JOURNAL OF BACTERIOLOGY, Dec. 1994, p. 7320–7327
0021-9193/94/$04.00+0

Vol. 176, No. 23

Alteration of the Specificity and Regulation of Fatty Acid Synthesis of *Escherichia coli* by Expression of a Plant Medium-Chain Acyl-Acyl Carrier Protein Thioesterase

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Received 17 June 1994/Accepted 28 September 1994

The expression of a plant (*Umbellularia californica*) medium-chain acyl-acyl carrier protein (ACP) thioesterase (BTE) cDNA in *Escherichia coli* results in a very high level of extractable medium-chain-specific hydrolytic activity but causes only a minor accumulation of medium-chain fatty acids. BTE's full impact on the bacterial fatty acid synthase is apparent only after expression in a strain deficient in fatty acid degradation, in which BTE increases the total fatty acid output of the bacterial cultures fourfold. Laurate (12:0), normally a minor fatty acid component of *E. coli*, becomes predominant, is secreted into the medium, and can accumulate to a level comparable to the total dry weight of the bacteria. Also, large quantities of 12:1, 14:0, and 14:1 are made. At the end of exponential growth, the pathway of saturated fatty acids is almost 100% diverted by BTE to the production of free medium-chain fatty acids, starving the cells for saturated acyl-ACP substrates for lipid biosynthesis. This results in drastic changes in membrane lipid composition from predominantly 16:0 to 18:1. The continued hydrolysis of medium-chain ACPs by the BTE causes the bacterial fatty acid synthase to produce fatty acids even when membrane production has ceased in stationary phase, which shows that the fatty acid synthesis rate can be uncoupled from phospholipid biosynthesis and suggests that acyl-ACP intermediates might normally act as feedback inhibitors for fatty acid synthase. As the fatty acid synthesis is increasingly diverted to medium chains with the onset of stationary phase, the rate of C_{12} production increases relative to C_{14} production. This observation is consistent with activity of the BTE on free acyl-ACP pools, as opposed to its interaction with fatty acid synthase-bound substrates.

The fatty acid biosynthesis pathway, comprising the sequential condensation of two-carbon units onto the growing fatty acyl chain, is universal. The four enzyme activities necessary for the extension and the acyl carrier protein (ACP) function can reside either as domains in one or two multifunctional polypeptides or as individual entities (16). The multidomain type has been termed the eukaryotic or type I fatty acid synthase and has been isolated from the cytosol of yeast and mammals. In contrast, in plants, *Escherichia coli*, and many other prokaryotes, each fatty acid synthase reaction is catalyzed by a discrete, monofunctional enzyme. In these organisms, the growing acyl chain is bound to ACP, which is itself a soluble polypeptide. This fatty acid synthase version has been termed prokaryotic, or type II. Thus, plant and prokaryotic fatty acid synthases resemble each other in structure, and the plant fatty acid synthase resides in the chloroplast (23), which is considered to be of prokaryotic origin. Therefore, the structural similarities may be due to a common ancestry of plant and prokaryotic fatty acid synthases (14, 17, 20). Currently, it is not known whether type II protein components exist in a dissociated state or whether they are noncovalently combined in a discrete enzyme complex (7, 10, 20, 32).

In plants a specific long-chain thioesterase terminates the acyl elongation process by hydrolysis of the acyl-ACP thioester; free fatty acid is then released from the fatty acid synthase (10). In *E. coli*, the long-chain acyl group is directly transferred from ACP to glycerol-3-phosphate by a glycerol-3-phosphate acyltransferase, and free fatty acids are not normally found as intermediates in lipid biosynthesis. Chain length is determined by the 3-ketoacyl-ACP synthases I and II and the glycerol-3-phosphate acyltransferase (14). As in most other organisms, the major end products of the plant and *E. coli* fatty acid synthase are usually 16- or 18-carbon fatty acids (7, 10).

However, certain plant species synthesize in developing seeds large amounts of predominantly medium-chain (C_8 to C_{14}) fatty acids, which are deposited in triglycerides for long-term carbon storage (25). We isolated a medium-chain acyl-ACP thioesterase (BTE) from predominately laurate (12:0)-producing oilseeds of California bay (*Umbellularia californica*) (9). Its cDNA was cloned and expressed in developing oilseeds of the plants *Arabidopsis thaliana* and *Brassica napus*. The BTE caused the accumulation of large amounts of laurate and, to a lesser extent, myristate (14:0) in the seeds. This demonstrated that a specialized medium-chain thioesterase can redirect a long-chain fatty acid synthase of a plant to medium-chain production (33).

In this report, we describe the expression of the plant enzyme BTE in the prokaryote *E. coli*, the resulting conversion of the *E. coli* fatty acid synthase to a nearly exclusive producer of medium-chain fatty acids, and the resulting impact on *E. coli* phospholipids and growth. In addition, our data allow an in vivo mechanistic study of the BTE's access to its substrates, the medium-chain acyl-ACPs.

MATERIALS AND METHODS

Plasmids: cloning strategy. A 1.2-kb *Xba*I DNA fragment from a full-length BTE cDNA clone (pCGN3822 [33]) was inserted into the plasmid pBS$^-$ (Stratagene) to form pCGN3823. This process resulted in a translational fusion between the N-terminal coding region of *lacZ* and the reading frame coding for the mature portion of BTE (amino acids 84 to

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382 [33]). The 324-amino-acid predicted fusion protein thus contained a 25-residue N-terminal domain coded by the vector.

Bacterial strains: growth. Bacteria were grown in LB (1% Bacto Tryptone, 0.5% Bacto Yeast Extract, 1% NaCl [pH 7]). For plasmid selection, 300 μg of penicillin per ml was added. Liquid cultures were shaken vigorously at the indicated temperatures.

The *E. coli* strains XL1-Blue (5), DH5α [F^- ϕ80d*lacZ*Δ M15 Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17* (r_K^- m_K^+) *supE44* λ^- *thi-1 gyrA96 relA1*], and K12Ymel (referred as Ymel) were used as *fad*$^+$ strains. The *E. coli* fatty acid-degradation mutant strain K27 (*fadD88*) (13) was obtained from the *E. coli* Genetic Stock Center, Yale University (CGSC 5478) and is here referred to as K27 (*fadD*). K27 (*fadD*) is a direct descendent of *E. coli* Ymel, which is a prototrophic wild-type strain (13, 24). In order to obtain pure strains harboring the BTE expression plasmid, primary $CaCl_2$ transformation colonies were resuspended and single colonies were selected after replating on selective medium.

Enzyme assays: lipid analysis. For determination of acyl-ACP hydrolytic activities, sedimented cells were sonicated in BTE assay buffer, and acyl-ACP hydrolysis was measured (8). For fatty acid analysis of total lipids, 4.5-ml samples of liquid cultures were acidified with 200 μl of acetic acid, supplemented with 0.1 mg of pentadecanoic acid as an internal standard, and partitioned with 10 ml of $CHCl_3$-CH_3OH (1:1 [vol/vol] [3]). The organic (lower) layer was rotary evaporated to near dryness, redissolved in 1 ml of 5% (vol/vol) H_2SO_4 in methanol, and incubated in a sealed vial at 90°C for 2 h. Fatty acid methyl esters were extracted with 300 μl of *n*-hexane after addition of 1 ml of 0.9% (wt/vol) NaCl in water. Gas-liquid chromatography was performed with 2 μl of the hexane solution on a Supelcowax 10 fused silica capillary column (Supelco, Bellefonte, Pa.) with a 30-m length, 0.25-μm diameter, and 0.25-mm film thickness. Instrument conditions were typically 42.3 ml/min for helium flow, 180°C for starting temperature (3 min), a 15-min ramp to 240°C, and holding at 140°C for 7 min. The fatty acid methyl esters were detected by flame ionization, all peaks were integrated, quantities were computed with reference to the internal standard, and the appropriate response factors were calculated (4, 29). The identity of the methyl esters of 12:1 and 14:1 in the gas chromatogram were determined by gas chromatography-mass spectrometry of a BTE-expressing bacterial culture extract (gas chromatography and gas chromatography-mass spectrometry data not shown).

For lipid class analysis, cultures were centrifuged to collect the cells. Lipids were extracted from the bacterial pellet with chloroform-methanol (2:1 [vol/vol]) and subjected to two-dimensional thin-layer chromatography (1). Areas containing the respective lipid classes were recovered, the lipids were eluted with chloroform, and the fatty acid compositions were determined (4). For the analysis of lipid A-bound fatty acids, the aqueous fraction of the Bligh-Dyer extract was dried down, and subsequently transesterification proceeded as described above. This procedure transesterifies all ester-bound fatty acids from lipid A, but the amide-linked hydroxymyristate molecules (50% of all hydroxymyristate [27]) are not accessible (data not shown).

RESULTS

Expression of BTE influences growth of *E. coli* cultures. To express BTE cDNA in several *E. coli* strains, we created a translational fusion of the BTE reading frame at a position coding for the N-terminal end of the mature protein by using the modified N-terminal coding sequence of an *E. coli lacZ* gene. A fusion protein with the expected molecular weight was detected in the BTE-transformed cells by anti-BTE antiserum (not shown). Also, a considerable amount of 12:0-ACP thioesterase activity, up to 1,000-fold above the control background level, could be measured in crude extracts of the BTE plasmid-harboring cells. In addition, the 14:0-ACP hydrolytic activity was markedly elevated and a slight increase in hydrolysis of 10:0-ACP was observed. The specificity of this novel activity is precisely that of the BTE purified from seeds (Fig. 1). Also unchanged relative to the seed enzyme were the apparent K_m for 12:0-ACP (1 μM) and the inhibition by 5 mM *N*-ethylmaleimide (9 [*E. coli* data not shown]). These results confirmed that no plant-specific factor or protein processing was necessary for authentic enzymatic competence of the BTE polypeptide.

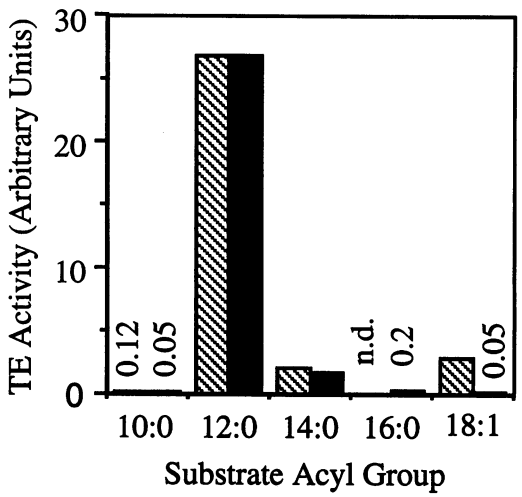

FIG. 1. Substrate specificities of BTE purified from California bay seed and expressed in *E. coli*. Bay seed enzyme (hatched columns) was purified through the ACP column step (9). At this stage of purification, the preparation was contaminated with residual 18:1-ACP thioesterase activity. BTE-transformed XL1-Blue cultures (solid columns) were grown at 37°C in LB to an optical density of 1 at 550 nm, and then they were grown for 2 h with 0.4 mM IPTG (isopropyl-β-D-thiogalactopyranoside) added. In order to compare the substrate specificies, the respective activity data were transformed into arbitrary units, achieving equal heights for 12:0 hydrolysis. In comparison with BTE lysates, lysates of control cultures (*E. coli* transformed with the vector alone) hydrolyzed acyl-ACPs with increasing activity as the chain length was increased from 10:0 to 18:1 (33), and the activities of an equal amount of lysate were very low compared with those of BTE-transformed cultures (10:0, 0.012; 12:0, 0.016; 14:0, 0.02; 16:0, 0.046; 18:1, 0.047). TE, thioesterase; n.d., not determined.

In liquid culture, we monitored the effects of BTE expression on bacterial growth at 30 and 37°C. During the log phase, growth of the BTE cultures was unimpaired relative to that of the respective controls in all strains tested. Most significantly, in the fatty acid degradation-deficient strain K27 (*fadD*) (13, 24), BTE expression affected cultures in the stationary phase and when grown on solid media. BTE-transformed cultures grown at 30°C reached stationary phase at about half the cell density of controls and accumulated a substantial amount of a methanol-soluble precipitate. At 37°C, the cultures never entered a stable stationary phase, but the titer of the plasmid-containing bacteria (as measured by antibiotic-resistant CFU) was arrested at 5×10^8 CFU/ml and subsequently dropped by several orders of magnitude during the following 5 h. In the

same time period, plasmid-free cells overgrew the cultures (data not shown).

On plates, colonies of the BTE-transformed strains grew more slowly than the respective controls and after prolonged incubation developed foci with faster growth. Especially, the colonies of BTE-transformed K27 (*fadD*) deposited large quantities of a granular material, and at 37°C growth was severely retarded. At this temperature, we observed extensive plasmid curing, and bacterial strains with altered colony phenotypes appeared (data not shown). Because the K27 (*fadD* BTE transformants were unstable at 37°C, all of the following analyses were conducted with cultures grown at 30°C.

BTE produces medium-chain fatty acids in *E. coli*. Fatty acids (as acyl groups) constitute about 8% of the total dry weight of *E. coli* cells and essentially are all found in membrane lipids (18). *E. coli* fatty acid composition is tightly regulated and is tuned to the temperature and age of the culture (6, 11). Under all conditions, laurate is found only in trace amounts; it is a minor fatty acid constituent of lipid A (27).

Normal *E. coli* strains are adapted for utilization of a medium rich in free fatty acids and carry an inducible fatty acid degradation pathway. After import, the fatty acids are activated by formation of coenzyme A thioesters, which can be used in β-oxidation or for lipid synthesis (2, 19). Therefore, it was conceivable that BTE-generated, intracellular free fatty acids could be immediately recycled in this way, leading to a diminished phenotype in *fad*$^+$ strains. In order to observe BTE's impact on the bacterial fatty acid synthase without interference from fatty acid degradation, we also established the BTE expression plasmid in *E. coli* K27 (*fadD*), a strain lacking acyl coenzyme A synthetase. This strain is unable to utilize free fatty acids when they are supplied in the medium (13, 24).

In Fig. 2, we compare the total fatty acyl compositions of stationary cultures of vector-transformed control strains with those of strains harboring the BTE expression plasmid. In Ymel (*fad*$^+$ [Fig. 2A]) and K27 (*fadD* [Fig. 2B]), vector-transformed controls produced fatty acyl profiles expected for *E. coli* grown at the respective temperature, with palmitate as the major component (6). BTE expression did not affect the total fatty acyl content in Ymel cultures (Fig. 2A), but it changed the composition significantly. The laurate content rose severalfold, and the myristate level was much reduced. The unsaturated medium-chain fatty acid 12:1, usually present only in traces, became prominent. Vaccenate (*cis*-11-octadecenoic acid [18:1]) replaced palmitate as the most prominent fatty acid in the culture. The appearance of 12:1 fatty acid suggests that BTE can act on 12:1-ACP, an intermediate absent from higher plants but available in the unsaturated fatty acid synthesis pathway of *E. coli* (7).

In contrast to the findings with Ymel, cultures of the BTE-expressing Ymel derivative K27 (*fadD*) produced much more fatty acid than the control. This dramatic increase, now in the absence of fatty acid degradation, was caused exclusively by the overproduction of medium-chain fatty acids, especially laurate (Fig. 2B). Laurate levels increased about 500-fold, while myristate levels increased to about 5% of the laurate levels. The ratio of 12:0 to 14:0 accumulation very closely reflected the in vitro hydrolytic specificity of BTE; its activity on 14:0-ACP is 5% of that on 12:0-ACP (Fig. 1). Also, 12:1 and 14:1, which were very minor components in the control cultures (Fig. 2B [also see Fig. 5C and E]), accumulated to considerable levels upon BTE expression, surpassing even those of the resident long-chain unsaturated fatty acids. Taking the mechanism of *E. coli* desaturation into account (7), the BTE, by intercepting the elongation of unsaturated acyl-ACP

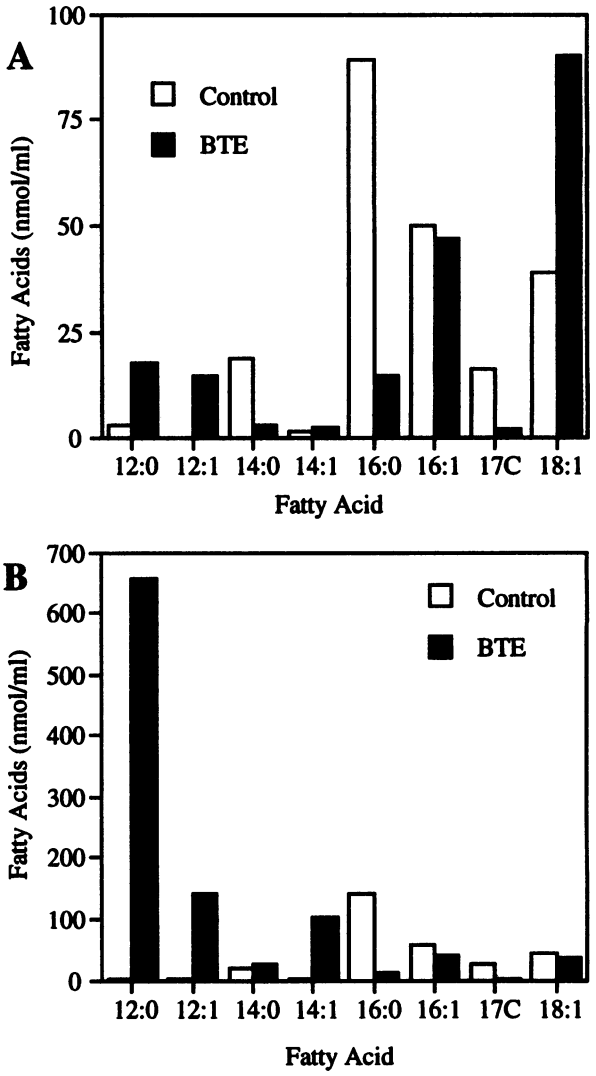

FIG. 2. Fatty acid composition of total cultures. Liquid cultures were grown overnight at 30°C, and subsequently the total fatty acyl composition of 4.5-ml cultures (cells and medium) was determined. (A) Bar graph display of the quantitated fatty acids found in Ymel (*fad*$^+$) transformed with the vector alone or with the BTE expression plasmid. (B) Total culture fatty acid compositions of K27 (*fadD*) transformed with the vector alone or with the BTE expression plasmid.

substrates, probably caused the accumulation of *cis*-5-dodecenoic acid (12:1) and *cis*-7-tetradecenoic acid (14:1). The significant differences in production rates of the four medium-chain fatty acids might reflect the different activities of BTE with the respective substrates, with 12:0-ACP hydrolysis being the most active (26). The expression of BTE also induced the appearance of several unidentified new compounds, as evidenced by the appearance of new peaks in the gas chromatogram of fatty acid methyl esters (results not shown).

When K27 (*fadD*) harboring the BTE expression plasmid was grown on solid medium, crystals appeared in the colonies and at the surface of the cell-free agar matrix (Fig. 3). These deposits were separated from bacteria, and their major fatty acid component was identified as laurate salts by fast atom bombardment-mass spectrometry (data not shown). Phase-contrast microscopic examinations of the cultures did not show

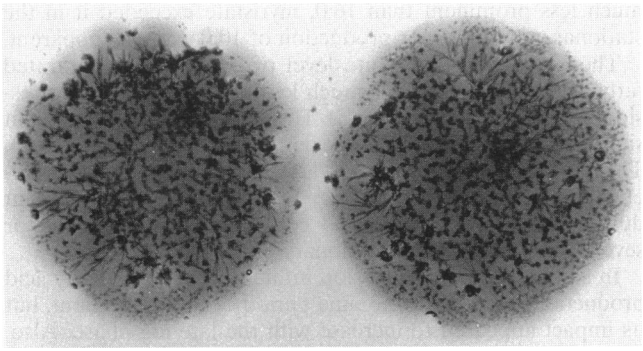

FIG. 3. Laurate crystals produced by *E. coli* BTE colonies. Colonies of K27 (*fadD*) BTE transformants that had been cultured on agar at 25°C for several days were photographed under transillumination. Two colonies are shown, each approximately 1 mm in diameter. The dark granular and needle-shaped structures in the colonies are predominantly laurate salts. Control colonies showed no internal detail under the same conditions.

any fragmented cells or any bacteria with intracellular granules. Also, the absence of significant amounts of protein in the medium indicated that the bacterial cells had stayed intact. We concluded that the bacteria probably secreted the free fatty acids into the medium by an undetermined mechanism, potentially by simply redirecting *fadL* (for a review of fatty acid transport, see reference 2), and upon reaching saturation, extracellular laurate salts crystallized.

BTE expression causes a shift to higher membrane lipid unsaturation. The major lipid components of *E. coli* membranes are phosphatidylethanolamine and phosphatidylglycerol, which contain predominantly long-chain fatty acyl moieties. Cardiolipin and lipid A account for minor fractions (7). In order to investigate whether the efficient interception of long-chain fatty acid biosynthesis influences the composition of the *E. coli* membrane lipids, we separated the latter from the free fatty acids by two-dimensional thin-layer chromatography.

When K27 (*fadD*) cultures were grown at 30°C, palmitate (16:0) was the most prominent phospholipid acyl group (Fig. 4), consistent with fatty acid profiles of other *E. coli* strains (15). After BTE expression, even though medium-chain fatty acids were produced in large excess (Fig. 2B), essentially none was incorporated into the phospholipids (Fig. 4). This is not surprising, because free fatty acids do not serve as substrates for lipid biosynthesis (24). In the phospholipid fraction, myristate and palmitate were reduced more than twofold, the proportion of palmitoleic acid (taken together with its cyclopropane derivative, *cis*-9,10-methylene hexadecenoic acid [17C]) was unaffected, and vaccenic acid became predominant. This trend to more unsaturated fatty acid accumulation was already observed with the total fatty acid profile of the *fad*$^+$ strain Ymel (Fig. 2A).

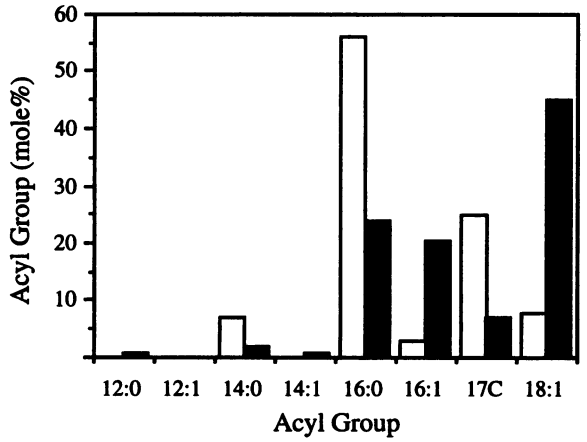

FIG. 4. Lipid composition of control and BTE-transformed K27 (*fadD*) cultures. Lipids from stationary cultures were isolated as described above. The fatty acid compositions of the phospholipids extracted from control (open columns) and the BTE-expressing strain (solid columns) are shown.

Typically, such a degree of phospholipid unsaturation can be induced by growing *E. coli* at 10°C (15), and it is believed to be a mechanism for maintaining proper membrane fluidity over a wide range of temperatures. The temperature stayed constant in our experiments, so the shift from palmitate to vaccenate as the predominant phospholipid fatty acid must have been caused by other factors. All of our data support the interpretation that the reduction of phospholipid-bound 14:0 and 16:0 resulted from the nearly complete redirection of saturated fatty acid biosynthesis from supplying acyl groups for lipids to the production of free laurate and myristate. Even though BTE interception of the unsaturated pathway was significant, the obvious scale-up of unsaturated fatty acid production by about threefold (Fig. 2B) allowed the nearly unimpaired synthesis of unsaturated acyl groups for lipid biosynthesis (as we observed [described below]).

BTE expression uncouples fatty acid production from phospholipid synthesis. Our data show that BTE expression drastically increased the total fatty acid production of the cultures. We also analyzed control and BTE-transformed K27 (*fadD*) cells at intervals during the growth cycle (Fig. 5). We monitored bacterial cell titer (CFU per milliliter) and fatty acid accumulation through 1 order of magnitude of exponential growth and into the stationary phase until no further increase in fatty acid accumulation could be observed.

Figure 5A correlates cell titer (solid symbols) with fatty acid accumulation (open symbols) of control and BTE-expressing strains. Growth during the log phase was not affected by BTE, and the culture reached the stationary phase at a titer twofold lower than that of the control. The BTE culture had a higher fatty acid content at all times, but the difference became much more pronounced with the onset of the stationary phase.

We also monitored the accumulation of each fatty acid in both cultures during the time course. In the control culture (Fig. 5B, saturated fatty acids; Fig. 5C, unsaturated fatty acids), palmitate was always by far the most prominent saturated fatty acid (Fig. 5B), with myristate and laurate about 1 and 2 orders of magnitude lower, respectively. The long-chain unsaturated fatty acids 16:1 and 18:1 were the two most prominent unsaturates, while medium chains existed only in traces (Fig. 5C). The results obtained with the BTE-expressing culture were completely different (Fig. 5D and E). Laurate was the most prominent saturated fatty acid at all sampling times (Fig. 5D). Initially about twice the palmitate level, it increased drastically during the late log phase and continued to accumulate for several hours into the stationary phase to a level 100 times higher than that of palmitate. Palmitate accumulation in the BTE culture appeared to be unaffected during the log phase, but its production was depressed drastically during the late log phase and all of the stationary phase. This suppression led to a nearly 10-fold reduction in the level of palmitate compared with that of the control. The accumulation kinetics for myristate approximated those of laurate at lower levels. Initially

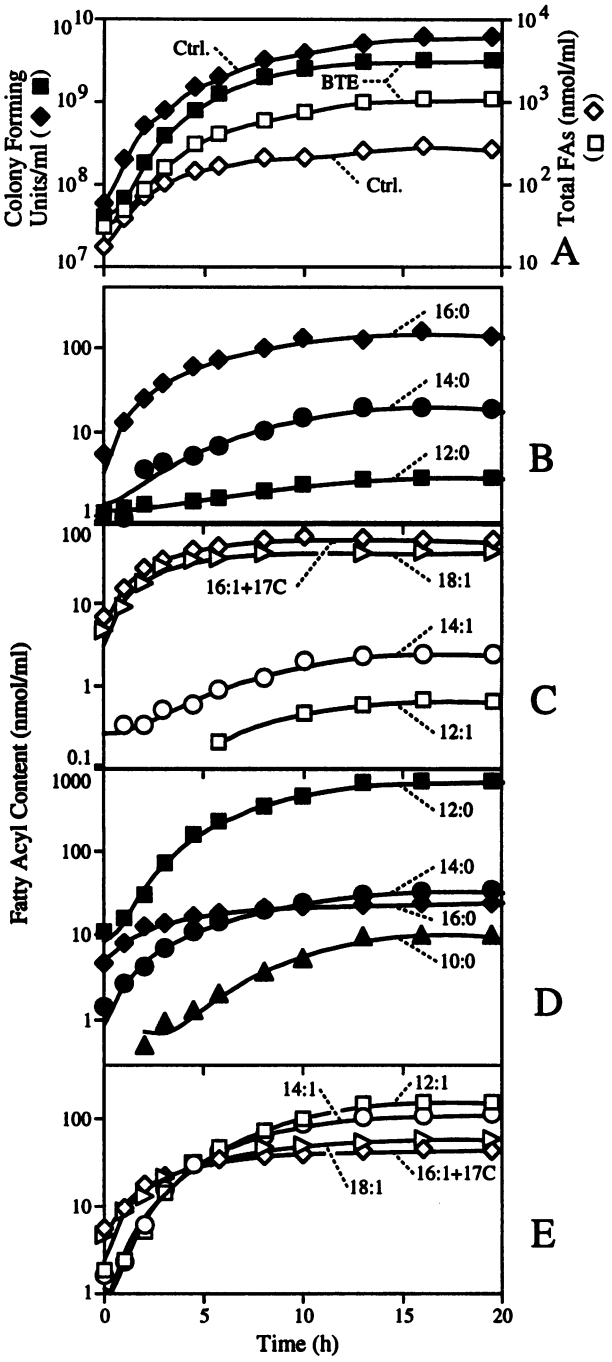

FIG. 5. Impact of BTE on growth and fatty acid (FA) composition of transformed bacterial cultures. Colonies of K27 (*fadD*) were resuspended in LB and grown under continuous shaking at 30°C for 2 h in order to enter the phase of exponential growth (time zero). Samples were drawn at indicated times. Bacterial titers (CFU) were determined by plating on antibiotic-free medium. Through the duration of the experiment, >99% of all colonies displayed the BTE phenotype (Fig. 3), indicating the presence of the BTE plasmid. Lipids were extracted from 4.5 ml of complete culture (cells and medium) in triplicate. The figure shows the averages of the triplicate primary data. (A) Bacterial titers and total fatty acid contents per milliliter of culture. (B to E) Quantitation of individual fatty acids. (B and C) Control. (D and E) BTE transformant. Ctrl., control.

much less prominent than 16:0, myristate exceeded it in the stationary phase. Minor production of 10:0 was also apparent.

The kinetics and absolute level of long-chain unsaturated fatty acid production were much less affected by BTE expression (compare Fig. 5C with 5E). The twofold lower levels might simply reflect the lower cell titer of the BTE culture (Fig. 5A). The medium-chain fatty acids 12:1 and 14:1, minor constituents in the control culture (Fig. 5C), became very prominent during the late log phase and continued to accumulate for several hours during the stationary phase (Fig. 5E).

In summary, BTE expression influenced bacterial fatty acid production throughout the time period of the experiment, but its impact appeared to increase with the late log phase. Also, fatty acid production continued for a longer period but ultimately ceased after several hours in the stationary phase. Most of the medium-chain accumulation occurred after the slowdown of bacterial replication.

In order to show this extended fatty acid production in the BTE-expressing culture more clearly, we determined the fatty acid synthesis rate over time. For this purpose, we assumed that in the absence of fatty acid degradation, the rate of fatty acid accumulation (as derived from data in Fig. 5) directly measured the rate of biosynthesis. In the control culture, the rate of fatty acid synthesis per cell fell sharply to almost 0 during the late log phase. In the BTE transformant, total fatty acid synthesis appeared not to be elevated much during exponential growth, but the cells reduced their fatty acid production at a much lower rate. Only after more than 5 h in the stationary phase had the production of new fatty acids essentially ceased (not shown).

In the BTE-transformed culture, laurate production represented about 50% of total saturated fatty acid biosynthesis during exponential growth, and it rapidly increased during the late log phase to more than 90%. The relative myristate synthesis rate dropped severalfold during this period. Because essentially no laurate and myristate were found in the lipid fraction in mature cultures (compare Fig. 2B with Fig. 4), practically all of the saturated medium-chain accumulation in the late log phase was caused by BTE's action. The action of BTE on the 12:0- and 14:0-ACP pools must have intercepted the saturated fatty acid pathway up to nearly 100% during this time period, decreasing the relative 16:0 production rapidly from 40% of the saturated pathway to almost 0. The proportion of 12:1 plus 14:1 increased from about 10% of the total unsaturated synthesis rates in the log phase up to nearly 90% in the early stationary phase. Because 12:1 and 14:1 were found essentially only as free fatty acids (Fig. 2B and 4), their values directly reflect BTE's interception of the unsaturated pathway.

We were also interested in studying the mechanism by which BTE redirects the resident fatty acid synthase to the production of medium chains. Currently, there are two different models under discussion (20, 21, 32). In one view, the medium-chain thioesterase simply acts on pools of freely diffusible acyl-ACPs. In this model, BTE has to compete with the ketoacyl-ACP synthases for the acyl-ACP intermediates (Fig. 6A), and the synthesis ratio of 12:0 to 14:0 should be dependent on the extent of interception at the 12:0 stage. This can be illustrated by kinetic simulation of such a pathway (8); the results of three simulations are shown in Fig. 6B. For each simulation, we specified K_m values for BTE acting on 12:0- and 14:0-ACP substrates (X and Y in Fig. 6A; see the actual ratios in Fig. 6B), and the concentration of BTE was then varied at a fixed rate of 12:0-ACP production. As expected, varying the concentration of BTE over a wide range caused a change in 12:0-ACP interception from nearly 0 to almost 100%. Starting from low interception levels, the 12:0/14:0 production ratio

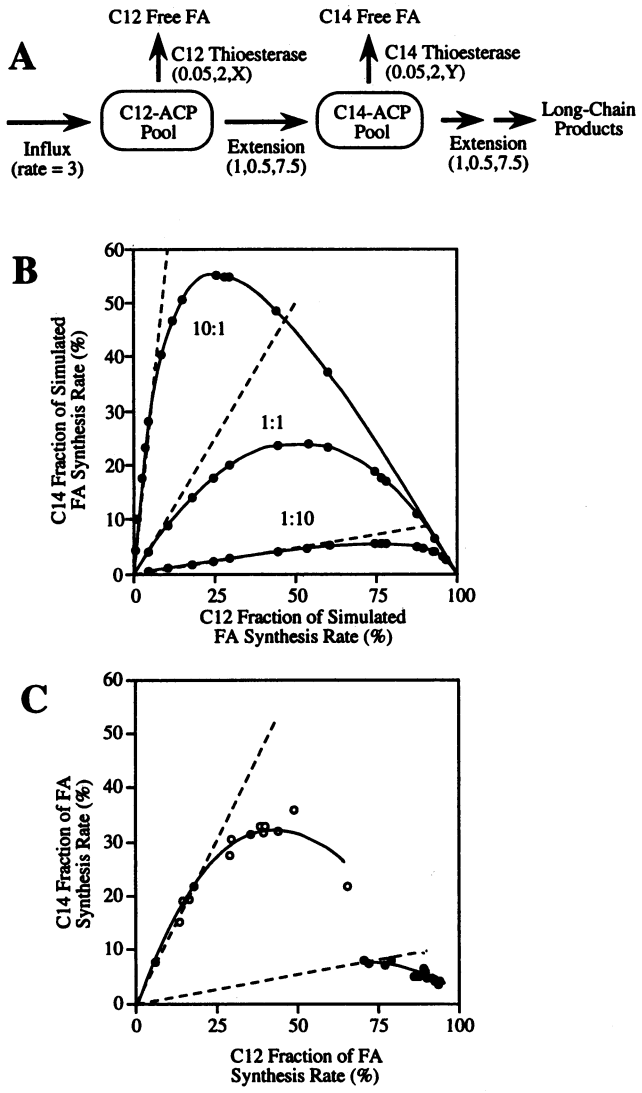

FIG. 6. Sequential action of BTE on the saturated and unsaturated fatty acid (FA) synthesis pathways. (A) Simulation of BTE action on C_{12}- and C_{14}-ACP substrates. The BTE is assumed to act on free pools of these substrates in competition with the fatty acid elongation cycle, here represented as a single reaction (8). For each thioesterase or elongation reaction, the values in parentheses indicate enzyme concentration, V_{max} and K_m (X and Y respectively). Michaelis-Menten kinetics are assumed throughout, and all units are arbitrary. [The kinetic constants are shown producing data for the lowest rate of C_{12} formation. Three sets of K_m ratios were used: K_m (C_{12}):K_m (C_{14}) ratios were 10:1, 1:1, and 1:10.] The simulation was constructed with Stella flux modeling software (High Performance Systems, Hanover, N.H.) running on a Macintosh SE/30 computer (Apple Computer, Cupertino, Calif.). Computation was by the Euler procedure, with a step time of 0.01. All progress curves were free of oscillations, and the use of smaller step times gave identical results. Simulations were run until the progress curves became linear, at which time the steady-state rates of C_{12}, C_{14}, and long-chain fatty acid production were noted. The BTE concentration was then progressively multiplied to simulate increased expression of the enzyme or decreased relative fatty acid synthase activity in order to obtain the rate data shown in panel B. The location (relative to the C_{12} and C_{14} axes) of the inflection point is determined by the C_{12}/C_{14} ratio of the thioesterase activities. Dotted lines in panels B and C represent the expected production ratios if the bacterial fatty acid synthase and BTE acted as a metabolon. Solid lines represent the pathway simulation. (C) Fatty acid synthesis rates observed with BTE expression in K27 (*fadD*). The rates of fatty acid production were derived from accumulation data of the total time course of the experiment (Fig. 5) by first subtracting adjacent fatty acyl contents from each other (average synthesis rates during the respective time period). Synthesis rates were recalculated to yield the fractions of the total output of the saturated (●) and unsaturated (○) fatty acid synthesis pathways for the respective fatty acids. This resulted in the average relative synthesis rates in the respective time periods. The C_{12} fractions of the fatty acid synthesis rates are plotted against the C_{14} fractions.

stayed constant until increasing 12:0-ACP interception caused the C_{14} rate to decrease. For each simulation, a specific inflection point was reached, with any further increase in 12:0-ACP interception then diminishing the rate of 14:0 production.

Alternatively, the so-called "metabolon" model (32) proposes an associated, multienzyme complex for the type II fatty acid synthase, functionally resembling the eukaryotic type I multidomain polypeptide. In such a situation, a specific thioesterase polypeptide associates with an individual fatty acid multienzyme (or metabolon) synthase during the fatty acid extension cycle. The growing acyl-ACP is presented to the thioesterase after each round of extension. Presumably, this mechanism applies to mammalian medium-chain fatty acid biosynthesis, in which a discrete medium-chain thioesterase polypeptide releases fatty acids from the multidomain type I fatty acid synthase (30). When applying the metabolon model to the interaction of BTE with the fatty acid synthase of *E. coli*, the BTE would cleave the thioester at the 12:0-ACP stage (most frequently) or one round of extension later at the 14:0-ACP stage. The ratio of 12:0 to 14:0 products would be determined solely by the invariant specificity of the BTE for 12:0-ACP relative to 14:0-ACP, because there would be no pools of free acyl-ACPs to contribute to the hydrolysis rates. This model therefore predicts that the ratio of 12:0 to 14:0 production would stay constant, no matter how much of the total acyl-ACP flux was diverted to the production of medium-chain fatty acids (Fig. 6B, dotted lines).

In BTE-transformed cells, the saturated and unsaturated 12:0- and 14:0-ACPs can serve as substrates for (i) chain elongation (subsequently accumulating as long-chain acyl groups of lipids), (ii) hydrolysis by BTE (diversion to a free fatty acid), or (iii) lipid formation. Because we intended to use total fatty acid accumulation data for testing the two models of BTE action, we had to consider the extent to which medium-chain acyl groups were found in lipids (option iii). We found that after BTE expression, practically all of the 12:0 accumulated as free fatty acid, with the phospholipid and lipid A contents of 12:0 remaining negligible (Fig. 4 [lipid A data not shown] [27]). Only after the colonies entered the late log phase was the total myristate level high enough so that most of it was present as free fatty acid (compare 14:0 and 16:0 levels in Fig. 2B and 4). (More than 95% of 16:0 was found in phospholipids [data not shown].) The unsaturated medium chain fatty acids 12:1 and 14:1 were found only in trace amounts in bacterial lipids (compare Fig. 2B with 4; also, no 12:1 or 14:1 was found in lipid A). Because acyl elongation was the only reaction competing with BTE in the unsaturated pathway, practically all 12:1 and 14:1 was made by BTE over the complete time course of the experiment.

Data obtained from the expression of BTE in *E. coli* clearly support the free acyl-ACP pool model. As discussed earlier, the relative rate of laurate production in the cultures climbed from about 50% of total saturated fatty acids during the log

phase to more than 90% during the transition to the stationary phase. In Fig. 6C, we use the data obtained in Fig. 5 to calculate the acyl fluxes for each time period and plot the experimentally determined fluxes in the same manner as the kinetic simulations of Fig. 6B. There was a curved relationship between the rates of 12:0 and 14:0 accumulation as the thioesterase activity increased and as a greater proportion of the total fatty acids being made became laurate (Fig. 6C). This result resembles the simulation of BTE action on free acyl-ACP pools and differs from the metabolon model, which predicts a fixed relationship between the rates of 12:0 and 14:0 production in such circumstances, the constant slope of the line being determined by the relative activities of the thioesterase on 12:0-ACP and 14:0-ACP (Fig. 6B).

The mechanism for BTE's redirection of the unsaturated fatty acid pathway appears to be very similar (Fig. 6C). At a low diversion of the pathway to 12:1 and 14:1, the 12:1/14:1 production ratio stayed fairly constant. But when the 12:1 diversion approached and exceeded 50%, progressively less 14:1 was produced relative to 12:1. Again, this behavior contradicts the metabolon model and is consistent with the action of BTE on free acyl-ACP pools.

DISCUSSION

By expressing BTE in transgenic plants (33) and now in *E. coli*, we have demonstrated that a plant medium-chain acyl-ACP thioesterase can redirect the type II fatty acid synthase of plants and bacteria from long-chain to medium-chain production. This contrasts with the apparent failure of a mammalian medium-chain thioesterase to impact the fatty acid synthase of plants (28) and *E. coli* (17). Together, these results from interkingdom switching of fatty acid synthase components further the idea that plant and bacterial type II fatty acid synthases are functionally related and are distinct from type I. In transgenic plants, BTE produced mainly 12:0 and about 10 times less 14:0 (33); in *E. coli*, this relationship was similar, with 14:0 being produced at about 5% of the level of 12:0. Because of the difference in the pathways for biosynthesis of unsaturated fatty acids in plants and in *E. coli* (7, 10), the expression of BTE in the latter also led to the production of 12:1 and 14:1 not found in transgenic plants.

The full extent of the BTE interference with *E. coli* fatty acid biosynthesis only became apparent in a bacterial strain unable to metabolize free fatty acids. In the K27 (*fadD*) strain, BTE-produced medium-chain free fatty acids could not be recycled, and they therefore accumulated to very high levels, exceeding the resident long-chain fatty acid production by almost 1 order of magnitude. Similar results were obtained in other genetic backgrounds. The *fad*$^+$ strains DH5α and XL1-Blue produced very little 12:0 (Fig. 1), while a strain with a different *fad* mutation, K19 (*fadE* [13]), replicated the K27 results (not shown). The striking differences between the fatty acid phenotypes obtained in *fad* versus *fad*$^+$ strains is therefore correlated with the absence or presence of a functional β-oxidation pathway for free fatty acids.

The nearly complete redirection of the saturated pathway to free fatty acids must have starved the cells for saturated acyl-ACP substrates needed for lipid biosynthesis. This is reflected in the significant reduction of myristate and palmitate in the phospholipid composition of the cultures (Fig. 4). The depletion of saturated acyl-ACP intermediates of C_{12} or greater chain length (22) or a resulting reduction of saturated acyl groups in phospholipids might have triggered an uncoupling of the fatty acid synthase from phospholipid synthesis. This would explain the extended commitment of fatty acid synthase activity into the stationary phase (Fig. 5). Such an uncoupling was shown recently by constructing an *E. coli* strain that produced fatty acids in the absence of phospholipid biosynthesis, albeit at a reduced rate (12). These authors argue that accumulation of long-chain acyl-ACP species might inhibit a key fatty acid synthetic enzyme(s) and thereby regulate flux via a feedback system. They also mention that expression of the periplasmic *E. coli* thioesterase I (31) in the cytoplasm also causes the production of large amounts of free fatty acids in the cultures (Cho and Cronan as cited in reference 12), which would be homologous to our results.

Diversion of much of the 12:0-ACP to free 12:0 would be expected to severely limit the supply of essential 16:0, and we can consider how the fatty acid biosynthesis pathways might respond to this problem. An overall increase in the common flux into both saturated and unsaturated pathways might restore 16:0 production but at the price of excessive production of unsaturated fatty acids. Regulation at the branching point of the saturated and unsaturated pathways, i.e., at the C_{10} β-hydroxyacyl-ACP intermediate (β-hydroxyacyl-ACP dehydrase; FabA [7]) might correct for this, enabling most of the increased flux to pass into the saturated branch. We observed a substantial change in the ratio of total saturated to unsaturated acyl groups (from 1/1 to 2/1), suggesting that this diversion was taking place. However, the resulting production of 16:0 was still insufficient to maintain the normal phospholipid composition, which shifted markedly toward unsaturated acyl groups.

It is possible that the fatty acid synthase enzymes catalyzing the acyl extension cycles are organized in a complex or metabolon and that the ketoacyl-ACP, hydroxyacyl-ACP, and enoyl-ACP intermediates of those cycles are transferred between them without the formation of significant free pools. However, the changing ratio of C_{12} to C_{14} production rates that we observed during the growth cycle is consistent with an action of BTE on free acyl-ACP pools in both the saturated and unsaturated pathways. Our results suggest that the acyl-ACP intermediates of medium-chain length do accumulate to the extent that they can be acted upon by the introduced BTE and that if an association between the BTE and enzymes of the fatty acid synthase does take place, it is certainly not obligatory. This illustrates how the introduction of BTE may be used as a novel kind of probe to perturb fatty acid biosynthesis in vivo to gain insight into pathway dynamics. Such studies have recently been extended by demonstrating that BTE depletes the acyl-ACP pools of C_{12} and greater acyl chain lengths and that its action results in the induction of acetyl coenzyme A carboxylase, an enzyme considered to be rate limiting and regulatory for fatty acid biosynthesis (22).

ACKNOWLEDGMENTS

We are indebted to Johanna Dimento for the analysis of phospholipids, Ed Cahoon (Michigan State University, East Lansing) for help with the development of the bacterial lipid extraction procedure, A. D. Jones (University of California, Davis) for generating the mass spectrometry data, and John Cronan (University of Illinois, Urbana) for advice. We thank colleagues at Calgene, Inc., and John Ohlrogge (Michigan State University, East Lansing) for critically reading the manuscript.

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