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Original Article

Characterization of 1-acyl-*sn*-glycerol-3-phosphate acyltransferase from a polyunsaturated fatty acid-producing bacterium, *Shewanella livingstonensis* Ac10

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Abbreviations:

acyl-ACP,	acyl-acyl carrier protein
acyl-CoA,	acyl-coenzyme A
EPA,	eicosapentaenoic acid
ESI-MS,	electrospray ionization-mass spectrometry
LPA,	1-acyl-*sn*-glycerol-3-phosphate
PA,	phosphatidic acid
PE,	phosphatidylethanolamine
PG,	phosphatidylglycerol
PlsC,	1-acyl-*sn*-glycerol-3-phosphate acyltransferase
TLC,	thin-layer chromatography

Summary

Shewanella livingstonensis Ac10, a psychrotrophic bacterium, produces the omega-3 polyunsaturated fatty acid eicosapentaenoic acid (EPA), as a fatty acyl chain of phospholipids at low temperatures. EPA is incorporated into the *sn*-2 position of phospholipids. 1-Acyl-*sn*-glycerol-3-phosphate acyltransferase (PlsC) catalyzes the acylation at the *sn*-2 position of 1-acyl-*sn*-glycerol-3-phosphate to form phosphatidic acid (PA). We found that 5 genes code for proteins homologous to *Escherichia coli* PlsC (named PlsC1 through PlsC5), suggesting that these PlsCs are involved in the synthesis of EPA-containing phospholipids. To examine the role of these putative PlsCs, we constructed the knockout mutants of each *plsC* gene (Δ*plsC*1 to Δ*plsC*5). In the mutant Δ*plsC*1, the amount of phospholipids containing EPA was less. Functional expression studies in a temperature-sensitive mutant of PlsC, *E. coli* JC201, showed that PlsC1 has a PlsC activity with a broad acyl-coenzyme A (acyl-CoA) specificity including EPA-CoA. These results indicate that PlsC1 is a key enzyme in the synthesis of EPA-containing PA in *S. livingstonensis* Ac10.

Introduction

Bacterial membranes consist of proteins and a phospholipid bilayer, and most membrane phospholipids are glycerolipids containing 2 fatty acyl chains. These phospholipid acyl chains play an important role in survival functions, such as adaptation to temperature[1, 2], osmotic pressure, solvent[3] and salinity[4] changes *in vivo* or in the passive permeability of hydrophobic molecules, active solute transport, and lipid protein interaction[5]. The adjustment in the fatty acid composition that maintains the biophysical properties of membranes is interpreted as an adaptation mechanism, which modifies the permeability and fluidity of the phospholipid bilayer to minimize energy expenditure, thus resulting in optimization of their growth. In addition to these biophysical properties of the membrane, some phospholipids containing specific acyl chain, such as the platelet activation factor and lung surfactant, can contribute to peculiar bioactivity in eukaryotes. However, in prokaryotes, such bioactive lipids have been scarcely reported except that the phosphatidylethanolamine (PE) containing specific acyl chains can act as an extracellular signaling molecule in *Myxococcus xanthus* [6] and *Pseudomonas aeruginosa*[7].

Eicosapentaenoic acid (EPA; 20:5) is an omega-3 polyunsaturated fatty acid, which is regarded as a vital nutrient for human health. Omega-3 polyunsaturated fatty acids, EPA and docosahexaenoic acid (22:6), have been shown to play beneficial roles in infant development, cancer, cardiovascular diseases, and mental illnesses[8].

Shewanella livingstonensis Ac10 has been used as a model organism for the investigation of microbial cold-adaptation mechanisms. *S. livingstonensis* Ac10 is a psychrotrophic bacterium isolated from Antarctic seawater, which produces the EPA as a fatty acyl chain of phospholipids at low temperatures. Previous studies have shown that the EPA-less mutant of this strain exhibits cold-sensitive phenotypes, which include growth retardation, defect in cell

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division, and development of intracellular membrane at 4°C, suggesting that EPA plays a role in membrane organization and cell division at low temperatures[9, 10]. In addition, a recently published study that used chemically synthesized fluorescent probes revealed that phospholipids containing an eicosapentaenyl group form a membrane microdomain at the cell division site of this strain, suggesting that EPA-containing phospholipids promote cell division[11]. However, despite the significance of phospholipids containing EPA in the cold adaptation of this strain, their biosynthesis mechanism is not well defined.

Phospholipid biosynthesis occurs primarily by *de novo* synthesis using glycerol-3-phosphate as an acyl acceptor and fatty acyl-acyl carrier protein (acyl-ACP) or fatty acyl-coenzyme A (acyl-CoA) as an acyl donor[12]. The reaction catalyzed by the first acyltransferase, encoded by the *plsB* gene in *Escherichia coli*, results in the transfer of an acyl group to the *sn*-1 position of glycerol-3-phosphate to form 1-acyl-*sn*-glycerol-3-phosphate (LPA)[13-15]. Subsequently, the second acyltransferase, encoded by *plsC*, transfers an acyl group to the *sn*-2 position of LPA to synthesize phosphatidic acid (PA)[16]. PA is then converted to PE, phosphatidylglycerol (PG), and cardiolipin. In *E. coli*, saturated fatty acids are mainly incorporated at the *sn*-1 position of phospholipids, and unsaturated fatty acyl chains at the *sn*-2 position[12]. EPA is exclusively restricted to the *sn*-2 position of phospholipids in *S. livingstonensis* Ac10, suggesting that the second acyltransferase, PlsC, is responsible for incorporation of EPA in *de novo* synthesis. In this study, 5 putative PlsCs, namely, PlsC1 through PlsC5, were identified in *S. livingstonensis* Ac10 and characterized. We found that PlsC1 is the most responsible for the synthesis of phospholipids containing EPA.

Materials and Methods

Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. A psychrotrophic bacterium, *S. livingstonensis* Ac10, was isolated from Antarctic seawater. Its rifampin-resistant mutant was used as the parental strain for gene disruption[9]. *S. livingstonensis* Ac10 and its derivatives were grown in Luria–Bertani (LB) media at 4°C, supplemented with rifampin (50 μg/ml) and kanamycin (40 μg/ml) as appropriate. *E. coli* S17-1/λ_{pir}[17] used as a donor cell of knockout vectors was grown in the same medium at 37°C. The acyltransferase complementation and activity assay were performed with the *E. coli* JC201 mutant strain[16], which is temperature-conditional in endogenous PlsC activity and able to grow at 30°C but not at 42°C. Bacteria carrying the plasmids were grown on an LB medium containing chloramphenicol (34 μg/ml) at 30°C.

Table 1 Strains and plasmids used in this study

Strain or plasmid	Description	Reference
Strains		
Escherichia coli		
S17-1/λ_{pir}	S17-1 derivative, host for pir-dependent plasmids	16
BL21	Source of *E. coli plsC* gene	
JC201	*plsC*(Ts) mutant	15
Shewanella livingstonensis Ac10		
Ac10-Rif^r	Parent strain, rifampin-resistant mutant of Ac10	8
Δ*plsC*1	Rif^r *plsC*1::pKNOCK-Km^r	This work
Δ*plsC*2	Rif^r *plsC*2::pKNOCK-Km^r	This work
Δ*plsC*3	Rif^r *plsC*3::pKNOCK-Km^r	This work
Δ*plsC*4	Rif^r *plsC*4::pKNOCK-Km^r	This work
Δ*plsC*5	Rif^r *plsC*5::pKNOCK-Km^r	This work
Plasmids		
pKNOCK-Km^r	RP4 oriT and R6K γ-ori; Km^r	16, 17
pSTVplsC1	pSTV28 harboring *plsC*1	This work
pSTVplsC1-His	pSTV28 harboring *plsC*1-hexa-His	This work
pSTVplsC2-His	pSTV28 harboring *plsC*2-hexa-His	This work
pSTVplsC3-His	pSTV28 harboring *plsC*3-hexa-His	This work
pSTVplsC4-His	pSTV28 harboring *plsC*4-hexa-His	This work
pSTVplsC5-His	pSTV28 harboring *plsC*5-hexa-His	This work
pSTVeplsC-His	pSTV28 harboring *E. coli plsC*-hexa-His	This work

Table 2 Primers used in this study

Primer	Sequence (5'→ 3')	Target gene
Used for gene disruption		
plsC1F	TGCACTGCAGTAAGCCAGTATCTTTGGTT	*plsC*1
plsC1R	CGCGGATCCTTTGACCAAATAGCGGCA	
plsC2F	CCGGAATTCTGTTCAGTTCACTTTGCTTAG	*plsC*2
plsC2R	AAACTGCAGCAGTTTATACAGTGGATCTTG	
plsC3F	CGGAATTCTACTGACGCTATTGTGCAAA	*plsC*3
plsC3R	AAACTGCAGGCCGCGAATAAACGGATTAC	
plsC4F	CGGAATTCGTTTATTATCAGCCTGTCGC	*plsC*4
plsC4R	AAACTGCAGCCAGCAACCTAGCCCCATTA	
plsC5F	CGGAATTCCGAAAATCAAAGGTTCAATCGC	*plsC*5
plsC5R	AAACTGCAGGGAAAGTCTAATGCCCACCA	
Used for expression in *E. coli*		
pETeplsCF	GGAATTCTATGCTATATATCTTTCGTC	*E. coli plsC*
pETeplsCR	CCGCTCGAGAACTTTTCCGGCGGCTTC	
pETplsC1F	GGAATTCCATATG CTGCTTATCGCTCGTTC	*plsC*1
pETplsC1R	CCGCTCGAGTGTTCGAGCTTTCAAGG	
pETplsC2F	GGAATTCCATATGTTCAGTTCACTTTGC	*plsC*2
pETplsC2R	CCGCTCGAGTCTCTGCTGATAATCAGG	
pETplsC3F	GGAATTCCATATGCCTAATCAACACC	*plsC*3
pETplsC3R	CCGCTCGAGATGTGATTTGATAGGC	
pETplsC4F	GGAATTCCATATGCTGGCTTTTTTACCTGG	*plsC*4
pETplsC4R	CCGCTCGAGTTGCTGTTGGTAAGC	
pETplsC5F	GGAATTCCATATGTCGCTGCTTTCGAAAATC	*plsC*5
pETplsC5R	CCGCTCGAGATCCTCTTGTTTTGCTTG	
pSTVeplsCF	GGAATTCTATGCTATATATCTTTCGTC	*E. coli plsC*
pSTVplsC1F	GGAATTCTATGCTGCTTATCGCTCGTTC	*plsC*1
pSTVplsC2F	GGAATTCTATGTTCAGTTCACTTTGC	*plsC*2
pSTVplsC3F	GGAATTCTATGCCTAATCAACACC	*plsC*3
pSTVplsC4F	GGGGTACCTATGCTGGCTTTTTTACCTGG	*plsC*4
pSTVplsC5F	GGAATTCTATGTCGCTGCTTTCGAAAATC	*plsC*5
pSTV6HisR	ACATGCATGCTCAGTGGTGGTG	Hexa-histidine tag
pSTVplsC1R	CGGAATTCTTATGTTCGAGTTTC	Native *plsC*1

Underlined bases indicate the recognition sites for restriction enzymes.

Construction of putative *pls*C knockout mutants

Putative *plsC* genes of *S. livingstonensis* Ac10 were disrupted by the integration of suicide plasmid pKNOCK-Km^r as previously reported[17, 18]. An internal region of each gene was amplified by PCR by using the primers listed in Table 2. Each PCR product was digested with BamHI/PstI and cloned into identical restriction sites of pKNOCK-Km^r to generate plasmids for knockout gene transfer. The plasmids obtained were introduced into competent *E. coli* S17-1/λ_{pir} cells and then transferred into the rifampin-resistant mutant of *S. livingstonensis* Ac10 by conjugation at 18°C on LB plates, overnight. Single crossover recombinants were selected on LB plates containing kanamycin (30 μg/ml) and rifampin (100 μg/ml). Representative colonies for each knockout type were verified by Southern blot hybridization.

Analysis of phospholipid composition by ESI-MS

S. livingstonensis Ac10 and its knockout mutant strains were grown to early stationary phase at 4°C. Phospholipids were extracted from the cells with methanol/chloroform (2:1, vol/vol) by the Bligh and Dyer procedure[19]. The total lipid extracts were analyzed by electrospray ionization-mass spectrometry (ESI-MS) with a triple-quadrupole Sciex API 3000™ LC/MS/MS System (Applied Biosystems, Foster City, CA) equipped with an ionspray ion source in the negative mode.

The total lipid extracts from Δ*plsC*1, Δ*plsC*4, and the wild-type strains were applied to a Silica gel thin-layer chromatography (TLC) plate and developed with a solvent (tetrahydrofuran/acetone/methanol/water, 50:20:40:8, vol/vol). The lipid spots were visualized under 365 nm UV lamp by spraying with a primuline solution (0.001% in 40:10 acetone/water, vol/vol). Most of the phospholipid classes found in these strains were PE and, to a lesser extent, PG. Both

PE and PG spots were scraped from the TLC plate, and extracted with chloroform/methanol (2:1, vol/vol) 3 times. The extracted PE and PG were analyzed by ESI-MS as described above. Reproducibility of the loss of EPA-containing and branched fatty acids-containing phospholipids was confirmed by three independent experiments.

Expression and complementation of PlsC in E.coli JC201

Genomic DNA was isolated from *S. livingstonensis* Ac10 and *E. coli* BL21 with the DNeasy Mini Kit (Qiagen Ltd, West Sussex, UK). The open reading frames corresponding to *plsC*1 to *plsC*5 and *E. coli plsC* genes were amplified from *S. livingstonensis* Ac10 and *E. coli* BL21, respectively, by PCR using the specific primer pairs listed in Table 2. The amplified DNA fragments of *plsC*1 to *plsC*5 and *E. coli plsC* genes were ligated into the corresponding NdeI/XhoI and EcoRI/XhoI sites of the pET21a vector (Novagen, Nottingham, UK), respectively, so that the hexa-histidine sequence was added in frame to the 3′ end of the open reading frame. At the same time, this also resulted in the start codon of the *plsC*1 gene changing from GTG to ATG. The hexa-histidine-tagged form of *plsC*1 to *plsC*5 and *E. coli plsC* genes were obtained by PCR using the above-described pET21a plasmids as templates and using the primer pairs listed in Table 2. The amplified DNA fragments were cloned in pSTV28 vector (Takara Bio, Otsu, Japan) using EcoRI/SphI or KpnI/SphI (for *plsC*4) restriction sites. The open reading frames corresponding to the native *plsC*1 was amplified from *S. livingstonensis* Ac10 genomic DNA. It was inserted into the EcoRI site of the pSTV28 plasmid. These pSTV28 vectors and control pSTV28 were each introduced into JC201. In the test for functional complementation, bacteria were grown on LB plates at 30°C and 42°C.

Bacteria grown in liquid cultures were used to obtain the membrane fraction as described below. A culture of JC201 cells in 5 ml of the medium, harboring each of the PlsC expression or empty vectors was grown for 16 h at 30°C. Cells were collected by centrifugation and were resuspended in 500 μl of 100 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM $MgCl_2$ and 1 mM phenylmethylsulfonyl fluoride. Cells were disrupted by sonication, and the total extract obtained was centrifuged at 20,000 × *g* for 5 min at 4°C to remove unbroken cells and debris. The supernatant was centrifuged again at 20,000 × *g* for 2 h at 4°C. The pellet containing the membranes was resuspended in 200 μl of the same buffer. The expression of recombinant proteins was confirmed by western blotting using a monoclonal antibody against the hexa-histidine sequence epitope (Nacalai Tesque, Kyoto, Japan). The membrane fraction was used in an assay for PlsC activity.

PlsC enzyme activity assay

The 45-μl volume of the reaction mixture used in the assay contained 100 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM $MgCl_2$, 0.1 mM 1-oleoyl lysophosphatidic acid (18:1-LPA, Avanti Polar Lipids), 370 Bq [^{3}H]-18:1 LPA (Perkin Elmer), 0.1 mM fatty acyl-coenzyme A (acyl-CoA), and 5 μl of recombinant JC201 membrane fraction (1 mg/ml protein). The mixture was incubated for 5 min at 18°C, and the reaction was terminated with chloroform and methanol (1:2, vol/vol). Lipids in the extract were subjected to TLC with the use of chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5, vol/vol). The spots were visualized by spraying with molybdenum blue reagent (Sigma, St. Louis, MO), identified by using a standard (1,2-dipalmitoyl-*sn*-glycero-3-phosphate), scraped off, and quantified by liquid scintillation counting using an LS 6500 multi-purpose scintillation counter (Beckman Coulter, Fullerton, CA) with 3 ml of Clear-Sol II (Nacalai Tesque).

Results and Discussion

A previous study by Lewin and co-workers revealed 4 regions of strong sequence homology, namely, blocks I–IV, which are characteristic of glycerolipid acyltransferase domain-containing proteins and deemed important for acyltransferase catalytic activity[20]. In our study, the genome survey of *S. livingstonensis* Ac10 revealed that this bacterium has 5 putative glycerolipid acyltransferase candidates, PlsC1 to PlsC5, which contain the highly conserved motifs I–IV (PlsC1, PlsC2, and PlsC3) or motifs I–III (PlsC4 and PlsC5) (Fig. 1). The protein sequence of these putative PlsC1 through 5 showed 45%, 13%, 13%, 17%, and 8% homology to PlsC from *E. coli*, respectively.

In order to understand the physiological role of these 5 PlsCs of *S. livingstonensis* Ac10, knockout mutants were created by disrupting the genes *plsC*1 to *plsC*5 individually, as described in the "Materials and Methods." The growth characteristic of the knockout mutants was determined at 4°C and 18°C. The knockout of putative *plsC*s did not affect growth rate at 18°C when compared to the wild type; in contrast, the Δ*plsC*1 mutant strain had a growth delay at 4°C. The doubling times of the wild-type and Δ*plsC*1 mu-

	Domain I	Domain II	Domain III	Domain IV
PlsC1	LA**NH**QNNF**D**LF	S**G**NILID**R**K	WI**F**P**EGTR**SRG	IV**P**VL
PlsC2	VGP**H**TSNW**D**FI	M**G**GSPVD**R**R	ALAP**EGTR**SP-	IV**P**IG
PlsC3	IA**NH**PTLV**D**VV	A**G**YI-PN**R**G	II**F**P**EGTR**TLT	IL**P**VV
PlsC4	IS**NH**VSGF**D**IA	LDMPFMD**R**T	INYV**EG**S**R**FTE	-----
PlsC5	IA**NH**QSWV**D**IL	LDFPFMR**R**Y	MN**F**V**EGTR**FTE	-----
***E.coli* PlsC**	IA**NH**QNNY**D**MV	T**G**NLLID**R**N	WM**F**P**EGTR**SRG	II**P**VC

Fig. 1 Conserved glycerolipid acyltransferase motifs used for the identification of putative acyltransferase from *S. livingstonensis* Ac10. The conserved residues in the specific motifs are highlighted in a boldface font.

tant strains were 15.6 and 28.7 h respectively, at 4°C.

To gain insight into the metabolic function of PlsC1 to PlsC5 in the cell, we analyzed and compared the lipid composition of the mutant and wild-type strains by ESI-MS. Considerable differences in the patterns of lipid species were observed in Δ*plsC*1 and Δ*plsC*4 mutants compared to wild type (data not shown). In order to detect specific perturbations and provide an insight into the molecular function of the encoded proteins, the major phospholipid classes, PE and PG molecular species, were analyzed by ESI-MS and compared from both the wild-type *S. livingstonensis* Ac10 and the Δ*plsC*1 and Δ*plsC*4 mutant strains (Fig. 2). The results of the comparative analysis showed that the knockout of *plsC*4 led to a significant decrease in both PE and PG containing 2 saturated fatty acyl chains (i13:0/i13:0, i13:0/14:0, i13:0/15:0, and i15:0/i15:0), accompanied by an increase in i15:0/16:1, 16:1/16:1, and 16:0/16:1 products. In the Δ*plsC*1 mutant, the percentage of the total EPA-containing PE and PG molecular species was markedly reduced. This specific change in phospholipid composition of mutant strains suggested that PlsC1 and PlsC4 were involved in lipid metabolism *in vivo*. In addition, the defect in the incorporation of EPA at the *sn*-2 position in both phospholipid classes suggests that PlsC1 acts as an acyltransferase responsible for the incorporation of EPA into phospholipids and is involved in the initial step in lipid biosynthesis, namely, *de novo* PA synthesis. This can be speculated because of the fact that PA is a key intermediate that serves as a general precursor for all phospholipids. PlsC1 is probably an acyltransferase in this pathway, specifically a 1-acyl-*sn*-glycerol-3-phosphate acyltransferase (PlsC), which completes the PA synthesis pathway by transferring an acyl-chain to the *sn*-2 position of LPA.

We tested the ability of the *plsC*1 to *plsC*5 constructs to encode proteins that possess PlsC enzymatic activity. A temperature sensitive mutant of PlsC, *E. coli* JC201, was used for this purpose. The JC201 strain grows at 30°C but not at 42°C due to the deficiency in PlsC activity. Thus, the test is based on the restoration of its growth at the non-permissive temperature. The full-length open reading frame of each of the 5 putative glycerolipid acyltransferases was

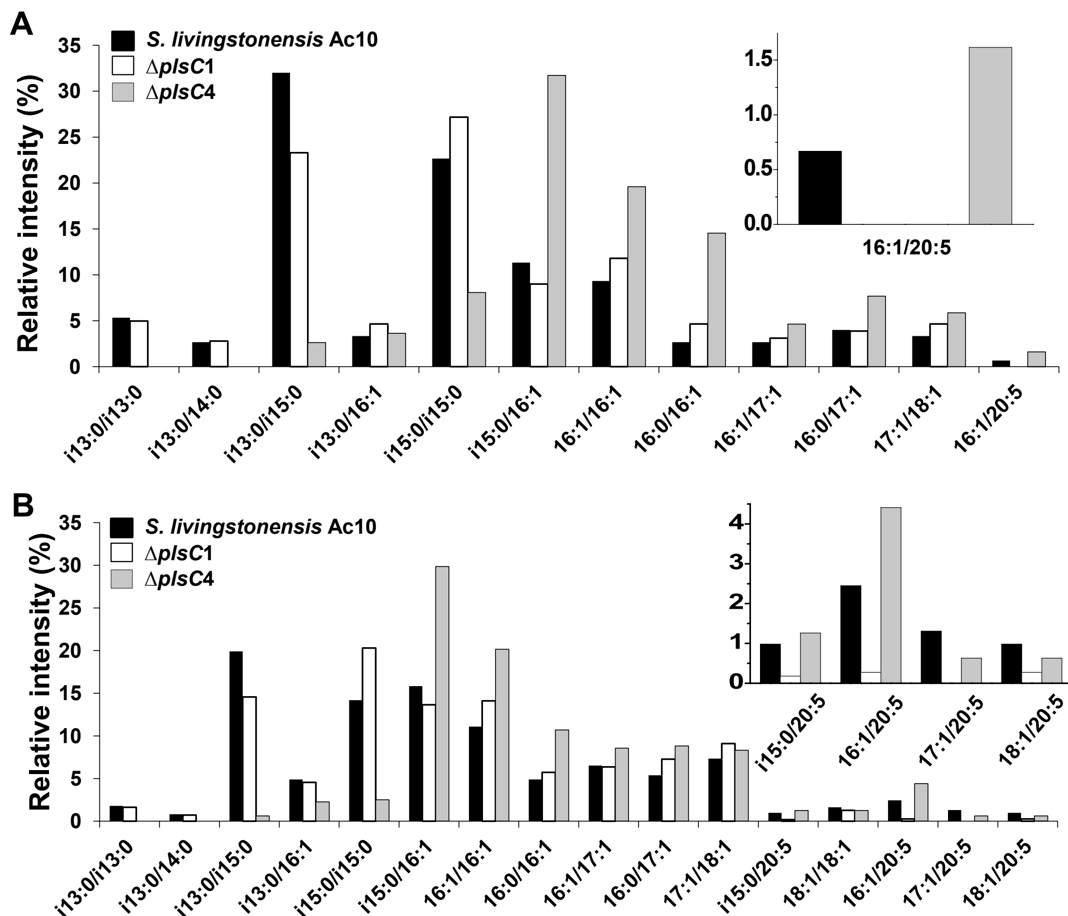

Fig. 2 Composition of PE (A) and PG (B) of *S. livingstonensis* Ac10 cultivated at 4°C. Data represent mean percentages of phospholipid molecular species derived from single samples of parental strain, and Δ*plsC*1 and Δ*plsC*4 mutants were harvested at mid-log phase. i13:0 represents isotridecanoic acid acid and i15:0 represents isopentadecaoic acid. Inset: a magnification of the plot showing EPA-containing phospholipids. Reproducibility of the loss of EPA-containing and branched fatty acids-containing phospholipids was confirmed by three independent experiments.

inserted into the expression vector pSTV28, as described in "Materials and Methods." We used JC201 transformants harboring an *E. coli plsC*-expressing plasmid, as a positive control and an empty pSTV28 vector as a negative control. Figure 3 shows that the vector expressing PlsC1 and hexa-histidine-tagged PlsC1 rescued the temperature sensitivity of *E. coli* JC201, but not the other putative PlsCs (PlsC2 to PlsC5). This result demonstrates that *plsC*1 of *S. livingstonensis* Ac10 indeed encodes a PlsC that can be substituted for the inactive bacterial PlsC in the mutant.

In the above experiment, obtained with a lipidomic approach using the *plsC*1 knockout mutant strain, the phospholipids containing EPA showed a significant decrease compared with the wild-type strain (Fig. 2). This result suggests that EPA-CoA is a preferential substrate for PlsC1; however, because EPA is not synthesized in *E. coli*, the JC201 complementation assay demonstrated that fatty acyl-CoAs other than EPA can be used by PlsC1 to synthesize PA.

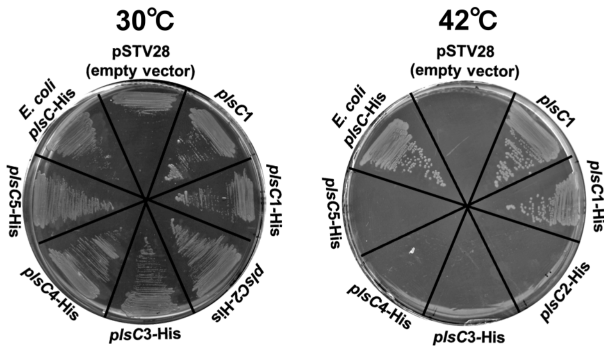

Fig. 3 Complementation analysis of *E. coli plsC* mutant after transformation with *plsC*1 and hexa-histidine-tagged *plsC*1 to *plsC*5 via plate assays. Expression of the PlsC1 and hexa-histidine-tagged PlsC1 in this mutant restores growth (*plsC*1 and *plsC*1-His), whereas the other putative *plsC*s genes do not complement for this mutation (*plsC*2 to 5-His). *E. coli plsC*-His, the strain carrying *E. coli* PlsC, was used as a positive control, and empty pSTV28 vector was used as a negative control.

Membrane fractions from *E. coli* JC201 expressing putative PlsCs were assayed for PlsC enzymatic activity by using labeled *sn*-1-18:1-LPA as the acyl acceptor and various kinds of molecular species of acyl-CoA as the acyl donor, to define the acyl donor specificity of PlsC1 (Fig. 4). The activity of JC201 PlsC was very low, and hence, it can be ignored when compared with the activity values for recombinant PlsCs expressed in this strain. This *in vitro* PlsC activity assay demonstrates that PlsC1 uses all the acyl-CoAs tested, and thus, it can be concluded that PlsC1 has activity toward a broad range of acyl-CoAs. The attached C-terminal hexa-histidine tag did not affect the activity of PlsC1. In addition, enzymatic activities weaker than that of PlsC1 were also detected in transformed JC201 membrane fractions with PlsC2 (approximately 60%) and PlsC5 (approximately 70%). Specificity of PlsC2 for fatty acyl-CoA substrates was similar to that of PlsC1 (Fig 4), whereas PlsC5 exhibited a preference for palmitoleoyl-CoA (Fig. 4B) and oleoyl-CoA (Fig. 4C) substrates.

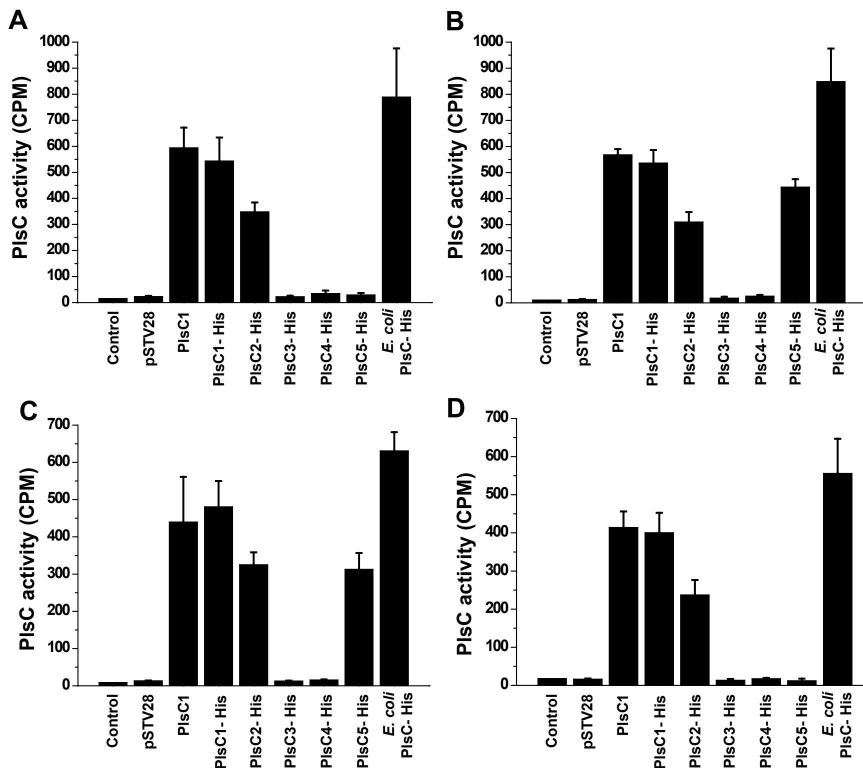

Fig. 4 Enzymatic assay for PlsC activity. PlsC activity in the membrane fraction from *E. coli* JC201 expressing PlsC1 or hexa-histidine-tagged PlsC1 to PlsC5 and *E. coli* PlsC were incubated with [^{3}H]-18:1 LPA as an acyl acceptor, and myristoyl-CoA (A), palmitoleoyl-CoA (B), oleoyl-CoA (C), or EPA-CoA (D) as an acyl donor. (Control, without membrane fraction; pSTV28, empty vector) After extraction and separation of the PA product, the radioactivity was measured. Values are presented as mean ± SEM; the data are representative of 3 independent experiments.

We currently do not have information on the structural determinants of the substrate specificity of each enzyme, which should be analyzed in future studies. Although the expression of PlsC2 and PlsC5 in JC201 dramatically increased the LPA acylation activity, reproducible complementation of the temperature-sensitive lesion was not obtained (Fig. 3). The reasons for the failure of these proteins to complement temperature sensitivity, despite the ability to synthesize PA could be due to the substrate specificity of PlsC2 and PlsC5, suggesting that these PlsCs may not have activity toward substrates that are required for the growth of *E. coli*.

The analysis of PE and PG molecular species showed that only the phospholipids containing EPA were markedly reduced in the PlsC1 knockout mutant strain (Fig. 2) despite PlsC1 having an activity toward a broad range of acyl-CoAs. This result indicated that PlsC2 and PlsC5 are able to compensate for specific defects in phospholipid biosynthesis caused by the absence of PlsC1 activity, except for the defect in synthesis of phospholipid molecular species containing EPA.

To summarize, all these results clearly suggest that EPA is mostly introduced into phospholipids in the *sn*-2 position during *de novo* synthesis by PlsC1 activity in *S. livingstonensis* Ac10. Although the PlsC4 knockout mutant strain showed a marked decrease in phospholipids with 2 saturated fatty acyl chains, the transformation of JC201 with PlsC4 did not rescue temperature sensitivity (Fig. 3B), and the membrane fraction from this JC201 strain did not have PlsC activity. These results suggest that PlsC4 could be involved in the maturation of phospholipids in a remodeling pathway to produce membrane diversity.

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