Journal of Industrial Microbiology & Biotechnology (2020) 47:1045–1057
https://doi.org/10.1007/s10295-020-02333-0

SIMB
Society for Industrial Microbiology and Biotechnology

ENVIRONMENTAL MICROBIOLOGY - ORIGINAL PAPER

Effects of a Δ-9-fatty acid desaturase and a cyclopropane-fatty acid synthase from the novel psychrophile *Pseudomonas* sp. B14-6 on bacterial membrane properties

Tae-Rim Choi[1] · Ye-Lim Park[1] · Hun-Suk Song[1] · Sun Mi Lee[1] · Sol Lee Park[1] · Hye Soo Lee[1] · Hyun-Joong Kim[1] · Shashi Kant Bhatia[1,2] · Ranjit Gurav[1] · Yoo Kyung Lee[3] · Changmin Sung[4] · Yung-Hun Yang[1,2]

Received: 9 July 2020 / Accepted: 17 November 2020 / Published online: 1 December 2020

Abstract

Psychrophilic bacteria, living at low and mild temperatures, can contribute significantly to our understanding of microbial responses to temperature, markedly occurring in the bacterial membrane. Here, a newly isolated strain, *Pseudomonas* sp. B14-6, was found to dynamically change its unsaturated fatty acid and cyclic fatty acid content depending on temperature which was revealed by phospholipid fatty acid (PLFA) analysis. Genome sequencing yielded the sequences of the genes Δ-9-fatty acid desaturase (*desA*) and cyclopropane-fatty acid-acyl-phospholipid synthase (*cfa*). Overexpression of *desA* in *Escherichia coli* led to an increase in the levels of unsaturated fatty acids, resulting in decreased membrane hydrophobicity and increased fluidity. Cfa proteins from different species were all found to promote bacterial growth, despite their sequence diversity. In conclusion, PLFA analysis and genome sequencing unraveled the temperature-related behavior of *Pseudomonas* sp. B14-6 and the functions of two membrane-related enzymes. Our results shed new light on temperature-dependent microbial behaviors and might allow to predict the consequences of global warming on microbial communities.

Keywords *Pseudomonas* strain · Phospholipid fatty acid · Genome sequencing · Hydrophobicity · Membrane fluidity

Introduction

The Arctic is home to various microbial strains, which can provide information regarding survival in harsh conditions [1]. The existence of cryophilic and cold-tolerant bacteria and the mechanisms by which these organisms adapt to fluctuations in temperature and osmolarity have prompted studies on general temperature-related survival mechanisms [2–6]. Previous studies have revealed several mechanisms of coping with different temperature changes, such as the expression of cold-shock proteins, changes in membrane fluidity, and the production of polyhydroxyalkanoates (PHAs) and exopolysaccharides (EPSs), and ongoing research aims to elucidate the different survival strategies [7, 8].

Phospholipid fatty acids (PLFAs) are a major component of the microbial cell membrane, and they can be analyzed to determine microbial community composition and to monitor dynamic changes in membrane properties. This is interesting, because the membrane is the essential first line of defense from the outer environment for both Gram-negative and Gram-positive bacteria [9]. Unlike total lipid analysis, PLFA analysis involves laborious fractionation steps and requires careful treatment of the highly unstable phospholipids [10]. However, it can provide direct information on the identity of membrane phospholipids, unlike total lipid analysis, which has difficulties in detecting some fatty acids that are sensitive to strong acid or high temperature [11, 12].

Pseudomonas is a widespread bacterial genus that exists in various temperature zones, produces PHAs and EPSs, and displays multiple hydrolase activities exploited for bioremediation. Therefore, *Pseudomonas* is a promising

✉ Yung-Hun Yang
seokor@konkuk.ac.kr

[1] Department of Biological Engineering, College of Engineering, Konkuk University, 1 Hwayang-dong, Gwangjin-gu, Seoul 05029, Republic of Korea

[2] Institute for Ubiquitous Information Technology and Applications, Konkuk University, Seoul 05029, Republic of Korea

[3] Korea Polar Research Institute, Incheon 21990, Republic of Korea

[4] Doping Control Center, Korea Institute of Science and Technology, Seoul 02792, Republic of Korea

genus for industrial use as well as for investigating different temperature-related behaviors [7, 13]. Previous studies have shown that bacterial lipid metabolism is altered in response to temperature and have attempted to identify the key players involved in mediating such changes in membrane fatty acids [13, 14]. Some studies have identified the enzymes Δ-9-fatty acid desaturase (DesA) and cyclopropane-fatty acid-acyl-phospholipid synthase (Cfa) as regulators of the levels of unsaturated fatty acids (UFAs) and cyclopropane-fatty acids (CFAs), respectively [15–17]. However, most studies concluded this on the basis of total lipid analysis, whose results can differ from those of PLFA analysis [18]. Moreover, a previous study has shown the function of *desA* in *Escherichia coli* through the addition of stearic and palmitic acid and total lipid analysis [19]. Therefore, although previous research provided some insights, there is a need to carry out in vivo experiments to verify the exact role of this desaturase in the remodeling of the cellular membrane.

In the current study, we characterized the newly identified *Pseudomonas* sp. B14-6 strain, isolated from arctic soil, which showed dynamic changes in membrane PLFA content, hydrophobicity, and fluidity upon changes in temperatures. We aimed to determine the function of the genes *desA* and *cfa* from this new strain using PLFA analysis and genomic sequencing. Our results are expected to provide insights into temperature-dependent membrane behavior and its key factors, and they might inspire the development of new methods for industrial use of microbes at harsh temperatures as well as shed some light on the ecological implications of global warming.

Materials and methods

Chemicals

All chemicals used in the present study were of analytical grade or higher. Fatty acids such as palmitic acid and stearic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Also, for the qualitative analysis of various fatty acids from bacteria, we purchased a bacterial acid methyl ester (BAME) mix from Sigma-Aldrich. Other chemicals used in the growth media were also purchased from Sigma-Aldrich or BD Difco (Franklin Lake, NJ, USA).

Screening of arctic strains

The bacterial strain *Pseudomonas* sp. B14-6 was isolated from arctic glacier soil collected from Midtre Lovénbreen, Ny-Ålesund in Svalbard. The arctic soils were diluted serially with 0.9% saline five-to-six times and spread on Luria–Bertani (LB) agar that was tenfold and 100-fold diluted to avoid nutrient shock to the arctic microbes. Two temperatures, namely 10 and 25 °C, were used for incubation and the colonies formed were isolated. The isolated microbes were subjected to 16S rRNA gene sequencing using primers 27F and 1492R (27F, 5′-AGAGTTTGATCCTGGCTCAG-3′; 1492R, 5′-GGTTACCTTGTTACGACTT-3′) by Cosmogenetech (Seoul, South Korea). The obtained partial sequences of 16S rRNA genes were compared to those included in the GenBank database using the Nucleotide BLAST tool (blast.ncbi.nlm.nih.gov/Blast.cgi). The identified partial sequence of the *Pseudomonas* sp. B14-6 16S rRNA gene was used for constructing a phylogenetic tree using a neighbor-joining method in a bootstrap test (2000 replicates) with the MEGA X software version 10.1 [20–23].

Strains and culture conditions

Isolated *Pseudomonas* sp. B14-6 was first cultured in various media such as LB broth (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L sodium chloride), Tryptic soy broth (TSB; 17 g/L tryptone, 3 g/L soytone, 2.5 g/L glucose, 5 g/L sodium chloride, and 2.5 g/L dipotassium phosphate), Terrific broth (TB; 24 g yeast extract, 12 g tryptone, 9.4 g/L dipotassium phosphate, 2.2 g/L potassium phosphate, and 4 ml/L glycerol), and Marine broth 2216 (MB; 5 g/L peptone, 1 g/L yeast extract, 0.1 g/L ferric citrate, 19.45 g/L sodium chloride, 5.9 g/L magnesium chloride, 3.24 g/L magnesium sulfate, 1.8 g/L calcium chloride, 0.55 g/L potassium chloride, 0.16 g/L sodium bicarbonate, 0.08 g/L potassium bromide, 0.034 g/L strontium chloride, 0.022 g/L boric acid, 0.004 g/L sodium silicate, 0.0024 g/L sodium fluoride, 0.0016 g/L ammonium nitrate, and 0.008 g/L disodium phosphate) to determine the optimal medium for further experiments [24]. All experiments were performed in duplicate. Cultures were incubated at 200 rpm at 10 or 25 °C and cell growth was measured every 24 h. Cell optical density was measured at a 600-nm wavelength using a spectrophotometer (TECAN, Männedorf, Switzerland). *E. coli* strains were cultured using LB media, supplemented with 50 μg/mL of kanamycin and 0.1 mM of IPTG if needed, at 37 °C. Cell growth in cultures containing palmitic acid and stearic acid was also compared at 48 h and 72 h. The optical density of the cultures was determined using a spectrophotometer at a 595-nm wavelength. The two membrane-related genes of *Pseudomonas* sp. B14-6 (*desA* and *cfa*) were cloned into pET24ma and transformed into *E. coli* for further experiments. Table 3 lists the strains used for gene overexpression and the primers used for cloning the two genes [25].

Whole-genome sequencing and genomic DNA assembly and annotation

The whole-genome sequencing of *Pseudomonas* sp. B14-6 was performed on a single-molecule real-time sequencer

(SMRT, 20 K, PacBio, Menlo Park, CA, USA) and de novo assembly was performed by DNALink (Seoul, South Korea). Samples were prepared by P6-C4 chemistry and sequenced using 1 SMRT cell with the MagBead OneCellPerWell v1 protocol (insert sizes, 20 kb; movie time, 1 × 240 min). De novo assembly was performed using the Hierarchical Genome Assembly Process (HGAP, version 2.3). As bacterial genomes and plasmids are typically circular, the forms were checked for each contig using the MUMmer software version 3.0 and, when applicable, one of the self-similar ends was trimmed for manual genome closure. As a result of form checking, we obtained a 6,766,722-bp long contig.

PLFA analysis

The PLFA composition of membranes of *Pseudomonas* sp. B14-6 and engineered *E. coli* was evaluated using PLFA analysis [26]. The strains were cultured in LB medium at 10 or 25 °C and 200 rpm for 72 h in duplicate. The culture broth was centrifuged (3500 g at 4 °C for up to 30 min) and pellets were washed twice with deionized water. The washed cells were transferred to a glass vial for lyophilization. Lipid extraction and further experiments were conducted based on the Bligh and Dyer method and the MIDI protocol [10, 27]. Briefly, 5 mL of the lyophilized culture of *Pseudomonas* sp. B14-6 or *E. coli* was used as the biomass to which both 2 mL of methanol and chloroform were added for lipid extraction from the cells. Thereafter, the mixture was vortexed and activated at 25 °C for 2 h. After the addition of 2 mL of distilled water to the mixture and vortexing, followed by centrifugation at 1500 g for 5 min, 2 mL of the liquid phase was transferred to glass vials and the sample was evaporated with N_2 gas and re-treated with 1 mL of chloroform for further steps. The sample containing the total lipid extract was subjected to column chromatography using silicic acid that binds to lipids [28]. Each lipid in the sample was then eluted using a different solvent; neutral lipids were eluted with chloroform, glycolipids were eluted with acetone, and phospholipids were eluted with methanol. Only methanol-dissolving phospholipids were collected in the glass vial and evaporated with N_2 gas. The quantification and identification of membrane phospholipids were carried out by gas chromatography–mass spectrometry (GC–MS). For mild alkaline methanolysis of phospholipids, 0.5 mL of methanol, 0.5 mL of toluene, and 1 mL of 0.3 M methanolic-KOH were added to the samples, which were subsequently incubated at 37 °C for 15 min [29, 30]. The organic phase was then extracted and transferred into clean borosilicate glass tubes containing 1 mg of Na_2SO_4. The resulting samples were analyzed through a GC–MS system (Perkin Elmer, Waltham, MA, USA) equipped with a fused silica capillary column (Elite-5 ms, 30 m, 0.25 mm, i.d. 0.25-µm film), and subjected to a linear temperature gradient for full resolution of fatty acids (120 °C held for 5 min, increased by 6 °C/min to 200 °C, increased by 2 °C/min to 220 °C, and then increased by 10 °C/min to 300 °C) [31]. The injector port temperature was set at 210 °C. Mass spectra were obtained by electron impact ionization at 70 eV, and scan spectra were obtained within the range of 45–400 m/z. Selected ion monitoring was used for the detection and fragmentation analysis of the major products [32].

Membrane characterization

Microbial adhesion to hydrocarbon (MATH) method

The relative cell surface hydrophobicity of the organisms was measured according to the protocol proposed by Aono et al. [33]. Briefly, cells from cultures in the stationary phase were harvested by centrifugation (3500 g, 10 min, 4 °C). The cells were then suspended in cold 0.8% saline, so that they reached an optical density of 0.6 at 660 nm (OD_{660}). The absorbance of this suspension was referred to as A1. Aliquots of the suspension (3 mL) were transferred to two glass tubes, and *n*-octane (0.6 mL) was added to one tube (sample) but not the other (control). Both suspensions were agitated vigorously for 2 min and then allowed to stand for 15 min to allow separation into n-octane and saline layers. Then, the OD_{660} of the saline phase of the sample (A2S) and of the control (A2C) was measured. The MATH value was calculated as the percentage of the decrease in turbidity of the saline phase, using the following equation: $\text{MATH} = \frac{\text{A2C}-\text{A2S}}{\text{A1}} \times 100$.

Membrane fluidity test

The membrane fluidity test was based on the fluorescence polarization by specific compounds [34]. Harvested cells were treated according to the protocol described by Royce et al. [35]. Briefly, the samples were washed twice in saline (pH 7.0), resuspended at a concentration of 1×10^8 cells/mL, and incubated at 37 °C for 30 min with 1,6-diphenyl-1,3,5-hexatriene (DPH; supplied by Life Technologies, Carlsbad, CA, USA) at a concentration of 0.2 µM (0.2 mM stock solution in tetrahydrofuran). Fluorescence polarization values were determined using a Synergy 2 Multi-Mode microplate reader (BioTek, Winooski, VT, USA) using sterile black flat-bottom Nunclon Delta-Treated 96-well plates (Thermo Fisher Scientific, Waltham, MA, USA). The filters used were a 360/40-nm fluorescence excitation filter and a 460/40-nm fluorescence emission filter (BioTek). The polarized excitation filter was set in vertical position, while the polarized emission filter was set either in vertical (I_{VV}) or horizontal (I_{VH}) position. The

polarization value (P) was calculated with the following formula: $P = \frac{(I_{VV} - I_{VH}G)}{(I_{VV} + I_{VH}G)}$, where G is the grating factor, assumed to be 1.

Results and discussion

Screening of arctic strains and genome sequencing of *Pseudomonas* sp. B14-6

To find out candidate arctic bacteria that could grow in both cold and warm conditions, we screened bacteria from an arctic soil sample collected from Midtre Lovénbreen. Based on their distinct growth phenotype on minimal medium, we obtained 42 different isolates (Table S1 and Table S2). These bacteria were subjected to 16S rRNA gene sequencing and classified into 14 genera accordingly. Then, we selected several bacteria that showed rapid growth in rich media and had similar 16S rRNA gene sequences, for an easier characterization. Finally, we selected the *Pseudomonas* sp. B14-6 strain, which showed high similarity to *Pseudomonas mandelii* JR-1 among published strains, the latter being a cold-adapted bacterium found in Korea and known to express an esterase [36]. *Pseudomonas* sp. B14-6 was found to contain proteases, as shown by plate assay (Table S3). Notably, *Pseudomonas* sp. B14-6 did not show high similarity to the *Pseudomonas* spp. (PAMC28618, PAMC28620, and PAMC28615) that we previously isolated from arctic soil [37, 38], but showed high similarity only to *P. mandelii* JR-1 and another new strain, *Pseudomonas* sp. B14-8 (Fig. 1a).

Next, we performed genomic sequencing of *Pseudomonas* sp. B14-6, which revealed a genome size of 6,766,122 bp (Fig. 1b and Table 1). The genome displayed a GC content of 59.14% and contained 6,326 predicted genes. Whole-genome sequencing data were deposited in GenBank under accession number CP053929 and in BioProject with the ID PRJNA634457. Despite the high 16S rRNA gene similarity, *Pseudomonas* sp. B14-6 had a smaller genome compared to that of *P. mandelii* JR-1, which shows a size of 7.1 Mb with 6374 coding genes [36]. Gene Ontology enrichment analysis based on biological processes indicated that genes were mostly related to metabolic processes (27.7%), cellular processes (21.3%), and single-organism processes (18.5%). These categories covered approximately 67.5% of the overall biological processes in which the genes were involved (Table 2). Furthermore, this strain could grow at both 10 and 25 °C and in various rich media, especially in terrific broth (Fig. 2).

Identification of two membrane fatty acid-modulating enzymes in the novel *Pseudomonas* strain

Bacteria that survive in arctic area possess survival strategies to adapt to the extreme temperature. To investigate the temperature-dependent behavior of the isolated *Pseudomonas* strain, changes in the composition of membrane lipids were observed [2]. To this purpose, PLFA analysis and total fatty acid analysis were applied [39]. As lipid analysis methods based on PLFA and total fatty acid analysis result in significant differences in the determination of fatty acid composition and extraction efficiency, and require different amounts of bacterial samples, since the two lipid analysis methods vary in derivatization procedures, especially in reaction temperature and pH [12], the two applied analytical methods showed totally different data. Total fatty acid extraction allowed highly sensitive detection of lipids from a small amount of samples, and could provide clear signal with relatively easy fatty acid methyl ester (FAME) derivatization, although it required high temperatures and an acidic pH for derivatization [11]. Unlike the total fatty acid extraction method, PLFA analysis has been widely used to discover and analyze biomarkers showing the composition of microbial communities in microbial and soil sciences [27]. In addition, this technique has been widely used for the estimation of the total bacterial biomass, and can indicate subtle and direct changes in the composition of cellular membranes [40, 41]. Indeed, although PLFA analysis needs relatively laborious processes, it can clearly show dynamic changes in the amount of important membrane phospholipids. Especially, to deal with degradable phospholipids, this technique requires an optimized derivatization method such as mild alkaline methanolysis. In fact, harsh reaction conditions, such as the high temperatures and acidic pH applied in total fatty acid analysis, lead to degradation of some fatty acids, such as CFAs, via cleavage of the cyclopropane ring by the acidic methanol used in the procedure [42]. Therefore, we applied PLFA analysis as a key tool to highlight the major bacterial markers showing important changes during shifts in temperature [11]. PLFA analysis revealed that the isolated strain produced more UFAs, such as C16:1ω9c, C16:1ω9t, and C18:1ω9t (palmitoleic acid, trans-palmitoleic acid, and elaidic acid), at 10 °C than at 25 °C (Fig. 3a). The ratio of UFAs among phospholipids increased from 26 to 81% when passing from 25 °C to 10 °C (Fig. 3b), and thus, the fluidity of the membrane was higher at 25 °C. Furthermore, when the temperature increased from 10 °C to 25 °C, the proportion of cyclic fatty acids (cyclo 17:0) increased from 0 to 25%. Cyclic PLFA forms are known to accumulate under stressful conditions, such as high salt concentrations and temperature changes, indicating that the new *Pseudomonas* strain seemed to recognize heat

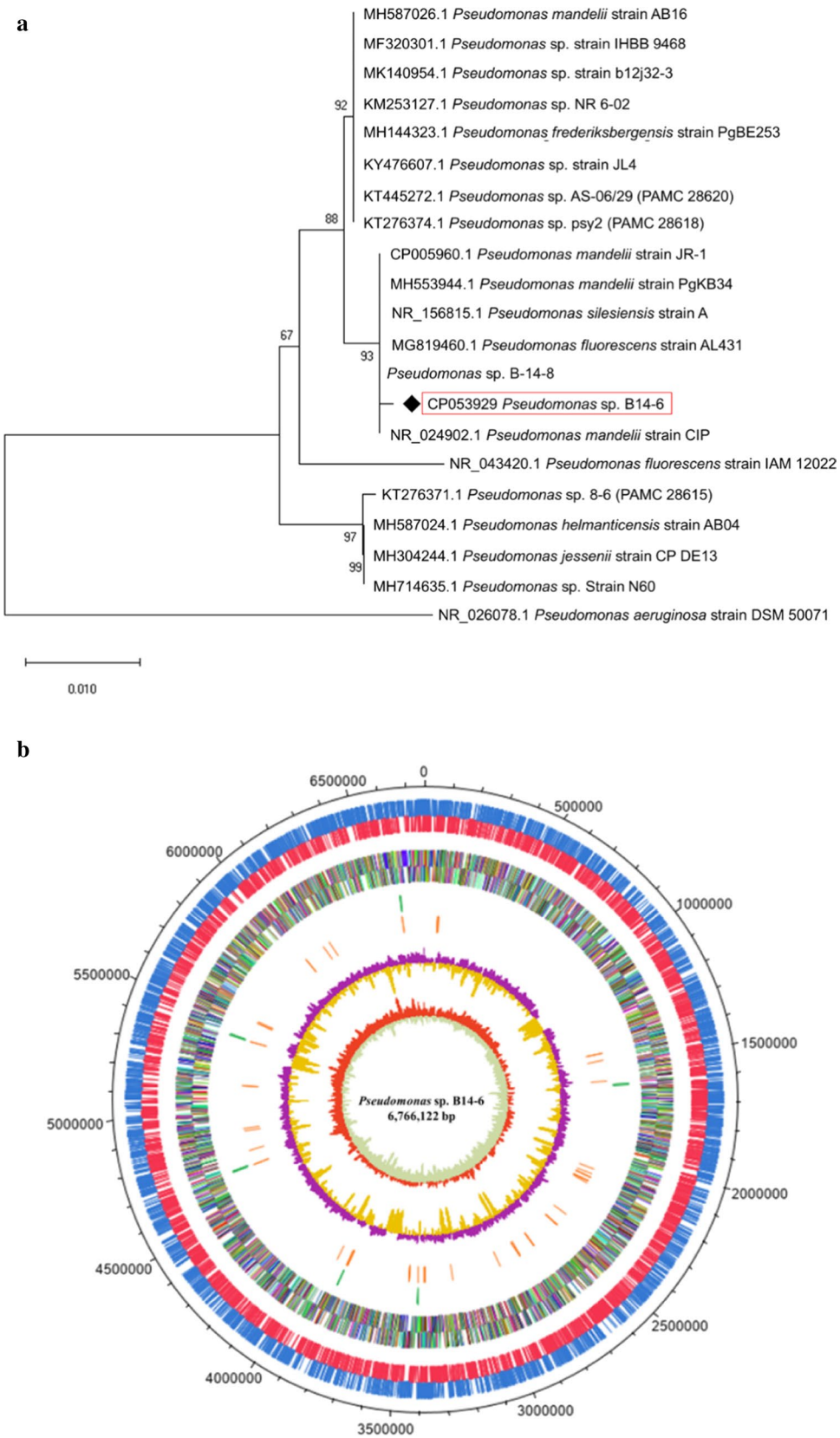

Fig. 1 Phylogenetic tree based on the 16S rRNA gene sequences (**a**) and genomic map (**b**) of *Pseudomonas* sp. B14-6

Table 1 Whole-genome sequencing data of *Pseudomonas* sp. B14-6

Attribute	Value
Genome size (bp)	6,766,122
GC content (%)	59.14
Number of rRNA genes	19
Number of tRNA genes	67
Number of genes	6,326
Cumulative gene length (bp)	5,831,520
Gene length/genome (%)	86.19

stress, despite showing relatively good growth at 25 °C [5, 9]. As expected, hydrophobicity was increased with increasing temperature (Fig. 3c), similarly to the trend observed in several other strains isolated from arctic soil (Fig S1). On the other hand, total lipid analysis indicated that the *Pseudomonas* sp. B14-6 strain produced more UFAs, namely C16:1ω9c, C16:1ω9t, and C18:1ω9t, at 25 °C than 10 °C (Fig. 3d). Interestingly, mesophilic *Pseudomonas* species such as *Pseudomonas stutzeri* and *Pseudomonas resinovorans* produced lower amounts of C16:1ω9 and C16:1ω7c at 37 °C than 25 °C, and thus differed from psychrophiles such as *Pseudomonas fluorescens* and *Pseudomonas putida* (Fig S2). Through PLFA analysis, we found that *Pseudomonas* sp. B14-6 displayed changes in membrane lipid composition in response to different temperatures, especially in the proportion of UFAs and cyclic fatty acids. We searched the newly sequenced *Pseudomonas* sp. B14-6 genome for genes related to these lipid species and found cyclopropane-fatty acid-acyl-phospholipid synthase (*cfa*) and Δ-9-fatty acid desaturase (*desA*) (Table S4). Alignment of Cfa and DesA amino acid sequences by BLAST showed high sequence similarity (up to 99%) to two correspondent enzymes of *P. mandelii* JR-1 (data not shown). To investigate the function of these enzymes, possibly related to changes in PLFA composition, we cloned both genes and introduced them in *E. coli* for overexpression (Table 3).

Effect of *desA* overexpression in *E. coli*

DesA is a desaturase mediating the synthesis of UFAs such as palmitoleic acid and oleic acid. In particular, DesA converts existing single-bonded fatty acyl chains to doubly bonded chains, especially palmitic acid [43]. Unlike DesA, DesB is an acyl-CoA Δ-9-fatty acid desaturase that can desaturate exogenous C16:0 and C18:0 fatty acids, thereby mainly producing C16:1ω9 and C18:1ω9. However, the *desB* gene was not clearly defined in the *Pseudomonas* sp. B14-6 genome, although this was found to contain another fatty

Table 2 Gene ontology enrichment analysis of *Pseudomonas* sp. B14-6 genes

Biological process	Number of genes	Number of genes (%)
Developmental process	24	0.23
Multicellular organismal process	2	0.02
Biological adhesion	11	0.10
Localization	798	7.54
Reproduction	4	0.04
Immune system process	2	0.02
Behavior	1	0.01
Response to stimulus	501	4.73
Biological regulation	744	7.03
Multi-organism process	9	0.09
Detoxification	2	0.02
Cellular process	2258	21.34
Single-organism process	1954	18.46
Metabolic process	2934	27.72
Regulation of biological process	723	6.83
Positive regulation of biological process	5	0.05
Negative regulation of biological process	20	0.19
Reproductive process	4	0.04
Cellular component organization or biogenesis	229	2.16
Signaling	303	2.86
Locomotion	55	0.52
	10,583	100.00

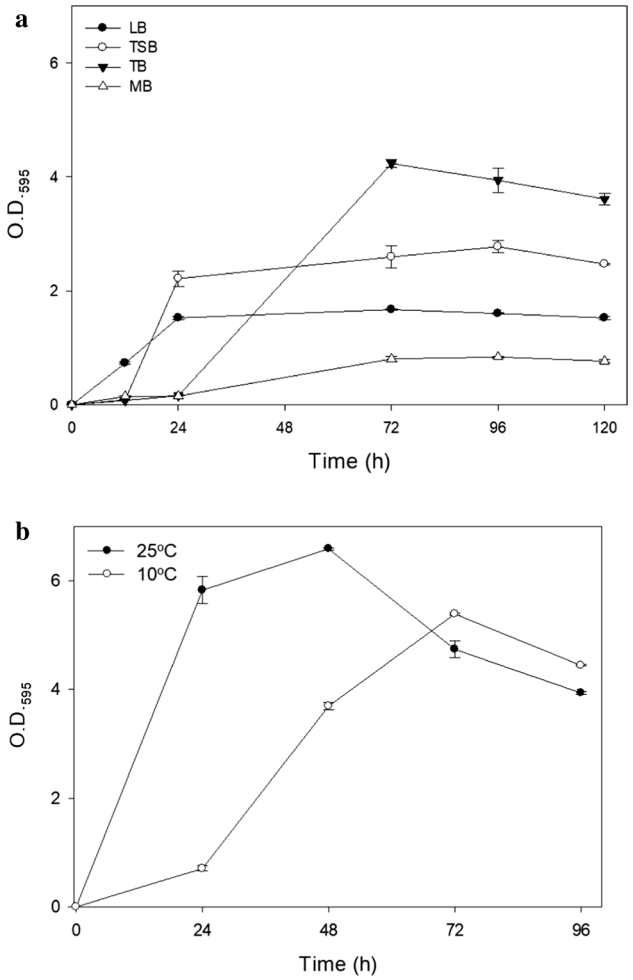

Fig. 2 Growth of *Pseudomonas* sp. B14-6 under different conditions. **a** Time-dependent growth of *Pseudomonas* sp. B14-6 in various media at 25 °C. **b** Temperature-dependent growth of *Pseudomonas* sp. B14-6 in Terrific broth

acid desaturase gene (Table S3) [26, 43]. Nevertheless, since we detected the *desA* gene in the genome of *Pseudomonas* sp. B14-6, we transferred it into *E. coli* using the pET24ma vector (DES100) and grew the cells to see the effect of *desA* overexpression on the membrane lipid composition. The overexpression of *desA* impaired the growth of *E. coli* at both 25 °C and 37 °C compared with the control strain BL21(DE3) containing empty pET24ma (BBL1) (Fig. 4a). Previously, UFAs, especially trans-form UFAs, were shown to counterbalance CFAs, which induced increased cell growth and slight changes in membrane fluidity [44]. In addition, it is generally acknowledged that UFAs inhibit bacterial growth through their incorporation into phospholipids, triggering membrane destruction due to the change in the UFA-to-CFA ratio [45, 46].

PLFA analysis indicated that low temperature led to increased levels of UFAs in both *desA*-overexpressing and control *E. coli* strains, while the overexpression of this desaturase further promoted the accumulation of UFAs such as C18:1ω9t and C16:1ω9c. Especially, at 25 °C, the content of C18:1ω9t increased from 14.86 to 39.18% whereas that of C16:1ω9c increased from 1.58 to 13.99% upon *desA* overexpression (Fig. 4b).

Although DesA has already been functionally characterized in *E. coli*, its effect was shown only by total lipid analysis and not PLFA analysis [19]. In addition, such previous functional study was performed with the addition of external substrates such as palmitic acid and stearic acid (C16:0 and C18:0 fatty acids), due to the difficulties of detecting significant changes in absence of external supplies. Conversely, in the current study, PLFA analysis could clearly show the function of DesA in vivo and its effect on PLFA composition. Indeed, we observed a clear increase in the peak associated with both C18:1ω9t and C16:1ω9c at both 25 °C and 37 °C (Fig. 5). Moreover, the overexpression of *desA* was associated with a decrease in hydrophobicity and an increase in fluidity of membranes, a result similar to that obtained with the addition of C18:0 and C16:0 substrates (Fig. 4c, d). Some reports showed that the *desA* gene is regulated by temperature changes in *Bacillus subtilis* [47]; however, there was no evidence of *desA* regulation by temperature in *Pseudomonas aeruginosa* [43]. Nevertheless, our results indicated that the activity of DesA in B14-6 is affected by temperature, similar to *B. subtilis*, although evidence at the molecular level is still lacking. Considering that the main function of total lipid is energy storage, while PLFAs are specifically cell membrane components, DesA might mediate changes in lipid composition associated with both storage molecules and structural membrane molecules, possibly critical under different stress conditions such as extreme temperatures.

Effect of *cfa* overexpression in *E. coli*

We detected CFAs such as cyclo 17:0 in *Pseudomonas* sp. B14-6 when we increased the growth temperature to 25 °C. To determine the CFA synthesis pathway in *Pseudomonas* sp. B14-6, we searched for the *cfa* gene in its genome, and overexpressed it in *E. coli* (CFA300). Next, we compared the effect of this *cfa* gene with that of *cfa* from *Halomonas* (CFA101), which was previously reported by our group [48], and *cfa* from *E. coli* (CFA200), both overexpressed in *E. coli*. Since we previously showed changes in PLFA composition upon *cfa* overexpression, we expected changes in the proportion of PLFAs in *E. coli* strains grown at different temperatures and overexpressing *cfa* genes from different sources, with consequent changes in growth; however, the various *cfa* genes did not exert different effects.

Nevertheless, the growth of *cfa*-overexpressing strains was increased at 25 °C and 37 °C compared with the empty-vector

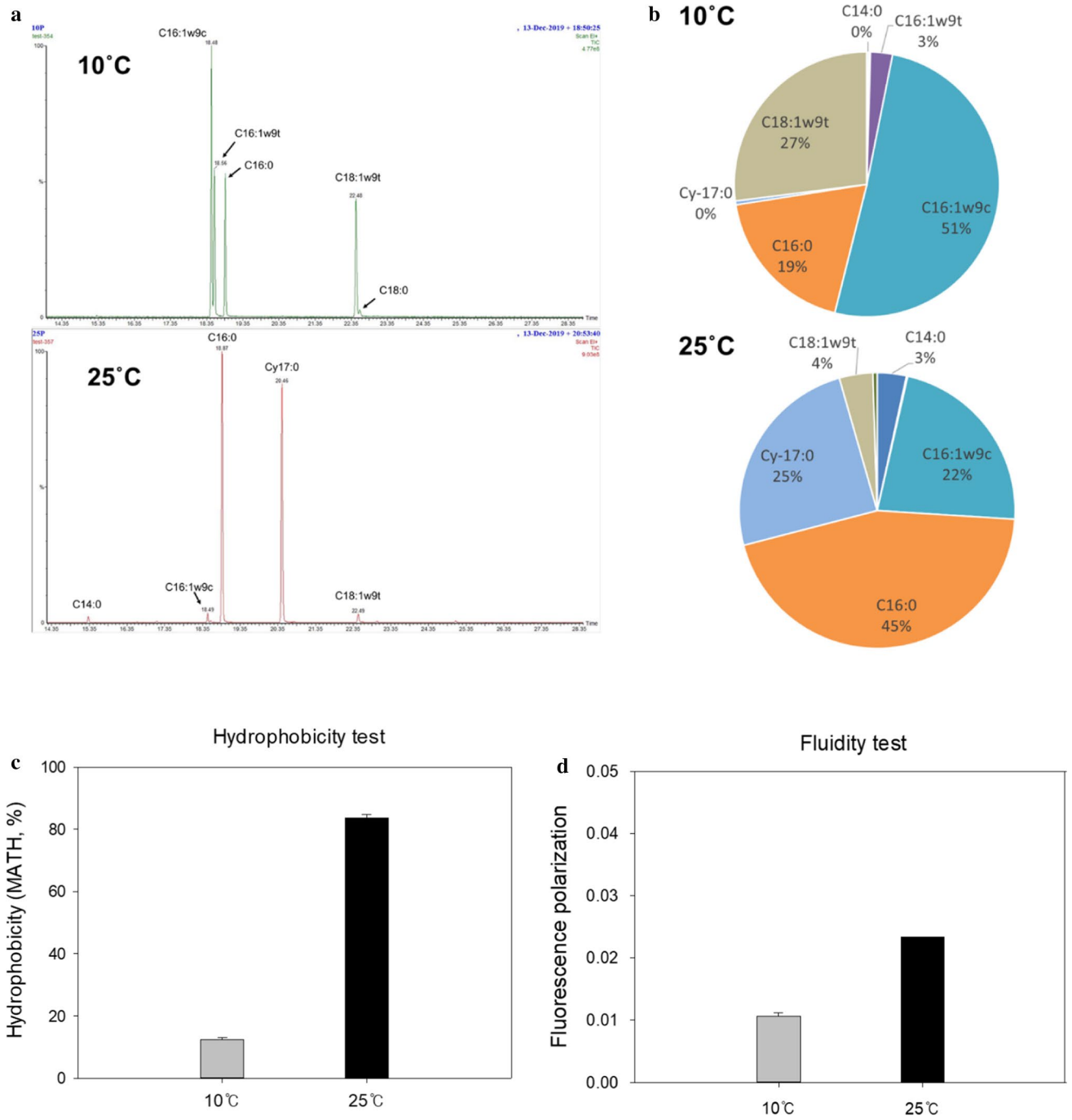

Fig. 3 PLFA analysis and characterization of membrane properties of *Pseudomonas* sp. B14-6. **a** GC chromatogram of PLFA and **b** phospholipid composition, and comparison of **c** membrane hydrophobicity and **d** membrane fluidity of *Pseudomonas* sp. B14-6 grown at 10 °C and 25 °C

control strain (BBL1). In particular, at 37 °C, both *Halomonas cfa* and *Pseudomonas cfa*-expressing cells (CFA101 and CFA300) showed significant better growth than BBL1 (Fig. 6a, b). Although CFA formation is energetically expensive, requiring three ATP molecules per cyclopropane ring, the presence of CFAs is generally assumed to enhance the chemical and physical stability of membranes. Specifically, CFAs have been hypothesized to improve resistance to superoxide, singlet oxygen, ozonolysis, and oxidative stress. It has also been suggested that CFAs may reduce the fluidity of membranes, thereby limiting their permeability to undesirable compounds ranging from a single protons (H^+) to butan-1-ol

Table 3 Strains, plasmids, and primers used in this study

Strain or plasmid	Description	Reference or source
Pseudomonas strains		
Pseudomonas sp. B14-6	Gram-negative, rod-shaped	This study
Pseudomonas stutzeri	Gram-negative, rod-shaped, KCTC1066	Korean Collection for Type Cultures (KCTC)
Pseudomonas resinovorans	Gram-negative, rod-shaped, KCTC12498	KCTC
Pseudomonas fluorescence	Gram-negative, rod-shaped, KCTC42821	KCTC
Pseudomonas putida	Gram-negative, rod-shaped, KCTC1751	KCTC
E. coli strains		
DH5α	F^- φ80*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ^-	Invitrogen
BL21(DE3)	F^- *omp*T *hsd*SB(*rB*$^-$, *mB*$^-$) *gal dcm*	Invitrogen
BBL1	*E. coli* BL21(DE3) harboring pET24ma	This study
CFA101	*E. coli* BL21(DE3) harboring pCFA100	[48]
CFA200	*E. coli* BL21(DE3) harboring pCFA200	[48]
CFA300	*E. coli* BL21(DE3) harboring pCFA300	This study
DES100	*E. coli* BL21(DE3) harboring pDES100	This study
Plasmids		
pET24ma	P15A ori *lac*I T7 promoter, kan^R	[51]
pCFA100	pET24ma carrying the PCR product of *cfa* amplification from *Halomonas socia*	[48]
pCFA200	pET24ma carrying the PCR product of *cfa* amplification from *E. coli*	[48]
pCFA300	pET24ma carrying the PCR product of *cfa* amplification from *Pseudomonas* sp. B14-6	This study
pDES100	pET24ma carrying the PCR product of *desA* amplification from *Pseudomonas* sp. B14-6	This study
Primers		
cfa F	5′-GCTGCTGGATCCATGCTCGCGCAACTTCCA-3′/	This study
cfa R	5′-GCTGCTTTCGAACTAAAGGTAGATGTCGTCTCGCGT-3′	This study
desA F	5′-GCTGCTGGATCCATGTGGTACGAAGGTTTTCTCGGCTTGTCG-3′/	This study
desA R	5′-GCTGCTCTCGAGTCAGGCAGCGGCAGGGCG-3′	This study

and possibly even some antibiotics [49, 50]. Based on these reports, *cfa*-overexpressing strains were expected to grow better than the control strain.

Notably, the Cfas introduced in *E. coli* did not show high amino acid sequence similarity among them (Fig. 6c), and *cfa* overexpression resulted in a somewhat promoted growth despite the presence of endogenous *cfa* in *E coli*. Although the *cfa* gene from *Pseudomonas* exerted a reduced effect on *E. coli* growth than *cfa* from *Halomonas*, the *Pseudomonas cfa* could be used to improve resistance and for other purposes, as Cfa exerts different effects on different molecules.

Conclusion

Here, we isolated a new bacterial strain, *Pseudomonas* sp. B14-6, from arctic soil, and found that this strain displayed changes in membrane lipid composition in response to different temperatures. Whole-genome sequencing revealed some genetic characteristics of *Pseudomonas* sp. B14-6 and unraveled two genes related to the membrane lipid composition. Among these, *desA* is related to the synthesis of UFAs such as palmitoleic acid and oleic acid. Indeed, overexpression of *desA* in *E. coli* resulted in a higher content of palmitoleic and oleic acids, as indicated by PLFA

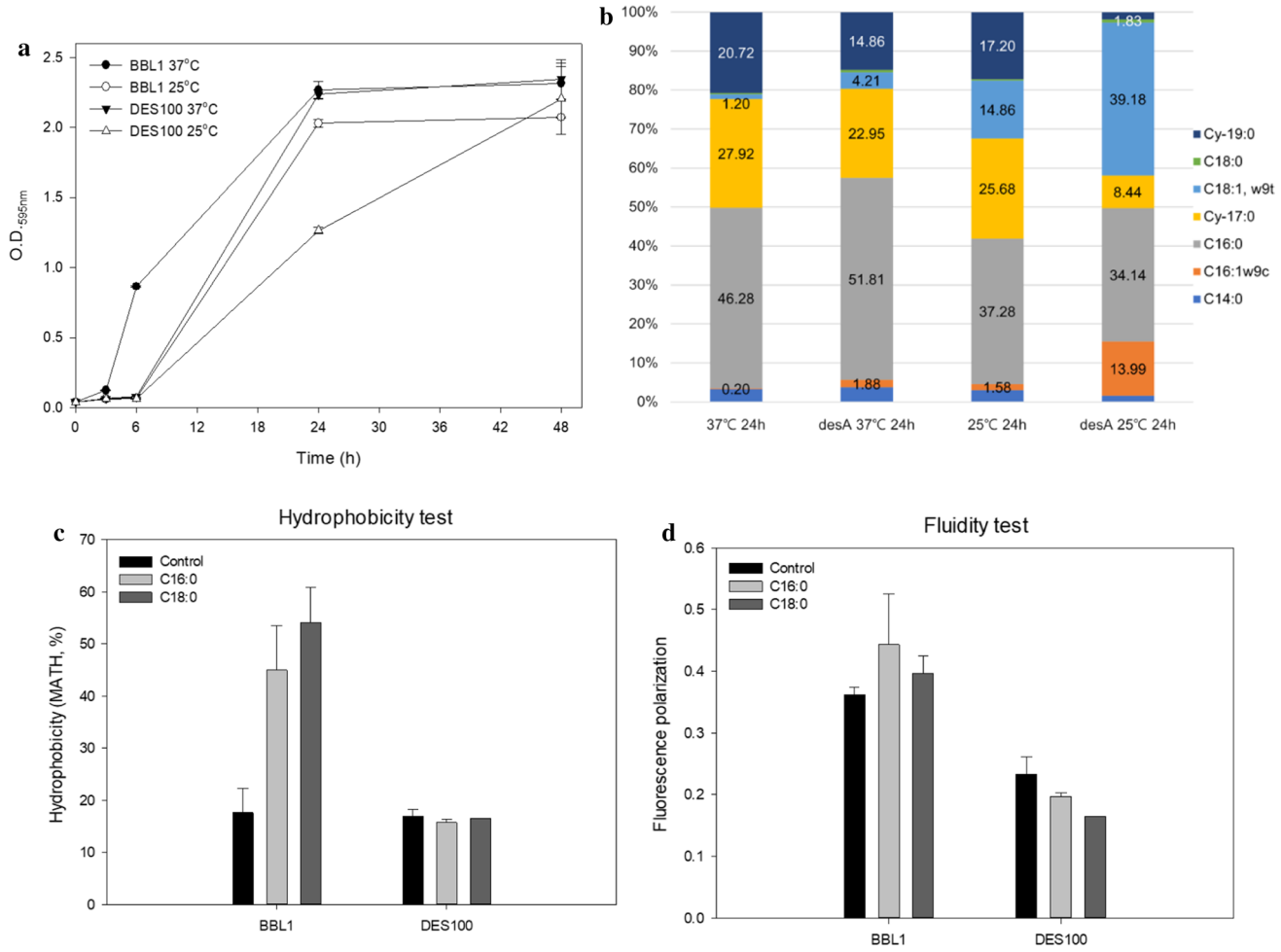

Fig. 4 Effects of *desA* overexpression in *E. coli*. **a** Growth of *desA*-overexpressing *E. coli* at 25 °C and 37 °C. **b** Changes in PLFA composition in response to temperature shift and *desA* overexpression. **c** Changes in membrane hydrophobicity and **d** membrane fluidity upon *desA* overexpression and supplementation with 1 mM stearic acid (C16:0) and palmitic acid (C18:0)

analysis, and different growth patterns at 25 °C. The second gene, *cfa*, is involved in CFA synthesis, especially at relatively high temperatures. Furthermore, the expression of *cfa* could affect cell resistance to stress conditions such as temperature changes.

As both Cfa and DesA triggered changes in membrane lipid composition in terms of phospholipid content, hydrophobicity, and fluidity, which led to changes in cell growth and resistance to environmental conditions, fine-tuning of the expression of these two genes may be critical to improve growth or production of metabolites upon culturing of bacterial cells under stressful or adverse conditions.

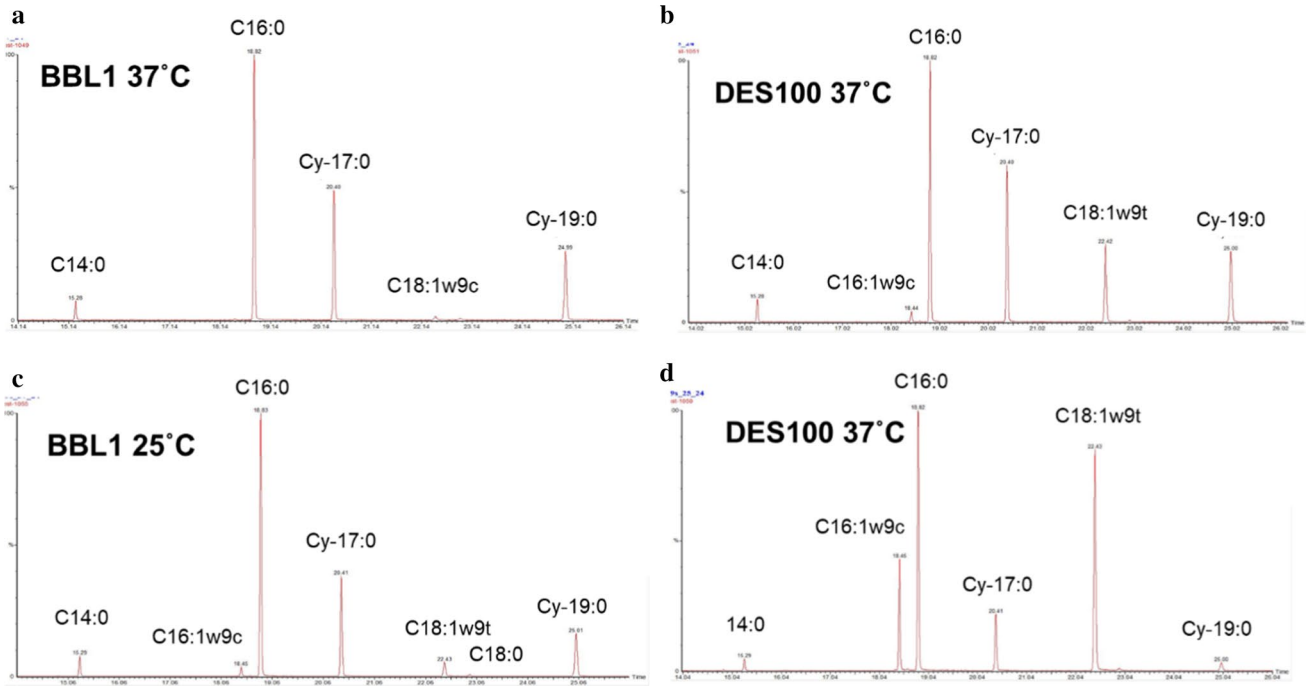

Fig. 5 PLFA chromatogram of *desA*-overexpressing *E. coli* at different temperatures. PLFA GC–MS chromatogram of *E. coli* with (**a**, **c**) or without (**b**, **d**) *desA* overexpression at (**a**, **b**) 37 °C and (**c**, **d**) 25 °C

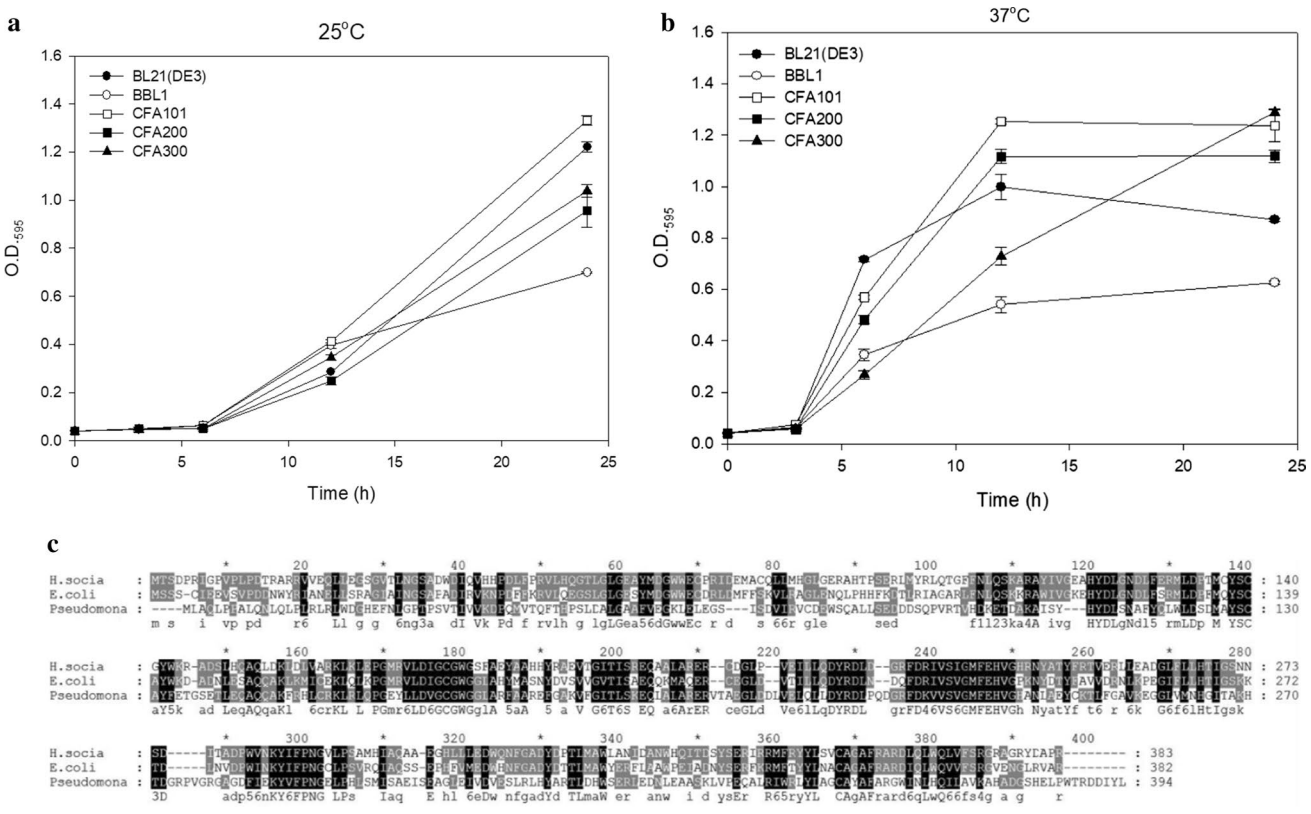

Fig. 6 Comparison of the effect of *cfa* genes from different bacterial species. Growth of *E. coli* strains transformed with *cfa* genes from various sources at **a** 25 °C and **b** 37 °C. **c** Sequence alignment of the Cfa proteins from three different species

Author contributions T-RC, YKL, and Y-HY contributed to the study conception and design. Material preparation, data collection, and analysis were performed by T-RC, Y-LP, H-SS, H-JK, and CS. Genetic works were performed by SML, SLP, and HSL. The first draft of the manuscript was written by T-RC and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding This study was supported by the National Research Foundation of Korea (NRF) (NRF-2019R1F1A1058805 and NRF-2019M3E6A1103979), and the Research Program to solve the social issues of the NRF funded by the Ministry of Science and ICT (2017M3A9E4077234). This work was also supported by the Polar Academic Program (PAP, PE20900).

Data availability The dataset generated during the current study is available in the GenBank repository under accession number CP053929 and in the BioProject repository under the ID PRJNA634457. Other datasets are available from the corresponding author on reasonable request.

Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

1. Harding T, Jungblut AD, Lovejoy C, Vincent WF (2011) Microbes in high arctic snow and implications for the cold biosphere. Appl Environ Microbiol 77:3234–3243
2. Zhang Y-M, Rock CO (2008) Membrane lipid homeostasis in bacteria. Nat Rev Microbiol 6:222
3. Yumoto I, Hirota K, Iwata H, Akutsu M, Kusumoto K, Morita N, Ezura Y, Okuyama H, Matsuyama H (2004) Temperature and nutrient availability control growth rate and fatty acid composition of facultatively psychrophilic Cobetia marina strain L-2. Arch Microbiol 181:345–351
4. Guillot A, Obis D, Mistou M-Y (2000) Fatty acid membrane composition and activation of glycine-betaine transport in Lactococcus lactis subjected to osmotic stress. Int J Food Microbiol 55:47–51
5. Chen YY, Gänzle MG (2016) Influence of cyclopropane fatty acids on heat, high pressure, acid and oxidative resistance in *Escherichia coli*. Int J Food Microbiol 222:16–22
6. Pini CV, Bernal P, Godoy P, Ramos JL, Segura A (2009) Cyclopropane fatty acids are involved in organic solvent tolerance but not in acid stress resistance in *Pseudomonas* putida DOT-T1E. Microb Biotechnol 2:253–261
7. Lee HJ, Rho JK, Yoon SC (2004) Growth temperature-dependent conversion of de novo-synthesized unsaturated fatty acids into polyhydroxyalkanoic acid and membrane cyclopropane fatty acids in the psychrotrophic bacterium *Pseudomonas* fluorescens BM07. J Microbio Biotechnol 14:1217–1226
8. Annous BA, Kozempel MF, Kurantz MJ (1999) Changes in membrane fatty acid composition of *Pediococcus* sp. Strain NRRL B-2354 in response to growth conditions and its effect on thermal resistance. Appl Environ Microbiol 65:2857–2862
9. Chang YY, Cronan JE (1999) Membrane cyclopropane fatty acid content is a major factor in acid resistance of *Escherichia coli*. Mol Microbiol 33:249–259
10. Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911–917
11. Drenovsky RE, Elliott GN, Graham KJ, Scow KM (2004) Comparison of phospholipid fatty acid (PLFA) and total soil fatty acid methyl esters (TSFAME) for characterizing soil microbial communities. Soil Biol Biochem 36:1793–1800
12. Fernandes MF, Saxena J, Dick RP (2013) Comparison of whole-cell fatty acid (MIDI) or phospholipid fatty acid (PLFA) extractants as biomarkers to profile soil microbial communities. Microb Ecol 66:145–157
13. Schweizer HP (2004) Fatty acid biosynthesis and biologically significant acyl transfer reactions in Pseudomonads, *Pseudomonas*. Springer, Berlin
14. Muñoz-Rojas J, Bernal P, Duque E, Godoy P, Segura A, Ramos J-L (2006) Involvement of cyclopropane fatty acids in the response of *Pseudomonas* putida KT2440 to freeze-drying. Appl Environ Microbiol 72:472–477
15. Garba L, Ali MSM, Oslan SN, Rahman RNZRA (2016) Heterologous expression of PA8FAD9 and functional characterization of a Δ9-fatty acid desaturase from a cold-tolerant *Pseudomonas* sp. A8. Mol Biotechnol 58:718–728
16. Grogan D, Cronan JE (1984) Cloning and manipulation of the *Escherichia coli* cyclopropane fatty acid synthase gene: physiological aspects of enzyme overproduction. J Bacteriol 158:286–295
17. Cronan JE, Reed R, Taylor F, Jackson M (1979) Properties and biosynthesis of cyclopropane fatty acids in *Escherichia coli*. J Bacteriol 138:118–121
18. Broadbent J, Oberg T, Hughes J, Ward R, Brighton C, Welker D, Steele J (2014) Influence of polysorbate 80 and cyclopropane fatty acid synthase activity on lactic acid production by Lactobacillus casei ATCC 334 at low pH. J Ind Microbiol Biotechnol 41:545–553
19. Heredia RM, Lucchesi GI (2019) Pseudomonas putida Δ9-fatty acid desaturase: gene cloning, expression, and function in the cationic surfactants stress. J Basic Microbiol 59:525–534
20. Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018) MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol 35:1547–1549
21. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425
22. Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791
23. Tamura K, Nei M, Kumar S (2004) Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc Natl Aca Sci 101:11030–11035
24. Dahal RH, Chaudhary DK, Kim D-U, Kim J (2020) Nine novel psychrotolerant species of the genus *Pedobacter* isolated from Arctic soil with potential antioxidant activities. Int J Syst Evo Microbiol 70:2537–2553
25. Kim J, Seo H-M, Bhatia SK, Song H-S, Kim J-H, Jeon J-M, Choi K-Y, Kim W, Yoon J-J, Kim Y-G (2017) Production of itaconate by whole-cell bioconversion of citrate mediated by expression of multiple cis-aconitate decarboxylase (cadA) genes in *Escherichia coli*. Sci Rep 7:1–9
26. Lawal Garba MSMA, Oslan SN, Rahman RNZRA (2016) Molecular cloning and functional expression of a Δ9-fatty acid desaturase from an antarctic *Pseudomonas* sp. A3. PLoS ONE 11(8):e0160681
27. Buyer JS, Sasser M (2012) High throughput phospholipid fatty acid analysis of soils. Appl Soil Ecol 61:127–130

28. Vorbeck ML, Marinetti G (1965) Separation of glycosyl diglycerides from phosphatides using silicic acid column chromatography. J Lipid Res 6:3–6
29. White D, Davis W, Nickels J, King J, Bobbie R (1979) Determination of the sedimentary microbial biomass by extractible lipid phosphate. Oecologia 40:51–62
30. Bhatia SK, Lee B-R, Sathiyanarayanan G, Song H-S, Kim J, Jeon J-M, Kim J-H, Park S-H, Yu J-H, Park K (2016) Medium engineering for enhanced production of undecylprodigiosin antibiotic in Streptomyces coelicolor using oil palm biomass hydrolysate as a carbon source. Bioresour Technol 217:141–149
31. Bhatia SK, Kim J, Song H-S, Kim HJ, Jeon J-M, Sathiyanarayanan G, Yoon J-J, Park K, Kim Y-G, Yang Y-H (2017) Microbial biodiesel production from oil palm biomass hydrolysate using marine *Rhodococcus* sp. YHY01. Bioresour Technol 233:99–109
32. Bhatia SK, Gurav R, Choi T-R, Han YH, Park Y-L, Park JY, Jung H-R, Yang S-Y, Song H-S, Kim S-H (2019) Bioconversion of barley straw lignin into biodiesel using *Rhodococcus* sp. YHY01. Bioresour Technol 289:121704
33. Aono R, Kobayashi H (1997) Cell surface properties of organic solvent-tolerant mutants of *Escherichia coli* K-12. Appl Environ Microbiol 63:3637–3642
34. Mykytczuk N, Trevors J, Leduc L, Ferroni G (2007) Fluorescence polarization in studies of bacterial cytoplasmic membrane fluidity under environmental stress. Prog Biophys Mol Biol 95:60–82
35. Royce LA, Liu P, Stebbins MJ, Hanson BC, Jarboe LR (2013) The damaging effects of short chain fatty acids on *Escherichia coli* membranes. Appl Microbiol Biotechnol 97:8317–8327
36. Jang S-H, Kim J, Kim J, Hong S, Lee C (2012) Genome sequence of cold-adapted *Pseudomonas* mandelii strain JR-1. Am Soc Microbiol 1:3263–3263
37. Park H-G, Sathiyanarayanan G, Hwang C-H, Ann D-H, Kim J-H, Bang G, Jang K-S, Ryu HW, Lee YK, Yang Y-H (2017) Chemical structure of the lipid A component of *Pseudomonas* sp. strain PAMC 28618 from thawing permafrost in relation to pathogenicity. Sci Rep 7:1–12
38. Sathiyanarayanan G, Bhatia SK, Kim HJ, Kim J-H, Jeon J-M, Kim Y-G, Park S-H, Lee SH, Lee YK, Yang Y-H (2016) Metal removal and reduction potential of an exopolysaccharide produced by Arctic psychrotrophic bacterium *Pseudomonas* sp. PAMC 28620. RSC Adv 6:96870–96881
39. Vestal JR, White DC (1989) Lipid analysis in microbial ecology. Bioscience 39:535–541
40. Ling Y, Li W, Tong T, Li Z, Li Q, Bai Z, Wang G, Chen J, Wang Y (2020) Assessing the microbial communities in four different daqus by using PCR-DGGE, PLFA, and biolog analyses. Pol J Microbiol 69:27
41. Chen H, Zhao X, Lin Q, Li G, Kong W (2019) Using a combination of PLFA and DNA-based sequencing analyses to detect shifts in the soil microbial community composition after a simulated spring precipitation in a semi-arid grassland in China. Sci Total Environ 657:1237–1245
42. Vulliet P, Markey S, Tornabene TG (1974) Identification of methoxyester artifacts produced by methanolic-HCl solvolysis of the cyclopropane fatty acids of the genus Yersinia. Biochim Biophys Acta Lipids Lipid Metab 348:299–301
43. Zhu K, Choi KH, Schweizer HP, Rock CO, Zhang YM (2006) Two aerobic pathways for the formation of unsaturated fatty acids in *Pseudomonas* aeruginosa. Mol Microbiol 60:260–273
44. Loffhagen N, Härtig C, Geyer W, Voyevoda M, Harms H (2007) Competition between cis, trans and cyclopropane fatty acid formation and its impact on membrane fluidity. Eng Life Sci 7:67–74
45. Jiang X, Duan Y, Zhou B, Guo Q, Wang H, Hang X, Zeng L, Jia J, Bi H (2019) The cyclopropane fatty acid synthase mediates antibiotic resistance and gastric colonization of *Helicobacter pylori*. J Bacteriol 201:e00374-e1319
46. Khulusi S, Ahmed H, Patel P, Mendall M, Northfield T (1995) The effects of unsaturated fatty acids on *Helicobacter pylori* in vitro. J Med Microbiol 42:276–282
47. Aguilar PS, Hernandez-Arriaga AM, Cybulski LE, Erazo AC, de Mendoza D (2001) Molecular basis of thermosensing: a two-component signal transduction thermometer in *Bacillus subtilis*. EMBO J 20:1681–1691
48. Choi T-R, Song H-S, Han Y-H, Park Y-L, Park JY, Yang S-Y, Bhatia SK, Gurav R, Kim HJ, Lee YK (2020) Enhanced tolerance to inhibitors of Escherichia coli by heterologous expression of cyclopropane-fatty acid-acyl-phospholipid synthase (cfa) from Halomonas socia. Bioproc Biosyst Eng 27:1–10
49. Shabala L, Ross T (2008) Cyclopropane fatty acids improve *Escherichia coli* survival in acidified minimal media by reducing membrane permeability to H+ and enhanced ability to extrude H+. Res Microbiol 159:458–461
50. Poger D, Mark AE (2015) A ring to rule them all: the effect of cyclopropane fatty acids on the fluidity of lipid bilayers. J Phys Chem B 119:5487–5495
51. Moon Y-M, Gurav R, Kim J, Hong Y-G, Bhatia SK, Jung H-R, Hong J-W, Choi TR, Yang SY, Park HY (2018) Whole-cell immobilization of engineered *Escherichia coli* JY001 with barium-alginate for itaconic acid production. Biotechnol Bioproc Eng 23:442–447