 2. Biosynthesis pathways of fungal penicillin and cephalosporin C and bacterial cephamycin (reviewed in Brakhage and Caruso, 2004).

(Fig. 2). All naturally occurring penicillins and cephalosporins produced by eukaryotic or prokaryotic microorganisms are synthesised from the same three amino acids, L-α-aminoadipic acid (L-α-AAA), L-cysteine and L-valine (Fig. 2). In fungi, the non-proteinogenic amino acid L-α-AAA is derived from the fungus specific aminoadipate pathway which leads to formation of lysine. It can also be provided by catabolic degradation of lysine although the contribution of this pathway to penicillin biosynthesis has not been clarified yet. In bacteria, a

specific pathway for formation of L-α-AAA for β-lactam biosynthesis has been found (reviewed in Brakhage, 1998).

In the first reaction of the cephalosporin and penicillin biosynthesis pathway, the amino acid precursors are condensed to the tripeptide δ-(L-α-aminoadipyl)-L-cysteine-D-valine (ACV). This reaction is catalysed by a single enzyme, δ-(L-α-aminoadipyl)-L-cysteine-D-valine synthetase (ACVS). ACVS is encoded by a single structural gene designated *acvA* (*pcbAB*) (Fig. 3). In the second step, oxidative ring closure of the linear tripeptide leads to formation of a bicyclic ring, i.e., the four-membered β-lactam ring fused to the five-membered thiazolidine ring which is characteristic of all penicillins. The resulting compound isopenicillin N (IPN) possesses weak antibiotic activity and is thus the first bioactive intermediate of both penicillin and cephalosporin pathways. This reaction is catalysed by isopenicillin N synthase (IPNS) encoded by the *ipnA* (*pcbC*) gene (Figs. 2 and 3). IPN is the branch point of penicillin and cephalosporin biosyntheses.

In the third and final step of penicillin biosynthesis, the hydrophilic L-α-AAA side chain of IPN is exchanged for a hydrophobic acyl group catalysed by acyl coenzyme A:isopenicillin N acyltransferase (IAT). The corresponding gene was designated *aatA* (*penDE*). In natural habitats penicillins such as penicillin F and K, which contain D3-hexenoic acid and octenoic acid as side chains, respectively, are synthesised. By supplying the cultivation medium with phenylacetic or phenoxyacetic acid, the synthesis can be directed mainly towards penicillin G and V, respectively (reviewed in Brakhage, 1999) (Fig. 2). The side chain precursors have to be activated before they become substrates for the IAT. It is generally believed that the activated forms of the side chains consist of their CoA-thioesters, but the mechanism behind this activation is still not fully elucidated (Evers et al., 2004).

The formation of hydrophobic penicillins has been reported in fungi only, notably *P. chrysogenum* and *A. nidulans*, whereas the hydrophilic cephalosporins are produced by both fungi and bacteria, e.g. *A. chrysoge-*

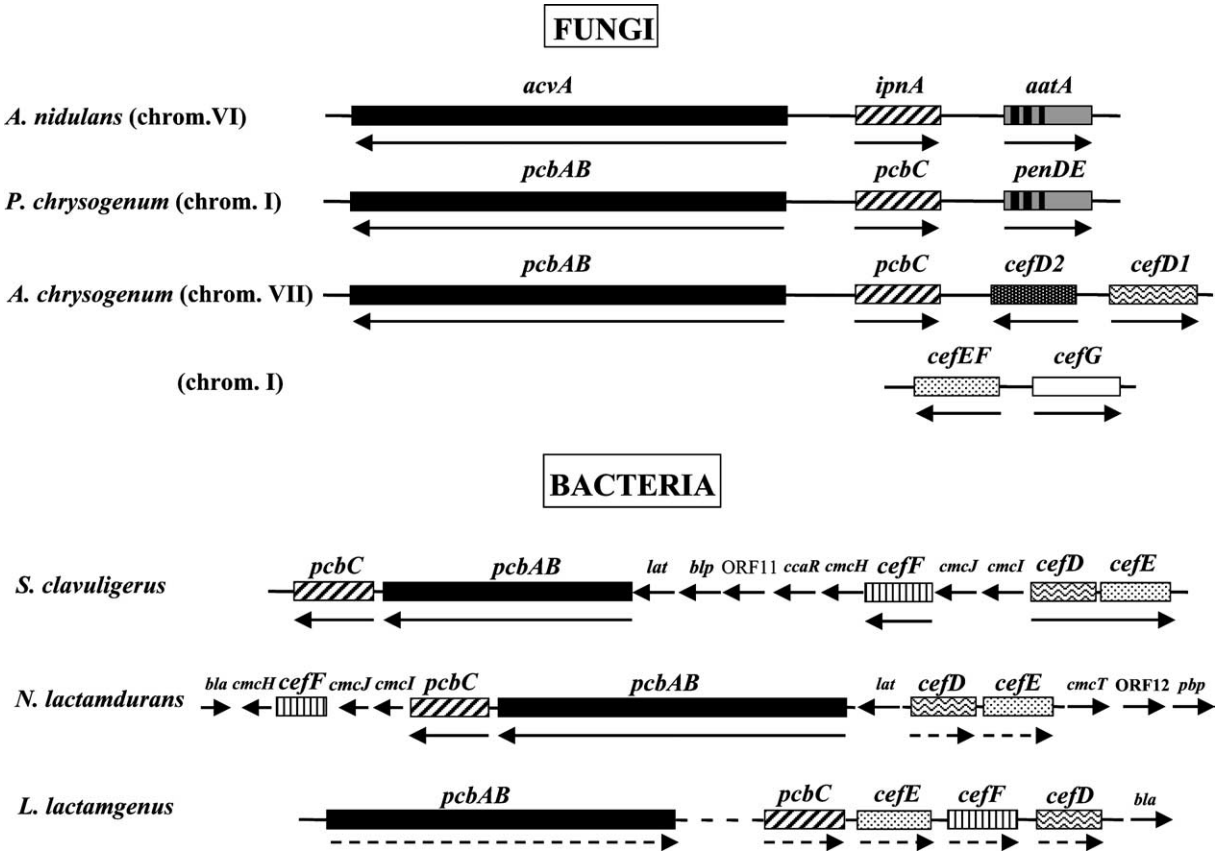

Fig. 3. β-Lactam biosynthesis gene cluster in fungi and bacteria (reviewed in Brakhage et al., 2004). The *A. chrysogenum* genes *cefD1* and *cefD2* are located next to the *pcbAB* and *pcbC* gene (Ullán et al., 2002). Bacterial genes with fungal homologs are boxed. The transcriptional orientation and the transcript units (Bacteria), as far as it has been determined, are indicated by arrows below the boxes. Arrows between boxes (Bacteria) and arrows with broken lines below boxes mark the orientation of genes. ORF specifies an open reading frame whose function is unknown. Abbreviations not mentioned in the text: *cmcT*, transmembrane protein; *L. lactamgenus*, *Lysobacter lactamgenus*; *N. lactamdurans*, *Nocardia lactamdurans*; *pbp*, penicillin-binding protein; *bla*, β-lactamase; *blp*, showing similarity to the extracellular β-lactamase inhibitory protein BLIP; ORF, open reading frame (reviewed in Brakhage et al., 2004).

num and *Streptomyces clavuligerus*, respectively (Figs. 1 and 2). The first step that commits the pathway to the production of cephalosporins is the isomerisation of the L-α-AAA side chain of IPN to the D-enantiomer to give penicillin N. This reaction is catalysed by an IPN epimerase system (Fig. 2). Penicillin N is the precursor for antibiotics containing the cephem nucleus, i.e. cephalosporins and cephamycins (7-methoxycephalosporins) produced by fungi and bacteria, respectively (Figs. 1 and 2). Penicillin N is converted to deacetoxycephalosporin C (DAOC) by DAOC synthetase (expandase) activity (in *A. chrysogenum* (Dotzlaf and Yeh, 1987); in *S. lactamdurans* it appears to be a DAOC synthase (Cortes et al., 1987)). This ring expansion step involves the oxidative opening of the thiazolidine ring to give upon reclosure the six-membered dihydrothiazine ring, which is characteristic of all cephalosporins. In the next step the methylgroup at carbon atom 3 of DAOC is hydroxylated/oxidized to form deacetylcephalosporin C (DAC). In *A. chrysogenum* both reactions are catalysed by a single enzyme, DAOC synthetase (expandase)/DAC hydroxylase, whereas in the bacterial cephalosporin-producing organism *S. clavuligerus* one enzyme for each reaction has been found (Figs. 2 and 3) (reviewed in Brakhage and Caruso, 2004).

In the last step of cephalosporin C biosynthesis, an acetyl moiety from acetyl coenzyme A is transferred to the OH group of DAC catalysed by the product of *cefG*, acetyl coenzyme A:DAC acetyltransferase (Fig. 2). Several cephalosporins have been isolated from a variety of microorganisms that differ from cephalosporin C in the substituent attached to the 3′C oxygen. Cephamycin C biosynthesis which has been studied best in *S. clavuligerus*, starts from the intermediate DAC. A carbamoyl group is attached to DAC to give *O*-carbamoyl-DAC (OCDAC). This reaction is catalysed by 3-hydroxymethyl ceph-3-em *O*-carbamoyltransferase which is encoded by the *cmcH* gene. Then, the C-7 is hydroxylated by the action of OCDAC hydroxylase encoded by *cmcI*. In the final step of cephamycin biosynthesis, the hydroxy group at C-7 is methylated to form cephamycin C (7-methoxycephalosporin) catalysed by cephamycin C synthetase. The corresponding gene is designated *cmcJ* (Alexander and Jensen, 1998) (Figs. 2 and 3).

3. Clustering of biosynthesis genes

The linkage of antibiotic-biosynthesis genes is a well-known phenomenon in many antibiotic-producing organisms (Fig. 3). It has been speculated that linkage has occurred during evolution owing to an ecological selective advantage (Martin et al., 1982). Seno and Baltz (1989) have suggested that coordinated regulation of antibiotic-biosynthesis genes could be achieved by organising the genes into large operons controlled by a single promoter. For example, genes of the actinorhodin biosynthesis pathway in *Streptomyces coelicolor* are clustered and expressed in several polycistronic messages (Malpartida and Hopwood, 1986). In eukaryotic fungi, however, β-lactam biosynthesis genes are transcribed separately, and are expressed from different promoters (Smith et al., 1990a; reviewed in Brakhage and Turner, 1995). Hence, in fungi, there is no obvious need for clustering and it thus seems more likely that linkage reflects a common ancestral origin, e.g., the transfer of a cluster or part of it from bacteria to fungi. However, there is no evidence that the acyl coenzyme A : isopenicillin N acyltransferase gene (*aatA*) has a close relative in modern prokaryotes, even though it is part of the cluster. This fact supports the hypothesis that linkage might also confer an ecological advantage to the eukaryotic fungi in their natural habitat, although the reason for this is not yet understood.

4. Evolution of β-lactam biosynthesis genes in fungi

4.1. Hypothesis on the origin of β-lactam biosynthesis genes in fungi

β-Lactam biosynthesis genes were found both in some bacterial species and in some fungi (Fig. 3). The availability of sequence information about bacterial and fungal genes led to speculations about their evolutionary relationship. Based on several observations, a horizontal transfer of β-lactam biosynthesis genes from bacteria to fungi during evolution has been proposed by several authors (Carr et al., 1986; Weigel et al., 1988; Landan et al., 1990; Peñalva et al., 1990; Aharonowitz et al., 1992). This hypothesis was questioned by Smith et al. (1992). The arguments in favour of a horizontal gene transfer are as follows. (i) *ipnA* genes of fungi and bacteria show high sequence similarities. More than 60% of the nucleotide bases and 50% of the deduced amino acids are identical. (ii) Bacterial as well as fungal β-lactam genes are organized in clusters. In bacteria, the β-lactam biosynthesis genes are organized into a single cluster, as are the penicillin biosynthesis genes in fungi (Fig. 3). The cephalosporin biosynthesis genes in *A. chrysogenum* are organized into two clusters located on different chromosomes (Fig. 3). This finding led to the assumption that the β-lactam biosynthesis genes were transferred as a single cluster from an ancestral prokaryote to a common ancestor of the β-lactam synthesising fungi. In the eukaryotic ancestor, the biosynthesis genes were split onto two chromosomes. One part encodes the early genes of β-lactam biosynthesis, the other the late genes. Later in the lineage an ancestor of *A. nidulans* and *P. chrysogenum* diverged from *A. chrysogenum* and has presumably lost the second cluster

with the genes for the late stage of cephalosporin biosynthesis. The third gene, *aatA*, was recruited during evolution to the biosynthesis gene cluster. It has all features of a eukaryotic gene, i.e., it contains introns and apparently, there is no obvious homolog in bacteria (Skatrud, 1991). Isopenicillin N already possesses weak antibiotic activity. However, the recruitment and involvement of AATA led to formation of penicillins with much higher antibiotic activity which could explain the selection pressure to add the third gene *aatA* to the genes *acvA* and *ipnA* (Fig. 3). (iii) The GC content in the third position of codons encoding the *ipnA* gene of *A. nidulans* and *P. chrysogenum* is unusually high and could indicate an evolutionary origin from streptomycetes which show GC contents of greater than 70% (Aharonowitz et al., 1992). (iv) Fungal *acvA* and *ipnA* genes do not contain introns indicating a bacterial origin of the genes (reviewed in Brakhage, 1998). (v) Gene transfer from bacteria to fungi is far more likely than the other way around because transfer of β-lactam biosynthesis genes from fungi to bacteria would have been lethal for bacteria without the presence of resistance mechanisms.

Based on the DNA sequences of *ipnA* genes from Gram-positive streptomycetes and fungi and a rate of nucleotide substitution of 10^{-9} nucleotide changes per site per year (Li et al., 1985), Weigel et al. (1988) proposed that the transfer occurred 370 million years ago. The cloning and sequencing of an *ipnA* gene from a Gram-negative bacterium, *Flavobacterium* sp., however, led to an extension/modification of the hypothesis of horizontal gene transfer. The *ipnA* gene of *Flavobacterium* sp. shares 69% sequence identity with the streptomycetes gene and 64–65% with the fungal genes (*A. chrysogenum, P. chrysogenum*) (Cohen et al., 1990). A reevaluation of the divergence times of organisms using a protein clock suggested that Gram-positive and Gram-negative bacteria split about 2 billion years ago, prokaryotes and a eukaryotic ancestor split about 3.2–3.8 billion years ago (Feng et al., 1997). If the gene transfer had occurred only 370 million years ago from streptomycetes to fungi as proposed by Weigel et al. (1988), it could be expected that the fungal and streptomycete genes show a greater homology than the Gram-positive (streptomycetes) and Gram-negative genes (*Flavobacterium* sp.). As outlined above, this is not the case (Cohen et al., 1990). Hence, Aharonowitz et al. (1992) suggested that multiple gene transfer events might have occurred from bacteria to fungi. It is difficult to imagine, however, why these multiple gene transfers then happened at about the same time what would be expected from the degree of similarity between the proteins of the various organisms. In addition, Smith et al. (1992) argued against a horizontal transfer. The authors criticised that the hypothesis of a horizontal gene transfer e.g. of the *ipnA* gene was made with a very limited data set and was based solely on assumptions about rates of change. They rooted the tree with two distantly related β-lactam biosynthesis enzymes. They compared the similarity of both isopenicillin N synthase (IPNS) of *A. nidulans*, *P. chrysogenum*, *A. chrysogenum*, *S. clavuligerus*, *S. anulatus* and *Flavobacterium* sp., and deacteoxycephalosporin C (DAOC) synthase of *S. clavuligerus* and *A. chrysogenum*. Based on these similarities, a tree arose with conventional evolutionary descent. The authors argued that the simplest interpretation is that the genes for the two enzymes are the result of a duplication that occurred before the prokaryote/eukaryote divergence. The topology of the tree rooted with the duplicated enzymes, the depth of the bacterial branches and the different orientations of genes in fungi and eubacteria all appear to be consistent with an ordinary evolution for IPNS. However, if the genes appeared very early in the evolution why have most of the eukaryotes and fungi lost the gene cluster? This question cannot be seriously answered at the moment.

4.2. Recruitment of trans-acting factors for fungal β-lactam biosynthesis genes

Within the last few years, several studies have indicated that the β-lactam biosynthesis genes of fungi are controlled by a complex regulatory network and a comparison with known regulators and DNA elements involved in the regulation of genes of primary metabolism is of interest. A variety of *cis*-acting DNA elements and regulatory factors is involved. As far as we know today, none of these regulatory factors is confined to β-lactam producing fungi. All of them represent wide-domain regulators, i.e., they also regulate other genes than the fungal β-lactam biosynthesis genes. Furthermore, the regulators so far identified are typical eukaryotic proteins, such as basic region helix–loop–helix proteins, CCAAT binding complex or zinc finger proteins (Table 1). Hence there is no hint for the presence of a regulator associated with the gene cluster and of possible prokaryotic origin.

The wide domain regulator PacC (Table 1) is the key player of the fungal pH regulation. At alkaline ambient pH, the *A. nidulans* PacC protein activates transcription of alkaline-expressed genes, e. g., of the alkaline phosphatase and protease genes *palD* and *prtA*, respectively, and also of the penicillin biosynthesis genes *ipnA* (Tilburn et al., 1995) and very likely of *acvA* (Then Bergh and Brakhage, 1998). The same is true for the CCAAT-binding complex AnCF, which was shown to exhibit positive effects on the expression of the penicillin biosynthesis genes *ipnA* and *aatA* (Litzka et al., 1998; Steidl et al., 1999). Because AnCF binds to CCAAT-containing sequences which are present in many eukaryotic gene promoters, it has been estimated that AnCF regulates more than 200 genes (Brakhage et al., 1999). However, deletion of subunit-encoding genes of AnCF

Table 1
Known *trans*-acting factors of penicillin/cephalosporin biosynthesis genes

Compound	Fungus/bacterium	Regulator	Structural class	Part of gene cluster	Reference
Penicillin	*A.nidulans*	PacC	Zinc finger	–	Tilburn et al. (1995)
		AnCF	CCAAT-binding complex	–	Steidl et al. (1999)
		AnBH1	bHLH	–	Caruso et al. (2002)
		VeA	No homology with any other protein of known function	–	Kato et al. (2003)
	P. chrysogenum	NRE	Zinc finger	–	Haas et al. (1995)
		PacC	Zinc finger	–	Suárez and Peñalva (1996)
Cephalosporin	*A. chrysogenum*	CpcR1	Ring finger	–	Schmitt and Kück (2000)
		Cre1	Zinc finger	–	Jekosch and Kück (2000)
		PacC	Zinc finger	–	Schmitt et al. (2001)
Cephamycin	*Streptomyces clavuligerus*	CcaR		+	Pérez-Llarena et al. (1997)

is not lethal (Papagiannopoulos et al., 1996) indicating that this regulatory complex is not essential for the direct survival of the cells and is only involved in the regulation of a certain subset of genes including some of the penicillin biosynthesis genes.

VeA is a light-dependent regulator of the sexual and asexual development in *A. nidulans*. This regulator is also involved in the regulation of the penicillin biosynthesis in *A. nidulans* (Kato et al., 2003; Spröte and Brakhage, unpublished results). Clearly, VeA can also be expected to act on a number of genes. Another regulator is represented by AnBH1 which is a basic-region helix–loop–helix (bHLH) protein. It was shown to bind to the promoter region of the *aatA* gene of *A. nidulans* and to act as a repressor of the *aatA* gene. So far, only two genes have been reported to be regulated by AnBH1, i.e., *aatA* and the *anbH1* gene itself (Caruso et al., 2002; Caruso and Brakhage, unpublished results). However, since the deletion of the *anbH1* gene appears to be lethal for the fungus (Caruso et al., 2002), it can be expected that more genes are regulated by AnBH1. There is data suggesting that the wide-domain regulatory protein NRE is involved in the regulation of the penicillin biosynthesis genes in *P. chrysogenum* (Haas and Marzluf, 1995). NRE is the homolog of the *A. nidulans* AreA protein. In *A. nidulans*, global nitrogen repression/derepression is mediated by the major positive control gene *areA* (Kudla et al., 1990). Cre1 of *A. chrysogenum* is the homolog of the *A. nidulans* CreA protein controlling carbon source regulation in *A. nidulans* (Bailey and Arst, 1975; Hynes and Kelly, 1977). Jekosch and Kück (2000a,b) showed that regulation of *A. chrysogenum ipnA* (*pcbC*) and *cefEF* gene expression is also mediated by Cre1 and therefore by glucose. Carbon catabolite regulation controls many genes including some of the cephalosporin biosynthesis genes in *A. chrysogenum*.

CpcR1 was identified by Schmitt and Kück (2000) as binding to a region in the promoter of the cephalosporin biosynthesis gene *pcbC* (*ipnA*). The polypeptide shows significant similarity to human transcription factors of the regulatory factor X (RFX) family 1. Members of this family possess a unique DNA binding and dimerisation domain. Although several proteins of this family have been identified in different organisms, only limited information is available on their function (Emery et al., 1996). In *A. chrysogenum*, based on the available data it was concluded that cephalosporin biosynthesis is regulated by a well-controlled or even functional redundant network of transcription factors, with CpcR1 being only one player within this process (Schmitt et al., 2004). CpcR1 can be expected to be involved in the regulation of other genes than the penicillin biosynthesis genes.

By contrast, in the Gram-positive bacterium *Streptomyces clavuligerus*, a regulatory gene (*ccaR*) of both the cephamycin and clavulanic acid biosynthesis was found to be located in the cephamycin biosynthesis gene cluster (Pérez-Llarena et al., 1997; Kyung et al., 2001) (Fig. 3). This gene shows high similarity to regulatory genes involved in the biosynthesis of other secondary metabolites in Streptomycetes, such as actinorhodin (Petrich et al., 1994). CcaR represents a pathway specific regulator controlling the β-lactam biosynthesis gene expression. There are additional regulatory circuits involved, e.g., the RelA dependent stringent response is involved in the regulation of cephamycin production in *S. clavuligerus* (Jones et al., 1996; Jin et al., 2004). The stringent response also represents a wide-domain regulatory network. The meaning of CcaR for this regulatory circuit is unknown yet.

As outlined above, the regulatory genes of the fungal β-lactam biosynthesis were neither part of the gene clusters nor pathway specific. This finding supports the hypothesis that upon horizontal gene transfer, existing regulatory genes/proteins were recruited to regulate the fungal β-lactam biosynthesis genes. This has guaranteed the expression of biosynthesis genes. It is conceivable that bacterial regulators were also transferred to fungi, but because they were not functional, they were lost during evolution. Alternatively, only parts of the β-lactam

biosynthesis gene cluster, e.g., only the *acvA* and *ipnA* gene, without the regulatory genes were transferred to fungi. The regulation was taken over by existing regulators.

Both aspects, i.e., the finding that wide-domain, not pathway specific regulators control the penicillin biosynthesis, and the assumption that horizontal gene transfer from bacteria to fungi could have occurred, are further supported by the notion that the transfer of the penicillin biosynthesis gene cluster into non-β-lactam producing fungi led to production of β-lactam antibiotics by these fungi. A cosmid clone containing the penicillin biosynthesis gene cluster from *P. chrysogenum* was used to transform the related filamentous fungi *Neurospora crassa* and *Aspergillus niger*, which do not produce β-lactam antibiotics. Both of the transformed hosts produced penicillin V. Assays of penicillin biosynthesis enzyme activity additionally demonstrated that they possessed ACVS, IPNS and AATA activity (Smith et al., 1990b). These findings were further supported by the observation that in *A. niger*, an *aatA-lacZ* gene fusion was expressed (Litzka et al., 1995). Hence, existing regulators have taken over the task to activate the expression of the penicillin biosynthesis genes.

The analysis of production strains led to the finding that between 8 and 16 copies are present in the high producer strain *P. chrysogenum* BW1890 (Smith et al., 1989). The*P. chrysogenum* high titer producing strains P-2 and AS-P-78 (old production strain) carry approximately 9 and 6 copies, respectively, of penicillin biosynthesis genes (Barredo et al., 1989). Fierro et al. (1995) showed that in the high titer *P. chrysogenum* strains E1 and AS-P-78 the amplifications are organized in tandem repeats. A conserved TTTACA hexanucleotide sequence may be involved in their generation. This TTTACA sequence borders the 106.5-kb long penicillin biosynthesis gene cluster in the wild-type strain NRRL 1951 and also the *P. notatum* strain ATCC 9478 (Fleming's isolate). The importance of this border hexanucleotide sequence was further supported by the analysis of independently isolated non-penicillin producing mutants of *P. chrysogenum*. All three mutants showed a deletion of the whole penicillin biosynthesis gene cluster at a specific site within the conserved hexanucleotide sequence (Fierro et al., 1996). It was suggested that this site may represent a hot-spot for site specific recombination after mutation with nitrosoguanidine, the process possibly being part of a fungal SOS system similar to that found in *E. coli* (Fierro et al., 1995, 1996). In other members of a strain improvement series, the length of the amplicon was found to be 57.5 kb. Furthermore, cDNA screening has failed to identify any further transcribed elements within the co-amplified region apart from those derived from the structural penicillin biosynthesis genes (Newbert et al., 1997). Taken together, these data indicated the presence of recombinogenic regions flanking the penicillin biosynthesis gene cluster (Fierro et al., 1995; Newbert et al., 1997). Possibly, these recombinogenic sites play a role in the horizontal transfer of the gene cluster. However, this assumption does not agree with the fact that the *aatA* gene is also part of the cluster flanked by the recombinogenic sites. Therefore, it could only be argued that a transferable cassette containing the penicillin biosynthesis genes has evolved after recruitment of the *aatA* gene to the cluster.

5. Conclusions

The question whether the fungal β-lactam biosynthesis genes were acquired by horizontal gene transfer from bacteria cannot be solved yet on basis of the current data. However, there are many arguments in favour of horizontal gene transfer, as outlined above. These arguments were further supported by the finding that in contrast to the bacterium *S. clavuligerus*, all regulators of fungal β-lactam biosynthesis genes represent wide-domain regulators which were recruited to also regulate the β-lactam biosynthesis genes. Moreover, the fungal regulatory genes are not part of the gene cluster. If bacterial regulators were co-transferred with the gene cluster from bacteria to fungi, most likely they would have been non-functional in eukaryotes and lost during evolution. Alternatively, it is conceivable that only a part of the β-lactam biosynthesis gene cluster was transferred to some fungi, e.g., the *acvA* and *ipnA* gene without a regulatory gene.

Acknowledgement

This work was supported by the Deutsche Forschungsgemeinschaft (DFG Schwerpunkt 1152 Evolution of Metabolic Diversity).

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