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Modification of the Fatty Acid Composition of *Escherichia coli* by Coexpression of a Plant Acyl-Acyl Carrier Protein Desaturase and Ferredoxin

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Expression of a plant Δ^6-palmitoyl (16:0)-acyl carrier protein desaturase in *Escherichia coli* resulted in the accumulation of the novel monounsaturated fatty acids Δ^6-hexadecenoic acid (16:1Δ^6) and Δ^8-octadecenoic acid. Amounts of 16:1Δ^6 accumulated by *E. coli* were increased more than twofold by the expression of a plant ferredoxin together with the Δ^6-16:0-acyl carrier protein desaturase.

Both plants and *Escherichia coli* contain type II fatty acid synthases (12, 17). In this biosynthetic system, dissociable enzymes catalyze the modification of fatty acids bound to acyl carrier protein (ACP). Enzymes of plant fatty acid synthase can use acyl chains attached to *E. coli* ACP as substrates for in vitro reactions (e.g., see references 7, 8, and 21), and at least several of these enzymes have been shown to function in *E. coli* (5, 9, 10, 25). Expression of plant acyl-ACP thioesterases, for example, can result in dramatic alterations in the amounts of certain fatty acids produced by *E. coli* (5, 9, 25).

Based on this knowledge, studies were initiated to develop an in vivo system to characterize the activities of plant acyl-ACP desaturases directly in *E. coli*. Such a system would involve monitoring changes in the unsaturated fatty acid composition of *E. coli* following expression of an acyl-ACP desaturase. This would provide a method to complement the more labor-intensive in vitro assays currently used to determine acyl-ACP desaturase activity (13). It would also allow rapid screening of acyl-ACP desaturases with altered activities (e.g., fatty acid chain length specificities) produced in site-directed mutagenesis studies. As a first step towards the development of an in vivo system for the characterization of plant acyl-ACP desaturase activity, we demonstrate in this report that a Δ^6-palmitoyl (16:0)-ACP desaturase from seed endosperm of *Thunbergia alata* (black-eyed Susan vine) (3) can function in *E. coli* to produce novel monounsaturated fatty acids. The activity of plant acyl-ACP desaturases requires molecular oxygen and is dependent on reduced ferredoxin (8, 16, 24). In this regard, we also show here that the amounts of monounsaturated fatty acids produced by the Δ^6-16:0-ACP desaturase in *E. coli* can be increased by the coexpression of a plant ferredoxin (Fd).

Plasmids and bacterial strains. Plasmids were constructed as described in Table 1. Studies were performed using *E. coli* BL21 (DE3) (22) harboring plasmids listed in Table 2.

Growth conditions. Bacterial cultures were started with the addition of 100 to 200 μl of *E. coli* BL21 (DE3)-derived strains (optical density at 600 nm ≈ 1.0) to 25 ml of Luria-Bertani media in 250-ml Erlenmeyer flasks. Media contained 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and appropriate antibiotics (100 μg of ampicillin per ml, 50 μg of kanamycin per ml, or 40 μg of chloramphenicol per ml). Cultures were incubated with shaking for 15 to 20 h at 20 to 22°C, resulting in optical densities at 600 nm of ≈0.5 to 0.8. Cells were then collected by centrifugation and washed with 50 mM Tris (pH 7.5)–5 mM EDTA.

Fatty acid analysis. Cell pellets were resuspended in 1 ml of 10% (wt/vol) boron trichloride in methanol (Alltech) and 100 μl of toluene and heated for 40 min at 90°C, and the resulting fatty acid methyl esters were extracted as described elsewhere (4, 15). Fatty acid methyl esters were analyzed by gas chromatography using a 5790II Hewlett Packard gas chromatograph and a DB23 column (60 m by 0.25 mm [inner diameter]; J&W Scientific). The oven temperature was programmed from 170°C (25-min hold) to 185°C at 2.5°C/min with a column head pressure of 55 kPa. Comparable results were obtained by transesterification using sodium methoxide (20) or by transesterification following total lipid extraction by the method described by Bligh and Dyer (1).

Unsaturated fatty acid methyl esters were partitioned into Δ^6-, Δ^8/Δ^9-, and Δ^{11}-monounsaturate-enriched fractions by argentation thin-layer chromatography (4, 14). Double-bond positions were then determined by gas chromatography-mass spectrometry analysis of dimethyl disulfide derivatives (26).

Measurement of Δ^6-16:0-ACP desaturase, Fd, and FNR expression. Expression of Δ^6-16:0-ACP desaturase was assessed by immunoblot analysis of total *E. coli* protein separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and probed with polyclonal antibodies raised against the Δ^6-16:0-ACP desaturase. Fd and Fd-NADP$^+$ oxidoreductase (FNR) expression was quantified by performing cytochrome *c* reduction assays with crude bacterial extracts (27). Expression of *Arabidopsis thaliana* Fd in *E. coli* could also be visualized by the presence of a pink hue in cell pellets.

E. coli BL21 (DE3) expressing the *T. alata* Δ^6-16:0-ACP desaturase cDNA behind the T7 RNA polymerase promoter accumulated two fatty acids not detected in control cultures (Fig. 1A and B). These fatty acids were identified by mass spectrometry as Δ^6-hexadecenoic acid (16:1Δ^6) and Δ^8-octadecenoic acid (18:1Δ^8) (Fig. 1C and D). The presence of 16:1Δ^6 in *E. coli* is consistent with the known in vitro activity of the Δ^6-16:0-ACP desaturase (3). It is presumed that 18:1Δ^8 results from the elongation of 16:1Δ^6-ACP in a manner analogous to the synthesis of *cis*-vaccenic acid (18:1Δ^{11}) from palmitoleoyl (16:1Δ^9)-ACP in *E. coli* (12).

Combined levels of 16:1Δ^6 and 18:1Δ^8 produced by *E. coli* expressing the Δ^6-16:0-ACP desaturase were 5 to 7 mol% of the total fatty acid content of cells (Table 2). To examine the

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TABLE 1. Bacterial plasmids used in this study

Plasmid	Description	Source or reference
pAtFd	pZL1 (Gibco BRL) containing *A. thaliana* Fd cDNA in *Not*I-*Sal*I site; Amp^r (GenBank accession no. T14207)	Arabidopsis Biological Resource Center
pET/Fd	pET3d (Novagen) containing *Nco*I-*Bam*HI fragment from pAtFd consisting of open reading frame of mature *A. thaliana* Fd; Amp^r	This study
pACYC/Fd	pACYC184 (New England Biolabs) containing *Bgl*II-*Hin*dIII fragment (which includes coding sequence of mature *A. thaliana* Fd behind the *s10* translation initiation site and the T7 RNA polymerase promoter) from pET/Fd cloned into the *Bam*HI-*Hin*dIII site; Cm^r	This study
pET/Delta6	pET3d containing coding sequence for the mature *T. alata* Δ^6-16:0-ACP desaturase cloned into the *Nco*I site; Amp^r	3
pKG1022	Contains *hok*/*sok* (*parB*) plasmid stabilization cassette linked to Kan^r marker (*aphA*); Kan^r	6
pET*hs*	pET3d containing *Pst*I fragment (which consists of *hok*/*sok* plasmid stabilization cassette and Kan^r marker) of pKG1022 resulting in loss of Amp^r; Kan^r	This study
pET/Delta6*hs*	pET/Delta6 containing *Pst*I fragment (which consists of *hok*/*sok* plasmid stabilization cassette and Kan^r marker) of pKG1022 resulting in loss of Amp^r; Kan^r	This study
pET/Delta6Fd	pET/Delta6 containing *Sal*I-*Bam*HI fragment (which includes T7 RNA polymerase promoter and coding sequence for mature *A. thaliana* Fd) of pET/Fd cloned into *Sal*I-*Bgl*II site; Amp^r	This study
pET/FNR	pET23b (Novagen) containing cDNA for *Zea mays* (maize) root FNR; Amp^r	18
pLac3a	pET3a with the T7 promoter replaced by the *lacUV5* promoter for *E. coli* RNA polymerase; 1,274-bp fragment (GenBank accession no. J01636; ECOLAC, bp 1 to 1274) containing the *lacI* gene and the *lacUV5* promoter inserted between the *Bgl*II and *Xba*I sites of pET3a; has the same strong translation initiation site as pET3a; Amp^r	M. Blewitt
pACYC/Lac3a	pACYC184 analog of pLac3a; *Bgl*II-*Hin*dIII fragment containing the expression signals from pLac3a inserted between the *Bam*HI and *Hin*dIII sites of pACYC184; Cm^r	This study
pACYC/LacFNR	pACYC/Lac3a containing *Xba*I fragment from pET/FNR cloned into *Xba*I site behind *lacUV5* promoter; Cm^r	This study

effect of plant Fd on this system, an *A. thaliana* Fd was coexpressed with the Δ^6-16:0-ACP desaturase. Fd-dependent cytochrome *c* reduction activity was more than sixfold higher in extracts of *E. coli* expressing *A. thaliana* Fd from pACYC/Fd than in extracts of cells expressing only the plant desaturase (data not shown). Accompanying expression of plant Fd and the Δ^6-16:0-ACP desaturase was a more than twofold increase in the amount of 16:1Δ^6 accumulated by *E. coli* (Table 2).

The presence of detectable amounts of 16:1Δ^6 and 18:1Δ^8 in *E. coli* expressing the desaturase alone suggests that coexpression of plant Fd is not essential for the activity of the desaturase in this system. *E. coli* contains low levels of [2Fe-2S]Fd (11, 23). However, *E. coli* Fd is of a structural class different from that of the [2Fe-2S] Fd found in plants (23). As such, the stimulation of 16:1Δ^6 and 18:1Δ^8 production by plant Fd suggests that either the form of Fd or the total amount of Fd or other reductant in *E. coli* limits desaturase activity in bacterial cells.

Acyl-ACP desaturase activity requires Fd in a reduced state (8, 16, 24). To increase levels of reduced Fd in *E. coli*, a plant FNR from maize root (19) was coexpressed with the Δ^6-16:0-ACP desaturase and *A. thaliana* Fd. Amounts of FNR were elevated 10-fold in cells expressing maize protein from pACYC/LacFNR, on the basis of cytochrome *c* reduction assays (data not shown). FNR expression, however, had little effect on the amounts of 16:1Δ^6 and 18:1Δ^8 produced by *E. coli* expressing the plant Fd and desaturase (Table 2). This suggests that conditions in *E. coli* cells are sufficient to maintain adequate levels of reduced Fd for the desaturase without the need for supplemental FNR activity or that NADPH levels in *E. coli* limit the in vivo activity of recombinant FNR.

Previously, it was reported that 16:1Δ^6 and 18:1Δ^8 production could not be detected in *E. coli* expressing the Δ^6-16:0-ACP desaturase at 37°C (3). Similar results were also obtained in this study, even in cells coexpressing *A. thaliana* Fd (data not shown). The lack of detectable amounts of 16:1Δ^6 and 18:1Δ^8 in cells induced at 37°C likely reflects the low expression levels of the desaturase in *E. coli* induced at this temperature (Fig. 2). As shown in Fig. 2, expression levels of the Δ^6-16:0-ACP desaturase were highest in cells induced at 22°C.

TABLE 2. Fatty acid composition of *E. coli* BL21 (DE3) expressing different plasmids[a]

Plasmid(s) carried by *E. coli* BL21 (DE3)	Fatty acid composition (mol% ± SD)						
	14:0	16:0	16:1Δ^6	16:1Δ^9	18:1Δ^8	18:1Δ^{11}	Other[b]
pET3d	5.3	27.8	ND[c]	21.1	ND	36.6	6.7
pET*hs* + pACYC/Fd	4.0 ± 0.1	34.7 ± 0.2	ND	25.6 ± 0.5	ND	32.0 ± 0.4	4.0
pET/Delta6*hs*	4.0 ± 0.7	21.2 ± 0.9	4.4 ± 0.1	25.8 ± 0.3	2.5 ± 0.2	40.0 ± 1.0	3.4
pET/Delta6*hs* + pACYC/Fd	4.9 ± 0.2	21.7 ± 0.2	11.3 ± 0.4	23.0 ± 0.5	3.1 ± 0.3	33.7 ± 0.3	2.7
pET/Delta6	4.3 ± 0.3	27.4 ± 1.2	3.2 ± 0.1	27.8 ± 2.2	1.4 ± 0.2	36.6 ± 0.9	3.0
pET/Delta6Fd	3.4 ± 0.2	25.2 ± 0.5	8.3 ± 0.3	22.5 ± 0.1	3.0 ± 0.3	32.6 ± 1.0	4.4
pET/Delta6Fd + pACYC/LacFNR	4.3 ± 0.5	21.3 ± 0.9	9.7 ± 0.2	22.6 ± 0.3	3.0 ± 0.5	35.5 ± 0.9	3.2

[a] Cells were grown in the presence of 0.1 mM IPTG at 20 to 22°C as described in the text. Fatty acid compositions were determined by gas chromatography following transesterification of lipids directly from harvested cells. Results are averages of measurements from three cultures of each strain.
[b] Includes 12:0, 18:0, and cyclopropane fatty acids.
[c] ND, not detected.

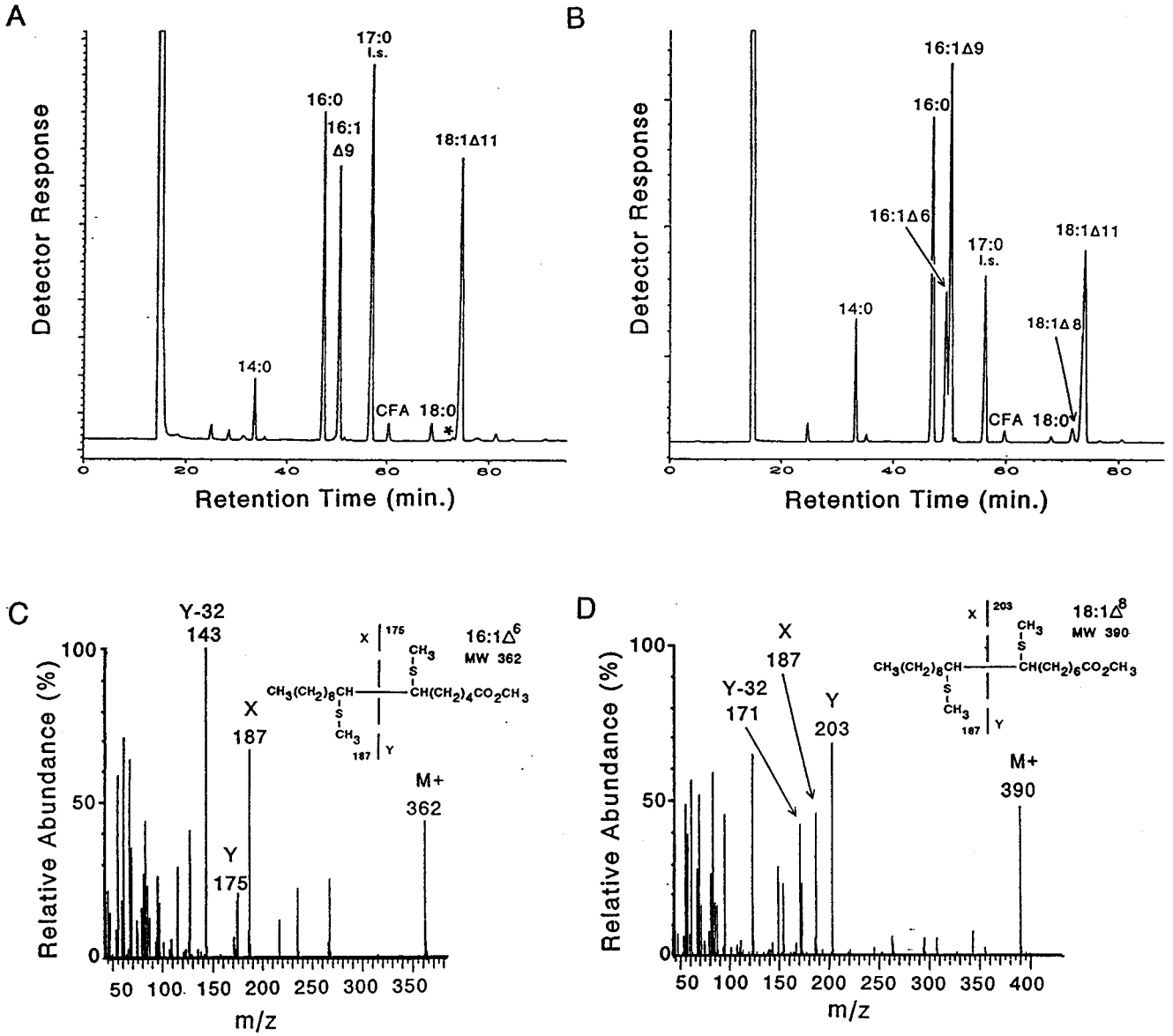

FIG. 1. Gas chromatograms of fatty acid methyl esters of *E. coli* BL21 cells transformed with pET3d (A) or cells expressing pDelta6*hs* plus pACYC/Fd (see Table 2) (B) and mass spectra of dimethyl disulfide derivatives of methyl-16:1Δ^6 (C) and methyl-18:1Δ^8 (D) from cells expressing the *T. alata* Δ^6-16:0-ACP desaturase. The asterisk in panel A indicates an unidentified compound with a mobility similar to that of methyl-18:1Δ^8 that was detected in fatty acid methyl esters of plasmid control cultures. This compound was typically present at low levels ($\leq$0.5 mol% of total fatty acid). Mass spectra were obtained with a Perkin-Elmer ITD ion trap detector. i.s., internal standard; CFA, cyclopropane fatty acid; MW, molecular weight.

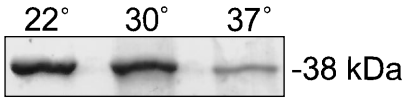

FIG. 2. Immunoblot of total protein extracts of *E. coli* BL21 (DE3) cells expressing the Δ^6-16:0-ACP desaturase at the temperatures indicated. Bacterial cells harboring pDelta6*hs* were grown at 37°C to an optical density at 600 nm of $\approx$0.4 and induced with 0.4 mM IPTG. Aliquots of induced cells were then incubated at either 22, 30, or 37°C for an additional 5 h. Total protein extracts of induced cells were separated by SDS-11% PAGE, transferred to nitrocellulose, and probed with polyclonal antibodies against the Δ^6-16:0-ACP desaturase. Each lane contained 15 μg of total protein.

In addition, it has been noted that expression of the Δ^9-stearoyl (18:0)-ACP desaturase, the enzyme associated with oleic acid synthesis in plants, does not alter the fatty acid composition of *E. coli* (24). On the basis of the results presented here, this finding is likely not due to an inability of the desaturase to function in *E. coli*. Instead, the lack of oleic acid production in bacteria expressing the Δ^9-18:0-ACP desaturase may be a result of the absence of available substrate for this enzyme. Ohlrogge et al. (18), for example, showed that long-chain acyl-ACP pools in *E. coli* K27 *fadD88* are enriched in moieties containing fatty acids of 16 carbon atoms or fewer but do not contain detectable amounts of 18:0-ACP. The methods presented here should therefore provide a general way to characterize desaturases that recognize acyl-ACP substrates of 16 carbon atoms or fewer.

Overall, these results indicate that the aerobic acyl-ACP desaturation system of plants can be superimposed on the anaerobic pathway of unsaturated fatty acid synthesis normally found in *E. coli* (2, 12).

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