Ecology and diversity of anaerobic alkali-thermophiles

J. Wiegel

Department of Microbiology and Center for Biological Resource Recovery, University of Georgia, Athens, GA 30602-2605, USA

ABSTRACT

We have recently isolated several genera and species able to grow under two extreme conditions: alkaline pH (above pH 9.0) and elevated temperatures (above 55^{o}C). They all belong phylogenetically to the Gram-type positive bacillus-clostridia subphylum. All of the isolated alkalithermophiles are characterized by relatively short doubling times with as short as 10 min exhibited by *Thermobrachium celere* growing optimally at 66^{o}C and pH^{66C} 8.5. *Clostridium paradoxum* (spore forming) and *C. thermoalkaliphilum* (non-sporulating) are the most alkaliphilic thermophiles and grow at pH values of up to pH^{60C} 10.3 (pH^{25C} 11.3). The various bacteria were isolated from a large variety of environments: e.g., *C. paradoxum* and *C. thermoalkaliphilum* were isolated only from mesobiotic sewage sludge containing up to 10^{4} CPU/ml sludge. The fast-growing *T. celere* was found in slightly acidic and slightly alkaline hot springs, manure compost and rivers from 4 continents. The constitutively lipolytic *Thermosyntropha lipolytica* was only isolated from an African soda lake.

Introduction

Recently, we described the first thermophilic anaerobic bacterium able to grow at alkaline pH values [5,6], thus extending the known conditions under which microorganisms demonstrate optimal growth. This can be demonstrated in graphs depicting the temperature optimum versus the pH-optimum for growth as shown for the subgroup of anaerobic thermophiles (Fig. 1).

Subsequently, a few more anaerobic alkaliphilic and alkalitolerant bacteria have been isolated from various sources [16](Fig. 1). We call these bacteria anaerobic alkalithermophiles, because three main parameters characterize the conditions for optimal growth: lack of the terminal electron acceptor oxygen, alkaline pH (above pH 8.0) and elevated temperatures (above 50^{o}C). Again, assuming that we only 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 as additional microorganisms are found in other extreme environments.

Biogeography

Since the conditions under which these bacterial alkalithermophiles grow best is a combination of extreme environmental conditions, we ask: does one find these anaerobic alkalithermophiles only in very specific niches which provide conditions where all three parameters are macroscopically close to the optimal growth conditions? Or alternatively, is it possible that these types of bacteria are present in all (alkaline) hot springs, or could they even be more or less ubiquitously distributed in non-alkaline and non-thermobiotic

Microbial Biosystems: New Frontiers
Proceedings of the 8th International Symposium on Microbial Ecology
Bell CR, Brylinsky M, Johnson-Green P (eds)
Atlantic Canada Society for Microbial Ecology, Halifax, Canada, 1999.

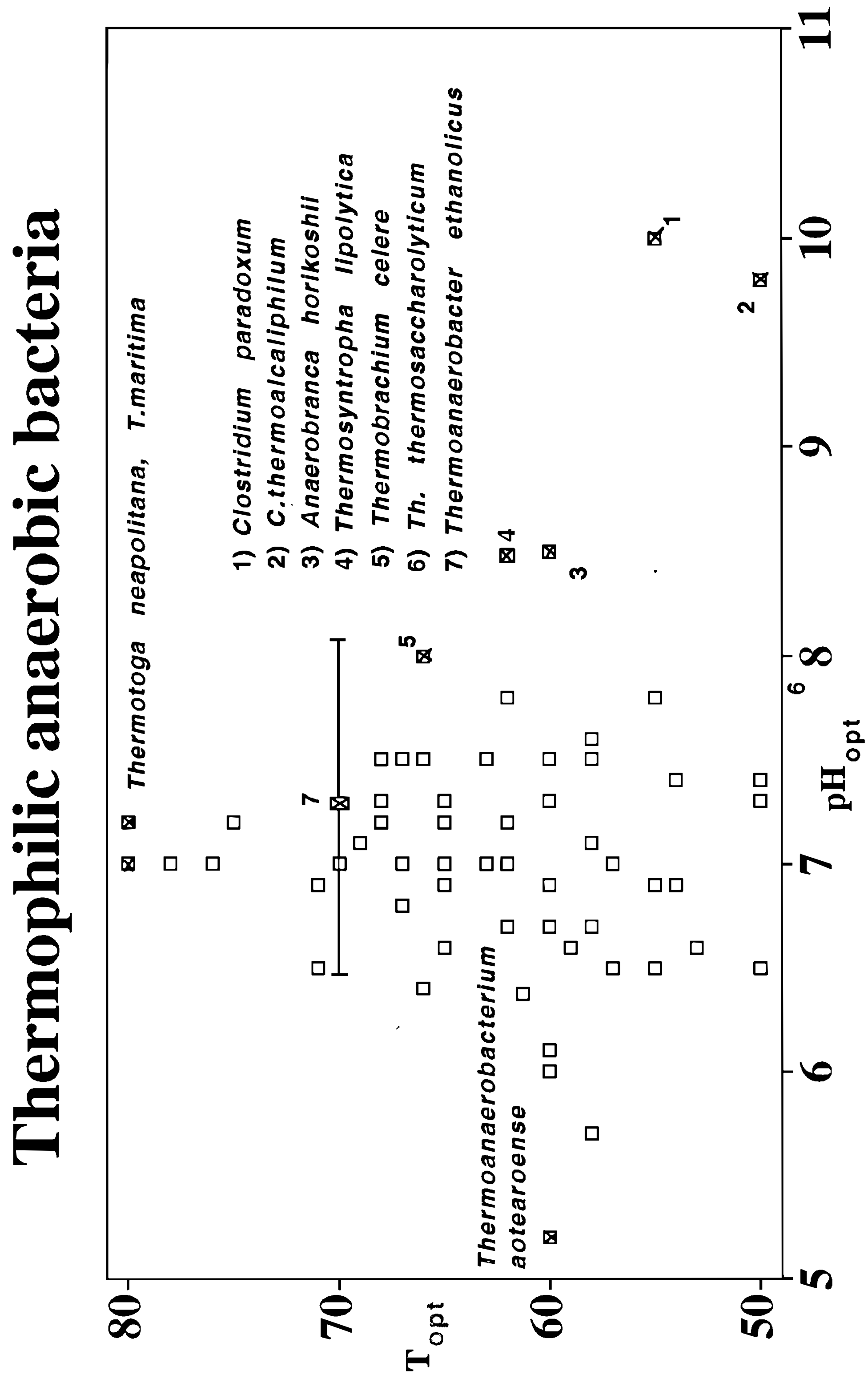

Fig. 1. Relationship between temperature optima (T_{opt}) and pH optima (pH_{opt}) for growth of anaerobic thermophilic bacteria. (See comment under Table 1).

environments? Biogeographical questions have been asked already many decades ago (Beijerinck; in 1) and more recently: "Is everyone everywhere, with only the geochemical and geo-physical parameters determining whether or not some organisms are prevalent in a given environment[11]?"

Temperature and pH boundaries for growth of microorganisms

The interest in extremophilic microorganisms is attributable to their ability to grow beyond the formerly assumed boundaries for life, as well as to the (potential) use of their enzymes in biotechnological applications. Presently the established temperature boundaries for observed growth of microorganisms are around a low of -12^{o}C for some fungi and a high of 113^{o}C for the anaerobic *Pyrococcus furiosus* and similar archaea [12]. Most microorganisms grow only within a relatively small temperature span (the difference between T_{min} and T_{max}) of 20 to 35^{o}C. A few bacteria, however, have been described as being able to grow within much wider spans of more than 35^{o}C. The archaeon *Methanobacterium thermoautotrophicum* --so far-- exhibits the most extended temperature span of about 55^{o}C (from 22^{o}C to 78^{o}C)[14,17]. Measurement of the marginal temperature data is not always straightforward. As a rule of thumb, the author usually uses incubation times of at least 200 times of the length of the shortest doubling time for determining growth at the lower and the upper temperatures, clearly a compromise between desired and practical circumstances (e.g., stability of the media containing complex nutrients for optimal growth). On the other hand, for subsurface bacteria *in situ* doubling times of thousand years have been estimated.

Even more problematic is the comparison of published growth-pH data for thermophiles since the method of determining the pH values are usually not given. A cited pH value could have been determined by measuring the pH at room temperature or at elevated temperatures with or without using a temperature probe and using a pH meter calibrated at room temperature, or (correct method for taking the temperature effect on the pK-values of the buffers and media into account) by measuring the pH at the elevated temperature using temperature equilibrated electrodes and pH-meters calibrated at that temperature. Although for neutral pH values, the errors are relatively small (usually below 0.3 pH units), at acidic or alkaline pH values, the temperature-induced errors can be larger than one pH unit [16]. To facilitate a more meaningful comparison of published data, we suggested that when extremophiles are described, that the temperature at which pH values were determined and the pH meter was calibrated are indicated as a superscript, e.g., pH^{60C}.

The pH ranges at which isolated microorganisms have been reported to grow range from slightly below pH 0 (aerobic thermophilic archaea) to around pH 12 (aerobic mesophilic bacteria, yeast). 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. Recently, the descriptions of three facultative anaerobic archaea, *Sulfurococcus mirabilis*, *S. yellowstonii*, and *Stygiolobus azoricus* (for citations see 16) have shown that this niche that was previously assumed to be exclusive is inhabited not only by the obligately aerobic archaea. It is interesting to note that no anaerobic eubacterium has yet been found which at or above 50^{o}C can grow below pH^{25C} 3.0, despite the fact that many geothermally heated features are more acidic than this. The slightly acidophilic

Thermoanaerobacterium aotearoense with a pH_{opt}^{60C} of 5.2 (growth range pH^{60C} 3.8 to 6.8) exhibits the most acidic pH optimum among the anaerobic thermophiles [7].

Presently, the most alkaliphilic prokaryotes are mesophilic bacteria belonging to the phylogenetic branch of *Bacillaceae* and archaeal isolates related to *Natronobacterium* and *Natronococcus*. Recently, the new genus *Bogoriella* and the novel species, *Dietzia natronolimnaios* have been described. More novel genera are expected to follow (Brian Jones, this volume). Again, so far(!), no truely thermophilic (T_{opt} > 60°C) alkaliphilic *Bacillus* species have been described. The highest growth temperature (T_{max}) for an aerobic bacterial alkaliphile growing above pH^{25C} 10 is 57°C (Bacillus sp. strain 221 [4]) and 63°C for an anaerobic bacterial alkaliphile (*Clostridium paradoxum*, pH^{55C}_{max} 10.4; pH^{25C}_{max} 11.3 [5,2]).

Alkalithermophilic anaerobes

The alkalithermophile subset is only a small group among the described extremophiles, although the author believes this is just the "tip of the iceberg" of existing alkalithermophiles. At this time no general conclusion about this group can be drawn, except that the distribution and the environments from which the various bacteria have been isolated are diverse. Apparently, little attention has been paid in the past to obtaining microorganisms able to grow at high pH and elevated temperatures. One possible reason is that it was generally assumed that microorganisms could not grow at elevated temperatures (e.g., above 60°C) and alkaline conditions (pH values above pH 10) since such conditions would place too great a strain on the stability and integrity of cell walls and membranes and energy metabolism. This assumption is obviously not true. Whether alkalithermophiles can be found in all groups remains to be seen. No aerobic archaean alkalithermophiles are yet known; the most alkaline pH_{opt} for aerobic thermophilic archaea is around 7.0, reported for *Aeropyrum pernix*. Among the aerobic bacterial thermophiles are a few with pH_{opt} around 8.5. The most alkaliphilic <u>an</u>aerobic thermophilic archaea are *Methanohalophilus zhilinae* (T_{max} 55°C pH_{max} <10.5, pH_{opt} 9.2) and *Methanobacterium thermoflexum* (T_{opt} 55°C; pH_{opt} 7.9-8.2); *M. wolfei* and some *M. thermoautotrophicum* strains previously described as *M. thermoalkaliphilum* (T_{max} <73°C, pH_{max} <10.0; pH_{opt} 7.5 to 8.5) (for references see 16). No extreme alkaliphilic, obligately <u>an</u>aerobic bacterial mesophiles have been validly published, but unidentified obligately anaerobic isolates growing as high as pH 11.3 [10] have been mentioned. The most alkaliphilic mesophile (validly published) bacterium growing under anaerobic conditions is the facultatively anaerobic *Amphibacillus xylanus*, growing between pH 8.0 and 10.0. Thus it was somewhat surprising to find the thermophilic anaerobic bacterium *C. paradoxum* able to grow at even more alkaline conditions: up to pH^{25C} 11.1 (pH^{60C} 10.3). *Thermopallium natronophilum* (*Thermotogales*) from the Kenyan-Tanzanian Rift Valley (East Africa [8]) is able to grow fermentatively at 70°C and pH^{25C} 10 (Brian Jones, pers. comm.).

Similar to most investigated microorganisms, the alkalithermophiles have very distinct pH_{opt} ranges of less than 0.5 pH units. However, some bacteria have an unusually wide pH range for their pH^{opt}, e.g., 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 [18].

Phylogenetically, all of the anaerobic alkalitolerant and alkalithermophilic bacteria we have isolated in our laboratory belong to the Gram-type positive bacillus-clostridia subphylum. In accordance with their difference in physiological properties, they are all well separated from each other in phylogenetic trees based on 16S rDNA sequence analysis.

Distribution of alkalithermophiles

Alkaliphilic environments have been reviewed previously (see Jones, this volume and cited therein). We have isolated alkalithermophiles and alkalitolerant thermophiles 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 [16]. This is not surprising since many thermophiles, especially sporulating species, have been isolated from many mesobiotic and psychrobiotic environments [15].

The spore-forming *Clostridium paradoxum* and the non-spore-forming *C. thermoalkaliphilum* are the most alkaliphilic (pH_{max}^{25C} of 11.1) anaerobic thermophiles. Surprisingly, they were isolated from various mesobiotic sewage facilities across the USA and from sewage plants from three continents (unpubl. results). The observed macroenvironmental temperature and pH values in several of these plants were never found to be above 35^{o}C and pH 7.8. Despite that the macroenvironmental parameters suggest that they would not provide growth conditions for these alkalithermophiles, enumerations for *C. paradoxum*- and *C. thermoalkaliphilum*-like bacteria revealed concentrations of up to 10,000 cells per ml in the anaerobic sludge, and in the aeration pools at up to 1,000 cell per ml sample [5]. Interestingly, we could not isolate them either from sediments or water samples of the Oconee river receiving the discharge of the Athens, GA (USA) sewage plant or from geothermally heated features of Yellowstone National Park, New Zealand, Italy, or Iceland. Only recently, we were able to isolate one strain belonging to *C. paradoxum* from one hot spring in Rotorua, New Zealand which had received grey water in the past, but –so far –not from the other springs. This 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 such as *Thermoanaerobacter thermohydrosulfuricus, C. paradoxum* should be widely distributed. *C. thermoalkaliphilum,* physiologically similar to *C. paradoxum,* except for being non-sporulating and motile throughout the different growth stages, has been isolated only from an Atlanta, GA, (USA) sewage plant [6].

The less alkaliphilic (pH_{max}^{60C} 10.2; pH_{opt}^{60C} 8.5), thermophilic (T_{max} 66^{o}C) bacterium *Anaerobranca horikoshii* has only been isolated from various springs located in the relatively restricted, geothermally heated area of less than 1 km^2 behind Old Faithful Hotel and the Ranger Station although samples of other hot springs within Yellowstone National Park, USA were tested. Notably, the pH-values of the *A. horikoshii* containing hot springs ranged from 8.7 down to 5.8 [3].

The only two alkalitolerant and alkaliphilic thermophilic anaerobes which have been exclusively isolated from an alkaline thermobiotic environment are the non sporulating *Thermosyntropha lipolytica* (and isolates similar to the three strains published) together with alkalitolerant strains of *M. thermoautotrophicum* [13] and the sporulating *'Thermoalcalibacter bogoriae'* (pH_{opt} 9.5; T_{opt} 50-55^{o}C [9]) from Lake Bogoria (Africa).

In contrast to all above described species, the alkalitolerant (pH_{max}^{66C} 9.5, pH_{opt}^{66C} 8.5) but more thermophilic (T_{max} 75°C) non sporulating *Thermobrachium celere*, has been found ubiquitously in nearly all tested sediments including mesobiotic river sediments from Argentina and Germany and lake sediments in the USA (including the alkaline Mono Lake, 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 *Tb. celere*. The habitats of other anaerobic alkalitolerant thermophiles are similar to those of the above-mentioned bacteria.

In summary, the anaerobic alkalithermophilic and alkalitolerant thermophilic bacteria are found frequently in non-alkaline environments and are as a group ubiquitous, although some species may be endemic to specific soda lakes or other specific environments. Thus, in response to the above question regarding biogeography and the quote from Bass Becking, some anaerobic alkalithermophiles are apparently everywhere, whereas others seem to be more endemic to specific environments. Alkalithermophiles are not the most alkaline bacteria nor the most thermophilic anaerobes, but they are the most thermophilic ones among anaerobic thermophiles or the most alkaliphilic ones among anaerobic alkaliphiles 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 (Table 1) and 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.

Table 1. Doubling times (t_d) of alkalithermophilic and alkalitolerant anaerobic thermophiles

	T_{opt}, (°C)	pH_{opt}^{25C}	t_d (min)
Clostridium paradoxum	56	10.1	16
C. thermoalcaliphilum	51	10.0	19 (- 21)*
Anaerobranca horikoshii	57±2*	9.0±0.2*	36 (- 70)*
Thermobrachium celere	66±2*	9.0±0.4*	10 (- 15)*
Thermotorquens fastidiosum'	66	8.6	30
Thermosyntropha lipolytica	64±2*	8.6±0.3*	60 (- 70)*

*Values for the doubling time (td) depend on the strain tested

References

1. Baas-Becking LMG. In:Geobiologie of inleiding tot de milieukunde. The Hague: WP van Stockum, NV Zoon,1934; pp. 12-15.
2. Cook GM, Russell JB, Reichert A, Wiegel J (1996) The intracellular pH of *Clostridium paradoxum*, an anaerobic, alkaliphilic and thermophilic bacterium. Appl. Environ. Microbiol. 62:4576-4579
3. Engle M, Li Y, Rainey F, DeBlois, S, Mai V, Reichert A, Mayer F, Messmer P, Wiegel J (1996) *Thermobrachium celere*, gen. nov., sp. nov., a fast growing thermophilic, alkalitolerant, and proteolytic obligate anaerobe. Int. J. Syst. Bacteriol. 46:1025-1033
4. Horikoshi K. 1990. Microorganisms in alkaline environments. Kodansha, Tokyo and literature cited therein.
5. Li Y, Mandelco L, Wiegel J (1993) Isolation and characterization of a moderately thermophilic anaerobic alkaliphile, *Clostridium paradoxum*, sp. nov. Int. J. Syst. Bacteriol. 43:450-460.
6. Li Y, Engle M, Mandelco L, Wiegel J (1994) *Clostridium thermoalcaliphilum* sp. nov., an anaerobic and thermotolerant facultative alkaliphile. Int. J. Syst. Bacteriol. 44:111-118.
7. Liu S-Y, Rainey FA, Morgan HW, Mayer F, Wiegel J (1996) *Thermoanaerobacterium aotearoense*, sp. nov., a slightly acidophilic, anaerobic thermophile isolated from various hot springs in New Zealand and emendation of the genus *Thermoanaerobacterium*. Int. J. Syst. Bacteriol. 46: 388-396
8. Meijer D, van Steenbergen R, Jones B (1996) Isolation and characterization of a novel alkaliphilic thermophilic bacterium from the soda lake environment belonging to the order *Thermotogales*. Conference Abstracts Thermophiles '96. Internat. Conference on "The Biology, Ecology, and Biotechnology of Thermophilic Microorganisms" September 1996, Athens, GA (USA) Abstr. # P1.A.4. University of Georgia.
9. Prowe, SG. (1996) Characterization of extracellular amylolytic enzymes and sodium coupled energy transduction of a newly isolated thermoalkaliphilic bacterium *Thermoalacalibater bogoriae*. Ph.D. dissertation. Technische Univ. Hamburg Harburg Germany.
10. Souza KA, Deal PH, Mack HM, Turnbill CE (1974) Growth and reproduction of microorganisms under extremely alkaline conditions. Appl. Microbiol. 28:1066-1968.
11. Staley JT (1997) Biodiversity:are microbial species threatened. Curr.Opinion in Biotechnol. 8:340-345
12. Stetter K O (1996) Hyperthermophilic Prokaryotes. FEMS Microbiol. Reviews, 18, 149-158
13. Svetlitshnyi V, Rainey F, Wiegel J (1996) *Thermosyntropha lipolytica* gen. nov. , sp. nov., a lipolytic, anaerobic, organoheterotrophic, alkalitolerant thermophile utilizing short- and long chain fatty acids in syntrophic co-culture with a methanogen. Int. J. Syst. Bacteriol. 46:1131-1137
14. Wiegel J (1990) Temperature spans for growth: A hypothesis and discussion. FEMS-Microbiol. Rev. 75:155-170.
15. Wiegel J (1992) The obligately anaerobic thermophilic bacteria. Chapter 6 In: Thermophilic Bacteria. J. K. Kristjansson (ed.), CRC-Press, Boca Raton, FL, pp. 105-184.

16. Wiegel, J (1998) Anaerobic alkalithermophiles, a novel group of extremophiles. Extremophiles 2:257-267 and literature cited therein.
17. Wiegel, J. (1998a) Lateral gene exchange, an evolutionary mechanism for extending the upper or lower temperature limits for growth of an microorganism? A hypothesis. In: Thermophiles: Key to Molecular Evolution and Origin of Life.(J. Wiegel and M. Adams (Eds) Chapter 11.Taylor & Francis, London. pp 177-185.
18. Wiegel J, Ljungdahl LG (1982) *Thermoanaerobacter ethanolicus* gen. nov., spec. nov., a new extreme thermophilic, anaerobic bacterium. Arch. Microbiol. 128, 343-348.