Incorporation of Exogenous Docosahexaenoic Acid into Various Bacterial Phospholipids

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Incorporation of exogenous docosahexaenoic acid (DHA) into bacterial phospholipids was examined as a method for DHA-linked phospholipid production. The cultivation of 23 bacterial strains in medium with DHA showed that an eicosapentaenoic acid-producing bacterium *Shewanella* sp. strain SCRC-2738 (strain SCRC-2738), *Bacillus subtilis* W23, *B. cereus*, an Antarctic marine bacterium strain S-7 (strain S-7), photosynthesis bacterium (PSB) *Rhodopseudomonas capsulatus* utilized for the production of larval marine fish, *Pseudomonas aeruginosa, Staphylococcus aureus, Serratia marcescens* and *Escherichia coli* K12 all incorporated DHA into their polar lipids. The polar lipids of the strain SCRC-2738, strain S-7, PSB and *E. coli* K12 were identified to be phospholipids. DHA was localized at the *sn*-2 position in the phospholipids of the four strains. Incorporation of exogenous DHA into their phospholipids produced an increase in saturated fatty acids and a decrease in monounsaturated fatty acids except *E. coli* K12. The strain SCRC-2738 incorporated the largest amount of DHA into their phospholipids among the tested bacterial strains in this study: DHA was 16% of the total fatty acids in the phosphatidylethanolamine (PE) and 29% in the phosphatidylglycerol (PG). In the PSB, incorporated DHA was 12% of the total fatty acids in the PE, 10% in the PG and phosophatidylcholine so that the PSB was nutritionally fortified.

KEY WORDS: Docosahexaenoic acid, eicosapentaenoic acid-producing bacterium, incorporation, phospholipids.

It is known that n-3 polyunsaturated fatty acids (PUFA) have many pharmacological and physiological effects, competing with arachidonic acid, an n-6 type PUFA, to reduce cardiovascular diseases (1), such as thrombosis and atherosclerosis, inflammation, carcinogenesis and systemic allergy. Especially docosahexaenoic acid (DHA) [22:6(n-3) represents chainlength, number of double bonds and, in parentheses, the position of the first double bond numbered from the methyl terminal of the fatty acid, DHA] also has other pharmacological functions (2,3), such as development of the brain and retina, that have not been recognized in other n-3 PUFA. Therefore, DHA is expected to be used as medicine and functional food. Recently, we have found that a large amount of DHA is contained in the orbital fat of marine fish, such as tunny and bonito, and we have established the production of highly purified DHA and bulk supplies of DHA (3). On the other hand, because a phospholipid is one of the vital components and has its own pharmacological effects, DHA-linked phospholipid (DHAPL) is a favorable DHA derivative as a medicine and functional food (4). Heretofore, many methods of DHAPL production, including chemical synthesis (5,6), enzymatic synthesis (7,8) and purification of natural DHAPL from marine fish and their eggs (9–12), have been studied and licensed. In this study, DHAPL production was examined by incorporation of exogenous DHA into bacterial phospholipids. Although it is known that microorganisms and cultured cells are able to incorporate exogenous fatty acids into their phospholipids (13–17), incorporation of exogenous DHA into bacterial lipids has not been examined. A *Shewanella* sp. (strain SCRC-2738) produces eicosapentaenoic acid (EPA) [20:5(n-3)] (18), one of the PUFA, so that the bacterium seems to be preferable for incorporation of exogenous DHA. It would be expected that simple molecular species of DHAPL are produced if an Antarctic marine bacterium (strain S-7) were to incorporate exogenous DHA in the phospholipids. Strain S-7 has a simple fatty acid composition and oleic acid [18:1(n-9)] makes up *ca.* 90% of the total fatty acids. The photosynthesis bacterium (PSB) *Rhodopseudomonas capsulatus* is commercially available and is used for the production of larval marine fish (19). However, because the bacterium has no n-3 type PUFA that are essential fatty acids (20–22) for marine fish (23), the bacterium is not a perfect nutrient. It would be expected that the bacterium is nutritionally fortified if the bacterium would incorporate exogenous DHA in its phospholipids. We, therefore, examined strains SCRC-2738, S-7 and PSB along with many other bacterial strains for the incorporation of exogenous DHA.

In these studies, we found that some non-PUFA-producing bacterial strains also incorporated exogenous DHA into their phospholipids. Strain SCRC-2738 incorporated the largest quantities of DHA among the strains examined: 16% of total fatty acids in phosphatidylethanolamine (PE) and 29% of that in phosphatidylglycerol (PG). Furthermore, PSB incorporated exogenous DHA so that the strain had a new nutritive value.

EXPERIMENTAL PROCEDURES

Chemicals. Phospholipase A_2 (bovine pancreas) was purchased from Sigma Chemical Co. (St. Louis, MO). Brain heart infusion, which contains infusion from calf brains and beef hearts, was from Difco Laboratories (Detroit, MI). Artificial seawater (ASW) was from Jamarin Laboratories (Osaka, Japan). Potassium docosahexaenoate of 79% purity was prepared from orbital fat by an ester exchange with 0.5 N sodium methylate, followed by purification through reverse-phase high-performance liquid chromatography and saponification by ethanolic KOH. The free acid of 94% purity was prepared from the potassium salt with acidification by 6 N HCl. All other reagents used in this study were of chemical grade and commercially available.

Strains and cultivation. Strain SCRC-2738 was grown in PY medium (1.0% peptone and 0.5% yeast extract in ASW diluted to half concentration). Bacterium strain S-7 was grown in PYM medium (1.0% peptone, 0.3% yeast extract, 0.3% meat extract and 3.0% NaCl in distilled water). PSB was grown in modified Niel's medium [0.3% peptone, 0.3% yeast extract, 0.3% sodium acetate, 0.05% K_2HPO_4, 0.02% $MgCl_2 \cdot 6H_2O$, 0.01% NaCl, 0.001% $FeCl_3 \cdot 6H_2O$ and 0.03% $(NH_4)_2SO_4$ in distilled water] *Escherichia coli* K12, *E. coli* B, *Pseudomonas aeruginosa, Serratia marcescens, Bacillus cereus, B. subtilis*

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W23, *B. subtilis* (natto) and *Staphylococcus aureus* were grown in BHI medium (3.7% BHI in distilled water). *Streptococcus thermophilus, S. faecium, S. faecalis, S. salivarius, S. lactis, Leuconostoc mesenteroides, Lactobacillus acidophilus, L. delbrueckii, L. plantarum, L. helveticus, L. bulgaricus* and *L. fermentum* were grown in Rogosa medium. The Rogosa medium consisted of 1% trypticase, 0.5% yeast extract, 0.3% tryptose, 0.3% K_2HPO_4, 0.3% KH_2PO_4, 0.2% triammonium citrate, 0.1% Tween 80, 2% glucose, 0.02% cystein and 5 mL salt solutions in 1000 mL distilled water. The salt solutions consisted of 11.5 g $MgSO_4 \cdot 7H_2O$, 0.68 g $FeSO_4 \cdot 7H_2O$ and 2.4 g $MnSO_4 \cdot 2H_2O$ in 100 mL distilled water. Strain SCRC-2738 was cultivated at 15°C while other strains were cultivated at 25°C. On initiation of the culture, 0.1% (w/w), potassium docosahexaenoate of 79% purity was added to a 5-mL scale culture of all strains. For analysis of the phospholipids, 0.05% (w/w) of potassium docosahexaenoate of 79% purity or DHA of 94% purity was added to a 100-mL scale culture of strain SCRC-2738 and *E. coli* K12 on initiation of the culture, while 0.05% (w/w) of DHA of 94% purity was added to a 100-mL scale culture of strain S-7 on initiation of the culture. However, 0.05% (w/w) of DHA of 94% purity was added to a 100-mL scale culture of PSB 1 d after initiation of the culture. The cultivation time of PSB was 2 d and those of the other strains were 12–20 h. The cells were harvested by centrifugation at 1,500 rpm at 4°C for 15 min and washed with 1.5% NaCl aqueous solution.

Extraction and analysis of lipids. Lipids were extracted from the wet cells according to Folch's *et al.'s* method (24) and were kept at −80°C. The polar lipids of all bacterial strains tested in this study were separated on silica gel-coated plates (Merck Art 5721; Merck, Darmstadt, Germany) developed with chloroform/methanol/water (65:15:1, vol/vol/vol) as the first screening for incorporation of exogenous DHA. The phospholipids and the products of phospholipase A_2 digestion of the phospholipids in the strain SCRC-2738, *E. coli* K12, strain S-7 and PSB were separated on the silica gel-coated plates developed with chloroform/methanol/water (65:25:4, vol/vol/vol).

The distribution of fatty acids at the *sn*-1 and *sn*-2 positions of the phospholipids was examined by enzymatic hydrolysis with phospholipase A_2 according to the method of Haverkate and van Deenen (25).

Analysis of fatty acids. The polar lipids, the phospholipids and the products of phospholipase A_2 digestion on the silica gel thin-layer chromatography (TLC) were removed from the plates, transferred to screw-capped test tubes and methanolyzed with 8% methanolic HCl at 80°C for 1 h. The fatty acid methyl esters were analyzed in a Shimadzu GC-9A gas chromatograph (Tokyo, Japan) equipped with an flame-ionization detector and a capillary column (0.25 mm × 25 m) coated with PEG 20M. The column temperature was kept at 180°C for the first minute, increased to 190°C at 1°C/min, kept for 2 min, increased to 210°C at 5°C/min, kept for 9 min, increased to 228°C at 6°C/min and then allowed to stand for 10 min.

RESULTS

In 5-mL scale culture, some strains had depressed growth with the addition of exogenous DHA in their medium: Optical densities (A_{610}) of *B. subtilis* W23 were 4.98 and 1.78, those of *P. aeruginosa* were 5.80 and 1.33, those of *S. aureus* were 2.44 and 1.00 and those of *S. faecalis* were 1.33 and 0.86 in the medium without and with DHA for the same cultivation time, respectively. Exogenous DHA was incorporated into polar lipids of strains SCRC-2738, strain S-7, PSB, *E. coli* K12, *P. aeruginosa, S. marcescens, B. cereus, B. subtilis* W23, *B. subtilis* (natto) and *S. aureus* (Table 1), but was not detected in the polar lipids of the other strains. The amounts of DHA incorporated into the polar lipids were *ca.* 20% of the total fatty acids in strain SCRC-2738, strain S-7 and *B. subtilis* W23 and were only slightly incorporated in *E. coli* K12, *P. aeruginosa, S. marcescens, B. cereus, B. subtilis* (natto) and *S. aureus. P. aeruginosa* and *B. subtilis* W23 incorporated much more exogenous DHA with cultivation in the presence of artificial seawater than in its absence.

The exogenous DHA-incorporated polar lipids of the strain SCRC-2738 and *E. coli* K12 were PE and PG; those of strain S-7 were PE, cardiolipin (CL) and PG; and those of PSB were PE, PG and phosphatidylcholine (PC). They were identified by Dittmer reagent, comparison of Rf values to authentics on silica-gel TLC and composition of their pulsed nuclear magnetic resonance spectra (data not shown). The polar lipids of *B. subtilis* W23 that incorporated more exogenous DHA among the strains examined were not phospholipids because of their negative reaction to Dittmer reagent. Because the separations of PG and PC of PSB, and of PG and CL of strain S-7, were not complete on silica-gel TLC, the fatty acid composition of these fractions could not be estimated separately. The ratio of incorporated DHA to total fatty acids was *ca.* 16% in PE and *ca.* 29% in PG for strain SCRC-2738 in the free acid (Fig. 1). These values were slightly higher than those in the potassium salt. Although the free acid was also more incorporated than the potassium salt in the case of *E. coli* K12, the ratio was lower than that of strain SCRC-2738, *ca.* 1% in PE and *ca.* 4% in PG (Fig. 2). While the ratio of monounsaturated fatty acids decreased with incorporation of exogenous DHA, that of saturated and branched fatty acids were increased for

TABLE 1

Incorporation of Exogenous Potassium Docosahexaenoate of 79% Purity into Polar Lipids of Various Bacterial Strains[a]

Bacteria	DHA (%)[b]
Shewanella sp. strain SCRC-2738	(18)[c]
Antarctic marine bacterium strain S-7	(19)
Rhodopseudomonas capsulatus	(5)
Escherichia coli K12	2 (1)
Pseudomonas aeruginosa	1 (6)
Serratia marcescens	1 (2)
Bacillus cereus	8 (8)
Bacillus subtilis W23	15 (21)
Bacillus subtilis (natto)	1 (1)
Staphylococcus aureus	3 (2)

[a]Exogenous potassium docosahexaenoate was added (0.1% w/w) to the culture weight.
[b]Ratio of docosahexaenoic acid (DHA) on fatty acid composition in polar lipids.
[c]The values in parentheses were obtained from medium prepared with 1/2 concentration of artificial seawater.

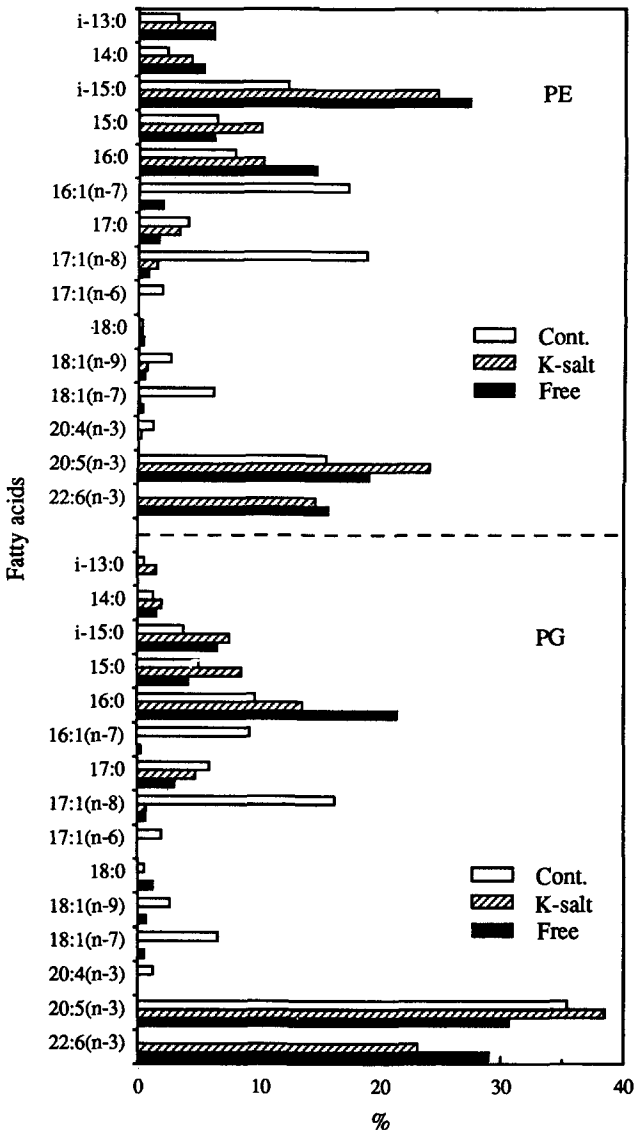

FIG. 1. Fatty acid compositions of phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) of strain SCRC-2738 incorporating exogenous docosahexaenoic acid (DHA). Cultivation was conducted without exogenous DHA (Cont.), with 0.05% potassium docosahexaenoate of 79% purity (K-salt) and with 0.05% DHA of 94% purity (Free) at 15°C. Fatty acids were represented by chainlength, numbers of double bonds and the position of the first double bond numbered from the methyl terminal. "i" represented a structural isomer containing a methyl branch next to the methyl terminal.

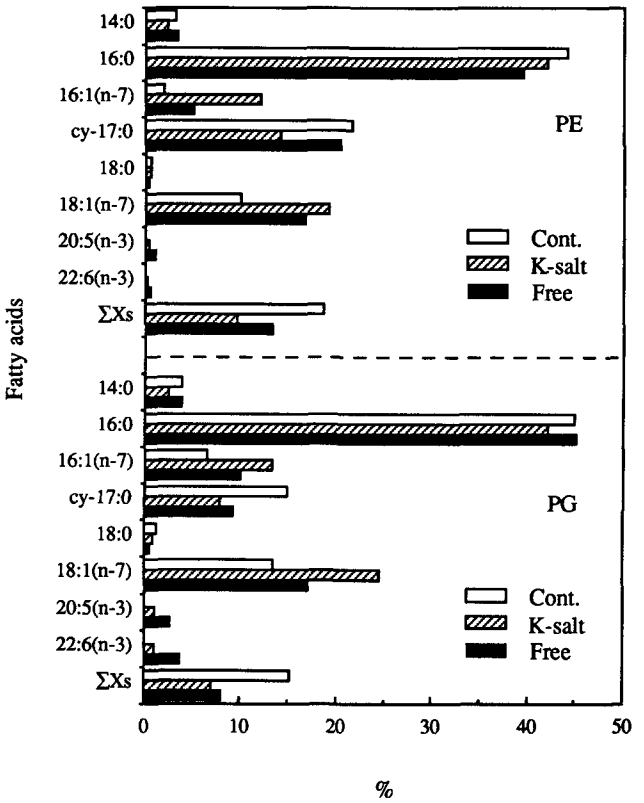

FIG. 2. Fatty acid compositions of PE and PG of *Escherichia coli* K12 incorporating exogenous DHA. Cultivation was conducted without exogenous DHA (Cont.), with 0.05% potassium docosahexaenoate of 79% purity (K-salt) and with 0.05% DHA of 94% purity (Free) at 25°C. "cy" represented a structural isomer containing cyclopropane ring. ΣXs was sum of unidentified fatty acids. See Figure 1 for abbreviations.

fatty acid compositions of phospholipids in strain SCRC-2738. A notable effect on fatty acid composition of phospholipids for incorporating DHA by *E. coli* K12 was not observed. In phospholipids of strain S-7 and PSB, incorporation of exogenous DHA showed the same effect as observed in strain SCRC-2738 on fatty acid composition and produced unidentified fatty acids (Fig. 3 and 4). In the phospholipids of exogenous DHA-incorporated *E. coli* K12, strain S-7 and PSB, EPA was detected. Although exogenous EPA, which was added to the culture with DHA, was less than 5% of DHA by weight, the amount of EPA detected was almost at the same level as that of DHA in the phospholipids of *E. coli* K12 and strain S-7.

Fatty acid compositions at the *sn*-1 and *sn*-2 positions were calculated to be 50% as the total fatty acid of each position. Exogenous DHA was strictly located at the *sn*-2 position in the phospholipids of strain SCRC-2738, *E. coli* K12 and PSB (Figs. 5, 6 and 8), while a small amount of exogenous DHA did exist at the *sn*-1 position in strain S-7 (Fig. 7). The unidentified fatty acid in strain S-7 was located at the *sn*-1 position, while the unidentified one in PSB was at the *sn*-2 position.

DISCUSSION

According to mechanisms of incorporation of exogenous fatty acids into the phospholipids of *E. coli* (26–28) and the biosynthesis of bacterial phospholipids (29), the amount of DHA incorporated seems to depend on the transit ability of the cell wall and cell membrane, the acyl-CoA synthesis ability at the inside of the inner cell membrane, the substrate selectivity and the affinity of the acyltransferase and acceptability of the cell membrane. The decrease in monounsaturated fatty acids instead of EPA incorporated with DHA in the phospholipids (Figs. 1, 3 and 4) implies that DHA can play some roles of monounsaturated fatty acids and different roles of EPA. This

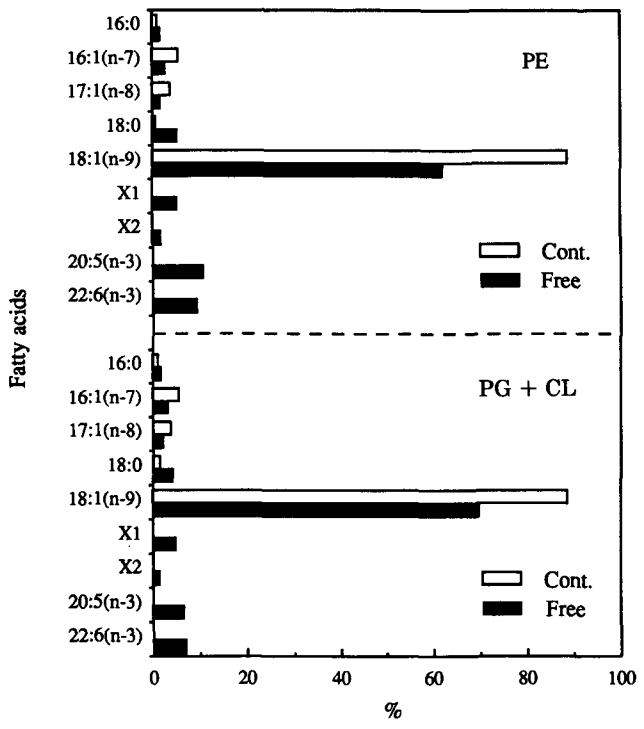

FIG. 3. Fatty acid compositions of PE and the sum of PG and cardiolipin (CL) of strain S-7 incorporating exogenous DHA. Cultivation was conducted without exogenous DHA (Cont.) and with 0.05% DHA of 94% purity (Free) at 25°C. X1 and X2 were unidentified fatty acids. See Figures 1 and 2 for abbreviations.

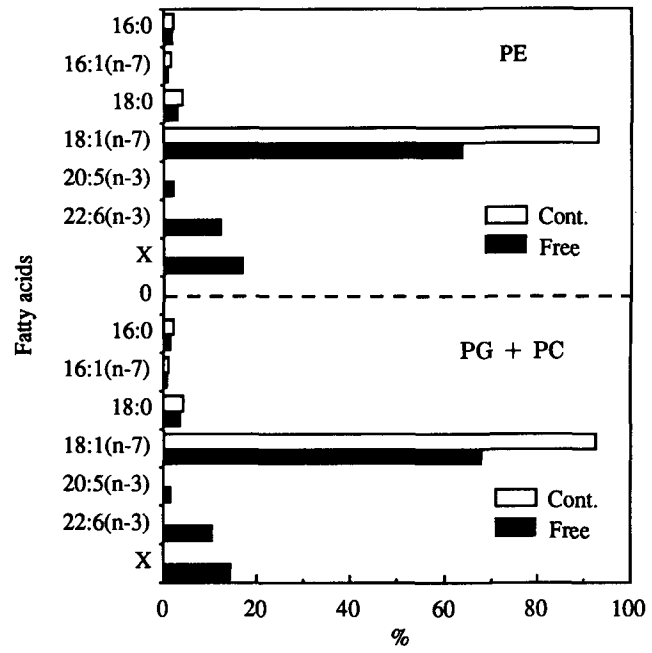

FIG. 4. Fatty acid compositions of PE and the sum of PG and phosphatidylcholine (PC) of strain photosynthesis bacterium *Rhodopseudomonas capsulatus* incorporating exogenous DHA. Cultivation was conducted without exogenous DHA (Cont.) and with 0.05% DHA of 94% purity (Free) at 25°C. X was unidentified fatty acid. See Figures 1 and 2 for abbreviations.

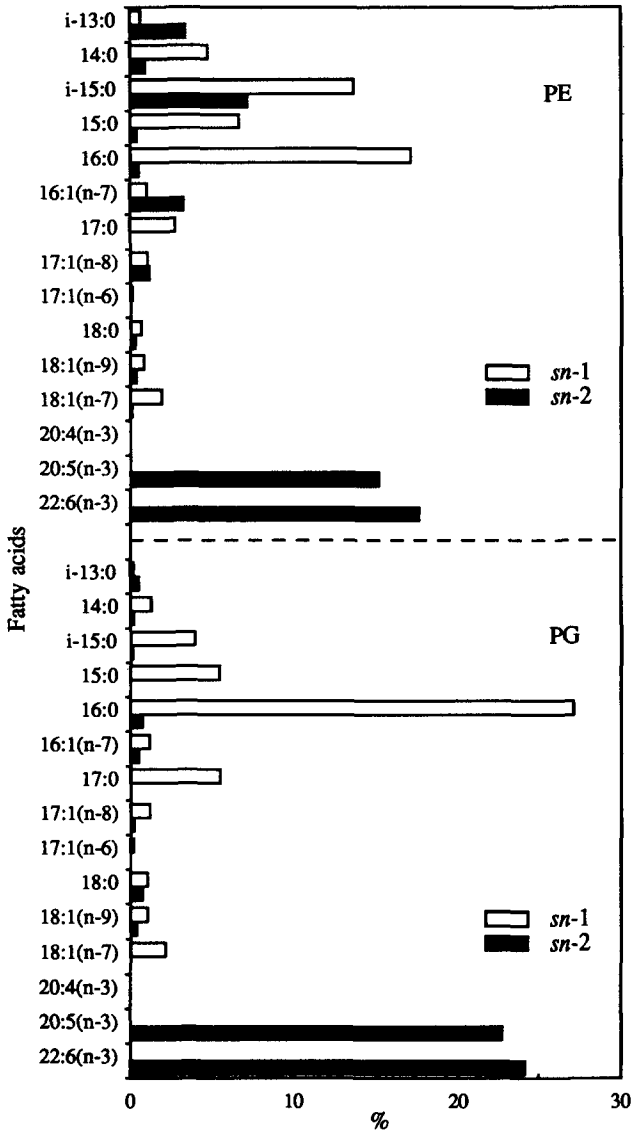

FIG. 5. Positional distribution of fatty acids in PE and PG of strain SCRC-2738 incorporating exogenous DHA. The fatty acid compositions at *sn*-1 and *sn*-2 positions were calculated as total of fatty acids to be 50% at *sn*-1 and *sn*-2 positions, respectively. See Figure 1 for abbreviations.

assumption is supported by information that DHA-linked PC is compactly packed in a cellular bilayer (30), and its phase transition temperature is the same as the 18:1(n-9)-linked PC and is higher than the 16:1(n-7) and 20:4(n-6)-linked PC (31–33). In *E. coli* K12 that has a large amount of saturated fatty acids, monounsaturated fatty acids did not decrease with exogenous DHA (Fig. 2). This result suggests that the unsaturation ratio of the bacterial cell membrane is one of the important factors for the incorporation ability of exogenous DHA. The amount of DHA incorporated in phospholipids was consistently higher in SCRC-2738 than in the other strains in this study (Table 1, Figs. 1, 2, 3 and 4). This result implies that the strain SCRC-2738 has some advantageous factors for incorporation of DHA, such as acyl-CoA synthesis ability and

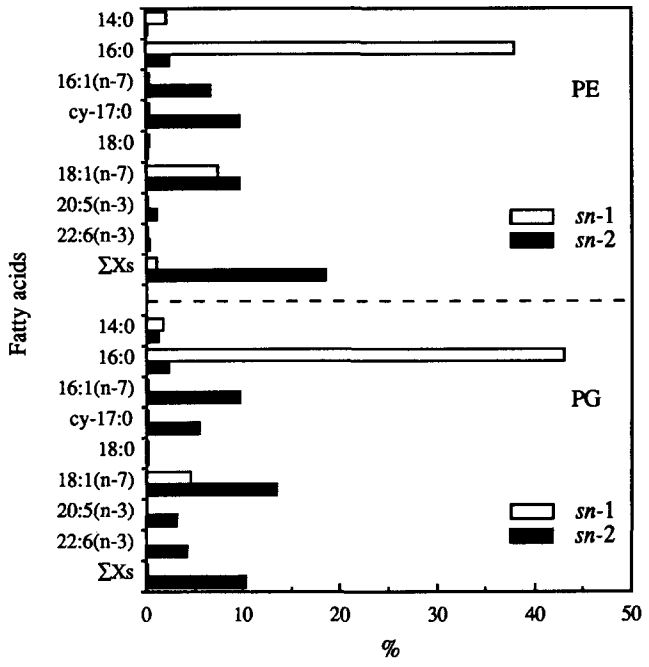

FIG. 6. Positional distribution of fatty acids in PE and PG of *E. coli* K12 incorporating exogenous DHA. The fatty acid compositions at *sn*-1 and *sn*-2 positions were calculated as described in the legend of Figure 5. See Figures 1 and 2 for abbreviations.

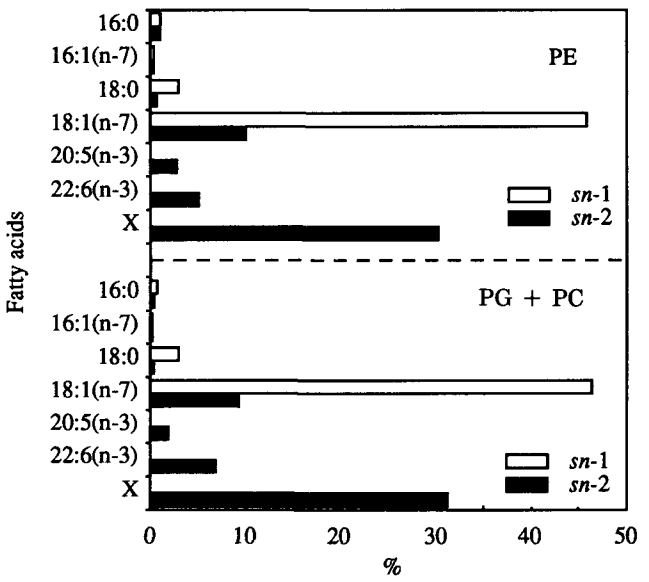

FIG. 8. Positional distribution of fatty acids in PE and in the sum of PG and PC of strain photosynthesis bacterium incorporating exogenous DHA. The fatty acid compositions at *sn*-1 and *sn*-2 positions were calculated as described in the legend of Figure 5. See Figures 1 and 4 for abbreviations.

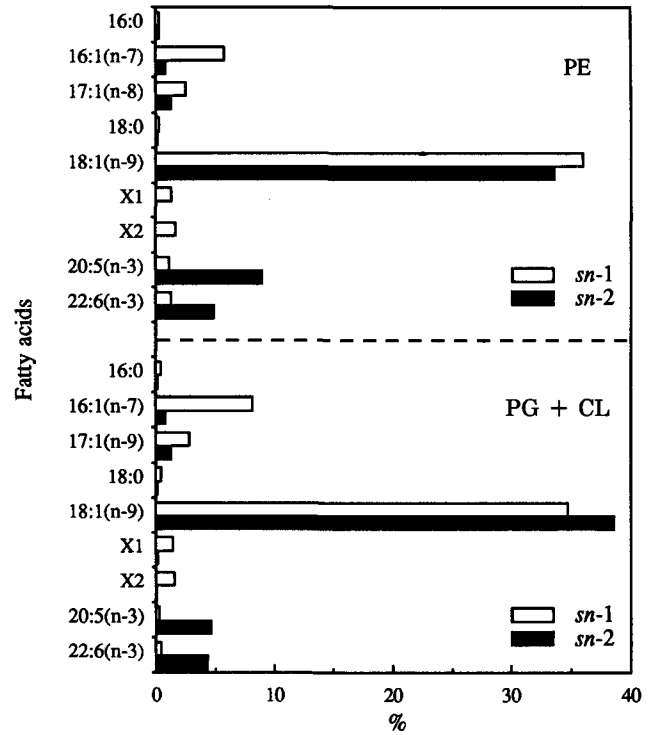

FIG. 7. Positional distribution of fatty acids in PE and in the sum of PG and CL of strain S-7 incorporating exogenous DHA. The fatty acid compositions at *sn*-1 and *sn*-2 positions were calculated as described in the legend of Figure 5. See Figures 1–3 for abbreviations.

affinity of acyltransferase, before esterification of DHA to the phospholipids.

In the phospholipids of both *E. coli* K12 and strain S-7, EPA was detected in almost the same amount as the incorporated exogenous DHA in spite of the fact that small amounts of exogenous EPA, in comparison with those of DHA, were used (Figs. 2 and 3). We also obtained other evidence that the amount of EPA detected in phospholipids was more than that of exogenous EPA added to the medium in strain S-7 (data not shown). These results suggested that the EPA in phospholipids of *E. coli* K12 and strain S-7 was derived from retroconversion of DHA-participating 2,4-dienoyl-CoA reductase (34–36).

The amount of DHA incorporated in the phospholipids of PSB was lower than that of S-7 in the 5-mL scale test but higher in the 100-mL scale test (Table 1 and Fig. 4). Strain PSB is a useful source of amino acids and vitamins for the production of rotifer *Brachionus plicatilis* and *Artemia salina* in that both plankton are commercially available for the production of larval marine fish. Strain PSB itself has no essential fatty acids, n-3 PUFA (25–27), for the production of larval marine fish (27). Therefore, the DHA-incorporated PSB that can be produced in a plant seems to be a new powerful nutrient for marine fish cultures.

The incorporated DHA is localized into the *sn*-2 positions of phospholipids of all examined strains (Figs. 5–8). This result was consistent with the natural tendency of phospholipids to locate higher unsaturated fatty acids into the *sn*-2 positions of phospholipids except in higher plants (37) and micro algae (38), which esterify their fatty acids to the *sn*-1 and *sn*-2 positions of lipids.

In conclusion, some bacterial strains incorporated exogenous DHA in their phospholipids, even in non-PUFA-producing bacteria, and decreased monounsaturated

fatty acids in their fatty acid compositions. Although the amounts of DHA incorporated in the phospholipids depend on cultivation conditions, concentration and chemical form of the exogenous DHA, strain SCRC-2738 incorporated the largest amounts of DHA and seems to be the best to produce DHAPL among all the test strains in this study. The DHA-incorporated strain PSB seems to be a powerful, new nutrient for marine fish cultures.

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