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Influence of growth conditions on fatty acid composition of a polyunsaturated-fatty-acid-producing *Vibrio* species

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Abstract The influence on fatty acid composition of growth medium composition and phase of growth during batch culture and of dilution rate and growth temperature during continuous culture was studied in the eicosapentaenoic-acid (20:5 n-3)-producing *Vibrio* CCUG 35308. In glucose-mineral medium, even-numbered normal fatty acyl residues, primarily 16:0, 16:1, 18:1, and 20:5, strongly dominated (ca. 90%), and the fatty acid profile remained practically unchanged throughout a batch-growth cycle. In nutrient broth, the contribution by "uncommon" fatty acids, mainly i-13:0, 15:0, i-15:0, and 17:1 was generally higher, and increased from 15.4% of total fatty acids in early exponential growth phase to 33.2% in the stationary phase. Reduction of the dilution rate in a chemostat from 0.27 to 0.065 h^{-1} also led to an almost threefold increase in the proportion of odd-numbered residues at the expense of the even-numbered normal ones. Contrary to this plasticity in the overall fatty acid profile influenced by variations in nutrient composition and availability, the level of eicosapentaenoic acid seemed exclusively dictated by growth temperature. The synthesis of this polyunsaturated fatty acid may be a key regulatory process in maintaining membrane fluidity.

Key words *Vibrio* · Polyunsaturated fatty acids · Eicosapentaenoic acid · Growth phase · Growth medium · Chemostat

Introduction

Since the work of Johns and Perry (1977), an increasing number of publications have emerged that demonstrate the presence of polyunsaturated fatty acids of the n-3 series as constituents of the membrane phospholipids of heterotrophic eubacteria. This trait seems especially prevalent among psychrotrophic/psychrophilic marine isolates. The reported contributions by polyunsaturated fatty acids to total fatty acids range from a few percent up to 40% in an *Alteromonas*-like strain isolated from Pacific mackerel (Yazawa et al. 1988b). In most cases, the fatty acid in question is eicosapentaenoic acid (Wirsen et al. 1987; Yazawa et al. 1988ab; Akimoto et al. 1990; Ringø et al. 1992; Nichols et al. 1994), but docosahexaenoic acid has also been found (DeLong and Yayanos 1986; Yano et al. 1994; Hamamoto et al. 1995). Significant amounts of α-linolenic acid seem principally confined to the cyanobacteria (Caudales and Wells 1992).

In the polyunsaturated fatty acid producers, as in bacteria in general, variation in the composition of fatty acyl residues has been related to a multitude of environmental factors. Increased levels of eicosapentaenoic acid and docosahexaenoic acid have been proposed by DeLong and Yayanos (1986) and Wirsen et al. (1987) as a means of maintaining membrane fluidity at high hydrostatic pressure. Furthermore, the influence of the composition, concentration, and salinity of the growth medium on polyunsaturated fatty acid content has been investigated (Johns and Perry 1977; Suzuki et al. 1991; Nichols et al. 1994). Temperature is, however, by far the most extensively studied environmental factor with these organisms. Adaptation to growth at lowered temperatures in eubacteria includes, in general, an increase in the unsaturation level of membrane fatty acyl residues – in the majority of strains by increasing the proportion of monounsaturated entities (Russel and Fukunaga 1990). The polyunsaturated fatty acid producers conform to this general pattern by having an increased proportion of these fatty acids at low growth temperatures (Wirsen et al. 1987; Yazawa et al. 1988a,b; Akimoto et al. 1990; Henderson et al. 1993; Nichols et al. 1994).

As a rule, studies of environmental influences on fatty acid composition have been carried out with batch-grown bacteria. An intrinsic problem with this approach is the lack of steady-state conditions throughout the growth cycle, i.e., the availability of nutrients becomes unbalanced

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toward the end of the exponential growth phase, often concomitant with changes in pH and oxygen tension of the medium. In addition, bacteria go through a series of adaptations when entering the stationary growth phase (Neidhardt et al. 1990). Each of these influences has been reported to affect the fatty acid composition in microorganisms (Oliver and Colwell 1973; Intriago and Floodgate 1991; Arneborg et al. 1993).

Jøstensen et al. (1990) isolated an eicosapentaenoic-acid-producing *Vibrio* species from the intestines of the freshwater salmonid fish *Salvelinus alpinus* L. In addition to the polyunsaturated fatty acid, which is predominantly situated in the *sn*-2 position of the phosphatidylglycerol phospholipid moiety, it produces a rather complex mixture of even-numbered (both *cis*- and *trans*-monounsaturated), odd-numbered, and branched fatty acids and responds to reduced growth temperatures by increasing the proportion of eicosapentaenoic acid and *trans*-palmitoleic acid (Henderson et al. 1993, 1995). In the present work, the effects of altering the growth medium and of sampling at different phases of the batch growth cycle on the fatty acid composition were investigated. In addition, the influences of variations in dilution rate and temperature during growth in continuous culture in a chemostat were examined.

Materials and methods

Bacterial strain and identification

Vibrio CCUG 35308 (Culture Collection of the University of Gothenburg) was originally obtained from the gut of arctic charr (*Salvelinus alpinus*, L) as described elsewhere (Jøstensen et al. 1990). It was classified as a *Vibrio* sp. according to the scheme of Muroga et al. (1987), by being a gram-negative, motile, oxidase-positive, fermentation-positive rod, sensitive to the O/129 reagent. A selection of 28 tests was included in a numerical taxonomic analysis based on the probability matrix for the identification of species of *Vibrio* and related genera (Bryant et al. 1986). Computer analysis (performed by H. Birkbeck, University of Glasgow) affiliated the strain to a hitherto unspeciated group of *Vibrio*, phenon 36, by a 98.9% probability score.

Batch culture

The *Vibrio* strain was grown at 10°C in 2 l baffled conical flasks in 1 l tryptic soy broth (Difco, Detroit, Mich., USA) or 1 l of the minimal medium M63 [100 mM KH_2PO_4, 75 mM KOH, 15 mM $(NH_4)_2SO_4$, 1 mM $MgSO_4$, 3.9 µM $FeSO_4$, pH 7.1, supplemented with 1 g/l glucose (Miller 1972)]. Cultures were inoculated with 0.1% (v/v) fresh bacterial culture and incubated on a rotary shaker. Cell concentration was assessed as optical density at 600 nm. Samples (1 ml) withdrawn for lipid analysis were immediately centrifuged at 6,000 × *g* for 10 min and washed once in the same volume of saline.

Continuous culture

The *Vibrio* sp. was grown in a 500 ml (350 ml working volume) chemostat (New Brunswick Scientific, New Brunswick, N. J., USA) with 30 g/l of tryptic soy broth as fresh medium. Agitation was set at 200 rpm. Culture medium was supplied by a peristaltic pump (Pharmacia, Sweden). To achieve oxygen saturation, sterile air was pumped through the reactor. Temperature was controlled by leading chilled water through heat-exchanging tubes in the reactor. The chemostat was inoculated with 0.1% (v/v) fresh bacterial culture and allowed to equilibrate for 24 h before the medium pump was started. After each change in growth conditions, the chemostat was equilibrated with at least 5 reactor volumes of medium before samples were withdrawn. Samples (1 ml) were collected from the overflow tube by dripping into glass vials embedded in ice at a temperature below –50°C, instantly freezing the sample. Thawed bacterial suspension was concentrated by centrifugation at 6,000 × *g* for 10 min at 4°C.

Lipid analyses

Fatty-acyl methyl esters were prepared by H_2SO_4-catalyzed transesterification (Christie 1982), involving direct methylation of freshly pelleted cells (Lambert and Wayne Moss 1983). The methyl esters were separated by gas chromatography (Hewlett-Packard, Model 5890, II, Waldbronn, Germany) using an SP-2330 (Supelco, Bellefonte, Pa., USA) capillary column (30 m × 0.25 mm i.d.) and helium as the carrier gas. The temperature program employed was 60°C for 1 min, followed by an increase of 20°C/min to 140°C for 3 min, and thereafter an increase of 10°C/min to 190°C for 3 min, followed by an increase of 10°C/min to 220°C for 8 min. Individual fatty-acyl methyl esters were identified by comparison with known standards and quantified using Hewlett-Packard Chem Station software.

Results

Growth medium composition

When grown in tryptic soy broth and in glucose-mineral medium M63, the *Vibrio* strain showed cell doubling times of 2.3 and 11.0 h, respectively, in the exponential growth phase at 10°C. The ratio of unsaturated to total fatty acids was influenced little by the choice of growth medium, being 58.6% in the complex broth and 57.3% in the minimal medium, and the difference in eicosapentaenoic acid levels was less than 0.5% (Table 1). The most distinct difference was a high occurrence of branched and odd-numbered fatty acids in the tryptic soy broth and a total domination by even-numbered normal residues in the glucose-mineral medium. Of the total fatty acids present in bacteria grown in M63 medium, 6.5% were branched or odd-numbered, whereas more than 30% of the total fatty acids in bacteria sampled during the last stages of the growth cycle in tryptic soy broth were branched or odd-numbered.

Growth phase

Samples of batch-grown bacteria in the two media were withdrawn in the early exponential growth phase (about 4 doublings after inoculation), late exponential growth phase, and after about 7 and 24 h into the stationary phase for tryptic soy broth and M63, respectively. As shown in Table 1, the level of eicosapentaenoic acid remained constant at about 6% and was unaffected by the growth phase. On the other hand, a steady decrease in the level of shorter even-numbered normal fatty acids (14:0, 16:0, and 16:1)

Table 1 Effect of growth medium (M63 glucose-mineral medium and tryptic soy broth) and growth phase (tryptic soy broth only) on fatty acid composition (wt% of total fatty acids) in total lipid of the *Vibrio* sp. grown in batch cultures at 10° C. Values are means ± standard deviations from duplicate experiments, each with samplings in triplicate (*i* iso, *nd* not detected)

Fatty acids	M63	Tryptic soy broth		
		Early exponential phase	Late exponential phase	Stationary phase
12:0	3.6 ± 0.3	2.8 ± 0.6	2.7 ± 0.4	2.2 ± 0.1
i-13:0	1.8 ± 0.1	4.8 ± 0.6	7.3 ± 0.4	7.2 ± 0.2
14:0	4.2 ± 0.1	5.1 ± 0.1	3.1 ± 0.1	2.2 ± 0.1
i-15:0	3.2 ± 0.1	10.6 ± 0.1	13.2 ± 0.1	13.4 ± 0.1
15:0	0.8 ± 0.1	nd	2.9 ± 0.1	2.9 ± 0.1
16:0	24.1 ± 0.4	16.8 ± 0.4	10.2 ± 0.2	8.9 ± 0.3
16:1 (n-9)	2.2 ± 0.1	3.6 ± 0.2	2.6 ± 0.1	2.0 ± 0.1
16:1 (n-7)	42.8 ± 0.3	42.0 ± 0.8	38.0 ± 0.5	33.3 ± 0.2
17:0	nd	nd	nd	1.2 ± 0.1
17:1 (n-8)	0.7 ± 0.1	nd	5.1 ± 0.2	6.9 ± 0.2
17:1 (n-6)	nd	nd	nd	1.6 ± 0.1
18:0	2.2 ± 0.1	2.3 ± 0.2	1.9 ± 0.1	0.7 ± 0.1
18:1 (n-9)	2.2 ± 0.1	1.7 ± 0.1	1.9 ± 0.1	2.1 ± 0.1
18:1 (n-7)	3.8 ± 0.1	4.5 ± 0.2	5.5 ± 0.2	6.7 ± 0.3
20:5 (n-3)	5.6 ± 0.1	5.8 ± 0.2	5.6 ± 0.2	6.0 ± 0.2
Sum even-numbered	90.7	84.6	71.5	64.1
Sum odd-numbered	1.5	nd	8.0	12.6
Sum branched	5.0	15.4	20.5	20.6
Unidentified	2.8	nd	nd	2.7

was balanced by an increased proportion of odd-numbered (15:0 and 17:1) and branched (i-13:0 and i-15:0) residues. The overall ratio of saturated to unsaturated fatty acids was not affected by the aging of the culture. When grown in M63, the bacteria kept a very stable fatty acid composition throughout the growth cycle, identical to that from the early exponential growth phase depicted in Table 1 (additional data not shown).

Continuous culture

The impact of nutrient-limited growth on the fatty acid composition was studied by establishing a continuous culture of the *Vibrio* strain at 10° C in a chemostat. Continuous growth in glucose-mineral medium at a low dilution rate and temperature was unsuccessful, due to precipitation of the medium. The dilution rate in tryptic soy broth was reduced stepwise from 0.27 h^{-1} via 0.13 h^{-1} to 0.065 h^{-1}. The highest of these rates was close to the instantaneous growth rate constant at unrestricted growth in batch culture of 0.30 h^{-1}. The resulting changes in the fatty acid profile were similar to those observed throughout the batch growth cycle (Table 2). The fraction of odd-numbered chain residues (15:0 and 17:1 isomers) increased from 7.9% at the highest dilution rate to 22.0% at the lowest, at the expense of the even-numbered normal ones. Only a slight increase in the proportion of branched fatty acids was observed, while the percentage of eicosapentaenoic acid was not affected.

The *Vibrio* strain was grown in the chemostat at 15, 10, 5 and 0° C at a constant dilution rate of 0.065 h^{-1}. The bacteria adapted to lowered temperature in the range 15 to 5° C by increasing the overall unsaturation level of the membrane fatty acids, primarily by increasing the proportion of eicosapentaenoic acid (Table 2). The even-numbered chain monounsaturated residues (16:1 and 18:1 isomers) showed a general increase, while the 17:1 isomers underwent an almost corresponding reduction. A further lowering of growth temperature to 0° C did not change the proportion of eicosapentaenoic acid or the fraction of even-numbered fatty acids, but led to an increase in branched residues, mainly i-13:0, at the expense of the odd-numbered ones.

Discussion

The variations in fatty acid composition of the present *Vibrio* species at a constant growth temperature, i.e., the alterations observed throughout the growth cycle in a complex medium and the overall simplicity of the fatty acid profile in glucose-mineral medium, are most conveniently interpreted as passive adaptations to a changing repertoire of nutrients. In complex medium, continuous changes of the composition can be expected to take place by unbalanced utilization of the various nutrients. Concomitantly, the range of biosynthetic precursors available for priming fatty acid synthesis should also vary. Influence of growth phase on fatty acid composition in complex media has been demonstrated earlier in gram-negative marine bacteria (Oliver and Colwell 1973), thermophilic bacteria (Nordström 1992) and in a polyunsaturated-fatty-acid-producing *Shewanella putrefaciens* (Nichols et al. 1994). When a single carbon and energy source was available at any phase of the growth cycle, as in the glucose-mineral medium, variation in fatty acid composition was eliminated. Consequently, no active regulatory pro-

Table 2 Effect of dilution rate and temperature on fatty acid composition (wt% of total fatty acids) in total lipid from the Vibrio sp. grown in continuous cultures at three different dilution rates (0.27 h^{-1}, 0.13 h^{-1}, 0.067 h^{-1}) at 10° C and at four different temperatures (15° C, 10° C, 5° C, 0° C) with a dilution rate of 0.067 h^{-1}. Values are means ± standard deviations from triplicate samplings (*i* iso, *nd* not detected)

Fatty acid	Dilution rates (h^{-1}) at 10° C			Temperatures (° C) at 0.067 h^{-1}		
	0.27	0.13	0.067	15	5	0
12:0	0.0 ± 0.0	1.9 ± 0.3	1.6 ± 0.1	0.9 ± 0.1	0.5 ± 0.2	2.4 ± 0.1
i-13:0	6.2 ± 0.5	7.5 ± 0.6	8.3 ± 0.2	8.8 ± 0.9	3.9 ± 1.3	7.7 ± 0.3
14:0	3.3 ± 0.1	2.6 ± 0.1	2.1 ± 0.1	2.3 ± 0.7	2.7 ± 0.2	4.7 ± 0.4
i-15:0	13.0 ± 0.1	12.8 ± 0.2	12.5 ± 0.2	11.7 ± 0.5	10.6 ± 0.7	12.5 ± 0.1
15:0	2.6 ± 0.1	5.1 ± 0.2	6.7 ± 0.1	11.3 ± 0.3	6.2 ± 0.1	5.5 ± 0.1
16:0	12.7 ± 0.3	10.7 ± 0.4	8.7 ± 0.2	6.9 ± 0.1	9.8 ± 0.2	8.8 ± 0.1
16:1 (n-9)	2.1 ± 0.3	4.9 ± 0.5	3.0 ± 0.1	2.8 ± 0.7	8.0 ± 0.6	6.6 ± 0.1
16:1 (n-7)	36.8 ± 1.2	27.9 ± 0.7	26.9 ± 0.9	21.1 ± 0.3	29.8 ± 1.3	28.4 ± 0.3
17:0	0.6 ± 0.1	1.4 ± 0.1	1.8 ± 0.1	0.8 ± 0.2	0.3 ± 0.0	nd
17:1 (n-8)	4.2 ± 0.3	8.0 ± 0.4	10.5 ± 0.1	16.6 ± 0.4	8.8 ± 0.3	6.2 ± 0.1
17:1 (n-6)	0.5 ± 0.1	1.3 ± 0.1	2.1 ± 0.1	2.4 ± 0.0	1.0 ± 0.0	0.4 ± 0.0
18:0	1.4 ± 0.1	0.5 ± 0.5	0.6 ± 0.1	3.3 ± 0.0	1.5 ± 0.0	1.1 ± 0.1
18:1 (n-9)	1.6 ± 0.1	1.1 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	nd
18:1 (n-7)	5.5 ± 0.2	4.6 ± 0.1	4.3 ± 0.1	2.6 ± 0.3	3.5 ± 0.1	4.1 ± 0.4
20:5 (n-3)	6.9 ± 0.3	6.8 ± 0.3	7.3 ± 0.2	4.4 ± 0.2	9.8 ± 1.0	10.4 ± 0.2
Sum even-numbered	70.3	61.0	55.2	45.1	66.4	66.5
Sum odd-numbered	7.9	15.8	21.1	31.1	16.3	12.1
Sum branched	19.2	20.3	20.8	20.5	14.5	20.2
Unidentified	2.6	2.9	2.9	3.3	2.8	1.2

cesses resulting in fatty acid modification seemed obligatory in the adaptation to stationary phase in this particular strain. A strong domination (about 90%) by even-numbered normal fatty acids in the minimal medium indicated that acetyl-coenzyme A, derived from the central fueling pathway, served almost exclusively as the priming molecule in fatty acid biosynthesis under these conditions. Similar trends have been observed in other bacteria. Chung et al. (1993) have demonstrated high levels of branched-chain fatty acids when *Rhodothermus marinus* is grown on yeast extract and tryptone. These residues decrease and are replaced by normal saturated fatty acids when sodium glutamate is used as sole source of carbon and energy. Likewise, when grown on acetate, a polyunsaturated-fatty-acid-producing strain of *S. putrefaciens* shows a higher level of even-numbered normal fatty acids than when cells are grown in Zobell's broth (Nichols et al. 1994).

As pointed out by Rose (1989), batch culture studies of environmental influences on microbial lipid composition are hampered by several limitations. Some of these, for example, distinguishing temperature effects from growth rate effects, can be avoided by the use of a chemostat culture. Very few studies of this kind have, however, been published on bacteria (Gill 1975; Arneborg et al. 1993). Growth of bacteria on a complex medium in a chemostat, as in the present work, is not a precisely defined system with respect to the nature of the growth-limiting substrate. On the other hand, to some extent it mimics conditions experienced by microbes in many natural environments, where they are faced with a multitude of nutrients, all at sub-saturation concentrations. By reducing the instantaneous growth rate, the severeness of nutrient limitation, and most likely also the relative composition of residual nutrients in the reactor, was altered. Changes were observed that are similar, but not identical to those observed with aging of batch cultures. The percentage of odd-numbered normal fatty acids increased at the expense of the even-numbered fatty acids, while the fraction of branched residues (in contrast to that observed in batch culture) remained rather constant. This suggests that fatty acid composition of a given strain of bacteria in natural environments can be substantially influenced by the nature and availability of nutrients present and can also be quite divergent from what is observed by conventional culture techniques in the laboratory.

In the present *Vibrio* strain, eicosapentaenoic acid was the exception to the rule by being practically unaffected by the choice of medium and the phase of growth during batch culture, and by dilution rate in a chemostat culture. The percentage of this fatty acid is also not influenced by the salinity of the growth medium, as shown by Henderson et al. (1993).

Most bacteria respond to decreasing growth temperatures by various means of counteracting the concomitant loss of membrane fluidity. This is most often achieved by increasing the fraction of unsaturated or branched fatty acids or by decreasing the average fatty acyl chain length (Russel 1984). Lipid class composition has also been shown to be influenced in a way that promotes the same objective (Bhakoo and Herbert 1979). In another study, it has been shown that when the growth temperature is reduced from 20 to 5° C, the *Vibrio* strain employed here increases its level of the phospholipid phosphatidylethanolamine and its fatty acid unsaturation through an increase in the fraction of eicosapentaenoic acid, whilst

keeping the percentage of total monounsaturated fatty acids rather constant (Henderson et al. 1993). Here we demonstrated the same pattern of changes when the temperature was lowered from 15 to 5°C at a constant and low dilution rate in the chemostat. This confirms that the influence of temperature on fatty acid composition, as reported by Henderson et al. (1993), is a genuine temperature effect, which is not blurred to any significant degree by concomitant alterations in growth rate, oxygen tension, or other parameters known to be influenced by growth temperature in batch cultures. A further increase in the level of eicosapentaenoic acid was not observed when the growth temperature was reduced from 5 to 0°C. In this temperature range, the bacteria seemed to maintain membrane fluidity by increasing the proportion of branched fatty acids, rather than by increasing the overall desaturation of the membrane.

According to the present work, the level of eicosapentaenoic acid in this *Vibrio* sp. seems to be exclusively dictated by growth temperature, implying that the regulation of its synthesis is the key process of membrane-fluidity adaptation to temperature, at least above 5°C. However, until a greater number of polyunsaturated-fatty-acid-producing strains have been investigated, the generality of this observation remains open to question.

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