Enhanced Triacylglycerol Metabolism Contributes to Efficient Oil Utilization and High Production of Salinomycin in *Streptomyces albus* ZD11

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Running title: Characterization of oil utilization in *S. albus* ZD11

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Abstract

Streptomyces is well-known for biosynthesis of secondary metabolites with diverse bioactivities. Although oils have been employed as carbon sources to produce polyketide antibiotics for several industrial Streptomyces strains, the intrinsic correlation between oil utilization and high production of antibiotics still remains unclear. In this study, we investigate the correlation between oil metabolism and salinomycin biosynthesis in Streptomyces albus ZD11 which employs soybean oil as the main carbon source. Comparative genomic analysis revealed the enrichment of genes related to triacylglycerol (TAG) metabolism in S. albus ZD11. Transcriptomic profiling further confirmed the enhancement of TAG metabolism and acyl-coenzyme A biosynthesis in S. albus ZD11. Multiple secreted lipases, which catalyze the TAG hydrolysis, were seen to be working in a synergistic and complementary manner in aiding the efficient and stable hydrolyzation of TAGs. Together, our study suggests that enhanced TAG hydrolysis and fatty acid degradation contribute to the high-efficiency of oil utilization in S. albus ZD11 in order to provide abundant carbon precursors for cell growth and salinomycin biosynthesis.

Importance

In order to obtain a high production of antibiotics, oils have been used as the main carbon source for some Streptomyces strains. Based on multi-omics analysis, this study provides insight into the relationship between triacylglycerol (TAG) metabolism and antibiotic biosynthesis in S. albus ZD11, an oil-preferring industrial Streptomyces strain. Our investigation into TAG hydrolysis gave the further evidence that this strain utilized complicated strategies enabling an efficient TAG metabolism. In addition, a novel secreted lipase was identified that exhibited highly hydrolytic activity towards medium- and long-chain TAGs. Our finding presents a good start to clarify the complicated relationship between TAG
catabolism and high antibiotic production in the industrial strains.

Keywords: triacylglycerol, fatty acid, lipase, antibiotics, metabolism
Introduction

*Streptomyces* is one of the most important genus *Actinomyces* due to its ability to produce varieties of secondary metabolites with diverse bioactivities. These include antibiotics, antitumor agents, immunosuppressive agents, and enzymes (1). Composition of the nutrients, such as carbon, nitrogen and inorganic ion, always affects the production of secondary metabolites in the genus *Streptomyces* (2).

Although sugars are the preferred carbon source for most microorganisms (2), several strains of *Streptomyces*, such as *Streptomyces clavuligerus* (clavulanic acid producer) (3), *Streptomyces hygroscopicus* (rapamycin producer) (4), and *Streptomyces tsukubaensis* (FK506 producer) (5), have been found to preferentially use oils as their carbon sources leading to higher production of antibiotics.

Since most of the oil-prefering *Streptomyces* strains are industrial strains (6, 7), the correlation between an efficient oil metabolism and the high production of antibiotics still remains unclear due to a relative lack of genomic information and the fairly low application levels for genetic manipulation techniques.

Triacylglycerols (TAGs) are the main ingredients of oils. The process of TAG catabolism normally comprises of three steps in *Streptomyces*. Initially, extracellular TAGs are hydrolyzed into free fatty acids (FFAs) and glycerol by the secreted lipases and esterases. FFAs are then absorbed by the cells through passive diffusion and/or protein-facilitated transport. This absorption process has been well characterized in *Escherichia coli* but remains unclear in *Streptomyces* (8). Finally, intracellular FFAs are degraded, mainly via the beta-oxidation pathway, resulting in the production of acetyl-CoA that can be easily transformed to acyl building blocks for antibiotic biosynthesis, especially polyketides (9). Acetyl-CoA can also be concurrently utilized in the biosynthesis of other lipids, such as phospholipids and neutral lipids, through the fatty acid (FA) biosynthetic pathway, which can then be used in cell growth or storage (10). For oil-utilizing *Streptomyces* and some other microorganisms, the activities of metabolic pathways
for TAGs, such as TAG hydrolysis and beta-oxidation pathways, are considered as the main factors affecting the efficiency of oil utilization (11-14). The hydrolysis of TAGs is the first rate-determining step for the oil-utilizing Streptomyces strains to utilize extracellular oils (13, 15). This process is mainly catalyzed by secreted lipases at the lipid-water interface (16). Lipase (EC 3.1.1.3) is a class of hydrolases that is widely found in bacteria, fungi, plants, and mammalian cells. Although several lipases from the Streptomyces strains have been reported, the relationship between lipase and secondary metabolism in the oil-utilizing Streptomyces strains remains obscure (17).

Streptomyces albus ZD11 is a derivative of an industrial salinomycin-producing strain which can produce up to 40g/L of salinomycin in a medium containing soybean oil (18). To investigate the mechanism of efficient conversion of TAGs to salinomycin in S. albus ZD11, whole genome sequencing and transcriptome sequencing were both performed in this study. The metabolic pathways for TAGs, including TAG hydrolysis, beta-oxidation and FA biosynthesis, were found to be enhanced in S. albus ZD11. Overall, 22 lipases were identified, of which 15 contained predicted secretory signal peptides. Among these, two secreted lipase genes, lip1 and lip2, showed increased expression levels upon soybean oil addition to the medium. The influence of these two lipases on salinomycin biosynthesis was evaluated and Lip1 was carefully characterized in vitro. Our results provide evidence that the first step of TAG catabolism, as catalyzed by lipase, is a complicated and complementary process in S. albus ZD11.

Results

Streptomyces albus ZD11 prefers using soybean oil as the carbon source in salinomycin biosynthesis.

Oils are often employed as primary or auxiliary carbon sources in the production of salinomycin and other antibiotics (19, 20). With its high salinomycin production and capability of soybean oil-utilization,
S. albus ZD11 was chosen as an ideal subject to explore the correlation between TAG metabolism and antibiotic biosynthesis. In this study, an equal carbon content of either glucose or soybean oil, was provided as the sole carbon source to evaluate salinomycin production in S. albus ZD11. As shown in Fig. 1A, the salinomycin yield in the medium containing soybean oil was up to 3.74 g/L at 120h. It is 11-fold higher than that in the medium containing glucose (0.33 g/L). Quantitative analysis of the carbon source consumption showed that more soybean oil (59.23%) than glucose (30.80%) had been consumed (Fig. 1B). The conversion ratio of soybean oil carbon to salinomycin carbon was 12.92% (3.08 mmol/mol), whilst that of glucose was 2.18% (0.52 mmol/mol). Comparison of salinomycin yield in the media, containing different concentrations of soybean oil or glucose, further confirmed that S. albus ZD11 is more inclined to utilize soybean oil than glucose (Fig. 1C).

To verify whether the oil-preferring property of S. albus ZD11 is distinctive, three Streptomyces type strains, Streptomyces coelicolor M145 (a Streptomyces coelicolor A3(2) derivative), Streptomyces albidoflavus J1074 and Streptomyces lividans TK24, were chosen to compare growth difference in the medium containing glucose or soybean oil as the sole carbon source. According to previous reports, whilst S. coelicolor M145 and S. lividans TK24 are able to utilize long-chain FAs or triolein (11, 13), we know of no prior report stating that S. albidoflavus J1074 can utilize oils. As shown in Fig. 1D, in the oil medium, the biomass of S. albus ZD11 was more than 2-fold those of the other three strains. In comparison, growth of S. albus ZD11 was a little worse than the other strains in the glucose medium. Among these four strains, only S. albus ZD11 grew much better in the oil medium than in the glucose medium, suggesting S. albus ZD11 might possess a distinct mechanism of highly-efficient oil utilization leading to an increased supply of acyl-coenzyme A (CoA) precursors, not only for its secondary metabolism but also for its primary metabolism.
Comparative genomic analysis reveals an abundant repertoire of genes related to TAG metabolism in *S. albus* ZD11.

In order to investigate why *S. albus* ZD11 can utilize oil more efficiently than the other three strains, whole genome sequencing was performed. *S. albus* ZD11 has a single linear chromosome of 8,317,371 bp with 7,159 predicted open reading frames (ORFs) and an average GC content of 72.64%. This linear chromosome is non-symmetrical due to the replication origin (*oriC*) and *dnaA* being located at about 207 kb right of the chromosome midpoint. Whole genome alignments between *S. albus* ZD11 and the other three strains mentioned above identified a 6.4 Mb core genome separated by a 0.7 Mb non-conserved region in *S. albus* ZD11. Two chromosomal ends and the 0.7 Mb non-conserved region constitute a 1.9 Mb non-core genome for *S. albus* ZD11 (Fig. 2A).

To look further into the relationship between functional genomics and highly-efficient oil utilization, the lipid metabolism related genes were analyzed according to the COG (Cluster of Orthologous Groups) database (21). In *S. albus* ZD11, more candidate proteins (257 predicted proteins) were clustered into lipid transport and metabolism category (category I, Fig. S1). Further metabolic pathway analysis based on KEGG (Kyoto Encyclopedia of Genes and Genomes) database (22) revealed that the number of genes involved in the TAG metabolism, especially the TAG catabolism including TAG hydrolysis and beta-oxidation, is significantly higher in *S. albus* ZD11 than in the other strains (Fig. 2B, Supplementary table 1). And the FA biosynthetic pathway is enhanced in the third step, which is catalyzed by 3-oxoacyl-ACP synthase (EC 2.3.1.41/179/180, Fig. 2C, Supplementary table 1). In addition, more than half of these TAG metabolism related genes are presented only in *S. albus* ZD11 and have no homolog identified in the other three strains, implying that they may play important roles in promoting the efficiency of TAG metabolism in *S. albus* ZD11.
Conversely, in the metabolic pathways for glucose such as the Embden-Meyerhof-Parnas (EMP) and pentose phosphate (PPP) pathways, the quantities of related genes presented in *S. albus ZD11* were generally lower, to varying degrees, than those found in the other three strains. In the EMP pathway, the gene counts of main reaction steps in *S. albus ZD11* were similar to or less than those in the other three strains resulting a relative lower level on the total number of these genes (Fig. 2D, Supplementary table 1). In addition, a notably lower gene count in the PPP pathway was observed as compared to the other strains (Fig. 2E, Supplementary table 1). This is consistent with the results of cell growth and salinomycin yield.

Pan-genome analysis was further employed to explore the specificity of *S. albus ZD11* in *S. albus*. All of 61,202 protein-coding genes from nine *S. albus* strains, are grouped into 24,858 clusters including 1,541 core gene clusters shared by the nine strains, and 15,994 unique gene clusters presented in only one strain (Fig. S2A). As compared with the other eight strains, more lipid transport and metabolism related genes are identified in *S. albus ZD11* and the unique genes occupy 52.14% of them (Fig. S2B). In addition, *S. albus ZD11* harbors more unique genes involved in the metabolic pathways for TAGs as compared with the other eight strains. But in the metabolic pathways for glucose, the quantities of total genes and unique genes are similar to or less than those in the other eight strains (Fig. S2C, Supplementary table 2). It further suggests the enhancement and specificity of TAG metabolism in *S. albus ZD11*.

**Transcriptomic profiling confirms the enhancement of TAG metabolism in *S. albus ZD11***.

Although more TAG metabolism related genes are located in the genome of *S. albus ZD11*, the transcriptional expression behavior of these genes remains unclear. Therefore, RNA-seq was performed to profile the transcriptomic changes in *S. albus ZD11*, when cultured in the presence of glucose or soybean oil. Comparative transcriptomic analysis result showed that up to 2,093 genes had changed
significantly in their expression levels between those in glucose and those in oil media (fold change > 2, p-value < 0.001). After filtering out low-expressed genes (FPKM < 10 in the both media), the remaining 1,860 genes exhibited differential expression levels (Fig. 3A). Among these genes, the expression levels of 655 genes were up-regulated while 1,205 genes were down-regulated, when *S. albus* ZD11 was cultured in the oil medium as compared to the glucose medium.

As expected, the total expression levels of the metabolic pathways for TAGs in *S. albus* ZD11 were up-regulated, and those of the metabolic pathways for glucose were down-regulated in the oil medium as compared to the glucose medium (Fig. 3B). The total expression level of all the predicted secreted lipase genes in the oil medium was 2.44-fold of that in the glucose medium and the total expression level of the beta-oxidation related genes was 2.81-fold as well. In the oil medium, the total expression level of genes involved in the FA biosynthetic pathway, of which only the third step is enhanced significantly according to comparative genomic analysis, also became 2.92-fold of that in the glucose medium. The activation of FA biosynthetic pathway might be due to the accumulation of intracellular acetyl-CoA resulting from the high efficiency of FA oxidation process in *S. albus* ZD11. This could also explain why *S. albus* ZD11 grows better in the oil medium as reflected by biomass. Conversely, the total expression levels of the EMP and PPP pathways showed obvious decreases in the oil medium. As shown in Fig. 3C, among the TAG hydrolysis, FA beta-oxidation and FA biosynthesis pathways, the expression levels of 52.86% of genes had increased (FPKM_{oil}/FPKM_{glucose} > 1.5) to varying degrees in the oil medium, whereas in the EMP and PPP pathways, 48.65% of genes had reduced their expression levels (FPKM_{oil}/FPKM_{glucose} < 0.67, Supplementary table 1).

The yield of salinomycin, as produced per liter of culture, is much higher in the oil medium than that in the glucose medium. This indicates that a large portion of acetyl-CoA generated by TAG catabolism is
converted to various acyl-CoA precursors for salinomycin biosynthesis. Therefore, the expression levels of genes participating in acyl-CoA biosynthesis were also analyzed. As shown in Fig. S3A, acetyl-CoA could be transformed to malonyl-CoA, methylmalonyl-CoA, or ethylmalonyl-CoA, either directly or through some simple reaction steps. The expression levels of genes encoding the acetyl-CoA carboxylase complex were upregulated dramatically in the oil medium, implying an increase in malonyl-CoA supply to support salinomycin or FA biosynthesis. And a notable increase in the expression levels of genes involved in the methylmalonyl-CoA or ethylmalonyl-CoA biosynthetic process was also observed (Fig. S3B, Supplementary table 3). The above results gave further evidence that the metabolic pathways for TAGs are enhanced in *S. albus* ZD11 and that acetyl-CoA generated by the beta-oxidation pathway could be directed to cell growth or secondary metabolic biosynthesis through the efficient acyl-CoA biosynthetic pathways.

*S. albus* ZD11 harbors at least 15 predicted secreted lipases that contribute to highly lipolytic activity.

In order to verify the hypothesis that enhanced TAG metabolism at genomic and transcriptomic levels results in high-efficiency of oil utilization in *S. albus* ZD11, the TAG hydrolysis process was chosen as the focus for investigation. Based on functional annotations in the PFAM database (23), twenty-two candidate lipases, belonging to α/β hydrolase family or SGNH hydrolase family, were predicted in *S. albus* ZD11. Among them, 15 secreted lipases were screened out according to analysis of signal peptide sequence (Table S1). In addition, the secreted lipases present in the other three strains were also extracted, of which 7, 10, and 10 were extracted from *S. albido flavus* J1074, *S. coelicolor* A3(2) and *S. lividans* TK24, respectively. Phylogenetic analysis showed that all secreted lipases identified from the four strains could be classified into three clades (Fig. 4A). Clade I contained 11 lipases which shared canonical
GXSXG pentapeptide around catalytic serine and belonging to the α/β hydrolase family. Six of these lipases were from *S. albus* ZD11 and the remaining five were from the other three strains. Clade II and III contained 14 and 17 lipases, respectively. The lipases from these two clades belong to the GDS(L) lipase family, a subfamily of the SGNH hydrolase family, and share a GDSX catalytic site motif. The low similarities of amino acid sequences among GDS(L) lipases probably resulted in the distribution into two clades on phylogenetic tree (24). Rhodamine B (RhB) plate assay was further performed to evaluate total lipolytic activity. As shown in Fig. 4B, the fluorescent halos formed by TAG hydrolysis around the aerial mycelia lawns of *S. albus* ZD11 were much larger than those around *S. albidoflavus* J1074. The other two strains failed to exhibit any discernible fluorescent halo. And the quantitative results further confirmed that the total lipolytic activity of *S. albus* ZD11 was higher than those of the other three strains, especially at the early stage of fermentation (Fig. 4C).

According to our transcriptomic data, eight secreted lipase genes from *S. albus* ZD11 could be expressed effectively in both growth media (FPKM value >= 10 either in oil or glucose medium). And the expression levels of these genes were reconfirmed by real-time quantitative PCR (RT-qPCR, Fig. 4D).

Two of these lipase genes, DUI70_6046 (named as *lip1*) and DUI70_4999 (named as *lip2*), showed significant increases in their expression levels (fold change > 2, p-value < 0.001) when *S. albus* ZD11 was cultured in the oil medium as compared to the glucose medium (Fig. S4). This implies that these two lipases may play functional roles in the hydrolytic process of TAGs.

**Lip1 and Lip2 play the functional roles in the hydrolytic process of TAGs.**

To further evaluate the functions of Lip1 and Lip2 in the hydrolytic process of TAGs, the Δlip1 and Δlip2 mutants were constructed. The result of RhB plate assay showed that the fluorescent halos formed by the Δlip1 and Δlip2 mutants were much smaller than those formed by the WT strain, indicating that the total...
lipolytic activities of the mutants had decreased (Fig. 5A). When each lipase gene was complemented into its deletion mutant, the total lipolytic activity showed a certain degree of recovery in both complementary mutant strains (Δlip1/plip1 and Δlip2/plip2). In the Δlip1/plip1 mutant, the total lipolytic activity recovered dramatically with fluorescent halos almost as large as those of the WT strain. However, lipolytic activity recovered only a little in the Δlip2/plip2 mutant. The result of expression level measurement showed that the incomplete recovery of the Δlip2/plip2 mutant for total lipolytic activity was due to the relatively lower expression level of complemented lip2 (Fig. S5A), indicating that the genomic context of complemented lip2 was different from its WT. In contrast, complemented lip1 showed a relatively higher expression level in the Δlip1/plip1 mutant (Fig. S5B). When both of the two lipase genes were disrupted in S. albus ZD11 (Δlip1/2 mutant), the total lipolytic activity decreased notably on the RhB plates, as expected (Fig. 5A). Further quantitative analysis revealed that the total lipolytic activity of the Δlip1/2 mutant was about 40% lower than that of the WT strain during the fermentation time course (Fig. 5B). Unexpectedly, the salinomycin yield and oil utilization efficiency of the Δlip1/2 mutant in flask fermentation did not change significantly (data not shown). Since there are still other secreted lipases in addition to Lip1 and Lip2 presented in S. albus ZD11, the remaining lipases might play complementary roles in TAG hydrolysis in the Δlip1/2 mutant. Among these remaining lipase genes, the expression levels of DUI70_0051, DUI70_2396, and DUI70_5339 in the Δlip1/2 mutant were more than 2-fold of those in the WT strain and that of DUI70_1978 was about 1.5-fold as well (Fig. 5C). This indicates that the secreted lipases in S. albus ZD11 could function in a synergistic way in order to maintain efficiently catalytic activity for hydrolyzing TAGs.

As Lip1 and Lip2 play the functional roles in S. albus ZD11, their catalytic activities were investigated in vitro. Both lip1 and lip2 were cloned from S. albus ZD11 and heterologously expressed in E. coli.
BL21(DE3). The signal peptides of these two lipases were truncated for intracellular soluble expression. The purified proteins of Lip1 and Lip2 were obtained with the aid of the chaperones DnaJ, DnaK and GrpE (25) (Fig. 6A and Fig. S6).

The effects of temperature, pH, and substrate to the activity of Lip1 or Lip2 were evaluated. As shown in Fig. 6B, Lip1 was active from 10 °C to 60 °C and the activity reached to peak at 35 °C with para-nitrophenyl palmitate (p-NPP) as the substrate. This temperature is consistent with the fermentation temperature of S. albus ZD11 (33 ± 1 °C). To determine the influence of pH on the activity of Lip1, triolein was selected as a substrate because the p-NPP method is affected by pH variation (26). As shown in Fig. 6C, Lip1 was active at pH from 6 to 9, with the optimum pH as 8.0. Furthermore, the substrate selectivity of Lip1 was detected by using TAGs with different carbon chain lengths. Our results showed that Lip1 preferred to hydrolyze medium- (C8-C12) and long-chain (> C12) TAGs (Fig. 6D). As oleic acid is one of the major FAs in soybean oil, Lip1 could play an important role in the process of soybean oil hydrolysis. Under the optimum reaction conditions, V_{max} and K_{m} of Lip1 were detected and calculated as 245.90 U/mg and 0.96 mM, respectively (Fig. 6E, Table 1). Compared to the previous reports (27-29), Lip1 exhibited the highest activity to triolein among the GDS (L) family members from the studied Streptomyces strains (27, 30). This gave increased support for the presence of an efficient oil metabolism in S. albus ZD11.

Discussion

As an industrial strain, S. albus ZD11 has an impressive ability to utilize oils efficiently, leading to a high production of salinomycin, the relationship between efficient TAG metabolism and high salinomycin production in S. albus ZD11 was investigated in this study. Multi-omics analysis results demonstrate that
TAG hydrolysis, beta-oxidation, and FA biosynthesis are all enhanced in S. albus ZD11. Taking the investigation into the first step of TAG catabolism as a main focus, it was found that multiple secreted lipases could work in a synergistic and complementary manner, contributing to highly efficient hydrolysis of extracellular TAGs. Among these lipases, Lip1 exhibited a highly lipolytic activity and was found to play an important role in the hydrolysis of soybean oil.

As shown in Fig. 7, we speculate that efficient hydrolysis of extracellular TAGs generates a large number of FFAs which are absorbed later by S. albus ZD11. These FFAs could be degraded by an enhanced beta-oxidation pathway to produce abundant acetyl-CoA, most of which is directly converted to various acyl-CoA precursors for salinomycin biosynthesis. This is consistent with the result in a recent report, it was confirmed that the efficient beta-oxidation would inhibit the activity of TCA cycle and direct the acetyl-CoA to polyketide biosynthesis (14). Simultaneously, with the aid of an enhanced FA biosynthetic pathway, S. albus ZD11 could utilize acetyl-CoA efficiently to synthesize other lipids such as the phospholipids and neutral lipids. These phospholipids could support fast cell growth and the neutral lipids could be used for polyketide biosynthesis during the stationary phase (14). Conversely, the glucose metabolism shows weaker tendency in S. albus ZD11 as compared to the other three Streptomyces strains, implying that S. albus ZD11 could reinforce its ability of oil utilization through the balance of reducing the capability of its glucose metabolism in order to maintain its competitiveness under special environmental conditions.

Microorganisms provide the widest source of lipases for biotechnological applications due to their stability, selectivity, and broad substrate specificity (31). Some lipases from Streptomyces species have been reported over the last thirty years (27-30). Ana Bielen and her colleagues characterized a GDS(L) lipase, SCO1725 from S. coelicolor A3(2), which shows a 63.94% sequence similarity with Lip1 (27).
However, Lip1 exhibited a 2.5-fold hydrolysis activity towards long-chain TAGs and a different optimum temperature, as compared to SCO1725. The expression level of lip2 was up-regulated significantly when S. albus ZD11 was cultured in the oil medium and the total lipolytic activity of the Δlip2 mutant had deceased dramatically. This implies that Lip2 plays an important role in TAG hydrolysis. However, the activity of Lip2 was not detected in vitro. Although most of bacterial lipases do not require cofactors (32), some lipases have been reported to require lipase-specific foldases that aid them in correct folding (33).

As Lip2 is a unique protein from S. albus ZD11 according to pan-genome analysis, there may therefore be some uncharacterized lipase-specific foldases participating in Lip2 folding.

As mentioned above, the salinomycin yield showed no obvious decrease in the Δlip1/2 mutant under our liquid growth conditions. Three reasons might lead to the appearance of this phenotype. Firstly, as multiple secreted lipases work in a synergistic and complementary way in S. albus ZD11, the remaining lipases might restore the lipolytic activity in the Δlip1/2 mutant to some extent. Secondly, TAG metabolism is a complicated process and a decrease in just one step may not significantly impair salinomycin production. Thirdly, given the much lower production of salinomycin in the lab (about 4 g/L) than that in the industrial production (40 g/L), the precursor supply might be still sufficient after deletion of these two lipase genes.

As the beta-oxidation pathway is highly important for TAG utilization, a preliminary study on 3-hydroxyacyl-CoA dehydrogenase, which catalyzes the third step of the beta-oxidation pathway, was also carried out by our group. Two highly expressed enzymes (DU170_0391 and DU170_0803), predicted to harbor both 3-hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase domains, were screened out according to the result of genome-wide mining. Homologs of these two enzymes in S. coelicolor A3(2) have been reported to participate in the degradation of FAs (34). Double deletion of these two genes
(DUI70_0391 & DUI70_0803) in S. albus ZD11 only led to about a 50% decrease in salinomycin yield (data not shown). This indicates although these two enzymes are very important for β-oxidation in S. albus ZD11, there are still some uncharacterized enzymes playing complementary roles. All above results suggest that digging and characterizing the novel functional proteins will be very helpful for understanding the intrinsic connection between efficient TAG metabolism and high antibiotic production in S. albus ZD11 and other oil-preferring industrial strains.

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Material and Methods

Bacterial strains and plasmids

All experiments were performed with the strains and plasmids as listed in Table 2. The mutants were derived from wild-type S. albus ZD11.

Media and growth conditions

S. albus ZD11 and its derivative strains were grown on ISP4 agar medium (BD, USA) for 7-8 days at 30°C for sporulation. Rhodamine B (RhB) agar medium (K$_2$HPO$_4$, 1 g; MgSO$_4$, 1 g; NaCl, 1 g; (NH$_4$)$_2$SO$_4$, 2 g; CaCO$_3$, 2 g; FeSO$_4$, 0.001 g; MgCl$_2$, 0.001 g; ZnSO$_4$, 0.001 g; agar, 20 g; soybean oil, 15 mL; RhB, 0.1 mg per liter) was used for lipolytic activity determining. The culture in Tryptic Soy Broth (TSB) medium (TSB, 30 g per liter) for 24 h was used to obtain high-quality genomic DNA for whole genome sequencing and genotype confirmation. The industrial seed medium (soybean powder, 30 g; glucose, 40
g; yeast extract, 10 g; CaCO\textsubscript{3}, 2 g per liter) was used for seed cultivation for 29 h. Ionic medium (NaCl, 2 g; KCl, 2 g; (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, 5 g; MgSO\textsubscript{4}, 0.1 g; K\textsubscript{2}HPO\textsubscript{4}, 0.2 g; CaCl\textsubscript{2}, 0.1 g; CaCO\textsubscript{3}, 5 g per liter) with soybean oil (oil medium) or glucose (glucose medium) containing an equimolar amount of carbon (2.7 M) was used for fermentation at 32 °C for 5 days. All *E. coli* strains were grown at 30 °C or 37 °C in Luria-Bertani (LB) medium (35). *E. coli* ET12567 (pUZ8002) was used for transferring DNA into *S. albus* by intergeneric conjugation (18). DNA manipulations were carried out according to standard procedures (36). When appropriate, apramycin, spectinomycin, kanamycin, or chloramphenicol (Sangon, China) was added to the medium at a final concentration of 50, 50, 50, or 25 μg/mL, respectively.

**Quantification of carbon source consumption**

To determine the glucose concentration in medium, the mycelia in fermented culture from the glucose medium were removed by a centrifugation at 12,000 \times g for 2 min. The supernatant was diluted 100 times with water and the glucose concentration was measured using an SBA-40D biosensor analyzer (Institute of Biology, Shandong Academy of Sciences, China). The soybean oil concentration in medium was measured using a method described previously (6).

**Whole genome sequencing and alignment**

The whole genome of *S. albus* ZD11 was sequenced using a PacBio RS II platform at the Beijing Genomics Institute (BGI, Shenzhen, China). Gene prediction was performed on a genome assembly using glimmer3 (http://www.cbcb.umd.edu/software/glimmer/) with Hidden Markov models. The databases, namely KEGG and COG, were used for general function annotation. Whole genome alignment was performed using MUMmer (version 4.0 beta) with mincluster = 65 and minmatch = 35 (37). Chromosomal sequences of *S. albidoflavus* J1074 (CP004370.1), *S. coelicolor* A3(2)...
(NC_003888.3) and *S. lividans* TK24 (NZ_CP009124.1) were obtained from GenBank in GenBank format.

**RNA-seq analysis**

The total RNA of *S. albus* ZD11 was prepared from the mycelia grown in ionic medium with soybean oil or glucose (0.45 M) as the sole carbon source. Two replicates of 40 μg (wet weight) mycelia were taken at 60h for both media. Total RNA isolation and RNA-seq were performed according to a previous report (38).

**Genome-wide mining and phylogenetic analysis of lipases**

To excavate the lipases in the *Streptomyces* strains, genome-wide mining of lipase was performed based on functional annotations in the PFAM database (version 32.0). Two clans, CL0264 (SGNH_hydrolase), CL0028 (AB_hydrolase), and five lipase families, LIP, Lipase_2, Lipase_GDSL, Lipase_GDSL_2 and Lipase_GDSL_3 were used to screen the putative lipases. Signal peptide analysis was performed on the SignalP web-sever (http://www.cbs.dtu.dk/services/SignalP/). All sequences of the lipase genes mentioned above were obtained from GenBank in FASTA format. Multiple sequence alignment (MSA) and phylogenetic tree construction were carried out using MAFFT on the EMBL web server (https://www.ebi.ac.uk/Tools/mafft/) with default parameter settings (39, 40). A Bootstrap N-J (Neighbor-Joining) tree was calculated using MEGA X (41) and the ITOL (Interactive Tree of Life) online software (https://itol.embl.de/) was used to landscape the phylogenetic tree (42).

**The total lipolytic activity and biomass determination**

For the total lipolytic activity determination, all *Streptomyces* strains were cultured in the oil medium...
containing 2.7 M carbon. The fermented culture was sequentially filtered using a piece of weighed filter cloth (300 mesh) and a 0.45 μm filter. Then the mycelium wrapped in the filter cloth was dried to a constant weight for biomass determination. Eight milliliters of filtered liquid were concentrated to 200 μL using an ultrafiltration centrifugal tube (10 KD, Millipore, USA). The concentrated liquid was employed for lipolytic activity determination using the end-point titration method with soybean oil (5%, vol/vol) as the substrate (43).

Constructions of gene disruption mutants

A Fosmid library was previously generated for the construction of S. albus ZD11 mutant (18). Fosmid SAF16H7, containing lip1, and SAF5A3, containing lip2, were used to construct the gene disrupted mutants using the PCR-Targeting system (44). Part of the Lip1 coding region (amino acids 10 to 254) and part of the Lip2 coding region (amino acids 16 to 237) were displaced by the apramycin and spectinomycin disruption cassette as described previously (45). All mutants were confirmed using PCR and DNA-sequencing analysis. Primer pairs for gene disruption and mutant confirmation are listed in Table S2.

Complementation of the lipase genes in the Δlip1 mutant and the Δlip2 mutant

The coding region of Lip1 or Lip2 with its native promoter and terminator was amplified and cloned into the EcoRV site of plasmid pSOK804H to generate the plasmid plip1 or plip2. For lip1, 305bp upward from the start codon and 300 bp downward from the stop codon was selected as its promoter and terminator, respectively. For lip2, 392 bp upward from the start codon and 385 bp downward from the stop codon were selected as its promoter and terminator, respectively. The complementary plasmid was transferred into the Δlip1 or the Δlip2 mutant, respectively, by intergeneric conjugation. The positive
transformants were identified by apramycin overlay and confirmed by PCR.

Expression and purification of recombinant proteins.

Signal peptide of either Lip1 (residues, 1-29) or Lip2 (residues, 1-30) was truncated when expressed in E. coli BL21(DE3). These two truncated sequences were amplified and cloned into pET28a to generate the recombinant plasmid pET-lip1 or pET-lip2, respectively. E. coli BL21(DE3)/pKJE7 was chosen for heterologous expression of the recombinant lipases. The overnight culture of recombinant E. coli BL21(DE3)/pKJE7 was inoculated into 200 mL liquid LB medium at a final optical density (OD) of 0.05 at 600 nm with appropriate antibiotics and 1 mM L-arabinose. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 0.1 mM when the culture was grown to OD₆₀₀ of 0.5-0.6 at 25 °C. After a further 12 h of incubation at 16 °C, the cells were harvested by centrifugation and disrupted by sonication on ice. The supernatant was then recovered by a centrifugation (4 °C, 13,000 ×g for 15 min).

Soluble Lip1 or Lip2 was purified using Ni-NTA His-bind resins as described by the manufacturer’s protocol (Novagen, USA).

Activity assays

Lipase activities at different temperatures were measured with para-nitrophenyl palmitate (p-NPP, Aladdin, China) as substrate according to Naeem Rashid et al (46). One enzyme unit (U) was defined as the amount of enzyme that produced 1 μmol of p-nitrophenyl per min. A modified colorimetric method was used to assess the effect of pH on the lipase activity (47). The experiment was performed at 35 °C in 50 mM different buffers (citrate (pH 5.0–6.0), sodium phosphate (pH 6.0–8.0), Tris-HCl (pH 8.0–9.0), Glycine-NaOH (pH 9.0–10.0)) containing 1% (wt/vol) gum arabic and 50 mM Triolein. Above mixture was emulsified by sonification for 4 min and cooled down on ice for 1 min, repeated three times.
Emulsified mixture (500 μL) was dispensed into an Eppendorf tube and pre-incubated for 5 min. Ten microliters of purified lipase were then added into the tube followed by another incubation (30 min). The reaction was ended with 500 μL of ethanol. The released FFAs were extracted with 1 mL of isooctane followed by a centrifugation at 12,000 × g for 10 min. Eight hundred microliters of the supernatant were mixed with 80 μL of cooper reagent (47) followed by another centrifugation at 12,000 × g for 5 min. The optical density of supernatant was measured at 715 nm using a UV spectrophotometer (UV-2450, Shimadzu, Japan). The end-point titration method was used to determine the substrate specificity (43).

Tributyrin, tricaprylin, trilaurin, and triolein were selected as the substrates and hydrolyzed at 35 °C for 30 min. A kinetic curve was also drawn using the end-point titration method at 35 °C, pH 8.0, with triolein as the substrate.

Analysis of the yield of salinomycin

A colorimetric assay based on the vanillin-sulfuric acid reagent was used to determine the production of salinomycin. At first, the fermentation broth (1 mL) was extracted using 9 mL of methanol for 12 h and centrifuged at 12,000 × g for 10 min. Four hundred microliters of the supernatant were mixed with 400 μL of methanol. To the mixture, 200 μL of vanillin-sulfuric acid reagent (vanillin, 3 g; methanol, 900 mL; concentrated sulfuric acid, 100 mL) was added and the mixture was then incubated at 60 °C for 30 min. The absorbance of the reaction product was measured at 520 nm using a UV spectrophotometer.

The OD value was used to represent the yield of salinomycin according to the standard curve of the standard salinomycin sample. HPLC analysis was used for precise determination as described previously (18).
The genome of *Streptomyces albus* ZD11 was deposited in the GenBank database under accession number CP033071. The sequence reads of RNA-seq were deposited in the SRA database under accession numbers SAMN12251778, SAMN12251779, SAMN12251780, SAMN12251781.

**Reference**


Table 1 Optimum reaction conditions for Lip1 and kinetic parameters of Lip1

<table>
<thead>
<tr>
<th>Optimum physical and chemical conditions</th>
<th>Enzyme kinetics *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td>pH</td>
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<tr>
<td>------------------</td>
<td>----</td>
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<tr>
<td>Lip1</td>
<td>35.0</td>
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</table>

a: Three replicates were performed to determine the kinetic parameters of Lip1.
Table 2. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strains or plasmids</th>
<th>Description</th>
<th>Reference</th>
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<tr>
<td><strong>strains</strong></td>
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<tr>
<td>ZD11</td>
<td>A derivative obtained with streak plate method from an industrial salinomycin-producing strain</td>
<td>CGMCC 4.7658</td>
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<td>( \Delta \text{lip1} )</td>
<td>ZD11 with disruption of lip1, spr</td>
<td>This study</td>
</tr>
<tr>
<td>( \Delta \text{lip2} )</td>
<td>ZD11 with disruption of lip2, spr</td>
<td>This study</td>
</tr>
<tr>
<td>( \Delta \text{lip1/plip1} )</td>
<td>( \Delta \text{lip1} ) harboring plip1</td>
<td>This study</td>
</tr>
<tr>
<td>( \Delta \text{lip2/plip2} )</td>
<td>( \Delta \text{lip2} ) harboring plip2</td>
<td>This study</td>
</tr>
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<td><strong>E. coli</strong> TG1</td>
<td>Host strain for DNA clone</td>
<td>Stratagene</td>
</tr>
<tr>
<td><strong>E. coli</strong> BL21(DE3)</td>
<td>Host strain for protein expression</td>
<td>Stratagene</td>
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<tr>
<td><strong>E. coli</strong> ET12567 (pUZ8002)</td>
<td>dam- dcm- strain containing helper plasmid pUZ8002</td>
<td>(44)</td>
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<td><strong>plasmids</strong></td>
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<td>pET28a</td>
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<td>plasmid with para-DnaK-DnaJ-GrpE</td>
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<td>pIJ779</td>
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<td>( \text{plip1} )</td>
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<td>( \text{plip2} )</td>
<td>Derived from pSOK804H carrying lip2 with native promoter and terminator</td>
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<tr>
<td>Fosmid SAF5A3</td>
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<td>pET-\text{lip2}</td>
<td>lip2 cloned into pET28a, kmr</td>
<td>This study</td>
</tr>
</tbody>
</table>

amr, ampicillin resistance; apr, apramycin resistance; spr, spectromycin resistance; kmr, kanamycin resistance; cmr, chloramphenicol resistance.
Fig. 1. *S. albus* ZD11 prefers soybean oil to glucose as a carbon source.

(A) Salinomycin yield in the liquid medium with soybean oil or glucose containing an equimolar amount carbon (2.7 M). (B) Consumption of soybean oil or glucose in the liquid medium at 120h. (C) Salinomycin yield in the liquid medium with different concentrations of carbon sources at 120h. (D) Comparison of cell growth among different *Streptomyces* strains in the oil medium containing 2.7 M carbon. Error bars indicate the SD of samples performed in triplicate.
Fig. 2 Genomic landscape of *S. albus* ZD11.

(A) Whole genome alignments between *S. albus* ZD11 and *S. albidoflavus* J1074, *S. coelicolor* A3(2), *S. lividans* TK24, respectively. Matches on the same strand are in red, and those on the opposite strand are in blue. Blue bars represent the conserved core regions on the *S. albus* ZD11 chromosome. Black arrow indicates the position of oriC. The quantities of genes involved in the catabolic pathways for TAGs (B), the FA biosynthetic pathway (C), the EMP pathway (D) and the PPP pathway (E). Quantity variance of genes corresponding to KEGG pathway as implicated for each biochemical reaction (k number) is color coded from 1 to 20. “Z”, “J”, “A” or “T” represents *S. albus* ZD11, *S. albidoflavus* J1074, *S. coelicolor* A3(2) or *S. lividans* TK24, respectively.
Fig. 3 Comparative transcriptomic profiling of \textit{S. albus} ZD11 based on the cultivation with different carbon sources. 

(A) Volcano map of differentially expressed genes (DEGs). Blue points represent down-regulated genes, red points represent up-regulated genes and grey points represent non-regulated genes. (B) The overall expression levels of the metabolic pathways for TAGs or glucose. Red or blue arrows represent the reaction steps involved in the metabolic pathways for TAGs or glucose, respectively. T\#, F\#, E\# and P\# represent the reaction steps involved in the TAG hydrolysis and FA beta-oxidation pathways, the FA biosynthetic pathway, the EMP pathway and the PPP pathway, respectively. The sum of FPKM values of genes in one pathway represents the total expression level of this pathway. (C) The expression levels and fold changes of genes involved in above metabolic pathways. RNA value is normalized log2 FPKM value and RNA fold change (RNA\_Fc) is normalized log2 ratio of FPKM values (FPKM\_oil/FPKM\_glucose). “G” or “S” represents the sample from the glucose or oil medium, respectively. “F” represents the fold change of gene expression level in the oil medium relative to that in the glucose medium.
Fig. 4 Secreted lipase scanning in S. albus ZD11.

(A) The phylogenetic relationship of the secreted lipases from the four *Streptomyces* strains. Grey solid circles represent the lipases from *S. albus* ZD11. Diameters of the light purple bubbles on the branches represent the bootstrap values of the N-J tree. Gene ID with “SCO”, “XNR” or “SLIV” prefix represents lipase from *S. coelicolor* A3(2), *S. albidosflavus* J1074 or *S. lividans* TK24, respectively. (B) The total lipolytic activity determination. Diameters of the fluorescent halos under UV represent lipolytic activities. (C) Quantification of the total lipolytic activity in the oil medium containing 2.7 M carbon. (D) Expression level analysis of secreted lipase genes based on RNA-seq and RT-qPCR data. Error bars indicate the SD of samples performed in triplicate.
Fig. 5 Multiple lipases work in a synergistic way to support efficient and stable TAG hydrolysis in *S. albus* ZD11.

(A) The total lipolytic activity as determined by the RhB plate assay. (B) Comparison of the total lipolytic activities between the Δlip1/2 mutant and WT strain in a liquid fermentation time course. (C) Expression level analysis of the remaining lipase genes in the Δlip1/2 mutant. RNA samples were isolated from 60h cultures. *hrdB* transcription was monitored and used as the internal control. Values are shown relative to those of the WT strain. Error bars indicate the SD of samples performed in triplicate.
Fig. 6 Determination of the hydrolytic activity of Lip1.

(A) SDS-PAGE for the truncated Lip1. Lane1-3, the purified truncated Lip1 with double 6 × His tag located at both ends of the peptide (lane1: 1.0 μg protein, lane2: 0.8 μg protein, lane 3: 0.5 μg protein).

Effects of temperature (B), pH (C) and substrate (D) on the activity of Lip1. C4, C8, C12 and C18 represent tributyrin, tricaprylin, trilaurin and triolein, respectively. (E) Kinetic curve for Lip1 activity.

Error bars indicate the SD of samples performed in triplicate.
Fig. 7 Proposed schematic diagram of the enhanced TAG metabolism in *S. albus* ZD11.

Green shading represents the enhanced TAG catabolism and precursor biosynthesis. Pink shading represents the enhanced FA biosynthesis. Dark blue arrows represent the enhanced reaction steps with grey arrows as the weakened reaction steps and dashed arrows as the multiple reaction steps.

Intermediates with red font are the direct precursors for salinomycin biosynthesis.