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A Desaturase Gene Involved in the Formation of 1,14-Nonadecadiene in *Synechococcus* sp. Strain PCC 7002

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The marine cyanobacterium *Synechococcus* sp. strain PCC 7002 synthesizes two alkenes, 1-nonadecene and 1,14-nonadecadiene. Whereas the genetic basis for the biosynthesis of the terminal double bond in both alkenes has been characterized, the origin of the internal double bond in 1,14-nonadecadiene has not. In this study, we demonstrate that a gene encoding an uncharacterized desaturase is involved in the formation of the internal double bond of 1,14-nonadecadiene. Further, at low temperatures, the desaturase gene is essential for growth, and in wild-type cells the levels of 1,14-nonadecadiene increase relative to that of cells grown at 38°C. These data suggest that 1,14-nonadecadiene plays a role in responding to cold stress.

Hydrocarbon biosynthesis is a common trait among both eukaryotes and prokaryotes that has gained significant attention for use in developing sustainable alternatives to petroleum (1–3). Although it has been known for decades that cyanobacteria accumulate hydrocarbons with C_{17} to C_{20} chains (4, 5), the cellular function of these molecules and the biosynthetic pathways responsible for their production remain poorly understood. A recent survey of sequenced cyanobacteria discovered that all strains possess one, but not both, of two pathways for producing saturated or unsaturated straight-chain hydrocarbons (6), indicating that hydrocarbons play an important role in cyanobacterial physiology. The majority of strains harbor the FAR/ADO pathway that was first characterized in *Synechococcus elongatus* PCC 7942 (7). The pathway synthesizes saturated alkanes (heptadecane and pentadecane) from acyl-acyl carrier proteins (acyl-ACP) via the action of an acyl-ACP reductase and an aldehyde-deformylating oxygenase. Conversely, a smaller percentage of cyanobacteria produce long-chain alpha-olefins via the Ols pathway, first characterized in *Synechococcus* sp. strain PCC 7002 (8). The Ols pathway is comprised of a single, multidomain enzyme (Ols) possessing modular organization similar to that of a polyketide synthase. Based on the protein's domain architecture and experimental feeding studies, Ols was predicted to produce alpha-olefins via elongation and decarboxylation of C_{18} acyl-ACP, the terminal product of fatty acid biosynthesis and substrate for phospholipid synthesis (8). Unlike other cyanobacterial strains that possess a more complex hydrocarbon profile in terms of length and degree of unsaturation (4, 5, 9), *Synechococcus* sp. strain PCC 7002 synthesizes only two alkenes: 1-nonadecene ($C_{19:1}$) and 1,14-nonadecadiene ($C_{19:2}$). The only difference between these compounds is the presence of an internal double bond at position 14 of the $C_{19:2}$ hydrocarbon. Whereas the biosynthesis of the terminal double bond in both hydrocarbons was explained by the decarboxylation and dehydration reactions performed by the Ols domains, the biosynthesis of the internal double bond in 1,14-nonadecadiene was not. In this study, we linked the presence of this internal double bond to a gene predicted to encode a desaturase. Further, we demonstrated an increase in $C_{19:2}$ abundance as an inverse function of temperature. This finding suggests that the hydrocarbons play a role in responding to cold stress similar to the way unsaturated fatty acid content in the cell membrane is modulated in many bacteria (10).

MATERIALS AND METHODS

Reagents, media, and growth conditions. Enzymes and reagents were purchased from New England BioLabs or Fisher Scientific unless otherwise noted. Oligonucleotides used in this study were purchased from Integrated DNA Technologies, Inc., and are listed in Table 1. Isolation of genomic DNA and purification of DNA fragments were performed with commercial reagents (Promega, Qiagen).

A wild-type strain of *Synechococcus* sp. strain PCC 7002 obtained from the Pasteur Culture Collection was grown photoautotrophically (140 μE m^{-2} s^{-1}) on solid medium A (11) (supplemented with 1 mg/ml $NaNO_3$) agar plates or in 20-ml liquid cultures sparged with air under constant illumination from cool-white lamps at the specified temperatures. When required, cultures were supplemented with streptomycin and/or kanamycin (final concentration, 100 μg/ml).

Strain construction. Knockout mutants were constructed by homologous recombination using a linear DNA fragment containing a resistance marker flanked by ~1,000 bases homologous to the regions flanking the corresponding gene. For the construction of desaturase knockouts (Table 2), the upstream and downstream flanking sequences of the desaturase genes (*desA*, SYNPCC7002_A2756; *desB*, SYNPCC7002_A0159; *desC*, SYNPCC7002_A2198; *desE*, SYNPCC7002_A2833; *desF*, SYNPCC7002_A1989) were amplified by PCR (Phusion polymerase) from genomic DNA. PCR products were digested with BamHI and PstI and gel purified. The *aadA* gene (Sm^r) was excised from plasmid pSRA81 (12) with PstI and BamHI. The fragments were mixed in a 3:1:3 ratio (left flank:Sm^r:right flank) and ligated with T4 DNA ligase. Ligation products were gel extracted, reamplified, and transformed into *Synechococcus* sp. strain PCC 7002 as described by Frigaard et al. (12).

For the construction of the *desE*-up strain, the upstream region of *desE* was replaced with the phycocyanin (*cpcBA*) promoter from *Synechocystis* sp. strain PCC 6803, a promoter that has been reported to have high activity in *Synechococcus* sp. strain PCC 7002 (13). The wild-type and Δ*ols* strains (8) were transformed with a DNA cassette assembled using the *in vitro* enzymatic assembly method described by Gibson et al. (14). The cassette contained an upstream flanking sequence of *desE*, the *aphII* gene

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TABLE 1 Oligonucleotides used in this study[a]

No.	Oligonucleotide	Sequence (5′→3′)
1	desA-a2	CCGTTCTGCAGGTTTCTTGGCGCAAGGGTTACAGCTTCC
2	desA-a1	GCAACCATGGGAAACCCAACGCAAGG
3	desA-b2	CCGTTGGATCCGTACGCTTCCATACCATGTTCACCAATATCG
4	desA-b1	CCTCACAGGTTCGGCCTACAGTGG
5	desB-a2	CCGTTCTGCAGCTTTACAACCCCTAATCCGCCTTTATTCATTTCC
6	desB-a1	CAGATCGAGGGGAACCTGGTTTGCG
7	desB-b2	CCGTTGGATCCCAACAACGCCTTGCAGAAAATCCCCAGC
8	desB-b1	CCAGTTTTAACAGACCTTGGGTAAAGGCTTC
9	desC-a2	CCGTTCTGCAGCCTTGTCACCTACGGCGAAGGTTGG
10	desC-a1	GGAATACACTGACGAATACCGCGATGGG
11	desC-b2	CCGTTGGATCCGGTAATTCCAATGCCGCCGGTAATCCAGT
12	desC-b1	GGTTTCTGGGCGAATTGCGATCTTGAGG
13	desE-a2	CCGTTCTGCAGATTGTTGTCCCCAAAGGGAAATATCTATTCCG
14	desE-a1	GACCCAGAAAACCCCGTAGAATAATCACG
15	desE-b2	CCGTTGGATCCAATGCTTTCCTAACGAGTTGAGAATATCTTCTATG
16	desE-b1	CGTCGATTTTGCCTCATTAATTTAGTTAAAGCAGC
17	desF-a2	CCGTTCTGCAGGAATAAATTTGCGTTTGATCATTACCGCCAATTC
18	desF-a1	GGTTGAAACCATTTAGGGAAACCCATACTG
19	desF-b2	CCGTTGGATCCGAATTCTCAAACAATAGAACAAGACAAAGGGGAATATC
20	desF-b1	CGAGGCAGGTTTTGAGAGCGTCAAC
21	desE-US-Fw	TATGCACATATGCGTCGATTTTGCCTCATTAATTTAGTTAAAGCAGC
22	desE-US-Rv	GCAGTTTCATTTGATGCTCGATGAGTTTTTCTAAGCTTTCCTAACGAGTTGAGAATATCTTCTATGAAACCG
23	KM-Fw	TTAGAAAAACTCATCGAGCATCAAATGAAACTGC
24	KM-Rv	GGACTCTTCTCTACAGGTGGGTATAGATTTGTTAAGCTTTGGCAGGATCCGGCTGCTAACAAA
25	Cpc-prom-Fw	CTTAACAAATCTATACCCACCTGTAGAGAAGAGTCC
26	Cpc-prom-Rv	GGGTCAAGAACGTTGCTGTAATGCGTCATGGAATTAATCTCCTACTTGACTTTATGAGTTGGG
27	desE-DS-Fw	ACGCATTACAGCAACGTTCTTGACCC
28	desE-DS-Rv	CTGCCGCCAGGCAAATTCTGTTTTATCCATATGCTAGGGATTGGCCGCGTTTTGTAGATC
29	pBAD18-Fw	GATAAAACAGAATTTGCCTGGCGGCAG
30	pBAD18-desE-USRv	GCTGCTTTAACTAAATTAATGAGGCAAAATCGACGCATATGTGCATAGGAGAAACAGTAGAGAGTTGCGATAAAAAGCG
31	pBAD18-NdeI-Fw	CATATGGATAAAACAGAATTTGCCTGGCGGCAG
32	pBAD18-A-acsRv	TAGGAGGTTACGGGGAAAAGCCAATAGGCATATGTGCATAGGAGAAACAGTAGAGAGTTGCGATAAAAAGCG
33	A-acsA-Fw	CCTATTGGCTTTTCCCCGTAACCTCCTA
34	A-acsA-Rv	GCAGTTTCATTTGATGCTCGATGAGTTTTTCTAACCTCGGCAGCAAAGTCTGGTG
35	KM-desE-Rv	GTTTCGGTGGTGACAGTTTCTGGGCTTTGGCAGGATCCGGCTGCTAACAAA
36	desE-comp-Fw	CCCAGAAACTGTCACCACCGAAAC
37	desE-comp-Rv	GTGTCGCCCACAATTTCCTGACCCCCAGGGCATCGTTTTAGCAACG
38	B-acs-Fw	GGTCAGGAAATTGTGGGCGACAC
39	B-acs-Rv	CTGCCGCCAGGCAAATTCTGTTTTATCCATATGCCAACAAGCCTTTGCCGCTGATC
40	AAS675-a1	TATGCACATATGCCTTCCATCACGTCGGCAGTAATTTC
41	AAS675-a2	GCAGTTTCATTTGATGCTCGATGAGTTTTTCTAACAAGCCGAAATCATGGCTACAATCCTAC
42	KM-675-Rv	GAAGATTCCGCCATTCGGATCGCCTTTGGCAGGATCCGGCTGCTAACAAA
43	AAS675-b1	GCGATCCGAATGGCGGAATCTTC
44	AAS675-b2	CTGCCGCCAGGCAAATTCTGTTTTATCCATATGCACTGAGGCCACATCCGTCAAAATC
45	pBAD18-675Rv	GAAATTACTGCCGACGTGATGGAAGGCATATGTGCATAGGAGAAACAGTAGAGAGTTGCGATAAAAAGCG
46	AAS1977-a1	TATGCACATATGGCAGTTCTGTAAGGCCCTACTAGAGG
47	AAS1977-a2	GCAGTTTCATTTGATGCTCGATGAGTTTTTCTAAGTTTTCGCAGAATGGGTCATGGTGG
48	KM-1977-Rv	GCAATTTTCATCGCCACCCTTTAGAGGCTTTGGCAGGATCCGGCTGCTAACAAA
49	AAS1977-b1	CCTCTAAAGGGTGGCGATGAAAATTGC
50	AAS1977-b2	CTGCCGCCAGGCAAATTCTGTTTTATCCATATGCATTGACCCCAGGCTCAATCAGATTCC
51	pBAD18-1977Rv	CCTCTAGTAGGGCCTTACAGAACTGCCATATGTGCATAGGAGAAACAGTAGAGAGTTGCGATAAAAAGCG

[a] Restriction sites are underlined. Oligonucleotides 1 to 20 were used to construct integration cassettes for creating the desaturase knockouts (a1 and a2 primers were used for the upstream region of each gene, b1 and b2 for the downstream region). Oligonucleotides 21 to 30 were used to construct integration cassettes for creating the *desE*-up strain. Oligonucleotides 31 to 39 and 23 were used to amplify DNA fragments for subsequent Gibson assembly of an integration cassette used to create the Δ*desE*+ complementation strain. Oligonocleotides 40 to 51 as well as 24 and 29 were used to construct integration cassettes for creating Δ*aas* strains.

TABLE 2 Cyanobacterial strains used in this study

Strain	Phenotype	Reference or source
Wild type	Wild-type *Synechococcus* sp. strain PCC 7002	Pasteur culture collection
Δ*desA*	Δ*desA*::*aadA*	This study
Δ*desB*	Δ*desB*::*aadA*	This study
Δ*desE*	Δ*desE*::*aadA*	This study
Δ*desF*	Δ*desF*::*aadA*	This study
desE-up	$\Phi(P_{cpcAB}$-*desE*)	This study
Δ*desE*+	Δ*desE*::*aadA* Δ*acsA*::*desE-aphII*	This study
Δ*ols*	Δ*ols*::*aadA*	8
Δ*ols-desE*-up	$\Phi(P_{cpcAB}$-*desE*) Δ*ols*::*aadA*	This study
Δaas_{675}	ΔSYNPCC7002_A0675::*aphII*	This study
Δaas_{1977}	ΔSYNPCC7002_A1977::*aphII*	This study

that confers Km^r (from pJ206 plasmid; DNA 2.0, Menlo Park, CA), the *cpcBA* promoter, and the 5′ end of *desE*.

The complementation strain (Δ*desE*+) was constructed by homologous recombination using a DNA cassette containing the native *desE* locus (including 390 bases immediately 5′ of the *desE* coding sequence predicted to encode the *desE* promoter), the *aphII* gene that confers Km^r, and the upstream and downstream flanking sequences of *acsA* (locus SYNPCC7002_A1838). The *acsA* locus (target for integration of *desE*) encodes an acetyl coenzyme A (CoA) ligase that confers sensitivity to exogenous acrylic acid. The conditional acrylic acid sensitivity has been used as a counterselection method to facilitate the integration of heterologous genes and segregation of the resulting mutants (15). Here, the heterologous DNA was assembled from four PCR products (primers are listed in Table 2) using the *in vitro* enzymatic assembly method described by Gibson et al. (14). The resulting DNA cassette was transformed into the Δ*desE* strain. Recombinants were selected on Km. Complete segregation of the mutants was verified by colony PCR.

The *aas* knockouts were created by homologous recombination of a linear targeting cassette. Here, plasmids (pBAD18 backbone) containing a kanamycin resistance cassette (*aphII* gene) and the upstream and downstream regions of the corresponding genes (SYNPCC7002_A0675 and SYNPCC7002_A1977) were assembled from PCR products (primers are listed in Table 2) using the *in vitro* enzymatic assembly method described by Gibson et al. (14). Each plasmid was linearized before transformation. Complete segregation of the mutants was verified by colony PCR. Mutant strains were grown in air-sparged, liquid cultures supplemented with pentadecanoic acid (final concentration, 20 mg/liter) at 38°C.

Lipid analysis (GC-MS). Cultures were grown to an optical density at 730 nm (OD_{730}) of ~1.0, centrifuged, resuspended in 3 ml of water, and extracted and analyzed by following previously described protocols (8, 16). Samples were analyzed using a Shimadzu GCMP QP2010S gas chromatograph mass spectrometer (GC-MS) equipped with an AOC-20i autoinjector and a Restek Rxi-5ms column (catalog no. 13423). The temperature program was a 100°C hold for 2 min, ramping up from 100°C to 150°C at 80°C per min, a hold for 4 min, ramping up from 150°C to 218°C at 4°C per min, ramping up from 218°C to 325°C at 80°C/min, and a hold at 325°C for 2.5 min. A sample injection temperature of 250°C and a volume of 1 μl was used, along with a 1:10 split ration. The MS was operated in scanning mode between 50 and 350 *m/z*. Quantification was achieved by comparison of integrated peaks with calibration curves of fatty acid methyl ester (FAME; Sigma) and 1-nonadecene standards (Fluka).

RESULTS AND DISCUSSION

Identification of a desaturase gene involved in alkene unsaturation. Given the lack of functional handles on the olefins, we hypothesized that the internal bond of 1,14-nonadecadiene would be present in the substrate of the Ols-catalyzed elongation-decarboxylation mechanism, i.e., an unsaturated C_{18} acyl-ACP. *Synechococcus* sp. strain PCC 7002 synthesizes lipids that incorporate 18-carbon fatty acids with zero, one, two, or three double bonds at the Δ9, Δ12, and Δ15 (or ω3) positions at the *sn*-1 position of lipids and C_{16} fatty acids containing zero or one double bond at the Δ9 position at the *sn*-2 position of lipids (17–19). These unsaturated and polyunsaturated fatty acids are essential constituents of polar glycerolipids and are used to control the fluidity of membranes in response to changes in temperature (10). Three acyl-lipid desaturases, encoded by *desA* (Δ12), *desB* (Δ15), and *desC* (Δ9), have been shown to be involved in the biosynthesis of the unsaturated fatty acids observed in *Synechococcus* sp. strain PCC 7002 (20–22). Two additional genes are predicted to encode uncharacterized desaturases, *desE* (SYNPCC7002_A2833) and *desF* (SYNPCC7002_A1989). We hypothesized that one of the five desaturases was responsible for the internal double bond in 1,14-nonadecadiene.

To test this hypothesis, a disruption mutant of each desaturase was constructed by homologous recombination using a knockout cassette consisting of an antibiotic resistance gene flanked by homology targeting sequences. After multiple attempts, we were unable to obtain a Δ*desC* mutant, suggesting that this mutation is lethal to the cells. The same problem was reported when trying to disrupt *desC* in *Synechocystis* sp. strain PCC 6803 (23). Conversely, fully segregated knockouts of *desA*, *desB*, *desE*, and *desF* were obtained after transformation and plating on the appropriate antibiotics. The observed fatty acid profiles of Δ*desA* and Δ*desB* mutants were consistent with past reports (20). Cells of the *desA* mutant contained 18:1(Δ9) but no 18:2(Δ9, Δ12) or 18:3(Δ9, Δ12, Δ15) fatty acids, and the cells of the *desB* mutant contained 18:1(Δ9) and 18:2(Δ9, Δ12) but no 18:3(Δ9, Δ12, Δ15) fatty acids. The hydrocarbon compositions of the Δ*desA*, Δ*desB*, and Δ*desF* mutants were indistinguishable from that of wild-type *Synechococcus* sp. strain PCC 7002 (data not shown). Conversely, the hydrocarbon extract of the Δ*desE* mutant contained no detectable $C_{19:2}$ alkene (Fig. 1). When the deletion was complemented by inserting *desE* under its native promoter in the *acsA* locus (a location used previously for making chromosomal insertions [15]), 1,14-nonadecadiene production was restored. These results sug-

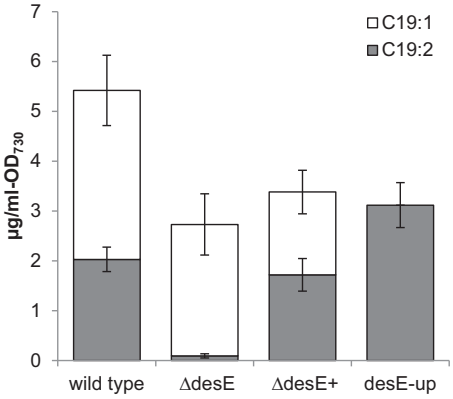

FIG 1 Comparison of the hydrocarbon composition from the wild-type, *desE* knockout (Δ*desE*), Δ*desE* complemented (Δ*desE*+), and *desE* upregulated (*desE*-up) strains. The deletion of the *desE* gene eliminated only the production of the hydrocarbon with the internal double bond ($C_{19:2}$). Cultures were grown autotrophically at 36°C with bubbling of air, and error bars represent the standard deviations from three biological replicates.

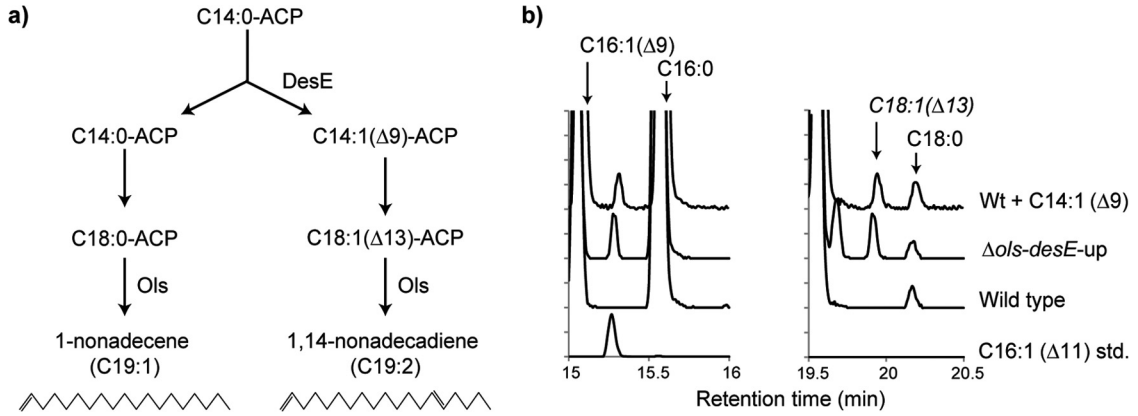

FIG 2 (a) Proposed route for alkene biosynthesis in *Synechococcus* sp. strain PCC 7002. DesE is required to place a double bond at the Δ9 position of the $C_{14:0}$ acyl-ACP substrate. The product subsequently is elongated to a $C_{18:1(\Delta 13)}$ acyl-ACP that would serve as the precursor for 1,14-nonadecadiene biosynthesis by Ols. (b) Comparison of the fatty acid profiles of *Synechococcus* sp. strain PCC 7002 and mutants. Feeding of the $C_{14:1(\Delta 9)}$ fatty acid to the wild-type strain and overexpression of *desE* in the Δ*ols* strain resulted in the formation of the $C_{16:1(\Delta 11)}$ fatty acid (retention time, 15.2 min) and the $C_{18:1(\Delta 13)}$ fatty acid (retention time, 19.9 min). Attempts to chemically complement the Δ*desE* mutant with $C_{14:1(\Delta 9)}$ fatty acid failed, as did attempts to chemically complement the Δ*ols* mutant with 1-nonadecene.

gest that the desaturase encoded by *desE* is responsible for the internal double bond in $C_{19:2}$. It should be noted that *desF* recently was reported to be essential for growth on plates under microoxic conditions (24), conditions that were not tested here.

Precursors for 1,14-nonadecadiene biosynthesis. The putative Ols pathway (8) calls for C_{18} acyl-ACP precursors to be processed via an elongation-decarboxylation mechanism catalyzed by a multimodular megasynthase. In the case of the $C_{19:1}$ hydrocarbon, a fully saturated acyl-ACP (18:0) would be the precursor. However, for the $C_{19:2}$ hydrocarbon to have an internal double bond at position 14, a C_{18} acyl-ACP with a double bond at position 13 would be required. This $C_{18:1(\Delta 13)}$ acyl-ACP could be directly synthesized by DesE acting on C_{18} acyl-ACP or could be the elongation product of a shorter unsaturated acyl-ACP. Potential DesE products consistent with this scheme include $C_{16:1(\Delta 11)}$, $C_{14:1(\Delta 9)}$, or $C_{12:1(\Delta 7)}$ acyl-ACPs. Compared to the Δ*desE* mutant extract, the lipid profiles of the wild-type strain contained no additional fatty acids, providing no assistance in determining the DesE substrate.

The amino acid sequence of DesE is similar to the Δ9 desaturases of *Rattus norvegicus* (rat) and *Saccharomyces cerevisiae* (34 to 38%) (25). DesE contains the conserved three-histidine cluster motifs observed in Δ9 desaturases: the HXXXXH motifs (residues 79 to 84) and two HXXHH motifs (residues 116 to 120 and 242 to 246). These histidine motifs are thought to bind iron atoms and play an important role in the introduction of the double bond in the hydrocarbon chains of fatty acids (19). The high degree of similarity of DesE to Δ9 desaturases suggested that a C_{14} acyl-ACP was the most likely substrate to be consistent with the formation of 1,14-nonadecadiene.

If true, the $C_{14:1(\Delta 9)}$ acyl-ACP synthesized by DesE would be elongated to a $C_{16:1(\Delta 11)}$ and a $C_{18:1(\Delta 13)}$ acyl-ACP that ultimately would be the substrate for Ols (Fig. 2a). The lack of these intermediates in the lipid extracts of the wild-type strain suggested that they are not accumulating to a detectable extent. Therefore, we increased the expression of *desE* by replacing its promoter with the strong P_{cpcBA} promoter from *Synechocystis* sp. strain PCC 6803 (13) (*desE*-up strain). The fatty acid profile of this strain contained no additional fatty acids compared to the wild type. Conversely, the hydrocarbon profile of *desE*-up was dramatically shifted from 35% $C_{19:2}$ hydrocarbon in the wild type to nearly 100% $C_{19:2}$ in the *desE*-up mutant (Fig. 1). These results suggest that the hydrocarbon profile is controlled by the relative level of Ols substrates and that the products of DesE are used exclusively in the formation of olefins. If this hypothesis is true, knocking out *ols* in the *desE*-up strain should result in the accumulation of unsaturated acyl-ACPs that ordinarily serve as intermediates in $C_{19:2}$ biosynthesis. A Δ*ols-desE*-up strain was constructed, and its fatty acid profile was analyzed. Compared to the wild-type strain, the extract of the Δ*ols-desE*-up mutant contained three additional peaks in its chromatogram at retention times of 15.2, 19.6, and 19.9 min. One (15.2 min) was identified as 11-hexadecenoic acid, $C_{16:1(\Delta 11)}$, by comparison to a commercial standard (Fig. 2b). The mass spectra for the other two peaks were consistent with C_{18} unsaturated fatty acids, but no commercial standard for the $C_{18:1(\Delta 13)}$ fatty acid was available for structural confirmation; in order to corroborate the identity of one of these peaks as the $C_{18:1(\Delta 13)}$ fatty acid, a different approach was used.

Exogenous fatty acids that are transported across the outer membrane can be incorporated into cyanobacterial lipid metabolism after being activated to the acyl-ACP form by an acyl-ACP synthetase (Aas). The Aas involved in this activation have been identified in *Synechocystis* sp. strain PCC 6803 and *Synechococcus elongatus* PCC 7942 (26). Although no Aas has been characterized in *Synechococcus* sp. strain PCC 7002, when pentadecanoic acid (C_{15}) was fed to this strain, we detected the formation of heptadecenoic acid (C_{17}) and 1-octadecene, showing that *Synechococcus* sp. strain PCC 7002 has the ability to elongate and incorporate exogenous fatty acids into lipid metabolism (8). Two enzymes with homology to the Aas from *Synechocystis* sp. strain PCC 6803 and *Synechococcus elongatus* PCC 7942 were identified in the genome of *Synechococcus* sp. strain PCC 7002 (SYNPCC7002_A0675 and SYNPCC7002_A1977). Knockout mutants were constructed for each gene (Δaas_{675} and Δaas_{1977} strains). After feeding C_{15} free fatty acid to each mutant, C_{17} fatty acid and 1-octadecene were not detected in the Δaas_{675} strain (Fig. 3) but were in the

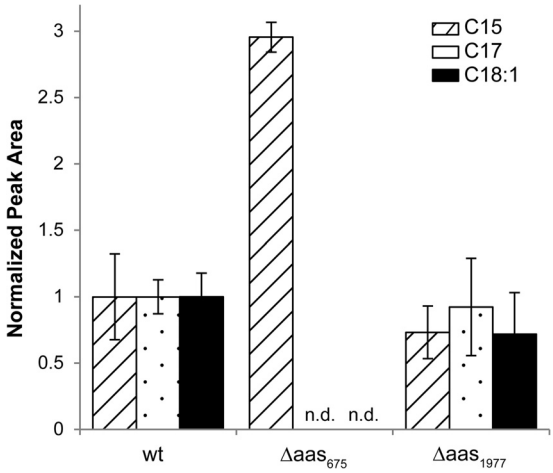

FIG 3 Heptadecanoic acid (C_{17}) and 1-octadecene ($C_{18:1}$) formation after the addition of pentadecanoic acid (C_{15}). The formation of C_{17} and $C_{18:1}$ was observed for the wild-type (wt) and Δ*aa*$_{1977}$ strains but not for the Δ*aas*$_{675}$ strain. Peak areas obtained from GC-MS analysis were normalized to the volume, OD_{730}, and the area of an internal standard; they are presented as a fraction of the wild-type-normalized area for the corresponding compound. Error bars represent the standard deviations from three biological replicates. n.d., not detected.

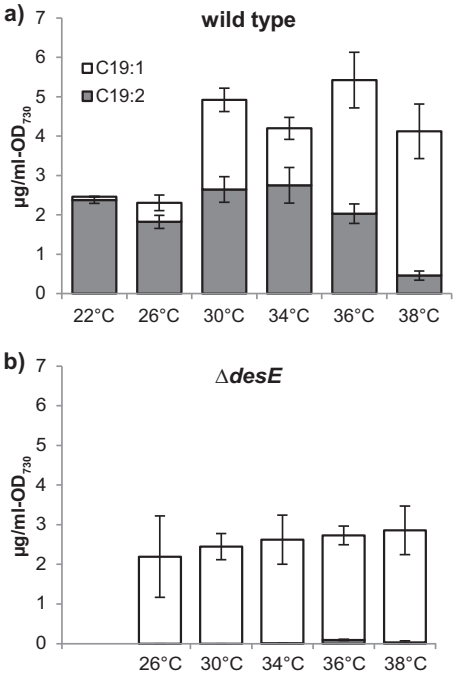

FIG 4 Comparison of the hydrocarbon composition from cultures grown at different temperatures. 1-Nonadecene ($C_{19:1}$) levels decrease at low temperatures for the wild-type strain (a) but not for the Δ*desE* strain (b). Error bars represent the standard deviations from three biological replicates.

Δ*aas*$_{1977}$ strain, suggesting that the Aas encoded by SYNPCC7002_A0675 is responsible for the activation of exogenous fatty acids to acyl-ACPs, and its activity is required for the formation of the alkenes from exogenous fatty acids.

Therefore, to corroborate the identity of one of the peaks observed in the Δ*ols-desE*-up strain as $C_{18:1(\Delta 13)}$ fatty acid, 9-tetradecenoic acid, $C_{14:1(\Delta 9)}$, was fed to wild-type *Synechococcus* sp. strain PCC 7002 (Fig. 2b). Once activated by the Aas, the elongation of the $C_{14:1(\Delta 9)}$ fatty acid should result in the formation of the $C_{16:1(\Delta 11)}$ and $C_{18:1(\Delta 13)}$ acyl-ACPs. After analysis, feeding *Synechococcus* sp. strain PCC7002 with $C_{14:1(\Delta 9)}$ resulted in the formation of two additional peaks with retention times of 15.2 min, corresponding to $C_{16:1(\Delta 11)}$, and 19.9 min (Fig. 2b); these peaks were not observed when $C_{14:1(\Delta 9)}$ was fed to the Δ*aas*$_{675}$ strain. These data suggest that the peak at 19.9 min observed in the Δ*ols-desE*-up strain is indeed the $C_{18:1(\Delta 13)}$ fatty acid and that DesE is acting on C_{14} acyl-ACP.

Efforts to heterologously express DesE in *Escherichia coli* (Mistic tagged [27] and His tagged under various conditions) failed to generate soluble protein or changes in the fatty acid profile (data not shown). The hydropathy profile of DesE shows two highly hydrophobic regions, suggesting that it is an integral membrane protein (25), and further optimization of the expression construct will be needed to confirm the biochemical activity of DesE.

Effect of temperature in alkene unsaturation. It is known that upon a downshift in temperature, expression of some desaturases is upregulated and the mRNA stability of desaturase genes is altered (22), resulting in an increase of the unsaturated lipids that are used to maintain membrane fluidity at low temperatures. In the case of *desE*, global transcriptome profiling via RNA-seq showed that its transcript levels increased approximately 2-fold in cells grown at 22°C compared to cells grown at 38°C (28). Also, it has been shown that *desE* transcripts had an estimated half-life of only 1 min at 38°C and 21 min at 22°C (25), suggesting that *desE* is regulated to respond to changes in temperature. To test if *desE* upregulation at low temperatures would affect the hydrocarbon composition of *Synechococcus* sp. strain PCC 7002, the wild-type strain was grown at different temperatures. At 38°C, the $C_{19:2}$ hydrocarbon represented only 11% of the total hydrocarbon pool, whereas at 22°C it was 96% (Fig. 4a). Except for 38°C, most of these differences were generated by a decrease in $C_{19:1}$ hydrocarbon rather than an increase of $C_{19:2}$. For the Δ*desE* strain, there was a slight decrease in $C_{19:1}$ at low temperatures; however, on average, hydrocarbon levels remained constant across all temperatures (Fig. 4b). Interestingly, no $C_{18:1(\Delta 13)}$ fatty acid was detected at any of the temperatures tested, suggesting that this intermediate is not incorporated into lipids but rather is exclusively converted to hydrocarbon by Ols. Given that *Synechococcus* sp. strain PCC7002 does not contain a pathway for fatty acid catabolism (e.g., beta-oxidation), other uses of the $C_{18:1(\Delta 13)}$ acyl chain are not obvious.

Although the physiological functions of hydrocarbons in cyanobacteria remain poorly understood, several hypotheses have been proposed, including intra- or interspecies chemical signaling, prevention of desiccation, enhanced buoyancy, and membrane fluidity/stability (6). The alkenes synthesized by *Synechococcus* sp. strain PCC 7002 are a significant portion of the total lipid content in this strain (for example, at 36°C the total alkene content is ~5.5 μg/ml-OD_{730} and the total fatty acid content is ~35 μg/ml-OD_{730}). Furthermore, olefins are found exclusively in the cell pellet and not in the spent media, indicating that olefins are not secreted outside the cell (results not shown). Therefore, the change in olefin composition with temperature suggested that these compounds are important for growth at low temperatures. To test this hypothesis, the growth of the wild-type, Δ*desE*, and Δ*ols* strains was compared at 38°C and 22°C on solid media. As

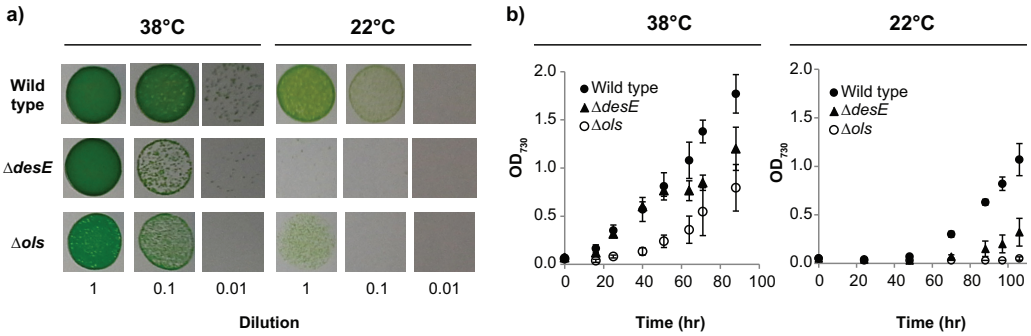

FIG 5 Effect of temperature on growth. (a) Cells were harvested from liquid cultures and suspended to the same initial cell concentration (OD_{730}, ~0.05) and then serially diluted (1:10). Seven μl of each dilution was plated on solid medium A (supplemented with 1 mg/ml $NaNO_3$), and cells were grown for 4 days at the specified temperatures. (b) Growth curves for the wild-type (wt), Δ*desE*, and Δ*ols* strains in liquid cultures.

can be seen in Fig. 5a, the three strains grew at 38°C after 4 days; at 22°C, however, only the wild-type strain was able to grow to comparable levels, suggesting that the alkenes are important for growth at low temperatures. Similar results were observed when the strains were grown in liquid cultures (Fig. 5b).

Conclusions. In summary, we demonstrated the involvement of a desaturase gene (*desE*) in the formation of the internal double bond in the 1,14-nonadecadiene synthesized by the cyanobacterium *Synechococcus* sp. strain PCC 7002. The amino acid sequence encoded by the *desE* gene shows a high degree of similarity to Δ9 desaturases, suggesting that its most likely substrate is a C_{14} fatty acid, which, after elongation to a $C_{18:1(\Delta 13)}$ fatty acid, would serve as the precursor for the formation of the hydrocarbon with the internal double bond at position 14. When expression of *desE* was increased by replacing its promoter, we observed the formation of only the $C_{19:2}$ (and not the $C_{19:1}$) hydrocarbon, suggesting that the products of DesE are earmarked for the hydrocarbon pathway. Moreover, since no hydrocarbons coming from the other unsaturated C_{18} fatty acids synthesized by *Synechococcus* sp. strain PCC 7002 have been detected, such as $C_{18:1(\Delta 9)}$ or $C_{18:2(\Delta 9,\ \Delta 11)}$, it seems that not only the presence of the additional unsaturation is important but also its location. Since the hydrocarbon content in *Synechococcus* sp. strain PCC 7002 represents about 10 to 15% of the total lipid content, the increase in $C_{19:2}$ (diunsaturated hydrocarbon) production and the essentiality of DesE at low temperatures suggests that hydrocarbons play a role in responding to cold, possibly in maintaining membrane fluidity.

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