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ORIGINAL PAPER

Cold adaptation of eicosapentaenoic acid-less mutant of *Shewanella livingstonensis* Ac10 involving uptake and remodeling of synthetic phospholipids containing various polyunsaturated fatty acids

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Abstract An Antarctic psychrotrophic bacterium, *Shewanella livingstonensis* Ac10, produces *cis*-5,8,11,14,17-eicosapentaenoic acid (EPA), a long-chain polyunsaturated fatty acid (LPUFA), as a component of membrane phospholipids at low temperatures. The EPA-less mutant generated by disruption of the EPA synthesis gene becomes cold-sensitive. We studied whether the cold sensitivity could be suppressed by supplementation of various LPUFAs. The EPA-less mutant was cultured at 6°C in the presence of synthetic phosphatidylethanolamines (PEs) that contained oleic acid at the *sn*-1 position and various C20 fatty acids with different numbers of double bonds from zero to five or *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA) at the *sn*-2 position. Mass spectrometric analyses revealed that all these fatty acids became components of various PE and phosphatidylglycerol species together with shorter partner fatty acids, indicating that large-scale remodeling followed the incorporation of synthetic PEs. As the number of double bonds in the *sn*-2 acyl chain decreased, the growth rate decreased and the cells became filamentous. The growth was restored to the wild-type level only when the medium was supplemented with phospholipids containing EPA or DHA. We found that about a half of DHA was converted into EPA. The results suggest that intact EPA is best required for cold adaptation of this bacterium.

Keywords Eicosapentaenoic acid · Docosahexaenoic acid · Polyunsaturated fatty acid · Phospholipid · Lipid remodeling · Cold adaptation · Psychrotroph · *Shewanella livingstonensis* Ac10

Abbreviations

EPA	*cis*-5,8,11,14,17-Eicosapentaenoic acid
DHA	*cis*-4,7,10,13,16,19-Docosahexaenoic acid
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
LPUFA	Long-chain polyunsaturated fatty acid
ESI-MS	Electrospray ionization mass spectrometry
GC-MS	Gas chromatography-mass spectrometry
LB	Luria-Bertani
T_m	Phase transition temperature
2-acyl-GPE	2-Acylglycerophosphoethanolamine
20:0	Eicosanoic acid
20:1	*cis*-11-Eicosenoic acid
20:2	*cis*-11,14-Eicosadienoic acid
20:3	*cis*-8,11,14-Eicosatrienoic acid
20:4	*cis*-5,8,11,14-Eicosatetraenoic acid (arachidonic acid)
20:5	*cis*-5,8,11,14,17-Eicosapentaenoic acid
22:6	*cis*-4,7,10,13,16,19-Docosahexaenoic acid
18:1	*cis*-9-Octadecenoic acid (oleic acid)

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18:2	*cis*-9,12-Octadecadienoic acid (linoleic acid)
α-18:3	*cis*-9,12,15-Octadecatrienoic acid (α-linolenic acid)
γ-18:3	*cis*-6,9,12-Octadecatrienoic acid (γ-linolenic acid)
15:0	Pentadecanoic acid
16:1	Hexadecenoic acid
17:0	Heptadecanoic acid
17:1	Heptadecenoic acid

Introduction

Cold-adapted marine bacteria belonging to the genus *Shewanella* produce *cis*-5,8,11,14,17-eicosapentaenoic acid (EPA, 20:5) as a component of phospholipids in the cell membrane (Yazawa et al. 1988). In contrast, bacteria that belong to the same genus but are not cold-adapted produce no or little EPA (Kato and Nogi 2001). The ability to produce ω-3 long-chain polyunsaturated fatty acids (LPUFAs), such as EPA and *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA, 22:6), is not limited to *Shewanella* bacteria but is also found in many other cold-adapted marine bacteria belonging to the genera of *Moritella*, *Photobacterium*, *Colwellia*, and *Psychromonas* (Allen et al. 1999; Fang et al. 2004; Kawasaki et al. 2002; Methe et al. 2005). These ω-3 LPUFA-producing bacteria possess a gene cluster coding for polyketide synthase-like proteins solely dedicated to the de novo synthesis of ω-3 LPUFAs (Metz et al. 2001; Valentine and Valentine 2004). The system for ω-3 LPUFA production in these bacteria is distinct from the system of fungi and nematodes, in which preexisting fatty acids are elongated and desaturated (Ratledge 2004). The bacterial system is unique in that ω-3 LPUFAs are produced by a system similar to the polyketide synthesis system independently of the conventional fatty acid synthesis system. These observations led to the hypothesis that ω-3 LPUFAs play a vital specific role in these bacteria, particularly in their cold adaptation. Indeed, we found that disruption of the EPA-synthesis genes in *Shewanella livingstonensis* Ac10, a psychrotrophic bacterium isolated from Antarctic seawater, induces cold sensitivity (Kawamoto et al. unpublished observations). Similarly, an EPA-less mutant of a piezotolerant and psychrotolerant bacterium, *Shewanella piezotolerans* WP3, shows much slower growth rate under low temperature or high pressure condition compared with the wild-type strain (Wang et al. 2008). It was also reported that EPA has an antioxidant function in *Shewanella marinintestina* IK-1 (Nishida et al. 2007).

These findings indicated that ω-3 LPUFAs play an important role as a membrane component in some cold-adapted bacteria. However, it is still not clear whether ω-3 LPUFAs are absolute requirements for these bacteria or their roles can be taken over by other unsaturated fatty acids such as *cis*-5,8,11,14-eicosatetraenoic acid (arachidonic acid, 20:4). In this study, we synthesized a series of phospholipids containing C20 fatty acids with different numbers of double bonds as well as DHA. We studied their effects on the low-temperature growth of the EPA-less mutant of *S. livingstonensis* Ac10. We found that the growth rate decreased as the number of double bonds in the acyl chain of the added phospholipid decreased and was restored to the wild-type level only when EPA- or DHA-containing phospholipid was supplemented to the medium. Interestingly, we found that half amount of the supplemented DHA was converted into EPA. The functions of EPA in *S. livingstonensis* Ac10 will be discussed on the basis of these findings.

Materials and methods

Materials

1-Oleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine was obtained from Avanti Polar Lipids (Pelham, AL). Eicosanoic acid (20:0), *cis*-11-eicosenoic acid (20:1), *cis*-11,14-eicosadienoic acid (20:2), *cis*-8,11,14-eicosatrienoic acid (20:3), arachidonic acid (20:4), and DHA (22:6) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) (Fig. 1). 1-Oleoyl-2-*cis*-5,8,11,14,17-eicosapentaenoyl-*sn*-glycero-3-phosphoethanolamine was synthesized as described (Hosokawa et al. 1995). Phospholipase D (type VII from *Streptomyces* species) was obtained from Sigma (St. Louis, MO, USA).

Synthesis of phosphatidylethanolamines containing C20 fatty acids and DHA

Phosphatidylcholines (PCs) with oleic acid at *sn*-1 and C20 fatty acid or DHA at *sn*-2 (Fig. 1) were synthesized from 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine by applying the published methods for the synthesis of PC (Gupta et al. 1977; Hubbell and McConnell 1971; Selinger and Lapidot 1966). Synthesized PCs were purified with a Sep-Pak Plus Silica Cartridge (Waters Corp., Milford, MA). Phosphatidylethanolamine (PE) was prepared by exchange of the head group of the corresponding PC using phospholipase D as described (Comfurius and Zwaal 1977). The structures of the synthesized PEs were verified by electrospray ionization mass spectrometry (ESI-MS) with a triple-quadrupole Sciex API 3000^{TM} LC/MS/MS System (Applied Biosystems, Foster City, CA) equipped with an ionspray ion source in the negative mode.

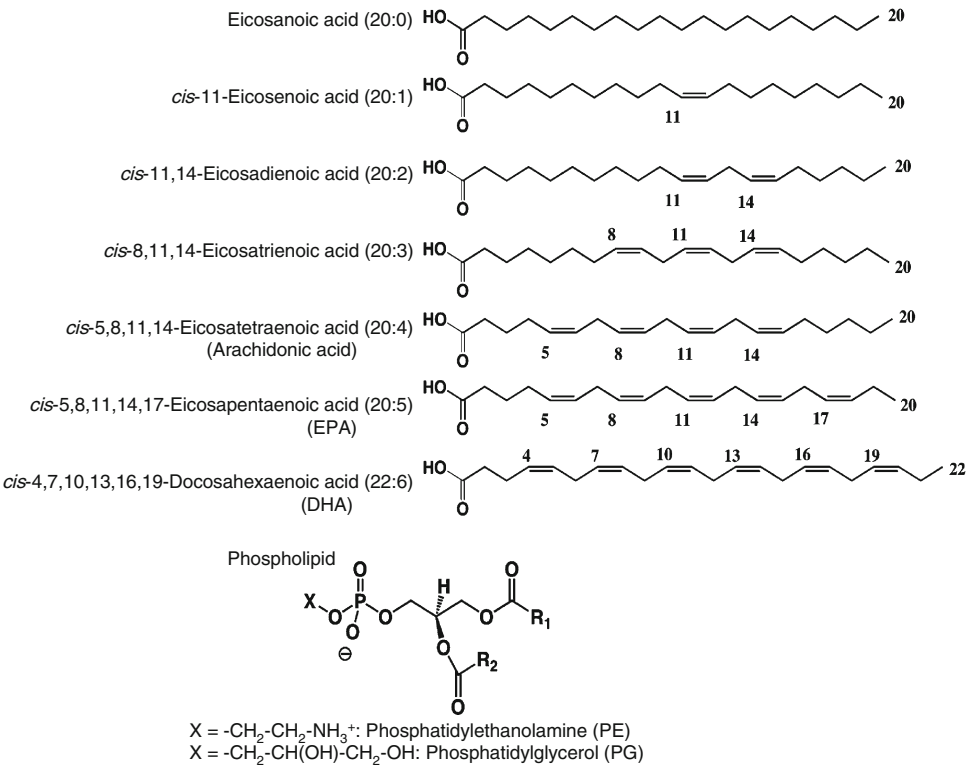


Fig. 1 Structures of fatty acids introduced into the *sn*-2 position of the synthetic phosphatidylethanolamines. R_1-CO- and R_2-CO- indicate the *sn*-1 and *sn*-2 acyl chains, respectively, of phospholipids

Bacterial strains and growth conditions

A rifampicin-resistant mutant of *S. livingstonensis* Ac10, a parent strain of the EPA-less mutant, was grown in 5 ml of a Luria-Bertani (LB) medium (pH 7.0) containing 50 μg ml^{-1} rifampicin for 48 h at 18°C. The EPA-less mutant constructed by the disruption of *orf5*, one of the genes required for EPA synthesis (Kawamoto et al. unpublished observations), was grown in an LB medium containing 50 μg ml^{-1} rifampicin and 40 μg ml^{-1} kanamycin for 48 h at 18°C. These seed cultures were used to inoculate 5 ml of LB media containing phospholipids synthesized as described above. The phospholipid solution in chloroform was dried in a sterilized tube with nitrogen gas and hydrated with 5 ml of an LB medium to a final concentration of 0.13 mM by vortexing. The cells were cultured at 6°C for 120 h to the stationary phase. Bio-photorecorder (Compact rocking incubator) TVS062CA (ADVANTEC Toyo, Tokyo, Japan) was used to monitor the growth of the cells at 6°C.

Analysis of phospholipids by ESI-MS

Phospholipids were extracted from the cells grown at 6°C with chloroform/methanol by the method of Bligh and Dyer (Bligh and Dyer 1959). Total phospholipids were analyzed by ESI-MS with an ionspray ion source in the negative mode. The fatty acyl residues in each molecular species were analyzed in the precursor ion scan mode.

Analysis of fatty acid methyl esters by gas chromatography-mass spectrometry

Fatty acid methyl esters were prepared from the cells grown at 6°C by the method of Fang et al. and analyzed with a gas chromatography-mass spectrometry (GC-MS) system (AutoSystem XL Gas Chromatograph, TurboMass Mass Spectrometer, Perkin Elmer, Wellesley, MA) equipped with a capillary column ULBON HR-1 (Shinwa Chemical Industries, Ltd., Kyoto, Japan) (Fang et al. 2004). Individual compounds were identified by comparing their retention times and mass spectra with those of the authentic compounds.

Results

Synthetic phospholipids were incorporated into the EPA-less mutant of *S. livingstonensis* Ac10 and remodeled

The EPA-less mutant and the parent strain of *S. livingstonensis* Ac10 were cultured at 6°C in an LB medium with or without supplementation of 0.13 mM PE containing

EPA, other C20 fatty acids, or DHA at the *sn*-2 position and oleic acid (18:1) at the *sn*-1 position. We used PEs that contain oleic acid at the *sn*-1 position because we found that supplementation of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine did not improve the growth of the EPA-less mutant at low temperatures (Kawamoto et al. unpublished observations). Thus, by using these synthetic PEs, we can determine the effects of the fatty acid at the *sn*-2 position on the low-temperature growth of the mutant. After cultivation, the cellular phospholipids were analyzed by ESI-MS. Figure 2a and b show the mass spectra (m/z = 720–825) of phospholipids from the parent strain and the EPA-less mutant, respectively, cultured without supplementation of the synthetic phospholipid. The following phospholipids containing EPA (20:5) were found in the parent strain: 15:0-20:5PE, 16:1-20:5PE, 17:1-20:5PE, 18:1-20:5PE, 15:0-20:5PG, 16:1-20:5PG, 17:0-20:5PG, 17:1-20:5PG, and 18:1-20:5PG (Fig. 2a, Table 1). The occurrence of these phospholipids was confirmed by precursor ion scanning. Of these EPA-containing PEs and PGs, 16:1-20:5PE and 16:1-20:5PG, respectively, were the most abundant. EPA-containing phospholipids were not found in the mutant (Fig. 2b).

When the EPA-less mutant was grown in a medium supplemented with synthetic 18:1-20:5PE, various EPA-containing phospholipids emerged. EPA was present not only in the PE species but also in the PG species, and acyl groups other than 18:1 were found in both of these EPA-containing PEs and PGs (Fig. 2c, Table 1). Of these, 16:1-20:5PE and 16:1-20:5PG, respectively, were the most abundant. The results indicated that the phospholipid added to the medium was incorporated into the cells and subsequently converted into other phospholipids via substitution of the polar head and acyl groups.

This mode of incorporation and conversion were observed for other exogenously applied synthetic phospholipids, i.e., 18:1-22:6PE, 18:1-20:4PE, 18:1-20:3PE, 18:1-20:2PE, 18:1-20:1PE, and 18:1-20:0PE. Figure 2d shows the mass spectrum of the phospholipids from the mutant cells grown in the presence of 18:1-22:6PE. After cultivation, the following phospholipids containing DHA (22:6) were found in the cells: 15:0-22:6PE, 16:1-22:6PE, 17:1-22:6PE, 18:1-22:6PE, 15:0-22:6PG, 16:1-22:6PG, 17:0-22:6PG, 17:1-22:6PG, and 18:1-22:6PG (Fig. 2d, Table 1). Notably, EPA-containing phospholipids were also produced in the cells. The composition of the molecular species of the EPA-containing phospholipids generated in the "EPA-less" mutant was similar to that of the parent strain (Fig. 2a). The occurrence of EPA was confirmed by GC-MS (Fig. 3a). The amount of EPA was almost equal to that of DHA. We tried to determine whether EPA was produced by the revertant of the mutant. In the presence of kanamycin, which suppresses the growth of the revertant, EPA-containing phospholipids were still

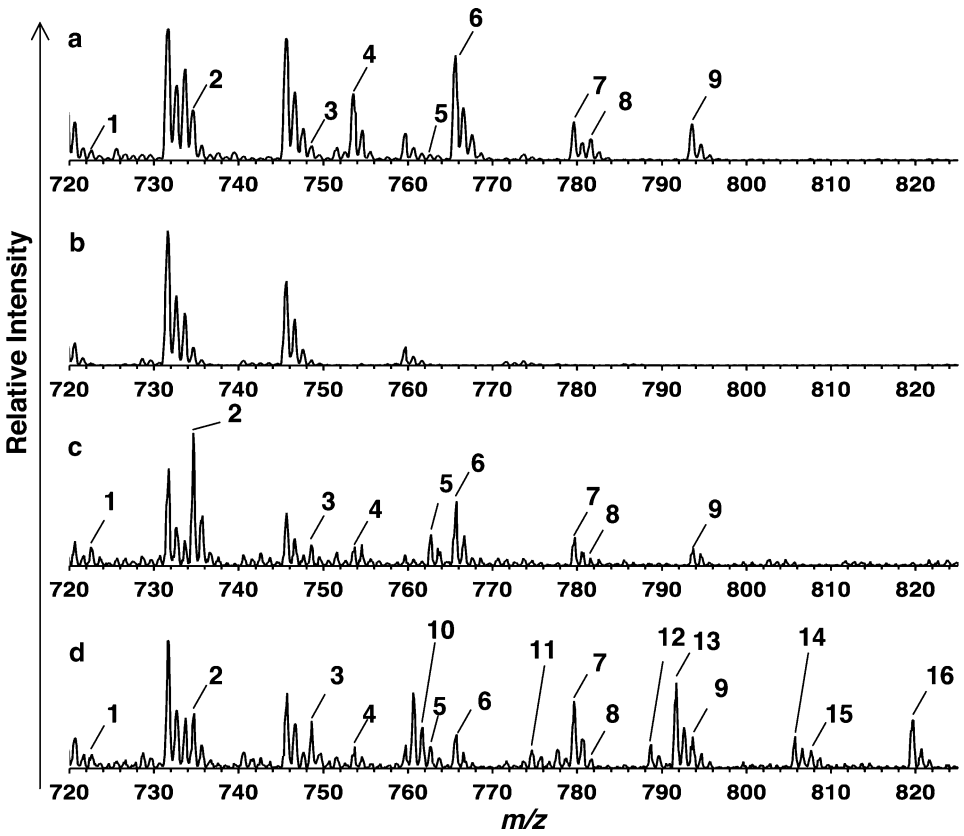


Fig. 2 ESI-MS analysis of phospholipids of the parent strain (**a**) and the EPA-less mutant (**b**, **c**, **d**) of *S. livingstonensis* Ac10. The cells were grown in an LB medium without supplementation of the synthetic phospholipids (**a**, **b**) and in the same medium supplemented with 18:1-20:5PE (**c**) or 18:1-22:6PE (**d**). The scan range was m/z 600–850. Mass spectra in the range of m/z 720–825, where differences among the spectra were found, are shown. The numbered peaks were identified as shown in Table 1

Table 1 Phospholipids containing EPA or DHA found in *S. livingstonensis* Ac10 grown with the synthetic phospholipids

Peak number[a]	Mass (*m/z*)	Phospholipid species	
1	722.6	15:0-20:5PE	
2	734.7	16:1-20:5PE	
3	748.6	17:1-20:5PE	15:0-22:6PE
4	753.7	15:0-20:5PG	
5	762.6	18:1-20:5PE	
6	765.7	16:1-20:5PG	
7	779.6	17:1-20:5PG	15:0-22:6PG
8	781.7	17:0-20:5PG	
9	793.6	18:1-20:5PG	
10	761.7	16:1-22:6PE	
11	774.6	17:1-22:6PE	
12	788.7	18:1-22:6PE	
13	791.7	16:1-22:6PG	
14	805.7	17:1-22:6PG	
15	807.6	17:0-22:6PG	
16	819.7	18:1-22:6PG	

[a] The peak numbers correspond to those in Fig. 2. Peaks 1–9 were found in the samples from the parent strain grown in an LB medium without supplementation of the synthetic phospholipids and the mutant grown in an LB medium supplemented with 18:1-20:5PE or 18:1-22:6PE. Peaks 10–16 were found in the sample from the mutant grown in an LB medium supplemented with 18:1-22:6PE. The acyl chain species in each phospholipid were confirmed by ESI-MS in the precursor ion scan mode

produced (data not shown). Thus, EPA was probably generated from DHA. The results suggest that EPA is a preferred ω-3 LPUFA for *S. livingstonensis* Ac10.

Chain shortening of the acyl group of the synthetic phospholipids was also observed for 18:1-20:2PE and 18:1-20:3PE (Fig. 3b). In these cases, linoleic acid (*cis*-9,12-octadecadienoic acid (18:2)) and γ-linolenic acid (*cis*-6,9,12-octadecatrienoic acid (γ-18:3)), respectively, were produced. About half of 20:2 was converted into 18:2, and about 30% of 20:3 was converted into γ-18:3. We did not observe conversion of 20:0, 20:1, 20:4, and 20:5 (EPA) into fatty acids that do not occur in the parent strain, although we cannot exclude the possibility that 20:0 and 20:1 were partially converted into 18:0 and 18:1, respectively, which preexisted in the cells.

Effects of externally added phospholipids on the growth of the EPA-less mutant

The EPA-less mutant of *S. livingstonensis* Ac10 grew more slowly than the parent strain at 6°C (Fig. 4), whereas the growth was normal at 18°C (data not shown), indicating that EPA-containing phospholipids play a vital role in the cold adaptation of this bacterium. We studied the effects of synthetic PEs on the growth of the EPA-less mutant at 6°C. When the medium was supplemented with 18:1-20:5PE, the growth was restored to almost the parent-strain level, confirming that the externally applied phospholipid containing EPA compensated for the loss of intrinsic EPA. The phospholipid containing DHA also almost completely complemented the growth defect although the initial growth rate was lower than that of the parent strain. In contrast, we found that 18:1-20:4PE and 18:1-20:3PE restored the growth only partially. The growth rate further decreased as the number of double bonds in the C20 acyl group of the added phospholipid decreased.

Effects of externally added synthetic phospholipids on the morphology of the EPA-less mutant

We examined the effects of the synthetic PEs on the morphology of the EPA-less mutant (Fig. 5). We found that the EPA-less mutant grown in the absence of synthetic phospholipids becomes filamentous at low temperatures, suggesting that the mutant has a defect in cell division. The cell length reverted to normal when 18:1-20:5PE or 18:1-22:6PE was supplemented to the medium. In contrast, supplementation with 18:1-20:0PE, 18:1-20:1PE, 18:1-20:2PE, and 18:1-20:3PE was rather deleterious: the cells became longer than in the absence of these phospholipids.

Discussion

Remodeling of synthetic phospholipids incorporated into *S. livingstonensis* Ac10

We found that synthetic PEs containing EPA and other long-chain fatty acids at the *sn*-2 position (Fig. 1) were incorporated into the EPA-less mutant of *S. livingstonensis* Ac10 and converted into various PE and PG species. While 18:1 was the *sn*-1 acyl chain of the added PEs, EPA and other long-chain fatty acids were found in the phospholipids containing 15:0, 16:1, 17:0, and 17:1 as well as 18:1 after cultivation (Fig. 2, Table 1). Thus, both the acyl group and the head group, or either of them, were replaced by others in the EPA-less mutant cells. Moreover, the kind of acyl chains and their relative abundance in these phospholipids were similar to those of the EPA-containing phospholipids in the parent strain.

Since PGs containing EPA and other long-chain fatty acids were found in the cells treated with the synthetic PEs, these PEs are supposed to be the substrate of phospholipase D. The occurrence of phospholipase D, which catalyzes the exchange of the polar head group of phospholipids, was recently demonstrated in a psychrophilic bacterium of the *Shewanella* genus (Tsuruta et al. 2007). A similar enzyme

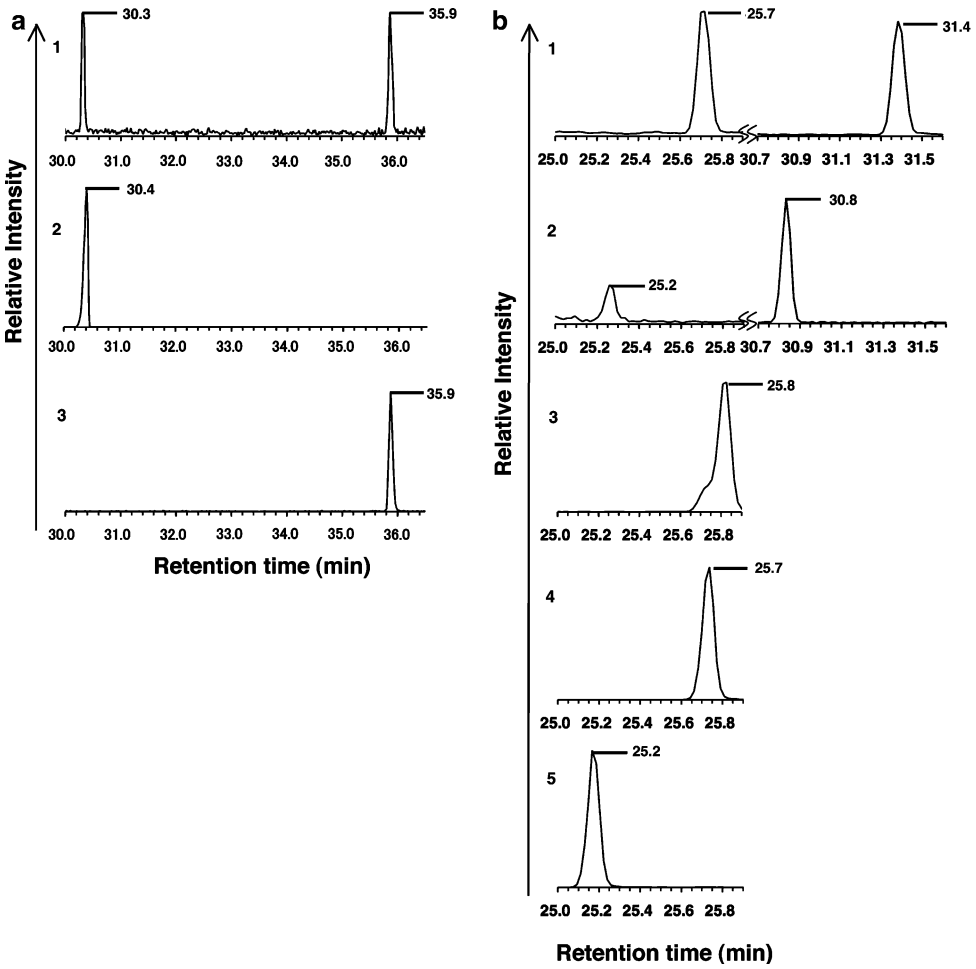


Fig. 3 GC-MS analysis of fatty acid methyl esters prepared from the EPA-less mutant grown with 18:1-22:6PE (**a-1**), 18:1-20:3PE (**b-1**), and 18:1-20:2PE (**b-2**) and methyl esters of authentic EPA (**a-2**), DHA (**a-3**), α-linolenic acid (α-18:3) (**b-3**), γ-linolenic acid (γ-18:3) (**b-4**), and linoleic acid (18:2) (**b-5**). The peaks at 30.3 min in **a-1**, 35.9 min in **a-1**, 25.7 min in **b-1**, 31.4 min in **b-1**, 25.2 min in **b-2**, and 30.8 min in **b-2** were identified as the methyl esters of EPA, DHA, γ-18:3, 20:3, 18:2, and 20:2, respectively, by co-chromatography with authentic compounds

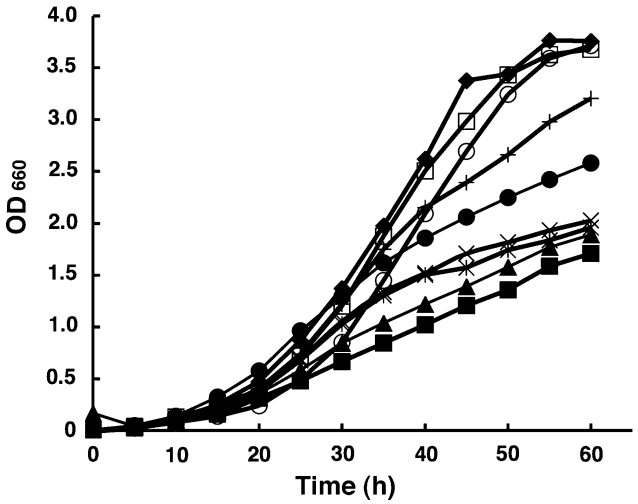
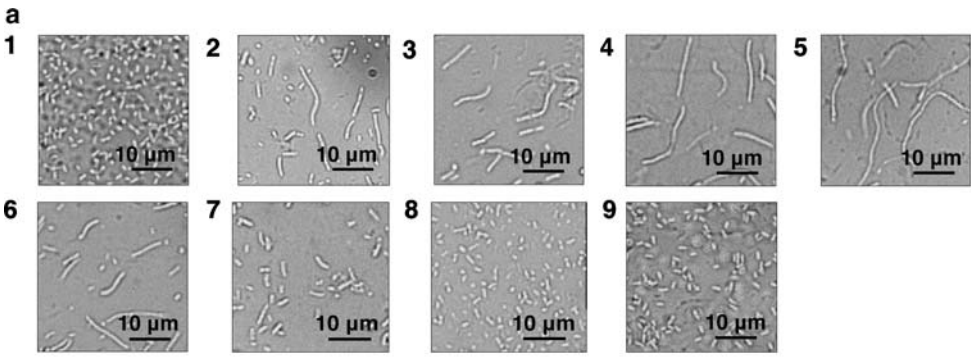


Fig. 4 Growth of the EPA-less mutant of *S. livingstonensis* Ac10 at 6°C in the medium supplemented with 18:1-20:5PE (*open square*), 18:1-22:6PE (*open circle*), 18:1-20:4PE (*plus*), 18:1-20:3PE (*closed circle*), 18:1-20:1PE (*cross*), 18:1-20:2PE (*asterisk*), and 18:1-20:0PE (*closed triangle*). The growth curves of the parent strain (*closed diamond*) and the EPA-less mutant grown in the absence of the synthetic phospholipid (*closed square*) are also shown as controls

might be present in *S. livingstonensis* Ac10. Alternatively, the applied PEs may be firstly hydrolyzed to phosphatidic acids and then converted into other phospholipids via CDP-diacylglycerol (Zhang and Rock 2008).

Regarding the exchange of acyl chains of phospholipids, various remodeling pathways have been reported so far. One of them found in bacteria involves phospholipid-protein acyltransferase and the bifunctional enzyme with 2-acylglycerophosphoethanolamine (2-acyl-GPE) acyltransferase and acyl-acyl carrier protein synthetase activities (Zhang and Rock 2008). The former enzyme catalyzes the conversion of PE into 2-acyl-GPE, and the latter catalyzes the *sn*-1 acylation of 2-acyl-GPE to produce PE. The formation of 2-acyl-GPE from PE may alternatively be catalyzed by outer membrane phospholipase A (Dekker 2000). This pathway involving the formation of 2-acyl-GPE may occur in *S. livingstonensis* Ac10 and account for the substitution of the acyl group at the *sn*-1 position in the synthetic phospholipids. However, this remodeling pathway does not explain the conversion of DHA, 20:3, and 20:2 at the *sn*-2 position into EPA, 18:3, and 18:2, respectively, (Fig. 3) because the acyl group at the *sn*-2



b

	Cell length (μm) (n = 20)
1 WT	2.1 ± 0.5
2 ΔEPA	4.2 ± 2.4
3 ΔEPA+18:1-20:0PE	5.8 ± 3.6
4 ΔEPA+18:1-20:1PE	13.6 ± 8.5
5 ΔEPA+18:1-20:2PE	10.4 ± 8.1
6 ΔEPA+18:1-20:3PE	7.2 ± 5.3
7 ΔEPA+18:1-20:4PE	2.5 ± 1.1
8 ΔEPA+18:1-20:5PE	1.9 ± 0.6
9 ΔEPA+18:1-22:6PE	1.8 ± 0.5

Fig. 5 **a** Morphology of the parent strain (WT) (**1**) and the EPA-less mutant (ΔEPA) (**2–9**) of *S. livingstonensis* Ac10 grown at 6°C. **b** Average length of the cells. The cells were grown in an LB medium without supplementation of the synthetic phospholipids (**1** and **2**) and in the same medium supplemented with 18:1-20:0PE (**3**), 18:1-20:1PE (**4**), 18:1-20:2PE (**5**), 18:1-20:3PE (**6**), 18:1-20:4PE (**7**), 18:1-20:5PE (**8**), and 18:1-22:6PE (**9**)

position is not released from the glycerol backbone in this remodeling pathway.

As the remodeling pathway involving the release of the *sn*-2 acyl group, the following two pathways may be proposed. In the first pathway, phospholipids are hydrolyzed to release the *sn*-2 acyl group by outer membrane phospholipase A, and the fatty acid produced is converted into acyl-CoA, which is introduced into 1-acyl-*sn*-glycerol-3-phosphate by the action of acyltransferase (Coleman 1992; Dekker 2000). Alternatively, in the second pathway, the *sn*-2 acyl group of phospholipids may be directly converted into acyl-CoA in an ATP-independent manner (Yamashita et al. 2001). We previously found that the phospholipid containing EPA is a better source of EPA than the free form of EPA and the methyl ester of EPA (Kawamoto et al. unpublished observations). This observation favors the second pathway, in which acyl-CoA is directly produced from phospholipids without activation of the free form of the fatty acid.

Acyl-CoA derived from the *sn*-2 acyl group of the synthetic phospholipids may be modified by the enzymes of the β-oxidation pathway and reductase before incorporation into 1-acyl-*sn*-glycerol-3-phosphate. One cycle of β-oxidation accounts for the conversion of 20:3 and 20:2 into γ-18:3 and 18:2, respectively. Conversion of DHA into EPA requires the reduction of the Δ4 double bond in addition to one cycle of β-oxidation. The reduction is probably catalyzed by 2,4-dienoyl-CoA reductase after introduction of the Δ2 double bond into the substrate by acyl-CoA dehydrogenase, which catalyzes the first step in β-oxidation (Tu et al. 2008).

Although direct biochemical and genetic evidence for these pathways is lacking, the occurrence of the genes coding for homologs of the abovementioned enzymes in the genome of *S. livingstonensis* Ac10 suggests that the synthetic phospholipids were processed through these remodeling pathways.

Effects of synthetic phospholipids on the growth and morphology of the EPA-less mutant of *S. livingstonensis* Ac10

The EPA-less mutant of *S. livingstonensis* Ac10 grows much more slowly than the parent strain at low temperatures (Fig. 4) and becomes filamentous probably due to a defect in cell division (Fig. 5). We investigated the structural requirement of fatty acyl chains for the growth and morphology of the EPA-less mutant. As a result, we found that the growth of the mutant was restored to the parent-strain level only when the medium was supplemented with phospholipids containing EPA itself or DHA. The latter was partially converted into EPA. Even arachidonic acid (20:4), which has four double bonds, did not completely complement the loss of EPA. Other unsaturated fatty acids,

such as 20:1 and 20:2, produced very small improvements in the growth and had significant negative effects on the morphology. The cells became much longer in the presence of phospholipids containing these fatty acids than in their absence, indicating their deleterious effects on cell division. These results suggest that EPA is best required for the cold adaptation of *S. livingstonensis* Ac10.

When supplemented with 18:1-20:0PE, 18:1-20:1PE, 18:1-20:2PE, and 18:1-20:3PE, the EPA-less mutant cells became longer than those in the absence of these phospholipids (Fig. 5), whereas these phospholipids can still improve the growth of the mutant on the basis of the optical density (Fig. 4). This seeming discrepancy may be partly due to higher light-scattering ability of longer filamentous cells compared with that of shorter cells. It should also be taken into account that the cell division was not completely inhibited even in the presence of these deleterious phospholipids. These factors may account for the increase of the optical density of the culture.

It is generally considered that LPUFAs, such as EPA, increase the membrane fluidity (Chintalapati et al. 2004; Russell 1997). Wang et al. systematically studied the effects of chain unsaturation of the *sn*-2 acyl chain on the phase transition temperature (T_m) of many phospholipids and showed that the T_m values of 20:0-20:0PE, 20:0-20:1PE, 20:0-20:2PE, 20:0-20:3PE, 20:0-20:4PE, and 20:0-20:5PE decrease continually with an increasing number of double bonds (Wang et al. 1999). Thus, it could be speculated that the low-temperature growth defect was caused by decreased membrane fluidity. However, we recently found that the membrane fluidity assessed by the diffusion of a small hydrophobic molecule, pyrene, in the cell membrane was not significantly decreased in the EPA-less mutant (Kawamoto et al. unpublished observations). It was also indicated that the high fluidity was ensured without EPA in the bilayer made from the lipids extracted from the EPA-less mutant. Compared with *Escherichia coli* phospholipids, which form a more rigid bilayer, both the mutant and the parent cells contained a large amount of *cis*-9-hexadecenoic acid (palmitoleic acid) and 13-methyltetradecanoic acid (isopentadecanoic acid), which are supposed to increase the membrane fluidity. While these amounted to as much as 43 and 27%, respectively, EPA constituted only 5% of the total fatty acids. Thus, EPA probably has a more specific physiological role than to contribute to bulk membrane fluidity in this bacterium. We found that the amounts of several membrane proteins are affected by the depletion of EPA (Kawamoto et al. unpublished observations). The results raised the possibility that direct interactions between EPA-containing phospholipids and membrane proteins play important roles in cold adaptation of *S. livingstonensis* Ac10. The partial complementation obtained by supplementation with C20 unsaturated fatty acids is more likely due to a structural resemblance between these fatty acids and EPA than to their abilities to increase the bulk membrane fluidity. In this respect, it is noteworthy that DHA was partially converted into EPA in the cells grown at low temperatures. EPA produced from DHA was incorporated into phospholipids without further processing by the β-oxidation enzymes and reductase. The results suggest that there is a mechanism by which EPA is preferentially incorporated into the cellular phospholipids. In order to determine whether the conversion of DHA into EPA is important for the cold adaptation of this bacterium or DHA itself can substitute for EPA, it would be necessary to use cells that lack the ability to convert DHA into EPA.

The structural element of EPA required for the cold adaptation of *S. livingstonensis* Ac10 is under further investigation using synthetic phospholipids with acyl chains possessing double bonds at various different positions.

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