NOVEL ANAEROBIC THERMOPHILIC BACTERIA;

INTRASPECIES HETEROGENEITY AND BIOGEOGRAPHY OF

THERMOANAEROBACTER ISOLATES FROM THE KAMCHATKA PENINSULA,

RUSSIAN FAR EAST

by

ISAAC DAVID WAGNER

(Under the Direction of Juergen Wiegel)

ABSTRACT

Thermophilic anaerobic prokaryotes are of interest from basic and applied scientific perspectives. Novel anaerobic thermophilic taxa are herein described are *Caldanaerovirga acetigignens* gen. nov., sp. nov.; and *Thermoanaerobacter uzonensis* sp. nov. Novel taxa descriptions co-authored were: *Thermosediminibacter oceani* gen. nov., sp. nov and *Thermosediminibacter litoriperuensis* sp. nov; *Caldicoprobacter oshimai* gen. nov., sp. nov. More than 220 anaerobic thermophilic isolates were obtained from samples collected from 11 geothermal springs within the Uzon Caldera, Geyser Valley, and Mutnovsky Volcano regions of the Kamchatka Peninsula, Russian Far East. Most strains were phylogenetically related to *Thermoanaerobacter uzonensis* JW/IW010^{T}, while some were phylogenetically related to *Thermoanaerobacter siderophilus* SR4^{T}. Eight protein coding genes, *gyrB*, *lepA*, *leuS*, *pyrG*, *recA*, *recG*, *rplB*, and *rpoB*, were amplified and sequenced from these isolates to describe and elucidate the intraspecies heterogeneity, α- and β-diversity patterns, and the spatial and physicochemical correlations to the observed genetic variation. All protein coding genes within

the *T. uzonensis* isolates were found to be polymorphic, although the type (i.e., synonymous/ nonsynonymous substitutions) and quantity of the variation differed between gene sequence sets. The most applicable species concept for *T. uzonensis* must consider metapopulation/ subpopulation dynamics and acknowledge that physiological characteristics (e.g., sporulation) likely influences the flux of genetic information between subpopulations. Spatial variation in the distribution of *T. uzonensis* isolates was observed. Evaluation of *T. uzonensis* α-diversity revealed a range of genetic variation within a single geothermal spring. β-diversity measurements revealed that while most of the molecular variance came from inter-regional comparisons, high diversity measures between populations within a region were also observed. Between geothermal springs from different regions, *T. uzonensis* genetic divergence was correlated with an increase in spatial separation. However, the trend was not observed when only the isolates between the geothermal springs within a region were considered. When 27 physicochemical properties from four geothermal springs in the Uzon Caldera were matched to a corresponding biological distribution pattern, high rank correlation values, calculated as Spearman's ρ, were observed. Together, these analyses suggest that the spatial variation of *T. uzonensis* was influenced by both environmental differences and spatial separation.

INDEX WORDS: thermophiles, microbial biogeography, microbial diversity, anaerobic thermophiles, biogeochemistry

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DEDICATION

For their constant support and encouragement, this dissertation is dedicated to my parents, Kim and David Wagner.

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CHAPTER 1

DISSERTATION STRUCTURE

The eight chapters and two appendices within this dissertation primarily relate to microbial diversity. Chapter 2 is a review of the literature and focuses on the diversity of validly published anaerobic thermophilic prokaryotes, and Chapter 8 is the concluding chapter where the major results of this dissertation are connected together. The remaining sections deal with two subtopics of microbial diversity: descriptions of novel bacteria, and quantifying and analyzing the spatial patterns of diversity observed for members of a particular phylogenetic clade.

Bacteria and archaea hold a large portion of Earth's carbon, have significant roles in biogeochemical cycles, and constitute a large fraction of life's genetic diversity (Whitman et al., 1998). Although the majority of microorganisms from the environment are presently uncultivated, culturing microorganisms, in particular novel taxa, is an indispensable requirement for the description of microbial diversity (Palleroni, 1997). Additionally, isolation and cultivation continues to be the foundation for testing the metabolic abilities of prokaryotes. Four chapters within this dissertation concern the description of novel anaerobic thermophiles. *Caldanaerovirga acetigignens* gen. nov., sp. nov. is described in Chapter 3; and *Thermoanaerobacter uzonensis* sp. nov. in Chapter 4. *Thermosediminibacter oceani* gen. nov., sp. nov. and *Thermosediminibacter litoriperuensis* sp. nov. are described in Appendix A, and *Caldicoprobacter oshimai* gen. nov., sp. nov., and the proposal of *Caldicoprobacteraceae* fam. nov. is described in Appendix B.

A fundamental issue in ecology relates to understanding the diversity of life and its distribution patterns at various spatial scales. Therefore, knowledge about the spatial patterns of diversity are critical to deciphering the forces shaping and maintaining the diversity of life (Zhou et al., 2008; and references therein). The diversity of a set of *Thermoanaerobacter* strains isolated from Kamchatkan geothermal springs are described in Chapters 5, 6, and 7.

The Kamchatka Peninsula, located in the Russian Far East, is a dynamic convergent margin where the subducting Pacific tectonic plate creates a region of high volcanic activity and related geothermal activity. The Uzon Caldera and nearby Geyser Valley regions of Kamchatka contain a variety of geothermal features and this location was chosen as a National Science Foundation-funded Microbial Observatory. An overall goal of the international and interdisciplinary Kamchatka Microbial Observatory was to correlate geochemical and microbial interactions in terrestrial geothermal systems. The isolation of >220 *Thermoanaerobacter* strains and the sequencing of the 16S rRNA and eight universally conserved protein coding genes are described in Chapter 5. This also includes the discussion concerning what the observed intraspecies heterogeneity suggests concerning the most applicable species concept for *T. uzonensis*. The population structure and spatial patterns of diversity based on the protein coding gene sequence heterogeneity of the *T. uzonensis* and *T. siderophilus* isolates are then described in Chapter 6. Lastly, the relationship between *T. uzonensis* genetic divergence and the spatial separation of the corresponding geothermal springs, and the relationship between the biological distribution pattern and the physicochemical properties of a set of geothermal springs located within the Uzon Caldera are examined in Chapter 7.

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CHAPTER 2

DIVERSITY OF THERMOPHILIC ANAEROBES[1]

[1] Wagner, I. D. & Wiegel, J. (2008). Diversity of thermophilic anaerobes. *Ann N Y Acad Sci* **1125**, 1-43.

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ABSTRACT

Thermophilic anaerobes are the group of *Archaea* and *Bacteria* which grow optimally at temperatures of 50 °C or higher and do not require the use of O_2 as terminal electron acceptor for growth. The prokaryotes with this type of physiology are studied for a variety of reasons, including: 1) to understand how life can thrive under extreme conditions, 2) for their biotechnological potential, and 3) because anaerobic thermophiles are thought to share characteristics with the early evolutionary life forms on Earth. Over 300 species of thermophilic anaerobes have been described; most were isolated from thermal environments, but some are from mesobiotic environments, and others are from environments with temperatures below zero. Within this overview the authors outline the phylogenetic and physiological diversity of thermophilic anaerobes as presently known; the purpose being to convey the incredible diversity and breadth of metabolism within this subset of anaerobic microorganisms.

INTRODUCTION

Bacteria and *Archaea* which grow optimally at elevated temperatures and do not require oxygen for growth are described as thermophilic anaerobes and the taxa having this physiology are of interest from basic and applied scientific perspectives. Since these prokaryotes grow optimally at elevated temperatures, thermophilic anaerobes are designated ‘extremophiles’ and are studied to understand how life can thrive in environments previously considered inhospitable to life. Such environments include volcanic solfatares and hot springs high in sulfur and toxic metals as well as abyssal hydrothermal vents with extremely high pressure and temperatures (Stetter, 1999, 2006a). Isolated species of thermophilic anaerobes include astonishing forms of life: for example, the mothercell of the alkalithermophile *Clostridium paradoxum* becomes

highly motile when sporulating (Li et al., 1993). and *Moorella thermacetica*-like strains have exceptionally heat-resistant spores with D_{10}-times of nearly 2 hours at 121 °C (Byrer et al., 2000). Also, *Pyrolobus fumarii* grows optimally at 106 °C (Blochl et al., 1997), and a recently isolated *Methanopyrus kandleri*-like strain grows at 122 °C under increased pressure (Takai et al., 2007). *Thermobrachium celere* strains have doubling times of about 10 minutes under optimal conditions (Engle et al., 1996), while the triple extremophile *Natranaerobius thermophilus* grows optimally simultaneously at high temperature (53 °C), high pH (9.5), and high salt concentration (3.3-3.9 M Na^+) (Mesbah et al., 2007b). Analyses of the biodiversity and patterns of biodiversity within thermal environments is an area of active research and continually expands as technology allows for novel approaches and more detailed analyses. Additionally, their thermostable enzymes, among other characteristics, make thermophilic anaerobes of significant interest for their biotechnological potential (Wiegel and Ljungdahl, 1986; Vieille and Zeikus, 2001; Unsworth et al., 2007).

Thermophilic anaerobes also attract research attention as it is assumed that they have properties similar, in various aspects, to those of the early evolutionary life forms on Earth.[2] There is little doubt that the first forms of life on Earth occurred at a time when significantly less oxygen was present. Apparent biogenic signatures have been dated to 3.85-3.8 billion years ago (Ga) and complex microfossil communities are dated to 3.5-3.4 Ga, while the accumulation of oxygen happened later, approximately 2.1 -2.3 Ga (Kasting, 1993; Baross, 1998). The early forms of life from which everything else then evolved, i.e., the progenotic life forms, were

[2] Further discussion of thermophiles in regard to the origin of life can be found in the conference proceeding book: Wiegel, J. and M. W. W. Adams. 1998. Thermophiles: The Keys to Molecular Evolution and the Origin of Life? Taylor & Francis Ltd. London.

therefore anaerobes (Canfield et al., 2006). Considering Earth history and these progenotic life forms, the authors believe that the present day life has several roots as proposed by Kandler (Kandler, 1998). In addition to having an anoxic origin, the present mainstream holds the opinion that life began at elevated temperatures, and was consequently thermophilic. While some contest this view of a thermophilic origin of life and postulate that prebiotic chemistry implies the emergence of living systems at a low temperature or a rapid selection for hyperthermophiles during the late bombardment (Miller and Lazcano, 1995; Forterre, 1998; Miller and Lazcano, 1998), the possibility of evolution from mesophily to hyperthermophily has been considered, but many believe the latter scenario is improbable (Wächtershäuser, 1998). Thus, the authors and most others posit that life began around 80 °C on clay or iron-sulfur mineral surfaces in shallow pools (Russell et al., 1998).

While the first forms of life no longer exist, natural thermal environments do still exist and some have properties similar to those environments where life assumingly first began. Many of these environments are characteristically anaerobic or have low levels of oxygen. The anaerobic feature can stem from a number of factors: remoteness of the environment from the atmosphere, low solubility of oxygen in water at elevated temperatures, hypersalinity, inputs of reducing gasses such as H_2 and H_2S, or the consumption of oxygen by aerobic microorganisms on or near the surface (Brock, 1970; Stetter, 1996). Broadly, natural thermobiotic environments are of terrestrial, marine, or subsurface nature. Terrestrial or continental geothermally heated futures include hot springs, geysers, solfatares, mud pools (‘mud pots’), and some solar-heated environments. One example, the Yellowstone National Park, USA, contains the highest concentration of terrestrial geothermal features on Earth (Smith and Siegel, 2000). Other locales of notable terrestrial thermal activity include Japan, New Zealand, Iceland, Hawaiian volcanoes,

various South Pacific Islands located at The Ring of Fire, and the Kamchatka Peninsula in the Russia Far East. Thermobiotic marine environments extend from geothermally-heated beaches to shallow hydrothermal vents to abyssal hydrothermal vents where water escapes at temperatures over 300 °C (Stetter, 2006b). Two examples of geothermally heated beaches are the one on the North Island of New Zealand and on SavuSavu (Fiji Islands); the geothermally heated spots are exposed at low tide and covered with water at high tide. Some shallow thermal marine systems where thermophilic anaerobes have been isolated include Volcano shore of Sicily and Lucrino Beach near Naples, Italy; Palaeochori Bay of Milos, Greece; coastal hot springs of Ibuski, Kangoshima Prefecture, Japan; marine solfataric fields of Kraternaya Cove, Ushishir Archipelago, Northern Kurils; a hydrothermal field at a depth of about 100 m at the Eyjafjörður fjord, Northern Iceland; and the Kolbeinsey Ridge located at a depth about 105 m, north of Iceland. First described in the 1970s on the Galapagos Rift (Corliss et al., 1979), deep-sea hydrothermal vent regions have been found and studied in the Pacific and Atlantic Oceans, e.g.; the 'Rainbow,' 'Snakepit,' and Logatchev hydrothermal vent regions of the Mid-Atlantic ridge; the 9 °N, 13 °N, and 21 °N deep-sea hydrothermal vent systems of the East Pacific Rise; the Iheya Ridge and Yonaguni Knoll IV of the Okinawa Trough; the Guaymas Basin, Gulf of California and recently hydrothermal vents were discovered as far north as the Mohns Ridge, Norway (Reed, 2006). Oil reservoirs, mines, and geothermal aquifers are examples of subsurface environments thermophiles populate. Species of the genera *Geotoga* and *Petrotoga* (family *Thermotogaceae* {*B*34}[3]) have thus far only been found in deep subsurface oil reservoirs, based

[3] The designation within the brackets corresponds to the particular thermophilic anaerobe-containing family as shown within Fig. 1., a 16S rDNA-based phylogenetic tree, and as listed within Table 1, which lists the validly described thermophilic anaerobes.

on this, it has been proposed that these taxa represent typical indigenous *Bacteria* in this particular ecosystem (Ollivier and Cayol, 2005). Geothermal aquifers such as The Great Artesian Basin of Australia, are regarded as being markedly different from volcanically-related hot springs, in that they have low flow rates and long recharge times (around 1000 years) which affect the microbial populations therein (Kimura et al., 2005b). Besides natural thermal environments, thermophilic anaerobes are also found within anthropogenically heated environments including coal refuse piles, compost heaps which not only contain sporeforming species but also methanogenic *Archaea* (Thummes et al., 2007). and nuclear power plant effluent channels. Contrary to expectations, thermophilic anaerobes have also been isolated from mesobiotic and even psychrobiotic environments: Two *Thermosediminibacter* species were isolated from ocean sediments of the Peru Margin at temperatures at or below 12 °C (Lee et al., 2005), uncharacterized *Thermoanaerobacter* species have been isolated from melted snow from Antarctica (J. Wiegel, unpublished results), alkalithermophiles have been isolated from many river sediments and wet meadow (Engle et al., 1996), and *Methanothermobacter thermoautotrophicus* and other methanogens can readily be found in lake sediments (e.g., Lake Mendota, Wisconsin, USA) and rivers in Northern Germany (Wiegel et al., 1981). Possible reasons for the presence of thermophilic anaerobes in environments where, considering their physiological properties they ought to not grow, include that the microorganisms are present but not growing in these environments, that they dispersed only transiently from other thermal environments, or, as the authors propose, that they are surviving in order to take advantage of temporary thermal microniches that become available when proteinaceous biomass is degraded (Engle et al., 1996).

Undoubtedly, the wide spectrum of properties of environments thermophilic anaerobes inhabit have been, and continues to be, of significance in the evolution and diversification of these microorganisms. The evolutionary implications are a fascinating topic alone. Nevertheless, the result of this diversification, i.e., the diversity of thermophilic anaerobes, is examined within this chapter. The goal herein is to provide an overview of the diversity of thermophilic anaerobes from phylogenetic and physiological perspectives and where appropriate, highlighting unique and noteworthy taxa.

MEASURING THE DIVERSITY OF THERMOPHILIC ANAEROBES

While biological diversity is usually considered to be a combination of two qualities of a population, species richness (the number of different kinds), and species evenness (the relative numbers of those present) (Magurran, 2004). the focus herein will primarily be species richness.. As such, this overview on the diversity of thermophilic anaerobes will be primarily limited to describing diversity as a function of what is known of these *Bacteria* and *Archaea* through studies on axenic cultures. The caveat is that most of the prokaryotes from any environment, including the thermal environments previously discussed, are presently uncultured (Hugenholtz, 2002). Consequently, our understanding of prokaryotic biodiversity and physiological properties are based on a very limited knowledge. Although culture-independent studies are not the focus of this chapter, such studies, for example analyses of environmental16S ribosomal RNA gene sequences, metagenomics (i.e., the sequencing of DNA isolated from an environment), and sequences of genes encoding particular functional proteins help, address this major limitation.

Culture-independent studies of terrestrial thermobiotic environments include sites in Yellowstone National Park (Reysenbach et al.; Barns et al., 1994; Hugenholtz et al., 1998;

Reysenbach et al., 2000b; Blank et al., 2002), hot springs of Japan (Yamamoto et al., 1998), hot springs of New Zealand (Hetzer et al., 2007), hot springs of Greenland (Roeselers et al., 2007), subterranean and terrestrial Icelandic hot springs (Skirnisdottir et al., 2000; Marteinsson et al., 2001), thermal subterranean Great Artesian Basin of Australia (Kimura et al., 2005a), and sun heated salt Lakes (Wadi An Natrun) in Egypt (Mesbah et al., 2007a), to name a few. Examples of deep-sea hydrothermal sites studied by culture-independent means include the Manus Basin near Papua New Guinea (Takai et al., 2001), the Suiyo Sea Mount and Myojin Knoll of the Ogasawara area and the hydrothermal fields at Iheya Basin of the Okinawa area, Japan (Takai and Horikoshi, 1999). Besides strictly culture-independent techniques, additional methods have been developed or employed to study the thermophilic anaerobes inhabiting thermobiotic environments, e.g., an in situ growth chamber deployed at a hydrothermal vent field revealed novel lineages (Reysenbach et al., 2000a). 16S rRNA hybridization probes developed for members of the *Thermoanaerobacter*/ *Caldanaerobacter* clade detected *Thermoanaerobacter* members from deep-sea hydrothermal regions, an environment not previously known to harbor *Thermoanaerobacter* species (Subbotina et al., 2003). Similarly, *Desulfotomaculum* 16S rDNA hybridization probes were employed to measure the change in abundance in thermophilic anaerobic digesters among other sites (Hristova et al., 2000).

Thermophilic anaerobes in pure culture are characterized through the polyphasic approach wherein phenotypic and genotypic/phylogenetic properties are examined (Vandamme et al., 1996; Stackebrandt et al., 2002). Phenotypic characteristics of interest for this discussion especially include oxygen relationships and metabolic properties such as energy production and carbon assimilation. Group-defining properties such as temperature growth range (e.g., T_{min}, T_{opt}, and T_{max}) and pH growth range (e.g., pH_{min}, pH_{opt}, and pH_{max}) are particularly important. These

values should be determined by measuring the doubling times over the range for growth specifically noting where growth was obtained and where growth was not obtained. (For example, in the authors' laboratory a shaking gradient incubator having 1-3 °C intervals is used to determine the temperature growth profile for a strain). Other properties such as salt tolerance and response to pressure are of importance, when considering thermophilic anaerobes from habitats such as hypersaline lakes and deep-sea hydrothermal regions. While genotypic characteristics such as G+C mol% and DNA-DNA relatedness between strains have been studied since the 1960s, in the past 20 years analysis of the 16S rRNA gene sequence has become standard and the analysis of housekeeping genes and whole-genome sequencing of prokaryotes is becoming increasingly common (Santos and Ochman, 2004). Known thermophilic anaerobes with available sequenced genomes are designated within Table 1 with the § symbol, but the continually increasing number can be obtained from the NCBI Taxonomy Database: http://www.ncbi.nlm.nih.gov/Taxonomy/. Analysis of the 16S rRNA gene sequence-based phylogenetic tree (Fig. 1), reveals that presently (November 2007), thermophilic anaerobes reside in 51 known prokaryotic families (Table 1). Still, this will undoubtedly change as novel thermophilic anaerobes are isolated and the subsequent phylogenetic reorganization of taxa proceeds.

OXYGEN RELATIONSHIP

As noted previously, many thermobiotic environments are either anaerobic or low in oxygen. Therefore, the expectation would be that thermophiles are predominantly anaerobic and indeed, this is what is seen. Most axenic thermophiles are anaerobic, or facultative aerobes (Brock, 1970; Stetter, 1996). The authors' definition of an anaerobe is the inability to use O_2 as

the terminal electron acceptor, even if they can grow in the presence of O_2 (i.e., an O_2-tolerant anaerobe). Facultative aerobes have the ability to utilize oxygen as a terminal electron acceptor and some obligately anaerobic thermophiles can survive exposure to oxygenic atmospheres, especially if they are metabolically inactive (e.g., at suboptimal temperatures, or with the absence of metabolizable substrates). In the absence of oxygen, respiring anaerobic or facultative aerobic thermophiles can utilize, via energy production through electron transport phosphorylation, a variety of compounds as electron acceptors, including: CO_2, CO, NO_3^-, NO_2^-, NO, N_2O, SO_4^{-2}, SO_3^{-2}, $S_2O_3^{-2}$, S^0, Fe (III), Mn (IV), and Mo (VI) (Amend and Shock, 2001). The energy gleaned from these respiratory pathways is in addition to the energy produced anaerobically through substrate level phosphorylation.

An examination of families of thermophilic anaerobes reveals that most (38 of 51) are presently composed solely of anaerobic taxa. While a majority of the cultured thermophilic *Bacteria* and *Archaea* are anaerobic, a relevant question is whether this observation accurately reflects the ecological situation in thermobiotic environments. Although this question is largely beyond the scope of this present chapter, culture-independent analyses have revealed that members of the *Aquificales*, (*Aquificaceae* {*B*32}), appear to be ubiquitous in terrestrial hot springs of Yellowstone National Park, as well as in Japan, Iceland, and Kamchatka. It has been postulated that these *Aquificales*, in particular the obligately aerobic *Thermocrinis ruber*-like microorganisms, are the primary producers (via chemoautotrophic hydrogen oxidation) in these ecosystems (Huber et al., 1998b; Blank et al., 2002).

TEMPERATURE RELATIONSHIP

Etymologically, thermophiles are those which 'love' heat, and *Bacteria* and *Archaea* with this physiology are further categorized according to their optimal (T_{opt}) and maximal (T_{max}) growth temperatures. The authors classify *Bacteria* and *Archaea* that have T_{opt} 50–64 °C as (moderate) thermophiles, those that have T_{opt} 65–79 °C as extreme thermophiles, and prokaryotes with $T_{opt} \geq 80$ °C as hyperthermophiles (Wiegel, 1990; Stetter, 1996; Wiegel, 1998a; Cavicchioli and Thomas, 2000). Although not considered true thermophiles, *Bacteria* and *Archaea* that grow optimally at mesophilic temperatures but have a T_{max} >50 °C are described as thermotolerant.

Thermophilic prokaryotes able to grow over a 35–40 °C temperature span are considered temperature-tolerant thermophiles (Wiegel, 1990). To the authors' knowledge, the record widest temperature growth range is 22-75 °C by a *Methanothermobacter thermautotrophicus*-like strain isolated from river sediment (J. Wiegel, unpublished). By comparison, some thermophilic anaerobes are reported with especially narrow temperature growth ranges; e.g., 42-55°C for *Anaerolinea thermolimosa* (Yamada et al., 2006), and 50-60 °C for *Anaerolinea thermophila* (Sekiguchi et al., 2003), two species from the family *Anaerolinaceae* {*B*27}.

Hyperthermophiles with $T_{opt} \geq 80$°C and T_{max} >100 °C, were first isolated by Stetter from the hot vents at Vulcano of the coast of Sicily, Italy, in 1981 (Stetter, 2006b). Isolates growing optimally above 100 °C are often found at deep-sea vents, or deep in terrestrial hot spring channels and sediments (Adams, 1994). This 'deep' need is ascribed to the increased pressure allowing for water to remain in liquid form at temperatures above 100 °C. Within the *Bacteria*, only the *Thermotogaceae* {*B*34} and *Aquificaceae* {*B*32} lineages contain hyperthermophilic members. Within the *Archaea*, taxonomic families containing hyperthermophlic taxa include:

Methanopyraceae {*A*10}, *Methanothermaceae* {*A*11}, *Methanocaldococcaceae* {*A*07}, *Thermococcaceae* {*A*08}, *Archaeoglobaceae* {*A*09}, *Sulfolobaceae* {*A*03}, *Desulfurococcaceae* {*A*01}, *Pyrodictiaceae* {*A*02}, *Thermofilaceae* {*A*04}, and *Thermoproteaceae* {*A*05} (see Table 1). Many species with extremely high temperature growth ranges and T_{opt} belong to the *Pyrodictiaceae* {*A*02}. *Pyrodictium abyssi* with a growth range of 80-110, T_{opt} 97°C (Pley et al., 1991); *Pyrodictium brockii* and *Pyrodictium occultum* both with T_{opt} of 105°C (Stetter et al., 1983); *Hyperthermus butylicus* with a reported T_{opt} 95-107 (Zillig et al., 1991); and *Pyrolobus fumarii* with a growth range of 90-113°C and T_{opt} 106 °C (Blochl et al., 1997). Very recently, Takai provided convincing evidence of a *Methanopyrus kandleri* strain isolated from a deep-sea hydrothermal region capable of growing under increased pressure at 122°C (Takai et al., 2007).

The base of the inferred 16S rRNA gene sequence phylogenetic tree contains hyperthermophilic taxa (Fig. 1). This is one line of evidence for the hypothesis that life evolved at a time of elevated temperature on Early Earth. However, thermophilic anaerobes are also found among branches of the phylogenetic tree containing predominantly mesophiles. These taxa having either evolved after a speciation event, during subsequent phyletic evolution, or they are remnants of the thermophilic origins of the clades. Because of the many specialized properties required for thermophilic life, thermophily is not a trait transferrable via horizontal gene transfer. In cases where thermophilic and mesophilic species exist within a genus, the ability to grow at elevated temperatures occurs via combinations of different properties, including the stabilization of intracellular compounds through binding of cations such as Ca^{2+}, insertion of additional hydrogen bridges in protein structures via the presence of increased acidic amino acids (such as substituting glutamine with glutamic acid), forming more-globular protein structures with additional hydrophobic regions inside, stabilization of membrane fluidity by changes in fatty

acid lengths and branching (Pond and Langworthy, 1987), and changes in proton permeability of the cytoplasmic membranes (van de Vossenberg et al., 1995). In cases where the addition of genes conferred higher growth temperatures, it is assumed that these enzymes removed the bottleneck of otherwise cryptic thermophiles (Wiegel, 1990, 1998b).

pH RELATIONSHIP

The pH growth minimum (pH_{min}), maximum (pH_{max}) and optimum (pH_{opt}) determine whether the prokaryote is characterized as acidophilic (pH_{opt} <5.0), acidotolerant (pH_{min} <4, pH_{opt} circumneutral), neutrophilic (pH_{opt} ~7.0), alkalitolerant (pH_{opt} < 8.5, pH_{max} >8.5) or alkaliphilic (pH_{opt} ≥8.5). The majority of known thermophilic anaerobes are neutrophilic. None of the known thermophilic anaerobic *Bacteria* grow below pH 3.0 (Wiegel, 1998a). To the authors' knowledge, the most acidophilic anaerobic thermophilic *Bacteria* presently are: *Thermoanaerobacterium aotearoense,* pH^{60C} range 3.8–6.8 and $pH^{60C}{}_{opt}$ 5.2 (Liu et al., 1996), *Thermoanaerobacterium aciditolerans* pH range 3.2-7.1, pH_{opt} 5.7 (Kublanov et al., 2007), and *Lebetimonas acidiphila,* pH range 4.2–7.0 and pH_{opt} (Takai et al., 2005). The most acidophilic thermophilic anaerobic *Archaea* belong to the *Thermoplasmataceae* {*A*06} and *Sulfolobaceae* {*A*03} families and nearly all are facultative aerobes, the most acidophilic being *Acidianus sulfidivorans* with pH growth range of 0.35-3.0, and pH_{opt} 0.8-1.4 (Plumb et al., 2007). One exception is the obligately anaerobic *Stygiolobus azoricus* of the *Sulfolobaceae* {*A*03}, which has a pH growth range of 1-5.5 and pH_{opt} 2.5-3 (Segerer et al., 1991).

In contrast to the aerobic and truly acidophilic *Archaea*, and to the alkalithermophilic *Bacteria* (Table 1), only a few known anaerobic thermophilic *Archaea* grow optimally at high pH. All are obligately anaerobic and reside within the *Euryarchaeota* clade; species from the

Methanobacteriaceae {*A*12}, *Methanothermobacter thermoflexus*, with pH_{opt} 7.9-8.1(Kotelnikova et al., 1993; Boone, 2001); from the *Thermococcaceae* {*A*08}, *Thermococcus acidaminovorans* with a pH range of 5-9.5 and pH_{opt} 9.0 (Dirmeier et al., 1998); *Thermococcus fumicolans*, with a growth range of pH 4.5-9.5 and pH_{opt} 8.5 (Godfroy et al., 1996); and *Thermococcus celer*, with pH_{opt} 8.5 (Zillig et al., 1983a); and *Thermococcus alcaliphilus*, with a pH range of 6.5-10.5 and pH_{opt} 9.0 (Keller et al., 1995).

Alkalithermophilic ($pH_{opt}^{55C} > 8.5$; $T_{opt} > 55°C$) anaerobic *Bacteria* are particularly intriguing because of the phylogenetic position of most known taxa and an observed physiological peculiarity. Anaerobic alkalithermophilic *Bacteria* taxa, while physiologically different, almost all belong to the Phylum *Firmicutes* (formerly called the Gram-type positive *Bacillus-Clostridium* group) phylogenetic subbranch {*B*07-14} (Wiegel, 1998a). The one known Gram-type negative exception is the non-validly published and poorly characterized *'Thermopallium natrophilum'* (Duckworth et al., 1996). Anaerobic alkalithermophilic *Bacteria* include: *Clostridium paradoxum*, the most alkaliphilic thermophile with a pH^{60C} growth range of 7-11.1, pH_{opt} 10.1 (Li et al., 1993); *Clostridium thermoalcaliphilum*, pH^{60C} range 7-11, pH_{opt} 9.6-10.1 (Li et al., 1994); as well as members of the genus *Anaerobranca* of the family *Syntrophomonadaceae* {*B*12}. Phylogenetically similar *Thermobrachium celere*-like strains have been isolated from a variety of niches including thermobiotic and mesobiotic and from alkaline and neutrophilic soils and sediments. Isolates from mesobiotic and neutrophilic niches exhibit shorter doubling times (10-15 minutes) compared to isolates from thermobiotic niches (25-40 minutes) (Engle et al., 1996; Wiegel and Kevbrin, 2004). *Natranaerobius thermophilus* (*Natranaerobiaceae* {*B*08}), isolated from an alkaline, hypersaline lakes of the Wadi An Natrun, Egypt, classifies within a novel order of the Class *Clostridia* in the Phylum *Firmicutes*. It is

alkalithermophilic (T_{opt} 53 °C, pH_{opt}^{55C} 9.5) and additionally halophilic growing optimally with 3.3 and 3.9 M Na^+ (Mesbah et al., 2007b). It is important to notice when determining the pH of alkalithermophiles (as well acidothermophiles) that this has to be done with temperature equilibrated electrodes and use of a pH meter standardized with buffers at the elevated temperature range (Wiegel, 1998a).

As with temperature growth ranges, there are thermophilic anaerobes with wide pH growth ranges spanning six or more pH units, e.g., *Thermococcus hydrothermalis* with a pH range of 3.5-9.5 (Godfroy et al., 1997), and *Pyrococcus glycovorans* with a pH growth range of 2.5-9.0 (Barbier et al., 1999). *Thermoanaerobacter ethanolicus* strain JW200 is another interesting example as it has an unusual pH profile with a broad and flat pH optimum from pH 5.5-8.5 (Wiegel and Ljungdahl, 1981). Thermophilic anaerobes have also been discovered with growth ranges of less than one pH unit, e.g., *Pelotomaculum thermopropionicum* with a pH range of 6.7-7.5 (Imachi et al., 2002), *Thermodesulfatator indicus* with a pH range of 6.0-6.7 (Moussard et al., 2004), and *Thermodesulfobacterium hydrogeniphilum* with a pH range of 6.3-6.8 (Jeanthon et al., 2002).

METABOLIC DIVERSITY OF THERMOPHILIC ANAEROBES

A majority of the axenic thermophilic anaerobic *Archaea* and *Bacteria* are chemoorganoheterotrophic, using organic compounds for carbon and energy. However, a variety of other metabolic strategies are seen within the set of thermophilic anaerobic prokaryotes, including chemolithoautotrophy, chemolithoheterotrophy, photoheterotrophy, and photoautotrophy. Considering these metabolisms and the variation that can be found within each type, an astonishing diversity of metabolism is observed within the thermophilic anaerobes.

Within this next section, an overview of commonly observed and unique physiologies of thermophilic anaerobes is provided. Amend and Shock have previously described thermophilic and hyperthermophilic energenic reactions in depth and their work is a key resource for the study of thermophilic metabolisms (Amend and Shock, 2001).

Chemoorganoheterotrophic metabolisms are further categorized, and include glycolytic, cellulolytic, lipolytic, and peptidolytic metabolisms. The Emden-Meyerhof and Entner-Doudoroff pathways are employed by glycolytic thermophilic anaerobes, but a variety of modifications have been discovered, particularly within the *Archaea* (Siebers and Schönheit, 2005). Principal fermentation products formed by glycolytic thermophilic anaerobes include acetate, lactate, ethanol, CO_2, and H_2 The production of ethanol by glycolytic and cellulolytic taxa has previously been studied (Wiegel, 1980). Cellulose is the most abundant renewable natural plant fiber and the degradation of cellulose coupled to the production of 'biofuels' such as ethanol by thermophilic anaerobes is an intensely studied and timely research area. This metabolism involving many unusual enzymes has been studied with axenic cultures as well as in co-culture. An example of the latter is the cellulolytic *Clostridium thermocellum* in culture with the glycolytic *Thermoanaerobacter ethanolicus* (Freier et al., 1988). Besides *Clostridium thermocellum*, other thermophilic species within the genus *Clostridium* are cellulolytic, these are: *C. sterocorium, C. thermolacticum, C. thermocopriae,* and *C. thermopapyrolyticum* (Mendez et al., 1991). Relatively recently, a hyperthermophilic archeaon, *Desulfurococcus fermentans*, was isolated with the ability to grow on cellulosic substrates (Perevalova et al., 2005). As with cellulose-degrading thermophilic anaerobes, xylanolytic thermophilic anaerobes generate interest since the conversion of xylan, a component of plant hemicellulose and the second-most abundant renewable polysaccharide in biomass, to useful products can possibly be coupled to increasing

the efficiency of processing lignocellulose and to production of energy from renewable resources (Biely, 1985). Xylan is widely used among thermophilic anaerobic *Bacteria*, especially among members of the *Firmicutes*. Lipolytic chemoorganotrophic thermophilic anaerobes include *Thermosyntropha lipolytica* which in co-culture syntrophically grows on saturated and unsaturated fatty acids with 4 to 18 carbon atoms and *Desulfurothermus naphthae* which can utilize long-chain fatty acids with 6 to 18 carbon atoms as well as alkanes having 6 to 14 carbon atoms (Svetlitshnyi et al., 1996; Alazard et al., 2003). While chemoorganoheterotrophic metabolisms appear to be common metabolic strategies for the axenic thermophilic anaerobes studied in the lab, the natural substrates for microorganisms are largely unknown (Amend and Shock, 2001); furthermore, culture independent analyses indicate that lithotrophic prokaryotes are the primary producers in certain thermobiotic environments, e.g., hot springs (Hugenholtz et al., 1998).

Among chemolithotrophic pathways, the methanogenic reaction, $4\ H_2 + CO_2 \rightarrow CH_4 + 2\ H_2O$, is well characterized and utilized by thermophilic taxa within the *Methanobacteriaceae* {*A*12}, *Methanothermaceae* {*A*11}, *Methanocaldococcaceae* {*A*07}, and *Methanococcaceae* {*A*13} (Amend and Shock, 2001). Another interesting chemolithotrophic metabolism of anaerobic thermophiles described relatively recently makes use of CO. CO is found as a normal component of escaping volcanic gas, of terrestrial and deep-sea hydrothermal origin (Sokolova et al., 2004a). Several thermophilic anaerobes have indeed been isolated which grow lithotrophically on CO, performing the metabolic reaction $CO + H_2O \rightarrow CO_2 + H_2$. Thermophilic anaerobes known to employ this strategy include *Desulfotomaculum carboxydivorans* (Parshina et al., 2005), *Carboxydothermus hydrogenoformans* (Svetlichny et al., 1991), *Thermolithobacter carboxydivorans* (Sokolova et al., 2007), *Carboxydocella thermautotrophica* (Sokolova et al.,

2002), *Thermincola carboxydiphila* (Sokolova et al., 2005), *Thermincola ferriacetica* (Zavarzina et al., 2007), *Caldanaerobacter subterraneus* subsp. *pacificus* (Fardeau et al., 2004), and *Thermosinus carboxydivorans* (Sokolova et al., 2004b). This same CO-utilizing reaction has also been observed within the *Archaea* in an isolate belonging to the genus *Thermococcus* (family *Thermococcaceae* {*A*08}) (Sokolova et al., 2004a). Another interesting chemolithotrophic strategy is employed by the acetogens using the Wood-Ljungdahl pathway (from the reaction: 4 H_2 + 2 CO_2 →Acetate + 2 H_2O). Both mesophilic and thermophilic taxa (e.g., *Moorella* species) are known to perform this reaction (for additional discussion, see the corresponding chapter within this book [author's note, this refers to: Drake, H.L., Gößner, A.S., and Daniel, S.L. (2008) Old Acetogens, New Light. *Annals of the New York Academy of Sciences* **1125**: 100-128.]).

Chemolithoheterotrophs generate energy chemolithotrophically and assimilate carbon heterotrophically. Thermophilic anaerobes with this metabolism include *Archaea*; *Archaeoglobus profundus*,(Burggraf et al., 1990a) and *Stetteria hydrogenophila* (Jochimsen et al., 1997), and *Bacteria; Desulfotomaculum alkaliphilum* (Pikuta et al., 2000a). *Desulfotomaculum carboxydivorans* (Parshina et al., 2005), *Thermincola carboxydiphila* (Sokolova et al., 2005), *Thermincola ferriacetica* (which can also grow chemolithoautotrophically) (Zavarzina et al., 2007), *Caldithrix abyssi* (Miroshnichenko et al., 2003a), *Vulcanithermus mediatlanticus* (Miroshnichenko et al., 2003d), and *Oceanithermus profundus* (Miroshnichenko et al., 2003c).

Two mechanisms for collecting light energy and converting it into chemical energy are known, one depends upon photochemical reaction centers containing (bacterio)-chlorophyll and the other employing rhodopsins (Bryant and Frigaard, 2006). However, to the authors'

knowledge, there are no rhodopsin-utilizing thermophilic anaerobes. The question of whether there is a biological explanation for this, e.g., that rhodopsin proteins do not function well at high temperature, or whether it is the result of a lack of searching for this particular physiology, appears unanswered at this time. However, there are phototrophic moderately thermophilic anaerobes (Table 1). Assumingly due to the temperature sensitivity of the photosystem, there are no known hyperthermophilic phototrophs (Amend and Shock, 2001). *Bacteria* with (bacterio)-chlorophyll containing photochemical reaction centers have been found within the *Cyanobacteria*, *Chlorobi*, *Proteobacteria*, *Chloroflexi* and *Firmicutes* (Bryant and Frigaard, 2006); the few presently known thermophilic anaerobic phototrophs are found within the latter three of the above phyla. *Thermochromatium tepidum,* of the *γ-Proteobacteria* clade (*Chromatiaceae* {*B*02}), is an anaerobe with T_{max} 57°C and T_{opt} 48-50°C and is one of the very few thermophiles capable of anaerobic photoautotrophic growth (Madigan, 1986; Imhoff et al., 1998). *Thermochromatium tepidum* is also capable of assimilating compounds such as pyruvate and is therefore also a photoheterotroph (Madigan, 1986; Imhoff et al., 1998). Four filamentous, gliding, moderately photoheterotrophic thermophilic facultative aerobes, *Roseiflexus castenholzii* (Hanada et al., 2002), *Chloroflexus aggregans* (Hanada et al., 1995), *Chloroflexus aurantiacus* (Pierson and Castenholz, 1974), and *Heliothrix oregonensis* (Pierson et al., 1984; Pierson et al., 1985), are found within the *Chloroflexaceae* {*B*29}. *Roseiflexus castenholzii, Chloroflexus aggregans,* and *Chloroflexus aurantiacus,* have a unique metabolic strategy as they grow photoheterotrophically under anaerobic conditions with available light, but also grow chemoorganotrophically under aerobic light or dark conditions (Pierson and Castenholz, 1974; Hanada et al., 1995; Hanada et al., 2002). Within the phylum *Firmicutes* (family *Heliobacteriaceae* {*B*13}), *Heliobacterium modesticaldum* is an obligately anaerobic

photoheterotroph which is also capable of growing chemoorganoheterotrophically (Kimble et al., 1995). *H. modesicaldum* is among the most recently discovered taxa containing (bacterio)-chlorophyll photochemical reaction centers, however, at present, they are poorly characterized (Bryant and Frigaard, 2006).

Although thermophilic anaerobes are most often studied as axenic cultures, this being a requirement for the valid publication of any prokaryotic taxon, some intriguing relationships have been observed for microorganisms with this physiology growing in co-culture. For example, in pure culture, the previously mentioned taxon *Thermosyntropha lipolytica* cannot utilize triacylglycerols or short- and long-chain fatty acids, but when grown in syntrophic coculture with *Methanobacterium* strain JW/VS-M29, *T. lipolytica* grows on triacylglycerols and linear saturated and unsaturated fatty acids with 4 to 18 carbon atoms (Svetlitshnyi et al., 1996). Many *Archaea* were initially described as being obligately dependent upon S^0 reduction for the production of energy (Adams, 1994). However, Bonch-Osmolovskaya and Stetter showed that some so-called 'sulfur-dependent' *Archaea* grew well in co-culture with hydrogen-utilizing thermophilic methanogens in the absence of sulfur. This is possible through interspecies hydrogen transfer whereby growth inhibiting hydrogen (from H^+ used as electron acceptor), is removed without sulfur serving as the electron acceptor (Bonch-Osmolovskaya and Stetter, 1991). The *Ignicoccus*–*'Nanoarchaeum'* system has been described as a symbiotic relationship. It was discovered that small cocci were attached to the larger cells of a strain of *Ignococcus* isolated from the Kolbeinsey ridge, north of Iceland. These tiny cocci could be isolated from the larger cells and subsequently studied, but grow only when attached to their host (Huber et al., 2002b). The genome sequence analyis of '*Nanoarchaeum'* showed that it is missing most of the enzymes required for non-parasitic growth (Waters et al., 2003).

The importance of sulfur in the metabolism of thermophilic anaerobes becomes evident considering that a majority of thermophiles (chemolithotrophs, as well as chemoheterotrophs) take advantage of the sulfur redox system. Amend and Shock posit that the most common energy-yielding reaction is possibly the reduction of elemental sulfur: $H_2 + S^0 \rightarrow H_2S$ (Amend and Shock, 2001). Indeed, the diversity of known thermophilic anaerobic taxa which utilize this strategy is notable: the sulfur reducing reaction has been reported within the *Pyrodictiaceae* {*A*02}, *Sulfolobaceae* {*A*03}, *Thermoanaerobacteriaceae* {*B*09}, *Thermoproteaceae* {*A*05}, *Aquificaceae* {*B*032}, *Desulfurellaceae* {*B*16}, *Desulfurococcaceae* {*A*01}, *Thermococcaceae* {*A*08}, *Thermoplasmataceae* {*A*06}, *Thermofilaceae* {*A*04}, and *Thermotogaceae* {*B*34}. Thermophilic, sulfate-reducing *Bacteria* have been isolated from a wide range of environments and many of these thermophiles belong to a phylogenetically coherent cluster of Gram-type positive, spore-forming *Desulfotomaculum* species of the *Firmicutes* (*Peptococcaceae* {*B*11}) (Stackebrandt et al., 1997; Goorissen et al., 2003). Thus, the role of sulfur in the metabolisms of thermophilic anaerobes can vary for different groups; it can be reduced, it can serve as an electron sink during fermentation, and it can also function as a terminal electron acceptor to allow sulfur respiration (Miroshnichenko et al., 1994).

Thermophilic anaerobic Fe (III)-reducing *Bacteria* and *Archaea* are found within nearly all thermobiotic environments and are usually respirationally diverse, capable of growing chemoorganotrophically with fermentable substrates or chemolithoautotrophically with molecular hydrogen (Slobodkin, 2005; Burgess et al., 2007; Zavarzina et al., 2007). Although only relatively recently described, a diverse set of thermophilic anaerobes are known to reduce Fe (III).

Families of the *Bacteria* with taxa known to reduce Fe (III) include the *Bacillaceae* {*B*15}, *Peptococcaceae* {*B*11}, *Thermoanaerobacteriaceae* {*B*09}, *Acidaminococcaceae* {*B*10}, *Syntrophomonadaceae* {*B*12}, *Deferribacteraceae* {*B*19}, *Hydrogenothermaceae* {*B*33}, *Thermotogaceae* {*B*34}, and the *Thermodesulfobacteriaceae* {*B*20}. Families of the *Archaea* with taxa known to reduce Fe (III) include the *Thermoproteaceae* {*A*05}, *Archaeoglobaceae* {*A*09}, and the *Thermococcaceae* {*A*08} (Slobodkin, 2005). *Geoglobus ahangari,* of the *Archaeoglobaceae* {*A*09} was reported as the first dissimilatory Fe (III)-reducing prokaryote obligately growing autotrophically on hydrogen (Kashefi et al., 2002). There are genera, such as *Thermoanaerobacter*, *Thermotoga*, and *Anaerobranca*, in which many of the species tested have been found to be capable of dissimilatory reduction of Fe (III) (Slobodkin, 2005), but overall it appears as though the ability to reduce Fe (III) does not correlate to an affiliation at the genus or sometimes even species level (Slobodkin, 2005). For example, while *Deferribacter abyssi* and *Deferribacter thermophilus* are closely related, having 98.1% 16S rRNA gene sequence similarity, *D. abyssi* is unable to reduce Fe (III) while it is a primary electron acceptor for *D. thermophilus* (Greene et al., 1997; Miroshnichenko et al., 2003b); Also, *Thermolithobacter ferrireducens* and *Thermolithobacter carboxydivorans* have 99% 16S rRNA gene sequence similarity to each other, however, *T. ferrireducens* is able to reduce Fe (III) but cannot assimilate CO, whereas *T. carboxydivorans* is able to assimilate CO but cannot reduce Fe (III) (Sokolova et al., 2007).

Besides Fe (III), the reduction of other metals coupled to the generation of energy by thermophilic anaerobes includes: Mn (IV), known to be used by *Acidianus infernus* (Segerer et al., 1986), *Thermoanaerobacter siderophilus* (Slobodkin et al., 1999), *Thermovenabulum ferriorganovorum* (Zavarzina et al., 2002), and *Deferribacter thermophilus* (Greene et al., 1997);

as well as Mo (VI), by *Acidianus brierleyi* (Brierley and Brierley, 1982). *Pyrobaculum arsenaticum* has the ability to grow chemolithoautotrophically by arsenate reduction, and both *Pyrobaculum arsenaticum* and *Pyrobaculum aerophilum* can use selenate, selenite, or arsenate chemolithoorganotrophically (Huber et al., 2000b). Thermophilic anaerobes are also known to reduce a number of other metals, possibly as a detoxification mechanism, e.g., *Thermoanaerobacter* strains isolated from Piceance Basin in Colorado were able to reduce Co (III), Cr (VI), and U (VI), in addition to Mn (IV), and Fe (III) (Roh et al., 2002).

UNIQUE PHYSIOLOGICAL ADAPTATIONS

In addition to these described characteristics- O_2-relationship, temperature and pH profiles, and metabolic strategies- a number of additional physiological properties of thermophilic anaerobes should be examined and thereby add to what is known about the diversity of thermophilic anaerobes. Nakagawa and Takai (2006) recently gave a detailed overview of the metabolic types isolated from deep-sea hydrothermal vents and are therefore not further discussed here. The NaCl optimum and tolerance of a prokaryote are often assessed. Thermophilic anaerobes of marine origin, for example, would be expected to grow best at marine salinity, around 3.5%. Prokaryotes which grow optimally with high salinity are referred to as halophiles, and halophilic thermophilic anaerobes are known, as well as halophilic alkalithermophiles which are further discussed in the chapter by Mesbah and Wiegel within this volume [author's note: this refers to: Mesbah, N.M., and Wiegel, J. (2008) Life at Extreme Limits. *Annals of the New York Academy of Sciences* **1125**: 44-57.].

Thermophilic anaerobes living at deep-sea hydrothermal vent sites must cope with the additional pressure exerted by the water column, therefore being piezotolerant or perhaps even

piezophilic. Both *Methanocaldococcus* (basonym *Methanococcus*) *jannaschii,* isolated from the 21°N East Pacific Rise deep-sea hydrothermal vent site, and *Thermococcus barophilus*, obtained from the Snakepit region of the Mid-Atlantic Ridge, grow faster under increased hydrostatic pressure (Miller et al., 1988; Marteinsson et al., 1999). At the optimal growth temperature of *Thermococcus barophilus*, the growth rate was more than doubled at elevated hydrostatic pressure (40 MPa) compared to low pressure (0.3 MPa) (Marteinsson et al., 1999). Furthermore, *Thermococcus barophilus* as well as '*Pyrococcus abyssi*' and *Pyrococcus* strain ES4, isolated from deep-sea hydrothermal vent sites, show an extension of their T_{max} with elevated hydrostatic pressure (Erauso et al., 1993; Holden and Baross, 1995; Marteinsson et al., 1999). Takai reported at the Thermophile 2007 Meeting that under hydrostatic conditions, a H_2/CO_2-utilizing, methanogenic, *Methanococcus kandleri*-like isolate was able to grow at 122 °C, and thus represents the new maximum temperature for sustaining life, i.e, for growing and multiplying (Takai et al., 2007).

Some of the isolated thermophilic anaerobes also possess ionizing radiation resistance; for example, *Tepidimicrobium ferriphilum* (family *Clostridiaceae* {*B*14}), isolated from a freshwater hot spring within the Barguzin Valley, Buryatiya, Russia (Slobodkin et al., 2006b). The level of natural radioactivity at hydrothermal vents can be 100 times greater than that at Earth's surface because of increased occurrence of elements such as ^{210}Pb, ^{210}Po, and ^{222}Rn (Cherry et al., 1992; Jolivet et al., 2003). Indeed, *Archaea* of the family *Thermococcaceae* {*A*08} *Thermococcus gammatolerans* and '*Thermococcus radiotolerans'* isolated from the Guaymas Basin, of the Gulf of California, and *'Thermococcus marinus,'* isolated from the Snakepit hydrothermal site of the Mid-Atlantic Ridge have γ-irradiation resistance (Jolivet et al., 2003; Jolivet et al., 2004).

CONCLUDING REMARKS

More than 300 species of thermophilic anaerobes have been described (Table 1). Many of the prokaryotes with this physiology were isolated from natural thermobiotic environments, e.g., terrestrial hot springs, deep-sea hydrothermal vents, and subsurface environments. Thermophilic anaerobes have also been isolated from anthropogenically-heated environments, as well as from mesobiotic environments such as rivers and lake sediment, and even psychrobiotic environments. The thermophilic anaerobes are metabolically diverse; a majority of isolated taxa are chemoorganoheterotrophic, though it is doubtful this reflects the ecological situation in most thermobiotic environments. The examples of the phylogenetic and physiological diversity of thermophilic anaerobes given herein are certainly not exhaustive. Beside the diversity found so far, there are physiological types among mesophiles and potentially as-yet unknown or only superficially described physiologies for which no thermophiles have been isolated probably due to a lack of serious isolation attempts. Thus, the authors believe that additional novel thermophilic anaerobes with unique and exciting properties will undoubtedly be isolated, especially when considering the small percentage of assumed existing prokaryotes on Earth which have been described (Whitman et al., 1998).

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Table 2.1. Validly described thermophilic anaerobes. Columns from left to right: Species, O_2-relationship and metabolism, Temperature range [optimum], pH range [optimum], and originally isolated from. Symbols and abbreviation: §, sequenced genome; AN, anaerobic; FAE, facultative aerobe; COH, chemoorganoheterotroph; CLA, chemolithoautotroph; F-CLA, facultative chemolithoautotroph; PH, photoheterotroph; CLH, chemolithoheterotroph; F-CLH, facultative chemolithoheterotroph; NR, not reported.

Bacteria; Proteobacteria; Gammaproteobacteria; Acidithiobacillales; Thermithiobacillaceae* {*B*01}; Genus: *Thermithiobacillus				
Thermithiobacillus tepidarius (Wood and Kelly, 1985; Kelly and Wood, 2000)	AN CLA	37-50 [43-45]	5.5-7.7 [6-7.5]	The Roman Bath, Avon, UK

Bacteria; Proteobacteria; Gammaproteobacteria; Chromatiales; Chromatiaceae* {*B*02}; Genus: *Thermochromatium				
Thermochromatium tepidum (Madigan, 1986; Imhoff et al., 1998)§	AN PA / PH	34-57 [48-50]	[7]	Mammoth Hot Spring, Yellowstone National Park, USA

***Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Burkholderiaceae* {*B*03}; Genera: *Thermothrix, Thiobacter* (unclassified *Betaproteobacteria*)**				
Thermothrix thiopara (Cadwell et al., 1976)	FAE F-CLA	55-85 [70-73]	[7]	Jemez Spring, New Mexico, USA
Thiobacter subterraneus (Hirayama et al., 2005)	AN CLA	35-62 [50-55]	5.2-7.7 [6.5-7]	Subsurface geothermal aquifer, Hishikari gold mine, Japan

Bacteria; Bacteroidetes/Chlorobi* group*; Bacteroidetes; Bacteroidetes; Bacteroidales; Bacteroidaceae* {*B*04}; Genera: *Acetomicrobium, Anaerophaga				
Acetomicrobium flavidum (Stoutschek et al., 1984)	AN COH	35-65 [58]	6.2-8	60°C biogas sewage fermentor
Acetomicrobium faecale (Winter et al., 1987)	AN COH	55-74 [70-73]	5.5-9 [6.5-7]	Mesophilically digested sewage sludge
Anaerophaga thermohalophila (Denger et al., 2002)	AN COH	37-55 [50]	NR	Blackish-oily sedimentary residues of an oil separation tank near Hannover, Germany

Bacteria; Spirochaetes; Spirochaetes; Spirochaetales; Spirochaetaceae* {*B*05}; Genus: *Spirochaeta				
Spirochaeta caldaria (Pohlschroeder et al., 1994)	AN COH	[48-52]	5.8-8.5 [7.2]	Cyanobacterial mat samples from Oregon and Utah, USA
Spirochaeta thermophila (Aksenova et al., 1992)	AN COH	40-73 [66-68]	5.9-7.7 [7.5]	Marine hot spring on the beach of an island from Kamchatka, also a hot spring, Raoul Island, New Zealand

Bacteria; Firmicutes; Clostridia; Halanaerobiales; Halanaerobiaceae* {*B*06}; Genus: *Halothermothrix				
Halothermothrix orenii (Cayol et al., 1994)§	AN COH	45-68 [60]	5.5-8.2 [6.5-7]	Chott El Guettar hypersaline lake, Tunisia

Bacteria; Firmicutes; Thermolithobacteria; Thermolithobacterales; Thermolithobacteraceae* {*B*07}; Genus: *Thermolithobacter				
Thermolithobacter ferrireducens (Sokolova et al., 2007)	AN F-CLA	50–75 [73]	6.5–8.5 [7.1–7.3]	Calcite Spring, Yellowstone National Park, USA
Thermolithobacter carboxydivorans (Sokolova et al., 2007)	AN CL	40-78 [70]	6.6 -7.6 [6.8–7.0]	Terrestrial hot spring at Raoul Island, Archipelago Kermadeck, New Zealand

Bacteria; Firmicutes; Clostridia; Natranaerobiales; Natranaerobiaceae* {*B*08}; Genus: *Natranaerobius				
Natranaerobius thermophilus (Mesbah et al., 2007b)§	AN COH	35-56 [53]	8.5-10.6 [9.5]	Sediment of alkaline, hypersaline lakes of the Wadi An Natrun

Bacteria; Firmicutes; Clostridia; Thermoanaerobacteriales; Thermoanaerobacteriaceae* {*B*09}; Genera: *Coprothermobacter, Gelria, Moorella, Thermacetogenium, Mahella, Thermoanaerobacterium, Thermoanaerobacter, Thermosediminibacter, Caldanaerobacter, Thermovenabulum, Tepidanaerobacter, Ammonifex, Thermanaeromonas, Thermhydrogenium				
Coprothermobacter platensis (Etchebehere et al., 1998)	AN COH	35-65 [55]	4.3-8.3 [7]	Methanogenic mesophilic reactor treating a protein-rich wastewater
Coprothermobacter proteolyticus (Ollivier et al., 1985; Rainey and Stackebrandt, 1993)§	AN COH	35-70 [63]	5-8.5 [7.5]	Thermophilic digester fermenting tannery wastes and cattle manure
Gelria glutamica (Plugge et al., 2002b)	AN COH	37-60 [50-55]	5.5-8 [7]	Thermophilic, syntrophic, propionate-oxidizing enrichment culture
Moorella glycerini (Slobodkin et al., 1997a)	AN COH	43-65 [58]	5.9-7.8 [6.3]	Calcite Spring area hot spring, Yellowstone National Park, USA
Moorella thermoautotrophica (Wiegel et al., 1981; Collins et al., 1994)	AN F-CLA	36-70 [55-58]	4.5-7.6 [5.7]	Yellowstone National Park, USA; also Georgia, Hawaii, USA, Zaire, Africa, and Germany

Moorella mulderi (Balk et al., 2003)	AN F-CLA	40-70 [65]	5.5-8.5 [7]	A methanol degrading enrichemnt culture obtrained from a thermophlic anaerobic reactor
Moorella thermoacetica (Fontaine et al., 1942; Collins et al., 1994) **§**	AN COH	45-65 [55-60]	NR	Horse manure
Thermacetogenium phaeum (Hattori et al., 2000)	AN F-CLA	40-65 [58]	5.9-8.4 [6.8]	Thermophilic anaerobic methanogenic reactor treating kraft-pulp production plant waste water, Japan
Mahella australiensis (Salinas et al., 2004)	AN COH	30-60 [50-60]	5.5-8.8 [7.5]	Riverslea oilfield, Bown-Surat Basin, Queensland, Australia
Thermoanaerobacterium thermosulfurigenes (Lee et al., 1993)	AN COH	55-75 [60]	4-7.6 [5.5-6.5]	Octopus Spring, Yellowstone National Park, USA
Thermoanaerobacterium saccharolyticum (Lee et al., 1993)	AN COH	45-70 [60]	5-7.5 [6]	Yellowstone National Park, USA
Thermoanaerobacterium xylanolyticum (Lee et al., 1993)	AN COH	45-70 [60]	5-7.5 [6]	Geothermal areas, Wyoming and Nevada, USA
Thermoanaerobacterium aotearoense (Liu et al., 1996)	AN COH	35-66 [60-63]	3.8-6.8 [5.2]	Geothermal hot springs, New Zealand
Thermoanaerobacterium polysaccharolyticum (Cann et al., 2001)	AN COH	45-70 [65-68]	5-8 [6.8-7]	Canning factory waste, Illinois, USA
Thermoanaerobacterium zeae (Cann et al., 2001)	AN COH	55-72 [65-70]	3.9-7.9	Canning factory waste, Illinois, USA
Thermoanaerobacterium aciditolerans (Kublanov et al., 2007)	AN COH	37-68 [55]	3.2-7.1 [5.7]	Isolated from a hydrothermal vent in the Orange Field, Uzon Caldera (Kamchatka, Far-Eastern Russia)
Thermoanaerobacterium thermosaccharolyticum (McClung, 1935; Collins et al., 1994)	AN COH	[55-60]	NR	Isolated from soil
Thermoanaerobacter brockii subsp. *lactiethylicus* (Cayol et al., 1995)	AN COH	37-75 [55-60]	[7.3]	Oil field of France and Cameroon, Africa

Thermoanaerobacter thermocopriae (Jin et al., 1988; Collins et al., 1994)	AN COH	47-74 [60]	6-8 [6.5-7.3]	Compost of cattle feces and grasses, at the University of Tokyo, other strains from camel feces, compost, soil, and a hot spring in Japan
Thermoanaerobacter brockii subsp. *finnii* (Cayol et al., 1995)	AN COH	40-75 [65]	[6.5-6.8]	Sediment sludge, Lake Kivu, Africa
Thermoanaerobacter acetoethylicus (Ben-Bassat and Zeikus, 1981; Rainey and Stackebrandt, 1993)	AN COH	[65]	5.5-8.5	Hot springs,Yellowstone National Park, USA
Thermoanaerobacter kivui (Leigh and Wolfe, 1983; Collins et al., 1994)	AN F-CLA	50-72 [66]	5.3-7.3 [6.4]	Lake Kivu sediment, Africa
Thermoanaerobacter wiegelii (Cook et al., 1996)	AN COH	38-78 [65-68]	5.5-7.2 [6.8]	Anthropogenically heated freshwater pool, Rotorua, New Zealand
Thermoanaerobacter thermohydrosulfuricus (Lee et al., 1993)	AN COH	37-78 [67-69]	5.5-9.2 [6.9-7.5]	Extraction juices from beet sugar factories; from mud and soil; from hot springs in Utah, Wyoming, and a sewage plant in Georgia, USA
Thermohydrogenium kirishiense (Zacharova et al., 1993)	AN COH	45-75 [65]	5-8 [7-7.4]	Industrial yeast biomass at the stages of thermal treatment
Thermoanaerobacter brockii subsp. *brockii* (Cayol et al., 1995)	AN COH	40-80 [65-70]	5.5-9.5 [7.5]	Washburn thermal spring, Yellowstone National Park, USA
Thermoanaerobacter italicus (Kozianowski et al., 1997)	AN COH	45-78 [70]	[7]	Thermal spas, water and mud samples, northern Italy
Thermoanaerobacter siderophilus(Slobodkin et al., 1999)	AN F-CLA	39-78 [69-71]	4.8-8.2 [6.3-6.5]	Hydrothermal vents near the Karymsky volcano, Kamchatka, Russia
Thermoanaerobacter mathranii (Larsen et al., 1997)	AN COH	50-75 [70-75]	4.7-8.8 [7]	Alkaline hot spring at Hverdagerdi-Hengil, Iceland
Thermoanaerobacter ethanolicus (Wiegel and Ljungdahl, 1981)§	AN COH	37-78 [69]	4.4-9.9 [5.8-8.5]	Hot springs, Yellowstone National Park, USA

Thermoanaerobacter pseudoethanolicus (Zeikus et al., 1980; Lee et al., 1993; Onyenwoke et al., 2007)§	AN COH	[65]	NR	Hot Spring, Yellowstone National Park, USA
Thermoanaerobacter sulfurigignens (Lee et al., 2007)	AN COH	34-72 [65]	4-8 [5.0-6.5]	Acidic volcanic steam outlet on White Island, New Zealand.
Thermoanaerobacter sulfurophilus (Bonch-Osmolovskaya et al., 1997)	AN COH	44-75 [55-60]	4.5-8.0 [6.9-7.2]	Cyanobacterial mat from a hot spring, Uzon Caldera, Kamchatka, Far East Russia
Thermosediminibacter litoriperuensis (Lee et al., 2005)	AN COH	43-76 [64]	5-9.5 [7.9-8.4]	Non-hydrothermal deep sea sediments of Peru Margin
Thermosediminibacter oceani (Lee et al., 2005)	AN COH	52-76 [68]	6.3-9.3 [7.5]	Non-hydrothermal deep sea sediments of Peru Margin
Caldanaerobacter subterraneus subsp. *subterraneus* (Fardeau et al., 2000; Fardeau et al., 2004)	AN COH	45-75 [65]	6-8.5 [7.5]	Oilfield reservoir, southwest France
Caldanaerobacter subterraneus subsp. *pacificus* (Sokolova et al., 2001; Fardeau et al., 2004)	AN F-CLA	50-80 [70]	5.8-7.6 [6.8-7.2]	Submarine hot vent in the Okinawa Trough
Caldanaerobacter subterraneus subsp. *tengcongensis* (Xue et al., 2001; Fardeau et al., 2004)§(Bao et al., 2002)	AN COH	50-80 [75]	5.5-9 [7-7.5]	Hot spring, Tengcong, China
Caldanaerobacter subterraneus subsp. *yonseiensis* (Kim et al., 2001; Fardeau et al., 2004)	AN COH	50-85 [75]	4.5-9 [6.5]	Hot water, mud, and soil from hot streams at Sileri, Java Island
Thermovenabulum ferriorganovorum (Zavarzina et al., 2002)	AN COH	45-76 [63-65]	4.8-8.2 [6.7-6.9]	Hydrothermal spring, Uzon Caldera, Kamchatka, Russia

Tepidanaerobacter syntrophicus (Sekiguchi et al., 2006)	AN COH	25-60 [45-50]	5.5-8.5 [6-7]	Sludges of thermophilic (55 °C) digesters that decomposed either municipal solid wastes or sewage sludge
Ammonifex degensii (Huber et al., 1996)§	AN F-CLA	57-77 [70]	5-8 [7.5]	Kawah Candradimuka crater, Dieng Plateau, Java, Indonesia
Thermanaeromonas toyohensis (Mori et al., 2002)	AN COH	55-73 [70]	5.5-8.5 [7.5]	Geothermal water at Toyoho Mine, Hokkkaido, Japan

Bacteria; Firmicutes; Clostridia; Clostridiales; Acidaminococcaceae* {*B*10}; Genus: *Thermosinus				
Thermosinus carboxydivorans (Sokolova et al., 2004b)§	AN CLA	40-68 [60]	6.5-7.6 [6.8-7]	Norris Basin hot spring, Yellowstone National Park, USA

Bacteria; Firmicutes; Clostridia; Clostridiales; Peptococcaceae* {*B*11}; Genera: *Desulfotomaculum, Pelotomaculum, Carboxydothermus, Thermincola				
Desulfotomaculum thermosapovorans (Fardeau et al., 1995)	AN COH	35-60 [50]	[7.2-7.5]	Mixed compost containing rice hulls and peanut shells
Desulfotomaculum alkaliphilum (Pikuta et al., 2000a)	AN CLH	30-58 [50-55]	8-9.15 [8.6-8.7]	Mixed cow/pig manure
Desulfotomaculum solfataricum (Goorissen et al., 2003)	AN COH	48-65 [60]	6.4-7.9 [7.3]	Solfataric mud pools, Krafla, northeast Iceland
Desulfotomaculum thermoacetoxidans (Min and Zinder, 1990)	AN COH	45-65 [55-60]	6-7.5 [6.5]	A thermophilic anaerobic digestor converting cellulosic waste to methane
Desulfotomaculum thermobenzoicum subsp. *thermobenzoicum* (Plugge et al., 2002a)	AN F-CLA	40-70 [62]	6-8 [7.2]	Thermophilic methane fermentation reactor treating kraft-pulp waste water
Desulfotomaculum thermobenzoicum subsp. *thermosyntrophicum* (Plugge et al., 2002a)	AN F-CLA	45-62 [55]	6-8 [7]	Thermophilic granular methanogenic sludge

Desulfotomaculum putei (Liu et al., 1997)	AN COH	22-65 [64]	6-7.8	Deep terrestrial rock; Taylorsville Triassic Basin, Virginia, USA
Desulfotomaculum australicum (Love et al., 1993)	AN F-CLA	40-74 [68]	5.5-8.5 [7-7.4]	Bore wells of the non-volcanically heated waters of the Great Artesian Basin, Australia
Desulfotomaculum geothermicum (Daumas et al., 1988)	AN F-CLA	37-57 [54]	6-8 [7.2-7.3]	Geothermally heated ground water, at a depth of 2500 m, Creil production well, France
Desulfotomaculum luciae (Karnauchow et al., 1992; Liu et al., 1997)	AN CLA	50-70 [60-65]	6.3-7.8	Hot spring, St. Lucia
Desulfotomaculum thermocisternum (Nilsen et al., 1996)	AN F-CLA	41-75 [62]	6.2-8.9 [6.7]	Brent group formation water originating 2.6 km below the sea floor, Norwegian sector, North Sea
Desulfotomaculum thermosubterraneum (Kaksonen et al., 2006)	AN F-CLA	50-72 [61-66]	6.4-7.8 [7.2-7.4]	Underground mine in a geothermally active region, Japan.
Desulfotomaculum carboxydivorans (Parshina et al., 2005)	AN CLH	30-68 [55]	6.8-8 [7.2]	Sludge from an anaerobic bioreactor treating paper mill wastewater
Desulfotomaculum kuznetsovii (Nazina et al., 1988; Turova et al., 2001)	AN F-CLA	50-85 [60-65]	NR	Thermal water sample from a spontaneous effusion from a rift in the Sukhumsk deposit
Desulfotomaculum nigrificans(Campbell and Postgate, 1965)	AN COH	[55]	NR	Spoiled food
Pelotomaculum thermopropionicum (Imachi et al., 2002)§	AN COH	45-65 [55]	6.7-7.5 [7]	Thermophilic upflow anaerobic sludge blanket reactor
Carboxydothermus ferrireducens (Slobodkin et al., 1997b; Slobodkin et al., 2006a)	AN COH	50-74 [65]	5.5-7.6 [6-6.2]	Hot springs of Yellowstone National Park, USA and New Zealand
Carboxydothermus hydrogenoformans (Svetlichny et al., 1991)§(Wu et al., 2005)	AN CLA	40-78 [70-72]	6.4-7.7 [6.8-7]	Freshwater hydrothermal springs, Kunashir Island, Kamchatka, Russia

Thermincola carboxydiphila (Sokolova et al., 2005)	AN CLH	37-68 [55]	6.7-9.5 [8]	Hot spring of the Baikal Lake region, Russia

Bacteria; Firmicutes; Clostridia; Clostridiales; Syntrophomonadaceae* {*B*12}; Genera: *Anaerobaculum, Syntrophothermus, Thermanaerovibrio, Carboxydocella, Anaerobranca, Thermosyntropha, Caldicellulosiruptor,				
Anaerobaculum thermoterrenum (Rees et al., 1997)	AN COH	28-60 [55]	5.5-8.6 [7-7.6]	Redwash oil field production fluids, Utah, USA
Anaerobaculum mobile (Menes and Muxi, 2002)	AN COH	35-65 [55-60]	5.4-8.7 [6.6-7.3]	Anaerobic wool-scouring wastewater treatment lagoon sludge, Trinidad, Uraguay
Syntrophothermus lipocalidus (Sekiguchi et al., 2000)	AN COH	45-60 [55]	5.8-7.5 [6.5-7]	Granular sludge of a thermophilic upflow anaerobic sludge blanket
Thermanaerovibrio velox (Zavarzina et al., 2000)	AN COH	45-70 [60-65]	4.5-8 [7.3]	Cyanobacterial mat, Uzon Caldera, Kamchatka, Russia
Thermanaerovibrio acidaminovorans (Baena et al., 1999)	AN COH	40-58 [55]	[6.5-8.1]	Granular methanogemic sludge from a sugar refinery, Breda, The Netherlands
Carboxydocella thermautotrophica(Sokolova et al., 2002)	AN CLA	40-68 [58]	6.5-7.6 [7]	Hot spring, Gyzer Valley, Kamchatka, Russia
Carboxydocella sporoproducens (Slepova et al., 2006)	AN F-CLA	50-70 [60]	6.2-8 [6.8]	Hot spring of Karymskoe Lake, Kamchatka Peninsula
Anaerobranca gottschalkii (Prowe and Antranikian, 2001)	AN COH	30-65 [50-55]	6-10 [9.5]	Hot inlet of Lake Bogoria, Kenya
Anaerobranca horikoshii (Engle et al., 1995)	AN COH	34-66 [57]	6.9-10.3 [8.5]	Thermal pools, Yellowstone National Park, USA
Anaerobranca californiensis (Gorlenko et al., 2004)	AN COH	45-70 [58]	8.6-10.4 [9-9.5]	Paoho Island hot springs Mono Lake, California, USA
Thermosyntropha lipolytica (Svetlitshnyi et al., 1996)	AN COH	52-70 [60-66]	7.15-9.5 [8.1-8.9]	Alkaline hot springs, Lake Bogoria, Kenya
Caldicellulosiruptor lactoaceticus (Mladenovska et al., 1995)	AN COH	50-78 [68]	5.8-8.2 [7]	Hveragerði alkaline hot spring, Iceland

Caldicellulosiruptor owensensis (Huang et al., 1998)	AN COH	50-80 [75]	5.5-9 [7.5]	Freshwater pond within the dry Owens Lake bed, California, USA
Caldicellulosiruptor kristjanssonii (Bredholt et al., 1999)	AN COH	45-82 [78]	5.8-8 [7]	Hot spring, Iceland
Caldicellulosiruptor saccharolyticus (Rainey et al., 1994)§	AN COH	45-80 [70]	5.5-8.0 [7.0]	Geothermal spring, Taupo, New Zealand
Caldicellulosiruptor acetigenus (Nielsen et al., 1993; Onyenwoke et al., 2006)	AN COH	50-78 [65-68]	5.2-8.6 [7.0]	Combined biomat and sediment from a slightly alkaline hot spring, Hverðagerdi, Iceland.
Thermovirga lienii (Dahle and Birkeland, 2006)	AN COH	37-68 [58]	6.2-8.0 [6.5-7]	Production water obtained from an oil reservoir in the North Sea

Bacteria; Firmicutes; Clostridia; Clostridiales; Heliobacteriaceae* {*B*13}; Genus: *Heliobacterium				
Heliobacterium modesticaldum (Kimble et al., 1995)§	AN PH & COH	25-56 [52]	[6-7]	Iceland, Yellowstone National Park, USA

Bacteria; Firmicutes; Clostridia; Clostridiales; Clostridiaceae* {*B*14}; Genera: *Alkaliphilus, Clostridium, Tepidibacter, Caloramator, Garciella, Caminicella, Caloranaerobacter, Thermobrachium, Thermohalobacter, Tepidimicrobium				
Clostridium isatidis (Padden et al., 1999)	AN COH	30-55 [50]	5.9-9.9 [7.2]	Fermenting woad vat
Clostridium thermoalcaliphilum (Li et al., 1994)	AN COH	27-57.5 [48-51]	7-11 [9.6-10.1]	Anaerobic digestor and aerobic oxidation basin, Municipal Sewage Plant, Atlanta, Georgia, USA
Clostridium straminisolvens (Kato et al., 2004)	AN COH	46-64 [50-55]	6-8.5 [7.5]	Cellulose-degrading bacterial community

Clostridium thermobutyricum (Wiegel et al., 1989)	AN COH	26-61.5 [55]	5.8-9 [6.8-7.1]	Horse manure
Clostridium paradoxum (Li et al., 1993)	AN COH	30-63 [55-56]	7-11.1 [10.1]	Municipal Sewage Plants, Athens and Atlanta, Georgia, USA
Clostridium thermopapyrolyticum (Mendez et al., 1991)	AN COH	45-66 [59]	NR	Sediment from a river bank, Buenos Aires, Argentina
Clostridium cellulosi (He et al., 1991)	AN COH	40-65 [55-60]	6.2-8.5 [7.3-7.5]	Cow manure compost
Clostridium stercorarium subsp. *stercorarium* (Madden, 1983; Fardeau et al., 2001)	AN COH	[65]	[7.3]	Compost heap
Clostridium stercorarium subsp. *leptospartum* (Toda et al., 1988; Fardeau et al., 2001)	AN COH	45-71 [60]	6.7-8.9 [7.5]	Cattle manure compost, Ehime prefecture, Japan
Clostridium stercorarium subsp. *thermolacticum* (Le Ruyet et al., 1985; Fardeau et al., 2001)	AN COH	50-70 [60-65]	6.8-7.4 [7.0]	Sludge from a mesophilic digester fed ground duck weed
Tepidibacter thalassicus (Slobodkin et al., 2003)	AN COH	33-60 [50]	4.8-8.5 [6.5-6.8]	Black smoker chimney, hydrothermal vent field, East Pacific Rise
Tepidibacter formicigenes (Urios et al., 2004a)	AN COH	35-55 [45]	5.0-8.5 [6.0]	Menez-Gwen hydrothermal site on the Mid-Atlantic Ridge
Caloramator proteoclasticus (Tarlera et al., 1997)	AN COH	30-68 [55]	6-9.5 [7-7.5]	Mesophilic granular methanogenic sludge
Caloramator coolhaasii (Plugge et al., 2000)	AN COH	37-65 [50-55]	6-8.5 [7]	Thermophilic methanogenic granular sludge
Caloramator viterbiensis (Seyfried et al., 2002)	AN COH	33-64 [58]	6-7.8 [6.5-7]	Hot spring at the Bagnaccio Spring, Viterbo, Italy
Caloramator indicus (Chrisostomos et al., 1996)	AN COH	[60-65]	6.2-9.2 [8.1]	Natural well of the artesian aquifer, Surat District, Gujarat State, India
Caloramator fervidus (Patel et al., 1987; Collins et al., 1994)	AN COH	37-80 [68]	5.5-9 [7-7.5]	Hot spring, New Zealand
Garciella nitratireducens (Miranda-Tello et al., 2003)	AN COH	25-60 [55]	5.5-9 [7.5]	A water separator collecting fluids, the SAMIII oil field, Gulf of Mexico

Caminicella sporogenes (Alain et al., 2002c)	AN COH	45-64 [55-60]	4.5-8 [7.5-8]	Deep-sea vent, East Pacific Rise
Caloranaerobacter azorensis (Wery et al., 2001b)	AN COH	45-65 [65]	5.5-9 [7]	Deep-sea hydrothermal chimney rocks, Mid-Atlantic Ridge
Thermobrachium celere (Engle et al., 1996)	AN COH	37-75 [62-65]	5-9.7 [8-8.5]	Geothermally and anthroprogenically heated environments on three continents
Thermohalobacter berrensis (Cayol et al., 2000)	AN COH	45-70 [65]	5.2-8.8 [7]	Solar saltern canal near Berre Lagoon, southern France
Tepidimicrobium ferriphilum (Slobodkin et al., 2006b)	AN COH	26-62 [50]	5.5-9.5 [7.8-8]	Freshwater hot spring at Barguzin Valley, Buryatiya, Russia

Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae* {B15}; Genera: *Anoxybacillus, Bacillus, Geobacillus, Vulcanibacillus				
Anoxybacillus pushchinoensis (Pikuta et al., 2000b)	AN COH	37-66 [62]	8-10.5 [9.5-9.7]	Manure from farms near Moscow, Russia
Bacillus infernus (Boone et al., 1995)	AN COH	[61]	[7.3-7.8]	Deep terrestrial rock; Taylorsville Triassic Basin, Virginia, USA
Bacillus thermoamylovorans (Combet-Blanc et al., 1995)	FAE COH	[50]	5.4-8.5 [7]	Palm wine collected in Rafisque, Senegal
Anoxybacillus ayderensis (Dulger et al., 2004)	FAE COH	30-70 [50]	6-11 [7.5-8.5]	Ayder hot spring, Rize province, Turkey
Anoxybacillus voinovskiensis (Yumoto et al., 2004)	FAE COH	30-64 [54]	7-8	Voinovskie Hot Springs, Kamchatka, Russia
Anoxybacillus kestanbolensis (Dulger et al., 2004)	FAE COH	40-70 [50-55]	6-10.5 [7.5-8.5]	Kestanbol hot spring, Canakkale province, Turkey
Anoxybacillus gonensis (Belduz et al., 2003)	FAE COH	40-70 [55-60]	6-10 [7.5-8]	Gonen hot spring, Balikesir province, Turkey
Anoxybacillus flavithermus (Pikuta et al., 2000b)	FAE COH	30-72 [60-65]	5.5-9 [7]	A hot spring, New Zealand

Geobacillus thermocatenulatus (Golovacheva et al., 1975; Nazina et al., 2001)	FAE COH	42-69 [55-60]	6.5-8.5	Thermal zone of the Tangan-Tau mountain, Southern Urals, Russia
Geobacillus thermodenitrificans (Manachini et al., 2000; Nazina et al., 2001)§(Feng et al., 2007)	FAE COH	45-70	6-8	Sugar beet juice from extraction installations; Austria
Geobacillus thermoleovorans (Merkel et al., 1978; Zarilla and Perry, 1987; Nazina et al., 2001)	FAE COH	35-78 [55-65]	[6.2-6.8]	Soil near hot water effluent, Bethlehem, PA, USA
Geobacillus uzenensis (Nazina et al., 2001)	FAE COH	45-65	6.2-7.8	The Uzen oilfield, Kazakhstan
***Vulcanibacillus modesticaldus* (L'Haridon et al., 2006a)**	AN COH	37-60 [55]	6-8.5 [7]	'Rainbow' Deep-sea hydrothermal vent field, Mid-Atlantic Ridge (36° 14' N 33° 54' W)

Bacteria*; *Proteobacteria*; delta/epsilon subdivisions; *Deltaproteobacteria; Desulfurellales; Desulfurellaceae* {*B*16}; Genera: *Desulfurella, Hippea				
Desulfurella kamchatkensis (Miroshnichenko et al., 1998)	AN F-CLA	40-70 [54]	[6.9-7.2]	The Pauzhetka hot spring, Kamchatka, Far East Russia
Desulfurella propionica (Miroshnichenko et al., 1998)	AN F-CLA	33-63 [55]	[6.9-7.2]	Cyanobacterial mat from sulfide-rich hot pond, Uzon Caldera, Kamchatka, Russia
Desulfurella acetivorans (Bonch-Osmolovskaya et al., 1990a)	AN COH	44-70 [52-57]	4.3-7.5 [6.8-7]	Hot water pool, Uzon Caldera, Kamchatka, Russia
Desulfurella multipotens (Miroshnichenko et al., 1994)	AN F-CLA	42-77 [58-60]	6.0-7.2 [6.4-6.8]	Green Lake, Raoul Island, Kermadec archipelago, New Zealand
Hippea maritima (Miroshnichenko et al., 1999)	AN COH	40-65 [52-54]	5.4-6.5 [5.8-6.2]	Shallow water hot vents, Bay of Plenty, New Zealand and Matupi Harbour, Papua New Guinea

Bacteria; Proteobacteria;* delta/epsilon subdivisions*; Epsilonproteobacteria; Nautiliales; Nautiliaceae* {*B*17}; Genera: *Nautilia, Lebetimonas, Caminibacter				
Nautilia lithotrophica (L'Haridon et al., 2002)	AN CLA	37-68 [53]	6.4-7.4 [6.8-7]	13 °N hydrothermal vent field, East Pacific Rise

Lebetimonas acidiphila (Takai et al., 2005)	AN CLA	30-68 [50]	4.2-7 [5.2]	TOTO caldera of the Mariana arc
Caminibacter mediatlanticus (Voordeckers et al., 2005)§	AN CLA	45-70 [55]	4.5-7.5 [5.5]	'Rainbow' deep-sea vent field, Mid-Atlantic Ridge
Caminibacter profundus (Miroshnichenko et al., 2004)	AN CLA	45-65 [55]	6.5-7.4 [6.9-7.1]	'Rainbow' deep-sea vent field, Mid-Atlantic Ridge
Caminibacter hydrogeniphilus (Alain et al., 2002b)	AN CLA	50-70 [60]	5-7.5 [5.5-6]	Deep-sea vent region, East Pacific Rise

Bacteria; Proteobacteria;* delta/epsilon subdivisions*; Epsilonproteobacteria; Campylobacterales; Hydrogenimonaceae* {*B*18}; Genus: *Hydrogenimonas				
Hydrogenimonas thermophila (Takai et al., 2004)	FAE CLA	35-65 [55]	4.9-7.2 [5.9]	Kairei deep-sea hydrothermal field, Central Indian Ridge

***Bacteria; Deferribacteres; Deferribacteres; Deferribacterales; Deferribacteraceae* {*B*19}; Genera: *Deferribacter; Flexistipes* also, *Caldithrix* (unclassified *Deferribacteres*)**				
Caldithrix abyssi (Miroshnichenko et al., 2003a)	AN CLH	40-70 [60]	5.8-7.8 [6.8-7]	Logatchev hydrothermal field, Mid-Atlantic Ridge
Deferribacter thermophilus (Greene et al., 1997)	AN COH	50-65 [60]	5-8 [6.5]	Produced formation water collected from a well in the Beatrice oil field
Deferribacter abyssi (Miroshnichenko et al., 2003b)	AN CLA	45-65 [60]	6-7.2 [6.5-6.7]	'Rainbow' vent field, Mid-Atlantic Ridge
Deferribacter desulfuricans (Takai et al., 2003a)	AN COH	40-70 [60-65]	5.0-7.5 [6.5]	From a black smoker vent from the hydrothermal fileds at the Suiyo Seamount in the Izu-Bonin Arc, Japan
Flexistipes sinusarabici (Fiala et al., 1990)	AN COH	30-53 [45-50]	6-8	Brine water samples of the Atlantis II Deep of the Red Sea, depth of 2000 m

Bacteria; Thermodesulfobacteria; Thermodesulfobacteria; Thermodesulfobacteriales; Thermodesulfobacteriaceae* {*B*20}; Genera: *Thermodesulfatator, Thermodesulfobacterium				
Thermodesulfatator indicus (Moussard et al., 2004)	AN CLA	55-80 [70]	6-6.7 [6.25]	The Kairei deep-sea hydrothermal vent field, Central Indian Ridge
Thermodesulfobacterium hydrogeniphilum (Jeanthon et al., 2002)	AN CLA	50-80 [75]	6.3-6.8 [6.5]	Deep-sea hydrothermal vent site, Guaymas Basin

Thermodesulfobacterium commune (Zeikus et al., 1983)§	AN COH	50-85 [70]	6.0-8.0	Ink Pot Spring, Yellowstone National Park, USA
Thermodesulfobacterium hveragerdense (Sonne-Hansen and Ahring, 1999)	AN COH	55-74 [70]	NR	Icelandic hot springs
Thermodesulfobacterium thermophilum (Rozanova and Pivovarova, 1988)	AN COH	45-85 [65]	NR	Strata water of the oil deposit on the Apsheron Peninsula, Caspian Sea

Bacteria; Proteobacteria;* delta/epsilon subdivisions*; Deltaproteobacteria; Desulfovibrionales; Desulfohalobiaceae* {*B*21}; Genus: *Desulfothermus				
Desulfothermus naphthae (Kuever et al., 2005)	AN COH	50-69 [60-65]	6.1-7.1 [6.5-6.8]	Guaymas Basin, Gulf of California
Desulfothermus okinawensis (Nunoura et al., 2007b)	AN COH	35-60 [50]	5.4-7.9 [5.9-6.4]	Black smoker chimney, deep-sea hydrothermal field, Southern Okinawa Trough

Bacteria; Proteobacteria;* delta/epsilon subdivisions*; Deltaproteobacteria; Syntrophobacterales; Syntrophobacteraceae* {*B*22}; Genus: *Desulfacinum, Thermodesulforhabdus				
Desulfacinum infernum (Rees et al., 1995)	AN F-CLA	40-65 [60]	6.6-8.4 [7.1-7.5]	Oil well of the Beatrice field platform, the North Sea near the coast of Scotland
Desulfacinum hydrothermale (Sievert and Kuever, 2000)	AN F-CLA	37-64 [60]	6-7.5 [7]	Shallow, submarine hydrothermal vent, Palaeochori Bay, Milos in the Aegean Sea
Thermodesulforhabdus norvegica (Beeder et al., 1995)	AN COH	44-74 [60]	6.1-7.9 [6.9]	Oil field water (Norwegian oil platform) from the North Sea

Bacteria; Nitrospirae; Nitrospira; Nitrospirales; Nitrospiraceae* {*B*23}; Genus: *Thermodesulfovibrio				
Thermodesulfovibrio islandicus (Sonne-Hansen and Ahring, 1999)	AN COH	45-70 [65]	NR	Icelandic hot springs
Thermodesulfovibrio yellowstonii (Henry et al., 1994)§	AN COH	40-70 [65]	[6.8-7]	Thermal vent, Yellowstone National Park, USA

Bacteria; Deinococcus-Thermus; Deinococci; Thermales; Thermaceae* {*B*24}; Genera: *Oceanithermus, Vulcanithermus				
Oceanithermus profundus (Miroshnichenko et al., 2003c)	FAE F-CLH	40-68 [60]	5.5-8.4 [7.5]	13° North hydrothermal vent field, East Pacific Rise, depth of 2600 m
Oceanithermus desulfurans (Mori et al., 2004)	FAE COH	30-65 [60]	6-8 [6.5]	Submarine hydrothermal field at the Suiyo Seamount in Izu-Bonin Arc, Western Pacific
Vulcanithermus mediatlanticus (Miroshnichenko et al., 2003d)	FAE F-CLH	37-80 [70]	5.5-8.4 [6.7]	Hydrothermal vent, Mid-Atlantic Ridge

Bacteria; Firmicutes; Clostridia; Thermoanaerobacteriales; Thermodesulfobiaceae* {*B*25}; Genus: *Thermodesulfobium				
Thermodesulfobium narugense (Mori et al., 2003)	AN CLA	37-65 [50-55]	4-6.5 [5.5-6]	Nogo hot spring, Miyagi Prefecture, Japan

Bacteria; Dictyoglomi; Dictyoglomi; Dictyoglomales; Dictyoglomaceae* {*B*26}; Genus: *Dictyoglomus				
Dictyoglomus thermophilum (Saiki et al., 1985)§	AN COH	50-80 [73-78]	5.9-8.3 [7]	Hot spring, Kumamoto Prefecture, Japan
Dictyoglomus turgidus (Svetlichny and Svetlichnaya, 1988)	AN COH	48-86 [72]	5.2-9.0 [7.0-7.1]	Oranzhevoe pole hot spring, Uzon Caldera, Kamchatka, Far Eas Russia

Bacteria; Chloroflexi; Anaerolineae; Anaerolinaeles; Anaerolinaceae* {*B*27}; Genus: *Anaerolinea,				
Anaerolinea thermolimosa (Yamada et al., 2006)	AN COH	42-55 [50]	6-7.5 [7]	Sludge from a thermophilic reactor in which wastewater from manufacture of a Japanese distilled alcohol is treated
Anaerolinea thermophila (Sekiguchi et al., 2003)	AN COH	50-60 [55]	6.0-8.0 [7.0]	Thermophilic upflow anaerobic sludge blanket reactor treating soybean-curd manufacturing waste water

Bacteria; Chloroflexi; Caldilineae; Caldilineales; Caldilineaceae* {*B*28} Genus: *Caldilinea				
Caldilinea aerophila (Sekiguchi et al., 2003)	FAE COH	37-65 [55]	7.0-9.0 [7.5-8.5]	Hot spring sulfur-turf, Japan.

Bacteria; Chloroflexi; Chloroflexi; Chloroflexales; Chloroflexaceae* {*B*29}; Genera: *Roseiflexus, Chloroflexus, Heliothrix				
Roseiflexus castenholzii (Hanada et al., 2002)§	FAE PH (anaerobic)	45-55 [50]	7-9 [7.5-8]	Hot spring, Nakabusa, Japan
Chloroflexus aggregans (Hanada et al., 1995)§	FAE PH (anaerobic)	[50-60]	7.0-9.0	Hot spring of the Okukinu Meotobuchi hot spring in Tochigi Perfecture, Japan
Chloroflexus aurantiacus (Pierson and Castenholz, 1974)§	FAE PH (anaerobic)	[52-60]	[8]	Hot spring in the canyon at Sokokura, Hakone district, Japan
Heliothrix oregonensis (Pierson et al., 1984; Pierson et al., 1985)§	FAE PH	[40-55]	NR	Hot spring near Warm Springs River, Oregon, USA

Bacteria; Chloroflexi; Thermomicrobia; Sphaerobacterales; Sphaerobacteraceae* {*B*30}; Genus: *Sphaerobacter				
Sphaerobacter thermophilus (Demharter et al., 1989)	AN COH	[55]	[8.5]	A laboratory-scale 60°C fermentor

Bacteria; Chloroflexi; Thermomicrobia; Thermomicrobiales; Thermomicrobiaceae* {*B*31}; Genus: *Thermomicrobium				
Thermomicrobium roseum (Jackson et al., 1973)§	AN COH	[70-75]	6-9.4 [8.2-8.5]	Hot spring, Yellowstone National Park, USA

***Bacteria; Aquificae; Aquificae; Aquificales; Aquificaceae* {*B*32}; Genera: *Hydrogenivirga, Aquifex, Desulfurobacterium* (unclassified *Aquificales*), *Balnearium* (unclassified *Aquificales*), *Thermovibrio* (unclassified *Aquificales*)**				
Hydrogenivirga caldilitoris (Nakagawa et al., 2004b)	AN CLA	55-77.5 [75]	5.5-8.3 [6.5-7]	Coastal hot spring, Ibuski, Kangoshima Prefecture, Japan.
Aquifex pyrophilus (Huber et al., 1992)	FAE CLA	67-95 [85]	5.4-7.5 [6.8]	Hot marine sediments at the Kolbeinsey Ridge, Iceland, 106 m deep

Desulfurobacterium thermolithotrophum (L'Haridon et al., 1998)	AN CLA	40-75 [70]	4.4-7.5 [6]	'Snake Pit' vent field, Mid-Atlantic ridge
Desulfurobacterium crinifex (Alain et al., 2003)	AN CLA	50-70 [60-65]	5.0-7.5 [6.0-6.5]	A Juan de Fuca Ridge hydrothermal vent sample (tubes of the annelid polychaete *Paralvinella sulfincola* attached to small pieces of hydrothermal chimney), depth of 1,581 m
Desulfurobacterium atlanticum (L'Haridon et al., 2006b)	AN CLA	50-80 [70-75]	5-7.5 [6.0-6.2]	Obtained from a deep-sea hydrothermal vent chimney at the Mid-Atlantic Ridge (23°N)
Desulfurobacterium pacificum (L'Haridon et al., 2006b)	AN CLA	55-85 [75]	5.5-7.5 [6-6.2]	Obtained from a deep-sea hydrothermal vent chimney at the East Pacific Rise (13°N)
Balnearium lithotrophicum (Takai et al., 2003b)	AN CLA	45-80 [70-75]	5-7 [5.4]	Deep-sea hydrothermal system, Suiyo Seamount
Thermovibrio ruber (Huber et al., 2002a)	AN CLA	50-80 [75]	5-6.5 [6]	Submarine hydrothermal vents in the Papua New Guinea region, type strain from sandy sediments taken from the beach off Lihir Island
Thermovibrio ammonificans (Vetriani et al., 2004)	AN CLA	60-80 [75]	5-7 [5.5]	Deep sea hydrothermal vent area, East Pacific Rise
Thermovibrio guaymasensis (L'Haridon et al., 2006b)	AN CLA	50-88 [75-80]	5.5-7.5 [6-6.2]	Obtained from a deep-sea hydrothermal vent chimney at Guaymas Basin

Bacteria; Aquificae; Aquificae; Aquificales; Hydrogenothermaceae* {*B*33}; Genera: *Hydrogenothermus, Sulfurihydrogenibium, Persephonella				
Hydrogenothermus marinus (Stohr et al., 2001)	FAE CLA	45-80 [65]	5-7	Vulcano Beach, Vulcano Island, Italy
Sulfurihydrogenibium subterraneum (Takai et al., 2003c; Nakagawa et al., 2005)	FAE CLA	40-70 [60-65]	6.4-8.8 [7.5]	Subsurface hot aquafier water in the Hishikari Japanese gold mine, Kagoshima Prefecture, Japan
Sulfurihydrogenibium azorense (Aguiar et al., 2004; Nakagawa et al., 2005)§	FAE CLA	50-73 [68]	5.5-7 [6]	near the Água do Caldeirão, Furnas, on São Miguel Island, Azores
Persephonella hydrogeniphila (Nakagawa et al., 2003)	FAE CLA	50-72.5 [70]	5.5-7.6 [7.2]	The hydrothermal field at the Suiyo Seamount in the Izu-Bonin Arc, Japan
Persephonella guaymasensis (Gotz et al., 2002)	FAE CLA	55-75 [70]	4.7-7.5 [6]	Deep-sea hydrothermal vent chimney in Guaymas Basin, Mexico

Persephonella marina (Gotz et al., 2002)	FAE CLA	55-80 [73]	4.7-7.5 [6]	9° North deep-sea hydrothermal vent, East Pacific Rise

Bacteria; Thermotogae; Thermotogae; Thermotogales; Thermotogaceae* {*B*34}; Genera: *Geotoga, Marinitoga, Petrotoga, Thermosipho, Thermotoga, Fervidobacterium				
Geotoga petraea (Davey et al., 1993)	AN COH	30-60 [45]	5.5-9 [6.5]	Oil field brines, Texas and Oklahoma, USA
Geotoga subterranea (Davey et al., 1993)	AN COH	30-55 [50]	5.5-9 [6.5]	Oil field brines, Texas and Oklahoma, USA
Marinitoga camini (Wery et al., 2001a)§	AN COH	25-65 [55]	5-9 [7]	Deep sea vent fields, Mid-Atlantic ridge
Marinitoga piezophila (Alain et al., 2002a)	AN COH	45-70 [65]	5-8 [6]	'Grandbonum' deep-sea-vent field site, East-Pacific Rise
Marinitoga hydrogenitolerans (Urios et al., 2004b)	FAE COH	35-65 [60]	4.5-8.5 [6]	Deep-sea hydrothermal chimney collected at the Rainbow field on the Mid-Atlantic Ridge
Marinitoga okinawensis (Nunoura et al., 2007a)	AN COH	30-70 [55-60]	5.0-7.4 [5.5-5.8]	deep-sea hydrothermal field in Yonaguni Knoll IV, Southern Okinawa Trough
Petrotoga mexicana (Miranda-Tello et al., 2004)	AN COH	25-65 [55]	5.8-8.5 [6.6]	Oil/water mixtures of production well heads in Tabasco, Gulf of Mexico, an offshore reservoir
Petrotoga miotherma (Davey et al., 1993)	AN COH	35-65 [55]	5.5-9 [6.5]	Oil field brines, Texas and Oklahoma, USA
Petrotoga olearia (L'Haridon et al., 2002)	AN COH	37-60 [55]	6.5-8.5 [7.5]	Oil/water mixtures of the Samotlor oilfields, western Siberia, Russia
Petrotoga sibirica (L'Haridon et al., 2002)	AN COH	37-55 [55]	6.5-9.4 [8]	Oil/water mixtures of the Samotlor oilfields, western Siberia, Russia
Petrotoga mobilis (Lien et al., 1998)§	AN COH	40-65 [58-60]	5.5-8.5 [6.5-7]	Oil reservoir production water from off-shore oil platforms, North Sea
Petrotoga halophila (Miranda-Tello et al., 2007)	AN COH	45-65 [55-60]	5.6-7.8 [6.7-7.2]	Oil-producing well of the Tchibouella offshore oilfield in Congo, West Africa
Thermosipho atlanticus (Urios et al., 2004b)	AN COH	45-80 [65]	5-9 [6]	Deep sea hydrothermal vent, Mid-Atlantic ridge
Thermosipho melanesiensis (Antoine et al., 1997)§	AN COH	45-80 [70]	3.5-9.5 [6.5-9.5]	Deep sea hydrothermal area, Lau Basin, southwest Pacific Ocean
Thermosipho geolei (Haridon et al., 2001)	AN COH	45-75 [70]	6-9.4 [7.5]	Production well-head oil/water mixture of a deep continental petroleum reservoir, western Siberia, Russia

Thermosipho japonicus (Takai and Horikoshi, 2000)	AN COH	45-80 [72]	5.3-9.3 [7.2]	Hydrothermal field, Iheya Basin of the Okinawa Trough, Japan
Thermosipho africanus (Huber et al., 1989; Ravot et al., 1996)	AN COH	35-77 [75]	6-8 [7.2]	Hydrothermal springs, Gulf of Tadjoura, Republic of Djibouti, Africa
Fervidobacterium gondwanense (Andrews and Patel, 1996)	AN COH	[65-68]	[7]	Great Artesian Basin, Australia
Fervidobacterium islandicum (Huber et al., 1990)	AN COH	50-80 [65]	6-8 [7]	Hot spring Hverageröi, Iceland
***Fervidobacterium nodosum* (Patel et al., 1985)**§	AN COH	41-79 [70]	6-8 [7]	Hot spring in New Zealand
Fervidobacterium pennavorans (Friedrich and Antranikian, 1996)	AN COH	50-80 [70]	5.5-8.0 [6.5]	Hot springs of the island San Miguel, Azores, Portugal
Fervidobacterium changbaicum (Cai et al., 2007)	AN COH	55-90 [75-80]	6.3-8.5 [7.5]	Hot spring, Changbai Mountains, China
Thermotoga lettingae (Balk et al., 2002)§	AN COH	50-75 [65]	6-8.5 [7]	Thermophilic, sulfate-reducing, slightly saline bioreactor
Thermotoga elfii (Ravot et al., 1995)	AN COH	50-72 [66]	5.5-8.7 [7.5]	African oil well
Thermotoga hypogea (Fardeau et al., 1997)	AN COH	56-90 [70]	6.1-9.1 [7.3-7.4]	Oil-producing well, Cameroon, central Africa
Thermotoga subterranea (Jeanthon et al., 1995)	AN COH	50-75 [70]	6-8.5 [7]	Oeep continental oil reservoir, East Paris Basin, France
Thermotoga thermarum (Windberger et al., 1989)	AN COH	55-84 [70]	5.5-9 [7]	Hot water and mud from the shore of Lac Abbe, Republic of Djibouti, Africa
Thermotoga maritima (Huber et al., 1986)§(Nelson et al., 1999)	AN COH	55-90 [80]	5.5-9 [6.5]	Geothermally heated sea floors, Italy and the Azores
Thermotoga petrophila (Takahata et al., 2001)§	AN COH	47-88 [80]	5.2-9 [7]	Production fluid of the Kubiki oil reservoir in Niigata, Japan
Thermotoga naphthophila (Takahata et al., 2001)	AN COH	48-86 [80]	5.4-9 [7]	Production fluid of the Kubiki oil reservoir in Niigata, Japan
Thermotoga neapolitana (Jannasch et al., 1988)§	AN COH	55-90 [80]	5.5-9 [7]	Shallow submarine hot springs, Lucrino Bay, Naples, Italy

***Archaea; Crenarchaeota; Thermoprotei; Desulfurococcales; Desulfurococcaceae* {A01}** **Genera: *Acidilobus, Staphylothermus, Ignicoccus, Desulfurococcus Thermosphaera, Sulfophobococcus, Stetteria, Thermodiscus,* (Also *Ignishpaera* of the *Ignisphaera* group)**				
Thermosphaera aggregans (Huber et al., 1998a)	AN COH	65-90 [85]	5-7 [6.5]	'Obsidian Pool' Yellowstone National Park, USA
Acidilobus aceticus (Prokofeva et al., 2000)	AN COH	60-92 [85]	2-6 [3.8]	A hot spring of the Mountnovski volcano; Kamchatka, Far East Russia
Staphylothermus marinus (Fiala et al., 1986)§	AN COH	65-98 [92]	4.5-8.5 [6.5]	Vulcano Island, Italy, also a deep-sea black smoker of the East Pacific Rise
Staphylothermus hellenicus (Arab et al., 2000)	AN COH	70-90 [85]	4.5-7 [6]	Palaeochori Bay, Milos, Greece
Ignicoccus islandicus (Huber et al., 2000a)	AN CLA	70-98 [90]	3.8-6.5 [5.8]	Submarine hydrothermal system, Atlantic; at the Kolbeinsey Ridge north of Iceland
Ignicoccus hospitalis (Paper et al., 2007)	AN CLA	73-98 [90]	4.5-7.0 [5.5]	Kolbeinsey Ridge, to the north of Iceland
Ignicoccus pacificus (Huber et al., 2000a)	AN CLA	75-98 [90]	4.5-7 [6]	Submarine hydrothermal system; black smoker samples, 9° N, 104° W, Pacific Ocean
Desulfurococcus fermentans (Perevalova et al., 2005)	AN COH	63-89 [80-82]	4.8-6.8 [6]	Freshwater hot spring of the Uzon Caldera, Kamchatka, Far East Russia
Desulfurococcus amylolyticus (Bonch-Osmolovskaya et al., 1988; Turova et al., 2000)	AN COH	68-97 [90-92]	5.7-7.5 [6.4]	Thermal springs of Kamchatka and Kunashir Islands, Far East Russia
Desulfurococcus mucosus (Zillig et al., 1982)	AN COH	[85]	[6]	Solfataric hot springs, Iceland
Desulfurococcus mobilis (Zillig et al., 1982)	AN COH	[85]	[6]	Solfataric hot springs, Iceland
Sulfophobococcus zilligii (Hensel et al., 1997)	AN COH	70-90 [85]	6.5-8.5 [7.5]	Hot spring near Hveragerdi, Iceland
Stetteria hydrogenophila (Jochimsen et al., 1997)	AN CLH	68-102 [95]	4.5-7.0 [6]	Marine hydrothermal sediment, Paleohori Bay, Milos, Greece.
Thermodiscus maritimus (Stetter, 1986; Burggraf et al., 1997; Stetter, 2001a)	AN COH	75-98 [90]	5-7 [5.5]	Submarine solfatara filed close to Vulcano, Italy
Ignisphaera aggregans (Niederberger et al., 2006)	AN COH	85-98 [92-95]	5.4-7 [6.4]	Geothermal Rotorua,Tokaanu New Zealand

Archaea; Crenarchaeota; Thermoprotei; Desulfurococcales; Pyrodictiaceae* {A02};** **Genera: *Pyrodictium, Hyperthermus, Pyrolobus				
Pyrodictium abyssi (Pley et al., 1991)	AN COH	80-110 [97]	4.7-7.1 [5.5]	Deep-sea hydrothermal areas of Guaymas Basin, Gulf of California and Kolbeinsey ridge, north of Iceland
Pyrodictium brockii (Stetter et al., 1983)	AN CLA	[105]	5-7 [5.5]	Shallow submarine solfataric field at Porto di Levante, Vulcano Island, Italy
Pyrodictium occultum (Stetter et al., 1983)	AN CLA	[105]	5-7 [5.5]	Shallow submarine solfataric field at Porto di Levante, Vulcano Island, Italy
Hyperthermus butylicus (Zillig et al., 1991)§	AN COH	[95-107]	[7]	Hydrothermally heated flat-sea sediments off the coast of São Miguel Island, Azores
Pyrolobus fumarii (Blochl et al., 1997)	FAE CLA	90-113 [106]	4.0-6.5 [5.5]	TAG site, Mid Atlantic Ridge,

Archaea; Crenarchaeota; Thermoprotei; Sulfolobales; Sulfolobaceae* {A03};** **Genera: *Stygiolobus, Sulfurisphaera, Acidianus				
Stygiolobus azoricus (Segerer et al., 1991)	AN CLA	57-89 [80]	1-5.5 [2.5-3]	Solfataric fields of São Miguel Island, Azores
Sulfurisphaera ohwakuensis (Kurosawa et al., 1998)	FAE COH	63-92 [84]	1-5 [2]	Hot springs of Ohwaku Valley, Hakone, Japan
Acidianus brierleyi (Segerer et al., 1986)	FAE CLA	45-75 [70]	1-6 [1.5-2]	Distant acidic geo- and hydrothermal heated areas
Acidianus ambivalens (Zillig et al., 1986; Fuchs et al., 1996)	FAE CLA	[80]	1-3.5	Leirhnukur fissure, Iceland
Acidianus infernus (Segerer et al., 1986)	FAE CLA	65-96 [90]	1-5.5 [2]	Italy, Solfatara Crater and Pisciarelli Solfatara, Naples
Acidianus sulfidivorans (Plumb et al., 2007)	FAE CLA	45-83 [74]	0.35–3.0 [0.8-1.4]	Solfatara on Lihir Island, Papua New Guinea

Archaea; Crenarchaeota; Thermoprotei; Thermoproteales; Thermofilaceae* {A04}** **Genus: *Thermofilum				
Thermofilum pendens (Zillig et al., 1983b)§	AN COH	[85-90]	[5]	Icelandic solfataras

Archaea; Crenarchaeota; Thermoprotei; Thermoproteales; Thermoproteaceae* {A05}** ***Genera: Thermoproteus, Pyrobaculum, Thermocladium, Caldvirga				
Thermoproteus uzoniensis (Bonch-Osmolovskaya et al., 1990b)	AN COH	74-102 [90]	4.6-6.8 [5.6]	Uzon Caldera, Kamchatka, Far-East Russia
Thermoproteus tenax (Zillig and Reysenbach, 2001)	AN F-CLA	[85]	[3.7-4.2]	Icelandic solfataric hot springs
Thermoproteus neutrophilus (Zillig, 1989; Zillig and Reysenbach, 2001)§	AN F-CLA	[85]	[6.8]	Hot spring, Iceland
Pyrobaculum oguniense (Sako et al., 2001)	AN COH	70-97 [90-94]	5.4-7.4 [6.3-7]	Terrestrial hot spring at Oguni-cho, Kumamoto Prefecture, Japan
Pyrobaculum arsenaticum (Huber et al., 2000c)§	AN F-CLA	68-100 [81]	NR	Hot water pond, Pisciarelli Solfatara, Naples, Italy
Pyrobaculum organotrophum (Huber et al., 1987)	AN COH	74-102 [100]	5-7 [6]	Boiling solfatic waters in Iceland, Italy, and the Azores
Pyrobaculum islandicum (Huber et al., 1987)§	AN F-CLA	74-102 [100]	5-7 [6]	Boiling solfataras and geothermal waters, Iceland
Pyrobaculum calidifontis (Amo et al., 2002)§	FAE COH	75-100 [90-95]	5.5-8.0 [7.0]	Terrestrial hot spring Calamba, Laguna, the Philippines
Pyrobaculum aerophilum (Volkl et al., 1993)§(Fitz-Gibbon et al., 2002)	FAE F-CLA	75-104 [100]	5.8-9 [7]	Boiling marine water hole, Maronti Beach, Ischia, Italy
Thermocladium modestius (Itoh et al., 1998)	FAE COH	45-82 [72]	2.6-5.9 [4]	Sites of volcanic activity, Japan
Vulcanisaeta souniana (Itoh et al., 2002)	FAE COH	65-89 [85]	3.5-5.0 [4.5]	Hot spring, Sounzan, Kanagawa, Japan
Vulcanisaeta distributa (Itoh et al., 2002)	FAE COH	70-92 [90]	3.5-5.6 [4.5]	Hot spring, Ohwakudani, Kanagawa, Japan
Caldivirga maquilingensis (Itoh et al., 1999)§	FAE COH	62-92 [85]	2.3 -6.4 [3.7-4.2]	Acidic hot spring in the Philippines

Archaea; Euryarchaeota; Thermoplasmata; Thermoplasmatales; Thermoplasmataceae* {A06};** **Genus: *Thermoplasma				
Thermoplasma acidophilum (Segerer et al., 1988)§(Ruepp et al., 2000)	FAE COH	45-63 [59]	0.5-4 [1-2]	Solfatara fields and self heated coal refuse piles
Thermoplasma volcanium (Segerer et al., 1988)§(Kawashima et al., 2000)	FAE COH	33-67 [60]	1-4 [2]	Submarine and continental solfataras at Vulcano Island, Italy; also from Java, Iceland and Yellowstone National Park, USA

Archaea; Euryarchaeota; Methanococci; Methanococcales; Methanocaldococcaceae* {A07}** **Genera: *Methanocaldococcus, Methanotorris				
Methanocaldococcus fervens (Jeanthon et al., 1999)	AN CLA	48-92 [85]	5.5-7.6 [6.5]	Deep-sea hydrothermal vent, Guaymas Basin, Gulf of California
Methanocaldococcus indicus (L'Haridon et al., 2003)	AN CLA	50-86 [85]	[6.5-6.6]	Deep-sea hydrothermal vent, Central Indian Ridge
Methanocaldococcus infernus (Jeanthon et al., 1998; Whitman, 2001c)	AN CLA	55-91 [85]	5.25-7.0 [6.5]	Deep-sea hydrothermal chimney sample collected on the Mid-Atlantic Ridge at a depth of 3000 m
Methanocaldococcus jannaschii (Jones et al., 1983; Whitman, 2001c)§ (Bult et al., 1996)	AN CLA	50-86 [85]	5.2-7.0 [6.0]	'White smoker' chimney on the 20°N East Pacific Rise
Methanocaldococcus vulcanius (Jeanthon et al., 1999)	AN CLA	49-89 [80]	5.2-7 [6.5]	Deep-sea vent, 13°N thermal field, East Pacific Rise
Methanotorris formicicus (Nakagawa et al., 2004a)	AN CLA	55-83 [75]	6-8.5 [6.7]	Deep-sea vent, Kairei field, Central Indian Ridge
Methanotorris igneus (Burggraf et al., 1990b; Whitman, 2001a)	AN CLA	45-91 [88]	5.0-7.5 [5.7]	Kolbeinsey ridge shallow (103 and 106 m) submarine hydrothermal system off Iceland

Archaea; Euryarchaeota; Thermococci; Thermococcales; Thermococcaceae* {A08};** **Genera: *Thermococcus, Pyrococcus, Palaeococcus				
Thermococcus sibiricus (Miroshnichenko et al., 2001)	AN COH	40-88 [78]	5.8-9 [7.5]	Samotlor oil reservoir, Western Siberia
Thermococcus zilligii (Ronimus et al., 1997)	AN COH	55-85 [75-80]	5.4-9.2 [7.4]	Terrestrial fresh water hot pool in New Zealand
Thermococcus profundus (Kobayashi et al., 1994)	AN COH	50-90 [80]	4.5-8.5 [7.5]	Deep-sea hydrothermal vent system, Middle Okinawa Trough
Thermococcus barossii (Duffaud et al., 1998)	AN COH	60-94 [82.5]	4-9 [6.5-7.5]	Hydrothermal vent flange formation, East Pacific Rise of the Juan de Fuca Ridge
Thermococcus siculi (Grote et al., 1999)	AN COH	50-93 [85]	5-9 [7]	Deep-sea hydrothermal vent; the Mid-Okinawa Trough
Thermococcus celericrescens (Kuwabara et al., 2007)	AN COH	50-85 [80]	5.6–8.3 [7.0]	Hydrothermal vent at Suiyo Seamount, Izu-Bonin Arc, Western Pacific Ocean
Thermococcus acidaminovorans (Dirmeier et al., 1998)	AN COH	56-93 [85]	5-9.5 [9]	Vulcano Island, Italy
Thermococcus aegaeus (Arab et al., 2000)	AN COH	50-95 [85]	4.5-7.5 [6]	Palaeochori Bay, Milos, Greece
Thermococcus alcaliphilus (Keller et al., 1995)	AN COH	56-90 [85]	6.5-10.5 [9]	Vulcano Island, Italy
Thermococcus atlanticus (Cambon-Bonavita et al., 2003)	AN COH	70-90 [85]	5-8 [7]	'Snakepit' hydrothermal vent region of the Mid-Atlantic ridge
Thermococcus barophilus (Marteinsson et al., 1999)§	AN COH	48-100 [85]	[7]	'Snakepit' hydrothermal vent region of the Mid-Atlantic ridge
Thermococcus chitonophagus (Huber et al., 1995)	AN COH	60-93 [85]	3.5-9 [6.7]	Guaymas Basin hydro-thermal vents, Gulf of California
Thermococcus fumicolans (Godfroy et al., 1996)	AN COH	73-103 [85]	4.5-9.5 [8.5]	Deep-sea hydrothermal vent, North Fiji Basin
Thermococcus litoralis (Neuner et al., 1990)	AN COH	50-96 [85]	4-8.5 [6]	Vulcano Island, Italy, alo submarine thermal spring at Lucrino beach, Naples
Thermococcus thioreducens (Pikuta et al., 2007)	AN COH	55–94 [83–85]	5.0–8.5 [7.0]	'Black smoker' chimney material from the 'Rainbow' hydrothermal vent site on the Mid-Atlantic Ridge (36.2°N, 33.9°W)

Thermococcus waiotapuensis (Gonzalez et al., 1999)	AN COH	60-90 [85]	5-8 [7]	Terrestrial freshwater hot spring, New Zealand
Thermococcus stetteri (Miroshnichenko et al., 1989)	AN COH	55-95 [75-88]	5.7-7.2 [6.5]	Marine solfataric fields of Kraternaya cove, Ushishir archipelago, Northern Kurils
Thermococcus pacificus (Miroshnichenko et al., 1998)	AN COH	70-95 [80-88]	6-8 [6.5]	Bay of Plenty, New Zealand
Thermococcus aggregans (Canganella et al., 1998)	AN COH	60-94 [88]	5.6-7.9 [7]	Guaymas Basin hydro-thermal vents, Gulf of California
Thermococcus celer (Zillig et al., 1983a)	AN COH	[88]	[8.5]	Vulcano Island, Italy
Thermococcus gammatolerans (Jolivet et al., 2003)	AN COH	55-95 [88]	[6]	Guaymas Basin, Gulf of California
Thermococcus gorgonarius (Miroshnichenko et al., 1998)	AN COH	68-95 [80-88]	5.8-8.5 [6.5-7.2]	Shore of Whale Island, New Zealand
Thermococcus guaymasensis (Canganella et al., 1998)	AN COH	56-90 [88]	5.6-8.1 [7.2]	Guaymas Basin hydrothermal vent site in the Gulf of California
Thermococcus hydrothermalis (Godfroy et al., 1997)	AN COH	55-100 [80-90]	3.5-9.5 [5.5-6.5]	21°N deep-sea hydrothermal vent region, East Pacific Rise
Thermococcus peptonophilus (Gonzalez et al., 1995)	AN COH	60-100 [85-90]	4-8 [6]	Izu-Borin forearc, also from the southern Mariana Trough
Thermococcus kodakarensis (Fujiwara et al., 1998; Atomi et al., 2004)§(Fukui et al., 2005)	AN COH	60-100 [85]	5-9 [6.5]	Solfatara on Kodakara Island, Kagoshima, Japan
Thermococcus coalescens (Kuwabara et al., 2005)	AN COH	57-90 [87]	5.2-8.7 [6.5]	Hydrothermal fluid obtained from Suiyo Seamount of the Izu-Bonin Arc
Pyrococcus furiosus (Fiala and Stetter, 1986)§(Maeder et al., 1999)	AN COH	70-103 [100]	5-9 [7]	Shallow marine hydrothermal system at Vulcano Island, Italy
Pyrococcus horikoshii (Gonzalez et al., 1998)§(Kawarabayasi et al., 1998; Maeder et al., 1999)	AN COH	80-102 [98]	5-8 [7]	Hydrothermal fluid samples obtained at the Okinawa Trough vents in the NE Pacific Ocean, at a depth of 1395 m
Pyrococcus glycovorans (Barbier et al., 1999)	AN COH	75-104 [95]	2.5-9 [7.5]	Deep-sea hydrothermal vent on the East Pacific Rise

Pyrococcus woesei (Zillig et al., 1987) Note: Kanoksilapatham et al. (2004), suggest reclassifying *Pyrococcus woesei* as *Pyrococcus furiosus* subsp. *woesei* (Kanoksilapatham et al., 2004)	AN COH	[100-103]	[6-6.5]	Marine solfataras at the northern beach of Porto di Levante, Vulcano Island, Italy
Palaeococcus ferrophilus (Takai et al., 2000)	AN COH	60-88 [83]	4-8 [6]	Hydrothermal vent chimney, Myojin Knoll; Ogasawara-Bonin Arc, Japan
Palaeococcus helgesonii (Amend et al., 2003)	FAE COH	45-85 [80]	5–8 [6.5]	Geothermal well on Vulcano Island, Italy

Archaea; Euryarchaeota; Archaeoglobi; Archaeoglobales; Archaeoglobaceae* {A09};** **Genera: *Archeoglobus, Geoglobus, Ferroglobus				
Archaeoglobus veneficus (Huber et al., 1997)	AN F-CLA	65-85 [75-80]	6.5-8 [7]	'Snake Pit' hydrothermoal of the Mid-Atlantic Ridge;
Archaeoglobus profundus (Burggraf et al., 1990a)	AN CLH	65-90 [82]	4.5-7.5 [6]	The Guaymas hot vent area; cores of hot sediment and active smoker chimneys
Archaeoglobus fulgidus (Stetter et al., 1987; Stetter, 1988)§(Klenk et al., 1997)	AN F-CLA	64-92 [83]	5.5-7.5	Marine hydrothermal systems at Vulcano island and at Stufe di Nerone, Naples, Italy
Geoglobus ahangari (Kashefi et al., 2002)	AN F-CLA	65-90 [88]	5-7.6 [7.0]	Guaymas Basin hydrothermal system, Gulf of California, at a depth of 2000 m
Ferroglobus placidus (Hafenbradl et al., 1996)	AN F-CLA	65-95 [85]	6.0-8.5 [7.0]	Shallow submarine hydrothermal systems at Vulcano island, Italy

Archaea; Euryarchaeota; Methanopyri; Methanopyrales; Methanopyraceae* {A10};** **Genus: *Methanopyrus				
Methanopyrus kandleri (Kurr et al., 1991)§(Slesarev et al., 2002)	AN CLA	84-110 [98]	5.5-7 [6.5]	Deep-sea sediment from the Guaymas Basin, Gulf of California, and from the shallow marine hydrothermal system of the Kolbeinsey ridge, Iceland

Archaea; Euryarchaeota; Methanobacteria; Methanobacteriales; Methanothermaceae* {A11};** **Genus: *Methanothermus				
Methanothermus sociabilis (Lauerer et al., 1986)	AN CLA	55-97 [88]	5.5-7.5 [6.5]	Hot waters and muds of Icelandic continental geothermal areas
Methanothermus fervidus (Stetter, 2001b)	AN CLA	55-97 [80-85]	NR	Icelandic solfatara fields at Keringarfjoll and Hveragerði

Archaea; Euryarchaeota; Methanobacteria; Methanobacteriales; Methanobacteriaceae* {A12};** **Genera: *Methanobacterium, Methanothermobacter				
Methanobacterium thermaggregans (Blotevogel and Fischer, 1985)	AN CLA	40-75 [65]	6.5-9 [7-7.5]	Mud from a cow pasture
Methanothermobacter defluvii (Kotelnikova et al., 1993; Boone, 2001)	AN CLA	[60-65]	[6.5-7.0]	Anaerobic sludge obtained from the pilot-scale plant digesting methacrylic waste water
Methanothermobacter marburgensis (Wasserfallen et al., 2000)	AN CLA	45-70 [65]	5.0-8.0 [6.8-7.4]	Mesophilic sewage sludge and hot springs
Methanothermobacter thermautotrophicus (Wasserfallen et al., 2000)§(Smith et al., 1997)	AN CLA	40-75 [65-70]	6.0-8.8 [7.2-7.6]	Anaerobic sewage sludge digestor
Methanothermobacter thermoflexus (Kotelnikova et al., 1993; Boone, 2001)	AN CLA	[55]	[7.9-8.2]	Anaerobic sludge obtained from the pilot-scale plant digesting methacrylic waste water
Methanothermobacter thermophilus (Laurinavichius et al., 1988; Boone, 2001)	AN CLA	45-65 [57]	7.0-8.5 [7.5]	Uzon Caldera, Kamchatka, Far East Russia
Methanothermobacter wolfei (Winter et al., 1985; Wasserfallen et al., 2000)	AN CLA	37-74 [55-65]	6.0-8.2 [7.0-7.5]	Mixture of sewage sludge and river sediment

Archaea; Euryarchaeota; Methanococci; Methanococcales; Methanococcaceae* {A13}; *Genus: Methanothermococcus				
Methanothermococcus thermolithotrophicus (Huber et al., 1982; Whitman, 2001b)§	AN CLA	30-70 [65]	6-8 [7]	Heated sea sediments near Naples, Italy
Methanothermococcus okinawensis (Takai et al., 2002)	AN CLA	40-75 [60-65]	4.5-8.5 [6-7]	Deep-sea vent at Iheya Ridge in the Okinawa Trough, Japan

Archaea; Euryarchaeota; Methanomicrobia; Methanosarcinales; Methanosarcinaceae* {A14}; Genera: *Methanosarcina, Methanomethylovorans				
Methanosarcina thermophila (Zinder et al., 1985)§	AN F-CLA	[50]	[6-7]	55 °C anaerobic sludge digestor
Methanomethylovorans thermophila (Jiang et al., 2005)	AN CLA	42-58 [50]	5-7 [6.5]	Thermophilic laboratory-scale upflow anaerobic sludge blanket reactor fed

Archaea; Euryarchaeota; Methanomicrobia; Methanosarcinales; Methermicoccaceae* {A15}; Genus: *Methermicoccus				
Methermicoccus shengliensis (Cheng et al., 2007)	AN CLA	50-70 [65]	5.5-8.0 [6.0-6.5]	Oil-production water of the Shengli oilfield, China

Archaea; Euryarchaeota; Methanomicrobia; Methanosarcinales; Methanosaetaceae* {A16}; Genus: *Methanothrix				
Methanothrix thermophila (Kamagata et al., 1992)§	AN COH	[55]	6.1-7.5 [6.7]	Mesophilic anaerobic sludge digestors

Archaea; Euryarchaeota; Methanomicrobia; Methanomicrobiales; Methanomicrobiaceae* {A17} Genus: *Methanoculleus				
Methanoculleus thermophilus (Rivard and Smith, 1982; Maestojuan et al., 1990)	AN F-CLA	37-65 [55]	6.18-7.82 [7]	Nuclear power plant effluent channel sediment

Fig. 2.1. Prokaryotic phylogenetic tree highlighting lineages with anaerobic thermophilic taxa. 16S rDNA-based phylogenetic tree, lineages with thermophilic or hyperthermophlic (T_{opt} 80°C or higher) anaerobic taxa are indicated with a bold text. Hyperthermophilic lineages are further differentiated with extra-bold branches. Sequences were aligned using ClustalW (Thompson et al., 1994), and the tree was constructed using the neighbor-joining method (Saitou and Nei, 1987), with the Jukes and Cantor distance corrections (Jukes and Cantor, 1969) through the PHYLIP program (Felsenstein, 2001). Scale bar = 5 nucleotide changes per 100 base pairs. When possible, the 16S rDNA sequence of the type species of the type genus of the family was used as a proxy for the prokaryotic family. If the nucleotide sequence of the type species was unavailable or of poor quality, a sequence from another member of the type genus was used. To our knowledge, no thermophilic anaerobes are present within the *Actinobacteria* or α-*Proteobacteria* phylogenetic groups. Within the shown β-*Proteobacteria* clade, thermophilic anaerobes have only been found within the *Burkholderiaceae*, and within the γ-*Proteobacteria*, thermophilic anaeorbes have only been found within the *Thermithiobacillaceae* and the *Chromatiaceae*. Designations within the brackets correspond to the thermophilic anaerobe-containing families as listed within Table 1 and as presented throughout the text. Family, species, and corresponding GenBank accession numbers for the 16S rDNA sequences used for the construction of the tree: *Acholeplasmataceae, Acholeplasma laidlawii* U14905; *Acidaminococcaceae* {*B*10}, *Acidaminococcus fermentans* X65935; *Acidimicrobiaceae, Acidimicrobium ferrooxidans* U75647; *Acidithiobacillaceae*, *Acidithiobacillus thiooxidans* Y11596; *Anaerolinaceae* {*B*27}, *Anaerolinea thermophila* AB046413; *Anaeroplasmataceae*, *Anaeroplasma abactoclasticum* M25050; *Aquificaceae* {*B*32}, *Aquifex pyrophilus* M83548; *Archaeoglobaceae* {*A*09}, *Archaeoglobus fulgidus* X05567; *Archangiaceae, Archangium gephyra* DQ768106; *Bacillaceae* {*B*15}, *Bacillus subtilis* AJ276351; *Bacteriovoracaceae, Bacteriovorax stolpii* AJ288899; *Bacteroidaceae* {*B*04}, *Bacteroides fragilis* X83935; *Bdellovibrionaceae*, *Bdellovibrio bacteriovorus* AJ292759; *Bifidobacteriaceae, Bifidobacterium bifidum* EF589113; *Burkholderiaceae*{*B*03}, *Burkholderia cepacia* U96927; *Caldilineaceae* {*B*28}, *Caldilinea aerophila*

AB067647; *Campylobacteraceae*, *Campylobacter fetus* L04314; *Caryophanaceae*, *Caryophanon latum* AJ491302; *Chlamydiaceae*, *Chlamydia trachomatis* D89067; *Chlorobiaceae*, *Chlorobium limicola* NZ_AAHJ01000048 (region 4935-6399); *Chloroflexaceae* {*B*29}, *Chloroflexus aggregans* D32255; *Chromatiaceae*{*B*02}, *Chromatium okenii* AJ223234; *Chrysiogenaceae*, *Chrysiogenes arsenates* X81319; *Clostridiaceae* {*B*14}, *Clostridium butyricum* AB075768; *Conexibacteraceae*, *Conexibacter woesei* AJ440237; *Coriobacteriaceae*, *Coriobacterium glomerans* X79048; *Cryomorphaceae*, *Cryomorpha ignava* AF170738; Cystobacteraceae, ytophaga hutchinsonii CP000383 (region 120118-121499); *Deferribacteraceae* {*B*19}, *Deferribacter thermophilus* U75602; *Deinococcaceae*, *Deinococcus radiodurans* Y11332; *Desulfarculaceae*, *Desulfarculus baarsii* M34403; *Desulfobacteraceae*, *Desulfobacter postgatei* AF418180; *Desulfobulbaceae*, *Desulfobulbus propionicus* AY548789; *Desulfohalobiaceae* {*B*21}, *Desulfohalobium retbaense* U48244; *Desulfomicrobiaceae*, *Desulfomicrobium baculatum* AF030438; *Desulfonatronaceae*, *Desulfonatronum lacustre* Y14594; *Desulfovibrionaceae*, *Desulfovibrio desulfuricans* AF192153; *Desulfurellaceae* {*B*16}, *Desulfurella acetivorans* X72768; *Desulfurobacteriaceae*, *Desulfurobacterium thermolithotrophum* AJ001049; *Desulfurococcaceae* {*A*01}, *Desulfurococcus mobilis* M36474; *Desulfuromonadaceae*, *Desulfuromonas acetoxidans* NZ_AAEW02000008 (region 189409-190966); *Dictyoglomaceae* {*B*26}, *Dictyoglomus thermophilum* X69194; *Entomoplasmataceae*, *Entomoplasma freundtii* AF036954; *Erysipelotrichaceae*, *Erysipelothrix rhusiopathiae* M23728; *Ferroplasmaceae*, *Ferroplasma acidiphilum* AJ224936; *Flavobacteriaceae*, *Flavobacterium anhuiense* EU046269; *Gemmatimonadaceae*, *Gemmatimonas aurantiaca* AB072735; *Geobacteraceae*, *Geobacter metallireducens* L07834; *Halanaerobiaceae* {*B*06}, *Halanaerobium praevalens* AB022034; *Halobacteriaceae*, *Halobacterium salinarum* AJ496185; *Halobacteroidaceae*, *Halobacteroides halobius* U32595; *Helicobacteraceae*, *Helicobacter pylori* Z25741; *Heliobacteriaceae* {*B*13}, *Heliobacterium sulfidophilum* AF249678; *Hydrogenimonaceae* {*B*18}, *Hydrogenimonas thermophila* AB105048; *Hydrogenothermaceae* {*B*33}, *Hydrogenothermus marinus* AJ292525; *Kofleriaceae*, *Kaistella flava* AM421015; *Ktedonobacteraceae*, *Ktedobacter racemifer* AM180156; *Lactobacillaceae*, *Lactobacillus delbrueckii* M58814; *Leptospiraceae*, *Leptospira*

interrogans Z12817; *Lysobacteraceae*, *Lysobacter enzymogenes* AY947529; *Methanobacteriaceae* {*A*12}, *Methanobacterium thermaggregans* AF095264; *Methanocaldococcaceae* {*A*07}, *Methanocaldococcus jannaschii* NC_000909 (region 157985-159459); *Methanococcaceae* {*A*13}, *Methanococcus vannielii* NC_009634 (region 155-1619); *Methanocorpusculaceae*, *Methanocorpusculum parvum* AY260435; *Methanomicrobiaceae* {*A*17}, *Methanomicrobium mobile* AY196679; *Methanoplanaceae*, *Methanoplanus limicola* M59143; *Methanopyraceae* {*A*10}, *Methanopyrus kandleri* AE010349 (region 6963-8474); *Methanosaetaceae* {*A*16}, *Methanosaeta thermoacetophila* AB071701; *Methanosarcinaceae* {*A*14}, *Methanosarcina thermophila* M59140; *Methanospirillaceae*, *Methanospirillum hungatei* AY196683; *Methanothermaceae* {*A*11}, *Methanothermus fervidus* M32222; *Methermicoccaceae* {*A*15}, *Methermicoccus shengliensis* EF026570; *Mycoplasmataceae*, *Mycoplasma mycoides* EU040177; *Myxococcaceae*, *Myxococcus fulvus* AB218224; *Nannocystaceae*, *Nannocystis exedens* AB084253; *Natranaerobiaceae* {*B*08}, *Natranaerobius thermophilus* DQ417202; *Nautiliaceae* {*B*17}, *Nautilia lithotrophica* AJ404370; *Nitrospinaceae*, *Nitrospina gracilis* L35504; *Nitrospiraceae* {*B*23}, *Nitrospira moscoviensis* X82558; *Opitutaceae*, *Opitutus terrae* AJ229235; *Oscillochloridaceae*, *Oscillochloris trichoides* AF093427; *Oscillospiraceae*, *Oscillospira guilliermondii* AB040495; *Parachlamydiaceae*, *Planctomyces brasiliensis* AJ231190; *Pasteuriaceae*, *Pasteuria hartismerei* AJ878853; *Patulibacteraceae*, *Patulibacter minatonensis* AB193261; *Peptococcaceae* {*B*11}, *Peptococcus niger* X55797; *Picrophilaceae*, *Picrophilus oshimae* X84901; *Planctomycetaceae*, *Planctomyces brasiliensis* AJ231190; *Planococcaceae*, *Planococcus citreus* X62172; *Polyangiaceae*, *Polyangium vitellinum* AJ233944; *Prochloraceae*, *Prochloron* sp. X63141; *Prochlorotrichaceae*, *Prochlorothrix hollandica* AJ007907; *Puniceicoccaceae*, *Puniceicoccus vermicola* DQ539046; *Pyrodictiaceae* {*A*02}, *Pyrodictium occultum* M21087; *Rubrobacteraceae*, *Rubrobacter radiotolerans* X87134; *Simkaniaceae*, *Simkania negevensis* U68460; *Solirubrobacteraceae*, *Solirubrobacter pauli* AY039806; *Sphaerobacteraceae* {*B*30}, *Sphaerobacter thermophilus* AJ420142; *Sphingobacteriaceae*, *Sphingomonas paucimobilis* U20776; *Spirochaetaceae* {*B*05}, *Spirochaeta bajacaliforniensis* AJ698859; *Spiroplasmataceae*, *Spiroplasma citri* AM157769; *Spirosomaceae*, *Spirosoma lingual* AM000023;

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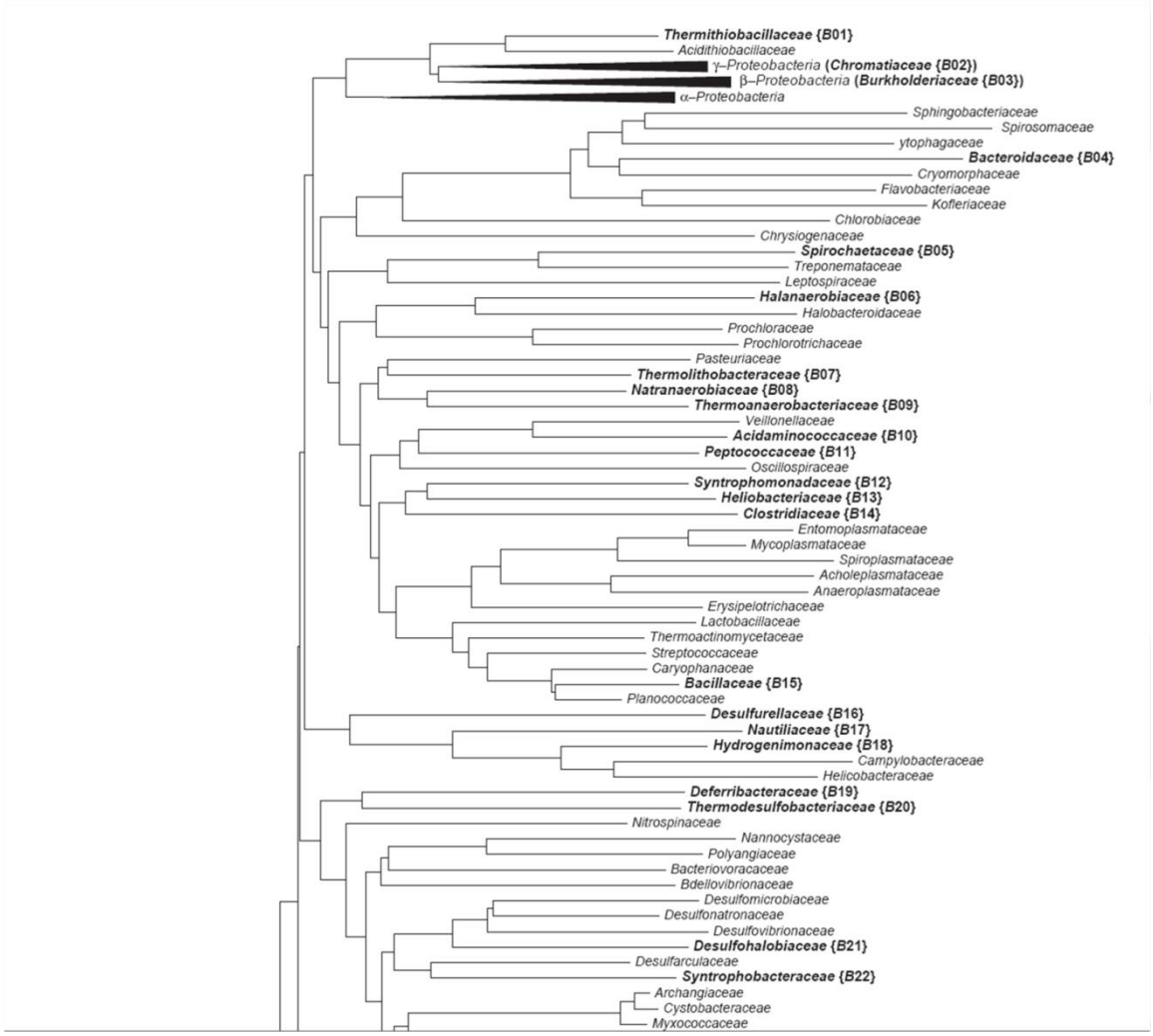
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Spirosomaceae
ytophagaceae
Bacteroidaceae {B04}
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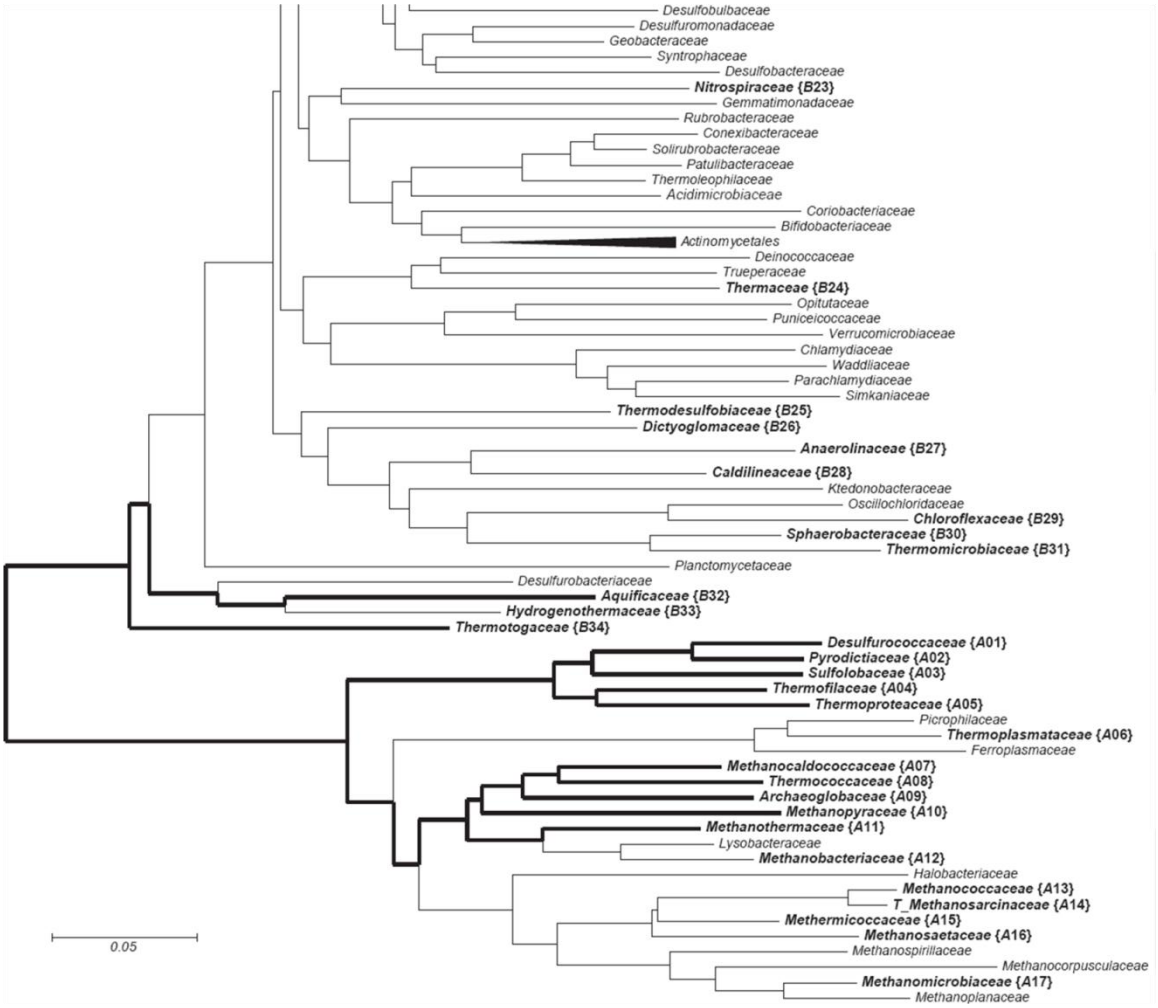
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Coriobacteriaceae
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Trueperaceae
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Parachlamydiaceae
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Thermodesulfobiaceae {B25}
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Methanomicrobiaceae {A17}
Methanoplanaceae
0.05

CHAPTER 3

CALDANAEROVIRGA ACETIGIGNENS GEN. NOV., SP. NOV., AN ANAEROBIC XYLANOLYTIC ALKALITHERMOPHILIC BACTERIUM ISOLATED FROM TREGO HOT SPRING, NEVADA, USA[4]

[4] Wagner, I. D., Ahmed, S., Zhao, W., Zhang, C. L., Romanek, C. S., Rohde, M. & Wiegel, J. (2009). *Caldanaerovirga acetigignens* gen. nov., sp. nov., an anaerobic, xylanolytic, alkalithermophilic bacterium isolated from Trego Hot Spring, Nevada, USA. *Int J Syst Evol Microbiol* **59**, 2685-2691.

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ABSTRACT

An anaerobic thermophilic bacterium, JW/SA-NV4^T, was isolated from a xylan-supplemented enrichment culture from Trego hot spring located within the Black Rock Desert (NV, USA). Cells were generally straight or slightly bent rod shaped 0.4-0.8 µm in width and 3-6 µm in length during exponential growth. Cells from stationary phase were variable in size and shape, showing curved or bent morphology. Motility was not seen and flagella were not observed in electron micrographs. Sporulation was not observed. Strain JW/SA-NV4^T stained Gram negative but is phylogenetically Gram-type positive. Growth at pH^{25C} 6.8-8.8, pH_{opt} = 8.4; no growth at 9.0 or above or at 6.5 or below. With glucose or xylose as the carbon source, strain JW/SA-NV4^T grew between 44- 74 °C, no growth at 76 °C or above and at 42 °C or below. However, the T_{opt} was 62 °C and 66 °C when grown on glucose and xylose, respectively. The shortest doubling time observed with glucose was approximately 4 hours, with xylose approximately 3.4 hours. Strain JW/SA-NV4^T tolerated an atmosphere containing up to 0.1% O_2; no growth at a gas atmosphere of 0.2% O_2. Chemoorganotrophic growth with xylose, glucose, mannnose, xylan, pyruvate, fructose, ribose, casamino acids, manitol, tryptone, peptone, cellobiose, and yeast extract. Grown in mineral media containing 1 g l^{-1}yeast extract as an electron donor, thiosulfate and sulfur were reduced to sulfide. G+C mol % content of the DNA was 38.6 (HPLC). 16S rRNA gene sequence analysis placed strain JW/SA-NV4^T within the order *Thermoanaerobacterales* and within the *Thermoanaerobacterales* Incertae Sedis Family III, specifically between taxa classified within the genera *Thermosediminibacter* and *Thermovenabulum*. The closest phylogenetic neighbors are *Thermosediminibacter oceani* JW/IW-1228P^T (94.2%) and *Thermosediminibacter litoriperuensis* JW/YJL-1230-7/2^T (94.0%) (Lee *et al.*, 2005). Based on physiological and genotypic characteristics, strain JW/SA-NV4^T

(JW/SA-NV4^T = DSM 18802^T = ATCC BAA-1454^T) is proposed as the type strain of a novel species in a novel genus *Caldanaerovirga acetigignens* gen. nov., sp. nov.

The numerous hot springs distributed throughout northern Nevada, USA, are the result of the Pacific and North American tectonic plates colliding and the circulation of meteoric waters along Basin and Range faults (Hose and Taylor, 1974; Pearson et al., 2004). Additionally, several of the hot springs in northern Nevada are alkaline from the bleaching of carbonate or silica-bearing rocks by hot water. Alkaline hot springs with pH values above 8.5 are less common than slightly- and strongly-acidic hot springs and are studied less frequently. One consequence is that alkalithermophiles, *Bacteria* and *Archaea* which grow at elevated temperatures (T_{opt} > 50 °C) and high pH ($pH_{opt} \geq 8.5$), have received less attention than the acidophilic or neutrophilic thermophiles (Wiegel, 1998; Kevbrin et al., 2004). Here we report on the isolation of a moderately alkalithermophilic anaerobe growing on hemicellulosic material. Xylan is a component of plant hemicellulose and the second-most abundant renewable polysaccharide. Xylanolytic microorganisms attract interest because the conversion of xylan to useful products can possibly be coupled to increasing the efficiency of processing lignocellulose (e.g., pulping processes) and to the production of energy from renewable resources (Biely, 1985; Himmel *et al.*, 2007). Xylan is widely used among thermophilic anaerobic *Bacteria*, especially among members of the *Firmicutes* but less studied in alkalithermophiles (Shao et al., 1995; Wiegel and Kevbrin, 2004; Wagner and Wiegel, 2008).

Water and sediment samples were collected from hot springs of northern Nevada at various sites, including Trego (4514476.9 N 321356.1 E, 46.8 °C, and pH 8.9). The samples were transferred to sterile anaerobic serum bottles, and sealed with butyl rubber stoppers.

Enrichment cultures were prepared from these Nevada Hot Spring samples using an anaerobic carbonate- and TAPS-buffered medium containing: 0.272 g l^{-1} KH_2PO_4, 0.5 g l^{-1} NH_4Cl, 0.5 g l^{-1} $(NH_4)_2SO_4$, 1.5 g l^{-1} $NaHCO_3$, 2.43 g l^{-1} TAPS buffer, 0.02 g l^{-1} $MgCl_2$, 0.015 g l^{-1} $CaCl_2$, 1.0 ml l^{-1} vitamin solution, and 1.0 ml l^{-1} Na_2SeO_3-Na_2WO_3 trace-element solution (Widdel and Bak, 1992) adjusted to either pH 8.5 or 9.5; 0.125 g l^{-1} cysteine HCl was added as a reducing agent. Xylose or xylan was added after autoclavation to a final concentration 0.3 g l^{-1} or 3.0 g l^{-1}, respectively. Initial incubations were performed at 62 °C. Following 5-7 days of incubation, growth was observed within the pH 8.5 xylan- or xylose-supplemented enrichment cultures from Trego hot spring. Agar (2.15% w/v)-shake-roll tubes were prepared, and single colonies from dilution series were sequentially isolated to obtain pure cultures (Ljungdahl and Wiegel, 1986). Culture purity was checked by phase contrast microscopy and analysis of the 16S rRNA gene sequence. Strain JW/SA-NV4^T was obtained and chosen for further study. For subsequent comparisons (see 16S rRNA gene sequence analysis below), *Thermosediminibacter oceani* strain JW/IW-1228P^T and *Thermosediminibacter litoriperuensis* strain JW/YJL-1230-7/2^T were obtained from laboratory stocks.

Within agar-shake-roll tubes, colonies of JW/SA-NV4^T were 0.1–1.5 mm in diameter and irregularly shaped. During exponential growth in a liquid medium, cells of strain JW/SA-NV4^T are uniformly straight or slightly bent rod shaped, 0.4-0.8 µm in width and 3-6 µm in length. Short chains of 2 or 3 cells are frequently observed (Fig 3.2a) but occasionally cells with lengths of 20-30 µm are observed during exponential growth. Cells from stationary phase cultures are variable in size and shape; most noticeably, cells are often curved or bent (Fig 3.2b). Strain JW/SA-NV4^T stained Gram negative but is phylogenetically Gram type positive (Wiegel, 1981). The formation of spores was not observed under various conditions including during growth on

glucose, beef extract, yeast extract supplied agar slants. No viability was observed when cultures were heated to 100 °C for 2 minutes as usual for thermophilic spore-forming anaerobic *Firmicutes*. Motility was not observed in cells from liquid culture and electron micrographs did not reveal flagella (Fig 3.2c).

Temperature profile, pH profile, NaCl tolerance, and substrate range were determined through measurement of ΔOD_{600} using a Spectronic 21 spectrophotometer (Bausch and Lomb). A temperature-gradient incubator (Scientific Industries) was used to determine the temperature range and optimum for growth of JW/SA-NV4^{T} using the carbonate- and TAPS-buffered basal medium complemented with 1.0 g l^{-1} yeast extract and either 3.0 g l^{-1} glucose or 3.0 g l^{-1} xylose. With glucose as a provided carbon source (pH^{25C} 8.1), strain JW/SA-NV4^{T} grew between 44-74 °C, optimally at about 61-62 °C; after more than 200 hours of incubation, no growth was observed at or below 42 °C, or at 77 °C or above. With xylose as the provided carbon substrate (pH^{25C} 8.2), strain JW/SA-NV4^{T} grew between 46 and 76 °C, optimally around 66 °C; after more than 300 hours of incubation, no growth was observed at or below 44 °C, or at 78 °C or above. In medium containing 1.0 g l^{-1} yeast extract and 3.0 g l^{-1} glucose (pH^{25C} 8.1) or xylose (ph^{25C} 8.2), the shortest doubling time was approximately 4 hours (glucose) and 3.4 hours (xylose). To determine the pH profile of JW/SA-NV4^{T}, 3 gl^{-1} glucose containing-media was buffered with 10 mM each of TES, TAPS and CAPS. Growth occurred between pH^{25C} 6.8-8.8, pH_{opt} = 8.4; no growth at 9.0 or above and at 6.5 or below. Strain JW/SA-NV4^{T} grew in medium containing up to 4.0% (w/v) NaCl by successive transferring growing culture to media with increased NaCl concentration; no growth was recorded at 4.5% (w/v) NaCl or higher.

Positive utilization of a carbon substrate was recorded for Strain JW/SA-NV4^{T} if ΔOD_{600} was twice that of cultures containing only the basal 0.5 g l^{-1} concentration of yeast extract

(ΔOD_{600} = 0.015). Strain JW/SA-NV4^T utilized xylose, glucose, mannnose, xylan, pyruvate, fructose, ribose, cellobiose, casamino acids, manitol, tryptone, peptone, yeast extract. Strain JW/SA-NV4^T did not utilize sucrose, glycine betaine, galactose, lactose, xylitol, sorbitol, inositol, arabinose, starch, or cellulose. Yeast extract is required for growth, with about 10 g l^{-1} saturation in media containing 5 g l^{-1} xylose. Acetate is the major fermentation product of strain JW/SA-NV4^T when grown with glucose as the carbon substrate with 2.2-2.4 mol acetate produced per mol glucose consumed suggesting a homoacetogenic fermentation, although a trace amount of lactate was occasionally detected (<0.1 mol lactate produced per mol glucose consumed; H_2 and CO_2 were not quantified) (Drake *et al.*, 2008). Fermentation products were similar when strain JW/SA-NV4^T was grown with either xylan or xylose.

Biochemical features of strain JW/SA-NV4^T, and phylogeneticly related taxa (see 16S rRNA sequence analysis below) *Thermosediminibacter oceani* JW/IW-1228P^T and *Thermosediminibacter litoriperuensis* JW/YJL-1230-7/2^T were tested using the api zym 25200 (bioMérieux) system (*Thermovenabulum ferriorganovorum* strain Z-9801^T is not available from DSMZ). For all three strains, strong positive reactions were observed for acid phosphatase and naphthol-AS-BI-phosphohydrolase. A strong α-glucosidase reaction was observed for *Thermosediminibacter oceani* JW/IW-1228P^T, but not for JW/SA-NV4^T or *Thermosediminibacter litoriperuensis* JW/YJL-1230-7/2^T (table 3.2).

The potential for strain JW/SA-NV4^T to utilize various electron acceptors was studied using the mineral media containing 1 g l^{-1}yeast extract as an electron donor. Thiosulfate (15 mM) and sulfur (0.96 g l^{-1}) were reduced to sulfide, as determined by the formation of CuS upon the addition of $CuSO_4$. Sulfate (15 mM) and sulfite (5 mM) were not reduced to sulfide. To determine the tolerance of oxygen by strain JW/SA-NV4^T, 140 ml of the inoculated mineral

media (containing 0.5 gL-1 yeast extract and 1 gL-1 glucose) were incubated in 1 l Media Storage Bottles Model 1395 (Pyrex) in the horizontal position. Media was prepared to be anoxic, by gassing with N_2 without reducing agents, and volumes of air were injected via syringes into the headspace. Liquid cultures of strain JW/SA-NV4^T tolerated an atmosphere containing up to 0.1% O_2, no growth was observed with an atmosphere of 0.2% O_2 or higher.

Lyophilized cell material was used to analyze the PLFA content of JW/SA-NV4^T. Lipid extraction was performed as previously described (Wagner *et al.*, 2008) based on the protocols of Zhang *et al.* (2004) and White *et al.* (1979). GC-MS analyses of FAME were performed on an HP5890 GC equipped with a 30-m, HP5-MSI column and programmable temperature vaporizing inlet and coupled to a HP5972 mass spectrometer. The major PLFA of strain JW/SA-NV4^T are i15:0, 46.8%; a15:0, 26.7%; 15:0, 6.3%; and 16:0, 5.5% (Table 3.1).

For cell wall analysis of strain JW/SA-NV4^T, peptidoglycan was isolated after disintegration of cells with glass beads in a Vibrogen cell mill (Edmund Bühler) and subsequently subjected to a trypsin digestion according to the method of Schleifer & Seidl (1985). Strain JW/SA-NV4^T has a peptidoglycan type A1γ. (Schleifer & Kandler, 1972; P. Schumann, pers. com.).

Genomic DNA For the analysis of G+C mol% of strain JW/SA-NV4^T was obtained through a large-scale phenol chloroform genomic DNA extraction; essentially, as described by Wilson (1997), but without performing the final cesium chloride gradient purification step described. The DNA G+C mol% was measured by the HPLC method of Mesbah *et al*. (1989), using the S1 nuclease (Invitrogen) and 0.3 M sodium acetate (pH 5.0) modifications described by Lee *et al*. (2005). The G+C mol% of strain JW/SA-NV4^T was 38.6; standard deviation 0.4 (n= 4).

Genomic DNA for amplification of the 16S rRNA gene sequence was extracted using the UltraClean DNA isolation kit (Mo Bio). Polymerase chain reactions were prepared with the bacterial domain-specific primer set 27F and 1492R (Lane, 1991), using GoTaq (Promega), and thermocycler conditions described previously by Lee *et al.* (2005). Amplification products were purified using the QIAquick PCR purification kit (QIAGEN) and sequenced by Macrogen. Due to unexplained difficulties with the 27F sequencing reaction, an additional reverse sequencing reaction was performed using a modified Univ907 R primer (5'- CGTCAATTCCTTTGAGTTT-3') (Amann *et al.*, 1992; Loy *et al.*, 2007). Sequencing results were aligned using Sequencher v4.1.4 (Gene Codes). A multiple sequence alignment was created with ClustalW (Thompson *et al.*, 1994) and subsequent phylogenetic analyses were performed using MEGA 4 software (Tamura *et al.*, 2007). Phylogenetic trees (Fig. 3.1) were inferred by the neighbor-joining method (Saitou and Nei, 1987) with the Jukes and Cantor model (Jukes and Cantor, 1969). The 16S rRNA gene sequence from JW/SA-NV4^T was deposited at GenBank (accession number EF530069). Phylogenetic analyses place strain JW/SA-NV4^T within the *Thermoanaerobacterales* Incertae Sedis Family III (Ludwig *et al.*, 2009) of the phylum *Firmicutes* (Fig. 3.1). The closest phylogenetic neighbors were the marine *Thermosediminibacter oceani* JW/IW-1228P^T(94.2%) and *Thermosediminibacter litoriperuensis* JW/YJL-1230-7/2^T (94.0%), both isolated from eastern equatorial Pacific sediment of the Peru Margin (Lee *et al.*, 2005), and *Thermovenabulum ferriorganovorum* Z-9801^T (90.3%), isolated from a terrestrial hot spring in the Uzon Caldera, Kamchatka, Far East Russia (Zavarzina *et al.*, 2002).

Nearly all of the validly described anaerobic alkalithermophilic *Bacteria* belong to the *Firmicutes* (Wiegel, 1998; Kevbrin et al., 2004); indeed, strain JW/SA-NV4^T belongs within this clade, specifically within the *Thermoanaerobacterales* Incertae Sedis Family III (Ludwig *et al.*,

2009). Within the *Thermoanaerobacterales* Incertae Sedis Family III, the *Thermoanaerobacterium*, *Caldicellulosiruptor*, and *Caldanaerobius* genera form well-defined phylogenetic clades (Fig. 3.1). The other validly published genera of the *Thermoanaerobacterales* Incertae Sedis Family III include *Thermosediminibacter*, *Thermovenabulum*, and *Tepidanaerobacter*. A hindrance to assigning a taxonomic affiliation for strain JW/SA-NV4^T is that at present there are only four validly published species classified within the three genera set of *Thermosediminibacter*, *Thermovenabulum*, and *Tepidanaerobacter*. Naturally, as additional species are described within these genera, a more complete view of their phylogenetic relationships will emerge. Strain JW/SA-NV4T has 94.2% similarity to *Thermosediminibacter oceani* JW/IW-1228P^T and 94.0% similarity to *Thermosediminibacter litoriperuensis* JW/YJL-1230-7/2^T. Although it is a topic of continued discussion, some microbial systematicists regard distance values of 5% as differentiating at the genus level (Schloss & Handelsman, 2004; references therein). The notion that strain JW/SA-NV4^T should be classified as a novel species within a novel genus is also supported by biochemical and physiological differences (Table 3.2). Thus,, we propose strain JW/SA-NV4^T belongs to a novel genus. The differences include the following properties. The *Thermosediminibacter* species have flagella (Lee *et al.*, 2005), whereas no flagella were observed for strain JW/SA-NV4^T. A small percentage of the cells in cultures of *Thermosediminibacter oceani*, *Thermosediminibacter litoriperuensis*, and *Thermovenabulum ferriorganovorans* exhibit a characteristic branched cell morphology (Zavarzina et al., 2002; Lee et al., 2005) which was not observed with cells of strain JW/SA-NV4^T. There are differences in PLFA profile of strain JW/SA-NV4^T and the *Thermosediminibacter* species. The 16:1 PLFA was detected for JW/SA-NV4^T, but was not in either *Thermosediminibacter oceani* JW/IW-1228P^T or

Thermosediminibacter litoriperuensis JW/YJL-1230-7/2^T; the 18:0 PLFA was found within *Thermosediminibacter oceani* JW/IW-1228P^T and *Thermosediminibacter litoriperuensis* JW/YJL-1230-7/2^T, but was not found in JW/SA-NV4^T; the a15:0 amount of 26.7% for JW/SA-NV4^T, with much lower values of 6.7% and 3.8% for *Thermosediminibacter oceani* JW/IW-1228P^T and *Thermosediminibacter litoriperuensis* JW/YJL-1230-7/2^T, respectively (table 3.1). There are also differences on the utilization of carbon substrates, e.g., both *Thermosedminibacter* species can utilize galactose and sucrose, whereas strain JW/SA-NV4^T cannot use either. The isolation sources of strain JW/SA-NV4^T was terrestrial hot spring whereas all the *Thermosediminibacter* strains were derived from marine sediment (Lee *et al.*, 2005). The difference of the G+C mol% of the genomic DNA from strain JW/SA-NV4^T and the *Thermosedminibacter* species are approximately 7%.

DESCRIPTION OF *CALDANAEROVIRGA* GEN. NOV.

Caldanaerovirga (cal.da.nae.ro.vir'ga. L. adj. *caldus* hot; Gr. pref. *an* not; Gr. n. *aer* air; L. fem. n. *virga* rod; N.L. fem. n. *Caldanaerovirga* rod that grows in the absence of air at elevated temperatures).

Anaerobic thermophilic, Gram-type positive, cell wall has a peptidoglycan type A1γ, belongs to the class *Clostridia*, G+C mol% around 38%. Type species is *Caldanaerovirga acetigignens*

DESCRIPTION OF *CALDANAEROVIRGA ACETIGIGNENS* SP. NOV.

Caldanaerovirga acetigignens (a.ce.ti.gig'nens. L. n. acetum vinegar; L. v. gignere to produce; N.L. part. adj. *acetigignens* vinegar- or acetic acid-producing).

During exponential growth, cells of strain JW/SA-NV4^{T} are straight or slightly bent rods of 0.4-0.8 µm in width and 3-6 µm in length. Cells from stationary phase are more variable in size and shape, showing a more expressed curved or bent morphology. Neither flagella or motility nor sporulation observed. Cells stain Gram negative but are phylogenetically Gram-type positive. Peptidoglycan type A1γ. Growth with glucose as carbon-and energy source occurs between pH^{25C} 6.8-8.8, pH_{opt} = 8.4; no growth after 200 h of incubations at 9.0 or above or at 6.5 or below. Strain JW/SA-NV4^{T} grows between 44- 74 °C (glucose); no growth after 300 h of incubation at 76 °C or above and at 42 °C or below. T_{opt} 61-62 °C and 66 °C when grown on glucose and xylose, respectively. The shortest doubling time observed was approximately 4 hours (glucose) and 3.4 hours (xylose). Chemoorganotroph. Utilization of xylose, glucose, mannnose, xylan, pyruvate, fructose, ribose, casamino acids, manitol, tryptone, peptone, cellobiose, and yeast extract. Acetate (2.2-2.4 mol/glucose) is the major fermentation product when grown with glucose suggesting a homoacetogenic fermentation. Yeast extract is required for growth. Does not utilize sucrose, glycine betaine, galactose, lactose, xylitol, sorbitol, inositol, arabinose, starch, or cellulose. Using mineral media containing 1 g l^{-1} yeast extract as an electron donor, thiosulfate and sulfur, but not sulfate or sulfite, are reduced to sulfide. Growth occurs in the presence of an atmosphere containing 0.1% O_2. Positive for acid phosphatase and naphthol-AS-BI-phosphohydrolase. Major PLFA s of strain JW/SA-NV4^{T} are i15:0, 46.8%; a15:0, 26.7%; 15:00, 6.3%; and 16:00, 5.5%. The G+C mol % content of the DNA was 38.6; standard deviation 0.4 (HPLC).

The type strain, JW/SA-NV4^{T} (= ATCC BAA1454^{T}, = DSM 18802^{T}), was isolated from Trego hot spring (4515379.772N 321439.459E), Black Rock Desert, northwestern Nevada, USA.

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Table 3.1. Phospholipid fatty acid contents (%) of strains JW/SA-NV4^T, JW/IW-1228P^{T}and JW/YJL-1230-7/2^T. JW/IW-1228P^{T}and JW/YJL-1230-7/2^T data from Lee *et al*. (2005). Abbreviation: ND, not detected.

Fatty acid methyl esters	*Caldanaerovirga acetigignens* JW/SA-NV4^T	*Thermosediminibacter oceani* JW/IW-1228P^T	*Thermosediminibacter litoriperuensis* JW/YJL-1230-7-2^T
i13:0	2.7	ND	ND
14:0	3.0	1.8	1.7
i15:0	46.8	56.2	16.7
a15:0	26.7	6.7	3.8
15:0	6.3	5.0	2.6
i16:0	ND	1.9	1.6
16:1ω9c	ND	5.6	19.9
16:1	2.8	ND	ND
16:00	5.5	7.5	15.5
i17:0	2.4	9.6	4.5
17:0	ND	1.0	ND
18:2 ω 6	ND	ND	1.4
18:1 ω 9c	3.9	3.3	20.3
18:1 ω 9t / 18:1 ω 7c[a]	ND	ND	6.3
18:0	ND	1.5	5.7

[a] **18:1 ω 9t/18:1 ω 7c** were unresolved in this analysis.

Table 3.2. Morphological and physiological characteristics of strain JW/SA-NV4^T and close relatives.				
Characteristic	*Caldanaerovirga acetigignens* JW/SA-NV4^T	*Thermosediminibacter oceani* JW/IW-1228P^T	*Thermosediminibacter litoriperuensis* JW/YJL-1230-7-2^T	*Thermovenabulum ferriorganovorum* Z-9801^T
Isolation source	Terrestrial hot spring, Nevada, USA	Subseafloor, Peru Margin	Subseafloor, Peru Margin	Terrestrial hot spring, Uzon Caldera, Russian Far East
Cell size (μm)	0.4-0.8 x 3-6	0.2–0.7 x 1.5–16	0.3–0.5 x 2–10	0.5–0.6 x 1.5–7.0
Flagella	No flagella observed	Flagella observed	Retarded peritrichous flagella	Peritrichous flagella
Temperature range (°C)	44- 74	52–76	43–76	45–76
Optimum temperature (°C)	61	68	64	63–65
pH range	6.8-8.8	6.3–9.3	5–9.5	4.8–8.2
Optimum pH	8.4	7.5	7.9–8.4	6.7–6.9
Salinity (% NaCl, w/v)	0-4.0	0–6.0	0–4.5	0–3.5
G+C content (mol%)	38.6	46.3	45.2	36
Gram stain	–	–	–	+
Spores observed	–	–	–	+
a-glucosidase	-	+	-	ND

Table 3.2 (continued). Morphological and physiological characteristics of strain JW/SA-NV4^T and close relatives.				
Characteristic	*Caldanaerovirga acetigignens* JW/SA-NV4^T	*Thermosediminibacter oceani* JW/IW-1228P^T	*Thermosediminibacter litoriperuensis* JW/YJL-1230-7-2^T	*Thermovenabulum ferriorganovorum* Z-9801^T
Utilization of Galactose	-	+	+	-
Glucose	+	+	+	-
Inositol	-	+	+	ND
Sorbitol	-	+	-	ND
Sucrose	-	+	+	+
Trehalose		+		ND
Xylitol	-	-	+	ND
16:1 PLFA	+	-	-	ND
18:0 PLFA	-	+	+	ND

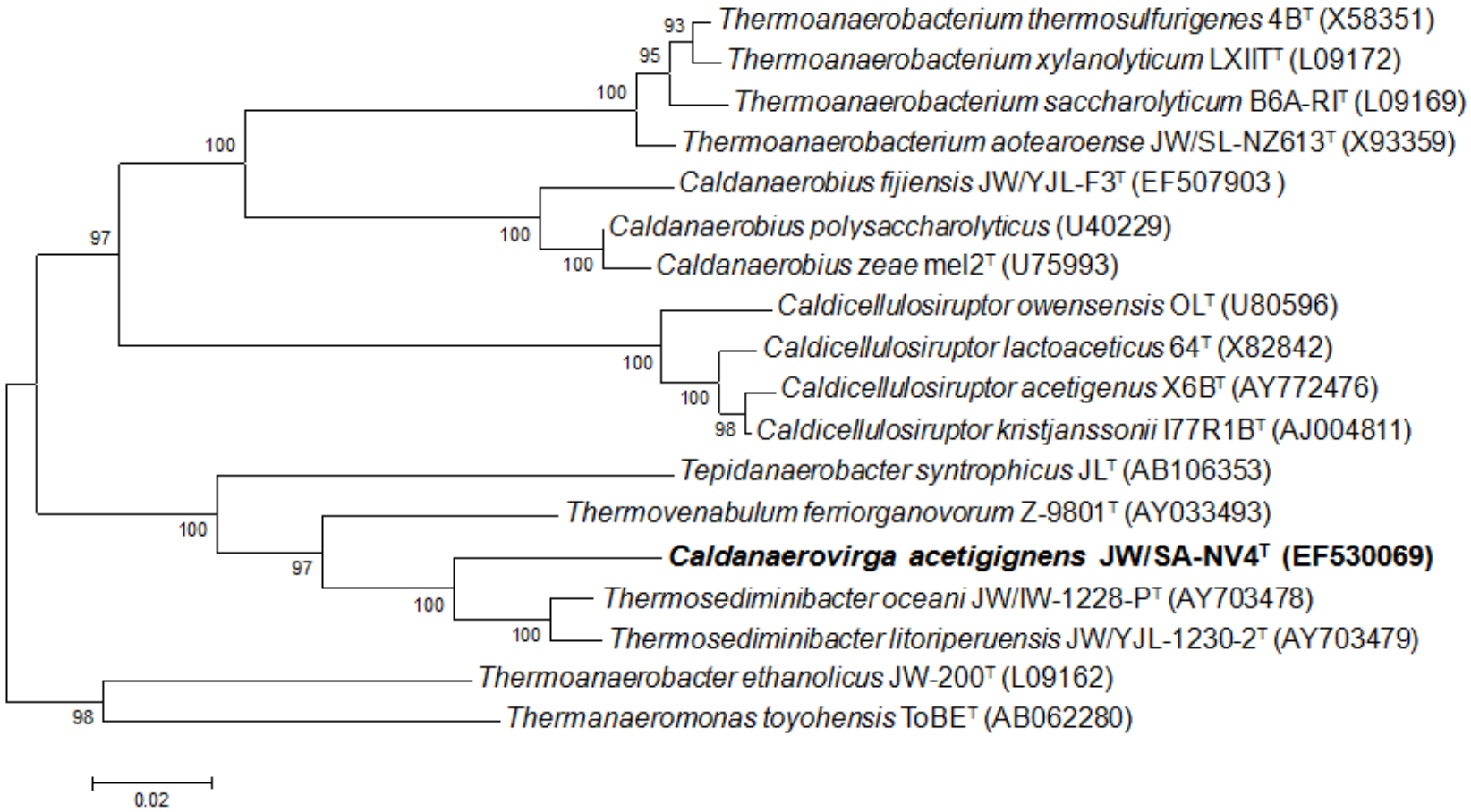

Fig. 3.1.16S rRNA gene sequence based phylogenetic dendrogram of strain JW/SA-NV4^T and related taxa. The tree was constructed using the neighbor-joining method (Saitou and Nei, 1987) and the Jukes and Cantor distance corrections (Jukes and Cantor, 1969). Numbers at the nodes represent bootstrap values as the percent of 1000 replicates. The scale bar indicates two nucleotide substitutions per 100 nucleotides. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). There were a total of 1836 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.*, 2007).

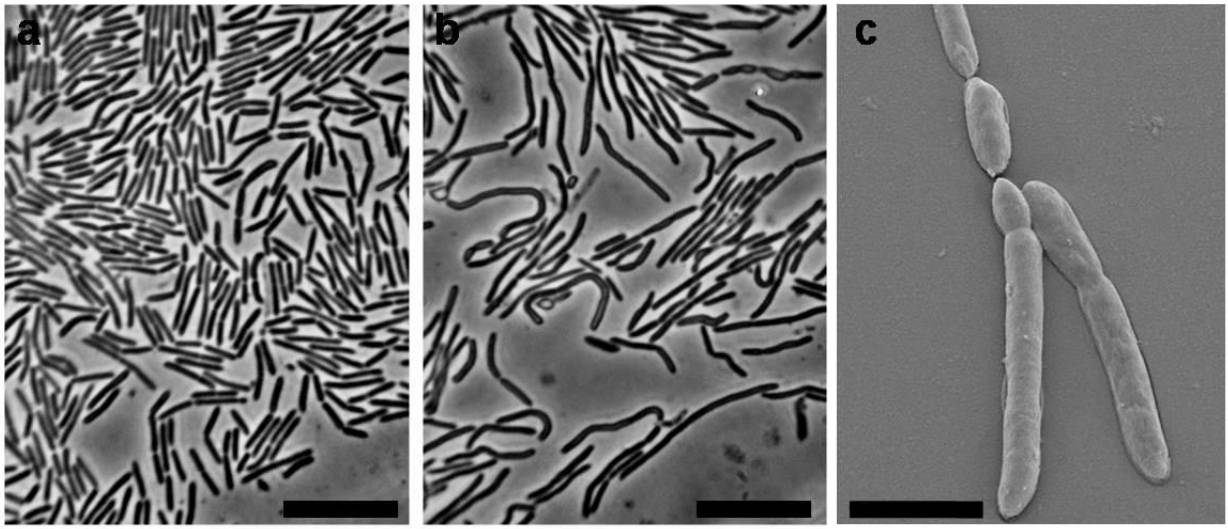

Fig. 3.2. Phase contrast and electron micrographs of strain JW/SA-NV4^T. Cells from exponential phase cultures (a), bar = 10 μm. Cells from stationary phase cultures (b)), bar = 10 μm. Cells from stationary phase cultures are more variable in size and shape compared to cells from exponential phase cultures which are generally straight or slightly bent rod shaped, 0.4-0.8 μm in width and 3-6 μm in length. Electron micrograph of strain JW/SA-NV4^T (c), bar(s) = 2 μm.

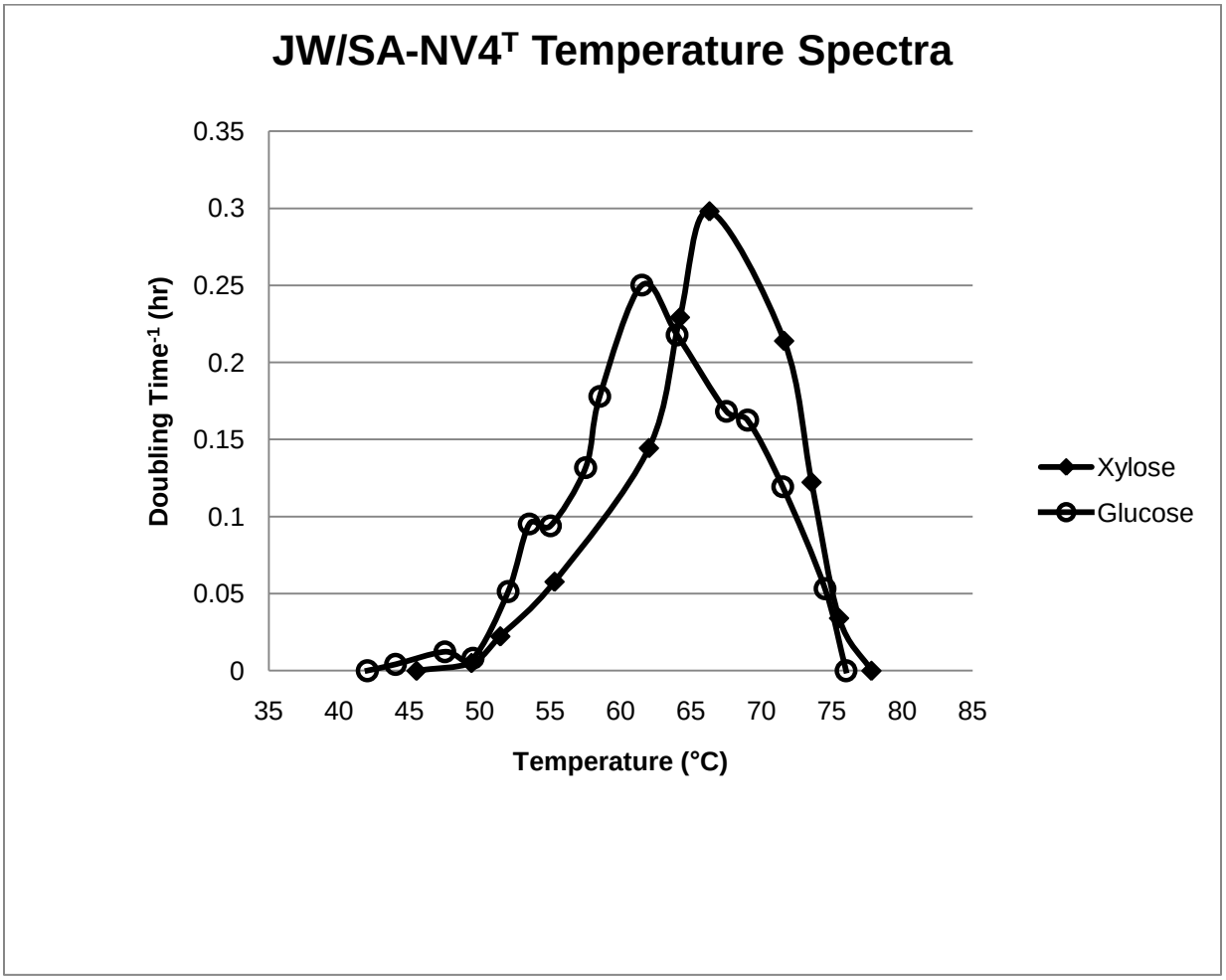

Fig. 3.S1. Temperature spectra of strain JW/SA-NV4^{T} when grown with glucose or xylose as the provided carbon substrate. With glucose as a provided carbon source (pH^{25C} 8.1), strain JW/SA-NV4^{T} grew between 44-74 °C, optimally at about 61-62 °C; after more than 200 hours of incubation, no growth was observed at or below 42 °C, or at 77 °C or above. With xylose as the provided carbon substrate (pH^{25C} 8.2), strain JW/SA-NV4^{T} grew between 46 and 76 °C, optimally around 66 °C; after more than 300 hours of incubation, no growth was observed at or below 44 °C, or at 78 °C or above.

CHAPTER 4

THERMOANAEROBACTER UZONENSIS SP. NOV., AN ANAEROBIC, THERMOPHILIC BACTERIUM ISOLATED FROM A HOT SPRING WITHIN THE UZON CALDERA, KAMCHATKA, FAR EAST RUSSIA[5]

[5] Wagner, I. D., Zhao, W., Zhang, C. L., Romanek, C. S., Rohde, M. & Wiegel, J. (2008). *Thermoanaerobacter uzonensis* sp. nov., an anaerobic thermophilic bacterium isolated from a hot spring within the Uzon Caldera, Kamchatka, Far East Russia. *Int J Syst Evol Microbiol* **58**, 2565-2573.

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ABSTRACT

Several strains of heterotrophic, anaerobic thermophilic bacteria were isolated from hot springs of the Uzon Caldera, Kamchatka, Far East Russia. Strain JW/IW010^{T} was isolated from a hot spring within the West sector of the Eastern Thermal field, near Pulsating Spring in the Winding Creek area. Cells of strain JW/IW010^{T} were straight to slightly curved rods, 0.5 µm in width and variable in length from 2 to 5 µm and occasionally up to 15 µm, and formed oval subterminal spores. Cells stained Gram-negative, but were Gram-type positive. Growth was observed between 32.5 and 69 °C with an optimum around 61 °C (no growth occurred at or below 30 °C, or at or above 72 °C). The pH$^{60\ °C}$ range for growth was 4.2–8.9 with an optimum at 7.1 (no growth occurred at or below pH$^{60\ °C}$ 3.9, or at 9.2 or above). The shortest observed doubling-time at pH$^{60\ °C}$ 6.9 and 61 °C was 30 min. Strain JW/IW010^{T} was chemo-organotrophic; yeast extract, peptone, Casamino acids and tryptone supported growth. Yeast extract was necessary for the utilization of non-proteinaceous substrates, and growth was observed with inulin, cellobiose, maltose, sucrose, glucose, fructose, galactose, mannose, xylose, trehalose, mannitol, pyruvate and crotonate. The G+C content of the genomic DNA of strain JW/IW010^{T} was 33.6 mol% (HPLC method). The major phospholipid fatty acids were iso-15 : 0 (53.5 %), 15 : 0 (11.8 %), 16 : 0 (7.3 %), 10-methyl 16 : 0 (7.3 %) and anteiso-15 : 0 (5.3 %). 16S rRNA gene sequence analysis placed strain JW/IW010^{T} in the genus *Thermoanaerobacter* of the family '*Thermoanaerobacteriaceae*' (*Firmicutes*), with *Thermoanaerobacter sulfurigignens* JW/SL-NZ826^{T} (97 % 16S rRNA gene sequence similarity) and *Thermoanaerobacter kivui* DSM 2030^{T} (94.5 %) as the closest phylogenetic relatives with validly published names. The level of DNA–DNA relatedness between strain JW/IW010^{T} and *Thermoanaerobacter sulfurigignens* JW/SL-NZ826^{T} was 64 %. Based on the physiological, phylogenetic and genotypic data, strain

JW/IW010^T represents a novel taxon, for which the name *Thermoanaerobacter uzonensis* sp. nov. is proposed. The type strain is JW/IW010^T (=ATCC BAA-1464^T=DSM 18761^T). The effectively published strain, 1501/60, of '*Clostridium uzonii*' [Krivenko, V. V., Vadachloriya, R. M., Chermykh, N. A., Mityushina, L. L. & Krasilnikova, E. N. (1990). *Microbiology* (English translation of *Mikrobiologiia*) **59**, 741–748] had approximately 88.0 % DNA–DNA relatedness with strain JW/IW010^T and was included in the novel taxon.

Abbreviations: AMC, 7-amino-4-methylcoumarin; 4MU, 4-methylumbelliferone; PLFA, phospholipid fatty acid

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain JW/IW010^T is EF530067.

The Uzon Caldera and Geyser Valley region of Kamchatka contain a wide variety of old and recently formed geothermal features in close proximity. Water and sediment samples were collected from hot springs of the Uzon Caldera and, concomitantly, temperature and pH measurements were taken and geochemical properties were assessed. For the isolation of novel anaerobic thermophilic prokaryotes, enrichment cultures were prepared from Uzon Caldera hot spring water and sediment samples using various carbon substrates, including methanol, crotonate, glucose, fructose and xylose.

The enrichment cultures were prepared using an anaerobic, carbonate-buffered basal medium [2 mM KH_2PO_4, 0.5 g NH_4Cl l^{-1}, 0.5 g $(NH_4)_2SO_4$ l^{-1}, 1.5 g $NaHCO_3$ l^{-1}, 0.1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1.0 ml vitamin solution l^{-1} and 1.0 ml Na_2SeO_3-Na_2WO_3 trace-element solution l^{-1} (Widdel & Bak, 1992)] at a $pH^{25\ °C}$ of 6, supplemented with 3 g carbon substrate l^{-1}

and 1 g yeast extract l^{-1}. From enrichment cultures with visible growth, pure cultures were obtained using serial dilutions in anaerobic agar-shake-roll tubes with the carbonate-buffered basal medium supplemented with 2.15 % agar (Ljungdahl & Wiegel, 1986) and multiple rounds of colony isolation. Purity was checked microscopically and by means of 16S rRNA gene sequencing.

Strains JW/IW007, JW/IW010^T and JW/IW015 were derived from water and sediment samples from a small pool 2 m east of Pulsating Spring (54.500 ° N 160.007 ° E), which had a temperature of about 77 °C and a pH of 5.6. These pools feed Winding Creek, which is located on the northern side of the central portion of the Uzon Eastern Thermal field and flows southwards into Chloride Lake. Strain JW/IW007 was isolated using methanol as the provided carbon substrate. Axenic cultures of strains JW/IW010^T and JW/IW015 were isolated using crotonate as the carbon substrate. Similarly, strain JW/HL-KA15 was isolated from a glucose-supplemented enrichment from the hot spring 'Blue Pool', located at the Northern Thermal field (78.8 °C and pH 6.3).

In order to check culture purity and for phylogenetic analyses, DNA was extracted using an UltraClean DNA isolation kit (Mo Bio) and 16S rRNA gene sequences were PCR-amplified and sequenced. Routine PCRs were prepared with the bacterial domain-specific primer set 27F and 1492R (Lane, 1991), using GoTaq (Promega) and the thermocycler conditions described previously by Lee et al. (2005)✦. The 16S rRNA gene sequence of strain JW/IW010^T was amplified using high-fidelity, PrimeStar HS polymerase (Takara Mirus Bio). Amplification with PrimeStar HS polymerase proceeded with thermocycler conditions of 10 s at 98 °C, 5 s at 58 °C and 90 s at 72 °C, repeated for a total of 30 cycles. The amplified 16S rRNA gene sequences were purified using a QIAquick PCR purification kit (Qiagen) and sequenced by Macrogen.

Sequencing results were aligned using Sequencher v4.1.4 (Gene Codes) and a multiple sequence alignment was created with CLUSTAL W (Thompson et al., 1994). Editing of alignments was performed using GeneDoc v2.6.001 (Nicholas & Nicholas, 1997). The phylogenetic tree (Fig. 4.1) was inferred by using the neighbour-joining method (Saitou & Nei, 1987) with the Jukes and Cantor model (Jukes & Cantor, 1969), using the PHYLIP v3.6a2.1 phylogenetic analysis package (Felsenstein, 2001).

From the 16S rRNA gene sequence analysis, strain JW/IW010^T and related strains were clearly placed in the genus *Thermoanaerobacter* of the family '*Thermoanaerobacteriaceae*' (*Firmicutes*) (Fig. 4.1). Subsequent 16S rRNA gene sequence-based phylogenetic analyses revealed that strain JW/IW010^T had a high similarity (99 %) to strain 1501/60 with the effectively, but not validly, published name '*Clostridium uzonii*' (Krivenko et al., 1990). '*C. uzonii*' has been described as a deeply branching member of the *Clostridium* cluster V, which contains the genus *Thermoanaerobacter* (Stackebrandt et al., 1999). Based on 16S rRNA gene sequence analysis, the closest phylogenetic relatives with validly published names were *Thermoanaerobacter sulfurigignens* JW/SL-NZ826^T (97 % similarity) (Lee et al., 2007a) and the acetogenic *Thermoanaerobacter kivui* DSM 2030^T (96 % similarity) (Onyenwoke & Wiegel, 2008). Strains 1501/60 (DSM 9752) and 1611 (DSM 9753), effectively described by Krivenko et al. (1990), were isolated from a mud cauldron and hydrothermal silt, respectively, within the Uzon Caldera and were obtained from the German Collection of Microorganisms and Cell Cultures for comparison with strain JW/IW010^T. *Thermoanaerobacter sulfurigignens* JW/SL-NZ826^T was obtained from laboratory stock.

Within agar-shake-roll tubes, colonies of strain JW/IW010^T were visible following overnight incubation at 60 °C; the colonies were circular, 1–2 mm in diameter, slightly convex

and creamy white in colour. When grown on plates of carbonate-buffered basal medium with 2.3 % agar within anaerobic jars at 60 °C, strain JW/IW010^T formed colourless, nearly transparent circular colonies up to 1 mm in diameter, whereas *Thermoanaerobacter sulfurigignens* JW/SL-NZ826^T formed circular colonies of up to 1 mm in diameter that were opaque and bluish white in colour. Cells of strain JW/IW010^T were rod-shaped, straight to slightly curved and approximately 0.5 µm in width and 2–5 µm in length (Figs 4.2 and 4.3). Occasionally, cells up to 15 µm in length were observed. Shorter or longer chains of cells frequently contained intermittent coccoid cells (Fig. 3), typical of *Thermoanaerobacter* (Wiegel & Ljungdahl, 1981). Gram stain reactions were performed on both early exponential and stationary growth phase cultures of strain JW/IW010^T using Fisherbrand Gram ✓ Control/Test Slides (Thermo Fisher Scientific). Strain JW/IW010^T stained Gram-negative under both growth conditions although phylogenetically it is Gram-type positive (Wiegel, 1981).

Sporulation of strain JW/IW010^T was observed, though only rarely. Spores were oval, located subterminal to terminal and caused the mother cell to swell (Fig. 4.2a). Viable subcultures were obtained from liquid cultures heated to 100 °C for 5 min. Although sporulation was rarely seen in strain JW/IW010^T, other *Thermoanaerobacter* species also produce spores infrequently, whereas sporulation has never been observed in other taxa of this genus, e.g. *Thermoanaerobacter ethanolicus* JW 200^T (Onyenwoke & Wiegel, 2008; Wiegel & Ljungdahl, 1981). Cells of strain JW/IW010^T had peritrichously inserted flagella (Fig. 4.3) and tumbling motility was observed when liquid cultures were viewed using phase-contrast microscopy. When grown in media containing 50 or 100 mM thiosulphate, cells of strain JW/IW010^T were more motile compared with cells grown in medium lacking thiosulphate.

The temperature and pH profiles, substrate range and tolerance of thiosulphate and sulphite were determined through measurement of ΔOD_{600} using a Spectronic 21 spectrophotometer (Bausch and Lomb). A temperature-gradient incubator (Scientific Industries) was used to determine the temperature range and optimum for growth of strain JW/IW010^T using the aforementioned basal carbonate-buffered medium complemented with 4 g glucose l^{-1} and 2 g yeast extract l^{-1}. Up to 96 h, growth was observed between 32.5–69 °C, with optimum growth at 61 °C; growth was not observed at or below 30 °C, or at or above 72 °C (Fig. 4.4a). Media used for the determination of the pH range of strain JW/IW010^T was buffered with 10 mM of each trisodium citrate, MES, Tris and Na_2CO_3, rather than $NaHCO_3$, and also contained 4 g glucose l^{-1} and 2 g yeast extract l^{-1}. Up to 75 h, growth was observed between an initial $pH^{60\ °C}$ of 4.2–8.9, with an optimum at 7.1; no growth was observed at or below $pH^{60\ °C}$ 3.9, or at 9.2 or above (Fig. 4.4b). The shortest doubling-time for strain JW/IW010^T was approximately 30 min and occurred at $pH^{60\ °C}$ 6.9 and 61 °C in medium containing 2 g yeast extract l^{-1} and 4 g glucose l^{-1}. No growth was observed in the absence of yeast extract while using glucose as a carbon source. Using 0.5 g yeast extract l^{-1} as the sole substrate led only to limited growth. Proteinaceous carbon sources added to the carbonate-buffered basal medium to a concentration of 5 g l^{-1} were tested for their ability to support the growth of strain JW/IW010^T. Growth was assumed when ΔOD_{600} increased or when cell numbers increased as determined through the use of phase-contrast microscopy. At 5 g l^{-1}, yeast extract (ΔOD_{600}=0.04), peptone (ΔOD_{600}=0.03), Casamino acids (ΔOD_{600}=0.03) and tryptone (ΔOD_{600}=0.03) served as sole carbon substrates, whereas beef extract did not support growth. The ability of strain JW/IW010^T to utilize other carbon substrates was determined with substrates at a concentration of 3 g l^{-1}, supplemented with 1 g yeast extract l^{-1}. Utilization was recorded as positive when ΔOD_{600} was twice that of cultures containing only

the basal (1 g l^{-1}) concentration of yeast extract. Growth of strain JW/IW010^T was observed with inulin, cellobiose, maltose, sucrose, glucose, fructose, galactose, mannose, xylose, trehalose, mannitol, pyruvate and crotonate. Growth was not observed with casein, cellulose, xylan, starch, olive oil, pectin, lactose, arabinose, rhamnose, ribose, xylitol, sorbitol, methanol, glucuronic acid or glycerate. Enhanced growth of strain JW/IW010^T was not observed in media containing a headspace of H_2/CO_2, in comparison with cultures grown in the same basal media containing 1 g yeast extract l^{-1}, with an N_2 gas headspace. When grown with 2 g glucose l^{-1} and 1 g yeast extract l^{-1}, strains JW/IW010^T and 1501/60 produced lactic acid, acetic acid, ethanol, CO_2 and H_2 as fermentation end products, though in different proportions. Strain JW/IW010^T primarily produced ethanol and acetate, and only a trace amount of lactic acid, yielding a simplified fermentation equation of: 1 glucose+0.85 $H_2O \rightarrow$ 0.85 acetic acid+1.15 ethanol+2 CO_2+1.7 H_2, whereas strain 1501/60 produced lactic acid, acetic acid and ethanol, yielding the simplified fermentation equation of: 1 glucose+0.6 $H_2O \rightarrow$ 0.84 lactic acid+0.6 acetic acid+0.56 ethanol+ 1.16 CO_2+1.76 H_2.

The phylogenetically close neighbour, *Thermoanaerobacter sulfurigignens* (two isolates), exhibits resistance to 1 M thiosulphate and 90 mM sulphite (Lee et al., 2007a). In contrast, strain JW/IW010^T tolerated only up to 200 mM thiosulphate (growth did not occur at or above 250 mM) and up to 40 mM sulphite (no growth at or above 50 mM) using mineral media supplemented with 1 g yeast extract l^{-1} and 1 g glucose l^{-1} as carbon and energy sources. The reduction of thiosulphate to sulphide was assessed by the use of lead acetate-impregnated paper. A culture that was supplemented only with yeast extract served as a control. In media containing 50 or 100 mM thiosulphate, and 1 g l^{-1} yeast extract and glucose, strain JW/IW010^T formed H_2S and elemental sulphur, observed as intracellular sulphur deposits, from thiosulphate. Sulphur

granula could be differentiated and from spores by the little to no swelling of the mother cell, occasional multiple inclusions per cell, and the late appearance of sulphur granula in the stationary phase after spore formation (Fig. 4.2a and b). The formation of elemental sulphur deposits in addition to H_2S formation is similar to what has been observed for *Thermoanaerobacter italicus* (Kozianowski et al., 1997), whereas *Thermoanaerobacter sulfurigignens* (Lee et al., 2007a, b) only produces elemental sulphur, similar to most species of *Thermoanaerobacterium* (Lee et al., 1993; Onyenwoke & Wiegel, 2008; Schink & Zeikus, 1983).

To compare the biochemical and enzymic features of strain JW/IW010^T with those of its closest recognized relative, *Thermoanaerobacter sulfurigignens* JW/SL-NZ826^T, the BBL Crystal RGP (Becton Dickinson) system was used. The procedures used were according to the manufacturer's instructions with the modification that cells were obtained via centrifugation of 250 ml cultures that were grown overnight (basal medium described previously, containing 2 g yeast extract l^{-1} and 4 g glucose l^{-1}). The cells were then washed twice with a solution of the basal medium that did not contain yeast extract or other carbon substrate. Under these conditions, both strain JW/IW010^T and *Thermoanaerobacter sulfurigignens* JW/SL-NZ826^T tested positive for the following: hydrolysis of the glycosidic bond of 4-methylumbelliferone (4MU) β-D-glucoside, hydrolysis of the amide bond of L-methionine-7-amino-4-methylcoumarin (AMC) and L-valine-AMC; hydrolysis of the aryl substituted glycoside of *o*-nitrophenyl β-D-glucoside and *p*-nitrophenyl β-D-glucoside, *p*-nitrophenyl β-D-glucoside, *p*-nitrophenyl phosphate, and *p*-nitrophenyl β-D-galactoside and *p*-nitrophenyl α-D-galactoside. Both taxa tested negative for the following: hydrolysis of the amide bond of L-proline-AMC, L-arginine-AMC, L-tryptophan-AMC and L-phenylalanine-AMC; hydrolysis of the glycosidic bond of 4MU β-cellobiose, 4MU-

phosphate, 4MU α -D-glucoside; utilization of arabinose, maltose, dextrin, mannitol, galactose, *N*-acetyl-D-glucosamine, trehalose, mannose, maltotriose, ornithine; the enzymic hydrolysis of the aryl substituted glycoside of *p*-nitrophenyl α -D-glucoside; and hydrolysis of urea and aesculin. However, the two taxa differed in that *Thermoanaerobacter sulfurigignens* JW/SL-NZ826^T hydrolysed the amide bond of L-pyrogluamic acid-AMC, whereas strain JW/IW010^T did not, and *Thermoanaerobacter sulfurigignens* JW/SL-NZ826^T hydrolysed the aryl substituted glycoside of *p*-nitrophenyl α -cellobiose, whereas JW/IW010^T did not.

Genomic DNA for the determination of the G+C content was extracted using a large-scale phenol/chloroform-based procedure essentially as described by Wilson (1997), without the final caesium chloride gradient purification step. The DNA G+C content was measured by using HPLC (Mesbah et al., 1989) with the modification of Lee et al. (2005), using S1 nuclease (Invitrogen) and 0.3 M sodium acetate (pH 5.0). The DNA G+C content of strain JW/IW010^T was 33.6 mol% (SD=0.4; *n*=9). Krivenko et al. (1990) reported the DNA G+C content of strain 1501/60 as being 34.4 mol%, determined using the thermal denaturation technique (Marmur & Doty, 1962). Using the HPLC-based method of Mesbah et al. (1989), the DNA G+C content of strain 1501/60 was 33.3 mol% (SD=0.08; *n*=4).

To assess the genotypic similarity between strain JW/IW010^T and *Thermoanaerobacter sulfurigignens* JW/SL-NZ826^T, and also strains JW/IW010^T and 1501/60, whole genome DNA–DNA relatedness and ΔT_m values were determined. DNA–DNA hybridizations performed at DSMZ revealed that strain JW/IW010^T and *Thermoanaerobacter sulfurigignens* JW/SL-NZ826^T had 64 % DNA–DNA relatedness, whereas strains JW/IW010^T and 1501/60 had 87–88.0 % DNA–DNA relatedness. Whole-genome ΔT_m values between strains JW/IW010^T, 1501/60 and *Thermoanaerobacter sulfurigignens* JW/SL-NZ826^T were determined using the fluorimetric,

SYBR Green I (Invitrogen)-based technique described by Gonzalez & Saiz-Jimenez (2005)✦. The mean ΔT_m between hybridized JW/IW010^T and JW/SL-NZ826^T DNA and homologous JW/IW010^T DNA was 5.85 °C (n=4). The mean ΔT_m between hybridized JW/IW010^T and 1501/60 DNA and homologous JW/IW010^T DNA was 4.95 °C (n=4). At present, ΔT_m values of >5 °C and DNA–DNA relatedness values of less than 70 %, considered along with phenotypic and chemotaxonomic differences, are acknowledged standards for the differentiation of species (Stackebrandt et al., 2002✦). In comparing strain JW/IW010^T with *Thermoanaerobacter sulfurigignens* JW/SL-NZ826^T, the ΔT_m value of 5.85 °C and DNA–DNA relatedness of 64 % indicated that strain JW/IW010^T and *Thermoanaerobacter sulfurigignens* JW/SL-NZ826^T represent separate species, which was also corroborated by phenotypic and chemotaxonomic differences. The ΔT_m value of 4.95 °C and DNA–DNA relatedness of 87–88 % demonstrate that strains JW/IW010^T and 1501/60 belong to the same species.

The phospholipid fatty acid (PLFA) contents of strain JW/IW010^T and the phylogenetically closely related taxon *Thermoanaerobacter sulfurigignens* JW/SL-NZ826^T were measured and compared. Cell material for lipid extraction was obtained according to previously described procedures (Zhang et al., 2004), based on the protocol of White et al. (1979). In brief, an overnight extraction commenced with a single-phase organic solvent system comprising chloroform, methanol and aqueous 50 mM phosphate buffer (pH 7.4) in a ratio of 1 : 2 : 0.8 (by vol.). Chloroform and nanopure water were then added to the extract to obtain a final ratio of 1 : 1 : 0.9 (by vol.), resulting in a two-phase system. Neutral lipids, glycolipids and polar lipids were fractionated using a silicic acid column (Guckert et al., 1985). The polar lipids were treated with mild alkaline methanolysis to produce fatty acid methyl esters. Analyses of the fatty acid methyl esters were performed using a 6890 gas chromatograph (Agilent) with a flame-ionization

detector, equipped with a 30 m DB-5 column (5 % phenyl) and programmable temperature vaporizing inlet. The temperature programme for samples in hexane was: starting at 60 °C, 10 °C min^{-1} to 180 °C, and 4 °C min^{-1} to 320 °C and hold for 20 min. GC-MS analyses of fatty acid methyl esters were performed using an HP5890 gas chromatograph (Hewlett Packard) equipped with a 30 m HP5-MSI column and programmable temperature vaporizing inlet and coupled to an HP5972 mass spectrometer. The major PLFAs for strain JW/IW010^T were iso-15:0 (53.5 %), 15:0 (11.8 %), 16:0 (7.3 %), 10-methyl 16:0 (7.3 %), and anteiso-15:0 (5.3 %). The major PLFAs for *Thermoanaerobacter sulfurigignens* JW/SL-NZ826^T were iso-15:0 (57.7 %), 15:0 (18.3 %), anteiso-15:0 (6.1 %), iso-17:0 (5.7 %), and 10-methyl 16:0 (5.5 %). PLFA 14:0 (1.7 %) was found within strain JW/IW010^T but was not detected for *Thermoanaerobacter sulfurigignens* JW/SL-NZ826^T, analysed at the same time. Whereas both strain JW/IW010^T and *Thermoanaerobacter sulfurigignens* JW/SL-NZ826^T contained 18:1ω9*c* (3.9 and 1.8 %, respectively), the saturated fatty acid 18 : 0 was only found within strain JW/IW010^T and 18:1ω 7*t* was only detected in *Thermoanaerobacter sulfurigignens* JW/SL-NZ826^T (Table 4.1). The PLFA content of strain 1501/60 was also analysed; as for strain JW/IW010^T, iso-15 : 0 was the major lipid (56.9 %); however, strain 1501/60 was found to have more of anteiso-15:0 (9.4 %) than 15:0 (1.1 %), in addition to having iso-16:0 (7.0 %) and anteiso-17:0 (6.3 %), which were not detected in strain JW/IW010^T or *Thermoanaerobacter sulfurigignens* JW/SL-NZ826^T (data not shown).

In addition, strain JW/IW010^T and *Thermoanaerobacter sulfurigignens* JW/SL-NZ826^T differed morphologically. Cells of strain JW/IW010^T were approximately 0.5 µm in width and 2–5 µm in length; occasionally cells up to 15 µm in length were observed. Cells of *Thermoanaerobacter sulfurigignens* JW/SL-NZ826^T were much more variable, between 0.3–

0.8 µm in diameter and 1.2–4.0 µm in length during exponential growth and, during stationary growth phase, the length of the cells increased up to 35 µm (Lee et al., 2007a). Certain characteristics between strain JW/IW010^T and *Thermoanaerobacter sulfurigignens* JW/SL-NZ826^T were similar; e.g. the measured DNA G+C content of 33.6 and 34.5 mol% (HPLC), respectively; cells of strains JW/IW010^T and JW/SL-NZ826^T stained Gram-negative; and the ability to use cellobiose, maltose, sucrose, xylose, galactose, mannitol and pyruvate for growth. However, these are characteristics held by many *Thermoanaerobacter* species. However, significant differences between strain JW/IW010^T and *Thermoanaerobacter sulfurigignens* JW/SL-NZ826^T include the optimum pH$^{60\ °C}$ for strain JW/IW010^T of 7.1 (Fig. 4.4b), whereas *Thermoanaerobacter sulfurigignens* JW/SL-NZ826^T had a broad optimum pH$^{60\ °C}$ of 5.0–6.5 (Lee et al., 2007a). Under optimal conditions, the doubling times for strain JW/IW010^T and related strains were approximately 0.5 h to less than 1 h, and thus are notably faster than the doubling times reported for *Thermoanaerobacter sulfurigignens* JW/SL-NZ826^T as well as for strain JW/SL-NZ824 (2.4 h) (Lee et al., 2007a). Metabolic differences between strains JW/IW010^T and *Thermoanaerobacter sulfurigignens* JW/SL-NZ826^T included that strain JW/IW010^T utilized Casamino acids as well as tryptone whereas *Thermoanaerobacter sulfurigignens* JW/SL-NZ826^T utilized neither (Lee et al., 2007a). *Thermoanaerobacter sulfurigignens* JW/SL-NZ826^T was also able to utilize both starch and lactose as growth substrates whereas strain JW/IW010^T could not (see Table 4.2). In addition, the thiosulphate and sulphite tolerance between strain JW/IW010^T and *Thermoanaerobacter sulfurigignens* strains JW/SL-NZ826^T and JW/SL-NZ824 is substantially different. Strain JW/IW010^T tolerated only up to 200 mM thiosulphate and 40 mM sulphite, whereas *Thermoanaerobacter sulfurigignens* exhibited a remarkable resistance to 1 M thiosulphate and 90 mM sulphite. Furthermore, strain

JW/IW010^T reduced thiosulphate to hydrogen sulphide and also converted thiosulphate to sulphur, whereas *Thermoanaerobacter sulfurigignens* could convert thiosulphate to elemental sulphur without any detectable sulphide production (Lee et al., 2007a). Differential characteristics of strain JW/IW010^T and *Thermoanaerobacter sulfurigignens* JW/SL-NZ826^T are summarized in Table 4.2.

In addition to strains JW/IW007, JW/IW015 and JW/HL-KA15, other isolates with a high similarity to strain JW/IW010^T (≥98 % 16S rRNA gene sequence similarity) have been found in hot springs throughout the Uzon Caldera. When the 16S rRNA gene sequences of strain JW/IW010^T and similar strains (data not shown) were compared with the sequence of the phylogenetically closely related recognized taxon *Thermoanaerobacter sulfurigignens* JW/SL-NZ826^T, noteworthy insertions/deletions were observed. A 7 bp region (nucleotides 183–189 of strain JW/SL-NZ826^T 16S rRNA gene sequence) is present within the *Thermoanaerobacter sulfurigignens* JW/SL-NZ826^T 16S rRNA gene sequence, but is absent from the 16S rRNA gene sequence of strain JW/IW010^T and 14 similar isolates from the Uzon Caldera. An additional 4 bp region (nucleotides 1428–1431 of the 16S rRNA gene sequence of strain JW/SL-NZ826^T) is also present within the *Thermoanaerobacter sulfurigignens* JW/SL-NZ826^T 16S rRNA gene sequence, but is absent from the 16S rRNA gene sequence of strain JW/IW010^T and 14 similar isolates from the Uzon Caldera.

Thus, strain JW/IW010^T is phenotypically, chemotaxonomically and phylogenetically different from the most closely related recognized species, *Thermoanaerobacter sulfurigignens*. For these reasons, strain JW/IW010^T is proposed to represent a novel species with the name *Thermoanaerobacter uzonensis* sp. nov. Strain 1501/60 was described by Krivenko et al. (1990)

as the type strain of the effectively published, but not recognized, taxon '*Clostridium uzonii*', which herewith is included in the novel taxon *Thermoanaerobacter uzonensis* sp. nov.

Description. of *Thermoanaerobacter uzonensis* sp. nov

Thermoanaerobacter uzonensis (u.zo.nen'sis. N.L. masc. adj. *uzonensis* pertaining to the Uzon Caldera, Kamchatka, Far East Russia).

Cells are straight to slightly curved rods, approximately 0.5 µm in width and 2–5 µm, and occasionally up to 15 µm, in length. Oval, subterminal to terminal, mother cell-distending spores are observed, though rarely. Cells have peritrichously inserted flagella and are motile. Cells stain Gram-negative and are Gram-type positive (Wiegel, 1981). Within agar-shake-roll tubes, colonies are circular, 1–2 mm in diameter, slightly convex and creamy white in colour. When grown on plates of carbonate-buffered basal medium with 2.3 % agar within anaerobic jars at 60 °C, forms colourless, nearly transparent circular colonies up to 1 mm in diameter. Temperature range for growth is 32.5–69 °C with an optimum at 61 °C (no growth is observed at 30 °C or below, or at 72 °C or above). $pH^{60\ °C}$ range for growth is 4.2–8.9 with an optimum at $pH^{60\ °C}$ 7.1 (no growth occurs at or below $pH^{60\ °C}$ 3.9, or at 9.2 or above). Chemo-organotrophic. Yeast extract, peptone, Casamino acids and tryptone support growth. In the presence of 0.1 % (w/v) yeast extract, growth occurs in the presence of inulin, cellobiose, maltose, sucrose, glucose, fructose, galactose, mannose, xylose, trehalose, mannitol, pyruvate and crotonate. Growth is not observed with beef extract, casein, cellulose, xylan, starch, olive oil, pectin, lactose, arabinose, rhamnose, ribose, xylitol, sorbitol, methanol, glucuronic acid or glycerate. Thiosulphate is converted to sulphide and also elemental sulphur. The G+C content of the genomic DNA of the

type strain is 33.6 mol% (SD=0.4; HPLC method). Major PLFAs are iso-15 : 0, 15 : 0, 16 : 0, 10-methyl 16 : 0 and anteiso-15 : 0.

The type strain, JW/IW010^T (=ATCC BAA-1464^T=DSM 18761^T), was isolated from a hot spring 2 m east of Pulsating Spring (54.500 ° N 160.007 ° E) in the Winding Creek area, on the northern side of the central portion of the Uzon East Thermal field, Kamchatka, Far East Russia. This species is apparently widespread throughout the Uzon Caldera in various hot springs. Additional strains are 1501/60 (DSM 9752) and JW/IW007, JW/IW015 and JW/HL-KA15 (available from the authors).

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thermohydrosulfuricus comb. nov., respectively; and transfer of *Clostridium thermohydrosulfuricum* 39E to *Thermoanaerobacter ethanolicus*. *Int J Syst Bacteriol* **43**: 41-51.

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Table 4.1. Phospholipid fatty acid contents (%) of strains JW/IW010^T and 1501/60. Abbreviation: ND, not detected.		
PLFA	**JW/IW010^T**	**1501/60**
iso-13:0	1.2	0.2
iso-14:0	ND	0.6
14:0	1.3	1.4
iso-15:0	74.3	56.9
anteiso-15:0	6.3	9.4
15:0	ND	1.1
iso-16:0	ND	7.0
16:1ω7c	1.3	0.1
16:0	1.5	5.0
iso-17:0	9.0	11.5
anteiso-17:0	1.3	6.3
18:1ω9c	2.0	0.1
18:00	1.8	0.4
Total	100	100

Table 4.2. Characteristics of JW/IW010^T, strain 1501/60, and selected members of the genus *Thermoanaerobacter*. Abbreviations: +, positive; -, negative; NR, not reported; RFE, Russian Far East; NZ, New Zealand; YNP, Yellowstone National Park. Characteristics of *Thermoanaerobacter* members from Onyenwoke & Wiegel (2007), and characteristics of strain 1501/60 come from our own studies and Krivenko et al. (1990).

	JW/IW010^T	**1501/60**	***T. sulfurigignens***	***T. kivui***	***T. ethanolicus*^{T}**
Temperature range, (optimum)	32.5-69, (61)	50-70, (65)	34-74, (63-65)	50-72, (66)	37-78, (69)
pH range, (optimum)	4.2-8.9, (7.1)	4.5-8.5, (7)	4.0-8.0, (4.8-6.5)	5.3-7.3, (6.4)	4.4-9.9, (5.8-8.5)
G+C mol%	33.6	33.3	34.5	38.0	32.0
Spore formation	+	+	+	+	-
Utilization of:					
Cellobiose	+	+	+	-	+
Maltose	+	+	+	-	+
Sucrose	+	+	+	-	+
Xylose	+	+	+	NR	+
Galactose	+	NR	+	-	+
Manitol	+	+	+	-	-
Pyruvate	+	-	+	+	+
Starch	-	+	+	-	+
Pectin	-	+	NR	-	NR
Lactose	-	+	+	-	+
Ribose	-	-	-	-	+
Autotrophic growth	-	-	-	+	-
Isolated from:	Uzon Caldera, RFE	Uzon Caldera, RFE	Acidic volcanic steam outlet on White Island, NZ	Sediments from Lake Kivu, Africa	An alkaline hot spring in White Creek, YNP, USA

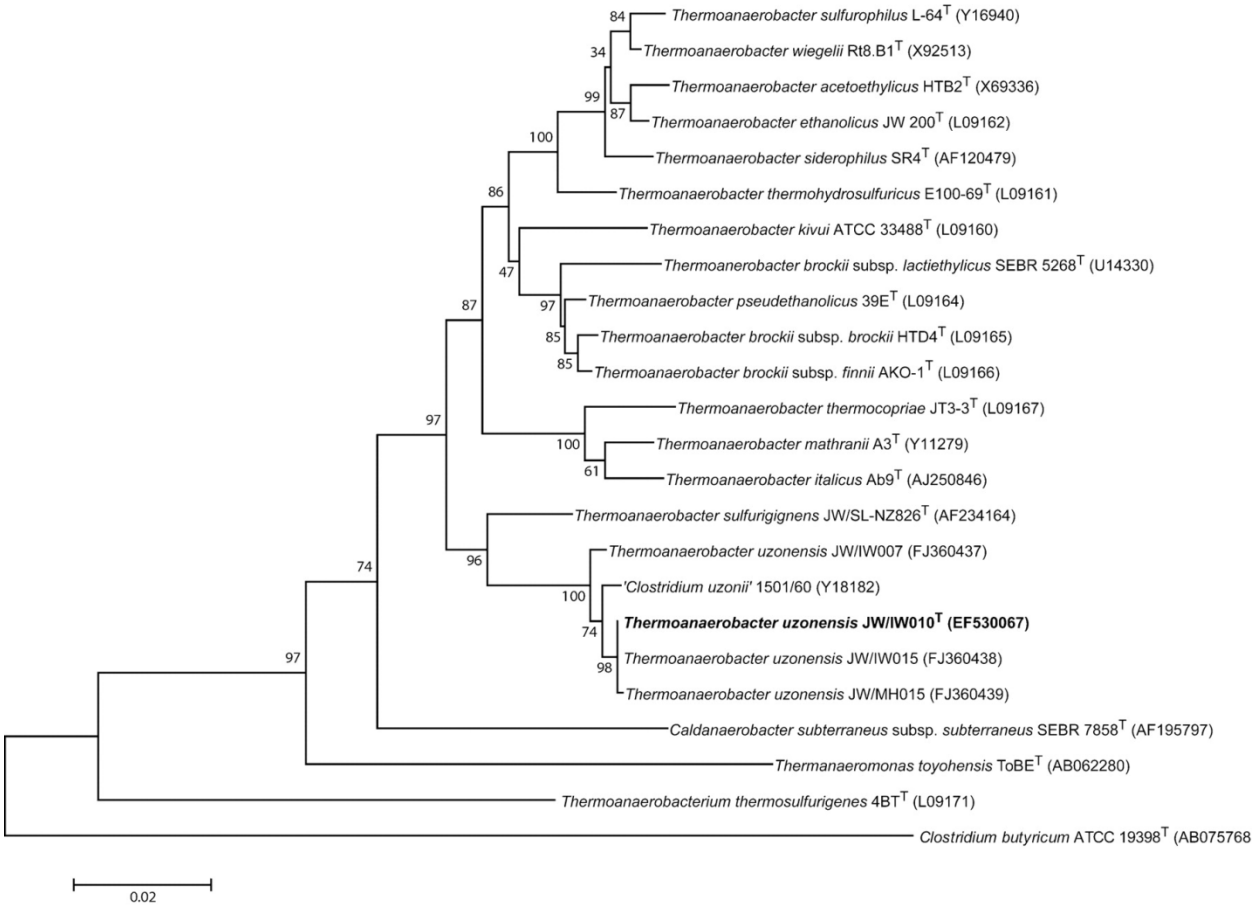

Fig. 4.1. 16S rRNA gene sequence-based phylogenetic dendrogram of strains JW/IW007, JW/IW010^T, JW/IW015 and JW/MH015 amongst members of the genus *Thermoanaerobacter* and selected members of the family '*Thermoanaerobacteriacae*'. *Clostridium butyricum* was used as an outgroup; numbers at nodes are bootstrap percentages (100 replicates). The tree was constructed using the neighbour-joining method (Saitou & Nei, 1987) and the Jukes and Cantor distance corrections (Jukes & Cantor, 1969). Bar, 2 nucleotide substitutions per 100 nucleotides.

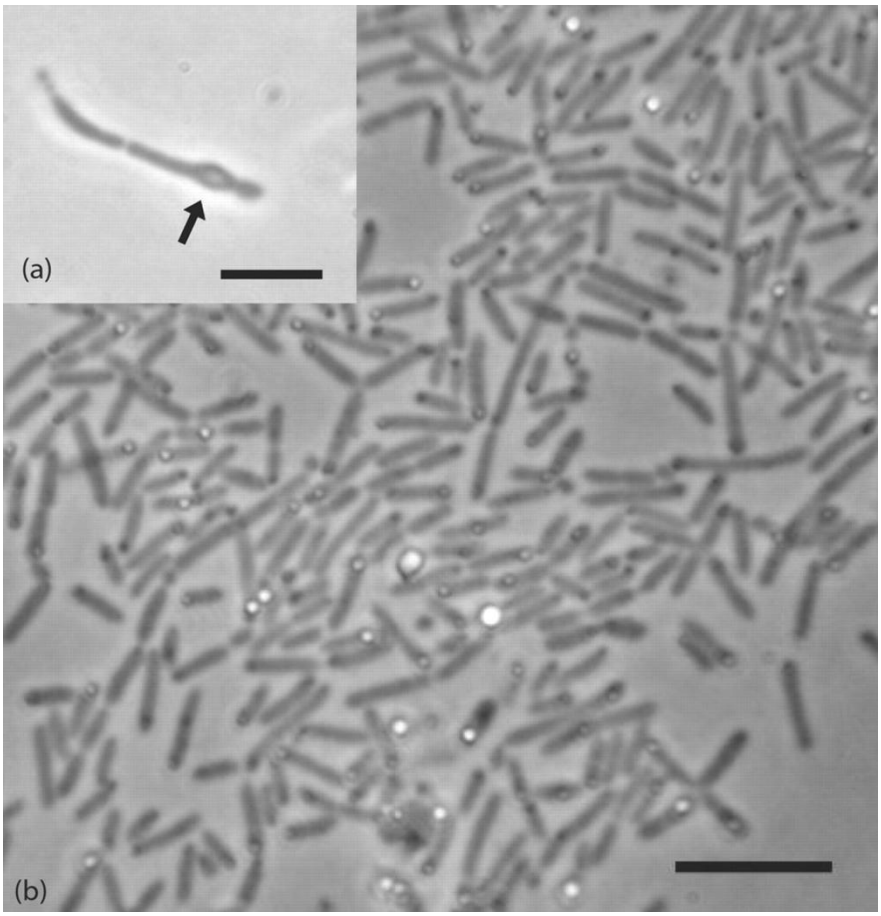

Fig. 4.2. Morphology of cells of strain JW/IW010^T, studied using an Olympus VANOX phase-contrast microscope (Tokyo, Japan). (a) Spore formation within a cell of strain JW/IW010^T. The arrow indicates the position of oval spores within the swollen mother cell. Bar, 5 µm. (b) Sulphur granula within cells of strain JW/IW010^T grown for approximately 24 h in medium containing 50 mM thiosulphate. Bar, 10 µm.

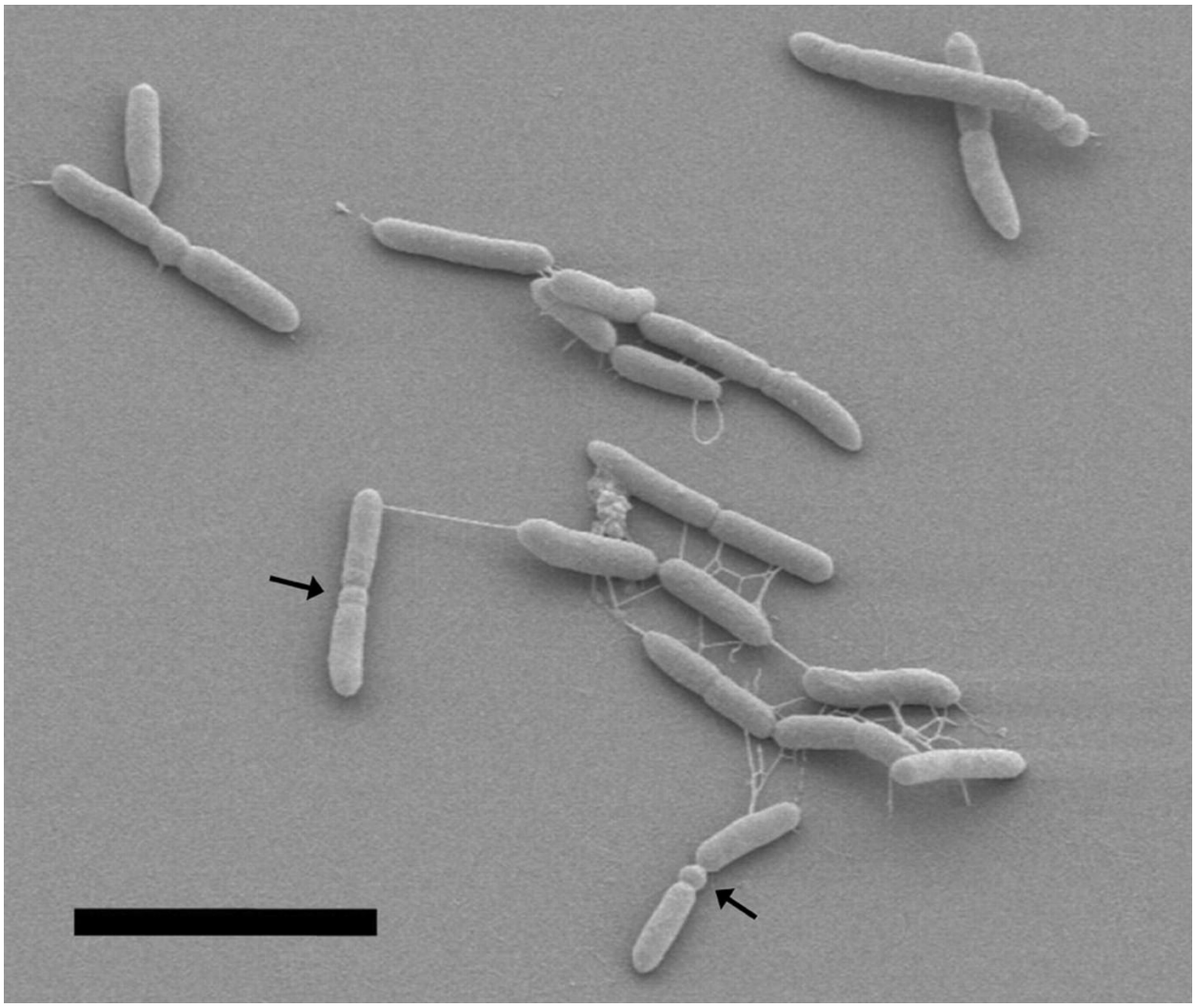

Fig. 4.3. Scanning electron micrographs of cells of strain JW/IW010^T. Arrows indicate characteristic coccoid cells observed within the chain. Bar, 5 µm.

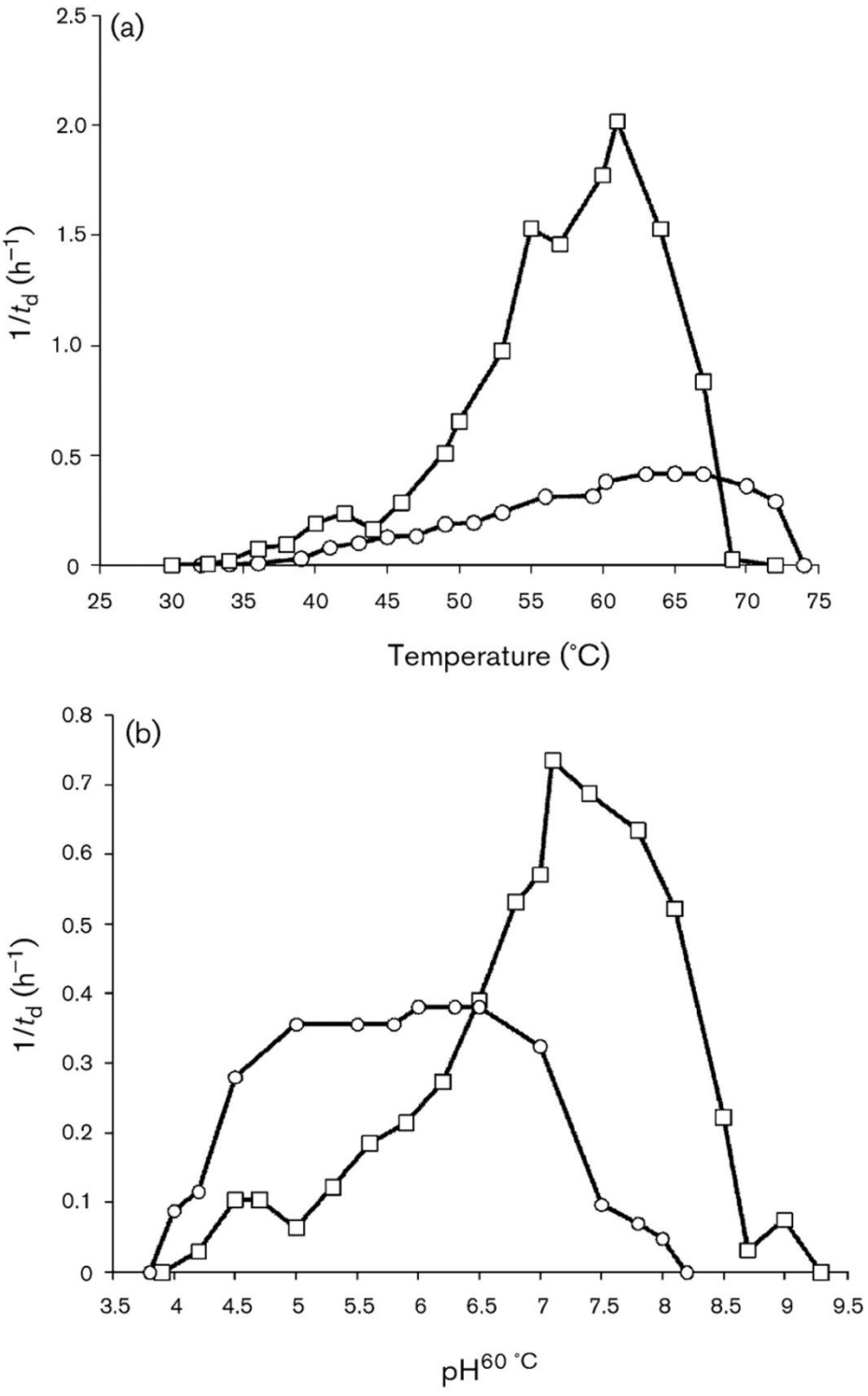

Fig. 4.4. Temperature and $pH^{60\ °C}$ profiles of strain JW/IW010^T and *Thermoanaerobacter sulfurigignens* JW/SL-NZ826^T. Growth rates over a range of temperatures (a) and $pH^{60\ °C}$ values (b) are shown. □, Strain JW/IW010^T; ○, *Thermoanaerobacter sulfurigignens* JW/SL-NZ826^T (data from Lee et al., 2007a)

CHAPTER 5

VARIATIONS OF EIGHT UNIVERSALLY CONSERVED PROTEIN CODING GENES AMONG 123 *THERMOANAEROBACTER UZONENSIS* ISOLATES FROM GEOTHERMAL SPRINGS OF KAMCHATKA, RUSSIAN FAR EAST[6]

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ABSTRACT

More than 220 thermophilic anaerobic bacteria were isolated from geothermal springs of the Kamchatka Peninsula, Russian Far East. Two hundred and eight of the isolates were found to be phylogenetically related to *Thermoanaerobacter uzonensis* (≥98% 16S rRNA gene sequence similarity). Eight universally conserved protein coding genes, *gyrB*, *lepA*, *leuS*, *pyrG*, *recA*, *recG*, *rplB*, and *rpoB*, were amplified and sequenced from 123 *T. uzonensis* isolates to assess the intraspecies variation. The protein coding genes were found to have divergent phylogenies. Heterogeneity was observed within all gene sequence sets, but the amount and type of variation differed. While evidence for linkage disequilibrium was apparent within the entire *T. uzonensis* population, it was absent in the *T. uzonensis* isolates from the seven geothermal springs of the Uzon Caldera where the protein coding gene sequence alleles appeared randomly associated. The intraspecies heterogeneity observed within this *T. uzonensis* cluster suggests that the most applicable species concept is one that takes into account subpopulation/ metapopulation dynamics and also acknowledges that phenotypic and physiological properties (e.g., endospore formation) influence how readily genetic information is shared between subpopulations.

INTRODUCTION

The concept with which microbiologists define a set of closely related bacteria or archaea, a cluster, as belonging to the same species has implications concerning how microbial diversity is defined, how it is analyzed, and what that diversity ultimately means. Practical applications are observed within industry, agriculture, and medicine (Fraser et al., 2009). Consequently, quantifying and assessing intraspecies heterogeneity is important. For example, when diagnosing clinical isolates, the differences between pathogenic and nonpathogenic strains classified within the same species can be of great significance (Welch et al., 2002). The considerable phenotypic differences that have been observed between closely related strains can influence the microorganism's fundamental niche and the microorganism's role in the biogeochemical cycles of that environment. *Thermolithobacter* strains having 99% 16S rRNA gene sequence similarity to each other were found to differ to the extent that some strains reduced Fe(III) but did not assimilate CO, whereas others strains assimilated CO but did not reduce Fe(III) (Sokolova et al., 2007). Differences of 30-60% in gene content have been reported within the genomes of strains within the same species (Welch et al., 2002; Konstantinidis and Tiedje, 2005).

Recently it has been questioned whether the current species definition, i.e., a species is a genomically coherent group of strains sharing a high degree of similarity considering several independent features tested under highly standardized conditions (Stackebrandt et al., 2002), meets the challenges of modern microbiology. Of particular concern is the emphasis placed on DNA-DNA reassociation values. For instance, the DNA-DNA hybridization technique is regarded as being arbitrary, too broad, cumbersome, indifferent to ecology and evolution, impractical for assigning a large collection of similar strains to species, and the presently used

value of 70% is not based on any particular theoretical justification (Achtman and Wagner, 2008; Fraser et al., 2009; Papke, 2009). Foreseeing these criticisms, the committee that developed the framework for the species definition noted that investigators are encouraged to develop new techniques to complement or supplant DNA-DNA reassociation (Stackebrandt et al., 2002).

A number of species concepts have been proposed for prokaryotes, and two that are commonly referenced are the revised Biological Species Concept (Dykhuizen and Green, 1991) and the Ecotype Species Concept (Cohan, 2001). Others have stated that the prokaryotic species concept should take into account qualities the metapopulation (Achtman and Wagner, 2008; Fraser et al., 2009). For example, Fraser and colleagues (2009) suggested that a species is the group of individuals that are similar enough, or interbreed enough, that individual variant genes compete directly for reproductive success. Achtman and Wagner (2008) have described a species as a metapopulation lineage; that is, connected subpopulations that extend through time .

Analyses with the 16S rRNA gene sequence remain an important tool for microbiologists. However, there are a number of disadvantages in using 16S rRNA gene sequences to assess the divergence between closely related taxa. The 16S rRNA gene sequence encodes a very small portion of the genome, and the rRNA does not encode a protein. Thus insertions and deletions can introduce problems for sequence alignments (Santos and Ochman, 2004). Additionally, the slow evolutionary rate of the 16S rRNA gene can complicate the differentiation of closely related strains. There are also questions concerning the degree to which rRNA sequences are resistant to lateral gene transfer (Cooper and Feil, 2004; Santos and Ochman, 2004). Perhaps the most unfavorable quality of the 16S rRNA gene sequence, with regard to certain phylogenetic analyses, is that multiple, variable rRNA operons can exist within a genome (Santos and Ochman, 2004). Data from the Ribosomal RNA Operon Copy Number

Database (http://ribosome.mmg.msu.edu/rrndb/) was used to calculate an average of 4.1 ribosomal operons per bacterial genome, although the authors of the study acknowledged that this value could be skewed higher than what actually exists in nature due to the types of microorganisms in the database (Case et al., 2007). Additionally, of the bacteria in the Ribosomal RNA Operon Copy Number Database with multiple rRNA operons, 62% were found to have some amount of intragenomic heterogeneity (Klappenbach et al., 2001; Case et al., 2007). Rather than focusing on the 16S rRNA gene sequence, the average sequence diversity between all orthologous genes in pairs of genome sequences was proposed as one way of assigning strains into the same, or different, species (Konstantinidis and Tiedje, 2005). The drawback of this approach is that genome sequencing, as a requirement for the description of a species, would be a significant challenge at this time (Achtman and Wagner, 2008).

The use of universally conserved protein coding genes, through phylogenetic analyses alone and in combination, was proposed as a way to address some of the disadvantages of 16S rRNA gene sequence analyses (Papke, 2009). Universally conserved protein coding genes are typically present as single copies within genomes, the genes are subject to very low rates of insertions and deletions, and nucleotide differences can be partitioned into synonymous and nonsynonymous substitutions (Santos and Ochman, 2004). Analyses with the universally conserved protein coding genes do have limitations. For example, there is no strict agreement regarding which genes to examine across species, and any homologous recombination and lateral gene transfer confounds subsequent phylogenetic analyses. Homologous recombination does influence bacterial population genetic structures, but the frequency of recombination within different taxa appears to vary (Smith et al., 1993; Achtman and Wagner, 2008).

Moreover, there is an important shortcoming concerning how the analyses with universally conserved protein coding genes contributes to the elucidation of intraspecies diversity. The universally conserved protein coding genes, as part of the "core" genome of a species, will not be as important for adaptation to different niches as the "variable" genome of a species (Medini et al., 2005; Fraser et al., 2009; Papke, 2009). That is, niche-specific traits often arise by the gain or loss of variable-genome genes, where the altered physiology allows cells to thrive in conditions that are hostile to parental strains (Retchless and Lawrence, 2007; references therein). Connecting these ideas to the proposed prokaryotic species concepts, some state that the prokaryotic species should accommodate the ecological distinctiveness of the microorganisms to be more reasonably predictive of the phenotype and the ecological potential of the species (Konstantinidis and Tiedje, 2005).

These limitations aside, analyses with 16S rRNA gene sequences and universally conserved protein coding gene sequences do provide insight into the intraspecies heterogeneity within a cluster of related strains. For a set of 123 *Thermoanaerobacter uzonensis* isolates obtained from geothermal springs within the Kamchatka Peninsula, the heterogeneity of eight universally conserved protein coding genes was determined, the divergence between protein coding gene sequence sets was compared, and linkage disequilibrium within the population was assessed.

MATERIALS AND METHODS

Sample collection

As part of the Kamchatka Microbial Observatory, mixed water and sediment samples were collected from Kamchatka geothermal springs during the 2005 and 2006 field seasons. The

water and sediment samples were transferred to sterilized 100 ml bottles, filled to the brim, sealed with butyl rubber stoppers, transferred to Athens, GA, USA, and stored at 4 °C.

Isolation of *Thermoanaerobacter* strains

At the University of Georgia, Athens, laboratory, 1 ml of mixed water/sediment was transferred to 50 ml Wheaton serum bottles containing 20 ml of a mineral medium (Wagner et al., 2008) supplemented with 1 $g \cdot l^{-1}$ glucose, 0.5 $g \cdot l^{-1}$ yeast extract, and 50 mM thiosulfate. Enrichment cultures were incubated at 62 °C for 48 h. A 10^{-1} dilution was prepared, streaked onto a 2.15% (w/v) agar plate of the same medium composition, and then incubated anaerobically at 62 °C for 48 hours. A single, isolated colony was selected and then re-streaked on a fresh agar plate containing the same mineral medium composition. The re-streaking process was repeated a minimum of two times. Analysis of the 16S rRNA gene was used to confirm culture purity. If nucleotide sequence ambiguities were observed during subsequent sequence analyses, single colonies were re-isolated and the genes were PCR-amplified and sequenced anew. Each isolate was derived from a separate enrichment culture.

Amplification of the 16S rRNA gene sequence

Genomic DNA was isolated with the UltraClean Microbial DNA Isolation kit (Mo Bio). The 16S rRNA gene sequence was amplified with the 27F and 1492R primers (Lane, 1991) using PrimeSTAR HS DNA Polymerase (Takara). The thermal cycler conditions for amplification were: 30 cycles of 98°C for 10 s, 58°C for 5 s, and then 72°C for 90 s. Purification of the amplification product and the subsequent sequencing reaction was performed by Macrogen USA (Rockville, MD).

Amplification of universally conserved protein coding gene sequences

The universally conserved protein coding genes analyzed in this study were selected from those suggested by Santos and Ochman (2004). Primers for the amplification of universally conserved protein coding genes from *Thermoanaerobacter* isolates were designed from the genes of representatives of the family *Thermoanaerobacteracae* with sequenced genomes, including, *Thermoanaerobacter pseudethanolicus* strain 39E (Refseq: NC_010321) and *Caldanaerobacter subterraneus* subsp. *tengcongensis* strain MB4 (Refseq: NC_003869). The oligonucleotide primers designed for the universally conserved protein coding genes are listed in Table 5.S1.

The Phusion High-Fidelity polymerase PCR Master Mix with HF Buffer (New England Biolabs) was used for the amplification of the universally conserved protein coding genes. Amplification was performed in a Mastercycler ep Gradient thermal cycler (Eppendorf). Conditions for the amplification of the *gyrB, lepA, leuS, pyrG, recG,* and *rpoB* gene sequences were: 98°C for 10 s; then 30 cycles of 98°C for 1 s, 56°C for 5 s, and 72°C for 20 sec; and then 72°C for 1 minute. Conditions for the amplification of the *recA* and *rplB* gene sequences were: 98°C for 10 s; then 30 cycles of 98°C for 1 s, 56°C for 5 s, and 72°C for 12 sec; and then 72°C for 1 minute. Purification of the amplification product and the subsequent sequencing reaction was performed by Macrogen USA (Rockville, MD).

Analysis of gene sequences

16S rRNA gene sequences were aligned and initially analyzed with Sequencher 4.1 (Gene Codes). Multiple sequence alignments were prepared with ClustalW (Larkin et al., 2007) or with NAST (DeSantis et al., 2006) through the greengenes web application (http://greengenes.lbl.gov/). Universally conserved protein coding gene sequences were initially

aligned with homologous sequences from the genomes of related *Thermoanaerobacteracae* and manually checked for spurious insertion or deletions. Protein coding gene sequences were then compared against each other and nucleotide polymorphisms found within only one gene sequence were reexamined for correctness. Multiple sequence alignments of the protein coding genes were routinely prepared with ClustalW (Larkin et al., 2007). Gene sequence polymorphism as nucleotide and amino acid sequence diversity measurements were calculated with DnaSP (Rozas and Rozas, 1999) or MEGA 4.1 (Tamura et al., 2007). Phylogenetic analyses were performed primarily with MEGA 4.1 (Tamura et al., 2007).

The nucleotide sequence polymorphism and diversity properties determined were: the total number of segregating (i.e., polymorphic) nucleotide sites, S_{nt}; the G+C mol% of the gene sequence set; the number of variant forms (i.e., alleles) of the gene, ntH; the average number of nucleotide differences, k; and the average number of nucleotide substitutions per site, π. Considering the deduced primary protein sequence, the diversity properties determined were: the total number of segregating amino acid residues, S_{aa}; the maximum number of amino acid differences within the protein coding gene sequence set, max ΔX; the average number of amino acid differences within the protein coding gene sequence set, mean ΔX; the average amino acid p-distance, the average proportion of of amino acid sites at which the sequences compared are different; and non-synonymous substitutions, d_S and d_N respectively, the Nei-Gojobori method was utilized with the Jukes-Cantor correction (Tamura et al., 2007). An amino acid substitution prediction method, SIFT, was used to assess whether an amino acid substitution would affect protein function (http://sift.jcvi.org/; Kumar et al., 2009). The amino acid residue changes in the primary protein sequence were considered from the perspective of the most-prevalent sequence variant.

The divergence observed within different gene sequence sets were compared through Mantel tests using the Primer 5 software (PRIMER-E Ltd). The dissimilarity matrices utilized were based on the Jukes and Cantor between-isolate genetic distances. Mantel tests were also employed to compare the G+C mol% differences between the universally conserved protein coding gene sequence sets and the rank correlation values (Spearman's ρ) obtained from comparisons of the Jukes and Cantor genetic distances between isolates.

The index of association, I_a, was used to measure linkage disequilibrium between *T. uzonensis* sequence variants at the eight universally conserved protein coding genes (http://www.mlst.net; Smith et al., 1993). Evidence for linkage disequilibrium was established if V_{obs} , the observed variance obtained from the actual data set, was greater than V_{max}, the maximum variance deteremined from 20, 100, or 1,000 datasets under the assumption of the random association of alleles.

RESULTS

Isolation of strains

Two hundred and twenty-seven anaerobic thermophilic heterotrophic bacteria were obtained using the isolation scheme described above. Of these isolates, 208 were found to have ≥98% 16S rRNA gene sequence similarity to *Thermoanaerobacter uzonensis* strain JW/IW010^T (Wagner et al., 2008), 14 isolates were found to have ≥97% 16S rRNA gene sequence similarity to *Thermoanaerobacter siderophilus* strain SR4^T (Slobodkin et al., 1999), and 5 isolates were phylogenetically similar to *Caloromator* species.

Universally conserved protein coding gene sequences analyzed

Preliminary phylogenetic analyses based on the 16S rRNA gene sequence revealed that *T. uzonensis* isolates from the same geothermal spring and region occasionally grouped together on phylogenetic trees (data not shown). A set of eight universally conserved protein coding genes (Santos and Ochman, 2004) were chosen to further assess the intraspecies divergence. The protein coding genes amplified, sequenced, and analyzed were: DNA gyrase subunit B (*gyrB*), GTP-binding protein LepA (*lepA*), leucyl-tRNA synthetase (*leuS*), CTP synthase (*pyrG*), bacterial DNA recombination protein RecA (*recA*), ATP-dependent DNA helicase RecG (*recG*), 50S ribosomal protein L2 (*rplB*), and RNA polymerase subunit B (*rpoB*). The inferred fragment of the gene sequence amplified from the *T. uzonensis* isolates varied from 0.46 (*leuS*) to 0.86 (*rpoB*) (Table 5.S1).

Overall heterogeneity measures between gene sequence sets

One way the heterogeneity of each *T. uzonensis* universally conserved protein coding gene sequence set was assessed was by enumerating the number of unique nucleotide- and amino acid sequence variant forms, ntH and aaH (Table 5.1 and Fig. 5.2a). The *rplB* gene sequence set was the least variable; the set had four nucleotide sequence variant forms and two primary protein sequence variant forms (Table 5.1). The *pyrG* gene sequence set had the most ntH, 27, whereas the *recG* gene sequence set had the most aaH, 19. The *recG* gene sequence was also distinctive in that almost every nucleotide sequence variant form had a corresponding deduced primary protein sequence variant form (Table 5.1, Table 5.S16). While the *recG* gene sequence set had the lowest ntH:aaH ratio, the *recA* gene sequence set had the highest; nine *T. uzonensis* *recA* ntH were found with only three aaH (Table 5.1).

Protein coding gene sequence heterogeneity was also calculated by determining the nucleotide and amino acid sequence polymorphism, specifically the average number of nucleotide differences, k, and the average number of nucleotide substitutions per site, π, (Fig. 5.1b). With respect to these diversity calculations, the protein coding gene sequence sets were grouped into three series. The first series contained genes with the least amount of nucleotide variation, *rplB*, *recA*, and *lepA*, where $k < 1.4$ and $\pi < 0.0015$. The genes *rpoB*, *leuS*, *gyrB*, and *pyrG* had $3.8 < k < 7$ and $0.004 < \pi < 0.0058$. The greatest variation was observed within the *recG* gene sequences where $k > 12.6$ and $\pi > 0.01$ (Fig. 5.1b).

Within most universally conserved protein coding gene sequence sets, the amino acid p-distance heterogeneity was approximately proportional to the nucleotide diversity (Fig. 5.1b). A notable exception was the *rpoB* gene sequence set, wherein the mean nucleotide diversity was 0.0038 while the average number of amino acid differences per site (amino acid p-distance) was 0.00053 (Table 5.1, Fig. 5.1b). As expected from these heterogeneity measures, the *rpoB* gene sequence set had the lowest d_N/d_S ratio. The synonymous substitutions rates differed across the universally conserved protein coding genes from 0.0003 (*rplB*) to 0.0262 (*recG*). The highest d_N values were found in the *pyrG* (0.0023) and *recG* (0.0056) gene sequence sets (Table 5.1).

The overall heterogeneity measures discussed above take into consideration the broad differences observed between the eight protein coding gene sequence sets analyzed from the *T. uzonensis* isolates. Specific differences that are observed within each protein coding gene sequence set, e.g., the ratio of synonymous to nonsynonymous substitutions, the inferred effect of the nonsynonymous substitution on the corresponding deduced primary protein sequence, and the sequence variant form observed within the largest fraction of *T. uzonensis* isolates, are described within the supplementary information section below.

Variation between *T. uzonensis* gene sequence sets

Pairwise comparisons of Jukes and Cantor genetic distances of the corresponding genes within the same *T. uzonensis* isolates, displayed as ordered pairs, qualitatively revealed no obvious linear relationships (Fig. 5.S1). The genetic divergences of the 16S rRNA gene and the universally conserved protein coding gene sequence sets were quantitatively compared using Mantel tests to determine the association between dissimilarity matrices of Jukes and Cantor distance values (Table 5.2). Only three pairwise comparisons had Spearman's ρ values >0.5, these were: *leuS* compared to *gyrB*, ρ = 0.58; *leuS* compared to *recA*, ρ = 0.58; and *recA* compared to *gyrB*, ρ = 0.54. Seven of the 28 universally conserved protein coding gene sequence comparisons have ρ values < 0.1 (Table 5.2).

The G+C mol% of the universally conserved protein coding gene sequence sets varied by up to 8% (Table 5.2, above diagonal). Using the Mantel test, the G+C mol% differences were compared to the Spearman's ρ values obtained between the corresponding gene sequence sets, essentially addressing whether there is a relationship between the divergence observed at the genetic level and the difference observed considering the ΔG+C mol%. The rank correlation value obtained from this comparison was close to zero (-0.064); i.e., there was no association between G+C mol% difference and Jukes and Cantor sequence divergence between the isolates of different gene sequence sets.

Assessing the influence of recombination on the *T. uzonensis* population structure

Linkage disequilibrium was assessed within the *T. uzonensis* population to determine whether the sequence variants of different protein coding genes were randomly associated, or whether there was a significant association between the different sequence variant forms when considering the eight universally conserved protein coding genes. From the analysis with the

entire *T. uzonensis* population I_a was 0.42, the V_{obs} was 1.96 and V_{max} obtained in 1000 trials was 1.38. As $V_{obs} > V_{max}$, evidence of linkage disequilibrium was detected. However, when only the *T. uzonensis* isolates from the seven geothermal spring within the Uzon Caldera region were analyzed I_a was -0.03, V_{obs} was 1.38, and V_{max} was 1.87 obtained in 1000 trials. As I_a was near zero and $V_{obs} < V_{max}$, linkage equilibrium was observed within the largest regional *T. uzonensis* subpopulation examined.

DISCUSSION

Thermoanaerobacter uzonensis strains were isolated from 10 geothermal springs in Kamchatka. A set of eight universally conserved protein coding genes (Santos and Ochman, 2004) were sequenced from these isolates to complement the information gained from an initial 16S rRNA gene sequence analyses and to further examine the intraspecies heterogeneity of this taxon. The universally conserved protein coding genes examined here were common to all *T. uzonensis* strains and were relatively conserved. *recG* was the most variable protein coding gene sequence set, and was found to have ΔX max = 10, and S_{aa} = 19 (Table 5.1). Thus, these genes are part of the *T. uzonensis* core genome. A goal of this work was to quantify the intraspecies diversity, however the most significant intraspecies differences are expected be found in the variable-genome genes. Assessing the variable-genome heterogeneity is a challenge, although whole-genome analyses have been performed within other phylogenetic clades with a small set of strains for this purpose (White et al., 2008; Reno et al., 2009). With that limitation noted, the heterogeneity within a set of *T. uzonensis* isolates was analyzed through sequencing of the 16S rRNA and the universally conserved protein coding genes. The variation within each protein

coding gene sequence set was quantified, the evolutionary divergence between the gene sequence sets was compared, and linkage disequilibrium within the population was assessed.

Culture independent analyses revealed that *Thermoanaerobacter* are present within geothermal springs of Kamchatka, although only as a relatively small proportion of the population (D. E. Crowe, unpublished data). The relative proportion of a taxon within a population may influence the intraspecies heterogeneity of the taxon. When a population size is small, it had been suggested that genetic diversity can more easily persist due to fluctuations in allelic frequency, rather than being eliminated by a periodic selection event (Achtman and Wagner, 2008). Such periodic selection event would "reset" the allelic diversity to zero and therefore all genes within a population should accumulate approximately the same number of neutral mutations (Papke, 2009).

The number of sequence variant forms varied for each protein coding gene sequence set within *T. uzonensis* population. For example, *pyrG* had the most nucleotide sequence variant forms, whereas *recG* had the most primary protein sequence variant forms, and *rplB* was found to have the fewest nucleotide- and amino acid sequence variant forms (Fig. 5.1). Collectors curves, generated from protein coding gene sequence variants, suggested that the isolation of additional *T. uzonensis* strains and subsequent sequencing of universally conserved protein coding genes would not result in additional unique sequence variants; the most-likely exceptions would be for the *pyrG* and *recG* genes (data not shown). Conversely, the observation that eight unique protein coding nucleotide sequence variant forms were only found in one isolate, JW/IW R649_9, indicated that additional sampling may result in new sequence variants for all universally conserved protein coding genes (strain JW/IW R649_9 is further discussed below).

For a set of *Halorubrum* isolates, the dominant *bop* allele was found in >85% of the strains (Papke, 2009). Based on this observation, Papke stated that the most reasonable explanation was that selection drove a particular allele to high frequency, but that recombination prevented a periodic selection event by continually spreading non-selected alleles randomly throughout the population (2009). Considering the protein coding genes within the *T. uzonensis* isolates, a particular nucleotide sequence variant was observed within >70% of the isolates with regard to the *rplB* and *gyrB* genes sequence sets (Tables 5.S2 and 5.S12). By comparison, within the *leuS*, *pyrG*, and *recG* gene sequence sets, all nucleotide sequence variant forms were found within < 31% of the *T. uzonensis* isolates (Tables 5.S10, 5.S14, and 5.S16). A predominant primary protein gene sequence variant, found within >70% of the isolates, was found within the *gyrB*, *lepA*, *recA*, *rplB*, and *rpoB* gene sequence sets (Tables 5.S2, 5.S4, 5.S6, 5.S8, and 5.S12).

The fixation, or near fixation, of a gene within a population is based upon the frequency of acquisition of DNA through homologous recombination and the frequency of any periodic selection events. It is generally acknowledged that homologous recombination has influenced bacterial population genetic structures, but that the frequency of this process varies between species (Smith et al., 1993; Achtman and Wagner, 2008). Frequent recombination will cause linkage equilibrium within a population of bacteria (Whitaker et al., 2005; Papke, 2009). To the authors' knowledge, there are no known viruses reported to associate with *Thermoanaerobacter* taxa, although viruses are a known component of terrestrial geothermal sites (Young et al., 2005). The association of *T. uzonensis* sequence variants with regard to the different protein coding genes was calculated. While evidence for linkage disequilibrium was observed when the entire *T. uzonensis* population was analyzed, the sequence variants appeared randomly associated when only the *T. uzonensis* isolates from the Uzon Caldera region were compared. Although it is

recognized that there are a number of possible explanations for the observation of linkage disequilibrium beside being a sign of little or no recombination within or between populations; e.g., sampling biases (Papke, 2009).

The universally conserved protein coding gene sequences of *T. uzonensis* strain JW/IW R649_9, were particularly distinctive in that each gene sequence was a unique variant found only within this isolate (Tables 5.S2, 5.S4, 5.S6, 5.S8, 5.S10, 5.S12, 5.S14, and 5.S16). Furthermore, within four of the eight universally conserved protein coding gene sequences sets, *lepA, leuS* , *pyrG*, and *recG*, the corresponding deduced primary protein sequence was also found only within this isolate (Tables 5.S4, 5.S6, 5.S8, and 5.S12). The *gyrB* nucleotide sequence variant number four (abbreviated *Tu gyrB* ntH_4) was only observed within strain JW/IW R649_9; and furthermore, was the only *gyrB* sequence variant found only within one isolate. Therefore, at least with regard to these eight loci, there is no evidence for recent homologous recombination between the larger *T. uzonensis* population and the lineage of this strain. A possible explanation is that strain JW/IW R649_9 was a recent migrant into the geothermal spring Resting Rock located in the Geyser Valley region of Kamchatka. That is, that Resting Rock was not the "original" geothermal spring of this lineage of strain JW/IW R649_9.

A protein coding gene sequence set could potentially have a large number of sequence variants, but a comparatively low number of segregating sites, whereas a different gene sequence set could potentially have a low number of sequence variants, but comparatively high number of segregating sites. For instance, within the *lepA* gene sequence set, 19 segregating sites were observed as were 11 nucleotide sequence variants. By comparison, 30 segregating sites were found within the *gyrB* gene sequence set but only six nucleotide sequence variants (Table 5.1). Within the *T. uzonensis* gene sequence set, the *gyrB* gene sequence set had a S_{nt}:ntH ratio of 5.

All other gene sequence sets had S_{nt}:ntH ratios < 2.6 (Table 5.1). This implied that the genetic divergence observed within *gyrB* gene sequences was due to allopatry or some other barrier to recombination (Whitaker, 2006); that if the sequence variants were readily shared between subpopulations the sequences would be less likely to be distinctly divergent (Chapter 6, Fig. 6.S1).

According to neutral theory (Kimura, 1968), synonymous substitutions (d_S) should not vastly differ across genes. If a periodic selection event had reset the diversity to zero, all alleles would therefore contain approximately the same number of neutral substitutions. However, comparison of the synonymous substitution values for these *T. uzonensis* protein coding gene sequences revealed a range of d_S values (Table 5.1). Within a set of *Halorubrum* strains, Papke (2009; references therein) similarly observed that different alleles contained different numbers of synonymous substitutions. The d_N differences may, in part, be due to differences with regard to the selective pressure for evolution placed on the gene. That is, the d_N values of < 0.001 for the *T. uzonensis* universally conserved protein coding genes *rplB*, *recA*, *rpoB*, and *lepA* were due to strong selective constraint, whereas the higher d_N values observed within the *pyrG* and *recG* gene sequence sets were due to a relaxation of the selective constraint (Table 5.1).

Beside considering d_N, S_{aa}, max ΔX; mean ΔX, and aaH (Table 5.1; supplementary information section), an amino acid substitution prediction method, SIFT, was employed to further investigate primary protein sequence heterogeneity (Kumar et al., 2009). Most amino acid substitutions were predicted to be tolerated, i.e., most substitutions were not predicted to affect protein function (Tables 5.S3, 5.S5, 5.S7, 5.S9, 5.S11, 5.S13, 5.S15, and 5.S17). The observation that most of the differences between primary protein sequences were predicted to be tolerated, was not unexpected considering that these genes are involved in transcription,

translational and regulatory pathways (Cooper and Feil, 2004). Since these genes were found within all strains, perform essential functions within the cell, and vary at most by 10 residues, and usually less than 5, within this set of 123 *T. uzonensis* isolates, the amino acid differences observed here arguably do not play a role in niche adaptation for these strains.

The conservation of gene order, or synteny, can also be used to deduce relationships and gene order trees have been used to resolve the phylogeny of closely related microorganisms (Coenye et al., 2005). The sequencing of genomes from *Thermoanaerobacter* species revealed that significant genome rearrangement was possible in these genomes. Curiously, the genomes from *Thermoanaerobacter* strains from geothermal environments appear to have genome arrangements that are highly conserved compared to *Thermoanaerobacter* strains from subsurface environments (C. L. Hemme, unpublished results).

For all gene sequence sets, the dissimilarity matrices describing the between-isolate Jukes and Cantor genetic distances were compared using the Mantel test. Most of the rank correlation values from these comparisons were low, and all ρ values < 0.6 (Table 5.2). However, results discussed above suggested that the protein coding genes analyzed here had divergent phylogenies. The differences in synonymous and nonsynonymous substitutions were observed beside different π values, which suggested that the protein coding genes are independently evolving; and considering the *T. uzonensis* populations within the Uzon Caldera region, the different sequence variants appear randomly associated, i.e., evidence for linkage disequilibrium was not observed. It therefore follows that the association between gene sequence sets would be weak if the corresponding protein coding genes had divergent phylogenies.

The sequencing and analysis of the 16S rRNA and eight universally conserved protein coding genes from a cluster of *T. uzonensis* strains isolated from geothermal springs of

Kamchatka, Russian Far East, provided insight into the intraspecies heterogeneity of this taxon. Specifically, these analyses of the gene sequences suggested the following five points: 1) that there had been no recent population-wide selection event for the *T. uzonensis* within the examined geothermal springs in Kamchatka, 2) that additional *pyrG* and *recG* sequence variants may be found with the isolation of additional *T. uzonensis* strains from the same geothermal springs, 3) that some protein coding genes appeared to be approaching fixation (*gyrB* and *rplB*), while most of the analyzed protein coding genes were not, 4) that the protein coding genes are independently evolving, and 5) that there is evidence for linkage disequilibrium considering the entire *T. uzonensis* population, but not when considering the largest regional *T. uzonensis* subpopulation.

T. uzonensis was found to inhabit spatially separated geothermal springs. Therefore, a populations within a particular geothermal springs can be considered a subpopulation and the collection of subpopulations can be considered the metapopulation. Since the sequence variants appear randomly associated within the regional group of *T. uzonensis* strains, there is strong evidence for the exchange of genetic information between geothermal springs. There may be particular physiological or metabolic characteristics that influence the ability of (sub)populations to remain connected to others and thus remain part of the metapopulation. One such trait would be endospore formation. Many of the described *Thermoanaerobacter* strains, including *T. uzonensis* strain JW/IW010^{T}, are known to form spores (Wagner et al., 2008). Therefore, the most applicable species concept for *T. uzonensis* is one that takes into account the relationship between populations in the context of the larger collection of populations.

The universally conserved protein coding gene sequence data from *T. uzonensis* isolates discussed herein could also be used to assess spatial patterns of diversity for this taxon.

Furthermore, the correlations between the genetic divergence and either the spatial separation of the geothermal springs and the physicochemical properties of the geothermal springs could also be examined.

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Table 5.1. Summary of *Thermoanaerobacter uzonensis* isolates universally conserved protein coding gene sequence heterogeneity

	gryB	*lepA*	*leuS*	*pyrG*	*recA*	*recG*	*rplB*	*rpoB*
Length (bp)	1111	1255	1040	1201	739	1227	501	911
N	123	123	123	123	123	123	123	122[†]
G+C mol %	35	36	39	38	39	36	37	31
S_{nt}	30	19	36	34	18	46	3	13
ntH	6	11	14	27	9	20	4	9
π (per site) *1000	5.35	1.05	4.93	5.84	1.24	10.34	0.61	4.18
k	5.94	1.32	5.13	7.01	0.91	12.68	0.31	3.8
d_N	0.0017	0.0003	0.0016	0.0023	0.0006	0.0056	0.0007	0.0003
d_S	0.0190	0.0036	0.0170	0.0184	0.0035	0.0262	0.0003	0.0186
d_N/d_S	0.089	0.083	0.097	0.12	0.16	0.22	2.74	0.014
ΔX max	5	5	8	5	2	10	1	2
ΔX mean	1.30	0.26	1.30	2.06	0.30	4.74	0.27	0.16
S_{aa}	7	8	13	13	2	20	1	3
aaH	5	7	10	14	3	19	2	4

[†]The isolate M504_35_2 had been removed from this analysis; the *rpoB* gene sequence from this isolate was similar to the corresponding gene from *T. siderophilus* strains isolated from Kamchatkan geothermal springs; see text for additional information.

Table 5.2. Comparisons of the divergence between the *Thermoanaerobacter uzonensis* universally conserved protein coding gene sequence sets. Below diagonal, Mantel tests of the divergence between universally conserved protein coding gene sequence sets comparisons between isolates with the sets. Rank correlation, Spearman's ρ, values are shown with the significance level of sample statistic, as a percentage, given in parentheses. Above diagonal, Δ G+C mol%.

	16S	***gyrB***	***lepA***	***leuS***	***pyrG***	***recA***	***recG***	***rplB***	***rpoB***
16S									
gyrB	0.022 (27.5)		0.01	0.05	0.04	0.05	0.01	0.03	0.03
lepA	0.031 (21.0)	0.389 (0.1)		0.03	0.02	0.03	0.02	0.01	0.05
leuS	0.071 (3.8)	0.579 (0.1)	0.271 (0.1)		0.01	0	0.06	0.02	0.08
pyrG	0.034 (18.4)	0.148 (0.4)	0.001 (46.3)	0.295 (0.1)		0.01	0.05	0.01	0.07
recA	0.041 (18.7)	0.538 (0.1)	0.29 (0.1)	0.577 (0.1)	0.396 (0.1)		0.06	0.02	0.08
recG	0.144 (0.1)	0.191 (0.1)	0.415 (0.1)	0.145 (0.1)	0.041 (11.0)	0.146 (0.2)		0.04	0.02
rplB	0.214 (0.1)	-0.141 (99.7)	0.038 (27.5)	-0.099 (97.2)	0.003 (45.7)	-0.098 (94.3)	0.138 (0.2)		0.06
rpoB	0.26 (0.1)	0.298 (0.1)	0.142 (0.1)	0.204 (0.1)	0.053 (6.5)	0.229 (0.1)	0.134 (0.1)	0.136 (0.3)	

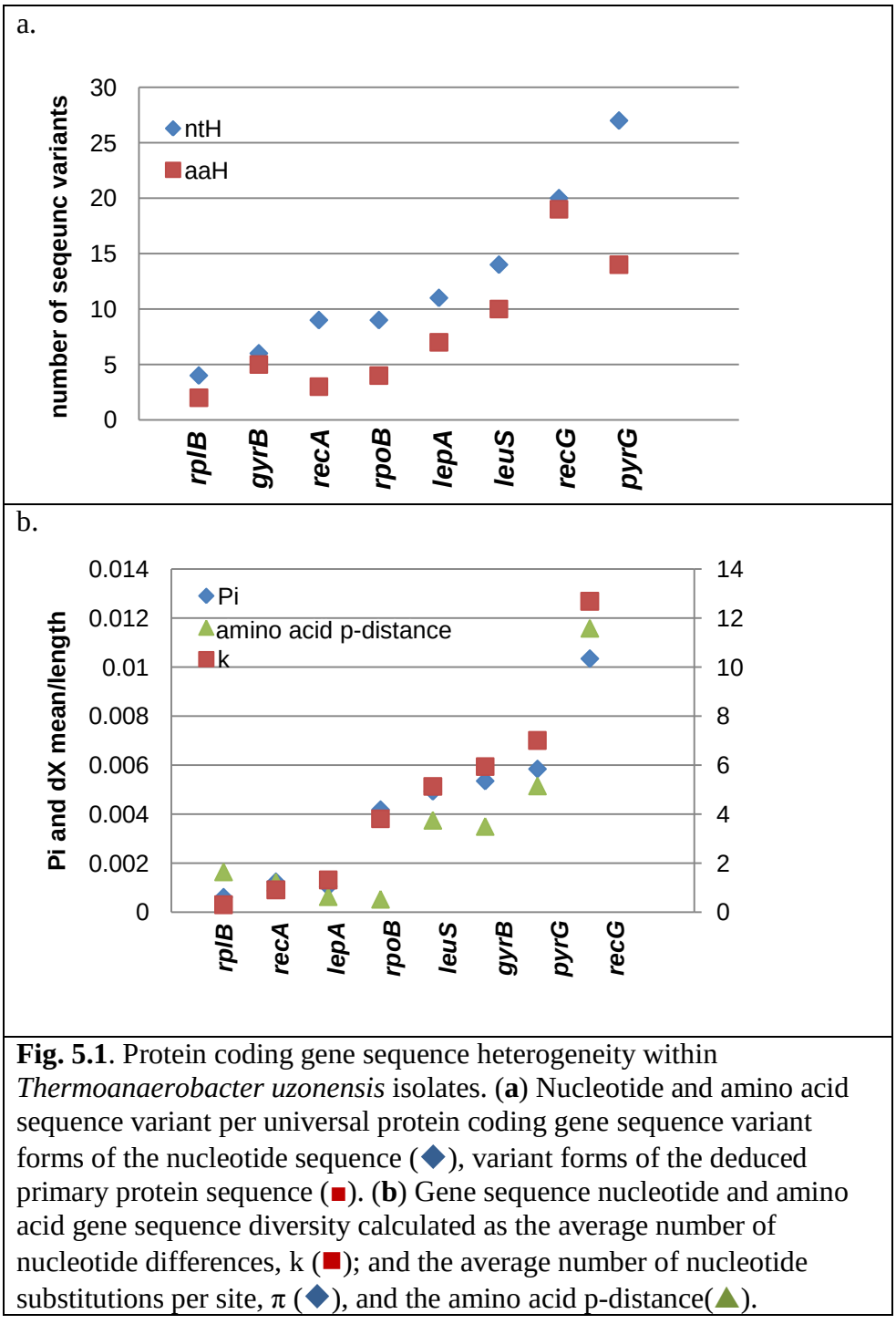

Fig. 5.1. Protein coding gene sequence heterogeneity within *Thermoanaerobacter uzonensis* isolates. (**a**) Nucleotide and amino acid sequence variant per universal protein coding gene sequence variant forms of the nucleotide sequence (◆), variant forms of the deduced primary protein sequence (■). (**b**) Gene sequence nucleotide and amino acid gene sequence diversity calculated as the average number of nucleotide differences, k (■); and the average number of nucleotide substitutions per site, π (◆), and the amino acid p-distance(▲).

SUPPLEMENTAL INFORMATION

***rplB*:** The gene sequence set with the least variability among the universally conserved protein coding genes in this study was the *rplB* gene sequence set. Specifically, the *rplB* gene sequence set had the lowest S_{nt}, ntH, and π values (Table 5.1). The *Tu rplB* ntH_2 nucleotide sequence variant was the most common, found within 82.1% of the *T. uzonensis* isolates (Table 5.S2). The *Tu rplB* aaH_1 primary protein sequence variant form was found within 82.9% of isolates the (Table 5.S2). The *rplB* gene sequence set from *T. uzonensis* isolates had lowest max ΔX value with only one amino acid difference between the gene sequences. This residue difference, A129T, changes the residue from a hydrophobic amino acid residue to a polar amino acid with an uncharged side group (Table 5.S3). The high d_N/d_S ratio was largely due to the *rplB* gene sequence set having low nucleotide and low amino acid diversity measures (Table 5.1).

***recA*:** The *recA* gene sequence set from the *T. uzonensis* isolates was the second-least divergent. The gene sequence set had 18 segregating sites between nine ntH with $\pi = 0.00124$ (Table 5.1). The overall d_N/d_S ratio for the *recA* gene sequences was 0.15. The *T. uzonensis* isolate *recA* gene sequence set was also one of the least divergent considering the amino acid diversity. The *recA* gene sequence set has only three primary protein sequence variant forms and max ΔX was two residues (Table 5.1). 68.3% of the *T. uzonensis* isolates had the *Tu recA* ntH_2 nucleotide sequence variant; 82.9% of isolates had the *Tu recA* aaH_2 variant of the protein sequence (Table 5.S4). Both amino acid residue differences were changes to other hydrophobic amino acid residues, specifically A107V and I226V. However, SIFT analyses indicated that the A107V substitution may affect protein function (Table 5.S5).

***lepA*:** The *lepA* gene sequence set from the *T. uzonensis* isolates had an overall d_N/d_S value of 0.08 (Table 5.1). The most common *lepA* nucleotide sequence variant observed was *Tu*

lepA ntH_3, which was found within 58.5% of the *T. uzonensis* isolates. The most common primary protein sequence variant was *Tu lepA* aaH_3, which was found in 88.6% of the *T. uzonensis* isolates (Table 5.S6). Some of the *lepA* amino acid residue differences changed the broad amino acid residue type, e.g., V147E, A509V, and L520S, and these substitutions may have altered the corresponding protein function (Table 5.S7).

***rpoB*:** The d_N/d_S ratio for the *rpoB* gene sequence set was 0.014, which, as mentioned previously, was the lowest value observed within the universally conserved protein coding gene sequence sets (Table 5.1). The amino acid sequence variant *Tu rpoB* aaH_3 was the most common, found within 91% of the *T. uzonensis* isolates, however, the most common nucleotide sequence variant, *Tu rpoB* ntH_3, was only found within 54.5% of the *T. uzonensis* isolates. (Table 5.S8). The three residue changes observed within the *T. uzonensis rpoB* genes were L144Y, K316E, and N335S (Table 5.S9). SIFT analyses predicted that the residue change at position 144 from Y to L in the *rpoB* gene sequence affected protein function. These *rpoB* gene sequence heterogeneity measures were for a set of 122 *T. uoznensis* isolates. The isolate JW/IW M504_35_2 was found to have a *rpoB* gene fragment identical to sequences found in *T. siderophilus* isolates (Table 5.S8); thus, an example of horizontal gene transfer.

***leuS*:** The *leuS* gene sequence set from *T. uzonensis* isolates had a relatively high number of segregating sites, 36. The *leuS* gene sequence set d_N/d_S value for the *T. uzonensis* isolates was 0.097 and the ΔX max. value was eight (Table 5.1). Of the 14 *leuS* nucleotide sequence variants, seven contained less than two isolates (Table 5.S10). 30.9% of isolates had the *Tu leuS* ntH_4 nucleotide sequence type and 41.5% of isolates have the *Tu leuS* aaH_2 primary protein sequence variant form (Table 5.S10). In analyzing the differences between the *leuS* deduced primary protein sequence variants, changes from asparagine to aspartic acid were observed at

positions 352 and 379 (Table 5.S11). Intriguingly, the change at position 379 was predicted to affect protein function whereas the change at position 352 was not (Table 5.S11).

***gyrB*:** Within the set of *gyrB* sequences from *T. uzonensis* isolates, the high ratio of segregating sites (S_{nt}) to nucleotide sequence variants (ntH), 30:6 was notable (Table 5.1). *T. uzonensis* isolates had six *gyrB* nucleotide sequence variants with five different primary protein sequence variants. The *Tu gyrB* ntH_1, aaH_1 sequence variant was the most common and 71.5% of the isolates had this version of the sequence (Table 5.S12). An asparagine reside at position 260 was only observed within the *Tu gyrB* ntH_5, aaH_4 sequence variant and the SIFT analysis indicated that the D260N amino acid residue differences may affect protein function (Table 5.S13).

***pyrG*:** The *pyrG* gene sequences from *T. uzonensis* isolates had the highest number of nucleotide sequence variant forms, 27, although the genes sequence set had fewer segregating sites (S_{nt}) than either the *leuS* or *recG* gene sequence sets (Table 5.1). The *pyrG* gene set had the second highest number of primary protein sequence variants, 14 (Table 5.1). Of the 27 *pyrG* nucleotide sequence variants, 14 contain ≤ 2 isolates (Table 5.S14). The *pyrG* nucleotide sequence variant form found within the most isolates was the *Tu pyrG* ntH_11 sequence variant, found in 9.8%; the largest *pyrG* amino acid sequence variant bin, *Tu pyrG* aaH_6, contained 23.6% of the isolates (Table 5.S15). As observed previously with SIFT analyses of the *leuS* primary protein sequence variants, the position of the amino acid substitution can be significant. The substitutions K183N and K399N were observed within *pyrG* deduced primary protein sequence variants. However, SIFT analysis suggested that the change at position 183 may affect protein function while the change at position 399 would be tolerated (Table 5.S15).

***recG*:** The *recG* gene sequence set from *T. uzonensis* isolates had the highest number of polymorphic nucleotide sites, S_{nt}, 46; the highest π value, 0.0103; and the second highest-number of nucleotide sequence variant forms, 20 (Table 5.1). The *recG* gene sequence set from *T. uzonensis* isolates had the second-highest maximum number of amino acid differences, 10, as well as the highest number of primary protein sequence variants, 19 (Fig. 5.1, Table 5.1). For the *recG* gene sequence sets, the ΔX mean value was conspicuously higher than for other universally conserved protein coding gene sets. The *recG* ΔX mean, 4.4, whereas *pyrG* ΔX mean 2.4 (Table 5.1). Of the observed *T. uzonensis recG* amino acid sequence variant bins, 6 of the 19 contain only a single isolate; 12 of 19 contain ≤ 5 isolates. The largest *recG* gene sequence variant bin, *Tu recG* ntH_10, aaH_10, contains 16.3% of the isolates (Table 5.S16). Considering the d_N/d_S ratio, the *recG* gene sequence had the second-highest value, behind the *rplB* gene sequence set discussed previously (Table 5.1). Considering the differences in the *recG* amino acid primary protein sequences, of the 20 S_{aa}, approximately half of the residue differences do not change the residue class. Five of the residue changes were predicted, thorough SIFT analyses, to affect protein function (Table 5.S17).

Table 5.S1. Oligonucleotide primers for the amplification of universally conserved protein coding genes from *Thermoanaerobacter* taxa

Gene	Primer name	Oligonucleotide sequence (5' to 3')	Average Gene Length analyzed (bp)	Inferred fraction of gene sequence analyzed
pyrG	TBIOG_pyrGF	AAGYCGCGGCMTATCAGTTGCWRT		
	TBIOG_pyrGR	TGGRTGRAAYTGGGAYGCYACAAA	1267	0.79
leuS	TBIOGEO_leuSF	GYTGYCAAACTGTTCTTGCAAACGARC		
	TBIOGEO_leuSR	TCATTCTGCTKCCATCAGGKCCCA	1132	0.46
gyrB	TBIOGEO_gyrBF	AGCSGTAAGAAARAGGCCAGGAAT		
	TBIOGEO_gyrBR	TYCCTCGKAGTGGAAGTATCGCTT	1170	0.62
recA	TBIOGEO_recAF	AGYCARATAGAGAGRCAGTTTGGC		
	TBIOGEO_recAR	CTCCATAGGAATACCAAGCACCAC	757	0.74
rplB	TBIOGEO_rplBR	GTGTCTTATARCCYAATGCAGGCT		
	TBIOGEO_rplBF	ATCTCCCGGCAGACGTCAAAT	655	0.79
rpoB	TBIOGEO_rpoBR	TCTCTAATGGCTGCWACAACYGGR		
	TBIOGEO_rpoBF	TACGTCCTGTACAAGTGGGCAACA	1294	0.86
recG	TBIOGEO_recGR	AAATTCTGACCTGCCAACTCTRCC		
	TBIOGEO_recGF	ACAGGYGYAGTAGARTTAGTSTGG	1310	0.64
lepA	TBIOGEO_lepAR	YTTCCCACCTGTCTCATGCGCTTT		
	TBIOGEO_lepAF	TTGAGGCGCAAACCCTTGCTAATG	1311	0.72

Table 5.S2. Distribution of *rplB* gene sequence variants among *Thermoanaerobacter uzonensis* isolates		
***rplB* gene sequence variant**	**No. of Isolates**	**Isolates**
Tu rplB ntH_1, aaH_1	1	B621_2rplB
Tu rplB ntH_2, aaH_1	101	A615_35rplB, A615_37rplB, A615_65rplB, B621_10rplB, B621_1rplB, B621_3rplB, B621_4rplB, B621_6rplB, B621_71rplB, B621_72rplB, B621_74rplB, B621_77rplB, B621_78rplB, B621_80rplB, B621_83rplB, B621_87rplB, B621_89_1rplB, B621_90rplB, B621_93rplB, B621_94rplB, H608_10rplB, H608_1rplB, H608_2rplB, H608_3rplB, H608_41rplB, H608_42-rplB_F_{H608 H608_4rplB, H608_6rplB, H608_71rplB, H608_72rplB, H608_77rplB, H608_78rplB, H608_79rplB, H608_81rplB, H608_84rplB, H608_85rplB, H608_88rplB, H608_91rplB, H608_93rplB, J614_60rplB, J614_61rplB, J614_62_1rplB, J614_63rplB, J614_65rplB, J614_7rplB, J614_9rplB, M504_30rplB, M504_31rplB, M504_33rplB, M504_35_2rplB, M504_36rplB, M504_37rplB, M504_38rplB, M504_39rplB, M504_5rplB, M504_7rplB, O629_42rplB, O629_47rplB, O629_48rplB, O629_51rplB, R649_40_2rplB, S648_4_2rplB, S648_62rplB, S648_7rplB, S648_9rplB, T515_10rplB, T515_1rplB, T515_3rplB, T515_40rplB, T515_41rplB, T515_42rplB, T515_44rplB, V634_10_2rplB, V634_1rplB, V634_2rplB, V634_3rplB, V634_5rplB, V634_6rplB, V634_71rplB, V634_73-rplB_F_{V634 V634_74rplB, V634_77rplB, V634_7rplB, V634_81rplB, V634_82rplB, V634_83rplB, V634_84rplB, V634_85rplB, V634_8rplB, V634_9rplB, Z606_36rplB, Z606_38rplB, Z606_3rplB, Z606_70rplB, Z606_71rplB_N, Z606_72rplB, Z606_73rplB, Z606_76rplB, Z606_77rplB, Z606_81rplB, Z606_9rplB
Tu rplB ntH_3, aaH_2	20	A615_31rplB, A615_32rplB, A615_39rplB, A615_62rplB, I502_40rplB, I502_41rplB, I502_42rplB, I502_43rplB, I502_45rplB, I502_46rplB, I502_48rplB, I502_49rplB, O629_40rplB, O629_43rplB, O629_44rplB, O629_45rplB, O629_49rplB, O629_50rplB, O629_6rplB, O629_7rplB
Tu rplB ntH_4, aaH_1	1	R649_9rplB

Table 5.S3. Summary of variable residues between the *Thermoanaerobacter uzonensis* deduced *recA* primary protein sequence variants. Position of the variable residue is listed vertically above the multiple sequence alignment. SIFT analyses were based on comparisons with 398 homologous primary protein sequences. The most common *rplB* sequence variant is shaded in grey.

	1 2 9
Tu rplB ntH_1, aaH_1	A
***Tu rplB* ntH_2, aaH_1**	**A**
Tu rplB ntH_3, aaH_2	T
Tu rplB ntH_4, aaH_1	A

SIFT analysis results:
Substitution at position 129 from A to T was predicted to be tolerated

Table 5.S4. Distribution of *recA* gene sequence variants among *Thermoanaerobacter uzonensis* isolates		
***recA* gene sequence variants**	**No. of Isolates**	**Isolates**
Tu recA ntH_1, aaH_1	3	B621_10recA, B621_6recA, B621_78recA
Tu recA ntH_2, aaH_2	84	A615_31recA, A615_32recA, A615_35recA, A615_37recA, A615_39recA, A615_62recA, A615_65recA, B621_1recA, B621_3recA, B621_4recA, B621_71recA, B621_72recA, B621_74recA, B621_77recA, B621_80recA, B621_83recA, B621_89_1recA, B621_90recA, B621_94recA, H608_3recA, H608_42recA, H608_4recA, H608_77recA, H608_78recA, H608_79recA, H608_91recA, H608_93recA, I502_40recA, I502_41recA, I502_42recA, I502_43recA, I502_45recA, I502_46recA, I502_48recA, I502_49recA, J614_60recA, J614_61recA, J614_62_1recA, J614_63recA, J614_65recA, J614_7recA, J614_9recA, O629_40recA, O629_42recA, O629_43recA, O629_44recA, O629_45recA, O629_47recA, O629_48recA, O629_49recA, O629_50recA, O629_51recA, O629_6recA, O629_7recA, T515_10recA, T515_3recA, T515_41recA, T515_42recA, T515_44recA, V634_10_2recA, V634_1recA, V634_2recA, V634_3recA, V634_5recA, V634_6recA, V634_71recA, V634_73recA, V634_74recA, V634_77recA, V634_7recA, V634_81recA, V634_82recA, V634_83recA, V634_84recA, V634_85recA, V634_8recA, V634_9recA, Z606_38recA, Z606_3recA, Z606_70recA, Z606_71recA, Z606_77recA, Z606_81recA, Z606_9recA
Tu recA ntH_3, aaH_2	1	H608_81recA
Tu recA ntH_4, aaH_2	1	T515_1recA
Tu recA ntH_5, aaH_2	3	Z606_36recA, Z606_72recA, Z606_73recA
Tu recA ntH_6, aaH_2	13	B621_87recA, B621_93recA, H608_10recA, H608_1recA, H608_2recA, H608_41recA, H608_6recA, H608_71recA, H608_72recA, H608_84recA, H608_85recA, H608_88recA, T515_40recA
Tu recA ntH_7, aaH_3	7	B621_2recA, R649_40_2recA, S648_4_2recA, S648_62recA, S648_7recA, S648_9recA, Z606_76recA
Tu recA ntH_8, aaH_3	10	M504_30recA, M504_31recA, M504_33recA, M504_35_2recA, M504_36recA, M504_37recA, M504_38recA, M504_39recA, M504_5recA, M504_7recA
Tu recA ntH_9, aaH_3	1	R649_9recA

Table 5.S5. Summary of variable residues between the *Thermoanaerobacter uzonensis* deduced *recA* primary protein sequence variants. Positions of variable residues are listed vertically above the multiple sequence alignment. SIFT analyses were based on comparisons with 398 homologous primary protein sequences. The most common *recA* sequence variant is shaded in grey.

	12
	02
	76
Tu recA ntH_1, aaH_1	AI
***Tu recA* ntH_2, aaH_2**	**VI**
Tu recA ntH_3, aaH_2	VI
Tu recA ntH_4, aaH_2	VI
Tu recA ntH_5, aaH_2	VI
Tu recA ntH_6, aaH_2	VI
Tu recA ntH_7, aaH_3	VV
Tu recA ntH_8, aaH_3	VV
Tu recA ntH_9, aaH_3	VV

SIFT analysis results:

Substitution at position 107 from V to A was predicted to affect protein function[a]

Substitution at position 226 from I to V was predicted to be tolerated

[a] This substitution may have been predicted to affect function just because the sequences used were not diverse enough; there was low confidence in this prediction.

Table 5.S6. Distribution of *lepA* gene sequence variants among *Thermoanaerobacter uzonensis* isolates		
***lepA* gene sequence variants**	**No. of Isolates**	**Isolates**
Tu lepA ntH_1, aaH_1	1	T515_41lepA
Tu lepA ntH_10, aaH_5	10	M504_30lepA, M504_31lepA, M504_33lepA, M504_35_2lepA, M504_36llepA, M504_37lepA, M504_38lepA, M504_39lepA, M504_5lepA, M504_7lepA
Tu lepA ntH_11, aaH_6	1	V634_83lepA
Tu lepA ntH_2, aaH_2	1	T515_44lepA
Tu lepA ntH_3, aaH_3	72	A615_31lepA, A615_32lepA, A615_35lepA, A615_37lepA, A615_39lepA, A615_62lepA, A615_65lepA, B621_10lepA, B621_1lepA, B621_2lepA, B621_3lepA, B621_4lepA, B621_6lepA, B621_71lepA, B621_74lepA, B621_78lepA, B621_80lepA, B621_87lepA, B621_89_1lepA, B621_90lepA, B621_93lepA, H608_10lepA, H608_1lepA, H608_2lepA, H608_3lepA, H608_41lepA, H608_42lepA, H608_4lepA, H608_6lepA, H608_71lepA, H608_72lepA, H608_77lepA, H608_79lepA, H608_81lepA, H608_84lepA, H608_85lepA, H608_88lepA, H608_91lepA, I502_40lepA, I502_41lepA, I502_42lepA, I502_43lepA, I502_45lepA, I502_46lepA, I502_48lepA, I502_49lepA, J614_60lepA, J614_61lepA, J614_63lepA, J614_65lepA, O629_42lepA, O629_47lepA, O629_48lepA, O629_51lepA, R649_40_2lepA, S648_62lepA, S648_7lepA, T515_10lepA, T515_1lepA, T515_3lepA, T515_40lepA, T515_42lepA, V634_1lepA, V634_2lepA, V634_6lepA, V634_7lepA, V634_85lepA, V634_9lepA, Z606_36lepA, Z606_72lepA, Z606_73lepA, Z606_76lepA
Tu lepA ntH_4, aaH_3	4	B621_72lepA, J614_62_1lepA, J614_7lepA, J614_9lepA
Tu lepA ntH_5, aaH_3	1	B621_77lepA
Tu lepA ntH_6, aaH_3	1	H608_78lepA
Tu lepA ntH_7, aaH_3	18	B621_83lepA, B621_94lepA, H608_93lepA, O629_40lepA, O629_43lepA, O629_44lepA, O629_45lepA, O629_49lepA, O629_50lepA, O629_6lepA, O629_7lepA, V634_10_2lepA, Z606_38lepA, Z606_3lepA, Z606_70lepA, Z606_71lepA, Z606_77lepA, Z606_9lepA
Tu lepA ntH_8, aaH_3	13	S648_4_2lepA, S648_9lepA, V634_3lepA, V634_5lepA, V634_71lepA, V634_73lepA, V634_74lepA, V634_77lepA, V634_81lepA, V634_82lepA, V634_84lepA, V634_8lepA, Z606_81lepA
Tu lepA ntH_9, aaH_4	1	R649_9lepA

Table 5.S7. Summary of variable residues between the *Thermoanaerobacter uzonensis* deduced *lepA* primary protein sequence variants. Positions of variable residues are listed vertically above the multiple sequence alignment. SIFT analyses were based on comparisons with ≥234 homologous primary protein sequences. The most common *lepA* sequence variant is shaded in grey.

	1113455
	4898902
	7805490
Tu *lepA* ntH_1, aaH_1	VQDVLAS
Tu *lepA* ntH_2, aaH_2	VQDVGAL
Tu *lepA* ntH_3, aaH_3	**VKDVGAL**
Tu *lepA* ntH_4, aaH_3	VKDVGAL
Tu *lepA* ntH_5, aaH_3	VKDVGAL
Tu *lepA* ntH_6, aaH_3	VKDVGAL
Tu *lepA* ntH_7, aaH_3	VKDVGAL
Tu *lepA* ntH_8, aaH_3	VKDVGAL
Tu *lepA* ntH_9, aaH_4	VKNVGVL
Tu *lepA* ntH_10, aaH_5	VKDIGAL
Tu *lepA* ntH_11, aaH_6	EKDVGAL

SIFT analysis results:

Substitution at position 147 from V to E was predicted to affect protein function

Substitution at position 188 from K to Q was predicted to be tolerated

Substitution at position 190 from D to N was predicted to be tolerated

Substitution at position 385 from V to I was predicted to be tolerated

Substitution at position 494 from G to L was predicted to affect protein function

Substitution at position 509 from A to V was predicted to affect protein function

Substitution at position 520 from L to S was predicted to affect protein function

Table 5.S8. Distribution of *rpoB* gene sequence variants among *Thermoanaerobacter uzonensis* isolates. The rows shaded grey are the isolates with *T. siderophilus rpoB* gene sequences..

***rpoB* gene sequence variants**	**No. of Isolates**	**Isolates**
Tu rpoB ntH_1, aaH_1	1	O629_40-rpoB
Tu rpoB ntH_2, aaH_2	4	B621_72rpoB, J614_62_1rpoB, J614_7rpoB, J614_9rpoB
Tu rpoB ntH_3, aaH_3	67	B621_10rpoB, B621_3rpoB, B621_4rpoB, B621_6rpoB, B621_71rpoB, B621_78rpoB, B621_80rpoB, B621_83rpoB, B621_87rpoB, B621_89_1rpoB, B621_90rpoB, B621_93rpoB, B621_94rpoB, H608_10rpoB, H608_1rpoB, H608_2rpoB, H608_3rpoB, H608_41rpoB, H608_42rpoB, H608_4rpoB, H608_6rpoB, H608_71rpoB, H608_72rpoB, H608_77rpoB, H608_78rpoB, H608_84rpoB, H608_85rpoB, H608_88rpoB, H608_91rpoB, H608_93rpoB, O629_43rpoB, O629_44rpoB, O629_45rpoB, O629_49rpoB, O629_50rpoB, O629_6rpoB, O629_7rpoB, T515_10rpoB, T515_1rpoB, T515_3rpoB, T515_40rpoB, T515_41rpoB, T515_42rpoB, T515_44rpoB, V634_1rpoB, V634_2rpoB, V634_3rpoB, V634_5rpoB, V634_6rpoB, V634_71rpoB, V634_73rpoB, V634_74rpoB, V634_77rpoB, V634_7rpoB, V634_81rpoB, V634_82rpoB, V634_83rpoB, V634_84rpoB, V634_85rpoB, V634_8rpoB, V634_9rpoB, Z606_38rpoB, Z606_3rpoB, Z606_70rpoB, Z606_71rpoB, Z606_77rpoB, Z606_9rpoB
Tu rpoB ntH_4, aaH_3	17	A615_37rpoB, A615_65rpoB, B621_2rpoB, B621_74rpoB, H608_79rpoB, H608_81rpoB, J614_60rpoB, J614_61rpoB, J614_63rpoB, J614_65rpoB, O629_51rpoB, S648_4_2rpoB, S648_9rpoB, V634_10_2rpoB, Z606_36rpoB, Z606_76rpoB, Z606_81rpoB
Tu rpoB ntH_5, aaH_3	25	A615_31rpoB, A615_32rpoB, A615_35rpoB, A615_39rpoB, A615_62rpoB, I502_40rpoB, I502_41rpoB, I502_42rpoB, I502_43rpoB, I502_45rpoB, I502_46rpoB, I502_48rpoB, I502_49rpoB, M504_30rpoB, M504_31rpoB, M504_33rpoB, M504_36rpoB, M504_37rpoB, M504_38rpoB, M504_39rpoB, M504_5rpoB, M504_7rpoB, R649_40_2rpoB, S648_62rpoB, S648_7rpoB
Tu rpoB ntH_6, aaH_4	1	B621_1rpoB
Tu rpoB ntH_7, aaH_4	3	B621_77rpoB, Z606_72rpoB, Z606_73rpoB
Tu rpoB ntH_8, aaH_4	1	R649_9rpoB
Tu rpoB ntH_9, aaH_3	3	O629_42rpoB, O629_47rpoB, O629_48-rpoB_
Ts rpoB ntH_1	4	M504_32rpoB, P635_9rpoB, R649_62_1rpoB, R649_65rpoB
Ts rpoB ntH_2	3	M504_35_2rpoB, R649_60rpoB R649_61rpoB

Table 5.S9. Summary of variable residues between the *Thermoanaerobacter uzonensis* deduced *rpoB* primary protein sequence variants. Positions of variable residues are listed vertically above the multiple sequence alignment. SIFT analyses were based on comparisons with ≥98 homologous primary protein sequences. The most common *rpoB* sequence variant is shaded in grey.

	133 **415** **465**
Tu *rpoB* ntH_5, aaH_3	YEN
Tu *rpoB* ntH_4, aaH_3	YEN
Tu *rpoB* ntH_3, aaH_3	**YEN**
Tu *rpoB* ntH_2, aaH_2	YKN
Tu *rpoB* ntH_7, aaH_4	YES
Tu *rpoB* ntH_1, aaH_1	LEN
Tu *rpoB* ntH_9, aaH_3	YEN
Tu *rpoB* ntH_8, aaH_4	YES

SIFT analysis results:

Substitution at position 144 from Y to L was predicted to affect protein function

Substitution at position 316 from E to K was predicted to be tolerated

Substitution at position 355 from N to S was predicted to be tolerated

Table 5.S10.. Distribution of *leuS* gene sequence variants among *Thermoanaerobacter uzonensis* isolates		
***leuS* gene sequence variant**	**No. of Isolates**	**Isolates**
Tu leuS ntH_1, aaH_1	1	B621_87leuS
Tu leuS ntH_2, aaH_2	12	B621_93leuS, H608_10leuS, H608_1leuS, H608_2leuS, H608_41leuS, H608_6leuS, H608_71leuS, H608_72leuS, H608_84leuS, H608_85leuS, H608_88leuS, T515_40leuS
Tu leuS ntH_3, aaH_2	1	A615_39leuS
Tu leuS ntH_4, aaH_2	38	A615_31leuS, A615_32leuS, A615_37leuS, A615_62leuS, B621_3leuS, B621_80leuS, B621_83leuS, B621_94leuS, H608_81leuS, I502_40leuS, I502_41leuS, I502_42leuS, I502_43leuS, I502_45leuS, I502_46leuS, I502_48leuS, I502_49leuS, O629_42leuS, O629_47leuS, O629_48leuS, T515_1leuS, T515_3leuS, V634_3leuS, V634_5leuS, V634_71leuS, V634_73leuS, V634_74leuS, V634_77leuS, V634_81leuS, V634_82leuS, V634_84leuS, V634_8leuS, Z606_38leuS, Z606_3leuS, Z606_70leuS, Z606_71leuS, Z606_77leuS, Z606_9leuS
Tu leuS ntH_5, aaH_3	33	B621_10leuS, B621_1leuS, B621_4leuS, B621_6leuS, B621_72leuS, B621_74leuS, B621_77leuS, B621_78leuS, H608_93leuS, J614_62_1leuS, J614_7leuS, J614_9leuS, O629_40leuS, O629_43leuS, O629_44leuS, O629_45leuS, O629_49leuS, O629_50leuS, O629_51leuS, O629_6leuS, O629_7leuS, T515_41leuS, T515_44leuS, V634_1leuS, V634_2leuS, V634_6leuS, V634_7leuS, V634_83leuS, V634_85leuS, V634_9leuS, Z606_36leuS, Z606_72leuS, Z606_73leuS
Tu leuS ntH_6, aaH_4	15	H608_3leuS, H608_42leuS, H608_4leuS, H608_77leuS, H608_78leuS, H608_91leuS, J614_60leuS, J614_61leuS, J614_63leuS, J614_65leuS, T515_10leuS, T515_42leuS, V634_10_2leuS, Z606_76leuS, Z606_81leuS
Tu leuS ntH_7, aaH_5	3	R649_40_2leuS, S648_62leuS, S648_7leuS
Tu leuS ntH_8, aaH_6	2	S648_4_2leuS, S648_9leuS
Tu leuS ntH_9, aaH_7	1	R649_9leuS
Tu leuS ntH_10, aaH_8	10	M504_30leuS, M504_31leuS, M504_33leuS, M504_35_2leuS, M504_36leuS, M504_37leuS, M504_38leuS, M504_39leuS, M504_5leuS, M504_7leuS
Tu leuS ntH_11, aaH_9	1	A615_35leuS
Tu leuS ntH_12, aaH_10	4	A615_65leuS, B621_71leuS, B621_89_1leuS, B621_90leuS
Tu leuS ntH_13, aaH_10	1	B621_2leuS
Tu leuS ntH_14, aaH_10	1	H608_79leuS

Table 5.S11. Summary of variable residues between the *Thermoanaerobacter uzonensis* deduced *leuS* primary protein sequence variants. Positions of variable residues are listed vertically above the multiple sequence alignment. SIFT analyses were based on comparisons with ≥62 related primary protein sequences. The most common *leuS* sequence variant is shaded in grey.

	1112223333345
	9990032567812
	5894536289933
Tu *leuS* ntH_1, aaH_1	AGDKEVLDSNELV
Tu *leuS* ntH_2, aaH_2	AEELDVLDSNELV
Tu *leuS* ntH_3, aaH_2	AEELDVLDSNELV
Tu *leuS* ntH_4, aaH_2	**AEELDVLDSNELV**
Tu *leuS* ntH_5, aaH_3	AEELDVLNSNELV
Tu *leuS* ntH_6, aaH_4	AEELDVLNSNEFV
Tu *leuS* ntH_7, aaH_5	AEELDVLNSDELA
Tu *leuS* ntH_8, aaH_6	AEELDVLNSDELV
Tu *leuS* ntH_9, aaH_7	AEELDVINSNELV
Tu *leuS* ntH_10, aaH_8	DEELDVLNSNELV
Tu *leuS* ntH_11, aaH_9	AEELDILNNNDLV
Tu *leuS* ntH_12, aaH_10	AEELDVLNNNDLV
Tu *leuS* ntH_13, aaH_10	AEELDVLNNNDLV
Tu *leuS* ntH_14, aaH_10	AEELDVLNNNDLV

SIFT analysis results:

Substitution at position 195 from A to D was predicted to be tolerated

Substitution at position 198 from E to G was predicted to affect protein

Substitution at position 199 from E to D was predicted to be tolerated

Substitution at position 204 from L to K was predicted to affect protein function

Substitution at position 205 from D to E was predicted to be tolerated

Substitution at position 233 from V to I was predicted to be tolerated

Substitution at position 326 from L to I was predicted to be tolerated

Substitution at position 352 from D to N was predicted to be tolerated

Substitution at position 368 from S to N was predicted to be tolerated

Substitution at position 379 from N to D was predicted to affect protein function

Substitution at position 389 from E to D was predicted to be tolerated

Substitution at position 413 from L to F was predicted to affect protein function

Substitution at position 523 from V to A was predicted to affect protein function

Table 5.S12. Distribution of *gyrB* gene sequence variants among *Thermoanaerobacter uzonensis* isolates		
***gyrB* gene sequence variant**	**No. of Isolates**	**Isolates**
Tu gyrB ntH_1, aaH_1	88	A615_31gyrB, A615_32gyrB, A615_35gyrB, A615_37gyrB, A615_39gyrB, A615_62gyrB, A615_65gyrB, B621_10gyrB, B621_1gyrB, B621_2gyrB, B621_3gyrB, B621_4gyrB, B621_6gyrB, B621_74gyrB, B621_77gyrB, B621_78gyrB, B621_83gyrB, B621_93gyrB, B621_94gyrB, H608_10gyrB, H608_1gyrB, H608_2gyrB, H608_3gyrB, H608_41gyrB, H608_42gyrB, H608_4gyrB, H608_6gyrB, H608_71gyrB, H608_72gyrB, H608_77gyrB, H608_78gyrB, H608_79gyrB, H608_81gyrB, H608_84gyrB, H608_85gyrB, H608_88gyrB, H608_91gyrB, H608_93gyrB, I502_40gyrB, I502_41gyrB, I502_42gyrB, I502_43gyrB, I502_45gyrB, I502_46gyrB, I502_48gyrB, I502_49gyrB, O629_40gyrB, O629_42gyrB, O629_43gyrB, O629_44gyrB, O629_45gyrB, O629_47gyrB, O629_48gyrB, O629_49gyrB, O629_50gyrB, O629_6gyrB, O629_7gyrB, T515_10gyrB, T515_40gyrB, T515_41gyrB, T515_42gyrB, T515_44gyrB, V634_1gyrB, V634_2gyrB, V634_3gyrB, V634_5gyrB, V634_6gyrB, V634_71gyrB, V634_73gyrB, V634_74gyrB, V634_77gyrB, V634_7gyrB, V634_81gyrB, V634_82gyrB, V634_83gyrB, V634_84gyrB, V634_85gyrB, V634_8gyrB, V634_9gyrB, Z606_36gyrB, Z606_38gyrB, Z606_3gyrB, Z606_70gyrB, Z606_71gyrB, Z606_76gyrB, Z606_77gyrB, Z606_81gyrB, Z606_9gyrB
Tu gyrB ntH_2, aaH_2	4	B621_71gyrB, B621_87gyrB, B621_89_1gyrB, B621_90gyrB
Tu gyrB ntH_3, aaH_3	8	B621_72gyrB, B621_80gyrB, J614_62_1gyrB, J614_7gyrB, J614_9gyrB, O629_51gyrB, Z606_72gyrB, Z606_73gyrB
Tu gyrB ntH_4, aaH_3	1	R649_9gyrB
Tu gyrB ntH_5, aaH_4	12	J614_60gyrB, J614_61gyrB, J614_63gyrB, J614_65gyrB, R649_40_2gyrB, S648_4_2gyrB, S648_62gyrB, S648_7gyrB, S648_9gyrB, T515_1gyrB, T515_3gyrB, V634_10_2gyrB
Tu gyrB ntH_6, aaH_5	10	M504_30gyrB, M504_31gyrB, M504_33gyrB, M504_35_2gyrB, M504_36gyrB, M504_37gyrB, M504_38gyrB, M504_39gyrB, M504_5gyrB, M504_7gyrB

Table 5.S13. Summary of variable residues between the *Thermoanaerobacter uzonensis* deduced *gyrB* primary protein sequence variants. Positions of variable residues are listed vertically above the multiple sequence alignment. SIFT analyses were based on comparisons with 397 homologous primary protein sequences. The most common *gyrB* sequence variant is shaded in grey.

	1122223 3434465 9888905
***Tu gyrB* ntH_1, aaH_1**	**RRDTEDD**
Tu gyrB ntH_2, aaH_2	GKDTEDD
Tu gyrB ntH_3, aaH_3	GRDTEDD
Tu gyrB ntH_4, aaH_3	GRDTEDD
Tu gyrB ntH_5, aaH_4	RRDTENE
Tu gyrB ntH_6, aaH_5	GRENDDE

SIFT analysis results:

Substitution at position 139 from R to G was predicted to be tolerated

Substitution at position 148 from R to K was predicted to be tolerated

Substitution at position 238 from D to E was predicted to be tolerated

Substitution at position 248 from T to N was predicted to be tolerated

Substitution at position 249 from E to D was predicted to be tolerated

Substitution at position 260 from D to N was predicted to affect protein function[a]

Substitution at position 355 from D to E was predicted to be tolerated

[a] This substitution may have been predicted to affect function just because the sequences used were not diverse enough; there was low confidence in this prediction.

Table 5.S14. Distribution of *pyrG* gene sequence variants among *Thermoanaerobacter uzonensis* isolates		
***pyrG* gene sequence variants**	**No. of Isolates**	**Isolates**
Tu pyrG ntH_1, aaH_1	3	B621_10pyrG, B621_6pyrG, B621_78pyrG
Tu pyrG ntH_2, aaH_1	1	B621_2pyrG
Tu pyrG ntH_3, aaH_2	1	B621_80pyrG
Tu pyrG ntH_4, aaH_3	4	B621_94pyrG, O629_42pyrG, O629_47pyrG, O629_48pyrG
Tu pyrG ntH_5, aaH_2	11	A615_31pyrG, A615_32pyrG, A615_62pyrG, I502_40pyrG, I502_41pyrG, I502_42pyrG, I502_43pyrG, I502_45pyrG, I502_46pyrG, I502_48pyrG, I502_49pyrG
Tu pyrG ntH_6, aaH_4	11	H608_10pyrG, H608_1pyrG, H608_2pyrG, H608_41pyrG, H608_6pyrG, H608_71pyrG, H608_72pyrG, H608_84pyrG, H608_85pyrG, H608_88pyrG, T515_40pyrG
Tu pyrG ntH_7, aaH_5	2	T515_1pyrG, T515_3pyrG
Tu pyrG ntH_8, aaH_6	1	B621_3pyrG
Tu pyrG ntH_9, aaH_7	1	H608_78pyrG
Tu pyrG ntH_10, aaH_8	2	T515_41pyrG, T515_44pyrG
Tu pyrG ntH_11, aaH_6	12	A615_39pyrG, A615_65pyrG, B621_74pyrG, Z606_36pyrG, Z606_38pyrG, Z606_3pyrG, Z606_70pyrG, Z606_71pyrG, Z606_72pyrG, Z606_73pyrG, Z606_77pyrG, Z606_9pyrG
Tu pyrG ntH_12, aaH_6	1	H608_93pyrG
Tu pyrG ntH_13, aaH_6	10	V634_3pyrG, V634_5pyrG, V634_71pyrG, V634_73pyrG, V634_74pyrG, V634_77pyrG, V634_81pyrG, V634_82pyrG, V634_84pyrG, V634_8pyrG
Tu pyrG ntH_14, aaH_9	7	V634_1pyrG, V634_2pyrG, V634_6pyrG, V634_7pyrG, V634_83pyrG, V634_85pyrG, V634_9pyrG
Tu pyrG ntH_15, aaH_8	1	H608_81pyrG
Tu pyrG ntH_16, aaH_6	5	B621_1pyrG, B621_71pyrG, B621_89_1pyrG, B621_90pyrG, B621_93pyrG
Tu pyrG ntH_17, aaH_6	1	B621_83pyrG
Tu pyrG ntH_18, aaH_10	2	B621_77pyrG, Z606_81pyrG
Tu pyrG ntH_19, aaH_10	6	A615_37pyrG, J614_60pyrG, J614_61pyrG, J614_63pyrG, J614_65pyrG, V634_10pyrG

Table 5.S14 (continued). Distribution of *pyrG* gene sequence variants among *Thermoanaerobacter uzonensis* isolates		
***pyrG* gene sequence variants**	**No. of Isolates**	**Isolates**
Tu pyrG ntH_20, aaH_11	1	H608_79pyrG
Tu pyrG ntH_21, aaH_10	7	H608_3pyrG, H608_42pyrG, H608_4pyrG, H608_77pyrG, H608_91pyrG, T515_10pyrG, T515_42pyrG
Tu pyrG ntH_22, aaH_11	11	B621_4pyrG, M504_30pyrG, M504_31pyrG, M504_33pyrG, M504_35_2pyrG, M504_36pyrG, M504_37pyrG, M504_38pyrG, M504_39pyrG, M504_5pyrG, M504_7pyrG
Tu pyrG ntH_23, aaH_11	14	B621_72pyrG, B621_87pyrG, J614_62_1pyrG, J614_7pyrG, J614_9pyrG, O629_40pyrG, O629_43pyrG, O629_44pyrG, O629_45pyrG, O629_49pyrG, O629_50pyrG, O629_51pyrG, O629_6pyrG, O629_7pyrG
Tu pyrG ntH_24, aaH_10	1	Z606_76pyrG
Tu pyrG ntH_25, aaH_12	5	R649_40_2pyrG, S648_4_2pyrG, S648_62pyrG, S648_7pyrG, S648_9pyrG
Tu pyrG ntH_26, aaH_13	1	R649_9pyrG
Tu pyrG ntH_27, aaH_14	1	A615_35pyrG

Table 5.S15. Summary of variable residues between the *Thermoanaerobacter uzonensis* deduced *pyrG* primary protein sequence variants. Positions of variable residues are listed vertically above the multiple sequence alignment. SIFT analyses were based on comparisons with ≥344 homologous primary protein sequences. The most common *pyrG* sequence variant is shaded in grey.

	112233334444
	9687811890139
	7730269999054
Tu *pyrG* ntH_1, aaH_1	TRKEEAYVKDTVE
Tu *pyrG* ntH_2, aaH_1	TRKEEAYVKDTV-
Tu *pyrG* ntH_3, aaH_2	TRKEEGYIKDTV-
Tu *pyrG* ntH_4, aaH_3	TRKEEGYINDTV-
Tu *pyrG* ntH_5, aaH_2	TRKEEGYIKDTV-
Tu *pyrG* ntH_6, aaH_4	TKKEEGYINDTV-
Tu *pyrG* ntH_7, aaH_5	TKKEEGYIKDTV-
Tu *pyrG* ntH_8, aaH_6	TRKEEAYIKDTVD
Tu *pyrG* ntH_9, aaH_7	TRNEEAYIKDTV-
Tu *pyrG* ntH_10, aaH_8	SRKEEAYIKDTV-
Tu *pyrG* ntH_11, aaH_6	**TRKEEAYIKDTV-**
Tu *pyrG* ntH_12, aaH_6	TRKEEAYIKDTV-
Tu *pyrG* ntH_13, aaH_6	TRKEEAYIKDTV-
Tu *pyrG* ntH_14, aaH_9	TRKEVAYIKDTV-
Tu *pyrG* ntH_15, aaH_8	SRKEEAYIKDTV-
Tu *pyrG* ntH_16, aaH_6	TRKEEAYIKDTV-
Tu *pyrG* ntH_17, aaH_6	TRKEEAYIKDTV-
Tu *pyrG* ntH_18, aaH_10	TRKGEAYIKDTV-
Tu *pyrG* ntH_19, aaH_10	TRKGEAYIKDTV-
Tu *pyrG* ntH_20, aaH_11	SRKGEAYIKDTV-
Tu *pyrG* ntH_21, aaH_10	TRKGEAYIKDTVE
Tu *pyrG* ntH_22, aaH_11	SRKGEAYIKDTV-
Tu *pyrG* ntH_23, aaH_11	SRKGEAYIKDTV-
Tu *pyrG* ntH_24, aaH_10	TRKGEAYIKDTVE
Tu *pyrG* ntH_25, aaH_12	SRKGEAYINDTL-
Tu *pyrG* ntH_26, aaH_13	SRKGEAHINDTVE
Tu *pyrG* ntH_27, aaH_14	TKKGEAYIKEAVE

SIFT analysis results:

Substitution at position 97 from T to S was predicted to be tolerated

Substitution at position 167 from R to K was predicted to be tolerated

Substitution at position 183 from K to N was predicted to affect protein function

Substitution at position 270 from E to G was predicted to be tolerated

Substitution at position 282 from E to V was predicted to be tolerated

Substitution at position 316 from A to G was predicted to be tolerated

Substitution at position 319 from Y to H was predicted to be tolerated

Substitution at position 389 from I to V was predicted to be tolerated

Substitution at position 399 from K to N was predicted to be tolerated

Substitution at position 409 from D to E was predicted to be tolerated

Substitution at position 410 from T to A was predicted to be tolerated

Substitution at position 435 from V to L was predicted to be tolerated

Substitution at position 494 from D to E was predicted to be tolerated

Table 5.S16. Distribution of *recG* gene sequence variants among *Thermoanaerobacter uzonensis* isolates		
***recG* gene sequence variants**	**No. of isolates**	**Isolates**
Tu recG ntH_1, aaH_1	1	O629_42recG
Tu recG ntH_2, aaH_2	1	R649_9recG
Tu recG ntH_3, aaH_3	3	H608_81recG, O629_47recG, O629_48recG
Tu recG ntH_4, aaH_4	12	A615_37recG, B621_3recG, B621_74recG, H608_79recG, O629_51recG, V634_1recG, V634_2recG, V634_6recG, V634_7recG, V634_83recG, V634_85recG, V634_9recG
Tu recG ntH_5, aaH_5	1	Z606_76recG
Tu recG ntH_6, aaH_6	1	Z606_81recG
Tu recG ntH_7, aaH_7	15	B621_1recG, B621_77recG, B621_87recG, B621_93recG, H608_10recG, H608_1recG, H608_2recG, H608_41recG, H608_6recG, H608_71recG, H608_72recG, H608_84recG, H608_85recG, H608_88recG, T515_40recG
Tu recG ntH_8, aaH_8	8	B621_2recG, H608_3recG, H608_42recG, H608_4recG, H608_77recG, H608_91recG, T515_10recG, T515_42recG
Tu recG ntH_9, aaH_9	1	H608_78recG
Tu recG ntH_10, aaH_10	20	A615_35recG, A615_39recG, A615_65recG, B621_10recG, B621_4recG, B621_6recG, B621_71recG, B621_78recG, B621_80recG, B621_89_1recG, B621_90recG, H608_93recG, J614_62_1recG, J614_7recG, J614_9recG, T515_41recG, T515_44recG, Z606_36recG, Z606_72recG, Z606_73recG
Tu recG ntH_11, aaH_8	2	T515_1recG, T515_3recG
Tu recG ntH_12, aaH_11	11	A615_31recG, A615_32recG, A615_62recG, I502_40recG, I502_41recG, I502_42recG, I502_43recG, I502_45recG, I502_46recG, I502_48recG, I502_49recG
Tu recG ntH_13, aaH_12	4	J614_60recG, J614_61recG, J614_63recG, J614_65recG
Tu recG ntH_14, aaH_13	2	B621_72recG, V634_10_2recG
Tu recG ntH_15, aaH_14	10	M504_30recG, M504_31recG, M504_33recG, M504_35_2recG, M504_36recG, M504_37recG, M504_38recG, M504_39recG, M504_5recG, M504_7recG
Tu recG ntH_16, aaH_15	5	Z606_38recG, Z606_3recG, Z606_71recG, Z606_77recG, Z606_9recG
Tu recG ntH_17, aaH_16	1	Z606_70recG

Table 5.S16 (continued). Distribution of *recG* gene sequence variants among *Thermoanaerobacter uzonensis* isolates		
***recG* gene sequence variants**	**No. of Isolates**	**Isolates**
Tu recG ntH_18, aaH_17	18	O629_40recG, O629_43recG, O629_44recG, O629_45recG, O629_49recG, O629_50recG, O629_6recG, O629_7recG, V634_3recG, V634_5recG, V634_71recG, V634_73recG, V634_74recG, V634_77recG, V634_81recG, V634_82recG, V634_84recG, V634_8recG
Tu recG ntH_19, aaH_18	5	R649_40_2recG, S648_4_2recG, S648_62recG, S648_7recG, S648_9recG
Tu recG ntH_20, aaH_19	2	B621_83recG, B621_94recG

Table 5.S17. Summary of variable residues between the *T. uzonensis* deduced *recG* primary protein sequence variants. Positions of variable residues are listed vertically above the multiple sequence alignment. SIFT analyses were based on comparisons with 13 homologous primary protein sequences. The most common *recG* sequence variant is shaded in grey.

	1111222223333444555
	5678125580569369122
	9734180821739659135
Tu *recG* ntH_1, aaH_1	RHIEYMVSHVGIQPGKNVGK
Tu *recG* ntH_2, aaH_2	RHIEYMVSQVGIQPGKTVGE
Tu *recG* ntH_3, aaH_3	RHIEYMVSQVGIQPGKNVGK
Tu *recG* ntH_4, aaH_4	RHIEYVVSQVGIQPGKNVGK
Tu *recG* ntH_5, aaH_5	RHIEYVVSQVGVKPRKNVGK
Tu *recG* ntH_6, aaH_6	RHIEYVVSQVGVKSGKNVGK
Tu *recG* ntH_7, aaH_7	RHIEYMMAQIGVKPGRNVGK
Tu *recG* ntH_8, aaH_8	RHIEYVVSQVGVKPGKNVGK
Tu *recG* ntH_9, aaH_9	RHVEYVVSQVGIKPGKNVGK
Tu *recG* ntH_10, aaH_10	**RHIEYVVSQVGIKPGKNVGK**
Tu *recG* ntH_11, aaH_8	RHIEYVVSQVGVKPGKNVGK
Tu *recG* ntH_12, aaH_11	RKVEYVVSQVGVKPGKNAGK
Tu *recG* ntH_13, aaH_12	RKVEYVVSQVGVKPGKNVGK
Tu *recG* ntH_14, aaH_13	RKVKYVVSQVGVKPGKNVGK
Tu *recG* ntH_15, aaH_14	KKIEYMMAQIGVKPGKNVRE
Tu *recG* ntH_16, aaH_15	RKVEYVVSQVGVKPGKNVRE
Tu *recG* ntH_17, aaH_16	RKVEYVVSQVVVKPGKNVRE
Tu *recG* ntH_18, aaH_17	RHVEYMMAQIGIKPGKNVGK
Tu *recG* ntH_19, aaH_18	RHVEYMMAQIGVKPGKNVGK
Tu *recG* ntH_20, aaH_19	RHVEHVMAQVGIQPGKNVGK

SIFT analysis results:

Substitution at position 159 from R to K was predicted to be tolerated
Substitution at position 167 from H to K was predicted to be tolerated
Substitution at position 173 from I to V was predicted to be tolerated
Substitution at position 184 from E to K was predicted to be tolerated
Substitution at position 211 from Y to H was predicted to be tolerated
Substitution at position 228 from V to M was predicted to be tolerated
Substitution at position 250 from V to M was predicted to be tolerated
Substitution at position 258 from S to A was predicted to be tolerated
Substitution at position 282 from Q to H was predicted to affect protein function
Substitution at position 301 from V to I was predicted to be tolerated
Substitution at position 357 from G to V was predicted to affect protein function
Substitution at position 363 from I to V was predicted to be tolerated
Substitution at position 399 from K to Q was predicted to be tolerated
Substitution at position 436 from P to S was predicted to affect protein function
Substitution at position 465 from G to R was predicted to affect protein function
Substitution at position 499 from K to R was predicted to be tolerated
Substitution at position 511 from N to T was predicted to be tolerated
Substitution at position 523 from V to A was predicted to be tolerated
Substitution at position 525 from G to R was predicted to affect protein function
Substitution at position 526 from K to E was predicted to be tolerated

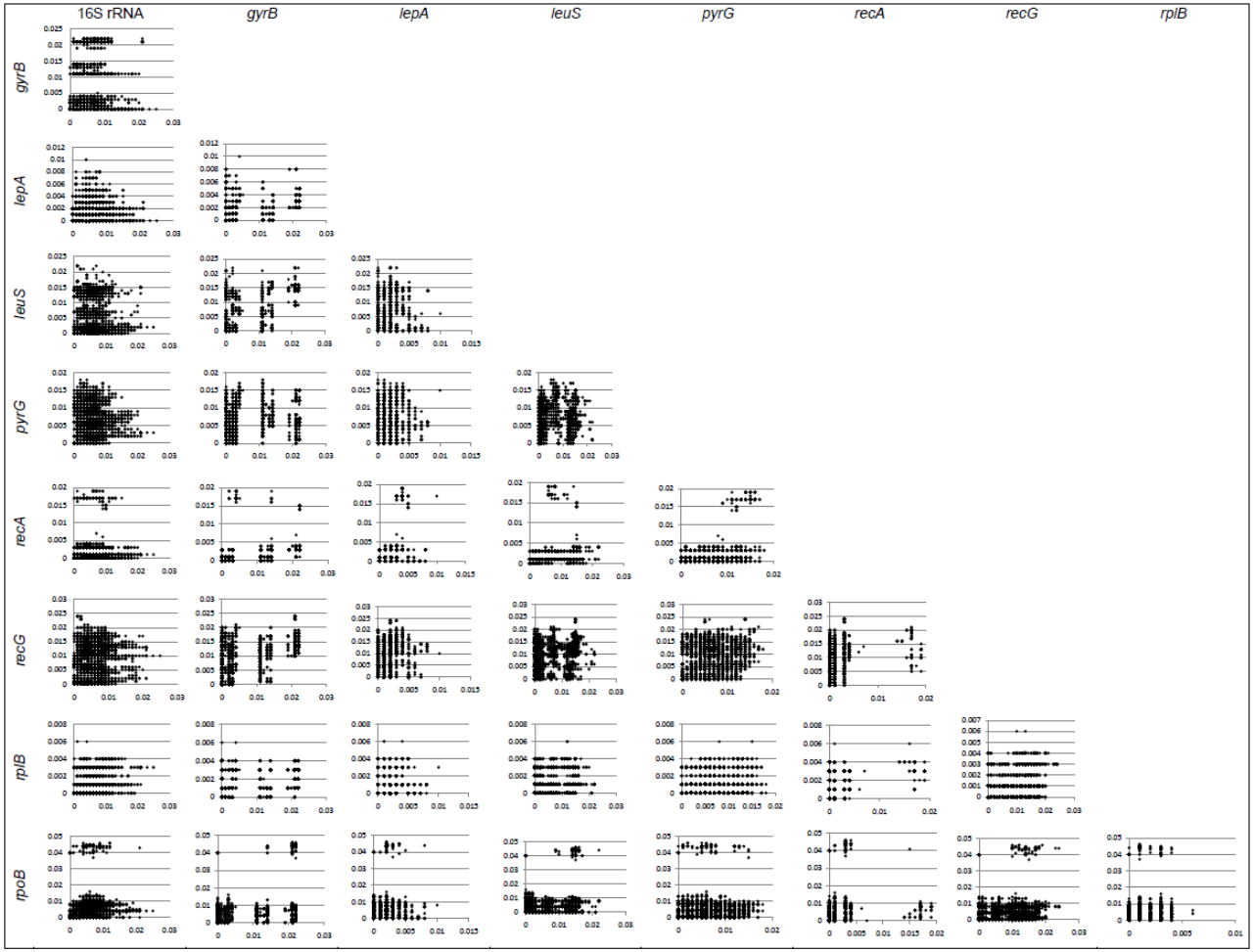

Fig. 5.S1. Pairwise comparisons of Jukes and Cantor genetic distances of the corresponding genes within the same *Thermoanaerobacter uzonensis* isolates displayed as ordered pairs. The genetic distance value of the x-axis corresponds to that of the gene given in the particular column; the genetic distance value of the y-axis corresponds to that of the gene given in the particular row.

CHAPTER 6

THE POPULATION STRUCTURE AND SPATIAL DIVERSITY OF *THERMOANAEROBACTER* ISOLATES FROM GEOTHERMAL SPRINGS OF KAMCHATKA, RUSSIAN FAR EAST, BASED ON ANALYSES WITH EIGHT UNIVERSALLY CONSERVED PROTEIN CODING GENES[7]

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SUMMARY

Strains phylogenetically related to *Thermoanaerobacter uzonensis* (≥98% 16S rRNA gene sequence similarity) and *Thermoanaerobacter siderophilus* (≥97% 16S rRNA gene sequence similarity) were isolated from 11 different geothermal springs within the Uzon Caldera, Geyser Valley, and Mutnovsky Volcano regions of the Kamchatka Peninsula, Far East Russia. Eight universally conserved protein coding genes, *gyrB, lepA, leuS, pyrG, recA, recG, rplB,* and *rpoB,* were sequenced to assess the interspecies diversity and the genetic structure of the population. A range of *T. uzonensis* intra-geothermal spring heterogeneity (α-diversity) was observed. Little to no variation was observed within gene sequences of *T. uzonensis* isolates from the geothermal springs Mutnovsky-4 and Arkashin 2005, which suggests that recent selective sweeps may have occurred. By comparison, all of the protein coding gene sequence sets from the *T. uzonensis* isolates from the Burlyashi spring were variable. β-diversity analyses revealed that the greatest genetic variation was between isolates from geothermal springs from different regions. Nucleotide sequence variants were regularly found within multiple isolates from one particular geothermal spring and not elsewhere. AMOVA revealed that 51.4% of variation was attributed to the diversity between regions, while 35.4% was attributed to the diversity within populations. Evidence for linkage disequilibrium within the *T. uzonensis* population was observed. Analyses suggested that multiple distinct niches are available for the *T. uzonensis* genotypes within many of the analyzed geothermal springs, or that the population may be structured into *T. uzonensis* genotypes with generalist or specialist characteristics. The *T. siderophilus* isolates were comparedto examine intraspecies variation within a different species of the same genus. The comparisons between *T. uzonensis* and *T. siderophilus* strains revealed that homologous universally conserved protein coding genes within different

species of the same genus are under different levels of evolutionary selection. These analyses also revealed and that lateral gene transfer of core-genome genes occurs.

INTRODUCTION

The Kamchatka Peninsula is located on the northern side of the Kurile-Kamchatka volcanic arc, and volcanic activity persists in this region, mainly in the East Kamchatka volcanic zone. Beside volcanic activity, over 130 groups of geothermal features, including terrestrial geothermal springs, fumaroles, and geysers, are found within Kamchatka (Karpov and Naboko, 1990). Several aerobic and anaerobic thermophilic prokaryotes have previously been isolated and described from geothermal springs of Kamchatka (Wagner and Wiegel, 2008; Kublanov et al., 2009), and recent massively parallel DNA pyrosequencing of the 16S rRNA V6 hypervariable region revealed diverse prokaryotic communities within analyzed geothermal springs of the Uzon Caldera region of Kamchatka (D. E. Crowe, unpublished results).

Members of the genus *Thermoanaerobacter* have been isolated from terrestrial geothermal sites, anthropogenic thermal sites, and some marine thermal sites (Subbotina et al., 2003; Prokofeva et al., 2005; Onyenwoke and Wiegel, 2008). Described *Thermoanaerobacter* strains are thermophilic, T_{opt}, 55-70 °C, and have a circumneutral pH_{opt}. Chemolithoautotrophic, organo- and chemolitho-heterotrophic metabolic strategies have been observed for *Thermoanaerobacter* species, and numerous carbon substrates and a diverse set of electron acceptors are utilized (Onyenwoke and Wiegel, 2008).

The type strains of *T. uzonensis* and *T. siderophilus* were both isolated from geothermal springs of Kamchatka (Slobodkin et al., 1999; Wagner et al., 2008).However, similar strains have been isolated elsewhere.

"Thermoanaerobacter inferii" strain AK15 (GenBank accession number EU262599) was isolated from a hot spring in Iceland, and has >98.5% 16S rRNA gene sequence identity to *T. uzonensis* JW/IW010^T. *Thermoanaerobacter* sp. 518-21 (GenBank accession number AY350592) was isolated from a chimney sample collected at the 13°N deep-sea hydrothermal vent field on the East Pacific Rise, and has 99.3% 16S rRNA gene sequence identity to *T. siderophilus* SR4^T (Prokofeva et al., 2005). Many of the described *Thermoanaerobacter* species form spores or contain sporulation-specific genes (Brill and Wiegel, 1997; Onyenwoke et al., 2004). Both *T. uzonensis* JW/IW010^T and *T. siderophilus* SR4^T form endospores (Slobodkin et al., 1999; Wagner et al., 2008).

The intraspecies heterogeneity within the *T. uzonensis* isolates from Kamchatka was described considering the 16S rRNA and eight universally conserved protein coding genes (Chapter 5). All of the *T. uzonensis* protein coding genes were polymorphic, although the type (e.g., synonymous or nonsynonymous substitutions) and quantity of the variation differed between genes. The observed intraspecies heterogeneity suggests that the most applicable species concept for the *T. uzonensis* cluster is one that considers subpopulation/metapopulation dynamics and the physiological properties that facilitate the exchange of genetic information between subpopulations (Chapter 5).

While a primary focus of the previous work was to quantify and describe the observed variation within the *T. uzonensis* cluster, the focus here is to elucidate the spatial patterns of diversity using the same protein coding gene sequence data. Spatial patterns of diversity were examined through a variety of approaches: i) the distribution of universally conserved protein coding gene sequence variants among *T. uzonensis* isolates was assessed, ii) the diversity of *T. uzonensis* isolates within and between geothermal springs was compared, iii) sequence types

were generated from the universally conserved protein coding gene sequence variants and then compared alone and as clusters of similar sequence types, and iv) the protein coding gene sequence heterogeneity of the *T. uzonensis* isolates was compared to the heterogeneity observed within the *T. siderophilus* isolates.

RESULTS

Isolation of *Thermoanaerobacter* strains

From mixed water and sediment samples, 208 strains with ≥98% 16S rRNA gene sequence similarity to *T. uzonensis* JW/IW010^T (Wagner et al., 2008), and 14 strains with ≥97% 16S rRNA gene sequence similarity to *T. siderophilus* SR4^T (Slobodkin et al., 1999), were isolated. *Thermoanaerobacter* strains were obtained from 11 different geothermal springs within the Uzon Caldera, Geyser Valley, and Moutnovsky Volcano regions of Kamchatka (Table 6.1, Fig. 6.1). Most, but not all, of the tested geothermal spring samples yielded *Thermoanaerobacter* isolates. Attempts to isolate *Thermoanaerobacter* strains from "Oil Pool" and K4 Well, both located in the Central Sector of the Eastern Thermal Field of the Uzon Caldera, were unsuccessful even though ≥ 12 enrichments where prepared from every geothermal spring sample. *Thermoanaerobacter* isolates were obtained from samples collected in 2005 and 2006 for the Thermophilny and Arkashin geothermal springs (Table 6.1).

Gene fragment length

The percentage of the gene sequence amplified varied from approximately 50% (*leuS*) to 90% (*rpoB*) (Table 6.S1). The analyzed length of the genes varied slightly depending on the set of isolates being examined. The gene sequence diversity measured and discussed below occasionally changed when longer fragments were analyzed. For example, when the

heterogeneity within the *lepA* gene sequence set from the *T. uzonensis* isolates from Mutnovsky-4 was assessed, there were no observed differences between the *lepA* gene sequences when the 1,255 bp section of the gene shared between all 123 isolates was examined. However, when the 1,319 bp section of the *lepA* gene shared between the 10 *T. uzonensis* Mutnovsky-4 isolates was examined three nucleotide sequence variant forms and two primary protein sequence variant forms were observed (Tables 6.S12 and 5.S6).

Spatial diversity based on *T. uzonensis* universally conserved protein coding gene sequences

Detailed information concerning the diversity of *T. uzonensis* isolates within each geothermal spring is provided in the supporting information section of this chapter. The heterogeneity of the different protein coding genes is discussed and the sequence variants found within particular sites are described. Tables containing the nucleotide- and primary protein sequence diversity calculations for the sets of isolates from each geothermal spring are also given (Tables 6.S2 through 6.S12) .

The diversity of *T. uzonensis* strains within a single geothermal spring (α-diversity) and the extent to which the diversity of multiple spatial units differed (β-diversity), were assessed. It is emphasized that these α- and β-diversity analyses were based on the available data and the possible undersampling of point diversity is acknowledged; i.e., that if additional *T. uzonensis* strains were isolated and analyzed, additional variant forms and sequence heterogeneity may be found. The correlation between geothermal spring diversity and the number of *T. uzonensis* strains isolated from the same geothermal spring was determined. The least squares fitting linear trendlines, calculated from comparisons of the number of isolates per geothermal spring set and either the average number of nucleotide sequence variants or the average number of primary

protein sequence variants in the same set, have R^2 values of 0.51 and 0.52, respectively. The comparison of eBURST groups to the number of *T. uzonensis* isolates from the same spring had a R^2 value of 0.34, and comparisons of protein coding gene average π values to the number of *T. uzonensis* isolates from the corresponding spring had a R^2 value of 0.11.

α-diversity based on *T. uzonensis* universally conserved protein coding genes

Variation was observed within all eight of the analyzed protein coding genes from the 18 *T. uzonensis* isolates from the geothermal spring Burlyashi; conversely, there was no variation observed within any of the protein coding genes from the eight Arkashin 2005 isolates (Fig. 6.2a). The average number of nucleotide sequence variant forms within the isolates from a geothermal spring varied from 5.25 (Burlyashi) to 1 (Arkashin 2005) (Fig. 6.2a). The isolates from six geothermal springs, ON1, Zavarzin, Vent 1 North, Pulsating Spring, Thermophilny 2005, and Arkashin 2006, had similar average numbers of nucleotide sequence variant forms values: $3 \geq$ average ntH > 2 (Fig. 6.2a). The *T. uzonensis* isolates from the sites Arkashin 2005, Mutnovsky-4, and Proximal to Salt Spring had the fewest nucleotide sequence types, with average ntH < 2 (Fig. 6.2a). A similar trend was observed for the intra-population average aaH values (Fig. 6.2a).

Intra-geothermal spring diversity calculated from individual number of nucleotide substitutions per site, π, values and as an average also revealed a range of genetic variation. The highest average π value was observed for the set of *T. uzonensis* isolates from the Burlyashi geothermal spring (Fig. 6.2b). A notable difference between these two α-diversity measurements is that the Arkashin 2006 isolates appeared to have relatively little diversity considering the number of nucleotide- and primary protein sequence variants (Fig. 6.2a), but the same set of isolates appeared relatively diverse considering the gene sequence π values and the average π

value (Fig. 6.2b). Meaning that for the Arkashin 2006 T. uzonensis isolates, there is relatively high variation observed, but only within a few of the protein coding gene sequence sets (Table 6.S7).

The eBURST program (Feil et al., 2004; Spratt et al., 2004) was utilized to cluster the sequence types of the *T. uzonensis* isolates into groups, where 6 of the 8 sequence variants (alleles) had to be identical to others in the group for inclusion (Table 6.2). The number of eBURST groups into which *T. uzonensis* isolates from a geothermal spring binned varied from one, for the isolates from the Arkashin 2005 and Mutnovsky-4, to 10 for the Burlyashi isolates (Table 6.2).

β-diversity based on *T. uzonensis* universally conserved protein coding gene sequences

The average nucleotide differences, k, within and between populations was determined as a way to compare the intra-geothermal spring diversity, the intra-regional diversity, and the inter-regional heterogeneity; examples are shown in Fig. 6.3. For the *gyrB*, *lepA*, *leuS*, *pyrG*, *recA*, *recG*, and *rpoB* gene sequence sets, as well as for comparisons with the concatenates of eight protein coding genes, the average number of nucleotide differences within a geothermal spring was always less than the average number of nucleotide differences considering all the isolates within a region, although the differences were usually not statistically significant (data not shown).With the exception of the *rpoB* gene sequence set, the differences in nucleotide divergence within a region were less than the nucleotide divergence between regions (examples shown in Fig. 6.3). The trend was not observed within the *rpoB* gene sequence set (Fig. 6.3c) because a particular nucleotide sequence variant form, *rpoB* nucleotide sequence variant #5 (abbreviated *Tu rpoB* ntH_5), was found within the *T. uzonensis* isolates from all three regions (Fig. 6.3, Table 6.S21).

Local:global taxa richness ratio

The number of protein coding gene sequence variants within the set of isolates from one geothermal spring was compared to the total number of gene sequence variants for the entire *T. uzonensis* population, per gene, as a way to assess local:global taxa richness. The local:global taxa richness ratios are given as ntH_S/ntH_T and aaH_S/aaH_T, where ntH_S was the number of nucleotide sequence variants observed within the population in a single geothermal spring, ntH_T was the total number of nucleotide sequence variants observed in the population, aaH_S was the number of primary protein sequence variants bserved within the population in a single geothermal spring, and aaH_T was the total number of primary protein sequence variants observed in the population. The histogram of the ntH_S/ntH_T and aaH_S/aaH_T values reveals that most values are ≤ 0.3 (Fig. 6.4). This showed that the number of ntH and aaH within the set of *T. uzonensis* isolates from one geothermal spring are a small proportion of the total number of ntH and aaH found within the entire *T. uzonensis* population. Most of the high ntH_S/ntH_T and aaH_S/aaH_T values come from comparisons involving the *rplB* gene sequence set, which had only four nucleotide- and two primary protein sequence variant forms (Table 6.S5).

Population pairwise F_{ST} values and AMOVA

Population pairwise F_{ST} values were calculated to quantify the proportion of genetic variation that lies between subpopulations within the total population (Table 6.3). The highest observed pairwise F_{ST} values came from comparisons of *T. uzonensis* populations from different regions. Of the 15 pairwise F_{ST} values >0.5, only one comparison is between isolates from geothermal springs within the same region; specifically Vent 1 North compared to Arkashin 2005 and 2006 (Table 6.3). AMOVA partitioned 51.4% of the variation to between region

groups, 13.2% among populations within regions, and 35.4% was attributed to variation within a population (Table 6.4).

Analyses with *T. uzonensis* sequence types

The number of nucleotide sequence variant forms within each *T. uzonensis* protein coding gene sequence set varied from 4 to 27 (Table 6.S5). Within the set of 123 *T. uzonensis* isolates, 51 unique nucleotide sequence variant combinations, or sequence types, were observed (Tables 6.S22, and 6.S23). While most of the sequence types were composed of a single isolate (37 of 51), four sequence types contained ≥ 8 isolates (Table 6.S13). This analysis revealed only three instances where *T. uzonensis* isolates derived from different geothermal spring samples had the same sequence type, specifically these were Arkashin 2005 and Arkashin 2006 isolates, Thermophilny 2005 and Thermophilny 2006 isolates, and isolates from the two Geyser Valley springs, Resting Rock and Proximal to Salt Spring (Table 6.S13).

The eBURST program was utilized to cluster the sequence types of the *T. uzonensis* isolates into groups, wherein 6 of the 8 sequence variants had to be identical to others in the group for inclusion (Table 6.4). eBURST Group 1 was the most diverse and contained isolates from six geothermal springs, all located within the Uzon Caldera (Table 6.4). Although Pulsating Spring and Arkashin were only approximately 150 m apart (Fig. 6.1), there was no overlap of isolates within eBURST groups (Table 6.4, Fig. 6.S9).

Inferred influence of recombination on the population structure of *T. uzonensis*

Linkage disequilibrium within the *T. uzonensis* population was assessed in two ways: considering the isolates from all 10 geothermal springs and considering the set of isolates from the 7 geothermal springs of the Uzon Caldera region (Table 6.7). Based on the set of 51 sequence types from the *T. uzonensis* isolates from all 10 geothermal springs, I_a was 0.42; V_{obs} was 1.96;

and V_{max} was 1.88 for 1,000 trials ($p<0.001$). As $V_{obs} > V_{max}$ for this set of isolates, evidence for linkage disequilibrium was observed. However, I_a was near zero (-0.032) considering the set 46 sequence types from the *T. uzonensis* isolates from the seven geothermal springs of the Uzon Caldera. Thus the different protein coding gene sequence variants appeared to be randomly associated throughout this regional *T. uzonensis* population (Table 6.7).

Comparison of *T. siderophilus* and *T. uzonensis* housekeeping gene sequence heterogeneity

Although > 90% of the *Thermoanaerobacter* strains isolated were phylogenetically related to *T. uzonensis,* strains related to *T. siderophilus* were also isolated from geothermal springs within the Uzon Caldera, Geyser Valley, and Mutnovsky Volcano regions (Table 6.1). The 16S rRNA and eight universally conserved protein coding genes were similarly amplified, sequenced, and analyzed from the set of *T. siderophilus* isolates (Table 6.S6). Two major observations were are made while comparing the heterogeneity of the housekeeping gene sequences of *T. siderophilus* to *T. uzonensis.* First, there was no variation within the *gyrB* gene sequences set from the *T. siderophilus* isolates while variation was observed within *gyrB* gene sequence set from the *T. uzonensis* isolates. Second, phylogenetic discontinuity within the *leuS* gene sequence set from the *T. siderophilus* isolates suggests that lateral gene transfers have occurred (Table 6.S6, Fig. 6.5).

DISCUSSION

Isolation of *Thermoanaerobacter* strains

Strains with high 16S rRNA gene sequence similarity to *Thermoanaerobacter* taxa were isolated from 11 geothermal springs in the Kamchatka Peninsula (Table 6.1). Since this study was based on isolated *Thermoanaerobacter* strains, it must be noted that the diversity of isolates

obtained may not accurately reflect the *Thermoanaerobacter* population within the geothermal springs. However, massively parallel DNA pyrosequencing of the 16S rRNA V6 hypervariable region revealed the presence of *Thermoanaerobacter* within geothermal springs of the Uzon Caldera (D. Crowe, unpublished results).

The protein coding gene fragment length analyzed in this present study varied from approximately 600 bp to 1,300 bp in length, or a fraction between 0.46 to 0.86 of the inferred gene sequence length (Table 6.S1). Thus, the individual nucleotide sequences that bin to the same variant form in the analyses described herein may not do so if the entire gene was sequenced and additional nucleotide differences were found. The number of *T. uzonensis* isolates from a single location varied (Table 6.S5); thus, the question of whether the geothermal spring sets with more isolates were more diverse than geothermal spring sets with fewer isolates was addressed. Based on comparisons between the number of strains isolated from a spring and the corresponding number of nucleotide- and primary protein sequence variant forms, π value averages, and the number of eBURST groups to which *T. uzonensis* isolates clustered, the resultant coefficient of determination values varied from 0.52 to 0.11. Thus, an increased number of isolates does not fully explain the observed differences in heterogeneity between the *T. uzonensis* isolates from different geothermal springs.

Universally conserved protein coding gene sequence distribution patterns

One goal of this study was to assess the distribution of universally conserved protein coding gene sequence variants among *T. uzonensis* isolates. There were many examples of particular nucleotide sequence variant forms found only within the set of isolates from one particular geothermal spring, the only exception was for the set of isolates from the Proximal to Salt Spring site. At times, this also included a unique corresponding primary protein sequence

variant. This qualitative observation suggested that some sequence variants are not randomly associated, but are rather structured among *T. uzonensis* subpopulations.

As part of the core genome of the species (Medini et al., 2005), it is unlikely that these protein coding gene sequences play a role in the adaptation the strain to the particular geothermal spring environment. Instead, genes involved in the adaptation of the strains to a particular habitat will most likely to be found within the variable genome (auxiliary gene) section of the pan-genome (Fraser et al., 2009; Papke, 2009). However, auxiliary genes that enhance the ecological specificity of a *T uzonensis* strain within a particular geothermal spring may be located near these universally conserved protein coding genes on the genome, and therefore, the protein coding gene is under strong selection due to the hitchhiking evolutionary process (Cooper and Feil, 2004). For example, almost all Vent 1 North *T. uzonensis* isolates have either *Tu pyrG* ntH_13 or *Tu pyrG* ntH_14, nucleotide sequence variants which were not found in isolates from any other geothermal spring (Table 6.S17). Auxiliary genes near *pyrG* in these Vent 1 North isolates may enhance the ecological specificity of the strains and recombination events that include a stretch of DNA with the *pyrG* gene result in less-fit strains. A related topic is the occurrence and frequency of homologous recombination within the *T. uzonensis* population. While this issue will be discussed in more detail below, here it is important to note that evidence for linkage disequilibrium within the entire *T. uzonensis* population was observed, although the alleles appeared randomly associated when only the Uzon Caldera isolates were analyzed (Table 6.7).

T. uzonensis α-diversity patterns

A primary objective of this study was to assess *T. uzonensis* patterns of spatial variation. To achieve this, a variety of α- and β-diversity analyses were performed considering the

universally conserved protein coding gene sequences. The intra-geothermal spring *T. uzonensis* isolate sets had a range of variation with regard to the number of nucleotide- and primary protein sequence variant forms (Fig. 6.2a). A range of variation was likewise observed when gene sequence nucleotide diversity was considered (Fig. 6.2b). AMOVA revealed that a relatively large percentage, 35.4%, of the variation was attributable to intra-geothermal spring variation among *T. uzonensis* isolate populations (Table 6.4). By nearly all measures, the set of *T. uzonensis* isolates from the Burlyashi spring appeared the most variable and this heterogeneity was likely influenced several interrelated factors. The Burlyashi geothermal spring is relatively large, and previous studies have reported that bacterial richness increased with volume (Green and Bohannan, 2006; references therein). The heterogeneity observed for *T. uzonensis* isolates from Burlyashi could be influenced by the complexity of the environment. Spatial and temporal variability at different scales relative to these isolates will result in an environment composed of multiple niches (Kassen, 2002).

By comparison, little or no intra-geothermal spring variability was observed within the protein coding genes of the isolates from the geothermal spring samples of Mutnovsky-4 and Arkashin 2005 (Tables 6.S6 and 6.S12). This lack of diversity could indicate that a selection event occurred prior to sampling and purged the nucleotide sequence variant diversity. An ecotype species concept has been proposed that incorporates selective sweeps that regularly purge the population of any diversity that might have accumulated (Cohan and Perry, 2007). While the ecotype species concept is a popular way to discuss prokaryotic evolution, speciation, and ecology, observations of repeated selective sweeps have been made in chemostats (Fraser et al., 2009), and there is a lack of understanding of how quickly sequence diversity is purged along with a lack of observations from nature that support complete purging (Achtman and Wagner,

2008). Although some geothermal springs have been described as having certain chemostat-like properties, e.g., Obsidian Pool in Yellowstone National Park (Shock et al., 2005), the Kamchatkan geothermal springs utilized in this study can generally be considered complex environments composed of multiple niches.

While the *T. uzonensis* isolates from the Mutnovsky-4 and Arkashin 2005 samples were found to be the least variable, the populations are not monophyletic. Considering the ability of cells to incorporate homologous DNA, monophyly is thought to be an unlikely property (Papke, 2009). Within the set of *T. uzonensis* isolates from Mutnovsky-4, a single nucleotide sequence variant form, found only in this set of isolates, was observed for the *gyrB*, *leuS*, *recA*, and *recG* gene sequence sets (Tables 6.S14, 6.S16, 6.S18, and 6.S19). However, nucleotide sequence variants within the *rplB* and *rpoB* gene sequence sets were found within isolates from Mutnovsky-4, Arkashin 2005, and other geothermal springs (Tables 6.S20 and 6.S21). This observation suggests that the *rplB* and *rpoB* genes are the result of recent homologous recombination events since neutral, synonymous substitutions were not observed. Both Mutnovsky-4 and Arkashin are relatively small geothermal springs, and as previously mentioned, relationships between bacterial richness and the volume of the environment have been reported (Green and Bohannan, 2006; references therein). While the geothermal spring Arkashin is relatively small, it is also geochemically distinct. The arsenic concentration measured within Arkashin in 2006 was 4252 mg kg^{-1} (Burgess, 2009). The prokaryotes withinArkashin, including *T. uzonensis,* assumedly have mechanisms that allow them to survive in this inhospitable environment, and it is likely that these mechanisms are the result of genes within their auxiliary genomes.

The observations regarding the α-diversity of *T. uzonensis* isolates described above provided the opportunity to consider the niche exclusion principle, i.e., that a single niche can support no more than one genotype (Kassen and Rainey, 2004). For example, within the phylogenetic tree based on concatenates of the eight protein coding genes (Fig. 6.6), two relatively distinct, phylogenetically separated clades were observed for the *T. uzonensis* isolates from Vent 1 North, ON1, Pulsating Spring, and Zavarzin. From this observation, the inference is that there were multiple distinct niches available for *T. uzonensis* within these geothermal springs. While between-year comparisons will be discussed in greater detail below, a point that has bearing here is that isolates from 2006 samples had the same sequence type (based on eight protein coding genes) as the isolates from the 2005 samples of the same geothermal spring (Table. 6.S13). Moreover, the particular sequence types were not found within isolates from any other geothermal spring (ST #1, for Arkashin 2005/2006; ST #20 and 21, for Thermophilny 2005/2006; Table. 6.S13). Thus, while multiple niches may be available within these geothermal springs, a particular niche may also remain temporally stable. On the other hand, results from multilocus sequencing analyses hint that the geothermal springs contain *T. uzonensis* with generalist or specialist characteristics. A portion of the isolates from most Uzon Caldera springs binned to eBURST Group 1 (Table 6.2). These eBURST groups may indicate that the strains isolated from the Kamchatkan geothermal spring can be categorized as either generalists (i.e., those found throughout the different geothermal springs, namely eBURST Group 1), or specialists which are only found within one geothermal spring (Table 6.2, Fig. 6.S9).

T. uzonensis β-diversity patterns

β-diversity analyses, particularly the local:global taxa richness ratio (Fig. 6.4), revealed that the diversity within a geothermal spring was usually a small proportion of the total diversity.

Although, it is noted that these results could change if additional unique gene sequence variant forms were found. AMOVA with the *T. uzonensis* protein coding gene sequence concatenates revealed that the largest proportion of the observed variation, 51.4%, was due to variation between geographic regions (Table 6.4). Similar patterns of spatial variation within thermal environments have been reported, for example, Whitaker and colleagues (2003), reported that AMOVA partitioned 73% of the molecular variance of *Sulfolobus* isolates among intercontinental regions. Analyses of *Rhodothermus* isolates at an intracontinental scale, specifically geothermal sites of Iceland, revealed that a large proportion of the observed diversity was due to variation between regions (Petursdottir et al., 2000).

The influence of recombination on the population structure of *T. uzonensis* was assessed and revealed evidence for linkage disequilibrium when the entire population was analyzed. However, linkage disequilibrium was not observed when only the *T. uzonensis* isolates from geothermal springs of the Uzon Caldera were analyzed (Table 6.7). This suggests that homologous recombination regularly occurs between *T. uzonensis* subpopulations in the Uzon Caldera (Table 6.7). This observation differs from what has been reported for aerobic thermophilic bacteria from Icelandic thermal sites (Petursdottir et al., 2000). Based on analyses with *Rhodothermus* isolates, Petursdottir and colleagues (2000) reported high intra-regional I_a values, which they attributed to clonal *Rhodothermus* populations. While the I_a value for *T. uzonensis* isolates of the Uzon Caldera was near zero, isolates from different geothermal springs of the region did not group together within any sequence type (Table 6.S13), eBURST groups regularly contained isolates from only one spring (Table 6.2, Fig. 6.S9), and particular sequence variant forms were often found only within the isolates from one particular spring (described in detail within the supporting information section below).

Between year comparisons

T. uzonensis isolates were obtained from samples of the Arkashin and Thermophilny geothermal springs collected in 2005 and 2006. Remarkably, the sequence types found in the isolates derived from the 2005 samples were, for both Arkashin and Thermophilny, also found in the isolates obtained from the samples collected in 2006 (Table. 6.S13). Notable between-year differences were also observed. Within the protein coding gene sequence sets of the Thermophilny isolates, there was a juxtaposition between years regarding whether or not heterogeneity was observed in the *gyrB, rplB,* and *rpoB* gene sequence sets (Tables 6.S3 and 6.S4). While there was no observed heterogeneity within any of the universally conserved protein coding gene sequence sets from the Arkashin 2005 isolates (Table 6.S6), variation was observed within protein coding gene sequence sets from the Arkashin 2006 isolates (Table 6.S7). Considering α-diversity based on protein coding gene sequence π value averages, the Arkashin 2006 isolates are among the most diverse intra-geothermal spring *T. uzonensis* set (Fig. 6.2b). The possibility that the lack of diversity observed within the Arkashin 2005 isolates was the result of a selective sweep having occurred was discussed above. With only eight isolates obtained from the Arkashin 2005 sample, it is also possible that heterogeneity would have been observed with additional isolates, or if different gene sequences were examined. The *leuS*, *pyrG*, and *recG* protein coding gene sequence sets within the *T. uzonensis* isolates from Arkashin 2006 each had a relatively high number of polymorphic sites, $\geq$16, and multiple nonsynonymous substitutions (Table 6.S7). It therefore follows that if a selection event led to the lack of variation observed within the Arkashin 2005 isolates, at least a portion of the increased heterogeneity observed within the 2006 isolates was due to either migration or homologous recombination events.

Comparison of spatial diversity patterns of *T. uzonensis* and *T. siderophilus*

Differences in the heterogeneity of the universally conserved protein coding gene sequences of *T. uzonensis* and *T. siderophilus* strains were observed, most notably with the *gyrB* and *leuS* gene sequence sets (Tables 6.S5 and 6.S6). The *leuS* gene sequence set from the 14 *T. siderophilus* isolates had six nucleotide sequence variants and 63 polymorphic sites (Table 6.S6). Phylogenetic analyses revealed discontinuity within the set of *leuS* gene sequences from *T. siderophilus* isolates, demonstrating that lateral gene transfer had occurred (Fig. 6.5). Genetic modification of microorganisms by lateral transfer is regarded as a widespread natural occurrence (Beiko et al., 2005), although some have noted that there is little evidence for recent, real-time evolution that is due to frequent and ongoing lateral gene transfer (Achtman and Wagner, 2008). Nonetheless, the stretches of foreign DNA observed within sequenced genomes implies that lateral gene transfer has significantly contributed to the natural diversity of prokaryotes (Fraser et al., 2009). Differences in the amount of intraspecies divergence among species within the same genus has previously been observed for *Thermus* strains isolated from Icelandic geothermal sites (Hreggvidsson et al., 2006). Analyses with bacteria of the family *Vibrionaceae* within the coastal waters revealed that closely related groups of bacteria can be ecologically divergent (Hunt et al., 2008). The type strains of *T. uzonensis* and *T. siderophilus* have physiological differences, for example, *T. siderophilus* SR4^T was found to grow on H_2/CO_2 in the presences of Fe(III) (Slobodkin et al., 1999), whereas growth on H_2/CO_2 was not observed for *T. uzonensis* JW/IW010^T (Wagner et al., 2008). Future studies could be developed to determine if and how *T. uzonensis* and *T. siderophilus* are ecologically divergent within the geothermal springs of Kamchatka. With additional *T. siderophilus* isolates, a thorough

comparison of the patterns of spatial variation among different *Thermoanaerobacter* species could also be performed.

CONCLUSIONS

An examination of eight universally conserved protein coding gene sequences from a set of *Thermoanaerobacter* isolates from geothermal springs in Kamchatka revealed patterns of spatial variation. Particular sequence variants were regularly observed within multiple isolates from one particular geothermal spring and not elsewhere. Evidence for linkage disequilibrium was also found considering the entire *T. uzonensis* population. A range of *T. uzonensis* intra-geothermal spring heterogeneity was observed. The observation that sets of isolates from several geothermal springs form two distinct clades on a prepared phylogenetic tree could indicate that there are multiple niches for *T. uzonensis* within a particular geothermal spring. On the other hand, the results from the clustering of sequence types via eBURST may indicate that the population is divided into more-generalized and more-specialized lineages. β-diversity analyses revealed that the greatest variation was between isolates from geothermal springs within different regions, although large differences within a region were also observed, e.g., the F_{ST} value of 0.56 calculated between the Vent 1 North and Arkashin *T. uzonensis* populations (Table 6.3). Comparisons of the local:global taxa richness revealed that the heterogeneity within the isolates of a particular spring was usually much less than the heterogeneity observed for the entire population (Fig. 6.4).

The size, volume, and type of geothermal spring may influence the *T. uzonensis* intraspecies variation observed therein and testable hypotheses could be developed to examine these relationships. The environmental and temporal variability of a geothermal spring

undoubtedly influence the array of niches in the environment. Additional insight concerning intra-geothermal spring niche adaptation and assessing specialist/generalist characteristics would come from genome sequencing or by assessing physiological, metabolic, and biochemical differences between isolates using, for example, Phenotype MicroArrays (Biolog, Inc.). Longitudinal studies could be employed to determine the occurrence or frequency of selective sweeps within the *T. uzonensis* population.

A notable difference in the observed variation between the *T. uzonensis* and *T. siderophilus* isolates was that there was no variation within the *gyrB* gene sequences of the *T. siderophilus* isolates, while variation within the *gyrB* of the *T. uzonensis* isolates was principally delineated by geographic region (Fig. 6.S1, Table 6.S14). Additionally, strong evidence of lateral gene transfer was seen within the *leuS* gene sequence set of the *T. siderophilus* isolates (Fig. 6.5). Together, these analyses suggest that the homologous core-genome genes within different species of the same genus are under different levels of evolutionary selection and that lateral gene transfer of core-genome genes occurs.

EXPERIMENTAL PROCEDURES

Sample collection and isolation of *Thermoanaerobacter* strains have been previously described (Chapter 5). Briefly, each isolate was derived from its own separate enrichment culture. Genomic DNA extraction, gene sequence amplification, and sequencing were performed using standard protocols (Table 6.S1; Chapter 5). The set of protein coding genes sequenced and analyzed were among those suggested by Santos and Ochman (2004); DNA gyrase subunit B (*gyrB*), GTP-binding protein LepA (*lepA*), leucyl-tRNA synthetase (*leuS*), CTP synthase (*pyrG*),

bacterial DNA recombination protein RecA (*recA*), ATP-dependent DNA helicase RecG (*recG*), 50S ribosomal protein L2 (*rplB*), and RNA polymerase subunit B (*rpoB*).

Within most analyses, sites with missing information in a multiple sequence alignments were not considering during calculations of the gene sequence diversity. However, the fragment length examined considering a subset of *Thermoanaerobacter* isolates was often slightly longer than the fragment length examined when the entire set of isolates was examined. Phylogenetic trees were generated with the Minimum Evolution method with Jukes and Cantor genetic distance calculations or with the Maximum Parsimony method using MEGA 4.1 (Tamura et al., 2007).

Gene sequence polymorphism, as nucleotide and amino acid sequence diversity measures, were calculated with DnaSP (Rozas and Rozas, 1999) or MEGA 4.1 (Tamura et al., 2007). The specific gene sequence polymorphism and diversity properties determined included: the total number of segregating (i.e., polymorphic) nucleotide sites, S_{nt}; the G+C mol% of the gene sequence set; the number of variant forms of the gene (i.e., different alleles or loci), ntH; the average number of nucleotide differences, k; and the average number of nucleotide substitutions per site, π.

The polymorphism and diversity properties calculated from the deduced primary protein sequence included: the maximum amino acid difference, max ΔX; the average amino acid difference, mean ΔX; and the number of variant forms of the primary protein sequence, aaH. To compare synonymous and non-synonymous substitutions, d_S and d_N respectively, the Nei-Gojobori method was utilized with the Jukes-Cantor correction (Tamura et al., 2007).

Diversity within a geothermal spring was assessed by: i) tallying the number of gene sequence sets wherein multiple nucleotide sequence variant forms (i.e., where ntH > 1) are

observed, ii) tallying the number of nucleotide- and primary protein sequence variant forms, and iii) by calculating the gene sequence heterogeneity, π, for each gene sequence set and as an average, considering the set of *T. uzonensis* isolates from each geothermal spring.

Diversity between geothermal springs and regions was assessed as i) the average proportion of nucleotide differences, k, between populations, ii) generating a local:global taxa richness ratio by comparing the number of nucleotide and primary protein sequence variant forms within a geothermal spring to the total number observed, iii) determining population pairwise F_{ST} values, and iv) performing analysis of molecular variance (AMOVA). The local:global taxa richness ratios are given as ntH_S/ntH_T and aaH_S/aaH_T, where ntH_S was the number of nucleotide sequence variants observed within the population in a single geothermal spring, ntH_T was the total number of nucleotide sequence variants observed in the population, aaH_S was the number of primary protein sequence variants bserved within the population in a single geothermal spring, and aaH_T was the total number of primary protein sequence variants observed in the population. Population pairwise F_{ST} values and the population genetic structure inferred by AMOVA were calculated using Arlequin ver 3.11 (Excoffier et al., 2005). The AMOVA and population pairwise F_{ST} value calculations were based on concatenates of the eight protein coding gene sequences. The eBURST program was used to cluster *T. uzonensis* sequence types (Feil et al., 2004; Spratt et al., 2004); the subsequent clusters of isolates were called eBURST Groups. Linkage disequilibrium was assessed through calculation of the index of association, I_a, the observed variance, V_{obs}, and the maximum variance observed with any of the randomized data sets, V_{max}, using the MLST database, http://www.mlst.net/ (Feil et al., 2004).

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Table 6.1. Summary of *Thermoanaerobacter* strains isolated from Kamchatkan geothermal springs						
Region	**Geothermal Spring (sample year)**	**T (°C)**	**pH**	**geothermal spring abbreviation**	***T. uzonensis* isolates**	***T. siderophilus* isolates**
Uzon Caldera	Arkashin (2005)	55-80	5.0-6.5	I502	10	
	Arkashin (2006)			A615	16	
	Burlyashi (2006)	58	7	B621	29	
	Jen Vent 2 (2006)	72-78	5.2	J614	16	
	ON1 (2006)	65-72	5.5	O621	16	
	Pippe's Bay (2006)	63-60	5.5-6	P635		3
	Thermophilny (2005)	62-65	7.4	T515	24	
	Thermophilny (2006)			H608	11	
	Vent 1 North (2006)	67	6	V634	31	
	Zavarzin (2006)	50-74	5.5-7.5	Z606	28	
Geyser Valley	Resting Rock (2006)	76	8-8.5	R649	6	9
	Proximal to Salt Spring (2006)	65-69	6.5	S648	8	
Mutnovsky Volcano	Mutnovsky-4 (2005)	70-89	6-6.5	M504	13	2

Table 6.2. eBURST Clustering of *Thermoanaerobacter uzonensis* isolates. Values correspond to the number of isolates from the particular geothermal spring (a row) that cluster to the particular eBURST group (the columns). Geothermal spring abbreviations are as follows: Arkashin 2006, A615; Arkashin 2005, I502; Burlyashi, B621; Thermophilny 2006, H608; Thermophilny 2005, T515; Pulsating Spring (Jen's Vent 2), J614; Mutnovsky, M504; ON1, O629; Proximal to Salt Spring, S648; Vent 1 North, V634; and Zavarzin, Z606. Far right column shows the total number of eBURST groups into which isolates from each geothermal spring binned.

	eBURST Group Number																										
Spring	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**Total:**
Z606	3	6																								1	1	4
T515	2					1	2												2									4
H608	2					10													5	1	1							5
J614				3	4																							2
I502										8																		1
A615	2									3	1	1																4
B621	6	2		1		1							1	1	3	1	1	1										10
O629	1							3	8																			3
V634	7				1																				10			3
R649																						1	1					2
S648																						2		2				2
M504			10																									1

eBURST group fraction color. The the fraction of isolates within a particular eBURST group that were isolated from the particular spring.			
0-.24	.25-.49	.50-.74	.75-1.0

Table 6.3. *Thermoanaerbacter uzonensis* population pairwise F_{ST} values. Based on concatenated sequences of eight universally conserved protein coding gene sequences; Number of usable loci for distance computation: 8865 Significant F_{ST} P values were calculated (significance level=0.05); values not significant are shaded grey. Geothermal spring abbreviations are as follows: Arkashin 2006, A615; Arkashin 2005, I502; Burlyashi, B621; Thermophilny 2006, H608; Thermophilny 2005, T515; Pulsating Spring (Jen's Vent 2), J614; Mutnovsky, M504; ON1, O629; Proximal to Salt Spring, S648; Vent 1 North, V634; and Zavarzin, Z606.

	A615 and I502	**B621**	**H608 and T515**	**J614**	**M504**	**O629**	**R649**	**S648**	**V634**	**Z606**
A615 and I502	0.00									
B621	0.32	0.00								
H608 and T515	0.46	0.10	0.00							
J614	0.47	0.21	0.36	0.00						
M504	0.83	0.68	0.78	0.78	0.00					
O629	0.30	0.11	0.21	0.16	0.59	0.00				
R649	0.66	0.45	0.63	0.45	0.75	0.23	0.00			
S648	0.77	0.53	0.67	0.61	0.87	0.29	0.36	0.00		
Z606	0.28	0.14	0.28	0.25	0.72	0.17	0.43	0.58	0.00	
V634	0.56	0.14	0.31	0.41	0.83	0.13	0.66	0.70	0.30	0.00

Table 6.4. *Thermoanaerobacter uzonensis* population genetic structure inferred by analysis of molecular variance (AMOVA). Columns show the percent of total variance attributed to the intra-population and its significance (P-value), based on 1023 permutations.

Source of variation	% of variance	P-value
Among regions	51.4	< 0.005
Among populations within regions	13.2	<0.0001
Within populations	35.4	<0.0001

Table 6.5. Summary of the overall universally conserved protein sequence heterogeneity of *Thermoanaerobacter uzonensis* isolates from Kamchatkan geothermal springs.

	gyrB	***lepA***	***leuS***	***pyrG***	***recA***	***recG***	***rplB***	***rpoB***
Length	1111	1255	1040	1201	739	1227	501	911
N	123	123	123	123	123	123	123	122†
G+C mol %	34.5	35.9	39.2	38.2	39.3	33.5	37.1	31.1
S_{nt}	30	19	36	34	18	46	3	13
ntH	6	11	14	27	9	20	4	9
π (per site) *1000	5.35	1.05	4.93	5.84	1.24	10.34	0.61	4.18
k	5.9	1.32	5.13	7.01	0.91	12.68	0.31	3.81
d_N	0.0017	0.0003	0.0016	0.0023	0.0006	0.0056	0.0007	0.0003
d_S	0.019	0.0036	0.017	0.018	0.0035	0.026	0.0003	0.019
d_N/d_S	0.089	0.083	0.097	0.12	0.16	0.22	2.74	0.014
ΔX max	5	5	8	5	2	10	1	2
ΔX mean	1.30	0.26	1.30	2.06	0.30	4.74	0.27	0.16
aaH	5	7	10	14	3	19	2	4

†The isolate M504_35_2 has been removed from this analysis; the *rpoB* gene sequence from this isolate is similar to the corresponding gene from *T. siderophilus* isolates that have also been isolated from Kamchatkan geothermal springs (Wagner, 2010).

Table 6.6. Summary of the overall universally conserved protein sequence heterogeneity of *Thermoanaerobacter siderophilus* isolates from Kamchatkan geothermal springs.

	gryB	***lepA***	***leuS***	***pyrG***	***recA***	***recG***	***rplB***	***rpoB***
N	14	13	14	15[a]	14	7	14	14
Length	1179	1319	1144	1281	747	1324	667	1325
Inferred portion of gene sequence	0.62	0.73	0.47	0.8	0.73	0.65	0.81	0.88
S_{nt}	0	4	63	16	2	6	3	2
G+C mol %	35.4	36	39.3	37.7	39.7	33.4	39.8	33
ntH	1	4	6	5	3	4	4	3
π (per site) *1000	0	0.47	17	4.79	0.9	1.44	1.66	0.44
k	0	0.6	19.5	6.1	0.7	1.9	1.1	0.6
d_N	0	0.00045	0.00216	0.00025	0.00093	0.00083	0.00029	0
d_S	0	0.00051	0.07269	0.02076	0.00081	0.00366	0.006	0.00203
d_N/d_S	NA	0.882	0.030	0.012	1.148	0.227	0.048	NA
ΔX max	0	3	4	1	1	3	1	0
ΔX mean	0	0.46	1.58	0.25	0.53	0.86	0.14	0
aaH	1	3	3	2	2	3	2	1

[a]While 14 strains with ≥97% 16S rRNA gene sequence similarity to the *T. siderophilus* strain SR4^T were isolated, on strain from Thermophilny 2005, JW/IW T515 8_2, was found to have a *pyrG* gene similar to those found in *T. siderophilus* isolates; all other genes in JW/IW T515 8_2 were similar to those found in *T. uzonensis* isolates.

Table 6.7. Assessment of linkage disequilibrium between *Thermoanaerobacter uzonensis* isolates from different regions of Kamchatka. I_a, the index of association; V_{obs}, the observed variance in the distribution of allelic mismatches in all pair-wise comparisons of the allelic profiles; V_{avg}, average variance from randomized data sets; V_{max}, the maximum variance from randomized data sets. Disequilibrium (D) or equilibrium (E); population tested was determined to be in D, or E if *V*obs < *V*max, even if the I_a was not zero.

Inclusion of *T. uzonensis* isolates from	I_a	V_{obs}	V_{avg}	V_{max}	**D/E**	**Significance value**
Uzon Caldera, Geyser Valley, and Mutnovsky	0.42	1.96	1.41	1.88	D	p< 0.001
Uzon Caldera and Mutnovsky	0.176	1.65	1.37	1.59	D**	p< 0.05
Uzon Caldera and Geyser Valley	0.28	1.80	1.43	1.75	D**	p < 0.01
Uzon Caldera	-0.032	1.38	1.39	1.63	E	NA

**Note that when 1000 random datasets are analyzed (the p< 0.001 significance level), $V_{obs} < V_{max}$ (thus, E) for the comparison between isolates from the Uzon Caldera and Mutnovsky regions, and between the and Uzon Caldera and Geyser Valley regions.

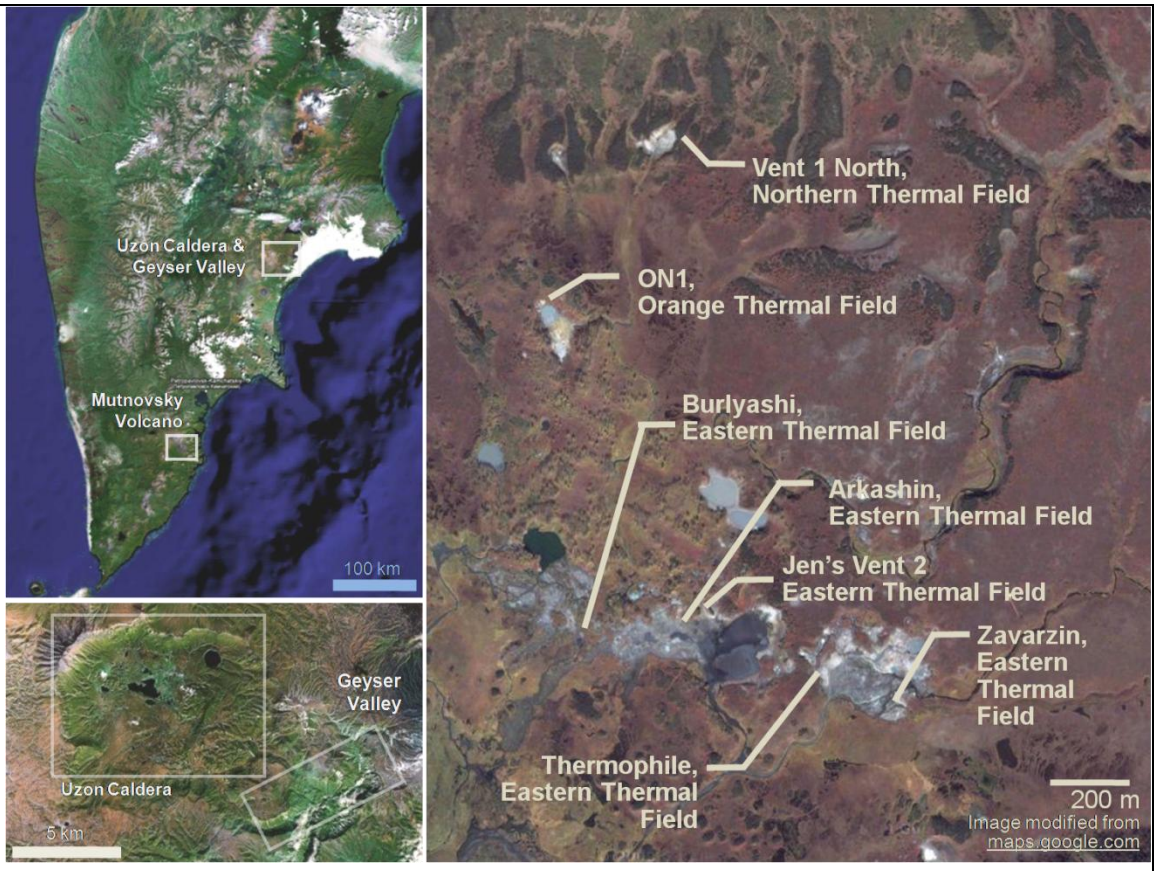

Fig. 6.1. Spatial location of the geothermal spring from which *Thermoanaerobacter uzonensis strains* were derived.

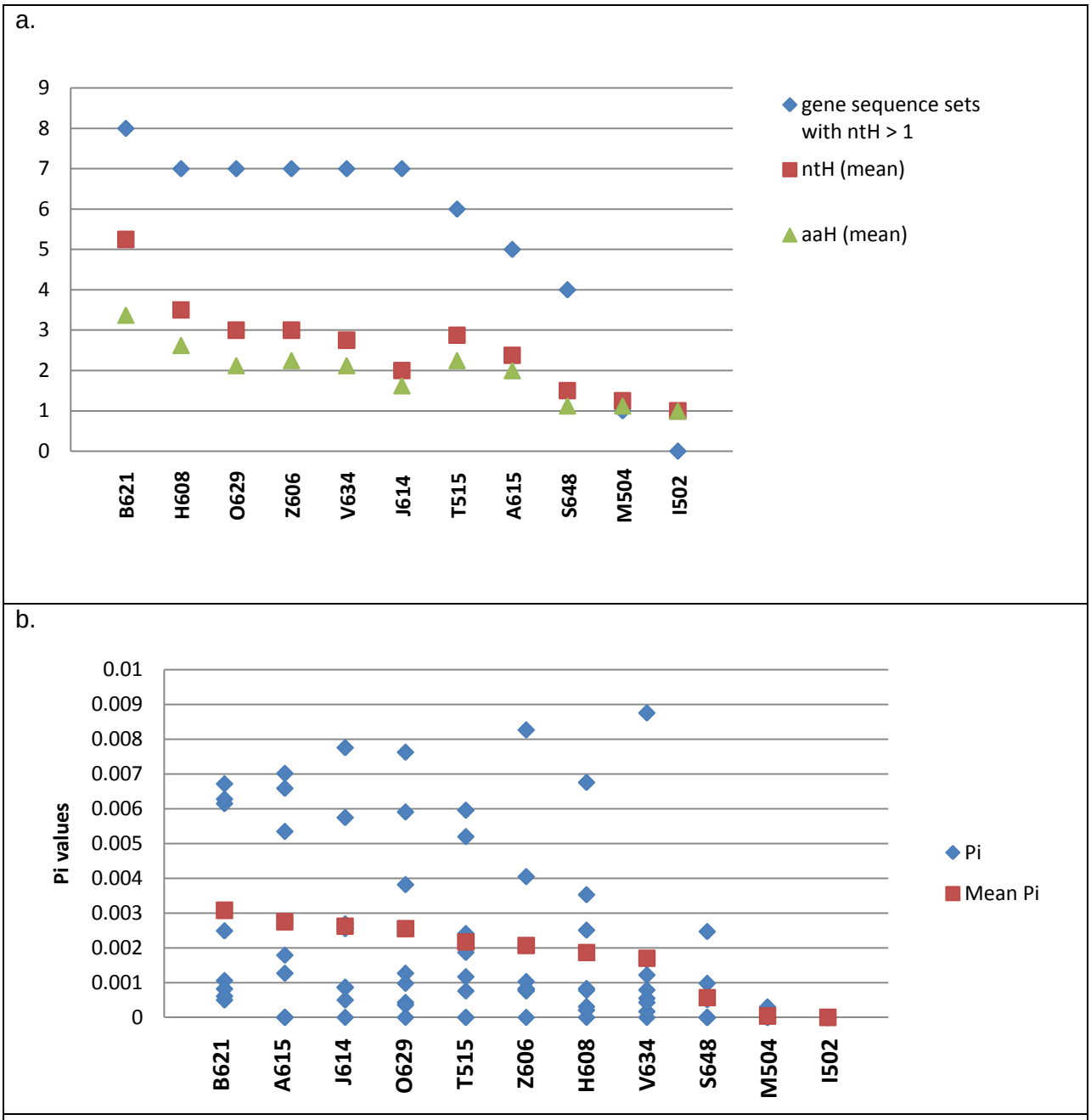

Fig. 6.2. *Thermoanaerobacter uzonensis* intra-geothermal spring heterogeneity. (a.) Number of gene sequence sets per geothermal spring where ntH > 1; 2. ntH (mean) per geothermal spring; 3. aaH (mean) per geothermal spring. (b.) π values for housekeeping gene sequence sets within the isolates from individual geothermal springs. Geothermal spring abbreviations are as follows: Arkashin 2006, A615; Burlyashi, B621; Thermophilny 2006, H608; Arkashin 2005, I502; Pulsating Spring (Jen's Vent 2), J614; Mutnovsky, M504; ON1, O629; Proximal to Salt Spring, S648; Thermophilny 2005, T515; Vent 1 North, V634; and Zavarzin, Z606.

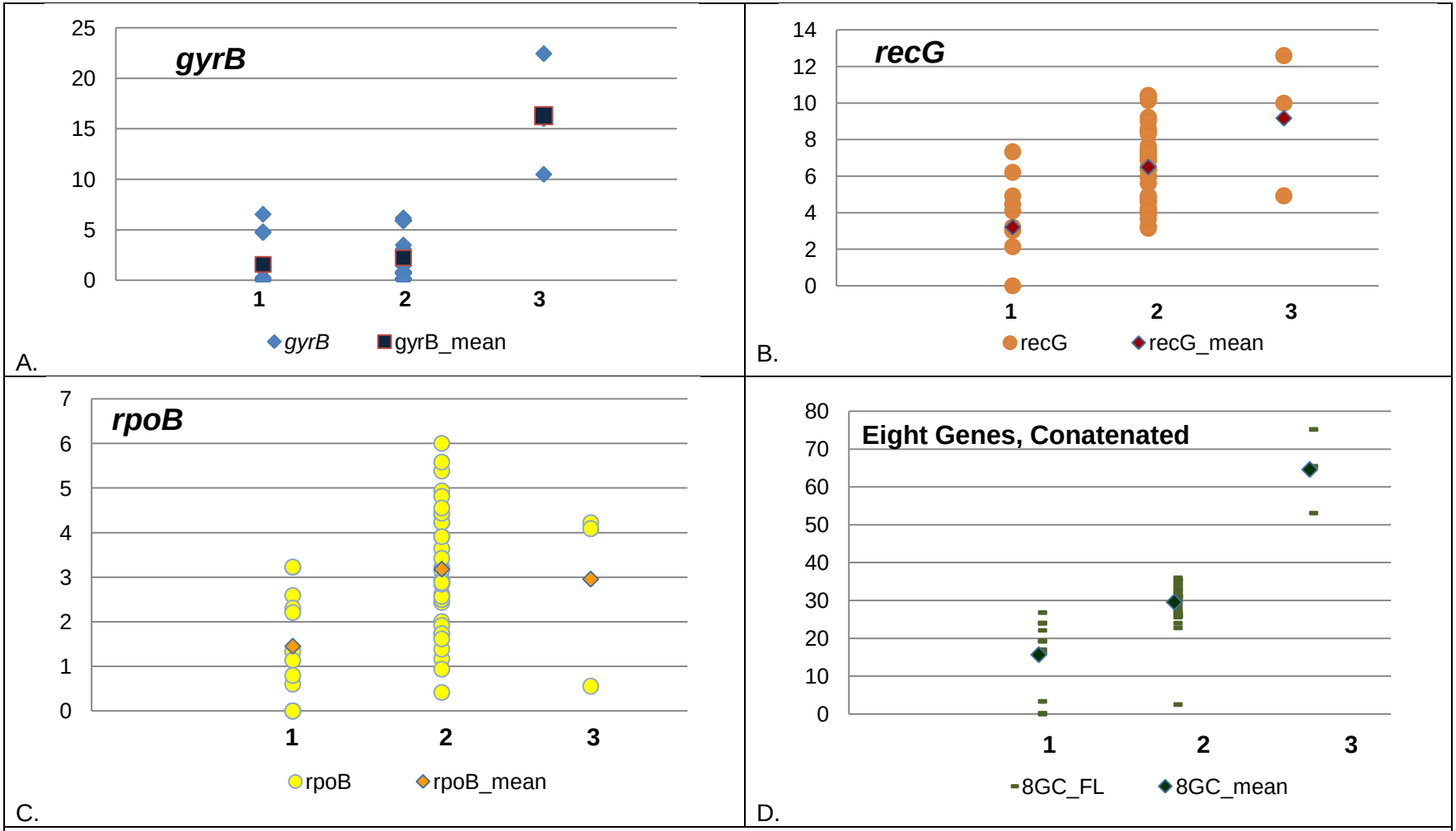

Fig. 6.3. Comparisons of *Thermoanaerobacter uzonensis* universally conserved protein coding gene sequence nucleotide divergence within a geothermal spring, between geothermal springs within the same region, and between geothermal springs of different regions. Y-axes: mean base differences per sequence, k. Labels on X-axes: Within a geothermal spring, 1; between geothermal springs within the same region, 2; and between geothermal springs of different regions. 3. Examples shown are based on (A) *gyrB*, (B) *recG*, (C) *rpoB*, and (D). eight protein coding genes, concatenated.

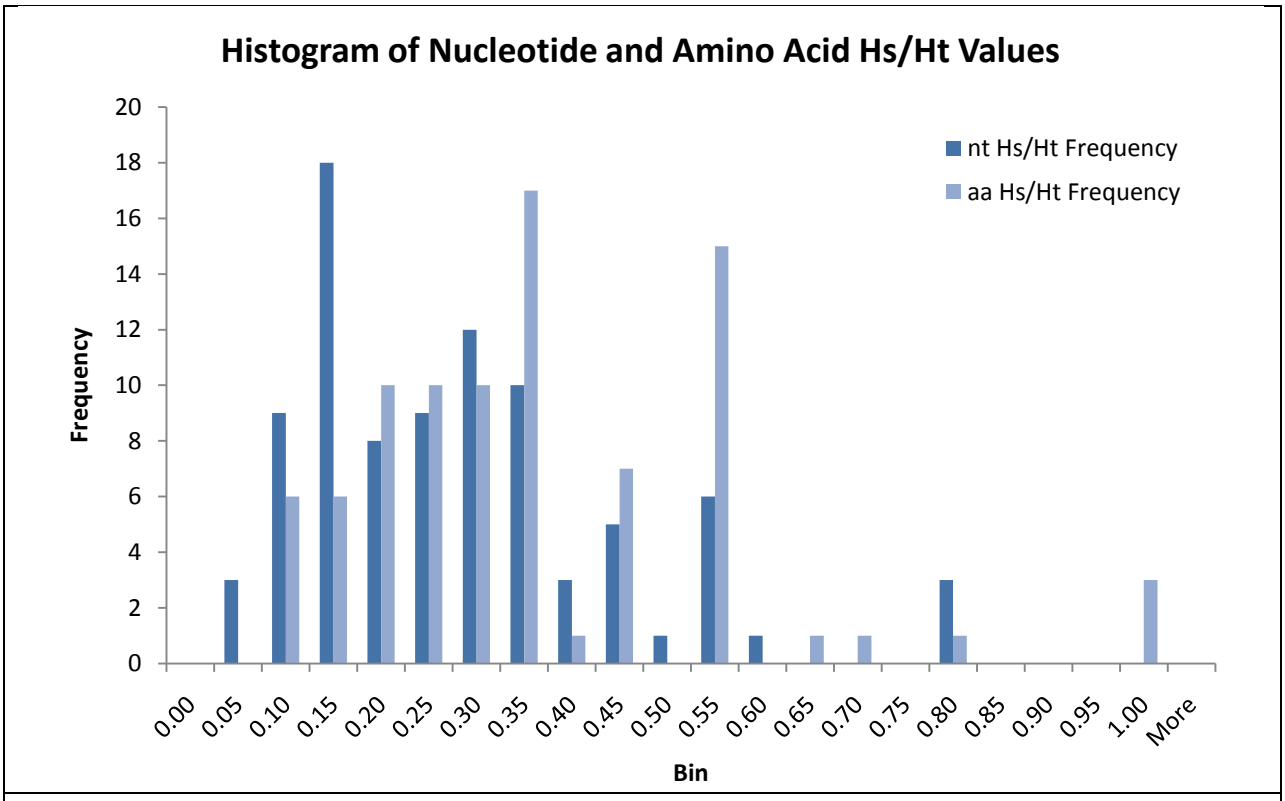

Fig. 6.4. *Thermoanaerobacter uzonensis* local:global taxa richness ratios shown as a histogram of ntH_S/ntH_T and aaH_S/aaH_T values.

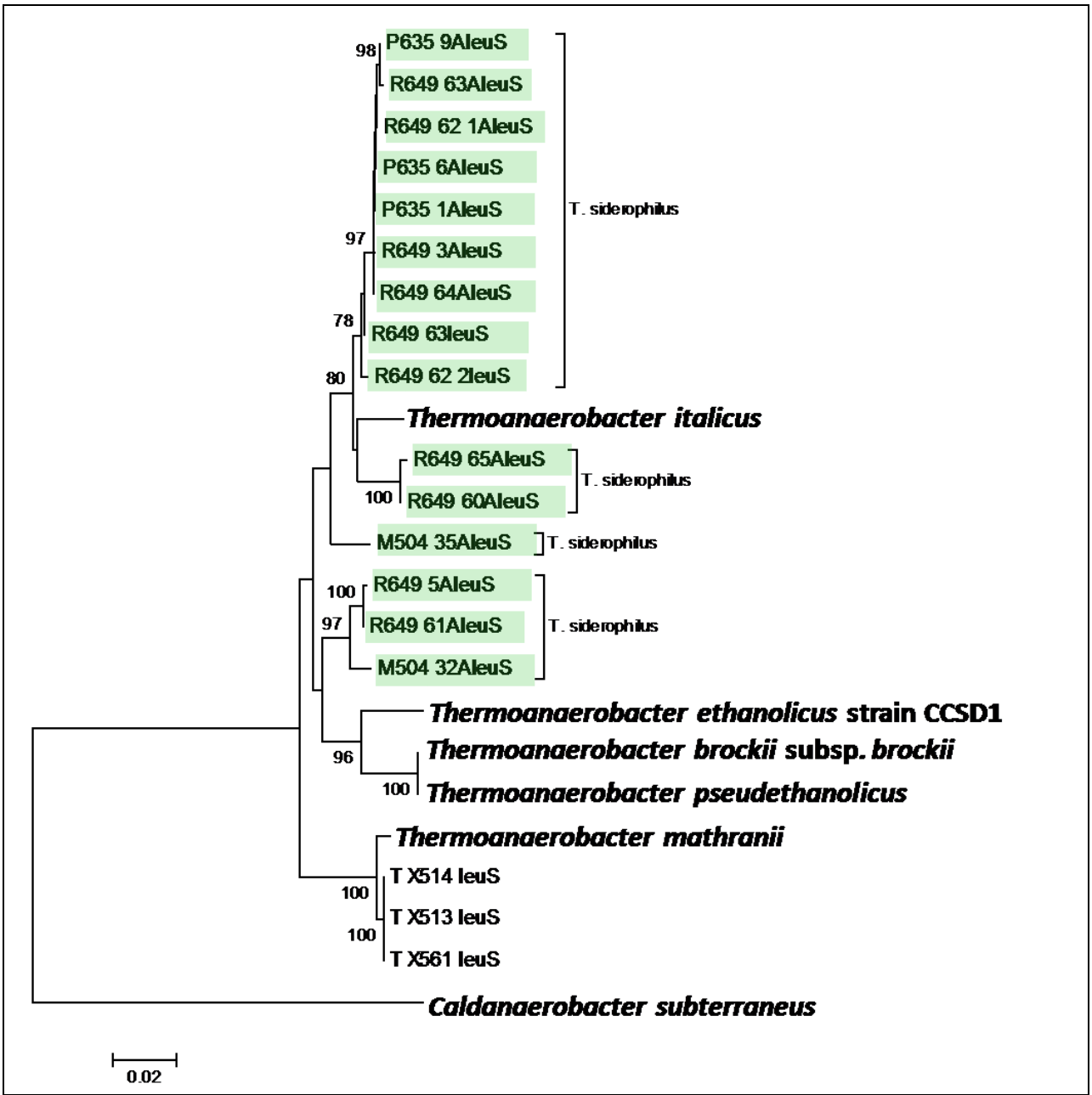

Fig. 6.5. Evolutionary relationships based on the *leuS* gene sequences of *Thermoanaerobacter siderophilus* isolates related *Thermoanaerobacteracae.* Genetic distances were calculated using the Jukes-Cantor method, and the evolutionary history was inferred using the Minimum Evolution method using MEGA 4.1 (Tamura et al., 2007). The optimal tree with the sum of branch length = 0.37 is shown and the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches. The *leuS* gene sequences from related taxa were *Thermoanaerobacter pseudethanolicus* ATCC 33223, GeneID:5873380; *Thermoanaerobacter italicus* Ab9, Thit_0284; *Caldanaerobacter* (basonym, *Thermoanaerobacter*) *subterraneus* subsp. *tengcongensis* strain MB4, locus_tag = TTE0324. The *leuS* nucleotide sequences of *Thermoanaerobacter ethanolicus* strain CCSD1, *Thermoanaerobacter brockii* subsp. *brockii*, and *Thermoanaerobacter mathranii* came from draft genomes sequences (C. L. Hemme, personal communication).

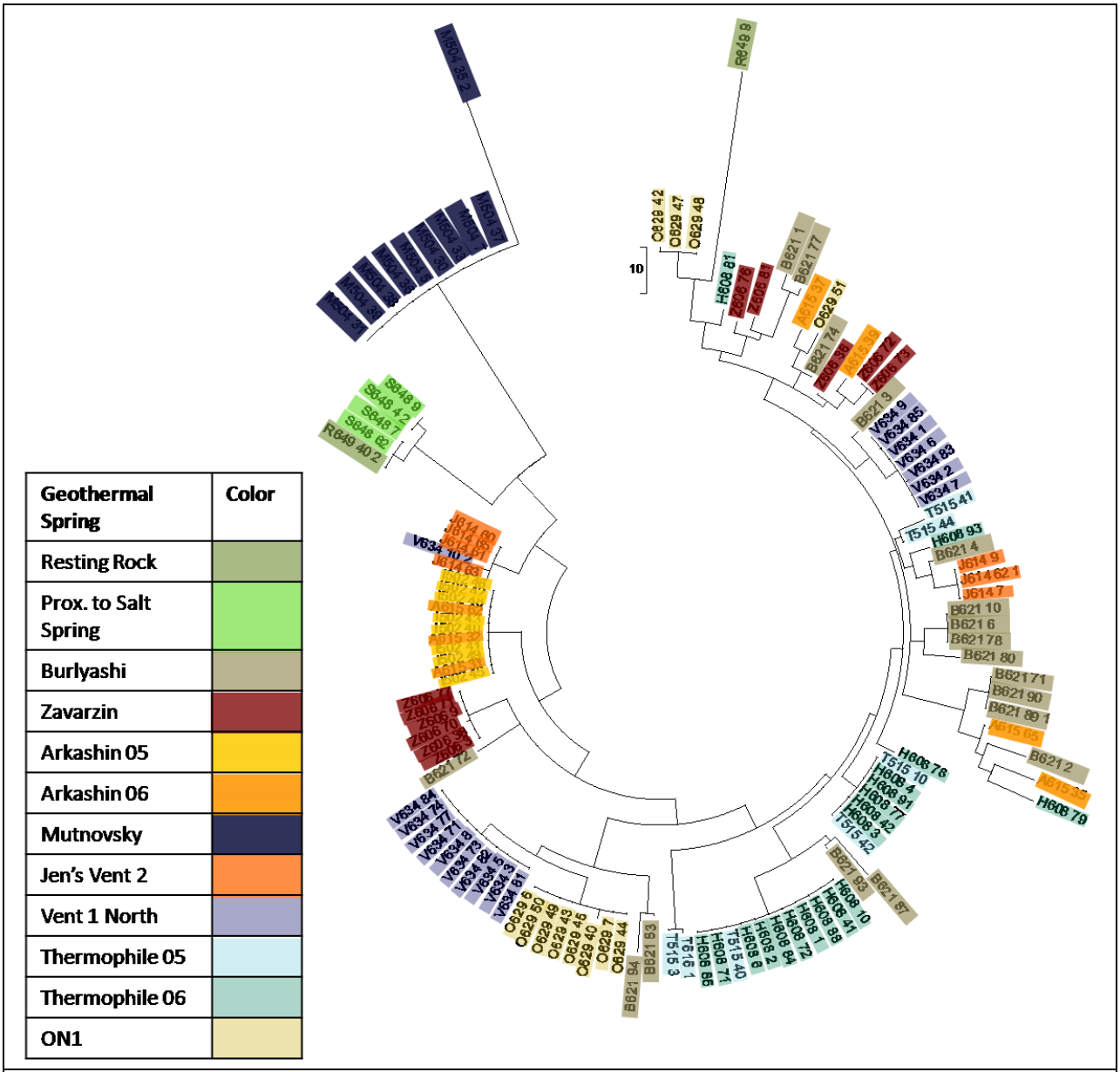

Fig. 6.6. Evolutionary relationships of 123 *T. uzonensis* isolates based on a concatenates of eight universally conserved protein coding genes. The evolutionary history was inferred using the Maximum Parsimony method. Tree #1 out of 285 most parsimonious trees (length = 530) is shown. The consistency index is 0.43 (0.32) and the retention index is 0.89 (0.89) for all sites and parsimony-informative sites in parentheses. The Maximum Parsimony tree was obtained using the Close-Neighbor-Interchange algorithm. There were a total of 7985 positions in the final dataset, out of which 145 were parsimony informative. Phylogenetic analyses were conducted in MEGA4.1(Tamura et al., 2007).

SUPPORTING INFORMATION

T. uzonensis isolates from the geothermal spring Zavarzin

The geothermal spring Zavarzin is located in the east sector of the Eastern Thermal field of the Uzon Caldera (Fig. 6.1). The eight universally conserved protein coding genes employed in this study have been sequenced from 11 isolates from Zavarzin. Nucleotide sequence polymorphisms were observed within all gene sequence sets except the *rplB* gene sequence set (Table 6.S2

A number of nucleotide- and primary protein sequence variant forms were found only within the *T. uzonensis* isolates from Zavarzin. Within the set of *T. uzonensis recA* gene sequences from the Zavarzin isolates, the nucleotide sequence variant form number 5 (abbreviated *Tu recA* ntH_5) was found only from 3 isolates from Zavarzin (Table 6.S18). Likewise, within the *recG* gene sequence set, 4 nucleotide- and primary protein sequence variant forms were only found within the *T. uzonensis* isolates from Zavarzin (Fig. 6.S6). The Zavarzin isolates were distributed among 4 eBURST groups (where 6 of 8 nucleotide sequence variants had to be identical to others in the group for inclusion), Group 26 and Group 27 were each composed of one Zavarzin *T. uzonensis* isolate (Table 6.2). The phylogenetic tree based on the concatenates of the eight protein coding genes from the *T. uzonensis* isolates revealed one clade of 6 Zavarzin isolates with nearly identical gene sequences, the remaining isolates were not grouped together, but were on the same branch of the phylogenetic tree (Fig. 6.6).

T. uzonensis isolates from the geothermal spring Thermophilny

The geothermal spring Thermophilny is located in the east sector of the Eastern Thermal Field of the Uzon Caldera (Fig. 6.1). The 8 universally conserved protein coding genes employed in this study have been sequenced from 7 isolates from the Thermophilny 2005 sample and 19

isolates from a sample collected in 2006. Variation was observed within the *gyrB* gene sequence set from the Thermophilny 2005 isolates (Table 6.S3), but there was no *gyrB* heterogeneity observed within the Thermophilny 2006 isolates (Table 6.S4). Conversely, there was no variation within either the *rplB* or *rpoB* gene sequence of the Thermophilny 2005 isolate sets (Table 6.S3), while variation was observed within these two protein coding genes as found within the Thermophilny 2006 isolate set (Table 6.S4). Although variation at the nucleotide level was observed within the *T. uzonensis* Thermophilny 2006 *rplB* or *rpoB* gene sequence sets, only synonymous substitutions were observed (Table 6.S4). Within the Thermophilny 2005 isolate set, two *gyrB* nucleotide sequence variants were observed, *Tu gyrB* ntH_1 and *Tu gyrB* ntH_5; however within the Thermophilny 2006 *T. uzonensis* isolates, only the *Tu gyrB* ntH_1 sequence variant was the observed (Table 6.S14). The gene sequence variants *Tu gyrB* ntH_1 and *Tu gyrB* ntH_5, found in the Thermophilny 2005 isolates, were notably divergent on the corresponding nucleotide sequence-based phylogenetic tree (Fig. 6.S1). The four *leuS* nucleotide sequence variant forms found within the Thermophilny 2005 isolates were also found in the Thermophilny 2006 isolates (Table 6.S16).

Two of the *lepA* sequence variants found only within *T. uzonensis* isolates from Thermophilny 2005 were notably divergent from the other *lepA* sequence variants, *Tu lepA* ntH_1, aaH_1 and to a lesser extent *Tu lepA* ntH_2, aaH_2 (Fig. 6.S2). The highest value observed *lepA* ΔX max value, within any individual geothermal springs, 3, was observed within the Thermophilny 2005 isolates. There were 2 *pyrG* sequence variant forms found only in the *T. uzonensis* isolates from Thermophilny 2005 and Thermophilny 2006 (Table 6.S17). The Thermophilny 2005 isolates were binned within 4 eBURST Groups, and the Thermophilny 2006 isolates were binned within 5 eBURST Groups. Thermophilny isolates were shared between

three eBURST groups, and eBURST Group 19 contains only Thermophilny 2005 and 2006 isolates (Table 6.2). Many of the Thermophilny 2005 and 2006 isolates were grouped together within two clades on the phylogenetic tree based on the concatenates of the eight protein coding genes (Fig. 6.6).

***T. uzonensis* isolates from the geothermal spring Pulsating Spring (Jen's Vent 2)**

Pulsating Spring, also called "Jen's Vent 2," feeds Winding Creek, which is located on the northern side of the central sector of the Eastern Thermal field of the Uzon Caldera (Fig. 6.1). The 8 universally conserved protein coding genes employed in this study have been sequenced from 7 isolates from Pulsating Spring. Nucleotide sequence heterogeneity was observed within all universally conserved protein coding gene sequence sets with the exception of *recA* (Table 6.S5). A single primary protein sequence variant was observed within the *recA*, *lepA* and *rplB* gene sequence sets (Table 6.S5). The other universally conserved protein coding gene sequence sets, *gyrB*, *leuS*, *pyrG*, *recG*, and *rpoB* have 2 nucleotide- and 2 primary protein sequence variant forms (Table 6.S5).

Although only 2 nucleotide sequence variant forms were found within the *gyrB* and *recG* genes from the Pulsating Spring *T. uzonensis* isolates, the corresponding S_{nt} values ≥ 13 (Table 6.S5). Within the *gyrB* gene sequence set, this high S_{nt} value was found to be result of divergent *gyrB* nucleotide sequence variant forms (Fig. 6.S1, Table 6.S14). None of the *T. uzonensis* isolates from Pulsating Spring had the *gyrB* ntH_1, aaH_1 sequence variant, which was found within isolates from all the other Uzon Caldera geothermal springs (Table 6.S14). The *Tu recG* ntH_13, aaH_4 nucleotide- and primary protein sequence variant was only found within 4 Pulsating Spring *T. uzonensis* isolates (Table 6.S19). The Pulsating Spring *T. uzonensis* isolates were binned within two eBURST groups and neither group exclusively contains Pulsating Spring

isolates (Table 6.2). Notably, none of the *T. uzonensis* isolates from Pulsating Spring bin to eBURST Group 1, a group composed of isolates from all other Uzon Caldera geothermal springs (Table 6.2).On the phylogenetic tree based on the concatenated sequence of the 8 protein coding gene sequences, the Pulsating Spring isolates for two phylogenetically separated clades (Fig. 6.6).

***T. uzonensis* isolates from the geothermal spring Arkashin**

The geothermal spring Arkashin was anthropogenically-generated and is located within the central sector of the Eastern Thermal Field of the Uzon Caldera (Fig. 6.1). The 8 universally conserved protein coding genes employed in this study have been sequenced from 8 isolates from the Arkashin sample collected in 2005 and 7 isolates from the sample collected in 2006. The set of Arkashin 2005 isolates were unique in that there was no heterogeneity observed within any of the universally conserved protein coding genes sequence sets (Table 6.S6). Heterogeneity was observed within 5 of the 8 universally conserved protein coding genes sequence sets of the Arkashin 2006 *T. uzonensis* isolates (Table 6.S7). The nucleotide sequence variant forms found within the Arkashin 2005 isolates were , in all cases, also found within the set of Arkashin 2006 isolates (see Table 6.S13). Within the Arkashin 2006 *T. uzonensis* isolate set the *leuS*, *pyrG*, and *recG* gene sequence sets, the S_{nt} values ≥ 16 (Table 6.S7). The Arkashin 2006 isolates *pyrG* ΔX max, 5, was the same as the overall *T. uzonensis pyrG* ΔX max (Tables 6. S7 and 6.S5).

The same *rpoB* nucleotide sequence variant, *Tu rpoB* ntH_5, was found within *T. uzonensis* isolates from Arkashin 2005 and 2006, as well as isolates from Mutnovsky-4, Resting Rock, and Salt Spring (Table 6.S21). There were two nucleotide sequence variant forms within the *pyrG* and *recG* gene sequence sets that were found only in isolates from Arkashin 2005 and

2006 Figs. 6.S4 and 6.S6). The eBURST Group 10 only contains the sequence types of Arkashin 2005 and 2006 isolates (Table 6.2). The phylogenetic tree based on the concatenates of the eight protein coding genes revealed that all Arkashin 2005 and three Arkashin 2006 isolates group together; the remaining Arkashin 2006 isolates appeared scattered throughout other branches of the phylogenetic tree (Fig. 6.6).

***T. uzonensis* isolates from the geothermal spring Burlyashi**

The geothermal spring Burlyashi is located within the central sector of the Eastern Thermal Field of the Uzon Caldera (Fig. 6.1). The 8 universally conserved protein coding genes employed in this study have been sequenced from 18 isolates from Burlyashi. Variation was observed within all eight of the universally conserved protein coding gene sequence sets. Moreover, by most diversity measures, the gene sequence sets from the Burlyashi *T. uzonensis* isolates have the greatest variation; these isolates have the highest number of nucleotide- and primary protein sequence variants, as well as the highest average π and highest ΔX mean values (Table 6.S5). While a single primary protein sequence variant was observed within the *lepA* and *rplB* gene sequence sets of the Burlyahsi *T. uzonensis* isolates, multiple primary protein sequence variants were observed within the other 6 universally conserved protein coding gene sequence sets (Table 6.S8). The *leuS*, *pyrG*, and *recG* gene sequence sets for Burlyashi *T. uzonensis* have ≥ 22 nucleotide polymorphic sites (Table 6.S8). The ΔX max value observed for the *leuS* B621 *T. uzonensis* isolates, 7, nearly matches the overall ΔX max value observed, 8 (Table 6.S8). The ΔX max value of observed within the Burlyashi *recG* gene sequence set, 8 residues, was the highest observed within any single intra-geothermal spring gene sequence set (Table 6.S8 and 6.S5).

Within the different universally conserved protein coding gene sequence sets, there were a number of instances where a particular gene sequence variant form was found only within the Burlyashi isolates; examples were seen within the *gyrB, leuS, pyrG, recA,* and *recG* gene sequence sets (Tables 6.S14, 6.S16, 6.S17, 6.S18, and 6.S19). The phylogenetic tree based on the *T. uzonensis leuS* gene sequence reveals that the *Tu leuS* nt_1, aaH_1, found within one Burlyashi isolate, was notably divergent from the other *leuS* nucleotide sequence variants (Fig. 6.S3). Seven of the 10 eBURST groups for the Burlyahsi *T. uzonensis* isolate set were composed of only a single isolate (Table 6.2). Six Burlyashi isolates bin to eBURST Group 1 which also contains isolates from most other Uzon Caldera geothermal springs from which *T. uzonensis* isolates were isolated (Table 6.2). While some of the *T. uzonensis* isolates from Burlyashi grouped together on the concatenated sequence-based phylogenetic tree, what was most notable were the other Burlyashi isolates that were scattered throughout the phylogenetic tree (Fig. 6.6).

***T. uzonensis* isolates from the geothermal spring ON1**

The geothermal spring ON1 is located on the northern side of the Orange Thermal Field of the Uzon Caldera (Fig. 6.1). The 8 universally conserved protein coding genes employed in this study have been sequenced from 12 isolates from ON1. The greatest gene sequence heterogeneity was observed within the *pyrG* and *recG* gene sequence sets, and there was no variation observed within the *recA* gene sequence set from the ON1 *T. uzonensis* isolates (Table 6.S9). The *recG* primary protein sequence variant of the ON1 *T. uzonensis* isolates vary by up to 6 amino acid residues; the sequences were , as expected, notably divergent on the corresponding nucleotide-sequence based phylogenetic tree (Table 6.S19, Fig. 6.S6). The other universally conserved protein coding gene sequence sets found within the ON1 *T. uzonensis* isolates, *gyrB, lepA, leuS, rplB,* and *rpoB* all have two primary protein sequence variants for which the ΔX max

between the sequence variants was 1 residue(Table 6.S9). Although there were 18 polymorphic nucleotide sites that correspond to 4 amino acid residue differences within the ON1 *pyrG* gene sequence set, there were only two primary protein sequence variants (Table 6.S9).

Eight of the 12 ON1 *T. uzonensis* isolates have the *Tu rplB* ntH_3, aaH_2 nucleotide- and primary protein sequence variant, which was elsewhere only found within the Arkashin 2005 and 2006 isolates (Table 6.S20). The *Tu rpoB* ntH_9 nucleotide sequence type, was found only in 3 ON1 *T. uzonensis* isolates (Table 6.S21). The ON1 *T. uzonensis* isolates were binned into three eBURST groups. One ON1 isolate binned to eBURST Group 1, which contains isolates from most of the other Uzon Caldera geothermal springs (Table 6.S2). The other two eBURST groups wherein the sequence types from ON1 isolates were found, Group 8 and Group 9 contain only ON1 isolates (Table 6.2). Most of the ON1 *T. uzonensis* isolates, with the exception of a single isolate, were found within two separated clades on the phylogenetic tree based on the concatenates of the eight protein coding genes (Fig. 6.6).

***T. uzonensis* isolates from the geothermal spring Vent 1 North**

The geothermal spring Vent 1 North is located in the Northern Thermal Field of the Uzon Caldera (Fig. 6.1). The 8 universally conserved protein coding genes employed in this study have been sequenced from 18 Vent 1 North *T. uzonensis* isolates. Apart from the *recA* gene sequence set, variation at the nucleotide level was observed within all other universally conserved protein coding gene sequence sets (Table 6.S10). As observed previously with *gyrB* gene sequence set from the Pulsating Spring and Thermophilny 2006 *T. uzonensis* isolates, the high *gyrB* S_{nt} value observed within the Vent 1 North isolates was due to the isolates having two notably different gene sequence variant forms, specifically *Tu gyrB* ntH_1 and *Tu gyrB* ntH_5 (Fig. 6.S1). The Vent 1 North *T. uzonensis gyrB, leuS, pyrG,* and *recG* gene sequence sets have the same number

of nucleotide sequence variants, ntH, as primary protein sequence variants, aaH (Table 6.S10). Within the Vent 1 North *T. uzonensis recG* gene sequence set the number of polymorphic sites, 28, was notably high. The corresponding Vent 1 North *T. uzonensis recG* gene sequence set π value was the highest observed in this universally conserved protein coding gene sequence study (Table 6.S10, Fig. 6.2b).

Ten Vent 1 North *T. uzonensis* isolates have the *Tu pyrG* ntH_13 sequence variant, and 7 isolates have the *Tu pyrG* ntH_14, aaH_9 sequence variant, both were found only in isolates from Vent 1 North (Table 6.S17). Assessing the eBURST clustering of sequence types, the Vent 1 North isolates were binned to three groups: eBURST Group 1, which contains isolates from many of the other Uzon Caldera geothermal springs; eBURST Group 5, wherein isolates from Jen's Vent 2 were also found; and eBURST Group 25, which contains 10 *T. uzonensis* isolates all from Vent 1 North (Table 6.2). As observed with the ON1 isolates, the Vent 1 North *T. uzonensis* isolates, with the exception of one isolate, were found within two separated clades on the phylogenetic tree based on the concatenates of the eight protein coding genes (Fig. 6.6).

T. uzonensis isolates from the geothermal springs within the Geyser Valley

T. uzonensis strains were isolated from samples collected from Resting Rock, and Proximal to Salt Spring, two geothermal springs within the Geyser Valley region (Fig. 6.1). The 8 universally conserved protein coding genes employed in this study have been sequenced from 2 isolates from Resting Rock and 4 isolates from Proximal to Salt Spring. One of the Resting Rock *T. uzonensis* isolates, JW/IW R649_9, was distinctive in that each universally conserved protein coding gene sequence from this isolates was a unique sequence variant and not observed within any other isolate (Wagner, 2010). Some of the sequence variants found within the isolate

JW/IW R649_9 were notably divergent, e.g., the *recA* nucleotide sequence variant form *Tu recA* ntH_9, aaH_3 (Fig. 6.S5).

Within the Proximal to Salt Spring isolates, there was no nucleotide sequence variation observed within the *gyrB*, *pyrG*, *recA*, or *recG* gene sequence sets (Table 6.S11). Considering the deduced primary protein sequence variant forms, the only the *leuS* gene sequence had aaH > 1. Although the diversity measures based on only 4 isolates must be viewed with caution as the sequences from additional isolates may change the results, what was intriguing from the data at hand was that the *pyrG* and *recG* gene sequence set show no variation (Table 6.S11). By comparison, throughout the entire *T. uzonensis* isolate set, these were the two most-variable universally conserved protein coding gene sequence sets (Table 6.S5).

All Proximal to Salt Spring *T. uzonensis* isolates have the *Tu gyrG* ntH_5, aaH_4 sequence variant form (Table 6.S14). Additionally there were nucleotide- and primary protein sequence variant forms within the *leuS*, *pyrG*, and *recG* gene sequence sets that were only found within *T. uzonensis* isolates from the Geyser Valley regions (Tables 6.S16, 6.S17, and 6.S19). The *pyrG* gene sequences found in the *T. uzonensis* isolates from the Geyser Valley geothermal springs were notable in that the clade they form was relatively divergent from related sequences (*Tu pyrG* ntH_25, aaH_12 and *Tu pyrG* ntH_26, aaH_13; Fig. 6.S4). The Proximal to Salt Spring isolates were found within 2 eBURST clusters; 2 isolates bin to eBURST Group 23 which also contains one of the Resting Rock isolates, and Group 24, which only contains 2 Proximal to Salt Spring isolates (Table 6.2). The phylogenetic tree based on the concatenates of the eight protein coding genes revealed that a group of 5 *T. uzonensis* Geyser Valley isolates form a distinct, separate phylogenetic clade; the remaining Geyser Valley isolate, JW/IW R649_9, was

phylogenetically divergent from all other *T. uzonensis* isolates and will be further discussed below (Fig. 6.6).

T. uzonensis isolates from the geothermal spring Mutnovsky-4

The geothermal spring Mutnovsky-4 is located near Mutnovsky Volcano, approximately 250 km southwest of the Uzon Caldera and Geyser Valley (Fig. 6.1). The 8 universally conserved protein coding genes employed in this study have been sequenced from 10 isolates from Mutnovsky-4. The universally conserved protein coding gene sequence sets from the *T. uzonensis* isolates from Mutnovsky-4 were usually composed of a single gene sequence variant (Table 6.S11), and many of these variant forms were notably divergent compared to the corresponding genes from Geyser Valley or Uzon Caldera geothermal spring-derived strains, most notably within the gene sequence sets *gyrB*, *leuS*, and *recG* (*Tu gyrB* ntH_6, Fig. 6.S1; *Tu leuS* ntH_10, Fig. 6.S3; and *Tu recG* ntH_15, Fig. 6.S6). An exception was observed within the *rplB* gene sequence set. The *Tu rplB* ntH_2, aaH_1 sequence variant was the predominant *rplB* sequence variant form found throughout the entire *T. uzonensis* population (Table 6.S20). Heterogeneity within the Mutnovsky-4 *T. uzonensis* gene sequence sets was only observed for the *lepA* gene sequences (Table 6.S12).

While all *T. uzonensis* Mutnovsky-4 isolates have *Tu pyrG* ntH_22, aaH_11, this sequence variant was not restricted to the isolates from this geothermal spring; the sequence was also found within one isolate from Burlyashi (Table 6.S17). The Mutnovsky-4 isolates were found to have a single sequence variant form, found only in the isolates from this geothermal spring, within the *gyrB*, *lepA*, *leuS*, *recA*, and *recG* protein coding gene sequence sets (Tables 6.S14, 6.S15, 6.S16, 6.S18, and 6.S19). Nine of the Mutnovsky-4 *T. uzonensis* isolates have the *Tu rpoB* ntH_5 sequence type (Table 6.S21), and one Mutnovsky-4 isolate was found to have a

rpoB gene sequence variant the same as one held by *T. siderophilus* strains isolated from Kamchatkan geothermal springs (i.e., the sequence variant form "R649_60rpoB" on Fig. 6.S8). The Mutnovsky-4 isolates bin to eBURST Group 3 (Table 6.2). The Mutnovsky-4 isolates form an exclusive clade based on the concatenates of the eight protein coding genes (Fig. 6.6). Within one *T. uzonensis* isolate, strain JW/IW M04_35_2, a lateral gene transfer event appears to have occurred which accounts for this isolate appearing notably divergent compared to other Mutnovsky-4 isolates (Wagner, 2010).

Table 6.S1. *Thermoanaerobacter uzonensis* universally conserved protein coding gene sequence length analyzed, inferred fraction of gene sequence analyzed, and oligonucleotide sequences for PCR amplification.

Gene	Average Gene Length analyzed	Inferred fraction of gene sequence analyzed	Primer name	Oligonucleotide sequence (5' to 3')
pyrG	1267	0.79	TBIOG_pyrGF	AAGYCGCGGCMTATCAGTTGCWRT
			TBIOG_pyrGR	TGGRTGRAAYTGGGAYGCYACAAA
leuS	1132	0.46	TBIOGEO_leuSF	GYTGYCAAACTGTTCTTGCAAACGARC
			TBIOGEO_leuSR	TCATTCTGCTKCCATCAGGKCCCA
gyrB	1170	0.62	TBIOGEO_gyrBF	AGCSGTAAGAAARAGGCCAGGAAT
			TBIOGEO_gyrBR	TYCCTCGKAGTGGAAGTATCGCTT
recA	757	0.74	TBIOGEO_recAF	AGYCARATAGAGAGRCAGTTTGGC
			TBIOGEO_recAR	CTCCATAGGAATACCAAGCACCAC
rplB	655	0.79	TBIOGEO_rplBR	GTGTCTTATARCCYAATGCAGGCT
			TBIOGEO_rplBF	ATCTCCCGGCAGACGTCAAAT
rpoB	1294	0.86	TBIOGEO_rpoBR	TCTCTAATGGCTGCWACAACYGGR
			TBIOGEO_rpoBF	TACGTCCTGTACAAGTGGGCAACA
recG	1310	0.64	TBIOGEO_recGR	AAATTCTGACCTGCCAACTCTRCC
			TBIOGEO_recGF	ACAGGYGYAGTAGARTTAGTSTGG
lepA	1311	0.72	TBIOGEO_lepAR	YTTCCCACCTGTCTCATGCGCTTT
			TBIOGEO_lepAF	TTGAGGCGCAAACCCTTGCTAATG

Table 6.S2. Summary of universally conserved protein sequence heterogeneity within the *Thermoanaerobacter uzonensis* isolates from the geothermal spring Zavarzin 2006.								
	gyrB	***lepA***	***leuS***	***pyrG***	***recA***	***recG***	***rplB***	***rpoB***
Length	1186	1324	1141	1302	762	1227	666	1337
N	11	11	11	11	11	11	11	11
S_{nt}	3	2	2	5	2	21	0	13
G+C mol %	35.2	35.9	39.5	38.6	39.4	33.5	38.6	32.8
ntH	2	3	3	3	3	5	1	4
π (per site)	0.00083	0.0008	0.00076	0.00103	0.00081	0.00827	0	0.00405
k	0.98	1.05	0.87	1.35	0.62	10.15	0	5.42
d_N	0.00036	0	0.00099	0.00033	0.00032	0.00419	0	0.00031
d_S	0.0025	0.0035	0	0.0035	0.0024	0.0215	0	0.018
d_N/d_S	0.14	NA	NA	0.095	0.13	0.20	NA	0.017
ΔX max	1	0	2	1	1	6	0	1
ΔX mean	0.33	0	0.87	0.33	0.18	3.16	0	0.33
aaH	2	1	3	2	2	5	1	2

Table 6.S3. Summary of universally conserved protein sequence heterogeneity within the *Thermoanaerobacter uzonensis* isolates from the geothermal spring Thermophilny 2005.								
	gyrB	***lepA***	***leuS***	***pyrG***	***recA***	***recG***	***rplB***	***rpoB***
Length	1191	1322	1141	1311	749	1346	679	1343
N	7	7	7	7	7	7	7	7
S_{nt}	13	8	3	17	2	9	0	0
G+C mol %	35	36	39.5	38.6	39.3	33.3	39	32.7
ntH	2	4	4	4	3	4	1	1
π (per site)	0.0052	0.0018	0.0012	0.0060	0.00076	0.0024	0	0
k	6.19	2.48	1.33	7.8	0.57	3.24	0	0
d_N	0.001	0.0015	0.0012	0.0024	0	0.0018	0	0
d_S	0.02	0.0033	0.0011	0.018	0.0032	0.0046	0	0
d_N/d_S	0.05	0.44	1.06	0.13	NA	0.4	NA	NA
ΔX max	2	3	2	4	0	6	0	0
ΔX mean	0.95	1.05	1.05	2.38	0	1.9	0	0
aaH	2	3	3	4	1	3	1	1

Table 6.S4. Summary of universally conserved protein sequence heterogeneity within the *Thermoanaerobacter uzonensis* isolates from the geothermal spring Thermophilny 2006.

	gyrB	***lepA***	***leuS***	***pyrG***	***recA***	***recG***	***rplB***	***rpoB***
Length	1124	1318	1141	1261	762	1339	503	1281
N	19	19	19	19	19	19	19	19
S_{nt}	0	3	16	22	2	16	1	5
G+C mol %	34.7	36	39.5	38.3	39.3	33.2	37.2	32.6
ntH	1	3	5	6	3	6	2	2
π (per site)	0	0.00031	0.0025	0.0068	0.00083	0.0035	0.00021	0.00078
k	0	0.41	2.87	8.53	0.63	4.73	0.11	0.99
d_N	0	0	0.0015	0.0024	0	0.0031	0	0
d_S	0	0.0014	0.0063	0.022	0.0035	0.005	0.00087	0.0036
d_N/d_S	NA	NA	0.23	0.11	NA	0.63	NA	NA
ΔX max	0	0	4	5	0	7	0	0
ΔX mean	0	0	1.29	2.34	0	3.27	0	0
aaH	1	1	5	5	1	6	1	1

Table 6.S5. Summary of universally conserved protein sequence heterogeneity within the *Thermoanaerobacter uzonensis* isolates from the geothermal spring Pulsating Spring (Jen's Vent 2) 2006.

	gyrB	***lepA***	***leuS***	***pyrG***	***recA***	***recG***	***rplB***	***rpoB***
Length	1178	1319	1144	1273	757	1291	664	1342
N	7	7	7	7	7	7	7	7
S_{nt}	16	2	1	6	0	13	1	6
G+C mol %	35	36	39.5	38.3	39.4	33.2	38.9	32.6
ntH	2	3	2	2	1	2	2	2
π (per site)	0.0078	0.00087	0.0005	0.0027	0	0.0058	0.00086	0.0026
k	9.14	1.14	0.57	3.43	0	7.43	0.57	3.43
d_N	0.0019	0	0.00065	0.00058	0	0.0023	0	0.00055
d_S	0.029	0.0038	0	0.0099	0	0.019	0.0035	0.0099
d_N/d_S	0.064	NA	NA	0.058	NA	0.12	NA	0.055
ΔX max	3	0	1	1	0	3	0	1
ΔX mean	1.71	0	0.57	0.57	0	1.71	0	0.57
aaH	2	1	2	2	1	2	1	2

Table 6.S6. Summary of universally conserved protein sequence heterogeneity within the *Thermoanaerobacter uzonensis* isolates from the geothermal spring Arkashin 2005.

	gyrB	***lepA***	***leuS***	***pyrG***	***recA***	***recG***	***rplB***	***rpoB***
Length	1129	1315	1141	1252	749	1344	668	1327
N	8	8	8	8	8	8	8	8
S_{nt}	0	0	0	0	0	0	0	0
G+C mol %	34.6	36	39.5	385	39.3	33.4	38.6	32.6
ntH	1	1	1	1	1	1	1	1
π (per site)	0	0	0	0	0	0	0	0
k	0	0	0	0	0	0	0	0
d_N	0	0	0	0	0	0	0	0
d_S	0	0	0	0	0	0	0	0
d_N/d_S	NA	NA	NA	NA	NA	NA	NA	NA
ΔX max	0	0	0	0	0	0	0	0
ΔX mean	0	0	0	0	0	0	0	0
aaH	1	1	1	1	1	1	1	1

Table 6.S7. Summary of universally conserved protein sequence heterogeneity within the *Thermoanaerobacter uzonensis* isolates from the geothermal spring Arkashin 2006.

	gyrB	***lepA***	***leuS***	***pyrG***	***recA***	***recG***	***rplB***	***rpoB***
Length	1186	1274	1141	1229	761	1344	673	1327
N	7	7	7	7	7	7	7	7
S_{nt}	0	0	16	16	0	19	2	5
G+C mol %	35.1	35.8	39.4	38.5	39.3	33.4	39	32.6
ntH	1	1	4	4	1	3	3	2
π (per site)	0	0	0.0066	0.0054	0	0.007	0.0013	0.0018
k	0	0	7.52	6.57	0	9.43	0.86	2.38
d_N	0	0	0.002	0.002	0	0.003	0.0011	0
d_S	0	0	0.023	0.017	0	0.022	0.0018	0.0083
d_N/d_S	NA	NA	0.083	0.12	NA	0.14	0.64	NA
ΔX max	0	0	4	5	0	5	1	0
ΔX mean	0	0	1.71	1.9	0	2.57	0.57	0
aaH	1	1	3	4	1	3	2	1

Table 6.S8. Summary of universally conserved protein sequence heterogeneity within the *Thermoanaerobacter uzonensis* isolates from the geothermal spring Burlyashi 2006.

	gyrB	***lepA***	***leuS***	***pyrG***	***recA***	***recG***	***rplB***	***rpoB***
Length	1179	1281	1040	1264	750	1326	665	1327
N	18	18	18	18	18	18	18	18
S_{nt}	4	4	22	23	3	33	2	13
G+C mol %	35	35.8	39.2	38.3	39.4	33.4	39.1	32.6
ntH	3	4	6	11	4	6	3	5
π (per site)	0.001	0.0005	0.0062	0.0063	0.00082	0.0067	0.00061	0.0025
k	1.25	0.64	6.4	7.93	0.61	8.92	0.41	3.31
d_N	0.00092	0	0.0022	0.0014	0.00071	0.0037	0	0.00031
d_S	0.0016	0.0022	0.02	0.023	0.0012	0.018	0.0025	0.011
d_N/d_S	0.57	NA	0.11	0.059	0.6	0.2	NA	0.029
ΔX max	2	0	7	4	2	8	0	2
ΔX mean	0.84	0	1.65	1.35	0.41	3.6	0	0.32
aaH	3	1	4	6	3	6	1	3

Table 6.S9. Summary of universally conserved protein sequence heterogeneity within the *Thermoanaerobacter uzonensis* isolates from the geothermal spring ON1 2006.

	gyrB	***lepA***	***leuS***	***pyrG***	***recA***	***recG***	***rplB***	***rpoB***
Length	1165	1317	1144	1247	757	1308	666	959
N	12	12	12	12	12	12	12	12
S_{nt}	3	4	1	18	0	25	2	10
G+C mol %	34.9	35.9	39.5	38.1	39.4	33.2	38.7	31.3
ntH	2	5	2	2	1	5	3	4
π (per site)	0.00043	0.0013	0.00036	0.0059	0	0.0076	0.00098	0.0038
k	0.5	1.67	0.41	7.36	0	9.98	0.65	3.67
d_N	0.00018	0.00016	0.00046	0.00171	0	0.00295	0.00097	0.00045
d_S	0.0013	0.005	0	0.021	0	0.025	0.001	0.016
d_N/d_S	0.14	0.032	NA	0.082	NA	0.12	0.93	0.028
ΔX max	1	1	1	4	0	6	1	1
ΔX mean	0.17	0.17	0.41	1.64	0	2.76	0.48	0.17
aaH	2	2	2	2	1	4	2	2

Table 6.S10. Summary of universally conserved protein sequence heterogeneity within the *Thermoanaerobacter uzonensis* isolates from the geothermal spring Vent 1 North 2006.								
	gyrB	***lepA***	***leuS***	***pyrG***	***recA***	***recG***	***rplB***	***rpoB***
Length	1180	1316	1143	1246	758	1271	670	1307
N	18	18	18	18	18	18	18	18
S_{nt}	13	5	2	8	0	28	1	5
G+C mol %	35	36	39.5	38.2	39.3	33.7	39.1	32.5
ntH	2	6	3	3	1	3	2	2
π (per site)	0.0012	0.00079	0.00055	0.0017	0	0.0088	0.00017	0.00043
k	1.4	1.05	0.63	2.07	0	11.1	0.11	0.56
d_N	0.00024	0.00022	0.00072	0.00064	0	0.0038	0	0
d_S	0.0048	0.0028	0	0.0051	0	0.027	0.00068	0.002
d_N/d_S	0.05	0.08	NA	0.125	NA	0.14	NA	NA
ΔX max	2	2	2	2	0	7	0	0
ΔX mean	0.22	0.22	0.63	0.61	0	3.4	0	0
aaH	2	3	3	3	1	3	1	1

Table 6.S11. Summary of universally conserved protein sequence heterogeneity within the *Thermoanaerobacter uzonensis* isolates from the geothermal spring Proximal to Salt Spring 2006.								
	gyrB	***lepA***	***leuS***	***pyrG***	***recA***	***recG***	***rplB***	***rpoB***
Length	1179	1319	1144	1277	762	1345	682	1350
N	4	4	4	4	4	4	4	4
S_{nt}	0	1	1	0	0	0	1	5
G+C mol %	34.9	36	39.5	38	39.5	33.1	38.9	32.8
ntH	1	2	2	1	1	1	2	2
π (per site)	0	0.00051	0.00058	0	0	0	0.00098	0.0025
k	0	0.67	0.67	0	0	0	0.67	3.33
d_N	0	0	0.00076	0	0	0	0	0
d_S	0	0.0022	0	0	0	0	0.004	0.011
d_N/d_S	NA	NA	NA	NA	NA	NA	NA	NA
ΔX max	0	0	1	0	0	0	0	0
ΔX mean	0	0	0.67	0	0	0	0	0
aaH	1	1	2	1	1	1	1	1

Table 6.S12. Summary of universally conserved protein sequence heterogeneity within the *Thermoanaerobacter uzonensis* isolates from the geothermal spring Mutnovsky-4 2005.

	gyrB	***lepA***	***leuS***	***pyrG***	***recA***	***recG***	***rplB***	***rpoB***
Length	1178	1319	1141	1275	764	1274	677	1341
N	10	10	10	10	10	10	10	9
S_{nt}	0	2	0	0	0	0	0	0
G+C mol %	35.1	35.9	39.2	38.2	39.4	33.3	39.3	32.7
ntH	1	3	1	1	1	1	1	1
π (per site)	0	0.0003	0	0	0	0	0	0
k	0	0.4	0	0	0	0	0	0
d_N	0	0.0002	0	0	0	0	0	0
d_S	0	0.00067	0	0	0	0	0	0
d_N/d_S	NA	0.3	NA	NA	NA	NA	NA	NA
ΔX max	0	1	0	0	0	0	0	0
ΔX mean	0	0.2	0	0	0	0	0	0
aaH	1	2	1	1	1	1	1	1

Table 6.S13. *Thermoanaerobacter uzonensis* unique sequence type combinations based on eight loci. Geothermal spring abbreviation are given in Table 6.1.

	A615	B621	H608	I502	J614	M504	O629	R649	S648	T515	V634	Z606
ST #1	**3**			**8**								
ST #2	**1**											
ST #3	**1**											
ST #4	**1**											
ST #5	**1**											
ST #6		**1**										
ST #7		**3**										
ST #8		**1**										
ST #9		**1**										
ST #10		**1**										
ST #11		**3**										
ST #12		**1**										
ST #13		**1**										
ST #14		**1**										
ST #15		**1**										
ST #16		**1**										
ST #17		**1**										
ST #18		**1**										
ST #19		**1**										
ST #20			**10**							**1**		
ST #21			**5**							**2**		
ST #22			**1**									
ST #23			**1**									
ST #24			**1**									
ST #25			**1**									
ST #26					**4**							
ST #27					**3**							

Table 6.S13 (continued). *T. uzonensis* unique sequence type combinations based on eight loci. Geothermal spring abbreviation are given in Table 6.1.

	A615	B621	H608	I502	J614	M504	O629	R649	S648	T515	V634	Z606
ST #28						**9**						
ST #29						**1**						
ST #30							**1**					
ST #31							**1**					
ST #32							**7**					
ST #33							**2**					
ST #34							**1**					
ST #35								**1**	**2**			
ST #36								**1**				
ST #37									**2**			
ST #38										**1**		
ST #39										**1**		
ST #40										**1**		
ST #41										**1**		
ST #42											**6**	
ST #43											**1**	
ST #44											**10**	
ST #45											**1**	
ST #46												**1**
ST #47												**5**
ST #48												**1**
ST #49												**2**
ST #50												**1**
ST #51												**1**

Table 6.S14. Distribution of *gyrB* nucleotide and primary protein sequence variants among the *Thermoanaerobacter uzonensis* isolates from Kamchatkan geothermal springs. Geothermal spring abbreviation are given in Table 6.1.

	Geothermal Spring											
Sequence Variant	**A615**	**B621**	**H608**	**I502**	**J614**	**M504**	**O629**	**R629**	**S648**	**T515**	**V634**	**Z606**
***Tu gyrB* ntH_1, aaH_1**	7	12	19	8			11			5	17	9
***Tu gyrB* ntH_2, aaH_2**		4										
***Tu gyrB* ntH_3, aaH_3**		2			3		1					2
***Tu gyrB* ntH_4, aaH_3**								1				
***Tu gyrB* ntH_5, aaH_4**					4			1	4	2	1	
***Tu gyrB* ntH_6, aaH_5**						10						

Table 6.S15. Distribution of *lepA* nucleotide and primary protein sequence variants among the *Thermoanaerobacter uzonensis* isolates from Kamchatkan geothermal springs. Geothermal spring abbreviation are given in Table 6.1.

	Geothermal Spring											
Sequence Variant	**A615**	**B621**	**H608**	**I502**	**J614**	**M504**	**O629**	**R649**	**S648**	**T515**	**V634**	**Z606**
***Tu lepA* ntH_1, aaH_1**										1		
***Tu lepA* ntH_2, aaH_2**										1		
***Tu lepA* ntH_3, aaH_3**	7	14	17	8	4		4	1	2	5	6	4
***Tu lepA* ntH_4, aaH_3**		1			3							
***Tu lepA* ntH_5, aaH_3**		1										
***Tu lepA* ntH_6, aaH_3**			1									
***Tu lepA* ntH_7, aaH_3**		2	1				8				1	6
***Tu lepA* ntH_8, aaH_3**									2		10	1
***Tu lepA* ntH_9, aaH_4**								1				
***Tu lepA* ntH_10, aaH_5**						10						
***Tu lepA* ntH_11, aaH_6**											1	

Table 6.S16. Distribution of *leuS* nucleotide and primary protein sequence variants among the *Thermoanaerobacter uzonensis* isolates from Kamchatkan geothermal springs. Geothermal spring abbreviation are given in Table 6.1.

	Geothermal Spring											
Sequence Variant	**A615**	**B621**	**H608**	**I502**	**J614**	**M504**	**O629**	**R649**	**S648**	**T515**	**V634**	**Z606**
***Tu leuS* ntH_4, aaH_2**	4	4	1	8			3			2	10	6
***Tu leuS* ntH_11, aaH_9**	1											
***Tu leuS* ntH_3, aaH_2**	1											
***Tu leuS* ntH_12, aaH_10**	1	3										
***Tu leuS* ntH_5, aaH_3**		8	1		3		9			2	7	3
***Tu leuS* ntH_13, aaH_10**		1										
***Tu leuS* ntH_1, aaH_1**		1										
***Tu leuS* ntH_2, aaH_2**		1	10							1		
***Tu leuS* ntH_6, aaH_4**			6		4					2	1	2
***Tu leuS* ntH_14, aaH_10**			1									
***Tu leuS* ntH_10, aaH_8**						10						
***Tu leuS* ntH_7, aaH_5**								1	2			
***Tu leuS* ntH_9, aaH_7**								1				
***Tu leuS* ntH_8, aaH_6**									2			

Table 6.S17. Distribution of *pyrG* nucleotide and primary protein sequence variants among the *Thermoanaerobacter uzonensis* isolates from Kamchatkan geothermal springs. Geothermal spring abbreviation are given in Table 6.1.

	Geothermal Spring											
Sequence Variant	**A615**	**B621**	**H608**	**I502**	**J614**	**M504**	**O629**	**R649**	**S648**	**T515**	**V634**	**Z606**
***Tu pyrG* ntH_1, aaH_1**		3										
***Tu pyrG* ntH_10, aaH_8**										2		
***Tu pyrG* ntH_11, aaH_6**	2	1										9
***Tu pyrG* ntH_12, aaH_6**			1									
***Tu pyrG* ntH_13, aaH_6**											10	
***Tu pyrG* ntH_14, aaH_9**											7	
***Tu pyrG* ntH_15, aaH_8**			1									
***Tu pyrG* ntH_16, aaH_6**		5										
***Tu pyrG* ntH_17, aaH_6**		1										
***Tu pyrG* ntH_18, aaH_10**		1										1
***Tu pyrG* ntH_19, aaH_10**	1				4						1	
***Tu pyrG* ntH_2, aaH_1**		1										
***Tu pyrG* ntH_20, aaH_11**			1									
***Tu pyrG* ntH_21, aaH_10**			5							2		
***Tu pyrG* ntH_22, aaH_11**		1				10						
***Tu pyrG* ntH_23, aaH_11**		2			3		9					
***Tu pyrG* ntH_24, aaH_10**												1
***Tu pyrG* ntH_25, aaH_12**								1	4			
***Tu pyrG* ntH_26, aaH_13**								1				
***Tu pyrG* ntH_27, aaH_14**	1											
***Tu pyrG* ntH_3, aaH_2**		1										
***Tu pyrG* ntH_4, aaH_3**		1					3					
***Tu pyrG* ntH_5, aaH_2**	3			8								
***Tu pyrG* ntH_6, aaH_4**			10							1		
***Tu pyrG* ntH_7, aaH_5**										2		
***Tu pyrG* ntH_8, aaH_6**		1										
***Tu pyrG* ntH_9, aaH_7**			1									

Table 6.S18. Distribution of *recA* nucleotide and primary protein sequence variants among the *Thermoanaerobacter uzonensis* isolates from Kamchatkan geothermal springs. Geothermal spring abbreviation are given in Table 6.1.

	Geothermal Spring											
Sequence Variant	**A615**	**B621**	**H608**	**I502**	**J614**	**M504**	**O629**	**R629**	**S648**	**T515**	**V634**	**Z606**
***Tu recA* ntH_1, aaH_1**		3										
***Tu recA* ntH_2, aaH_2**	7	12	8	8	7		12			5	18	7
***Tu recA* ntH_3, aaH_2**			1									
***Tu recA* ntH_4, aaH_2**										1		
***Tu recA* ntH_5, aaH_2**												3
***Tu recA* ntH_6, aaH_2**		2	10							1		
***Tu recA* ntH_7, aaH_3**		1						1	4			1
***Tu recA* ntH_8, aaH_3**						10						
***Tu recA* ntH_9, aaH_3**								1				

Table 6.S19. Distribution of *recG* nucleotide and primary protein sequence variants among the *Thermoanaerobacter uzonensis* isolates from Kamchatkan geothermal springs. Geothermal spring abbreviation are given in Table 6.1.

	Geothermal Spring											
Sequence Variant	**A615**	**B621**	**H608**	**I502**	**J614**	**M504**	**O629**	**R649**	**S648**	**T515**	**V634**	**Z606**
***Tu recG* ntH_1, aaH_1**							1					
***Tu recG* ntH_2, aaH_2**								1				
***Tu recG* ntH_3, aaH_3**			1				2					
***Tu recG* ntH_4, aaH_4**	1	2	1				1				7	
***Tu recG* ntH_5, aaH_5**												1
***Tu recG* ntH_6, aaH_6**												1
***Tu recG* ntH_7, aaH_7**		4	10							1		
***Tu recG* ntH_8, aaH_8**		1	5							2		
***Tu recG* ntH_9, aaH_9**			1									
***Tu recG* ntH_10, aaH_10**	3	8	1		3					2		3
***Tu recG* ntH_11, aaH_8**										2		
***Tu recG* ntH_12, aaH_11**	3			8								
***Tu recG* ntH_13, aaH_12**					4							
***Tu recG* ntH_14, aaH_13**		1									1	
***Tu recG* ntH_15, aaH_14**						10						
***Tu recG* ntH_16, aaH_15**												5
***Tu recG* ntH_17, aaH_16**												1
***Tu recG* ntH_18, aaH_17**							8				10	
***Tu recG* ntH_19, aaH_18**								1	4			
***Tu recG* ntH_20, aaH_19**		2										

Table 6.S20. Distribution of *rplB* nucleotide and primary protein sequence variants among the *Thermoanaerobacter uzonensis* isolates from Kamchatkan geothermal springs. Geothermal spring abbreviation are given in Table 6.1.

	Geothermal Spring											
	A615	**B621**	**H608**	**I502**	**J614**	**M504**	**O629**	**R649**	**S648**	**T515**	**V634**	**Z606**
***Tu rplB* ntH_3, aaH_2**	4			8			8					
***Tu rplB* ntH_2, aaH_1**	3	17	19		7	10	4	1	4	7	18	11
***Tu rplB* ntH_1, aaH_1**		1										
***Tu rplB* ntH_4, aaH_1**								1				

Table 6.S21. Distribution of *rpoB* nucleotide and primary protein sequence variants among the *Thermoanaerobacter uzonensis* isolates from Kamchatkan geothermal springs. Geothermal spring abbreviation are given in Table 6.1. The isolate M504_35_2 has been removed from this analysis; the *rpoB* gene sequence from this isolate is similar to the corresponding gene from *T. siderophilus* isolates that have also been isolated from Kamchatkan geothermal springs (Wagner, 2010)

	Geothermal Spring											
	A615	**B621**	**H608**	**I502**	**J614**	**M502**	**O629**	**R649**	**S648**	**T515**	**V634**	**Z606**
***Tu rpoB* ntH_1, aaH_1**							1					
***Tu rpoB* ntH_2, aaH_2**		1			3							
***Tu rpoB* ntH_3, aaH_3**		13	17				7			7	17	6
***Tu rpoB* ntH_4, aaH_3**	2	2	2		4		1		2		1	3
***Tu rpoB* ntH_5, aaH_3**	5			8		9		1	2			
***Tu rpoB* ntH_6, aaH_4**		1										
***Tu rpoB* ntH_7, aaH_4**		1										2
***Tu rpoB* ntH_8, aaH_4**								1				
***Tu rpoB* ntH_9, aaH_3**							3					

Table 6.S22. *Thermoanaerobacter uzonensis* sequence types based on the eight universally conserved protein coding genes. Sequence Type, ST.

ST	***gyrB***	***lepA***	***leuS***	***pyrG***	***recA***	***recG***	***rplB***	***rpoB***
1	1	1	1	1	1	1	1	1
2	1	1	2	2	1	2	2	1
3	1	1	1	3	1	3	2	2
4	1	1	3	4	1	2	1	1
5	1	1	4	4	1	2	2	2
6	1	1	5	6	1	4	2	4
7	1	1	5	5	2	2	2	3
8	1	1	6	7	3	5	3	2
9	1	1	1	8	1	3	2	3
10	1	1	5	9	1	2	2	3
11	2	1	4	6	1	2	2	3
12	3	2	5	10	1	6	2	5
13	1	1	5	4	1	3	2	2
14	1	3	5	11	1	4	2	6
15	3	1	1	12	1	2	2	3
16	1	4	1	13	1	7	2	3
17	2	1	7	10	4	4	2	3
18	1	1	8	6	4	4	2	3
19	1	4	1	14	1	7	2	3
20	1	1	8	15	4	4	2	3
21	1	1	9	16	1	5	2	3
22	1	5	9	17	1	8	2	3
23	1	1	10	18	1	3	2	2
24	1	1	1	19	5	9	2	2
25	1	4	5	20	1	2	2	3
26	4	1	9	3	1	10	2	2
27	3	2	5	10	1	2	2	5
28	5	6	11	9	6	11	2	1
29	5	6	11	9	6	11	2	10
30	1	4	5	10	1	12	1	7
31	1	1	1	14	1	13	2	8
32	1	4	5	10	1	12	1	3
33	1	1	1	14	1	9	2	8
34	3	1	5	10	1	3	2	2
35	4	1	12	21	3	14	2	1
36	6	7	13	22	7	15	4	9

Table 6.S22 (continued). *Thermoanaerobacter uzonensis* sequence types based on the eight universally conserved protein coding genes. Sequence Type, ST.

ST	***gyrB***	***lepA***	***leuS***	***pyrG***	***recA***	***recG***	***rplB***	***rpoB***
37	4	8	14	21	3	14	2	2
38	4	1	1	23	8	16	2	3
39	4	1	1	23	1	16	2	3
40	1	9	5	24	1	2	2	3
41	1	10	5	24	1	2	2	3
42	1	1	5	25	1	3	2	3
43	4	4	9	3	1	6	2	2
44	1	8	1	26	1	12	2	3
45	1	11	5	25	1	3	2	3
46	1	1	5	4	9	2	2	2
47	1	4	1	4	1	17	2	3
48	1	4	1	4	1	18	2	3
49	3	1	5	4	9	2	2	6
50	1	1	9	27	3	19	2	2
51	1	8	9	11	1	20	2	2

Table 6.S23. Distribution of *Thermoanaerobacter uzonensis* isolates among sequence types based on eight universally conserved protein coding genes. Sequence Type, ST.

ST	***T. uzonensis* isolates**		**ST**	***T. uzonensis* isolates**
1	A615_31, A615_32, A615_62, I502_40, I502_41, I502_42, I502_43, I502_45, I502_46, I502_48, I502_49		27	J614_62_1, J614_7, J614_9
2	A615_35		28	M504_30, M504_31, M504_33, M504_36, M504_37, M504_38, M504_39, M504_5, M504_7,
3	A615_37		29	M504_35_2
4	A615_39		30	O629_40
5	A615_65		31	O629_42
6	B621_1		32	O629_43, O629_44, O629_45, O629_49, O629_50, O629_6, O629_7
7	B621_10, B621_6, B621_78		33	O629_47, O629_48
8	B621_2		34	O629_51
9	B621_3		35	R649_40_2, S648_62, S648_7
10	B621_4		36	R649_9
11	B621_71, B621_89_1, B621_90		37	S648_4_2, S648_9
12	B621_72		38	T515_1
13	B621_74		39	T515_3
14	B621_77		40	T515_41
15	B621_80		41	T515_44
16	B621_83		42	V634_1, V634_2, V634_6, V634_7, V634_85, V634_9
17	B621_87		43	V634_10_2
18	B621_93		44	V634_3, V634_5, V634_71, V634_73, V634_74, V634_77, V634_81, V634_82, V634_84, V634_8
19	B621_94		45	V634_83
20	H608_10, H608_1, H608_2, H608_41, H608_6, H608_71, H608_72, H608_84, H608_85, H608_88, T515_40		46	Z606_36
21	H608_3, H608_42, H608_4, H608_77, H608_91, T515_10, T515_42		47	Z606_38, Z606_3, Z606_71, Z606_77, Z606_9
22	H608_78		48	Z606_70
23	H608_79		49	Z606_72, Z606_73
24	H608_81		50	Z606_76
25	H608_93		51	Z606_81
26	J614_60, J614_61, J614_63, J614_65			

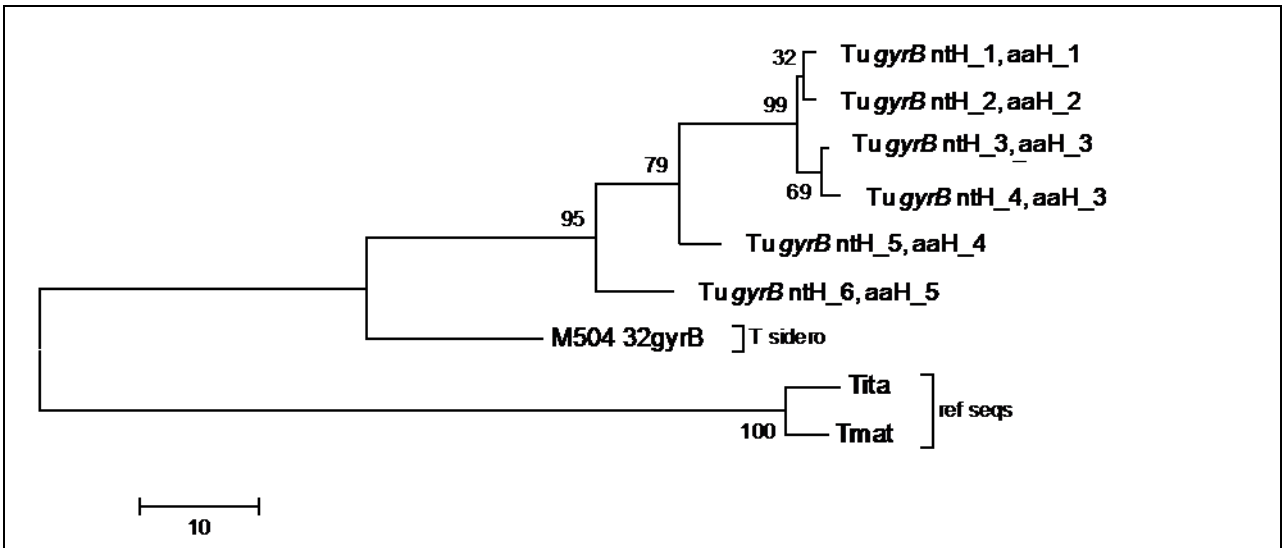

Fig. 6.S1. Phylogenetic tree based on the six *Thermoanaerobacter uzonensis gyrB* nucleotide sequence variants and the *gyrB* nucleotide sequences of related taxa. The evolutionary history was inferred using the Maximum Parsimony method. Tree #1 out of 3 most parsimonious trees (length = 166) is shown. For parsimony-informative sites, the consistency index is 0.89 and the retention index is 0.9. The Maximum Parsimony tree was obtained using the Close-Neighbor-Interchange algorithm with search level 3. Branch lengths are in the units of the number of changes over the whole sequence. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches . Phylogenetic analyses were conducted in MEGA4.1(Tamura et al., 2007).

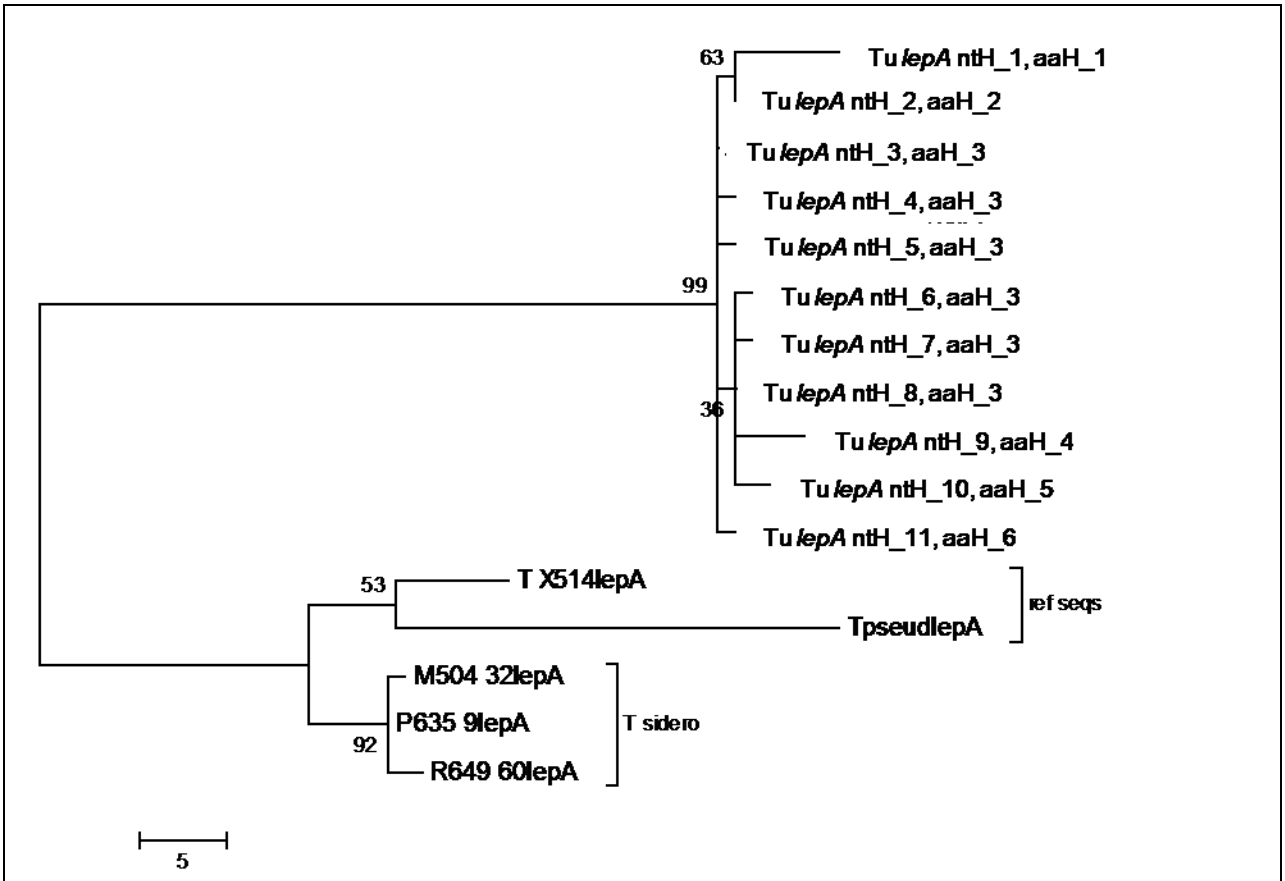

Fig. 6.S2. Phylogenetic tree based on the 11 *Thermoanaerobacter uzonensis lepA* nucleotide sequence variants, and the *lepA* nucleotide sequences of related taxa. The evolutionary history was inferred using the Maximum Parsimony method. Tree #1 out of 179 most parsimonious trees (length = 118) is shown. For parsimony-informative sites, the consistency index is 0.9 and the retention index is 0.97. The Maximum Parsimony tree was obtained using the Close-Neighbor-Interchange algorithm. Branch lengths are in the units of the number of changes over the whole sequence. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. Phylogenetic analyses were conducted in MEGA4.1(Tamura et al., 2007).

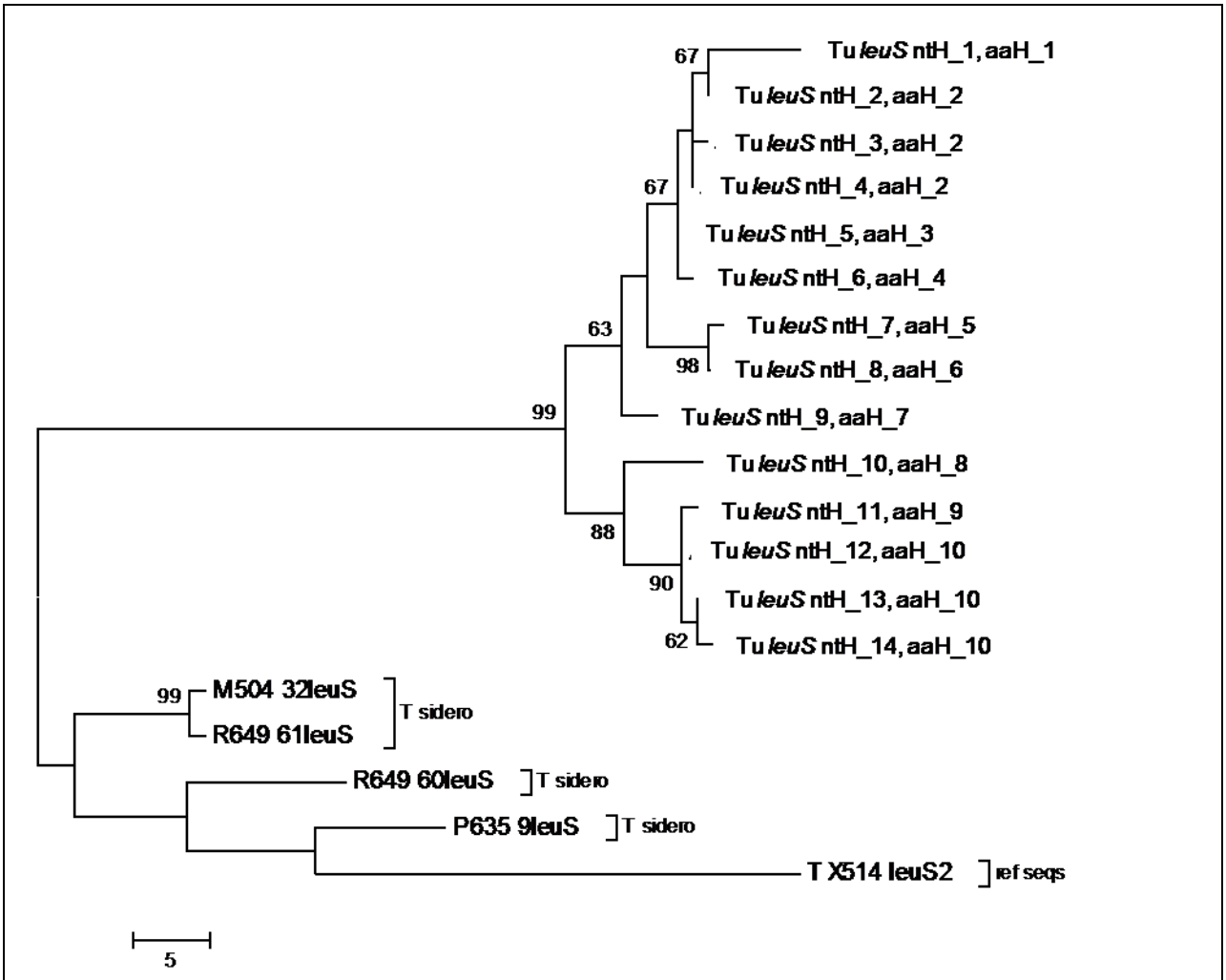

Fig. 6.S3. Phylogenetic tree based on the 14 *Thermoanaerobacter uzonensis leuS* nucleotide sequence variants and the *leuS* nucleotide sequences of related taxa. The evolutionary history was inferred using the Maximum Parsimony method. Tree #1 out of 27 most parsimonious trees (length = 153) is shown. For parsimony-informative sites, the consistency index is 0.7 and the retention index is 0.87. The Maximum Parsimony tree was obtained using the Close-Neighbor-Interchange algorithm with search level 3. Branch lengths are in the units of the number of changes over the whole sequence. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. Phylogenetic analyses were conducted in MEGA4.1(Tamura et al., 2007).

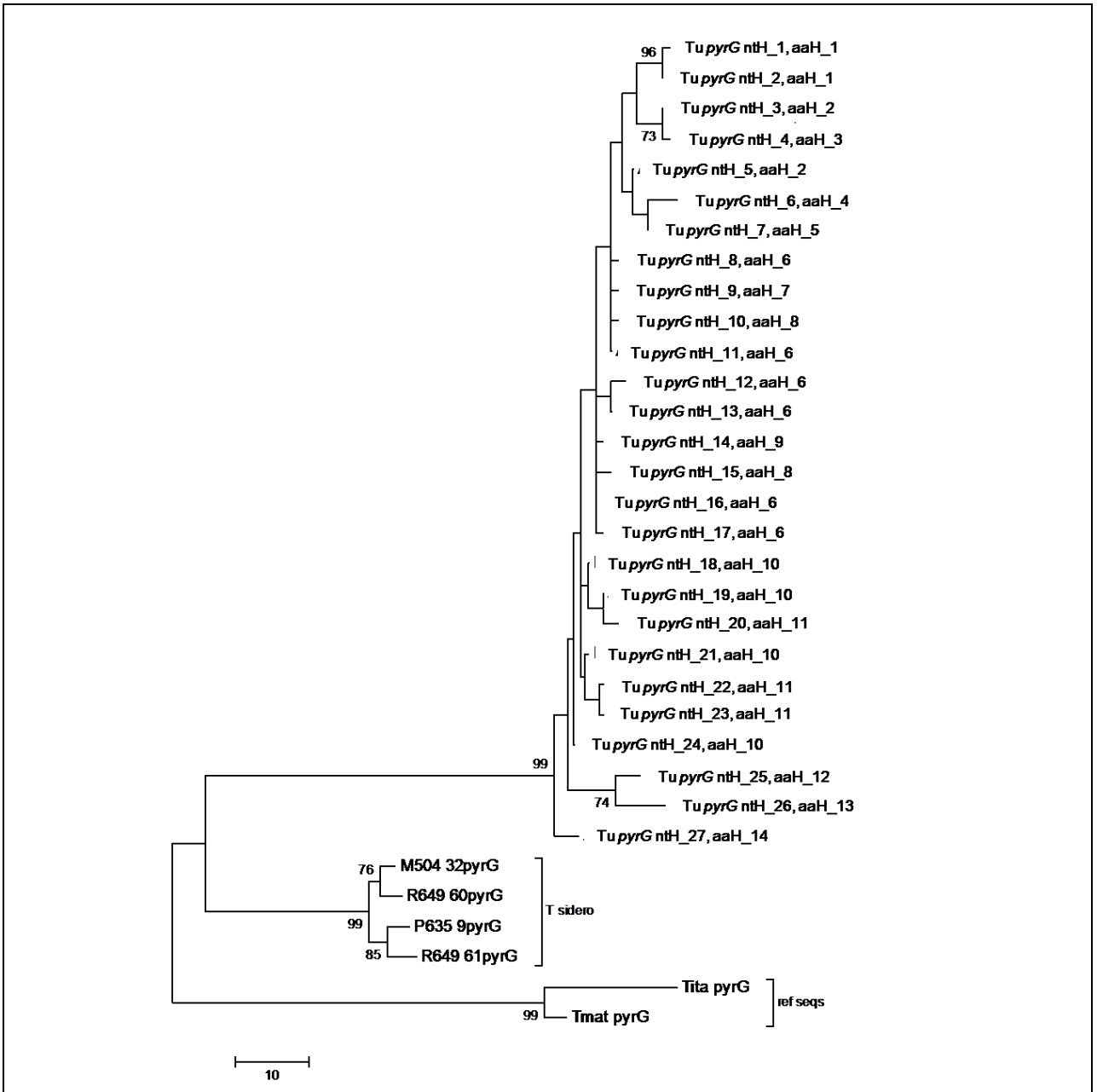

Fig. 6.S4. Phylogenetic tree based on the 27 *Thermoanaerobacter uzonensis pyrG* nucleotide sequence variants and the *pyrG* nucleotide sequences of related taxa. The evolutionary history was inferred using the Maximum Parsimony method. Tree #1 out of 46 most parsimonious trees (length = 229) is shown. For all parsimony-informative sites, the consistency index is 0.73 and the retention index is 0.88. The Maximum Parsimony tree was obtained using the Close-Neighbor-Interchange algorithm with search level 3. Branch lengths are in the units of the number of changes over the whole sequence. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. Phylogenetic analyses were conducted in MEGA4.1 (Tamura et al., 2007).

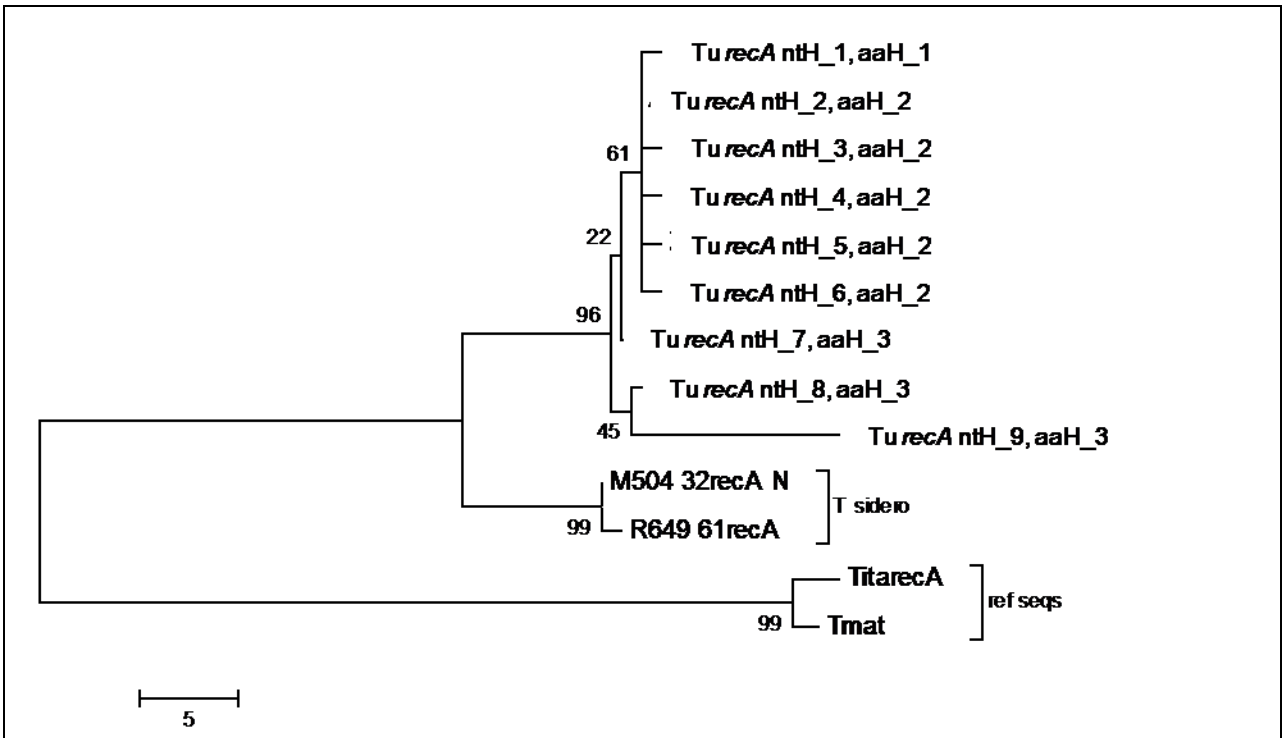

Fig. 6.S5. Phylogenetic tree based on the nine *Thermoanaerobacter uzonensis recA* nucleotide sequence variants and the *recA* nucleotide sequences of related taxa. The evolutionary history was inferred using the Maximum Parsimony method. Tree #1 out of 111 most parsimonious trees (length = 97) is shown. For parsimony-informative sites, the consistency index is 0.94 and the retention index is 0.95. The Maximum Parsimony tree was obtained using the Close-Neighbor-Interchange algorithm with search level 3. Branch lengths are in the units of the number of changes over the whole sequence. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. Phylogenetic analyses were conducted in MEGA4.1 (Tamura et al., 2007).

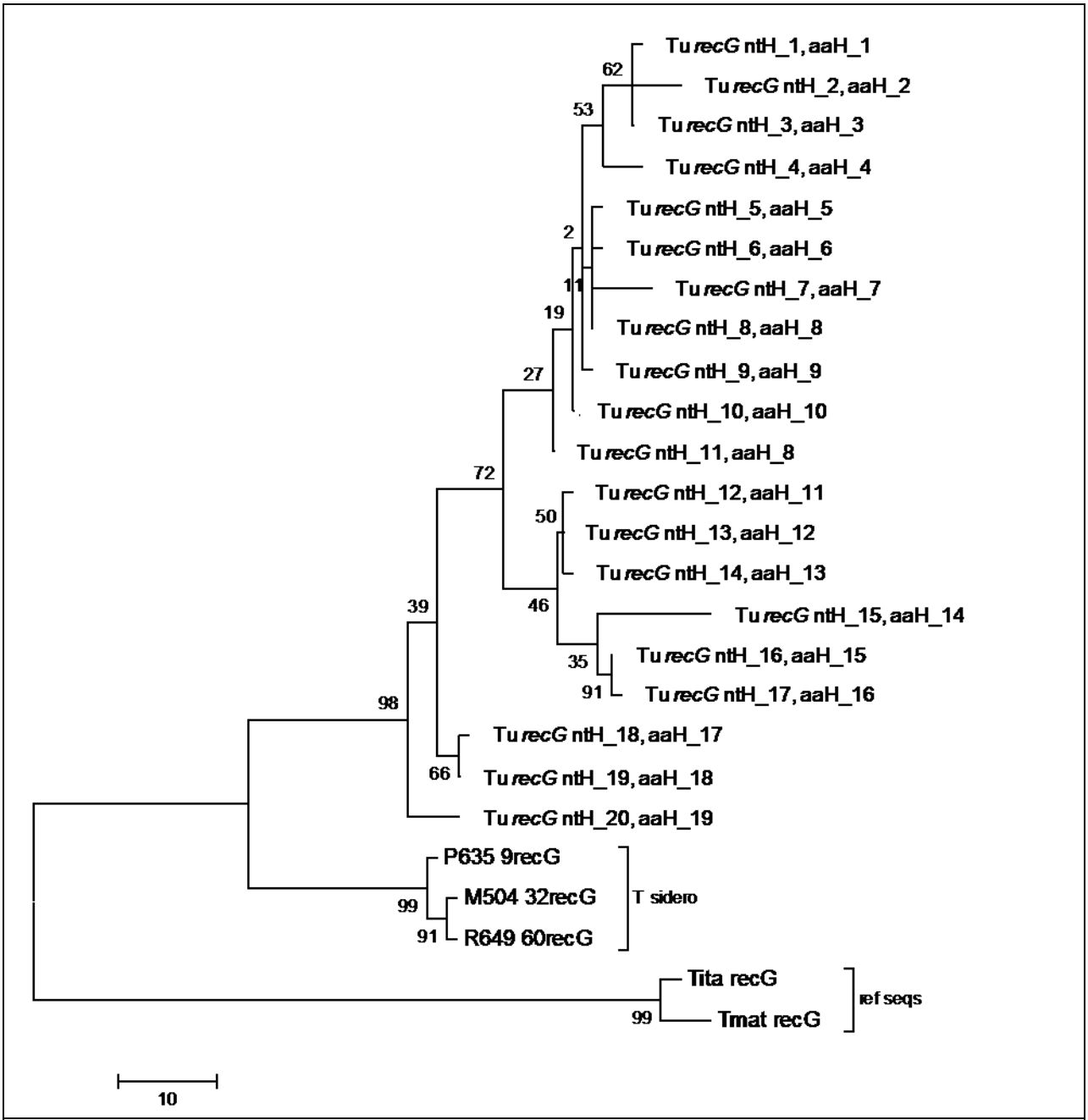

Fig. 6.S6. Phylogenetic tree based on the 20 *Thermoanaerobacter uzonensis recG* nucleotide sequence variants and the *recG* nucleotide sequences of related taxa. The evolutionary history was inferred using the Maximum Parsimony method. Tree #1 out of 40 most parsimonious trees (length = 209) is shown. For parsimony-informative sites, the consistency index is 0.75 and the retention index is 0.88. The Maximum Parsimony tree was obtained using the Close-Neighbor-Interchange algorithm with search level 3. Branch lengths are in the units of the number of changes over the whole sequence. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. Phylogenetic analyses were conducted in MEGA4.1 (Tamura et al., 2007).

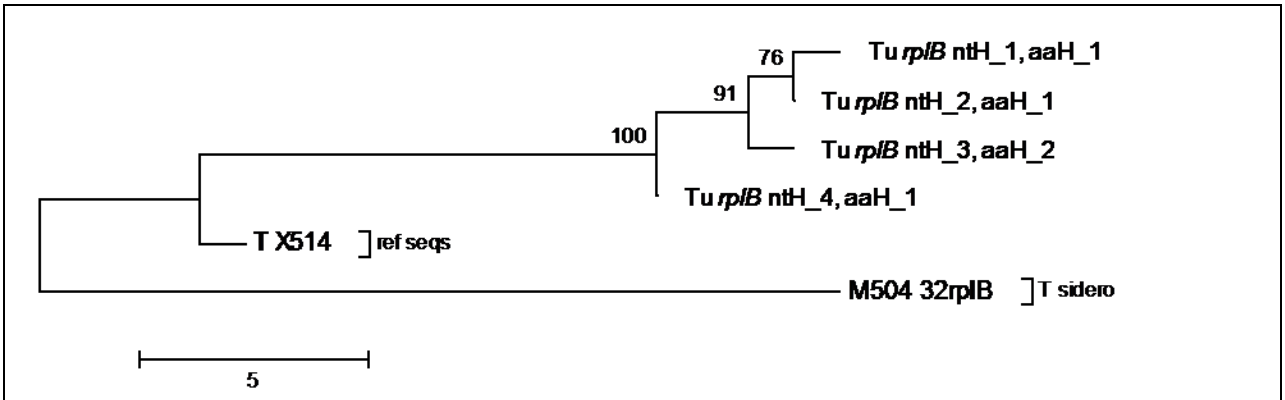

Fig. 6.S7. Phylogenetic tree based on the four *Thermoanaerobacter uzonensis rplB* nucleotide sequence variants and the *rplB* nucleotide sequences of related taxa. The evolutionary history was inferred using the Maximum Parsimony method. The most parsimonious tree with length = 37 is shown. For parsimony-informative sites, the consistency index is 1.0 and the retention index is 1.0. The Maximum Parsimony tree was obtained using the Close-Neighbor-Interchange algorithm with search level 3. Branch lengths are in the units of the number of changes over the whole sequence. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. Phylogenetic analyses were conducted in MEGA4.1 (Tamura et al., 2007).

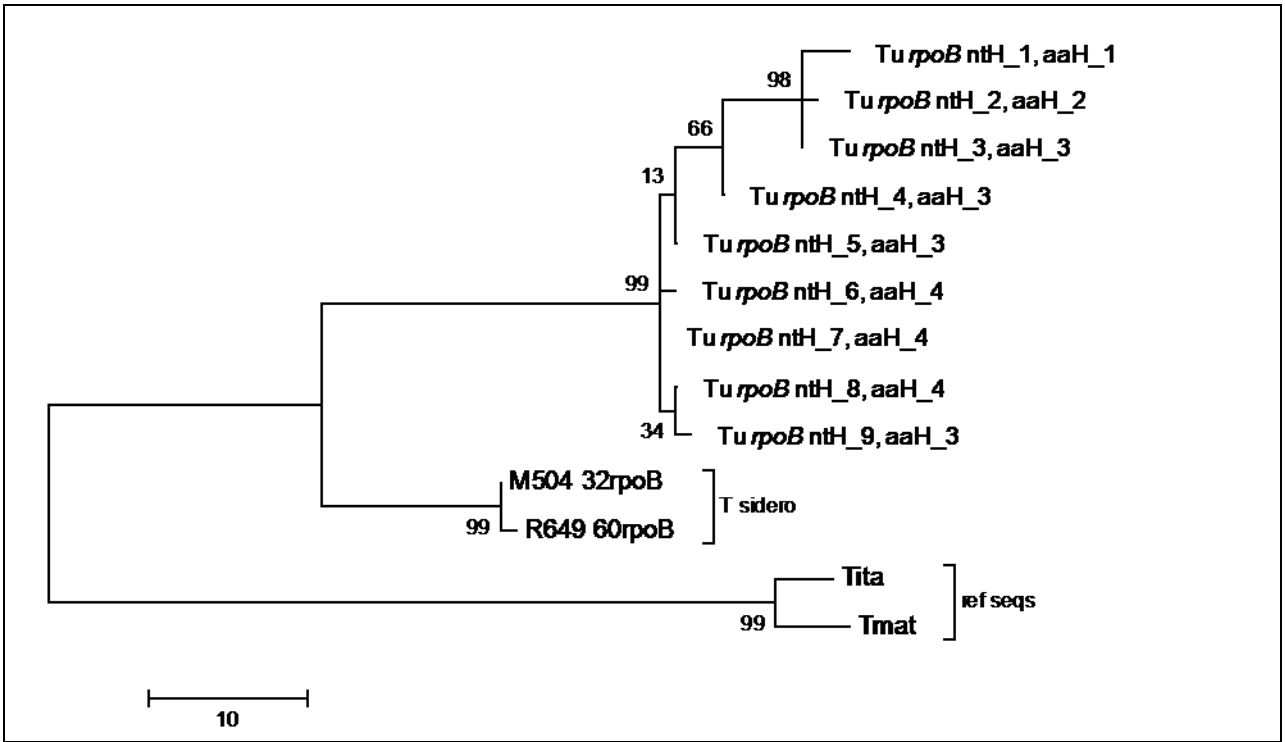

Fig. 6.S8. Phylogenetic tree based on the nine *Thermoanaerobacter uzonensis rpoB* nucleotide sequence variants and the *rpoB* nucleotide sequences of related taxa. The evolutionary history was inferred using the Maximum Parsimony method. Tree #1 out of 46 most parsimonious trees (length = 121) is shown. For parsimony-informative sites, the consistency index is 0.94 and the retention index is 0.96. The Maximum Parsimony tree was obtained using the Close-Neighbor-Interchange algorithm with search level 3. Branch lengths are in the units of the number of changes over the whole sequence. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. Phylogenetic analyses were conducted in MEGA4.1 (Tamura et al., 2007).

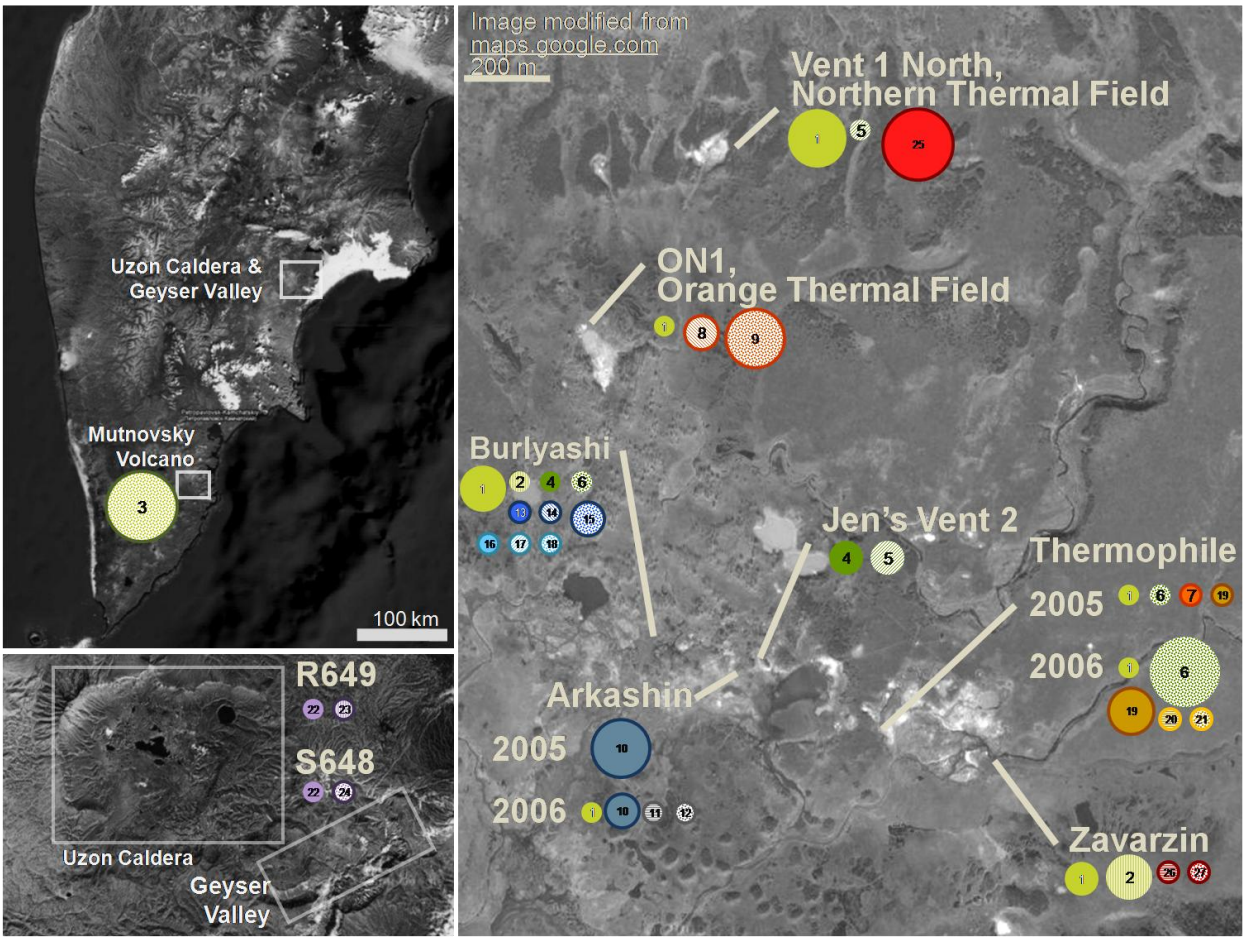

Fig. 6.S9. Distribution of the *Thermoanaerobacter uzonensis* isolates eBURST group, based on eight universally conserved protein coding genes, among geothermal springs of Kamchatka, Russian Far East. Information from Table 6.2. Sizes correspond to number of isolates binned within the eBURST group; bolded ring indicates that the isolates with the particular eBURST group were only isolated from that particular geothermal spring.

CHAPTER 7

SPATIAL AND PHYSICOCHEMICAL CORRELATIONS TO THE GENETIC DIVERSITY OF *THERMOANAEROBACTER UZONENSIS* ISOLATES FROM GEOTHERMAL SPRINGS OF THE UZON CALDERA, GEYSER VALLEY, AND MUTNOVSKY REGIONS OF KAMCHATKA, RUSSIAN FAR EAST[8]

[8] Isaac D. Wagner, Douglas E. Crowe, Christopher S. Romanek, Paul A. Schroeder, Chuanlun L. Zhang, and Juergen Wiegel

To be submitted to: Environmental Microbiology

SUMMARY

Analyses of the 16S rRNA and eight universally conserved protein coding gene sequences from *Thermoanaerobacter uzonensis* isolates from 10 geothermal springs in Kamchatka revealed patterns of spatial variation. A relationship was observed between *T. uzonensis* genetic divergence and the spatial separation of the corresponding geothermal springs. The between-group average Jukes and Cantor genetic distance values compared to the spatial separation of the geothermal springs had an R^2 value of 0.88, obtained from a least squares fit logarithmic trendline. However, a similar relationship was not observed when only intra-regional *T. uzonensis* populations were considered. High Spearman's ρ rank correlation values were obtained when comparing 27 physicochemical properties from four geothermal springs in the Uzon Caldera region of Kamchatka to the biological distribution pattern based on 105 *T. uzonensis* isolates from the same springs. A ρ value of 1.0 was observed with aluminum (Al), and for some other combinations of physicochemical properties. While Al has no known biological essentiality, Al has been shown to influence the availability of phosphate. Aluminum might have also co-varied with properties that were not measured. Together, these analyses suggest that the spatial variation of *T. uzonensis* isolates was influenced by both environmental differences and spatial separation of the geothermal springs.

INTRODUCTION

Until the end of the 20th century, a prevailing assumption was that geographic barriers between habitats of the same type did not exist for microorganisms (de Wit and Bouvier, 2006). Notably, this assumption was based on observation; for example, representatives belonging to the species *Sulfolobus acidocaldarius* and *Sulfolobus solfataricus* were each isolated from North

American and Italian sources (Zillig et al., 1980). However, this longstanding viewpoint has been amended with advances in DNA sequencing that have improved the ability to analyze small genetic differences, across multiple genes, within many strains isolated from geographically separated regions.

Biogeographical differences have been observed for a number of *Bacteria* and *Archaea*. Intercontinental examples include *Synechococcus* spp. and *Oscillatoria amphigranulata* from hot springs within North America, Japan, and New Zealand (Papke et al., 2003), and *Sulfolobus* isolates from North America (Yellowstone National Park and Lassen Volcanic National Park USA), Kamchatka (Uzon Caldera/Geyser Valley and Mutnovsky Volcano regions), and western Iceland (Whitaker et al., 2003). Spatial patterns of diversity within a region have also been observed and examples include *Rhodothermus marinus* isolates from geothermal sites in Iceland (Petursdottir et al., 2000), and taxa belonging to the genus *Sulfurihydrogenibium* from thermal springs of Yellowstone National Park (Takacs-Vesbach et al., 2008).

While most reports that have described biogeographical differences focused on genetic differences between strains, phenotypic differences have been reported for strains of the extremely halophilic bacterium *Salinibacter ruber* isolated from Mediterranean, Atlantic, and Peruvian salterns (Rosselló-Mora et al., 2008). Many, though not all, of the microorganisms for which biogeographical differences have been observed are extremophiles. The habitats of extemophiles, e.g., terrestrial geothermal springs or hypersaline salterns, are often confined to comparatively small and spatially separated areas; as such, these environments have insular qualities (Petursdottir et al., 2000; Rosselló-Mora et al., 2008).

Studies of the biogeography of prokaryotes are interconnected with the ideas concerning ecological diversity, community assemblage patterns, the scaling of microbial diversity, and the

mechanisms of speciation and evolution (Green and Bohannan, 2006; Martiny et al., 2006; Horner-Devine et al., 2007). A framework of hypotheses regarding microbial biogeography have been proposed, essentially related to two questions, i) do microbial assemblages differ in different locations, and ii) if they do differ by location, is the spatial variation attributable to environmental factors, historical incidences, or both (Martiny et al., 2006)?

More than 130 groups of geothermal features, including terrestrial geothermal springs, fumaroles, and geysers, are found within the Kamchatka Peninsula (Karpov and Naboko, 1990). From Kamchatkan geothermal springs, over 220 *Thermoanaerobacter* strains were isolated from mixed water and sediment samples (Chapter 5). Isolates were obtained from geothermal springs within the Uzon Caldera, Geyser Valley, and Mutnovsky Volcano regions. Most isolates were found to be phylogenetically related to *Thermoanaerobacter uzonensis*, having ≥98% 16S rRNA gene sequence similarity to strain JW/IW010^{T} (Wagner et al., 2008), although 14 isolates were related to *Thermoanaerobacter siderophilus* strain SR4^{T} (Slobodkin et al., 1999). *T. uzonensis* isolates were obtained from 10 of the examined Kamchatkan geothermal springs.

Fragments of eight universally conserved protein coding genes, *gyrB*, *lepA*, *leuS*, *pyrG*, *recA*, *recG*, *rplB*, and *rpoB* (Santos and Ochman, 2004), were amplified and sequenced from the *Thermoanaerobacter* isolates (Chapter 5). Analyses with these protein coding genes from the *T. uzonensis* isolates revealed i) polymorphism existed within all gene sequence sets, although the heterogeneity varied in type and quantity, ii) that there was a range of variation within the isolates derived from one geothermal spring, and iii) that while most of the intraspecies variance came from inter-regional comparisons, high diversity measures between populations within a region were also observed (Chapter 6). The previous analyses also revealed patterns of spatial variation with regard to the *T. uzonensis* isolates from different geothermal springs of Kamchatka

(Chapter 6). Using the same set of *T. uzonensis* isolates, the two goals here are to i) assess the correlation between the genetic divergence between isolates and the spatial separation of the geothermal springs, and ii) assess the correlation between the observed biological diversity pattern and the physicochemical differences considering a subset of geothermal springs from the Uzon Caldera region of Kamchatka.

RESULTS

T. uzonensis genetic distance correlations to the spatial separation of the populations

Thermoanaerobacter isolates were derived from geothermal springs separated by distances that varied between approximately 140 m and 250 km (Chapter 6, Fig. 6.1, Table 7.S1). The correlation between genetic diversity of 123 *T. uzonensis* isolates and the spatial separation of the geothermal springs from which strains were derived was assessed in two ways, i) considering the between-group average Jukes and Cantor genetic distance and ii) considering the population pairwise F_{ST} values (Fig. 7.1). Between geothermal springs from different regions, the *T. uzonensis* genetic divergence does increase with spatial separation. The logarithmic trendline based on the between-group average Jukes and Cantor genetic distances and the spatial separation of the geothermal springs ($y = 0.001\ln(x) - 0.0022$), had a R^2 value of 0.88 (Fig. 7.1a). A similar trend was observed when population pairwise F_{ST} values were considered (logarithmic trendline $y = 0.0785\ln(x) - 0.2158$), although a lower R^2 value, 0.63, was obtained (Fig. 7.1b). However, when the correlation assessments are restricted to *T. uzonensis* populations within a region, comparisons of spatial separation and the between-group average Jukes and Cantor distance and the population pairwise F_{ST} values had R^2 values of 0.03 and 0.01, respectively (Fig. 7.1).

Comparison of genetic divergence between populations and the physicochemical properties

The BIO-ENV procedure (Primer v5, Primer-E Ltd.) was used to assess the relationship between the biological diversity pattern and the physicochemical properties of the geothermal spring (Clarke and Ainsworth, 1993). Specifically, a distribution pattern based on 105 *T. uzonensis* strains from four geothermal springs located within the Uzon Caldera was compared to a set of 27 physicochemical properties, The analysis revealed that many comparisons had high rank correlation values (Table 7.2). Most notably, the comparison between the biotic matrix and Al was found to have a ρ value of 1.0, meaning that the Al data completely explained the observed biological pattern. The combination of Al and P also had a ρ value of 1.0, as did many of the combinations of four abiotic variables (Table 7.2). Physicochemical properties that were repeatedly found, alone or in combination, to have high Spearman's ρ values are listed in Table 7.S2.

DISCUSSION

Previously, the intraspecies diversity of *T. uzonensis* strains was examined using eight universally conserved protein coding genes sequences (Chapter 5). A phylogenetic tree based on universally conserved protein coding gene sequence concatenates from the *T. uzonensis* isolates showed that to a great extent, the isolates group according to region (Chapter 6, Fig. 6.6). Moreover, the *T. uzonensis* isolates often cluster according to the particular geothermal spring from which they were derived (Chapter 6). Although 13 *Sulfolobus* strains from the Uzon Caldera and Geyser Valley group clustered together (Whitaker et al., 2003), strains of *T. uzonensis* the Uzon Caldera and Geyser Valley generally formed separate clades on the phylogenetic tree (Chapter 6, Fig. 6.6).

While Petursdottir et al. (2000), Hreggvidsson et al. (2006), and Papke et al. (2003), have described patterns of diversity within and between spatially separated microbial populations, they did not quantitatively describe any relationship between genetic divergence and spatial separation. Whitaker and colleagues (2003) clearly showed a relationship between genetic divergence and intercontinental geographic isolation. A clear relationship is similarity observed considering the genetic divergence of *T. uzonensis* isolate populations between regions within the Kamchatka Peninsula (Fig. 7.1).

The population pairwise F_{ST} values Whitaker and colleagues (2003), calculated from the *Sulfolobus* populations of the Uzon Caldera:Geyser Valley and Uzon Caldera/Geyser Valley:Mutnovsky were 0.14 and 0.59 respectively. By comparison, the same analyses with the *T. uzonensis* populations described herein gave pairwise F_{ST} values of 0.41 and 0.59 (data not shown). The F_{ST} parameter provides a measure of population differentiation based on variance in genetic diversity within and between groups of strains. Therefore, what was especially intriguing from the comparison of pairwise F_{ST} values from the *T. uzonensis* and *Sulfolobus* populations was that the *T. uzonensis* Uzon Caldera:Geyser Valley F_{ST} value was higher even though the *T. uzonensis* isolates presumably form spores (Wagner et al., 2008; Chapter 6). Sporulation would allows strains to more easily survive dispersal to new environments, whereas *Sulfolobus* isolates have no know spore-like state (Whitaker et al., 2003). Considering the set of *T. uzonensis* isolates from the Uzon Caldera and Geyser Valley, linkage disequilibrium was not observed when 1000 randomized datasets were examined ($p<0.001$); however, $V_{obs} > V_{max}$ when 20 randomized datasets were examined ($p <0.01$) (Chapter 6, Table 6.7). These analyses suggest that recombination does occur between the *T. uzonensis* populations within and between the

geothermal springs of the Uzon Caldera and Geyser Valley, but that there is also some association between the alleles at different loci.

However, caution is needed when making direct comparisons between the results obtained in the studies discussed above. Although, Whitaker and colleagues (2003) obtained *Sulfolobus* isolates from the Uzon Caldera, Geyser Valley, and Mutnovsky Volcano regions of Kamchatka, and *T. uzonensis* isolates were obtained from geothermal springs within the same three regions (Chapter 6), differences between the studies include: number of isolates examined, the number geothermal springs within a region sampled, the concatenated gene sequence length, and the number of polymorphic nucleotide sites. Additionally, the term "region" is subjective; e.g., Whitaker et al. (2003) referred to the Uzon Caldera and Geyser Valley as one region; within this work, the authors consider the Uzon Caldera and Geyser Valley two separate regions.

Papke and colleagues (2003) surveyed the diversity of cyanobacteria from geothermal springs of North America, Japan, and New Zealand, primarily though the use of a culture-independent 16S rRNA gene sequence analysis approach. While the analyses with cyanobacteria from geothermal springs revealed distribution patterns, there was no strong association observed between distribution of genotypes and the chemical character of hot springs based on 20 chemical parameters measured from 47 geothermal sites (Papke et al., 2003). In contrast, strong associations were observed between the biological diversity pattern based on the genetic divergence of 105 *T. uzonensis* isolates and 27 physicochemical properties measured from the corresponding springs (Table 7.2). Moreover, the rank correlation results imply that the physicochemical data fully explained the observed biological diversity pattern within the springs examined.

When *T. uzonensis* isolates were binned to different eBURST groups (Feil et al., 2004; Spratt et al., 2004), none of the isolates from Pulsating Spring and Arkashin grouped together, although the geothermal springs are located in the central sector of the Eastern Thermal Field separated by <140 m (Chapter 6, Fig. 6.1, Table S1). Although a diverse set of *T. uzonensis* isolates were readily isolated from Burlyashi, *Thermoanaerobacter* isolates were not obtained from the geothermal spring "Oil Pool" located approximately 50 m north of the Burlyashi spring. These initial results suggested that environmental heterogeneity influenced the distribution of *T. uzonensis* genotypes.

The physicochemical properties with high rank correlations to the biotic matrix (Table 7.2) were interpreted in three ways, i.e., by assessing i) which measured property is the most parsimonious, ii) which physicochemical properties are repeatedly seen as having high rank correlations alone or in combination (Table 7.S2), and iii) which physicochemical properties could be biologically relevant with regard to the distribution of microbial taxa.

The most parsimonious physicochemical measurement that explains the observed biological pattern is the aluminum concentration (Al ppm). Within the geothermal springs analyzed, the Al concentration varied from below the detectable limit, in Thermophilny 2006, to 0.26 ppm in Vent 1 North (Table 7.1). While Al is normally regarded as being biologically inert, i.e., that there is a lack of any known biological essentiality of aluminum, a biogeochemical cycle for Al has been discussed (Exley, 2003). More specifically, there appears to be a relationship between Al, P, and Si (Exley, 1998). In laboratory-grown cultures of diatoms, aluminum was shown to limit the biological availability of phosphate (Exley et al., 1993). Notably, within the studied geothermal springs, there is an inverse relationship between the concentrations of Al and P. The high rank correlation observed for Al could also be due to Al

having co-varied with abiotic properties that did influence the intraspecies diversity patterns but were not included in the physicochemical analysis.

Beside Al, there were other abiotic factors that were repeatedly found to have high rank correlation values, either alone or in combination (Table 7.S2). Physicochemical properties that that were repeatedly observed as having high rank correlation values included P, Ba, NO_2, T°, H_2S, and pH (Table 7.S2). Notably, this list includes several biologically important compounds such as P, NO_2, and H_2S (Amend and Shock, 2001). However, these abiotic factors may have co-varied with the physicochemical properties that did influence the distribution of *T. uzonensis* but were not measured.

The list of physicochemical properties that are repeatedly seen with high rank correlations, alone or in combination, includes compounds previously implicated as having an influence on the distribution of prokaryotes within thermal environments. For example, the concentration of sulfide was reported to influence the predominant *Aquificales* within different geothermal springs of southwestern Iceland (Skirnisdottir et al., 2000). Within geothermal springs of Yellowstone National Park, USA, Mathur and colleagues (2007) reported that the bacterial diversity correlated strongly with the predominant mineral chemistry of the sediment. Specifically, they observed differences with regard to the predominant bacterial group between sampled acidic thermal springs with sulfur-rich sediments, iron-rich sediments, and mixed iron-sulfur sediments (Mathur et al., 2007).

The data presented here revealed that there was a strong correlation between genetic divergence and the corresponding spatial separation of the geothermal springs (Fig. 7.1). For a subset of geothermal springs, high rank correlation values were observed between the physicochemical measurements and the biological diversity pattern. Based on these results, the

spatial variation of *T. uzonensis* isolates appears to have been influenced by both environmental heterogeneity and the spatial separation of the geothermal springs.

Sulfurihydrogenibium spp. can constitute up to 95% of the biomass at some geothermal sites (Takacs-Vesbach et al., 2008; references therein). In contrast, *Thermoanaerobacter* appear to represent a minor percentage of the bacterial population, although culture-independent analyses have shown that *Thermoanaerobacter* taxa are present within Kamchatkan geothermal springs (D. E. Crowe, unpublished). The combination of dispersal ability and population density of a taxon are expected to govern its rate of colonizing new and distant habitats (Martiny et al., 2006). Papke and colleagues (2003) also stated that microorganisms with different phylogenies may have different dispersal and/or invasiveness capabilities. An obvious example is endospore formation, which increases the ability of members of the taxon to survive dispersal over great distances. As mentioned above, sporulation was observed within the type strain of *T. uzonensis* (Wagner et al., 2008).

Proposed trait-based approaches to microbial biogeography have the potential to advance ecological theory and predict responses to environmental change (Green et al., 2008). Previous analyses considering the clustering of *T. uzonensis* sequence types with eBURST and phylogenetic analyses based on concatenated gene sequences have suggested that the *T. uzonensis* population may be structured into generalists/specialists and/or that *T. uzonensis* are adapted to multiple niches within a single geothermal site (Chapter 6). Future analyses should include assessing the physiological and metabolic traits among *Thermoanaerobacter* isolates which would provide additional insight concerning the intraspecies diversity, but were outside the scope of this investigation.

Microorganisms from geothermal sites have been described as being particularly well suited for studies concerning ecological diversity and mechanisms of speciation and evolution because of their special habitat structure (Petursdottir et al., 2000). Future analyses concerning the biogeography of microorganisms from geothermal sites should include a larger number of intraregional springs analyzed and a larger array of geochemical measurements. These studies should then be combined with laboratory-based physiological analyses to test hypotheses generated from the spatial diversity patterns described herein and elsewhere.

EXPERIMENTAL PROCEDURES

Sample collection and *Thermoanaerobacter* isolation

Sample collection and isolation of *Thermoanaerobacter* strains were described in Chapter 5. In brief, the strains were isolated using a mineral medium (Wagner et al., 2008) supplemented with 1 $g \cdot l^{-1}$ glucose, 0.5 $g \cdot l^{-1}$ yeast extract, and 50 mM thiosulfate. Axenic cultures were obtained by repeatedly isolating single colonies on agar plates kept in anaerobic jars and incubated at 62°C. Each isolates was obtained from its own separate enrichment culture. Genomic DNA extraction, gene sequence amplification, and sequencing were performed using standard protocols (Chapter 5). The set of protein coding genes analyzed were among those suggested by Santos and Ochman (2004); DNA gyrase subunit B (*gyrB*), GTP-binding protein LepA (*lepA*), leucyl-tRNA synthetase (*leuS*), CTP synthase (*pyrG*), bacterial DNA recombination protein RecA (*recA*), ATP-dependent DNA helicase RecG (*recG*), 50S ribosomal protein L2 (*rplB*), and RNA polymerase subunit B (*rpoB*).

Geothermal spring spatial separation and measurement of physicochemical properties

Spatial separation of geothermal spring was measured using ArcView software (ESRI Co.) with a QuickBird Satellite Image (Satellite Imaging Corp.). The distances between geothermal springs that included the springs of the Geyser Valley and Mutnovsky region were measured using tools within the Google Earth software (http://earth.google.com; Google Inc.). Temperature and pH were measured at the sample collection location. In the field, Hach kits (Hach Co.) were used to measure SO_4, H_2S, and Fe(total). The concentrations of cations and anions were measured from acidified water samples analyzed at the University of Georgia, Athens; cations were measured via inductively coupled plasma mass spectrometry (ICP-MS), and anions were measured via ion chromatography (IC). To measure $\delta^{18}O$, δD, samples were extracted using standard temperature conversion elemental analyzer (TCEA) protocols and measured via dual inlet mass spectrometry.

Analysis of the *Thermoanaerobacter uzonensis* population genetic diversity

The *T. uzonensis* between-group average Jukes and Cantor genetic distance values and the pairwise F_{ST} values, were calculated from concatenated sequences of the eight universally conserved protein coding genes. The set contained 123 *T. uzonensis* isolates and when all positions containing gaps and missing data were eliminated from the dataset, there were a total of 7,985 nucleotide positions. MEGA 4.1 was used to calculate the between population average Jukes and Cantor genetic distance values (Tamura et al., 2007), and Arlequin 3.1 was used to calculate the population pairwise F_{ST} values (Excoffier et al., 2005). To assess linkage disequilibrium within the *T. uzonensis* population, I_a, V_{obs}, and V_{max} values were determined using the MLST database, http://www.mlst.net/ (Feil et al., 2004).

Comparison of the genetic divergence between populations and the physicochemical properties of the hot springs

A dissimilarity matrix based on the genetic divergence between *T. uzonensis* populations was generated for subsequent comparisons with the dissimilarity matrices based on the physicochemical properties from the geothermal springs. From a set of 105 *T. uzonensis* isolates from the geothermal springs Burlyashi, Thermophilny 2006, Vent 1 North, and Zavarzin, the Jukes and Cantor genetic distance values were calculated from concatenates of the *gyrB*, *leuS*, *pyrG*, and *recA* genes sequences. The concatenated sequences were grouped at the 0.002 level using DOTUR (Schloss and Handelsman, 2005), a taxa abundance table was constructed, and a Bray-Curtis dissimilarity matrix was then generated (Magurran, 2004).

The Primer v5 BIO-ENV procedure (Primer-E Ltd.) was used to assess the agreement between the differences in biological diversity, i.e., the Bray-Curtis similarity matrix, and the abiotic properties of the geothermal springs (Clarke and Ainsworth, 1993). A set of 27 physicochemical measurements were available from the Burlyashi, Thermophilny 2006, Vent 1 North, and Zavarzin geothermal springs (Table 7.1). Data describing concentrations were log transformed prior analysis. Spearman's ρ values were calculated between the biotic and abiotic similarity matrices taking into account combinations of one to four physicochemical variables.

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Tables

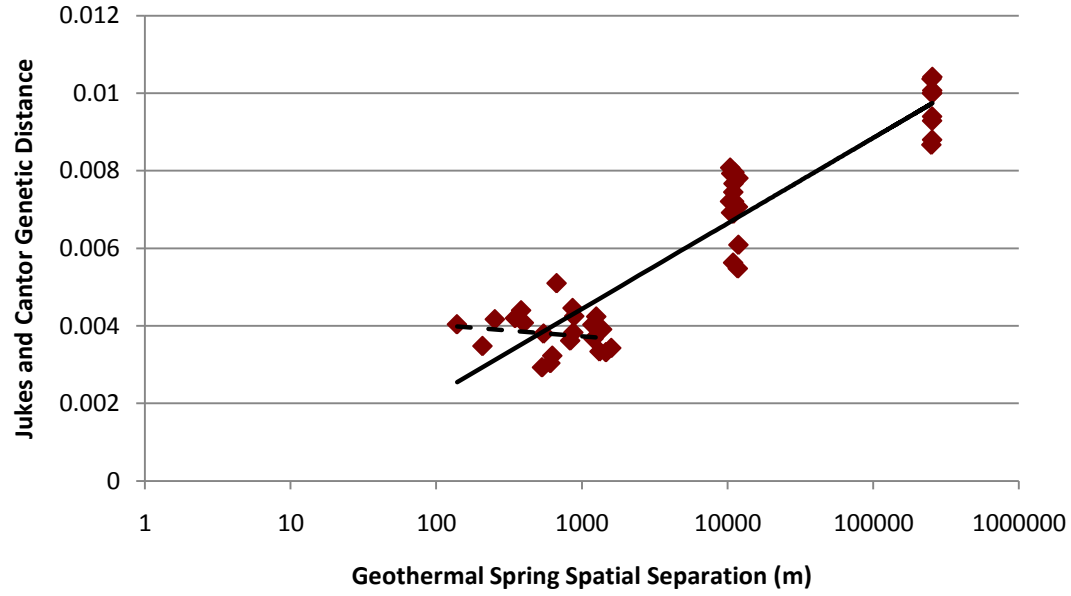
Table 7.1. Physicochemical measurements from four geothermal springs from the Uzon Caldera, Kamchatka. Abbreviations of geothermal springs: Burlyashi 2006 (B621), Thermophilny 2006 (H608), Vent 1 North 2006 (V634), and Zavarzin 2006 (Z606). Measurements given as 0 are below detection limit .

Geothermal Spring	pH	T°	Al ppm	As ppm	B ppm	Ba ppm	Ca ppm	Fe ppm	K ppm	Mg ppm	Mn ppm	Na ppm	P ppm	Pb ppm
Burlyashi	6.1	94	0.01	0.52	11.1	0.08	34.27	0.21	22.31	8.09	0.29	213.5	0.27	0.11
Thermophilny	4.4	75	0	0.15	5.32	0.06	34.15	0	15.44	11.12	0.6	131.1	0.38	0.02
Vent 1 North	5.8	40	0.26	0.02	0.94	0.22	32.96	0.41	14.5	7.02	0.62	40.61	0.2	0.03
Zavarzin	5.8	54	0.02	0.03	0.72	0.05	19.93	0	5.53	6.04	0.43	34.83	0.26	0

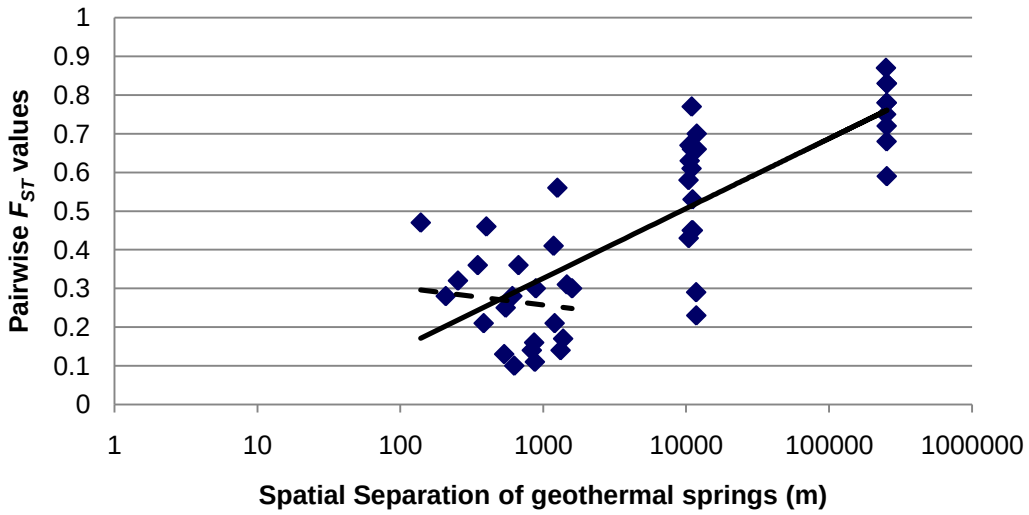
Geothermal Spring	Sb ppm	Si ppm	Sr ppm	Li ppm	Hg ppm	SO_4 ppm	Cl ppm	δ18O	δD	H_2S	NO_2	NH_4	ALK
Burlyashi	0.06	87.19	130	0.754	0	230.2	251	-7.9	-92	2.1	0.006	15	130
Thermophilny	0	109.4	150	0.444	0	19.91	90	-15.7	-112	1.375	0.008	2.3	265
Vent 1 North	0	116.3	180	0.014	0	132.69	35	-15.8	-115	8	0.003	7	110
Zavarzin	0.35	72.99	90	0.035	0	53.47	17	-14.2	-107	0.2	0.004	18	195

Table 7.2. Summary of Spearman's ρrank correlation results comparing the physiochemimcal properties to the observed *Thermoanaerobacter uzonensis* biological diversity pattern within four geothermal springs of the Uzon Caldera. Rank correlation values (calculated as Spearman's ρ), ρ; abiotic variable(s), AV.

1 Abiotic Variable		2 Abiotic Variables		3 Abiotic Variables				4 Abiotic Variables			
AV	**ρ**	**AV**	**ρ**	**AV**	**ρ**	**AV**	**ρ**	**AV**	**ρ**	**AV**	**ρ**
Al	**1.00**	**Al, P**	**1.00**	**pH, T°, H_2S**	**0.94**	**Al, P, Li**	**0.94**	**pH, Al, Ba, NO_2**	**1.00**	**Al, P, Sb, dD**	**1.00**
H_2S	**0.66**	**Al, NO_2**	**0.94**	**pH, Li, H_2S**	**0.94**	**Al, P, Sr**	**0.94**	**pH, Al, Ba, Na**	**1.00**	**Al, P, Sb, d18O**	**1.00**
Ba	**0.60**	**Ba, P**	**0.94**	**T°, Al, NO_2**	**0.94**	**Al, P, Si**	**0.94**	**pH, Al, Li, H_2S**	**1.00**	**Al, P, NO_2, ALK**	**1.00**
P	**0.54**	**Ba, NO_2**	**0.94**	**T°, Al, P**	**0.94**	**Al, P, NO_2**	**0.94**	**pH, Al, Sr, Li**	**1.00**	**Mn, P, H_2S, NO_2**	**1.00**
NO_2	**0.49**	**Al, Mg**	**0.83**	**T°, Ba, P**	**0.94**	**Al, P, H_2S**	**0.94**	**T°, Al, P, Si**	**1.00**	**pH, T°, Al, Sr**	**0.94**
Fe	**0.29**	**P, H_2S**	**0.83**	**T°, Ba, NH_4**	**0.94**	**Al, Si, NO_2**	**0.94**	**T°, Ba, Mg, ALK**	**1.00**	**…**	
T°	**0.14**	**Al, Fe**	**0.77**	**T°, Ba, NO_2**	**0.94**	**Al, NO_2, ALK**	**0.94**	**T°, Ba, P, Si**	**1.00**		
ALK	**0.14**	**…**		**T°, P, Si**	**0.94**	**Ba, Mn, P**	**0.94**	**T°, P, Si, ALK**	**1.00**		
Li	**0.14**			**T°, P, Sr**	**0.94**	**Ba, Na, P**	**0.94**	**Al, B, Fe, NH_4**	**1.00**		
NH_4	**0.09**			**Al, Ba, P**	**0.94**	**Ba, P, NO_2**	**0.94**	**Al, Ba, P, NO_2**	**1.00**		
...				**Al, Ba, Mg**	**0.94**	**Ba, P, H_2S**	**0.94**	**Al, Ca, Mn, P**	**1.00**		
				Al, Ba, NH_4	**0.94**	**Mn, H_2S, NO_2**	**0.94**	**Al, Fe, Li, NH_4**	**1.00**		
				Al, Ba, NO_2	**0.94**	**pH, Al, Ba**	**0.89**	**Al, Mn, H_2S, NO_2**	**1.00**		



a.



b.

Fig. 7.1. Genetic divergence between *Thermoanaerobacter uzonensis* populations compared to spatial separation of the geothermal springs. (a) Between-group average Jukes and Cantor genetic distance values. The number of base substitutions per site using the Jukes and Cantor method from averaging over all sequence pairs between groups compared to the spatial separation of the geothermal springs. Trendlines were: overall : y = 0.001ln(x) - 0.0022 (R^2 = 0.875); Intraregional: y = -2E-07x + 0.004 (R^2 = 0.03). (b) Population pairwise F_{ST} values compared to the spatial separation of the geothermal springs. Trendlines were: overall: y = 0.0785ln(x) - 0.2158 (R^2 = 0.63); intraregional:y = -3E-05x + 0.3003 (R^2 = 0.01).

SUPPLEMENTARY INFORMATION

Supplementary Tables

Table 7.S1. Spatial separation (m) of the Kamchatkan geothermal springs from which *Thermoanaerobacter uzonensis* isolates were derived

	Arkashin 2006	Burlyashi	Thermophilny 2006	Arkashin 2005	Pulsating Spring (Jen's Vent 2)	Mutnovsky	ON1	Resting Rock	Prox. to Salt Spring	Thermophilny 2005	Vent 1 North
Arkashin 2006											
Burlyashi	253										
Thermophilny 2006	400	627									
Arkashin 2005	1	253	400								
Pulsating Spring (Jen's Vent 2)	139	383	348	139							
Mutnovsky	253120	252959	253157	253120	253206						
ON1	887	874	1202	887	864	253674					
Resting Rock	10983	11145	10597	10983	10948	250958	11780				
Prox. to Salt Spring	10944	11089	10560	10944	10915	250298	11757	671			
Thermophilny 2005	400	627	1	400	348	253157	1202	10597	10560		
Vent 1 North	1256	1323	1463	1256	1182	254206	533	11865	11870	1463	
Zavarzin	608	832	208	608	546	253189	1378	10425	10397	208	1592

Table 7.S2. Frequency of abiotic factors observed in Table 7.2			
Al	33	NH_4	5
P	27	Fe	4
Ba	20	Sr	4
NO_2	16	Mg	3
T°	14	Na	2
H_2S	10	Sb	2
pH	8	B	1
Li	6	Ca	1
Si	6	$\delta^{18}O$	1
ALK	5	δD	1
Mn	5		

CHAPTER 8

DISSERTATION CONCLUSIONS

"Microbial communities are highly diverse, a fact that demands both description and explanation" wrote Kassen and Rainey (2004). Isolation and cultivation continue to be the foundation for testing metabolic abilities and for in-depth genomic studies of individual members of microbial communities (Cardenas and Tiedje, 2008). Novel anaerobic thermophilic taxa were herein described. *Caldanaerovirga acetigignens* gen. nov., sp. nov.; and *Thermoanaerobacter uzonensis* sp. nov. were descriptions written by the author. Co-authored novel taxa descriptions were *Thermosediminibacter oceani* gen. nov., sp. nov and *Thermosediminibacter litoriperuensis* sp. nov, as well as *Caldicoprobacter oshimai* gen. nov., sp. nov.

Understanding the spatial patterns of diversity are critical to deciphering the forces shaping and maintaining the diversity of life (Zhou et al., 2008; references therein). More than 220 anaerobic thermophilic isolates were obtained from samples collected from 11 geothermal springs within the Uzon Caldera, Geyser Valley, and Mutnovsky Volcano regions of the Kamchatka Peninsula, Russian Far East. Subsequent 16S rRNA gene sequence analyses revealed that most strains were phylogenetically related to *Thermoanaerobacter uzonensis* JW/IW010^T, although some strains were related to *Thermoanaerobacter siderophilus* SR4^T. To describe and elucidate the intraspecies heterogeneity, α- and β-diversity patterns, and the spatial and physicochemical correlations to the observed genetic variation, eight universally conserved protein coding genes were amplified and sequenced from the *T. uzonensis* and *T. siderophilus* strains. The eight protein coding genes examined were: DNA gyrase subunit B, *gyrB*; GTP-

binding protein LepA, *lepA*; leucyl-tRNA synthetase, *leuS*; CTP synthase, *pyrG*; RecA bacterial DNA recombination protein, *recA*; ATP-dependent DNA helicase RecG, *recG*; 50S ribosomal protein L2, *rplB*; and RNA polymerase subunit B, *rpoB*. In total, more than 1,500 protein coding gene sequences from *T. uzonensis* isolates and more than 100 from *T. siderophilus* isolates were analyzed.

Variation was observed within all of the protein coding gene sequence sets examined in the *T. uzonensis* isolates. However, the type of substitution, e.g., synonymous or nonsynonymous, and quantity of the variation differed between gene sequence sets. The *T. uzonensis* protein coding genes appear to have divergent phylogenies, e.g., the genes appear to be independently evolving and evidence for homologous recombination and lateral gene transfer was observed. The most applicable species concept for *T. uzonensis* must consider metapopulation/ subpopulation dynamics and acknowledge that physiological characteristics (e.g., sporulation) likely influences the flux of genetic information between subpopulations.

Spatial variation patterns were observed for the set of *T. uzonensis* isolates. Evaluation of *T. uzonensis* α-diversity revealed a range of genetic variation within a single geothermal spring. β-diversity measurements revealed that while most of the molecular variance came from inter-regional comparisons, high diversity measures between populations within a region were also observed. Analyses suggested that multiple niches could be available for the *T. uzonensis* genotypes within many of the analyzed geothermal springs, or that the population may be structured into *T. uzonensis* genotypes with generalist and specialist characteristics.

The set of *T. siderophilus* isolates allowed for comparisons of intraspecies variation within a phylogenetically close taxon. No variation between the *gyrB* gene sequences of the *T. siderophilus* isolates was observed whereas the *gyrB* variation of *T. uzonensis* isolates was

mostly delineated by geographic region. Evidence for lateral gene transfer was observed within the *T. siderophilus leuS* gene sequence set. These comparisons suggested that the homologous core-genome genes within different species of the same genus are under different levels of evolutionary selection and that lateral gene transfer of core-genome genes occurs.

Between geothermal springs from different regions, there is a strong correlation between *T. uzonensis* genetic divergence and spatial separation of the corresponding geothermal springs. However, the trend was not observed when only the isolates from geothermal springs within a region were considered. When 27 physicochemical properties from four geothermal springs in the Uzon Caldera were matched to a corresponding biological distribution pattern, high rank correlation values, calculated as Spearman's ρ, were observed. A ρ value of 1.0 was observed with aluminum (Al), and for some other combinations of physicochemical properties. While Al has no known biological essentiality, Al has been shown to influence the availability of phosphate, although Al might have also co-varied with properties that were not measured. Together, these analyses suggest that the spatial variation of *T. uzonensis* was influenced by both environmental heterogeneity and spatial separation of the geothermal springs.

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APPENDIX A

THERMOSEDIMINIBACTER OCEANI GEN. NOV., SP. NOV. AND *THERMOSEDIMINIBACTER LITORIPERUENSIS* SP. NOV., NEW ANAEROBIC THERMOPHILIC BACTERIA ISOLATED FROM PERU MARGIN[9]

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ABSTRACT

A new group of anaerobic thermophilic bacteria was isolated from enrichment cultures obtained from deep sea sediments of Peru Margin collected during Leg 201 of the Ocean Drilling Program. A total of ten isolates were obtained from cores of 1–2 m below seafloor (mbsf) incubated at 60°C: three isolates came from the sediment 426 m below sea level with a surface temperature of 9°C (Site 1227), one from 252 m below sea level with a temperature of 12°C (Site 1228), and six isolates under sulfate-reducing condition from the lower slope of the Peru Trench (Site 1230). Strain JW/IW-1228P from the Site 1228 and strain JW/YJL-1230-7/2 from the Site 1230 were chosen as representatives of the two identified clades. Based on the 16S rDNA sequence analysis, these isolates represent a novel group with *Thermovenabulum* and *Caldanaerobacter* as their closest relatives. The temperature range for growth was 52–76°C with an optimum at around 68°C for JW/IW-1228P and 43–76°C with an optimum at around 64°C for JW/YJL-1230-7/2. The pH25C range for growth was from 6.3 to 9.3 with an optimum at 7.5 for JW/IW-1228P and from 5 to 9.5 with an optimum at 7.9–8.4 for JW/YJL-1230-7/2. The salinity range for growth was from 0% to 6% (w/v) for JW/IW-1228P and from 0% to 4.5% (w/v) for JW/YJL-1230-7/2. The G+C content of the DNA was 50 mol% for both JW/IW-1228P and JW/YJL-1230-7/2. DNA–DNA hybridization yielded 52% similarity between the two strains. According to 16S rRNA gene sequence analysis, the isolates are located within the family, *Thermoanaerobacteriaceae*. Based on their morphological and physiological properties and phylogenetic analysis, it is proposed that strain JW/IW-1228P^{T} is placed into a novel taxa, *Thermosediminibacter oceani*, gen. nov., sp. nov. (DSM 16646^{T}=ATCC BAA-1034^{T}), and JW/YJL-1230-7/2^{T} into *Thermosediminibacter litoriperuensis* sp. nov. (DSM 16647^{T} =ATCC BAA-1035^{T}).

INTRODUCTION

It is estimated that the number of prokaryotes in the deep subsurface sediment can make up more than 60% of the global number of prokaryotes (Whitman et al. 1998). They also represent 10–35% of the total biomass on the Earth (Parkes et al. 2000; Whitman et al. 1998). Recent studies showed microbial community in deep subsurface sediments may affect atmospheric carbon stocks and climate change (D'Hondt et al. 2002 and literature cited therein). Despite their significant impacts on Earth's surface chemistry and climate (Dickens 2001), and oceanic alkalinity (D'Hondt et al. 2002), little is known about the phylogenetic, metabolic, and physiological diversity of the deep subsurface microbiota.

Due to the low culturability and viability (Cragg et al. 1990), the study of individual microorganisms in deep subsurface biosphere is a challenge. Temperature, one of the limiting factors also affects bacterial distributions in the deep subsurface sediments. Temperature rises as the depth increases, and may be responsible for the limitation of the organic matters. However, the presence of a significant bacterial population has been reported in deep sea sediments (Parkes et al. 1994; Cragg et al. 1996). Besides their potential for environmental and biotechnological applications, the isolation and characterization of thermophilic microorganisms from marine deep subsurface sediments can provide better understanding of metabolic and biogeochemical influences of the indigenous microorganisms on the ecosystem.

The Ocean Drilling Program (ODP) Leg 201 primarily focused on the microbial communities in deep sea sediments, at a series of sites in the eastern equatorial Pacific, the Peru Basin, and the Peru Margin. To determine whether thermophilic microorganisms can survive at suboptimal temperatures in marine sediments over long periods of time, an attempt was made to isolate thermophilic anaerobes from sediment samples collected at various depths and thus of

increasing age at the equatorial Pacific sites and at the Peru Margin sites. Here, we report the identification of several isolates representing two novel thermophilic anaerobes from the upper layers of the Peru Margin sediment.

METHODS

Collection of inocula

Core samples were collected from Eastern Equatorial Pacific and Peru Margin during the ODP cruise Leg 201 in February/March 2002 as described in detail in D'Hondt et al. (2003). The testing for drilling fluid contamination is described by House et al. (2003). The characteristics of different sites and drilling holes from which core samples were used and the schemes for microbial analysis are given in detail by Shipboard Scientific Party (sections ldquoMicrobiologyrdquo, 2003b, c, d, e). The incubations of interest for this report are those denoted by the incubation temperature 60°C. Basically after the cores were retrieved on board and cut in about 1.5-m sections, subsections were made in the cold room from which subsamples were aseptically cored from the center using sterile 60-ml syringes or 5-ml syringes (for smaller subsamples) from which the tips were cut off. These cores were extruded into sterile glass vials under a stream of sterile anaerobic nitrogen gas, the vials were closed with a butyl rubber stopper and stored until use.

Enrichment and isolation

Two different approaches were used to obtain the here described new isolates (a) inoculation and incubation of enrichments and MPNs (five tenfold dilutions in triplicates) performed immediately after recovering the core sample on board of the ship including using heterotrophic glycolytic media (b) inoculation and incubation on shore after obtaining the

samples (2.5–4 months after collection and storing at about 4°C under an oxygen-free nitrogen atmosphere) using a sulfate-reducing media for enrichments.

(a) On board procedure: After the core samples were collected, samples were either suspended in oxygen-free saline under an atmosphere of nitrogen which then were used for inoculation or samples were transferred via a sterile spatula into the incubation tubes using basically the Hungate procedure to keep samples and media anaerobic (Ljungdahl and Wiegel 1986). The equivalence of 0.8–1 ml solid core sample was inoculated in Balch tubes containing 9 ml of pre-reduced anaerobic heterotrophic sea salt media of pH60C (Wiegel 1998) 8.0 and 8.8 and supplemented with 0.05% yeast extract and 0.2% each of glucose, fructose and mannose (hexose media), or xylose and ribose (pentose media) with or without 25 mM thiosulfate as additional electron acceptor, respectively. Basic Sea Salt media (full strength SSM) contained 40 g Sigma sea salt, 2 mM Na2HPO4, 2.5 ml/l modified (Ni, W) Wolfe's mineral and 5 ml Wolfe's vitamin solution (Freier et al. 1988), 0.1% NH4Cl and 25 mM Na2CO3. 2 mM each of Na2S and cystein HCl were used as reducing agents (Ljungdahl and Wiegel 1986). From the second subculture made on board, inocula were made on shore into the half diluted sea salt media containing only one of the carbon sources, and the subcultures were also incubated at 60°C.

(b) On shore procedure: Aseptically collected core samples (Shipboard party 2003a) were inoculated into 150 ml serum bottles containing either a phosphate-buffered basal medium or a carbonate-buffered medium (Widdel and Bak 1992) under anaerobic condition by using the modified Hungate technique (Ljungdahl and Wiegel 1986). The phosphate-buffered basal media contained the following (gram per liter of deionized water unless otherwise indicated): NaH2PO4, 5 mM; Na2HPO4, 15 mM; NH4Cl, 0.5; (NH4)2SO4, 0.5; NaCl, 10; MgSO4 7H2O, 0.01; CaCl2, 0.01; trace element solution, 5 ml; vitamin solution, 1 ml; yeast extract, 5;

resazurin, 1 mg; cysteine HCl, 0.05. The carbonate-buffered medium was amended by adding 1% NaCl and 0.1 mM ferric citrate. The pH25C (Wiegel 1998) was adjusted to 7.3 (at 25°C) for a phosphate-buffered basal media and to either 7.0 or 8.0 (at 25°C) for a carbonate-buffered medium before degassing, and cysteine was added after degassing with N2.

Pure cultures were isolated by using agar-shake-roll technique and were usually grown in 10 ml medium in Balch tubes closed with black butyl rubber and aluminum crimps. All incubations were done at 60°C and 80°C. Pure cultures were usually grown in the sea salt medium containing only 1% sea salt or NaCl.

Determination of growth

The growth of isolates was determined by direct cell count using microscopy and by measuring the optical density (OD) at 600 nm using Spectronic 21 spectrophotometer (Bausch and Lomb, Rochester, NY, USA).

Microscopy

The morphology was studied by light and electron microscopy using an Olympus VANOX phase-contrast microscope and JEM-1210 Transmission Electron Microscope (JEOL Inc., Tokyo, Japan), respectively. Phase-contrast micrographs of bacteria were taken using agar-coated slides. Cells used for negative staining were from both early exponential growth phase and stationary growth phase.

Effect of temperature, pH, and salinity

The temperature-gradient incubator (Scientific Industries Inc., Bohemia, NY, USA) was used to determine the temperature range for growth of each isolate. To determine the pH optimum, the various pH values were determined at the optimum growth temperature (Topt) as described by Wiegel (1998). Media for the pH range determination were buffered with 10 mM

each of MES, HEPES, and TAPS in combination with 2 mM phosphate. Various NaCl and KCl (ratio of 9:1) concentrations were added to the basal medium (minus NaCl) to obtain the ranges of salinity supporting growth.

Range of substrate utilization

The ability of the isolates to grow on potential carbon sources was assayed using the phosphate-buffered basal medium as described above but with only 0.02% yeast extract. The cultures were incubated and observed for more than 2 weeks, and the utilization was judged positive if the OD of the culture was twice above the value of control culture containing only yeast extract.

Electron acceptors

The potential use of various electron acceptors was studied using the basal medium (1% NaCl) containing 0.3% yeast extract as an electron donor. Cultures in the exponential growth phase in the basal medium without any additional electron acceptors were used as the inocula (2% v/v). The electron acceptors tested were fumarate (20 mM), sulfate (20 mM), sulfite (2 mM), thiosulfate (20 mM), elemental sulfur (20 mM), nitrate (20 mM), amorphous Fe (III) oxide (90 mM), Fe (III) citrate (20 mM), AQDS (10 mM), and MnO_2 (10 mM). The use of electron acceptors was determined by measuring growth (OD600), sulfide, ammonium or nitrite production, or color-change, respectively.

Analytical techniques

The concentration of dissolved and precipitated sulfides was determined by the CuSO4 spectrophotometric assay (Cord-Ruwisch 1985). Nitrate reduction was performed as previously described (Finegold and Baron 1986). Ferric ion was monitored by measuring ferrous ion production using the Ferrozine assay (Dailey and Lascelles 1977).

Phospholipid fatty acid analysis

Samples were extracted by a single-phase organic solvent system comprised of chloroform, methanol, and aqueous 50 mM PO_4 buffer (pH 7.4) in the ratio of 1:2:0.8 (v/v/v; White et al. 1979). After extracting overnight, equal volumes of chloroform and nanopure water were added to the extractant, resulting in a two-phase system. The lower organic (lipid-containing) phase was collected and concentrated to yield the total lipid extract. The concentrated lipid extract was fractionated on a silicic acid column into neutral lipids, glycolipids, and polar lipids (Guckert et al. 1985). The phospholipid fatty acid (PLFA) in the polar lipid fraction were subjected to a mild alkaline methanolysis to produce fatty acid methyl esters and then the hydroxyl groups converted to the silyl ethers prior to GC and GC-MS analyses.

G+C content of genomic DNA

The DNA was extracted from each isolate using DNeasy Tissue Kit (Qiagen Inc., Valencia, CA, USA). The guanine plus cytosine (G+C) content was measured by HPLC as described previously (Mesbah et al. 1989) with the modification of using S1 nuclease (Invitrogen Co., Carlsbad, CA, USA) and 0.3 M sodium acetate (pH 5.0).

DNA–DNA hybridization

DNA–DNA hybridization was performed by the German culture collection (DSMZ). DNA was isolated using a French pressure cell (Thermo Spectronic) and was purified by chromatography on hydroxyapatite as described by Cashion et al. (1977). DNA–DNA hybridization was carried out as described by De Ley et al. (1970), with the modifications described by Huss et al. (1983), using a model Cary 100 Bio UV/VIS-spectrophotometer

equipped with a Peltier-thermostatted 6×6 multicell changer and a temperature controller with in-situ temperature probe (Varian).

16S rRNA gene sequence determination and phylogenetic analyses

The DNA was extracted as described above and amplified with bacterial domain-specific primer set for 16S rDNA, 27 forward and 1492 reverse (Lane 1991). The PCR amplification was carried out as described previously (Wise et al. 1999). PCR products were purified using QIAquick PCR Purification Kit (Qiagen) and sequenced by Macrogen Inc. (Seoul, Korea). The Similarities of partial sequences were determined using the Sequencher v4.0.5 (Gene Codes Co., Ann Arbor, MI, USA). Retrieved 16S rDNA sequences, 1,386 for JW/IW-1228P and 1,402 for JW/YJL-1230-7/2 were analyzed using BLAST (basic local alignment search tool) and then aligned manually using ClustalX v1.81 (Thompson et al. 1997) to create a multiple sequence alignment. Phylogenetic trees were inferred by the neighbor-joining method (Saitou and Nei 1987) using the model of Jukes and Cantor (Jukes and Cantor 1969), with the phylogenetic analysis package PHYLIP v3.6a2.1 (Felsenstein 2001).

Nucleotide sequence accession number

The 16S rDNA sequences of both strains JW/IW-1228P and JW/YJL-1230-7/2 were submitted to GenBank and assigned accession number AY703478 and AY703479, respectively.

RESULTS AND DISCUSSION

Enrichment and isolation

Samples from the Site 1225, 1226, 1227, and 1228 were inoculated shortly after collecting the core samples into the basal sea salt medium containing 0.05% (w/v) yeast extract and 0.2% each of glucose, fructose and mannose, or xylose and ribose at 60°C. Samples from the

Site 1230 and 1231 were inoculated into the bicarbonate-buffered medium containing acetate or lactate with 28 mM sulfate and incubated at 60°C and 80°C, respectively. Positive enrichment cultures were obtained only at 60°C, but at various pHs (7.0, 7.3, 7.8, 8.0). After three rounds of purification procedures using the agar-shake-roll tube technique (Ljungdahl and Wiegel 1986) a total of ten pure isolates were obtained. Besides the below-described pure isolates, at the shipboard positive enrichments were additionally obtained from site 201-1228-2H (565–582 cm below seafloor) and from site 201-1226E-1H-2 (75–80 cm below sea floor). The first subculture of the latter enrichment had produced after about 8 days 40 mM lactate, 10 mM acetate, and 5 mM formate (Arthur Spivack, personal communication). However these two enrichments were not any longer viable after returning to the University of Georgia. Three of the ten purified isolates had been obtained from the heterotrophic media originally inoculated with sediment from core 201–1227D-1H-1 (ca. 131–138 cm below seafloor) (Site 201-1227, Trujillo Basin on the Peru continental shelf, sea floor 426 m below sea level and a mudline temperature of 9°C) and one from core 201-1228E-1H-1 (136–143 cm below seafloor; Site 201-1228 outer shelf edge of the Peruvian high productivity upwelling system with a sea floor 252 m below sea level and a mud line temperature of 12°C). The strains were designated as JW/IW-1227G (glucose-supplemented media), JW/IW-1227M (mannose- supplemented media), JW/IW-1227X (xylose-supplemented media) and JW/IW1228P (pyruvate-supplemented media). Growth was obtained only in the first two dilution tubes from MPN experiments, indicating less than 100 viable cells/ml sediments. Six isolates were obtained from the medium for sulfate reducers inoculated on shore with samples from Site 201-1230 (lower slope of the Peru Trench, with a mudline temperature of 2°C, 5,086 m below sea level). The strains were designated as JW/YJL-1230-7/1, JW/YJL-1230-7/2, JW/YJL-1230-7/3 (all isolated from media with a pH25C 7.0), JW/YJL-

1230-8/1, JW/YJL-1230-8/2, and JW/YJL-1230-8/3 (isolated from media with a pH25C 8.0). Post cruise enrichments using the medium for sulfate reducers were also set for the samples from 201-1227A-2H-5 (section 64–78 cm), 201 1229-2H-2 (section 64–78 cm), 201 1230A 2H –2 (section 82–75 cm), 201-1230A 11H-2 (section 60–67 cm), 201-1230A 12H-3 (section 64–78 cm), and 201-1230A 15H-3 (section 27–34 cm). Incubations of enrichment cultures at 60°C and 80°C yielded no visible growth.

Colony and cell morphology

Based on 16S rDNA sequence analysis and growth parameters during isolation, two of the isolates were chosen for more detailed characterizations: JW/IW-1228P and JW/YJL-1230-7/2. In agar-roll-tube cultures, the colonies appeared after 2–3 days. The colonies were irregular shaped with 0.1–1.5 mm in diameter. Vegetative cells of strain JW/IW-1228P grown in liquid cultures were straight, sometimes highly elongated rods with 0.2–0.7 mgrm in diameter and 1.5–16 mgrm in length, which occurred singly, in pairs or in chains (Fig. A1a) and staining Gram-negative. Without agitation, cells grown in liquid cultures had the tendency to elongate, form chains or/and aggregates and to flocculate. In the late-exponential or stationary growth phase, cells started to yield swollen ends and bulging sections throughout the elongated cells, and the cytoplasm became granular and heterogeneous, and eventually formed autoplasts (L-shaped cells) (Fig. A1b). Cells of JW/YJL-1230-7/2 isolated under sulfate-reducing conditions, were straight rods, with a diameter of 0.3–0.5 mgrm and 2.0–10.0 mgrm in length (Fig. A1c), and thus were less elongated than the JW/IW-1228P. Cells occurred singly, in pairs, or in chains and stained Gram-negative. Strain JW/YJL-1230-7/2 also produced swollen ends, but infrequently formed autoplasts (Fig. A1d). Electron microscopy revealed both strains were flagellated. Strain JW/YJL-1230-7/2 had 2–4 long peritrichous flagella with a periodicity (wavelength) of ~1–1.3

mgrm (Fig. A1e). Less than 1% cells of strain JW/IW-1228P and up to 5% cells of strain JW/YJL-1230-7/2 exhibited branched cell morphology (Fig. A1). The occurrence of spores was not detected by microscopy or by heat treatment (10 min at 100°C). Despite flagellation no motility besides tumbling was detected by microscopy for any of the strains.

Temperature, pH, and salinity ranges

The temperature range for growth at pH25C 7.8 of strain JW/IW-1228P was 52–76°C, with an optimum at 68°C and 1.6 h doubling time. No growth was detected at above 78°C or below 50°C. Strain JW/YJL-1230-7/2 grew at 45–75°C, with an optimum at 64–65°C. No growth was detected at above 76°C or below 43°C. Due to bioturbation of the sediment it is difficult to exactly determine how long the bacteria have survived in the sediment at temperatures below the minimal growth temperatures determined under laboratory conditions. The minimal growth temperature determined under laboratory conditions is probably higher than in vivo where bacteria could sustain at doubling times of many months, but those growth rates are usually not measured in the laboratory. The age of the sediments from which the samples were taken (Skilbeck, personal communication) is estimated to be around 30,000–50,000 years for 201-1226-1H-1 (no pure isolate obtained), 2,000 years for 201-1228E-1H-1 (strain JW/IW-1228P), and 201-1228E-2H-1 around 50,000 years (no pure isolate obtained). The age of sediments from 201-1230A-1H-1 from which strain JW/YJL-1230-7/2 came is estimated to be between 10,000 years and 15,000 years. Considering that the samples came from sediments hundreds to several tens of thousands of years old (Pleistocene) one could postulate minimal maintenance metabolisms or/and extremely slow growth aiding in survival at temperatures below the lower temperature limit determined in the laboratory. At 60°C, the pH range for JW/IW-1228P was 6.3–9.3, with an optimum at pH25C 7.5. Strain JW/YJL-1230-7/2 grew at pH25C

6.2–9.1, with an optimum at pH 7.9–8.4. The salinity range for JW/IW-1228P was from 0% to 6.0% (w/v), with an optimum at 1%. No growth was detected at 7% (w/v) and above. Strain JW/YJL-1230-7/2 has salinity range from 0% to 4.5% (w/v), with an optimum at 0.5–2%. No growth was detected at 5% (w/v) and above.

Substrate utilization

Strain JW/IW-1228P grew well on Casamino acids, fructose, glucose, mannose, sucrose and xylose (0.2%, w/v). It showed very weak growth on Difco Beef extract, tryptone, lactate, pyruvate, methanol, inositol, manitol, sorbitol, cellobiose, maltose, raffinose and trehalose (0.2%, w/v). However, strain JW/YJL-1230-7/2 utilized tryptone, acetate, lactate, inositol, manitol, xylitol, fructose, galactose, glucose, mannose, raffinose, sucrose and xylose (0.2%, w/v) in the presence of 0.1% yeast extract, but showed only little growth on this relatively broad substrate spectrum. This might indicate the survival strategy of the isolate in unfavorable oligotrophic environment. Both strains required yeast extract for growth. There was no indication of growth either under aerobic condition or under chemolithoautotrophic conditions using H2/CO2 (80:20, v/v) in the presence of 0.02% yeast extract and in the presence or absence of Fe(III). The main fermentation end product from glucose was acetate in both strains. Propionate, isobutyrate, and isovalerate were also detected in small amounts.

Electron acceptors

In the presence of yeast extract (0.3%, w/v) as a sole carbon source and electron donor, both strains reduced thiosulfate and elemental sulfur to sulfide, and Mn(IV)O2. There was no indication of Fe(III) reduction, however, the supplementation of 0.1 mM of ferric citrate enhanced growth of both strains. The selective utilization of Mn(IV) ions as an e– acceptor, together with the 4–6% NaCl tolerance can be taken as a hint that the isolates are indeed marine

bacteria, although it is very possible that the strains originated from the terrestrial geothermal features of Peru.

Phospholipid fatty acid composition

Both strains were differentiated clearly by the composition of their PLFA profiles (Table A1). While the most abundant fatty acid in strain JW/IW-1228P was i15:0, strain JW/YJL-1230-7/2 contained the four major fatty acids, i15:0, 16:1w9c, 16:0 and 18:1w9c. The polyunsaturated PFLA 18:2w6 was found in minor amounts only in strain JW/YJL-1230-7/2. Cyclopropane fatty acids, a possible biomarker for Gram-type negative bacteria (Zelles 1997), were not observed in either strain, which is in agreement that the isolates are Gram-type positive bacteria (Wiegel 1981).

DNA base composition

The G+C contents of the genomic DNA of both strain JW/IW-1228P and JW/YJL-1230-7/2 were 50 mol% (HPLC). The G+C mol% contents of the 16S rDNA of strain JW/IW-1228P and JW/YJL-1230-7/2 were 60 and 61, respectively. The DNA–DNA hybridization tests resulted in a re-association value of around 52%, which confirmed that strains JW/IW-1228P and JW/YJL-1230-7/2 are not related at the species level (Wayne et al. 1987).

16S rRNA gene sequences and phylogenetic analyses

Almost complete 16S rRNA gene sequences of strain JW/IW-1228P and JW/YJL-1230-7/2 were determined, comprising 1,386 (47–1457 based on E. coli numbering) nucleotides and 1,402 (47–1475 based on E. coli numbering) nucleotides, respectively. When compared, both sequences were about 98.3% similar to each other. All partial sequences of JW/IW-strains and all of the JW/YJL-strains clustered together, respectively (data not shown). According to BLASTN search, these isolates represent a novel group with *Thermovenabulum* and *Caldanaerobacter* as

their closest relatives. The closest relative of strain JW/IW-1228P is *Thermovenabulum ferriorganovorum* (AY033493) with a G+C mol% of 36 showing 98% similarity for the first 88 bp, 37 gaps, and then 93% similarity to the rest of 1,204 bp. The probability score (P score) from BLASTN results for strain JW/YJL-1230-7/2, however, showed anaerobic syntrophic bacterium OL (GenBank accession number; AB106354) as the closest relative. When compared to *T. ferriorganovorum*, the sequences of the novel isolates showed two gap regions (data not shown). When the gaps were included in this analysis, strain JW/IW-1228P and JW/YJL-1230-7/2 showed 90.3% and 89.7% similarity, respectively to *T. ferriorganovorum*. When unalignable regions eliminated, strain JW/IW-1228P and JW/YJL-1230-7/2 showed 93.7 and 94.4% similarity, respectively to *T. ferriorganovorum*. In a phylogenetic tree constructed by the neighbor-joining method (Fig. A2), both strains had the same closest relative, *T. ferriorganovorum*. The 16S rDNA sequences place the new isolates within the radius of the family *Thermoanaerobacteriaceae*. Based on the 16S rDNA sequences analysis and the difference of about 14 mol% in the G+C content of the DNA in respect to the closest identified relative, *Thermovenabulum*, the novel strains cannot be assigned to any of the genera in the family. In addition, the novel strains differ in morphology and physiology from the closest relative *Thermovenabulum* (Table A2). Thus, isolates JW/IW-1228P and JW/YJL-1230-7/2 together with the related strains are placed in the new genus *Thermosediminibacter*. Based on the phenotypic differences between the two strains, the DNA–DNA hybridization analysis, and the fatty acid composition, the strains are placed into two different species.

DESCRIPTION OF *THERMOSEDIMINIBACTER* GEN. NOV.

Thermosediminibacter (Ther.mo.se.di.mi.ni.bac'ter. Gr. Adj. thermos, hot; L. neut. n. sediment -inis, sediment; N. L. masc. n. bacter (from Gr. neut. n. bactron), a rod or staff; N. L. masc. n. *thermosediminibacter*, thermophilic rod from sediment, referring to its origin and growth temperature)

The genus *Thermosediminiibacter* belongs to the low G+C, Gram-type positive Bacillus-Clostridium subphylum. Habitat: so far only isolated from ocean subsurface sediments. The cells are straight rod to curved, and swollen and subsequently form autoplasts (L-shaped) in the late-exponential or stationary phase of growth. Anaerobic and thermophilic chemoorganotrophs. Yeast extract is required for growth. No growth on H2/CO2 (80:20, v/v). The G+C mol% of the DNA is around 50. The type species is *Thermosediminibacter oceani*.

DESCRIPTION OF *THERMOSEDIMINIBACTER OCEANI* SP. NOV.

Thermosediminibacter oceani (o.ce.an'i. L. masc. n. oceanus, ocean; L. gen. masc. n. oceani, of an ocean, referring to its origin from the ocean)

The cells are straight to curved rods, 0.2–0.7 mgrm in diameter and 1.5–16 mgrm in length. Cells occur singly, in pairs, or in chains and stain Gram-negative. Cells tend to elongate and form aggregates. Flagella observed. The temperature range for growth is 52–76°C, with an optimum at around 68°C. The pH25C range for growth is from 6.3 to 9.3, with an optimum at 7.5. The salinity range for growth is from 0 to 6% (w/v), with an optimum at 1%. In the presence of 0.02% yeast extract, casamino acids, beef extracts, tryptone, cellobiose, fructose, galactose, glucose, maltose, mannose, raffinose, sucrose, trehalose, xylose, methanol, inositol, manitol, sorbitol, lactate, pyruvate serve as carbon and energy. Thiosulfate, elemental sulfur, and MnO2

can serve as e– acceptors. No indication of sulfate or Fe(III) reduction. The most abundant fatty acid is i15:0. The G+C content of the genomic DNA is 50 mol% (HPLC). The type strain is JW/IW-1228PT (DSM 16646T, ATCC BAA-1034T).

DESCRIPTION OF *THERMOSEDIMINIBACTER LITORIPERUENSIS* SP. NOV.

Thermosediminibacter litoriperuensis (li.to.ri.pe.ru.en'sis. L. neut. n. litus–oris, the seashore, seaside, beach, coast; N. L. masc. adj. peruensis, pertaining to Peru; N. L. masc. adj. litoriperuensis, of a Peruvian coast, referring to its origin from the coast of Peru)

The cells are straight to slightly curved rod, 0.3–0.5 mgrm in diameter and 2.0–10.0 mgrm in length. Cells occur singly, in pairs, or in chains and stain Gram-negative. Retarded peritrichous flagella detected. The temperature range for growth is 43–76°C with an optimum at around 64°C. The pH25C range for growth is from 5 to 9.5 with an optimum at 7.9–8.4. The salinity range for growth is from 0 to 4.5% (w/v), with an optimum at 0.5–2%. Substrates utilized include yeast extract, tryptone, acetate, lactate, inositol, manitol, xylitol, fructose, galactose, glucose, mannose, raffinose, sucrose and xylose. The major fatty acids are i15:0, 16:1w9c, 16:0 and 18:1w9c with small amount of the polyunsaturated PFLA, 18:2w6. Thiosulfate, elemental surfur, MnO_2 can function as e– acceptors. There was no indication of sulfate or Fe(III) reduction. The G+C content of the genomic DNA was 50 mol% (HPLC-method). The type strain is JW/YJL-1230-7/2T (DSM 16647T, ATCC BAA-1035T).

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Table A1. Phospholipid fatty acid contents (%) of strains JW/IW-1228P and JW/YJL-1230-7/2.

Fatty acid methyl esters	**JW/IW-1228P**	**JW/YJL-1230-7/2**
14:0	1.8	1.7
i15:0	56.2	16.7
a15:0	6.7	3.8
15:00	5	2.6
i16:0	1.9	1.6
16:1w9c	5.6	19.9
16:0	7.5	15.5
i17:0	9.6	4.5
17:0	1	ND[b]
18:2w6	ND	1.4
18:1w9c	3.3	20.3
18:1w9t/18:1w7c[a]	ND	6.3
18:0	1.5	5.7

[a]18:1w9t/18:1w7c are unresolved in this analysis. [b]ND, Not detected.

Table A2. Morphological and physiological characteristics of strains JW/IW-1228P and JW/YJL-1230-7/2 and their closest relatives. Strains: 1, *Thermosediminibacter oceani* JW/IW-1228P, this study; 2, *Thermosediminibacter litoriperuensis* JW/YJL-1230-7/2, this study; 3, *Thermovenabulum ferriorganovorum* Z-9801, Zavarzina et al. (2002); 4, *Caldanaerobacter subterraneus* subsp. *Tengcongensis* MB4, Xue et al. (2001).

Character	**1**	**2**	**3**	**4**
Source	Subseafloor	Subseafloor	Hot spring	Hot spring
Cell size (μm)	0.2–0.7x1.5–16	0.3–0.5x2–10	0.5–0.6x1.5–7.0	0.5–0.6x1–10
Temperature range (°C)	52–76	43–76	45–76	50–80
Optimum temperature (°C)	68	64	63–65	75
pH range	6.3–9.3	5–9.5	4.8–8.2	5.5–9.0
Optimum pH	7.5	7.9–8.4	6.7–6.9	7.0–7.5
Salinity (% NaCl, w/v)	0–6.0	0–4.5	0–3.5	0–2.5
Optimum salinity	1	0.5–2	NR[a]	0.2
G+C content (mole%)	50	50	36	33
Gram stain	–	–	+	–
Spores observed	–	–	+	–
Reduction of iron (III)	–	–	+	NR
Requirements for growth	Yeast extract	Yeast extract	H_2, Fe(III), yeast extract	Yeast extract

[a]*NR*, Not reported.

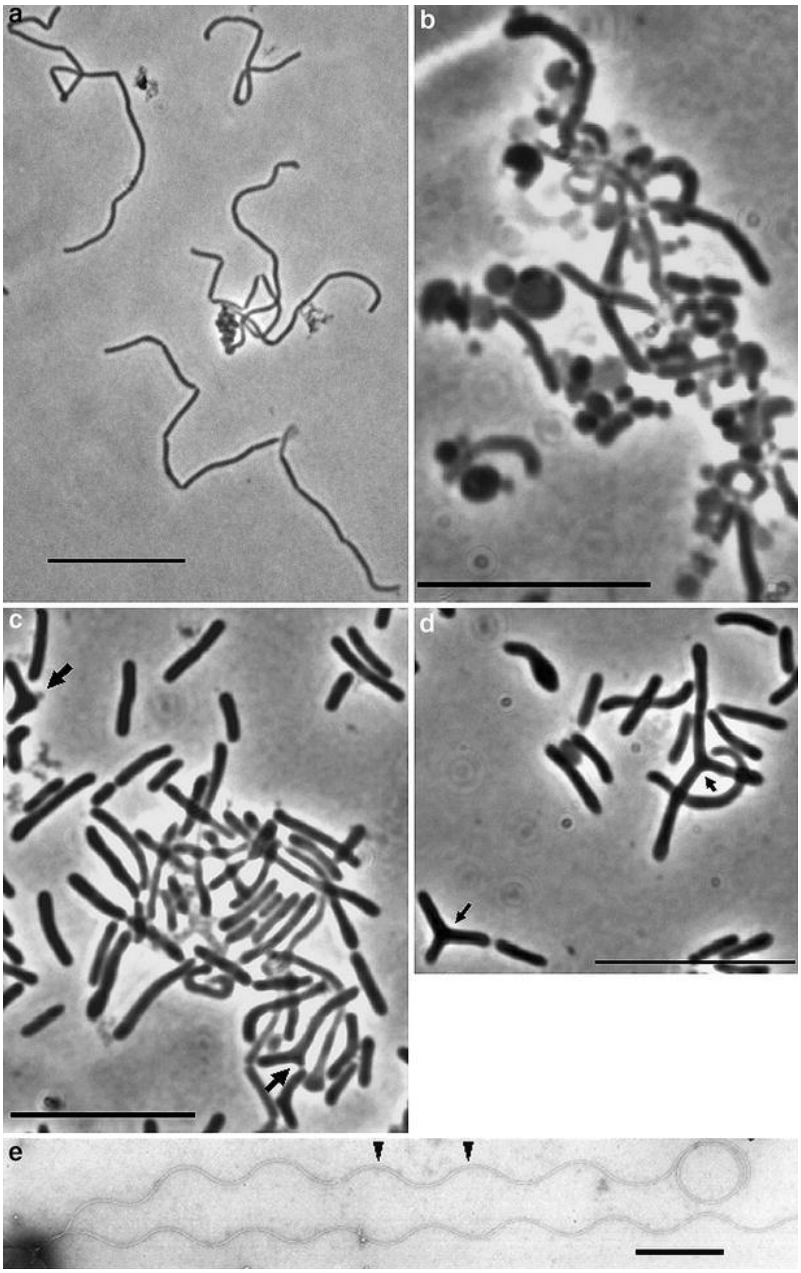

Fig. A1. Micrographs of JW/IW-1228-P and JW/YJL-1230-7/2. Cells from mid-exponential growth phase of JW/IW-1228P (**a**), late-exponential growth phase of JW/IW-1228P exhibiting partly swollen cells and L-form-like cells (**b**), mid-exponential growth phase of JW/YJL-1230-7/2 (**c**), late-exponential growth phase of JW/YJL-1230-7/2 with primary branches (**d**), TEM of JW/YJL-1230-7/2, *Arrows* point to branched cells. **e** *Arrowhead* indicates periodicity. Bars, 10 (**a, b, c, d**) and 1 (**e**) μm.

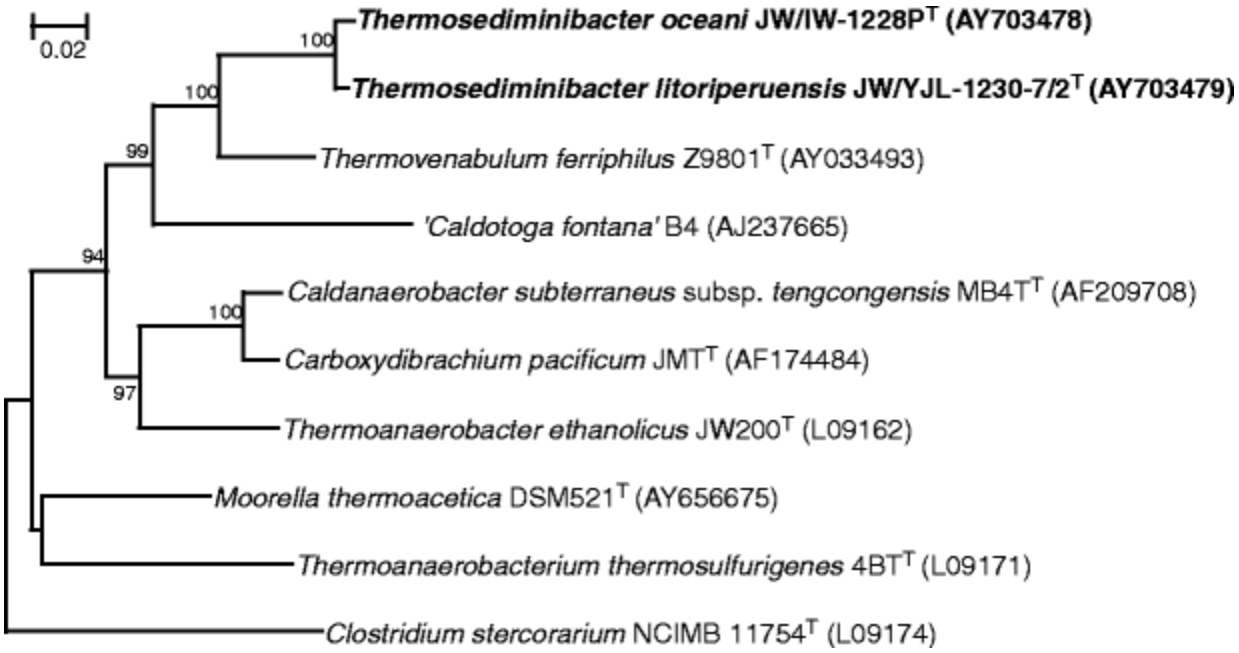

Fig. A2. A phylogenetic dendrogram based on 16S rDNA sequence showing the positions of strains JW/IW-1228P and JW/YJL-1230-7/2 (*boldface text*) amongst members of the family *Thermoanaerobacteriaceae*. The tree was constructed using Neighbor-joining method with Jukes and Cantor distance corrections. *Numbers at the nodes*represent the bootstrap values (% of 1,000 replicates); values above 90% were considered significant. The scale bar indicates two nucleotide substitutions per 100 nucleotides.

APPENDIX B

CALDICOPROBACTER OSHIMAI GEN. NOV., SP. NOV., AN ANAEROBIC, XYLANOLYTIC, EXTREMELY THERMOPHILIC BACTERIUM ISOLATED FROM SHEEP FECES AND PROPOSAL OF *CALDICOPROBACTERACEAE* FAM. NOV.[10]

[10] Yokoyama, H., Wagner, I. D. & Wiegel, J. (2009). *Caldicoprobacter oshimai* gen. nov., sp. nov., an anaerobic, xylanolytic, extremely thermophilic bacterium isolated from sheep faeces, and proposal of Caldicoprobacteraceae fam. nov. *Int J Syst Evol Microbiol* **60**, 67–71.

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ABSTRACT

An obligately anaerobic, xylanolytic, extremely thermophilic bacterium, strain JW/HY-331^T, was isolated from sheep feces collected from a farm at the University of Georgia, USA. Cells of strain JW/HY-331^T stained Gram-positive, were catalase negative, and non-motile rods. Single terminal endospores (0.4-0.6 μm in diameter) swelling the mother cell. Growth ranges were 44-77 °C (optimum 70 °C at pH70 °C 7.2) and pH70 °C 5.9-8.6 (optimum 7.2 at 70 °C). Salt tolerance was 0-2.0% (w/v) NaCl. No growth observed at at 42 °C or below or at 79 °C or above and at pH70 °C 5.7 and below or 8.9 and above. In the presence of 0.3% yeast extract and 0.1% tryptone, strain JW/HY-331^T utilized as carbon and energy source xylose, glucose, galactose, cellobiose, raffinose, or xylan, but not dextran, potato soluble starch, CM-cellulose, cellulose powder, casein, and Casamino acids. Fermentation products from glucose were lactate, acetate, ethanol, CO2, and H2. The G+C content of genomic DNA was 45.4 mol% (HPLC). Major cellular fatty acids were iso-C17:0, iso-C15:0, and anteiso-C17:0. No respiratory quinones were detected. The cell-wall structure was a single layer (Gram-type positive) of the peptidoglycan type A1γ; the cell-wall sugars were galactose and mannose. Based on 16S rRNA gene sequence analysis, '*Catabacter hongkongensis*' (85.4%), *Caloramator fervidus* (84.2%), and *Caloranaerobacter azorensis* (83.4%) were the closest relatives but only distantly related to strain JW/HY-331^T. On the basis of physiological, chemotaxonomic, and phylogenetic data, the isolate JW/HY-331^T (=DMS 2165 = BAA-1711) is proposed as the type strain of *Caldicoprobacter oshimai* gen. nov. sp. nov., placed in *Caldicoprobacteraceae* fam. nov. within the order *Clostridiales* of the Phylum *Firmicutes*.

Several moderate thermophilic 45 anaerobic bacteria with growth optima at 50-65 °C have been isolated from animal feces. Examples include *Clostridium thermocellum* from cow and horse manure, *Moorella thermoacetica* from horse feces, and *Thermoanaerobacter thermocopriae* from camel feces (Wagner & Wiegel, 2008). However, comparatively little is known about the occurrence of anaerobic extreme thermophiles with growth optima above 65 °C in animal feces. A study has reported the presence of an anaerobic extreme thermophile, *Caldanaerobacter subterraneus*, in cow feces (Yokoyama *et al.*, 2007b), implying that dormant extreme thermophiles might prevail in animal feces. Many validly published anaerobic, thermophiles can degrade hemicellulosic material, the second most abundant component of plant fiber, however the other extremely thermophilic hemicellulytic bacteria were not isolated from animal feces but from hot springs or oil-producing wells (Wagner & Wiegel, 2008).

Samples of sheep, horse, pig and cow feces were collected at different locations of University of Georgia, USA, farms, and were stored anaerobically at 4 °C until use. The basal medium I consisted of (per liter distilled water): 0.25 g Na2PO4-7H2O, 0.1 g NH4Cl, 0.18 g MgCl2-6H2O, 10 mg FeCl2-4H2O, 1 mg MnCl2-4H2O, 0.2 g NaCl, 0.2 g KCl, 1 mg resazurin, 1 g cysteine-HCl, and 40 mM HEPES. For enrichment cultures, the basal medium I was supplemented with 0.2% yeast extract, 0.1% tryptone, 0.5% birch wood xylan, and 0.5% beech wood xylan, and adjusted at a pH75 °C 7.0 (Wiegel, 1998) with 10N NaOH. Approximately 0.5 g of the mixed feces were inoculated into 10 ml aliquots of the anaerobic medium. After one week of incubation at 74 °C, 1 ml was transferred into fresh medium, and incubated again for a week. Only the sample inoculated with the sheep feces yielded growth. The transfer procedure was further repeated three times. Pure cultures were obtained by the modified phytagel-shake roll-tube technique (Ljungdahl & Wiegel, 1986) using 5% phytagel, and 0.12% MgCl2-6H2O

and .the basal medium I supplemented with 0.25% yeast extract, 0.1% tryptone, 0.05% xylose, 0.5% birch wood xylan, and 0.5% beech wood xylan. White to creamy round colonies (0.5-1.3 mm in diameters) developed after 8 days of incubation at 70 °C. Strain JW/HY-331^T (DSM 21659) was obtained by repeating three times rounds of single-colony isolations.

The basal medium II (pH70 °C 7.2) containing (per liter distilled water) 54 mg Na_2PO_4-$7H_2O$, 0.1 g NH_4Cl, 0.18 g $MgCl_2$-$6H_2O$, 4 mg $Fe(NH_4)SO_4$-$6H_2O$, 1.5 mg $CaCl_2$-2H2O, 1 mg $MnCl_2$-$4H_2O$, 0.5 g NaCl, 0.2 g KCl, 1 mg resazurin, 1 g cysteine-HCl, and 20 mM HEPES, was used in the morphological analyses. For the correct pH measurement under the applied conditions and the superscript notation see Wiegel (1998). For light microscopy, cells of strain JW/HY-331^T were cultured in liquid xylan medium (0.5% yeast extract, 0.25% tryptone, 0.25% from birch wood, and 0.25% beech wood xylan). The cells were mounted on an agarose-coated glass slides, and observed with a VONOX (Olympus) phase-contrast microscope. For transmission electron microscopy (TEM), cells cultured with a glucose medium (0.5% yeast extract, 0.25% tryptone, and 0.5% glucose) were ultra-thin sectioned, and stained with 2% uranyl acetate and a lead staining solution (Sigma-Aldrich). The sections were observed with a JEM-1200 EX (JEOL). Vegetative cells were straight to curved rods, 0.4-0.5 μm in diameter and 4-14 μm in length, and appeared singly, or in pair (Fig. B1a). No active mobility was detected. Sporulating cells were observed in stationary growth phase. They formed single spherical endospores (0.4-0.6 μm in diameter) in a swollen terminus (Fig. B1b). In the death phase, the swollen termini were separated from the rod (Fig. B1c), and subsequently the spores were released from the swollen body. Many cells failed to form a spore and became spheroplast-like round cells. Based on microscopic observations of transferred cells into fresh media, it appeared that the spheroplast–like cells were not viable. Vegetative cells stained Gram positive, and the

cell-wall structure was of a single layer (Gram-type positive), as judged by TEM (Fig. B1d-e). Analyses of cell-wall peptidoglycan by TLC and GC (Mackenzie, 1987; Schleifer & Kandler, 1972) revealed the presence of amino acids (molar ratio) alanine (1.0), glutamic acid (1.0), and *meso*-diaminopimelic acid (1.1), indicating the peptidoglycan type A1γ. The cell-wall sugars were galactose and mannose, as determined by TLC (Staneck & Roberts, 1974).

Until otherwise stated, the glucose medium (mentioned above) was used in the physiological analyses. Strain JW/HY-331^T was obligately anaerobic, no growth in pink (=oxidized resazurin) media, and negative for both catalase and oxidase. Temperature range for growth at pH70 °C 7.2 was 44-77 °C with an optimum of 70 °C (no growth at 42 °C or below or at 79 °C or above), and pH70 °C range for growth at 70 °C was 5.9-8.6 with an optimum 7.2 (no growth at 5.7 or below or 8.9 or above). Salinity tolerance was 0-2.0% (w/v) NaCl (no growth at 2.5% or above). Shortest observed doubling time was 48 min under the optimal growth condition (70 °C at pH70 °C 7.2). Substrate utilization was analyzed using the basal medium II supplemented with 0.3% yeast extract, 0.1% tryptone, and 0.5% substrates tested. No substantial growth was observed in 0.3% w/v yeast extract- and 0.1% w/v tryptone-containing medium without addition of another substrate; however yeast extract was required for measurable growth xylose, glucose, galactose, fructose, mannose, cellobiose, lactose, raffinose, and xylan from birch wood, beech wood and oat spelt. Growth on all three xylans was confirmed by measuring the resulting fermentation products lactate, acetate, and ethanol and the intermittent accumulation of the hydrolysis product xylose. No growth was observed with dextran, soluble potato starch, CM-cellulose, cellulose powder, cellulose (filter paper), sorbitol, mannitol, lactate, acetate, ethanol, butanol, peptone, casein, gelatin, or Casamino acids. In the presence of 0.3% yeast extract and 0.1% tryptone and using a gas phase of 70% H2 and 30% CO_2, no autotrophic growth was

observed. No growth enhancement (or formation of the corresponding reduced products) was observed with the possible electron acceptors 20 mM Na2SO4 (sulfide), 5 mM or 2.5 mM Na2SO3 (sulfide), 20 mM Na2S2O3 (sulfide or sulfur), 20 mM NaNO3 (nitrite), or 5 mM NaNO2 (ammonium, N_2). Fermentation endproducts from glucose were quantified using the basal medium II supplemented with 0.3% yeast extract, 0.1% tryptone, and 0.5% glucose. Approximately 0.6 mol lactate, 0.3 mol acetate, 0.5 mol ethanol, 0.8 mol CO2, and 0.4 mol H2 were detected per mol-glucose degraded fermentation. Without the addition of glucose to the medium, only traces of the fermentation products were observed.

The DNA G+C content was 45.4 mol%, as determined by HPLC (Mesbah *et al.*, 1989). For the analysis of cellular fatty acids, the fatty acid methyl esters were prepared from a freeze-dried sample, and analyzed by GC (Guckert *et al.*, 1985). Major cellular fatty acids (>10%) were branched- and saturated-fatty acids with odd numbers of carbon: iso-C17:0 (31.1%), iso-C15:0 (23.7%), and anteiso-C17:0 (12.9%). Minor cellular fatty acids (>1%) were iso-C17:0 3OH (8.1%), C16:0 (6.8%), anteiso-C15:0 (5.7%), iso-C16:0 (2.4%), and C17:0 141 2OH (1.9%). Trace cellular fatty acids (<1%) were iso-C11:0, anteiso-C11:0 , iso-C13:0, C14:0, iso-C15:0 3OH, C16:0 3OH, iso-C16:0 3OH, C17:0 2OH, C18:0, iso-C18:0, -C18:1ω7*c*, -C18:1ω9*c*, iso-C19:0, iso-C16:0 3OH, and C20:4ω6, 9, 12, 15*c*. No respiratory quinones were detected by a combination method of TLC and HPLC (Tindall, 1990a, Tindall, 1990b).

A nearly complete 16S rRNA gene sequence was amplified by PCR with the primer set of 27F and 1492R (Lane, 1991), and sequenced by Macrogen (Seoul, Korea). Sequences of the closest relatives were retrieved by a BLAST search, and aligned by a program CLUSTAL W (Thompson *et al.*, 1994). The distant matrix was calculated by the Jukes-Cantor model (Jukes & Cantor, 1969) using MEGA 4 (Tamura *et al.*, 2007). Phylogenetic trees were constructed by the

neighbour-joining method (Saitou & Nei, 1987). Phylogenetic analyses using the 16S rRNA gene sequence revealed strain JW/HY-331^T belongs to the order *Clostridiales*, however, strain JW/HY-331^T does not group within any validly published family within the order *Clostridiales*. Interestingly, strain JW/HY-331^T showed the highest identity, 99.5%, to an unidentified clone OTU4 (AB286973), which was detected in enrichment cultures at 75 °C from cow feces, collected in Japan (Yokoyama *et al.*, 2007a) (Fig.2). Based on the phylogenetic and physiological similarities, the unidentified clone OTU4 and strain JW/HY-331^T belongs to the same species, which assumingly prevails in herbivore feces. Strain JW/HY-331^T also showed a high similarity to unidentified clones Hb (EF661580) (97.7%) and LNE-5 (AY531642) (95.7%) (Hobel *et al.*, 2004). These unidentified clones were detected in thermophilic enrichment cultures inoculated with bioreactor sludge and a sample from a hot spring, respectively. Among isolates with effectively and validly published names, strain JW/HY-165 331^T showed the highest identities to '*Catabacter hongkongensis*' (85.4%) (Lau *et al.*, 2007), *Caloramator fervidus* (84.2%) (Patel *et al*., 1987), and *Caloranaerobacter azorensis* (83.4%) (Wery *et al.*, 2001). Both *Caloramator fervidus* and *Caloranaerobacter azorensis* belong to the family *Clostridiaceae*, whereas '*Catabacter hongkongensis*' is currently proposed to belong to a novel family '*Catabacteriaceae'*, consisting of a single genus and single species, within the order *Clostridiales*. '*Catabacter hongkongensis*' isolated from a clinical blood sample is a non spore-forming mesophile, significantly different from strain JW/HY-331^T in physiological characteristics. Both *Caloramator fervidus* and *Caloranaerobacter azorensis* are anaerobic thermophiles, similar to strain JW/HY-331^T (Table B1). However *Caloramator fervidus*, isolated from a hot spring, differs from strain JW/HY-331^T in Gram staining, DNA G+C mol% content, and morphology. *Caloranaerobacter azorensis*, isolated from a deep-sea vent, differs from strain

JW/HY-331^T in salinity requirement for growth, spore formation, cell-wall structure, and DNA G+C mol%.

In the 16S rRNA-based phylogenetic tree, strain JW/HY-331^T forms a separate branch within the order *Clostridiales*, and clusters only with unidentified clones (Fig. B2). Strain JW/HY-331^T is only distantly related to the single member of the family '*Catabacteriaceae'*, but the identity to the type species *'Catabacter hongkongensis'* is only 85.4%, suggesting that it belongs to a different family. Strain JW/HY-331^T shows even lower similarities (76-81%) to all other type genera of the families within the order *Clostridiales*. On the basis of these physiological, chemotaxonomic, and phylogenetic data, strain JW/HY-331^T is proposed as the type strain of the novel taxon *Caldicoprobacter oshimai* gen. nov. sp. nov. and *Caldicoprobacter* as the type genus for the novel family *Caldicoprobacteraceae* fam. nov. within the order *Clostridiales* of the Phylum *Firmicutes*.

DESCRIPTION OF *CALDICOPROBACTER* GEN. NOV.

Caldicoprobacter (cal.di.co.pro.bac'ter. L. adj. caldus hot, G. n. kopros dung, N.L. masc. n. bacter rod; N.L. masc. n. *Caldicoprobacter* a rod from dung growing at elevated temperatures).

Cells are Gram-staining positive, spore-forming, non-motile, and straight to curved rods. Negative for catalase and oxidase. Thermophilic. Neutrophilic with a pH range for growth of 5-9. Cellular fatty acids are mainly composed of 15 and 17 carbon-containing saturated- and branched-fatty acids. The peptidoglycan type is A1γ. Anaerobic chemo-organotrophs. The type species is *Caldicoprobacter oshimai* sp. nov.

DESCRIPTION OF *CALDICOPROBACTER OSHIMAI* SP. NOV.

Caldicoprobacter oshimai (o.shi'ma.i. N.L. gen. masc. n. oshimai, of Oshima, named after the Japanese microbiologist Tairo Oshima, in honor of his many contributions to the knowledge of thermophiles and their biochemistry).

Cells are non-motile, and straight to curved rods, 0.4-0.5 μm in diameter and 4-14 μm in length, and appeared singly or in pair and staining Gram positive. Cell wall type is A1γ. Main cell wall sugars are galactose and mannose. Spherical endospores (0.4-0.6 μm in diameter) are formed in terminal position causing swelling of the mother cell. Non sporulating cells form protuberances in stationary growth phase and convert then to spheroplast-like round cells, which are not viable. Obligately anaerobic and negative for catalase and oxidase. Extremely thermophilic growing in a temperature range of 44-77 °C (optimum 70 °C) at pH70 °C 7.2 °C (no growth at 42 °C or below or at 79 °C or above), and neutrophilic with a pH70 °C range for growth at 70 °C of 5.9-8.6 with an optimum at 7.2 (no growth at pH70 °C 5.7 and below or 8.9 and above). The salinity tolerance is 0-2.0% (w/v) NaCl. Xylose, glucose, galactose, fructose, mannose, cellobiose, lactose, raffinose, and xylan are utilized as carbon and energy source but not dextran, potato soluble starch, CM-cellulose, cellulose powder, cellulose (filter paper), sorbitol, mannitol, lactate, acetate, ethanol, butanol, peptone, casein, gelatin, and Casamino acids. The fermentation endproducts from glucose are lactate, acetate, ethanol, CO2, and H2. The DNA G+C content is 45.4 mol%. The major cellular fatty acids are iso-C17:0, iso-C15:0, and anteiso-C17:0.

The type strain, JW/HY-331^T (=DSM 21659 = ATCC BAA-1711), was isolated from sheep feces collected from a farm at the University of Georgia, USA.

DESCRIPTION OF *CALDICOPROBACTERACEAE* FAM. NOV.

Caldicoprobacteraceae (Cal.di.co.pro.bac.te.ra.ce'ae. N.L. masc. n. *Caldicoprobacter*, type genus of the family; suff.-aceae, ending to denote a family; N.L. fem. pl. n. *Caldicoprobacteraceae*, the *Caldicoprobacter* family).

Comprised of spore-forming members exhibiting Gram-type positive rod-shaped cells with anaerobic chemo-organotrophic metabolism.The DNA G+C content is around 45 mol%. Belongs to the order *Clostridiales* within the class *Clostridiae* of the Phylum *Firmicutes.* The type genus is *Caldicoprobacter.*

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Table B1. Phenotypic comparison of strain JW/HY-331^T with its closest relatives with validly published names. Strains: 1, JW/HY-331^T; 2, *Caloramator fervidus* ATCC 43204^T (data from Patel *et al.*, 1987); 3, *Caloranaerobacter azorensis* MV1087^T (Wery *et al.*, 2001). +, Positive; –, negative; ND, no data available. All strains are positive for xylan utilization.

Characteristic	**1**	**2**	**3**
Length of rods (μm)	4–12	2–5	0.5–2
Motility	–	Weak	+
Gram stain	+	–	–
Cell-wall structure	Single layer	Single layer	Double layer
Spore formation	+	+	–
DNA G+C content (mol%)	45.4	39	27
Temperature range (optimum) (°C)	44–77 (70)	43–74 (68)	45–65 (65)
$pH^{70\ °C}$ range (optimum)	5.9–8.6 (7.2)	5.5–8.5 (7–7.5)	5.5–9.0 (7)
NaCl range (%, w/v)	0–2.0	ND	0.65–6.5
Starch utilization	–	+	+

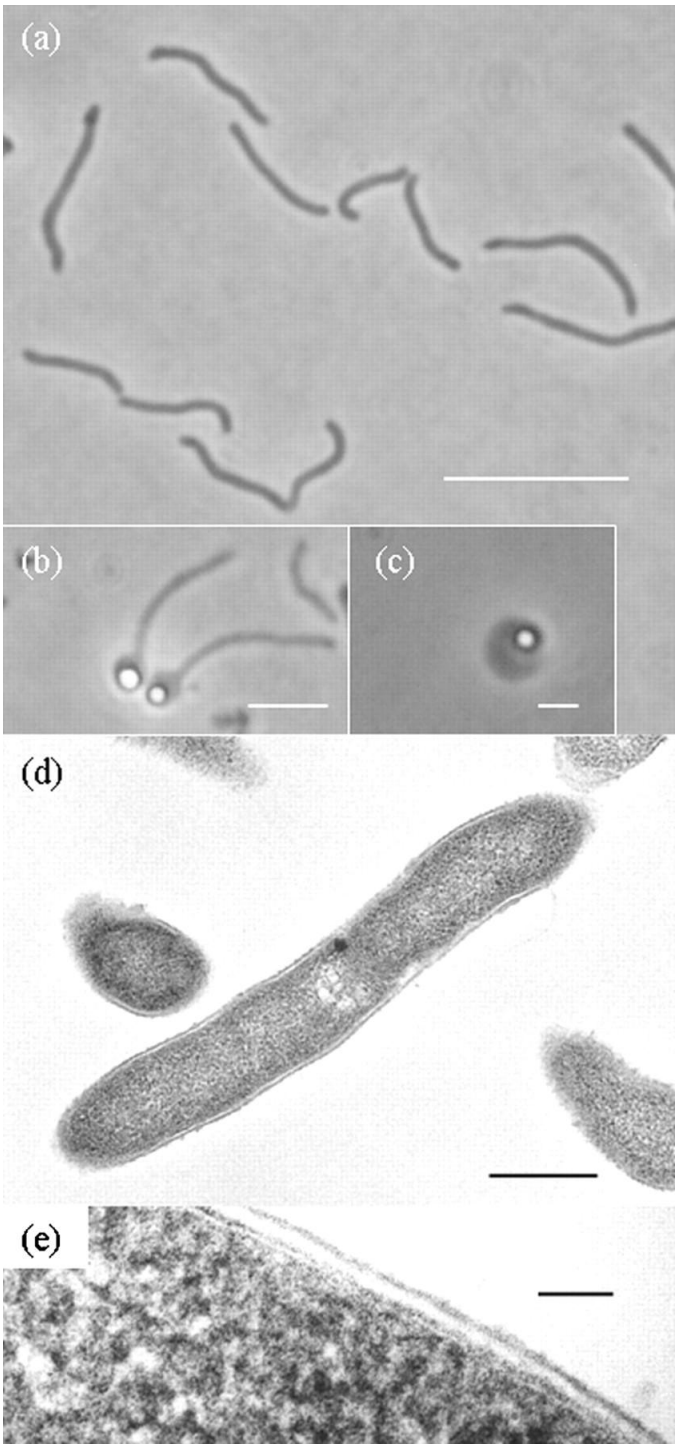

Fig. B1. Morphology of strain JW/HY-331^{T}. (a–c) Phase-contrast micrographs of vegetative cells (a), sporulating cells (b) and a released spore (c). (d) TEM image of ultrathin-sectioned cells. (e) Enlargement of (d), showing the cell-wall structure. Bars, 10 μm (a), 5 μm (b), 2 μm (c), 500 nm (d) and 50 nm (e).

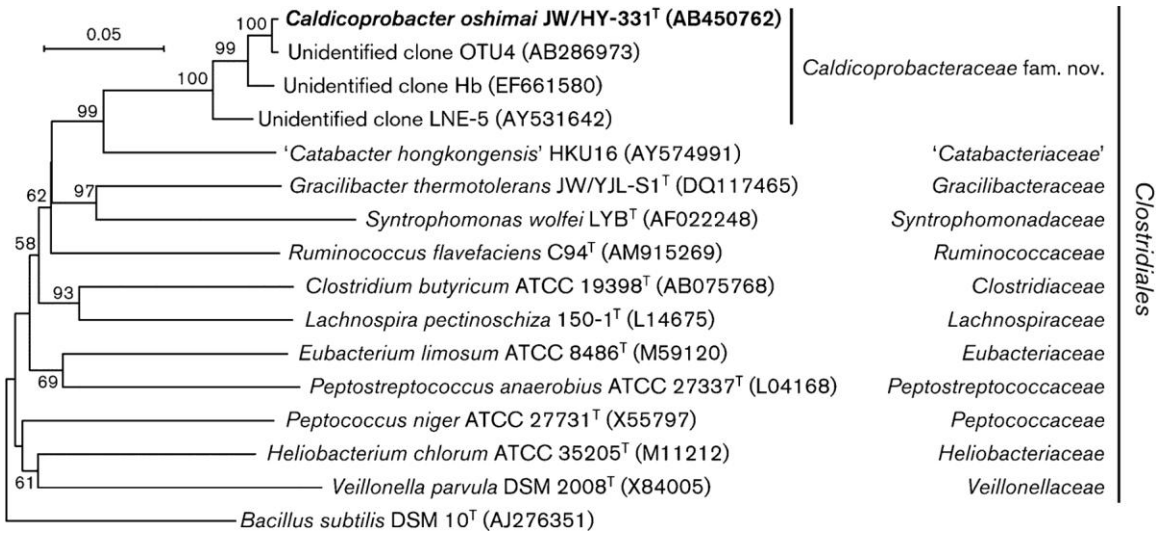

Fig. B2. Neighbour-joining tree generated using 16S rRNA gene sequences, showing the relationships of strain JW/HY-331^T to its closest relatives and the type species of type genera for families within the order *Clostridiales*. Numbers at nodes represent bootstrap percentages (above 50 %) from 1000 replicates. Bar, 0.05 substitutions per nucleotide position.