Studies on 1-acyl-*sn*-glycerol-3-phosphate acyltransferase of *Shewanella livingstonensis* Ac10

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Phospholipid *de novo* biosynthesis occurs primarily by 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. 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). Subsequently, the second acyltransferase, 1-acyl-*sn*-glycerol-3-phosphate acyltransferase encoded by *plsC*, transfers an acyl group to the *sn*-2 position of LPA to synthesize phosphatidic acid (PA). PA is then converted to phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin.

Shewanella livingstonensis Ac10, a psychrotrophic bacterium isolated from Antarctic seawater, is a model organism for investigation of microbial cold-adaptation mechanisms. This strain produces eicosapentaenoic acid (EPA) as a fatty acyl chain of phospholipids at low temperatures, which plays a role in membrane organization and cell division. However, despite the importance of phospholipids containing EPA in the cold adaptation of this strain, their biosynthesis mechanism is not well defined. EPA is exclusively found at the *sn*-2 position of phospholipids in *S. livingstonensis* Ac10. Thus, PlsC is supposed to be responsible for the *de novo* synthesis of EPA-containing phospholipids. In this research, I studied catalytic function and subcellular localization of PlsC of *S. livingstonensis* Ac10 to elucidate the biosynthesis mechanism of EPA-containing phospholipids.

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

S. livingstonensis Ac10 has five genes coding for proteins homologous to *E. coli* PlsC (named PlsC1 through PlsC5). Each of the *plsC* genes was disrupted. The amount of phospholipids containing EPA was remarkably decreased only in the *plsC1*-disrupted strain, suggesting that PlsC1 is responsible for incorporation of EPA into phospholipids. I performed functional expression assay for these putative PlsCs of *S. livingstonensis* Ac10 by using a temperature-sensitive mutant of PlsC, *E. coli* JC201. The JC201 strain grows at 30°C but not at 42°C due to the deficiency in the PlsC activity. The plasmids expressing PlsC1 and *E. coli* PlsC, as a positive control, but not an empty vector, as a negative control, rescued the temperature sensitivity of *E. coli* JC201. This result demonstrated that *plsC1* of *S. livingstonensis* Ac10 indeed encodes 1-acyl-*sn*-glycerol-3-phosphate acyltransferase that can substitute for the inactive PlsC in *E. coli* JC201. Membrane fractions from these transformed *E. coli* JC201 were assayed for PlsC enzymatic activity by using radiolabeled 1-oleoyl-*sn*-glycerol-3-phosphate as the acyl acceptor and various kinds of acyl-CoAs as the acyl donor to define the acyl donor specificity of PlsC1. The PlsC activity of the host cell 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 demonstrated that PlsC1 uses all the acyl-CoAs tested including EPA-CoA and has a broad substrate specificity. These results indicate that EPA is introduced into phospholipids at the *sn*-2 position during *de novo* synthesis mostly by PlsC1 in *S. livingstonensis* Ac10.

2. Subcellular localization and physiological function of 1-acyl-*sn*-glycerol-3-phosphate acyltransferase in *Shewanella livingstonensis* Ac10

The *plsC1* knockout mutant strain (Δ*plsC1*) of *S. livingstonensis* Ac10, in which EPA-containing phospholipids were remarkably decreased, became filamentous at 4°C similarly to the EPA-deficient strain grown under the same conditions. Lack of EPA-containing phospholipids and defect in cell division observed for Δ*plsC1* were suppressed by expression of PlsC1. In contrast, when *E. coli* PlsC was expressed in the Δ*plsC1* mutant strain, the cells remained filamentous though EPA-containing phospholipids were produced.

To gain further information on the difference between PlsC1 of *S. livingstonensis* Ac10 and *E. coli* PlsC, I determined subcellular localization of these enzymes in the Δ*plsC1* mutant strain by immunofluorescence microscopy. The immunofluorescence staining of PlsC1 showed the localization of this enzyme in the middle of the cells, which corresponded to the nucleoid occlusion site. On the other hand, this localization was not observed in Δ*plsC1* expressing *E. coli* PlsC. These results suggested that EPA-containing phospholipids synthesized by PlsC1 at the middle of the cells are important for cell division of *S. livingstonensis* Ac10. In addition, mid-cell localization of PlsC1 was not observed in the EPA-less mutant (Δ*orf5*) generated by the gene disruption of one of the EPA-biosynthesis genes, *orf5*, suggesting that subcellular localization of PlsC1 depends on the presence of EPA-biosynthesis enzyme.

3. Preparation of monoclonal antibody that recognizes EPA and its application to analysis of subcellular localization of EPA

Long-chain omega-3 polyunsaturated fatty acids, EPA and docosahexaenoic acid (DHA), play beneficial roles in human health, such as infant development and prevention of cancer, cardiovascular disease, and mental illnesses. These fatty acids also play beneficial roles in bacterial physiology. Previous studies reported that EPA plays a role in cell division of *S. livingstonensis* Ac10, and a chemically synthesized fluorescent phospholipid probe containing an eicosapentaenyl group forms a membrane microdomain at the cell division site. To ascertain whether endogenous EPA-containing phospholipids are accumulated at the cell division site, I developed a novel monoclonal antibody against EPA through hybridoma technology. Rats were immunized with EPA coupled to bovine serum albumin (BSA) modified with glutaraldehyde and L-cysteine. Enzyme-linked immunosorbent assay (ELISA) was performed with a hybridoma culture medium supernatant containing anti-EPA monoclonal antibody in the presence of 0.5% *N*-lauroyl sarcosine, which facilitates access to hydrophobic antigens. This monoclonal antibody was bound to EPA-Cys-BSA conjugate and showed less reactivity to other fatty acid-Cys-BSA conjugates (18:1, 18:2, 18:3, 20:0, 20:4, and DHA-Cys-BSA). Pre-incubation of the antibody with oleic acid did not affect its reactivity to EPA-Cys-BSA conjugate but abolished low-level cross-reactivity with other fatty acid-Cys-BSA conjugates. Pre-incubation with EPA eliminated all the reactivities. These results demonstrate that this monoclonal antibody can bind to immobilized EPA and also recognize free EPA.

To determine subcellular localization of EPA-containing molecules, immunofluorescence staining was performed for *S. livingstonensis* Ac10 by using this monoclonal antibody. The immunofluorescence signals were concentrated at the middle of the cells and the polar regions. In the mutant that lacks a key enzyme for the synthesis of EPA-containing phospholipids, Δ*plsC1*, immunofluorescence localization at the mid-cell and polar regions was not observed, though fluorescence signal was evident in entire cells in comparison to the EPA-less mutant strain (Δ*orf5*). Thus, the fluorescence localization at the mid-cell and polar regions in the wild-type strain probably

corresponds to the localization of the EPA-containing phospholipids. The anti-EPA monoclonal antibody developed in this study would be valuable to reveal the localization and function of EPA or EPA-containing molecules in various organisms.

I further performed immunofluorescence staining of EPA-containing molecules in Δ*plsC1* expressing PlsC1 or *E. coli* PlsC. Intense fluorescence signal was observed at the mid-cell and polar regions when PlsC1 was expressed in the Δ*plsC1* mutant strain, whereas the localization of fluorescence signal was not observed in the Δ*plsC1* expressing *E. coli* PlsC. This difference may be due to the difference in subcellular localization of PlsC1 and *E. coli* PlsC: PlsC1 is localized at the mid-cell region, whereas *E. coli* PlsC is widely distributed in the Δ*plsC1* mutant strain. The occurrence of the microdomain enriched in EPA-containing phospholipids n at the cell division site of *S. livingstonensis* Ac10 depends on the appropriate localization of PlsC1.