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Original Article

Regulatory mechanism of membrane protein production in an EPA-producing bacterium, *Shewanella livingstonensis* Ac10

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Summary

A psychrotrophic Gram-negative bacterium, *Shewanella livingstonensis* Ac10, inducibly produces eicosapentaenoic acid (EPA) as a component of membrane phospholipids at low temperatures. We disrupted the genes required for EPA biosynthesis and found that the levels of the outer membrane porin homolog, Omp417, were markedly decreased in the EPA-less mutant (*Δ*EPA). To examine the effects of EPA on the folding of Omp417, *in vitro* refolding of recombinant Omp417 was carried out with liposomes in the presence or absence of EPA-containing phospholipids (EPA-PLs). Tryptophan (Trp) fluorescence dynamics of Omp417 reconstituted into liposomes with or without EPA-PLs demonstrated that the presence of EPA-PLs did not affect the local environments of Omp417 Trp residues. This result suggests that EPA-PLs are not involved in the folding of this protein at low temperatures. On the other hand, the transcription of *omp417* was suppressed in the *Δ*EPA mutant, and the amount of *omp417* transcript in the *Δ*EPA mutant was less than 2% of that in the wild type strain. To analyze the effects of EPA-PLs on *omp417* expression, exogenous supplementation of EPA to *Δ*EPA cells and rescue of *Δorf2* cells, a gene-disrupted mutant of a phosphopantetheinyl transferase required for the *de novo* synthesis of EPA, by using an *orf2*-expression vector were performed. Although these treatments restored EPA-PLs in the *Δ*EPA mutant, the transcriptional defect was not restored. These results suggest that the suppression of the transcription of *omp417* is not due to the lack of EPA, but due to the insertion of a knockout plasmid for EPA-biosynthesis genes into the chromosome.

Abbreviations:
LB, Luria-Bertani
EPA, eicosapentaenoic acid
EPA-PLs, EPA-containing phospholipids
SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis
PEPE, 1-palmitoleoyl-2-eicosapentaenoyl-*sn*-glycero-3-phosphatidylethanolamine
DPPC, 1,2-dipalmitoleoyl-*sn*-glycero-3-phosphocholine
DPPE, 1,2-dipalmitoleoyl-*sn*-glycero-3-phosphatidylethanolamine
PEPG, 1-palmitoleoyl-2-eicosapentaenoyl-*sn*-glycero-3-phosphatidylglycerol
DPPG, 1,2-dipalmitoleoyl-*sn*-glycero-3-phosphatidylglycerol
Trp, tryptophan
PUFAs, polyunsaturated fatty acids
DHA, docosahexaenoic acid
PCR, polymerase chain reaction
qRT-PCR, quantitative reverse transcription polymerase chain reaction

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Introduction

Eicosapentaenoic acid (EPA), an omega-3 polyunsaturated fatty acid, plays a beneficial role in human health[1]. EPA is present as acyl chains in membrane phospholipids, which serve as precursors of various eicosanoids. EPA might also affect the physicochemical properties of the cell membrane to modulate the functions of membrane-associated proteins.

In prokaryotes, EPA plays important physiological roles in survivability. Various marine Gram-negative bacteria, isolated from cold and high-pressure environments such as deep sea and the polar regions, produce polyunsaturated fatty acids (PUFAs) such as docosahexaenoic acid (DHA) or EPA. A psychrotrophic bacterium, *Shewanella livingstonensis* Ac10, isolated from Antarctic seawater, produces EPA as a component of membrane phospholipids at low temperatures. A gene cluster is involved in the synthesis of EPA in *S. livingstonensis* Ac10. Five genes in the cluster, *orf2*, *orf5*, *orf6*, *orf7*, and *orf8*, show sequence similarity to polyketide-synthesis genes and are essential for the production of EPA in this bacterium[2].

The EPA-less mutant (*Δ*EPA) generated by disruption of the EPA-biosynthesis genes showed growth retardation and formed filamentous cells at 4℃ but not at 18℃. Supplementation of EPA complemented the growth defects of the *Δ*EPA cells. These results suggested that EPA has an important role in the cold adaptation of this bacterium[2, 3]. EPA may regulate membrane fluidity[4]; however, there is no significant difference in membrane fluidity between the *Δ*EPA and the parent strains[2]. Nevertheless, the lack of EPA affected the composition of membrane proteins in *S. livingstonensis* Ac10, indicating that EPA affects the behavior and functions of membrane proteins at low temperatures without affecting bulk membrane fluidity in this bacterium[2]. Here, we found that the levels of the major outer membrane protein, Omp417, were markedly decreased in *Δ*EPA cells, suggesting that EPA is involved in the production of this protein. In this article, we focused on the effects of the lack of EPA on the synthesis of Omp417, and found that targeted gene disruption of EPA-biosynthesis genes exerts a negative transcriptional effect on the production of Omp417.

Materials and methods

Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. A rifampicin-resistant mutant of *S. livingstonensis* Ac10 and *Δ*EPA were grown in Luria-Bertani (LB) broth at 4℃. Seed cultures of the parent strain and *Δ*EPA mutant were cultivated at 18℃. When required, antibiotics were added to the medium at the following concentrations: rifampicin (50 μg/ml), kanamycin (30 μg/ml), and chloramphenicol (30 μg/ml) for the parent strain and *Δ*EPA mutant. *Escherichia coli* S17-1/λ_{pir} was used as a donor cell for conjugative transformation of the *orf2*-complemented vector. *E. coli* DH5*α* cells were used as the host for plasmid construction. *E. coli* BL21(DE3) cells were used for recombinant-Omp417 production. These *E. coli* cells were grown in LB medium at 37℃. When required, antibiotics were added to the medium at the following concentrations: kanamycin (30 μg/ml), chloramphenicol (30 μg/ml), and ampicillin (30 μg/ml).

Construction of the *orf2*-complementation vector

The bacterial strains, plasmids, and primers used in this study are listed in Table 1 and Table 2. A complementation vector harboring *orf2* and its promoter, p*Nat-orf2*, was constructed as follows. The gene fragment composed of the *orf2* gene and its 1000 base pair 5' upstream region, including the predicted promoter, was amplified using primers (pJRD-orf2-MluI (f) and pJRD-orf2-SpeI (r)) listed in Table 2 by polymerase chain reaction (PCR) with KOD-plus DNA polymerase (Toyobo, Osaka, Japan). The amplified product was digested with MluI and SpeI, ligated to plasmid pJRD-Cm^r (Table 1), and digested with the same restriction enzymes to get an expression plasmid p*Nat-orf2*. The purified plasmid was introduced into *E. coli* S17-1/λ_{pir} cells for conjugation. The transformant of *Δorf2* harboring an *orf2*-complementation plasmid was selected using chloramphenicol (30 μg/ml) plate. The inserted fragment in the vector was confirmed by sequence analysis.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis for the membrane protein composition of *S. livingstonensis* Ac10 and the ΔEPA mutant

The parent strain and the EPA-less mutant of *S. livingstonensis* Ac10 were cultivated at 4℃ and collected in the log phase, $OD_{600} \approx 0.5$; early-stationary phase, $OD_{600} \approx 1.0$; and stationary phase, $OD_{600} \approx 2.0$. Cells obtained from 4 ml of culture were resuspended into 20 mM Tris-HCl buffer (pH 8.0) and disrupted by sonication. Crude membrane proteins were collected by centrifugation at 20,300×*g* for 30 min at 4℃ and solubilized with 50 μl of 2% (w/v) *N*-lauroylsarcosine sodium salt (Sigma, St. Louis, MO). Samples were not heated before SDS-PAGE.

Table 1 Strains and plasmids used in this study

Strains or Plasmids	Description	References
Strains		
E. coli		
DH5*α*	F^-, *deoR*, *endA1*, *gyrA96*, *hsdR*17(rk^-mk^+), *recA1*, *relA1*, *supE*44, *thi-1*, *Δ*(*lacZYA-argF*) U169, (Phi80*lacZΔ*M15)	
S17-1/λ_{pir}	S17-1 derivative, host for *pir*–dependent plasmids	5
BL21 (DE3)	F^- *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* (DE3)	
S. livingstonensis		
Ac10	Antarctic seawater isolate, Rif^r	2
Δorf2	Rif^r; *orf2* ::pKNOCK-Km^r	2
Δorf5	Rif^r; *orf5* ::pKNOCK-Km^r	2
Δorf6	Rif^r; *orf6* ::pKNOCK-Km^r	2
Δorf7	Rif^r; *orf7* ::pKNOCK-Km^r	2
Δorf8	Rif^r; *orf8* ::pKNOCK-Km^r	2
Plasmids		
pJRD215-Cm^r	Cm^r; A broad-host-range vector	6
p*Nat-orf2*	pJRD215-Cm^r derivative harboring *orf2* and its 1000-bp upstream region	This work
pET-21a (+)	Amp^r; T7 promoter, expression vector	
pET-21a-Omp417	Expression of Omp417 as inclusion body	This work

Table 2 Primers used in this study

Primer name	Sequence (5'-3')	Target gene
Used for construction of plasmid for gene complementation		
pJRD-orf2-MluI (f)	CCGACGCGTTGAATGTGTTTAGGCTATAG	*orf2*
pJRD-orf2-SpeI (r)	GGACTAGTTTAAGACTCCTTGTATACAT	*orf2*
Used for construction of plasmid for expression in *E. coli*		
C417NP	GGAATTCCATATGATCGAAGTTTATAAAGATG	*omp417*
C417NH	GGAATTCTTAAAGAGTGAAACGAATACCAATG	*omp417*
Used for Real-Time RT-PCR		
16S-F	GAGCGGCGGACGGGTGAGTA	*16s rRNA*
16S-R	GAGTTAGCCGGTCCTTCTTCTGTA	*16s rRNA*
rtPCR417F	AAACCGTTCAGTACCGTAACAG	*omp417*
rtPCR417R	GATGTTCCACCGTTACCAAAGG	*omp417*
RT-Omp176-F	GGCCAGTTACGTAAAGCTGA	*omp176*
RT-Omp176-R	GCTGCAGCTACGTAGTAAGG	*omp176*

Underlined sequences indicate the recognition sites for restriction enzymes.

Expression and purification of Omp417

To obtain recombinant Omp417 as an inclusion body as described previously[7], the gene coding for mature Omp417 without its signal peptide, as confirmed by N-terminal amino acid sequence analysis of native Omp417, was amplified from *S. livingstonensis* Ac10 genomic DNA by PCR using the primers C417NP and C417NH listed in Table 2 with KOD-plus DNA polymerase. The amplified product was digested with NdeI and EcoRI and ligated to plasmid pET-21a(+) (Novagen, Madison, WI) (Table 1) digested with the same restriction enzymes to get expression plasmid pET-21a-Omp417. pET-21a-Omp417 was introduced into *E. coli* BL21(DE3), and the transformant was cultivated at 37℃ in 300 ml LB broth until the culture OD_{600} reached ≈ 1.0. The cultivation temperature was then lowered to 25℃. After the cells were cultivated for 1 h to allow them to adapt to lower temperatures, isopropyl 1-thio-*β*-D-galactoside was added at a final concentration of 0.4 mM, and the expression of Omp417 was induced at 20℃ overnight. Cells were collected by centrifugation at 8,000×*g* for 30 min at 4℃ and washed with 0.9% (w/v) NaCl solution. The cell pellet was suspended in 30 ml of 20 mM Tris-HCl buffer (pH 8.0) and disrupted by sonication until its viscosity was reduced. Inclusion bodies were washed five times with 2% (v/v) Triton X-100 (Nacalai Tesque, Kyoto, Japan) to remove contaminating proteins and then washed three times with TED buffer (20 mM Tris-HCl, 2 mM EDTA, and 2 mM DTT (pH 8.0)) to remove the remaining detergent. The purified inclusion bodies were resuspended in 5 ml TED buffer and stored at

−80℃. The N-terminal amino acid sequence of the isolated protein was analyzed to confirm that the purified protein was the mature form of Omp417.

Synthesis of EPA-containing phospholipids

To mimic the cell membrane environment of *S. livingstonensis* Ac10, 1-palmitoleoyl-2-eicosapentaenoyl-*sn*-glycero-3-phosphatidylethanolamine (PEPE) was synthesized as described previously[3]. 1,2-Dipalmitoleoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-dipalmitoleoyl-*sn*-glycero-3-phosphatidylethanolamine (DPPE) were purchased from Avanti Polar Lipids (Alabaster, AL). PEPE and DPPC were converted to 1-palmitoleoyl-2-eicosapentaenoyl-*sn*-glycero-3-phosphatidylglycerol (PEPG) and 1,2-dipalmitoleoyl-*sn*-glycero-3-phosphatidylglycerol (DPPG), respectively, by using phospholipase D (Sigma) as described previously[3]. The molecular masses of synthesized phospholipids were verified by electrospray ionization-mass spectrometry (ESI-MS) with a triple-quadrupole PE-Sciex API 3000 liquid chromatography-tandem mass spectrometry system (Applied Biosystems, Foster City, CA).

Liposomes preparation

Liposomes made from DPPE and DPPG (1:1 M ratio) and those containing PEPE and PEPG (2.5 molar% each) were prepared as previously reported with a slight modification[7]. Phospholipids (4 μmol) dissolved in chloroform were dried to a thin film in glass tubes with a nitrogen gas stream and then placed under vacuum for more than 30 min to remove residual chloroform. The dried lipid films were hydrated in 400 μl of TED buffer and agitated by vortexing for at least 30 min, and then sonicated in a bath sonicator until the solutions became translucent. The liposome solutions were equilibrated to room temperature overnight before use.

In vitro reconstitution of Omp417

Purified inclusion bodies of Omp417 were dissolved in 8 M urea and centrifuged at 8,000×*g* for 30 min to remove insoluble materials. The protein concentration was measured with the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA) and adjusted to a final concentration of 0.1 mM. *In vitro* reconstitution was performed by diluting unfolded Omp417 to 10-fold into the liposome solution at 4℃.

Tryptophan fluorescence spectroscopy

A time course of the folding of Omp417 was monitored using tryptophan (Trp) fluorescence measurements. Trp fluorescence measurements were carried out with an RF-5300 PC (Shimadzu, Kyoto, Japan). The slit width was 5 nm for excitation and 5 nm for emission. The excitation wavelength was 290 nm, and the emission spectrum was measured from 300 nm to 380 nm. The buffer containing liposomes alone was measured and subtracted as background.

Extraction of total RNA

The EPA-less mutants of *S. livingstonensis* Ac10 (*Δorf2*, *Δorf5*, *Δorf6*, *Δorf7*, and *Δorf8*), obtained by the disruption of one of the genes required for EPA biosynthesis, were used for real-time quantitative reverse transcription PCR (qRT-PCR) analysis. Seed cultures of the parent strain and mutant strains were cultivated at 18℃ and inoculated in 6 ml of LB broth. When required, kanamycin (30 μg/ml), chloramphenicol (30 μg/ml), EPA, and oleic acid (Wako Pure Chemical Industries, Ltd., Osaka, Japan) were added to the medium. EPA and oleic acid solutions in chloroform were dried in a glass tube with nitrogen gas, dissolved in 60 μl of ethanol, and subsequently hydrated with 6 ml LB broth to a final concentration of 130 μM. The cells were cultured at 4℃ until the OD_{600} of the culture reached about 1.0. Total RNA was isolated with the High Pure RNA Isolation Kit (Roche, Mannheim, Germany) from the parent strain and the mutant strains. Residual DNA in the RNA preparations was removed by incubating with 10 U of RNase-free DNase I (TaKaRa, Shiga, Japan) at 37℃ for 1 h. RNA was dissolved in 0.1% diethyl pyrocarbonate-treated water and stored at −80℃. Total RNA was quantified using a spectrophotometer.

Analysis of transcription levels of *omp417* and *omp176* genes by qRT-PCR

qRT-PCR was carried out using the SuperScript III Platinum SYBR Green One-step qRT-PCR Kit (Invitrogen, Carlsbad, CA) and the Mx3000P Multiple Quantitative RT-PCR system (Stratagene, La Jolla, CA). RT-PCR was performed as previously reported[8]. The primers used are listed in Table 2. The threshold cycle (Ct) value for each sample was normalized with the Ct value for 16S rRNA.

Results

Disruption of the genes for EPA biosynthesis decreased the levels of a major membrane protein

To determine the effect of EPA deficiency on the composition of proteins in the cell membrane, the crude membrane proteins of the parent strain (WT) and the EPA-less mutant, *Δorf5*, were analyzed by SDS-PAGE (Fig. 1A, B).

The levels of a protein of estimated molecular mass of approximately 37 kDa were markedly decreased in the *Δorf5* strain (Fig. 1A). The decrease in the level of this protein in the *Δorf5* cells was observed in the log, early stationary, and stationary phases at 4℃ (Fig. 1A). By peptide mass fingerprinting analysis, this protein was identified as a homolog of bacterial outer membrane porin, which shows sequence similarity to *E. coli* OmpC (25% identity) and was named Omp417. The amount of Omp417 was also decreased in other EPA-less mutants, *Δorf2*, *Δorf6*, *Δorf7*, and *Δorf8*, which were generated by targeted gene disruption of each of the EPA-biosynthesis genes (Fig. 1B).

EPA-containing phospholipids did not affect *in vitro* folding of Omp417

Previously, we demonstrated that EPA affects *in vitro*

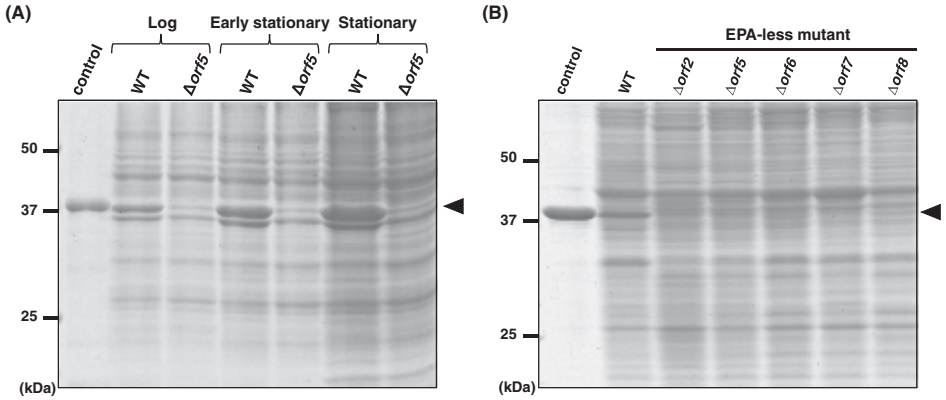

Fig. 1 SDS-PAGE analysis of the crude membrane extracts from *S. livingstonensis* Ac10 and the EPA-less mutants
(A) Cells were grown at 4℃. The crude membrane proteins of the parent strain (WT) and *Δorf5* harvested in log phase, early stationary phase, and stationary phase were analyzed by SDS-PAGE. Purified Omp417 was loaded as a control. Arrowhead represents the position of Omp417.
(B) WT and the EPA-less mutants, *Δorf2*, *Δorf5*, *Δorf6*, *Δorf7*, and *Δorf8*, were grown at 4℃, and crude membrane proteins were prepared from cells harvested in stationary phase. Purified Omp417 was loaded as a control. Arrowhead indicates the position of Omp417.

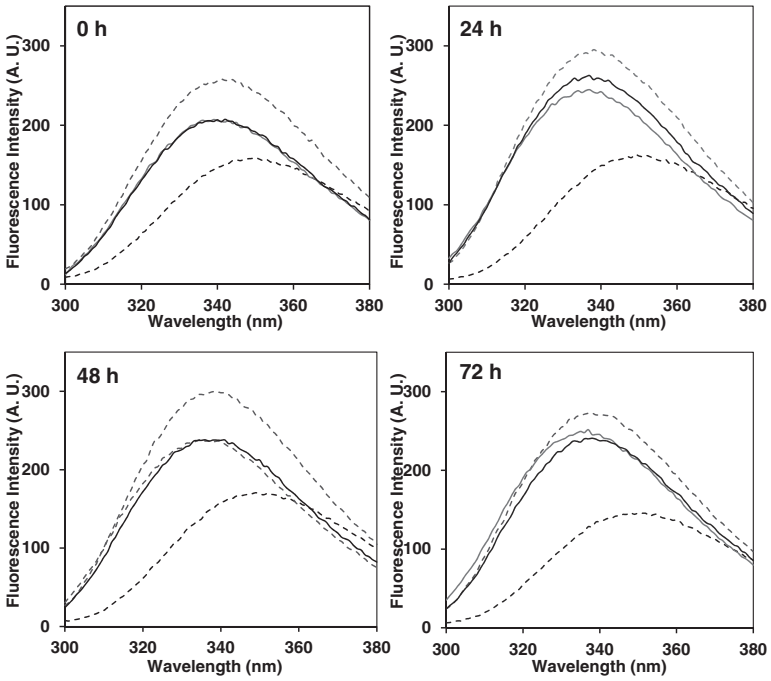

Fig. 2 Tryptophan fluorescence analysis to monitor *in vitro* refolding of Omp417
Tryptophan (Trp) fluorescence spectra of Omp417 refolded in DPPE/DPPG liposomes with (black solid line) and without (grey solid line) 2.5 mol% PEPE and 2.5 mol% PEPG for 0 h, 24 h, 48 h, and 72 h at 4℃ were monitored. Trp fluorescence spectra of Omp417 denatured by 8 M urea (black dotted line) and that of Omp417 diluted without liposome (grey dotted line) are shown. The excitation wavelength was 290 nm and the Trp fluorescence of Omp417 was measured at 300–380 nm. The reproducibility was confirmed by three independent experiments.

folding of the cold-inducible outer membrane protein, Omp74. Moreover, EPA-containing phospholipids (EPA-PLs) facilitate the membrane insertion and secondary structure formation of Omp74[7]. As with Omp74, EPA-PLs might interact with Omp417 and affect the folding of this protein at low temperatures. To evaluate the effects of EPA-PLs on the folding of Omp417, *in vitro* reconstitution experiments were performed.

Trp fluorescence spectroscopy is widely used to monitor protein folding. Omp417 has seven Trp residues in the predicted extracellular region and transmembrane region (PRED-TMBB: http://bioinformatics.biol.uoa.gr/PRED-TMBB/). Using Trp fluorescence measurements, we monitored the folding of Omp417 at 4℃ (Fig. 2). The Trp fluorescence spectrum of denatured Omp417 showed an emission maximum at about 350 nm. When Omp417 was diluted in the presence or absence of liposomes, a blue-shifted emission was observed; the emission maximum was shifted to 340 nm. The fluorescence spectrum of diluted Omp417 showed higher fluorescence intensities than those obtained from denatured Omp417. In the presence of liposomes, the folded Omp417 showed a lower fluorescence intensity than the hydrated Omp417 without liposomes, indicating that the environments of the Trp residues in Omp417 folded in the lipid environment are different from those in Omp417 in the hydrated state. Prolonged incubation of Omp417 with liposomes with or without EPA-PLs demonstrated that EPA-PLs did not change the local environments of the Trp residues.

Disruption of the EPA-biosynthesis gene suppresses the production of Omp417 at the transcriptional level

To assess the effects of the disruption of the genes for EPA biosynthesis on the expression of Omp417, we determined the transcript levels of *omp417* and *omp176* in WT and *Δorf5* cells by qRT-PCR. A gene product of *omp176* was identified as a cold-inducible protein of *S. livingstonensis* Ac10; the lack of EPA significantly decreased the amount of this protein at 4℃[2], suggesting that EPA is involved in the production of Omp176 at low temperatures. Transcript levels of *omp176* in *Δ*EPA cells were similar to those of WT at 4℃ (Fig. 3A). Under the same conditions, the relative amount of *omp417* transcript in *Δ*EPA cells was less than 2% of that of the WT.

Five genes named *orf2*, *orf5*, *orf6*, *orf7*, and *orf8* are essential for the biosynthesis of EPA in *S. livingstonensis*

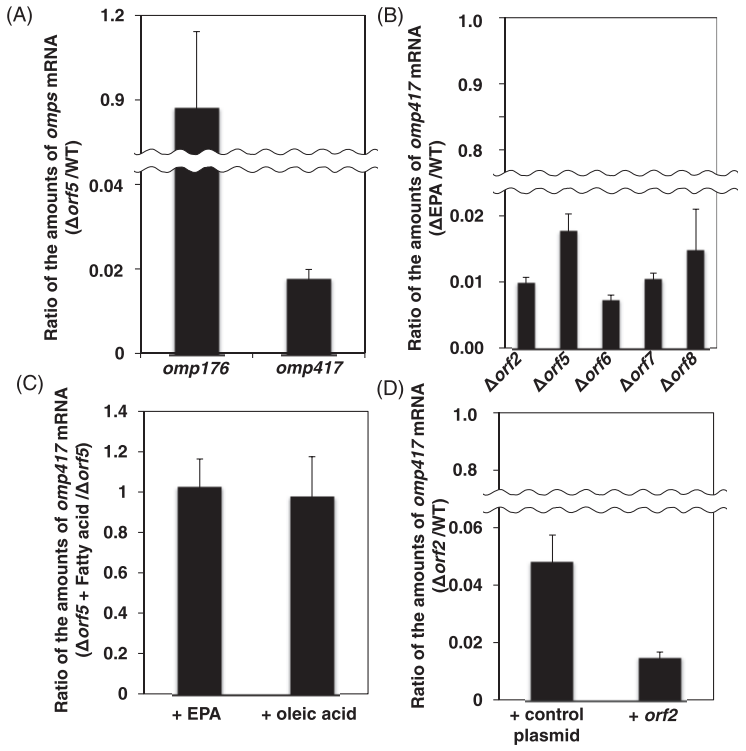

Fig. 3 Transcription levels of *omp417* in WT and *Δ*EPA cells
(A) Transcription levels of *omp417* and *omp176* in WT and *Δorf5* cells were analyzed by qRT-PCR. (B) Relative levels of *omp417* in each of the EPA-less mutants, *Δorf2*, *Δorf5*, *Δorf6*, *Δorf7*, and *Δorf8*, grown at 4℃. (C) Effects of fatty acid supplementation on transcription of *omp417*. *Δorf5* cells were grown with 130 μM of EPA- or oleic acid-containing media, and the total RNA extracts were analyzed by qRT-PCR. (D) Transcription levels of *omp417* in *Δorf2* cells carrying pJRD-Cm^r or p*Nat-orf2* were analyzed by qRT-PCR. The integrities of RNA samples were confirmed using denaturing agarose gel electrophoresis (data not shown).

Ac10[2]. To confirm whether gene disruption of each of these EPA-biosynthesis genes suppresses the transcription of *omp417* at 4℃, the amount of *omp417* mRNA in each of the EPA-less mutants was determined (Fig. 3B). In all mutants, the amount of *omp417* transcript was decreased to less than 2% of that in the WT.

To examine whether the lack of endogenously synthesized EPA suppresses the production of Omp417 at the transcriptional level, the exogenous supplementation of EPA to *Δorf5* was performed, and the mRNA levels of *omp417* were determined. When EPA was exogenously supplemented to *Δorf5* cells, EPA was incorporated into the cells and converted into the acyl chains of membrane phospholipids (data not shown). With supplemented EPA, the growth retardation and the abnormal morphology of *Δorf5* cells were suppressed. Total RNA was extracted from *Δorf5* cells grown with either EPA or oleic acid as a negative control and analyzed by qRT-PCR. The mRNA levels of *omp417* in *Δorf5* cells grown with fatty acid supplementation were similar to mRNA levels of cells grown without fatty acid supplementation (Fig. 3C), indicating that the exogenous supplementation of EPA restored the growth of *Δorf5* cells but did not reverse the decrease in the transcription of *omp417*.

Functional gene complementation of *Δorf2,* a gene-disrupted mutant of a phosphopantetheinyl transferase required for the *de novo* synthesis of EPA, was performed using an expression vector for *orf2* under the control of its putative promoter. The recovery of the EPA-biosynthesis activity of the complemented strain was confirmed by the analysis of the fatty acid composition of this strain by GC-MS (data not shown). The effects of EPA, which is endogenously synthesized in *Δorf2* cells, on the transcription of *omp417* were then analyzed. The mRNA levels of *omp417* in *Δorf2* cells harboring a control plasmid were about 5% of that of the WT. When an *orf2*-expression vector was introduced into *Δorf2* cells, this mutant synthesized EPA, and the production of EPA in the mutant was similar to that of WT (data not shown). The mRNA levels of *omp417* in the rescued strain was about 2% of that of the WT (Fig. 3D). These results demonstrated that neither exogenous supplementation of EPA in the EPA-less cells nor endogenous synthesis of EPA in *Δorf2* cells restored the mRNA levels of *omp417*.

Discussion

In this study, we focused on the production of membrane proteins in an EPA-less mutant of *S. livingstonensis* Ac10. ΔEPA cells show several defects at low temperatures such as abnormal morphology, growth retardation, and changes in the levels of various membrane proteins[2]. Here, we found a major outer membrane protein, Omp417, which was significantly decreased in the ΔEPA mutants. Omp417 is one of the major membrane proteins of *S. livingstonensis* Ac10 grown at 4℃, which shares a 25% sequence identity with an outer membrane porin, OmpC, of *E. coli* (ADU34074). The OmpC family of proteins plays critical roles in the incorporation of hydrophilic solutes into the cells[9].

In the production of bacterial outer membrane porins, the phospholipid composition directly affects the efficiency of membrane protein insertion and the folding of nascent membrane proteins. For example, in ΔEPA cells, the cold-inducible porin homologs, Omp74, lacks conformational diversity *in vivo*[7]. However, EPA-PLs did not affect the folding of Omp417, suggesting that the defect of Omp417 production did not occur at the posttranslational level.

In bacteria, expression of genes coding for outer-membrane porins is regulated by the growth conditions (e.g. temperature, osmotic condition, etc.) depending on the functions of the transcription regulatory proteins[10]. The EnvZ-OmpR system and various other transcription regulation systems are involved in the expression of porin genes[11]. In *S. livingstonensis* Ac10, EPA and the EPA-biosynthesis enzyme complex might regulate the expression of *omp417* at low temperatures. To test this hypothesis, we analyzed the transcription of *omp417* and found that both exogenous supplementation of EPA to ΔEPA cells and functional gene complementation in *Δorf2* cells did not restore the defects in expression of *omp417*. As a control, a gene-disrupted mutant of alanine dehydrogenase (*Δald*) was constructed, which has been identified as a cold-inducible protein in this strain[12]. We found that the mRNA levels of *omp417* were similar to those of the WT strain grown under the same conditions (data not shown). These results imply that targeted gene disruption of each of the EPA-biosynthesis genes has a specific effect on *omp417* transcription.

We generated the *S. livingstonensis* Ac10 EPA-less mutant by homologous recombination with a gene knockout plasmid, pKNOCK, for each of the EPA-synthesis genes on chromosomal DNA[2]. It is known that targeted gene disruption sometimes affects the expression of adjacent genes, which are co-expressed as a transcribed unit. However, *omp417* is not included in the EPA-biosynthesis gene cluster, and the distance between *omp417* and the gene cluster is longer than 21 kbp, according to the draft genome sequence of this strain. Thus, it is unlikely that *omp417* is co-transcribed with EPA-biosynthesis genes or

that disruption of the EPA-biosynthesis genes causes a polar effect on the expression of *omp417*.

One possible explanation for the defects in *omp417* transcription is that targeted gene disruption of EPA-biosynthesis genes causes changes to the conformation of the chromosome. Recently, it has been reported that bacterial chromosomal conformation and gene position in the bacterial chromosome affects gene expression[13–15]. Therefore, the expression of *omp417* might be linked to chromosomal conformation in the EPA-biosynthesis gene cluster, and the expression of *omp417* might be controlled by the spatial relationship between *omp417* and the EPA-biosynthesis gene cluster on the chromosome. The details of the molecular mechanism regulating the expression of *omp417* in the presence or absence of EPA-biosynthesis genes are still unclear and further studies will be necessary. In conclusion, this study shows the presence of a unique gene expression system of an outer membrane protein that is affected by the structure of EPA-biosynthesis gene cluster.

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