Production of Eicosapentaenoic Acid from Marine Bacteria

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ABSTRACT: A marine bacterium, judged as a new species close to *Shewanella putrefaciens*, was isolated from the intestinal contents of the Pacific mackerel. The isolated strain SCRC-2378 produced eicosapentaenoic acid (EPA) as the sole polyunsaturated fatty acid, which amounted to 24–40% of the total fatty acid in the cell, which corresponded to 2% of dry cell weight. Under the optimal growth conditions (pH 7.0, 20°C, and grown aerobically for 12–18 h), the yield of SCRC-2738 reached 15 g of dry cells/L or 2×10^{10} viable cells/mL. EPA existed as phospholipid and was found in the *sn*-2 position of phosphatidylethanolamine and phosphatidylglycerol. The 38 kbp (1,000 base pairs) genome DNA fragment was cloned from SCRC-2738 and expressed in *Escherichia coli*, which resulted in the production of EPA. The nucleotide sequence of the 38 kbp DNA fragment was determined. The DNA fragment contains eight open reading frames, and three of them possess homology with enzymes involved in fatty acid synthesis. Thus, it may be possible that these EPA biosynthesis genes are applied for EPA production in yeasts or higher plants, and offer a new method for EPA synthesis as new foods containing EPA.
Lipids 31, S-297–S-300 (1996).

Eicosapentaenoic acid (EPA) is an important component in the cell membranes of animals, and its pharmacological functions are well known (1). The principal bioactivities associated with EPA are summarized in Table 1. The pharmacological functions of EPA have generally been explained from the viewpoint of an antagonistic action against arachidonic acid (2–7), especially in the case of coronary vascular disease.

The origin of EPA has been explained by a series in the marine food chain as follows: microalgae → phytoplankton → zooplankton → small fish → larger fish and marine animals. It is generally accepted that the phytoplankton are the primary producers of EPA. However, it was thought EPA may also be produced by some of the symbiotic microorganisms which are present in the intestines of blue-back fish rich in EPA, in addition to being produced by the food chain series described previously. Thus, our search for EPA-producing bacteria started, and we succeeded in finding bacteria which produced EPA. We first cloned the EPA biosynthesis gene cluster from the EPA-producing bacteria, and its analysis is in progress.

Abbreviations: EPA, eicosapentaenoic acid; GLC, gas–liquid chromatography; kbp, 1,000 base pairs; ORF, open reading frame; PE, phosphatidylethanolamine; pEPA, the plasmid isolated from strain No. 95 of SCRC-2738; PG, phosphatidylglycerol.

TABLE 1
Distribution of Fatty Acids in Phospholipids in SCRC-2738

	Fatty acid composition (mol%)			
	Phosphatidylethanolamine		Phosphatidylglycerol	
Fatty acids	*sn*-1 (α)	*sn*-2 (β)	*sn*-1 (α)	*sn*-2 (β)
14:0	3.2	0.8	1.1	0.4
i-15:0[a]	11.5	7.2	4.5	2.1
15:0	0.5	0.3	3.4	0.4
16:0	12.1	0.6	10.7	1.6
16:1	6.8	20.9	8.0	11.5
i-17:0	0.6	0	0.9	0
17:0	1.8	0	2.7	0.3
17:1[b]	4.0	6.4	5.7	6.1
17:1[b]	1.6	0	2.2	0.3
18:0	0.4	0.1	0.6	0.3
18:1(9)	1.1	1.0	1.5	1.2
18:1(11)	6.1	0.7	7.8	1.1
20:5	0.1	11.8	1.0	24.7

[a]*iso*-Type fatty acid.
[b]Isomers in position of double bond.

METHODS

The intestinal contents of the marine animals were diluted with sterilized seawater and the diluted contents were cultured in dozens of agar media for the selection of bacterial colonies. Various conventional media supplemented with artificial seawater (Jamarin Laboratory, Osaka, Japan) were used (8,9). Colonies formed on various agar media were cultivated singly in the liquid media to test for the presence of EPA in the cellular lipid. Each EPA-positive strain was tested immediately for bacteriological purity. The promising strains were then subjected to tests involving about 60 items for classification and identification and, after testing, were subcultured and stored.

The lipids of bacterial cells were extracted by the Folch method (chloroform/methanol, 2:1, vol/vol). The total lipid extract was then saponified and the free fatty acids obtained were methylated with CH_2N_2 and analyzed by gas–liquid chromatography (GLC) equipped with a capillary column.

Chromosomal DNA was isolated from strain SCRC-2738, an EPA-producing bacteria. The DNA was partially digested with Sau3AI and fractionated by size on 0.3% agarose gel (Sigma, St. Louis, MO). The fragments longer than 20 kbp (kbp = 1,000 base pairs) were recovered by electroelution and then ligated with BamHI-digested and dephosphorylated cosmid pWE15. The ligated DNA were packaged into the phage heads and transfected into *Escherichia coli* AG1. The trans-

formants were selected for expression of ampicillin resistance and cultivated singly in the liquid media to test for the presence of EPA in the cellular lipid.

The plasmid was isolated from an EPA-producing clone and its insert DNA fragment was subcloned for sequencing. The nucleotide sequence was determined by the dideoxy chain termination procedure.

RESULTS

About 50,000 bacterial strains were isolated from the intestines of various marine fish and other marine animals. Each of those strains was cultured as a single clone and was assessed for EPA levels by GLC analysis. EPA-producing bacteria were isolated with relatively high frequency from the blue-black fish containing high levels of EPA in their oils.

There were also several new species of marine bacteria which were phylogenetically similar to *Shewanella putrefaciens*. Among them, the intestinal strain SCRC-2738, isolated from a horse mackerel (9), had the highest EPA productivity. An electronmicrograph of SCRC-2738 (Fig. 1) indicates its size and presence of multiple flagella.

The fatty acid composition of the bacterium was simple and characteristic (10)—EPA was the only polyunsaturated fatty acid detected in significant quantities. In addition to EPA, several monoenoic acids and saturated fatty acids of the C_{13}–C_{18} range were detected. The percentage of EPA to the total fatty acids ranged from 25–40%, depending on the conditions of cultivation. In the fatty acid composition of SCRC-2738, almost none of the polyunsaturated fatty acids, varying from $C_{18:2}$ to $C_{20:4}$, were detected. This fact could be due to a high level of activity of the series of biosynthetic enzymes producing EPA.

We concluded that 90–95% of the EPA was in the form of phospholipids, and the free fatty acid fraction accounted for the remaining 5–10%. Furthermore, EPA was distributed almost equally between phosphatidylethanolamine (PE) and phosphatidylglycerol (PG). The PE and PG were studied further to determine the positional specificity of the EPA within the molecules. The PE and PG fractionated by thin-layer chromatography were isolated and mixed with snake venom phospholipase. It was found that the EPA in both PE and PG was specifically bound by an ester bond to the *sn*-2 position (Table 1).

The SCRC-2738 was cultivated in a medium (half-strength, artificial seawater containing 1–3% peptone and

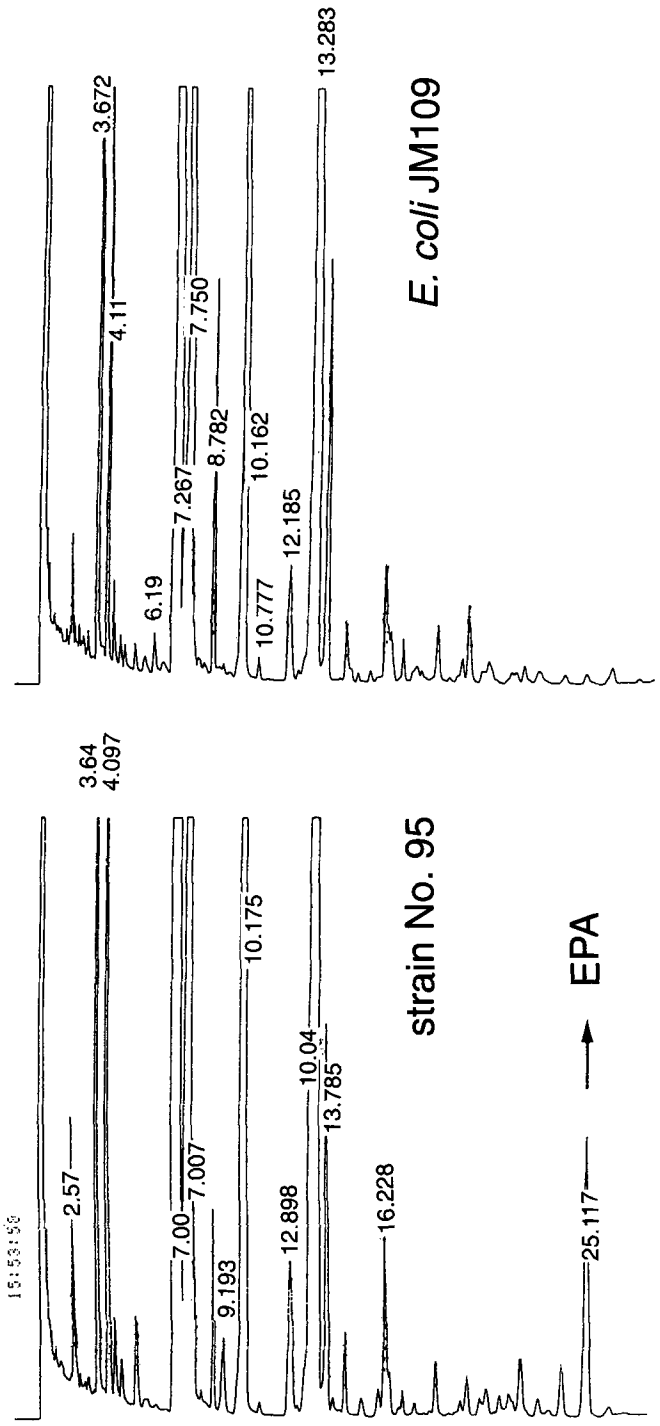

FIG. 1. Transmission electron micrograph of SCRC-2738 with negative staining.

FIG. 2. Gas–liquid chromatography of fatty acid methyl esters from the cells of strain No. 95 and *Escherichia coli* JM109. EPA, eicosapen-

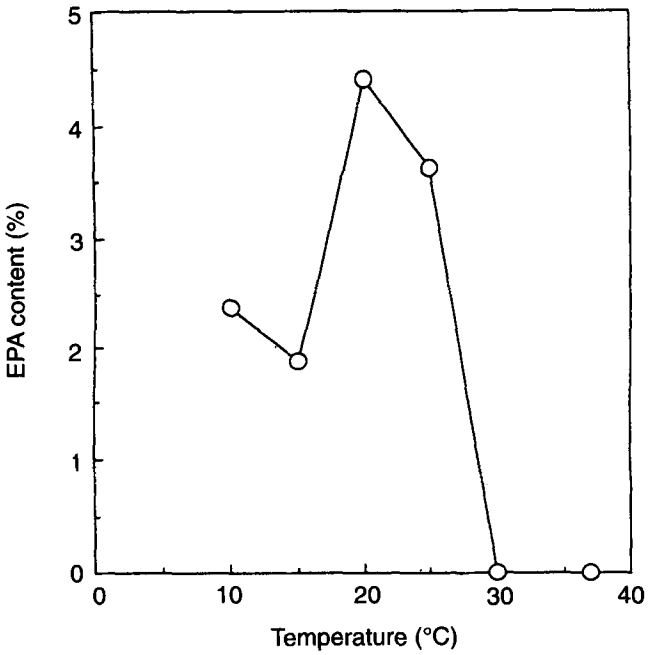

FIG. 3. Effect of growth temperature on EPA content in total fatty acids of strain No. 95. Abbreviation as in Figure 2.

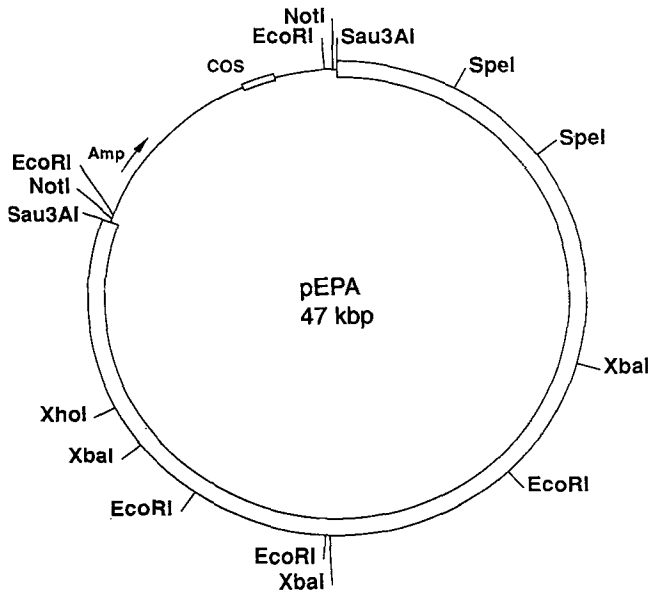

FIG. 4. Restriction map of plasmid pEPA. Abbreviation as in Figure 2.

0.5–1% yeast extract, pH 7.0) at 10 to 15°C with plenty of aeration. Under these conditions, 200–400 mg EPA was produced per liter of culture in 8 to 12 h (11). Furthermore, good EPA productivity was obtained when corn steep liquor or marine industrial waste liquid was used as a carbon source, thereby considerably reducing the overall fermentation costs. In this case, lactic acid was used to prevent the production of NH_3 and the increase of pH during cultivation. It may be possible to further reduce the cost of production by using a fed-batch culture, a high-density culture, and/or gene technology.

A genome DNA library of the SCRC-2738 was constructed and screened by EPA production. In a total of 390 analyzed clones, only strain No. 95 showed EPA productivity (Fig. 2). This means that strain No. 95 has the complete plasmid containing EPA biosynthesis genes.

It is known that growth temperature affects cellular EPA contents in SCRC-2378. As the cultivation temperature increased above 25°C, the productivity of EPA decreased, and no EPA production was observed at 30°C or higher temperature. The study of recombinant strain No. 95 indicated similar results (Fig. 3).

The plasmid was isolated from strain No. 95 and was designated pEPA; pEPA contains a 38 kbp genome DNA fragment from SCRC-2738 in the BamHI site of 8.8 kbp cosmid pWE15 (Fig. 4). The 38 kbp DNA fragment was subcloned and its nucleotide sequence was determined. In this fragment, eight open reading frames (ORF) larger than 1.0 kbp existed. To determine the ORF responsible for EPA synthesis, several deletion mutants of pEPA were constructed. pEPA-d1, which lacks XhoI-AscI fragment containing ORF1, indicates EPA

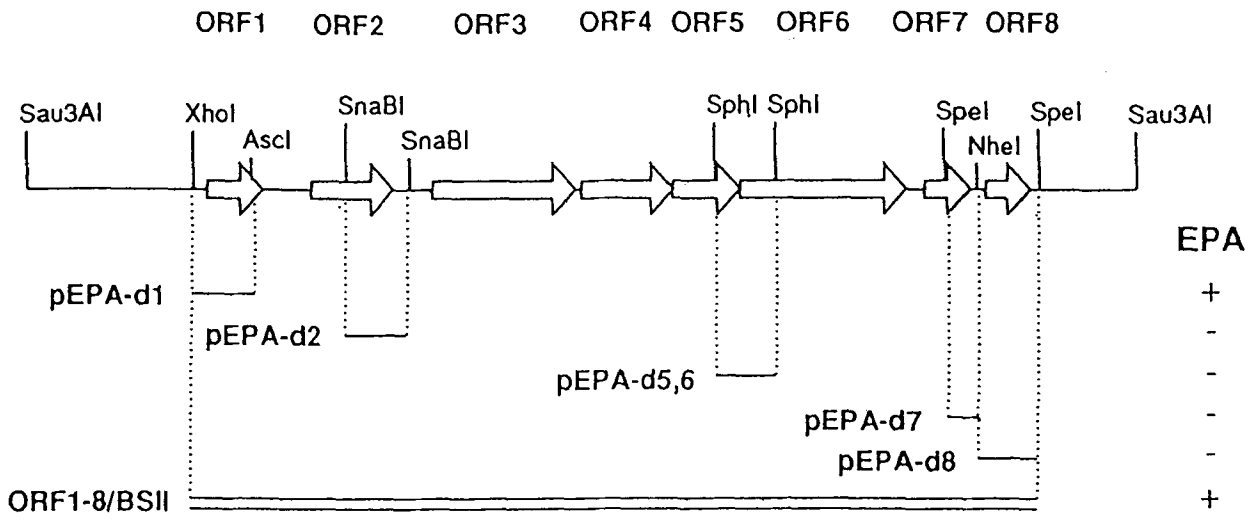

FIG. 5. EPA productivity of deletion mutants of pEPA. ORF, open reading frame; other abbreviation as in Figure 2.

TABLE 2
Homologous Enzymes with Open Reading Frames (ORF) of pEPA[a]

ORF no.	Enzymes (origin)
3	3-Oxoacyl-ACP synthase I (*Escherichia coli*) 26.9%/290aa
3	Malonyl CoA-ACP transacylase (*E. coli*) 29.1%/265aa
4	3-Oxoacyl-ACP reductase homologous protein (*Saccharopolyspora erythraea*) 30.5%/167aa
6	3-Oxoacyl-ACP synthase (Barley chloroplast) 29.1%/234aa
6	3-Hydroxydecanoyl-ACP dehydratase (*E. coli*) 31.0%/142aa

[a]pEPA (eicosapentaenoic acid) is the plasmid isolated from recombinant *E. coli* strain No. 95.

FIG. 6. Application of EPA biosynthesis genes for EPA production in yeast or plant. Abbreviation as in Figure 2.

productivity. However, pEPA-d2 (lacking ORF2), pEPA-d5,6 (lacking ORF5 and ORF6), pEPA-d7 (lacking ORF7), and pEPA-d8 (lacking ORF8) lost EPA productivity (Fig. 5). These results suggest that these ORF, except for ORF1, are involved in EPA synthesis. The deletion studies of other ORF are in progress.

A homology search of amino acid sequences deduced from the nucleotide sequences of the ORF with proteins in our database was done. ORF3, ORF4, and ORF6 showed a similarity with several enzymes involved in fatty acid synthesis (Table 2). This finding suggests that these ORF are involved in fatty acid *de novo* synthesis or in carbon chain elongation. Therefore, it is suggested that the ORF coding desaturase genes exist in the remaining ORF. The function of these ORF may be revealed by further studies. It may be possible that these EPA biosynthesis genes are applied for EPA production in yeast or plant, and offer a new method for polyunsaturated fatty acid synthesis (Fig. 6).

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