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REVIEW ARTICLE

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Evolution of the clusters of genes for ß-lactam antibiotics: a model for evolutive combinatorial assembly of new ß-lactams

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Summary β-Lactam antibiotics are produced by prokaryotic and eukaryotic organisms. The genes for β-lactam biosynthesis are organized in clusters but the location of the different genes is not identical. Biosynthesis genes are clustered with genes for resistance (*bla*, *pbp*) and for the efflux of the antibiotic (*cmc*T) in prokaryotes. Comparison of proteins reveals much larger differences for primary metabolism enzymes than for β-lactam biosynthesis enzymes in producing organisms. This suggests a horizontal transfer of the β-lactam antibiotic biosynthesis genes.

Key words β-Lactam antibiotics · Microbial evolution · Gene clusters · *Streptomyces* · *Penicillium*

The biosynthetic pathways of penicillins, cephalosporins and cephamycins are well known and most of the enzymes for the biosynthesis of these three classes of β-lactams have been now purified and characterized biochemically. However, the biosynthetic pathways for the more complex β-lactam (clavulanic acid, clavams and carbapenems) are still poorly known. Most of the genes for penicillins, cephalosporins and cephamycins have been cloned and sequenced. Comparison of the nucleotide and deduced amino acid sequences for the ACV tripeptide synthetase and the isopenicillin N synthase of prokaryotes (*Streptomyces*, *Nocardia* and *Lysobacter* species) and eukaryotes (*Penicillium*, *Cephalosporium* and *Aspergillus* species) has revealed a large conservation, suggesting that the genes encoding those enzymes were transferred horizontally from prokaryotic to eukaryotic β-lactam producers about 370 millions years ago, much later than the divergence of the prokaryotic and eukaryotic kingdoms. The horizontal transfer hypothesis is supported by the lack of introns in the *pcb*AB and *pcb*C genes in fungi, despite the large size (about 11 kb) of the *pcb*AB gene. The bacterial β-lactam clusters contain, in addition, genes for precursor (α-aminoadipic acid) formation and antibiotic exportation as well as resistance genes. Genes for α-aminoadipic biosynthesis were not transferred to the eukaryotic organisms since this precursor is synthesized by a different pathway in eukaryotic cells; furthermore, the β-lactam resistance genes are not needed in fungi. By contrast, the fungal β-lactam cluster has evolutively incorporated introns containing functional "late genes" that are not present in the bacterial β-lactam clusters. Many other genes of the bacterial clusters which are not available in fungi provide interesting tools for combinatorial construction of β-lactams with improved pharmacological activities.

Biosynthetic pathways for β-lactam antibiotics

β-Lactam antibiotics—penicillins, cephalosporins and cephamycins— are produced by soil microorganisms. Particularly relevant among β-lactam producing organisms are the filamentous fungi *Penicillium chrysogenum* and *Cephalosporium acremonium*, industrial producers of penicillin G and

cephalosporin C respectively [1], the actinomycetes *Streptomyces clavuligerus* and *Nocardia lactamdurans* [22, 28] and also the Gram-negative soil bacteria *Lysobacter lactamgenus* and *Flavobacterium* sp., producers of cephamycins and cephabacins [23]. Other β–lactam compounds such as the tabtoxin, produced by the plant pathogenic bacteria *Pseudomonas tabaci*, are not considered in this study because the genes forming the β–lactam nucleus have not yet been cloned.

A simplified version of the biosynthetic pathway for β–lactam antibiotics is depicted in Fig. 1. For more detailed information on genes and enzymes involved in these pathways, several reviews can be consulted [2, 20, 21].

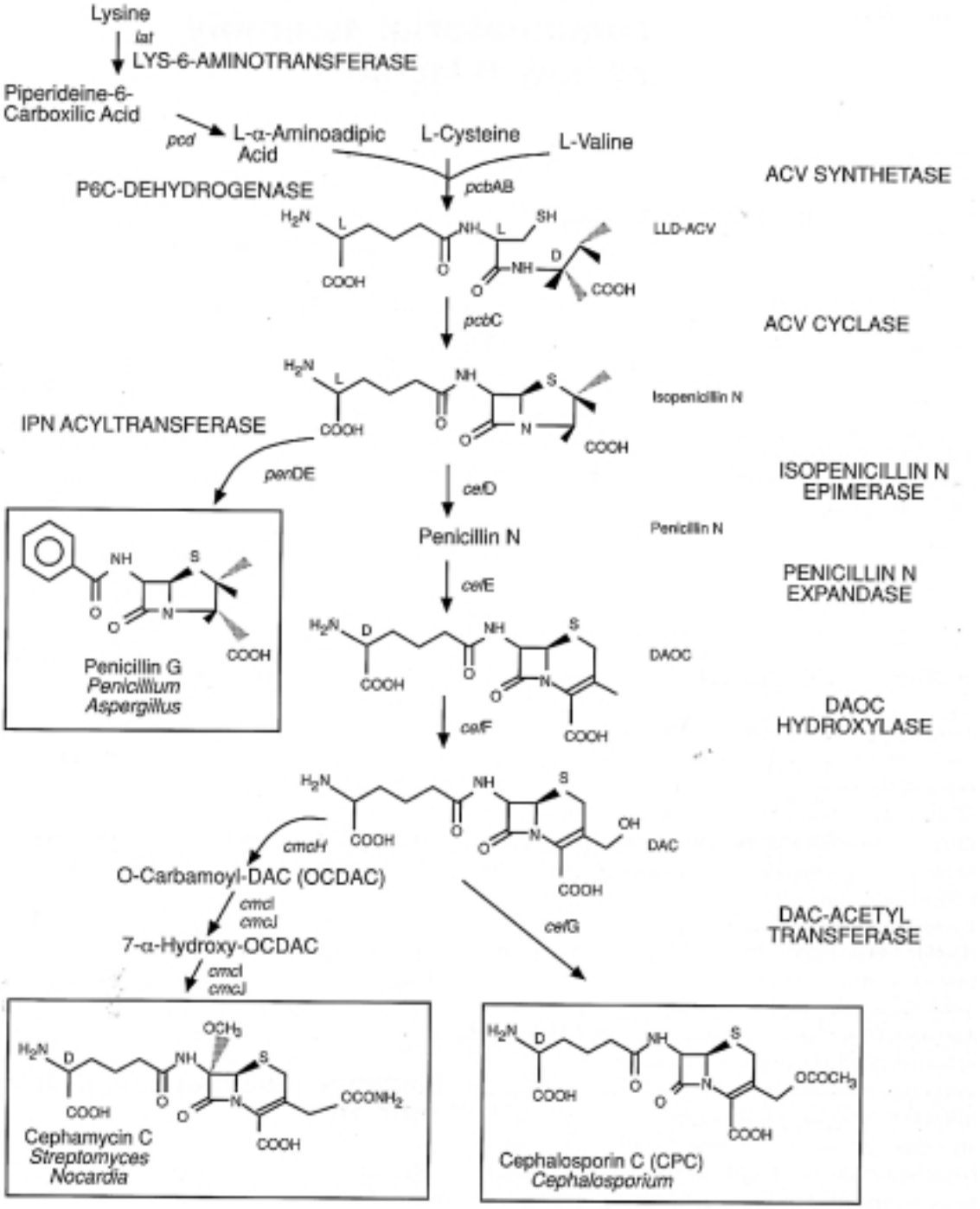

Fig. 1 Biosynthetic pathway for penicillin, cephalosporin and cephamycin. The enzymatic activities are indicated in capital letters and the genes involved in italics

The initial two steps of the pathway are common for penicillin, cephalosporin and cephamycin biosynthesis; in the first the multidomain enzyme α–aminoadipyl-cisteinyl-valine synthetase (ACVS) condensates the amino acids α–aminoadipic acid, cysteine and valine to form the tripeptide α–aminoadipyl-cysteinyl-valine (ACV). Then this peptide is cyclized by ACV cyclase to form the bioactive molecule of isopenicillin N. Penicillin producers interchange the α–aminoadipic lateral chain of isopenicillin N with phenylacetyl-CoA to give penicillin G (Fig. 1).

The pathway in cephalosporin and cephamycin producers continues after isopenicillin N to form the isomer penicillin N; then the five-membered thiazolidin ring is expanded by the penicillin N expandase (also named deacetoxycephalosporin C synthase) to the six-membered dihydrothiazinic ring in the deacetoxycephalosporin C (DAOC). The last intermediate common to cephalosporins and cephamycins is deacetyl-cephalosporin C (DAC), which is finally acetylated at 3' by *C. acremonium* to form cephalosporin C. In actinomycetes and Gram-negative bacteria, the deacetoxycephalosporin C is methoxylated at C-7 giving the characteristic 7-methoxy-cephem nucleus, common to all cephamycins, which can be further modified at C-3' to form different types of cephamycins and cephabacins. Therefore, the β–lactam pathway is longer in prokaryotes than in eukaryotic organisms, and probably, although it is not completely known, it is even larger in Gram-negative bacteria.

β-Lactams gene clusters organization

Genes for β–lactam antibiotic biosynthesis are clustered both in filamentous fungi and bacteria [2]. The organization common to all the β–lactam producers includes the genes *pcb*AB for ACV synthetase and *pcb*C for ACV cyclase (Fig. 2). In all producers of penicillin G an additional gene, *pen*DE, is located downstream of *pcb*C. Cephalosporin producers possess additional genes for the medium steps of the pathway leading from penicillin N to deacetoxycephalosporin C. The cluster is larger in actinomycetes, and Gram-negative bacteria include, genes for steps involved in the biosynthesis of the rare amino acid precursor α–aminoadipic acid, genes for the enzymes involved in late modifications of the cephamycin molecule (late genes), and additional genes for resistance, regulation and exportation of the antibiotic.

The organization of the clusters is specially well known in *S. clavuligerus* and *N. lactamdurans* (Fig. 2). In *N. lactamdurans*, the β–lactam cluster extends about 34 kb of contiguous DNA [6–10]. In *S. clavuligerus*, the cephamycin cluster [16, 17, 19] is associated to the cluster of the β–lactamase inhibitor clavulanic acid in a supercluster of at least 45–50 kb [24, 29] and both compounds are coordinately regulated.

As indicated previously, the **early genes** common to all producers are *pcb*AB and *pcb*C. In prokaryotes, formation of

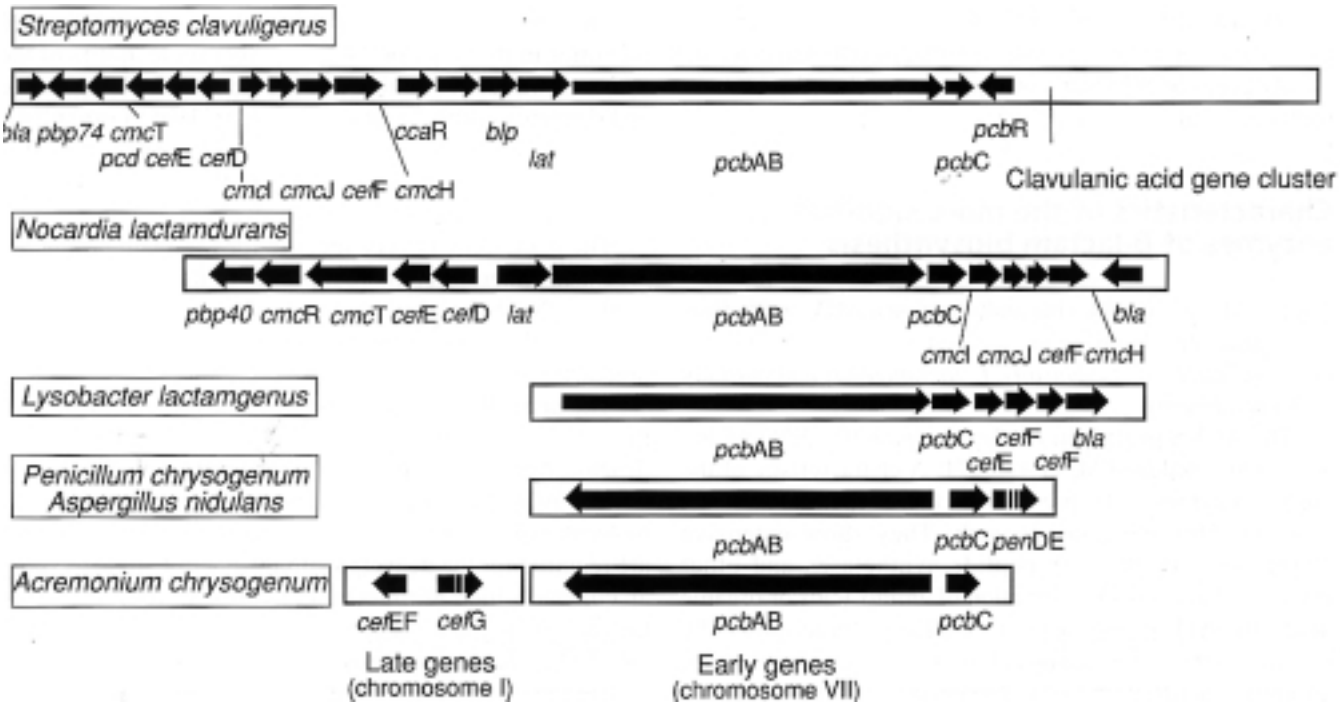

Fig. 2 Organization of the clusters of genes for the β-lactam antibiotic in prokaryotic (*Streptomyces clavuligerus, Nocardia lactamdurans, Lysobacter lactamgenus*) and eukaryotic (*Penicillium chrysogenum, Aspergillus nidulans, Acremonium chrysogenum*) organisms

the α–aminoadipic acid (α–AAA) precursor requires two enzymes: (i) lysine-6-aminotransferase, which forms α–aminoadipic acid semialdehyde (which is spontaneously cyclized to piperideine-6-carboxylic acid) from lysine [19] and (ii) piperideine-6-carboxylate dehydrogenase, which converts the semialdehyde into α–AAA [13]. Genes encoding both enzymes (*lat* and *pcd*) are present in the *S. clavuligerus* gene cluster, being the *lat* gene directly associated to the *pcb*AB gene [6, 19].The *lat* gene is not present in fungi, which obtain α–AAA as an intermediate in lysine biosynthesis.

Genes for the **medium steps** of the pathway (*cef*D, *cef*E) are located together and transcribed usually as an operon in actinomycetes and bacteria. In cephalosporin producing *C. acremonium*, the *cef*EF gene has been located linked to *cef*G (encoding the last step of *Cephalosporium* specific pathway [27]); it is present in a different chromosome (chromosome I in *C. acremonium* C10) than the genes for the early steps (present in chromosome VII).

Genes for **late steps** (*cmc*I, *cmc*J, *cef*F, *cmc*H), specific for cephamycin production, are clustered and show the same organization both in *S. clavuligerus* and *N. lactamdurans* [10, 21], but are not present in filamentous fungi. Little information about the genes for late steps is known in Gram-negative bacteria.

An interesting aspect of the clusters that may have evolutive relevance is the absence of introns in fungal genes. Even the long *pcb*AB gene (11.3 kb) lacks introns. Introns are present in genes for specific steps in the fungal biosynthetic pathways. Such is the case of the *pen*DE gene, encoding the acyltransferase involved in lateral chain interchange in the penicillin producers [4] and of *cef*G, involved in the acetylation at C-3' in *C. acremonium* [14]. This indicates that *pen*DE and *cef*G are clearly genes of eukaryotic origin, whereas the others have a bacterial origin.

Characteristics of the more significant enzymes of ß-lactam biosynthesis

The *pcb*AB gene encodes the multifunctional **ACV synthetase**. This gene has been sequenced in *P. chrysogenum*, *C. acremonium*, *N. lactamdurans*, *L. lactamgenus* and partially in *S. clavuligerus*.

The ACVS protein in *P. chrysogenum* has 3792 amino acids and a deduced M_r of 425,971. A characteristic of the ACVS enzymes is the presence of three repeated domains of about 500 amino acids each. They show extensive similarity with *Bacillus* peptide synthetases and other enzymes that carry out activation of amino acids or organic acids by ATP-pyrophosphate exchange reactions. The enzymes activate the amino acids α–AAA, cysteine and valine as amino acid adenylates, epimerize L-valine to the D configuration and polymerize the amino acids to give the LLD-ACV tripeptide, which is released from the enzyme by its own thioesterase activity [2]. In some aspects, the large peptide synthetases, which are known to be able to activate up to 11 amino acids (in the case of cyclosporin synthetase), resemble very primitive ribosomes that synthesize peptides without a mRNA template.

Comparison of the ACVS amino acid sequences shows that the enzyme of *L. lactamgenus* has a 54.5% identity with the enzyme of *N. lactamdurans*—over the whole sequence of the protein—and percentages of 41.3 to 42.3 identity with the enzymes of *C. acremonium*, *P. chrysogenum* and *A. nidulans*.

The microbial **ACV cyclases** form a family of closely related proteins. They cyclize rather specifically the ACV peptide using molecular oxygen, α–ketoglutarate and ascorbate as cofactors, belonging to a narrow group of related dioxygenases [21]. Genes from fungi (*P. chrysogenum*, *C. acremonium* and *A. nidulans*), Gram-positive actinomycetes (four different *Streptomyces* sp. and *N. lactamdurans*) and two Gram-negative bacteria (*L. lactamgenus* and *Flavobacterium* sp.) possess amino acid identities in the order of 57 to 77%, and aproximately 40% of the amino acid changes at other positions are conservative. The conservation in cyclases sequence is stricking. The sequence identity is found throughout the whole protein, making it difficult to identify functionally important domains for enzyme activity [2]. Recently the enzyme has been crystallized and the amino acids involved in the active center identified. Relatedness to other characterized proteins is low. Only some plant proteins such as giberellin 3-β-hydroxylase (an enzyme known to require also iron and ascorbate), 1-aminocyclopropane 1-carboxylate oxidase (an enzyme involved in the biosynthesis of ethylene) and flavonol synthases possess amino acid identities in the order of 24–27%. Also some motifs of the cyclases are related to those of the penicillin N expandase and deacetoxycephalosporin C hydroxylase in organisms producing cephalosporins and cephamycins, suggesting that all these enzymes recognize the β–lactam nucleus of their respective substrates.

The *pcb*C gene is very specific for β–lactam producing organisms; therefore, internal probes of this gene are being used in DNA hybridization screens of culture collections and natural isolates as a valuable approach to detect β–lactam producing organisms.

The **penicillin N expandase** converts the five-membered thiazolidine ring of penicillin N to a six membered dihydrothiazinic ring, forming deacetoxycephalosporin C. In common with the ACV cyclase the expandase is an iron, α–ketoglutarate, molecular oxygen dependent oxygenase. The same cofactors are required for the **deacetylcephalosporin C hydroxylase**, an enzyme which introduces an hydroxyl group at C-3' in the cepham ring of deacetoxycephalosporin C. The expandase and the hydroxylase proteins are very similar; they have been isolated and separately purified in prokaryotes such as *S. clavuligerus* [3] and *N. lactamdurans* [20]. However, this is not the case with the enzyme of *C. acremonium* , in which both

activities remained physically linked up to electrophoretic homogeneity of one unique purified protein [12]. Genes encoding the expandase (*cef*E) and hydroxylase (*cef*F) activities have been cloned and sequenced in *S. clavuligerus*, *N. lactamdurans* and *L. lactamgenus*. Both genes have large similarity (71% identity in nucleotides for the *S. clavuligerus* genes) reflecting the common cofactors and similar substrates of both enzymes, which share a 59% amino acid identity. Moreover, a low expandase activity (3%) has been detected in the DAOC hydroxylase, and the expandase has also been found to hydroxylate DAOC with low efficiency [3]. In the case of *C. acremonium*, a gene, *cef*EF, was cloned and sequenced [27]. The *cef*EF gene possesses a large nucleotide identity to both the *cef*E (57%) and the *cef*F (54%) genes of *S. clavuligerus* and other prokaryotes, and it confers expandase and hydroxylase activities to transformants of *E. coli*. Initial kinetic studies indicate the presence of a common active site for both expandase and hydroxylase activities in fungial. This indicates that the three different enzymes represent evolutionary products from a common ancestral gene. The gene may have duplicated in prokaryotes (after horizontal transfer to fungi) and one copy might have evolved to specialize in expandase activity while the second copy specialized in hydroxylase activity [3].

Evolution of biosynthetic genes

When cyclases from Gram-positive, Gram-negative and eukaryotes are compared the percentage of amino acid identity is in the order of 70–75% within the actinomycetes, and decreases to 60% in relation to the cyclases of Gram-negative bacteria and to 57–59% when compared with the eukaryotic cyclases from filamentous fungi. The percentages of identical amino acid are similar when ACV synthetases, epimerases or expandases are compared (Table 1). A comparison of enzymes for primary metabolism, such as threonine synthase, aspartic semialdehyde dehydrogenase, homoserine synthase, or of enzymes for the arginine biosynthesis (see Table 1) was made between actinomycetes (*N. lactamdurans*, *S. clavuligerus*, *Mycobacterium tuberculosis*), a model Gram-positive bacteria (*B. subtilis*), a model Gram-negative bacteria (*E. coli*) and a model eukaryotic microorganism (*S. cerevisiae*). Surprisingly, the percentages of amino acid identity reveal much larger differences for the primary metabolism enzymes than for β–lactam biosynthesis enzymes. Actinomycetes proteins show about 60% identity to homologus proteins of other actinomycetes (*M. tuberculosis*), 30–50% identity to the protein of *B. subtilis*, 27–40% identity with those of *E. coli* and 24–35% identity with the homologous proteins of *S. cerevisiae*.

Two plausible explanations have been advanced in relation to these results [2, 5, 18]. The similarity might result from a slow and constant rate of change in the genes, reflecting perhaps some essential function or selective advantage for survival, none of which has any experimental support yet. Alternatively, the high similarity may have resulted from a horizontal gene-transfer, probably from the bacterial lineage to the fungal lineage after the prokaryote-eukaryote split. Based on the divergence of the cyclases sequence, Weigel et al. [30] estimated that the gene transfer event took place about 370 million years ago, well after the proposed time of the

Table 1 Percentage of identical amino acids in enzymes for primary metabolism and for β-lactams biosynthesis in different organisms*

A	Enzymes for β-lactam biosynthesis	Actinomycetes					Gram-negative		Eukaryotes		
		1	2	3	4	5	6	7	8	9	10
	ACVS	100	70.5	–	–	–	54.5	–	44.3	43.6	43.8
	Cyclase	100	75.2	77.0	72.6	71.5	59.7	59.7	57.2	59.6	57.6
	Epimerase	100	63.2	–	–	–	52.4	–	–	–	–
	Expandase	100	70.1	–	–	–	57.2	–	–	–	60.1
	Hydroxylase	100	77.7	–	–	–	55.5	–	–	–	56.0

B	Primary metabolism enzymes	Actinomycetes			Gram-positive	Gram-negative	Eukaryotes	
		1	2	11	12	13	14	15
	ThrC	100	–	67.0	52.0	28.0	–	–
	Asd	100	–	45.7	37.2	33.7	–	–
	Hom	100	–	62.0	42.0	25.3	23.8	–
	ArgC	–	100	61.0	37.0	41.0	32.0	33.3
	ArgD	–	100	59.2	33.3	42.4	27.0	–
	ArgG	–	100	67.5	52.3	27.0	35.5	–

*Percentages of amino acids identity of (A) Enzimes for β-lactam biosynthesis: (1) *N. lactamdurans*, (2) *S. clavuligerus*, (3) *S. jumonjinensis*, (4) *S. griseus*, (5) *S. lipmanii*, (6) *L. lactamgenus*, (7) *Flavobacterium* sp., (8) *P. chrysogenum*, (9) *A. nidulans*, and (10) *C. acremonium*. (B) Enzymes of primary metabolism: (1) *N. lactamdurans* (2) *S. clavuligerus* (11) *Mycobacterium tuberculosis* (12) *Bacillus subtilis* (13) *Escherichia coli* (14) *Saccharomyces cerevisiae* and (15) *Neurospora crassa*. The proteins compared are: threonine synthase (ThrC), aspartic semialdehyde dehydrogenase (Asd), homoserine synthase (Hom), N-acetylglutamylphosphate reductase (ArgC), arginine succinate synthetase (ArgG) and ornithine acetyl transferase (ArgJ).

prokaryote-eukaryote split (about 2000 million years ago), although other authors differ in the time of the transfer event [5] (Fig. 3). Additional evidence for the horizontal transfer is the narrow distribution in nature of β–lactam-producing species, most of them soil organisms, the presence of β–lactam gene clusters in fungal species (a rare event in fungal genes) and the lack of introns in the fungal genes *pcb*C, *pcb*AB, *cef*E/F common to all cephalosporin producers. The larger size of the bacterial clusters point to the bacterial origin of the transfer event that may have resulted in truncated clusters in the filamentous fungi. Probably the transfer event took place more than once. This may explain the presence of genes in two different fungal chromosomes (early and medium step genes) in *C. acremonium*. The transfers may also have been followed by a reorganization, which explains the different transcriptional directions of the *pcb*AB and the *pcb*C genes in prokaryotes and eukaryotes. Finally, the content of G+C in fungal β–lactam antibiotics genes is somewhat higher than in the overall genome, suggesting that they might proceed from the high G+C (70%) containing actinomycetes.

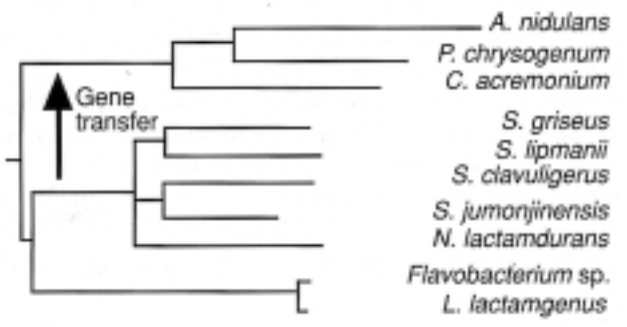

Fig. 3 Horizontal transfer of β-lactam genes, based in DNA sequences identities (Modified from [30])

Additional genes in actinomycete cluster

The search in the cephamycin C cluster of actinomycetes reveals a great complexity which is really striking in *S. clavuligerus*. The presence of resistance genes in antibiotic biosynthesis clusters is a well- known fact [11]. They protect the producing organisms from the deletereous effect of the antibiotic they are producing. In fact, *S. clavuligerus* or *N. lactamdurans* are remarkably resistant to β-lactams (MICs in the order of 200 mg/ml for penicillin G and 900 mg/ml for cephalosporin C in solid cultures). Genes probably involved in the export of cephamycin C (*cmc*T) have been found in the cephamycin C gene clusters of *N. lactamdurans* and *S. clavuligerus*. The homologous genes present in other antibiotic gene clusters are responsible in different degrees for the resistance to the antibiotic produced, but since cephamycin antibiotics have their targets located in the outer envelope of the cell, the *cmc*T gene is not expected to play a major role in resistance. Probably of most interest for resistance to cephamycin are the penicillin-binding proteins (PBPs). There are two genes per PBP in the *S. clavuligerus* gene cluster, they are called *pcb*R and *pbp*74 [24]. Both of them encode high molecular PBPs, and in the case of *pcb*R, it is known that the disruption of this gene leads to an increase in MIC for penicillin G and cephalotin. The PBP encoded by *N. lactamdurans pbp*40 (previously named *pbp*) is known to have much more affinity for penicillin G than for cephamycin C [9].

Classical resistance to β–lactam antibiotics in microorganisms is provided by β–lactamases. However, the presence of genes for β–lactamases seems contradictory to the production of β–lactam antibiotics which are irreversibly inactivated by these enzymes. In spite of this, genes for β–lactamases have been found in the cephamycin clusters of *S. clavuligerus*, *N. lactamdurans* and *L. lactamgenus* [9, 15, 26]. The presence of a β–lactamase gene is specially striking in *S. clavuligerus*, a strain that produces the β–lactamase inhibitor clavulanic acid and two β–lactamases inhibitory proteins (BLIP and BLP) encoded by the genes *blip*, of unknown location, and *blp*, which is present in the cephamycin gene cluster [25].

The enzymes of both actinomycetes are classical type A β–lactamases with the characteristic consensus sequences ^{70}STFK, ^{130}SDG, ^{166}EPELN and ^{234}KTG. The enzyme of *S. clavuligerus* has been well studied [26]. It is active against penicillin G (K_m, 11 μM), sensible to clavulanic acid and BLIP, it shows a low PBP activity and is surprisingly active against cephoxitin (a semi-synthetic cephamycin) but not against cephamycin C. β–Lactamase activity is not detected in *S. clavuligerus* cultures although the gene is continuously expressed in the cells, as measured by Northern analysis. Disruption of the *bla* gene does not affect the MIC for penicillin G, but it modifies the onset of sporulation in solid cultures and slightly affects cell morphology. A phylogenetic analysis of the β–lactamases of *S. clavuligerus* and *N. lactamdurans* indicates that they are clustered with the homologous proteins of *Streptomyces badius*, *Actinomadura* R39 and the two β–lactamases described in *Streptomyces cacaoi* strains [26]. However, this does not mean that all β–lactamase containing actinomycetes produce β–lactam antibiotics as shown by the lack of hybridization with the *pcb*C gene encoding the ACV cyclase.

The β–lactamase gene of *Lysobacter lactamgenus* encodes a 41,876 Da protein similar to *amp*C cephalosporinases and cephamycinases. The gene restores the cephalosporin resistance to an *E. coli amp*C negative mutant. The class A β–lactamases *S. clavuligerus* and *N. lactamdurans* have low identity in amino acids with the homologous enzyme of *L. lactamgenus*. A phylogenetic tree of the *Lysobacter* β–lactamase and other cephalosporinases and cephamycinases reveals that the protein of *Lysobacter* is located between the group of FOX

cephamycinases and the group containing CMY-2, CMY-3, Bil-1 and the *amp*C of *C. freundii* (Fig. 4), far from the *amp*C group of β–lactamases, which is poorly active against cephamycins or the class A β–lactamases of *N. lactamdurans* and *S. clavuligerus*.

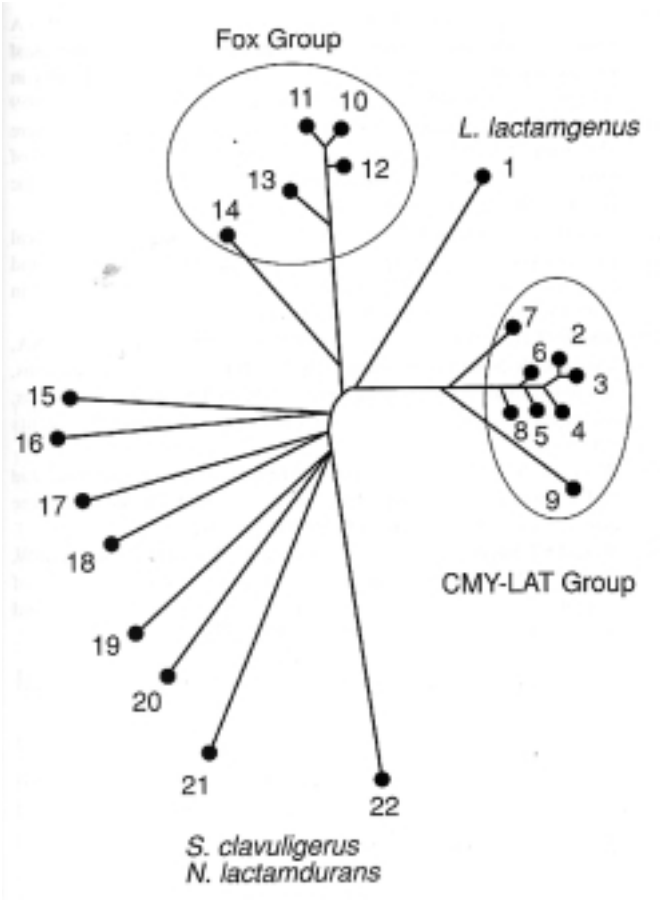

Fig. 4 Unrooted phylogenetic tree of 20 class C (numbers 1–20) β-lactamases and the class A β-lactamases of *N. lactamdurans* (21) and *S. clavuligerus* (22). The dendogram was calculated with the Clustal V and the neighbour-method. The numbers correspond to the following β-lactamases: (1) *Lysobacter lactamgenus* (X56660), (2) LAT-2 (X97039), (3) *Salmonella senftemberg* (U77414), (4) CMY-2 (X91840), (5) CMY-3 *Proteus mirabilis* (Y15130), (6) BIL-1 (X74512), (7) *amp*C of *C. freundii* (X03866), (8) *amp*C of *E. cloacae* (X07274), (9) *E. cloacae* (D44479), (10) FOX-2 (Y10282), (11) FOX (X77455), (12) FOX-3 (Y11068), (13) CEPS of *A. sobria* (X80277), (14) *K. pneumoniae* (U58492), (15) MOX-1 (D13304), (16) *K. pneumoniae* (X92508), (17) *Psychrobacter immobilis* (X83586), (18) *amp*C of *S. marcescens* (AB08454), (19) *amp*C of *E. coli* (J01611), (20) *amp*C of *P. aeruginosa* (X54719), (21) *N. lactamdurans* (X57310), and (22) *S. clavuligerus* (Z54190)

The presence of all these genes for resistance in the cephamycin biosynthesis clusters and the well conserved organization of large DNA fragments for β–lactam antibiotic biosynthesis in prokaryotes (30–50 kb) point out the fact that these compounds probably had a different and essential role at some moment in the evolution of microorganisms present in the soil. The existence of toxins with β–lactam structure in plant pathogenic bacteria may be an example of this role. The β–lactamase genes, which confer a selective advantage for prokaryotes, were promiscuously transferred to other bacterial strains. Eukaryotes acquired a selective advantage when they received β–lactam biosynthesis genes, but interestingly they did not require the transfer of resistance genes, being naturally resistant to β–lactams.

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