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STUDY ON MICROBIAL DIVERSITY AND BIOTECHNOLOGICAL POTENTIAL OF THE ISOLATED *FIRMICUTES* STRAINS FROM TAO DAM HOT SPRING IN THAILAND

A THESIS

BY

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10 ส.ค. 2555

Presented in Partial Fulfillment of the Requirements for the
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AN ABSTRACT

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Various kinds of hot springs and pools are located in Thailand. More than hundred of them are distributed throughout Thailand. However, only a few of them had been investigated the microbial diversity. Tao Dam hot spring was discovered in 2007 and contained sulfide-rich with pH above 7. This phenomenon is an interesting characteristic for investigation the microbial diversity of this spring, due to the sulfide-rich hot springs with a basic pH are very rare on earth. This was the first report of a survey of the microbial community of Tao Dam hot spring. The spring has a high microbial diversity. The amount of the bacteria is approximately ten times greater than the archaea. Tao Dam spring is a rich source of thermophiles, especially the bacteria in the phylum Proteobacteria and also the archaea in the phylum Euryarchaeota. Methanogens are most found in the archaeal community. The three main pathways of methane production may exist in the Tao Dam hot spring. Perhaps, the hydrogenotropic methanogenesis pathway is the major pathway of the methane production in Tao Dam hot spring. A novel *Anoxybacillus* strain TD5 has been isolated and characterized from the Tao Dam hot spring. Aminoacylase, laccases, lipases and xylanases genes, which are discovered from the Tao Dam's strains, are the good candidates for the novel enzymatic genes and enzymes. Moreover, the pectate lyase activities are represented from some Tao Dam's strains, which have not been reported elsewhere before. They have highly potential to be a good pectate lyase producer, which contain the heat-stable properties. Moreover, the strains, which were isolated from the Tao Dam hot spring, contain some others enzymatic genes such as D-arabinose isomerase, nitrilase, protease and L-rhamnose isomerase.

การศึกษาความหลากหลายของจุลินทรีย์ และแนวโน้มเพื่อการนำไปใช้ประโยชน์ทางชีวภาพจากแบคทีเรียในกลุ่ม *Firmicutes* ที่คัดแยกได้จากบ่อน้ำพุร้อนเต่าดำ, ประเทศไทย

บทคัดย่อ

ของ

มนตรี ยะสาวงษ์

เสนอต่อบัณฑิตวิทยาลัย มหาวิทยาลัยศรีนครินทรวิโรฒ เพื่อเป็นส่วนหนึ่งของการศึกษา
ตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต สาขาวิชาอณูชีววิทยา
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ประเทศไทยมีบ่อน้ำพุ และบ่อน้ำร้อนประเภทต่างๆอยู่เป็นจำนวนมากกว่าร้อยแห่ง แต่มีเพียงบ่อน้ำพุ และบ่อน้ำร้อนจำนวนไม่กี่แห่งเท่านั้น ที่ได้มีการนำศึกษาการกระจายตัวทางชีวภาพของจุลินทรีย์ ในปี 2007 ได้มีการค้นพบบ่อน้ำพุร้อนเต่าดำ ซึ่งบ่อน้ำพุร้อนแห่งนี้มีความน่าสนใจเพื่อที่จะนำไปศึกษาการกระจายตัวทางชีวภาพของจุลินทรีย์ ทั้งนี้เนื่องมาจากบ่อน้ำพุร้อนแห่งนี้มีน้ำพุร้อนที่มีส่วนประกอบของซัลไฟด์ในปริมาณสูงแต่กลับมี pH ที่เป็นด่างซึ่งบ่อน้ำพุร้อนที่มีสภาพเช่นนี้พบได้น้อยมากในธรรมชาติ ดังนั้นรายงานฉบับนี้จึงเป็นรายงานฉบับแรก ที่ได้นำเสนอการศึกษาเรื่องการกระจายตัวของจุลินทรีย์ของบ่อน้ำพุร้อนเต่าดำ จากการศึกษาพบว่าบ่อน้ำพุร้อนแห่งนี้มีการกระจายตัวของจุลินทรีย์สูงมาก อีกทั้งยังได้พบว่ามีปริมาณของแบคทีเรียสูงกว่าอาร์เคียประมาณสิบเท่า บ่อน้ำพุร้อนแห่งนี้ถือได้ว่าเป็นแหล่งของแบคทีเรียที่ชอบร้อนโดยเฉพาะอย่างยิ่งแบคทีเรียในไฟลัม Proteobacteria และอาร์เคียในไฟลัม Euryarchaeota ซึ่งถูกพบเป็นจำนวนมากในบ่อน้ำพุร้อนแห่งนี้ นอกจากนี้ยังพบอีกว่าอาร์เคียในกลุ่มของ methanogen ยังถูกพบเป็นสัดส่วนที่มากที่สุดในกลุ่มของอาร์เคียด้วยกัน ทั้งนี้จึงทำให้สามารถทำนายได้ว่าบ่อน้ำพุร้อนแห่งนี้มีการสร้างแก๊สมีเทนโดยอาร์เคียในกลุ่ม methanogen โดยขบวนการสร้างแก๊สมีเทนแบบ hydrogenotrophic และจากการศึกษาอนุกรมวิชานของแบคทีเรียทำให้ค้นพบแบคทีเรียสายพันธุ์ใหม่ชื่อว่า *Anoxybacillus* sp. สายพันธุ์ TD5 ทั้งนี้จากการศึกษาในส่วนอื่นๆ ยังทำให้ค้นพบยีนที่สร้างเอนไซม์ที่มีความสำคัญทางด้านอุตสาหกรรมจากแบคทีเรียอีกหลายสายพันธุ์ ที่แยกได้จากบ่อน้ำพุร้อนเต่าดำอีก เช่น ยีนที่สร้างเอนไซม์ aminoacylase, laccases, lipases และ xylanases ซึ่งยีนเหล่านี้มีแนวโน้มสูงที่จะเป็นยีนชนิดใหม่ และจากการศึกษาการสร้างของเอนไซม์ pectate lyase จากแบคทีเรียสายพันธุ์อื่นที่แยกได้จากบ่อน้ำพุร้อนแห่งนี้ ทำให้พบว่าเอนไซม์ดังกล่าวมีความเป็นไปได้อย่างสูงที่จะเป็นเอนไซม์ชนิดใหม่ และมีคุณสมบัติคงตัวได้ดีในสภาวะที่มีอุณหภูมิสูง เนื่องจากในปัจจุบันยังไม่มีรายงานฉบับใดรายงานว่าแบคทีเรียสายพันธุ์ดังกล่าวสามารถสร้างเอนไซม์ชนิดนี้ได้ และนอกจากนี้ยังพบว่าแบคทีเรียบางสายพันธุ์ที่แยกได้จากบ่อน้ำพุร้อนเต่าดำยังมียีนสำหรับการสร้างเอนไซม์บางชนิดที่มีประโยชน์ทางด้านอุตสาหกรรมอีก เช่น ยีนที่สร้างเอนไซม์ D-arabinose isomerase, nitrilase, protease และ L-rhamnose isomerase.

The dissertation titled

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by

Montri Yasawong

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TABLE OF CONTENTS

Chapter	Page
1 INTRODUCTION	1
2 LITERATURE REVIEW	2
Hydrothermal systems	2
Industrial relevance of thermophiles and their enzymes	6
Polymer degrading thermoactive enzymes cellulose hydrolyzing enzymes	7
Cellulose hydrolyzing enzymes	7
Endoglucanases	8
β-Glucosidases	9
Xylanase	10
Starch processing enzymes	11
α-Amylases	11
β-Amylases	12
Glucoamylases	13
α-Glucosidases	14
Pullulanases	14
Pectin degrading enzymes	16
Proteases	17
Biocatalysis with nonpolymeric compounds	18
Lipases and esterases	18
Glucose and arabinose isomerase	20
Nitrile-degrading enzymes	21
Thermophiles as cell factories	22
Biomining	22
Lipids and peptides	22
Hydrogen production	23

TABLE OF CONTENTS (continued)

Chapter	Page
2 (Continued)	
Molecular and genomic methods	24
The molecular context of microbial diversity	24
Interpretation of molecular diversity	25
Specific nucleic acid-based methods	27
Microbial diversity and indexes of environmental change	29
Quantitative measures of species diversity	31
Phylogenetic analysis	35
The rationale for phylogenetic trees	36
Multiple sequence alignments	38
Constructing phylogenetic trees from aligned sequences	39
Interpreting phylogenetic trees	40
3 MATERIALS AND METHODS	42
Samples collection	42
Isolation of bacterial strains and cultivation	42
DNA extraction and standard PCR amplification	42
Quantitative real time PCR (Q-PCR)	43
Q-PCR standard curves	44
Single strand conformation polymorphism	44
Preparation of SSCP gel	44
Preparation of single-stranded DNA (ssDNA)	44
Running SSCP gel	45
Gel fixation	45
Gel staining	45
Gel developing	45

TABLE OF CONTENTS (continued)

Chapter	Page
3 (Continued)	
Stopping the developing reaction	45
Gel preservation	45
Cloning of PCR products	45
Sequence analysis	46
Computation analysis for the 16S rRNA and *mcrA* library	46
Screening of commercialize enzymes from the isolates	46
Pectate lyase assay	47
Determination of bacterial morphology	48
Biochemical test	49
Chemotaxonomy	49
Determination of base composition	49
DNA-DNA hybridization	50
Phylogenetic analysis for bacterial systematic	50
4 RESULTS	52
Microbial diversity	52
SSCP analysis	52
Clone libraries and phylogenetic analysis	53
Richness estimation	59
Quantitative PCR	61
Bacterial systematic	62
Bacterial isolation	62
Comparison of 16S rRNA genes	63
Phylogenetic analysis	64
Molecular systematic	65

TABLE OF CONTENTS (continued)

Chapter	Page
4 (Continued)	
Physiological and biochemical characteristics	66
Biotechnology	73
Functional screening of commercialize enzymes	73
Screening of commercialize enzymatic genes	73
5 DISSCUSSION	75
Microbial diversity	75
Bacterial systematics	79
Biotechnology	79
Conclusion	81
REFERENCES	82
APPENDICES	105
CURRICULUM VITAE	109

LIST OF TALES

Table		Page
1	The major categories of national ecological indicators recommended by the National Research Council	35
2	PCR primers for microbial diversity study	43
3	PCR primers for enzymatic genes screening	47
4	Species richness of 16S rRNA gene libraries	53
5	Species richness of mcrA gene library	57
6	Identification of 16S rRNA gene sequences of Tao Dam's strains using EzTaxon	63
7	Pairewised comparison of the 16S rRNA gene of the bacterial strains that belonging to the members of the genus *Anoxybacillus* using MatGat program	64
8	Cellular fatty acid components of strain TD5 and relative type strains from the genus *Anoxybacillus*	66
9	Phenotypic characterization of strain TD5 and closely related species of genus Anoxybacillus	71
10	Functional screening of Tao Dam's strains	73
11	Identity of enzymatic genes of Tao Dam's strains and its closet relative species from BLAST search	74

LIST OF FIGURES

Figure		Page
1	A hypothetical hydrothermal deep-sea vent system	2
2	Some examples of hot spring and thermophiles	5
3	SSCP profiles of 16S rRNA gene from Tao Dam hot spring sediment	52
4	Phylogenetic tree of archaeal 16S rRNA gene from Tao Dam hot spring	54
5	Abundancy of archaeal 16S rRNA gene library	55
6	Phylogenetic tree of bacterial 16S rRNA gene from Tao Dam hot spring	56
7	Abundancy of bacterial 16S rRNA gene library	57
8	Phylogenetic tree of methanogens mcrA gene from Tao Dam hot spring	58
9	Abundancy of methanogenic archaeal mcrA gene library	59
10	Rarefaction curves analysis of 16S rRNA gene libraries	60
11	Rarefaction curve analysis of mcrA gene library	61
12	Results from Q-PCR analysis	62
13	Phylogenetic tree relationships between strain TD5 and related *Anoxybacillus* species on the basis of 16S rRNA gene sequences	65
14	Scanning electronic micrograph of strain TD5	67
15	Electronic micrograph of an ultrathin-sectioned cell of strain TD5	68
16	Proportion of proteobacterial clones of the 16S rRNA gene library	76

CHAPTER I

INTRODUCTION

More than hundred hot springs are distributed throughout Thailand, where the surface temperatures range from 40-100°C at a pH of 6.4-9.5. Microbial communities of these springs (like Bor Khlueng in the Ratchaburi province) are proposed to comprise a rich diversity of both unique bacterial and archaeal species which posses interesting enzymes[1]. The thermophilic components of these communities are particularly interesting because of the unique stability of their enzymes at elevated temperatures, extremes pH and high pressure, making them valuable resources for industrial enzymes[2]. Of special interest is the thermoactivity and thermostability of these enzymes in the presence of high concentrations of organic solvents, detergents and alcohol and are thus expected to be a powerful tool in harsh industrial biotransformation processes[3, 4]. The application of robust enzymes and microorganisms for the sustainable production of chemicals, biopolymers, materials and fuels from renewable resources, also defined as industrial biotechnology, will offer great opportunities for various industries. The utmost is the reduction of waste, energy input and raw material, and the development of highly efficient and environmentally friendly process[5]. The microbial diversity of Tao Dam hot spring (a spring only discovered in recent years) has not been previously investigated and may be a candidate for novel enzyme-producing thermophiles. Moreover, the investigation is aiming to discover novel enzymatic genes or activities from the microbial strains, which will be isolated from the Tao Dam hot spring.

CHAPTER II

LITERATURE REVIEW

Hydrothermal systems

In 1979 when John Corliss and co-workers published their discovery of animal and bacterial communities living within active hydrothermal vent systems on the Galápagos Rift[6] they initiated a flurry of research into cataloging the life forms that survive under such seemingly inhospitable conditions.

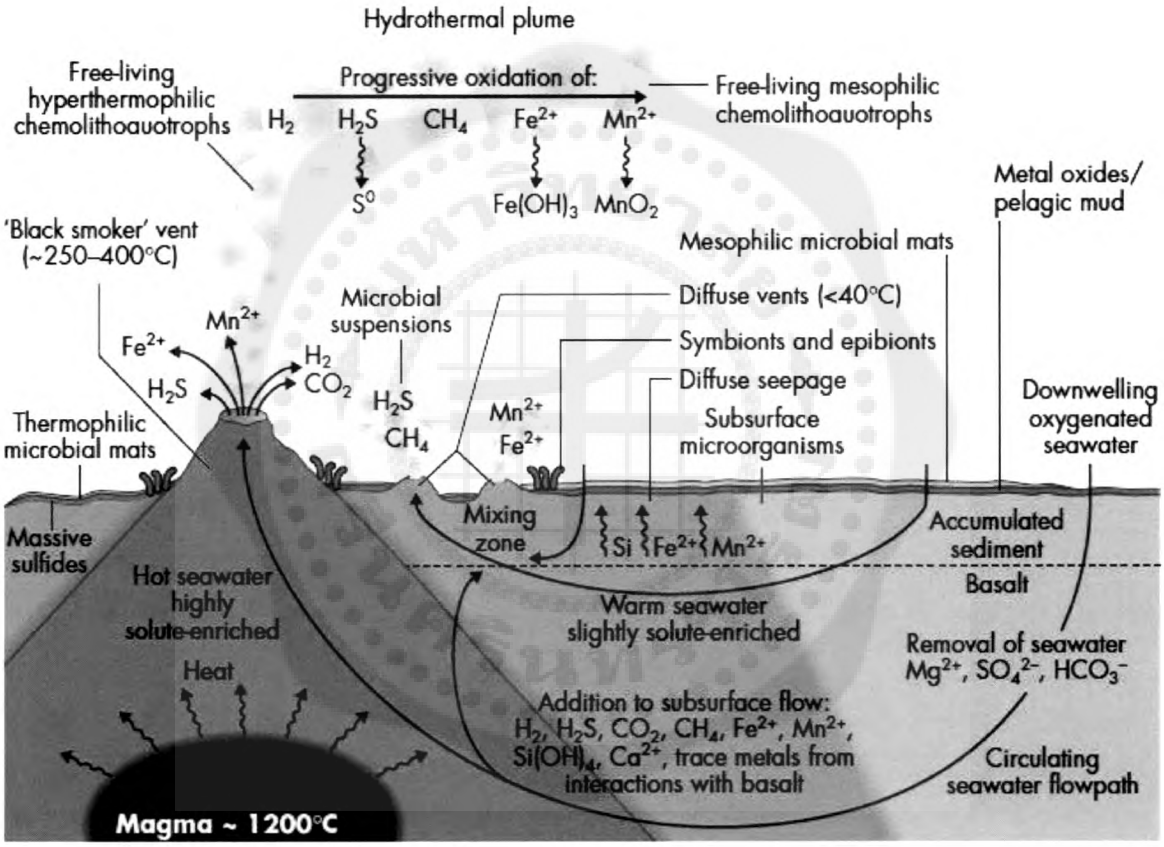


Figure 1 A hypothetical hydrothermal deep-sea vent system, showing the emission of a number of reduced gases and solutes from high temperature (black smoker-type) chimneys, warm water vents, and diffuse seepages from fissures underlying sediment. These reductants serve as primary sources of energy for a variety of free-living, benthic and/or sub-seafloor hyperthermophilic to mesophilic bacterial and archaeal communities[7].

Growing at pressures equivalent to water depths in excess of 2500 meters, and around vents emitting fluids as hot as 380°C, are a community of bacteria and archaea feeding off the toxic emissions from hydrothermal vents (Figure 1).

Although the hyperthermophiles do not live in the superheated fluid, they locate themselves along a gradient where the hot water mixes with cold bottom seawater, for instance, as free-living cells in the buoyant metal-laden plumes, and even within the walls and adjacent sediments of the so-called "black smoker" chimneys (Figure 1). Microorganisms also grow profusely as mats around warm water vents (<40°C), where the effluent is sourced from a subsurface mixing zone of upwelling hot hydrothermal fluids and downwelling cold, oxygenated seawater. Observations of dense, white-colored (sulfur-rich) suspensions of cells being dispersed as plumes from the warm vents further suggest that a substantial microbial community exists in the porous lava and sedimentary layers below the seafloor[8]. The primary producers in that community obtain their energy chemolithoautotrophically by exploiting the steep chemical gradients and localized redox disequilibrium that result from the discharge of reduced, solute-rich hydrothermal effluent into an oxygenated environment[9, 10]. Hydrogen sulfide (H_2S or HS^-, depending on fluid pH) is quantitatively the most important electron donor for chemosynthesis, and it is not uncommon to observe centimeter-thick mats of sulfur-oxidizing bacteria, such as *Beggiatoa* sp. growing at the vent periphery[11]. Other reductants, such as hydrogen gas (H_2), elemental sulfur (S^0), thiosulfate ($S_2O_3^{2-}$) and methane (CH_4) support localized chemolithoautotrophic communities. Some vents instead emit high concentrations of dissolved ferrous iron (Fe^{2+}) and manganous manganese (Mn^{2+}), but low H_2S. Such sites are typically characterized by thick accumulations of ferric oxyhydroxide (e.g., $Fe(OH)_3$) and manganese oxide (MnO_2), and it has been proposed that mineralization is partly the result of the activity of metal-oxidizing bacteria[12, 13]. In O_2-depleted niches, chemolithoautotrophic sulfur-reducing and methane-generating bacteria respire using emitted H_2, and either S^0 or CO_2, respectively, while the organic substrates provided by the autotrophic communities serve as food for a variety of heterotrophs[14]. Significant chemical energy to support biomass production is further available in the form of mineral substrates. This energy can be harnessed from the oxidation of seafloor hydrothermal sulfide deposits[15] or from particles of elemental sulfur, metal sulfides, Fe^{2+}, and Mn^{2+} entrained within, or settling out of hydrothermal plumes[16]. In fact, the latter study estimated that the total primary productivity potential in the plume may represent a significant fraction of the

organic matter present in the deep sea. In addition to the microbial communities, there are a number of benthic animals that exist within a few meters of the vents. They consist of tube worms and bivalves that often contain endosymbiotic chemolithoautotrophic bacteria that reside within their tissues. At sulfide-rich vents, the host tube worm provides shelter and gases to sulfur-oxidizing bacteria (e.g., O_2 and H_2S via an unusual hemoglobin), while the bacteria supply organic carbon. This activity leads to elemental sulfur and iron sulfide mineral precipitation within the organic tissues of the animals, in effect fossilizing part of the tube[17]. In areas where methane is abundant, a symbiotic relationship exists between giant mussels and methane-oxidizing bacteria. Still other bacteria act as epibionts, growing attached to the outer surface of the host[18]. Despite the current excitement garnered by new discoveries at deep-sea vents, much of our actual understanding regarding the phylogenetic diversity of hydrothermal communities comes from detailed studies at the more accessible terrestrial hot springs (Figure 2). Nearly one hundred thermophilic to hyperthermophilic species have been described in the past decade, and many of the isolated genera were completely unknown until a few years ago. A case in point is the discovery of two uncultured organisms at Yellowstone National Park, USA, that branch so deeply in the archaeal tree that a new phylum-level designation, "*Korarchaeota*," was subsequently proposed[19]. Recently, a very small (400 nm wide) member of the archaea, "*Nanoarchaeum equitans*," was identified and also proposed as forming a new phylum in the archaea, the *Nanoarchaeota*[20]. The purported deepest-branching bacteria, the *Aquificales*, and the highest temperature cyanobacteria have similarly been isolated from a variety of hot spring sites[10]. Viruses showing a wide range of morphology have additionally been found in nearly boiling waters at Yellowstone, hosted by hyperthermophilic archaea, further highlighting our very limited understanding of microbial diversity[21]. Aside from the ecological discoveries, hot springs offer us the unique opportunity to determine the processes of fossilization because many vents emit high concentrations of dissolved silica that encrust living organisms in a manner reminiscent of early Earth. And, from a biotechnology standpoint, a hot spring near Great Fountain Geyser in Yellowstone, was the source of the culture of *Thermus aquaticus* that is used to make *Taq* polymerase, a key constituent of the polymerase chain reaction. One of the most significant outcomes from studies of microbial ecology at hydrothermal systems is the hypothesis that life originated in a hot aqueous setting. The most persuasive argument supporting this notion, is the recognition that the deeply-

rooted lineages within the universal phylogenetic tree are all represented by archaea and bacteria that grow in hot water[22].

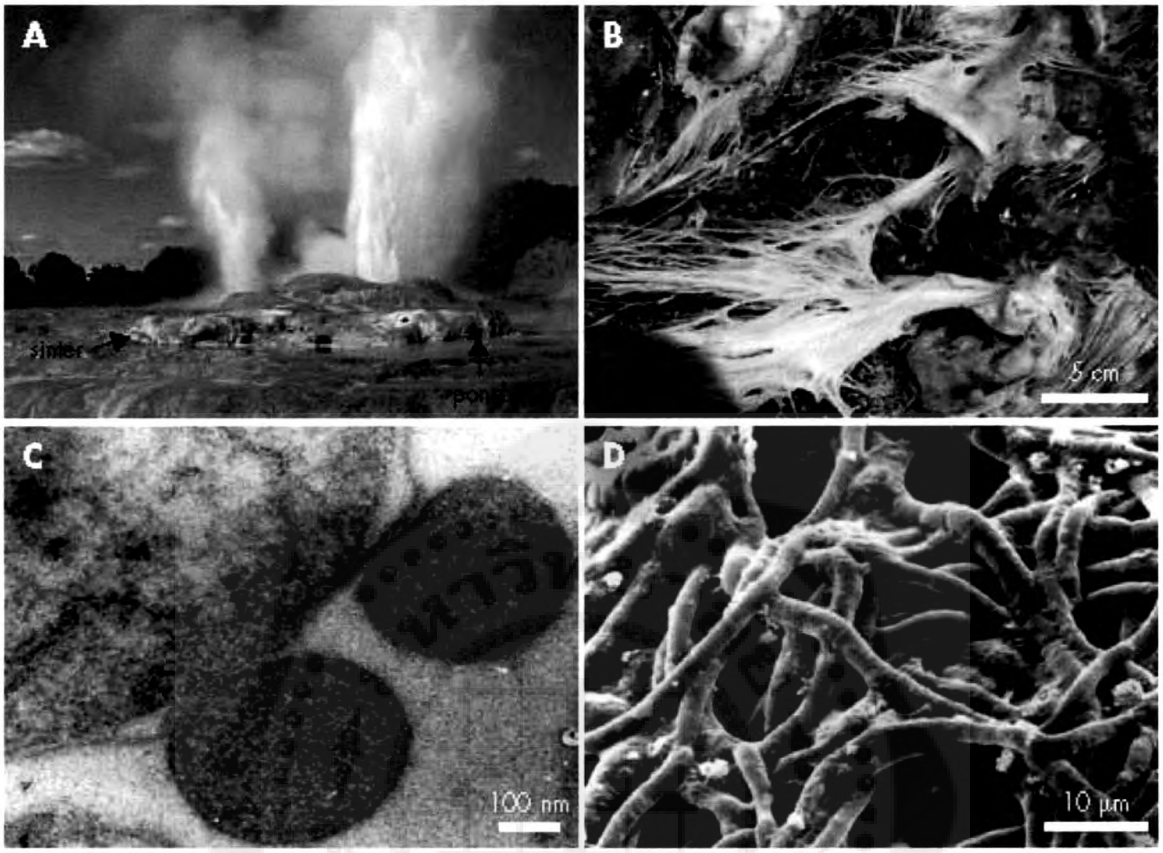


Figure 2 Some examples of hot spring and thermophiles. (A) The Whakarewa Geyser in New Zealand, showing extensive silica sinter deposits flanking the vent. Growing in the geothermal fluids is a diverse microbial community, ranging from hypethermophiles near the vent to mesophilic cyanobacteria, algae, and fungi downstream. (B) White bacterial streamers of the deeply-branching S^0-oxidizing *Aquificales* predominate, the high-temperature microbial community at Angel Terrace hot spring, Yellowstone National Park (courtesy of Anna-Louise Reysenbach). (C) Transmission electron micrograph (TEM) of two newly described archaea, "*Nanoarchaeum equitans*" attached to the surface of a larger archaeal cell (courtesy of Rheinhard Rachel, Harald Huber and Karl Stetter). (D) SEM image of silica-encrusted cyanobacteria *Calothrix* growing in 30°C geothermal waters from Krisuvik, Iceland (courtesy of Vernon Phoenix)[7].

Furthermore, the rather short phylogenetic distances between contemporary hyperthermophilic archaea, with disparate chemolithoautotrophies, suggests that major

evolutionary adaptations have not been required, perhaps because the archaea evolved in a hot setting and they remain there today. By contrast, the moderately thermophilic and mesophilic bacteria (i.e., Gram positives, proteobacteria, cyanobacteria, etc.) represent long lineages that reflect higher rates of rRNA sequence evolution[23]. However, it must be pointed out that the hot origin hypothesis is controversial, and the precise branching is still likely to be modified because a number of recent studies have suggested that the branching of these deeply rooted genera may be misplaced[7].

Industrial relevance of thermophiles and their enzymes

Thermophilic archaea and bacteria that are able to grow at temperatures up to 110°C and at extremes of pH (thermoacidophiles and thermoalkaliphiles) are an interesting source of stable enzymes (extremozymes). These are in general superior to the traditional biocatalysts, because they provide proteins with unique properties and many show reasonable activity even at 120°C, at pH values between 0 and 3, pH 9 to 11 and in the presence of organic solvents (up to 99%). Aiming at the production of high-value products particularly for the chemical, pharmaceutical, cosmetic, food, feed, beverage, paper and textile industries, robust enzymes from thermophiles are gaining significant interest. There is an increasing interest in the utilization of renewable sources to satisfy the exponentially growing energy needs. Therefore, efficient enzyme systems are also needed for the breakdown of plant biomass, which contains complex substrates such a cellulose, hemicellulose, lignin, fats and oils. Microorganisms living in extreme habitats are an ideal source for polymer degraders, which allow to perform biotransformation reactions under nonconventional conditions under which many proteins are completely denatured[7].

The unique stability of enzymes from thermophiles at elevated temperatures (up to 110°C), extremes of pH and high pressure (up to 1000 bar) makes this group of organisms a valuable resource for industrial enzymes[2]. Of special interest is the thermoactivity and thermostability of these enzymes in the presence of high concentrations of organic solvents, detergents and alcohols. Therefore, they are expected to be a powerful tool in industrial biotransformation processes that run at harsh conditions[3, 4]. For the successful exploitation of extremophiles and their enzymes a number of problems, however, have to be resolved. These include the development of efficient cloning and expression systems, especially for hyperthermophilic archaea, and improved cultivation techniques. It is a well known fact that the limitation of the fossil

resources in the future will require a new approach to meet the challenges of the next decades. In order to ensure the supply of raw materials for the chemical and pharmaceutical industries and for fuel production a new strategy based on biomass has to be developed. New technologies should allow the efficient conversion of renewable resources which contain polymeric substrates, for example, cellulose, hemicellulose, lignin, and fats and oils to high value products. The application of robust enzymes and microorganisms for the sustainable production of chemicals, biopolymers, materials and fuels from renewable resources, also defined as industrial (white) biotechnology, will offer great opportunities for various industries. The utmost aim will be the reduction of waste, energy input and raw material and the development of highly efficient and environmentally friendly processes. Most of the industrial enzymes known to date have been derived from bacteria and fungi[5]. The global annual enzyme market has been estimated to be around 5 billion euros and the market for products derived from enzymes in more than 30-fold. In the case of thermophiles, only few enzymes, however, have found their way to the market. Concerted efforts and interdisciplinary approach from academia and industry are required in order to deliver tailor made industrial enzymes. In order to meet the future challenges, innovative technologies for the production of new generation of enzymes and bioprocesses are needed[7].

Polymer degrading thermoactive enzymes cellulose hydrolyzing enzymes

Cellulose hydrolyzing enzymes

Cellulose, which is the most abundant organic biopolymer in nature, consists of glucose units linked by β-1,4-glycosidic bonds with a polymerization grade of up to 15,000 glucose units. The molecular weight of cellulose has been estimated to vary from about 50,000 to 2,500,000 in different species. Naturally occurring cellulose is structurally heterogeneous and has both amorphous and highly ordered crystalline regions. The higher crystalline regions are more resistant to enzymatic hydrolysis. Therefore, complex enzyme systems with different specificities are needed to efficiently hydrolyze cellulose. Cellulose can be hydrolyzed into glucose by the synergistic action of different enzymes: endoglucanase (cellulase), exoglucanase (cellobiohydrolase), and β-glucosidase (cellobiase). Endoglucanase (EC 3.2.1.4) hydrolyzes cellulose in a random manner as endo-hydrolase producing various oligosaccharides, cellobiose, and glucose. Exoglucanases, (EC 3.2.1.91) hydrolyze β-1,4d-glycosidic linkages in cellulose and cellotetraose, releasing cellobiose from the nonreducing end of the chain. β-

glucosidases (EC 3.2.1.21) catalyze the hydrolysis of terminal, nonreducing β-d-glucose residues releasing β-d-glucose[7].

Endoglucanases

Several cellulose degrading enzymes from various thermophilic organisms including archaea and bacteria have been investigated[7]. Thermostable endoglucanases, which degrade β-1,4 or β-1,3 linkages of β-glucans and cellulose, have been identifi ed in few archaea such as *Pyrococcus furiosus, Pyrococcus horikoshii,* and *Sulfolobus solfataricus*[2]. The purified recombinant endoglucanase from the hyperthermophilic archaeon *P. furiosus* is active at 100°C and hydrolyzes β-1,4 but not β-1,3 glycosidic linkages with the highest specifi c activity on cellopentaose and cellohexaose[24]. Another thermoactive glucanase (laminarinase) (T_{opt} 100°C) from this strain catalyzes the hydrolysis of mixed-linked oligosaccharides with both β-1,4 and β-1,3 specificities[25]. The E170A mutant of the enzyme is additionally active as a glycosynthase, catalyzing the condensation of α-laminaribiosyl fluoride to different acceptors at pH 6.5 and 50°C[26]. Depending on the acceptor, the synthase generates either β-1,4 or β-1,3 linkage. A recombinant endoglucanase from *P. horikoshii* was also characterized[27]. This enzyme is active even towards crystalline cellulose. Its activity was recently improved by protein engineering[28]. This enzyme is expected to be useful in biopolishing of cotton products. Very recently, an acid-stable endoglucanase from the thermoacidophilic archaeon *S. solfataricus* P2 was cloned and expressed in *Escherichia coli*[29]. The purified recombinant enzyme with optimal activity at 80°C and pH 1.8, hydrolyses carboxymethylcellulose and cello-oligomers, with cellobiose and cellotriose as main products. This extracellular enzyme could be applicable for the large-scale hydrolysis of cellulose under acidic conditions. Bacteria belonging to the genera *Thermotoga*, *Thermobifida*, *Rhodothermus*, and *Clostridium* are also good cellulose degraders[30-35]. Thermostable endoglucanases from *Thermotoga maritima* and *Thermotoga neapolitana* are rather small with a molecular mass of 27 kDa and are optimally active at 95°C to 106°C and between pH 6.0 and 7.0[33]. Cellulase and hemicellulase genes have been found to be clustered together on the genome of the thermophilic anaerobic bacterium *Caldocellum saccharolyticum*, which grows on cellulose and hemicellulose as sole carbon sources[36]. The gene for one of the cellulases was isolated and was found to consist of 1,751 amino acids. This is the largest cellulase gene sequenced to date. Another large cellulolytic enzyme with the ability to hydrolyze microcrystalline cellulose was isolated from the extremely

thermophilic bacterium *Anaerocellum thermophilum*[37]. This enzyme has an apparent molecular mass of 230 kDa and exhibits significant activity towards Avicel. It is most active towards soluble substrates such as carboxymethylcellulose and β-glucan. Maximal activity is at pH 5 to 6 and 85°C to 100°C. The thermophilic bacterium *Rhodothermus marinus* produces a thermostable endoglucanase, with a temperature optimum of around 80°C[31]. A 100-kDa protein with endoglucanaseactivity was purified from Triton X-100 extract of cells of the thermoacidophilic Gram-positive bacterium *Alicyclobacillus acidocaldarius*[38]. The enzyme exhibits activity towards carboxymethylcellulose and oat spelt xylan with pH and temperature optima of pH 4 and 80°C, respectively. Remarkable stability was observed at pH values between 2 and 6 and 60% of activity was retained after incubation at 80°C for 1 h. Another glucanase purified from the same microorganism is less acidstable, having maximum activity at pH 5.5[39].

β-Glucosidases

Unlike endoglucanases, several β-glucosidases have been characterized from archaea. These enzymes have been detected in strains of the genera *Sulfolobus, Pyrococcus,* and *Thermosphaera*. The β-glucosidase from *P. furiosus* is very stable with optimal activity at 103°C and it also exhibits a β-mannosidase activity[40]. The β-glucosidase from *S. solfataricus* MT4 is very resistant to various denaturants with activity up to 85°C. The gene for this β-glucosidase has been cloned and expressed in *E. coli* and *Saccharomyces cerevisiae*[41]. Using a mixture of both β-glucosidases from *P. furiosus* and *S. solfataricus* an ultra high-temperature process for the enzymatic production of novel oligosaccharides from lactose was developed [19]. For the production of glucose from cellobiose a bioreactor system with immobilized recombinant β-glucosidase from *S. solfataricus* was developed[42]. The system runs at a high flow rate and has a high degree of conversion, productivity, and operational stability. The thermoactive β-glucosidase from *P. horikoshii* is active in organic solvents and it synthesizes a heterosaccharide with high optical purity[2]. There is a great demand for robust cellulolytic enzymes especially for the efficient bioconversion of plant material to utilizable monomeric and oligomeric sugar molecules. The bottleneck for the successful application of the enzymes is making the complex substrate available to the enzymes. This limiting step can be overcome by the application of chemical, physical and enzymatic methods including high pressure, temperature, ionic solvents, extraction with supercritical fluids and enzymes. The combination of these parameters will make the

substrate accessible to enzymes and allow the conversion of renewable biomass (lignocelluloses) to high value products for various applications such as improvement of juice yield, effective color extraction of juices and fuel (ethanol) production. To date more than 40 million tons of ethanol is produced by fermentation with an estimated growth rate of 10% to 20% per year. Other suitable applications of cellulases include the pretreatment of cellulose biomass and forage crops to improve nutritional quality and digestibility. Due to the limitation of fossil resources it is expected that cellulases will be useful tools for the saccharification of agricultural and industrial wastes and production of fine chemicals. It has been estimated that around 40% of the bulk chemical produced today can be derived from plant waste material[7].

Xylanase

There are a variety of thermophilic bacteria and archaea that are able to utilize xylan as carbon and energy source. Xylan is a heterogeneous molecule that constitutes the main polymeric compound of hemicellulose, a fraction of the plant cell wall, which is a major reservoir of fixed carbon in nature. The main chain of the heteropolymer is composed of xylose residues linked by β-1,4-glycosidic bonds. Approximately half of the xylose residues have substitution at O-2 or O-3 positions with acetyl-, arabinosyl-, and glucuronosyl-groups. The complete degradation of xylan requires the action of several enzymes. The endo- β-1,4-xylanase (EC 3.2.1.8) hydrolyzes β-1,4-xylosydic linkages in xylans, while β-1,4-xylosidase (EC 3.2.1.37) hydrolyzes β-1,4-xylans and xylobiose by removing the successive xylose residues from the nonreducing termini. To date only few hyperthermophilic archaea that are able to grow on xylan and secrete thermoactive xylanolytic enzymes[43, 44]. Among the thermophilic archaea, a xylanase from *Pyrodictium abyssi* has been characterized with an optimum temperature of 110°C, which is one of the highest values reported for a xylanase[45]. The crenarchaeon *Thermosphaera aggregans* was shown to grow on heat-treated, but not native, xylan[46]. The xylanase from *Thermococcus zilligii* AN1 is active up to 100°C, can attack different xylans and is not active towards cellulose[47]. Recently, a thermoactive endoxylanase from *S. solfataricus* was purified and characterized[43]. The products of xylan hydrolysis are xylooligosaccharides and xylobiose. Thermophilic anaerobic bacteria such as *Clostridium, Dictyoglomus, Rhodothermus, Thermotoga,* and *Thermus* are also able to secrete heat stable xylanases[3, 48-51]. These enzymes are active between 80°C and 105°C and are mainly cell associated and most probably localized within the toga, which covers the cells. Several genes encoding thermostable

xylanases, for example, *T. maritima,* have been already cloned and expressed in *E. coli*. Robust xylanases are attractive candidates for various biotechnological applications and can be used also in combination with other depolymerases. Enzymes from bacteria and fungi are already produced on industrial scale and are used as food additives in poultry, for increasing feed efficiency diets and in wheat flour for improving dough handling and the quality of baked products. In the last decade, the major interest in thermostable xylanases was in enzyme-aided bleaching of paper. The chlorinated lignin derivatives generated by this process constitute a major environmental problem. Recent investigations have demonstrated the feasibility of enzymatic treatments as alternatives to chlorine bleaching for the removal of residual lignin from pulp. A treatment of craft pulp with cellulase-free thermostable xylanases leads to a release of xylan and residual lignin without undo loss of other pulp components. Xylanase treatment at elevated temperatures opens up the cell wall structure, thereby facilitating lignin removal in subsequent bleaching stages and thus enhance the development of environmentally friendly processes[52].

Starch processing enzymes

The starch processing industry, which converts starch into more valuable products such as dextrins, glucose, fructose, maltose, and trehalose, utilize microbial thermostable enzymes. In all starch converting processes, high temperatures are required to liquefy starch and make it accessible to enzymatic hydrolysis. The synergetic action of thermostable amylolytic enzymes brings an advantage to those processes, lowering the cost of sugar syrup production. The use of thermostable enzymes can lead to other valuable products, which include innovative starch-based materials with gelatin-like characteristics and defi ned linear dextrins that can be used as fat substitutes, texturizers, aroma stabilizers, and prebiotics[53].

α-Amylases

α-amylase (α-1,4-glucan-4-glucanohydrolase; EC 3.2.1.1), hydrolyzes linkages in the starch polymer, which leads to the formation of linear and branched oligosaccharides. The sugar reducing groups are liberated in the α-anomeric configuration. Most of starch hydrolyzing enzymes belongs to the α-amylase family that contains a characteristic catalytic (β/α)8-barrel domain. Throughout the α-amylase family, only eight amino acid residues are invariant, seven at the active site and a glycine in a short turn. It seems that the ability of hyperthermophilic archaea to utilize

starch is more frequent than cellulose[7]). Extremely thermostable α-amylases have been characterized from a variety of hyperthermophilic archaea belonging to the genera *Methanocaldococcus, Pyrococcus*, and *Thermococcus*[2]. The thermostability of the enzymes is often enhancing in the presence of divalent metal ions. The optimal temperatures for the activity of these enzymes range between 80°C and 100°C. The high thermostability of the pyrococcal extracellular α-amylase (thermal activity even at 130°C and after autoclaving for 4 h at 120°C) and α-amylase from *Methanocaldococcus jannashii* (temperature optimum 120°C, half-life of 50 h at 100°C) makes these enzymes interesting candidates for industrial application[54]. The extreme marine hyperthermophilic archaeon *P. abyssi* can grow on various polysaccharides and also secretes a heat stable α-amylase[45]. α-amylases with lower thermostability have been isolated from the archaea *Thermococcus profundus*, *Thermococcus kodakaraensis* and the extreme thermophilic bacteria *Dictyoglomus thermophilum, T. maritima, Thermus filiformis,* and *R. marinus*[3, 52, 55, 56]. In general the bacterial enzymes are less thermoactive than the archaeal enzymes. Similar to the amylase from *Bacillus licheniformis*, which is commonly used in liquefaction of starch in the industry, most of the enzymes from extreme thermophilic bacteria require calcium for activity. The use of α-amylases in detergents for medium-temperature laundering demands enzymes with high stability and activity at alkaline conditions. Therefore, extracellular enzymes from thermoalkaliphiles are good candidates for application in laundry and dish washing. The enzyme from the thermoalkaliphilic bacterium *Anaerobranca gottschalkii* is optimally active at pH 8.0 and has high transglycosylation activity on maltooligosaccharides. Interestingly, the enzyme exhibits also significant β-cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19) activity[57]. On the other hand, enzymes that are active at high temperatures but low pH are also of interest for application, for example, textile and beverage industries. An acid-stable amylase was purified and characterized from the thermoacidophilic bacterium *Alyciclobacillus acidocaldarius*[58]. This enzyme with a molecular mass of 160 kDa exhibits highest activity at pH 3.0 and 75°C[58].

β-Amylases

β-amylase (1,4-alpha-d-glucan maltohydrolase; EC 3.2.1.2), hydrolyzes 1,4-alpha-d-glucosidic linkages in polysaccharides removing successive maltose units from the nonreducing ends of the chains. For the efficient production of maltose syrups an additional debranching enzyme is needed. To date only few thermoactive bacterial β-amylases are known[52]. The enzyme from *T. maritima* is active in the absence of

calcium at low pH (pH 4.3-5.5) and high temperature (95°C)[52]. The enzyme from *Thermoanaerobacter thermosulfurigenes* retains 70% of its activity at pH 4.0 and 70°C[52].

Glucoamylases

Unlike α-amylase, the production of glucoamylase seems to be rare in archaea and bacteria[7]. Glucoamylases (EC 3.2.1.3) hydrolyze terminal α-1,4-linked-d-glucose residues successively from nonreducing ends of the chains, releasing β-d-glucose. An ideal catalyst for starch liquefaction should be optimally active at 100°C and pH 4.0 to 5.0 without requirement of calcium ions for the stabilization of the enzyme. Recently, it has been shown that also the thermoacidophilic archaea *Thermoplasma acidophilum*, *Picrophilus torridus*, and *Picrophilus oshimae* produce heat and acid stable extracellular glucoamylases. The purified archaeal glucoamylases are optimally active at pH 2 and 90°C. Catalytic activity is still detectable at pH 0.5 and 100°C[59]. These enzymes are more thermostable than already described glucoamylases from bacteria, yeasts, and fungi. They are of interest for application in the beverage industry. However, the lack of suitable genetic methods for thermoacidophiles have precluded structural studies aimed to discover their adaptation at very low pH. Recently, the gene encoding a putative glucoamylase from *S. solfataricus*, was cloned and expressed in *E. coli*, and the properties of the recombinant protein were examined in relation to the glucose production process[60]. This recombinant enzyme is extremely thermostable, with an optimal temperature at 90°C; however, it is most active in a slight acidic pH range from 5.5 to 6.0. The tetrameric enzyme liberates β-d-glucose from maltotriose, and the substrate preference for maltotriose distinguishes this enzyme from fungal glucoamylases. Genome analysis of other thermocidophiles revealed further putative glucoamylases, which were cloned and expressed in *E. coli*. Thus, the recombinant intracellular glucoamylase from *M. jannashii* is active at pH 6.5 and 80°C[61]. In our laboratory, the intracellular glucoamylases from the extreme thermoacidophiles *P. torridus* and *T. acidophilum* have been recently cloned and expressed in *E. coli*; the recombinant enzymes are optimally active at 50°C to 75°C and pH 5. Thermophilic anaerobic bacteria such as *Clostridium thermohydrosulfuricum*, *Clostridium thermosaccharolyticum,* and *Thermoanaerobacterium thermosaccharolyticum* produce glucoamylases, which have been purified and characterized[62, 63].

α-Glucosidases

α-glucosidases (EC 3.2.1.20) attack the α-1,4 linkages of oligosaccharides and unlike glucoamylases, α-glucosidase prefers smaller oligosaccharides, for example, maltose, maltotriose and liberates glucose with an α-anomeric configuration. An intracellular and an extracellular α-glucosidases have been purified and characterized from archaea, belonging to the genera *Pyrococcus*, *Sulfolobus*, and *Thermococcus*[3, 46, 64-67]. The enzymes exhibit optimal activity at pH 4.5 to 7.0 over a temperature range from 105°C to 120°C. An α-glucosidase gene and flanking sequences from *S. solfataricus* were cloned in *E. coli* and the product was characterized[67]. The purified recombinant enzyme with a calculated size of 80.5 kDa hydrolyzes *p*-nitrophenyl-d-glucopyranoside. At pH 4.5 it exhibits a pH optimum for maltose hydrolysis. Unlike maltose hydrolysis, glycogen was hydrolyzed efficiently at the intracellular pH of the organism (pH 5.5). The recombinant α-glucosidase exhibits greater thermostability than the native enzyme, with a half-life of 39 h at 85°C at a pH of 6.0. Less thermostable α-glucosidases were detected in the bacteria *Thermoanaerobacter ethanolicus*[64, 68], *T. thermosaccharolyticum*[62], *T. maritima*[69], and archaea *Ferroplasma acidiphilum*[70]. The α-glucosidase from the extreme acidophilic archaeon has maximal activity at pH 2.4 to 3.5 (>70% activity at pH 1.5). Iron was found to be essential for enzymatic activity and His30 was shown to be responsible for iron binding[7].

Pullulanases

Enzymes capable of hydrolyzing α-1,6 glucosidic bonds in pullulan and branched oligosaccharides are defined as pullulanases. On the basis of substrate specificity and product formation, pullulanaseshave been classified into three groups: pullulanase type I, pullulanase type II and pullulan hydrolases (type I, II, and III). Pullulanase type I (EC 3.2.1.41) specifically hydrolyzes the α-1,6-linkages in pullulan as well as in branched oligosaccharides (debranching enzyme), and its degradation products are maltotriose and linear oligosaccharides, respectively. Pullulanase type I is unable to attack α-1,4-linkages in α-glucans. Pullulanase type II (amylopullulanase) attacks α-1,6-glycosidic linkages in pullulan and branched polysaccharides. Unlike pullulanase type I, this enzyme also attacks α-1,4-linkages in branched and linear oligosaccharides and is able to fully convert polysaccharides (e.g., amylopectin) to small sugars (e.g., glucose, maltose, and maltotriose) in the absence of amylases. Thermoactive pullulanase type II from the archaea *Desulfurococcus mucosus*, *P. furiosus*, *P. woesei*, and *Thermococcus hydrothermalis*, have been reported to have

temperature optima between 85°C and 105°C[3], as well as remarkable thermostability even in the absence of substrate and calcium ions. In the presence of calcium ions pullulanase activity was also detected at 130°C to 140°C[71]. Site-directed mutagenesis performed on pullulanase from *T. hydrothermalis* reveals that the residues E291 and D394 are critical for the pullulanolytic and amylolytic activities of the pullulanase[72]. The crucial role of E291 as the catalytic nucleophile has been also confirmed for the pullulanase from *P. furiosus*[73]. The apparent catalytic efficiencies (Kcat/Km) of mutants E291Q and D394N on pullulan were 123 and 24 times lower than that of the native enzyme. The hydrolytic patterns for pullulan and starch were the same, while the hydrolysis rates differed as reported. Therefore, these data strongly suggest that the bifunctionality of the pullulanase type II is determined by a single catalytic center. Due to the dual specificity of pullulanases type II to degrade both α-1,4- and α-1,6-glucosidic linkages they cannot be used as debranching enzymes in maltose and glucose syrup production. The archaeal enzymes are promising candidates to optimize starch liquefaction for the production of maltose, maltotriose, and maltotetraose syrups. The aerobic thermophilic bacterium *Thermus caldophilus* GK-24 produces a thermostable pullulanase of type I when grown on starch[64]. The pullulanase is optimally active at 75°C and pH 5.5, is thermostable up to 90°C, and does not require calcium ions for either activity or stability. The first debranching enzyme (pullulanase type I) from anaerobic thermophilic bacteria was found in *Fervidobacterium pennivorans.* The recombinant enzyme forms long chain linear polysaccharides from amylopectin. A similar enzyme was also characterized from *T. maritima.* All known bacterial debranching enzymes are active in the acidic or neutral pH range. Until very recently, no reports have been presented on the ability of thermoalkaliphiles to produce heat and alkaline stable pullulanase type I. After sequencing the whole genome of the thermoalkaliphile *A. gottschalkii*, a pullulanase encoding gene was cloned and expressed in *E. coli*, this enzyme is optimally active at pH 8.0 and 70°C[74]. The third class of pullulan-hydrolyzing enzymes includes pullulan hydrolases type I, II, and III. Pullulan hydrolases type I and II are active towards α-1,4 linkages of amylose, starch, pullulan, but are unable to hydrolyze α-1,6 linkages. An exception is pullulan hydrolase type III. This enzyme attacks α-1,4 as well as α-1,6 linkages of pullulan. The enzymes from *P. furiosus* (AmyL), *Thermococcus aggregans* and *T. profundus*[3, 75] exhibit maximal activity at 90°C and pH 5.5 to 6.5 and are stable for several hours at 95°C to 100°C. In addition, the pullulan hydrolase from *P. furiosus* degrades β-cyclodextrin.

Pectin degrading enzymes

Pectin is an important plant material that is present in the middle lamellae as well as in the primary cell walls. This biopolymer is a branched heteropolysaccharide consisting of a main chain of α-1,4- d-polygalacturonate, which is partially methyl esterified. Along the chain, l-rhamnopyranose residues are present that are the binding sites for side chains composed of neutral sugars. Pectin is degraded by pectinolytic enzymes, which can be classified into two major groups. The first group comprises methylesterases, which function is to remove the methoxy groups from pectin. The second group comprises the depolymerases (hydrolases and lyases), which attack both pectin and pectate (polygalacturonic acid). Interestingly, no reports are available on the production of pectinolytic enzymes by thermophilic archaea. The enzymes usually act at alkaline pH and are calcium dependent. Thus, a spore forming anaerobic microorganism *Thermoanaerobacter italicus* is able to grow at 70°C on citrus pectin and pectate. After growth on citrus pectin, two pectate lyases were induced, purified and biochemically characterized[76]. Both enzymes display similar catalytic properties and can function at temperatures up to 80°C. An increase in the enzymatic activity of both pectate lyases was observed after the addition of calcium ions. The ability of the hyperthermophilic bacterium *T. maritima* to grow on pectin as a sole carbon source coincides with the secretion of an extracellular pectate lyase. The corresponding gene was functionally expressed in *E. coli* as the first heterologously produced thermophilic pectinase[77]. Highest activity was demonstrated on polygalacturonic acid, whereas pectins with an increasing degree of methylation were degraded at a decreasing rate. The tetrameric enzyme requires calcium ions for stability and activity. The enzyme is highly thermoactive and thermostable, operating optimally at 90°C and pH 9.0, with a half-life for thermal inactivation of almost 2 h at 95°C, and an apparent melting temperature of 102.5°C. With polygalacturonic acid PelA has a unique eliminative exo-cleavage pattern liberating unsaturated trigalacturonate as the major product. *T. maritima* also produces exopolygalacturonase, which has been rarely described in bacteria[78]. Pectin degrading enzymes from the bacteria of the genus *Clostridium* are not very thermostable[52]. Enzymatic pectin degradation is widely applied in food technology processes, as in fruit juice extraction and wine making, in order to increase the juice yield, to reduce its viscosity, improve color extraction from the fruit skin and to macerate fruit and vegetable tissues[7].

Proteases

Proteases, which are involved in the conversion of proteins to amino acids and peptides, have been classified according to the nature of their catalytic site in three groups: serine, cysteine, aspartic, or metalloproteases. Proteases and proteasomes play a key role in the cellular metabolism of archaea and a variety of heat-stable proteases has been identified in hyperthermophilic archaea belonging to the genera *Aeropyrum*, *Desulfurococcus*, *Sulfolobus*, *Staphylothermus*, *Thermococcus*, *Pyrobaculum*, and *Pyrococcus*(7). It has been found that most proteases from extremophilic archaea and bacteria belong to the serine type, and are stable at high temperatures even in the presence of high concentrations of detergents and denaturing agents[3, 68]. Those properties of extracellular serine proteases are reported in a number of *Thermococcus* species and could be well illustrated by the extracellular enzyme from *Thermococcus stetteri*, which is highly stable (half-life of 2.5 h at 100°C) and resistant to chemical denaturation such as 1% SDS[5]. Heat-stable serine proteases were isolated from the cell-free supernatant of the hyperthermophilic archaea *Desulfurococcus* strain Tok12S1 and *Desulfurococcus* sp. SY[3]. A globular serine protease from *Staphylothermus marinus* was found to be extremely thermostable and is heat-resistant up to 125°C in the stalk-bound form[79]. A novel intracellular serine protease (pernisine) from the aerobic hyperthermophilic archaeon *Aeropyrum pernix* K1 is active at 90°C. The enzyme has a broad pH profile with an optimum at pH 9.0 for peptide hydrolysis[80, 81]. A gene encoding a serine protease, named aereolysin has been cloned from *P. aerophilum* and the protein was modeled based on structures of subtilisin-type proteases[82]. Multiple proteolytic activities have been observed in *P. furiosus*. The cell-envelope associated serine protease of *P. furiosus* called pyrolysin was found to be highly stable with a half-life of 20 min at 105°C[83]. Proteases have also been characterized from the thermoacidophilic archaea *S. solfataricus*[84] and *Sulfolobus acidocaldarius*[82]. The enzyme from *F. pennivorans* is able to hydrolyze feather keratin forming amino acids and peptides. The enzyme, which has been named fervidolysin, is optimally active at 80°C and pH 10.0[85]. The gene encoding fervidolysin was cloned and successfully expressed in *E. coli*. The gene encodes for a 73-kDa fervidolysin precursor, a 58-kDa mature fervidolysin, and a 14-kDa fervidolysin propeptide. Using site-directed mutagenesis, the active-site histidine residue at position 79 was replaced by an alanine residue. The resulting fervidolysin showed a single protein band corresponding in size to the 73-kDa fervidolysin precursor, indicating that its proteolytic cleavage resulted from

an autoproteolytic process. From the thermoacidophile *Alicyclobacillus sendaiensis*, a novel thermostable collagenolytic member of the serine-carboxyl proteinase family was characterized[86]. This enzyme, with a molecular mass of 37 kDa, can be applied for the production of peptides from collagen. Organic solvents resistant aminopeptidase was described from the hyperthermophilic bacterium *A. aeolicus*[87]. Indeed, the *P. furiosus* strain contains at least 13 different proteins with proteolytic activity. The amount of proteolytic enzymes produced worldwide on commercial scale is the largest. Heat stable proteases are useful enzymes, especially for the detergent industry. Serine alkaline proteases from thermophiles could be used as additives for laundering, where they have to resist denaturation by detergents and alkaline conditions. Proteases are also applied for peptide synthesis using their reverse reaction, mainly because of their compatibility with organic solvents. A number of heat-stable proteases are now used in molecular biology and protein chemistry. The protease S from *P. furiosus* is used to fragment proteins before peptide sequencing (TaKaRa Biomedicals). Carboxyand aminopeptidases from *P. furiosus* and *S. solfataricus* are used for protein N- or C-terminal sequencing[88, 89].

Biocatalysis with nonpolymeric compounds

Lipases and esterases

Lipases hydrolyze triglycerides to glycerol and fatty acids and are also able to catalyze the reverse reaction in the presence of organic solvents. Lipases are an important group of biotechnologically relevant enzymes and they find applications in food, dairy, detergent, and pharmaceutical industries. Lipases produced by microbes and specifically bacterial lipases play a vital role in commercial ventures. Lipases are generally produced on carbon sources, such as oils, fatty acids, glycerol, or tweens in the presence of an organic nitrogen source. Bacterial lipases are mostly extracellular and are produced by submerged fermentation. Most lipases can act in a wide range of pH and temperature, though alkaline bacterial lipases are more common. Bacterial lipases generally have temperature optima in the range 30°C to 60°C. Lipases are serine hydrolases and have high stability in organic solvents. In addition, some lipases exhibit chemo-, regio-, and enantioselectivity. Very recently, five anaerobic thermophilic bacteria were found to produce extremely heat stable lipases. In the field of industrial biotechnology, also esterases are gaining increasing attention because of their application in organic biosynthesis. In aqueous solution, esterases catalyze the

hydrolytic cleavage of esters to form the constituent acid and alcohol, whereas in organic solutions, transesterification reaction is promoted. Both the reactants and the products of transesterification are usually highly soluble in the organic phase and the reactants may even form the organic phase itself. Esterases from archaea *A. pernix, Pyrobaculum calidifontis,* and *Sulfolobus tokodaii* exhibit high thermoactivity and thermostability and are active also in a mixture of a buffer and water-miscible organic solvents, such as acetonitrile and dimethyl sulfoxide[2]. The optimal activity for ester cleavage of the esterase from *S. tokodaii* strain 7 is at 70°C and pH 7.5 to 8.0. From the kinetic analysis, *p*-nitrophenyl butyrate is the better substrate than caproate and caprylate[89]. The *P. furiosus* esterase is the most thermostable (a half-life of 50 min at 126°C) and thermoactive (temperature optimum 100°C) esterase known to date[90]. A carboxylesterase from *P. calidifontis,* stable against heating and organic solvents, is active towards tertiary alcohol esters, a very rare feature among previously reported lipolytic enzymes[91]. A novel thermostable esterase from *A. pernix* K1 with an optimal temperature at 90°C exhibits additionally a phospholipase activity[92]. In our laboratory two thermoactive esterases from the thermoacidophilic archaeon *P. torridus* have been recently characterized after successful expression in *E. coli* (Antranikian, unpublished results). Both esterases are active at 50°C to 60°C and neutral pH. A gene coding the esterase from *Archaeoglobus fulgidus* was subjected to error-prone PCR in an effort to increase the low enantioselectivity towards the racemic mixture of *p*-nitrophenyl-2-chloropropionate to produce the *S*-2- chloropropionic acid. This compound is an important intermediate in the synthesis of some optically pure compounds, such as a herbicide mecoprop[93]. A double mutant, Leu101Ile/Asp117Gly was obtained with increased preference in the opposite direction. The esterase from *S. solfataricus* P1 has been studied in detail for the chiral resolution of 2-arylpropionic esters[94]. Thus, the application of the esterase toward *R,S*-naproxen methyl ester yields highly optically pure *S*-naproxen [ee(p) > 90%][94, 95]. The enzyme is activated by DMSO to various extents, due to small changes in the enzyme structure resulting in an increase in its conformational flexibility. Thus, the addition of cosolvents, which is useful for solubilization of hydrophobic substrates in water, also serves as activators in applications involving thermostable biocatalysts at suboptimal temperatures[96]. Interestingly, experimental data on kinetic resolution of α-arylpropionic acid revealed that a carboxylesterase from *S. solfataricus* P2 hydrolyzes the *R*-ester of racemic ketoprofen methylester with enantiomeric excess of 80%[60]. A gene encoding a thermostable

esterase was cloned from the bacterium *Thermoanaerobacter tengcongensis* and over-expressed in *E. coli*. The recombinant esterase, with a molecular mass of 43 kDa hydrolyzes tributyrin but not olive oil. The esterase is optimally active at 70°C (over 15 min) and at pH 9. It is highly thermostable, with a residual activity greater than 80% after incubation at 50°C for more than 10 h[97].

Glucose and arabinose isomerase

Glucose isomerase or xylose isomerase (d-xylose ketol-isomerase; EC 5.3.1.5) catalyzes the reversible isomerization of d-glucose and d-xylose to d-fructose and d-xylulose, respectively. The enzyme has the largest market in the food industry because of its application in the production of high-fructose corn syrup (HFCS). Glucose isomerases are widely distributed in mesophilic microorganisms and intensive research efforts were directed towards improving their suitability for industrial application. In order to reach fructose concentration higher than 55% the reaction must approach 110°C. Improved thermostable glucoses isomerases have been engineered from mesophilic enzymes[98]. The gene encoding a xylose isomerase of *Thermus flavus* AT62 was cloned and the DNA sequence was determined. The enzyme has an optimum temperature at 90°C and pH 7.0; divalent cations are required for enzyme activity[99]. *Thermoanaerobacterium* strain JW/SL-YS 489 forms a xylose isomerase, which is optimally active at pH 6.4 and 60°C or pH 6.8 and 80°C. Like other xylose isomerases, this enzyme requires divalent cations for thermal stability (stable for 1 h at 82°C in the absence of substrate). The gene encoding the xylose isomerase of *Thermoanaerobacterium* strain JW/SLYS 489 was cloned and expressed in *E. coli*[52]. Comparison of the deduced amino acid sequence with sequences of other xylose isomerases showed that the enzyme has 98% homology with a xylose isomerase from a closely related bacterium *Thermoanaerobacterium saccharolyticum* B6A-RI. A thermostable glucose isomerase was purified and characterized from *T. maritima*. This enzyme is stable up to 100°C, with a half-life of 10 min at 115°C[52]. Interestingly, the glucose isomerase from *T. neapolitana* displays a catalytic efficiency at 90°C, which is 2 to 14 times higher than any other thermoactive glucose isomerases at temperatures between 60°C and 90°C[100]. Arabinose isomerase (EC 5.3.1.4) catalyzes the reversible isomerization of arabinose to ribulose. Thermoactive enzymes have been reported to convert d-galactose to d-tagatose, a novel and natural sweetener[101]. Such enzymes have been described from the thermophilic bacteria *A. acidocaldarius*, *Geobacillus*

stearothermophilus, *Thermoanaerobacter mathranii*, *T. maritima,* and *T. neapolitana*[102-105].

Nitrile-degrading enzymes

Nitrile-degrading enzymes are of considerable importance in industrial biotransformations, and to date several processes have been developed for chemical and pharmaceutical industries for the production of optically pure compounds, drugs, acrylic, and hydroxamic acids[106]. Nitrile degrading enzymes play also a significant role in the protection of the environment due to their capability to eliminate highly toxic nitriles. Thermostable amidases and nitrilases are gaining more attention, especially in enzymatic processes in mixtures of organic solvent or in the formation of highly pure products with a concomitant reduction of wastes. Amidases (EC 3.5.1.4) catalyze the conversion of amides to the corresponding carboxylic acids and ammonia. Nitrilases (EC 3.5.5.1) are thiol enzymes that convert nitriles directly to the corresponding carboxylic acids with release of ammonia. A number of bacterial amidases and nitrilases have been purified and characterized. Very little, however, is known on the enzymes that are active at high temperatures. Amidases are highly *S*-enantioselective, usually forming the optically pure acids with an enantiomeric excess above 99%. The only amidase derived from archaea is the amidase from the thermoacidophile *S. solfataricus*[3]. This enzyme is *S*-stereoselective with a broad substrate spectrum and is optimally active at 95°C[107]. Very recently, the first thermoactive and thermostable amidase from the thermophilic actinomycete *Pseudonocardia thermophila* has been purified and characterized[106]. The amidase is active at a broad pH (pH 4-9) and temperature range (40-80°C) and has a half-life of 1.2 h at 70°C. The amidase has a broad substrate spectrum, including aliphatic, aromatic, and amino acid amides. The amidase is highly *S*-stereoselective for 2-phenylpropionamide with an enantiomeric excess of >95% at 50% conversion of the substrate. Recently, the first archaeal nitrilase from the hyperthermophile *Pyrococcus abyssi*, regiospecific towards aliphatic dinitriles, was cloned and characterized in our laboratory[108]. The enzyme is highly thermostable, having a half-life at 90°C for 6 h. Thermoactive nitrilases described so far were isolated from the bacteria *Acidovorax facilis* 72 W and *Bacillus pallidus* Dac521[108].

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Thermophiles as cell factories

Biomining

The development of industrial mineral processing has been established in several countries, such as South Africa, Brazil, and Australia. Iron- and sulfur-oxidizing microorganisms are used to release occluded gold from mineral sulfi des. Most industrial plants for biooxidation of gold-bearing concentrates have been operated at 40°C with mixed cultures of mesophilic bacteria of the genera *Thiobacillus* or *Leptospirillum*. In subsequent studies a dissimilatosy iron-reducing archaea *Pyrobaculum islandicum* and *P. furiosus* were shown to reduce gold chloride to insoluble gold[109]. The potential of thermophilic sulfi de-oxidizing archaea in copper extraction has attracted interest due to the efficient extraction of metals from sulfide ores that are recalcitrant to dissolution[42]. The acidophilic archaea *Sulfolobus metallicus* and *Metallosphaera sedula* tolerate up to 4% of copper and have been exploited for mineral biomining[110]. The efficiency of copper extraction from chalcopyrite by thermoacidophilic archaea was influenced by the characteristics of mineral concentrates. Between 40% and 60% copper extraction was achieved in primary reactors and more that 90% extraction in secondary reactors with overall residence times of about six days[110]. The handling and recycling of spent tyres are a significant and worldwide problem. The reuse of rubber material is preferable from an economic and environmental point of view. The anaerobic sulfate-reducing thermophilic archaeon *P. furiosus* was investigated for its capacity to desulfurize rubber. The tyre rubber treated with *P. furiosus* for 10 days was subsequently vulcanized with virgin rubber material (15% w/w). This results in the desulfurization of ground rubber and leads to a product with good mechanical properties[111]. The thermoacidophilic archaeon *S. acidocaldarius* has been also tested for desulfurization of rubber material[111].

Lipids and peptides

Liposomes are artificial spherical closed vesicles consisting of one or more lipid bilayers. Liposomes made from ether phospholipids have been studied extensively over the last thirty years as artificial membrane models with remarkable thermostability and tightness against solute leakage. Considerable interest has been generated for applications of liposomes in medicine, including their use as diagnostic agents, as carrier vehicles in vaccine formulations, or as delivery systems for drugs, genes or cancer imaging agents[112]. In general, archaeosomes (liposomes from archaea) demonstrate higher stability to oxidative stress, high temperature, alkaline pH, to attack

of phospholipases, bile salts, and serum proteins. Some archaeosome formulations can be sterilized by autoclaving without problems of fusion or aggregation of the vesicles. The uptake of archaeosomes by phagocytic cells can be up to 50-fold higher than that of conventional liposomes[113]. Crystalline cell surface layers (*S*-layers) that are composed of protein and glycoprotein subunits are one of the most commonly observed cell envelope structures of bacteria and archaea. *S*-layers could be produced in large amounts by continuous cultivation of *S*-layer-carrying microorganisms and used as isoporous ultrafiltration membranes or as matrices for immobilization of biologically active macromolecules such as enzymes, ligands or mono- and polyclonal antibodies[114]. *S*-layers have been shown to be excellent patterning structures in molecular nanotechnology due to their high molecular order, high binding capacity and ability to recrystallize with perfect uniformity on solid surfaces, at the water/air interface or on lipid films. The two-dimensionally organized *S*-layers of *S. acidocaldarius* are suggested to be of practical use as biomimetic templates for material deposition and fabrication of advanced materials[114]. The production of antibiotic peptides and proteins is a near-universal feature of living organisms regardless of phylogenetic classification. Antimicrobial agents from bacteria and eucarya have been studied for more than 50 years. However, thermophilic archaea and bacteria are just in the beginning of investigation for the production of peptide antibiotics. A variety of halocins have been detected in halophilic archaea. These antimicrobial agents are diverse in size, consisting of proteins as large as 35 kDa and peptide "microhalocins" as small as 3.6 kDa[115]. Microhalocins with unclear mechanism of action are hydrophobic and robust, withstanding heat, desalting and exposure to neutral residues and are not cationic. The microhalocins S8 and R1 lack the biochemical and structural properties demonstrated by other antibiotics, suggesting that their mechanisms of action should be novel. The halocin H7 has been suggested for reducing injury during organ transplantation. Archaeocins are also produced by a thermoacidophilic *Sulfolobus* strain. The 20 kDa protein antibiotics are not excreted and are associated with small particles apparently derived from the cells *S*-layer[115].

Hydrogen production

There is an increasing interest in the utilization of renewable sources to satisfy the exponentially growing energy needs. Products of anaerobic fermentation include ethanol, methane, and hydrogen. Research on biological hydrogen production became attractive due to the possible use of biohydrogen as a clean energy carrier and

raw material. The production of hydrogen in photobiological or heterotrophic fermentation routes depends on supply of organic substrates and could be therefore ideally suited for coupling energy production with treatment of organic wastes. A two-stage fermentation system was constructed for the production of biohydrogen from keratin-rich biowaste[116]. First, the bacterial strain *B. licheniformis* KK1 was employed to convert keratin-containing waste into a fermentation product that is rich in amino acids and peptides. In the next stage the thermophilic anaerobic archaeon *T. litoralis* was fermentated on the hydrolysate and hydrogen was produced. Also archaeal hydrogenases have been the target of intensive research. A cytosolic NiFe-hydrogenase from the hyperthermophilic archaeon *T. kodakaraensis* is optimally active at 90°C for hydrogen production with methyl viologen as the electron carrier[117]. A membrane bound NiFe-hydrogenase, responsible for hydrogen production, was also identified in the anaerobic bacterium *T. tengcongensis*[118, 119]. Other thermophilic bacteria of the order Thermotogales have also demonstrated the ability to produce hydrogen[120-122].

Molecular and genomic methods

The ultimate determinants of microbial diversity are the molecules which, by virtue of their fixed structures and variable functions, can generate and maintain differences between lineages of microorganisms. Therefore, methods designed to evaluate the relationships among these molecules are directly relevant to the recognition of diversity and the classification of newly discovered species. This will present an overview of new molecular approaches for investigating microbial communities from the perspective of diversity assessments. The strengths and limitations of these methods are explored through comparisons with microscopic and cultivation methods. Ideally, molecular methods are best used to complement other methods[123].

The molecular context of microbial diversity

Unlike other branches of ecology, the theoretical discord between the fundamental approaches of reductionism and holism has not produced a seemingly irreconcilable schism in the discipline of microbial ecology. The substantive issues that divide these scientific worldviews do exist in microbiology; however, as the primary domain of molecular biology, there is a near unanimous acceptance of the tenet that meaningful answers to important questions can be gained through exclusive analyses of

the molecular components of microbial cells. The molecular approach has been enormously powerful for approaching key questions in microbial physiology and genetics at the single-species level. The analysis of multi-species microbial communities required further innovation in methodological tools to facilitate the proper application of molecular techniques. For example, the variable nature of microbial sensitivity to cell lyses techniques, differences in the tenacity of cellular adsorption to abiotic particles, and the disproportionate representation of individual species in microbial community gene pools, all posed specific but surmountable challenges for the reproducible application of routine molecular techniques to natural microbial communities[124].

The advent within the past three decades of molecular tools for investigating microbial diversity has led to a cadre of specialists trained exclusively to analyze nucleic acids, lipids, or proteins. Unfortunately, narrow "molecular" specialization may have slowed the pace at which the relatively slippery microbial species concept may finally be settled[125]. The elucidation of the major driving processes behind microbial speciation, and the relationship between molecular diversity and functional diversity may have been slowed by the dominance of new and practical but non-comprehensive methodological approaches[126]. The pace may have been slow, but it is difficult to envisage a path different from molecular assessment that could have produced the vast amount of new information on the structure and organization of microbial communities[10]. Developments in molecular microbial ecology have provoked new questions about microbial diversity and about the appropriate level of analysis within the "central dogma" of molecular biology upon which the concept of microbial speciation can be rooted. Regardless of the level of analysis and the independence of specialized research approaches, there is general agreement that robust answers to questions regarding various dimensions of microbial diversity will require coincident assessment of different biochemical molecules. Knowledge of the relative abundance of molecules unique to specific phylogenetic groups, and the clarification of exchange mechanisms that regulate their distribution are needed to support inferences about the sensitivity of microbial diversity to environmental pressures and the process of natural selection[127].

Interpretation of molecular diversity

In a landmark publication, Emile Zukerkandl and Linus Pauling proposed the use of polymeric biomolecules, specifically nucleic acids and proteins, to reconstruct evolutionary distances and phylogenetic relationships among organisms. The proposal

was based on the recognition that the cumulative effect of random mutations, which occur as a result of infidelity in the molecular aspects of genetic replication, produces distinct lineages of organisms that may be tracked chronologically[128]. This observation led directly to the invention, calibration, and subsequent revisions of the "molecular clock" that has been used extensively for phylogenetic analysis of several groups of organisms including bacteria, fungi, viruses, and microscopic eukaryotes[129-133]. The characterization of differences in the sequence of molecules that form polymeric cell components in different organisms has progressed from simple descriptions to the level of predictive assessments based on large collations of phylogenetically conserved and unique sequence databases. It is not surprising, however, that not all seriously proposed molecular sequence information is relevant for phylogenetic analysis. The shortcomings of "molecular clocks" include lateral genetic exchange among distantly related organisms, limited penetration of molecular sequences in different branches of the universal phylogenetic tree, and disproportionately high rates of mutation in certain sequences due to sporadic integration of exogenous fragments associated with mobile genetic elements such as transposon, plasmids, and viral genomes[125, 134, 135].

To establish the link between molecular diversity and the observable phenotypic differences among species, it is necessary to define limits for each category of molecular sequence data. For example, the redundancy inherent in genetic transcription and translation means that a certain level of difference is tolerated at the nucleotide sequence level among individuals of the same species. Conversely, differences at the level of protein structure and function typically produce differences in ecological fitness among organisms inhabiting similar environments. These molecular differences are, presumably, the fundamental determinants of speciation and niche diversity in prokaryotic populations. Large differences in protein function also relate to differences in the structures and functions of secondary macromolecules such as fatty acids and carbohydrates that have also been compiled and used successfully for assessing diversity in microbial communities. Methods for extracting and analyzing polymeric molecules in microbial communities have been the driving force behind strategies for assessing microbial diversity[136]. These methods are at different stages of development, and in many cases, they have not been used concurrently for investigating the linkages between microbial diversity and ecosystem function[123].

Specific nucleic acid-based methods

Insight into species diversity and ecological interactions within microbial communities has been acquired through the application of techniques based on the complementary nature of the double-helix structure of DNA[137, 138]. The recognition of single-stranded RNA molecules by partially complementary strands of DNA to form RNA-DNA hybrid molecules has also contributed to molecular techniques available for assessing diversity[139, 140]. These hybridization and reassociation reactions have been enhanced by advances in polymerase chain reaction (PCR) and fine-scale sequencing to provide information on the level of relatedness among well-characterized species and on the composite characteristics of microbial community gene pools[125]. Single-stranded nucleic acid probes labeled with radioactive or fluorescent substrates have been instrumental in tracking the spatial and temporal distribution of specific microorganisms across geographic gradients in many environments[141-144]. These assessments advanced knowledge on the relative abundance of microbiological niches involved in ecosystem processes such as nutrient cycling, toxic chemical biodegradation, and biological population dynamics[145, 146]. There are several variations on the theme of "nucleic acid sequence analysis and hybridization", which have facilitated the measurement of microbial diversity with implications for the discovery of new taxonomic groups. The most salient of these technical approaches are described in the following paragraphs.

DNA reassociation kinetics was originally developed to assess intra-genomic complexity in eukaryotic organisms and the technique has been adapted for the measurement of diversity in natural microbial communities containing chromosomes of different sizes and gene composition. The result of DNA reassociation kinetics experiments is the length of time it takes for a mixed pool of single-stranded nucleic acids to hybridize, forming double-stranded molecules. The length of time is proportional to the concentration of complementary sequences in the pool. The method produces a quantitative measure of heterogeneity in a sample of DNA pooled from various sources. The main contribution of this approach has been estimates of the number of genomes per unit of environmental samples such as soil or water[147].

Restriction fragment length polymorphism (RFLP) refers to the technique where differences in the distribution of DNA sequences recognized by restriction enzymes are displayed through electrophoretic resolution. RFLP patterns have been used extensively for mapping the level of relatedness among isolated organisms and for tracking the

influence of genetic exchange mechanisms on the dissemination of "marked" genes in a microbial community[148].

Terminal restriction fragment length polymorphism (T-RFLP) depends on the amplification of a region of the small subunit of the ribosomal RNA (SSU-RNA) gene using primers that are fluorescently labeled at the 5' end. The PCR products are then subjected to RFLP preferably with a tetrameric restriction enzyme (Noble *et al.*, 1998). If, for example, primers specific for the SSU-RNA genes of major bacterial phylogenetic categories are used, T-RFLP can provide detailed information on the diversity of bacterial species in DNA samples extracted from microbial communities. T-RFLP has also been used for analyzing the distribution of functional genes in ecosystems. Marsh and colleagues[149] have published a web-based interactive program for selecting combinations of primers and restriction enzymes that give optimum conditions for microbial community analysis.

Denaturing or temperature gradient gel electrophoresis (D/TGGE) allows the detection of very small differences in the sequence of nucleotides within phylogenetically conserved genes[150]. The technique depends on physical conditions that retard the movement of DNA molecules through a gel due to partial melting within discrete regions of double-stranded DNA. The melting is caused by gradients of elevated temperature or increasing concentrations of denaturing agents such as urea and formamide. Typically, fragments larger than 500 base pairs cannot be separated with this technique, a restriction that limits the detail of phylogenetic information generated[151].

Single-strand conformation polymorphism (SSCP) detection has been applied in conjunction with PCR using primers specific for variable regions of ribosomal RNA genes to fingerprint changes in microbial population structure in response to variable environmental parameters. The technique can separate single-stranded DNA (ssDNA) molecules on the basis of fragment length and conformation because ssDNA folds to form stable structures that are defined by sequence composition. Small differences in these "conformers" can be resolved electrophoretically[152].

PCR-amplicon length heterogeneity (PCR-ALH) has been used for comparative assessments of diversity within microbial communities as they change across geographic gradients (Fisher and Triplett, 1999). The technique is based on the amplification of 16S-23S rRNA interspacer regions resulting in DNA fragments of different lengths. The different size fragments are subsequently separated electrophoretically[146, 153]. Fragment length can vary by as much as 1.2 Kb, but the

technique is limited in the ability to resolve long fragments that differ in sequence identity by few nucleotide bases.

Low molecular weight (LMW) RNA pattern analysis depends on the size distribution of transfer RNA and 5S ribosomal RNA molecules in species within a heterogeneous microbial community. The composition of LMW RNA is not affected by environmental conditions, and it is therefore a stable measure of species occurrence. The resolution of LMW RNA extracted from a complex natural environmental sample requires sophisticated pattern recognition programs to identify the presence of known species, and the possible discovery of novel species[154].

Reverse sample genome probing (RSGP) depends on the extraction of bulk DNA from environmental samples, and the use of this community gene pool to probe immobilized DNA that has been extracted from known microbial species[155]. The technique is particularly sensitive to the need to track specific organisms in different ecological contexts, and to identify dominant species within perturbed ecosystems.

Microbial diversity and indexes of environmental change

The international Convention on Biological Diversity (CBD) defines biodiversity as "the variability among living organisms from all sources including, *inter alia*, terrestrial, marine, and other aquatic ecosystems and the ecological complexes of which they are part"[156]. For those concerned with quantitative assessments of biological diversity, the key issue in the CBD definition is how to measure variability. The development of robust indicators of biodiversity has been fraught with problems for several reasons pertaining to the different scales at which biodiversity is usually presented. For example, there are no universally accepted discrete boundaries between functional genes, between species, or between ecosystems. Therefore, it is not possible to quantify the absolute numbers of varieties within each of these three categories across phylogenetic domains. Perlman and Adelson[157] have alleged that the current definitions of biodiversity on the basis of genes, species, and ecosystems fail both in theory and practice because they do not recognize the conceptual difficulties inherent in these constituent terms; they ignore practical and technical problems involved in making real-world inventories; they do not account for the lack of commensurability between different scales; and they make no distinctions in the worth of biodiversity units within each scale of assessment. Despite these shortcomings, attempts to develop quantitative indexes of biological diversity that are sensitive to environmental change have relied on

three major concepts, namely scale, component, and viewpoint[158]. The scale aspect focuses on the criteria of species richness and the geographical distributions of individuals among the species (evenness). Species richness within a local ecosystem is referred to as alpha diversity. The variation in alpha diversity among ecosystems within the same landscape is referred to as beta diversity, and, when measurable, gamma diversity represents species richness at the regional and global scales. Gamma diversity is sensitive primarily to phenomena that impact the environment at the global level (e.g. major climate shifts) as opposed to local scale impacts such as erosion, fires, the introduction of new invasive species, and exposure to toxic chemical pollutants, all of which influence alpha and beta diversities. Applications of the component concept in assessing biodiversity are aimed at empirically determining the minimum viable number of species necessary to maintain ecosystem functions. Finally, the viewpoint concept, perhaps the vaguest and most subjective of the three criteria, refers to the necessity of juxtaposing alternative viewpoints in assessing biodiversity, including but not limited to utilitarian, practical, and aesthetic viewpoints[159, 160]. Not all these criteria apply uniformly to the assessments of microbial diversity without major refinements. For example, in estimating microbial species richness, it is frequently not possible to account for all species in an environmental sample. Therefore, carefully defined subsets are designated as indicator or surrogate species, and these are monitored to represent overall diversity[161, 162]. Species "weighting" can also be used as a shortcut to arrive at a quantitative estimate of changing diversity in microbial communities where only a fraction of existing species have been characterized. For example, the presence and relative population densities of organisms known to have unique ecological functions (e.g. nitrogen fixation or biodegradation of a particular toxic compound) are assigned larger weights than the presence of organisms whose ecological functions are either unknown or not immediately relevant for the purpose of the biodiversity assessment. Weighting is facilitated by the construction of phylogenetic trees describing the relationships between microorganisms present in a particular habitat. Phylogenetic trees provide a visual representation of the level of redundancy that may exist within groups and among distantly related species. Changes in environmental conditions that are likely to affect most members of phylogenetic groups can also be weighted more highly than changes that affect a fraction of members belonging to phylogenetic groups with several representatives[123].

Quantitative measures of species diversity

No single quantitative index of biological diversity can capture the magnitude and direction of environmental change. However, there is no shortage of ecological techniques for monitoring changes in the biological diversity of specific ecosystems, although most of the available field methods were designed for macroscopic organisms with fairly well-known spatial distributions and physiological characteristics (Purvis and Hector, 2000). There are several reasons why the tools that have been developed for assessing biodiversity in plant and animal communities are not appropriate for measuring microbial diversity. Ecologists have traditionally relied on integrating multiple biotic and abiotic indexes to explain past trends in environmental parameters and to account for trajectories of change. The simplest measures of species diversity rely only on the number of species (*s*) and the total number of individ uals representing all the species (*N*). For example, the Margalef index (*Dm*) computes the species diversity according to the following equation[163, 164]:

$$D_m = (s-1) \div \sqrt{\log N}$$

The Margalef index and similar indexes do not support the differentiation of communities that have identical *s* and *N* values because the evenness of the distribution of individuals within the communities is not considered. To correct this shortcoming, some investigators have introduced the concept of species dominance into measures of diversity. For example, Simpson[165] demonstrated that if two individuals are selected randomly from a community, the probability (P_d) that the two individuals belong to the same species is a measure of dominance (d_s), and it is given by the following equation:

$$d_s = 1 - P_d = 1 - \sum n_i(n_i - 1) \div N(N-1)$$

where n_i is the abundance of individual members belonging to species *i*. Simpson's measure of dominance has been modified to compute species diversity as follows:

$$D_s = 1 \div P_d = N(N-1) \div \sum n_i(n_i - 1)$$

Simpson's diversity index has been referred to as the probability of an interspecific encounter, which expresses the number of times required to select two independent

individuals at random from the community before both are found to belong to the same species[166, 167]. Estimates of *Ds* are based on the assumption that the data on the number of species and abundance of individual members are derived from randomly collected environmental samples. However, in cases where it is possible to conduct an exhaustive sampling of a community (e.g. microcosm experiments), where direct molecular methods that can capture the complete spectrum of diversity are used, or where other non-random methods are applied, modified Simpson's indexes of dominance (λ) and diversity (Δ_s) are represented as follows:

$$\Delta_s = 1 \div \lambda = N^2 \div \sum n_i^2$$

These probability-based measures of species diversity have not found as much use as measures based on the concept of uncertainty as defined by information-theoretic indexes.

In a community with relatively low species diversity, there is a relatively high level of certainty that the identity of a species selected at random can be predicted. Conversely, in a relatively diverse community, the level of certainty in predicting the species identity of a randomly selected individual is low. The Shannon diversity index (*H'*) is perhaps the best known of diversity measures rooted in information theory[168, 169]:

$$H' = -\sum p_i \log p_i$$

where *pi* is the fraction of the total number of individuals in the community that belong to species *i*. The equation can be rewritten to facilitate the calculation of *H'* without the need to convert abundances (n_i) to proportions (p_i) as follows:

$$H' = (N \log N - \sum (n_i \log n_i)) \div N$$

Skujins and Klubek[170] used Shannon's index to investigate the correlations between the diversity of microbial populations, the nitrification potential, changes in organic carbon content, and nitrogen content pools of soils along a montane sere consisting of meadow-aspen-fir-spruce (a sere is the sequence of communities that develops through the process of ecological succession). These investigators

demonstrated that when organic carbon increased along the sere from 2.15 to 26.8%, and total nitrogen increased from 0.13 to 0.98%, the index of microbial diversity according to Shannon's formula (*H'*) increased from 0.87 to 1.28. Furthermore, there was a statistically significant correlation between *H'* and organic carbon and total nitrogen (r^2 = 0.99 and 0.98, respectively). The conclusion that may be reached from this assessment is limited because it includes both species richness and evenness in the same index of diversity[171, 172]. It is possible to express evenness as a separate measure by determining the closeness between observed species abundances and the abundance associated with a hypothetical situation with maximum possible species diversity. For example, the maximum possible species diversity (H_{max}) is observed only when the set of *N* individuals is distributed evenly among *s* number of species, or

$$n_i = N \div s$$

The maximum possible Shannon's diversity index can thus be expressed as:

$$H_{\max} = (\log N! - (s-r)\log c! - r\log(c+1)!) \div N$$

where *c* is the integer value of $N \div s$, and *r* is the remainder. The index of evenness (*E'*) or relative diversity is expressed by the ratio of observed diversity (*H'*) and maximum diversity (*Hmax*):

$$E' = H' \div H_{\max}$$

In a bid to develop a conceptual model for evaluating ecological indicators, the US National Research Council (NRC) identified ecosystem productivity, the capacity to capture solar energy and store it as carbon-based molecules, as the foundation upon which specific categories of ecological indexes should be erected. Since ecosystem productivity is influenced by biodiversity, temperature, moisture, and soil fertility, indexes developed to monitor changes in each of these categories must be clearly related to changes in other categories, and more directly to measures of productivity[159].

Although the specific coverage of microbial diversity is slim in the NRC report, it is arguable that microorganisms are active participants in all the recommended ecological indicators (Table 1). For example, changes in land-use patterns are probably the largest anthropogenic impact on ecosystems. These changes result in the

modification of several environmental factors that affect microbial diversity. The conversion of native soil biological communities to agricultural practices dominated by single plant species, supported by periodical application of nitrogen and phosphorus-based fertilizers and pesticides, is bound to change the microbial diversity of soils. The larger the proportion of Earth's terrestrial surface is devoted to monoculture-type farming, the larger still will be the impact on the abundance and distribution of specific types of soil microorganisms. Similarly, changes in the indicators of ecological capital such as nutrient run-off, which can lead to the eutrophication of receiving water systems, are typically accompanied by changes in the population diversity of photosynthetic and heterotrophic microorganisms[173]. Even more profoundly, changes in any indicator of ecosystem functioning are likely to be rooted in microbial activities through their involvement in the biogeochemical cycles.

The large ratio of surface area to mass that is characteristic of many microorganisms renders them highly sensitive to changing environmental conditions, particularly in the aquatic environment. In addition, the situation of many microorganisms at the base of important ecological processes suggests that indicators of microbial community diversity and function can serve as early warning systems for environmental changes that may result in major ecological catastrophes. Therefore, interest in developing biochemical and molecular indicators or biosensors useful for monitoring microbial communities is intensifying, and the results of this concerted effort have produced robust tools, although in many cases, these tools are adapted for specific contemporary environmental issues. The following paragraphs address case studies focusing on the relevance of microbial diversity to these environmental issues, however, the need for cross-cutting indicators of microbial diversity and function is paramount, and should be acknowledged as a major direction for future research.

Table 1 The major categories of national ecological indicators recommended by the National Research Council[159]. Although not explicitly stated, the numbers, forms, and functions of microorganisms are intricately connected to the larger scale indicators that might seem more directly connected to the desired target information

Target information	**Indicators**
Extent and status of ecosystems	Land cover and land use
Ecological capital	Total species diversity
	Native species diversity
	Nutrient run-off
	Soil organic matter
Ecological functioning	Carbon storage
	Production capacity
	Net primary production
	Lake trophic status
	Stream oxygen
	Nutrient use efficiency
	Nutrient balance

Phylogenetic analysis

The establishment of quantifiable similarities and differences among various microbial taxonomic groups is one of the most thrilling endeavors encountered in investigations of microbial diversity. Distinctive characteristics are increasingly recognized at the genomic level, and rapid progress in the sequencing of microbial genomes has energized research interests in the comparative analysis of differences and similarities at this level. However, not all differences in genomic sequence contribute to the observable differences in microbial phenotypes that are interpreted to represent diversity. The availability of extensive databases for nucleic acid and amino acid sequences has necessitated the development of tools for “mining” the databases, and representing information on differences and similarities in a way that contributes to the understanding of diversity beyond genetic potential. Phylogenetic analysis requires an equivalent level of skill for the analysis of environmental samples to discover novel organisms and to produce molecular sequence data. Phylogenetics is generally at the

center of empirical strategies designed to produce meaningful information on how microbial communities are organized[123].

The rationale for phylogenetic trees

The rapid accumulation of nucleic acid and protein sequence datasets in publicly available formats represents the pinnacle of reductionism as a dominant analytic approach in the biological sciences. The remarkable success of this approach has necessitated the invention of reliable strategies for comparing sequence information generated from various sources. Methods were also needed for integrating such comparative assessments into the analytic frameworks being developed to explore phenotypic diversity and evolutionary convergence of microbial traits that support adaptation in various ecological contexts. Systematists who focus on microorganisms are generally interested in reconstructing phylogenetic relationships among extant taxonomic groups, and occasionally in identifying the position of ancestral species in the lineage of more complex organisms. Phylogenetic trees are designed to reveal these relationships in the simplest way possible without compromising the understanding of the complicated mechanisms underlying the translation of genetic potential into phenotype expression. Methods for constructing phylogenetic trees emerged before the proliferation of molecular sequencing. The use of morphological and physiological characteristics made it relatively easy to recognize differences among groups of organisms within the major phylogenetic branches. It is more difficult to recognize such differences in phylogenetic trees constructed with molecular sequence information. Powerful computing capabilities are now required and they must still be trained by expert knowledge systems to produce meaningful information from the routine evaluation of multiple databases compiled for numerous phylogenetic groups. The construction of phylogenetic trees with molecular sequence data is increasingly appealing to microbiologists because of the reputation of this technique as a very powerful way to visually represent fine-scale differences in biological diversity[174-177]. Nucleic acid and protein sequences are being generated far in advance of useful information about phenotypic characteristics associated with specific molecular signatures. This knowledge gap has led, in some cases, to the attribution of specific functions to protein molecules solely on the basis of sequence homologies[178]. The negative consequences of assigning function to nucleic acid and protein sequences are exacerbated by assumptions that the true function of the first protein to be sequenced

within a group has been categorically determined. Furthermore, assumptions that similar proteins perform identical functions in different organisms dealing with different ecological constraints are questionable at best. Although the construction of a phylogenetic tree can facilitate the detection of relative homology between newly identified proteins and the sequence of molecules associated with specific phenotypes, post-translational modification and protein folding may obscure the straightforward interpretation of sequence data to predict ecological functions. Early attempts at quantitative comparisons of molecular sequences involved simple expression of pairwise homologies as percent similarities. This strategy became limited in its application by the expansive database which precluded the publication of exhaustive estimates of homologs within phylogenetic categories. Therefore, multiple alignment programs were invented[179, 180]. Multiple alignments involve the construction of a "guide tree" from all possible pairwise comparisons; however, such trees do not truly reflect the domains that are homologous across all the sequences used for pairwise comparisons[178]. The construction of "true" phylogenetic trees was necessitated in part by the desire to avoid errors in the interpretation of guide trees constructed by pairwise comparisons. The most challenging - and time-consuming - aspect of producing phylogenetic trees is the sequencing of nucleic acid or protein molecules that will be compared to sequences already existing in available databases. Sequencing is now mostly automated but protein sequences are still routinely inferred from structural DNA sequences, although perfect correspondence should not be assumed. The recognition of bioinformatics, functional genomics, and proteomics as fully fledged sub-disciplines of molecular biology has intensified progress toward more robust approaches for translating genetic potential, as recorded in nucleic acid sequences, into functional diversity in microbial communities[181]. The increasing production of molecular sequences and their storage in electronic databases by numerous investigators necessitated the development of computer-assisted search engines with user-defined search parameters for retrieving sequence data. One of the most widely used computer software programs for exploring and downloading molecular sequences is BLAST[182]. BLAST is managed by the United States government through the National Center for Biotechnology Information under the auspices of the National Institutes of Health. Nucleic acid ("blastn") or protein ("blastp") sequences may be uploaded into BLAST for comparison with other sequences preexisting in the database. Alternatively, deposited sequences may be recalled to the query form through "Accession" numbers allocated to specific sequences deposited by

investigators. The result of a query submitted to BLAST is a list of sequences in the database, which are judged to be related to the specific target sequence. The judgment of relatedness is identified by the "bit score" and the "E value" attributed to sequences related to the target. For nucleic acid sequences, the bit score is derived through a summation of 1 and 0 values assigned to each match and mismatch, respectively, and through subtracting penalties for gaps in the sequence. In the case of protein sequences, the nature of mismatch is taken into consideration when assigning the bit score. BLAST presents the related sequences in descending order of bit-score values. In general, high bit scores represent sequences that are more closely related to the target sequence. The E value is a probabilistic estimate of the chance occurrence of identified matches in the database. Small E values indicate a high level of confidence in the inference that the similarities between two sequences are due more to common descent than to chance. Programs such as BLAST recover more sequences than are needed for constructing robust phylogenetic trees. Therefore, it is necessary to set a limit on the minimum level of sequence relatedness (bit score) to be included in the analysis. The limit can be set at naturally occurring gaps in the E-value score, below which there is a high possibility of chance occurrence of sequence similarities. Selected sequences can then be downloaded and saved in formats (e.g. "FASTA") that facilitate subsequent multiple sequence alignments[123].

Multiple sequence alignments

Molecular sequences that are deposited into public databases are typically byproducts of research projects designed to explore fundamental questions about the relationship between specific genotypes and protein function in broadly defined ecological contexts. Therefore, it is probable that sequence data begins and ends at different locations in different genomes. To compare sequences from different sources and different organisms reliably, it is important to ensure that the sequences are aligned in a realistic manner to avoid spurious correlations that can nullify inferences regarding gene flow and protein function. The quality of sequence alignment is quite likely the most important aspect of constructing reliable phylogenetic trees. The alignment of molecular sequences from different sources is aided by computer algorithms, although the process usually requires user-specified parameters that influence the rigor of the alignment. Several software programs have been developed to assist in the alignment of molecular sequences. The "Clustal" series is one of the most widely used programs

in microbiology, and it is available at http://inn-prot.weizmann.aci.il/software/ClustalX.html. "CodonAlign" is useful for converting protein sequence alignments to DNA alignments[178]. Most programs begin with pairwise comparisons among sequences in the database, followed by the production of a guide tree, which is then used to create multiple alignments. The existence and length of gaps between two sequences are "penalized" to optimize the reliability of alignment scores. Ideally, the outcome of multiple alignments should produce meaningful data regarding the relative biochemical significance of motifs or domains in protein sequences that are conserved across phylogenetic groups. The recognition of these motifs can lead to testable hypotheses about protein function[123].

Constructing phylogenetic trees from aligned sequences

The construction of phylogenetic trees proceeds according to one of two basic approaches, namely "tree searching" and "algorithms". Popular tree-searching methods, including maximum parsimony, maximum likelihood, and Bayesian analysis, generate several preliminary trees from an aligned sequence database, and then use certain criteria to select the best tree. Algorithmic methods, including neighbor joining and the less common unweighted pair-group method with arithmetic mean (UPGMA), use an algorithm to construct a single tree from the database. The algorithmic methods are also referred to as "distance methods" because they are based on matrices generated from computed pairwise differences or "distances" between aligned sequences. Several software programs are available to support the construction of phylogenetic trees, using one or more of the methods described in the paragraph above. Some programs (e.g. ClustalX) designed primarily for producing sequence alignments can also produce phylogenetic trees, although it is generally preferred to import aligned sequences for use by programs designed explicitly for phylogenetic tree construction because they tend to offer more user-specified options for guiding the format of the tree. Among the most commonly used programs are PAUP*[183]; MrBayes[184]; PUZZLE, which uses the maximum-likelihood technique[185]; and Phylogeny Inference Package (PHYLIP)[186]. For a thorough discussion of advantages and disadvantages of these methods and tutorials on their uses, consult Hall[178].

Interpreting phylogenetic trees

The ideal outcome of the phylogenetic tree construction process is the revelation of branching order and distance from a common ancestor(s) that describes the evolutionary pathway of sequences represented in the database. The branch order is characterized by branches from internal nodes representing the common ancestor of two or more external nodes, also known as clades or phyletic groups. The distance is estimated from the number of molecular substitutions that have occurred along a branch. The reliability of inferences regarding evolutionary distance on the basis of molecular substitutions can be compromised by genetic exchange events that cut across clades. The interpretation of molecular clocks based on sequence divergence has been debated extensively in the literature[129, 176, 177, 187-193]. It is not always possible to identify the single common ancestor for all the taxonomic groups represented in a phylogenetic tree. This implies that the "direction" of evolution is difficult to obtain from phylogenetic analysis based on a single gene or protein. There is usually not sufficient information to "root" a phylogenetic tree on the basis of available sequences. However, trees can be rooted if there is independent and complementary evidence from other sources about the evolutionary relationship among the clades under investigation. For example, if other biochemical evidence or the fossil record points to a particular organism as evolutionarily "older" than the other organisms in the database, then it is reasonable to select the oldest organism as the "outgroup", which is used as the root of the tree, but this strategy must be justified rigorously. It is not easy to achieve this justification unequivocally for microorganisms because of the scant fossil record and the possibility of lateral gene flow. The possible number of rooted trees increases considerably as the number of taxonomic groups being compared increases according to the following equation:

$$T_r = (2t-3)!\div 2^{t-2}(t-2)!$$

where T_r is the number of possible rooted tree and t is the number of taxonomic groups[178].

According to this equation, approximately 34.5 million rooted trees are possible for a phylogenetic analysis based on sequences from 10 taxonomic groups. The difficulty of rooting phylogenetic trees is compounded by the questionable reliability of groupings or

the assignment of specific sequences to a given clade, as they are presented in the tree. Some investigators are interested primarily in the reliability of the branching order, whereas others are more interested in group associations. The reliability of phylogenetic trees is typically estimated by a randomized sampling technique known as "bootstrapping", where the frequency of occurrence of a given clade with the same component sequences occurring over 100 to 1,000 bootstrap trials is determined. Bootstrap values reflect the level of confidence attributable to branches of the phylogenetic tree, and high values (e.g. >95%) inspire confidence; whereas low values (e.g. <30%) inspire little confidence in the phylogenetic groupings. It is not unusual to discover that bootstrap values are uneven across phylogenetic branches represented in a tree. Branches with unusually low bootstrap values may reflect considerable genetic exchange events within those branches. The occurrence of horizontal genetic exchange events has made it difficult to construct "universal" trees for prokaryotes.

In a little more than two decades, molecular approaches for exploring microbial diversity have developed rapidly, emerging from rudimentary comparisons of gross DNA homologies that were inferred from hybridization signal intensities, to the level of computer-assisted processing of molecular sequences to generate fine-scale phylogenetic trees. The rapid accumulation of molecular sequence data engendered the discipline of bioinformatics, and innovative tools developed in that discipline have become indispensable for rapid analysis of microbial diversity. The reliability of phylogenetic trees depends first on the integrity of the sequence data used to generate the tree. Molecular sequencing is now mostly automated, but there remains a certain level of uncertainty, and caution must be exercised in using sequences from sources other than the investigator's own laboratory. There is no single "best" technique for producing phylogenetic trees. It is to be expected that the proliferation of techniques will continue, but in all likelihood, some convergence will occur to minimize the sources of error in each technique. Finally, the interpretation of phylogenetic trees and their use in generating testable hypotheses is where most of the intellectual effort remains for students of microbial diversity. Microorganisms do not generally leave fossils, and genetic exchange is rampant. Therefore, the reconstruction of microbial phylogenetic relationships based on molecular sequence information can be challenging, however, the strategy provides a rich opportunity for exploring the relationship between genotype and phenotype across groups of organisms with fascinating natural histories shaped by ecological adaptation and speciation over geological time frames[123].

CHAPTER III

MATERIALS AND METHODS

1. Samples collection

Sediment samples were collected from Tao Dam hot spring, where locates in a remote region of Wangchao national park, Kampangpetch province of Thailand. At the time of sampling, the surface temperature of the spring was ranged from 68-75°C with a pH of pH 8.0. The spring was discovered only recently and contains mineral (sulfide-rich) water. Sediments were collected at a depth of 10 cm (68°C), 30 cm (72°C) and 60 cm (75°C). Samples were pooled and transported on ice to laboratory for subsequent DNA extraction and a proportion placed at -80°C for long-term storage.

2. Isolation of bacterial strains and cultivation

Sediment samples were diluted and spread on solid medium. Tenfold serial dilution of sediment was carried out with phosphate buffer saline pH 8. The dilutions (100 μl) were spread on R2A agar (Difco, USA). Plates were incubated for overnight at 55°C. Colonies with interesting characters were selected and subcultured on R2A medium as previous described. The isolated strains were transferred to 30% of glycerol and stored at -80 °C.

3. DNA extraction and standard PCR amplification

Environmental DNA was extracted from 10g of sediment using DNA Clean Mega Soil Kit (MoBio, USA) using bead-beating method that according to the manufacturer's instructions. For the 16S rDNA libraries, mcrA gene library and SSCP, The specific primers and annealing temperature were used according to Table 2. Each 50 μl of PCR reaction contained 10 ng of template DNA, 10 pmol of each primer, 80 μM dNTP, 1×*Taq* buffer B (EuRx, Poland) and 1.25 U *Taq* DNA polymerase (EuRx, Poland). Cycling parameters consisted of an initial denaturation of 94°C for 3 min, followed by denaturation 30 cycles of 94°C for 30 sec, annealing for 30 sec, extension 72°C for 90 sec, and a final extension step of 72°C for 3 min. PCR products were purified using MinElute (QIAGEN, Germany) using spin column according to the manufacturer's instructions.

Table 2 PCR primers for microbial diversity study

Primers	Sequence 5'⟶3'	Target group	Annealing (°C)	Amplicon (bp)	Reference
16S rRNA libraries					
23f	TCYGGTTGATCCTGCC	archaea	55	893	(194)
ARC915	GTGCTCCCCCGCCAATTCCT				(195)
27f	AGAGTTTGATCMTGGCTCAG	bacteria	52	1,466	(196)
1492r	TACGGYTACCTTGTTACGACTT				
mcrA gene library					
Me1f	GCMATGCARATHGGWATGTC	methanogens	50	850	(197)
Me2r	TCATKGCRTAGTTDGGRTAGT				
SSCP					
ARC333F-P	TCCAGG CCCTACGGG G	archaea	55	529	(195)
ARC915F	GTGCTCCCCCGCCAATTCCT				
Com1-P	CAGCAGCCGCGGTAATAC	bacteria	55	380	(198)
Com2	CCGTCAATTCCTTTGAGTTT				
M894F-P	CTTAAAGGAATTGGCGGGGGA	methanogens	60	483	This study
M1376R	GGGCGGTGTGTGCAAGGAGCA				
Q-PCR					
364F	CGGGGYGCASCAGGCGCGAA	archaea	60	570	(199)
943R	GTGCTCCCCCGCCAATTCCT				
519F	CAGCMGCCGCGGTAANWC	bacteria	48	385	(196)
907R	CCGTCAATTCMTTTRAGTTT				
MSL812F	GTAAACGATRYTCGCTAGGT	methanogens	50	348	(200)
MSL1159R	GGTCCCCACAGWGTACC				
Sequencing					
M13F	GTTTTCCCAGTCACGAC	vary	60	vary	Fermentas
M13R	CAGGAAACAGCTATGAC				

4. Quantitative real time PCR (Q-PCR)

Q-PCR reaction mixture (25 μl) contained: 12.5 μl SYBR Green Ready Mix (Bio-Rad, USA), 4.0 μl $MgCl_2$ (25 mM), 0.25 μl of 50 μM of each primer (Table 2), 0.25 μl of (20 μg/ μl) BSA (Bovin Serum Albumin), 0.25 μl FITC (fluorescein isothiocyanate) (1/1000), and 2 ng of DNA template. Cycling conditions consisted of an initial denaturation at 94°C for 3 min, followed by 45 cycles of denaturation at 94°C for 30 sec, annealing for 1 min, and extension at 72°C for 1.5 min. This was followed by a final extension step at 72°C for 3 min. PCR was carried out in iCycler thermal cycler (Bio-Rad, USA). Q-PCR assayed with C_T values over 40 were considered negative. For each PCR run, a negative (no-template) control was used to test for false-positive

results or contamination. The presence of nonspecific products or primer dimers was confirmed by observation of a single melting peak in a melting curve analysis.

4.1 Q-PCR standard curves

The strain *Methanosarcina barkeri* DSM 800 was used as a template to generate the standard curves for the quantification of archaea and methanogens, and *Anoxybacillus flavithermus* DSM 2641 for bacteria. The experiments were carried out in triplicate for each strain. Appropriate dilutions of the samples were sometimes necessary prior to the amplification in order to fit the C_T value in the linear range of each standard curve. For each strain, different 16S rRNA gene copy numbers in a range of 10^0-10^7 orders were amplified by Q-PCR after serial dilutions.

5. Single strand conformation polymorphism

5.1 Preparation of SSCP gel

The cooling plate was cleaned with 70% ethanol and 1 ml of repel saline. The gel plate was first cleaned with 70% ethanol, and subsequently with a mixture of 2.5 ml absolute ethanol, 25 μl of bind saline and 25 μl of 100% acetic acid. Both plates were allowed to dry for 10 min at the room temperature. The cooling and gel plate were assembled to be a block for the polyacrylamide (PA) gel. SSCP analysis was performed as it was previously described (Schwieger, 1998). PA gel contained 12 ml of H_2O, 6 ml of MDE® gel solution (Lonza, Germany), 2 ml of 10× TBE buffer, 80 μl of $(NH_4)_2S_2O_8$ and 8 μl of TEMED (Bio-Rad, Germany). The mixture was load on the gel block as previous described and incubated at room temperature for 3 h until the PA gel was completely polymerized.

5.2 Preparation of single-stranded DNA (ssDNA)

The reaction solution contained 1× lambda exonuclease buffer (NEB, England), 5 units of Lambda exonuclease (NEB, England) and 1 μg of purified PCR product. The solution was incubated at 37°C for 2 h. The ssDNA was purified using MinElute (QIAGEN, Germany) according to the manufacturer's instructions. The purified ssDNA was dried in a vacuum (SpeedVac, Germany) for 30 min. The ssDNA pellet was resuspended in 2 μl of deionized water and 2 μl of the SSCP denaturating buffer (95% Formamide; 10 mM NaOH; 0.05% Xylene cyanol; 0.05% bromophenol blue). The mixture was incubated for 2 min at 95°C and chilled on ice.

5.3 Running SSCP gel

The electrophoresis was performed in a Pharmacia Multiphor II apparatus (LKB Bromma, Sweden). One litre of 1×TBE buffer was loaded into upper and lower part of SSCP chamber. The 4 μl of the mixture from step 5.2 was loaded into the PA gel from step 5.1. The electrophoresis was performed at 400 V for 16 hours at 20°C.

5.4 Gel fixation

After 16 h of the electrophoresis, the PA gel was fixed in fixation solution. The solution contained 50 ml of acetic acid and 450 ml of water. The PA gel was incubated on shaking plate for 30 min at room temperature. The fixed-PA gel was washed using 500 ml of water for 5 min. The washing step was repeated for 3 times.

5.4 Gel staining

The fixed-PA gel was stained using silver nitrate solution. The solution contained 0.5 g of silver nitrate, 500 μl of formaldehyde and 500 ml of water. The gel was stained for 30 min with shaking.

5.5 Gel developing

The stained-PA gel was washed with 500 ml of water for 20 sec. The gel was developed in 500 ml of developing solution. The solution contained 12.5 g of sodium carbonate, 500 μl of formaldehyde, 500 μl of (2%w/v) $Na_2O_3S_2$ and 500 ml of water. The gel was developed for 1-3 min with shaking.

5.6 Stopping the developing reaction

The developing solution was discarded and the stopping solution was added to stop the reaction. The developing reaction was stop using stopping solution, which contained 10 g of glycine, 2.5 g of EDTA and 490 ml of water. The reaction was remained on shaker for 10 min.

5.7 Gel preservation

The stopping solution from step 5.6 was removed. 500 ml of preservation solution and cellophane paper was added. The solution contained 50 ml of glycerol and 450 ml of water. The reaction was remained on shaker for 10 min.

6. Cloning of PCR products

PCR products (100 ng) were ligated in pGEM-T vector system II (Promega, Germany) and transformed into *Escherichia coli* JM109 competent cells according to the manufacturer's instructions. Transformants were spread on LB agar with Ampicilin/IPTG/X-Gal. Plates were incubated overnight at 37°C. Positive clones were

selected and cultured overnight at 37°C in LB broth with Ampicilin. Recombinant plasmid was extracted using Montage Plasmid Miniprep$_{96}$ Kit (Millipore, Germany) according to the manufacturer's instructions.

7. Sequence analysis

Recombinant plasmid was sequenced using M13 primers (Table 2) with Big Dye reagent kit (ABI, Germany). Sequencing was performed on ABI Genetic Analyzer (AB3130XL) (ABI, Germany). To confirm the identity of nucleotides, each clone was sequenced twice. Chimeric artifacts were removed using Bellerophon[201] and Mallard[202]. Non-chimeric sequences were subsequently compared to the sequences deposited to Genbank using BLAST[203].

8. Computation analysis for the 16S rRNA and *mcr*A library

Sequences were aligned using MUSCLE 3.7[204] with closely related strains and environmental clones. Evolutionary models for the data sets were calculated using Modeltest 3.8[205]. Bayesian inference (BI) was carried out with MrBayes 3.1.2[184]. Bayesian posterior probabilities (BPP) were obtained by performing two separate runs with four Markov chains (MC). Each run was conducted with 4×10^6 generations and sampled every 100 generations. Convergence was checked by examining the generation plot visualized with TRACER 1.4[206] and computing the potential scale reduction factor (PSRF) with the sump command in MrBayes 3.1.2. A consensus tree was calculated after discarding the first 25% of the iterations as burn-in.

Rarefaction analysis and richness estimation were performed using DOTUR program[207]. Jukes-Cantor evolutionary distances were calculated using DNADIST of Phylip 3.68 package[186].

9. Screening of commercialize enzymes from the isolates

For the functional screening, isolates were grown in M9 broth containing per liter: 1 g Ammonium chloride, 6 g di-sodium hydrogen phosphate, 3 g potassium dihydrogen phosphate, 0.5 g sodium chloride, for the final pH of 7.4 ± 0.2 at 37°C. A total of 100 ml of the medium were distributed to 500 ml flask. To each flask, one of the following substrates was added: agarose, cellulose and pectate and was incubated at 55°C, 150 rpm overnight.

The gene screening of the isolates was carried out by amplifying specific genes with the given primer sets (Table 3). To design the primers, sequences of the isolates were compared against those from Genebank and the closest relative selected. The specificity of the primers for annealing was determined using temperature gradient PCR in an Eppendorf 5341 thermocycler (Eppendorf, Germany).

Table 3 PCR primers for enzymatic genes screening

No.	Target genes	Code	Sequence (5'⟶3')	Annealing (°C)	Amplicon (bp)
1	Aminoacylase	AacyF	TGTAAGCCGAACGGAGATTG	55	907
		AacyR	AAACGTGGCGCACAGGTATT		
2	D-Arabinose isomerase	GaraiF	TGCTGGTGCTATGGAACT	50	1,360
		GaraiR	GCGTGATTAAATCGGCTC		
3	Laccase	AlaccF	ATACGTTCGCTCGGAACACC	54	651
		AlaccR	GTTTGATGGATGGGAAGACG		
4	Lipase	AlipF	CACCACGAAATCCAAATC	50	963
		AlipR	AAGCATCAGACGGAAAGG		
5	Nitrilase	GnitF	TTTTATTCCCGCTTATCCTA	50	474
		GnitR	GTTGACTGCCAAGTTTCCCT		
6	Protease	AznpF	AAGTGGAGGAAACGGCAATA	57	454
		AznpR	AGCATATCCGCTTCGAGAAA		
7	Pectate lyase	BpelF	ACTCGGGCAAAGTAAATC	50	426
		BpelR	CGTTCAGGCTGTGGTAAA		
8	Superoxide dismutase	AsodF	CATTCGGAGCCATCAGTTGT	55	333
		AsodR	CAGGTGGATGTATGGAAGAAAA		
9	L-Rhamnose isomerase	GrhiF	GCTGAAGAATAAGGCAG	50	933
		GrhiR	ATTGCACCGAAAGGATAAGT		
10	Xylanase	AxylF	AATCAAGTCCCTTCCTCC	50	1,027
		AxylR	TTTGTCGCTCGTTCTACC		

10. Pectate lyase assay

1% of AZ Rhamnogalacturonan (Megazyme, Ireland) was added to R2A agar. Plates were incubated overnight at 55°C. Clear halo zone would appear when pectate lyase was produced. Positive clones were inoculated in 100 ml of M9 medium (pH 8) plus sodium pectate (Sigma, Germany). Flask was incubated at 55°C and shaking 150 rpm overnight. Bacterial cell was removed by centrifugation at 8,000 rpm for 10 min. Supernatant was collected for photometry assay. 0.5 ml of supernatant was added to 2.5 ml of substrate (60 mM Tris-HCl pH 8.0, 0.6 mM $CaCl_2$, 0.24% (w/v) sodium pectate). The reaction was incubated at 55°C for 2 hours. Pectate lyase activity was

assay using spectrophotometer at 232 nm of wavelength. Pectate lyase from *Aspergillus* sp. (Megazyme, Ireland) was used as a positive control. Sterile water and substrate were used as negative control.

11. Determination of bacterial morphology

The Gram-staining was performed using a commercial kit (Sigma, Germany) according to manufacturer's instructions. Bacterial morphology was characterized by Dr. Manfred Rohde, department of medical microbiology, Helmholtz centre for infection research, Braunschweig, DE-38124, Germany. Morphology was examined after cultivation of the strains at 55°C in R2A medium overnight under Zeiss field emission scanning electron microscope Gemini DSM852 (Zeiss, Oberkochen, Germany) and Zeiss transmission electron microscope TEM910 (ProScan, 1024×1024, Scheuring, Germany). Preparation for field emission scanning electron microscopy (FESEM), samples were fixed with a solution containing 2% glutaraldehyde and 5% formaldehyde in cacodylate buffer (0.1 M cacodylate, 0.09 M sucrose, 0.01 M $MgCl_2$, 0.01 M $CaCl_2$, pH 6.9) for 1 h on ice and washed with TE buffer (20 mM TRIS, 2 mM EDTA, pH 6.9). The fixed bacteria were placed onto poly-L-lysine coated cover slips, left for 5 min, washed once in TE buffer and fixed with 2% glutaraldehyde. After washing with TE buffer samples were dehydrated with a graded series of acetone (10, 30, 50, 70, 90, 100%); each step for 10 min on ice, the last 100% acetone step was performed at room temperature. Samples were then critical-point-dried with liquid CO_2 (CPD 030, Bal-Tec, Liechtenstein) mounted onto aluminium stubs and sputter coated with gold (SCD 500, Bal-Tec, Liechtenstein) before examination in a Zeiss field emission scanning electron microscope Gemini DSM852 (Zeiss, Oberkochen, Germany) at an acceleration voltage of 5 kV using the Everhart-Thornley SE-detector and the inlens SE-detector in a 50:50 ratio. Images are stored onto a 230 MB MO-disk. Embedding of bacteria for transmission electron microscopy, samples were fixed in a fixation solution containing 5% formaldehyde and 2% glutaraldehyde in cacodylate buffer (0.1M cacodylate, 0.01 M $CaCl_2$, 0.01 M $MgCl_2$, 0.09 M sucrose, pH 6.9) and washed with cacodylate buffer. Samples were then osmificated with 1% aqueous osmium for 1 h at room temperature, washed and pellets of the samples were embedded in 2% water agar and cut into small cubes. Dehydration was achieved with a graded series of acetone (10%, 20%, 50%) for 30 min on ice followed by contrasting with 2% uranyl acetate in 70% acetone for overnight at 4°C and further dehydrated with 90% and 100% acetone. Samples in the

100% acetone step were allowed to reach room temperature and were infiltrated with the epoxy resin according to Spurr's formular for a medium resin[208]; 1 part 100% acetone/1 part resin for overnight, 1 part 100% acetone/2 parts resin for 8 h, pure resin for overnight and several changes the following 2 days. Samples were then transferred to resin filled gelatine capsules and polymerized for 8 h at 75°C. Ultrathin sections were cut with a diamond knife, picked up with formvar-coated copper grids (300 mesh) and counterstained with 2% aqeuous uranyl acetate and lead citrate. After air-drying samples were examined in a Zeiss transmission electron microscope TEM910 at an acceleration voltage of 80 kV. Images were recorded digitally with a Slow-Scan CCD-Camera (ProScan, 1024×1024, Scheuring, Germany) with ITEM-Software (Olympus Soft Imaging Solutions, Germany).

12. Biochemical test

The temperature ranges for growth were examined from 30-65°C on R2A agar plates (Difco, USA). Phenotypic of TD5 was investigated using API 20E and API 50CHB (bioMérieux, Nuertingen, Germany) at 55°C, according to the manufacturer's instructions.

13. Chemotaxonomy

Analysis of cellular fatty acid was performed at DSMZ (Braunschweig, Germany) according to the Sherlock Microbial Identification System manual (version 4.5; Microbial ID, Newark, DE). The profiles of cellular fatty acid were compared to TSBA40 library version 4.10 (Microbial ID, Newark, DE).

14. Determination of base composition

Genomic G+C ratio was examined using HPLC by DSMZ (Braunschweig, Germany). For the DNA base compositions, the bacterial cells were disruption with French pressure cell. The DNA was purified on hydroxyapatit according to the procedure of Cashion[209]. The DNA was hydrolyzed with P1 nuclease and the nucleotides dephosphorylized with bovine alkaline phosphatase[210]. The resulting deoxyribonucleotides were analyzed by HPLC (Shimadzu, Japan). The HPLC chromatography was performed at 45°C. The 10 µl of samples were injected to the reversed-phase column (Varian, Germany). The mobile phase consisted of 40:1 (v/v) of 0.3 M $(NH_4)H_2PO_4$ and acetonitrile. The flow rate was 1.3 ml/min (adapted from

Tamaoka and Komagata[211]). The G+C content was calculated from the ratio of deoxyguanosine (dG) and thymidine (dT) according to the method of Meshbah[210].

15. DNA-DNA hybridization

The DNA-DNA hybridization was examined between strain TD5 and closest phylotype (*A. gonensis* NCCB 100040^T). The procedure was carried out as described by De Ley[212] under consideration of the modifications described by Huss[213] using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6×6 multicell changer and a temperature condition controller with in-situ temperature probe (Varian, Germany).

16. Phylogenetic analysis for bacterial systematics

DNA extraction was performed using MasterPure™ Gram Positive DNA Purification Kit (Biozym Scientific, Germany) according to the manufacturer's instructions. Primers 27f and 1492r (Table 2) were used for bacterial 16S rDNA amplification. Total volume (50 μl) of PCR reaction contained 10 ng of DNA, 20 pmol of each primer, 80 μM dNTP, 1×*Taq* reaction buffer and 1.25 U *Taq* DNA polymerase (EuRx, Poland). PCR started by heating reactions to 94°C for 2 minutes, followed by 30 cycles of 30 second at 94°C, 30 second at 52°C, and 1 minute at 72°C, ending with a final step at 72°C for 3 minutes. PCR product was purified using MinElute PCR Purification Kit (QIAGEN, Germany). DNA sequencing was carried out directly on purified PCR product. Sequencing primers were 27f and 1492r. Big Dye v. 1.1 reagents (Applied Biosystem) was used for the sequencing. Analysis was performed on an ABI Genetic Analyzer sequencer (AB3130XL).

Sequences of 16S rRNA were identified using EzTaxon[214]. The 16S rRNA gene sequences of the subject strain and other closest relative strains were aligned using MAFFT 6 (Q-INS-I strategy)[215]. The pairwise similarity of 16S rRNA gene was analyzed using MatGat 2.02 (global alignment algorithm)[216]. The best-fit evolutionary model of the data set was calculated using Modeltest 3.8[205]. The bayesian inference (BI) was carried out with MrBayes 3.1[184]. The Bayesian posterior probabilities (BPP) were obtained by performing two separate runs with four Markov chains (MC). Each run of MC was conducted with 10×10^6 generations and sampled every 100 generations. The convergence of results was checked by examining the generation plot, which visualized by TRACER 1.4[206]. The potential scale reduction factor (PSRF) was computed using

the sump command in MrBayes 3.1. A consensus tree was calculated after discarding the first 10% of the iterations as burn-in.

CHAPTER IV

RESULTS

1. Microbial diversity

1.1 SSCP analysis

The 16S rRNA genes were amplified using universal primers for bacteria, archaea and methanogens. The SSCP profile exhibited resolve bands in Figure 3.

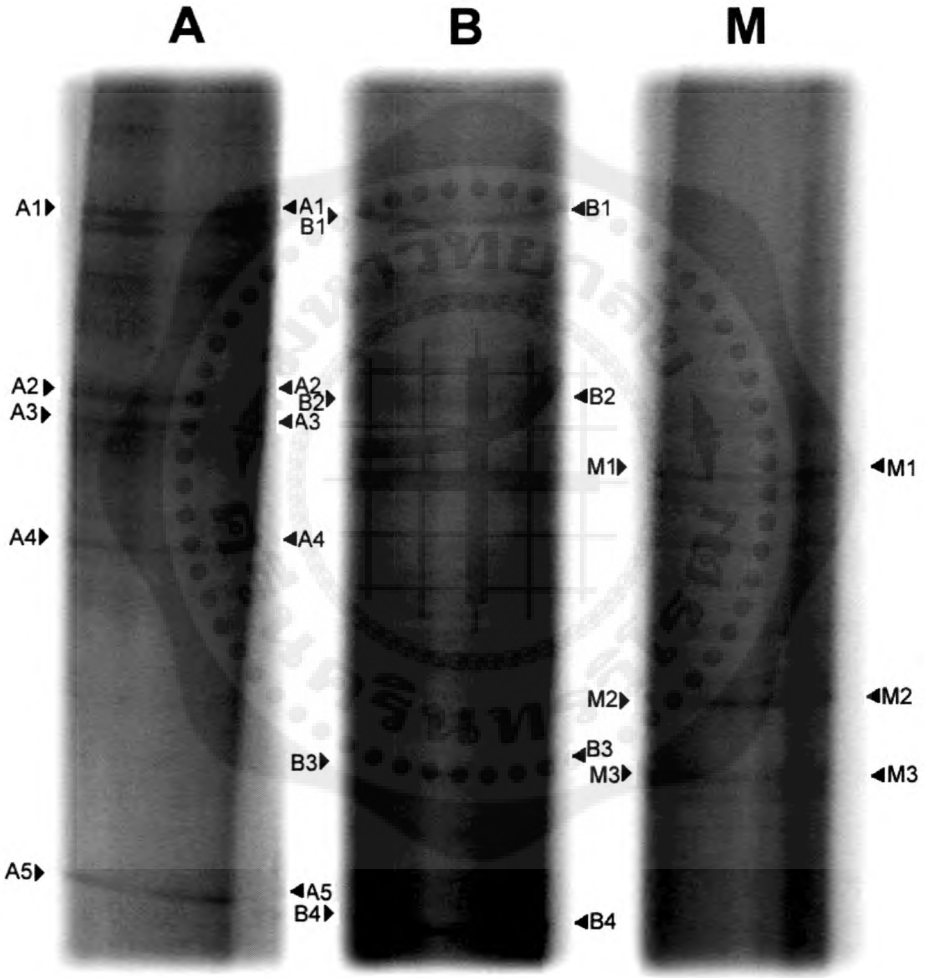


Figure 3 SSCP profiles of 16S rRNA gene from Tao Dam hot spring sediment. A) archaea, B) bacteria and M) methanogens.

The lane A represented the archaeal SSCP profile. The bands A1, A2, A3 and A5 were closely related to uncultured crenarchaeaote. The band A4 was only related to uncultured archaeon. The lane B represented the bacterial SSCP profile. The band B1

was identified as uncultured *Chloroflexi*, B2 as *Diatom* sp., B3 as *Geobacter argillaceus* and B4 as *Geobacter pelophilus*. The lane M represented the methanogens SSCP profile. The band M1 was identified as uncultured *Methanosarcinaceae* archaeon, M2 as uncultured *Methanosaeta* sp. and M3 as *Methanobacterium bryantii*.

1.2 Clone libraries and phylogenetic analysis

The positive clones were selected and analysed from two 16S rRNA gene libraries and one *mcrA* library. For the 16S rRNA libraries, 196 clones of archaeal and 198 clones of bacterial library were selected for sequencing as same as 192 clones of *mcrA* library. The sequences from the selected clones were identified using BLAST. The sequences, which contained chimera or low quality, were removed before performed the other computational analysis. The high quality of DNA sequences contained 141 of archaeal, 158 of bacterial and 177 of *mcrA* clones. The sequences were assigned to OTUs using DOTUR program (Table 4).

The archaeal 16S rRNA gene library was assigned to 45 OTUs (Table 4) that represented the phyla *Crenarchaeota* and *Euryarchaeota* (Figure 4 and 5). *Crenarchaeaota* were found to be the most abundant (41.1% of the total archaeal clone library), although they were all related to uncultured crenarchaeote. The phylum Euryarchaeota was strongly dominated by methanogens belonging to the orders *Methanosarcinales* (74.7% of the total methanogens sequences), *Methanomicrobiales* (19.7% of the total methanogens sequences) and *Methanocellales* (5.6% of the total methanogens sequences). Among the archaeal sequences, 5.7% branched with the order Thermoplasmatales and 2.8% of *Euryarchaeota*.

Table 4 Species richness of 16S rRNA gene libraries

Library	OTUs	ACE	BRE	Chao1	Jack	SW
Archaea	45	69.20	53.73	75.00	75.73	3.42
Bacteria	112	448.03	147.01	367.94	369.36	4.47

ACE abundance-based coverage estimator; BRE bootstrap richness estimators; Chao1 Chao richness estimator; Jack Jackknife richness estimator; SW Shannon weaver index of diversity

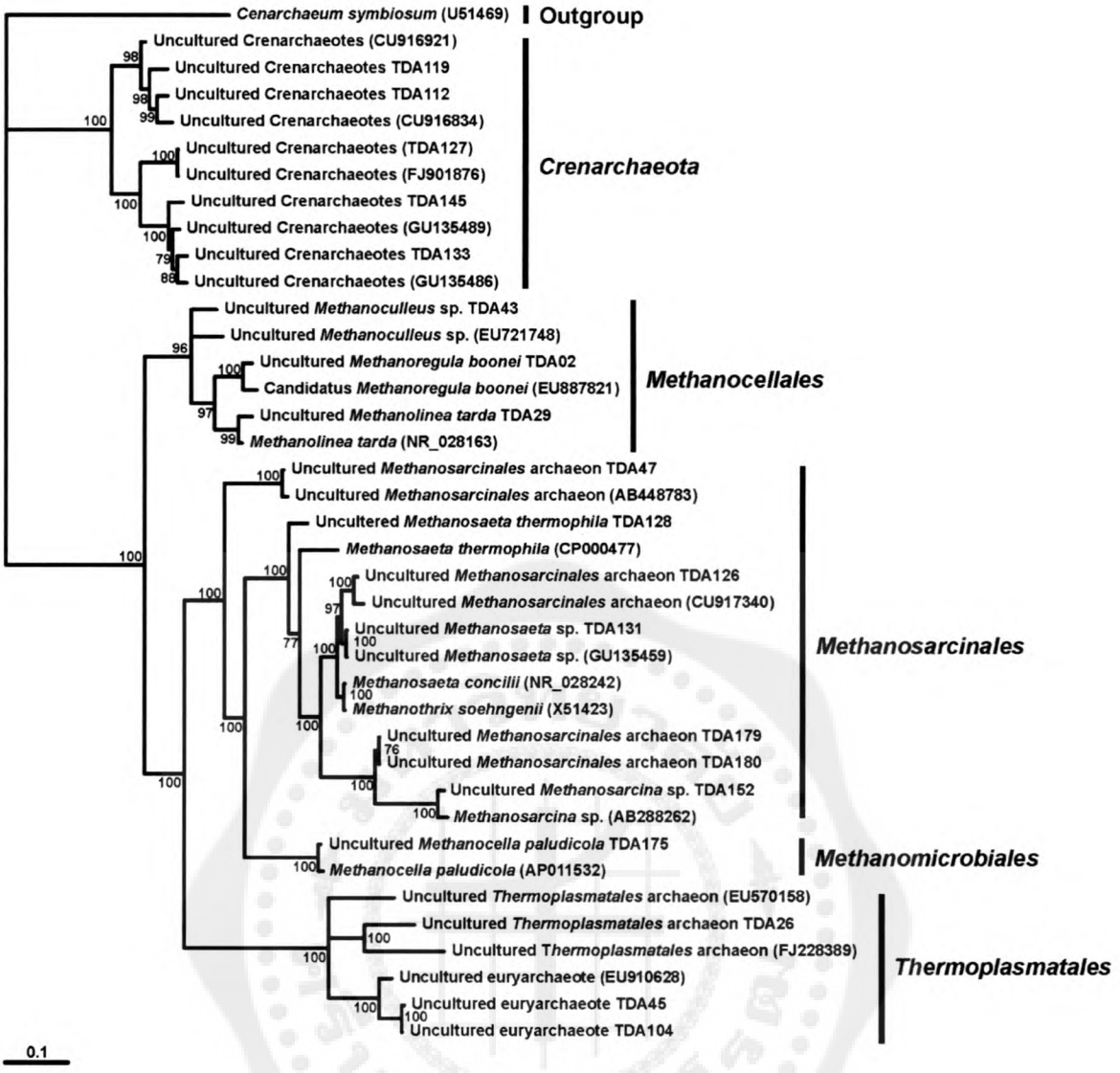


Figure 4 Phylogenetic tree of archaeal 16S rRNA gene from Tao Dam hot spring.

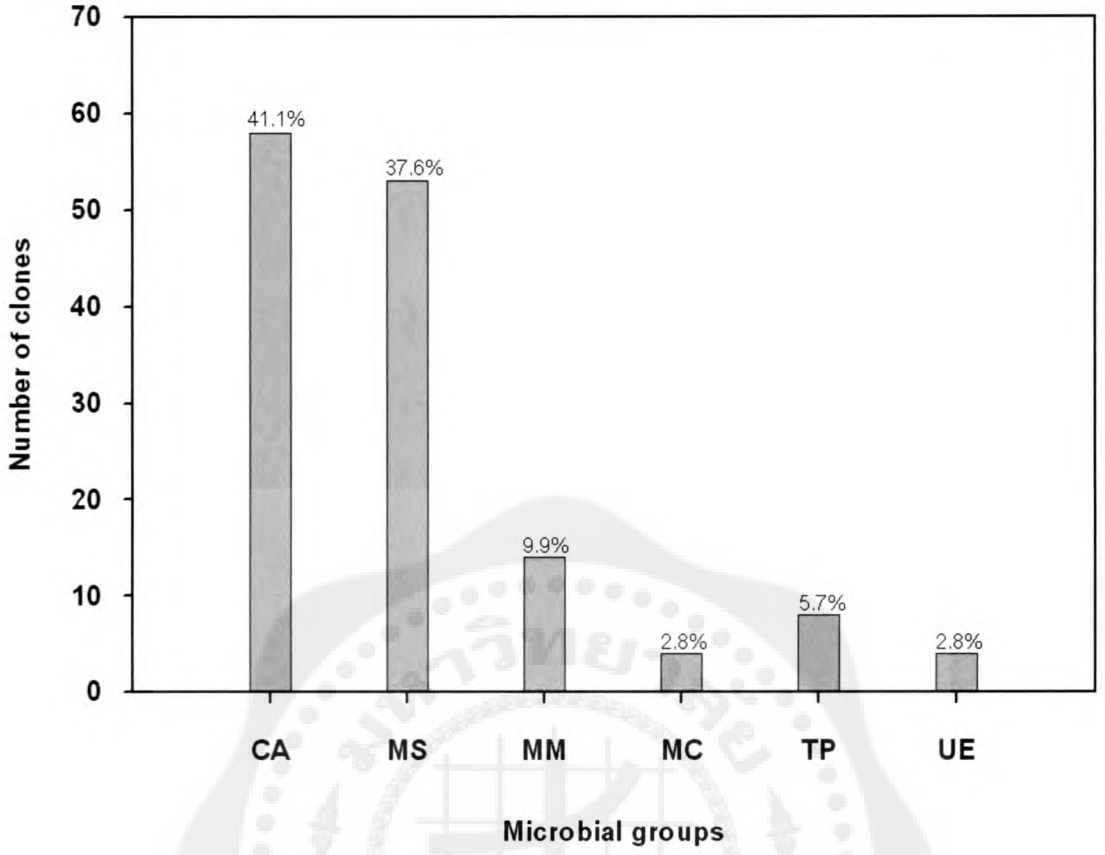


Figure 5 Abundancy of archaeal 16S rRNA gene library: CA: *Crenarchaeota*; MS: *Methanosarcinales;* MM: *Methanomicrobiales;* MC: *Methanocellales*; TP: *Thermoplasmatales;* EU: uncultured *Euryarchaeote*.

In comparison, sequences from the bacterial 16S rRNA gene library were diverse to 112 OTUs (Table 4) and comprised 13 groups representing *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Chlorobi*, *Cyanobacteria*, *Deferribacteres*, *Firmicutes*, *Nitrospirae*, *Planctomycetes*, *Proteobacteria*, *Spirochetes*, *Verrucomicrobia*, and uncultured bacteria (Figure 6 and 7). Proteobacteria represented the main bacterial group with 55.1% of the total bacterial community base on the bacterial clone library. In this group, *Alpha-* and *Beta- Proteobacteria* were dominant, with 36.9% and 26.2% of the total *Proteobacteria* respectively. *Epsilonproteobacteria*, with only 6% of the total *Proteobacteria* community, was the less abundant.

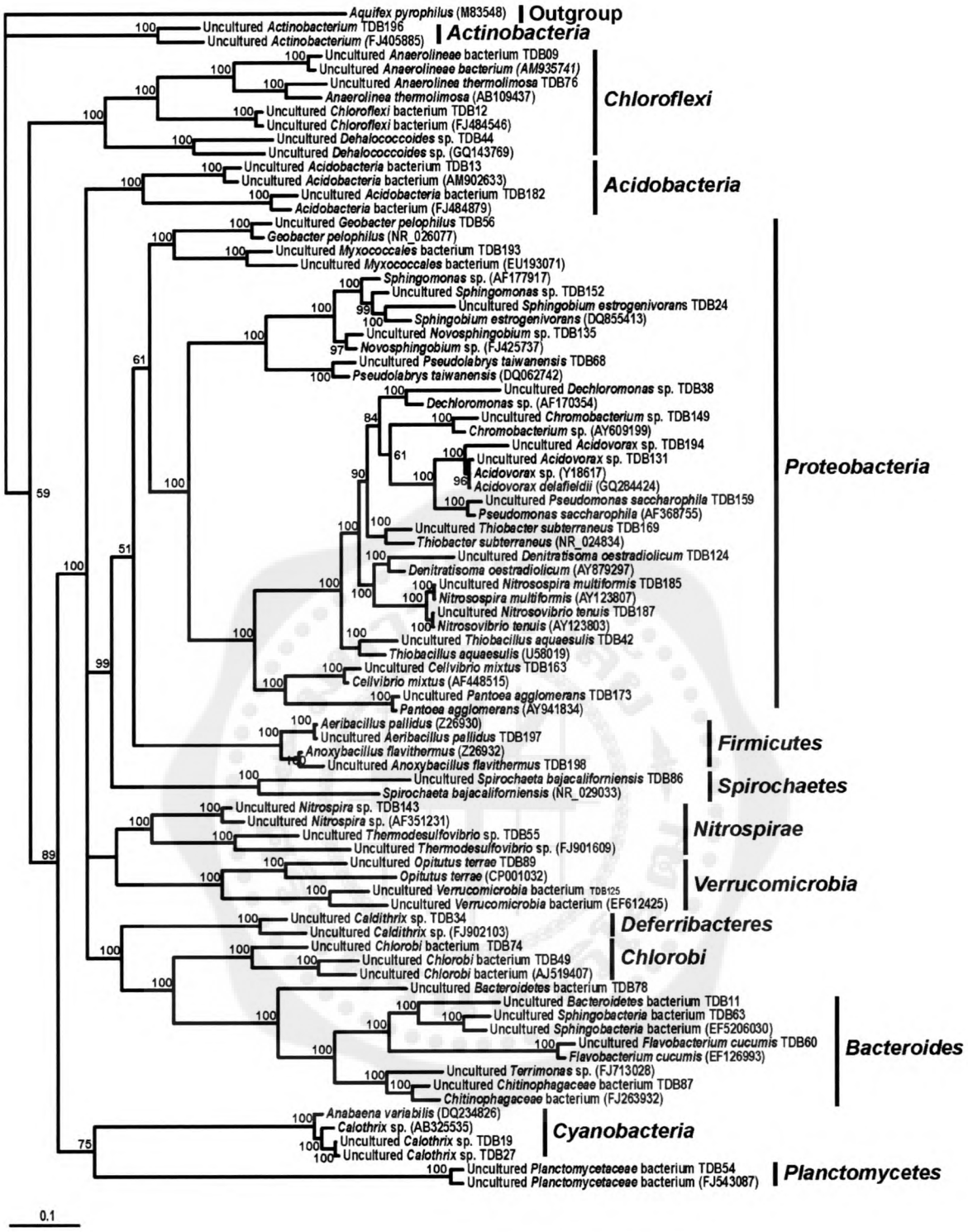


Figure 6 Phylogenetic tree of bacterial 16S rRNA gene from Tao Dam hot spring.

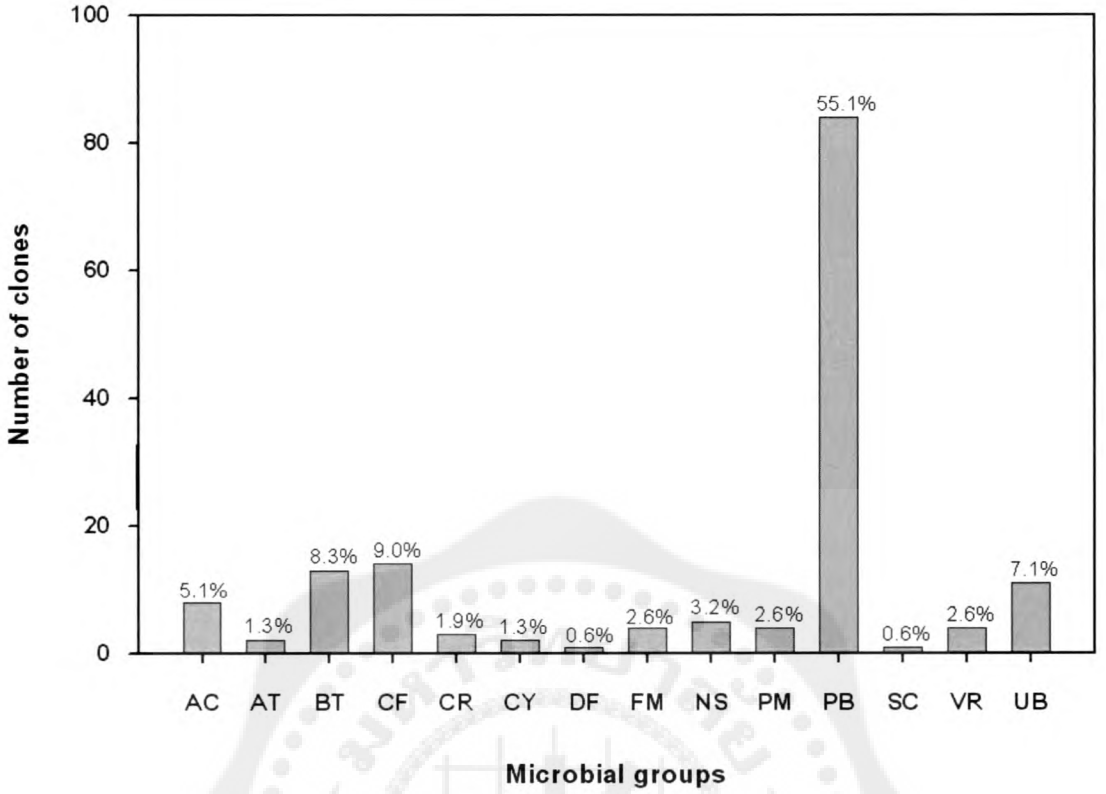


Figure 7 Abundancy of bacterial 16S rRNA gene library: AC: *Acidobacteria;* AT: *Actinobacteria*; BT: *Bacteroidetes;* CF: *Chloroflexi;* CR: *Chlorobi*; CY: *Cyanobacteria*; DF*: Deferribacteres;* FM: *Firmicutes*; NS: *Nitrospirae;* PM: *Planctomycetes;* PB: *Proteobacteria;* SC: *Spirochaetes;* VR: *Verrucomicrobia*; UB: uncultured bacteria.

The *mcrA* library was assigned to 45 OTUs (Table 5) that represented the orders *Methanobacteriales*, *Methanocellales*, *Methanomicrobiales* and *Methanosarcinales* (Figure 8 and 9). The *mcrA* library was dominated by *Methanomicrobiales* (51.41%), *Methanomicrobiales* (37.29%), *Methanosarcinales* (10.17%) and *Methanocellales* (1.13%).

Table 5 Species richness of mcrA gene library

Library	OTUs[a]	ACE	BRE	Chao1	Jack	SW
Methanogen	45	68.88	53.66	59.25	64.00	3.16

ACE abundance-based coverage estimator; BRE bootstrap richness estimators; Chao1 Chao richness estimator; Jack Jackknife richness estimator; SW Shannon weaver index of diversity

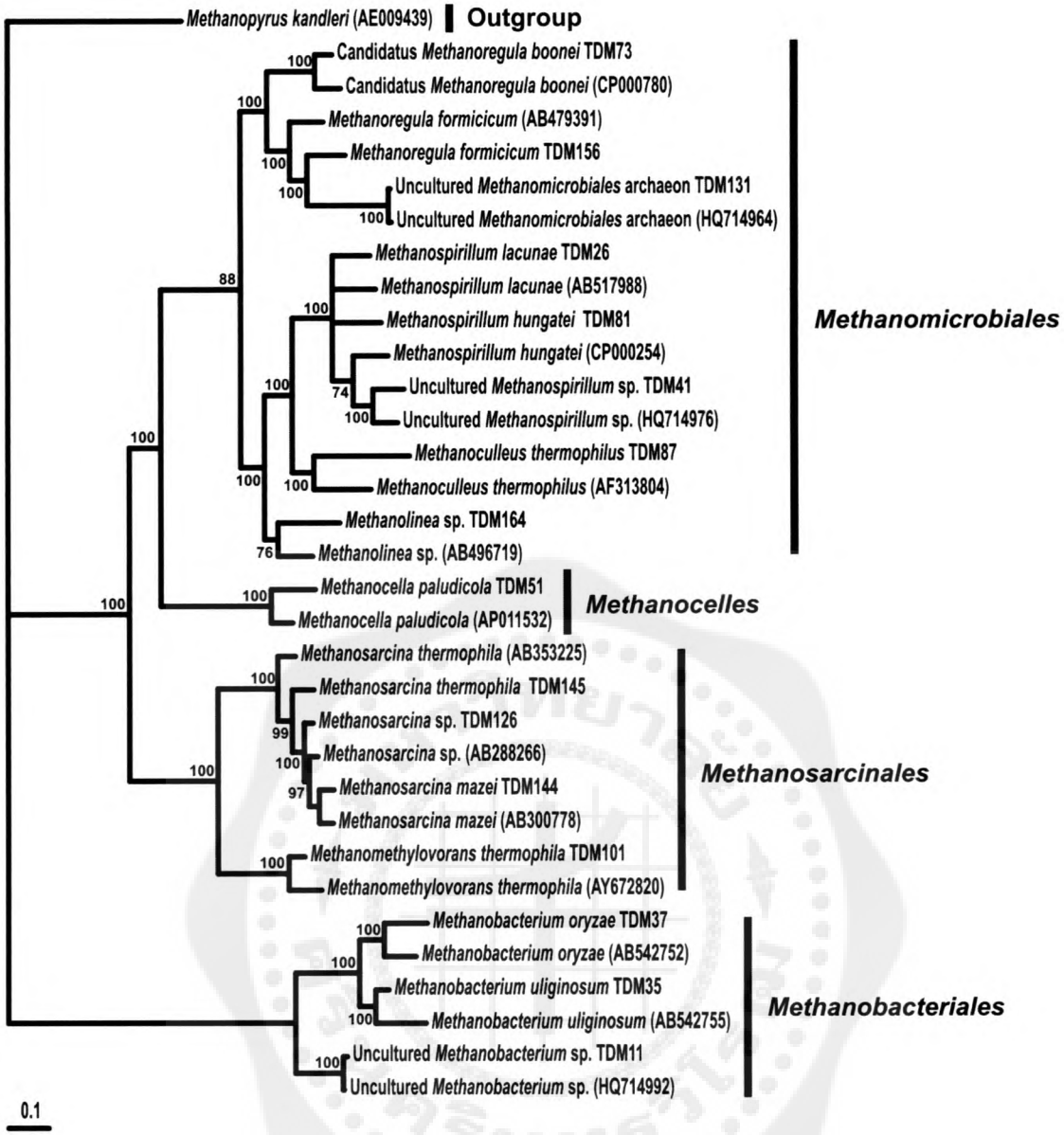


Figure 8 Phylogenetic tree of methanogens mcrA gene from Tao Dam hot spring.

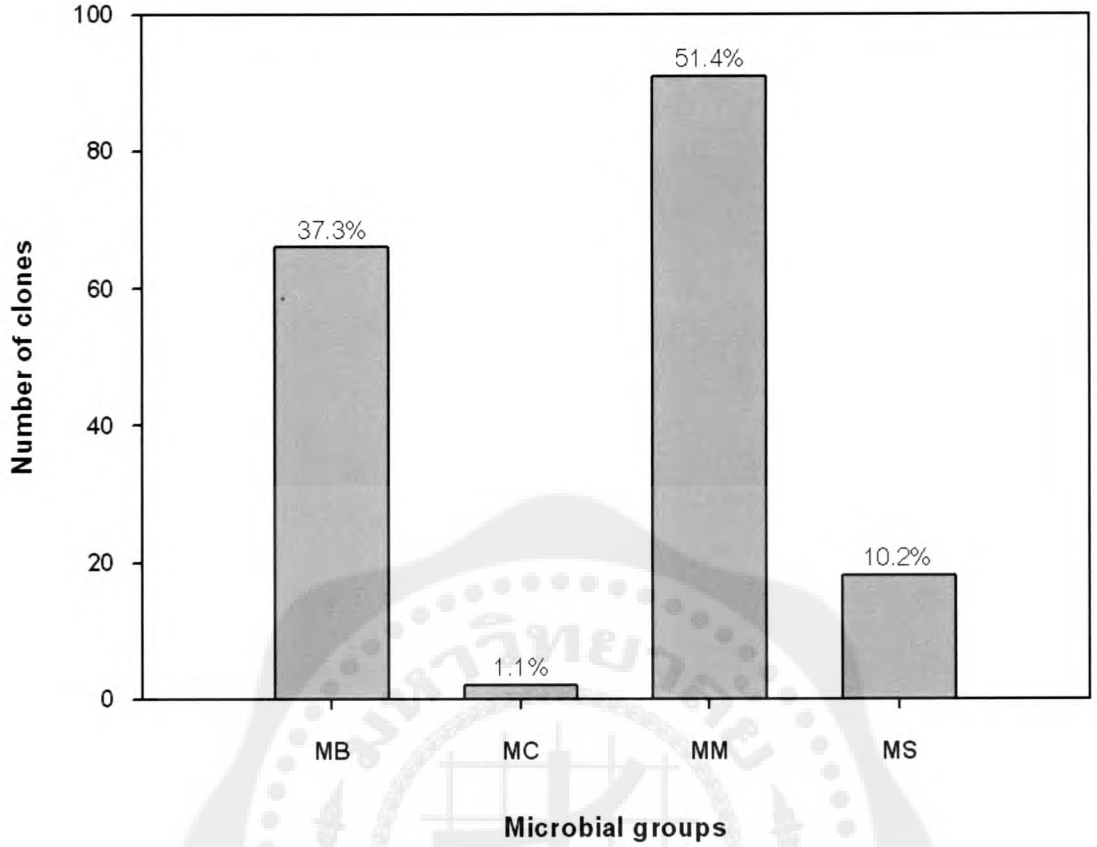


Figure 9 Abundancy of methanogenic archaeal mcrA gene library: MB: *Methanobacteriaes*; MC: *Methanocellales*; MM: *Methanomicrobiales;* MS: *Methanosarcinales*.

1.3 Richness estimation

The diversity indices and the richness estimators were calculated and the rarefaction curve was constructed using DOTUR program. Rarefaction curves, which constructed from 16S rRNA gene sequences of archaeal and bacterial library, had shown in Figure 10. Figure 11 was the the rarefaction curve, which constructed from mcrA gene sequences of the methanogens library.

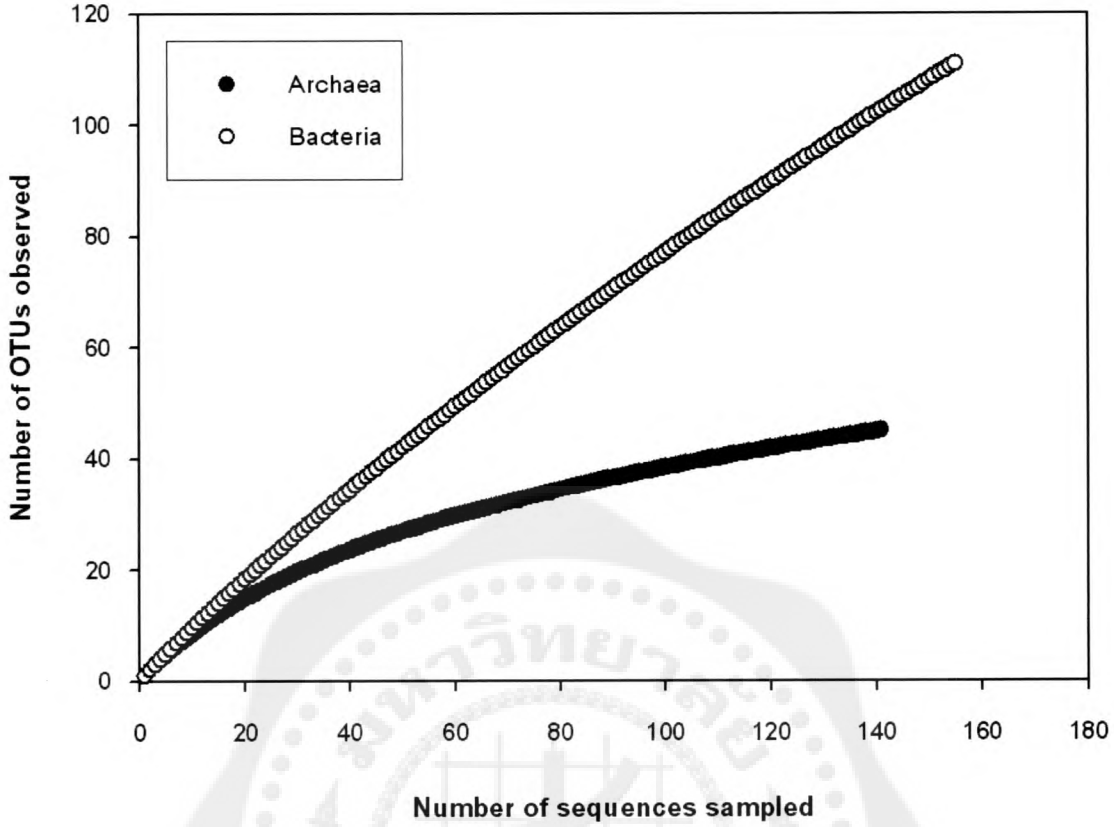


Figure 10 Rarefaction curves analysis of sequences from A) archaeal and B) bacterial 16S rRNA gene library at 0.03% distance level.

Diversity indices and richness estimators are useful to compare the relative complexity of two or more communities and to estimate the completeness of sampling of a community[207]. Once DOTUR assigns sequences to OTUs, it performs a random sampling without replacement procedure. The probability of drawing a representative from an OTU is the number of times the OTU was observed divided by the total number of sequences in the library[207]. Rarefaction curves using an evolutionary distance of 0.03 (equal to three mismatches per 100 bp) were computed using DOTUR (Figure 7). Species richness was estimated using the abundance-based coverage estimator (ACE), bootstrap richness estimators (BRE), Chao1 richness estimator (Chao1), Jackknife richness estimator (Jack) and Shannon weaver index of diversity (SW) (Table 3). This analysis indicated that the diversity of the bacterial clone library was higher than that of the archaeal clone library.

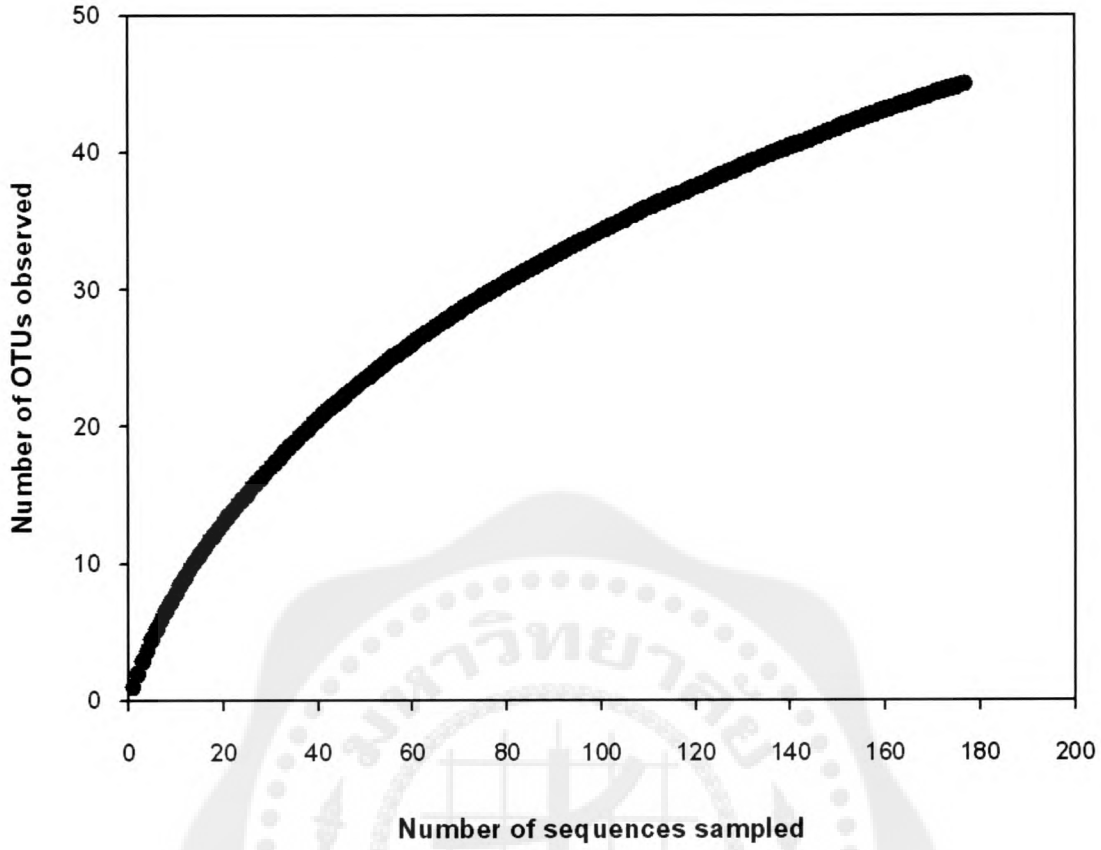


Figure 11 Rarefaction curve analysis of mcrA gene library at 0.03% distance level.

1.4 Quantitative PCR

To select the linear standard curves, r^2 of the slope was chosen for the value at least equal to 0.993. The number of 16S rRNA gene copies was determined by comparing the C_T values of each sample to their corresponding standard curves. The microbial community in Tao Dam hot spring contained 90.8% of bacteria and 9.2% of archaea (Figure 12). The quantity of bacteria was 10 times higher than that observed for the archaea. The archaeal communities were estimated to comprise 26.57% methanogens (equivalent to 2.5% of the total prokaryotic community of Tao Dam hot spring).

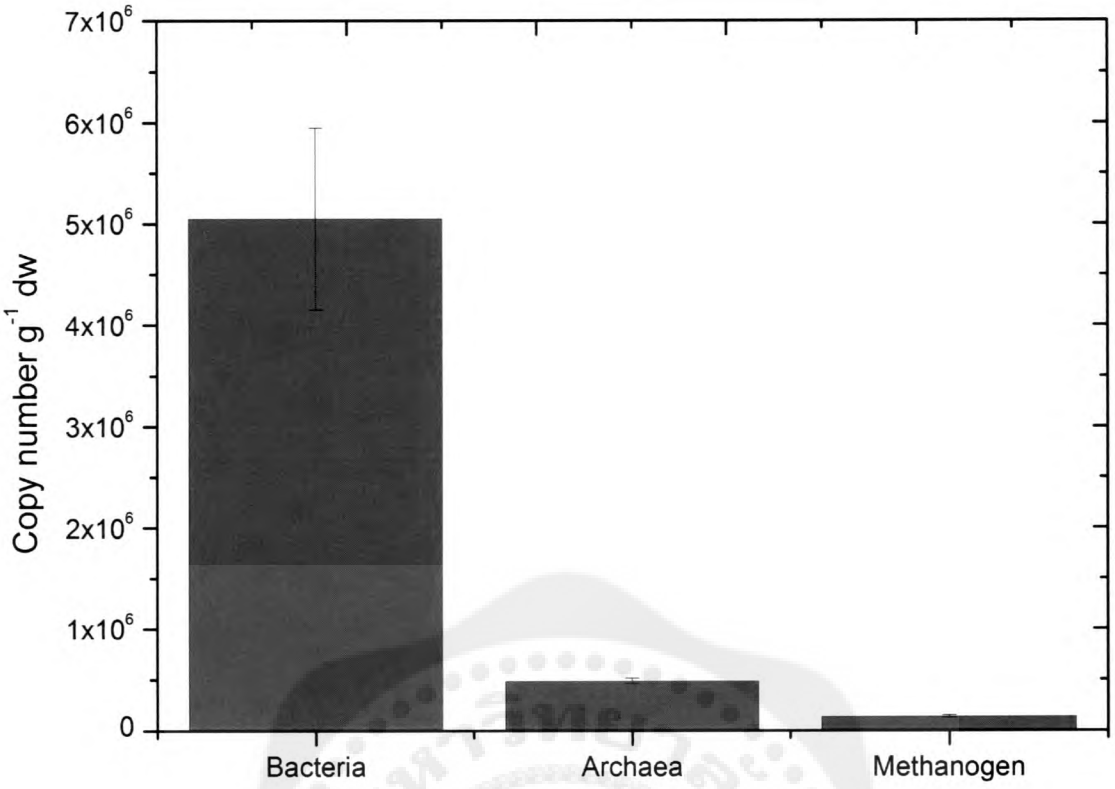


Figure 12 Results from Q-PCR analysis. Proportional of bacteria, archaea and methanogens from Tao Dam hot spring.

2. Bacterial systematics

2.1 Bacterial isolation

Nine bacterial strains were isolated from Tao Dam hot spring. All of the isolated were Gram's stain positive. The strains were rod-shaped and aerobically growth. They were able to growth on R2A medium at 55°C for overnight. The analysis of 16S rRNA gene could affiliate four strains to the genus Aeribacillus, three strains to the genus Anoxybacillus and two strains to the genus Bacillus (Table 6).

Table 6 Identification of 16S rRNA gene sequences of Tao Dam's strains using EzTaxon[214]

Strains	Affiliation	Accession No.	Family	Identity (%)
TD1	*Aeribacillus pallidus*	Z26930	*Bacillaceae*	99.67
TD2	*Aeribacillus pallidus*	Z26930	*Bacillaceae*	99.73
TD3	*Aeribacillus pallidus*	Z26930	*Bacillaceae*	98.27
TD4	*Anoxybacillus flavithermus*	AF004589	*Bacillaceae*	98.23
TD5	*Anoxybacillus kamchatkensis*	AF510985	*Bacillaceae*	98.43
TD6	*Anoxybacillus pushchinoensis*	AJ010478	*Bacillaceae*	98.65
TD7	*Bacillus licheniformis*	CP000002	*Bacillaceae*	99.72
TD8	*Aeribacillus pallidus*	Z26930	*Bacillaceae*	99.81
TD9	*Bacillus licheniformis*	CP000002	*Bacillaceae*	99.59

The strain TD5 was chosen for bacterial systematics because it could produce several types of commercialize enymes (Table 10-11). Moreover, the strain TD5 had low identity of 16S rRNA when compared with the type strain of *Anoxybacillus kamchatkensis* (98.43%).

2.2 Comparison of 16S rRNA genes

The 16S rRNA gene sequence of the strain TD5 was performed pairwised comparison between the members of bacterial strains in the genus Anoxybacillus (Table 7). At the time of study, there were eleven bacterial strains in the genus Anoxybacillus. They were *A. amylolyticus*, *A. ayderensis*, *A. bogrovensis*, *A. contaminans*, *A. flavithermus*, *A. gonensis*, *A. kamchatkensis*, *A. kestanbolensis*, *A. pushchinoensis*, *A. rupiensis* and *A. voinovskiensis*. The genus Anoxybacillus is affiliated to the family Bacillaceae, order Bacillales, class Bacilli and phylum Firmicutes[217]. The strain TD5 shared 85.53-94.75% of 16S rRNA gene identity to the members of the genus Anoxybacillus (Table 7). The closet relative strain of the strain TD5 was A. *kamchatkensis* (94.75%). *A. bogrovensis* (85.53%) had the lowest 16S rRNA gene identity to the strain TD5.

Table 7 Pairewised comparison of the 16S rRNA gene of the bacterial strains that belonging to the members of the genus Anoxybacillus using MatGat program[216]

		1	2	3	4	5	6	7	8	9	10	11	12
A. contaminans	1		98.15	98.35	96.96	95.38	92.46	90.11	89.83	87.55	87.01	84.56	83.20
A. amylolyticus	2	98.15		98.48	95.84	96.36	91.72	89.42	88.82	86.86	86.13	83.48	83.91
A. voinovskiensis	3	98.35	98.48		95.64	97.66	93.49	91.12	90.77	88.53	87.98	85.37	84.45
A. kanchatkensis	4	96.96	95.84	95.64		94.33	94.75	92.28	91.28	89.79	88.27	86.08	85.31
A. rupiensis	5	95.38	96.36	97.66	94.33		92.37	90.05	89.44	87.28	86.53	84.25	84.07
Strain TD5	6	92.46	91.72	93.49	94.75	92.37		93.01	92.19	90.54	88.85	87.02	85.53
A. ayderensis	7	90.11	89.42	91.12	92.28	90.05	93.01		98.37	95.22	93.88	92.30	89.78
A. flavithermus	8	89.83	88.82	90.77	91.28	89.44	92.19	98.37		94.44	94.44	92.64	89.49
A. gonensis	9	87.55	86.86	88.53	89.79	87.28	90.54	95.22	94.44		96.89	92.82	92.39
A. kestanbolensis	10	87.01	86.13	87.98	88.27	86.53	88.85	93.88	94.44	96.89		92.88	91.59
A. pushinoensis	11	84.56	83.48	85.37	86.08	84.25	87.02	92.30	92.64	92.82	92.88		88.34
A. bogrovensis	12	83.20	83.91	84.45	85.31	84.07	85.53	89.78	89.49	92.39	91.59	88.34	

2.2 Phylogenetic analysis

The same evolution model was chosen by Akaike information criteria (AIC) and hierarchical likelihood ratio tests (hLRT). The best-fit model of the data set was general time reversible plus proportion invariant (I) and gamma distribution (G) (GTR+I+G). The average standard deviation of split frequencies and PSRF value from data set were approximately 0.001 and 1.000 respectively. The generation plot (data not shown) had shown that the two runs of the analysis were convergent. Thus, the 10×10^6 generations of MCMC analysis was sufficient and the two runs of the analysis were converged onto the stationary distribution. Strain TD5 was clustered with *A. gonensis* NCCB 100040^T, which supported by 64% of clade credibility (Figure 13).

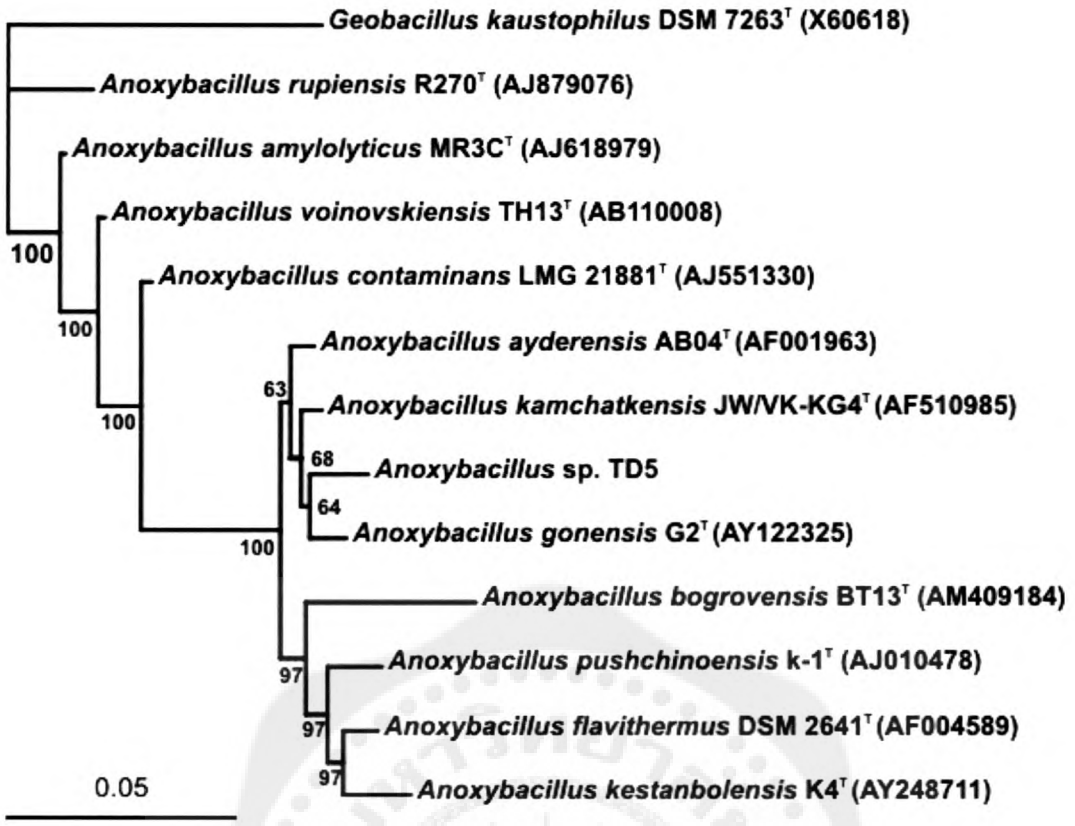


Figure 13 Phylogenetic tree relationships between strain TD5 and related *Anoxybacillus* species on the basis of 16S rRNA gene sequences. The tree was constructed using the Bayesian inference method. *G. kaustophilus* was used as an outgroup.

2.2 Molecular systematic

The DNA G+C content of the strain TD5 was 42.1 mol %. DNA-DNA hybridization (DDH) was 11.2% (15.9%) between the strain TD5 and *A. gonensis* NCCB 100040^T. Cellular fatty acid methyl esters from whole-cell fatty acids were performed according to the method described in the Sherock Microbial Identification System manual (version 4.0; MIDI). The fatty acids pattern of strain TD5^T has shown in Table 8. The major cellular fatty acids were $C_{15:0}$ iso (45.41%), $C_{16:0}$ (20.19%), and $C_{17:0}$ iso (10.89%). $C_{15:0}$ iso was found as the major cellular fatty acid of all strains of genus *Anoxybacillus* (Table 8). Unlike the other strains of genus *Anoxybacillus*, only strains TD5^T contained $C_{16:0}$ as the second predominant cellular fatty acid (Table 8).

Table 8 Cellular fatty acid components of strain TD5 and relative type strains from the genus Anoxybacillus

Fatty acid	1	2	3	4
$C_{14:0}$ iso	0.28	1.25	1.3	-
$C_{14:0}$	6.83	1.18	1.3	0.30
$C_{15:0}$ iso	45.41	65.19	54.7	52.81
$C_{15:0}$ anteiso	4.10	2.64	8.0	1.64
$C_{15:0}$	0.34	1.12	-	0.31
$C_{16:0}$ iso	1.78	5.99	7.1	2.01
$C_{16:0}$ anteiso	-	-	-	-
$C_{16:0}$	20.19	2.38	1.9	5.44
$C_{17:0}$ iso	10.89	11.96	3.9	33.55
$C_{17:0}$ anteiso	5.39	3.29	-	3.94
$C_{17:0}$ anteiso A	-	0.82	-	-
$C_{17:0}$	-	-	-	-
$C_{17:1}$ ω5c	-	2.63	-	-
$C_{17:1}$ iso ω7c	0.92	-	-	-
$C_{17:1}$ anteiso	-	-	7.1	-
$C_{17:1}$ anteiso A	0.50	-		-
$C_{17:1}$	-	-	2.6	-
$C_{16:1}$ ω7c/$C_{15:0}$ iso 2OH	3.37	-		-

Strains: 1, TD5 (this study); 2, *A. gonensis*[218]; 3, *A. voinovskiensis*[219]; 4, *A. rupiensis*[220]

2.3 Physiological and biochemical characteristics

Cells are Gram-positive, nonmotile, endospore-forming rods, 0.9-1.1 × 3.0->5.0 μm in size (Figure 14 and 15), occurring singly or in pairs. After aerobic growth at 55°C for 24 h, colonies on R2A agar are 1-2 mm in diameter, round with creamy colonies. Endospore is oval or cylindrical shaped, which locates at the terminal or subterminal of the cells in swollen sporangium. The strain is able to produce yellow pigment. Reactions are positive for catalase and oxidase. Growth is not observed at 30°C. The temperature ranges for growth are 40-65°C. The optimum temperature for growth is 55-60°C. Weakly growth is observed at 65°C. The strain is able to hydrolyzed starch and gelatin (Table 9). The strain is able to utilize ribose, D-xylose, galactose,

glucose, fructose, mannitol, *N*-acetyl-glucosamine, esculin, salicin, cellobiose, maltose, melibiose, sucrose, trehalose, glycogen and gluconate, but not erythritol, D-arabinose, L-arabinose, L-xylose, adonitol, βMeDxyloside, mannose, sorbose, rhamnose, dulcitol, inositol, sorbitol, αMeDMannoside, αMeDGlucoside, amygdalin, arbutin, lactose, inulin, melezitose, raffinose, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, 2Keto-gluconate and 5Keto-gluconate.

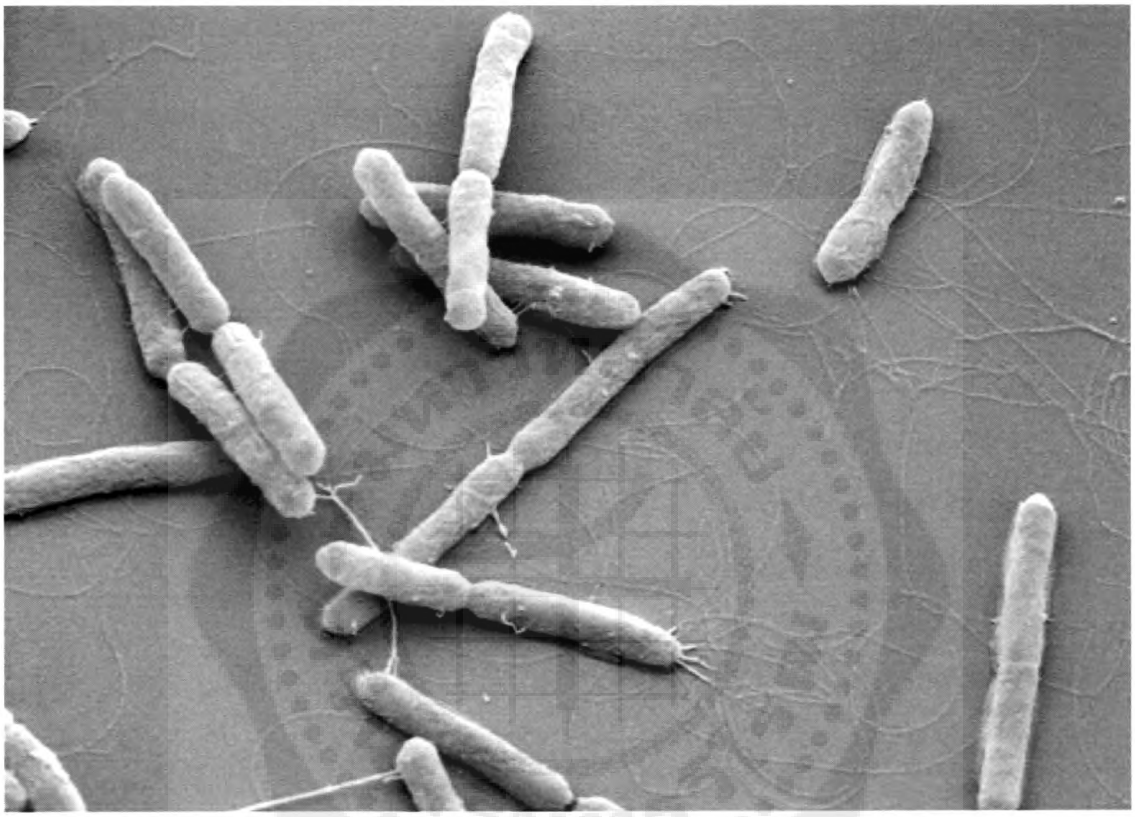


Figure 14 Scanning electronic micrograph of strain TD5.



Figure 15 Electronic micrograph of an ultrathin-sectioned cell of strain TD5.

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Table 9 Phenotypic characterization of strain TD5 and closely related species of genus Anoxybacillus

Characteristic	**1**	**2**	**3**	**4**	**5**	**6**
Size (μm)	0.9-1.1 × 3.0->5.0	1.0 × 2.5-8.8	0.4-0.6 ×1.5-5.0	0.7-1.5 × 3.3-7.0	0.75 × 5.0	0.55 × 4.60
Motility	+	+	-	+	+	+
Spore shape	oval/cylindrical	oval	ND	oval/cylindrical	spherical	spherical
DNA G+C content (mol%)	42.1	42-43	43.9	41.7	57	54
Temperature for growth (°C):						
Range	40-65	37-66	30-64	35-67	40-70	30-70
Optimum	55-60	57-62	54	55	55-60	50
Catalase	+	-	+	+	w	+
Oxidase	+	-	+	ND	+	ND
Substrates used:						
Arabinose	-	-	+	ND	-	+
Glucose	+	+	+	+	+	+
Ribose	+	w	+	+	ND	ND
Mannose	-	-	ND	ND	ND	+
Rhamnose	-	-	-	-	ND	-
Mannitol	+	+	-	+	+	+
Sorbitol	-	-	+	-	ND	+

Table 9 (Continue)

Characteristic	**1**	**2**	**3**	**4**	**5**	**6**
Sucrose	+	+	+	-	+	+
Raffinose	-	-	-	-	+	+
Hydrolysis of:						
Gelatin	+	-	-	ND	+	+
Starch	+	-	-	+	+	+

Strains designations: 1, strain TD5 (this study); 2, *A. kamchatkensis*[221]; 3, *A. voinovskiensis*[219]; 4, *A. rupiensis*[220]; 5, *A. gonensis*[218]; 6, *A. ayderensis*[222]

+ positive, w weakly positive, - negative, ND not determine

3. Biotechnology

3.1 Functional screening of commercialize enzymes

Nine isolates were obtained from the Tao Dam hot spring in Thailand. They were subcultured from well isolated colonies. To identify the isolates, a comparative analysis of their 16S rRNA genes against the genes from the data base was done and thus, they could be affiliated to a specific group. The isolates were found to belong to the genera *Aeribacillus*, *Anoxybacillus* and *Bacillus*. Each isolate was growth on different carbon source including agarose, cellulose and pectate (Table 10). For the strain TD7 and TD9, the growth was observed on three above mentioned carbon sources used. Other isolates (TD1-TD6 and TD8) were found to grow only on cellulose and pectate.

Table 10 Functional screening of Tao Dam's strains

Substrate	TD1	TD2	TD3	TD4	TD5	TD6	TD7	TD8	TD9
Agarose	-	-	-	-	-	-	+	-	+
Cellulose	+	+	+	+	+	+	+	+	+
Pectate	+	+	+	+	+	+	+	+	+

3.2 Screening of commercialize enzymatic genes

Partial sequences of enzymatic genes were amplified and sequencing from the nine strains, which isolated from Tao Dam hot spring (Table 11). The genes were identified using BLAST. There were laccase, superoxide dismutase, aminoacylase, lipase, xylanase, nitrilase, protease, pectate lyase, D-arabinose isomerase and L-rhamnose isomerase (Table 11). Laccase and superoxide dismutase belong to enzyme class I. Aminoacylase, lipase, xylanase, nitrilase and protease belong to enzyme class III. Pectate lyase belongs to enzyme class IV. D-Arabinose isomerase and L-rhamnose isomerase belong to enzyme class V.

Table 11 Identity of enzymatic genes of Tao Dam's strains and its closet relative species from BLAST search

Enzyme	Strain	Class	Closet relative strain	Identity (%)	
				DNA	Protein
Aminoacylase	TD5	III	*Anoxybacillus flavithermus*	88	94
D-Arabinose isomerase	TD1	V	*Aeribacillus pallidus*	100	100
	TD2		*Aeribacillus pallidus*	99	100
	TD3		*Aeribacillus pallidus*	99	99
Laccase	TD4	I	*Anoxybacillus flavithermus*	86	86
	TD6		*Anoxybacillus flavithermus*	86	86
Lipase/Esterase	TD4	III	*Anoxybacillus flavithermus*	86	87
	TD6		*Anoxybacillus flavithermus*	86	87
Nitrilase	TD1	III	*Aeribacillus pallidus*	99	100
	TD2		*Aeribacillus pallidus*	99	100
Protease	TD4	III	*Anoxybacillus flavithermus*	90	96
	TD5		*Anoxybacillus flavithermus*	90	96
	TD6		*Anoxybacillus flavithermus*	88	96
Pectate lyase	TD7	IV	*Bacillus licheniformis*	100	100
	TD9		*Bacillus licheniformis*	100	100
Superoxide dismutase	TD5	I	*Anoxybacillus flavithermus*	NS	94
L-Rhamnose isomerase	TD1	V	*A. pallidus* / *B. subtilis*	95	98
	TD2		*A. pallidus* / *B. subtilis*	95	98
Xylanase	TD4	III	*Anoxybacillus flavithermus*	91	93
	TD5		*Anoxybacillus flavithermus*	86	90
	TD6		*Anoxybacillus flavithermus*	91	93

CHAPTER V
DISCUSSION

1. Microbial diversity

Tao Dam hot spring contains various kinds of archaea and bacteria. There are two phyla of archaea, thirteen phyla and one unidentified group of bacteria, which discovered from Tao Dam hot spring 16S rRNA gene libraries. Microbial diversity of the spring is high especially in bacteria, which is greater than the archaea approximately five times by comparing the Choa1 value. The amount of bacteria is also greater than archaea around ten times. This result was similar to that obtained by Jannasch[223] who found approximately 10:1 ratio of bacteria to archaea in hydrothermal fluids[223]. These results are able to assume that the bacteria will play the important role of this ecosystem. Bacterial clones belonging to the phylum Proteobacteria shared more than half of the total number of the bacterial 16S rRNA clones of Tao Dam hot spring. Proteobacteria may drive the main function of the bacterial community of Tao Dam hot spring. This evidence from Tao Dam hot spring was similar to the results obtained in Yellowstone hot spring[224], where the proteobacterial clones were also the dominant bacterial group, but in opposition to the results from Bor Khlueng hot spring in Thailand, in which the acidobacterial clones were found to be the dominant bacterial group[1]. That might be because of the acidic pH of Bor Khlueng hot spring (pH 6.6)[1] compare to that of Tao Dam hot spring (pH 8.0). The microbial diversity may strongly influenced by the pH of the ecosystem. Within the domain Bacteria, the phylum Proteobacteria constitutes at present the largest and phenotypically most diverse phylogenetic lineage. In 2002, the phylum Proteobacteria consist of more than 460 genera and more than 1,600 species, scattered over five major phylogenetic lines of descent known as the classes "Alphaproteobacteria," "Betaproteobacteria," "Gammaproteobacteria," "Deltaproteobacteria" and "Epsilonproteobacteria".

"Alphaproteobacteria" and "Betaproteobacteria" were the major groups of the proteobacterial clones, which characterized from the Tao Dam 16S rRNA library (Figure 16). The majority of the "Alphaproteobacteria" are rod-shaped, but cocci and curved, spiral, stalked, budding and prosthecate forms do also occur. Some are phototrophic purple nonsulfur (PNS) bacteria (such as *Rhodospirillum* and *Rhodobacter*), whereas others are chemolithotrophs (e.g., the nitrite-oxidizing *Nitrobacter*) or

chemoorganotrophs (e.g., *Sphingomonas* and *Brucella*). The bacteria in the family Sphingomonadaceae were most found in the bacterial 16S rRNA library of Tao Dam hot spring, especially the bacterial strains in the genus Sphingomonas. Sphingomonads shared 67.7% of the total alphaproteobacterial clones with found in Tao Dam 16S rRNA library. Generally, the family Sphingomonadaceae containing glycosphingolipids in their cell envelopes belong to the phylogenetic lineage of the sphingomonads, forming a versatile group of aerobic bacteria occurring in various environments such as soil, water and clinical specimens. This lineage corresponds to the α-4 subgroup. The genus Sphingomonas contained at least 20 species and was recently split on the basis of phylogenetic and chemotaxonomic analyses into four different genera[225]. Some of the species belonging to these genera can metabolize various aromatic compounds and possess promising biotechnological properties.

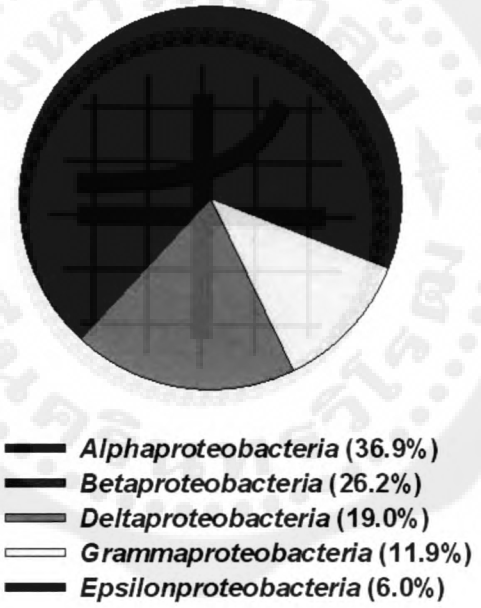


Figure 16 Proportion of proteobacterial clones of the 16S rRNA gene library.

Most of the betaproteobacterial clones of the Tao Dam hot spring were affiliated to the order Burkholderiales, which shared 50.0% of the total betaproteobacterial clones. The "Betaproteobacteria" (at least 75 genera and 220 species) clearly represent a monophyletic group within the larger phylogenetic lineage composed of the β-γ proteobacterial complex, named *Chromatibacteria* by Cavalier-Smith[226]. From the metabolic, morphological and ecological viewpoint, the "Betaproteobacteria" are very

heterogeneous. They contain some purple nonsulfur (PNS) phototrophs, various chemolithotrophs, some methylotrophs, a great number of chemoorganotrophs, some nitrogen-fixing bacteria, and some important plant-, human- and animal pathogens. Their morphologies can vary from rods or cocci to spiral and sheathed cells. Some members of the "Betaproteobacteria" are of biotechnological interest owing to their biodegradation properties. Recently nitrogen-fixing "Betaproteobacteria" were described that nodulate the roots of legumes[227, 228].

Of the archaea, methanogens were dominant and were estimated to consist of 50.3% of the total archaeal population according to archaeal 16S rRNA gene clone library. Preliminary analysis of the 16S rRNA library, the archaeal from the order Methanosarcinales was found as the dominant group among the methanogens. It accounted for 37.6% of the total methanogenic community. However, the deep analysis by using the mcrA gene library represented *Methanomicrobiales* (51.4%) and *Methanomicrobiales* (37.3%) as the major group of the methanogens.

The order Methanomicrobiales can be distinguished from other methanogens by growth properties, cell wall and lipid composition, and rRNA gene sequence. All *Methanomicrobiales* can use H_2 + CO_2 as a substrate for methanogenesis, most species can utilize formate, and many species utilize alcohols[229]. They cannot use acetate and methylated C-1 compounds such as methanol, methylamines and methyl sulfides as substrates for methanogenesis, and this property distinguishes them from the order Methanosarcinales. Notably, even though the order Methanomicrobiales cannot use acetate as a substrate for methanogenesis, many species require acetate as a carbon source. The absence of hydroxyarchaeol in their lipids further distinguishes them from the order Methanosarcinales[230]. In addition, all of the order Methanomicrobiales so far examined contain aminopentanetetrols, galactose and glycerol in their lipids, and aminopentanetetrols are unique to this taxon. The absence of pseudomurein further distinguishes the order Methanomicrobiales from both the order Methanobacteriales and Methanopyrales. Based upon the phylogeny of the 16S rRNA genes as well as phenotypic and genotypic characteristics, the order Methanomicrobiales has been divided into three families and nine genera of hydrogenotrophic methanogens[229, 231-233]. Twenty-four species have been described so far within this order[229].

Members of the order Methanobacteriales are distinguished from other methanogens by their limited range of catabolic substrates, their morphology, lipid

composition, and rDNA sequence. The Methanobacteriales are generally hydrogenotrophic, using H_2 to reduce CO_2 to CH_4. Some members of this order can use formate, CO, or secondary alcohols as electron donors for CO_2 reduction. However, members of one genus within this order, *Methanosphaera*, use H_2 to reduce methanol to methane. The predominant cell wall polymer of the order Methanobacteriales is pseudomurein, which distinguishes this order from the order Methanomicrobiales. Lipids composing the cell membranes include caldarchaeol and myo-inositol. Cells usually stain Gram positive and are generally rod-shaped, often forming chains or long filaments up to 40 μm in length. The order Methanobacteriales is divided into two families, the Methanobacteriaceae and Methanothermaceae, on the basis of phylogenetic analysis of 16S rRNA gene sequences as well as phenotypic characteristics[234].

Furthermore, there were 10.2% of archaeal clones that affiliated to the order Methanosarcinales. The order Methanosarcinales is one of the five orders of methanogens. These microbes catalyze the terminal step in the degradation of organic matter in anoxic environments where light and terminal electron-acceptors other than CO_2 are limiting. Representatives of the order Methanosarcinales are widespread and cosmopolitan in anaerobic environments. They are found in freshwater, marine environments and extremely halophilic sediments as well as in anaerobic sludge digestors and the gastrointestinal tracts of animals. Use of molecular techniques has also identified the order Methanosarcinales in the deep terrestrial subsurface environments such as in the gold mines in South Africa. Members of the order Methanosarcinales are coccoids, pseudosarcinae, or sheathed rods. Most cells have a protein cell wall, and some are surrounded by a sheath or acidic heteropolysaccharide; no peptidoglycan or pseudomurein is present. Hydroxyarchaeol is usually present in the lipids, which contain myo-inositol, ethanolamine and glycerol as polar headgroups. Cells are very strictly anaerobic and obtain energy with the concomitant production of methane. The order Methanosarcinales has the widest substrate range of methanogens: many can grow by reducing CO_2 with H_2, by dismutating methyl compounds, or by the splitting of acetate. Some species can use only one of those catabolic schemes, but others can use all three.

From the results above can predict that the hydrogenotrophic, acetotrophic and methylotrophic methanogenesis pathways are existed in Tao Dam hot spring. The hydrogenotrophic methanogenesis pathway may be the major pathway of methane

production of the Tao Dam hot spring. This assessment is consistent with the results obtained by Sandbeck and colleagues, who detected the presence of methanogenesis in Octopus hot spring algal-bacterial mats[235].

2. Bacterial systematic

The genus Anoxybacillus is a member of bacterial family Bacillaceae[217]. The type species of the genus is *A. pushchinoensis*[236]. *A. pushchinoensis* was isolated from equine and porcine manure in Moscow[236]. The genus was first described in year 2000 and emended in 2003 by Pikuta and colleagues[236, 237]. Currently, there are 11 validation species affiliated to the genus Anoxybacillus[217]. The genus is defined as Gram-positive, rod-shaped and non-motile bacteria[236]. The members of the genus contain one endospore at the terminal of the cells[237]. The genus is aerotolerant anaerobes or facultative anaerobe[237]. Also, the genus is chemoorganotropic, with a fermentative metabolism[237]. DNA G+C content is 42-57 mol%[237]. The genus is alkaliphilic or alkalitolerant and thermophilic[237]. Oxygen and catalase reactions are variable[237]. Strain TD5 is Gram-positive, rod-shaped, nonmotile and endospore forming bacterium. The strain was isolated from sediments of Tao Dam hot spring in Thailand. Strain TD5 is thermophilic, facultatively anaerobic and chemoorganotrophic bacterium, with fermentative metabolism. The 16S rRNA gene of the strain TD5 was 95.6% similar to the type strain of *A. kamchatkensis*. However, the phylogenetic analysis clustered the strain together with *A. gonensis* NCCB 100040^{T}. DNA-DNA hybridization values between the strain TD5 and *A. gonensis* NCCB 100040^{T} were 11.2% and 15.9% for the second replication. DNA G+C content of strain TD5 was 42.1 mol%. The major cellular fatty acids were $C_{15:0}$ iso (45.41%), $C_{16:0}$ (20.19%), and $C_{17:0}$ iso (10.89%). According to the studied, the isolate will represent a novel *Anoxybacillus* species, which proposes the new name as *Anoxybacillus thermophilus* sp. nov.

3. Biotechnology

Various kinds of enzymatic genes are discovered from the bacterial strains, which were isolated from the Tao Dam hot spring. The themostable properties of the enzymes are expected, because they are isolated from the themophiles. Some of them have a potential to be a novel enzymatic genes and enzymes such as the laccase and lipase genes, which were characterized from the bacterial strain TD4 and 6. Laccases (EC 1.10.3.2) form a family of multi-copper oxidases capable of catalyzing the oxidation

of many redox-active substances (particularly phenols) with the concomitant reduction of O_2 to water. The enzymes, detected in various bacteria, fungi, plants, and insects, are postulated to play an important role in many cellular or microbial activities such as biosynthesis of plant cell wall, phytopathogenesis, degradation or humification of wood material, and pigmentation of bacterial/fungal spore. Extensive characterization of laccase has been carried out in the past decades[238]. The ability of laccases to catalyze the oxidation (by O_2) of various substances of industrial and other interest has driven intensive research efforts to exploit and develop these enzymes as industrial catalysts. Based on the aims, the reported application trials could be divided mainly into degradation (of waste, pollutants, and so forth), transformation (of raw materials into functional materials), and sensing, covering industrial-technical, specialty chemical synthesis, environmental, food, medicinal and personal care fields. Laccases are exploited to replace hazardous/expensive chemicals, save on energy/resources consumption, create novel functionalities, and reduce detrimental impacts on the environment[238].

Lipases (EC 3.1.1.3) are generally versatile enzymes that accept a broad range of substrates (i.e. aliphatic, alicyclic, bicyclic and aromatic esters, thioesters, activated amines) whilst maintaining high regio-, chemo- and enantioselectivity. The stability of most lipases in organic solvents paves the way for their exploitation in organic synthesis: in esterification, transesterification, aminolysis and oximolysis reactions[239]. Such properties make lipases key players in the industrial enzyme sector[240-244].

The strain TD5 contains a novel aminoacylase gene, due to the analysis of the gene sequence. However, the function of this gene is still unknown and needed more study for the further applications. Aminoacylases (EC 3.5.1.14) have the chiral specificity in the synthesis of acylated amino acids. So, they are attractive candidates for application in fine chemistry[245].

The strains TD4-6 also have a high potential to have the novel xylanase genes and enzymes. Xylanases (endo-β-1,4-xylanases, EC 3.2.1.8) cleave the xylan backbone into smaller oligosaccharides. They are the key enzymes for xylan degradation and differ in their specificities toward the xylan polymer. Many cleave only at unsubstituted regions whereas others have a requirement for side chains in the vicinity of the cleaved bonds[246]. Most xylanases are also active on xylooligomers of degree of polymerization greater than 2, showing increasing affinity for xylooligomers of increasing length.

Xylanases are endo type enzymes that hydrolyse internal linkages in xylan and act by a random attack mechanism yielding a mixture of xylooligosaccharides from the polymer.

Futhermore, the activity of pectate lyase is detected from the strains TD1-9. Together with the bacterial systematic study, all strains except TD7 and TD9 are the good candidate for the pectate lyase production, because this is the first time that the activity of the pectate lyase are discovered from the bacteria in the genus Aeribacillus and Anoxybacillus. However, the molecular study of the pectate lyase gene from them is needed for the further applications. Pectate lyases (EC 4.2.2.2), otherwise known as pectate transeliminases, catalyse the eliminative cleavage of de-esterified pectin, which is a major component of the primary cell walls of many higher plants[247]. The backbone of pectic, polysaccharides is built of blocks of α-1,4 linked polygalactosyluronic acid residues interspersed with regions of alternating galactosyluronic acid and rhamnosyl residues[248]. Cleavage by pectate lyase requires the presence of calcium ions and generates oligosaccharides with unsaturated galacturonosyl residues at their non-reducing ends.

Besides, the strains, which were isolated from the Tao Dam hot spring, contain some others enzymatic genes such as D-arabinose isomerase, nitrilase, protease and L-rhamnose isomerase.

4. Conclusion

This was the first report of a survey of the Tao Dam hot spring sediment microbial community. This spring is the rich source of the thermophiles, which contain some valuable enzymatic genes and enzymes. The methanogenesis pathways of this spring are an interesting subject for the further studies, due to the knowledge of such kind of the studies are still rare and insufficient. Moreover, the Tao Dam hot spring has highly potential to become a biotechnological resource for the industrial applications.

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APPENDICE

APPENDICE

Materials

Absolute ethanol	Sigma
Acetic acid	Sigma
Acetone	Sigma
Agarose	Bio-Rad
Ammonium chloride	Sigma
Ammonium persulfate	Sigma
Ampicillin	Fermentas
API 20E	BioMérieux
API 50CHB	BioMérieux
AZ Rhamnogalacturonan	Megazyme
Bacto agar	Difco
Bacto™ peptone	Difco
Boric acid	Sigma
Bromophenol blue	Sigma
Cacodylate	Sigma
Calcium chloride	Sigma
Chloroform	Sigma
Di-sodium hydrogen phosphate	Sigma
Deoxynucleotide triphosphates (dNTPs)	Fermentas
100 bp DNA ladder	Fermentas
Ethylenediaminetetraacetic acid (EDTA)	Sigma
Fluorescein isothiocyanate (FITC)	Bio-Rad
Formamide	Sigma
Formaldehyde	Sigma
Glutaraldehyde	Sigma
Glycerol	Sigma
Glycine	Sigma
Hydrochloric acid	Sigma
Isoropyl-b-D-Thiogalactopyranoside (IPTG)	Sigma

Lambda exonuclease	Invitrogen
Lysozyme	Sigma
Magnesium chloride	Sigma
MDE® gel solution	Lonza
Pectate lyase	Megazyme
Phenol	Sigma
Phenol/chloroform/Isoamyl alcohol pH 7.8	Sigma
Phusion (*Taq*) DNA polymerase	Finyzmes
Potassium dihydrogen phosphate	Sigma
2-Propanol	Sigma
Proteinase K	Sigma
R2A agar	Difco
SDS (Sodium dodecyl sulfate)	Merck
Sodium acetate	Merck
Sodium carbonate	Sigma
Sodium chloride	Merck
Sodium hydroxide	Sigma
Sodium pectate	Sigma
Sucrose	Sigma
SYBR Green Ready Mix	Bio-Rad
Taq polymerase	EuRx
TEMED	Fisher Bioreagents
Tris-base	Sigma
Tris-acetate EDTA buffer	Sigma
TBE buffer BlueView™	Sigma
Xylene cyanol	Fulka
Yeast extract	Biomark

Reagents

LB Broth

NaCl	(171 mM)	10	g
Paptone	(1% w/v)	10	g
Yeast extract	(0.5% w/v)	5	g
Deionized H_2O adjust volume to		1000	ml

Phosphate buffer (200 mM), pH 7.0

NaH_2PO_4	(200 mM)	230	ml
Na_2HPO_4	(200 mM)	770	ml
Deionized H_2O adjust volume to		1000	ml

Tris-EDTA (TE) buffer, pH 8.0

EDTA	(100 mM)	0.01	ml
Tris	(1 M)	1.0	ml
H_2O (distilled water) adjust volume to		100	ml

Tris-HCl buffer (200 mM), pH 8.0

Tris-HCl	(50 mM)	24.4	g
H_2O (distilled water) adjust volume to		100	ml

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