Co-transcribed Genes for Long Chain Polyunsaturated Fatty Acid Biosynthesis in the Protozoon Perkinsus marinus Include a Plant-like FAE1 3-Ketoacyl Coenzyme A Synthase*

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The marine parasitic protozoon Perkinsus marinus synthesizes the polyunsaturated fatty acid arachidonic acid via the unusual alternative Δ8 pathway in which elongation of C18 fatty acids generates substrate for two sequential desaturations. Here we have shown that genes encoding the three P. marinus activities responsible for arachidonic acid biosynthesis (C18 Δ9-elongating activity, C20 Δ8 desaturase, C20 Δ5 desaturase) are genomically clustered and co-transcribed as an operon. The acyl elongation reaction, which underpins this pathway, is catalyzed by a FAE1 (fatty acid elongation 1)-like 3-ketoacyl-CoA synthase class of condensing enzyme previously only reported in higher plants and algae. This is the first example of an elongating activity involved in the biosynthesis of a polyunsaturated fatty acid that is not a member of the ELO/SUR4 family. The P. marinus FAE1-like elongating activity is sensitive to the herbicide flufenacet, similar to some higher plant 3-ketoacyl-CoA synthases, but unable to rescue the yeast elo2 elo3Δ mutant consistent with a role in the elongation of polyunsaturated fatty acids. P. marinus represents a key organism in the taxonomic separation of the single-celled eukaryotes collectively known as the alveolates, and our data imply a lineage in which ancestral acquisition of plant-like genes, such as FAE1-like 3-ketoacyl-CoA synthases, occurred via endosymbiosis.

Although attempts have been made to identify and breed Dermo-resistant strains of C. virginica, the basis on which P. marinus is such an effective and prevalent pathogen of the eastern oyster is unclear (3). One unusual aspect of P. marinus is its use of the so-called alternative Δ8 pathway to synthesize long chain polyunsaturated fatty acids (LC-PUFAs), such as arachidonic acid (ARA) (20:4 5,8,11,14n-6 (4, 5), which are well known precursors of the eicosanoid class of signaling molecules (such as prostaglandins, thromboxanes, and leukotrienes) (5). It has been hypothesized that fatty acids such as ARA play a key role in the free-living (merotent) infectious stage of the P. marinus lifecycle (6). There is ongoing debate as to the prevalence of the alternative Δ8 pathway in which 18:2n-6 and 18:3n-3 are elongated to generate C20 fatty acid substrates for a specific Δ8 desaturase (from which the pathway derives its name) followed by Δ5 desaturation. This Δ8 pathway therefore differs from the predominant conventional pathway found in the vast majority of LC-PUFA-synthesizing organisms, where 18:2n-6 and 18:3n-3 undergo Δ6 desaturation followed by elongation prior to Δ5 desaturation (7). Currently, only three unrelated unicellular organisms have been demonstrated to contain genes encoding the alternative Δ8 pathway (Euglena gracilis, Acanthamoeba castellanii, and Isochrysis galbana) (8). Metabolic evidence for this Δ8 pathway in mammals, in particular diseased or cancerous cells, has been presented but also disputed (reviewed in Ref. 10). More recently, the presence of the alternative pathway in P. marinus has also been demonstrated biochemically with the free-living merotent stage of this protozoon synthesizing arachidonic acid from the C18 precursor oleic acid via elongation of 18:2n-6 (11). In addition to its commercial significance as a pathogen of shellfish, P. marinus represents a key species in the taxonomic division of the alveolates. In some taxonomies, P. marinus has been accorded its own branch within the alveolates (the perkinsozoa) (12), but a sublineage more closely related to dinoflagellates has also been proposed on the basis of multiple molecular phylogenies (13). However, the evolutionary path of this organism is still unclear and the basis of ongoing debate.

A number of recent studies have attempted to produce LC-PUFAs such as ARA in transgenic plants with the aim of providing an alternative, sustainable source of these important fatty acids (14, 15). In particular, the use of the alternative Δ8

* This work was supported by a grant-in-aid from the Biotechnology and Biological Sciences Research Council (UK). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank/EBI Data Bank with accession number(s) DQ508730, DQ508731, and DQ508732.

1 Partially supported by the European Union FP6 Project LIPGENE (FOOD-CT-2003-505944).

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pathway appears to represent a successful approach to bypassing some of the endogenous bottlenecks that constrain the high level production of LC-PUFAs (14, 16). With this in mind, we considered that the LC-PUFA biosynthetic pathway of *P. marinus* might provide additional insights into this process. Here we report the identification of an entirely new configuration of the alternative Δ8 pathway, which represents an additional level of diversification in known examples of LC-PUFA biosynthetic pathways.

**EXPERIMENTAL PROCEDURES**

**Growth and Harvesting of *P. marinus***—*P. marinus* cells were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). *P. marinus* meronts were grown in ATCC Medium 1886 and prepared as described by the manufacturer. Meronts were inoculated (~1 × 10⁶ ml⁻¹) and cultivated in 10-ml aliquots of medium in T-10 tissue culture flasks at 25 °C. Meronts at exponential growth phase (7 or 9 days old) were harvested by centrifugation at 200 × g for 5 min.

**Nucleic Acid Manipulation and PCR-based Cloning**—Total RNA was extracted from cells using an RNeasy mini kit (Qiagen). First strand cDNA was synthesized from total RNA using the SMART RACE cDNA amplification kit (BD Biosciences) according to the manufacturer’s instructions and used for PCR-based cloning. Single-stranded cDNAs were amplified with primers specific to each desaturase gene as follows. The reactions were heated to 95 °C for 2 min followed by 30 cycles at 94 °C for 30 s, 30 s at temperatures ranging from 55 to 72 °C according to the primer design and 72 °C for 2 min, and then a single step at 72 °C for 10 min. PCR amplification products were cloned into TOPO vector (Invitrogen) and verified by sequencing. cDNAs were amplified with specific primers to synthesize full-length copies of the putative desaturases. Gene-specific primers were designed to the 5'- and 3'- ends of the coding regions of the corresponding nucleotide sequences with restriction sites to facilitate cloning into the yeast expression vector. Forward primers for cloning into yeast expression vector pYES2 (Invitrogen) were designed to contain a G/A at position −3 and a G at position +4 to improve translation initiation in eukaryotic cells.

**Sequence of PCR Oligonucleotides Used in This Work**—The following gene-specific primers were used: Elo2For (5'-ATGC-AAGTTCCCGGAGCATCACTCC-3'), Elo2Rev (5'-GTTCGGCATCAATATTTGCACTACC1ACCC-3'), Des1Afor (5'-ATGACTCTCTACAACCTACTGTCGA-3'), Des1Arev (5'-CTTTAGGATGTGGAAGGTACGAC-3'), Des2For (5'-AGAACTCTCTTACCC1ACCTACGAGG-3'), and PemaDes2-Rev (5'-CATTTCCACTATGGAACAGCAGCAGCA-3'). The following yeast expression primers were used (restriction sites are shown in bold): KpnElo2For (5'-TTGGTACCATGAGGATT-TCCGGGAG-3'), SacElo1Rev (5'-GGATCTCTTACCGGAT-CATTATTTGACAC-3'), KpnDes1For (5'-CCCGTAC-TGCTACTTCAACACTACTGTCG-3'), SacDes1Rev (5'-GGAGCTCTCTTACCTAGCAAGAATCTCTGTAGTG-TACGC-3'), KpnDes2For (5'-CCGTACGGTACCTTACGCACGACTGTCG-3'), and SacDes2Rev (5'-GGAGGCGCTCCTTACCTACGAGG-3').

**Flufenacet Inhibition of Elongation in Yeast**—Flufenacet (20–500 μM) (Sigma-Aldrich) was added to 25 ml of yeast culture transformed with pYES2PmFAE at the time of induction. After 48 h of incubation time, the yeast cells were processed as described above, and fatty acid composition was compared with cell-expressing PmFAE grown in the absence of herbicide. Data presented represent the mean of three independent experiments with two technical replicates for each concentration tested. Inhibition of the herbicide was expressed at percentage of inhibition of the elongation of C₁₈ Δ9 desaturated substrate compared with the control treatment.

**Yeast Mutant Complementation**—The *P. marinus* FAE ORF was cloned into pADH for expression and complementation assays. This construct was introduced in elo2Δ/elo3Δ yeast strain TDY7005. Rescue of the mutant was carried out as described previously (17).

**Fatty Acid Analysis**—Fatty acids were extracted and methylated using standard protocols. Total fatty acids were gas chromatographed using methyl ester derivatives.

**RESULTS AND DISCUSSION**

The genome sequence of *P. marinus* was examined for the presence of genes likely to encode LC-PUFA biosynthetic activities. Searches of the shotgun-sequenced unassembled reads were carried out via the WU-BLAST2 tool on The Institute for Genomic Research (TIGR) *P. marinus* genome site using *N*-terminal cytochrome *b₅* fusion desaturases containing *Pfam* domains 00173 and 00487 and ELO/SUR4-elongating activity (containing *Pfam* domain 01151) as templates. These two components (desaturase, ELO-like elongating activity) are absolutely conserved in all known examples of the aerobic PUFAs biosynthetic pathway (14, 18). This search identified one
FIGURE 1. Phylogenetic relationship of *P. marinus* desaturases. A, sequence comparison of *P. marinus* cytochrome *b*_{5} fusion desaturases (designated PmD8, PmD5) present on genomic clone 104730867. The three conserved histidine motifs are boxed, the conserved heme-binding motif (His-Pro-Gly-Gly) of the cytochrome *b*_{5} fusion domain is underlined. B, phylogenetic tree of front-end desaturases, including *P. marinus* sequences PmD8 and PmD5 (bold italics) compared with known examples of alternative pathway Δ8 desaturases (bold) and other protozoa (31) (*L. major*, asterisk and italics). Sequence notation is fully described in Refs. 9 and 30 using an abbreviation of the binomial name (e.g. *A. castellanii* Δ8 desaturase = AcD8). The clustering of higher plant *b*_{5} fusion desaturases is shown.
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genomic association with the two known examples of the alternative Δ8 pathway C20 Δ8 desaturases from *E. gracilis* and *A. castellanii* (9) (Fig. 1B).

Further analysis of the sequence of clone 1047306867 identified one additional ORF also in close proximity (150 bp upstream) to these two desaturase-like genes. This ORF showed similarity to condensing enzymes involved in the biosynthesis of long chain fatty acids, indicating the genomic clustering of the two distinct classes of ORFs involved in this biochemical process (desaturases, elongating activity) and their organization in a distinct linear manner (illustrated in Fig. 2A). Perhaps more surprisingly, this putative condensing enzyme was not a member of the ELO/SUR4 family normally associated with this pathway but rather a member of the FAE1-like (22) class of 3-koacyl-CoA synthase (KCS) activities (Pfam cd00831.2), which until now have only been functionally described in higher plants and photosynthetic algae (and as such, have been assumed to be specific to the plant kingdom) (23). The FAE1 gene of *Arabidopsis thaliana* has been demonstrated biochemically to be a

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**FIGURE 2. Genomic organization and relationship of *P. marinus* FAE1-like KCS.** *A*, diagrammatic representation of the clustering of *P. marinus* genes involved in PUFA biosynthesis. Distance between the KCS and desaturase 1 (PmD8) is 150 bp, and 117 bp between desaturase 1 and desaturase 2 (PmD5). No other genes are predicted to be present on this 18-kb fragment. *B*, phylogenetic tree of FAE-like KCS-condensing enzymes. Munich Information Centre for Protein Sequences codes for the 21 Arabidopsis FAEs are given; other designations are GeneDb accessions. The *P. marinus* FAE is in *bold italics*, *E. histolytica*, *D. discoideum* and *P. tricornutum* (AY746358) FAE1-like ORFs are in *italics*. Two relevant Arabidopsis KCSs (FAE1 and CUT1) are annotated (see Fig. 5).

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genomic fragment (TIGR designation 1047306867) of 18 kb, which reported significant similarity to N-terminal cytochrome $b_5$ fusion desaturases associated with LC-PUFA biosynthesis. Closer inspection of the deduced amino acid sequence of this particular clone in fact revealed the presence of two cytochrome $b_5$ fusion desaturases in close proximity to one another. The deduced ORFs of these two putative LC-PUFA desaturases were separated by 117 bp, and neither gene appeared to contain introns. Although clearly related to each other, the two putative desaturases were only 29% identical, 40% similar (Fig. 1A). However, they both contain the diagnostic histidine boxes associated with LC-PUFA desaturases (including the variant Gln→His substitution in the third box) as well as an N-terminal cytochrome $b_5$ domain containing the highly conserved heme binding motif His-Pro-Gly-Gly (Fig. 1A) (19). These two ORFs were placed in a phylogenetic tree of known cytochrome $b_5$ fusion desaturases as a tool to infer biochemical function (Fig. 1B). This failed to reveal any obvious lineage but grouped the two *P. marinus* cytochrome $b_5$ fusion desaturases with Δ5 and Δ6 desaturases from the parasitic protozoa *Leishmania major* (20) and the algae *Thraustochytrium* sp. and *Ostreococcus tauri* (21). In contrast, the higher plant cytochrome $b_5$ fusion desaturases clearly form a distinct branch (19). As can also be seen, neither of the *P. marinus* desaturases showed any close phylo-

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To confirm the predicted function of the *P. marinus* ORFs in the biosynthesis of arachidonic acid, the individual coding sequences for the three genes were cloned into yeast expression vectors. As shown in Fig. 4, this functional characterization in yeast demonstrated LC-PUFA biosynthetic activities for these *P. marinus* ORFs. The first ORF, the FAE1-like KCS, was shown to encode an elongating activity for Δ9-desaturated C18 fatty acids, converting 18:2 9,12-Δ6 and 18:3 9,12,15Δ-3 to yield the desaturation products (data not shown). The second cytochrome *b* fusion desaturase PmD8, which lies adjacent to the 3′ end of the PmFAE gene, was shown to encode a C20 Δ8 desaturase, the activity associated with the alternative pathway of PUFA biosynthesis. This enzyme utilized 20:2 11,14Δ-6 and 20:3 11,14,17Δ-3 to yield the desaturation products 20:3 8,11,14Δ-6 and 20:4 8,11,14,17Δ-3. When this desaturase was co-expressed with PmFAE (generating the n-3 C20 substrate *in vivo*), this latter fatty acid (20:4Δ-3) was observed (Fig. 4C) and its identity confirmed by gas chromatography/mass spectrometry (data not shown). The second cytochrome *b* fusion desaturase PmD5 was identified as a C20 Δ5 desaturase, (Fig. 4D) responsible for the conversion of the C20 products of the Δ8 desaturase to ARA (n-6) and eicosapentaenoic acid (20:5 5,8,11,14,17Δ-3; eicosapentaenoic acid), respectively (the latter product is shown in Fig. 4B). Such activity represents the first reaction in the alternative Δ8 pathway for PUFA biosynthesis (also detailed in Fig. 4), but the only previously identified C18 Δ9-elongating activity was the structurally unrelated ELO/SUR4-like ORF from *I. galbana* (26). The *P. marinus* C18 Δ9-elongating activity (hereafter designated PmFAE) was tested with other substrates such as γ-linolenic acid and stearidonic acid but found to have no activity toward C18 Δ6-desaturated fatty acids. The first cytochrome *b* fusion desaturase PmD8, which lies adjacent to the 3′ end of the PmFAE gene, was shown to encode a C20 Δ8 desaturase, the activity associated with the alternative pathway of PUFA biosynthesis. This enzyme utilized 20:2 11,14Δ-6 and 20:3 11,14,17Δ-3 to yield the desaturation products 20:3 8,11,14Δ-6 and 20:4 8,11,14,17Δ-3. When this desaturase was co-expressed with PmFAE (generating the n-3 C20 substrate *in vivo*), this latter fatty acid (20:4Δ-3) was observed (Fig. 4C) and its identity confirmed by gas chromatography/mass spectrometry (data not shown). The second cytochrome *b* fusion desaturase PmD5 was identified as a C20 Δ5 desaturase, (Fig. 4D) responsible for the conversion of the C20 products of the Δ8 desaturase to ARA (n-6) and eicosapentaenoic acid (20:5 5,8,11,14,17Δ-3; eicosapentaenoic acid), respectively (the latter product is shown in Fig. 4D). Fig. 4D not only confirms the activity of PmD5 as a C20 Δ5 desaturase but also demonstrates that co-expression of all three *P. marinus* ORFs (PmFAE, PmD8, and PmD5) resulted in the successful heterologous reconstitution of the alternative LC-

such as trypanosomes (25). No information exists regarding *P. marinus* transcriptional systems, although the proximity of the three genes makes such a process likely. Therefore, two primer pairs were designed to (a) the 3′ end of the FAE1-like ORF and 5′ end of the first desaturase ORF and (b) the 3′ end of the first desaturase ORF and the 5′ end of the second desaturase ORF (Fig. 3). Thus, if these three genes were co-transcribed as a single transcript, it would be possible to generate two discrete PCR products of (461 and 424 bp). As shown in Fig. 3, such products were amplified with the identity of these PCR products confirmed by cloning and sequencing. In view of the possibility that these PCR products were derived from genomic DNA still present in the cDNA template used for amplification, control primers were designed to another (uncharacterized) FAE-like gene we identified on *P. marinus* TIGR clone 22547 (hence designated FAE225a). This FAE-like gene contained three small introns (totaling ~185 bp) and allowed us to determine whether our *P. marinus* cDNA contained any gDNA. cDNA derived from total RNA treated with DNaseI only yielded a control product of ~315 bp (Fig. 3), whereas cDNA that was derived from untreated RNA also contained a higher product of 498 bp (data not shown). Because the data presented in Fig. 3 clearly show amplification of PCR products with primers spanning the three cluster genes as well as the absence of any contaminating gDNA (as judged by the amplification of only spliced FAE225a), we believe this provides good evidence that this *P. marinus* gene cluster is indeed co-transcribed.

![Genomic organization of *P. marinus* PUFA biosynthetic ORFs.](image)

**FIGURE 3.** Genomic organization of *P. marinus* PUFA biosynthetic ORFs. Co-transcription of the three *P. marinus* was defined by cDNA PCR. Total RNA was treated with DNaseI and then used to synthesize cDNA. Primers designed to the regions indicated (Faefor, D8rev, D8for, D5rev) amplified two discrete PCR products of the predicted size. A control primer pair that recognized a region of a second FAE1-like gene (FAE225a), which contained three small introns, was used to confirm the absence of contaminating gDNA; a cDNA-derived product was ~315 bp in size compared with the predicted gDNA product of 498 bp. DNA size markers are shown.
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PUFA biosynthetic Δ8 pathway. The substrate preference of these three activities was also tested by individual expression of these activities in yeast. As can be seen in Table 1, although PmFAE and PmD8 showed a slight preference for \( n-3 \) substrates, PmD5 showed preference for \( n-6 \) substrates.

Based on the above results, the three clustered \( P. marinus \) genes encode all of the primary biosynthetic activities required for the synthesis of ARA and eicosapentaenoic acid via the alternative Δ8 pathway. Because these three genes are co-transcribed (Fig. 3), they represent a PUFA biosynthetic operon. Moreover, the three genes are structurally positioned in the same order as their encoded biochemical reactions proceed in the alternative Δ8 pathway (i.e. elongation, Δ8 desaturation, and Δ5 desaturation); however, whether this represents some aspect of selection pressure is currently unclear.

The functional identification and characterization of this \( P. marinus \) PUFA biosynthetic operon raises a number of intriguing points. First, the involvement of a FAE1-like KCS in LC-PUFA biosynthesis is remarkable, given the taxonomic distribution of FAE1-like orthologs. Moreover, these FAE1-like genes normally found only in photosynthetic organisms are required for the synthesis of saturated and monounsaturated long chain fatty acids primarily used in storage lipids and cuticular waxes (27), with no previously characterized examples shown to be involved in the elongation of polyunsaturated fatty acids (23). This is therefore the first observation of the aerobic biosynthesis of LC-PUFAs using a FAE1-like KCS-elongating activity rather than the ELO/SUR4 system. Second, we confirmed the presence of the alternative Δ8 pathway for the biosynthesis of LC-PUFAs in \( P. marinus \). This represents a new variant form of this pathway because of the presence of the higher plant-like PmFAE. This also implies the independent evolution of the alternative Δ8 pathway in the few organisms that contain it, because the only other example of this activity is an ELO/SUR4 ortholog from \( I. galbana \) (26). Therefore, the \( P. marinus \) pathway represents a new configuration of PUFA biosynthetic activities. Interestingly, the two cytochrome \( b_5 \) fusion desaturases present in the \( P. marinus \) PUFA operon show only very low levels of similarity with each other (Fig. 1). However, the lack of introns in these two genes precludes any additional insights into the evolution of these two activities, as does their lack of any obvious promoter sequences.

Equally intriguing is the genomic organization of the \( P. marinus \) alternative pathway genes as a structured operon, which could indicate the co-evolution of all three genes. The presence of the FAE1-like KCS points toward an ancestral gene transfer from a photosynthetic alga, also implied by the phylogenetic association with the \( P. tricornutum \) FAE. Whether such a genetic exchange would involve the nuclear transfer of the entire operon or just the
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TABLE 1
Percentage of substrate converted by P. marinus enzymes, expressed in yeast (mol % of total fatty acids)

Exogenous substrates (500 μM) were supplied to yeast cultures induced for expression of pYES-ORFs by the addition of galactose. Total fatty acids were methylated and analyzed by gas chromatography. The preferential substrate (n-3 versus n-6) is shown in bold.

| Substrate     | 18:2n-6 | 18:3n-3 | 20:2n-6 | 20:3n-3 | 20:3n-6 | 20:4n-3 |
|---------------|---------|---------|---------|---------|---------|---------|
| PemaFAE       | 4.1 ± 0.5 | 6.0 ± 0 |         |         |         |         |
| PemaD8        | 11.5 ± 0  | 12.4 ± 0 |         |         |         |         |
| PemaD5        | 16.81 ± 0.1 | 10.9 ± 0.5 |         |         |         |         |

FAE1-like ORF is unknown, although this particular gene has significantly diverged in function from other FAE1-like condensing enzymes. Because this is the first description of all three activities of the alternative Δ8 pathway from the same organism, further speculation is not possible. P. marinus has been reported to contain vestigial plastid-like structures (implying previous endosymbiosis), and its lineage has been closely linked with dinoflagellates (13). In that respect, we have also intrinsically identified several additional FAE1-like KCS genes in the recently released genome sequence of the draft P. marinus genome sequence (e.g. TIGR clone 22547 contains two FAE-like genes). Equally, we have also detected candidate genes for ELO/SUR4-type elongating activities (e.g. TIGR P. marinus clone 7619 that contains two ELO-like genes). It is interesting to note that, although both the FAE1-like and ELO/SUR4 genes are present as tandem pairs, the genes are ~1.5 kb apart in both cases, making it less likely they are co-transcribed. The function of these various elongating activities has not yet been determined. We have also surveyed the genomic organization of a number of other P. marinus candidate genes for fatty acid metabolic activities and did not identify any more examples of co-clustering of different genes.

Further evidence for the unique nature of the P. marinus FAE1-like elongating activity was provided by the inability of PmFAE to complement yeast mutants lacking the ELO2p- and ELO3p-elongating activities and therefore defective in the synthesis of saturated very long chain fatty acids present in sphingolipids. It has been shown that higher plant FAE1-like KCS ORFs can rescue the otherwise lethal yeast elo2Δ/elo3Δ double mutant, even though the two enzymes are structurally unrelated (17). However, as can be seen in Fig. 5, the PmFAE was unable to substitute for either ELO2 or ELO3, in contrast to two Arabidopsis genes At2g16280 (CUT1) and At4g34520 (FAE1), both involved in the elongation of C18-saturated and non-monounsaturated fatty acids. In that respect, the PmFAE Δ9-elongating activity behaves in a similar manner as that observed for the ELO/SUR4-like PUFA-elongating activity from Caenorhabditis elegans PEA1 (7), which also fails to rescue the elo2Δ/elo3Δ double mutant (Fig. 5). This indicates that the primary function of the P. marinus FAE is likely to be the biosynthesis of LC-PUFAs as opposed to sphingolipids and highlights its functional divergence from higher plant FAE-like genes.

The observation of PUFA biosynthesis catalyzed by a FAE1-like KCS not only indicated the presence of a plant-like elongation system in P. marinus but also suggested a possible target for chemical inhibition. Recent studies on the FAE1-like KCSs of Arabidopsis have shown that the K3 class of anilide herbicides can act as potent inhibitors of the very long chain fatty acid elongation reactions catalyzed by these condensing enzymes (29). Expression in yeast of several Arabidopsis FAE1-like KCS activities were inhibited by K3 herbicides, such as flufenacet (N-(4-fluorophenyl)-N-(1-methylethyl)-2-(5-fluorooromethyl-1,3,4-thiadiazol-2-yl oxy)acetamide), although this compound had no effect on the endogenous yeast ELO/SUR4 elongases (30). We therefore examined the sensitivity of PmFAE to flufenacet using similar methods, examining the
inhibition of elongation of substrate fatty acids such as 18:2 9,12 and 18:3 9,12,15. In view of the divergent nature of PmFAE (both in terms of function and primary sequence), it was not obvious whether the effect of anilide herbicides on this prostet enzyme would be similar to that observed for the higher plant enzymes. However, application of flufenacet (in the range 50–500 μM) to yeast cultures expressing PmFAE resulted in a strong inhibition of the C18 Δ9-elongating activity (76% inhibition at 50 μM, max inhibition 89% at 500 μM). Based on these data, it may also be likely that flufenacet would inhibit the FAE1-like elongating activities present in E. histolytica and D. discoideum. In the case of P. marinus, it may be possible to use K3 anilide herbicides to control their infection of oysters, as the toxicity of compounds such as flufenacet, although severe toward terrestrial and aquatic plants, shows much reduced (several orders of magnitude) toxicity to non-photosynthetic organisms including the eastern oyster (see www.epa.gov/opprd001/factsheets/flufenacet.pdf). An analogous approach to controlling P. marinus has recently been proposed using the type II fatty acid synthase inhibitor triclosan (31). Alternatively, the knowledge that flufenacet disrupts the P. marinus FAE elongase, and by implication the synthesis of ARA, may serve as the basis for chemical intervention via anilide herbicides. Given the ecological importance of oyster population via Dermo infection (1, 3), these approaches may prove important in dealing with both direct and consequence problems associated with this disease.

As mentioned above, the alternative Δ8 pathway has been shown to function efficiently in transgenic plants (9, 14, 16). However, these previous studies have utilized the ELO/SUR4 Δ9-elongating activity from I. galbana (14, 16, 26). The use of the P. marinus FAE1-like Δ9-elongating activity may represent an additional approach with which to enhance the accumulation of LC-PUFAs in transgenic plants.

In conclusion, we have identified a PUFA biosynthetic operon that encodes for all of the primary enzymatic components of the alternative Δ8 pathway. Unusually for a PUFA biosynthetic pathway, this operon contains an elongating activity that belongs to the FAE1-like class of higher plant 3-ketoacyl-CoA synthases. Our data therefore imply that the alternative Δ8 pathway has independently evolved on at least two occasions and, in the case of P. marinus, may have involved the acquisition of the plant-like FAE1 activity by horizontal gene transfer (presumably through endosymbiosis of a photosynthetic alga). The data presented here also provide new insights into the metabolism of P. marinus, including possible targets for chemical intervention via anilide herbicides. Given the ecological problems such as increased algal blooms associated with loss of oyster population via Dermo infection (1, 3), these approaches may prove important in dealing with both direct and consequence problems associated with this disease.

Acknowledgments—We thank John Pickett for critical reading of the manuscript and BASF Plant Sciences (Limburgerhof, Germany) for support. We thank Teresa Dunn for generously providing yeast strains used in this study. Preliminary sequence data were obtained from The Institute for Genomic Research. Sequencing of P. marinus was accomplished with support from National Science Foundation.

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