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Structure and regulation of the omega-3 polyunsaturated fatty acid synthase genes from the deep-sea bacterium *Photobacterium profundum* strain SS9

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Omega-3 polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (20:5*n*-3; EPA) and docosahexaenoic acid (22:6*n*-3; DHA) have been shown to be of major importance in the promotion of cardiovascular health, proper human development and the prevention of some cancers. A high proportion of bacterial isolates from low-temperature and high-pressure marine environments produce EPA or DHA. This paper presents the sequence of a 33 kbp locus from the deep-sea bacterium *Photobacterium profundum* strain SS9 which includes four of the five genes required for EPA biosynthesis. As with other bacterial *pfa* (*p*olyunsaturated *f*atty *a*cid) genes, the deduced amino acid sequences encoded by the SS9 genes reveal large multidomain proteins that are likely to catalyse EPA biosynthesis by a novel polyketide synthesis mechanism. RNase protection experiments separated the SS9 *pfa* genes into two transcriptional units, *pfaA–C* and *pfaD*. The *pfaA* transcriptional start site was identified. Cultivation at elevated hydrostatic pressure or reduced temperature did not increase *pfa* gene expression despite the resulting increase in percentage composition of EPA under these conditions. However, a regulatory mutant was characterized which showed both increased expression of *pfaA–D* and elevated EPA percentage composition. This result suggests that a regulatory factor exists which coordinates *pfaA–D* transcription. Additional consideration regarding the activities required for PUFA synthesis is provided together with comparative analyses of bacterial *pfa* genes and gene products.

Keywords: eicosapentaenoic acid, *pfa* genes, hydrostatic pressure, polyketide synthase, ribonuclease protection assay

INTRODUCTION

Omega-3 polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (20:5*n*-3: EPA) and docosahexaenoic acid (22:6*n*-3; DHA) are essential components of many animal membrane lipids, well documented for their beneficial effects in human health and role as precursors for many hormone and hormone-like regulatory molecules (Angerer & Schacky, 2000; Lauritzen *et al*., 2001; Sauer *et al*., 2001). PUFAs can be obtained directly from dietary sources or synthesized by chain elongation and aerobic desaturation of pre-existing fatty acids such as linoleic acid (18:2*n*-6) and α-linolenic acid (18:3*n*-3) (Beaudoin *et al*., 2000; Parker-Barnes *et al*., 2000). Marine algae and numerous fungal micro-organisms produce significant quantities of PUFAs via aerobic pathways involving elongation/desaturation (Bajpai & Bajpai, 1993).

PUFAs were once thought to be absent in bacterial membranes (Erwin & Bloch, 1964), but numerous bacterial species of marine origin have now been shown to produce very-long-chain PUFAs such as EPA and

Abbreviations: ACP, acyl carrier protein; AT, acyl CoA:ACP transacylase; CLF, chain length factor; DHA, docosahexaenoic acid; DH/I, dehydratase/isomerase; EPA, eicosapentaenoic acid; ER, enoyl reductase; FAS, fatty acid synthase; KR, β-ketoacyl-ACP reductase; KS, β-ketoacyl-ACP synthase; PKS, polyketide synthase; PPTase, phosphopantetheinyl transferase; PUFA, polyunsaturated fatty acid; RPA, ribonuclease protection assay.

The GenBank accession numbers for the sequences reported in this paper are AF409100 and AF467805.

DHA. Such isolates have been found to be particularly prevalent in high-pressure, low-temperature deep-sea habitats and permanently cold marine environments (DeLong & Yayanos, 1986; Nichols *et al*., 1993; Yano *et al*., 1997). The enrichment of PUFA-producing strains from these environments has led to speculation that PUFA synthesis is an important adaptation for countering the effects of elevated hydrostatic pressure and low temperature on membrane fluidity or phase. In strains which have been analysed, PUFA synthesis undergoes temperature-dependent and, for deep-sea isolates, pressure-dependent regulation. Typically, as cultivation temperature is decreased, and/or pressure increased, PUFA incorporation into membrane phospholipids is enhanced. This modulation is thought to maintain appropriate membrane physical structure (Russell & Nichols, 1999). However, for at least one psychrotolerant piezophilic (high-pressure-adapted) deep-sea bacterium, *Photobacterium profundum* strain SS9, growth at high pressure and low temperature does not depend upon PUFA synthesis (Allen *et al*., 1999).

A variety of bacterial fatty acid biosynthetic mechanisms exist which vary with taxonomic identity and class of fatty acid product (Cronan & Rock, 1996; Fujii & Fulco, 1977; Rawlings, 1998). Some reports have suggested bacterial omega-3 PUFA production to be mediated by undefined desaturases (Russell & Nichols, 1999; Tanaka *et al*., 1999; Watanabe *et al*., 1997). However, sequence studies of bacterial genes required for PUFA biosynthesis have gradually led to a reappraisal of this view. Initial insight into the genetics of bacterial PUFA synthesis was gained by the cloning and analysis of a 38 kbp genomic fragment from the EPA producer *Shewanella* sp. strain SCRC-2738 (Yazawa, 1996). Five *Shewanella* genes, designated ORFs 2, 5, 6, 7 and 8, were shown to be necessary for recombinant EPA synthesis in *Escherichia coli* and in the marine cyanobacterium *Synechococcus* sp. (Takeyama *et al*., 1997; Yazawa, 1996). A subsequent analysis of the predicted amino acid sequences of the products of these genes indicated that they are most related to microbial polyketide synthase (PKS) complexes and fatty acid synthase (FAS) enzymes (Metz *et al*., 2001). PKS enzymes catalyse the synthesis of a wide array of complex natural products by the repetitive condensation and processing of simple monomeric substrates in a process resembling fatty acid synthesis (Hopwood & Sherman, 1990). In addition to the *Shewanella* sp. SCRC-2738 sequences, related genes partially responsible for PUFA production have been analysed from the DHA-producing bacterium *Moritella marina* strain MP-1 (formerly *Vibrio marinus*) (Tanaka *et al*., 1999) and from a DHA-producing thraustochytrid marine protist belonging to the genus *Schizochytrium* (Metz *et al*., 2001).

Recently, Metz *et al*. (2001) reported biochemical analyses of PUFA production in *E. coli* strains harbouring *Shewanella* sp. SCRC-2738 DNA and in the *Schizochytrium* species. Consistent with the examination of enzyme domains, isotopic labelling studies provided compelling support for a PKS-like pathway of PUFA synthesis in both systems studied (Metz *et al*., 2001). However, whereas considerable advances have been made towards a mechanistic understanding of microbial PUFA production, very little is known about the regulation of PUFA synthesis. The present study reports the cloning and molecular analysis of genes responsible for EPA synthesis, herein referred to as *pfa* (polyunsaturated fatty acid) genes, from the deep-sea bacterium *P. profundum* strain SS9. Transcriptional regulation of the SS9 *pfaA–D* genes was analysed as a function of varying temperature and hydrostatic pressure, and SS9 mutants containing polar insertions in two *pfa* genes were used to verify gene function and to help delineate the transcriptional organization of the *pfa* operon. Furthermore, an SS9 mutant that overproduces EPA was characterized and found to upregulate *pfa* gene transcription.

METHODS

Strains and growth conditions. All strains and plasmids used in this study are listed in Table 1. *P. profundum* strains were routinely cultured at 15 °C, 1 atmosphere (1 atm = 0·101 MPa) in 2216 Marine Medium (28 g litre; Difco). All temperature experiments (15 and 4 °C) were conducted aerobically in 2216 Marine Medium. For solid media, agar (Difco) was added at 17 g l^{-1}. Antibiotics kanamycin (50 μg ml^{-1} for *E. coli*, 200 μg ml^{-1} for *P. profundum* strains), rifampicin (100 μg ml^{-1}) and chloramphenicol (20 μg ml^{-1}) were added to media when required. All antibiotics were obtained from Sigma. High-pressure cultivation of *P. profundum* strains for growth studies, fatty acid analyses and extraction of RNA was as previously described (Allen *et al*., 1999).

Construction of an SS9 genomic fosmid library. Preparation of a genomic library of *P. profundum* strain DB110 in the pFOS1 vector (Kim *et al*., 1992) was performed as described by Stein *et al*. (1996). Briefly, high-molecular-mass genomic DNA was isolated from strain DB110 by lysis and extraction in agarose plugs and subsequently digested with *Sau*3A. DNA fragments between 35 and 45 kbp were purified following gel electrophoresis using the GeneClean Spin Kit (Bio 101). Size-selected DNA was ligated into the *Bam*HI site of pFOS1 vector arms, *in vitro* packaged using Gigapack III XL packaging extracts (Stratagene), and transfected to *E. coli* DH10B. Approximately 960 fosmid clones were individually picked into 96-well microtitre dishes containing LB medium plus 20 μg chloramphenicol ml^{-1} and 10% (v/v) glycerol and stored at −80 °C until further analysis.

DNA hybridizations and fosmid clone manipulations. Fosmid library clones were replicated onto MagnaCharge (MSI, Westboro, MA, USA) nylon filters and hybridized to DNA probes obtained from internal fragments of SS9 *pfa* genes using standard protocols (Sambrook *et al*., 1989). Previously we reported the cloning of an 885 bp internal fragment of the SS9 *pfaA* gene (designated ORF 3/4 in the previous report) making use of arbitrary primers derived from the *Shewanella* sp. SCRC-2738 EPA gene sequence (GenBank accession no. U73935) (Allen *et al*., 1999). An internal fragment of SS9 *pfaD* was subsequently obtained using primers ORF9-3 (5′-CGTTGAAGCATCAGCTTTCTT-3′) and ORF9-2 (5′-TACGCCCATCTCGAACATATC-3′) derived from SCRC-2738 EPA gene sequence. The resultant 571 bp PCR product contained a high degree of similarity, 79% identity at the DNA level, to the SCRC-2738 *pfaD* homologue (designated ORF7; Yazawa,

Table 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
Photobacterium profundum		
SS9	EPA$^+$; wild-type strain	DeLong *et al.* (1997)
DB110	Lac$^-$ Rif^r; SS9 derivative	Chi & Bartlett (1993)
EA2	EPA-overproducing chemical mutant	Allen *et al.* (1999)
EA10	*pfaA* insertion mutant (EPA$^-$)	Allen *et al.* (1999)
EA50	*pfaD* insertion mutant (EPA$^-$)	This study
Shewanella sp.		
SC2A	EPA$^+$	DeLong *et al.* (1997)
Plasmids		
pFOS1	Fosmid cloning vector	Kim *et al.* (1992)
pFOS8E1	Fosmid clone containing SS9 *pfaA–D*	This study
pMUT100	Mobilizable suicide plasmid, Km^r	Brahamsha (1996)
pCR2.1	PCR cloning vector, Km^r	Invitrogen
pDP18	*In vitro* transcription vector	Ambion

1996)). Filters were initially probed using the internal fragment of SS9 *pfaA*, stripped of bound probe, and reprobed with the *pfaD* probe. Of the 42 clones to which both probes hybridized, fosmid 8E1 (the clone with the smallest insert size of 33·1 kbp) was selected for further analysis and sequencing. Fosmid DNA was purified using the Qiagen Plasmid Midi kit and digested with *Not*I to excise the cloned insert. A subclone library of fosmid 8E1 was prepared by digesting the *Not*I insert with *Sau*3A, size selecting for 1–2 kbp fragments, and ligation into *Bam*HI-digested pUC18. Plasmid minipreparations of the fosmid 8E1:pUC18 subclones were prepared and sequenced using pUC18-specific primers flanking the cloned inserts.

DNA sequencing and analysis. Double-stranded DNA sequencing reactions were performed using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) and run on an Applied Biosystems model 373 DNA sequencing system. Initial sequence analysis and contig assembly was performed using Sequencher 3.1 software (Gene Codes Corp.). Additional sequence needed to fill in contig gaps was obtained using sequence-specific oligonucleotide primers and sequencing of PCR products. Global similarity searches were performed using the BLAST network service (Altschul *et al.*, 1997). Multiple alignments were performed using ClustalW (Higgins & Sharp, 1988) in conjunction with GeneDoc software (Nicholas & Nicholas, 1997). Domain arrangement analyses of the predicted amino acid sequences of the *pfa* genes were conducted using the ProDom database of protein domain families (Corpet *et al.*, 1999), the Conserved Domain Database with Reverse Position Specific BLAST (Altschul *et al.*, 1997) and the ISREC ProfileScan server (http://hits.isb-sib.ch/cgi-bin/hits_motifscan).

Insertional inactivation mutagenesis. Following previously published procedures (Allen & Bartlett, 2000; Allen *et al.*, 1999), insertional inactivation mutagenesis was performed targeting the SS9 *pfaD* gene. Briefly, an internal fragment of *pfaD* was PCR-amplified using primers ORF9-3 and ORF9-2 (sequences listed above; amplified region corresponding to 23708–24278 of GenBank accession no. AF409100), cloned into the pCR2.1-TOPO vector (Invitrogen) and subcloned into the mobilizable suicide vector pMUT100 (Brahamsha, 1996). The *pfaD*:pMUT100 construct was introduced into SS9 from *E. coli* by conjugal transfer as described by Chi & Bartlett (1993). Kanamycin-resistant exconjugants arose from plasmid integration into the SS9 chromosome in a single crossover event yielding strain EA50, with *pfaD* insertionally inactivated. The site of plasmid insertion was verified by PCR amplification of a portion of the *pfaD* gene using primers located upstream of the insertion site together with pMUT100-specific primers.

Cloning of phosphopantetheinyl transferase (PPTase) genes. The *Bacillus subtilis sfp* gene (GenBank accession no. X63158) was isolated from *B. subtilis* by PCR amplification of the complete gene using primers 5′-TGCTGAATTATGCTGTGGCAAGGC-3′ and 5′-GCTTCTCGAAATGATGTTCCCCGG-3′. In attempts to isolate PUFA synthase PPTase gene sequences, degenerate PCR primers were designed to conserved PPTase motifs found by alignment of known PPTase protein sequences including the PUFA synthase PPTase of *Shewanella* sp. strain SCRC-2738 (ORF2; GenBank accession no. U73935). Template DNA isolated from a variety of EPA-producing bacterial strains was employed; however, only DNA from the psychrotolerant, moderate piezophile *Shewanella* sp. strain SC2A (DeLong *et al.*, 1997) yielded an amplification product of the expected size relative to the SCRC-2738 sequence. Using forward primer 5′-GGCGATAAAGGYAARCCK-3′ and reverse primer 5′-CAACGHTCRATRTCWCCACC-3′ a 212 bp product was sequenced whose deduced amino acid sequence showed 48 % identity and 61 % similarity over 72 amino acids to the SCRC-2738 ORF2 product. In order to obtain flanking DNA sequence, primers internal to the SC2A PPTase sequence were designed for inverse PCR (Ochman *et al.*, 1990) and an SC2A cosmid library (Chilukuri & Bartlett, 1997) was screened using the SC2A PPTase internal fragment. Colony hybridizations were performed according to standard protocols (Sambrook *et al.*, 1989). Plasmid DNA was isolated from positively hybridizing clones and sequenced. The complete sequence of the *Shewanella* sp. strain SC2A PPTase is deposited under GenBank accession no. AF467805.

Fatty acid analyses. Extraction and analysis of fatty acid methyl ester preparations via combined gas chromatography-mass spectroscopy were performed as previously described (Allen *et al*., 1999). Fatty acids are denoted as number of carbon atoms : number of double bonds.

RNA isolation and ribonuclease protection assay (RPA) analyses. Total RNA was extracted from mid-exponential-phase *P. profundum* strains grown at various temperatures and pressures using the RNAzol B method (Tel-Test, Friendswood, TX, USA). [α-^{32}P]UTP-labelled RNA probes were synthesized using the T7 RNA polymerase MAXIscript *in vitro* transcription kit (Ambion, Austin, TX) and RPAs were performed using the RPA III kit according to the manufacturer's protocols (Ambion). Probe template preparations involved the PCR amplification of fragments of SS9 *pfa* genes, cloning of PCR products into the pCR2.1-TOPO vector and subsequent subcloning of inserts into the pDP18 transcription vector (Ambion). The sizes of full-length probes and protected fragments were as follows (the positions of *pfa* RPA probes are indicated with reference to GenBank accession no. AF409100): *pfaA* 377/268 bp (9422–9689), *pfaB* 465/322 bp (15332–15653), *pfaC* 477/344 bp (19133–19476), *pfaD* 477/344 bp (23385–23728), *pfaA/B* 520/264 bp (15020–15283), *pfaB/C* 516/260 bp (17129–17388), *pfaC/D* 511/255 bp (23052–23306). RNA probes were purified from denaturing acrylamide continuous gels, co-precipitated with 10 μg total RNA and hybridized overnight at 45 °C. Following RNase treatment, protected fragments were separated on denaturing acrylamide gels (5 % acrylamide/8 M urea) against undigested probe and appropriate controls. For detection of probes and protected fragments, gels were transferred to filter paper and exposed to X-ray film overnight. [α-^{32}P]UTP-labelled RNA Century Markers (Ambion) were used as size standards.

Primer extension analysis. Primer extension analysis of the SS9 *pfaA–D* genes was performed using the Primer Extension System-AMV Reverse Transcriptase kit (Promega). RNA was isolated from SS9 strain DB110 using the RNAzol B method. Multiple *pfa* extension primers were tested; however, only *pfaA* primers 5′-GCCATGCCAACAATCGCAAT-3′ (position 7529–7548) and 5′-GTTGCGATTAGGCAACTGGTGA-3′ (position 7379–7400) yielded extension products. Primers were end-labelled using [γ-^{32}P]ATP and T4 polynucleotide kinase. Labelled primers were annealed to 40 μg SS9 RNA and extended using AMV-RT. For DNA sequencing, *pfaA* plasmid templates were constructed using DNA amplified with primers 5′-AACCTCTTGCTCCAGTGATTG-3′ and 5′-TATCACGGTCGTATGTTTCCG-3′ (amplified fragment position 7067–7856), cloned into pCR2.1, and sequenced using the labelled primers used for primer extension in conjunction with the *fmol* DNA Cycle Sequencing System Kit (Promega). DNA fragments were resolved on 8 M urea/8 % polyacrylamide gels.

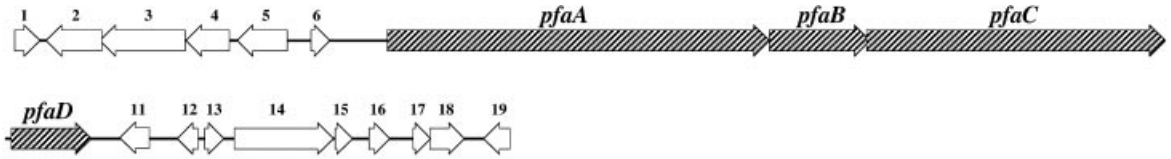
RESULTS

Isolation and sequence analysis of the *P. profundum* strain SS9 *pfa* gene cluster

The isolation of SS9 genes required for EPA synthesis (termed *pfaA–D*; *p*olyunsaturated *f*atty *a*cid) first involved the generation of a large insert fosmid library of SS9 genomic DNA and the subsequent probing of this library using a partial fragment of the SS9 *pfaA* gene previously isolated (Allen *et al*., 1999). Based on omega-3 PUFA synthase sequence information from *Shewanella* sp. SCRC-2738 (GenBank accession no. U73935) and *Moritella marina* (GenBank accession no. AB025342), the size of the SS9 gene cluster was predicted to be approximately 18 kbp. In order to optimize identification of library clones containing the complete EPA gene cluster from SS9, clones hybridizing positively with the proximal *pfaA* gene fragment were subsequently probed using an internal fragment of the distal SS9 *pfaD* gene. Fosmid 8E1 (insert size of 33100 bp) was found to positively hybridize to both probes and was selected for sequencing. Fig. 1 shows the genetic organization of the 19 predicted ORFs identified within this sequence as well as the size and similarity of these ORFs to other sequences present in GenBank based on BLAST searches. All SS9 sequences have been deposited in GenBank under accession number AF409100.

The SS9 *pfaA–D* genes span a region of 17347 bp (*pfaA* 7722 bp, *pfaB* 2100 bp, *pfaC* 5877 bp, *pfaD* 1635 bp). The deduced amino acid sequence of the SS9 *pfa* genes had a high degree of similarity and identity to *pfa* homologues in *Shewanella* and *Moritella* (% identity/similarity): SS9 *pfaA* – 62 %/72 % *Shewanella*, 46 %/61 % *Moritella*; SS9 *pfaB* – 44 %/58 % *Shewanella*, 18 %/33 % *Moritella*; SS9 *pfaC* – 67 %/77 % *Shewanella*, 45 %/60 % *Moritella*; SS9 *pfaD* – 82 %/88 % *Shewanella*, 62 %/76 % *Moritella*. Phylogenetically, SS9 is closely related to both *Shewanella* and *Moritella*, and the high degree of similarity of the SS9 and *Shewanella pfa* gene products likely reflects functional relatedness of the products (i.e. EPA vs DHA). In addition, the predicted proteins possess strong similarity to PKSs from *Streptomyces*, *Mycobacterium* and *Bacillus*, as well as cyanobacterial heterocyst glycolipid synthases and eukaryotic FAS enzymes. Comparison of ORFs in SS9, *Shewanella* and *Moritella* flanking their respective *pfa* gene clusters showed no sequence conservation with the exception of SS9 ORF6, which showed a high degree of identity (85 %) to an ORF also located immediately upstream of *pfaA* in *Moritella* (ORF7; GenBank accession no. AB025342). The function of this gene is not known but it possesses homology to a hydroxyacyl-CoA thioesterase (product of the *fcbC* gene) of *Pseudomonas* sp. DJ-12 (Chae *et al*., 2000).

Of the 19 ORFs identified, seven did not possess any significant homology to GenBank sequences and were noted as hypothetical proteins. Upstream of the SS9 *pfa* genes is a putative methyl-accepting chemotaxis protein (similar to *Vibrio cholerae* protein VCA093) and a cluster of four genes which appear to be arranged in an operon structure. Included in this cluster is a putative *fabH* paralogue. We denote this gene as a putative paralogue because a homologue of *fabH* has previously been cloned and sequenced from SS9, located within a distinct cluster of genes involved in saturated and monounsaturated fatty acid synthesis, an organization found in numerous γ-proteobacteria (our unpublished results). In addition, putative haloalkane dehalogenase, acyl-CoA ligase, and steroid dehydrogenase genes were identified within this upstream cluster. Based on BLAST searches, each of these four genes displays the highest similarity to genes in the plant pathogen *Xylella*



ORF	Size (bp)	Similarity
1	513 partial	putative methyl-accepting chemotaxis protein (*Vibrio cholerae* protein VCA093)
2	1116	putative steroid dehydrogenase (*Xylella fastidiosa* protein XF1970)
3	1755	putative acyl-CoA ligase (*Xylella fastidiosa* protein XF2276)
4	915	putative haloalkane dehalogenase (*Xylella fastidiosa* protein XF1965)
5	1071	putative *fabH* paralog; 3-oxoacyl-ACP synthase III (*Xylella fastidiosa* protein XF1970)
6	402	ORF6; *Moritella marina* ORF7 homolog; possible hydroxyacyl-CoA thioesterase
7	**7722**	***pfaA*; Eicosapentaenoic acid (20:5n-3) fatty acid synthase A**
8	**2100**	***pfaB*; Eicosapentaenoic acid (20:5n-3) fatty acid synthase B**
9	**5877**	***pfaC*; Eicosapentaenoic acid (20:5n-3) fatty acid synthase C**
10	**1635**	***pfaD*; Eicosapentaenoic acid (20:5n-3) fatty acid synthase D**
11	630	ORF11; hypothetical protein
12	414	ORF12; hypothetical protein
13	243	ORF13; hypothetical protein
14	2046	alkaline serine protease (*Vibrio metschnikovii vapT*)
15	393	ORF15; hypothetical protein
16	477	ORF16; hypothetical protein
17	384	ORF17; hypothetical protein
18	762	ORF18; hypothetical protein
19	556 partial	formyltetrahydrofolate deformylase; purine biosynthesis; (*Pseudomonas aeruginosa purU*)

Fig. 1. *P. profundum* strain SS9 *pfa* gene cluster and flanking DNA. Graphic map showing the organization of the 19 ORFs identified from the sequencing of the 33 100 bp genomic insert of fosmid 8E1 together with the size of the identified ORFs and their similarity to sequences present in GenBank based on BLAST searches. All SS9 sequences have been deposited under GenBank accession no. AF409100.

fastidiosa (GenBank accession no. AE003849). Unlike SS9, however, none of these genes are linked in the *Xylella* genome. Downstream of the SS9 *pfa* cluster numerous ORFs of unknown function were identified as well as an alkaline serine protease (homologue of *Vibrio metschnikovii vapT*) and a formyltetrahydrofolate deformylase gene (homologue of *Pseudomonas aeruginosa purU*) involved in purine biosynthesis.

Analysis of SS9 EPA biosynthetic enzymes

Similarity searches of the SS9 *pfa* gene products revealed significant matches to numerous multifunctional enzyme complexes involved in such processes as polyketide antibiotic synthesis (Hopwood & Sherman, 1990; Pfeifer & Khosla, 2001), eukaryotic fatty acid synthesis (Beaudoin *et al*., 2000; Parker-Barnes *et al*., 2000) and heterocyst glycolipid synthesis (Campbell *et al*., 1997). Domain analyses within individual *pfa* gene products also revealed numerous enzyme domains characteristic of functions present in bacterial type II fatty acid synthesis. This type of organization is similar to type I PKSs, multifunctional enzymes containing sets of FAS-related activities for successive rounds of polyketide chain elongation and derivatization (Rawlings, 2001). Fig. 2 shows the domain organization of the SS9, *Shewanella* and *Moritella pfaA–D* deduced amino acid sequences. Seven enzyme domains were identified within the *pfa* products: β-ketoacyl-ACP synthase (KS), acyl CoA-ACP transacylase (AT), acyl carrier protein (ACP), β-ketoacyl-ACP reductase (KR), chain length factor (CLF; possible malonyl-ACP decarboxylase activity – Bisang *et al*., 1999), β-hydroxyacyl-ACP dehydratase/isomerase (DH/I) and enoyl reductase (ER).

The domain organization of the SS9 and *Shewanella pfa* products was identical with the exception of one additional ACP domain found in *Shewanella* PfaA (six ACPs compared to five in SS9 PfaA). Pfa domains of the DHA-producing *Moritella* differed from the EPA-producing SS9 and *Shewanella* products by the inclusion of one additional KS (or CLF) domain in PfaB. CLF domains are homologous to KS domains but the KS active site has a conserved cysteine residue whereas in CLF a glutamine residue exists (Bisang *et al*., 1999). In *Moritella*, PfaB has a region homologous to the C-terminal domain of KS but lacks an active-site sequence. Similarly, the second KS-like domain present in *Moritella* PfaC lacks both a KS active-site motif and the diagnostic glutamine residue of CLF.

Interestingly, growth of SS9 in the presence of the fungal antibiotic cerulenin, a potent irreversible inhibitor of fatty acid biosynthetic condensing enzymes such as KAS I and KAS II (Omura, 1981), has no effect on EPA production (Allen *et al*., 1999). This resistance could result from blocked access of cerulenin to the Pfa KS active sites, reflecting structural differences between the Pfa KS domains and type II KAS enzymes.

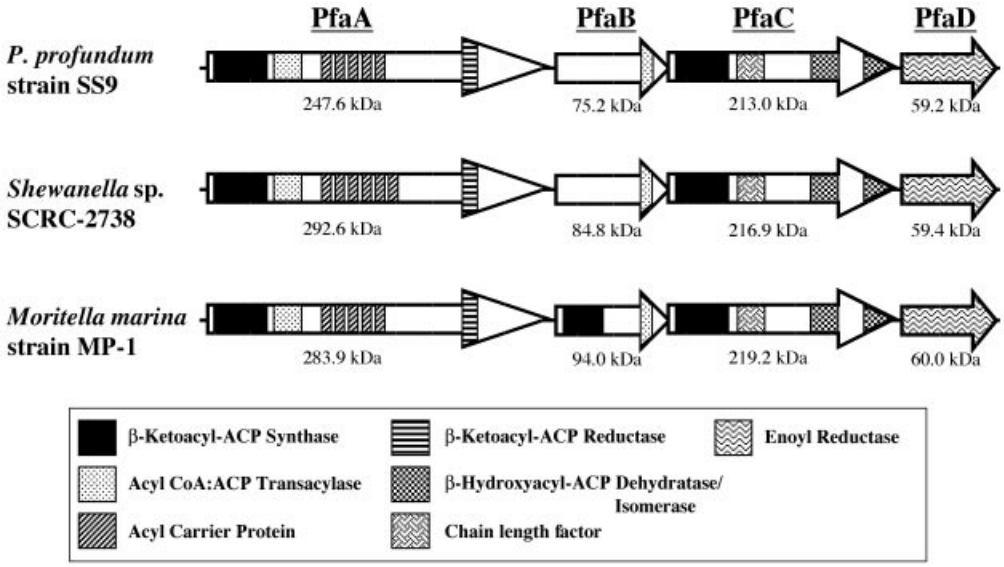


Fig. 2. Comparison of enzyme domains identified within bacterial *pfaA–D* gene products. *P. profundum* strain SS9 and *Shewanella* sp. strain SCRC-2738 produce EPA whereas *M. marina* strain MP-1 produces DHA. Enzyme domains (represented as filled regions) were identified within individual gene products by conserved motif database searches as described in Methods. The predicted sizes of deduced amino acid sequences are noted beneath each product.

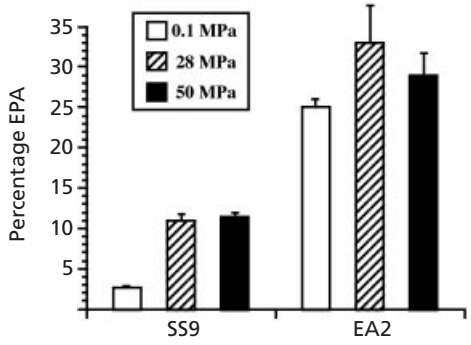


Fig. 3. Effect of varying hydrostatic pressure on EPA percentage composition in wild-type SS9 and chemical mutant strain EA2. Fatty acids were recovered and analysed from mid-exponential-phase cells cultivated at the corresponding pressure (9 °C) as described in Methods. Data represent mean percentage composition (by weight) ± standard deviation ($n = 3$).

EPA synthesis in SS9 and overproducing strain EA2

The percentage composition of EPA present in SS9 membranes undergoes temperature- and pressure-dependent modulation (Allen *et al*., 1999). An increase in cultivation pressure from 0·1 MPa to 28 MPa [0·1 MPa = 1 atm = 1 bar] results in nearly a fourfold increase in EPA percentage composition (Fig. 3). Similarly, EPA percentage composition undergoes moderate increase in response to reduced cultivation temperature, i.e. 15 °C to 4 °C (Allen *et al*., 1999). Fig. 3 shows the percentage composition of EPA as a function of varying hydrostatic pressure in wild-type SS9 and an SS9 mutant strain found to overproduce EPA. This strain, designated EA2, was isolated as an oleic acid (18:1*n*-9)-requiring auxotrophic chemical mutant (Allen *et al*., 1999). Strain EA2 constitutively produces EPA at a level nearly fivefold that of wild-type SS9 grown at atmospheric pressure (Fig. 3).

Transcripitonal analyses of the SS9 *pfa* operon: ribonuclease protection assays and primer extension

RPAs were performed on SS9 *pfa* genes with RNA isolated from strains cultivated at various hydrostatic pressures and temperatures (Fig. 4). RPA analyses were chosen due to the potential large size of transcripts and indication of relatively weak expression of the *pfa* genes from prior Northern blot analysis attempts. No differences in the relative transcript abundance of any of the four *pfa* genes were detected as a function of varying temperature (15 °C vs 4 °C, Fig. 4B, lanes 3 vs 4). RNA extracted from cells cultivated at varying hydrostatic pressure (0·1 MPa vs 28 MPa) revealed different results. When cells were grown at elevated pressure, transcript abundance of all *pfa* genes was considerably less than when cells were grown at atmospheric pressure (Fig. 4B, lanes 5 vs 6), a confounding result given the observed increase in EPA percentage composition as a function of increased hydrostatic pressure (Fig. 3). RPA analyses of *pfa* gene expression were also performed for the EPA-overproducing strain EA2 (Fig. 4B, lanes 7). Results for EA2 revealed dramatic upregulation of *pfa* gene transcription compared to wild-type SS9 cultivated under identical conditions (15 °C, 0·1 MPa).

In order to delineate the transcriptional organization of the SS9 *pfa* gene cluster, RPA analyses were performed on RNA extracted from SS9 grown at 15 °C, 0·1 MPa,

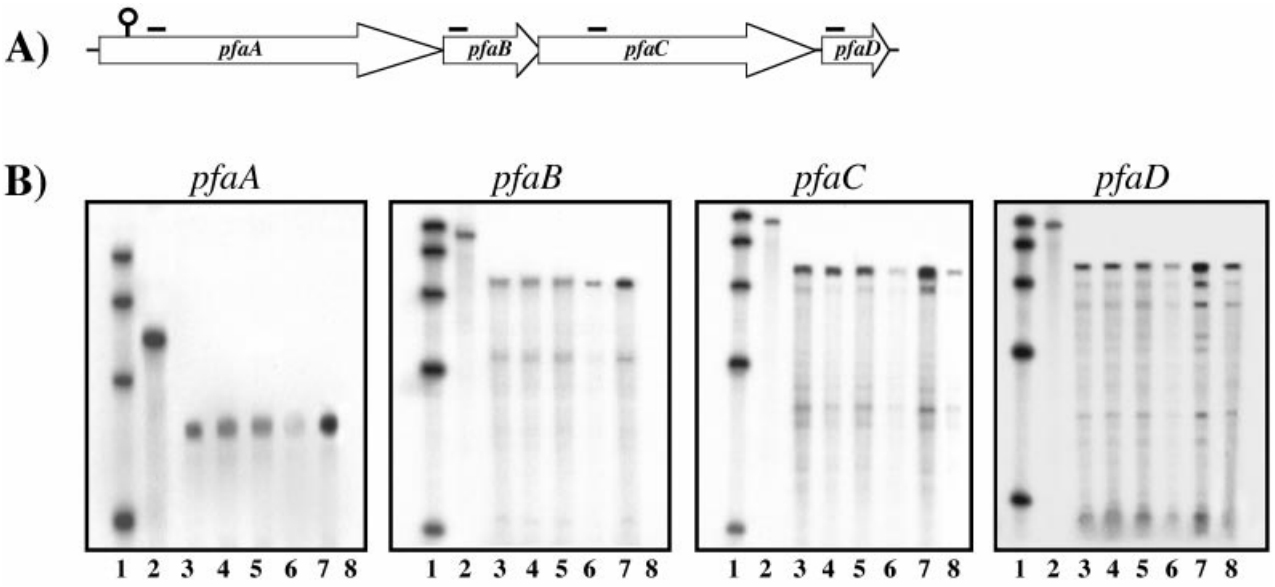


Fig. 4. RPA analyses of SS9 *pfaA–D* gene transcription. (A) Schematic representation of *pfa* operon showing location of RPA probes (lines) and site of *pfaA* insertion in EPA defective mutant strain EA10 (lollipop). (B) RPA results of *pfaA–D* expression. RPA analyses were performed as described in Methods with 10 μg total RNA using ^{32}P-labelled riboprobes. Lanes: 1, RNA Century size markers (500, 400, 300, 200, 100 bases); 2, unprotected full-length probes; 3, SS9 at 15 °C; 4, SS9 at 4 °C; 5, SS9 at 0·1 MPa (9 °C); 6, SS9 at 28 MPa (9 °C); 7, EA2 (EPA-overproducing chemical mutant) at 15 °C; 8, EA10 (*pfaA* insertion strain) at 15 °C.

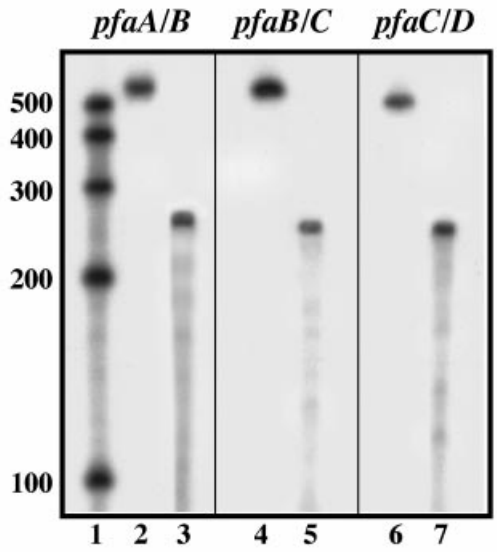


Fig. 5. RPA analysis of SS9 *pfa* gene linkage: *pfaA/B*, *pfaB/C*, *pfaC/D*. RPA probes were designed spanning the intergenic regions of adjacent *pfa* genes. RNA was extracted from SS9 cultivated at 15 °C. Lanes: 1, RNA Century size maker; 2, 4 and 6, unprotected full-length probes; 3, 5 and 7, protected fragments.

using probes spanning the putative intergenic regions of the *pfa* genes (i.e. *pfaA/B*, *pfaB/C*, *pfaC/D*; Fig. 5). Linkage RPAs showed protected fragments of the expected size, indicating that adjacent *pfa* genes are co-transcribed or that promoters for downstream genes are present upstream of the probe-binding sites. Furthermore, transcript analyses were performed using RNA extracted from the *pfaA* insertion strain EA10. This strain contains a pMUT100 (Brahamsha, 1996) insertion in the 5′ end of *pfaA*. EA10 RPA analyses were used to determine whether there was transcriptional linkage between *pfaA* and any of the downstream *pfa* genes. Using RNA from EA10 no RNA protection was detected with the *pfaA* or *pfaB* probes. These results indicate that transcription upstream of *pfaA* drives *pfaA* and *pfaB* co-transcription. Analysis of *pfaC* expression in this strain showed a faint protected fragment suggesting the presence of a weak promoter located in the region spanning the sites of the *pfaB* and *pfaC* probes. Finally, a *pfaD* product of comparable intensity was present using RNA from wild-type SS9 or EA10, indicating the likelihood of a promoter between the region of the *pfaC* and *pfaD* probes. RPA analyses thus revealed two major promoter regions and one minor promoter region driving expression of the SS9 *pfa* gene cluster, giving rise to *pfaA–C*, *pfaC* and *pfaD* transcripts.

In order to localize these promoter regions, primer extension reactions were performed at multiple sites within each of the *pfa* genes. All attempts to determine transcriptional start sites within *pfaB*, *pfaC* and *pfaD* failed to reveal extension products even with RNA concentrations up to 100 μg total RNA. The lack of detection of *pfaC* and *pfaD* transcription initiation sites, despite the evidence for *pfaC* and *pfaD* promoters, presumably stems from low transcript abundance or stability. However, the transcriptional start upstream of

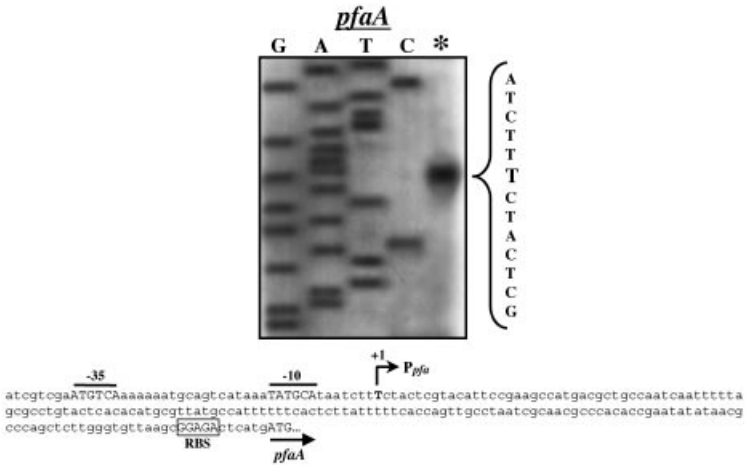


Fig. 6. Determination of SS9 *pfaA* transcriptional start site by primer extension analysis. The *pfaA* extension product (lane denoted by an asterisk) was detected using the ^{32}P-labelled primer located at position −75 to −54 relative to the translational start of *pfaA*. Dideoxy DNA sequencing reaction mixtures using the same primer were electrophoresed in parallel (lanes G, A, T and C). The nucleotide sequence of the transcribed strand immediately flanking the transcriptional start T is bracketed to the right of the sequencing ladder. The nucleotide sequence and promoter features of the upstream *pfaA* region are shown below the primer extension analysis results.

pfaA was readily mapped (Fig. 6). *pfaA* primer extensions were performed with primers corresponding to positions +75 to +94 and −75 to −54 with respect to the 5′ end of *pfaA*. Both primers provided identical results indicating transcription initiation 167 bp upstream of the *pfaA* ATG translational start (Fig. 6 shows results obtained with the −75 to −54 primer). Putative −35 (ATGTCA) and −10 (TATGCA) regions were identified as well as the putative ribosome-binding site (GGAGA) located at position −11 to −7.

Insertional inactivation mutagenesis of the SS9 *pfaD* gene

In an analogous manner to the isolation of *pfaA* insertion mutant EA10, a mutant containing an insertionally inactivated *pfaD* was constructed, resulting in strain EA50. This *pfaD* mutant, like the *pfaA* insertion strain, did not produce EPA under any culture conditions, nor did it accumulate any fatty acids that could serve as intermediates in the EPA biosynthetic pathway (data not shown). Thus, both *pfa* operons are required for EPA biosynthesis. Mutant EA50 also shared with EA10 the phenotype of no growth defect when cultivated at elevated hydrostatic pressures or reduced temperatures despite its gross alteration in fatty acid composition.

Introduction of PPTase genes into *E. coli* harbouring SS9 *pfaA–D*

In an attempt to achieve recombinant EPA production in *E. coli* we sought to obtain a gene encoding the last remaining enzyme activity needed: PPTase activity. PPTase genes were recovered from both the EPA-producing deep-sea *Shewanella* sp. strain SC2A (DeLong *et al*., 1997) and *B. subtilis*. Based on BLAST analyses, the SC2A putative PPTase shares 55% identity (89/161 amino acids) with the *Shewanella* sp. strain SCRC-2738 PUFA synthase PPTase (ORF2) shown to be required for recombinant EPA production in *E. coli* host strains (Metz *et al*., 2001). Introduction of either the SC2A PPTase gene or the *sfp* gene, the surfactin-synthetase-activating PPTase of *B. subtilis* (Nakano *et al*., 1992), into *E. coli* strains expressing SS9 *pfaA–D* from pFOS8E1 consistently failed to yield recombinant EPA synthesis.

DISCUSSION

Progress towards understanding microbial PUFA production has benefited from the recent identification and characterization of genes involved in their synthesis (Metz *et al*., 2001; Tanaka *et al*., 1999; Yazawa, 1996). These analyses have provided clues to the mode of synthesis, although biochemical investigations will be needed to resolve many aspects of the pathway. The organization of microbial PUFA synthases is in stark contrast to known eukaryotic PUFA synthesis mechanisms involving elongases and position-specific, oxygen-dependent desaturases (Napier & Michaelson, 2001; Parker-Barnes *et al*., 2000). Furthermore, numerous PUFA-producing bacterial strains, including SS9, are capable of producing PUFAs under strictly anaerobic conditions, thus precluding the involvement of an oxygen-dependent mechanism (our unpublished results; Metz *et al*., 2001; Nichols *et al*., 1992). Based on the identification of multiple enzyme domains within *pfa* gene products and ^{13}C-labelling studies (Metz *et al*., 2001) it is now evident that PUFA production shares many features with polyketide synthesis. Analysis of the four SS9 *pfa* gene products has revealed 10 regions possessing enzyme activities akin to those found in PKS and FAS systems (Fig. 2). The activities of these integrated domains include condensation reactions (KS domains), acyl CoA:ACP transfer reactions (AT), multiple acyl carrier protein domains (ACP), ketoacyl reduction reactions (KR), chain length factor domains (CL) presumably involved in decarboxylation reactions, dehydratase/isomerase reactions (DH/I), and enoyl reduction reactions (ER). These domains presumably catalyse the reiterative steps in the processing of the growing acyl chain, where each condensation reaction is followed by complete or abbreviated reductive reaction cycles of ketoreduction, dehydration/isomerization, and enoyl reduction.

The ability to introduce multiple double bonds into a single acyl chain in the absence of desaturation reactions likely arises from the activities of the DH/I domains present in the microbial PUFA synthases (bacterial PfaC homologues and *Schizochytrium* ORF C). Such dehydration/isomerization reactions would be analogous to those catalysed by FabA (β-hydroxydecanoyl-ACP dehydratase) in bacterial monounsaturated fatty acid synthesis (Cronan & Rock, 1996). By dehydration of the β-hydroxyacyl-ACP substrate, product of β-ketoacyl-ACP synthase condensation and subsequent reduction of the β-ketoester by β-ketoacyl-ACP reductase, a *trans* double bond is introduced into the growing acyl chain. In selective rounds of acyl chain elongation these double bonds are either preserved by isomerization to the *cis* form to form an unsaturated acyl-ACP or reduced by an enoyl reductase to a saturated acyl-ACP. Metz *et al.* (2001) propose a hypothetical pathway for EPA synthesis in *Shewanella* sp. SCRC-2738 wherein position-specific isomerases (*trans*-2,*cis*-3 and *trans*-2,*cis*-2) are involved in *trans*/*cis* double bond isomerization. Such a mechanism may be consistent with two DH/I domains being present in PfaC homologues (Fig. 2). Alternatively, the two DH/I domains could be analogous to the FabA and FabZ β-hydroxyacyl-ACP dehydratase isozymes found in *E. coli* which differ in reactivity and specificity (Heath & Rock, 1996).

Unique to microbial PUFA synthases is the presence of clustered repetitive ACP domains (Fig. 2): SS9 *pfaA* possesses five ACP domains, *Shewanella* sp. has six, *Moritella* has five, and *Schizochytrium* has nine (Metz *et al.*, 2001; Tanaka *et al.*, 1999). Intermediates in the biosynthetic process are presumably bound to these ACP domains as thioesters with AT domains being required for the loading of the starter and extender units. The significance of the disparity in the number of ACP repeats among the PUFA synthase systems is unknown.

Currently, we have been unable to achieve recombinant EPA synthesis in *E. coli* with the introduced SS9 *pfaA–D* genes. This shortcoming arises from the need for an additional gene whose product is required for the post-translational modification of the constituent ACP domains present in PfaA. This activity is achieved by a PPTase which converts apo-ACP to its active holo-form by transfer of a 4′-phosphopantetheinyl moiety from coenzyme A to ACP (Lambalot *et al.*, 1996). In *Shewanella* sp. SCRC-2738 a fifth gene, designated ORF2, required for recombinant EPA synthesis in *E. coli* has been identified as a PPTase and is located within close proximity of the *pfaA–D* operons (Metz *et al.*, 2001). Unlike SCRC-2738, the PUFA synthase PPTase is unlinked from the other *pfa* genes in SS9 and *Moritella*. We have been unable to clone the ORF2 homologue from either SS9 or *Moritella*. During attempts to obtain recombinant EPA synthesis, we introduced two PPTase genes into *E. coli* harbouring SS9 *pfaA–D*, *B. subtilis sfp* and a *Shewanella* ORF2 homologue. The *B. subtilis sfp* gene, involved in surfactin biosynthesis, encodes a PPTase with a broad substrate recognition spectrum (Nakano *et al.*, 1992; Reuter *et al.*, 1999). In addition, a PPTase homologue which contained a high degree of identity to *Shewanella* sp. SCRC-2738 ORF2 was cloned and sequenced from the EPA producer *Shewanella* sp. strain SC2A. Expression of either of these genes in *E. coli* harbouring SS9 *pfaA–D* failed to yield recombinant EPA synthesis, suggesting a high degree of specificity of individual PPTases to their cognate ACPs.

While substantial progress has been made towards a mechanistic understanding of microbial PUFA synthesis, very little information exists regarding the regulation of bacterial PUFA production. In those organisms that have been studied, modulation of PUFA percentage composition occurs during changes in cultivation temperature or pressure. For example, growth of SS9 at a hydrostatic pressure of 28 MPa results in an approximately fourfold increase in EPA percentage composition relative to growth at 0·1 MPa (Fig. 3). At the outset of our studies, one possibility was that this modulation was the result of transcriptional regulation of the EPA biosynthetic genes.

RPA analyses performed on each of the SS9 *pfa* genes using RNA extracted from SS9 cells cultivated at various temperatures and pressures revealed that the *pfa* genes are not transcriptionally regulated in an adaptive manner in response to these parameters (Fig. 4). The observed reduction in *pfa* transcript abundance at elevated pressure is confounding and could result from diminished transcription initiation or increased transcript turnover at high pressure. Numerous prokaryotic species regulate percentage composition of particular membrane fatty acids in response to cultivation parameters. In *E. coli* increased *cis*-vaccenic acid (18:1*n*-11) composition at low temperature is an intrinsic property of the fatty acid biosynthetic enzyme KAS II (β-ketoacyl-ACP synthase II), product of the *fabF* gene, and a similar regulatory mechanism may account for increased *cis*-vaccenic acid composition at high pressure in SS9 (Allen & Bartlett, 2000; Cronan & Rock, 1996). In both bacteria, *fabF* is not transcriptionally regulated and, at least for the *E. coli* enzyme, it is the relative activity of the enzyme at different temperatures that is responsible for the increased production of 18:1 at low temperature. Hence, the possibility exists that PUFA synthases exhibit temperature/pressure-responsive characteristics.

Transcriptional analyses indicate that the *pfa* gene cluster is organized into two operons, *pfaA–C* and *pfaD* (Figs 4 and 5). Evidence in support of this conclusion includes the presence of overlapping start/stop codons of adjacent genes, RPA results with probes spanning intergenic regions, and transcript analyses of a strain containing a polar insertion within *pfaA*. The transcriptional start of *pfaA* has been mapped to 169 bp upstream of the translational start (Fig. 6).

Results from SS9 suggest that the pathway for PUFA synthesis is separate and distinct from the type II FAS producing monounsaturated and saturated fatty acids (Allen & Bartlett, 2000; Allen *et al.*, 1999). Many of the type II FAS genes have been cloned and sequenced from

SS9 and *Moritella* (Allen & Bartlett, 2000; Tanaka *et al*., 1999). Metz *et al*. (2001) reported a probable PUFA synthetic mechanism reliant on malonyl-CoA derived from acetate as would be expected for the type II FAS system. An interesting question is the 'cross-talk' that exists between the two systems with regard to coordinated expression and lipid incorporation. Some initial insight into this interplay has been provided by *pfa* transcript analysis of an SS9 mutant strain, designated EA2, originally isolated as an oleic acid auxotrophic chemical mutant that overproduces EPA nearly fivefold compared to wild-type SS9 (Fig. 3). In addition, this strain greatly underproduces monounsaturated fatty acids (MUFAs) (Allen *et al*., 1999). *pfa* transcript analyses in this strain reveal substantial *pfaA–D* overexpression relative to wild-type SS9 (Fig. 4). While the nature of the mutation in this strain has yet to be resolved, two opposing hypotheses can be proposed. Either this strain harbours a lesion resulting in decreased MUFA production which results in compensatory increases in *pfa* transcription and EPA synthesis, or the mutation results in overexpression of both *pfa* operons and the cellular response is decreased MUFA synthesis. Both models require the presence of a transcription factor that modulates *pfa* gene expression.

The high degree of sequence similarity between the bacterial (*Shewanella* sp SCRC-2738, *M. marina* and SS9) and the eukaryotic microbe *Schizochytrium pfa* genes suggests the possible involvement of horizontal gene transfer in the acquisition of the *pfa* gene clusters in the marine environment. However, among the three bacterial strains whose *pfa* gene clusters have been cloned and sequenced no sequence conservation flanking the *pfa* clusters is observed with the exception of a single undefined ORF located upstream of *pfaA* in SS9 and *Moritella*. Furthermore, there is no apparent GC bias among the *pfaA–D* genes nor is there indication of flanking genes possessing functions which could facilitate horizontal transfer.

Located upstream of the SS9 *pfa* cluster resides an intriguing cluster of four genes which appear to be organized into a possible operon (Fig. 1). Included in this cluster is a putative *fabH* (3-oxoacyl-ACP synthase III; KAS III) paralogue. This *fabH* paralogue is distinct from the *fab* cluster *fabH* homologue, involved in type II fatty acid biosynthesis initiation, which we have cloned and sequenced from SS9 (our unpublished results). Multiple *fabH*-like sequences have been identified in a few bacterial species including *B. subtilis* (*yjaX*, GenBank accession no. F69842, and *yhfB*, Y14083) and *V. cholerae* (GenBank accession nos A82423 and H82128). Within this cluster also reside a putative haloalkane dehalogenase, a probable acyl-CoA ligase, and a putative steroid dehydrogenase/isomerase. Proteins of the hydroxysteroid dehydrogenase/isomerase family (Labrie *et al*., 1992) are unusual in prokaryotic organisms, with only a single other bacterial homologue having been identified in the plant pathogen *Xylella fastidiosa* (GenBank accesssion no. AE004004). The sequence and possible operon structure of this gene cluster suggest that their products could function in a common metabolic process including some aspect of fatty acid physiology. Curiously, upstream of the *M. marina pfa* gene cluster lie two genes presumably involved in fatty acid metabolism as well, a 3-ketoacyl-CoA thiolase/acetyl-CoA acetyltransferase and a probable lipid A acyltransferase (ORFs 1 and 3, GenBank accession no. AB025342).

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