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# Exploring the function of acyltransferase and domain replacement in order to change the polyunsaturated fatty acid profile of *Schizochytrium* sp.

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ABSTRACT

Omega-3 fatty acids have received considerable attention due to their substantial health benefits. The aim of this study was to investigate the roles of acyltransferase (AT) domain and replace it for regulating fatty acid profile in *Schizochytrium* sp.. Herein, *Schizochytrium* sp. was engineered via gene deletion of AT and replacement of the native AT with its homologue, Shew-AT domain from *Shewanella* sp.. The docosahexaenoic acid (DHA) content in total fatty acids of the AT deficient strain observably decreased from 49.52% to 35.2% and the strain exhibited a low growth rate. Replacement with the Shew-AT gene recovered the cell growth and led to a high DHA content, with 3.7 times more eicosapentaenoic acid (EPA). This study will expand knowledge for synthesis of polyunsaturated fatty acid and facilitate the design of microbes with high yields of omega-3 fatty acids as a source of these valuable compounds for nutritional improvement.

## 1. Introduction

Long-chain polyunsaturated fatty acids (LC-PUFAs), especially eicosapentaenoic acid (EPA, 20:5, n-3) and docosahexaenoic acid (DHA, 22:6, n-3), are essential to human health, providing important neural and cardiovascular benefits throughout life [1]. Because of their unique physiological effects, they have been widely applied in the food industry, for medical treatment, and in many other fields. Currently, PUFAs are mainly produced from fish, but the supply of high quality fish oil is in significant decline due to ocean pollution and depleted stocks of fish [2]. Fortunately, prokaryotic and eukaryotic microorganisms capable of producing EPA and DHA in high amounts have emerged as an attractive alternative for LC-PUFAs production [3].

*Schizochytrium* sp., a marine microalga, synthesizes DHA through a mode of polyketide biosynthesis that differs from the classic aerobic fatty acid elongation and desaturation pathways generally observed in the well-studied producer *Mortierella alpina* [4]. Polyketide synthase (PKS)-like enzymes, known as PUFA synthases, contain a specific isomerase subunit which catalyzes a 2,3-trans/cis isomerization after reduction and dehydration steps, synthesizing DHA or docosapentaenoic acid (DPA) in *Schizochytrium* sp. [5]. In addition to *Schizochytrium* sp., many bacteria can also synthesize PUFAs through the PKS pathway. These include *Shewanella pneumatophori*, *Photobacterium profundum* and *Moritella marina* [6,7,8]. Intriguingly, different polyunsaturated fatty acid synthases have similar domains, but the detailed protein sequences and structures are totally different, which might be highly related to the final fatty acid composition. For instance, *Shewanella marinintestina* sp. and *Shewanella schlegeliana* sp. can only synthesize EPA, while some deep sea bacteria only synthesize DHA [9,10]. However, *Schizochytrium* produces both DPA and DHA [11]. These findings suggest that the differences in fatty acid composition in different microbes may be determined by a catalytic domain or be the result of differences in gene organization.

Recently, the use of the synthetic capacity of PKS enzymes in order to develop improved microbial strains, as well as characterization of the involved synthetic pathways has become a hot research topic. Many PUFA synthetic gene clusters have been transferred and expressed in *Escherichia coli*, rendering it capable to synthesize EPA or DHA [12,13]. In 2001, three genes encoding PKS was discovered in *Schizochytrium* by Metz [14]. Then Hauvermale et al. suggested that *Schizochytrium*

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contained fatty acid synthase (FAS) in addition to PKS. *Schizochytrium* also contained some elongation and desaturase enzymes, but these enzymes were not a complete series. Therefore, it was recognized that *Schizochytrium* synthesized saturated fatty acids by FAS and synthesized LC-PUFAs by PKS [15].

Acyltransferase (AT) carries out a number of distinct acyl transfer reactions. In *Schizochytrium* sp., the AT located in the second domain of PUFA synthase subunit ORFB harbors a special structure comprising a GxSxG motif, which might be involved in the release of PUFAs [16]. However, the mechanism by which PUFAs are released by PKS is still unclear. In mammalian systems, the final products are released as free fatty acids by thioesterase, but PUFA synthases have been characterized to contain an AT domain with no assigned function could be a candidate determinant to release products from the ACP domain as free LC-PUFAs in *Schizochytrium* [17]. AT may potentially possess a function similar to thioesterase. In addition, many other metabolites, such as lovastatin, squalestatin, and fumonisin were also shown to be released by the AT domain [18]. Fisch et al. conducted rational KS (ketosynthase), AT, DH (dehydration), C-Met (*c*-methylation) domain swaps of polyketide synthases in *Aspergillus oryzae*, which successfully changed the methylation pattern and chain length and produced reprogrammed compounds [19]. Liu et al. exchanged SAT (start unit ACP transacylase), KS, AT domain in the nonreduced polyketide synthase AfoE with domain from nonreduced polyketide synthase AN3386, which changed chain length and created new polyketides [20]. However, whether the AT domain is responsible for fatty acid release and determination of their composition remains uncharacterized in *Schizochytrium*.

In this study, to determine whether the AT domain is responsible for fatty acid species composition, we disrupted the AT gene of *Schizochytrium* by recombination-based insertion of a zeocin resistance gene, creating the AT deficient strain (AT-DS). Subsequently, the Shew-AT gene from *Shewanella* sp. SCRC-2738 driven by an ubiquitin promoter-terminator system was specifically incorporated into the AT gene locus by homologous recombination, creating the Shew-AT replacement strain (ShAT-RS). Finally, cell growth, fatty acid composition, and expression levels of key enzymes were compared between the wild type strain (WTS), AT-DS and ShAT-RS, with the aim of accessing the roles of the AT domain in DHA synthesis and the regulation of fatty acid composition in *Schizochytrium* sp..

## 2. Material and methods

### 2.1. Microorganisms and culture conditions

*Schizochytrium* sp. HX-308(CCTCC M209059), preserved in the China Centre for Type Culture Collection (CCTCC) [21], was used in the present study. This strain was preserved in 20% (v/v) glycerol at − 80 °C. The seed culture medium and the conditions were the same as those used in our previous study [22]. The culture preserved in a glycerol cryotube was inoculated into a 250 mL flask with 50 mL medium and cultivated for 48 h. The seed culture (1% v/v) was then transferred to a 500 mL shake flask containing 100 mL medium and incubated for 24 h. *E. coli* DH5α was used for general cloning procedures. Luria-Bertani medium was used for *E. coli* seed cultures.

### 2.2. Plasmid construction

Plasmids and primers used in this study are listed in Table 1. Plasmid extraction, agarose gel electrophoresis, and other DNA manipulations were carried out using standard protocols [23]. Sequence encoding the ORFB-AT domain is position 2701-4200 of AF378328. As shown in Fig. 1, the AT upper and downstream fragments were amplified from *Schizochytrium* sp. HX-308 by PCR using primers ATup-S to ATdown-A, and cloned into the vector pMD19-T Simple, generating pMD19-ATup and pMD19-ATdown, respectively. After confirming the ligation products by sequencing (Genewiz, Suzhou, China), the fragments for homologous recombination were cut from pMD19-ATup and pMD19-ATdown using restriction enzymes (*Not*I, *Xba*I, *Eco*RI, *Kpn*I) and cloned into the PBS-Zeo vector (including pTEF1 promoter, zeocin resistance gene, and CYC1TT terminator and pGAP promoter), resulting in the targeting vector PBS-Zeo-AT. The Shew-AT gene from *Shewanella* sp. SCRC-2738 was provided as synthetic DNA (Genewiz, Suzhou, China), and ligated into pUC57 Simple, generating PUC-Shew-AT. The ubiquitin promoter, Shew-AT coding sequence (CDS) and ubiquitin transcription terminator were amplified from the corresponding parent plasmids (ubiquitin promoter from pUC-promoter, Shew-AT CDS from pUC-Shew-AT, ubiquitin terminator from pUC-terminator), using PCR using primers Promoter-S to Terminator-A. Subsequently, overlap extension PCR was applied to splice the above three fragments in specific order, making the full-length product promoter-Shew-AT-terminator. The spliced fragment was then digested with *Xba*I and *Bam*HI, and the digested fragment was inserted into the multiple cloning site (MCS) of PBS-Zeo-AT, creating the final multiple-gene co-expressing plasmid PBZ-Shew-AT.

### 2.3. Transformation of Schizochytrium sp.

Transformation of *Schizochytrium* sp. was conducted using a modified method published previously [24] as follows: 10 mL of cell suspension was withdrawn with a pipette and transferred to an ice-cold centrifuge tube. The cells were collected by centrifugation (5000 g, 4 °C, 10 min), washed with ice-cold distilled water, followed by 1 M sorbitol, and finally resuspended in 1 M sorbitol. PBS-Zeo-AT plasmid DNA was linearized by restriction digestion with *Not*I and *Kpn*I, and the digested DNA was extracted from an agarose gel following electrophoresis. The purified fragments were subsequently transformed to *Schizochytrium* sp. via electroporation (0.75 KV,200 Ω,50 μF). After electroporation, 1 mL seed medium was added and the cells incubated (170 rpm, 30 °C, 1 h) to promote regeneration. The putative knockout strains were selected on solid media containing 20 μg/mL zeocin at 30 °C, and confirmed by genomic DNA extraction and PCR verification. The finally confirmed recombinant strain lacking the AT domain was designated AT-DS. The plasmid PBZ-Shew-AT was used to transform *Schizochytrium* on the basis of PBS-Zeo-AT using the same method. The finally confirmed recombinant strain harboring the Shew-AT domain was designated ShAT-RS.

### 2.4. Analytical methods

Aliquots comprising 10 mL fermentation broth were used to determine cell dry weight gravimetrically. The cells were transferred to a centrifuge tube using a pipette and centrifuged for 5 min at 4500 g. Afterwards, the complete cell mass was transferred to a filter paper which was dried at 60 °C to constant weight and weighted. The method of fatty acid methyl ester (FAME) preparation was the same as used in our previous study [25]. The concentration of glucose in the fermentation broth was measured using a bioanalyzer equipped with a glucose oxidase electrode (SBA−40C, Institute of Biology, Shandong Academy of Sciences, China).

### 2.5. Real-time quantitative PCR

After three generations of cultivation, samples of each strain were obtained at the lag (24 h) phases at the third generation and RNA was isolated using the Rapid fungal RNA extraction kit (Zoonbio Biotechnology, Nanjing, China). cDNA was obtained using the TUREscript cDNA Synthesis Kit (Zoonbio Biotechnology, Nanjing, China) and used for quantitative PCR analysis. Four target genes were tested in the present study: FAS (GenBank accession number DJ335030.1), ORFA (GenBank accession number AF378327.2), ORFB, (GenBank accession number AF378328.2), and ORFC (GenBank accession number