1 **Title**

2 **Combination of Atmospheric and Room Temperature Plasma (ARTP) mutagenesis,**

3 **Genome shuffling and Dimethyl sulfoxide (DMSO) feeding to improve FK506**

4 **production in *Streptomyces tsukubaensis***

5 Authors

6 Liting Ye[a*], Ruifang Ye[a*], Fengxian Hu[a], Guozhu Wang[a]

7 Affiliations

8 a State Key Laboratory of Bioreactor Engineering, East China University of Science and

9 Technology, 130 Meilong Road, Shanghai 200237, China

10 * Corresponding author:Ruifang Ye

11 E-mail addresses: rfye@ecust.edu.cn

12 * Corresponding author:Liting Ye

13 E-mail addresses: litingYeee@163.com

14 Phone: +86-21-64253286

15 Fax: +86-21-64253286

16

Abstract

FK506 is a clinically important macrocyclic polyketide with immunosuppressive activity produced by *Streptomyces tsukubaensis*. However, the production capacity of the strain is very low. To improve production, atmospheric and room temperature plasma (ARTP) mutagenesis was adopted to get the initial strains used in genome shuffling (GS). After three rounds of GS, *S. tsukubaensis* R3-C4 was the most productive strain, resulting in a FK506 concentration of 335 μg/mL, 2.6 times than that of the original wild-type strain. Moreover, exogenous DMSO 4 % (v/v) addition could induce efflux of FK506 and increased FK506 production by 27.9% to 429 μg/mL. Finally, analyses of the differences in morphology, fermentation characteristics and specific gene expression levels between *S. tsukubaensis* R3-C4 and the wild-type strain revealed that R3-C4 strain: has hampered spore differentiation, thicker mycelia and more red pigment, which are likely related to the downregulation of *bldD* and *cdgB* expression. In addition, the expression levels of *fkbO*, *fkbP*, *dahp*, *pccB* and *prpE* all showed up-regulation at diverse degrees compared to the wild-type *S. tsukubaensis*. Overall, these results show that a combined approach involving classical random mutation and exogenous feeding can be applied to increase FK506 biosynthesis and may be applied also to the improvement of other important secondary metabolites.

Keywords：*Streptomyces tsukubaensis*；ARTP mutation；genome shuffling；DMSO

Introduction

FK506 (also named tacrolimus) is a 23-membered polyktide macrolide with immunosuppressant activity, which was originally isolated from the soil bacterium *S.tsukubaensis* in 1987 (Kino et al., 1987). FK506 has gradually replaced the utilization of cyclosporine (CsA) since its FDA approval in 1994, and now it becomes one of the best-selling medicines for treating patients after solid organ transplantation in the world (Meier-Kriesche et al., 2006). FK506 has an immunosuppressive effect of 10 to 100 times that of CsA. In clinical practice, it is widely used in the prevention of organ transplant rejection, but also in the treatment of neonatal Rh hemolysis and autoimmune diseases such as allergic dermatitis and eczema (Thomson et al., 2006).

Biosynthesis of FK506 is initiated by polyketide synthase (PKS) catalyzed condensation. The chorismic acid under the action of FkbO forms the starting unit 4,5-dihydroxycyclohex-1-enecarboxylic acid (DHCHC). Then DCHCHC and ten extender units, namely, two malonyl-CoA, five methylmalonyl-CoA, two methoxymalonyl-ACP, and a unique allylmalonyl-CoA extender unit are incorporated into nascent polyketide chain. The linear polyketide chain is followed by incorporation of lysine-derived pipecolic acid and a cyclization step, mediated by FkbP peptide synthetase, resulting in the earliest macrolactone intermediate of FK506. Finally, post-PKS processing reactions are catalyzed by a specific methyl transferase (FkbM) and oxidoreductase (FkbD) resulting in FK506 (Andexer et al., 2011; Mo et al., 2011).

In order to meet the increasing demand of FK506, considerable effort has been invested

over the past few years, many important genes of the biosynthetic cluster have been elucidated (Mo et al., 2011; Motamedi et al., 1998; Kosec et al., 2012). Medium optimization (Kim and park 2007), exogenous feeding (Huang et al., 2013; Huang et al., 2013; Yan 2015), metabolic pathway modification (Chen et al., 2013; Chen 2015) and the traditional breeding methods such as mutagenesis (Du et al., 2014) have also been carried out. Recently, atmospheric and room temperature plasma (ARTP) mutagenesis has been proven to be an effective tool to generate stable high-yield mutant strains for microorganism breeding (Ottenheim et al., 2018). Nevertheless, these approaches and techniques are still limited and inefficient to obtain the desirable high-producing phenotype. More importantly, the demand to engineer more complex phenotypes requires a more combinatorial approach.

Genome shuffling (GS) has been demonstrated to be an excelent evolutionary tool for the rapid improvement of both antibiotic production and complex cellular phenotypes. As an efficient technology, it offers the advantage of simultaneous changes at different sites throughout the whole genome even without necessity of detailed genetic and network information. Currently, the method has been successfully used in many organisms to increase their metabolite productivity (Li et al., 2012; Wang et al., 2012; Hui 2006; Chen et al., 2009).

In this study, we first explored the conditions for ARTP mutagenesis and protoplast preparation, regeneration, lethal and fusion of *S. tsukubaensis* No.9993. Then, mutants were used as the initial strains for GS, and a FK506 high-yielding strain was obtained by high-throughput screening. As far as we know, Dimethyl sulfoxide (DMSO) is a polar aprotic solvent, which can be used as a permeable protectant and has a certain effect on changing the

permeability of biofilms to the electrolytes, metabolites, drugs and toxicants (Santos et al., 2003; Meravý et al., 1988). Therefore, the method of adding a certain concentration of DMSO was adopted to further improve the FK506 productivity by promoting its efflux from cells. So far, there is report of an increase in FK506 production after adding DMSO at the mid-exponential growth phase in *S. tsukubaens*is (Martínez-Castro et al., 2013). Subsequently, the differences in the morphology, fermentation characteristics and expression levels of key genes in the metabolic pathway between recombinant and wild-type strain were analyzed, which might provide novel information on the metabolic mechanism of FK506 biosynthesis and further guide the strain development and fermentation technology improvement.

Materials and Methods

Microorganisms

The strain *S. tsukubaensis* No.9993 (the wild type strain) was used throughout this work, which was stocked in our laboratory.

Medium and culture conditions

S. tsukubaensis No.9993 was cultured for sporulation on ISP4 agar (1) plates consisting of 1% soluble starch, 0.1% K_2PO_4, 0.1% $MgSO_4$, 0.1% NaCl, 0.2% $(NH_4)_2SO_4$, 0.2% $CaCO_3$, 0.001% $MnCl_2$, 0.001% $FeSO_4$, 0.001% $ZnSO_4$, 1.5% agar at pH 7.0 for sporulation. A square shaped spore mass with a side length of 0.5cm was then inoculated into 30 mL of seed medium (2), containing 1.0% glucose, 1.0% tryptone, 1.0% glycerol, 0.5% yeast exact, at pH

7.4, and was cultured at 28℃, and 220 rpm for 48 h. Subsequently, 0.5mL of the seed culture was used for the inoculation of fermentation medium (25mL). The fermentation medium (3) contained 0.6% glucose, 0.15% yeast exact, 1.23% soybean meal, 2.5% groundnut meal, 0.5% K_2HPO_4, 3.0% corn starch with pH adjusted to 7.6-7.8 before autoclaving. Fermentation was performed at 28℃, and 220 rpm for 168h.

The R2YE regeneration medium (4) contained glucose 10 g/L, tryptone 4 g/L, yeast extract 3 g/L, sucrose 103 g/L, potassium sulfate 0.25 g/L, magnesium chloride hexahydrate 2.03 g/L, trace elements 2 mL, potassium dihydrogen phosphate 5 g/L, anhydrous calcium chloride 36.8 g/L, Tris-HCl 25 g/L, agar powder 20 g/L.

Both slant and plate cultures were incubated for 5-7 days at 28℃.

High-throughput screening method

1 mL of seed medium was added to each well of the aseptic 48-well microtiter-plate, the spore mass contained in the ISP4 agar piece within 0.5cm in diameter were inoculated into each well (controls were set), covered and incubated at 28℃for 48 h, by shaking at 250 rpm/min. 2% (v/v) of the seed culture was transferred into one corresponding well of microtiter plates with 0.6mL of sterile fermentation medium. The cultivation was carried out at 28℃for 7d by shaking at 250 rpm.

After the fermentation, 0.9mL methanol was added into each well of microtiter plates, which was shaken for 10min and then allowed to stand for 1h. After centrifuging at 3000rpm for 10min, the supernatant was filtered with 0.22 μm filter membranes and the yield of FK506 was determined by high-performance liquid chromatography (HPLC).

In addition, the method of deep-well plates culture was optimized because of the difference of dissolved oxygen in different positions of deep-well plates, observed during the experiment. Controls must be set in each circle of the microtiter plates (Figure S1), and the measured yield of FK506 should be compared with that of the controls in the corresponding circle.

Analytical Methods

FK506 production of the strain was measured by HPLC, equipped with a Zorbax SB-C18 packing column (4.6×150 nm, 5 μm; Agilent, USA). The mobile phase was composed of 0.1% H_3PO_4:acetonitrile = 32:68 (v/v) with a flow rate of 1 mL/min. The column temperature was 50℃, and the detection wavelength was 205nm (Yeiwei et al., 2006). However, in order to measure extracellular FK506 production, the fermentation broth was directly sampled to centrifuged for 5min at 10000rpm and 4℃, without adding methanol. And the supernatant was filtered and measured by HPLC after filtering.

Bacterial concentrations were measured as PMV (Pacaked Mycelia Volume) following the procedure: took 10mL of fermentation broth into a clean 15mL centrifuge tube and centrifuged at 3000rpm for 10min. The volume of supernatant was measured with a measuring cylinder, denoted as V_1.

$$\mathrm{PMV}(\%) = \frac{10mL - V_1}{10mL} \times 100$$

The medium of *S. tsukubaensis* is a complex medium, which contains insoluble substances of soybean powder. Therefore, this method only obtains rough values, but it can be used to estimate PMV due to its simple operation.

Procedures for ARTP mutagenesis

Spores grown on ISP4 medium for 3-5 days were scraped and washed with 0.85% sterile saline solution and then diluted to an OD_{600} of 0.6-0.8 before mutagenesis. ARTP mutagenesis was performed using the ARTP breeding system (ARTP-III, Sichuan Siqingyuan Biotechnology Co., Ltd., Wuxi, China). Pure helium was used as the working gas at a flow rate of 0.8 SLPM (Standard Liters Per Minute). The RF power input was 100W. The distance (D) between the plasma torch nozzle exit and the sample plate was 4mm and the plasma jet temperature was below 30℃. To provide different dosages of the active species in the plasma jet region, 10μL of the spore suspension was pipetted onto the stainless minidisk and then exposed to ARTP jet for 0, 30, 45, 60, 75, 90s, respectively. After mutagenesis, the cells were eluted with sterile saline solution into a 1.5 mL centrifuge tube, Then the mutated cell suspension was diluted gradiently with normal saline solution to a suitable concentration, spread on the ISP4 agar plates and grown at 28℃ for 5-7 days.

Genome shuffling

GS was carried out using the described methods with modifications (Wang et al., 2017; Liu et al., 2020). Spore of the starting strains were inoculated in 50mL Erlenmeyer flasks containing 30mL of seed medium and cultured at 28℃ and 220rpm for 2days. Subsequently, 1% (v/v) of the above seed medium was used for the inoculation of the second seed medium, the composition of which is the same of the seed medium except for the presence of glycine 0.5%. After incubation for 1d, mycelia were harvested by centrifugation at 3000 rpm for 10

161 min, washed twice with P buffer (containing sucrose 103 g/L, potassium sulfate 0.25 g/L,
162 magnesium chloride hexahydrate 2.03 g/L, trace elements 2 mL, potassium dihydrogen
163 phosphate 5 g/L, anhydrous calcium chloride 36.8 g/L, Tris-HCl 25 g/L). Then lysozyme
164 (Shanghai Macklin Biochemical, Co. Ltd., Shanghai, China) was added at a final
165 concentration of 1 mg/mL, protoplasts were harvested by centrifuging at 800rpm at room
166 temperature after digesting at 32°C for 1 h, protoplasts were divided into 2 allicuots, one was
167 used for UV inactivation for 10 min and the other was heated at 60℃ for 10 min. Equal
168 volume of protoplasts of four target strains were mixed in 5 mL 50% PEG1000 fusion
169 solution and incubated at 32°C for 5 min, collected and washed twice with 5 mL buffer P. The
170 protoplast solution after the fusion process was diluted and spreaded on the regeneration
171 R2YE plate, and then single colonies with good growth were picked and transferred to ISP4
172 plates to restore the growth.

173 **Assay of gene expression by quantitative real-time RT-PCR (qRT-PCR)**

174 Gene transcription profile was determined by qRT-PCR using the SuperReal PreMix
175 Plus kit (TIANGEN). The samples were isolated from the wild-type and the fusion strain
176 R3-C4 of *S. tsukubaensis* at 48, 72 and 96h, as well as before and after DMSO addition. Total
177 DNA was extracted using Trizol reagent (TIANGEN) according to the manufacturer's
178 instructions. Primers were designed (Supplementary Table S1) based on published genome
179 sequence. The RNA 16s was used as an internal control. Data analysis was performed
180 according to the comparative C_T method. Fold change of the gene expression of the
181 recombinant strain was calculated by normalizing the value of the control wild-type.

qRT-PCR experiments were done in triplicate, using RNA samples from three independent cultures.

Scanning electron microscopy (SEM) sample processing method

The model of scanning electron microscope (SEM) used in this study is Hitachi S3400N. The method of sample processing is to soak the agar blocks with spores, dug on the ISP4 plates, into 2.5% glutaraldehyde solution for fixing. After being placed overnight, the blocks were air-dried on the clean bench. After cutting the dried sample to a suitable size, the conductive adhesive is used to stick the sample on sample table of SEM. After freeze-drying with liquid nitrogen, the gold is sprayed onto the surface of the sample to increase its electrical conductivity.

Results

Mutant screening

Based on preliminary experiments, ARTP mutagenesis was performed on *S. tsukubaensis* No.9993. To isolate high FK506 producing strains from 450 mutants after the ARTP treatment, the strains were subjected to deep-well microtiter-plate cultivation combined with HPLC. Shake flask fermentation was then performed to verify the candidate mutants identified in the 48-deep-well microtiter-plate cultivation. As a result, four ARTP mutants (YB-2830, YB-6009, YB-1513, and YB-9009) were selected due to their improved productivity (compared to the wild type) and genetic stability (Table 1). The highest FK506

201 production from YB-2830 reached to 175 μg/mL, which represented a 37.2% increase
202 compared to the wild-type strain. These results indicated that ARTP mutagenesis is a
203 promising mutagenesis tool for industrial applications.

204 **High-yield FK506 producing strain by GS**

205 The genome shuffling method seems useful to improve FK506 biosynthesis of
206 microorganism (Du et al., 2014). Recombinant strains have potential for increased FK506
207 production. For this reason, three successive rounds of GS were carried out with four strains:
208 two ARTP mutants (YB-1513 and YB-2830) and their respective descendants (YB-1514 and
209 YB-2809). As shown in Figure 1 (and Table S2), the FK506 production increased gradually
210 after each round of GS. After three rounds of protoplast fusion, three highest FK506 yield
211 strains were selected from 478 colonies. The most productive strain, named R3-C4, yielded a
212 concentration of 335μg/mL FK506, which was 2.6-fold of that of the wild-type strain. Genetic
213 stability is a very important criteria for high-yielding strains originated from various
214 treatments of mutagenesis or recombination (Luo et al., 2012). Hence, the genetic stability of
215 these high yield fusion strains by three successive cultivations and shake flask fermentation
216 was evaluated (Table 2). The range of production levels among three generations ranged from
217 325 ± 3.4 to 335 ± 5.8 μg/L, suggesting that the hereditary characteristics of the high FK506
218 producing recombinant R3-C4 strain were stable.

The differences in solid and liquid media between the high-yield FK506 recombinants and the wild-type strain

It was found in slant culture that there were obvious differences in morphology, fermentation characteristics and specific gene expression levels between high-yield FK506 recombinants and wild-type strain. Therefore, the dynamics in bacterial growth were compared among recombinants (R3-A31, R3-B9, R3-C4) and wild-type strain. In slant culture, the recombinants showed a relatively slower growth and a bald colony phenotype, suggesting a blockage in aerial mycelium and spore formation (Fig.2). The wild-type strain was covered with abundant long spore chains separated by fully developed septa, however, the R3-C4 strain only produced stronger and tighter wound mycelium and merely few spore chains were visible after 4 days of growth (Fig.3). The comparison suggested a blockage in spore differentiation. Furthermore, recombinants also produce more red pigments than the wild type strain (Fig.3).

In addition, there was a significant difference in the fermentation broth character between wild-type and R3-C4 strain, of which the fermentation broth was thicker and reddish (Fig. 3a). According to the PMV measurement during fermentation the increased FK506 production of R3-C4 strain was partly due to the increased biomass (Fig. 3b).

Transcriptional differences of key genes between R3-C4 and wild-type strain

In this study, transcription level of two fkb cluster genes (*fkbO*, *fkbP*), two transporter genes (*drrA*, *drrB*), three enzyme genes in metabolic pathway (*dahP, pccB, prpE*), and two

239 regulatory genes (*bldD, cdgB*) were investigated to explain the possible mechanism of the
240 increasing FK506 productivity of recombinants R3-C4. The transcription levels of *fkbO* and
241 *fkbP* were 9.8-fold and 4.1-fold up-regualted in R3-C4 strain at 96h compared to those of
242 wild-type strain (Fig. 3c), speculating that more FK506 was synthesized by recombinant in
243 the fermentation process, possibly because of more cyclized linear polyketide chain and a
244 large number of precursors were supplied for FK506 biosynthesis.

245 Previous studies revealed that *bldD* is a transcriptional regulator essential for
246 morphological development and antibiotic production in *Streptomyces coelicolor* (Elliot et al.,
247 1998; Elliot et al., 2001). It was found in *S. coelicolor* that *bldD* gene encodes a small
248 molecule protein BldD that can bind to DNA. In addition to binding to its own promoter
249 region to block its own transcriptional expression, BldD protein can also bind to the
250 promoters of *bldN*, *whiG* and *sigH*, to affect their expression, and then regulates the synthesis
251 of antibiotics in *S. coelicolor*. In addition, the regulatory mechanism of *bldD* on aerial hyphae
252 formation of *S. coelicolor* has also been well studied (Elliot et al., 1998). Meanwhile, the
253 regulatory effect of *bldD* on morphological differentiation and secondary metabolism has also
254 been found in other Streptomyces. For example, on the cellular differentiation gene *amfTSBA*
255 in *Streptomyces griseus* (Ueda et al., 2005). The transcription of *bldD* of R3-C4 at 48h was
256 60% lower than that of the wild-type strain (Fig. 3c), which meant that the transcription level
257 of *bldD* in *S. tsukubaensis* No.9993 was positively correlated with sporulation. Additionally,
258 combining the above results with the increased FK506 production, it can be concluded that
259 the regulation of *bldD* for morphological development was negatively correlated with the

biosynthesis of FK506 in *S.tsukubaensis* No.9993.

Propionyl-CoA synthase encoded by *prpE* can use propionate to form propionyl-CoA, which can be transformed into methylmalonyl-CoA under the catalyzation of Propionyl-CoA carboxylase encoded by *pccB* (Mo et al., 2009). The transcription levels of *pccB* and *prpE* of R3-C4 strain were 5.5 and 2.6 times higher than those of wild-type strain at 96h (Fig. 3c). *dahp* is responsible for encoding the first rate-limiting enzyme of shikimic acid pathway, 3-deoxy-7-phosphohetulonate synthase (DAHP synthase). The transcription of *dahp* in R3-C4 strain was 3.0 times higher than that in wild-type strain at 96h (Fig. 3c).

Secondary messengers (such as (p)ppGpp and c-di-GMP) play important roles in antibiotic biosynthesis and morphological differentiation in actinomycetes, and the intracellular levels of c-di-GMP can control the developmental switch between vegetative growth and sporulation by binding to BldD (Schumacher et al., 2017; Zhen et al., 2019). Moreover, diguanylate cyclases (DGCs) are responsible for the synthesis of c-di-GMP and coordinates with phosphodiesterases (PDEs) to control the intracellular c-di-GMP level (Jenal et al., 2017). It was found in this study that the transcription level of *cdgB* (a diguanylate cyclase encoding gene) in R3-C4 strain reached its highest level at 72 h, which was 3.6 times than that of the wild-type strain (Fig. 3d). Therefore, it is speculated that the increased FK506 production of R3-C4 strain might be resulted from the up-regulation of *cdgB* that improved the level of c-di-GMP. In addition, differences in morphological differentiation between R3-C4 strain and wild-type strain analyzed above may also be related to *cdgB*.

Effect of DMSO on FK506 production

Some researchers investigated the effects of DMSO at different concentrations on metabolic activity of cultured tobacco cells. It has been found that DMSO could promote the effloxation of proteins and phenols by changing the permeability of cell membranes in short-term culture (Meravý et al., 1988). Careful application of this solvent increased uptake of nutrients from the medium affecting in this way cell metabolism (Fritsch et al., 1988). Moreover, previous studies have reported that tacrolimus production could be improved by 64.7% in the DMSO and La combined treatment by enhancing the metabolic pathway from acetyl-CoA to malonyl-CoA (Cheng et al., 2018). Therefore, DMSO was chosen to further enhance the FK506 productivity of recombinant R3-C4.

A range of DMSO concentrations from 1 to 5% (v/v) were added to the cultures of R3-C4 strain, resulting in a significant increase of FK506 yields (Fig. 4a). The highest FK506 yield, 429μg/mL, was achieved with a treatment of 4 %(v/v) DMSO. This FK506 yield shows an increase of the 27.8% over the control and a 3.4-fold enhancement in relation to the wild-type strain. Nevertheless, the PMV of the samples treated by DMSO was lower than that of the control (Fig. 4b), which revealed that the increase in FK506 production was not caused by biomass. But in previous study (Martinz-Castro et al., 2013), DMSO addition did not affect biomass formation. The different results may be caused by different fermentation medium or different addition amount of DMSO or other reasons. The yield of intracellular and extracellular FK506 were measured separately both before and after 4 %(v/v) DMSO treatment (Figure 4C). It was shown that the extracellular content of FK506 in the control

sample without DMSO was close to zero, but those of the samples treated by DMSO increased with the extension of fermentation time and reached the maximum value at 96h, after which the external FK506 remained stable or slightly decreased. We hypothesize that exogenous DMSO addition is likely to change the permeability of cell membrane, inducing efflux of the intracellular metabolite FK506, and reducing the product feedback inhibition.

Effect of DMSO on expression level of key genes

The expression levels of FK506 biosynthetic genes (*fkbO, fkbP*) and putative transporter genes (*drrA, drrB*), which were determined in *S.tsukubaensis* by sequence alignment with *Streptomyces peucetius*, were investigated at 96h of fermentation by using qRT-PCR. There is a significant up-regulation in the expression of *fkbO* and *fkbP* in the DMSO addition condition compared to that of the control without DMSO (Fig. 4d). The formation of initial macrolactone intermediate of FK506 (preFK506) is followed by the incorporation of pipecolate and a cyclization step, mediated by FkbP peptide synthetase (Huang et al., 2013). Chorismatase (FkbO) is encoded by *fkbO*, which is the rate-limiting enzyme in the first step of FK506 biosynthesis (Du et al., 2014). It is suggested that the transcription of *fkbO* is regarded as a signal of FK506 overproduction (Yan Sun, 2015). Therefore, the up-regulation of *fkbO* and *fkbP* expression in R3-C4 was probably a reason of an increased FK506 production of R3-C4 strain with DMSO treatment. Notably, it was observed that after adding DMSO, the gene expression level of *drrA* (ABC transporter ATP-binding protein encoding gene) and *drrB* (ABC transporter permease encoding gene) increased 2.0 times and 2.6 times compared to that of control at 96h. According to the results, we speculated that DMSO can

activate the expression of membrane protein, promote efflux of intracellular FK506, reduce feedback inhibition of product, and increase the yield of FK506.

Discussion

GS has become a promising technique to enhance secondary metabolite production in a variety of microorganisms since it was discovered (Lv et al., 2013; Zhang et al., 2013; Zhao et al., 2012). As an artificial evolution method, it addresses the limitations of classical strain improvement, and can greatly improve the frequency of desired mutation in microbial cells (Tong et al., 2018). Meanwhile, the novel ARTP mutagenesis technology provides a mutational library containing multiple positive mutants for GS. Furthermore, when GS and ARTP are combined with the improved high-throughput screening method, the strains with improved phenotype can be screened efficiently and rapidly.

In this study, ARTP mutagenesis technology was first applied for preliminary improvement in the low FK506 producing wild-type *S. tsukubaensis* strain, and the yield of FK506 was increased by 37.2% after the mutagenesis. We also investigated the preparation, regeneration, lethal and fusion conditions of *S.tsukubaensis* No.9993 protoplasm system, reaching the fusion rate of 73.5% which was not further optimized due to time constraints. Meanwhile, from our observations, we suggest that protoplast should not be stirred during UV inactivation, because the shear force brought by the agitator may damage the protoplast, since the lack of cell wall weakens the cells and hampers regeneration.

Then, after three rounds of GS performed on four ARTP mutants, a high-yielding and

genetic stable strain R3-C4 was successfully obtained, whose production reached 335μg/mL, 2.6-fold enhancement of the wild-type strain, indicating that the combination of ARTP mutagenesis and GS is effective in microbial breeding.

In addition, by investigating the morphological differentiation we found that there was a significant decrase in growth, stronger mycelium formation and inhibited spore differentiation, which was possibly related to downregulation of *bldD* and *cdgB* in R3-C4 compared to the wild type. Combined with previous research (Mo et al., 2009; Schumacher et al., 2017), the regulatory mechanism of *cdgB* and c-di-GMP on morphological differentiation and antibiotic synthesis of *S.tsukubaensis* needs further investigation. Of course, there may also other growth factors and genes associated with morphology (such as *whiB* and *adpA*) (Davis et al., 1992; Xuliang et al., 2019) and sporulation (Dongxu et al., 2018), which were not studied in this study. Moreover, it was found that in slant culture and fermentation, high-yielding fusion strains, altered the secondary metabolism, and tacrolimus production was enhanced, also producing a pink pigment, which deserved further analysis.

The DrrAB system represents the simplest form of an ABC drug transporter, which is believed to be a single-drug transporter, assembled from two molecules of DrrA (the catalytic subunit) and DrrB (integral membrane subunit) (Kaur et al., 1998). However, DrrAB system turns out to be as a multidrug transporter like most MDR proteins in *Streptomyces peucetius* reported in the later studies (Wen et al., 2014). ABC transporter ATP-binding protein (DrrA) and ABC transporter permease gene (DrrB) were detected in *S.tsukubaensis* No.9993 by homologous sequence alighment. Although there was no significant difference in R3-C4 and

the wild-type in terms of expression levels of *drrA* and *drrB* (Fig 3), it was found that the expression levels of both genes were up-regulated after adding DMSO to optimize the FK506 productivity of R3-C4 strain. Therefore, including the change of extracellular FK506 yield before and after the addition of DMSO, we hypothesized that the DrrAB efflux system is associated with the transportation of tacrolimus in *S. tsukubaensis*. Therefore, genetic engineering on *drrA* and *drrB* to increase the expression may be an effective way to further increase the yield of FK506, but many other experiments are needed to confirm this hypothesis. In addition, it has been reported that adding low-concentration of DMSO can promote the production of antibiotics by strengthening primary metabolic pathways and antibiotics biosynthesis precursor pathways (Cheng et al., 2018; Cheng et al., 2019). Since there was a report of the increase of FK506 in *S.tsukubaensis* by the addition of DMSO, the mechanism of action of DMSO has not been further studied (Martinez-Castro et al., 2013).

In conclusion, the combination of ARTP and GS and the findings above have provided an approach to obtain FK506 overproducing strains. The method utilized in this study can also be applied in other studies to improve antibiotic yields in other actinobacteria. However more detailed mechanisms need to be examined through additional experiments.

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Table 1 The result of each generation by ARTP mutagenesis

Strains	Preliminary screening (μg/mL)	Generation F_1(μg/mL)	Generation F_2(μg/mL)	Generation F_3(μg/mL)	Increased ratios
No.9993	119	129±2.1	125±6.9	129±3.5	-
YB-2830	181	175±3.2	173±2.8	177±1.3	37.2%
YB-6009	142	148±5.4	148±3.6	153±2.9	18.6%
YB-1513	139	143±3.6	144±5.0	149±7.1	15.5%
YB-9009	139	144±1.7	142±2.1	144±5.9	11.6%

The data of preliminary screening was measured after high-throughput cultivation, and the FK506 production of every generation was measured after shake flask fermentation. The values are presented as the mean±standard deviations from three independent experiments.

Table 2 The stability of high yield fusion strain

Strain	Production of 1st generation μg/mL	Production of 2nd generation μg/mL	Production of 3rd generation μg/mL	Improvement （fold）
R3-C4	335±5.8	326±7.9	325±3.4	1.64
R3-B9	303±12.6	304±3.7	289±6.3	1.39
R3-A31	308±5.5	306±2.1	290±11.4	1.43

The values are presented as the mean±standard deviations from three independent experiments.

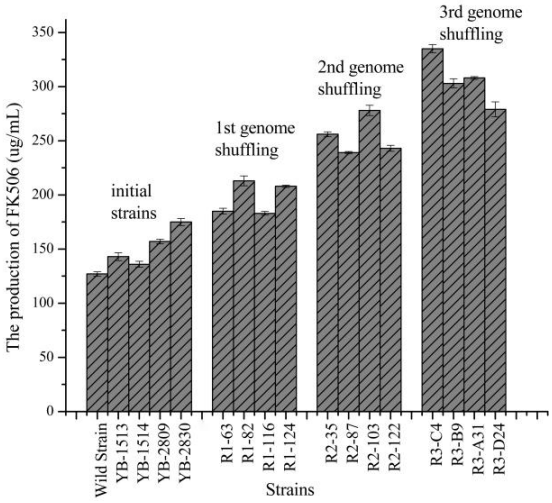
Figure Captions

Fig 1 The result of three rounds of protoplast fusion. The figure shows the FK506 production of wild-type strain, the four initial strains used for GS (YB-1513, YB-1514, YB-2809, YB-2830), and four strains with the highest FK506 yield per round of GS.

Fig 2 The mycelium and spore chains seen by SEM. a: The mycelium and spore chains seen at 5000 x (left: *S. tsukubaensis* R3-C4; Right: the wild-type strain No.9993); b: The mycelium and spore chains seen at 10000 x (left: *S. tsukubaensis* R3-C4; Right: the wild-type strain No.9993).

Fig 3 Difference between S. tsukubaensis R3-C4 and the wild-type strain. a: Different Color of fermentation broth. The images were photographed at final fermentation. WT represent for the wild-type strain. b: The dynamic change curve of PMV during fermentation, values were obtained from three independent experiments. c: Difference of key gene expression between R3-C4 strain and wild-type strain, values were obtained from three independent experiments. d: Difference of key gene expression between R3-C4 strain and wild-type strain, values were obtained from three independent experiments.

Fig 4 Effects of adding DMSO during fermentation. a: The effect of DMSO on the FK506 production; b: The effect of DMSO on PMV; c: The effect of DMSO addition on the FK506 content in the intracelular and extracelular media; d: The effect of DMSO on *fkbO*、*fkbP*、*drrA*、*drrB* gene expression at 96h by qRT-PCR. All values were obtained from three independent experiments.



601

602 **Fig.1**

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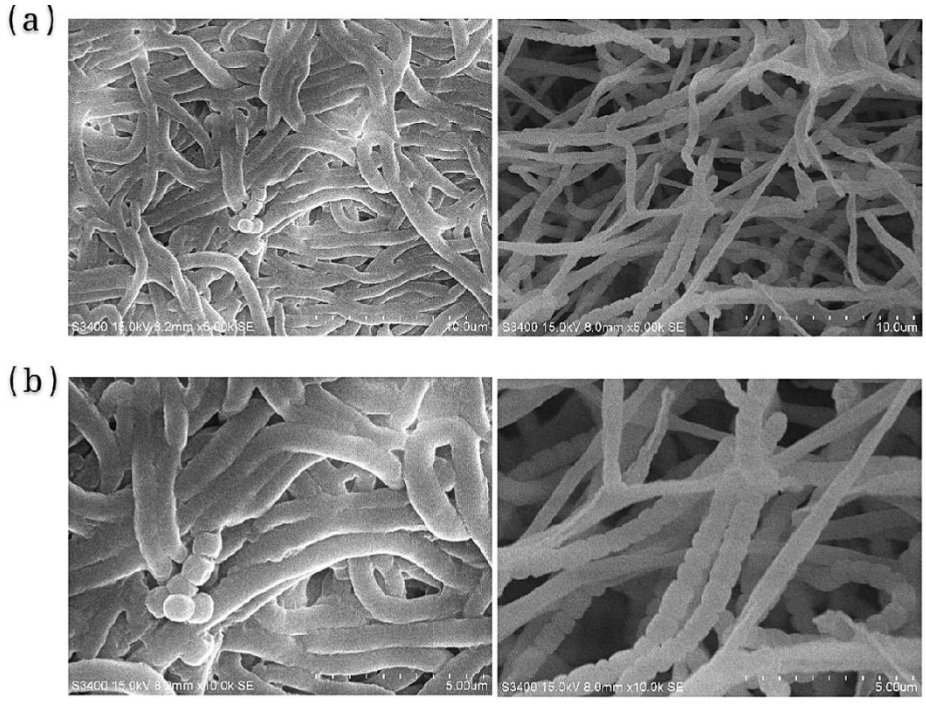

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607 **Fig.2**

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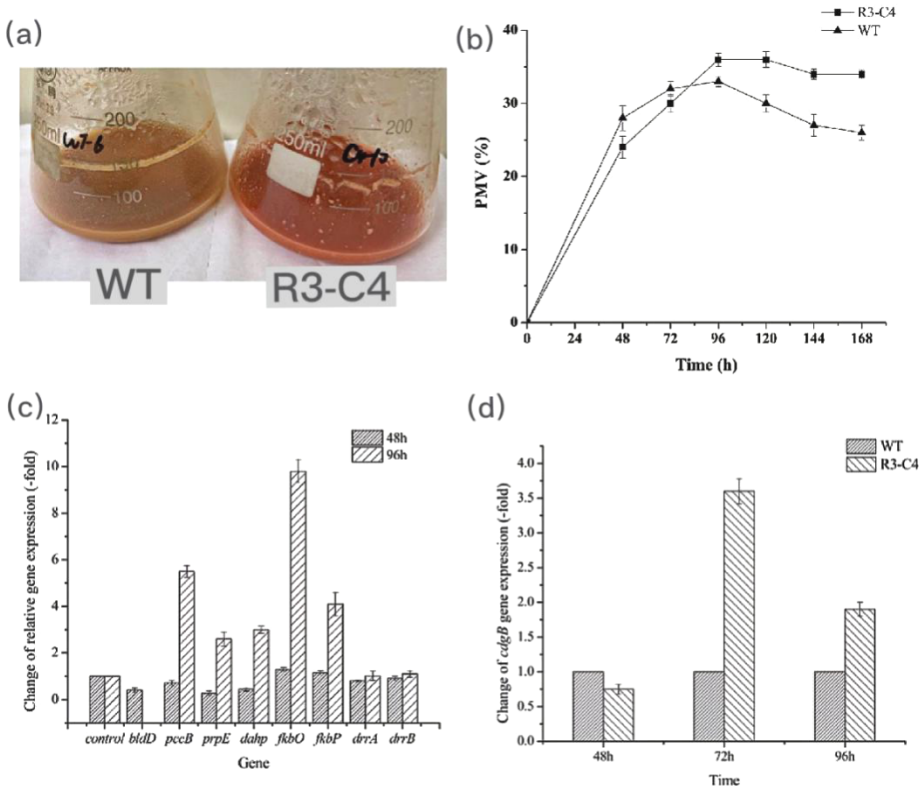

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612 **Fig.3**

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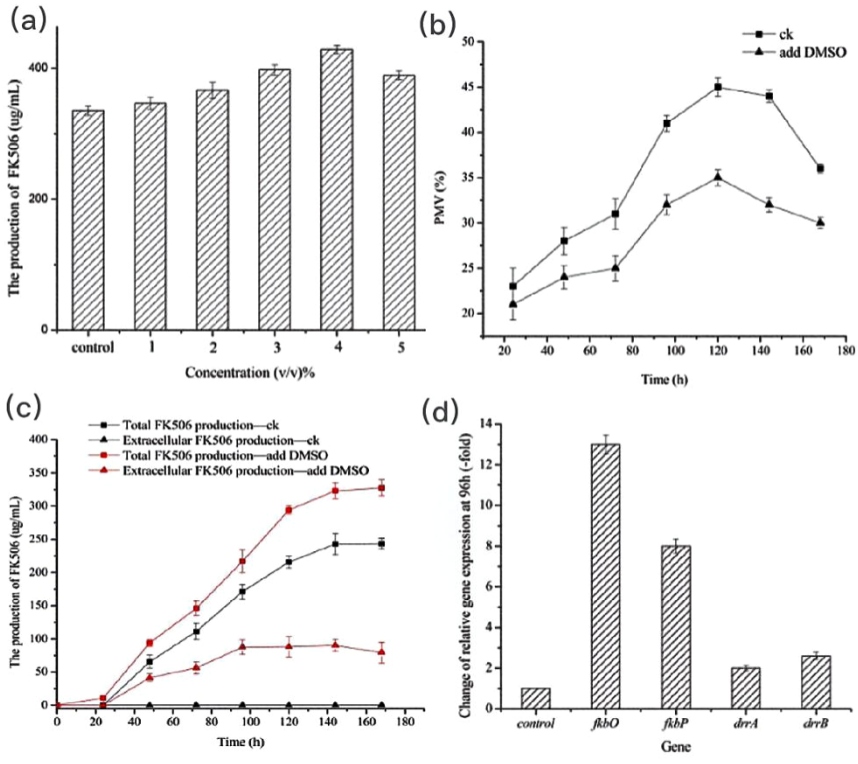

614

615 **Fig.4**

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Supplementary Materials

Table S1 The primers used for qRT-PCR

引物	序列 5’-3’
RT-*fkbP*-F	GCACCGACGAGTACCTGTTC
RT-*fkbP*-R	GCGTTGATCCCACTGATGTA
RT-*fkbO*-F	GCTGCCCGTCGAGACACT
RT-*fkbO*-R	TCCGCATAGGTGAGTGAGGT
RT-*dahp*-F	CTTCGTGAAGTCGTCCCCCT
RT-*dahp*-R	CTCGTGGGAGGCGAAGAAC
RT-*drrA*-F	CGCAAGAGGTACGGGGAGAAG
RT-*drrA*-R	TGTCCTGCGTCGAACTTCAG
RT-*drrB*-F	ATGGTCGGCTATCTCCTGGG
RT-*drrB*-R	AAGGCGAAGACGAGCAGTAG
RT-*bldD*-F	AGCACACCCCAGTTGATGAG
RT-*bldD*-R	GACCATCCAGTCCCAGCG
RT-*cdgB*-F	TCCTCGGTGTGATCTCCGT
RT-cdg*B*-R	CCAGCGCCCTTTGCATATTT
RT-*pccB*-F	TACGGCCAGAAGATCGTCAA
RT-*pccB*-R	GAAGATCTCGCCGTACATCCC
RT-*prpE*-F	TCCTCGGTGTGATCTCCGT
RT-*prpE*-R	ATCGGCCGTACACCAGTAGA
RT-*16sRNA*-F	GCATCTGTGGTGGTTGAAAG
RT-*16sRNA*-R	CGTGTCTCAGTCCCAGTGTG

Table S2 The results of three rounds of protoplast fusion

Strains	Production ug/mL	Strains	Production ug/mL	Strains	Production ug/mL	Strains	Production ug/mL
Wild-type	127±2.1	1st round		2nd round		3rd round	
YB-1513	143±3.6	R1-63	185±2.7	R2-35	256±2.2	R3-C4	335±3.9
YB-1514	136±2.8	R1-82	213±4.5	R2-87	239±1.0	R3-B9	303±4.2
YB-2809	157±2.0	R1-116	183±1.7	R2-103	278±4.7	R3-A31	308±1.3
YB-2830	175±3.2	R1-124	208±1.1	R2-122	243±2.8	R3-D24	279±6.9

The values are presented as the mean±standard deviations from three independent experiments.

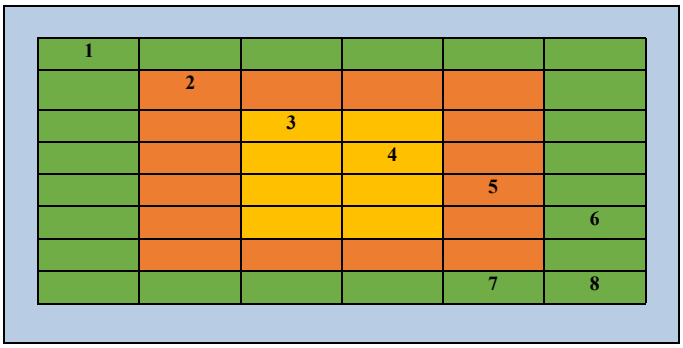

Fig. S1 Schematic diagram of strains setting in 48-deep-well plate

There are 48 squares of color in the figure, representing 48 holes of deep-well-plate. According to the different colors, they are divided into three circles, and each circle has a control (the position with marked number in the figure). Through the comparison of 1 and 8, 2 and 5, 3 and 4, 6 and 7, the parallelism of the experiment can also be shown.