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RESEARCH ARTICLE

Thermotrophy Exploratory Study

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HIGHLIGHTS

Experiments with certain microorganisms in the absence of oxygen showed that life can utilize heat from the ambient environment as a source of energy.

ABSTRACT

The question of whether environmental heat energy could be utilized as a source of energy for biological metabolism is the center of this exploratory research. In 1979, this author postulated a hypothesis for the existence of thermotrophs that could isothermally utilize environmental heat energy as a source of their energy on Earth. According to this hypothesis, the thermotrophs could be the first primitive forms of life in the early Earth environment. The chemotrophs and phototrophs that we currently are all well familiar with might have been evolved somehow from the primitive thermotrophs. Furthermore, all the organisms currently regarded as the "chemotrophs" and "phototrophs" could actually be the mixed trophy types containing thermotrophic features: thermo-chemotrophs and thermo-phototrophs. Energetic analysis with the thermodynamic first law indicated that the anaerobic acetate-utilizing methanogenic archaea *Methanosarcina* could be a "living fossil specimen" of the thermotrophs. Experiments with enriched acetate-utilizing methanogen, including *Methanosarcina,* has, for the first time, demonstrated that their anaerobic metabolism was indeed associated with isothermal environmental heat utilization, resulting in their liquid culture temperature to change (decrease) by about −0.10 °C and sometimes drop by as much as −0.45 °C observed in the experiments. The mean temperature change (drop) was −0.25 ±0.06 °C.

KEYWORDS

Type-B thermotrophy, isothermal environmental heat energy utilization, thermotrophic function, methanogen, anaerobic fermentative hydrogen-producing bacteria, thermotrophs, thermo-chemotrophs, thermo-phototrophs.

Hypothesis for the Existence of Thermotrophic Organisms on Earth

American scientists Harada and Fox in 1958 performed an experiment in which they heated a mixture of amino acids and found polypeptides formed from many of the amino acids (Harada & Fox, 1958). It indicated that the use of environmental heat energy could promote the formation of complicated organic complexes from simple organic substrates, which appeared to resemble quasi-life-like organic matter, including polypeptides. If environmental heat energy could be involved in the formation of "quasi-life-like organic matters" before the first primitive forms of life emerged on Earth, why would life no lon-

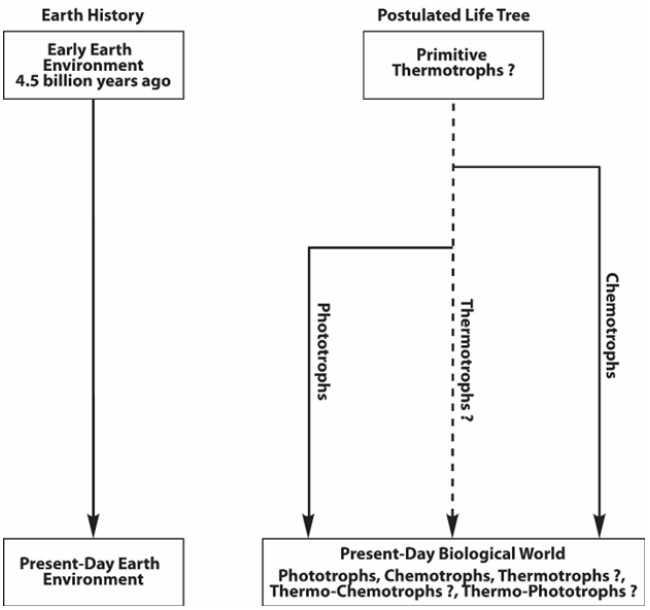

Figure 1. Illustration of the postulated hypothesis for the existence of thermotrophic life on Earth.

ger use heat energy as a source of its energy? Or, among some of the organisms (especially the primitive forms of life such as the primitive archaea including the extreme thermophiles), would they still retain their early evolution-historical features of thermotrophy that isothermally utilizes environmental heat energy as an energy source for their metabolism? This was one of the questions that came to the author's mind more than 40 years ago.

Furthermore, heat energy widely exists in the natural environment. In view of molecular dynamics, heat energy is the thermal molecular motion kinetic energy. Meanwhile, the molecular motion energy also represents the motion of the fundamental particles (including electrons) of the molecules. The motion of the fundamental particles can emit electromagnetic waves known as blackbody heat radiation (Planck, 1910). A 25 °C (T=298 °K) object can emit electromagnetic waves with their peak emission wavelength (λ_{peak}) of about 6.66 μm, which is in the infrared wavelength domain. The infrared peak wavelength (λ_{peak}) has an inverse relationship with the temperature (T). Infrared and visible light are both electromagnetic waves, which are fundamentally the same, except for their difference in wavelength. It is known that the dominant photosynthetic pigments in higher plants are chlorophyll-a molecules that have absorbance peaks in the blue and red regions of sunlight. The higher plant photosystem-I reaction center pigment (a dimer of chlorophyll-a molecules) has a light absorbance peak wavelength of around 700 nm, which is near the infrared domain. Some of the photosynthetic bacteria can utilize near-infrared radiation with a wavelength of < 1000 nm (certain bacteriochlorophyll shows absorption maxima around 805 nm and absorbs the light in the 800–1040 nm range). With these early perspectives, the author was wondering whether there could be some thermotrophic organisms that could utilize infrared (environmental heat energy) as a source of energy for their metabolism.

Inspired by the preliminary analysis above, the author in 1979 postulated the following thermotrophic life hypothesis (Figure 1) in regard to the question of whether there could be thermotrophic metabolism in the biological world.

According to the thermotrophy hypothesis, in addition to the currently known chemotrophs and phototrophs, there could exist another type of organism — "thermotrophs," which might have been one of the earliest forms of life on Earth. This hypothesis was based on the knowledge of the early Earth environment (Owen et al., 1979) that was likely quite hot (Woese, 1979), and its early atmosphere was reductive in nature containing no molecular oxygen and no ozone layer. Consequently, at that time, solar radiations, including UV light, x-ray, γ-ray, and cosmic radiations, could directly reach the Earth's surface, which could quickly destroy life's DNA (if any) on the early Earth's surface. From there, one could imagine that the land surface of the early Earth was probably life-less for a very long time; the sea surface at that early time was also likely lifeless. The high-energy radiations, including UV light, x-ray, and γ-ray could penetrate quite deep into the seawater so that life probably could not exist at any places on the early Earth surface where sunlight and high-energy radiations could hit; Consequently, at that early time, photosynthetic organisms could hardly exist there either.

However, in the deep dark ocean where the life-destroying high-energy solar radiations could not reach, there may be a quite different story. Especially near the deep ocean volcanic vents, the hot and relatively stable deep ocean environment could be a favorable birthplace for the formation of the first primitive forms of thermotrophic life: the primitive thermotrophs. Therefore, the thermotrophs could be born as the first primitive forms of life in a quite surprising manner in the early stage of the Earth's history when it was still quite hot, and its early atmosphere was reductive with no molecular oxygen and no ozone layer. It could be possible for the thermotrophs to first occupy the ocean floor and then further expand from the sea floor to the other living spaces on Earth while evolving themselves to produce more-advanced forms of life, including the chemotrophs and phototrophs and the possible mixed trophy types including thermo-chemotrophs (Lee, 2020a, 2021a, 2021b) and thermo-phototrophs (Jennings et al., 2018) that carry certain thermotrophic functions.

After the life-protecting ozone layer was formed in the Earth's atmosphere, life in all its forms: thermotrophs, chemotrophs, and phototrophs and their combined types, including thermo-chemotrophs, thermo-phototrophs, and thermo-chemo-phototrophs had the greater opportunities in expanding to nearly all areas of the Earth natural environment. As the Earth got cooler, however, the environmental conditions could become less favorable to the thermotrophs but more favorable to the phototrophs and chemotrophs, especially their combined types, including thermo-phototrophs, thermo-chemotrophs, and thermo-chemo-phototrophs. For example, the phototrophs (and/or thermo-phototrophs), owning to their advantage of utilizing energy-rich visible photons, have now apparently occupied the Earth's surfaces, including the oceans and land areas by their competing advantage against the primitive thermotrophs. However, under the present-day Earth conditions, it should still be possible to find some thermotrophs in certain special places, such as in the deep water/mud, anaerobic digestors, deep soils, and/or earth subsurface where the phototrophs could not compete with them.

In the present-day Earth environment, it should be possible to also find thermotrophs' derivatives such as thermo-phototrophs, thermo-chemotrophs, and thermo-chemo-phototrophs. This hypothesis for the postulated existence of thermotrophic organisms is illustrated in Figure 1.

The disclosure of the thermotrophic life hypothesis here is to encourage the scientific community to consider support for research on this topic of fundamental importance. Hopefully, this could stimulate scientific discussions on how to re-evaluate the effects of environmental heat energy on life for its possible applications. For example, one of the main goals for the agriculture system is to harvest and convert sunlight energy into the chemical energy of foods. Currently, only about 1-3% of the sunlight energy that reaches the crop field can be converted into biomass energy through photosynthesis (Edreira et al., 2020) (Keller et al., 2022); the remainder (over 90%) of the light energy is lost as the dissipated heat (infrared) into the Earth environment and the out space, which is quite wasteful. If we could somehow find a way to utilize not only the visible light energy but also the environmental heat energy for crop production, then it could fundamentally improve the yields for food and energy production.

Notably, inspired by the recent discovery on thermotropy (Lee, 2020a, 2021b), a novel invention on asymmetric-function-gated isothermal electricity generation (Lee, 2019b) has now been made to isothermally utilize the limitless environmental heat energy alone (without requiring any fossil fuel energy) for production of isothermal electricity, which could power many electric devices including (but not limited to) mobile phones, laptops, cars, and trains. It could potentially help liberate all people from their dependence on fossil fuels (Lee, 2022b). Therefore, this line of research and development may have much broader fundamental and practical implications, including its applications to solve the energy crisis and reduce greenhouse-gas emissions for a sustainable future to control climate change on Earth (Lee, 2021a, 2022a).

Preliminary Thermodynamic Evidence for the Existence of Thermotrophic Metabolism

In the present-day biological world, does there really exist any such a thermotrophic organism that can isothermally utilize environmental heat as a source of energy for its metabolism? According to our analysis with the thermodynamic first law, the anaerobic methanogenic archaea that utilize acetate as the sole organic carbon source for their growth and production of methane and carbon dioxide may represent "a living fossil specimen" of the thermotrophs.

The methanogenic bacteria (Fox et al., 1977), such as *Methanosarcina sp*., can grow in an anaerobic minimal culturing medium that contains acetic acid (CH_3COOH) as the sole organic carbon source for their growth and production of methane (CH_4) and carbon dioxide (CO_2) (Weimer & Zeikus, 1978a, 1979). Note, the production of methane (CH_4) and carbon dioxide (CO_2) from acetic acid (CH_3COOH) is an endothermic reaction process that requires heat energy (ethalpy change: ΔH = +3.77 kcal/mol) from the environment:

$$CH_3COOH \rightarrow CH_4 + CO_2 \; - 3.77 \text{ kcal/mol} \qquad (1)$$

The methanogen's anabolism, which utilizes acetic acid (CH_3COOH), mineral nutrients (N, P, K, Ca, Mg, S, Fe, Zn, Cu, Mo, and etc.) under anaerobic conditions to synthesize cellular materials including sugar, lipids, proteins, and nucleotides for cell growth, also requires exogenous energy input from the environment. Since both its catabolic production of methane (CH_4) and carbon dioxide (CO_2) and its anabolism in synthesizing the cellular materials from acetic acid (CH_3COOH) are endothermic, the total metabolic process of *Methanosarcina* must thus be endothermic, requiring exogenous energy input. Consequently, based on the first law (conservation of mass and energy), the required energy input, in this case, must be from the isothermal utilization (absorption) of heat energy from the environment. This thus provides the thermodynamic first-law evidence for the existence of ther-

motrophic metabolism in the anaerobic methanogenic *Methanosarcina* cells.

The predicted isothermal utilization (absorption) of environmental heat energy through thermotrophic metabolism is expected to lower the temperature in the liquid *Methanosarcina* cell culture. This predicted isothermal utilization (absorption) of environmental heat energy by *Methanosarcina* was experimentally demonstrated, as described near the end of this article. Briefly, the *Methanosarcina* cells were enriched from a liquid inoculum of a methane-producing anaerobic digestor using a minimal liquid culture medium containing acetate as the sole source of carbon under the anaerobic conditions. The experimental observation demonstrated that the metabolic activities in *Methanosarcina* liquid cell culture indeed resulted in a substantial temperature change (drop) by about −0.10 °C (and sometimes drop as much as −0.45 °C) compared with the control (liquid medium only without cells). This is a significant experimental observation since it has, for the first time, demonstrated that the anaerobic methanogenic archaea *Methanosarcina* cells might indeed represent a "living fossil specimen" of the thermotrophs on Earth.

In addition, our early analysis (Lee, 1983) indicated that certain anaerobic H_2-producing bacteria (Anukam et al., 2019; Turker et al., 2008) are also likely to carry the thermotrophic function capable of isothermally utilizing environmental heat energy in a way similar to that of the methanogenic archaea *Methanosarcina* cells. For example, the anaerobic hydrogen-producing "S" bacteria can utilize ethanol (CH_3CH_2OH) to produce molecular dihydrogen (H_2) and acetic acid (CH_3COOH) according to the following process reaction:

$$CH_3CH_2OH + H_2O \rightarrow CH_3COOH + 2H_2 \text{ -19.16 kcal/mol} \quad (2)$$

This catabolic production of CH_3COOH and H_2 from CH_3CH_2OH (Eq. 2) is endothermic (ΔH = +19.16 kcal/mol) (Lee, 1983). The "S" bacteria anabolism, which anaerobically utilizes ethanol (CH_3CH_2OH) and mineral nutrients (N, P, K, Ca, Mg, S, Fe, Zn, Cu, Mo) to synthesize cellular materials including sugar, lipids, proteins, and nucleotides for cell growth, also requires energy input. Consequently, based on the first law of thermodynamics, the anaerobic hydrogen-producing "S" bacteria are also likely to carry thermotrophic function for isothermal utilization (absorption) of heat energy from the environment.

Similarly, certain propanoic acid-utilizing, H_2-producing, and acetic acid-producing bacteria anaerobically utilize propanoic acid (CH_3CH_2COOH) to produce acetic acid (CH_3COOH), hydrogen (H_2), and carbon dioxide (CO_2) according to the following process reaction:

$$CH_3CH_2COOH + 2H_2O \rightarrow C_3COOH + 3H_2 + CO_2 \text{ -48.93 kcal/mol} \quad (3)$$

This catabolic production of acetic acid, hydrogen, and carbon dioxide from propanoic acid (Eq. 3) is clearly endothermic (ΔH = +48.93 kcal/mol; ΔG^o = + 13.59 kcal/mol; $\Delta G^{o\prime}$ = + 18.24 kcal/mol) (Lee, 1983); Their anabolism that anaerobically utilizes propanoic acid (CH_3CH_2COOH) and mineral nutrients to synthesize cellular materials for cell growth certainly also requires additional energy input. Based on the thermodynamic first law, their required energy must be from the isothermal utilization (absorption) of heat energy from the environment. Therefore, this analysis result also indicated the existence of thermotrophic metabolism in the propanoic acid-utilizing, H_2-producing, and acetic acid-producing bacteria.

Furthermore, certain butanoic acid-utilizing, H_2-producing, and acetic acid-producing bacteria anaerobically

Table 1. The published data of physical chemistry constants (from the 55th edition Handbook of Chemistry and Physics 1974–1975) (Weast, 1974 -1975) that were employed in the calculation for the enthalpy changes (ΔH) in the anaerobic process reactions of Eqs. 1–4.

Chemical formula	Name	State	Temperature °C	$-\Delta H_{Combustion}$ kcal/mol
CH_3COOH	Acetic acid	Liquid	25	209.02
CH_3CH_2OH	Ethanol	Liquid	25	326.48
CH_3CH_2COOH	Propanoic acid	Liquid	25	365.03
$CH_3CH_2CH_2COOH$	Butanoic acid	Liquid	20	521.90
CH_4	Methane	Gas	25	212.79
H_2	Hydrogen	Gas	25	68.31
CO_2	Carbon dioxide	Gas	25	0
H_2O	Water	Liquid	25	0

utilize butanoic acid ($CH_3CH_2CH_2COOH$) to produce acetic acid (CH_3COOH) and hydrogen (H_2) according to the following process reaction:

$$CH_3CH_2CH_2COOH + 2H_2O \rightarrow 2CH_3COOH + 2H_2 \ -32.77 \text{ kcal/mol} \quad (4)$$

This catabolic production of acetic acid, hydrogen, and carbon dioxide from butanoic acid (Eq. 4) is clearly endothermic (ΔH =+ 32.77 kcal/mol; ΔG^o =+ 16.13 kcal/mol; $\Delta G^{o'}$ =+ 11.50 kcal/mol) (Lee, 1983); their anaerobic utilization of butanoic acid and mineral nutrients to synthesize cellular materials for cell growth apparently requires additional energy from isothermal utilization (absorption) of heat energy from the environment. This analysis result indicated the existence of thermotrophic metabolism in the butanoic acid-utilizing, H_2-producing, and acetic acid-producing bacteria.

The enthalpy changes (ΔH) for each of Eqs. 1–4 above were calculated using the published data of physical chemistry constants from the 55th edition Handbook of Chemistry and Physics 1974–1975 (Weast, 1974 -1975) as listed in Table 1.

Recently, a new type of energy process called "Type-B energetic process" (Lee, 2021a) has been identified through the author's research work in alkalophilic bacteria (Lee, 2019d, 2020a) and mitochondria (Lee, 2019c, 2021b) that were shown to isothermally utilize environmental heat in driving ATP synthesis. The findings showed that the bacteria and mitochondria commonly regarded as "chemotrophs" are now known to also contain thermotrophic functions. That is, they actually are the mixed trophy type of "thermo-chemotrophs," which well supports the thermotrophic life hypothesis (Figure 1) as proposed by Lee in 1979.

Notably, Jennings et al. have also independently identified that the photosynthetic systems can also isothermally utilize environmental heat energy (Jennings et al., 2018). This independent finding (Jennings et al., 2018) also seems to implicate that the "phototrophs" are also likely to be the "thermo-phototrophs" as predicted in the Lee 1979 hypothesis (Figure 1) as well.

The Lee Thermotrophy Exploratory Research Group first published the idea of thermotrophs in an article titled "There may be thermotrophic type of life on Earth" in the 1983 Chinese journal *Potential Science* in Beijing, China (Lee, 1983). The 1983 Lee team article with thermodynamic energy analysis showed the possibility for the existence of thermotrophic life, which possesses the ability to "resist (disobey) the second law of thermodynamics," and is thus capable of "utilizing dissipated environmental heat energy as a source of energy for their metabolisms" (Lee, 1983 p. 1). It postulates that the "thermotrophic life forms may stay in the places where phototrophs could not compete with them, such as at the bottom of natural water bodies (ponds, rivers, lakes, and oceans) and deep soil earth layers." The article points out that "the anaerobic propanoic acid-utilizing and hydrogen (H_2)-producing and acetate-generating bacteria, and the anaerobic butanoic acid-utilizing and hydrogen-producing and acetate-generating bacteria may also be considered as some of the examples for the thermotrophic type of life in the present-day Earth environment" (Lee, 1983 p. 1).

The 1983 Lee team article (Lee, 1983) further draws attention to the fact that the physiological Gibbs free energy change in the anaerobic bacteria catabolism from propanoic acid to produce molecular hydrogen, acetate, and carbon dioxide has a substantial positive value ($\Delta G^{o'}$ = +18.24 kcal/mol) ; and so does that in the catabolism from butanoic acid to produce molecular hydrogen and acetate with a substantial positive change of the physiological Gibbs free energy ($\Delta G^{o'}$ =+ 11.50 kcal/mol). Consequently, the Lee team postulated that the total entropy in the anaerobic bacteria catabolic processes decreases instead of increasing (Lee, 1983). Since the anabolism (biosynthesis) per se is known as a process from disorder to order (with decreasing entropy of the system), the article concludes that the total entropy of the anaerobic H_2-producing bacterial life, including both their catabolism and anabolism also decreases, which could not be explained by the second law of thermodynamics. Thus, the article points out that the second law may not be fully applicable to the life systems. It also envisions that it will be possible for human society to isothermally utilize the limitless environmental heat energy to do useful work through the mimics and application of thermotrophic functions (Lee, 1983).

Notably, more than 20 years later, in a 2007 publication (Sheehan, 2007), Dr. Daniel Sheehan also excellently introduced a similar idea, "thermosynthetic life," which bears a strong resemblance to Lee's concept of "thermotrophic life" published in 1983 (Lee, 1983). Apparently, Dr. Sheehan seemed not aware of the 1983 Lee team publication (Lee, 1983) and came up with a somewhat similar idea for thermotrophy. In the 2007 "thermosynthetic life" article (Sheehan, 2007), Dr. Sheehan proposed an intriguing superthermal membrane capacitor where certain mobile electrons are hypothesized to climb the electrostatic potential across a biomembrane "against the potential gradient via multiple small, diffusive sub-k_BT steps" using thermal energy alone. This was proposed as a physical model with "a 3-D pyramidal array of charge transport molecules" through "an electrically conducting molecular ladder spanning the membrane from pyramidal base

to vertex" for the conversion of thermal energy into biochemical work (Sheehan, 2007 pp. 1779,1785). Although such a proposed thermal energy-driven pyramidal array charge transport for electrocapacitor energization has never been observed in the biological world so far, it remains still as an interesting research topic that should be encouraged to explore.

The protonic biomembrane capacitors (Lee, 2019a, 2020b) that Lee recently identified in bacteria and mitochondria are now known to charge up through a chemical (redox) energy-driven electron-transport-coupled proton translocation process (Lee, 2020a, 2021b), which is different from Sheehan's proposed thermal energy-driven pyramidal array charge transport mechanism (Sheehan, 2007). In the protonic biomembrane capacitor system (Lee, 2019a, 2020b), it is the use of the thermal kinetic energy (k_BT) associated with transmembrane-electrostatically localized protons through asymmetric membrane structures that enables a thermotrophic function in isothermal utilization of environmental heat energy to make ATP for cellular activities (Lee, 2020a, 2021a, 2021b) (Guan, 2022).

Based on the finding of protonic thermotrophic function, Lee has recently identified two thermodynamically distinct types (A and B) of energetic processes naturally occurring on Earth (Lee, 2021a). Type-A energetic processes, such as the classical heat engines, ATP hydrolysis, and many of the known chemical, electrical, and mechanical processes, apparently follow well the second law of thermodynamics; Type-B energetic processes, such as the newly discovered protonic thermotrophic function that isothermally utilizes environmental heat energy to do useful work in driving ATP synthesis, which follows the first law of thermodynamics (conservation of mass and energy), but do not have to be constrained by the second law, owning to their special asymmetric functions (Lee, 2022a).

Remarkably, Dr. Anthonie Muller excellently proposed a "heat engine"-based "thermosynthesis" hypothesis (Muller, 1996; Muller, 2012), which appears well in line also with the Lee concept of "thermotrophic life" (Lee, 1983) and the Sheehan model of "thermosynthetic life" (Sheehan, 2007); they all pointed to the same big direction that life could utilize heat energy as a source of energy to do useful work. However, there is also some fundamental difference here: Muller's "thermosynthesis" model requires a "thermal gradient" or "temperature cycling" (Muller, 1985; Muller, 2009; Muller & Schulze-Makuch, 2006), whereas both the Lee concept of "thermotrophic life" and the Sheehan model of "thermosynthetic life" may be classified as a Type-B process that isothermally utilizes environmental heat energy to do useful work without requiring any thermal gradient. That is, according to the classification of Type A and B energetic processes (Lee, 2021a, 2022a), Muller's "thermosynthesis" model would represent a Type-A thermotrophy while both the Lee concept of "thermotrophic life" and the Sheehan model of "thermosynthetic life" would be classified as the Type-B thermotrophy. Probably, it is debatable or discussable whether or not Muller's "thermosynthesis" model as Type-A thermotrophy could really operate in any biological cells with no substantial temperature gradient under the nearly constant temperature conditions in today's Earth environments. Nevertheless, Muller's "thermosynthesis" model is also valuable since it is a testable hypothesis and may also have potential implications in helping to explain some of the possible roles that heat energy could probably play in regard to the origin of life including the RNA world (Muller, 2005).

The following section of this paper reports the experimental demonstrations of the isothermal heat absorption (utilization) by anaerobic acetate-utilizing methane-producing bacteria that were performed at the former Zhejiang Agricultural University in 1979–1982. This experimental work through enrichment of anaerobic acetate-utilizing methanogenic bacteria and monitoring of their heat absorption has, for the first time, demonstrated that the methanogen *Methanosarcina* cells indeed absorb heat energy from the liquid culture environment, which may now be considered as the first experimental evidence for the methanogenic archaea as "a living fossil specimen" of thermotrophs that manifests the predicted Type-B thermotrophic activities (Figure 1 and Eq. 1).

EXPERIMENTAL DEMONSTRATION OF HEAT ABSORPTION BY ANAEROBIC ACETATE-UTILIZING METHANOGEN

Enrichment Culturing of Anaerobic Acetate-Utilizing Methane-Producing Bacteria

A minimal culturing medium (also known as the "#16" liquid medium) comprising acetate as the sole source of organic carbon was made and employed to enrich anaerobic acetate-utilizing methane-producing bacteria (methanogen) from a liquid inoculum of a methane-producing anaerobic digestor at the former Zhejiang Agricultural University. The "#16" liquid medium was made from distilled water by adding the following ingredients (per 2 liters (L) of the liquid medium): 31.298 gram (g) of $Ca(CH_3COO)_2$, 0.128 g of CH_3COOH, 1.0820 g of NH_4CH_3COO, 0.0300 g of $MgCl_2$, 0.4000 g of K_2HPO_4, 0.4000 g of $Na_2S.9H_2O$, 0.2000 g of yeast powder, 2.5 ppm of Fe, 0.5 ppm of B, 1.5 ppm of Mn, 1 ppm of Zn, 0.25 ppm of Cu, 0.25 ppm of Mo, 0.25 ppm of Vitamin B12, 0.5 ppm of

Vitamin B2, 0.75 ppm of Vitamin B1, 0.75 ppm of Vitamin B6, and 50 ppm of Vitamin C.

The experimental enrichment culturing of acetate-utilizing methane-producing bacteria was conducted employing the technique of anaerobic liquid culturing, as illustrated in Figure 2, using liter-size glass bottles as culturing reactors that were filled with the "#16" liquid medium. Each of the liquid culturing bottle reactors was sealed with an airtight rubber cap that was installed with a clinic glass cylinder with a needle to collect any gas product (including CH_4 and CO_2) from the headspace of the anaerobic liquid culture in each bottle reactor. The anaerobic liquid conditions were established by adding small amounts of reducing chemical compound ($Na_2S.9H_2O$) for the elimination of any residual O_2 by its reduction chemistry in the liquid medium so that the redox potential (E_h) of the liquid culturing medium was maintained at about – 100 mV, which was measured with a redox-measuring electrometer.

Note the acetate-utilizing methanogen cells that the enrichment culturing experiment was designed to enrich were known to be strictly anaerobic bacteria (archaea such as *Methanosarcina*). They cannot grow under aerobic conditions since they could be inactivated by any exposure to air oxygen. Under the strictly anaerobic conditions (the redox potential (E_h) of the liquid culturing medium was maintained at about – 100 mV), no other organisms except the acetate-utilizing methanogen cells could utilize the substrate acetate as shown in the ingredients of the "#16" liquid medium. Therefore, it was crucial to strictly keep the liquid culturing medium under anaerobic conditions, although sterilization of the liquid media in bottles was typically performed by autoclaving as well. The anaerobic conditions were accomplished and maintained through: 1) the use of reducing chemical compound ($Na_2S.9H_2O$) in the liquid medium as listed in the ingredients of the "#16" liquid medium; 2) the removal (or minimizing) of any residual air O_2 from the reactor headspace by filling the bottle reactor with the anaerobic liquid medium to near its full capacity (Figure 2); and 3) using airtight rubber caps to seal the anaerobic liquid medium bottles.

The anaerobic selective enrichment culturing experiment began with inoculation using a clinic needle injection of about 0.1 ml of the liquid inoculum (from the former Zhejiang Agricultural University's methane-producing anaerobic digestor) into a liter-size glass bottle containing about 1 liter of the "#16" liquid medium through its airtight rubber cap.

After the selective enrichment culturing under the anaerobic liquid incubation conditions for 30 days, the

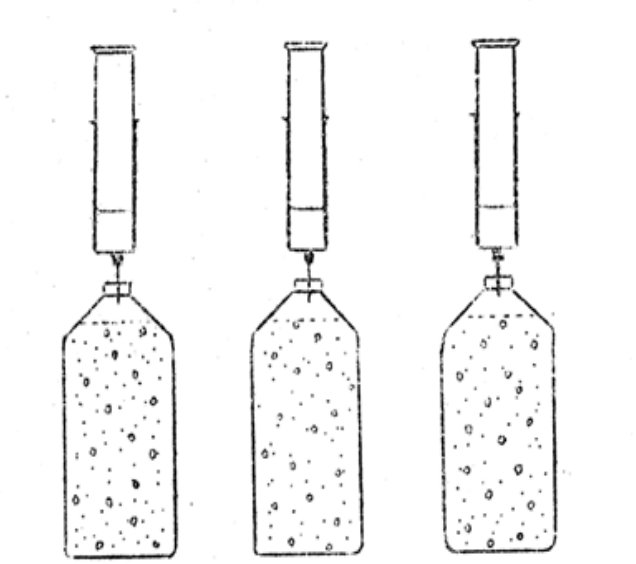

Figure 2. The anaerobic acetate-utilizing methane-producing bacteria enrichment experiment using liter-size glass bottle culturing reactors filled with the "#16" liquid medium and each sealed with an airtight rubber cap installed with a clinic glass cylinder with a needle to collect any gaseous product (including CH_4 and CO_2) from the headspace of the anaerobic liquid culture.

enrichment of acetate-utilizing methane-producing bacteria was finally accomplished. During this anaerobic liquid incubation process, about 40.5% of the acetate in the culturing reactor liquid medium was consumed, which was estimated from the amounts of gaseous products CO_2 and CH_4 as produced by the liquid culture. Its total gas production was 10,896 mL per L of the enriched methanogen liquid culture medium. About 74% of the collected gaseous products were determined to be methane (CH_4). Microscopic examination of the microorganisms in the enriched methanogen liquid culture showed that the microbes were mainly *Methanosarcina sp*. cells, as shown in Figure 3. The observed characteristics of "multicellular aggregates" (Figure 3) matched excellently with those reported by independent researchers (Maeder et al., 2006; Sowers et al., 1993) in a low-saline culture medium like our "#16" liquid medium.

Experiments Demonstrating Isothermal Heat Absorption of Enriched Acetate-Utilizing Methane Producing Bacteria

To demonstrate the isothermal heat absorption from the predicted thermotrophic activities, the methanogenic bacteria *Methanosarcina* cells obtained through the enrichment culturing above were subsequently used here to inoculate a freshly prepared acetate-based minimal cul-

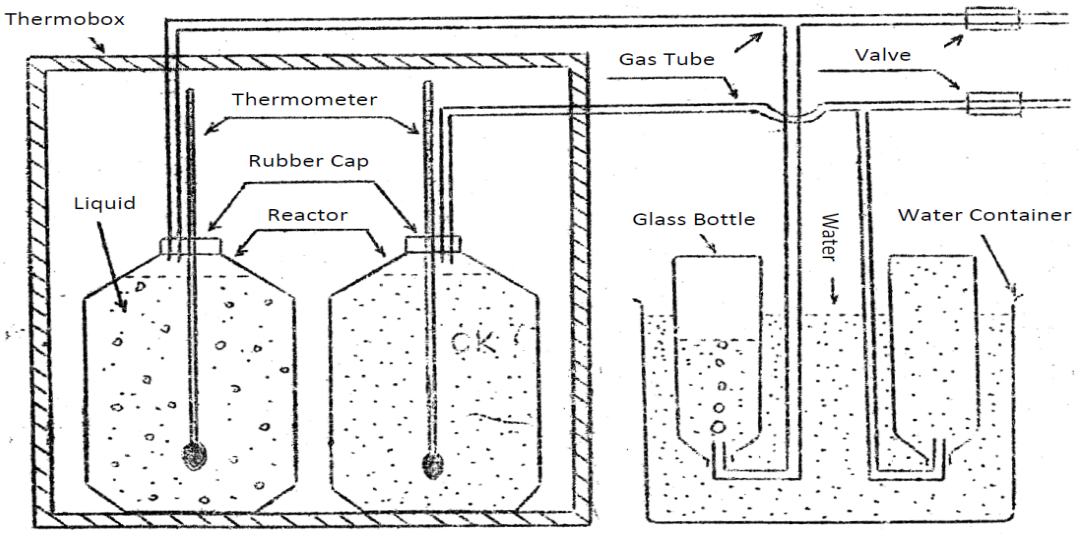

Figure 4. Illustration for the experimental apparatus comprising two 45-cm long Beckman mercury-based thermometers (temperature measuring range from 0 to 50 °C with detection precision of ± 0.01 °C) installed with two 5-bl size thermo-insulating bottle reactors through their rubber caps to monitor temperature changes in each of the two reactors. Each of the two thermobottle reactors was connected through the rubber cap with its plastic tubing system comprising a set of a tube valve and a gas-collecting bottle placed in a water tank to collect any gaseous product from the reactor headspace.

turing medium (also known as the "#16" liquid medium) in one of the two 5-bl size thermo-insulating bottle reactors (Figure 4), in comparison with the other reactor as a control that had the same physical setup and contained the same volume of the "#16" liquid medium except without the methanogen inoculation.

As shown in Figure 4, a special experimental apparatus was set up in a laboratory at the former Zhejiang Agricultural University. This experimental apparatus comprised two 45-cm long Beckman mercury-based thermometers (temperature measuring range from 0 to 50 °C with detection precision of ± 0.01 °C) that were installed with two 5-bl size thermo-insulating bottle (thermobottle) reactors through their rubber caps, respectively, to monitor temperature changes in each of the two reactors in real-time. Note the two 5-bl size thermobottle reactors were made of special heat-insulating double-glass walls with internal reflective coating and vacuum insulation to minimize heat exchange across the reactor walls.

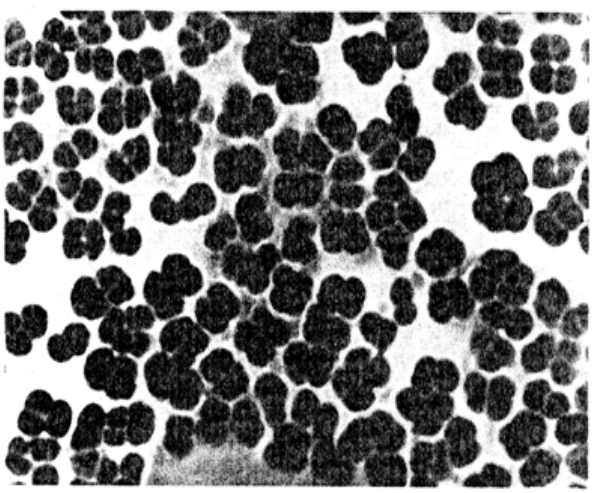

Figure 3. Microscopic imaging (1:8710) of the experimentally enriched acetate-utilizing methane-producing microbes that were identified mainly as *Methanosarcina sp*. cells.

Furthermore, to better control the experimental temperature and minimize heat exchange between the inside and outside of the thermobottle reactors so that the predicted methanogen isothermal heat absorption effect could be more clearly measured, the two thermobottle reactors were both placed in the same electric thermal box (Model GQ70B) with the thermostat of the electric thermal box set at the desirable physiology temperature of 37 °C for the methanogen activities (Weimer & Zeikus, 1978b).

In addition, the headspace for each of the two thermo-insulating bottle reactors was connected through its rubber cap with a plastic tubing system comprising a set of a tube valve and a gas-collecting bottle placed in a water tank to collect any gaseous product from the reactor headspace as shown in Figure 4.

At the beginning of each of the experiments, all the reactor items (including the thermobottle reactors and the "#16" liquid medium) were pre-heat equilibrated at

the temperature of about 37 °C. The two thermobottle reactors were filled with the "#16" liquid medium to the same liquid level, leaving a small (minimal) headspace for the possible gaseous product collection. Then, only one of the two thermobottle reactors was inoculated with the enriched methanogenic bacteria *Methanosarcina* cells while keeping the other reactor containing the same "#16" liquid medium (without methanogen inoculation) to serve as a control.

As illustrated in Figure 4, the experimental measurements for the methanogen isothermal heat absorption effect were performed by comparatively monitoring the temperature changes in the anaerobic liquid culture medium within each of the two thermo-insulating bottle reactors with and without the enriched methanogenic bacteria *Methanosarcina* cells.

The experiments were repeated four times. For each replication experiment, only the liquid culture of the enriched methanogenic bacteria *Methanosarcina* cells from the treatment reactor was swapped with the pure "#16" liquid medium (without inoculation) of the control reactor, while experimental apparatus including the two thermobottle reactors were remained (unchanged) at their same position. For example, the second reactor (marked "CK" in Figure 4), once used for a control, was subsequently used as a treatment reactor to be filled

Table 2. The results of the four replication experiments assessing the isothermal heat absorption of enriched acetate-utilizing methanogen, including *Methanosarcina sp*., by monitoring the methanogen gas production and their liquid medium temperature changes in comparison with the control (liquid medium without methanogen).

Replication of experiments	Time (hr)	Methanogen T (°C)	Control T (°C)	Δ T (°C)	Methanogen gas production rate (ml/L.hr)
I	0	36.96	36.96	0	0
I	7.25	36.90	36.97	-0.07	27.4
I	18.60	36.84	36.96	-0.12	27.2
I	23.25	36.82	36.96	-0.14	18.2
I	31.00	36.84	36.98	-0.14	16.6
I	42.75	36.34	36.98	-0.14	19.6
I	50.25	36.81	36.99	-0.18	24.6
II	0	36.88	36.88	0	0
II	19.47	36.77	36.80	-0.03	30.4
II	24.47	36.78	36.82	-0.04	20.0
II	34.47	36.80	36.89	-0.09	13.6
II	43.47	36.80	36.92	-0.12	13.4
II	53.47	36.80	36.97	-0.17	20.4
II	65.97	36.80	37.01	-0.21	31.0
II	70.97	36.80	37.03	-0.23	34.1
II	82.72	36.68	37.02	-0.34	47.0
II	93.47	36.35	36.80	-0.45	58.2
III	0	36.70	36.70	0	0
III	15.75	36.71	36.73	-0.02	24.0
III	25.75	36.65	36.70	-0.05	27.6
III	28.75	36.70	36.75	-0.05	33.4
III	39.25	36.72	36.78	-0.06	19.0
III	48.00	36.70	36.80	-0.10	13.4
III	50.25	36.68	36.80	-0.12	11.2
IV	0	36.70	36.70	0	0
IV	10.75	36.60	36.70	-0.10	28.0
IV	22.75	36.20	36.40	-0.20	42.0
IV	25.75	36.18	36.42	-0.24	58.0
IV	35.09	36.14	36.37	-0.23	4.6

with the #16" liquid medium inoculated with the enriched methanogenic bacteria *Methanosarcina* cells; while the first thermobottle reactor was then used as a control to be filed with the pure #16" liquid medium without any methanogens. In this way, any possible systematic error of the experimental apparatus comprising the thermobottle reactors, thermometers, and the other items of the system would be eliminated. Furthermore, during the 34 days of the experiment period, an additional 22.2 g of 36% acetic acid and 44.4 g of $Ca(CH_3COO)_2$ were gradually supplemented into the liquid culture for the enriched methanogenic *Methanosarcina* cells (typically at the beginning of each replication experiment) to maintain their active methane-producing metabolism for the replication experiments. A total of 25,685 ml of the gaseous product was generated per 2 L of the enriched methanogenic *Methanosarcina* cells liquid culture. About 74% of the collected gas volume was determined to be methane (CH_4). The temperature changes from the methanogen isothermal heat absorption, as observed through the four replication experiments, are presented in Table 2. At the end of the experiment, the anaerobic methanogenic bacteria in the experimental reactor were verified again by microscopic examination to be primarily the *Methanosarcina* cells as before.

As expected, we observed no gas production in the control thermo-insulating bottle reactor that contained the same volume of the pure "#16" liquid medium without any methanogen cells.

Statistical analysis of the experimental data (Table 2) showed that the temperature in the methanogen liquid culture medium (methanogen + "#16" liquid medium) decreased substantially in comparison with that of the control ("#16" liquid medium only). That is, the temperature of the active methanogen liquid culture medium consistently dropped by about −0.10 °C; the biggest observed temperature drop was as much as −0.45 °C. Taking the terminally observed temperature change from each of the four replication experiments, the mean temperature change (drop) and standard error (SEM) were calculated to be −0.25 ±0.06 °C. This experimental result (Table 2) supports the thermotrophic life hypothesis (Figure 1) since the observed isothermal heat absorption (utilization) was consistent with the predicted methanogen thermotrophic activities (Eq. 1).

In summary, the energy required for the anaerobic acetate-utilizing methane-producing bacteria *Methanosarcina* cells appeared indeed from the isothermal utilization (absorption) of environmental heat energy within the anaerobic acetate-based liquid medium (the "#16" liquid medium); When the anaerobic acetate-utilizing methane-producing *Methanosarcina* cells isothermally utilizing the environmental heat energy within the "#16" liquid medium, it caused the liquid temperature to consistently drop (observed as the temperature changes of about −0.10 °C and sometimes as much as −0.45 °C) in the experiments. Based on the terminally observed temperature changes from the four replication experiments, the mean temperature change (drop) and standard error (SEM) were calculated to be −0.25 ±0.06 °C as a result of the anaerobic acetate-utilizing methanogen activities.

As a conclusion, based on the observed isothermal utilization (absorption) of environmental heat energy, the anaerobic acetate-utilizing methanogenic archaea, including *Methanosarcina* may now be considered as "a living fossil specimen" of the thermotrophs (Figure 1) that manifest the predicted "Type-B" thermotrophic activities.

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AUTHOR CONTRIBUTIONS

Lee conceived the original idea for the thermotrophy hypothesis first in 1979, and he subsequently obtained research resource support (including materials & supplies, instruments, equipment, and lab spaces) from the former Zhejiang Agriculture University's Department of Agronomy and the Office of Research Administration

to conduct this exploratory research project to test the thermotrophy hypothesis. Lee then organized and led a group of seven fellow undergraduate students known as the "Thermotrophy Exploratory Research Group" and conducted the experimental study during a period from 1979–1982 at the former Zhejiang Agriculture University, Hangzhou, China. Lee designed and performed research, analyzed data, and wrote the article.

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