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Cyclopropane Ring Formation in Membrane Lipids of Bacteria

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NATURAL DISTRIBUTION OF CYCLOPROPANE FATTY ACIDS

Discovery

In a study of the fatty acid composition of *Lactobacillus arabinosus* published in 1950, Hofmann and Lucas (43) reported the isolation by fractional distillation of a novel fatty acid, which they named lactobacillic acid. In a series of studies, Hofmann and coworkers subsequently demonstrated this to be *cis*-11,12-methylene octadecanoic acid, a 19-carbon cyclopropane analog of *cis*-vaccenic acid, the major unsaturated fatty acid (UFA) of *L. arabinosus* phospholipid (44). The development of gas chromatography subsequently made fatty acid analysis of complex lipids routine, and lactobacillic acid and other cyclopropane fatty acids (CFAs) were observed in the phospholipids of many bacteria (Table 1).

Since the amino acid sequences of enzymes catalyzing cyclopropane ring formation seem highly conserved (see the section on mechanistic aspects inferred from molecular sequence data, below), genomic sequence analysis promises to aid the identification of CFA producers and nonproducers in the future. If a bacterium lacks a gene encoding a homologous protein, we may be able to deduce that the bacterium is unable to synthesize CFAs. This appears to be the case for *Haemophilus influenzae* Rd (28) (for which we, however, have been unable to find a published fatty acid analysis). However, the converse argument, i.e., that the presence of certain sequence motifs implies the ability to make CFAs, may not be as strong. This is based on the observation that mycobacteria have been found to harbor some homologs of CFA synthase genes (see the section on mechanistic aspects inferred from molecule sequence data, below) which probably encode enzymes that catalyze other mechanistically related C_1 additions to UFA. Nevertheless, the finding that proteins catalyzing cyclopropane ring formation

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TABLE 1. Distribution of CFAs among bacterial genera[a]

CFA production		UFA but no CFA production	
Gram positive	Gram negative	Gram positive	Gram negative
Arthrobacter	*Alcaligenes*	*Corynebacterium*	*Leptospira*
Bfidobacterium	*Azotobacter*	*Streptomyces*	*Moraxella*
Clostridium	*Bordetella*		*Neisseria*
Lactobacillus	*Campylobacter*		*Rhodobacter*[b]
Pediococcus	*Caulobacter*		*Spirochaeta*
Streptococcus	*Chlorobium*		*Treponema*
	Citrobacter		
	Enterobacter		
	Helicobacter[c]		
	Klebsiella		
	Nitrobacter		
	Proteus		
	Pseudomonas		
	Rhizobium		
	Salmonella		
	Serratia		
	Thiobacillus		
	Vibrio[d]		
	Yersinia		

[a] These data represent a selection of those compiled by Lechivalier (57), except as noted. Only one member of the genus needs to have been shown to contain CFAs in order to be listed as a CFA-positive species.

[b] Reference 18.

[c] Reference 41.

[d] Reference 38.

from bacteria as diverse as *Escherichia coli* and *Mycobacterium* have highly conserved amino acid sequences suggests that new CFA biosynthetic enzymes will be identified by these conserved regions. Indeed, an unpublished open reading frame (ORF3 of GenBank accession no. X55074) from *Pseudomonas putida* seems almost certain to encode a CFA synthase, as does a 89-residue partial open reading frame from *Citrobacter freundii* (GenBank accession no. U09771) that is 70% identical to the *E. coli* CFA synthase C terminus. The recently reported genomic sequence of *Helicobacter pylori* (90a) contains a putative CFA synthase. Only one such sequence is found in the genome, eliminating the possibility that the conflicting fatty acid compositional data for *H. pylori* (see below) is due to synthesis of both CFA and methoxy fatty acids under different culture conditions.

Bacteria

The list of bacterial genera known to produce CFAs (Table 1) includes gram-positive and gram-negative genera comprising strict anaerobes, aerotolerant anaerobes, facultative anaerobes, microaerophiles, and obligate aerobes. This list is by no means exhaustive, and the complicating factors discussed below it difficult to completely rule out the synthesis of CFAs in some cases where they have not been observed. Certain prokaryotes lack CFAs for the simple reason that UFAs, the precursor of CFAs (see the section on enzymology of CFA biosynthesis, below), are themselves absent. Many thermophilic bacteria and gram-positive bacteria (and all members of the *Archaea*) fall into this category. However, CFAs have not been detected by gas chromatographic analysis of several bacteria that contain appropriate phospholipid UFA moieties (Table 1). In particular, one of these organisms, *Rhodobacter sphaeroides*, was grown under diverse metabolic regimens and examined closely by our coworkers at the University of Illinois. The available data thus indicate that the ability to synthesize CFAs occurs widely but not universally among the major bacterial lineages. This pattern seems consistent with the possibility that cyclopropane ring formation is an ancient characteristic generally associated with the occurrence of UFAs in the membrane. In considering these studies of lipid composition, it should be noted that the ability of a given bacterial strain to produce CFAs can go undetected for at least two reasons. One is loss of the cyclopropane ring during the conversion of lipid fatty acid moieties to the methyl esters, the form in which fatty acids are generally analyzed. Certain reagents commonly used to generate methyl esters catalyze the opening of cyclopropane rings to give various branched and methoxy products. Indeed, the boron halides BF_3 and BCl_3 have actually been used to convert CFAs to branched-chain acids as a means of assigning the acyl chain positions of cyclopropane rings by mass spectrometry (64). Assertions that these reagents can be applied in such a way as to avoid attack of cyclopropane rings have been challenged (72), and the volatility of the reagents makes control of the boron trihalide concentration intrinsically difficult. Another popular reagent, HCl in anhydrous methanol, also attacks cyclopropane rings, although to a lesser extent than the boron reagents (72). This may explain the fact that whereas *H. pylori* has been reported to contain methoxy fatty acids (45), more recent work done without acid-catalyzed methylation showed the presence of CFAs and the absence of methoxy fatty acids (41). We therefore believe that reports of the occurrence of methoxy fatty acids in bacteria be considered suspect if an acid catalyst was used (72). The best-characterized reagent known to give efficient methyl esterification of CFA in complex lipids is sodium methoxide in anhydrous methanol. It should be noted, however, that this reagent is suitable only for transesterification and will not methylate free fatty acids. Sodium (or potassium) hydroxide in methanol can be used in a similar manner. Although 1 mol of water is generated for each mole of methoxide formed, this is probably of little consequence in most applications.

The dependence of CFA synthesis on physiological conditions is a second reason that the lack of CFAs in cells grown under a given set of conditions may not indicate the intrinsic lack of the ability to synthesize CFAs. This caveat is illustrated by *Azotobacter vinelandii*, which forms special resting structures, called cysts, following nutritional down-shift. Whereas vegetative cells of *A. vinelandii* lack CFAs (and thus were often used as the lipid substrate in early enzymological studies), cysts contain CFAs as their major fatty acids (84).

Eukaryotes

CFAs and the structurally related cyclopropene fatty acids have also been found in a few eukaryotic organisms. (Cyclopropene fatty acids are made by desaturation of CFAs [56].) At least four genera of trypanosomatid protozoa (*Crithidia*, *Herpetomonas*, *Leptomonas*, and *Leishmania*) synthesize CFAs (27, 63), as does the slime mold *Physaram polycephalum* (65). A 19-carbon CFA constitutes a large proportion of the fatty acid content of the tropical millipede *Graphidostreptus umuliporus* and occurs in other millipedes of the same order (Spirostreptida). However, this fatty acid has been found only in the females of these species (73). Both cyclopropane and cyclopropene fatty acids have been identified in the seed oils of various plants of the order Malvales (13). In general, the natural distribution of CFAs among eukaryotes appears much more isolated and sporadic than among bacteria.

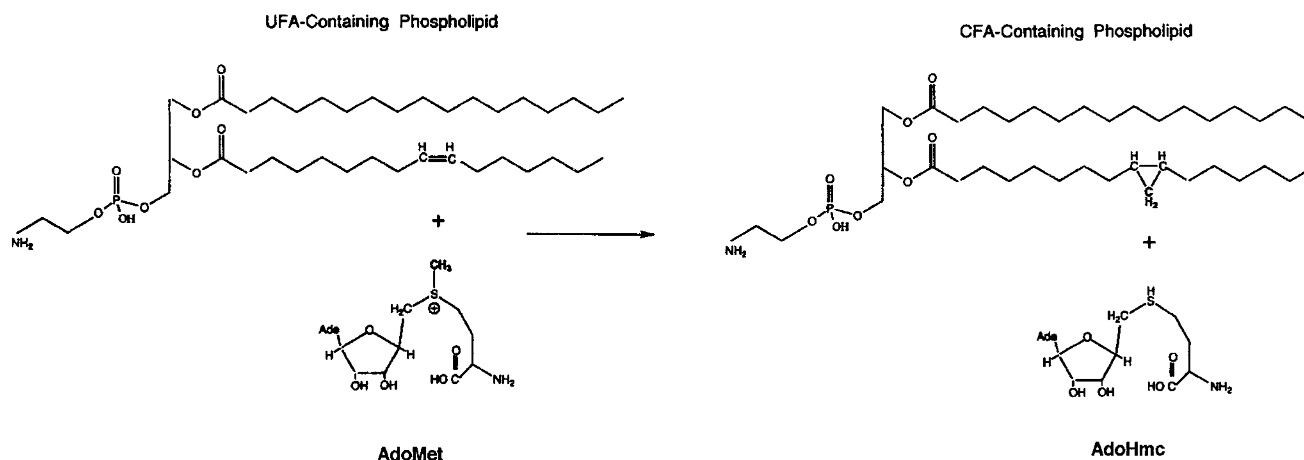

FIG. 1. Structures of CFA synthase substrates and products. The phospholipids shown (phosphatidylethanolamines) are typical components of the membrane lipids of gram-negative bacteria. AdoHmc, *S*-adenosyl-L-homocysteine.

BIOSYNTHETIC PATHWAY

Precursors

Three CFAs predominate in bacterial membrane lipids: *cis*-9,10-methylene hexadecanoic acid, *cis*-11,12-methylene octadecanoic acid (lactobacillic acid), and *cis*-9,10-methylene octadecanoic acid (also called dihydrosterculic acid by analogy to the corresponding cyclopropene fatty [sterculic acid] from seed oils). Natural CFAs have the *cis* configuration about the cyclopropane ring (15) and occur only in organisms that synthesize the structurally homologous UFA, i.e., palmitoleic (*cis*-9-hexadecenoic acid), *cis*-vaccenic (*cis*-11-octadecenoic acid), and oleic (*cis*-9-octadecenoic acid) acids, respectively. Furthermore, CFAs typically appear rather abruptly at a particular stage in the growth of batch cultures (see the section on temporal regulation, below). This appearance coincides with a corresponding decrease in the UFA content of cells. This correlation provided evidence in vivo that bacterial CFAs are synthesized directly from UFAs by modification of the *cis* double bond (15, 56).

C_1 Donor

The immediate source of the C_1 unit which forms the cyclopropane ring is *S*-adenosylmethionine (AdoMet). Early studies used auxotrophic mutants of *Enterobacter aerogenes*, whose growth is dependent upon exogenous AdoMet. Feeding cultures with [*methyl*-^{14}C]AdoMet resulted in formation of radiolabeled CFAs, whereas exogenous, nonradioactive L-methionine failed to dilute the isotopic label in CFAs (71). Infection of *E. coli* cultures with wild-type phage T3 (which encodes an AdoMet hydrolase that specifically destroys AdoMet) blocked CFA synthesis in vivo, whereas infection with a mutant of phage T3 that lacked the hydrolyase failed to block CFA synthesis (16). Finally, cell-free systems that synthesize CFAs in vitro all demonstrate an absolute requirement for AdoMet (88, 98), and AdoMet analogs inhibit the reaction in vitro (see the section on CFA synthase inhibitors, below).

C_1 Acceptor

Several lines of evidence show that the form of the UFA which accepts the C_1 unit is the full-length fatty acid esterified to phospholipid (Fig. 1). Biotin-starved *Lactobacillus* cultures (which cannot synthesize fatty acids), as well as UFA auxotrophic mutants of *E. coli*, readily incorporate exogenously supplied UFAs and (given the proper position of a *cis* double bond) can form CFAs in the absence of fatty acid synthesis de novo (15, 44). *E. coli* UFA auxotrophs grown with palmitoleate ($C_{16:1}$) and then switched to oleate ($C_{18:1}$) supplementation before the onset of CFA formation (see the section on physiological aspects of CFA formation, below) efficiently converted the palmitoleolyl acyl chains, previously incorporated into phospholipid, to the 17-carbon CFA (15). Also, several enterobacteria, when grown at low temperatures, contain palmitoleic acid but not the C_{17} CFA derivative in the lipid A portion of lipopolysaccharide. Since the fatty acid moieties of lipid A and phospholipids are derived from the same pathway, the lack of CFA in lipid A under these conditions indicates that methylene addition occurs after completion of the acyl chains. Finally, phospholipids have been shown to be necessary substrates for CFA synthesis in vitro. Homogeneous enzyme preparations efficiently synthesize CFA in vitro by using only AdoMet and vesicles of purified phospholipid as substrates (92). Moreover, Thomas and Law (90) demonstrated that cell extracts of *Clostridium butyricum* can use 1,2-di-(9-octadecenyl)-3-(2-aminoethyl)phosphorylpropane, the diether analog of phosphatidylethanolomine, as a substrate for CFA synthesis. Since the ether linkage cannot be cleaved under the assay conditions, this result conclusively ruled out the participation of a free fatty acid, even as a short-lived intermediate, in the synthesis of CFAs.

Reaction Mechanism

Formation of a cyclopropane ring in a linear monoene appears to proceed via a carbocation intermediate (58, 95). The detailed mechanism has yet to be established, but isotopic labeling studies allow certain other mechanistic possibilities to be discarded. For example, *Lactobacillus* cells convert oleic acid deuterated at both vinyl positions to CFAs which retain deuterium at both positions. This effectively rules out reaction intermediates, such as exomethylene or cyclopropene compounds, that retain the UFA double bond following C_1 transfer (6, 75). Trideuterated methyl groups, supplied to cells as [*methyl*-D_3]methionine, have been found to be incorporated into CFAs with some isotopic dilution. This has been interpreted as evidence of a slow transfer of the methyl group

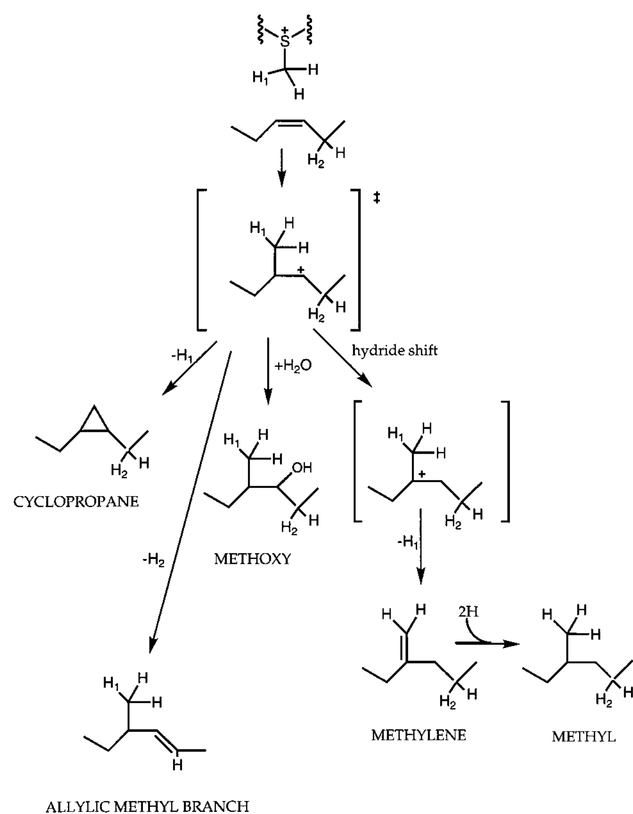

FIG. 2. Probable mechanisms for C_1 additions to double bonds based on the proposal of Lederer (58) for sterol methyltransferases.

followed by a fast but partially reversible protonation (7). The configuration of CFA formed in vivo is >99% *cis* (15), implying a lack of rotation about the olefin carbons during catalysis.

The currently accepted mechanism is given in Fig. 2. Following separation of the AdoMet sulfonium group from its counterion, the π electrons of the double bond attack the sulfonium cation, leading to the initial carbocation. The carbocation can then be quenched by a variety of pathways depending on the nature of the active-site residues. Abstraction of a methyl proton from the initial carbocation will give the cyclopropane ring (Fig. 2).

Alternative reactions of the initial carbocation yield other lipid products. The addition of water, for example, will yield a methoxy fatty acid. Alternatively, biosynthesis of the methyl fatty acid tuberculosteric acid, which proceeds via a branched methylene precursor, would require a hydride shift to the more stable tertiary carbocation followed by abstraction of a methyl proton as in cyclopropane formation. This mechanism is supported by the labeling studies discussed above and by studies showing similar rates of proton exchange during cyclopropane and tuberculostearic acid synthesis (7). Another possible fate for the carbocation would be loss of an adjacent carbon chain proton to give a *trans* olefin with an adjacent methoxy group (58). A *Mycobacterium tuberculosis* gene, called *mms1*, probably encodes such an activity. When overproduced in the host organism, the gene product leads to increased levels of *trans* olefins with an allylic methyl branch (96). The double bond would then be converted to a *trans* cyclopropane ring by an unknown methylene transferase.

The proposed mechanism allows some predictions concerning the active site of these enzymes (6, 7). First, the site could be expected to contain a hydrophobic region due to the nature of the lipid substrates and the high methyl transfer reactivity of separated ion pairs in such environments. Second, the active site should contain one or two basic functions (depending on the product formed), such as a lysine ε-amino group or possibly a thiol. One would bind the counterion of the sulfonium group, whereas the second would abstract the proton. Dougherty (22) argues convincingly that one or more active-site aromatic residues may stabilize and align the methyl sulfonium moiety of AdoMet via π-cation interaction. This means of stabilizing the sulfonium cation would be compatible with both a highly hydrophobic active site (see the section on CFA synthase inhibitors, below) and the active-site configurations of other AdoMet-dependent methyl transferases (22).

ENZYMOLOGY OF CFA BIOSYNTHESIS

Enzyme Assay and Stabilization

Synthesis of CFAs in vitro was first achieved by Law and coworkers with extracts of *Serratia marcescens* and *Clostridium butyricum* (56). Extracts from both organisms catalyzed the transfer of radioactive methyl groups from AdoMet to saponifiable lipid, but only the *Clostridium* extract required exogenous lipid dispersions to demonstrate activity (98). The assay method of Taylor and Cronan (88) employs liposomes of *E. coli* phospholipid containing UFAs and measures the incorporation of radioactive methyl groups (from labeled AdoMet) into trichloroacetic acid (TCA)-insoluble material rather than into saponifiable lipid. Substrate vesicles are most conveniently prepared from polar lipid extracts of a CFA-deficient mutant of *E. coli* (88).

Repeated attempts to characterize and purify bacterial CFA synthases provide abundant, albeit largely unpublished, evidence of the instability of these enzymes. The magnitude of the problem for enzymological studies was illustrated by the data of Taylor and Cronan (88). When crude extracts of *E. coli* were freed of endogenous lipid by ultracentrifugation, less than 1% of the initial CFA synthase activity remained after a 30-min incubation at 37°C. This instability has severely hampered purification and detailed studies of the enzyme, such that most features of its catalytic mechanism have been deduced from studies of CFA synthesis in vivo and from partially purified enzyme preparations.

Substrate Specificity

A rather lax requirement for the position of the *cis* double bond has been demonstrated by supplementing UFA auxotrophs of *E. coli* K-12 with individual UFA isomers. The *cis*-9, *cis*-10, and *cis*-11 isomers of 16:1 and 18:1 UFAs are converted to CFA in vivo (60, 69). However, *cis* double-bond positions close to the ends of a C_{18} fatty acyl chain (60, 69) are not converted. UFAs of the *trans* configuration are also not converted (81), although *trans* CFAs can be made by chemical synthesis. CFA synthase from *C. butyricum* shows strong stereoselectivity for fatty acyl esterification to the glycerol backbone: only *sn*-1,2-disubstituted glycerol phosphate derivatives serve as substrates in vitro (90). CFA synthesis in most organisms seems to prefer unsaturated fatty acids in the *sn*-2 position of the phospholipid backbone (the acyl chain closer to the phosphate moiety) (42a). However, most of this apparent specificity can be attributed to fact that position 2 is greatly enriched in palmitoleyl groups, the preferred substrate of most CFA synthases. When position 1 is occupied by a UFA (as in cells grown at low temperature) the UFA is *cis*-vaccenic acid,

a rather poor substrate for most CFA synthases. An exception is *C. butyricum* phosphatidylethanolamine, in which most of the palmitoleyl moieties are located in position 1. In vitro, the *C. butyricum* CFA synthase has a definite but not absolute specificity for UFA moieties located in the *sn*-1 position (42a). Further experiments with chemically synthesized phospholipids should be done to more reliably document the positional specificity of these enzymes.

Interactions of CFA Synthase with Phospholipid Vesicles

Active CFA synthase of *E. coli* is recovered in the cytoplasmic fraction of cell homogenates, but the enzyme does associate reversibly with membrane fragments and with phospholipid vesicles. This reversible association is the only generally effective means of stabilizing CFA synthase identified thus far (88).

An electrostatic mode of interaction between the enzyme and a phospholipid bilayer has been inferred by differences in enzyme activity toward liposomes of differing phospholipid head group compositions. Thomas and Law (90) observed that vesicles composed of phosphatidylglycerol mixed with phosphatidylethanolamine were better substrates than were vesicles of either pure lipid. Furthermore, activity with pure phosphatidylethanolamine vesicles was stimulated by sodium dodecyl sulfate, whereas activity of pure phosphatidylglycerol vesicles was stimulated by Ca^{2+} ions. Similarly, Taylor and Cronan (88) found high activity with vesicles composed of phosphatidylglycerol, no activity with cardiolipin vesicles, and intermediate activity with vesicles composed primarily of phosphatidylethanolamine. Alternatively, these three phospholipids (the normal lipids of *E. coli* membranes) were presented as a mixture, chromatographically resolved following the enzyme assay, and independently assayed for CFA formation. In this case, all three phospholipids were cyclopropanated with the same efficiency. This result demonstrated that the difference in reactivity toward vesicles of pure lipids is not due to properties of the individual phospholipid species and therefore reflects the substrate suitability (presumably the surface properties) of the entire vesicle.

In addition to its apparent electrostatic interaction with the surface of substrate vesicles, CFA synthase exhibits a selectivity for acyl chains. As a result, the enzyme reversibly associates only with vesicles that contain UFA or CFA in the phospholipid. This has been demonstrated on the basis of three different criteria. Vesicles containing only saturated fatty acids (SFA) do not stabilize the enzyme (88), whereas UFA- (or CFA)-containing vesicles do. Furthermore, vesicles containing only SFA do not bind the enzyme, as demonstrated by flotation of CFA synthase activity in sucrose gradients, whereas UFA- or CFA-containing vesicles bind the enzyme effectively (88). Finally, vesicles containing only SFA do not protect the enzyme from inactivation by an irreversible inhibitor, whereas UFA- or CFA-containing vesicles do protect the enzyme (92). The apparent acyl-chain selectivity cannot be attributed to phase properties of the respective lipid bilayer interiors. Vesicles of phospholipid containing branched-chain fatty acids, which have fluidity properties comparable to those containing UFA, also failed to float the enzyme in density gradients (88). Furthermore, substrate vesicles whose UFA compositions had been artificially adjusted and had been confirmed to result in widely different phase transition temperatures yielded essentially identical Arrhenius curves for enzymatic cyclopropanation (88). It should also be noted that high-level production of an integral membrane protein has been reported to inhibit CFA synthesis in *E. coli*, suggesting that a grossly altered bacterial inner membrane can impede CFA synthase in vivo (67).

The evidence that CFA synthase of *E. coli* is a soluble, cytoplasmic enzyme which associates reversibly with substrate vesicles raises questions about the topology of its catalysis. Partially purified CFA synthase can apparently cyclopropanate both inner and outer leaflets of unilamellar vesicles without penetrating or permeabilizing the bilayer (88). This result remains to be confirmed with homogeneous enzyme preparations, but it seems to support the idea that CFA synthase may act in a manner analogous to the DNA methyltransferases which methylate the adenine and cytosine rings of double-stranded DNA (11, 12, 79). These enzymes gain access to a base by flipping the base out of the DNA double helix. This dramatic protein-induced DNA distortion, first reported for *Hha*I DNA methyltransferase, appears to be a common mechanism used by various proteins that act on DNA bases. This precedent reinforces the possibility that CFA synthase flips an acyl chain out of the lipid bilayer, catalyzes cyclopropanation, and then releases the chain for return to the lipid bilayer. Indeed, a flip-out mechanism for CFA synthesis may be less energetically demanding than methylation of duplex DNA, since no hydrogen bonds need be broken. In any event, cyclopropane formation in gram-negative bacteria probably involves a significant rate of phospholipid exchange between the cytoplasmic membrane and the inner leaflet of the outer membrane (55). This is implied by the cytoplasmic location of CFA synthase and the kinetics of CFA appearance in total membrane phospholipids in the absence of significant growth and phospholipid biosynthesis (see the section on temporal regulation, below).

CFA Synthase Inhibitors

Several inhibitors of CFA synthase activity have been identified, some of which show promise as probes of active-site structure. Three structural analogs of AdoMet inhibit the CFA synthases of *Lactobacillus plantarum* and *E. coli* (in order of increasing potency): *S*-adenosyl-L-homocysteine (the nucleoside product of the reaction), sinefungin, and A9145C (two antifungal drugs) (83, 89, 92). Sulfhydryl-modifying reagents, particularly 5,5′-dithiobis(2-nitrobenzoic acid), rapidly inactivate the CFA synthase of *E. coli*, suggesting that a cysteine residue may play an essential role in catalysis (88, 98).

Although the inactivation by dithionitrobenzoate can be reversed by the subsequent addition of a suitable reducing agent, the Michael adduct formed on the enzyme by *N*-ethylmaleimide is irreversible (87). This property has enabled chemical probing of the immediate environment of the essential sulfhydryl by using a homologous series of *N*-alkylmaleimides. The effectiveness of these inhibitors, indicated by the pseudo-first-order rate constant of enzyme inactivation, increased exponentially as the hydrocarbon chain length was increased from four to seven methylene units (92). This result indicates the localization of the *N*-alkylmaleimide in a hydrophobic cleft or pocket near the essential sulfhydryl group. In addition, the ability of various agents to protect the essential sulfhydryl was measured. The soluble substrate (AdoMet) was ineffective, as were vesicles containing only SFA chains. However, vesicles containing either CFA or UFA protected the enzyme against inactivation, and in the case of UFA vesicles, the concentration dependence of protection resembled the concentration dependence of catalysis (88, 92). The available data thus indicate that the essential sulfhydryl lies near a hydrophobic site of the enzyme which binds only UFA or CFA chains (as would be predicted for the active site) or which is otherwise protected only when the enzyme binds to phospholipid bilayers. Buist and Pon (8) reported that CFA synthesis in *L. plantarum* con-

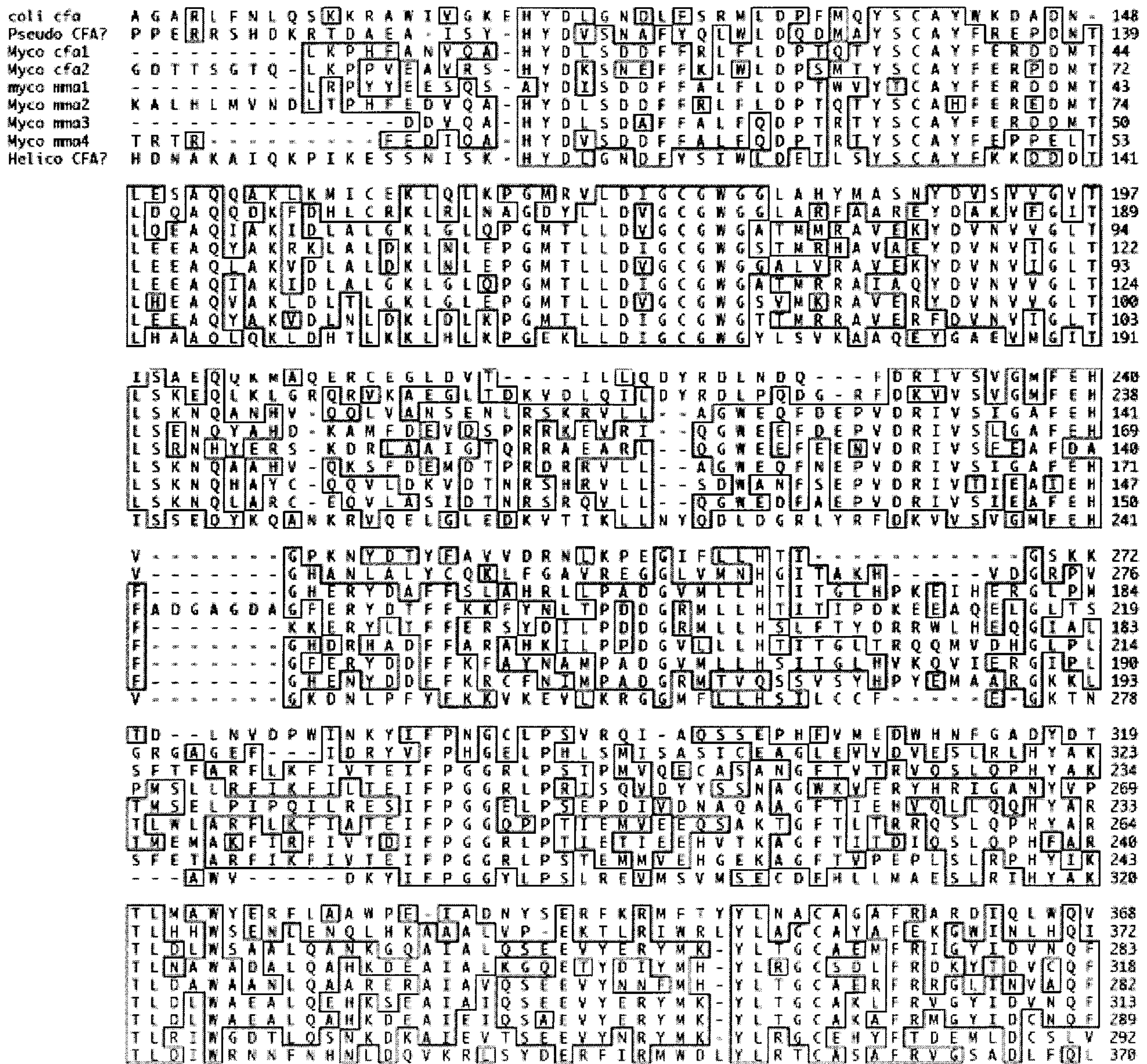

FIG. 3. Amino acid sequence comparisons of CFA synthase and related enzymes. Residues identical to *E. coli* Cfa (coli cfa) are boxed. The second and last lines are putative Cfa synthases from *P. putida* and *H. pylori*, respectively, whereas the remaining sequences are from *M. tuberculosis* (see the section on mechanistic aspects inferred from molecular sequence data for details).

verts two octadecenoic acid isomers (oleic acid and *cis*-vaccenic acid) in a manner that suggests that different faces of the double bonds of the two isomers might interact with the active site. However, these data could also be explained by the presence of two CFA synthases, one specific for each of the two isomers.

Mechanistic Aspects Inferred from Molecular Sequence Data

The structural gene for the CFA synthase of *E. coli* has been cloned, based on its ability to complement CFA-deficient mutants and to elevate the level of enzyme activity when present in multiple copies (33). Due to the availability of the cloned structural gene, the CFA synthase of *E. coli* was the first cyclopropane ring-synthetic enzyme of known amino acid sequence, and it remains the only such protein that has been purified to homogeneity (92). Alignment of the *E. coli* CFA synthase sequence with its close relatives indicates that three cysteines are conserved (Fig. 3). Two of these (C176 and C354) have been changed to alanine by site-directed mutagenesis, with the result that both mutant proteins retain activity (10). Although the third cysteine residue has not yet been mutated, this result raises the possibility that the sulfhydryl group is simply located adjacent to the active site and does not otherwise participate in catalysis. Thus, inhibition of the enzyme by sulfhydryl reagents may reflect steric hindrance at the active site rather than covalent modification of a catalytic nucleophile or base.

Upon publication of the *E. coli* CFA synthase sequence (92), we tentatively identified a region as an AdoMet-binding motif based on sequence similarity to a subset of methyltransferases. This region, which corresponds to motif I of the alignments of

Kagan and Clarke (51), is conserved in the related mycobacterial enzymes and has since been found in a large number of AdoMet-dependent methyltransferases, particularly sterol methytransferases. Motifs II and III of Kagan and Clarke (51) are also present in the CFA synthase enzyme family. However, analysis of the available crystal structures (80) indicated that the methyltransferase active-site structure can be attained by markedly different amino acid sequences. Moreover, since the three motifs involved in methyl transfer are not contiguous blocks of primary sequence, they are not the easiest of motifs to recognize.

The deduced sequence of *E. coli* CFA synthase has also facilitated the identification of several enzymes that catalyze cyclopropane ring formation in slow-growing pathogenic mycobacteria (Fig. 3). These bacteria have, in addition to phospholipids and glycolipids typical of gram-positive bacteria (to which they are related), large amounts of high-molecular-weight, α-branched, β-hydroxylated fatty acids called mycolic acids. Mycolic acids are specific to mycobacteria and related organisms and are hypothesized to form an unusually impermeable membrane around the cell (68), thereby giving these bacteria their characteristic acid-fast staining properties. The acyl chains of mycolic acids can be very long (up to 90 carbon atoms) and can carry a variety of functional groups. Three of these groups (cyclopropane rings, methyl branches, and methoxy groups) are derived by the addition of a methylene or methyl from AdoMet to a *cis* double bond. Three *M. tuberculosis* genes, *cma1*, *cma2*, and *mma2*, have been isolated that give cyclopropane mycolic acids upon introduction into a fast-growing nonpathogenic mycobacterium, *M. smegmatis*, which normally lacks mycolic acid cyclopropane rings (95, 97). The first such gene, *cma1*, was isolated by screening a cosmid bank of *M. tuberculosis* DNA fragments for those that altered the lipid composition of *M. smegmatis* (97). The product of one of these genes was found to have 34% amino acid identity to *E. coli* CFA synthase (Fig. 1) and was subsequently used to isolate several other *M. tuberculosis* genes based on sequence homology (29, 95). All three genes were shown to give cyclopropane-modified mycolic acids upon introduction into *M. smegmatis*. Enzyme specificity seems to account for the presence of at least three cyclopropane mycolate synthases in *M. tuberculosis*. The *cma1*-encoded enzyme converts the *cis* double bond distal from the head group to a *cis* cyclopropane ring, whereas the *cma2*-encoded enzyme acts on the proximal double bond (29, 95). The protein encoded by the *mma2* gene also converts the proximal double bond to a *cis* cyclopropane ring, but only if the distal position is occupied by an oxygenated group (95).

The studies on mycobacteria also uncovered another set of genes (present in both *M. tuberculosis* and *M. bovis*) that encode methoxymycolates upon introduction into *M. smegmatis* (23, 95). All of the proteins encoded by these genes are highly homologous to one another (>52%) and to *E. coli* CFA synthase. The finding that the introduction of highly homologous genes into *M. smegmatis* can result in conversion of a double bond to a cyclopropane ring in one case or to a methoxy group in a second case supports the idea of a common reaction intermediate, first proposed by Lederer (58) and summarized in Fig. 2. It should be noted that none of the published data on the mycobacterial enzymes have been obtained by the usual direct means, i.e., enzyme assay in vitro. Thus, an enzyme present in *M. smegmatis* (rather than an intrinsic activity of the *M. tuberculosis* protein) could account for the hydration needed in the synthesis of the methoxy species. Direct demonstration of these enzyme activities by in vitro assay would very welcome, and the work is in progress (3). It is not clear at which stage(s) of mycolate synthesis the cyclopropane and methoxy functions are introduced, but is possible that the corresponding substrates will be technically tractable. Barry and coworkers (3), for example, have developed a promising in vitro system in which the AdoMet methyl carbon is incorporated into a C_{40} to C_{50} intermediate that may be protein bound.

It should be noted that although the nonmycolate lipids of *Mycobacterium* spp. would seem to be suitable substrates for CFA formation, CFAs have not been found in these lipids. In particular, the data of Akamatsu and Law (1, 2) have been cited as demonstrating the absence of CFA in the nonmycolate lipids. We note, however, that these workers reported only the lack of detectable CFA synthesis in an in vitro system; the question thus seems to warrant further examination. In any event, the nonmycolate lipids do contain another type of AdoMet-modified fatty acid, tuberculosteric acid (10-methyloctadecanoic acid). The synthesis of these lipids has been studied both in vivo (58) and in vitro (1, 2). The methyl carbon and two of the three methyl protons have been shown to be derived from AdoMet, and the in vitro substrate is oleic acid esterified to phospholipid. This reaction thus seems very likely to be catalyzed by an enzyme similar to the CFA and cyclopropane mycolate synthases. The addition reaction first gives a compound with a methylene branch at C-10, and this double bond is then reduced in the presence of NADPH to give tuberculosteric acid. The methylene-branched intermediate has been demonstrated (58) and could readily result from proton abstraction from a carbocation intermediate common to C_1 transfers to UFA (Fig. 2).

GENETICS OF CFA FORMATION IN *ESCHERICHIA COLI*

Mutants Deficient in CFA Synthase

Using chemical mutagenesis and a tritium suicide selection, Taylor and Cronan (86) isolated two mutant strains of *E. coli* which fail to produce CFA due to very low CFA synthase activity. Characterization of these mutants demonstrated that the inability to make CFA is not deleterious to growth and does not alter the phospholipid composition. Indeed, the absence of any phenotypic property other than fatty acid composition hampered the genetic analysis of cyclopropane ring formation and provided no new means of isolating additional mutants (87). Later isolation of Tn*10* insertions near the *cfa* locus of *E. coli* (see the section on chromosomal location of *cfa*, below) allowed additional *cfa* mutants to be isolated by selecting for excision of the Tn*10* element. The resulting mutants, bearing chromosomal rearrangements, were considered null (knockout) mutants. They synthesized no CFA detectable by a sensitive radiochemical incorporation assay, whereas *cfa* mutant FT17 made a small amount of CFA that was detectable by this assay (36). Also, strains containing a targeted disruption of the CFA gene have recently been constructed (10).

Isolation and Analysis of the *cfa* Gene

CFA-deficient mutants of *E. coli* have no known phenotype useful in routine genetic analysis. A method of screening colonies via incorporation of radioactive methyl groups into acid-insoluble material, followed by autoradiography, proved instrumental in both the isolation of *cfa* mutants (34, 86) and the isolation of the *cfa* gene (33). However, the method is rather tedious, requires a methionine auxotroph, and has limited accuracy (it identified only one of two *cfa*-complementing plasmids present in a collection of recombinant plasmids, for ex-

ample [33]). Much of the genetic analysis of CFA formation in *E. coli* has therefore depended upon fatty acid analysis (by gas chromatography) of phospholipids extracted from individual liquid cultures as the most reliable means of scoring bacterial clones for a functional *cfa* gene.

The *cfa* gene was first isolated on a chromosomal DNA fragment of approximately 19 kbp in a recombinant ColE1 plasmid (pLC18-11). Deletion and subcloning procedures showed that the *cfa* gene was less than about 1.5 kbp and encoded a protein of approximately 40 kDa (32, 35, 92). The *cfa* gene was sequenced and gave a 382-amino-acid product that matched the N-terminal sequence of purified enzyme (92). The only sequence homology to other proteins so far identified (except for other putative CFA synthases [29]) consists of three short motifs, conserved among AdoMet-specific enzymes, which constitute a putative AdoMet-binding site (51, 92). The deduced molecular weight of the protein (43, 93) is in reasonable agreement with the mobility of purified CFA synthase in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Size exclusion chromatography provided an estimate of 90 kDa for the native enzyme in cell extracts (88), suggesting that the catalytically active species may be a dimer of the polypeptide encoded by the *cfa* gene. Confirmation of this estimate remains problematic, however. Either the protein must remain bound to lipid vesicles, which invalidates such measurements, or it must be released from vesicles, in which case an enzyme with compromised activity is studied.

Chromosomal Location of *cfa*

Cotransduction frequencies, cloning results, and three-factor crosses placed *cfa* between the closest loci identified at the time, *sodB* and *ksgB* (34). More recently, systematic sequencing of the *E. coli* genome placed *cfa* at min 37.6 (bp 1744243 to 1745388) of the chromosome between the *ydhC* and *ribC* loci (5). It should be noted that a cloning artifact that gave a portion of *cfa* linked to a gene at min 67 has now been corrected (26).

PHYSIOLOGICAL ASPECTS OF CFA FORMATION

Temporal Regulation

Early studies of CFA-producing bacteria found that these modified fatty acids first appear in the late exponential or early stationary phase of growth. In *Azotobacter vinlandii*, CFAs are made only during encystment (84). The basis of the timed appearance of CFAs is reported to be the induction of CFA synthase in several bacteria, including *Pseudomonas* spp. (19, 46), *Proteus vulgaris* (47), and *L. plantarum* (88), but growth-phase-specific induction of the enzyme was not obvious in early studies of *E. coli* (88). It has more recently been shown that *E. coli* produces a sharp peak of CFA synthase activity, which is easily missed, in the transition from exponential growth to stasis (93). Furthermore, modest elevation (about 10-fold) of CFA synthase activity by a multicopy plasmid is sufficient to cause *E. coli* to produce high levels of CFAs in the exponential phase, when they normally do not accumulate (33). These results suggest that the primary determinant of CFA synthesis in *E. coli*, and presumably in other bacteria, is specific activity of the enzyme in vivo.

Influence of Culture Conditions

In addition to the growth phase, various culture parameters have been observed to determine the extent of cyclopropanation of the bacterial membrane lipid. Knivett and Cullen (53), for example, demonstrated that a suitable combination of carbon source, medium pH, and degree of aeration can exert a greater effect on CFA formation than does the growth phase. Culture conditions which enhanced CFA formation in their study of fermentor-grown cultures included a low pH, a high incubation temperature, a high Mg^{2+} concentration, and a low aeration rate. Under these conditions, exponentially growing cells contained larger amounts of CFAs than did stationary-phase cells grown under the adverse conditions.

Analogous effects on the specific activity of CFA synthase have been observed for medium pH with *L. plantarum* (39) and for aeration with *Pseudomonas denitrificans* (46), although the latter situation is complex with regard to the role of the carbon source (47). Enhancement of CFA formation in *E. coli* by increasing the medium osmolarity has been reported (61) but could not be confirmed (37).

Influence of Genetic Background

An additional determinant of CFA synthase activity, which has been difficult to characterize, can be broadly described as the genetic background. Although it has not been systematically studied, the variation in CFA synthase specific activities among *E. coli* strains is considerable; 10-fold differences between otherwise identical, stationary-phase cultures are common, and a 70-fold difference between two strains has been found to persist despite a variety of genetic and physiological manipulations (see the section on regulatory mechanisms, below). Variants of other bacteria which consistently produce abnormally low levels of CFAs have also been fortuitously found (14, 50). Since this apparently genetic variation in activity can exceed that caused by growth phase or culture conditions (33, 92), it must be considered physiologically significant, despite the uncertainty of its biochemical basis in some situations.

Regulatory Mechanisms

The available data allow at least three mechanisms to be implicated in determining the CFA synthase level of an *E. coli* cell.

Stringent control. Starvation of growing cells for amino acids, the classical means of inducing the stringent response, has been observed to induce CFA synthase and CFA formation in normal (*rel*$^+$) *E. coli* strains. In "relaxed" (*relA*) mutants, this induction was not seen (30, 85). These observations are consistent with the fact that a *relA* mutant, fortuitously included in a study of CFA synthase amplification by plasmids, yielded low specific activities in both unamplified and plasmid-amplified stationary-phase cultures (33). It is not clear, however, whether the stringent response functions independently of *rpoS* control (see below) in influencing CFA synthase levels.

Stationary-phase (RpoS) control. Analysis of *cfa* transcription has revealed two functional promoters of the CFA synthase gene. The distal promoter resembles *E. coli* promoters recognized by the σ^{70} subunit of RNA polymerase and initiates transcription in all phases of growth. The proximal promoter, however, becomes active only in the transition to the stationary phase; it lacks a σ^{70} consensus structure but has a sequence resembling promoters recognized by the alternate RNA polymerase submit σ^{38} (93). This alternate sigma factor, the product of the *rpoS* gene, is known to be responsible for the expression of an array of genes whose products accumulate in stationary-phase cells (99). Moreover, the level of active RpoS is itself regulated in a complex manner by environmental conditions, including osmotic strength, cell density, and temperature (76). A two-component regulatory system and proteolytic

inactivation of RpoS protein seem to contribute to this regulation of RpoS itself (76).

The presence of the two *cfa* promoters suggests a mechanism for the observed rapid induction of CFA synthase (and of CFA accumulation) in the transition to the stationary phase. The basal transcription rate of *cfa* has been found to increase severalfold due to transcription from the second promoter as the RpoS protein (38) accumulates. This increase coincides with the slowing of growth and phospholipid synthesis, which probably enhances the relative rate at which the fatty acid composition is altered. The finding also links CFA synthesis in a more concrete and (potentially) informative way to other, coordinately regulated molecular changes in the *E. coli* cell. The array of RpoS-regulated genes identified to date has no marked relevance to lipid metabolism or membrane properties per se. The RpoS response encompasses diverse enzymatic and cellular activities and can be presumed to reflect multiple adaptations of fundamental cellular functions. Genetic evidence for the pervasive physiological impact of this global response can be inferred from the fact that it appears to be deleterious under certain conditions; specifically, prolonged incubation of nongrowing cultures selects particular mutant alleles of *rpoS* (99). Various *rpoS* mutations are widely distributed among strains of *E. coli* K-12 (21, 99), apparently due to inadvertent selection under laboratory conditions. Indeed, even different stocks of strain W3110, a prototypic wild-type strain, show three different RpoS patterns (49). One stock synthesized a truncated RpoS polypeptide that also has two mutations in the N-terminal region, a second stock failed to transcribe the *rpoS* gene, and a third stock encoded a normal RpoS protein.

Synthesis and accumulation of the RpoS protein are determined, in part, by ppGpp, the guanosine tetraphosphate responsible for the stringent response (54). Thus, the observed effect of *relA* mutations on CFA synthesis may be mediated by RpoS rather than by the classical stringent response.

Proteolysis. After its induction in batch culture, CFA synthase activity declines sharply. Since this decline occurs in the absence of net growth and protein synthesis and is not observed for chloramphenicol acetyltransferase expressed from the *cfa* promoters, it represents inactivation of the enzyme itself (93). CFA synthase has been modified by the addition of a short carboxy-terminal peptide sequence which enables labeling by biotinylation in vivo. Pulse-chase labeling of this slightly modified protein demonstrated a half-life of less than about 5 min in vivo (93). Such rapid turnover is in no way characteristic of *E. coli* proteins generally, although it is observed for certain regulatory proteins (31).

In addition to its role in the temporal control of CFA synthesis, proteolysis may underlie much of the observed variation of CFA synthase activity among bacterial strains. For example, our own unpublished results show that two strains of *E. coli* K-12, C600 and AMA1004 (9), produce very different levels of CFA synthase. This difference (which averages about 70-fold over multiple cultures grown under various physiological conditions) cannot be attributed to a differential efficiency of *cfa* expression between the two strains, since a plasmid-borne *cfa-lacZ* fusion protein was expressed equally well in both strains under these conditions. The difference also cannot be attributed to differences in the catalytic efficiencies of the CFA synthase made by the two strains, since the ratio persists when a high-copy-number cfa^+ plasmid, pGI22, is introduced into both (37). At present, therefore, we attribute this difference to differential stability (presumably reflecting proteolysis) of CFA synthase in the two strains.

What Physiological Roles Do CFAs Play?

Cyclopropane ring formation in bacterial membranes should have a high energetic cost. A typical *E. coli* cell contains about 4×10^7 acyl chains (66), of which nearly half can be UFAs, virtually all of which are subsequently converted to CFAs under suitable conditions (53). By reasonable estimates, each AdoMet molecule that is recycled from *S*-adenosylhomocysteine after cyclopropanation costs the cell three ATPs (91). In view of this investment in chemical-bond energy, it was at one time hypothesized that CFAs may act as a specialized storage form of activated C_1 units which, due to the strained nature of the cyclopropane ring, might be used in subsequent methylation reactions. However, the methylene carbons of the CFAs of *E. coli* were found to be stably retained in the fatty acids both during normal growth and during starvation of a methionine auxotroph for methionine (15).

The combination of the energetic expense of CFA synthesis, its timing (i.e., immediately before growth cessation), and its sensitivity to culture conditions have historically been interpreted as evidence that the presence of CFAs (or absence of UFAs) adapts the cell for adverse conditions normally encountered in the stationary phase or beyond. However, a clear picture of this protection which CFA formation is supposed to confer has eluded several generations of researchers using a variety of experimental approaches.

Membrane fluidity. A prominent theme among the various hypotheses put forward is that CFA formation changes the fluidity or other physical properties of bacterial membranes in a biologically relevant way. Unfortunately, for each measurement that detects a physical change due to cyclopropane formation, another detects little or no change or even a contrary effect. At least two studies have found that hydrated synthetic phosphatidylcholines have higher thermotropic phase transition (melting) temperatures when UFAs are replaced with CFAs (62, 82). This observation may not be relevant to bacterial membranes, however, since the synthetic lipids studied were *sn*-1,2-di-unsaturates. As a rule, bacteria have SFAs rather than UFAs in the *sn*-1 position; they also grow at temperatures well above the phase transition temperatures of their lipids (17). A similar analysis of hydrated phosphatidylcholines or phosphatidylethanolamines with SFA in the *sn*-1 position indicated the opposite effect, i.e., a significant decrease in the transition temperature when UFAs are replaced by CFAs (74). In addition, the overall breadth of the transition was found to increase. These results indicate that CFAs pack more poorly into the acyl chain array of the phospholipid bilayer than do UFAs (74). This may be significant, since the primary function of monoenoic fatty acids is believed to be disruption of ordered acyl chain packing to ensure fluidity of biological membranes (17).

Studies of acyl chain dynamics by nuclear magnetic resonance spectroscopy (NMR) indicate that cyclopropane rings restrict the overall mobility and disorder of the acyl chain between the *cis* segment and the polar head group, compared to *cis* double bonds (24). Within a biologically relevant temperature range, the *cis*-cyclopropane ring appears to reduce the effects of temperature on membrane fluidity and to restrict the propagation of motion from one end of the acyl chain to the other (25). Deuterium NMR has demonstrated a similar effect, albeit of rather modest magnitude, in isolated membranes of *Acholeplasma laidlawii* whose fatty acyl composition was experimentally manipulated (48). However, another NMR technique detected little difference in the behavior of CFA- and UFA-containing lipids in such membranes (59). A decreased fluidity has also been reported upon distal cyclopro-

panation of mycolic acids (29, 58a), and a decreased thermal transition has been found upon an increase in the content of *trans*-mycolic acids (a mixture of olefin and cyclopropane chains) (29), but these data are difficult to interpret due to the lack of defined structural studies of mycolic acids.

Chemical stability. Despite the high strain imposed by C—C bond angles in the cyclopropane ring, CFAs, once formed, appear to be stable membrane components. Upon return to exponential growth, the CFAs are merely diluted by the synthesis of new lipids that lack CFAs (15, 84). To our knowledge, the only report of reversal of cyclopropane ring formation comes from a very recent study on a mutant of *Pseudomonas putida* that has gained tolerance to toluene (78). Upon exposure to toluene, CFAs disappeared coincident with an increase in *cis*-UFAs. The resulting *cis*-UFAs were then converted to the *trans* isomers, which are thought to protect the organism from toluene. However, conversion of one molecule to another was not directly demonstrated, and the loss of CFAs occurred only upon addition of toluene, raising the possibility that cyclopropane rings were cleaved by a separate cellular function coinduced with the toluene degradation enzymes. Moreover, similar experiments on other toluene-resistant strains of *P. putida*, in which CFA levels were found to increase (or remain unchanged) upon toluene addition, have been reported (42, 94). Unfortunately, interpretation of these studies suffers from the use of the known cyclopropane-cleaving reagent, BF_3, to form the methyl esters analyzed.

Rather than being labile, CFAs appear to be considerably less reactive than the corresponding UFAs toward certain forms of oxidation (56). Unlike UFAs, CFAs resist ozonolysis and other mild oxidative treatments (13), as well as photochemical generation of singlet oxygen (36). Thus, absence of dramatic effects on the physical properties of biological membranes could be interpreted as evidence that the primary function of CFA formation is to change chemical properties of the membrane without changing physical properties. Harley et al. (40) provided some experimental support for this idea. They produced *E. coli* cells with various fatty acid compositions (containing either CFAs or monoenoic or polyenoic fatty acids) by appropriate supplementation of a UFA auxotroph. They then subjected the cells, harvested in both exponential and stationary phases, to hyperbaric oxygen (20-atm) and monitored cell survival. CFA-containing cells survived better than did monoene-containing cells, which in turn fared better than did cells containing polyene fatty acids (which *E. coli* does not produce). The biochemical basis of the effect remains unclear, however, since it could not be attributed to oxidative decomposition of the membrane lipids themselves (40). In more recent work, Yuan et al. (97) expressed in *M. smegmatis* an *M. tuberculosis* gene that converted the mycolic acids of the host organism to cyclopropane derivatives (which *M. smegmatis* normally lacks) and reported that this strain was more resistant to hydrogen peroxide than was the parental *M. smegmatis* strain. However, high concentrations of peroxide were needed, and no direct action of peroxide on the mycolic acids was demonstrated. It should also be noted that the relevance of this heterologous system to *M. tuberculosis* pathogenesis has not been established.

Genetic tests of physiological impact. The availability of *cfa* mutant strains, Tn*10* insertions near *cfa*, and various genetic constructs containing the *cfa* gene provide potentially rigorous means of testing the physiological significance of CFAs in *E. coli*. In particular, by introducing otherwise identical *cfa*-containing and *cfa*-defective plasmids into a *cfa* null host, isogenic pairs of strains can be constructed in which the greatest attainable difference in the CFA content can be achieved independently of the growth phase (33, 36). This allows rigorous criteria to be applied in evaluating an apparent benefit or disadvantage of CFAs in vivo. For example, the effect should depend only on the fatty acid composition; i.e., it should occur in different phases of growth and in various genetic backgrounds (distinct strains) of *E. coli*.

Despite (or, more accurately, due to) the rigor of such genetic approaches, physiological functions of CFAs in *E. coli* have not been convincingly elucidated. Neither the complete absence of active CFA synthase and CFAs nor their overproduction in all phases of growth has been found to affect the growth rate, growth yield, or survival under laboratory culture conditions (33, 86). The effects of various chemical and environmental stresses, including sunlight, photosensitizers, solvents, and oxidizing agents, on survival have similarly been tested, with no apparent difference due to the CFA content (36). So far, differential survival of isogenic wild-type and *cfa*-defective *E. coli* strains has been observed only in 20% (vol/vol) ethanol and during repeated freeze-thaw treatments. Although observed in different genetic backgrounds, these two effects remain difficult to interpret in mechanistic terms (36).

CONCLUSION AND FUTURE DIRECTIONS

Diverse bacteria, as they encounter starvation or other forms of growth stasis, modify their membrane lipid in situ by converting the phospholipid UFA chains to CFA chains. The modifying enzyme was first characterized in the 1960s but has been purified to homogeneity from *E. coli* only recently, due to its remarkable instability in vitro and in vivo. Although the enzyme is a soluble protein located in the bacterial cytoplasm, it catalyzes the addition of a methylene group from the small soluble molecule AdoMet to the *cis* double bond of a UFA chain esterified to membrane phospholipid. Association of the enzyme with phospholipid bilayers is reversible and discriminating, requiring the presence of acyl chains of appropriate geometry within the bilayer. This combination of properties implies a unique mode of protein-lipid interaction. Determination of the nucleotide sequence of *cfa* and availability of purified enzyme opens the door to more detailed molecular analysis of this interaction. However, the instability of *E. coli* CFA synthase persists in purified preparations (92), implying that the analyses will not necessarily be straightforward.

The physiological rationale for cyclopropane ring formation in bacterial membranes remains obscure. Many plausible hypotheses have been raised, but they generally appear to have failed under the scrutiny afforded by genetic manipulation of CFA synthesis in *E. coli*. We note, however, that a role in the long-term survival of nongrowing cells remains a valid, if rather vague, working hypothesis, as illustrated by the identification of a role for RpoS in CFA formation. Perhaps the elucidation of new biochemical aspects of stressed bacteria (which probably best represent the state of bacteria in nature) will help shed light on the logic of CFA formation. We note, for example, that the synthesis of *trans*-UFAs by isomerization of *cis*-UFAs has been observed in various bacteria, where it is associated with prolonged starvation, low-temperature growth in marine environments, or growth stasis imposed by high concentrations of phenolic solvents (38, 52, 70). This compositional change appears to result from enzymatic *cis-trans* isomerization of phospholipid acyl chains in situ. It has been interpreted as the means by which the bacterial cell can decrease membrane fluidity (or some related property) when the absence of fatty acid biosynthesis precludes changes of acyl composition via the normal route (52). The analogy of this phenomenon to CFA formation seems intriguing. Unfortunately, the physiological

significance of *trans*-UFAs in bacterial membranes itself remains obscure. Whether an *E. coli* UFA auxotroph tolerates (i.e., survives) the incorporation of *trans*-UFAs in membrane phospholipid depends on mutations of a gene which has been mapped but not biochemically characterized (20).

Although identifying the biochemical and physiological consequences of CFA in *E. coli* does not appear especially straightforward at present, it nevertheless seems strategic for elucidating the function of CFAs, in particular bacterial and protozoan pathogens, for which experimental methods are much more limited. Cyclopropanation of the mycolic acids is common among pathogenic mycobacteria but rare among saprophytic species. Furthermore, *M. tuberculosis* cyclopropanates its mycolic acids at two positions, and two distinct enzymes, with sequence homology to *E. coli* CFA synthase, seem to be required (29). Among *Helicobacter* isolates, those identified as gastric colonizers tend to make large amounts of CFAs whereas those identified as intestinal colonizers generally do not (41). Results such as these seem to suggest that CFAs play a role in the physiology of pathogenesis, but physiological and genetic analyses of the pathogens remain extremely difficult. For example, it has been possible to inhibit CFA synthesis in the trypanosomatids *Crithidia* and *Leishmania* by administering thiastearic acids, but this treatment undoubtedly has other, unidentified biochemical consequences on the pathogen and remains unable to demonstrate an effect of CFAs in vivo (4, 77).

Finally, one may speculate that a biological effect of CFAs might have been missed in laboratory studies of pure microbial cultures because the effect is not on the producing organism. For example, the slime mold *Physarum polycephalum* produces a lyso-phosphatidic acid that competitively inhibits mammalian DNA polymerases of the α-family. Two structural determinants of the inhibitory activity have been identified: a cyclic 2,3-phosphodiester on the glycerol backbone, and a *cis*-cyclopropane ring in the fatty acyl chain (65). This phenomenon awaits detailed evaluation; it is not clear, for example, whether this lipid is part of a chemical defense or acts as some form of intracellular regulator. It seems significant, however, that mammalian DNA polymerases of other families are insensitive to this lipid and that the precise stereochemistry of both the cyclic phosphodiester and the cyclopropane ring appears necessary for the inhibition of sensitive DNA polymerases (65).

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