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Identification and functional expression of a Δ9 fatty acid desaturase from the marine bacterium *Pseudoalteromonas* sp. MLY15

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ABSTRACT

A gene putatively encoding a Δ9 desaturase-like protein was cloned from the isolated marine bacterium *Pseudoalteromonas* sp. MLY15. The 1134 bp open reading frame, designated as *PhFAD9*, codes for a 377 amino acid peptide with a molecular weight of 43.4 kDa. The protein was supposed to be a membrane-bound desaturase and its possible topology model was predicted using the Phobius program. The PhFAD9 protein was confirmed to be functional with high Δ9 desaturase activity when expressed in *Escherichia coli*. The *PhFAD9 E. coli* transformant accumulated palmitoleic acid, which accounted for 91.7% of the cellular C16 fatty acids after 2 h of induction. The ability for bioconversion of stearic acid to oleic acid was also demonstrated by supplementing the medium with exogenous stearic acid.

1. Introduction

cis-Unsaturated fatty acids (UFAs) have profound effects on the fluidity and function of biological membranes. Because the steric hindrance imparted by the rigid kink of the *cis*-double bond results in much poorer packing of the acyl chains, UFAs have a much lower transition temperature than saturated fatty acids. Most living organisms, ranging from bacteria to humans, respond to low temperature stress by using an oxygen-dependent fatty acid desaturation pathway to increase the relative UFA content of membrane lipids so that the biological membrane can be remained in the correct physical state for good physiological function [1]. Understanding the mechanisms involved in the adaptive regulation of membrane lipid composition requires the identification of those enzymes which mediate the critical steps in synthesis of UFAs before uncovering the factors which influence their specific activity and expression [2].

The fatty acid desaturases, which catalyze the desaturation reactions by introducing double bonds into fatty acyl chains, can be divided into two evolutionary unrelated classes: one is the class of soluble acyl-ACP desaturase, the other consists of membrane-bound enzymes including the acyl-CoA desaturase and the acyl-lipid desaturase [3]. The membrane class is more widespread in nature [4]. The fact that they are found in cyanobacteria [5] and yeasts [2] suggests that they likely arose earlier than the soluble plastid desaturases found in higher plants [6]. Bacteria have an alternative pathway to produce UFAs without desaturase [7,8], however, some reports suggest that the integral membrane desaturases are utilized in bacteria more extensively than initially thought. The cold shock-induced, membrane-bound desaturase from *Bacillus subtilis* has regioselectivity of Δ5 [9]. A model of the regulatory pathway involving the Δ5 desaturase was proposed to adapt to temperature shifts by inserting double bonds into the membrane acyl-chain [10]. From the results of a systematic analysis of fatty acid desaturases found in the genome data of 397 organisms, some other bacterial sequences, falling into the same group composing of desaturases from plants and cyanobacteria, are good candidates to be desaturases [11]. A membrane-bound Δ9 desaturase in *Mycobacterium tuberculosis* was experimentally verified [12]. Two aerobic desaturases were added to the enzymes used for fatty acid metabolism in *Pseudomonas aeruginosa* and these alternate pathways provide a mechanism to modify existing membrane phospholipid fatty acids and to produce UFAs from exogenous saturated fatty acids [13,14]. Recently, we also reported the identification and functionally characterization of a membrane-bound Δ9 desaturase from *Psychrobacter urativorans* DSM 14009 [15].

UFAs, especially monounsaturated fatty acids, were important for growth of the marine bacteria [16]. The biosynthesis of UFAs generally was initiated by a Δ9 desaturases in the desaturase-mediated pathway [4]. Although sequences from some marine bacteria with significant similarity to Δ9 fatty acid desaturase genes have been submitted to the public databases, their

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function in most cases has not yet experimentally demonstrated. In the present work, we succeeded in cloning a gene encoding a putative desaturase from the identified strain *Pseudoalteromonas* sp. MLY15, which was isolated from sea water and had a relatively high UFA content. We determined its function by heterologous expression in *Escherichia coli*. The expression product was revealed to have a high Δ9 fatty acid desaturase activity and classified as a membrane desaturase. The fact that the Δ9 desaturase might be used in a complementary pathway for UFA synthesis by some marine bacteria would contribute to the study of adaptation to cold marine conditions.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth media

The isolated strain MLY15 from sea water was cultured at 20 °C and in a medium containing (L^{-1}) 5 g tryptone, 10 g yeast extract, 10 g NaCl, 3 g $MgSO_4{\cdot}6H_2O$, 1 g $Na_2SO_4{\cdot}10H_2O$, 1 g K_2HPO_4, 1 g NH_4NO_3, 5 mL corn steep liquor and 18 g agar at pH 7.0, which was identified as *Pseudoalteromonas* sp. MLY15 based on 16S rDNA sequence analysis and its morphological and physiological characteristics defined in the Bergey's Manual of Systematic Bacteriology [17]. *E. coli* DH5α (Clontech, Staint-Germain-en-Laye, France) strain and pMD18-T vector (TaKaRa, Dalian, China) were used in all routine DNA manipulations and cloning procedures. *E. coli* Rosetta (DE3) (Novagen, Merck Chemicals Ltd., Nottingham, United Kingdom) was used as the host for the pET21a (+) (Novagen, Darmstadt, Germany) plasmid containing the desaturase gene from *Pseudoalteromonas* sp. MLY15. The recombinant *E. coli* strains were grown on Luria–Bertani broth containing the required antibiotics.

2.2. PCR-based cloning

Genomic DNA of *Pseudoalteromonas* sp. MLY15 was obtained using the standard phenol/chloroform precipitation protocol [18]. For cloning of the desaturase gene from strain MLY15, the CODEHOP primers of DE1 (CGGATCCAC-CACAGGCA(A/T/C/G)GT(A/T/C/G)GA(C/T)(A/G)A) and DE2 (CGAA-GATGTGGTGGAAGTTGTG(A/G)TA(A/T/C/G)CC(C/T)TC), which had been previously designed for cloning of the Δ9 desaturase gene from *P. urativorans* DSM 14009 [15], were used. PCR amplification was carried out in a total volume of 50 μL containing 1 μL of genomic DNA, 1 μL of Taq DNA polymerase (Fermentas, St. Leon-Rot, Germany), 5 μL of 10× Taq buffer (with $MgCl_2$), 4 μL of dNTP (2.5 mM each), and 1 μL of the above primers (20 mM), under the following procedure: initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 45 s, 50 °C for 1 min and 72 °C for 1 min, and a final extension at 72 °C for 4 min. The amplified 400 bp PCR fragment was sequenced after DNA agarose gel purification (TaKaRa, Dalian, China).

Subsequently, two primers, AGTTGTACGCTGAGATAGAGAAT and CATATTGGTATGTCACGGGTGTT, were designed according to the flanking regions of the putative desaturase gene in *Pseudoalteromonas haloplanktis* TAC125 (Genbank accession no. CR954246) and based on the sequence information of the obtained PCR fragment. The PCR mixture and amplification conditions were the same as described above with the exception of the annealing temperature (52 °C). The amplified 1.2-kb PCR fragment purified by gel-extraction was cloned into the pMD18-T vector and sequenced. As a result, the nucleotide sequence containing 1134 bp of the putative desaturase gene from *Pseudoalteromonas* sp. MLY15 was determined.

2.3. Topology prediction

The distribution of the hydrophobic amino acids was analyzed using Kyte-Doolittle hydropathy scale with a window size of 19 (http://www.expasy.ch/cgi-bin/protscale.pl) [19]. Topology predictions were done using HMMTOP [20,21], TMHMM [22], DAS [23], TMpred [24], SOSUI [25], TopPred [26] and Phobius [27].

2.4. Expression of the desaturase gene in E. coli

For cloning of the target gene the following primers were designed: CGGGATCC*ATG*AACAAACCACCACT and CCG-CTCGAG*TTA*ACTAAAAGAGGCTT containing ATG start and TAA stop sites (italicized), and BamHI and XhoI cloning sites (underlined), respectively. PCR was performed under the same conditions as described above except for using Pfu DNA polymerase (Fermentas, St. Leon-Rot, Germany). The resultant PCR product was isolated and digested with BamHI and XhoI restriction endonucleases (TaKaRa, Dalian, China) and then ligated into the BamHI/XhoI sites of pET21a (+) vector to generate an expressing construct, which was transformed into *E. coli* Rosetta (DE3) by $CaCl_2$ method [18]. Positive transformants screened on the LB agar plates with ampicillin and chloromycetin resistance were further validated by control PCR and subsequent sequencing.

For the expression of this putative desaturase gene in *E. coli*, individual colonies of *E. coli* Rosetta (DE3) cells transformed with the recombinant plasmid were grown overnight in 5 mL LB medium containing 100 μg/mL ampicillin and 34 μg/mL chloromycetin at 30 °C. Then aliquots of the cultures were taken to inoculate 35 mL of fresh LB medium supplemented with corresponding antibiotics. Cells were cultivated until the OD_{600} value reached 0.8–1, then isopropyl-β-D-thiogalacto-pyranoside (IPTG) was added at a final concentration of 0.4 mM and the incubation was shifted to 25 °C. At various intervals after addition of IPTG, the cells were harvested from 35 mL culture. In order to test the activity of the overexpressed product to stearic acid, cultures were supplemented with 400 μM stearic acid at inoculation and collected after 4 h of induction. Centrifugation was done at 8228 × *g* for 5 min at 4 °C. The pellets were washed with 0.9% NaCl solution and stored at −70 °C until use. Control experiments were performed under the same experimental conditions using *E. coli* transformants containing empty plasmid pET21a (+).

Table 1
Fatty acid compositions of *Pseudoalteromonas* sp. MLY15 under the different temperature conditions

Temperature conditions	Fatty acids (%)					Unsaturation (%)	UFA/SFA
	15:0	16:1	16:0	17:1	17:0		
4 °C, 96 h	9.3	44.0	9.7	32.8	4.2	76.8	3.3
20 °C, 48 h + 4 °C, 48 h	10.8	38.4	10.5	34.8	5.5	73.2	2.7
4 °C, 48 h + 20 °C, 48 h	13.6	35.1	12.8	31.5	7.0	66.6	2.0
20 °C, 96 h	12.1	34.0	11.8	34.8	7.3	68.8	2.2

2.5. Total cellular fatty acid analysis

The fatty acid compositions of *Pseudoalteromonas* sp. MLY15 and the recombinant *E. coli* cells were analyzed according to the MIDI protocol (Microbial Identification System, Microbial ID Inc., Newark, Delaware, USA). Fatty acid methyl esters (FAMEs) were separated and analyzed by Gas chromatography–mass spectrometry (GC–MS) (Thermo Finnigan TRACE DSQ GC/MS, USA). The GC was equipped with a 30 m DB-5 ms capillary column (internal diameter 0.25 mm, film thickness 0.25 μM) using helium as carrier gas with a linear velocity of 1.0 mL/min. The column temperature program was composed of an initial temperature at 100 °C, ramping at 25 °C/min to 200 °C, followed by heating until 300 °C with 10 °C/min. The injector temperature and transfer-line temperature were both 250 °C. Ionization energy was 70 eV. The ion-source temperature was 250 °C. Scan mass range was 50–400 amu.

3. Results and discussion

3.1. Bacterium identification

The bacterial 16S rDNAs of stain MLY15 amplified by PCR using primers BSF8/20 (AGAGTTTGATCCTGGCTCAG) and BSR1541/20 (AAGGAGGTGATCCAGCCGCA) [28] were cloned into the pMD18-T vector (TaKaRa) and sequenced by Invitrogen Corporation, Shanghai, China. 1528 bp of the obtained partial 16S rDNA sequence showed 99.87% identity with *Pseudoalteromonas* sp. ER72M2 16S ribosomal RNA gene (Genbank accession no. AF155038) by performing a BLAST search using default parameter. The morphological and physiological characteristics of this strain also supported the result that it belonged to *Pseudoalteromonas* genus, which was thus named as *Pseudoalteromonas* sp. MLY15 afterwards [29].

3.2. Fatty acid composition analysis of Pseudoalteromonas sp. MLY15

Pseudoalteromonas sp. MLY15 was cultured on the media plates under four different temperature conditions. As shown in Table 1, this strain contained five kinds of fatty acids. Grown either at 20 °C or 4 °C, the unsaturation degree of its cellular fatty acids was more than 65%. UFA/SFA was 2.2 at 20 °C while it was 3.3 at 4 °C. When the growth temperature was changed from 20 °C to 4 °C, UFA/SFA was close to that at 4 °C. A similar effect was observed when it was changed from 4 °C to 20 °C. These results proved that *Pseudoalteromonas* sp. MLY15 had a relatively high UFA content and reposed to growth temperature changes by altering its fatty acid composition. More UFAs were synthesized in the cells at lower temperatures.

3.3. Cloning of desaturase gene from Pseudoalteromonas sp. MLY15

A DNA fragment of about 400 bp was amplified from *Pseudoalteromonas* sp. MLY15 using CODEHOP primers DE1 and DE2 [30]. The amplified DNA-fragment showed a very high identity (99%) to a putative desaturase gene in *P. haloplanktis* TAC125. Therefore, another pair of primers were derived from the flanking regions to get the full gene sequence. The acquired nucleotide sequence had an open reading frame of 1134 bp, designated as *PhFAD9*, encoding a Δ9 fatty acid desaturase-like protein with 377 amino acid residues, a calculated molecular weight of 43.4 kDa and a theoretical isoelectric point of 9.45. A Blast search (http://www.ncbi.nlm.nih.gov/blast/) revealed that the primary structure of this putative desaturase was almost the same as that in *P. haloplanktis* TAC125 (Genbank accession no. YP341374) which has not been characterized, except for K304R. Phylogenetic analysis also demonstrated the homology between PhFAD9 and the putative fatty acid desaturase from other marine bacteria (Fig. 1). The three histidine-rich motifs containing eight histidine residues were found as HXXXXH (58–63), HXXHH (95–99) and HXXHH (233–237), which are typical among membrane-bound acyl-CoA and acyl-lipid desaturases [31]. The importance of the conserved histidine residues was studied by site-directed mutagenesis of stearoyl-CoA desaturase of rat [31] and of the Δ12 acyl-lipid desaturase of *Synechocystis* sp. PCC 6803 [32]. They are potential ligands for iron atoms and believed to be responsible for di-iron binding at the catalytic center.

3.4. Topology prediction of the putative desaturase

While essentially no three-dimensional structural information is available for these difficult-to-purify enzymes, to date, experimental evidence on the topology of membrane enzymes that contain the conserved His-motifs, mainly derived from studies performed in acyl-lipid Δ5 desaturase from *B. subtilis* [35], membrane-bound alkane hydroxylase of *Pseudomonas oleovorans* [36] and mouse stearoyl-CoA desaturase [37], has provided a model that might be extended to most membrane desaturases.

The distribution of the hydrophobic amino acids of the putative desaturase from *Pseudoalteromonas* sp. MLY15 was shown as Fig. 2A. A variety of tools are available to predict the topology of

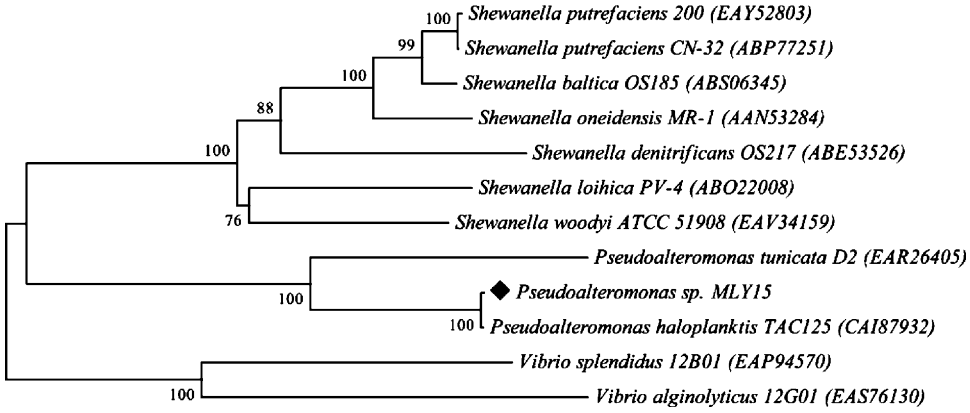

Fig. 1. Phylogenetic relationship between the putative desaturase amino acid sequences from the isolated *Pseudoalteromonas* sp. MLY15 and other marine bacteria. The GenBank accession numbers for the selected sequences are shown in parentheses. Sequence alignment and phylogenetic tree construction were done by clustalx 1.81 [33] and MEGA 3.1 [34] using default parameters.

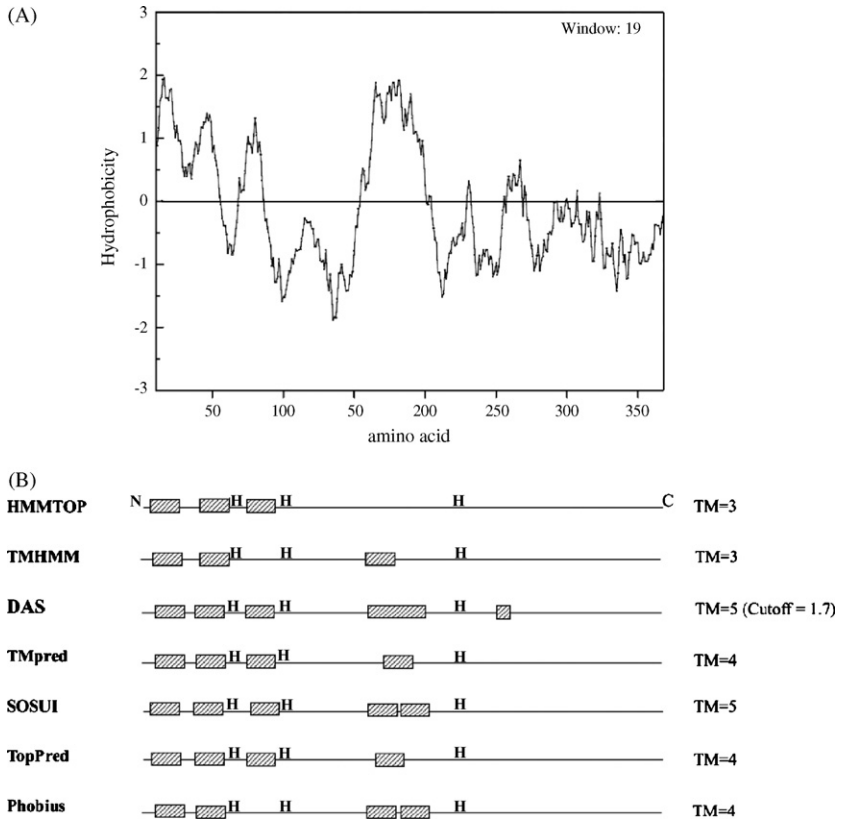

Fig. 2. Hydropathy plot and comparison of the predicted topology of the putative desaturase in *Pseudoalteromonas* sp. MLY15. (A) Kyte–Doolittle hydropathy plot derived from the amino acid sequence of the desaturase in *Pseudoalteromonas* sp. MLY15 using a window of 19. (B) The results from seven types of prediction methods (two hidden Markov model (HMM) methods, HMMTOP [20,21] and TMHMM [22], the Dense Alignment Surface (DAS) [23], TMpred [24], SOSUI [25], TopPred [26] and Phobius [27]). The TM domain is represented as a rectangle and the number of predicted TM domain is indicated for each. The positions of His-motifs are represented as the bold **H**.

transmembrane proteins. However, few of the currently best known and most widely used methods can give the reasonable topology model for this *Pseudoalteromonas* desaturase with the default parameters (Fig. 2B), including TMHMM that was thought to be the best performing transmembrane prediction program [38]. Considering that three conserved His-motifs would be on the same side of the cytoplasm to form the potential di-iron active site, Phobius is probably the best candidate tool here for this enzyme that predict a common membrane topology consistent with the experimental results [35,37]. The deduced protein contained four hydrophobic domains between amino acids 7–30, 36–57, 155–174 and 180–199, which would be long enough to span the membrane bilayer twice, with both the N- and C-termini facing the cytosol.

3.5. *In vivo study of Δ9 desaturase expressed in E. coli*

To determine the function of the Δ9 desaturase-like protein, the *PhFAD9* gene was cloned into pET21a (+) vector and expressed in *E. coli* Rosetta (DE3) as described in Section 2. At various intervals after addition of IPTG, the cultures were collected and their fatty acid compositions were analyzed by GC–MS. The conversion ratio of cellular palmitic acid into palmitoleic acid was time-dependent (Fig. 3), indicating that it can be controlled through expression time. The desaturation level of C16 fatty acids was 91.7% while the control was 47.3% when measured only 2 h after induction (Fig. 3). The accordingly ratios of palmitoleic to palmitic acid were 11 and 0.9, respectively.

As the candidate substrate stearic acid for Δ9 desaturase was not present as the major component fatty acid in *E. coli* cells [39], the recombinant strain and the control were inoculated to the media without and with stearic acid at the final concentration of 400 μM. The partial patterns of cellular fatty acids for 4 h after induction were shown in Fig. 4. The great changes in the pattern of palmitic and palmitolic acid between the *PhFAD9* transformant and the control strain, whether the stearic acid was added or not, still supported that the PhFAD9 protein was capable of using palmitic acid. GC–MS analysis of FAMEs revealed that a small and obvious fatty acid peak corresponding to oleic acid methyl ester, which accounted for 3% of

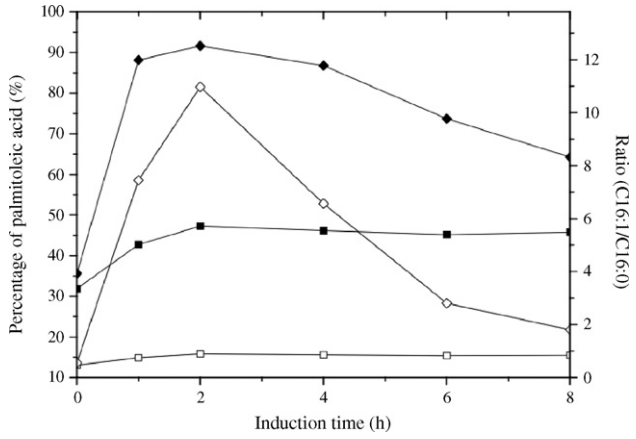

Fig. 3. The unsaturation degree of cellular C16 fatty acids in *E. coli* Rosetta (DE3) cells transformed with the control vector pET21a (+) (■, □) or with the *PhFAD9* construct (♦, ◊). (■) and (♦) represent the percentage of palmitoleic acid in cellular C16 fatty acids; (□) and (◊) represent the ratio of palmitoleic to palmitic acid. Cells were collected at different intervals after induction.

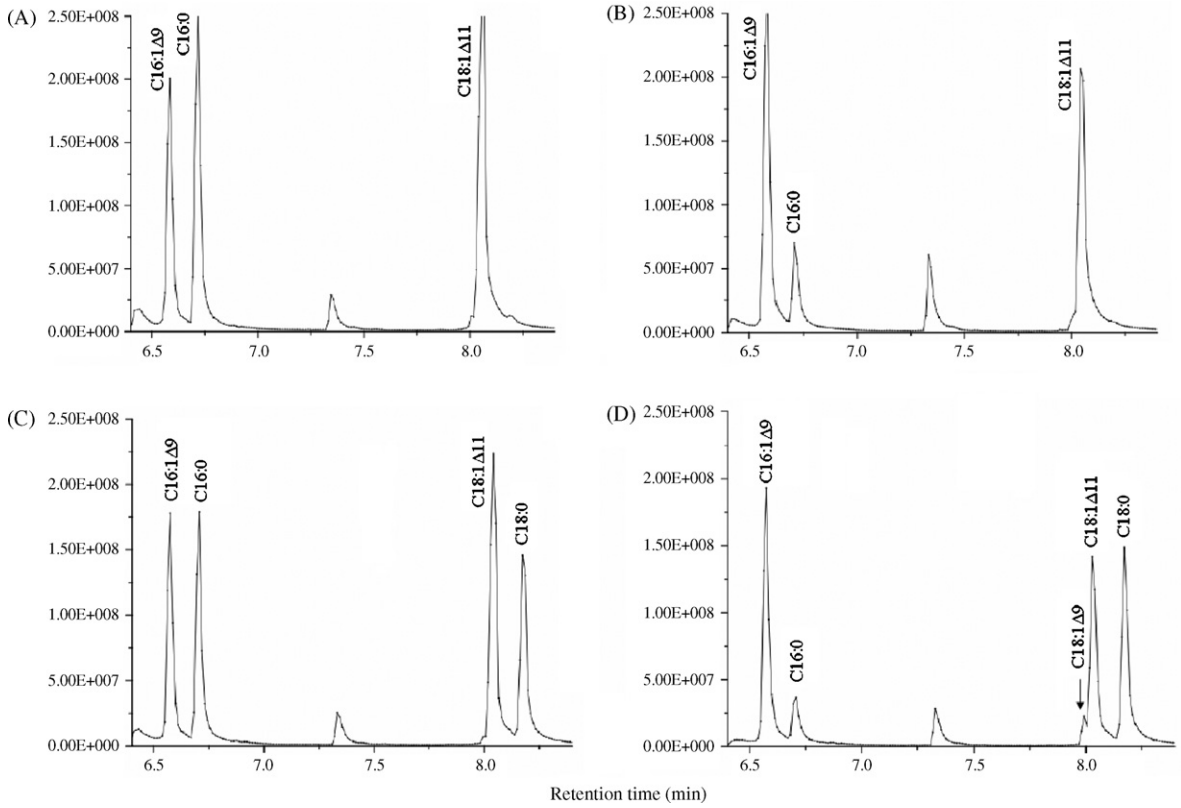

Fig. 4. Gas chromatograms of methyl esters of fatty acids obtained from *E. coli* transformants. The control strains (A) and (C), and the *PhFAD9* transformants (B) and (D) were cultivated at 25 °C for 4 h after induction. For (C) and (D) stearic acid was added to the media at the final concentration of 400 μM. The arrowhead indicates the position of oleic acid methyl ester. The other peaks at the retention time of 6.45 min and 7.37 min were identified as 3-hydroxymyristic acid methyl ester and cyclopropane fatty acid methyl ester, respectively, which came from the *E. coli* biomass.

the total fatty acids in the recombinant cells, was detected in case that the media were supplemented with stearic acid. These results demonstrated that the product encoding by *PhFAD9* gene was a Δ9 fatty acid desaturase, which can be actively expressed in *E. coli* and had the ability to catalyze the conversions from palmitic and stearic acid to palmitoleic and oleic acid, respectively. It was supposed that the exogenous stearic acid was incorporated into the membrane of *E. coli* before it was desaturated by the Δ9 desaturase. If the amount of the incorporated stearic acid in the membrane was not limited to the desaturation reaction, this enzyme probably preferred palmitic acid to stearic acid *in vivo*.

4. Conclusion

The tolerance of organisms to chilling determines their choice of natural habitat. The related molecular mechanism for cold adaptation has attracted great interest for several years [40]. UFAs play a key role for the maintenance of appropriate fluidity and thereby the correct function of biological membranes. The genes and the gene products involved in fatty acid biosynthesis and metabolism need to be identified and characterized as a prerequisite for comprehensive research into these mentioned processes. In this study, we identified a marine bacterium belonging to the genus *Pseudoalteromonas*, which grows at 20 °C [17]. A gene encoding a Δ9 fatty acid desaturase involved in monounsaturated fatty acid biosynthesis in the identified strain *Pseudoalteromonas* sp. MLY15 has been cloned and functionally expressed in *E. coli*. Enzymatic activity of the target protein was confirmed by GC–MS analysis of the cellular fatty acid composition, to demonstrate that this enzyme can effectively catalyze the desaturation of both palmitic acid and stearic acid. This work should be useful for further research of the genetic engineering about UFA production [41] and the improvement of cold-tolerant organisms for some specific purposes.

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