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Freeze-Fracture Planes of Methanogen Membranes Correlate with the Content of Tetraether Lipids

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Methanospirillum hungatei **GP1 contained 50% of its ether core lipids (polar lipids less head groups) as tetraether lipids, and its plasma membrane failed to fracture along its hydrophobic domain during freeze-etching. The membrane of *Methanosaeta* ("*Methanothrix*") *concilii* did not contain tetraether lipids and easily fractured to reveal typical intramembranous particles. *Methanococcus jannaschii* grown at 50°C contained 20% tetraether core lipids, which increased to 45% when cells were grown at 70°C. The frequency of membrane fracture was reduced as the membrane-spanning tetraether lipids approached 45%. As the tetraether lipid content increased, and while fracture was still possible, the particle density in the membrane increased; these added particles could be tetraether lipid complexes torn from the opposing membrane face. The diether membrane (no tetraether lipid) of *Methanococcus voltae* easily fractured, and the intramembranous particle density was low. Protein-free liposomes containing tetraether core lipids (ca. 45%) also did not fracture, whereas those made up exclusively of diether lipids did split, indicating that tetraether lipids add considerable vertical stability to the membrane. At tetraether lipid concentrations below 45%, liposome bilayers fractured to reveal small intramembranous particles which we interpret to be tetraether lipid complexes.**

Methanogens demand strict anaerobiosis to grow and to generate large amounts of methane. It has been speculated that a membrane-disrupting effect of methane may be countered by their membrane composition, which includes some of the most unique lipids found in procaryotic cells (8, 9, 14, 16, 20, 23, 24). In a general sense, most of these lipids are similar to the phospholipids found in eubacteria in that they possess single or bipolar head groups and underlying hydrophobic alkyl chains. However, the chemistry of these moieties is markedly different from that of the familiar eubacterial or eucaryotic lipids (e.g., the alkyl chains are phytanyl substituents and are linked to the head group portion by ether linkages). Nonetheless, in aqueous environments these lipids still arrange themselves into bilayered membrane structures, with the polar head groups towards the aqueous phase and the alkyl chains intermingling to produce a central hydrophobic domain along the membrane's length (7). In thin sections these membranes cannot be distinguished from their eubacterial counterparts (1).

Freeze-etching has traditionally been the electron microscopical technique of choice to visualize bacterial membranes (19). This is because when cells are fractured while frozen, they often cleave along the hydrophobic domain of their constituent membranes. In this case, integral proteins and their boundary lipids can be seen as intramembranous particles (1). In fact, a limited amount of physical information is also gathered, because cells fracture along the lines of least bond energy (1, 19) and the hydrophobic interactions between opposing alkyl chains within membranes are easily broken. If membranes are difficult to fracture, the bonding forces between these chains must be greater than normal. For this reason, freeze-etching is a method whereby unusual bonding between polar head groups can be visualized. Since certain methanogens possess tetraether lipids which probably span the membrane (9, 16), their plasma membrane should be difficult to fracture. In fact, regions of the membrane with high concentrations of these lipids should be virtually cemented together by covalent linkage and should not fracture. This expectation is strengthened by the finding that tetraether lipid-rich *Thermoplasma* membranes do not exhibit a fracture plane (16). However, it is difficult to interpret much of the past freeze-fracture data on methanogen cells in this context, since the frequency of intramembrane fractures compared with cross fractures is rarely reported (21).

Bacteria, growth conditions, lipid analyses, liposome formation, and electron microscopy. All methanogens were grown in liquid media in pressure bottles containing 80% H_2 and 20% CO_2 (vol/vol) in the head space, as detailed by Breuil and Patel (6), until a mid- or late exponential growth phase was attained. *Methanospirillum hungatei* GP1 (DSM 1101) was grown at 37°C in mineral salts SA medium (6). *Methanosaeta* ("*Methanothrix*") *concilii* GP6 (18) (DSM 3671) was grown at 37°C under N_2 in acetate medium (10), and *Methanococcus jannaschii* JAL-2 (DSM 2661) was grown at 50, 65, and 70°C in defined medium as described by Ferrante et al. (12). *Methanococcus voltae* PS (DSM 1587) was grown in Balch medium-3 at 35°C.

Lipid analyses were performed as described by Sprott et al. and Ferrante et al. (10, 12, 23, 24), and the tetraether lipid content was estimated as a dry-weight percentage of the total core lipids (total polar lipids less head groups) by a radiotracer method (24).

For liposome preparation, polar lipids were obtained from the total lipid extracts by precipitating thrice from cold acetone. The polar lipid (ca. 2 g) dissolved in chloroform-methanol (2:1, vol/vol) was applied to a column of Silica Gel G (2 by 5 cm), and lipids were eluted with 125 ml of

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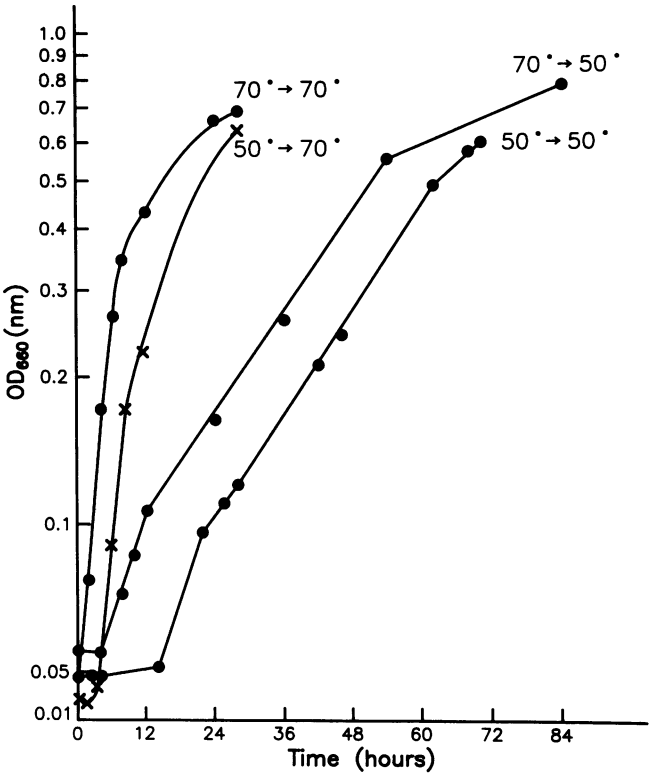

FIG. 1. Growth curves of *M. jannaschii* cultivated at 50 and 70°C. The first number beside each curve is the temperature at which the inoculum was grown, and the second number is the growth temperature for the illustrated growth curve. Optical densities at 660 nm (OD_{660}) are shown.

chloroform-methanol (2:1, vol/vol) followed by 125 ml of chloroform-methanol (1:2, vol/vol). Pooled eluates were concentrated by flash evaporation, and silica particles were removed by a two-phase Bligh-Dyer procedure (5).

For freeze-etching, each culture was harvested at an optical density at 660 nm of 0.3 to 0.4 (Fig. 1; the growth of *M. jannaschii* at 50 and 70°C is shown to reveal temperature effects), pelleted by centrifugation (16,000 × *g*), and divided in half; each half was suspended for 10 min in either deionized water or 10% (vol/vol) glycerol as a cryoprotectant. The cryoprotectant did not affect the membrane fracture frequency or the membrane particle density in any of the cultures used. Cell suspensions were centrifuged, and the pellet was scraped onto gold planchets, plunge-frozen in propane at −196°C, and fractured in a Balzers BA 360M freeze-etcher at −100°C. Etching times were kept to a minimum of 15 s at 10^{-6} torr (ca. 10^{-3} Pa). The fracture faces were shadowed with platinum (Pt) and coated with carbon (C) so that Pt-C replicas could be obtained after H_2SO_4 and hypochlorite treatment. The replicas were picked up on 400-mesh uncoated copper grids and imaged in a Philips EM300 transmission electron microscope operating at 60 kV under standard conditions with the cold trap in place. Each replica was visually scanned, and ca. 100 separate fractured cells in each sample were imaged for statistical analysis.

***M. concilii* and *M. hungatei*.** The structures of the sheathed methanogens *M. concilii* and *M. hungatei* are very complicated (2–4, 17, 26), and this article concentrates only on the plasma membrane. In thin section, this membrane in both methanogens was a typical tripartite bilayer with two densely stained regions (the lines of polar head groups) enclosing a translucent inner (hydrophobic) domain (Fig. 2 and 4). Each membrane was overlaid with multiple additional envelope layers which have previously been identified (2–4).

TABLE 1. Tetraether lipid content and freeze-fracture data

Species	Tetraether lipid content (%)[a]	% of membrane fractures per 100 cells[b]	Intramembrane particle density (no. per 250 nm^2)[c]
M. concillii	0	70	370
M. hungatei	50	0	ND[d]
M. jannaschii			
Grown at 50°C	20	46	320
Grown at 70°C	45	11	600
M. voltae	0	67	260

[a] From references 7, 11, 22, and 23.
[b] Approximately 100 cells were analyzed in each freeze-etching.
[c] Particles were counted in a total area of 250 nm^2 on the membranes of each of 10 cells and then averaged. The standard deviation was less than 5% in each freeze-etching.
[d] ND, not determinable.

Freeze-fracture of *M. concilii* frequently cleaved the plasma membrane, exposing dispersed intramembrane particles in both cryoprotected and noncryoprotected cells (Fig. 5). These membranes contained 70% diether lipids and 30% *sn*-3-hydroxydiether lipids (11). The membrane lipids of *M. hungatei* contained 50% diether lipids and 50% tetraether lipids, and no membrane fractures were seen in either the glycerol-treated or the non-glycerol-treated samples. The most frequently encountered fracture was along the surface of the sheath, which is the outermost envelope layer of this bacterium (Fig. 3). There seemed to be a broad correlation between the presence of tetraether lipids and the inability of a membrane to fracture.

***M. jannaschii* and *M. voltae*.** *M. jannaschii* was chosen to investigate this finding further, since the proportion of tetraether lipids of this methanogen can be manipulated by changing the growth temperature (24). *M. voltae* was used as a control, since it contains exclusively diether lipids (7, 22) and, like other mesophilic methanococci, fractures through the membrane (13, 15). *M. jannaschii* grown at 50°C contained only a small amount of tetraether lipids (ca. 20% [by weight] of total ether core lipids), and at 70°C this increased to 45%. Shifting the growth temperature from 50 to 70°C decreased both the lag period and the doubling times of the cells; similar culture cell densities were achieved at both temperatures (Fig. 1). In thin section, the plasma membrane of *M. jannaschii* (Fig. 6) resembled those of *M. hungatei* and *M. concilii* (Fig. 2 and 4). Cells grown at 50°C were found to fracture frequently along the plasma membrane, exposing randomly distributed, intramembranous particles similar to those seen in Fig. 7 with *M. voltae* (Table 1). The frequencies of intramembranous particles within methanogen membranes were as follows: *M. concilii* > *M. jannaschii* (50°C) > *M. voltae*. When comparing 50 and 70°C mid-exponential-phase cells, at 70°C the number of intramembranous particles had nearly doubled (from 320 to 600 particles per 250 nm^2) and the tetraether lipid content approached 45%; concomitantly, the frequency of membrane fractures declined dramatically (Table 1). Those fractures of membranes which were seen at this time were only small regions of the entire membrane and had intramembrane particle densities

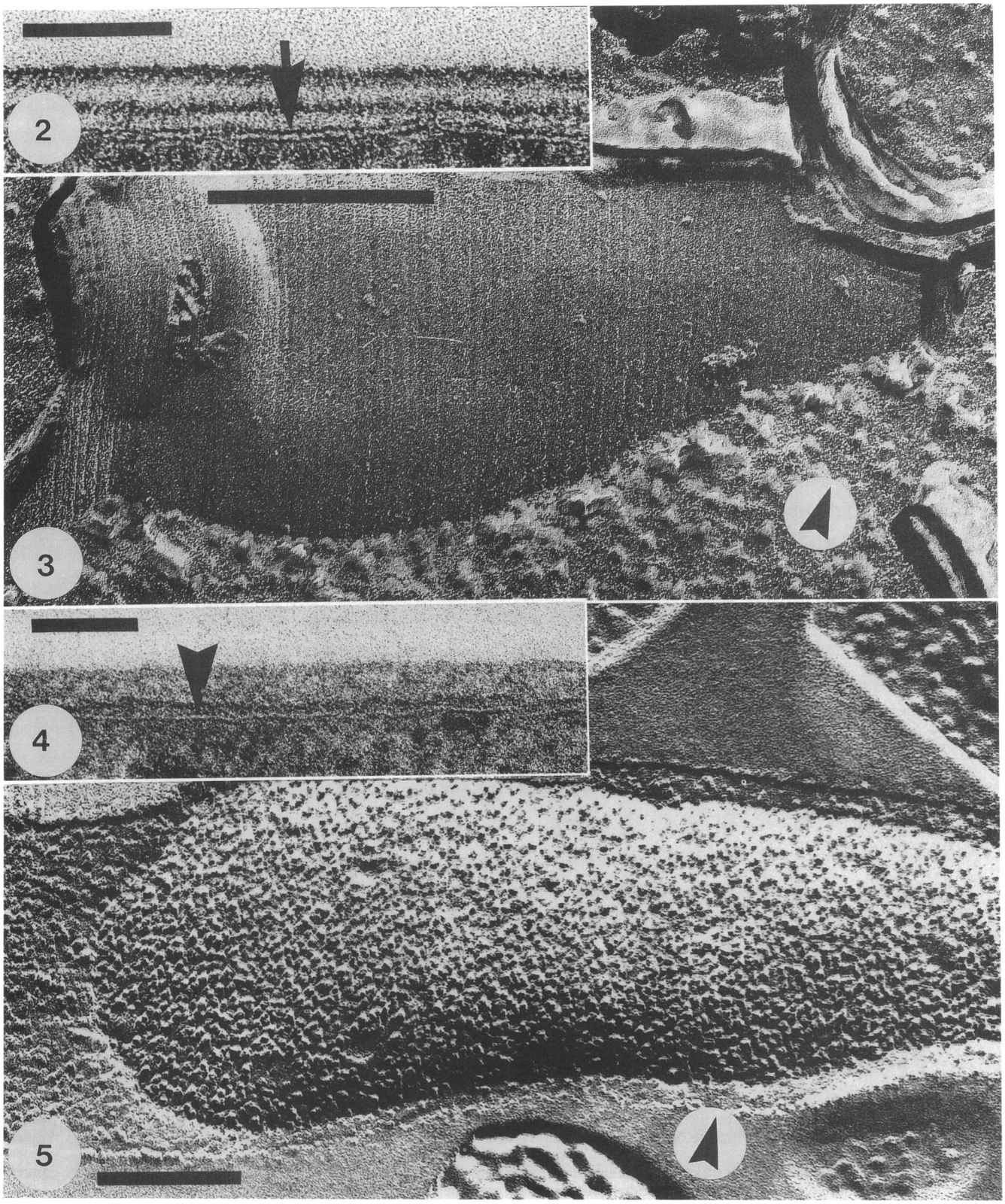

FIG. 2. Thin section through a portion of the cell envelope of *M. hungatei* chemically fixed and processed as outlined in reference 3. The plasma membrane is a typical tripartite bilayer (arrow), above which the cell wall and sheath are seen. Bar = 100 nm.

FIG. 3. The typical convex freeze-fracture of *M. hungatei* occurs along the outer surface of the sheath, which is seen here. The arrowhead denotes shadow direction. Bar = 350 nm.

FIG. 4. Thin section through a portion of the cell envelope of *M. concilii* fixed and processed as outlined in reference 4. The plasma membrane (arrowhead) is tripartite with the sheath plus amorphous material above it. Bar = 100 nm.

FIG. 5. Some of the fractures in *M. concilii* typically cleave the plasma membrane and show numerous intramembranous particles on the convex surface. The arrowhead denotes shadow direction. Bar = 350 nm.

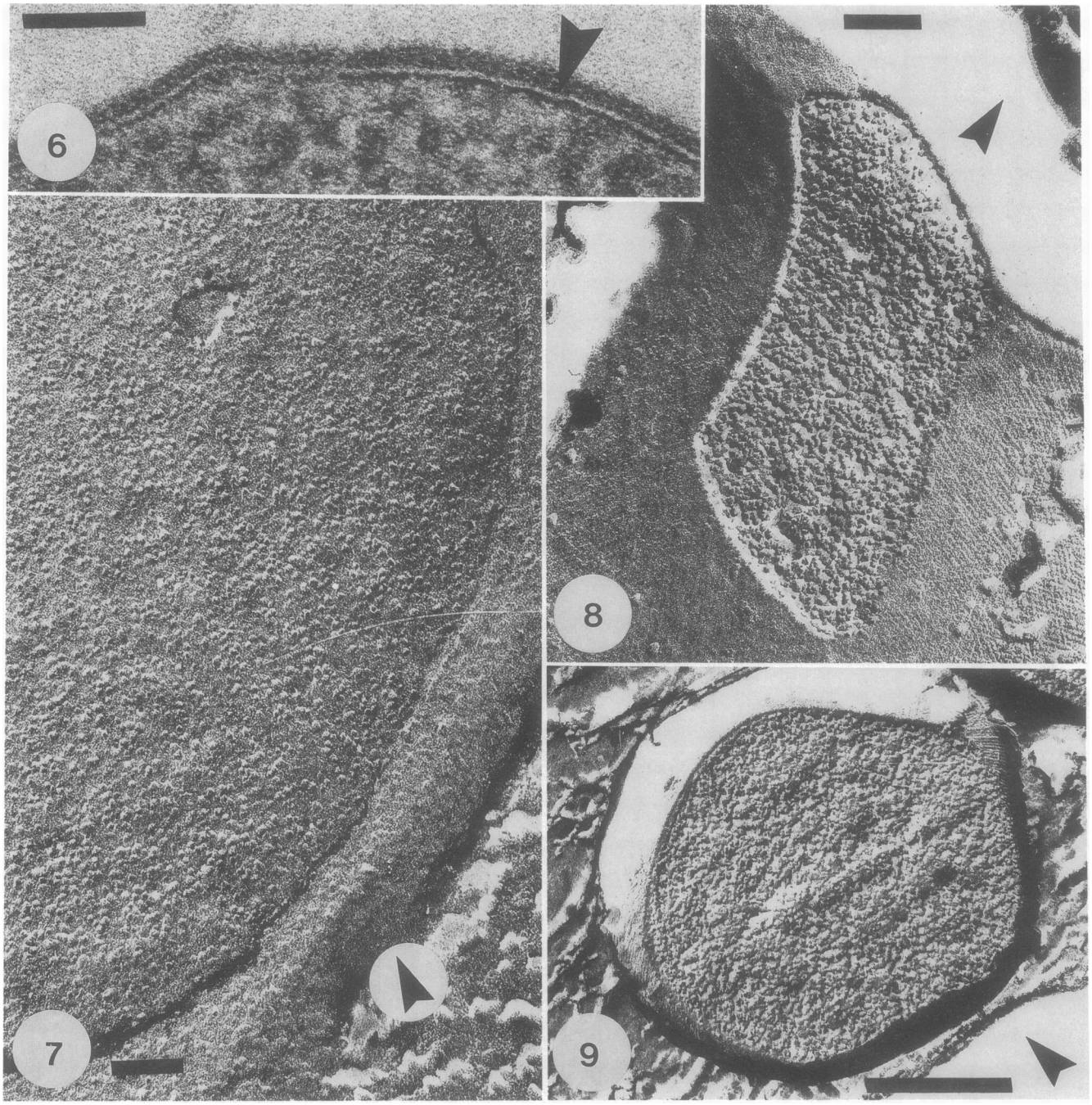

FIG. 6. Thin section of a portion of the cell envelope of *M. jannaschii* at 50°C, processed as described in reference 3. The plasma membrane (arrowhead) is a typical tripartite bilayer with a regularly structured S layer above it. *M. jannaschii* and *M. voltae* at 70°C looked very similar to this. Bar = 100 nm.

FIG. 7. Freeze-fracture through the plasma membrane of *M. voltae*, showing a low density of intramembranous particles on the convex surface. This was a common fracture surface for this bacterium. The arrowhead denotes shadow direction. Bar = 200 nm.

FIG. 8. As *M. jannaschii* was shifted from 50 to 70°C, the tetraether lipid concentration increased and only some fractures occurred through the plasma membrane. These fractures showed an extremely high concentration of intramembranous particles on the convex face. The surface S layer with its periodicity can be seen as the outermost layer on the cell. The arrowhead denotes shadow direction. Bar = 200 nm.

FIG. 9. For 70°C *M. jannaschii* virtually no internal membrane fractures were seen, only fractures along the S layer or cross fractures as seen here. The arrowhead denotes shadow direction. Bar = 1 μm.

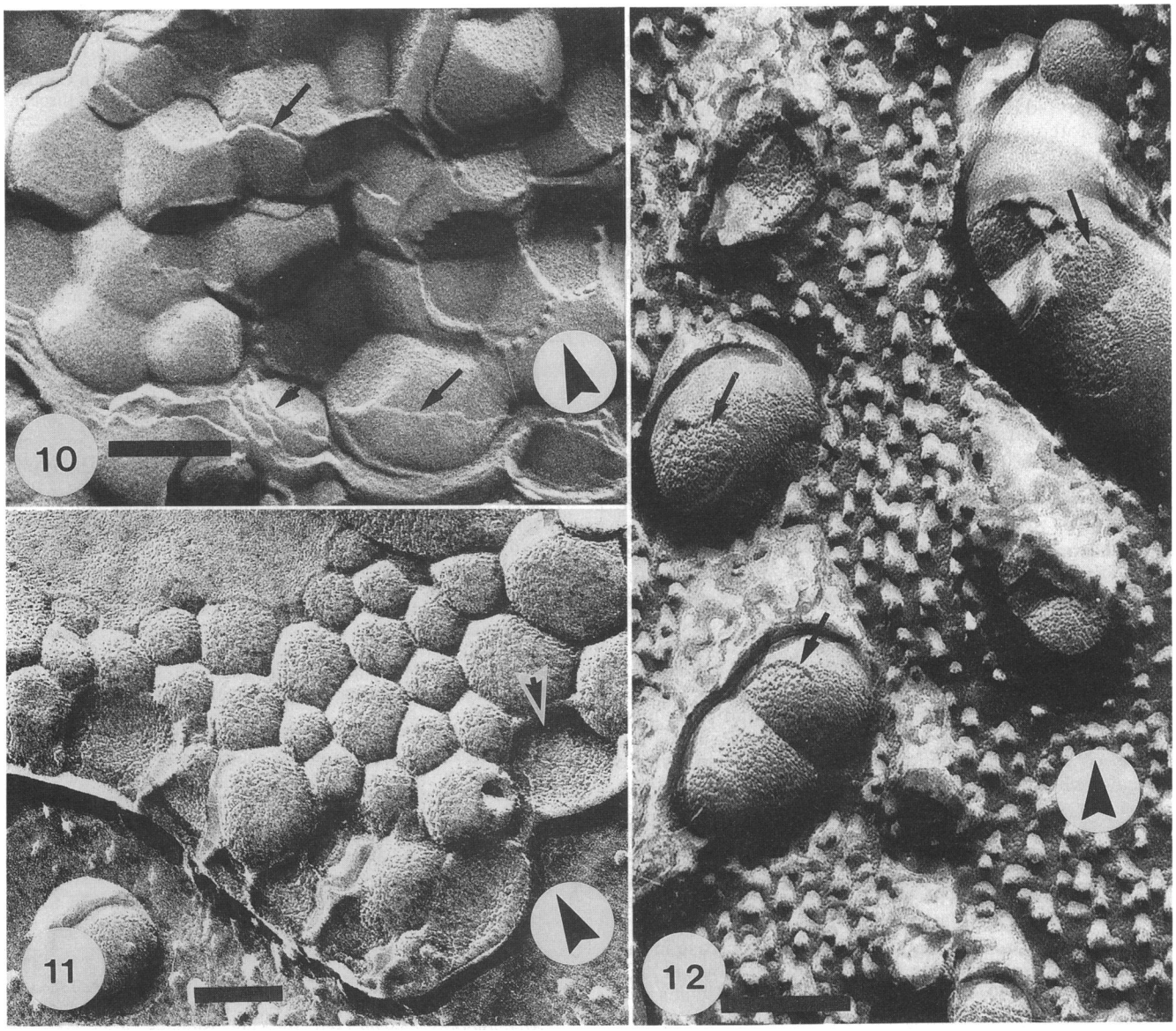

FIG. 10. Freeze-fractured liposomes from *M. voltae*, consisting of >90% diether and <10% hydroxydiether lipids (no tetraether lipids). Notice the internal bilayer fractures (arrows) and their smooth surfaces. The arrowhead denotes shadow direction. Bar = 50 nm.

FIG. 11. Freeze-fractured liposomes from 65°C *M. jannaschii*, consisting of ca. 45% tetraether lipids. The remaining lipids were macrocyclic diether and standard diether lipids. Notice that most liposomes cleaved along their outer surfaces, although a few cross-fractured (arrow) so that their inner hydrophilic surfaces could be seen after etching. The arrowhead denotes shadow direction. Bar = 100 nm.

FIG. 12. Freeze-fractured 50°C *M. jannaschii* liposomes containing ca. 20% tetraether lipids. Notice that small internal bilayer fractures occur and that intramembrane particles (arrows) can be seen in these proteinless structures. The arrowhead denotes shadow direction. Bar = 100 nm.

of ca. 600 particles per 250 nm^2 (Fig. 8). Most fractures of cells were cross fractures which exposed the cytoplasm (Fig. 9) or fractures along the surface of the S layers (similar to those in Fig. 8, but without membrane fractures). As seen with *M. concilii* and *M. hungatei*, there was a strong correlation between the abundance of tetraether lipids and the way that the plasma membrane fractured.

Virtually all of the membrane ether lipids of *M. voltae* are diether lipids composed of 2,3-di-*O*-phytanyl-*sn*-glycerol, and a lesser amount (<10%) are 3- and 3′-hydroxydiether lipids (22, 23). *M. voltae*'s membranes easily fractured in both glycerol- and non-glycerol-treated cells and had particle densities (Table 1) below those seen in *M. concilii*. Therefore, both of these tetraether lipid-negative methanogens possessed fracturable membranes and intermediate intramembrane particle densities (Table 1).

Liposomes. For cells of *M. jannaschii* there was also the possibility that growth shifts from 50 to 70°C induced the formation of new membrane-spanning proteins which might contribute to the lack of membrane fracture; this could also contribute to increased particle densities in the hydrophobic membrane face (Fig. 8). To check for this possibility, freeze-fractures were compared for liposomes prepared from total polar lipid extracts of *M. hungatei*, *M. jannaschii* grown at

65°C, and *M. voltae*. Freeze-fractures of the *M. voltae* preparation frequently cleaved through the hydrophobic domain of the reconstituted bilayer (Fig. 10). Since there was no protein in these preparations, the fractures were smooth and were devoid of particles usually attributed to intramembranous protein. *M. concilii* liposomes were similar. Liposomes made from 65°C *M. jannaschii* lipids, containing a high proportion of tetraether lipids, showed virtually no internal membrane cleavages; these liposomes usually fractured along their outer surface or cross-fractured (Fig. 11). Those of *M. hungatei*, which also contained a high proportion of tetraether lipids, fractured in a similar manner. Interestingly, when liposomes prepared from 50°C *M. jannaschii* lipids were cleaved, small fracture zones along the hydrophobic domain were seen and these contained small particles (Fig. 12). Since these 50°C liposomes possessed no protein and since the internal fracture faces were particulate as compared with those of *M. voltae* (cf. Fig. 10 and 12), it was apparent that these 50°C particles must be tetraether lipid complexes which had been ripped from the upper face. These could be seen only if the tetraether lipid concentration within the liposome bilayer was low enough to allow fracture (i.e., 50°C = 20% tetraether lipids); higher concentrations made cleavage impossible (i.e., 65 to 70°C = 45% tetraether lipids). Such tetraether lipid particles may resemble the lipidic particles seen in freeze-fractures under certain conditions with ester liposomes (25).

Interpretations. Since their first identification, the unique chemical differences of tetraether membrane lipids have intrigued researchers. Not only do they possess the archaeobacterial features of phytanyl alkyl chains and ether linkages, but also opposing head groups of tetraether lipids are actually covalently attached to one another to form a single molecule which spans the membrane. There have been relatively few physical studies on the dynamic nature of these unusual lipids (9, 14); the bulkiness of the molecule should inhibit lateral and rotational motion, thereby making the membrane more rigid. Tetraether lipids may also affect the motional attributes of smaller neighboring lipids. Presumably, these effects also have some input to gel-liquid phase transitions, explaining why there is an increase in the tetraether lipid concentration as *M. jannaschii* grows at high temperature. Clearly, an abundance of these molecules will also increase vertical stability across the bilayer and inhibit the membrane's capacity to freeze-fracture. Membranes containing large quantities of tetraether lipids were very difficult to fracture (Table 1), and those of *M. jannaschii* had increased particle densities (Table 1). This increase in particle density correlates with an increase in the tetraether lipid content from 20 to 45%. It is possible that a proportion of the particles are actually intact tetraether lipid complexes which have been pulled out of the bilayer as the membrane fractured. Therefore, at low tetraether lipid concentrations (i.e., *M. jannaschii* at 50°C), when the membrane would still have a tendency to split, there would be an increased particle density (above that produced by membrane proteins alone) as the lipid was torn from the overlying membrane face. At high tetraether lipid concentrations, fractures would be only rare and would have high particle densities for the same reason. Strong evidence for these interpretations of whole-cell fractures was obtained by fracturing unilamellar liposomes prepared from total polar lipid extracts of these methanogens. If the reasoning that some of these particles are actually tetraether lipids and that they cannot be distinguished from protein is correct, then, like protein, the lipid must be randomly distributed throughout the membrane.

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