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Title

Cloning and characterization of Δ6/Δ5 fatty acyl desaturase (Fad) gene promoter in the marine teleost *Siganus canaliculatus*

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Running Title: Analysis of rabbitfish Δ6/Δ5 *Fad* promoter

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Abbreviations

ALA, α-linolenic acid (18:3n-3)

AP1, activated protein 1

ARA, arachidonic acid (20:4n-6)

C/EBP, CCAAT enhancer binding protein

DHA, docosahexaenoic acid (22:6n-3)

DR1, direct repeat 1 element

EFA, essential fatty acid

EPA, eicosapentaenoic acid (20:5n-3)

Fad, fatty acyl desaturase

FAS, fatty acid synthase

GATA-2, GATA binding protein 2

HEK 293T cell, human embryonic kidney 293T cell

HNF4α, hepatocyte nuclear factor 4α

LC-MS, liquid chromatography coupled with tandem mass spectrometry

LC-PUFA, long-chain polyunsaturated fatty acids

LNA, linoleic acid (18:2n-6)

NF-1, nuclear factor 1

NF-Y, nuclear factor Y

PPARγ, peroxisome proliferator activated receptor γ

PUFA, polyunsaturated fatty acids

Sp1, stimulatory protein 1

SRE, sterol regulatory element

TF, transcription factor

TSS, transcription start site

UTR, untranslated region

Abstract

The rabbitfish *Siganus canaliculatus* was the first marine teleost demonstrated to have the ability of biosynthesizing long-chain polyunsaturated fatty acids (LC-PUFA) from C_{18} PUFA precursors, and all genes encoding the key enzymes for LC-PUFA biosynthesis have been cloned and functionally characterized, which provides us a potential model to study the regulatory mechanisms of LC-PUFA biosynthesis in teleosts. As the primary step to clarify such mechanisms, present research focused on promoter analysis of gene encoding Δ6/Δ5 fatty acyl desaturase (Fad), a rate-limiting enzyme catalyzing the first step in the conversion of C_{18} PUFA to LC-PUFA. First, 2044 bp promoter sequence was cloned by genome walking, and the sequence from -456 bp to + 51bp was determined as core promoter by progressive deletion mutation. Moreover, binding sites of transcription factors (TF) such as CCAAT enhancer binding protein (C/EBP), nuclear factor 1 (NF-1), stimulatory protein 1 (Sp1), nuclear factor Y (NF-Y), activated protein 1 (AP1), sterol regulatory element (SRE), hepatocyte nuclear factor 4α (HNF4α) and peroxisome proliferator activated receptor γ (PPARγ) were identified in the core promoter by site-directed mutation and functional assays. Moreover, NF-1 and HNF4α were confirmed to interact with the core promoter region by gel shift assay and mass spectrometry. This is the first report of the promoter structure of a Δ6/Δ5 Fad in a marine teleost, and a novel discovery of NF-1 and HNF4α binding to the Δ6/Δ5 *Fad* promoter.

Key words

Fatty acyl desaturase; LC-PUFA biosynthesis; transcriptional regulation mechanism; rabbitfish *Siganus canaliculatus*; marine teleost; NF-1; HNF4α

1. Introduction

It is known that long-chain polyunsaturated fatty acids (LC-PUFA) such as eicosapentaenoic (EPA; 20:5n-3), docosahexaenoic (DHA; 22:6n-3) and arachidonic (ARA; 20:4n-6) acids are essential fatty acids (EFA) for normal growth and development of vertebrates including fish. Freshwater fish generally have the ability to convert linoleic (LNA; 18:2n-6) and α-linolenic (ALA; 18:3n-3) acids into LC-PUFA, and thus these two C_{18} precursors can satisfy the EFA requirements of these species. On the contrary, most marine teleosts require LC-PUFA as EFA due to the low or absent capability of LC-PUFA biosynthesis (Yone, 1978; Watanabe, 1982 ; Kanazawa, 1985). Accordingly, fish oil (FO) rich in LC-PUFA must be added to compound feed for the aquaculture of marine fish. The limited resource and high price of FO seriously restricted the development of marine aquaculture. Consequently, many studies have been conducted to develop feasible and sustainable alternatives of FO, and vegetable oils (VO) which is rich in C_{18} precursors but devoid of LC-PUFA, have been considered as the prime candidates (Turchini and Francis, 2009). However, replacement of dietary FO by VO results in reduced flesh n-3 LC-PUFA contents of farmed fish, which could significantly compromise their nutritional quality for humans, and possibly have other negative effects on fish growth and health (Turchini and Francis, 2009; Geay et al., 2015). Over the last decade, therefore, considerable efforts have been made to understand the molecular basis of endogenous LC-PUFA biosynthesis, so as to facilitate the efficient and effective utilization of sustainable plant lipid sources while maintaining the nutritional quality of farmed fish, especially marine teleosts.

The biosynthesis of LC-PUFA from C_{18} precursors requires a series of desaturations and

elongations catalyzed by fatty acyl desaturases (Fad) and elongases of very long-chain fatty acids (Elovl), respectively (Sprecher, 1981). At present, most studies have focused on the cloning and characterization of genes encoding key enzymes in LC-PUFA biosynthesis, including Fad and Elovl. Irrespective of specificity, Fad in teleosts all belong to the FADS2 gene cluster, which includes Δ6 Fad, Δ6/Δ5 Fad, Δ5 Fad and Δ4 Fad. So far, Δ6 Fad has been found in more than fifteen fish species (Castro et al., 2016), while Δ6/Δ5 Fad has been reported in one marine teleost, rabbitfish (*Siganus canaliculatus*) (Li et al., 2008), and four freshwater fish including zebrafish (*Danio rerio*) (Hastings et al., 2001), Mexican whitefish (*Chirostoma estor*) (Jorge Fonseca-Madrigal, 2014), Nile tilapia (*Oreochromis niloticus*) (Tanomman et al., 2013) and striped snakehead (*Channa striata*) (Kuah et al., 2016). Δ5 Fad is reported in Atlantic salmon (*Salmo salar*) (Hastings et al., 2004) and rainbow trout (*Oncorhynchus mykiss*) (Abdul Hamid et al., 2016). Δ4 Fad is identified in vertebrates first by our group in rabbitfish (Li et al., 2010), followed by Senegalese sole (*Solea senegalensis*) (Morais et al., 2012), Mexican silverside (*Chirostoma estor*) (Jorge Fonseca-Madrigal, 2014), striped snakehead (*Channa striata*) (Kuah et al., 2015), and primates, the baboon (Park et al., 2015).

The accumulating studies demonstrated that the activity of Fad was regulated mainly at the transcription level (Nakamura and Nara, 2004). For examples, the expression of Atlantic salmon Δ6 *Fad* was up-regulated by replacement of dietary FO with VO (Zheng et al., 2004). Moreover, the transcription activity of Atlantic salmon Δ6 *Fad* promoter was much higher than that of marine fish Atlantic cod (Zheng et al., 2009). In contrast, the mRNA expression of Atlantic cod Δ6 *Fad* did not respond to dietary lipid and its activity was also weak (Tocher

et al., 2006). The expression of European sea bass Δ6 *Fad* was increased by VO treatment, however, its activity and protein content were not changed, suggesting a potential regulatory mechanism of Δ6 Fad activity at post-transcriptional level (Geay et al., 2010). However, the detailed regulatory mechanisms of LC-PUFA biosynthesis in teleosts are largely unknown at present, which are important for increasing the efficient and effective utilization of sustainable VO in feed while maintaining the nutritional quality of farmed fish, especially marine teleosts.

Rabbitfish *S. canaliculatus* is an economically important marine teleost, which is widespread along the Indo-West Pacific coast and farmed in southeastern Asia including China. What is worthy specially mentioned is that *S. canaliculatus* was the first marine teleost demonstrated to have the ability of biosynthesizing LC-PUFA from C_{18} PUFA precursors (Li et al., 2008; Xie, 2015). Moreover, all genes encoding the key enzymes for LC-PUFA biosynthesis have been cloned and functionally characterized including a Δ4 Fad (the first report in vertebrates) and a Δ6/Δ5 Fad (the first report in marine teleosts) (Li et al., 2010), and two elongases (Elovl4 and Elovl5) (Monroig et al., 2012). These provide us a good model for investigating the regulatory mechanisms of LC-PUFA biosynthesis in teleosts. Recently, studies at transcriptional levels have demonstrated the roles of HNF4α and Lxr-Srebp in *Fad* expression (Dong et al., 2016; Zhang et al., 2016a). At post-transcriptional level, miR17 targeted at the 3' untranslated region (3' UTR) of Δ4 *Fad* and downregulated its gene expression, and miR-33 was involved in the regulation of LC-PUFA biosynthesis probably through targeting *insig1* in rabbitfish (Zhang et al., 2016b). Moreover, the structure of rabbitfish Δ4 *Fad* promoter was characterized and HNF4α was identified as a TF of *Fad*

genes, for the first time in vertebrates (Dong et al., 2016) .

Δ6/Δ5 Fad is a rate-limiting enzyme catalyzing the first step in the conversion of C_{18} PUFA to LC-PUFA, and thus understanding the regulatory mechanisms of this gene is important for comprehensively exploring the regulation mechanisms of LC-PUFA biosynthesis in teleost. As the first step to the goal, the present study focused on the promoter structure analysis of rabbitfish Δ6/Δ5 *Fad* gene, including the cloning of promoter sequence, determination of core promoter region by progressive deletion mutation, identification of possible binding sites for TFs by a combination of bioinformatics analysis and site-directed mutagenesis. Moreover, TFs interacting with core promoter were confirmed by gel shift assay and liquid chromatography coupled with tandem mass spectrometry (LC-MS). The results will increase our understanding on the regulatory mechanisms of LC-PUFA biosynthesis in vertebrates, which at last contribute to the optimization and/or enhancement of the LC-PUFA pathway in teleosts.

2. Materials and methods

2.1 Ethics statement

Nan Ao Marine Biology Station (NAMBS) of Shantou University provided wild rabbitfish for the present study. In order to minimize the suffering of rabbitfish, 0.01% 2-phenoxyethanol (Sigma-Aldrich, St. Louis, MO, USA) was used for anesthesia. Based on the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978), all the experiments with fish in the present research were assessed as correct and reasonable by the Institutional Animal Care and Use Committee

of Shantou University (Guangdong, China).

2.2 Cloning of Δ6/Δ5 Fad gene promoter

Genomic DNA was extracted from muscle tissue of rabbitfish *S. canaliculatus* (Joseph Sambrook, 2001) and used for thermal asymmetric interlaced PCR (TAIL PCR) to clone the 、Δ6/Δ5 Fad gene promoter with forward primer AP4 in the Genome Walking Kit (TaKaRa, Dalian, China) and three reverse primers SP1, SP2, SP3 (Table 1), which was designed from the cloned Δ6/Δ5 Fad mRNA of *S. canaliculatus* (GenBank: EF424276.2). The PCR method was the same as we used before (Dong et al., 2016). Fragments of upstream sequence were purified and inserted into pMD18-T vector (TaKaRa, Dalian, China), and sequenced (Shanghai Sangon Biotech Co., Ltd, China).

2.3 Construction of progressive deletion mutants from Δ6/Δ5 Fad gene promoter

With the sequence data from promoter cloning, another three sense primers (DF3, DF2, DF1) with an *Eco*RV site and an antisense primer DR with a *Hin*dIII site (Table 1) were designed to obtain the accurate sequence of the full-length promoter fragment (D3: 2044 bp) and two progressive deletion fragments (D2: 1085 bp, D1: 578 bp). The PCR was performed with genomic DNA as template by high-fidelity 2× *Pfu* PCR Master Mix (Tiangen Biotech, Beijing, China). The PCR product and pGL4.10 [luc2] vector (Promega Corporation, Madison, WI, USA) were both digested by restriction endonuclease *Eco*RV and *Hin*dIII (New England Biolabs, Ipswich, MA, UK), then ligated by T4 DNA ligase (New England Biolabs, Ipswich, MA, UK). The upstream sequence in the insert fragments D3, D2 and D1 was of

-1415 bp, -456 bp, and +51 bp length to the putative transcription start site (TSS) +1, respectively (Fig. 1). All the recombinant plasmids were isolated using the High Pure Plasmid Isolation Kit (Roche, Mannheim, Germany).

2.4 Bioinformatic analysis of Δ6/Δ5 *Fad* promoter and site-directed mutant construction

The 5' flanking sequence of Δ6/Δ5 *Fad* was analyzed by TRANSFAC® and TF binding®. The rabbitfish Δ6/Δ5 *Fad* promoter was compared with the Δ4 *Fad* promoter sequence of *S. canaliculatus* (Dong et al., 2016), Δ6 *Fad* promoter sequences of *Dicentrarchus labrax* (Geay et al., 2012), *Gadus morhua* (Zheng et al., 2009), *Salmo salar* (Zheng et al., 2009), and *Homo sapiens* (Tang et al., 2003), and Δ6/Δ5 desaturase promoter sequence from *D. rerio* genome (*D. rerio* strain Tuebingen chromosome 25 genomic scaffold, Zv9 scaffold3372), the conservative elements of rabbitfish Δ6/Δ5 *Fad* was identified by alignment with the above *Fad* sequences (BioEdit v7.0.9, Tom Hall, Department of Microbiology, North Carolina State University, USA).

Site-directed mutation of recombinant plasmids was carried out to detect the potential functions of predicted TF elements on core promoter activity. For the Δ6/Δ5 *Fad* promoter, site-directed mutants were designed and produced with Muta-direct™ site-directed mutagenesis kit (SBS Genetech, Shanghai, China) from the deletion mutant D2 construct (pGL4.10-D2) which was treated as wild-type. The mutation site of elements was designed in the middle of the primer, and the corresponding TFs included CCAAT enhancer binding protein (C/EBP), nuclear factor 1 (NF-1), stimulatory protein 1 (Sp1), nuclear factor Y (NF-Y), sterol regulatory element (SRE), activated protein 1 (AP1) and peroxisome

proliferator activated receptor γ (PPARγ) (Table 2). The detail procedure of PCR was the same as we did previously (Dong et al., 2016). The mutants were isolated with High Pure Plasmid Isolation Kit (Roche, Mannheim, Germany).

2.5 Cell culture and Dual luciferase reporter assay

Human embryonic kidney (HEK 293T) cells (Chinese Type Culture Collection, Shanghai, China) were seeded in 96-well cell culture plates with 100 μl high glucose Dulbecco's Modified Eagle Medium (Gluta-MAX DMEM) (Gibco, Life Technologies, USA) supplemented with 10 % fetal bovine serum (FBS, Sijiqing Biological Engineering Material Company, Hangzhou, China) per well for plasmid transfection in dual luciferase reporter assay. After growing for 24 h to 80 % confluence, 100 ng of reporter firefly luciferase construct (progressive deletion or site directed mutants) and 0.01 ng of internal control vector pGL4.75 (Promega Corporation, Madison, WI, USA) was co-transfected into the cells with 0.25 μl Lipofectamine® 2000 Reagent (Invitrogen, Carlsbad, CA, USA) per well, and empty vector pGL4.10 (D0 group) was set as a negative control. Cells were transfected with plasmid complex in triplicate in three independent experiments. Twenty-four h after transfection, media was replaced with 75 μl fresh medium and luciferase assays were performed 24 h later (48 h after transfection) with the Dual-Glo™ luciferase assay system (Promega, Madison, WI, USA). Chemical luminescence intensity was detected in duplicate readings using a microplate reader (Infinite M200 Pro, Tecan, Switzerland), and promoter activity was calculated from the luminescence ratio of firefly:renilla luciferase for each construct.

2.6 Gel shift assay and LC-MS

To determine interaction between TFs and the core promoter of the Δ6/Δ5 *Fad* gene, Beyotime Nuclear Extract Kit (Beyotime, China) and Non-Interference Protein Assay Kit (Sangon, China) were used to extract and quantify nucleus and cytoplasmic proteins from rabbitfish liver, then a 5' end biotin-labeled probe of 270 bp covering the TF elements (C/EBP, NF-1, Sp1, NF-Y, SRE, AP1 and PPARγ) was designed and incubated with rabbitfish liver proteins. The experimental probe was produced in a 50 μl PCR reaction system including 18 μl dd H_2O, 25 μl 2× *Pfu* PCR Master Mix (TIANGEN, China), 3 μl 10 mM 5' end biotin-labeled forward primer, 3 μl 10 mM 5' end biotin-labeled reverse primer and 1 μl pGL 4.10-D2 recombinant plasmid (100 ng/μl), with the competitor probe produced in the same system using unlabeled primers. PCR conditions were one cycle (95 °C for 3 min), 35 cycles (94 °C for 30 sec, 51 °C for 30 sec, 72 °C for 40 sec), one cycle (72 °C for 5 min) and one cycle (10 °C for 5 min). The detail experimental condition of EMSA was the same as we did previously (Dong et al., 2016). The reaction was carried out in lane A (no proteins, 5' biotin labeled probe), lane B (liver cytoplasmic proteins, 5' biotin labeled free probe), lane C (liver cytoplasmic proteins, unlabeled competitor probe, 5' biotin labeled free probe), lane D (liver nucleus proteins, 5' biotin labeled probe), lane E (liver nucleus proteins, unlabeled competitor probe, 5' biotin labeled probe) (Fig. 5).

To confirm the TFs in rabbitfish nucleus, the proteins for LC-MS detection were isolated by PureProteome™ Streptavidin Magnetic Beads (Millipore, Bedford, MA, USA) and Amicon® Ultra-0.5 Centrifugal Filter Devices (Millipore, Bedford, MA, USA). The procedure detail for LC-MS was the same as our previous study (Dong et al., 2016).

2.7 Statistical analysis

Data in present study was presented as means ± SEM (n = 3). One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test (software Origin 7.0) were used to analyze promoter activity, with a significance of $P < 0.05$ applied to all statistical tests.

3. Results

3.1 The structure of rabbitfish Δ6/Δ5 *Fad* gene promoter

A 2044 bp length of 5' flanking sequence from the initiation codon ATG of the Δ6/Δ5 *Fad* gene was cloned and treated as the promoter candidate (Fig. 1). The first base of the first non-coding exon was defined as +1 in the sequence and assumed as the putative transcription start site (TSS). One 500 bp intron was found between two noncoding-exons, which were 100 bp and 29 bp, respectively. Based on progressive deletion analysis of 5' flanking sequence of the Δ6/Δ5 *Fad* gene promoter, it was shown that, compared with the activity of original promoter candidate (D3), deletion of fragment from -1415 bp to -457 bp (construct D2) caused an increase of promoter activity, while deletion of fragment from -456 bp to +51 bp (construct D1) caused a significant decrease of promoter activity, suggesting that the core promoter region was located at -456 bp to +51 bp.

3.2 Possible TF binding sites in the core promoter of rabbitfish Δ6/Δ5 *Fad* gene

Bioinformatics analysis using TRANSFAC® and TF binding® software indicated that there were five predicted TF binding sites including C/EBP, NF-1, Sp1, AP1 and PPARγ in the

core promoter region of rabbitfish Δ6/Δ5 *Fad* (Table 2). In addition, two highly conserved elements including NF-Y and SRE were also identified in the core promoter region of rabbitfish Δ6/Δ5 *Fad* according to the promoter alignment among Δ6/Δ5 *Fad* and Δ4 *Fad* of *S. canaliculatus*, Δ6 *Fad* of *D. labrax*, *G. morhua*, *S. salar* and *H. sapiens*, and Δ6/Δ5 *Fad* of *D. rerio* (*D. rerio* strain Tuebingen chromosome 25 genomic scaffold, Zv9 scaffold3372) (Fig. 2). The relative location of seven potential TF binding sites in the rabbitfish Δ6/Δ5 *Fad* promoter is shown in Fig. 3.

3.3 Confirmation of TF binding sites in the core promoter by site-directed mutagenesis

According to the information on the above predicated TF binding sites in the core promoter, a series of site-directed mutants was constructed and transfected into HEK 293T cells for determining effects on transcriptional activity. Compared with wild type D2, mutation of TF binding sites for C/EBP, NF-1, Sp1, AP1, PPARγ, NF-Y and SRE resulted in significantly decreased transcriptional activity ($P < 0.05$) (Fig. 4), which suggested that these TF binding sites were important for maintaining promoter activity.

3.4 Demonstration of TFs in liver interacting with the TFs binding sites in the core promoter by Gel shift assay and LC-MS analysis

To further confirm whether the TFs identified *in vitro* were present in rabbitfish liver, Gel shift assay was performed with liver cytoplasmic and nucleus proteins. The electrophoretic results showed that a gel shift band was observed only in lane D with liver nucleus proteins

and 5' biotin labeled probe, which indicated interaction between nucleus proteins and the core promoter of rabbitfish Δ6/Δ5 *Fad* gene (Fig. 5). No gel shift band was seen in lane A without protein, lane B or C with liver cytoplasmic proteins, or lane E with liver nucleus proteins, unlabeled competitor probe and 5' biotin labeled probe. The results suggested there were nucleus proteins in rabbitfish liver that could bind to the core promoter of rabbitfish Δ6/Δ5 *Fad*.

For further identification of the DNA-protein complex isolated from lane D, the digested protein sample was analyzed by LC-MS and the IPI Zebrafish database (40470 seqs) (ftp://ftp.ebi.ac.uk/pub/databases/IPI). The binding proteins were identified and classified by GO (Gene Ontology) and COG (Cluster of Orthologous Group of proteins), respectively (Fig. 6). There were 259 identified spectra in a total of 14730 spectra, and 85 identified proteins from a total of 147 peptides. Among them, HNF4α and NF-1 protein fragments were identified (Table 3), while the predicted TFs including C/EBP, Sp1, AP1, PPARγ, NF-Y and SREBP-1c were not found.

4. Discussion

The present study identified the structure of the rabbitfish Δ6/Δ5 *Fad* promoter. Previously, several vertebrate *Fad* gene promoters had been reported including that of Δ4 *Fad* from *S. canaliculatus* recently characterized by our group (Dong et al., 2016), and those of Δ6 *Fad* genes from *D. labrax* (Geay et al., 2012), *G. morhua* (Zheng et al., 2009), *S. salar* (Zheng et al., 2009), *H. sapiens* (Tang et al., 2003), and the Δ6/Δ5 *Fad* from *D. rerio*. These provided a

detailed background for promoter analysis including core region, TF binding sites and the discovery of a possible role for HNF4α and NF-1 in rabbitfish Δ6/Δ5 *Fad* transcription.

As to the core region, differences existed within and between species. The rabbitfish Δ6/Δ5 *Fad* core promoter was located from -456 bp to +51 bp, while in Δ4 *Fad* it was from -262 bp to +203 bp, and Δ6 *Fad* of *D. labrax, G. morhua, S. salar* and *H. sapiens* was located from -194 bp, -167 bp, -546 bp, -385 bp to TSS, respectively. Alignment of the mentioned *Fad* core promoter sequences above demonstrated that NF-Y and SRE binding sites were highly conserved among these genes. Other than NF-Y and SRE, binding sites of other TFs in the *Fad* core promoter varied with species. A C/EBPα element was located in the *G. morhua* Δ6 *Fad* promoter at -99 bp to TSS, and an Sp1 element was situated in the *S. salar* Δ6 *Fad* promoter at -314 bp to TSS (Zheng et al., 2009). While in human Δ6 *Fad* promoter there were five Sp1 elements, moreover, a DR-1 element (PPARα binding) from -385 to -373 bp was reported (Tang et al., 2003). The present study showed that potential binding sites for C/EBP, NF-1, Sp1, NF-Y, AP1, PPARγ and SRE existed in rabbitfish Δ6/Δ5 *Fad* promoter. Thus, the promoter of rabbitfish Δ6/Δ5 *Fad* had the same binding site for C/EBP in common with the *G. morhua* Δ6 *Fad* promoter, a similar Sp1 element to those of *S. salar* and *H. sapiens* Δ6 *Fad* promoters, and similar PPRE (peroxisome proliferator response element) with *H. sapiens* Δ6 *Fad* promoter (PPARα element in *H. sapiens* Δ6 *Fad* promoter and PPARγ element in rabbitfish Δ6/Δ5 *Fad* promoter). Moreover, rabbitfish Δ6/Δ5 *Fad* and Δ4 *Fad* promoter shared the same elements for C/EBP, NF-1, NF-Y and SRE, while there were special binding sites for Sp1, AP1 and PPARγ in Δ6/Δ5 *Fad*, GATA binding protein 2 (GATA-2) and HNF4α in Δ4 *Fad*. Whether these reported transcription factors are involved in the regulation of rabbitfish

Δ6/Δ5 *Fad* transcription requires to be further clarified.

HNF4α is a positive regulator in LC-PUFA *de novo* biosynthesis, which could activate various genes involved in lipid and cholesterol metabolism such as ApoCIII, Cyp7α hydroxylase, fatty acid synthase (FAS) and stearoyl-CoA desaturase (SCD) (Hayhurst et al., 2001; Lu et al., 2001; Odom et al., 2004; Adamson et al., 2006). The binding of unsaturated fatty acids like ALA, EPA and DHA to HNF4α would repress its influence on activating transcription (Rachel Hertz, 1998). A previous study on human Δ6 *Fad* promoter found that HNF4α did not affect Δ6 *Fad* promoter activity when co-transfecting CV1 cells expression vector of human Δ6 *Fad* promoter and HNF4α (Tang et al., 2003). Recently, our group first discovered that HNF4α was a transcription factor of vertebrate Fad gene (Δ4 Fad) as identified in *Siganus canaliculatus* (Dong et al., 2016). Interestingly, present research also identified HNF4α fragment in the core promoter region of rabbitfish Δ6/Δ5 *Fad*, suggesting that it was a potential regulatory TF in LC-PUFA biosynthesis, and a further study was required for clarification.

HNF4α and PPARγ might be involved in the transcriptional regulation of rabbitfish Δ6/Δ5 *Fad* by competition for DR-1 element. It was reported that HNF4α and PPARα shared a similar consensus binding site, a direct repeat 1 (DR1) element (AGGTCA n-AGGTCA or variants thereof) in the promoter or intron of genes such as acyl-CoA oxidase and acyl-CoA thioesterase I, and that they could compete for the DR-1 site when involved in the regulation of target genes (Nishiyama et al., 1998; Rachel Hertz, 1998; Mandard et al., 2004; Dongol et al., 2007). However, the predicted DR-1 element in the human Δ6 *Fad* promoter was only regarded as a PPRE element since the Δ6 *Fad* promoter activity did not response to

pcDNA3.1-HNF4α over-expression (Tang et al., 2003). The present study predicted a PPARγ site in rabbitfish Δ6/Δ5 *Fad* promoter, which was quite similar to DR-1 element, and further identified such site was important for Δ6/Δ5 *Fad* promoter activity. However, based on the proteomic data of LC-MS analysis, PPARγ was not identified, while HNF4α was not predicted, but a protein fragment of such TF was identified in rabbitfish Δ6/Δ5 *Fad* promoter. This might be due to competition for the DR-1 element between PPARγ and HNF4α in the transcription of rabbitfish Δ6/Δ5 *Fad*. Although some of the other predicted TFs including C/EBP, Sp1, NF-Y, AP1, and SREBP-1c could not be identified by LC-MS, NF-1 was another protein fragment identified, indicating its potential regulatory role in rabbitfish Δ6/Δ5 *Fad* transcription. Further investigations are required to clarify the regulatory function of NF-1 in the rabbitfish Δ6/Δ5 *Fad* transcription.

In summary, the present study characterized the structure of the *S. canaliculatus* Δ6/Δ5 *Fad* gene promoter, which is the first report of a Δ6/Δ5 *Fad* gene promoter in marine teleost. Moreover, HNF4α and NF-1 were confirmed as potential TFs of Δ6/Δ5 *Fad* gene. These results might provide a new background for regulatory mechanism of LC-PUFA biosynthesis in marine teleost.

Competing Interests

The authors declared that there are no competing interests.

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Author Contributions

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Contributed reagents/materials/analysis tools: Yang Liu, Qinghao Zhang and Cuihong You

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Figure Legends

Figure 1. Functional analysis of *Siganus canaliculatus* Δ6/Δ5 *Fad* gene promoter. On the left, D3 represents whole promoter region, while D2 and D1 are the progressive deletion mutants, and D0 is the negative control without promoter region. The white boxes stand for non-coding exons, and black bar between the two exons represented the intron. The first base of first 5' non-coding exon is assumed to be +1, the other sequence is numbered relative to the transcription start site (TSS). Promoter activity of each construct is represented as the value normalized activity (Firefly luciferase: Renilla luciferase) on the right. The letters a, b, c and d indicate significant differences of promoter activity relative to each other ($P < 0.05$).

Figure 2. The core promoter regions alignment of *Siganus canaliculatus* Δ6/Δ5 *Fad* with *S. canaliculatus* Δ4 *Fad* promoter (Dong et al., 2016), Δ6/Δ5 *Fad* promoter from *Danio rerio* genome data and Δ6 *Fad* promoter from *Dicentrarchus labrax* (Geay et al., 2012), *Gadus morhua*, *Salmon salar* (Zheng et al., 2009) and *Homo sapiens* (Tang et al., 2003). NF-Y and SRE are identified with shading. The predicted binding sites of PPARγ in Δ6/Δ5 *Fad* promoter and HNF4α in Δ4 *Fad* promoter are also labelled with gray.

Figure 3. The distribution of possible transcription factors binding sites predicted by corresponding online software in *Siganus canaliculatus* Δ6/Δ5 Fad core promoter region. The underlined sections represent the probe region of gel shift assay in Fig. 5.

Figure.4. Effects of site-directed mutation of possible transcription factors binding sites on the promoter activity of *Siganus canaliculatus* Δ6/Δ5 *Fad* gene. The letters a, b, c and d indicate significant differences relative to each other ($P < 0.05$).

Figure 5. The gel shift assay of *Siganus canaliculatus* Δ6/Δ5 *Fad* core promoter with protein from liver extract. The reaction was performed in the following lane, A (no proteins, 5' biotin

labeled probe), B (liver cytoplasmic proteins, 5’ biotin labeled free probe), C (liver cytoplasmic proteins, unlabeled competitor probe, 5’ biotin labeled free probe), D (liver nucleus proteins, 5’ biotin labeled probe), E (liver nucleus proteins, unlabeled competitor probe, 5’ biotin labeled probe).

Figure 6. Classification of nucleus proteins in core promoter of *Siganus canaliculatus* Δ6/Δ5 *Fad*. COG (Cluster of Orthologous Group of proteins) method is used to identify the proteins isolated from lane D in gel shift assay. The number above each column represents the amount of identified proteins.

Table 1. Primers used in present study.

Subject	Primers	Nucleotide sequence
TAIL PCR for 5' flanking sequence cloning	SP1	5'-GTCTGCTTTCCTATGGTCACGTTG-3'
	SP2	5'-GTGCTCCGTCCACATTCGAGTT-3'
	SP3	5'-TCATCCTCCTCAAATAGG -3'
Pfu PCR for deletion mutant construction	DF3	5'-CCCGATATCTTGCTGACGTAAAGTGTGGA-3'
	DF2	5'-CCCGATATCGGCCATTTGATTAACTCTGCT-3'
	DF1	5'-CCCGATATCGGAGCACGGTCAACGTGAC-3'
	DR	5'-CCCAAGCTTCATCTTCACTGCTGTCTCTGCTT-3'
EMSA for gel shift	BF (5'biotinlabeled)	5'-GTATTTCATCAGACTGTTTCCGT-3'
	BR (5'biotinlabeled)	5'-CAAACGTTGTCTGCTTTCCTAT-3'
	UF (5' unlabeled)	5'-GTATTTCATCAGACTGTTTCCGT -3'
	UR (5' unlabeled)	5'-CAAACGTTGTCTGCTTTCCTAT-3'

Restriction sites underlined are *Eco*RV (5'-GATATC-3') and *Hin*dIII (5'-AAGCTT-3') in expression vector pGL4.10

Table 2. The data of TF binding sites in rabbitfish Δ6/Δ5 *Fad* core promoter.

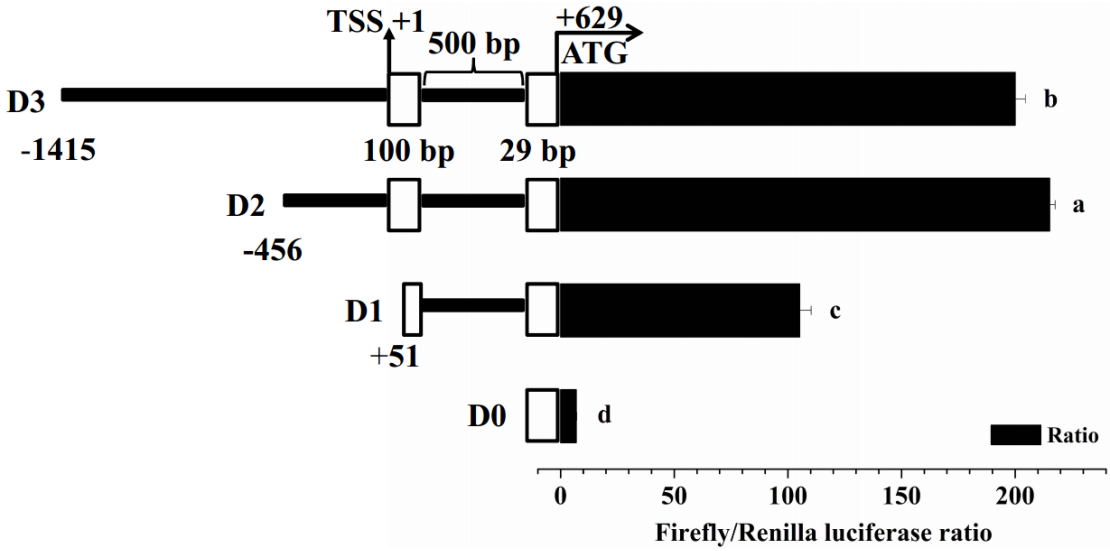
TF	Software	Position	Predicted element	Mutation site
C/EBP	TF binding®	-164	GTATTTCATCAGAC	GTATTTCATCAGAC→×
NF-1	TF binding®	-138	TCTGGGCGCGCAGGCGAC	TGGGC→×
Sp1	TF binding®	-144	TGGGCGCGC	GG→TT
NF-Y	Sequence alignment	-59	GCGCCCATTGG	GCGCCCATTGG→×
SRE	Sequence alignment	-26	CTCGAATGATCGGCTCGGAATTT	CTCGAATGATCGGCTCGGAATTT→×
AP1	TF binding®	-13	ACTGAATCAGT	ACTGAATCAGT→×
PPARγ	TRANSFAC®	+53	AGCACGGTCAACGTGACCATAGG	AGCACGGTCAACGTGACCATAGG→×

The position of each element is numbered relative to supposed transcription start site (TSS). The bases underlined are conservative part in mutation, × means deletion. TF binding®, TRANSFAC® are two software tools online used for the prediction.

Table 3. Proteomics information of TFs identified by LC-MS

Transcription factor	NF-1	HNF4α
Protein fragments	LDLVMVILFK	QQLLVLVEWAK

Figure. 1.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+629
ATG
D3
-1415
100 bp
29 bp
b
D2
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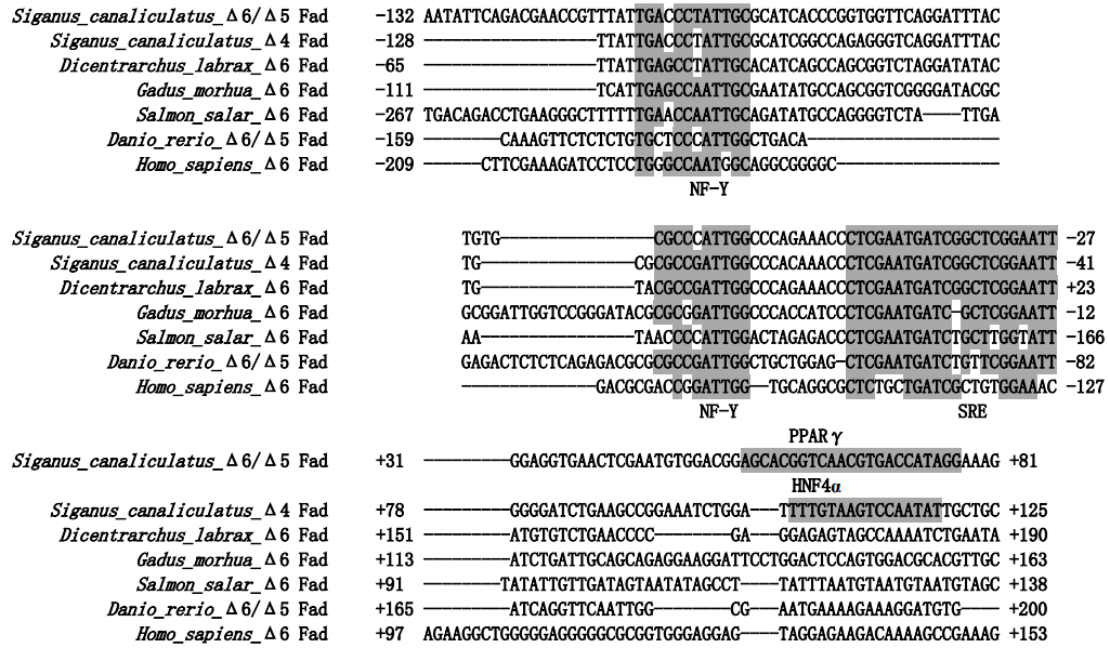
a
D1
+51
c
D0
d
Ratio
0
50
100
150
200
Firefly/Renilla luciferase ratio

Figure. 2.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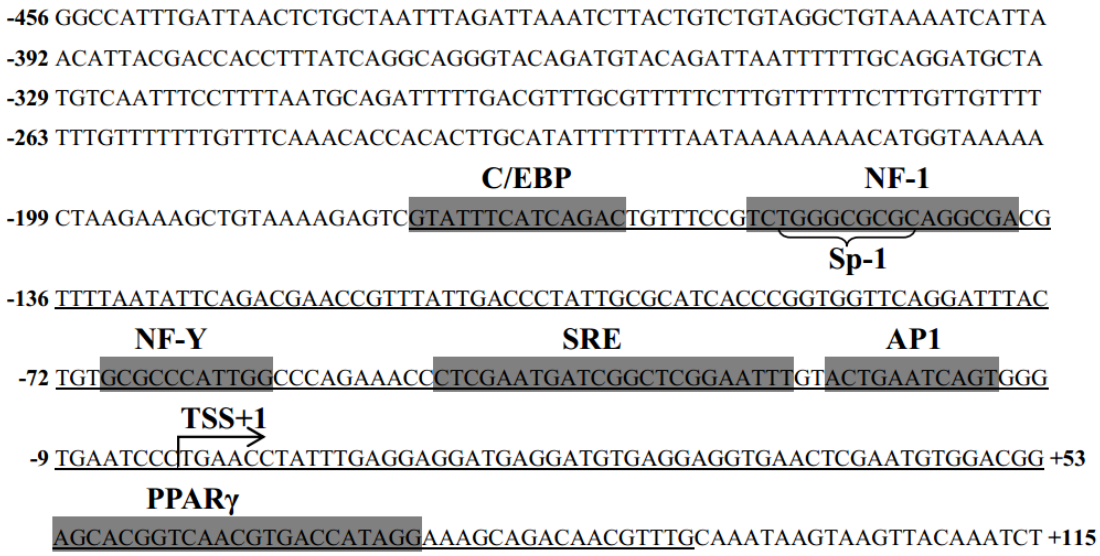
Dicentrarchus_labrax_Δ6 Fad -65 TTATTGAGCCTATTGCACATCAGCCAGCGGTCTAGGATATAC
Gadus_morhua_Δ6 Fad -111 TCATTGAGCCAATTGCGAATATGCCAGCGGTCGGGGATACGC
Salmon_salar_Δ6 Fad -267 TGACAGACCTGAAGGGCTTTTTTGAACCAATTGCAGATATGCCAGGGGTCTA TTGA
Danio_rerio_Δ6/Δ5 Fad -159 CAAAGTTCTCTCTGTGCTCCCATTGGCTGACA
Homo_sapiens_Δ6 Fad -209 CTTCGAAAGATCCTCCTGGGCCAATGGCAGGCGGGGC
NF-Y
Siganus_canaliculatus_Δ6/Δ5 Fad TGTG CGCCCATTGGCCCAGAAACCCTCGAATGATCGGCTCGGAATT -27
Siganus_canaliculatus_Δ4 Fad TG CGCGCCGATTGGCCCACAAACCCTCGAATGATCGGCTCGGAATT -41
Dicentrarchus_labrax_Δ6 Fad TG TACGCCGATTGGCCCAGAAACCCTCGAATGATCGGCTCGGAATT +23
Gadus_morhua_Δ6 Fad GCGGATTGGTCCGGGATACGCGCGGATTGGCCCACCATCCCTCGAATGATC GCTCGGAATT -12
Salmon_salar_Δ6 Fad AA TAACCCCATTGGACTAGAGACCCTCGAATGATCTGCTTGGTATT -166
Danio_rerio_Δ6/Δ5 Fad GAGACTCTCTCAGAGACGCGCGCCGATTGGCTGCTGGAG CTCGAATGATCTGTTCGGAATT -82
Homo_sapiens_Δ6 Fad GACGCGACCGGATTGG TGCAGGCGCTCTGCTGATCGCTGTGGAAAC -127
NF-Y
SRE
PPARγ
Siganus_canaliculatus_Δ6/Δ5 Fad +31 GGAGGTGAACTCGAATGTGGACGGAGCACGGTCAACGTGACCATAGGAAAG +81
HNF4α
Siganus_canaliculatus_Δ4 Fad +78 GGGGATCTGAAGCCGGAAATCTGGA TTTTGTAAGTCCAATATTGCTGC +125
Dicentrarchus_labrax_Δ6 Fad +151 ATGTGTCTGAACCCC GA GGAGAGTAGCCAAAATCTGAATA +190
Gadus_morhua_Δ6 Fad +113 ATCTGATTGCAGCAGAGGAAGGATTCCTGGACTCCAGTGGACGCACGTTGC +163
Salmon_salar_Δ6 Fad +91 TATATTGTTGATAGTAATATAGCCT TATTTAATGTAATGTAATGTAGC +138
Danio_rerio_Δ6/Δ5 Fad +165 ATCAGGTTCAATTGG CG AATGAAAAGAAAGGATGTG +200
Homo_sapiens_Δ6 Fad +97 AGAAGGCTGGGGGAGGGGGCGCGGTGGGAGGAG TAGGAGAAGACAAAAGCCGAAAG +153

Figure. 3.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C/EBP
NF-1
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Sp-1
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NF-Y
SRE
AP1
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TSS+1
-9 TGAATCCCTGAACCTATTTGAGGAGGATGAGGATGTGAGGAGGTGAACTCGAATGTGGACGG +53
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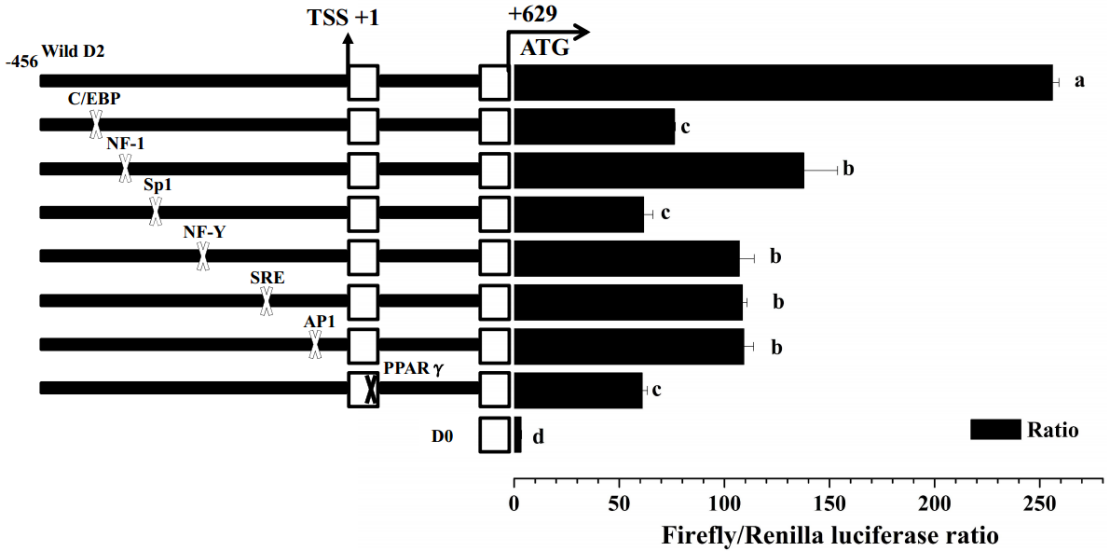
Figure. 4.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+629
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Wild D2
C/EBP
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Sp1
NF-Y
SRE
AP1
PPAR γ
D0
a
c
b
c
b
b
b
c
d
Ratio
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50
100
150
200
250
Firefly/Renilla luciferase ratio

Figure. 5.

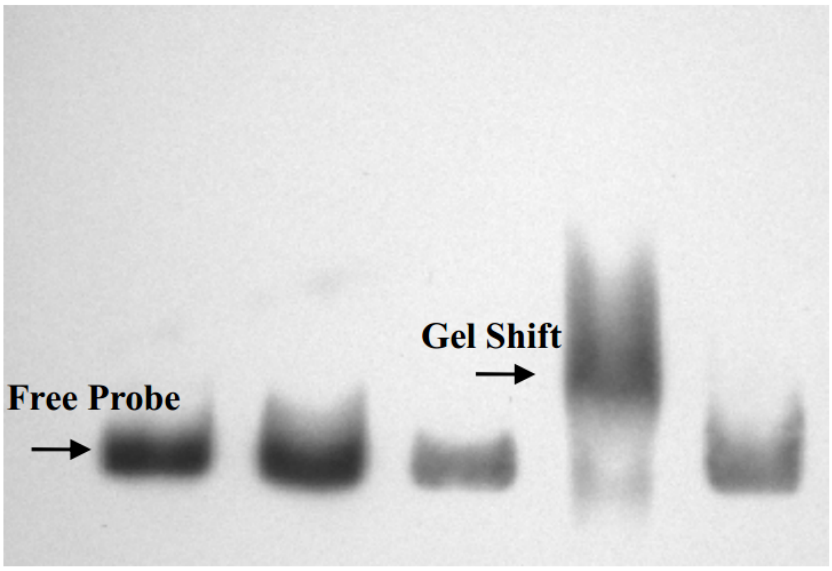
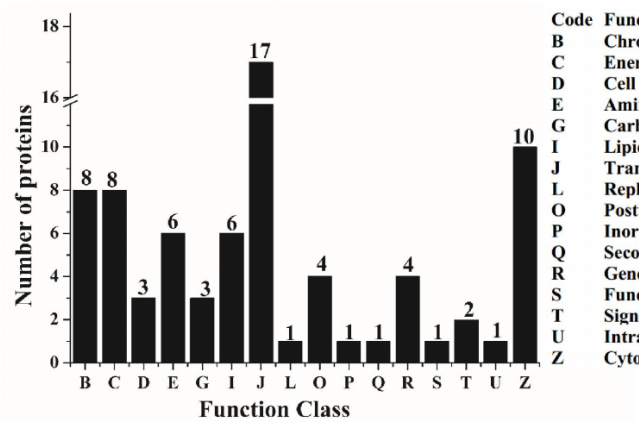


Figure. 6.



Code	Functional-Categories
B	Chromatin structure and dynamics
C	Energy production and conversion
D	Cell cycle control, cell division, chromosome partitioning
E	Amino acid transport and metabolism
G	Carbohydrate transport and metabolism
I	Lipid transport and metabolism
J	Translation, ribosomal structure and biogenesis
L	Replication, recombination and repair
O	Posttranslational modification, protein turnover, chaperones
P	Inorganic ion transport and metabolism
Q	Secondary metabolites biosynthesis, transport and catabolism
R	General function prediction only
S	Function unknown
T	Signal transduction mechanisms
U	Intracellular trafficking, secretion, and vesicular transport
Z	Cytoskeleton