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ORIGINAL PAPER

Enhancement of polyunsaturated fatty acid production by Tn5 transposon in *Shewanella baltica*

Mitra Amiri-Jami · Haifeng Wang · Yukio Kakuda · Mansel W. Griffiths

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Abstract Transposon Tn5 mutagenesis was used to generate random mutations in *Shewanella baltica* MAC1, a polyunsaturated fatty acid (PUFA)-producing bacterium. Three mutants produced 3–5 times more eicosapentaenoic acid (EPA 20:5 *n*–3) compared to the wild type at 10°C. One of the mutants produced 0.3 mg EPA g^{-1} when grown at high temperature (30°C). Moreover, 2 mg docosahexaenoic acid (DHA 22:6 *n*–3) g^{-1} was produced by *S. baltica* mutants at 4°C. Sequencing of insertion mutation(s) showed 96% homology to trimethylamine *N*-oxide (TMAO) reductase gene and 85% homology to rRNA operons of *E. coli*. Tn5 transposon mutagenesis therefore is a suitable technique to increase PUFA formation in bacteria.

Keywords Docosahexaenoic acid · Eicosapentaenoic acid · *Shewanella* · Transposon mutagenesis

M. Amiri-Jami · H. Wang · Y. Kakuda · M. W. Griffiths
Department of Food Science, University of Guelph, N1G 2W1 Guelph, ON, Canada

H. Wang · M. W. Griffiths (✉)
Canadian Research Institute for Food Safety, University of Guelph, N1G 2W1 Guelph, ON, Canada
e-mail: mgriffit@uoguelph.ca

Introduction

Polyunsaturated fatty acids (PUFAs), in particular omega-3 fatty acids (ω-3 FAs) such as eicosopentaenoic acid (EPA; 20:5 *n*–3) and docosahexaenoic acid (DHA; 22:6 *n*–3), have received much attention for their remarkably wide range of physiological (Fontani et al. 2005) and clinical effects (Siddiqui et al. 2004; MacLean et al. 2006). Currently, the main sources of dietary EPA and DHA are marine fish. However, declining fish populations and an undesirable fishy flavor and odor in fish oil have led to an interest in alternative sources of EPA and DHA. These sources include fungi, marine algae, diatoms, and some bacteria (*Shewanella* and *Colwellia*) (Russell and Nichols 1999; Satomi et al. 2003). Attention has been focused on bacterial production of EPA and DHA for two main reasons. Firstly, bacteria can be easily cultivated in simple media which makes them a prime candidate for industrial production of PUFAs. Secondly, it is possible to genetically manipulate bacteria in order to increase synthesis of PUFAs.

A bacterium, isolated from mackerel entrails in our laboratory (Cadieux et al. 1998), was identified as *Shewanella baltica* MAC1 (M. Amiri and M. W. Griffiths, unpublished data). This bacterium has been of interest because it has the capability of synthesizing PUFA (Cadieux et al. 1998). To increase PUFA production in *Shewanella* cells,

changes to the growth media and incubation conditions have been investigated (Cadieux et al. 1998; Morita et al. 2005). However, Tn5 transposon mutagenesis has not been reported as a means for increasing PUFA production. The Tn5 transposon is particularly useful for insertion mutagenesis of a variety of Gram-negative bacteria (Lewenza et al. 2005). De Lorenzo et al. (1990) constructed a collection of Tn5-derived mini-transposons that simply generate random mutations. The mini-transposons consist of genes specifying resistance to antibiotics such as kanamycin and chloramphenicol as selection markers and an unique *Not*I cloning site flanked by 19-base pair terminal repeat sequences of Tn5 (De Lorenzo et al. 1990). The delivery system employed for all the Tn5 transposons is the suicide pUT plasmid. Plasmid pUT is only maintained and replicated in π protein-producing bacteria. When pUT is transferred from a donor strain to a recipient strain without the gene encoding the π protein, pUT cannot replicate and only Tn5 is inserted in the genome of the recipient strain (Llamas et al. 2000). In this study, Tn5 transposon mutagenesis was investigated as a new strategy to enhance PUFA production in *Shewanella* MAC1. The benefits of having mutant MAC1 with ability to produce more PUFA are described.

Materials and methods

Bacterial strains and growth conditions

Escherichia coli containing pUT mini-Tn5 Km2 (obtained from the Institute of Infection and Immunity, Nottingham, UK) and *Shewanella baltica* MAC1 were the donor and recipient strains, respectively. Wild-type MAC1 was grown in Marine Broth (MB) 2216 (Difco) and Marine Broth supplemented with 50 mg kanamycin ml^{-1} (MBK) was used for selection of the insertion mutants. Sixty selected mutants and the parent *Shewanella* MAC1 were grown in 5 ml MBK or MB overnight at 30°C with shaking at 70 rpm. Then 200 ml of broth were inoculated with 1 ml of each overnight culture. Cells were incubated at 10°C on a rotary shaker (70 rpm) for 2–3 days to an OD at 600 nm of 2. Furthermore, three high EPA-producing mutants were incubated at 4 and 30°C under the same conditions explained above.

Insertion mutagenesis

To generate insertion mutants, the pUT mini-Tn5 Km2 transfer plasmid was used. For conjugation, the donor strain was grown overnight in 5 ml Luria broth (Fisher) containing kanamycin at 10 mg ml^{-1} at 37°C. The recipient strain was grown overnight in 5 ml MB at 30°C. The rest of the steps for generating mutants were performed as described previously (Winson et al. 1998).

Fatty acid extraction and analysis

The cells were harvested by centrifugation at 28,000 *g* for 17 min at 10°C, washed with ultrapure water twice and freeze-dried overnight. Total lipids from freeze dried cells were extracted by the method of Cadieux et al. (1998). The fatty acid methyl esters were analyzed by GC using a BPX70 capillary column (60 m × 0.22 mm). The injector and detector were maintained at 260 and 280°C, respectively. The oven was programmed to increase from 110°C to 230°C. The initial time was set for 2 min, program rate for 4 min and final time for 10 min. Peak areas were quantified by a data processor. The fatty acid methyl esters were identified by comparison of their retention times with those of known standards. Internal standards for PUFA peaks were also used. Furthermore, EPA production was confirmed by GC/MS using a Varian Saturn 2000 GC-mass spectrometer with a Varian CP-Sil 5CB-MS 30 m × 0.25 m column (Varian Canada, Mississauga, ON, Canada). The oven was programmed to increase from 125°C to 250°C at 4°C min^{-1}. Compounds were identified by comparison of relative retention time and mass spectra with that of pure standard EPA.

Site mapping of insertion mutagenesis

GenomicPrep Cells and Tissue DNA Isolation Kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) was used to extract the genomic DNA of wild MAC1 and mutant MAC1. Primers,

5′-CGAGCTCGAATTCGGCCTAG-3; 5′-CTG CAGGCATGCAAGCTTCG-3′; 5′-GCCGAAC TTGTGTATAAGAGTC-3′ and 5′-GCCAGAT CTGATCAAGAGAC-3′ were designed according to sequences at either end of the kanamycin resistance genes and terminal sequences of the mini transposon Tn5 (Karlyshev et al. 2000). Single-Primer Polymerase Chain Reaction was used to locate the insertion position of the mini-Tn5 in mutants as described previously (Karlyshev et al. 2000). The PCR product was separated from the reaction mix by running a 1% agarose gel. DNA bands exclusively appearing in mutant lanes were excised, and DNA fragments in the gel pieces were extracted using a commercial Qiagen Quick Gel Extraction kit (Qiagen Inc., Mississauga, ON, Canada). Purified PCR fragments were ligated with linear pGEM-T Easy Vector (Promega Corporation; Fisher Scientific Co.) in 1× ligase buffer and were transformed into high efficiency competent JM 109 cells by the heat shock transformation method. Colonies containing the PCR insert were selected and were purified by subculturing on LB agar plates (Difco) supplemented with 50 μg ampicillin ml^{-1}, 80 μg X-Gal ml^{-1} and 0.5 mM IPTG. Plasmids in the selected clones were extracted and sent for sequence analysis to Guelph Molecular Supercentre, Guelph, Canada.

Results and discussion

Effect of insertional mutagenesis on EPA-production

Mini-Tn5 random insertion mutagenesis resulted in more than 500 mutants. Sixty mutants were selected randomly and they were tested for PUFA production. Wild type and all mutated MAC1 were capable of synthesizing EPA and linolenic acid (LNA; 18:3 *n*–3) at 10°C but three mutants showed significantly higher EPA production than the wild-type MAC1. These mutants, which were designated as mutants a, b and c, produced 3–5 times more EPA than that of parent (Fig. 1). Amounts of EPA in these three selected mutants were confirmed in three independent experiments. Since there is a significant difference ($P < 0.01$) in the amount of EPA

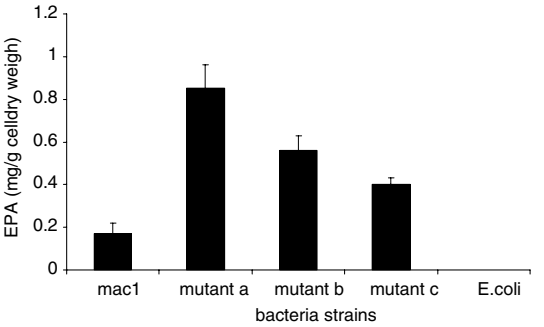

Fig. 1 Mean of EPA concentrations in 0.2 g cell dry weight of wild type MAC1 and three mutated MAC1 cultured at 10°C in three independent experiments. EPA concentrations are expressed in mg and were calculated from the area under the GC chromatogram peak corresponding to EPA

production between mutants and wild-type MAC1, particularly at 10°C, this may suggest that the insertion of mini-Tn5 affected the enzyme activity related to biosynthesis of EPA. These mutants were confirmed to have insertion(s) in their genomic DNA by antibiotic resistance and PCR. The mini-transposon is located on an R6K-based suicide delivery plasmid, which can be maintained only in donor strains producing the R6K-specified π protein (Llamas et al. 2000). When the suicide plasmids are transferred into a recipient cell without π protein, the plasmids will not be amplified but chromosomally located genes will be expressed.

Polyunsaturated fatty acids production by wild type and mutant at different temperatures

Mutants were normally grown at 10°C. However, differences in the cell membrane fatty acid composition, particularly EPA, docosapentaenoic acid (DPA; 22:5*n*–3, a precursor of DHA), DHA and LNA (18:3*n*–3) acid concentrations, were studied to investigate PUFA production by mutants at 4 and 30°C. Wild type and mutant strains cultured at 4°C produced higher amounts of EPA compared to cells grown at 10°C (Table 1). Mutant (a) produced 10-fold more DPA than wild type MAC1 at 4°C (Table 2). In addition, mutants produced 0.04 to 0.2% (w/w) DHA at 4°C (Table 2). The ability of bacteria to adapt their membrane lipid composition in

Table 1 Cells dry weight and total lipid contents of the wild type MAC1 and mutants at different temperatures

Parameter	MAC1			Mutant a			Mutant b			Mutant c		
	4°C	10°C	30°C	4°C	10°C	30°C	4°C	10°C	30°C	4°C	10°C	30°C
Biomass[a]	0.9[d]	1.1	0.85	0.9	1	0.85	0.88	1.05	0.85	0.9	1.08	0.87
Lipid yield[b]	0.032	0.036	0.03	0.032	0.036	0.03	0.032	0.036	0.031	0.032	0.036	0.030
EPA in-Biomass[c]	2.4	1	0	6	4.8	0.3	4	3.8	0	3.85	2.9	0

[a]g dry cells 1^{-1}

[b]g lipid 1^{-1}

[c]mg EPA g^{-1} dry cells

[d]Values are means of three samples

Table 2 Fatty acid compositions (wt%) of the lipid from wild type MAC1 and mutants

Fatty acid	MAC1 (%)			Mutant[a] (%)			Mutant[b] (%)			Mutant[c] (%)		
	4°C	10°C	30°C	4°C	10°C	30°C	4°C	10°C	30°C	4°C	10°C	30°C
14:0	4.9[a]	4.0	8.4	4.4	4.0	7.0	4.8	3.8	7.8	4.9	4.02	8.2
14:1n7	2.0	1.5	3.5	1.6	1.3	3.1	1.9	1.42	3.45	2	1.09	3.5
15:0	15	13	19	14.8	11.9	18.8	15	12.7	19	14.5	13	18.2
16:0	0.69	1.1	0.95	0.65	0.95	0.96	0.68	1.1	1.07	0.70	1.03	1
16:1n7c	8.6	7	14.8	8	6.1	15.2	8.7	6.5	14.7	8.5	6.7	15.8
17:0	36.9	42	30.7	36.6	39.3	30.5	36.9	40.8	29.7	36.3	41	29.3
18:0	2.31	3.2	6.8	2.2	3.4	7.8	2.3	3.2	7.86	2.3	3	7.4
18:1n9c	1.7	2.8	1.5	1.6	3.0	1.8	1.71	2.5	2.3	1.68	2.3	2
18:2n6c	6.1	9.4	1.6	6.06	9.2	1.5	6	9	1.3	6	9.09	1.5
18:3n3	0.05	0.3	0.35	0.06	0.4	0.4	0.05	0.36	0.38	0.053	0.4	0.35
20:5n3[b]	3.5	1.5	0	5.5	6.0	0.2	4.85	4.5	0	4.9	3.8	0
22:5n3	0.03	0	0	0.3	0	0	0.08	0	0	0.08	0	0
22:6n3	0	0	0	0.04	0	0	0.2	0	0	0.07	0	0
Others[c]	17.2	14.5	12.5	18	14.5	12.5	16.83	14.2	12.4	17.7	14.5	12.7

[a]Values are means of three samples

[b]Eicosapentaenoic acid (EPA)

[c]Includes: 13:0; 15:1n8c; 16:1n9c; 17:1n8c; 18:1n9t; 18:1n7c; 18:2n6t; 21:0 and some traces which were less than 0.1%

response to changes in environmental temperature has been well documented (Valentine and Valentine 2004). This adaptation usually involves a change in unsaturated fatty acid concentrations in the membrane. The proportion of PUFA in membrane lipids indicates that they are likely to be part of a homeoviscous adaptive response to regulate membrane fluidity. Moreover, PUFA make the cellular membranes more permeable at cold temperatures (Valentine and Valentine 2004). The relative amount of EPA in mutant MAC1 decreased as the growth temperature was increased to 30°C and this is similar to results reported previously (Watanabe et al. 1996). Therefore, it can be concluded that the adaptation of wild-type and mutant MAC1 to growth at low temperatures was related to changes in the relative amount of cellular PUFA, particularly EPA. EPA also regulates membrane fluidity of the scallop (*Placopecten magellanicus*) in response to cold (Hall et al. 2002). Wild type MAC1 does not produce EPA at 30°C while mutant (a) was able to produce EPA at this temperature (Table 1). Therefore, this indicates the strong effect of insertion mutagenesis in enhancement of EPA production.

The total fatty acid composition of the wild type and mutants was studied when they were grown at different temperatures (Table 2). The number of fatty acid peaks was the same for MAC1 and mutants at 4, 10 and 30°C. At 30°C, more long chain saturated fatty acids and short

chain unsaturated fatty acids were detected. Apart from EPA, no significant differences were seen for the fatty acid composition of the wild type MAC1 and mutants at 10, 4 and 30°C (Table 2).

Genetic analysis of mutants

Four different primers were used in the single primer PCR method. Forty PCR generated fragments (500–2,000 bp) were isolated from mutants, which were absent in the wild type MAC1 (Fig. 2). Three non-specific common fragments were seen in wild type and mutant MAC1. The extra bands indicate that amplification resulted in predominantly Tn5-specific PCR products. Ten plasmids containing different PCR fragments were sent for sequencing. A search of the Genbank database revealed that one of the sequences related to mutant (a) showed 96% homology to the DNA sequence of the trimethylamine *N*-oxide (TMAO) reductase encoding gene (*tor*A) from *Shewanella massilia* (data not shown). The TorA enzyme is responsible for TMAO reduction. This enzyme has a high substrate specificity and can only reduce TMAO efficiently as a natural compound (Bordi et al. 2003). Various bacteria grow anaerobically using TMAO which acts as an alternative terminal electron acceptor of a respiratory transport chain (McCrindle et al. 2005). Furthermore, it is reported that TMAO is involved in the respiratory pathway and increases the ability for anaerobic growth of bacteria (Carpentier et al. 2005). *Shewanella* MAC1 is a facultative anaerobic bacterium. It can be speculated that *Shewanella* MAC1 might use both aerobic and anaerobic pathways to synthesize fatty acids. It is possible that the mini Tn-5 insertion disrupted the *tor*A gene encoding TorA enzyme involved in the TMAO reductase respiratory pathway. Therefore, the level of TMAO might have increased in bacterial cells and, consequently, the anaerobic pathway of bacterial fatty acid biosynthesis might be elevated. This phenomenon might be a reason for higher EPA production by mutant (a). In addition, the anaerobic pathway, using the polyketide synthase (PKS) route, is responsible for bacterial PUFA biosynthesis (Ratledge 2004). Another sequence related to the mutant had 85% homology with rRNA operons of *E. coli*. Since rRNA is involved in protein synthesis, it is possible that insertion of mini Tn5 in this region led to changes in the regulation of the enzyme(s) responsible for PUFA production.

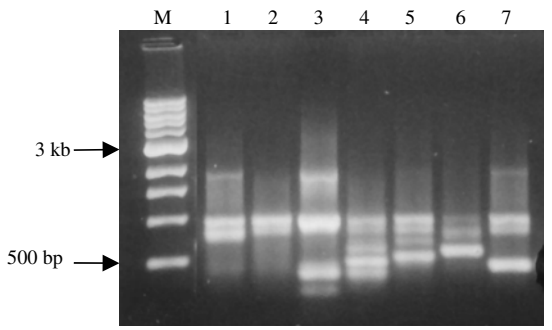

Fig. 2 Gel electrophoresis of PCR products obtained using primer 2. M: 1 kb ladder; lane 1: MAC1; lanes 2–7 mutants

Conclusion

The PUFA production in bacteria usually can be increased by changing the growth media and incubation conditions. However, the use of the Tn5 transposon is a great advantage to generate mutants with ability to produce higher level of PUFA or to find a new strain that can produce PUFA at high temperature (30°C). In addition, the PUFA production in PUFA-producing mutants can be further enhanced by changing the composition of growth media and cultural conditions to make them a better candidate for use in industry.

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