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Morphological Variation of New *Thermoplasma acidophilum* Isolates from Japanese Hot Springs

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We isolated 12 strains of *Thermoplasma acidophilum* from hot springs in Hakone, Japan. *T. acidophilum* strains showed morphological variation in the crystal-like structure in the cell and the fibrous structure on the cell surface. Two strains tested were sensitive to novobiocin. However, a novobiocin-resistant mutant was obtained by spontaneous mutation.

Thermoplasma sp. is a thermoacidophilic archaebacterium. Its cells are highly irregular in shape as a result of the absence of cell walls. The cytoplasmic membrane consists mainly of tetraether lipid. The organism can grow anaerobically in the presence of elemental sulfur (15). On the basis of these features, it was proposed that *Thermoplasma* sp. is a host cell of the endosymbiosis theory of eukaryotic cells (8, 13). Two species have been reported for the genus *Thermoplasma*. The first species, *Thermoplasma acidophilum*, was reported by Darland et al. (2). The type strain (ATCC 25905) of this species was isolated from self-heated coal refuse piles. The second species, *Thermoplasma volcanium*, was isolated from hot springs and reported by Segerer et al. (15). Ohba and Oshima have previously isolated *Thermoplasma* sp. from a hot spring in Japan (11). We isolated several new *Thermoplasma* strains from Japanese hot springs. They showed significant morphological variations.

Isolation and growth characteristics of strains. Several water samples were taken from hot sulfur springs at Ooakudani solfataric field in Hakone, Japan. The original temperatures were between 53 and 63°C, and the pHs were between 2.5 and 3.5. All of the samples were carried to our laboratory without any temperature control within a day. Samples were enriched and cultured in a medium consisting of the following compounds (in grams per liter): yeast extract (Difco), 1.0; Casamino Acids (Difco), 1.0; $(NH_4)_2SO_4$, 1.3; NaCl, 0.2; KH_2PO_4, 0.3; $MgSO_4 \cdot 7H_2O$, 0.25; $CaCl_2 \cdot 2H_2O$, 0.05; vancomycin, 1.1 (pH 1.8) (11). The culture was incubated at 56°C. It is expected that no other thermophilic bacteria can grow under these conditions except *Thermoplasma* spp. because the growth of thermoacidophilic bacilli or *Sulfolobus* sp. will be inhibited by vancomycin, at 1.1 g/liter, or by a high phosphate concentration, 0.3 g/liter, respectively (2, 16).

Colonies were isolated by plating them on medium that was solidified with 0.6% Gelrite and 3 mM $CaCl_2$. After incubation at 56°C for about 30 days, colonies of the fried-egg morphology with diameters of about 1 mm were observed. Twelve strains (HO-01, HO-42, HO-51, HO-52, HO-62, HO-63, HO-66, HO-67, HO-101, HO-103, HO-121, and HO-122) were isolated from different samples.

All of the isolates required yeast extract for their growth. For anaerobic culture, the medium was supplemented with elemental sulfur and resazurin. A rubber-stoppered culture bottle (30 ml) was filled with 10 ml of the medium, and the gas phase was replaced with H_2. All of the isolates tested were able to grow under the anaerobic conditions in the presence of elemental sulfur. Formation of H_2S in the gas phase was detected by the darkening of the lead acetate paper. These characteristics matched well those of the genus *Thermoplasma* (1, 2). The optimum growth temperature of the isolates was between 57 and 58°C. The optimum temperature is similar to that of *T. acidophilum* but slightly higher than that of *T. volcanium.*

G+C content. The G+C content of DNA was estimated to be 49% ± 1% on the basis of the thermal denaturation temperature (9) in 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate). This value is slightly higher but close to that reported previously for *T. acidophilum* (46%) and significantly higher than that for *T. volcanium* (38%) (15). From the bacteriological criteria, 12 isolates were identified as *T. acidophilum.*

Sequence determination of the 16S rRNA. RNA was extracted with phenol from about 100 mg (wet weight) of cells by the method described by Lane et al. (7). Partial 16S rRNA sequence was determined by the primer extension method with reverse transcriptase (7). Three oligonucleotides, deoxy(ACG GGCGGTGTGTGC), deoxy(CAATTCCTTTAAGTTC), and deoxy(TACCGCGGCGGCTGGC), were used as sequencing primers. Partial 16S rRNA sequences were determined for three isolates, HO-101, HO-122, and HO-51. Three regions of HO-101 rRNA, i.e., positions 262 to 442, 598 to 833, and 1083 to 1330 (665 bases in total), three regions of HO-122 rRNA, i.e., positions 207 to 444, 595 to 838, and 1100 to 1325 (708 bases in total), and one region of HO-51 rRNA, i.e., positions 1102 to 1321 (220 bases in total), were sequenced. The three strains had the same sequences, and the sequences determined were the same as the corresponding parts of the *T. acidophilum* 16S rRNA sequence reported for strain 122-1B2 (type strain = ATCC 25905 = DSM 1728 [12]).

Morphology. Thin sections of cells were stained with uranyl acetate and lead nitrate for transmission electron microscopy (JEOL 1200EX). For scanning electron microscopy, colonies were fixed with 1% glutaraldehyde and dehydrated by washing with an increased concentration series of ethanol. After critical-point drying, the colonies were coated with gold.

The cells of the isolates showed an irregular shape with no detectable cell wall under an electron microscope (Fig. 1A and B), although some differences were noted between isolates. Some isolates had fibrous structures at the surfaces of the cells (Fig. 1C and D), and others contained crystal-like structures

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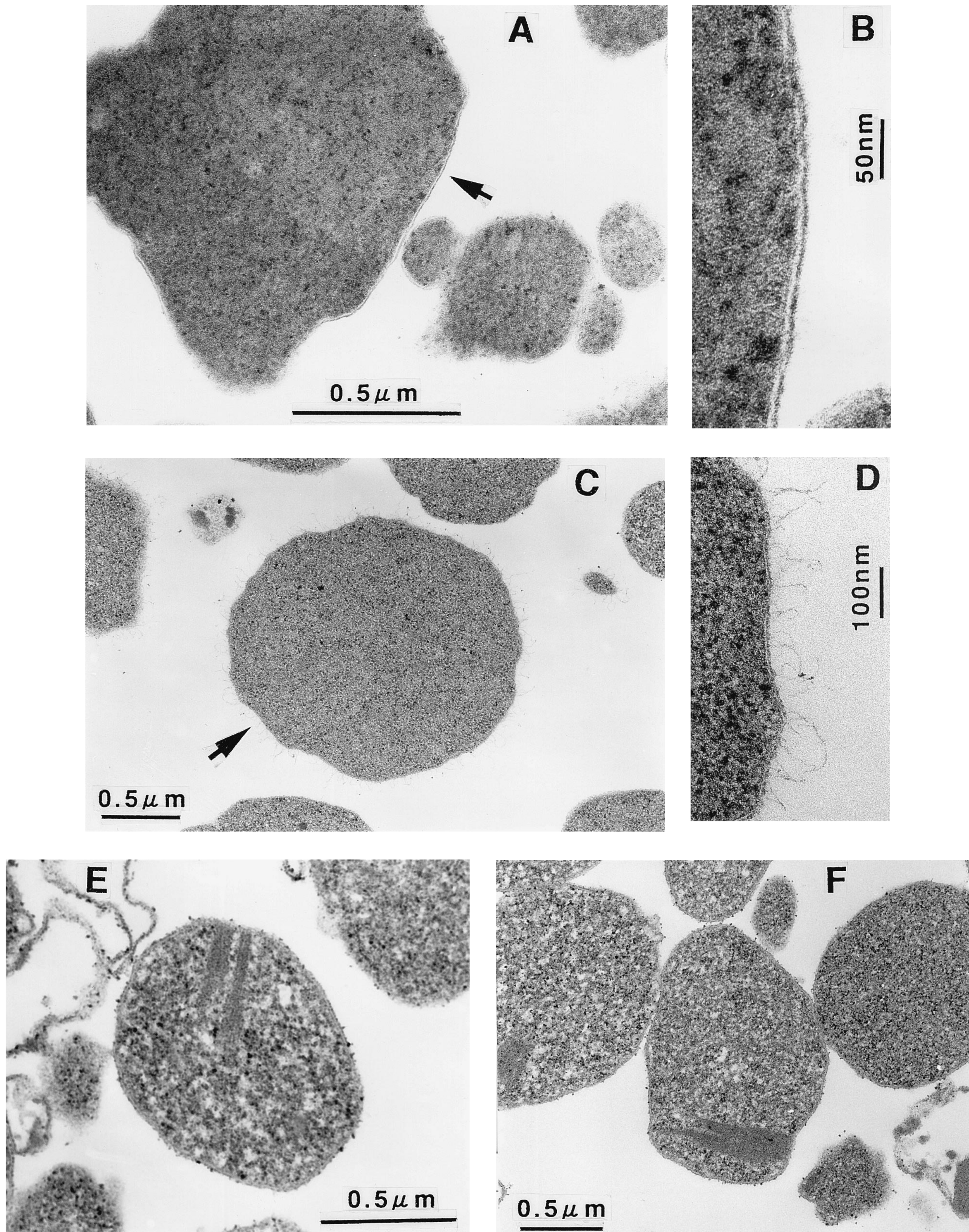

FIG. 1. Electron micrographs of isolates. (A) Thin section of strain HO-101; (B) enlargement of the area indicated by the arrow in panel A, showing the triple-layered membrane structure; (C) strain HO-121; (D) enlargement of the area indicated by the arrow in panel C; (E) strain HO-01; (F) strain HO-63.

TABLE 1. Morphological variation of new isolates of *T. acidophilum*

Strain	Presence of[a]:	
	Crystal-like structure	Fibrous structures at the surface
HO-01	+	−
HO-42	+	−
HO-62	−	−
HO-63	+	−
HO-121	+	++

[a] +, present; ++, present at high levels; −, absent.

(Fig. 1E and F). HO-121 had both characteristics, but HO-62 had neither structure (Table 1).

The presence of a cytoskeleton has been discussed (14); however, no intracellular structure has been reported for *Thermoplasma* spp. The cell division mechanism of *Thermoplasma* spp. is not known. Since *Thermoplasma* cells have no cell walls, some machinery must be required for cell division and separation of DNA into daughter cells. The crystal-like structure found in our study might be related to the cell division mechanism. The function of the crystal-like structure will be studied in the future.

There are several large differences between archaebacteria and eukaryotes. Cell size and genome size are significantly different between archaebacteria and eukaryotes. If the cells of the common ancestor of archaebacteria and eukaryotes could fuse, the fusion of the cells could be responsible for the process of increasing their cell and genome sizes. Figure 2 shows the network structure of the cells in a colony formed on a Gelrite plate. A similar structure has been reported previously for *T. acidophilum* (10) and *Pyrodictium oculutum* (17). The formation of the network structure may be the intermediate state of the fusion process of the cells.

Although the bacteriological characteristics and the 16S rRNA sequences indicate that these isolates can be classified into a single species, *T. acidophilum*, the isolates differ from strain to strain with respect to morphological features as mentioned above (Table 1). These morphological differences between the strains were observed in the same cultural stage, the late log phase, and under the same conditions. Since we cannot totally dismiss the possibility of morphological variation occurring during the cultural stage and under these conditions, experiments are in progress to check this possibility.

Effects of heavy metal ions on growth. If some of the strains show different sensitivities to heavy metal ions, the gene responsible for the resistance can be used as a marker for gene manipulation. Cells of the early stationary phase (2×10^5) were inoculated onto Gelrite plates. The test plates contained heavy metal ions at the following concentration: 0.01, 0.05, 0.1, 0.5, 1.0, or 5.0 mM. The plates were incubated at 56°C, and growth was scored after 10 days. The effect of eight heavy metal ions (Zn, Ni, Co, Cu, Ag, Pb, and As) on growth of the strains was tested (Table 2). The influence spectrum resembled that reported for *Sulfolobus* spp. (3). However, the resistance to cadmium was considerably higher than that of *Sulfolobus* spp. No significant difference was found among strains.

Effects of antibiotics on growth. Novobiocin is known to inhibit the growth of *T. acidophilum* (2). The growth of two strains, HO-62 and HO-63, was inhibited by novobiocin at 0.01

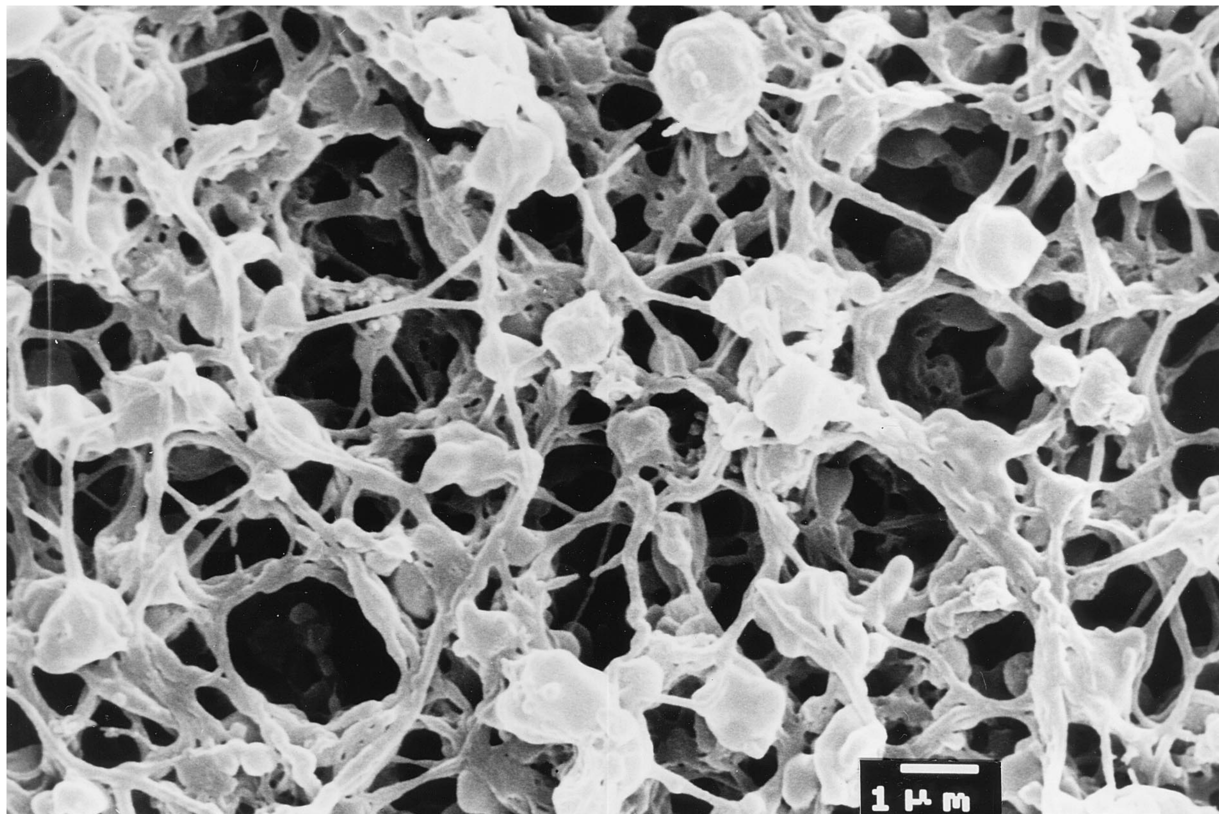

FIG. 2. Scanning electron micrograph of strain HO-51.

TABLE 2. Inhibitory effect of heavy metal ions

Isolate	MIC (mM)							
	$ZnSO_4$	$NiSO_4$	$CoCl_2$	$CdCl_2$	$CuSO_4$	$AgNO_3$	$Pb(CH_3COO)_2$	$NaAsO_2$
HO-01	5	0.1	0.01	10	1	1	1	5
HO-42	5	0.1	0.01	10	1	1	1	1
HO-51	5	0.1	0.01	10	1	1	1	5
HO-52	5	0.1	0.01	10	1	1	1	5
HO-62	10	0.1	0.01	10	1	1	1	1
HO-63	10	0.1	0.01	10	1	1	1	1
HO-66	10	0.1	0.01	10	1	1	1	1
HO-67	10	0.1	0.01	10	1	1	1	1
HO-101	5	0.1	0.01	10	1	1	1	5
HO-103	5	0.1	0.1	50	1	1	1	5
HO-121	10	0.5	0.1	10	1	1	1	1
HO-122	5	0.5	0.1	10	1	1	1	1

mg/ml. These two isolates were more sensitive than the *T. acidophilum* type strain, ATCC 25905, which requires a MIC of 0.1 mg/ml.

Selection of novobiocin-resistant mutant. For the purpose of obtaining a novobiocin-resistant strain, we incubated strain HO-62 in medium containing 0.01 mg of novobiocin per ml. Growth was noted after 3 weeks of incubation. The concentration of the antibiotic was gradually increased. A mutant that could grow in medium that contains 2.0 mg of novobiocin was isolated. Figure 3 shows the growth curve of the novobiocin-resistant strain HO-62N1C and the wild-type strain HO-62. Growth of the wild-type strain was hardly detected for 190 h, indicating that the frequency of spontaneous occurrence of novobiocin-resistant mutation in the wild-type strain is very low.

In the case of halophilic archaebacteria, a novobiocin-resistant strain was isolated, and the resistance is due to a mutation in the gene of DNA gyrase B subunit (5). The host-vector system was constructed with this gene as a marker (6). The novobiocin-resistant *Thermoplasma* strain may contribute to the development of a gene manipulation system in thermoacidophilic archaebacteria.

Several *Thermoplasma* strains have been isolated from samples collected in the nearby hot springs. These strains were identified as *T. acidophilum* by the bacteriological criteria. Partial sequences of 16S rRNA also supported the identification of the three strains. The morphological variations in the closely related strains will provide a good system for the molecular analysis of the structural differences.

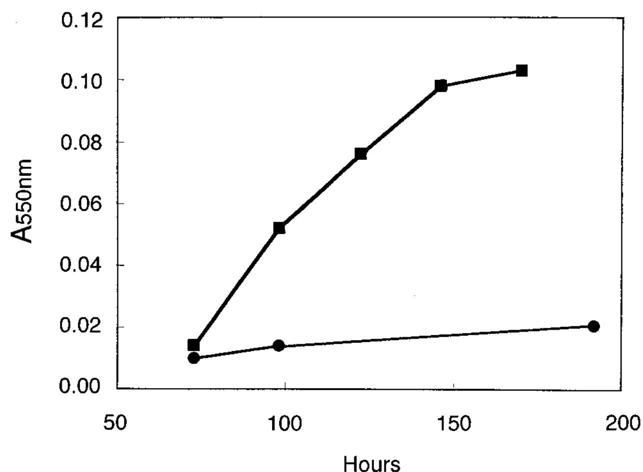

FIG. 3. Effect of novobiocin on the growth of the wild-type HO-62 (circles) and the novobiocin-resistant mutant strain HO-62N1C (squares) of *T. acidophilum*. A mid-log-phase culture of each strain was used to inoculate the medium containing novobiocin (2.0 mg/ml). The culture was incubated at 56°C, and the growth was monitored by measuring the A_{550}.

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