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Novel chemobiosynthetic approach for exclusive production of FK506

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ABSTRACT

FK506, a widely used immunosuppressant, is produced by industrial fermentation processes using various *Streptomyces* species. Independently of the strain, structurally related compound FK520 is co-produced, resulting in complex and costly isolation procedures. In this paper, we report a chemobiosynthetic approach for exclusive biosynthesis of FK506. This approach is based on the *Streptomyces tsukubaensis* strain with inactivated *allR* gene, a homologue of crotonyl-CoA carboxylase/reductase, encoded in the FK506 biosynthetic cluster. This strain produces neither FK506 nor FK520; however, if allylmalonyl-S-N-acetylcysteamine precursor is added to cultivation broth, the production of FK506 is reestablished without FK506-related by-products. Using a combination of metabolic engineering and chemobiosynthetic approach, we achieved exclusive production of FK506, representing a significant step towards development of an advanced industrial bioprocess.

1. Introduction

FK506, also known as tacrolimus, is widely used as immunosuppressant after allogeneic kidney, liver and bone marrow transplantation as well as for treatment of inflammatory skin diseases and eczema (Husain and Singh, 2002; Stuetz et al., 2006). The immunosuppressants FK506 and cyclosporine share a similar mechanism of action as they both inhibit the calcineurin phosphatase and thereby reduce T-cell mediated immune response by decreasing the production of interleukin-2 (Liu et al., 1991). FK506 is in many clinical aspects superior to cyclosporin, therefore market share and production volumes of FK506 have been increasing constantly in recent years (Haddad et al., 2006; Webster et al., 2005). FK506 and its structural analogue FK520 (ascomycin) as well as biogenetically similar rapamycin (sirolimus) are secondary metabolites belonging to the group of macrolactones. They are synthesised by combined polyketide synthase (PKS)/non-ribosomal peptide synthetase (NRPS) enzymatic complexes. PKS and NRPS are polyfunctional enzymes comprising different enzymatic domains organised in sets of modules. PKS incorporates specific carboxylic acid-derived extender units into polyketide chains and mediates subsequent reductive reactions (Hopwood, 1997; Staunton and Weissman, 2001). In contrast, NRPS incorporates structurally diverse amino acids or amino acid-analogues into non-ribosomally synthesised peptides (Koglin and Walsh, 2009).

Biosynthesis of FK506 macrolactone is initiated by PKS-catalysed condensation of an unusual shikimic acid-derived starter unit 4,5-dihydroxycyclohex-1-enecarboxylic acid and 10 extender units, namely, two malonyl-CoA, five methylmalonyl-CoA, two methoxymalonyl-ACP and an unusual allylmalonyl-CoA extender unit are incorporated into nascent polyketide chain. This is followed by incorporation of lysine-derived pipecolic acid and a cyclization step, mediated by the NRPS gene *fkbP*, resulting in the earliest macrolactone intermediate of FK506. Finally, post-PKS processing reactions are catalysed by a specific methyl transferase and oxido-reductase resulting in FK506 (Goranovič et al., 2010; Gregory et al., 2006; McDaniel et al., 2005; Motamedi et al., 1996).

Structures of FK506 and FK520 differ only in the side chain at the carbon 21 where the former bears an allyl moiety and the latter an ethyl moiety (Fig. 1). The biosynthetic and evolutionary aspects of this structural difference were revealed by sequencing of biosynthetic gene clusters from various *Streptomyces* strains, producing either compound, *Streptomyces.* sp. MA6548 and *S. hygroscopicus* var. *ascomyceticus* (Motamedi and Shafiee, 1998; Wu et al., 2000). Genes involved in the supply of the corresponding extender units for polyketide chain synthesis, allylmalonyl-CoA or ethylmalonyl-CoA, were found to have a crucial influence in determining whether FK506 or FK520 is produced by individual microbial strain. In FK520 producing *Streptomyces hygroscopicus*

Abbreviations: PKS, polyketide synthase; NRPS, non-ribosomal peptide synthetase; ACP, acyl carrier protein; ccr, crotonyl-CoA reductase/carboxylase; SNAC, N-acetylcysteamine; AT4, acyltransferase domain of the fourth module of FK506 PKS; FK506D, 37,38-dihydro-FK506

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var. *ascomyceticus* several genes, among them a homologue of crotonyl-CoA reductase (ccr), have been identified that are involved in the biosynthesis of ethylmalonyl-CoA, a 4-carbon extender unit (Wu et al., 2000). Apart from being an extender unit for polyketide biosynthesis, ethylmalonyl-CoA is also a key metabolite of the recently identified "ethylmalonyl-CoA pathway," involved in acetate assimilation in diverse bacterial species (Erb et al., 2007). In contrast to FK520 producing strains, we have recently identified a group of genes in the FK506 biosynthetic cluster of *Streptomyces tsukubaensis* NRRL 18488, termed the "all subcluster" (Goranovič et al., 2010). We have demonstrated that the "all-subcluster" contains genes involved in the biosynthesis of the 5-carbon extender unit giving origin to the allyl side chain in FK506 (Fig. 2a). We have proposed a biosynthetic pathway of this extender unit, which begins with an unusual diketide synthase-mediated condensation of 2-carbon and 3-carbon compounds and continues with subsequent reduction of the resulting β-keto group. Further details of this biosynthetic pathway were subsequently determined in a closely related FK506-producing organism *Streptomyces* sp. KCTC11604BP, most notably the origin of the terminal double bond of the allylmalonyl-CoA extender unit, was elucidated (Mo et al., 2011). In both streptomycetes a ccr homologue, termed *allR* (Goranovič et al., 2010) or *tscC* (Mo et al., 2011), was found to be encoded among the "all" genes, suggesting its involvement in reductive carboxylation of the 5-carbon precursor in this biosynthetic pathway.

A number of independently isolated streptomycete species have been identified as FK506 producers such as *Streptomyces* sp. MA6858 ATCC 55098 (Motamedi et al., 1996), *S. tsukubaensis* NRRL 18488 (Edmunds and Grassberger, 1990), *Streptomyces clavuligerus* CKD 1119 (Kim and Park, 2007), *S. clavuligerus* KCTC 10561BP and *Streptomyces glaucescens* MTCC 5115 (Kumar et al., 2007). Based on the patent and scientific literature, several analogues (by-products) of FK506 are co-produced during the fermentation process and the impurity profile for all FK506 producers is relatively similar. Independently of the strain background, industrial processes for production and isolation of FK506 have been hampered by at least two mayor FK506-related analogues: FK520 and 37,38-dihydro-FK506 (FK506D) (Fig. 1). Relatively high proportion of FK520 produced by the FK506-producing strains is regularly observed in fermentation broths, often reaching over 20% of the total FK506 content, suggesting that ethylmalonyl-CoA may be a readily available metabolite in FK506-producing organisms and is likely competing with allylmalonyl-CoA for incorporation into the polyketide chain at C21 carbon atom. In addition to FK520, FK506D is also produced, but in rather lower ratios compared to FK520 (Fig. 1), presumably by incorporation of propylmalonyl-CoA. The presence of these impurities significantly increases the cost of FK506 downstream processes. Several downstream approaches have been developed to ensure the desired purity of FK506 in large industrial scale, and they usually involve steps of repetitive extractions using organic solvents and/or chromatographic separation (Cabri et al., 2006; Cvak et al., 2007), significantly increasing the costs of the final product. In addition, these procedures present a heavy environmental burden.

In this paper, we report for the first time how metabolic pathways leading to either allylmalonyl-CoA or ethylmalonyl-CoA are interrelated in an FK506 producing organism. Based on our findings a novel chemobiosynthetic strategy is presented, whereby *S. tsukubaensis* may synthesise FK506 exclusively and in the absence of FK520. Our results present a promising potential for development of an industrially scalable process for exclusive production of FK506 or biogenetically similar compounds.

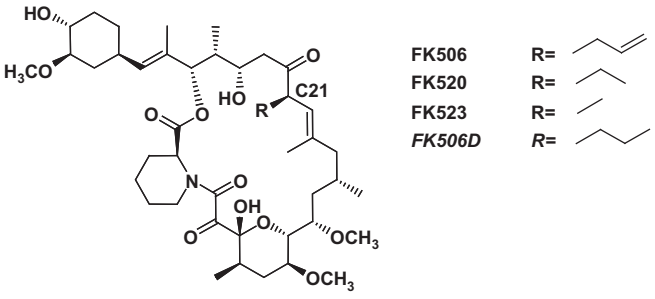

Fig. 1. Structure of FK506 and its analogues (by-products) produced by *S. tsukubaensis* and structurally differing at the position of the carbon 21.

2. Materials and methods

2.1. Bacterial cultures, culture conditions and detection of metabolites

S. tsukubaensis NRRL 18488 strain was used for all cultivations and genetic manipulations. For spore stock preparation *S. tsukubaensis* strains were cultivated as a confluent lawn on the ISP4 agar sporulation medium (Kieser et al., 2000) for 8–14 day at 28 °C. For liquid cultures spores of *S. tsukubaensis* strains were inoculated in seed medium VG3 (Goranovič et al., 2010) and incubated at 28 °C and 250 rpm for 24–48 h. The above seed culture was used for the inoculation of a 250 ml Erlenmeyer flask containing 50 mL of production medium PG3 (Goranovič et al., 2010). Cultivation was carried out at 28 °C, 250 rpm for 6–7 days. Apramycin (50 μg/mL) and thiostrepton (25 μg/mL) were added to the solid and liquid media after sterilisation, as required.

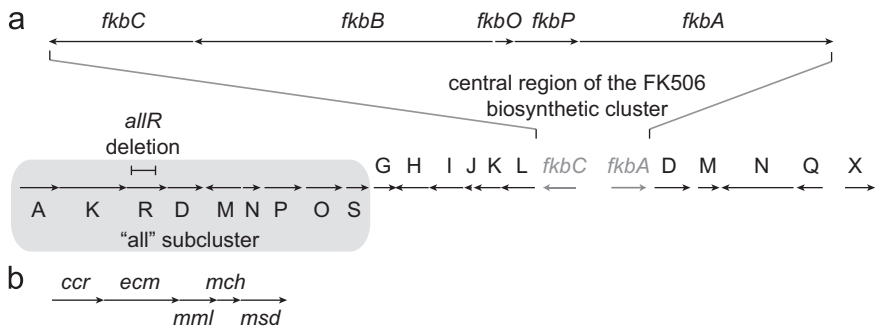

Fig. 2. (a) Schematic representation of the FK506 biosynthetic cluster of *S. tsukubaensis*. Genes of the left fringe of the cluster, also termed as the »all« subcluster, are marked in grey and deletion of the central part of the *allR* gene is shown. (b) Schematic representation of the genes involved in the ethylmalonyl-CoA metabolic pathway in genomes of *Streptomyces* species, apparently located on a single transcription unit. The gene homologues are marked as follows: *ccr*—crotonyl-CoA carboxylase/reductase, *ecm*—ethylmalonyl-CoA mutase, *mml*—L-malyl-CoA/ß-methylmalyl-CoA lyase, *mch*—mesaconyl-CoA hydratase and *msd*—methylsuccinyl-CoA dehydrogenase.