African Journal of Microbiology Research Vol. 6(49), pp. 7545-7549, 25 December, 2012
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR12.1226
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

Cloning and expression analysis of *accD* gene in *Chlorella prototheco*ides FACHB-2

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Accepted 22 November, 2012

Acetyl-CoA carboxylase (ACCase) is one of the key enzymes that participate in lipid biosynthesis; a detailed understanding of its regulation mechanism is of potential value for lipid production in oil-producing species. In this study, a 1.5 kb fragment of *accD* gene from *Chlorella prototheco*ides FACHB-2 was cloned, and the expression levels of it under autotrophic and heterotrophic conditions were investigated through quantitative real-time PCR technique. The results show that the expression levels of *accD* gene under heterotrophic conditions were higher than under autotrophic conditions, higher at the stationary phases than at exponential phases, and accordingly the lipid contents increased significantly. So, it can be concluded that expression of *ACCase* gene plays an important role in regulating the lipid contents of *Chlorella prototheco*ides FACHB-2 under different cultivation conditions.

Key words: Acetyl-CoA carboxylase, *Chlorella prototheco*ides, lipid synthesis, quantitative real-time PCR, transcription.

INTRODUCTION

Many species in the genus *Chlorella* have received considerable attention for being able to accumulate neutral triacylglycerols, an ideal feedstock for biodiesel production (Feng et al., 2011; Hsieh et al., 2012; Mujtaba et al., 2012; Wang et al., 2012; Xu et al., 2006; Xiong et al., 2010; Xie et al., 2012). Compared with large amounts of work focusing on improving the lipid content from technical aspects, investigation on the regulation mechanism of lipid synthesis has somewhat fallen behind. Metabolic flux analysis in *Chlorella prototheco*ides revealed that the carbon flux targeting into lipid synthesis was via acetyl-CoA, but no further research was conducted about the molecular mechanism (Miao et al., 2006).

Acetyl-CoA carboxylase (ACCase), one of the key enzymes in lipid synthesis pathway, catalyzes the carboxylation of acetyl-CoA to malonyl-CoA, and has been proved to have vital influence on lipid content (Sasaki et al., 2004). It was reported in another algal species *Chlorella sorokiniana* that increased expression levels of accD (heteromeric acetyl-CoA carboxylase beta subunit) might account for the increased lipid content in stationary phase of mixotrophic growth (Wan et al., 2011). The species in the genus *Chlorella* are usually from different circumstances, and the same metabolic pathways in two species often response differently to the same environmental condition (Cha et al., 2011). To our knowledge, no work on the role of ACCase in *C. prototheco*ides has been reported.

For the purpose of recognizing the regulation mechanism of ACCase in *C. prototheco*ides FACHB-2, in this work the coding sequence of *accD* gene was cloned, and the relationship between expression levels of this gene and the lipid contents was also investigated.

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Table 1. Primers used in this experiment.

Primer	Sequences (5'➔3')
accdF0	TTAGARTTTMRAGATCAAAAAGC
accdR0	CCRGCAAAMCCAATTAAAGCTTT
accdF1	AAGATGCTCAAGAAAGAACCGG
accdR1	TACACCGCCTGTTGTCGGAG
SMARTer II oligo	AAGCAGTGGTAACAACGCAGAGTACXXXXX
3′-RACE Primer A	AAGCAGTGGTAACAACGCAGAGTAC$(T)_{30}$VN
5′-RACE Primer A	$(T)_{25}$VN
UPM	(aatacgactcactatagggc)AAGCAGTGGTATCAACGCAGAGT
accdF2	TTATGTCAATTCTTTCTTGGATT)
accdR2	TCAGCGTGTAAAAGTCTTAACTCG
accdF3	AAGATGCTCAAGAAAGAACCGG
accdR3	CCATGCTTCCGCCCATAAAG
18s rDNA F	TTGACGGAAGGGCACCA
18s rDNA R	CACCACCCATAGAATCAAGAAAGAG

MATERIALS AND METHODS

The strain and cultivation conditions

The green microalga *C. protothecoides* FACHB-2 originally obtained from the Freshwater Algae Culture Collection of the Institute of Hydrobiology, Chinese Academy of Sciences, was cultured with light in KNOP medium (Reski et al., 1985). For autotrophic cultivation, it was inoculated in flask (250 mL) containing 100 mL medium, and cultured in 12 h:12 h light-dark cycle at a light intensity of 40 μmol/m^2s. For heterotrophic cultivation, it was inoculated in medium containing 10 g/L glucose, and cultivated under darkness. All the cultures were kept at 28°C, and were monitored periodically for the presence of bacteria by microscope (Olympus BX51, Japan). Samplings were carried out every two days to measure the growth at 600 nm using a UV/visible spectrophotometer (Unico Instrument Co., Ltd, UV-2000).

RNA preparation and cDNA synthesis

Total RNA from algal cells was extracted using Trizol following the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). cDNA was prepared using StarScript First-strand cDNA Synthesis Kit (GenStar Biosolutions Co., Ltd), with 2 μg of purified RNA as the template.

Molecular cloning of *accD* gene

Degenerate primers (accdF0, accdR0) were designed for the amplification of partial cDNA from *C. protothecoides* FACHB-2. The primers were designed based on the highly conserved nucleotide sequences reported for the *accD* genes from four kinds of algae: *Chlorella variabilis* (GI: 325296270), *Chlorella vulgaris* C-27 (GI: 2224352), *Parachlorella kessleri* (GI: 254798616) and *Chlorococcum humicola* (GI: 325462158) (Table 1). The PCR program included denaturation at 94°C for 5 min, followed by 30 cycles of 30 s at 94°C, 30 s at 50°C, 30 s at 72°C, and an extension step of 5 min at 72°C. The PCR products were cloned into the pMD18-T vector (Takara Dalian, China) and sequenced commercially.

Based on the partial cDNA sequence obtained from the above RT-PCR reaction, *accD* gene specific RACE primers (accdF1, accdR1) were designed to amplify the full-length cDNA sequence (Table 1). 3'-RACE and 5'-RACE PCR were conducted with the SMART™ RACE cDNA Amplification kit (Clontech Laboratories, CA, USA) according to the manufacturer's instructions.

The 3'-RACE and 5'-RACE PCR products were gel-purified and sequenced as previously described. One pair of specific primers (accdF2, accdR2) (Table 1) was designed from the sequences of the 5′ and 3′RACE fragments to amplify a full-length cDNA of *accD* gene.

Quantitative real-time PCR analysis of *accD* gene

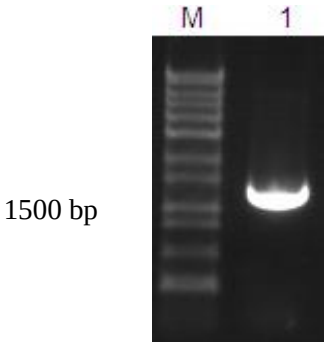
The autotrophic and heterotrophic algal cells were collected at the middle stage of exponential and stationary phases respectively. RNA preparation and cDNA synthesis were as described above.

The primers (accdF3, accdR3) used for quantitative real-time PCR experiments were designed based on the sequence of *accD* gene we cloned (Table 1). 2 μl of 1:10 diluted cDNA was used as the template for real-time PCR experiments. Real-time PCR reaction was performed in a total volume of 50 μl mixture containing 0.4 μM of each primer and 25 μl of 2 × SYBR Green PCR Master Mix (Takara, Japan) on an ABI 7500 sequence detection system (Applied Biosystems, USA). The PCR program consisted of a preliminary step of 1 min at 95°C followed by 40 cycles at 95°C for 30 s and at 60°C for 30 s. The oligonucleotides used for endogenous control (reference gene) expression analysis were designed on the basis of 18s *rDNA* (Table 1). Each primer pair was tested for specificity by melting analysis.

Gene expression levels were normalized against the average Ct (cycle threshold) values for the internal control gene (*18s rDNA*), Ct values were extracted using the SDS 2.3 software (Applied Biosystems). Data analysis was performed using the ΔΔCt method (Livak et al., 2001).

Lipid content detection

Collection of algal cells was as described above. Lipid in the algal cells was extracted according to the microwave oven method (Lee et al., 2010). 0.2 g dry algae were moistened and treated with the microwave oven (NN-K573, Panasonic) at 500 watts for 50 s, then



a

AGAAAAAAAATTTTTTAGAATAAAAAAGAGGAGTTTTTATGTCAATTCTTTCTTGGATTGAA
AATCAACGAAAATTAAAATTATTGAATGCACCTAAATATAATCAGCCGGAGTCTGATGGAAG
TCAAGGGCTTTGGACAAGATGTGATCATTGTGGGGTCATTTTATATATTAAACATTTAAAAG
AAAATCAACGTGTTTGTTTTGGTTGTGGTTACCATCTACAAATGAGTAGTACTGAACGAATC
GATTATTTAATAGATACAGGTACTTGGCGTCCTTTTGATGAAACTGTTTCTCCTTGTGATCC
ACTCGAATTTCGAGATCAAAAAGCCTATACAGAAAGATTAAAAGATGCTCAAGAAAGAACCG
GTTTACAAGATGCTGTTCAAACGGGAACCGGACTTCTTGATGGTATTCCAGTAGCGTTAGGC
GTAATGGATTTTAACTTTATGGGCGGAAGCATGGGCTCTGTTGTTGGCGAAAAAATTACTCG
CTTAATTGAATATGCAACTCAAGAAGGATTACCCGTTATTTTAGTTTGTGCTTCGGGTGGAG
CTCGCATGCAAGAAGGAATTTTAAGTTTAATGCAAATGGCTAAAATCTCGGCTGCTCTTCAT
GTTCATCAAAATTGTGCTAAACTTTTATATATTTCAGTTTTGACTTCTCCGACAACAGGCGG
TGTAACCGCAAGTTTTGCTATGTTAGGTGACCCTATTTTTGCTGAACCGAAAGCCCTTATTG
GTTTTGCTGGTCGAAGAGTTATTGAACAAACTTTGCAAGAACAATTACCTGATGATTTTCAA
ACAGCCGAATATTTATTGCATCATGGTCTTCTTGATTTAATTGTTCCACGTTCTTTTTTAAA
ACAAGCTTTATCAGAAACTATTACATTGTACAAAGAAGCTCCGCTCAAAATGCAAGGCCGAA
TTCCATACGGTGAACGAGGTCCCCTTACCAAGATTCGTGAAGAACAGCTCCGTCGTTTTGCA
AAAGCACCAAAAAATCCACAATATAGTAATTTAGTTGCCGAATTTGAACAGTTGTTGGAATT
GCTAACATCAGATAAAAACATGCTATCTTCAGTTGATGTGTTCGCTCCAGAAACAGTAAATA
AAGCTTTTGAATTAGCTTGTGGATCACAAACTCGTCTTGATTGGTTAAATGATAAAACGAAT
CAATTTCGTCTTCGACCAGTTTTTTAGTTAGAAGAACGAGTTAAGACTTTTACACGCTGAAA
GGAATCCCAAAAATGAAATTTTAGGCATCAGGCTCAAAGGGTAAAGGGTCTCCTCCCGTTTT
TGTTTACAAAAACGGGACCAATTGGAGGAGAAATTTTATTTTTCTAGTTTTGGACAACTAAT
ATAATAAATAAGGCAAAATTTTTGTCACAAACAAACTAGAATTGGGCTAAAAAAGACTAGTG
TATCAATAAAAATTAAGGCAAACTCTCTAGCCTAAAAAAATAACTTTGTTCTTCCAGTTTTT
GTAATCAAAAACAGG

b

Figure 1. Cloning of accD gene from C. protothecoides FACHB-2. a) The PCR products of accD gene. M, DNA Marker (10000,8000,6000,5000,4000,3000,2000,1500,1000,750,500 bp); lane 1, the PCR products of accD gene. b) The nucleotide sequence of accD gene. The coding region was shadowed.

blended with 3 mL chloroform/methanol (2 : 1), shaken for 20 min and centrifuged (8000 rpm) for 10 min. The supernatant was collected in a pre-weighed tube, and after evaporating to constant weight at 60°C, it was weighed to calculate the total lipid content.

Statistical analysis

The statistical program SPSS (Ver 13.0) was used to collate and analyze all the collected data. The standard deviation (STDEV) and Student's t test were used to evaluate the divergence and significance of values between different samples. Significance was accepted at $p<0.05$.

RESULTS

Cloning of *accD* gene from *C. protothecoides* FACHB-2

With the degenerate primers designed according to the conserved sequences of *accD* genes from several algal

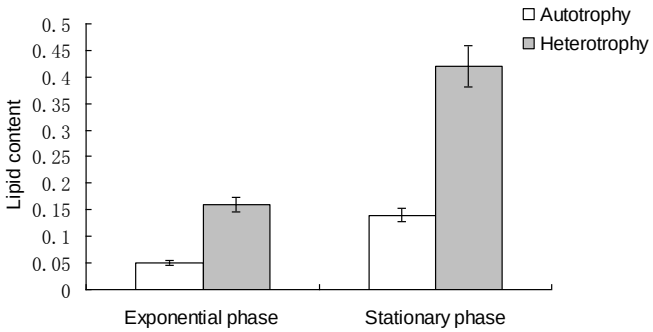


Figure 2. Comparison of *C. protothecoides* FACHB-2 lipid contents under autotrophic and heterotrophic culture conditions.

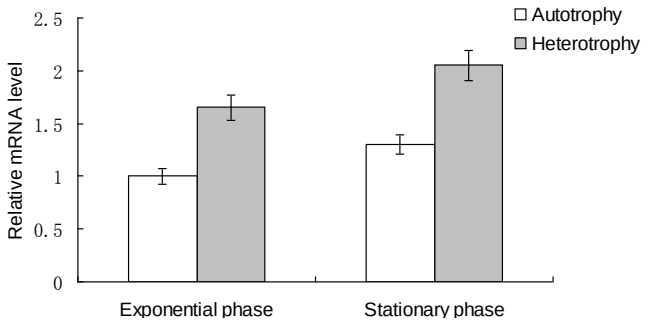


Figure 3. Comparison of *C. protothecoides* FACHB-2 *accD* mRNA levels under autotrophic and heterotrophic culture conditions. The mRNA level was determined by the ΔΔCt method. The bars indicated standard deviations from triplicate assays.

species, a fragment of 440 bp was obtained, sequenced and verified by bioinformatic analysis. With this sequence information, specific primers were designed for 5′ and 3′RACE of *accD* gene, and a 1 kb 3'-RACE fragment as well as a 800 bp 5'-RACE fragment was cloned and sequenced; Subsequently, according to their sequence information, a 1503 bp long full-length cDNA of *accD* gene was successfully amplified (Figure 1a). Its nucleotide sequence was submitted to GenBank under accession No JN831941 (Figure 1b).

Comparison of the algal lipid contents under autotrophic and heterotrophic conditions

Cultivation conditions could influence many aspects of the alga, such as growth rate, chlorophyll content and lipid content. Our analysis showed that the lipid contents under heterotrophic culture conditions were much higher than those under autotrophic culture conditions, reaching nearly 50% of the dry weight at the stationary phases; and under all the conditions, the lipid contents at stationary phases were all higher than at exponential phases, respectively (Figure 2).

Quantification of *accD* gene transcripts under autotrophic and heterotrophic conditions

The lipid contents of the alga under autotrophic and heterotrophic conditions were different, in order to investigate the role of ACCase in lipid accumulation, the mRNA levels of *accD* gene under these conditions were compared.

The expression levels of *accD* gene under different conditions were calculated based on the $\otimes\otimes C_T$ value for each sample. The results showed that the expression levels of *accD* gene under heterotrophic conditions were higher than under autotrophic conditions, and higher at the stationary phases than at the exponential phases, showing good consistence with the lipid contents (Figures 2 and 3).

DISCUSSION

There are two types of ACCases found in nature, and only the heteromeric ones catalyze the *de novo* fatty acid biosynthesis. It is composed of four subunits: one (accD) is encoded by the plastid genome, and the other ones (accA, accB, accC) are encoded by the nuclear genome. The activity of this enzyme can be regulated through many ways, such as transcription, post-transcription, RNA editing, or post-translation (Sasaki et al., 2004). The transcription of the four genes appears to be coordinated, and maybe the level of accD subunit is a determinant of ACCase level (Madoka et al., 2002; Ke et al., 2000), so the transcription abundance of *accD* gene can reflect the response of ACCase to changing culture conditions.

Since it is considered to be very important in regulating fatty acid and thus oil biosynthesis, the genetics of ACCase has been well studied in many plants; as for oleaginous microalgae, only Wan et al. (2011) reported recently in *C. sorokiniana* that increased lipid contents in stationary phases of mixotrophic growth were related to increased expression levels of *accD* gene. Our results showed that the expression levels of *accD* gene began to increase during the exponential phases under heterotrophic cultivations, but elevated expression of this gene did not lead to elevation of lipid contents in the same degree as in the stationary phases. At exponential phases, the carbon source had to meet the need of rapid growth, while at stationary phases, the carbon flux mainly turned to lipid synthesis.

Transcription of genes in many organisms have been found to be carbon-source dependent (Busti et al., 2010; Poças-Fonseca et al., 2000), so elevated levels of *accD* gene under heterotrophic cultivations should be caused by the utilization of glucose. Besides that, glucose might also regulate the activities of many other genes, and then lead to many changes to the cells, such as the decline of

the chlorophyll contents under heterotrophic cultivations, which indicated the decrease of chloroplasts. Recent research indicated that an autophagy-like mechanism played a critical role in the photosynthesis-fermentation conversion and the production of lipids in *Chlorella*; many autophagy related genes were found to be expressed when changing to the heterotrophic medium (Jiang et al., 2012). In the meantime, it should be pointed out that the decreasing of chloroplasts did not mean the reduction of the copies of *accD* gene, since under this condition, the chloroplasts were just changed to another kind of plastids (Ahmad et al., 1990).

Then what improved expression levels of *accD* gene at the stationary phases under autotrophic conditions? Previous work suggested that high ratio of carbon to nitrogen in culture media could promote the lipid accumulation in the cells (Cho et al., 2011). When the stationary phases were reached, many nutritional components in the media were nearly used up, including the nitrogen sources, so decreased nitrogen concentration led to an elevated ratio of carbon to nitrogen, then improved expression levels of *accD* gene. In the same way, under heterotrophic cultivations, the ratio was mainly lifted by supply of glucose.

In conclusion, transcription of *accD* gene in response to different cultivation conditions played an important role in regulating the activity of ACCase, thus influenced the carbon flux distribution. This may be of value in designing strategies for improving lipid synthesis in *C. protothecoides*. Furthermore, whether the activity of ACCase in this species is also regulated via other manners needs further research.

ACKNOWLEDGEMENTS

This work was supported by the National Key Technology R&D Program of China (No. 2011BAD14B04), Shandong Provincial Science Foundation, China (No.ZR2009EM012); Shandong High School Science and Technology Planning Project (No. J10LC53), the Open Project Program of State Key Laboratory of Freshwater Ecology and Biotechnology (No.2011FB18), and the Open Project Program of State Key Laboratory of marine bioactive substances (No. MBSMAT-2010-05).

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