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TRANSMETHYLATION REACTIONS IN BACTERIAL LIPIDS

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SUMMARY

Lipids of *Escherichia coli*, *Serratia marcescens* and *Agrobacterium tumefaciens* grown in the presence of methyl-labeled methionine have been examined. *A. tumefaciens* produces lipids containing *N*-methylethanolamine, *N*,*N*-dimethylethanolamine and choline with labeled methyl groups as well as labeled cyclopropane acids. The rate of formation of methylated bases parallels growth, while the rate of cyclopropane acid formation does not. In all three organisms, cyclopropane acid formation takes place mainly in late logarithmic growth and in the stationary phase. Hydrogen as well as carbon of methionine methyl groups is incorporated into cyclopropane rings. A mutant organism blocked in methionine synthesis forms cyclopropane acids from methionine methyl groups, the extent of incorporation being undiluted by a number of known one-carbon donors.

INTRODUCTION

The occurrence, structure and biosynthetic origin of a 19-carbon fatty acid containing a cyclopropane ring emerged from the extensive studies of HOFMANN *et al.*[1,2] on the fatty acids of lactobacilli. Chemical, physical and biological properties of this acid indicate its identity with D- or L-*cis*-11,12-methylene octadecanoic acid[3,4]. Isotopic tracer experiments revealed that the carbon chain of this compound arises from *cis*-octadec-11-enoic acid (*cis*-vaccenic acid), the predominant 18-carbon mono-olefinic fatty acid of these organisms[5]. The methylene bridge was shown to arise from one-carbon donors in whole cells[6].

A 17-carbon homologue of lactobacillic acid was found in *Escherichia coli*[7,8] and identified as *cis*-9,10-methylene hexadecanoic acid[9]. Other organisms in addition to the aforementioned which contain cyclopropane acids are *Agrobacterium tumefaciens*[10], *Serratia marcescens*[11,12], *Clostridium butyricum*[13], pleuropneumonia-like organisms[14], *Streptococcus lactis*[15], and *Aerobacter aerogenes*[16]. It is interesting to note that, of these organisms, two have been found to contain methylated derivatives of ethanolamine in the phospholipids; choline in *A. tumefaciens*[17] and *N*-methylethanolamine in *C. butyricum*[18].

In this paper we wish to report studies on the incorporation of methyl groups of methionine into bacterial lipid components.

EXPERIMENTAL

Materials

All radioactive compounds were purchased from New England Nuclear Corp. Radioactive samples were counted in a Packard Tri-Carb instrument using a toluene system for lipid samples and a polyether system (diethylene glycol dimethyl ether–dioxane–anisole (12.5:75:12.5) for water-soluble samples.

E. coli mutant 205-2 was a gift of Professor B. D. DAVIS.

Methods

E. coli B and *Serratia marcescens* (ATCC 8195) were grown in an inorganic salts (amounts in g/l: NH_4Cl, 2; $(NH_4)_2SO_4$, 0.2; $Na_2HPO_4 \cdot 7H_2O$, 11.4; KH_2PO_4, 3; NaCl, 3; $MgSO_4 \cdot 7H_2O$, 0.1; $CaCl_2$, 0.01; $FeSO_4 \cdot 7H_2O$, $5 \cdot 10^{-5}$) medium with 2% glucose and other compounds as indicated. *E. coli* 205-2 requires *p*-aminobenzoic acid as well as methionine in the growth medium[19]. *Agrobacterium tumefaciens* TT105 was grown on the inorganic medium of STARR[20]. Growth was estimated by turbidity readings in a Klett colorimeter using a 66 filter. Cells were harvested by centrifugation. Lipids were extracted and washed by the method of FOLCH, LEES AND SLOANE-STANLEY[21]. Extracted lipids or whole cells were saponified with 10–20% KOH in 50% methanol. After extraction of the alkaline solution with petroleum ether to remove unsaponifiable material, the aqueous phase was acidified and the acidic lipid fraction was extracted with ethyl ether.

Fatty acids were converted to methyl esters with freshly distilled diazomethane. Gas–liquid chromatographic analysis was performed with a Research Specialties Co. instrument equipped with an ionization detector. In most cases the liquid phase was 5% diethylene glycol succinate on chromosorb W packed in a 6 ft × 0.25 in stainless-steel column. The esters were collected in cooled U-shaped tubes[22] with 50–80% efficiency. Collection efficiency of commercial samples of radioactive fatty acid esters of known purity also varied over a considerable range depending upon the technique used. The assumption is made that the efficiency of collection for each ester is equal within a given run, and that radioactivity not accounted for escaped collection. Estimation of the amount of each acid present is based upon peak area as determined by the method of CARROLL[23].

Phospholipid bases were freed from lipids by hydrolysis with 1 N HCl and were separated by paper chromatography, using the system of BREMER *et al.*[24]. The bases were visualized by first spraying with a solution of ninhydrin in pyridine to develop spots for ethanolamine and *N*-methylethanolamine, followed by exposure to iodine vapors and spraying with starch to develop spots for *N*,*N*-dimethylethanolamine and choline. Radioactivity on paper strips was determined with a Nuclear-Chicago paper-strip counter.

RESULTS

Incorporation of methionine methyl groups into bacterial lipids

Cells of the organisms grown in synthetic media containing L-[*Me*-^{14}C]methionine or L-[*Me*-^{3}H]methionine were harvested after maximal growth. The incorporation of radioactivity into various lipid fractions is shown in Table I. In the case of *E. coli* and

S. marcescens, most of the radioactivity enters the fatty acid fraction. The small amount found in the non-saponifiable lipid fraction in *S. marcescens* may be due to the formation of the methoxyl groups of coenzyme Q, which is found in large quantities in this organism[25].

Considerable radioactivity from the lipids of *A. tumefaciens* is found in the water-soluble portion after hydrolysis. The aqueous portion of an acid hydrolysate of

TABLE I

INCORPORATION OF METHIONINE METHYL GROUPS INTO BACTERIAL LIPIDS

Organisms were grown in synthetic medium with the amounts of radioactive compounds indicated. At maximal growth, cells were harvested and either saponified directly or extracted with chloroform–methanol. Fatty acids were isolated and chromatographed as described in the text.

Organism	*Compound administered*	*Volume of culture (ml)*	*Lipid extract (disintegrations/min)*	*Non-saponifiable fraction (disintegrations/min)*	*Fatty acid fraction (disintegrations/min)*	*Per cent of radioactivity in fatty acids*		
						*17:0 Δ**	*19:0 Δ**	*Others*
Escherichia coli 205-2	L-[*Me*-^{14}C]methionine 35 μC, 15 μmoles	125	$9 \cdot 10^6$	**	$8 \cdot 10^6$	90	7	3
Serratia marcescens ATCC 8195	L-[*Me*-^{14}C]methionine 1 μC, 0.2 μmole	10	***	10^3	$3 \cdot 10^5$	89	8	3
	L-[*Me*-^{3}H]methionine 2.7 μC, 0.2 μmole	10	***	10^3	$4 \cdot 10^3$	85	9	6
Agrobacterium tumefaciens TT105	L-[*Me*-^{14}C]methionine 10 μC, 2 μmoles	100	$3 \cdot 10^6$	**	$2 \cdot 10^6$	17	72	11

* The Δ sign is introduced into the conventional fatty acid abbreviation system to denote a cyclopropane ring.

** Not counted.

*** Whole cells saponified.

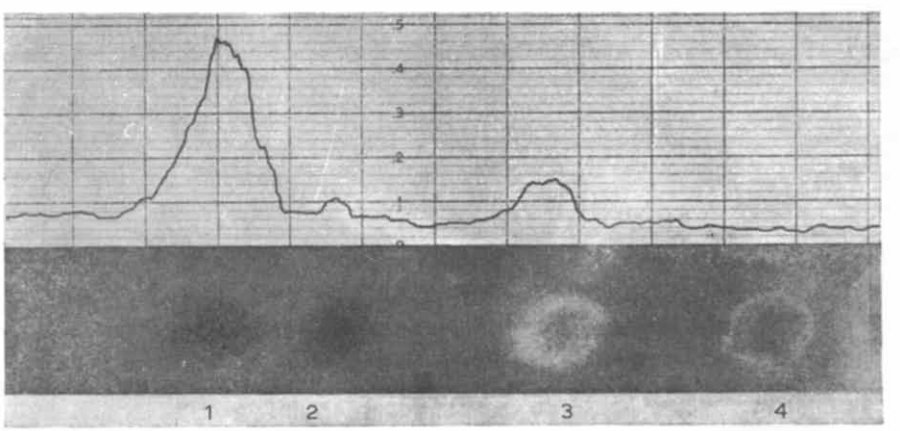

Fig. 1. Paper chromatography of phospholipid bases from *A. tumefaciens*. Phospholipids extracted from cells grown in the presence of L-[*Me*-^{14}C]methionine were hydrolyzed with acid and the bases were chromatographed as described in the text. Upper portion: level of radioactivity; lower portion: stained paper strip. 1, choline; 2, *N*,*N*-dimethylethanolamine; 3, *N*-methylethanolamine; 4, ethanolamine.

A. tumefaciens lipids extracted from cells grown in the presence of L-[*Me*-^{14}C]methionine was mixed with carrier bases and chromatographed on paper. The resulting strip was scanned for radioactivity and stained to reveal the bases (Fig. 1). Virtually all of the radioactivity lies in the three methylated bases, *N*-methylethanolamine, *N*,*N*-dimethylethanolamine and choline. Choline was further identified by elution from the paper strip, dilution with carrier choline and formation and recrystallization of the Reineckate salt. The other methylated bases were eluted, diluted with the appropriate carrier, converted to choline[24] and similarly characterized as the Reineckate salt. The results are summarized in Table II.

TABLE II

CHARACTERIZATION OF METHYLATED PHOSPHOLIPID BASES FROM *Agrobacterium tumefaciens*

Bases eluted from paper strips were counted and diluted with 50 μmoles of the appropriate carrier. Monomethyl and dimethyl derivatives of ethanolamine were converted to choline by addition of 0.5 ml 1 N NaOH and 0.4 ml 1 M CH_3I in ethanol. After shaking for 1 h at 45°, choline was precipitated as the Reinecke salt, which was recrystallized 3 times from acetone–propanol.

Base	*Total radioactivity eluted from paper strips (counts/min)*	*Expected specific activity of Reineckate (counts/min/mg)*	*Found specific activity of Reineckate (counts/min/mg)*
Choline	50 000	2200	2300
N,*N*-Dimethylethanolamine	3 700	160	180
N-Methylethanolamine	14 000	610	430

Formation of cyclopropane fatty acids during growth in batch cultures

Aliquots of bacterial cells were taken at various periods during the growth of batch cultures, and the methyl esters of fatty acids obtained after saponification were analyzed by gas–liquid chromatography (Tables III and IV).

TABLE III

FORMATION OF CYCLOPROPANE ACIDS IN *Escherichia coli* AS A FUNCTION OF GROWTH

E. coli B was grown at 37° in 200 ml of synthetic medium containing 20 μC (9 μmoles) of L-[*Me*-^{14}C]-methionine. Turbidity was determined by the use of a colorimeter tube fused to the culture flask. 10-ml aliquots were withdrawn at the indicated times, the cells were harvested and the fatty acids were isolated, counted for radioactivity and analyzed as described in the text.

Culture age (h)	*Growth (Klett units)*	*Radioactivity in fatty acid fraction (disintegrations/ml of culture)*	*Fatty acid composition (%)*					
			16:0	*16:1*	*17:0 Δ**	*18:1*	*19:0 Δ**	*Others***
1.8	16	$1.2 \cdot 10^3$	37	14	3	23	2	21
5.8	135	$3.6 \cdot 10^4$	33	5	19	23	3	17
6.6	226	$3.9 \cdot 10^4$	38	1	24	3	22	12
24	283	$4.0 \cdot 10^4$	45	2	20	—	15	18
50	355	$4.0 \cdot 10^4$	43	—	20	—	17	20
122	370	$4.0 \cdot 10^4$	41	1	24	2	24	8

* See note to Table I.

** Saponification of whole cells frees lauric, myristic and hydroxymyristic acid from the cell-wall lipopolysaccharide[8]. These components are not found in lipids extracted with solvents prior to saponification.

TABLE IV

FATTY ACID COMPOSITION OF *Serratia marcescens* LIPIDS AS A FUNCTION OF GROWTH

Ten 2-l flasks, each containing 500 ml of medium, were inoculated with 50 ml of a fully grown culture of *S. marcescens* and shaken at 30°. Flasks were removed at the indicated times and the cells were harvested, washed and extracted as described. Total dry weight was determined by summing the weight of the extracted lipids and of the dried, defatted residue.

Age of culture (h)	*Dry weight of cells (mg/l)*	*Lipids*		*Fatty acid composition (%)*				
		Weight (mg/l)	*Per cent of cell dry weight*	*16:0*	*16:1*	*17:0 Δ**	*18:1*	*19:0 Δ**
2.7	40	3.4	8.5	46	28	14	12	—
4.4	97	10	10	46	32	11	12	—
5.7	243	19	8.2	44	31	10	14	—
6.9	642	42	6.8	46	32	10	11	—
9.4	1230	51	4.1	48	14	27	10	—
28	2200	143	6.8	47	—	44	—	8
151	1665	125	7.5	47	—	44	—	9

* See note to Table I.

Batch cultures of *S. marcescens* reached maximal growth between 9 and 28 h, and the mono-olefinic acids were completely converted to cyclopropane fatty acids during this period. Batch cultures of *E. coli* reached maximal growth between 24 and 50 h, but the incorporation of ^{14}C-methyl groups and the conversion of mono-olefinic acids to cyclopropane acids were complete in 7 h. There appears to be no degradation of cyclopropane acids even after 150 h.

Cells in early logarithmic phase of growth produce and store some cyclopropane acids, but in late logarithmic growth and in the stationary phase the reaction proceeds at the same or possibly at an accelerated rate, until virtually all of the olefinic acids have been "saturated" by the insertion of a methylene bridge. Furthermore, it is evident in both organisms that the synthesis of the 17-carbon cyclopropane acids proceeds either earlier or at a faster rate than the synthesis of the 19-carbon cyclopropane acids.

Formation of cyclopropane acids and methylated bases in Agrobacterium tumefaciens

Since this organism uses methionine methyl groups in reactions involving fatty acids and phosphatide bases, it was of interest to compare the rates of synthesis. Aliquots from a batch culture of *A. tumefaciens* grown in synthetic medium containing L-[*Me*-^{14}C]methionine were extracted and the lipids were hydrolyzed with acid so that incorporation of isotope into both cyclopropane acids and methylated bases could be measured (Fig. 2). The rate of formation of methylated phospholipid bases is nearly parallel to growth, while the rate of cyclopropane acid formation is more nearly linear and continues long after the growth of cells has ceased.

Methyl donor in the synthesis of cyclopropane fatty acids

E. coli 205-2 requires L-methionine for growth and lacks the ability to convert one-carbon compounds to methyl groups of methionine[19]. This mutant utilizes the methyl group of methionine for synthesis of cyclopropane fatty acids (Table I). Similar experiments with a methionine-requiring mutant (*E. coli* 113-3) were reported

by O'LEARY[26]. These results indicate the transfer of methyl groups of methionine rather than one-carbon units at some other oxidation level; thus there is only a slight incorporation of formate into the methylene bridge of lactobacillic acid[6].

It is possible that the methyl groups of methionine are oxidized or converted to another intermediate (*e.g.*, formaldehyde) prior to the transfer to mono-olefinic acids.

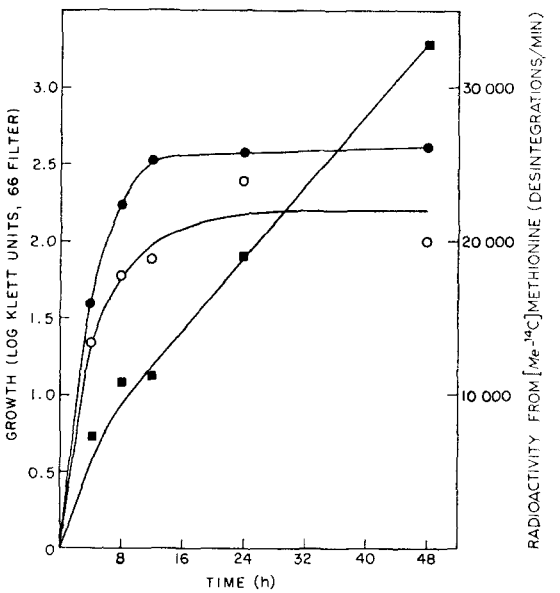

Fig. 2. Time course of formation of cyclopropane acids and methylated bases in *A. tumefaciens*. Cells were grown at 30° in 670 ml of synthetic medium containing 10 μC (4.3 μmoles) L-[*Me*-^{14}C]-methionine. 100-ml aliquots were withdrawn at the times indicated. Extracted lipids were hydrolyzed with 0.3 N HCl at 60° for 2 h. Both the fatty acid fraction and the ether-extracted aqueous solution containing the bases were counted for radioactivity. ●—●, growth; ■—■, fatty acids; O—O, methylated bases.

A series of competitive experiments using a number of unlabeled one-carbon donors in the presence of L-[*Me*-^{14}C]methionine (Table V) indicates that no dilution of radioactivity occurs during the incorporation into fatty acids. It is therefore improbable that any of the compounds tested serves as an intermediate between the methyl group of methionine and the methylene bridge of cyclopropane fatty acids.

DISCUSSION

Liver microsomes catalyze the conversion of phosphatidylethanolamine to phosphatidylcholine by means of transmethylation reactions involving *S*-adenosylmethionine[27–29]. Phospholipid derivatives of *N*-methylethanolamine and *N,N*-dimethylethanolamine are probably intermediates in this process. It appears that phosphatidylcholine synthesis in *A. tumefaciens* follows a similar course. Accumulation of methylated phospholipid bases in a choline-less mutant of *Neurospora crassa* has been reported, but it was observed that only choline and ethanolamine could be found in the lipids of the "wild-type" organism[30]. *Clostridium butyricum* accumulates a phospho-

lipid containing *N*-methylethanolamine but is devoid of *N,N*-dimethylethanolamine and choline[18].

The biosynthesis of the cyclopropane ring in bacterial fatty acids constitutes a novel and unusual process. As the extensive studies of HOFMANN *et al.* have shown, the essence of the reaction is the insertion of a methylene bridge arising from a methionine methyl group across the double bond of a mono-olefinic acid or derived

TABLE V

COMPETITION BETWEEN METHIONINE METHYL GROUPS AND OTHER ONE-CARBON DONORS AS PRECURSORS OF CYCLOPROPANE FATTY ACIDS

Tubes containing 10 ml of synthetic medium, 20 μg *p*-aminobenzoic acid, 1.5 μmoles (10^5 counts/min) L-[*Me*-^{14}C]methionine and 2 μmoles of unlabeled test substances were inoculated with one drop of a fully grown culture of *E. coli* mutant 205-2. The cultures were grown aerobically for 18 h at 37°. Cells were harvested and saponified, and fatty acids were isolated as described.

Addition	*Radioactivity in fatty acid fraction (counts/min)*
None	$1.3 \cdot 10^4$
Sodium propionate	$1.3 \cdot 10^4$
L-Serine	$1.4 \cdot 10^4$
Sodium formate	$1.4 \cdot 10^4$
Formaldehyde	$1.5 \cdot 10^4$
Methanol	$1.5 \cdot 10^4$
Glycine	$1.5 \cdot 10^4$

lipid[2,6]. The results reported in this paper augment this basic information in several ways. Competition experiments with known one-carbon donors and use of a mutant organism blocked in methionine synthesis strengthen the assertion that the process of cyclopropane ring formation involves the direct transfer of a methyl group to a fatty acid derivative. The fact that some of the hydrogen of the methyl group is retained in the product is in accord with this idea. No attempt to determine the amount of hydrogen retained was made, since an isotope effect in the breakage of the carbon–tritium bonds could render the result doubtful.

No direct evidence as to the nature of the fatty acid derivative which participates in the reaction is provided by these experiments. However, KANESHIRO AND MARR[17] found that phosphatidylethanolamine from late exponential phase cells contained both unsaturated and cyclopropane acids. This compound constitutes over 70% of the cellular lipids. Since all of the unsaturated acids are eventually converted to cyclopropane acids, either unsaturated fatty acids are split from phosphatidylethanolamine, converted to cyclopropane acids and re-incorporated into phospholipid, or the conversion takes place at the phospholipid level. Experiments favoring the latter alternative will be described in a future publication.

The role of the cyclopropane fatty acids in the bacterial cell remains a more obscure question. The studies of HOFMANN *et al.*[2,31] established the biotin-sparing activity of cyclopropane fatty acids in lactobacilli. Unsaturated fatty acids have similar biotin-sparing activity, and present knowledge of the role of biotin in fatty acid biosynthesis[32] permits a reasonable explanation of this observation. It is probable that cells require a phospholipid with certain physical properties to fulfil a structural

function, and these properties are available when either unsaturated or cyclopropane fatty acids are present in the molecule.

In this regard it is of interest that soluble phospholipid micelles can be obtained from bacterial phospholipids containing unsaturated fatty acids or containing only saturated and cyclopropane fatty acids using the procedure of FLEISCHER AND KLOUWEN[33]. These authors have reported that phospholipids containing saturated fatty acids are not appreciably solubilized and relate this to the function of unsaturated fatty acids in mitochondrial phospholipids. Therefore, the "saturation" of unsaturated fatty acids in phospholipids by insertion of a methylene bridge does not alter certain important physical properties (*e.g.*, ability to form soluble lipid micelles). This may indicate merely that cyclopropane formation does not interfere with the normal function of unsaturated acids. The fact that many microorganisms, *e.g.*, some streptococci[34] or *Azotobacter agilis*[17], are devoid of cyclopropane acids accords with this notion.

No completely satisfactory proposal concerning the function of cyclopropane fatty acids can be made with information presently available. There appears to be no metabolic turnover of these structures (Tables II and III). It can be conjectured that the reaction merely serves to remove some undesirable metabolic product such as *S*-adenosylmethionine or unsaturated acids. This would seem less economical than the well-controlled bacterial metabolism usually permits. Alternately it can be suggested that unsaturated fatty acids in the membranes of bacteria are labile to either oxygen in aerobic bacteria or free radicals in anaerobic organisms. Since bacteria are not known to contain lipid antioxidants, saturation with cyclopropane rings could prevent lipid peroxidation. It is difficult to rationalize the limited distribution of these compounds with this hypothesis.

The precursor-product relationship between olefinic acids and cyclopropane acids and the fact that the rate of the conversion is not parallel to the rate of olefinic acid synthesis suggest an infinite number of possibilities for experimentally determined fatty acid compositions of bacterial cells which contain these compounds. It seems desirable in reporting composition of fatty acid in organisms displaying this phenomenon to establish the final concentration of the various acids in late stationary phase cells where cyclopropane ring formation has proceeded to completion.

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