Biotechnology Letters (2005) 27: 389–393
DOI 10.1007/s10529-005-1532-4

Enhancement of polyunsaturated fatty acid production by cerulenin treatment in polyunsaturated fatty acid-producing bacteria

Naoki Morita[1,*], Takanori Nishida[2], Mika Tanaka[2], Yutaka Yano[3] & Hidetoshi Okuyama[2]

[1]*Research Institute of Genome-based Biofactory, National Institute of Advanced Industrial Science and Technology (AIST), Toyohira-ku, Sapporo 062-8517, Japan*
[2]*Laboratory of Environmental Molecular Biology, Graduate School of Environmental Earth Science, Hokkaido University, Kita-ku, Sapporo 060-0810, Japan*
[3]*National Research Institute of Fisheries Science, Kanazawa-ku, Yokohama, Kanagawa 236-8648, Japan*
**Author for correspondence (Fax: +81-11-857-8992; E-mail: morita.n@aist.go.jp)*

Received 18 January 2005; Accepted 19 January 2005

Key words: cerulenin, docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), polyunsaturated fatty acid (PUFA), PUFA-producing bacteria

Abstract

When docosahexaenoic acid (DHA)-producing *Moritella marina* strain MP-1 was cultured in the medium containing 0.5 μg cerulenin ml^{-1}, an inhibitor for fatty acid biosynthesis, the cells grew normally, but the content of DHA in the total fatty acids increased from 5.9–19.4%. The DHA yield of *M. marina* strain MP-1 cells also increased from 4 to 13.7 mg l^{-1} by cerulenin treatment. The same effect of cerulenin was observed in eicosapentaenoic acid (EPA)-producing *Shewanella marinintestina* strain IK-1 grown in the medium containing 7.5 μg cerulenin ml^{-1}, and the cerulenin treatment increased the EPA yield from 1.6 to 8 mg l^{-1}. The use of cerulenin is, therefore, advantageous to increase the content of intracellular polyunsaturated fatty acids (PUFA) in particular PUFA-containing phospholipids in bacterial cells.

Introduction

Numerous bacterial strains that produce polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA; 20:5 n−3) and docosahexaenoic acid (DHA; 22:6 n−3), have been isolated from environments such as the deep-sea, low-temperature regions like the polar areas, and intestines of marine animals (Nichols *et al.* 1999, Valentine & Valentine 2004). EPA and DHA have been used as biomarkers for the phylogenetic classification of psychrophilic and piezophilic bacteria because of their limited habitat (Russell & Nichols 1999, Kato & Nogi 2001). On the other hand, these PUFA-producing bacteria are potent biological sources of PUFAs instead of fish oils. However, optimization of PUFA production in PUFA-producing bacteria has so far been confined to screening and isolation of the strains that accumulate high levels of PUFAs in cells (Bajpai & Bajpai 1993, Nichols *et al.* 1999, Singh & Ward 1997).

Until recently, the pathway of fatty acid formation in all microorganisms was considered to be by the routes involving a fatty acid synthetase (FAS) complex to provide the starting fatty acids with 16 or 18 carbon atoms for subsequent desaturation and elongation reactions. Previous works (Morita *et al.* 1999, Tanaka *et al.* 1999) showed that DHA-producing *Moritella marina* strain MP-1 has at least two distinct genomic organizations for fatty acid biosynthesis. One is a *fab* (fatty acid biosynthesis) gene cluster, which partially encodes enzymes that belong to FAS. The other is a novel DHA biosynthetic gene cluster, which encodes putative proteins and enzymes involved in DHA biosynthesis. The genomic

organization of the DHA biosynthetic gene cluster is similar to that of polyketide synthase (PKS). The FAS and PKS-like systems were more likely to be independently functioning to synthesize fatty acids *de novo* in the *M. marina* strain MP-1 (Morita *et al.* 2000).

When DHA- or EPA-producing bacteria were treated with cerulenin, an inhibitor for fatty acid biosynthesis, the biosynthesis of monounsaturated fatty acids was preferentially inhibited concomitantly the proportion of PUFAs increased (Allen *et al.* 1999, Fang *et al.* 2004, Morita *et al.* 2000). Since these observations would be caused by different cerulenin sensitivity of two separate biosynthetic pathways, cerulenin or other FAS inhibitor(s) might be a potent agent to increase the intracellular amount of PUFAs in PUFA-producing bacteria. However, utilization of any inhibitors of fatty acid biosynthesis *de novo* has never been reported for the strategy to produce PUFAs in bacteria.

In this study, we analyzed profiles of growth and fatty acids of DHA-producing *M. marina* strain MP-1 and EPA-producing *Shewanella marinintestina* strain IK-1 in order to optimize the enhancement of PUFA production after the cerulenin treatment. The benefits of treating PUFA-producing bacteria with cerulenin for provision of PUFAs and PUFA-containing phospholipids are described.

Materials and methods

Bacterial strain and culture conditions

DHA-producing *M. marina*, formally known as *Vibrio marinus* (Urakawa *et al.* 1998), strain MP-1 (ATCC 15381) was purchased from ATCC, Rockville, MD. It and *S. marinintestina* strain IK-1 (Satomi *et al.* 2003) were grown in LB [1% (w/v) Tryptone (Difco), 0.5% (w/v) yeast extract (Difco)] medium containing 3% (w/v) NaCl with shaking at 10 and 20 °C, respectively. The growth of cells was monitored turbidometrically at 600 nm. The antibiotic, cerulenin (Sigma), in 50% (v/v) ethanol was added to culture medium at various concentrations prior to cultivation. The antibiotic, triclosan, was obtained from Wako Pure Chemicals, Osaka, Japan.

Lipid and fatty acid analysis

The cells in late growth phase were collected by centrifugation at 5000 × *g*, washed in 0.5 M NaCl three times, and collected cells were lyophilized. Total lipids from lyophilized cells were extracted by the method of Bligh & Dyer (1959). Fatty acid methyl esters (FAMEs) were prepared by reacting extracted lipids with 10% (v/v) acetyl chloride in methanol at 90 °C for 3 h in a test tube with Teflon-lined caps with a known amount of heneicosanoic acid (21:0) as an internal standard. FAMEs were extracted three times with hexane, and hexane layer was evaporated by vacuum centrifugation. The FAME residue was then redissolved in an appropriate volume of hexane. Analysis of the FAME was performed by gas chromatograph equipped with a capillary column [BPX70; 25 m by 0.22 mm (internal diameter); SGE, Austin, TX]. Helium was used as the carrier gas. The injector and the detector were maintained at 260 °C and the column oven was programmed to increase from 160 to 240 °C at 10 °C min^{-1} and then maintained at 240 °C for 7 min. Peak areas were quantified by Shimadzu C-R8A. Compounds were identified by comparison of their retention times with those of known standards and confirmed by GC/MS using Saturn 2000 ion trap mass spectrometer (Varian, Inc. Walnut Creek, CA) connected to Varian 3800 gas chromatograph equipped with BPX70 capillary column [25 m by 0.22 mm (internal diameter)]. The oven was programmed to increase from 80 to 240 °C at 4 °C min^{-1}. Helium was used as the carrier gas. GC/MS was operated at an ionization voltage of 70 eV and a trap temperature at 230 °C with a mass range of 40–400 atomic mass units.

Results and discussion

Effect of cerulenin treatment on the growth and fatty acid composition of Moritella marina *strain MP-1 cells*

Figure 1 shows the growth profile of *M. marina* strain MP-1 cells grown at 10 °C in the medium containing various concentrations of cerulenin. The cells grown in the medium containing 1 μg cerulenin ml^{-1} displayed growth ability essentially identical to non-treated cells. However, the cells

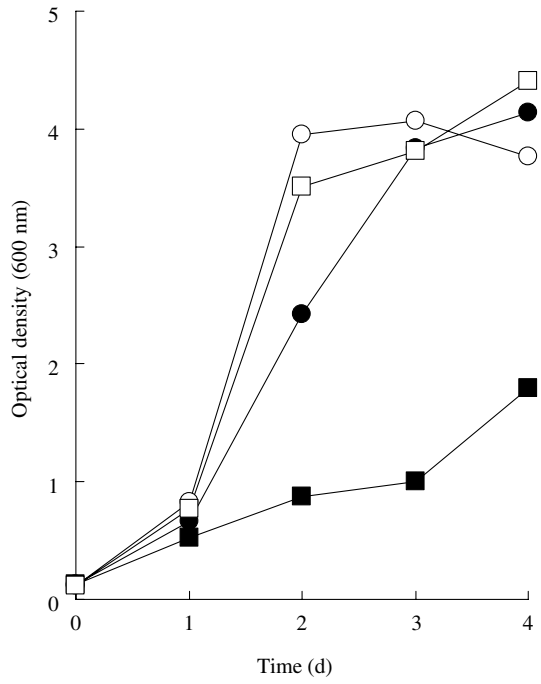

Fig. 1. Growth characteristics of *M. marina* strain MP-1 grown in the medium containing various concentrations of cerulenin. □, One μg ml^{-1}; ○, 1 μg ml^{-1}; ●, 5 μg ml^{-1}; ■, 10 μg ml^{-1}.

grown in the medium containing 5 and 10 μg cerulenin ml^{-1} decreased their growth rate. Since the cells treated with 5 and 10 μg cerulenin ml^{-1} also had a decreased yield (data not shown), we further characterized DHA production of *M. marina* strain MP-1 cells treated with cerulenin at under 5 μg ml^{-1}.

When DHA-producing *M. marina* strain MP-1 was cultured in the medium containing cerulenin at less than 5 μg ml^{-1} (0, 0.1, 0.5, 1 and 2.5 μg ml^{-1}) at 10 °C, the cerulenin-treated cells grew almost identical to non-treated cells, however the growth of cells treated with 2.5 μg cerulenin ml^{-1} was slightly inhibited (data not shown).

Table 1 shows that fatty acid compositions of *M. marina* strain MP-1 grown in the medium containing cerulenin up to 2.5 μg ml^{-1} at 10 °C. Levels of octadecenoic (18:1, 1.7–0.1%) and hexadecenoic acids (16:1, 58.5–17.2%) decreased and those of dodecanoic (12, 1–6.7%), tetradecenoic (14:1, 2.1–9.8%) and tetradecanoic acids (14, 10.3–32.5%) increased markedly with an increase in cerulenin concentration. The level of hexadecanoic acid (16) changed only slightly at any cerulenin concentration examined. DHA level was increased from 5.9% up to 19.4% by cerulenin at 0.5 μg ml^{-1}, however, DHA level decreased to 14.8% at 2.5 μg cerulenin ml^{-1}.

Effect of cerulenin treatment on the amount of biomass, lipids and DHA in M. marina *strain MP-1 cells*

Biomass production, lipid and DHA yields obtained from *M. marina* strain MP-1 cells grown in the medium containing cerulenin up to 2.5 μg ml^{-1} at 10 °C are summarized in Table 2. The cell biomass was 2.6 g dry cells l^{-1} when cells were grown in the medium containing cerulenin at 0.1, 0.5 and 1 μg ml^{-1}. This value was almost the same as that of non-treated cells. The lipid yield (g l^{-1}) was unchanged by the concentration of cerulenin in the range from 0 to 1 μg ml^{-1}. Biomass and lipid yield were scarcely influenced by the concentration of cerulenin to the medium up to 1 μg ml^{-1}, indicating no effects of cerulenin to the growth of *M. marina* strain MP-1 cells. The highest DHA yield at 13.7 mg l^{-1} was obtained from cells treated with cerulenin at 0.5 μg ml^{-1}. This yield was more than three times that obtained from non-treated cells. All these results clearly show that the optimization of cerulenin treatment enhances DHA production in *M. marina* strain MP-1 cells.

Effect of cerulenin treatment on the amount of biomass, lipids and EPA in Shewanella marinintestina *strain IK-1 cells*

Table 3 shows biomass production, lipid and EPA yields obtained from EPA-producing *S. marinintestina* strain IK-1 cells grown in the medium containing 0, 5 and 7.5 μg cerulenin ml^{-1}. The cells treated with cerulenin at 5 and 7.5 μg ml^{-1} displayed growth ability essentially identical to non-treated cells (data not shown). The cell biomass was 2.8 g dry cells l^{-1} when cells were grown in the medium containing cerulenin at 5 and 7.5 μg ml^{-1}. This value was almost the same as that of non-treated cells. The lipid yield (g l^{-1}) was decreased (252–182 g l^{-1}) as the cerulenin concentration increased, whereas EPA yield (mg l^{-1}) was increased (1.6–8 mg l^{-1}). The EPA yield was increased five times by cerulenin treatment. Since cerulenin inhibits phospholipid synthesis as well,

Table 1. Effect of cerulenin on fatty acid compositions of DHA-producing *M. marina* strain MP-1 at 10 °C.

Fatty acid	Cerulenin (μg ml^{-1})				
	0	0.1	0.5	1	2
12	1 [a]	1.7	4.6	6.1	6.7
14	10.3	15.9	23.8	26.9	32.5
14:1	2.1	4.6	6.4	7.7	9.8
16	12.2	11.3	12.1	11.3	14.1
16:1	58.5	52	28	22	17.2
18	–[b]	0.1	0.2	0.3	0.4
18:1	1.7	1	0.3	0.2	0.1
22:6	5.9	8	19.2	19.4	14.8
Others	8.3	5.5	5.9	6.6	4.9

[a]Values are means of three samples.
[b]Below the limit of detection.

cerulenin-treated cells may decrease the amount of their intracellular lipids.

All these results indicate that cerulenin treatment is also effective on enhancement of EPA production in EPA-producing *S. marinintestina* strain IK-1.

Other antibiotics

We have examined another antibiotic, triclosan, a broad-spectrum antimicrobial agent that inhibits lipid biosynthesis in *Escherichia coli*, probably by action upon enoyl–acyl carrier protein reductase (FabI) (McMurry *et al*. 1998). However, triclosan did not have the same effect as cerulenin on enhancement of PUFA production in *M. marina* strain MP-1 and *S. marinintestina* strain IK-1 cells (data not shown). It would be worth trying to see if other antibiotics or chemicals that inhibit fatty acid biosynthesis have the same effect of cerulenin or not on enhancement of PUFA production in PUFA-producing bacteria. For example, thiolactomycin (Oishi *et al*. 1982), which acts on the same enzyme on which cerulenin acts, would be a potent antibiotic to be examined.

Table 2. Effect of cerulenin on DHA production by DHA-producing *M. marina* strain MP-1 at 10 °C.

Parameter	Cerulenin (μg ml^{-1})				
	0	0.1	0.5	1	2.5
Biomass (g dry cells l^{-1})	2.5[a]	2.6	2.6	2.6	1.3
Lipid yield (g l^{-1})	0.2	0.2	0.2	0.2	0.1
DHA in biomass (mg g^{-1} dry cell)	1.6	2.4	5.3	5	4.1
DHA yield (mg l^{-1})	4	6.3	13.7	13.2	5.2

[a]Values are means of three samples.

Table 3. Effect of cerulenin on EPA production by EPA-producing *Shewanella marinintestina* strain IK-1 at 20 °C.

Parameter	Cerulenin (μg ml^{-1})		
	0	5	7.5
Biomass (g dry cell l^{-1})	2.6[a]	2.8	2.8
Lipid yield (mg l^{-1})	252	234	182
EPA in biomass (mg g^{-1} dry cell)	0.6	2.6	2.9
EPA yield (mg l^{-1})	1.6	7.3	8

[a]Values are means of three samples.

Possible use of PUFA-producing bacteria as a PUFA source

PUFAs, such as EPA and DHA, being supplied to the market are almost entirely extracted from the fish oil as PUFA-containing triacylglycerol. However, it has become apparent that PUFA-containing phospholipids have their specific physiological functions (Hibino & Tanaka 1994, Yazawa & Masuzawa 1991, Tanaka *et al*. 1993). Although the productivity of PUFAs in PUFA-producing bacteria seems lower than that in algae and fungi, PUFA-producing bacteria could be promising sources of high purity PUFA itself and PUFA-containing phospholipids because of their lipid characteristics as: (1) almost all cellular lipids are occupied by phospholipids, and (2) EPA or DHA is a sole PUFA found in PUFA-producing bacteria and extremely few other PUFAs are found in their membrane lipids. These characteristics make them attractive as alternative of fish oil.

Conclusion

The PUFA production in microorganisms usually starts from the selection of the strains that especially highly accumulate PUFAs. As we described here, however, the intracellular amount of DHA or EPA is able to be increased in cells of DHA-producing *M. marina* strain MP-1 and EPA-producing *S. marinintestina* strain IK-1, respectively, by adding a certain amount of cerulenin into culture medium. Furthermore, cerulenin treatment would be effective among certain kinds

of eukaryotic microorganisms, such as thraustochytrids that are the potential PUFA producers for industrial use (Singh & Ward 1997) since in the DHA-synthesizing thraustochytrid *Schizochytrium*, PUFA biosynthesis appears to occur by a bacterial-like PKS system (Ratledge 2004).

In conclusion, the use of cerulenin treatment is advantageous to enhance the intracellular PUFA production in PUFA-producing bacteria. These findings should significantly contribute to produce PUFA-related lipids in bacteria as well as eukaryotic microorganisms.

Acknowledgement

We are grateful to Ms Yumiko Kayukawa for her helpful assistance in bacterial cultivation and the extraction of lipids and fatty acids.

References

Allen EE, Facciotti D, Bartlett DH (1999) Monounsaturated but not polyunsaturated fatty acids are required for growth of the deep-sea bacterium *Photobacterium profundum* SS9 at high pressure and low temperature. *Appl. Environ. Microbiol.* **65**: 1710–1720.

Bajpai P, Bajpai PK (1993) Eicosapentaenoic acid (EPA) production from microorganisms: a review. *J. Biotechnol.* **30**: 161–183.

Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. *Can J. Biochem. Physiol.* **37**: 911–917.

Fang J, Kato C, Sato T, Chan O, McKay D (2004) Biosynthesis and dietary uptake of polyunsaturated fatty acids by piezophilic bacteria. *Comp. Biochem. Physiol. B. Biochem. Mol. Biol.* **137**: 455–461.

Hibino H, Tanaka Y (1994) Molecular structure and physiological functions of phospholipid containing docosahexaenoic acid. *J. Jpn. Oil Chem. Soc.* **43**: 687–697 (in Japanese).

Kato C, Nogi Y (2001) Correlation between phylogenetic structure and function: example from deep-sea Shewanella. *FEMS Microbiol. Lett.* **35**: 223–230.

McMurry LM, Oethinger, M, Levy SB (1988) Triclosan targets lipid synthesis. *Nature* **394**: 531–532.

Morita N, Tanaka M, Okuyama H (2000) Biosynthesis of fatty acids in the docosahexaenoic acid-producing bacterium *Moritella marina* strain MP-1. *Biochem. Soc. Transact.* **28**: 943–945.

Morita N, Ueno A, Tanaka M, Ohgiya S, Hoshino T, Kawasaki K, Yumoto I, Ishizaki K, Okuyama H (1999) Cloning and sequencing of clustered genes involved in fatty acid biosynthesis from the docosahexaenoic acid-producing bacterium, *Vibrio marinus* strain MP-1. *Biotechnol. Lett.* **21**: 641–646.

Nichols D, Bowman J, Sanderson K, Nichols CM, Lewis T, McMeekin T, Nichols PD (1999) Developments with antarctic micoorganisms: culture collections, bioactivity screening, taxonomy, PUFA production and cold-adapted enzymes. *Curr. Opin. Biotechnol.* **10**: 240–246.

Oishi H, Noto T, Sasaki H, Suzuki K, Hayashi T, Okazaki H, Ando K, Sawada M (1982) Thiolactomycin, a new antibiotic. I. Taxonomy of the producing organism, fermentation and biological properties. *J. Antibiot* (*Tokyo*). **35**: 391–395.

Ratledge C (2004) Fatty acid biosynthesis in microorganisms being used for single cell oil production. *Biochimie* **86**: 807–815.

Russell NJ, Nichols DS (1999) Polyunsaturated fatty acids in marine bacteria – a dogma rewritten. *Microbiology* **145**: 767–779.

Satomi M, Oikawa H, Yano Y (2003) *Shewanella marinintestina* sp. nov., *Shewanella schlegeliana* sp. nov. and *Shewanella sairae* sp. nov., novel eicosapentaenoic acid-producing marine bacteria isolated from sea-animal intestines. *Int. J. Syst. Evol. Microbiol.* **53**: 491–499.

Singh A, Ward OP (1997) Microbial production of docosahexaenoic acid (DHA, C22:6). *Adv. Appl. Microbiol.* **45**: 271–312.

Tanaka M, Ueno A, Kawasaki K, Yumoto I, Ohgiya S, Hoshino T, Ishizaki K, Okuyama H, Morita N (1999) Isolation of clustered genes that are notably homologous to the eicosapentaenoic acid biosynthesis gene cluster from the docosahexaenoic acid-producing bacterium *Vibrio marinus* strain MP-1. *Biotechnol. Lett.* **21**: 939–945.

Tanaka Y, Soda Y, Ando S (1993) Modulation by phospholipids of acetylcholine release from mouse brain synaptosome. *Neurochem. Res.* **18**: 847 (in Japanese).

Urakawa H, Kita-Tsukamoto K, Steven SE, Ohwada K, Colwell RR (1998) A proposal to transfer *Vibrio marinus* (Russell 1891) to a new genus *Moritella* gen. nov. as *Moritella marina* comb. nov. *FEMS Microbiol. Lett.* **165**: 373–378.

Valentine RC, Valentine DL (2004) *Omega*-3 fatty acids in cellular membranes: a unified concept. *Prog. Lipid Res.* **43**: 383–402.

Yazawa K, Masuzawa Y (1991) Phospholipids – Physiological activity of phospholipids. *J. Jpn. Oil Chem. Soc.* **40**: 845–857 (in Japanese).