Eco. Env. & Cons. 27 (3) : 2021; pp. (1186-1195)

ISSN 0971–765X

Identification of the Archaeon *Picrophilus torridus* isolated and cultured from Tangkuban Perahu and Dieng Plateau on Java Island, Indonesia

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(Received 15 November, 2020; Accepted 7 January, 2021)

ABSTRACT

Indonesia is a tropical country consisting of about 17,000 islands and located in the ring of fire that is rich in geothermal activities and biodiversity, from the deepest seas to the mountain peaks. Knowledge about microbial ecology, especially concerning Archaea is still scarce in Indonesia. These microorganisms often live in extreme areas at hot temperatures and acidic conditions of volcano craters and solfatara mudholes and fumaroles. Sampling from Tangkuban Perahu and Dieng Plateau and subsequent culturing at 60 °C and pH around 1 now led to the identification of *Picrophilus torridus*, a member of the order *Thermoplasmatales*. The *Picrophilus* genus had not yet been reported from Indonesia, before.

Key words : *Thermoplasmatales, Picropilus torridus, Dieng Plateau, Tangkuban Perahu, Volcano craters.*

Introduction

Indonesia is rich in archaeal habitats and an ideal place to collect archaeal samples from nature. However, investigation of archaea is still at the beginning and continuous long-term research projects on these domestic organisms have not yet been established.

Huber *et al.* (1991) reported on thermophilic archaea in Indonesian hydrothermal areas. On Java island, they isolated hyperthermophilic and thermoacidophilic archaea such as *Thermoplasma, Thermoproteus, Acidianus*, and *Desulfurococcus* species from hot springs and solfatara fumaroles on Dieng Plateau (DP) and Tangkuban Perahu (TP) (Huber *et al.*, 1991). Moreover, huge methane sources under the East Java Sea appear due to methanogenic archaea (Noble and Henk, 1998).

From 1994 to date, some archaeal technologies have been transferred to Indonesia in cooperation with German scientists (Freisleben, 1994; Freisleben 1999; Freisleben 2012; Freisleben and Deisinger, 1998), especially the isolation and culture of archaea from TP (Handayani *et al.*, 2012; Malik *et al.*, 2014), lipid extraction from archaeal membrane and purification of tetraether lipid (Antonopoulos *et al.*, 2013; Freisleben, 2019) and its application for stable liposomes (Oertl *et al.*, 2016). Since only few microorganisms have been identified, very limited information

is available on the wide range of archaea on TP and even less from other hyperthermophilic or thermoacidophilic solfatara fields and volcanoes in Indonesia. Hence, the purpose of this investigation was to further isolate, culture and identify archaeal species from TP and DP on Java island.

The main objective of future research is the application of these microorganisms for biomedical purposes as suitable candidates for drug and vaccine delivery systems (Freisleben, 2000) and possibly – with modifications – also for gene therapy (Balakireva and Balakirev, 2000). Another aspect of the nano-biotechnical use of these archaea will be for metal leaching in waste management and biomining (Ni *et al.*, 2018; Dopson *et al.*, 2004; Dopson and Holmes; Zhu *et al.*, 2013), monomolecular thin layer coating (Vidawati *et al.*, 2011; Liefeith *et al.*, 2018) for the construction of light-driven biological batteries (Freisleben *et al.*, 1995) or for the in vitro production of biological compounds like peptides and proteins (Berhanu *et al.*, 2019).

Materials and Methods

Sampling

During 2019, samples were taken from 3 mudholes on TP located at Domas Crater, with temperatures of 60, 80, and 100 °C, named TP1, TP2, and TP3, respectively. On DP, samples were drawn from two mudholes (Fig. 1) located at Sikidang Crater, named DP1 (temperature of 60 °C) and DP2 (80°C). At all locations from which samples were drawn pH was 2 or below, measured with pH universal indicator strip Merck (Merck KGaA, Darmstadt, Germany).

Fig. 1. Sample taken during sampling at Dieng Plateau on August 24, 2019

Culture

Culture flasks on a shaker

We followed the procedure reported by Malik *et al.* (2014). Once a week cells were subcultured at 60 °C and at pH around 1 (clearly below pH 2) and then stored at -4 °C (= stock culture A). Into a 1-Liter or 2-Liter flask 100 or 200 mL (10% of total volume) stock culture A were inoculated on a B. Braun HT shaker and incubated at 120 UpM and at 60 °C (Figure 2).

Fig. 2. (A) Culture flasks

(B) Incubator with shaker

Composition of the medium

84 % Freundt's solution
5% glucose solution (stock solution 200 g/l)
1% difco yeast extract (stock solution 200 g/l)
10% inoculum

During up to 10 serial cultures, growth temperature was kept at 60 °C and pH around 1; the addition of the yeast extract increased pH, which had to be compensated by adjusting the pH back below 2 by cautious and simultaneous addition of sulfuric acid. Measuring of pH in the culture medium during the growth was done together with the determination of optical density (OD) using a Shimadzu photometer at wavelength λ of 578 nm.

In addition to OD, the solution was checked before inoculation and in the last culture sample before harvesting, under the phase-contrast microscope at 400x magnification, especially for contamination, e.g., by *Bacillus acidocaldarius,* which organism might be able to grow into the culture under the given condition. This contamination can be detected microscopically as much larger cells than *Thermoplasma* and *Picrophilus*.

Molecular genetic identification

DNA extraction, polymerase chain reaction (PCR, DNA sequencing and TEM analysis

For identification, we used cultures from samples TD1 and DP1. Fifty milliliters of culture medium were centrifuged and the pellet containing the archaeal cells was washed with phosphate-buffered saline (PBS). We continued with the Geneaid™ DNA Isolation Kit (Geneaid Biotech Ltd, New Taipe City, Taiwan) to extract the genomic DNA of the microorganism. Concentration and purity were determined by means of a Denovix™ DS-11 FX spectrophotometer. Subsequently, PCR was conducted with a miniPCR™ thermal cycler machine and DNA sequencing detection following the PCR method of Malik *et al.* (2014). For identification of the species by DNA sequencing, the PCR product was sent to Macrogene Laboratory, Korea, and to 1st Base Laboratory, Malaysia, to compare the result and blast the DNA sequence by Blast Nucleotide NCBI free analysis. The results presented here are from sample TP1.

We analyzed the structure of *Picrophilus torridus* by Transmission Electronic Microscopy (TEM) at University Gadja Mada, Yogyakarta.

Results

Sampling and culture growth

Culture growth of samples from TP and DP was conducted sequentially up to 10 generations. Every 5 days, 10% of the volume of the culture of microorganisms was taken and inoculated into the medium for the next generation. The OD was checked spectrophotometrically for growth density. Figure 3 shows the growth of the archaea.

Microscopical analysis using phase-contrast microscopes showed the growth of living microorganisms, shape and size resembling the microscopical picture of *Thermoplasma* cultures (Figure 4 A, B).

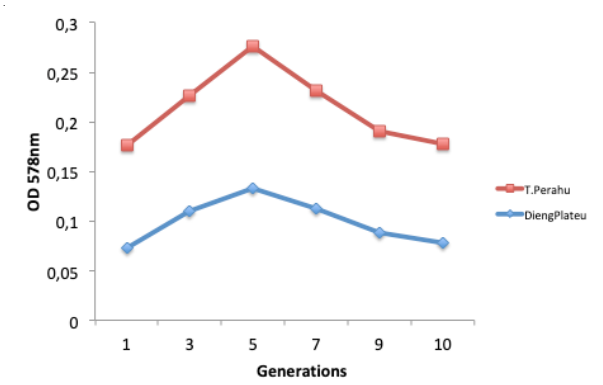


Fig. 3. Optical density (OD) at λ = 578 nm; serial culture generations of archaea from Tangkuban Perahu (TP1) and Dieng Plateau (DP1).

Phase-contrast microscopy was conducted in two laboratories exerting identical results, at the Faculty of Health Sciences, Universitas Islam Negeri Syarif Hidayatullah in Jakarta, and at the Center for Infectious Diseases, University of Heidelberg, Germany. Figure 4 (A, B) was taken by a camera-connected Nikon Eclipse Ts2 phase-contrast microscope showing living cells resembling archaea by size and appearance.

The transmission electron microscopy revealed the S-layer around the cell as reported (Schleper *et al.*, 1995). The central brighter part in the cytoplasm possibly indicates a vacuole.

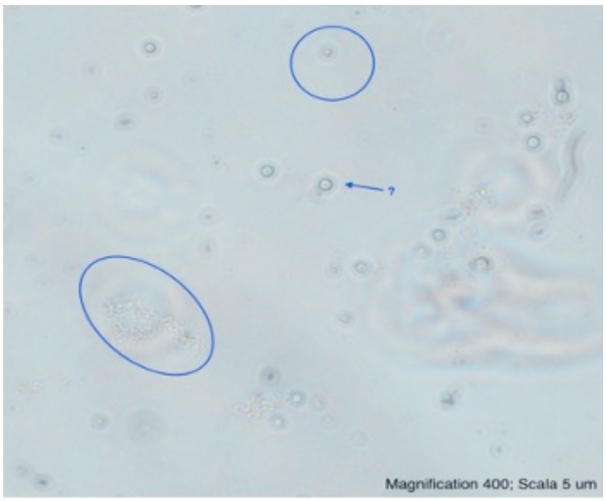


Fig. 4a. Phase-contrast microscopy of the 5th serial culture generation in early logarithmic phase. The arrow (→) and the upper circle show single cells with a diameter of about 1 µm; the lower oval circle surrounds living cell conglomerations.

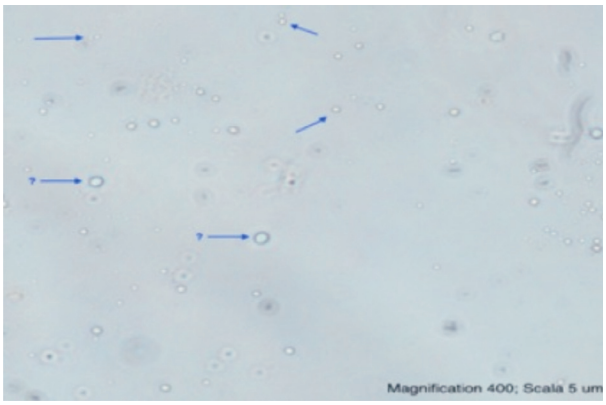


Fig. 4b. Phase-contrast microscopy of the 5th generation from serial archaeal cultures in late logarithmic phase. The arrows without question mark show single cells. Figure 4 A, B were taken by Nikon Eclipse Ts2; magnification x 400.

Molecular genetic identification

Before sequencing, concentration and purity of the DNA samples were measured. In Figure 5A, the graph the DNA concentration of the sample from Tangkuban Perahu (TP1) shows 1058 ng/µl with a genome purity of 2.17 (OD 260/280), whereas the sample from Dieng Plateau (DP1) was 762 ng/µl with genome purity of 2.09. The molecular genetic identification followed the methods of Malik *et al.*, (2014) who analyzed the 16S rRNA genes of *Thermoplasma* species. Figure 5B shows the major PCR product with an approximate length of 700 bp. Further analysis of the PCR product by DNA sequencing identified *Picrophilus torridus*. Figures 5C and 5D show the sequence chromatograph and Blast nucleotide result after DNA sequencing. The alignment of the PCR product showed 99% similarity with *Picrophilus torridus* 16S ribosomal RNA. The phylogenetic relationship of *Picrophilus* is closest with *Thermoplasma* (Figure 6).

The GC content of 16S rRNA was calculated as

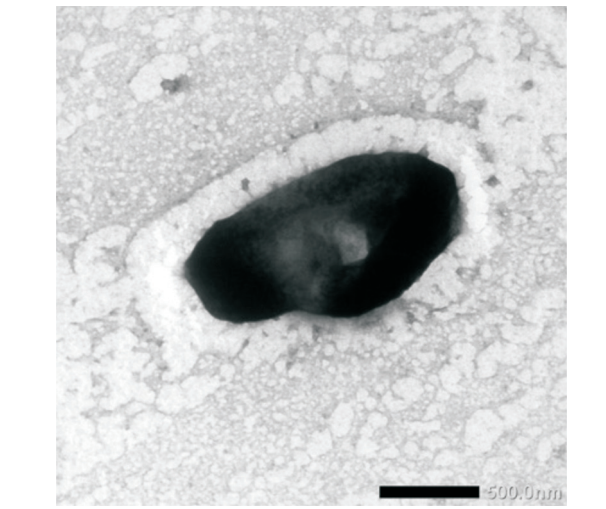


Fig. 4c. Transmission electron micrograph (TEM) of *Picrophilus*, six[th] generation, negatively stained with uranyl acetate 1%; pleomorphic shape, size: 1.4x0.75 µm, S-layer clearly visible. TEM accomplished with a JEM 1400, JEOL USA, by the UGM-TEM Service Center, Universitas Gadjah Mada (UGM), Yogyakarta, Indonesia.

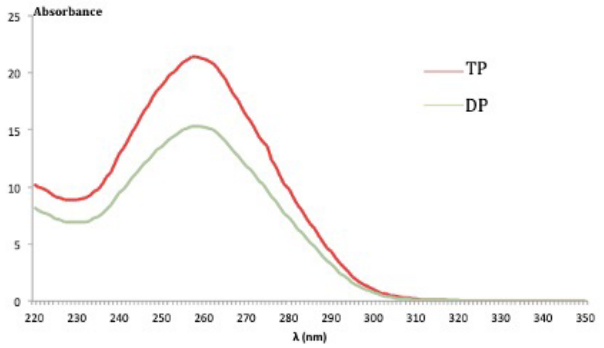


Fig. 5a. The absorbance graph of concentration of the genome samples (an absorbance value of 1.5 using the Smart Path 0.02 mm pathlength is equivalent to a 1 cm (10 mm) pathlength absorbance value of 750).

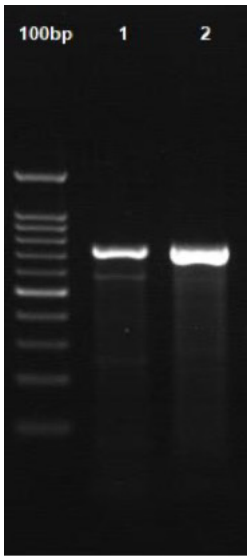
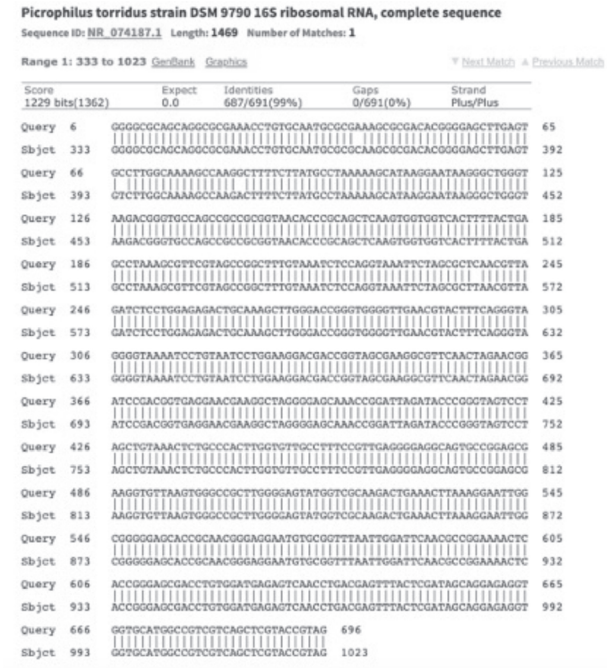


Fig. 5b. PCR product on agarose gel 1.3%; a volume of 1 µL sample (0.5 µg) was loaded per lane; 100bp, DNA ladder, lanes 1 and 2 are PCR products (1 = DP1; 2 = TP1).

Fig. 5c. Sequence chromatogram of the PCR product by Geneious Prime Software; green color represents thymine (T); yellow, guanine (G); red, adenine (A); blue, cytosine (C). Highlighted parts mean that the chromatogram could not separate clearly the signal from noise and the software had to remove the noise.



Picrophilus torridus strain DSM 9790 16S ribosomal RNA, complete sequence

Sequence ID: NR_074187.1 Length: 1469 Number of Matches: 1

Range 1: 333 to 1023 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
1229 bits(1362)	0.0	687/691(99%)	0/691(0%)	Plus/Plus

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Query  6     GGGGCGCAGCAGGCGCGAAACCTGTGCAATGCGCGAAAGCGCGACACGGGGAGCTTGAGT  65
Sbjct  333   GGGGCGCAGCAGGCGCGAAACCTGTGCAATGCGCGCAAGCGCGACACGGGGAGCTTGAGT  392
Query  66    GCCTTGGCAAAAGCCAAGGCTTTTCTTATGCCTAAAAAGCATAAGGAATAAGGGCTGGGT  125
Sbjct  393   GTCTTGGCAAAAGCCAAGACTTTTCTTATGCCTAAAAAGCATAAGGAATAAGGGCTGGGT  452
Query  126   AAGACGGGTGCCAGCCGCCGCGGTAACACCCGCAGCTCAAGTGGTGGTCACTTTTACTGA  185
Sbjct  453   AAGACGGGTGCCAGCCGCCGCGGTAACACCCGCAGCTCAAGTGGTGGTCACTTTTACTGA  512
Query  186   GCCTAAAGCGTTCGTAGCCGGCTTTGTAAATCTCCAGGTAAATTCTAGCGCTCAACGTTA  245
Sbjct  513   GCCTAAAGCGTTCGTAGCCGGCTTTGTAAATCTCCAGGTAAATTCTAGCGCTTAACGTTA  572
Query  246   GATCTCCTGGAGAGACTGCAAAGCTTGGGACCGGGTGGGGTTGAACGTACTTTCAGGGTA  305
Sbjct  573   GATCTCCTGGAGAGACTGCAAAGCTTGGGACCGGGTGGGGTTGAACGTACTTTCAGGGTA  632
Query  306   GGGGTAAAATCCTGTAATCCTGGAAGGACGACCGGTAGCGAAGGCGTTCAACTAGAACGG  365
Sbjct  633   GGGGTAAAATCCTGTAATCCTGGAAGGACGACCGGTAGCGAAGGCGTTCAACTAGAACGG  692
Query  366   ATCCGACGGTGAGGAACGAAGGCTAGGGGAGCAAACCGGATTAGATACCCGGGTAGTCCT  425
Sbjct  693   ATCCGACGGTGAGGAACGAAGGCTAGGGGAGCAAACCGGATTAGATACCCGGGTAGTCCT  752
Query  426   AGCTGTAAACTCTGCCCACTTGGTGTTGCCTTTCCGTTGAGGGGAGGCAGTGCCGGAGCG  485
Sbjct  753   AGCTGTAAACTCTGCCCACTTGGTGTTGCCTTTCCGTTGAGGGGAGGCAGTGCCGGAGCG  812
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Sbjct  813   AAGGTGTTAAGTGGGCCGCTTGGGGAGTATGGTCGCAAGACTGAAACTTAAAGGAATTGG  872
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Sbjct  873   CGGGGGAGCACCGCAACGGGAGGAATGTGCGGTTTAATTGGATTCAACGCCGGAAAACTC  932
Query  606   ACCGGGAGCGACCTGTGGATGAGAGTCAACCTGACGAGTTTACTCGATAGCAGGAGAGGT  665
Sbjct  933   ACCGGGAGCGACCTGTGGATGAGAGTCAACCTGACGAGTTTACTCGATAGCAGGAGAGGT  992
Query  666   GGTGCATGGCCGTCGTCAGCTCGTACCGTAG  696
Sbjct  993   GGTGCATGGCCGTCGTCAGCTCGTACCGTAG  1023
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Fig. 5d. Blast DNA sequencing result from TP1: 99% similarity with *Picrophilus torridus* strain DSM 9790 ribosomal RNA.

55% matching reported NCBI Reference Sequence: NR_074187.1 (Subhrayeti *et al.*, 2017).

Discussion

Huber *et al*. (1991) reported the isolation of archaeal *Thermoplasma* strains from DP - in Sileri Crater (Kawah Sileri) from a tropical swamp with an original temperature of 32 °C and pH of 3.5 and in Sikidang Crater (Kawah Sikidang) from several samples with original pH of 1.5-3 and temperatures of 27-50°C - and on TP from Domas Crater (Kawah Domas) solfatara fumaroles several samples at original pH of 1.5-3 and temperatures from 32 to 58 °C. It was concluded that these places may be natural biotopes for these thermo-acidophilic archaea. Only few of these samples were aerobic, most of them anaerobic (or microaerobic), because of their highly sulfuric environment. Higher temperatures up to 94 °C and a pH range from 1 to 5.5 had also been investigated, possibly containing *Acidianus, Thermoproteus, Desulfurococcus* spp., and a *Sulfolobus*-shaped coccoid novel archaeal metal mobilizer which had not been further characterized. *Sulfolobus* species isolated from TP grew in aerobic cultures on S° and yeast extract (Handayani *et al.*, 2012). In the attempts to further characterize archaeal isolates cultured from Indonesian samples *Th. acidophilum* and *Th.volcanium* have been identified (Malik *et al.*, 2014).

Now, we identified an archaeon from TP and DP, *Picrophilus torridus*, the closest relative to *Thermoplasma* species. *Picrophilus* species had not been reported from Indonesia before. The samples grow in cultures of Freundt's medium with a strongly acidic pH around 1 and a growth temperature around 60 °C, i.e., under conditions where only mesothermal acidophilic archaea can exist. *Thermoplasma* species are naturally living anaerobically, but can facultatively grow also in microaerobic culture environment (Freisleben *et al.*, 1994), while *Picrophilus torridus* is living aerobically at 60°C and at pH around 1 or even below.

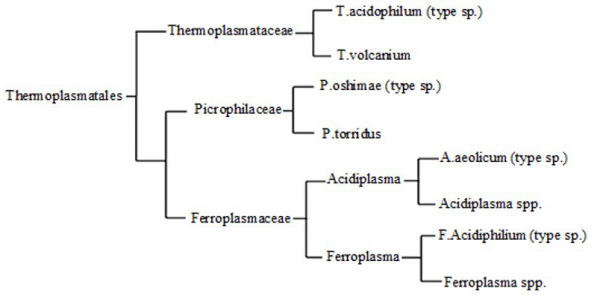

Fig. 6. The phylogeny of *Thermoplasma* spp. and *Picrophilus* spp.

Figure 6 shows the phylogeny of *Picrophilus*

torridus and *Thermoplasma* spp. (*Th. volcanium* and *Th. acidophilum*). Both archaea are in the phylum of *Euryarchaeota,* class *Thermoplasmata* and order *Thermoplasmatales.* The latter divides into several families, *inter alia*, *Picrophilaceae, Ferroplasmaceae,* and *Thermoplasmataceae*. *Picrophilus torridus* and *Th. acidophilum* display significant homology in 66% of all genes (Fütterer *et al.,* 2004).

Since most data are available from TP, we will focus on this volcanic area with three craters in close vicinity. Sampling was conducted mainly at the Domas Crater from solfataric fumaroles and mud holes. They reach from a lower part with temperatures between 40 and 50 °C, which we did not use for sampling because too many tourists were soaking their feet, rather started sampling above 50 °C. From the lower part of the crater the mud fumaroles and hot springs reach gradually higher temperatures to above 90 °C at the upper crater rim (Handayani *et al.,* 2012), where the access is normally restricted to the public because of the hot temperatures and poisonous gas. The pH varies across the solfatara field from 1 to 5 and the original growth environment from anaerobic in most cases to only a few aerobic conditions (Huber *et al.,* 1991; Handayani *et al.,* 2012)

For the microorganisms Domas Crater and the other two near-by craters on TP provide a wide range of slightly different thermo-acidophilic growth conditions and multifold possibilities for vertical and horizontal genetic exchange and adaptation. This offers almost the entire order of *Thermoplasmatales* the possibility to grow, as well as *Crenarchaeota*, e.g., *Sulfolobus* species (Handayani *et al.,* 2012). With *S. solfataricus P. torridus* shares 58% genetic homology (Fütterer *et al.,* 2004).

Concerning the cell membrane, the major difference between *Picrophilus* spp. and most other *Thermoplasmatales* is that the former have an S-layer, whereas the latter don't; so, the cytoplasmic membrane is naked and protects the interior milieu of pH 5.5 towards the outer sulfuric acidic of pH 2 at temperatures up to 60 °C (Searcy, 1976). This means, the membrane has to maintain a pH gradient of roughly 3.5 pH magnitudes. The same applies for *Picrophilus torridus* with a cytoplasmic pH of 4.6 and an Environmenta lpH around 1 (Fütterer *et al.,* 2004). The question about the function of S-layers was discussed in general and specifically in archaea. S-layers may participate in the shape maintenance or stabilization, but are not shape-determining (Engelhardt, 2007). Engelhardt (2007) differentiates primary or primordial and secondary or acquired functions. The former may apply to archaea and hence to *Picrophilus*: mechanical, osmotic, and thermal cell stabilization. It is primarily the cytoplasmic membrane that must cope with the low environmental pH, maintenance of the pH gradient of about 3.5-4 magnitudes and also with elevated temperatures. The experiments with bilayer and monolayer model membranes and recrystallized bacterial S-layer showed non-specific association and loss in membrane fluidity and flexibility, increased stability and heat resistance. Although these experimental conditions cannot be directly transferred to archaeal wild life, similar effects can be assumed.

Although phylogenetically more distant, *Sulfolobus* spp. grow under similar conditions, at external pH 2.5-3 and exert an internal pH of 6.5 (She *et al.,* 2001; Moll and Schäfer, 1988). All these archaea have in common tetraether lipids with a majority of caldarchaeol, or in case of *Sulfolobus*, calditoglycerocaldarchaeol (Sugai *et al.,* 1995). Although these lipids vary in the number of

Table 1. Typical characteristics

Genus	Growth pH opt	Inner pH	Opt growth temperature	Cell wall/ S-layer	Main polar lipid caldarchactidylglycerol/ *calditoglycero-caldarchacol	References
Ferroplasma	17	5.6	35°C	None	β-D-Glucopyranosyl	(Batrakov *et al.,* 2002; Golyshina *et al.,* 2000)
Acidiplasma	15	5.6	42°-45°C	None	β-Galactopyrasosyl	(Golyshina *et al.,* 2009)
Thermoplasma	1.5-2	5.5	56°-59°C	None	β-L-Gulopyranosyl	(Swain *et al.,* 1997)
Picrophilus	0.7	4.6	60°C	S-Layer	β-D-Glucopyranosyl	(Schleper *et al.,* 1995)
Sulfolobus	2.5-3	6.5	70°-80°C	S-Layer	*calditol	(Moll and Schäfer, 1988; Sugai *et al.*, 1995; and Gambacorta *et al.,* 2002)

pentacycles (0-8) and their sugar components (glucose, galactose and mannose, in case of *Thermoplasma*, gulose), but the biphytanyl tetraether cores are crucial to these lipids, as well as to lipoglycans and lipopolysaccharides and appear necessary to keep the pH gradient across the cytoplasmic membrane (Stern *et al.,* 1992; Shimada *et al.,* 2002). Lipoglycans and lipopolysaccharides are in close association with the S-layer and seem necessary for its attachment to and anchoring in the cytoplasmic membrane (Fütterer *et al.,* 2004; Engelhardt, 2007).

Comparison of the characteristics in Table 1 suggests that moderately thermophilic archaea do obviously not need a cell wall, albeit very low environmental pH of 1.5-2, but higher temperatures above 60°C need additional stabilization by the S-layer of *Picrophilus* or *Sulfolobus* spp. Archaeal cell wall as reported in *Sulfolobales* is dominated by an S-layer on the outside of the cytoplasmic membrane. In other words, *Sulfolobus* and *Picrophilus,* both contain S-layers (Klingl, 2014) which has also been termed "cell wall" in literature.

The S-layer of *Picrophilus* was isolated and described to exert as a regular and filigreed lattice of tetragonal symmetry with additional brush-like polysaccharide structures on the outer surface (Schleper *et al.,* 1995). On the other hand, pili or flagella, often observed in cell-wall-less *Thermoplasmatale*s have not been detected in *Picrophilus* (Schleper *et al.,* 1995; Schleper *et al.,* 1996).

Archaea appear to have (at least) two ways to cope with elevated growth temperatures, firstly, via modification of the tetraether / diether lipid composition (Siliakus *et al.,* 2017), especially by the number of pentacycles in their biphytanyl chains (Uda *et al.,* 2001; Shimada *et al.,* 2008) and secondly, by adding S-layers on the outside of their cytoplasmic membranes. However, heat resistance may not be the only function and reason for archaeal S-layers.

On the other hand, also low environmental pH may not be a major reason for archaeal S-layers (Golyshina and Timmis, 2005; Golyshina *et al.,* 2016) (or only in the combination of both, elevated temperature and extremely low pH).

Lysis experiments with *Th. acidophilus* under various conditions showed highest membrane stability at pH 4 (Freisleben, 2019; Luthfa *et al.,* 2015). *Picrophilus oshimae* appears to lose viability and cell integrity above pH 4, because of impaired barrier function of the cytoplasmic membrane (van de Vossenberg *et al.,* 1998).

At pH 4, membrane stability is high (or even highest), although growth optimum is at much lower pH. Energy is necessary to maintain membrane structure and integrity at extreme acidity, which was confirmed for *Thermoplasma* by computer simulation (Luthfa *et al.,* 2015). This fact certainly applies also to other microorganisms growing at extremely low pH. Proton-driven ATPases and transport/antiport systems have been reported to maintain the pH gradient across the archaeal cytoplasmic membranes and heat-stable cytoplasmic enzymes with low pH optimum, close to the outside pH, which enable these archaea coping with extreme environmental conditions (Schleper *et al.,* 1995; Fütterer *et al.,* 2004). However, all these mechanisms would be worthless, if the tetraether lipids were not present to seal cytoplasmic membranes against proton leakage (Zeng *et al.,* 2018), which was demonstrated also in liposomal membranes made up from archaeal tetraether lipids (Freisleben *et al.,* 1995; Komatsu and Chong, 1998; Elferink *et al.,* 1994).

Culture growth conditions have been varied with archaea mainly in pH and temperature showing different lipid composition, number of pentacycles and phase transition patterns (Freisleben, 2019; Dopson *et al.,* 2004; Dopson and Holmes, 2014; Zhu *et al.,* 2013; Freisleben *et al.,* 1994; Uda *et al.,* 2001; Shimada *et al.,* 2008; Golyshina *et al.,* 2016; Luthfa *et al.,* 2015; Ernst *et al.,* 1998). Additional modifications in culture conditions (culture media, oxygen supply, nutrients, etc.) have been made to detect their impact on membrane composition. Although artificial growth conditions cannot be directly transferred to wild life, the natural environment of archaea certainly provides much broader variations and challenges (Tierney *et al.,* 2015; Kaur *et al.,* 2015). Modification of the genetic and enzymatic pattern and especially of the membrane composition and its transport systems is necessary for adaptation to environmental challenges of the natural habitats as well as artificial culture growth conditions. Even different growth phases yield different lipid composition (Elling *et al.,* 2014).

Conclusion

For the first time, the thermo-acidophilic archaeon *Picrophilus torridus* was isolated and cultured from Tangkuban Perahu and Dieng Plateau on Java Island, Indonesia. Identification was accomplished by

genetic alignment, phase contrast and transmission electron microscopy. Membrane characteristics of thermo-acidophilic archaea are discussed.

Acknowledgement

The authors are grateful to the Faculty of Medicine Syarif Hidayatullah Jakarta and Ministry of Religion Affairs for their support in facilities and funding. Moreover, the authors want to thank Universitas Gadjah Mada (UGM), Yogyakarta, TEM Service Center for accomplishing the electron microscopy and to Anke-Mareil Heuser and Frauke Müksch, Center for Infectious Diseases, University of Heidelberg, for assistance with phase contrast microscopy.

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