Metal Salts Requisite for the Production of Eicosapentaenoic Acid by a Marine Bacterium Isolated from Mackerel Intestines

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Metal salts important for the growth and 5,8,11,14,17-*cis*-eicosapentaenoic acid (EPA) production of a bacterium isolated from Pacific mackerel intestines were investigated at 25°C in culture media containing 1.0% peptone and 0.50% yeast extract, and the composition of an optimum culture medium was determined. This bacterium could grow in the media in which sodium chloride was the sole added inorganic component. By raising the concentration of sodium chloride from 1.2 to 2.4%, the yield of bacterial cells increased and the yield of EPA reached a maximum at 2.0% NaCl concentration. In contrast to calcium chloride, potassium chloride and magnesium chloride as second metal salts promoted the growth of this bacterium at relatively low concentrations without inhibiting the accumulation of EPA. The yield of EPA reached its maximum value of 51.9 mg/L of culture broth at 8 hr at 2.0% NaCl, 0.15% KCl and 0.16% $MgCl_2$ concentrations. This yield of EPA was 20% greater than that obtained with Jamarin S artificial sea water.

KEY WORDS: *Alteromonas putrefaciens*, bacterium, eicosapentaenoic acid, fatty acids, lipids, mackerel.

5,8,11,14,17-*cis*-Eicosapentaenoic acid (EPA) is a C_{20} polyunsaturated fatty acid effective for the prevention and cure of thrombosis and arteriosclerosis (1). The microbial production of EPA by a marine alga, *Chlorella minutissima* (2), a freshwater alga, *Monodus subterraneous* (3), and *Mortierella* fungi (4–6) has thus been investigated extensively.

Recently, Yazawa and co-workers (7–9) investigated the production of EPA by a marine bacterium isolated from the intestinal contents of Pacific mackerel *Pneumatophorus japonicus*. This bacterial strain, tentatively named SCRC-2738, was gram-negative, obligately aerobic, and motile by means of peritrichous flagella, and was identified to be a new species close to *Alteromonas putrefaciens*. This bacterial strain required natural or artificial sea water (ASW) for growth (8). In our previous study on the optimization of cultivation conditions, we found that Jamarin S ASW, which was neither diluted nor concentrated, was optimum for the growth and EPA production by SCRC-2738 (9). In the present paper, we have investigated the metal salts in the ASW, which are important for the growth of this bacterial strain and its EPA production.

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MATERIALS AND METHODS

The bacterial strain SCRC-2738 was from the stock in Sagami Chemical Research Center, Kanagawa, Japan. Its cultivation was carried out aerobically at 25°C in a 500-mL shaking flask with reciprocal shaking of 120 strokes/min. Culture media containing 1.0% peptone and 0.50% yeast extract in aqueous solutions of metal salts (pH 7.0, 100 mL) were employed. Growth was followed turbidimetrically at 610 nm and was stopped at the late logarithmic growth stage (6 hr). The EPA productivity of SCRC-2738 with the optimum culture medium was determined by use of a 3-L jar fermenter equipped with four baffles.

Peptone, yeast extract, and artificial sea water (Jamarin S) were purchased from Kyokuto Pharmaceutical Industries Co. Ltd. (Tokyo, Japan), E. Merck (Darmstadt, West Germany), and Jamarin Laboratory (Osaka, Japan), respectively. Metal salts used were of guaranteed grade. The cells were harvested by centrifugation at 6,000 rpm for 25 min and were dried at room temperature under vacuum after being washed twice with 50% ASW (artificial sea water prepared by mixing Jamarin S with an equal volume of distilled water). The content of EPA and the fatty acid composition in dry cells were determined by means of methanolysis and gas-liquid chromatography, as reported previously (9). Both the yields of bacterial cells and EPA are expressed as the mass obtained per liter of culture broth. In the comparative studies, cultivations were always carried out concurrently by use of the same precultures. The standard deviation of the yield of bacterial cells was 5%.

RESULTS

Effect of the concentration of sodium chloride. Jamarin S ASW contains 0.799% Na^+, 1.455% Cl^- and such minor components as Mg^{2+}, S, Ca^{2+}, and K^+. We first investigated the effect of the concentration of sodium chloride on the cultivation of SCRC-2738 (Fig. 1). It should be noted that this bacterial strain could grow in the media in which sodium chloride was the sole added inorganic component (Fig. 1). When the concentration of sodium chloride was 1.2%, the yield of bacterial cells was 1.39 g/L of culture broth. However, the yield increased when the concentration of sodium chloride was raised and reached 2.38 g/L at 2.4%. This bacterial strain contains stearic acid $C_{18:0}$, oleic acid $C_{18:1}$, cis-vaccenic acid $C_{18:1}$, linoleic acid $C_{18:2}$, and EPA $C_{20:5}$ in addition to C_{13} to C_{17} saturated and unsaturated fatty acids (7–8). The cellular EPA content, the total fatty acid content and the yield of EPA reached maximum values of 7.5 mg/g of dry cells, 35.0 mg/g of dry cells and 17.2 mg/L of culture broth, respectively, at 2.0% NaCl concentration (Fig. 1). This concentration of sodium chloride corresponds to 0.786%

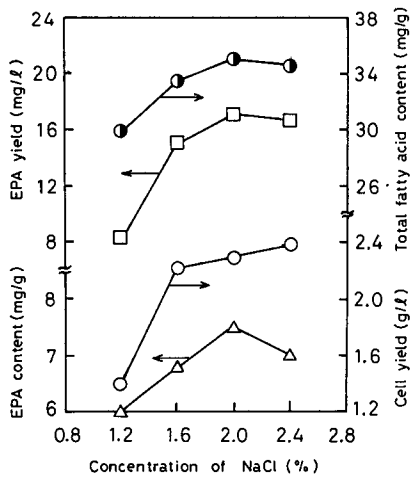

FIG. 1. Effect of the concentration of sodium chloride on the cultivation of SCRC-2738. Culture medium: peptone 1.0%, yeast extract 0.50%, initial pH 7.0. Cultivation: 25°C, 6 hr. ○: Cell yield, △: EPA content, □: EPA yield, ◑: total fatty acid content.

Na^+. This value is almost equal to the concentration of Na^+ in Jamarin S ASW (0.799%).

Table 1 summarizes the cellular fatty acid compositions of SCRC-2738 grown at different concentrations of sodium chloride. For C_{13} to C_{17} fatty acids, the cellular compositions of fatty acids having odd numbers of carbon atoms ($C_{13:0}$, $C_{15:0}$, $C_{17:0}$, and $C_{17:1}$) decreased when the concentration of sodium chloride was raised from 1.2 to 2.4% whereas those of fatty acids having even numbers of carbon atoms ($C_{14:0}$, $C_{16:0}$ and $C_{16:1}$) increased with such a rise in the concentration of sodium chloride. For C_{18} to C_{20} fatty acids, the combined cellular composition of $C_{18:1}$, $C_{18:2}$ and $C_{20:5}$ fatty acids was 27.4% at 1.2% NaCl. However, it increased when the concentration of sodium chloride was raised and reached a maximum 29.6% at 2.0% NaCl. The unsaturation index of cellular fatty acids, which is the averaged number of carbon-carbon double bonds involved in one molecule of fatty acid accumulated, also showed a maximum 1.26 at 2.0% NaCl concentration (Table 1).

Effect of second metal salts. We investigated the effect of second metal salts on the cultivation of SCRC-2738 at 2.0% NaCl. Magnesium chloride, magnesium sulfate, calcium chloride and potassium chloride were used as second metal salts. The concentrations of these second metal salts in the culture media were based on the levels of these metal ions in Jamarin S ASW. As summarized in Table 2, the yield of bacterial cells, the cellular EPA content and the total fatty acid content were 2.41 g/L of culture broth, 7.5 mg/g of dry cells and 35.5 mg/g of dry cells, respectively, in the absence of second metal salts. However, they decreased to 1.57 g/L, 6.2 mg/g and 28.5 mg/g, respectively, when magnesium chloride was added. A similar effect can be seen for magnesium sulfate. However, these two magnesium salts did not affect markedly the cellular fatty acid composition (*e.g.*, EPA content: 21.2% ↔ 21.8 and 22.3%, Table 2, Fig. 2). When the concentration of magnesium chloride was lowered to 0.08%, however, the yield of bacterial cells increased to 2.75 g/L of culture broth without any special change in the EPA and total fatty acid contents (7.6 and 35.2 mg/g). The yield of EPA was thus 20.9 mg/L of culture broth.

The yield of bacterial cells increased from 2.41 to 2.79 g/L of culture broth by the addition of calcium chloride (Table 2). However, this addition caused a decrease in the cellular EPA content without any deteriorative effect on the total fatty acid content (EPA content: 7.5 mg/g → 6.1 mg/g, Table 2, Fig. 2). The cellular compositions of $C_{15:0}$, $C_{17:0}$ and $C_{17:1}$ fatty acids increased (total: 28.3% → 40.4%) with simultaneous decreases in those of $C_{16:0}$, $C_{16:1}$, $C_{18:1}$ and $C_{20:5}$ acids (total: 63.4% → 50.8%) (Fig. 2). Potassium chloride promoted growth a little and had no particular inhibiting effect on the accumulation of fatty acids (Table 2, Fig. 2).

Effect of the concentrations of potassium chloride and magnesium chloride. We further studied the effect of the concentrations of potassium chloride and magnesium chloride on the production of EPA at 2.0% NaCl. The yield of bacterial cells increased when potassium chloride was added and reached 2.76 g/L of culture broth at 0.20% (Fig. 3). Although the cellular EPA content showed a maximum value of 7.8 mg/g of dry cells at 0.10% KCl concentration, the yield of EPA reached its maximum value of 20.9 mg/L of culture broth at 0.15%. The cellular total fatty acid content changed similarly as in the case of the cellular EPA content (Fig. 3). With the exception of the combined cellular composition of $C_{18:1}$, $C_{18:2}$ and $C_{20:5}$ fatty acids, the cellular fatty acid composition was nearly the same as that of SCRS-2738 grown at 0% KCl concentration. The combined cellular composition of $C_{18:1}$, $C_{18:2}$ and $C_{20:5}$ fatty acids was 28.8% at 0% KCl and it reached a maximum 32.4% at 0.15% KCl concentration. At this concentration of potassium chloride, the unsaturation index of cellular fatty acids reached a maximum value of 1.30 as well. These behaviors are similar to those in the case of sodium chloride (Fig. 1, Table 1).

TABLE 1

Cellular Fatty Acid Compositions of SCRC-2738 Grown at Different Concentrations of Sodium Chloride[a]

NaCl concentration (%)	Cellular fatty acid composition (%)[b]												U.I.[c] (−)
	$C_{13:0}$	$C_{14:0}$	$C_{15:0}$	$C_{16:0}$	$C_{16:1}$	$C_{17:0}$	$C_{17:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{20:5}$	Others	
1.2	5.2	1.2	24.4	13.3	13.1	2.8	9.3	3.1	7.2	0	20.2	0	1.16
1.6	4.9	2.1	20.3	15.4	18.3	2.3	7.6	0.7	7.4	0.3	20.3	0.3	1.21
2.0	4.4	2.4	17.7	17.7	18.6	2.4	5.9	0.9	8.0	0.3	21.3	0.4	1.26
2.4	4.2	2.4	16.8	19.6	19.7	1.6	4.9	1.1	8.9	0.2	20.2	0.4	1.22

[a]Culture conditions. See Figure 1.

[b]Weight per cent.

[c]Unsaturation index of cellular fatty acids = Σ (mole fraction of fatty acid) × (number of C=C double bonds in a fatty acid molecule).

TABLE 2

Results of Cultivation with Culture Media Supplemented with Sodium Chloride and a Second Metal Salt[a]

Second metal salt concentration (%)	Cell yield[b] (g/L)	Fatty acid content (mg/g)[c]		EPA content[d] (%)	EPA yield[e] (mg/L)
		EPA	Total		
None	2.41	7.5	35.5	21.2	18.1
$MgGl_2$ 0.59	1.57	6.2	28.5	21.8	9.7
$MgSO_4$ 0.50	1.42	5.9	26.4	22.3	8.4
$CaCl_2$ 0.18	2.79	6.1	34.3	17.8	17.0
KCl 0.06	2.51	7.4	33.2	22.3	18.6

[a]Culture medium: peptone 1.0%, yeast extract 0.50%, sodium chloride 2.0%, initial pH 7.0. Cultivation: 25°C, 6 hr.

[b]Cell yield per one liter of culture broth.

[c]Content of EPA or total fatty acids in one gram of dry cells.

[d]Weight composition of EPA in total fatty acids.

[e]EPA yield per one liter of culture broth.

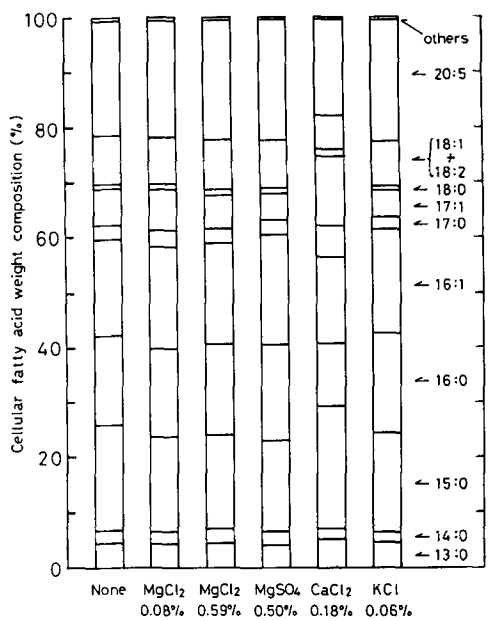

FIG. 2. Cellular fatty acid compositions of SCRC-2738 grown in the culture media supplementd with sodium chloride and a second metal salt. Cultivation conditions: See Table 2.

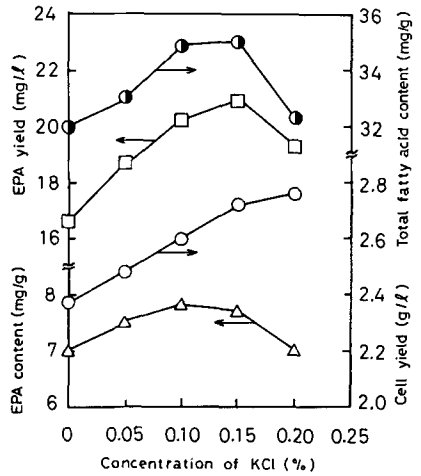

FIG. 3. Effect of the concentration of potassium chloride on the cultivation of SCRC-2738. Culture medium: peptone 1.0%, yeast extract 0.50%, sodium chloride 2.0%, initial pH 7.0. Cultivation: 25°C, 6 hr. For symbols used, see Figure 1.

The effect of the addition of magnesium chloride on the cultivation of SCRC-2738 was similarly investigated at 2.0% NaCl and 0.15% KCl concentrations. The yields of bacterial cells and EPA and the total fatty acid content reached maximum values of 3.34 g/L, 26.5 mg/L of culture broth and 35.4 mg/g of dry cells, respectively, at 0.16% $MgCl_2$ concentration and then decreased gradually with further rise in the concentration of magnesium chloride (Fig. 4).

Determination of EPA productivity. The EPA productivity of SCRC-2738 was determined by use of a 3-L jar fermenter with the culture medium containing 2.0% NaCl, 0.15% KCl and 0.16% $MgCl_2$ as the added inorganic components, and was compared with that obtained with the culture medium prepared from Jamarin S ASW (Table 3). Nissan disfoam FDS-2224 was used as the defoaming agent and the cultivation was carried out at the optimum culture conditions already determined in our previous paper (9).

The yield of bacterial cells, the cellular EPA and total fatty acid contents and the yield of EPA obtained with the former culture medium were always greater than those obtained with the latter culture medium. The yield of EPA with the former culture medium reached its maximum value 51.0 mg/L of culture broth at 8 hr. This EPA productivity is 1.2 times greater than that obtained with the culture medium prepared from Jamarin S ASW (43.6 mg/L, Table 3).

DISCUSSION

We discuss the roles of metal salts in relation to fatty acid-synthesis in SCRC-2738. Based on the observed effects of the addition of metal salts on the total fatty acid content and the cellular fatty acid composition (see Results), the metal salts used in the present work can be divided

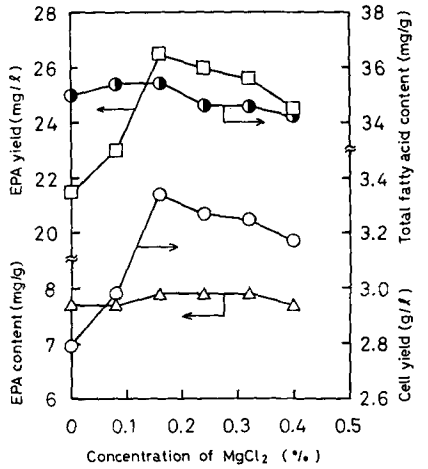

FIG. 4. Effect of the concentration of magnesium chloride on the cultivation of SCRC-2738. Culture medium: peptone 1.0%, yeast extract 0.50%, sodium chloride 2.0%, potassium chloride 0.15%; initial pH 7.0. Cultivation: 25°C, 6 hr. For symbols used, see Figure 1.

into the following three groups: group 1 — NaCl and KCl, group 2 — $MgCl_2$ and $MgSO_4$, and group 3 — $CaCl_2$.

Group-1 metal salts are usually important for organisms for controlling the osmotic pressure of cell sap in the cytoplasm and for the participation in the neurotransmission through ion-channel mechanism. However, these metal salts seem to affect the content and composition of cellular fatty acids in SCRC-2738 through the fluidity of cellular membrane.

In our previous papers (7-9), both the cellular EPA content and the total fatty acid content increased when cultivation temperature was lowered. Hence the cellular fatty acids involved in SCRC-2738 were concluded to occur as the components of cellular membrane lipids. In general, the fluidity of cellular membrane increases when the content and degree of unsaturation of the lipids involved increase, and bacteria tend to maintain the fluidity of cellular membrane to be maximum in the given environmental and physiological conditions (10). In the present work, the observation of the maximum values of the total fatty acid content and the unsaturation index of cellular fatty acids at 2.0% NaCl concentration (Fig. 1, Table 1) indicates that the fluidity of the cellular membrane of SCRC-2738 grown at 25°C is maximum at this concentration of sodium chloride. As pointed out previously, SCRC-2738 is a marine bacterium and the Na^+ concentration in the culture medium containing 2.0% NaCl was nearly equal to that in Jamarin S ASW. Thus, we believe that sodium chloride affects the production of EPA through controlling the fluidity of the cellular membrane. The role of another group-1 metal salt, KCl, could be similar to that of sodium chloride (Fig. 3). The variation of fatty acid composition in *Saccharomyces rouxii* in response to the concentration of sodium chloride in the culture medium was also explained in terms of the fluidity of cellular membrane (11).

Magnesium ions in group-2 metal salts are usually important for organisms as cofactors in the metabolism of glucose (12). In our bacterium SCRC-2738, the mechanism of ATP (adenosine 5′-triphosphate) synthesis is still unknown. However, these group-2 and group-3 metal salts ($MgCl_2$, $MgSO_4$ and $CaCl_2$) seem to directly affect the fatty acid synthesis in SCRC-2738. Polyunsaturated fatty acids in bacteria are usually synthesized from acetyl coenzyme A by the *de novo* synthesis route (13). Acetyl coenzyme A carboxylase catalyzes the initial committed step of *de novo* fatty acid synthesis, namely the carboxylation of acetyl coenzyme A to form malonyl coenzyme A. For this carboxylation step, acetyl coenzyme A carboxylase usually requires such bivalent metal ions as Mg^{2+}, Mn^{2+} or Co^{2+} as cofactors (14). However, these bivalent metal ions have in turn an inhibiting effect on the carboxylation reaction at relatively higher concentrations: no inhibiting effect was observed at 8mM Mg^{2+} (0.08% $MgCl_2$) (14).

In the present work, the concentration of Mg^{2+} in the culture medium (Table 2) was 61.9 mM ($MgCl_2$) or 41.6 mM ($MgSO_4$), respectively. Thus we have no experimental evidence for the participation of Mg^{2+} ions in the fatty acid synthesis in SCRC-2738. However, the observed decrease in the total fatty acid content caused by the addition of magnesium salts (Table 2) is explainable based on the inhibiting effect of Mg^{2+} on the initial step of *de novo* fatty acid synthesis. No particular change in cellular fatty acid composition in the presence of magnesium salts and the observed increase in the total fatty acid content at relatively low concentrations of magnesium chloride (Figs. 2 and 4) support this view. Comparison of the cell yield and the total fatty acid content between Table 2 and

TABLE 3

Comparison of the Production of EPA Between NaCl-KCl-$MgCl_2$ and ASW Culture Media in the pH-Controlled Jar Fermenter[a]

	NaCl-KCl-$MgCl_2$ culture medium[b] cultivation time (hr)				Jamarin S ASW culture medium[c] cultivation time (hr)			
Production of EPA	7	8	9	10	7	8	9	10
Cell yield (g/L)[d]	4.74	5.31	5.56	5.62	4.39	4.95	5.29	5.45
EPA content (mg/g)[e]	9.4	9.6	8.3	8.1	9.2	8.8	7.9	7.6
Total fatty acid content (mg/g)[f]	44.5	42.6	41.3	42.8	38.6	39.2	37.8	37.1
EPA yield (mg/L)[g]	44.6	51.0	46.2	45.5	40.4	43.6	41.8	41.4

[a]Cultivation; 25°C, air 1.0 L/min, agitation 450 rpm.

[b]Culture medium (1 L): peptone 1.0%, yeast extract 0.50%, NaCl 2.0%, KCl 0.15%, $MgCl_2$ 0.16%, defoaming agent 0.01%, pH 7.0.

[c]Culture medium (1 L): peptone 1.0%, yeast extract 0.50%, defoaming agent, 0.01%, Jamarin S ASW, pH 7.0.

[d,e,f,g]See Table 2.

Figure 4 suggests that K^+ ions reduce the inhibiting effects caused by Mg^{2+} ions.

Nearly the same values of the total fatty acid content in SCRC-2738 grown in the presence and absence of calcium chloride (Table 2) suggest that this salt did not inhibit the fatty acid synthesis in SCRC-2738. This could be due to the relatively low concentration of Ca^{2+} ions in the culture medium (16.2 mM, Table 2). As shown by the increases in the cellular compositions of $C_{15:0}$, $C_{17:0}$ and $C_{17:1}$ fatty acids at the sacrifice of $C_{16:0}$, $C_{16:1}$, $C_{18:1}$ and $C_{20:5}$ acids (Fig. 2), however, Ca^{2+} ions appear to cause the scission of carbon-carbon bonds during the *de novo* EPA synthesis in SCRC-2738. At any rate, the above finding made in this study indicates that the culture medium prepared from the aqueous solution of sodium chloride, potassium chloride and magnesium chloride is better than that prepared from Jamarin S ASW for the production of EPA by SCRC-2738.

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