

Anaerobic Bacteria from Hypersaline Environments

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INTRODUCTION

Scientific interest in extremophilic microorganisms, especially hyperthermophiles, thermoacidophiles, archaeobacterial anaerobes, and hyperhalophiles, has recently increased (50). One reason for this interest is the need to understand the biochemical mechanisms involved under extreme conditions because of possible biotechnological use of enzymes and molecules from such organisms.

Research on microorganisms from extreme environments also intensified with the recognition of a third domain of life (*Archaea*) by Woese et al. (102). Indeed, extreme ecosystems often yield aerobic and anaerobic archaeobacteria. Furthermore, extreme environments such as subthermal vents exhibit the primitive physicochemical conditions compatible with those present during the origin of life.

Among extremophilic bacteria, thermophiles are the most intensively studied. In contrast, less attention has been paid to halophilic microorganisms. Up to now, microbiological research in this area focused on the aerobic, halophilic microflora; relatively little work was reported on the anaerobic halophiles or their breakdown of organic matter under anaerobic conditions.

The inland lakes of the world may include some of the most extreme natural environments (Dead Sea, Great Salt Lake) for halophilic microorganisms. Such halophilic lakes are subject to high rates of evaporation because of high temperatures. Estuaries and particularly shoreline rockpools exposed to intensive evaporation can also become extremely saline. Human activity

also creates highly saline habitats such as solar salterns, which may have an NaCl concentration at saturation in some ponds.

In contrast to halotolerant bacteria, which do not require NaCl for growth but can grow under saline conditions, halophiles must have NaCl for growth. Halophiles can be classified into three groups on the basis of their response to NaCl (45): (i) the slight halophiles (most rapid growth at 2 to 5% NaCl [0.34 to 0.85 M]), (ii) the moderate halophiles (most rapid growth at 5 to 20% NaCl [0.85 to 3.4 M]), and (iii) the extreme halophiles (most rapid growth at 20 to 30% NaCl [3.4 to 5.1 M]).

In this paper, we focus on the strictly anaerobic halophiles, namely the fermentative, sulfate-reducing, homoacetogenic, and methanogenic bacteria, and their involvement in oxidizing organic carbon and energy sources. The anaerobic phototrophic bacteria are discussed primarily in relation to their ecology and halophily because of the extensive published information available on them (6, 8, 42, 77). Strictly aerobic bacteria and aerobic bacteria capable of facultative anaerobic growth (27) are not included.

CHARACTERISTICS OF HYPERSALINE ECOSYSTEMS

Physicochemical Characteristics

Hypersaline ecosystems (inland lakes, marine salterns) show a great variability in ionic composition, total salt concentration, and pH. Several lakes (Big Soda Lake, Mono Lake, Soap Lake) from the great basin of the western United States, with salinity ranging from 8.9 to 10% (wt/vol), are highly alkaline with pH values of 9 to 10 (59). In contrast, the Great Salt Lake, the Dead Sea, and the Orca Basin in the Gulf of Mexico, which are hypersaline, with total salt content over 20%, have pH values around 7.0 (59). In some hypersaline ecosystems, including the Great Salt Lake and Lake Assal (East Africa), Na⁺

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and Cl^- are the predominant ions in solution (67). In the Great Salt Lake, Na^+ and Cl^- concentrations are 105.4 and 181 g/liter, respectively (67). Cl^- ions dominate (224.9 g/liter) in the Dead Sea, where equivalent concentrations of Na^+ (40.1 g/liter) and Mg^{2+} (44 g/liter) are found (67). Ca^{2+} may be an important component of the water, reaching 17.2 g/liter in the Dead Sea. High pH values in brines result in the absence of divalent cations such as Mg^{2+} and Ca^{2+} . Sulfate is an important electron acceptor, involved in the mineralization of organic matter in hypersaline ecosystems. Its concentration varies from 0.48 g/liter in the Dead Sea to 21.22 g/liter in Soap Lake (59). The low levels of dissolved sulfate observed in the Dead Sea (0.48 g/liter) or in the Great Salt Lake (2.68 g/liter) probably result from its precipitation.

Marine salterns are manmade systems where the evaporating sea water is sequentially pumped through a succession of ponds with increasing salinity. This process results in the sequential precipitation of calcium compounds (CaSO_4 , CaCO_3) and NaCl (for more details, see reference 33).

The dynamics of salinity and ion composition in marine salterns implies a versatile microflora which adapts to salt stress from halotolerance to extreme halophily. The marine origin and possibly the soil origin of adapted microorganisms raise the problem of survival, resistance, and bacterial mutability in such biota.

Sources of Organic Matter

Hypersaline ecosystems are generally inhabited by a limited variety of life forms. The upper limit of salt concentration for vertebrates (*Tilapia* spp.) is about 10%. Above this level, only invertebrates such as brine shrimp (*Artemia salina*) or brine flies, algae (*Dunaliella salina*), bacteria (members of the families *Halobacteriaceae* and *Haloanaerobiaceae*, methanogens, etc.), and cyanobacteria (*Oscillatoria* spp.) have been reported.

In addition to organic matter originating from the dead cells and metabolites of halophilic organisms growing in hypersaline environments, algae and plants growing nearby may input organic matter into the hypersaline system. This was reported in a hypersaline lake of Africa, when a marked increase in water level at the beginning of the rainy season led to the submersion and death of the vegetation growing on the banks of the lake (85).

However, invertebrates, algae, and prokaryotes are the major sources of oxidizable compounds in these environments. The recent isolation of a chitinolytic bacterium (47) from a solar saltern (*Haloanaerobacter chitinovorans*) was expected because of the presence of brine shrimp and brine flies, which contain large quantities of chitin. Cyanobacteria and members of the *Halobacteriaceae* at high salt concentrations may add significant quantities of organic matter from decomposition of their cell walls, which are composed of sugars, proteins, and lipids.

Organic osmolytes which maintain cell turgor pressure under high salt concentration also contribute to the overall carbon cycle in hypersaline ecosystems. Besides potassium, halophilic bacteria accumulate low-molecular-weight organic compounds (glycine betaine, for example) to adapt to osmotic stress. This was reported for prokaryotic microorganisms such as *Ectothiorhodospira* (23) and *Methanohalophilus* (43, 44, 86, 87) species and for cyanobacteria (81). Glycine betaine, β -glutamine, *N*-acetyl- β -lysine, and even carbohydrates (α -glucosylglycerate) also accumulated under increasing salt concentrations (21, 65, 86). In cyanobacteria, sucrose and trehalose accumulated (81) in osmotically stressed cells, whereas glycerol is the major compound synthesized by *Dunaliella* spp. in

response to high salt concentration (25). Finally, a cyclic amino acid called ectoin was isolated and identified from extremely halophilic *Ectothiorhodospira* spp. (21, 22).

Thus, it appears that a wide range of substrates is available in hypersaline environments and that, in spite of the extremely unfavorable environmental conditions, microorganisms have responded by metabolizing and growing on these substrates. This agrees with the diversity of carbon and energy sources used by currently isolated halophilic microorganisms.

Oxidation of Organic Matter

Increasing salt concentrations result in an abnormal accumulation of H_2 (105) and diverse volatile fatty acids (VFA) in sediments (41, 64). These results suggest that oxidation of organic matter is incomplete at high levels of NaCl compared with that in other ecosystems (digestors, marine ecosystems, etc.) in which acetate and $\text{H}_2 + \text{CO}_2$ are used to produce CH_4 or are oxidized by sulfate-reducing bacteria when sulfate is available. At salinities higher than 15‰, the mineralization of organic compounds is limited by poor rates or the complete absence of sulfate reduction or methanogenesis from H_2 and acetate (64). Accumulation of H_2 and VFA indicates that catabolism via interspecies H_2 transfer hardly occurs in hypersaline environments. For example, the Great Salt Lake contains up to 200 μM dissolved H_2 in sediments (105). Even if the possibility of oxidizing VFA exists, the process is slow compared with fermentation of carbohydrates by most anaerobes. Similar results were obtained from Dead Sea sediments which were stimulated to reduce sulfate with H_2 and formate but not with acetate, propionate, or lactate (64). Enrichments from sediments of a hypersaline lake in Senegal containing 340 g of salts per liter indicated that acetate was produced from cellulose degradation and accumulated in the culture (53). Neither acetate, propionate, nor butyrate was metabolized 2 months after addition to enrichment cultures.

Microbial Diversity

Marine salterns are habitats for a large variety of halophilic or halotolerant bacteria that develop throughout the entire gradient of salt concentration. In the first ponds most bacteria are slightly halophilic, whereas in the intermediary ponds, where the seawater is concentrated to a salinity of about 10 to 20‰ NaCl , most of the bacteria are moderately halophilic. This intermediate environment contains the greatest numbers of organisms. The last ponds are inhabited by extremely halophilic organisms including aerobic members of the *Archaea* (105) from the genera *Halobacterium*, *Natronobacterium*, *Haloferax*, and *Haloarcula* in addition to several species pertaining to the *Bacteria* and *Eucarya*. Only one methanogenic species of the *Archaea* was reported to grow optimally at NaCl concentrations over 20‰ (111).

Most of the extremely halophilic anaerobic members of the *Bacteria* (which are genetically completely different from the members of the *Archaea*) were isolated from anoxic hypersaline environments. Among them, two bacterial groups are well represented: the fermentative bacteria belonging to the family *Haloanaerobiaceae* (66, 69) and the phototrophic sulfur-oxidizing bacteria of the family *Ectothiorhodospiraceae* (29, 30).

The phototrophic sulfur-oxidizing bacteria grow at the anoxic sediment surface in a narrow zone containing sulfide and receiving light. They use sulfide as an electron donor for photosynthesis. The sediment of the marine salterns is anoxic and rich in sulfides in all ponds throughout the salinity gradient, either at the salinity of seawater or up to NaCl saturation. Consequently, various kinds of phototrophic sulfur-

oxidizing bacteria are encountered in the different ponds. Most of them originate from the marine environment and tolerate salt concentrations up to 8 to 10% NaCl; therefore they grow in the first ponds. Some of them are moderately halophilic or extremely halophilic and populate the higher-salt ponds.

The sulfide from the anoxic sediments is produced mainly by sulfate reduction. Sulfate is one of the major inorganic compounds of seawater (25 mM). It is concentrated in the salterns up to its saturation and precipitated in the form of calcium sulfate (gypsum). Consequently, it is never a limiting factor for sulfate reduction in the salterns and serves as the final electron acceptor for sulfate-reducing bacteria. These bacteria may metabolize low-molecular-weight organic compounds produced as metabolic end products of aerobic or fermentative halophilic organisms. Although sulfate-reducing bacteria are present in the various ponds of the salterns, very few have been isolated. Both phototrophic and sulfate-reducing bacteria contribute to the turnover of the sulfur cycle in the anoxic zones of the hypersaline environments.

STRICT ANAEROBES INVOLVED IN HYPERSALINE ECOSYSTEMS

Fermentative Bacteria

To date, six anaerobic fermentative genera, containing nine species, have been described (Table 1). Two of them are homoacetogens. Six species belong to the newly described family *Haloanaerobiaceae*, as indicated by their unique 16S rRNA oligonucleotide sequences (66, 69). Fermentation patterns and DNA-DNA homologies showed that *Halobacteroides lacunaris* belongs to the family *Haloanaerobiaceae* (110). *Haloanaerobacter chitinovorans* (47) and *Haloicola saccharolytica* (113) are also typical representatives of this family because of their obligate halophily, anaerobic mode of life, gram-negative cell wall structure, and low G+C content of the DNA.

All isolates of the *Haloanaerobiaceae* family ferment carbohydrates, except *Acetohalobium arabaticum* (112). *Halobacteroides halobius*, *Halobacteroides lacunaris*, *Sporohalobacter lortetii*, *Sporohalobacter marismortui*, and *Haloanaerobacter chitinovorans* use starch. *Haloanaerobium praevalens* degrades pectin. *Sporohalobacter marismortui* and *Haloanaerobacter chitinovorans* use glycogen and chitin, respectively (Table 1). The existence of the cellulose degrader "*Halocella cellulolytica*" was recently reported (113). Cellulase activity was also demonstrated in a hypersaline African lake (53). *Sporohalobacter* species differ from all other species in being sporogenous (63, 66, 70). Their separation from *Clostridium* species was ascertained by the structure of the cell envelope and from comparative 16S rRNA cataloging (70). Motility by peritrichous flagella was shared by most species except *Haloanaerobium praevalens* (106). Several strains are considered moderate halophiles, with most rapid growth from 3 to 15% NaCl. *Halobacteroides lacunaris* and *Haloanaerobacter chitinovorans* are extreme halophilic bacteria since they grow most rapidly at 18% NaCl and have an upper limit of growth at 30% NaCl. Although *Acetohalobium arabaticum* grows rapidly at 18% NaCl, it has an upper growth limit of only 25% NaCl.

Fermentation patterns clearly distinguish the six genera so far described. *Halobacteroides* species (71, 82, 83, 109, 110) are characterized by acetate, ethanol, and H₂-CO₂ production from glucose, whereas *Haloanaerobium* species (106) produce acetate, propionate, butyrate and H₂-CO₂. *Haloanaerobacter chitinovorans* (47) accumulates acetate and H₂-CO₂ with additional formation of isobutyrate. The spore-forming *Sporoha-*

lobacter lortetii and *Sporohalobacter marismortui* (63, 66, 70) oxidize carbohydrates to a mixture of VFA with gas including isobutyrate for the former species and formic acid and ethanol for the latter.

In classifying these species, phenotypic differences in the use of substrates were clearly established (Table 1). In addition, several new strains of strictly anaerobic halophiles were isolated and studied in our respective laboratories, and their characterization is in progress (data not shown). Of these, strain H168 (13) represents the first true thermophilic (growing up to 68°C, with most rapid growth at 60°C) halophilic fermentative anaerobe described at this time and should be classified in a new genus.

Homoacetogenic Bacteria

Haloicola saccharolytica (113) exhibits a homoacetogenic pathway of metabolism on glucose. Only *Acetohalobium arabaticum* (112) reduces CO₂ to acetate. *Haloicola saccharolytica* (113) ferments carbohydrates and *N*-acetylglucosamine at an optimum NaCl concentration of 10%, with a range of 3 to 30% (Table 1). *Acetohalobium arabaticum* (112) grows on betaine and trimethylamine at NaCl concentrations ranging from 10 to 25% (Table 1). With an optimum NaCl concentration between 15 and 18%, this isolate is considered a possible competitor of sulfate-reducing bacteria (SRB) for H₂, depending on the affinity of its hydrogenase(s) with hydrogen.

Sulfate-Reducing Bacteria

The SRB form an ecophysiological group with the common property of using sulfate as the main electron acceptor during anaerobic metabolism. They are recognized as strict anaerobes, although metabolic activity in the presence of oxygen was recently reported (17, 19). Most may also use thiosulfate, sulfite, or sulfur as electron acceptors, and fewer also use nitrate or fumarate. When a sulfur compound is used as the electron acceptor, the final product is hydrogen sulfide, which is excreted into the environment. Generally, they are chemoor-ganotrophs which use low-molecular-weight organic compounds, such as lactate, pyruvate, ethanol, and VFA, or H₂ as electron donors. Few can use fatty acids and degrade them completely to CO₂, alcohols up to C₃ or sugars (glucose, fructose), and, in some cases, specific organic compounds such as indole, phenol, or catechol. Organic compounds also serve as carbon sources; a few sulfate reducers are autotrophs which use CO₂ as sole carbon source.

Metabolically, SRB differ from each other by oxidizing organic electron donors either completely or incompletely. Species which exhibit incomplete oxidation produce low-molecular-weight fatty acids, mainly acetate, as the end product of metabolism.

The physiology and systematics of the SRB are well reviewed and discussed (96–99). Bacterial sulfate reduction is an important process of mineralization of organic matter in anoxic environments, especially in marine and hypersaline systems (5, 36, 38, 41, 56, 64, 105). In the marine environment, sulfate reduction occurs mainly in the anoxic sediment or in the bottom anoxic waters of stratified lagoons and is performed by halotolerant or slightly halophilic sulfate reducers belonging to many different species and genera. The slightly halophilic sulfate reducers have been allocated to the genera *Desulfovibrio*, *Desulfobacter*, *Desulfococcus*, *Desulfosarcina*, *Desulfobacterium*, and *Desulfonema* and grow optimally at salinities ranging from 1 to 4% NaCl (Table 2).

Biological sulfate reduction was observed in hypersaline ecosystems (53, 56, 105) containing large amounts of sulfate.

TABLE 1. Comparative properties of haloanaerobic fermentative bacteria

Property	Characteristic or value in ^a :								
	<i>Halobacteroides halobius</i> (71, 109)	<i>Halobacteroides acetoeithylicus</i> (82)	<i>Halobacteroides lacunaris</i> (110)	<i>Haloanaerobium praevallens</i> (106)	<i>Haloanaerobacter chitinovorans</i> (47)	<i>Haloincola saccharolytica</i> (113)	<i>Sporohalobacter lortetii</i> (63, 70)	<i>Sporohalobacter marismortui</i> (70)	<i>Acetohalobium arabaticum</i> (112)
Morphology	Flexible rods	Rods	Flexible rods	Rods	Rods	Rods	Rods	Rods	Curved rods
Size (μm)	0.3-0.5 \times 2-20	0.4-0.7 \times 1-1.6	0.7-1 \times 0.5-6	0.5 \times 1.5	0.5 \times 1.4-8	0.5-0.7 \times 1-1.5	0.5-0.6 \times 2.5-10	0.6 \times 3-13	0.7-1 \times 2-5
Gram stain	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Spores	-	-	-	-	-	-	-	-	-
Motility	+	+	+	-	+	+	+	+	+
NaCl range (%)	8.0-30.0	6.0-20.0	5.0-30.0	2.0-30.0	3.0-30.0	3.0-30.0	4.0-15	3.0-18.0	10.0-25.0
NaCl optimum (%)	9.0-15.0	10.0	15.0-18.0	12.5	12.0-18.0	10	8.0-9.0	3.0-12.0	15.0-18.0
Temp range ($^{\circ}\text{C}$)	30-47	15-45	25-52	15-50	25-50	15-47	25-52	25-50	ND-47
pH range	ND ^b	5.4-8.0	6.0-8.0	6.0-8.0	ND	6.0-8.0	ND	ND	5.6-8.4
Doubling time (h)	1-2	7.8-9.5	2.9-4.5	4	2.5-2.9	3.9	8	ND	ND
Habitat	Dead Sea-Lake Sivash	Oil well	Lake Chokcrack	Great Salt Lake	Solar saltern	Lake Sivash	Dead Sea	Dead Sea	Lake Sivash
G+C (mol%)	30.7	32.0	32.4	27.0	34.8	31.3	31.5	29.6	33.6
Substrates used									
Carbohydrates	+	+	+	+	+	+	+	+	-
Amino acids	-	-	-	+	ND	-	ND	-	+
<i>N</i> -Acetylglucosamine	-	+	-	+	+	+	ND	ND	ND
Starch	+	-	+	-	±	+	+	+	ND
Chitin	ND	-	-	-	+	-	ND	-	ND
Cellulose	ND	ND	ND	ND	-	ND	ND	ND	ND
End product(s) from glucose	Acetate, ethanol, H ₂ +CO ₂	Acetate, ethanol, H ₂ +CO ₂	Acetate, ethanol, H ₂ +CO ₂	Acetate, butyrate, propionate, H ₂ +CO ₂	Acetate, isobutyrate, H ₂ +CO ₂	Acetate	Acetate, ^c propionate, isobutyrate, butyrate, isovalerate, H ₂	Acetate, ethanol, butyrate, formate, H ₂ +CO ₂	Acetate, ^d trimethylamine ^d

^a Numbers in parentheses after bacterial names are the references from which the data were gathered.^b ND, not determined.^c In rich medium, sugar poorly used.^d From betaine degradation.

TABLE 2. Grouping of halophilic sulfate-reducing bacteria^a

Species	Salinity range (% NaCl)	Salinity optimum (% NaCl)
Slight halophiles		
<i>Desulfovibrio desulfuricans</i> subsp. <i>aestuarii</i>	0.5–6	2.5
<i>Desulfovibrio salexigens</i>	0.5–12	2–4
<i>Desulfovibrio giganteus</i>	0.2–6	2–3
<i>Desulfobacter postgatei</i>	0.5–4	0.7
<i>Desulfobacter latus</i>		2
<i>Desulfobacter curvatus</i>		2
<i>Desulfobacter hydrogenophilus</i>		1
<i>Desulfococcus multivorans</i>		0.5
<i>Desulfococcus niacini</i>		1.5
<i>Desulfosarcina variabilis</i>		1.5
<i>Desulfobacterium autotrophicum</i>		2
<i>Desulfobacterium vacuolatum</i>		2
<i>Desulfobacterium phenolicum</i>		2
<i>Desulfobacterium indolicum</i>		2
<i>Desulfonema limicola</i>		1.5
<i>Desulfonema magnum</i>		2–3
Moderate halophiles		
<i>Desulfovibrio halophilus</i>	3–18	6–7
<i>Desulfohalobium retbaense</i>	3–25	10

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However, only a limited range of substrates including H₂, formate, and lactate were involved in this process (53, 64, 105). No evidence of oxidation of VFA such as acetate by sulfate reducers has so far been demonstrated at high salinities, indicating that the ambient strains are mainly incomplete oxidizers. This process favors the accumulation of acetate in sediments, as reported by Oren (64), who suggested that salinity may have some role in limiting the decomposition of VFA. Increasing NaCl concentrations up to 30% resulted in unusual levels of acetate up to 800 μM (64). These results show that the complete oxidation of organic matter is drastically reduced by high salt concentration. At salinities above 15%, sulfate reduction and methanogenesis functioned very poorly (64).

Only a few moderately halophilic sulfate reducers were isolated from different kinds of hypersaline environments including marine salterns. Trüper (92) isolated a few SRB from hot brines in the Red sea. One of them tolerated up to 17% NaCl and seemed similar to *Desulfovibrio halophilus*, a moderately halophilic sulfate reducer recently isolated by Caumette et al. (11) from the hypersaline Solar Lake in Sinai. Cord-Ruwisch et al. (15) isolated several strains of SRB from hypersaline oil field water containing about 10% NaCl. One isolate, a lactate- and fatty acid-oxidizing strain, grew slowly at concentrations up to 27% but has not been described in more detail. Another halophilic SRB species, *Desulfovibrio salexigens*, does not grow at concentrations higher than 12% NaCl (80).

Very recently, a second moderately halophilic sulfate reducer was fully described and characterized as a new species of a new genus, *Desulfohalobium retbaense* (58). It was isolated from the small hypersaline Retba Lake in Senegal. The organism was a nonsporulating motile straight rod. This isolate

TABLE 3. Main characteristics of sulfate reducers isolated from hypersaline ecosystems

Property	Characteristic or value in ^a :	
	<i>Desulfovibrio halophilus</i> (11)	<i>Desulfohalobium retbaense</i> (58)
Morphology	Vibrio	Rod
Size (width × length) (μm)	0.6 × 2.5–5	0.7–0.9 × 1–3
Gram stain	Negative	Negative
Motility	1 polar flagellum	1–2 polar flagella
Temp optimum (°C)	35–40	37–40
Salinity optimum (%)	6–7	10
pH optimum	6.5	6.5–7.0
DNA content (%G+C)	60.7	57.1
Habitat	Solar Lake	Lake Retba
Electron donor (with sulfate)		
H ₂	+	+
Formate	+	+
Ethanol	+	+
Pyruvate	+	+
Lactate	+	+
Acetate	–	–
Electron donors (without sulfate)		
Pyruvate	–	+
Malate	–	–
Electron acceptors		
Sulfate	+	+
Sulfite	+	+
Thiosulfate	+	+
Sulfur	+	+

^a Numbers in parentheses after bacterial names are the references from which the data were gathered.

and *Desulfovibrio halophilus* represent the only two moderately halophilic species so far reported (Table 3), although many other halophilic sulfate reducers should exist in hypersaline environments. Both species have a salinity range between 3 and 18 to 20% NaCl, with their most rapid growth between 6 and 10% NaCl. For electron donors, they use a limited number of organic compounds, such as lactate, pyruvate, and formate, and H₂. They can use acetate as a carbon source in the presence of H₂ as an electron donor. *Desulfovibrio halophilus* and *Desulfohalobium retbaense* incompletely oxidized lactate or ethanol to acetate. *Desulfohalobium retbaense* differed from *Desulfovibrio halophilus* in its morphology, salinity range, fermentation of pyruvate, and the presence of desulfurubidin instead of desulfoviridin as the dissimilatory bisulfite reductase. The G+C content of the DNA of *Desulfovibrio halophilus* was higher than that of *Desulfohalobium retbaense* (Table 3).

The 20% upper limit of NaCl concentration for growth of *Desulfovibrio halophilus* demonstrates that this isolate may play an important ecological role in its environment, where the NaCl concentration varied from 6 to 20%. The salt content of the environment from which *Desulfohalobium retbaense* was isolated (34%) shows that this bacterium cannot be very active in this ecosystem, preferring ecological niches with lower concentrations of NaCl. However, the artificial conditions in which this strain was maintained are probably different from the mineral or organic content of the original water and could influence the response of the isolate to salt stress.

The osmoregulation of these bacteria is not well elucidated. Recent experiments demonstrate that *D. halophilus* cannot synthesize compatible solutes and is able to grow in defined mineral media by accumulating salts into the cytoplasm (20).

Phototrophic Bacteria

The anoxygenic phototrophic bacteria constitute a physiological group of microorganisms that share the common property of anoxygenic photosynthesis (1, 26, 27, 42, 76–78, 91, 94). They are divided into purple and green bacteria according to their respective light-harvesting pigments, bacteriochlorophylls and carotenoids, which transfer electrons via a photosystem and a cyclic chain of electron transport. Consequently, in contrast to cyanobacteria (oxygenic phototrophic bacteria), which use water as an electron donor and produce oxygen during their photosynthesis, the anoxygenic phototrophic bacteria may use H_2 , organic compounds, or reduced sulfur compounds as electron donors; they live in anoxic environments reached by light. When reduced sulfur compounds are used, they form the various corresponding oxidized sulfur compounds, the final product being sulfate.

Anoxygenic phototrophic bacteria often develop as dense layers in a wide variety of anoxic, generally poorly illuminated environments found in the metalimnia or hypolimnia of stratified waters or at the sediment surface where sufficient light is present. Most blooms of phototrophic bacteria are seen as colored biomasses of mainly purple or green sulfur bacteria. In addition to requiring anoxic conditions and photosynthetically active radiation, phototrophic purple and green sulfur bacteria need a suitable electron donor such as hydrogen sulfide. Most of the hydrogen sulfide in anoxic layers is biogenic, with the exception of that in sulfur springs and hydrothermal vents. In anoxic sediments, hydrogen sulfide is formed from bacterial degradation and fermentation of sulfur-containing proteins or from reduction of sulfate or sulfur by sulfate- or sulfur-reducing bacteria (96). Sulfate reduction can produce more than 95% of the biogenic sulfide found in anoxic layers of sulfate- or sulfur-rich habitats. Shallow coastal marine environments with salinities ranging from brackish to hypersaline are ideal habitats for these phototrophs. In such environments, the purple and green sulfur bacteria are distributed according to vertical oxygen, sulfide, and light gradients.

In coastal anoxic sediments the oxic/anoxic interface (chemocline or redoxcline) is generally found within the first millimeter or centimeter (5, 36, 84). The narrow interface between the oxygen and sulfide layers often reveals a transition zone of less than 1 mm, free of both compounds (38), in contrast to stratified lakes, where both compounds can be found in a large transition layer.

Oxygen residing in the overlying water column usually does not penetrate deeper than 2 mm into the sediment, although in sediment covered by cyanobacterial or algal mats it can be detected as deep as 10 mm (35, 36, 38). Below such depths, oxygen is depleted as a consequence of both chemical combination with sulfide and consumption by different heterotrophic and chemotrophic organisms, particularly the aerobic colorless sulfur-oxidizing bacteria. In many shallow-water sediments, adequate photosynthetically active radiation reaches depths of 2 to 8 mm (18, 37, 38). The blue and green parts of the light spectrum penetrate less deeply than do the red and the near-infrared light, which are used by phototrophic bacteria. Light penetration into sediments depends on the overlying water depth; near-infrared light penetrates sediments under very shallow water (less than 50 to 100 cm in depth) only. In waters deeper than 2 to 4 m, only wavelengths between 450 and 550 nm reach the sediment surface; however, they can be used by phototrophic bacteria which have bacteriochlorophylls and specific carotenoids as light-harvesting pigments.

Some phototrophic bacteria from marine coastal environments are halotolerant up to 2 to 4% NaCl, but strictly

halophilic purple or green bacteria have frequently been isolated. These latter organisms generally exhibit optimal growth at salinities between 2 and 5% NaCl and are slightly halophilic organisms (Table 4). They are abundant in the first ponds of marine salterns connected to the sea, where the seawater is concentrated to about 6 to 8% NaCl.

In contrast, only a few purple bacteria have thus far been isolated from hypersaline habitats and some green sulfur bacteria have been observed (16, 24) but not isolated. Most of the purple bacteria isolated from hypersaline ponds in marine salterns are moderately halophilic, with optimal growth at salinities between 6 and 11% NaCl (Table 4). They belong to the genera *Rhodospirillum*, *Chromatium*, *Thiocapsa*, and *Ectothiorhodospira*. The most common organisms isolated so far are *Chromatium salexigens* (9), *Thiocapsa halophila* (10), and *Rhodospirillum salinarum* (55).

Extremely halophilic purple bacteria have most commonly been isolated from alkaline brines in athalassohaline environments such as desert lakes (31, 32). They require about 20 to 25% NaCl for optimal growth. They belong to the family *Ectothiorhodospiraceae*.

In these hypersaline environments, phototrophic bacteria control their osmoregulation by synthesis or uptake of compatible solutes that accumulate in their cytoplasm. Among the compatible solutes they use, the most common is glycine betaine. However, most of the purple and green bacteria are able to accumulate sugars (trehalose or sucrose), and some of them accumulate N-acetylated compounds such as N-acetylglutamyl glutamic acid (Table 5).

The extremely halophilic purple bacteria synthesize another type of compatible solute (ectoine), which is an amino acid derivative (93). The biosynthetic pathway of ectoine in *Ectothiorhodospira halochloris* has been identified recently (75).

Methanogenic Bacteria

Methanogenesis in hypersaline ecosystems was first reported in a submarine brine pool in the gulf of Mexico (4). A strictly methylotrophic activity related to methanogens but in the absence of H_2 and acetate oxidation was demonstrated in an alkaline lake (60) and in the Great Salt Lake (79, 105). Identical results were obtained in Lake Retba in Senegal, West Africa (53). Microbiological studies in these ecosystems confirmed the presence of methanogens using methylated compounds (48, 51, 64, 73, 95, 108). All these results suggest that the use of methylotrophic substrates by methane-producing bacteria in halophilic environments probably predominates over H_2 and acetate utilization.

Oremland and King (59) reported the production of methane from H_2 plus CO_2 in a lake containing 9% NaCl. Although the isolation of a hydrogenotrophic halophilic methanogen was reported (103), this strain was never mentioned again and never verified. A halotolerant hydrogenotrophic methanogenic rod growing in up to 5% NaCl was recently isolated and characterized (57); it uses H_2 plus CO_2 , formate, and CO_2 plus 2-propanol with a doubling time of 10 h under optimal conditions. To our knowledge, the highest NaCl concentration so far reported for the methanogens using H_2 or formate is 8.3% (28). However, considering the multiplicity of halophilic ecosystems from the physicochemical point of view, further investigations will probably lead to isolation of hydrogenotrophic bacteria growing at higher salt concentrations. At present, H_2 does not appear to be an important source of energy for methanogenesis in hypersaline environments.

Because of the high sulfate concentration in hypersaline environments, it is not surprising that sulfate reducers may

TABLE 4. Halophilic phototrophic bacteria grouped according to their salt requirement and classification of halophilic organisms^a

Type of halophiles	Species	5	10	15	20	25 % NaCl
Slight halophiles	<i>Chromatium buderi</i>	—	—	—	—	—
	<i>Chloroherpeton thalassium</i>	—	—	—	—	—
	<i>Ectothiorhodospira mobilis</i>	○	—	—	—	—
	<i>Rhodobacter sulfidophilus</i>	○	—	—	—	—
	<i>Pelodictyon phaeum</i>	—	—	—	—	—
	<i>Rhodospseudomonas marina</i>	—	—	—	—	—
	<i>Ectothiorhodospira vacuolata</i>	—	—	—	—	—
	<i>Prostecochloris phaeoasteroidea</i>	—	—	—	—	—
	<i>Thiorhodovibrio winogradskyi</i>	○	—	—	—	—
	<i>Chlorobium chlorovibrioides</i>	—	—	—	—	—
	<i>Chromatium purpuratum</i>	○	—	—	—	—
	<i>Rhodobacter adriaticus</i>	—	—	—	—	—
	<i>Prostecochloris aestuarii</i>	—	—	—	—	—
	<i>Chromatium vinosum</i> HPC	○	—	—	—	—
<i>Lamprobacter modestohalophilus</i>	—	—	—	—	—	
Moderate halophiles	<i>Rhodospirillum mediosalinum</i>	—	○	—	—	—
	<i>Rhodospirillum salexigens</i>	—	○	—	—	—
	<i>Ectothiorhodospira marismortui</i>	—	○	—	—	—
	<i>Thiocapsa halophila</i>	—	○	—	—	—
	<i>Chromatium salexigens</i>	—	—	○	—	—
	<i>Ectothiorhodospira abdelmalekii</i>	—	—	—	○	—
Extreme halophiles	<i>Ectothiorhodospira halophila</i>	—	—	—	○	—
	<i>Ectothiorhodospira halochloris</i>	—	—	—	—	○

^a Adapted from reference 8 with permission of the publisher. Data gathered from references 6, 9, 10, 68, and 72. Symbol: o, optimum salinity.

outcompete methanogens for H₂ (49, 96) since marine and halophilic methanogens are not known for their ability to compete for H₂. What is surprising is the inability of native SRB to use up all available H₂ under hypersaline conditions. Low-molecular-weight methyl compounds such as the methylamines are probably the major substrates for methanogenesis; no evidence of sulfate reduction with methylamines as electron donors has been established.

Halophilic methylotrophic methanogens have been reported among three recognized genera and five species (Table 6). The phenotypic characteristics of “*Methanohalococcus alcaliphilum*” (54), and “*Halomethanococcus doii*” (104) could place these species in the genus *Methanohalophilus*, but 16S rRNA oligonucleotide sequences and DNA-DNA hybridizations are needed to establish their valid taxonomic position. However,

“*Halomethanococcus doii*” is apparently no longer available in culture. The halotolerant *Methanohalophilus oregonensis* (48) was recently reclassified as *Methanohalobium oregonensis* (2). The five species of halophilic methanogens are obligately moderate halophilic cocci, with the exception of the extreme halophile *Methanohalobium evestigatum* (111). Marked differences in optimum pH and temperature for growth were observed. Two species are alkaliphiles (*Methanosalsus zhilinaeae* and “*Methanohalococcus alcaliphilum*”), and the others are neutrophiles. *Methanosalsus zhilinaeae* and *Methanohalobium evestigatum* are moderately thermophilic bacteria with temperature optima of 45 and 50°C, respectively (Table 6); two other species are mesophilic. Significant differences were also observed in the G+C content, which ranged from 38 to 48.5%. These findings indicate that more basic studies are needed to define the

TABLE 5. Compatible solutes synthesized and glycine betaine taken up by different halophilic phototrophic bacteria grown with 0.5 or 1.5 M NaCl in synthetic medium^a

Bacterium	NaCl concn (M)	Solute(s) synthesized	Glycine betaine uptake ^b
<i>Thiocapsa roseopersicina</i> OP1	0.5	Sucrose	+
<i>Thiocapsa roseopersicina</i> 5811	0.5	Sucrose	++
<i>Thiocapsa roseopersicina</i> 5911	0.5	Sucrose	++
<i>Thiocapsa halophila</i> SG 3202	1.5	Betaine sucrose <i>N</i> -acetylglutaminyglutamine amide	++++
<i>Amoebobacter roseus</i> 6611	0.5	Sucrose	+++
<i>Thiocystis violaceae</i> 2311	0.5	Sucrose	+
<i>Chromatium minus</i> 1211	0.5	Sucrose betaine	+++
<i>Chromatium vinosum</i> D	0.5	Sucrose	+
<i>Chromatium</i> NCIMB 8379	1.5	Sucrose betaine	+++
<i>Chromatium salexigens</i> SG 3201	1.5	Betaine sucrose <i>N</i> -acetylglutaminyglutamine amide	++++
<i>Chlorobium limicola</i> Kios 6230	0.5	Trehalose	+++
<i>Chlorobium vibrioforme</i> 6030	0.5	Trehalose	++

^a Reprinted from reference 8 with permission of the publisher. Courtesy of Rod Herbert.

^b +, weak uptake; ++, good uptake; +++, very good uptake.

TABLE 6. Comparative properties of methanogens isolated from hypersaline ecosystems

Property	Characteristic or value in ^a :				
	<i>Methanohalophilus mahii</i> (74)	<i>Methanohalophilus halophilus</i> ^b (107)	<i>Methanohalophilus portucalensis</i> (2)	<i>Methanosalsus zhilinae</i> (3, 52)	<i>Methanohalobium evestigatum</i> (111)
Morphology	Irregular cocci	Irregular cocci	Irregular cocci	Irregular cocci	Irregular cocci
Size (wide × length) (μm)	0.8 × 1.8	0.5 × 2	0.6 × 2.0	0.75 × 1.5	1
Optimal temp (°C)	35	26–36	40	45	50
Optimal pH	7.5	6.5–7.4	6.5–7.5	9.2	7.0–7.5
Optimal NaCl (%)	12	7–9	3–12	4	24
Type of halophilism	Moderate	Moderate	Moderate	Moderate	Extreme
Habitat	Great Salt Lake	Saline cyanobacterial mat	Salinarium	Bosa Lake	Saline lagoon, Crimea
Substrates used					
H ₂ +CO ₂	–	–	–	–	–
Acetate	–	–	–	–	–
Methylamines	+	+	+	+	+
Methanol	+	+	+	+	+
DNA content (%G+C)	48.5	44	43–44	38	ND ^c

^a Numbers in parentheses after bacterial names are the references from which the data were gathered.

^b Renamed from *Methanococcus halophilus* (101).

^c ND, not determined.

methanogenic halophilic genera (89). Since the substrates used as energy sources by these strains are identical and are limited to methyl compounds, traditional phenotypic cultural and substrate characteristics are too few and too restrictive to classify these organisms properly.

COMPETITION BETWEEN METHANOGENS AND SULFATE REDUCERS

Sulfate-reducing bacteria are known to outcompete methanogens for different energy sources when sulfate is not limiting in the ecosystem. This is observed in marine environments, where H₂ and acetate are used mainly via sulfate reduction (59, 61). Nevertheless, methanogenesis occurs in these environments where methanogens use methylamines, which are considered noncompetitive substrates because their use by sulfate reducers has never been described.

In hypersaline ecosystems, which contain larger amounts of sulfate than marine ecosystems, competition for substrates might be amplified; the major pathway for H₂ oxidation is via sulfate reduction. However, this does not imply the absence of hydrogenotrophic methanogens. For example, hydrogenotrophic methanogens belonging to the family *Methanomicrobiaceae* (88, 100) or *Methanococcaceae* (14, 34) have been isolated from marine environments.

In the native pelagic sediment of Mono Lake, the upper limit of NaCl concentration for H₂ utilization by methanogens was reported as 9% (59). This suggests that both sulfate reducers and methanogens have similar apparent *K_s* values for H₂ (59). When the NaCl concentration is above 15%, the methanogenic activity from H₂ as electron donor is low or not expressed. Thus, the persistence of methanogens in hypersaline environments is related to the presence of noncompetitive substrates such as methylamines, which originate mainly from the breakdown of osmoregulatory amines. This leads to the hypothesis that methanogenesis does not contribute to the mineralization of carbohydrates at NaCl concentration higher than 15%. Above this concentration, sulfate reduction is probably the main way to oxidize H₂ and occupies a terminal function in the degradation of carbohydrates. However, this function decreases concurrently with fatty acid accumulation when salt concentration increases.

Therefore in hypersaline ecosystems, the NaCl concentration drastically affects the distribution and functioning of both

methanogens and sulfate reducers. Sulfate reducers remain somewhat more active with regard to H₂ metabolism, but the methanogens may also remain active by using specific organic compounds at the higher NaCl concentrations. In most ecosystems, anaerobic mineralization of organic matter leads to production of the simplest compounds: CO₂, CH₄, and H₂S. However, this probably does not apply to hypersaline sediments, in which the high salt content leads to the accumulation of VFA and H₂.

EXAMPLE OF MASS BLOOM DEVELOPMENTS OF PHOTOTROPHIC BACTERIA IN MARINE SALTERNS

In the marine salterns of Salins-de-Giraud, located on the Mediterranean French coast in the Rhone Delta, microbial mats of oxygenic and anoxygenic phototrophic bacteria were observed underneath a gypsum crust in ponds with salinities ranging from 13 to 20%. These mats have been investigated during the last 5 years (7, 9, 10, 12). They are composed of cyanobacteria and phototrophic purple bacteria organized in laminated thin layers as shown in Fig. 1. Above the gypsum crust, a brown layer 2 to 5 mm deep is composed of unicellular cyanobacteria of the group *Aphanothece* embedded in a mucoid substance. Below the gypsum crust, a green layer 2 mm deep composed of the filamentous cyanobacterium *Phormidium* overlies a pure layer of phototrophic bacteria. This latter layer is 2 to 4 mm thick and is composed mainly of purple sulfur bacteria of the family *Chromatiaceae*. These mats are fully developed during the spring and summer season.

Recent investigations showed that the purple sulfur bacteria grew by using the sulfide from sulfate reduction occurring in the underlying sediment (12). In the first 5 cm of this sediment, sulfate reduction occurred at very high rates. On the basis of incubation with ³⁵S, the calculated sulfate reduction rates were about 40 μmol · cm⁻³ · day⁻¹ or 2,000 mmol · m⁻² · day⁻¹ (calculated on area surface basis) at the top 5 cm of sediment. In the purple layer, sulfide oxidation measured by microelectrodes (12) was calculated at about 12 μmol · cm⁻³ · h⁻¹ in the 3-mm depth of the red layer. This value could be 300 to 400 mmol · m⁻² · day⁻¹ assuming a photosynthetic period of 8 to 10 h. From such observations it is evident that the sulfide produced is not completely reoxidized by the phototrophic purple bacteria. Cyanobacteria could be involved in the reoxidation either by producing oxygen which chemically reacts with

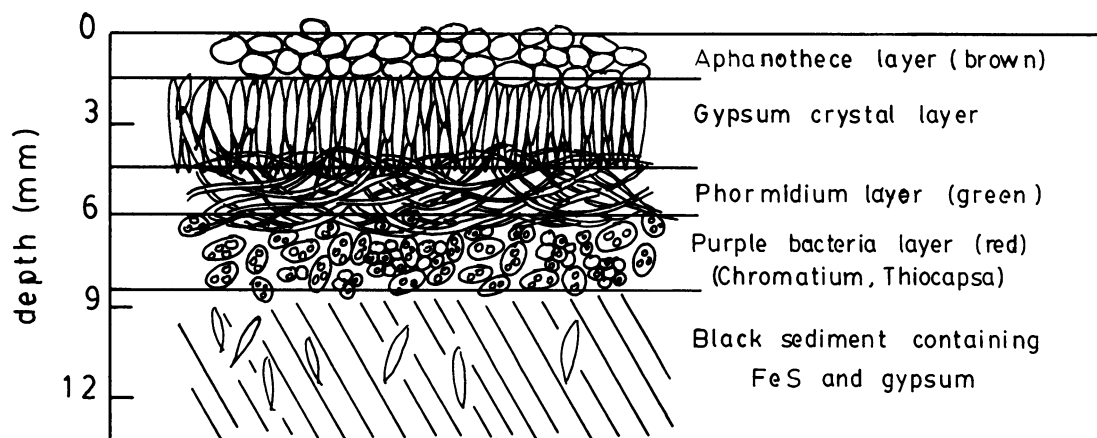


FIG. 1. Scheme of microbial mat structure in the marine saltern of Salins-de-Giraud. Reprinted from references 8 and 12 with permission of the publishers.

sulfide or by anoxygenic photosynthesis. Microprofiles of oxygen and sulfide in the mats support this observation: during daylight, sulfide was detected only in the deeper layers below the purple bacteria, whereas during the night until early morning, sulfide was present in the whole mat up to the gypsum crust (12), thus forming an anoxic environment for cyanobacteria.

From the purple layer two new species of halophilic bacteria belonging to the family *Chromatiaceae* were isolated. *Chromatium salexigens* (9) and *Thiocapsa halophila* (10) grew at salinities between 4 and 20% NaCl with optimal growth at 6 to 10% NaCl in synthetic media. Thus, they are well adapted to their environments, where salinities ranged from 13 to 20% of total salinity. Both organisms are able to use sulfide, sulfate, sulfur, or thiosulfate as the electron donor and CO_2 as the carbon source. They can also use some organic compounds, namely acetate and pyruvate.

In the mats the phototrophs grew by using the light wavelengths that reached the purple layer. During maximum daylight, the light intensity reaching the purple layer was about 460 lux (i.e., 0.1 to 0.5% of photosynthetically active radiation at the sediment surface). Both types grew well at such a light intensity. Their growth rate at optimum light intensity (1,000 lux) was 0.030 h^{-1} . It decreased to 0.018 h^{-1} at 460 lux. The bacteria were well adapted to low light intensity since they also grew at 25 lux with a growth rate of 0.006 h^{-1} (9, 10). As discussed above and presented in Table 5, both types of bacteria synthesized or took up compatible solutes for their osmoregulation processes.

From the sediment of these hypersaline environments, a few strains of halophilic *Desulfovibrio* species were also isolated. They were very similar to *Desulfovibrio halophilus* SL 8903 isolated from the hypersaline solar lake in Sinai (11). The new strains (SG 3802 and SG 3805), members of the species *Desulfovibrio halophilus*, have a salinity range from 1 to 17% NaCl and optimal growth at salinities between 4 and 6% NaCl in synthetic media. Like the purple sulfur bacteria, they were well adapted to the salinity range of the ponds in the marine salterns of Salins-de-Giraud. They used lactate, pyruvate, formate, ethanol, propanol, and H_2 as energy sources. They also used acetate as a carbon source in the presence of H_2 ; acetate is a major product of fermentation processes that take place in these hypersaline anoxic sediments.

Recent investigations showed that these halophilic sulfate reducers did not synthesize compatible solutes and accumu-

lated salt when they grew in mineral media with lactate as the carbon and energy source (20). Further investigations are in progress to verify whether they can take up compatible solutes as do heterotrophic bacteria such as members of the *Enterobacteriaceae* (46). If this hypothesis is verified, various new halophilic sulfate reducers could be isolated with the help of compatible solutes in the defined culture media.

CONCLUSION

Microbiological anaerobic studies in hypersaline habitats revealed the presence of heterotrophs growing on a wide range of substrates, including polymers such as starch, glycogen, pectin, cellulose, and chitin. In these habitats methanogens appeared to be restricted to oxidizing methyl compounds with little or no role in the metabolism of H_2 or acetate. Indeed, no methanogenesis occurred via H_2 oxidation above 15% NaCl. Sulfate reducers from hypersaline areas most probably out-competed methanogens for H_2 . In this way, the microbial community of hypersaline environments was close to that observed in a variety of marine systems (39, 40, 62, 97). However, in contrast to marine environments, acetate degradation was never described for any moderate or extreme halophilic microorganism, either by sulfate reduction or by acetate fermentation.

Halophilic sulfate-reducing bacteria metabolized a few substrates such as H_2 plus CO_2 , formate, lactate, pyruvate, and ethanol and performed an incomplete oxidation of lactate and ethanol. These specific metabolic properties (fermentation and sulfate reduction) resulted in the accumulation of acetic acid and other VFA. The accumulation was positively correlated with the salt concentration of the ecosystem. Increased salinities notably reduced the turnover of VFA by anaerobic bacteria.

The nature of metabolites produced by fermentative isolates may lead to interrelationships with either methanogens or SRB. Several fermentative strains produced ethanol, a common substrate for sulfate reducers. Furthermore, pectinolytic activity was detected in *Haloanaerobium praevalens*, which probably resulted in the formation of methanol (90), which is used by *Methanohalophilus* species to produce CH_4 . The most important source of methylamines for methanogens was probably glycine betaine, an osmoregulator widespread among eukaryotes and prokaryotes living in hypersaline conditions, and trimethylamine oxide, an osmolyte in fish. This was

supported by the recent isolation of a homoacetogenic bacterium cleaving glycine betaine to acetate plus trimethylamine (112).

Research in hypersaline habitats appears promising from the biotechnological, microbiological, biochemical, and phylogenetic points of view. The discovery of the new family *Haloanaerobiaceae* and the rather few anaerobic microorganisms currently characterized provide incentives to search for new types of bacteria. Studies of these halophiles may also provide biochemists with organisms from which new polymeric substances, enzymes, or osmolytes of industrial interest could be produced. Basic studies on novel osmolytes may improve the understanding of mechanisms involved in osmoregulation at high salt concentrations.

The simultaneous presence of aerobic and anaerobic archaeobacteria in extreme environments (hyperthermophilic, hyperhalophilic) early in the evolution of the Earth might strengthen phylogenetic studies to clarify the transition steps from the anoxic to the aerobic life within the *Archaea* domain.

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