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<th>Significance of Antioxidative Function of Eicosapentaenoic and Docosahexaenoic Acids in Marine Microorganisms</th>
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Various marine organisms, from animals to bacteria, have n-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFAs) with 20 or 22 carbon atoms such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (7). In mammals and fish, DHA accumulates as the most abundant fatty acid among membrane phospholipids in the brain and the eye, as in land animals (11, 16). Therefore, EPA and DHA, as well as arachidonic acid (n-6), attract special interest for their physiologically important functions in humans (17, 22) and even in animals (8) and are used in pharmaceuticals and/or food supplements.

EPA and DHA are distributed universally in marine seaweeds and microalgae (7). Some marine psychrophilic and/or piezophilic bacterial species and several enteric species of marine fish and other animals can produce these LC-PUFAs (12, 53). Bacterial EPA and DHA are found exclusively in cell membrane phospholipids, and their content accounted for up to 30% (wt/wt) of the total fatty acids (38). In eukaryotic microorganisms, these n-3 LC-PUFAs are distributed in phospholipids and storage lipids (31, 50). The content of DHA in phosphatidylethanolamine and triacylglycerol of some marine thraustochytrids was more than 50 and 40%, respectively, of their totals (31, 39).

In poikilothermic organisms, EPA and DHA, like other PUFAs, are generally considered to play an important role in maintaining optimal membrane fluidity as acyl components of structural lipids under low-temperature or high-hydrostatic pressure conditions (12, 48, 52). However, whether this is their primary function has not been clarified: first, because EPA-deficient mutants derived from EPA-producing psychrophilic bacteria do not necessarily become cold sensitive (2), and second, because the ecological distribution of n-3 LC-PUFA-producing bacteria and eukaryotic microorganisms is not limited to cold environments. Interestingly, heterocont algae that include extremely high levels of DHA in storage triacylglycerol and in membrane phospholipids have been isolated even from tropical and subtropical marine environments (33, 39, 42, 50), and some n-3 LC-PUFA-producing bacteria have mesophilic growth temperatures (21).

In general, polyunsaturated fatty acids, including n-3 LC-PUFAs, are among the molecules most susceptible to oxygen and reactive oxygen species (ROS) (19). However, there is a growing body of evidence that n-3 LC-PUFAs and other LC-PUFAs are rather stable when they are in vivo against oxidative stress caused by ROS (see below and also references 5, 29, and 54). Although they are not LC-PUFA, linoleic and linolenic acids, which are the most common modulator of membrane fluidity in poikilotherms, are requisite for optimum growth, respiration, and photosynthesis (47) and for protecting the photosynthetic machinery against salt-induced damages in cyanobacteria (1). However, no clear definite role of these PUFAs has been elucidated. We review here the physiological functions and particularly the antioxidative effects of EPA and DHA in bacterial and other microbial systems in marine environments.

**DISTRIBUTION OF EPA AND DHA**

EPA and DHA are sometimes called “marine lipids” because of their preferential distribution in marine environments. Most marine animals, protozoa, seaweeds, and microalgae, and some bacterial species, have EPA and/or DHA (7, 8, 14, 17, 33). It is noteworthy that EPA and DHA are scarcely detectable in the higher land plants and terrestrial algae and that some marine seed plants contain EPA (45). Only EPA is found in some ferns and mosses and in freshwater diatoms (3). Interestingly, for green algae these n-3 LC-PUFAs are found in their marine forms but not in their terrestrial ones (3). Nichols (32) listed 20 LC-PUFA-producing bacterial species in six genera isolated from marine environments. Eighteen of them are halophilic but two, *Shewanella frigidimarina* and *Shewanella japonica*, are not. Although *S. frigidimarina* and *S. japonica* can grow in the absence of NaCl (nonhalophilic), they grow well at 9% NaCl (9) and 1 to 3% NaCl (21), respectively. Cyanobacteria, to our knowledge, have no n-3 LC-PUFAs, irrespective of their sources.

As many marine animals lack the ability to synthesize n-3 LC-PUFAs de novo (32), they rely on a dietary supply of n-3 LC-PUFAs. Microalgae have been regarded as the principal producer of n-3 LC-PUFAs in marine food webs (32), where some marine bacteria and marine animals’ enteric bacteria that have EPA or DHA may contribute as primary producers of these n-3 LC-PUFAs. Considering their preferential distribution in marine organisms, it is likely that the presence of LC-PUFAs is closely associated with a marine habitat.

**OXIDATIVE STABILITY OF EPA AND DHA IN BIOLOGICAL SYSTEMS**

PUFAs, including EPA and DHA, are cellular compounds that are easily oxidized when exposed to air or dissolved in organic solvents, because they have many bisallylic hydrogen atoms (19). However, in the aqueous system PUFAs are stable against peroxidation (5, 29, 54). In liposomes made of phos-
pholipids, higher unsaturation of fatty acids leads to their higher oxidative stability (5). Although the molecular mechanism is not known, the oxidative stability of PUFAs, when they are present as mass substance (in the bulk phase), is entirely different from that seen in aqueous biological systems.

**ANTIOXIDATIVE FUNCTIONS**

Recently, the antioxidative function of EPA was investigated using EPA-producing bacterial recombinant systems. Escherichia coli can be conferred with the ability to synthesize EPA de novo (10% or more of total fatty acids [28, 34]) by being transformed with the clustered EPA biosynthesis genes (pfaA, pfaB, pfaC, pfaD, and pfaE) derived from EPA-producing marine bacteria. The pfa genes encode proteins involved in the biosynthesis of EPA and DHA in a polyketide biosynthesis mode (28, 40, 41). This is a quite different biosynthetic way from that found in eukaryotic organisms, where PUFAs are synthesized by combination of desaturation and chain elongation of fatty acids (18). To investigate the antioxidative function of EPA, Nishida et al. (36, 37) utilized EPA-producing E. coli strains DH5α and UM2 that had been transformed with pfaA-E genes from marine Shewanella pneumatophori SCRC-2738. In the two recombinants, EPA protected cells from the effects of exogenous H₂O₂, namely, growth inhibition, carbonylation (oxidation) of cellular proteins, and breakage of the cell structure. Treating transformed cells that possess EPA with H₂O₂ did not result in notable peroxidation of the EPA. The catalase activity of E. coli DH5α cells was affected neither by transforming the cells with the pfa genes nor by treating them with H₂O₂. These results suggested that EPA had an antioxidative effect against exogenous H₂O₂. The transformant catalase-deficient mutant E. coli UM2, which included EPA, had consistently lower intracellular concentrations of H₂O₂ than the control strain, which did not include EPA, when treated exogenously with H₂O₂. Hence, EPA in E. coli transformants served to shield the membrane against the H₂O₂ molecule. Almost the same results have been obtained using the marine Shewanella marinintestina IK-1, which naturally produces EPA (ca. 20%), and its EPA-deficient mutant IK-1Δ8 (38). The antioxidative effects of EPA can be relatively easily evaluated by growth inhibition testing on plates using E. coli transformants and S. marinintestina IK-1 strains (Fig. 1).

Although there are no data to show the molecular mechanism of the membrane-shielding effects of EPA in bacterial systems, the inherent molecular structure of the phospholipids having EPA and hexadecanoic or hexadecenolic acids might be involved in the antioxidant mechanism. Thus, phospholipids with DHA or arachidonic acid have a more highly packed structure than those with less unsaturated fatty acids, and lipid membranes consisting of phospholipids with saturated fatty acids such as hexadecanoic acid (16:0) and LC-PUFAs such as DHA and probably EPA may form more hydrophobic interfaces between the phospholipid bilayers (25, 43, 44). This hydrophobic interface of the cell membrane might prevent the entry of the hydrophilic H₂O₂ molecule. The in vivo antioxidative function of EPA probably arises through a combination of EPA and its counterpart fatty acid of the phospholipid molecules to shield membranes against the effects of exogenous ROS (Fig. 2). More hydrophobic interfaces between the artificial phospholipid bilayers can be formed using phospholipid consisting of equimolar 16:0 and arachidonic acid or equimolar 16:0 and DHA (43). The similar structure could be generated in E. coli transformant cells with EPA and EPA-producing S. marinintestina, although their EPA levels were ca. 20%, respectively. E. coli DH5α transformants with more EPA became more resistant against the treatment with exogenous H₂O₂ (34, 35), suggesting that the integrity or strength of hydrophobic interfaces between the phospholipid bilayers might be dependent on the content of LC-PUFAs of membrane phospholipids.

The membrane-shielding effects of n-3 LC-PUFAs have been shown only for bacterial cells producing EPA (36–38). However, our preliminary result showed that E. coli cells carrying pfa genes responsible for DHA biosynthesis became more resistant to exogenous H₂O₂ than cells not possessing DHA (T. Nishida and H. Okuyama, unpublished results). Therefore, this function would be common for EPA and DHA. It is necessary to investigate whether LC-PUFAs other than EPA and DHA have similar effects.
The antioxidative functions of DHA that are not based on its membrane-shielding effects against ROS have been reported in animals and their cultured cells (6, 20). In animal cells, docosahexanoids, such as 17β-hydroxy-DHA, an oxidized metabolite of DHA, enhance catalase activity or the production of reduced glutathione; here, the docosahexanoids function as signal molecules. In addition, these docosahexanoids inhibit the expression of proapoptotic proteins (such as BCl-1 and Bax) that are induced by ROS (15). According to Araseki et al. (4), highly unsaturated fatty acids such as DHA are more resistant to lipid peroxidation caused by exogenous H$_2$O$_2$ in human hepatoma cells, which is analogous to the finding in *E. coli* transformant cells that produce EPA or DHA. Considering the molecular structure and specific properties of phospholipids with n-3 LC-PUFAs, their membrane-shielding function against exogenous ROS might also operate in eukaryotes, including marine algae.

Cellular EPA might have an antioxidative effect against intracellular ROS, because in the absence of extracellular H$_2$O$_2$, EPA decreased the protein carbonyl content in *E. coli* DH5α cells transformed with *pfa* genes (36). Oxidative stress causing the carbonylation of proteins occurs even in *E. coli* DH5α cells that carry a vector, that grow in the presence of antibiotics, or that grow in low-temperature conditions (36). This stress was relieved by EPA. Although no other direct data supporting the antioxidative shielding effects of EPA against endogenous ROS were obtained, the speculation described above can be supported by the fact that marine raphidophycean flagellates (*Chlorella*) that produce high levels of ROS to kill fish are EPA-accumulating microalgae (26) and that high levels of ROS are not toxic to ROS-producing cells. External stimuli (environmental stresses) also induce the generation of various ROS in organisms (30, 46, 49). Therefore, the membrane-shielding effects of LC-PUFAs appear to operate generally against endogenously produced ROS and can protect the membrane proteins (proteins inside and outside organelles for eukaryotes) from being damaged oxidatively.

Although not all psychrophilic marine bacteria necessarily have n-3 LC-PUFAs, genome projects for such bacteria, including *Cobwellia psychroerythraea*, *Desulfotalea psychrophila*, and *Flavobacterium psychrophorum*, demonstrate that these bacteria commonly have a wide variety of proteins involved in antioxidation (10, 13). This is likely because low temperature increases the solubility of oxygen and other ROS (13). Therefore, for at least n-3 LC-PUFA-producing psychrophilic bacteria such as *C. psychroerythraea*, these PUFAs (DHA for *C. psychroerythraea*) should function as antioxidative components in cell membranes under their natural low-temperature environments.

**ECOLOGICAL SIGNIFICANCE OF EPA AND DHA IN MARINE ENVIRONMENTS**

According to Lesser (24), the production of ROS is prevalent in the world’s oceans and oxidative stress is an important component of the stress response in marine organisms. In marine systems, the absorption of solar radiation by dissolved organic matter in seawater leads to the photochemical production of diverse reactive transients including ROS. Of these, H$_2$O$_2$ has the longest lifetime in seawater and the highest steady-state concentration of 10$^{-7}$ M; it can pass readily through biological membranes (24). Hence, marine organisms must be exposed to exogenous ROS including H$_2$O$_2$. In addition, some phytoplanktons (microalgae), such as dinoflagellates, produce high levels of ROS, such as superoxide anions, hydroxyl radicals, and H$_2$O$_2$ under normal physiological conditions, and these are involved in killing fish and other organisms exposed to them (26, 51). Since marine organisms cannot avoid these challenges by biotic and abiotic ROS, the membrane-shielding effects of n-3 LC-PUFAs likely operate as a primary protective “breakwater” for all marine microorganisms possessing them.

**CONCLUSION AND PERSPECTIVES**

The n-3 LC-PUFAs such as EPA and DHA are distributed preferentially in marine environments. Their primary producers are limited to microalgae and probably to some psychrophilic, piezophilic, or halophilic bacteria. These unsaturated fatty acids can be provided to all marine animals via food webs. Given their chemical stability against oxidation by ROS in organisms and the natural generation of biotic and abiotic ROS in seawaters, EPA and probably DHA could operate as antioxidative components in marine biological systems. In bacteria, and probably in microalgae, membrane phospholipids with EPA and/or DHA would function as shield molecules against such oxidative challenges exogenously and endogenously raised in marine environments.

When EPA-producing *E. coli* recombinant cells were treated with butyl hydroperoxide, a biologically inert analog of H$_2$O$_2$, the same results as with H$_2$O$_2$ were obtained (35). However, more varieties of ROS and hydrophilic and hydrophobic compounds leading to endogenous generation of ROS must be...
tested to investigate the substrate specificity of the membrane-shielding effects of n-3 LC-PUFAs using these E. coli recombinant systems. The use of marine and nonmarine bacteria that inherently produce EPA or DHA as experimental materials might help illustrate the in situ function of n-3 LC-PUFAs in marine environments, although, to our knowledge, no EPA- or DHA-producing nonmarine bacteria are available yet.

Another approach to prove the membrane-shielding effect of n-3 LC-PUFAs and probably arachidonic acid is the usage of artificial membranes as a model system. Since this effect is a purely physical function, the same membrane-shielding effect against ROS should be observed in liposomes made of phospholipids containing LC-PUFAs. Inclusion of a soluble protein(s) (and hydrophobic proteins) in the liposomes would make it possible to know how the protein can be oxidized by exogenously added ROS. In addition, physical structures of liposomes could be analyzed much more easily and precisely by instrumental analysis such as proton magnetic resonance and X-ray diffraction compared to biological membranes (43). Taken together, the structural hindrance of the cell membranes containing LC-PUFAs against ROS might be evidenced as a principal contribution factor in the stability of LC-PUFAs in biological (aqueous) systems.

As stated above, low temperature and oxidative stress are interrelated (46, 49), and the increased oxidative stresses caused by low temperature could be relieved by n-3 LC-PUFAs (36). However, it has not been elucidated whether salinity is directly related to oxidative stress. According to Leblanc et al. (23), alkyl hydroperoxide reductase was induced commonly (36). However, it has not been elucidated whether salinity is caused by low temperature could be relieved by n-3 LC-PUFAs (36).

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