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Modular Synthase-Encoding Gene Involved in α-Olefin Biosynthesis in *Synechococcus* sp. Strain PCC 7002▽†

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A gene involved in the production of medium-chain α-olefins was identified in the cyanobacterium *Synechococcus* sp. strain PCC 7002. The gene encodes a large multidomain protein with homology to type I polyketide synthases, suggesting a route for hydrocarbon biosynthesis from fatty acids via an elongation decarboxylation mechanism.

Biological hydrocarbons are a promising alternative to petroleum-based liquid transportation fuels as they would be compatible with current engines and distribution systems yet be sourced from renewable substrates. Although it has been known for decades that some species of bacteria, including *Synechococcus* sp. strain PCC 7002 (formerly *Agmenellum quadruplicatum*), can synthesize hydrocarbons (20), until recently, very little was known about their biosynthesis. Enzymes responsible for producing three types of hydrocarbons (alkanes, internal olefins, and α-olefins) have recently been identified. Cyanobacterial alkanes can be derived from fatty acids by decarbonylation of the corresponding aldehydes (16). Alkenes with internal double bonds can be generated from the head-to-head condensation of fatty acids; genes involved in this pathway have been described for *Micrococcus luteus* (2). Recently, a P450 fatty acid decarboxylase was reported to be involved in α-olefin biosynthesis in *Jeotgalicoccus* sp. (15). In this study, we analyzed the hydrocarbon profile of the cyanobacterium *Synechococcus* sp. PCC 7002 and demonstrated the involvement of a gene with modular organization, similar to a polyketide synthase, in the synthesis of medium-chain α-olefins. Feeding studies suggested that the putative enzyme used an elongation-decarboxylation mechanism to convert fatty acyl-acyl carrier proteins (fatty acyl-ACPs) to α-olefins.

PCC 7002 was reported to synthesize two C_{19} alkenes, but nothing was known about their structure (20). In order to characterize these compounds, cultures were grown photosynthetically (140 μE/m^2/s) at 35°C in 100 ml of medium A (18) to an optical density at 730 nm (OD_{730}) of 0.2, and cell pellets were subjected to lipid extraction and analysis by gas chromatography-mass spectrometry (GC-MS) (12). Two major peaks were observed at 14.87 min and 15.15 min (Fig. 1) whose mass spectra (see Fig. S2 in the supplemental material) were consistent with a 19:2 and a 19:1 hydrocarbon, respectively. In order to determine the position of the double bonds, the hydrocarbon mixture was derivatized with dimethyl disulfide (DMDS) (19) and analyzed by GC-MS. The spectrum for the DMDS adduct of the $C_{19:1}$ hydrocarbon showed molecular ions corresponding to a terminal double bond (Fig. S3). The compound was confirmed as 1-nonadecene by comparison with a commercial standard. The spectrum for the DMDS adduct of the $C_{19:2}$ hydrocarbon also showed ions consistent with a terminal double bond. To identify the position of the internal double bond, hydrocarbons were purified over silica gel and subjected to permanganate/periodate oxidation (8). The mass spectra of the major resulting peak were consistent with a 13-carbon dicarboxylic, dimethyl ester (Fig. S4), suggesting that the 19:2 species is 1,14-nonadecadiene, a compound previously observed in a distinct isolate of *Synechococcus* sp. (8).

Analysis of the PCC 7002 fatty acid profile indicated that the largest species were C_{18} (13). Therefore, it is unlikely that a decarboxylase like the one described for *Jeotgalicoccus* sp. is involved in the biosynthesis of the C_{19} α-olefins. Biochemical characterization of lysates from the microalgae *Botryococcus braunii* suggested that α-olefins can also be formed from fatty acids by an elongation-decarboxylation mechanism (21), resulting in α-olefins one carbon larger than the fatty acid substrate. However, the enzymes involved in this conversion remain unknown. In search for additional genes involved in α-olefin formation, we considered the biosynthesis of curacin A, a natural product isolated from the marine cyanobacterium *Lyngbya majuscula* (4) that contains a terminal double bond. Curacin A is synthesized by enzymes encoded by a 64-kb gene cluster comprising nine polyketide synthases and one nonribosomal peptide synthase module. Biochemical characterization (9) showed that the last module, encoded by *curM*, is responsible for forming the terminal double bond.

On the basis of these observations, we hypothesized that enzymes with homology to CurM could be involved in the biosynthesis of the α-olefins observed in PCC 7002. We used the basic local alignment search tool (BLAST) from NCBI to look for homologs to CurM in PCC 7002. This search identified an open reading frame encoding a protein with 45% amino acid sequence identity to CurM (SYNPCC7002_A1173, here referred to as the *ols* protein for olefin synthase). Several motif sequences commonly found in polyketide synthases were identified within *ols* (Fig. 2). Overall, *ols* encodes a protein with

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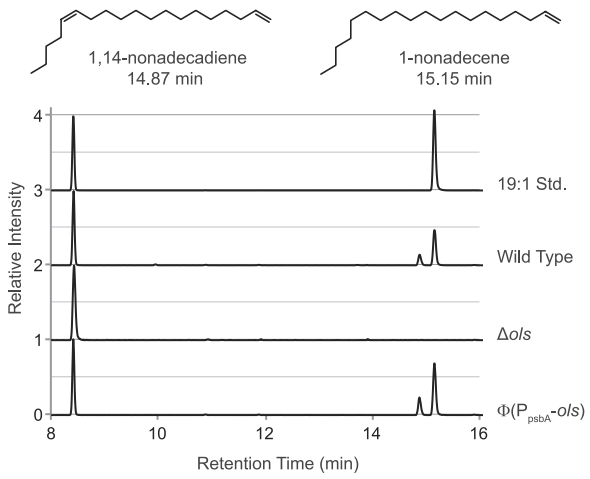


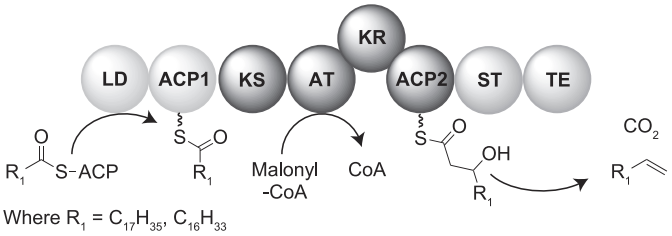
FIG. 1. Comparison of hydrocarbon extracts from the wild-type and mutant strains of PCC 7002. GC-MS signal was normalized to the height of an internal standard peak (hexadecane, 8.3 min). Two hydrocarbons, 1,14-nonadecadiene and 1-nonadecene, were identified in extracts of the wild type and the promoter replacement $\Phi(P_{psbA}$-ols) mutant but not in extracts of the Δ*ols* mutant.

C-terminal domain architecture that is highly similar to CurM, including the polyketide elongation module and terminal olefin forming domains. Unlike for CurM, the N terminus of Ols contains two additional domains, which are predicted to comprise a loading module (Fig. 2). We hypothesize that substrates are loaded onto the ACP1 domain by the ATP consuming loading domain (LD). Once loaded, the central extension module (ketosynthase [KS], acyltransferase [AT], ketoreductase [KR], ACP) would add two carbons from malonyl-coenzyme A (CoA) (a malonyl-CoA recognition motif was found with in the AT domain) (Fig. 2a) to the acyl-substrate and reduce the β-keto group to a β-hydroxyl. The presence of a sulfotransferase (ST) domain adjacent to ACP2 suggests that it activates the β-hydroxyl group via sulfation. Activation is required to drive subsequent dehydration and decarboxylation reactions that could be catalyzed by the C-terminal thioesterase (TE) domain analogous to the final reactions performed by CurM.

To confirm the involvement of *ols* in α-olefin biosynthesis, a fully segregated null mutant of the *ols* gene was made by homologous recombination of a linear DNA fragment containing a streptomycin resistance cassette flanked by 1,000 bases homologous to the regions flanking *ols* (7). Mutants were verified by PCR (Fig. 3). Table 1 gives the oligonucleotide sequences used in construction and verification. The mutant strain (Δ*ols*) was grown under conditions identical to those used for the wild-type strain and subjected to hydrocarbon analysis. Hydrocarbon extracts of Δ*ols* did not contain any detectable olefins (Fig. 1). Moreover, a deletion of only 1,000 bp at the 5′ end of the gene corresponding to the putative loading domain gave identical results (not shown). No significant differences were observed when the fatty acid profiles of the wild type and the mutant strain were compared (not shown).

To demonstrate a positive correlation between the *ols* gene and olefin production, the 250 bases immediately upstream of the *ols* coding sequence were replaced with the sequence that controls transcription of *psbA* in *Amaranthus hybridus* (6). A fully segregated mutant harboring the promoter replacement $\Phi(P_{psbA}$-*ols*) was obtained and verified by PCR (Fig. 3). Hydrocarbon extracts from cultures of the mutant strains contained significantly increased titers of each olefin (Fig. 1). A 2-fold increase in 1-nonadecene production and a 5-fold increase in 1,14-nonadecadiene were observed in cultures grown at 35°C in medium A (Table 2). mRNA was extracted from each culture using the Trizol 95 method (14). Quantitative PCR of *ols* mRNA, using primers that amplified a short 104-bp

a)



b)

LD	ACP1	KS	Domain
ATP/AMP motif (3)	Ppant binding (5)	KS motif (1, 5)	Function
YTSGTTGXPKGV-----GYGXTE	GXDSL	TVDTGCSSSLV	Consensus
YTSGSTGDPKGV-----CYGMAE	GLDSV	SIDTACSSSLV	Ols sequence
168 320	634	841	

AT	KR	ACP2	Domain
Malonyl-CoA recognition (10)	NADP(H) binding site (17)	Ppant binding (5)	Function
ETGYAQXXXXXQXAXFGLL--------GHSXG	GXGXXGXXXA	GXDSL	Consensus
ETVYTQPLLFAVEYAIAQLW--------GHSVG	GLGAIGRKIA	GMDSL	Ols sequence
1256 1287	1783	2061	

FIG. 2. (a) Domain organization and proposed mechanism of a putative olefin synthase encoded by *ols*. (b) Partial sequence alignments of Ols with consensus polyketide synthase domain motifs. Numbers correspond to the amino acid positions in Ols. References are in parentheses. Ppant, phosphopantetheine.

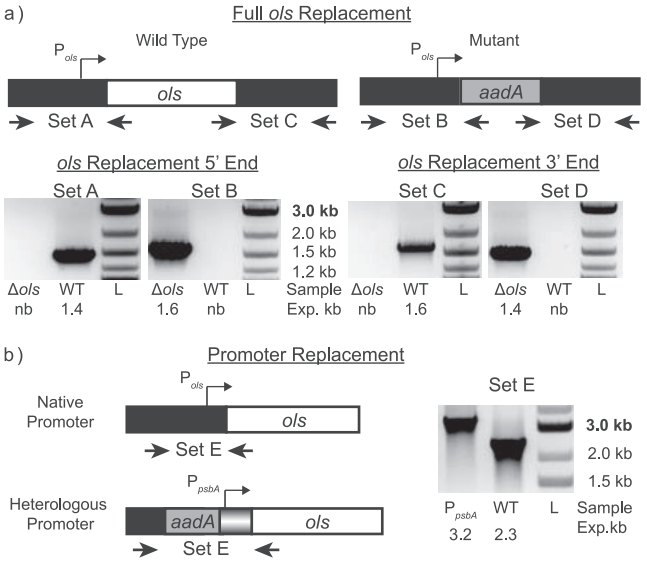


FIG. 3. Confirmation of PCC 7002 mutants by PCR. (a) Primers amplifying the 5′ and 3′ junctions of *ols* (sets A and C) and the expected integrated resistance cassette (sets B and D) were used to generate PCR products specific to each strain. Gels of each PCR confirm the replacement of *ols* with a streptomycin resistance cassette (*aadA*). (b) Primers flanking the promoter of *ols* were used to confirm the size of the genomic sequence on both the wild type and the $\Phi(P_{psbA}$-*ols*) mutant. The larger size of mutant PCR product is due to the presence of an *aadA* expression cassette positioned upstream of P_{psbA}. nb, no band; exp, expected.

segment at the 3′ end of *ols*, confirmed a 2.2-fold increase in mRNA in the promoter replacement mutant relative to the level for wild-type PCC 7002 (Table 2).

Further analysis of hydrocarbon extracts of the wild-type and mutant strains revealed trace amounts of two additional hydrocarbons (at 12.6 and 12.8 min) that also disappeared in extracts of the Δ*ols* strain and increased in extracts of the $\Phi(P_{psbA}$-*ols*) mutant (Fig. 4). We identified the latter of the two compounds as 1-octadecene by its mass spectrum and by comparison with a commercial standard. The mass spectrum of the first compound was consistent with octadecadiene, but the compound was present in insufficient quantities to confirm its structure. On the basis of the proposed mechanism for α-olefin formation, we hypothesized that heptadecanoic acid ($C_{17:0}$) might be the substrate for 1-octadecene formation. In order to test this hypothesis, we fed $C_{17:0}$ to each of the three strains (final concentration, 0.1 mM) and cultures were grown to an OD_{730} of 0.3. We observed increases in the peak areas for 1-octadecene in both the wild-type and the $\Phi(P_{psbA}$-*ols*) strains

TABLE 2. Olefin production and olefin synthase expression[a]

Description or genotype	Estimated $C_{19:2}$ concn (μg/ml/OD_{730} unit)[b]	$C_{19:1}$ concn (μg/ml/OD_{730} unit)	RNA level relative to WT level[c]
WT	0.15 ± 0.06	1.60 ± 0.24	1.00 ± 0.10
Δ*ols*	ND	ND	NA
Δ*ols*-LD[d]	ND	ND	0.07 ± 0.01
$\Phi(P_{psbA}$-*ols*)	0.75 ± 0.13	3.45 ± 0.71	2.20 ± 0.30

[a] ND, not detected; NA, not applicable. Data represent averages of results from three biological replicates ± standard deviations.
[b] Concentrations of 1,14-nonadecadiene were estimated from a dilution series of 1-nonadecene analytical standards.
[c] *ols* RNA levels, determined by quantitative PCR (qPCR), were normalized to the amount of *petB* mRNA in each sample and compared to the wild-type-PCC7002 ratio.
[d] Loading domain disruption mutant.

TABLE 1. Oligonucleotides used[a]

No.	Oligonucleotide name	Sequence
1	OFE7002-Sp-a2	CCGTTCTGCAGCCTGTGAATGGAAATTCTGGACTCCGTATCC
2	OFE7002-Sp-a1	CCAACCGGAGGTTGAAGCGGACTACA
3	OFE7002-Sp-BamHI-b2	CCGTTGGATCCGCAAAGTGCAGTCCGAAAACCCGTAAATATTAGATCC
4	OFE7002-Sp-BamHI-b1	GCCAAGTCAAAGGGTTTCTGGGCATGG
5	OFE7002-Sp-b2	CCGTTGAATTCCAAGGGACAGAAACAACGGTGACCTTGG
6	OFE7002-Sp-b1	GGGAAAACGACAACTGAGACCCACCAC
7	Prom-sw-a2	CCAGAATTCCGGAGCTTCATCCTGGGGACAATGG
8	Prom-sw-a1	GCTTTCAGCCCACCTGTTCCCAATATGC
9	Prom-sw-b1	CCAAGGTCACCGTTGTTTCTGTCCCTTG
10	Prom-Amar-b2-1	GAGACAGGATGAGGATCGTTTCGCATGGTTGGTCAATTTGCAAATTTCGTCGATCTGC
11	Prom-Amar-b2-2	CTGTTGAATAACAAGGACGGATCTGATCAAGAGACAGGATGAGGATCGTTTCGCATG
12	Prom-Amar-b2-3	GTTGACACGGGCGTATAAGACATGTTATACTGTTGAATAACAAGGACGGATCTGATCAAG
13	Prom-Amar-b2-4	CCACTGCAGGATCTCAATGAATATTGGTTGACACGGGCGTATAAGACATGTTATACTG
14	Gaz7002-Seq2-Rv	CGTTGATCGCCTTTAGCCACC
15	aadA-Rv2	GCAAGATAGCCAGATCAATGTCGATCGTG
16	Gaz7002-Seq11	CCCAAAGACCTCTCGGCGTTC
17	aadA-Fw2	GACATTCTTGCAGGTATCTTCGAGCCAGC
18	SYNPCC7002_A1173-RVT	TTATTGTGTTTTGGGTACAGG
19	petB-RVT	TTACAAAGGACCAGAAATACC
20	SYNPCC7002_A1173-RTF	TGGCATTAGCAGACGACGTTACCT
21	SYNPCC7002_A1173-RTR	TGGAGATCAGCAGGGCGGTTAAAT
22	petB-RT-Fw	GATTCGCAATGACCTTCTAC
23	petB-RT-Rv	CCAGTAATCCAAGTCAGCTC

[a] Restriction sites used in cloning are underlined. Oligonucleotides 1 to 4 were used for making the *ols* knockout cassette, oligonucleotides 1, 2, 5, and 6 for making the loading domain knockout cassette, oligonucleotides 10 to 13 for making the *Amaranthus* promoter (P_{psbA}) replacement cassette, oligonucleotides 7 to 9 and 13 for promoter replacement, oligonucleotides 14 to 17 for screening of mutant strains, and oligonucleotides 18 to 23 for quantitative reverse transcription-PCR (qRT-PCR).

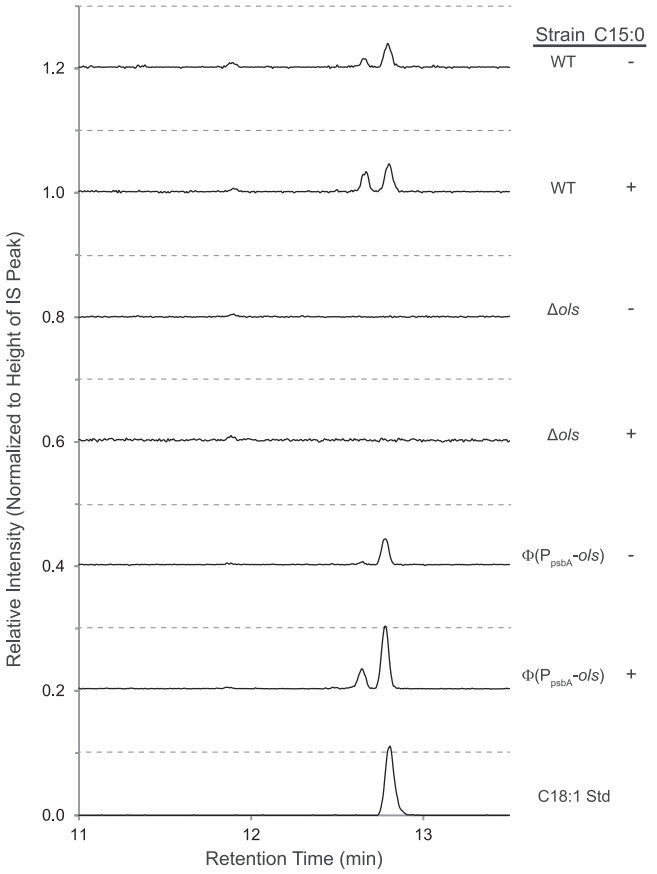


FIG. 4. Comparison of hydrocarbon extracts from wild type (WT), *ols* deletion mutant (Δ*ols*), and Φ(P_{psbA}-*ols*) strains of PCC 7002 supplemented with pentadecanoic ($C_{15:0}$) acid. The GC-MS signal was normalized to the height of an internal standard peak (hexadecane, 8.3 min). Supplementation of cultures with heptadecanoic ($C_{17:0}$) resulted in similar traces (not shown). Supplementation of odd chain fatty acids resulted in increased production of 1-octadecene ($C_{18:1}$) and a compound consistent with a doubly unsaturated 18-carbon hydrocarbon ($C_{18:2}$). No peaks corresponding to a 1-hexadecene analytical standard were observed in any of the extracts.

(not shown). These results, combined with the fact that fatty acids no larger than C_{18} have been observed in PCC 7002 (13), suggest that elongation of fatty acids is required for α-olefin formation. Interestingly, feeding of pentadecanoic acid ($C_{15:0}$) did not result in the formation of 1-hexadecene but in an increase of 1-octadecene as well (Fig. 4). PCC 7002 contains an ortholog of an acyl-ACP synthetase responsible for activation of exogenous free fatty acids (11). Therefore, it is likely that an acyl-ACP is the direct substrate of the *ols* gene product. Further *in vitro* work will be required to confirm this hypothesis.

In summary, we have identified a novel biological route for producing hydrocarbons in cyanobacteria. While the highest observed hydrocarbon titer, approximately 4.2 μg/ml/OD_{730} unit, was suboptimal for commercial production, increasing the supply of fatty acid substrates via metabolic engineering and enhancing the rate of carbon dioxide uptake may increase olefin titers. In addition, the combinatorial nature of polyketide synthesis raises the possibility of using CurM or Ols to produce a wide range of industrially relevant α-olefins. The N-terminal loading domain's substrate specificity could be altered to prefer shorter-chain-length fatty acids, thereby generating olefins with sizes similar to those of diesel constituents. Alternatively, the existing loading domain could be replaced with modules from other natural product pathways that act on short organic and amino acids to yield higher-value chemical building blocks.

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