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Identification and functional characterisation of genes encoding the omega-3 polyunsaturated fatty acid biosynthetic pathway from the coccolithophore *Emiliania huxleyi*

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ABSTRACT

The Prymnesiophyceae coccolithophore *Emiliania huxleyi* is one of the most abundant alga in our oceans and therefore plays a central role in marine foodwebs. *E. huxleyi* is notable for the synthesis and accumulation of the omega-3 long chain polyunsaturated fatty acid docosahexaenoic acid (DHA; $22{:}6\Delta^{4,7,10,13,16,19}$, $n-3$) which is accumulated in fish oils and known to have health-beneficial properties to humans, preventing cardiovascular disease and related pathologies. Here we describe the identification and functional characterisation of the five *E. huxleyi* genes which direct the synthesis of docosahexaenoic acid in this alga. Surprisingly, *E. huxleyi* does not use the conventional Δ6-pathway, instead using the alternative Δ8-desaturation route which has previously only been observed in a few unrelated microorganisms. Given that *E. huxleyi* accumulates significant levels of the Δ6-desaturated fatty acid stearidonic acid ($18{:}4\Delta^{6,9,12,15}$, $n-3$), we infer that the biosynthesis of DHA is likely to be metabolically compartmentalised from the synthesis of stearidonic acid.

1. Introduction

The Prymnesiophyceae phytoplankton *Emiliania huxleyi* is a member of the coccolithophore family, most notable for their ornate calcium carbonate extracellular platelets known as coccoliths. *E. huxleyi* is a cosmopolitan oceanic species and is almost certainly one of the most abundant organisms present in our seas (Iglesias-Rodriguez et al., 2008). *E. huxleyi* also forms blooms, the largest of which occur in the North Atlantic; remarkably, the refractive properties of the coccoliths allows these blooms to be visualised remotely from orbiting satellites (Smyth et al., 2002). *E. huxleyi* is known to synthesise and accumulate omega-3 long chain polyunsaturated fatty acids (LC-PUFAs) such as eicosapentaenoic acid (EPA; $20{:}5\Delta^{5,8,11,14,17}$, $n-3$) and docosahexaenoic acid (DHA; $22{:}6\Delta^{4,7,10,13,16,19}$, $n-3$; abbreviated to DHA) (Bell and Pond, 1996) which are health-beneficial compounds found in marine foodwebs (Williams and Burdge, 2006). There is now good evidence of the importance of EPA and DHA in the human diet, with both these fatty acids playing a role in preventing cardiovascular disease and associated precursor states such as metabolic syndrome (Riediger et al., 2009). Given its global abundance, it is likely that *E. huxleyi* is a significant source of omega-3 LC-PUFA biosynthesis in the marine environment.

In view of the utility and value of omega-3 LC-PUFAs, there is considerable interest in understanding the biosynthesis of these fatty acids (Sayanova and Napier, 2004; Domergue et al., 2005a,b). Work over the last decade by ourselves and others have identified two archetypal enzyme activities which underpin the aerobic synthesis of EPA and DHA, namely the so-called "front-end" cytochrome b5-fusion desaturases (which insert double bonds between the carboxyl ground and pre-existing unsaturations) and polyunsaturated fatty acid-specific elongases (which condense acyl-substrates with malonyl-CoA to chain-elongate the fatty acid by two carbons) (reviewed in Venegas-Calerón et al., 2010). Examples of desaturases and elongases involved in the synthesis of omega-3 LC-PUFAs have been cloned and functionally characterised from a range of organisms including diatoms, oomycetes, fungi, mosses and animals (Domergue et al., 2002, 2003; Pereira et al., 2004; Tonon et al., 2005). The predominant sequence of enzymatic reactions required to convert C_{18} fatty acids to C_{20}+PUFAs commences with the introduction of a double bond at the Δ6-position, followed by C2-chain elongation and a second desaturation at the Δ5 position in the C_{20} acyl chain, generating EPA from α-linolenic acid (ALA; $18{:}3\Delta^{9,12,15}$, $n-3$) and

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arachidonic acid (ARA, 20:4$\Delta^{5,8,11,14}$, $n-3$) from linoleic acid (LA; 18:2$\Delta^{9,12}$, $n-6$). A number of examples of an alternative configuration of this pathway have been observed, in which ALA first undergoes C_2-elongation prior to two sequential desaturations (at the Δ8, then Δ5 positions), with this pathway reported in *Euglena gracilis* (Wallis and Browse, 1999), *Isochrysis galbana* (Qi et al., 2002), *Pavlova salina* (Zhou et al., 2007; Robert et al., 2009) and *Perkinsus marinus* (Venegas-Calerón et al., 2007). The evolutionary significance of this alternative configuration is unclear, but given the sequence similarity between the various desaturases (Δ6, Δ8, Δ5) it is likely that they have evolved from a common ancestor. Conversely, this similarity means that, in the absence of functional characterisation, it is not possible to infer (on the basis of deduced amino acid sequence) which pathway is present in any particular complete genome sequence. Equally, the conversion of EPA to the highly-unsaturated fatty acid DHA is also carried out by related desaturases and elongases, again complicating the annotation of predicted genes.

We examined the genome sequence of *E. huxleyi* as a potential source of genes encoding the biosynthesis of omega-3 LC-PUFAs. Previous studies have reported the accumulation of significant levels of DHA and the unusual highly unsaturated omega-3 C_{18} PUFA octadecapentaenoic acid (OPA; 18:5$\Delta^{3,6,9,12,15}$, $n-3$) in this alga (Bell and Pond, 1996), and preliminary examination of partially assembled genome scaffolds indicated the presence of sequences with similarity to cytochrome b_5-fusion desaturases and elongases associated with omega-3 LC-PUFA biosynthesis. Here, we describe the systematic identification and characterisation of this pathway in *E. huxleyi*.

2. Results

2.1. Fatty acid and acyl-CoA composition of E. huxleyi

Previous reports have indicated the presence of significant levels of omega-3 LC-PUFAs in *E. huxleyi* (Bell and Pond, 1996; Evans et al., 2009). To confirm these observations, we analysed by GC–FID and GC–MS the fatty acid methyl esters of total lipids from an actively growing culture of *E. huxleyi* (CCMP1516). As can be seen in Fig. 1A, significant levels of DHA were detected, similar to that reported by Bell and Pond (1996). The most abundant fatty acid was OPA, followed by myristic acid (14:0), DHA and stearidonic acid (SDA; 18:4$\Delta^{6,9,12,15}$, $n-3$). Thus, these data confirmed the presence of omega-3 LC-PUFAs in *E. huxleyi*, and also were indicative (on the basis of discrete desaturation products) of the presence of an aerobic pathway (Lippmeier et al., 2009). To gain further evidence for such a biosynthetic route, and to provide additional insights into the metabolic channelling of acyl-intermediates in *E. huxleyi*, we used the highly sensitive HPLC method of Larson and Graham (2001) to measure the acyl-CoA pool of this alga. As shown in Fig. 1B, the total acyl-CoA pool of *E. huxleyi* indicates the presence of multiple different fatty acids linked to CoA, again indicative of the sequential biosynthesis of omega-3 LC-PUFAs via aerobic desaturation and elongation. Significant levels of C_{18} acyl-CoAs were detected, with the levels of C20/C22 PUFA-CoAs notably lower. Perhaps of most interest was the absence of OPA from the acyl-CoA pool, even though other unsaturated C_{18} fatty acids such as ALA and SDA were present.

2.2. Identification of candidate E. huxleyi genes for omega-3 LC-PUFA biosynthesis

The genome sequence of *Emiliania huxleyi* strain CCMP1516 generated by JGI (http://genome.jgi-psf.org/Emihu1/Emihu1.home.html) was searched with previously functionally characterised sequences (desaturases, elongases) known to be involved in the biosynthesis of omega-3 LC-PUFAs. The current release (v1.0) of the *E. huxleyi* genome comprises 167.7 Mb of DNA sequence predicted to contain 39,126 genes. The top-scoring predicted *E. huxleyi* open reading frames are listed in Table 1 for the desaturases and elongases required for the synthesis of DHA. Most notably, the presence of a clear ortholog (*E. huxleyi* protein ID #433098) of an *Isochrysis galbana* C_{18} elongase (Qi et al., 2002) associated with the "alternative pathway" was detected, indicating that *E. huxleyi* might utilise this configuration of the omega-3 LC-PUFA biosynthetic pathway to generate EPA. Commensurate with this, a predicted cytochrome b_5-fusion desaturase (*E. huxleyi* protein ID #216445) showed similarity with the *Euglena* C_{20} Δ8-desaturase (Wallis and Browse, 1999), though as noted above, such similarities are ambiguous in the absence of functional characterisation. Further support for the presence of the alternative pathway in *E. huxleyi* is evidenced by the absence of any clear orthologs of the C_{18} Δ6-elongase in the genome. As will be discussed below, the presence of significant levels of SDA might be expected to be associated with the presence of a Δ6-desaturase. Examination of v1.1 release of the *E. huxleyi* genome sequence failed to identify a predicted ORF with significant similarity to known Δ6-desaturases; one predicted ORF (ID #417285) contained the diagnostic histidine box motifs associated with front-end desaturases but lacked the N-terminal cytochrome b5-domain. As shown in Table 1, nominal candidate orthologs for C20 Δ5-desaturase, C22 Δ4-desaturase and C20 Δ5-elongase activities were also detected in the genome sequence of *E. huxleyi*.

2.3. Functional expression in yeast of candidate desaturases

The predicted functions of the candidate *E. huxleyi* ORFs listed in Table 1 were investigated by expression studies in *Saccharomyces cerevisiae*. Synthetic codon-optimised genes for each ORF were chemically generated (Genscript), based on the predicted gene model(s) available on the JGI genome portal. The deduced amino acid sequences were manually queried against known desaturases and elongases, to determine the precision of automatically predicted intron/exon junctions. This was further validated by querying genomic derived predictions against *E. huxleyi* EST (cDNA) sequences. These synthetic ORFs were then cloned into the galactose–inducible yeast expression vector pYES2 (Invitrogen) and expressed in *S. cerevisiae* in the presence of a range of potential fatty acid substrates (see below for details). Total fatty acid methyl esters from these yeast cells were then analysed by GC–FID and the identity of novel peaks confirmed by GC–MS and co-migration with authentic standards. As shown in Fig. 2, expression of a synthetic ORF encoding *E. huxleyi* Protein 443389, predicted to encode a C20 Δ5-desaturase of 455 amino acids (aa), confirmed the enzymatic capability to convert exogenously supplied substrate dihomo gamma-linolenic acid (DHGLA; 20:3$\Delta^{8,11,14}$, $n-6$) to the Δ5-desaturated form ARA (Fig. 2C). In the absence of galactose, the exogenous substrate DHGLA is not converted to ARA (Fig. 2B), and neither substrate or product are present in untransformed yeast, which has a very simple FAMe profile, predominantly comprising of 16:0, 16:1, 18:0 and 18:1 (Fig. 2A). No activity was detected against exogenous LA, ALA, GLA, SDA, ARA or EPA. Thus, on the basis of these results, *E. huxleyi* Protein #443389 was designated EhDes5. It should be noted that EhDes5 also was active against 20:4$n-3$ as a substrate, generating EPA (data not shown). As can be seen in Fig. 2C, EhDes5 displays good activity in yeast, with C20 Δ5-desaturated fatty acid products accumulating to 6.5% of total fatty acids, with 50–60% of exogenous substrate conversion. In addition, the fatty acid composition for different neutral and phospholipid species of yeast cells expressing EhDes5 in the presence of exogenous DHGLA was determined (Table 2). This indicated that Δ5-desaturation products