

Hyperthermophilic Archae- and Eubacteria occurring within Indonesian Hydrothermal Areas

by

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With 3 Figures and 6 Tables

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Abstract

From 85 samples taken during cruise 45B of the R/V SONNE within the Sunda Arc subduction zone and from solfatara fields in Java, thermophilic and hyperthermophilic archae- and eubacteria were isolated. The archaeobacteria belong to the genera *Methanobacterium*, *Methanolobus*, *Methanosarcina*, *Acidianus*, *Thermoproteus*, *Desulfurococcus*, *Thermoplasma* and to two up to now unknown genera of hyperthermophilic marine heterotrophs and continental metal mobilizers. The eubacterial hyperthermophilic isolates are a new species of *Thermotoga* and a novel strictly anaerobe, thriving by H_2/NO_3^- -autotrophy.

1. Introduction

The most extremely thermophilic organisms known up to now belong to the archaeobacteria, the third kingdom of life besides the eubacteria and the eukaryotes (WOESE et al., 1978). These hyperthermophiles are the archaeobacterial sulfur metabolizers and some methanogens, growing optimally at temperatures above 80°C (STETTER, 1986). *Thermotoga maritima*, the only hyperthermophilic species known in eubacteria, represents the deepest phylogenetic branch-off within its kingdom (HUBER et al., 1986b; WOESE, 1987). Due to their exceptional position in the evolution of life and their outstanding cellular properties, hyperthermophiles are of great interest for microbiology and biotechnology.

Different hyperthermophilic archaeobacteria have been isolated from continental and submarine hydrothermal systems at the Azores, East Pacific Rise, Iceland, Italy, Japan, Yellowstone National Park (USA), and New Zealand (BROCK, 1978; STETTER, 1986; STETTER et al., 1987). Since hydrothermal biotopes are very distant and hyperthermophiles are unable to grow at normal environmental temperatures, the question of spreading arises. Although very few systematic studies on the distribution of hyperthermophiles were carried out up to now, there is evidence for the existence of endemic groups: The *Methanothermaceae* were up to now only found in the southwest of Iceland (LAUERER et al., 1986), while *Pyrodictium*, *Pyrococcus*, and *Archaeoglobus* could only be isolated in Vulcano, Italy (STETTER, 1982; FIALA & STETTER, 1986; STETTER et al., 1987). During the R/V SONNE cruise 45B in Indonesia within marine areas of active volcanic activity along the Sunda Arc subduction zone and at solfatara fields in Bali and Java, attempts were made to address the following points: a) existence of novel hyperthermophilic archae- and eubacteria in Indonesia, b) distribution of hyperthermophilic archaeobacteria within Indonesian hydrothermal systems and solfatara fields, c) dissemination of hyperthermophilic archaeobacteria between different hydrothermal systems through cold sea water and d) survival of hyperthermophiles in cold deep-sea sediments. Here we report on the first results of our screening program.

2. Results and Discussion

2.1 Sampling

During cruise SO-45B, ten anaerobic samples (each 100 ml) of shallow (depth: 30 to 82 m; $t = 20$ to 16°C) and deep sea (depth: 1283 to 1743 m; $t = 2.9$ to 3.7°C) sediments were taken from box grabs, piston- and box cores at different stations north, east and south of Sumbawa and in the Komodo-Rinja region. Thirteen additional sediment samples were taken by a syringe (STETTER, 1982) within the Satonda Crater lake (depth = 0.6 to 2 m; $t = 32^{\circ}\text{C}$) and from shallow submarine hot springs (depth: 0 to 2 m; $t = 75 - 96^{\circ}\text{C}$) at the beach of Sangeang Island, about 7 km north of Kelapa village. The freshly taken samples were collected in 100 ml storage bottles and oxygen was reduced immediately by addition of sodium dithionite and sodium sulfide until the redox indicator resazurin added before became colorless (STETTER, 1982). The bottles were tightly stoppered and stored at 4°C . Eight

Table 1
Samples taken from Hot Springs and Continental Solfataras Fields in Bali and Java

Abbreviations: TB = Toye Bunkah; KS = Kawah Sikidang; KC = Kawah Candradimuka;
SL = Kawah Sileri; KD = Kawah Domas; KB = Kawah Badak; KDj = Kawah Djarian;
C = Ciater; GG = Garung Gede.

| Designation | | Original | | Designation | | Original | |
|-------------|-----|----------|-----------------------------|-------------|-----|----------|-----------------------------|
| | | pH | Temp ($^{\circ}\text{C}$) | | | pH | Temp ($^{\circ}\text{C}$) |
| TB | 1 | 7 | 42 | KD | 1 | 3 | 91 |
| | 2 | 7 | 39 | | 2 | 2 | 94 |
| | | | | | 3 | 5 | 67 |
| KS | 1 | 1.5 | 93 | 4 | 2 | 46 | |
| | 2 | 1.5 | 92 | 5 | 2 | 86 | |
| | 3* | 3 | 22 | 6* | 1.5 | 32 | |
| | 4* | 3 | 92 | 7 | 2 | 70 | |
| | 5 | 1.5 | 50 | 8 | 4 | 94 | |
| | 6* | 2 | 24 | 9 | 3 | 50 | |
| | 7* | 3 | 27 | 10 | 3 | 50 | |
| | 8 | 3.5 | 51 | 11 | 3 | 58 | |
| | 9 | 7 | 92 | 12 | 3 | 93 | |
| | 10* | 3 | 50 | 13 | 3 | 92 | |
| | 11 | 3 | 50 | | | | |
| | 12 | 2.5 | 92 | KB | 1* | 1 | 90 |
| | 13 | 3.5 | 22 | | 2 | 1.5 | 68 |
| | 14* | 3.5 | 22 | | 3 | 2 | 94 |
| 15 | 5 | 92 | | | | | |
| 16* | 3 | 30 | KDj | 1* | 3 | 92 | |
| 17* | 2 | 30 | | 2 | 3 | 93 | |
| | | | | 3 | 5.5 | 92 | |
| KC | 1 | 7.5 | 88 | 4 | 1.5 | 92 | |
| | 2 | 6 | 90 | 5 | 3.5 | 94 | |
| | 3 | 5 | 60 | 6 | 1.5 | 90 | |
| | 4 | 7.5 | 80 | | | | |
| SL | 1* | 6.5 | 55 | C | 1 | 3 | 42 |
| | 2 | 6.5 | 62 | | 2 | 3 | 43 |
| | 3* | 6 | 55 | | | | |
| | 4* | 6.5 | 40 | GG | 1 | 6.5 | 56 |
| | 5* | 3.5 | 32 | | 2 | 6.5 | 56 |
| | 6 | 5.8 | 72 | | | | |
| | 7 | 3.5 | 32 | | | | |

* aerobic sample

samples of sea water (depth: 14 to 2000 m) were deoxygenated as described for the sediments. In another experiment, 3-5 l of the anaerobic water samples were passed through millipore ultrafilters (pore width: 0.4 μm) in order to concentrate the microorganisms. The filter concentrates were then stored anaerobically in tightly closed 20 ml storage tubes.

During land expeditions anaerobic and aerobic water and mud samples were taken from the following places (Table 1): Lake Batur, Bali: Toye Bungkah Hot Springs (TB); Dieng Plateau, Java: Kawah Sikidang Crater (KS), Kawah Candradimuka Crater (KC), and Kawah Sileri Hot Lake (SL); Tangkuban Prahur, Bandung, Java: Kawah Domas Crater (KD; altitude: 1500 m above sea level), Kawah Badak Crater (KB), and Kawah Djarian Crater (KDj); Ciater, Bandung, Java: Ciater Hot Springs (C); Garung Gede, Java: Hot Waterfall (GG). All samples were carried back to the laboratory by airplane without temperature control. Growth experiments were performed at 20, 37, 60, 85, 100 and 110°C and at pH 2, 5.5, 7, and 8.5 with various organic and inorganic substrates (e.g. FIALA & STETTER, 1986).

2.2 Archaeobacterial Isolates from Sea Sediments and Sea Water Samples from the Sumbawa and Komodo-Rinja Areas

In order to detect hyperthermophiles possibly existing and spreading between zones of active submarine volcanism, water and sediment samples with low original temperatures were incubated anaerobically at high temperatures in the presence of substrates suitable for such organisms. No hyperthermophiles could be enriched.

Table 2

Mesophilic Methanogenic Archaeobacteria isolated from Sea Sediments with Low Original Temperatures from the Sumbawa and Komodo-Rinja areas.

| Sample | Station | Water depth (m) | Original temperature (°C) | Description | Isolate |
|-----------------------------------|---------------------------------------|-----------------|---------------------------|-------------------------------------------|---------------------------------------------------------------------------------------------|
| FS - 1 | 101 - 2 | 1735 | 2.9 | greyish-green sediment | <i>Methanosarcina sp.</i> |
| FS - 6 <i>Methanolobus sp.</i> | 107 - 2 | 82 | 16 | greyish sediment with concretions | |
| FS - 7 <i>Methanolobus sp.</i> | 113 - 1 | 45 | 19 | greyish sediment banded hematite | |
| FS - 14 | Satonda Crater Lake, Stat. 16 | 0 | 27 | black mud in spring | <i>Methanolobus sp.</i> |
| FS - 15 | Satonda Crater Lake, embarkation site | 2 | 32 | black sediment | <i>Methanolobus sp.</i> |
| FS-15a | Satonda Crater Lake, bottom | 60 | 29 | black sediment, contains H ₂ S | <i>Methanosarcina sp.</i> enrichment culture: novel irregular leach-shaped methanogen |

Although only relatively few samples were taken, this result suggests that (a) these sediments did not contain significant amounts of hyperthermophiles which survived at low temperatures in the resting state and (b) the surrounding water contains less than 1 cell of hyperthermophiles/5 liters. Possibly, much larger quantities of sea water within hydrothermally active areas have to be filtered (e.g. cubic meters) in order to detect spreading cells of hyperthermophiles. When these samples were incubated at 30°C, mesophilic strictly anaerobic methanogenic archaeobacteria were enriched from some sediments and were purified by plating (Table 2). The physiological properties and morphology suggest that these methanogenic isolates belong to the genera *Methanosarcina*, *Methanobacterium*, and *Methanolobus* (BALCH et al., 1979; KÖNIG & STETTER, 1982). *Methanolobus* is an obligate methylotroph which was up to now isolated only once from a black marine sediment in Tindari, Sicily (KÖNIG & STETTER, 1982). Our results suggest that it may be common in marine sediments. A novel, highly irregular ("leach"-shaped) methanogen from Lake Satonda was up to now obtained only in syntrophic mixed culture with *Clostridia*.

2.3 Hyperthermophilic Archaeobacterial and Eubacterial Isolates from the Shallow Submarine Hot Springs at the Beach of Sangeang Island

From sample SG 7 (original temperature = 90°C), a new coccoid strictly anaerobic heterotrophic hyperthermophilic archaeobacterium was isolated under anaerobic growth conditions at 90°C in artificial sea water (SME; STETTER et al., 1983) supplemented with yeast extract (0.1 %) and elemental sulfur. Cells of the isolate are motile cocci, about 0.5 to 2 µm in diameter, occurring mainly in pairs, and are monopolar monotrichous flagellated (Fig. 1). Complex organic nutrients such as

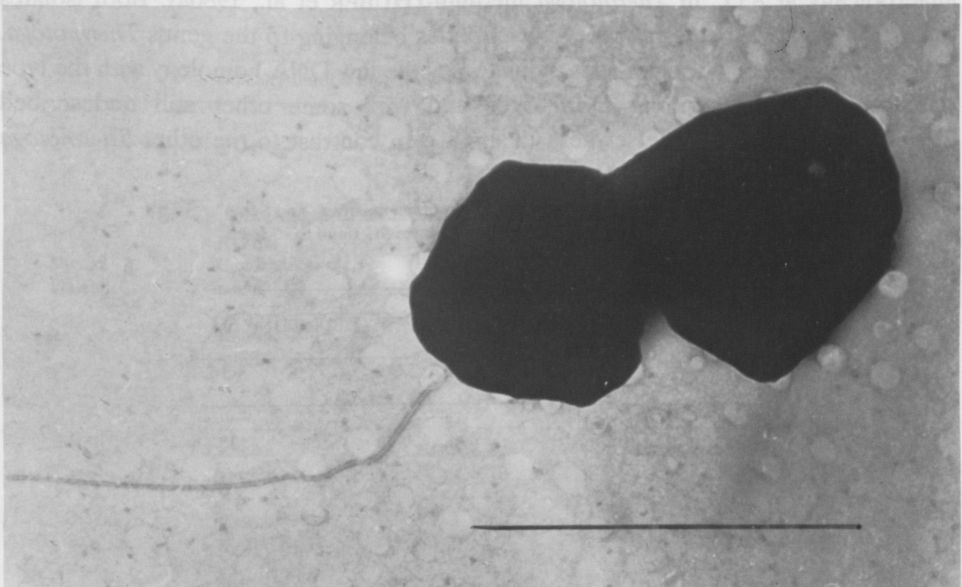


Fig. 1 Dividing cell of the hyperthermophilic archaeobacterial isolate SG 7. EM micrograph, negative-staining. Bar, 1 µm.

yeast extract, pepton, meat extract and tryptone serve as energy source for growth. Elemental sulfur is not essential for growth. Although S⁰ is not necessary, H₂S is formed in its presence instead of H₂ (up to 3 μmoles/ml culture medium). The new isolate grows between 46 and 96°C with an optimum at around 88°C. Growth occurs in the presence of 0.7 to 8 % NaCl at a pH between 5.0 and 8.0. The DNA of isolate SG 7 shows a G + C-content of 40 mol% and is therefore different from the similarly shaped *Thermococcus celer* (56 mol% G + C; ZILLIG et al., 1983) and *Pyrococcus furiosus* (37 mol% G + C; FIALA & STETTER, 1986). By DNA-DNA-hybridization (Table 3) no specific phylogenetic relationship of SG 7 with either genus could be

Table 3
DNA Homology (%) between the New Archaeobacterial Isolate SG 7, *Thermococcus celer*, and *Pyrococcus furiosus*.

| Filter-bound DNA of | ³² P - labelled DNA of | | |
|----------------------------|-----------------------------------|---------------------|--------------|
| | <i>Tc. celer</i> | <i>Pc. furiosus</i> | Isolate SG 7 |
| <i>Thermococcus celer</i> | (100) | 2 | 7 |
| <i>Pyrococcus furiosus</i> | 2 | (100) | 7 |
| Isolate SG 7 | 6 | 12 | (100) |

detected, indicating that the Sangeang isolate most likely represents a new genus of hyperthermophilic archaeobacteria.

From samples SG 1 and SG 7 (both with original temperatures of 90°C), rod-shaped cells with a characteristic outer sheath could be enriched and isolated anaerobically at 85°C in *Thermotoga* medium (HUBER et al., 1986a). Both isolates turned out to be eubacterial hyperthermophiles belonging to the genus *Thermotoga*. They represent a new species as is indicated by the low DNA homology with the type strain *Thermotoga maritima* DSM 3109 and with some other still undescribed *Thermotoga* isolates from elsewhere (Table 4). In contrast to the other *Thermotoga*

Table 4
DNA Homology (%) between *Thermotoga maritima* and Some Recent *Thermotoga* Isolates and Isolates SG 1 and SG 7 from Sangeang.

| Filter-bound DNA of | ³² P - labelled DNA of | |
|----------------------------|-----------------------------------|------|
| | SG 1 | SG 7 |
| <i>Thermotoga maritima</i> | 15 | 13 |
| Isolate NS-E (Italy) | 55 | 40 |
| Isolate RQ 2 (Azores) | 12 | 15 |
| Isolate RQ 7 (Azores) | 55 | 38 |
| Sangeang isolate SG 1 | (100) | 82 |

strains, the Indonesia isolates grow in aggregates (up to 15 cells) and can therefore be easily distinguished.

2.4 Methanogenic Isolates from the Toye Bungkah Hot Springs, Bali

From anaerobic samples taken from the black mud of two ponds at the hot spring of Toye Bungkah, Lake Batur, Bali (TB 1, 2; Table 1), a member of the genus *Methanobacterium* (BALCH et al., 1979) was isolated from the pond close to the origin of the spring (TB 1). From the second pond (TB 2), a *Methanobacterium* sp. similar to TB 1 and a *Methanosarcina* sp. were isolated. The determination of their exact taxonomic position is in progress.

2.5 Hyperthermophilic and Thermophilic Archae- and Eubacteria Isolated from Solfatara Fields at the Dieng Plateau and Tangkuban Prahau, Java

In Java, hot springs and solfatara fields at the Dieng Plateau, Tangkuban Prahau, Ciater and Garung Gede (Table 1) were investigated for thermophilic anaerobic and aerobic archae- and eubacteria (Table 5). At the Dieng Plateau, samples were taken from three different types of solfatara fields: (a) Kawah Sikidang with strongly acidic water and mudholes of up to boiling temperatures, (b) Kawah Candradimuka with neutral to slightly acidic almost boiling hot springs and (c) Kawah Sileri with neutral to slightly acidic muddy blackish water with temperatures around 65°C.

Table 5

Hyperthermophilic and Thermophilic Archaeobacterial and Eubacterial Isolates from Hot Springs and Mudholes in Java.

| Area | Name of solfatara field | Isolates from samples | Genus to (°C): | Growth up to (°C): |
|------------------|-------------------------|-----------------------|------------------------------------------------------------|-----------------------|
| Dieng Plateau | Kawah Sikidang | KS 1, 2, 12, 15 | Acidianus (infernus?) | 96 |
| | | KS 9, 15 | Thermoproteus sp. | 96 |
| | | KS 9, 15 | Desulfurococcus sp. | 96 |
| | | KS 5, 8, 11 | Thermoplasma sp. | 67 |
| | Kawah Candradimuka | KC 1, 2, 4 | Thermoproteus sp. | 96 |
| | | KC 1, 2 | Desulfurococcus | 96 |
| | | KC 4 | novel unnamed H ₂ /NO ₃ ⁻ | 85 |
| | Kawah Sileri | SL 7 | Thermoproteus sp. | 67 |
| | | SL 3 | vibrio-shaped novel eubacterial metal mobilizer | 50 |
| | Kawah Domas | | KD 1, 2, 3, 5 | Acidianus (infernus?) |
| KD 2, 5, 7 | | | Thermoplasma sp. | 67 |
| Tangkuban Prahau | Kawah Badak | KB 3 | Acidianus (infernus?) | 96 |
| | | KB 1 | coccoid novel archaeobacterial metal mobilizer | 80 |
| | Kawah Djarian | KDj 3 | Acidianus (infernus?) | 96 |
| | | KDj 3, 5 | Thermoproteus sp. | 96 |

Within the Tangkuban Prahua area, samples were collected at three different solfatara fields mainly with strongly acidic pH and very high temperatures: (a) Kawah Domas with many strongly gassed water and mudholes, (b) Kawah Badak with many fumaroles and very few tiny waterholes, and (c) Kawah Djarian situated within a rain forest with trees decomposing within boiling holes of sulfur mud. In addition, samples were taken from an acidic warm spring in Ciater and from a hot waterfall with neutral pH at Garung Gede.

No archaeobacterial isolates were obtained from the samples of Ciater and Garung Gede. From the acidic hot springs and mudholes at the Dieng Plateau and at Tangkuban Prahua members of the genus *Acidianus* were isolated in ALLEN's medium (ALLEN, 1959; Table 5). These are chemolithoautotrophic facultative aerobes, growing by oxidation or reduction of elemental sulfur, depending on the redox potential (SEGERER et al., 1985; SEGERER et al., 1986a). All isolates grow at temperatures of at least 96°C and are by this feature similar to the type species *Acidianus infernus* (isolated in Italy). Again from the acidic hot springs of this area, many *Sulfolobus* enrichment cultures could be obtained aerobically on S⁰ and yeast extract (data not shown) which have not been further characterized up to now. From a hot acidic waterhole (KB 1; Table 5) in Kawah Badak, a novel coccoid *Sulfolobus*-shaped (BROCK, 1978) archaeobacterial metal mobilizer was isolated, suitable for microbial leaching of sulfidic ores at high temperatures. The organism grows chemolithoautotrophically at temperatures up to 80°C with pyrite, chalcopyrite and sphalerite as energy sources. After 1 week at 80°C, cell densities of 10⁸/ml are obtained in the laboratory, indicating very vigorous growth on ores compared to other thermophilic ore leachers (HUBER et al., 1986a).

The less acidic to neutral hot samples contained anaerobic, sulfur respiring, rod-shaped organisms, growing at 96°C in ALLEN's medium (ALLEN, 1959) in the presence of yeast extract, pepton and elemental sulfur. Elemental sulfur was replaceable by thiosulfate (0.1 %). The rods exhibit true branching and terminal spherical bodies ("golf clubs") which are typical for members of the genus *Thermoproteus* (ZILLIG et al., 1981). In the same enrichment cultures, also coccoid archaeobacteria were found, thriving organotrophically by S⁰-respiration at temperatures up to 96°C (Table 5). Most likely, they belong to the genus *Desulfurococcus* (ZILLIG et al., 1982).

From locales with strongly acidic pH and moderately hot temperatures (50°C), cell wall-less (Fig. 2) highly irregular coccoid thermoacidophilic archaeobacteria growing up to 67°C could be enriched in DARLAND's medium (DARLAND et al., 1970). They were cloned by plating on medium solidified by 10 % starch. After 4 days of incubation at 60°C in the presence of a CO₂/air atmosphere (50:50), small (0.2 mm ϕ) "fried-egg"-shaped colonies became visible (Fig. 3). Very surprisingly, the isolates turned out to be close relatives of members of the genus *Thermoplasma*. This genus was up to now found almost exclusively within smoldering coal refuse piles (DARLAND et al., 1970; BROCK, 1978). In contrast to *Thermoplasma acidophilum* which is the only species of *Thermoplasma* described up to now, the Indonesian

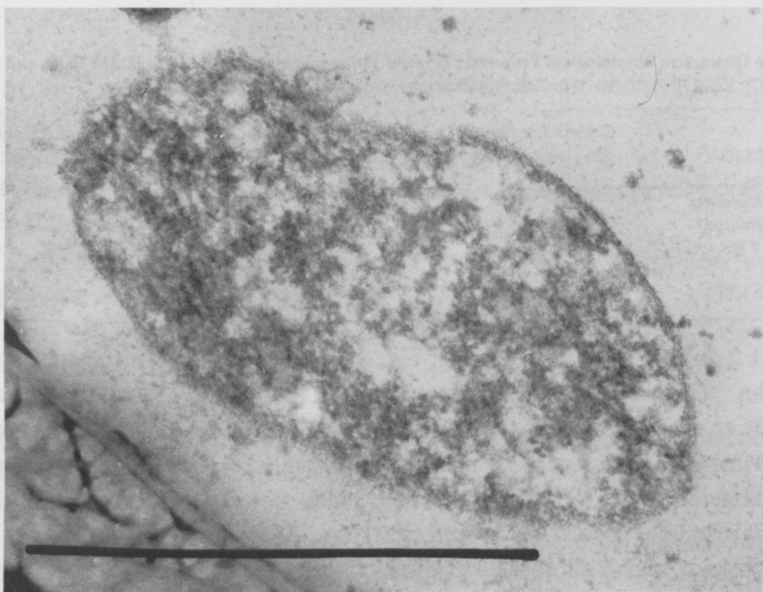


Fig. 2 Cell of the *Thermoplasma* isolate KD 3. EM micrograph, ultrathin section. Bar, 1 μ m.

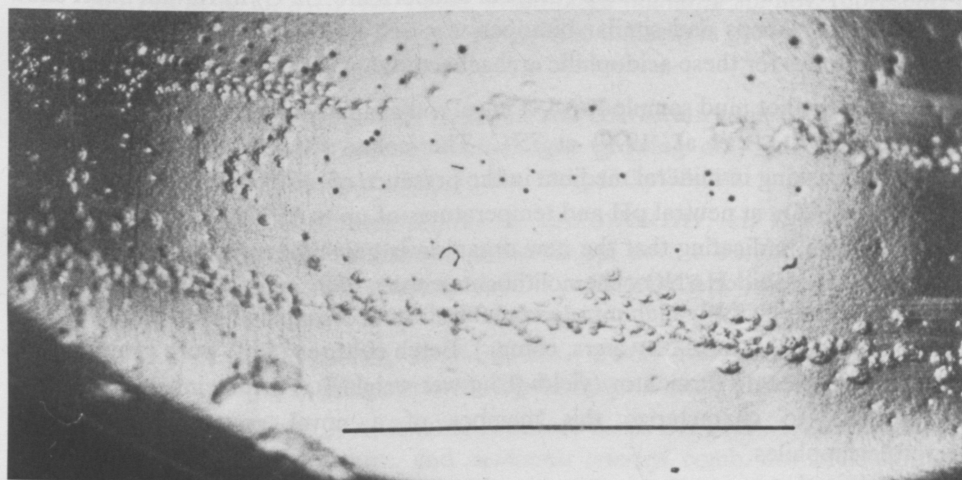


Fig. 3 Colonies of the *Thermoplasma* isolate KD 3 on a starch plate. Bar, 1 cm.

isolates show a G + C-content of their DNA of 40 mol% (instead of 46%). In DNA-DNA-hybridization experiments all 7 Indonesian isolates turned out to be genetically identical (100 % DNA homology; data not shown), while they exhibited no significant homology (18 %; not shown) with *Thermoplasma acidophilum*. Cell extracts of the Indonesian isolates show serological cross-reaction with antibodies prepared against the histone-like protein of *Thermoplasma acidophilum* DSM 1728, indicating a phylogenetic relationship (STEIN & SEARCY, 1978). Therefore, the Indonesian isolates represent a new species of *Thermoplasma*. The new species can be distinguished from the type strain also by physiological properties, for example by their lower minimal and higher maximal growth temperatures, their inability to grow

Table 6

Some Distinctive Physiological Properties between *Thermoplasma acidophilum* (DSM 1728) and the Indonesian *Thermoplasma*-like Isolate (KD 3)

| Strain | Growth temperature (°C) | | | pH for growth | | | NaCl requirement (%) | | |
|------------------------------------------|-------------------------|-----|-----|---------------|-----|-----|----------------------|------|-----|
| | min | opt | max | min | opt | max | min | opt | max |
| <i>Thermoplasma acidophilum</i> DSM 1728 | 45 | 60 | 63 | 0.5 | 2 | 4 | 0.01 | 0.25 | 1.0 |
| Isolate KD 3 | 32 | 65 | 67 | 2 | 4 | 4 | 0.01 | 0.03 | 0.5 |

below pH 2 and their lower salt tolerance (Table 6). Similar to the type strain, the Indonesian *Thermoplasma* isolates are organotrophic facultative anaerobes (SEGERER et al., 1986b). The isolation of members of *Thermoplasma* in Indonesia demonstrates that solfatara fields are the (most likely primary) biotope of this group of archaeobacteria. This finding is in line with the recent isolation of different *Thermoplasma*-like organisms within solfatara fields in Vulcano, Solfatara, the Azores, Iceland and Yellowstone National Park (SEGERER et al., in preparation). The ability to grow within the mesophilic temperature range and the isolation of strain SL 7 from a tropical swamp (original temperature: 32°C) in Kawah Sileri show that tropical swamps and similar biotopes, e.g. self-heating organic waste, may be further biotopes for these acidophilic archaeobacteria.

From the hot mud sample KC 4, a novel rod-shaped bacterium was obtained on medium 1 (BALCH et al., 1979) at 75°C. The isolate (KC 4; Table 5) is a strict anaerobe, growing in mineral medium in the presence of molecular hydrogen, nitrate (0.1 %) and CO₂ at neutral pH and temperatures of up to 85°C. Ammonia is formed during growth, indicating that the new organism is gaining energy by the up to now unknown anaerobic H₂/NO₃⁻-chemolithoautotrophy. This way of nutrition is made possible by the relatively high nitrate content of up to 5 µmoles/l present in this type of hot springs (G. LIEBEZEIT, pers. comm.). Batch cultures (50 l) were grown within an enamel-protected fermentor (yield: 0.5 g wet weight/l). Further investigations are in progress to characterize this member of a novel group of eubacterial hyperthermophiles.

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