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SHORT COMMUNICATION

Archaeal Life on Tangkuban Perahu- Sampling and Culture Growth in Indonesian Laboratories

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The aim of the expedition to Tangkuban Perahu, West Java was to obtain archaeal samples from the solfatara fields located in Domas crater. This was one of the places, where scientists from the University of Regensburg Germany had formerly isolated Indonesian archaea, especially *Thermoplasma* and *Sulfolobus* species but not fully characterized. We collected five samples from mud holes with temperatures from 57 to 88 °C and pH of 1.5-2. A portion of each sample was grown at the University of Regensburg in modified Allen's medium at 80 °C. From four out of five samples enrichment cultures were obtained, autotrophically on elemental sulphur and heterotrophically on sulfur and yeast extract; electron micrographs are presented. In the laboratories of Universitas Indonesia the isolates were cultured at 55-60 °C in order to grow tetraetherlipid synthesizing archaea, both *Thermoplasmatales* and *Sulfolobales*. Here, we succeeded to culture the same type of archaeal cells, which had been cultured in Regensburg, probably a *Sulfolobus* species and in Freundt's medium, *Thermoplasma* species. The harvested cells are documented by phase contrast microscope equipped with a digital camera. Our next steps will be to further characterize genetically the cultured cells from Tangkuban Perahu isolates.

Key words: Archaea, Sulfolobus, Thermoplasma, Tangkuban Perahu, Indonesian volcanoes, tetraether lipid

INTRODUCTION

Sulfolobales (Huber & Prangishvili 2004) and *Thermoplasmatales* (Huber & Stetter 2001) are archaea with unique tetraetherlipids, which have raised our interest in biomedical and biotechnical applications (Freisleben 1999). *Sulfolobus* species had been found in solfatara fields and mud springs around the world. The first representative described was *Sulfolobus acidocaldarius* isolated from Yellowstone Park (Brock *et al*. 1972). Cell wall-less *Thermoplasma acidophilum* was first isolated by Darland *et al*. (1970) from sulfuric acid milieu in self-heated coal refuse piles. Later, other *Sulfolobus* strains were isolated, among them *Sulfolobus solfataricus* (Zillig *et al*. 1980) and *Thermoplasma volcanium* spp. from solfataric hot springs in Italy (Segerer *et al*. 1988). Solfataric environment appears to be the natural habitat also of *Thermoplasma acidophilum* (Yasuda *et al*. 1995). In Indonesia both, *Sulfolobus* and *Thermoplasma* species, which have not yet been further characterized, have been reported from Tangkuban Perahu (Huber *et al*. 1991) an easily accessible volcano in West Java island, south of Jakarta, near the city of Bandung. Growth of *Thermoplasma acidophilum* was achieved in fermentors under laboratory conditions at pH 1.5 to 2.0 and an optimal growth temperature of 59 °C (Freisleben *et al*. 1994), whereas *Sulfolobus* strains optimally grows around 75-80 °C and a pH between 2.5 and 3.5 (Brock *et al*. 1972). *Sulfolobus* strains have become a major source of tetraether lipid, rather than *Thermoplasma*, although the latter lacks a cell wall, and thus the membrane lipids are 'naked' and easily accessible. Both *Sulfolobales* and *Thermoplasmatales* members grow autotrophically metabolizing elemental sulfur, but can also grow mixo- and heterotrophically, from anoxic to oxic conditions (Huber *et al*. 1991).

Interest was raised in Indonesia for domestic archaea more than ten years ago, when one of us (HJF) had been asked to write a review about *Thermoplasma* and tetraether lipid for this journal (Freisleben 1999). Soon after, an excursion with sampling on Tangkuban Perahu followed, but attempts to grow archaea from these samples in Indonesian laboratories did not succeed. New excursions to one of the craters on Tangkuban Perahu, Kawah Domas (KD, Domas crater) yielded samples, which have been transferred to cultures in the laboratories of

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Universitas Indonesia in cooperation with the Archaea Centre at the University of Regensburg, Germany.

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 & Sprott 2008; Thavasi *et al*. 2008; Vidawati *et al*. 2010).

With our own research, we hope to induce further interest for the investigation and application of extremophilic archaea in the Pacific region (Karner *et al*. 2001) and especially in Indonesia (Noble & Henk 1998).

MATERIALS AND METHODS

Sampling. Sampling was carried out on April 22, 2011 ("first sampling") and on June 11, 2012 ("second sampling") in the Indonesian volcano Tangkuban Perahu located in West Java, Bandung. Samples were taken from solfatara fields in the Domas crater (Kawah Domas, KD), one of several craters on this volcano.

In the crater, there was a distinct and constant smell of H_2S from sulfuric fumes. Apart from the odor, yellow stains of molecular sulfur were present on the rock formations in Kawah Domas, which further strengthened the notion of a geothermal solfatara field with many hot springs and mud holes. The lower section of the crater exerted lower temperatures than the hot springs located in the higher region, the geothermal vents originating rather from the top of the crater than from the bottom. A barrier was set at the top region to keep visitors from reaching the high temperature hot springs. Temperatures measured by us varied from 48 to 88 °C.

Most of the samples collected here were obtained from sources where acidic steam was rising from the mud holes and hot springs. Previous reports regarding archaeal habitats have shown that the growth temperature of *Thermoplasma* species are well around 50 up to 60 °C (Huber *et al.* 1991; Yasuda *et al*. 1995) confirmed in fermentor growth (Freisleben *et al*. 1994), whereas *Sulfolobus* species grow preferably at higher temperatures around 75-80 °C (Brock *et al*. 1772). Hence, we took mud and water samples from five different mud holes and hot springs with temperatures above 50 °C. Samples were collected into 140 ml screw-capped glass bottles.

Culture Experiments in Regensburg. The samples in the screw capped culture bottles were transported to the laboratories of Universitas Indonesia in Jakarta-Depok at ambient temperature, all bottles were filled to the top and firmly closed. An aliquot of each sample was then transferred to screw cap centrifuge bottles, filled to the top, firmly closed and taken to the University of Regensburg, Germany for growth in culture medium.

From the first sampling (April 22, 2011), five samples from Kawah Domas, Tankuban Perahu KD1–KD5 were grown aerobically in two different media, modified Allen medium (Allen 1959) and Darland's medium (Zillig *et al*. 1980) under various conditions (Table 1).

Medium Preparation. The composition of 1 litre Allen medium (MAL) is 1.30 g $(NH_4)_2SO_4$, 0.25 g $MgSO_4 \cdot 7H_2O$, 0.095 g KH_2PO_4, 0.07 g $CaCl_2 \cdot 2H_2O$, 0.02 g $FeCl_3 \cdot 6H_2O$ and added with stock solution (1 mg/ml) of 14.5 ml $Na_2B_4O_7 \cdot 10H_2O$, 0.45 ml $ZnSO_4 \cdot 7H_2O$, 0.25 ml $CuCl_2 \cdot 2H_2O$, 0.03 ml $Na_2MoO_4 \cdot 2H_2O$, 0.01 ml $CoSO_4 \cdot 7H_2O$, 1.75 ml $KAl(SO_4)_2 \cdot 12H_2O$ and 0.6 ml $(NH_4)_2Ni(SO_4)_2$. All substances were dissolved and diluted to 1 l in aqua bidest, then pH of the solution was adjusted to 2.0-3.0 with H_2SO_4 10% (v/v).

The composition of 1 litre Darland's medium(DL) is 3.00 g KH_2PO_4, 1.02 g $MgSO_4 \cdot 7H_2O$, 0.25 g $CaCl_2 \cdot 2H_2O$, 0.20 g $(NH_4)SO_4$, 1.00 g yeast extract (YE) and 10.0 g glucose (glc). All substances were dissolved to 1 l in aqua bidest; the pH was adjusted to 2 (10% H_2SO_4 (v/v)).

All media containing S^o (molecular sulfur) were autoclaved at 110 °C for one hour; media without S^o were sterilized at 121 °C for 20 min.

Culture Experiments in Jakarta-Depok. Culture samples from Regensburg were transferred back to Jakarta and grown in modified Allen medium (Allen 1959), cultured autotrophically and mixotrophically in an incubator at 60 °C with a shaker at 110 rpm.

After 3 weeks, samples were harvested and examined by means of 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.

From the second sampling (June 11, 2012), two samples were grown micro-aerobically in Freundt's medium (Freisleben *et al*. 1994) in closed 1-litre culture bottles at 55 °C and a pH of 1.5 at 110 rpm. Harvesting after 72 h yielded cellular growth as examined and documented with the same Olympus device as above. The culture medium was composed of 1 l Freundt's medium, 200 ml of a solution

Table 1. Various growth conditions of samples cultured aerobically in modified Allen's medium (MAL) and Darland's medium (DL)

Sample	Medium	Addition of	Growth temperature (°C)
KD1	MAL pH 2.5	S^o	80
		S^o + 0.02% YE	
KD2	MAL pH 2.5	S^o	80
		S^o + 0.02% YE	
KD3	MAL pH 2.5	S^o	80
		S^o + 0.02% YE	
KD4	MAL pH 2.5	S^o	80
		S^o + 0.02% YE	
KD5	MAL pH 2.5	S^o	80
		S^o + 0.02% YE	
KD1	DL pH 2.0	0.5% Glc + 0.1% YE	60
KD2			50
	DL pH 2.0	0.5% Glc + 0.1% YE	60
KD3			50
	DL pH 2.0	0.5% Glc + 0.1% YE	60
KD4			50
	DL pH 2.0	0.5% Glc + 0.1% YE	60
KD5			50
	DL pH 2.0	0.5% Glc + 0.1% YE	60
			50

YE = yeast extract, Glc = glucose, volume of medium = 20 ml per serum bottle.

containing glucose (20 g) and Difco yeast extract (DYE,1 g) and 50 ml inoculum from KD samples.

RESULTS

First Sampling and Growth of *Sulfolobus.* The condition of mud and water samples from five different mud holes and hot springs were: KD1= 68 °C, pH = 1.5, turbid; KD2 =57 °C, pH = 1.5, sediment; KD3 = 85 °C,pH = 1.5, clear; KD4 = 84 °C, pH = 1.5, sediment, but less than in KD2 and KD5 = 88 °C, pH = 2.0, clear. The letters KD denote the location of the site, i.e. Kawah Domas, and the number represents the source from which we collected the samples. The sample KD2 with the lowest temperature was obtained from a hot spring located at the lower valley of the crater, where people were having quick baths and soaking their feet. Sample KD5 was obtained from a spring located near the hazardous region at the top of the crater. Samples KD1 and KD3 were collected from small mud holes in the middle part of the crater (Figure 1) while KD4 was drawn from a hot spring in the upper region located near KD5 and used by tourists to boil eggs.

Culture Growth in Regensburg. Five samples from Kawah Domas, Tangkuban Perahu KD1–KD5 were grown aerobically in two different media, modified Allen medium (Allen 1959) and Darland's medium (Zillig *et al.* 1980) under various conditions (Table 1). Growth temperatures were between 50 and 80 °C.

From more than 20 enrichment attempts, eight cultures at 80 °C (KD1 through KD4; Table 1 no. 1-4) exerted microbial growth in modified Allen's medium (MAL) with elemental sulfur (S^o) only (e.g., KD1a, "a" from autotrophic) and elemental sulfur (S^o) with yeast extract (YE, e.g., KD1m, "m" from mixotrophic), four each. Transmission electron micrographs (TEM) were taken. Figure 2 shows isolates KD3a and KD4m, i.e., one example of each growth condition, autotrophic and mixotrophic.

Culture Growth in Jakarta-Depok. The same isolates (KD1-4), which had already been cultured successfully in Regensburg were cultured in the laboratories at UI in Jakarta -Depok at 60 °C autotrophically (e.g., KD3a) and mixotrophically (e.g., KD4m), observed by phase contrast microscopy and documented by a digital camera system as shown in Figure 3.

Second Sampling and Growth of *Thermoplasma.* The second sampling on TP yielded 3 samples, KD2/1 (pH 1.5; T 57 °C), KD2/2 (pH 1.5; T 52 °C) and KD2/3 (pH 2.0; T 82 °C). Samples KD2/1 and KD2/2 were expected to contain *Thermoplasma*, whereas KD2/3 should contain *Sulfolobus*. Hence, KD2/1 and KD2/2 were used to grow *Thermoplasma* cultures and KD2/3 was stored as a back-up for *Sulfolobus* cultures. *Thermoplasma* was grown in closed culture bottles filled between half and two-thirds with medium at a pH of 1.5 at 55 °C. Closed culture bottles are the easiest way to provide limited air access to the medium (micro-aerobic condition): the air in the bottle provides enough oxygen, but its concentration slowly decreases in the medium in parallel with cell growth (Freisleben *et al.* 1994). The pH below 2 ensures that under these conditions–apart from *Thermoplasma*–only one other microorganism may grow, *Bacillus acidocaldarius*, which can easily be differntiated from Thermoplasma in the microscope by its shape and much larger size.

Figure 1. Measuring mudholetemperature before taking sample KD3.

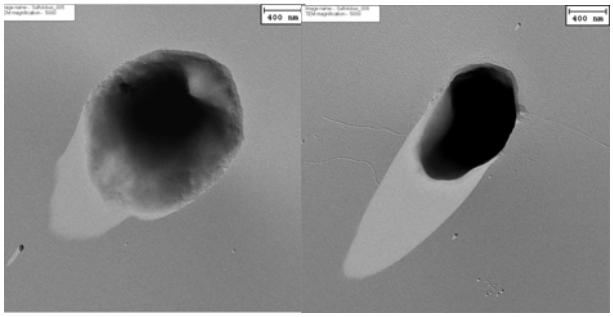

Figure 2. Transmission electron microscopy of isolates KD3a (Sulfolobus_005) and KD4m (Sulfolobus_008); cell diameter is around 1.5 µm (platinum shadowed).

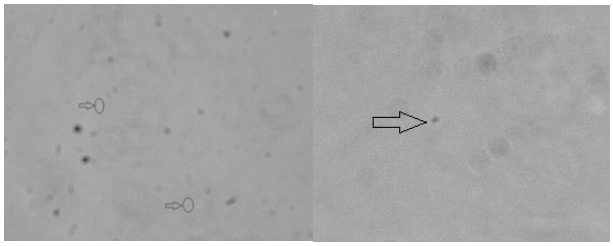

Figure 3. Phase contrast microscopy of isolates KD3a (Sulfolobus_ 005) and KD4m (Sulfolobus_ 008).

DISCUSSION

From five samples isolated from Kawah Domas on Tangkuban Perahu in the first sampling, four enrichment cultures were obtained, obviously with the same type of cells under autotrophic and mixotrophic culture conditions and limited oxygen supply. From our experience with culture media, growth conditions and shape, we had most probably isolated and cultured a *Sulfolobus* species; the culture temperature of 80 °C used in Regensburg hints to *Sulfolobales*.

At 60 °C, the growth temperature used in Jakarta, *Sulfolobales* do not grow optimally; these conditions were originally adjusted to culture *Thermoplasma*, which do not grow above 62 °C. Hence, with these temperature conditions, we intend to cover growth of both species.

In their excursion to Tangkuban Perahu, Huber *et al.* (1991) had collected samples from 13 hot springs and mud holes in Domas crater and additional nine samples from

two other craters on this volcanoe, most of them were anaerobic. The temperatures measured were 32-94 °C and pH between 1 and 5.5. From all three craters on TP, they isolated members of the genus *Acidianus*, chemolithoautotrophic and facultative aerobes, which grow on elemental sulfur at temperatures of up to 96 °C and are thus similar to *Acidianus infernus* isolated in Italy (Segerer *et al*. 1985; Segerer *et al*. 1986).

Moreover, *Sulfolobus* species isolated from Tangkuban Perahu grew in aerobic cultures on S° and yeast extract (Huber *et al*. 1991), but have not been further characterized. Now, we present the first data on laboratory growth in Indonesia and further characterization will follow as soon as we have obtained sufficient cell mass.

Of further interest is a novel coccoid *Sulfolobus*-shaped thermophilic ore leaching archaeon, which was isolated from a hot acidic waterhole in one of the craters on TP and which grows chemolithoautotrophically in the laboratory at temperatures up to 80 °C (Huber *et al*. 1986; Huber *et al*. 1991). It will need further investigation to identify and characterize these archaea in our own isolates from TP and subsequent cultures in our laboratories.

The three archaeal cell types above exert similar growth conditions so that the differentiation and characterization of these organisms will be one direction of our future research. Our special interest aims to the growth and characterization of cell wall-less *Thermoplasmatales* isolated on TP from locations with strongly acidic pH and moderately hot temperatures up to 67 °C. Huber *et al*. (1991) could enrich these highly irregular coccoid thermoacidophilic archaea in Darland's medium (Darland *et al*. 1970). Cell extracts of these isolates showed phylogenetic relationship to the genus *Thermoplasma* (Stein & Searcy 1978), especially to *Thermoplasma acidophilum* (DSM 1728), which strain we had formerly grown in our laboratories in Frankfurt in Freundt's medium (Freisleben *et al*. 1994). From this experience, we decided to continue our growth experiments in Jakarta-Depok with Freundt's medium and succeeded in June 2012 with the new samples from TP.

Thermoplasma isolates from solfatara fields around the world including Indonesia differ in their DNA GC-content of 40 mol % from *Thermoplasma acidophilum*, which exerts 46 mol %, but also from the Italian *Thermoplasma volcanium* isolate (DSM 4299), which has a GC content of 38% (Segerer *et al*. 1988). Since Indonesian *Thermoplasma* isolates appear to be genetically homogeneous and differ from all other samples isolated so far, they may represent an unknown genotype of *Thermoplasma* (Segerer *et al*. 1988). Hence, it is our aim to enrich growth in culture and to obtain sufficient cell mass for the genetic characterization of our isolates from Tangkuban Perahu.

Currently, archaea and their components are subject to intense investigation and incorporation to already developed technology in order to enhance the capability under extreme conditions. Archaeal metal leaching enzymes remain active under extremely high temperatures (Schiraldi *et al*. 2002). Bacteriorhodopsin, an ion pump found in halophilic archaea, is incorporated into solar cells to improve light capture (Thavasi *et al*. 2008) and can be used as artificial biological battery for light-driven ATP production after co-reconstitution into archaeal tetraether lipid liposomes (Freisleben *et al*. 1995).

In our research project, we will attempt to utilize the membrane of archaea for the design and development of archaeosomal drug, gene and vaccine delivery systems (Krishnan & Sprott 2008; González-Paredes *et al*. 2011; Li *et al*. 2011). It was demonstrated that archaeal tetraether lipid forms stable model membranes (Vidawati *et al*. 2011) and liposomes (Freisleben 2000; Patel *et al*. 2000).

Indonesia as a hot bed of habitats is ideal for the growth of archaea; but until now, research on these domestic organisms has not yet been established. With our multidisciplinary team of researchers, we intend to develop such applications from native Indonesian archaea, with the assistance of the Archaea Centre of the University of Regensburg in Germany.

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