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Methyl Sterol and Cyclopropane Fatty Acid Composition of *Methylococcus capsulatus* Grown at Low Oxygen Tensions

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Methylococcus capsulatus **contained extensive intracytoplasmic membranes when grown in fed-batch cultures over a wide range of oxygen tensions (0.1 to 10.6%, vol/vol) and at a constant methane level. Although the biomass decreased as oxygen levels were lowered, consistently high amounts of phospholipid and methyl sterol were synthesized. The greatest amounts of sterol and phospholipid were found in cells grown between 0.5 and 1.1% oxygen (7.2 and 203 μmol/g [dry weight], respectively). While sterol was still synthesized in significant amounts in cells grown at 0.1% oxygen, the major sterol product was the dimethyl form. Analysis by capillary gas chromatography-mass spectrophotometry showed that the phospholipid esterified fatty acids were predominantly 16:0 and 16:1 and that the hexadecenoates consisted of *cis* Δ9, Δ10, and Δ11 isomers. At low oxygen tensions, the presence of large amounts (25%) of cyclopropane fatty acids (cy17:0) with the methylene groups at the Δ9, Δ10, and Δ11 positions was detected. Although the Δ9 monoenoic isomer was predominant, growth at low oxygen levels enhanced the synthesis of the Δ10 isomers of 16:1 and cy17:0. As the oxygen level was increased, the amount of cyclopropanes decreased, such that only a trace of cy17:0 could be detected in cells grown at 10.6% oxygen. Although *M. capsulatus* grew at very low oxygen tensions, this growth was accompanied by changes in the membrane lipids.**

Methanotrophic bacteria are characterized by extensive intracytoplasmic membrane systems. It has been suggested (31) that the intracytoplasmic membrane plays some role in the oxidation of methane, and indeed, the facultative methanotroph *Methylobacterium organophilum* exhibits intracytoplasmic membranes only when grown on methane (22).

The lipid composition of methane oxidizers is unique in several respects. Methyl sterols have been shown to be present in *Methylococcus capsulatus* and *Methylobacterium organophilum* (1, 3, 22). In *M. capsulatus*, both 4α-methyl and 4,4-dimethyl sterols are synthesized in significant quantities (0.22% of the dry weight). While the phospholipid base content of these organisms is not unusual compared with that of other bacteria which contain intracytoplasmic membrane systems (9, 11, 18, 33), the fatty acid moieties are distinct. Two types of membrane arrangement are observed in methane oxidizers, and lipid analysis has shown that the fatty acid composition correlates with the membrane type (4, 18, 28, 30–32). Type I methanotrophs (i.e., *M. capsulatus*), which possess vesicular disks of bundled membranes distributed throughout the cell, synthesize saturated and monounsaturated fatty acids with a C_{16} chain. Type II organisms (i.e., *Methylobacterium organophilum*) have a system of paired peripheral membranes and are composed predominantly of monounsaturated C_{18} fatty acids.

In the type II methanotrophs *Methylosinus trichosporium* OB3b and *Methylobacterium organophilum*, the amount of internal membrane apparent in electron micrographic thin sections increases when cells are grown under oxygen-limiting conditions (22, 29). For *Methylobacterium organophilum*, Patt and Hanson (22) correlated the increase in internal membrane with an increase in the total lipid and methyl sterol extracted.

M. capsulatus, a type I organism, synthesizes more sterol than does *Methylobacterium organophilum* (2.2 mg/g versus 0.3 μg/g [dry weight] of cells). In a preliminary study, a slight increase in sterol and in an unidentified fatty acid was observed when *M. capsulatus* was grown at low oxygen tensions (L. L. Jahnke, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, K210, p. 206). In the present study, we identified this unknown fatty acid as a mixture of cyclopropane fatty acid isomers and showed that cyclopropane synthesis increased with decreasing oxygen tension. In addition, we describe further changes that occurred in the membrane lipids of this organism when cells were grown at very low oxygen concentrations.

MATERIALS AND METHODS

Growth conditions. *M. capsulatus* (Bath) was grown in fed-batch cultures at 37°C with a nitrate-mineral salts medium (34). Jacketed-side-arm 3-liter flasks (Wheaton Industries, Millville, N.J.) were modified with additional side ports, which allowed for a constant flow of gas (120 ml/min) through four sintered-glass bubbling tubes and which permitted culture sampling and the monitoring of oxygen by a model 900 dissolved-oxygen electrode (New Brunswick Scientific Co., Inc., Edison, N.J.). Gas mixtures were controlled with a rotometer (Matheson Gas Products, Secaucus, N.J.) and monitored by gas chromatography (GC) analysis (15). The proportions of methane (50%) and carbon dioxide (1%) were kept constant, and oxygen was varied with helium as a diluent. Under these conditions, the concentration of dissolved oxygen decreased to zero (<0.1%) at the onset of exponential growth and remained so until the culture entered the stationary phase, when the dissolved oxygen readings increased to the initial values. Cells were harvested by centrifugation at 6,000 × *g* for 10 min as they entered the stationary phase. Cell pellets were washed by suspending

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them in cold distilled water and centrifuging them a second time. Pellets were frozen with liquid nitrogen and lyophilized.

Lipid extraction and fractionation. Lyophilized cells were extracted by the modified Bligh and Dyer procedure of Kates (16). In initial experiments no C_{27} sterol was detected, and in subsequent analyses, an internal standard (1 μg of β-cholestanol per mg [dry wt]) was added before extraction. Nonlipids were removed by the procedure of Folch (16). Phospholipid phosphate and fatty acid determinations were made on samples of the total lipid extract. For the phosphate analysis, the phospholipid was digested with perchloric acid at 165°C with a Tecam dry block, and phosphate was determined by the Bartlett method (5). Phosphatidylcholine was used for a digestion control. Fatty acid methyl esters of the total lipid were prepared by acid methanolysis with synthetic diarachidoyl phosphatidylcholine as an internal standard (23).

The remaining extract was reduced in volume by evaporation under N_2 and was suspended in cold acetone, which precipitated the phospholipids (16). The neutral lipids in the acetone supernatant were separated on thin-layer Silica Gel G plates (E. Merck, EM Laboratories, Elmsford, N.Y.), which were developed to a height of 15 cm twice with methylene chloride (26). Plates were sprayed with rhodamine 6G, and the areas corresponding to cholestanol (R_f, 0.15), 4α-methyl sterol (R_f, 0.21), and dimethyl sterol (R_f, 0.25) were located by UV fluorescence. The sterol zone was transferred to extraction tubes and eluted from the silica gel with ethyl acetate. Acetate derivatives of sterols were prepared by dissolving this eluted sample in acetic anhydride-pyridine (5:1, vol/vol) and heating it at 60°C for 1 h. The acetylated sterols were dried under nitrogen and dissolved in methylene chloride for GC analysis.

GC analysis. Fatty acid and sterol samples were analyzed on a Sigma 3B gas chromatograph (The Perkin-Elmer Corp., Norwalk, Conn.) equipped with a flame ionization detector and a 3390A integrator (Hewlett-Packard Co., Palo Alto, Calif.). The column used for the routine separation of the methylated fatty acids was stainless steel (0.125 in. by 6 ft [0.3175 by 182.88 cm]) packed with 20% DEGS on 80/100 Chrom WAW (Alltech Associates, Inc., Applied Science Labs, State College, Pa.) at 165°C with a carrier (helium) gas flow of 30 ml/min. The separation of acetylated sterols was performed with a nickel column (0.125 in. by 10 ft [0.3175 by 304.8 cm]) packed with 3% OV 17 on 100/120 Gas-Chrom Q (Alltech) at 250°C with a 30-ml/min He flow. Methyl and dimethyl sterols were quantified based on the internal standard (cholestanol). These procedures routinely resulted in at least a 90% recovery of the cholestanol.

GC-mass spectrophotometry of fatty acids. Methylated fatty acids were prepared from the phospholipid fraction by a mild alkaline methanolysis procedure (10). Determinations of the ring position in the cyclopropyl fatty acids and the geometry and position of the double bonds in the monounsaturates were performed with a 5995A GC-mass spectrophotometer (Hewlett-Packard) fitted with a direct capillary inlet and a nonpolar, cross-linked methyl silicone capillary column (10). The position of the cyclopropyl ring was verified by hydrogenation of the methylated fatty acids with Adam's catalyst (PtO_2) and glacial acetic acid under a 140-kPa hydrogen atmosphere with a Parr hydrogenation apparatus to form the branched-chain derivatives (10). For the analysis of the double-bond position and configuration, the dimethyl disulfide adducts of monounsaturated methyl fatty acids were formed by the method of Dunkelblum et al. (7), which was slightly modified (P. D. Nichols, J. B. Guckert, and D. C. White, J. Microbiol. Methods, in press).

TABLE 1. Sterol and phospholipid composition of *M. capsulatus* grown at low oxygen concentrations

Oxygen concn[a] (%, vol/vol)	Biomass (mg/liter)[b]	Sterol (μmol/g [dry wt] of cells)[c]		Phospholipid (μmol/g [dry wt] of cells)[d]
		Methyl	Dimethyl	
10.6	336	2.9	2.7	188
2.5	244	2.8	3.8	193
1.1	148	5.6	1.6	200
0.5	104	4.8	2.2	203
0.2	58	1.4	3.8	177
0.1	21	1.2	2.9	174

[a] Cells were grown and oxygen concentrations were determined as described in the text. The data for cells grown at 0.2, 0.5, 1.1, and 10.6% oxygen were obtained by averaging results of two experiments (range for all duplicate analyses was <±9%).
[b] Cells (dry weight) harvested from growth medium.
[c] Calculated from cholestanol response and the molecular weights of methyl and dimethyl sterols (400 and 414, respectively), determined by gas chromatography-mass spectrophotometry.
[d] Assuming 1 μmol of phosphate per μmol of phospholipid.

Fatty acid nomenclature. Fatty acids are designated by the total number of carbon atoms:number of double bonds, followed by the position of the double bond relative to the Δ (carboxylic) end of the molecule. Cyclopropane fatty acids are designated with the prefix cy, with the ring position relative to the carboxylic end of the molecule.

Materials. Organic solvents were glass distilled and high-pressure-liquid-chromatography grade and were purchased from Alltech. 9,10-Methylene hexadecenoic acid was obtained from Applied Science Labs, Inc., State College, Pa. (currently Alltech). β-Cholestanol and diarachidoyl phosphatidylcholine were purchased from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Sterol and phospholipid content. When *M. capsulatus* was grown over a wide range of oxygen concentrations in fed-batch cultures (Table 1), methyl sterols were synthesized at appreciable levels. The cell yield (biomass) decreased as the oxygen concentration was decreased. Since methane was maintained at 50% of the gas flow, the decrease in biomass was most probably a result of oxygen limitation. The maximum quantity of sterol, however, did not coincide with maximum cell yield; the greatest concentration of sterol was extracted from cells grown in the 0.5 to 1.1% oxygen range. As oxygen levels were decreased below 0.5%, both the total sterol content and the proportion of 4α-methyl sterol declined. Although the proportion of methyl sterol decreased in cells grown with 2.5 and 10.6% oxygen, this was only true of cells in the exponential or early stationary phase. When *M. capsulatus* was harvested in the late stationary phase, methyl sterol was 89.1% of the total sterol. When cells were grown at or below 0.2% oxygen, the proportion of dimethyl sterol was independent of the growth phase and was always the major end product of sterol synthesis. Large quantities of phospholipid (174 to 203 μmol/g [dry wt]) were characteristic of cells grown at all oxygen levels into the early stationary phase of growth, and approximately 30 μmol of phospholipid per μmol of sterol was typical of cells grown with 0.5% oxygen or higher.

Fatty acid composition. GC analysis of the fatty acid obtained from cells grown at high and low oxygen concen-

TABLE 2. Fatty acid composition of *M. capsulatus* grown at various oxygen concentrations

Fatty acid	Isomer	% of total phospholipid fatty acid[a] at oxygen concn (%, vol/vol):	
		7.9	0.2
14:0		5.5	7.0
16:0		40.9	39.5
16:1	Δ9c[b]	31.2	18.4
	Δ10c	10.4	5.1
	Δ11c	8.3	4.7
	Δ11t[c]	0.1	0.1
cy17:0	Δ9,10	1.7	10.1
	Δ10,11	0.6	13.4
	Δ11,12	ND[d]	0.3

[a] When the total fatty acid for 7.9 and 0.2% oxygen was 337 and 335 μmol/mg (dry weight) of cells, respectively.
[b] c, *cis*.
[c] t, *trans*.
[d] ND, None detected.

trations showed an additional fatty acid peak from cells grown at 0.2% oxygen. This peak had an identical retention time to that of authentic 9,10-methylene hexadecanoic acid, and the mass spectra of the compound showed it to be a mixture of three positional isomers of the C_{17} cyclopropyl fatty acid with the methylene group at Δ9,10; Δ10,11; and Δ11,12 (data not shown). At high oxygen tensions (10.6%), which are routinely used to cultivate methane-oxidizing organisms in batch cultures, a normal type I methanotrophic fatty acid pattern was observed, with 35.7% saturated and 61.0% monounsaturated C_{16} predominating; only 0.1% of the total fatty acid was cy17:0. As oxygen was reduced, the amount of monounsaturated C_{16} decreased and the cyclopropane fatty acids increased concomitantly (Table 2). Since cyclopropane fatty acids are normally synthesized by bacteria during the onset of the stationary phase of growth, an analysis of *M. capsulatus* grown well into the stationary phase with 10.6% oxygen (a biomass of 779 mg [dry wt]/liter) was made. Only a slight increase (1.8% of the total fatty acid) in cy17:0 was observed in such cells.

Biosynthesis of the cyclopropane ring occurs by the introduction of a methylene bridge across the double bond of a monoenoic fatty acid (24). Although the synthesis of *trans* unsaturated fatty acids has been reported (18) for *M. capsulatus*, under our growth conditions, only Δ9, Δ10, and Δ11 unsaturates of the *cis* configuration and a small amount of *trans* Δ11 were synthesized (Table 2). Most interestingly, the position of the methylene group of the cyclopropanes reflected the double-bond positions of the 16:1 monounsaturates. Although very little of the Δ11,12 isomer could be detected under either a high or low oxygen concentration, at the former, the amount of Δ9,10 and Δ10,11 cy17:0 isomers reflected the relative ratio of the precursor monoenoic isomers (3:1). At low oxygen levels, however, because of the greater synthesis of cy17:0 Δ10,11 fatty acid, the ratio of Δ9 to Δ10 isomeric products decreased.

DISCUSSION

M. capsulatus grew by the oxidation of methane over a wide range of oxygen concentrations and synthesized high levels of phospholipid and sterol. Higgins et al. (12) suggested that dissolved oxygen tension may control the synthesis of the internal membrane of methanotrophic bacteria. In *Methylobacterium organophilum*, oxygen concentration affected the amount of membrane and total lipid (22). In our experiments, although cell yields and growth rates diminished as oxygen concentrations were lowered, electron micrographs revealed that growth was accompanied by the synthesis of an extensive intracytoplasmic membrane (unpublished data). No quantitative differences were observed in the amount of membrane in *M. capsulatus* grown at high or low oxygen concentrations, which correlated with the large quantities of lipid extracted from such cells. Although the biomass yields varied from 21 to 336 mg (dry wt) per liter of medium, phospholipid ranged from only 174 to 203 μmol/g (dry wt) and attained an apparent maximum when cells were grown between 0.5 and 1.1% oxygen. Similar large amounts of phospholipid are obtained from *Methylobacterium organophilum* grown at low oxygen, a condition known to enhance membrane synthesis in this organism (22). Such phospholipid levels are high compared to those of other methanotrophs and of *M. capsulatus* (116 μmol/g) grown under conditions known to limit intracytoplasmic membrane synthesis (9, 13, 18).

Although the quantitative differences in total phospholipid and sterol observed in this study would not in themselves indicate a major alteration of the cellular membrane of *M. capsulatus*, the qualitative changes in the lipid components that were observed, particularly below 0.5% oxygen, could affect membrane function. 4α-Methyl sterol has been identified as the predominant sterol of *M. capsulatus*, and 4,4-dimethyl sterol (15 to 30%) has been found to be the lesser one (3). At 0.2% oxygen, the total amount of sterol decreased and dimethyl sterol became the major end product of sterol synthesis. The initial steps in the synthesis of sterol in eucaryotic organisms require oxygen for the epoxidation of squalene and the demethylation of lanosterol (2). These oxidative processes result in the methyl sterols characteristic of *M. capsulatus*. Since the total level of sterol decreased as the proportion of dimethyl sterol increased, this effect may reflect a higher oxygen requirement for the second demethylation step. Methyl sterols are normally found only as intermediates in sterol synthesis, and methanotrophic bacteria are the only procaryotic organisms known to accumulate these sterols as end products (1, 3, 22). Methane-oxidizing bacteria also synthesize hopanoids by an anaerobic cyclization of squalene, and these compounds have been proposed as evolutionary precursors to oxidatively synthesized sterol (25, 27). In this respect, the synthesis of methyl sterol by *M. capsulatus* at 0.1% oxygen was of interest, since the optimum oxygen concentration for sterol synthesis in yeast occurs in this range (14, 15). *Methylobacterium organophilum* synthesizes the greatest amount of methyl sterol at low oxygen levels, and although the small amounts of sterol present in this species probably play no structural role in membranes, this sterol may have another function. Indeed, small amounts of specific sterols have been shown to act synergistically with other lipids to effect major metabolic changes in mycoplasma and yeast sterol auxotrophs (2).

This first report of the synthesis of large quantities of cyclopropane fatty acids in a methanotrophic bacterium appears to entail the greatest alteration of bulk membrane lipid in these cells. Cyclopropane fatty acids occur in a wide variety of bacteria and have been reported in trace quantities in other methanotrophic bacteria (21, 28, 32). Makula (18) did not observe cyclopropanes in *M. capsulatus*; however,

this was probably due to the relatively high oxygen level (30:70, methane-air) used to grow those cells. Reduced oxygen has been implicated in the synthesis of cyclopropane fatty acids in other organisms (20), and it is apparent from our experiments that low oxygen tension resulted in substantial synthesis of these fatty acids in our methanotroph. Ohlrogge et al. (20) suggested that the increased synthesis of cyclopropane fatty acid during the stationary phase was due to diminished oxygen tension. In our experiments with *M. capsulatus*, dissolved oxygen increased during the stationary phase and may account for the low amounts of cyclopropanes observed during this growth phase. The function of cyclopropane fatty acids has not been resolved (24). The physical properties of cyclopropane and unsaturated fatty acids have been shown to be very similar, although it has been proposed that cyclopropane fatty acids increase the organizational stability of membranes (6, 17). Presumably, such a membrane characteristic is essential for the growth of *M. capsulatus* at very low oxygen concentrations.

Positional isomers of unsaturated fatty acids are not common in bacteria, but they have been reported in a number of organisms, including *Bacillus* sp. (Δ8, Δ9, and Δ10) and mycobacteria (Δ9 and Δ10) (8). Makula (18) reported that the monounsaturates of *M. capsulatus* were composed of the multiple positional isomers Δ8, Δ9, Δ10, and Δ11 in approximately equal proportions of the *cis* and *trans* configurations. However, under our growth conditions, no Δ8 isomers and only a small amount of one *trans* isomer, Δ11, were observed in *M. capsulatus*. In this regard, it should be noted that Makula reported growing this methanotroph at a temperature (30°C) considerably lower than its optimum growth temperature (37°C). Macdonald et al. (17) have shown that *trans*-substituted unsaturates may affect membrane fluidity by altering the membrane lipid gel-to-liquid-crystalline phase transition temperature. Additionally, Fulco (8) has reviewed a number of factors, including temperature, which have been shown to affect unsaturated fatty acid synthesis. Indeed, *M. capsulatus* grown at different oxygen concentrations in our growth experiments showed variation in the positional isomers. At high oxygen levels, Δ9 was the predominant isomer. However, at a low oxygen concentration, when the cyclopropanes are considered as products of the unsaturated isomers, the increased synthesis of the Δ10 isomer was apparent. Whether this was due to the increased synthesis of Δ10 hexadecenoic acid or to a preference of the cyclopropane synthase for the Δ10 isomer as a substrate is not certain. In a study of the specificity for ring formation in *Escherichia coli*, Marinari et al. (19) found that while the Δ9, Δ10, and Δ11 hexadecenoic acids were all methylenated, the best substrate for cyclopropane synthesis was the naturally occurring Δ9 isomer.

Our findings demonstrate that *M. capsulatus*, while capable of growth and membrane synthesis at low oxygen concentrations, regulates the synthesis of its membrane lipids in response to such limiting growth conditions.

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