Characterization of *Escherichia coli* Mutants Completely Defective in Synthesis of Cyclopropane Fatty Acids

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The synthesis of cyclopropane fatty acids (CFA) in bacteria represents a biochemically and physiologically unique membrane modification whose importance for the cell remains unknown, despite extensive study of a Cfa⁻ mutant of *Escherichia coli* and of the cloned *cfa* gene. Recently we reported the isolation of new Cfa⁻ mutants (D. W. Grogan and J. E. Cronan, Jr., *Mol. Gen. Genet.* 196:367-372, 1984). Molecular-genetic and biochemical analysis indicated that these were null mutants of the *E. coli cfa* locus which were formed by inversions of a chromosomal segment. Isogenic Cfa⁺ and Cfa⁻ strains were constructed from one such mutant and subjected to various stress conditions. In nearly all cases, both strains responded equally, but certain treatments, such as repeated freezing and thawing, favored the survival of Cfa⁺ strains over Cfa⁻ strains. Though not essential, CFA thus appeared to play some beneficial role (or roles) in the bacterial cell.

Many eubacteria modify their membrane phospholipids during the onset of stationary phase by converting the unsaturated fatty acid (UFA) moieties of these lipids to the corresponding cyclopropane fatty acids (CFA) (3). These moieties are formed by a soluble enzyme, CFA synthase, which requires a soluble substrate, *S*-adenosylmethionine, and an insoluble substrate, a fatty acid double bond in the hydrophobic interior of the lipid bilayer. The catalytic mechanism implies unique topology of lipid-protein interactions at the inner face of the cytoplasmic membrane (27). Because CFA synthase acts upon phospholipid bilayers rather than free fatty acids, CFA formation represents a postsynthetic *in situ* modification of the cell membrane. The process requires considerable energy and is subject to physiological and temporal regulation in a variety of bacteria (3, 16). A biological function of CFAs has not been established, however. Neither removal (via mutation) nor overproduction (via genetic amplification) of CFA synthase impairs growth or survival of *Escherichia coli* under an assortment of experimental conditions (12, 26).

As a means of genetic mapping, we previously isolated a Tn10 insertion near the CFA synthase gene (*cfa*) of *E. coli* K-12 (13). Selecting for loss of Tn10, which generates localized chromosomal deletions and inversions (18), yielded CFA-deficient isolates at a frequency of about 1% (13). Such Cfa⁻ strains should be null mutants, i.e., completely devoid of CFA synthase activity. Since it is possible that a very low level of UFA-to-CFA conversion *in vivo* may suffice for some cellular function of CFA, null mutants would provide a superior means of reevaluating the possible biological role of CFA in *E. coli*. In this study, we further characterized the *E. coli cfa* gene and showed that the Tn10-catalyzed lesions which inactivated this gene were inversions. We also tested the effect of the complete absence of CFA on growth and cell viability under a variety of experimental conditions.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study, all derivatives of *E. coli* K-12, are listed in Table 1. Plasmid pGI13, phage constructs, and scoring of clones for *cfa* were described previously (11, 12).

For survival tests, cultures were grown to late stationary phase in R broth (12) (plus 0.1 mg of sodium ampicillin per ml for plasmid-containing strains) and the fatty acid compositions were determined by using gas chromatography (12). The resulting cell suspensions were diluted into distilled water or 10 mM Tris hydrochloride (pH 7.6) (1:100 or greater factor) and treated at 25°C, unless otherwise noted. Freeze-thaw samples were frozen at -20°C and thawed at 30°C. Illumination with near-UV light (400 to 300 nm) was performed by the method of Klamen and Tuveson (17). Cells spread on plates containing medium E (28) plus 0.3% xylose, 0.1% casein hydrolysate, 5 µM riboflavin, and 1% *N,N,N',N'*-tetramethylethylenediamine (for production of superoxide) were illuminated for up to 20 h with a 30-W fluorescent desk lamp (Ken-Rad Cool White) at 50 cm. Aqueous solutions (0.02%) of eosin Y (for production of singlet oxygen) were illuminated at 15 to 20 cm. Suitable samples of liquid suspensions were plated on R agar at 37°C for viable cell counts.

DNA hybridization analysis. Chromosomal DNA, extracted by a rapid procedure (4), was processed by the blotting technique of Southern (25) by using 0.5% high-melting-point agarose gels (Sigma type V, Sigma Chemical Co., St. Louis, Mo.). After transfer to nitrocellulose, the DNA fragments were hybridized with 10⁶ to 10⁷ cpm of *cfa*-specific probe at 65°C. Probe I was prepared by incorporating [³²P]dCTP into *Eco*RI-linearized pGI13 to approximately 10⁸ cpm/µg by the T4 DNA polymerase method (20) and then purifying the 1.1-kilobase-pair (kbp) *Pst*I-*Pst*I segment by digestion and preparative electrophoresis. Probe II was prepared from *Hind*III-linearized pGI30 and [³²P]dATP, yielding a specific radioactivity of approximately 10⁷ cpm/µg, and was used without further purification.

Radiochemical determination of CFA levels. The appropriate *metB* strains were grown 5 to 12 h beyond the stationary phase in methionine assay medium (Difco Laboratories, Detroit, Mich.) containing 20 µg (0.2 µCi) of L-[methyl-¹⁴C]methionine per ml. Total phospholipid was extracted from each culture, 200 µg of methyl arachidate (C_{20:0} methyl ester) was added, and the phospholipid acyl groups were converted to methyl esters by transesterification with NaOCH₃. The esters were treated with mercuric acetate and passed over small silica gel columns to remove unsaturated

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TABLE 1. Bacterial strains

Designation	Relevant genotype ^a	Derivation (Reference)
AMA1004	F ⁺ (⁷) DE(<i>lacIPOZ</i>)C29 <i>leuB6 trpC9830 hsdR rpsL</i>	(2)
FT1	F ⁻ <i>proC32 purE42 metE70 metB1 lysA32 trpE38 rpsL109</i>	(26)
FT17	F ⁻ <i>cfa-1</i> , other markers as in FT1	(26)
GI21	F ⁻ <i>cfa-1 rpsL109</i>	KL96 × FT17
GI42	F ⁻ <i>zdg-299::Tn10</i> , other markers as in FT17	(13)
GI76	F ⁻ <i>ksgB1 zdg-299::Tn10</i>	(13)
GI76-13 ^b	F ⁻ <i>ksgB1 cfa-2^c</i>	Tet ^s of GI76 (13)
GI76-14 ^b	F ⁻ <i>ksgB1 cfa-3^d</i>	Tet ^s of GI76 (13)
GI76-16 ^b	F ⁻ <i>cfa-4^d</i>	Tet ^s of GI76 (13)
GI100	F ⁻ DE(<i>lpp</i>)254 <i>zdg-299::Tn10 cfa-1</i>	P1(GI42) × GI86 (13)
GI107	F ⁻ <i>ksgB1</i>	Tet ^s of GI76 (13)
GI109	Hfr (PO2A) <i>aroD zdg-299::Tn10 lpp-3</i>	Pps ⁺ AroD ⁺ Tet ^r of E610 (13)
GI20	Hfr (PO2A)	P1(GI107) × GI109
GI121	Hfr (PO2A) <i>cfa-2^c</i>	P1(GI76-13) × GI109
GI122	Hfr (PO2A) <i>cfa-3^d</i>	P1(GI76-14) × GI109
KL96	Hfr (PO44)	Laboratory collection

^a IN, Inversion; DE, deletion.

^b Formerly called GI76Δ13, etc. (13).

^c Alternate designation, IN(*cfa-zdg-299::Tn10*)200.

^d Alternate designation, IN(*cfa-zdg-299::Tn10*)201.

^e Alternate designation, IN(*cfa-zdg-299::Tn10*)202. This rearrangement also inactivates the *ksgB1* allele, conferring a kasugamycin-sensitive phenotype (13).

fatty esters and polar contaminants from saturated and cyclopropane fatty esters (8). The mass of each component in the resulting samples was determined by gas chromatography (flame ionization detector coupled to an electronic integrator) based upon the arachidate internal standard.

Construction of recombinant plasmids. Plasmid pGI22 (Cfa⁺) was constructed by inserting the 1.8-kbp *Cla*I-*Cla*I fragment of plasmid pGI13 (12) into the *Cla*I site of plasmid pBR322. Plasmid pGI30 (Cfa⁻) was constructed by inserting the small (about 200 base pair [bp]) *Sph*I-*Ava*I restriction fragment of pGI22 into the gene encoding Tc^r of plasmid pBR322. A *cfa-lacZ* gene fusion was constructed by ligating *Pst*I-digested pMC1871 DNA (2) with *Pst*I-digested pGI22 DNA. Transformation of strain AMA1004 yielded several hundred Ap^r colonies, two of which became light blue after prolonged incubation on R broth agar containing 0.4% 5-bromo-4-chloro-3-indolyl-β-D-galactoside. These two plasmids were scored as Cfa⁻. Mutagenesis of either strain with a frame-shift mutagen, ICR-191, yielded increasing numbers of strongly Lac⁺ (dark blue) colonies with increasing mutagen concentration. This suggested that in these plasmids, the *lacZ* gene was inserted in the proper orientation within *cfa* but was out of frame with respect to *cfa* translation. A -1 frameshift introduced by excision of 19 bp between the *Bam*HI sites of the *cfa-lacZ* junction region (2) had no effect, but a +1 frameshift, introduced by inserting a 10-bp *Hind*III linker into the *Sma*I site of the junction, yielded the strongly Lac⁺ plasmid, pGI29. This plasmid was found to be Cfa⁻ and to encode a polypeptide possessing β-galactosidase activity and migrating with an apparent molecular weight of about 130,000 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

RESULTS

Molecular characterization of *E. coli cfa* gene. Subclones of previously described Cfa⁺ recombinant plasmids (12) have facilitated refinement of the physical map of the *E. coli cfa* region by standard methods of functional analysis. The results show that recognition sites for *Sph*I, *Pst*I, *Ava*I, and *Xho*I occur within the *cfa* gene, whereas the nearby *Bgl*II or *Cla*I sites lie outside the functional gene (Fig. 1). This

implies that *cfa* occupies less than about 1,600 bp, corresponding to a maximum polypeptide molecular weight of about 59,000. The direction of *cfa* transcription was established by the construction of a plasmid-borne *cfa-lacZ* gene fusion (pGI29). The position of the internal *Sst*I site (2) showed that the *lacZ* "cartridge" transcribed from the *Cla*I-proximal to the *Bgl*II-proximal end of *cfa* (Fig. 1).

The iron-containing superoxide dismutase gene (*sodB*) of *E. coli* was localized to a region 5 to 6 kbp leftward from *cfa* by the agreement between cloning and restriction data of Nettleton et al. (22), Sakamoto and Touati (23), and that of Fig. 1. Restriction sites lying outside the region corresponding to known plasmid clones (approximately -11 to +3 kbp in Fig. 1) were determined by blot hybridization (see below). These data, together with a similar map (21) centered about the Braun lipoprotein structural gene (*lpp*, about 36.8 min), give a physical map for most of the 36- to 37-min interval of the *E. coli* chromosome.

Molecular characterization of novel *cfa* lesions. In a previous study (13) we recovered nine Tet^s Cfa⁻ isolates of strain GI76. To determine the type of rearrangements responsible for the Cfa⁻ phenotype, we monitored the chromosomal restriction fragments homologous to the *cfa* locus via blot hybridization (25). Hybridization of probe I to chromosomal *Pst*I fragments (Fig. 2A) showed that the *cfa* region persisted in all cases but that the Cfa⁻ isolates fell into two groups: (i) those bearing probe I sequences on the original 1.1-kbp fragment (isolates 6 and 14) and (ii) those bearing probe I sequences on a 6-kbp *Pst*I fragment (isolates 7 through 13). Having been physically distinguished, the lesions of strains GI76-13 and GI76-14 were designated *cfa-2* and *cfa-3*, respectively. (The remaining isolates were not further characterized, since they may have represented siblings of the former two strains.) Subsequently, gas-chromatographic analysis showed isolate GI76-16, originally retained because of its kasugamycin-sensitive phenotype, to be Cfa⁻. The lesion in this phenotypically distinct strain was designated *cfa-4*.

A more detailed analysis of the *cfa-2*, *cfa-3*, and *cfa-4* lesions used the small, *cfa*-internal probe II (Fig. 1). Strain GI76-16 was of special interest because its phenotype sug-

FIG. 1. Physical-genetic map of the *E. coli* *cfa* region. The heavy horizontal line represents the region of the *E. coli* chromosome from about 36 to 36.5 min (13). Half-brackets illustrating formation of chromosomal inversions which inactivate *cfa*, a reference scale arbitrarily centered about the *Xho*I site within *cfa* (11), and the intervals corresponding to hybridization probes I and II are shown above the physical map. Beneath the map are indicated the positions of genetic markers and an enlargement of the 1,600-bp fragment borne by the *Cfa*⁺ plasmid pGI22 (see Materials and Methods). The site of *lacZ* insertion and direction of its transcription in the *Lac*⁺ *Cfa*⁻ derivative pGI29 is also shown. Restriction site abbreviations: A, *Ava*I; B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hind*III; M, *Mlu*I; P, *Pst*I; R, *Rsa*I; S, *Sal*I; Sp, *Sph*I; SS, *Sst*I; X, *Xho*I. Dots (•) indicate that only the sites closest to the *cfa* gene have been shown.

gested an extensive chromosomal deletion. However, hybridization showed that all three mutants retained *cfa* sequences (Fig. 2B and C). To determine the specific rearrangements giving rise to the *Cfa*⁻ phenotype and thereby locate *zdg-299::Tn10* on the physical map, the changes in restriction patterns (Table 2) were compared to the physical map (Fig. 1). The position of *zdg-299::Tn10* relative to *cfa*, the type of rearrangement (inversion or deletion), and the position of probe-homology (inside or outside the affected interval) allowed six formal possibilities; the available data discredited all but one. Deletions entering the *cfa* gene from the left (*sodB* side) would not have preserved the probe I

interval intact. Corresponding deletions from the right (*ksgB* side) would have eliminated probe II homology in strain GI76-13 and in general could not have shortened the *cfa*-bearing *Sal*I fragment by more than 3 kbp. Inversions to the right leaving probe II homology in place and inversions to the left including (and thus relocating) probe II homology were also precluded by this latter criterion. Finally, inversions to the left leaving probe II homology in place could have shortened the *Cla*I fragment by no more than about 0.7 kbp. Furthermore, leftward displacement of the *Bgl*II site by such an inversion required a separation greater than 27 kbp between *cfa* and *zdg-299::Tn10*, which fit poorly with a

FIG. 2. Genomic blot hybridization of *cfa* mutants. Selected autoradiograms show *Pst*I fragments hybridizing probe I (A), *Eco*RI fragments hybridizing probe II (B), and *Sal*I fragments hybridizing probe II (C). Corresponding bacterial strains (GI76 and derivatives [13]) are indicated below each lane. Numbers to the left of each panel indicate positions and size (kilobase pairs) of molecular weight markers. (The heavy 4.3-kbp bands shown in panel A are an internal molecular weight marker [pBR322] added to each chromosomal DNA preparation, and do not represent *cfa*-homologous fragments.)

TABLE 2. Summary of genomic hybridization analysis

DNA fragment ^a	Mol wt (kbp) ^b of fragment in strain:			
	GI76	GI76-13	GI76-14	GI76-16
<i>Pst</i> I ^b	1.1	6	1.1	ND ^c
<i>Eco</i> RI	19	16	16	16
<i>Sal</i> I	18	7.8	7.0	7.4
<i>Bgl</i> II	12	15	15	15
<i>Cla</i> I	11.5	10.0	10.0	10.0

^a Fragments bearing *cfa* homology.^b Data for *Pst*I were determined by using *cfa* probe I. All other data were determined by using probe II.^c ND, Not determined.

maximum separation of about 13 kbp calculated from genetic linkage. Hybridization data from the three *cfa* mutants was thus consistent only with rightward inversions relocating most of the probe II sequences (illustrated by overhead half-brackets in Fig. 1). This placed *zdg-299::Tn10* near the right end of the restriction map; the *Eco*RI site in this region may accordingly mark the location of the *Tn10* Tet^r gene (6).

Confirmation of phenotype. Because the nature of *Tn10*-catalyzed lesions implied gross disruption of the affected coding regions, we expected a complete loss of *cfa* gene function in these mutants. Stationary-phase cultures of all such strains lacked detectable levels of CFA as assayed by gas chromatography (i.e., <0.3% by weight of total fatty acid). To obtain a more sensitive estimation, CFA levels of certain strains were measured via radiochemical labeling (Table 3). The results show that *cfa-1* mutant FT17 made a detectable, albeit very small, quantity of CFA. The *cfa-2* and *cfa-3* strains, however, yielded background levels of ¹⁴C incorporation. (The noise level imposed by impurities or randomization of the ¹⁴C labeled under these conditions was illustrated by the presence of radioactive label in the unsaturated fatty ester portion [Table 3]. The signal-to-noise ratio was not improved by increasing the specific radioactivity of the methionine label 100-fold.) These data indicated that stationary-phase *cfa-2* and *cfa-3* mutants contained no CFA. These alleles thus qualified as null mutations by biochemical as well as genetic criteria.

Physiological consequences of *cfa* null mutations. Unlike *cfa-1* mutant FT17, which was generated by chemical mutagenesis (26), *cfa-2* and *cfa-3* strains proved to be completely defective in CFA production. Their absolute and presumably discrete lesions thus provided a means to unequivocally test the physiological functions of *cfa*. We measured growth of isogenic, prototrophic strains (GI120, GI121, and GI122) in

medium E (28) under a variety of conditions, including anaerobic (H₂-N₂ atmosphere), aerobic, and carbon source "shift-down" (glucose-to-acetate) conditions. No significant differences in lag periods, growth rates, or total yields were observed. We also tested the influence of CFA upon the viability of stationary-phase cells under various forms of stress, emphasizing treatments likely to affect membranes. To achieve the greatest difference in CFA content, we used strain GI121 (*cfa-2*) as host for the multicopy, Ap^r plasmid pGI5 (*Cfa*⁺) or pGI6 (*Cfa*⁺) (12). Gas-chromatography analysis of GI121(pGI5) and GI121(pGI6) cultures showed that none and 89%, respectively, of the phospholipid UFA had been converted to CFA in late stationary phase.

The viability of both strains decayed exponentially when cells were stored in H₂O at 4, -20, or 42°C, or at 30 to 35°C under direct sunlight (treatments listed in order of increasing rate constants). When exposed to toluene, chloroform, 5 mM cumene hydroperoxide, 50 mM *t*-butyl hydroperoxide, or systems generating singlet oxygen, superoxide, or both (including near-UV light), the death rates were nonexponential, increasing with time of exposure. With these and several other bacteriocidal treatments, the *Cfa*⁺ cultures displayed no significant survival advantage over *Cfa*⁻ cultures.

Two treatments which did favor *Cfa*⁺ over *Cfa*⁻, repeated freezing and exposure to 20% ethanol, have been illustrated in Fig. 3. Results similar to Fig. 3A were obtained with other *Cfa*⁺ and *Cfa*⁻ strains of *E. coli*, indicating that the apparent survival advantage conferred by CFA in vivo was general. To test the effect in mixed populations, strains FT1 and KL96 were transduced to Tet^r with bacteriophage P1 grown on strain GI100 (*cfa-1*) and about 100 colonies from each cross were pooled and grown to stationary phase. The viable cells in these FT1- and KL96-derived pools consisted of 29 and 15% *cfa*⁺ cells, respectively. After five freeze-thaw cycles, the surviving population consisted of 53 and 63% *cfa*⁺ cells, respectively. This indicated a selective advantage of CFA-containing over CFA-deficient cells in a mixed population, although the bias was modest when compared with overall killing (>99%). The effect of ethanol was more difficult to interpret. Figure 3B shows that killing rates of GI121(pGI5) and GI121(pGI6) equalized during the course of treatment, so that the difference was primarily one of initial lag. Also, in contrast to that of freeze-thaw treatment, the differential effect of ethanol proved very sensitive to genetic background and did not correlate well with actual fatty acid composition (data not shown).

DISCUSSION

Our data indicate that *cfa* occupies less than about 1,600 bp of the *E. coli* chromosome and that it is transcribed in the

TABLE 3. Radiochemical determination of CFA levels

Strain	Mass (μg) ^a of following fatty acid in sample:		Amt (counts/min) of following fatty acid incorporated:		Specific radioactivity (cpm/μg) of CFA	Calculated mass (μg) of CFA	% of cellular fatty acids as CFA	No. of CFA molecules/cell ^c
	C _{16:0}	17 cyc	C _{16:0} + 17 cyc	UFA ^b				
GI76	52.1	5.0	260,000	17,800	52,000		4.5	2.0 × 10 ⁶
GI107	58.5	5.7	301,000	14,700	52,800		4.5	2.0 × 10 ⁶
FT17	45.1	<0.2	130	5,200	52,400 ^d	2.7 × 10 ⁻³	3 × 10 ⁻³	1,200
GI76-13	53.8	<0.2	11	1,500	52,400 ^d	<2 × 10 ⁻⁴	<2 × 10 ⁻⁴	<90
GI76-14	93.7	<0.2	16	1,400	52,400 ^d	<3 × 10 ⁻⁴	<2 × 10 ⁻⁴	<70

^a Measurements are described in Methods. Small amounts of myristic (C_{14:0}) and lactobacillic (19 cyc) esters have been neglected. Cyc, Cyclopropane.^b Included as an index of nonspecific labeling (see text).^c Assumes 4.4 × 10⁷ acyl chains per cell (15).^d Average of specific radioactivities of GI76 and GI107.

FIG. 3. Killing by freeze-thaw treatments and 20% ethanol. Dilute suspensions of strains GI121(pGI5) (Cfa⁻) (□ or □) and GI121(pGI6) (Cfa⁺) (▨ or ■) were repeatedly frozen and thawed (see Materials and Methods) over a period of 10 days (A) or added to one-fourth volume absolute ethanol (B). Viable titers were determined as described in Materials and Methods.

clockwise direction. The maximum polypeptide size dictated by purely genetic considerations conflicts with that proposed by Taylor and Cronan based upon partial enzyme purification (27) but is consistent with evidence that native CFA synthase is multimeric (D. W. Grogan and J. E. Cronan, Jr., manuscript in preparation). Similarly, the *cfa-lacZ* fusion shows earlier indirect evidence of *cfa* transcription direction to be in error (12).

Although the difficulties of scoring *cfa* has precluded the use of most classical genetic techniques, we found excision of a closely linked Tn10 insertion very effective in the brute-force isolation of *cfa* mutants (13). Among these, we identified three distinct alleles. These null mutations apparently represent inversions with endpoints within *cfa* and the *zdg-299::Tn10* insertion. (The phenotype of mutant GI76-16 indicates additional inactivation of *ksgB*, which is extremely close to *zdg-299::Tn10* [13].) Complementation tests involving the *cfa-1*, *cfa-2*, and *cfa-3* alleles and various *cfa*⁺, *cfa*⁻ λ phage (11) confirmed that these three alleles inactivated the same diffusible product of the cloned *cfa* gene (data not shown). Thus, all available genetic evidence argues that *cfa* is the structural gene for the cyclopropane fatty acid synthase of *E. coli* and that the enzyme is nonessential.

Although cyclopropane fatty acids appear in many bacteria, their physiological role remains unclear. Investigators have proposed various functions for CFA synthesis; these tend to invoke either (i) alteration of the membrane's physical-structural properties or (ii) protection of the UFA double bonds against chemical attack. The first line of argument seems weakened by the similarity between thermotrophic phase transitions of UFA- and CFA-containing phospholipid (24). More recently, however, nuclear magnetic spectroscopy has revealed a chain-restraining effect of the cyclopropane ring (5). This superficially resembles the effect of cholesterol in eucaryotic membranes and may have biological relevance.

The attraction of chemical-protection arguments stems from the reactivity of olefins toward activated oxygen species (ozone, superoxide, and singlet oxygen) and the propen-

sity of biological and photochemical processes for generating these species (1, 7). Although studies have demonstrated the deleterious effects of such activated species upon model and biological membranes in vitro (9, 10), we know of no direct experimental evidence that conversion of UFA to CFA in vivo protects bacterial cells from membrane oxidation. Studies implicating UFA as potentiators of oxygen-related killing (14, 17) have compared unequally perturbed systems (a UFA auxotroph was grown on different fatty acid supplements, for example), and other parameters, notably growth phase, were found to be greater determinants of resistance than fatty acid composition. Furthermore, the ability of a bacteriocidal agent to oxidize UFA-containing phospholipid does not guarantee the biological relevance of membrane degradation. We found that illuminated eosin Y solutions (a source of singlet oxygen [19]) degraded vesicles of Cfa⁻ *E. coli* phospholipid and killed viable cells and that the effect required both oxygen and illumination. However, Cfa⁺ and Cfa⁻ cultures were killed with similar kinetics, whereas *recA* cultures (deficient in DNA repair) appeared to be dramatically more sensitive than either Cfa⁺ Rec⁺ or Cfa⁻ Rec⁺ (data not shown).

Analysis of *cfa* mutants (null mutants in particular) represents the method of choice for identifying physiological roles of CFA. Such roles have proven remarkably elusive (12, 26). Our finding that *cfa*⁻ *E. coli* strains were more sensitive than isogenic *cfa*⁺ strains to freeze-thaw treatment is the first evidence that CFA protect bacteria from environmental stress. The ill-defined nature of freeze-thaw stress makes it difficult to understand in molecular terms, however, and many other treatments of potential relevance to CFA remain untested. Thus, whereas a biological role for these unusual fatty acids has been indicated, its elucidation promises to remain a tedious and empirical undertaking.

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LITERATURE CITED

- Baxter, R. M., and J. H. Carey. 1983. Evidence for photochemical generation of superoxide in humic waters. *Nature* (London) **306**:575-576.
- Casadaban, M. J., A. Martinez-Arias, S. K. Shapiro, and J. Chou. 1983. Beta-galactosidase gene fusions for analyzing gene expression in *Escherichia coli* and yeast. *Methods Enzymol.* **100**:285-293.
- Cronan, J. E., Jr. 1968. Phospholipid alterations during growth of *Escherichia coli*. *J. Bacteriol.* **95**:2054-2061.
- Davis, R. W., C. Bostein, and J. R. Roth. 1980. Advanced bacterial genetics, p. 120-123. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Dufour, E. J., I. C. P. Smith, and H. C. Jarrell. 1984. Role of cyclopropane moieties in the lipid properties of biological membranes: a ²H NMR structural and dynamical approach. *Biochemistry* **23**:2300-2309.
- Foster, T. J., M. A. Davis, D. E. Roberts, K. Takeshita, and N. Kleckner. 1981. Genetic organization of transposon Tn10. *Cell* **23**:201-213.
- Fridovitch, I. 1974. Superoxide dismutases. *Adv. Enzymol.* **41**:35-97.
- Fulco, A. J., and K. Bloch. 1964. Cofactor requirements for the formation of Δ⁹-unsaturated fatty acids in *Mycobacterium phlei*.

- J. Biol. Chem. **239**:993-997.
9. Garnier-Suillerot, A., L. Tosi, and E. Paniago. 1984. Kinetic and mechanism of vesicle lipoperoxide decomposition by Fe(II). *Biochim Biophys. Acta* **794**:307-312.
 10. Girotti, A. W., and J. P. Thomas. 1984. Damaging effects of oxygen radicals on resealed erythrocyte ghosts. *J. Biol. Chem.* **259**:1744-1752.
 11. Grogan, D. W., and J. E. Cronan, Jr. 1983. Use of lambda plasmids for deletion mapping of non-selectable markers cloned in plasmids. *Gene* **22**:75-83.
 12. Grogan, D. W., and J. E. Cronan, Jr. 1984. Cloning and manipulation of the *Escherichia coli* cyclopropane fatty acid synthase gene: physiological aspects of enzyme overproduction. *J. Bacteriol.* **158**:286-295.
 13. Grogan, D. W., and J. E. Cronan, Jr. 1984. Genetic characterization of *Escherichia coli* cyclopropane fatty acids (*cfa*) locus and neighboring loci. *Mol. Gen. Genet.* **196**:367-372.
 14. Harley, J. B., G. M. Santangelo, H. Rasmussen, and H. Goldine. 1978. Dependence of *Escherichia coli* hyperbaric oxygen toxicity on the lipid acyl chain composition. *J. Bacteriol.* **134**:808-820.
 15. Ingraham, J. L., O. Maaloe, and F. C. Neidhart. 1983. Growth of the bacterial cell, p. 3. Sinauer Associates, Inc., Sunderland, Mass.
 16. Jacques, N. A. 1982. Relationship between cyclopropane synthetase and the formation of cyclopropane fatty acids by *Proteus vulgaris* grown under various respiratory conditions. *J. Gen. Microbiol.* **128**:177-184.
 17. Klamen, D. L., and R. W. Tuveson. 1982. The effect of membrane fatty acid composition on the near-UV (300-400 nm) sensitivity of *Escherichia coli* K1060. *Photochem. Photobiol.* **35**:167-173.
 18. Kleckner, N., K. Reichardt, and D. Botstein. 1979. Inversions and deletions of the *Salmonella* chromosome generated by the translocatable tetracycline resistance element Tn10. *J. Mol. Biol.* **127**:89-115.
 19. Kopecky, K. R., and H. J. Reich. 1965. Reactivities in photosensitized olefin oxidations. *Can. J. Chem.* **43**:2265-2270.
 20. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 21. Nakamura, K., E. T. Katz-Wurtzel, R. M. Pirtle, and M. Inouye. 1979. Restriction enzyme cleavage sites surrounding the structural gene for the lipoprotein of the *Escherichia coli* outer membrane. *J. Bacteriol.* **138**:715-720.
 22. Nettleton, C. J., C. Bull, T. O. Baldwin, and J. A. Fee. 1984. Isolation of the *E. coli* iron superoxide dismutase gene: evidence that intracellular superoxide concentration does not regulate oxygen-dependent synthesis of manganese superoxide dismutase. *Proc. Natl. Acad. Sci. USA* **81**:4970-4973.
 23. Sakamoto, H., and D. Touati. 1984. Cloning of the iron superoxide dismutase gene (*sodB*) in *Escherichia coli* K-12. *J. Bacteriol.* **159**:418-420.
 24. Silvius, J. R., and R. N. McElhaney. 1979. Effects of phospholipid acyl chain structure on thermotropic phase properties. II. Phosphatidylcholines with unsaturated or cyclopropane acyl chains. *Chem. Phys. Lipids* **25**:125-134.
 25. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**:503-517.
 26. Taylor, F., and J. E. Cronan, Jr. 1976. Selection and properties of *Escherichia coli* mutants defective in the synthesis of cyclopropane fatty acids. *J. Bacteriol.* **125**:518-523.
 27. Taylor, F. R., and J. E. Cronan, Jr. 1979. Cyclopropane fatty acids synthase of *Escherichia coli*: stabilization, purification, and interaction with phospholipid vesicles. *Biochemistry* **15**:3292-3300.
 28. Vogel, H. J., and D. M. Bonner. 1956. Acetyl-ornithinase of *Escherichia coli*: partial purification and some properties. *J. Biol. Chem.* **218**:97-106.