Lipase Activity and Tacrolimus Production in *Streptomyces clavuligerus* CKD 1119 Mutant Strains

KIM, HYUNG SOO AND YOUNG IN PARK*

Department of Biotechnology, School of Life Science and Biotechnology, Korea University, Seoul 136-713, Korea

Abstract The effect of carbon sources on tacrolimus production by a mutant strain of *Streptomyces clavuligerus* CKD 1119, an isolate from soil, was examined. Among the carbohydrates and oils tested in this work, a mixed carbon source of soluble starch and corn oil was the best. An analysis of the culture kinetics also showed that, in contrast to the carbohydrates, the corn oil was consumed later in the antibiotic production phase, implying that the oil substrate was the principal carbon source for the biosynthesis of tacrolimus, and this was directly proven by experiments using ^{14}C-glucose and ^{14}C-oleate substrates. Furthermore, corn oil induced the formation of lipase by the mutant strain, whereas the addition of glucose significantly repressed lipase activity. The lipase activity exhibited by the FK-506-overproducing mutants was also observed to be directly proportional to their tacrolimus yield, indicating that a high lipase activity is itself a crucial factor for tacrolimus production. A feasibility study with a 200-l pilot-scale fermentor and the best strain (Tc-XII-15322) identified in this work revealed a high volumetric and specific productivity of about 495 mg/l and 0.34 mg/mg dry mycelium, respectively.

Keywords: Tacrolimus, lipase activity, *Streptomyces clavuligerus*

Tacrolimus (FK-506), an antibiotic with immunosuppressant activity, was first discovered in 1984 from the fermentation broth of *Streptomyces tsukubaensis* No9993 (soil sample, Tsukuba, Japan) by Fujisawa scientists [9]. Their subsequent characterization and *in vitro* and *in vivo* immuno-pharmacological tests of the novel immunosuppressive agent, and the extensive clinical trials initiated in 1989 at the University of Pittsburgh, revealed that the neutral macrolide antibiotic had a superior potency relative to cyclosporin A as regards the prevention of graft rejection and treatment of autoimmune diseases, plus fewer side effects [7, 23]. Thus, because of the clinical importance described

above, many attempts have been made to develop a more efficient and economical process for the industrial production of FK-506. These include studies on screening for new FK-506-overproducing strains, strain improvement based on classical and genetical mutageneses, and optimization of the medium composition and culture conditions [6, 13, 24]. In a recent report by the current authors, a novel FK-506-overproducing strain of *Actinomycetes* was isolated from a soil sample taken from Chungnam prefecture, and the isolate identified as a strain of *Streptomyces clavuligerus* and named *Streptomyces clavuligerus* CKD 1119.

Similar to many other antibiotic fermentations, the isolate was found to attain the highest FK-506 titer when cultured in a complex medium containing both soluble starch and corn oil as the carbon source. Lipase is responsible for the breakdown of triacyl glycerols, the main constituent of oils, and may also play a role in the import of fatty acids into cells [4, 5, 11]. As such, the enzyme is a prerequisite for the utilization of an oil-supplemented substrate. Of note, the fatty acids thus transported into cells are known to be broken down *via* the β-oxidation pathway to produce acetyl or propionyl coenzyme A, which are reported to be the precursors for the biosynthesis of polyketide antibiotics [12, 22]. As expected, *S. clavuligerus* CKD 1119 was also observed to produce a cell-associated form of lipase, like various other *Streptomyces* species [25, 26].

Accordingly, this study attempted to demonstrate whether the same situation mentioned above was also true for tacrolimus biosynthesis by *S. clavuligerus* CKD 1119 mutant strains. In addition, the detailed link between the lipase activity and the specific productivity of FK-506 in the mutants was also investigated.

MATERIALS AND METHODS

Microorganisms

FK-506-overproducing mutant strains of *Streptomyces clavuligerus* CKD 1119 previously isolated from soil by the current authors were used throughout this study. Stock

*Corresponding author
Phone: 82-2-3290-3425; Fax: 82-2-923-2983;
E-mail: yipark@korea.ac.kr

cultures of these strains were maintained at 4°C on a YM medium (4 g/l soluble starch, 10 g/l malt extract, 4 g/l yeast extract, 0.05 g/l tacrolimus, 20 g/l agar).

Media and Cultural Conditions

To obtain seed cultures of the *S. clavuligerus* CKD 1119 mutant strains, 0.2 ml of frozen mycelia (about 10^9 spores/ ml) was thawed and transferred into a 500-ml Erlenmeyer flask containing 50 ml of a seed medium and incubated at 28°C for 2 days on a reciprocal shaker at 220 rpm. The seed medium consisted of 20 g/l soluble starch, 10 g/l cotton seed meal, 10 g/l corn steep liquor, 10 g/l dried yeast, 2 g/l $CaCO_3$, and 1 g/l SAG-471, and was adjusted to pH 7.0 after autoclaving. Batch cultures were then grown in a 100-ml Erlenmeyer flask containing 20 ml of a production medium at 25°C for 8 days on a reciprocal shaker at 220 rpm, or in a 7-l jar fermenter (KF-7, KoBio Tech, Korea) containing 4.5 l of the production medium, consisting of 50 g/l soluble starch, 5 g/l corn steep powder, 30 g/l cotton seed meal, 30 g/l corn oil, 2 g/l malonic acid, 0.5 g/l methionine, 0.3% (v/v) ethanol, 0.1 g/l $FeSO_4 \cdot 7H_2O$, 1 g/l $CaCO_3$, 0.2 g/l NaH_2PO_4, and 1 g/l SAG-471.

Cell Growth Measurement

The cell growth was followed by determining the mycelial dry weight. The mycelium was separated from the culture broth (20 ml) by centrifugation at 9,600 ×g for 20 min, and then the resulting pellet was washed twice with *n*-butanol-ethanol (1:1, v/v) (10 ml) and once with water (10 ml), and dried to a constant weight at 80°C. The viscosity was determined using a Brookfield Viscometer (Loughton, U.K.) at 20 rpm and room temperature.

Quantitative Analysis of Carbon Sources

The culture broth was centrifuged at 9,600 ×g (Vision Scientific Co. Ltd, model VS5500 CF) for 20 min and 1 ml of the supernatant transferred to a 50-ml volumetric flask. After adding 5 ml of 1.5 N HCl, each sample was boiled for 2 h, and then 5 ml of 1 N NaOH was added and the volume adjusted to 50 ml using a phosphate buffer (pH 8.0; 12.8 g/l KOH and 27.2 g/l KH_2PO_4). After filtering, the concentration of sugar was measured using a sugar analyzer (YSI model 2700, U.S.A.).

Oil Measurement

The residual oil concentrations in the culture broth were assayed using a modified version of the procedure developed by Omura *et al.* [20]. Two ml of the culture broth was added to a 1% Adecatol solution in a 50-ml mess flask, and then centrifuged after shaking for 10 min [sample solution (SA)]. Reaction mixture 1 (RM-1) contained a 0.2 M K-Na phosphate buffer (pH 7.5), 10 mM $MgCl_2$, 10% Triton X-100, 0.1 M ATP (pH 7.5), 10 mM CoA (pH 7.5), and 50 μ/ml acyl-CoA synthetase; RM-2 contained a 0.2 M

K-Na phosphate buffer (pH 7.5), 0.3% 4-aminoantipyrine, 0.2% phenol, 5.0% NaN_3, 0.25% *N*-ethylmaleimide, 100 μ/ml peroxide, and 20 μ/ml acyl-CoA oxidase; RM-3 contained a 0.2 M K-Na phosphate buffer (pH 7.5) and 800 μ/ml lipase; and the standard solution (ST) used was 0.1% oleic acid. RM-3 was preincubated for 3 min at 37°C and the reaction initiated by the addition of an SA or distilled water for a blank reaction (BR). After 10 min, RM-1 was added to the SA and BR, plus 0.2% Triton X-100 was added to the ST, and then the reaction mixtures were incubated for a further 10 min. Thereafter, RM-2 was added and the incubation continued for another 10 min, at which point, the OD was measured with a UV spectrophotometer (HP 8453, 500 nm) and the quantity of oil calculated using the following equation:

$$\frac{(SA - BR)}{(ST - BR)} \times \text{dilution rate (g/l)}$$

Enzyme Activity Detection and Activity Assay

The lipase activity was determined at 30°C using a Metrohm pH-stat system. The substrate was composed of 15 ml of tributyroylglycerol and 50 ml of an emulsifier agent (17.9 g NaCl, 0.41 g KH_2PO_4, 540 ml glycerol, 6 g arabic gum, and distilled water) in a final volume of 1 l [17]. The emulsion was mechanically stirred and the free fatty acids released were titrated by the addition of 50 mM sodium hydroxide to maintain a constant pH at an end-point value of 7.0. One unit of lipase activity corresponded to the amount of enzyme that catalyzed the hydrolysis of 1 μM tributyroylglycerol/min [10]. To investigate the location of the lipase activity in the cells, the lipase activity was measured in the whole broth, supernatant, and pellet samples, plus whole broth that had been subjected to sonication using a Soniprep 150 (Sanyo) at an 8-μm setting for 2×10 min [16].

Selection of Mutants with Enhanced Lipase Activity

The *S. clavuligerus* Tc-IX-12303 strain, a drug-resistant mutant with enhanced tacrolimus productivity isolated in this work, was used as the starting strain for the isolation of modified strains capable of producing higher levels of lipase activity. The mutants were obtained by inoculating the starting strain after mutagenesis by UV irradiation onto selective plates containing 1.0 g/l Tween 80, 0.5 g/l $CaCl_2$, and 0.01 g/l methyl red as the indicator. After 72 h of incubation, the diameters of the halo zones surrounding the colonies were measured, and the colony that formed the largest clear zone was selected.

Assay of ^{14}C-labeled Tacrolimus

Cells of the tacrolimus-overproducing strain grown in the production medium for 72 h were aseptically collected and washed with distilled water and suspended in sodium phosphate (pH 7.0) to make a 150-ml cell suspension.

Fifty ml of the resting cell suspension was then incubated in a 500-ml flask for 22 h on a rotary shaker at 220 rpm and 25°C, in the presence of labeled [1-^{14}C]glucose or [1-^{14}C]oleate (Amersham Biosciences, 50 μCi). Thereafter, the cells were harvested, suspended in 50 ml of MeOH, shaken for 1 h, and centrifuged. Normal-phase silica gel column chromatography was then applied to the supernatant, followed by alumina column chromatography. The eluate was washed with *n*-hexane and the tacrolimus-containing solvent layer concentrated to dryness. The radioactivity of the tacrolimus produced was measured with a liquid scintillation counter (Beckman LS 1801) using a Bio-Safe II biodegradable counting cocktail (Research Products International Corp.).

Quantification of Tacrolimus

An HPLC analysis was conducted according to the method described by Akashi *et al.* [3]. The sample extraction was performed for 60 min from 5 ml of a culture broth prepared as described above using 20 ml of 60% (v/v) methanol, followed by centrifugation at 10,000 rpm for 10 min. An aliquot of the resulting supernatant was then analyzed using an HPLC equipped with a Whatman Partsil 5 ODS-3 analytical column (4.6×250 mm, particle size 5 μm) maintained at 60°C, as described previously.

Cultivation in Scaled-up Pilot

A scaled-up fermentation was performed at 25°C in a 200-l pilot fermentor (KoBio Tech, Korea) with a working volume of 120 l. The dissolved oxygen was continuously monitored using an oxygen probe (Ingold, Urdorf, Switzerland), while the stirrer speed ranged from 200 to 400 rpm to ensure a minimal level of dissolved oxygen at 30% saturation. The air flow was fixed at 1.0 vvm and the pH automatically maintained at 6.8±0.2 by the addition of 25% NH$_4$OH. The pilot was inoculated with a seed culture (inoculum size: 10%), and then the biomass and lipase accumulation were monitored as a function of time until the lipase production in the culture broth reached its maximal value.

RESULTS AND DISCUSSION

Selection of Tacrolimus-resistant Mutant

Streptomyces clavuligerus Tc-V-05813 is a mutant strain that was previously selected as being resistant to both ethionine and cyclosporine A and with a significantly improved tacrolimus productivity, as seen in Fig. 4A. Nonetheless, this mutant strain still shows a considerable degree of cell lysis and viability loss, especially in the secondary metabolite production phase, with a concomitant decline in the antibiotic titer. A potential reason for this culture profile is suspected to be the toxic effect of tacrolimus accumulated in the
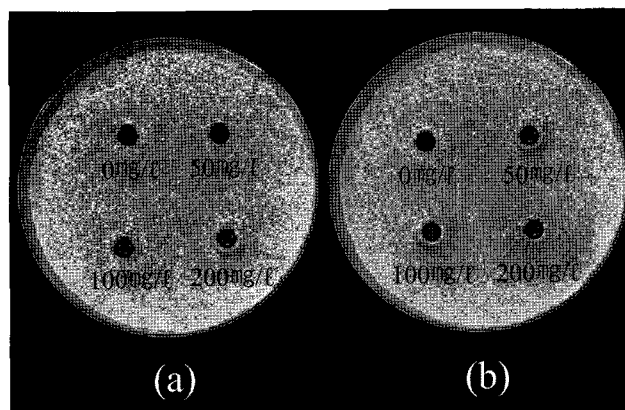

Fig. 1. Cell growth inhibition of tacrolimus-resistant mutant Tc-IX-12303 (a) and its parent strain Tc-V-05813 (b) by tacrolimus.
The tacrolimus concentrations added to cylindrical wells in a solid culture of each strain were 0, 50, 100, and 200 mg/l, and the clear zones around the wells reflect the cell growth inhibition due to tacrolimus.

cells, as in the case of the overproduction of cephalosporin by *Streptomyces clavuligerus* [1]. Therefore, the first task of this work was to obtain a new mutant of the Tc-V-05813 strain that was resistant to a higher level of tacrolimus. As shown in Fig. 1, the tacrolimus-resistant mutant Tc-IX-12303, obtained *via* two successive classical mutation and selection programs, exhibited no remarkable growth inhibition up to 200 mg/l tacrolimus. In contrast, the original strain, Tc-V-05813, exhibited growth inhibition even with a tacrolimus concentration of 50 mg/l under the experimental conditions used in this work. As expected, the selected tacrolimus-resistant mutant showed an increased tacrolimus yield, which was about 1.4-fold higher than that with the original strain, as indicated in Fig. 4A. The Tc-IX-12303 strain was then used in most of the subsequent experiments, unless otherwise specified.

Selection of Carbon Sources

It is well established that selecting the appropriate carbon source can have a significant effect on the peak productivity of secondary metabolites, as well as the raw material costs [15, 21]. Therefore, to understand the detailed effect of carbon sources on FK-506 formation, shake-flask cultures were carried out with the Tc-IX-12303 strain using preculture media supplemented with several different carbohydrates and oil substrates, either alone or in combination. Not surprisingly, when tested as the sole carbon source, the carbohydrates supported higher levels of mycelial growth than the oil substrates, where the highest dry cell weight (DCW) value of about 1.1 mg/ml was achieved with dextrin after a culture time of 6 days. Soluble starch was the best among the carbohydrates examined for FK-506 production, giving a peak titer of 145 mg/l tacrolimus after 8 days of cultivation. In contrast, significantly higher peak
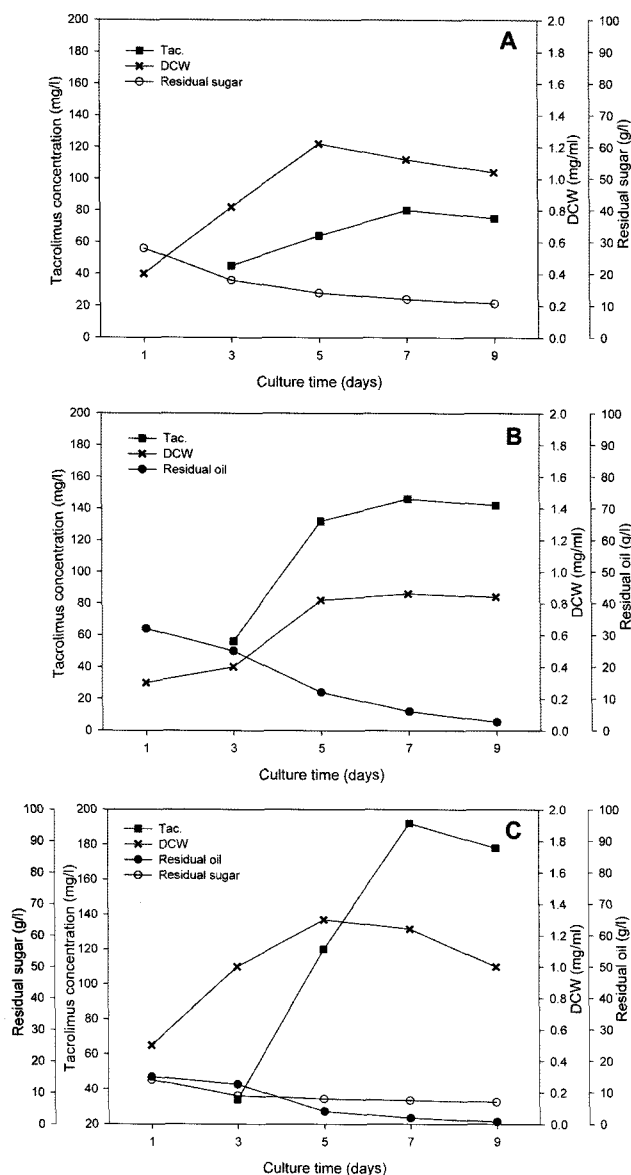
Fig. 2. Effect of carbon sources on cell growth and tacrolimus production in *S. clavuligerus* Tc-IX-12303

The fermentation was carried out in a 7-1 mini jar fermentor containing 4.5 l of the production medium at pH 6.8 and a stirrer speed of 500–900 rpm. The carbon sources supplied in the medium were 3.0% soluble starch (A), 3.0% corn oil (B), and 1.5% of each in a combined substrate (C).

titers were observed in the cultures supplemented with oil, where the highest antibiotic production of 235 mg/l was achieved with corn oil after a culture time of 8 days (detailed data not shown).

As seen in Fig. 2, although the cultures containing soluble starch or a combination of soluble starch and corn oil supported nearly the same level of mycelial growth (DCW of about 1.3 mg/ml), the highest yield (about 190 mg/l) after 8 days of cultivation and an extended duration of FK-506 production were observed in the culture containing mixed carbon sources. Furthermore, the figure shows that

the consumption rate of the oil substrate was closely correlated with the specific productivity of tacrolimus, while soluble starch was mostly utilized earlier during the initial growth phase. Therefore, these results imply that the catabolites of the oil substrate may have been used as the principal precursors for tacrolimus biosynthesis in the *S. clavuligerus* mutant cells.

Biosynthetic Precursor of Tacrolimus

The FK-506 fermentation profile shown in Fig. 2 strongly suggests that the catabolites of corn oil may be utilized as the biosynthetic precursors of the carbon backbone of FK-506, as in the case of the biosynthesis of polyketide antibiotics [10, 14]. Thus, in an attempt to confirm the above suggestion, two separate sets of cultures were carried out under the same conditions, as described in Materials and Methods. In one experiment, the mixed carbon source consisted of ^{14}C-labeled glucose and unlabeled cold oleate, one of the most abundant fatty acid components associated with plant oils, whereas the carbon source in the other experiment consisted of cold glucose and ^{14}C-oleate. As presented in Table 1, the radioactivity measured for the tacrolimus produced with the carbon source containing ^{14}C-oleate was estimated to be about 5.2-fold higher than that for the tacrolimus synthesized on the medium containing ^{14}C-glucose. Therefore, when taken together, these results confirm that the catabolites of the oil substrate were the principal components for tacrolimus biosynthesis by the *S. clavuligerus* strain.

Localization of Lipase Activity

As described above, the addition of corn oil to the starch-based medium significantly increased the titer of FK-506 without promoting further mycelial growth of *S. clavuligerus* Tc-IX-12303. However, oils are insoluble in water and their components have to be extracellularly hydrolyzed prior to absorption, and thus the organism under cultivation must produce a stable lipase that can efficiently hydrolyze the oil substrate. Hence, as a first step to studying the biosynthesis of lipase in *S. clavuligerus* cells, the

Table 1. Incorporation of [1-^{14}C]glucose and [1-^{14}C]oleate into tacrolimus.

Substrates	Specific activity of purified tacrolimus (nCi/mmol)
[1-^{14}C]Glucose, 10 µCi[a]+oleate[b]	0.22
Glucose[b]+[1-^{14}C]oleate, 10 µCi[a]	1.14

[a]Specific activity of [1-^{14}C]glucose and [1-^{14}C]oleate: 50 µCi/µmol.
[b]0.1% cold glucose and 0.1% cold oleate, respectively.
Cells grown in the production medium for 72 h were aseptically collected, washed with distilled water, and suspended in sodium phosphate (pH 7.0). Fifty ml of the resting cell suspension was then incubated in a 500-ml flask for 22 h on a rotary shaker at 220 rpm and 25°C in the presence of labeled [1-^{14}C]glucose, 10 µCi, and cold oleate, or cold glucose and [1-^{14}C]oleate, 10 µCi, as the substrates.

**Table 2.** Detection of lipase activity in tacrolimus fermentation samples of *S. clavuligerus* Tc-IX-12303.

	S. clavuligerus Tc-IX-12303
Whole broth	+
Supernatant	-
Resuspended pellet	+
Whole broth sonicated	-

+, Activity detected; -, no activity detected.

localization of lipase in relation to the cells was examined using the *S. clavuligerus* Tc-IX-12303 strain. To detect the cell-associated enzyme, whole broth, supernatant, pellets resuspended in deionized water, and sonicated whole-broth samples were prepared, as described in Materials and Methods, and the resulting samples analyzed for their lipase activity. As presented in Table 2, lipase activity was only detected in the whole broth and resuspended pellets with similar enzyme activity values for both samples. Thus, when taken together, these results clearly indicate that the lipase activity produced by the tacrolimus-overproducing strain was not extracellular, but rather associated with the mycelium, as previously reported for many other lipase-producing microorganisms, including a strain of *S. clavuligerus* [27, 28].

Induction and Repression of Lipase Synthesis

Next, to gain some basic information on the regulation of lipase biosynthesis in the *S. clavuligerus* strain under the
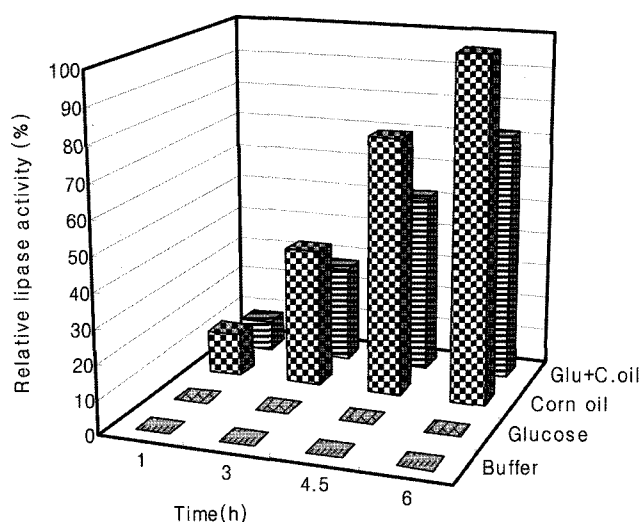
Fig. 3. Induction and repression of lipase formation by washed mycelium of *S. clavuligerus* Tc-IX-12303 with corn oil and glucose.

Eight g of wet mycelium prepared from a 2-day-old shake flask culture in a basal medium (100 ml per 500-ml flask) was washed twice with distilled water. The washed cells were then aseptically incubated for 22 h at 30°C and 120 rpm in 80 ml of a 0.05 M sodium phosphate buffer (pH 7.0) with 0.5% (w/v) corn oil and 0.5% (w/v) glucose. The values shown are the means from three independent experiments.

conditions of no mycelial growth, washed cells from a 2-day-old culture were incubated in a 0.05 M sodium phosphate buffer (pH 7.0) with 0.5% (w/v) corn oil or 0.5% (w/v) glucose, or a mixture of the two carbon sources (each 0.25%) at 30°C for 6 h. As shown in Fig. 3, lipase expression was induced when the oil substrate was present, whereas significant repression was observed with the addition of glucose. These induction and repression patterns are already known as the most common regulation mechanisms, although the mechanisms involved in lipase biosynthesis have been reported to vary widely depending on the microorganism [8, 19].
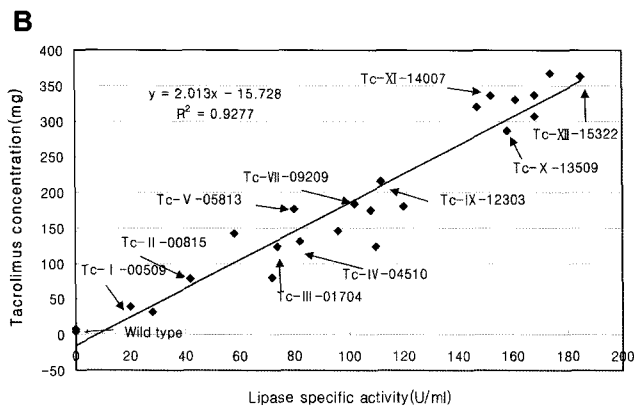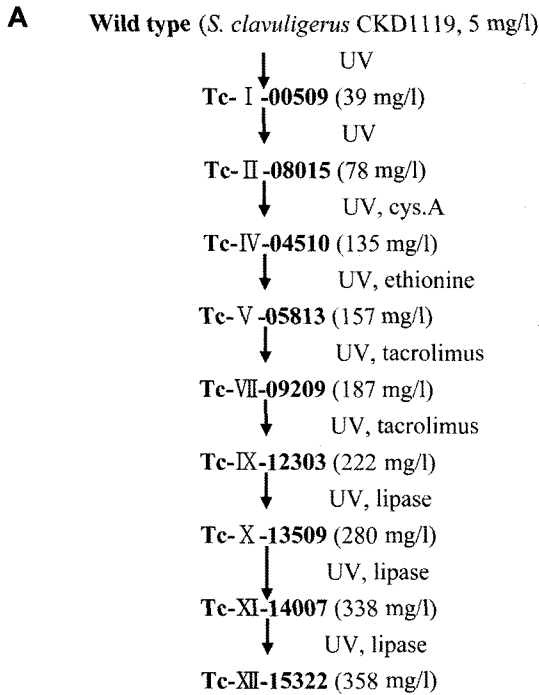
Relationship Between Lipase Activity and Tacrolimus Productivity

Lipase, the first enzyme involved in the catabolism of oil substrates, has also been reported to play a potential role in importing the fatty acids released from the enzymatic reaction into cells [18]. Furthermore, the current work demonstrated that the oil supplement had a remarkable effect on improving both the peak titer and the specific productivity of tacrolimus by the *S. clavuligerus* strain, indicating that lipase played a central role in the biosynthesis of FK-506 by the test strain. Notwithstanding, little research has been published on the lipolytic activity in *Streptomyces*, despite their widespread use in antibiotic production. Thus, it may be very important to define the precise relationship between the levels of lipase activity and the tacrolimus productivity of FK-506-overproducing strains.

Therefore, this study generated a pool of diverse mutants possessing different capabilities to produce lipase based on iteration of the classical mutagenesis process, as described in Materials and Methods (Fig. 4A). The resulting mutant strains were then cultured in 100-ml flasks containing 20 ml of production media at 25°C for 8 days, and their lipase activity and FK-506 productivity determined. Thereafter, the specific lipase activity estimated for the mutants was plotted against the FK-506 productivity attained by the respective strains. Quite remarkably, as shown in Fig. 4B, the FK-506 productivity was observed to increase linearly when the lipase specific activity increased, from a non-detectable level (the value for the wild type) up to 187 U/ml (for Tc-XII-15322), thereby strongly suggesting that lipase activity was the limiting factor for tacrolimus biosynthesis, even in the mutant strain revealing the highest FK-506 productivity in this study. Consequently, this implies a potential for further improvement of FK-506 productivity through strain modification to enhance the capability of lipase production. Furthermore, the present results indicate that a high lipase activity is itself a crucial factor in acquiring a high productivity of FK-506.
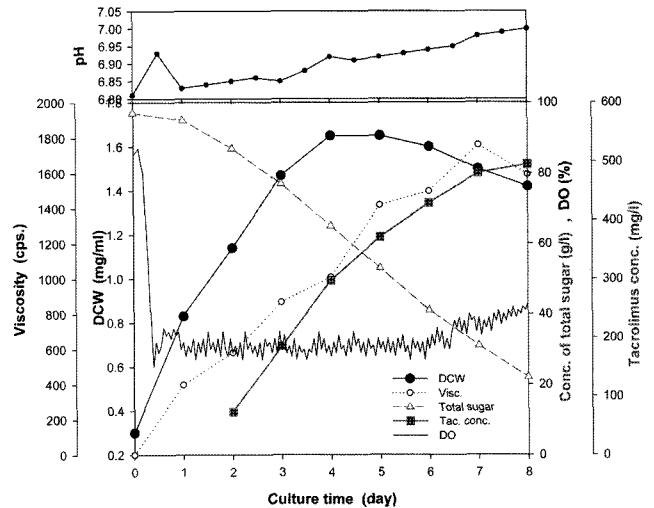
Pilot-scale Fermentation

Finally, to assess the feasibility of applying this process to industrial mass production, a pilot-scale fermentation

A   Wild type (*S. clavuligerus* CKD1119, 5 mg/l)

$\downarrow$ UV

Tc- I -00509 (39 mg/l)

$\downarrow$ UV

Tc- II -08015 (78 mg/l)

$\downarrow$ UV, cys.A

Tc-IV-04510 (135 mg/l)

$\downarrow$ UV, ethionine

Tc-V -05813 (157 mg/l)

$\downarrow$ UV, tacrolimus

Tc-VII-09209 (187 mg/l)

$\downarrow$ UV, tacrolimus

Tc-IX-12303 (222 mg/l)

$\downarrow$ UV, lipase

Tc-X -13509 (280 mg/l)

$\downarrow$ UV, lipase

Tc-XI-14007 (338 mg/l)

$\downarrow$ UV, lipase

Tc-XII-15322 (358 mg/l)

B

Fig. 5. Time course of tacrolimus fermentation by *S. clavuligerus* Tc-XII-15322 on pilot scale.

The fermentation was carried out in a 200 l pilot fermentor, and the culture broth (50 ml) sampled at the time points indicated in the figure and analyzed for the content of tacrolimus (■), cell growth (●), residual sugar (△), viscosity (○), pH, and dissolved oxygen.

Fig. 4. A. Genealogy of tacrolimus-producing *Streptomyces clavuligerus* CKD 1119 mutant strains. The tacrolimus yields indicated were determined by analyzing samples taken from shake flask cultures carried out at 25°C and 220 rpm for 8 days in a production medium. **B.** Relationship between lipase specific activity and tacrolimus production in mutants of *Streptomyces clavuligerus* CKD 1119. All the strains used in this study were grown in a 100-ml Erlenmeyer flask containing 20 ml of a production medium at 25°C for 8 days on a reciprocal shaker at 220 rpm, and the crude lipase activity measured in a whole-broth sample prepared from a culture of each mutant strain.

experiment was performed using the *S. clavuligerus* Tc-XII-15322 strain and a 200-l pilot fermentor with a working volume of 120 l of the production medium. The cultivation was carried out at 25°C for 8 days with an aeration rate of 1 vvm and 400 rpm. As seen in Fig. 5, the maximum cell mass (DCW of 1.65 mg/ml) was achieved after 4 days cultivation, while the highest tacrolimus production was observed after 8 days of cultivation. The key

fermentation parameters, the volumetric productivity and specific productivity of tacrolimus, were determined to be 495 mg/l and 0.34 mg/mg-dry mycelium, respectively. This tacrolimus yield was estimated to be about 100-fold higher than that for the wild-type strain, and about a 2.1 times higher level than that obtained from the flask fermentation.

In conclusion, approaches to develop this process for an economical mass production system could include further strain improvement with a higher capacity of lipase production without changing any other factors critical for FK-506 production, such as mycelium fragmentation and the length of the FK-506 production period, along with a further detailed and systematic optimization of the environmental conditions for the scaled-up fermentation process.

Acknowledgment

REFERENCES

1. Aharonowitz, Y. and A. L. Demain. 1978. Carbon catabolite regulation of cephalosporin production in *Streptomyces clavuligerus*. *Antimicrob. Agents Chemother.* **14:** 159–164.

2. Ahn, J. O., H. W. Jang, H. W. Lee, E. S. Choi, S. J. Haam, T. K. Oh, and J. K. Jung. 2003. Overexpression of thermoalkalophilic lipase from *Bacillus stearothermophilus*

L1 in *Saccharomyces cerevisiae. J. Microbiol. Biotechnol.* **13:** 451–456.

3. Akashi, T., T. Nefuji, M. Yoshida, and J. Hosoda. 1996. Quantitative determination of tautomeric FK-506 by reversed-phase liquid chromatography. *J. Pharmaceut. Biomed. Anal.* **14:** 339–346.

4. Berk, P. D. 1996. How do long-chain free fatty acids cross cell membranes? *Proc. Soc. Exp. Biol. Med.* **212:** 1–4.

5. Cavanagh, M. F., A. P. Ison, M. D. Lilly, and S. W. Carleysmith. 1994. The utilization of lipids during *Streptomycete* fermentations. IChemE Res. Event, Two-Day Symp. Institute of Chemical Engineering, Rugby, UK. **1:** 226–228.

6. Cheigh, C.-I., S.-J. Lee, Y.-R. Pyun, D.-J. An, Y.-S. Hwang, Y. Chung, and H. Park. 2005. The effect of carbon sources on nisin z biosynthesis in *Lactococcus lactis* subsp. *lactis* A164. *J. Microbiol. Biotechnol.* **15:** 1152–1157.

7. Dumont, F. J., M. R. Melino, M. J. Staruch, S. L. Koprak, P. A. Fischer, and N. H. Sigal. 1990. The immunosuppressive macrolides FK506 and rapamycin act as reciprocal antagonists in murine T cells. *J. Immunol.* **144:** 1418–1424.

8. Harwood, J. 1989. The versatility of lipase for industrial uses. *Trends Biochem. Sci.* **14:** 125–126.

9. Hatanaka, H., T. Kino, M. Asano. T. Goto, H. Tanaka, and M. Okuhara. 1988. FK506 related compounds produced by *Streptomyces tsukubaensis.* No. 9993. *J. Antibiot.* **42:** 620–622.

10. Hopwood, D. A. 1997. Genetic contributions to understanding polyketide synthases. *Chem. Rev.* **97:** 2485–2497.

11. Huge-Jensen, B., D. R. Galluzzo, and R. G. Jensen. 1987. Partial purification and characterization of free and immobilized lipases from *Mucor miehei. Lipids* **22:** 559–565

12. Kim, J. C., S. W. Kang, J. S. Lim, Y. S. Song, and S. W. Kim. 2006. Stimulation of cephalosporin C production by *Acremonium chrysogenum* M35 with fatty acids. *J. Microbiol. Biotechnol.* **16:** 1120–1124.

13. Kino, T., H. Hatanaka, S. Miyata, N. Inamura, M. Nishiyama, T. Yajima, T. Goto, M. Okuhara, M. Kohsaka, and H. Aoki. 1987. FK-506, a novel immunosuppressant isolated from a *Streptomyces.* I. Fermentation, isolation, physico-chemical and biological characterization. *J. Antibiot.* **40:** 1249–1255.

14. Kino, T., T. Hatanaka, S. Miyata, N. Inamura, N. Nishiyama, T. Yajima, T. Goto, M. Okuhara, M. Kohsaka, H. Aoki, and T. Ochiai. 1987. FK-506: A novel immunosuppressant isolated from a *Streptomyces.* II. Immunosuppressive effect of FK-506 *in vitro. J. Antibiot.* **40:** 1256–1265.

15. Kosugi, Y. and H. Suzuki. 1973. Fixation of cell-bound lipase and properties of the fixed lipase as an immobilized enzyme. *J. Ferment. Technol.* **51:** 895–903.

16. Large, K. P., N. Mirjalili, M. Osborne, L. M. Peacock, V. Zormpaidis, M. Walsh, M. E. Cavanagh, P. F. Leadlay, and A. P. Ison. 1999. Lipase activity in *Streptomycetes. Enz. Microb. Technol.* **25:** 569–575.

17. Longo, M. A. and D. Combes. 1995. A novel chemoenzymatic glycosylation strategy: Application to lysozyme modification. *FEBS Lett.* **375:** 63–66.

18. Macrae, A. R. and R. C. Hammond. 1988. Present and future applications of lipases. *Biotechnol. Genet. Eng. Rev.* **3:** 193–217.

19. Macris, J. B., E. Kourentzi, and D. G. Hatzinikolaou. 1996. Studies on localization and regulation of lipase production by *Aspergillus niger. Process Biochem.* **31:** 807–812.

20. Omura, S., Y. Tanaka, C. Kitao, H. Tanaka, and Y. Iwai. 1980. Stimulation of leucomycin production by magnesium phosphate and its relevance to nitrogen catabolite regulation. *Antimicrob. Agents Chemother.* **18:** 691–695.

21. Park, Y. S., I. Momose, K. Tsunoda, and M. Okabe. 1994. Enhancement of cephamycin C production using soybean oil as the sole carbon source. *Appl. Microbiol. Biotechnol.* **40:** 773–779.

22. Reynolds, K. A. and A. L. Demain. 1997. Rapamycin, FK506, and ascomycin-related compounds, Chapter 17, pp. 497–520. *In* William Strohl (ed.), *Biotechnology of Antibiotics.* Marcel Dekker, New York.

23. Sawada, S., G. Suzuki, Y. Kawase, and F. Takaku. 1987. Novel immunosuppressive agent, FK506. *In vitro* effects on the cloned T cell activation. *J. Immunol.* **139:** 1797–1803.

24. Shafiee, A. and H. Motadedi. 1997. Chemical and biological characterization of two FK-506 analogs produced by targeted gene disruption in *Streptomyces* sp. MA6548. *J. Antibiot.* **50:** 418–423.

25. Stuer, W., K. E. Jae, and U. K. Winkler. 1986. Purification of extracellular lipase from *Pseudomonas aeruginosa. J. Bacteriol.* **168:** 1070–1074.

26. Sugiura, T., T. Ota, and Y. Minoda. 1975. Effects of fatty acids, lipase activator, phospholipids and related substances on the lipase production by *Candida paralipolytica. Agric. Biol. Chem.* **39:** 1689–1694.

27. Tsujisaka, Y., M. Iwai, J. Fukumoto, and Y. Okamoto. 1973. Induced formation of lipase by *Geotrichum candidum* link. *Agric. Biol. Chem.* **37:** 837–842

28. Watanabe, N., Y. Ota, Y. Minoda, and K. Yamada. 1977. Isolation and identification of alkaline lipase producing microorganisms, cultural conditions and some properties of crude enzymes. *Agric. Biol. Chem.* **41:** 1353–1358.