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Methanogens and the Diversity of Archaebacteria

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INTRODUCTION

The concept proposed by Woese and associates in the late 1970s reclassified all living organisms into three primary kingdoms: eucaryotes, eubacteria, and archaebacteria (127, 519, 522). The procaryotic organisms were considered to comprise two phylogenetically distinct groups: the eubacteria, which consist of the traditional bacterial groups including the photosynthetic bacteria, blue-green bacteria, endospore-forming bacteria, actinomycetes, and spirochetes; and archaebacteria, which consist of the methanogenic bacteria, the extremely halophilic bacteria, *Thermoplasma* spp., and the "thermoacidophiles," now recognized as the extremely thermophilic archaebacteria. Indeed, Woese's discovery and concept were both revolutionary and exciting. They were primarily based on homologies of partial sequences of the 16S ribosomal ribonucleic acid (rRNA) of many diverse procaryotes and the corresponding small-subunit rRNA of eucaryotes.

Perhaps even more important, Woese's data and concept brought a sense of harmony and clarity to an otherwise confusing and misleading state of bacterial systematics. Although many species of exceptional bacteria had been studied in detail before Woese's proposal and had been recognized by certain scientists as being unusual, their phylogenetic affiliation with other organisms was uncertain. For example, *Halobacterium* (now classified among the archaebacteria) had previously been classified together with eubacterial gram-negative aerobic rods, such as *Pseudomonas*, *Azotobacter*, and *Acetobacter* (284). However, even at that time it was known that *Halobacterium* possessed characteristics distinguishing it from other gram-negative aerobic rods, including major differences in cell wall structure and membrane lipids composed of C_{20} isoprenoid glycerol diethers (267, 421). Thus, "supporting evidence" for the concept of archaebacteria existed almost 20 years before Woese's proposal.

The other archaebacteria had also been considered different from typical bacteria in various respects. The methane-producing bacteria were the first extensively studied archaebacteria. Previously dispersed among other well-characterized bacteria on the basis of morphological criteria, the methanogens were reorganized as a coherent taxonomic group by H. A. Barker in 1956. Barker, a pioneer in the field of methanogen research, recognized "the striking physiological characteristics of all members of the group" (19). The subsequent demonstration of an array of novel coenzymes in methanogens by Wolfe and his co-workers provided further support for Barker's conclusions (12).

In the early 1970s, Brock and associates reported the existence of and subsequent isolation of two more "exceptional" organisms: *Sulfolobus*, an aerobe isolated from a Yellowstone hot spring; and *Thermoplasma*, a thermoacidophilic mycoplasmalike bacterium isolated from burning coal refuse piles (41, 68). These unique organisms were later demonstrated to contain distinctive cell envelopes and membrane lipids containing C_{40} tetraethers (279, 280). At that time, it was postulated that the presence of the unusual envelope and cell membrane constituents was a result of an adaptation to their extremely thermoacidophilic environment (40).

However, a short time after the isolation of *Thermoplasma*, an interesting discovery was made which further distinguished it from typical bacteria. Searcy discovered a histonelike protein in *Thermoplasma* sp. (413). The association of histones with nuclear deoxyribonucleic acid (DNA) was thought to be characteristic of eucaryotes and had not been previously observed in procaryotes. Searcy postulated that the *Thermoplasma* histone functioned to stabilize DNA either by conferring greater thermostability at specific DNA replication sites or by protection from depurination, which may occur at in situ growth conditions of high temperature and low pH (413). Based on these observations, Searcy postulated that *Thermoplasma* sp. was only distantly related to either eucaryotes or typical procaryotes. Nevertheless, it was not possible at that time to justify uniting *Thermoplasma* with the halobacteria and the methanogens.

The unification of these groups of exceptional bacteria in the archaebacterial kingdom provides an opportunity to reevaluate previous work in light of this evolutionary relationship. Moreover, it has stimulated many new investigations on these unique bacteria. The purpose of this review is to examine some of this new information to determine more precisely the relationship between the methanogens and the other archaebacteria. Previously, the methanogens had been considered a remarkable example of procaryotic diversity. Should they now be considered a remarkable example of archaebacterial diversity? To achieve this goal, an overview of the archaebacteria and what is known about their biochemistry is presented. On this foundation, the unique features of the methanogens are discussed.

ARCHAEBACTERIA

Diversity of Methanogens

Among the archaebacteria, the methane-producing bacteria are currently the only recognized group that can be termed cosmopolitan. Although a metabolically restricted group, methanogens exhibit extreme habitat diversity; species have been isolated from virtually every habitat in which anaerobic biodegradation of organic compounds occurs, including freshwater and marine sediments, digestive and intestinal tracts of animals, and anaerobic waste digesters (208, 325, 431, 504, 546). Additional isolates have also been obtained from extreme environments such as geothermal springs and both shallow and deep-sea hydrothermal vents (179, 207, 446). Physiologically, methanogens are represented by extremely thermophilic, moderately thermophilic, and many mesophilic isolates. Perhaps one of the few unexplored methanogen habitats lies in the productive psychrophilic waters of Antarctica. Other major groups of archaebacteria, the extreme halophiles, *Thermoplasma*, and the extreme thermophiles are represented to date by species which are restricted to "extreme" (high-salt or high-temperature) habitats. A summary of the described species of methanogens is presented in Tables 1 to 3.

Methanogens likely owe their cosmopolitan status to their unique mode of energy metabolism: methane generation. In most instances, compounds which serve as substrates for methanogenic bacteria, including H_2, formate, acetate, methanol, and methylamines, are produced as end products of various eubacterial and eucaryotic fermentations and anaerobic oxidations of both complex and simple organic compounds (525). In nature, competition among microorganisms for these methanogenic substrates may exist, especially in sulfate- or nitrate-rich habitats (14, 515, 516); however, symbiotic methanogenic associations have been described, and anaerobic "microzones," where methanogens can effectively compete for available substrates, probably exist (44, 303, 526, 527). This postulate is supported by evidence of the ubiquitous distribution of methanogens in

TABLE 1. Summary of characteristics of methanogenic archaebacteria, order *Methanobacteriales*[a]

Archaebacteria	Morphology	Substrates	G+C (mol%)	Temp optimum (°C)	pH optimum	Cell envelope composition	Major membrane isoprenoid	Reference(s)
Family *Methanobacteriaceae*								
Methanobacterium formicicum	Rod	H_2, formate	40.7	37	7.0	Pseudomurein	C_{20} + C_{40}	12
M. bryantii	Rod	H_2	32.7	38	7.0	Pseudomurein	C_{20} + C_{40}	12
M. thermoautotrophicum	Rod	H_2	49.7	65–70	7.2–7.6	Pseudomurein	C_{20} + C_{40}	546
M. wolfei	Rod	H_2	61	55–65	7.0–7.5	Pseudomurein	ND[b]	517
M. thermoaggregans	Rod	H_2	42	65	7.0–7.5	ND	ND	32
M. thermoalcaliphilum	Rod	H_2	38.8	60	7.5–8.5	ND	ND	33
Methanobrevibacter ruminantium	Rod	H_2, formate	30.6	38	7.2	Pseudomurein	C_{20} + C_{40}	431
M. smithii	Rod	H_2, formate	31	38	6.9–7.4	Pseudomurein	C_{20} + C_{40}	325
M. arboriphilus	Rod	H_2	29	30–37	7.5–8.0	Pseudomurein	C_{20} + C_{40}	544
Methanosphaera stadtmaniae	Coccus	H_2 + methanol	25.8	36–40	6.5–6.9	Pseudomurein	ND	323, 324
Family *Methanothermaceae*								
Methanothermus fervidus	Rod	H_2	33	83	6.5	Pseudomurein + protein	C_{20}	446

[a] Also refer to references 460, 504, and 514 and consult text.
[b] ND, Not determined.

anaerobic habitats. One interesting aspect of methanogen symbioses involves "interspecies hydrogen transfer," a term coined by Wolin to describe H_2 transfer between specific ruminal bacteria, resulting in more favorable energetics for the H_2-producing species and a change in the distribution of fermentation products toward more oxidized compounds (45, 185). In some instances, for example the anaerobic oxidation of butyric acid by *Syntrophomonas wolfei*, H_2 transfer is an obligate requirement because of thermodynamic considerations (314). The ecology of methanogens has been extensively reviewed (302, 460, 514) and will not be discussed further here.

Methanogen diversity is dramatically illustrated by the variety of morphological, physiological, and biochemical characteristics of the described species (Tables 1 to 3). All basic morphological types are represented, including cocci and packets of cocci (pseudosarcina), rods of varying length and figure, and a spirillum. Within the order *Methanobacteriales*, all species described prior to 1984 were rod shaped. Recently, divergence within that group is indicated by the description of *Methanosphaera stadtmaniae*, which exhibits definite coccoid morphology (324). In addition, *Methanosphaera stadtmaniae* deviates from the typical mode of energy metabolism of the *Methanobacteriales* in that H_2-mediated methanol reduction to methane occurs; this isolate cannot grow by the typical mode of CO_2 reduction to CH_4 by H_2. Although peculiarities exist, *Methanosphaera stadtmaniae* is phylogenetically grouped with species of *Methanobacteriales* based on cell envelope composition and 16S rRNA homology (324).

Within the order *Methanococcales*, all six species described to date (Table 2) exhibit coccoid morphology and were isolated from marine habitats (504). Two species are thermophilic, and one of these, *Methanococcus jannaschii*, exhibits deep divergence within the group, as indicated by 16S rRNA homology (207). Further, *Methanococcus jannaschii* is characterized by its high temperature optimum (85°C), its short doubling time (25 min), and the presence of a unique membrane constituent not found in any other species of archaebacteria (55). All mesophilic species of the *Methanococcales* appear to be closely related to each other and differ mainly by nutritional characteristics (504).

The third methanogen order, *Methanomicrobiales*, consists of the most diverse assemblage of methanogens of the three orders described. All morphological types are represented, both mesophilic and thermophilic species exist, and

TABLE 2. Summary of characteristics of methanogenic archaebacteria, order *Methanococcales*, family *Methanococcaceae*[a]

Archaebacteria	Morphology	Substrates	G+C (mol%)	Temp optimum (°C)	pH optimum	Cell envelope composition	Major membrane isoprenoid	Reference(s)
Methanococcus vannielii	Coccus	H_2, formate	32.5	36–40	7.0–9.0	Protein	C_{20}, tr C_{40}	12
M. voltae	Coccus	H_2, formate	29.6	32–40	6.7–7.4	Protein	C_{20}	505
M. maripaludis	Coccus	H_2, formate	33.4	38	6.8–7.2	Protein	ND[b]	208
M. deltae	Coccus	H_2, formate	33.6	37	ND	ND	ND	56
M. thermolithotrophicus	Coccus	H_2, formate	33.6	65	6.5–7.5	Protein	C_{20}	179
M. jannaschii	Coccus	H_2	31	85	6.0	Protein	Cyclic diether, C_{20} + C_{40}	55, 207

[a] Also refer to references 12 and 504 and consult text.
[b] ND, Not determined.

TABLE 3. Summary of characteristics of methanogenic archaebacteria, order *Methanomicrobiales*[a]

Archaebacteria	Morphology	Substrates	G+C (mol%)	Temp optimum (°C)	pH optimum	Cell envelope composition	Major membrane isoprenoid	Reference(s)
Family *Methanomicrobiaceae*								
Methanomicrobium mobile	Rod	H_2, formate	48.8	40	6.1–6.9	Protein	C_{20} + C_{40}	357
M. paynteri	Rod	H_2	44.9	40	6.5–7.0	ND	ND	375
Methanogenium cariaci	Coccus	H_2, formate	51.6	20–25	6.8–7.3	Protein	C_{20} + C_{40}	382
M. marisnigri	Coccus	H_2, formate	61.2	20–25	6.2–6.6	Glycoprotein	C_{20} + C_{40}	382
M. olentangyi	Coccus	H_2	54.4	37	ND	ND	ND	56
M. tatii	Coccus	H_2, formate	54	40	7.0	Glycoprotein	ND	539
M. limicola	Planes	H_2, formate	47.5	40	7.0	Glycoprotein	C_{20} + C_{40}	513
M. thermophilicum	Coccus	H_2, formate	59	55	7.0	ND	C_{20} + C_{40}	376
M. frittonii	Coccus	H_2, formate	49.2	57	7.0–7.5	Protein	ND	157
Methanospirillum hungatei	Curved rod	H_2, formate	45	30-37	6.6–7.4	Protein, sheath	C_{20} + C_{40}	122
Family *Methanosarcinaceae*								
Methanosarcina barkeri	Coccus, packets	H_2, Me, $MeNH_2$, Ac	39	35	7.0	HPS + protein	C_{20} + C_{25}	12, 168
M. mazei	Coccus	H_2, Me, $MeNH_2$, Ac	42	40	6.0–7.0	HPS	C_{20} + C_{25}	301
M. thermophila	Coccus	H_2, Me, $MeNH_2$, Ac	42	50	6.0–7.0	HPS	ND	559, 560
M. acetivorans	Coccus	Me, $MeNH_2$, Ac	42	40	6.5–7.0	Protein	ND	433
Methanococcoides methylutens	Coccus	Me, $MeNH_2$	42	35	7.0–7.5	Protein	C_{20}	434
Methanolobus tindarius	Coccus	Me, $MeNH_2$	40	25	6.5	Glycoprotein	C_{20} + C_{25}	259
Methanococcus halophilus	Coccus	Me, $MeNH_2$	ND	26–36	6.5–7.4	ND	ND	548
Halomethanococcus mahi	Coccus	Me, $MeNH_2$	48.5	35	7.5	ND	ND	354
Methanothrix soehngenii	Rod	Ac	51.9	37	7.4–7.8	Protein; sheath	C_{20}	183
Methanothrix sp.	Rod	H_2, Ac	ND	60	ND	ND	ND	557

[a] Also refer to references 12, 436, 504, and 514 and consult text. Abbreviations: ND, Not determined; Me, methanol; $MeNH_2$, methylamines; Ac, acetate; HPS, heteropolysaccharide.

substrates for carbon-energy metabolism are varied (Table 3). Substrates include H_2 plus CO_2, acetate, and methylated compounds such as methanol and mono-, di-, and trimethylamines. Some variation in membrane lipid and cell envelope composition exists within the order, but as with *Methanobacteriales* and *Methanococcales*, these constituents exhibit typical archaebacterial traits. Aspects of cell envelope and cell membrane constituents of archaebacteria are discussed in a later section.

Thermoplasma and the Extreme Thermophiles

Thermoplasma and *Sulfolobus* spp. were isolated and described by Brock and associates (39, 41, 68) in the early 1970s and were distinguished from "typical" procaryotes by their ability to grow at low pH and high temperature. These novel genera were subsequently placed in the thermoacidophilic branch of archaebacteria described by Woese (519). Both *Thermoplasma* and *Sulfolobus* spp. are aerobic, thermophilic bacteria isolated from environments of high temperature and low pH. Thus, these organisms were termed thermoacidophiles. Since their discovery, several new isolates have been described (445, 448, 549, 550, 552) that are related to *Sulfolobus* spp. but which do not exhibit low pH optima. Because all species are thermophilic and capable of sulfur metabolism, the term sulfur-dependent archaebacteria or extreme thermophiles is currently used to describe these new isolates and *Sulfolobus* spp. (448). *Thermoplasma* spp. are no longer grouped with the sulfur-dependent archaebacteria but instead is considered to be a distant relative to the methanogens based upon key characteristics of 16S rRNA sequences (536).

Thermoplasma sp. was originally isolated by Darland et al. (68) from a burning coal refuse pile and more recently from hot springs (35, 349). The organism, represented by the species *Thermoplasma acidophilum* (Table 4), is wall-less, grows aerobically at 59°C and pH 1 to 2 by heterotrophic metabolism, and contains C_{40} hydrocarbons as the major glycerol ether membrane lipid (279). *Thermoplasma acidophilum* represents the only thoroughly characterized archaebacterial mycoplasma, although one additional methanogenic species has been described to be mycoplasmalike (383).

The extreme thermophiles are currently represented by 13 species contained within two orders, the *Sulfolobales* and the *Thermoproteales* (Table 4); species of these groups are primarily distinguished by morphology and by their mode of energy generation. Species of *Sulfolobales* are aerobic thermoacidophiles of coccoid morphology which grow at the expense of sulfur oxidation or by an unknown fermentation of organic compounds, including amino acids, yeast extract, and sugars (448). Most species grow within the pH range of

TABLE 4. Summary of characteristics of *Thermoplasma* sp. and the sulfur-dependent, extremely thermophilic archaebacteria[a]

Bacteria	Morphology	Relation to O_2	G+C (mol%)	Temp optimum (°C)	pH optimum	Metabolism	Reference(s)
Order *Thermoplasmales*							
Thermoplasma acidophilum	Coccus	Aerobe	46	59	1–2	Oxidation of organics	68, 448
Order *Sulfolobales*							
Sulfolobus acidocaldarius	Coccus	Aerobe	37–41	70–75	2–3	Oxidation of organics, S°	35, 41
S. solfataricus	Coccus	Aerobe	36–40	87	3.5–5	Oxidation of Y extract, S°	448
S. brierleyi	Coccus	Aerobe	31–37	70	1.5–2	Oxidation of Y extract, S°	39, 448
Order *Thermoproteales*							
Thermoproteus tenax	Thin rod	Anaerobe	55.5	88	5.5	S° respiration, auto & hetero	123, 553
T. neutrophilus	Thin rod	Anaerobe	56.2	85	6.8	H_2 + S° autotrophy	123, 553
Thermophilum pendens	Thin rod	Anaerobe	57.4	88	5.5	S respiration of organics	549
Desulfurococcus mucosus	Coccus	Anaerobe	50.8	85	6.0	S respiration of organics	552
D. mobilis	Coccus	Anaerobe	50.8	ND	ND	S respiration of organics	552
Thermococcus celer	Coccus	Anaerobe	50.8	92	5.8	Respiration of organics ± S	550
Thermodiscus maritimus	Disk	Anaerobe	53	87	5.5	Respiration of organics ± S	123, 448
Pyrodictium occultum	Disk, filaments	Anaerobe	62	105	6.5	H_2 + S° autotrophy	445
P. brockii	Disk, filaments	Anaerobe	51	105	5–7	H_2 + S° autotrophy	445

[a] Also refer to references 40, 123, 349, 444, 448, 477, 520, and 536 and consult text. Abbreviations: Y extract, yeast extract; S, sulfur; auto, autotrophic; hetero, heterotrophic. ND, Not determined.

2 to 3.5 and at temperatures of 70 to 90°C. Species capable of chemolithotropic metabolism oxidize elemental sulfur to sulfuric acid as their primary energy source (41). Several species have been described in detail (Table 4).

Species of *Thermoproteales*, on the other hand, are more diverse and are represented by six genera (Table 4); all species are thermophilic anaerobes which grow optimally at pH values of 5.5 to 6.5. Further, the characterized species have diverse morphologies and obtain energy predominantly by a form of sulfur metabolism. Most species have temperature optima at 90°C and above (448).

Energy metabolism among the *Thermoproteales* is varied. *Thermoproteus tenax* is capable of chemolithotrophic energy metabolism via reduction of sulfur by H_2 or by sulfur respiration of various organic compounds, including glucose, yeast extract, ethanol, and formate (553). *Thermoproteus neutrophilus*, however, is an obligate chemolithotroph. Three additional genera, *Thermophilum*, *Desulfurococcus*, and *Thermococcus*, are not known to grow by a chemolithotrophic mode of metabolism. Instead, most species obtain energy by sulfur respiration of organic compounds such as yeast extract, peptides, and protein or by an unknown means of fermentation (448, 549, 550, 552). Details of other physiological, biochemical, and molecular characteristics will be discussed elsewhere; an excellent recent review has been presented by Stetter and Zillig (448).

Two additional genera, *Thermodiscus* and *Pyrodictium*, have been recently isolated from a submarine thermophilic habitat by Stetter and co-workers (123, 445) and are characteristic of the extreme thermophiles. *Thermodiscus* is a flat, disk-shaped organism that grows optimally at 87°C and pH 5.5. This isolate grows by heterotropic sulfur respiration of yeast extract or by an unknown fermentative metabolism in the absence of sulfur and H_2. *Pyrodictium* sp. is a very unusual and most interesting isolate; it is capable of growth at 110°C and pH 5 to 7, although optimal growth occurs at 105°C. It is the most thermophilic organism described to date (445) and obtains energy by hydrogen-sulfur autotrophy in a manner apparently analogous to *Thermoproteus* species. A summary of the characteristics of *Thermoplasma* and species of the extreme thermophiles is presented in Table 4.

All species of *Thermoplasma* and the extremely thermophilic branch of archaebacteria described to date are restricted to a thermophilic mode of growth and metabolism. Diversity within the group exists, as demonstrated primarily by the variety of morphological characteristics, different modes of energy metabolism, and various physiological parameters (temperature, pH) of the species. Additional characteristics, including cell envelope and cell membrane constituents, will be discussed in other sections.

Extremely Halophilic Archaebacteria

Extreme halophiles are represented by a restricted number of species of procaryotes which survive and grow in habitats approaching the saturation point of NaCl. Eubacterial extreme halophiles include the photosynthetic bacterium *Ectothiorhodospira* and the actinomycete *Actinopolyspora*. The term "halobacteria" is commonly used to denote only the extremely halophilic archaebacteria (266, 284).

Halobacteria were identified as archaebacteria in the late 1970s and consisted of two genera, *Halobacterium* and *Halococcus* (284). These organisms have characteristic archaebacterial properties, including the presence of ether-linked isopranyl polar lipids and the lack of a typical murein-containing cell envelope (216, 443). In addition, 16S rRNA catalogs indicate that halobacteria are most closely related to the methanogenic branch of the archaebacteria (127).

Species of halobacteria are aerobic chemoorganotrophs that grow optimally at NaCl concentrations of 16 to 26% (wt/vol) and contain carotenoids, predominately bacterioruberin, which give halobacteria their reddish pigmentation (170, 268, 449). Many species also contain satellite DNA (made of several extrachromosomal DNA fragments) in

TABLE 5. Summary of characteristics of extremely halophilic archaebacteria[a]

Archaebacteria	Morphology	G+C (mol%)		Temp optimum (°C)	pH optimum	Optimal NaCl (M)	Substrates	Reference(s)
		Major DNA	Minor DNA					
Halobacterium salinarium	Rod	66–68	57–59	50	7.2	3.5–4.3	Amino acids	266
H. saccharovorum	Rod	ND[b]	ND	50	ND	3.5–4.5	Carbohydrates	266
H. vallismortis (maris-mortui)	Pleomorph	ND	ND	40	7.0	4.3	Carbohydrates	346
H. volcanii	Disk, oval	63–66	55	45	6.8	1.8–2.5	Carbohydrates	266
H. mediterranei	Rod	60	None	35	6.5	3	Carbohydrates & amino acids	378
H. sodomense	Rod	68	None	40	ND	1.7–2.5	Carbohydrates	351
Halococcus morrhuae	Coccus	65	53.4	30–37	7.2	3.5–4.5	Amino acids	266, 443
Natronobacterium gregoryi	Rod	65	None	37	9.5	3	Carbohydrates	470
N. magadii	Rod	63	49.7	37–40	9.5	3.5		470
N. pharaonis	Rod	64.3	51.9	45	8.5–9	3.5	Organic acids	432, 470
Natronococcus occultus	Coccus	64	55.7	35–40	9.5	3.5–4	Carbohydrates	470

[a] Also refer to references 170, 266, 284, and 469 and consult text.
[b] ND, Not determined.

addition to their major DNA component (266, 328). As a group, halobacteria exhibit diversity in their mode of energy generation, and all species are capable of aerobic respiration of sugars, amino acids, or other low-molecular-weight organic compounds. In the presence of light and low partial pressures of O_2, many species synthesize a novel purple pigment, bacteriorhodopsin, which functions as an alternative means of energizing the cell membrane for adenosine triphosphate (ATP) synthesis and active transport (283, 449). Finally, a limited number of species of halobacteria are capable of anaerobic growth in the presence of nitrate or by fermentation (158, 284).

The genus *Halobacterium* consists of eight described species, three of which, *H. salinarium*, "*H. halobium*," and "*H. cutirubrum*," were recently reclassified as one species (*H. salinarium*) based on similarities in physiological characteristics and 16S rRNA catalogs (127, 284). All species of *Halobacterium* described to date are rod or disk shaped, grow optimally near 45°C at neutral pH and NaCl concentrations of 16 to 26%, and have similar DNA guanine-plus-cytosine content (G+C). With the exception of *H. salinarium*, species of *Halobacterium* utilize various carbohydrates as growth substrates. *H. salinarium* may grow aerobically or via anaerobic metabolism of arginine (158). Species diversity among *Halobacterium* is indicated by both metabolic and physiological characteristics (Table 5).

The genus *Halococcus* consists of only one species described to date. *Halococcus morrhuae* is a nonmotile, mesophilic coccus capable of aerobic growth on amino acids or via reduction of nitrate to nitrite (266, 284). In addition to morphological differences, *Halococcus morrhuae* is distinguished from other halobacteria by the presence of a sulfated heteropolysaccharide as its major cell wall component (443).

Recently, two novel genera of alkaliphilic halobacteria, *Natronobacterium* and *Natronococcus*, were described by Tindall et al. (469, 470). These genera share similar characteristics with other halobacteria, including a common mode of energy generation, temperature optima, NaCl requirements, and the presence of the pigment bacterioruberin. However, the newly described genera have an obligate requirement for high pH (optimum near 9.5) and low Mg^{2+} concentrations. Further, ether lipids of the haloalkaliphiles are composed of C_{25} isopranyl constituents in addition to the C_{20} isopranyl lipids commonly found in other halobacteria (78, 81). Three species of *Natronobacterium* (*Natronobacterium gregoryi*, *N. magadii*, and *N. pharaonis* [formerly "*Halobacterium pharaonis*"]) and one species of *Natronococcus* (*Natronococcus occultus*) have been described to date (432, 470). A summary of their characteristics is presented in Table 5.

This brief description of extremely halophilic archaebacteria nonetheless should illustrate their diversity by the variety of morphological, physiological, and metabolic characteristics of the described species.

THE ARCHAEBACTERIAL RIBOSOME

Comparative studies of the ribosome and its components were instrumental in formulation of the archaebacterial hypothesis. Many of the detailed phylogenetic studies on archaebacteria have continued to be concerned with different aspects of the ribosome. Therefore, it is appropriate to describe these extensive studies before discussing other aspects of archaebacterial metabolism.

The general structure of ribosomes of archaebacteria closely resembles the eubacterial ribosome. They are approximately the same size, 70S, and contain only three rRNAs, 23S, 16S, and 5S (20, 87, 487). No evidence has been obtained for a 5.8S rRNA, as is found in eucaryotes (487). Like the eubacterial ribosome, ribosomes from archaebacteria dissociate into two components, the 30S and 50S subunits. The 16S rRNA is part of the 30S subunit; the 5S and 23S rRNAs are part of the 50S subunit. In most archaebacteria, the numbers of proteins associated with each subunit agree well with the number found in the eubacterial subunits (399, 453). In a few cases, an unusually large number of proteins are associated with the 30S subunit in archaebacteria (399, 400). Whether or not the increased

mass of the 30S subunit is functionally significant or artifactual is not known.

rRNA

Since partial sequencing of the 16S rRNA led to formulation of the archaebacterial concept in 1977 (519), full sequencing of at least nine 16S rRNAs from archaebacteria has been completed. In addition to full sequences from each of the major groups of methanogens (199, 285, 536), four halobacterial sequences (151, 181, 286, 305) and two sequences from the extreme thermophiles (293, 350) have been obtained. The complete sequence data support the previous hypotheses of an extensive diversity within the archaebacteria and a clear distinction between the archaebacterial kingdom and the eubacterial and eucaryotic kingdoms. Likewise, the full sequence data support the division of the archaebacteria into two phyla (536). One phylum is composed of the methanogens, halobacteria, and *Thermoplasma* spp. The second phylum consists of the aerobic and anaerobic extreme thermophiles. In addition, the complete sequence data demonstrate the specific relationship of the halobacteria to one group of the methanogens, the *Methanomicrobiales* (536). This relationship was ambiguous in the partial sequencing data (12).

The full sequence data also revealed that extensive homology exists among rRNAs from all sources. Partial sequencing data of rRNA had previously identified the extremes of procaryotic diversity. Therefore, full sequences were obtained from organisms representative of the full extent of the diversity of microorganisms. The homology of the archaebacterial sequences to *Escherichia coli* sequences is between 59 and 63%. For comparison, within both the archaebacterial and eubacterial kingdoms, sequence homologies of >70% are encountered. When compared with homologous regions from the *Dictyostylium discoideum* 18S RNA, archaebacterial RNAs are 54 to 56% homologous. The homology between *D. discoideum* and *E. coli* RNAs is somewhat lower, 53%, which suggests that the archaebacterial RNA may more closely resemble the ancestral progenote small-subunit RNA than either eubacterial or eucaryotic RNAs (521). These sequence homologies are further supported by examination of selected regions which are highly homologous within the RNAs from all kingdoms (151, 286). A comparison of 30 such regions demonstrated a closer homology between the archaebacterial and eubacterial than the eucaryotic regions in most cases. However, in a significant number of comparisons, the archaebacterial sequences more closely resembled the eucaryotic sequences than the eubacterial ones. Only one case of close homology between the eubacterial and eucaryotic sequences to the exclusion of the archaebacterial sequences was observed (286). The extensive homology in primary structure is also reflected in the proposed secondary structures of small-subunit rRNA from the three kingdoms (521). Thus, many of the structural features of RNAs are conserved even among the most diverse organisms known.

High amounts of homology have also been observed in other rRNAs. The 5S rRNA has been studied extensively (126). Although it is much smaller and more variable than the 16S RNA, the proposed secondary structure of archaebacterial 5S RNAs closely resembles the structure of RNAs from the other kingdoms. In addition to features commonly associated with either the eubacterial or the eucaryotic form, it also contains features uniquely associated with archaebacterial RNAs (126). The number of 23S rRNAs sequenced is much smaller than either the 16S or 5S rRNAs. Like the 16S rRNA, the 23S rRNAs of *Methanococcus* and *Halobacterium* spp. contain about 60% sequence homology to the RNA from *E. coli* (200, 304). While some regions are very similar to the eubacterial RNA, a number of insertions are present whose locations coincide with the location of insertions found in eucaryotic RNAs. An intron has also been identified in the *Desulfurococcus* 23S rRNA gene (243). This intron contains the consensus sequence necessary for correct splicing of rRNA introns in eucaryotes and a large open reading frame. In these and other respects, the archaebacterial intron resembles eucaryotic introns.

The organization of the structural genes for rRNAs is highly conserved in eubacteria and eucaryotes. The gene organization for a number of archaebacteria has been determined by restriction endonuclease mapping and, in some cases, DNA sequencing. Usually the gene order is similar to that found in eubacteria, 16S-23S-5S (171, 197, 345). The exception is *Thermoplasma acidophilum*, where it was not possible to link the genes by restriction endonuclease mapping (478). In the methanogens and halobacteria, the genes which have been sequenced appear to form a single operon, and potential transfer RNA (tRNA) genes are found between the 16 and 23S rRNA genes and, in some cases, distal to the 5S rRNA gene (60, 181, 198–200, 285, 304, 305). Moreover, noncoding regions of the operon have the potential to form secondary structures very similar to those necessary for maturation of rRNAs in eubacteria (60, 200, 285, 286, 304). These aspects of the gene organization in archaebacteria are very similar to the organization in eubacteria. However, *Methanococcus vannielii*, in addition to having four ribosomal operons of the general eubacterial organization, also possesses one unlinked 5S rRNA gene, which is a feature commonly found in eucaryotes (197). This 5S rRNA gene appears to be part of an operon containing seven potential tRNA genes and the necessary promoter, termination, and processing sites (510). While these features tend to argue against it being nonfunctional or a pseudogene, conclusive proof is not currently available.

In the extreme thermophiles, the gene order is conserved, but the distance between the genes is highly variable (345). The distance between the 16S and 23S rRNA gene varies from 57 bases to >2 kilobases. For two organisms with short intergenic spacers which have been sequenced, no potential tRNA genes have been found (293, 350). The distance between the 5S and 23S rRNA genes varies from <1 to about 11 kilobases. Moreover, additional copies of unlinked 5S rRNA genes are present in some organisms. These results suggest that the location of the 5S rRNA gene is not highly conserved in this phylum of the archaebacteria (345).

tRNA

A number of tRNAs or their genes have been sequenced, including 33 tRNAs for 18 amino acids from *Halobacterium volcanii* (149). The general secondary structure of archaebacterial tRNAs is similar to the eubacterial and eucaryotic tRNAs. However, many of the structural details are unique to the archaebacteria (150, 152). For instance, the sequence TΨCG, which is nearly universal in eubacteria and eucaryotes, is not present (152). Instead, the sequence 1-methylΨΨCG is present in most archaebacterial tRNAs. 1-MethylΨ is an interesting minor nucleotide because it is a structural analog of ribothymidine, which is found at the same position in most other tRNAs. Other minor nucleotides in archaebacterial tRNAs have eucaryotic characteristics.

For example, the minor base 1-methyladenine is common to eucaryotic and archaebacterial tRNAs but not eubacterial tRNAs (150, 244). Likewise, the initiator tRNA is methionyl-methionyl-tRNA (Met-tRNA^{Met}) and not *N*-formylmethionyl-tRNA^{Met}. However, the archaebacterial initiator tRNA is unique in having a 5′-triphosphate terminus. Other similarities have also been found in the structure of tRNA genes. Like the eucaryotic genes, most of the sequenced tRNA genes in archaebacteria lack the 3′-CCA end (510). In addition, introns have been identified in the tRNA^{Trp} gene of *Halobacterium* spp. and the tRNA^{Ser} and tRNA^{Leu} genes of *Sulfolobus* spp. (59, 209). In all of these cases, the processing sites occur at positions analogous to the locations of processing sites in eucaryotes.

Similar comparisons have been made for the aminoacyl-tRNA synthetases of archaebacteria. The enzyme which has been purified to homogeneity, Phe-tRNA synthetase from *Methanosarcina barkeri*, resembles the eubacterial and eucaryotic enzymes in terms of quaternary structure and catalytic properties (373, 374). Like the synthetases from higher eucaryotes, the archaebacterial enzyme uses a pretransfer-proofreading mechanism to obtain specificity for charging tRNA^{Phe} (373). The enzymes from eubacteria and yeasts use a post-transfer-proofreading mechanism. In spite of these functional similarities, there is little or no immunological cross-reactivity of archaebacterial aminoacyl-tRNA synthetase with the enzymes from other kingdoms (372–374).

Heterologous charging of tRNAs by synthetases from different kingdoms has been used to determine other possible homologies. In general, synthetases are most active on tRNAs from organisms within the same kingdom (372). However, archaebacterial synthetases have slightly more activity with eucaryotic tRNAs than eubacterial tRNAs. Moreover, both eubacterial and eucaryotic synthetases are more active with archaebacterial tRNAs than tRNAs from each other (271, 372). These results are consistent with the analysis of 16S rRNAs described above, where the archaebacterial structure appears to be more closely related to the common ancestor of the three kingdoms than either the eubacterial or eucaryotic RNAs (521).

Ribosomal Proteins

The ribosomal proteins of archaebacteria have been best characterized for "*Halobacterium cutirubrum*" (308, 310, 453, 531). However, comparisons with eubacterial and eucaryotic ribosomes are hampered by the complexity of the ribosome and incomplete sets of data on ribosomal proteins from other kingdoms. Although immunological and protein sequence data have identified a number of homologies with eubacterial and eucaryotic proteins, these studies are far from complete (9, 241, 308, 310, 348, 398, 401, 402, 531). The early observation that ribosomal proteins of archaebacteria are unusually acidic is well correlated with the intracytoplasmic concentrations of ions in halobacteria and some methanogens and is probably not of major phylogenetic significance (196). The archaebacterial ribosomal "A" proteins, whose complete sequence is known, has substantial homology to the eucaryotic "A" protein and much less homology to the eubacterial equivalent, L12 (308, 309, 348). Similarly, the archaebacterial 5S RNA-binding proteins have more sequence homology to the eucaryotic than to the eubacterial proteins (9, 241). In contrast, other archaebacterial proteins which are homologous to the eubacterial proteins S9 and S15 have no known homologies to eucaryotic proteins (342, 430). In any case, both immunological and protein sequence data support the hypothesis that archaebacteria form a separate kingdom distinct from other bacteria and eucaryotes (309, 401).

Elongation Factors and Antibiotics

Translation in eubacteria and eucaryotes requires two elongation factors, which are highly conserved. Similar factors have been highly purified from a number of archaebacteria (238, 247). Like the eucaryotic translocation factor, the archaebacterial aEF-2 contains a post-translationally modified histidinyl residue, diphthamide, which is adenosine diphosphate (ADP) ribosylated by diphtheria toxin (237, 247, 353). Although the amino acids surrounding the diphthamide are highly conserved in archaebacteria and eucaryotes, the rate of modification by the toxin is much faster in the latter (247, 353). Diphthamide is not present in the analogous eubacterial protein EF-G (353). In spite of the similarities between archaebacterial and eucaryotic translocation factors, these proteins do not substitute for each other or for eubacterial EF-G during in vitro polypeptide synthesis with ribosomes from each kingdom (248). The aminoacyl-tRNA binding factor from archaebacteria, aEF-1α, also resembles the eubacterial and eucaryotic factors in molecular weight and ability to bind guanosine diphosphate and guanosine triphosphate (247). In vitro, the archaebacterial aEF-1α is interchangeable with the eucaryotic EF-1α and probably the eubacterial EF-Tu (247). In contrast, the eucaryotic and eubacterial aminoacyl-tRNA binding factors are rarely interchangeable.

Functional relationships of archaebacterial ribosomes have been further explored with antibiotics. In an in vitro polypeptide synthesis assay prepared from *Sulfolobus* extracts, 57 of 60 antibiotics were not inhibitory at low concentrations, regardless of their normal site of inhibition or selectivity to eubacterial or eucaryotic translation (49). Similar results have been obtained in vitro with methanogen ribosomes (34, 106, 182). Some antibiotics, for example neomycin, are inhibitory only at concentrations high enough to inhibit eucaryotic as well as eubacterial polypeptide synthesis (34, 49, 106). Other antibiotics which are also somewhat inhibitory at high concentrations include gentamicin, paromomycin, thiostrepton, anisomycin, tetracycline, puromycin, and α-sarcin. Many other common eubacterial or eucaryotic antibiotics are ineffective even at extremely high concentrations (34, 49, 106, 128, 182).

Evolution of the Ribosome

In summary, several themes recur in the large body of work on the archaebacterial ribosome. First, the archaebacterial hypothesis has been validated repeatedly. Thus, the bacteria identified as archaebacteria have more in common with each other than with either the eubacteria or eucaryotes. This result is common to comparisons of all aspects of the ribosome including rRNAs, ribosomal proteins, elongation factors, and aminoacyl-tRNA synthetases.

A second major conclusion is that the translation systems of all three kingdoms are homologous. Therefore, it is reasonable to believe that the ribosome evolved only once. This conclusion is convincingly impressive when viewed from the context of the rRNA data, from which it is reasonable to believe that a sampling of the entire spectrum of organisms on earth has been obtained. If the homologies observed in modern organisms are representative of a com-

mon ancestor, this progenote appears to have been fairly sophisticated. The minimum size of the ribosome was established as well as the core sequence of the two large rRNAs. The catalytic properties of the elongation factors and the aminoacyl-tRNA synthetases were defined. Some of the RNA post-transcriptional modification and protein post-translational modification systems were developed. The gene order and processing sites of the rRNAs were also set within limits. In fact, much of the complex apparatus required for the synthesis and function of the modern ribosome appears to have been formed.

The third recurring theme is the impression that the archaebacteria are more closely related to the eucaryotes than the eubacteria are related to the eucaryotes. The most conservative interpretation of this data would be that the archaebacteria more closely resemble the progenote than either the eubacteria or eucaryotes. However, two qualifications are important. First, no matter what the relationship between archaebacteria and eucaryotes is on the molecular level, both the archaebacteria and eubacteria share the procaryotic cellular organization. Thus, they are specialized for life as small, unicellular organisms. This feature is as important in understanding these bacteria as their phylogeny. Second, the only component of the ribosome which has been studied in depth in the eubacteria is the 16S rRNA. For other aspects of ribosome structure and function, most of the information comes from studies of *Escherichia* and *Bacillus* species. It is not clear that these organisms are representative of the full extent of eubacterial diversity (127, 523). Thus, the eubacterial ribosome is only partially described. Moreover, as demonstrated by a recent report of a eucaryote which lacks a 5.8S rRNA, this kingdom also has a few surprises left (489). Thus, understanding the phylogenetic relationships between archaebacteria and the other kingdoms requires more comparative studies on the nature of eubacteria and eucaryotes.

OTHER ASPECTS OF ARCHAEBACTERIAL BIOCHEMISTRY

The perspective gained from reviewing the comparative studies of the ribosome will be useful for understanding other aspects of archaebacterial metabolism. Thus, we will proceed from other molecular aspects, including transcription and DNA structure, to general metabolism and, finally, cellular lipids and envelopes. We expect many of these systems to be homologous to eubacterial and eucaryotic systems. The question that remains is the extent of homology.

Transcription

The DNA-dependent RNA polymerase has been purified from at least eight archaebacteria (300, 369, 447, 457, 551, 554–556). These include representatives of the major groups of methanogens, halophiles, and extreme thermophiles. In striking contrast to the eubacterial enzyme, the polymerase from archaebacteria is very complex and contains at least eight polypeptides. In this respect, it resembles the eucaryotic enzyme. Other similarities to the eucaryotic enzyme include the stimulation of the archaebacterial polymerase by silybin and its insensitivity to rifampin and streptolydigen (409). Moreover, antisera to the eucaryotic polymerases cross-react with the archaebacterial enzymes (180, 410). Unlike eucaryotes, only a single polymerase has been found in any archaebacterium, and the archaebacterial polymerases are insensitive to α-amanitin (554).

In the halobacteria, the start sites for transcription have been identified for the bacteriopsin gene and the rRNA gene cluster (70, 73). In neither case has a typical eucaryotic or eubacterial RNA polymerase-binding site been found. However, these regions do contain features common to other promoter regions, i.e., inverted repeats and adenine-thymine-rich regions (306). A possible archaebacterial RNA polymerase recognition site, G/A-A-G-T-T/A-A, has been proposed 24 to 35 bases upstream of the start sites of transcription, at a position analogous to recognition sites in other promoters (70, 73). An extensive comparison of DNA sequences upstream from 15 open reading frames (primarily from insertion elements of halobacteria and methanogens) suggests that a more general consensus promoter is 5′-GAANTTTCA and 5′-TTTTAATATAAA (155). A similar promoter is found in *Drosophila* heat shock genes.

Possible termination sites have also been identified (70, 181, 334, 510). A common feature is a G-C-rich inverted repeat followed by an adenine-thymine-rich region. For a highly expressed operon in *Methanococcus* spp. consecutive thymidines following the inverted repeat are the site of termination (334). These features resemble termination regions in eubacteria.

Like the messenger RNA in eubacteria, the apparent messenger RNA in *Methanococcus* spp. is not capped, and a small fraction contains short polyadenylated sequences at their 5′ end (43). Messenger RNAs also contain potential ribosome-binding sequences which are complementary to the 3′ termini of the 16S rRNA (36, 58, 90, 155). In addition to the rRNA genes, a number of other probable operons have been identified (155, 252). These features closely resemble the eubacterial system of transcription and translation.

DNA Structure and Replication

The general structure of the genome of archaebacteria resembles the eubacterial genome (245, 327, 329). The complexity varies between about 40 and 90% of the complexity of the *E. coli* genome and corresponds to a genome size of 1.0×10^9 to 2.4×10^9 daltons. Because the *E. coli* genome is larger than average, the number of genes in archaebacteria is probably comparable to the number in eubacteria.

The DNA composition is also similar to that of eubacteria. The G+C content varies over most of the range observed in eubacteria. The modified bases common in eubacteria (5-methylcytosine, 4-methylcytosine, and N^6-methyladenine) are present (111). Likewise, a number of restriction endonucleases have been purified (312, 368, 403). In *Halobacterium* spp. there is additional evidence for restriction-modification systems similar to those found in eubacteria (64).

Extrachromosomal DNA is common in archaebacteria. Cryptic plasmids have been reported in methanogens (316, 467, 528). A covalently closed circular prophage has also been observed in *Sulfolobus* spp. (307, 537), This prophage integrates into a specific site in the chromosome, and it is inducible by ultraviolet radiation, like some eubacterial phages (307). A variety of other phages have also been isolated from *Halobacterium* and *Thermoproteus* spp. (190, 356, 408, 474, 475, 490). Their morphologies vary from lambdalike to rod shaped. So far, only DNA-containing phages have been identified.

Although some progress has been made on genetic systems in *Halobacterium* spp. (321), the lack of well-developed

genetic systems has limited functional studies of archaebacterial DNA. Comparison of the amino acid sequence of bacterio-opsin to the DNA sequence of the gene demonstrated that all 45 of the codons which appeared in the gene were read in the normal fashion (90). Although introns have been observed in genes for tRNA from several archaebacteria and the 23S rRNA of *Desulfurococcus* spp. (59, 209, 243), introns have not been reported in genes for proteins so far.

In "*Halobacterium halobium*," it has been possible to correlate the high frequency of spontaneous mutations in pigmentation and gas vacuole formation with alterations in DNA structure. The DNA in this archaebacterium consists of high moles percent G+C DNA derived from the chromosome and low moles percent G+C satellite DNA (328). The low-moles percent G+C DNA contains a large, low-copy-number plasmid (pHH1), a heterogeneous collection of very low-copy-number covalently closed circular DNA, and large fragments of linear DNA which are also derived from the chromosome (91, 360, 361, 495). High rates of loss (10^{-2} to 10^{-4} per generation) of gas vacuoles, ruberin synthesis, and bacterio-opsin synthesis are correlated with spontaneous insertions of DNA into plasmid pHH1 (362, 495) and the bacterio-opsin gene in the chromosome (22, 69, 424). Similar insertions, as well as deletions and inversions, are also correlated with the high amount of structural variability in the genome of phage ϕH (404–407). Moreover, the genome of "*H. halobium*" contains many families of mobile, repeated sequences, some of which are conserved in other halobacteria (386, 387). Thus, this remarkable mutation frequency is explained by the presence of a large number of different transposable elements, many of which are found in more than one copy, which resemble the transposable elements found in eubacteria (69, 91, 360, 404–406, 424, 530). Although transposable elements have been identified in other archaebacteria, it is not clear that the extreme variability observed in "*H. halobium*" is typical (154, 155). Certainly, it is not common in eubacteria.

DNA polymerases have been purified from *Halobacterium*, *Methanococcus*, and *Sulfolobus* species (246, 339, 538). Two of the enzymes are sensitive to aphidicolin, an inhibitor of the eucaryotic type-α DNA polymerase (339, 538). Moreover, growth of methanogens and halobacteria are also sensitive to aphidicolin (125, 395, 538). However, the reported molecular weights of the aphidicolin-sensitive polymerases are not similar so it is not clear that these enzymes are equivalent. An aphidicolin-resistant DNA polymerase has been purified from *Sulfolobus acidocaldarius* (246). A similar polymerase is also present in extracts of "*Halobacterium halobium*" (339). The *Sulfolobus* enzyme is composed of a single polypeptide and has 3′→5′ exonuclease activity. It may have a nonreplicative function (246).

Sulfolobus spp. also contain a variety of topoisomerases (240). One of these, a reverse gyrase, is unusual in that it is a type I topoisomerase which requires both divalent cations and ATP to introduce positive superhelical turns in DNA (338). Whether reverse gyrase is unique to archaebacteria or yet to be discovered in eubacteria and eucaryotes is not known. *Sulfolobus* spp. also contain a type II topoisomerase (240).

A histonelike protein has been described in *Thermoplasma acidophilum* (71, 413). This basic protein resembles eucaryotic histones in amino acid composition and ability to protect small DNA fragments from nuclease digestion. Although it has some slight sequence homology with eucaryotic histones 2A and 3, it has much more sequence homology with the eubacterial DNA-binding proteins HU-1 and HU-2 (72). Other nucleoproteins have been identified in *Sulfolobus*, *Methanosarcina*, and *Methanobacterium* species (51, 142, 468). Antiserum to the *Thermoplasma* protein does not cross-react with these other nucleoproteins. Moroever, these proteins have different molecular weights and amino acid compositions. Therefore, the *Thermoplasma* protein does not appear to be typical of other archaebacterial nucleoproteins. This result is somewhat surprising because eubacterial nucleoproteins are highly conserved (468).

Energy Metabolism

A proton motive force (PMF) composed of either a transmembrane electrical potential or a proton gradient has been well documented in archaebacteria. In "*Halobacterium halobium*," the coupling of the PMF to the phosphorylation of ADP and active transport has been reviewed (283). In methanogens, the coupling of the PMF to ATP synthesis is still controversial (for a review see reference 63). However, even in methanogens the PMF is likely to play a central role in other aspects of bioenergetics.

For comparative purposes, respiratory systems which might be similar to eubacterial systems for generating the PMF are of special interest. Respiration has been described for three archaebacteria: *Halobacterium*, *Sulfolobus*, and *Thermoplasma* spp. In *Halobacterium* sp., type *b*, *a*, *o*, and *c* cytochromes have been identified, and two type *b* cytochromes have been solubilized from the membrane (52, 153, 169, 282). Electron transport appears to follow the "normal" pattern: reduced nicotinamide adenine dinucleotide (NADH) → cytochrome *b* → cytochrome *c* → cytochrome *a* or cytochrome *o* → O_2. Reduction of oxygen is sensitive to carbon monoxide and cyanide, and the oxidation of cytochrome *b* is sensitive to antimycin A and 2-heptyl-4-hydroxyquinoline *N*-oxide. In *Sulfolobus* sp. cytochrome *c* is not detectable and respiration is also insensitive to antimycin A and 2-heptyl-4-hydroxyquinoline *N*-oxide (5). The electron transport chain is composed of cytochrome *b*, cytochrome *a*, and probably caldariellaquinone, a sulfur-containing quinone (5, 54, 77). In spite of some unusual features, there is no evidence that these electron transport chains are uniquely archaebacterial. The diversity of electron transport chains in eubacteria is well recognized, and the variations observed in *Sulfolobus* and *Halobacterium* spp. are not exceptional (204).

Although a type *b* cytochrome and quinones have been identified in *Thermoplasma* sp., its respiration may be atypical (173, 414). *Thermoplasma* sp. maintains a large pH gradient, which is about the same magnitude as eubacterial acidophiles (322). However, the PMF may not be linked to ATP synthesis (414). Instead, it has been proposed that substrate-level phosphorylation is the major route of ATP synthesis and that the PMF is used primarily for ion transport (414, 415). Moreover, soluble oxidases similar to eucaryotic microbodies may be responsible for 80% of the total respiratory activity. However, evidence in support of the hypothesis is still preliminary, and a more thorough biochemical characterization of respiration in *Thermoplasma* spp. will be necessary to establish this evolutionary relationship.

In addition to oxidative phosphorylation, *Halobacterium* spp. are capable of photophosphorylation and substrate-level phosphorylation at the expense of arginine (158). The arginine fermentation via ornithine and ornithine carbamoyl transferase appears to be homologous to the eubacterial fermentation (88, 89).

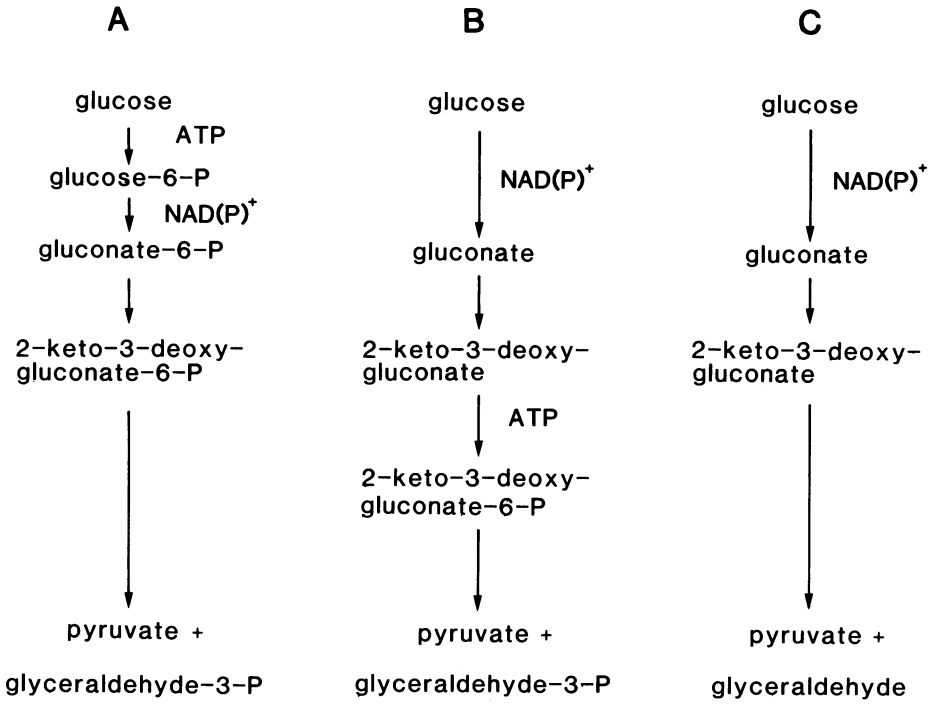


FIG. 1. Modifications of the Entner-Doudoroff pathway in archaebacteria. (A) Pathway common in *Pseudomonas* spp. and other eubacteria; (B) pathway found in *Halobacterium* sp. and *Clostridium aceticum*; (C) pathway found in *Sulfolobus* and *Thermoplasma* spp.

Photophosphorylation has been reviewed recently (283, 450, 451). Although the major pigment-protein complex, bacteriorhodopsin, is strikingly similar to the mammalian eye pigment rhodopsin (for a review, see reference 340), an evolutionary relationship is unproven. Although both photosystems contain the pigment retinal, different isomers are used. In the halobacterial system, retinal and a lysyl residue of bacterio-opsin form a Schiff's base. The absorption of light converts all-*trans*-retinal to 13-*cis*-retinal and causes deprotonation of the Schiff's base. Protons are then translocated through the membrane. In the eye, light converts 11-*cis*-retinal to an all-*trans* configuration. Then conformational changes of the protein opsin expose an enzymatic activity on the membrane surface, which in turn activates a second messenger for the visual response. Thus, the mechanisms of the two photosystems are fundamentally different.

Nevertheless, the secondary structure of the two proteins may be similar. The bacterio-opsin consists of seven α-helices which are stacked in the membrane. Although the amino acid sequence of bacterio-opsin has no significant homology to the sequence of opsin, the predicted secondary structure of opsin also contains seven α-helices in a similar orientation. Thus, it is possible to argue either convergent or divergent evolution (450). Moreover, halobacteria contain other retinal-based photosystems, the chloride pump halorhodopsin and the sensory pigment "slow rhodopsin." Therefore, it is also possible that these pigments might have specific homologies to rhodopsin that are not apparent in bacteriorhodopsin (450).

Carbon Metabolism

Some of the major pathways of glucose catabolism have been investigated in the heterotrophic archaebacteria. *Halobacterium* spp. use a variation of the Entner-Doudoroff pathway shown in Fig. 1 (471). Glucose is oxidized and dehydrated to 2-keto-3-deoxygluconate prior to the kinase and aldolase reactions. A similar pathway has also been demonstrated during the fermentation of gluconate by *Clostridium aceticum* and a few other eubacteria (4). In *Sulfolobus* and *Thermoplasma* spp., glucose is oxidized via an additional modification of the Entner-Doudoroff pathway (46, 80). Glucose is oxidized to pyruvate and glyceraldehyde via 2-keto-3-deoxygluconate without the formation of any phosphorylated intermediates. In *Thermoplasma* sp., glyceraldehyde is further oxidized to pyruvate via glycerate and phosphoenolpyruvate (46). Key enzymes of the Embden-Meyerhof and pentose phosphate pathways are apparently absent. These results are surprising because they suggest that the Entner-Doudoroff pathway may have arisen early in evolution (80). However, a great deal more characterization of the protein catalysts will be necessary to eliminate the possibility of convergent evolution.

Methanogens generally do not take up sugars, and hexose biosynthesis occurs via classical gluconeogenesis (131, 133, 191). Fructose bisphosphate aldolase and phosphatase have been demonstrated as well as the other enzymes of gluconeogenesis. The aldolase is only detectable when assayed in the biosynthetic direction, which is consistent with its physiological function. Moreover, the phosphatase is insensitive to the common effectors fructose bisphosphate and adenosine monophosphate. Instead, regulation of the pathway may occur primarily at the phosphoenolpyruvate synthase reaction (118). These results are also consistent with the ^{13}C-labeling pattern (104). The classical pathway for gluconeogenesis is closely related to the Embden-Meyerhof pathway of glycolysis. Therefore, it is curious that modifications of the Entner-Doudoroff pathway are also found in close relatives like the halobacteria.

Glycogen has been identified as a major reserve material in some methanogens as well as the extreme thermophiles

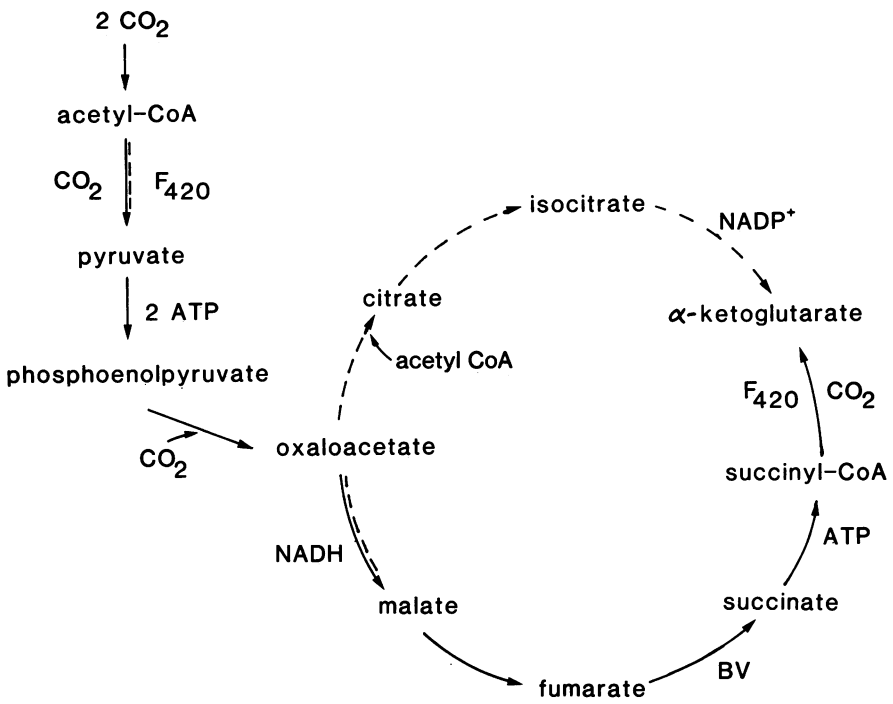


FIG. 2. Pathway of autotrophic CO_2 assimilation and the incomplete TCA cycle in *Methanobacterium thermoautotrophicum* and *Methanosarcina barkeri* (130, 131, 496). Solid lines refer to the reductive incomplete TCA cycle in *Methanobacterium thermoautotrophicum*, and broken lines refer to the reactions demonstrated in *Methanosarcina barkeri*. BV, Benzyl viologen.

(256, 258). Similarly, glycogen is also found in some eubacteria, i.e., the cyanobacteria. From the viewpoint of carbohydrate metabolism, glycogen catabolism in methanogens is of great comparative interest. Thus, it would be of interest to determine if an Entner-Doudoroff-like pathway is also present in the methanogens. Poly(β-hydroxybutyrate), the common reserve material in eubacteria, is also found in certain sugar-utilizing halobacteria (120). Thus, it is not possible to conclude that glycogen is the only likely reserve material in the ancestors for both kingdoms.

Methanobacterium sp. also contains high concentrations of a novel compound, cyclic 2,3-diphospho-D-glycerate (215, 417, 418). In cell extracts, it is formed from phosphoenolpyruvate, but its role in carbohydrate metabolism is uncertain (117). When phosphate and hydrogen are abundant, the levels of cyclic 2,3-diphospho-D-glycerate can reach 5% of the cell dry weight (419, 420). Proposed roles for this compound include phosphate storage or being an intermediate in lipid biosynthesis and gluconeogenesis.

The enzymes of the tricarboxylic acid (TCA) cycle are present in both heterotrophic and autotrophic archaebacteria. The complete catabolic cycle is probably present in *Halobacterium*, *Sulfolobus*, and *Thermoplasma* spp. (2, 415). *Sulfolobus* sp. may also use a reductive (reverse) TCA cycle for autotrophic growth (214). Two anabolic variations of the incomplete TCA cycle are found in methanogens (Fig. 2). *Methanobacterium* sp. uses an incomplete reverse TCA cycle for α-ketoglutarate synthesis via succinyl-coenzyme A (CoA) (130–132). An incomplete reverse TCA cycle is also consistent with ^{13}C-labeling data obtained from *Methanospirillum* and *Methanococcus* spp., which are representatives of the two additional major groups of themethanogens (103, 104). In contrast, *Methanosarcina* sp. has an incomplete oxidative TCA cycle in which α-ketoglutarate is formed from citrate (496). The pathway of succinyl-CoA biosynthesis has not been determined, although malate is formed by the reduction of oxaloacetate. However, methanogens synthesize tetrapyrroles via the C-5 pathway from α-ketoglutarate (see below), so the anabolic requirement for succinyl-CoA may be greatly reduced. In any case, the modifications of the TCA cycle in archaebacteria are not surprising considering the diversity of forms of the cycle found in eubacteria (251).

A number of enzymes from the TCA cycle have been characterized. The citrate synthase from halobacteria, *Sulfolobus*, and *Thermoplasma* resemble the enzymes from eucaryotes and the gram-positive eubacteria (50, 65, 144, 166). Their molecular weights are all between 83,000 and 112,000, which is much lower than those of the enzymes from gram-negative eubacteria. The sensitivity to possible effectors such as ATP, NADH, and α-ketoglutarate are also comparable to the sensitivity of enzymes from other kingdoms.

The isocitrate dehydrogenase from *Sulfolobus* sp. is active with both $NADP^+$ and NAD^+, even though the affinity form $NADP^+$ is greater than that for NAD^+ (67). Eubacteria normally contain an NADP-specific enzyme. In eucaryotes, a NADP-specific enzyme is found in addition to a NAD-specific enzyme associated with the mitochondria. Although an isocitrate dehydrogenase has also been purified from a halobacterium, little information on its catalytic properties was determined (177, 178). Therefore, it is not known if the dual specificity of the *Sulfolobus* enzyme is a general property of the archaebacterial isocitrate dehydrogenase.

The succinate thiokinases of halobacteria, *Sulfolobus*, and *Thermoplasma* are specific for adenine nucleotides, as areenzymes from gram-positive eubacteria (65). The molecular weight of the *Thermoplasma* enzyme is also similar to that of the enzymes from gram-positive eubacteria. How-

ever, the halobacterial enzymes are much larger, as are the enzymes of the gram-negative eubacteria (65, 498).

In halobacteria, most of the succinate dehydrogenase activity is associated with the cell envelope (139). However, the enzyme has been purified from the soluble fraction, which represents 10 to 20% of the total activity. The soluble dehydrogenase has a molecular weight of 90,000, as do the membrane-associated eubacterial and mitochondrial enzymes. However, it is not known whether or not the soluble enzyme is identical to the membrane-associated form in halobacteria.

The malate dehydrogenase from several halobacteria and a methanogen have been characterized (174, 175, 319, 320, 439, 452). The molecular weights and subunit compositions of the archaebacterial enzymes are very similar to the eubacterial and eucaryotic enzymes. The kinetic mechanism and stereospecificity for NAD^+ are also the same (138, 452). However, the methanogen malate dehydrogenase is somewhat unusual in that it is essentially unidirectional for oxaloacetate reduction, which is consistent with its physiological function (452). This result is explained by an unusually low affinity for NAD^+.

In the heterotrophic archaebacteria *Thermoplasma*, *Sulfolobus*, and *Halobacterium* spp., the pyruvate and α-ketoglutarate oxidoreductases are ferredoxin dependent, like the enzymes from some anaerobic eubacteria (232–234). In *Methanobacterium* sp., where ferredoxins have not been found, the equivalent biosynthetic enzymes pyruvate and α-ketoglutarate synthases are dependent on the deazaflavin coenzyme F_{420} (543). Archaebacterial 2-oxoacid oxidoreductases contain thiamine pyrophosphate like the familiar pyruvate dehydrogenase complexes of aerobic eubacteria and mitochondria, but they lack lipoic acid and flavins. Instead, they contain two 4Fe:4S clusters (233). Because the ferredoxin-dependent oxidoreductases are found in both archaebacteria and eubacteria, it has been proposed that it is an ancestral form of the enzyme (235). Moreover, the complex pyruvate dehydrogenase of aerobic eubacteria may have evolved in response to the introduction of oxygen to the atmosphere later in the earth's history (235). However, dihydrolipoamide dehydrogenase, a specific component of pyruvate dehydrogenase, has also been detected in halobacteria (2, 66). The catalytic mechanism and molecular weight of the halobacterial enzyme closely resemble the enzymes from aerobic eubacteria and mitochondria. Thus, without more comparative information on the distribution of these enzymes, speculation on their evolution is premature.

A number of ferredoxins which may be linked to the 2-oxoacid oxidoreductases have been described in archaebacteria. Two types have been found. In halobacteria, 2Fe:2S ferredoxins have been isolated from "*Halobacterium halobium*" and a *Halobacterium* sp. from the Dead Sea (236, 499). Although somewhat larger than the 2Fe:2S "plant" ferredoxins, these ferredoxins have amino acid sequence homology (about 70 amino acid residues are similar or identical) and immunological cross-reactivity to the cyanobacterial ferredoxins from the same family (134, 160, 161). Both halobacterial ferredoxins also contain an unusual *N*-acetyl-lysyl residue. Although they can partially substitute for the plant ferredoxin in photosynthesis, their physiological function is probably in 2-oxoacid oxidation or nitrate reduction (232, 499, 500). In other respects, their midpoint potential and secondary structure more closely resemble the 2Fe:2S ferredoxin from *E. coli* or the eucaryotic adrenodoxin (499).

The second type of archaebacterial ferredoxin includes the 8Fe:8S ferredoxins from *Thermoplasma*, *Sulfolobus*, and *Desulfurococcus* spp. (232). Although less than one-half the size, the 3Fe:3S ferredoxin from *Methanosarcina* sp. has some similar features (162, 163). These ferredoxins have some homologies to other bacterial ferredoxins, especially the *Methanosarcina* ferredoxin and the clostridial 4Fe:4S ferredoxin (163, 476). They also have some unique features. The *Sulfolobus* ferredoxin contains an N^6-monomethyl-lysyl residue (326). The *Thermoplasma* ferredoxin is fairly large and contains 142 amino acid residues (491). Thus, it has been proposed that the diversity of archaebacterial ferredoxins argues for multiple phyletic origins of these proteins (235, 491). Some of the ferredoxins would then have been acquired late in the evolution of the bacterial group (235). While this argument is attractive, it is important to note that the archaebacterial ferredoxins contain only 59 to 142 amino acid residues. Therefore, the phylogenetic information is very limited. In size, they are similar to the 5S rRNAs. Even though the sequences of a great many more archaebacterial and eubacterial 5S rRNAs are known than for the ferredoxins, it has been difficult to establish evolutionary relationships between very distantly related bacteria (126).

Biosynthetic Pathways

The most extensive investigations of biosynthetic pathways have been ^{13}C-labeling experiments (103, 104). From these studies, the labeling of most of the amino acids in methanogens is consistent with familiar pathways. In a few cases, these pathways have been confirmed by enzymatic assays. Thus, most of the enzymes for arginine biosynthesis via the ornithine acetyltransferase pathway (as opposed to the *N*-acetylglutamate synthase pathway) have been demonstrated (88, 89, 317). Some of the enzymes of the diaminopimelate pathway of lysine biosynthesis have also been demonstrated in *Methanobacterium* sp. (11). In contrast, isoleucine is not synthesized from threonine, as is common in eubacteria and eucaryotes. Instead, isoleucine is synthesized from pyruvate and acetyl-CoA via citramalate (95, 105). In addition, isoleucine may also be formed from exogeneous propionate or 2-methylbutyrate (94, 103, 505). Although not common, these alternative pathways have been described in a variety of eubacteria and in *Saccharomyces* sp.

The ^{13}C-labeling patterns of the nucleosides are also consistent with established pathways. In *Methanospirillum* sp., the carbon atoms of the pyrimidines and purines are probably obtained from aspartate and serine plus glycine, respectively (104). However, in *Methanobacterium* sp. C_1 groups may also be contributed from the methanogenic pathway (see below). Ribose is probably formed by the oxidative decarboxylation of hexose (104). Deoxyribonucleotides are probably formed by reduction of the ribonucleosides (438).

Lipids

Several recent reviews have extensively described details of archaebacterial lipid structure and composition (78, 277). The intention of this section is to describe predominant aspects of methanogen and related archaebacterial membrane lipids and to discuss some interesting recent discoveries.

Polar lipids. Polar membrane lipids of archaebacteria, although diverse in structure and composition, are primarily

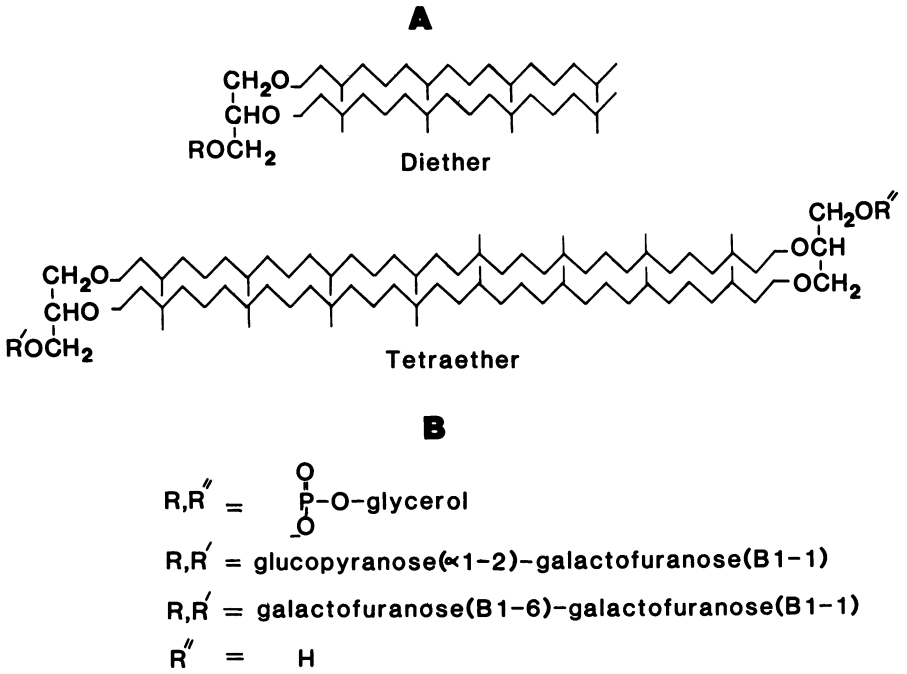


FIG. 3. Characteristic polar lipid constituents of methanogenic archaebacteria. (A) Representative structure of diphytanyl and dibiphytanyl glycerol lipids; R, R′, or R″ represents polar head group, or H. (B) Polar head groups found as constituents of complex diether and tetraether polar lipids of *Methanospirillum hungatei* (270).

isopranyl glycerol ether derivatives (78, 277, 472). The ester-linked fatty acyl glycerol derivatives common in the eubacteria and eucaryotes do not occur in the archaebacteria. In archaebacteria, the alkyl chains linked to the glycerol are either phytane (C_{20} isoprenoid) or biphytane (C_{40} isoprenoid). The structures of the diphytanyl glycerol ethers and the dibiphytanyl diglycerol tetraethers are presented in Fig. 3. The archaebacterial lipids also differ from the eubacterial and eucaryotic lipids by the glycerol moiety having the 2,3-*sn*-stereo configuration rather than the common 1,3-*sn*-configuration (Fig. 4) (219, 277).

Glycolipids and phospholipids, containing carbohydrate and phosphoderivatives analogous to constituents of eubacterial and eucaryotic lipids, are also prevalent forms of archaebacterial polar lipids. The polar lipid composition of one methanogen species, *Methanospirillum hungatei*, has been determined (270); the composition is presented in Fig. 3B. *Methanospirillum hungatei* polar lipids consist of both the diether and tetraether glycerols linked to various combinations of (i) galactofuranose and glucopyranose or (ii) phosphate or both. The predominant polar lipid constituents are diether glycolipids (31%), diglycerol tetraether glycolipids (0.3%), diether phospholipids (10%), and tetraether phosphoglycolipids (58%). It seems predictable that methanogens in the groups *Methanobacteriaceae*, *Methanothermaceae*, and *Methanomicrobiaceae*, which contain significant amounts of the diether and tetraether glycerolipids, will also contain polar lipids analogous to those described for *Methanospirillum hungatei*. Members of *Methanococcales* and *Methanosarcinaceae*, in which only glycerol diethers are predominant constituents of polar lipids, may consist primarily of diether glycolipids and diether phospholipids.

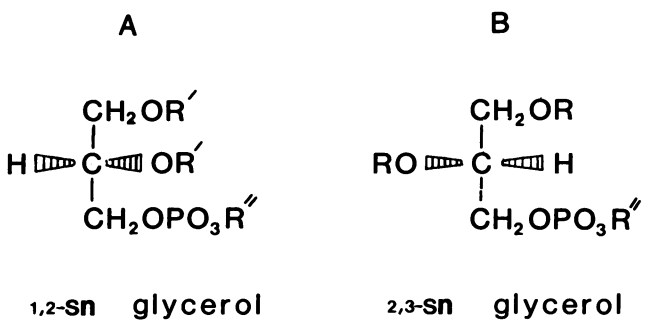


FIG. 4. Representative configurations of (A) eubacterial and eucaryotic acylglycerol polar lipids and (B) archaebacterial alkylglycerol polar lipids. R represents alkyl group, R′ represents acyl group, and R″ represents polar head group. Note difference in stereochemistry of glycerol.

Although the glycerol diether and diglycerol tetraether are predominant constituents of archaebacterial polar lipids, diversity exists within these characteristic structures. Polar lipids of the extreme halophiles closely resemble the C_{20} polar lipids of methanogens, but differ in the nature of the attached polar head groups (217–219, 221). In addition, a C_{25}-hydrocarbon constituent has been reported to occur in haloalkaliphilic archaebacteria (81). Membranes of the extreme thermophiles *Sulfolobus* and *Thermoplasma* spp. consist of glycolipids and phospholipids containing primarily tetraether (C_{40} biphytanyl) chains with one to four cyclopentyl rings (75, 76, 78) within the alkyl chains (Fig. 5A). To date, cyclopentyl rings within the biphytanyl chains have only been detected in one methanogen, *Methanosarcina barkeri* (78). One extremely thermophilic methanogen, *Methanococcus jannaschii*, is distinctly different from the other methanogens and archaebacteria since its major membrane lipid is a glycerol diether referred to as a macrocyclic diether (55). This diether consists of two C_{20} phytanyl chains condensed together and linked to a glycerol (Fig. 5B).

Since the molecular dimensions of membranes composed

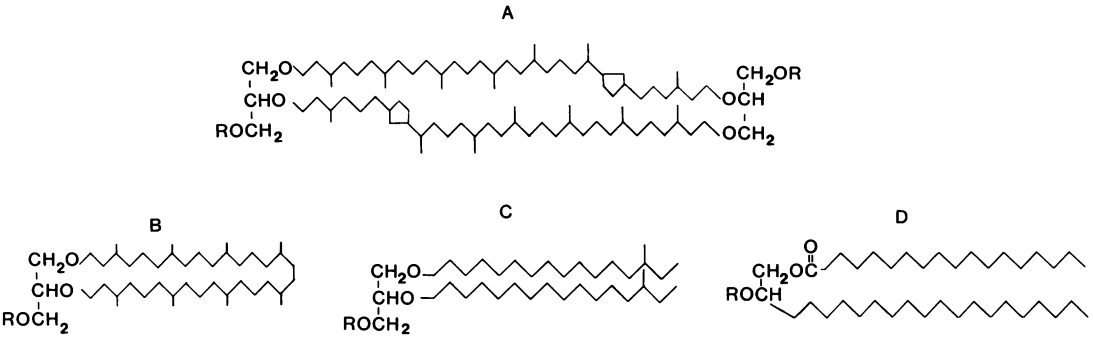


FIG. 5. Atypical polar lipids of archaebacteria and eubacteria. (A) Representative structure of a diglycerol tetraether polar lipid containing cyclopentyl rings. (B) Structure of the novel macrocyclic glycerol diether from *Methanococcus jannaschii*. (C) Glycerol diether from *Thermodesulfotobacterium commune*; *sn*-1,2-glycerol diether containing anteiso- C_{17} hydrocarbon. (D) Postulated structure of the major long-chain 1,2-diol of *Thermomicrobium roseum*, containing the predominant (C_{18}) fatty acyl side chain. (R represents polar head group.)

of tetraether lipids are equivalent to the dimensions of traditional membrane lipid bilayers, it has been postulated that archaebacterial membranes consist of a "monolayer" of tetraethers and bilayers of diethers (276, 277, 281, 352). For many archaebacteria, tetraethers comprise a significant part of their membrane lipids. It has been predicted that the diglycerol tetraether membrane confers greater thermostability due to its "rigid" monolayer structure (277); further, internal cyclization of the hydrocarbon chains may also increase membrane thermostability. In addition, the unusual macrocyclic diether membrane component of the extremely thermophilic *Methanococcus jannaschii* likely confers greater membrane thermostability than the corresponding glycerol diether. Thus, it appears that extremely thermophilic archaebacteria have evolved different mechanisms of adaptation to life at high temperature.

The isopranyl glycerolipids of archaebacteria are unique, and the lack of fatty acid ester-linked glycerolipids is a criterion distinguishing archaebacteria from eubacteria (519). However, some eubacteria contain similar lipids. Major membrane components of the thermophilic eubacterium *Thermodesulfotobacterium commune* are glycerolipids containing ether-linked, iso and anteiso branched alkyl chains (278) (Fig. 5C). No isoprenoid glycerol ethers are found. These atypical glycerol alkylethers are not found elsewhere in procaryotes, and *T. commune* is not an archaebacterium. *T. commune* glycerol diethers likely function in a fashion analogous to membrane constituents of archaebacteria which contain diphytanyl ether glycerolipids. However, the diethers of *T. commune* are stereochemically identical to ester-linked diacylglycerols of eubacteria. This result suggests that the pathway of lipid biosynthesis in *T. commune* evolved from the typical eubacterial glycerol ester biosynthetic pathway rather than the archaebacterial pathway of glycerol ether biosynthesis.

A new class of membrane lipids have been recently discovered in *Thermomicrobium roseum*, a thermophilic heterotroph which also lacks a typical peptidoglycan cell wall like the archaebacteria (365). *Thermomicrobium roseum* membrane lipids are devoid of glycerol-derived lipids and have long-chain diols to which a fatty acid is ester linked (Fig. 5D). The major fatty acids are 12-methyl C_{18} and *n*-C_{18}; no isoprenoid branched glycerol ethers are detected. Although analogous to ester-linked glycerolipids of eubacteria, this class of diol-derived membrane constituents is unique, and they are likely to represent a divergence from typical eubacterial lipid biosynthesis. *Thermomicrobium roseum* and *Thermodesulfotobacterium commune* both are thermophiles and have temperature optima near 75°C (186, 542); perhaps these organisms diverged early in eubacterial evolution and synthesized thermostable membrane constituents in response to their environment. Because these newly described lipid constituents appear to be restricted to only a few procaryotic species, they may be adaptive traits rather than representative constituents of early eubacterial lipids.

The presence of isopranyl ether-linked lipids as major membrane constituents of archaebacteria may indeed be a reflection of their origin and prevalence in extreme habitats (high temperature, low pH, high salt). Greater chemical stability is afforded by the ether linkage of the alkyl chains to a glycerol molecule. Mesophilic species of archaebacteria (including species of methanogens and extreme halophiles) are indeed numerous; thus, it cannot be ruled out that some other unknown factor may have directed the evolution of biosynthesis of this unique class of membrane constituents in archaebacteria. However, the mesophilic archaebacterial species could be descendants of their thermophilic relatives (K. O. Stetter, personal communication). Within the methanogens, for example, every taxonomic family is represented by at least one thermophilic species. Although no thermophilic species of extreme halophiles are known to date, these organisms are considered to be close relatives of methanogens (127, 536).

Nonpolar lipids. Neutral lipids in archaebacteria may comprise as much as 30% of the total lipids (472, 473) and thus are a significant component of their membranes. Isoprenoid and hydroisoprenoid hydrocarbons that vary in length from C_{15} to C_{30} are major constituents of neutral lipids in all archaebacteria examined to date (277). Hydrogenated analogs of C_{20} and C_{30} isoprenoid hydrocarbons constitute the predominant neutral lipid components in most methanogens. In *Methanosarcina barkeri*, saturated analogs of C_{25} isoprenoids predominate (78, 176, 473).

Among the extreme thermophiles and halophiles examined, squalene and a spectrum of hydrogenated squalene analogs were found (219, 277). *Thermoplasma* sp., on the other hand, contained only squalene (176). Neutral lipids of extreme halophiles, however, are characteristically different from other archaebacterial species. Isoprenoids less than C_{30} in length are not found in the halophiles; in addition to the predominant C_{30} derivatives, halophiles possess C_{40} and C_{50} carotenoids (219, 268, 269). Bacterioruberins (hydroxylated C_{50} carotenoids) are the prevalent carotenoid components in these organisms and bestow on halophiles their distinctive

red color. Other minor neutral lipid components are found in various species of archaebacteria and will not be discussed in detail. However, it is worth noting that various quinones have been detected as nonpolar lipid constituents in species of halophiles, *Thermoplasma*, and *Sulfolobus*; several of these quinones have not been found elsewhere (54, 77). One interesting example is a C_{30} sulfur-containing quinone from *Sulfolobus* sp. named caldariellaquinone (77). Additional unusual nonpolar lipid constituents of *Sulfolobus* and *Thermoplasma* spp. include alkylbenzenes of variable structure (281). These compounds are not common but have been detected in sediments, crude oils, and other procaryotes, including species of methanogens, thermophilic clostridia, and *Thermomicrobium roseum* (G. Holzer, personal communication).

Lipid biosynthesis. Although great diversity exists among archaebacterial lipids, the central theme is the presence of isoprenoid hydrocarbons as major constituents of polar and neutral lipids. Although at different concentrations, the ubiquitous presence of isoprenoid hydrocarbons in biological systems suggests that a common mode of biosynthesis may occur in all organisms. Evidence supports this concept. The mevalonate pathway is well established for isoprenoid biosynthesis (for subsequent production of sterols, carotenoids, etc.) in eucaryotes and eubacteria. Evidence for the mevalonate pathway in archaebacteria has been demonstrated by the direct incorporation of [^{14}C]-mevalonate into lipid constituents of *Halobacterium*, *Sulfolobus*, and *Thermoplasma* spp. (74, 220, 280). The ^{13}C-labeling pattern of the phytanyl side chains of the lipids is also consistent with established pathways. The phytanyl side chain is probably formed by decarboxylation of mevalonate in methanogens as well as other archaebacteria (79, 104). These results indicate a common mechanism for the formation of the basic hydrocarbon precursors in all organisms.

The variety of isoprenoids found in archaebacteria no doubt represents the diversity of this bacterial group; the basic C_5 (isopentenyl) skeleton generated by the mevalonate pathway may be condensed to form C_{10} (geranyl), C_{15} (farnesyl), and C_{20} (geranylgeranyl) constituents. These hydrocarbons may give rise to diverse hydrocarbon structures by tail-to-tail or head-to-tail condensation of combinations of the precursors (78, 219, 277). Squalene (C_{30}) biosynthesis seems to occur by a tail-to-tail condensation of farnesyl groups (277). C_{25} isoprenoids, found abundantly in some methanogens and other archaebacteria (78), arise from the head-to-tail condensation of a C_{20} and a C_5 moiety or via the tail-to-tail condensation of a C_{15} and a C_{10} moiety (277). The formation of the C_{40} biphytanyl hydrocarbon occurs by head-to-head condensation and subsequent reduction of two C_{20} phytanes, which is opposite of the tail-to-tail linkage in the formation of the carotenoid precursor in nonarchaebacterial species (75, 76). Addition of pentacyclic rings to tetraethers of the extreme thermophiles may occur via reductive steps of the biphytanyl chains, the mechanism of which is unknown. Most, if not all, of these mechanisms are probably specific biosynthetic traits of the diverse archaebacteria; however, the pathway for the formation of the basic hydrocarbon skeletons appears to be common in eucaryotes and procaryotes.

Fatty acids, which are the predominant constituents of eubacterial glycerolipids, are also found in archaebacteria, although at relatively low levels (219, 277, 472). For example, 1 to 10% of the polar lipid fraction of some methanogens consist of C_{15} to C_{18} free fatty acids (472). Synthesis of fatty acids appears to occur by the traditional malonyl-CoA pathway for eubacteria, as indicated by the incorporation of [^{14}C]acetate into the fatty acids of *Halobacterium* sp. (220). Although a limited number of studies have been performed, the mode of biosynthesis of both isoprenoid hydrocarbons and fatty acids in archaebacteria appears to be similar to the pathways in eubacteria and eucaryotes. The major difference in archaebacteria seems to be in regulation of the biosynthetic pathways toward increased isoprenoid hydrocarbon production and decreased fatty acid synthesis.

Cell Envelopes

Archaebacteria, and especially methanogens, exhibit great diversity in cell envelopes, ranging from simple, nonrigid surface layers consisting of protein or glycoprotein subunits in certain methanogens, extreme halophiles, and extreme thermophiles to a rigid "pseudomurein" sacculus, analogous to eubacterial murein, in the methanogen order *Methanobacteriales* (212, 213, 253–255). Neither muramic acid nor D-amino acids, which are typical components of eubacterial cell walls, have been detected in any archaebacterium to date. This distinguishing feature lends further support to the distinction of eubacteria and archaebacteria.

Cell envelopes of archaebacteria may be categorized into three characteristic classes according to their major cell envelope constituent: (i) pseudomurein; (ii) protein or glycoprotein layers; and (iii) heteropolysaccharides. Moreover, some archaebacteria contain complex envelopes composed of more than one of each of these constituents.

Pseudomurein. Members of the gram-positive *Methanobacteriales* are the only archaebacterial species that possess a pseudomurein-type cell wall analogous to eubacterial murein (211, 213). Pseudomurein differs from eubacterial murein in that (i) L-talosaminuronic acid is substituted for muramic acid; (ii) different sequences of amino acids (L configuration of alanine, glutamic acid, and lysine, mainly) are constituents of peptides involved in the glycan polymer crosslinking; and (iii) the chemical bonds between the sugar moieties of alternating *N*-acetylglucosamine and *N*-acetyltalosaminuronic acid are probably β(1-3) linkages instead of β(1-4) glycosidic linkages which occur in eubacterial murein (213, 254). The pseudomurein composition is variable among the species of *Methanobacteriales* mainly with respect to amino acid components of the attached peptides and, in some cases, by replacement of glucosamine with galactosamine (255). Antibiotics such as vancomycin and penicillin, which affect eubacterial cell wall biosynthesis by interfering with reactions involving D-alanine, do not effect biosynthesis of methanogen pseudomurein (156, 167).

Eubacterial murein and methanogen pseudomurein appear to be analogous based on function, chemical composition, and primary structure. To determine if this apparent homology extended to the secondary structure, Leps et al. (294) made predictions of possible secondary structures of pseudomurein and murein heteropolymer based on conformational energy calculations. Pseudomurein, consisting completely of repeating β(1-3)-linked *N*-acetylglucosamine-*N*-acetyltalosaminuronic acid, exhibits striking similarities to the eubacterial murein three-dimensional structure (Fig. 6). Notable similarities include an extended chain conformation and the orientation and flexibility of peptide attachment sites. In contrast, the predictions of Leps et al. (294) of pseudomurein three-dimensional structure with alternating β(1-3) and α(1-3) linkages, as proposed by Konig et al. (254), indicate important differences when compared with murein.

These include low conformational flexibility of the peptide-binding region and an unusual zig-zag pseudomurein chain shape. Leps et al. (294) concluded that the repeating β(1-3) structure is favored due to the similarities in the established secondary structure of murein. If they are correct, it is most interesting that members of phylogenetically distinct groups, eubacteria and archaebacteria, evolved cell wall polymers of such striking similarity.

Protein cell envelopes. The second, but most predominant, type of cell wall found in archaebacteria consists of a layer of protein or glycoprotein subunits external to the cell membrane. This cell wall type occurs in many species of methanogens and extreme halophiles, and it is found in all members (described to date) of the extreme thermophiles (213). In many species, the surface layer (S-layer) of protein or glycoprotein is easily disrupted (549, 553). Most species of *Methanococcaceae* and *Methanomicrobiaceae* are lysed by detergents or solutions of low osmolality. Members of the extreme thermophiles, however, possess detergent resistant S-layers composed of glycoprotein. In addition, the S-layers from the extreme thermophile *Thermoproteus tenax* are resistant to many proteases (213). In this case, some type of strong chemical or physical interaction between subunits must occur to confer such stability. Three-dimensional models of the *Sulfolobus* S-layer glycoprotein illustrate the presence of a highly porous matrix with large channels, but these channels are too large to function as a molecular sieve for low-molecular-weight compounds (213, 461).

The carbohydrate components of the glycoproteins in archaebacterial S-layers vary greatly. *Methanolobus tindarius* contains only glucose as a constituent of its surface glycoprotein (213, 259), while the glycoprotein from *Thermoproteus tenax* contains glucose, arabinose, mannose, and rhamnose as predominant constituents. "*Halobacterium halobium*" S-layers are composed of a sulfated glycopeptide similar in structure to animal glycosaminoglycans (511, 512).

One member of the *Methanobacteriales*, the extremely thermophilic *Methanothermus fervidus*, has a pseudomurein cell envelope covered by a layer of protein subunits (446). Perhaps the S-layer provides greater thermostability for *Methanothermus fervidus* and is an adaptive trait in response to environmental factors.

S-layers are not unique to archaebacteria; in fact, protein-containing S-layers are found in diverse species of eubacteria (426), and little difference in the chemical compositions of S-layers of eubacteria and archaebacteria have been detected. In general, S-layers from both groups are composed of proteins which are rich in acidic amino acids and have a low percentage of sulfur-containing amino acids (426). S-layers of one archaebacterium, *Sulfolobus acidocaldarius*, however, deviate from this trend in that amino acids containing hydroxyl groups predominate over acidic amino acids (426).

Heteropolysaccharide and complex cell envelopes. A third distinguishing cell wall type found in archaebacteria is thus far restricted to *Halococcus* and *Methanosarcina* spp. The thick, rigid cell wall structure of *Halococcus* sp. consists of a sulfated heteropolysaccharide containing *N*-glycyl-D-glucosamine and glycyl peptide bridges (396). An analogous structure is found in methanogens belonging to the genus *Methanosarcina*, which usually grow in spherical packets and exhibit an unusually thick, rigid outer envelope. The structural wall in *Methanosarcina* is a polymer of D-glucuronic acid and *N*-acetylgalactosamine, which is similar to animal chondroitin (213). The constituents of this polymer in *Methanosarcina* sp. are not known to be sulfated as in the case of *Halococcus* sp.

Methanospirillum hungatei and *Methanothrix soehngenii* are characterized by complex cell envelopes containing a thin, fibrillar outer sheath surrounding an electron-dense inner wall (540, 541). Isolated sheath material consists of protein and possibly glycoprotein, as indicated by the presence of amino acids and neutral sugars as sheath hydrolysis products (212, 213). The inner wall of *Methanothrix* sp. is involved in septum formation during cell division as indicated by electron microscopy (540); this phenomenon has not been observed in *Methanospirillum* sp. Little else is known about this unusual cell wall structure.

Comparison with eubacterial cell envelopes. From the preceding discussion, it is apparent that great diversity exists in cell envelopes of archaebacteria; indeed, the methanogens alone possess all cell wall types. The lack of muramic acid (and thus typical murein structure) in cell walls is not restricted only to archaebacteria. For example, cell walls of *Thermomicrobium roseum*, an anerobic thermophilic eubacterium, are composed primarily of protein with the amino acids proline, glutamic acid, glycine, and alanine predominating (318). Only very low levels of galactosamine and muramic acid were detected, and glucosamine was absent. This gram-negative bacterium thus possesses an atypical cell wall structure composed mainly of a protein polymer. It is interesting to note that this thermophilic organism also contains atypical membrane lipids (see above). *Planctomyces* and *Pasteuria* are two genera of budding, nonprosthecate bacteria which also were recently shown to be void of peptidoglycan (257). Although these organisms are not closely related phylogenetically to archaebacteria (based on 16S rRNA homology), it was postulated that they may be descendants of a eubacterial group that diverged early in eubacterial evolution (442). These limited examples demonstrate that great diversity in cell envelope type exists among the procaryotes. Moreover, the types of envelopes common in the archaebacteria occur at a low frequency in the eubacteria.

Other Topics

Nitrogen metabolism. In addition to ammonia and a few organic nitrogen sources, methanogens can also use molecular nitrogen (21, 23, 37, 335, 504). Although not investigated in detail, the nitrogenase in methanogens must have some homology with the eubacterial enzyme because DNA from methanogens hybridizes with the structural genes for the eubacterial nitrogenase (423). In *Methanobacterium* sp., at low ammonia concentrations, the glutamine synthetase/glutamate synthase system is the primary pathway of ammonia assimilation. The glutamine synthetase has been purified, and its quarternary structure and absence of adenylation resemble the enzymes from gram-positive eubacteria (24). At higher ammonia concentrations, alanine dehydrogenase is probably also important in nitrogen assimilation (228). In *Methanosarcina* sp., only the glutamine synthetase/glutamate synthase system has been documented (228). The glutamate synthase was dependent on flavins or coenzyme F_{420}. A NAD(P)-dependent glutamate dehydrogenase could not be demonstrated. In contrast, both glutamate dehydrogenase and alanine dehydrogenase have been documented in halobacteria. The quaternary structure of the halobacterial glutamate dehydrogenase suggests a specific relationship to the enzyme from plants (288). The alanine dehydrogenase is monomeric, unlike the complex enzyme

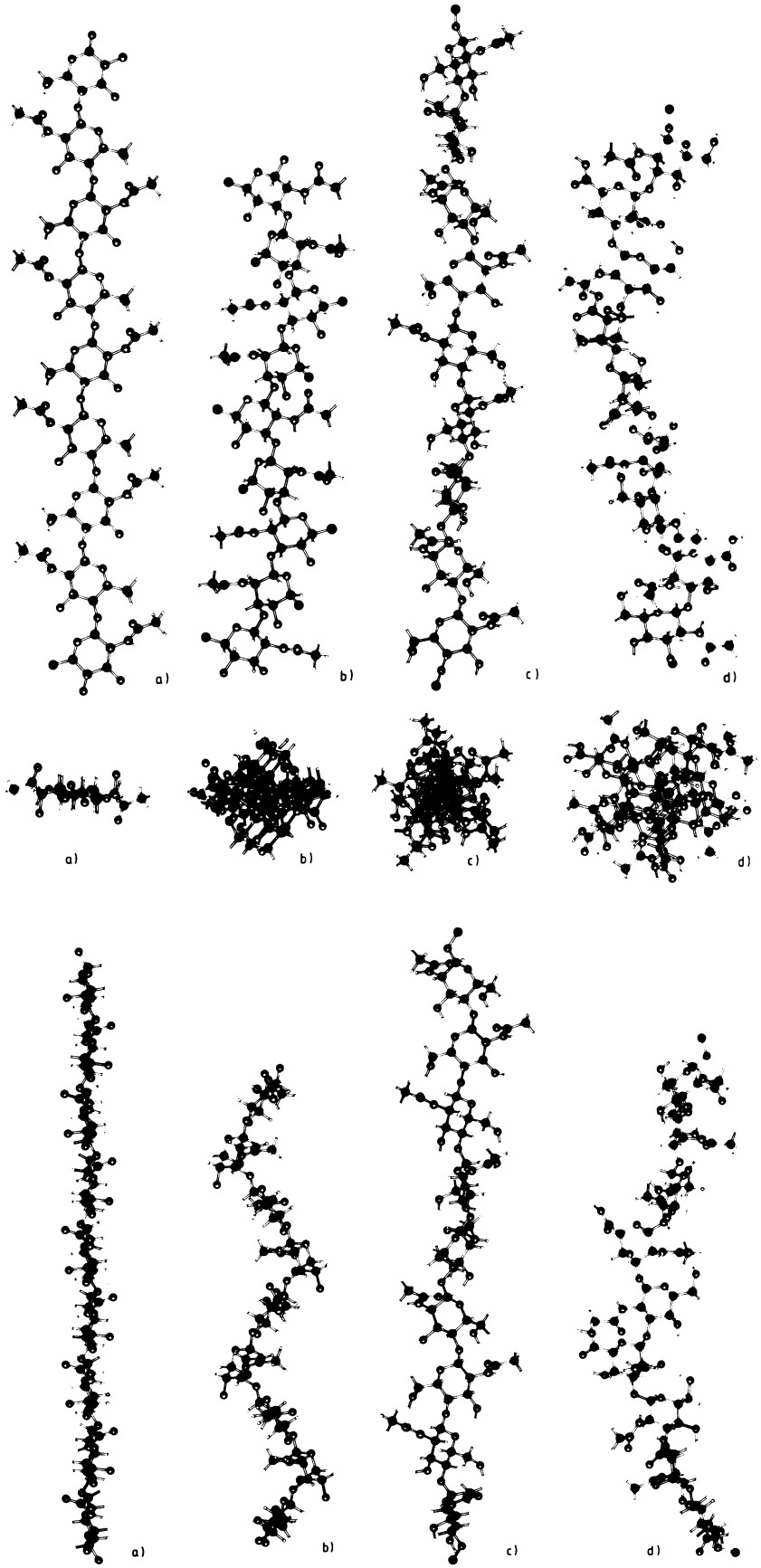


FIG. 6. Favored conformations for the sugar strands comprising various types of cell walls. (a) Crystalline chitin; (b) pseudomurein, alternating β(1,3) and α(1,3) linkage; (c) eubacterial murein; and (d) pseudomurein, all β(1,3) linkage. Upper panel represents front view, middle panel represents view along the axis of the sugar chain, and bottom panel represents side view. Reproduced from Eur. J. Biochem. with permission of Leps et al. (294).

from *Bacillus* sp. (230). Otherwise, its kinetic properties resemble the activity identified in extracts of *Methanobacterium* sp.

Superoxide dismutase. Superoxide dismutase has been characterized in *Methanobacterium* and *Thermoplasma* spp. (242, 332, 416). Both enzymes are tetramers and contain Fe and Zn. Otherwise, they resemble the dimeric, Fe-containing eubacterial enzymes in terms of subunit molecular weight, amino acid composition and catalytic features. Even though the eucaryotic enzyme also contains Zn (as well as Cu), this feature does not suggest a specific evolutionary relationship (242).

Protein phosphorylation. A common regulatory mechanism in eucaryotes is protein phosphorylation. It is also found in eubacteria, where its importance is less certain. Protein phosphorylation has been observed in *Sulfolobus* sp. (425). Phosphoserine and phosphotyrosine are the major modified amino acids, and only a few proteins are phosphorylated. Unlike eucaryotes, the ribosomal proteins and RNA polymerase are not phosphorylated. In vitro, phosphorylation is not effected by cyclic adenosine monophosphate, and typical substrates for the eucaryotic protein kinases such as casein and histones are not modified. In these respects, the *Sulfolobus* protein kinase(s) resembles the eubacterial systems.

7S RNA. Archaebacteria contain a small stable 7S RNA which is not associated with the ribosome (299). The sequence of the RNA from *Halobacterium* sp. has some homology to the 7S-L RNA from mammalian cells (330, 331). Because the 7S-L RNA is a component of the mammalian signal recognition particle, the halobacterial RNA may also function in protein export. Moreover, the halobacterial RNA has no sequence homology with *Escherichia* 6S nonribosomal RNA, which is not part of a procaryotic signal recognition particle.

Gas vesicles. Gas vesicles are composed of a small hydrophobic protein in *Halobacterium*, *Methanosarcina*, and cyanobacteria (8, 547). These intracellular structures provide buoyancy to aquatic procaryotes. The N-terminal sequences of the archaebacterial and eubacterial gas vesicle proteins are homologous (492). Whether this homology is an example of convergent evolution or conservation of an ancestral feature is not known.

Summary

In conclusion, the comparative biochemistry of archaebacteria is an astonishing, thorough confirmation of the principle of the unity of biochemistry developed by Kluyver and van Niel (249). As stated by van Niel, "These points support, in an almost compulsory manner, the concept of the relatedness of all forms of life and the monophyletic origin of the multitude of living organisms known to us today" (249, p. 156). Thus, although there are some unusual features, archaebacterial metabolism and physiology are remarkably similar to eubacterial processes. Familiar pathways of carbohydrate catabolism, nitrogen assimilation, and biosynthesis are found. Moreover, the structural and catalytic properties of many of the enzymes closely resemble either eubacterial or eucaryotic enzymes.

Are the methanogens, then, just a "variation of a theme"? Obviously, the answer is yes. The methanogens are unique to the archaebacteria in the same way that photosynthetic bacteria are unique to the eubacteria. Both groups have remarkable adaptations to a particular mode of energy metabolism. Nevertheless, both groups have retained many fundamental properties common to other members of their respective kingdoms. The important question which then remains is the nature of the methanogenic variation. This question will be discussed in detail in the next section.

Prior to the recognition that the archaebacteria were distantly related to other bacteria, comparative biochemistry focused on the differences between eubacteria and eucaryotes. Such comparisons were cumbersome because of the enormous differences in cell structure and tissue differentiation between the two groups. The discovery of two procaryotic kingdoms and a detailed proposal for their phylogeny may make comparative biochemistry a more profitable enterprise (127). Thus, it may be possible to trace the evolution of metabolic pathways in procaryotes to an extent never before thought possible. Some good examples are the recent investigations on the regulation of aromatic amino acid biosynthesis and DNA adenine methylation (16, 48).

Moreover, it may be possible to determine the mechanisms of procaryotic evolution. For example, it has been proposed that genetic information may have been transmitted "horizontally" between major procaryotic groups (235). Thus, new metabolic capabilities may have been acquired late in the evolution of a procaryotic group, much like the acquisition of the mitochondrion and the chloroplast by eucaryotes (141). Although it is an attractive hypothesis, there is only one case for which the acquisition of genetic information by procaryotes in nature has been well documented. Although not proven, it is likely that *Photobacterium* sp. acquired a eucaryotic-type copper/zinc superoxide dismutase late in its evolution (15).

However, the alternative hypothesis, that most or all genetic information is transmitted from prior generations, or "vertically," is not certain either. Moreover, it also raises interesting difficulties. For instance, both glycogen and poly(β-hydroxybutyrate) are reserve materials in archaebacteria and eubacteria. Was the ability to synthesize and then metabolize these compounds acquired independently or were they properties of a common ancestor? A more striking example may be the apparent homology between pseudomurein in *Methanobacterium* sp. and murein in eubacteria. On one hand, it seems unlikely that the ability to synthesize these complex polymers could have evolved more than once. On the other hand, it seems unlikely that, if a common ancestor possessed a murein or pseudomurein wall, it would have been lost by most of the archaebacteria. These difficulties do not discredit the hypothesis. Rather they illustrate the need for more detailed information on procaryotic metabolism and evolution. Many of these apparent difficulties may disappear upon further characterization of the biosynthetic pathways for glycogen, poly(β-hydroxybutyrate), and pseudomurein in archaebacteria, which may be very different from eubacterial pathways. In addition, if the rate of the acquisition of new metabolic capabilities by procaryotes was known, speculation on convergence in bacterial evolution may be more profitable.

These contrasting points of view have important implications. For instance, the 16S rRNA of *Halobacterium* sp. is more closely related to the RNA of the methanogens in the order *Methanomicrobiales* than the RNAs of other aerobic archaebacteria (536). Moreover, sequences of the 5S rRNA suggest that the relationship is even more specific and that the halophiles are closely related to the family *Methanosarcinaceae* to the exclusion of the family *Methanomicrobiaceae* (126). If respiration in *Halobacterium* sp. is homologous to respiration in other archaebacteria, as it appears to be, and if respiration was acquired vertically, then the

ancestral methanogen must have possessed some respiratory ability. An important implication of this hypothesis is that the ecology of the ancestral methanogen would have been very different from modern organisms. Moreover, some of this metabolic capability may remain in modern methanogens. The presence of cytochromes, ferredoxin, and gas vesicles in both *Halobacterium* and *Methanosarcina* spp. could be vestiges of this versatile ancestor. This illustration further demonstrates how knowledge of procaryotic evolution might enhance our understanding of modern microorganisms.

THE PROCESS OF METHANOGENESIS

Microorganisms producing methane as the end product of metabolism benefit by the production of cellular energy during the catabolism of extremely simple substrates at low reduction potential. Because of the overall similarities between archaebacterial and eubacterial physiology, the pathway of methane synthesis in archaebacteria might be expected to resemble familiar C-1 biochemistry. In fact, methane synthesis per se is not unique, and some anaerobic eubacteria evolve small amounts of methane during their normal metabolism (366). However, only the methanogenic archaebacteria are known to couple methane synthesis to energy generation. Therefore, the pathway in methanogenic archaebacteria must contain some novel features. An exploration of these novel features is the goal of the remaining discussion. Moreover, it is expected that the ability to obtain energy for growth might be a result of a few very specific adaptations. Other unique features of methanogens may represent secondary modifications of common biochemical pathways in response to this novel metabolic capability.

Early work on these bacteria suggested that the methanogens were highly diverse in metabolic potential, as well as in other aspects of their biology. The unity within the group was pointed out by H. A. Barker, who noted the numerous resemblances among them (19). In particular, he hypothesized that a central pathway from carbon dioxide to methane with the one-carbon unit bound to a carrier(s) would explain many of the observations in hand. When it was shown that the substrates for methanogenesis were limited to carbon dioxide plus hydrogen, formate, methanol, methylamines, acetate, and carbon monoxide, it became clear that the central metabolic pathway in methanogens involved the stepwise reduction of a one-carbon unit which was derived from the growth substrate.

The process of methanogenesis has proven to be rich in biochemical information. The first part of this section will discuss the coenzymes of methanogens. Six of the first seven are unique to methanogens. The rest are present in other organisms. The structures of these compounds and the roles they play in functions other than methanogenesis are discussed, followed by what is known about their biosynthesis. Second, the pathways to methane, from carbon dioxide, formate, methanol and methylamines, and acetate are outlined. The major thrust of this examination is to point out similarities and differences among the methanogens, other archaebacteria, eubacteria, and eucaryotes.

Cofactors of Methanogens

Coenzyme F_{420}. Cells of methanogens can be recognized by their strong autofluorescence under oxidizing conditions. The major contributors to this phenomenon are coenzyme F_{420} and the methanopterin derivatives. Coenzyme or factor

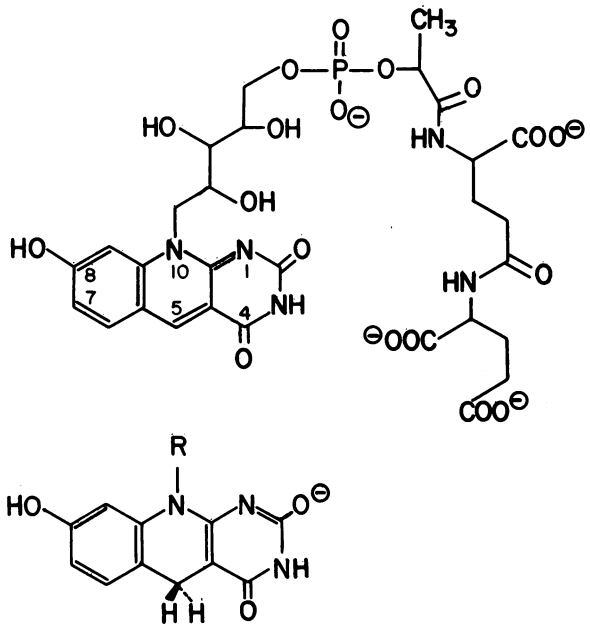


FIG. 7. Structure of coenzyme F_{420}. The upper structure is coenzyme F_{420}. Derivative F^+ consists of the chromophore, ribitol side chain, and the phosphate (deazaflavin mononucleotide analog), and derivative FO consists of the chromophore and ribitol side chain only (deazariboflavin analog) (98). The lower structure is the reduced chromophore.

F_{420} was found in all methanogen cells examined at levels ranging from 1.2 (*Methanobrevibacter ruminantium*) to 65 (*Methanobacterium thermoautotrophicum*) mg of coenzyme per kg of cell dry weight (98–100). The compound has an absorption maximum at 420 nm, hence its name. The absorbance is lost upon reduction. Chemical degradation studies, combined with nuclear magnetic resonance, infrared, and ultraviolet spectroscopy, yielded the structure of coenzyme F_{420} shown in Fig. 7 (98–100). *Methanosarcina barkeri* contains a form of factor F_{420} which contains additional glutamyl residues linked to the distal glutamate (99). The 5-deazariboflavin chromophore, called FO, is a derivative of 7,8-didemethyl-8-hydroxy-deazariboflavin. FO was the first example of a deazaflazin in naturally occurring systems, although pharmaceutical chemists had synthesized it some years previously. Confirmation of its structure was provided by chemical synthesis (10).

The midpoint redox potential, E_0', of −340 to −350 mV is consistent with the role of coenzyme F_{420} as a low-potential electron carrier (188). Because it functions solely in two-electron transfer reactions, coenzyme F_{420} is similar to the nicotinamide cofactors. It transfers the hydrogen from C-5 to the oxidant without exchange with solvent protons (124, 535). Acting as the first shuttle for electrons from hydrogen, coenzyme F_{420} replaces ferredoxin in methanogens. In fact, many methanogens lack ferredoxin, and the acetoclastic *Methanosarcinaceae*, which do make ferredoxin, contain little coenzyme F_{420} (99, 162, 163).

A number of enzyme activities in methanogens are coupled to the oxidation or reduction of coenzyme F_{420}. Hydrogenase (187, 480, 533), formate dehydrogenase (205, 392, 479), carbon monoxide dehydrogenase (62), $NADP^+$ reductase (479, 480, 535), pyruvate synthase, and α-ketoglutarate synthase (131, 543) are some of the activities

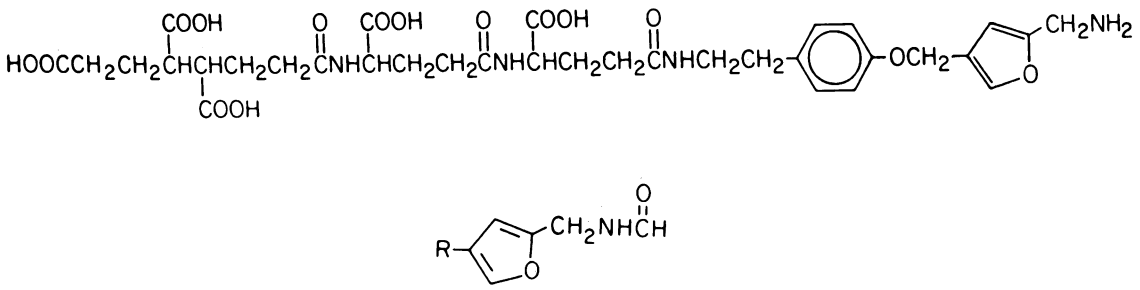


FIG. 8. Structure of MFR (carbon dioxide reduction factor). The structures of MFR and formyl-MFR from *Methanobacterium thermoautotrophicum* proposed by Leigh et al. (290). Reproduced from *The Bacteria*, vol. 8 (504) with permission of Academic Press, Inc.

which have been shown to catalyze coenzyme oxidation or reduction. Recently, coenzyme F_{420} was shown to participate directly in the reduction of CO_2 to CH_4 by the demonstration of 5-deazaflavin-dependent methylenetetrahydromethanopterin dehydrogenase activity in *Methanobacterium thermoautotrophicum* (159).

Some structure-function relationships have been studied with coenzyme F_{420}. The hydrolytic product FO is a substrate for hydrogenase (187, 533). Similarly, Yamazaki et al. (534) showed that the 7-demethyl-8-hydroxy structure is important for activity of the $NADP^+$ reductase, and this enzyme does not require the N-10 side chain of the natural cofactor. This finding is significant in light of the description of new coenzyme F_{420}-derived chromophores (factors F_{390}) in *Methanobacterium* cells stressed with oxygen (165). Factors F_{390} are coenzyme F_{420} adducts having adenosine 5'-phosphate or guanosine 5'-phosphate in phosphodiester linkage to the 8-hydroxy group. The functional significance of these adducts is unknown, but it could entail regulation of metabolism at some level, such as control of enzyme activity (e.g., the $NADP^+$ reductase would not be active with factors F_{390}), or, since the compounds accumulate in response to oxygen, as an alarmone (165).

Because of their abundance, the deazaflavins are clearly a critical factor in the physiology of methanogens. The discovery of naturally occurring 5-deazaflavins in other organisms suggests that the chemistry of the compound has been useful in other types of metabolism. For example, *Streptomyces griseus* photoreactivating enzyme, which is involved in DNA repair, possesses an 8-hydroxy-5-deazaflavin chromophore (101, 102). It is possible that the participation of 5-deazaflavin in photoreactivation processes is a widespread phenomenon since the action spectra for DNA photorepair in a number of organisms, ranging from cyanobacteria to green algae, mosses, and a midge (*Smittia* sp.), are consistent with the presence of a 5-deazaflavin chromophore (100, 101). Recently, the action spectrum for photorepair of ultraviolet-treated *Methanobacterium thermoautotrophicum* was also shown to be consistent with the involvement of a 5-deazaflavin (239).

The redox chemistry of the deazaflavins is also utilized in other metabolic pathways. In the eubacterium *Streptomyces aureofaciens*, synthesis of the antibiotic chlortetracycline requires a two-electron reduction of dehydrochlortetracycline to chlortetracycline. The cofactor utilized in this step, called cosynthetic factor 1, is identical to fragment FO of coenzyme F_{420} (313). A search among the actinomycete group of eubacteria showed that some genera (*Streptomyces*, *Mycobacterium*, and *Nocardia*) contain 5-deazaflavins at levels <1% of those found in *Methanobacterium* spp. (61). Because 5-deazaflavins are found in the archaebacteria and the eubacteria (and probably the eucaryotes), it is likely that 5-deazaflavin chemistry is an ancient biological character.

The biosynthesis of coenzyme F_{420} is of interest because of the resemblance of the molecule to flavins and pteridines. Evidence that the ring system is derived from the purine nucleus was obtained from labeling experiments with C-2- or C-8-labeled guanine (189). C-2 was retained in coenzyme F_{420}, but C-8 was lost. This result is consistent with the previously described pathways of flavin and pterin biosyntheses in eubacteria and suggests that a similar pathway is used in coenzyme F_{420} biosynthesis in methanogens. Rapidly growing cultures of *Methanobacterium thermoautotrophicum* also secrete significant levels of fragment FO (the riboflavin analog), which may be a biosynthetic intermediate in the synthesis of the complete compound (231).

MFR (carbon dioxide reduction factor). When extracts of *Methanobacterium thermoautotrophicum* capable of producing methane from carbon dioxide and hydrogen were chromatographed on Sephadex G-25, they were resolved to a low-molecular-weight fraction which contained a carbon dioxide reduction factor (381). A second coenzyme, methanopterin, was also resolved from this same fraction (292). The structure determined for purified carbon dioxide reduction factor is shown in Fig. 8; the compound was renamed methanofuran (MFR) (290). The 2,4-disubstituted furan moiety and the distal 4,5-dicarboxy octanoate moiety are unique in natural systems. So far, MFR has been found only in methanogens. Representatives of all three orders of methanogens were found to contain MFR at levels ranging from 0.5 to 2.5 mg/kg of cell dry weight (206). The other bacteria tested, including three eubacteria, an extreme thermophile, and a halobacterium, did not contain MFR (206).

The function of MFR as a formyl carrier in methanogenesis and acetogenesis is outlined below (291). Other functions of MFR and the pathway of its biosynthesis are not yet known.

Methanopterin. A blue fluorescent compound in *Methanobacterium thermoautotrophicum* was called factor F_{342} because it had an absorbance maximum at 342 nm (147). Factor F_{342} is a 7-methylpterin derivative and thus related to folic acid (26, 225). The structure of the native compound, called methanopterin, is given in Fig. 9C (482). A slightly modified form, called sarcinapterin because of its discovery in *Methanosarcina barkeri*, contains a glutamyl residue esterified to the hydroxyglutarate moiety (Fig. 9D; 482). The structures of methanopterin and sarcinapterin are somewhat reminiscent of the polyglutamate derivatives of folic acid because they contain additional charged groups distal to the pteridine moiety (26). Folylpolyglutamates are the forms of folate used in vivo in eubacteria and eucaryotes and in

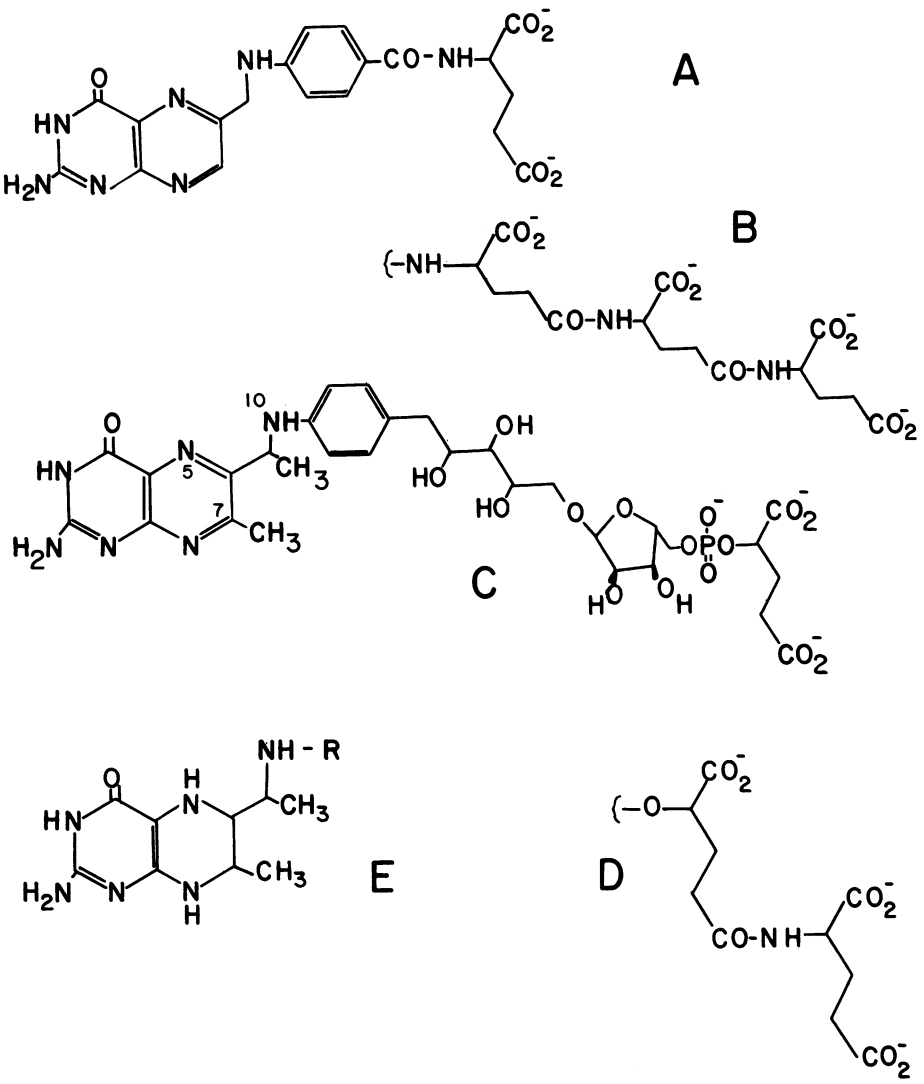
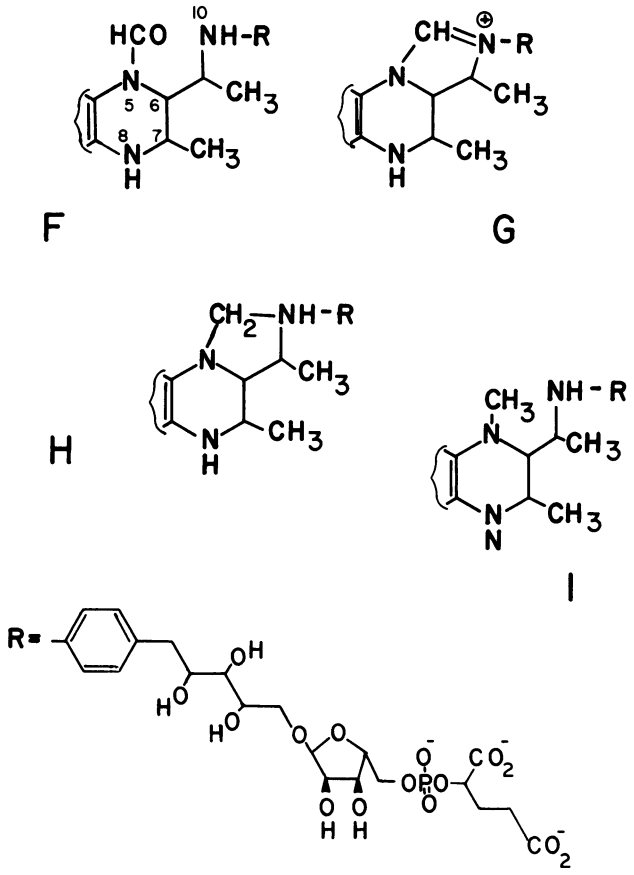


FIG. 9. Structures of folate and methanopterin derivatives. (A) Folic acid (pteroyl glutamate); (B) pteroyl triglutamate ($PteGlu_3$); (C) methanopterin (MPT); (D) sarcinapterin; (E) H_4MPT; (F) 5-formyltetrahydromethanopterin ($HCO\text{-}H_4MPT$); (G) 5,10-methenyltetrahydromethanopterin [5,10-(=CH—)H_4MPT^+]; (H) 5,10-methylenetetrahydromethanopterin ($CH_2{=}H_4MPT$); (I) 5-methyltetrahydromethanopterin ($CH_3\text{-}H_4MPT$) (26, 482, 524).

general bind more tightly to enzymes than the monoglutamate derivative (26). The structure of the triglutamate form of folic acid is shown in Fig. 9B.

The 7-methylpterin nucleus of methanopterin is unusual, but not unique. The chemistry of folic acid and its derivatives in one-carbon reduction reactions is well understood. There is no reason to believe that methanopterin functions differently from folate (113, 481, 488). Evidence has accumulated showing clearly that the reduced form of methanopterin, tetrahydromethanopterin (H_4MPT), is active in one-carbon transfers in methanogens. The formyl, methenyl, methylene, and methyl derivatives are intermediates of CH_4 synthesis (112, 114, 483). Details about this role of the coenzyme are given below. Other functions of H_4MPT in methanogen metabolism are beginning to come to light. The total synthesis of acetate by the autotroph *Methanobacterium thermoautotrophicum* has been shown to require H_4MPT (275). The synthesis of serine may also require methylenetetrahydromethanopterin (116). In eubacteria and eucaryotes, serine is made from glycine and methylenetetrahydrofolate, another analogous reaction.

Before the presence of methanopterin was known in methanogens, the possible role of tetrahydrofolate in these organisms was tested by assaying for enzymatic activities, using folates as a substrate. Ferry et al. (121) demonstrated that extremely low levels of tetrahydrofolate-dependent formyltetrahydrofolate synthetase and methylenetetrahydrofolate reductase were present in methanogen extracts. The later demonstration of methylenetetrahydromethanopterin dehydrogenase (F_{420} dependent) in *Methanobacterium thermoautotrophicum* suggests that the earlier results were due to cross specificity of the enzyme (159). Formyl-H_4MPT synthetase activity has not been demonstrated, and other reactions with H_4MPT analogous to tetrahydrofolate reactions in eubacteria and eucaryotes need to be examined. It will be of interest to determine if they are



catalyzed by a multifunctional enzyme such as occurs in eucaryotes (355).

Biosynthesis of methanopterin must be somewhat different from folate biosynthesis since methanogens for the most part are resistant to sulfanilamide (167). Folate biosynthesis has provided a framework for study of methanopterin synthesis (42). When *Methanobacterium thermoautotrophicum* (222) or *Methanobrevibacter ruminantium* (502) were fed deuterated acetate, the enrichments of the hydrogen atoms in methanopterin and guanine were similar. Therefore, the pteridine moiety of methanopterin may be derived from guanosine triphosphate in a manner analogous to biosynthesis of folate (42, 222, 502). The methyl moiety at C-7 of the pteridine system is derived from [*methyl*-^{2}H]methionine, while the origin of the methyl moiety at C-9 is not known (503). White (502) showed that *p*-aminobenzoic acid (PABA) is a precursor of the arylamine (*p*-aminophenyltetrahydroxypentane) moiety of methanopterin, and it appears to be utilized as a sugar-phosphate adduct. This reaction is analogous to the terminal step in formation of folate in which *p*-aminobenzyl glutamate is condensed with a pteridine. However, this step is apparently sulfanilamide insensitive in methanogens. Keller et al. (222) reached a similar conclusion about the genesis of the arylamine portion of methanopterin by comparing the fate of label from deuterated acetate in tyrosine and methanopterin. Thus, it is clear that the aromatic ring is derived from an early intermediate of the shikimate pathway, and methanopterin is made via pathways analogous to pathways for folate and pteridine biosyntheses in other organisms.

Coenzyme M. Another coenzyme unique to methanogens

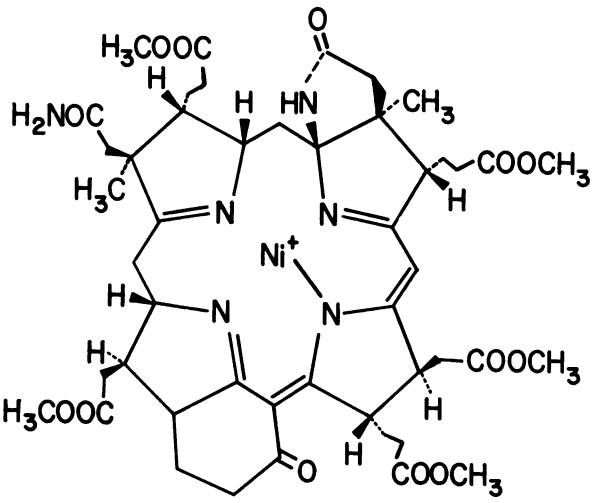


FIG. 10. Methylated chromophore of factor F_{430}. The structure of the derivative isolated from *Methanobacterium* sp. is that proposed by Pfaltz et al. (359). Reproduced from *The Bacteria*, vol. 8 (504) with permission of Academic Press, Inc.

and which functions as a carrier of methyl groups in the methanogenic pathway is coenzyme M (2-mercaptoethanesulfonic acid). The structure of coenzyme M is $HS\text{-}CH_2\text{-}CH_2\text{-}SO_3^-$ (459). The disulfide form $(\text{-}S\text{-}CH_2CH_2SO_3^-)_2$ and two mixed disulfides containing coenzyme M combined with unidentified second compounds have also been described (13, 459). Methyl coenzyme M ($CH_3\text{-}S\text{-}CoM$, 2-methylthioethanesulfonic acid, $CH_3\text{-}S\text{-}CH_2CH_2\text{-}SO_3^-$) is reduced to methane and HS-CoM at the terminal step in the methanogenic pathway (148, 311). Coenzyme M is also a growth factor for a number of different isolates of *Methanobrevibacter ruminantium*, but most methanogens can synthesize it (297).

The biosynthesis of coenzyme M has been studied with heavy isotope-labeled precursors (501). The incorporation of labeled acetate, sulfite, and sulfolactate into coenzyme M and the pattern of label retained was consistent with a biosynthetic pathway from 3-sulfopyruvate through sulfoacetaldehyde followed by formation of the thiol bond, possibly via a new mechanism (501).

Factor F_{430}. The requirement for nickel in growth of *Methanobacterium thermoautotrophicum* led to the discovery that the major sink for this metal was the yellow compound factor F_{430} (82, 83, 506). (Other roles for nickel are discussed below.) Biosynthetic and structural studies led to the proposal that the compound was a nickel-containing tetrapyrrole. The structure of a chemically methylated derivative, factor F_{430} M, is presented in Fig. 10 (359). The unique structure has a uroporphinoid (type III) skeleton and is related to the porphyrin and corrin ring systems (462, 463).It is the first biologically occurring nickel tetrapyrrole described and appears to be unique to methanogens (83).

Factor F_{430} is the yellow prosthetic group of component C of the $CH_3\text{-}S\text{-}CoM$ methylreductase system (107, 108, 333). Although early work suggested that factor F_{430} may contain coenzyme M or a lumazine derivative, more recent studies have failed to confirm these observations (164, 184, 223, 224, 227). However, coenzyme M is tightly associated with the protein component C (164, 184). Results of resonance raman spectroscopy suggested that there is an axial ligand to the nickel ion (394). Studies of the function of factor F_{430} have

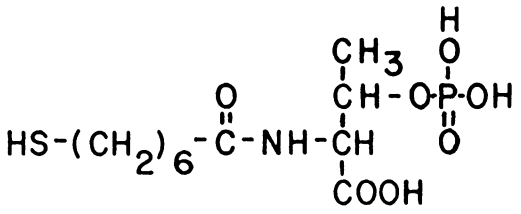


FIG. 11. Structure of component B from *Methanobacterium* sp. Proposed by Noll et al. (347).

shown that it is required as part of the terminal step of methane production (109).

The biosynthesis of factor F_{430} was shown to proceed from 5-aminolevulinate (462). 5-Aminolevulinate is synthesized by the C-5 pathway common in plants and many eubacteria (135). Gilles and Thauer demonstrated that uroporphyrinogen III is an intermediate in the biosynthesis of the coenzyme in cells of *Methanobacterium thermoautotrophicum* (136). When cultures were limited for nickel, labeled 5-aminolevulinate accumulated in uroporphyrinogen III. When nickel was added, factor F_{430} was formed from the uroporphyrinogen III. Enzyme activities of 5-aminolevulinate dehydratase, hydroxymethylbilane synthase, and uroporphyrinogen III synthase were present in detergent-permeabilized cells. Thus, the intermediates 5-aminolevulinic acid, porphobilinogen, uroporphyrinogen I, and uroporphyrinogen III lie on the pathway of factor F_{430} biosynthesis. It therefore appears that all porphinoid compounds in biology (chlorophylls, hemes, sirohemes, cobalamins, and now nickel tetrapyrroles) are synthesized from common intermediates (136).

Component B of the methylreductase system. Component B of the CH_3-S-CoM methylreductase system (148) is a colorless cofactor whose structure and function have been quite elusive (347). The compound is required for methanogenesis from CH_3-S-CoM in crude cell extracts resolved of low-molecular-weight compounds. Noll et al. (347) have recently determined that the structure of the active component of fractions containing component B is 7-mercaptoheptanoylthreonine phosphate (Fig. 11). Although the function of component B is unknown, it contains a thiol which could participate in methyl or electron transfer reactions.

Mobile factor. *Methanomicrobium mobile*, a ruminal isolate, requires a low-molecular-weight growth factor found in ruminal fluid or in boiled cell-free extracts of methanogens (357). The structure of this oxygen-sensitive, acid-stable compound has not been determined. Mobile factor may be a novel vitamin or coenzyme because low levels support growth of *Methanomicrobium mobile* and it has been found only in extracts of methanogens and not eubacteria (R. S. Tanner and R. S. Wolfe, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982 , I90, p. 109).

Other coenzymes in methanogens. The previous sections dealt with newly described compounds unique to methanogens or which were first described in these cells and later found in eubacteria or eucaryotes (e.g., factor F_{420}). The methanogens also contain a number of familiar coenzymes carrying out major metabolic roles.

Thiamine. The B vitamins in *Methanobacterium* and *Methanococcus* spp. have been assayed with a *Leuconostoc* sp. indicator strain (289). Vitamin B_1 (thiamine) was present at 1 to 2 μg/g of cell dry weight, a level about 20% of that found in several species of eubacteria. Presumably, it is required by pyruvate and 2-ketoglutarate synthases (543). Thiamine is also required for growth of a newly isolated methanogen, *Methanosphaera stadtmanii* (324). The biosynthetic pathways of thiamine in eubacteria and eucaryotes have been studied, and one fundamental difference is the origin of the carbon atoms at C-2 and C-4 of the pyrimidine moiety. In *E. coli* and *Salmonella typhimurium*, C-2 is derived from formate; in the eucaryote *Saccharomyces cerevisiae*, C-4 is derived from formate (532). It will be of interest to determine if methanogens utilize the "eubacterial" or "eucaryotic" pathway for synthesis of the pyrimidine moiety of thiamine.

Riboflavin. Vitamin B_2 (riboflavin) was found in methanogens at about 40 μg/g of cell dry weight, which is about the same level found in eubacteria. Thus, familiar isoalloxazine chemistry as well as deazaisoalloxazine chemistry are utilized by methanogens. Flavin adenine dinucleotide is present in membrane fractions of *Methanobacterium byrantii* (272) and is a component of the coenzyme F_{420}-reducing hydrogenase of *Methanobacterium thermoautotrophicum* (187). The flavin-adenine dinucleotide in the hydrogenase of *Methanobacterium formicicum* can be resolved from the enzyme by chromatography and is required for the enzyme to reduce the electron acceptor, coenzyme F_{420} (343). Other flavin-dependent enzymes include NADH oxidase (diaphorase) from *Methanospirillum hungatei*, formate dehydrogenase of *Methanobacterium formicicum*, and the CH_3-S-CoM methylreductase system of *Methanobacterium* sp. (315, 337, 391, 392).

Pyridoxine. Pyridoxine (vitamin B_6) was detected in methanogens at 1 to 6 mg/g of cell dry weight (289). Presumably this vitamin is utilized in its familiar biochemical function as pyridoxal phosphate by transaminases and other enzymes of amino acid metabolism (493).

Corrins. Vitamin B_{12}-like cobamides are present in large amounts in methanogens. In the mixed culture "*Methanobacillus omelianskii*," Lezius and Barker demonstrated that the major cobamides were 5-hydroxybenzimidazole derivatives rather than the common 5,6-dimethylbenzimidazole derivatives found in vitamin B_{12} (295). Pure cultures of methanogens also contain large amounts of 5-hydroxybenzimidazolyl cobamides (261, 363, 364, 508). Although the 5-hydroxybenzimidazolyl cobamide is apparently unique to methanogens, other modifications of the benzimidazolyl side chain of corrins are common in anaerobic eubacteria. Therefore, this feature is not of great phylogenetic importance.

Cobamides are implicated in methanogenesis from methanol (30, 485, 529), acetate (97, 229), and presumably methylamines. They also have a marked stimulatory effect upon methanogenesis from CH_3-S-CoM in extracts of *Methanobacterium bryantii* (509). In the autotrophic methanogens, corrins play a central role in acetate synthesis similar to their function in the acetogenic eubacteria (172, 296).

The biosynthesis of 5-hydroxybenzimidazolylcobamide is consistent with established pathways in eubacteria (393). The carbons from glycine are incorporated into 5-hydroxybenzimidazole, and the corrin is formed from glutamate. These results are consistent with the biosynthesis of 5-hydroxybenzimidazole via guanosine and biosynthesis of tetrapyrroles from 5-aminolevulinate via the C-5 pathway (135, 393).

Biotin. The B vitamin biotin is present in *Methanobacterium thermoautotrophicum* at about 40 μg/g of cell dry weight, a level between 1/3 and 1/20 that present in representative eubacteria (289). Biotin is also a required growth factor for *Methanococcoides methylutens* (435).

Niacin. Nicotinic acid (the B vitamin niacin) was present in acid-hydrolyzed extracts of *Methanococcus* spp. at levels on the order of those in eubacteria (about 200 μg/g of cell dry weight [289]). In *Methanobacterium thermoautotrophicum*, the levels were much lower, about 20 μg/g (289). Clearly, a role of nicotinic acid is in the function of the several pyridine nucleotide-linked enzymes described in methanogens.

Pantothenate. Levels of pantothenic acid are quite low in methanogens compared with the eubacteria tested (1 to 8 μg/g of cell dry weight in two methanogens; 200 to 400 μg/g of cell dry weight in four eubacteria) (289). Pantothenate is strongly stimulatory for the growth of *Methanococcus voltae* (505). The role of panthothenate is almost certainly in formation of coenzyme A. The pathway of pantothenate synthesis in methanogens is not known. In *E. coli*, the hydroxymethyl carbon is formed in a methylenetetrahydrofolate-mediated reaction (367). It will be interesting to determine if methylene-H_4MPT in methanogens functions in a similar manner.

Folate. Folic acid levels in methanogens determined by bioassay are extremely low (289). The slight vitamin activity present in *Methanobacterium thermoautotrophicum* and *Methanococcus voltae* (0.8 to 2% of that found in *E. coli*) presumably resulted from the ability of methanopterin or a pterin precursor in methanopterin biosynthesis to substitute for folic acid at low efficiency. The indicator strain did not respond to PABA, pterin, pterin-6-carboxylic acid, or a combination of all known products of folate metabolism, including serine, methionine, thymidine, adenine, guanine, and pantothenate (289). PABA is a required growth factor for *Methanomicrobium mobile* (Tanner and Wolfe, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982) and *Methanosarcina* sp. strain TM-1 (336). Growth of *Methanosarcina* sp. strain TM-1 is inhibited by sulfanilamide. The inhibition is reversed by PABA (336). This result suggests that a step analogous to the sulfanilamide-sensitive step of folate biosynthesis in eubacteria exists in this strain of *Methanosarcina*. Support for this idea comes from the finding that PABA functions as a precursor to methanopterin in *Methanobacterium* and *Methanobrevibacter* spp. (502).

Molybdopterin and proteinaceous cofactors. Other cofactors in methanogens include a molybdopterinlike component of formate dehydrogenase and the proteinaceous cofactors ferredoxin and thioredoxin (162, 163, 390, 392, 397). Molybdopterin is a component of formate dehydrogenase from *Methanobacterium formicicum*, and preliminary studies suggest that it is analogous to the molybdopterin cofactor of xanthine oxidase and nitrate reductase. Ferredoxins function as electron carriers in the acetoclastic methanogens (162, 163). Thioredoxin was isolated from *Methanobacterium thermoautotrophicum*. The peptide of M_r 12,000 was functional in an assay with *E. coli* ribonucleotide diphosphate reductase. The ability of the methanogen protein to act as cofactor to a eubacterial enzyme was taken to indicate the conserved nature of this cellular component (397).

Overview of Methanogenesis

Methanogens are limited to simple growth substrates and do not gain a rich living. A comparison of the free energies of hydrolysis of ATP (−31.8 kJ/mol) and those of methane formation from the substrates hydrogen and carbon dioxide (−135.6 kJ/mol of CH_4), formate (−130.1 kJ), methanol (−104.9 kJ), methylamines (about −74 kJ), carbon monoxide (−196.7 kJ), and acetate (−31.0 kJ) leads to the conclusion that only small amounts of energy are available to these organisms (63, 464). The bioenergetics of methanogenesis have been thoroughly reviewed (63, 226). The brief discussion which follows points out major findings that have begun to clarify how the anabolic needs of these organisms are met. The following sections outline the biochemistry of this system.

Clearly the membranes of methanogens are important in energy coupling through familiar chemiosmotic mechanisms. Most of the data obtained to date may be reconciled with a model that includes a membrane-associated, proton-dependent adenosine triphosphatase coupled to a sodium/proton antiporter. However, there are some differences in the results obtained in the various genera. In *Methanobacterium thermoautotrophicum*, hydrogenase and adenosine triphosphatase are associated with internal cellular membranes. A hydrogen-dependent chemiosmotic potential could drive ATP synthesis (85). Since these cells contain an ADP/ATP translocase sensitive to atractyloside, an inhibitor of the mitochondria ADP/ATP translocase, the notion of an archaebacterial "methanochondrion" organelle was proposed (63, 84). The idea of an internal chemisomotic potential associated with the machinery of methanogenesis has other support, although other interpretations of these data could be made (388, 389, 437). However, not all methanogens contain internal membranous structures, and the cytoplasmic membrane must play a major part in the bioenergetics of most species (441).

A number of workers have measured the PMF of methanogens and have shown that it is composed of both a ΔpH and a $\Delta\psi$. The internal pH appears to be controlled at about pH_i 6.7; $\Delta\psi$ varies from −80 to −200 mV in different organisms (47, 63, 193). Cation pumping is essential for maintenance of the PMF. Sodium is also required for methanogenesis (63, 195). A sodium/proton antiporter in *Methanobacterium thermoautotrophicum* is implicated in methanogenesis and ATP synthesis (412). The Na^+/H^+ antiporter is inhibited by ammonia, harmoline, and amiloride. These inhibitors also inhibit the eucaryotic Na^+/H^+ antiporters (412). Ammonium and potassium antiports have also been observed in *Methanospirillum* and *Methanosarcina* spp. (440).

The coupling of ATP synthesis to methanogenesis is controversial at present. Although ATP synthesis in *Methanosarcina* spp. is dependent on sodium ions and an energized membrane (28, 29), investigators have not been able to demonstrate conclusively that a PMF is required in *Methanobacterium* spp. (57, 411). In *Methanococcus* spp., substrate-level phosphorylation is proposed as an alternative means of ATP synthesis (57, 274). However, substrate-level phosphorylation coupled to methanogenesis is only inferred from very indirect evidence. Thus, the different hypotheses may arise from artifacts associated with differences in the experimental systems. For instance, the location of methanogenesis on intracyctoplasmic vesicles in *Methanobacterium thermoautotrophicum* may explain a number of anomolous effects of uncouplers in that system (63, 388, 437).

Active transport has been described in methanogens (63). For example, coenzyme M is transported by *Methanobrevibacter ruminantium* in an energy-dependent process (13). Nickel is transported by *Methanobacterium bryantii* in a sodium-linked process (194). In *Methanococcus voltae*, transport of amino acids is by sodium symport (195). Thehalophilic archaebacteria also transport amino acids by sodium symport, and the sodium gradient is maintained by an Na^+/H^+ antiporter (25, 143).

Methanogenesis is required for growth of methanogens. However, inhibition of growth does not necessarily curtail

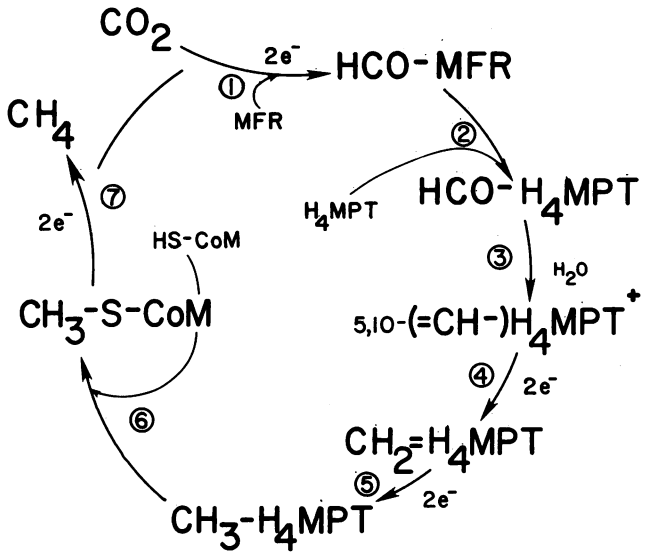


FIG. 12. Pathway from CO_2 to CH_4 in *M. thermoautotrophicum*.

methanogenesis. Uncoupling of growth from methanogenesis may be obtained by treatment with uncouplers, phosphate limitation, or treatment with the base analog 5-fluorouracil (7, 63, 427; D. P. Nagle, Jr., A. Eisenbraun, and R. Teal, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, I-50, p. 173).

Hydrogenase is central to methanogen bioenergetics. Hydrogenases from all bacteria contain iron-sulfur centers essential for enzyme activity (1). In addition, methanogen hydrogenases contain a redox-active nickel (3, 107, 140, 273, 463). The redox-active nickel has been further characterized by electron paramagnetic resonance and optical spectroscopy (140, 203, 250). This exciting result was the first time that the oxidation reduction properties of nickel had been observed in a biological system and prompted searches to find out if this metal was present in other hydrogenases. Redox-active nickel was subsequently detected in the periplasmic hydrogenase of the anaerobe *Desulfovibrio gigas* (287). Nickel was also found in hydrogenases of the aerobic chemolithotroph *Alcaligenes eutrophus* and the phototroph *Rhodopseudomonas capsulata* (53, 129), and it appears to be a common component of uptake hydrogenases.

Methanobacterium formicicum contains two hydrogenases, which differ in electron acceptor specificity (F_{420} or dyes) and subunit structure (187, 202). The hydrogenase from *Methanococcus vannielii* contains selenocysteine in one of its subunits (533). Some methanogens utilize formate in addition to hydrogen. Two forms of formate dehydrogenase have been isolated from *Methanococus vannielii*. Only one form has been obtained from *Methanobacterium formicicum*. Enzymes from both bacteria reduce the deazaflavin coenzyme F_{420} (205, 390–392). A molybdopterin similar to the cofactor of xanthine oxidase and nitrate reductase is found in the formate dehydrogenase from *Methanobacterium formicicum*.

Methanogenesis from CO_2 and H_2

The pathway of CO_2 reduction to CH_4 as it is now understood is given in Fig. 12. This figure summarizes a large body of work (113, 524, 525). The central theme is that three coenzymes unique to methanogens are the one-carbon carriers during the sequential reduction of CO_2 to CH_4. The coenzymes are MFR, H_4MPT, and coenzyme M. The terminal reduction of CH_3-S-CoM to CH_4 by hydrogen involves two additional cofactors, component B and factor F_{430}, whose functions are unknown.

The fixation and initial reduction of carbon dioxide are not well understood (step 1 of Fig. 12). The product, formyl-MFR, has been identified in Sephadex G25-treated cell-free extracts of *Methanobacterium thermoautotrophicum* (291). When formyl-MFR is added to methanopterin-replete extracts, the formyl moiety is converted to methane. Particularly intriguing about this initial CO_2 reduction is the lack of a requirement for stoichiometric amounts of ATP and a requirement for CH_3-S-CoM reduction. Therefore, methanogenesis is drawn as a cycle. The stimulation of methane formation from carbon dioxide by methyl coenzyme M was first observed by R. P. Gunsalus, so the phenomenon has been called the "RPG effect" (145). Although poorly understood, the coupling of CH_3-S-CoM reduction and CO_2 fixation may conserve the free energy released from methanogenesis for activation of CO_2. Compounds which are C-1 donors for CH_3-S-CoM synthesis (serine, pyruvate, formaldehyde) also stimulate CO_2 reduction to methane (380).

The transfer of the formyl equivalent from formyl-MFR to H_4MPT has been demonstrated (step 2). The enzymatic reaction yields 5-formyl-H_4MPT (M. I. Donnelly and R. S. Wolfe, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, I56, p. 174). Step 3 of the pathway is catalyzed by the cyclohydrolase which reversibly converts 5-formyl-H_4MPT to 5,10-methenyl-H_4MPT (86). The analogous enzyme in clostridia and yeasts, methenyltetrahydrofolate cyclohydrolase, forms 10-formyltetrahydrofolate (26, 494). The 10-formyltetrahydrofolate is utilized in biosynthetic pathways in these organisms. In methanogens, about 95% of the CO_2 reduced is converted to methane via 5-formyl-H_4MPT. It has been suggested that biosynthetic one-carbon units might be derived from the 10-formyl derivative, which could be formed nonenzymatically or by an enzyme not yet described (86).

The enzyme 5,10-(=CH—)H_4MPT$^+$ dehydrogenase (step 4) was initially demonstrated during studies on methanogenesis from formaldehyde in crude cell extracts of *Methanobacterium* spp. (115, 379). CH_2=H_4MPT was formed nonenzymatically by condensation of formaldehyde and H_4MPT, a reaction analogous to the condensation of formaldehyde and tetrahydrofolate (112, 114, 210). Under an N_2 atmosphere, *Methanobacterium* cell-free extracts converted 3 mol of CH_2=H_4MPT to 2 mol of 5,10-(=CH—)H_4MPT$^+$ and 1 mol of methane. Thus, electrons from the oxidation of the methylene moiety to a methenyl moiety (the reverse of step 4) were utilized for methanogenesis (115). Under a hydrogen gas atmosphere, formaldehyde was totally converted to methane. Nonphysiological substrates for methanogenesis (e.g., thiazolidine and $HOCH_2$-S-CoM) could also function as formaldehyde donors (116). Methylene-H_4MPT dehydrogenase has been isolated from *Methanobacterium thermoautotrophicum*. Coenzyme F_{420} is the specific electron carrier in this reversible reaction (159). This represents the first time that the deazaflavin cofactor has been directly implicated in methanogenesis.

Step 5, catalyzed by methylene-H_4MPT reductase, has not been demonstrated directly. Transfer of the methyl equivalent from CH_3-H_4MPT to HS-CoM has been postulated, but not demonstrated (step 6).

The terminal step of this pathway (step 7), the methyl coenzyme M methylreductase reaction, has been studied in

detail. The complete methylreductase reaction is written below, showing protein components above the arrow and coenzymes below it:

$$CH_3\text{-S-CoM} + H_2 \xrightarrow[B,\ F_{430},\ FAD,\ Mg,\ ATP,\ B_{12}]{A1,\ A2,\ A3,\ C} CH_4 + HS\text{-CoM}$$

Components A, B, and C were resolved by anoxic column chromatography of crude extracts of *Methanobacterium thermoautotrophicum* (148). Component C is central to the reaction. An oxygen-stable protein, it was purified to homogeneity and shown to contain 2 mol of factor F_{430} per mol of enzyme. A multimeric protein with a native molecular weight of 300,000, it has the subunit structure α-2, β-2, γ-2. The relative molecular weights of the subunits are 68,000, 45,000, and 38,500, respectively (108, 110). Native component C also contains coenzyme M (164, 227). The other known proteinaceous components of the methyl coenzyme M reductase system are called A1, A2, and A3, which were resolved by anoxic chromatography of component A (337). Components A1 and A3 are oxygen labile and have not yielded further to purification. Component A1 contains a deazaflavin-reducing hydrogenase, although the requirement for this activity in the methylreductase reaction has not been demonstrated. Component A2, an oxygen-stable protein, has been purified to homogeneity and found to be a colorless protein of M_r 59,000 (384). It is required at far lower levels than component C in the assay system. It has not been possible to define the role of component A2, although it may be involved in the ATP-dependent activation of the methylreductase system (384).

The cofactors required by the methylreductase system have also been identified. Component A was resolved of flavin adenine dinucleotide, which is required by the purified methylreductase system (337). Component B, whose function is not known, was described above. Another factor in this reaction that is not fully understood is the stimulation by cobamides. The rate of the methylreductase reaction was stimulated strongly by all corrins tested, suggesting a possible nonphysiological role of these compounds (509). Moreover, reduced corrins can drive the methylreductase reaction in the presence of partially purified components C and B and the absence of other electron donors (6).

The role of ATP in this reaction is also not fully understood. It acts as a "primer" for the system, and many moles of methane are produced per mole of ATP added (146, 377). ATP can be removed from extracts of *Methanobacterium bryantii* with a hexokinase-ATP trap before initiation of the methylreductase reaction. These extracts are active in producing methane in the absence of ATP, but the "activated state" is unstable and exhibits a half-life of <15 min. These results suggest that ATP is required to activate a protein or coenzyme in the methylreductase system (507). Because ATP is not required for the corrin-dependent reaction, ATP may in fact be required for coupling to the physiological electron donor, H_2.

Methanogenesis from Methanol and Methylamines

Methanogenesis from methanol or methylamines in the absence of hydrogen requires that the methyl carbon be dismutated to carbon dioxide and methane. Organisms of the family *Methanosarcinaceae* are capable of this reaction. Extracts of *Methanosarcina barkeri* form methane from methanol (31). The evidence indicates that cobamides are involved in the activation of methanol. The formation of CH_3-S-CoM as an intermediate in methanogenesis from methanol was demonstrated, and less than stoichiometric amounts of ATP were required for formation of CH_3-S-CoM (422). Two separate methyl transfer reactions are involved in this process (30, 364, 484–486). Methyl transferase 1 (MT_1) contains an oxygen-sensitive cobamide. In the presence of methanol, ATP, and magnesium, the cobamide is methylated. MT_1 also catalyzed the methylation of free cobamide. Reduced ferredoxin is required for the activation of MT_1. Methyl transferase 2 catalyzes transfer of the methyl moiety from MT_1 or cobamides to HS-CoM.

Trimethylamine-grown cells contain an enzymatic activity which catalyzes the transfer of a methyl group from trimethylamine to HS-CoM, forming dimethylamine and CH_3-S-CoM (341). Catalytic amounts of ATP and the presence of reductant (hydrogen or NADPH) are required by dialyzed extracts. The role of cobamides in the reaction has been suggested. The trimethylamine:HS-CoM methyltransferase activity was not found in cells grown on methanol, suggesting that this enzyme system was induced (341).

The dismutation of methanol, methylamines, or acetate (following section) to methane and carbon dioxide in the absence of hydrogen requires a system of electron carriers. Members of the *Methanosarcinaceae*, the only methanogens capable of these dismutations, are the only methanogens which possess membrane-bound cytochromes (264, 265). Two type *b* cytochromes ($E_{m1} = -325$ mV; $E_{m2} = -183$ mV) are present in methanol-grown cells. Acetate-grown cells contain an additional type *b* cytochrome ($E_{m3} = -250$ mV) as well as a type *c* cytochrome (265). Cytochromes are important in the respiration of aerobic archaebacteria. Althoug the involvement of the cytochromes in the redox processes of methanogenesis is likely, it has not been demonstrated directly. In support of this contention, the methanol-utilizing member of the *Methanobacteriaceae* (*Methanosphaera stadtmaniae*) is only capable of growth on methanol plus hydrogen and contains no cytochromes (324). Presumably, reducing equivalents are generated by hydrogenase.

Methanogenesis from Acetate

Acetoclastic methanogenesis is dependent upon the ability of the cell to cleave the acetate molecule, reduce the methyl equivalent, and oxidize the carboxyl equivalent. The standard free energy of this reaction (−31 kJ/mol of methane) is nearly equal to that required for synthesis of a molecule of ATP from ADP and inorganic phosphate (+31.8 kJ/mol) (462). Thus, acetoclastic methanogenesis is not a lucrative means to make a living when compared with methanogenesis form one-carbon substrates. If better growth substrates are available, the cell will utilize acetate for biosynthesis and form methane via the energetically more favorable pathways (27, 119, 429, 558). The control of cellular switching from the use of one substrate to another is not yet understood.

The mechanism of methanogenesis from acetate has begun to be uncovered. Coenzyme M has been identified as a carrier of the methyl equivalent from acetate (260, 298), and the methyl coenzyme M methylreductase system is involved in conversion of the methyl moiety to methane (260, 344). Cobamides are also implicated in the process because corrinoid antagonists inhibit methane production from acetate but not from H_2 plus CO_2 (97, 229). The oxidation of the carboxyl carbon may involve carbon monoxide dehydrogenase (CODH). This conclusion is based on the following observations. Cyanide inhibits methanogenesis from acetate or carbon monoxide. Cyanide also inhibits cell carbon

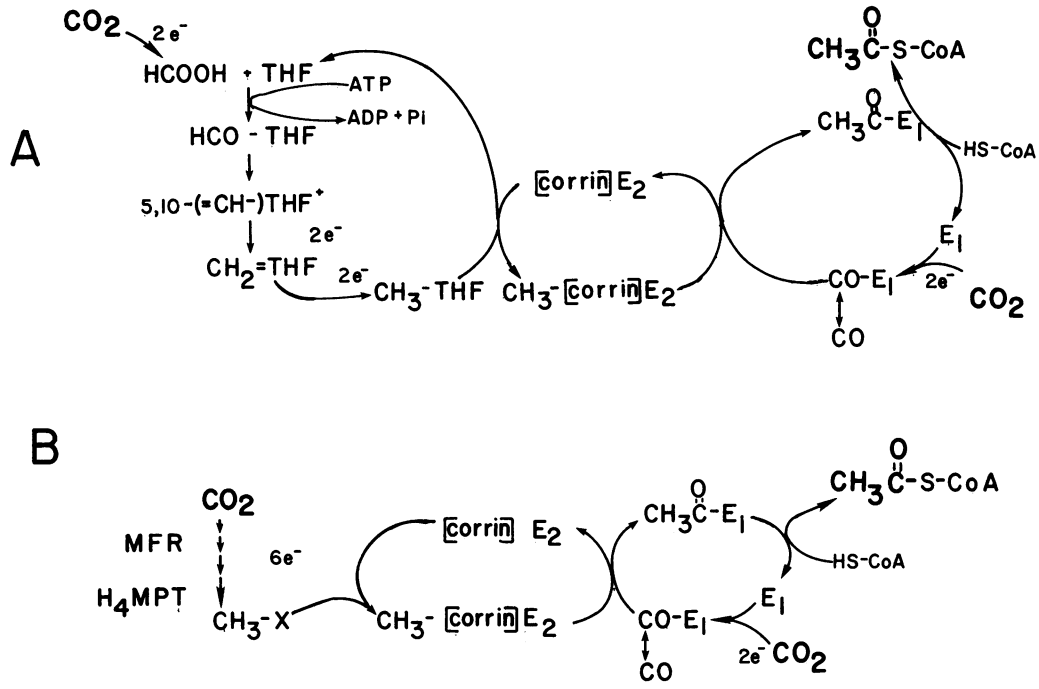


FIG. 13. Total synthesis of acetate in (A) *Clostridium thermoaceticum* and (B) *M. thermoautotrophicum*. Tetrahydrofolate is abbreviated THF. E_1 represents the nickel-containing CODH, and E_2 (corrin) represents the cobamide-containing protein involved in methyl transfer.

synthesis (via acetyl-CoA) from H_2 plus CO_2 but not methanogenesis from H_2 plus CO_2 (96, 428). The carboxyl of acetate is in enzymatic equilibrium with CO_2 and CO by an exchange reaction catalyzed by *Methanosarcina* extracts (96). CO provided to these extracts is oxidized and provides reducing equivalents to the methylreductase system (344). Moreover, removal of CO dehydrogenase activity by immunoprecipitation with antibodies to the highly purified CODH prevents acetate cleavage by *Methanosarcina* extracts (262, 263).

The role of membranes in acetate-driven methanogenesis was demonstrated by Baresi. The pelleted membrane fraction from acetate-grown *Methanosarcina* cells is sufficient to produce methane from acetate under an N_2 atmosphere at rates approaching in vivo rates (18). A hydrogen atmosphere inhibited methane formation from acetate; instead, the methyl groups of acetate were oxidized to CO_2. Acetate-grown cells contain hydrogenase and methylreductase at levels similar to those in hydrogen-grown cells (17). A membrane-free, soluble system for acetate cleavage under a hydrogen atmosphere has also been developed. The methyl moiety is transferred to the methylreductase system and reduced by electrons from soluble reductants (260).

The similarities between acetate cleavage and acetate synthesis in methanogens are apparent (see below). The *Methanosarcinaceae* are capable of growth by acetate cleavage, but during growth on H_2 plus CO_2 many are able to synthesize acetate autotrophically. The other methanogens are specialists and are limited to growth on single-carbon substrates. They synthesize acetate autotrophically in many cases, but they do not cleave it.

Autotrophy in Methanogens

Methanogen autotrophy is a newly recognized type, in which the key intermediate of carbon fixation is acetyl CoA and the first known CO_2 fixation product is formyl-MFR (291, 385). Formation of acetyl CoA in methanogens is clearly interlinked with catabolism: CO_2 is both the primary electron acceptor and precursor to all anabolites. In *Methanobacterium* spp., about 95% of the carbon dioxide fixed is used for energy production, and 5% is used for biosynthesis (455, 465, 545).

The de novo synthesis of acetate involves anabolic and catabolic intermediates and cofactors unique to methanogens. A simplified version of acetogenesis in *Methanobacterium thermoautotrophicum* is shown in Fig. 13. The converging pathways of C-1 and C-2 of acetate are clearly seen (455). CODH (E_1 in the figure) is centrally involved in acetate synthesis as shown by in vivo experiments. Carbon monoxide is incorporated into acetate, and the carboxyl of acetate exchanges with free CO (454, 456). A small amount of CO is also synthesized by autotrophic methanogens (93). Methanogens which are incapable of autotrophic growth lack CODH. This finding explains their dependence on preformed acetate (38).

The methyl carbon of acetate is derived from the H_4MPT-dependent series of reactions that lead to methane (275). The direct involvement of H_4MPT in acetogenesis in vitro was demonstrated (275). The role of cobamides is also certain (229). Details concerning the transfer of the methyl moiety and the identity of the other individual components remain unknown.

The total synthesis of acetate by methanogens is strikingly analogous to the total synthesis of acetate by certain eubacteria: *Clostridium thermoaceticum*, *Eubacterium limosum*, *Butyribacterium methylotrophicum*, *Acetobacterium woodii*, and *Desulfovibrio baarsii* (92, 192, 296, 358, 370, 371, 465, 545). Many of these organisms are capable of autotrophic growth on hydrogen and carbon dioxide (or carbon monoxide alone), and all of them carry out the total

synthesis of acetate from CO_2. The culmination of a number of years of work by Wood, Ljungdahl, and co-workers has been the elucidation of this complete pathway in *C. thermoaceticum* shown in Fig. 13 (296, 358, 371). Enzymes of each of the steps shown have been purified to homogeneity and studied in detail. The methyl and carboxyl carbons are derived from separate, converging pathways. CO_2 is reduced via formate dehydrogenase, and then bound to H_4-folate by the ATP-dependent formyltetrahydrofolate synthetase. The carbon is then reduced in a stepwise series of reductions to the methyl level in CH_3-H_4-folate. The source of the carboxyl carbon is CO_2 or pyruvate. However, carbon monoxide will substitute for pyruvate as a carbonyl donor. The terminal step of acetate synthesis, in which the methyl moiety is condensed with a carbonyl equivalent, occurs through the nickel-containing CODH (370, 371). This is a new concept, since for some time it was believed that a cobamide enzyme-mediated methyl transfer, and not CODH-mediated carbonyl transfer, was the central step in acetogenesis.

Modifications of the pathway unique to methanogens are the ATP-independent activation of CO_2 by the methylreductase and formation of formyl-MFR. The sparing of ATP by utilizing a portion of the free energy of the methylreductase reaction is a beneficial adaptation.

Thus, cobamide and CODH-dependent acetate syntheses occur in bacteria of quite diverse groupings: methanogens, acetogens, clostridia, and *Desulfovibrio* spp. This pathway may be used catabolically or anabolically, depending upon the physiological circumstances and the organism (458). Despite variations, such as the replacement of folate with methanopterin, and the differences in the first steps of the pathway (requiring ATP in the eubacterial case and methyl coenzyme M reduction in the methanogen case) and other twists that may occur in the diverse eubacterial acetate synthesizers, the central mechanism of acetogenic autotrophy is conserved. Nickel-containing CODH seems to be the key enzyme which forms acetyl-CoA from methyl and carboxyl equivalents from divergent pathways.

CONCLUSIONS

Methanogens and the process of methanogenesis have proven to be rich in new biological and biochemical phenomena often considered to be novelities. However, these organisms and their biochemistry are not simply curiosities. Rather, they are specializations for a unique form of energy metabolism, a phenomenon common in eubacteria. Thus, the major roles of the unique coenzymes found in methanogens are played, for the most part, in the methanogenic pathway. In methanogens which fix carbon dioxide autotrophically, some of the available free energy of the methanogenic reaction is utilized to form the first fixation product (a novel reaction in which activation of carbon dioxide requires no ATP). The later steps of methanogen autotrophy are remarkably parallel to those of acetate synthesis in acetogenic eubacteria. Both pathways require CODH, slightly different forms of corrin, and H_4MPT (methanogens) or tetrahydrofolate (eubacteria).

Reactions of intermediary metabolism known in the methanogens appear to be analogous to those found in eubacteria and eucaryotes. In cases where the novel methanogen cofactors are utilized, the pathways are formally equivalent.

The broad biochemical and phylogenetic diversity within the methanogens is exceeded by the even broader relationships among the archaebacteria. The grouping of the order *Methanomicrobiales* with the extreme halophiles suggested by rRNA sequence data is supported by the presence of several other biological characters, including respiratory chains, cell wall structure, gas vesicles, and common storage products. The remaining two orders of methanogens are more distantly related to the halophiles, a conclusion consistent with their biological characters. The extreme thermophiles are phylogenetically quite distinct from the other archaebacterial groups. However, more data are needed on aspects of metabolism of the extreme thermophiles to clarify the relatedness of this group.

The biology of methanogens will reveal information of great interest in itself. The general areas of coenzyme, metal, and membrane biochemistry have already been greatly enlarged. Further investigations of the biochemistry and physiology of the extreme thermophiles also have great promise. In addition, the basic framework of archaebacterial biology may be examined in the light of phylogenetic relationships provided by rRNA data. Certain key questions may now be clearly addressed. These include the following. Were biological properties common to distantly related microorganisms acquired by vertical or horizontal transfer, or were they convergently evolved? Does horizontal transfer of information over broad phylogenetic distances occur in contemporary time? Can the evolution of biological pathways be followed through evolutionary time (201)? Can it be determined which biological properties, such as sulfur metabolism, methanogenesis, or extreme thermophily, were characters of the progenitor cell (518)?

Because the archaebacteria possess much in common with eubacteria and eucaryotes and yet are unique unto themselves, a deeper insight into the genesis of microorganisms may be gained. Although substantial progress has been made in understanding bacterial diversity, many exciting questions are unanswered. The comments of K. V. Thimann in 1955 remain true today.

"The many physiological and ecological types. . . .and the striking differences between the bacteria and the other orders, make one wonder whether bacteriology has as yet done any more than scratch the surface of the subject. Perhaps for many physiologists this represents the fascination of the bacteria, that although they are so complexly interrelated, yet they comprise modes of life more varied than all the other plant types together. They offer an outstanding example of the evolutionary success which attends upon versatility. Perhaps it is because of this very versatility that, though they have been intensively studied, they remain so profoundly unknown" (466, p. 713).

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