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

Eric E. Allen and Douglas H. Bartlett

Omega-3 polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (20:5n-3; EPA) and docosahexaenoic acid (22:6n-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 (polyunsaturated fatty acid) 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
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, *Schizochytrium* *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 kb 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 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 thaurochtyrid 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* (polysaturated 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−1; 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 litre−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 pFOSI 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 Sau3A. DNA fragments between 35 and 45 kb were purified following gel electrophoresis using the GeneClean Spin Kit (Bio 101). Size-selected DNA was ligated into the BamHI site of pFOSI vector arms, in vitro packaged using Gigapack III XL packaging extracts (Stratagene), and transfectioned 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 clone libraries 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′-TACGCCATCTCGAAGCATATC-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, 1996).
Global similarity searches were performed using the oligonucleotide primers and sequencing of PCR products. and contig assembly was performed using Sequencher 3.1 model 373 DNA sequencing system. Initial sequence analysis (Applied Biosystems) and run on an Applied Biosystems BigDye Terminator Cycle Sequencing Ready Reaction kit server (http: Specific 1999), the Conserved Domain Database with Reverse Position ProDom database of protein domain families (Corpet et al. 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).

**Table 1. Strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference or source</th>
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<td>profundum</td>
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<td>SS9</td>
<td>EPA⁺; wild-type strain</td>
<td>DeLong et al. (1997)</td>
</tr>
<tr>
<td>DB110</td>
<td>Lac⁻ Rif⁺; SS9 derivative</td>
<td>Chi &amp; Bartlett (1993)</td>
</tr>
<tr>
<td>EA2</td>
<td>EPA-overproducing chemical mutant</td>
<td>Allen et al. (1999)</td>
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<tr>
<td>EA10</td>
<td>pfaA insertion mutant (EPA⁻)</td>
<td>Allen et al. (1999)</td>
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<td>EA50</td>
<td>pfaD insertion mutant (EPA⁻)</td>
<td>This study</td>
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<td><strong>Shewanella sp.</strong></td>
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<td>SC2A</td>
<td>EPA⁺</td>
<td>DeLong et al. (1997)</td>
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<td><strong>Plasmids</strong></td>
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<td>Fosmid cloning vector</td>
<td>Kim et al. (1992)</td>
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<tr>
<td>pFOS8E1</td>
<td>Fosmid clone containing SS9 pfaA–D</td>
<td>This study</td>
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<tr>
<td>pMUT100</td>
<td>Mobilizable suicide plasmid, Km⁺</td>
<td>Brahamsha (1996)</td>
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<tr>
<td>pCR2.1</td>
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<td>Invitrogen</td>
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<tr>
<td>pDP18</td>
<td>In vitro transcription vector</td>
<td>Ambion</td>
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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-TOP 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 EA30, 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 phosphopantetheinyll transferase (PPTase) genes. The Bacillus subtilis spf gene (GenBank accession no. X63158) was isolated from B. subtilis by PCR amplification of the complete gene using primers 5'-TGCTGAATTATGCTGTG-GCAAGGC-3' and 5'-GCTTTCGAAATGATGTTCCC-GG-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'-GGCCGATAAAGGYAARCC-3' and reverse primer 5'-CAACGHTCRT-RTCWCCACC-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 spectrometry 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-<sup>32</sup>P]UTP-labelled RNA probes were synthesized using the T7 RNA polymerase MAXiscript in vitro transcription kit (Ambion, Austin, TX) and RPA 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). Primers 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-<sup>32</sup>P]UTP-labelled RNA Fatty acid analyses. Extraction and analysis of fatty acid methyl ester preparations via combined gas chromatography-mass spectrometry were performed as previously described (Allen et al., 1999). Fatty acids are denoted as number of carbon atoms:number of double bonds.

**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_ ; polysaturated fatty acid) 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 _pfa_ 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 PKs from _Streptomyces_, _Mycobacterium_ and _Bacillus_, as well as cyanobacterial heterocyst glycolipid syntheses 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 hydroxacyl-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 methyl-accepting chemotaxis protein (product of the _fcbC_ gene) of _Pseudomonas_ sp. DJ-12 (Chae et al., 2000).
EPA synthesis in a deep-sea bacterium

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 33100 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 de-formylase 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. 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.
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**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.

<table>
<thead>
<tr>
<th>Enzyme Domain</th>
<th>PfaA</th>
<th>PfaB</th>
<th>PfaC</th>
<th>PfaD</th>
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<tr>
<td>Size (kDa)</td>
<td>247.6</td>
<td>75.2</td>
<td>213.0</td>
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</table>

**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).

**Transcrptional 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.

**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 (18:1n-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).
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 32P-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
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 (ATGTC) 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 inser tionally 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 set out to obtain a gene encoding the last 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 *S. marinoff*, 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 13C-labeling 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.

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**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 5′-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.
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 or 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′-phosphopantetheiny1 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 (18:1n-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 (MUFA) (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) parologue. This fabH parologue 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 (ypjX, GenBank accession no. F69842, and yfbB, Y14083) and V. cholerae (GenBank accession nos A82423 and H82128). Within this cluster also resides 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 accession 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/acyl-CoA acetyltransferase and a probable lipid A acyltransferase (ORFs 1 and 3, GenBank accession no. AB025342).

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