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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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 Δ⁶-palmitoyl (16:0)-acyl carrier protein desaturase in Escherichia coli resulted in the accumulation of the novel monounsaturated fatty acids Δ⁶-hexadecenoic acid (16:1Δ⁶) and Δ⁸-octadecenoic acid. Amounts of 16:1Δ⁶ accumulated by E. coli were increased more than twofold by the expression of a plant ferredoxin together with the Δ⁸-16:0-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 synthases 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 Δ⁶-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 Δ⁶-16:0-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 trifluoride 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 Δ⁶-, Δ⁸/Δ⁶-, and Δ¹₁-monounsaturate-enriched fractions by argentation thin-layer chromatography (4, 14). Double-bond positions were then determined by gas chromatography-mass spectrometry analysis of dimethyl sulfoxide derivatives (26).

Measurement of Δ⁸-16:0-ACP desaturase, Fd, and FNR expression. Expression of Δ⁸-16:0-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 Δ⁸-16:0-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 Δ⁸-16:0-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 Δ⁶-hexadecenoic acid (16:1Δ⁶) and Δ⁸-octadecenoic acid (18:1Δ⁸) (Fig. 1C and D). The presence of 16:1Δ⁶ in E. coli is consistent with the known in vitro activity of the Δ⁸-16:0-ACP desaturase (3). It is presumed that 18:1Δ⁸ results from the elongation of 16:1Δ⁶-ACP in a manner analogous to the synthesis of cis-vaccenic acid (18:1Δ⁹) from palmitoleoyl (16:1Δ⁸)-ACP in E. coli (12).

Combined levels of 16:1Δ⁶ and 18:1Δ⁸ produced by E. coli expressing the Δ⁸-16:0-ACP desaturase were 5 to 7 mol% of the total fatty acid content of cells (Table 2). To examine the...
effect of plant Fd on this system, an A. thaliana Fd was coex- pressed with the Δ^8-16:0-ACP desaturase. Fd-dependent cyto-chrome 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^8-16:0-ACP desaturase was a more than twofold increase in the amount of 16:1^Δ^8 accumulated by E. coli (Table 2).

The presence of detectable amounts of 16:1^Δ^8 and 18:1^Δ^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 stimulated of 16:1^Δ^8 and 18:1^Δ^8 production by plant Fd sug- gests 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^8-16:0-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 as- say (data not shown). FNR expression, however, had little effect on the amounts of 16:1^Δ^8 and 18:1^Δ^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 16:1^Δ^8 and 18:1^Δ^8 production could not be detected in E. coli expressing the Δ^8-16:0-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 16:1^Δ^8 and 18:1^Δ^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^8-16:0-ACP desaturase were highest in cells induced at 22°C.

### Table 1. Bacterial plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Source or reference</th>
</tr>
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<tbody>
<tr>
<td>pAtFd</td>
<td>pZL1 (Gibco BRL) containing A. thaliana Fd cDNA in NotI-SalI site; Amp^R (GenBank accession no. T14207)</td>
<td>Arabidopsis Biological Resource Center</td>
</tr>
<tr>
<td>pET/Fd</td>
<td>pET3d (Novagen) containing NcoI-BamHI fragment from pAtFd consisting of open reading frame of mature A. thaliana Fd; Amp^R</td>
<td>This study</td>
</tr>
<tr>
<td>pACYC/Fd</td>
<td>pACYC184 (New England Biolabs) containing BglII-HindIII 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 BamHI-HindIII site; Cm^R</td>
<td>This study</td>
</tr>
<tr>
<td>pET/Delta6</td>
<td>pET3d containing coding sequence for the mature T. alata Δ^8-16:0-ACP desaturase cloned into the Ncol site; Amp^R</td>
<td>3</td>
</tr>
<tr>
<td>pKG1022</td>
<td>Contains hok/sok (parB) plasmid stabilization cassette linked to Kan^R marker (aphA; Kan^R) of pKG1022 resulting in loss of Amp^R; Kan^R</td>
<td>6</td>
</tr>
<tr>
<td>pET/BglI</td>
<td>pET3d containing PstI fragment (which consists of hok/sok plasmid stabilization cassette and Kan^R marker) of pKGG1022 result</td>
<td>This study</td>
</tr>
<tr>
<td>pET/Delta6-Fd</td>
<td>pET/Delta6 containing Sall-BamHI fragment (which includes T7 RNA polymerase promoter and coding sequence for mature A. thaliana Fd) of pET/Fd cloned into Sall-BglII site; Amp^R</td>
<td>This study</td>
</tr>
<tr>
<td>pET/FNR</td>
<td>pET23b (Novagen) containing cDNA for Zea mays (maize) root FNR; Amp^R</td>
<td>18</td>
</tr>
<tr>
<td>pLac3a</td>
<td>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 BglII and Xbal sites of pET3a; has the same strong translation initiation site as pET3a; Amp^R</td>
<td>M. Blewitt</td>
</tr>
<tr>
<td>pACYC/Lac3a</td>
<td>pACYC184 analog of pLac3a; BglII-HindIII fragment containing the expression signals from pLac3a inserted between the BamHI and HindIII sites of pACYC184; Cm^R</td>
<td>This study</td>
</tr>
<tr>
<td>pACYC/LacFNR</td>
<td>pACYC/Lac3a containing Xbal fragment from pET/FNR cloned into Xbal site behind lacUV5 promoter; Cm^R</td>
<td>This study</td>
</tr>
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### Table 2. Fatty acid composition of E. coli BL21 (DE3) expressing different plasmids

<table>
<thead>
<tr>
<th>Plasmid(s) carried by E. coli BL21 (DE3)</th>
<th>14:0</th>
<th>16:0</th>
<th>16:1^Δ^8</th>
<th>16:1^Δ^9</th>
<th>18:1^Δ^8</th>
<th>18:1^Δ^9</th>
<th>Other^a</th>
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<tbody>
<tr>
<td>pET3d</td>
<td>5.3</td>
<td>27.8</td>
<td>ND^a</td>
<td>21.1</td>
<td>ND^a</td>
<td>36.6</td>
<td>6.7</td>
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<tr>
<td>pET3d + pACYC/Fd</td>
<td>4.0</td>
<td>34.7</td>
<td>21.2</td>
<td>25.6</td>
<td>25.8</td>
<td>40.0</td>
<td>3.4</td>
</tr>
<tr>
<td>pET3d + Delta6</td>
<td>4.0</td>
<td>34.7</td>
<td>21.2</td>
<td>25.6</td>
<td>25.8</td>
<td>33.7</td>
<td>2.7</td>
</tr>
<tr>
<td>pET3d + Delta6 + pACYC/Fd</td>
<td>4.0</td>
<td>34.7</td>
<td>21.2</td>
<td>25.6</td>
<td>25.8</td>
<td>33.7</td>
<td>2.7</td>
</tr>
<tr>
<td>pET3d + Delta6</td>
<td>4.0</td>
<td>34.7</td>
<td>21.2</td>
<td>25.6</td>
<td>25.8</td>
<td>33.7</td>
<td>2.7</td>
</tr>
<tr>
<td>pET3d + Delta6 + pACYC/Fd</td>
<td>4.0</td>
<td>34.7</td>
<td>21.2</td>
<td>25.6</td>
<td>25.8</td>
<td>33.7</td>
<td>2.7</td>
</tr>
<tr>
<td>pET3d + Delta6 + pACYC/LacFNR</td>
<td>4.0</td>
<td>34.7</td>
<td>21.2</td>
<td>25.6</td>
<td>25.8</td>
<td>33.7</td>
<td>2.7</td>
</tr>
</tbody>
</table>

^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 transfermalization 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.
In addition, it has been noted that expression of the \( \Delta^9 \)-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 \( \Delta^9 \)-16:0-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.

FIG. 1. Gas chromatograms of fatty acid methyl esters of \( E. coli \) BL21 cells transformed with pET3d (A) or cells expressing pDelta6his plus pACYC/Fd (see Table 2) (B) and mass spectra of dimethyl disulfide derivatives of methyl-16:1\( ^\Delta^6 \) (C) and methyl-18:1\( ^\Delta^8 \) (D) from cells expressing the \( T. alata \) \( \Delta^6 \)-16:0-ACP desaturase. The asterisk in panel A indicates an unidentified compound with a mobility similar to that of methyl-18:1\( ^\Delta^8 \) 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 \( \Delta^6 \)-16:0-ACP desaturase at the temperatures indicated. Bacterial cells harboring pDelta6his 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 \( \Delta^6 \)-16:0-ACP desaturase. Each lane contained 15 μg of total protein.
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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REFERENCES


