

The *Escherichia coli* highly expressed *entD* gene complements the *pfaE* deficiency in a *pfa* gene clone responsible for the biosynthesis of long-chain n-3 polyunsaturated fatty acids

Shinji Sugihara¹, Yoshitake Orikasa² & Hidetoshi Okuyama^{1,2}

¹Course in Environmental Molecular Biology and Microbial Ecology, Division of Biosphere Science, Graduate School of Environmental Science, Hokkaido University, Kita-ku, Sapporo, Japan; and ²Laboratory of Environmental Molecular Biology, Faculty of Environmental Earth Science, Hokkaido University, Sapporo, Japan

Correspondence: Hidetoshi Okuyama, Laboratory of Environmental Molecular Biology, Faculty of Environmental Earth Science, Hokkaido University, Sapporo 060-0810, Japan. Tel.: +81 11 706 4523; fax: +81 11 707 2347; e-mail: hoku@ees.hokudai.ac.jp

Received 27 December 2009; revised 25 February 2010; accepted 3 April 2010.
Final version published online 4 May 2010.

DOI:10.1111/j.1574-6968.2010.01987.x

Editor: Robert Gunsalus

Keywords

ASKA clone; EntD; eicosapentaenoic acid; *pfaE*; phosphopantetheinyl transferase; polyunsaturated fatty acid.

Introduction

In some marine bacteria and eukaryotic microorganisms, long-chain n-3 polyunsaturated fatty acids (n-3 PUFAs), such as the eicosapentaenoic acid (EPA) and the docosahexaenoic acid (DHA), are synthesized *de novo* via a polyketide biosynthesis pathway (Metz *et al.*, 2001; Orikasa *et al.*, 2006a, b, c). Five genes (*pfaA*, *pfaB*, *pfaC*, *pfaD* and *pfaE*) that are involved in the biosynthesis of EPA or DHA have been cloned from bacteria (Tanaka *et al.*, 1999; Allen & Bartlett, 2002; Orikasa *et al.*, 2004). Similar eukaryotic genes have been cloned from heterokont algae such as *Schizochytrium* (Metz *et al.*, 2001) and *Ulkenia* (Luy *et al.*, 2009), although their gene structures are different from those of bacteria. The bacterial gene structures and domain structures of all *pfa* genes that are essential for the biosynthesis of EPA and DHA are well conserved (Okuyama *et al.*, 2007).

Among the five *pfa* genes, the *pfaE* gene encodes an Sfp-type phosphopantetheinyl transferase (PPTase) of approximately

Abstract

The *Escherichia coli entD* gene, which encodes an Sfp-type phosphopantetheinyl transferase (PPTase) that is involved in the biosynthesis of siderophore, is available as a high-expression ASKA clone (pCA24N::*entD*) constructed from the *E. coli* K-12 strain AG1. In *E. coli* DH5 α , pCA24N::*entD* complemented a *pfaE*-deficient clone that comprised *pfaA*, *pfaB*, *pfaC* and *pfaD*, which are four of the five *pfa* genes that are responsible for the biosynthesis of eicosapentaenoic acid derived from *Shewanella pneumatophori* SCRC-2738. Sfp-type PPTases are classified into the EntD and PfaE groups, based on differences between their N-terminal-domain structures. Here, we showed that all Sfp-type PPTases may have the potential to promote the biosynthesis of long-chain n-3 polyunsaturated fatty acids.

30 kDa, which catalyses phosphopantetheinylation via transfer of the 4'-phosphopantetheine prosthetic group from coenzyme A to a conserved serine residue in the carrier proteins, thus converting these proteins from their inactive 'apo' forms into their active 'holo' forms (Gehring *et al.*, 1998). Orikasa *et al.* (2006b) classified Sfp-type PPTases into two groups: the first includes PPTases that are involved mainly in the biosynthesis of n-3 PUFAs, while the second includes PPTases that are involved principally in polyketide and/or nonribosomal peptide synthesis. The Sfp-type PPTases have three conserved domains: P1, P2 and P3 (Weissman *et al.*, 2004). The P1 and P3 domains are responsible for coenzyme-A binding and domains P2 and P3 are responsible for Mg²⁺ binding (Reuter *et al.*, 1999; Chirgadze *et al.*, 2000). However, the PPTases that are required for the biosynthesis of n-3 PUFAs (i.e., PfaEs) are different from the other Sfp-type PPTases in some aspects: the P1 domain at their N terminus can be separately recognized as P1a and P1b in PfaE and is highly conserved

among PfaEs. Moreover, PfaEs have an additional conserved P0 domain (L/VRxL/VLS) (where x is a nonconserved amino acid) upstream of P1a (Orikasa et al., 2006a).

The second representative group of PPTase includes the EntD protein of *Escherichia coli*, which is responsible for the synthesis of the siderophore enterobactin (Hantash et al., 1997). Interestingly, the genome of *Photobacterium profundum* SS9, which is an EPA-producing deep-sea bacterium, includes only one Sfp-type PPTase gene that was categorized into this second group (the EntD type; Sugihara et al., 2008). These findings suggest that this Sfp-type PPTase of *P. profundum* (SS9 PPTase) may be involved in the production of EPA, together with the other *pfa* genes (*pfaA*, *pfaB*, *pfaC* and *pfaD*) (Allen & Bartlett, 2002) located in the *P. profundum* SS9 genome (Vezzi et al., 2005).

Previously, we provided evidence that the SS9 PPTase gene complemented a *pfaE*-deficient *pfa* gene clone, pDHA3, which carried only *pfaA*, *pfaB*, *pfaC* and *pfaD* derived from the DHA-producing *Moritella marina* MP-1 (Sugihara et al., 2008). However, there is no evidence that *pfaE* is replaced with the *E. coli entD* gene. In the past, *E. coli entD* was considered as not being responsible for the biosynthesis of n-3 PUFAs, as neither EPA nor DHA was detected in any *E. coli* recombinant cells that carried vectors harbouring *pfaE*-deficient *pfa* genes prepared from *Shewanella pneumatophori* SCRC-2738 (Orikasa et al., 2004), *M. marina* MP-1 (Tanaka et al., 1999; Orikasa et al., 2006a, b) and *P. profundum* SS9 (Allen & Bartlett, 2002).

To elucidate whether *pfaE* is replaced with *entD*, we used the ASKA clone pCA24N::*entD*, which is a plasmid that expresses *entD* at high levels. This clone was obtained from the cloning vector collection of the *E. coli* Strain National BioResource Project (<http://www.shigen.nig.ac.jp/ecoli/strain/top/top.jsp>). In this study, pCA24N::*entD* was coexpressed with pEPA Δ 1,2,3, which was a pWE15 cosmid clone carrying an EPA biosynthesis gene cluster that lacked *pfaE* from *S. pneumatophori* SCRC-2738 (Orikasa et al., 2004).

Materials and methods

Bacterial strains and culture conditions

The bacterial strains and vectors used in this study are listed in Table 1. *Escherichia coli* DH5 α recombinant cells were precultivated in Luria–Bertani (LB) medium supplemented with the indicated antibiotics at 37 °C for 16 h under shaking at 160 r.p.m. Portions of the culture were then transferred to the same medium and grown at 20 °C for 72 h, for EPA production.

Plasmid preparation and transformation

The ASKA library is a comprehensive *E. coli* K-12 ORF plasmid library in which one gene was cloned into each *E. coli* strain via gene cloning at the Nara Institute of Science and Technology (Kitagawa et al., 2005). The *E. coli* strain K-12 carrying pCA24N::*entD* was obtained from the National BioResource Project. The ASKA clone library is based on the *E. coli* K-12 strain AG1 and individual genes were cloned into the pCA24N vector (see Table 1).

Escherichia coli K-12 cells carrying pCA24N::*entD* were grown at 30 °C for 16 h in LB medium. pCA24N::*entD* was isolated using the mini-prep method and was used to transform *E. coli* DH5 α cells carrying pEPA Δ 1,2,3 using the heat-shock method. The transformed *E. coli* DH5 α cells were grown in LB medium containing ampicillin at 50 $\mu\text{g mL}^{-1}$ and chloramphenicol at 30 $\mu\text{g mL}^{-1}$ at 20 °C for 72 h with shaking.

Fatty-acid analysis and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of proteins

Transformed *E. coli* DH5 α cells were collected by centrifugation. The precipitated cells were washed and were then directly subjected to methanolysis using 10% v/v acetyl chloride in methanol at 100 °C for 1 h. The resulting fatty-acid methyl esters were analysed by gas–liquid chromatography

Table 1. Strains and vectors used in this study

Strain/plasmid/cosmid	Relevant characteristics	Source
<i>Strain</i>		
<i>Escherichia coli</i> DH5 α	<i>deoR</i> , <i>endA1</i> , <i>gyrA96</i> , <i>hsdR17</i> (rK ⁻ /mK ⁺), <i>recA1</i> , <i>phoA</i> , <i>relA1</i> , <i>thi-1</i> , Δ (<i>lac ZYA-argF</i>), U169 ϕ 80d/ <i>lacZ</i> Δ M15, F ⁻ , λ ⁻ , <i>supE44</i>	Takara Bio*
<i>E. coli</i> K-12 strain AG1	<i>recA1</i> , <i>endA1</i> , <i>gyrA96</i> , <i>thi-1</i> , <i>hsdR17</i> (rK ⁻ /mK ⁺), <i>supE44</i> , <i>relA1</i> ; provided as a host of pCA24N:: <i>entD</i>	Kitagawa et al. (2005)
<i>Plasmid/cosmid</i>		
pEPA Δ 1,2,3	pWE15 carrying an EPA gene cluster that lacks <i>pfaE</i> from <i>S. pneumatophori</i> SCRC-2738	Orikasa et al. (2004)
pCA24N:: <i>entD</i>	pCA24N carrying <i>entD</i> from <i>E. coli</i> K-12 strain AG1	Kitagawa et al. (2005)

*Takara Bio Inc. (Tokyo, Japan).

and GC/MS using the mode of electron impact, as described by Orikasa *et al.* (2006a).

The proteins produced by the recombinant cells were analysed by SDS-PAGE 7 h after treatment with or without 0.3 mM isopropyl- β -D-thiogalactopyranoside (IPTG), as described previously (Orikasa *et al.*, 2006a, c). The concentration of the proteins was estimated using the method of Bradford (1976).

Results and discussion

Coexpression of pCA24N::entD with pEPA Δ 1,2,3 in *E. coli* DH5 α cells

pCA24N::entD was used to transform *E. coli* DH5 α cells carrying pEPA Δ 1,2,3. GC/MS analysis of fatty-acid methyl esters prepared from *E. coli* DH5 α cells that carried pCA24N::entD plus pEPA Δ 1,2,3 revealed the presence of an unknown peak with a retention time of 30.2 min (Fig. 1a), which was not detected in *E. coli* DH5 α host cells carrying

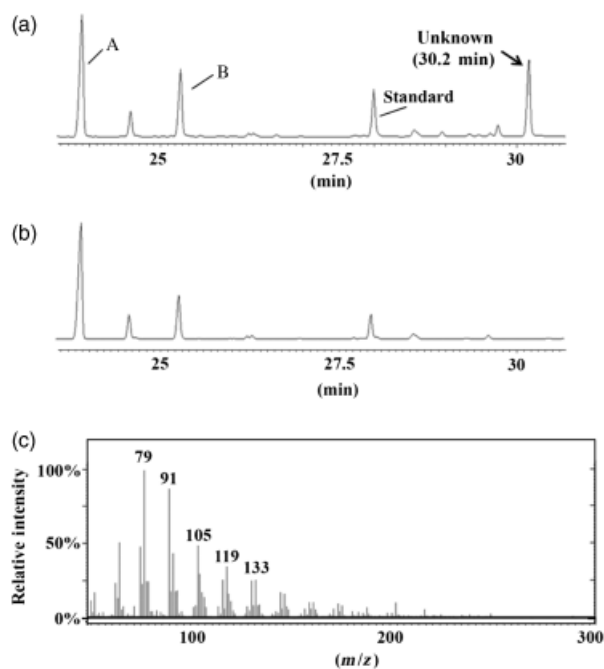


Fig. 1. Gas chromatograms of total fatty-acid methyl esters prepared from *Escherichia coli* DH5 α recombinants, and electron-impact MS of the unknown fatty-acid peak. *Escherichia coli* carrying pEPA Δ 1,2,3 plus pCA24N::entD (a) and *E. coli* carrying pEPA Δ 1,2,3 (b). Mass spectrum of the unknown peak with a retention time of 30.2 min detected in (a). Peaks A and B are those of *cis*-vaccenic and 3-hydroxyl tetradecanoic acids, respectively. Heneicosanoic acid (21:0) was used as an internal standard. *Escherichia coli* DH5 α cells carrying pEPA Δ 1,2,3 and cells carrying pEPA Δ 1,2,3 plus pCA24N::entD were cultivated at 20 °C for 72 h in LB medium containing ampicillin at 50 μ g mL $^{-1}$ and ampicillin at 50 μ g mL $^{-1}$ and chloramphenicol at 30 μ g mL $^{-1}$, respectively.

only pEPA Δ 1,2,3 (Fig. 1b). The retention time of the unknown peak was the same as that of the methyl ester of authentic EPA (data not shown). The GC/MS profile of the unknown peak shown in Fig. 1c was typical of methylene-interrupted PUFAs, and analysis of the fragmentation profile using a program from the National Institute of Standard and Technology Databases (<http://www.nist.gov/srd/htm>) indicated that the profile of this unknown component was closest to that of EPA. Based on these results, this compound was identified as EPA methyl ester. The content of EPA was $9.2 \pm 0.2\%$ of the total fatty acids from cells grown at 20 °C for 72 h. PUFAs other than EPA were not detected.

Expression of the EntD protein in *E. coli* DH5 α cells

Figure 2 shows the SDS-PAGE profiles of *E. coli* DH5 α cells carrying either pEPA Δ 1,2,3 or pEPA Δ 1,2,3 plus pCA24N::entD, in the presence or absence of IPTG. A significantly denser

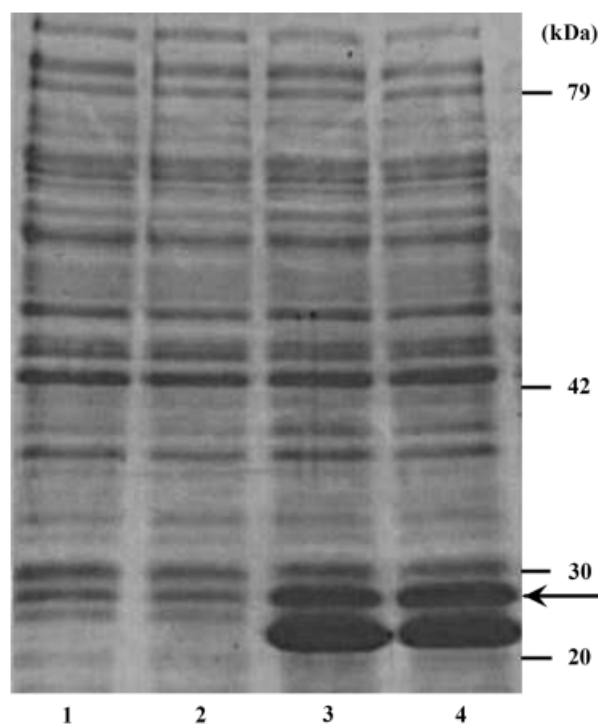


Fig. 2. SDS-PAGE analysis of EntD at high levels in *Escherichia coli* DH5 α cells carrying pEPA Δ 1,2,3 plus pCA24N::entD. *Escherichia coli* DH5 α cells carrying pEPA Δ 1,2,3 and not treated with IPTG (lane 1); *E. coli* DH5 α cells carrying pEPA Δ 1,2,3 and treated with IPTG (lane 2); *E. coli* DH5 α cells carrying pEPA Δ 1,2,3 plus pCA24N::entD and not treated with IPTG (lane 3); and *E. coli* DH5 α cells carrying pEPA Δ 1,2,3 plus pCA24N::entD and treated with IPTG (lane 4). Recombinant cells were grown at 37 °C for 7 h. The arrow indicates the band corresponding to EntD, at 26.1 kDa. Fifty micrograms of protein were loaded onto each lane. The dense band detected below EntD corresponds to chloramphenicol acetyltransferase derived from the pCA24N vector.

band of 26 kDa was detected only in recombinant cells carrying pEPA Δ 1,2,3 plus pCA24N::*entD* in the presence and absence of IPTG (lanes 3 and 4, indicated by arrows). Although the intensity of this band was slightly stronger in cells treated with IPTG than that observed in cells not treated with IPTG, it is evident that pCA24N::*entD* can be highly expressed without induction by IPTG. There is no information regarding whether or not ASKA library plasmids are expressed at a low temperature without an inducer (see Kitagawa *et al.*, 2005). However, it is interesting to note that the EPA biosynthesis gene cluster from *Shewanella oneidensis* MR-1 cloned under the *lacZ* promoter on a high copy number plasmid, pBluescript SK(+), was highly expressed in the absence of IPTG in *E. coli* (Lee *et al.*, 2008), which was assessed by the high content of EPA produced at 20 °C. Thus, the inducer (IPTG)-independent leaky expression of the ASKA library plasmid would be due to the low-temperature effects on this plasmid.

The *entD* gene sequence encodes a predicted protein of 23 579 Da. The size detected for the induced band (EntD; 26 kDa) corresponds to the protein with a His tag and four and five spacer amino-acid sequences at its N and C termini, respectively (Kitagawa *et al.*, 2005). Native EntD was not detected in cells carrying only pEPA Δ 1,2,3 (lanes 1 and 2 of Fig. 2). According to Armstrong *et al.* (1989), no native band of EntD was detected in *E. coli* strains by SDS-PAGE, unless it was overexpressed in the T7 promoter-directed high-expression system. The present results suggest that PfaE can be replaced by significantly higher levels of EntD. An undetectable level of expression of the native *entD* gene product of host *E. coli* DH5 α cells (lanes 1 and 2 of Fig. 2) was insufficient to complement pEPA Δ 1,2,3 lacking *pfaE*. The difference in the N-terminal domain structure between EntD and PfaE, and the addition of a His tag and of spacer amino-acid sequences to EntD would affect its affinity for its substrates, i.e. coenzyme A and/or a conserved serine residue in carrier proteins (such as acyl carrier proteins). This would be the most relevant reason for the partial replacement of PfaE with high levels of EntD. However, we have no idea how the addition of a His tag and of spacer amino-acid sequences to native EntD affects the structure and the catalytic activity of the Pfa enzyme complex. The *pfaE* from the EPA biosynthesis genes is compatible with that from the DHA biosynthesis genes (Orikasa *et al.*, 2006a, c). It should be noted that the Sfp-type PPTases responsible for the biosynthesis of siderophores (and probably other polyketide compounds) and those responsible for the biosynthesis of n-3 PUFAs from terrestrial and marine bacteria, respectively, are partially compatible.

The PPTase involved in the production of EPA in *P. profundum* SS9 is an EntD-type enzyme (see above and Sugihara *et al.*, 2008). This suggests that the PfaA–D proteins of this bacterium do not need high levels of EntD

to synthesize EPA in an *E. coli* recombinant. Considering that the *entD* gene is expressed under low-iron conditions (Armstrong *et al.*, 1989), no production of EPA in the *E. coli* recombinant grown in nutrient broth (2216 Marine Medium, Difco; Allen & Bartlett, 2002) might be caused by lack of the EntD protein.

The dense band detected below EntD corresponded to chloramphenicol acetyltransferase derived from the pCA24N vector, as assessed from its amino-acid sequencing.

Acknowledgements

This work was partly supported by the National Institute of Polar Research. pEPA Δ 1,2,3 was kindly provided by the Sagami Chemical Research Center, Ayase 252-1193, Japan.

Statement

For the present address of Yoshitake Orikasa, please contact the author Hidetoshi Okuyama.

Re-use of this article is permitted in accordance with the Terms and Conditions set out at <http://www3.interscience.wiley.com/authorresources/onlineopen.html>

References

- Allen EE & Bartlett DH (2002) Structure and regulation of the omega-3 polyunsaturated fatty acid synthase genes from the deep-sea bacterium *Photobacterium profundum* strain SS9. *Microbiology* **148**: 1903–1913.
- Armstrong SK, Pettis GS, Forrester LJ & McIntosh MA (1989) The *Escherichia coli* enterobactin biosynthesis gene, *entD*: nucleotide sequence and membrane localization of its protein product. *Mol Microbiol* **3**: 757–766.
- Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254.
- Chirgadze NY, Briggs SL, McAllister KA, Fischl AS & Zhao G (2000) Crystal structure of *Streptococcus pneumoniae* acyl carrier protein synthase: an essential enzyme in bacterial fatty acid biosynthesis. *EMBO J* **19**: 5281–5287.
- Gehring AM, Mori I & Walsh CT (1998) Reconstitution and characterization of the *Escherichia coli* enterobactin synthetase from EntB, EntE, and EntF. *Biochemistry* **37**: 2648–2659.
- Hantash FM, Ammerlaan M & Earhart CF (1997) Enterobactin synthase polypeptides of *Escherichia coli* are present in an osmotic-shock-sensitive cytoplasmic locality. *Microbiology* **143**: 147–156.
- Kitagawa M, Ara T, Arifuzzaman M, Ioka-Nakamichi T, Inamoto E, Toyonaga H & Mori H (2005) Complete set of ORF clones of *Escherichia coli* ASKA library (a complete set of *E. coli* K-12 ORF archive): unique resources for biological research. *DNA Res* **12**: 291–299.

- Lee S-J, Kim CH & Seo P-S (2008) Enhancement of heterologous production of eicosapentaenoic acid in *Escherichia coli* by substitution of promoter sequences within the biosynthesis gene cluster. *Biotechnol Lett* **30**: 2139–2142.
- Luy M, Rusing M & Kiy T (2009) PUFA-PKS genes from *Ulkenia*. US Patent Application Publication No. US 20090093033 A1.
- Metz JG, Roessler P, Facciotti D *et al.* (2001) Production of polyunsaturated fatty acids by polyketide synthases in both prokaryotes and eukaryotes. *Science* **293**: 290–293.
- Okuyama H, Orikasa Y, Nishida T & Morita N (2007) Bacterial genes responsible for the biosynthesis of eicosapentaenoic and docosahexaenoic acids and their heterologous expression. *Appl Environ Microb* **73**: 665–670.
- Orikasa Y, Yamada A, Yu R, Ito Y, Nishida T, Yumoto I, Watanabe K & Okuyama H (2004) Characterization of the eicosapentaenoic acid biosynthesis gene cluster from *Shewanella* sp. strain SCRC-2738. *Cell Mol Biol* **50**: 625–630.
- Orikasa Y, Nishida T, Hase A, Watanabe K, Morita N & Okuyama H (2006a) A phosphopantetheinyl transferase gene responsible for biosynthesis of *n*-3 polyunsaturated fatty acids from *Moritella marina* strain MP-1. *FEBS Lett* **580**: 4423–4429.
- Orikasa Y, Nishida T, Watanabe K, Morita N & Okuyama H (2006b) Phosphopantetheinyl transferase genes essential for biosynthesis of polyunsaturated fatty acids and their domain structures and compatibility. *Current Advances in the Biochemistry and Cell Biology of Plant Lipids: Proceedings of the 17th International Symposium on Plant Lipids, East Lansing, Michigan, July 2006* (Benning C & Ohlrogge J, eds), pp. 169–173. Aardvark Global Publishing Company, LLC, Salt Lake City, UT.
- Orikasa Y, Nishida N, Yamada A, Yu R, Watanabe K, Hase A, Morita N & Okuyama H (2006c) Recombinant production of docosahexaenoic acid in a mode of polyketide biosynthesis in *Escherichia coli*. *Biotechnol Lett* **28**: 1841–1847.
- Reuter K, Mofid MR, Marahiel MA & Ficner R (1999) Crystal structure of the surfactin synthetase-activating enzyme Sfp: a prototype of the 4'-phosphopantetheinyl transferase superfamily. *EMBO J* **18**: 6823–6831.
- Sugihara S, Orikasa Y & Okuyama H (2008) An EntD-like phosphopantetheinyl transferase gene from *Photobacterium profundum* SS9 complements *pfa* genes of *Moritella marina* strain MP-1 involved in biosynthesis of docosahexaenoic acid. *Biotechnol Lett* **30**: 411–414.
- 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.
- Vezi A, Campanaro S, D'Angelo M *et al.* (2005) Life at depth: *Photobacterium profundum* genome sequence and expression analysis. *Science* **307**: 1459–1461.
- Weissman KJ, Hong H, Oliynyk M, Siskos AP & Leadlay PF (2004) Identification of a phosphopantetheinyl transferase for erythromycin biosynthesis in *Saccharopolyspora erythraea*. *Chembiochem* **5**: 116–125.