DISTRIBUTION OF THERMOPHILIC BACTERIA IN GIFU PREFECTURE : I. A PRELIMINARY REPORT ON THERMOPHILES COLLECTED IN THE OKUHIDA SPA AREA

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SUMMARY

Thirteen samples of hot water and one sample of hot sand were collected in the Okuhida spa area. Thirteen of them contained rod bacteria which grew in both a rich medium and a synthetic medium. Without individual species or strains being isolated, the bacteria contained in each sample were analyzed for their growth conditions and biochemical characteristics.

Many thermophilic algae, principally blue-green algae and bacillariophyceae, can be found in the spas of Gifu prefecture, as reported by Emoto in his study of the distribution of thermophilic organisms in spas throughout Japan [1]. Three bacteria have also been reported for Gifu prefecture : *Gallionella ferruginea CHOLODNY, Leptothrix trichogenes CHOLODNY,* and *Leptothrix ochracea KUETZING,* all of which are rod shaped iron bacteria having filamentous structures. However, many bacteria are reported for other prefectures. The discrepancy stimulated us to investigate the distribution of bacteria in Gifu prefecture and to characterize the biochemical behavior of the bacteria found. The Okuhida spa area was chosen because it has the largest amount of hot water and the highest temperatures.

Investigation of thermophiles is very interesting because it can yield information about thermophilic mechanisms. Also, since the biological substances produced by these organisms, such as enzymes, are very stable not only against heat but against other denaturing agents as well[2,3], research may lead to valuable applications of these substances to human life. In fact, in Japan, Oshima and Imahori[4] have characterized an extremely thermophilic bacterium isolated at Mine spa in Izu peninsula, *Thermus thermophilus* strain HB8. This bacterium has been employed to study the heat resisting mechanism of a cell membrane[5,6], the properties of cytochromer c oxidase[7], and a DNA binding protein[8]. This paper reports the results of a preliminary investigation into the growth conditions and biochemical characteristics of yet-unisolated bacteria living in hot water in the Okuhida spa area.

MATERIALS AND METHODS

Chemicals ····· Polypeptone (Daigoeiyo Chemical Co. Ltd., Osaka), yeast extract (Kyo-

kuto Seiyaku Co. Ltd., Tokyo), casamino acids (Difco Laboratories, Detroit, Michigan, U. S. A.), agar (Wako Pure Chemical Industries, Ltd., Osaka), and gelatin (Hayashi Pure Chemical Industries, Ltd., Osaka) were obtained from commercial sources.

Collection of samples ····· Hot water was collected at 13 sites in the Okuhida spa area. The temperature of source water and the pH of the samples determined in the laboratory are shown in Table I. Portions (about 100 ml) of sample water were immediately concentrated approximately 10-times with a Toyo Membrane filter (Type TM-4). The concentrated samples were frozen with dry ice and were lyophilized in the laboratory for preservation. One sample (No. 14, Table I) of hot sand, which was obtained at the bottom of a hot pool (No.7, Table I), was treated in the same way except for concentration. One spoonful of wet sand was subjected to lyophilization after being kept in powdered dry ice.

Culture of bacteria ····· Five ml of the nutritional medium described by Oshima *et al*[4]. were added to the lyophilized materials. The medium contained 0.8% polypeptone, 0.4% yeast extract, and 0.3% NaCl. The pH of the medium was adjusted at room temperature to 7.5 with 1N solutions of NaOH and HCl ("PYN medium"). Stainless-steel capped culture tubes containing the medium were stood in an oven kept at 60℃. After one day's incubation in the oven, one drop of the turbid culture was transferred to a fresh medium. This inoculation was repeated several times to remove the non-biological turbidity which was present in the sample water. Several one-ml portions of each culture were lyophilized and the cells were kept at −50℃ over silica gel until use. When long term culturing (several days or longer) was done, distilled water was added to the culture medium at appropriate intervals to compensate for water lost by evaporation. Cell growth was monitored by measuring absorbance at 650 nm in a spectrophotometer (Model 139, Hitachi Perkin-Elmer) using an F_2 filter. A linear relationship between the cell concentration and the absorbancy was observed in the range of absorbance between 0.02 and 0.12. By counting the cell number with a hematometer, it was estimated that 1.0 unit of absorbance corresponded to 2.8×10^8 cells per ml.

Microscopic observations ····· Flagella staining, spore staining, and gram staining were carried out by the methods of Toda, Dorner, and Hucker, respectively. Cells were usually observed at a magnification of 600 or 1,000.

RESULTS

Microscopic observations ····· Only rod bacteria grew from any of the samples except from No. 10, the sample obtained from a water-pool directly connected to a well pump. All rods were around 0.5—1.0 μm in diameter. The lengths of bacteria cultured in PYN medium are shown in Table I. These values are larger than those of aerobic mesophilic rods and similar to the values reported for an extremely thermophilic bacterium[9]. Accurate comparison, however, will require more detailed investigation under strict conditions concerning such things as the composition of the culture medium and age of cells observed.

Growth of bacteria ····· Optimal conditions for the growth of the bacteria are also summarized in Table I. Detailed data concerning the growth-temperature relationship are shown in Fig. 1, in which the concentration of bacteria is expressed as absorbance at 650 nm. The height of each curve attained in 27 hrs' stand culture roughly represents the growth of the

Table I Properties of sample water collected, growth conditions, and length of rod bacteria

Sample No.	Source		Growth		Cell length
	Temp(℃)	pH	Optium Temp(℃)	Optium pH	(μm)
1	56	8.1	50	6.0—7.0	5—7
2	37	7.9	60	7.0	5—7
3	49	7.7	60 (50)	7.0	5—7
4	62	8.1	50	7.0	7
5	52	7.1	60	7.0	5—14
6	42	7.5	60	7.0—7.5	4—12
7	71	7.9	50	7.0	4—12
8	65	7.9	55	6.5—7.0	5—10
9	39	7.9	50	7.5—8.0	5—10
10	86	7.6	—	—	—
11	65	7.7	60	6.0, 9.0	5—7
12	56	7.9	60 (50)	7.0	5—10
13	62	8.4	55 (50)	7.0	3—10
14	(71)	(7.9)	55	7.0	3—5

The pH of sample water was determined at room temperature (around 30℃) in the laboratory. The values of the optimum pH for growth are those adjusted at the room temperature when the culture media were prepared.

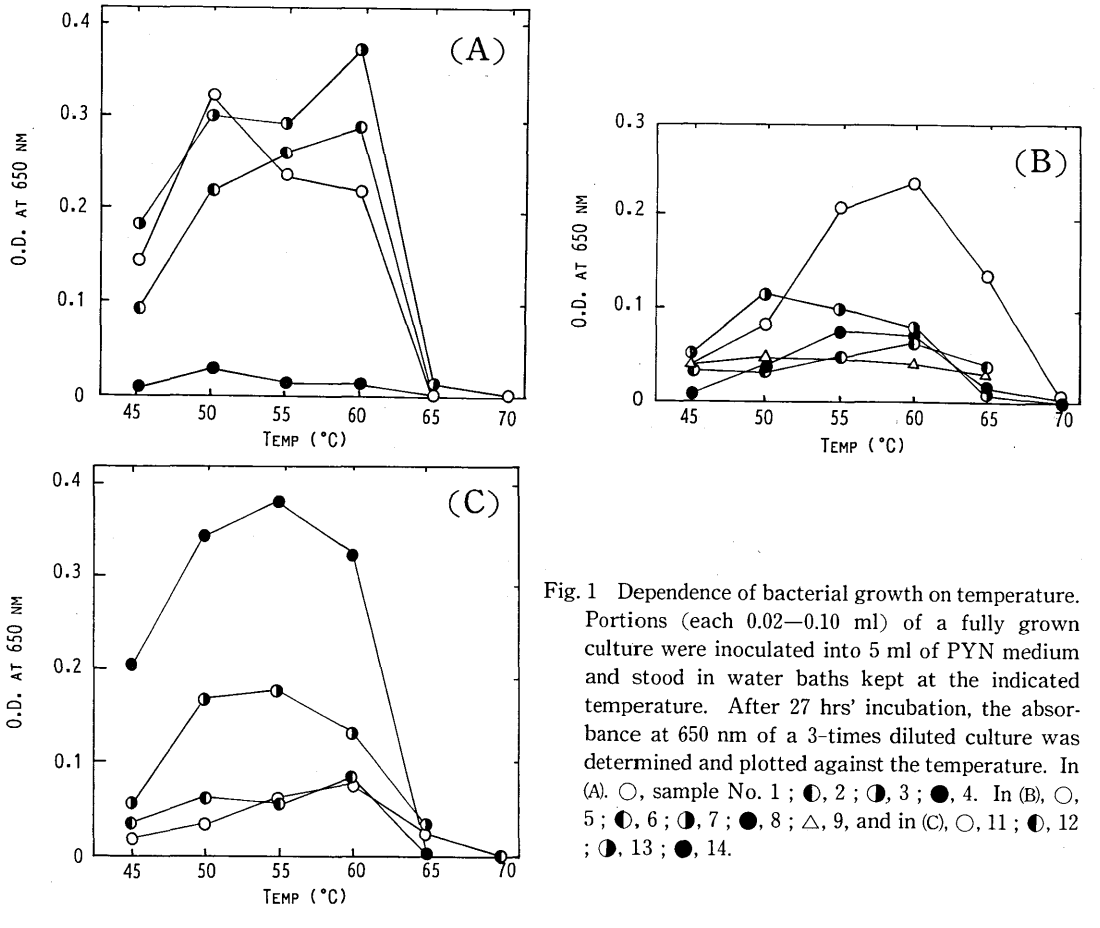

Fig. 1 Dependence of bacterial growth on temperature. Portions (each 0.02—0.10 ml) of a fully grown culture were inoculated into 5 ml of PYN medium and stood in water baths kept at the indicated temperature. After 27 hrs' incubation, the absorbance at 650 nm of a 3-times diluted culture was determined and plotted against the temperature. In (A). ○, sample No. 1 ; ◐, 2 ; ◑, 3 ; ●, 4. In (B), ○, 5 ; ◐, 6 ; ◑, 7 ; ●, 8 ; △, 9, and in (C), ○, 11 ; ◐, 12 ; ◑, 13 ; ●, 14.

bacteria of each sample at the indicated temperature, since the growth of cells at each peak was ascertained, by prolonged culturing, to be the maximum under the conditions. It is clear that the bacteria contained in each sample grew best under temperature conditions the same as or similar to those of the source water from which they came in all but a few cases (No. 2, 6, and 9). Bacteria in samples No. 3 and 12 presented two absorbance peaks, clearly indicating the presence of at least two different species or strains of rod bacteria. Growth rates were also observed at various pHs. Bacteria in all the samples except for No. 11 showed nearly symmetric growth curves (data not shown) with one optimum pH value (Table I). Two optimum pH values were obtained for sample No. 11, one at pH 6.0 and the other at fairly alkaline pH 9.0 (Fig. 2).

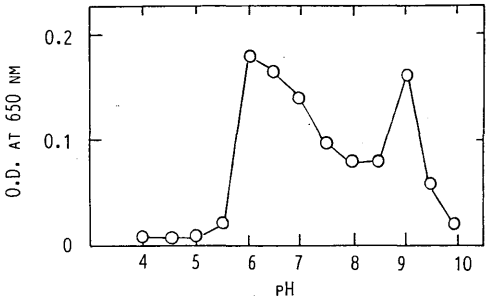

Fig. 2 Dependence on pH of growth of bacteria contained in sample No. 11. Portions (each 0.05 ml) of a fully grown culture of the bacteria in sample No. 11 were inoculated into 5 mls of PYN medium at varying pHs as indicated, and the culture tubes were stood at 60℃. Determination of absorbance was carried out in the same way as in Fig. 1.

Bacterial growth in the presence of 2% NaCl was examined using PYN medium. Bacteria in all the samples except No. 5, 6 and 12 could grow well at this concentration, but no bacteria grew in any sample with 5% NaCl in the PYN medium.

Biochemical characteristics of bacteria ····· The gelatin liquefaction ability of the bacteria was tested by inoculating one drop of fully-grown culture of bacteria into 5 ml of PYN medium containing 3% gelatin. After 7 days' incubation at 60℃, the cultures were taken up into Pasteur pipets (7095B-9, Corning Glass Works, New York) and the pipets were stood in ice-water for 20 min. Liquefaction of the gelatin was confirmed by checking whether the culture dropped

Table II Summary of three biochemical characteristics examined

Sample No.	Gelatin liquefaction	KNO_3 reduction	H_2S production
1	+	−	+++
2	+	+	++
3	+	+	−
4	−	+	−
5	−	+	++
6	+	+	−
7	+	+	+++
8	+	−	−
9	+	−	+
11	+	−	++
12	+	+	++
13	+	−	+
14	+	+	+++

Generally (+) means positive result and (−) negative result. In the sulfide production assay, a double or triple mark means stronger production of sulfide relative to that in single mark cases.

down through the pipets or remained in them when the caps were removed. The results are shown in Table II.

Nitrate-reducing ability of the bacteria was investigated with 0.1% KNO_3 added to the medium. After 2 days' culture of the bacteria at 60℃, the presence of KNO_2, the reduction product, was ascertained with α-naphthylamine and sulphanyl acid. Bacteria in 8 samples had reduced nitrate (Table II).

Indole production from polypeptone was tested after 2 days' culture in PYN medium. The presence of indole was detected by Ehrlich's method using ρ-dimethylaminobenzaldehyde and potassium peroxodisulfate. Bacteria in only one sample, No. 14, had produced indole.

Urea decomposing ability was examined with 2% urea added to the culture medium. After the bacteria were well grown (2 days' incubation), 30 μl of 0.2% methylred solution was added to the cultures. No bacteria were observed to decompose urea.

Production of H_2S was examined with 1% L-cysteine-HCl added to the culture medium. Strips of filter paper treated with a solution containing 10% lead acetate and 10% glycerol were hung in the culture tubes so that PbS would form on the strip once H_2S was liberated from the cysteine by the bacteria and its black color indicate the occurrence of H_2S production. These results are also shown in Table II.

Utilization of sugars by bacteria was investigated by identifying acid production in the culture medium. Twice diluted PYN medium, the pH of which was adjusted to 7.0, was supplemented with a sugar at a concentration of 0.5% and bromphenolblue as a pH indicator at a concentration of 0.002%. The medium (5 ml) was inoculated with one drop of fresh culture of bacteria and incubated at 60℃. Change in color to yellow was observed daily, with the results summarized in Table III. The same results were obtained when a synthetic medium for yeast[10] was employed as a basal medium without either $CaCl_2$ or glucose added, in place of the

Table III Summary of acid production assay

Sample	Sugars				
	Without	Glucose	Maltose	Sucrose	Mannitol
1	—	—	—	—	—
2	slow	fast	—	fast	slow
3	—	fast	slow	fast	slow
4	—	fast	—	—	slow
5	slow	—	—	—	—
6	—	slow	—	fast	—
7	slow	fast	fast	—	—
8	—	—	—	—	—
9	—	slow	fast	slow	slow
11	slow	fast	fast	fast	fast
12	—	—	—	—	—
13	—	—	—	—	—
14	slow	fast	—	slow	fast

fast : color change in 2 days
slow : color change in 4 days

One drop of fresh culture of each sample was inoculated into 5 ml of the test medium and the cells cultured at 60℃.

PYN medium. As seen in the table, bacteria of 5 samples (No. 1, 5, 8, 12, and 13) produced no acid from either of the sugars tested.

All the bacteria were aerobic and grew only on the surface of both liquid culture media and agar media. Thus there was no way to test whether these bacteria produce gas from sugars.

DISCUSSION

Only rod bacteria grew in a rich medium at 60℃ from water samples collected in the Okuhida spa area of Gifu prefecture. In some samples, particularly in Nos. 5—7, filamentous structures were also observed. This fact suggests that the iron bacteria reported by Emoto[1] are contained in these samples. Biochemical characterization of these bacteria was carried out before any isolation of species or strains in order to draw out all detectable and interesting characteristics of organisms. It must be noted of course that this type of investigation may fail to uncover interesting characteristics of some of the bacteria due to possible interference by the presence of other bacteria.

Results of experiments on growth conditions of the bacteria showed that almost all organisms grew best under conditions the same as or very similar to those of the source water in which they lived. Thus all bacteria observed in this study can be classifed as moderate thermophiles according to Oshima[11]. It was also shown that all bacteria prefer neutral pH except those in sample No. 11. Casamino acids added to the medium at a concentration of 0.6% in place of polypeptone were also good nutrients for the bacteria in all the samples shown in Table I. Bacteria in all the samples also grew well in a synthetic medium[10] from which $CaCl_2$ was omitted so that pH could be adjusted to 7.0 without forming precipitates. It was also confirmed using bacteria of sample No. 11 that vigorous shaking at 60℃ enhanced the growth of bacteria 4—5 times.

That the temperatures of sample (Table I) were lower than the values (around 97℃) known for hot spring water in this area will have to be explained. Samples were collected in small, natural pools formed where water spilled from springs or wells. Sample No. 10 had a high temperature, however, no organism grew from this sample under the conditions employed, since this sample was obtained in an artificial pool built of concrete and connected to a hot well pump. No bacteria grew at temperatures higier than 70℃ (Fig. 1) and at temperatures lower than 35℃ (data not shown).

It is very interesting that bacteria of sample No. 11 grew only with two optimum pHs. This may simply imply that this sample contains two distinctly different bacteria. Even if so, why the two bacteria, having optimum pH for growth at 6.0 and 9.0, occur in water of pH 7.7 will have to be considered. Moreover, that a thermophile preferred an alkaline pH for growth is physiologically interesting and there is the possiblility of utilizing the products of these bacteria, since they seem to be stabl at an alkaline pH.

A gelatin liquefaction test showed positive results for almost all bacteria when gelatin was added to the PYN medium at a concentration of 3% (Table II). Bacteria in only two samples (No. 1 and 11) grew in a PYN medium containing gelatin at a concentration of 30% and these also liquefied gelatin completely in 2—3 days. These samples are likely to contain some interesting bacteria which produce proteases with high activities. In fact, a culture medium of

a bacterium isolated from sample No. 11 was ascertained, after concentration, to be effective in hydrolyzing synthetic substrates for collagenase[12,13], so the bacterium may produce a collagenase. Purification and characterization of the hydrolyzing activity is now under way.

Although some problems are associated with the nitrate reduction test employed here, it can safely be said that bacteria showing positive results can reduce nitrate to nitrite. For the organisms with negative results, care must be taken : either they could not catalyze this step, or the nitrite formed did not accumulate in the culture medium at the time of the test, which probably would have been due to the activity of some of the components of the reduction system.

These investigations into the growth properties and biochemical characteristics of thermophilic bacteria have suggested at least the presence of many species or strains of rods in hot water in the Gifu area, although only three have been reported[1]. Secondly, some of the organisms that will be isolated are likely to have utilizable products.

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