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Biology, Ecology, and Biotechnological Applications of Anaerobic Bacteria Adapted to Environmental Stresses in Temperature, pH, Salinity, or Substrates

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INTRODUCTION

To address the mechanisms used by specialized groups of chemoorganotrophic anaerobes that exist under extreme environments, it is pertinent to outline the evolution of the Earth and the sequence of events which led to the biosphere and the kinds of bacteria we have today. It is generally agreed that life arose when the Earth was cooling and occupied by numerous volcanic features. The early atmosphere was reducing and free of oxygen and contained H₂, CH₄, CO, NH₃, HCN, and H₂S. One line of evolutionary speculation rests on the assumption that among organisms living today, obligate anaerobic bacteria are the simplest in structure and biochemistry and are the most closely related to the earliest forms of life. Although archaeobacterial anaerobes are considered to have predated eubacterial anaerobes, more conclusive evidence is required to answer the question of which bacterial kingdom evolved first (464).

Many laboratory experiments have demonstrated the synthesis of organic matter under conditions simulating those of primitive Earth. Such processes were inhibited by trace amounts of oxygen, suggesting that life arose under anoxic conditions. The starting materials for such synthesis included H₂S, CO, and HCN. Although these three compounds are poisonous gases for most aerobic organisms, they are important metabolites for certain anaerobic bacterial species. It is assumed that these gases and organic matter chemically derived from them were the energy substrates utilized by the first kinds of anaerobic bacteria on Earth. Further evolution gave rise to anoxic phototrophs, and the continued diversification of anaerobic bacteria is thought to have occurred before the development of aerobic photosynthesis, which gave rise to an oxygen-rich atmosphere. Phototrophic bacteria will not be covered here, but some phototrophic species can grow as chemoorganotrophic anaerobes in CO or under halophilic or thermophilic growth conditions.

The purpose of this text is to review recent understanding of the biology of anaerobic bacteria that have evolved to grow under extreme physiological and biochemical condi-

tions. By extreme conditions we mean those which are far from the normal conditions used to describe the origins of physiological biochemistry (i.e., pH, neutral; temperature, 37°C; atmosphere, aerobic; salinity, 1.5%; substrate, glucose). These normal growth conditions were used to understand the foundations of how normal cells (i.e., animal cells, *Escherichia coli*, and *Bacillus subtilis*) function. In recent years we have learned that prokaryotic microorganisms can differ from eukaryotic cells in part because they have adapted to grow under extreme growth conditions of temperature (>100°C), salinity (saturated NaCl), pH (<2.0, >10), and substrate stress (limited chemical free energy or on toxicants). These kinds of extreme microbial growth conditions are found in exotic environments which appear limited today but which were thought to be much more widespread on primitive Earth.

Detailed understanding of the biology of anaerobic bacteria adapted to environmental stresses has developed relatively recently when compared with the body of knowledge of aerobic microorganisms that grow under extreme environmental conditions. Excellent reviews that deal with microbial adaptation to extreme environments, primarily with aerobic bacteria, have been published (224, 233, 400). In general, the physiological processes for adaptation to environmental stress in anaerobic bacteria seem to have evolved differently from those in aerobic bacteria for two major reasons. First, anaerobes are energy limited during the chemoorganotrophic growth mode because they cannot couple dehydrogenation reactions to oxygen reduction and gain a high level of chemical free energy (425). Second, growth of most chemoorganotrophic anaerobes (except for methanogens) is naturally associated with the generation of toxic end products (e.g., organic acids or alcohols, HS⁻), which requires that anaerobic species develop some sort of dynamic adaptation mechanism or tolerance to their catabolic end products.

Table 1 summarizes the kinds of anaerobes that will be reviewed here. Thermoanaerobes have received the most attention to date and display greater species diversity than do aerobic thermophiles. We now know that hyperthermo-

TABLE 1. Comparison of requirements for optimal growth of anaerobes adapted to extreme environmental conditions

Class	Growth requirement	Species example (reference)
Thermophile	High temperature (>60°C)	<i>Thermoanaerobacter brockii</i> (497)
Halophile	High salinity (≥10% NaCl)	<i>Haloanaerobium praevalens</i> (498)
Acidophile	High acidity (external pH ≥2.0, internal pH <6.0)	<i>Sarcina ventriculi</i> (391)
Alkaliphile	High alkalinity	<i>Methanohalophilus zhilinae</i> (276)
Syntroph	Derives limited free energy and forms inhibitory catabolites (<10 kJ/mol of substrate consumed; H ₂ , HCOOH, acetate formed), grows with a metabolic partner	<i>Syntrophospora bryantii</i> (509)
CO utilizer or dehalogenator	Derives energy from substrate detoxification (CO as energy source)	<i>Butyribacterium methylophilicum</i> (268)

TABLE 2. Habitats of thermophiles

Habitat	Isolation temp (°C)	Organism present	Optimal temp for organism (°C) ^a	Reference
Stratal water in Caspian Sea	84	<i>Desulfurovibrio thermophilus</i>	65 (45–85)	356
Yellowstone water and mud	45–60	<i>Thermoanaerobacter ethanolicus</i>	69 (37–78)	454
Geothermally heated sea sediments	56–100	<i>Thermotoga maritima</i>	80 (55–90)	174
Water well, hot mud	80–101	<i>Thermotoga thermarum</i>	70 (55–84)	461
Thermal spring on Kamchatka	70	<i>Thermoanaerobium lactoethylicum</i>	65–70 (42–75)	485
Submarine solfataric field	112	<i>Hyperthermus butylicus</i>	95–106 (max. 107) ^b	518
Icelandic hot spring	88	<i>Fervidobacterium islandicum</i>	65 (50–80)	177
Hydrothermal vents	98	<i>Caldococcus litoralis</i>	88 (55–100)	418
Submarine vent system	90	<i>Methanococcus igneus</i>	88 (45–91)	68
Smokers, sediments from hydrothermal systems	7–140	<i>Archaeoglobus profundus</i>	82	69

^a Values in parentheses represent the temperature range for growth.

^b Maximum temperature for growth.

anaerobic species (e.g., *Pyrococcus furiosus*) that grow above 100°C exist. In some cases, thermoanaerobes are somewhat easier to grow at extremely high temperature than are aerobes, because medium evaporation problems caused by aeration at high temperature does not occur. Certain anaerobic species have more recently been examined in relation to their unique mechanism of adaptation to high salt or low pH. Syntrophic, CO-utilizing, and dehalogenating anaerobes represent different physiological classes of extreme anaerobes whose aerobic counterparts have not yet been recognized. Syntrophic anaerobes degrade substrates with such limited free energy (e.g., fatty acids, organic acids, and aromatic substrates) that they require a partner species (i.e., a methanogen or sulfate reducer) to remove the catabolic end products for energy conservation and growth. CO-utilizing and dehalogenating anaerobes actually derive energy from extremely toxic substrates (e.g., CO or chlorinated aromatics) which inhibit the growth of many aerobic forms of life. At present the described haloanaerobes and thermoanaerobes display obligate growth requirements for high salt and high temperature, respectively, whereas, the described acidophiles, syntrophs, and CO utilizers and dehalogenators appear facultative as natural groups. This text will review the ecology and diversity, the physiology and biochemistry, the stress adaptation mechanisms, and the biotechnological features of anaerobes capable of growth under extreme conditions.

THERMOPHILES

Ecology, Diversity, and Taxonomy

A number of natural habitats of high temperature exist, ranging from Sun-heated soils and litter with temperatures of 60 to 70°C to erupting volcanoes, reaching 1,000°C. A question of considerable interest centers on the upper temperature limit for life. As noted by Brock, "Bacteria are able to grow. . . at any temperature at which there is liquid water, even in pools which are above the boiling point" (50). Until recently, liquid-water environments with the highest known naturally occurring temperatures were close to sea level and hence had boiling points around 100°C. Over the past decade, habitats have been found at the bottom of the oceans with temperatures up to 350°C, raising questions about the possibility of life in these environments. Hyperthermoanaerobic species that grow above 100°C have been isolated from deep-sea thermal vents (403). Since covalent bonds within proteins, RNA, DNA, ATP, and NADP will

undergo hydrolysis at 250°C, and since tertiary structure forces for most macromolecules are altered at much lower temperatures, the upper temperature limit for life is expected to be higher than 100°C but far lower than 250°C (52, 450).

Thermophilic aerobic and anaerobic sporeforming bacteria were isolated from self-heating soil environments before the 1940s (53). About two decades ago, nonsporulating aerobic bacteria were detected in thermal springs of constantly high temperature, and the first thermophilic organism *Sulfolobus acidocaldarius*, an archaeobacterium with a temperature optimum above 80°C and an upper growth temperature at around 92°C, was isolated (54). Another archaeobacterium, *Methanobacterium thermoautotrophicum*, was the first nonsporulating thermophilic anaerobe described (506). The diversity and ecology of thermoanaerobes in thermal-spring environments was first described in 1979 (487, 497). Since then, hyperthermophilic anaerobes have been isolated from continental and submarine volcanic areas, such as solfataric fields, geothermal power plants, and geothermally heated sea sediments and hydrothermal vents (400, 406). Several recent and more comprehensive reviews on thermophiles and their biology have been published (53, 400, 416).

In general, moderate thermophiles are primarily eubacteria and display optimal growth between 60 and 80°C, whereas hyperthermophiles are primarily archaeobacteria and grow optimally at 80°C or above. Table 2 outlines sites from which thermoanaerobes have been isolated. Interestingly, some organisms have been isolated from areas with temperatures much higher than their maximum growth temperature, e.g., *Hyperthermus butylicus* (518) and *Fervidobacterium islandicum* (177), which suggests that in these environments the organisms may not be actively growing. The same could be true for the organisms isolated from temperatures much below their growth temperature optimum, such as *Archaeoglobus profundus* (69).

Some of the hydrothermal areas in Yellowstone National Park (Fig. 1) contain dense mats of layered microbial communities, where the volcanic source water provides constant high temperatures and mineral nutrients which enable a complete thermophilic bacterial ecosystem to become established. These thermophilic bacterial mats usually consist of primary producers (i.e., thermophilic cyanobacteria and green phototrophic bacteria) in the upper layers, where light can penetrate, and diverse species of chemoorganotrophic thermoanaerobes (i.e., hydrolytic, fermentative, methanogenic, and sulfate-reducing species) in the lower layers, where decomposition of primary production occurs (444,



FIG. 1. Octopus Hot Spring in Yellowstone National Park, Wyo. This was the sampling site for the isolation of many moderate thermoanaerobes. Here, thermophilic, photosynthetic bacteria grow and are degraded by diverse thermoanaerobic bacteria that use all types of biomass-based macromolecules and biochemicals as energy sources.

487, 489). These thermal spring mats, as well as volcanic waters, sediments, soils, and sewage sludge rich in organic matter, are good source materials for enrichment of moderate thermoanaerobes.

The organisms isolated from the microbial mats in the hot springs at Yellowstone National Park participate in the anaerobic food chain and represent members of each of the various trophic levels, including saccharolytic bacteria, acetogens, sulfur metabolizers, and methanogens (489). The saccharolytic clostridia utilize components from the algal mat, forming products which can act as substrates for the sulfur-metabolizing organisms, with methanogens forming the terminal reaction in the food chain with the formation of methane. An unusual feature unique to the microbial ecosystem in the algal mat is the fact that acetate was not significantly transformed into methane, unlike the situation in neutral, mesophilic, freshwater environments, where the majority of carbon and electrons become methane (489). The phototrophs in the algal bacterial mat actively incorporate acetate (369), which makes any methanogenesis from this precursor insignificant to total carbon mineralization.

Enumeration studies of the microbial population in Octopus Spring algal-bacterial mat estimated more than 10^9 chemoorganotrophic hydrolytic bacteria and 10^6 chemolithotrophic methanogenic bacteria per gram (dry weight) of the mat (489). A prevalent chemoorganotrophic obligate anaerobe, subsequently described as *Thermobacteroides acetoethylicus* (27), was isolated from the algal-bacterial mat, as were *Thermoanaerobacter brockii* (formerly *Thermoanaerobium brockii* [245]) and *Thermoanaerobacter thermohydrosulfuricus* (formerly *Clostridium thermohydrosulfuricum* [245]) (493). *Methanobacterium thermoautotrophicum* was isolated as the abundant chemolithotrophic methanogen (493). Analysis of the physiological properties of these anaerobic bacteria isolated from the 65°C site in the Octopus Spring mat shows that they are adapted to the described environmental temperature. The growth temperature optima of these isolated methanogenic and hydrolytic strains coin-

cided with the environmental temperature and the estimated optimum temperature for in situ methanogenesis (493).

Most of the eubacterial anaerobic thermophiles are chemoorganotrophic in their metabolism (Table 3). *Acetogenium kivui*, *Clostridium thermoaceticum*, *Clostridium thermoautotrophicum*, *Desulfotomaculum thermoacetoxidans*, *Thermodesulfotobacterium commune*, and *Thermosiphon africanus* are also chemolithotrophic. *Thermobacteroides proteolyticus* is proteolytic and grows poorly on sugars (315). The other proteolytic thermophile is *Thermosiphon africanus*, which also requires CO_2 and is inhibited by H_2 in the absence of sulfur (176). The eubacterial thermoanaerobes belong to nearly the same range of nutritional categories as do mesophilic bacteria. *Carboxydotherrmus hydrogeniformans* is limited to growing on CO and produces H_2 and CO_2 as end products (419).

The moderate thermophilic archaeobacteria include the methanogens with temperature optima from 50°C (for *Methanosarcina thermophila*) to 88°C (for *Methanothermobacter sociabilis* and *Methanococcus igneus*) (Table 4). A novel group of methanogens, *Methanopyrus* spp., has been isolated from sediment samples. *Methanopyrus* strain AV19 is the most hyperthermophilic methanogen isolated to date and demonstrates that methanogenesis can occur at temperatures above 100°C (Table 4) (173). Thermophilic methanogens are found in mesophilic and thermophilic anaerobic digestors, muds, sediments, and hydrothermal vents. A number of the methanogens are obligate autotrophs, with the majority using formate and several species, including *Methanosarcina* strain CHT155 and *M. thermophila*, utilizing more complex organic compounds such as methylamine and trimethylamine.

Perhaps the most interesting group of thermophiles is the hyperthermophiles (Table 5), since the isolation of these organisms has caused us to reevaluate possible habitats for microorganisms and has increased the high-temperature limits at which life is known to exist. Of these organisms, the one with the highest growth temperature reported to date is

TABLE 3. Characteristics of eubacterial thermoanaerobes

Organism	Optimum mode of nutrition	Fermentation products	Isolation/habitat	Growth conditions	Reference(s)
<i>Acetogenium kivui</i>	Sugars, H ₂ + CO ₂ , formate, CO, pyruvate	Acetate	Lake in South Africa	66°C, pH 6.4	250, 251
<i>Acetomicrobium faecalis</i>	Chemorganotroph, variety of hexoses and pentoses	Lactate, acetate, ethanol, H ₂ + CO ₂	Sewage sludge	70–73°C, pH 6.5–7.0	462
<i>Acetomicrobium flavidum</i>	Variety of sugars	Acetate, CO ₂ + H ₂	Thermophilic sewage sludge fermentor	58°C, pH 6.2–8.0	392
<i>Acetothermus paucivorans</i>	Glucose, fructose	Acetate, CO ₂ + H ₂	Sewage sludge	58°C, pH 7–8	101
<i>Carboxydotherrnus hydrogenoformans</i>	CO	H ₂ + CO ₂	Hot swamp on Kunashir Island	70–72°C, pH 6.8–7.0	419
<i>Clostridium fervidus</i>	Glucose, maltose, mannose, xylan, starch, pyruvate	Acetate, CO ₂ + H ₂ , other minor products	Hot spring in New Zealand	68°C, pH 7.0–7.5	331
<i>Clostridium stercorarium</i>	Cellulose, xylan, soluble sugars	Acetate, lactate, ethanol, H ₂ + CO ₂	Compost heap	65°C, pH 7.3	270
<i>Clostridium thermoacetium</i>	H ₂ + CO ₂ or CO (chemolithotroph), pyruvate, glucose (chemoorganotroph)	Acetate, CO ₂	Horse manure	55–60°C, pH NR	138
<i>Clostridium thermoautotrophicum</i>	CO ₂ + H ₂ or CO, glucose, other sugars	Acetate, H ₂	Mud and wet soils	55–60°C, pH 5.7	453
<i>Clostridium thermobutyricum</i>	Soluble sugars	Butyrate, CO ₂ + H ₂ , minor amounts of acetate and lactate	Horse manure	55°C, pH 6.8–7.1	456
<i>Clostridium thermocellum</i>	Cellulose, cellobiose, hemicellulose, glucose, fructose, formate, galactose, methanol, glycerate	Acetate, lactate, ethanol, H ₂ + CO ₂	Sewage digester sludge	60–64°C, pH 7.0	441
<i>Clostridium thermocopriae</i>	Cellulose and wide variety of sugars, S ⁰	Ethanol, acetate, butyrate, lactate, H ₂ , CO ₂ , H ₂ S	Feces, soil, hot spring in Japan	60°C, pH 6.5–7.3	194
<i>Clostridium thermohydro-sulfuricum</i>	Starch, cellobiose, glucose, xylose and other soluble sugars, sulfite, thiosulfate	Ethanol, lactate, acetate, H ₂ + CO ₂ , H ₂ S	Hot springs, soil, juices extracted from beets during sugar manufacture	68°C, pH 6.9–7.5	213, 458
<i>Clostridium thermolacticum</i>	Variety of carbohydrates	Lactate; minor amounts of ethanol, acetate, H ₂ + CO ₂	Widespread, sediments anaerobic digestors, cattle manure	60–65°C, pH 7.0–7.2	252
<i>Clostridium thermopalmarium</i>	Sugars	Butyric acid; H ₂ ; CO ₂ ; trace acetate, lactate, ethanol	Palm wine in Senegal	55°C, pH 6.6	238
<i>Clostridium thermosaccharolyticum</i>	Dextrin, pectin (electron donors); sulfite and thiosulfate (electron acceptors)	Acetate, butyrate, lactate, ethanol, H ₂ + succinate, H ₂ S	Soil, extraction juice in beet sugar factories	55–62°C, pH NR	163, 238, 282
<i>Clostridium thermosuccinogenes</i>	Sugars	Formate, acetate, lactate, succinate, H ₂	Cow manure, beet pulp, soil, mud	58°C, pH 7.6	112
<i>Thermoanaerobacterium thermosulfurigenes</i>	Pectin, starch, xylose, glucose, etc., thiosulfate	Ethanol, acetate, lactate, H ₂ + CO ₂ , S ⁰	Algal mat in hot spring in Yellowstone	60°C, pH 5.5–6.5	376
<i>Desulfotomaculum geographicum</i>	Lactate, ethanol, fructose, saturated fatty acids, H ₂ , CO ₂ , sulfate, sulfite	Acetate, H ₂ S	Geothermal groundwater	54°C, pH 7.3–7.5	88
<i>Desulfotomaculum kuznetsovii</i>	Formate, acetate, propionate, various alcohols, sulfate, sulfite, thiosulfate	CO ₂ , H ₂ S	Thermal mineral waters in the Sukhnsk deposit	60–65°C, pH NR, 2–39% NaCl	304

<i>Desulfotomaculum nigrificans</i>	Lactate, pyruvate, sulfate	H ₂ S	Soils, compost heaps, anaerobic digestion, thermal-spring water	55°C, pH NR	70
<i>Desulfotomaculum thermoacetoxidans</i>	Lactate, pyruvate, ethanol, H ₂ + CO ₂ ; sulfate, thiosulfate, S ⁰ (electron acceptors)	Acetate, H ₂ S	Thermophilic anaerobic bioreactor	55–60°C, pH 6.5	292
<i>Desulfotomaculum thermobenzoicum</i>	Benzoate, some fatty acids and alcohols; electron acceptors include sulfite, sulfite, thiosulfate, and nitrate	Acetate, CO ₂	Thermophilic methane fermentation reactor	62°C, pH 7.2	422
<i>Desulfurella acetivorans</i>	Acetate, S ⁰ (electron acceptors)	H ₂ S + CO ₂	Hot spring in Kamchatka	50–59°C, pH 6.8–7.0	39
<i>Desulfurovibrio thermophilus</i>	Pyruvate, formate + acetate, lactate, sulfate	H ₂ S	Water samples from a petroleum stratum	65°C, pH NR	356, 357
<i>Dictyoglomus thermophilum</i>	Chemoorganotroph	Acetate, ethanol, lactate, CO ₂ + H ₂	Japanese hot spring	78°C, pH 7.0	367
<i>Fervidobacterium islandicum</i>	Cellulose, sugars, pyruvate, S ⁰	Lactate, acetate, ethanol, H ₂ + CO ₂ , H ₂ S	Icelandic hot spring	65°C, pH 7.2	177
<i>Fervidobacterium nodosum</i>	Range of carbohydrates	Lactate, acetate, H ₂ + CO ₂ , low levels of ethanol	Hot spring New Zealand	65–70°C, pH 7.0–7.5	329
<i>Thermoanaerobacter ethanolicus</i>	Starch, lactose, cellobiose, various hexoses and pentoses, pyruvate; does not utilize cellulose	Ethanol + CO ₂ , low levels of acetate and lactate	Hot springs Yellowstone	69°C, pH 5.8–8.5	454
<i>Thermoanaerobacter finlayii</i>	Glucose, mannose, various soluble sugars, pyruvate	Ethanol + CO ₂ , low levels of lactate and acetate	Sediment sludge in an African lake	65°C, pH 6.5–6.8	377
<i>Thermoanaerobacter Brockii</i>	Starch, sucrose, maltose, lactose, cellobiose and glucose, thiosulfate	Ethanol, lactate, acetate, H ₂ + CO ₂ , H ₂ S	Hydrothermal area in Yellowstone	65°C, pH 7.5	497
<i>Thermoanaerobium lactoethylicum</i>	Starch, glucose and other sugars, S ⁰	Ethanol, acetate, lactate, H ₂ , CO ₂ ; byproducts include propionate, butyrate, isovalerate, and H ₂ S	Hot spring in Kamchatka	65°C, pH 7.0	219
<i>Thermobacteroides acetoethylicus</i>	Starch, glucose and other soluble sugars, thiosulfate	Ethanol, acetate, H ₂ , CO ₂ , butyrate, isobutyrate, H ₂ S	Hydrothermal area in Yellowstone	65°C, pH 5.5–8.5	27
<i>Thermobacteroides leptospartum</i>	Soluble sugars	Ethanol, acetate	Cattle compost	60°C, pH 7.5	433
<i>Thermobacteroides proteolyticus</i>	Yeast extract, peptone, casein, gelatin, Trypticase peptone	Acetate, H ₂ + CO ₂	Thermophilic digester fermenting tannery wastes and cattle manure	63°C, pH 7.5	315
<i>Thermodesulfobacterium commune</i>	Pyruvate, lactate, H ₂ (electron donors); sulfite, thiosulfate (electron acceptors)	Acetate, H ₂ + CO ₂ , H ₂ S	Hydrothermal area in Yellowstone	70°C, pH 6.0–8.0	495
<i>Thermotoga maritima</i>	Starch, glycogen, glucose, other soluble sugars, sulfur, cysteine	Lactate, acetate, CO ₂ + H ₂ , H ₂ S	Heated sea floors in Italy and the Azores	80°C, pH 6.5, 2.7% salt	174
<i>Thermosipho africanus</i>	Yeast extract, peptone, tryptone, CO ₂ , cysteine, S ⁰	H ₂ S	Marine hydrothermal area at OBook, Africa	75°C, pH 7.2, 0.11–3.6% NaCl	176
<i>Thermotoga neapolitana</i>	Variety of carbohydrates, S ⁰	H ₂ S	Heated marine sediment, Vulcano submarine thermal vent at Lucrino, Italy	80°C, pH 7.0	25, 191
<i>Thermotoga thermanum</i>	Variety of carbohydrates	NR ^a	African hot springs at Lac Abbé, Italy	70°C, pH 7.0, NaCl optimum at 0.35%	461

^a NR, not reported.

TABLE 4. Characteristics of thermophilic methanogenic archaeobacteria

Organism	Mode of nutrition	Isolation/habitat	Optimal growth conditions	Reference(s)
<i>Methanobacterium</i> strain CB12	H ₂ + CO ₂ , formate	Sludge sample from a mesophilic biogas plant, China	56°C, pH 7.4	510
<i>Methanobacterium</i> strain FTF	Formate, H ₂ + CO ₂	Thermophilic digester	55°C, pH 7.5	436
<i>Methanobacterium thermoaggregans</i>	Obligate autotroph, H ₂ + CO ₂	Mud from cattle pasture	65°C, pH 7.0–7.5	35
<i>Methanobacterium thermoalcalphilum</i>	Obligate autotroph, H ₂ + CO ₂	Biogas plant	60°C, pH 7.5–8.5	36
<i>Methanobacterium thermoautotrophicum</i>	Obligate autotroph, H ₂ + CO ₂	Sewage sludge, hydrothermal areas in Yellowstone	65–75°C, pH 7.2–7.6	506
<i>Methanobacterium thermoformicum</i>	H ₂ + CO ₂ , formate	Thermophilic manure digester	55°C, pH 7–8	513
<i>Methanobacterium wolfei</i>	Obligate autotroph, H ₂ + CO ₂	Sewage sludge and river sediment	55–65°C, pH 7.0–7.5	463
<i>Methanococcus</i> strain AG86	H ₂ + CO ₂	Hydrothermal vent	85°, pH 6.5, 3% NaCl	508
<i>Methanococcus igneus</i>	H ₂ + CO ₂	Submarine vent	88°C, pH 5.7, 1.8% NaCl	68
<i>Methanococcus jannaschii</i>	2–3% NaCl is required; Obligate autotroph, H ₂ + CO ₂ ; sulfide is required for growth	Deep-sea white smoker (East Pacific Rise)	85°C, pH 6.0	196
<i>Methanococcus thermolithotrophus</i>	Formate, H ₂ + CO ₂ , grows optimally in 4% salt	Thermal heated sea sediments, Naples	65°C, pH 7.0	175
<i>Methanogenium frittonii</i>	H ₂ + CO ₂ , formate	Nonthermal freshwater sediments	57°C, pH 7.0–7.5	156
<i>Methanogenium thermophilicum</i>	Formate, H ₂ + CO ₂ , 0.2 M salt optimum	Marine cooling channel of nuclear power plant	55°C, pH 7.0	349, 484
<i>Methanogenium</i> strain UCLA	Formate, H ₂ + CO ₂	Anaerobic sludge digester	55–60°C, pH 7.2	128
<i>Methanopyrus</i> strain AV19	H ₂ + CO ₂	Sediment samples at Guaymas Basin hot vents	98°C, pH NR, ^b 1.5% NaCl	173
<i>Methanosarcina</i> strain CHTI 55	Acetate, methanol, methylamines	Thermophilic digester	57°C, pH 6.8	435
<i>Methanosarcina thermophila</i>	Acetate, methanol, methylamine, trimethylamine, H ₂ + CO ₂	Sludge from thermophilic digester	50°C, pH 6–7	527, 528
<i>Methanothermus fervidus</i>	Obligate autotroph, H ₂ + CO ₂	Terrestrial solfataric muds, Iceland	83°C, pH 6.5	405
<i>Methanothermus sociabilis</i>	Obligate autotroph, H ₂ + CO ₂	Terrestrial solfataric muds	88°C, pH 6.5	237
<i>Methanotherx thermoacetophila</i>	Not defined (manure extract, N ₂)	Soil, mud, water, and algal-bacterial mats from thermal springs in Kamchatka	62°C, pH NR	311, 312, 330

^b NR, not reported.

Pyrodictium occultum, which grows at temperatures up to 110°C (403).

Hyperthermophiles have been isolated from many different habitats. *Thermoproteus*, *Thermofilum*, and *Desulfurococcus* spp. are relatively widespread in their distribution and share habitats. These organisms can be isolated in Iceland, Italy, the Azores, and the United States from various solfataric springs with environmental temperatures between 55 and 100°C and pH values between 3 and 7. In similar hydrothermal habitats, with a pH above 5.5, *Thermophilum* and *Desulfurococcus* spp. can also be found along, with *Methanothermus* spp. (400). Illustrating the specific distribution of some of these hyperthermophiles, *Methanothermus* spp. could not be isolated from other solfataric areas such as in Yellowstone Park, Italy, and the Azores (400).

Pyrodictium Brockii, *Pyrodictium occultum* (403), *Pyrococcus woesei* (520), and *Pyrococcus furiosus* (130) were isolated from a shallow submarine solfataric field in Vulcano, Italy, which consisted of sandy sediments through which hot seawater and volcanic gases emanated. Although

Pyrodictium spp. were found in this area, isolates could not be obtained from similar solfataric areas in other parts of Italy (400). *Thermoproteus maritimus*, like *Pyrodictium Brockii* and *Pyrodictium occultum*, has been isolated only from the submarine solfataric field close to Vulcano (134), where the organisms occur together, although their distribution within the habitat is different. Unlike *Pyrodictium* species, *Thermoproteus maritimus* does not occur at temperatures about 95°C. *Pyrobaculum islandicum* and *Pyrobaculum organotrophum* were isolated from superheated neutral to slightly alkaline anaerobic solfataric waters. *Pyrobaculum islandicum* was isolated from sites in Iceland, whereas *Pyrobaculum organotrophicum* was more widespread, being isolated in Iceland, Italy, and the Azores. Because of their low salt tolerance, it was concluded that the organisms do not grow within submarine hydrothermal systems and are adapted to the low-salt continental solfataric springs (172).

The hyperthermophilic, anaerobic archaeobacteria have some very unusual morphological features. The sulfur-metabolizing archaeobacteria are coccoid, floc-shaped, or disc-shaped organisms that stain gram negative, and they have

TABLE 5. Characteristics of hyperthermophilic archaeobacteria

Organism	Mode of nutrition	Fermentation products	Isolation/habitat	Optimal growth conditions	Reference(s)
<i>Archaeoglobus fulgidus</i>	CO ₂ + H ₂ , simple organic compounds including glucose and sulfate required	H ₂ S + CO ₂ , CH ₄ (trace)	Marine hydrothermal systems in Vulcano and Stufe di Nerone, Italy	83°C, pH 5.5–7.5	401, 404, 507
<i>Archaeoglobus profundus</i>	H ₂ , acetate, lactate, pyruvate, yeast extract, sulfate	H ₂ S	Smokers, sediments from deep-sea hydrothermal system	82°C, pH 6.0, 1.8% NaCl	69
<i>Caldococcus litoralis</i>	Peptides, S ⁰	H ₂ S	Hydrothermal vents	88°C, pH 6.4, 2.5% NaCl	418
<i>Desulfurococcus amylolyticus</i>	Starch, pectin, glycogen, and peptides; facultative sulfur reducer	H ₂ S	Thermal springs on Kanchatka	90–92°C, pH 6.4	38
<i>Desulfurococcus mobilis</i>	Organic matter, S ⁰ , peptides	H ₂ S + CO ₂	Terrestrial, solfataric muds (Iceland and United States)	85°C, pH 5.5–6.0	521
<i>Desulfurococcus mucosus</i>	S ⁰ , peptides	H ₂ S + CO ₂	Terrestrial, solfataric muds (Iceland and United States)	85°C, pH 5.5–6.0	521
<i>Desulfurococcus saccharovorans</i>	Glucose, yeast extract, S ⁰	NR ^a	Terrestrial, solfataric muds (Iceland and United States)	85°C, pH NR	400
<i>Hyperthermus butylicus</i>	Peptides, S ⁰	CO ₂ , butanol, acetate, propionate, H ₂ S	Submarine solfataric field	95–107°C, pH 7.0	518
<i>Pyrobaculum islandicum</i>	H ₂ , complex organic compounds, reduces various sulfur compounds	H ₂ S + CO ₂	Terrestrial, solfataric mud holes (Azores, Italy, and Iceland), geothermal plant	100°C, pH 6.0	172
<i>Pyrobaculum organotrophum</i>	Complex organic materials but not sugars, S ⁰ , L-cysteine (electron acceptors)	H ₂ S + CO ₂	Terrestrial, solfataric mud holes (Azores, Italy, and Iceland), geothermal plant	100°C, pH 6.0	172
<i>Pyrococcus furiosus</i>	Starch, maltose, peptone, complex organic substrates, S ⁰	H ₂ + CO ₂ , H ₂ S	Marine solfataric mud (Italy)	100°C, pH 7.0	130
<i>Pyrococcus woesei</i>	Polysaccharides yeast extract, S ⁰ , H ₂	H ₂ S + CO ₂	Marine solfataric mud (Italy)	100–103°C, pH 6.0–6.5, 2% NaCl	520
<i>Pyrodictium abyssi</i>	Heterotrophic growth, yeast extract, gelatine, starch, formate, S ⁰	Isovalerate, isobutyrate, butanol, CO ₂ , H ₂ S	Marine hot abyssal	97°C, pH 5.5, 2% NaCl	339
<i>Pyrodictium brockii</i>	H ₂ + CO ₂ + S ⁰	H ₂ S + CO ₂	Marine solfataric mud (Italy)	105°C, pH 5.5, 1.5% NaCl	403
<i>Pyrodictium occultum</i>	H ₂ + CO ₂ + S ⁰	H ₂ S + CO ₂	Marine solfataric mud (Italy)	105°C, pH 5.5, 1.5% NaCl	403
<i>Staphylothermus marinus</i>	Obligate heterotroph, complex organic substrates, S ⁰	Acetate, isovalerate, CO ₂ + H ₂ S	Marine solfataric mud (Italy); deep-sea vents (East Pacific Rise)	92°C, pH 6.5, 1.5% NaCl	131
<i>Thermococcus celer</i>	Peptides, protein, S ⁰	CO ₂ + H ₂ S	Marine solfataras (Italy and Azores)	88°C, pH 5.8, 3.8% NaCl	519
<i>Thermococcus litoralis</i>	Yeast extract, peptone, tryptone, meat extract, casein, S ⁰	H ₂ S	Shallow submarine solfataras (Naples and Vulcano, Italy)	88°C, pH 6.0, 6.5% NaCl	305
<i>Thermococcus stetteri</i>	Peptone, starch, pectin, S ⁰	H ₂ S, CO ₂ , acetate, isobutyrate + isovalerate	Marine solfataric fields of Kratmaya Cove	73–77°C, pH 6.5, 2.5% NaCl	293
<i>Thermodiscus maritimus</i>	Yeast extract, H ₂ + S ⁰	CO ₂ + H ₂ S	Marine solfataras (Italy only)	88°C, pH 5.0, 2% NaCl	134, 400
<i>Thermofilum librum</i>	Obligate heterotroph, S ⁰	H ₂ S	Terrestrial solfataras (Italy, Azores, Iceland, and United States)	NR	400
<i>Thermofilum pendens</i>	Peptides, S ⁰ + H ₂ S	CO ₂ + H ₂ S	Terrestrial solfataras (Italy, Azores, Iceland, and United States)	85–90°C, pH 5–6	517
<i>Thermoproteus neutrophilus</i>	H ₂ + CO ₂ + S ⁰ , acetate	H ₂ S + CO ₂	Terrestrial solfataras (Italy, Azores, Iceland, and United States)	88°C, pH 5.5–7.5	134, 370, 400
<i>Thermoproteus tenax</i>	Facultative autotroph, CO ₂ , CO, organic compounds, requirement for S ⁰ and H ₂ S	H ₂ S + CO ₂	Terrestrial solfataras (Italy, Azores, Iceland, and United States)	88°C, pH 5.0	134, 522, 523
<i>Thermoproteus uzoniensis</i>	Peptides, S ⁰	Acetate, isobutyrate, isovalerate, H ₂ S	Hot springs and soil	90°C, pH 5.6	37

^a NR, not reported.

envelopes of protein subunits, termed S-layers, which cover their cytoplasmic membranes. Some isolates are motile by means of flagella. Cell division does not occur by septum formation; instead, budding or constriction results in new cells (402). This is also the mode of cell division in the eubacterium *Thermotoga* (174).

Although many eubacteria and archaeobacteria possess crystalline S-layers, the mass distribution of the S-layers from *Thermoproteus tenax* and *Thermoproteus neutrophilus* have unique features (290). The S-layer proteins are highly stable, maintain their structural integrity under extreme environmental conditions, and resist dissociation by high temperature, chemical treatment, or mechanical disruption (290). Possession of such a coat suggests an adaptive mechanism to the extreme environment in which these organisms exist and could have a barrier function against both external and internal factors. Thin sections of cells of *Thermoproteus tenax* and *Thermoproteus neutrophilus* have shown that the S-layers are the only cell wall component, supporting the hypothesis that S-layers play a major role in the determination of cell shape (290). The thermophilic saccharolytic clostridia have a type of S-layer lattice which provides a characteristic taxonomic feature. All strains of *Clostridium thermohydrosulfuricum* exhibit hexagonal lattices, whereas *Clostridium thermosaccharolyticum* strains have square lattices (163). The S-layer protein has a similar amino acid composition and is glycosylated (390).

Pyrodictium occultum and *Pyrodictium brockii* grow as a mold-like layer on sulfur, and the cells are irregularly disc shaped and dish shaped, with granules of sulfur frequently seen sticking to the fibers, whose production may confer an adaptational advantage to the organism in trapping nutrients. The extremely thermophilic eubacterial *Thermotoga* species have a very interesting cellular ultrastructure, unlike that of eubacteria and resembling some of the extremely thermophilic archaeobacteria. *Thermotoga thermarum*, *Thermotoga neapolitana* (461), and *Thermotoga maritima* (174) are surrounded by a characteristic "toga," a sheath-like structure, ballooning over the ends of the rods, which resembles an S-layer (389). All three *Thermotoga* species have lipids which appear to be unique among the eubacteria (174, 191, 461). *Thermosipho africanus* is a member of the new genus *Thermosipho* and represents a second genus within the order *Thermotogales*. The cells possess a surrounding sheath with ballooning ends similar to that of the *Thermotoga* spp. In contrast to *Thermotoga* spp., however, up to 12 cells are covered by one tube-like sheath (176).

Aerobic thermophiles fall into two distinct ranges of pH optima: habitats with pH values of 1.5 to 4, at which the thermoacidophiles exist, and pH 5.8 to 8.5, at which the neutrophilic and alkalophilic organisms thrive. The eubacterial thermoanaerobes have optimum growth at pH values around neutral (Table 3), with the exception of *Clostridium thermoautotrophicum*, which has a pH optimum of 5.7 (453) and *Thermoanaerobium thermosulfurigenes* (formerly *Clostridium thermosulfurigenes* [245]), which grows optimally at pH 5.5 to 6.5 (376).

The methanogenic bacteria share the unique ability of being able to synthesize methane from various compounds such as CO₂ plus H₂, formate, methylamines, methanol, and acetate. The thermophilic methanogens have the same metabolism as do their mesophilic counterparts, sharing the same substrate range and end product formation. *Methanococcus jannaschii* and *Methanococcus* strain AG86, however, differ from other methanococci in not utilizing formate (Table 4).

Examination of the habitat of hyperthermophiles from the submarine hydrothermal vents and continental solfataras illustrates the role of these organisms in carbon cycling within that domain. The hydrothermal vents do not receive sunlight as a result of their deep location, have low levels or an absence of organic nutrients, and are rich in H₂S, Mn, H₂, CO, CH₄, and a variety of other inorganic nutrients. The hyperthermophiles form part of the base of the food chain, utilizing H₂, CO₂, and CO and forming products which feed the animal community associated with the vents, thus contributing to a unique ecosystem independent of the Sun. Therefore, hyperthermophiles are well adapted to their environment, being primarily autotrophic, nonphotosynthetic, and capable of growing in the high-temperature environment of the vent. A number of the hyperthermophilic archaeobacteria are able to utilize carbon dioxide as their sole carbon source, obtaining energy from the oxidation of hydrogen by sulfur with the production of hydrogen sulfide (134). Interestingly, *Thermoproteus tenax* is the only thermophilic archaeobacterium to utilize CO (Table 5). *Thermoproteus neutrophilus*, *Thermoproteus tenax*, *Archaeoglobus fulgidus*, and *Pyrobaculum islandicum* are facultative autotrophs, and the remaining organisms are obligately heterotrophic (Table 5). Only a few of the organisms can utilize sugars, including *Desulfurococcus saccharovorans*, *Pyrococcus furiosus*, *Thermofilum librum*, *Pyrococcus woesei*, and *Archaeoglobus fulgidus*, although most of the organisms can utilize organic matter. *Archaeoglobus fulgidus* forms trace amounts of methane via an unknown pathway, and, similar to methanogens, *Archaeoglobus* cells fluoresce at 420 nm, indicating the presence of factor 420 (404). The other hyperthermophiles produce CO₂ and H₂S, and *Staphylothermus marinus*, *Thermococcus stetteri*, and *Thermoproteus uzoniensis* produce acids and *Hyperthermus butylicus* produces acids and alcohol (Table 5).

The evolution and taxonomy of thermophiles is an area that is receiving increasing attention. The moderate and hyperthermophiles are not well characterized taxonomically at the DNA-DNA hybridization level, but their evolutionary relatedness has been examined. By using 16S rRNA sequence comparison, an archaeobacterial phylogenetic tree has been proposed by Woese (464), with *Desulfurococcus*, *Sulfolobus*, *Pyrodictium*, and *Thermoproteus* forming one branch and the remaining thermophiles forming the branch containing methanogens, halophiles, and the two thermophiles *Thermoplasma* and *Thermococcus*. The branching of *Thermococcus* from the main methanogen branch is sufficiently deep to suggest that it may represent a third major archaeobacterial lineage (464).

The thermophilic eubacteria have been classified on the basis of morphology, in particular the ability to form spores, and on biochemical differences. More recently, partial 16S rRNA sequences have been compared from eight thermoanaerobes, seven of which were isolated from cyanobacterial mats in hot springs at Yellowstone National Park (18). *Thermobacteroides acetoethylicus* clusters tightly with two other asporogenous isolates, *Thermoanaerobium brockii* and *Thermoanaerobacter ethanolicus*, and with *Clostridium thermohydrosulfuricum*. The acetogens *Clostridium thermoautotrophicum* and *Clostridium thermoaceticum* form another tight cluster, and *Clostridium thermosulfurigenes* forms a distinct branch between the two clusters (18).

DNA-DNA hybridization techniques have been especially successful in resolving taxonomic relatedness among various strains and species within the same genus and have been used to investigate the taxonomic relationships of xyl-

TABLE 6. Proposed nomenclature for xylanolytic thermophilic anaerobic bacterial species of uncertain phylogenetic affiliation

Group	Proposed name	Former name	Strain	Habitat
I	<i>Clostridium thermocellum</i>	<i>Clostridium thermocellum</i>	LQRI	Farm soil
II	<i>Thermoanaerobacterium thermosulfurigenes</i>	<i>Clostridium thermosulfurogenes</i>	4B	Thermal spring
	<i>Thermoanaerobacterium xylanolyticum</i>	None ^a	LX-11	Thermal spring
	<i>Thermoanaerobacterium saccharolyticum</i>	None ^a	B6A-RI	Thermal spring
		<i>Thermoanaerobacter</i>	B6A	
III	<i>Thermoanaerobacter Brockii</i>	<i>Thermoanaerobium Brockii</i>	HTD4	Thermal spring
	<i>Thermoanaerobacter thermohydrosulfuricus</i>	<i>Clostridium thermohydrosulfuricum</i>	E100-69	Farm soil
	<i>Thermoanaerobacter ethanolicus</i>	<i>Thermoanaerobacter ethanolicus</i> ,	JW200,	Thermal spring
		<i>Clostridium thermohydrosulfuricum</i>	39E	

^a New isolates.

analytic thermoanaerobic species (Table 6) (245). This study found three independent groups of saccharolytic thermoanaerobes that form ethanol and lactate. On the basis of these findings, new taxonomic assignments for these organisms have been proposed, and the three groups represent three separate genera: *Clostridium* (group I), *Thermoanaerobacterium* (group II), and *Thermoanaerobacter* (group III). *Clostridium thermocellum* LQRI was the least closely related to the other seven strains and is placed in group I, retaining its original taxonomic assignment. Group II includes new isolates *Thermoanaerobacterium saccharolyticum* B6A-RI, *Thermoanaerobacterium xylanolyticum* LX-11, and *Thermoanaerobacterium thermosulfurigenes* 4B. Group III includes *Thermoanaerobacter Brockii*, *Thermoanaerobacter ethanolicus* JW200, *Thermoanaerobacter ethanolicus* 39E (formerly *Clostridium thermohydrosulfuricum* 39E), and *Thermoanaerobacter thermohydrosulfuricus* E100-69 (Table 6). Species in these three groups displayed very low homology with any species in the other groups.

These studies illustrate that although morphological and biochemical characteristics as a means of taxonomic assignment are important, DNA-DNA hybridization and 16S rRNA cataloging may prove more accurate in determining the relatedness of organisms from extreme environments. At present, spore formation is considered an important taxonomic criterion to distinguish between organisms, but often sporulation can be observed only under specialized laboratory conditions, and some anaerobes previously classified as nonsporeformers, such as *Sarcina ventriculi* (214, 266) and *Thermoanaerobacter Brockii* (81), have recently been found to sporulate, making this misleading for taxonomic assessment. 16S rRNA sequencing revealed that clostridia do not form one phylogenetic homogenous family but six sublines, which embrace both sporeforming and nonsporeforming species (74).

Archaeobacteria are considered to be the most primitive group of organisms, from which evolved eubacteria and eukaryotes as two distinct lines. The archaeobacteria consist of extreme halophiles, methanogens, and hyperthermophiles. It has been suggested that *Archaeoglobus fulgidus* could be a possible biochemical missing link among archaeobacteria, representing a transition form between an anaerobic thermophilic sulfur-based type of metabolism as found in *Thermococcus* spp. and methanogenesis as found in *Methanococcus* spp. (1). *Thermotoga maritima* represents one of the deepest-known branches in the eubacterial line of descent as measured by rRNA sequence comparisons and strongly suggests that eubacteria arose from a thermophilic ancestor (2). Further studies of the phylogenetic relation-

ships between thermophiles and anaerobes will provide information on evolution and development of the three kingdoms, archaeobacteria, eubacteria, and eukaryotes.

Physiology, Biochemistry, and Genetics

Overview. Interestingly, thermoanaerobes have been used as model systems to provide information which has formed the basis for our understanding of major metabolic groups of anaerobes such as methanogens, acetogens, and ethanol producers. Thermoanaerobes grow readily, and the enzymes of these model organisms are thermostable and hence easy to work with, which has helped prove their metabolic pathways for carbon utilization and end product formation.

Catabolism and autotrophy of methanogens and acetogens. (i) **Methanogenesis and autotrophy.** Methanogens which grow on H₂ plus CO₂ utilize unique C₁ metabolic pathways (32, 353, 500). CO₂ fixation into methane and cell carbon was elucidated by detailed studies on *Methanobacterium thermoautotrophicum* (141, 496), one of the most extensively studied methanogens. This species grows well on H₂-CO₂ and poorly on CO as the sole carbon and energy sources (87, 506). *M. thermoautotrophicum* synthesizes cell precursors by C₁ transformation reactions that schematically resemble those of acetogens in the utilization of certain catabolic reactions for anabolism. A methyl-carbonyl condensation reaction for C₂ synthesis is catalyzed by CO dehydrogenase, which also functions in the synthesis of the carbonyl group that becomes the C-1 of acetyl coenzyme A (acetyl-CoA) or acetate (202, 411). The synthesis of oxaloacetate from pyruvate and the synthesis of glutamate differ in methanogenic species. *M. thermoautotrophicum* synthesizes glutamate reductively via fumarate reductase and α -ketoglutarate dehydrogenase (500). To date it has not been demonstrated that a methanogenic species has a complete tricarboxylic acid cycle. *M. thermoautotrophicum* synthesizes oxaloacetate via phosphoenolpyruvate carboxylase (202), and, although the thermophilic methanococci lack this enzyme, their pathway of carbon assimilation resembles that in *M. thermoautotrophicum*.

Two hydrogenases have been purified and characterized from extracts of *M. thermoautotrophicum* (139, 218, 443). More recently, genes encoding a hydrogenase from *M. thermoautotrophicum* were cloned and sequenced (344) and were found to be tightly linked to an adjacent gene whose product was predicted to contain six randomly repeated polyferredoxin-like domains which could contain as many as 48 iron atoms in 12 Fe₄S₄ clusters. This is the first example of a polypeptide containing multiple, tandemly repeated

bacterial ferredoxin-like domains (344). Two of the genes encoding the methyl viologen-reducing hydrogenase of *M. thermoautotrophicum* encode the same conserved cysteinyl- and histidinyll-containing peptides found in the small and large subunits of all the eubacterial (NiFe)-hydrogenases. These archaeobacterial genes must therefore have evolved from the same ancestral sequence that gave rise to the genes which encode hydrogenases in many eubacteria (343).

Formylmethanofuran:tetrahydromethanopterin formyltransferase (FTRase) has been demonstrated to be an essential enzyme in the biosynthesis of methane (109) and has been cloned and sequenced (102). The sequence was not homologous to any other sequenced proteins, including proteins which use pterin substrates, and suggests that FTRase has a novel pterin-binding site (102). Comparison of the predicted amino acid sequences for the five methyl reductase genes from the thermophiles *Methanothermobacter feravidus* and *Methanobacterium thermoautotrophicum*, and the mesophiles *Methanococcus vannielii* and *Methanosarcina barkeri* showed a greater percent identity between the genes from the two thermophiles (446). The genes encoding the four largest subunits of the RNA polymerase of *Methanobacterium thermoautotrophicum* were found to be more strongly homologous to the eukaryotic than the eubacterial RNA polymerase genes (31).

(ii) **Acetogenesis and autotrophy of *C. thermoacetikum*.** Homoacetogens share with methanogens the ability to grow well on H₂ plus CO₂ and poorly on CO (204) and to form acetyl CoA by using a CO-dependent pathway involving CO dehydrogenase (470). *Clostridium thermoacetikum* and *Clostridium thermoautotrophicum* are homoacetogens and synthesize acetate from C₁ compounds by the recently established Wood pathway of acetyl CoA synthesis, named after Harland G. Wood for his significant contributions to this field (469). This pathway was largely established by studies with *C. thermoacetikum*, which synthesizes acetate from CO₂ but was considered to be a heterotroph until (i) hydrogenase activity was detected in cell extracts (111) and (ii) it was found that the organism can grow with CO or CO₂-H₂ as carbon and energy sources (204). More recently, several of the genes encoding enzymes in the Wood pathway have been cloned and sequenced, including the thermostable formyltetrahydrofolate synthetase (FTHFS) (263). A high level of amino acid sequence conservation between the *C. thermoacetikum* FTHFS and the same enzyme from *Clostridium acidurici* and the FTHFS domains of the *Saccharomyces cerevisiae* C₁-tetrahydrofolate synthetases was found, and the hydrophobicity profiles of the two clostridial enzymes were very similar and did not support the idea that large hydrophobic domains play an important role in thermostabilizing the *C. thermoacetikum* FTHFS (263). The five genes which encode the key proteins involved in acetyl CoA synthesis in *C. thermoacetikum*, CO dehydrogenase (CODH), the corrinoid/FeS protein, and methyltransferase were cloned into *Escherichia coli* (350). Both the corrinoid/FeS protein and CODH, although expressed at high levels and with identical subunit molecular weights in *E. coli*, were inactive and less heat stable than were the native enzymes from *C. thermoacetikum* (350).

(iii) **Novel properties of sulfur/sulfate/thiosulfate reducers and other species.** Almost half of the eubacterial thermophiles form H₂S from the reduction of elemental sulfur, sulfite, thiosulfate, or sulfate (Table 3); they include organisms from the genera *Clostridium*, *Desulfurella*, *Desulfotomaculum*, *Ferrobacterium*, *Thermoanaerobium*, *Thermobacteroides*, *Thermosiphon*, and *Thermotoga*. *Ther-*

moanaerobacterium thermosulfurigenes (376) and *Thermoanaerobacterium saccharolyticum* B6A-RI and LX-11 (Fig. 2) are the only thermophiles that produce elemental sulfur from thiosulfate, which is deposited on the cell surface and in the culture medium (245). These organisms use sulfur compounds as electron acceptors, which is a marked difference from anaerobic phototrophs, which use sulfur compounds as electron donors.

All of the hyperthermophiles, with the exception of the methanogens, utilize elemental sulfur as a catabolic electron acceptor, with the formation of H₂S (Table 5). This type of chemolithoautotrophy, the sulfur respiration, and the mechanisms involved could be primeval and should be considered possible precursors of other types of hydrogen oxidation or respiration (134). Of the methanogens, *Methanococcus jannaschii* has an obligate requirement for sulfide (196), as does *Methanobacterium thermoautotrophicum* (352). These two organisms, together with *Methanococcus thermolithotrophicus*, are able to grow with mercaptans, such as methanethiol and ethanethiol, and with dimethyl sulfoxide (341). Of the archaeobacterial thermophiles, only *Methanococcus thermolithotrophicus* can utilize sulfate (86), a growth characteristic which is far more common among the eubacterial thermophiles. The sulfate reducer *Thermodesulfotobacterium commune* has a novel type of bisulfite reductase, differing from the homologous enzymes by its adsorption spectrum, siroheme content, and thermostability (159).

Ethanolic fermentation of saccharides. Thermoanaerobes have also been used as model organisms to study saccharide fermentations for ethanol production (494). Unlike yeasts and *Zymomonas mobilis*, which possess pyruvate decarboxylase for the metabolism of pyruvate to acetaldehyde and then subsequent reduction to ethanol, the alternative route for ethanol production involves acetyl-CoA as an intermediate in the metabolism of pyruvate to acetaldehyde. Thermoanaerobes have provided a great deal of information on ethanol fermentation by the pathway with acetyl-CoA as an intermediate (230), which can be applied to understanding ethanol production in other anaerobic bacteria, including *Clostridium thermocellum*, *Clostridium thermosaccharolyticum*, *Thermoanaerobacter thermohydrosulfuricus*, *Thermoanaerobacter brockii*, and *Thermoanaerobacter ethanolicus*. Ethanol yields of heterofermentations vary considerably with the specific growth conditions used. The biochemical basis for different reduced end product ratios of thermophilic ethanol producers that contain the same glycolytic pathways is related to subtle differences in the specific activities and regulatory properties of the enzymes which control electron flow during fermentation. Similarly specific changes in culture conditions such as temperature and pH influence the rate and direction of the enzymatic machinery responsible for end product formation.

(i) **Comparison of carbon and electron flow in *Thermoanaerobacter brockii* and *Clostridium thermocellum*.** Metabolic control of end product formation in thermophilic ethanol-producing organisms has been best characterized in *Thermoanaerobacter brockii* and *Clostridium thermocellum* (26, 230). Fermentation of cellobiose under identical conditions yielded a reduced product ratio (in micromoles) of 224:20:352 (ethanol to H₂ to lactic acid) in *T. brockii* and a ratio of 157:285:24 in *C. thermocellum*. The ethanol yield was higher in *T. brockii* as a consequence of electron flow from pyruvate to ethanol via pyruvate-ferredoxin reductase, ferredoxin NADP reductase, and both NAD- and NADP-acetaldehyde reductase. Ferredoxin-NAD reductase and NADP-acetaldehyde reductase were not detected in cellobiose-grown *C.*

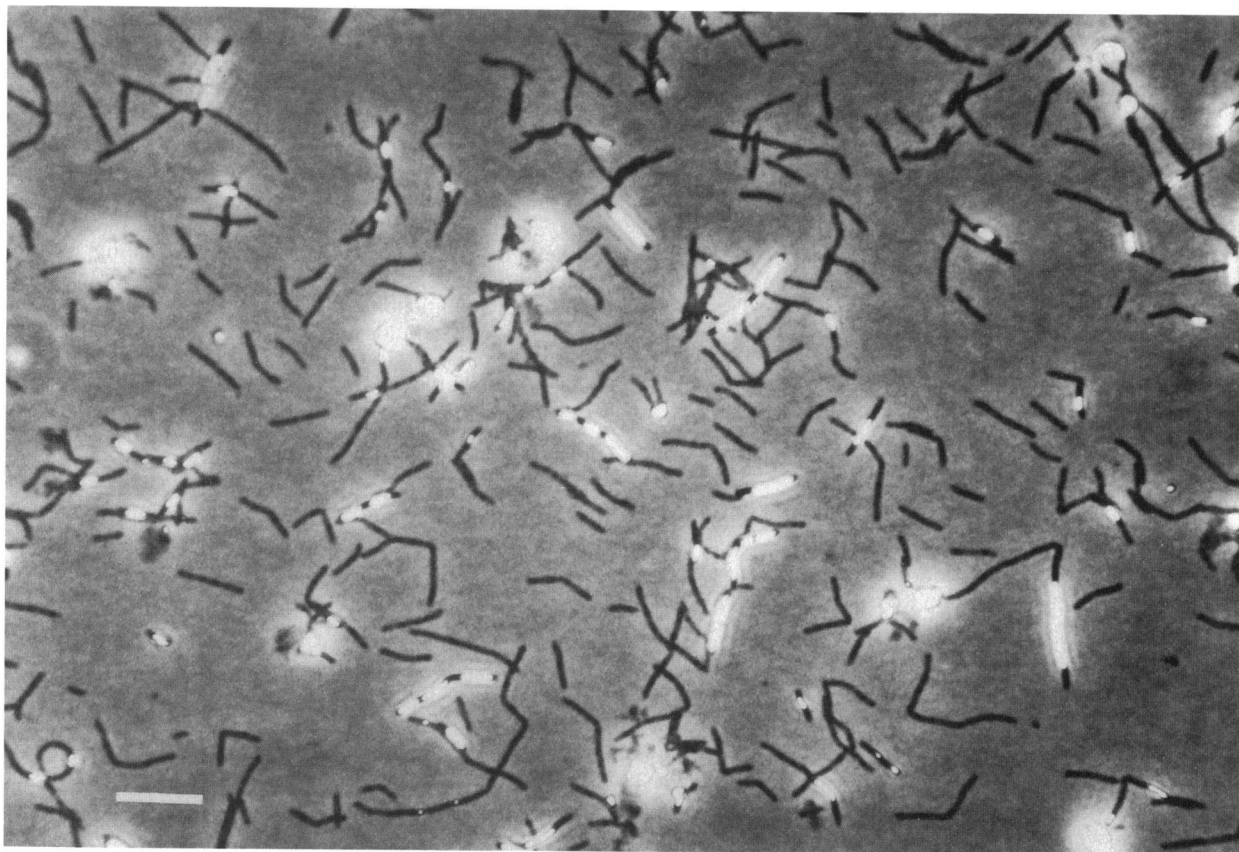


FIG. 2. Phase-contrast photomicrograph of sulfur-depositing cultures of *Thermoanaerobacterium saccharolyticum* B6A-R1. Note that the phase-bright sulfur accumulates in the medium and on the cells. Bar, 3 μm . Reprinted from references 244 and 245 with permission.

thermocellum cells. The hydrogen yield was higher in *C. thermocellum* because of the higher hydrogenase activity and the absence of electron flow from reduced ferredoxin to lactate or ethanol. The higher lactic acid yield of *T. brockii* was related to the ninefold-higher level of fructose 1,6-diphosphate within the cells. Hence, lactic acid production was regulated in part by the amount of allosteric activator (i.e., fructose 1,6-diphosphate) present for lactate dehydrogenase activity. The amount of ethanol produced by *T. brockii* is related to the intracellular FDP concentration. During growth on starch, the FDP concentration was 25 times lower and the ethanol/lactate fermentation ratio was 4 times higher than that observed on glucose (26).

(ii) **Properties of alcohol dehydrogenases from thermoanaerobes.** A variety of alcohol dehydrogenases which couple to either NAD^+ or NADP^+ have been detected in thermophilic anaerobes. Both *Thermoanaerobacter brockii* HTD4 and *Thermoanaerobacter ethanolicus* 39E contain a novel, thermoactive NADP-linked alcohol dehydrogenase that is absent in other ethanol-producing thermoanaerobes such as *Clostridium thermocellum* and *Thermobacteroides acetioethylicus*, in addition to a NAD-linked alcohol dehydrogenase (232). The secondary alcohol dehydrogenase from *Thermoanaerobacter brockii* couples to NADP^+ and has been studied in the greatest detail. This enzyme, termed NADP-linked alcohol-aldehyde/ketone oxidoreductase, showed highest activity with secondary alcohols, had moderate activity with ketones, and was least active with primary alcohols (232). The substrate specificity and reversibility of

this enzyme appear well related to the metabolic functions of the enzyme during growth. In *Thermoanaerobacter brockii* ethanol is formed as a consequence of electron flow from pyruvate to ethanol via pyruvate-ferredoxin reductase, ferredoxin-NAD and -NADP reductase, and both NAD and NADP acetaldehyde reductase (230). In addition to providing two possible routes for ethanol formation, by coupling to either NAD^+ or NADP^+ , enabling the organism to continue active metabolism with changing levels of nucleotide pools, the NADP-linked alcohol-aldehyde/ketone oxidoreductase can operate in the reverse direction. Ethanol can be oxidized by acetaldehyde, or, if ketones are added to the medium of glucose-fermenting cells, stoichiometric amounts of the respective alcohols are formed (232).

Thermoanaerobacter ethanolicus JW200 has two different alcohol dehydrogenases, and these primary and secondary alcohol dehydrogenases have also been purified and studied (59). The secondary alcohol dehydrogenase is synthesized early during growth and has a relatively low K_m for acetaldehyde (44.8 mM) in comparison with that of the primary alcohol dehydrogenase ($K_m = 210$ mM), and its activity is enhanced by pyruvate, supporting the idea that the enzyme is responsible for ethanol formation at least during the early part of growth. The primary alcohol dehydrogenase is formed late in the fermentation and may be involved in converting alcohols to aldehydes to be used as alternative energy sources (59).

(iii) **Novel properties of other ethanol-producing species.** *Thermoanaerobacter ethanolicus* 39E has a low tolerance

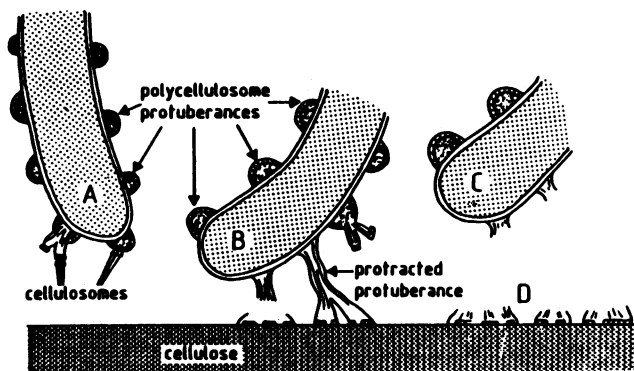


FIG. 3. Cellulosome of *Clostridium thermocellum*. Interaction of *Clostridium thermocellum* cells with cellulose is mediated by protracted polycellulosome protuberances. Cell A, prior to contact; cell B, following contact; cell C, following attachment. Bar, 1.0 μm . Reprinted from reference 361 with permission.

for ethanol, with growth inhibition occurring at 2% (wt/vol) ethanol. An ethanol-tolerant strain (39EA), which was tolerant to 4% (wt/vol) ethanol at 60°C and produced ethanol under these conditions (265), was selected; another strain (H8) requires 4% (wt/vol) ethanol for optimal growth and grows in the presence of 8% (wt/vol) ethanol but produces lactic acid at high solvent concentration (385). The mechanism for moderate (i.e., 4%) ethanol tolerance in *T. ethanolicus* 39EA was a lack of detectable levels of ferredoxin-NAD reductase and NAD-linked alcohol dehydrogenase activities, which were present in the wild-type strain (264). As a consequence of these enzymatic differences, the pool levels of NADH did not increase, preventing inhibition of glyceraldehyde-3-phosphate dehydrogenase. End product inhibition in *T. ethanolicus* appears to be a consequence of reverse electron flow, since the wild-type strain can consume both hydrogen and ethanol as electron donors, a process which also occurs in other glucose-fermenting thermoanaerobes including *Thermoanaerobacter brockii* (230).

The mechanism of high (i.e., 8%) ethanol tolerance of *T. ethanolicus* is related to the unique transmembrane lipids (C_{30} to C_{34} fatty acids) which are present. Alcohol increases membrane fluidity, and these transmembrane lipids may serve to reduce fluidity and maintain membrane integrity (165).

Biopolymer degradation mechanisms. Thermoanaerobes possess amyolytic, xylanolytic, and cellulolytic enzyme activities. A great deal of our understanding of cellulose degradation has come from studying the cellulose system in anaerobes, in particular *Clostridium thermocellum*. In general, hydrolytic enzymes from moderate thermoanaerobes appear to be largely cell associated and are secreted when the growth substrate is limiting.

(i) **Cellulolytic system of *C. thermocellum*.** Perhaps the biopolymer degradation system which has the greatest significance, not only to thermophiles but also to anaerobic and aerobic mesophiles, is the cellulase complex produced by *C. thermocellum*. In this organism, as well as in other cellulolytic organisms, although good growth occurs on crystalline cellulose, relatively little extracellular cellulase is found. During the course of studies on the cellulase system of *C. thermocellum*, a very high molecular weight, multifunctional, multienzyme complex was purified and has been termed the cellulosome (Fig. 3) (226, 228, 229). In addition to containing most of the endoglucanase activity in the organ-

ism, the cellulosome was responsible for the adhesion of *C. thermocellum* to the insoluble cellulosic substrate. Interestingly, the purified cellulosome (228) had all the properties of the crude cellulase system of *C. thermocellum* (307).

The presence of such an enzyme complex does not appear to be limited to *C. thermocellum* or to cellulases. The cellulosome has been shown to be an integral part of at least five different strains of *C. thermocellum* (225), and in addition, a variety of cellulolytic bacteria have been shown to possess cellulosome-like complexes, including mesophilic anaerobic bacteria and one aerobic organism (225), illustrating that the cellulosome may be a widespread system for the degradation of cellulose. Recently, xylanase activity from *C. thermocellum* was found to be localized in both the cellulosome and the noncellulosome fractions (297).

Probably the largest undertaking with recombinant DNA technology has been the cloning of the cellulase genes from *C. thermocellum* into *E. coli*. The work has been carried out mainly by the group at the Institut Pasteur (291) and has demonstrated that the cellulase genes are not clustered and that they constitute about one-third of the *C. thermocellum* genome. However, a cellulolytic system with the unique ability to degrade crystalline cellulose, as found in the parent organism, has not been reconstructed, despite the cloning of about 20 genes.

(ii) **Amyolytic systems of thermoanaerobes.** *Thermoanaerobacter ethanolicus* 39E readily degrades starch and possesses pullulanolytic and amyolytic activities with unique thermoactive and thermostable characteristics (181). The organism contains α -glucanase and trehalase activities (179). A novel amylopullulanase activity that cleaves α -1,4 linkages in starch and α -1,6 linkages in pullulan has been characterized from this organism (277, 279, 363). *Thermoanaerobacter ethanolicus* 39E was also found to have a cyclodextrinase activity, which displayed greater hydrolysis activity on α - and β -cyclodextrins than on starch (364). This enzyme cleaves cyclodextrin in a multiple-attack manner by opening up the ring of the cyclic dextrin and then degrading the linear dextrin molecules to smaller molecules. In addition, this organism displays an α -glucosidase activity which is distinct from amylopullulanase and cyclodextrinase, having no activity on the substrates for these enzymes but cleaving maltose (365). The starch-hydrolyzing system of this organism differs from the systems found in aerobic mesophilic bacteria or fungi which contain α -amylase and glucoamylase. The α -glucosidase of *T. ethanolicus* 39E should play an important role in the formation of glucose by acting on low-molecular-weight oligosaccharides produced by the action of the amylopullulanase.

Amylopullulanase has been purified from a number of strains of *T. thermohydrosulfuricus* E100-69, including E101-69 (288) and 39E (363), and has a unique mode of action in that it displays both α -amylase and pullulanase activities (277, 278, 288), acting on amylose (α -1,4 linkages) and specifically hydrolyzing the α -1,6 linkages in pullulan. The gene encoding this amylopullulanase in *T. ethanolicus* 39E has been cloned and expressed in *E. coli* and *B. subtilis*, with the resulting protein being thermostable and having a single active site with dual specificity (277, 279). Pullulanase activities have also been reported to be produced by other strains of *T. ethanolicus* (9, 287, 338) and by *T. thermohydrosulfuricus* E100-69, in which α -amylase and pullulanase activities are attributed to a single enzyme with two active sites.

Pullulanase from *T. brockii* has also been cloned into *E. coli* and *B. subtilis* (79). In the host organism, the enzyme was glycosylated and thermostable, properties which were

absent after cloning and expression in *B. subtilis*. The enzyme also has dual specificity, cleaving both α -1,6 glucosidic linkages in pullulan and α -1,4 linkages in starch, demonstrating for the first time that a single enzyme could have two catalytic activities (79). Other amylopullulanases from *Clostridium thermosulfurigenes* EM1, *Thermoanaerobacterium thermosulfurigenes* 4B *Clostridium thermosaccharolyticum*, *Thermoanaerobacter finnii*, *Thermobacteroides acetoethylicus*, and *Dictyoglomus thermophilum* also have this dual specificity (8). An enzyme having dual specificity for pullulan and starch has also been purified from *Thermoanaerobacterium thermosulfurigenes* (395) and cloned into *E. coli*. There was no difference in the temperature optimum and thermostability between the original and the cloned enzyme (67).

Conclusive evidence for the presence of one single active site with an enzyme having the ability to cleave both α -1,6 and α -1,4 bonds was demonstrated with detailed sequence analysis of the amylopullulanase gene from *Thermoanaerobacter ethanolicus* 39E (277, 279). The active site for both α -amylase and pullulanase was located by using nested deletion mutants, and sequence comparison with α -amylases identified four conserved peptide regions responsible for catalysis and substrate binding. That pullulanase activity was also present within the DNA fragment was shown by using site-directed mutagenesis, whereby substitution of Asp-625 and Asp-734 with Asn or Glu and substitution of Glu-657 with Gln or Asp resulted in loss of both α -amylase and pullulanase activity (277, 279), demonstrating that the same amino acids were involved in catalysis.

The synthesis of amylopullulanase in this organism was inducible and subject to catabolite repression. Catabolite repression-resistant mutants which displayed improved starch metabolism in terms of enhanced rates of growth, ethanol production, and starch consumption were isolated (183). In chemostat cultures, both wild-type and mutant strains produced amylopullulanase at high levels in starch-limited chemostats but not in glucose- or xylose-limited chemostats. The enzyme was excreted into the medium when the organism was grown in continuous culture under maltose-limited conditions and was cell bound during batch culture (277). The presence of cell surface structures as visualized by scanning electron microscopy coincided with cell-bound amylopullulanase and could represent an enzyme complex responsible for starch hydrolysis (277, 279).

In *Thermoanaerobacter thermohydrosulfuricus* E100-69, the formation of pullulanase was dependent on growth and occurred predominantly in the exponential phase. The enzyme was largely cell bound during growth of the organism on 0.5% starch. When the organism was grown in continuous culture on a defined medium containing growth-limiting amounts of starch, pullulanase and α -amylase activities were overproduced and a partial disintegration of the cell surface layer occurred, associated with the formation of membrane blebs and extracellular vesicles (10).

Thermoanaerobacterium thermosulfurigenes 4B produces an extracellular β -amylase, which has been purified and characterized (362, 386). Higher levels of β -amylase were produced by *Thermoanaerobacterium thermosulfurigenes* 4B in continuous culture at optimal pH and temperature for growth of the organism and enzyme production, and high substrate concentrations were used (309). Hyun and Zeikus (180, 182) found that β -amylase synthesis in *Thermoanaerobacterium thermosulfurigenes* 4B was inducible and subject to catabolite repression. A hyperproductive mutant which produced eightfold more β -amylase than the wild type

did was isolated, and synthesis of the enzyme was both constitutive and resistant to catabolite repression.

Coculture of *Thermoanaerobacterium thermosulfurigenes* with *Thermoanaerobacter ethanolicus* 39E demonstrated that starch fermentation dramatically improved as a consequence of the coordinate action of amyolytic enzymes and synergistic metabolic interactions between the two species. In monoculture, neither species completely degraded starch, whereas in coculture the substrate was completely hydrolyzed. In monoculture starch fermentation, *Thermoanaerobacter ethanolicus* produced lower levels of pullulanase and glucoamylase, whereas *Thermoanaerobacterium thermosulfurigenes* produced lower levels of β -amylase and glucoamylase. In coculture fermentation, improved starch metabolism by each species occurred, with increased amounts and rates of starch consumption, amylase production, and ethanol formation (182). Therefore, it would appear that these thermoanaerobes have different starch-degrading enzymes.

(iii) **Xylanolytic systems of thermoanaerobes.** Little is known about the hemicellulases from thermophilic anaerobic bacteria that grow rapidly on insoluble xylan. Wiegel et al. (455, 459) studied a variety of thermophilic, anaerobic, saccharolytic bacteria, including *Thermoanaerobacter ethanolicus*, *Thermobacteroides acetoethylicus*, *Thermoanaerobacter Brockii*, and *Clostridium thermocellum*, which all ferment xylan, albeit very slowly. Studies were also conducted on *Thermoanaerobacter* strain B6A (now *Thermoanaerobacterium saccharolyticum* B6A [245]), an organism isolated from an algal mat present in Big Spring, Thermopolis, Wyo. (449), which was shown to extensively degrade xylan (447). Later, *Thermoanaerobacterium saccharolyticum* B6A was shown to possess a number of saccharidases, including amylase, glucose isomerase, and high levels of endoxylanase (241).

Recently a new isolate, *Thermoanaerobacterium saccharolyticum* B6A-RI, was isolated from Frying Pan Springs in Yellowstone National Park (245). This organism has very active xylanases but is not cellulolytic, and it produces endoxylanase, β -xylosidase, arabinofuranosidase, acetyl esterase, and xylose isomerase, with the first three enzymes produced coordinately (247). Negatively charged cell surface structures were visualized in cells growing on xylan, and they coincided with the production of cell-bound endoxylanases and the ability of cells to bind tightly to xylan. From transmission electron micrographs, these protruberances on the cell surface appeared to be part of the S-layer and may be analogous to the cellulosome, but they were specific for xylan adhesion and hydrolysis (246).

Thermoanaerobacterium saccharolyticum B6A-RI produces multiple endoxylanases, which are glycosylated and thermostable. Endoxylanase (*xynA*), β -xylosidase (*xynB*) and xylose isomerase (*xylA*) genes were cloned in *E. coli* and sequenced, and the expressed enzymes were purified and characterized (249). *xynB* and *xynA* are closely situated on the chromosome of *Thermoanaerobacterium saccharolyticum* B6A-RI. The deduced amino acid sequence of xylose isomerase showed very high homology to those from other thermoanaerobes but not from mesophilic aerobes. *xynA* was similar to genes from family F β -glycanases grouped by hydrophobic cluster analysis. This multiple alignment of amino acid sequences revealed six highly conserved motifs, which included the consensus sequence ITEL D, in the catalytic domain. Three aspartic acids, two glutamic acids, and one histidine were conserved in all six enzymes aligned, and they were targeted for analysis by site-specific mutagenesis. Substitution of Asp-612 by Asn, Glu-508 by Gln, and

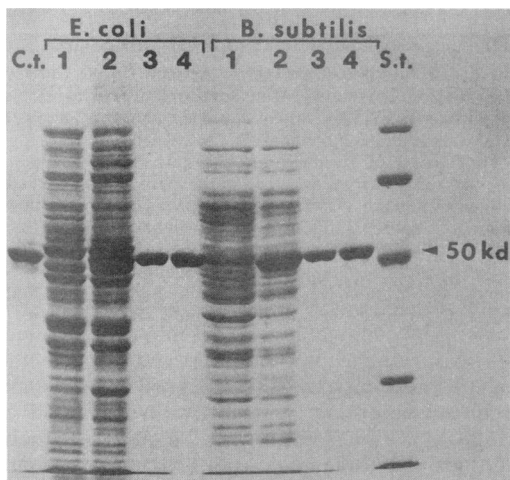


FIG. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of thermophilic glucose isomerase activity fractions showing single-step heat treatment purification of recombinant *E. coli* (lane 3) and *B. subtilis* (lane 3). Lanes: C.t., glucose isomerase purified from *C. thermosulfurogenes*; 1, whole-cell preparation from *E. coli* W595(pHSG262) or *B. subtilis* NA1(pTB523) that did not carry the DNA insert; 2, whole-cell preparation from *E. coli* W595(pCG138) or *B. subtilis* NA1(pMLG1); 3, soluble fraction from heat-treated cell extract of *E. coli* W595(pCG138) or *B. subtilis* NA1(pMLG1); 4, glucose isomerase purified from *E. coli* W595(pCG138) or *B. subtilis* NA1(pMLG1); S.t., molecular weight standards (from the top: phosphorylase *b*, 97,400; bovine serum albumin, 66,200; ovalbumin, 42,700; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500). kd, kilodaltons. Reprinted from reference 240 with permission.

His-539 by Asn had little effect on the enzyme activity, whereas substitution of Asp-504 and Asp-569 by Asn and of Glu-567 by Gln completely destroyed endoxylanase activity and implicated these amino acids in a general acid catalysis mechanism (244, 249).

Glucose/xylose isomerase from *Thermoanaerobacterium thermosulfurigenes* 4B has been purified (242) and cloned and expressed in *E. coli* and *B. subtilis* (240). Glucose/xylose isomerase from *T. thermosulfurigenes* 4B was found to be thermostable and required metal ions for enzyme activity and stability (242). The protein produced from the cloned gene was found to be thermostable, yielding a rapid purification procedure involving heating the *E. coli* or *B. subtilis* cell extract at 85°C for 15 min to generate 80% pure glucose/xylose isomerase (Fig. 4). Sequence comparison of the deduced amino acid sequence from the structural gene (*xyiA*) for glucose/xylose isomerase from *T. thermosulfurigenes* 4B showed higher homology with those of the thermolabile glucose/xylose isomerase from *B. subtilis* (70%) and *E. coli* (50%) than with those of thermostable xylose isomerases from the aerobes *Ampullariella* spp. (22%), *Arthrobacter* spp. (23%), and *Streptomyces violaceoniger* (24%) (239). By using site-directed mutagenesis, histidine residues at four different positions in the *T. thermosulfurigenes* 4B enzyme were individually modified, with the finding that the His-101 was part of the catalytic site (239).

Adaptation Mechanisms

Overview. Comparison of growth characteristics of thermophilic bacteria with temperature optima between 55 and

70°C shows that the growth rate in complex media ranges between 11 and 16 min, in contrast to the mesophiles *E. coli* and *B. subtilis*, with generation times of 21 and 26 min, respectively (50).

With temperature there is no dispute about the internal environment experienced by the organism, since this is an environmental parameter which cannot be regulated, and therefore a means of adaptation is to possess cellular components which are stable to temperature. A large number of enzymes from thermophiles have been found to be very thermostable (502, 503), and interestingly, these enzymes also have unusual stability to organic solvents, detergents, proteolytic agents, and pH extremes (135).

A variety of enzymes have been purified and characterized from moderate thermoanaerobic bacteria (Table 6). These include endoglucanase (308), alcohol dehydrogenase (232), β -amylase (386), amylopullulanase (363), and xylose (glucose) isomerase (242). Detailed structural studies of amino acid sequence and three-dimensional structure of thermophilic enzymes have been performed to find an explanation of their stability at high temperature. Indeed, it is unlikely that one mechanism could account for such stability in diverse enzymes, and current evidence suggests that enzyme stability is a result of multiple factors including intramolecular hydrophobic interactions, hydrogen bonding, β -turns, disulfide bonds, metal binding, glycosylation, and stabilizing cofactors such as polyamines. In general, enzymes from thermoanaerobes display temperature optima for activity that are close to or above the optimum temperature for growth of the organism.

The molecular mechanism to explain why thermophilic enzymes are active at high temperatures but not low temperatures has not been established. A number of genes encoding enzymes from thermoanaerobes have been cloned into either *E. coli* or *B. subtilis*, including those encoding cellulases and xylanase from *Clostridium thermocellum* (297), β -amylase from *Thermoanaerobacterium thermosulfurigenes* (210), xylose isomerase from the same organism (240), a pullulanase from *Thermoanaerobacter brockii* (79), and endoxylanase, β -xylosidase, xylose isomerase (244, 249), and amylopullulanase from *Thermoanaerobacterium saccharolyticum* B6A-RI (248). Comparison of the cloned enzyme properties with those of the host enzyme did not reveal one unifying factor which could account for thermostability.

One interesting difference between moderate thermophiles and hyperthermophiles is the lack of spore formation in the latter group. To date, spore formation has not been reported in hyperthermophiles, although many of the moderate thermoanaerobes form them. It is possible that there is a great need for a cell to be able to sporulate when existing in an environment such as the hydrothermal vents, where the temperature differences can be much more extreme than, for example, some of the niches inhabited by the moderate thermophiles.

The extreme heat resistance of spores from thermophilic anaerobes including *Clostridium thermosaccharolyticum* (479), *Desulfotomaculum nigrificans* (108), *Clostridium thermocellum* LQR1, *Thermoanaerobacterium thermosulfurigenes* 4B, and *Thermoanaerobacter ethanolicus* 4B (184) has been reported, and the spores of the last organism were more heat resistant than the spores of *Bacillus stearothermophilus*, which is commonly used as the standard to judge autoclaving procedures for materials used in microbial culture work.

Thermostable enzymes. (i) **General features.** Table 7 lists

TABLE 7. Enzymes from anaerobic moderate thermophiles

Enzyme	Optimal temp (°C)	Thermostability	Organism	Reference
Xylose/glucose isomerase	80	22 min at 90°C	<i>Thermoanaerobacterium thermosulfurigenes</i>	242
Endo-acting amylopullulanase	75	5 h at 70°C, 45 min at 75°C	<i>Thermoanaerobacterium saccharolyticum</i> B6A	360
Pullulanase	90	30 min at 95°C	<i>Clostridium thermohydrosulfuricum</i> Z 21-109	363
Cyclodextrinase	65	402 min at 60°C, 180 min at 65°C, 75 min at 70°C	<i>Thermoanaerobacter ethanolicus</i> (39E)	364
Pullulanase	80	17 min at 85°C, 5 min at 90°C	<i>Thermoanaerobium</i> strain ToK6-B1	337
NADP-linked alcohol-aldehyde/ketone oxidoreductase	65	22 min at 91°C	<i>Thermoanaerobacter brockii</i>	232
L-Malate:NADP ⁺ oxidoreductase	40	10 min at 72°C	<i>Clostridium thermocellum</i>	231

some representative examples of enzymes characterized from moderate thermoanaerobes, and Table 8 lists the characteristics of all enzymes isolated to date from anaerobic hyperthermophiles. In general, the enzymes studied from moderate thermoanaerobes are active at temperatures above the optimum growth temperature and are thermostable. As would be expected, the enzymes from hyperthermophiles have higher temperature optima and increased thermostability than do enzymes from moderate thermoanaerobes. All of the extracellular enzymes are insensitive to oxygen, as are some of the cytoplasmic enzymes, depending on whether the reaction occurs at a low redox potential. Two hydrogenases from *Methanococcus jannaschii* have temperature optima corresponding to the growth temperature optima of 85°C, and although one is relatively thermostable, with a half-life of 37 min at 85°C, the F₄₂₀-reactive hydrogenase has a half-life of only 1.2 min (383). The hydrogenase from *Pyrodictium brockii* is structurally and functionally similar to hydrogenase from the mesophile *Bradyrhizobium japonicum*, differing only in their temperature optima (336), and further comparisons may provide insight into the mechanisms for thermophilicity.

Amylolytic enzymes from *Pyrococcus furiosus* all have temperature optima of at least 100°C and exhibit remarkable stability (Table 7). The α -glucosidase activity has a broad temperature optimum from 105 to 115°C and a half-life of 46

to 48 h at 98°C (83). The hydrogenase from this organism has a similar temperature optimum and possesses properties of hydrogenases found in some aerobic and anaerobic eubacteria and archaeobacteria, providing further support for the hypothesis that the extremely thermophilic, sulfur-metabolizing bacteria have an ancient and primitive phenotype (58).

The recent observation that the genes encoding a number of hydrogenases from thermoanaerobes are linked to adjacent genes encoding a polyferredoxin was an unusual finding. This polyferredoxin has been found in *Methanobacterium thermoautotrophicum* (344) and *Methanothermobacter fervidus* (398), and both polyferredoxins are predicted to contain six domains, suggesting that the hexameric structure is significant. Although some amino acids differ when the two sequences are compared, these changes do not compromise the predicted formation of 12 Fe₄S₄ centers and the predominantly α -helical structure (398). The polymeric nature of the polypeptide suggests that electrons could be transferred from one Fe₄S₄ center of the molecule to the next, possibly spanning the membrane. Alternatively, such a cellular component may provide a very reduced environment, protecting methanogens from brief exposure to oxygen (344).

(ii) **Unique catalytic activities.** One unique feature of the secondary NADP-linked alcohol dehydrogenase from *Thermoanaerobacter brockii* was that the secondary alcohol

TABLE 8. Enzymes from anaerobic hyperthermophiles

Enzyme	Optimal temp (°C)	Thermostability (<i>t</i> _{1/2})	Organism	Reference
Hydrogenase (H ₂ evolution)	95	2 h at 100°C	<i>Pyrococcus furiosus</i>	58
Hydrogenase (H ₂ oxidation)	-87	NR ^a	<i>Pyrodictium brockii</i>	336
Hydrogenase				
F ₄₂₀ reactive	80-90	3 h at 70°C	<i>Methanococcus jannaschii</i>	383
F ₄₂₀ nonreactive	80	9 h at 70°C	<i>Methanococcus jannaschii</i>	383
Glyceraldehyde-3-phosphate dehydrogenase	ND ^b (substrate was unstable)	44 min at 100°C	<i>Pyrococcus woesei</i>	530
DNA-dependent RNA polymerase	86	135 min at 100°C	<i>Thermoproteus tenax</i>	522
α -Glucosidase	>115	1 h at 105°C	<i>Pyrococcus furiosus</i>	57
Pullulanase	105	30 min at 105°C	<i>Pyrococcus furiosus</i>	57
α -Amylase	>108	30 min at 105°C	<i>Pyrococcus furiosus</i>	57
Amylase complex	NR	2 h at 90°C	<i>Dictyoglomus thermophilum</i>	215
Amylase	100	2 h at 120°C	<i>Pyrococcus furiosus</i>	217
α -Glucosidase	105-115	48 h at 98°C	<i>Pyrococcus furiosus</i>	83
Serine protease	115	4 h at 100°C	<i>Pyrococcus furiosus</i>	117
Lactate dehydrogenase	>98	150 min at 90°C	<i>Thermotoga maritima</i>	473
ATP sulfurylase	90	NR	<i>Archaeoglobus fulgidus</i>	84
Aldehyde ferredoxin oxidoreductase	>90	NR	<i>Pyrococcus furiosus</i>	302

^a NR, not reported.

^b ND, not determined.

dehydrogenase activity temperature dependence was biphasic with a notable deflexion point at near 50°C, although the Q_{10} values both below and above the 50°C breakpoint were above 2.0 (334). The reasons for the observed biphasic Arrhenius plots for the *Thermoanaerobacter brockii* enzyme could be related to the differential effect of temperature on binding parameters in the enzyme-substrate-NADP(H) complex (529). Alternatively, the temperature dependence may indicate a conformational change of the enzyme at 50°C, and this effect has been reported for some other thermophilic enzymes (529). Another enzyme from a thermoanaerobe which has been shown to have a biphasic temperature dependence is the amylopullulanase from *Thermoanaerobacter ethanolicus*, with a deflexion point at 65°C (279).

Another interesting feature of the starch-degrading enzymes is the dual specificity of amylopullulanase, which appears to be common for this enzyme from a number of different thermoanaerobes. Sequencing data of the gene encoding this enzyme and site-directed mutagenesis have shown that the dual activity is due to the action of only one enzyme, and biochemical studies have supported the hypothesis that only one active site is involved (277, 279). It is possible to speculate that because thermoanaerobes evolved under energy-limiting conditions, the possession of enzymes with an active site with more than one catalytic function is an advantage. Since thermoanaerobes are considered to have evolved earlier than aerobes, enzymes with these dual activities would also be expected to be present in the latter organisms, although this has not yet been documented.

Membranes and other cell components. One adaptive mechanism that thermophiles possess is a membrane which can maintain integrity at high temperatures. The archaeobacterial thermophiles have higher proportions of tetraether relative to diether lipid than the mesophilic archaeobacteria do, but this alone cannot account for thermostability, since, for example, *Pyrodictium occultum* possesses only 45% tetraethers (403). Although *Thermoanaerobacterium commune* is a eubacterium with peptidoglycan in the cell wall, the lipids of this organism have been shown to comprise unique nonisoprenoid branched glycerol diethers and monoethers that have not been previously detected in other organisms (234). The presence of glycerol diethers suggests that this organism may have had an evolutionary episode similar to archaeobacteria, but, from the structure of the components, this organism may be more similar to a eubacterium capable of ether lipid biosynthesis (234). Another unusual lipid is found in *Thermoanaerobacter ethanolicus* and *Thermoanaerobacterium thermosulfurigenes*. They contain C_{30} dicarboxylic acids, which are formed by head-to-head condensation of *iso*-branched C_{15} fatty acids (235). It remains to be shown whether these lipids span the membrane to form a monolayer which would help to maintain membrane integrity.

The cell components of hyperthermophiles appear to be remarkably thermostable, demonstrating their adaptation to growth and survival at extreme temperatures. *Pyrococcus furiosus* grows optimally at 100°C and contains the most thermostable ferredoxin reported to date; the protein was stable after 24 h of incubation at 95°C (11). Another ferredoxin which contains one basic amino acid, lysine, has been isolated from *Methanococcus thermolithotrophicus* (158); like the *Clostridium thermocellum* and *Thermodesulfotobacterium commune* ferredoxins (136, 328), it differs from ferredoxins of other heat-stable clostridia (123, 482) in that it does not contain histidine.

Methanopyrus strain AV 19 contains high levels of 2,3-

diphosphoglycerate (1.1 M) compared with levels found in mesophilic and thermophilic methanogens (173). Since this component is thought to contribute to the thermostability of enzymes (380), high levels might be expected in *Methanopyrus* strain AV 19, the most thermophilic methanogen isolated to date. This view is further supported by the finding that the resistance of *Methanothermus fervidus* proteins to thermal denaturation may result more from interactions with the high level (300 mM) of 2,3-diphosphoglycerate present in the cytoplasm of the organism than from any inherent stability provided by their primary amino acid sequences (398).

Little is known about the molecular biology of hyperthermophiles, in contrast to moderate thermophiles. One interesting feature which is thought to be a hallmark of hyperthermophilic archaeobacteria is the presence of reverse gyrase, which has been found to be widely distributed in phylogenetically distinct organisms (46). The presence of reverse gyrase activity is thought to be linked to high-temperature growth, particularly above 70°C, since it causes positive supercoiling, which would stabilize the DNA. This activity appears to be restricted to the archaeobacterial kingdom (46).

The first restriction map of an archaeobacterial chromosome has been determined from *Thermococcus celer*, revealing three important features: first, the chromosome is arranged in a circular form, which was not known previously for archaeobacteria; second, it is composed of a single DNA molecule; and third, it is relatively small (310). Another study has determined the sequence, organization, and transcription of the rRNA operon and the downstream tRNA and protein genes in *Thermophilum pendens*, and phylogenetic trees derived from these findings placed *Thermophilum pendens* close to *Thermoproteus tenax* (211).

Fukusumi et al. (142) have cloned the heat-stable amylase gene from the hyperthermophile *Dictyoglomus thermophilum* into *E. coli*; the resulting protein was thermostable and had similar temperature and pH optima (90°C and 5.5) to those of the native enzyme (215).

Comparison with aerobic thermophiles. From the numerous studies undertaken, the proteins and enzymes from thermoanaerobes appear to be very thermostable and well suited to function under the environmental conditions found in the ecological niches inhabited by these organisms. This includes both anabolic and catabolic enzymes of methanogens and biopolymer-fermenting thermoanaerobes. Enzymes from thermoanaerobes, unlike some enzymes from aerobic thermophiles (120), do not appear to have high turnover rates. Therefore, catalytic efficiency is not achieved by increased rates of protein synthesis but, rather, by possession of proteins with thermostability and high catalytic activity, thereby providing these organisms growing under energy-constrained conditions with an advantage over their aerobic counterparts.

Biotechnological Features

Overview. Thermophilic bacteria have considerable process advantages over mesophilic microorganisms; these include high growth rates, facilitated end product recovery, increased process stability, reduced process (utility) costs, and the ability to directly ferment complex plant polymers such as cellulose and starch (264, 306, 487, 494). Elevated temperatures are advantageous for maintaining anaerobic conditions and ensuring the growth of anaerobes, since the solubility of oxygen is relatively low. The main attraction of

using anaerobes centers around their mode of metabolism and the formation of fermentation products which could be of biotechnological importance. Most thermoanaerobic strains described to date, however, possess a low product tolerance (265, 494). Thermophiles present different physiological and technological difficulties, which must be overcome before an industrial process can be designed and compared with those for aerobes.

Alcohol and organic-acid fermentations. For an excellent review of the use of thermophiles to produce fuels and chemicals, the reader is referred to an article by Weimer (448). The oil crisis of the mid-1970s directed technologies toward the use of renewable resources to produce chemicals that were an attractive alternative to processing oil. In the mid-1980s this approach had dwindled with the drop in oil prices, but the need to intensify the utilization of renewable resources in the future remains, because of the finite supply of economic oil. Industrial alcohol production by fermentation was an outgrowth of the alcoholic-beverages industry. The cost of alcohol is largely in the raw materials, although steam, labor, and waste treatment costs are important. Of the 300 million gallons (1,135 million liters) of industrial ethanol produced in the United States in the late 1970s, only 23% was produced by fermentation, whereas over 60% (857 million liters) of the industrial ethanol produced in Europe was produced by fermentation. This dramatically changed in the early 1980s, with fermentation alcohol dominating all markets in 1985 (264).

In the United States, use of ethanol as a fuel is almost wholly confined to its use as an octane enhancer in the higher grade of gasoline. Currently, this fuel ethanol is obtained from both fermentation and chemical synthesis. However, because of the significant cost advantage of fermentation ethanol over its chemically synthesized competitor, projected new plant construction in the United States will be of the fermentation type only.

The most quantitative use of the fermentation ethanol may be as a source of ethylene, which is the largest volume organic chemical produced by the chemical process industry. Ethylene has a wide variety of uses in the production of both polymers (e.g., polyethylene) and monomer derivatives (e.g., ethylene glycol, vinyl chloride, and styrene). Significant improvements in dehydration technology and increases in petroleum prices are likely to make ethylene obtained from fermentation ethanol a viable business opportunity.

All of the fermentation ethanol produced in the United States is made by yeast fermentation. Using thermophilic bacteria for ethanol production has been the focus of study for a number of research groups. There are an increasing number of patented processes concerning the use of thermophilic microorganisms, including ethanol fermentation (77, 257, 259, 260, 504). There are a number of advantages to using a thermophilic rather than a mesophilic process. First, the elevated temperature should facilitate recovery of the volatile ethanol. Second, the heating costs of thermophilic fermentations will probably be lower than the cooling costs of mesophilic fermentations. Third, many of the thermoanaerobes possess greater substrate versatility than mesophilic anaerobes do. *Clostridium thermocellum*, *Clostridium thermohydrosulfuricum*, *Clostridium thermosaccharolyticum*, *Clostridium thermosulfurogenes*, *Thermoanaerobium brockii*, *Thermobacteroides acetoethylicus*, and *Thermoanaerobacter ethanolicus* use a wide range of substrates, from polymeric carbohydrates such as cellulose, pectin, xylan, and starch to mono- and disaccharides such as glucose, cellobiose, xylose, and xylobiose. The main fermentation

product is ethanol, but acetate, lactate, carbon dioxide, and hydrogen are also formed in various ratios (with the exception that *Thermobacteroides acetoethylicus* which has not been shown to produce lactic acid).

The control of end product formation in thermoanaerobes has received much attention (264). At present, one of the major limitations to their use is the variability in end product ratios and yields, which are affected by species, enzyme complement, and environmental conditions. Certain strains of *Clostridium thermohydrosulfuricum* and *Thermoanaerobacter ethanolicus* have the best conversion of carbohydrates to ethanol, forming 1.6 to 1.9 mol/mol of glucose fermented (264). At high glucose concentrations, these thermoanaerobes and *Thermoanaerobacter brockii* produce more lactate, probably as a result of increased levels of sugar phosphates, which cause an increase in the levels of fructose diphosphate, leading to the activation of lactate dehydrogenase (146, 230). Similar high ethanol and hydrogen concentrations also reduce the yield of ethanol (264) owing to the flexibility of the carbon and electron flow pathways, which may possess many reversible enzyme systems (230, 264).

Acetic acid is one of the largest chemical intermediates in the United States with an annual use of 2,700 million lb (1,225 million kg). It is currently made by carboxylation of methanol or oxidation of butane. Dilute food-grade acetic acid, or vinegar, is produced (80 million lb/year [36 million kg/year]) solely by microbial action because of governmental regulatory requirements. This microbial fermentation is carried out by aerobic mesophiles belonging to the genus *Acetobacter*. Other microbial systems merit further attention as a means of producing acetic acid. Bacteria of particular interest include the homoacetate fermenters, which produce considerably higher yields of acetic acid from carbohydrates than the *Acetobacter* process does. The homoacetogens include both mesophiles and thermophiles, and examples of the latter group include *Clostridium thermoacetatum*, *Clostridium thermoautotrophicum*, and *Acetogenium kivui*. These thermophiles and mesophiles lack the acidophilic characteristics of the aerobic acidophiles used for vinegar production. Fermentations involving homoacetogens occur at pH values above the pK_a of acetic acid, and economic recovery of the acetic acid is not currently possible. Therefore, attempts have been made to optimize homoacetogenic fermentations for low pH and high total acetate concentration by using *Clostridium thermoaceticum* (378, 379).

There is increasing interest in the use of calcium magnesium acetate as an environmentally benign, biodegradable deicer for roads, bridges, underground pipes, etc., avoiding the corrosion problems caused by the use of chloride salts. *Clostridium thermoaceticum* and *Clostridium thermoautotrophicum*, as homoacetogens, could be potential producers of calcium magnesium acetate, with the complete conversion of hydrolyzed cornstarch to acetate as the only product (258).

Recent market developments in the food industry have increased the demand for naturally derived flavors and ingredients. Butyric acid, butyrate esters, and other derivatives are important flavor ingredients in many natural and processed foods. *Clostridium thermosaccharolyticum* produces butyric acid as the fermentation product during the exponential phase (170), although there are a number of mesophilic butyrate-producing anaerobes which may prove more suitable for this process, such as *Clostridium thermobutyricum*, which produces butyrate as 90% of its end product.

```

CGGATTTTAAATTTGOTAGAAATATAATATAATATTAATTTGTTGGACAGACAAACGA 60
-35                               -10
      HindIII
ATAGAAGGAGGAAGCTTTATGAATAAATATTTGAGAACGTATCTAAAATAAAATATGAA 120
R B S      MetAsnLvsTyrPheGluAsnValSerLysIleLysTyrGlu

```

FIG. 5. Nucleotide and deduced amino acid sequence of *Thermoanaerobacterium thermosulfurigenes* xylose isomerase (*xyIA*) gene. The putative -35 and -10 sequences in the promoter region and the putative ribosome-binding site (RBS) are underlined. At the -10 site a double TATA box is present. The underlined amino acids represent the N-terminal sequence. Reprinted from reference 240 with permission.

Lactic acid is also produced commercially by fermentation with homofermentative lactobacilli. L-Lactic acid is produced by numerous thermophilic bacteria such as *Clostridium thermocellum*, *Thermoanaerobacter thermohydrosulfuricus* E100-69, *Thermoanaerobacter ethanolicus* 39E, *Thermoanaerobacter brockii*, and *Thermoanaerobacter ethanolicus*. These organisms all produce lactic acid as one of a number of fermentation products. For industrial production of acids, the major cost is that of product recovery; in this regard *Clostridium thermolacticum* may prove to be a suitable organism for lactate production since lactate is the major end product.

Thermophilic enzymes and genes. Thermoanaerobes produce many thermostable enzymes but in low yields. Nonetheless, the genes encoding these enzymes can be cloned and overproduced in mesophilic, aerobic industrial hosts. Work on glucose isomerase has shown the uniqueness of exploiting thermophilic genes. First, the promoter of glucose isomerase

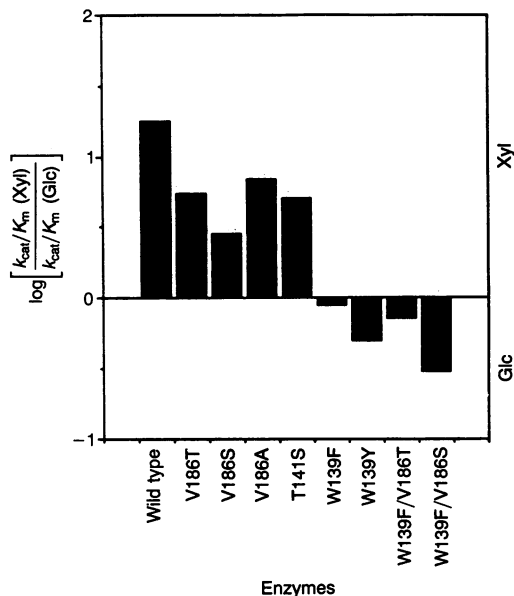


FIG. 6. Redesigned active site of glucose isomerase from *Thermoanaerobacterium thermosulfurigenes*. Diagram illustrating amino acid changes of substrate preference from xylose (Xyl) to glucose (Glc) associated with amino acid substitutions in the substrate-binding pocket of xylose isomerase. The ratios of catalytic efficiency (k_{cat}/K_m) of enzymes with xylose versus those with glucose are expressed on a logarithmic scale. The negative values shown by factitious enzymes indicate more favored enzyme specificity toward glucose than xylose, which is required of "true" glucose isomerase. Amino acids are indicated by the single-letter code. Reprinted from reference 289 with permission.

was novel, having a double TATA box (Fig. 5), and high levels of enzyme were produced. Second, the gene was expressed in both gram-positive and gram-negative hosts. Third, greater than 95% purification was accomplished for recombinant *E. coli* or *B. subtilis* by a single-step high-temperature treatment which also inactivated host proteases. Finally, true glucose isomerase was augmented by using site-directed mutagenesis techniques because of active-site homology to the mesophilic xylose isomerase crystal structure (Fig. 6).

Amylolytic enzymes constitute an important group of industrial enzymes. Three types of enzymes, endoamylase (α -amylase), exoamylase (β -amylase, glucoamylase), and debranching enzymes (pullulanase, isoamylase), are involved in the production of sugars from starch. Bioprocessing of starch usually involves two steps, liquefaction and saccharification, which are both run at high temperatures. Therefore, there is a need for thermostable saccharolytic enzymes to run the saccharification reaction at a higher temperature (higher than 60°C). Amylosaccharidases from thermoanaerobes such as amylopullulanase may find an application in starch conversion biotechnologies because of their novel activity, extreme thermostability and thermoactivity, and pH compatibility.

The broad specificity of the secondary alcohol dehydrogenase from *Thermoanaerobacter brockii* makes it interesting as an industrial ketone/aldehyde/alcohol oxidoreductase, and it has been patented (501). The enzyme displays extraordinary activity at moderate temperature and high substrate concentration or at moderate substrate concentration and high temperature. The enzyme is very thermostable, with activity decreasing only at temperatures greater than 86°C (232). It is of interest to biotechnology because of its ability to form specialty chiral chemical products from a wide variety of substrates (227, 503). It could also be used to manufacture the natural component of the perfume civet, representing an application of both applied enzymology and organometallic chemistry (200).

Anaerobic waste treatment. Thermophilic anaerobic digestors (55°C) are used successfully worldwide to digest agricultural and municipal waste to methane (193), and a number of these processes have been patented (78, 212). The use of anaerobes for waste treatment has a number of advantages over aerobic processes, including low production of excess sludge, low nutrient requirement, no energy requirement for aeration, high volumetric organic loading rates, potential recovery of energy in the formed biogas, degradation of some toxic compounds such as halogenated compounds which are recalcitrant to aerobic degradation, and the ability to preserve anaerobic sludge for a long time without serious reduction of activity. These features apply to both mesophilic and thermophilic anaerobic digestors; however, one important advantage of using a digester at elevated temperatures is the loss of possible pathogens which would otherwise reduce the usefulness of the resulting sludge.

The complete anaerobic digestion of complex organic compounds (polysaccharides, proteins, and lipids) requires at least three trophic groups of microorganisms, which form a microbial food chain or web and include hydrolytic-fermentative bacteria, syntrophic acetogenic bacteria, and methanogenic bacteria. The process begins with the breakdown of cellulose, starch, and hemicellulose to sugars, alcohols, acids, H₂, and CO₂ by the hydrolytic-fermentative bacteria into simple organic compounds (formate, acetate, propionate, butyrate, ethanol, etc.). The anaerobic thermophiles important to the biomethanation process include the

TABLE 9. Substrates utilized by dehalogenating and CO-utilizing organisms

Substrate	Location	Organisms capable of utilizing CO or halogenated compounds	Reference(s)
CO	Volcanic gas, thermal springs, soil, synthesis gas	<i>Butyribacterium methylotrophicum</i> , <i>Peptostreptococcus productus</i> , <i>Clostridium ljungdahlii</i>	16, 261, 268, 421
PCP	Soil, wastewater, subsurface water	<i>Acetobacterium woodii</i> , <i>Butyribacterium methylotrophicum</i> , <i>Eubacterium limosum</i> , <i>Methanobacterium ivanovii</i> , <i>Methanobacterium formicicum</i> T1N, <i>Methanosarcina barkeri</i>	33
PCE	Soil, wastewater, subsurface water	<i>Desulfomonile tiedjei</i> , <i>Methanosarcina</i> strain DCM, <i>Methanosarcina mazei</i> S6	91, 124, 125
TCA	Soil, wastewater, subsurface water	<i>Clostridium</i> sp.	143
CCl ₄	Wastewater, subsurface water	<i>Acetobacterium woodii</i> , <i>Clostridium thermoaceticum</i> , <i>Desulfobacterium autotrophicum</i>	119

biopolymer degraders *Clostridium thermocellum*, *Clostridium thermosaccharolyticum*, and *Clostridium thermo-hydro-sulfuricum* (457, 524).

The syntrophic acetogenic bacteria then convert the metabolic products of the first group (such as ethanol, propionate, butyrate, valerate, isovalerate, benzoate) into directed methanogenic precursors, acetate and hydrogen or formate. These bacteria are referred to as syntrophic bacteria because they can convert the above substrates only in association with a hydrogen-utilizing partner, usually a methanogen (or sulfate reducer if sulfate is available) to prevent accumulation of the intermediate product H₂ or formate and, as a result, to keep the conversion thermodynamically favorable (65, 428, 487). The thermophilic syntrophic acetogens are not well characterized, but the predominant organisms include *Clostridium thermoautotrophicum* and *Desulfotomaculum nigrificans* (457).

The third group contains the methanogenic bacteria. Methanogens utilize the acetate, H₂-CO₂, and formate produced by the other trophic groups to produce methane and CO₂. The important thermophilic methanogens include *Methanobacterium thermoautotrophicum*, *Methanosarcina* spp., and *Methanotherix* spp. (457, 525, 526).

Waste products from processes which operate at high temperature can also be treated by using thermophilic anaerobic digestors, because cooling costs will be reduced since the temperature needed to operate the thermophilic digester is higher than that needed for mesophilic digestors. Waste processes such as coffee water waste, sugar manufacture, and vinasse (the wastewater of alcohol distilleries) can be treated by thermophilic anaerobic digestion (452).

A new type of anaerobic composting which is being used in Europe is dry anaerobic composting, a novel, high-quality dry fermentation process for the composting of household refuse (90). This process has a number of good features, including energy recovery in the form of methane, low running costs because mixing is not required, very high reduction of pathogens, and no production of wastewater. Also, the final product is a dry and very stable compost. Because of the shortage of landfill space in The Netherlands, this process, although expensive, is government subsidized and is used to compost household refuse.

DEHALOGENATING AND CARBON MONOXIDE-UTILIZING ORGANISMS

Ecology, Diversity, and Taxonomy

Overview. The organisms described here include bacteria with the ability to degrade a toxic or hazardous compound or

to modify it to other compounds that are nontoxic (Table 9). These, in general, include organisms that readily degrade high levels of (CO) and halogenated compounds. An early indication of CO metabolism by anaerobic bacteria came in 1931, when Fischer et al. (133) reported that CO was converted to CH₄ and CO₂ by sewage sludge and mixed cultures of methanogens. Since then, a number of methanogens in pure culture have been shown to utilize CO and possess a CO-metabolizing system which is constitutive (87). CO oxidation to CO₂ also occurs in acetogenic bacteria, sulfate-reducing bacteria, and clostridia.

Utilization of halogenated compounds involves the process of reductive halogenation, which is the replacement of a dehalogen substituent of a molecule with a hydrogen atom. In all known biological examples of this activity, the halogen is released as a halide ion. This process makes many xenobiotic compounds less toxic and more readily degradable and appears to be the essential primary step in anaerobic degradation of halogenated aromatic compounds. Anaerobic reductive dehalogenation is the only known biodegradation mechanism of certain significant environmental pollutants, such as highly chlorinated biphenyls, hexachlorobenzene, and tetrachloroethylene.

There have been limited studies on acidogenic and methanogenic anaerobes that consume high levels of CO in addition to their ability to grow and ferment other single-carbon and multicarbon compounds. The habitats of cofermenting anaerobes are very diverse, with CO-fermenting organisms being isolated from sewage digester sludge, chicken manure, horse manure, the rumen, and volcanic features. Interestingly, in all these habitats CO is not the principal source of carbon (Table 10).

Most of the studies of detoxification of halogenated compounds have involved mixed-culture consortia rather than pure cultures. Many chlorinated organic compounds are not biodegraded under anaerobic conditions, often because the chlorine substitutions prevent ring cleavage and subsequent dechlorination. Recently, however, certain chlorinated aromatic compounds have been shown to be dechlorinated in anaerobic habitats such as sediment, flooded soil, and digested sludge. These chemicals include chlorinated benzoates (168, 413, 414), chlorinated phenols (48, 49, 186, 303), some of the pesticides such as diuron [3-(3,4-dichlorophenyl)-1,1-dimethylurea] (12), techlofthalam [*N*-(2,3-dichlorophenyl)-3,4,5,6-tetrachlorophthalic acid] (209), chloronitrofen (4-nitrophenyl-2,4,6-trichlorophenyl ether) (481), and 2,4,5-trichlorophenoxyacetic acid (415). In all of these cases, chlorine is removed from the aromatic ring before ring cleavage, which is in contrast to aerobic metabolism of chlorinated compounds. This reaction (a reductive dechlori-

TABLE 10. CO-utilizing anaerobic bacteria

Organism	Substrates	CO fermentation products	Isolation/habitat	Optimal growth conditions	Reference(s)
<i>Acetobacterium woodii</i>	Fructose, glucose, CO, methanol	Acetate	Aquatic and marine anaerobic sediments	30°C, pH 6.6–6.8	15, 56
<i>Acetogenium kivui</i>	Sugars, pyruvate, formate, CO ₂ + H ₂ , CO	Acetate	Lake in South Africa	66°C, pH 6.4	251
<i>Butyribacterium methylotrophicum</i>	Glucose, methanol, formate, CO	Acetate, butyrate, butanol, ethanol	Sewage digester	37°C, pH 7.0–7.2	153, 268, 471
<i>Carboxydotherrnus hydrogeniformans</i>	CO	H ₂ + CO ₂	Hot swamp on Kunasyhir Island	70–72°C, pH 6.8–7.0	419
<i>Clostridium formicoaceticum</i>	Fructose, ribose, pyruvate, CO	Acetate, CO ₂	Sewage, pond, and ditch mud	37°C, pH 7.0–7.2	7, 100
<i>Clostridium ljungdahlii</i>	CO, fructose	Ethanol, acetate	Chicken waste	37°C, pH 4.0–5.0	16, 439
<i>Clostridium pasteurianum</i>	Glucose, pyruvate, CO	CO ₂	Soil	37°C, pH 7.0–7.2	140
<i>Clostridium thermoaceticum</i>	Glucose, CO ₂ + H ₂ , CO	Acetate, CO ₂	Horse manure	55–60°C, pH 7.0–7.2	100, 204
<i>Clostridium thermoautotrophicum</i>	Glucose, CO ₂ + H ₂ , CO	Acetate + H ₂	Mud and wet soils	55–60°C, pH 5.7	453
<i>Eubacterium limosum</i>	Glucose, methanol, formate, CO	Acetate, CO ₂	Sheep rumen	37°C, pH 7.0–7.2	145
<i>Methanosarcina barkeri</i>	Acetate, H ₂ + CO ₂ , CO	Methane, H ₂	Sewage digester, animal waste lagoons, freshwater and marine mud	30–40°C, pH 6.8–7.2	61, 313
<i>Peptostreptococcus productus</i>	Glucose, fructose, H ₂ + CO ₂ , CO	Acetate, CO ₂	Sewage digester, human colon and feces	37°C, pH 6.5–6.8	144, 261
<i>Thermoproteus tenax</i>	Organic compounds, CO ₂ , CO	H ₂ S + CO ₂	Terrestrial solfataras	88°C, pH 5.0	134

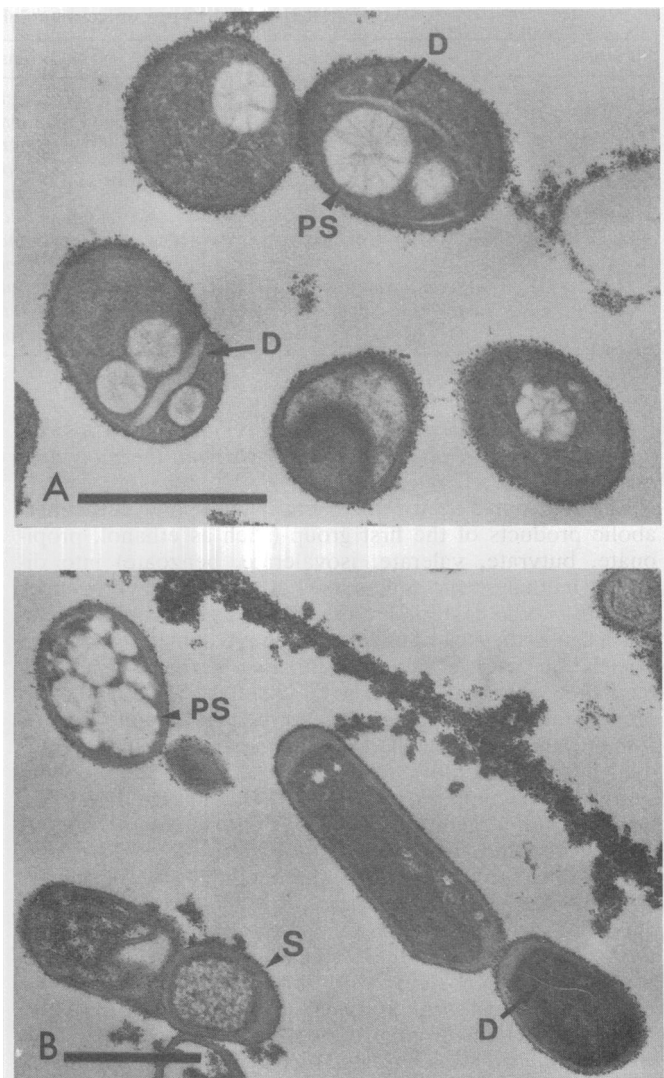


FIG. 7. Transmission electron micrograph of *Butyribacterium methylotrophicum* Marburg. (A) Vegetative cells. Bar, 1 μ m. (B) Sporulating cells with mature spores in left lower corner. Bar, 2 μ m. Reprinted from reference 491 with permission.

nation) is therefore important, since it has the potential for making some of the highly chlorinated, serious pollutants less persistent and less toxic.

Both groups of organisms oxidizing CO or performing dehalogenation reactions are taxonomically diverse, containing both archaeobacteria and eubacteria and including methanogens, sulfate reducers and acetogens. Interestingly, some of the organisms are both CO utilizers and capable of dehalogenation reactions.

CO-utilizing bacteria. Although CO is not a major intermediate in anaerobic ecosystems, it is present at low levels as a by-product. Some of these organisms such as *Butyribacterium methylotrophicum*, *Eubacterium limosum*, *Peptostreptococcus productus*, *Clostridium thermoaceticum*, *Clostridium ljungdahlii*, *Desulfovibrio vulgaris*, and *Methanosarcina barkeri* can utilize CO as an energy source, whereas others such as *Clostridium pasteurianum* can metabolize CO but grow only in the presence of an additional

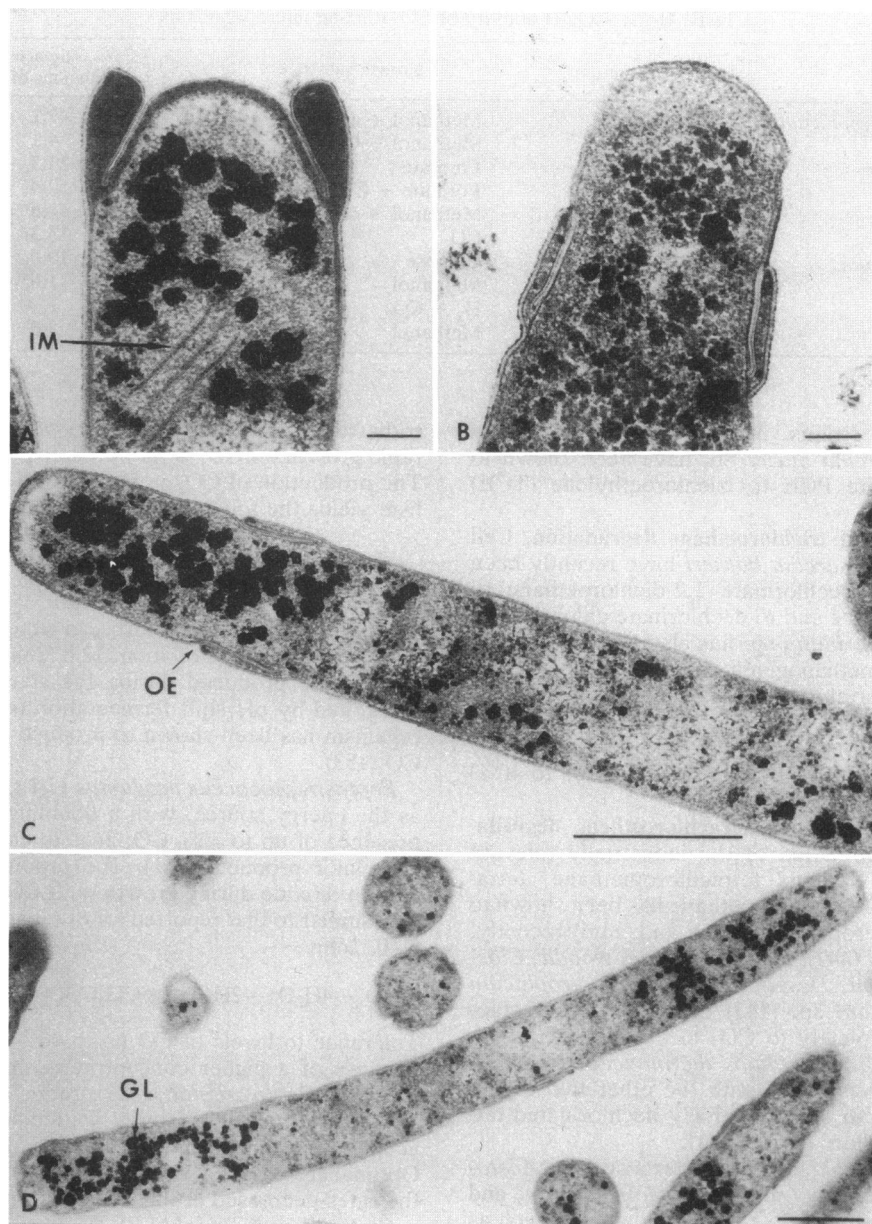


FIG. 8. Transmission electron micrographs of *Desulfomonile tiedjei* showing the collar in various locations and internal structures. OE, open end of collar; IM, internal membrane; GL, glycogen bodies. Bars, 0.1 μ m (panels A and B) and 0.5 μ m (panels C and D). Reprinted from reference 294 with permission.

energy source. *Butyribacterium methylotrophicum* was isolated from a sewage digester in Marburg, Germany (Fig. 7). Initially the Marburg strain did not grow on CO alone but did consume CO during growth on a variety of substrates in the presence of a 100% CO gas phase. However, Lynd et al. (268) selected a strain (CO strain) that grew vigorously on CO alone. The ability of the CO strain to grow on CO was stable through multiple transfers in the absence of CO. *Carboxydotherrnus hydrogeniformans* has an obligate dependence on CO and is found in hydrothermal springs (419). Another clostridium species capable of utilizing CO is *Clostridium ljungdahlii*, which was isolated from chicken waste (439). A sheep rumen isolate, *Eubacterium limosum*, which resembles *Butyribacterium methylotrophicum*, is capable of

growth on CO, but its growth has not been tested beyond 75% CO in the gas phase (145). *Peptostreptococcus productus* U-1 was isolated from an anaerobic sewage digester and grew rapidly with CO as the energy source (261).

Anaerobes that perform dehalogenations. (i) **PCE degradation.** The first anaerobic bacterium available in pure culture that was able to reductively dechlorinate aromatic compounds was strain DCB-1, isolated from a sewage sludge bacterial consortium (384). The bacterium is a gram-negative, nonsporulating, obligately anaerobic rod with an unusual morphological feature, an invagination of the cell wall, or "collar" (Fig. 8); it has been taxonomically described as *Desulfomonile tiedjei* (91, 384). This organism significantly dechlorinates perchloroethylene (PCE) (125).

TABLE 11. CODH activity of CO-utilizing microorganisms

Organism	Strain	Growth substrate	Sp act ($\mu\text{mol}/\text{min}/\text{mg}$ of protein)	Reference
<i>Butyribacterium methylotrophicum</i>	Marburg	Methanol + acetate + CO ₂	47.2	205
		Methanol + CO	4.1	205
		Formate	23.3	205
	CO adapted	Formate + CO	9.4	205
		Methanol + acetate + CO ₂	41.6	205
		CO	13.3	205
<i>Clostridium thermoaceticum</i>		Glucose	10.0	100
<i>Methanosarcina barkeri</i>	MS	Methanol	1.05	223
		H ₂ + CO ₂	1.10	223
	Acetate adapted	Methanol	5.0	223

Two *Methanosarcina* strains, *Methanosarcina* sp. strain DCM and *Methanosarcina mazei* S6, have been shown to reductively dechlorinate PCE to trichloroethylene (TCE) (124, 125).

(ii) **Dichloroethane and trichloroethane degradation.** Cell suspensions of *Methanosarcina barkeri* have recently been shown to reductively dechlorinate 1,2-dichloroethane to ethylene and chloroethane and to dechlorinate chloroethane to ethane (164). A *Clostridium* sp. has also been isolated in pure culture from a methanogenic tricarboxylic acid- and trichloroethane-transforming mixed culture which was able to dehalogenate chlorinated aliphatic compounds. 1,1,1-Trichloroethane was completely transformed (99.5%) by reductive dehalogenation to 1,1-dichloroethane (30 to 40%) (143).

(iii) **Tetrachloromethane and tetrachloroethene degradation.** Microbial transformation of the industrially relevant polychlorinated hydrocarbons tetrachloromethane, tetrachloroethylene, and 1,1,1-trichloroethane has been shown to occur only under anaerobic conditions and, until recently, only in mixed cultures (442). *Acetobacterium woodii*, *Clostridium thermoaceticum*, *Desulfobacterium autotrophicum* (119), and a *Clostridium* sp. (143) transformed tetrachloromethane either completely to CO₂ in the case of *Acetobacterium woodii* and *Clostridium thermoaceticum* or to dichloromethane, as was found with the other two organisms. A *Methanosarcina* sp. reductively dechlorinated tetrachloroethene to trichloroethene (124).

(iv) **PCP degradation.** Acetogenic bacteria such as *Acetobacterium woodii*, *Butyribacterium methylotrophicum*, and *Eubacterium limosum* and sulfate-reducing bacteria such as *Desulfovibrio vulgaris* have been found to degrade pentachlorophenyl (PCP) in the presence of a cosubstrate (Table 9) (33). Methanogens such as *Methanobacterium ivanovii*, *Methanobacterium formicicum* T1N, and *Methanosarcina barkeri* also transformed PCP while growing on H₂CO₂ and acetate (33).

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CO fermentation. Anaerobic CO fermentations are preferable for production of relatively reduced biochemicals, since no available electrons are lost to oxygen. These fermentations typically yield carbon dioxide, cell mass, and a relatively reduced product such as CH₄, H₂, acetate, butyrate, butanol, or ethanol. Prolific growth with carbon monoxide as the only apparent source of carbon and energy qualitatively distinguishes CO utilization by *Butyribacterium methylotrophicum* and *Carboxydotherrnus hydrogenoformans* from CO utilization by methanogens and CO-utilizing clos-

tridia. *Carboxydotherrnus hydrogenoformans* has the most rapid growth on CO, with a doubling time of 120 min (419). The production of CO₂ + H₂ as products from CO metabolism yields the following stoichiometry:



Butyribacterium methylotrophicum can convert CO, as the sole carbon and energy source, to either acetate or butyrate, with a doubling time of about 12 h (268). The ratio of acetate to butyrate produced during the stationary phase can be controlled by pH shift fermentation (471). In addition, this organism has been shown to produce butanol directly from CO (153).

Peptostreptococcus productus U-1 grows rapidly with CO as the energy source, with a doubling time of 1.5 h in the presence of up to 50% CO; acetate and carbon dioxide are the major products (261). The production of acetate and carbon dioxide during growth with CO yielded a stoichiometry similar to that reported for other acetogenic CO utilizers (140, 268): -



Tolerance to levels of CO up to 90% were achieved at the expense of a higher concentration of yeast extract in the medium. *Peptostreptococcus productus* Marburg grew on 50% CO as the sole energy source with the formation of acetate and carbon dioxide, with a doubling time of 3 h. Optimal growth rates were observed with 50 to 70% CO, and the rates decreased at higher concentrations (144).

Clostridium ljungdahlii PETC produces acetate and ethanol from CO, CO₂, and H₂ (16). However, under optimal growth conditions, the organism produces acetate in preference to ethanol, with acetate/ethanol product ratios as high as 20:1 (439). Heterotrophically, strain PETC utilizes arabinose, ethanol, fructose, glucose, pyruvate, and xylose and produces a mixture of acetate and ethanol (110). *Clostridium ljungdahlii* has been suggested to be preferentially a proteolytic rather than a saccharolytic organism (110). *Clostridium thermoaceticum* was culturally adapted to grow on CO as the energy source (204), and *Eubacterium limosum* metabolizes CO as an energy source under CO partial pressures of below 0.75 atm (75.9 kPa) (145).

Carbon monoxide dehydrogenase. Growth of acetogens on CO as the sole energy source can be expected because of favorable energetics of CO fermentations, provided that the organism has tolerance to CO toxicity (145, 261, 268, 500). Acetogenic bacteria, which grow on CO as the sole energy source, possess carbon monoxide dehydrogenase (CODH) (Table 11) and have been used as a tool to study the role of

CO as the precursor for the synthesis of the carboxyl group of acetate. Carbon monoxide in a bound form (rather than formate) was suggested as an intermediate in the biosynthesis of the carboxyl group of acetate (95, 96, 99, 141, 500). The function of CODH in acetogens *in vivo* appears to be more extensive than the oxidation of CO to CO₂ plus electrons, or H₂ (98, 203, 268, 269), as indicated by (i) *in vitro* results indicating that CODH is one component necessary for the conversion of methyl-tetrahydrofuran and either CO or the carboxyl group of pyruvate into acetyl-CoA; (ii) higher specific activities of the enzyme when *Butyrivibacterium methylotrophicum* was grown with methanol rather than CO as the energy substrate (Table 11) and the presence of significant amount of the enzymes in all acetogens tested, regardless of the growth substrates; and (iii) preferential *in vivo* incorporation of CO into the acetate carboxyl group by *Acetobacterium woodii* and *Butyrivibacterium methylotrophicum*. Furthermore, the effects of removal of nickel on inhibition of the fermentation of fructose by *A. woodii* are consistent with a role for CODH in the synthesis of the acetate carboxyl group (97). Preliminary experiments with cell suspensions of *Peptostreptococcus productus* incubated with labeled CO and unlabeled CO₂ or vice versa indicated that this organism mediates the incorporation of CO in the free form into acetate (94).

Dehalogenation. (i) **PCE dechlorination by *D. tiedjei*.** *Desulfomonile tiedjei* reductively dehalogenates *meta*-substituted halobenzoates and also reduces sulfate, sulfite, and thiosulfate as electron acceptors. The bacterium requires nicotinamide, 1,4-naphthoquinone, and thiamine for optimal growth in a defined medium. It can grow autotrophically on H₂-CO₂ with sulfate or thiosulfate as terminal electron acceptors. It can also grow heterotrophically with several methoxybenzoates, formate plus sulfate, or benzoate plus sulfate, and it ferments pyruvate to acetate and lactate in the absence of other electron acceptors (91). *D. tiedjei* DCB-1 specifically metabolizes halobenzoates and preferentially removes *meta*-substituted halogens (93). The type of halogen and the presence of other aryl substituents influence the dehalogenation rate (254). The reaction has been implicated as a major energy source for *D. tiedjei* grown in a defined triculture (104, 105). In pure culture, high concentrations of formate, H₂, or a variety of sulfur oxyanions inhibit the dehalogenation reaction (254). Observations that the presence of a *para*-amino or -hydroxy group inhibited the rate of dechlorination suggest that the rate-limiting step in the reductive dechlorination of 3-chlorobenzoate is a nucleophilic attack on the negatively charged electron cloud around the benzoate nucleus (106). *D. tiedjei* can also reductively dehalogenate nonaromatic compounds better than other anaerobes can (125, 412). The highest rate of dechlorination of PCE was obtained by *D. tiedjei* DCB-1, which dechlorinates 3-chlorobenzoate. The rate of dechlorination of PCE by DCB-1 during active growth was 2.34 nmol of TCE per mg of protein per day (125). The rate of dechlorination of PCE by DCB-1 was approximately 3 to 5 times that observed for *Methanosarcina* sp. strain DCM and *Methanosarcina mazei*. TCE was stoichiometrically recovered from PCE (125). DeWeerd and Suffita (92) characterized the aryl reductive dehalogenation activity in cell extracts of *D. tiedjei*. The rate of dehalogenation was proportional to the amount of protein in the assay mixture. The substrate specificity for aryl dehalogenation activity for various aromatic compounds in *D. tiedjei* cell extracts was identical to that of whole cells. Dehalogenation was 10-fold greater in cells cultured in the presence of 3-chlorobenzoate, suggesting that the aryl deha-

logenation activity was inducible. Aryl reductive dehalogenation in cell extracts was inhibited by sulfite, sulfide, and thiosulfate but not sulfate (92).

(ii) **PCE dechlorination by *Methanosarcina* spp.** Growth of *Methanosarcina* sp. strain DCM on methanol, acetate, methylamine, and trimethylamine resulted in PCE dechlorination (124). The reductive dechlorination of PCE occurred only during methanogenesis, and no dechlorination was found when CH₄ production ceased. The extent of PCE dechlorination was dependent on the amount of methanogenic substrate (methanol) consumption. Since the amount of TCE formed per millimole of CH₄ formed remained essentially constant over a 20-fold range of methanol concentration, it seemed that reducing equivalents for PCE dechlorination are derived from CH₄ biosynthesis. It is likely to enhance the extent of chloroethylene dechlorination by stimulating methanogenesis. It was proposed that electrons transferred during methanogenesis are diverted to PCE by a reduced electron carrier involved in methane formation (124).

(iii) **CCl₄ dechlorination.** Total transformation of tetrachloromethane was shown by *Desulfobacterium autotrophicum*, *Acetobacterium woodii*, and *Clostridium thermoaceticum* (119). Reduction of CCl₄ by *Desulfobacterium autotrophicum* required 18 days of incubation, and *Acetobacterium woodii* was able to degrade 80 μM CCl₄ completely within 3 days (119). Trichloromethane accumulated as a transient intermediate, but the only chlorinated methanes recovered at the end of the incubation period were 8 μM dichloromethane and traces of chloromethane. Therefore, 90% of the CCl₄ was degraded to unknown products. Growing cultures of *Acetobacterium woodii* converted 92% of added ¹⁴CCl₄ to nonhalogenated products. Much of the initial radioactivity (67%) was recovered as CO₂, acetate, pyruvate, and cell material; the remainder included an unknown, hydrophobic material and CH₂Cl₂ (119). On the basis of the reactivity of halomethanes with cobamides, and because all three organisms contain cobamides, it has been suggested that cobamides could be involved in the dehalogenation of CCl₄.

(iv) **Metabolism of tetrachloromethane.** Egli et al. (119) have proposed a pathway for CCl₄ metabolism by *Acetobacterium woodii* that comprises at least two sequences. In the first sequence, corrinoid enzymes putatively catalyze CCl₄ reduction to trichloromethane, dichloromethane, and chloromethane. In the second sequence, a substitutive branch transforms CCl₄ into CO₂ by a series of unknown reactions, which do not cause a net change in the oxidation state of the carbon atom. Carbon dioxide is then assimilated by the acetyl-CoA pathway. In the reductive pathway, CCl₄ and the other chlorinated methanes serve as electron acceptors. Under anaerobic conditions in reduced buffer, suspensions of *Acetobacterium woodii*, *Desulfobacterium autotrophicum*, or *Methanobacterium thermoautotrophicum* degraded CCl₄ by both reductive and substitutive mechanisms (118). The products formed included less highly chlorinated methanes and CO₂. Cell extracts of *Acetobacterium woodii* degraded tetrachloromethane in a manner similar to that in whole cells but at a lower rate (63 versus 140 μkat/kg of protein) (118). When *Methanobacterium thermoautotrophicum* or *Acetobacterium woodii* was autoclaved, reductive dechlorination was partly abolished, whereas substitutive dechlorination was retained. Trichloromethane was oxidized to CO₂ by both native and autoclaved cells of *Acetobacterium woodii*. Halomethanes are therefore degraded anaero-

bically by reductive, substitutive, and oxidative mechanisms (118).

Adaptation Mechanisms

Overview. CO utilization appears to occur within a taxonomically diverse group of anaerobes, including both eubacteria and archaeobacteria. Although CO is considered to be one of the gases present in Earth's early atmosphere, the findings that both ancient and more recently evolved bacteria can utilize this substrate and that CO utilization does not coincide with the presence of CO as a major component in the niche these organisms occupy, suggest that the ability to utilize CO may be adventitious. Dehalogenation by anaerobes also occurs within a number of unrelated organisms and may be due to a nonspecific mechanism, i.e., a process which has another physiological role but also can be involved in dehalogenation reactions.

Substrate versatility. Single-carbon compounds occur in nature at all redox states of carbon with oxidation numbers from -4 to $+4$. Among the anaerobic bacteria metabolizing single carbon compounds, homoacetogenic bacteria are perhaps the most versatile ones with respect to the range of substrates utilized, followed by methanogenic bacteria and sulfate-reducing bacteria. Competition for these single-carbon compounds between these groups of anaerobes, therefore, often yields results very different from those obtained in similar experiments with hydrogen or acetate as substrates. Anaerobic bacteria use C_1 compounds as an electron donor and/or electron acceptor in their energy metabolism. In contrast to the aerobic organisms, the free-energy changes associated with these redox reactions are only very small, for example, the conversion of CO and H_2O to CO_2 and H_2 ($\Delta G^\circ = -20$ kJ/mol).

The acetogenic bacteria which can use CO as a sole carbon and energy source can also normally utilize other single-carbon compounds such as formate, methanol, and CO_2-H_2 , as well as multicarbon compounds such as glucose. Therefore, these organisms have a very versatile enzyme machinery which helps them to survive under different environmental conditions. These types of bacteria can convert CO to CO_2 and H_2 , and they couple this reaction with the phosphorylation of ADP.

Because chlorobenzoates are not substrates normally available in natural habitats, the ability of *Desulfomonile tiedjei* to catabolically utilize chlorobenzoates is probably not due to natural selection for such utilization. Catabolism of chlorobenzoates is probably due to a respiratory system, either known or novel, having another terminal acceptor. Stevens et al. (407) have shown that *D. tiedjei* can utilize sulfoxy anions by a respiratory process resembling that found in sulfate-reducing bacteria. Elaboration of the respiratory system of *D. tiedjei* still remains to be done, together with coupling of that system to reduce dechlorination. Dolfing (103) has shown that reductive dechlorination of 3-chlorobenzoate is coupled to ATP production in *D. tiedjei*. In another study, Mohn and Tiedje (295) have shown that *D. tiedjei* conserves energy for growth by coupling formate and probably H_2 oxidation to reductive dechlorination. Since formate, hydrogen, and acetate are present in anaerobic habitats, it is likely that acetogenic and, to a certain extent, methanogenic bacteria have adapted to these conditions over time and are able to carry out reductive dechlorination. The catabolic machinery of these types of bacteria is not inhibited by halogenated compounds. More recently, it has been shown that reductive dechlorination supports ATP

synthesis in *D. tiedjei* (296). Uncouplers and ionophores can decrease the efficiency of dechlorination-dependent ATP synthesis, suggesting the involvement of a proton motive force (PMF) in coupling dechlorination and ATP synthesis. An imposed proton gradient causes ATP synthesis, further supporting the existence of a proton-driven ATPase (296).

Comparison with aerobic dehalogenators and CO utilizers.

(i) **CO-utilizing microorganisms.** The mechanism of CO oxidation does not appear to be uniform. Anaerobic bacteria and hydrogen-oxidizing bacteria catalyze the oxidation of CO to CO_2 via a dehydrogenase (480), and the O in CO_2 is derived from water. However, methane-oxidizing bacteria and CO-utilizing carboxydobacteria mediate the reaction via a monooxygenase and a CO oxidase, respectively, and the second oxygen in CO_2 is derived from O_2 (127). Interestingly, in these organisms the oxidation of CO to CO_2 does not appear to be the physiological function of this enzyme system, and its true role has yet to be established.

(ii) **Dehalogenation by microorganisms.** The initial reactions involved in dehalogenation of halogenated compounds appear to be similar in both aerobic and anaerobic organisms. Both groups of organisms can utilize mono- and dihalogenated hydrocarbons, but microbial transformation of the industrially relevant polychlorinated hydrocarbons tetrachloromethane, tetrachloroethylene, and 1,1,1-trichloroethane is catalyzed only under anaerobic conditions.

Biotechnological Features

CO utilization. Probably the most versatile of these unicarbonotrophic anaerobic bacteria, *Butyribacterium methylotrophicum* is capable of producing not only acetate and butyrate but also ethanol and butanol. The CO strain of this organism can convert CO to one or more of these products depending on the growth and fermentation conditions. If there is any potential application for bioconversion of coal-derived synthesis gas, it will most probably involve an acidogenic fermentation with a C_1 metabolism by one of these acetogenic bacteria. Although production of acetic acid from synthesis gas can be a practical process, acetic acid has a rather low commercial value and is currently produced chemically in large volume (188, 490). However, alcohols produced from acetic and butyric acid precursors are potential liquid fuel additives, industrial solvents, and intermediates for further processing to ethylene or butadiene, any or all of which could become commercially attractive products.

Biodegradation of halogenated compounds. Anaerobic biodegradation of halogenated benzoates and phenols is a new field of research in microbiology. Considerable efforts are underway to understand the mechanisms of dehalogenation and cleavage of the aromatic ring. From biotechnological perspectives, it may turn out that anaerobic microorganisms are better suited than aerobes to removing halogenated aromatic compounds from industrial effluents. Sahn et al. (366) have suggested that this could be due to the polymerization of haloaromatic compounds when degraded by aerobic bacteria, difficulty in electrophilic attack of oxygen on aromatic structures with an increase in the degree of halogenation of an aromatic ring and decrease in the electron density of the aromatic nucleus, and finally to the high affinity of anaerobes for haloaromatics (414), making it possible to remove even trace levels of haloaromatics by using anaerobic treatment technology.

Some recent work has demonstrated the potential of anaerobes for use in detoxification of aromatic waste. Syn-

TABLE 12. Chemical free-energy change for reactions catalyzed by syntrophic acetogenic bacteria in anaerobic ecosystems in the absence of sulfate and nitrate

Reaction ^a	ΔG° (kJ) per reaction (standard conditions) ^b	$\Delta G'$ per reaction (in situ conditions) ^c
1. Ethanol + 2HCO ₃ ⁻ → acetate ⁻ + 2 formate ⁻ + H ₂ O + H ⁺	+7.0	
2. Ethanol + H ₂ O → acetate ⁻ + 2H ₂ + H ⁺	+9.6	-49.8
3. Butyrate ⁻ + 2H ₂ O → 2 acetate ⁻ + 2H ₂ + H ⁺	+48.1	-29.2
4. Propionate ⁻ + 3H ₂ O → acetate ⁻ + HCO ₃ ⁻ + 3H ₂ + H ⁺	+76.1	-8.4
5. Benzoate ⁻ + 6H ₂ O → 3 acetate ⁻ + 3H ₂ + CO ₂ + 2H ⁺	+53.0	
6. Succinate ²⁻ + 4H ₂ O → acetate ⁻ + 2HCO ₃ ⁻ + 3H ₂ + H ⁺	+56.1	
7. 2 Butyrate ⁻ + 2HCO ₃ ⁻ → 2 acetate ⁻ + H ⁺ + 2 formate ⁻ + isobutyrate ⁻	+43.5	
8. 2 Isobutyrate ⁻ + 2HCO ₃ ⁻ → 2 acetate ⁻ + H ⁺ + 2 formate ⁻ + butyrate ⁻	+49.5	
9. Butyrate ⁻ + isobutyrate ⁻ + 4H ₂ O → 4 acetate ⁻ + 4H ₂ + 2H ⁺	+98.2	
10. Butyrate ⁻ + isobutyrate ⁻ + 4HCO ₃ ⁻ → 4 acetate ⁻ + 4 formate ⁻ + 2H ⁺	+93.0	
11. Palmitate + 14H ₂ O → 8 acetate ⁻ + 7H ⁺ + 14H ₂	+351.5	

^a Acetogenic CO₂ reduction (reaction 1) yields -1.3 kJ more free energy per 2 mol of reducing equivalents than reaction 2 does. All syntrophic acetogenic reactions could involve production of either H₂ + CO₂ or formate.

^b From reference 429 with data from references 253 and 425.

^c From reference 85.

trophic biomethanation granules, which contain unique syntrophic acetogenic and methanogenic species (187, 474, 478), were used for the further design of new granules by the introduction of other species with specific degradative and detoxifying functions. This approach was used to make dechlorinating syntrophic biomethanation granules which were able to dechlorinate PCP, PCE, and TCE and mineralize chlorophenols to methane and CO₂ (34). These granules have great potential in waste treatment because (i) their large size means that washout from the culture vessel does not occur as readily as with free organisms, (ii) their presence as part of a consortium results in a stable population, and (iii) most importantly, incorporation of different organisms with specific attributes can result in the design of granules specific for each type of waste.

SYNTROPHS

Ecology, Diversity, and Taxonomy

Overview. The syntrophic (syn, Greek: together; trophein, Greek: eat) bacteria metabolize hydrolytic fermentation products in an anaerobic food chain. They are composed of obligate proton- and carbonate-reducing acetogens and certain sulfate-reducing species which grow by transferring electrons to methanogens when SO₄ is absent. These syntrophic acetogens oxidize saturated fatty acids, ethanol, and benzoate and produce acetate together with a hydrogen- or formate-utilizing partner. Some syntrophic acetogenic bacteria also ferment xenobiotic compounds such as polyethylene glycol and phenolic compounds (114, 372, 373, 414, 420).

From energetic analysis, the free energy available for acetogenesis from ethanol, propionate, or butyrate, with either hydrogen or formate as an intermediate, is not thermodynamically favorable under standard conditions (Table 12). Low levels of hydrogen and formate have to be maintained (10⁻³ to 10⁻⁵ atm of H₂ [1.01 to 0.01 kPa]), via methanogenesis or sulfate reduction during acetogenesis, to allow the reaction to proceed. Hydrogen production and utilization can profoundly influence the course of fermentations in anaerobic ecosystems (178), and low hydrogen partial pressures exist in all syntrophic acetogenic cultures (23, 60). In addition, perturbation with high partial pressures of H₂ inhibit syntrophic butyrate degradation until hydrogen is consumed to a low level (4, 115).

In addition to hydrogen, formate was recently suggested to be an intermediate in syntrophic ethanol degradation and fatty acid degradation (41, 326, 428, 429). The concept of interspecies electron transfer with formate as the electron-carrying intermediate requires that formate be synthesized from bicarbonate by syntrophic acetogens and oxidized back to bicarbonate by formate-utilizing methanogens or sulfate reducers to maintain a low formate concentration (428, 429). This concept appears to be possible when syntrophic acetogenic isolates are associated with hydrogen- and formate-utilizing syntrophic partners, either *Methanobacterium*, *Methanospirillum*, or *Desulfovibrio* spp. In addition, hydrogen production was observed when formate was added to a culture of *Syntrophomonas wolfei*, indicating that this syntrophic butyrate-degrading organism appeared to have formate dehydrogenase activity (41); however, there are some exceptions to this proposed suggestion. For example, syntrophic butyrate degraders which do not utilize formate were isolated with *Methanobacterium thermoautotrophicum* (3, 160). In the latter case, formate cannot serve as an intermediate during interspecies electron transfer. Furthermore, the mechanism of inhibition of H₂ has not been well explained by present interspecies formate transfer models. Absolute evidence of formate as an intermediate and the relationship between formate and hydrogen during syntrophic acetogenesis requires further evaluation.

Only a limited number of obligate syntrophs have been isolated in the last 12 or 13 years, mainly because of their slow growth and difficulty in maintenance. They have been isolated mainly from sewage sludge, anaerobic digestors, and other anaerobic habitats (Table 13). The isolation of the "S" organism from *Methanobacillus omelianskii* was the first documentation of an H₂-producing acetogenic bacterium (65). The methanogen was thought to oxidize ethanol to acetate and reduce CO₂ to CH₄, but this fermentation was shown to be carried out by a syntrophic association of two bacterial species. The S organism oxidizes ethanol to acetate, and the electrons are used to reduce protons to H₂, while the methanogen uses the H₂ to reduce CO₂ to CH₄. Hydrogen inhibits the growth of the S organism on ethanol, and good growth occurs only when the H₂-utilizing organism is present (65, 342).

Acid-utilizing bacteria. (i) **Acetate utilizers.** Zehnder and Koch (486) reported the coisolation of a thermophilic, two-membered culture consisting of a eubacterial rod which

TABLE 13. Syntrophic acetogenic bacteria

Organism	Substrates	Fermentation products	Isolation/habitat	Optimal growth conditions	Syntrophic partner	Reference(s)
<i>Syntrophomonas wolfei</i> subsp. <i>wolfei</i>	Ethanol Butyrate	Acetate, H ₂ Acetate, H ₂	<i>Methanobacillus omelianskii</i> Digester sludge	37°C 30–37°C	<i>Methanobacterium</i> sp. <i>Desulfovibrio</i> sp., <i>Methanospirillum hungatei</i> , <i>Methanobacterium formicicum</i>	65, 342 41, 262, 284
<i>Syntrophomonas wolfei</i> subsp. <i>saponavida</i>	C ₄ –C ₁₈ straight-chain fatty acids	Acetate, CO ₂	Digester sludge	30–37°C	<i>Desulfovibrio</i> sp., <i>Methanospirillum hungatei</i>	262
<i>Syntrophomonas saporovans</i>	C ₄ –C ₁₈ linear saturated fatty acids	Acetate, H ₂	Digester sludge	35°C, pH 7.3	<i>Desulfovibrio vulgaris</i> , <i>Methanospirillum hungatei</i>	355
<i>Syntrophospora bryantii</i>	Butyrate, 2-methylbutyrate	Acetate, H ₂ , propionate	Marine and freshwater mud	28–34°C, pH 6.5–7.5	<i>Desulfovibrio</i> sp., <i>Methanospirillum hungatei</i>	408, 509
<i>Syntrophobacter wolinii</i>	Propionate	Acetate, H ₂	Sewage digester	37°C, pH 6.8–7.2	<i>Desulfovibrio</i> sp.	40
<i>Syntrophus buswellii</i>	Benzoate	Acetate, H ₂ , CO ₂ , formate	Sewage digester	37°C, pH 7.2	<i>Desulfovibrio</i> sp.	298, 299
<i>Syntrophococcus sucromutans</i>	Fructose	Acetate, H ₂	Rumen	35–42°C, pH 6.4	<i>Methanobrevibacter smithii</i>	222
<i>Pelobacter carbinolicus</i>	Ethanol, 1,2-propanediol, 2,3-butanediol	Acetate	Anaerobic digestors, marine sediments	35°C	<i>Methanobrevibacter arboriphilus</i>	113, 121
<i>Desulfovibrio vulgaris</i>	Ethanol	Acetate, CO ₂	Freshwater and brackish water mud; marine sediments	34–37°C	<i>Methanosarcina barkeri</i>	63, 437

oxidized acetate to H₂ and CO₂ and an H₂-CO₂-utilizing methanogen, *Methanobacterium* sp. strain THF. The coculture grew at 60°C and produced nearly equimolar amounts of CH₄ from acetate. By using ¹⁴C-labeled radiotracers, it was shown that both methyl and carboxyl carbons of acetate were oxidized by the coculture to CO₂ and that CO₂ was reduced to CH₄. This acetate-oxidizing rod-shaped bacterium has been isolated and found to grow acetogenically on H₂-CO₂ (243).

(ii) **Propionate utilizers.** *Syntrophobacter wolinii* was the first syntrophic propionate-degrading culture that was isolated from methanogenic enrichments from an anaerobic municipal sewage digester in association with an H₂-utilizing, sulfate-reducing *Desulfovibrio* sp. (40). A mixed culture of *Desulfovibrio* sp. and *Methanosarcina barkeri* 227 has also been shown to produce methane from propionate in the presence but not in the absence of sulfate (155). Two syntrophic propionate-oxidizing strains, one sporeforming and the other nonsporeforming, have recently been isolated from syntrophic biomethanation granules (476–478) and remain to be taxonomically characterized. One of the strains (strain PT) seems to be a sulfate reducer as well.

(iii) **Butyrate utilizers.** Since isolation of the *S* organism, syntrophic acetogenic bacteria that degrade propionate, butyrate, and isobutyrate have been isolated. The first such butyrate-degrading bacterium was *Syntrophomonas wolfei*, which was isolated from anaerobic digester sludge (284, 285) in a syntrophic coculture with *Methanospirillum hungatei*. This is a nonsporulating, gram-negative, slightly helical, rod-shaped bacterium. A sporeforming butyrate degrader, *Syntrophospora bryantii* (408, 509), which oxidizes even-numbered fatty acids of up to 10 carbon atoms to acetate and H₂ and odd-numbered fatty acids with up to 11 carbon atoms (including 2-methylbutyrate) to acetate, propionate, and H₂, was isolated from marine and freshwater mud samples. Other sporeforming butyrate-oxidizing strains include strains SF-1 (384), BH (476, 478), and one isolated by Tomie et al. (434). Isolation of cocultures containing a butyrate-oxidizing strain at thermophilic temperatures in syntrophic association with the hydrogen-utilizing methanogenic bacterium *Methanobacterium thermoautotrophicum* (3, 160) provides evidence for the existence of thermophilic, obligately syntrophic, acetogenic bacteria similar to those isolated under mesophilic conditions.

Reversible isomerization of isobutyrate to butyrate, which was postulated by Zinder et al. (526), has been observed in enrichment cultures (430). Subsequently, this was confirmed by Stieb and Schink (410) when they developed a methanogenic enrichment with isobutyrate as the sole source of carbon and energy. They, however, were unable to purify the primary isobutyrate-fermenting bacteria from this enrichment. Oxidation of isobutyrate by strain SF-1 as reported by Shelton and Tiedje (384) has not been confirmed. Jain et al. (189) have reported the isolation of an isobutyrate-oxidizing strain in a triculture with *Methanobacterium formicicum* and *Methanosarcina mazei*. This nonsporeforming, rod-shaped, syntrophic acetogen reversibly isomerizes isobutyrate to butyrate and has shown degradation of isobutyrate to acetate via butyrate.

(iv) **Long-chain fatty acid utilizers.** An anaerobic, obligately syntrophic, fatty acid-degrading, acetogenic bacterium, *Syntrophomonas saporovans* OM, was isolated on calcium laureate medium from an oleate enrichment (355). This organism is a short, slightly curved, gram-negative rod which can use only protons as electron acceptors. It ferments all linear saturated fatty acids with 4 to 18 carbon

atoms in coculture with a hydrogen-utilizing partner. Some mono- and diunsaturated long-chain fatty acids (oleate, elaidate, and linolenate) are also oxidized. *Syntrophomonas wolfei* subsp. *saponavida* SD2, obtained from digester sludge in coculture with the H₂-utilizing bacterium *Desulfovibrio* sp. strain G-11, utilizes long-chain saturated fatty acids and catabolizes C₉ to C₁₈ saturated fatty acids (262).

(v) **Benzoate and 3-chlorobenzoic acid utilizers.** Compounds other than fatty acids have been shown to be degraded as a result of syntrophic associations. A consortium of bacteria was required for anaerobic degradation of benzoate to methane (129). An anaerobic bacterium capable of benzoate degradation to acetate and, presumably, CO₂ and H₂ or formate was isolated in syntrophic association with a sulfate-reducing or methanogenic bacterium (299) and was named *Syntrophus buswellii* (298). A complex microbial consortium that degrades 3-chlorobenzoic acid involves seven bacteria, including a syntrophic benzoate-utilizing bacterium, a spore-forming butyrate-utilizing syntrophic bacterium, and a butyrate-utilizing syntrophic bacterium that does not form spores (384). These syntrophic associations are indicative of the complex and coupled metabolic interactions that occur in anaerobic environments.

Clostridium bryantii (408) has been renamed *Syntrophospora bryantii* on the basis of a 16S rRNA sequence analysis of a crotonate-grown pure culture (509). In the past, the classification of *C. bryantii* could be based only on its morphological characteristics, and it was difficult to characterize the organism at the molecular level because of the unavailability of a pure culture. Although *C. bryantii* is similar to *Syntrophomonas wolfei* in the unique syntrophic fatty acid-degrading function, these organisms differ other in their substrate range and especially in their morphology (284, 408). The former is a sporeformer with a gram-positive cell wall ultrastructure, and the latter is a nonsporeformer with a gram-negative cell wall ultrastructure. However, successful growth of *C. bryantii* in pure culture has enabled the study of its G+C content. The results of 16S rRNA sequence analyses indicated that, as a member of the gram-positive eubacteria phylum, this species is not closely related to any of the species in the cluster of typical clostridia with which it was compared or to any other clusters (subdivisions) in the gram-positive phylum with which it was compared. It was closely related to *Syntrophomonas wolfei*, which is a new member of the gram-positive phylum. Since there is no precedent for including ultrastructurally different gram-positive and gram-negative bacteria in the same genus, *Clostridium bryantii* was placed in a new genus, *Syntrophospora*, as *Syntrophospora bryantii*.

Sulfate-reducing bacteria. As well as reducing sulfate in monoculture, sulfate-reducing bacteria can grow in syntrophic associations in the presence of sulfate. They utilize H₂ and formate as electron donors to reduce sulfate when grown together with syntrophic acetogens that degrade benzoate (298, 299) and fatty acids with three or more carbons (40, 284, 408). Degradation of acetate and methanol via sulfate reduction was also demonstrated by using a coculture of *Desulfovibrio vulgaris* and *Methanosarcina barkeri* (335). Methanol can be degraded to CO₂ via sulfate reduction by a coculture of *Desulfovibrio vulgaris* and the homoacetogen *Sporomusa acidovorans* (82).

In the absence of sulfate, certain sulfate-reducing bacteria, such as *Desulfovibrio* spp., can grow together with H₂-utilizing methanogens to convert ethanol or lactate to acetate syntrophically (63, 283, 437). Syntrophic conversion of formate to methane was also observed in a syntrophic associ-

ation consisting of an H₂-utilizing methanogen, *Methanobacterium bryantii*, and *Desulfovibrio vulgaris*, which can split formate to hydrogen and CO₂ (154). It was proposed that syntrophic hydrogen production by sulfate-reducing bacteria in association with hydrogen-dependent methanogens occurred in the sediments from Lake Mendota (80). No mention of sulfate-reducing bacteria capable of syntrophically catabolizing volatile fatty acids (VFAs) has been reported. VFAs such as propionate and butyrate are thought to be converted only by syntrophic acetogens in concert with H₂-utilizing methanogens (40, 284, 285, 408, 451).

Physiology and Biochemistry

Inhibition of growth of syntrophs. (i) **Inhibition by H₂ or formate.** The isolation of bacteria that degrade fatty acids anaerobically required that an H₂-utilizing bacterium be included in the isolation media, since these reactions are thermodynamically unfavorable unless the hydrogen or formate concentration is very low. This hypothesis is based on the observation that syntrophic acetogenic bacteria can usually be maintained in coculture with methanogenic or sulfate-reducing bacteria (271, 468). Some syntrophic acetogenic bacteria can be grown in pure culture, however, either with sulfate as a terminal electron acceptor, e.g., *Desulfovibrio* spp., or with alternate carbon and energy sources, such as pyruvate for the S organism, ethylene glycols for *Pelobacter* sp. or *Syntrophomonas wolfei*, and crotonate for *Syntrophomonas bryantii* (63, 121, 342, 373).

(ii) **Inhibition by VFAs.** Volatile fatty acids are recognized as key intermediates in anaerobic digestion. Under active digestion, acetate rarely accumulates to concentrations greater than 1 to 10 mM, although its degradation accounts for about 70% of the methane formed. Other VFAs are usually found in lower concentrations. However, high levels of VFAs do accumulate when anaerobic digestors are stressed by high organic loading rates or short retention times. Until recently, syntrophic acetogenic bacteria could be grown only in coculture with an H₂-utilizing bacterium, so it was not possible to determine whether the syntrophic bacterium was affected by high levels of organic acid anions. Since *Syntrophomonas wolfei* can now be grown in pure culture, the effects of organic acid anions on its growth were determined by varying the initial concentration of the acid anion in the medium (22). Higher initial acetate concentrations decreased the butyrate degradation rate and yield of cells of *S. wolfei* per butyrate degraded, and acetate concentrations above 25 mM inhibited crotonate-using pure cultures and cocultures of *S. wolfei*. Benzoate and lactate inhibited the growth of *S. wolfei* on crotonate in pure culture and coculture. Lactate was an effective inhibitor of *S. wolfei* cultures at concentrations greater than 10 mM. High concentrations of acetate and lactate altered the electron flow in crotonate-catabolizing cocultures, resulting in the formation of less methane and more butyrate and caproate (22). The inclusion of the acetate-utilizing *Methanosarcina barkeri* in a methanogenic butyrate-catabolizing coculture of *S. wolfei* and *Methanospirillum hungatei* increased both the yield of *S. wolfei* cells per butyrate degraded and the efficiency of butyrate degradation. Butyrate degradation by acetate-inhibited cocultures occurred only after the addition of *Methanosarcina barkeri*.

(iii) **Inhibition by acetate.** Similarly, failure in isolation of isobutyrate-oxidizing cultures could be attributed to acetate inhibition in a coculture with only an H₂-utilizing methanogen or a sulfate reducer. This hypothesis was confirmed

when inclusion of an acetate-utilizing methanogen, *Methanosarcina mazei*, in a coculture with an H₂- and formate-utilizing methanogen, *Methanobacterium formicicum* yielded an isobutyrate-utilizing syntrophic strain (189). Ahring and Westermann (5) have also shown that butyrate consumption in a thermophilic coculture with *Methanobacterium thermoautotrophicum* was inhibited by increasing concentrations of acetate and that inhibition by acetate was gradually reversed when the acetate-utilizing methanogen was included in the coculture.

Nutrient influence on syntrophs. Evidence for the existence of two subgroups of propionate users in a sulfate-limited digester was found by kinetic analysis (161). The first subgroup, with a μ_{\max} of 0.0054/h, was reported as being similar to *Syntrophobacter wolinii*, and the second was a faster subgroup, with a μ_{\max} of 0.05/h and was not related to a known bacterium. Coculture of a sulfate-reducing bacterium, *Desulfovibrio* sp., with *Methanosarcina barkeri* 227 was able to produce CH₄ from propionate in the presence of sulfate if sufficient ferrous iron was added to the medium to trap the soluble sulfides produced from sulfate (155). In the absence of ferrous iron, soluble sulfides inhibited the acetate-clastic reaction. Boone and Xun (43) used enrichment cultures to determine the conditions promoting the fastest methanogenic propionate degradation and growth, by adapting the cultures to various physical and chemical conditions and measuring the specific growth rate. They found that the fastest growth of propionate oxidizers occurred at pH 6.8 to 8.5 and 32 to 45°C. The propionate enrichment culture growing in a medium without any added organic growth factors (except for a very small amount of EDTA, [5 mg/liter]) grew as rapidly as those with added yeast extract and Trypticase peptone. Addition of 10 mM FeCl₂ and 10 mM Na₂S reduced the rate of propionate degradation by about 30%. Addition of 20 mM CaCO₃ also reduced the rate of propionate degradation by about 20%. When 20 mM formate was added to the pH 7.2 culture 3 days after transfer, propionate degradation was severely inhibited. Elevated acetate concentration (20 mM), but not NaCl (20 mM), caused a moderate inhibitory effect on the propionate-oxidizing bacteria (43). Inhibition of propionate oxidation by acetate concentrations in the digesting sludge was also observed by Kaspar and Wuhrmann (199) and Zehnder and Koch (486).

Roy et al. (354) established two highly purified syntrophic associations degrading stearate and oleate, with *Methanospirillum hungatei* as the syntrophic partner that removed H₂ from the system. During growth on long-chain fatty acids, supplementation of the culture medium with calcium chloride was an absolute requirement. Saturated long-chain fatty acid degradation occurred in the presence of equinormal amounts of calcium (fatty acid/Ca ratio, 2:1), whereas oleate degradation took place only in the presence of an equimolar amount of calcium (fatty acid/Ca ratio, 1:1).

Tricultures of syntrophs. Kinetics of butyrate, acetate, and hydrogen metabolism were determined with butyrate-limited chemostat grown tricultures of a thermophilic butyrate-utilizing bacterium together with *Methanobacterium thermoautotrophicum* and the TAM organism, a thermophilic acetate-utilizing methanogenic rod (4). The K_m s for butyrate, acetate, and dissolved hydrogen were 76 μ M, 0.4 mM, and 8.5 μ M, respectively. Butyrate and hydrogen were metabolized to a concentration of less than 1 μ M, whereas acetate uptake usually ceased at a concentration of 25 to 75 μ M, indicating a threshold level for acetate uptake. Acetate is reported to be the first volatile fatty acid to accumulate

after a decrease in retention time or an increase in organic loading of a digester.

Fatty acid-degrading pathways. (i) **Butyrate degradation.** *Syntrophomonas wolfei* is an interspecies H₂-transfer-dependent, proton-reducing bacterium that catabolizes short-chain fatty acids only in association with H₂-using bacteria. It grows very slowly in coculture with *Methanospirillum hungatei* with butyrate as the energy source, since the degradation of butyrate by the coculture releases a very small amount of free energy (425). Assuming that a free energy change of about 45 kJ mol⁻¹ is required to synthesize 1 mol of ATP (425) and considering the fact that the free energy released during butyrate degradation must support the growth of two organisms, little energy is available for growth of *S. wolfei*. Also, it has been proposed that energy is required to produce H₂ from electrons generated in the oxidation of butyryl-CoA to crotonyl-CoA (426), suggesting that even less of the energy generated in butyrate metabolism is available to support the growth of *S. wolfei*.

S. wolfei oxidizes butyrate as well as the straight-chain, monocarboxylic, saturated C₅ to C₈ fatty acids and isoheptanoate in coculture with a single H₂-utilizing *Methanospirillum hungatei* culture. Wofford et al. (466) selectively lysed the cells of *S. wolfei* by lysozyme treatment and found that the cell extracts had high specific activities of all four β -oxidation enzymes, acyl-CoA dehydrogenase, enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, and 3-keetoacyl-CoA thiolase. The acyl-CoA dehydrogenase activity was high when a C₄ but not a C₈ or C₁₆ acyl-CoA derivative served as the substrate. *S. wolfei* cell extracts had high CoA transferase specific activities and no detectable acyl-CoA synthetase activity, indicating that fatty acid activation occurred by transfer of CoA from acetyl-CoA. The presence of phosphotransacetylase and acetate kinase activities indicates the existence of substrate-level phosphorylation in *S. wolfei* cells.

(ii) **Propionate degradation.** The pathway of propionate degradation was studied by Koch et al. (216), using propionate as the sole organic carbon and energy source in a highly enriched mixed methanogenic culture. By using ¹⁴C-radiotracers, propionate was first metabolized to acetate, carbon dioxide, and hydrogen by nonmethanogenic organisms. The carbon dioxide originated exclusively from the carboxyl group of propionate, whereas both [2-¹⁴C]propionate and [3-¹⁴C]propionate led to the production of radioactive acetate. The methyl and carboxyl groups of the acetate produced were equally labeled regardless of whether [2-¹⁴C]propionate or [3-¹⁴C]propionate was used, suggesting that propionate degradation occurred through a randomized pathway. Mucha et al. (300) studied the anaerobic degradation of propionate to acetate and methane by a defined coculture of *Syntrophobacter wolinii* and *Desulfovibrio* strain G11 and by a thermophilic, methanogenic consortium T13. Their experiments involving labeled propionate produced evidence for a propionate degradation pathway that involves methylmalonyl-CoA as an intermediate. The degradation of [1-¹⁴C]propionate led exclusively to the formation of ¹⁴CO₂ by *S. wolinii*/*Desulfovibrio* strain G11 and to the formation of ¹⁴CH₄ by the methanogenic consortium T13. The conversion of either [2-¹⁴C]propionate or [3-¹⁴C]propionate resulted in uniformly labeled acetate, which was further converted by the methanogenic consortium T13 to yield equivalent amounts of ¹⁴CO₂ and ¹⁴CH₄. Using cell extracts of pure culture of *Desulfovibrio* strain G11 as a control, most of the enzymes involved in the methylmalonyl-CoA pathway for propionate oxidation, including a propio-

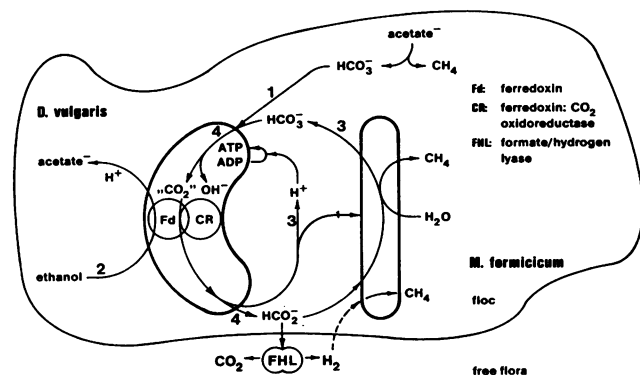
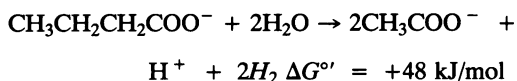


FIG. 9. Hypothetical model illustrating the principles of the bicarbonate-formate electron shuttle mechanism for explaining the control of interspecies electron flow during syntrophic ethanol conversion to methane during syntrophic growth of *Desulfovibrio vulgaris* and *Methanobacterium formicicum* in an anaerobic digester floc. Reprinted from reference 429 with permission.

nyl-CoA:oxaloacetate transcarboxylase, were recently demonstrated in *S. wolinii* (169).

Adaptation Mechanisms

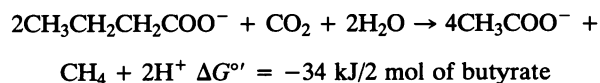
Cocultures of obligately syntrophic anaerobic bacteria such as those for butyrate oxidation or propionate oxidation appear to act as a single organism with the combined metabolic activities of both bacteria. These associations involve fatty acid-oxidizing bacteria in coculture with H_2 -utilizing methanogens or sulfidogens. The fermentation of butyrate to acetate and hydrogen is a highly endergonic reaction under standard conditions:



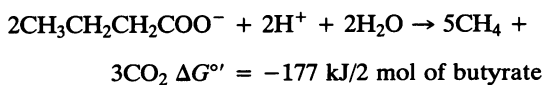
This reaction can occur only if the equilibrium is shifted to the right side, e.g., by removal of one of the reaction products:



The overall reaction can therefore be written as follows:



However, since under the natural conditions acetate produced is further converted to CH_4 , the yield and energy balance for the whole process of butyrate conversion to methane can be written as follows:



Since the production of methane from butyrate will involve three different bacteria, the syntrophic acetogenic bacteria have to share the fraction of total energy (fractions of ATP equivalents) available with H_2 -utilizing and acetate-utilizing methanogenic bacteria. The cooperation between the partner organisms in such syntrophic relationships is optimal if the diffusion distance for transfer of metabolites (H_2 , acetate) is as small as possible (374); therefore, juxtapositioning

of the organisms would be the most beneficial and appears to occur in many syntrophic associations.

Thiele and Zeikus (428, 429) have shown that formate may be an important extracellular intermediate in methanogenic systems. They have proposed a hypothetical model (Fig. 9), which illustrates the bicarbonate-formate electron cycle for control of interspecies electron flow. This model is quite different from that described for interspecies H_2 transfer mechanisms (467), since bicarbonate is a substrate for the acetogens, not the methanogens. The juxtaposition of *Desulfovibrio vulgaris* and *Methanobacterium formicicum* in flocs enables methanogenic CO_2 regeneration to be coupled with acetogenic CO_2 reduction to formate, which itself serves as the mediator of interspecies electron flow. Energy conservation in the acetogen is postulated to occur as a consequence of putative cytoplasmic carbonic anhydrase and a membrane-linked CO_2 reduction, which leads to generation of an alkaline cell interior, an acidic exterior, and a PMF to drive membrane-coupled ATP synthesis. The model shows the dynamics of formate metabolism in the digester ecosystem. More than 90% of the CO_2 -dependent syntrophic ethanol conversion to methane occurs via interspecies formate transfer within flocs, whereas less than 10% occurs via interspecies H_2 transfer caused by cleavage of floc-excreted formate in the soluble digester phase to H_2 and CO_2 (428, 429).

The importance of formate as an extracellular intermediate in methanogenic systems was subsequently confirmed by Jain et al. (189), using a defined triculture in which formate inhibited oxidation of butyrate as well as isobutyrate in a consortium that included a butyrate-isobutyrate-oxidizing strain IB, the H_2 -utilizing *Methanobacterium formicicum*, TIN, and the acetate-utilizing *Methanosarcina mazei* T18. Electron transfer from NADH of fatty acid-oxidizing bacteria to F_{420} of methanogens requires several steps: the reduction of an extracellular electron carrier (H^+ or HCO_3^- to H_2 or HCOO^-), the diffusion of that carrier into the bulk aqueous phase, the diffusion from the bulk aqueous phase to the methanogen, and the subsequent oxidation of that electron carrier, forming reduced F_{420} . Thus, the flux of hydrogen (or formate) is directly proportional to the surface area of the producing bacterium and the concentration difference at the surfaces of both partners and is inversely proportional to the diffusion distance (374).

It has been experimentally demonstrated that if the two syntrophic partners in a syntrophic ethanol-oxidizing coculture are separated by a dialysis membrane, the doubling time of both the partners increased from about 7 h to about 14 to 18 h and the levels of H_2 in the compartment of the syntrophic acetogen increase (409), verifying the importance of juxtapositioning of the syntrophs.

It is likely that in triculture of an isobutyrate-butyrate degrader with an acetate-utilizing and an $H_2 + \text{CO}_2$ and formate utilizing methanogen, the isobutyrate-butyrate degrader may be able to derive more energy from butyrate metabolism when an acetate-utilizing organism is present. This adds to the concept that any particular organism may be incapable of generating the energy required for growth but that combinations of organisms may together do so, in a nice example of "social biology."

On the basis of the assumption that formate is the intermediary metabolite of syntrophic oxidation of butyrate-isobutyrate, a simulation curve illustrating free-energy changes under different isobutyrate and butyrate ratio has been constructed (Fig. 10) (475, 478). In this simulation (pH 7.0), the total amount of isobutyrate plus butyrate is 10 mM

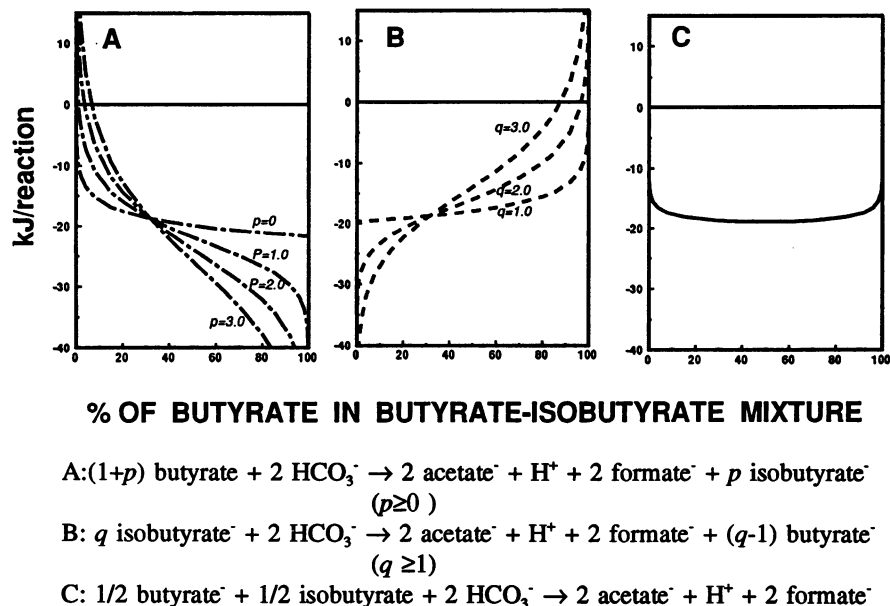


FIG. 10. Free-energy change of isomerization coupled with butyrate oxidation versus the percentage of butyrate in a butyrate-isobutyrate mixture, based on interspecies formate transfer. Reprinted from references 475 and 478 with permission.

and the concentrations of formate, acetate, and bicarbonate are assumed to be 5 mM, 2 μM , and 50 mM, respectively. Curve A describes the free-energy change of the isomerization from isobutyrate to butyrate coupled with syntrophic butyrate conversion (Table 12, reaction 8). This reaction is thermodynamically possible when the isobutyrate level is more than 6% in the mixture of isobutyrate and butyrate. This way, energetically there are three areas for possible isomerization coupled with syntrophic butyrate degradation: (i) where the butyrate level is much lower than the isobutyrate level and only isomerization from isobutyrate to butyrate occurs, (ii) where the butyrate level is much higher than the isobutyrate level and only isomerization from butyrate to isobutyrate is favorable, and (iii) where reversible isomerization occurs. Curve C represents the energy change of the isomerization of isobutyrate and butyrate coupled with syntrophic oxidation of butyrate and illustrates that the three possible reaction areas also exist and the most favorable free energy for the reverse isomerization between isobutyrate and butyrate is obtained at the isobutyrate and butyrate equilibrium (475, 478).

Propionate and longer-chain fatty acids are ecologically much more important as intermediates in the process than lactate or ethanol, but only a few documented species can degrade these compounds. As discussed above, the problem can be explained on a thermodynamic basis. We have also discussed that by adding an acetate-utilizing methanogen to a fatty acid-degrading coculture, the syntrophic acetogenic bacteria are able to derive more energy by increased transformation reactions. It is therefore tempting to speculate that syntrophic acetogenic bacteria are adapted to these energy stresses in unique anaerobic niches and are able to thrive in the company of methanogenic or sulfate-reducing bacteria that are able to participate in interspecies formate or hydrogen transfer. Seitz et al. (381, 382) determined the Gibbs free energy available to both ethanol oxidation and H_2 oxidation at steady state and the free energy shared among syntrophic partners when an ethanol-oxidizing, proton-reducing *Pelo-*

bacter acetylenicus strain was grown in coculture with either *Acetobacterium woodii*, *Methanobacterium bryantii*, or *Desulfovibrio desulfuricans*. The Gibbs free energy ($\Delta G = -16.3$ kJ/mol of ethanol) available to *Pelobacter acetylenicus* and its maximum yield coefficient (Y_{max}) (1.7 to 2.2 g/mol of ethanol) were almost constant in the different cultures. *Pelobacter acetylenicus* shared 44 to 67% of the total biomass produced, whereas it shared only 19, 23, and 37% of the total ΔG available from ethanol oxidation coupled to sulfate

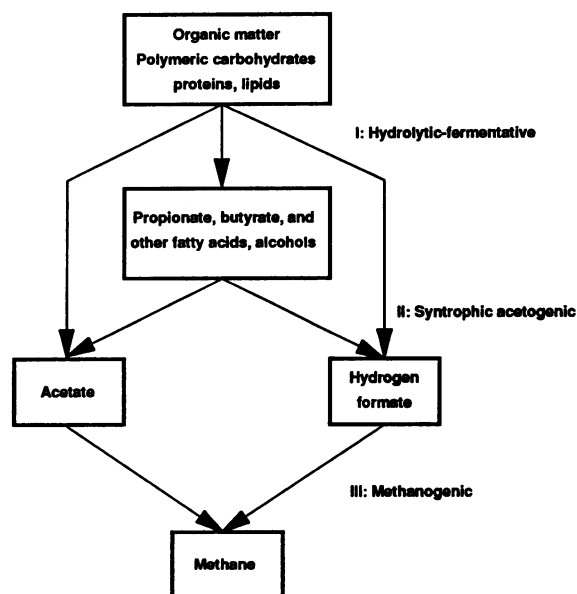


FIG. 11. Carbon flow scheme in complete mineralization of organic matter to methane and carbon dioxide. Syntrophs function to degrade end products of the hydrolytic-fermentative bacteria and transfer these as the energy sources for methanogens.

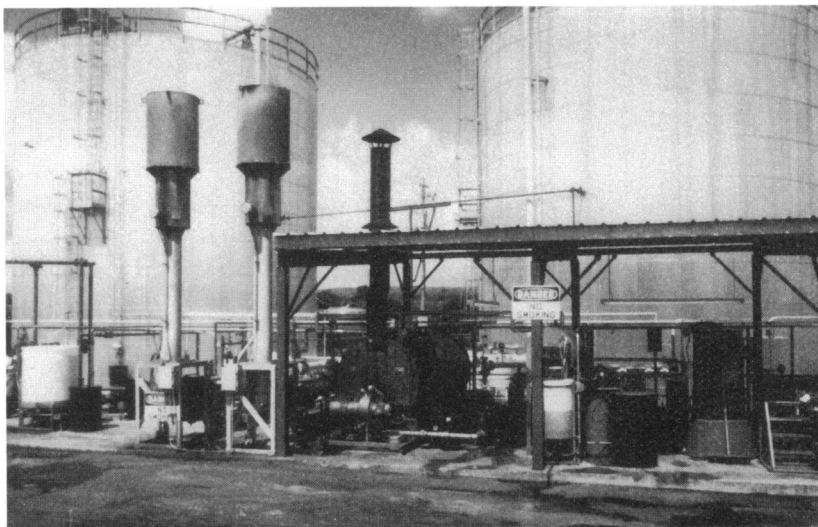


FIG. 12. Anaerobic digester with a two-phase reactor system, with hydrolytic organisms producing fatty acids in the first tank and syntrophs and methanogens in the second tank consuming the fatty acids and producing methane.

reduction, methanogenesis, and homoacetogenesis, respectively (382). The residual 63 to 81% of the total available ΔG was shared by the H_2 oxidizers, which exhibited Y_{max} values of 6.6 g/mol of acetate for *Acetobacterium woodii*, 3.8 g/mol of sulfide for *Desulfovibrio desulfuricans*, and 2.2 g/mol of CH_4 for *Methanobacterium bryantii*.

Biotechnological Features

Overview. Complete anaerobic digestion of complex organic matter (polysaccharides, proteins, and lipids) requires at least three groups, including hydrolytic-fermentative microorganisms, syntrophic acetogenic bacteria, and methanogenic bacteria (Fig. 11 and 12). The hydrolytic-fermentative organisms convert a variety of complex organic matter into formate, acetate, propionate, and other acids and alcohols, hydrogen, and carbon dioxide. The syntrophic acetogens convert some of these metabolic products into direct methanogenic precursors (acetate, hydrogen, or formate). The third trophic group includes the methanogens, which utilize these precursors to produce methane and CO_2 .

Syntrophic biomethanation granules. Biomethanation granules offer a unique biotype in which all the trophic groups responsible for complete mineralization of complex organic matter to methane and carbon dioxide can be present within the same bacterial consortium or ecosystem. Development and growth of conventional granular sludge (i.e., UASB granules) is important for high-rate anaerobic digestion systems. Syntrophic biomethanation (SB) granules have been developed (427) for high-rate VFA turnover in two-phase anaerobic digestion systems (Fig. 13 and 14). These improved granules function with high chemical oxygen demand turnover rates and high volumetric gas production rates. SB granules are smaller and more dense than UASB granules, and this improves diffusion limitations. They also contain novel adhesive methanogenic and syntrophic butyrate- and propionate-degrading bacteria that are present at very high cell densities (474, 478). It is speculated that interspecies distances are minimized in these granules by juxtapositioning mechanisms which maximize interspecies metabolite transfer between syntrophic fatty acid degraders and methanogens (187).

The utilization of syntrophic acetogenic and methanogenic bacteria in SB granules lacking hydrolytic fermentative bacteria may be advantageous, since this granular culture has been selected for high-rate VFA degradation and may be used as a second-stage inoculum for treating any waste material provided that another starter is used in the first phase. Two separate microbial phases enable maximization of rate-limiting biodegradation reactions and can eliminate adverse conditions, which may include shock loading and toxic compounds, from inactivating the sensitive syntrophic acetogenic and methanogenic populations. In addition, syntrophic interactions between H_2 - and formate-producing acetogenic and CO_2 -reducing methanogens are of tremendous importance in controlling the rate-limiting metabolic steps of VFA degradation in anaerobic digestors. It can be concluded that a syntrophic microniche is dynamically related to the optimized biomethanation performance of the entire ecosystem. This understanding can be used to develop new biomethanation systems for toxic and residual wastes (187).

ACIDOPHILES AND ALKALIPHILES

Ecology and Diversity

Overview. Bacteria have been found in environments with pH values from approximately 1 up to 11, but most organisms grow between pH 4 and 8, with optimum growth at pH 7 (233). Natural environments with acidic pH values of 3 to 4 are relatively common; they include thermal springs, soils and lakes, peat bogs, and certain gastrointestinal environments. Naturally occurring alkaline environments result from the presence of large amounts of sodium carbonate and other salts. Although many organisms have the ability to grow or survive extremes in pH outside of their optimum range for growth, only a few bacteria grow optimally at acidic or alkaline pH.

In considering pH extremes, it is important not to overlook microenvironments in which the pH can be very different from the gross pH measurements of the environment as a whole. Such variations can arise from the biological activities of organisms in close association, whereby the metabolic activities of one organism alter the pH of the

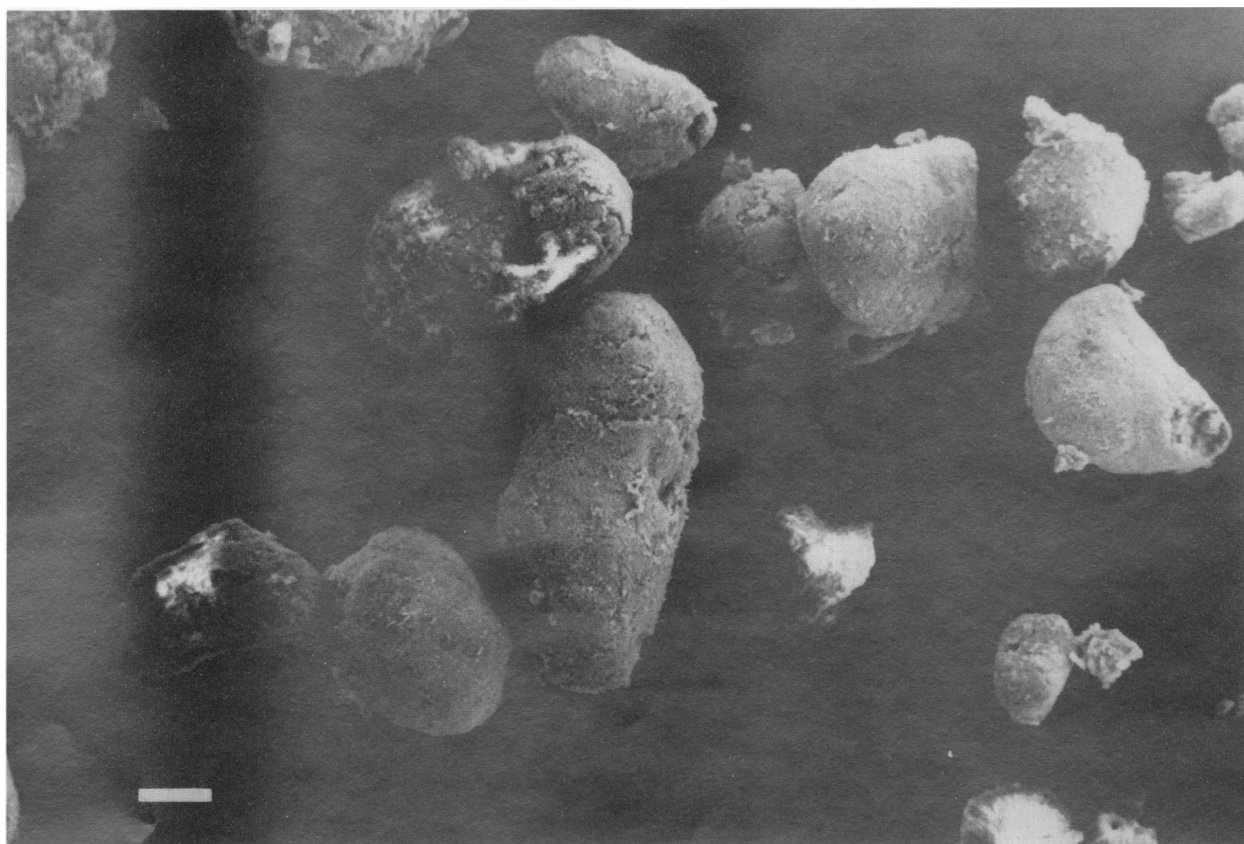


FIG. 13. Scanning electron micrograph of syntrophic biomethanation granules made up of syntrophiles and methanogens. Scale bar = 1,000 μm . Reprinted from reference 478 with permission.

microniche. This is particularly true of the pH of soil, which is a heterogeneous, discontinuous structured environment consisting of microhabitats of variable sizes differing in nutrient content, O_2 levels, and pH. Even though pH measurements of a given soil are usually the gross measurement, microniches may occur in soil, differing by several pH units either more alkaline or acidic from the average value obtained (107).

Gastrointestinal ecosystems. The ruminal environment is known to undergo fluctuations in pH in response to changes in both diet composition and the activities of ruminal microorganisms. It has been clearly established that pH affects the efficiency of growth of individual species (358). However, because of the extensive buffering capacity of the rumen, gross pH changes will be relatively short in duration.

The human gastrointestinal tract varies greatly in pH, with the stomach being the most acidic environment. The most important factor controlling the flora of the stomach is pH. In the absence of food the pH can be low (approximately 1.5), but with food ingestion the pH is approximately 4.0 and active colonization occurs. Organisms present include the facultative anaerobes, streptococci, lactobacilli, and the obligate anaerobe *Sarcina ventriculi*. Presumably, if these organisms are to survive in the gastrointestinal tract, they must possess means by which to survive the acidic pH in the stomach or the alkaline pH further down the gastrointestinal tract. The extreme pH tolerance of *S. ventriculi*, with growth occurring at pH 1.0 to pH 9.8 (72), would tend to support the pH-tolerant nature of organisms which exist in environments with extremes in pH.

Anaerobes make up about half of the microorganisms present in the biliary tract, with *Clostridium perfringens* being the most common anaerobe, although large numbers of *Bacteroides fragilis* can also be frequently found. It is likely that these anaerobes are metabolically active and that they are involved in the pathogenesis of biliary tract inflammation because of their ability to deconjugate bile acids, releasing the more toxic nonconjugated form (45).

Anaerobes are also found in the distal small bowel, where the redox potential is -150 mV. The cecum has a lower redox potential of -200 mV, and in this organ the total number of anaerobes is larger than the total number of aerobic organisms, although presumably the facultative anaerobes are growing fermentatively (45). Studies made with organisms isolated from the intestinal tract have shown that pH is very important in the induction and production of enzymes. The pH of the intestinal tract varies greatly from the acidic conditions found in the stomach to the alkaline conditions of the duodenum. Bacterial amino acid decarboxylases are not produced until the pH falls much below neutral pH, and deaminases are produced only at alkaline pH values (162).

Fermentation of carbohydrate in the colon causes the pH to fall to about 4.8. Continuous-culture studies on colonic bacteria grown from pH 7 to 5 indicated that there is a population change, with clostridia surviving better at pH 7 and only lactobacilli and bifidobacteria being recovered at pH 5. Propionic acid and acetic acid production was favored at pH 6, and ammonia production was unchanged with changing pH (116).

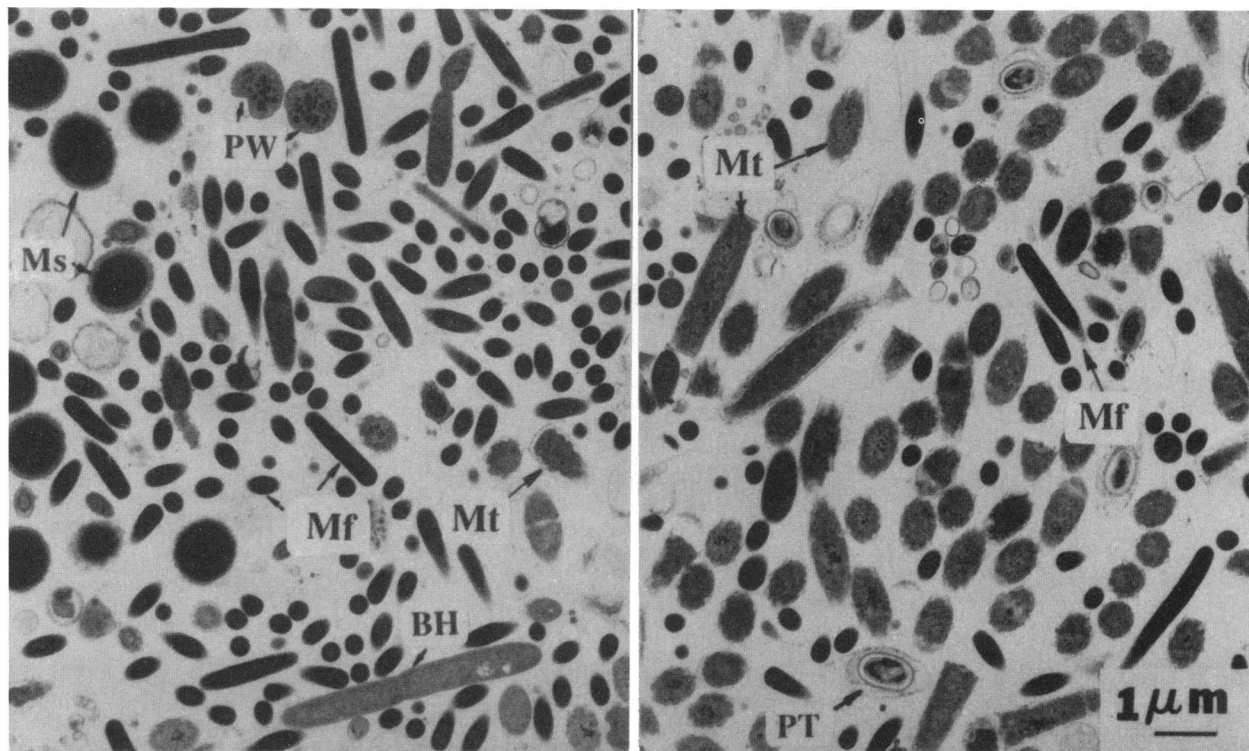


FIG. 14. Transmission electron micrographs of syntrophic biomethanation granules. Mf, *Methanobacterium formicum*-like rods; Mt, *Methanotrix*-like rods; Ms, *Methanosarcina mazei*-like cocci; BH, sporeforming *Syntrophospora bryantii*-like rods; PT, sporeforming syntrophic propionate degrader; PW, nonsporeforming propionate degrader. Note that the syntrophiles are surrounded by H_2 - and formate-consuming methanogens. Reprinted from reference 478 with permission.

Carbohydrate fermentation. Spoilage in the food industry is a major problem, and one of the important factors influencing microbial growth in food is pH. Most food spoilage bacteria do not grow at pH values below 5, and the pH can be lowered by the addition of acid or by microbial action with lactic acid, acetic acid, and propionic acid bacteria, with growth ceasing once the pH drops below 4. Growth of *Clostridium botulinum* in foods usually results in overt spoilage due to the production of gas, volatile organic acids, and proteolytic enzymes; occasionally, botulinum toxin is produced. It was thought that this organism could not grow and form toxins below pH 4.6; however, one study showed that at pH 4.0 *C. botulinum* grows and produces toxin (340), indicating its acidophilic nature.

Ensiling has been used for crop preservation for over 3,000 years. In ensiling, a crop is harvested directly and sealed to create anaerobic conditions. During storage, sugars and some organic acids in the crop are fermented by lactic acid bacteria, producing lactic acid as well as acetic acid, ethanol, and other minor products and thus reducing the pH to between 3.8 and 5.0. The low pH decreases plant enzyme activity and prevents the proliferation of detrimental anaerobic microorganisms, especially clostridia and enterobacteria, and the anaerobic environment prevents aerobic spoilage and heating of the crop. The main organisms responsible for this process include *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Streptococcus* spp. These bacteria are all microaerophilic, gram-positive nonsporeformers. Clostridia are the most detrimental to the ensiling process; some clostridia will ferment amino acids to amines and ammonia, whereas others will ferment lactate to butyrate, and in

general these organisms are inhibited at pH values around 4 (301).

Methanobacterium arbophilicum is found in wet wood in living trees which were sites for methanogenesis; it has a pH optimum for growth of 7.5 to 8.5 (499). Large numbers of anaerobic bacteria, including heterotrophic and methanogenic species, were routinely detected in wetwoods from cottonwood, elm, and willow (505). The pH of wetwood varies from acidic to alkaline depending on the tree species, and both extremes in pH could occur in the same tree (445), demonstrating the ability of anaerobic bacteria to be metabolically active in environments with pH values differing from the optimum growth pH.

Sarcina maxima was first observed in spontaneously souring flour paste; it is commonly found on the hull or outer coat of cereal grains such as wheat, oat, rice, and rye. It was isolated in pure culture and studied by Smit (391). This organism is capable of growth from pH values near 1.0 up to 9.8 (72), and it produces acetate, butyrate, H_2 , and CO_2 . Both *Sarcina ventriculi* and *Sarcina maxima* appear to be unique in their ability to tolerate such a broad range in pH, and this raises questions as to any structural and biochemical differences between cellular components, in particular the membranes of these organisms and those of less acid-tolerant bacteria.

Protein fermentation. In anaerobic digestion processes, complex substrates (carbohydrates, proteins, and lipids) are hydrolyzed and degraded to methane and carbon dioxide by a mixed population (488). Hydrolytic species which ferment various saccharides in anaerobic digestion have been well characterized, but proteolytic species such as those which



FIG. 15. Crystal Lake Bog located in northern Wisconsin. The sediments of the bog are high in organic matter, and both carbon degradation and hydrogen metabolism are inhibited by low pH (pH 3.8), which occurs as a result of lack of buffering capacity. This bog is a natural niche for *Sarcina ventriculi*.

degrade collagen or gelatin have only recently been investigated. Gelatin is a heterogeneous degradation product of collagen with respect to both N- and C-terminal amino acids. Bacteria which utilize collagen also have the ability to consume gelatin, but not all gelatin degraders consume collagen.

Pathogenic *Clostridium histolyticum* is the only *Clostridium* species that displays active extracellular collagenase activity. *Clostridium proteolyticum* and *Clostridium collagenovorans* were isolated from an anaerobic digester and are prevalent organisms among collagen- and gelatin-degrading organisms in waste-processing digestors (190). Proteolytic clostridia are able to excrete proteases to digest proteins, with the formation of branched-chain fatty acids from the corresponding amino acids, which is characteristic for this type of fermentation (286).

Sedimentary ecosystems. Sediments from oligotrophic, eutrophic, and dystrophic lake systems associated with acid peat bogs were found to have pH values from 4.9 to 8.0. Dissolved-hydrogen concentrations and hydrogen transformation kinetic parameters were measured to assess the influence of pH on hydrogen metabolism, with the finding that the overall rate of hydrogen metabolism was lowered as a result of decreased sediment pH, although activity was detected at low pH values, with the type of ecosystem (i.e., eutrophic, oligotrophic or dystrophic), having as much effect as the pH (148).

The pH optimum for methane production in acidic peat bogs in Minnesota was 6.0 and was well above the in situ pH of 3.8. Addition of acetate to peat sediments inhibited methanogenesis, leading to the conclusion that carbon dioxide and hydrogen were the dominant precursors of methane in this environment (460).

The dynamics of anaerobic digestion were examined in the low-pH sediments (pH 4.9) of Crystal Lake Bog in Wisconsin (Fig. 15). *Lactobacillus*, *Clostridium*, and *Sarcina ventriculi* strains were isolated from the bog and were found to grow at pH values as low as 4.7, 3.9, and 2.0, respectively (149). The pH of the bog sediment altered anaerobic digestion processes such that total biocatalytic activity is lower, but the general carbon and electron flow pathways are

similar to neutral anoxic sediments. The hydrolytic, hydrogen-producing acetogenic and the methanogenic populations appeared to be the most sensitive to low pH (149). *Sarcina ventriculi* has been cultivated from garden soil (24), sand (391), river mud (73) and acid peat bog sediments (149). The presence of this organism in acidic and alkaline soils and its ability to grow optimally over a broad pH range (150) demonstrate its ability to adapt to changes in pH.

Alkalophilic methanogenesis has been reported when methane formation was observed following incubation of slurries from Big Soda Lake, Nev. (316). The optimum pH for methane production from methanol was 9.7, which is higher than the pH ranges reported for growth of pure cultures of methanogens (14). A number of methanogens have pH optima above neutral, including a number of strains of *Methanobacterium alcaliphilum*, one of which has a pH optimum of 7.5 to 8.5 (36) four others have alkaline pH optima for growth, and the type strain has a pH optimum of pH 8.1 to 9.1 (472). *Methanobacterium arbophilicum* grows optimally at pH 7.5 to 8.0 (499), and *Methanococcus vanielii* has a pH optimum of 7.0 to 9.0 (397).

A number of methanogens were isolated from sediments of lakes in Egypt (42). The pH values of these lakes were in the range of 8.3 to 9.3, with one lake having a pH of 9.7. Four of the strains were obligately halophilic and had pH optima around 8.3; they belong to a new species, *Methanobacterium alcaliphilum* (472). *Methanohalophilus zhilinae* was isolated from the same site and has the highest pH optimum reported, pH 9.2. This organism is also halophilic, requiring 0.7 M salt for growth (276). *Methanohalophilus oregonense*, a methylotrophic methanogen, was isolated from an alkaline saline aquifer with a pH of 10, 3 m below the ground near Alkali Lake; it grows optimally at a pH of 8.4 to 9.0 and fails to grow below pH 7.6 (256).

A number of anaerobic, halophilic eubacteria have been isolated from hypersaline alkaline environments. These organisms were all gram-negative, nonsporeforming rods. Three of the organisms (strains SS-11, SS-15, and SS-21) were isolated from the upper layers of muds with a pH of between 6.5 and 8.5 to 9.5 in the Salton Sea, had an optimum pH for growth of 7.5 to 8.0, and produced acidic fermenta-

tion products, with one strain also forming ethanol. Another isolate (strain M-20) was obligately alkalophilic, not growing below neutral pH and having a pH optimum of 8.5 to 9.5, produced acids and ethanol as fermentation products, and was isolated from the surface sediments in Big Soda Lake, Nev., from a site with a pH of 9 to 10 (167, 387). The organism with the highest optimum pH for growth, of 8.5 to 10.0, was strain KY-402, isolated from sediment from Lake Magadi, Kenya (167). Another obligately alkalophilic anaerobe was isolated from Aqua de Ney spring, which had an average pH of 11.5, and the organism grew over a pH range of 8.0 to 11.3, with a pH optimum of 9.5 (393). It is important to note that although these organisms grow optimally at high salt concentrations, alkaliphily is not typical of halophilic anaerobes. Indeed, the obligately halophilic anaerobes taxonomically described to date all grow at neutral pH or below.

Thermophilic ecosystems. Octopus Springs in Yellowstone Park has a pH of 8.3 (51); however, the thermophilic organisms isolated from this environment have pH optima ranging from neutral for *Clostridium thermohydrosulfuricum* (458), *Thermoanaerobacter brockii* (497), and *Methanobacterium thermoautotrophicum* (506) to acidic pH values of 5.5 to 6.5 for *Thermoanaerobacterium thermosulfurigenes* (376). The pH measurements made in the spring probably represent the gross pH and do not take into account micro-niches within the algal mat whereby acid-producing organisms will lower the pH and methane-forming organisms will increase the pH. Indeed, pH measurements taken from the microbial mats by using microelectrodes have shown that even over a distance as small as 3 mm, the pH can range as much as 2 pH units (55).

Many extremely thermophilic archaeobacteria are acidophilic and have been isolated from environments with pH values ranging from acid to alkaline, illustrating the discrepancy between the pH of the isolation source and the optimal pH of the organism. *Pyrobaculum islandicum* and *Pyrobaculum organotrophicum* occur in neutral to slightly alkaline boiling solfataric waters (172). *Desulfurococcus mobilis*, *Desulfurococcus mucosus* (521), and *Thermofilum pendens* (517) were isolated from areas with pH values ranging from 2.2 to 6.5. *Thermodiscus maritimus* occurs in hot seawater with a pH of 6.5, and *Thermoproteus tenax* was isolated from a mud hole with a pH of 5.5 (134). Despite the range in pH of the isolation sites, *Pyrodictium brockii* and *Pyrodictium occultum* (403), *Thermococcus celer* (519), and the above extreme thermophiles have pH optima for growth around 5.0 to 6.0.

Physiology and Metabolism

Overview. The ability of anaerobic bacteria to adapt to changing pH conditions is illustrated by the fact that few species are described as acidophilic or alkaliphilic or are isolated from environments with extremes in pH. There is, however, a growing amount of literature from physiological and biochemical studies that these supposedly neutrophilic bacteria can not only tolerate pH extremes but also adapt to them.

Anaerobes will often experience changes in environmental pH as a consequence of growth and the production of fermentation products, causing acidification of the surrounding environment. Often a change in environmental pH is accompanied by a shift in fermentation carbon and electron flow pathways. If an organism produces only acidic fermentation products, the acid with the lower pK_a will often no

longer be formed to prevent further acidification. For organisms with a mixed-acid fermentation, growth at low pH often results in the cessation of acid production and a shift to alcohol formation.

Acidophilic fermentations. (i) **Acetone and butanol fermentation.** Probably the organism which has been investigated most intensively with respect to the mechanisms in which pH affects metabolism is *Clostridium acetobutylicum*. This organism has two growth phases. During the first phase of growth the pH of the medium drops from around pH 6 to 4.5 and the internal pH decreases but does not go below 5.5 (152, 171, 423). Accumulation of acetic and butyric acid within the cell at an internal pH of 5.5 is thought to be a trigger for the switch from acid to solvent production (13, 151, 166, 281).

In *C. acetobutylicum*, the change from the acidogenic phase to the solventogenic phase of growth is accompanied by alterations in levels of key catabolic enzymes. The highest activity of phosphotransacetylase, phosphotransbutyrylase, acetate kinase, and butyrate kinase was found in cells which carried out an acetate-butyrate fermentation, compared with levels in solvent-forming cells (6, 157). The levels of butyraldehyde and butanol dehydrogenases, enzymes involved in solvent formation, were found in small amounts exclusively in solvent-forming cells, together with high levels of CoA-transferase and acetoacetate decarboxylase, enzymes involved in acetone formation (6). Synthesis of NADH-rubredoxin oxidoreductase increased significantly from the acidogenic to the solventogenic phase of growth, and it was suggested that this enzyme could play some role in the deacidification mechanism in relation to proton transport (272). The specific hydrogen production rates of *C. acetobutylicum* decreased as the culture became solventogenic and were accompanied by a corresponding decrease in the specific activity of hydrogenase (207). These biochemical changes contribute to the physiological changes observed in *C. acetobutylicum*. During the acidogenic phase of growth, hydrogenase levels are high and the organism has a very efficient route for the disposal of internal protons by producing gaseous hydrogen generated through the action of pyruvate dehydrogenase. Solventogenesis is associated with a decrease in hydrogen production and an increase in the level of solvent-forming enzymes, providing a means of diverting intracellular protons and electrons to neutral solvents (207).

(ii) **Ethanol fermentation.** *Sarcina ventriculi* produces acetate, ethanol, formate, hydrogen, and carbon dioxide as fermentation products (71). When it is grown at neutral pH, acetate, ethanol, and formate are formed in equal concentrations, and with a decrease in pH the ethanol/acetate ratio increases significantly (150, 267, 399). In another anaerobe, *Streptococcus bovis*, the growth yield and the production of acetate, ethanol, and formate decreased at pH values less than 6.5, whereas the production of lactate increased (132).

In *Sarcina ventriculi* the levels of acetate kinase and alcohol dehydrogenase do not appear to be regulated as a function of environmental pH; instead, regulation appears to involve the central part of the pathway of glucose metabolism through to fermentation products, rather than the terminal stages of the pathway. Significant differences in levels of pyruvate dehydrogenase, acetaldehyde dehydrogenase, and pyruvate decarboxylase were observed in cells grown at low and neutral pH. The former two enzymes were present at the highest levels in cells grown at neutral pH, diverting carbon and electron flow through to acetate and ethanol by the oxidative decarboxylation of pyruvate by pyruvate dehydrogenase, whereas at low pH carbon flowed via pyruvate

decarboxylase to ethanol (267). This allows a much tighter control on regulation of carbon and electron flow by the involvement of key branch point enzymes rather than the entire pathway.

(iii) **Lactate, propionate, and succinate fermentations.** Lactic acid bacteria are one of the few groups of anaerobic bacteria known to grow in moderately acidic environments, and their specific growth rates and the magnitude of their PMFs are markedly lowered under moderately acidic conditions than under neutral conditions. *Lactobacillus helveticus* is a homolactic acid producer, and the growth and glucose catabolism of this organism are dynamically related to internal and external protonated acid concentrations which control energy flux and the mechanism of energy conservation. Since this organism makes only lactic acid, alteration of the fermentation product profile to less acidic products is not possible. During growth on glucose, a rapid drop in pH was paralleled by a decrease in cytoplasmic pH, substrate-product flux, PMF, and the $\text{NADH}_2/\text{NAD}^+$ ratio. A new passive mechanism for energy conservation in *L. helveticus* is suggested that is based on coupling the electroneutral efflux of lactic acid from the cytoplasm, with the generation of a proton gradient in a process dependent on the cytoplasmic buffering capacity but independent of a lactate-specific carrier molecule (147).

Relatively little is known about regulatory processes in anaerobic bacteria that produce propionic acid. *Propionispira arboris*, isolated from the wetwood of living trees, ferments lactate and a variety of saccharides with the formation of propionate, acetate, and CO_2 as the major fermentation products (375). Addition of hydrogen to cultures of *Propionispira arboris* led to a near homopropionate fermentation (432), a finding that has not been reported previously for other anaerobes. This shift in metabolism was found to be due to competition between hydrogenase and the pyruvate-ferredoxin oxidoreductase for oxidized ferredoxin. When sufficient hydrogen is present, the carrier is over-reduced and there is no oxidized form of ferredoxin to accept electrons from pyruvate oxidation. Therefore, acetate formation is inhibited and the electrons required for reduction of pyruvate to propionate come from hydrogen (431).

Succinate is formed from sugars by *Propionibacterium* species, typical gastrointestinal bacteria such as *E. coli*, *Pectinatus* spp., *Bacteroides* spp. (371), *Anaerobiospirillum* spp. (89), *Ruminococcus* spp., *Succinivibrio* spp. (64), and *Succinimonas* spp. (62). Succinate can also be formed from the reduction of aspartate, fumarate, or malate by *Wolinella* sp. (185).

Anaerobiospirillum succiniciproducens ferments glucose with the formation of succinate, acetate, lactate, and ethanol. The distribution of fermentation products was influenced by the level of CO_2 and the culture pH. Growth at pH 6.2 and under conditions of excess CO_2 resulted in the formation of succinate as the main fermentation product, with lower levels of acetate. In contrast, growth at pH 7.2 with limiting levels of CO_2 resulted in lactate formation in addition to lower levels of succinate and acetate (368). The succinate yield and the yield of ATP per mole of glucose were significantly enhanced under excess CO_2 - HCO_3^- growth conditions and suggests that there exists a threshold level of CO_2 for enhanced succinate production in *Anaerobiospirillum succiniciproducens*. Regulation of carbon and electron flow was found to be in the levels of phosphoenolpyruvate carboxykinase, which increased under excess CO_2 -

HCO_3^- growth conditions while lactate dehydrogenase and alcohol dehydrogenase levels decreased, resulting in increased succinate production (368).

Alkaliphilic fermentations. For anaerobic alkaliphilic fermentations it is important to make the distinction between organisms capable of growth with an initial pH which is above neutral and true alkaliphiles. Many anaerobes are capable of growth above neutral pH; however, through fermentation and production of acidic end products the pH can rapidly decrease to a value below neutral, which is more favorable for growth. True alkaliphiles grow optimally with a pH value that is maintained above neutral; they include relatively few obligate anaerobes.

Clostridium proteolyticum was isolated from a chicken manure digester, and *Clostridium collagenovorans* was isolated from a sewage sludge digester. Both organisms are proteolytic, fermenting gelatin, collagen, azocoll, peptone, Trypticase tryptone, cooked meat and poly-pep, with the main fermentation products being acetate and CO_2 . The optimum pH for growth of these organisms was 6.0 to 8.0, and the pH remained neutral throughout the fermentation because of the formation of ammonia, even though high levels of acetate and occasionally butyrate were formed (190). Therefore, these organisms differ from other clostridia in their ability to grow at high pH. Both organisms produce an active collagenase, and, besides *Clostridium histolyticum*, these are the only described *Clostridium* species which produce this enzyme. Collagenase is a very important enzyme since it can hydrolyze collagen and release peptides from animal protein, a property not found with normal proteases (190).

The alkaliphilic halophilic anaerobes isolated by Horikoshi et al. (167) fermented glucose, fructose, dextrin, and soluble starch; in addition, strains SS-21 and M-20 utilized many other monosaccharides and disaccharides. Strains SS-11, SS-15, KY-402, and M-20 all produced acetate, propionate, butyrate, H_2 , and CO_2 from glucose, and M-20 also formed lactate. Strain SS-21 formed acetate, ethanol, lactate, pyruvate, H_2 , and CO_2 (167).

Sulfate is often present in hypersaline environments, and under such conditions methylamines and other methylated compounds such as dimethylsulfide are the major methanogenic precursors, with methanogenesis from acetate, formate, or H_2 not being significant. In this regard the substrate range of methanogens isolated from highly saline environments may depend on the presence or absence of sulfur. For example, the alkaliphile *Methanohalophilus oregonense* was isolated from a sulfate-containing environment; it utilizes trimethylamine, grows slowly on methanol or dimethylsulfide, and cannot utilize H_2 - CO_2 , formate, or acetate (256).

The physiological requirement of four strains of *Methanobacterium alcaliphilum* may reflect the low level of sulfate in their ecological niche. These strains were isolated from low-salt environments in lakes in the Wadi el Natrun of Egypt, produced methane only from hydrogen and carbon dioxide, and did not utilize acetate, trimethylamine, methanol, or formate (42). *Methanohalophilus zhilinae* was also isolated from high-salt sediments from the same lakes; it catabolized trimethylamine and methanol but not H_2 - CO_2 , formate, or acetate, and it had a much higher tolerance for salt (276). Therefore it would seem that these methanogens occupy different ecological niches of the lake according to their substrate ranges and salt tolerance.

Adaptation Mechanisms

Overview. For organisms which either exist in environments of high or low pH or experience changes in pH during growth, several survival strategies exist. Either conditions are maintained as close to homeostasis as possible with the expenditure of energy, or cytoplasmic conditions adjust to adapt to changes in environmental pH, which requires less expenditure of energy. Anaerobes derive substantially smaller amounts of energy from a given substrate than do aerobes (425), and anaerobes appear to have adapted to pH extremes by mechanisms which do not involve high-energy-dependent processes.

Internal pH and maintenance of PMF. From measurements of the intracellular pH of a number of bacterial species, regulation of cytoplasmic pH would appear to be under stringent control (44, 327). Much of the earlier work on pH homeostasis has been performed with aerobic organisms, including obligate acidophiles growing optimally in the pH range 2 to 4 with an internal pH from 5.5 to 6.7 (280) and alkaliphiles which maintain their intracellular pH up to 1.5 units more acidic than the external pH (221). It is only recently that anaerobes have received attention, and studies indicate that intracellular pH is not maintained at a constant value in these organisms.

During growth of *Clostridium pasteurianum*, the external pH decreased from 7.1 to 5.1 and the internal pH simultaneously changed from 7.5 to 5.9 (348). *Clostridium thermoaceticum* (17) and *Clostridium acetobutylicum* (152) have been shown to maintain a Δ pH of only approximately 1.0, with decreasing pH. When *Sarcina ventriculi* grew at neutral pH (7.0) the internal pH of the cell was 7.1, and with a decrease in the external pH to 3.0 the internal pH became 4.3. The PMF increased as a function of decreasing medium pH, and very little change in the membrane potential was observed over a wide range of pH values (150). When *Streptococcus bovis* was subjected to changes in external pH from 6.5 to 5.0, the cytoplasmic pH changed from 6.8 to 5.6 (359).

Methanogens also experience changes in internal pH with alteration in environmental pH; for example, when grown on H₂ and CO₂ over a pH range of 5.0 to 8.1, *Methanobacterium bryantii* does not maintain a constant cytoplasmic pH. Cells grown at optimum pH had a PMF consisting predominantly of the membrane potential, but those grown at nonoptimal pH generated a transmembrane pH gradient which was insufficient to maintain a constant internal pH (396). *Methanospirillum hungatei* and *Methanobacterium thermoautotrophicum* were grown at an external pH range of 5.8 to 7.8, across which the internal pH was always alkaline with respect to the external pH, except at pH 6.7, when the two were equal (192).

The production of weak acids such as acetic and butyric acids results in loss in maintenance of a constant internal pH with respect to changes in external pH (17, 171, 423). The undissociated forms of these acids are able to freely permeate through the cytoplasmic membrane, accumulate inside the cell at large Δ pH values, and decrease the internal pH (201). Thus, anaerobes have to adapt to changes in environmental pH because of their fermentative metabolism.

Oral streptococci show a range of acid tolerance. Generally *Streptococcus mutans* is the most aciduric, whereas strains of *Streptococcus sanguis* have relatively low acid tolerance. Glycolysis is reduced by one-half of its maximal value in *S. mutans* under experimental conditions at pH 5.0 (30), whereas the corresponding pH for this to occur in *S.*

sanguis is 6.2 (29). This difference is not inherent in the acid resistance of the glycolytic enzymes from the two species, which is very similar (66), but, rather, is due to the relative abilities of their membranes to maintain a transmembrane pH difference. It seems that the proton-translocating F₁F₀-ATPases are involved in the acid tolerance of oral streptococci, maintaining the Δ pH across the cell membrane. The pH optima of about 7.5 and 6.0 for ATPase activities of *S. sanguis* and *S. mutans*, respectively, reflect the differences in acid tolerances of these bacteria and the permeability of the cells to protons (29). For *S. mutans*, acid adaptation would be expected to be important for ecological competitiveness because the organism requires an acidified environment to be able to compete effectively with less acid-tolerant organisms such as *S. sanguis* (47).

Lactobacillus casei is the most acid-tolerant organism in dental plaque, and among the gram-positive organisms, *Actinomyces viscosus* is the most acid sensitive. The characteristics of *Lactobacillus casei* contributing to acid tolerance include somewhat greater resistance of membranes and enzymes to acid damage, a lower pH optimum of the membrane-associated enzyme, and greater capacities of cells to expel protons at low pH, which is related to an increase in ATPase levels (28).

Membrane and cellular components. Due to varying internal pH values with changes in environmental pH, anaerobes use a number of mechanisms for overcoming the detrimental effects of pH extremes. The phenomenon of altering carbon and electron flow through the cell can be regarded as an adaptation to changing pH conditions. Other cellular processes also affected by pH include cellular morphology, membrane structure, and protein synthesis.

Clostridium acetobutylicum (195), *Lactobacillus bulgaricus* (347), and *Sarcina ventriculi* (266) all undergo morphological changes in response to changes in environmental pH. With *Clostridium acetobutylicum*, the acidogenic phase of growth causes a decrease in the medium pH from 6.8 to around 5.0, and when the switch to the solventogenic phase occurs at low pH, the vegetative rods are converted to clostridial forms (195). Membrane composition in *Clostridium acetobutylicum* is also altered in response to increasing levels of solvents. Cells from the solventogenic phase of growth have thicker walls and are more resistant to shear forces and cell wall-hydrolyzing enzymes than are acidogenic cells (388).

When *Lactobacillus bulgaricus* is grown at low pH (around pH 4.5), it exists as rods with a relatively small number of cells per chain. With increasing medium pH, filamentous cell growth occurs with addition of cells to the chain, and when the pH reaches 8.0, the chains become folded into clumps. Autolytic activity is needed to dechain *Lactobacillus bulgaricus* cells, and alkaline pH causes the failure in synthesis of these dechaining enzymes rather than inhibition of enzyme activity (347).

Sarcina ventriculi grows as uniform cells in a tetrad at acidic pH; as the pH increases the cells become larger and cell division becomes irregular, resulting in large numbers of cells within each packet, and at alkaline pH the cells sporulate (Fig. 16 and 17) (266). Spore formation can be regarded as a mechanism of adaptation to pH extremes. The role of sporulation in the life cycle of *Sarcina ventriculi* could be a survival mechanism, being triggered by passage of the organism from the acidic environment of the stomach to the alkaline conditions of the small intestine. Soil is another natural environment for *Sarcina ventriculi*, and this can vary from acidic to alkaline conditions; such pH variation may

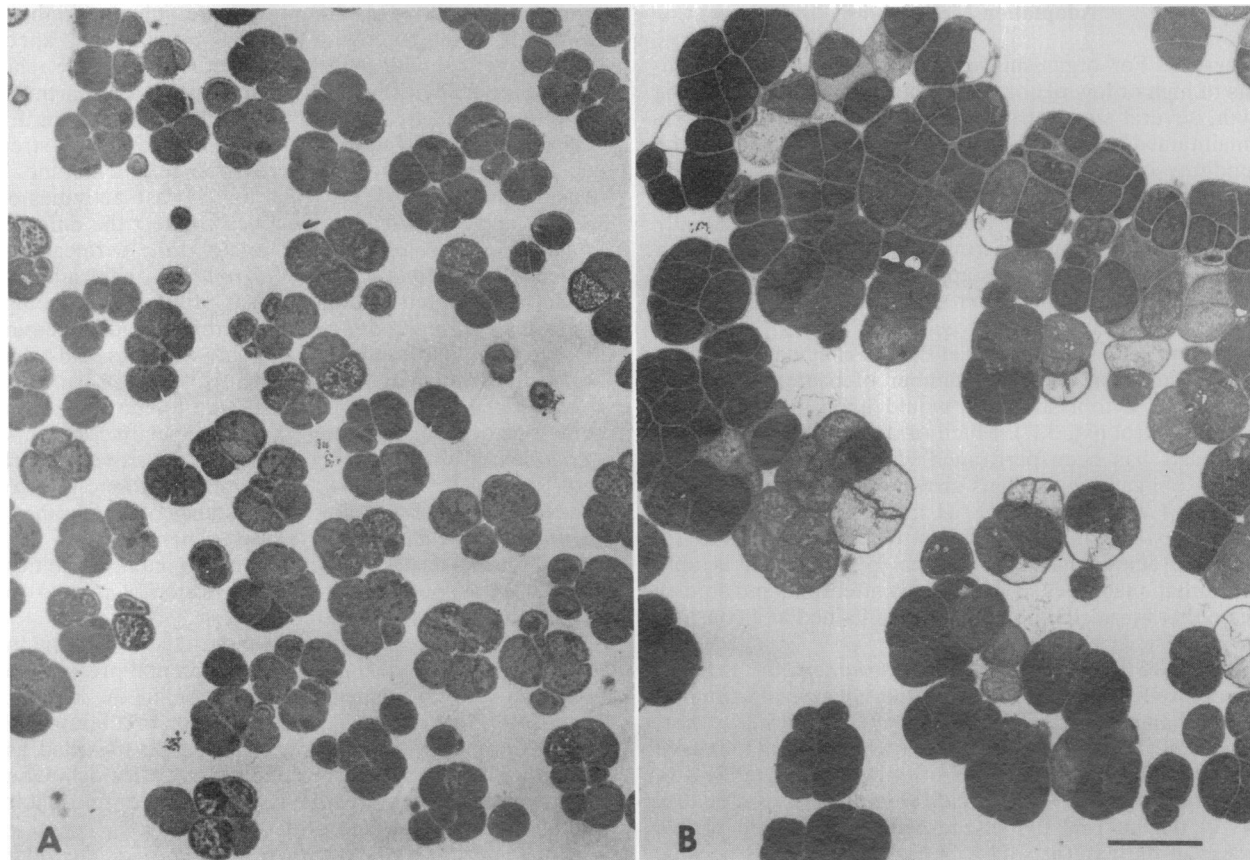


FIG. 16. Electron micrographs of grazing cells of *Sarcina ventriculi* in thin section. (A) Cells from batch culture at a constant pH of 3. (B) Cells grown at a constant pH of 7. Bar, 5 μ m.

trigger sporulation of this organism in soil and sedimentary environments.

Changes occurred in the lipid composition and structure in *Sarcina ventriculi* in response to pH. At neutral pH, the predominant membrane fatty acids ranged in chain length from C₁₄ to C₁₈. However, during growth at pH 3.0, a family of unique very long chain α,ω -dicarboxylic fatty acids containing 32 to 36 carbon atoms are present and account for 50% of the total membrane fatty acids. Because of their more hydrophobic nature, these unusual fatty acids may negate the damage to membrane stability caused by deleterious fermentation products (acetic acid, formic acid, and ethanol) formed during growth and may prevent the uptake of metabolic acids which would dissipate the PMF at low pH (198). Because anaerobes exist under energy-constrained conditions, rather than consuming ATP to pump out protons, it may be more beneficial for these organisms to change their membrane composition with changes in pH. Characterization of the membrane lipids of other anaerobes will determine how widespread this phenomenon is and may lead to the discovery of novel lipids.

Another organism found in the alimentary canal is *Clostridium welchii*, which also appears to have an adaptation mechanism to ensure survival of pH extremes in the absence of active growth. The vegetative cells from the stationary phase of growth of this food-poisoning organism were more resistant to low pH values of 1.5 to 3.6 than were cells from the exponential phase; this could represent a possible survival mechanism during passage of the organism through the

stomach, ensuring large numbers of viable organisms necessary to initiate subsequent infections (417).

Comparison with aerobic acidophiles and alkaliphiles. Recently there have been reports on the effect of changes in environmental pH on gene expression in aerobic organisms, with a number of new proteins synthesized which could be a means of protecting and maintaining homeostasis within the cell. If *Salmonella typhimurium* is exposed to acidic conditions (pH 3.3), an acid tolerance response is evoked with the production of proteins thought to be a specific defense mechanism for acid (137). Genetic response systems enable *E. coli* to adapt to changes in its environment, surviving rapid alteration in external pH (516) and adapting to the presence of weak acids which depress internal pH, by developing pH taxis and weak-acid-repellent responses (206).

Preliminary studies with anaerobes suggest that these organisms are able to adapt to changes in environmental conditions, such as pH, which is a variable environmental parameter inherent to their mode of life. When subjected to pH changes, aerobic organisms will tend to change their metabolism to tolerate the changes, until a favorable pH occurs, rather than to adapt. Carbon flow in *Sarcina ventriculi* appears to be regulated at the level of gene expression and protein synthesis, since changes in levels of protein synthesized occur in response to changes in environmental pH, rather than to alteration or inhibition of enzyme activity (267). Butanol exposure of *Clostridium acetobutylicum* causes changes in protein synthesis (424), and from changes

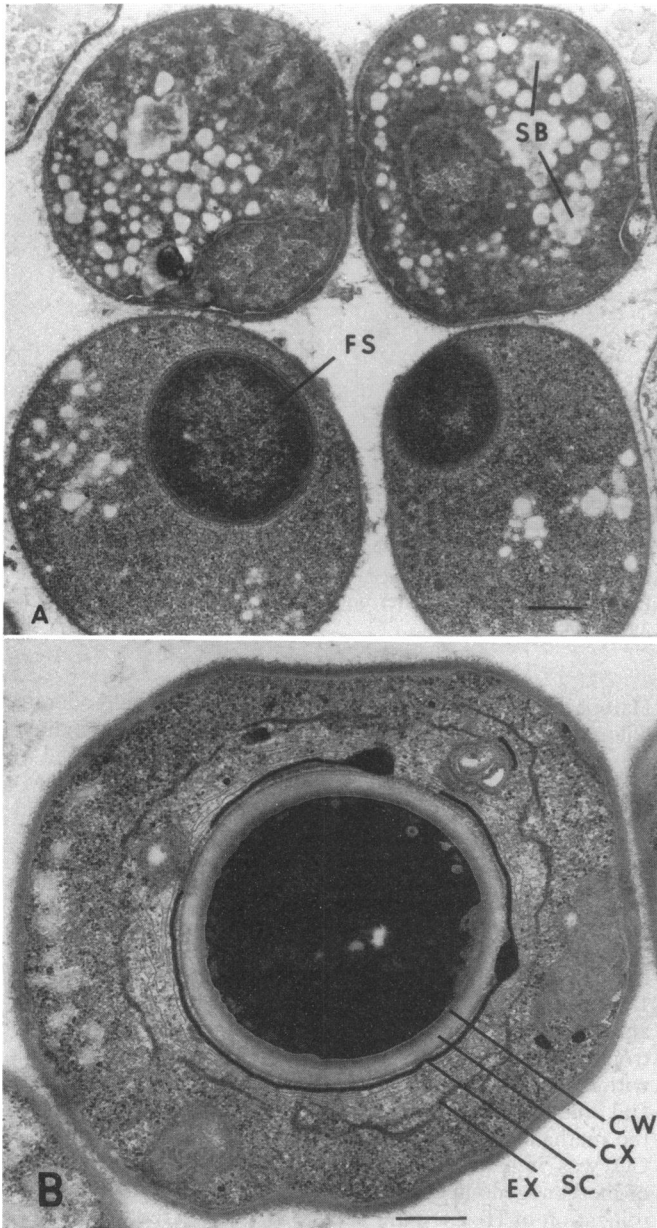


FIG. 17. Electron micrographs of *Sarcina ventriculi* in thin section. (A) Cells in various stages of forespore (FS) development, with storage bodies. (B) Mature spore within the sporangium. The exosporium (EX) surrounds the spore coat (SC), which encloses the cortex (CX) and the cell wall (CW). Scale bar, 0.5 μ m. Reprinted from reference 266 with permission.

in levels of key enzymes involved in end product formation, alteration in carbon and electron flow appears to be regulated by protein synthesis rather than enzyme activity. Studies to demonstrate that changes in enzyme activity are reflected by comparable changes in the amount of corresponding mRNA will provide conclusive evidence that these organisms are able to adapt to changing conditions of environmental pH by regulation of gene expression. The types of changes made by anaerobes in response to altered environmental pH can be related to adaptation of their existing metabolism rather than to the induction of systems designed

to protect the organism against stress conditions, which appears to be the approach used by aerobic organisms.

Biotechnological Features

Overview. Organic acids are used in large volume by both the food and chemical industries. Currently, citric acid, gluconic acid, and itaconic acid are produced by fermentation. However, other organic acids such as acetic, propionic, butyric, fumaric, succinic, maleic, and lactic acids can also be produced from biomass, wood hydrolysate, starch, molasses, etc., by fermentation technology.

Acidogenic fermentation of carbohydrates to volatile organic acids is well known (488). In general, anaerobic bacterial fermentations are of interest to developing biotechnology because of the wide range of products formed and substrates fermented, the high substrate-to-product conversion yields and rates, and the potential for enhanced process stability and product recovery as a result of the physiological diversity of anaerobes.

Higher-value organic acids. The key nonvolatile organic acids that have large economic potential for value-added products are lactic acid and C_4 dicarboxylic acids. Lactic acid fermentation has been known for over 100 years, and lactic acid has a long history of use in the food industry. It has flavoring and preservation properties and is used as an acidulant, particularly in dairy products, confectionery, beverages, pickles, bread, and meat products. Potassium lactate is becoming increasingly important as a replacement for sodium chloride in low-salt or salt-free food products. Lactic acid is also used in the pharmaceutical and cosmetic industries. The cyclic dimers of lactic acid are used in the synthesis of adsorbable surgical sutures and slow-release drugs. Crude grades of lactic acid are used for the deliming of hides in the leather industry. Calcium lactate is used in powder form as an animal and poultry feed supplement. Also, various derivatives of lactic acid are used in the production of plastics (188).

Homofermentative lactic acid bacteria produce predominantly only lactate with trace or no other end products. Some of the common lactic acid bacteria include *Lactobacillus casei*, *Lactobacillus pentosus*, *Lactobacillus leichmannii*, *Lactobacillus acidophilus*, *Lactobacillus delbrueckii*, *Lactobacillus bulgaricus*, *Streptococcus cremoris*, *Streptococcus lactis*, *Streptococcus diacetylactis*, *Sporolactobacillus* sp., and *Pediococcus* sp. Fermentation lactic acid has a worldwide market size of only 50 million to 60 million lb (23 million to 27 million kg), even though large-volume chemicals can be produced from lactic acid (255).

Succinic acid is a specialty chemical with numerous current and potential uses in the textile, plastic and resin, detergent, and food industries. Although it is currently produced by hydrogenation of maleic anhydride, it can be produced by fermentation at a very competitive price. Succinic salts may also be used as additives to animal feed for ruminants and monogastrics such as pigs. Because of its simple structure as a linear saturated dicarboxylic acid, succinic acid can be used as an intermediate to produce many aliphatic chemicals, and carboxylic acids and esters are versatile reactants used in the manufacture of automobiles, plastics, electronics, paper, textile, furniture, and paint (188).

Acetone-butanol-ethanol fermentation. Until World War I, all acetone produced in the United States was obtained by the dry distillation of calcium acetate from the pyrolignous acid fraction of wood distillates. During the war there were



FIG. 18. South Arm of Great Salt Lake, Utah. A salt crust is present just below the water surface, and an oil-drilling platform is in the background. Because of the high growth and sedimentation of halophilic phototrophs in the water column, the salt-saturated sediments contain very large amounts of organic matter and large numbers of haloanaerobic bacteria.

great shortages of acetone in the manufacture of munitions, and at that time Wiezmann developed a process involving the fermentation of starch grains by *Clostridium acetobutylicum*. This fermentation gave approximately 2 parts butanol to 1 part acetone and 1 part ethanol. A market for butanol arose from the development of fast-drying nitrocellulose lacquers for the automotive industry, and butanol became the more important product. New cultures using molasses gave more desirable butanol-to-acetone ratios of 3:1. During the 1940s and 1950s the petrochemical routes replaced the process fermentative routes in the United States and Europe. Recently South Africa has stopped using fermentation, and China is the only current user of the fermentation technology.

New high-technology improvements have been achieved so that fermentation-derived butanol can compete with the current cost of chemical-derived butanol. Development of solvent-tolerant asporogenous strains and the use of metabolic controls in continuous multistage fermentations allowed for higher butanol concentrations and chemical yields from glucose. Development of more energy-efficient product separations by modifications in distillation technology has lowered the cost of end product recovery (492).

Fermented foods. Acid-tolerant bacteria are used in a number of foods. German bakeries use souring bacteria including *Lactobacillus brevis*, *Lactobacillus fermentans*, *Lactobacillus plantarum*, and *Lactobacillus casei* to obtain the flavor of sour rye bread (333). The dough for rye bread is fermented to form lactic and acetic acid in a ratio of 4:1 for optimum flavor. Growth of the organisms accelerates until the dough pH is near 4.0, and then both populations become stationary while the pH drops further.

HALOPHILES

Ecology, Diversity, and Taxonomy

Overview. Halophiles include prokaryotic bacteria and eukaryotic algae which have adapted to live in environments with salt concentrations above those found in seawater.

Halophilic bacteria fall into either the group called moderate halophiles, which include organisms growing in sodium chloride concentrations ranging from 2 to about 20% (0.3 to 3.4 M), or extreme halophiles, requiring at least 15% (2.6 M) sodium chloride for growth (236). Although the ecology and physiology of the aerobic population of hypersaline habitats have been studied, relatively little is known about the role of anaerobic organisms in such environments.

Ecology and diversity. The ecology of the anoxic zone of the Great Salt Lake has been studied from samples taken from the South Arm with a salinity greater than 20% (Fig. 18). Degradation of organic matter in the sediment was attributed to bacterial action, by the enumeration of various trophic groups. From the sediments, enrichment cultures with medium containing 15% salt yielded hydrolytic bacteria and a lactate-metabolizing, sulfate-reducing population, but the methanogenic population consuming methanol and H_2 - CO_2 was present at only low levels (489). The small numbers of methanogens in the Great Salt Lake are probably due to utilization of H_2 - CO_2 and acetate by sulfate reducers, leaving methylamine or methanol to be utilized by the methanogens, as these compounds were demonstrated to be methanogenic precursors in situ (489). These findings suggest that the ecological niche for the methanogens in salt environments is different from their role in nonhalophilic ecosystems. Rather than utilizing the terminal fermentation products of many anaerobic bacteria, including acetate, H_2 - CO_2 , and formate, these organisms utilize methanol and methylamines.

Methane formation has been found in other hypersaline environments with salt concentrations of around 2.0 M (208). Several strains of methanogenic bacteria were isolated from lagoons in Crimea that ranged in salinity from 2.2 to 30%, with some strains growing at 25% salt (512). It was from this location that the first extremely halophilic methanogen, *Methanohalobium evestigatum* (514), was isolated. In these hypersaline environments, methylamines were the methanogenic precursors, and acetate, H_2 - CO_2 , and formate were not utilized (208, 512). Also isolated from cyanobacterial

mats present in this environment were several extremely halophilic methanogens, and an acetogen *Acetohalobium arabaticum* (515). It is proposed that in this ecological niche, betaine produced as an osmoregulant by aerobic halophilic eubacteria is consumed by *Acetohalobium arabaticum*, producing acetate and mono-, di-, and trimethylamines, which form the substrates for *Methanohalophilus* and *Methanohalobium* species, leading to methane formation (515).

Biological sulfate reduction is active in surface sediments in the Great Salt Lake, and enrichment cultures of sulfate reducers in hypersaline lactate medium contained both sporeforming rods and short motile rods (489). Other hypersaline environments have been reported as ecosystems for sulfate reduction, including the Dead Sea and salterns (318, 321). Although sulfate reduction in such ecosystems occurs, until recently the organisms participating in these reactions had not been isolated in pure culture, and so nothing was known of their physiology and biochemistry. *Desulfohalobium retbaense* is a moderately halophilic sulfate-reducing bacterium and was isolated from Retba Lake, a pink hypersaline lake in Senegal. The organism grows optimally in 1.8 M NaCl, but in the hypersaline lake, which has a salt concentration of 5.8 M, the organism would be existing under salt concentrations which are inhibitory for growth (314). Another moderately halophilic sulfate-reducing bacterium, *Desulfovibrio halophilus*, was isolated from a microbial mat of Solar Lake, Sinai (75). The organism grows in a salinity range of 3 to 18% NaCl, with optimum growth occurring at 6 to 7% NaCl. *Desulfovibrio halophilus* is found in stagnant anoxic mud and sediments in hypersaline environments such as evaporitic lagoons and coastal salterns at salt concentrations of about 6 to 20% (75). Therefore, the salt concentrations enabling the growth of this organism presumably mean that the organism is metabolically active in these habitats.

A number of halophilic, anaerobic eubacteria have been isolated and studied, although to a much lesser extent than their aerobic counterparts. *Haloanaerobium praevalens* was isolated from surface sediment (10 m) from the Great Salt Lake at a site where the salinity of the overlying water is more than 20%; it grows optimally at 13% salt (2.2 M) (498). Oren et al. isolated a number of moderately halophilic anaerobes including *Sporohalobacter lortetii* (formerly *Clostridium lortetii*), growing optimally at 1.4 to 1.5 M NaCl (317), and *Halobacteroides halobius*, growing optimally at 1.5 to 2.5 M NaCl (325), from sediments from the Dead Sea, and *Sporohalobacter marismortui*, growing optimally in 0.5 to 2.0 M NaCl (324), from anaerobic black sediments of a salt flat on the shore of the Dead Sea. Another eubacterium, *Halobacteroides acetothylicus* was isolated from deep-subsurface brine waters (1,600 ft [488 m]) associated with injection water filters on offshore oil rigs in the Gulf of Mexico; it grows optimally in 1.7 M NaCl (345). The moderate halophile *Clostridium halophilium*, requiring 1.0 M NaCl for optimum growth, was isolated from anoxic mud sediments of hypersaline environments of the Maldive Islands and Solar Lake, Egypt, as well as marine sediments of the North Sea, Germany (126).

Recently, four chemoorganotrophic halophiles were isolated from the hypersaline surface sediments from the evaporating closed lagoon at the rim of Salton Sea, Calif., and from Big Soda Lake, Nev. Interestingly, although these organisms are obligately anaerobic, they were isolated from the aerobic zone of the ecosystem. All of the isolates were gram-negative, motile, nonsporeforming, moderately halophilic eubacteria and required a minimum concentration of 3

to 10% NaCl for growth. Three of the strains grew optimally in salt concentrations between 5 and 10%, and the other strain grew between 12 and 20% salt (387).

Many of the hyperthermophiles have been isolated from marine environments and have a requirement for salt; however, because of the low concentration needed (1.5 to 3.8%), these organisms cannot be considered true moderate halophiles, with the possible exception of *Thermococcus litoralis*, which grows in 6.5% NaCl (305).

The moderate halophiles *Methanobolus tindarius* (220) and *Methanococcoides methylutens* (394) have been isolated from a number of different hypersaline environments and have optimum growth at 0.49 and 0.4 M salt, respectively. *Methanogenium cariaci* was isolated from sediment collected from the Cariaco Trench and grows optimally in 0.46 M salt (351). *Methanohalophilus zhilinae* has an optimum salt requirement for growth of 0.7 M (276). *Methanococcus halophilus* was isolated from mats of cyanobacteria found between modern stromatolites in Shark Bay, Australia, and required 1.2 M NaCl for optimum growth (511). *Halomethanococcus mahi* is also a moderate halophile, growing optimally between 1.0 and 2.0 M NaCl; it was isolated from the Great Salt Lake (332). The methanogen strain SF1 was isolated from the sediment of a solar salt pond and had an optimum salt concentration for growth of 2.1 M (275). The organism with the highest salt requirement for growth is *Halomethanococcus doii*, with optimum growth at 3.0 M NaCl; it was isolated from a salt pond near San Francisco (483).

Taxonomy. The halophilic methanogens belong to the most metabolically unusual of the methanogen groups, the family *Methanosarcinaceae*, a subdivision of the order *Methanomicrobiales*. All morphological types are represented in this group and include both mesophilic and thermophilic species. A variety of substrates are used by these organisms, and both halophiles and nonhalophiles are included (197).

Four different genera are recognized among the halophilic anaerobic eubacteria: *Clostridium*, *Sporohalobacter*, *Halobacteroides*, and *Haloanaerobium*. With the exception of *Clostridium halophilium*, all of these haloanaerobic eubacteria are gram-negative rods, forming mixed fermentation products from glucose, and are differentiated on the basis of their salt tolerance range, catabolism, habitat, DNA base composition, and spore formation. Analysis of the 16S rRNA of *Halobacteroides halobius* (320), *Sporohalobacter lortetii*, *Haloanaerobium praevalens*, and *Sporohalobacter marismortui* shows that these organisms are related to each other (318, 324), and the findings that *Halobacteroides halobius* (320) and *Sporohalobacter marismortui* (324) produce endospores and that *Haloanaerobium praevalens* hydrolyzes *N*'-benzoyl-D-arginine-*P*-nitroanilide (323) demonstrate that these organisms share properties characteristic of endosporeforming bacteria. *Clostridium halophilium* is a gram-positive rod-shaped sporeformer (126). This organism belongs to the group of clostridia (74) that produce acid from glucose, do not hydrolyze gelatin, and do not digest meat. Further taxonomic characterization is required to determine how closely related this organism is to the other anaerobic halophilic eubacteria.

Physiology and Metabolism

Growth features. The physiological requirements of haloanaerobes for salt reflect the chemical composition of their environment. The Dead Sea contains an extremely high

concentration of Mg^{2+} (1.8 M), and *Halobacteroides halobius* isolated from this environment tolerates high magnesium concentrations, with Mg^{2+} replacing the high Na^+ requirement (325). *Sporohalobacter lortetii* and *Sporohalobacter marismortui* isolated from the same environment have a low tolerance for magnesium (up to 0.5 M), and it is thought that the former organism can grow only slowly in its habitat (317). *Halobacteroides acetoethylicus* was isolated from deep subsurface waters which contained only low levels of potassium (0.02 M) and magnesium (0.119 M) compared with the concentrations of sodium (1.34 M) and chlorine (1.57 M). The organism grows optimally in medium with a similar composition to the environment from which it was isolated, i.e., high levels of NaCl and low levels of potassium, and does not grow in medium with greater than 0.2 M Mg^{2+} (345).

Metabolic types. The haloanaerobic eubacteria produce a mixed-acid fermentation together with H_2 and CO_2 from glucose. The end products acetate and butyrate are produced by *Sporohalobacter lortetii* (317) and *Haloanaerobium praevalens* (498), whereas *Halobacteroides halobius* (325) and *Halobacteroides acetoethylicus* (345) ferment glucose with the formation of acetate and ethanol, and *Sporohalobacter marismortui* produces formate in addition to the other fermentation products (324).

Clostridium halophilum is capable of carrying out the Stickland reaction, degrading betaine in the presence of L-alanine or other suitable electron donors with the production of acetate, CO_2 , ammonia, and trimethylamine. In addition, this organism is able to ferment carbohydrates, including a number of disaccharides, monosaccharides, and sugar alcohols, and grows on pyruvate. Fermentation products from glucose include acetate and ethanol in equal ratios and lower levels of butyrate and lactate, and propionate was produced from growth on threonine (126). The halophilic sulfate reducer *Desulfhalobium retbaense* is a strict chemorganotroph, incompletely oxidizing lactate, ethanol, and pyruvate to acetate and CO_2 . Sulfate, sulfite, thiosulfate, and elemental sulfur were used as electron acceptors and were reduced to H_2S . During growth on lactate in the presence of hydrogen as the energy source, longer cells (up to 20 μm in length) are found (314).

Halophilic methanogens all fall into the same physiological group with respect to substrate utilization, with pure-culture studies confirming the environmental observations that methylamines are the methanogenic precursors. *Methanococcus halophilus* (511), *Methanolobus tindarius* (220), *Methanococcoides methylutens* (394), *Halomethanococcus mahi* (332), *Halomethanococcus doii* (483), and strain SF1 (275) are all obligately methylotrophic, growing only on methylamines or methanol but not on the common methanogenic substrates including hydrogen plus carbon dioxide, formate, and acetate. These organisms represent a distinct physiological group within the methanogenic bacteria and can be described as the halophilic, methylotrophic, nonaceticlastic methanogens.

Adaptation Mechanisms

Internal salt concentration. In general obligate halophiles have evolved two different strategies for growth in an environment of concentrated NaCl. The internal salt concentration can be maintained at a level comparable to that of the environment, or the organism can actively exclude NaCl and produce an organic-compatible osmoregulant such as betaine (438). Intracellular salt concentrations in *Haloanaer-*

obium praevalens, *Halobacteroides halobius*, and *Halobacteroides acetoethylicus* were similar (319, 346). In these haloanaerobes, the chlorine levels inside the cells were comparable to those in the media and osmoregulants were not produced, demonstrating a marked difference from haloaerobic eubacteria, in which chlorine is excluded (76, 273).

The intracellular salt content of the halophilic aerobic or anaerobic archaeobacteria and haloanaerobic eubacteria is dependent on the external NaCl concentration, with an increase in external NaCl concentration resulting in a corresponding increase in intracellular salt concentration. The total internal concentration of monovalent ions either equals or exceeds the external concentration (21, 319, 346). When growing at their optimal NaCl concentration, *Haloanaerobium praevalens* and *Halobacteroides halobius* have approximately equal intracellular concentrations of Na^+ and K^+ (319). This is in contrast to *Halobacteroides acetoethylicus*, in which Na^+ is the predominant intracellular ion (346). In the haloaerobic eubacteria which have NaCl within the cell, an increase in salt concentration above the optimum for growth will not result in a corresponding increase in the internal salt content (273, 274), suggesting that, unlike their anaerobic counterparts, aerobic eubacteria are able to control the internal salt concentration.

Enzymes. To date little work has been done on biochemical mechanisms used by haloanaerobes to adapt to their environment. However, the halophilic eubacteria do maintain high intracellular concentrations of salt, and presumably the cellular processes have adapted to functioning in such an environment. Comparison of the effect of salt on the *in vitro* activities of a number of enzymes from *Halobacteroides acetoethylicus* revealed some striking similarities with enzymes from halophilic, aerobic archaeobacteria.

Catabolic enzymes from *Halobacteroides acetoethylicus* were found to all require salt for activity, although the optimum salt concentration varied depending on the enzyme (346). Several of the enzymes, including glyceraldehyde-3-phosphate dehydrogenase, alcohol dehydrogenase, and uptake hydrogenase, showed maximum activity at concentrations of Na^+ similar to or above those found in the cell. Pyruvate dehydrogenase was unlike the other enzymes in that maximum activity was shown at K^+ concentrations equivalent to those *in vivo*. Although the enzymes varied in activity in the absence of salt, the presence of either Na^+ or K^+ stimulated activity in all cases and alcohol dehydrogenase activity increased with increasing salt concentrations.

The tolerance by enzymes of *Halobacteroides acetoethylicus* to concentrations of salt above those normally found within the cells reveals a similarity to enzymes from haloaerobic archaeobacteria (20, 122, 236). Studies of the effect of salt on enzymes from haloaerobic eubacteria have shown that most of the enzymes were active at salt concentrations below those found inside the cell (19, 20).

Membranes and other cell components. Analysis of the membranes by either electron microscopy or lipid composition has not revealed the presence of components or ultrastructural differences that could account for the ability of haloanaerobes to inhabit ecological niches high in salt. The halophilic methanogens *Methanococcus halophilus* (511), *Methanolobus tindarius* (220), and *Methanogenium cariaci* (351) all had cell walls which were thin, osmotically sensitive, and sensitive to detergents.

Electron micrographs of the cell wall of *Halobacteroides halobius* showed a clear double-track membrane in contact with the cell cytoplasm, and external to this membrane was another double-track membrane, which apparently formed

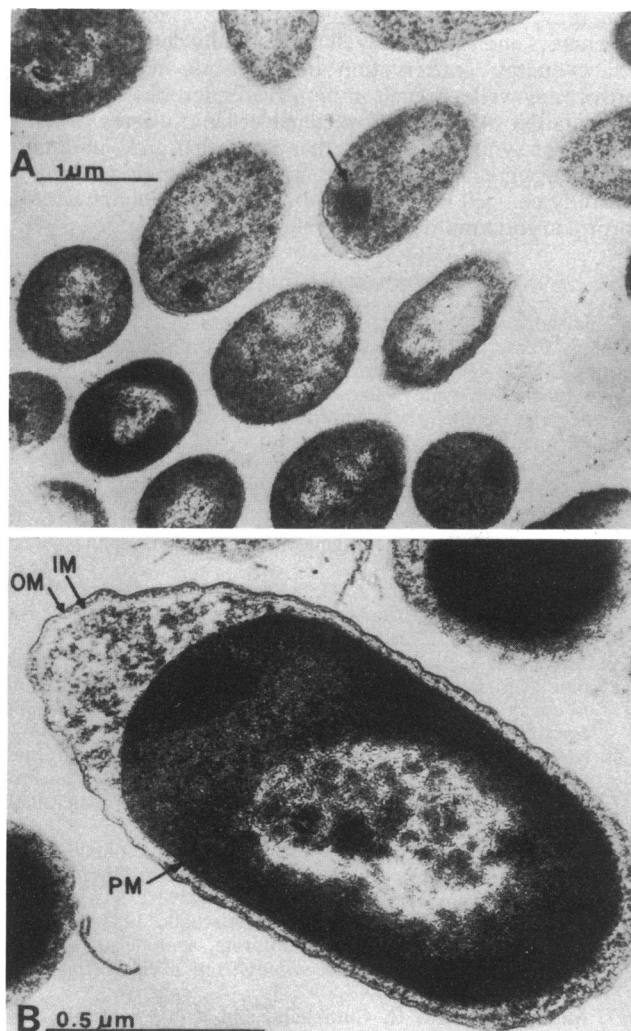


FIG. 19. Transmission electron micrographs showing the internal cell architecture of *Halobacteriodes acetoehtylicus*. (A) Arrows indicate crystalline inclusion structures. Bar, 1 μm . (B) Outer-wall envelope profile layers indicating the outer membrane (OM), inter-wall material (IM), and plasma membrane (PM). Bar, 0.5 μm . Reprinted from reference 345 with permission.

the outer layer of the cell envelope. Sensitivity of the cells to penicillin implied the presence of peptidoglycan, although a prominent peptidoglycan layer could not be seen in electron micrographs (325). In *Haloanaerobium praevalens* the lipid content did not change with changes in salt concentration, and therefore the membrane may not play a major role in the ability of the organism to grow over an extended range of salt concentrations (498). Electron-microscopic observations of thin sections revealed that cells of *Halobacteriodes acetoehtylicus* had a typical gram-negative layered cell envelope but contained unusual internal crystal-like forms (345), although it is not known whether these contribute to or counteract the high internal salt concentration (Fig. 19).

Biotechnological Features

Higher-value organic acids. Organic acid-producing fermentations, e.g., those producing acetic acid, propionic acid, and lactic acid, are generally limited by low pH, and if

the pH is controlled, they are limited by species tolerance to the salt of the organic acid. The potential for producing concentrated organic-acid salt solutions by anaerobes has not been reported, although 15% (wt/vol) monosodium glutamate is currently being produced from an aerobic organism. Organic solvent-producing fermentations such as ethanol and butanol are generally limited by solvent concentrations that are economically recoverable and by species that are tolerant to the solvent. Solvent-producing haloanaerobes could have higher tolerance and lower energy requirements for solvent recovery because of less process water at higher salt concentration. The processing of certain organic industrial wastes can be associated with both high salinity and anoxic conditions, e.g., spent liquors from the pulp and paper industry and oil shale rock refining wastes from the synfuel industry. Effective biodegradation of these wastes may require salt-tolerant populations of anaerobic bacteria.

Coal gasification and waste treatment. The biological aspects of anaerobic breakdown of aromatic compounds to biogas under hypersaline conditions are as good as unknown. In addition, the process of methanogenesis from more easily degradable substrates at high salt concentrations is not completely understood. One proposal for the biogasification of chemically pretreated lignite was to use underground salt caverns as cheaply available bioreactors. This would depend on the biological conversion of lignite breakdown products to methane at high salt concentrations, since salt would dissolve from the walls of the caverns. The findings showed that although a potential for methane formation from lignite breakdown products at high salt concentrations does exist, the process is not expected to occur above 16% salt and that the methane yields obtained were very low (322).

Wastewater from industrial processes can often contain salt, and the dominant genus in a bioreactor for treatment of such water is highly dependent on the wastewater composition. In a reactor treating wastewaters from the tuna-processing industry, the dominant methanogens were mainly *Methanogenium* spp., although *Methanobacterium* and *Methanosarcina* spp. were also present. The dominance of *Methanogenium* spp. is probably due to the high salt content of the wastewater (440). Possibly the halophilic methanogens described above have some application in anaerobic digestion of waste high in salt.

CONCLUSIONS AND FUTURE RESEARCH DIRECTIONS

The known diversity of microbial life has been greatly expanded by studies of anaerobic bacteria that proliferate in extreme environments. This feature has been best exemplified by the vast array of moderate and extreme thermophilic species of eubacteria and archaeobacteria which have been recently described. The recently recognized phenotypic and phylogenetic diversity of methanogens is composed in part of thermophilic, halophilic, and alkaliphilic species (32). Continued studies of the diversity of anaerobic species that live in thermophilic, halophilic, acidophilic, and alkaliphilic environments, as well as examination of anaerobic species that grow on toxic chlorinated or nitrile-containing compounds or on limiting substrates such as aromatic compounds or VFA, should yield many new species of bacteria yet to be discovered. As a group, these kinds of anaerobes are considered to be extremophiles by the authors. These organisms have adapted to growth under extreme environ-

mental conditions, although their habitat or isolation site may not necessarily reflect their ability to tolerate and proliferate under adverse conditions. Aerobic extremophiles which have adapted to grow or exist under extreme environments have been termed superbugs by Horikoshi et al. (167), and they represent the aerobic counterparts to the anaerobes described here.

The first forms of life arose when environmental conditions on Earth were anaerobic and thermophilic. The recent phylogenetic tree for life, as proposed by Woese et al. (465), is composed of three kingdoms: the Bacteria, the Archaea, and the Eucarya. Quite interestingly, the most divergent members of both the Bacteria and the Archaea are *Thermoproteus* and *Pyrodictium* species, both of which are thermoanaerobes. At this point, more studies are in order to answer the question whether the thermophilic Archaea or the thermophilic Bacteria arose first. At the molecular level, the members of the Archaea appear in some ways more similar to those of the Eucarya than to those of the Bacteria (465). Indeed, some thermophilic eubacteria such as *Thermodesulfotobacterium commune* have ether-linked lipids like members of the Archaea but also have 16S rRNA sequences more homologous to members of the Bacteria. Further studies on diversity of anaerobic extremophiles may uncover ancestor organisms to both of these life forms.

The correct taxonomic assignments for most anaerobic extremophiles await further detailed study at the molecular level. The pioneering studies of Woese et al. (465) on 16S rRNA sequencing has helped establish classification above the genus and species level. More detailed studies on DNA-DNA homology are in order to establish correct genus and species assignments of the anaerobic species described. Recently, this approach has been used to distinguish *Clostridium*, *Thermoanaerobacter*, and *Thermoanaerobacterium* as three different genera of thermoanaerobic eubacteria that ferment saccharides into H₂+CO₂, ethanol, lactic acid, and acetic acids (245).

The physiological mechanisms used anaerobic members of the Bacteria and Archaea for adaptation to extreme environmental conditions appear to involve novel stabilization mechanisms for both proteins and lipids. This occurs in lieu of production of labile macromolecules, producing general intracellular protectants such as osmoregulants or pumping H⁺ out of the cells, which would require extensive energy expenditure that is limited in anaerobes when compared with aerobic life forms. Considerably more work is required to demonstrate the molecular mechanisms which account for how these organisms and their enzymes and lipids function under extreme conditions.

Extreme conditions are generally the preferred conditions (i.e., high temperature, high salinity, low or high pH) for industrial processes because they maximize reaction rates and product yields and/or facilitate process stability. It is for these reasons that more research on the anaerobes described in this review and their enzymes is important to industrial biotechnology. Recently, the stable enzymes of hyperthermophiles have become an important topic for applied research. This has resulted in new or improved enzymes for starch processing and for production of chiral chemicals. Future applied research should also extend to acidophiles, alkaliphiles, and halophilic anaerobes and their enzymes, which remain overlooked and understudied.

Anaerobic waste treatment systems are important because they do not require O₂ or mixing and can generate methane as a vendible by-product. Many highly toxic chlorinated compounds can only be readily degraded by reductive

anaerobic microbes. Continued studies on anaerobic dehalogenators and syntrophs will undoubtedly demonstrate their exact energy conservation mechanisms, which are not proven, as well as result in improved microbial systems for remediation of toxic and residual organic wastes. Finally, studies of syntrophs and methanogens that grow in defined granular aggregates can serve as a general biological model to study cell-cell interactions and metabolic communication in prokaryotic multicellular systems.

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