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Methylation of the Cellular Lipid of Methionine-requiring *Agrobacterium tumefaciens*

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Mutants of *Agrobacterium tumefaciens* requiring methionine for growth on a solid basal medium were induced by the use of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. In addition to the difference of mutant strains, the extent of methionine dependency differed in a liquid basal medium and in the presence of aspartate or fumarate. When (^{14}C-methyl)-methionine was added to strain WM-11 growing in a prescribed basal medium, incorporation of ^{14}C into the cellular "residue" fraction and polar "N-methylated" lipid fraction depended strictly on cellular growth and on external methionine concentration. However, a net synthesis of the "cyclopropane" fatty acid fraction occurred even during the maximal stationary phase if excess methionine was present.

Methylation of cellular lipid from the methyl group of methionine has been investigated with methionine auxotrophs of *Escherichia coli* (13, 14, 17) and of *Neurospora crassa* (11, 12). Nyc and co-workers (9, 18, 19) used choline-requiring *N. crassa* to control the transmethylation into *N*-methylated phospholipid. However, the methylation of lipid in *Agrobacterium tumefaciens* has been analyzed only with wild-type strains (6, 8, 14, 21). With agrobacteria, Goldfine and associates (6, 8) showed that *N*-methylated phospholipid resides in the cell membrane. Transmethylation to synthesize cyclopropane fatty acids (2, 10, 16, 17, 26, 29) from monounsaturated fatty esters also occurs at the phospholipid level but can be clearly distinguished from the *N*-methylation of the polar $-NH_2$ moiety (14). These investigations suggested that transmethylation proceeding from methionine substrate into lipid residing in the cell membrane is of two distinct types, methyl addition to *cis*-monoenes and to polar $-NH_2$ moieties.

This paper proposes to define the growth substrate and conditions necessary for the occurrence of two different types of lipid methylation with mutants of *A. tumefaciens*. Mutants that require methionine for growth were acquired by exposure to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) (1, 22). These methionine-requiring strains were used to study the cellular synthesis of *N*-methylated lipid and cyclopropane fatty ester. This report suggests that ^{14}C-methylations of cellular lipid can be controlled with exogenous methionine.

MATERIALS AND METHODS

Isolation of methionine-requiring strains. The wild-type *A. tumefaciens* (NRRL B-36) designated strain W was treated with 200 μg of NTG mutagen per ml of cellular suspension. The selection procedure outlined by Adelberg et al. (1) was followed, with modification of the media and allowance for differences in growth rate between *E. coli* and *A. tumefaciens*. *A. tumefaciens* strain W was grown aerobically at 25 C in Nutrient Broth with 200 mg of yeast extract per liter. After treatment with NTG, the filtered cells were washed with a synthetic basal medium (BM). The cells were then incubated in the BM containing methionine (300 mg per liter) or other growth factors for 8 hr before dilution with saline and selective plating.

A methionine-requiring strain designated WM-1 was isolated by replica plating (15). Strain WM-11 was selected from a NTG-mediated mutation of parent strain WM-1. The WM strains were cultured on Nutrient Agar slants containing yeast extract and were plated in replica at least once more to assure stability of the methionine requirement on solid media.

Culture of strains. Methionine dependency varied between strains as well as between media. For example, replication of WM strains on solid-agar plates (Table 1) disclosed a difference from liquid cultures. Replica plating of discrete colonies was a more reliable index of the methionine requirement than use of liquid cultures.

The basal medium (BM) for *A. tumefaciens* (24, 25) was modified to contain 5 g of sucrose, 1.5 g of NH_4Cl, 5 g of K_2HPO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, and 1.4 g of KOH per liter at *p*H 7.0. To each liter of BM, 1 ml of a solution of trace metals was added containing: $Na_2B_4O_7 \cdot 10H_2O$, 44 μg; $CaCO_3$, 250 μg; $CuSO_4 \cdot 5H_2O$, 40 μg; $FeSO_4 \cdot 7H_2O$, 2,480 μg; KI, 1.3 μg; $MnSO_4 \cdot 4H_2O$, 81 μg; $Na_2MoO_4 \cdot 2H_2O$, 25 μg;

TABLE 1. *Requirement for methionine and aspartic acid when WM strains (Agrobacterium tumefaciens) are replica-plated*

Supplement to BM[a]	Replicated colonies[b] of strain	
	WM-1	WM-11
Yeast extract (control)	23	113
Aspartic acid	55	2
Methionine	55	106
Aspartic acid and methionine	55	118

[a] Each amino acid was added to a synthetic basal medium (BM) in a concentration of 300 mg per liter; yeast extract was 500 mg per liter.

[b] Overnight cells grown on BM slants supplemented with yeast extract were diluted in saline and plated on BM with aspartic acid and methionine. The plates with 20 to 80 colonies were then replicated.

and $ZnSO_4 \cdot 7H_2O$, 260 μg. All supplements to the BM, except the yeast extract, were added at a final concentration of 300 mg per liter; the yeast extract supplement was 200 mg per liter. Cultures were incubated on a rotary shaker at 25 C, and growth was estimated turbidimetrically with a Klett no. 66 filter. The viable count of cells was approximately proportional to 200 Klett units (50 units gave 8.0×10^8 viable cells per ml), and the dry weight of cells was approximately proportional to 300 Klett units (50 units gave 0.20 mg of dry weight per ml).

^{14}C-methyl incorporation into cellular components. Strains W, WM-1, and WM-11 grown overnight on Nutrient Agar slants supplemented with yeast extract were suspended in saline. Each 250-ml Erlenmeyer flask with a sidearm cuvette contained a 4% inoculum of cells suspended in saline and 40 ml of BM having 0.6 μc of (^{14}C-methyl) methionine (0.8 mg of L-methionine per liter of medium). Growth was measured turbidimetrically, and ^{14}C-methyl incorporation into cellular components from different growth phases was determined with four samples of 10 ml each. With nonlabeled methionine (300 mg per liter), a dilution effect of ^{14}C-methyl incorporation was expected if the synthesis of cellular components depended on exogenous methionine.

Since the cellular components were diminutive during the early phase of growth, each 10-ml sample was mixed at ice-bath temperature with 5 ml of carrier cells grown to the maximal stationary phase. A procedure has been described (14) for the separation of cellular components of *A. tumefaciens* into (i) cellular residue after $CHCl_3$-methanol (2:1) extraction of lipid, (ii) fatty acid (cyclopropane) fraction by diethyl ether extraction of the acid-hydrolyzed lipid, and (iii) water-soluble (*N*-methylated) fraction of the acid-hydrolyzed lipid.

Special chemicals. NTG was purchased from K&K Laboratories, Inc. (Plainview, N.Y.).

(^{14}C-methyl) methionine from Volk Radiochemical Co. (Skokie, Ill.) was diluted to a specific activity of 2.8 μc per μmole of L-methionine. The ^{14}C-methyl incorporation into the cellular residue and water-soluble fractions was determined in a Beckman LS-100 scintillation counter with a fluid system containing naphthalene (100 g), 2,5-diphenyloxazole (5 g), and Cab-O-Sil (5 g; Beckman Instrument Co., Fullerton, Calif.) per liter of dioxane. For the ^{14}C detection in fatty acids, the dried samples were dissolved in toluene containing 2,2-*p*-phenylene-bis(5-phenyloxazolyl) benzene (50 mg per liter) and 2,5-diphenyloxazole (4 g per liter).

RESULTS

Methionine requirement for growth. Strains W, WM-1, and WM-11 were grown on Nutrient Agar slants containing yeast extract (24 hr at 25 C) and were diluted in saline. The cellular suspensions were then transferred to a liquid BM with various concentrations of exogenous methionine. Figure 1 indicates that the growth of both W and WM-1 was repressed by increasing concentrations of methionine, whereas WM-11 showed a methionine requirement of approximately 50 mg per liter for maximal growth. The growth response of mutant strain WM-1 to methionine was extremely variable in a liquid medium (Fig. 1), in contrast to colonies on solid media (Table 1).

Growth rate of strains. Maximal growth rates of both strains W and WM-1 were the same whether or not methionine was supplemented to the BM. However, the growth rate of strain WM-11 with a limited concentration of external methionine [0.8 mg of (^{14}C-methyl) methionine per liter of medium] was considerably less. With excess methionine (300 mg per liter), the maximal

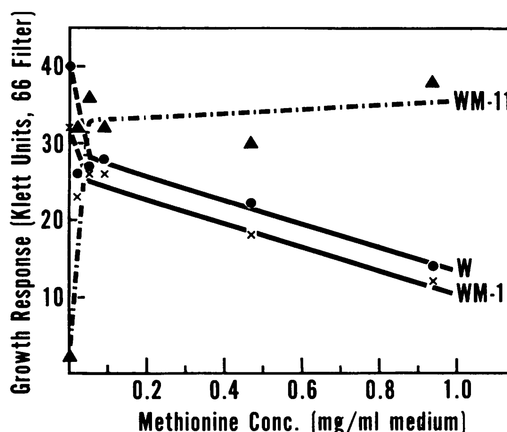

FIG. 1. *Growth response of Agrobacterium tumefaciens to methionine during exponential-growth phase (24-hr incubation aerobically in a synthetic basal medium at 25 C). Strain W (●), strain WM-1 (×), and strain WM-11 (▲). Fifty Klett units gave 0.20 mg (dry weight) of cells per ml.*

growth rate was approximately that of strains W and WM-1 (Fig. 2).

Substrate requirement for growth. Since the synthesis of methionine can be separated into three metabolic pathways, depending on the origin of the carbons and sulfur (27), other compounds besides methionine were added to the liquid BM. Table 2 indicates that the growth of strain W was repressed by homocysteine, cysteine, or methionine. Strain WM-1 showed slight stimulation with aspartic acid, methionine (note contrast to Fig. 1), and sodium thiosulfate. However, strain WM-11 consistently exhibited a marked stimulation by exogenous methionine. Other differences of strain WM-11 included a complete lack of repression with cysteine and an efficient repression of growth with aspartic acid (Table 1).

Since the growth response of strain WM-1 to exogenous methionine varied, further analysis was conducted by supplementing the liquid BM with vitamins (40 to 200 μg per liter of medium), sugars (3 g per liter), other organic carbon sources (1 g per liter), nucleic acids (150 mg per liter), and amino acids (300 mg per liter). None of the vitamins tested (vitamin B_{12}, folic acid, thiamine, pyridoxine, pantothenic acid, *p*-aminobenzoic acid, biotin, riboflavine, and niacin) affected growth appreciably. Of the sugars (glucose, galactose, fructose, mannose, and ribose), only ribose affected growth by a slight repression of 0.5 time (Klett turbidity ratio of the supplemented culture/control culture). When other organic carbon sources (malate, succinate, fumarate, and α-ketoglutarate) were added to the BM, only fumarate was observed to stimulate (3.2 times) appreciably. When a fragment of nucleic acid (adenosine, guanine, thymine, and uracil) was added, only adenosine repressed growth to 0.4 time. Guanine, thymine, and uracil stimulated growth slightly (1.2 to 2.0 times). Of the amino acids (cysteine, cystine, tryptophan, glutamic acid, lysine, valine-isoleucine, arginine, and aspartic acid), only arginine (1.5 times) and aspartic acid (2.0 times) stimulated growth of strain WM-1. Cysteine, glutamic acid, and lysine showed varied degrees of repression (0.2 to 0.5 time).

TABLE 2. *Growth response of strains W, WM-1, and WM-11 to compounds related to the synthesis of methionine*

Supplements to BM[a]	Turbidity ratio of supplemented cultures/control cultures[b] of strain		
	W	WM-1	WM-11
Yeast extract	2.7	5.0	7.0
Methionine	0.6	1.6	9.7
Aspartic acid	1.7	2.4	0.8
Methionine-aspartic acid	1.1	1.4	13.3
Betaine	0.5	0.8	0.6
Homocysteine	0.3	0.5	0.8
Cysteine	0.2	0.3	1.1
Sodium thiosulfate	0.9	2.0	0.9

[a] Each supplement to the basal medium (BM), with the exception of yeast extract, was added in a final concentration of 300 mg per liter; yeast extract was 200 mg per liter.

[b] The inocula were cellular suspensions in saline from 24-hr cultures grown on Nutrient Agar slants supplemented with yeast extract. Cultures were incubated at 25 C for approximately 12 hr (early exponential growth), and growth was measured turbidimetrically with a Klett no. 66 filter. The control cultures were grown on liquid BM without supplements.

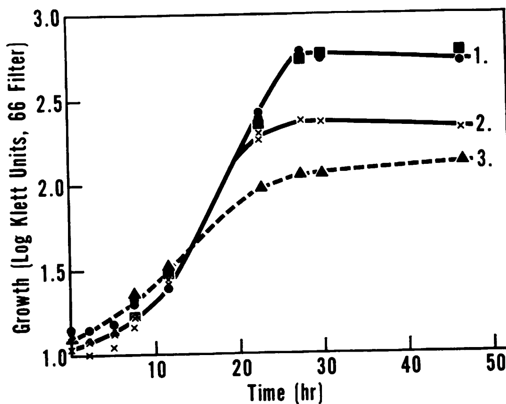

FIG. 2. *Growth cycle of Agrobacterium tumefaciens strains in the presence of limited (0.8 mg per liter) or excess (300.8 mg per liter) methionine in a synthetic basal medium. Curve 1, growth of strain W with limited or excess methionine (●) and strain WM-11 with excess (■) methionine. Curve 2, growth of strain WM-1 with limited or excess methionine (X), and curve 3, growth of strain WM-11 with a limited amount of methionine (▲). Fifty Klett units gave 0.20 mg (dry weight) of cells per ml.*

^{14}C-methyl incorporation into cellular lipid. If cellular synthesis depended on exogenous methionine, unlabeled methionine (300 mg per liter) was expected to dilute ^{14}C-methyl incorporation throughout the growth cycle. Furthermore, the ^{14}C-methyl of methionine incorporated into the cell can be separated into (i) a cellular residue, (ii) a fatty acid (cyclopropane) fraction, and (iii) a water-soluble (*N*-methylated) fraction of hydrolyzed lipid (14).

With strains W and WM-1, exogenous unlabeled methionine reduced ^{14}C-methyl incorporated into the cellular residue and cyclopropane fraction only through the mid-exponential growth phase. Although this dilution effect was observed

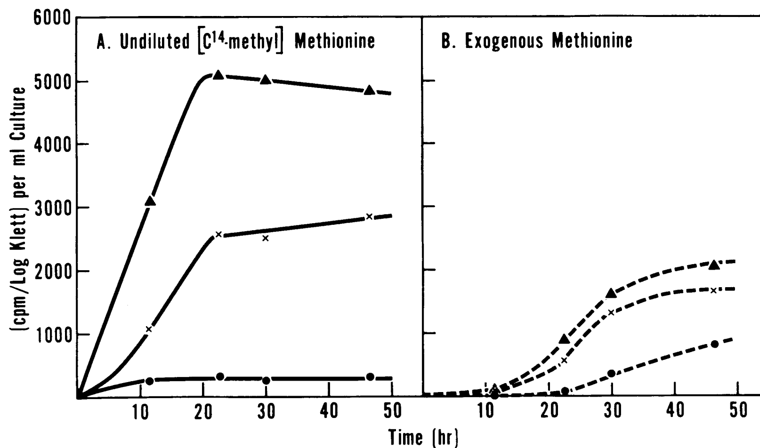

FIG. 3. *¹⁴C-methyl incorporated into fatty acid (cyclopropane) fraction (●), N-methylated lipid fraction (X), and cellular residue (▲) of strain WM-11 of Fig. 2, when methionine (0.8 mg per liter) was limited (A) and when methionine (300.8 mg per liter) was in excess (B). Fifty Klett units gave 0.20 mg (dry weight) of cells per ml.*

in the *N*-methylated lipid fraction of strain WM-1 also, exogenous methionine had no effect on the *N*-methylated lipid of the wild type (strain W). No dilution effect was detected in all three methylated components when growth approached the stationary phase and thereafter. Therefore, the control of cellular methylation with exogenous methionine is tenuous with strains W and WM-1.

In contrast, the methionine-dependent strain WM-11 incorporated ^{14}C-methyl uniformly throughout the growth cycle, as indicated by the dilution effect of Fig. 3. Excess unlabeled methionine diluted ^{14}C-methylation of both the cellular residue and the *N*-methylated lipid fraction throughout the growth cycle. However, the synthesis of cyclopropane fatty acid was unlike the other cellular components. After the stationary phase (30 hr), exogenous unlabeled methionine stimulated rather than reduced ^{14}C transmethylation into fatty acids. The culture (Fig. 3A) with limited methionine (0.8 mg per liter) ceased transferring ^{14}C-methyl to the fatty acid component within 10 hr.

DISCUSSION

Since the synthesis of methionine involves three diverse cycles (27), methionine auxotrophs may be conveniently divided into organisms with (i) defects in methylation, e.g., vitamin B_{12} and adenosyl-methionine requiring mutants (4, 7, 20, 28); (ii) defects in sulfur metabolism (11); and (iii) defects of the 4-C metabolism, e.g., aspartic acid (3, 5, 23). This investigation suggests that strain WM-1 is probably defective in the metabolism of the 4-C backbone, since aspartic acid, fumaric acid, and thiosulfate were the only compounds tested that stimulated growth. However, strain WM-11 showed an abnormally large demand for the intact methionine molecule; even cysteine and homocysteine failed to repress its growth. Consistent with the requirement for methionine, strain WM-11 was repressed efficiently with aspartic acid.

In batch cultures, exogenous unlabeled methionine was used to control the extent of ^{14}C-methyl incorporation into the cellular constituents (residue fraction) of strain WM-11 as well as its polar lipid (*N*-methylated) fraction. In contrast, excess unlabeled methionine stimulated ^{14}C-methyl incorporation into the fatty acid (cyclopropane) fraction of stationary-phase cultures. Previous studies with methionine auxotrophs of *E. coli* (13, 14, 17) have shown that cyclopropane fatty acid is synthesized by the transfer of the methyl group of methionine. Nevertheless, the relation of cyclopropane fatty acid synthesis to cellular growth is not direct and is thus specified as an "accessory biochemical character" (13). The use of continuous cultures is contemplated to control the methylation of phospholipid in *A. tumefaciens*.

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