Streptomyces tsukubensis VKM Ac-2618D—an Effective Producer of Tacrolimus

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Abstract—The Streptomyces sp. VKM Ac-2618D strain has been identified, and its morphological and physiological features have been studied in relation to the production of the immunosuppressant tacrolimus. The phenotypic variability of the strain was analyzed, and a dissociant with a high level of tacrolimus production was selected. Based on a comprehensive study of morphological, physiological, and chemotaxonomic properties and on phylogenetic analysis, the strain was named Streptomyces tsukubensis VKM Ac-2618D. The strain genome contains the full version of the tacrolimus biosynthetic gene cluster. The advantages of fed-batch cultivation mode for tacrolimus biosynthesis are shown. The results broaden the understanding of the characteristics of polyketide biosynthesis and can be used in the development of technology for tacrolimus production.

Keywords: Streptomyces tsukubensis, tacrolimus, FK-506, dissociant, genome, biosynthesis

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INTRODUCTION

Soil actinobacteria of the Streptomyces genus are the subject of active research due to their unique ability to synthesize biologically active substances, including antibiotics, antineoplastic agents, immunosuppressants, and antifungal and anthelminthic compounds, as well as herbicides and other substances [1, 2].

Tsukuba macrolide immunosuppressant FK-506 (Tacrolimus) was detected for the first time in 1984 in Streptomyces tsukubensis culture broth as a result of screening by the Fujisawa Pharmaceutical Co. (now Astellas Pharma Inc.). The producer strain was isolated from a soil sample in the Tsukuba region (Japan), and tacrolimus became the first known immunosuppressant with a macrolide structure [3, 4]. Tacrolimus is currently recognized as the most effective immunosuppressive agent and is widespread in various fields of medicine: transplantology [5, 6], dermatology [7], the treatment of autoimmune [8] and viral [9] diseases, etc. The range of its clinical application is constantly expanding. Recent studies indicate its effectiveness in combination with anti-inflammatory corticosteroid drugs in the treatment of patients with serious complications caused by the new coronavirus infection (COVID-19) [11].

All of the known tacrolimus producers belong to the Streptomyces genus. A strain capable of synthesizing tacrolimus was discovered in 1984. It was patented first as S. tsukubensis 9993 [3, 4] and deposited later as S. tsukubensis NRRL 18488. Despite the number of publications on the features of tacrolimus biosynthesis by the NRRL 18488 strain, the species did not receive a proper taxonomic description until 2013. Due to a deep study of the morphological and physiological properties of the 9993T = NRRL 18488T (S. tsukubensis) strain, which had become typical by that time, its phylogenetic position was confirmed and its species name S. tsukubensis was clarified [12].

Many aspects of tacrolimus biosynthesis by various S. tsukubensis strains have been elucidated to date: the features of the genome structure have been clarified; the gene cluster encoding enzyme systems for tacrolimus biosynthesis and examples of obtaining mutant producer strains have been described [13, 14]; the rela-
tionship of tacrolimus biosynthesis and central cellular metabolism has been studied [15, 16]; and approaches are being developed to increase the productivity of strains via the creation of nutrient media and optimization of the culturing conditions [17, 18]. However, the productivity levels of some strains are not yet sufficient, and the formation of side products such as ascomycin (FK-520), which complicates the isolation and purification of the tacrolimus substance to pharmacopeial quality, as well as the unstable biosynthesis of the target compound in known producers, are key problems in tacrolimus production that are still to be resolved. There is practically no information in the literature on the morphostructural characteristics of the known producers and the effect of their phenotypic variability on the biosynthesis of the target compound.

The **Streptomyces** sp. VKM Ac-2618D strain is a highly active tacrolimus producer. The technique to obtain tacrolimus based on the use of this strain and various types of polymeric sorbents [19] made it possible for the first time to discover the effect of stimulation of tacrolimus biosynthesis by yeast [20], to propose some new solutions based on the addition of high- and low-molecular starches of various structures [21] that increase the tacrolimus yield, and to obtain a substance of pharmacopeial quality [22]. However, there is a significant gap due to the lack of an evidence base for the reliable identification of the strain species. In addition, the practical use of the strain was also complicated by the low stability of the activity associated with the productivity levels of some strains are not yet sufficient, and the formation of side products such as ascomycin (FK-520), which complicates the isolation and purification of the tacrolimus substance to pharmacopeial quality, as well as the unstable biosynthesis of the target compound in known producers, are key problems in tacrolimus production that are still to be resolved. There is practically no information in the literature on the morphostructural characteristics of the known producers and the effect of their phenotypic variability on the biosynthesis of the target compound.

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The goal of the present work was to identify the tacrolimus-producing strain and to study its morphological and physiological properties associated with tacrolimus production.

**EXPERIMENTAL**

**Reagents**

Soluble starch (Kupavnareaktiv, Russia), glucose and α-asparagine (Dia-M, Russia), baker’s yeast (Saff-Moment, France), corn extract (Sigma-Aldrich, United States), malt extract (Fluka, United States), L-lysine monohydrochloride (PanReac, United States), a sorbent of XAD type (Sigma-Aldrich), *Aspergillus oryzae* α-amylase (Serva, Germany), and the tacrolimus reference preparation for HPLC (Zhejiang Hisun Pharmaceutical Co. Ltd., China) were used in this work. The rest of the reagents and solvents were of chemically pure or pure for analysis grades from Russian manufacturers.

**Microorganism and Conditions for Its Maintenance**

The **Streptomyces** sp. strain VKM Ac-2618D was received from the All-Russia Collection of Microorganisms of the Institute of Biochemistry and Physiolog-
The phylogenetic analysis of Streptomyces sp. VKM Ac-2618D was carried out with the MEGA 4 software package [31]. Phylogenetic analysis and dendrogram building were according to the manufacturer’s recommendations. The purified preparations of cell walls were obtained with a known method [25]. The amino-acid composition of peptidoglycans was determined with a Hitachi amino acid analyzer (Japan). Isoprenoid quinones were isolated with the protocol [26]; the menaquinone composition was determined via mass-spectrometry (MAT 8430, Finnigan MAT, Germany). The fatty-acid composition was analyzed as described in [27]. Methyl esters of fatty acids were studied with a Perkin-Elmer F-17 gas chromatograph (Perkin Elmer, Germany). The enzymatic activity of Streptomyces sp. VKM Ac-2618D was assessed with API ZYM express tests (bioMerieux) according to the manufacturer’s recommendations. The content of GC pairs was evaluated based on the DNA melting temperature [28, 29].

**Phylogenetic Analysis**

Chromosomal DNA was isolated and purified with a modified method [30]. The DNA concentration was measured on a Shimadzu UV-160 spectrophotometer (Japan). The 16S RNA was amplified on a Tertsyk amplifier (DNK-Teknologii, Russia). The universal bacterial primers 27f (AGAGTTTGATC(A/C)TGGCTCAG) and 1492r (ACGG(С/Т)TACCTTGTTACGACTT) were used for this purpose. The nucleotide sequences of the 16S RNA gene were analyzed with an ABI PRISM 3730 automatic sequencer (Applied Biosystems, Terminator v.3.1) of the 16S RNA were analyzed with an ABI PRISM 3730 automatic sequencer (Applied Biosystems, Terminator v.3.1) according to the manufacturer’s recommendations. The enrichment and dendrogram building were carried out with the MEGA 4 software package [31] and the neighbor-joining method [32].

The phylogenetic position of the strain was confirmed via full-genome sequencing and subsequent annotation. The library of DNA fragments 300–400 bp long was obtained with a NEBNext Ultra II DNA Library Prep Kit for Illumina (Illumina, United States). The library was sequenced on HiSeq 2500 with the use of a HiSeq Rapid PE Cluster Kit v2 (Illumina) and a HiSeq Rapid SBS Kit v2 (Illumina) (500 cycles). The quality of the obtained reads was assessed with FastQC 0.11.8 [33]. Adapter fragments were eliminated from the initial reads and the reads containing artifact sequences and PhiX were filtered with BBDuk 38.35. After that, the reads were purified of possible contamination with human DNA. To this end, the reads were mapped with BBMap 38.35 in the human genome and excluded if their identity with this genome exceeded 95%. Then, low-quality reads were eliminated with BBDuk 38.35. The purified reads were assembled with the help of SPAdes 3.13.0 [34]; the resulting contigs were removed if their size was less than 200 bp. The genome was annotated with the NCBI Prokaryotic Genomes Automatic Annotation Pipeline service. The average nucleotide identity based on BLAST values (ANIb) and digital DNA-DNA hybridization (dDDH) values were calculated with the JSpecies 1.2.1 software [35] and the GGDC 2.1 service [36], respectively.

**Analysis of the Bound Glucose Content**

For this purpose, culture-broth samples were hydrolyzed with 3 M trifluoroacetic acid at 100°C for 3 h. The bound glucose was quantified with the method in [37]. The sugar concentration was measured on a carbohydrate analyzer (Biotronik, Germany) at a wavelength of 570 nm.

**Analysis of the Tacrolimus Content**

The compound was determined via HPLC as described previously [20, 21].

**Statistical Analysis**

The experimental data were obtained in triplicate. Results are presented as means with sample standard deviations.

**RESULTS AND DISCUSSION**

**Strain Taxonomic Position**

The Streptomyces sp. strain VKM Ac-2618D forms on solid media in dense, opaque colonies with pronounced differentiation of the structural surface. The colonies range in color from pinkish orange to brick orange. The strain releases a pinkish orange pigment into the medium (Fig. 1a); it is characterized by the development of a branched substrate mycelium, which differentiates into straight chains of cylindrical spores with a smooth surface (Fig. 1b).

Analysis of the Streptomyces sp. VKM Ac-2618D cell-wall composition revealed the presence of LL-diaminopimelolic acid and a lack of diagnostic sugars, which is characteristic of type-I cells. The bulk of fatty acids in cell walls is represented by anteiso-C15:0 (17.05%), iso-C15:0 (15.16%), and iso-C16:0 (15.11%). The main menaquinones are MK-9 (H8) and MK-9 (H6).

The strain actively utilized D-glucose, dextrin, glycerol, starch, D-ribose, and N-acetylgalactosamine, while poor growth was observed on maltose and salicin. High esterase, leucine-arylamidase, naphthol-AS-BI-phosphohydrolase, and α-glycosidase activities were also typical for the strain.

Preliminary analysis of the 16S rRNA sequences showed high similarity (99.9%) of Streptomyces sp. VKM Ac-2618D with the typical Streptomyces tsukubensis strain NRRL 18488T (Fig. 2).
We recently performed full-genome sequencing and genome annotation to confirm the taxonomic identification of the VKM Ас-2618D strain [38]. The genomic sequence of the strain was deposited in the DDBJ/ENA/GenBank as SGFG00000000. The size of the *Streptomyces* sp. VKM Ac-2618D genome was shown to be 7.93 Mb with an average G+C content of 71.9%. The number of candidate protein-encoding genes was 6265 (1227 of which code for hypothetical proteins); 66 tRNA, 23 whole and partial rRNA, and 3 ncRNA were also found.

In this work, we carried out a comparative analysis of the genomes of the VKM Ac-2618D strain and the typical *S. tsukubensis* strain NRRL 18488T (CP029157.1-CP029159.1). It was shown that the values of ANI and dDDH between the sequences of these genomes were 99.99 and 99.9%, respectively, which is much higher than the threshold values for species dis-
tinction (95–96% for ANI and 70% for dDDH). This indicated that the VKM Ac-2618D strain belongs to the species *Streptomyces tsukubensis*.

Detailed analysis of the genome VKM Ac-2618D sequence also made it possible to detect the gene cluster of the tacrolimus biosynthesis.

It is known that the cluster of the tacrolimus biosynthesis genes encodes the polyketide synthase I—nonribosomal peptide synthetase (PKSI-NRPS) system. Two types of this cluster have been described: those with a reduced gene set, which including 19 genes (*S. tacrolimicus* and *S. kanamyceticus*) strains, and those with the complete (extended) gene set, which contains 25–26 genes (*S. tsukubaensis* and *Streptomyces* sp. KCTC 11604BP strains) [39]. The VKM Ac-2618D genome was shown to contain seven genes of the so-called full version of the tacrolimus biosynthesis cluster (Fig. 3) that are absent in the short version typical of the *S. tacrolimicus* ATCC 55098 and *S. kanamyceticus* KCTC 9225 producer strains.

Similar clusters with the full gene set for tacrolimus biosynthesis were found in the *S. tsukubensis* NRRL 18488T, *S. tsukubensis* L19, and *Streptomyces* sp. KCTC 11604BP strains [40]. We previously noted differences between the gene clusters in the *S. tsukubensis* VKM Ac-2618D and *S. tsukubensis* NRRL 18488T strains; however, the later publication of the full genome sequence for the *S. tsukubensis* NRRL 18488T (CP029157.1–CP029159.1) strain changed the situation. Comparative analysis of the genomic data available by the time of the present work publication showed that the gene clusters of tacrolimus synthesis in the *S. tsukubensis* VKM Ac-2618D (SGFG0100008.1:83700–166586), *S. tsukubensis* NRRL 18488T (CP029159.1:c7554111–7471225), and *Streptomyces* sp. KCTC 11604BP (HM116537.1:8712–91598) strains are completely identical.

**Phenotypic Variability and Tacrolimus Biosynthesis**

A high degree of morphological and physiological heterogeneity of the population (or phenotypic dissociation) is a typical feature of streptomycetes. This characteristic is expressed in the appearance of morphologically different colonies from a single predecessor colony, which are designated below as dissociants. Four dissociants with different morphologies were observed in the studied strain when grown on MDA (Fig. 4a).

Cultures grown from single colonies of different dissociants were analyzed for their capacity for tacrolimus production. The differences in tacrolimus productivity accounted for 30–86% (Fig. 4b).

The highest biosynthetic potential was characteristic of dissociant D2, which was represented by round-shaped colonies with a diameter of 7–13 mm of a bright-brick color with a dry, opaque surface. It grew weakly in agar but rose noticeably above its surface and had a central depression of a rounded or irregular shape, bounded by a roller. The lateral slopes of the colonies had a comb-wrinkled structure; the colonies were surrounded by a thin, flattened, hyaline layer of dull yellow or pastel orange; the spore formation was weak or completely absent (Fig. 4a). The dissociant provided a high and stable tacrolimus yield. We determined and optimized the conditions to maintain a high biosynthetic activity in dissociant D2 and to minimize the influence of dissociation on biosynthesis. This was achieved with the storage of dissociant D2 at -70°C with monitoring of its biosynthetic potential via plating on MDA medium and a series of alternating passages on liquid and solid media. As a result, the number of colonies of dissociant D2 with low activity during cultivation on solid MDA after growth in a liquid medium did not exceed 1–2%.

Earlier, we showed the positive effect of fed-batch cultivation on tacrolimus biosynthesis, which was expressed by a more than twofold increase in the immunosuppressant yield as compared to the batch-growth regime [21]. In this work, we assessed micro- and macromorphological changes in the producer’s mycelium. During the first two days, the mycelium biomass grew by 22–25 times (Fig. 5a), which was accompanied by an almost twofold decrease in the carbon source content (Fig. 5b). The amount of biomass then decreased (Fig. 5a), which indicated the beginning of the stationary growth phase and tacrolimus biosynthesis (Fig. 5b). The starch-fed batch mode provided a smoother decrease in biomass during tacrolimus biosynthesis as compared to batch mode; the average difference in biomass content was 36% (Fig. 5a).

According to SEM data, the 1-day-old mycelium developing during tacrolimus biosynthesis in batch mode was represented by hyphae with a smooth outline without signs of destruction (Fig. 6a).

On the sixth day (the stage of active biosynthesis), the structure of the productive mycelium hyphae changed from cylindrical to sinuous; the clarity of
their boundaries and the cytoplasm density were lost, and empty hyphal envelopes appeared (Fig. 6b). On the tenth day (the maximal production of tacrolimus and a decrease in the biosynthesis rate), enhancement of destructive processes were observed. This was expressed by a severe curvature of the hyphal profile and loss of the integrity of part of the mycelium (empty envelopes) (Fig. 6c).

Similar changes in the mycelium structure were also observed as a result of fed-batch cultivation; however, the degradation was less pronounced in this case (Figs. 6d–6f). The formation of sporiferous cultures was not observed during submerged growth and biosynthesis of the target product.

Fed-batch cultivation did not give the expected effect of pronounced mycelium intactness; however, it was accompanied by a higher level of the tacrolimus production (Figs. 5b, 6d–6f). The tacrolimus yield during fed-batch cultivation was higher than the 100% titer in control and amounted to 500 ± 47 and 245 ± 22 mg/L in fed-batch and batch modes, respectively. In terms of biomass content, the difference was not so large, only 36%.

The different levels of the tacrolimus production may be a consequence of the different amounts of NADPH in each of the used cultivation modes. It is known that the biosynthesis of most antibiotics (including tacrolimus) is an energy-consuming process, in which NADPH acts as the main reducing cofactor. In most microorganisms, the largest portion of NADPH is synthesized through the pentosophosphate pathway (PPP), which involves the glucose-6-
phosphate dehydrogenase and phosphogluconate dehydrogenase activities. For instance, it was shown that the carbon flow through the PPP stimulated an increase in methylenomycin production by *S. coelicolor* [41]. It is most likely that the products of the enzymatic hydrolysis of starch, which was also introduced during tacrolimus biosynthesis by *S. tsukubensis* VKM Ac-2618D, were metabolized through the PPP with the direct uptake of synthesized NADPH for the needs of biosynthetic processes.

On the one hand, the synthesis of antibiotics depends on the occurrence of both NADPH and ATP, and, on the other hand, a high energetic charge in the cell inhibits its secondary metabolism [42]. The fact that the derepression of the genes of antibiotic biosynthesis is due to the decrease in the intracellular ATP supports the latter statement. For instance, it was reported that a high intracellular content of ATP is required for the synthesis of gramicidin C by a *B. brevis* strain. Conversely, the presence of reduced NADPH has an extremely strong effect on tetracycline synthesis that the tetracycline yield increases with decreases in the intensity of the TCA reactions [43]. A positive relationship was established between the production of

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**Fig. 6.** Morphological changes in *S. tsukubensis* VKM Ac-2618D during tacrolimus biosynthesis: (a–c) batch cultivation mode, (d–f) fed-batch cultivation mode; (a) and (d) 1 day; (b) and (e) 6 days; (c) and (f) 10 days (scanning electron microscopy, scale bar is 5 μm). Arrows indicate destructive changes, such as hyphal profile curvature and loss of hyphal intracellular content.
FK-506 and the accumulation of some metabolites of both PPP and TCA [15, 16]. At the same time, the presence of inorganic phosphate at high concentrations in the medium is a negative factor for tacrolimus biosynthesis by S. tsukubaensis NRRL 18488T, which is usually the result of intracellular ATP formation [18].

We previously showed that baker’s yeasts inactivated via medium sterilization have a stimulating effect on tacrolimus biosynthesis [20]. On the first day of cultivation, inactivated yeasts were represented by whole cells with intact cell walls and internal contents, while noticeable destructive changes were observed by the tenth day of biosynthesis. Empty yeast-cell envelopes appeared at the phase of active tacrolimus synthesis; these structures significantly differed from cells of saccharomycetes on the first days of incubation, when intracellular contents were seen in them. The yeast cells remained unchanged in the control medium without inoculation with Streptomyces culture.

The final stage of tacrolimus biosynthesis under the studied cultivation modes (Fig. 5b) was characterized by the predominance of mycelial destruction over the growth processes (Fig. 6).

CONCLUSIONS

Thus, the Streptomyces sp. strain VKM Ac-2618D was identified as S. tsukubaensis VKM Ac-2618D based on a complex of chemotaxonomic and molecular biological studies, as well as in silico DNA-DNA hybridization assay. An in-depth analysis of the genome showed the presence of a cluster for tacrolimus biosynthesis with a full set of genes (26 pieces), which correlated with a high biosynthetic activity of the strain. The phenotypic variability of the strain was studied, and the D2 dissociant with the highest tacrolimus productivity was selected. The advantages of fed-batch cultivation over batch mode were shown; it was assumed that these advantages are mainly associated with the formation of higher amounts of cofactors required for the FK-506 biosynthesis, in particular, NADPH. The results can be used for the development of a tacrolimus production process.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflicts of interest.

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