Selection and Properties of *Escherichia coli* Mutants Defective in the Synthesis of Cyclopropane Fatty Acids

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Mutants of *Escherichia coli* K-12 defective in the synthesis of cyclopropane fatty acids (CFA) have been selected and isolated by a L-[*methyl-³H*]methionine suicide procedure. Two mutants were isolated. Stationary-phase cultures of both mutants contain <0.7% of the CFA content found in the parental strain. The CFA deficiency is attributed to a deficiency of CFA synthetase activity. Extracts of both mutants contain less than 10% of the CFA synthetase activity found in extracts of the parental strain. Experiments in which parental and mutant extracts were mixed indicate that the lack of activity in the mutant strains is not due to an inhibitor of CFA synthetase present in the mutant extracts. We have not yet detected a physiological phenotype for these mutants. These strains grow normally at various temperatures in a variety of media. We have tested survival (colony-forming ability) in response to (i) prolonged incubation in stationary phase, (ii) exposure to drying, and (iii) exposure to detergents, heavy metals, low pH, high salt concentration, and a variety of other environmental conditions. The survival of both mutants is identical to that of the parental strain under all conditions tested. The compositions (excepting the CFA deficiency) and metabolic turnover rates of the phospholipids of both mutant strains are indistinguishable from those of the wild-type strain. The transport of several amino acids also seems normal in these mutants.

Cyclopropane fatty acids (CFAs) have been found in a wide variety of bacterial species (for review, see reference 13), in protozoa (4), in some seed oils and plant leaves (4, 17), in females of spirostreptid millipedes (27), and in sheep rumen tissues (3). In bacteria these acids have been demonstrated by Law and co-workers (18, 19) to be formed by the transfer of the methyl group of S-adenosyl-L-methionine (SAM) to the double bond of an unsaturated fatty acid moiety of a phospholipid (for review, see reference 18). In *Escherichia coli* and other species of bacteria, this conversion from unsaturated to CFA has been shown to occur preferentially in the late exponential and stationary growth phases and results in most of the unsaturated phospholipid acyl moieties being converted to CFA (6, 19). Despite the widespread occurance and the timed appearance of CFAs, their physiological role in bacteria remains a mystery. Some of the roles suggested have been: (i) prevention of catabolic degradation of fatty acids in stationary phase (6), (ii) as a sink for methyl groups (8), and (iii) in chemotaxis or transport (1, 5) (since SAM is thought to be involved in these membrane-localized events). Kito and co-workers (16) have suggested that

CFAs modify the fluidity of the membrane; however, physical measurements make this hypothesis unlikely (23, 26). A more common suggestion is that the cyclopropane group protects unsaturated fatty acids from oxidation or other chemical destruction (18).

In this paper we report the isolation of mutants of *E. coli* that are unable to form CFAs owing to a lack of the biosynthetic enzyme, CFA synthetase. These mutants were used to test various hypotheses concerning the role of CFAs in cell physiology.

MATERIALS AND METHODS

Bacterial strains. Strain FT1 has the following relevant genotype: F⁻, *proC32*, *purE42*, *metE70*, *metB1*, *lysA23*, *thi-1*, *trpE38*, *str-109*. This strain was derived from strain X478 (2) by mating with strain CY78 (8) and selecting for Leu⁺ streptomycin-resistant recombinants. These recombinants were scored for strains requiring either both vitamin B12 (the *metE* phenotype) and homocysteine (the *metB* phenotype) or L-methionine for growth.

Strains FT16 and FT17 are CFA⁻ derivatives of strain FT1 derived by the [*methyl-³H*]methionine suicide selection reported below.

Media. The minimal medium used was medium E (28) supplemented with glucose or glycerol (0.4%),

thiamine (1 μg/ml), and the required L-amino acids and adenine sulfate at 100 μg/ml each. The R broth medium has been described previously (21).

Strain FT1 was treated with N-methyl-N-nitro-N-nitrosoguanidine as described by Oeschger and Berlyn (21) and grown overnight in minimal medium at 30 C. The cells were diluted 100-fold in 10 ml of R broth and grown to about 5×10^8 cells/ml at 30 C. The cells were collected by centrifugation, washed twice with medium E, and suspended in 10 ml of medium E supplemented only with glucose (0.4%), L-valine (100 μg/ml), and DL-serine hydroxamate (100 μg/ml). After 20 min of shaking at 37 C, the culture was divided into two portions. One portion of the culture was supplemented with 2 μg of L-[methyl-^{3}H]methionine per ml (11 Ci/mmol). The second portion received 2 μg of nonradioactive L-methionine per ml. Both cultures were shaken at 37 C for 2 h, filtered on a 0.45-μm membrane filter (Millipore Corp.), and then washed with 10 ml of medium E followed by 10 ml of cold distilled water. The washed cells were suspended in 5 ml of medium E and stored at 4 C. After various intervals of storage, the cell suspension was plated for viable cells on R broth plates at 30 C.

Enzyme preparation. Cultures were grown on minimal medium at 37 C to the stationary growth phase and disrupted in 0.1 M tris(hydroxymethyl)-aminomethane-chloride (pH 8.0) in a French pressure cell operated at 18,000 lb/in². Unbroken cells were removed by centrifugation at $10,000 \times g$ for 10 min. Protein was determined by the biuret procedure (9).

CFA synthetase assay. The assay mixture (total volume, 0.2 ml) contained: 0.05 M tris(hydroxymethyl)aminomethane-chloride, pH 8.0, 0.5 mM [methyl-^{3}H]SAM (25 μCi/μmol); 1 mg of a sonicated dispersion of Azotobacter agilis phospholipids per ml (which lack CFA); 1 to 25 mg of enzyme protein per ml; and 0.1 U of an S-adenosyl-L-homocysteine hydrolase from E. coli (10). This hydrolase cleaves S-adenosyl-L-homocysteine to adenine and ribosylhomocysteine (but has no activity on SAM) and was added to prevent possible product inhibition of the enzyme by S-adenosyl-L-homocysteine. The reaction was incubated at 37 C for 30 min. Incorporation of [^{3}H]methyl groups into lipid was then determined by a modification of the filter disk assay of Goldfine (12). Samples of the incubated reaction mixture were pipetted onto Whatman 3MM filter disks, dried in a stream of hot air, and immersed in a solution of 10% trichloroacetic acid. The filters were then placed in boiling 5% trichloroacetic acid for 4 min and washed with 5% trichloroacetic acid and twice with distilled water (each wash was for 15 min). The filters were dried, and the radioactivity was determined in Aquasol scintillation fluid (New England Nuclear Corp.). A unit of activity is 1 pmol of CFA formed per min.

The S-adenosyl-L-homocysteine hydrolase preparation was purified through the first ammonium sulfate step described by Duerre (10). The active fraction was desalted and adsorbed to a hydroxyapatite (Bio-Gel HTP) column which had been equilibrated in 0.025 M potassium phosphate, pH 7.5. The column was eluted with a gradient of 0.025 to 0.50 M in potassium

phosphate, pH 7.5. The enzyme eluted from this column was assayed essentially as described by Duerre (10). This preparation was free of CFA synthetase. A unit of hydrolase activity is 1 μmol of S-adenosyl-L-homocysteine cleaved per h at 37 C.

Lipid analyses. The methods used to extract and hydrolyze phospholipids and to perform the chromatographic analyses were those previously described (8).

RESULTS

Isolation of CFA-deficient mutants. The method used to isolate mutants deficient in CFA synthesis is a modification of the tritium suicide method, which we previously used in the isolation of several classes of mutants in lipid synthesis (7, 9, 14). The present selection method is based on the killing of wild-type cells as the result of the incorporation of [^{3}H]methyl groups into CFA.

A suicide procedure requires the specific biosynthetic incorporation of large amounts of a tritiated precursor into the molecule of interest and a method to remove intracellular pools of the unincorporated radioactive precursor.

The first condition was satisfied by labeling a metB metE methionine auxotroph with [methyl-^{3}H]methionine under conditions that inhibited protein synthesis (the met double auxotroph was used to preclude selection of met$^+$ revertants). Methionine incorporation into protein was inhibited by starvation of the culture for several amino acids, addition of serine hydroxamate (a specific inhibitor of seryl-transfer ribonucleic acid synthetase [24, 25]), and a nutrient shiftdown. The pool of unincorporated intracellular [^{3}H]methionine was removed by washing the cells with cold distilled water (7, 9). This labeling protocol resulted in a cell suspension containing 0.15 dpm of tritium per cell, 70% of which was in CFA.

Upon storage of the suspension of radioactive cells at 4 C, viability was steadily lost (Fig. 1). After 100 days of storage, the cell suspension was plated for single colonies on minimal medium at 30 C. The resulting colonies were tested for temperature sensitivity on R broth plates at 42 C (<5% of the colonies were temperature sensitive). Each isolate was also tested for CFA synthesis (the ability to incorporate [methyl-^{3}H]methionine into phospholipid) at 37 C (the seven temperature-sensitive survivors were tested 15 min after shift from 30 to 42 C). Two (strains FT16 and FT17) of the 133 isolates tested were found to be deficient in CFA synthesis, and neither of these strains was temperature sensitive in growth or CFA synthesis.
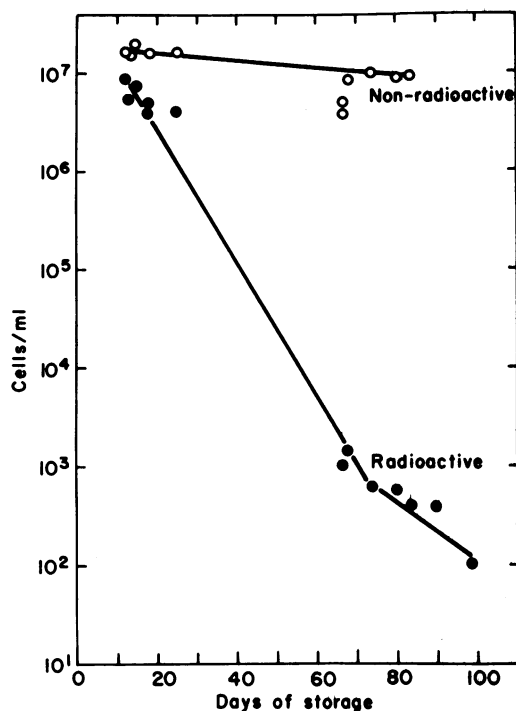
FIG. 1. *Survival of radioactive and nonradioactive cultures of strain FT1. See Materials and Methods for experimental details. Symbols:* ●, *radioactive culture;* ○, *nonradioactive (sham) culture.*

Characterization of the mutants. (i) CFA synthesis in vivo. Cultures of strains FT16 and FT17 as well as their parent, FT1, were grown under various culture conditions, and their fatty acid compositions were analyzed by gas-liquid chromatography (Table 1). Both mutants had extremely low levels of CFA when compared to cultures of the parental strain grown under identical conditions. The amount of CFA de-

tected in the mutants approached the limit of detection by gas-liquid chromatography (0.5 to 1% of the total fatty acids). Therefore, to obtain a more valid estimate of the residual CFA synthesis in these strains, a more sensitive technique was used.

In these experiments, strains FT1, FT16, and FT17 were grown to stationary phase in the presence of [*methyl*-^{3}H]methionine. The lipids were extracted and analyzed by thin-layer chromatography. The area of the chromatogram corresponding to the phospholipid fraction was scraped from the plate, and the radioactivity was determined. The phospholipid fraction from both mutants contained <0.7% of the radioactivity incorporated by an identical culture of the wild-type strain (Table 2).

(ii) CFA synthesis in vitro. Strains FT16 and FT17 and their parent strain FT1 were assayed for CFA synthetase as described in Materials and Methods. Disrupted cell preparations (which contain both membranous and soluble activity) were examined (Table 3). The specific activities of both of the mutant strains were <10% of the wild-type activity. When a parental extract was mixed with an equal amount of mutant extract protein, no inhibition of the parental extract was observed (Table 3). Therefore, both mutants are deficient in CFA synthetase activity.

Tests for physiological functions of CFA. The CFA$^-$ phenotypes of strains FT16 and FT17 enabled us to seek the physiological functions of CFA in a straightforward manner. However, we have not yet found any physiological difference between the mutants and their parent strain, FT1.

The growth rates and final extents of growth of strains FT16 and FT17 in a variety of media were identical to that of strain FT1 (Fig. 2). The

TABLE 1. *Fatty acid content of CFA-deficient and parental cultures*

Fatty acid[a]	Wt % of methyl esters							
	A[a]			B[a]			C[a]	
	FT1	FT16	FT17	FT1	FT16	FT17	FT1	FT17
Myristic	3.3	1.4	3.1	3.0	1.4	2.4	3.9	3.8
Palmitoleic	1.4	21.4	23.6	7.7	28.2	27.8	34.4	36.3
Palmitic	43.8	54.2	51.8	41.0	44.3	43.7	38.9	37.3
cis-9,10-methylene hexadecanoic	31.6	0.6	0.6	26.8	<0.5	<0.5	2.8	<0.5
cis-vaccenic	2.1	22.4	19.8	16.6	25.0	24.7	18.6	21.7
Stearic	0.5	<0.5	0.6	1.1	1.1	1.4	1.4	0.9
Lactobacillic	17.3	<0.5	<0.5	3.8	<0.5	<0.5	<0.5	<0.5

[a] Culture conditions: A, Stationary-phase cells grown in glucose minimal medium at 37 C; B, stationary-phase cells grown in R broth at 28 C; C, early log-phase cells grown at 37 C in R broth.

TABLE 2. *Incorporation of L-[methyl-³H]methionine into phospholipid*[a]

Strain	No. of cells extracted	nmol of methyl groups incorporated	nmol incorporated/10⁹ cells
FT1	5.3×10^9	11.4	2.15
FT16	4.9×10^9	0.05	0.011
FT17	3.4×10^9	0.05	0.014

[a] Cultures of each strain were grown in parallel to late stationary phase in R broth supplemented with L-[methyl-³H]methionine. The lipids were extracted and chromatographed as previously described (6, 8). The areas of silica gel corresponding to the phospholipid fraction were scraped from the plate and counted.

TABLE 3. *CFA synthetase activities of the parental and the mutant strains*

Extract assayed[a]	Sp act (U/mg of protein)	% of parental activity
Expt 1		
FT1	14.7	100
FT16	1.4	9.5
FT17	0.81	5.5
Expt 2		
FT1	10.60	100
FT16	1.02	9.6
FT17	0.02	1.9
FT1 + FT16[b]	6.04	56.6
FT1 + FT17[b]	5.95	55.7

[a] Experiments 1 and 2 were performed with extracts from two enzyme preparations, each from different cultures of each strain.

[b] Equal amounts of protein from the parent and a mutant were mixed and then assayed. The total protein concentration was the same as the other assays in experiment 2. A similar experiment performed in the absence of exogenous lipids gave a similar result.

hypothesis most commonly advanced for the function of CFA is to somehow protect the cellular membrane during stationary phase. We have therefore compared the viability of the parental strain, FT1, with that of mutant strain FT17 during prolonged incubation in stationary phase (Fig. 3). No difference was found between mutant and wild type. We have also tested the survival of strains FT1 and FT17 to drying on filter paper. Again, no differences were found (Fig. 4).

Jungkind and Wood (15) have reported a mutant of *Streptococcus faecalis* that has an abnormally low CFA content. This mutant was shown to be more sensitive than its parental
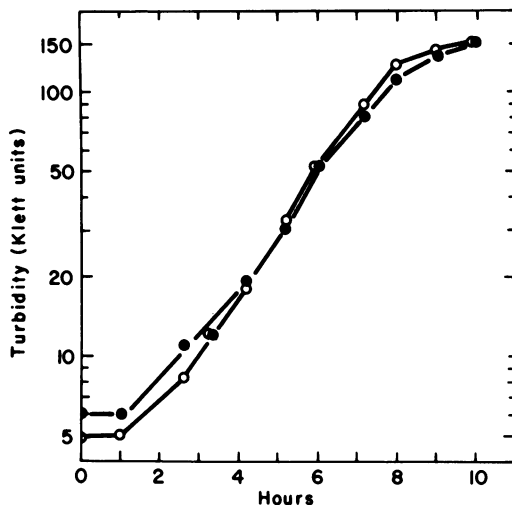

FIG. 2. *Growth curves of strains FT1 and FT17. The curves shown are for growth in minimal medium with glycerol as carbon source at 37 C. One Klett unit equals a density of about 5×10^6 cells/ml. Symbols: ●, strain FT1; ○, strain FT17. Strain FT16 gave a growth curve identical to that shown for strain FT17.*
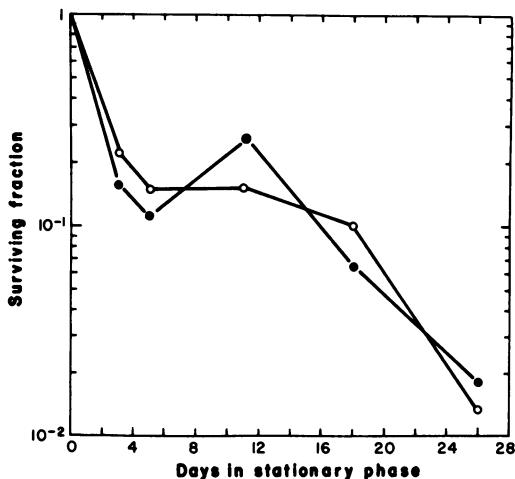

FIG. 3. *Survival of strains FT1 and FT17 in stationary phase. Cultures grown in R broth were sampled after various intervals in stationary phase and plated for colony counts on R broth plates at 37 C. Symbols: ●, strain FT1; ○, strain FT17.*

strain to exposure to 0.1 M lactate (pH 4), to high NaCl concentrations, to various detergents, and to amethopterin (17). We tried these same conditions as well as some variations and found no detectable differences between strain FT1 and strains FT16 and FT17. We have also tested the relative sensitivities of the parent and mutant strains toward rapid freezing and thawing and to lysis of spheroplasts by Brij 58 (11).
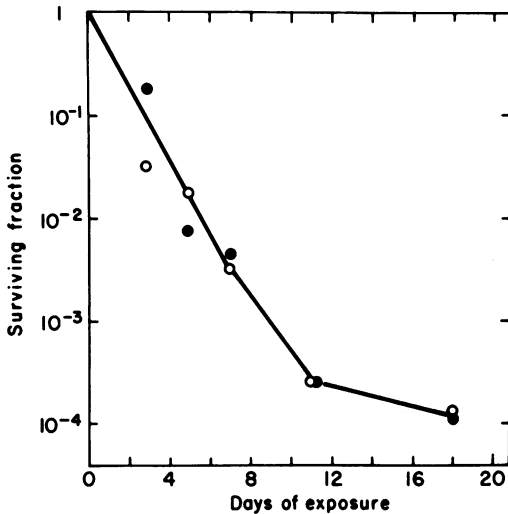
FIG. 4. *Survival of strains FT1 and FT17 on filter paper. Samples (0.1 ml) of a stationary-phase culture grown in R broth were pipetted onto sterile 3MM filter paper disks (24 mm in diameter). The disks were stored in an empty petri dish at room temperature. At various times the cells were resuspended from the disk in medium E, diluted, and plated on R broth plates at 37 C. Symbols: ●, strain FT1; ○, strain FT17.*

No differences were noted among the strains.

Cronan (6) suggested that CFA may play a role in the determination of the rate of phospholipid turnover in *E. coli*. More recent experiments from this laboratory indicated that this was not the case for the polar group of the phospholipid molecules (8). However, we have tested the turnover of the fatty acyl chains of the phospholipids of the parental and mutant strains. The turnover of the acyl chains was identical in all three strains (data not shown).

CFA formation has also been suggested to play a functional role in the active transport of leucine, lysine, and methionine (5). Our strains require lysine, methionine, and two other amino acids for growth, and thus transport of these amino acids did not seem impaired. In addition, we directly assayed the transport of leucine and found no differences in the rate of transport of this amino acid in strains FT1 and FT17 (assayed at a saturating amino acid concentration [data not shown]).

Chemotaxis is known to require methionine (1). CFA formation seems a reasonable site for the function of methionine in the chemotaxic response. However, strains FT1, FT16, and FT17 showed similar chemotaxic responses on the tryptone motility plates of Ordal and Adler (22).

DISCUSSION

The mutant strains FT16 and FT17 are almost completely deficient in the ability to form CFAs (Tables 1 and 2). This deficiency can be attributed to a lack of CFA synthetase activity (Table 3). The presence of only low levels of CFA synthetase activity in these mutants is not due to an inhibitor of the reaction, which is present only in mutant extracts (Table 3). These mixing experiments also rule out hypotheses that would explain a lack of CFA synthetase activity by differences in endogenous phospholipid substrates in the mutant and parental extracts. Another possible hypothesis is that strains FT16 and FT17 are unable to trigger CFA formation at the onset of stationary phase. However, exponential-phase cultures of the mutants are also deficient in CFA content (Table 1). Furthermore, Cronan (6) has shown that similar levels of CFA synthetase activity are found in cells from all growth phases. We have obtained similar results (unpublished data) using the improved assay described in this paper.

Owing to these considerations, it therefore seems likely that the phenotype of these mutants is due to a defect in the gene coding CFA synthetase. However, owing to our inability to find an in vivo phenotype for these mutants, we are unable to prove this point by a biochemical and genetic characterization.

The isolation of mutants of *E. coli* defective in CFA synthesis allows several conclusions concerning the biosynthesis of these acids. Our finding that the mutants are deficient in CFA synthetase indicates that this enzyme is indeed involved in the synthesis of these acids. Furthermore, these mutants were isolated at a frequency of 10^{-7} and thus are very probably due to a single mutation. Since these mutants form no cyclopropane acids, this finding suggests that *E. coli* contains a single CFA synthetase, which catalyzes the synthesis of both the 17-carbon and 19-carbon CFAs and also forms cyclopropane acids in both positions 1 and 2 of the substrate phospholipid molecules.

Our failure to find a physiological phenotype for the CFA-deficient mutants presents an apparent paradox. Why does *E. coli* form CFA in a metabolically expensive process (three adenosine 5'-triphosphate molecules are required per cyclopropane ring formed) if it does not require these molecules? We can conceive of two possible general explanations. First, the presence of CFA molecules may not be strictly required but may only provide a small advan-

tage, which may be reinforced by natural selection. However, this explanation is unattractive owing to the energetic expense of the CFA synthetase reaction. Another possibility is that we have not yet found the proper stress to reveal the function of these acids. It should be noted that *E. coli* survives in a number of natural environments (e.g., soil, the colon, natural water), and hence there may be a complex stress present in such an environment that strongly selects those organisms able to synthesize CFA.

ACKNOWLEDGMENTS

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