Presence of methyl sterol and bacteriohopanepolyol in an outer-membrane preparation from *Methylococcus capsulatus* (Bath)

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Cytoplasmic/intracytoplasmic and outer membrane preparations of *Methylococcus capsulatus* (Bath) were isolated by sucrose density gradient centrifugation of a total membrane fraction prepared by disruption using a French pressure cell. The cytoplasmic and/or intracytoplasmic membrane fraction consisted of two distinct bands, Ia and Ib (buoyant densities 1.16 and 1.18 g ml⁻¹, respectively) that together contained 57% of the protein, 68% of the phospholipid, 73% of the ubiquinone and 89% of the CN-sensitive NADH oxidase activity. The only apparent difference between these two cytoplasmic bands was a much higher phospholipid content for Ia. The outer membrane fraction (buoyant density 1.23–1.24 g ml⁻¹) contained 60% of the lipopolysaccharide-associated, β -hydroxypalmitic acid, 74% of the methylsterol, and 66% of the bacteriohopanepolyol (BHP); phospholipid to methyl sterol or BHP ratios were 6:1. Methanol dehydrogenase activity and a c-type cytochrome were also present in this outer membrane fraction. Phospholipase A activity was present in both the cytoplasmic membrane and outer membrane fractions. The unique distribution of cyclic triterpenes may reflect a specific role in conferring outer membrane stability in this methanotrophic bacterium.

Introduction

Methanotrophs are Gram-negative bacteria capable of growth on methane as their sole source of carbon and energy. Electron micrographs reveal not only the presence of an outer and an inner (cytoplasmic) membrane, but additionally a complex internal (intracytoplasmic) membrane system (Davies & Whittenbury, 1970). Analysis of the lipid extracts of intact cells demonstrated the presence of a group of membrane lipids, the hopanoids, in all methanotrophic bacteria (Rohmer *et al.*, 1984). The principal hopanoid in methanotrophs is an amphiphilic molecule consisting of a pentacyclic triterpene nucleus linked to a polyhydroxylated side chain referred to as bacteriohopanepolyol (BHP). Some evidence suggests that these molecules function as membrane stabilizers in much the same manner as sterols do in eukaryotes (Ourisson *et al.*, 1987). The synthesis of hopanoids is considered to have evolved prior to sterols because of the widespread distribution of

hopanoids among prokaryotes, and because sterol synthesis requires oxygen and hopanoid synthesis does not. Indeed, hopanoids have been proposed as the phylogenetic precursors of the eukaryotic steroids (Rohmer *et al.*, 1979).

The synthesis of cyclic triterpenes in *M. capsulatus* is of particular evolutionary interest, because it is the only known bacterium to synthesize large amounts of both hopanoids and sterols (Ourisson *et al.*, 1987). This bacterium can cyclize squalene to diploptene and 2,3-epoxysqualene to lanosterol (Rohmer *et al.*, 1980). In *M. capsulatus*, the primary hopanoid product is a BHP molecule with an aminopentol side chain (Neunlist & Rohmer, 1985), and the primary sterol products are 4,4-dimethyl and 4 α -methyl sterols (Bird *et al.*, 1971). These methyl sterols are the products of the incomplete demethylation of lanosterol and represent only the initial enzymic steps of the C₂₇ sterol pathway that results in the synthesis of cholesterol in many eukaryotes (Bloch, 1983).

Few data are available on the lipid composition of isolated membranes in methanotrophs, particularly with regard to the cyclic triterpenes. Separation of the outer and cytoplasmic/intracytoplasmic membranes has been attempted for *Methylomonas methanica*, but no lipid

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Abbreviations: BHP, bacteriohopanepolyol; KDO, 2-keto-3-deoxyoctonate.

analysis was reported (Chemina & Trotsenko, 1981). Some preliminary, qualitative evidence suggests that the BHP of a facultative methanotroph, *Methylobacterium organophilum*, is present in the isolated membranes (Hancock & Williams, 1986). The presence of such unusual lipids in methanotrophic membranes poses many interesting questions about their relationship to the physiology of these bacteria and the evolution of membrane function. As a first effort to understand their role, we have attempted to separate the inner (cytoplasmic and/or intracytoplasmic) and outer membranes of *M. capsulatus*, and have isolated an outer membrane fraction highly enriched in both BHP and methyl sterols.

Methods

Organism and growth conditions. *Methylococcus capsulatus* (Bath) was obtained from the American Type Culture Collection (ATCC 33009). Bacteria were grown at 37 °C in 5.5 l defined nitrate mineral salts medium containing 10 µg CuCl₂ l⁻¹ with a continuous stream (100 ml min⁻¹) of methane/air/CO₂ (49:49:2) as previously described (Jahnke & Nichols, 1986).

Membrane preparation. All procedures were performed at 4 °C. Bacteria were harvested by centrifugation at 6000 g for 10 min. Cells (4–5 g wet wt) were washed once with distilled water, suspended in water, and treated with a Sorvall Omnimixer blender to remove capsular material (Schnaitman, 1970). Cells were recovered by centrifugation and suspended to 20 ml with 20 mM-HEPES buffer (Research Organics) (pH 7.4). When methanol dehydrogenase was to be analysed, the buffer was made 10 mM with respect to methanol prior to cell breakage (Ghosh & Quayle, 1981). Approximately 1 mg each of pancreatic ribonuclease and deoxyribonuclease were added to the suspension, and the cells were broken by one passage through an Aminco French pressure cell operated at 103 MPa. The broken cell suspension was centrifuged twice at 6000 g for 10 min to remove whole cells and debris. The supernatant (SII) was centrifuged for 60 min at 197000 g. The resultant pellet (TM) was suspended with HEPES buffer containing 34% (w/w) sucrose and was applied to the top of a 28 ml, 35% to 55% (w/w) continuous sucrose gradient. Samples were centrifuged in a Beckman SW27 rotor at 25000 r.p.m. for 16 h. The four observed bands were collected by immersing a 15-gauge cannula through the gradient and withdrawing fractions from the bottom by means of a peristaltic pump. Sucrose density was determined with a refractometer. The fractions containing individual bands were pooled, and the sucrose was diluted at least two-fold with HEPES buffer containing NaCl (final concentration adjusted to 50 mM), then centrifuged for 60 min at 200000 g. The resulting pellets were rinsed with, then suspended in, a small amount of HEPES buffer. Phospholipase and NADH oxidase assays, and lipid extraction were done with freshly prepared material; samples were frozen at -20 °C for methanol dehydrogenase, protein and cytochrome analyses. In some experiments designed to minimize the potential for membrane hybridization, the total membrane fraction for application to the continuous sucrose gradients was prepared by breaking cells using a HEPES buffer containing 20% sucrose, and then preparing the crude membranes by the discontinuous gradient method of Ishidate *et al.* (1986).

Lipid extraction, separation, and analysis. Lipids were extracted from the freshly prepared membrane fractions with a one-phase Bligh &

Dyer (1959) extraction as modified by Kates (1986). The residue was recovered by centrifugation before initiating phase separation and was used for β -hydroxy fatty acid analysis (see below). The total lipid extract was analysed for phospholipid phosphate (Dittmer & Wells, 1969), and phospholipid esterified fatty acid by mild-alkaline methanolysis (White *et al.*, 1979). Polar (phospholipid and BHP) and neutral (sterol and ubiquinone) lipids were separated by precipitation of the polar lipids in cold acetone (Summons & Jahnke, 1992). The BHP was recovered by Bligh & Dyer (1959) extraction of the acetone precipitate and was converted to its hopanol derivative according to procedure II of Rohmer *et al.* (1984). Neutral lipids were further separated on silica gel G thin-layer plates (Merck) developed to a height of 15 cm twice with methylene chloride in a paper-lined tank. The 4 α -methyl and 4,4-dimethyl sterols (R_f 0.21 and 0.25, respectively) were recovered and analysed as previously described (Jahnke & Nichols, 1986). The ubiquinone (Q-8), which was an intense yellow band (R_f 0.73), was recovered from the silica gel by elution with chloroform, and identified by its ultraviolet absorption spectrum in absolute ethanol (Crane & Barr, 1971), and its behaviour on Whatman KC18 reverse-phase thin-layer plates (Collins, 1985). Quantification of the ubiquinone was based on an oxidized minus borohydride reduced extinction coefficient at 275 nm of $\Delta\epsilon_{\text{nm}} = 12.7$ (Crane & Barr, 1971).

Lipopolysaccharide content. The lipopolysaccharide (LPS) content of TM was determined by analysis of the 2-keto-3-deoxyoctonate (KDO) and β -hydroxy fatty acids. KDO was measured by the method of Karkhanis *et al.* (1978) using a lyophilized TM preparation. The β -hydroxy fatty acids were analysed using the solvent-extracted membrane residue by the method of Nichols *et al.* (1987). Methyl esters of the hydroxy fatty acids were prepared using boron trifluoride-methanol (O'Brien & Rouser, 1964). The methylated β -hydroxy fatty acids were isolated by TLC (R_f 0.22) in the above CH₂Cl₂-TLC system, and analysed by gas chromatography. Hydroxy fatty acid identification was based on comparison of relative TLC mobility and GC retention times with data for standards obtained from Supelco and Ultra Scientific.

Gas chromatographic analysis. Normal and hydroxy fatty acids, methyl sterols and BHP hopanol derivatives were analysed on a Perkin-Elmer Sigma 3B gas chromatograph equipped with a flame ionization detector and fused silica megabore columns (J & W Scientific). Methylated fatty acids were separated on a 30 m DB-23 operated at 155 °C. Acetates of the methyl sterols and the hopanols were prepared (Jahnke & Nichols, 1986) and were separated on a 30 m DB-5 operated at 295 °C. Quantification of methyl sterol, β -OH palmitate and phospholipid fatty acids was based on recovery of internal standards [β -cholestanol, β -OH myristate and (diarachidoyl)-phosphatidylcholine, respectively].

Enzyme assays. NADH oxidase activity was measured at 22 °C in the presence and absence of 10 mM-KCN by recording the decrease in absorbance at 340 nm in incubation mixtures containing 120 µM-NADH, 20 mM-HEPES (pH 7.4) and membrane fraction. Methanol dehydrogenase was measured as described by Ghosh & Quayle (1981). Phospholipase activity was measured as the decrease in esterified fatty acid over 20 min at 37 °C. The substrate, endogenous phospholipid, was prepared by heating a crude membrane fraction at 90 °C for 5 min. Incubation mixtures contained this crude phospholipid (approximately 6 µmol esterified fatty acid), 100 µg Triton X-100, 75 mM-CaCl₂, 20 mM-HEPES (pH 7.4) and membrane fraction in 1 ml. Incubations were stopped at zero, 10 min and 20 min intervals by addition of methanol/chloroform. An internal standard (diarachidoyl)phosphatidylcholine, was added, the phospholipid was extracted, and the amount of esterified fatty acid was determined by the mild-alkaline methanolysis procedure of White *et al.* (1979).

Gel electrophoresis. The polypeptide composition of the isolated membrane fractions was analysed by SDS-PAGE on 12% (w/v) gels, 0.75 mm thick, in a discontinuous buffer system (Laemmli, 1970) at a constant 45 mA, with water cooling. The gels were stained for protein with Coomassie Brilliant Blue R. Low-molecular-mass markers (Dalton VII-L, Sigma) were used as standards. Membrane proteins were solubilized by heating at 100 °C for 3 min in 1% (w/v) SDS sample buffer (Laemmli, 1970).

Cytochrome spectra. Cytochrome spectra of the isolated membrane fractions were measured at room temperature in an Aminco DW2A dual wavelength/split-beam spectrophotometer operated in the split beam mode. Reduced *minus* oxidized spectra were obtained by reducing one cuvette with a few grains of Na₂S₂O₄. Reduced *plus* CO *minus* reduced spectra were obtained by reducing both cuvettes with Na₂S₂O₄, and bubbling a steady stream of CO through one cuvette for 30 s. The CO-treated cuvette was left in the dark for 15 min before recording the spectra.

Electron microscopy. Pellets of the isolated membrane fractions were fixed at 4 °C with 0.5% OsO₄ for 30 min, or with 2% (w/v) glutaraldehyde for 30 min, followed by 0.5% OsO₄ plus 0.8% K₃Fe(CN)₆ for 30 min (McDonald, 1984). Both fixation procedures were followed by *en bloc* staining with 2% (w/v) aqueous uranyl acetate for 2 h at 4 °C. Reagents were prepared in 0.02 M-HEPES (pH 7.4). Samples were dehydrated in increasing concentrations of ethanol, followed by butyl glycidyl ether, then embedded in Quetol 651. Sections were stained with uranyl acetate-lead citrate and examined in a Philips 300 electron microscope.

Protein was determined by the bicinchoninic acid procedure using bovine albumin as the standard (Smith *et al.*, 1985).

Results

Separation and characterization of membrane fractions

Centrifugation of TM on continuous sucrose density gradients resulted in four visible bands. The upper two

bands, designated Ia and Ib, had buoyant densities of 1.16 and 1.18 g ml⁻¹, respectively. Centrifugation of bands Ia or Ib yielded a pellet that was straw-coloured upon suspension. The bottom band, III, was considerably broader, ranging from a buoyant density of 1.23 to 1.24 g ml⁻¹, and yielded a membranous material with a distinct pinkish coloration. Band II had an intermediate density (1.21 g ml⁻¹) and had a similar pinkish coloration.

Analysis of the TM fraction (197 000 g pellet) yielded almost equimolar amounts of KDO and β -hydroxypalmitic acid. Hydroxy fatty acids have been reported to be associated with LPS isolated from *M. organophilum* (Hancock & Williams, 1986), and in methanotrophs, such as *M. capsulatus*, β -OH palmitic acid is the predominant acid found (Nichols *et al.*, 1985; Bowman *et al.*, 1991). Because of our requirement in these experiments for large amounts of material for lipid analyses, it was convenient to use the lipid-extracted residue for β -OH fatty acid analysis as a marker for the outer membrane. In our experiments, virtually all of the β -hydroxy acid was palmitic, only a small amount of β -hydroxymyristate (0.5% of the total) was detected in the absence of added internal standard.

The results of the chemical and enzyme analyses on the isolated membrane fractions are shown in Table 1. β -Hydroxy fatty acid and ubiquinone were used routinely as indicators for the outer and cytoplasmic membranes, respectively. In four experiments, Ia and Ib together accounted for $77 \pm 3\%$ of the ubiquinone. This observation, coupled with the presence of cyanide-sensitive NADH oxidase activity and with the relatively small amounts of β -hydroxy fatty acid, suggest that the

Table 1. Chemical composition and enzyme activities of isolated membrane fractions from *M. capsulatus*

Data reported are for the total membrane material recovered by centrifugation of gradient fractions and its relative distribution. Recovery of applied TM material was 76% or greater with the exception of protein (56%) and methanol dehydrogenase (67%). Results are means of three experiments.

Component	Total Recovery	Distribution (%)			
		Ia	Ib	II	III
Protein	40.3*	25	32	16	27
Phospholipid	17.7†	39	29	16	17
Phospholipase	5.2‡	16	27	27	30
Ubiquinone	0.4†	37	36	18	9
NADH oxidase (CN sensitive)	0.8‡	47	42	8	3
β -Hydroxy fatty acid	1.3†	3	8	29	60
Methanol dehydrogenase	11.3‡	7	9	41	42

* Total recovered protein expressed in mg.

† Total recovered material expressed in μ mol.

‡ Total recovered activity expressed in μ mol product min⁻¹.

Fig. 1. SDS-PAGE of the membrane fractions. Total membranes from a 200000 *g* pellet (TM), and isolated membranes from four visible bands (Ia, Ib, II & III) on sucrose density gradients. Each sample contained 20 μ g protein. Molecular mass markers (kDa) are shown on left.

material recovered from bands Ia and Ib represents the cytoplasmic and/or intracytoplasmic membrane(s). In contrast, band III contained most of the β -hydroxy fatty acid, suggesting the presence of the outer membrane. Both the methyl sterol and BHP showed a distribution similar to that of β -hydroxy fatty acid (Table 2).

The distribution of membrane components between the low density (Ia + Ib) and high density (III) membranes was highly reproducible. Some variation was observed in the distribution of the low density material between Ia and Ib; however, Ia consistently contained higher levels of phospholipid than Ib. Based on a molecular mass of 692 Da for the major phospholipid in *M. capsulatus* (dipalmitoyl)phosphatidylethanolamine (Makula, 1978), the phospholipid content of Ia would be 0.49 ± 0.05 mg (mg protein) $^{-1}$ as compared to a value of 0.24 ± 0.06 for Ib and 0.24 ± 0.03 for III ($n = 3$).

The material recovered in band II appeared to be a mixture of the membrane material present in bands I and III based on the biochemical characteristics in Table 1

Table 2. Lipid composition of isolated membrane fractions

The results are from an experiment in which band II was not observed, but the lipid data were similar in four experiments. Data are presented as nanomoles of membrane lipid recovered by centrifugation of the designated gradient fraction. Recovery of the phospholipid, methyl sterol and BHP applied to gradients was 74%, 100% and 94%, respectively.

Component	Composition		
	Ia	Ib	III
Protein*	8.4	11.9	14.5
Phospholipid	5300	3030	4530
Ubiquinone	165	177	57
β -Hydroxy fatty acid	110	75	970
Methyl sterol	141	136	809
Bacteriohopanepolyol	225	135	712

* Total recovered protein expressed in mg.

and the SDS-PAGE analysis of membrane proteins (Fig. 1). The amount of material recovered in II varied widely between experiments; in some, band II was not observed, and in others it accounted for as much as 40% of the total recovered protein and phospholipid. A substantial increase in band II resulted when cells were ruptured by a second passage through the French pressure cell. Preparation of the total membrane material using discontinuous gradients, which are reported to decrease non-specific membrane adhesion (Ishidate *et al.*, 1986), had no effect on the presence of this band in continuous gradients.

Phospholipase activity, which is normally associated with the outer membrane in Gram-negative bacteria, was found in both the upper (Ia and Ib) and lower (III) bands in this organism (Table 1). This phospholipase activity was lost upon freezing; storage at -20°C for 4 d resulted in a decrease of 60% in the activity associated with Ia and Ib, while essentially no activity remained in III. Endogenous phospholipase activity was not normally a problem in the preparation of these membranes, however, when TM was prepared according to the methods of Ishidate *et al.* (1986), large amounts of free fatty acids were observed in the neutral lipid fraction.

As in previous reports (Wadzinski & Ribbons, 1975; Patel & Felix, 1976), a significant portion of the methanol dehydrogenase in these cells was membrane-bound. The TM fraction contained 58% of the methanol dehydrogenase activity present in SII; the remaining activity was recovered in the 197000 *g* supernatant. Of the methanol dehydrogenase activity present in TM, 67% was recovered in the membrane pellets after

centrifugation of the gradient fractions, a major portion in fraction III (Table 1). In one experiment, an attempt was made to collect the upper and lower zones of band III. The upper (1.23 g ml^{-1}) portion had a slightly higher specific activity than the lower (1.24 g ml^{-1}) portion; $441 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$ versus $285 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$, respectively.

Membrane protein patterns

The protein composition of the membrane fractions was analysed by SDS-PAGE (Fig. 1). Ia and Ib were indistinguishable from each other and contained a number of distinct proteins (Fig. 1, proteins e and f). Under the conditions employed, a number of similar bands appear in both I and III, however, at least seven bands (Fig. 1, a, b, d, h, i, j and k) appear significantly enriched in fraction III.

Membrane cytochromes

The predominant species of cytochrome in methanotrophic bacteria are *c*-types having an α -peak at 552–553 nm (Davey & Mitton, 1973; Tonge *et al.*, 1974). In our experiments, a cytochrome with an α -peak at 553 nm was the major component in the membranes of Ia, Ib and III (Fig. 2a). Based on an extinction coefficient for the α -peak of $\Delta\epsilon = 27.6$ (Wood, 1984), 54% of the membrane-associated cytochrome *c* was recovered in Ia and Ib, $2.1 \text{ nmol (mg protein)}^{-1}$, and 34% in III, $2.6 \text{ nmol (mg protein)}^{-1}$. A distinct peak at 604 nm and a shoulder in the Soret region at 445 nm in the spectra of Ia and Ib indicated the presence of an *aa*₃-type cytochrome (Chance & Williams, 1955; Tonge *et al.*, 1974) in this membrane fraction. A slight asymmetry in the 560 nm region of the α -band may also indicate the presence of a small amount of *b*-type cytochrome in Ib. Reduced *plus* CO *minus* reduced difference spectra of both Ib and III (Fig. 2b) showed a Soret peak at 415 nm and a trough in the α -band region, indicating the presence of a *c*-type, low-spin CO-complex (Wood, 1984). Based on an ϵ (α -peak– α -trough) = $25 (\text{mM cm})^{-1}$ for a *c*_{oo} (Wood, 1984), 37% and 12% of the *c*-type cytochrome in Ib and III, respectively, binds CO.

Electron micrographs

Thin sections of membrane fractions Ia (Fig. 3a) and Ib (Fig. 3b) showed many faintly-stained unit membrane vesicles filled with a darkly stained material. While Ib was dominated by these osmophilic structures, Ia contained many empty, irregular-shaped vesicles and a variety of convoluted, stacked membrane structures

Fig. 2. Absorption spectra of the membrane fractions. (a) $\text{Na}_2\text{S}_2\text{O}_4$ -reduced *minus* oxidized difference spectra of Ib (0.9 mg ml^{-1}) and III (0.6 mg ml^{-1}). (b) $\text{Na}_2\text{S}_2\text{O}_4$ -reduced *plus* CO *minus* $\text{Na}_2\text{S}_2\text{O}_4$ -reduced difference spectra of Ib and III.

(Fig. 3a, enlargement) which were not apparent in Ib. Thin sections of fraction III (Fig. 3c) showed coiled, C-shape and vesicular structures characteristic of Gram-negative cell wall (Osborn *et al.*, 1972; Ishidate *et al.*, 1986). The darkly-stained double track of the outer membrane and the underlying dense murein layer were clearly present in all fields.

Visualization of the membrane vesicles in Ia or Ib was highly dependent on the fixation method. No membrane structure was observed when glutaraldehyde was used prior to OsO_4 . We found it was necessary to fix Ia and Ib directly with OsO_4 , or when glutaraldehyde was used, to post-fix the membranes with $\text{OsO}_4/\text{K}_3\text{Fe}(\text{CN})_6$ according to the method of McDonald (1984). Fixation method was less critical for preparation of fraction III.

Fig. 3. Electron micrographs of thin sections of membrane fractions. Fraction Ia (a) showing two fields characteristic of this material, one of the irregular-shaped and vesicular structures, and the other (higher-magnification insert) of a stacked unit membrane structure (arrow); fraction Ib (b) showing membrane-bounded, darkly stained material; and fraction III (c) showing outer membrane (om) and underlying murein layer (arrowhead). Pellets were fixed with glutaraldehyde, followed by OsO_4 plus $\text{KFe}(\text{CN})_6$ as described in text. Bar, 100 nm.

Discussion

Separation of the cell envelope of *M. capsulatus* by sucrose density gradient centrifugation resulted in isolation of two membrane types that in many respects are similar to the outer and cytoplasmic membranes isolated by Osborn *et al.* (1972). The two bands of buoyant densities 1.16 and 1.18 (Ia and Ib, respectively) were enriched in a number of electron transport components (cytochrome aa_3 , NADH oxidase and ubiquinone), while band III, buoyant density 1.23–1.24, contained most of the LPS-associated β -hydroxy fatty acids. The SDS-PAGE protein patterns and electron micrographs clearly demonstrate that I and III are unique membrane fractions.

It is well established that phospholipase A is present in the outer membrane of a variety of bacteria (Osborn *et al.*, 1972; Scott *et al.*, 1976; Booth & Curtis, 1977); however, in this methanotroph, phospholipase activity was distributed throughout the membrane fractions. The only other report of a similar distribution is in *Myxococcus xanthus* (Orndorff & Dworkin, 1980). In *M. organophilum*, a facultative methanotroph, phospholipase A is located in the outer membrane; however, the cells for that study were grown under conditions where intracytoplasmic membrane is not produced (Hancock & Williams, 1986). Localization of the enzymes responsible for membrane degradation in the intracytoplasmic membrane would be consistent with the high degree of variation observed in the internal membrane morphology of this organism (Hyder *et al.*, 1979; Prior & Dalton, 1985). Presumably, the rapid turnover involved in the synthesis and degradation of these internal membranes would require an active phospholipase.

Several ultrastructural studies of methanotrophs have shown invaginations of the cytoplasmic membrane seemingly connected to the internal bundles (Davies & Whittenbury, 1970; DeBoer & Hazeu, 1972; Saralov *et al.*, 1985). Indeed, Davies & Whittenbury (1970) have observed in thin sections of disrupted cells that the intracytoplasmic membrane bundles tend to remain in groups accompanied by pieces of cytoplasmic membrane. Localization of the cytochrome oxidase in the intracytoplasmic membrane has been reported based on cytochemical tests (Monosov & Netrusov, 1976), thus the presence of cyanide-sensitive NADH oxidase and cytochrome aa_3 in Ia and Ib suggests that some portion of this material represents the intracytoplasmic membrane observed in the electron micrographs of cells grown under these conditions (Jahnke & Nichols, 1986). The most significant difference between these two upper bands was the higher phospholipid content of Ia, which could reflect the presence of specific membrane domains

associated with the areas of tight curvature in the internal membrane bundles. This is true of artificial membrane vesicles where the ratio of phospholipid in the outer and inner leaflets is known to be related to the degree of curvature (Cullis & Hope, 1985), and is also consistent with the highly convoluted membranes apparent in electron micrographs of Ia.

Cytochromes are not normally found in the outer membrane of other Gram-negative bacteria. The presence of cytochrome *c* in fraction III suggests the presence of some cytoplasmic or pericytoplasmic-associated membrane. The presence of cytochrome in a similar outer membrane preparation from *Methylomonas albus* has also been observed (personal communication, M. L. P. Collins, University of Wisconsin, Milwaukee, USA). In methylo-trophs, methanol dehydrogenase activity is localized in the periplasmic space, where it is thought to function by interaction with cytochrome *c* on the outer face of the cytoplasmic membrane (Anthony, 1986). The presence of membrane-bound methanol dehydrogenase and cytochrome *c* in fraction III may merely represent two classes of membrane with similar buoyant densities. Alternatively, it could also be explained by adhesion zones between the outer and cytoplasmic membranes. Such adhesion zones have been documented in Gram-negative bacteria (Bayer, 1979; MacAlister *et al.*, 1983) and have been observed in electron micrographs of methanotrophs (Saralov *et al.*, 1985). A slightly higher specific activity for methanol dehydrogenase in the lower density portion of band III does indeed suggest the presence of a subfraction more highly enriched in some such pericytoplasmic membrane fragment.

The enrichment of BHP and methyl sterol in the outer membrane fraction of *M. capsulatus* suggests a specific function for these molecules. Hancock & Williams (1986) suggested that the exceptional stability of the outer membrane of *M. organophilum* to detergent might be due to the presence of hopanoids; indeed, hopanoids and methyl sterols appear to play a reinforcement role in membranes (Dahl *et al.*, 1980; Benz *et al.*, 1983). Both the hopanoids in *Zymomonas mobilis* (Bringer *et al.*, 1985) and sterols in yeast (Thomas *et al.*, 1978) have been implicated in the ethanol resistance of these micro-organisms. The ratio of phospholipid to either BHP or sterol in fraction III is approximately 6:1, and given the nature of the mixture of membrane types in this band, may well be lower in some specific membrane domain. This suggests that both of these cyclic triterpenes are important for the integrity of some outer and/or pericytoplasmic membrane function. Given the hydrophobic nature of a substrate such as methane, the presence of BHP and methyl sterol in the membranes of the cell envelope may relate to the problems commensurate with growth on such a compound.

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References

- ANTHONY, C. (1986). Bacterial oxidation of methane and methanol. In *Advances in Microbial Physiology*, pp.113–210. Edited by A. H. Rose & D. W. Tempest. London: Academic Press.
- BAYER, M. E. (1979). The fusion sites between outer membrane and cytoplasmic membrane of bacteria: their role in membrane assembly and virus infection. In *Bacterial Outer Membranes*, pp. 167–202. Edited by M. Inouye. New York: John Wiley & Sons.
- BENZ, R., HALLMANN, D., PORALLA, K. & EIBL, H. (1983). Interaction of hopanoids with phosphatidylcholines containing oleic and ω -cyclohexyldodecanoic acid in lipid bilayer membranes. *Chemistry and Physics of Lipids* **34**, 7–24.
- BIRD, C. W., LYNCH, J. M., PIRT, S. J., REID, W. W., BROOKS, C. J. W. & MIDDLEDITCH, B. S. (1971). Steroids and squalene in *Methylococcus capsulatus* grown on methane. *Nature, London* **230**, 473–474.
- BLIGH, E. G. & DYER, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemical Physiology* **37**, 911–917.
- BLOCH, K. (1983). Sterol structure and membrane function. *Critical Reviews in Biochemistry* **14**, 47–92.
- BOOTH, B. R. & CURTIS, N. A. C. (1977). Separation of the cytoplasmic and outer membrane of *Pseudomonas aeruginosa* PAO-1. *Biochemical and Biophysical Research Communications* **74**, 1168–1176.
- BOWMAN, J. P., SKERRATT, J. H., NICHOLS, P. D. & SLY, L. I. (1991). Phospholipid fatty acid and lipopolysaccharide acid signature lipids in methane-utilizing bacteria. *FEMS Microbiology Ecology* **85**, 15–22.
- BRINGER, S., HÄRTNER, T., PORALLA, K. & SAHM, H. (1985). Influence of ethanol on the hopanoid content and the fatty acid pattern in batch and continuous cultures of *Zymomonas mobilis*. *Archives of Microbiology* **140**, 312–316.
- CHANCE, B. & WILLIAMS, G. R. (1955). Respiratory enzymes in oxidative phosphorylation. II. Difference spectra. *Journal of Biological Chemistry* **217**, 395–407.
- CHEMINA, E. V. & TROTSSENKO, Y. A. (1981). Isolation and identification of *Methylomonas methanica* membranes. *Microbiology* **49**, 735–739.
- COLLINS, M. D. (1985). Isoprenoid quinone analyses in bacterial classification and identification. In *Chemical Methods in Bacterial Systematics*, pp. 267–287. Edited by M. Goodfellow & D. E. Minnikin. London: Academic Press.
- CRANE, F. L. & BARR, R. (1971). Determination of ubiquinones. *Methods in Enzymology* **18**, 137–165.
- CULLIS, P. R. & HOPE, M. J. (1985). Physical properties and functional roles of lipids in membranes. In *Biochemistry of Lipids and Membranes*, pp. 25–72. Edited by D. E. Vance & J. E. Vance. Menlo Park, California: Benjamin/Cummings Publishing Co.
- DAHL, C. E., DAHL, J. S. & BLOCH, K. (1980). Effect of alkyl-substituted precursors of cholesterol on artificial and natural membranes and on the viability of *Mycoplasma capricolum*. *Biochemistry* **19**, 1462–1467.
- DAVEY, J. F. & MITTON, J. R. (1973). Cytochromes of two methane-utilizing bacteria. *FEBS Letters* **37**, 335–337.
- DAVIES, S. L. & WHITTENBURY, R. (1970). Fine structure of methane and other hydrocarbon-utilizing bacteria. *Journal of General Microbiology* **61**, 227–232.
- DEBOER, W. E. & HAZEU, W. (1972). Observations on the fine structure of a methane-oxidizing bacterium. *Antonie van Leeuwenhoek* **38**, 33–47.
- DITTMER, J. C. & WELLS, M. A. (1969). Quantitative and qualitative analysis of lipids and lipid components. *Methods in Enzymology* **14**, 482–530.

- GHOSH, R. & QUAYLE, J. R. (1981). Purification and properties of methanol dehydrogenase from *Methylophilus methylotrophus*. *Biochemical Journal* **199**, 245–250.
- HANCOCK, I. C. & WILLIAMS, K. M. (1986). The outer membrane of *Methylobacterium organophilum*. *Journal of General Microbiology* **132**, 599–610.
- HYDER, S. L., MEYERS, A. & CAYER, M. L. (1979). Membrane modulation in a methylotrophic bacterium *Methylococcus capsulatus* (Texas) as a function of growth substrate. *Tissue and Cell* **11**, 597–610.
- ISHIDATE, K., CREEGER, E. S., ZRIKE, J., DEB, S., GLAUNER, B., MACALISTER, T. J. & ROTHFIELD, L. I. (1986). Isolation of differentiated membrane domains from *Escherichia coli* and *Salmonella typhimurium*, including a fraction containing attachment sites between the inner and outer membranes and the murein skeleton of the cell envelope. *Journal of Biological Chemistry* **261**, 428–443.
- JAHNKE, L. L. & NICHOLS, P. D. (1986). Methyl sterol and cyclopropane fatty acid composition of *Methylococcus capsulatus* grown at low oxygen tensions. *Journal of Bacteriology* **167**, 238–242.
- KARKHANIS, Y. D., ZELTNER, J. Y., JACKSON, J. J. & CARLO, D. J. (1978). A new and improved microassay to determine 2-keto-3-deoxyoctonate in lipopolysaccharide of Gram-negative bacteria. *Analytical Biochemistry* **85**, 595–601.
- KATES, M. (1986). Techniques of lipidology: isolation, analysis and identification of lipids. In *Laboratory Techniques in Biochemistry and Molecular Biology*, vol. 3. Edited by R. H. Burdon & P. H. van Knippenberg. Amsterdam: Elsevier.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.
- MACALISTER, T. J., MACDONALD, B. & ROTHFIELD, L. I. (1983). The periseptal annulus: an organelle associated with cell division in gram-negative bacteria. *Proceedings of the National Academy of Sciences of the United States of America* **80**, 1372–1376.
- McDONALD, K. (1984). Osmium ferricyanide fixation improves microfilament preservation and membrane visualization in a variety of animal cell types. *Journal of Ultrastructure Research* **86**, 107–118.
- MAKULA, R. A. (1978). Phospholipid composition of methane-utilizing bacteria. *Journal of Bacteriology* **134**, 771–777.
- MONOSOV, E. Z. & NETRUSOV, A. I. (1976). Localization of energy generators in methane-oxidizing bacteria. *Microbiology* **45**, 518–523.
- NEUNLIST, S. & ROHMER, M. (1985). Novel hopanoids from the methylotrophic bacteria *Methylococcus capsulatus* and *Methylomonas methanica*. *Biochemical Journal* **231**, 635–639.
- NICHOLS, P. D., SMITH, G. A., ANTORTH, C. P., HANSON, R. S. & WHITE, D. W. (1985). Phospholipid and lipopolysaccharide normal and hydroxy fatty acids as potential signatures for methane-oxidizing bacteria. *FEMS Microbiology Ecology* **31**, 327–335.
- NICHOLS, P. D., MANCUSO, C. A. & WHITE, D. C. (1987). Measurement of methanotroph and methanogen signature phospholipids for use in assessment of biomass and community structure in model systems. *Organic Geochemistry* **11**, 451–461.
- O'BRIEN, J. S. & ROUSER, G. (1964). Analysis of hydroxy fatty acids by gas-liquid chromatography. *Analytical Biochemistry* **7**, 288–296.
- ORNDORFF, P. E. & DWORKIN, M. (1980). Separation and properties of the cytoplasmic and outer membranes of vegetative cells of *Myxococcus xanthus*. *Journal of Bacteriology* **141**, 914–927.
- OSBORN, M. J., GANDER, J. E., PARIS, E. & CARSON, J. (1972). Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. *Journal of Biological Chemistry* **247**, 3962–3972.
- OURISSON, G., ROHMER, M. & PORALLA, K. (1987). Prokaryotic hopanoids and other polyterpenoid sterol surrogates. *Annual Review of Microbiology* **41**, 301–333.
- PATEL, R. N. & FELIX, A. (1976). Microbial oxidation of methane and methanol: crystallization and properties of methanol dehydrogenase from *Methylosinus sporium*. *Journal of Bacteriology* **128**, 413–424.
- PRIOR, S. D. & DALTON, H. (1985). The effect of copper ions on membrane content and methane monooxygenase activity in methanol-grown cells of *Methylococcus capsulatus* (Bath). *Journal of General Microbiology* **131**, 155–163.
- ROHMER, M., BOUVIER, P. & OURISSON, G. (1979). Molecular evolution of biomembranes: structural equivalents and phylogenetic precursors of sterols. *Proceedings of the National Academy of Sciences of the United States of America* **76**, 847–851.
- ROHMER, M., BOUVIER, P. & OURISSON, G. (1980). Non-specific lanosterol and hopanoid biosynthesis by a cell-free system from the bacterium *Methylococcus capsulatus*. *European Journal of Biochemistry* **112**, 557–560.
- ROHMER, M., BOUVIER-NAVE, P. & OURISSON, G. (1984). Distribution of hopanoid triterpenes in prokaryotes. *Journal of General Microbiology* **130**, 1137–1150.
- SARALOV, A. I., KRYLOVA, I. N., SARALOVA, E. E. & KUZNETSOV, S. I. (1985). Distribution and species composition of methane-oxidizing bacteria in lake waters. *Microbiology* **53**, 695–700.
- SCHNAITMAN, C. A. (1970). Examination of the protein composition of the cell envelope of *Escherichia coli* by polyacrylamide gel electrophoresis. *Journal of Bacteriology* **104**, 882–889.
- SCOTT, C. C. L., MAKULA, R. A. & FINNERTY, W. R. (1976). Isolation and purification of membranes from a hydrocarbon-oxidizing *Acinetobacter* sp. *Journal of Bacteriology* **127**, 469–480.
- SMITH, P. K., KROHN, R. I., HERMANSON, G. T., MALLIA, A. K., GARTNER, F. H., PROVENZANO, M. D., FUJIMOTO, E. K., GOEKER, N. M., OLSON, B. J. & KLENK, D. C. (1985). Measurement of protein using bicinchoninic acid. *Analytical Biochemistry* **150**, 76–85.
- SUMMONS, R. E. & JAHNKE, L. L. (1992). Hopenes and hopanes methylated in ring-A: Correlation of the hopanoids from extant methylotrophic bacteria with their fossil analogues. In *Biological Markers in Sediments and Petroleum*, pp. 182–200. Edited by M. Moldowan. Englewood Cliffs, NJ: Prentice Hall.
- THOMAS, D. S., HOSSACK, J. A. & ROSE, A. H. (1978). Plasma-membrane lipid composition and ethanol tolerance in *Saccharomyces cerevisiae*. *Archives for Microbiology* **117**, 239–245.
- TONGE, G. M., KNOWLES, C. J., HARRISON, D. E. F. & HIGGINS, I. J. (1974). Metabolism of one carbon compounds: cytochromes of methane- and methanol-utilizing bacteria. *FEBS Letters* **44**, 106–110.
- WADZINSKI, A. M. & RIBBONS, D. W. (1975). Oxidation of C₁ compounds by particulate fractions from *Methylococcus capsulatus*: properties of methanol oxidase and methanol dehydrogenase. *Journal of Bacteriology* **122**, 1364–1374.
- WHITE, D. C., DAVIS, W. M., NICKELS, J. S., KING, J. D. & BOBBIE, R. J. (1979). Determination of the sedimentary microbial biomass by extractable lipid phosphate. *Oecologia* **40**, 51–62.
- WOOD, P. M. (1984). Bacterial proteins with CO-binding b- or c-type haem functions and absorption spectroscopy. *Biochimica et Biophysica Acta* **768**, 293–317.