FK506 Synthesis through Sigma Factor Enhancement: Pathway to Improving Affordable Access to Viable Organ Transplants?

Shiva Niakan

Hearts, kidneys and livers are the most common organs transplanted. If an organ transplant is to be successful, the patient receiving the transplant must take immunosuppressive medication to prevent the immune system from attacking the new organ. There are numerous challenges for patients who require organ transplants. There are limited numbers of organ donors, and successful transplants require finding a donor who matches the recipient. If the donor does not match the recipient, the recipient's body will likely reject the organ, even with immune-suppressant medications. But even with a good tissue match, successful organ transplants often require immune suppressants, and these, too, are in limited supply and costly to produce, which serves to restrict patient access to organ transplants.

Organ donors may be living or dead. Donors who are deceased generally die from the cessation of brain activity. Most tissues and organs must be recovered within 24 hours of heart failure. Once recovered, organs must be transplanted quickly. Tissues, on the other hand, can be preserved for up to five years and kept in storage. In addition, a number of grafts can be retrieved from just one sample of tissue. For these reasons, tissue transplants are much more common than organ transplants. The need for organ transplants is often more urgent than tissue transplants, and for a variety of reasons they can also be more problematic to pull off successfully.

The kidney is the most commonly transplanted organ. With the increased incidence of diabetes and obesity, the need for organ transplants has increased dramatically. Type II diabetes and obesity are prevalent health issues and can lead to problems like heart disease and kidney failure. Because of this, we can see why efforts to increase our access to viable organ transplants are a growing and critical focus of contemporary medicine.

Because the demand for replacement organs surpasses available supply, scientists are investigating possible ways to grow organs and tissues from stem cells. These stem cells can be embryonic or harvested from the recipient's own body. Use of tissue and cells from the recipient will greatly reduce the potential for problems caused by the patient's immune system. However, stem-cell science is still developing and has not yet established viable options for patients in need of new organs. In the meantime, we rely on organ and tissue donors to provide our available supply. This leaves us with the problem of organ rejection, which occurs when a patient's immune system attacks the transplanted organ. Immune suppressants are utilized to lower the activity of the patient's immune system, increasing the success rate of organ transplants. FK506 is a key component of these immune suppressant drugs, but supplies of this component are limited and can be expensive to produce. The challenge of finding an affordable way to synthesize increased quantities of FK506 also offers the prospect of improving affordable access to organ transplants, at least until breakthroughs in stem cell science can support viable efforts to generate new organs and tissues from a patient's own stem cells. The following research project aims to address this challenge by identifying a cost-effective process for producing FK506 synthesis.

The soil bacterium *Streptomyces tsukubaensis* produces FK506, a polyketide

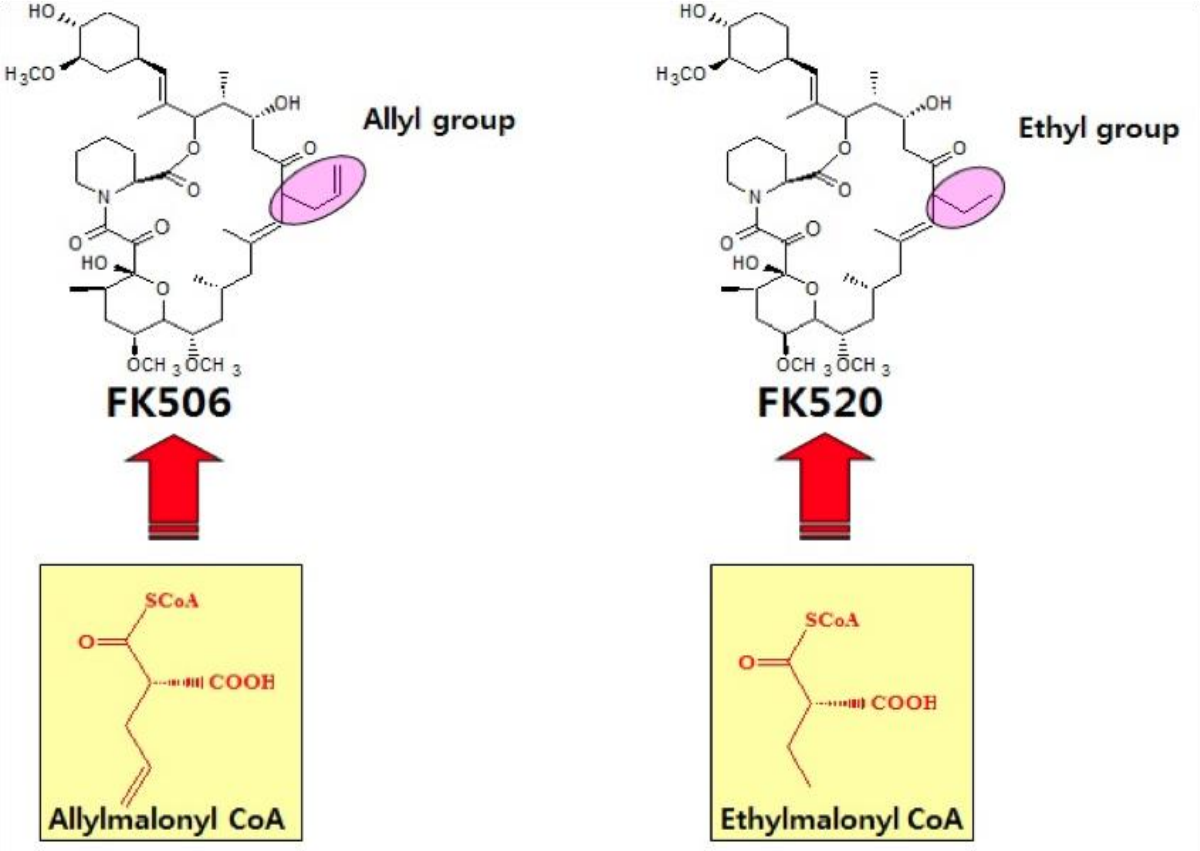
with immunosuppressant properties. FK506 (Tacrolimus) is used to prevent organ rejection after a transplant surgery. It accomplishes this by lowering the activity of the patient's immune system. Tacrolimus is also used for treatment of atopic dermatitis, an autoimmune disease. This type I polyketide is commercially available at a cost of roughly US $100,000 per kilogram, and its production is only available through fermentation.

In the early years of transplant activity, the patient's immune system made transplantation impossible except in identical twins. In the 1960's a medication was developed that helped suppress the immune system, but rejection rates were still at a high rate (generally 70-80%). As more medications like Tacrolimus were discovered, rejection rates were gradually lowered, and now run around 10-15%. But because production of FK506 has not kept pace with demand, medications like Tacrolimus have been prohibitively costly. With increases in the synthetic production of FK506, the cost of Tacrolimus could be lowered and the supply increased, making organ transplants a viable option for more people in need.

Strains of *Streptomyces tsukubaensis* known to produce FK506 also produce FK520, which is a structural analog of FK506. The research project I have been involved with aims to increase FK506 production through combined inactivation of ICM and overexpression of sigma factor. Another goal of the project is to confirm that overexpression of sigma factor actually increases FK506 and FK520 through up-regulation of the FK506 biosynthetic gene cluster.

FK506 (Tacrolimis) is an important 23-membered polyketide macrolide with immunosuppressant activity. *Streptomyces* sp. KCCM 11116P, the FK506 producer, is isolated from 10,000 library strains. The FK506 biosynthetic gene cluster from *Streptomyces* sp. KCCM 11116P (wild type) contains four putative allylmanoly-CoA biosynthetic genes *fujA, fujB, fujC*, and *fujD*, which were identified by protein homology search. We also noticed that there exists a putative gene for ECF σ-factor at the beginning of the cluster, which may initiate and enhance the transcription of the cluster with RNA polymerase. Extracytoplasmic function (ECF) sigma factors have different protein sequences compared to most other sigma factors, and they are known to function in cell signaling. As soon as an extracellular signal triggers the receptor, ECF sigma factor activates and commences transcription. We noticed that these genes were actively expressed in *Streptomyces* sp. KCCM 11116P by RT-PCR. When we overexpressed the ECF σ-factor in a replicate plasmid, we noticed that transcription levels of the genes inside the cluster increased substantially. This result suggests that putative ECF σ-factor regulates the transcription of the FK506 gene cluster in *Streptomyces* sp. KCCM 11116P and may play an important role in producing higher amounts of FK506.

FK506 and FK520 are structural analogs of one another, the difference being that the substituent present on Carbon 21: FK506 has an allyl group whereas FK520 has an ethyl group. During FK506 synthesis, ethylmalonyl-CoA and allylmalonyl-CoA compete with each other to produce either of 2 possible products. We hypothesize that by decreasing the ethylmalonyl-CoA quantity, the production of FK506 will decrease, leaving only allylmalonyl-CoA to react and synthesize the desired product.



In the biosyntheis pathway of ethylmalonyl-CoA, a crucial enzyme is present, called ICM. If this enzyme is deactivated, then the synthesis of ethylmalonyl-CoA cannot take place. If the synthesis of ethylmalonyl-CoA is halted, then allylmalonyl-CoA will not have its competitor, resulting in the production of more FK506. Prior research has shown that when ICM deactivation was achieved by in-frame gene deletion, FK520 production decreased by 35% and FK506 production increased by 220%.

Transcription has three crucial parts: initiation, elongation and termination. RNA polymerase complex, with many subunits, is the primary component during transcription. However, the sigma factor is the only component that can initiate transcription.

Extracytoplamismic function (ECF) sigma factors comprise a sequence that differs from most other sigma factors, and they usually outnumber all other types of sigma factors combined. ECF sigma factors are small regulatory proteins. We found that when these proteins were overexpressed in FK506 and FK520, the result was an increase yield in both polyketides: FK506 synthesis increased by 150% and FK520 synthesis increased by 100%. To achieve optimum FK506 synthesis, we combined ICM inactivation and ECF sigma factor overexpression. This was accomplished in three steps:

Step I: Overexpression of ECF RNA polymerase sigma factor in the ICM inactivated FK506 producer. The overexpression vector of ECF RNA polymerase sigma factor was transferred into the ICM inactivated FK506 producer using the E. coli-Streptomyces intergeneric conjugation method.

Step II: Quantification of FK506 production in the ECF RNA polymerase

sigma factor overexpressed-ICM inactivated FK506 producer. To quantify FK506 production in the ECF RNA polymerase sigma factor overexpressed-ICM inactivated FK506 producer, this strain was fermented for 5 days, its culture broth will be extracted by methanol, and the extracts will be analyzed by HPLC (High Performance Liquid Chromatography).

Step III: Confirmation of the function of ECF RNA polymerase sigma factor as an upregulator in the FK506 biosynthetic gene cluster. To confirm whether ECF RNA polymerase sigma factor induces the FK506 biosynthetic gene cluster, the gene expression level of PKS (Polyketide Synthase), which is located in the FK506 biosynthetic gene cluster, was checked and compared in wild-type and ECF RNA polymerase sigma factor overexpressed FK506 producer by RT-PCR (Reverse Transcription-PCR).

The wild-type strain was cultured in production medium, and the transcription levels of allylmalonyl-CoA biosynthetic genes were confirmed at 16h, 20h, 24h, 48h, and 72h by RT-PCR. Each primer for RT-PCR was designed to produce 500 bp of DNA band. RNeasy Mini Kit (Qiagen) was used to isolate total RNA from samples, and Maxima first strand synthesis kit was used to make cDNA. 500 ng of RNA template used for cDNA synthesis, and 1ul of cDNA was used for PCR. PCR was performed for 38 cycles.

ECF σ-factor was cloned into integrative plasmid pSET152 with constitutive *ermE** promoter introduced into *Streptomyces* sp. KCCM 11116P by *E. coli-Streptomyces* conjugation. As controls, we also included strains with or without the plasmid pSET152. Each strain was cultured in R2YE medium, and the transcription level of each gene was confirmed at 72 h by RT-PCR. Primers for RT-PCR were designed to produce approximately 500 bp of DNA band. RNeasy Mini Kit (Qiagen) was used to isolate total RNA from samples, and Maxima first strand synthesis kit was used to make cDNA. 500 ng of RNA template was used for cDNA synthesis, and 1ul of cDNA was used for PCR. PCR was performed for 31 cycles.

For this study, the transcription level of biosynthetic genes (*fujA, fujB, fujC*, and *fujD*) of the FK506 wild-type was analyzed and compared. As a result, the transcription level of biosynthetic genes was enhanced with ECF σ-factor overexpression, relative to wild-type. These results also show that gene cluster productivity is enhanced by the ECF σ-factor