REVIEW

Juergen Wiegel

Anaerobic alkalithermophiles, a novel group of extremophiles

Abstract Although some anaerobic and aerobic meso-philes have long been known to grow at alkaline pH (above 9.5), little was known until recently about thermophilic alkaliphiles, termed now alkalithermophiles. This minireview describes presently known and recently validly described anaerobic alkalithermophilic bacteria ($pH_{opt}^{55C} > 8.5$; $T_{opt} > 55°C$) and alkalitolerant thermophiles ($pH_{opt}^{55C} < 8.5$ but pH_{max}^{55C} above 9.0). Some of these are widely distributed, but others have been isolated (thus far) only from one specific location. This novel group of anaerobic bacteria is comprised of physiologically different genera and species which, so far, all belong to the Gram-type positive *Bacillus-Clostridium* phylogenetic subbranch. An interesting feature of these anaerobic alkalithermophiles is that most of the isolates have short doubling times. The fastest growing among them are strains of *Thermobrachium celere*, with doubling times as short as 10 min while growing above pH 9.0 and above 55°C.

Key words Alkaliphil* · Anaerob* · Thermophil* · *Clostridium paradoxum* · *Clostridium thermoalkaliphilum* · *Thermobrachium celere* · *Anaerobranca horikoshii* · *Thermosyntropha lipolytica* · *Caloramator* · pH stasis · Internal pH · Isolation · Phylogeny · Biodiversity

Introduction

This review focuses on a novel group of thermophilic anaerobic bacteria, called alkalithermophiles (or thermoalkaliphiles). Recently, the first thermophilic anaerobic bacteria able to grow at alkaline pH values were described, thus extending the known conditions under which microorganisms demonstrate optimal growth (Li et al. 1993, 1994). Subsequently, a few more anaerobic alkaliphilic and alkalitolerant bacteria have been isolated from various sources. These bacteria are compared with respect to their basic properties and their distribution. Additionally, preliminary results of studies on the internal pH and energy metabolism are discussed.

Assuming that we know less than 5% of the existing microorganisms, one can expect that the presently observed boundaries for growth of microorganisms – and thus boundaries for life – will be further extended in the future when additional microorganisms are found in other extreme environments. This minireview is just a snapshot of the beginning of the ongoing discovery of microorganisms belonging to the group of anaerobic alkalithermophiles.

Temperature and pH boundaries for growth of microorganisms

Temperature

Microorganisms can grow in a wide range of temperatures (Wiegel 1990). Temperatures around 10°–40°C (mesobiotic) and pH values about 6.0–8.0 (i.e., near neutral) are regarded as normal conditions, that is, from the human point of view and from growth conditions of *Escherichia coli*. All other conditions are regarded as extreme, an obviously artificial classification (Table 1). In recent years, various extremophilic microorganisms including alkaliphiles and extreme- and hyperthermophiles have attracted considerable attention (e.g., Van der Oost et al. 1996; Canganellat Wiegel 1993; Antranikian 1996). The interest in these microorganism is attributable to their ability to grow beyond the formerly assumed boundaries for life, as well as the use (or potential) use of their enzymes in biotechnological applications. The taq polymerase from *Thermus aquaticus* is the best known example of a successful application of an enzyme from a thermophile.

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**Table 1.** Definitions of alkaliphiles and thermophiles

Thermophiles	T_{min} –	$T_{opt} > 50°C$	$T_{max} > 60°C$
Extreme thermophiles	T_{min} usually $> 35°C$	$T_{opt} \geq 65°C$	$T_{max} > 70°C$
Hyperthermophiles	T_{min} usually $> 60°C$	$T_{opt} > 80°C$	$T_{max} > 85°C$
Alkalitolerant organisms	pH_{min} [a]	$pH_{opt} < 8.5$ [a]	$pH_{max} \geq 9.0$ [a]
Alkaliphiles		$pH_{opt} \geq 8.5$ [a]	$pH_{max} \geq 10.0$ [a]
Facultative	$pH_{min} < 8.0$ [a]		
Obligate	$pH_{min} > 8.0$ [a]		

[a] The values depend on the temperature at which the pH was determined and the pH meter was calibrated. Depending on the medium, temperature, and pH range, the differences can be as great as 1 pH unit (see text and Fig. 2). Because these conditions are frequently not stated, the presented data from the literature are difficult to compare.

Presently, the widely accepted temperature boundaries for observed growth for microorganisms are about a low of $-12°C$ for some fungi and a high of 113°C for the anaerobic archaean *Pyrococcus furiosus* and similar Archaea (Stetter 1996). Whereas, the majority of known and isolated bacteria grow best in the mesobiotic range, with an optimal temperature range for growth (T_{opt}) above 20° and below 45°C, many extremophiles (especially in respect to temperature) have been isolated during the last 15 years. Included are thermophilic bacteria with T_{opt} in the range between 50° and 75°C and extreme thermophilic and hyperthermophilic bacteria and archaea with T_{opt} above 75° and up to 105°C and T_{max} about and above 100°C (Wiegel 1992; Stetter 1996). Although most microorganisms grow only within a relatively small temperature span (the difference between T_{min} and T_{max}) of 20°–35°C, a few bacteria have been described as being able to grow within much wider spans, of more than 35°C. So far, the microorganism with the most extended temperature span, of about 55°C (from 22°C to 78°C), is the archeaon *Methanobacterium thermoautotrophicum* (Wiegel 1990, 1998).

pH

There is an inherent problem with comparing published growth pH data for thermophiles. Many investigators do not specify how the given pH values were determined. Specifically, the values could have been determined by measuring at room temperatures or at elevated temperatures with or without using a temperature probe and using a pH meter calibrated at room temperature or, the correct method, measuring the pH at the elevated temperatures using temperature-equilibrated electrodes and pH meters calibrated at the same temperature. For neutral pH values the error for differently determined values is frequently relatively small (usually less than 0.3 pH units). However, at acidic or alkaline pH values the error can be larger than 1 pH unit (Fig. 1). To facilitate comparison of published data, one needs to know under which conditions the pH was determined. Thus, we have suggested that in the future when extremophiles are described, the temperature (or other important parameters) at which the pH values were determined and at which the pH meter was calibrated are indicated as a superscript, e.g., pH^{60C}.
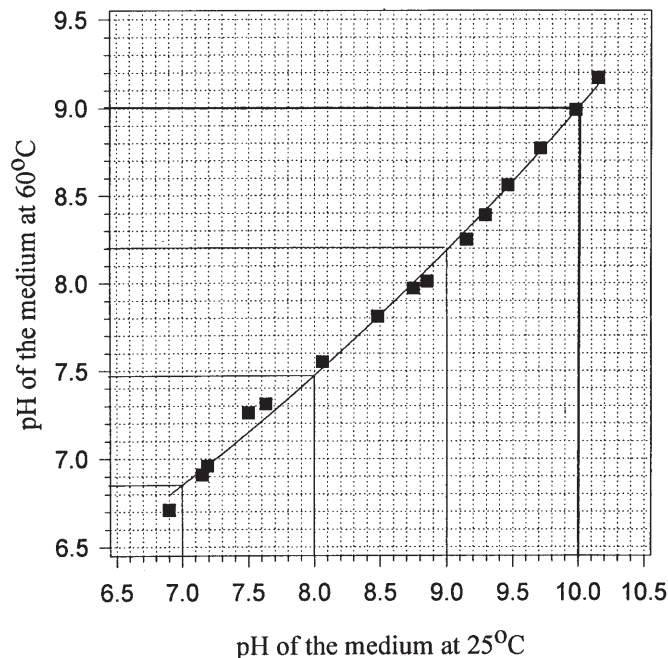
Fig. 1. Relationship of pH values measured at 25°C and various growth temperature using the medium used for *Thermosyntropha lipolytica* (without the olive oil). The pH was first determined at 25°C using the pH meter (containing a temperature probe) calibrated at 25°C, and then at 60°C with the pH meter calibrated at 60°C after the electrode equilibrated at the new temperature for at least 30 min

The pH ranges at which presently isolated microorganisms can grow range from slightly below pH 0 (aerobic archaea) to around pH 12 (aerobic mesophilic bacteria, yeast). Most of the known bacteria and anaerobic archaea grow optimally at about neutral pH values (e.g., pH_{opt} between pH 5.5 and 7.5). It is interesting to notice that – so far – all obligately aerobic thermophilic archaea are acidothermophiles, except for the recently isolated *Aeropyrum*, a neutrophilic thermophilic archaeon. This group is presently composed of more than 15 species, which contains the most acidophilic microorganisms among the Bacteria and Archaea (*Picriphilus oshimae* and *P. torridus*, aerobic thermophilic archaea with a pH_{opt} about 0.5) (Schleper et al. 1995). The number of known species in this group, similar to all other groups of thermophiles, increases

Fig. 2. Relationship between temperature optima (T_{opt}) and pH optima (pH_{opt}) for growth of anaerobic thermophilic bacteria (see footnote to Table 1)
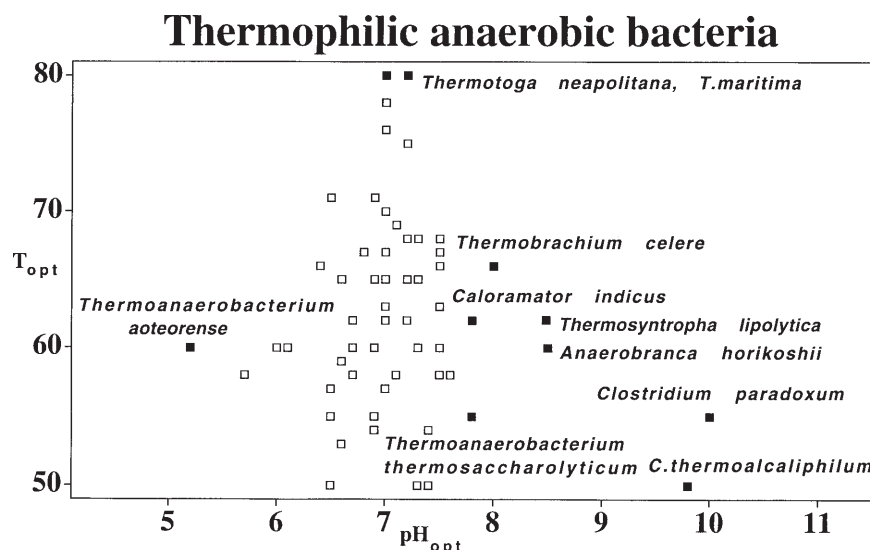
constantly. The descriptions of three facultative anaerobic archaea, *Sulfurococcus mirabilis* (Golovacheva et al. 1985), *Sulfurococcus yellowstonii* (Karavaiko et al. 1994), and *Stygiolobus azoricus* (Segerer et al. 1991) have shown that this previously assumed exclusive niche is not inhabited only by obligately aerobic archaea. As more and more researchers are becoming interested in studying the various groups of extremophiles, the original clustering of known microorganisms in specific pH and temperature ranges will probably disappear as more microorganisms are isolated. In this context, it is interesting to note that no anaerobic eubacterium has yet been found that can grow at the elevated temperatures of above 50°C at pH values below pH 3.0 despite the fact that many geothermally heated features are more acidic than this. Among the anaerobic thermophiles, *Thermoanaerobacterium aotearoense* with a pH_{opt}^{60C} of 5.2 (growth range, pH^{60C} 3.8–6.8) has the most acidic pH optimum, but this classifies it only as a slightly acidophilic microorganism (Liu et al. 1996). (Fig. 2)

To date, the most alkaliphilic microorganisms are all mesophilic bacteria, and many are species of the genus *Bacillus*. Again, the dominance by *Bacillus* species is now starting to disappear; e.g., recently a new genus, *Bogoriella*, has been described and a new alkaliphilic species, "*Dietzia natronolimnaios*" (Brian Jones, personal communication), and more are expected to follow. *Bogoriella caseilytica* is an interesting alkaliphilic actinomycete with a pH_{opt} between 9.0 and 10.0 that was isolated from samples from the soda lakes in the Kenyan-Tanzanian Rift valley (Groth et al. 1997). It is surprising, that no true thermophilic alkaliphilic *Bacillus* species have been described; however, they should exist. The highest growth temperature (T_{max}) for an aerobic alkaliphile growing above pH 10 is 57°C (*Bacillus* sp. strain 221; Horikoshi 1990). For the anaerobic bacteria, this temperature is 63°C (*Clostridium paradoxum*; Li et al. 1993).

The novel subset alkalithermophiles is still a small group (Tables 2 and 3). As indicated, no aerobic thermophilic archaea is found in this group; the most alkaline pH_{opt} for

aerobic thermophilic archaea was reported for *Aeropyrum pernix* with a pH_{opt} about 7.0 (Sako et al. 1996). Among the aerobic bacterial thermophiles are a few with pH_{opt} around 8.5 (Table 2). Among the anaerobic thermophilic archaea *Methanohalophilus zhilinae* (T_{max} 55°C, $pH_{max} < 10.5$, pH_{opt} 9.2; Mathrani et al. 1988), *Methanobacterium thermoflexum* (T_{opt} 55°C, pH_{opt} 7.9–8.2; Kotelnikova et al. 1993), *Methanobacterium wolfei*, and some *Methanobacterium thermoautotrophicum* strains previously described as *M. thermoalkaliphilum* ($T_{max} < 73°C$, $pH_{max} < 10.0$, pH_{opt} 7.5–8.5) (Winter et al. 1985; Zeikus and Wolfe 1972; Blotevogel et al. 1985) are the most alkaliphilic. Among the obligately anaerobic bacterial mesophiles, no extreme alkaliphiles have been validly published. Aside from several unidentified obligately anaerobic isolates growing at pH as high as 11.3 (Souza et al. 1974), the most alkaliphilic (validly published) bacterium growing under anaerobic conditions is the facultatively anaerobic *Amphibacillus xylanus*, growing between pH 8.0 and 10.0. The most alkaliphilic anaerobic alkalithermophilic bacterium is *Clostridium paradoxum* (Fig. 2), which is described next. It can grow at pH^{25C} up to 11.1 (pH^{60C} 10.3). Apparently, until very recently little attention has been paid to obtaining microorganisms able to grow at high pH and elevated temperatures. One of the reasons might be that for a long time it was assumed that bacteria or archaea could not grow at elevated temperatures (e.g., above 60°C) and alkaline conditions (pH values >10) because such conditions would place too great a strain on the stability and integrity of cell walls and membranes. This assumption is obviously not true. Meijer et al. (1996) reported on the isolation of '*Thermopallium natronophilum*' from the Kenyan-Tanzanian Rift Valley (East Africa). The bacterium Gram-stains negative and is able to grow fermentatively at 70°C and pH^{25C} pH 10 (Brian Jones, personal communication) and belongs, according to the 16S rDNA sequence analysis, to the *Thermotogales*. Thus it is expected that upon further efforts to isolate these extremophiles from the appropriate environments (e.g.,

the African soda lakes with hot springs), more alkalithermophiles will be found, and probably with even higher $pH^{opt/max}$ values and temperatures than presently known.

Many of the most thoroughly investigated published microorganisms have very distinct pH_{opt} ranges of less than 0.5 pH units. The alkalithermophiles described here do belong in this group. However, some bacteria have an unusually wide pH range for their pH^{opt} of about 3.0 pH units. One example is the thermophilic anaerobe *Thermoanaerobacter ethanolicus*, which grows at 69°C in the pH^{25C} range of 4.4 to 9.8 and has the same doubling time in the pH range between pH^{25C} 5.5 and 8.5 (Wiegel and Ljungdahl 1982). Thus, growth conditions for this microorganism span from the range of the so-called slightly acidophiles to the range of alkalitolerant bacteria (Tables 1, 2, 3).

Distribution of alkalithermophiles

For descriptions of the main alkaliphilic environments, the reader is referred to previously published reviews (e.g., Horikoshi 1990; Grant et al. 1990). Similar to the occurrence of thermophilic bacteria and archaea with a $T_{max} < 80°C$ in many mesobiotic environments (e.g., the nonspore-forming bacterium *Thermus* sp. in water pipes as well as hot springs and the archaea *Methanobacterium thermoautotrophicum* in cold lakes, but also alkalithermophiles and alkalitolerant thermophiles have been isolated from a great variety of environments including mesobiotic sewage sludge, river and lake sediments, microbially heated compost, and geothermally heated springs with a wide variety of environmental pH values.

The most alkaliphilic (pH_{max}^{25C} of 11.1) anaerobic thermophiles, the spore-forming *Clostridium paradoxum* and the non-spore-forming *Clostridium thermoalkaliphilum* were isolated from various mesobiotic sewage facilities across the United States and from sewage plants from three continents (unpublished results), where the macroenvironmental temperature and pH were never found to be above 35°C and pH 7.8, respectively. Enumerations for *C. paradoxum*- and *C. thermoalkaliphilum*-like bacteria revealed that these bacteria are present in concentrations up to 10000 cells/ml in the anaerobic sludge, but also in the aeration pools with up to 1000 cells/ml of sample (Li et al. 1993). *C. paradoxum*, however, could not be isolated from sediments or water samples of the Oconee River, which receives the discharge of the plant in Athens, GA, nor from the geothermally heated features of Yellowstone National Park or Iceland. Only recently were we able to isolate one strain belonging to *C. paradoxum* from a hot spring in Rotorua, New Zealand (Wiegel and Rainey unpublished results), which had received gray water in the past, but so far not from the other springs. This result is somewhat surprising because nearly all cells in a culture sporulate easily at the end of the exponential phase and thus, similar to other thermophilic spore-formers, should be widely distributed.

However, it is possible that in many natural environments the cells do not sporulate. This possibility is indicated by the observations that under laboratory conditions when employing media with pH values below pH^{25C} 7.5 or below 50 mM NaCl concentrations, the sporulation frequency drops sharply. At pH 7.0 and below and also below 10 mM NaCl, spores were not detectable. The macroenvironmentally determined pH, temperature, and sodium-potassium concentrations (usually about 3–10 mM) in most sewage plants harboring this bacterium were far below the optimum for growth and below the concentrations required for motility and sporulation. Interestingly, the onset of sporulation by *C. paradoxum* also changes the motility behavior of the cells. In the exponential growth phase, cells are only sluggishly motile, mainly exhibit tumbling, whereas the sporulated cells exhibiting very short tumbling phases and long swim phases with a speed up to 15 μm/s, allowing the sporulated cells to find more suitable conditions. They swim in both directions with respect to the cell poles, i.e., independently of where the terminal spore was located (Li et al. 1993).

Clostridium thermoalkaliphilum has been only isolated from one (Atlanta, GA, USA) sewage plant (Li et al. 1994). Except for being nonsporulating, its growth parameters are nearly identical to those of *C. paradoxum*. However, this species is motile throughout the exponential growth phase. The motility again is strongly influenced by pH and temperature. The relationship of motility to pH and temperature more or less parallel the corresponding curve for the inverse doubling times. Because little motility is seen in the absence of glucose, the motility pattern seems to be in this bacterium primarily a function of available energy and not a direct response to adverse growth conditions as in *C. paradoxum*. The observed difference in the distribution of these two similar alkalithermophiles could result from thus far limited efforts to isolate these bacteria and to the lack of determinations using more sensitive molecular methods.

In contrast to these two alkaliphiles, the less alkaliphilic (pH_{max}^{60C} 10.2; pH_{opt}^{60C} 8.5), thermophilic (T_{max} 66°C) bacterium *Anaerobranca horikoshii* has been so far only isolated from various springs located in a relatively restricted, geothermally heated area of less than 1 km² within Yellowstone National Park, WY, USA (area behind Old Faithful Hotel and the Ranger Station). Notably, the pH values of these springs ranged from 8.7 down to 5.8, and this species was isolated from springs with an alkaliphilic as well as the acidic pH (Engle et al. 1995).

The only two alkalitolerant and alkaliphilic thermophilic anaerobes that have been exclusively isolated from an alkaline thermobiotic environment are the nonsporulating *Thermosyntropha lipolytica* (and isolates similar to the three strains published) together with alkalitolerant strains of *M. thermoautotrophicum* (Svetlitshnyi et al. 1996) and the sporulating "*Thermoalcalibacter bogoriae*" LBS3 (pH_{opt} 9.5; T_{opt} 50–55°C; Prowe 1996; Prowe et al. 1996) from Lake Bogoria (Africa).

In contrast to all the above described species, the alkalitolerant (pH_{max}^{66C} 9.5, pH_{opt}^{66C} 8.5) but more thermophilic (T_{max} 75°C) *Thermobrachium celere* has been found

Table 2. Thermophilic Bacteria and Archaea able to grow optimally at or above pH 7.8[a,b]

Group of microorganisms	pH$_{opt}$	T$_{opt}$	Reference
Methanogenic Archaea			
Methanohalophilus zhilinae	9.2	55	Mathrani et al. (1988)
Methanobacterium thermoflexum	7.9–8.2 (8.1)	55	Kotelnikova et al. (1993)
Methanobacterium thermoautotrophicum	7.7–7.8 for		Zeikus and Wolfe (1972)
	(syn. *M. thermoalcaliphilum*)	65	Blotevogel et al. (1985)
Anaerobic Archaea			
Thermococcus alcaliphilus	9.0	8.5	Keller et al. (1995)
Thermococcus fumicolans	8.5		Godfroy et al. (1996)
Aerobic Archaea			
None			
Anaerobic Bacteria			
(See Table 3)			
Aerobic and facultative anaerobic bacteria			
Bacillus pallidus	8.0–9.0 (8.5)	60–65 (63)	Scholz et al. (1987)
Bacillus thermocloaceae	8.0–9.0 (8.5)	55	Demharter and Hensel (1989)
Sphaerobacter thermophilus	8.5	55	Demharter et al. (1989)
Thermomicrobium roseum	8.2–8.5 (8.4)	70–78 (74)	Jackson et al. (1973)
Isosphaera pallida	7.8–8.8	40–55	Giovannoni et al. (1987)
"*Bacillus caldotenax*"	8.0	80	Heinen and Heinen (1972)
Chloroflexus aurantiacus	8.0	52–60 (56)	Pierson and Castenholz (1974)
Meiothermus chliarophilus	8.0	50	Nobre et al. (1996)
Meiothermus ruber	8.0	60	Nobre et al. (1996)
Meiothermus silvanus	8.0	55	Nobre et al. (1996)
Thermus oshimai	7.0–8.0 (range)	70	Williams et al. (1996)
Bacillus thermoaerophilus	7.0–8.0 (range)	40–60 (range)	Meier-Stauffer et al. (1996)
Rubrobacter xylanophilus	7.0–8.0	60	Carreto et al. (1996)

[a] For the anaerobic alkalithermophiles, see Table 3.
[b] The published pH data are difficult to compare because the reviewer does not know, for most of the microorganisms, how the pH value was determined (see text). When known, the temperature for the measurement inclusive for the calibration of the pH meter is given as a superscript after the pH value.

Table 3. Thermophilic anaerobic bacteria able to grow above pH 8.5 and having pH optima for growth above 7.7 and temperature optima above 50°C

Genus and species	pH$_{opt}$[a]	pH range[a]	T$_{opt}$ (°C)	Temperature range (°C)	Doubling times[b] (min)	Distribution/ origin of isolation	Reference
Clostridium paradoxum	10.1[25C] 9.3[55C]	7.0–11.1[25C] 6.9–10.3[55C]	56	30–63	16–20	Sewage plants, contaminated hot springs	Li et al. (1993)
C. thermoalacaliphilum	9.8[25C] 9.2[50C]	7.0–11[25C]	51	27–57.5	20	Sewage Plant, Georgia, USA	Li et al. (1993)
Anaerobranca horikoshii	8.5[60C]	6.9–10.3[60C]	57	34–66	30–40	Yellowstone National Park, USA	Engle et al. (1995)
Thermosyntropha lipolytica	8.1–8.9[25C] 7.6–8.1[60C]	7.1–9.5[25C]	60–66	52–70	50–60	Soda lake (Bogoria), Kenya	Svetlitshnyi et al. (1996)
Thermobrachium celere	8.2[66C]	5.4–9.5[66C]	66	43–75	10–25	Ubiquitous	Engle et al. (1996)
Caloramator indicus	8.1	6.2–9.2	60–65	37–75	20	Deep-seated aquifer, India	Chrisostomos et al. (1996)
Thermoanaerobacter ethanolicus	5.5–8.5[25C]	4.5–9.8[25C]	69	35–78	90	Yellowstone National Park, USA (slightly alkaline and acidic springs)	Wiegel and Ljundgahl (1982)
Thermoananerobacter thermosaccharolyticum	7.8	(6.0)–8.5[25C]	55	35–67	80–150	Ubiquitous	Collins et al. (1994)

[a] When known, the temperature for the measurement inclusive for the calibration of the pH meter is given as superscript. The published pH data are difficult to compare because of uncertainties about the pH determinations (see footnote in Table 1 and text).
[b] If different strains were tested and they showed different doubling times, the observed range is given.

ubiquitously, although no sporulation for any of the strains has been observed. This bacterium was found in nearly all tested sediments including mesobiotic river sediments from Argentina and Germany and lake sediments in the United States (including the alkaline Mono Lake, CA, USA), in man-made thermobiotic environments such as horse manure compost, and in various geothermally heated environments such as the hot springs in New Zealand and Italy. Similar isolates obtained from Yellowstone National Park samples are still under taxonomic investigation but they seem to be closely related to *Thermobrachium celere*.

The habitats of the other anaerobic alkalitolerant thermophiles (see Table 1) are similar to those of the above-mentioned bacteria. Examples are *Thermoanaerobacterium* (*Clostridium*) *thermosaccharolyticum*, which is ubiquitous (known in the canning industry as swelling can bacterium), and conversely the above-mentioned *Thermoanaerobacter ethanolicus* with the extremely broad pH optimum, from 5.5. to 8.5, which was only isolated from a slightly alkaline and from a slightly acidic hot spring of Yellowstone National Park (Wiegel and Ljungdahl 1982).

In summary, the anaerobic alkalithermophilic and alkalitolerant thermophilic bacteria are found frequently in nonalkaline environments and are as a group ubiquitous, although some species might be endemic to specific soda lakes or other specific environments. One of the reasons that several of these alkalithermophilic bacteria are found in environments with macroenvironmental conditions less suitable for optimal growth may lay in the fact that they are all facultative alkaliphiles and that their doubling times are as short as 10 min (see following); thus, if temporary microenvironmental conditions occur which are suitable for growth, these bacteria are apparently able to respond quickly and to take advantage of such a situation. However, several of these ubiquitously found species are nonsporulating under laboratory test conditions.

Diversity of anaerobic alkalithermophiles

Phylogeny

All the anaerobic alkalitolerant and alkalithermophilic bacteria belong to the Gram-type-positive *Bacillus-Clostridia* subphylum, although well separated from each other in 16S rDNA sequence-based phylogenetic trees (Fig. 3), i.e., the isolated bacteria belong to different groups in the classification scheme of Collins et al. (1994). *Thermobrachium celere* and *Caloramator indicus* are very closely related and thus should be at least combined in one genus. There are some differences in the G + C mol% content and some other features; thus, DNA-DNA hybridization should be done. The sporulating *Clostridium paradoxum* and the nonsporulating *C. thermoalkaliphilum* also are closely related and differ in about 2 per 100 nucleotides in their 16S rDNA gene sequence. Although closely related, the two species differ not only in sporulation (which is really no longer a reliable phylogenetic marker) (Collins et al.
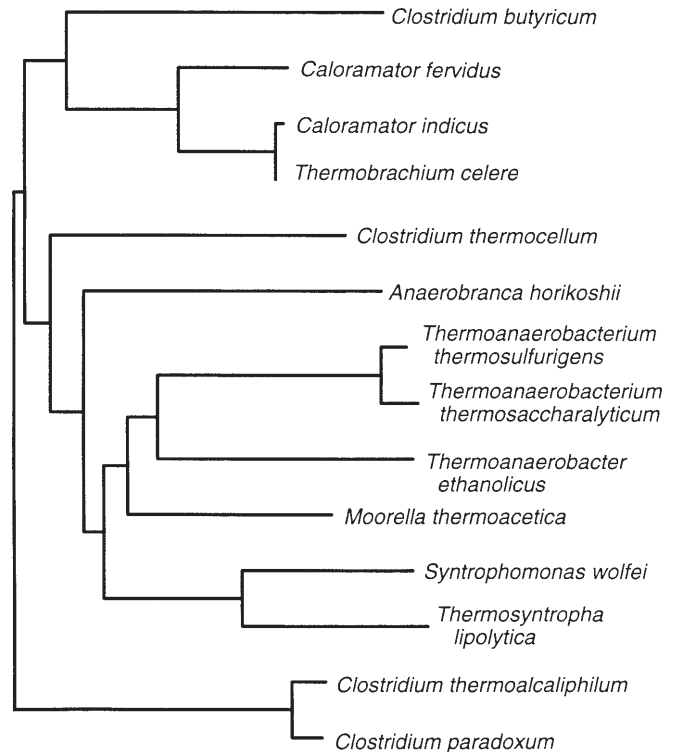

Fig. 3. Phylogenetic tree for anaerobic alkalithermophiles and alkalitolerant thermophiles based on the 16S rDNA sequence comparisons

1994; Brill 1997; Brill and Wiegel 1997) but also differ in their cell wall type. *C. paradoxum* has the meso-diaminopimilic acid direct (A1γ *m*-DAP) type, *C. thermoalkaliphilum* exhibits the more complex L-ornithine-D-aspartate (A4β) type, a situation that is unusual among such closely related species. Neither species belongs to group 1 clostridia (Collins et al. 1994) containing the *Clostridium* species sensu stricto, and the author will rename them after additional species belonging to this group have been found.

The other described alkalithermophiles are clearly separated from each other. *Thermobrachium* is 9% and about 15% apart from the alkalitolerant *Caloramator fervidus* and *Clostridium paradoxum*, respectively. *Anaerobranca horikoshii* is even more distantly related and is about 18–20% different in the nucleotide sequence compared to those of *C. paradoxum* (Fig. 3).

Morphology

Clostridium paradoxum, *C. thermoalkaliphilum*, and *Thermosyntropha lipolytica* are slightly irregular rods with retarded peritrichously inserted flagella. The cells of all three species stain Gram negative at all growth phases although they are Gram-type positive (Wiegel 1981; Wiegel and Quandt 1982) in agreement with their phylogenetic position. Cells of *Thermobrachium celere* and *A. horikoshii* strains that stain Gram positive, for which only tumbling motility has been observed, exhibit a truly branched mor-

phology as indicated by electron micrographs of ultrathin sections. However, only 10% of the cells in a culture of *A. horikoshii* exhibit the branched morphology. After being under culture for more than 3 years in the laboratory, branching of *T. celere* cells has become a rare event, whereas shortly after the isolation of these strains, more than 10% of the cells were branched. The extent and frequency of branching was in neither genus markedly influenced by the incubation temperature or pH of the growth medium.

From the six most alkaline species that are presently validly published, only *C. paradoxum* and *Caloramator indicus* have been observed to form spores (in addition the unpublished '*Thermoalcalibacter bogoriae*'). For the other four mentioned species, sporulation has never been observed, although, as we have recently shown (Brill and Wiegel 1998, unpublished results), all four contain three representative sporulation-specific genes, i.e., genes for the regulator spoOA, the small acid-soluble proteins, and the dipimelic acid synthetase. This finding, in agreement with the above-mentioned phylogenetic position of these bacteria, indicates that they are sporulating bacteria in origin (Brill and Wiegel 1997). All five species exhibit in one form or another the formation of irregular cells such as club-shaped cells (*Clostridium paradoxum*, *C. thermoalkaliphilum*), round cells (*T. celere*), or lemon-shaped cells (*C. paradoxum*). In addition, at high pH values (pH^{60C} above 9.8) *C. paradoxum* grows in long, sparsely septated, filamentous cells. Although all other species exhibit the typical septation mode for cell division, cells of *C. paradoxum* show a combination between a pinching and the septation mode (Li et al. 1993).

Physiology

With respect to temperature optima and pH optima, there are distinct differences among the described alkalithermophiles (see Fig. 2). The distribution in the graph indicates that the higher the T_{opt} (or T_{max}) for a species, the lower the pH_{opt} (or pH_{max}). When different strains of a species were compared, this trend was also observed (Fig. 4). Whether this is a true trend or an artifact of too few isolates remains to be seen.

The described alkaliphilic and alkalitolerant thermophilic anaerobes (Fig. 2) are all heteroorganotrophs that to some degree differ in their substrates utilized and products formed. The author believes the similarities and dissimilarities largely result from the employed enrichment and isolation conditions. All isolated alkalithermophiles require yeast extract as a supplement of the medium that could not be substituted by vitamins or trace elements (both were regularly included in the media used) but could be substituted by tryptone and some times by peptone.

All strains of *C. paradoxum* and *C. thermoalkaliphilum* are peptidolytic and caseinolytic, as they were isolated on milk powder and casein in the presence of yeast extract and/or tryptone. All tested strains, however, in the presence of yeast extract or tryptone also could utilize glucose, fructose, maltose, and sucrose as well as pyruvate. The fermentation products from yeast extract in the absence of glucose are acetate and H$_2$ and CO$_2$ at pH^{25C} 7.5 (but not CO$_2$ HCOO$^-$ at pH^{25C} 10.1) and traces of isovalerate and lactate. Also, no detectable amounts of CO$_2$ HCOO$^-$ were produced at the higher pH when glucose was utilized. In both cases an unknown product of substantial amounts (based on carbon- and electron balance) is produced. *Thermobrachium celere*, again because of the employed isolation procedures, is a proteolytic bacterium that grows with yeast extract as sole carbon and energy source. In the presence of 0.1% yeast extract it also can utilize several sugars (glucose, fructose, galactose, and maltose), but does not utilize pyruvate.

Anaerobranca horikoshii is, in contrast, nearly exclusively (based on tested substrates) a peptidolytic bacterium that does not utilize sugars or even pyruvate. However, it can utilize fumarate as alternate electron acceptor, forming succinate in stoichiometric amounts. Fermentation products during growth on yeast extract were acetate, H$_2$, and CO$_2$.

Thermosyntropha lipolytica is physiologically somewhat different from the other bacteria. As the name indicates it is a lipolytic bacterium, and it constitutively produces alkaline

Fig. 4. Comparison of pH (*left*) and temperature optima (*right*) of (three strains were exemplarily chosen) strains of *Clostridium paradoxum* suggests that the higher the T_{opt} of a strain is, the lower is its pH_{opt}
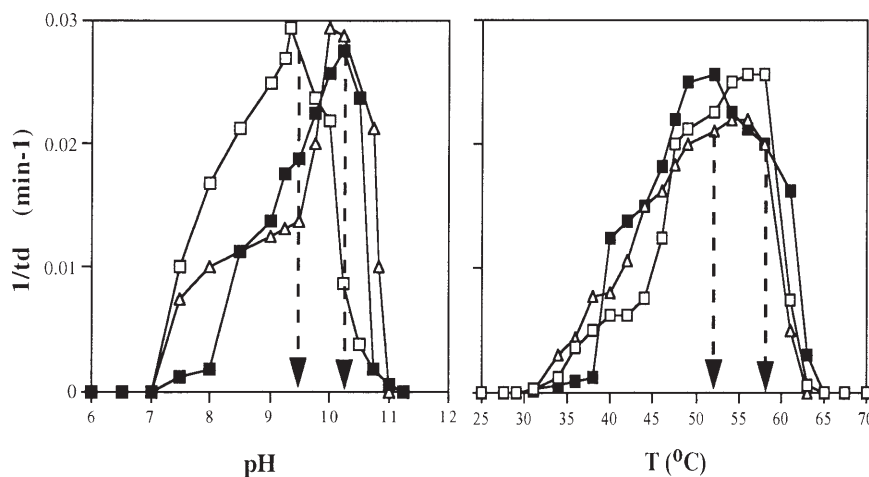
thermostable lipase activity. Because it does not utilize the formed glycerol but rather the fatty acids, it is only able to grow on lipids in syntrophic cultures either with a methanogen or a sulfate reducer. The hydrogen-utilizing partners are required to keep the hydrogen concentration low enough to make the thermodynamically unfavorable fatty acid degradation feasible. During growth on yeast extract or other complex media such as tryptone, a hydrogen gas atmosphere was not inhibitory. *Th. lipolytica* is the only thermophilic syntrophic anaerobe known. Originally it was isolated from a methanogenic coculture enriched on commercial olive oil. The methanogen was a *Methanobacterium thermoautotrophicum*-like archaeon able to grow well at pH 9.0. The methanogenic reassociated coculture utilizes, besides the triolein (olive oil), trilaurin, tributyrin, tripalmetin, tristearin, and triolein. The formation and utilization of the fatty acids also requires the presence of Ca ion in equimolar concentrations to the fatty acid content of the lipid. From the three purified strains, strain JW/SV-265^{T} was further characterized, and it can utilize aliphatic fatty acids from a carbon chain length of 4 (butyrate, but not isobutyrate or hydroxybutyrate), up to a length of 18 (stearate) including the unsaturated acids (e.g., oleate, linoleate) but not arachidate ($C_{20:0}$). None of the branched fatty acids tested were utilized. Utilization of butyrate, valerate, and caproate was slow. Even-numbered fatty acids led to the production of acetate and methane (in the methanogenic coculture) whereas odd-numbered acids yielded additionally one propionate per degraded fatty acid.

In pure culture, the bacterium can grow on the characteristic substrate for syntrophic anaerobes, crotonate. Compared to 2–5 weeks for mesophilic syntrophs, the thermophile grows relatively fast on crotonate, yielding dense cultures and colonies within 7 days. Minor growth was also obtained with ribose, xylose, pyruvate, and betaine. Using these substrates no methane was produced in the coculture, indicating that no significant amounts of hydrogen were produced under these conditions (Svetlitshnyi et al. 1996). A physiologically similar strain was isolated as a stable methanogenic fermentor culture run to degrade fat. Further characterizations are needed to clarify the relationship between these two lipolytic microorganisms (Blotevogel, personal communication).

Utilization of biopolymers

Although we obtained enrichments of alkaline cellulolytic thermophiles growing above pH^{25C} 9.0, none of our isolated strains exhibited a thermostable cellulase with significant activities at pH values at pH^{25C} 10 or above. Also, none of the above described alkaliphilic or alkalitolerant thermophiles utilized xylan, starch, or pectin to a detectable extent. However, *Caloramator* sp. and *C. thermosaccharolyticum* do hydrolyze xylan, and the latter also can utilize starch. The slightly alkalitolerant thermophiles "*Clostridium pseudocellulolyticum*" strain JW/SD-EPP100 (pH^{55C} range 6.5–9.2, pH$^{55C}_{opt}$ 7.8, T$_{max}$ 64°C; isolated from an alkaline hot pool at Mickey Hot Springs, OR, USA) and

"*Thermotorquereum fastidiosum*" strain JW/SD-NDF190 (pH^{66C} range 6.2–9.2, pH$^{66C}_{opt}$ 7.8, T range 50–70°C; T$_{opt}$ 66°C; isolated from compost, Athens, GA, USA) have alkaline stable xylanases with a pH optimum around pH 9.0–10 and 6.5, respectively (DeBlois 1996; DeBlois and Wiegel 1992). Despite the neutral pH optimum of the "*T. fastidiosum*" xylanase, its pH-dependent formation has a maximum around pH 8.3. The xylanase from "*C. pseudocellulolyticum*" is interesting with respect to various aspects. Its formation is regulated similarly to cellulases and is induced by the presence of lactose and cellobiose. It also binds highly to cellulose. However, the strain does not exhibit any cellulase activity or even carboxymethyl cellulase (CMC) activities and subsequently does not grow on any cellulosic substrates (DeBlois 1996; DeBlois and Wiegel 1995). The 16S rDNA sequence analysis places this bacterium together with other cellulolytic thermophiles such *Clostridium thermocellum, C. stercorarium*, and *C. thermolacticum* (F. Rainey, personal communication), which may explain the unusual properties of the xylanase and suggest the possibility that this organism might be a natural cellulase-negative mutant of a so far unknown cellulolytic alkalitolerant thermophile.

Antibiotic sensitivity

The antibiotic sensitivity spectrum of the alkalithermophiles does not show any particularities and is similar to those of other bacteria from the phylogenetic *bacillus-clostridia* branch. *C. paradoxum* was, at both pH^{25C} 7.1 and 10.1, sensitive toward (e.g., 25 µg/ml) chloramphenicol, erythromycin, tetracycline, monensin, gramicidin S, and lasalocid, and against 50 µg/ml of gentamicin; *C. thermoalcaliphilum* was sensitive toward 50 µg/ml penicillin G, ampicillin, metroimidazole, gentamicin, and rifampin; and *Thermosyntropha lipolytica* was sensitive to 50 µg/ml ampicillin, chloramphenicol, kanamycin, neomycin, rifampin, and vancomycin. However, the testing of the antibiotic resistance pattern is presently equivocal because the stability of the antibiotics under the growth conditions of combined high temperatures and alkaline pH values is not known (Peteranderl et al. 1990); this is, however, currently under investigation.

Intracellular pH and ΔpNa-dependent membrane potential

Alkaliphiles must control their internal pH to allow metabolism to proceed optimally and to maintain a proper gradient between external pH and cytosolic pH. As van de Vossenberg et al. (1995) demonstrated, thermophiles have to handle an increased permeability of the membrane, especially for protons and other monovalent ions such as sodium. The permeability for protons in organisms growing at neutral pH values is about a hundred to a thousand fold higher than for sodium. Alkalithermophiles, however, face a high sodium permeation, especially if they grow in soda

lakes or similar environments, because of the high concentrations of sodium ion. Aerobic mesophilic alkaliphiles usually use a Na^+/H^+ antiporter system to maintain their pH stasis of a more acidic cytosolic pH in combination with a respiration-dependent pumping of H^+ to maintain their electrochemical gradient of protons. In anaerobic alkalithermophiles, sodium ions are extruded by a net Na^+-ATPase. The resulting membrane potential (inside negative) pulls in the proton, thus leading to a reversed pH gradient (inside acidic) and an electrochemical proton gradient of 0 mV.

So far we have only investigated the intracellular pH of one of the alkalithermophiles, *C. paradoxum*, which has the highest pH_{max} of all the isolated anaerobic alkalithermophiles. In addition to our reports, Prowe et al. (1996) reported on the sodium-coupled energy transduction in the moderately thermophilic, anaerobic alkaliphilic 'Thermoalcalibacter bogoriae' strain LBS3 and on its uptake of amino acids via sodium symport mechanisms. Furthermore, Speelmans et al. (1993, 1995; Zu Bentrup et al. 1997) have investigated energy transduction in the neutrophilic *Caloramator* (Basonym *Clostridium*) *fervidus*. This bacterium is closely related to the fast-growing, slightly alkalitolerant thermophilic *Thermobrachium celere*. This anaerobic thermophile does not regulate its internal pH, has an energy transduction that is dependent on sodium ions as coupling factors, and contains a sodium-ion pumping V_1V_o-ATPase complex, but apparently lacks sodium proton antiporter systems. Zu Bentrup et al. 1997. Whether these are more general properties of similar anaerobic thermophiles and of other alkalithermophilic anaerobes is not known because very little is known about internal pH and energy mechanisms for anaerobic thermophilic glycolytic and peptidolytic bacteria.

In contrast to aerobic alkaliphilic *Bacillus* strains (Horikoshi 1990), the anaerobic alkalithermophilic bacteria do not adjust their media pH to the optimal pH if incubated in media with pH values above or below the optimum. Because the anaerobic metabolism produces acids as fermentation products, the pH of cultures utilizing sugars becomes progressively more acidic. None of the isolated alkalithermophiles produce solely pH-neutral compounds, nor even switch to increased amounts of alcohols to avoid further acidification. Only the slightly alkalitolerant *Thermoanaerobacterium thermosaccharolyticum* can produce larger amounts of ethanol and even up to 40 mM butanol under special conditions (Freier et al. 1989). The alkalitolerant anaerobic thermophiles *Caloramator indicus* (Chrisostomos et al. 1996) and *Thermobrachium celere* can produce smaller amounts of ethanol. Thus, it appears that most of the alkaliphilic and alkalitolerant thermophilic anaerobes are growing in environments where the acid formation causes no serious limitation on their growth and survival.

Cells of *Clostridium paradoxum* exhibit – very similarly to other cells of other alkaliphilic bacteria – a lower internal pH than the pH in the medium (Cook et al. 1996). This characteristic leads to an inverted pH gradient and usually to a lower electrochemical proton gradient for driving ATP synthesis. As described here, the organism might rely on a high sodium ion electrochemical gradient. The internal pH of *C. paradoxum* changes with the external pH in the medium, and thus no pH stasis was observed. The ΔpH was zero at pH^{55C} 7.0 and 10.9, i.e., at the pH boundaries for growth, but the ΔpH (inside acid) was highest (ΔpH 1.3) in the pH range for optimal growth, pH^{55C} 9.0–9.5. At the external pH^{55C} of 10.1, the internal pH^{55C} was 9.8, slightly higher than has been observed for other alkaliphiles (Cook et al. 1996; Krulwich 1995; Krulwich and Guffanti 1992). The ΔΨ and the ZΔpH are maximal at the pH optimum for the bacterium and reach values around −120 mV and +80 mV, respectively. Consequently, the electrochemical proton gradient is about −40 mV. Depending on the pH, potassium ions were concentrated from the external concentration of about 5 mM to a cytoplasmic concentration of maximal 200 mM around the pH optimum for growth. In contrast to the potassium ions, the internal sodium concentration was maintained at about 20 mM while the medium contained 100 mM sodium ion. Thus, the sodium ion electrochemical gradient $[\Delta\Psi + Z \log(Na^+_{in}/Na^+_{out})]$ is $-120\,mV - 60 \log 5\,mV$ and thus has a significant value. For *C. paradoxum* cells incubated at the external pH^{55C} range of 7.8–10.3, the ATP concentration remained about 1 mM. The assumption of the importance of the sodium ion electrochemical gradient is supported by the observations of growth inhibition on the addition of monensin, an ionophore that dissipates sodium gradients. In accordance with these findings with *C. paradoxum*, Prowe et al. (1996) showed for 'Thermoalcalibacter bogoriae' that the ATPase activity in inside-out vesicles was stimulated by sodium and lithium ions and that the uptake of most amino acids was strictly sodium dependent. The authors concluded that the primary mechanism of energy transduction in this anaerobic alkalithermophile is dependent on sodium cycling. The uptake of leucine (the only amino acid studied in more detail) was highest at suboptimal pH values for growth and utilized a sodium/leucine symport mechanism (Prowe et al. 1996).

Further studies on these bacteria as well as on the fast-growing alkalitolerant anaerobic thermophile *Thermobrachium celere*, for example, are necessary to obtain a better picture of how the alkalithermophiles cope with the extreme conditions of alkaline pH values at elevated temperatures. Furthermore, it will be interesting to compare the mechanisms in aerobic alkalithermophiles, microorganisms to be isolated in the future.

Potential of Industrial Applications

Clostridium paradoxum, *C. thermoalkaliphilum*, *T. celere*, and *A. horikoshii* were isolated during a survey for anaerobic thermophiles harboring alkalistable and thermostable proteases for use in washing detergents. *Thermosyntropha lipolytica* was isolated during search for a suitable lipase, also as a component for washing detergent formulations. This bacterium produces an alkali-stable, thermostable,

washing detergent-stable lipase activity with an alkaline pH optimum for initial activity. The pH and temperature profiles for the lipase activity in all three isolates indicates that *Th. lipolytica* produces a more neutral and a more alkaline lipase. We are in the process of cloning the enzyme for further testing because the wild-type activities are too low for commercial testing. All the proteolytic isolates produced proteases sensitive to EDTA, indicating that they were metalloproteases that are not suitable for the formulation of washing detergents although some of them had good protease activity at pH values above pH 9.0.

Other enzymes of industrial interest are alkaline-stable, low molecular weight xylanases for the pulp and paper industry. Although several of the cultures can grow on xylan, none of the described bacteria exhibited high alkaline xylanase activities in culture filtrates passed through filters with a cut-off of MW 30 000. We presently have some stable enrichment cultures containing substantial xylanase activities at 60°C at pH^{25C} above 10. However, the isolates obtained so far had only fewer alkaline xylanases (DeBlois and Wiegel 1992, 1995; DeBlois 1996).

Conclusion

The alkalithermophilic and the alkalitolerant thermophilic anaerobes are neither the most thermophilic nor the most alkaliphilic microorganisms known. However, they are the most alkaliphilic among the anaerobic thermophiles ($\Rightarrow$alkalithermophiles) or, differently expressed, the most thermophilic among the alkaliphiles ($\Rightarrow$thermoalkaliphiles). Their description extended the range of conditions under which microorganisms are known to thrive optimally, that is, the combination of thermobiotic temperatures with alkaline pH. So far, they are all organoheterotrophs. One of their interesting features is the fast growth rates of most isolates, with doubling times as short as 10 min. It is expected that in the future we will see the description of additional, and probably physiologically more diverse, bacterial and probably archaeal alkalithermophiles, including the aerobic thermophilic counterparts to the anaerobic thermoalkaliphiles described here. This novel group of thermophilic alkaliphiles is interesting for future physiological studies and has a high potential for being the best source for alkalistable thermophilic enzymes in industrial applications.

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