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Gene clusters for β -lactam antibiotics and control of their expression: why have clusters evolved, and from where did they originate?

Summary. While β -lactam compounds were discovered in filamentous fungi, actinomycetes and gram-negative bacteria are also known to produce different types of β -lactams. All β -lactam compounds contain a four-membered β -lactam ring. The structure of their second ring allows these compounds to be classified into penicillins, cephalosporins, clavams, carbapenems or monobactams. Most β -lactams inhibit bacterial cell wall biosynthesis but others behave as β -lactamase inhibitors (e.g., clavulanic acid) and even as antifungal agents (e.g., some clavams). Due to the nature of the second ring in β -lactam molecules, the precursors and biosynthetic pathways of clavams, carbapenems and monobactams differ from those of penicillins and cephalosporins. These last two groups, including cephamycins and cephabacins, are formed from three precursor amino acids that are linked into the α -aminoadipyl-L-cysteiny-D-valine tripeptide. The first two steps of their biosynthetic pathways are common. The intermediates of these pathways, the characteristics of the enzymes involved, the lack of introns in the genes and bioinformatic analysis suggest that all of them should have evolved from an ancestral gene cluster of bacterial origin, which was surely transferred horizontally in the soil from producer to non-producer microorganisms. The receptor strains acquired fragments of the original bacterial cluster and occasionally inserted new genes into the clusters, which once modified, acquired new functions and gave rise to the final compounds that we know. When the order of genes in the *Streptomyces* genome is analyzed, the antibiotic gene clusters are highlighted as gene islands in the genome. Nonetheless, the assemblage of the ancestral β -lactam gene cluster remains a matter of speculation. [*Int Microbiol* 2006; 9(1):9-19]

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Classical and novel β -lactam families

The β -lactam antibiotics, like many other secondary metabolites, have highly unusual chemical structures, very different from those of classical primary metabolites [31]. Understanding the biosynthesis and the molecular genetics of these compounds has been a challenge for microbiologists over the last few decades, and only a few of the impressive array of secondary metabolites is known at the molecular level. A comprehensive study of these compounds will surely require many more decades of research.

The structure of the β -lactam antibiotic penicillin consists of a bicyclic penam nucleus formed by a β -lactam ring and a

thiazolidine ring containing a sulfur atom and an acyl side-chain bound to the amino group present at C-6. The two rings are produced by *Penicillium* and *Aspergillus* species, as well as a few other ascomycetes [24]. A second compound with β -lactam structure, produced by *Acremonium chrysogenum*, was discovered in 1955 [38] and chemically characterized as cephalosporin C, a molecule in which the five-membered thiazolidine ring of penicillin is replaced by a six membered dihydrothiazine ring forming the cephem nucleus (Fig. 1). Cephalosporin C has an α -aminoadipyl side-chain attached at the C-7 amino group, which is identical to that of penicillin N but different from those of other penicillins. Both β -lactam compounds, penicillin and cephalosporin C, are of

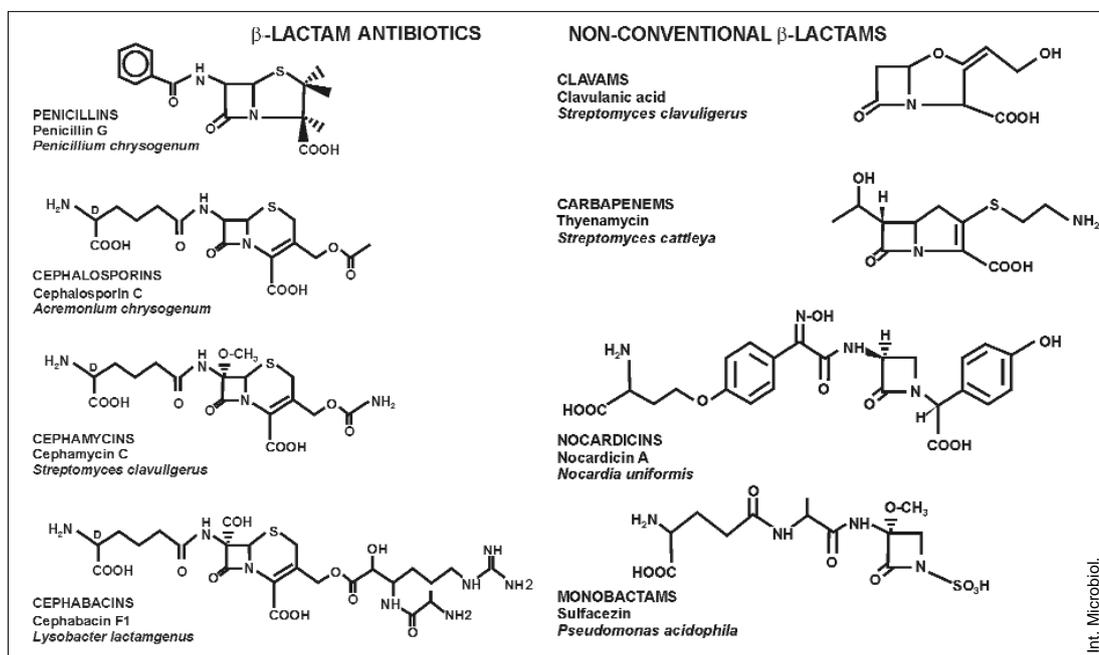


Fig. 1. Structures of classical β -lactam antibiotics (left) and novel non-conventional lactams (right). The generic names of the families, specific names of the structures, and producer microorganisms are indicated.

great clinical interest as inhibitors of peptidoglycan biosynthesis in bacteria.

Over the last three decades, a wide array of compounds belonging to the same family of molecules has been discovered using new targets and screening techniques. Some of these compounds are modified cephalosporins, such as the cephamycin family, in which the cephem nucleus contains, in addition to the α -aminoadipyl side-chain, a methoxyl group at C-7. This methoxyl group renders the cephamycin structure insensitive to hydrolysis by most β -lactamases. Cephamycins are produced by different actinomycetes species. In other β -lactams, as in the family of cephabacins, produced by gram-negative bacteria, a formyl group is frequently present at C-7, and different peptides are attached to the C-3 carbon of the dihydrothiazine ring in the cephem nucleus. All the above-mentioned groups, produced either by filamentous fungi or by bacteria, have a common mode of action, similar precursors, and partially overlapping biosynthetic pathways [1]. In addition to these classical β -lactam compounds, many non-conventional β -lactam structures have been discovered and characterized since 1970. Some of them, including carbapenems and nocardicins, also inhibit peptidoglycan biosynthesis. Others, such as clavulanic acid, are weak antibiotics but potent β -lactamase inhibitors, or have antifungal activity (i.e., some clavams). These non-conventional β -lactams contain a β -lactam ring and they usually have a distinct bicyclic structure. The second ring in the molecule of clavulanic acid and other clavams is an oxazolidinonic ring that includes oxygen instead of a sulfur atom, as occurs in classical β -lactams (Fig. 1). The members of carbapenem and the olivanic acid family have a carbapenem ring containing a carbon atom instead of sulfur.

Thienamycin is the model structure of this family. Finally, there are many compounds with monocyclic structure that contain only the β -lactam ring and different side-chains (they are known as monobactams). Some monobactams, as is the case of the nocardicins, are produced by actinomycetes [2], but others, such as sulfacezin, are produced by proteobacteria [19].

Non-conventional β -lactam antibiotics are molecules of scientific and industrial interest, and their biochemistry and molecular genetics are being actively investigated by several groups [10,30,36,39,45,49]. The precursors and biosynthetic pathways of non-conventional β -lactam antibiotics are different from those of classical β -lactams. The enzymes, genes, and gene organization are also different [17]. For example, the specific enzyme involved in the formation of the β -lactam ring in non-conventional β -lactams is not a dioxygenase, as occurs in the classical β -lactam antibiotics, but more closely resembles asparaginases or asparagine synthetases [3], suggesting that those compounds should have a different evolutionary origin. Up to now, non-conventional β -lactam antibiotics have never been found to be produced by eukaryotic cells. The genetic information encoding enzymes for the biosynthesis of the different β -lactam families is restricted to a few taxonomic groups [1]. This raises the question of the evolutionary origin of these genes (see below).

Penicillin, cephalosporin and cephamycin biosynthesis pathways

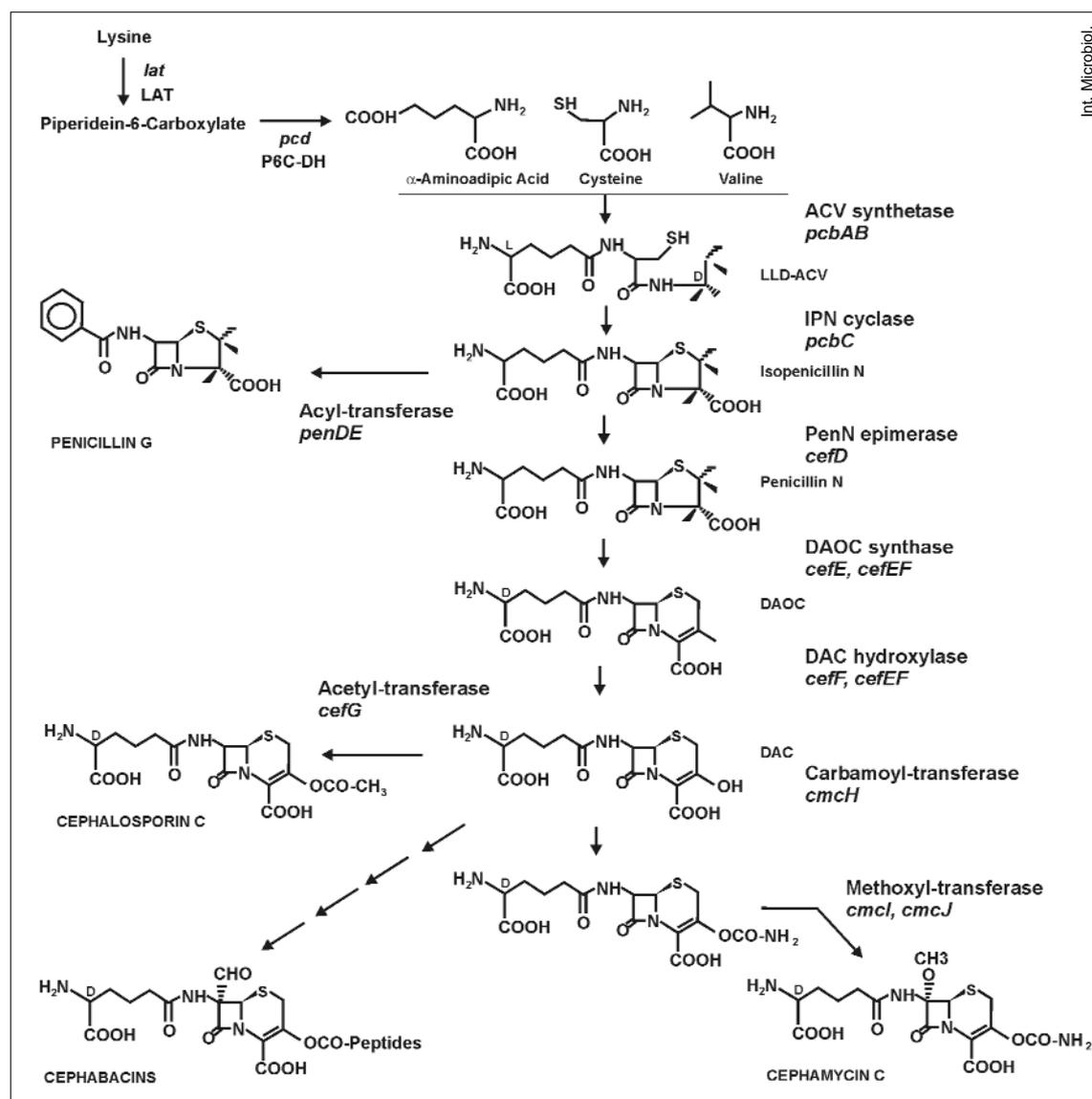
A brief description of the biochemical pathways leading to classical β -lactam antibiotic biosynthesis is provided here. Several

previous reviews have described the specific steps in greater detail [27,29,32]. The formation of β-lactam compounds proceeds through “early biosynthetic steps”, “intermediate steps”, “late (‘decorating’) steps,” and reactions for the formation of the specific precursors. Depending on the compound formed in the pathway, more or fewer steps will be required—penicillin biosynthesis being the shortest pathway, and cephamycin and cephabacins pathways being the longest ones.

Precursors. Three aminoacids, L-α-aminoadipic acid, L-cysteine, and L-valine are always the precursors of the basic structure of all classical β-lactam antibiotics (Fig. 2). Of these three precursor amino acids, L-valine and L-cysteine are common, whereas L-α-aminoadipic acid is a non-proteinogenic amino acid that must be synthesized by a specific pathway. In fungi, α-aminoadipic acid is an intermediate of

the lysine biosynthesis pathway [4]. In addition, lysine is catabolized to α-aminoadipic acid in *Penicillium chrysogenum* by: (i) an ω-aminotransferase, encoded by the *oat1* gene, which is induced by lysine, and (ii) by a reversal of the lysine biosynthesis pathway catalyzed by the enzymes saccharopine dehydrogenase/saccharopine reductase [13,35]. Gene *oat1* is present in neither penicillin nor cephalosporin gene clusters [37], and only one gene encoding a saccharopine dehydrogenase, potentially involved in the α-aminoadipic acid biosynthesis, has been located in the amplified region containing the penicillin biosynthesis cluster in *P. chrysogenum*.

In β-lactam-producing actinomycetes, lysine is converted into α-aminoadipic acid semialdehyde by lysine-6-amino-transferase (LAT) [5,54]. This enzyme and its encoding gene (*lat*) are only found in β-lactam-producing microorganisms, and the presence of this enzyme activity or positive hybridi-



Int. Microbiol.

Fig. 2. Biosynthetic pathway of penicillins, cephalosporins, cephamycins, and cephabacins.

zation with the *lat* gene are good indicators for detecting novel β -lactam producers. The LAT-reaction product, α -aminoadipic semialdehyde, cyclizes spontaneously to form piperidine-6-carboxylate (PCD) [16] and later is oxidized to α -aminoadipic acid by a piperidine-6-carboxylate dehydrogenase, encoded by the *pcd* gene [43]. Both *lat* and *pcd* are located in the cephamycin gene cluster in *Streptomyces clavuligerus*.

Early steps in the formation of β -lactam antibiotics. Two enzymatic steps are common to all β -lactam producers and result in the formation of isopenicillin N, the first compound in the pathway with antibiotic activity. The first enzyme of the pathway is δ -(L- α -aminoadipyl)-L-cysteiny-D-valine (ACV) synthetase (ACVS), a non-ribosomal peptide synthetase [31]. ACV synthetases are very large multifunctional proteins (about 460 kDa) encoded by 11-kb intron-free genes named *pcbAB*. This enzyme uses ATP to sequentially activate the three amino-acid substrates to form aminoacyl-adenylates, then binds them to the enzyme as thioesters, epimerizes the L-valine to the D-valine configuration, and finally links the three amino acids to form the peptide L- δ -(α -aminoadipyl)-L-cysteiny-D-valine. The peptide is released from the enzyme by the action of an internal thioesterase, another of the activities of the ACVS complex. ACVSs have three well-conserved domains, specific to activate each amino acid [31]. Several research groups are attempting to construct hybrid *pcbAB* genes containing genetic domains from other peptide synthetases in order to obtain modified β -lactam antibiotics.

The second enzyme in the pathway is isopenicillin N (IPN) synthase (also named ACV cyclase), encoded by the *pcbC* gene. IPN synthases are intermolecular dioxygenases that require Fe^{2+} , molecular oxygen, and ascorbate for their activities. These enzymes remove four hydrogens from the ACV tripeptide, forming directly the bicyclic structure of isopenicillin N. The cyclase of *P. chrysogenum* has been crystallized [46], and knowledge of the enzyme's protein structure has been an important step in obtaining basic information to produce, by mutagenesis, modified cyclases able to recognize other peptides. The process of direct formation of the bicyclic structure of isopenicillin N differs from that of the other non-conventional β -lactam antibiotics, which first form the β -lactam ring and then, using a different enzyme, cyclize the monocyclic intermediate to form the second ring (oxazolidinic or carbapenem) present in the molecule, although it is probable that the two steps are coupled *in vivo*. Monobactam producers lack the ability to close the second ring of the nucleus.

In addition to the *pcbAB* and *pcbC* genes common to bacteria and filamentous fungi, penicillin producers (i.e., *Penicillium*, *Aspergillus*) contain a third gene in the penicillin

gene cluster, *penDE*. This gene is of eukaryotic origin (it contains three introns) and encodes an isopenicillin N acyltransferase, an enzyme that hydrolyzes the α -aminoadipic side-chain of the isopenicillin N and introduces instead a chain from an acyl activated compound, most frequently phenylacetyl-CoA to produce penicillin G. This gene is not present in either cephalosporin-C or cephamycin-producing microorganisms.

Intermediate steps. Isopenicillin N is converted into the D-isomer, penicillin N, by all cephalosporin and cephamycin producers. This conversion is carried out in a single step in bacterial strains. The enzyme responsible for this epimerization was first purified from actinomycetes [26,56], and the gene encoding its activity, *cefD*, was found to be located in the cephamycin gene cluster [6,23]. The bacterial *cefD*-encoded protein is a pyridoxal phosphate-dependent enzyme of about 43 kDa. However, attempts to find a homologous *cefD* gene and a clear epimerase activity in *A. chrysogenum* failed for many years. Purification of the *A. chrysogenum* "epimerase" proved to be difficult, non-reproducible, and unreliable. In 2002, a significant breakthrough in our understanding of cephalosporin formation occurred when we found that the epimerization reaction was different in eukaryotic and prokaryotic microorganisms (Fig. 3). The epimerization of isopenicillin N in *A. chrysogenum* is encoded by two linked genes, *cefD1-cefD2*, located in the early cephalosporin gene cluster. Transcriptional studies on *A. chrysogenum* revealed the presence of two transcripts in the region downstream of *pcbC* [55]. Sequencing of the region confirmed the presence of two open reading frames (ORF) separated by a bidirectional promoter region. The first, *cefD1*, has five introns and encodes a 71-kDa protein with homology to fatty acid acyl-CoA synthetases. The second, *cefD2*, has one intron and encodes a protein with high homology to α -methylCoA racemases of eukaryotic origin. Disruption of either of these ORFs results in lack of cephalosporin C production, loss of isopenicillin N epimerase activity, and the accumulation of isopenicillin N in the culture. The proposed epimerization in fungi includes three biochemical steps: (i) CefD1 converts isopenicillin N into isopenicillinyl N-CoA; (ii) CefD2 isomerizes the compound into penicillinyl N-CoA (Fig. 3); and (iii) penicillinyl N-CoA is probably released from the enzyme by a thioesterase. Indeed, ACVS contains a thioesterase domain, although it is unclear whether it works on the penicillinyl N-CoA. Another gene encoding a separate thioesterase has been located 1 kb downstream of *cefG*, in the late cephalosporin gene cluster, but it has not yet been characterized.

The next step in the cephalosporin/cephamycin pathway is expansion of the five-membered thiazolidine ring of penicillin N to a six-membered dihydrothiazine ring. The enzyme responsible for this important conversion is the deacetoxy-

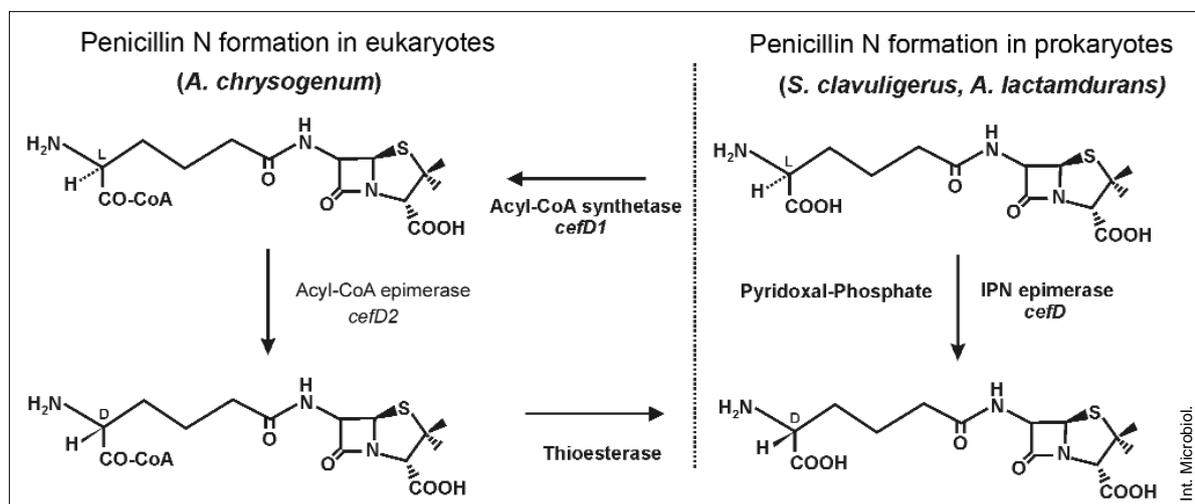


Fig. 3. Epimerization of the isopenicillin N to penicillin N in eukaryotic (left) and prokaryotic (right) cells.

cephalosporin C (DAOC) synthase commonly known as expandase. This protein is an intermolecular dioxygenase very similar to ACV cyclase. It requires Fe^{2+} , molecular oxygen, and α -ketoglutarate to form DAOC and succinic acid. The expandase does not recognize the isomer isopenicillin N, penicillin G, or deacylated 6-aminopenicillanic acid (6-APA) as substrates. Expandase from *S. clavuligerus* has been crystallized, and the gene *cefE* was introduced in *Penicillium chrysogenum*, leading to the biosynthesis of adipyl-7-aminodeacetoxycephalosporanic acid (adipyl-7-ADCA) and adipyl-7-ACA, compounds that can be transformed into the economically relevant 7-ADCA and 7-ACA intermediates (reviewed in [11]).

Interestingly, the expandase from *Acremonium chrysogenum* is able to catalyze also the next step of the pathway, i.e., the hydroxylation at C-3 that produces deacetylcephalosporin C (DAC). However, in cephamycin- and cephabacin-producing organisms, two different genes, *cefE* and *cefF*, encode enzymes that carry out these two sequential steps. Genes *cefE* and *cefF* encode proteins with about 70% identity in amino acids, and which are 60% identical to the protein encoded by *cefEF* in fungi. It is likely that a gene duplication event served as the origin of the two genes, which subsequently became specialized in their different functions (expandase and hydroxylase). In fact, the two enzymes have related molecular mechanisms, although each has retained approximately 10% of the residual activity of the other one. This is a very interesting example of enzyme "specialization" to perform different, although mechanistically related, reactions.

Late steps. The final step in cephalosporin C biosynthesis is the conversion of DAC to cephalosporin C by a DAC-acetyltransferase that uses acetyl-CoA as donor of the acetyl group. This 49-kDa enzyme, encoded by the *cefG* gene [18], is evolu-

tionarily similar to O-acetyl homoserine acetyl transferases. The *cefG* gene contains two introns and is linked to *cefEF*, but in the opposite orientation. Bioinformatic analysis of *cefG* revealed 55% identical residues with the *met2* genes of *Aspergillus fumigatus* and *Ascobolus immersus*. The *met2* genes encode O-acetyl homoserine acetyl transferase activity involved in sulfur-containing amino-acid biosynthesis. However, mutations disrupting *cefG* do not lead to methionine auxotrophy, which suggests that there is a separate *met2* in *Acremonium chrysogenum*. A detailed phylogenetic analysis of the CefG protein revealed that it is, indeed, a member of the serine/homoserine acetyl transferase class. However, it seems to be a specialized enzyme, different from that of primary metabolism, that has evolved to form part of the "late" cephalosporin gene cluster. The weak nature of the *cefG* promoter causes a bottleneck of this enzymatic step in the pathway. Replacing the *cefG* promoter by the glyceraldehyde-3-phosphate dehydrogenase promoter (*gpd*) of *Aspergillus nidulans* or the glutamate dehydrogenase (*gdh*) promoter of *P. chrysogenum* improved cephalosporin C production by two- to three-fold.

In cephamycin-producing actinomycetes, two sequential enzymatic reactions—an hydroxylation and the transfer of a methyl group to the hydroxyl present at C-7—are carried out by a CmcI-CmcJ complex. This complex co-purifies by immunoaffinity chromatography and binds S-adenosylmethionine and deacetylcephalosporin C, in contrast to the isolated CmcI and CmcJ purified proteins, which do not have binding activity [8,12]. In addition, the product of the *cmcH* gene carries out a carbamoyl transfer reaction [9]. The final steps of cephabacin biosynthesis have been deduced from the presence of several genes in the cephabacin gene cluster encoding non-ribosomal peptide synthetases. Their protein products are probably involved in the formation of the lateral chain in C-3 [21,50].

Gene clusters for β -lactam antibiotic biosynthesis: possible transmission by horizontal transfer

Genes for β -lactam biosynthesis are clustered in all producer strains, whether they are eukaryotic or prokaryotic microorganisms (Fig. 4). With minor differences between strains, the *pcbC-pcbAB* genes are always grouped and are located next to the *penDE* gene in penicillin producers. In *A. chrysogenum*, the “early” gene cluster, located in chromosome VII (4.6 Mb), contains the genes *pcbAB* and *pcbC*, encoding the enzymes for the first two steps of the pathway; *cefD1* and *cefD2*, responsible for the epimerization of isopenicillin N; and *cefT*, which encodes a transmembrane protein needed for putative exportation of the antibiotic. The “late” gene cluster, located in chromosome I (2.2 Mb), contains the genes *cefEF* and *cefG*, whose protein products are involved in the final steps of cephalosporin biosynthesis. A similar gene organization, *pcbAB-pcbC-cefD2*, is found in *Kallichroma tethys*, a wood-inhabiting marine fungus phylogenetically related to *A. chrysogenum* [20,33].

The largest β -lactam clusters are those of cephamycin- and cephabacin-producing bacteria [28]. In *S. clavuligerus*, the cephamycin C gene cluster is adjacent to the clavulanic acid gene cluster. The entire supercluster of cephamycin C-clavulanic acid extends for about 50 kb. This organization of the biosynthetic genes of both antibiotics in a supercluster occurs also in other clavulanic-acid-producing strains, including *Streptomyces jumonjinensis* and *Streptomyces katsurahamus*.

Analysis of the distribution of the amino-acid sequence of the enzymes involved in β -lactam biosynthesis (ACV syn-

thetase, IPN synthase, CefE, CefF) in bacteria reveals that their amino-acid identities range from 70 to 75%, while their identity to homologous proteins in β -lactam-producing eukaryotes ranges from 57 to 60%. These percentages of identity between prokaryotic and eukaryotic homologous proteins are higher than those of primary metabolism enzymes, including enzymes for amino-acid biosynthesis. This finding led to the proposal that either horizontal gene transfer took place in the soil about 370 million years ago [57] or multiple gene transfer events occurred from bacteria to fungi [1], conferring upon the latter the ability to produce β -lactam antibiotics to several genera of fungi. This theory is also supported by the lack of introns in *pcbAB* (despite its large size, 11.5 kb), *pcbC*, and *cefEF*, while specific genes absent in bacterial β -lactam clusters but present in penicillin and cephalosporin biosynthesis clusters in *Penicillium* and *Acremonium*, such as *penDE*, *cefG*, *cefD1*, and *cefD2*, contain introns.

Ecological advantages of β -lactam gene clustering: antibiotic resistance genes in β -lactam clusters

The presence of genes for β -lactam antibiotics, and therefore the ability to produce these compounds, confer a major ecological advantage to soil fungi, which are thus able to protect nutrient sources that would otherwise be used by fast-growing bacteria. In prokaryotes, the advantage of producing β -lactams is less clear. Since most bacteria are sensitive to β -lactams, producer bacteria might therefore be killed by their own antibiotics; however, β -lactam-producing bacteria are somehow less sensitive to β -lactams. Frequently, β -lactam

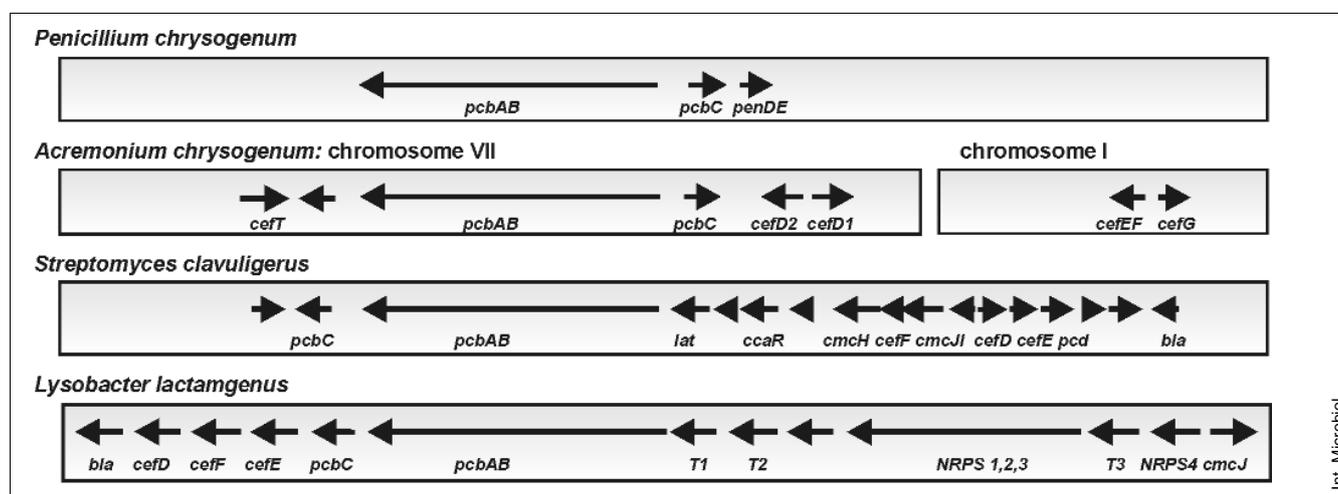


Fig. 4. Cluster of genes for β -lactam biosynthesis in the fungi *Penicillium chrysogenum* and *Acremonium chrysogenum*, the actinomycete *Streptomyces clavuligerus*, and the gram-negative bacterium *Lysobacter lactamgenus*. Note that *pcbAB* and *pcbC* have different orientations in prokaryotic and eukaryotic producers. The NRPS1 to NRPS4 genes present in the *L. lactamgenus* cluster encode non-ribosomal peptide synthetases, putatively involved in the tailoring of the side chain at C-3; T1, T2, and T3 are genes encoding ABC-transport proteins.

clusters include genes for β-lactamases and penicillin-binding proteins (PBP) [7,41]. Class A β-lactamase, present in the cephamycin cluster of *S. clavuligerus*, is a typical penicillinase, inactive against both cephalosporins and cephamycins. *S. clavuligerus* is a natural cephalosporin-resistant strain (up to 1 mg cephalosporin/ml), but it is sensitive to 1000-fold less penicillin G. Cephalosporin resistance correlates with the ability of secreting cephamycin or cephalosporin intermediates [34] by cephamycin producers. It seems that cephamycin C is of ecological importance to this strain in natural habitats in which it competes with cephamycin-sensitive bacteria. The β-lactamase present in the cephamycin cluster protects the producer strain against intermediate penicillins formed in the pathway and against penicillins released either by secretion (intermediate penicillins are indeed partially secreted) or by cell lysis, but it is not active against cephamycins. The cluster of cephabacin biosynthesis genes in *Lysobacter lactamgenus* also contains a class A β-lactamase, but in this case the enzyme resembles typical cephalosporinases. The substrate specificity of this “cephalosporinase-like” enzyme and its role in the biosynthesis of cephabacins therefore deserve to be studied in more detail.

In addition, the cluster of genes for β-lactam biosynthesis contains genes encoding proteins that appear to be involved in antibiotic secretion from cells. Such proteins are membrane proteins of the major facilitator family (MFs). *A. chrysogenum* contains a gene, *cefT1*, located downstream of *pcbAB* and in the opposite orientation, encoding a protein with 12-transmembrane domains (TMs) bearing the characteristic motifs of Drug:H⁺ antiporters of the 12-TMs class. Targeted inactivation of *cefT1* does not affect significantly cephalosporin biosynthesis. This might be due to the presence of an additional *cefT3* gene similar to *cefT1* located downstream of *cefD1*. Amplification of *cefT1* results in a 100% increase in cephalosporin C production, which supports the importance of these genes in antibiotic secretion or in the regulation of antibiotic biosynthesis [34]. Current information does not exclude the possibility that either CefT1 or homologous transmembrane proteins are involved in the transduction across the membrane of signal molecules controlling antibiotic biosynthesis rather than in the secretion of β-lactam antibiotics.

Similar *cmcT* genes are also present in the cephamycin C clusters of *S. clavuligerus* and *Amycolatopsis lactamdurans*. Both genes encode 14-TMs proteins of the MFs family that are 73% identical among themselves. Hybridization studies using probes internal to *cmcT* gave positive hybridization with total DNA from all the β-lactam producers tested (*Streptomyces cattleya* 8057, *S. griseus* 3851, *S. lipmanii* 3584) but not with the β-lactam non-producer *S. lividans* J11326. Transmembrane proteins of the 14-TMs class are also present in the sequenced genomes of the actinomycetes

Streptomyces coelicolor (SCO2309) and *Streptomyces avermitilis* (SAV5867), but they are only 48% identical to *cmcT*, which supports the conclusion that CmcT proteins of the cephamycin clusters are specific cephamycin exporters. A significant number of *cmcT*-related genes encoding proteins of the MFs family have been described in different antibiotic-producing actinomycetes [31]. The closest *cmcT* relative (55% amino-acid identity) known so far is the *entT* (Q9KHJ5) gene of *Streptomyces maritimus* present in the enterocin (a polyketide antibiotic) gene cluster.

Amplification of the penicillin gene cluster in penicillin-overproducing strains

Hybridization studies of total DNA from penicillin low-producing, medium-producing, and high-producing strains using probes for the *pcbC*, *pcbAB* or *penDE* genes showed that high-producing strains have more copies of the three genes. A 106.5-kb DNA region was found to be amplified in tandem repeats (five to six copies in the improved producing strain *P. chrysogenum* AS-P-78, and up to fourteen in the industrial *P. chrysogenum* E1 strain [14]. Amplification always occurs in chromosome I (Fig. 5). *P. notatum* ATCC 9478 (the strain isolated by Fleming), and the wild-type *P. chrysogenum* NRRL1951 contain a single copy of the 106.5-kb region. That region is bounded by a hexanucleotide sequence, TTTACA/T, which appears to be a site-specific sequence for recombination. Some high-producing strains, such as *P. chrysogenum* E1, contain more copies in tandem of a shorter 56.8-kb DNA region (internal to the 106.5-kb region). This 56.8-kb region carries the penicillin biosynthesis genes and is bounded either by the TTTACA/T sequence or its complementary TGTA AA [14]. It seems that this amplification arose through the mutagenesis process used in the screening programs by researchers in industry to obtain high-yield-producing strains. The loss of that 56.8-kb DNA fragment in strain *P. chrysogenum* npe10, obtained in our laboratory, and in several mutants isolated in industrial companies, results in penicillin-non-producing mutants [14]. This type of iterated DNA amplification has not been found in cephalosporin-producing *A. chrysogenum*, nor in the penicillin-producing *Aspergillus nidulans*.

Transcriptional analysis of the 56.8-kb amplified region revealed the presence of at least eight transcripts expressed in penicillin-producing conditions. The entire 56.8-kb region has been sequenced, and computer analysis showed a total of 16 ORFs, including the three structural genes involved in penicillin biosynthesis (F. Fierro and J.F. Martín, unpublished results). Studies to determine whether ORFs present in the amplified region are related to penicillin biosynthesis are underway. One of these genes encodes a saccharopine dehy-

drogenase, an enzyme involved in the conversion of α -amino-adipic acid into lysine, and might play a role in penicillin biosynthesis by contributing to the formation of α -amino-adipic acid from lysine by the reverse lysine pathway.

Production of penicillin by fungi growing on food products

The mycobiota of food involved in the ripening and development of the specific flavor in cured meats, such as salami or cecina, includes several *Penicillium* species. Strains of *Penicillium nalgiovense*, the strain used as starter for cured and fermented meat products, as well as *Penicillium griseofulvum* and *Penicillium verrucosum* have been tested by hybridization for the presence of genes for penicillin biosynthesis. A cluster of genes almost identical to that in *P. chrysogenum* was found in *P. nalgiovense* and *P. griseofulvum*, while a truncated cluster is present in *P. verrucosum* [24]. The clusters of *P. nalgiovense* and *P. griseofulvum* are functional—as shown by the production of penicillin and detected by high-performance liquid chromatography (HPLC) or bioassay of the surface of salami and cecina—despite the high salt concentrations in some of these meat products (Fig. 6). Starter cultures are essential in the food industry to prevent undesirable fungi or bacteria from growing on the surface of fermented meats and also to provide the characteristic white “velvet-like” surface to these products. In our laboratory we obtained *P. nalgiovense* strains disrupted in the *pcbAB* gene [25] that behave like the wild-type strain in their ability to colonize meat and provide it with certain organoleptic properties, but they do not produce penicillin, thus preventing allergic reactions and the increasing penicillin-resistance of human pathogenic bacteria. The production of antibiotics by mycobiota growing in different natural habitats may be more widespread than expected. In fact, the expression of otherwise silent genes encoding secondary metabolites is favored in natural solid substrates (e.g., foods).

The same argument applies to the production of other secondary metabolites, such as mycotoxins, by those fungi.

Regulation of β -lactam biosynthesis

Transcriptional analysis of the penicillin gene cluster indicates that the *pcbC* and *pcbAB* genes are expressed from a bidirectional promoter region of 1013 bp (see Fig. 4). The two genes show a similar pattern of temporal expression and regulation, which suggests their coordinated expression. The third penicillin gene (*penDE*) carries its own promoter. These promoter regions are under the control of a variety of regulatory mechanisms. For example, in *P. chrysogenum*, alkaline pH results in positive regulation of the three promoters. Multiple regulatory sequences have been described in the *pcbAB-pcbC* bidirectional promoter region [22]; seven Pac sequences recognized by the pH-dependent transcriptional regulator PacC [53]; six CreA sites for binding of the general carbon catabolite regulatory protein CreA; six NRE sequences, putatively involved in nitrogen repression; and six CCAAT boxes for the binding of the wide domain trimeric regulator AnCF [51]. By coupling the bidirectional *pcbAB-pcbC* promoter to reporter genes followed by sequential deletion of the promoter, three boxes (A, B, C) were defined as essential for optimal expression of the reporter genes [22]. Boxes A and B formed clear protein–DNA complexes, as demonstrated by changes in their electrophoretic mobility. Deletion of box A decreases transcriptional activity of the promoter by 41%. A palindromic heptanucleotide sequence, TTAGTAA, is the binding site for the transcriptional activator PTA1. Box A also contains four of the putative CreA binding sites, which have the consensus sequence SYGGRG. Box B contains a CreA site, three putative recognition sites for PacC, and is bordered by two NRE consensus sequences. However, little is known about the nature of the modulator proteins—such as PTA1—that control the expression of this promoter.

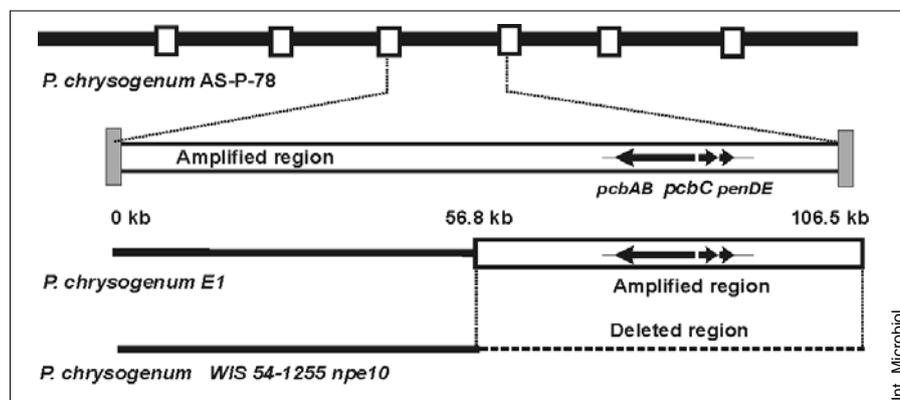
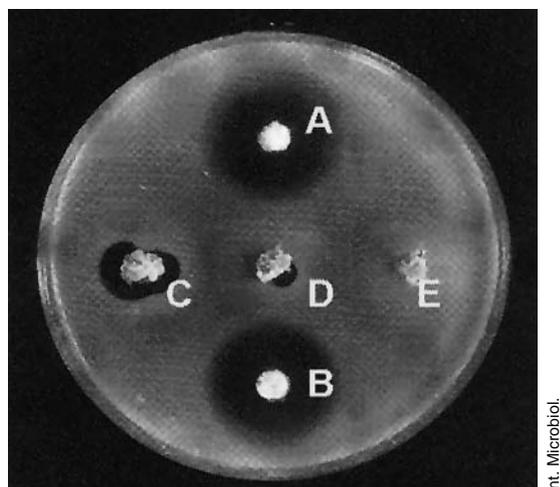


Fig. 5. Amplified region containing the penicillin biosynthesis genes in the improved producing strain *Penicillium chrysogenum* AS-P-78, the high-producing strain *P. chrysogenum* E1, and deletion present in the non-producing strain *Penicillium chrysogenum* npe10.



Int. Microbiol.

Fig. 6. Bioassays of antibiotic produced in situ by *Penicillium nalgioense*. Direct assays of soft, semi-cured salami. (A, B) different samples of salami casing, (C) outer salami layer, (D) inner salami layer, (E) inner core of salami.

Regulation in prokaryotic β-lactam producers is completely different than in eukaryotes. In cephamycin-producing *S. clavuligerus*, a gene present in the cluster, *ccaR*, encodes a SARP-type protein (*Streptomyces* antibiotic regulatory proteins). These proteins are specific activators of antibiotic biosynthesis gene clusters in *Streptomyces*. Disruption of *ccaR* results in mutants unable to produce clavulanic acid and cephamycin C; the production of both compounds is restored by re-transformation with the entire gene [42]. Mobility-shift experiments indicate that CcaR binds both to its own promoter and to the bidirectional *cefD-cmI* promoter. Binding of CcaR to the *cefD-cmI* bidirectional promoter, which controls early, intermediate, and late steps of cephamycin biosynthesis, increases the expression of the genes involved [47]. The clavulanic-acid cluster contains the gene *claR*, encoding a LysR-type regulatory protein [40,44]. Mutants disrupted in *claR* do not produce clavulanic acid but are still able to produce cephamycin C. Binding of CcaR to promoters of genes involved in clavulanic acid has never been found, but quantitative-PCR expression of *claR* in *ccaR*-null mutants indicates that its expression is low (M.T. López-García, unpublished results), which suggests that a cascade of regulatory proteins connect CcaR and *claR*.

In addition to the region for CcaR binding, the promoter of *ccaR* contains a 26-bp ARE sequence located 890 bp upstream of the initial ATG start codon. ARE sequences (consensus sequence TNANAWACNNACYNNCGGTTTKTTT) have been reported to be binding sites for Brp proteins (butyrolactones receptor proteins) [15]. The ARE sequence of *S. clavuligerus* is functional and binds a Brp protein expressed in *Escherichia coli* from the *S. clavuligerus* *brp* gene [48]. However, in addition to Brp, the sequence is a binding site for other proteins, as shown by gel mobility assays using cell extracts from a *brp*-null mutant of *S. clavuligerus*. The role of

butyrolactones in *Streptomyces* antibiotic production has been reported in the case of streptomycin, pristinamycin, and tylosin [52], but butyrolactones do not appear to be synthesized in *S. clavuligerus* or other cephamycin C producers.

Concluding remarks

The presence of gene clusters for β-lactam antibiotic biosynthesis confers an ecological advantage to β-lactam-producing fungi or bacteria living in the soil. It appears that different fungal strains received large intron-less DNA fragments of the β-lactam gene cluster from soil bacteria, and subsequently attached other fungal genes (containing introns) to the fragment of the original bacterial antibiotic gene clusters to produce new and more potent antibiotics, such as penicillin G and cephalosporin C. These final compounds are of the utmost interest in the pharmaceutical industry. The penicillin-overproducing strains used industrially contain multiple copies of the penicillin gene cluster, which was amplified by random mutagenesis and selection during strain improvement programs. A worrying aspect of β-lactam production by fungi is that these gene clusters are expressed in the mycobiota involved in the ripening of cured meat products (e.g., salami), which results in a potential problem in that selection of β-lactam-resistant bacterial strains is thereby favored. The regulation of β-lactam biosynthesis is beginning to be elucidated and is clearly different in bacterial and fungal β-lactam producers.

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Complejos génicos ("clusters") de antibióticos β-lactámicos y control de su expresión: ¿por qué se han originado, y de dónde proceden?

Resumen. Las β-lactamas fueron descubiertas en hongos filamentosos, pero se sabe que los actinomicetos y algunas bacterias gram negativas también producen diferentes tipos de β-lactamas. Todas las β-lactamas contienen un anillo de cuatro miembros. La estructura del segundo anillo permite clasificarlas en penicilinas, cefalosporinas, clavamas, carbapenemas o monobactamas. La mayoría de estos compuestos inhiben la síntesis de la pared celular bacteriana, pero algunos se comportan como inhibidores de β-lactamasas (por ejemplo, el ácido clavulánico), o incluso como agentes antifúngicos (algunas clavamas). Debido a la naturaleza del segundo anillo en la molécula de las β-lactamas, los precursores y las vías biosintéticas de clavamas, carbapenemas y monobactamas son diferentes de los de penicilinas y cefalosporinas. Las moléculas de estos dos grupos, incluyendo cefamicinas y cefabacinas, están formadas por tres aminoácidos precursores que se unen para formar el tri péptido α-aminoadipil-L-cisteinil-D-valina. Las primeras dos etapas de la biosíntesis de las cefamicinas y las cefabacinas son comunes. Los intermediarios de estas vías, las características de las enzimas que intervienen en ellas, la falta de intrones en los genes y el análisis bioinformático sugieren que se originaron a partir de un complejo génico ("cluster") ancestral de origen bacteriano, que fue transferido horizontalmente en el suelo desde los microorganismos productores a los no productores. Las cepas receptoras adquirieron fragmentos del complejo génico bacteriano original y ocasionalmente insertaron en él nuevos genes; dichos genes, una vez modificados, adquirieron funciones nuevas y dieron lugar a los nuevos compuestos finales que conocemos. Cuando se analiza el orden de los genes en el genoma de *Streptomyces*, los complejos génicos de la síntesis de antibióticos destacan como islas en el genoma. Sin embargo, la forma en que se ensambló el complejo génico ancestral para β-lactamas sigue siendo motivo de conjeturas. [*Int Microbiol* 2006; 9(1):9-19]

Palabras clave: antibióticos β-lactámicos · biosíntesis de antibióticos · regulación metabólica · complejos génicos ("clusters") · evolución microbiana

Complexos génicos ("clusters") de antibióticos β-lactámicos e controle de sua expressão: por que se originaram, e de onde procedem?

Resumo. As β-lactamas foram descobertas em fungos filamentosos, mas sabe-se que os actinomicetos e algumas bactérias gram-negativas também produzem diferentes tipos de β-lactamas. Todas as β-lactamas possuem um anel de quatro membros. A estrutura do segundo anel permite classificar estes compostos em penicilinas, cefalosporinas, clavamas, carbapenemas ou monobactamas. A maioria das β-lactamas inibem a síntese da parede celular bacteriana, mas algumas se comportam como inibidoras de β-lactamasas (por exemplo, o ácido clavulânico) ou inclusive como agentes fungicidas (algunas clavamas). Devido à natureza do segundo anel da molécula das β-lactamas, os precursores e as vias biosintéticas de clavamas, carbapenemas e monobactamas são diferentes das de penicilinas e cefalosporinas. As moléculas nestes dois grupos, incluindo cefamicinas e cefabacinas, são formadas por três aminoácidos precursores que se unem para formar o tri péptido α-aminoadipil-L-cisteinil-D-valina. As primeiras duas fases da biosíntese dos cefamicinas e cefabacinas são comuns. Os intermediários destas vias, as características das enzimas que intervêm nelas, a falta de íntrons nos genes e a análise bioinformática sugerem que todas elas se originaron a partir de um complexo génico ("cluster") ancestral de origem bacteriano, que foi transferido horizontalmente no solo dos microorganismos produtores aos não produtores. As cepas receptoras adquiriram fragmentos do complexo génico bacteriano original e ocasionalmente inseriram nele novos genes, os quais, uma vez modificados, adquiriram funções novas e deram lugar aos novos compostos finais que conhecemos. Quando se analisa a ordem dos genes nos genomas de *Streptomyces*, os complexos génicos para sínteses de antibióticos destacam-se como ilhas no genoma. No entanto, a forma em que se encaixou o primeiro complexo génico ancestral para β-lactamas continua sendo motivo de conjeturas. [*Int Microbiol* 2006; 9(1):9-19]

Palavras chave: antibióticos β-lactámicos · biosíntese de antibióticos · regulamento metabólico · complexos génicos ("clusters") · evolução microbiana