Minireview

Antibiotics – cloning of biosynthetic pathways

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Biosynthetic pathways leading to antibiotics have often been found to be clustered, and new organizational forms of multifunctional enzymes have been discovered. Such polyenzymes accomplish the synthesis of complex metabolites such as peptides or polyketides by a sequence of enzymatic reactions. So, reactions leading to the tripeptide precursor of β-lactam antibiotics, ACV, or to the cycloundecapeptide cyclosporine have been fused into single polypeptide chain synthetases, respectively. In certain isofunctional sites restricted similarities have been detected.

Antibiotic; β-lactam; Polyketide; Synthetase; Synthase; Multienzyme

1. INTRODUCTION

Many significant advances in the antibiotic field have come through the application of molecular genetic tools for localizing the respective biosynthetic genes and their regulatory regions. The current main themes are the elucidation of regulatory networks on one side, and the analysis of sequential biosynthetic pathways on the other. Structural genes for enzyme systems comprising a pathway are assembled in clusters of varying complexity and sometimes extrachromosomal location. Resistance factors are frequently included in such clusters, and the genetic exchange of such DNA-elements is discussed. Although biosynthetic pathways seem to be divergent depending on the structural class of compound produced, new organizational forms of enzymes have been detected, mainly in the classes of synthases and synthetases. This multifunctional organization, however, is observed in varying degrees of complexity. As sequence data become available now, similarities between various enzyme systems appear to be of a complex nature.

We point here to some recent developments in the field and apologize for not citing all of those who prepared the path to that stage as in a review type of presentation.

2. β-LACTAM BIOSYNTHETIC GENES AND PEPTIDE SYNTHETASES

Following the characterization and cloning of the key enzyme isopenicillin N synthase (IPNS) catalyzing the formation of the β-lactam ring system [1], a variety of IPNS from microbial sources has been obtained [2–7], and evidence for a gene cluster directing the formation of this class of modified peptides is emerging [8–10]. Besides the classical fungal producers *Penicillium chrysogenum*, *Cephalosporium acremonium* and *Aspergillus nidulans*, IPNS from *Streptomyces clavuligerus*, *Streptomyces lipmanii*, and *Streptomyces jumjinensis* have been cloned [5,6]. as well as from *Flavobacterium* sp., a Gram-negative bacterium [7]. The significant homologies of these unique enzymes have aided the isolation of their respective structural genes. Yair Aharonowitz discussed the comparative analysis of the sequences that reveal a surprisingly high similarity. Especially two regions containing cysteine residues are highly conserved. The high GC-content of the IPNS-genes in fungi have been interpreted as to indicate a tranfer of bacterial genes [4,11,12]. Miguel Penalva compared their evolutionary distances with the corresponding 5 S RNA genes and discussed multiple horizontal gene transfers from *Streptomycetes* to *Ascomycotina*. This transfer hypothesis is being now reconsidered regarding the ACV-synthetase and acyl transferase genes, and the adaption of these clusters to the eucaryotic transcriptional machinery, especially with regard to the integration in the developmentally regulated networks. The regulation of expression of the penicillin genes in *Aspergillus nidulans* has been studied by Penalva by the use of lacZ-constructs.

Again the search in β-lactam nonproducers with the IPNS-gene probes reveals the presence of apparently *silent* genes, as has already been shown for the phenoxazinone synthase in *Streptomyces lividans* [13,14]. This enzyme catalyzes the oxidative dimerization of the ac-

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tinomycin precursor acyl-pentapeptide in *Streptomyces antibioticus*. Other examples of such silent genes that have been activated in *S. lividans* by transformation of the regulatory *afsB*-gene from *S. coelicolor* are the formation of a-factor, actinorhodin and undecylprodigiosin [15]. The cluster of the peptide-related phosphinotricinyl-D-alanyl-D-analine (bialaphos, from *Streptomyces hygrocopicus*) has been well characterized by Julian Thompson's group [16]. Besides genes coding for biosynthesis and self-resistance, it contains a regulatory protein (*brp*) acting on the expression of 6 of the 13 genes. D. Noack and colleagues discussed studies on the peptide-related nourseothricin produced by *Streptomyces noursei*. Self-resistance by acetylation is mediated by two independent resistance genes (*natI/II*), showing similarities to other amino-acetyl transferases. Genetic instability leads to the loss of antibiotic production followed by the decrease in resistance level in a second segregation step. Complementation cannot be achieved by *natI* or *natII* alone, but requires an additional gene.

In his studies on the ultrastructure of penicillin production in *Penicillium chrysogenum*, Wieslaw Kurzatkowski has demonstrated the cellular complexity of the overproduction of a metabolite. The biosynthetic machinery forming penicillin G is found to be located within Golgi-type vesicles [17], and their formation corresponds to the synthetic activity.

The recent isolation of ACV synthetase from *Aspergillus nidulans* has grouped this multienzyme into the class of peptide synthetases [18]. This tripeptide synthetase represents one of the most simple cases for these enzyme systems, catalyzing sequential reactions by integration of the respective enzyme activities into multienzymes of varying complexity [19]. The most complex system described so far is the cyclosporin synthetase from *Beauveria nivea* [20]. This multienzyme contains all functions required to form the cyclic undecapeptide immunosuppressor with seven N-methylated positions from the respective amino acids, *S*-adenosylmethionine and ATP as energy source. So altogether 40 catalytic reactions are assembled on a single polypeptide of approximately 800 kDa.

The structural genes for the multienzymes forming gramicidin S and tyrocidine have also been shown to be organized in clusters in *Bacillus brevis* [21,22]. In addition, a new gene has been identified in the gramicidin S-cluster (*grs*), with significant similarity to mammalian thioesterases (*grsT*) [21]. The possible function of this enzyme in peptide biosynthesis is now under investigation. From the sequences obtained so far from the multienzymes GS1 (*grsA*), GS2 (*grsB*), TY1 (*tycA*), and TY2 (*tycB*) [21–24] gene and protein structures are corresponding, and no introns or rearrangements on the nucleic acid level have been found. Similarities of these peptide synthetases with other synthases and synthetases are now becoming evident.

3. POLYKETIDE SYNTHASES

Polyketides as polycondensation products of activated C_2-units are assembled on synthases. These multienzymes or enzyme complexes share at least 3 different catalytic sites, the acyl and malonyl transferase, and the β-ketoacyl synthase. In addition, the cofactor 4′-phosphopantetheine is involved in the transport of intermediates between modification and extension sites. This cofactor is generally an intrinsic function of the integrated type of synthase, and also present in peptide synthetases. It is found to be attached to a protein moiety similar to the acyl carrier protein known from most bacterial and plant fatty acid synthase complexes.

With respect to antibiotics or *special metabolites* two main lines have been followed in the last years. There has been considerable work on clustered biosynthetic genes of several systems in *Streptomycetes*, and there has been a comparative study on the multienzymes forming the patulin precursor 6-methyl salicylic acid (MSA) and fatty acids respectively, in *Penicillium patulum* by Eckehard Schweizer and his colleagues. The MSA-synthase (MSAS) is a tetra- or hexamer of a multifunctional 188 kDa-polypeptide. Six of the 7 partial activities linked on this peptide chain catalyze identical reactions as comparable sites in fatty acid synthases (FAS); differences exist, of course, in the sequence of these reactions. Following the characterization and sequence determination of the FAS from *P. patulum* [25], Schweizer et al. have now succeeded in the isolation of the MSAS-gene from the same strain [26]. Quite unexpectedly, there are only few restricted sequence similarities between these two synthases, residing in the catalytic transferase and condensation sites, and in the binding site of pantetheine. Thus, contrary to expectations, functional identities have not been conserved at the protein level, even in the same organism.

Contrary to such integrated systems in fungi, which closely resemble the situation found for peptide synthetases isolated from fungi forming enniatin, beauvericin and cyclosporin [19], bacterial polyketide-forming systems are complex enzyme systems rather than multifunctional polyenzymes [27,28]. Several research groups have made significant contributions to these and other systems analysing the respective antibiotic biosynthetic gene clusters in *Streptomycetes*. By sequence similarities ACP and acyl transferase sites have been located in the erythromycin, granaticin and tetracenomycin clusters, respectively [29–32]. In contrast to this genetic approach, V. Behal and colleagues have characterized the last two enzymes of the tetracycline pathway [33,34], and have determined the level of anhydrotetracycline oxygenase in a high producer strain of *Streptomyces aureofaciens* to 1.64% of the total protein. This is more than 10-fold the level of alanine or valine dehydrogenase in the same organism,

and indicates a direct correlation between secondary metabolism enzyme and production. This suggests the direct application of genetic engineering techniques in strain improvements.

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