AEM Journals.ASM.org

MINIREVIEW

Life under Multiple Extreme Conditions: Diversity and Physiology of the Halophilic Alkalithermophiles

Noha M. Mesbah[a] and Juergen Wiegel[b]

Department of Biochemistry, Faculty of Pharmacy, Suez Canal University, Ismailia, Egypt,[a] and Department of Microbiology, University of Georgia, Athens, Georgia, USA[b]

Around the world, there are numerous alkaline, hypersaline environments that are heated either geothermally or through intense solar radiation. It was once thought that such harsh environments were inhospitable and incapable of supporting a variety of life. However, numerous culture-dependent and -independent studies revealed the presence of an extensive diversity of aerobic and anaerobic bacteria and archaea that survive and grow under these multiple harsh conditions. This diversity includes the halophilic alkalithermophiles, a novel group of polyextremophiles that require for growth and proliferation the multiple extremes of high salinity, alkaline pH, and elevated temperature. Life under these conditions undoubtedly involves the development of unique physiological characteristics, phenotypic properties, and adaptive mechanisms that enable control of membrane permeability, control of intracellular osmotic balance, and stability of the cell wall, intracellular proteins, and other cellular constituents. This minireview highlights the ecology and growth characteristics of the extremely halophilic alkalithermophiles that have been isolated thus far. Biochemical, metabolic, and physiological properties of the extremely halophilic alkalithermophiles are described, and their roles in resistance to the combined stressors of high salinity, alkaline pH, and high temperature are discussed. The isolation of halophilic alkalithermophiles broadens the physicochemical boundaries for life and extends the boundaries for the combinations of the maximum salinity, pH, and temperature that can support microbial growth.

Extremophiles are prokaryotes which not only survive but grow optimally under conditions considered harsh and inhospitable from both the anthropogenic point of view and that of common mesophilic and neutrophilic microorganisms.

This minireview focuses on a group of extremophiles called the halophilic alkalithermophiles and on some aspects of their physiology and taxonomic distribution. The halophilic alkalithermophiles are a unique group of polyextremophiles that grow optimally under the combined conditions of extreme salinity, alkaline pH, and elevated temperature. The isolation and description of these recently discovered polyextremophiles have extended the known physical and chemical boundaries for life and have provided excellent models for the study and characterization of novel physiologies and biochemical pathways (2, 3).

HALOPHILIC ALKALITHERMOPHILES

Many definitions have been proposed that classify microorganisms according to their requirement or tolerance for salt, alkaline pH, and temperature (2, 3, 6, 9, 14, 19, 35, 36). Since the minimum, optimum, and maximum salt concentration, pH, and temperature for growth are dependent upon each other and can vary with changes in the growth medium composition, it is difficult to delineate sharp boundaries for what a halophilic alkalithermophile is. Due to article length limitations, this discussion is limited to the extremely halophilic alkalithermophiles. The definition described by Bowers and Wiegel is used: microorganisms that grow optimally at or above Na^+ concentrations of 1.7 M, pH greater than or equal to 8.5, and temperatures greater than or equal to 50°C are deemed halophilic alkalithermophiles (3).

For halophilic alkalithermophiles, care should be taken when data regarding marginal and optimal growth are determined and described, as the value for one parameter is affected by another. For example, the measured pH value of a medium is dependent on the temperature at which the measurement was made due to the temperature dependence of the pK_a values of different medium components (13, 36). Thus, the pH of the medium determined at room temperature is usually different from that determined at a higher growth temperature, using temperature-calibrated electrodes, preheated standards, and a pH meter calibrated at that temperature. At acid or alkaline pH values, this difference can be larger than 1 pH unit. To facilitate comparison of published data, it is necessary to know the conditions under which the pH was determined. Thus, Wiegel proposed to indicate the temperature at which the pH measurement was taken and the pH electrode calibrated as a superscript, e.g., $pH^{55°C}$ (36).

Another problem that arises when the optimal, minimal, and maximal pH values for growth of a halophilic alkalithermophile are being determined is the "Na^+ error." In solutions with high Na^+ concentrations, Na^+ can be read as H^+ by the pH electrode; this is even more pronounced at high pH values (>10). This effect can be minimized by the use of a Na^+-less sensitive glass combination electrode, e.g., one made with alkaline high-temperature glass, or an alternative solid-state electrode (for a detailed discussion, see reference 17 and references therein). Finally, when the [Na^+] range and optimum for growth of a halophilic alkalithermophile are being described, it is necessary to define the medium composition used for this determination. It is preferable that this medium be minimal and, provided that they are not required for growth, free of organic compounds (e.g., yeast extract, tryptone, Casamino Acids). These organic compounds can be used by bacteria as sources for synthesis of compatible solutes, which can alter the observed [Na^+] optima, maxima, and minima for growth. Furthermore, it is preferable to use different K^+ concentrations

Published ahead of print 6 April 2012

Address correspondence to Noha M. Mesbah, noha_mesbah@pharm.suez.edu.eg.

doi:10.1128/AEM.00050-12

TABLE 1 Growth conditions of halophilic alkalithermophiles

Species	Source	Na^+ concn (M)		pH ($T_{measured}$ [°C])		Temp (°C)	
		Optimum	Range	Optimum	Range	Optimum	Range
Bacteria							
Natranaerobius thermophilus	Wadi An Natrun, Egypt	3.3–3.9	3.1–4.9	9.5 (55)	8.5–10.6 (55)	53	35–56
Natranaerobius trueperi	Wadi An Natrun, Egypt	3.8	3.1–5.4	9.5 (55)	8.0–10.8 (55)	52	26–55
Natronovirga wadinatrunensis	Wadi An Natrun, Egypt	3.9	3.3–5.3	9.9 (55)	8.5–11.5 (55)	51	26–56
"*Natranaerobius jonesii*"	Lake Magadi, Kenya	3.9	3.1–5.0	10.5 (60)	8.5–11.5 (60)	66	47–71
"*Natranaerobius grantii*"	Lake Magadi, Kenya	4.3	2.9–sat.	9.5 (45)	7.5–10 (45)	46	31–52
Halonatronum saccharophilum	Lake Magadi, Kenya	2.05	0.5–2.9	8.5 (room temp)	7.7–10.3 (room temp)	55	18–60
Dichotomicrobium thermohalophilum	Solar Lake near Eilat (Sinai)	2.4	1.4–3.8	8.5 (room temp)	5.8–9.5	50	20–65
Archaea							
Natronolimnobius aegyptiacus	Wadi An Natrun, Egypt	4.5	2.9–5.5	9.5 (55)	7.3–10.8 (55)	54–57	29–65
Natrialba hulunbeirensis	Soda lake, China	3.4	2.0–5.1	9.0 (room temp)	8.5–10.5	50	20–55

when measuring the Na^+ optima, maxima, and minima for growth, as K^+ can drastically influence the optimal and maximal Na^+ concentrations required for growth.

HALOPHILIC ALKALITHERMOPHILES AND THE PHYLOGENETIC TREE

Halophilic alkalithermophiles are found within both the *Bacteria* and *Archaea* (Table 1) (2, 3, 14, 16). Of the halophilic alkalithermophilic *Bacteria*, the anaerobic halophilic alkalithermophiles fall into the class *Clostridia*, phylum *Firmicutes* (Fig. 1). Among the *Halanaerobiales*, *Halonatronum saccharophilum* is the only halophilic alkalithermophile. The order *Natranaerobiales*, comprised of the genera *Natranaerobius* and *Natronovirga*, contains mainly anaerobic halophilic alkalithermophiles (Fig. 1). The genus *Natranaerobius* consists of three thermophiles, *N. thermophilus*, *N. trueperi*, and the unpublished "*Natranaerobius jonesii*." While not a thermophile, the thermotolerant "*Natranaerobius grantii*" is included in the discussion due to its unique [Na^+] and pH optima for growth, which distinguish it from other extremely halophilic bacteria. The halophilic alkalithermophiles of the *Natranaerobiales*, *N. thermophilus*, *N. trueperi* and *Natronovirga wadinatrunensis*, were isolated from the alkaline, hypersaline lakes of the Wadi An Natrun. Microbial work in the Wadi An Natrun has been extensively reviewed by Oren (22).

The aerobic halophilic alkalithermophile *Dichotomicrobium thermohalophilum* belongs to the alphaproteobacteria. It was isolated from the hypersaline Solar Lake, Sinai, Egypt (4).

Among the *Archaea*, only two species are extremely halophilic, obligately alkaliphilic, and thermophilic: *Natrialba hulunbeirensis* and "*Natronolimnobius aegyptiacus*" (3, 38). Both belong to the order *Halobacteriales*, which is composed of extreme halophiles and halophilic alkaliphiles.

MORPHOLOGICAL FEATURES OF HALOPHILIC ALKALITHERMOPHILES

Cells of *H. saccharophilum* are motile and slender during the exponential growth phase, ranging between 0.4 and 0.6 μm in width and 3.5 and 3.7 μm in length (40). At the late exponential growth phase, the cells curve in a horseshoe manner and lyse. *H. saccharophilum* is peritrichous. Spores are observed at the late exponential growth phase and are located at the cell poles (40). Cells of the *Natranaerobius* spp. as well as of *Nv. wadinatrunensis* are also short and slender, 0.3 to 0.6 μm wide and 2 to 5 μm long, during the exponential growth phase (12, 16). The cells of *N. thermophilus*, *N. trueperi*, and *Nv. wadinatrunensis* all are single, and form chains on entry into stationary phase. No active motility was observed for all three strains under phase-contrast microscopy, and neither flagella nor spores were observed in electron micrographs (12, 16).

D. thermohalophilum is characterized by tetrahedral cell morphology with 1 to 4 hyphae. The hyphae show dichotomous branching and contain poly-beta-hydroxybutyrate granules (4).

GROWTH CHARACTERISTICS AND METABOLISM OF HALOPHILIC ALKALITHERMOPHILES

All halophilic alkalithermophiles (*Bacteria* and *Archaea*) show optimal growth at Na^+ concentrations greater than 2 M, pH values greater than 8.5, and temperatures greater than or equal to 50°C (Table 1). The halophilic alkalithermophiles of the genera *Natranaerobius*, *Natronovirga*, and *Halonatronum* are obligately anaerobic chemoorganotrophs, living by fermentation of sugars. *H. saccharophilum* has a broad substrate utilization spectrum and catabolizes glucose, fructose, sucrose, maltose, starch, glycogen, and *N*-acetyl-D-glucosamine (40). The doubling time of *H. saccharophilum* under optimal growth conditions is 2.5 h. The maximum optical density (OD) (at 600 nm) reached by *H. saccharophilum* in the presence of glycogen was 0.38 at the optimal [Na^+], pH, and temperature for growth (40).

N. thermophilus, the type species of the genus *Natranaerobius*, utilizes cellobiose, fructose, ribose, trehalose, xylose, Casamino Acids, peptone, and trimethylamine as carbon and energy sources, where its maximal optical density for growth is 0.20 to 0.30. Growth of *N. thermophilus* is particularly enhanced in the presence of pyruvate (OD_{600}, 0.30 to 0.4; maximum of 4×10^8 cells/ml) (12). *N. thermophilus* grows with a doubling time of 3.5 h in the presence of 0.2% (wt/vol) pyruvate, 0.5% (wt/vol) yeast extract, 0.5% (wt/vol) tryptone, and 3.5 M Na^+, at $pH^{55°C}$ 9.5 at 53°C. *Nv. wadinatrunensis* similarly catabolizes glucose, fructose, trehalose, mannose, pyruvate, Casamino Acids, acetate, galactose, sucrose, and lactose (16). It grows with a shorter doubling time under optimal conditions (2 h) but reaches smaller cell densities (OD_{600}, 0.2). The anaerobic halophilic alkalithermophiles conserve energy by substrate-level phosphorylation through mixed-acid fermentation pathways with mainly acetate, CO_2, hydrogen,

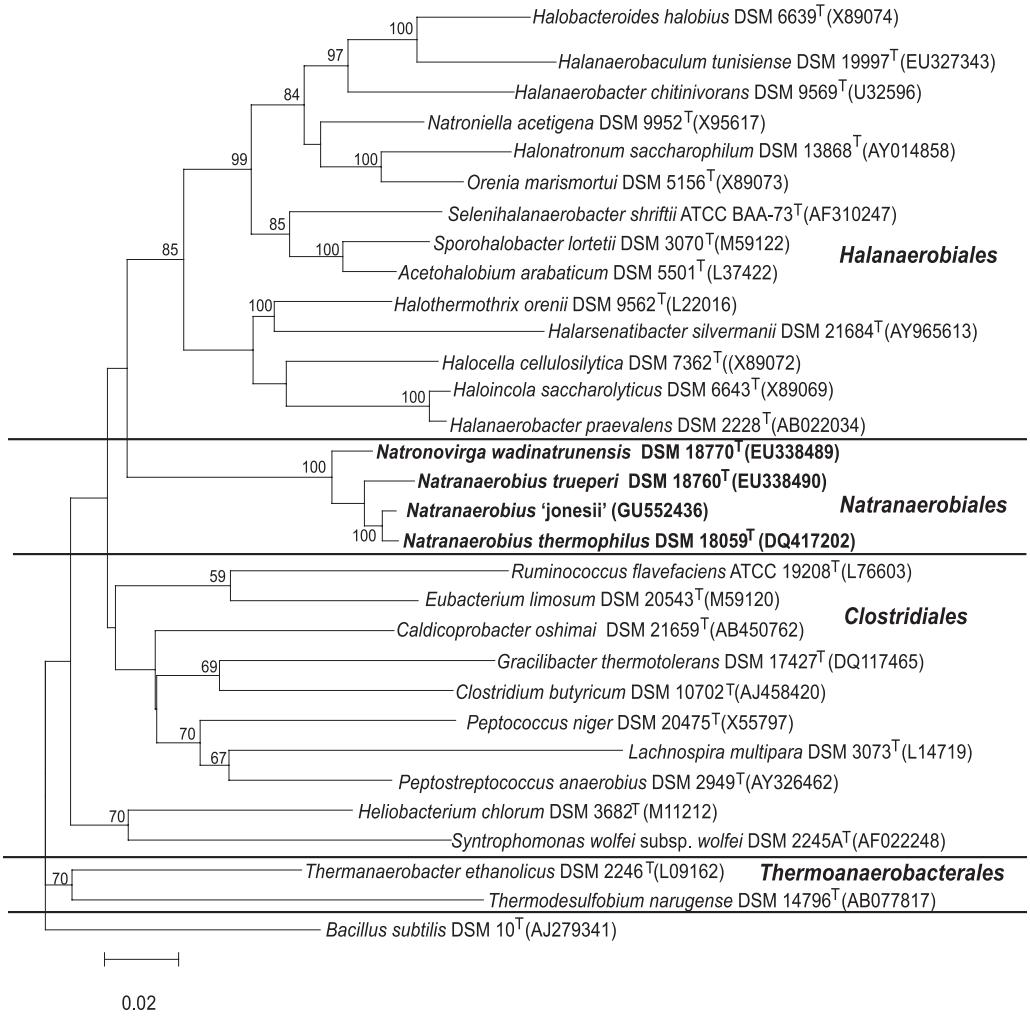


FIG 1 Neighbor-joining tree based on 16S rRNA gene sequences showing the position of the anaerobic halophilic alkalithermophiles in relation to type species of the type genera of families within the class *Clostridia*. GenBank accession numbers for the sequences are in parentheses. The tree was rooted with the 16S rRNA of *Bacillus subtilis* DSM 10^T as the outgroup. Numbers at nodes denote bootstrap values based on 100 replicates; only values greater than 50 are shown. Bar, 2 nucleotide substitutions per 100 nucleotides.

or formate as the main fermentation product (12, 16, 40). All four anaerobic halophilic alkalithermophiles have an obligate requirement for yeast extract and tryptone or Casamino Acids. The anaerobic halophilic alkalithermophiles of the *Natranaerobiales* require at least 0.2% (wt/vol) yeast extract and tryptone each in the growth medium, suggesting that these species are unable to synthesize *de novo* some amino acids and essential vitamins and/or cofactors.

The anaerobic halophilic alkalithermophiles can grow by anaerobic respiration, though they show poor growth in the presence of nonfermentable carbon sources (12, 16, 40). *N. thermophilus* has been shown to reduce fumarate (20 mM), thiosulfate (20 mM), nitrate (20 mM), and iron(III) citrate (20 mM) (12). *N. trueperi* reduces only nitrate, whereas *Nv. wadinatrunensis* reduces both nitrate and manganese oxide (16). All three species grow with extended doubling times (>5 h) and do not reach optical densities greater than 0.15 in the presence of these electron acceptors. In line with this, synthesis of ATP by the Na^+-coupled F-type ATPase of *N. thermophilus* proceeded very slowly in the presence of a membrane potential and a sodium motive force, suggesting that it is probably not used for ATP synthesis (15). *Halonatronum saccharophilum* was able to reduce elemental sulfur to hydrogen sulfide but was unable to reduce sulfate, sulfite, or thiosulfate (40). The poor growth of the isolated halophilic alkalithermophiles using anaerobic respiration suggests that anaerobic respiration might be energetically unrewarding under combined hypersaline, alkaline, and thermal conditions.

Insights from genomic data corroborate the above observations. The genome sequence of *N. thermophilus* contains homologs of enzymes catalyzing a complete glycolytic pathway and a nonoxidative pentose phosphate pathway. The genome sequence also contained a gene for a putative pyruvate-formate lyase. Indeed, acetate and formate were the major fermentation products identified when *N. thermophilus* was grown with sucrose as a carbon source (12). Analysis of the genome sequence of *N. thermophilus* showed that it had homologs for leucine dehydrogenase, asparagine synthase, and cysteine synthase, enzymes involved in the biosynthesis of valine, leucine, isoleucine, alanine, aspartate,

glutamate, asparagine, and cysteine. *N. thermophilus* apparently does not have the genetic ability to synthesize aromatic amino acids. *N. thermophilus* possesses genes encoding enzymes for the biosynthesis of the cofactors riboflavin and thiamine but lacks the genetic ability to synthesize folate. These deficiencies can explain the obligate requirement for a complex growth medium.

The question of how halophilic alkalithermophiles can regenerate cellular NAD^+ in the apparent absence of electron transport arises. Genome analysis of *N. thermophilus* showed homologs for enzymes of a reverse citric acid cycle. This cycle most likely plays the major role in regenerating cellular NAD^+ in *N. thermophilus* in lieu of electron transport (39).

In contrast to the taxa discussed above, *Dichotomicrobium thermohalophilum* is obligately aerobic and, similar to other alphaproteobacteria, is chemoorganotrophic, utilizing yeast extract, acetate, malate, succinate, and amino acids as carbon and nitrogen sources (4). It grows optimally at pH^{RT} 8.5 and 50°C in the presence of 2.4 M Na^+ (4).

The aerobic archaeon "*Natronolimnobius aegyptiacus*," similar to other *Archaea* of the order *Halobacteriales*, is a chemoorganotroph capable of utilizing a wide range of organic compounds, including crotonate and inulin (K. J. Bowers and J. Wiegel, unpublished data). Besides oxygen, only nitrate was observed to serve as an electron acceptor. Similarly, *Natrialba hulunbeirensis* is strictly aerobic and chemoorganotrophic and can also utilize nitrate as an electron acceptor (38).

CHEMOTAXONOMIC CHARACTERISTICS OF HALOPHILIC ALKALITHERMOPHILES

The cell wall is directly exposed to both hypersaline and alkaline environments. Protoplasts of aerobic alkaliphilic *Bacillus* spp. are not stable in alkaline environments, indicating that the cell wall plays a protective role during alkaline stress (5). It was also found that acidic amino acids in the cell wall of *Bacillus halodurans* play a role in supporting growth at alkaline pH (5).

The peptidoglycan hydrolysate of *Nv. wadinatrunensis* was of the type α-4-β and does not contain detectable amounts of isomers of diaminopimelic acid (16). It contains the amino acids aspartic acid, glutamic acid, ornithine, alanine, and glycine. The peptidoglycan hydrolysate from cells of *Natranaerobius* spp. contained the amino acids lysine, alanine, glycine, and glutamic acid with the approximate molar ratio 1:1.5:0.8:1.0 (*N. thermophilus*) and alanine, glycine, serine, aspartate, glutamate, and ornithine (*N. trueperi*). As with *Nv. wadinatrunensis*, no isomers of diaminopimelic acid were detected in hydrolysates from either microorganism. The amount of peptidoglycan isolated from 5 g (dry weight) of cells of *N. thermophilus* was very small and did not allow further studies of the cell wall structure (P. Schuman, personal communication). The absence of peptidoglycan from cell walls of *Natranaerobius* spp. is in contrast to extreme alkaliphilic bacilli, which have been reported to possess cell walls with structures similar to that of neutrophilic *Bacillus* spp. (5). Since in alkaliphilic *Bacillus* spp., the cell wall undoubtedly plays a role in survival under alkaline stress, further studies are necessary to delineate the cell wall structures of the remaining isolated halophilic alkalithermophiles and the hypothetical role of the cell wall in coping with the combination of multiple stressors.

Analysis of the phospholipid fatty acid (PLFA) profile for *N. thermophilus*, *N. trueperi*, and *Nv. wadinatrunensis* showed that the polar and neutral fatty acid compositions were similar, with iso- and, to a lesser extent, anteiso-branched 15:0 fatty acids dominating (Table 2). Unexpectedly, the PLFA profile of *N. thermophilus* did not change significantly in response to either suboptimal pH or alkaline stress ($pH^{50°C}$ 8.5 and 10.5, respectively) or relative hyposalinity and hypersalinity (3.3 and 4.8 M Na^+, respectively). The PLFA profiles of the three halophilic alkalithermophiles do not show the hallmarks of thermophilic bacteria: there are no fatty acids longer than 18 carbons and only small amounts of some branched and unsaturated fatty acids. This could explain why most of the anaerobic halophilic alkalithermophiles are moderate thermophiles, showing no growth at temperatures greater than 60°C (Table 1). An exception to this is "*Natranaerobius jonesii*," which grows optimally at 3.9 M Na^+, $pH^{60°C}$ 10.5, and 66°C; the maximum temperature for growth is 71°C (Table 1). The PLFA profiles for "*N. jonesii*" and the thermotolerant "*N. grantii*" have not been determined. Since membrane composition plays a role in limiting the upper growth temperature for thermophiles, it will be interesting to compare the PLFA profile of "*N. jonesii*" with profiles of the other, relatively less thermophilic haloalkaliphiles.

Polyamines, low-molecular-weight aliphatic polycations, are found in the cells of all living organisms and are often used for chemotaxonomic characterization of microorganisms. The most common polyamines in microorganisms are putrescine (1,4-diaminobutane), spermine, and spermidine. Polyamines are positively charged, thus they bind to macromolecules such as DNA, RNA and proteins. Polyamines are involved in numerous processes and are reported to play critical roles in the stabilization of nucleic acids and proteins during exposure to extremes of temperature, either hot or cold (29). Polyamines stabilize DNA and RNA in cells of thermophiles; the presence of the polyamine tetrakis(3-aminopropyl)ammonium and spermidine is critical for protein biosynthesis in *Thermus thermophilus* near the optimal growth temperature of 65°C (30, 32).

Analysis of the polyamine contents of *N. thermophilus*, "*N. grantii*," and "*N. jonesii*" showed the presence of spermine, spermidine, and putrescine, in addition to two unidentified polyamines (Table 3). One of the unidentified polyamines had an elution time that coincides with that of tetrakis(3-aminopropyl)ammonium, a branched polyamine found in hyperthermophiles (30). Interestingly, "*N. jonesii*," with the highest growth temperature optimum and maximum, had the highest content of this particular polyamine, suggesting that it could play a role in thermal adaptation in this particular microorganism. Analysis of the changes in intracellular concentrations of polyamines in response to changing temperature and genetic analyses are needed to determine the precise role each polyamine plays in the adaption of the halophilic alkalithermophiles to high temperature in the presence of high salinity and alkaline pH.

INTRACELLULAR ION CONTENT OF THE HALOPHILIC ALKALITHERMOPHILES

Intracellular Na^+ and K^+ concentrations have been measured in cells of the type species of the genus *Natranaerobius*, *N. thermophilus*, and in both "*N. jonesii*" and "*N. grantii*." All three species maintained an intracellular [K^+] between 200 and 300 mM (extracellular [K^+] ~ 8 mM). This level was maintained even at an extracellular K^+ concentration of 400 mM. Furthermore, when measured in exponentially growing cells, this intracellular concentration did not change significantly in response to alkaline (close to pH_{max}) or more neutral pH (close to pH_{min}), hyper- or

TABLE 2 Membrane lipid composition of *N. thermophilus*, *N. trueperi*, and *Nv. wadinatrunensis*[a]

Lipid[b]	% in:		
	N. thermophilus	*N. trueperi*	*Nv. wadinatrunensis*
Polar lipids			
Terminally branched saturated fatty acids			
il14:0	0.4	1.1	0.5
i15:0	77.2	81.1	80.4
a15:0	11.3	12.2	9.4
i16;0	0.9	0.5	0.6
i17:0	1.8	0.5	0.9
a17:0	0.9	0.2	0.3
Monoenoic fatty acids			
16:1ω7c	0.7	0.3	0.7
18:1ω9c	0.3	0.0	0.0
18:1ω7c	0.3	0.0	0.0
Branched monoenoic fatty acids			
i17:1ω7c	1.2	0.8	2.2
Normal saturated fatty acids			
14:0	2.4	2.5	2.6
15:0	0.3	0.1	0.6
16:0	2.1	0.6	1.4
17:0	0.1	0.0	0.0
18:0	0.4	0.1	0.4
21:0	0.0	0.0	0.0
22:0	0.0	0.0	0.0
Polyenoic fatty acids			
18:2ω6	0.0	0.0	0.0
Neutral lipids			
Terminally branched saturated fatty acids			
il14:0	0.0	0.0	0.0
i15:0	54.4	76.3	68.1
a15:0	11.5	10.0	7.5
i16;0	1.8	1.0	0.0
i17:0	5.9	2.3	5.3
a17:0	2.4	0.6	0.0
Monoenoic fatty acids			
16:1ω7c	2.7	1.1	0.0
18:1ω9c	3.0	0.6	0.0
18:1ω7c	1.7	0.0	0.0
Branched monoenoic fatty acids			
i17:1ω7c	2.4	2.8	9.6
Normal saturated fatty acids			
14:0	2.2	2.5	0.0
15:0	0.0	0.0	0.0
16:0	7.3	2.2	7.1
17:0	0.0	0.0	0.0
18:0	4.8	0.6	2.4
21:0	0.0	0.0	0.0
22:0	0.0	0.0	0.0
Polyenoic fatty acids			
18:2ω6	0.0	0.0	0.0

[a] All cultures were grown at 52°C and $pH^{55°C}$ 9.5 in the presence of 3.9 M Na^+.
[b] Numbers denote the percentages of total polar and neutral lipids, respectively.

TABLE 3 Polyamines of anaerobic halophilic alkalithermophiles

Polyamine	Intracellular concn (nmol/mg [dry wt])		
	Natranaerobius thermophilus	"*Natranaerobius grantii*"	"*Natranaerobius jonesii*"
Putrescine	22.8	81.0	68.2
Spermidine	45.5	87.7	153.7
Spermine	59.0	31.2	43.9
N^1-Aminopropylagmatine[a]	22.8	12.6	153.7
Unknown	7.9	53.8	22.0

[a] Further structural analyses are needed for confirmation.

hyposalinity (2a, 11). These observations suggest a strong regulation of the intracellular K^+ concentration to maintain a K^+-homeostasis.

Intracellular [Na^+] in all three species ranged between 6 and 10 mM and did not change significantly in response to changing salinity (3.1 to 4.5 M Na^+). However, intracellular [Na^+] in *N. thermophilus* changed during alkaline stress: at $pH^{50°C}$ 10.3 (close to the pH_{max} for growth), intracellular Na^+ increased to 30 mM, indicating weakening of the stress-coping mechanisms.

All three *Natranaerobius* species have an obligate requirement for chloride in their growth medium. Interestingly, the species with the highest temperature optimum, "*N. jonesii*," requires the greatest amount of Cl^- in its growth medium (1.4 M), with no growth occurring below 1.2 M Cl^-, whereas "*N. grantii*," the thermotolerant species with the lowest growth temperature optimum, requires only 0.2 M Cl^- for optimal growth, with no growth at 0.1 M Cl^- or below. The moderate thermophile *N. thermophilus* requires an intermediate concentration of Cl^- for growth, showing optimal growth at 1.2 M Cl^-, with no growth at or below 1.0 M Cl^- (Bowers and Wiegel, unpublished). The exact relationship between intracellular chloride accumulation and growth salinity is yet to be discerned. Roeßler and Müller reported on the chloride dependence of the moderate halophile *Halobacillus halophilus* (26). A chloride regulon regulates the transcription as well as activation of a number of genes and proteins, some of which play roles in osmoadaptation (25). An example of upregulated genes includes that for glutamine synthetase, which is responsible for the synthesis of glutamine and glutamate. Glutamine and glutamate are the major compatible solutes utilized at intermediate salinities (1.0 to 1.5 M NaCl). In *H. halophilus*, induction of this enzyme as well as its enzymatic activity is strictly dependent on Cl^- (28). It was proposed that *H. halophilus* senses the salt concentration of its environment through sensing the chloride concentration (27).

Halophiles must maintain their cytoplasm at least isosmotic with their surrounding medium (21). Two different strategies are used by halophiles to balance their cytoplasm osmotically with their surrounding medium. The first mechanism, the "salt-in" strategy, involves accumulation of molar concentrations of potassium and chloride, so that the cytoplasm becomes isosmotic with the extracellular medium. This method is commonly used by the most extreme halophiles, with [Na^+] optima greater than 2.5 M. The extremely halophilic *Archaea* of the *Halobacteriales* accumulate up to 2 M potassium in their cytoplasm (21). In contrast to Na^+, a high concentration of intracellular K^+ is not toxic to these cells. The second strategy, the "compatible solute" strategy, involves biosynthesis and/or accumulation of low-molecular-weight organic osmotic solutes in the cytoplasm (21, 24).

During the symposium "General and Applied Aspects of Halophilic Microorganisms," held in Alicante in 1989 (31), H. G. Trüper summarized in four postulates what was known at the time about the presence, distribution, and biosynthesis of organic compatible solutes. The second postulate stated, "Archaeobacteria and anaerobic fermenting eubacteria are incapable of synthesizing organic compatible solutes." To our knowledge, no cases of anaerobic fermenting halophilic bacteria that produce organic compatible solutes have been previously reported in the literature. High concentrations of Na^+, K^+, and Cl^- (3.0 to 4.5 M K^+, 2.3 to 3.6 M Cl^-, and 0.8 to 1.6 M Na^+) have been measured inside the cells of *Halanaerobium praevalens*, *Halanaerobium acetethylicum*, and *Halobacteroides halobius*. These concentrations are high enough to be at least isosmotic with the extracellular medium (20). However, in the anaerobic extremely halophilic alkalithermophiles, even at their highest measured concentrations, 293 mM K^+ and 1.2 M Cl in *N. thermophilus* are not sufficient to osmotically balance the extracellular Na^+ concentration of 3.3 M. It follows that the anaerobic halophilic alkalithermophiles must use an additional strategy or strategies to adapt to high salinity.

Analysis of the complete genome sequence of *N. thermophilus* showed that it contains genes encoding glycine sarcosine methyltransferase and sarcosine dimethylglycine methyltransferase (both catalyzing synthesis *de novo* of the solute glycine betaine), 15 genes encoding glycine betaine ABC transporters, four genes encoding glycine betaine/L-proline ABC transporters, and three genes encoding betaine/carnitine/choline transporters (39). Physiological assays showed that when this organism is grown at 52°C and $pH^{55°C}$ 9.5, the intracellular concentration of glycine betaine increases more than 2-fold, from 400 mM at 3.3 M Na^+ to 1 M at 4.5 M Na^+ (B. Zhao and J. Wiegel, unpublished data). Intracellular concentration of the amino acid glutamate (the only amino acid present in millimolar amounts in the cells) also increased greatly, from 19 mM in the presence of 3 M Na^+ to 200 mM at 4.5 M Na^+. The intracellular concentration of glycine betaine was affected only by changes in the extracellular Na^+ concentration, not by alterations in either temperature or extracellular pH. On the other hand, intracellular concentration of glutamate increased almost 3-fold in response to an increase in temperature. These data indicate that the accumulation of the amino acid plays roles in the adaptation to both high salinity and temperature. Furthermore, they indicate that the anaerobic extremely halophilic alkalithermophile *N. thermophilus* utilizes combinations of both the "salt-in" and "compatible-solute" strategies for osmotic adaptation. It is possible that for the halophilic alkalithermophiles, alkaline pH and high temperature make the accumulation of high molar concentrations of intracellular ions, particularly K^+ and Cl^-, difficult. Maintenance of ion gradients necessitates an impermeable cell membrane. It is known that permeability of the cell membrane to ions increases with both increasing temperature and pH (7, 33). The accumulation of ions inside the cytoplasm is the result of the cooperative action of different ion pumps, antiporters, and transport proteins which rely on active transport and/or the transmembrane electrochemical potential.

That being said, data on intracellular pH, presence, and concentration of intracellular compatible solutes and intracellular ion concentrations of other halophilic alkalithermophiles are not available at this time. Thus, this point should be revisited in the future when more of these data become available. It is evident, though, that the presence of molar concentrations of glycine be-

taine in cells of the anaerobe *N. thermophilus* necessitates a modification of Trüper's second postulate to allow for at least a few fermentative extremely halophilic bacteria producing compatible solutes.

DESICCATION, RADIATION, AND UV RESISTANCE OF THE HALOPHILIC ALKALITHERMOPHILES

Prokaryotes adapt to desiccation using different mechanisms, including sporulation (18), production of extracellular polysaccharides (23), and increasing intracellular solute concentrations to equilibrate the cytoplasm with hypertonic surroundings (34). *N. thermophilus* and related species are exposed to periods of intense solar radiation and desiccation in their natural habitats due to scarce precipitation and arid climates. Spores have not been observed in any of the anaerobic halophilic alkalithermophiles of the *Natranaerobiales*, either on entry into stationary phase, in response to heat treatment, or in response to variations in growth medium. The three species of the genus *Natranaerobius* and *Nv. wadinatrunensis* are extremely sensitive to desiccation; of about 5×10^9 cells, none were culturable after 1 h of anaerobic desiccation over $CaCl_2$ (2a). Furthermore, cultures of *N. thermophilus* exposed to 1 kGy of gamma radiation had a survival rate of only 0.01%, and cultures exposed to 3 kGy gamma radiation were no longer viable (2a). The resistance of *N. thermophilus* to gamma radiation was only slightly higher than that of *Escherichia coli*.

In contrast to what has been observed for radiation and desiccation sensitivity, both *N. thermophilus* and *Nv. wadinatrunensis* show remarkable resistance when exposed to the three wavelengths of UV A, B, and C radiation (385, 312, and 254 nm, resulting in irradiance levels of 0.35, 0.38, and 0.645 mW cm^{-2}, respectively). Both organisms showed between 40 and 80% survival after 28 h of exposure (J. Blamey, personal communication). Under the same test conditions, *E. coli* showed no survival after 2 h of exposure. The presence of several putative DNA repair systems in the genome of *N. thermophilus* could explain its resistance to UV radiation. Analysis of the genome sequence of *Natranaerobius thermophilus* showed that it possesses homologs for the *recFOR*, *radA*, and *radC* genes, in addition to homologs for DNA mismatch repair proteins MutS and MutN. Functional genetic analyses are needed to confirm the role of each gene in the UV resistance of *N. thermophilus*.

Induction of DNA double-strand breaks and formation of artifacts in DNA, such as dimers and modified bases, have been observed in microorganisms exposed to desiccating conditions and gamma radiation (8). Efficient DNA repair upon rehydration is critical for maintaining DNA fidelity in other desiccation-resistant species, such as *Halobacterium* NRC1 and *Deinococcus radiodurans* (8, 37). It is hypothesized that adaptation to desiccation with the resulting DNA damage is the source of resistance to gamma radiation, since cells are not naturally exposed to ionizing radiation (10). Thus, it would be logical that *N. thermophilus* is susceptible to both desiccation and ionizing radiation. However, a recent study concluded that there is not a direct correlation between desiccation tolerance and tolerance to radiation among hyperthermophilic archaea (1). It is possible that desiccation or a decrease in water activity in *N. thermophilus* results in irreversible destabilization and denaturation of intracellular proteins. Consistent with its being a halophile, the proteome of *N. thermophilus* is acidic, with an isoelectric point between 4 and 5. Intracellular proteins thus must be stabilized by polar and ionic interactions, which are disrupted by the absence of water. Further, it is possible that desiccation causes irreversible alkalinization of the cytoplasm, which results in inactivation of critical enzymatic machinery. Despite the presence of genes encoding putative proteins necessary for homologous recombination in the genome of *N. thermophilus*, it appears that, even if they are upregulated in response to desiccation or ionic radiation, DNA repair is not sufficient to rescue *N. thermophilus* from their damaging effects.

CONCLUSIONS

Halophilic alkalithermophiles are an unusual group of extremophiles, termed polyextremophiles, which are capable of robust growth under the combined extremes of high salinity, alkaline pH, and elevated temperature. The anaerobic strains isolated thus far all belong to two novel genera within the novel order *Natranaerobiales*. These halophilic alkalithermophiles display a number of unique physiological characteristics. They show limited metabolic diversity; the majority of the isolated polyextremophiles are chemoorganotrophs and show poor growth in the presence of nonfermentable carbon sources. In contrast to what has been presumed for anaerobic halophiles, the anaerobic, fermenting halophilic alkalithermophiles adapt to osmotic stress by storing both compatible solutes and potassium ions in their cytoplasm. The anaerobic halophilic alkalithermophiles have unusual cell wall structure, presumably contributing to the stabilization of the membranes and thus to the necessary cytoplasm acidification and maintenance of ion gradients across the membrane. The anaerobic halophilic alkalithermophiles do not show unusual phospholipid fatty acid profiles, indicating that the combined extremes of high salt, alkaline pH, and elevated temperature do not significantly influence the membrane lipid composition. It is anticipated that the currently known halophilic alkalithermophiles are only a small example of polyextremophiles existent in nature and that further study of extreme environments will extend the known limits of combined high $[Na^+]$, elevated temperature, and alkaline pH that support life. It is hoped that this minireview on these interesting microorganisms will entice others to investigate polyextremophiles further.

ACKNOWLEDGMENTS

We thank Jenny Blamey for assistance with the UV resistance experiment, Baisuo Zhao for providing data on compatible solute accumulation, and Tairo Oshima for performing polyamine analysis. Karen Bowers performed experiments on the desiccation and, together with Michael Daly, experiments on the gamma radiation resistance of *N. thermophilus*.

The research presented herein was supported by grants MCB-060224 and INT-021100 from the National Science Foundation and AFOSR 033835-01 from the Air Force Office of Scientific Research to Juergen Wiegel.

REFERENCES

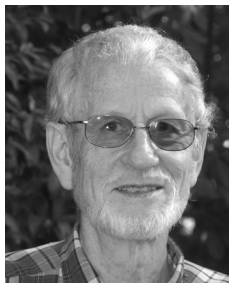
1. **Beblo K, et al.** 2011. Survival of thermophilic and hyperthermophilic microorganisms after exposure to UV-C, ionizing radiation and desiccation. Arch. Microbiol. **193**:797–809.
2. **Bowers KJ, Mesbah NM, Wiegel J.** 2009. Biodiversity of polyextremophilic bacteria: does combining the extremes of high salt, alkaline pH and elevated temperature approach a physico-chemical boundary for life? Saline Systems **5**:9.

2a. **Bowers KJ.** 2010. M.S. thesis. University of Georgia, Athens, GA.

3. **Bowers KJ, Wiegel J.** 2011. Temperature and pH optima of extremely halophilic *Archaea*: a mini-review. Extremophiles **15**:119–128.
4. **Hirsch P, Hoffman B.** 1989. *Dichotomicrobium thermohalophilum* gen.

nov., spec. nov., budding prosthecate bacteria from the Solar Lake (Sinai) and some related strains. Syst. Appl. Microbiol. **11**:291–301.
5. **Horikoshi K.** 2011. General physiology of alkaliphiles, p 99–118. *In* Horikoshi K, Antranikian G, Bull AT, Robb FT, Stetter KO (ed), Extremophiles handbook. Springer, Tokyo, Japan.
6. **Kevbrin VV, Romanek CS, Wiegel J.** 2004. Alkalithermophiles: a double challenge from extreme environments, p 395–412. *In* Seckbach J (ed), Origins. Kluwer Academic Publishers, Dordrecht, the Netherlands.
7. **Konings WN, Albers S-V, Koning S, Driessen AJM.** 2002. The cell membrane plays a crucial role in survival of bacteria and archaea in extreme environments. Antonie Van Leeuwenhoek **81**:61–72.
8. **Kottemann M, Kish A, Ilooamusi C, Bjork S, DiRuggiero J.** 2005. Physiological responses of the halophilic archaeon *Halobacterium* sp. strain NCR1 to desiccation and gamma radiation. Extremophiles **9**:219–227.
9. **Kushner DJ.** 1978. Life in high salt and solute concentrations, p 317–368. *In* Kushner DJ (ed), Microbial life in extreme environments. Academic Press, London, United Kingdom.
10. **Mattimore V, Battista JR.** 1996. Radioresistance of *Deinococcus radiodurans*: functions necessary to survive ionizing radiation are also necessary to survive prolonged desiccation. J. Bacteriol. **178**:633–637.
11. **Mesbah NM, Cook GM, Wiegel J.** 2009. The halophilic alkalithermophile *Natranaerobius thermophilus* adapts to multiple environmental extremes using a large repertoire of $Na^+(K^+)/H^+$ antiporters. Mol. Microbiol. **74**:270–281.
12. **Mesbah NM, Hedrick DB, Peacock AD, Rohde M, Wiegel J.** 2007. *Natranaerobius thermophilus* gen. nov. sp. nov., a halophilic, alkalithermophilic bacterium from soda lakes of the Wadi An Natrun, Egypt, and proposal of *Natranaerobiaceae* fam. nov. and *Natranaerobiales* ord. nov. Int. J. Syst. Evol. Microbiol. **57**:2507–2512.
13. **Mesbah NM, Wiegel J.** 2006. Isolation, cultivation and characterization of alkalithermophiles. Methods Microbiol. **35**:451–468.
14. **Mesbah NM, Wiegel J.** 2008. Life at extreme limits: the anaerobic halophilic alkalithermophiles. Ann. N. Y. Acad. Sci. **1125**:44–57.
15. **Mesbah NM, Wiegel J.** 2011. The Na^+-translocating F_1F_o-ATPase from the halophilic, alkalithermophile *Natranaerobius thermophilus.* Biochim. Biophys. Acta **1807**:1133–1142.
16. **Mesbah NM, Wiegel J.** 2009. *Natronovirga wadinatrunensis* gen. nov. sp. nov. and *Natranaerobius trueperi* sp. nov., two halophilic alkalithermophilic microorganisms from soda lakes of the Wadi An Natrun, Egypt. Int. J. Syst. Evol. Microbiol. **59**:2042–2048.
17. **Mesmer RE.** 1991. Comments on A new approach to measuring pH in brines and other concentrated electrolytes by K. G. Knauss, T. J. Wolery, K. J. Jackson. Geochim. Cosmochim. Acta **55**:1175–1176.
18. **Nicholson WL, Munakata N, Horneck G, Melosh HJ, Setlow P.** 2000. Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. Microbiol. Mol. Biol. Rev. **64**:548–572.
19. **Oren A.** 2006. Life at high salt concentrations, p 263–282. *In* Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebrandt E (ed), The prokaryotes: a handbook on the biology of bacteria: ecophysiology and biochemistry, 3rd ed, vol 2. Springer, New York, NY.
20. **Oren A.** 2000. Life at high salt concentrations. *In* Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebrandt E (ed), The prokaryotes: an evolving electronic resource for the microbiological community, 3rd ed, release 3.1. Springer-Verlag, New York, NY. [Online.]
21. **Oren A.** 2008. Microbial life at high salt concentrations: phylogenetic and metabolic diversity. Saline Systems **4**:2.
22. **Oren A.** 2012. Two centuries of microbiological research in the Wadi Natrun, Egypt: a model system for the study of the ecology, physiology, and taxonomy of haloalkaliphilic microorganisms. *In* Seckbach J, Oren A, Stan-Lotter H (ed), Polyextremophiles—organisms living under multiple stress, in press. Springer, Dordrecht, the Netherlands.
23. **Potts M.** 1994. Desiccation tolerance of prokaryotes. Microbiol. Rev. **58**:755–805.
24. **Roberts MF.** 2005. Organic compatible solutes of halotolerant and halophilic microorganisms. Saline Systems **1**:5.
25. **Roeßler M, Müller V.** 2002. Chloride, a new environmental signal molecule involved in gene regulation in a moderately halophilic bacterium. J. Bacteriol. **184**:6207–6215.
26. **Roeßler M, Müller V.** 1998. Quantitative and physiological analyses of chloride dependence of growth of *Halobacillus halophilus.* Appl. Environ. Microbiol. **64**:3813–3817.
27. **Saum SH, Müller V.** 2008. Regulation of osmoadaptation in the moderate halophile *Halobacillus halophilus*: chloride, glutamate and switching osmolyte strategies. Saline Systems **4**:4.
28. **Saum SH, et al.** 2006. Biochemical and molecular characterization of the biosynthesis of glutamine and glutamate, two major compatible solutes in the moderately halophilic bacterium *Halobacillus halophilus.* J. Bacteriol. **188**:6808–6815.
29. **Tabor CW, Tabor H.** 1985. Polyamines in microorganisms. Microbiol. Rev. **49**:81–99.
30. **Terui Y, Otnuma M, Hiraga K, Kawashima E, Oshima T.** 2005. Stabilization of nucleic acids by unusual polyamines produced by an extreme thermophile, *Thermus thermophilus.* Biochem. J. **388**:427–433.
31. **Trüper HG, Severin J, Wolhfarth A, Müller E, Galinski EA.** 1991. Halophily, taxonomy, phylogeny and nomenclature, p 3–7. *In* Rodriguez-Valera F (ed), General and applied aspects of halophilism. Plenum Press, New York, NY.
32. **Uzawa T, Hamasaki N, Oshima T.** 1993. Effects of novel polyamines on cell-free polypeptide synthesis catalyzed by *Thermus thermophilus* HB8 extract. J. Biochem. **114**:478–486.
33. **van de Vossenberg JL, Driessen AJ, Grant WD, Konings WN.** 1999. Lipid membranes from halophilic and alkali-halophilic *Archaea* have a low H^+ and Na^+ permeability at high salt concentration. Extremophiles **3**:253–257.
34. **Welsh DT.** 2000. Ecological significance of compatible solute accumulation by microorganisms: from single cells to global climate. FEMS Microbiol. Rev. **24**:263–290.
35. **Wiegel J.** 2011. Anaerobic alkaliphiles and alkaliphilic poly-extremophiles, p 81–97. *In* Horikoshi K, Antranikian G, Bull AT, Robb FT, Stetter KO (ed), Extremophiles handbook. Springer, Tokyo, Japan.
36. **Wiegel J.** 1998. Anaerobic alkalithermophiles, a novel group of extremophiles. Extremophiles **2**:257–267.
37. **Xu G, et al.** 2008. RecO is essential for DNA damage repair in *Deinococcus radiodurans* J. Bacteriol. **190**:2624–2628.
38. **Xu Y, et al.** 2001. *Natrialba hulunbeirensis* sp. nov. and *Natrialba chahannaoensis* sp. nov., novel haloalkaliphilic archaea from soda lakes in Inner Mongolia Autonomous Region, China. Int. J. Syst. Evol. Microbiol. **51**:1693–1698.
39. **Zhao B, et al.** 2011. Complete genome sequence of the anaerobic, halophilic, alkalithermophile *Natranaerobius thermophilus* JW/NM-WN-LF^T. J. Bacteriol. **193**:4023–4024.
40. **Zhilina TN, Garnova ES, Tourova TP, Kostrikina NA, Zavarzin GA.** 2004. *Halonatronum saccharophilum* gen. nov. sp. nov.: a new haloalkaliphilic bacterium of the order *Halanaerobiales* from Lake Magadi. Microbiology **70**:64–72.

Noha M. Mesbah is an assistant professor of biochemistry at the Faculty of Pharmacy, Suez Canal University, in Ismailia, Egypt. She obtained her bachelor's degree in pharmacy from Suez Canal University (2002) and her Ph.D. from the University of Georgia (2008) in Juergen Wiegel's lab, studying the biochemical characteristics and physiology of the polyextremophilic halophilic alkalithermophiles. While at the University of Georgia, she isolated and validly described the novel halophilic, alkaliphilic, and thermophilic order *Natranaerobiales*. She was a postdoctoral research associate in the laboratory of Juergen Wiegel (2008 to 2009), where she studied the biochemical characteristics of the F-type ATPase from the halophilic alkalithermophile *Natranaerobius thermophilus*. She began her academic career at Suez Canal University at the end of 2009; her current research pursuits involve the purification and characterization of salt-, alkali-, and temperature-stable enzymes with potential industrial uses.



Juergen Wiegel, Distinguished Research Professor, emeritus, at the Departments of Microbiology and of Biochemistry & Molecular Biochemistry at the University of Georgia, received his M.S. (Dipl. Chem.) in organic chemistry and his Ph.D. (Dr. Rer. Nat.) and Habilitation (Dr. Sci.) from the Microbiology Department of the University Göttingen. While in Göttingen he described the novel nitrogen-fixing chemolithoautotrophic genus *Xanthobacter*. At the University of Georgia, working as a postdoc with L. G. Ljungdahl, he isolated the ethanolic *Thermoanaerobacter ethanolicus* JW200. For the last 35 years he has worked on various aspects of anaerobic thermophiles, including (i) describing novel taxa and their ecology, biodiversity, physiology, and biochemistry, (ii) developing a rudimentary genetic system, and (iii) exploring potential industrial applications. The latter includes the use of enzymes from thermophiles degrading biopolymers (cellulose, hemicellulose, and lipids). A second work area dealt with the anaerobic degradation of halogenated aromatic compounds, including polychlorobiphenyls (PCBs), and the cofactor-independent carboxylation of phenol.