

Characterization of *Thermoplasma* Species Cultured from Sampling on Tangkuban Perahu, Indonesia

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Archaea is an organism with unique features because of its ability to inhabit extreme conditions. Our expeditions to Tangkuban Perahu, West Java aimed to obtain archaeal strains from the solfataric fields located in Domas crater. From the samples, we intended to culture *Thermoplasma* species growing around 55 °C below pH 2, which until now have not yet been fully characterized. We collected five samples from mud holes with temperatures from 52 °C to 57 °C and pH below 2. In serial cultures of up to 8 transfers in Freundt's medium we grew tetraetherlipid synthesizing *Thermoplasma* species as documented by phase contrast microscope. Total membrane lipid extracts were analysed by thin layer chromatography; the pattern matched total lipid extracts from *Thermoplasma acidophilum* DSM 1728 membranes. For confirmation, 16S rDNA identification performing PCR and sequencing were carried out. Analysis using BLAST showed *T. acidophilum* identities as the highest similarity of 99%, followed by *T. volcanium*, also with 99% similarity (AN KF776908 and AN KF776909). This is the first report of culturing cell-wall-less thermoacidophilic archaea, in particular *Thermoplasma* species in Indonesian laboratories.

Key words: archaea, Indonesian volcanoes, Tangkuban Perahu, tetraether lipid, *Thermoplasma*

Archaea merupakan organisme dengan fitur unik karena kemampuannya mendiami kondisi ekstrem. Ekspedisi ke Tangkuban Perahu, Jawa Barat dilakukan dengan tujuan untuk memperoleh galur archaea dari ladang solfatara yang terletak di kawah Domas. Dari sampel yang diperoleh selanjutnya dilakukan pengulturan spesies *Thermoplasma* pada kondisi berkisar 55 °C dan di bawah pH 2; pengulturan seperti ini belum dilaporkan ada yang melakukan. Sebanyak lima sampel diambil dari lubang lumpur dengan suhu berkisar 52 °C sampai dengan 57 °C dan pH di bawah 2 telah diperoleh. Dalam kultur seri hingga 8 kali transfer menggunakan media Freundt telah berhasil diperoleh kultur spesies *Thermoplasma* sintesis tetraether lipid yang kemudian di dokumentasikan menggunakan mikroskop fase kontras. Ekstrak total lipid membran dianalisis dengan kromatografi lapis tipis; pola yang didapat sesuai dengan jumlah pola ekstrak lipid membran *Thermoplasma acidophilum* DSM 1728. Untuk konfirmasi, identifikasi menggunakan 16S rDNA juga dilakukan dengan PCR dan kemudian dilanjutkan dengan sequencing DNA. Analisis menggunakan BLAST menunjukkan identitas *Thermoplasma acidophilum* dengan kesimilaritas tertinggi 99 %, dan identitas *Thermoplasma volcanium* juga dengan kesimilaritas tertinggi 99 % (AN KF776908 dan AN KF776909). Penelitian ini merupakan pengulturan archaea termoasidofilik tanpa dinding sel yang pertama di laboratorium di Indonesia, khususnya spesies *Thermoplasma*.

Kata kunci: archaea, gunung api Indonesia, lipid tetraeter, Tangkuban Perahu, *Thermoplasma*

Recently, we reported on isolates from Tangkuban Perahu, a volcano in West Java and culture growth in laboratories in Regensburg, Germany, and Depok, Indonesia (Handayani *et al.* 2012). We could demonstrate growth of *Sulfolobales* (Huber and Prangishvili 2004) in Regensburg by electron microscopy and obviously cultured members of the order *Thermoplasma* (Huber *et al.* 2002) in Depok, but the latter could not be characterized

(Handayani *et al.* 2012). Now, we focused on the isolation and culture growth of *Thermoplasma* species, e.g. *T. acidophilum* and *T. volcanium*. These organisms are cell wall-less thermoacidophilic archaea with unique tetraetherlipids, which have raised our interest in biomedical and biotechnical applications (Freisleben 1999). *Thermoplasma acidophilum* was first isolated by Darland *et al.* (1970) from sulfuric acid milieu in self-heated coal refuse piles. Later, *T. volcanium* *spp.* was found in solfataric hot springs (Segeer *et al.* 1988). Solfataric environments appear to be the natural habitat also of *T. acidophilum* (Yasuda

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et al. 1995). In Indonesia *Thermoplasma* species, which have not yet been further characterized, have been reported from TangkubanPerahu (Huber *et al.* 1991), an easily accessible volcano in West Java, South of Jakarta, near the city of Bandung.

Formerly, growth of *T. acidophilum* DSM 1728 was achieved in fermenters under laboratory conditions at pH 1.5 to 2.0 and an optimal growth temperature of 59 °C (Freisleben *et al.* 1994) as a major source of tetraether lipid, because the organism lacks a cell wall, and thus the membrane lipids are 'naked' and easily accessible. In nature, *Thermoplasma* members grow autotrophically metabolizing elemental sulfur, but they can also grow mixo- and heterotrophically, from anoxic to oxic conditions (Huber *et al.* 1991).

It is intended to optimize growth conditions, to identify and characterize the archaeal cells and to extract and purify their tetraether lipids for application in the biomedical field (e.g. liposomes, archaeosomes as drug and vaccine delivery systems) and in nanotechnology (e.g., monomolecular thin film surface coating) (Freisleben *et al.* 1995; Bakowsky *et al.* 2000; Patel *et al.* 2000; Schiraldi *et al.* 2002; Krishnan and Sprott 2008; Thavasiet *et al.* 2008). In the present study, the identification by molecular techniques performing PCR and sequencing was used. We applied a pair of primers designed by aligning 16S rRNA genes specifically targeting the gene encoding for 16S rRNA, followed by DNA sequence analysis using BLAST, as well as Clustal W and TreeView X^R.

MATERIALS AND METHODS

Sampling. On June 11, 2012 (samples KD 1,2) and January 21, 2013 (samples KD 3,4,5) sampling was carried out on the Indonesian volcano Tangkuban Perahu located in West Java, Bandung. Samples were taken from solfatara fields in the Domas crater (Kawah Domas, KD), under similar conditions as described by Handayani *et al.* 2012. In contrast to the former sampling the temperature range of our new samplings was quite lower; all samples were taken from mud holes between 52 °C and 57 °C, because we wanted to concentrate on *T. acidophilum* and *T. volcanium* species, which are known to grow in this temperature range.

The samples were obtained from acidic mud holes and warm springs from where a strong smell of H₂S was rising. Previous reports regarding archaeal habitats had shown that the growth temperature of *Thermoplasma* species is around 50 °C up to 60 °C (Huber *et al.* 1991; Yasuda *et al.* 1995) which was also

confirmed in fermentor growth (Freisleben *et al.* 1994). None of samples from five different mud holes and warm springs had a pH above 2; KD1 and KD5 were sampled at 57 °C; KD2 at 52 °C; KD3 at 56 °C; and KD4 at 54 °C. Samples were collected into 140 mL screw-capped glass bottles, filled to the top and firmly closed.

Freundt's medium was prepared according to Freisleben *et al.* (1994). All substances were dissolved to 1 L in aquabidest; the pH was adjusted to 2 with 10% H₂SO₄(v/v). The medium was autoclaved at 121 °C for 20 min.

Culture. The culture medium was composed of 1 L Freundt's medium, 200 mL of a solution containing glucose (20 g) and Difco yeast extract (DYE, 1 g) and 50 mL inoculum from KD samples. Aliquots of 300 mL were cultured micro-aerobically in closed 500 mL-culture bottles at pH 1-2 in an incubator at 55 °C with a shaker at 110 rpm. The culture bottles were prepared with a syringe through the rubber top for limited ("micro-aerobic") oxygen supply.

After 5 to 7 d the cultures were examined and documented with an Olympus Phase Contrast Microscope Model BX41-32000-2. Photos were taken using a Digital Microscope Camera Model Dp20 with its manufacturer-provided Camera Software. Cell counts were accomplished by means of a Neubauer Chamber. For serial cultures, aliquots of 30 mL were transferred to new culture bottles containing 300 mL of Freundt's medium pre-heated to 55 °C. Additional purification steps, e.g. either by plating, were not carried out since we considered enrichment by 8 serial transfers sufficiently selective.

Subsequently, cells from transfers 5 to 8 were harvested for further examination, such as genetic characterization and extraction of total membrane lipids. Aliquots of cells harvested from these cultures were kept as frozen stocks (*Thermoplasma* MBFKD-W2 and *Thermoplasma* MBFKD-B2) in the repository of the Laboratory of Microbiology and Biotechnology, Faculty of Pharmacy, Universitas Indonesia.

Extraction of Total Membrane Lipids from Cells (Antonopoulos *et al.* 2013). One gram of cells (Freisleben *et al.* 1994) was extracted three times with a total amount of 65 mL chloroform/methanol 1:1 (v/v). The cells were centrifuged after each extraction step and finally the combined extracts centrifuged at 1500 × g for 30 min.

Removal of Hydrophilic Contaminants by Two Phase Separation. For removal of hydrophilic-contaminants the lipid extracts were made biphasic by

the addition of chloroform and 0.1 M aqueous NaCl solution to achieve a ratio of chloroform/methanol/salt solution of 2:1:0.8 (v/v/v) (Folch *et al.* 1957). The separated chloroform phase was extracted for a second time with 1/4 of its volume methanol/aqueous NaCl 1:1 (v/v). The lower phase was filtered by a phase separation paper (MN 616wa Macherey-Nagel, Düren, Germany) and evaporated to dryness in a Rotavapor-R (Büchi, Flawil, Switzerland) with repeated addition of chloroform/methanol 3:1 in order to readily remove the water.

Thin Layer Chromatography. Thin layer chromatography (TLC) was carried out on 0.25 mm layers of silicagel (Merck, Darmstadt, cut to 10×5 cm) and developed in chloroform/methanol/water 65:25:4 (v/v/v).

Lipids were detected with sulphuric acid/methanol 1:9 (v/v) and heating at 140 °C. If heating was accomplished slowly the isoprenoid-derived lipids showed characteristic colours of red, brown or yellow before turning to black.

For comparison, total lipid extracted from *Thermoplasma acidophilum* DSM 1728 (Freisleben *et al.* 1994) was chromatographed under the same condition. Apart from the total lipid extract of strain DSM 1728, we had a fraction with mild hydrolyzation of the phosphoester of MPL (Antonopoulos *et al.* 2013). In TLC the respective band of MPL disappeared and instead, the MGL band at the front increased (Antonopoulos *et al.* 2013).

Molecular Genetic Identification DNA Extraction. Genomic DNA extraction were performed as described (Herrera and Cockell 2007; Bergmann *et al.* 2010) modified by the following procedure: After harvesting by centrifugation at 1 500 ×g for 30 min, the cells were homogenized and washed twice with 500 and 750 µL STET buffer, respectively (NaCl 100 mM; Triton X-100 5% v/v; EDTA 1 mM; Tris-HCl 10 mM, pH 8.0) by centrifugation for 3 min at 23 °C. The pellet was re-suspended in 557 µL of the same buffer and incubated with 10 µL lysozyme solution (10 mg lysozyme from chicken egg white (Sigma, St. Louis, MO) in 1 mL Tris-HCl 10 mM, pH 8) and 4 µL proteinase K (25 mg ml⁻¹) in the water bath for 1 h at 37 °C.

Subsequently, 65 µL of 5M NaCl and 80 µL of 4% (w/v) hexadecyltrimethylammonium bromide, CTAB (Sigma, St. Louis, MO) were added, vortexed, incubated in the water bath at 65 °C and after addition of 4 µL proteinase K further incubated in the shaking water bath at 37 °C for 1 h. The samples were taken from the water bath, cooled to RT, and incubated with RNase in the water bath at 37 °C for 15 min. DNA was

precipitated by adding 650 µL chloroform-isoamyl alcohol (24:1, v/v), vortexing for 10 sec and centrifugation at 1200 ×g for 20 min.

Again, 65 µL of 5M NaCl and 80 µL CTAB 4% were added, vortexed and incubated in the water bath at 65 °C. Supernatant was carefully removed into new Eppendorf cups. The pellet was re-suspended in 650 µL chloroform-isoamyl alcohol, vortexed for 10 sec and centrifuged at 1 200 ×g for 20 min. The supernatant was carefully removed into new Eppendorf cups.

Into 500 µL of supernatant, 400 µL of cold isopropanol were added and shaken very gently until white threads became visible. Concentrations measured in our samples were between 29.55 and 54.55 µg mL⁻¹. The DNA precipitates were dried *in vacuo* in a desiccator for 10 min, then re-suspended in 20 µL TE buffer (10 mM Tris-HCl, 1 mM disodium EDTA, pH 8.0) and stored at -20 °C until used.

Identification Using Polymerase Chain Reaction (PCR). For PCR identification of archaeal isolates, we applied primer design as reported (Slobodkina *et al.* 2004; Baker *et al.* 2001), modified to our condition. First, six *Thermoplasma* species 16S rDNA sequences (*T. acidophilum*, 2 sequence data, and *T. volcanicum*, 4 sequence data) were downloaded from NCBI database and aligned using Clustal W2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). Second, by using the *T. volcanicum* 16S rRNA gene sequence (accession number AJ299215.1) as reference, we designed the primers performing Clone Manager Suite^R. The result was further analyzed using the program available at <http://sg.idtdna.com/analyzer/Applications/OligoAnalyzer/>. The forward primer was 5'-GGAGATGGACTCTGAGACAACAG-3' and the reverse primer 5'-CTACGGTACGAGCTGACGACG-3'. PCR was run in a reaction mixture (50 µL) containing approximately 0.20 µg genomic DNA, 8 pmol of each primer, 1 µL 10× buffer, 1.6 mM MgSO₄, 2 mM of each deoxyribonucleotide triphosphate (dNTPs), and 1 U of KOD DNA polymerase on a thermal cycler. The temperature profile was as follows: initial DNA denaturation for 4 min at 95 °C; then 34 cycles of denaturation at 94 °C for 30 s and 58 °C primer annealing for 30 s, extension at 70 °C for 45 s, and final extension at 70 °C for 4 min.

Visualization of PCR products. Amplification products (2 µL) were visualized in agarose gel according to standard protocols. For casting the gels, agarose was dissolved in 1% Tris-acetate-EDTA (TAE) buffer (Cytrynet *et al.* 2000). Ethidium bromide was added prior to gel casting with very careful handling.

After running the gels for 30 min on a horizontal gel electrophoresis device, DNA bands could be visualized on UV transilluminator connected to a documentation system. The PCR product was further analyzed by DNA sequencing (1st BASE, Singapore).

Analysis of DNA Sequence Data and Nucleotide Sequence Accession Numbers. The DNA sequence information obtained was analyzed by BLASTserver maintained at the National Center for Biotechnology Information, Bethesda, MD (<http://www.ncbi.nlm.nih.gov>), i.e. nucleotide BLAST. Furthermore, the partial 16S rDNA sequences were analyzed by using Clone Manager Suite^R and aligned with known 16S rDNA of *Thermoplasma* species downloaded from database GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>).

The search for similarity or homology of DNA sequences was done on-line using the BLAST server maintained at the National Center for Biotechnology Information (NCBI), Bethesda, MD (<http://www.ncbi.nlm.nih.gov>). To check the relationship of our strain to other existing *Thermoplasma* species, we performed neighbor-joining method by Clustal W^R to create a phylogenetic tree of closely related 16S rDNA sequences. The tree was edited by means of TreeViewX^R software.

The DNA sequences obtained in this study have been deposited under GenBank AN KF776908 and AN KF776909 in NCBI database from where they can be uploaded by employing BankIt^R.

RESULTS

Culture Condition. From KD samples up to 8 serial cultures were grown under conditions, which are preferred by *Thermoplasma* species, i.e., microaerophilically at 55 °C in Freundt's medium at pH below 2. To follow the culture growth, samples were taken from the cultures at times indicated and optical density (OD) was read at λ 578nm. Fig 1 shows KD3 culture development of transfers 1, 3, and 5 over a period of two weeks. We observed a lag phase of 5 days in culture 1, reduced to 3 days after five transfers with OD<0.1, then a log/exponential phase to 7-8 d and OD=0.35. Highest OD of 0.4 was measured in the culture after 5 transfers on day 10, which was already considered as late stationary phase, where the OD already started to decrease. From growth behaviour, it was decided that cultures should be harvested not later than on day seven (between 160 and 170 hours). Fig 2 shows serial culture 3 in the phase contrast microscope. Diameter determination ranged from 0.9 to 1.7 μ m with a mean value of 1.2 μ m. Counts in the Neubauer Chamber resulted in 57×10^6 cells mL⁻¹.

Cells were harvested on the 7th day of culture and used for the extraction of total membrane lipids and the extraction of DNA.

Comparison of Total Lipid Extract. From two serial cultures total lipid was extracted according to

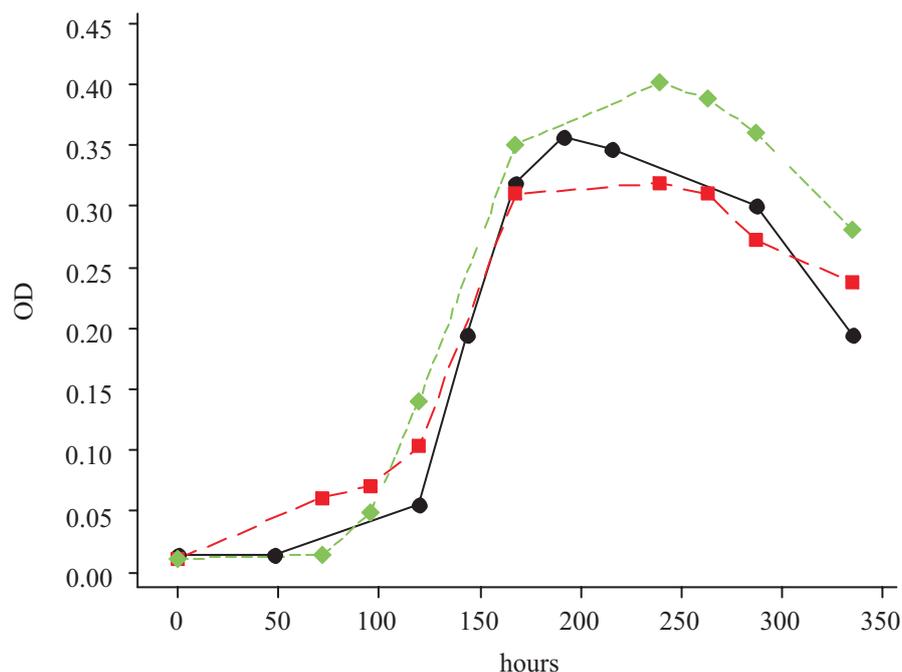


Fig 1 Serial cultures from KawahDomas KD3 sample to optimize growth conditions for *Thermoplasma* species. Depicted are generations 1, 3, and 5 monitored by OD at λ 578 nm. —●— : KD3 Gen 1, —■— : KD3 Gen 3, and —◆— : KD3 Gen 5.

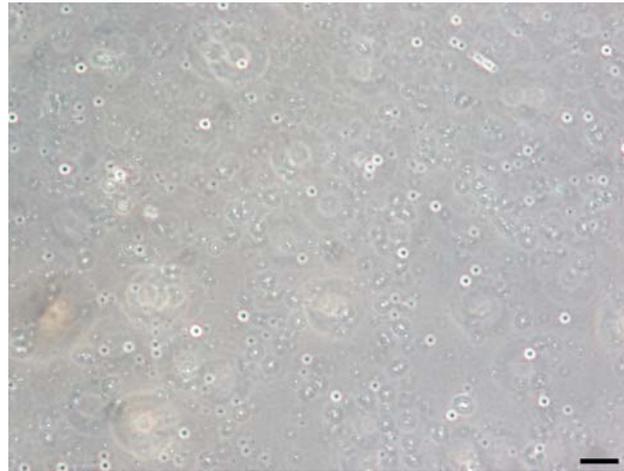


Fig 2 Phase contrast image of the third serial culture of sample KD3. Magnificationx200 (scale bar = 5 μ m), characteristic cells of *Thermoplasma*.

Antonopoulou *et al.* (2013) and compared to total lipid extract from *T. acidophilum* DSM 1728. The result is shown in Fig 3. Mild hydrolysis of the main tetraetherglycophospholipid (MPL) splits the phosphoester and yields the main tetraetherglycolipid (MGL). This reaction demonstrates the position of the two lipids in the chromatogram. The band of MGL at

the top of the chromatogram mixes with the apolar dye present in the total membrane lipid extract from all cultures. In general, the pattern of the extracts from TP isolates matches exactly that of *T. acidophilum* DSM 1728 (Antonopoulou *et al.* 2013).

**Primer for PCR and Molecular Identification.
Alignment of *T. acidophilum* and *T. volcanium* 16S**

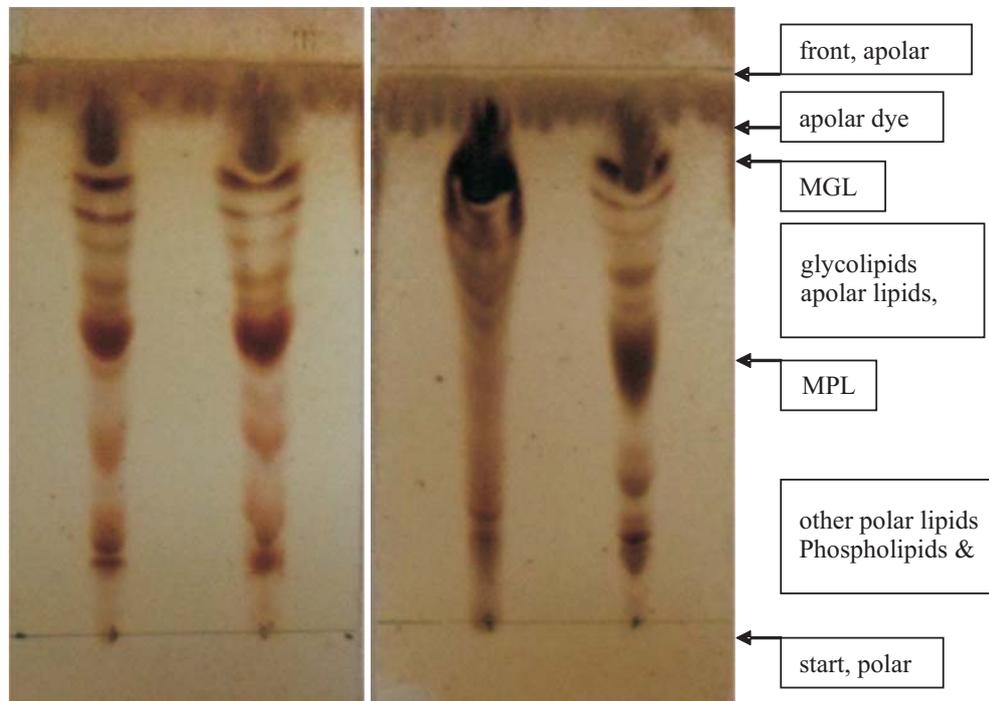


Fig 3 Total membrane lipid extracts from cultures MBFKD-W2 and MBFKD-B2 (left plate, lanes 1 and 2) of sample KD3; Fig. 3(b) (right plate, lanes 3 and 4) show the comparison of the total membrane extract from *Thermoplasma acidophilum* strain DSM 1728. Lane 3; the phosphoesters or the tetraetherglycophospholipids of the latter had been mildly hydrolyzed; the phospholipid bands in the middle part of the chromatogram are not present, especially the thickest band of the main phospholipid (MPL) disappeared. Instead, the band of the main glycolipid at the top of the chromatogram is much thicker than in the other lanes. Lane 4 is total membrane extract from *Thermoplasma acidophilum* grown under conditions for maximum MPL yield (Freisleben *et al.* 1994; Antonopoulou *et al.* 2013). MPL, main polar lipid = main (glyco)phospholipid; MGL, main glycolipid.

rRNA Genes. Several 16S rRNA gene sequences of *Thermoplasma* species were aligned by performing Clustal W. Using *T. volcanium* (AJ299215) as reference for primer design the resulting target region was between nt255 and nt978. The pair of primers was designed from the regions at nt 255-277 and nt 956-978, both for forward and reverse primers.

Two PCR products (W2 and B2) were generated and chosen for DNA sequencing after gel visualization as shown in Fig 4, each was obtained from two genomic DNA samples extracted from two different cell cultures but from the same starter culture.

Alignment of the first PCR product exerted 99% similarity with *T. acidophilum*, followed by *T. volcanium* with the same percentage (99%). The tree of

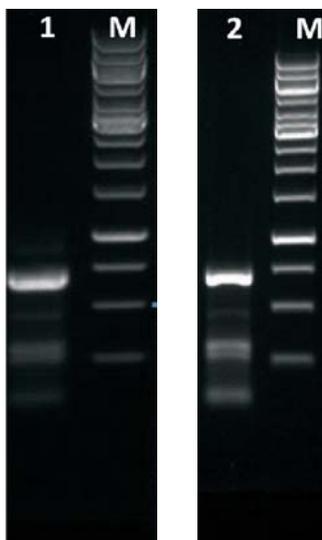


Fig 4 Agarose gel electrophoresis of 16S rDNA PCR of genomic DNAs; lane 1 = MBFKD-W2; lane 2 = and MBFKD-B2; lanes M = DNA ladder (250 - 1000 kb).

phylogenetic relationship (Fig 5) shows the closest NR 028235 and M38637.1, which are both *T. acidophilum*. Hence, we conclude that our cultures from KD isolates contain *Thermoplasma* strains belonging to the species *T. acidophilum*, but also contain *T. volcanium* strains.

DISCUSSION

From our samples we grew serial enrichment cultures with up to 8 transfers. As expected, growth behavior did not change significantly in the serial cultures. We used the medium and pH according to the culture conditions of Freisleben *et al.* (1994); however, growth temperature was slightly lower (55°C) than the optimum laboratory temperature of 59 °C. Limited air

(oxygen) supply was obtained by a syringe through the tight rubber stopper of the half-litre culture bottles (empty half-litre aquabidest bottles filled 2/3 volume with culture medium). Cultures can certainly still be optimized for faster growth to higher cell concentration and higher contents of desired lipids, e.g. MPL or MGL.

Our main intention is to obtain special tetraether lipids from these cells. Hence, we extracted the membranes with the method described in Antonopoulos *et al.* (2013) and compared the extracts from cultured Kawah Domas samples with those from *T. acidophilum* strain DSM 1728. To denote the position of the main glycolipid (MPL) in TLC, we applied mild hydrolysis to split the phosphoester in MPL (the only ester in the compound) to yield the main glycolipid MGL (Antonopoulos *et al.* 2013). Hence, the thick band of MPL in TLC should disappear and concomitantly, the band of MGL should become thicker (see TLC, right plate, lane 3). The total membrane extract contains a contaminating yellow-brownish apolar dye almost co-migrating with the solvent front and merging with the high amounts of MGL in lane 3. Smaller amounts of the latter separate from the dye (TLC lanes 1 and 2). Summarizing this part of our study we can state that the TLC pattern of total membrane extracts from cultured KD isolates matches exactly the pattern obtained from *T. acidophilum* DSM 1728 extracts indicating that we cultured *Thermoplasma* species from the isolates.

In Indonesian biotopes *Thermoplasma* species have not been further identified except for Huber *et al.* (1991). Hence, it is essential to compare their results with ours. Starting from the description of isolate sampling, the location and the habitat conditions were identical with ours: solfataric mud holes with strongly acidic pH and moderately hot temperatures around 50 °C (in our case pH 2 and 52-57 °C). Huber *et al.* (1991) enriched [citation] “cell wall-less highly irregular coccoid thermo acidophilic archaea” in Darland’s medium (Darland *et al.* 1970); we used Freundt’s medium as published (Freisleben *et al.* 1994).

Huber *et al.* (1991) cloned the cultured microorganisms on starch-solidified medium at 60 °C under an air-reduced atmosphere and obtained small “fried egg”-shaped colonies after 4 d of incubation. The authors concluded that the Indonesian *T. volcanium* strain differed from *T. volcanium* strains isolated from other places in the world, but had also some indication of *T. acidophilum*: Cell extracts of their isolate KD3 DSM 4300 showed serological cross-reaction with antibodies prepared against the histone-

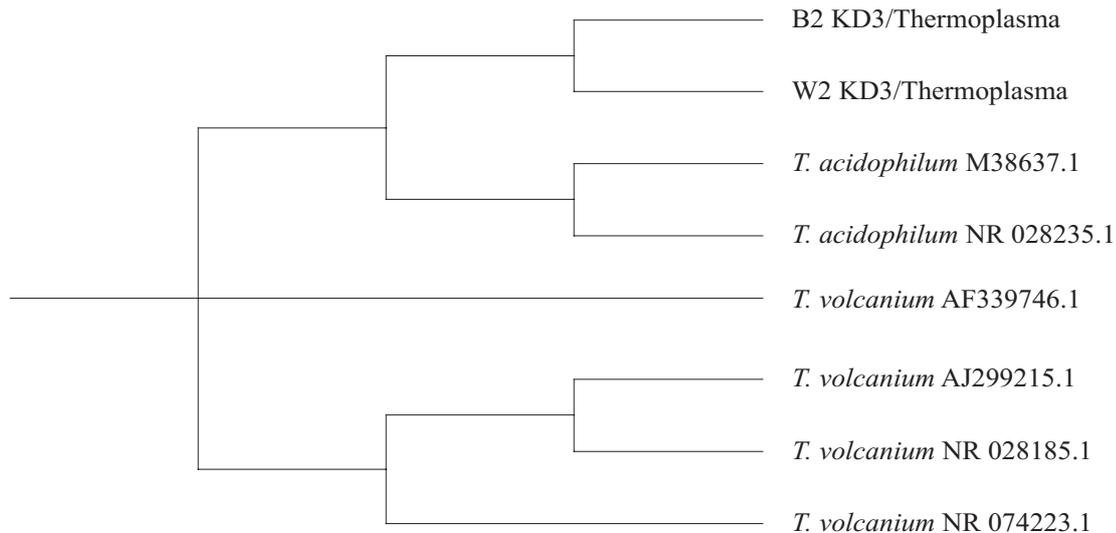


Fig 5 Phylogenetic tree of *Thermoplasma* species with strains MBFKD-W2 and MBFKD-B2 from isolate KD3, Domas Crater, TangkubanPerahu, West Java, Indonesia. The tree was created by alignment using Clustal W2 and by performing neighbor-joining method.

like protein of *T. acidophilum* (DSM 1728).

From our results, we interpret that our samples from Kawah Domas (TP) and subsequent cultures contain both, *T. acidophilum* and *T. volcanium* and we assume that they can be found together also in other Indonesian biotopes. This clarifies the question raised by Huber *et al.* (1991) concerning the above cross-reaction. Furthermore, the authors discuss the differences in GC-content of 46% in *T. acidophilum* and 38% in *T. volcanium* isolated so far from several habitats around the world, whereas the Indonesian isolate KD3 DSM 4300 exerted a GC-content of 40%. No wonder that GC-content is in between, since we found both species.

Outlook. It is our intention to scale-up *Thermoplasma* cultures sampled from Tangkuban Perahu in fermenters to obtain sufficient amounts of tetraether lipid for the production of acid-stable liposomes or archaeosomes which can be applied to oral drug and vaccine delivery. Indonesia, a hotspot of extremophilic archaea has been left behind for decades in this area of research. We hope that our successful cultivation and identification of *Thermoplasma* will induce enthusiasm to further investigate extremophilic forms of life from biotopes in this country.

Note. It should be mentioned that it was not intended to have the same isolate ID in our study as Huber *et al.* (1991), i.e., “KD3”. We had various isolates with varying pH and temperature, numbered KD1-KD5 in three samplings; our KD3 from the last sampling on January 21st, 2013 turned out successful in culturing *Thermoplasma* species. From other samples, we managed to culture *Sulfolobus* (Handayani *et al.*

2012). Hence, the identity of “KD3” with the one of Huber *et al.* (1991) is unintentional.

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