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Isolation and Characterization of an Extremely Thermophilic Sulfur-metabolizing Bacterium from a Deep-sea Hydrothermal Vent System

KWAK, YI-SEONG*, TETSUO KOBAYASHI[1], TERUHIKO AKIBA[1] KOKI HORIKOSHI[1] AND YOUNG-BAE KIM[2]

Korea Ginseng and Tobacco Research Institute, Taejon 305-34, Korea
[1]The Institute of Physical and Chemical Research, Wako, Saitama 351-01, Japan
[2]Department of Food Technology, Korea University, Seoul 136-701, Korea

A water sample was taken from a black smoker chimney of a deep-sea hydrothermal vent by using an unmanned submersible "Dolphin 3K". The temperature of the hydrothermal fluid from the black smoker was 276°C. After isolation by repeated serial dilutions, An extremely thermophilic bacterial strain was selected. The strain designated as DT1331, was an anaerobic, non-motile, coccoid shaped bacterium with about 0.5 to 1.0 μm in diameter. The strain DT1331 could grow up to 93°C, but the optimum temperature of this strain was 80°C. The growth occurred in the pH range of 4.5 to 8.5 and the optimum pH was 6.0. The strain DT1331 required 1% to 5% NaCl for growth and cell lysis was observed below 1% NaCl concentration. The bacterium could grow on polypeptides such as tryptone, peptone, soytone and on proteins such as casein or gelatin. However, no growth was observed on single amino acids, sugar and organic acids. Hydrogen gas was detected slightly during growth. This bacterium obligately required elemental sulfur and hydrogen sulfide gas was produced during growth.

Hydrothermal vent area at deep-sea is one of the extreme aquatic environments, where the hydrothermal fluid of high temperature (>100°C) and a high content of sulfide are erupted from chimneys into the sea water (7, 10, 16). These deep-sea volcanic systems are characterized by a wide variation in temperatures and chemical properties according to the vent environments. Because of the pressure at the depths of these vent environments, water can exist in the liquid form at temperatures near 0 to those greater than 400°C (12). It has been reported that such a habitat offered unique opportunities for investigating life under the extremes of temperature and pressure (1, 2).

The isolation of thermophilic bacterial from a deep-sea hydrothermal vent area has proven to be challenging not only because of the difficulty in getting access to the sites and perhaps the stresses which the bacteria undergo during retrieval but also because the bacteria grow in enrichment cultures yet cannot be subcultured (12). In spite of these difficulties, a number of extremely thermophilic bacteria have recently been isolated and described (4, 8, 9). In addition to methane producing bacteria, they also include sulfur-dependent archaebacteria, such as the obligately lithotrophic genus *Phyrodictium* (14) and numerous heterotrophic cocci capable of both sulfur respiration and fermentation (3, 4, 15, 19, 20).

The characterization of these organisms has led to new insights into many aspects of microbial taxonomy and evolution (17). So far, most of these bacteria have been isolated from the shallow solfataric water holes of a volcano (13).

The purpose of this study was to isolate the extremely thermophilic bacteria that can grow at high temperatures above 80°C from a black smoker structure at deep-sea and then to investigate their morphological, cultural and physiological characteristics which will provide ecological and evolutional informations on these rare microorganisms.

MATERIALS AND METHODS

Collection of Samples and Enrichment.

Samples were taken from a black smoker chimney at Minami-ensei Knoll in Japan (Fig. 1) using an unman-

*Corresponding Author
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Fig. 1. The black smoker chimney structure erupting hot water from the deep-sea hydrothermal vent at Minami-ensei Knoll. The photograph was taken by the Japan marine science and technology center (JAMSTEC).

ned submersible "Dolphin 3K". The sample was put into 50 m*l* screw cap bottles, sodium sulfide was added to final concentration of 0.25% as a reducing agent and then the sample was stored at 4°C (12).

Media

The medium (M14) used throughout the experiment was consisted of an artificial sea water containing NaCl 25.0 g, $(NH_4)_2SO_4$ 1.0 g, KH_2PO_4 0.05 g, $FeSO_4 \cdot 7H_2O$ 0.001 g, $MgSO_4$ 2.0 g, KCl 0.5 g, $CaCl_2$ 0.01 g, Trytone 5.0 g, Yeast Extract 1.0 g, Na_2S 0.4 g, S^0 10.0 g, Resazurine·Na 0.001 g per liter. To this medium was added 10 m*l* of trace element solution ($CuCl_2$ 10 mg, $ZnSO_4$ 100 mg, $CoSO_4$ 5 mg, $MnCl_2$ 200 mg, Na_2MoO_4 100 mg, KI 5 mg, NaBr 50 mg, $Na_2B_4O_7$ 200 mg, LiCl 50 mg, Na_2WO_4 5 mg, Na_2SeO_4 5 mg, $SrCl_2$ 5 mg, $VoSO_4$ 5 mg, $NiCl_2$ 10 mg, $BaCl_2$ 10 mg, $BaCl_2$ 5 mg per liter) and 5 m*l* of vitamin solution (Thiamine·HCl 50 mg, Pyridoxine·HCl 100 mg, Pyridoxamine·HCl 30 mg, Pyridoxal·HCl 30 mg, Calcium D-pantothenate 50 mg, Riboflavin 20 mg, Nicotinic acid 100 mg, *p*-Aminobenzoic acid 10 mg, d-Biotin 0.1 mg, Folic acid 1 mg, Cyanocobalamin 0.1 mg, Lipoic acid 5 mg per liter).

Elemental sulfur was added because in the case of other thermophilic bacteria isolated from deep-sea, it played an important role in their growth (18). $Na_2S \cdot 9H_2O$ was added as a reducing agent in order to achieve reducing condition of hydrothermal vent fluid (12) and resazurin was used as a redox indicator. Trace element and vitamin mixture were added since some thermophilic bacteria require them for growth (4).

Enrichment and Cultivation

One m*l* of each smaple was added to a 13 m*l* serum bottle containing the medium (M14). The remaining head space was filled with medium M14 as much as possible to remove air. The bottles were incubated at 85°C and 95°C until bacterial growth was observed. The pH was adjusted to 6.0 with 1 N H_2SO_4 and 1 N NaOH. The strain was routinely cultivated in 13 m*l* or 20 m*l* serum bottles with butyl rubber stopper. The isolated strain was checked for homogeneity by observing their morphological characteristic under various growth conditions. Sulfur was removed by filtration and cells were separated by centrifugation (12).

Isolation

Attempts to obtain isolated colonies on agar or Gelite plates (18) were unsuccessful, thus a dilution to extinction technique was employed to obtain a pure culture (12). The thermophilic bacteria was isolated by repeated 3 : 1 serial dilution. Media and culture conditions were the same as described above.

Determination of Growth

Growth of isolated culture was determined by counting cell numbers using a counting chamber (Herbers bacterial counting chamber) under phase contrast microscopy (12).

Physiological Charateristics

The presence of hydrogen sulfide was determined by the formation of a dark precipitate in the culture (11). The formation of H_2 was determined by gas chromatograpy (SHIMADZU, JAPAN) with a thermal conductivity detector and column (oven temperature 120 °C, injection temperature 150°C). The upper limit temperature was determined by using a shaking oil bath. To investigate how much the growth is dependent on the pH, the pH of the medium was adjusted with 1 N H_2SO_4 or 1 N NaOH. NaCl was used to examine the effect of salinity and nutritional requirements were investigated by substituting tryptone and yeast extract for other possible energy sources.

Sulfur Requirements

Possible electron acceptors were added to the sulfur-free medium (12). To examine the effect of elemental sulfur on growth, cells were cultivated in medium with or without sulfur. And the head space was degassed continuosly with nitrogen or argon gas to remove hydrogen sulfide gas.

RESULTS AND DISCUSSION

Enrichment and Isolation

Samples were obtained at hydrothermal vents at Minami-ensei knoll in Japan using an unmanned submersible Dolphin 3K. Hydrothermal activity at this area was described in 1991 (5). The temperature of the hydrothermal fluid was 276°C at maximum. Collected samples were enriched as described in Materials and Methods. After two days, abundant growth of cocci was observed in a sample, which was hydrothermal

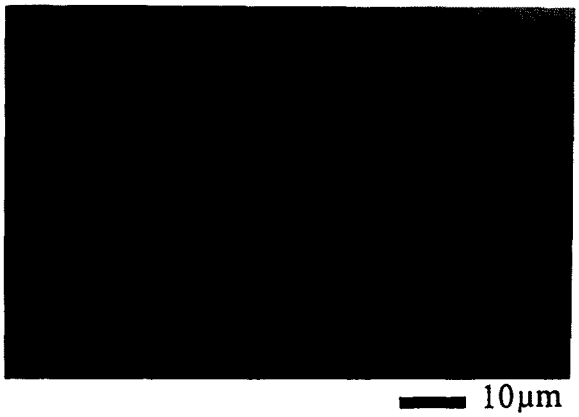

Fig. 2. Differential interference micrograph of deep-sea thermophilic bacterium DT1331.

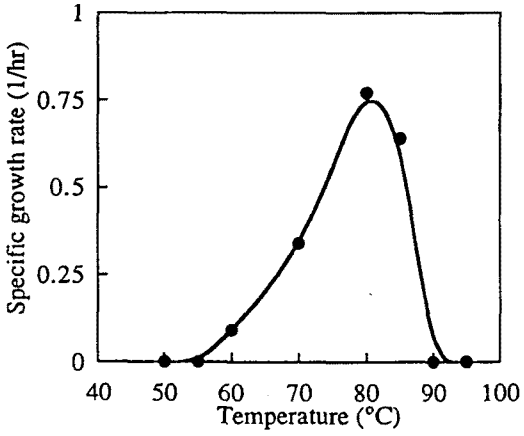

Fig. 3. Effect of temperature on specific growth rates of deep-sea thermophilic bacterium DT1331.

materials collected from a black smoker. A strain of cocci was isolated by the method of repeated serial dilutions. The strain showing growth at the highest dilution rate was designated as strain DT1331.

Morphology

Microscopic inspection exhibited a coccoid type organism resembling the morphology of *Desulfurococcus* (8), *Thermococcus* (12) and *Pyrococcus* (4), which are extremely thermophilic archaebacteria. The strain DT 1331 was an anaerobic, non-motile, coccoid shaped bacterium with 0.5 to 1.0 μm in diameter. No flagella or pili were observed. No septa formation in dividing cells have been observed. Cells multiply most likely by constriction (Fig. 2).

Growth Characteristics

To date, the highest growth temperature for an organism was 110°C for the archaebacterium *Pyrodictium brockii* (14). All organisms known to grow optimally above 75°C were archaebacteria until recent reports of the eubacterium *Thermotoga maritima* growing optimally at 80~85°C (6). The strain DT1331 was able to grow up to 93°C and no growth was observed below 50°C. The optimum temperature was 80°C. At high temperatures above 90°C. DT1331 grew very slowly (Fig. 3). This strain grew in the pH range ,of 4.5 to 8.5 with the optimum pH at 6.0 (Fig. 4).

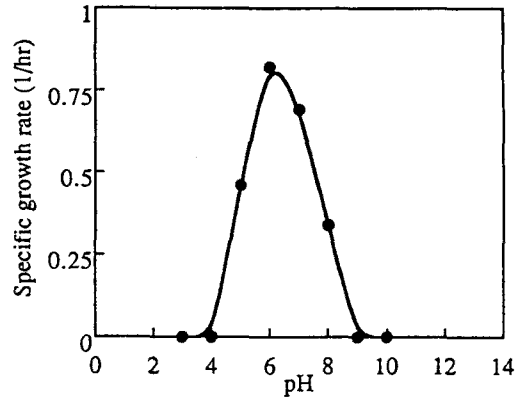

Fig. 4. Effect of pH on specific growth rates of deep-sea thermophilic bacterium DT1331.

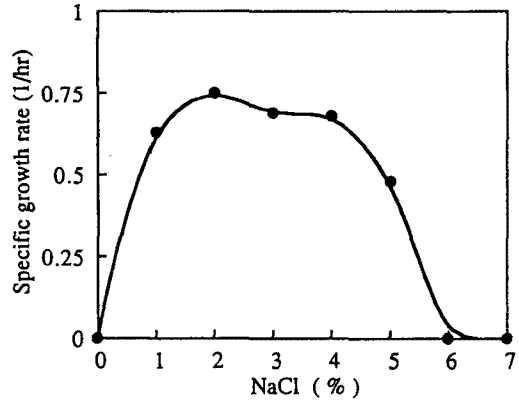

Fig. 5. Effect of NaCl on specific growth rates of deep-sea thermophilic bacterium DT1331.

The pH of the fluids from submarine hydrothermal vents was known to be neutral to slightly acidic (5). DT1331 required 1 to 5% NaCl for growth. The optimum concentration was around 2 to 4% (Fig. 5). Cell lysis was observed below 1% NaCl.

The generation time of strain DT1331 was 50 minute at 80°C, in the medium containing 2% NaCl and 1% sulfur (pH 6.0). The generation time was very short comparing with other extremely thermophilic bacterium. In the case of exteremely thermophilic bacterium *Thermococcus* celer (19), the generation time was 70 min. The lag phase of strain DT1331 lasted about 3 hours and the maximum cell concentration was approximately 1.5×10^8 cells/ml (Fig. 6).

Nutritional Requirements

The isolate grew chemoorganotrophically like some

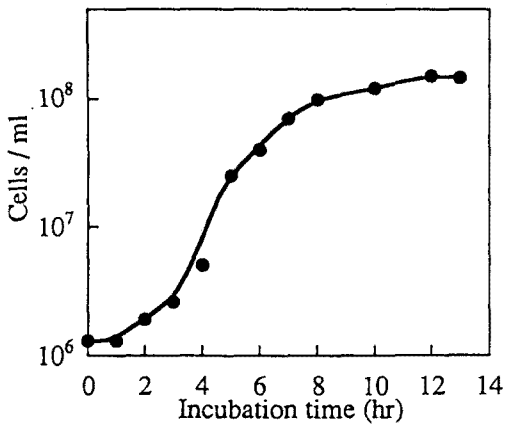

Fig. 6. The growth curve of deep-sea thermophilic bacterium DT1331 under optimal conditions (Temp. 80°C, pH 6.0, NaCl 2.0%, Sulfur 1.0%).

Table 1. The nutritional requirements properties of deep-sea thermophilic bacterium DT1331

Substrates	Growth
Monosaccharides	−
Disaccharides	−
Polysaccharides	−
Organic acids	−
Single amino acid	−
Casamino acid	−
Casamino acid+Tryptophan	+
Proteins (Casein, Gelatin)	+
Peptides (Tryptone, Peptone, Soytone)	+
Yeast extract	+
Vitamin mixture	+
Growth in absence of S^0	−
H_2S production	+

of the related thermophiles such as *Thermococcus* (19), *Pyrococcus* (4) and *Desulfurococcus* (8). It grew well on the medium containing tryptone and elemental sulfur. The bacterium was capable of growing on polypeptides such as tryptone, peptone, soytone, also and on proteins such as casein or gelatin.

Growth was also observed on casamino acids supplemented with tryptophan. However, no growth was observed on any single amino acids. Addition of vitamin mixture strongly stimulated the cell growth. No growth was observed on sugars (glucose, galactose, fructose, ribose, sorbose, xylose, arabinose, sucrose, lactose, trehalose, cellobiose), acetate, ethanol, methanol, starch, cellulose, xylan and on casamino acids (Table 1).

Sulfur Requirements

This bacterium obligately required elemental sulfur and produced hydrogen sulfide during growth. Elemental sulfur could not be replaced with other possible electron acceptors such as SO_2, SO_3, SO_4, S_2O_3 or NO_3 unlike other extremely thermophilic archaebacteria *Desulfurococcus* (8) and *Pyrococcus* (4). It has been found that, with the exception of extermely thermophilic archaebacteria *Thermococcus stetteri* (12), elemental sulfur usually was required in growth concomitant with the formation of H_2S. DT1331 also produced H_2S during growth and a trace amount of H_2 was identified (Fig. 7). It has been known that heterotrophic "S^0-dependent" archaebacteria gain energy by a mixture fermentative and respiratory types of metabolism. Sulfur serves as a hydrogen acceptor. Considering the high temperature and the presence of reducing gas in the black smoker fluid (12), DT1331 seems well to be adapted to the deep-sea hydrothermal vent environment. The results presented in this study suggested that DT1331 is a member of the extremely thermophilic sulfur metabolizing marine bacteria.

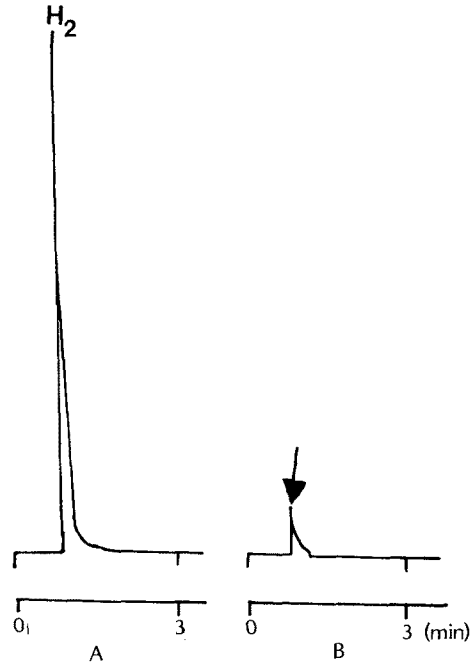

Fig. 7. Gas chromatogram of the gas product of deep-sea thermophilic bacterium DT1331.
Arrow indicates H_2 gas produced by thermophilic bacterium DT1331. A: Hydrogen gas standard, B: Gas product.

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