Eicosapentaenoic Acid Productivity of the Bacteria Isolated from Fish Intestines

Kazunaga Yazawa,^{*1} Keiko Araki,^{*1} Kazuo Watanabe,^{*1}

Chikako Ishikawa,^{*1} Akira Inoue,^{*1} Kiyosi Kondo,^{*1}

Shugo Watabe,^{*2} and Kanehisa Hashimoto^{*2}

(Received April 4, 1988)

Attempts were made to find eicosapentaenoic acid(EPA)-producing microorganisms. Out of some 7,000 bacterial strains screened, 112 of them showed more or less this activity. Most of them were isolated from intestinal contents of marine fishes which were known to contain high levels of EPA in their body lipid. A bacterial strain isolated from the Pacific mackerel *Pneumatophorus japonicus* showed a high productivity of EPA (3.8 to 10.8 mg/g dry cells) and also a high ratio of EPA to total fatty acids (27.8 to 36.3%). The strain was Gram-negative, motile, obligate aerobic and short rod-shaped, which we judge it to be a new species close to genus *Alteromonas*.

The epidemiological investigation on Greenland Eskimos by Dyerberg and co-workers^{1,2)} demonstrated that EPA is effective for prevention and cure of thrombosis, arteriosclerosis and subsequent blood-circulatory diseases. In other words, EPA is allegedly important for us to maintain homeostasis.^{1,3-6)} Some marine fishes also require EPA as essential, and usually contain this fatty acid at a fairly high level.^{7,8)}

It is generally accepted that EPA in fish comes from marine algae and phytoplankton via the food chain.⁹⁾ Recently, Wirsen *et al.*¹⁰⁾ have claimed that a psychrophilic and barophilic marine bacterium collected from the deep sea contained "a C_{20:5} polyunsaturated fatty acid" of unknown structure. Many organisms, such as bacteria, fungi, plants, and even animals are known to maintain the optimal fluidity and function as the environmental temperature or pressure varies, by altering proportions of unsaturated fatty acids.^{11,12)} This situation prompted us to search for EPA elaborators from marine microorganisms inhabiting fish intestines and other environments. We have already isolated some EPA-producing bacteria and reported the data elsewhere.¹³⁾ Further screening for EPA-producers was performed and this report deals with the results obtained, along with identification of a bacterium which showed a notable productivity of EPA.

Materials and Methods

Some 7,000 strains of marine microorganisms were isolated from various marine sources such as intestinal contents of fish and shellfish, zooplankton, seawater and sediment (Table 1). Bacterial colonies were picked up from such samples by the usual method using the following media; GAM "Nissui", Nutrient agar "Nissui", Salt polymyxin "Nissui", TCBS agar "Eiken", BTB Teepol agar "Nissui", Mannitol salt agar "Eiken", NAC agar "Eiken", Potato dextrose agar "Nissui", Sabouraud agar "Nissui", Czapek Dox agar "Nissui", Bacto-Marine agar 2216, Malt agar "Nissui", and Trypto-Soy agar "Eiken". Each strain was cultured at 25°C for 2 days in "P-Y-M-Glucose medium" which contained 1% peptone, 0.5% yeast extract, 0.25% meat extract and 2% glucose in 1/2 concentration of an artificial seawater ("Jamarin S", Jamarin laboratory, Osaka). Cells were harvested by centrifugation and extracted with chloroform-methanol (2:1, v/v). The extract was evaporated to eliminate the organic solvents. The resulting lipid was saponified by the usual method, and analyzed for fatty acids by gas liquid chromatography (GLC) after methylation with diazomethane. Sterols and other non-saponifiable matters were checked by thin-layer chromatography (TLC) using; n-hexane: ethyl ether: formic acid (80: 20: 0.2, v/v) as

^{*1} Sagami Chemical Research Center, Nishi-Ohnuma, Sagami-hara, Kanagawa 229, Japan (矢澤一良, 荒木恵子, 渡部和郎, 石川千夏子, 井上 玲, 近藤 聖: 相模中央化学研究所).

^{*2} Laboratory of Marine Biochemistry, Faculty of Agriculture, University of Tokyo, Bunkyo, Tokyo 113, Japan (渡部終五, 橋本周久: 東京大学農学部).

Table 1. Details of the fish and other samples used

	Place and date of collection
Fishes:	
Pacific mackerel <i>Pneumatophorus japonicus</i>	Saganoseki, Oita Pref., Feb. 1987
Horse mackerel <i>Trachurus japonicus</i>	Ajiro, Kanagawa Pref., Sept. 1986
Sardine <i>Sardinops melanosticta</i>	Onahama, Fukushima Pref., April 1987
Flying fish <i>Cypselurus heterurus dodderleini</i>	Masuda, Shimane Pref., July 1987
Saury-pike <i>Cololabis saira</i>	Hokkawa, Shizuoka Pref., May 1987
Yellowtail <i>Seriola quinqueradiata</i>	Masuda, Shimane Pref., July 1987
Anchovy <i>Engraulis japonica</i>	Kamakura, Kanagawa Pref., Nov. 1986
Pike <i>Sphyræna pinquís</i>	Masuda, Shimane Pref., July 1987
Alaska pollack <i>Theragra chalcogramma</i>	Yoichi, Hokkaido, Jan. 1987
Chum salmon <i>Oncorhynchus keta</i>	Sanriku, Iwate Pref., Dec. 1986
Invertebrates:	
Squid <i>Doryteuthis bleekeri</i>	Hokkawa, Shizuoka Pref., May 1987
Krill Euphausiacea	Onahama, Fukushima Pref., April 1987
Seawater	Nagai, Kanagawa Pref., Oct. 1986
Sediment	Nagai, Kanagawa Pref., Oct. 1986

solvent.

Transmittance electron microscopy was performed on a TEM-100CX (JEOL, negative stain) by the usual method. Biological and biochemical characters of a bacterium were examined according to the Bergey's manual.¹⁴⁾

Results and Discussion

Results showed that 112 out of some 7,391 strains tested produced EPA. One hundred and four strains of them were isolated from intestinal contents of marine fishes such as Pacific mackerel, horse mackerel, sardine, flying fish and saury-pike, all of which usually contain EPA at a high level. On the contrary, few EPA-producing microorganisms were isolated from other organisms, seawater, *etc.* (Table 2). This fact suggests that the said fishes directly utilize EPA produced by those bacteria, in addition to EPA supplied by the food chain.

One of the EPA elaborators isolated from Pacific mackerel exhibited a remarkably high ability when cultured in P-Y-M-Glucose medium at 25°C for 18 h. This strain, tentatively named SCRC-2738, was Gram-negative, rod-shaped, motile by means of peritrichous flagella, and essentially required a natural or artificial seawater and oxygen for growth.

SCRC-2738 strain which was 0.5–1.5 µm diameter and 1.5–2.5 µm length (Fig. 1), was examined for 60 items of various biological and biochemical characters. Over 50 out of 60 items of characters of our strain were the same as those

Table 2. Detection of EPA-producing bacteria in marine fishes

	EPA-producing strains	/Strains tested
Pacific mackerel	40 (8)* ¹ /1403	(2.9%)* ²
Horse mackerel	36 (1)/1346	(2.6%)
Sardine	11 (4)/ 748	(1.5%)
Flying fish	12 (3)/ 650	(1.8%)
Saury-pike	5 (2)/ 295	(1.7%)
Yellowtail	4 (0)/ 328	(1.2%)
Anchovy	2 (2)/ 360	(0.6%)
Pike	1 (0)/ 333	(0.3%)
Alaska pollack	1 (0)/ 424	(0.2%)
Chum salmon	0 / 280	
Squid	0 / 536	
Krill	0 / 268	
Seawater	0 / 196	
Sediment	0 / 224	
	112 (20)/7391	

*¹ Highly producing strains (> 10 mg/l).

*² Frequency of EPA-producing strains.

of *Alteromonas putrefaciens*. As shown in Table 3, the strain was generally similar to *Alt. putrefaciens*^{14,15)} except for several important characters such as ornithine decarboxylase activity, citrate utilization, seawater requirement, and growth at 37°C.

In addition, both bacteria distinctly differed from each other in respect of flagellation: Our strain was peritrichous, whereas the cited and any other *Alteromonas* strains so far reported not. On the other hand, genus *Alcaligenes* has peritrichous flagella. However, genus *Alcaligenes* and SCRC-2738 distinctly differed from

Fig. 1. Electron micrograph of a new EPA-producing bacterium SCRC-2738.

Fig. 2. EPA-production and fatty acid composition of the bacterium SCRC-2738 which was cultivated at 25°C for 18 h or 4°C for 5 days.

each other in respect of G+C contents of DNA (*Alcaligenes* had 56–70%, whereas SCRC-2738 had 45.0%), and other important characters such as gelatin hydrolysis, DNase and growth at 37°C. The facts that SCRC-2738 required seawater and did not grow at 37°C, along with the fact that it contained no sterols in the cell as demonstrated by TLC (data not shown), indicate that our strain SCRC-2738 was a marine bacterium. Based on these results, we have concluded that SCRC-2738 strain is similar to, but not identical with, genus *Alteromonas*. The strain showed the optimum growth when cultured at the initial

pH 7.0 and at 25°C for 18 h aerobically, resulting a yield of 7 g dry cells/l medium, or 2×10^{10} viable cells/ml. It showed a doubling time of 30 min on the exponential phase. The amount of EPA produced increased in parallel with cell growth, reaching a maximum of 26.1 mg/l or 3.8 mg/g dry cells, on the late exponential phase. The strain did not grow at all at pH 5 or 10, and at 37°C.

GLC demonstrated that this bacterium exclusively possessed EPA (27.8%) as a polyunsaturated acid, in addition to oleic (10.2%), palmitoleic (25.9%) and myristoleic (17.1%) as

monounsaturated acids, palmitic (15.6%) and myristic (3.4%) as saturated acids, along with less than 5% of unidentified fatty acids. Even traces of other polyunsaturated fatty acids such as arachidonic, linolenic and linoleic acids were not detected. The strain disclosed an EPA productivity of 30.3 mg/l (10.8 mg/g dry cells) and the EPA to total fatty acid ratio of 36.3%, when cultivated at 4°C for 5 days (Fig. 2). These results suggest that the increase of EPA might be important for the bacterium to maintain cell fluidity at lower temperatures.¹⁸⁻¹⁹⁾

As described above, the bacterial strain SCRC-2738 contained EPA as the sole polyunsaturated acid, lacking in any fatty acids having (n-3) and (n-6) structure. This suggests that the desaturating and/or elongating enzymes for the precursors of EPA are quite active in this bacterium.¹⁹⁾

Further investigations are needed to clarify the metabolism, biological activities, and ecological significance of EPA in the marine host animals.

We are indebted to Dr. K. Yamasato and Miss M. Akagawa, Institute of Applied Microbiology, University of Tokyo, for kind advices on identification of the bacterium.

References

- 1) J. Dyerberg, H. O. Bang, E. Stofferson, S. Moncada and J. R. Vane: *Lancet*, ii, 117-119 (1978).
- 2) J. Dyerberg and H. O. Bang: *Haemostasis*, **8**, 227-233 (1979).
- 3) T. O. Von Rossonczy, A. Ruiter, H. C. Brongeeest-Schoute, C. M. Van Gent and R. J. J. Hermus: *Amer. J. Clin. Nutr.*, **31**, 1340-1346 (1978).
- 4) P. Singer, M. Wirth, S. Voigt, E. Naumann, S. Zimontkowski, I. Hajdu and W. Goedicke: *Atherosclerosis*, **49**, 99-108 (1983).
- 5) P. Singer, M. Wirth, S. Voigt, S. Zimontkowski, W. Goedicke and H. Heine: *Biomed. Biochim. Acta*, **43**, s421-s425 (1984).
- 6) Y. Terano, A. Hirai, T. Hamazaki, S. Kobayashi, T. Fujita, Y. Tamura and A. Kumagai: *Atherosclerosis*, **46**, 321-331 (1983).
- 7) S. Murota and I. Morita: *Igakunoayumi*, **130**, 787-798 (1984).
- 8) Y. Ohno and I. Hara: *Yukagaku*, **30**, 784-790 (1981).
- 9) M. Kayama, Y. Tsuchiya and J. F. Mead: *Nippon Suisan Gakkaishi*, **29**, 452-458 (1963).
- 10) C. O. Wirsén, H. W. Jannasch, S. G. Wakeham and E. A. Canel: *Current Microbiol.*, **14**, 319-322 (1987).
- 11) A. A. Yayanos and E. F. Deloy: *Science*, **228**, 1101-1102 (1985).
- 12) A. G. Marr and J. L. Ingraham: *J. Bacteriol.*, **84**, 1260-1267 (1962).
- 13) K. Yazawa, K. Araki, N. Okazaki, K. Watanabe, C. Ishikawa, A. Inoue, N. Numao and K. Kondo: *J. Biochem.*, **103**, 5-7 (1988).
- 14) P. Baumann, M. J. Gauthier, and L. Baumann: in "Bergey's Manual of Systematic Bacteriology" (ed. by N. R. Krieg and J. G. Holt) Vol. 1, Williams and Wilkins, London, 1984, pp. 343-352.
- 15) M. T. MacDonell and R. R. Colwell: *System. Appl. Microbiol.*, **6**, 171-182 (1985).
- 16) R. D. Jones and F. G. Prahl: *Mar. Ecol. Prog. Ser.*, **26**, 157-159 (1985).
- 17) Y. Hasegawa, N. Kawada and Y. Nosoh: *Arch. Mikrobiol.*, **126**, 103-108 (1980).
- 18) M. Oshima and A. Miyakawa: *Lipids*, **9**, 476-480 (1974).
- 19) J. Gellerman and H. Schlenk: *Biochim. Biophys. Acta*, **573**, 23-30 (1979).