Eicosapentaenoic Acid (EPA) Biosynthetic Gene Cluster of *Shewanella oneidensis* MR-1: Cloning, Heterologous Expression, and Effects of Temperature and Glucose on the Production of EPA in *Escherichia coli*

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Abstract The putative EPA synthesis gene cluster was mined from the entire genome sequence of *Shewanella oneidensis* MR-1. The gene cluster encodes a PKS-like pathway that consists of six open reading frames (ORFs): ORFSO1602 (multi-domain beta-ketoacyl synthase, KS-MAT-4ACPs-KR), ORFSO1600 (acyl transferase, AT), ORFSO1599 (multi-domain beta-ketoacyl synthase, KS-CLF-DH-DH), ORFSO1597 (enoyl reductase, ER), ORFSO1604 (phosphopentetheine transferase, PPT), and ORFSO1603 (transcriptional regulator). In order to prove involvement of the PKS-like machinery in EPA synthesis, a 20.195-kb DNA fragment containing the genes was amplified from *S. oneidensis* MR-1 by the long-PCR method. Its identity was confirmed by the methods of restriction enzyme site mapping and nested PCR of internal genes *orf*SO1597 and *orf*SO1604. The DNA fragment was cloned into *Escherichia coli* using cosmid vector SuperCos1 to form pCosEPA. Synthesis of EPA was observed in four *E. coli* clones harboring pCosEPA, of which the maximum yield was 0.689% of the total fatty acids in a clone designated 9704-23. The production yield of EPA in the *E. coli* clone was affected by cultivation temperature, showing maximum yield at 20°C and no production at 30°C or higher. In addition, production yield was inversely proportional to glucose concentration of the cultivation medium. From the above results, it was concluded that the PKS-like modules catalyze the synthesis of EPA. The synthetic process appears to be subject to regulatory mechanisms triggered by various environmental factors. This most likely occurs via the control of gene expression, protein stability, or enzyme activity.

Keywords: *Shewanella oneidensis* MR-1, polyunsaturated fatty acids, eicosapentaenoic acid, EPA biosynthetic gene cluster, polyketide synthase-like pathway

INTRODUCTION

Polyunsaturated fatty acids (PuFAs) such as arachidonic acid (AA, 20:4 ω3), eicosapentaenoic acid (EPA, 20:5 ω3), and docosahexaenoic acid (DHA, 22:6 ω3), are critical components of the glycolipids and phospholipids that compose plasma membranes. They also act as precursors to certain hormones and the signaling molecules eicosanoids [1-3]. Additionally, these fatty acids are commonly known to provide beneficial effects in the prevention and treatment of heart disease, high blood pressure, inflammation, and certain types of cancer [4-7].

PuFAs are typically synthesized via iterative reactions of the elongation and desaturation on the saturated fatty acid palmitic acid (PA, 16:0) by fatty acid synthase (FAS) [8-10]. Recently, a novel alternative pathway for the bio-synthesis of C_{20+} PuFAs has been suggested in some marine microorganisms [11-13]. This pathway was first proposed by Metz *et al.* for *Shewanella putrefaciens* SCRC-2738. This pathway employs polyketide synthase (PKS)-like modules, instead of the multiple desaturase and elongase enzymes. The PKS-like modules were assumed to directly condense acetyl units to C_{20+} poly-ketides. Each step of the two-carbon extension consists of sequential catalysis by ketoreductase, dehydratase, and/or enoyl reductase, leading to the partial or complete reduction of the keto group. Therefore, in addition to the structural simplicity of enzymatic complexes, the PKS-like pathway has an advantage over the desaturase-elongase pathway. In this unique process, a *cis*-double bond is inserted by aerobic desaturation after the complete reduction of keto group, thus conserving cellular reduction energy (1 NADPH every double bond) [14].

Shewanella oneidensis MR-1, a gram-negative facultative anaerobic proteobacterium, is capable of growing under a variety of conditions. In particulary, *S. oneidensis*

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MR-1 is reportedly able to growth at a fairly low temperature (3°C) [15]. The production of EPA was observed in this genus [16], where this PuFA likely contributes to maintaining membrane fluidity during growth at low temperatures. EPA synthesis and growth of *S. oneidensis* MR-1 has been characterized at various temperatures [17]. EPA was hypothesized to be produced via the PKS-like pathway, and the putative gene cluster was mined from the genome sequence. *Escherichia coli* is a useful strain in the fermentation [19] and has a powerful genetic tool system [20,21]. Therefore, in order to confirm the role of the PKS-like modules in EPA production, the gene cluster encoding PKS-like modules was cloned from *S. oneidensis* MR-1 and expressed in the heterologous host *E. coli*.

MATERIALS AND METHODS

Bacterial Strains, Plasmid, and Culture Media

S. oneidensis MR-1 was cultivated in a Luria Bertani (LB) medium at 30°C with shaking speed of 150 rpm. For the cloning of the EPA synthesis gene from *S. oneidensis* MR-1, *E. coli* strain XL1-Blue MR was used as the cloning host and cosmid SuperCos1 was and the vector. Ampicillin was added to the LB medium at a concentration of 50 μg/mL to select the cosmid plasmid.

Amplification of EPA Synthesis Gene Cluster

Genomic DNA of *S. oneidensis* MR-1 was purified using a Wizard® Genomic DNA Purification Kit (Promega) according to the manufacturers' protocol. Purified genomic DNA was quantified on an agarose gel (0.3%) and used as the template for long-PCR to amplify the EPA synthesis gene cluster. A set of primers was designed based on the genome sequence encoding PKS-like modules. This sequence showed high homology to the EPA synthesis gene cluster of *S. putrefaciens* SCRC-2738 (U73935), with a P1 of 5'-TAT<u>GCGGCCGC</u>TTAGCA-GCGTTGCAGAGGTTTG-3' and a P2 of 5'-TAT<u>GCG-GCCGC</u>TTAGTCAGCCAAACTAGCCGCTA-3'. To clone PCR products, the restriction site of *Not*I (underlined) was added to the primers. Long-PCR was performed using LA-Taq (Takara). The initial denaturation was carried out at 94°C for 5 min. This was followed by 30 cycles of denaturation at 94°C for 20 sec and annealing at 55°C for 30 sec. Annealing was followed by an extension at 68°C for 18 min and a final extension at 68°C for 25 min.

The amplified DNA fragments with the expected size of 20.195 kb were purified. Mapping of restriction sites of *Bgl*II and *Nde*I on the DNA fragments (Fig. 2) was carried out to confirm the correct amplification of the targeted DNA sequences. Primers used in nested PCR of internal genes on the DNA sequences *orf*SO1597 and *orf*SO1604 are shown in Fig. 2.

Cloning of EPA Synthesis Gene Cluster

The 20.195 kb DNA fragment was cloned into *E. coli* XL1-Blue using cosmid SuperCos1. The DNA fragment was treated with the restriction enzyme *Not*I, dephosphorylated with alkaline phosphatase, and was ligated with cosmid vector SuperCos1. This vector had been previously digested with *Xba*I and dephosphorylated with alkaline phosphatase. It was then cleaved with *Not*I. The ligated products were packaged *in vitro* to bacteriophage lambda particles using an *in vitro* packaging kit (Gigapack III Gold Packaging Extract, Stratagene) and introduced into *E. coli* cells by transfection. *E. coli* clones having the plasmid DNA, designated pCosEPA, were selected on an LB agar plate supplemented with antibiotic ampicillin (50 μg/mL).

Production of EPA in Transgenic *E. coli*

To induce the functional expression of genes encoding PKS-like modules, the *E. coli* clones harboring pCosEPA were cultivated in LB medium supplemented with ampicillin (50 μg/mL) at 25°C for 32 h. The cells were then transferred into a fresh medium and further cultivated for 36 h at 20°C. The fatty acid composition of the cultivated *E. coli* clones was analyzed by the method described below.

To examine effects of temperature and glucose concentration on the production of EPA, the *E. coli* clone (9704-23) was cultivated in LB medium at various cultivation temperatures (15, 20, 25, or 30°C) and with glucose concentrations (0, 0.05, and 0.2%).

Analysis of Fatty Acids Compositions

The *E. coli* cells were harvested from 50 mL of culture broth by centrifugation at 4,800 rpm for 15 min at 4°C. The cell pellet was washed twice with 1 M sorbitiol solution and dried for 3 h at 60°C. The dried cells were then suspended in 3 mL of 5% methanolic HCl and heated at 90°C for 1 h in sealed tubes. Fatty acid methyl esters were extracted from the cells with 0.6 mL hexane and were applied to a gas chromatograph (GC, Hewlett Packard 6890, USA) equipped with a flame-ionized detector (FID) and a DB23 (30 m × 0.25 mm × 0.26 m, Agilent Technologies, USA) capillary column. The column temperature was raised from 150°C (2 min) to 270°C (2 min) at 7°C per minute [22,23]. All the results presented were obtained from at least three independent experiments. Average values are shown.

RESULTS AND DISCUSSION

Organization of EPA Biosynthetic Gene Cluster

Recently, an alternative pathway for the synthesis of polyunsaturated fatty acids (PuFAs) has been found in some marine prokaryotic and eukaryotic microorganisms. In this pathway, eicosapentaenoic acid (EPA, 20:5 ω3) and docosahexaenoic acid (DHA, 22:6 ω3) are catalyzed by polyketide synthase (PKS)-like modules. Yazawa and collaborators have initially isolated a 38-kb gene cluster

Fig. 1. Organization of genes and domains of EPA biosynthesis gene cluster from *S. oneidensis* MR-1. Abbreviations: ER = enoyl reductase, DH = dehydratase, CLF = chain length factor, KS = 3-ketoacylsynthase, AT = acyltransferase, KR = 3-ketoacyl reductase, ACP = acyl carrier protein, MAT = malonyl-CoA acyltransferase, TR = transcriptional regulator, and PPT = 4-phosphopantetheine transferase.
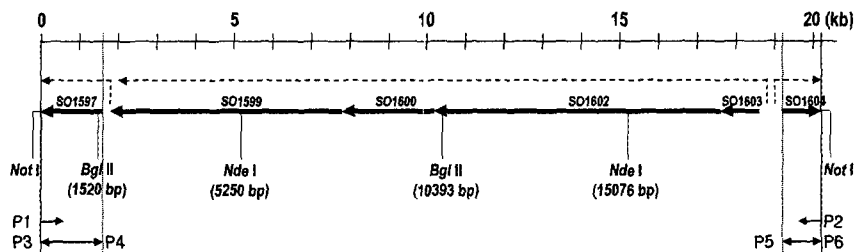

Fig. 2. PCR amplified DNA fragment (20.195 kb). Bent and dashed arrows indicate putative transcripts. Restriction enzyme sites for mapping and primers used for long-PCR (P1 and P2) and nested PCR (P3 and P4 for *orf*SO1597 and P5 and P6 for *orf*SO1604) are presented.

from *S. putrefaciens* SCRC-2738. The expression of these genes resulted in the production of EPA in the heterologous host *E. coli* [11,18]. Five open reading frames (ORFs) were identified to be required for EPA production in *E. coli*. A gene cluster was also identified from the whole genome sequences of fatty acid-producing *S. oneidensis* MR-1 that included correspondents to the five ORFs [17].

The ORFs from the *Schwanella* species are believed to build a polyketide synthase (PKS)-like mechanism employing ORFSO1602 (multi-domain beta-ketoacyl synthase, KS-MAT-4ACPs-KR), ORFSO1600 (acyl transferase, AT), ORFSO1599 (multi-domain beta-ketoacyl synthase, KS-CLF-DH-DH), ORFSO1597 (enoyl reductase, ER), and ORFSO1604 (phosphopentetheine transferase, PPT) in *S. oneidensis* MR-1 (Fig. 1).

Expression of the gene cluster encoding PKS-like modules was predicted to occur as at least two divergent transcripts consisted of *orf*SO1604 and *orf*SO1603-SO1602-SO1600-SO1599-*orf*SO1597 (but *orf*SO1597 may be transcribed apart from upstream genes) (Fig. 2). OrfSO1603, a homolog of the transcriptional regulator, possibly controls gene expression. However, the signal or the regulatory mechanism is unclear.

Amplification and Cloning of EPA Biosynthetic Gene Cluster

In order to confirm the role of PKS-like modules in EPA synthesis and to analyze the function of each module and domain, the gene cluster was cloned from *S. oneidensis* MR-1. The 20.195 kb DNA fragment, which contained six genes consisting of the PKS-like modules from genomic DNA, was amplified by long-PCR method (Fig. 2). For success with the long-PCR method, chromosomal DNA of *S. oneidensis* MR-1 was moderately prepared using a Wiz-

ard® Genomic DNA Purification Kit (Materials and Methods). This procedure included protein removal via a salt precipitation step, which protected against shearing of the genomic DNA. Band Doctor Reagent solution was used to overcome the problems caused by high GC concentration or interference from the secondary structure of the genomic DNA. An additional extension reaction catalyzed by LA-Taq polymerase was carried out at 68°C for 50 sec per 1 kb DNA to minimize any errors that may have occurred during the long-PCR process.

Correct amplification of the targeted DNA fragment was confirmed by nested PCR amplification of *orf*SO1597 and *orf*SO1604 genes located on each end of the gene cluster (Fig. 2). Additionally, mapping of the restriction enzyme sites was performed on the PCR-amplified DNA fragment was performed (Fig. 2). As expected, DNA was cut into fragments with sizes of 1,520, 8,873, and 9,802 bp by *Bgl*II (Fig. 2C). Fragments of 5,250, 9,826, and 5,119 bp were created by *Nde*I (Fig. 2).

The DNA fragment digested with *Not*I and the dephosphorylated DNA fragments were inserted into the cosmid vector SuperCos1 predigested with *Xba*I and *Not*I. The ligated DNAs were packaged using an *in vitro* λ bacteriphage packaging system, and were transfected into *E. coli* XL1-Blue MR. Approximately 800 colonies of *E. coli* cells with the cosmid plasmids, known as pCosEPA, were obtained. Among them, 30 transformants were selected and subjected to further analysis.

Production of EPA in Transgenic *E. coli*

To examine the production of EPA by the functional expression of cloned genes, *E. coli* clones harboring plasmid pCosEPA were cultivated at 20°C for 34 h. The fatty acid composition of the cells was analyzed by gas chromatography (GC). EPA production was detected in four *E. coli* cell
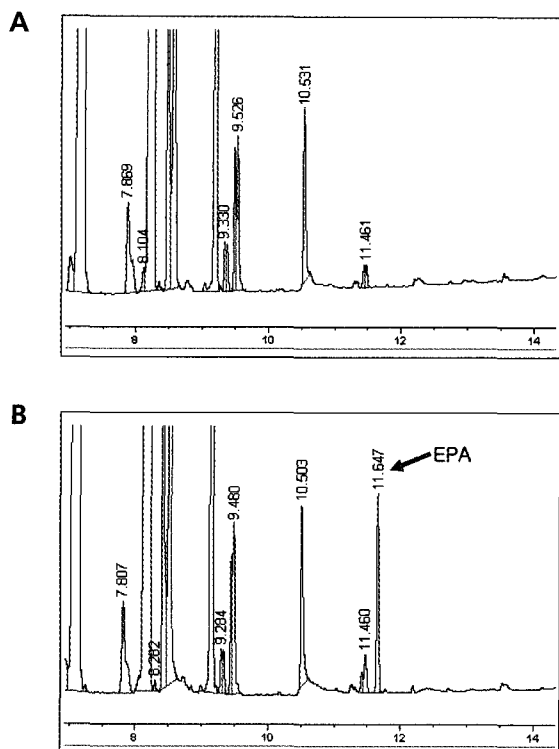
Fig. 3. Gas chromatography of fatty acid methyl esters. (A) *E. coli* XL1-Blue MR-1 containing cosmid vector SuperCos1. (B) *E. coli* XL1-Blue MR-1 containing pCosEPA.

Fig. 4. Levels of EPA in *E. coli* strain XL1-Blue MR clones transformed with pCosEPA. Transformed *E. coli* cells were grown at 20°C.

lines (Fig. 3): 9704-13, 9704-20, 9704-21, and 9704-23 (Fig. 4). This clearly indicates that the PKS-like modules are involved in the production of EPA in *S. oneidensis* MR-1. The maximum yield of EPA obtained in 9704-23 was 4.5 times higher than that in the native host. This may be simply explained by the increase in the copy number of the genes encoding PKS-like modules in the *E. coli* host. On the other hand, the yield of EPA was shown to vary in the four clones, suggesting that mutation(s) of the genes that encode PKS-like modules were introduced during the amplification process. Further analyses on the mutation(s) could provide clues for understanding the relationship between function and structure of the PKS-like modules.

Effect of Temperature on EPA Production in *E. coli*

The production yield of EPA catalyzed by the PKS-like modules in *S. oneidensis* MR-1 cells was found to depend on the cultivation temperature (Fig. 5) [17]. The maximum yield of EPA production was approximately 0.6% at 4°C, with slightly lower yields of approximately 0.15% at 15 and 22°C, and there was no production of EPA at 30°C or higher. It was concluded that PUFAs likely played a role in maintaining the membrane fluidity at low temperatures. The effect of cultivation temperature on the heterologous production of EPA in *E. coli* was also examined. The *E. coli* clones harboring pCosEPA showed extremely slow growth at the low temperature condition of 10°C. It was not possible to measure the yield of EPA
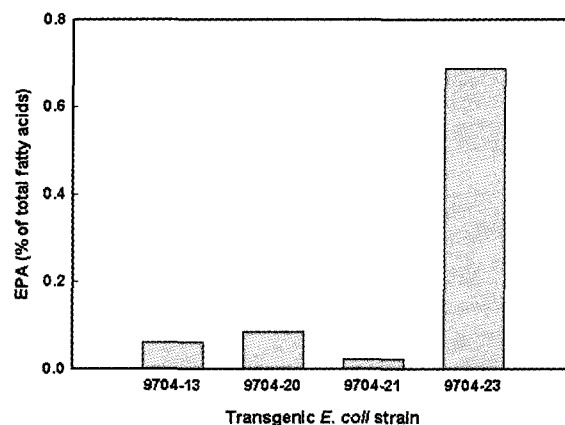
at this under such conditions, primarily for the reason that the cell mass was insufficient for an analysis of the fatty acid composition. Analysis was also hindered by a prolonged cultivation time of more than seven days, resulting in the probable loss of large-sized plasmids in the clones. The maximum yield of EPA production was observed in *E. coli* cells grown at 20°C (Fig. 5). Interestingly, at 15°C, the production yield was relatively lower compared to that at 20°C, and was a similar level to that seen at 25°C (Fig. 5). A similar result was reported by Yazawa *et al.* [11] in the heterologous production of EPA in *E. coli* carrying pEPA containing similar genes from *S. putrefaciens* SCRC-2738, although the cause of this was unclear. At a high temperature condition of 30°C or higher, production of EPA in the *E. coli* clone was completely abolished. This may be explained by the temperature-dependent control of the gene expression or by the thermolabile characteristic of the PKS-like modules.

Effect of Glucose Concentration on EPA Production in *E. coli*

Cytosolic acetyl-CoA and malonyl-CoA that are derived from glucose are known to be utilized in the enzymatic reaction of PKS-like modules as substrates of the synthesis of PUFAs. From this viewpoint, the effects of glucose concentration on the production yield of EPA in *E. coli* clones were examined. Unexpectedly, the production yield decreased as glucose concentration increased in the culture medium (0, 0.05, and 0.2%) (Fig. 6). This suggests that the production of EPA is subject to regulation by various environmental factors.

CONCLUSION

A gene cluster believed to participate in the biosynthesis of EPA was identified from the whole genome sequence of *S. oneidensis* MR-1. This was done through computational analysis based on the EPA biosynthetic
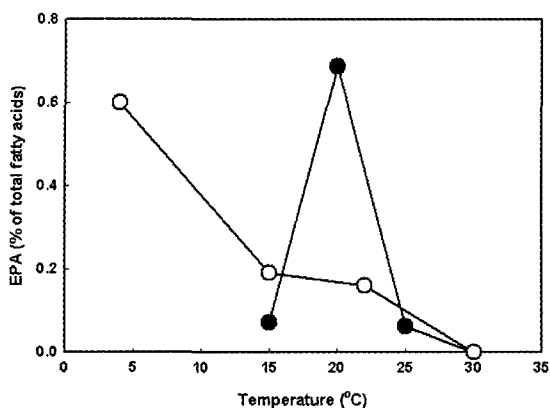
Fig. 5. Effects of growth temperature on EPA levels in total fatty acids of *E. coli* strain 9704-23 (closed circle) or *S. oneidensis* MR-1 (open circle).
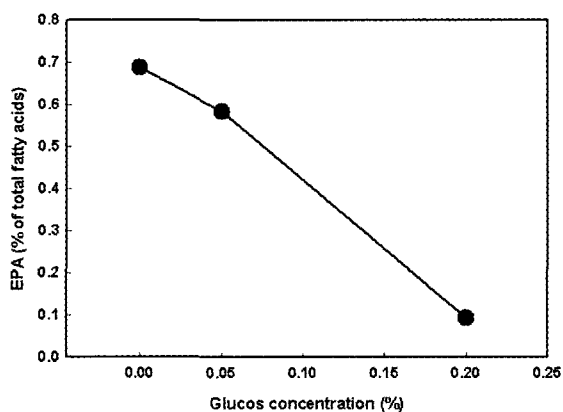

Fig. 6. Effects of glucose concentration on EPA levels in total fatty acids of *E. coli* strain 9704-23.

genes of *S. putrefaciens* SCRC-2738. The gene cluster, containing five genes encoding PKS-like modules and a transcription factor, was amplified by long-PCR and cloned using the cosmid vector SuperCos1. This resulted in the appearance of *E. coli* clones harboring pCosEPA. Production of EPA was observed in the *E. coli* clones, confirming that the PKS-like modules are involved in the synthesis of EPA. The production yield of EPA was affected by the cultivation temperature of heterologous host *E. coli*, showing the maximum yield at 20°C and no production at 30°C or higher. Additionally, the production yield was inversely proportional to glucose concentration. These results indicate that the production of EPA is controlled by various environmental factors, most likely via the control of gene expression, protein stability, or enzyme activity.

REFERENCES

[1] Lauritzen, L., H. S. Hansen, M. H. Jorgensen, and K. F.

Michaelsen (2001) The essentiality of long chain n-3 fatty acids in relation to development and function of the brain and retina. *Prog. Lipid Res.* 40: 1-94.

[2] Braden, L. M. and K. K. Caroll (1986) Dietary polyunsaturated fat in relation to mammary carcinogenesis in rats. *Lipids* 21: 285-288.

[3] Abbey, M., P. Clifton, M. Kestin, B. Belling, and P. Nestel (1990) Effect of fish oil on lipoproteins, lecithin: cholesterol acyltransferase, and lipid transfer protein activity in humans. *Arteriosclerosis* 10: 85-94.

[4] Sauer, L. A., R. T. Dauchy, and D. E. Blask (2001) Polyunsaturated fatty acids, melatonin, and cancer prevention. *Biochem. Pharmacol.* 61: 1455-1462.

[5] Angerer, P. and C. von Schacky (2000) n-3 polyunsaturated fatty acids and the cardiovascular system. *Curr. Opin. Clin. Nutr. Metab. Care* 3: 439-445.

[6] Tresguerres, J. A. F., V. Lahera, and M. V. Cachofeiro (1989) Dietary essential polyunsaturated fatty acids and blood pressure *News Physiol. Sci.* 4: 64-68.

[7] Fiocchi, A., M. Sala, P. Signoroni, G. Banderal, C. Agostoul, and E. Riva (1994) The efficiency and safety of γ-linolenic acid in the treatment of infantile atopic dermatitis. *J. Int. Med. Res.* 22: 2-32.

[8] Chung, T.-H., S.-J. Lee, H.-J. Oh, G.-J. Kim, and B.-K. Hur (2005) Cloning of a Δ5 desaturase from *Thraustochytrium* sp. 26185 and functional expression in *Pichia pastoris. Korean J. Biotechnol. Bioeng.* 20: 93-99.

[9] Wallis, J. G., J. L. Watts, and J. Browse (2002) Polyunsaturated fatty acid synthesis: what will they think of next? *Trends Biochem. Sci.* 27: 467-473.

[10] Qiu, X. (2003) Biosynthesis of docosahexaenoic acid (DHA, 22:6-4, 7,10,13,16,19): two distinct pathways. *Prostaglandins Leukot. Essent. Fatty Acids* 68: 181-186.

[11] Yazawa, K. (1996) Production of eicosapentaenoic acid from marine bacteria. *Lipids* 31: S297-S300.

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

[13] Kaulmann, U. and C. Hertweck (2002) Biosynthesis of polyunsaturated fatty acids by polyketide synthases. *Angew. Chem. Int. Ed.* 41: 1866-1869.

[14] Metz, J. G., P. Roessler, D. Facciotti, C. Levering, F. Dittrich, M. Lassner, R. Valentine, K. Lardizabal, F. Domergue, A. Yamada, K. Yazawa, V. Knauf, and J. Browse (2001) Production of polyunsaturated fatty acids by polyketide synthases in both prokaryotes and eukaryotes. *Science* 293: 290-293.

[15] Abboud, R., R. Pora, V. Souza-Egipsy, C. S. Giometti, S. Tollaksen, J. J. Mosher, R. H. Findlay, and K. H. Nealson (2005) Low-temperature growth of *Shewanella oneidensis* MR-1. *Appl. Environ. Microbiol.* 71: 811-816.

[16] Kato, C. and Y. Nogi (2001) Correlation between phylogenetic structure and function: examples from deep-sea *Shewanella. FEMS Microbiol. Ecol.* 35: 223-230.

[17] Jeong, Y.-S., S.-K. Song, S.-J. Lee, and B.-K. Hur (2006) The growth and EPA synthesis of *Shewanella oneidensis* MR-1 and expectation of EPA biosynthetic pathway. *Biotechnol. Bioprocess Eng.* 11: 127-133.

[18] Orikasa, Y., A. Yamada, R. Yu, Y. Ito, T. Nishida, I. Yu-

moto, K. Watanabe, and H. Okuyama (2004) Characterization of the eicosapentaenoic acid biosynthesis gene cluster from *Shewanella* sp. strain SCRC-2738. *Cell. Mol. Biol.* 50: 625-630.

[19] Choi, J.-I., S. Y. Lee, K. Shin, W. G. Lee, S. J. Park, H. N. Chang, and Y. K. Chang (2002) Pilot scale production of poly(3-hydroxybutyrate-*co*-3-hydroxy-valerate) by fed-batch culture of recombinant *Escherichia coli. Biotechnol. Bioprocess Eng.* 7: 371-374.

[20] Choi, J.-I. and S. Y. Lee (2004) High level production of supra molecular weight poly(3-hydroxybutryrate) by metabolically engineered *Escherichia coli. Biotechnol. Bioprocess Eng.* 9: 196-200.

[21] Yoon, S.-H., C. Li, Y.-M. Lee, S.-H. Lee, S.-H. Kim, M.-S. Choi, W.-T. Seo, J.-K. Yang, J.-Y. Kim, and S.-W. Kim (2005) Production of vanillin from ferulic acid using recombinant strains of *Escherichia coli. Biotechnol. Bioprocess Eng.* 10: 378-384.

[22] Lepage, G. and C. C. Roy (1984) Improved recovery of fatty acid through direct transesterification without prior extraction or purification. *J. Lipid Res.* 25: 1391-1396.

[23] Kang, K.-Y., D.-H. Ahn, S.-M. Jung, D.-H. Kim, and B.-S. Chun (2005) Separation of protein and fatty acids from tuna viscera using supercritical carbon dioxide. *Biotechnol. Bioprocess Eng.* 10: 315-321.