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Author(s)	Nishida, Takanori; Hori, Ryuji; Morita, Naoki; Okuyama, Hidetoshi
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RESEARCH LETTER

Membrane eicosapentaenoic acid is involved in the hydrophobicity of bacterial cells and affects the entry of hydrophilic and hydrophobic compounds

Takanori Nishida^{1§†}, Ryuji Hori^{2§}, Naoki Morita³ & Hidetoshi Okuyama^{1,2}

¹ Laboratory of Environmental Molecular Biology, Faculty of Environmental Earth Science, Hokkaido University, Kita-ku, Sapporo 060-0810, Japan; ² Course in Environmental Molecular Biology and Microbial Ecology, Division of Biosphere Science, Graduate School of Environmental Science, Hokkaido University, Kita-ku, Sapporo 060-0810, Japan; ³ Research Institute of Genome-based Biofactory, National Institute of Advanced Industrial Science and Technology (AIST), Toyohira-ku, Sapporo 062-8517, Japan

Correspondence: Hidetoshi Okuyama, Laboratory of Environmental Molecular Biology, Faculty of Environmental Earth Science, Hokkaido University, Sapporo 060-0810, Japan. Tel.: +81 11 706 4523; fax: +81 11 707 2347; e-mail: hoku@ees.hokudai.ac.jp

[§]These two authors contributed equally to this work.

[†]The present address: Akita Research Institute for Food and Brewing (ARIF), Araya-machi, Akita 010-1623, Japan

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membrane-shielding effect; *Shewanella*; hydrophobic uncouplers

Running title: Bacterial cell hydrophobicity and membrane transport

Abstract

Eicosapentaenoic acid (EPA)-producing *Shewanella marinintestina* IK-1 (IK-1) and its EPA-deficient mutant IK-1Δ8 (IK-1Δ8) were grown on microtiter plates at 20 °C in nutrient medium that contained various types of growth inhibitors. The minimal inhibitory concentrations of hydrogen peroxide and *tert*-butyl hydroxyl peroxide were 100 μM and 1 mM, respectively, for IK-1 and 10 μM and 100 μM, respectively, for IK-1Δ8. IK-1 was much more resistant than IK-1Δ8 to the four water-soluble antibiotics (ampicillin sodium, kanamycin sulfate, streptomycin sulphate, and tetracycline hydrochloride) tested. In contrast, IK-1 was less resistant than IK-1Δ8 to two hydrophobic uncouplers, carbonyl cyanide *m*-chloro phenyl hydrazone (CCCP) and *N,N'*-dicyclohexylcarbodiimide (DCCD). The hydrophobicity of the IK-1 and IK-1Δ8 cells grown at 20 °C was determined using the bacterial adhesion to hydrocarbon method. EPA-containing (~10% of total fatty acids) IK-1 cells were more hydrophobic than their counterparts with no EPA. These results suggest that the high hydrophobicity of IK-1 cells can be attributed to the presence of membrane EPA, which shields the entry of hydrophilic membrane-diffusible compounds, and that hydrophobic compounds such as CCCP and DCCD diffuse more effectively in the membranes of IK-1, where they can fulfil their inhibitory activities, than in the membranes of IK-1Δ8.

Introduction

We reported previously that long-chain polyunsaturated fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) exert an antioxidative function in bacterial cells based on the cell membrane-shielding effect (Nishida *et al.*, 2006b; 2007; Okuyama *et al.*, 2008). The cell membrane-shielding effect is defined as a structural function of cell membrane phospholipids acylated in combination with a polyunsaturated fatty acid and a medium-chain saturated or monounsaturated fatty acid such as hexadecanoic acid (16:0) or hexadecenoic acid (16:1). In this structure, a more hydrophobic interface (region) of alkyl chain can be formed between the phospholipid bilayer (Rajamoorthi *et al.*, 2005; Okuyama *et al.*, 2008), and this hydrophobic structure hinders the entry of extracellular hydrophilic compounds such as hydrogen peroxide (H₂O₂). We showed that entry of H₂O₂ molecules through the cell membrane is prevented in *Escherichia coli* cells transformed with the EPA biosynthesis *pfa* genes (Nishida *et al.*, 2006a; 2006b) and in naturally EPA-producing *Shewanella marinitestina* IK-1 (Nishida *et al.*, 2007). Treatment of these bacterial cells possessing EPA with H₂O₂ maintained the intracellular concentration of H₂O₂ in these cells at a lower level than that in the reference cells without EPA. The resultant generation of protein carbonyls by H₂O₂ was suppressed to a lesser extent in cells with EPA than in cells without EPA.

Because the structure of membrane phospholipids comprising long-chain polyunsaturated fatty acids shields the entry of reactive oxygen species (ROS) such as H₂O₂, such a membrane structure should accelerate the diffusion into and capture at the membrane of hydrophobic compounds such as *N,N'*-dicyclohexylcarbodiimide (DCCD). Bacterial cells normally contain saturated and monounsaturated fatty acids with chain lengths up to C18, and one may speculate that the presence of C20 or C22 fatty acids in the cell membrane would increase the hydrophobicity of the cell and that the membrane-shielding effect of EPA and DHA could be evaluated by measuring the

hydrophobicity of the cell membranes, although this viewpoint has not been explored experimentally.

We investigated the effects of various types of hydrophilic and hydrophobic growth inhibitors on EPA-producing *S. marinintestina* IK-1 (Satomi *et al.*, 2003) and its EPA-deficient mutant strain IK-1Δ8 (Nishida *et al.*, 2007) in microtitre plates. These growth inhibitors included two water-soluble ROS, four types of water-soluble antibiotics, and two types of ethanol-soluble hydrophobic oxidative phosphorylation-uncoupling reagents. To evaluate whether the hydrophobicity of the two strains is associated with the inhibitory effects of each compound on the growth of these bacteria, cell hydrophobicity was measured by the bacterial adhesion to hydrocarbon (BATH) method (Rosenberg *et al.*, 1980).

Materials and methods

Reagents

Ampicillin sodium, kanamycin sulphate, tetracycline hydrochloride, carbonyl cyanide *m*-chloro phenyl hydrazone (CCCP), DCCD, and H₂O₂ (30%) were purchased from Wako Pure Chemicals Industries (Osaka, Japan). Streptomycin sulphate and *tert*-butyl hydroxyl peroxide (*t*-BHP) were obtained from Sigma-Aldrich Japan (Tokyo) and Kishida Chemical Company (Osaka), respectively.

Bacterial strains and cultivation

EPA-producing *S. marinintestina* IK-1 (IK-1; Satomi *et al.*, 2003) and its EPA-deficient mutant *S. marinintestina* IK-1Δ8 (IK-1Δ8; Nishida *et al.*, 2007) were used in this study. IK-1Δ8 is a knockout mutant that had been introduced with pKNOCK-Cm at the *pfaD* gene among the five *pfaA*, *pfaB*,

pfaC, *pfaD*, and *pfaE* genes responsible for biosynthesis of EPA. IK-1 was pre-cultured by agitation at 180 rpm in Luria–Bertani medium containing 3.0% (weight in volume) NaCl at 20 °C, and IK-1Δ8 was pre-cultured in the same medium that contained chloramphenicol at 50 μg mL⁻¹. When both cells were cultivated in microtitre plates, the same Luria–Bertani medium containing no antibiotics was used.

Growth inhibition testing

To perform growth inhibition tests, 96-well microtitre plates (0.35 mL well⁻¹; Iwaki, Tokyo) were used. IK-1 and IK-1Δ8 cells were grown for 24 h at 20 °C until the early stationary phase. The OD of cultures was adjusted to 1.0 at 600 nm (OD₆₀₀) with the same medium. One hundred microlitres of these cultures was diluted with 100 mL of medium. The calculated OD₆₀₀ of the diluted cultures was about 0.01. One hundred eighty microlitres of diluted IK-1 and IK-1Δ8 cultures were mixed with 20 μL of aqueous solutions containing various concentrations of growth inhibitors: H₂O₂ and *t*-BHP as ROS, and ampicillin, kanamycin, streptomycin, and tetracycline as antibiotics. CCCP and DCCD were dissolved in absolute ethanol. Two-microlitre aliquots of CCCP and DCCD were mixed with 198 μL of diluted IK-1 or IK-1Δ8 cultures. After inoculation, the plates were incubated at 20 °C for four days. Cell growth was monitored visibly, and the growth was estimated by scanning the bottom face of the microtitre plates with a scanner (type GT-F500, Epson, Tokyo). Because IK-1Δ8 has a chloramphenicol-resistant cartridge on its chromosome, chloramphenicol was added only during pre-cultivation and not during cultivation in the microtitre plates to cultivate IK-1 and IK-1Δ8 under the same conditions. IK-1Δ8 cells grown in medium containing no chloramphenicol contained no EPA (Nishida *et al.*, 2007).

Analytical procedures

The hydrophobicity of the bacterial cells was estimated by the BATH method (Rosenberg *et al.*, 1980). IK-1 and IK-1 Δ 8 cells were washed twice with 50 mM HEPES buffer (pH 8.0) containing 0.5 M NaCl. The OD₆₀₀ of the cell suspensions was adjusted to 1.0 using the same buffer. Cell suspensions of 1.8 mL in volume were overlaid with 0.3 mL of *n*-hexadecane, incubated for 10 min at 37 °C, and then mixed with a vortex for 2 min. The cell solutions stood for 15 min at room temperature, and 100 μ L of the lower (water) layer was withdrawn and its OD₆₀₀ was measured using a spectrophotometer.

The fatty acids of cells were analysed as methyl esters by gas–liquid chromatography, as described previously (Orisaka *et al.*, 2006).

Results

Effects of ROS and antibiotics on growth of cells with and without EPA

The minimum inhibitory concentrations of individual compounds were compared between IK-1 and IK-1 Δ 8. The minimum inhibitory concentrations of H₂O₂ and *t*-BHP were 100 μ M and 1 mM, respectively, for IK-1 and 10 μ M and 100 μ M, respectively, for IK-1 Δ 8 (Fig. 1a). IK-1 was more resistant to the two ROS tested than was IK-1 Δ 8. The same tendency was observed when cells of IK-1 and IK-1 Δ 8 were treated with various kinds of water-soluble antibiotics including ampicillin sodium, kanamycin sulphate, streptomycin sulphate, and tetracycline hydrochloride. The results are summarized in Table 1.

Effects of hydrophobic uncoupling compounds on growth of cells with and without EPA

The proton ionophore, CCCP, and ATP synthase inhibitor, DCCD, are water-insoluble and ethanol-soluble compounds. CCCP and DCCD were dissolved in absolute ethanol. The final concentration of ethanol in the culture medium was 1% (volume in volume), and this concentration of ethanol had no effect on the growth of IK-1 or IK-1 Δ 8. The minimum inhibitory concentrations of CCCP and DCCD were 1 μ M and 1 mM, respectively, for IK-1 and 10 μ M and > 10 mM, respectively, for IK-1 Δ 8 (Fig. 1b and Table 1). Although the growth of IK-1 Δ 8 at 1 mM and 10 mM DCCD seemed lower than that at \leq 0.1 mM DCCD after four days at 20 °C (Fig. 1b), prolonged incubation of all IK-1 Δ 8 cultures at a DCCD concentration of \leq 10 mM produced almost the same turbidity. In contrast, growth of IK-1 was never observed at a concentration of DCCD of \geq 1 mM.

Hydrophobicity of bacterial cells with and without EPA

The cell surface hydrophobicity is expressed as the percent adhesion of bacterial cells to water measured by the BATH method (Rosenberg *et al.*, 1980). In cells grown at 20 °C, the values were 94% \pm 1% and 99% \pm 1% for IK and IK-1 Δ 8, respectively: the surface hydrophobicity was greater in IK-1 cells, in which EPA comprised 8% of the total fatty acids, than in IK-1 Δ 8 cells.

Discussion

IK-1 with EPA was more resistant than IK-1 Δ 8 with no EPA to H₂O₂ and to *t*-BHP, an analogue of H₂O₂ (Fig. 1a and Table 1), suggesting that catalases or other H₂O₂-decomposing enzymes are not

involved in the resistance of IK-1. The finding that IK-1 was slightly more resistant to all the water-soluble antibiotics tested than was IK-1Δ8 (Table 1) suggests that hydrophilic compounds other than ROS may be prohibited from entering the cell through the cell membrane by the membrane-shielding effect more efficiently in IK-1 than in IK-1Δ8 cells, as was the case for hydrophilic ROS. However, in Gram-negative bacteria, hydrophilic antibiotics with molecular weight less than about 600 pass non-specifically through porin channels on the outer membrane and not by diffusion (Nikaido & Vaara, 1985) and the compounds that enter the cells can be pumped out from the cells (Walsh, 2000; Martinez *et al.*, 2009). Therefore, the membrane-shielding effects of EPA are not necessarily involved directly in the higher resistance to these antibiotics in IK-1 cells. However, because the entry of streptomycin sulphate, whose molecular weight (1457.4) is much higher than 600, must not depend on porin channels but must involve electrostatic interactions between the antibiotic and the negatively charged lipopolysaccharide (Magalhães & Blanchard 2009), the uptake of this compound could be affected directly by the membrane-shielding effect.

Kawamoto *et al.* (2009) suggested recently that EPA affects the synthesis of some membrane proteins at low temperature (4 °C) in the cold-adapted bacterium *Shewanella livingstonensis* Ac10 because the protein levels decreased in EPA-deficient mutants of this strain. One such protein (Omp_C176) is inducibly produced in parental cells at 4 °C (Kawamoto *et al.*, 2007). It was suggested that the stability of Omp_C176 and other outer membrane proteins at low temperature depends on EPA-containing phospholipids and that such proteins facilitate the membrane passage of hydrophilic nutrients through porin (Kawamoto *et al.*, 2009). However, this would not be applicable to IK-1 for the following reasons. First, IK-1 and IK-1Δ8 were cultivated at 20 °C in this study. Second, the effects on the stability and abundance of porin proteins such as Omp_C176, which should accelerate the entry of both nutrients and growth inhibitors with molecular weight less than about 600 into cells possessing EPA and thereby induce greater resistance to antibiotics in IK-1 cells

with EPA than in IK-1Δ8 with no EPA, are controversial. Third, an *E. coli* recombinant with EPA grown at 20 °C was also more resistant to water-soluble antibiotics than was the control *E. coli* recombinant with no EPA (R. Hori, T. Nishida, and H. Okuyama, unpublished results).

One principal strategy for bacterial survival against drugs such as antibiotics is an ability to pump these compounds out of the cell (Walsh, 2000; Martinez *et al.*, 2009). Although we have no biochemical or molecular evidence, it is possible that EPA (and other polyunsaturated fatty acids) increases the activity of membrane efflux pumps in EPA-producing bacteria; the synthesis of some porin proteins is accelerated in EPA-producing *S. livingstonensis* Ac10 (Kawamoto *et al.*, 2009). Interestingly, a group of proteins whose concentrations are decreased by EPA depletion in the mutant of *S. livingstonensis* Ac10 include a tentative TolC family protein. It is known that TolC is involved in efflux of enterobactin (Bleuel *et al.*, 2005) and various types of drugs (Nikaido 1996; Blair & Piddock, 2009) across the outer membrane in *E. coli*. Therefore, EPA may affect the synthesis of some efflux proteins or protein structures, irrespective of temperature.

According to Andersen and Koeppe (2007), the lipid bilayer thickness correlates with membrane protein functions. Interestingly, polyunsaturated fatty acids such as DHA, EPA, and arachidonic acid may modulate membrane protein functions, including various channel and enzyme activities, through bilayer-mediated mechanisms that do not involve specific protein binding but rather changes in bilayer material properties (e.g., thickness, curvature, elastic compression, and bending modulus) in prokaryotic and eukaryotic systems. Compounds that pass through the membrane via porin, by diffusion, or by other ways can be pumped out of bacterial cells through these efflux pump proteins whose functions could be altered by changes in non-specific physical properties of the phospholipid bilayer. The possibility cannot be excluded that the bilayer structure of phospholipids with a hydrophobic alkyl region (interface), which is generated by EPA-containing

phospholipids, affects the efflux pumping activity of compounds including growth inhibitors through the membranes.

The hydrophobicity or hydrophilicity of microbial cells varies between microbial species, and these properties are associated with various functions (see Rosenberg & Doyle, 1990). IK-1 cells had a higher hydrophobicity than did IK-1Δ8 cells. No difference in the phospholipid composition was observed between IK-1Δ8 and IK-1 cells (Nishida *et al.*, 2007), suggesting that fatty acid composition (i.e., the presence of EPA) leads to higher hydrophobicity of IK-1 cells. Phospholipid bilayers with a hydrophobic interfacial region are permeable to hydrophobic molecules, and the permeability is greater for more hydrophobic solutes (Cohen & Bangham, 1972). This would be applicable primarily to the outer membrane of Gram-negative bacteria, where the lipid bilayer is formed, because the outer leaflet comprises mainly lipopolysaccharide and the inner leaflet mainly phospholipids (Nikaido & Vaara, 1985), and to the cell membrane (inner membrane), where the outer and inner leaflets comprise phospholipids. The membrane-shielding effect of EPA-containing phospholipids would operate in both the outer and inner cell membranes. The lower minimum inhibitory concentrations of CCCP and DCCD (Table 1) for IK-1 demonstrate that these hydrophobic compounds tend to remain and to operate in the more hydrophobic cell membrane. These data indicate that the fatty acid composition of the outer and inner membranes should be analysed separately. According to Allen *et al.* (1999), a deep sea EPA-producing bacterium, *Photobacterium profundum* SS9, includes almost similar levels of EPA (~5% of total fatty acids) in the outer and inner membranes.

Interestingly, EPA-producing mutants of the eukaryotic monocellular alga eustigmatophyte, *Nannochloropsis oculata*, become more resistant to higher concentrations of cerulenin and erythromycin, both of which are slightly water soluble, compared with the wild type (Chaturvedi & Fujita, 2006). For example, the growth of wild-type cells was inhibited completely by cerulenin at a

concentration of 25 μM , but cerulenin at 75 μM had no effect on the growth of the mutant (CER1). Although the involvement of the membrane-shielding effect of EPA has not been investigated, it may be operative even in eukaryotic organisms. The wild type of this alga contains 16:0 (17% of total), 16:1 (17%), and EPA (38%) as the major fatty acids, and the contents of EPA and 16:0 and 16:1 fatty acids increase in antibiotic-resistant mutants.

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Table 1. Effects of various hydrophilic and hydrophobic compounds on the growth of cells of *S. marinintestina* IK-1 and its EPA-deficient mutant IK-1Δ8.

	Solvent	MICs of various compounds	
		IK-1	IK-Δ8
Reactive oxygen species (MW)			
H ₂ O ₂ (34.0)	Water	100 μM	10 μM
<i>t</i> -BHP (90.1)	Water	1 mM	100 μM
Antibiotics			
Ampicillin sodium (371.4)	Water	1000 μg mL ⁻¹	500 μg mL ⁻¹
Kanamycin sulphate (582.6)	Water	250 μg mL ⁻¹	100 μg mL ⁻¹
Streptomycin sulphate (1457.4)	Water	300 μg mL ⁻¹	150 μg mL ⁻¹
Tetracycline hydrochloride (480.9)	Water	6 μg mL ⁻¹	3 μg mL ⁻¹
Oxidative phosphorylation uncouplers			
CCCP (204.1)	1% ethanol ^a	1 μM	10 μM
DCCD (206.3)	1% ethanol ^a	1 mM	> 10 mM

MW, molecular weight; MICs, minimal inhibitory concentrations; *t*-BHP, *tert*-butyl hydroxyl peroxide; CCCP, carbonyl cyanide *m*-chloro phenyl hydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide.

^a Final concentration.

Figure legends

Fig. 1. Effects of concentration of various compounds on the growth of *S. marinintestina* IK-1 and its EPA-deficient mutant IK-1Δ8. (a) Hydrogen peroxide (H₂O₂) and *t*-butyl hydroperoxide (*t*-BHP); (b) carbonyl cyanide *m*-chloro phenyl hydrazone (CCCP) and *N,N'*-dicyclohexylcarbodiimide (DCCD). Cells were grown for four days at 20 °C.

Fig. 1.

