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Research paper

Motifs in the C-terminal region of the *Penicillium chrysogenum* ACV synthetase are essential for valine epimerization and processivity of tripeptide formation

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ABSTRACT

The first step in the penicillin biosynthetic pathway is the non-ribosomal condensation of L-α-aminoadipic acid, L-cysteine and L-valine into the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV). This reaction is catalysed by the multienzyme ACV synthetase (ACVS), which is encoded in the filamentous fungus *Penicillium chrysogenum* by the *pcbAB* gene. This enzyme contains at least ten catalytic domains. The precise role of the C-terminal domain of this multidomain NRPS still remains obscure. The C-terminal region of ACVS bears the epimerase and the thioesterase domains and may be involved in the epimerization of LLL-ACV to LLD-ACV and in the hydrolysis of the thioester bond. In this work, the conserved motifs ^{3371}EGHGRE^{3376} (located in the putative epimerase domain) and ^{3629}GWSFG^{3633} (located in the thioesterase domain) were changed by site-directed-mutagenesis to LGFGLL and GWAFG, respectively. In addition, the whole thioesterase domain (230 amino acids) and the different parts of this domain were deleted. The activity of these mutant enzymes was assessed *in vivo* by two different procedures: i) through the quantification of bisACV produced by the fungus and ii) by quantifying the benzylpenicillin production using tailored strains of *P. chrysogenum*, which lack the *pcbAB* gene, as host strains. All indicated mutant enzymes showed lower or null activity than the control strain confirming that E3371, H3373, R3375 and E3376 belong to the epimerase active centre. Different fragments included in the C-terminal region of ACVS control thioester hydrolysis. Overexpression of the sequence encoding the ACVS integrated thioesterase domain as a separate (stand-alone) transcriptional unit complemented mutants lacking the integrated thioesterase domain, although with low ACV releasing activity, suggesting that the stand-alone thioesterase interacts with the other ACVS domains.

1. Introduction

Non-ribosomal peptide synthetases (NRPS) are multimodular enzyme systems involved in the biosynthesis of thousands of peptide secondary metabolites [1,2]. Due to the large size and multiple enzyme activities their catalytic sites are still poorly known. The *in vitro* activities of these enzymes are very poor because they lose their native structure easily. However, molecular genetic approaches allow their characterization *in vivo*.

Penicillium chrysogenum is a filamentous fungus used for the industrial production of penicillins [3] and some cephalosporin derivatives [4,5]. Penicillins and cephalosporins are β-lactam antibiotics formed from a common intermediate; the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV). ACV can be found either in a reduced monomeric form or as an oxidised dimer (disulphide bisACV), which is the predominant ACV form released to the culture broths under strongly oxidative (aerated) conditions [6,7]. Intracellularly, the ACV is kept in the reduced form by the thioredoxin and thioredoxin reductase system, since only the reduced monomer is substrate for the next enzyme of the β-lactam biosynthetic pathway; IPN synthase [8].

ACV is synthesized by non-ribosomal condensation of three amino acids; L-α-aminoadipic acid, which is an intermediate in the lysine biosynthetic pathway, L-cysteine and L-valine [9]. Amino acid condensation is catalysed by the non-ribosomal multidomain ACV synthetase (ACVS), which requires ATP and Mg^{2+} [10,11]. The ACVS, encoded by the *pcbAB* gene [12], is a large protein with a mass of 405–425 kDa depending on the microorganism [10,13,14].

Abbreviations: ACV, α-aminoadipyl-cysteinyl-valine; ACVS, ACV synthetase; NRPS, non-ribosomal peptide synthetase.

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The unusually long (11376 bp) *P. chrysogenum pcbAB* gene lacks introns and encodes a protein of 3791 amino acids [12]. The *pcbAB* gene is linked to the *pcbC* and *penDE* genes, which are clustered together with other ORFs in a DNA region amplified in tandem repeats in high-penicillin producing strains [15–17].

The ACVS is able to catalyse multiple activities including substrate amino acids adenylation, peptide-bond formation, epimerization and tripeptide release by an integrated thioesterase [9,13], since it contains three different modules each of approximately 1000 amino acids. The domains of each module of the NRPSs are partially conserved among themselves [2]. Each module contains adenylate-forming (designated A for activation), aminoacyl (or peptidyl) carrier (T, for thiolation) and condensation (C) domains consisting of conserved motifs arranged in the characteristic order ATC, ATC, AT [9,18] (Fig. 1A).

The A domain is involved in ATP binding and amino acid adenylate formation and contains a region determining the substrate amino acid specificity. The aminoacyl carrier module (thiolation) bears a conserved serine residue that binds a thiol-containing phosphopantetheine co-factor derived from CoA [19]. Phosphopantetheine is added to the apoprotein by an enzyme known as a 4′-phosphopantetheine transferase (PPTase) [20,21]. The gene encoding the stand-alone PPTase involved in the activation of the *P. chrysogenum* ACVS has been cloned [21]. The C domains of ACVS are responsible for the condensation of two activated amino acids on adjacent modules, and catalyse the elongation reaction [22].

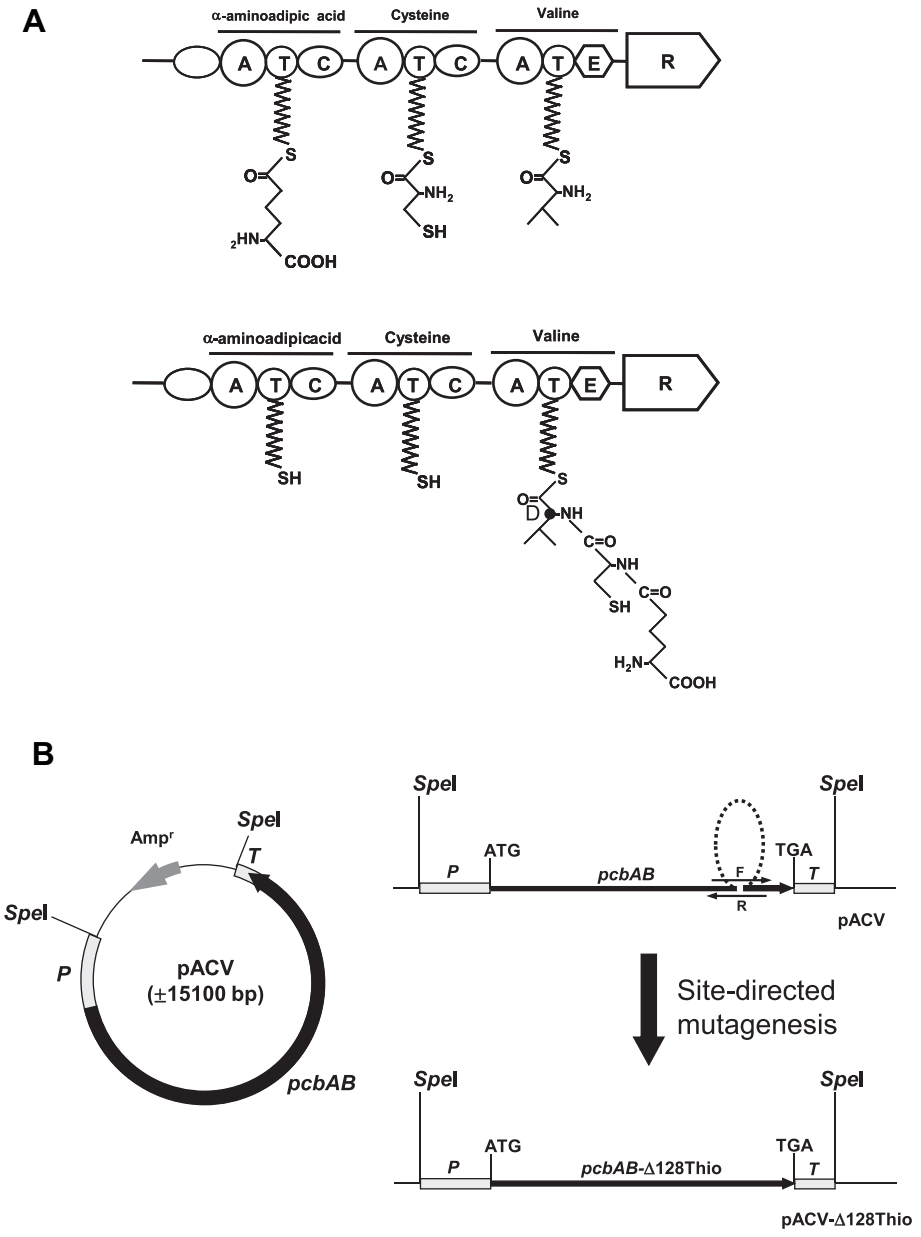

Fig. 1. Schematic representation of ACVS and plasmid pACV. A) Multidomain organization of ACVS. Each amino acid to be condensed is recognized by one module. Activation (A), thiolation (T) and condensation (C) domains are represented inside each module. At the end of the Valine module the epimerisation domain (E) is shown. The thioesterase domain (R) is located at the C-terminal end of ACVS. The three amino acids L-α-aminoadipic acid, L-cysteine and L-valine are shown bound to the phosphopantetheine arm, which is linked to the T domain of each module. Note that at the end of the process the tripeptide linked to the valine module already has the LLD-configuration (black dot). B) Representation of plasmid pACV and scheme of the strategy followed to generate deletions in the 3′ region of the *pcbAB* gene. Construction of plasmid pACV-Δ128Thio was chosen as example. P: promoter region; T: terminator region; F: forward primer; R: reverse primer. The fragment to be deleted is drawn as a discontinuous line loop.