深海熱水噴出域および海底火山活動域における
化学合成硫黄細菌の採集

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掘越弘毅

化学合成硫黄細菌を以下の3種の海底試料から分離した：(1)チューブワーム血液
このチューブワームは沖縄トラフ伊平屋海間の熱水活動域で採取した；(2)鷲見
島湾の海底噴気活動（“たぎり”）域の底泥；(3)手石海丘の火山底泥。手石海丘は
1989年7月に誕生した海底火山である。分離された硫黄細菌の特徴を調べたと
ころ、次のような結果が得られた：(1)チューブワーム血液中には少なくとも11種類の
細菌が存在していた。その中の1種の増殖最適温度が0.5℃から、この細菌は海水塩
分よりもむしろチューブワーム組織の塩分に適応していると考えられる。(2)化学合
成硫黄細菌はほとんどが無色硫黄細菌にもかかわらず，“たぎり” 域から分離され
た硫黄細菌は赤～紫（540nmに吸収極大）の色素を生産した。(3)手石海丘から分離
された硫黄細菌の増殖最適塩分は0.6％であったが、手石海丘の位置（沖合約2 km
から想定して、河口域由来の硫黄細菌と考えられる。

Collection of Chemosynthetic Sulfur Bacteria from
a Hydrothermal Vent and Submarine Volcanic Vents

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and Deep Star Group*6

Abstract

Chemosynthetic sulfur bacterial strains were isolated from: 1) blood of tube
worms inhabiting a hydrothermal vent area of Iheya Depression in the mid
Okinawa Trough, 1400m deep; 2) a submarine volcanic vent in Kagoshima

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Bay, where submarine volcanic activity called *tagiri* is known, 200m deep; and 3) a submarine crater of *Teishi* Knoll, which erupted in 1989, 115m deep. Their characteristics (e.g. colony and cell morphology, Gram staining, and growth physiology) were examined. The results were as follows.

1) At least eleven bacterial species were found in tube worm blood, one of which was studied with physiological emphasis. It grew best at 43°C, 0.6% salinity and pH 7.0 at 1 atm pressure. Its slightly halophilic growth suggests its adaptation to the salinity of tube worm tissue rather than the seawater salinity.

2) A sulfur bacterial strain isolated from a *tagiri* vent produced red to purple pigment. The pigment had an absorption peak at 540nm in chloroform extract of cell suspension. Since colored chemosynthetic sulfur bacteria have been little reported, its taxonomy and physiology would be of great importance and interest.

3) A sulfur bacterial strain was isolated from sediment inside the *Teishi* Knoll crater. Its optimum salinity of 0.6% for growth and location of the knoll only 2km off shore suggest the strain possibly of estuarine origin.

### 1. Introduction

Sulfur bacteria are thought to play essential roles not only in supporting biological communities at hydrothermal vents (e.g. Karl et al., 1980; Baross and Deming, 1985; Tuttle, 1985), but also in mediating geochemical cycles of sulfur and inorganic compounds through oxidation and reduction there. For example, it was estimated that $1.5 \times 10^6$ tons of $S^2-$sulfur at hydrothermal vents, about a half of total emission, may be used for sulfur bacterial chemosynthesis (Jannasch, 1989). While such bacterial processes in sulfur and carbon cycling at hydrothermal vents have been discussed (e.g. Jannasch and Mottl, 1985), hydrothermal activity itself appears a source of carbon dioxide as well as sulfides to the oceanic environment (e.g. Sakai et al., 1990; Honda, 1990).

As bacterial chemosynthetic carbon fixation has been suggested to make not a negligible contribution to marine carbon pathways (Karl et al., 1984), sulfur bacterial chemosynthesis could be thought as a major factor affecting the geochemical dynamics of carbon and sulfur (e.g. Wirsen et al., 1986).

A number of bacteria mediating the sulfur cycle through reduction and oxidation have been observed at and isolated from hydrothermal vents and plume, most of which are thermophilic archaeabacteria (Table 1; Prieur et al., 1981, for eastern Pacific). However, more sulfur bacterial species are expected to occur, and collection of sulfur bacteria is still needed to study carbon and sulfur cycling in more detail.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Description</th>
<th>Reference</th>
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<tbody>
<tr>
<td><em>Pyrodinium occultum</em></td>
<td>Submarine vent</td>
<td>Sutter (1982)</td>
</tr>
<tr>
<td>PL-19</td>
<td>Submarine vent</td>
<td>Sutter et al. (1983)</td>
</tr>
<tr>
<td>P. brockii</td>
<td>Submarine vent (Italy, 2-10 m deep)</td>
<td>Sutter et al. (1983)</td>
</tr>
<tr>
<td>Archaebacteria</td>
<td>Submarine vent</td>
<td>Sutter et al. (1983)</td>
</tr>
<tr>
<td>Thermoproteus teuaux</td>
<td>Submarine vent (Iceland)</td>
<td>Fischer et al. (1983)</td>
</tr>
<tr>
<td>T. neutrophilis</td>
<td>Hot spring (Iceland)</td>
<td></td>
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<tr>
<td>T. sp. Hz</td>
<td>Sulfate mud (Iceland)</td>
<td></td>
</tr>
<tr>
<td>T. sp. Bo5</td>
<td>Hot spring (California)</td>
<td></td>
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</tbody>
</table>
A hydrothermal vent area in the mid-Okinawa Trough is an excellent site of geochemical and microbiological interest. Hydrothermal fluids vented there are rich in carbon dioxide and hydrogen sulfide at the same time (Sakai et al., 1990). From water and organisms collected there, several sulfur bacterial strains were isolated (Naganuma et al., 1990b; Table 1). Some of the strains were from tube worms, mussels and limpets, but it is likely that they were epibiotic, not symbiotic (see Gaill et al., 1984; Alayse-Danet et al., 1985). There is still need to collect bacterial samples from truly inside the bodies of tube-worms or other host animals.

On the other hand, whether the collected sulfur bacteria were epibiotic or truly endosymbiotic, collection of sulfur bacteria from shallow water vents, as a counterpart of deep-sea hydrothermal vents, is required to see biological similarities and differences of vent-associated communities.

Kagoshima Bay, southwest Japan, is a candidate for the counterpart. Sakura-jima, an active volcano in Kagoshima Bay, shows submarine vent activity, so called tagiri in Japanese (“boiling” or “seething” in English). High concentrations of hydrogen sulfide were reported for the volcanic gas from submarine vents and ambient seawater up to 0.8% (v/v) and 7.1 ppm (w/v), respectively (e.g., Kagoshima Prefecture, 1978). And seemingly vent-associated mud-tubes were directly observed by a manned submersible Hakuyo. The mud-tubes were reported 5 to 10 cm long and 1 cm wide, and an annelid inhabited inside each tube (Kagoshima Prefecture, 1978). These observations suggest the close resemblance to deep-sea hydrothermal biological communities except hydrostatic pressure.

A recent tagiri survey with Dolphin 3K, a remotely operated vehicle, showed that the tagiri vents were still active with high temperatures of up to 105°C in sediment and high concentrations of hydrogen sulfide and methane (Hashimoto et al., 1990; Osaka et al., 1990).

Dolphin 3K also targeted at a newborn submarine volcano. An eruption in June 1989 resulted in the formation of Teishi Knoll, located 2 km off east coast of Izu Peninsula, about 100 km south-
west of Tokyo. The survey of the Teishi Knoll crater, about 13 months after the eruption, recorded high temperatures of up to 70°C or more in crater sediment and images of shimmering water (Naka and Deep Sea Research Group, 1990). The case of Teishi Knoll must be of special interest because it has been being studied from the very birth or earlier. From biological points of view, it seems to be an excellent field for studying the settlement and succession of hydrothermal vent-associated organisms (see Hessler, 1987).

During the surveys of the Iheya hydrothermal vents, the Tagiri vents in Kagoshima Bay and the Teishi crater vent, biological and microbiological samples were collected. Although elevated bacterial biomass and growth stimulated with thiosulfate addition were found in hydrothermal plume waters (e.g. Winn et al., 1986; Naganuma et al., 1989), vent sediment and macro-organisms, which seem to harbor more abundant and diverse bacteria, were principally collected for this study. The article reports preliminary results of isolation and characterization of sulfur bacterial strains from those samples of 1) blood of tube worms inhabiting a deep-sea hydrothermal vent area (Iheya); and 2) sediments of submarine volcanic vent areas (tagiri and Teishi).

2. Materials and Methods

2.1. Collection of tube worm blood and bacteria occurring therein

A deep-sea diving to a hydrothermal biological community in the mid-Okinawa Trough was made in May 1990 by a submersible Shinshii 2000 (JAMSTEC). The hydrothermal site is located in Iheya Depression, at 27°33.0'N, 126°58.1'W, 1390-1400m deep (Naganuma et al., 1990b; Table 2).

Individuals of tube worms that inhabited the edge of fissures venting hot water of 100°C or more were collected without heavy damage by the submersible’s manipulator, and put in a polyvinyl container for thermal insulation. Hydrostatic pressure at the site, about 140atm, was not retained.

Immediately after retrieval, a sheath tube of each tube worm was dissected and removed to expose whole part of its soft tissue: the anteriormost part (prosoma), a short forepart (mesosoma), a trunk that was the longest part of the body (metasoma) and posterior portion (opisthosoma) (terminology, according to Brusca and Brusca, 1990). Most of the tube worms were “thin” type ones (Naganuma et al., 1990b). The body length was 10 to 30cm and the width was less than 5mm.

The bodies were rinsed with 75% ethanol to decontaminate epibiotic microorganisms. Each body was cut at the top trunk, and the blood (and possibly other coelomic fluid) was collected in a serum tube with the body inverted. About 1ml of blood was obtained from a 10cm-long, and 1.5ml from a 20cm-long. Every care was taken to avoid the chance of contamination by epibiotic bacteria.

Each 0.1ml of the blood was spread on a 1.5% agar plate containing : 0.1% peptone (designated P) or 1% sodium thiosulfate pentahydrate (40mM Na2S2O3; T) or 1% sodium sulfide nonahydrate (42mM Na2S; S) or their combinations of P + T, P + S and T + S, in seawater. Portions of the blood were also added to a liquid medium containing 1% sodium sulfide nonahydrate in seawater. The inoculated plates and the liquid medium were kept at 4°C for 4weeks including the cruise, then at 27°C at laboratory.

2.2. Collection of sulfur bacteria from sediments of submarine volcanic vents

Sediment samples were collected by an unmanned
The sulfur bacterial isolates were cultured both organically with 0.1% peptone and inorganically with 40mM thiosulfate. The organically grown cultures were mainly used for characterization of their diagnostic features, most of which were examined with commercially available kits: O/F Test (Eiken Kagaku, Tokyo) for oxidative/fermentative test; Oxidase Disk and Cytochrome Disk (Eiken Kagaku, Tokyo) for cytochrome χ oxidase test; ID Test NF-18 and EB-20 (Nissui Seiyaku, Tokyo) for enzyme activities (urease, β-galactosidase, phenylalanine deaminase, lysin decarboxylase, arginine dihydrolase and ornithine decarboxylase), hydrogen sulfide production, indole production, esculin hydrolysis, Voges-Proskauer test, gelatin liquefaction and nitrate reduction. Other items, e.g. gram staining, catalase activity and motility, were examined by ordinary methods (Smibert and Krieg, 1981).

Growth responses to temperature, salinity and pH were examined for the cultures with an organic medium (0.1% peptone) at or near the optimum conditions for each strain, if data available. Otherwise, bacterial strains were cultured principally at 37°C, 3% NaCl, and pH 7.4. growth was measured by absorbance at 660nm with 1-cm light path.

In cases the numbers of bacterial cells were directly counted by epifluorescence microscopy combined with membranese filtration (Hobbie et al., 1977).

3. Results

3.1. Bacterial strains from tube worm blood (agar plate incubation)

From tube worm blood sample, 10 distinguishable bacterial colonies were observed. The colonies were not observed during the first 4 weeks at 4°C, but then grew to visible sizes soon after transferring to 27°C. They were differentiated from each other by color, shape and margin of colonies (Table 3). The same descriptions in the table do not fully express subtle variation recog-
Table 3 Observation of colonies formed by bacterial strains from tube-worm blood.

<table>
<thead>
<tr>
<th>TW-</th>
<th>Color</th>
<th>Shape</th>
<th>Margin</th>
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</thead>
<tbody>
<tr>
<td>2</td>
<td>pink</td>
<td>circular</td>
<td>entire</td>
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<tr>
<td>4</td>
<td>white</td>
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<tr>
<td>14</td>
<td>yellow</td>
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nizable by careful observation. For example, strains TW-4 and TW-13 could be distinguished by: more white margin for TW-4; and creamy white and more elevated colony for TW-13. Also TW-7 and TW-8 were separated by: thin yellow for TW-7; and yellow to orange for TW-8. And TW-12 and TW-14 were differentiated by elevated and flat colonies, respectively. Difference in colony shapes between punctiform and circular was not very essential, because it was more arbitrary than color and margin.

There are missing numbers following "TW-" (e.g. TW-1 is missing), which dropped off during subculture, despite every effort to maintain them. Most of the "droppers off" formed white or yellow filamentous or rhizoid colonies.

Physiological and biochemical examination confirmed the distinction of the 10 colony types based on colony observation. First, gram-stain differentiated TW-7 from other yellow, circular colony types (Table 4). Other features in table 4 (spore formation, oxidative/fermentative test, catalase activity and oxidase activity) could not support the colony grouping, except cell morphology grouping out TW-02 (sphere) and TW-07 (short rod) from others (rods).

Examination on enzyme activities supported the separation within white, entire margin colonies (TW-04, TW-05, TW-09 and TW-13; Table 5). Also among yellow colony group, TW-8, was separated from TW-12 and TW-14 (Table 5). TW-12 and TW-14 were distinguished by the ability of nitrate reduction (to nitrite) for TW-12 and inability for TW-14 (Table 6).

Table 5 Enzymatic characterization of bacterial strains from tube-worm blood. Cat, catalase; Ox, oxidase; ADH, arginine dihydrolase; β-Gal, β-galactosidase; LDC, lysin decarboxylase; ODC, ornithine decarboxylase; PDA, phenylalanine deaminase; Ure, urease. Designations of "+" and "−" are positive and negative enzyme activities, respectively.

<table>
<thead>
<tr>
<th>TW-</th>
<th>Cat</th>
<th>Ox</th>
<th>ADH</th>
<th>Gel</th>
<th>LDC</th>
<th>ODC</th>
<th>PDA</th>
<th>Ure</th>
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Table 6 Metabolic activities characterizing bacterial strains from tube-worm blood. Esc, esculin hydrolysis; Gel, gelatin liquefaction; H₂S, hydrogen sulfide production; Indol, indole production; NR, nitrate reduction; VP, Voges-proskauer test. Designations of "+" and "−" are positive and negative metabolic activities, respectively.

<table>
<thead>
<tr>
<th>TW-</th>
<th>Esc</th>
<th>Gel</th>
<th>H₂S</th>
<th>Indol</th>
<th>NR</th>
<th>VP</th>
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Thus grouping of the 10 colony types were confirmed by combination of several physiological and biochemical characteristics. All of them grew aerobically. Most of the 10 types could grow on inorganic media designated T, S, and/or T + S (see Materials and methods): TW-02, TW-05 and TW-07 could grow on mixotrophic media of P + T and/or P+S. But TW-09 formed colonies exclusively on the organic P medium. Inferred from colony counts of the primary inoculation, TW-09 occurred in tube worm blood in not a small number, more than $10^3$ cells ml$^{-1}$. The comparable abundance was shown only by TW-04 growing on a wide range of media as P, T, P+T, P+S and T +S.

### 3.2. A sulfur bacterial strain from tube worm blood (liquid culture)

A sulfur bacterial strain, TW-A, was isolated from liquid culture inoculated with tube worm blood. TW-A, an aerobic straight rod (Photo 1), was predominant in the liquid culture, but did not form colonies in detectable size and number on the agar plates of first tube worm blood inoculation. When portions of the liquid culture were streaked or spread on organic (0.1% peptone) or inorganic (40mM thiosulfate) agar plates, it did form colonies on both types of plates. The colony and cell morphology, gram-staining, and other basic characteristics of TW-A are summarized in Table 7. These characteristics suggested TW-A be different from the bacteria of ten different colony types mentioned above.

Growth was indicated as cell abundance after a given time of incubation, and the cell abundance was measured by colormetric absorbance at 660nm. A relationship of absorbance to cell abundance of TW-A was calibrated with a culture grown on 0.1% peptone. A good linear relationship was seen for the cell abundance up to $1.5 \times 10^8$ cells ml$^{-1}$, and it was expressed as:

$$[A_{660}] = 0.007 + 7.9 \times 10^{-10} \text{ [cell ml}^{-1}]$$

$r = 0.971 \ (n = 16)$

where $A_{660}$ represents the absorbance at 660nm, $r$ is the correlation coefficient, and $n$ is the number of samples.

Growth curves, i.e. increase or decrease of the absorbance in time-course, were also examined for 5 incubation ways (Fig. 1) at $37^\circ$C: 1) reciprocal shaking with baffled flasks (filled circle);
2) reciprocal shaking with Erlenmeyer flasks (open square); 3) reciprocal shaking with test tubes (triangle); 4) gyratory shaking with Ehlenmeyer flasks (open circle); and 5) stand still (filled square). The maximum abundance and fastest growth was shown for the reciprocal shaking with baffled flasks. Therefore other growth experiments employed this incubation way for mostly 12 hours, but growth at different temperatures was measured after 24 hours of stand still incubation.

Growth response of TW-A to temperature, salinity (as concentration of sodium chloride) and pH was examined. First, in a temperature range from 12°C to 42°C, it grew best at 43°C (Fig. 2). The optimum temperature was nearer the maximum temperature for growth, about 47°C, than the minimum temperature for growth, below 12°C, which is very usual as seen in most "textbooks".

The optimum salinity for TW-A growth was 0.6% NaCl, which is 1/5 of seawater strength. At seawater salinity, between 3 and 3.5% NaCl, the growth was about 1/5 of the highest growth (Fig. 3).

The optimum pH for growth was between 7.0 and 7.8. The pH range for growth could be roughly depicted 4 and 11 (Fig. 4).

![Fig. 2 Growth of a sulfur bacterium TW-A at different temperatures. The culture was grown on 0.1% peptone at 0.6% NaCl and pH 7.4.](image)

![Fig. 3 Growth of a sulfur bacterium TW-A at different salinities (NaCl concentrations). The culture was grown on 0.1% peptone at 37°C and pH 7.4.](image)

![Fig. 4 Growth of a sulfur bacterium TW-A at different pH. The culture was grown on 0.1% peptone at 37°C at 0.6% NaCl.](image)

3.3. A sulfur bacterium from Kagoshima Bay Tagiri vent

Bacterial abundances in surface and bottom water of Kagoshima Bay were determined. The counts were \(4 \times 10^6\) cells ml\(^{-1}\) for the surface water and \(7 \times 10^4\) cells ml\(^{-1}\) for the bottom water at 200m deep. Both counts were within the range of known bacterial abundance for the depths (e.g. Naganuma et al., 1990a).

At the edge of a bubbling vent, white flocculate material swaved by the upward movement of gas and water was observed, and part of the flock was collected. The flock was composed of filamentous microorganisms branching and bundling each other, with length varying from tens nm to mm.
(Photo 2). Some of the filaments have clear septa partitioning a filament into compartments (Photo 3). Each compartment was 20-50\mu m long and 20-30\mu m wide. Deposition of particles (inclusions) within the compartments could be observed. Unfortunately, isolation of these filamentous microorganisms is under way. However, we could observe minor restoration of white floculate formation on and in the sediment sample kept in 50-ml tubes. Cultivation of them will be reported elsewhere.

A sulfur bacterial strain, Tag-1, was isolated from a thiosulfate agar plate inoculated with the submarine vent sediment of Kagoshima Bay. Tag-1, an aerobic short rod, could grow on inorganic media containing 40mM sodium thiosulfate, but grew better on organic media containing peptone and/or yeast extract up to 2% concentration. Its colony and cell morphology, gram staining, and other basic features are summarized in Table 8.

Characteristically, both colonies and liquid culture of Tag-1 presented red to purple pigmentation (Figure 5). Chloroform extract of the pigmentation was associated with mucoid matter that covered colony surface or liquid surface. At the later stationary phase of growth in liquid culture, the pigmented covering sank down to the bottom. The pigmented mucoid matter was most likely polysaccharides, because it disappeared or thinned with removing polysaccharides from cell suspension by CTAB extraction (CTAB, hexadecyltrimethyl ammonium bromide; according to Frederick et al., 1989).

Growth of Tag-1 at different temperatures after 24 hours of stand incubation is shown in Fig. 6. The optimum temperature for growth was 27°C.

![Absorbance vs Temperature](image)

**Fig. 5** Absorption spectra with cell suspension of a sulfur bacterial strain Tag-1 at different incubation times and with chloroform extract from the suspension. The culture was grown on 0.1% peptone at 37°C, 2% NaCl, and pH 7.4.

**Fig. 6** Growth of a sulfur bacterial strain Tag-1 at different temperatures. The culture was grown on 0.1% peptone at 2% NaCl and pH 7.4.
The growth response to salinity (% NaCl) after 12 hours of shaken incubation revealed the optimum salinity for growth was 2% (Fig. 7). Little or no growth was seen at 0% salinity, while 1/2 of maximum growth was maintained at 4.5%, about 1.5 times of seawater strength (Fig. 7).

3.4. A sulfur bacterium from Teishi Knoll vent

The mortar-like crater of Teishi Knoll was heavily covered and mostly flattened with sediment, compared with a towed camera survey in September 1989 (Deep Sea Research Department, unpublished). The sedimentation observed this time was possibly due to fine particles drifted together by a series of typhoons in July and August 1990 (Naka and Deep Sea Research Group, 1990).

Exposed rocks and stones were observed outside the crater and sporadically inside it. Their surfaces were colonized by polychaete worms, which had been rarely seen by the previous camera survey a year before. This worm, forming a calcareous tube, was thought to belong to Serpulolid (e.g., *Hydroides*, *Spirobranchus*, *Pomatoleos*) which are commonly seen.

Sulfur bacterial colonies formed by the first inoculation did not necessarily represent a single clone population, but a mixed population of sulfur bacteria and others, some of which were extremely elongated (Photo 4). After a series of subculturing, a sulfur bacterial strain was isolated from the crater sediment. This sulfur bacterium, Tei-1, formed relatively large white colonies of 5mm or more in diameter on inorganic agar plates containing 40mM sodium thiosulfate. Its colony and cell morphology, gram staining, and other basic features are summarized in Table 9.

<p>| Table 9 General characterization of a sulfur bacterium Tei-1 from sediment of an submarine volcanic vent of Teishi Knoll. Abbreviations are the same as in Tables 5 and 6. |</p>
<table>
<thead>
<tr>
<th>Colony color</th>
<th>Colony shape</th>
<th>Colony margin</th>
<th>Cell shape</th>
<th>Gram stain</th>
<th>Spore formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creamy</td>
<td>Circular</td>
<td>Entire</td>
<td>Straight</td>
<td>Negative</td>
<td>No</td>
</tr>
<tr>
<td>Oxidative</td>
<td>Fermentative</td>
<td>Cat</td>
<td>Oxi</td>
<td>ADH</td>
<td>+Gal</td>
</tr>
<tr>
<td>Oxidative</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Esc</td>
<td>Gel</td>
<td>H$_{2}$S</td>
<td>Indol</td>
<td>NR</td>
<td>VP</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Growth of Tei-1 showed a mesophilic nature with the optimum temperature for growth of 37°C (Fig. 8). The minimum and maximum temperatures for growth would be just below 5°C and just above 55°C, respectively.

Fig. 8 Growth of a sulfur bacterium Tei-1 at different temperatures. The culture was grown on 0.1% peptone at 0.6% NaCl and pH 7.4.
The growth response to salinity (% NaCl) was slightly halophilic rather than moderately halophilic. The optimum salinity for growth was 0.6%, but 1/3 to 1/2 of the maximum growth was sustained at seawater strength (3 to 3.5%) or more (Fig. 9). Contrary, its growth at 0% salinity was only 1/10 or less of the maximum growth, which suggests its NaCl requirement for the growth.

4. Discussion

Sulfur bacterial strains TW-A and Tei-1 could be very tentatively assumed *Thiobacillus* species, though some major essential characteristics, e.g. ubiquinone and fatty acid composition, were not fully examined. However, a preliminary estimation of G+C moles % of their chromosomal DNA was 65-69 for TW-A and 53-55 for Tei-1, and the values were within those known for *Thiobacillus* species ranging from about 50 to 70. Other sulfur bacterial taxa, *e.g.* Thiotrix, Thiomicrospira and Sulfothobus, are known to have G+C moles % of less than 50. In any case, as the genus *Thiobacillus* is thought to be a complex taxon consisting of at least 6 to 9, or more, distinct subgroups (Hutchinson et al., 1969; Katayama-Fujimura et al., 1982), further examination and consideration are needed to classify TW-A and Tei-1. [The G+C moles % were measured according to Tamaoka and Komagata (1984) with the support of Dr. Jin Tamaoka (RIKEN).]

4.1. Bacteria in tube worm blood

Ten bacterial strains of different colony types and a sulfur bacterial strain from liquid culture were isolated from tube-worms blood (Tables 3 to 6). We cannot say the “blood” was vascular blood or mixed with coelomic fluid. However, it was reported for a tube worm, *Riftia pachyptila*, that both vascular blood and coelomic fluid contained hemoglobin (Albéric, 1986). And the “blood” we collected was actually bright red. Thus it can be said that bacterial strains were isolated from, at least, hemoglobin containing fluid.

In any case, at least eleven bacterial strains were shown to occur in tube worm blood. This number was larger than we had expected, and a question was asked: Do these eleven strains make any contribution to host tube worms through chemosynthetic processes? Although chemosynthetic activity possibly by endosymbiotic sulfur bacteria in tube worm trophosomes has been reported (e.g. Fisher and Chidress, 1984), other roles of bacteria in tube worm blood should be discussed as well. For example, character and function of two gram-positive strains (Table 4) would be of particular interest, since the sulfur oxidizing bacteria are known gram-negative and symbiotic sulfur oxidizing bacteria in *Mytilus spinifera* (bivalve) gills were reported gram-negative (Dando et al., 1985). Then, what is the function of gram-positive bacteria in tube worm blood? Do they operate another mode of chemosynthesis?

Another interest of bacterial symbiosis is how host animals, e.g. tube worms, control the number and activity of symbionts. Not all the symbionts would be sulfur bacteria nor contribute to host diet. Moreover, some of them could be parasitic, or even pathogenic. In some polychaete worms, phagocytosis is a major defense mechanism against bacteria invading their blood, and the defence would be occasionally in vain (Dales, 1984). Then what defense mechanism(s) do tube worms possess? How can they distinguish commensal
symbionts from parasitic or invading ones?

One of the eleven bacterial strains, TW-A from liquid culture, was studied with physiological emphasis. Mesophilic growth of TW-A (Fig. 2) could be considered adaptive to a hydrothermal vent condition. Although high-pressure effects on growth-temperature relationship were not examined in this study, we would suppose that TW-A adaptation to deep-sea hydrothermal vent conditions could be confirmed by future pressure studies. That is, the optimum temperature would shift to the higher range with the increase of pressures, and the optimum pressure would be high, at least higher than 1 atm.

Growth of TW-A on different salinity was impressively slightly halophilic (Fig. 3). The optimum salinity was 0.6% NaCl. Ambient salinity of the vent area was nothing far from usual (deep-) seawater salinity of 3-3.5%. The slightly halophilic nature of TW-A is considered due to: 1) its terrestrial or estuarine origin; or 2) its adaptation* to the salinity of tube worm blood. Unfortunately, enough quantity of the blood for salinity measurement was not available. However, while coelomic fluid and blood of marine invertebrates were reported to have similar salt concentration (isosmotic) and composition (isotonic) to seawater, their tissues showed 1/10 to 1/20 salt concentration of seawater, i.e. 0.3 to 0.8% NaCl equivalent (Hammel, 1980). Thus it is suggested that TW-A adapted more to tissue salinity than blood salinity of tube worms, and that TW-A was possibly a true endosymbiotic sulfur bacterium.

4.2. White flock and a sulfur bacterium of Tagiri vents

White flocculate matter at the edge of tagiri vents was composed of filamentous microorganisms. Though isolation and cultivation of them is under way, some of them were suggested to belong to Beggiatoa, sulfur oxidizing filamentous bacteria, by morphological observation (Miura and Tagiri Research Group, 1990). But it was not clear which filaments were supposed Beggiatoa by Miura's group.

We observed filaments with inclusions inside which are often seen with cultured Beggiatoa (Photo 3). However, there were rather negative observations of the occurrence of clearer septa, longer septum-to-septum distance, and wider filament for the species of Beggiatoa except B. gigantea. Filaments of B. gigantea were described as 26-55μm wide with cell segments of 5-13μm long in an old Bergey's Manual (of Determinative Bacteriology, 8th ed.; Buchanan and Gibbons, 1974), but there is little reference to it in the latest Bergey's Manual (of Systematic Bacteriology, Vol. 3; Staley et al., 1989). In contrast, recently found deep-sea Beggiatoa spp. were relatively larger as up to 42μm (Nelson et al., 1989). Moreover Beggiatoa of virtually monocultural colony at a deep-sea vent were up to 122μm wide and probably 30cm long (Jannasch et al., 1989). These findings suggest our flock would consist of such large Beggiatoa at least in part. In addition, Jacq et al. (1987) reported Beggiatoa-like, large filaments at a hydrothermal vent off southern California. The filaments seemed very similar to our flock organisms in terms of their large size and intracellular inclusions, and Jacq et al. also hesitated to state that the filament was Beggiatoa because of its extremely large size. In any case, isolation and cultivation of the tagiri flock microorganisms are strongly required.

A sulfur bacterial strain, Tag-1, was isolated from the tagiri vent sediment in Kagoshima Bay. One of major characteristics of Tag-1 is its red to purple pigmentation (Table 8, Fig. 5). Most of the sulfur bacteria, which can obtain CO₂-fixation energy from oxidation of sulfur and/or inorganic sulfur compounds, are known to be colorless.

* The word of "adaptation" is used here in a broad sense for the consequence of acclimatization of populations (physiological change), or selection among populations (structural change), or both (e.g. Atlas, 1986).
They are termed chemolithoautotrophs.

Colored, purple and green, sulfur bacteria can also oxidize sulfur and/or inorganic sulfur compounds, but they are photosynthetic organisms (photolithoautotrophs). In addition, most of colored sulfur bacteria are known anaerobic, with the only exception of *Erythrobacter* being obligately aerobic (Shiba and Shimidu, 1982). However, an absorption spectrum of *Erythrobacter* cell suspension is different from that of Tag-1 (Fig. 5). Thus classification of Tag-1, a colored aerobic chemosynthetic sulfur bacteria, would be of great interest and importance.

4.3. A sulfur bacterium from Teishi Knoll

A sulfur bacterial strain, Tei-1, was isolated from sediment inside the Teishi Knoll crater. Tei-1 was able to form fast-growing colonies on either organic or inorganic media. Table 9 suggests Tei-1 is enzymatically more active and versatile than TW-A and Tag-1. In fact, Tei-1 showed relatively strong activities of amylase and protease (Photo 5), while TW-A and Tag-1 displayed relatively a weak amylase activity and little or no protease activity (data are not shown). Tei-1's large colonies and fast growth on both organic and inorganic media would be sustained by that versatility.

One of its marked characteristics is slightly halophilic growth. Though Tei-1 could hardly grow in fresh water, its optimum salinity for growth was only 0.6%, which is about 1/5 strength of seawater. This slight halophilism suggests Tei-1 of estuarine origin. In fact, Teishi Knoll is located only 2km off shore of Ito, a well-known spa resort for hot springs on and in shore. And one of the authors (T. N.) had isolated sulfur bacteria from an estuary near by Ito (unpublished). Therefore there is a possibility that sulfur bacteria from the in-shore hot springs were drifted to Teishi Knoll and started colonizing a new habitat.

It is a very rare opportunity to monitor biological succession of an submarine volcanic vent from the very first. Continued surveys for years are strongly expected.

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Reference


*DEEP stands for Deep-sea Environment Exploration Program.


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Photo 1  Scanning electron micrograph of a sulfur bacterium, TW-A, from tube-worm blood of a mid-Okinawa Trough hydrothermal vent. Bar, 1nm.

Photo 2  Filaments of the white flocculate material collected from the edge of a vent in Kagoshima Bay. Clear septa and inclusions of filaments are shown. Bar, 100nm.

Photo 3  Fluorescence photomicrograph of flocculate material collected from the edge of a vent in Kagoshima Bay. The major filament was about 3nm in full length. Filaments were stained with 0.01% acridine orange.

Photo 4  Scanning electron micrograph of a mixed bacterial population from a submarine volcano Teishi Knoll. Rod-like cells, at least some of them, were sulfur-oxidizing. Bar, 2.5nm.
Photo 5  Colonies of a sulfur bacterium Tei-1 grown on agar plates containing 0.2% blue starch (left) and 0.2% litmus milk (right), both with 0.1% peptone. Clear zones around colonies suggest the bacterial excretion of amylase (left) and protease (right).